Analysis of Hemopoietic Malignancy in

$lgH_\mu TLX1^{Tg}Prkdc^{Scid/Scid}$ Mice

by

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A thesis submitted in conformity with the requirements
for the Degree of Doctor of Philosophy

Institute of Medical Science

University of Toronto

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*IgHμ-TLX1*<sup>Tg</sup>*Prkdc*<sup>Scid/Scid</sup> Mice

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Abstract

The non-cluster homeobox gene *TLX1* was initially identified at the breakpoint of the *t*(10;14)(q24;q11) chromosome translocation in the malignant cells. Three independent research groups have generated transgenic mouse models expressing *TLX1* in various hemopoietic cells resulting in development of B cell lymphoma and T-ALL. T-ALL tumours were characterized as immature Double Negative or/and Double Positive phenotypes. In addition, all *TLX1* transgenic mice showed a long latency for leukemia development suggesting requirement of additional genomic abnormalities for the conversion of premalignant cells to full-blown malignancy.

We hypothesized that unresolved double strand DNA breaks might act as additional genetic mutation and contribute to T-ALL progression in *TLX1* overexpressed thymocytes. To address this hypothesis, we generated double mutant *IgHμ-TLX1*<sup>Tg</sup>*Prkdc*<sup>Scid/Scid</sup> mice, which accelerated the development of leukemia relative to *Prkdc*<sup>Scid/Scid</sup> littermates. We identified that multiple genes associated with chromosome segregation, apoptosis and cell cycle progression were aberrantly expressed in *IgHμ-TLX1*<sup>Tg</sup>*Prkdc*<sup>Scid/Scid</sup> premalignant thymocytes. We found that
IgHμ-TLX1\textsuperscript{Tg}\textsuperscript{Prkdc}\textsuperscript{Scid/Scid} thymocytes were prone to chromosome instability suggesting malfunction of the mitotic spindle assembly checkpoint.

In addition to T-ALL, 46\% of IgHμ-TLX1\textsuperscript{Tg}\textsuperscript{Prkdc}\textsuperscript{Scid/Scid} mice developed Acute Myeloid Leukemia, suggesting that the cancer initiating effects of TLX1 are not limited to cells of lymphoid origin. Transplantation experiments revealed that T cell acute lymphoblastic leukemia initiating cells (T-ALL-ICs) reside in the thymus of IgHμ-TLX1\textsuperscript{Tg}\textsuperscript{Prkdc}\textsuperscript{Scid/Scid} mice and T-ALL-ICs were enriched in the c-kit\textsuperscript{+}CD44\textsuperscript{+}CD25\textsuperscript{−} fraction. We showed that T-ALL tumour cells from IgHμ-TLX1\textsuperscript{Tg}\textsuperscript{Prkdc}\textsuperscript{Scid/Scid} were transplantable and there was a tendency for the latency period of T-ALL development to be reduced with secondary and tertiary transplantations.

We demonstrated that premalignant IgHμ-TLX1\textsuperscript{Tg}\textsuperscript{Prkdc}\textsuperscript{Scid/Scid} myeloid progenitors exhibited deregulated apoptosis and proliferation. Collectively, our studies demonstrate that TLX1 expression in DNA-PK-deficient cells activates multiple oncogenic pathways leading to apoptosis resistance, accelerated proliferation and deregulation of the spindle assembly checkpoint. We propose that activation of the same pathways supporting survival and proliferation in various cells may be indicative of the universal principles driving TLX1-induced tumourogenesis. Our data provide clinically relevant information of the molecular mechanisms involved in the pathogenesis of leukemia that makes IgHμ-TLX1\textsuperscript{Tg}\textsuperscript{Prkdc}\textsuperscript{Scid/Scid} mouse model a powerful tool to explore potential treatment options directed to delay disease progression.
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<th>Definition</th>
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<tr>
<td>Aldh1</td>
<td>Aldehyde dehydrogenase gene</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute Lymphoblastic Leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute Myeloid Leukemia</td>
</tr>
<tr>
<td>AML-L-IC</td>
<td>AML-Leukemia Initiating Cells</td>
</tr>
<tr>
<td>ANK</td>
<td>Ankyrin repeat domain</td>
</tr>
<tr>
<td>ANT-C</td>
<td>Antennepedia Complex</td>
</tr>
<tr>
<td>APC</td>
<td>Anaphase Promoting Complex</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>B-ALL</td>
<td>B-Cell Acute Lymphoblastic Leukemia</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>B/F/B</td>
<td>Breakage/Fusion/Bridge</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BX-C</td>
<td>Bithorax Complex</td>
</tr>
<tr>
<td>Cdks/Cdc</td>
<td>Cyclin-dependent kinases/Cell division control protein</td>
</tr>
<tr>
<td>CCNB1</td>
<td>Cyclin B1 gene</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunopresipitation</td>
</tr>
<tr>
<td>CLP</td>
<td>Common Lymphoid Progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>Common Myeloid Progenitor</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>DEGs</td>
<td>Differentially Expressed Genes</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DN</td>
<td>Double Negative thymocytes subpopulation, eg. DN1, Double Negative 1</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco’s Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>EGIL</td>
<td>European Group for Immunological Characterization of Leukemias</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>FBS</td>
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<td>DNA-PKcs</td>
<td>DNA-PK catalytic subunit</td>
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<td>dsDNA breaks</td>
<td>Double Strand DNA breaks</td>
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<td>Endoplasmic Reticulum</td>
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<td>Early Thymocyte Progenitors</td>
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<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<td>FDR</td>
<td>False Discovery Rate</td>
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<tr>
<td>FISH</td>
<td>Fluorescent <em>in situ</em> Hybridization</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>G0</td>
<td>Gap0 phase of cell cycle</td>
</tr>
<tr>
<td>G1</td>
<td>Gap1 phase of cell cycle</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>G2</td>
<td>Gap 2 phase of cell cycle</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene Set Enrichment Assay</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HOM-C</td>
<td>Homeotic Complex</td>
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<tr>
<td>HOX</td>
<td>Homeobox</td>
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<tr>
<td>HOM</td>
<td>Homeotic Complex</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscov’s Modified Dulbecco’s Medium</td>
</tr>
<tr>
<td>ICN</td>
<td>Intracellular domain of Notch</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgHµ</td>
<td>Immunoglobulin Heavy Chain – µ subunit</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin, eg. IL-3, Interleukin-3</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>Lck</td>
<td>Lymphocyte-specific protein tyrosine kinase</td>
</tr>
<tr>
<td>L-IC</td>
<td>Leukemia Initiating Cell</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph Node</td>
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<tr>
<td>LOH</td>
<td>Loss Of Heterozygosity</td>
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<tr>
<td>LTR</td>
<td>Long Terminal repeats</td>
</tr>
<tr>
<td>M</td>
<td>Mitotic phase of cell cycle</td>
</tr>
<tr>
<td>mAIDS</td>
<td>murine Acquired Immunodeficiency Syndrome</td>
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<tr>
<td>MCC</td>
<td>Mitotic Checkpoint Complex</td>
</tr>
<tr>
<td>MEFs</td>
<td>Mouse Embryonic Fibroblasts</td>
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<tr>
<td>MLL</td>
<td>Mixed Lineage Leukemia gene</td>
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<tr>
<td>MPF</td>
<td>Maturation Promoting Factor</td>
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<tr>
<td>MPP</td>
<td>Multi-potent progenitor</td>
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<tr>
<td>MSigDB</td>
<td>Molecular Signatures Database</td>
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<tr>
<td>NES</td>
<td>Normalized Enrichment Score</td>
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<tr>
<td>NHEJ</td>
<td>Nonhomologous End Joining</td>
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<tr>
<td>NK</td>
<td>Natural Killer</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<tr>
<td>PIM</td>
<td>PBX-Interacting Motif</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
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<tr>
<td>PP</td>
<td>Protein Phosphatase, eg. PP1A, Protein Phosphatase 1A</td>
</tr>
<tr>
<td>Pre-TCR</td>
<td>pre-T Cell Receptor</td>
</tr>
<tr>
<td>Prkdc&lt;sup&gt;Scid/Scid&lt;/sup&gt;</td>
<td>DNA-PKcs deficiency, eg. Prkdc&lt;sup&gt;Scid/Scid&lt;/sup&gt; mice</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>quantitative Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoid Acid</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RPs</td>
<td>Ribosomal Proteins</td>
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<tr>
<td>RSS</td>
<td>Recombination Signal Sequences</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>S</td>
<td>Synthesis phase of cell cycle</td>
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<tr>
<td>SAC</td>
<td>Spindle Assembly Checkpoint</td>
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<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency Syndrome</td>
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<tr>
<td>Ser</td>
<td>serine</td>
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<tr>
<td>SKY</td>
<td>Spectral Karyotyping</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SP</td>
<td>Single Positive thymocytes subpopulation, e.g. SP CD4+, SP CD4&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>t(</td>
<td>translocation, eg. t(10;14), translocation between chromosomes 10 and 14</td>
</tr>
<tr>
<td>TALE</td>
<td>Three-Amino-acid-Loop-Extension family of homeodomain proteins</td>
</tr>
<tr>
<td>T-ALL-ICs</td>
<td>T Cell Acute Lymphoblastic Leukemia Initiating Cells</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T-cell Acute Lymphoblastic Leukemia</td>
</tr>
<tr>
<td>TAN1</td>
<td>Truncated NOTCH1 protein</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl Transferase</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TTA</td>
<td>Tetracycline Transactivator</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endophelial Growth Factor</td>
</tr>
<tr>
<td>WHO</td>
<td>The World Health Organization</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>Wt1</td>
<td>Wilm’s tumour 1</td>
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</table>
Chapter 1

General Introduction
1.1. The homeobox gene family

1.1.1. Class I homeobox gene clusters

In mammals there are 39 HOX genes that are organized into four clusters at four different chromosomal loci (HOXA, HOXB, HOXC and HOXD). The number of genes within each cluster varies from 9 to 13 genes. Every HOX gene is identified by its location within each cluster. For example, genes positioned at the 3' end of the A cluster are named HOXA1 to HOXA8, while genes located at the 5' end are HOXA9 to HOXA13. The genes in the four clusters are subdivided into paralogous groups of genes based on the similarity of the sequences and position within the cluster (Figure 1.1) (Carroll, 1995; Kappen, Schughart, & Ruddle, 1989; Krumlauf, 1992; Ruddle et al., 1994).

For example, genes with the same position number (HOXA4, HOXB4, HOXC4 and HOXD4) belong to the same paralogue. To date, thirteen paralogous groups have been defined and each paralogue contains from two to four genes. Genes in the same paralogues group share more homology relative to genes of the same cluster. Based on this finding, it has been suggested that HOX genes of one paralogous group share related functions. For example, mutations in HoxA3 and HoxD3 result in distinct phenotypes, while, HoxA3 and HoxD3 double mutant mice exhibit more severe defects (Condie & Capecchi, 1993; Chisaka &Capecchi, 1991; Condie & Capecchi, 1994). This has led to the hypothesis that the ancestral HOM-C cluster underwent duplication and insertion into a new chromosomal location. The different numbers of genes within individual paralogous groups may have resulted from the incomplete duplication of the entire gene cluster during evolution. Alternatively, it is also possible that all genes were originally duplicated, but function of some genes was dispensable and redundant genes were lost during evolution (Krumlauf, 1992). The amplification of HOX genes is thought to be a vital event for adaptive evolution (Kappen, Schughart, & Ruddle, 1989; Schughart, Kappen, & Ruddle, 1989; Kappen & Ruddle, 1993).
**Figure 1.1. The HOX gene family.** Schematic representation of relationship between Drosophila homologs in the HOM-C cluster to the four mouse HOX genes clusters. HOX genes are shown as colored boxes in their order on the chromosome. Orthologous genes between Drosophila and mouse, and paralogous mouse genes are shown color-coded. This picture was adapted from Meyers 2007.
A new cluster of 12 related HOX genes was recently identified on the X chromosome. These genes were shown to be expressed in the reproductive tissues of male and female mice. These reproductive HOX (Rhox1-Rhox12) genes are expressed in a cell type-specific manner mostly in Sertoli cells regulating the expression of somatic-cell gene products critical for germ cell development and in the ovary and placenta (MacLean & Wilkinson, 2010). Drosophila melanogaster has a single Rhox gene. Only two RHOX orthologs were detected in human and a large Rhox gene cluster was detected in rats. The variability in number on Rhox genes between primate and rodent species has been suggested to be related to the greater reproductive capacity of rodents (Wayne, MacLean, Cornwall, & Wilkinson, 2002).

1.1.2. The role of homeobox genes during embryogenesis

A single gene whose expression is both necessary and sufficient to trigger activation of many other genes has been defined as a master control gene, or master switch. Homeobox (HOX) genes are master control genes that play critical roles in regulating patterning and axial morphogenesis in vertebrates during embryogenesis. Identification of HOX genes revealed that very different body plans of insects and vertebrates are achieved by evolutionary conserved networks which display similar regulatory interactions (Duboule & Dolle, 1989; Lewis, 1978; McGinnis, Levine, Hafen, Kuroiwa, & Gehring, 1984; McGinnis, Garber, Wirz, Kuroiwa, & Gehring, 1984). Initially identified in Drosophila, HOX genes have also been detected in cephalopods, primitive chordates, mammals and humans (Callaerts et al., 2002; Garcia-Fernandez & Holland, 1994; Boncinelli et al., 1989; Duboule & Dolle, 1989; Carrasco, McGinnis, Gehring, & De Robertis, 1984; Levine, Rubin, & Tjian, 1984). Expression of HOX genes is required from early embryogenesis until the end of development. Since the genomes of all bilateral organisms contain HOX genes, their role as regulators of patterning body formation appears to have been conserved throughout evolution.

Eight HOX genes are organized in one cluster in a head-to-tail orientation and are located on the right arm of chromosome 3 (3R) within the Drosophila genome. This cluster is subdivided into two regions. Genes of the first region, termed the Antennapedia complex (ANT-C), are involved during embryogenesis in specification of the anterior thorax and head. The second region, the Bithorax (BX-C) complex, specifies the posterior thoracic and abdominal segments of the developing embryo. The genes of each complex exhibit spatial colinearity, which means there is a correlation between the physical location of each HOX gene on the
chromosome relative to its site of expression on the anterior-posterior axis of the developing embryo. Thus, expression of \textit{HOX} genes from the 5’ to 3’ region along the gene cluster correlates with the tail-to-head orientation of the developing embryo. Within each complex, \textit{HOX} genes located at the 3’ end of the cluster are activated first, whereas genes located at the 5’ end are transcribed afterwards (Figure 1.2). This spatial colinearity was first described in Drosophila and later demonstrated in vertebrates (Duboule & Dolle, 1989; Lewis, 1978).

Improper expression of even a single class I \textit{HOX} gene has been shown to result in abnormal body plan development. For example, homozygous loss of function of the twin of eyeless (toy) gene within the ANT-C results in a “headless” phenotype and \textit{Ant}^{73} gain-of-function mutations transform antennae into middle legs (Kaufman, Lewis, & Wakimoto, 1980; Lewis, 1978). Mutations of BX-C genes affect the posterior portion of the body. Deletion of the Bithorax gene complex, for example, transforms all posterior body segments into reiterated segments with structures of the second thoracic segments (Gehring, 1987). Duplication of Ultrabithorax, a region of the BX-C complex, converts hind wings into halteres whereas gain-of-function of \textit{Abd-A} results in loss of legs from the abdominal segments (Gehring, Kloter, & Suga, 2009).

1.1.3. Homeodomain-containing transcription factors

\textit{HOX} genes contain a common 180 bp DNA sequence encoding the 60-amino acid homeodomain that forms a DNA-binding helix-turn-helix motif. The homeodomain consists of a flexible N-terminal region followed by \(\alpha\)-helical region, \(\beta\)-turn and another \(\alpha\)-helical region (Gehring et al., 1990; Sharkey, Graba, & Scott, 1997). During DNA binding, the two alpha helices make contact with the DNA and are linked by the \(\beta\)-turn. The N-terminal of the homeodomain is required for high affinity association with DNA. Early data indicated that homeodomain binds the DNA recognition sequence TAAT through amino acid residues 3, 5, within the N-terminal and 47 and 51 within the third helix of the homeodomain. New data obtained using high resolution analysis of sequence preferences indicated that roughly half of the Hox subclasses interact with a canonical TAAT core, while other homeodomains preferentially bind other sequences. Moreover, many homeodomains bind both canonical and additional sequences (Berger et al., 2008).

Homeodomain-containing transcription factors bind DNA in a sequence-specific manner, although the specificity of target sequences usually requires association with other
transcription factors. The high specificity and affinity of homeodomain-containing transcription factors are provided by association with proteins which are designated as HOX cofactors. Among the best-characterized HOX cofactors are the Three-Amino-acid-Loop-Extension (TALE) family of homeodomain proteins. For example, a member of the TALE family of class II homeodomain transcription factors, PBX1, interacts with class I HOX proteins belonging to paralogues 1 to 10 through a highly conserved hydrophobic hexapeptide sequence (Chang et al., 1995). The six amino acids of the hexapeptide sequence are located outside the homeodomain and are connected to it through a linker arm of variable length (Bondos, Tan, & Matthews, 2006). In this motif, tryptophan and methionine are present for all HOX gene products of paralogous 1–8. Mutational analysis of HoxA1 has revealed that the tryptophan and methionine residues of the conserved hexapeptide are critical for the cooperative interaction between HoxA1 and Pbx1 (Chang et al., 1995). Other members of the TALE HOX subfamily, MEIS1 and PKNOX (PREP), are binding partners of HOX proteins belonging to paralogues 9-13 (Shen et al., 1997; Knoepfler, Calvo, Chen, Antonarakis, & Kamps, 1997). MEIS1 and PKNOX1 interact with PBX and HOX family members and bind similar DNA sequences but play opposing roles in tumour development. Thus, elevated levels of MEIS1 accelerate development of HOX- and MLL-induced leukemias. In contrast, diminished expression of PKNOX1 has been linked with cancer development (Bisaillon, Wilhelm, Krosl, & Sauvageau, 2011). TALE proteins interact with each other via the Homothorax domain of MEIS and the PBC-A domain of PBX. These interactions promote the stability of the heterodimers (Rieckhof, Casares, Ryoo, Abu-Shaar, & Mann, 1997). This complex forms heterodimers with Class I HOX proteins to regulate expression of downstream target genes (Jacobs, Schnabel, & Cleary, 1999). Combined, three MEIS family members, two PKNOX proteins, and four PBX gene products in addition to combinatorial diversity of HOX proteins may form numerous regulatory complexes targeting numerous downstream target genes with high specificity.

1.1.4. Regulation of the expression of HOX genes

The regulation of expression of HOX genes is a complex process requiring numerous regulatory proteins. Retinoic acid (RA) has been shown to be an activator of HOX gene transcription. Thus, RA-responsive elements (RARE) have been identified in the 3’ region of murine HoxA1 and HoxB1 genes (Marshall et al., 1994; Studer, Popperl, Marshall, Kuroiwa, & Krumlauf, 1994; Frasch, Chen, & Lufkin, 1995). The AP-2 transcription factor, in combination with RA, is also
important in the expression of some HOX genes since elimination of an AP-2 binding site within the HOXA4 promoter region resulted in delayed RA-induced expression of HOXA4. Thus co-
activation of HOXA4 is regulated by RA and AP-2 (Doerksen, Bhattacharya, Kannan, Pratt, & Tainsky, 1996). More recent studies have revealed that RA upregulates expression of only anterior 3’ Hox genes from clusters A, B, and C, but downregulates expression of posterior 5’ Hox genes from clusters A, B, C, and D. In addition, the RA pathway targets HOX genes in
selected cell types. Although the role of RA regulated expression of HOX genes in adult
hemopoiesis has been established, more recent studies have indicated that HOX genes
expression during primitive hemopoiesis is not regulated by RA (Szatmari, Iacovino, & Kyba,
2010).

Figure 1.2. Schematic representation of regulation of the HoxD cluster of the mouse.
On the left 3’ of the clusters is the hypothetical regulatory mechanism accounting for the
temporally sequential induction of 3’ (early) to 5’ (late) genes. EE stands for “Early Enhancer”.
The grey box RARE is retinoic acid response elements. Only the 3’ RARE is represented in the
case of mouse. On the right, 5’ of the vertebrate cluster, is the regulatory region (GCR) that mediates
digit-specific expression of the 5’ Hoxd genes. Arrows represent transcriptional stimulation.
The colors reflect the paralogy relationship between Hox genes on the different cluster.
The interruptions of the “genomic line” between EE and the 30 end of the Hox clusters and between
the 50 end of the vertebrate cluster and GCR indicates a long distance.
Adopted from Jacqueline Deschamps, Current Opinion in Genetics & Development 2007
Other regulatory proteins have been shown to regulate HOX expression. For example, expression of HOXB2 is mediated by GATA-1 (Vieille-Grosjean, Roullot, & Courtois, 1992). The polycomb family of proteins, which are involved in the remodeling of chromatin to a transcriptionally silent state, repress HOX gene expression via negative elements in their promoters (Bienz & Muller, 1995; Simon, 1995; Sessa et al., 2007). The zinc finger-containing transcription factor, Krox20, mediates expression of HoxA2 and HoxB2 in the developing brain of mice (Sham et al., 1993). Transcriptional activation of Hox genes is regulated by association of CDX proteins with TTTATG Cdx-binding sites (Tabaries et al., 2005). Two Cdx4-binding sites have been identified in the 5′ regulatory region of mouse Hoxa9 gene. One region contains a tandem repeat unit while the other consists of a single Cdx-binding site (Yan et al., 2006). Moreover, Cdx4 changes the levels of expression of Hoxb4, Hoxb3, Hoxb8 and Hoxa9. These HOX genes are involved in the maintenance of HSCs and immature progenitors (Davidson et al., 2003). HOXA10 has been identified as a direct Cdx4 target in myeloid progenitor cells. In these studies, overexpression of Cdx4 activated the HOXA10 promoter and increased HoxA10 expression (Bei et al., 2011).

The spatial folding of chromatin or chromatin looping has been proposed as another possible mechanism of regulation and coordination of genes expression. Recent data indicated that chromatin looping may participate silencing genes of Hox clusters to prevent improper activation by irrelevant cellular pathways (Pindyurin & van, 2011). Thus, genes at the 5′-end of the HoxA cluster were brought together by a series of loops in human NT2/D1 and THP-1 cells when they are not expressed (Ferraiuolo et al., 2010; Fraser et al., 2009). Another binding protein, the CTCF, was identified as a candidate mediating DNA loops in all HOX clusters (Ferraiuolo et al., 2010).

1.1.5. Class II homeobox genes

In addition to class I HOX genes that are organized in clusters in the genomes of most organisms, a large number of HOX genes are dispersed throughout the genome. These homeodomain-containing genes are referred to as noncluster, class II genes. Comparisons of murine and Drosophila class II HOX genes have revealed up to 80% homology at the amino acid level suggesting class II homeodomain-containing genes, similar to Class I genes, have been conserved during evolution thereby suggesting overlapping developmental functions (Joyner & Martin, 1987). Class II HOX genes are classified into families based on the presence of specific
motifs or domains (Krumlauf, 1992; Kessel & Gruss, 1990; Gehring, 1987). Some examples of Class II families include the HOX11/TLX family, TALE and LIM-only domain homeodomain transcription factors. The HOX11/TLX family includes HOX11/TLX1, HOX11L1/TLX2 and HOX11L2/TLX3 (here and after TLX1, TLX2 and TLX3, correspondingly). This family is defined by the presence of a threonine rather than the more common valine or isoleucine at position 247 within the third helix of the homeodomain (Dube et al., 1991). Both TLX1 and TLX3 have been shown to play a role in the development of T-ALL (Dube et al., 1991; Ferrando et al., 2002). The TALE homeodomain family is defined by a three amino acid loop extension between helix1 and helix2 of the homeodomain (discussed in the Homeodomain-Containing Transcription Factors section). Members of this family include PBX1, PBX2, PBX3 and PBX4, MEIS1, MEIS2 and MEIS3, and PKNOX1 and PKNOX2 (Monica, Galili, Nourse, Saltman, & Cleary, 1991; Chang et al., 1997; Ferretti et al., 2006). PBX1 have been shown to contribute of pre-B cell leukemia (Kamps, Look, & Baltimore, 1991). The LIM-only domain proteins, another Class II family of homeodomain-containing transcription factors, play an initiating role in the development of various cancers including leukemia (Rabbitts et al., 1997). Due to the role of HOX genes as regulators of cellular differentiation and proliferation I would like to elaborate their role in normal hemopoiesis and leukemogenesis.

1.1.6. Role of the HOX genes in hemopoiesis

The first data describing the HOX gene expression in blood cells were obtained in experiments with cell lines of both human and murine origin. These data indicated that some homeobox genes were predominantly lineage restricted, while expression of other HOX genes was detected in cells of diverse phenotypes (Mathews, Detmer, Boncinelli, Lawrence, & Largman, 1991; Shen et al., 1989; Shen, Largman, Lowney, Hack, & Lawrence, 1989; Petrini et al., 1992). Studies of purified hemopoietic progenitors from normal human bone marrow demonstrated that HOX genes of the A, B and C clusters were expressed in hemopoietic stem cells (HSC) with a marked reduction in expression of HOXA, HOXB and HOXC genes as stem cells progressed to the progenitor stage, while HOXD was silenced in hemopoietic progenitors. Furthermore, although HOXA and HOXB genes located at the 3’ region of the gene clusters were expressed in enriched subpopulations of human CD34+ bone marrow cells, their expression was downregulated as these cells progressed to erythroid and myeloid progenitors. In contrast, HOX genes located at 5’ region of the gene cluster are silenced in hemopoietic progenitors and active
as cells progressed to more mature stages (Sauvageau et al., 1994). However, this rule is not universal with some genes showing nearly equivalent levels of expression in both immature and more mature progenitors. For example, HOXA10 is expressed at comparable levels in CD34+ and in cells restricted to the myeloid lineage, while the elevated expression of HOXB3 and HOXB4 in CD34+ cells is downregulated during maturation (Shen et al., 1989; Sauvageau et al., 1994; Pineault, Helgason, Lawrence, & Humphries, 2002). Moreover, HOXB4 is a key factor required for self-renewal of HSCs and a number of the HOXB genes is expressed in natural killer (NK) cells (Thorsteinsdottir, Sauvageau, & Humphries, 1999; Petrini et al., 1992; Hills et al., 2011). In contrast, HOXC4 is silent in CD34+ progenitors, but activated as cells progress to the early stages of T and B maturation (Lawrence et al., 1993). Expression of HOXD cluster genes in HSCs and progenitors has not been detected.

More recent studies have confirmed that the majority of Hox genes (at least 22 of the 39 Hox genes) are expressed in murine CD34+ cells with genes of the A and B clusters being more predominant in immature cells with downregulation being associated with lineage commitment (Grier et al., 2005; Arigropoulos & Humphries, 2007). Mature B- and T-lymphocytes and monocytes express the highest levels of HOXA and HOXC, while expression of HOXD and HOXB is reduced 10 to 100 fold (Morgan & Whiting, 2008). Additional studies have shown that Hoxa cluster genes are predominantly expressed during differentiation and that, overall, expression of the majority of Hox genes (27/39) is decreased during hemopoietic commitment. Only expression of HOXA9 – HOXA13 is maintained in cells throughout all hemopoietic differentiation (Wheadon et al., 2011). To summarize, expression of the majority of Hox genes is restricted to undifferentiated or proliferating progenitor cells, with genes of 5’ HoxA cluster being the exception.

HOXA9 – HOXA13 genes are expressed in both progenitors and lineage committed cells. Studies of hemopoiesis in mice overexpressing Hox genes by retroviral vector infection of hemopoietic cells, or in genetically modified mice by either homologous recombination or pronuclear injection, have provided additional evidence that normal hemopoiesis is sensitive to the levels of expression of Hox genes. Deregulated expression of a single Hox gene can affect one or several hemopoietic lineages. For example, overexpression of HoxB3 resulted in a block in lymphopoiesis, whereas elevated expression of HoxB4 increased self-renewal of HSCs (Sauvageau et al., 1997; Bjornsson et al., 2003). By contrast, overexpression of either HOXA10 or HOXB6 in murine hemopoietic cells resulted in a block in the differentiation of lymphocytes, impaired erythropoiesis, initiation of myeloproliferative disorders and an increased propensity to
leukemia (Lawrence, Sauvageau, Humphries, & Largman, 1996; Owens & Hawley, 2002; Grier et al., 2005; Perkins & Cory, 1993).

In addition, mice lacking the HoxA9 gene showed reduced numbers of granulocytes and lymphocytes, HoxA5 deficient mice showed reduced numbers of granulocytes and monocytes and HoxC8 null mice exhibited impaired erythropoiesis and development of granulocyte/macrophage progenitors (Fuller et al., 1999; Shimamoto et al., 1999; Lawrence et al., 1997). Hox knockout mouse models have also revealed that Hox genes have redundant and cross regulatory functions. For example, enforced overexpression of HoxB4 resulted in a profound expansion of HSCs (Buske et al., 2002; Zhang et al., 2006). However, HoxB4 knockout mice exhibited a subtle reduction in HSCs and progenitor numbers suggesting that other genes compensated for the loss of HoxB4.

Redundancy of Hox genes functions was confirmed in HoxB3/HoxB4 double mutant mice where the phenotype exhibited a pronounced decrease in the numbers of HSC (Brun et al., 2004; Bjornsson et al., 2003). However, these studies indicated that HoxB3 and HoxB4 are required, but not necessary, for normal HSC function and that even after loss of two homeobox genes their function can still be compensated for by the self-renewal function of other Hox genes. Moreover, studies in HoxB1-HoxB9 knockout mice revealed that nearly all Hoxb cluster genes are dispensable for normal hematopoiesis. Thus, HoxB1-HoxB9 knockout HSCs competed with wild-type HSCs in competitive repopulation assays. However, gene expression profiling of HoxB1-HoxB9 null fetal liver cells revealed downregulation of most HoxA genes, and upregulation of three HoxC genes, including the HoxB4 paralogue, HoxC4 (Bijl et al., 2006). Combined, these studies provided evidence that Hox genes are redundant and cross-regulatory mechanisms effectively regulate their functions among clusters in the blood cells of different origin suggesting that deregulation of these regulatory mechanisms may alter expression of the homeobox genes and contribute to malignancy.

1.1.7. Role of the homeobox genes in cancer and leukemia

Normal embryogenesis and neoplasia share many pathways that control pattern formation. For example, deregulation of Wnt and Hedgehog pathways was detected in medulloblastoma, basal cell carcinoma and medulloblastoma (Reya & Clevers, 2005; Taipale & Beachy, 2001). Homeobox genes represent another example of the close relationship between embryo- and cancerogenesis. Numerous homeobox genes have been found to be aberrantly expressed in a
wide range of solid tumours. For example, aberrant expression of nine HOX genes (HOXA10, HOXA11, HOXB8, HOXB9, HOXC8, HOXC10, HOXD10, HOXD11 and HOXD13), was detected in the lung cancers, but not in normal lungs (Tiberio et al., 1994). Downregulation of HOXB5 with a loss of p53 and upregulation of HOXA1 were detected in breast cancer and in cervical cancer, respectively (Raman et al., 2000; Shim, Zhang, Rhee, & Lee, 1998). Overexpressions of HOXB6, HOXB8, HOXC8, HOXC9 and HOXB2, HOXB5 and HOXB9 genes were reported in colorectal and in renal cancer, respectively (Vider et al., 1997; Cillo et al., 1992). Finally, deregulated expression of the cluster C homeobox genes was detected in melanoma (HOXC10, HOXC11, HOXC13) and in prostate cancer (HOXC1-HOXC6, HOXC8) (Cillo et al., 1996; Waltregny, Alami, Clausse, de, & Castronovo, 2002; Miller et al., 2003).

Aberrant expression of homeobox genes is also a frequent event in hemopoietic malignancies. Thus, expression of HOX genes from paralogous groups A, B and C has been detected in normal hematopoietic cells, while expression from all clusters has been demonstrated in leukemic cells. The first demonstration that dysregulated expression of HOX genes was associated with leukemia came from studies in which activation of Hoxb8 resulted in a block in the maturation of the myeloid leukemia cell line, WEHI-3B (Blatt, Aberdam, Schwartz, & Sachs, 1988). Additional studies revealed that coactivation of the interleukin-3 (IL-3) and Hoxb8 provided autocrine stimulation for the myeloid leukemic cells (Perkins, Kongsuwan, Visvader, Adams, & Cory, 1990). The oncogenic potential of the coexpression of Hoxb8 and IL-3 was further confirmed after transplantation of Hoxb8 and IL-3 overexpressed bone marrow progenitors into mice. Thus, mice that were transplanted with murine bone marrow cells overexpressing Hoxb8 alone initiated leukemia after a long latency period, while mice transplanted with bone marrow cells overexpressing both Hoxb8 and IL-3 rapidly developed transplantable myeloid leukemia (Perkins & Cory, 1993).

Further evidence for the involvement of HOX genes in leukemia came from studies involving the BXH-2 mouse strain. These mice spontaneously activate endogenous murine leukemia virus and develop myeloid leukemia with high incidence. Proviral activation of the class I HOX genes, Hoxa7 and Hoxa9, and the noncluster class II homeobox gene Meis1 was discovered in myeloid leukemia cells obtained from terminal BXH-2 mice. These data indicated that Hoxa7 and Hoxa9 cooperate with Meis1 in leukemia formation and provided a first evidence of noncluster homeobox cofactors as accelerators of leukemic transformation (Nakamura, Largaespada, Shaughnessy, Jr., Jenkins, & Copeland, 1996). In addition, coexpression of HOXA7, HOXA9 and MEIS1 has also been detected in human Acute Myeloid Leukemia (AML)
suggesting that this collaboration is relevant to human leukemogenesis (Afonja et al., 2000). Dysregulated expression of \textit{HOXA9} in patients with AML was shown to be strongly correlated with poor prognosis, suggesting a principal role of \textit{HOXA9} in myeloid leukemia development (Sitwala, Dandekar, & Hess, 2008).

Chromosomal translocations generating fusion proteins have been reported in many types of hemopoietic malignancies. For example, as a result of the \textit{t}(7;11)(p15;p15) chromosomal translocation detected in the malignant cells of patients with AML, the nucleoporin gene, \textit{NUP98}, is juxtaposed with \textit{HOXA9} (Nakamura et al., 1996). This fusion oncoprotein, consisting of the N-terminal domain of NUP98 fused to the C-terminal domain of HOXA9 including the homeodomain, exhibits increased protein stability. Enforced expression of \textit{NUP98-HOXA9} in NIH 3T3 fibroblasts was sufficient for anchorage-independent growth on soft agar and transformation (Kasper et al., 1999). The direct involvement of NUP98-HOXA9 in the pathogenesis of AML was demonstrated after transplantation of retrovirally infected bone marrow cells expressing \textit{NUP98–HOXA9}. Recipient mice developed a myeloproliferative disease that eventually progressed to AML after a long latency (Kroon, Thorsteinsdottir, Mayotte, Nakamura, & Sauvageau, 2001). Another translocation, the \textit{t}(2;11)(q31;p15) chromosomal translocation, fusing \textit{NUP98} with \textit{HOXD11} was identified in patient with AML (Taketani et al., 2002). Combined, these findings support the hypothesis that \textit{NUP98} collaborates with class I \textit{HOX} genes in the pathogenesis of AML.

Additional evidence for the involvement of \textit{HOX} genes in leukemia has come from the characterization of translocation breakpoints in lymphoid malignancies. For example, the \textit{t}(1;19)(q23;p13.3) translocation, detected in 25\% of pediatric patients with pre-B cell ALL, results in the fusion of E2A, a protein involved in the regulation of transcription and the class II homeodomain-containing transcription factor PBX1 (Sang et al., 1997). The chimeric protein combines the DNA-binding capacity of PBX1 and the transactivation activity of E2A. The E2A-PBX1 protein acts as a DNA-binding activator of transcription leading to the transcriptional regulation of a new set of genes that normally are not expressed in B cells that eventually results in leukemia (Kamps, Murre, Sun, & Baltimore, 1990). The oncogenicity of the E2A-PBX1 fusion protein was demonstrated after injection of malignant blasts carrying the \textit{t}(1;19)(q23;p13) translocation obtained from patients with pre-B-ALL into \textit{PrkdScid/Scid} mice which rapidly developed B cell Acute Lymphoblastic Leukemia (B-ALL) (Uckun et al., 1993). Additional studies reported that mice transplanted with bone marrow cells retrovirally transduced with an \textit{E2A-PBX1}-expressing vector developed a myeloproliferative disorder while \textit{E2A-PBX1}
transgenic mice rapidly died of CD4+CD8+ immature T cell lymphoma (Kamps & Baltimore, 1993; Dedera et al., 1993).

The molecular mechanisms of E2A-PBX1 in the development of AML and T-ALL in mice remain unclear. It has been suggested that in the case of E2A-PBX1-initiated AML, the E2A-PBX1 fusion protein may compete more successfully than PBX1 for specific DNA recognition sequences which regulate transcription of PBX1 downstream target genes. Alternatively, overexpression of the E2A-PBX1 chimeric protein might reduce the relative amounts of E2A or PBX1 proteins below the level that is required for normal differentiation.

E2A-PBX1 transgenic mice expressed the chimeric E2A-PBX1 homeodomain protein under the control of Eμ immunoglobulin heavy chain enhancer that is active not only in all stages of B cell development, but also in the early stages of T cell development and, it was speculated that this expression of E2A-PBX1 in immature thymocytes contributed to the development of T-ALL. Combined, these data reveal the leukemic potential of the E2A-PBX1 fusion protein in pre-B, myeloid and T-cells.

The transcription factor mixed lineage leukemia (MLL) gene is located at chromosome 11q23 and in fact is a human homolog of the TRX gene, which regulates HOX genes by maintaining transcription in the later developmental stages (Simon & Tamkun, 2002). Its protein includes several domains which are involved in the remodeling of the chromatin structure, in the epigenetic regulation of transcription by methylation and in recruiting chromatin remodeling complexes to specific chromosomal regions (Tkachuk, Kohler, & Cleary, 1992; Dimartino & Cleary, 1999; Nilson et al., 1996). MLL is required for normal hemopoiesis and is frequently involved in translocations associated with the development of leukemia. Chromosomal abnormalities involving 11q23 occur in up to 70% of infant cases of ALL, and in approximately 10% of all other ALL cases (Armstrong & Look, 2005). Leukemic cells carrying translocations involving MLL express surface markers of both the lymphoid and the myeloid lineage. The chromosomal rearrangements fuse N-terminal sequences of MLL with the C-terminus of different genes. The MLL gene has been identified in 73 different translocations. The MLL fusion protein deregulates expression of downstream target genes primarily through upregulation of A-cluster HOX genes which abnormal expression was detected in T- and B-cell ALL (Muntean & Hess, 2012). Furthermore, chromosome translocations juxtaposing MLL-HOXA9 and MLL-MEIS1 have been reported in malignant cells of patients with AML (Wong, Iwasaki, Somervaille, So, & Cleary, 2007). A summary of the impact of homeobox genes function mutations on hematopoiesis is indicated in Table 1.1. Combined, the above studies confirm the
critical role that both class I and class II homeodomain-containing transcription factors play in normal and malignant hemopoiesis.

Table 1.1. A summary of impact of HOX genes mutations on hemopoiesis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Impact of the malfunctioned gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoxb8</td>
<td>Gain</td>
<td>Block in the maturation of the myeloid leukemia cell line</td>
<td>Blatt C et al 1988</td>
</tr>
<tr>
<td>Hoxa1, Hoxa9, Meis1</td>
<td>Gain</td>
<td>Induction of the myeloid leukemia in BXH-2 mice</td>
<td>Nakamura T et al 1996</td>
</tr>
<tr>
<td>HOXA9</td>
<td>Gain</td>
<td>Poor prognosis in patients with AML</td>
<td>Sitwala K et al 2008</td>
</tr>
<tr>
<td>NUP98-HOXA9 fusion</td>
<td>Gain</td>
<td>A myeloproliferative disease and AML</td>
<td>Kroon E et al 2001</td>
</tr>
<tr>
<td>NUP98-HOXD11 fusion</td>
<td>Gain</td>
<td>AML</td>
<td>Taketani T et al 2002</td>
</tr>
<tr>
<td>E2A-PBX1</td>
<td>Gain</td>
<td>Pre B cell ALL, myeloproliferative disorder, T-ALL</td>
<td>Sang B et al 1997</td>
</tr>
<tr>
<td>MLL-HOXA cluster genes fusions</td>
<td>Gain</td>
<td>AML, B cell ALL, T-ALL</td>
<td>Armstrong S et al 2005</td>
</tr>
<tr>
<td>MLL-MEIS1</td>
<td>Gain</td>
<td>AML</td>
<td>Wong P et al 2007</td>
</tr>
</tbody>
</table>
1.2. Lymphoid Development

1.2.1. B cell development

The generation of B lymphocytes from committed progenitor cells is a complex process involving the transit of cells through several critical stages of development. During development different stages of B-cells can be defined by the use of a combination of cell-surface markers and the status of their immunoglobulin heavy chain (Ig\(\mu\)) or light chain (Ig\(\kappa\) or Ig\(\lambda\)) gene rearrangements. B cell development begins in the fetal liver and continues in the bone marrow. Within bone marrow, B cells are represented as pro-B, pre-BI, large pre-BII, small pre-B-II and immature B cells (LeBien, 2000; Ogawa, Ten, & Melchers, 2000).

Early pro-B cells, or pre-pro-B cells, are the earliest B-lineage specified cells and are defined by the phenotype B220\(^+\)CD19\(^−\)c-kit\(^+\)CD43\(^+\)CD25\(^−\)IgM\(^−\)IgD\(^−\). These B-lineage progenitors still retain the potential to differentiate to B-cells, T-cells, dendritic cells (DC), and natural killer (NK) cells. Pre-pro-B cells have not initiated immunoglobulin gene rearrangements. Late pro-B cells are phenotypically similar to the early pro-B cells but they have initiated expression of CD19 (B220\(^+\)CD19\(^+\)c-kit\(^+\)CD43\(^+\)CD25\(^−\)IgM\(^−\)IgD\(^−\)) and progress to the pre-BI cell stage, with cells of this maturation stage being expressed CD19 (B220\(^+\)CD19\(^+\)c-kit\(^+\)CD43\(^+\)CD25\(^−\)IgM\(^−\)IgD\(^−\)). In addition, pre-BI cells have completed Ig rearrangement of the \(D_H\) to \(J_H\) loci and have initiated rearrangement of the immunoglobulin \(V_H\) to \(DJ_H\) that commits cells to the B cell lineage (Ten, Melchers, & Rolink, 1995).

Pre-BI cells which have successfully undergone V(D)J rearrangements of their Ig heavy chain contain Ig\(\mu\) protein in their cytoplasm (c\(\mu\)\(^+\) cells). These cells progress to become large pre-BII cells, which phenotypically are identified as B220\(^+\)CD19\(^+\)c-kit\(^−\)CD43\(^−\)CD25\(^+\)IgM\(^−\)IgD\(^−\). Large pre-BII cells are characterized by V(D)J rearranged heavy chain, but the light chain gene loci remains in the germ line configuration. At the large pre-BII cell stage, cells undergo several rounds of division, with 60%–70% of the cells being in the S, G2 or M phases of the cell cycle (Karasuyama et al., 1994; Nishimoto et al., 1991).

After proliferation, large pre-BII cells differentiate into small pre-BII cells, which have the same cell-surface phenotype as their larger precursors. However, rearranged Ig\(\kappa\) and Ig\(\lambda\) transcripts are detected in small pre-BII cells (Engel, Rolink, & Weiss, 1999).

Pre-BII cells that have undergone a functional Ig light chain rearrangement express the heavy and light Ig proteins on the cell surface. These immature B cells are phenotypically
characterized as B220⁺CD19⁺c-kit⁻CD43⁻CD25⁻IgM⁺IgD⁻. Differentiation to immature IgM⁺ cells is dependent upon the quality of the produced IgL chains. Each IgL chain must be capable of pairing with the IgH chain protein already expressed and reaching the cell surface as a functional B-cell receptor (BCR). Once a functional BCR is expressed at the cell surface, B cells undergo negative selection of the immunoglobulin repertoire by external ligands. IgH/IgL dimer must not form a surface BCR capable of recognizing self-antigens. If autoantigens are recognized, then further development is arrested (Allman, Srivastava, & Lindsley, 2004). Once a non-auto-reactive BCR molecule is expressed, cells leave the bone marrow and migrate to the spleen, where they are called transitional B cells. In the spleen transitional B cells mature into B220⁺CD19⁺c-kit⁻CD43⁻CD25⁻IgM⁺IgD⁺ cells.

1.2.2. T cell development

T cell development is a tightly regulated, multistep process that involves the intrathymic differentiation, proliferation and selection of T cell precursors. The earliest thymocyte progenitors, referred to as early thymocyte progenitors (ETP), arise from the thymus seeding progenitors that migrate from the bone marrow to the thymus, which provides a specialized microenvironment for their specification and maturation. These cells are characterized by high expression of the c-kit receptor and low expression of the interleukin 2α receptor (IL-2R α) also referred to as CD25 (Bhandoola, Sambandam, Allman, Meraz, & Schwarz, 2003). ETPs have the potential to become natural killer cells and dendritic cells when transferred intravenously into an irradiated host and good myeloid potential, but they have minimal potential to differentiate to B cells (Wu, Antica, Johnson, Scollay, & Shortman, 1991; Bell & Bhandoola, 2008). Furthermore, they have lost their ability for self-renewal indicating that they are not hemopoietic stem cells.

Thymocytes are primarily subdivided into double negative (DN), double positive (DP) and single positive (SP) populations based on their expression of the co-receptors CD4 and CD8. The most immature thymocytes lack expression of both CD4 and CD8 and are therefore called double negative (DN) thymocytes. During maturation, immature DN thymocytes pass through four stages, termed DN1, DN2, DN3 and DN4. These stages are defined by differential expression of the CD44 and CD25 receptors. Thus, DN1 thymocytes are CD44⁺CD25⁻, DN2 cells are phenotypically defined as CD44⁺CD25⁺, DN3 stage thymocytes are CD44⁺CD25⁺, while DN4 thymocytes lack expression of both CD25 and CD44 and are defined as CD44⁻
CD25. DN1 thymocytes are multipotent that, in addition to being able to progress in their maturation to the DN2 stage, they still have the potential to differentiate into dendritic cells, natural killers or macrophages (Wu, Antica, Johnson, Scollay, & Shortman, 1991). ETPs comprise a subset of DN1 cells and are most enriched for T-cell potential.

DN2 stage thymocytes or pro-T cells, initiate recombination of the β locus of the T cell receptor. At this stage, progenitors lose the ability to differentiate into B cells, but still support commitment to dendritic cells, and natural killer cells but not macrophages.

There are two kinds of T cells (αβ and γδ) and several models have been proposed to explain how the αβ- versus γδ-lineage decision is made. The earliest model proposed that developing thymocytes are predisposed to develop into γδ-T cells and therefore αβ-T cells will develop only in the case where γ- and δ- chains fail to undergo productive rearrangements (Pardoll et al., 1987). A subsequent model proposed that specification is predetermined and commitment to the αβ-T cell lineage occurs prior to TCR expression (Ishida et al., 1990). A third model proposed that the strength of the signal delivered by the TCR in developing T cells impacts the outcome of the lineage selection process. If the γδ TCR provides a stronger signal, cells differentiate to γδ T cells, whereas weaker signals associated with pre-TCR signaling promote the development of αβ thymocytes (Hayes, Li, & Love, 2005).

During the third stage of DN thymocyte maturation, the progenitors are referred to as early pre-T cells or DN3. CD44CD25+CD3+ thymocytes have three choices, to pass either β- or γδ TCR selections or undergo apoptosis. At this stage, thymocytes complete T cell receptor (TCR) β recombination and a functional TCR-β protein is expressed at the cell surface. At this stage the pre-TCR complex is an 80 kDa protein complex consisting of the TCR-β protein covalently coupled with pre-Tα and the CD3-δ, -ε, -γ and TCR-ζ molecules (Saint-Ruf et al., 1994; Malissen et al., 1995). This process, termed β-selection, selects thymocytes that have undergone productive TCR-β rearrangement and assembly of a surface pre-T cell receptor (pre-TCR) complex and represents a critical checkpoint in thymocyte development. DN3 thymocytes that do not express a functional TCR-β protein at their cell surface are induced to undergo apoptosis. Expression of the pre-TCR complex inhibits recombination of the second TCR-β gene locus, signals the downregulation of expression of CD25, initiates recombination of the TCR-α locus and stimulates the proliferation and continues maturation of DN3 thymocytes (Von & Fehling, 1997).
β-selection begins at the DN3 stage and is completed during the DN4 (CD44−CD25−CD3−) stage. This process is regulated by a complex signal transduction mechanism. Lymphocyte-specific protein tyrosine kinase (Lck), which is associated with the intracellular region of the TCR, mediates recruitment of tyrosine kinases ZAP-70 and Syk to the receptor complex. ZAP-70/Syk phosphorylates the downstream linker for activation of T cells (LAT), the adaptor molecules SLP-76, and PLCγ1. This results in Ca2+ mobilization and the activation of protein kinase C (PKC) and Ras which then activates expression of genes involved in cell survival, differentiation and proliferation (Michie & Zuniga-Pflucker, 2002).

DN4 thymocytes progress to the double positive CD4+CD8+CD3lo (DP) thymocyte stage. DPs represent approximately 85% of all thymocytes. During this stage, rearrangement of TCRα is completed and the pre-TCR is replaced with the αβ TCR complex (Love & Bhandoola, 2011). When the αβ TCR complex is expressed, cells upregulate CD3. DP thymocytes move into the thymic cortex, where they will undergo positive selection via interaction with MHC with self-antigens expressed by thymic cortical epithelial cells. Positively selected cells move to the medullar area, where they undergo negative selection.

In general, both positive and negative selections can be described as the selection of thymocytes having the ability to interact with self-ligands with moderate affinity. Positive selection occurs if the TCR can interact with MHC class I or class II molecules. If it can, the DP thymocyte is positively selected. Negative selection selects thymocytes expressing a TCR that interacts with self-antigens. If there is a strong affinity/avidity for the antigens, the cell undergoes apoptosis (Starr, Jameson, & Hogquist, 2003; Love & Bhandoola, 2011). After positive and negative selection, αβ-T cells mature into CD4+CD3high or CD8+CD3high single positive (SP) T cells comprising the majority of functional mature thymocytes. In addition, the continued development of T cells along the αβ-lineage fate requires constant Notch signals, while the adoption of the γδ-lineage fate does not (Ciofani, Knowles, Wiest, Von, & Zuniga-Pflucker, 2006).

Mature thymocytes are either CD4+ or CD8+ single positive T cells. CD4+ thymocytes, also referred to as helper T cells, consist of several subsets, with each subset having a specific function. These subsets are Th1, Th2, Th3, Th17, induced regulatory T and TR1 cells. Briefly, Th1 cells are critical for immunity to intracellular microorganisms and Th2 cells are involved in immunity against extracellular pathogens. Abnormal activation of Th1 cells results in autoimmune diseases, while over reactivity of Th2 cells causes asthma (Mosmann & Coffman, 1989; Paul & Seder, 1994). Th3 and TR1 cells produce growth factor β (TGF-β) and IL-10,
respectively (Chen, Kuchroo, Inobe, Hafler, & Weiner, 1994; Groux et al., 1997). Th17 cells are involved in protection against bacteria and fungi (Weaver, Harrington, Mangan, Gavrieli, & Murphy, 2006). Induced regulatory T cells are a component of the immune system which performs a negative regulation of other immune cells (Shevach, 2000).

CD8$^+$ T cells, which are also referred to as cytotoxic cells, induce the death of malignant cells and virus infected cells. The TCR expressed by CD8$^+$ T cells recognizes the foreign antigens that initiate clonal proliferation. As a result of clonal proliferation, antigen-specific T cells rapidly increase and become more than 50% of splenic CD8$^+$ T cells (Busch, Pilip, Vijh, & Pamer, 1998). Activated CD8$^+$ T cells eliminate cells expressing foreign antigens using two molecular pathways: the granule exocytosis pathway, which is dependent on the pore forming molecule perforin, or by upregulation of FasL (CD95L), which initiates apoptosis of target cells (Berke, 1995). Once cells expressing the foreign antigen have been eliminated, some of the T cells involved in the immune response become memory T cells but the majority undergo apoptosis (Murali-Krishna et al., 1998).

### 1.3. T-Cell Acute Lymphoblastic Leukemia

#### 1.3.1. Introduction

Acute Lymphoblastic Leukemia (ALL) is a malignant disorder of lymphoid progenitor cells blocked at a specific stage of maturation which is characterized by uncontrolled cell proliferation (Sawyers, Denny, & Witte, 1991). ALL is the most common childhood cancer affecting children younger than 19 years of age. The World Health Organization (WHO) currently recognizes three main ALL subgroups: B-ALL not specified, B-ALL with recurrent genomic abnormalities and T-ALL. In addition, ALLs are further classified based on the degree of differentiation of the lymphoblasts according to immunophenotype (Paolini et al., 2011). The disease has a relatively good prognosis for children with up to 80% patients being relapse-free. However, less favorable outcomes occur for adults although the recent chemotherapy regimes have resulted in up to 50% long-term complete remission for adults (Bassan et al., 2009; Bassan & Hoelzer, 2011).

ALL originates from various genetic lesions that occur in the bone marrow progenitors committed to differentiate to the T-cell or B-cell lineage. As a result of these lesions, cells acquire the capacity for unlimited self-renewal and lose the potential to differentiate. Cancer
initiating mutations can also occur in hemopoietic stem cells with mutations blocking cell maturation and differentiation (Pui, Robison, & Look, 2008). Clonal expansion or accumulation of transformed immature B- or T-cells in the bone marrow and dissemination of tumour cells to distant organs via the blood and lymph vessels result in the suppression of normal hemopoiesis and malfunction of these organs.

The etiology of ALL is largely unknown. A minority of cases (<5%) is associated with exposure to carcinogens including pesticides, solvents or chemicals, environmental factors including ionizing or non-ionizing radiation or with an inherited predisposing genetic syndromes. Pesticides, solvents or chemicals are the most common risk factors associated with paediatric ALLs (Freedman et al., 2001; Zahm & Ward, 1998). The relationship between the degree of ionizing radiation and childhood leukemia is well established. The risk of leukemia development is dependent on the dose of radiation and the duration of exposure (Miller, 1967). Epidemiologic studies revealed an association between non-ionizing electromagnetic fields and childhood leukemia. For example, pooled analysis of records of nine studies indicated that for children who were exposed with residential magnetic field ≥0.4μT during the year prior to diagnosis had an increased relative risk of developing ALL (Ahlbom et al., 2000).

Genetic predispositions also account for some cases of ALL. For example, 4%-5% of ALL cases are associated with Down’s syndrome, Bloom’s syndrome ataxia-telangiectasia and Nijmegen breakage syndrome (Pui, Robison, & Look, 2008). Retrospective analyses of archived neonatal blood and studies of leukemia in twins have identified leukemia-specific fusion genes, hyperdiploidy or translocations involving immunoglobulin or T-cell-receptor loci. These studies suggest the possibility that prenatal predisposition accounts for some cases of childhood leukemias (Maia et al., 2003). However, variable latency periods and clinical outcomes indicate the requirement of additional postnatal mutations trigger leukemic transformation. Other factors increasing the risk for childhood ALL initiation include high birth weight, parental occupation, parental tobacco or alcohol use, maternal diet and prenatal vitamin use (Hjalgrim et al., 2003; Buffler, Kwan, Reynolds, & Urayama, 2005).

In contrast to the etiology, the pathogenesis of many ALL subtypes has been well-studied and genetic abnormalities as well as several deregulated molecular pathways contributing to the leukemic phenotype have been identified. For example, chromosomal translocations represent the most common genetic abnormalities in ALL and, according to the WHO classification, are a defining signature of both T- and B-cell ALL. The most common recurrent translocations identified in patients with B-ALL are t(12;21)(p13;q22),
t(9;22)(q34;q11), t(1;19)(q23;p13) and translocations involving the MLL gene, located at 11q23 and any one of a number of different fusion partners (Pui, Robison, & Look, 2008; Radich, 2001; Meyer et al., 2005; Piccaluga et al., 2006). Aneuploidy and cooperating mutations also play important roles in the progression of B cell leukemia (Moorman et al., 2003; Mullighan et al., 2007).

1.3.2. Leukemia initiating cells (L-ICs)

Leukemias arise from the malignant transformation of hemopoietic stem cells or more mature progenitors through the accumulation of genomic mutations and chromosomal rearrangements conferring a selective advantage in growth control, resistance to apoptosis and a block in differentiation. Leukemia initiating cells (L-ICs) are functionally defined by their ability to initiate tumours in xenografts or immunodeficient mice with high efficiency. Similar to normal HSC, L-ICs exhibit unlimited self-renewal potential and the capacity to generate a variety of tumour subpopulations. However, unlike highly regulated stem cells, L-ICs exhibit uncontrolled self-renewal and produce daughter cells which are blocked at immature stages of differentiation. Although L-ICs comprise only a small subset of bulk tumour cells, they are responsible for leukemia initiation, progression and metastasis. This is in contrast to the bulk tumour cells which are characterized by limited proliferation potential and inability to form a new tumour at distant organs (Ward & Dirks, 2007; Reya, Morrison, Clarke, & Weissman, 2001; Zhou et al., 2009). In terms of the mechanism, two types of events which may generate L-ICs were proposed. The first type is when mutations and other genetic alterations allow to bypass the restraints of growth and apoptosis characteristic of normal stem cells and to block differentiation of HSC progeny. The second type of events may occur during differentiation, when progenitors are arrested in their immature state and restore self-renewal ability characteristic of HSC.

The L-IC model was first suggested by Dick et al. in studies with human Acute Myeloid Leukemia (AML) (Bonnet & Dick, 1997; Hope, Jin, & Dick, 2004). In series of transplantation assays of flow‐sorted AML cells into NOD/SCID mice, they showed that most L-ICs resided within the CD34⁺CD38⁻ fraction and were capable of inducing leukemia, whereas recipients of the more mature CD34⁺CD38⁺ tumour fraction remained healthy for the observation period. More recent data, however, demonstrated the ability of CD34⁺CD38⁺ as well as CD34⁻CD38⁺ cells to initiate AML after transplantation into femur of NOD/SCID/γ2 null recipients pretreated with injection of anti-CD122, which depletes natural killers and macrophages (Taussig
et al., 2008; Taussig et al., 2010). Some of these populations appear to be susceptible to a particular type of genetic alteration and were associated with specific disease subtypes. For instance, only CD34+CD38- blasts were able to initiate BCR-ABL-positive B-cell ALL in NOD/SCID mice (Cobaleda et al., 2000). Similarly, CD34+CD38<sup>low</sup>CD19+ leukemic blasts carrying TEL-AML fusion produced B-cell leukemia in the immunodeficient mice (Castor et al., 2005; Hong et al., 2008). Consistent with the low frequency of L-ICs detected in AML, B-cell ALL initiating cells comprised a minority (less than 1%), of the bulk tumour cells as shown by their ability to maintain long-term cultures of B acute lymphoblastic leukemia cells and by serial transplantations of flow-sorted malignant cells in NOD/SCID recipients (Cox, Diamanti, Evely, Kearns, & Blair, 2009). Subsequent studies demonstrated, however, that similar to AML, B-cell leukemia-inducing cells were found within CD34+CD19-, CD34+CD19+ and CD34-CD19+ populations, suggesting their heterogeneity (Le et al., 2008).

Detection of the T-cell Acute Lymphoblastic Leukemia Initiating Cell (T-ALL-IC) was hampered for a long time by difficulties in maintaining primary cultures of T-ALL cells and due to poor engraftment of human T cells in mice. Therefore, the immunophenotype of T-ALL-ICs has not been well characterized. Although first reports described CD34+CD4- and CD34+CD7- cells derived from human flow-sorted T-ALLs as a potential source of T-ALL-ICs (Cox et al., 2007), other groups experienced difficulties both in expanding T-ALL cells in vitro under similar conditions and in demonstrating the existence of T-ALL-ICs in immunodeficient mice (Ma et al., 2002; Coustan-Smith et al., 2009). These limitations indicated requirements for more reliable assays and implementation of more stringent flow-sorting criteria. More recently, Campana et al. reported that pediatric T-ALL-ICs exhibited an early thymocyte cell surface phenotype (CD1a<sup>-</sup>, CD8<sup>-</sup>, CD5<sup>lo</sup>), along with expression of the stem cell and myeloid antigens, CD117 and CD11b, respectively, indicating that the L-IC may be progenitors migrating from the bone marrow to the thymus (Steele et al., 1997). This is also in agreement with other reports demonstrating that T-ALL-ICs reside within the very immature c-kit<sup>+</sup> leukemic cell fractions (Guo et al., 2008). Finally, Dick et al. reported that the CD7<sup>-</sup>CD1a<sup>-</sup> cell subpopulation derived from human T-ALL patients were able to expand in OP9-DL1 stromal co-cultures, and engraft and initiate T-ALL in NOD/SCIDIL2Rγ<sup>null</sup> recipients (Chiu, Jiang, & Dick, 2010).

The influence of genetic abnormalities on thymic progenitors with enhanced disease potential was assessed by using various T-ALL mouse models. For instance, Pten inactivation in HSCs resulted in the enrichment of c-kit<sup>+</sup>CD3<sup>+</sup>Lin<sup>-</sup> T-ALL-ICs and T-ALL development (Guo et al., 2008). Interestingly, the degree of dephosphorylation of β-catenin in c-kit<sup>+</sup>CD3<sup>+</sup>Lin<sup>-</sup>
cells was significantly increased, with conditional inactivation of β-catenin significantly reducing the incidence of T-ALL, suggesting a role for β-catenin in generating T-ALL-ICs. Using the LMO2 transgenic mouse model in which this transcription factor was constitutively expressed in thymocytes, an increase in self-renewal of preleukemic thymocytes was demonstrated. This suggests that LMO2 supports the self-renewal of T-ALL-IC, thereby allowing the accumulation of genomic abnormalities in immature T cells and facilitating malignant transformation (McCormack et al., 2010). Similarly, LMO1 or its nuclear partner SCL/TAL1 induced proliferation of primitive thymocyte progenitors in transgenic mice and increased numbers of self-renewing T-ALL-ICs (Tremblay et al., 2010). Interestingly, 75% of TAL1/LMO2 transgenic mice developing T-ALL also exhibited spontaneous mutation of Notch. It is becoming increasingly clear that Notch plays an important role in T-cell transformation, since inhibition of Notch by γ-secretase reduced the frequency of the thymic progenitor T-ALL-IC population (Tatarek et al., 2011). (Notch1 and its role in the development of thymocytes and T-ALL will be discussed in the 1.4. section). Altogether, these studies support the existence of the T-ALL-IC, but a clearer understanding of the precise origin, immunophenotype and major genetic aberrations generating T-ALL-IC remain to be elucidated.

1.3.3. T-cell Acute Lymphoblastic Leukemia

T-cell Acute Lymphoblastic Leukemias (T-ALL) is a lymphoid malignancy characterized by the accumulation of immature lymphopoietic blast cells in the bone marrow (Pui, Relling, & Downing, 2004). It is often associated with an enlarged thymus and with early spread to the cerebrospinal fluid. T-ALLs comprise about 25% of all cases of ALL and affect mostly children and adolescents (more boys than girls). When treated with combination chemotherapy, the clinical outcomes for T-ALL are less favourable for T-ALL than for B-ALL (Uckun et al., 1997; Pui & Evans, 2006). The difficulty in treating T-ALL is due, in part, to the heterogeneity of the disease and the differential responsiveness of the different forms of T-ALL to chemotherapy. Among several classifications, the most commonly used is the European Group for Immunological Characterization of Leukemias (EGIL) classification. EGIL is based on the expression on T-lymphoblasts of markers specific for the stages of normal T cell development. In this classification scheme, thymic blasts are categorized into four subgroups. The first, defined as TI or the pro T-ALL subgroup, consists of the most immature thymic blasts and includes cells that express the CD7 marker. The second subgroup, TII or pre T-ALL, is
composed of less immature thymic blasts and includes cells that coexpress a combination of CD2, CD5, and/or CD8. The third subgroup is TIII or cortical T-ALL. Cells which are included in this subgroup also express CD1a. The fourth subgroup, termed TIV or mature T-ALL, includes cells which are positive for surface expression of CD3 and lack expression of CD1a (Bene et al., 1995). The ability to classify T-ALL subtypes was enhanced by the ability to identify non-random, clonal karyotypic abnormalities in 66%-78% of T-ALL patients (Douet-Guilbert et al., 2004; Johansson, Mertens, & Mitelman, 2004). Classification of mouse lymphoid neoplasms according to their clinical features, morphology, histopathology and genetic features was proposed in Bethesda (Herbert C. Morse III et al., 2002). While recognizing species specific differences, this classification has numerous similarities to the classification of human lymphoid tissues.

T-ALL is commonly associated with genomic abnormalities resulting in the dysregulated expression of oncogenic transcription factors in thymocyte progenitors. The most common abnormalities are chromosome rearrangements involving the α- and δ- T cell receptors (TCR) loci at 14q11.2, the β-TCR at 7q35, and the γ-TCR located on the short arm of chromosome 7 at band p14-15 (Paolini et al., 2011). These translocations place transcription factors under the regulatory control of the TCR. Translocations juxtaposing transcription factors with TCR loci have been identified in 75% of all T-ALL cases.

Other chromosomal abnormalities found in malignant cells of patients with T-ALL include duplication or deletion of chromosomes (aneuploidy), translocations resulting in the creation of fusion proteins, deletion, duplication or amplification of genes and activating or inactivating genetic mutations. Genomic imbalances due to changes in chromosome numbers are a common characteristic of T-ALL. Thus, aneuploidy was detected in 20% of biopsies obtained from T-ALL patients, with hyperdiploidy being detected in 15% and hypodiploidy being detected in 5% of T-ALL patients (Douet-Guilbert et al., 2004). Genomic deletions were also defined as T-ALL initiation events. These deletions typically lead to the loss of function of tumour suppressor genes. For example, deletion on chromosome 9 results in the loss of the tumour suppressor gene CDKN2A and two other related genes, p14/ARF and p15/CDKN2B. These genes encode critical factors involved in the regulation of cell cycle and apoptosis, with their deletion leading to loss of control cell cycle of at the G1 stage (Kitagawa et al., 2002). Chromosomal deletions may also result in ectopic expression of transcription factors acting as oncogenes. Thus, a deletion on chromosome 11 (del(11)(p12p13)) results in loss of a negative regulatory region upstream of the transcription factor LMO2, causing activation of the proximal
LMO2 promoter. Activated LMO2 has been detected in about 4% of paediatric T-ALL patients (Van et al., 2006). Another transcription factor, TAL1, is activated as a result of a genomic 90 kb deletion (del(1)(p32)) in up to 30% of T-ALLs. This deletion disrupts TAL1 in its 5' portion and juxtaposed TAL1 to the 5' part of SIL, thereby forming a SIL-TAL-1 chimeric gene (Janssen, Ludwig, Sterry, & Bartram, 1993).

Duplications and amplifications of genes have also been identified in T-ALL. For example, duplication of MYB occurs in up to 9% of paediatric cases of T-ALL while the amplification of an ABL1 variant fusion gene, NUP214-ABL1, is detected in the malignant cells of 6% of adult patients with T-ALL (Clappier et al., 2007; Graux et al., 2004). Activating or inactivating mutations are often associated with other genomic abnormalities. For example, activating mutations of the NOTCH1 signalling pathway, which plays a critical role during the early stages of T-cell development, are commonly detected in both paediatric and adult forms of T-ALL (Weng et al., 2004). Mutations or homozygous deletion of FBW7, an ubiquitin ligase implicated in NOTCH turnover, were detected in 8% of primary T-ALL. Activation of NOTCH is associated with a favourable prognosis (Asnafi et al., 2009). Inactivating mutations of the X-linked PHF6 gene, a gene which acts as a tumour suppressor gene, are detected in 16% of paediatric and in 38% of adult cases of T-ALL (Van et al., 2010). Table 1.2 provides a list of many of the frequent genetic abnormalities associated with T-ALL.
### Table 1.2. Most frequent genetic abnormalities in T-ALL

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Gene Locus</th>
<th>Gene Product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(10;14)(q24;q11)</td>
<td>HOX11;TCRδ</td>
<td>homeodomain-containing transcription factor</td>
<td>Dube et al., 1991</td>
</tr>
<tr>
<td>t(7;10)(q35;q24)</td>
<td>HOX11;TCRα</td>
<td>homeodomain-containing transcription factor</td>
<td>Hatano et al., 1991</td>
</tr>
<tr>
<td>t(11;14)(p15;q11)</td>
<td>LMO1;TCRδ</td>
<td>LIM-domain only protein</td>
<td>McGuire et al., 1989</td>
</tr>
<tr>
<td>t(11;14)(p13;q11)</td>
<td>LMO2; TCRδ</td>
<td>LIM-domain only protein</td>
<td>Royer-Pokora 1991</td>
</tr>
<tr>
<td>t(1;14)(p32;q11)</td>
<td>TAL1; TCRδ</td>
<td>bHLH transcription factor</td>
<td>Begley et al., 1989</td>
</tr>
<tr>
<td>t(7;9)(q34;q32)</td>
<td>TAL2; TCRβ</td>
<td>bHLH transcription factor</td>
<td>Xia et al., 1991</td>
</tr>
<tr>
<td>inv(7)(p15q32)</td>
<td>HOXA; TCRβ</td>
<td>homeodomain-containing transcription factor</td>
<td>Speleman et al., 2005</td>
</tr>
<tr>
<td>t(7;19)(q23;p13)</td>
<td>LYL1; TCRβ</td>
<td>bHLH transcription factor</td>
<td>Millentin et al., 1989</td>
</tr>
<tr>
<td>t(5;14)(q35;q32)</td>
<td>HOX11L2; BCL11B</td>
<td>homeodomain-containing transcription factor</td>
<td>Bernardal et al., 2001</td>
</tr>
<tr>
<td>t(1;14) or del1(p32)</td>
<td>SIL-TAL1</td>
<td>fusion protein</td>
<td>Bash et al., 1995</td>
</tr>
<tr>
<td>9(q34) episomes</td>
<td>NUP214-ABL1</td>
<td>fusion protein</td>
<td>Graux et al., 2004</td>
</tr>
<tr>
<td>t(10;11)(p13;q14)</td>
<td>CALM-AF10</td>
<td>fusion protein</td>
<td>Berger et al., 1989</td>
</tr>
<tr>
<td>t(11;19)(q23;p13)</td>
<td>MLL-ENL</td>
<td>fusion protein</td>
<td>Rubnitz</td>
</tr>
<tr>
<td>Genetic Aberration</td>
<td>Gene</td>
<td>Function</td>
<td>Year</td>
</tr>
<tr>
<td>-------------------</td>
<td>----------</td>
<td>---------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>del(9)(p21)</td>
<td>CDKN2A</td>
<td>cyclin-dependent kinase</td>
<td>1996</td>
</tr>
<tr>
<td>dup(6)(q23)</td>
<td>MYB</td>
<td>transcription factor</td>
<td>2002</td>
</tr>
<tr>
<td>mutation</td>
<td>NOTCH1</td>
<td>transmembrane receptor</td>
<td>2007</td>
</tr>
<tr>
<td>mutation</td>
<td>FBW7</td>
<td>ubiquitin ligase</td>
<td>1991</td>
</tr>
<tr>
<td>mutation</td>
<td>JAK1</td>
<td>kinase</td>
<td>2007</td>
</tr>
<tr>
<td>mutation</td>
<td>PTEN</td>
<td>tumour suppressor</td>
<td>2008</td>
</tr>
<tr>
<td>mutation</td>
<td>p53</td>
<td>tumour suppressor</td>
<td>2009</td>
</tr>
<tr>
<td>mutation</td>
<td>PHF6</td>
<td>transcription factor</td>
<td>2010</td>
</tr>
</tbody>
</table>

This table was modified from Graux C. 2011 (Graux, 2011).

1.4. NOTCH1

1.4.1. Introduction

As was discussed in the T cell Acute Lymphoblastic Leukemia section and was shown in Table 1.2, there are a large variety of genetic aberrations that contribute to the transformation of T cells. Gene expression profiling has facilitated the classification of T-ALL patients into subgroups reflecting genomic defects. Some of these subgroups exhibit unique aberrations, whereas other aberrations are shared among several groups (Ferrando et al., 2002). Hyperactivation of NOTCH1 has been found in more than 50% of patients with T-ALL, indicating the importance of this pathway in the pathogenesis of this disease (De et al., 2010).
The function of NOTCH1 during T lymphocyte development and its role in leukemogenic progression will be discussed in this section.

### 1.4.2. Notch1

The Notch signaling pathway is an evolutionarily conserved signaling pathway that is active in many cell types at different stages during development. Mutation of genes involved in this pathway leads to diseases in various organs and tissues supporting the critical role of Notch signaling in various cell types (Andersson, Sandberg, & Lendahl, 2011). As such, mice haploinsufficient for Notch1 exhibit supernumerary hair cells in the inner ear (Zhang, Martin, Kelley, & Gridley, 2000). Haploinsufficiency of NOTCH1 in humans was detected in aortic valve disease and haploinsufficiency of NOTCH2 was observed in Alagille syndrome (Garg, 2006; McDaniell et al., 2006).

Notch signaling is mediated by Notch ligands and Notch receptors. Notch ligands are single-pass transmembrane proteins with a DSL (Delta-Serrate-LAG-2) domain and varying numbers of EGF-like repeats. In mammals, there are five canonical ligands Jagged-1, Jagged-2, DLL1, DLL3 and DLL4 which are divided into two classes: the Delta/Delta-like and the Serrate/Jagged class. The Jagged-1 and Jagged-2 ligands are distinct from DLL1-DLL4 ligands and are united in the same class due to presence of additional cysteine rich repeats domain close to the transmembrane domain. These ligands that bind to the four Notch receptors are referred to as Delta-like 1–4 (DL1-DL4).

The Drosophila Notch receptor has an extracellular domain (186 kDa) and a smaller intracellular domain (ICD) approximately 100 kDa. The extracellular domain consists of 36 contiguous epidermal growth factor (EGF) repeats that mediate ligand binding. A distinctive feature of these repeats is the presence of six conserved cysteine residues. In addition, Notch receptors have LNR motifs and the heterodimerization domain (Wharton, Johansen, Xu, & Artavanis-Tsakonas, 1985). The Notch ICD is a monomer containing two domains. One is a CDC10 ankyrin repeat domain (ANK), which interacts with CSL and the other domain is a PEST domain which is enriched in proline, glutamate, serine and threonine residues. PEST is indispensable for the ICN degradation (Kopan, 2002).

Briefly, Notch1 is synthesized in the endoplasmic reticulum (ER) as a single protein (pre-Notch), which is transported to the Golgi network, where it undergoes post-translational modification. After cleavage, pre-Notch1 is converted to a heterodimeric receptor which is
transported to the plasma membrane. Notch signaling is activated by binding of Notch ligands to the Notch receptor. The binding results in the cleavage of Notch receptor by the extracellular ADAM proteinase and γ-secretase complex. This leads to the removal of the extracellular domain of Notch, which continues to interact with the ligand. The ligand and the Notch1 extracellular domain are endocytosed by the ligand-expressing cell and targeted for lysosomal degradation. The intracellular domain of Notch1 (ICN) translocates to the nucleus where it binds the DNA-binding transcriptional repressor CSL (CBF1/Suppressor of Hairless/LAG-1). Binding of ICN to CBF1 induces the dislocation of the Mint corepressor and the recruitment of the Mastermind mastermind-like 1 (MAML1) co-activators. MAML1 binds to ICN in the ICN-CSL-DNA complex, thereby converting the CSL repressor into the transcriptional activator. Disassembly of the ICN-CSL-DNA complex results in ubiquitination and proteosomal degradation of ICN by the E3 ubiquitin ligase Fbw7, which targets the PEST domain on the C terminal of ICN (Fryer, White, & Jones, 2004).

To date, several gene targets of Notch1 signaling involved in T lymphopoiesis have been identified. One target, Hairy/Enhancer of Split (HES), belonging to the family of bHLH-type transcriptional repressors, is important in T cell lineage commitment. Notch1 signaling activates HES which leads to repression of HES target genes (Wendorff et al., 2010). Another Notch1 signaling target gene is Ptcra (Smelty et al., 2010). The Ptcra protein, together with TCRβ and CD3, forms the pre-T-cell receptor complex, which regulates early T-cell development. Expression of CD25 in primary T cells is also targeted by Notch1 (Adler et al., 2003).

Notch1 inactivation causes a complete block in T lineage development, indicating that other Notch family members can not compensate for the loss of Notch1 in thymocyte development (Radtke et al., 1999). Data obtained from experiments with transgenic mice demonstrated that bone marrow–derived ETPs must activate Notch1 soon after entering the thymus to suppress their B cell potential and undergo T lineage specification. For example, incomplete inhibition of Notch1 activity in ETPs resulted in development of both B and T cells in the thymus (Tan, Visan, Yuan, & Guidos, 2005). Complete ablation of Notch1 in bone marrow precursors led to the development of B rather than T cells in the thymus (Radtke et al., 2000; Koch et al., 2001). Moreover, deletion of Notch1 in DN3 thymocytes (after loss of B cell potential) impairs TCR-β selection and production of double-positive (DP) thymocytes, indicating the necessity of Notch1 signaling after T cell commitment has taken place (Wolfer, Wilson, Nemir, MacDonald, & Radtke, 2002). Conversely, experiments with cell lines demonstrated that overexpression of the Notch1 intracellular domain or the culture of lymphoid
progenitors with bone marrow stromal cells expressing the Notch ligand, Delta-like 1, inhibited B cell development and promoted thymus-independent development (Pui et al., 1999; Schmitt & Zuniga-Pflucker, 2002). Combined these data indicate that signaling through the Notch1 receptor at the earliest stages of thymocyte development is an absolute requirement for progenitors to choose a T cell fate in the thymus.

Other Notch receptors, including Notch-2, and Notch-4, are expressed in endothelial cells suggesting a role in vascular development (Liu et al., 2003). Expression of Notch4 was also detected in human bone marrow CD34+ and CD34- cell populations (Karanu, Yuefei, Gallacher, Sakano, & Bhatia, 2003). Mice deficient in Notch2del1/Notch2del1 exhibited severe defects in vasculature development in the glomerulus, heart, and eye and embryos were not viable (McCright et al., 2001). In contrast, high levels of Notch2 expression caused T-cell leukemia in mice, whereas low levels of Notch2 expression enhanced the frequency of the CD8+ thymocyte subpopulation indicating its role in thymocyte development (Witt, Hurez, Swindle, Hamada, & Klug, 2003). T-ALL also developed in the mice with a constitutively activated Notch3 intracellular domain expressed in thymocytes (Bellavia et al., 2002). Given the importance of Notch signaling during organogenesis including T cell development, it is not surprising that mutations in genes encoding Notch signaling components have been implicated in a wide spectrum of tumours and in T cell acute lymphoblastic leukemia.

1.4.3. Role of the Notch1 in the development of solid tumours and leukemias

Recent application of the whole genome sequencing to various human cancers has detected somatic mutations in NOTCH1 receptor genes in various tumours expanding the role of NOTCH1 in tumorigenesis. For example, numerous data indicated overexpression of NOTCH1 in breast cancer accompanied by deregulated expression of H-RAS and MYC (Weijzen et al., 2002; Robinson et al., 2011; Klinakis et al., 2006). Two independent groups identified NOTCH1 loss-of-function in the head and neck squamous cell carcinomas (Demehri, Turkoz, & Kopan, 2009; Stransky et al., 2011). Truncating NOTCH1 mutations were also detected in primarily cutaneous and lung squamous cell carcinomas (Wang et al., 2011). Finally, NOTCH1 gain-of-function mutations were reported in B cell chronic leukocytic leukemia and mantle cell
lymphoma cased by PEST degron deletion and NOTCH1 locus demethylation, respectively (Di et al., 2009; Sportoletti et al., 2010; Kridel et al., 2012).

Although NOTCH2 is expressed in HSC and NOTCH3 is abundant in the thymus, only mutations in NOTCH1 have, to date, been reported in T-ALL. Until recently, evidence for the role of Notch1 in the development of T-ALL was limited to studies reporting the presence of the t(7,9)(q34;q34.3) translocation. This chromosomal translocation juxtaposed the TCRβ enhancer to a region of intron 24 of the NOTCH1 gene thereby resulting in the expression of a truncated NOTCH1 protein, TAN1. TAN1 functions as a constitutively active form of NOTCH1 (Ellisen et al., 1991). The oncogenic potential of TAN1 was confirmed in mice transplanted with TAN1-expressing hemopoietic progenitor cells. Half of these mice developed immature T-cell leukemia with immunophenotypes ranging from Thyl.2+CD4−CD8−DN cells to predominantly Thyl.2+CD4−CD8+DP cells (Pear et al., 1996). Detection of gain-of-function mutations in NOTCH1 found in more than 50% of human T-ALLs, revealed the prominent role of aberrant NOTCH1 expression in the pathogenesis of T-ALL (Weng et al., 2003). The majority of these mutations cluster in two regions either at the PEST or the heterodimerization domain of NOTCH1. PEST mutations cluster at the C-terminus of the NOTCH1 receptor resulting in the deletion of the PEST domain which normally regulates ICN degradation (Chiang et al., 2006). These mutations lead to increased stabilization of the ICN and, as a result, increased activity of NOTCH1. Another region of NOTCH1 mutations was detected within exons 26 or 27, which encode the N and C-terminal heterodimerization domain subunits. Mutations within this region consist of insertions or deletions that maintain the reading frame and induce ligand-independent NOTCH1 signaling (Malecki et al., 2006). In addition, a small proportion of primary human T-ALLs carry mutations in both the heterodimerization and PEST domains (Weng et al., 2004).

Notch signaling has been shown to regulate the expression of target genes that support the growth of malignant T cells. NOTCH1 upregulates expression of IL7Ra, an important mediator of thymocyte proliferation and down-regulates the tumour suppressor gene PTEN via HES1 (Gonzalez-Garcia et al., 2009; Palomero et al., 2007a). Overexpression of NOTCH1 in immature thymocytes inhibits E2A, suggesting that loss of E2A may contribute to the development of T-ALL. The finding that a high proportion of E2A-deficient mice developed T-ALL supports the hypothesis that downregulated activity of E2A by NOTCH1 contributes to the initiation of T-ALL (Bain et al., 1997). NOTCH1 also directly binds to the promoter region of cyclin D3 which is highly expressed in Notch1-dependent leukemic mice (Joshi et al., 2009). Since cyclin D3 promotes progression of cells through G1/S checkpoint of the cell cycle, it has
been suggested that Notch1 targeted expression of cyclin D3 contributes to T-ALL by
deregulating this cell cycle check point. Combined, the above studies indicate that aberrant
activation of Notch1 signaling and the dysregulation of NOTCH1 target genes play a critical
role in the etiology and pathogenesis of T-ALL.

1.5. \textit{Prkdc}^{Scid/Scid} mice as a model to study role of NHEJ proteins deficiency in leukemia initiation

1.5.1. Introduction

Since \textit{in vivo} experimentation on humans is constrained by ethical concerns, mice provide an
alternative, cost-effective, highly reproducible model to study factors related to the development
of hemopoietic malignancies and the preclinical efficacy of potential therapies for these
diseases. Moreover, the ability to manipulate the genomes of mice to generate mice with
targeted mutations or deregulated expression of specific genes has provided important insight
into many of the genes responsible for hemopoietic malignancies. In this section I will discuss
CB17-Prkdcscid (here and after \textit{Prkdc}^{Scid/Scid}) mice. These mice are deficient in the DNA-PK
catalytic subunit (DNA-PKcs) which ligates double strand DNA breaks (dsDNA breaks)
initiated during V(D)J recombination. To accomplish my project, \textit{Prkdc}^{Scid/Scid} mice were used
to generate double mutant mice deficient in DNA-PK and expressing elevated levels of the class
II homeobox gene, TLX1, in lymphocytes.

1.5.2. V(D)J recombination

V(D)J recombination is the lymphoid-restricted rearrangement process of gene segments
(variable [V], diversity [D] and joining [J]), which form the variable domains of
immunoglobulin (Ig) and T-cell receptor (TCR) gene loci. V(D)J recombination is initiated
when the recombination activating genes, \textit{Rag}1 and \textit{Rag}2, recognize and bind to recombination
signal sequences (RSS) flanking all V, D and J gene segments. RSS are composed of seven
conserved nucleotides, a heptamer, adjacent to the V, D and J regions. The heptamer is followed
by a spacer containing either 12 or 23 nonconserved nucleotides which in turn is followed by a
conserved 9 nucleotide nonamer. The RSSs are located on the 3’ end (downstream) of V regions
and the 5’ regions (upstream) of J regions. The heptamer and nonamer sequences are the sites
that are brought together during V-D-J recombination. In addition, only heptamer – nonamer pairs with dissimilar spacer RSSs are efficiently recombined (i.e. one with a spacer of 12 nucleotides will be recombined with one that has a spacer containing 23 nucleotides). This is known as the 12/23 rule of recombination (or “one turn/two turn” rule). Thus, the RAG proteins bind and bring a 12RSS and a 23RSS into close proximity. After binding, the RAG proteins nick the partner RSS and catalyze the coupled cleavage of both RSSs. This reaction yields a pair of blunt, 5’-phosphorylated signal ends and a pair of coding ends that terminate in covalently sealed DNA hairpin structures (Figure 1.3) (McBlane et al., 1995). After the initiation of the dsDNA breaks by RAG1 and RAG2 proteins, the DNA breaks are ligated by the Nonhomologous DNA End Joining (NHEJ) repair process. Similar to other DNA repair processes, three enzymatic activities are required to repair double strand breaks by the NHEJ pathway: a) nucleases to remove nucleotides for the adjacent DNA, b) polymerases to fill in or to repair the segment of DNA, and c) a ligase to restore the phosphodiester backbone.
Figure 1.3. 12/23 Rule.
RSSs are located 3' of each V gene segment, both 5' and 3' of each D segment, and 5' of each J segment. Recombination recognition sequences have a heptamer located next to the coding sequence, followed by a spacer of 12 or 23 nonconserved nucleotides, followed by a conserved nonamer. RAG enzymes bind to RSSs only if one has a 12 nucleotide spacer and the other a 23 (12/23 rule) and perform a first single strand nick at the border between the coding segment and the RSS. V(D)J recombinase makes double-stranded breaks in DNA between the recognition sequence and a V, D or J coding sequence. The two broken ends are then joined (VDJ).
This illustration was modified from Immunology, 2007.
The protein complex responsible for the repair of the dsDNA breaks initiated by RAG proteins consists of multiple proteins including Ku70/Ku80, DNA-PKcs, Artemis, XRCC4, DNA Ligase IV, Cernunnos/XLF protein and pol X polymerases. The Ku proteins, Ku70/Ku80, are the first proteins to bind the open ends of the DNA. Ku70/Ku80 improves the binding equilibrium of the nuclease, polymerases and ligase of the NHEJ DNA end repair (Povirk, 2006). The hairpin opening is regulated by the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) and Artemis which form a structure-specific endonuclease (Ma, Pannicke, Schwarz, & Lieber, 2002). DNA-PKcs is a DNA end-dependent serine/threonine kinase which phosphorylates Artemis. Once phosphorylated, Artemis acquires endonucleolytic activity and resects the hairpins.

The polymerases for NHEJ, including the pol X polymerases, pol μ pol λ and terminal deoxynucleotidyl transferase (TdT), catalyze the filling of double strand breaks gaps (Povirk, 2006). Signal and coding ends are then processed and joined by DNA Ligase IV, XRCC4 and the Cernunnos/XLF protein. DNA ligase IV ligates double-stranded DNA breaks; XRCC4 stabilizes and improves its joining activity (Grawunder et al., 1997; Modesti, Hesse, & Gellert, 1999). Cernunnos/XLF stimulates the ability of the XRCC4-DNA ligase IV complex to ligate the DNA in the presence of the physiologic Mg$^{2+}$ (Gu, Lu, Tsai, Schwarz, & Lieber, 2007).

1.5.3. The role of the Ig and TCR recombination in lymphoid malignancies

A major feature of malignancies involving B or T cells is the presence of chromosomal rearrangements involving either the *TCR* or *Ig* genes loci. These rearrangements juxtapose cellular oncogenes with the transcriptional regulatory regions of the immunoglobulin or *TCR* genes thereby resulting in the inappropriate or ectopic expression of oncogenes involved in the control of proliferation or differentiation in lymphoid cells. For example, the t(10;14)(q24;q11) or t(7;10)(q35;q24) translocations places the class II homeodomain-containing transcription factor *TLX1* under the transcriptional control of *TCRα/δ* or *TCRβ* chain regulatory region, respectively. These translocations result in the development of T-ALL (Dube et al., 1991; Hatano, Roberts, Minden, Crist, & Korsmeyer, 1991).

Although V(D)J recombination is normally limited to antigen receptor loci, the RAG proteins can mediate illegitimate V(D)J recombination events outside antigen receptor loci.
It has been hypothesized that this illegitimate recombination occurs by two mechanisms. In one model, the RAG proteins assemble a synaptic complex containing an authentic RSS and a functional cryptic RSS (cRSS) adjacent to a proto-oncogene and subsequently mediate cRSS cleavage through the standard nick-hairpin mechanism. This is “type 1” translocation (targeting mistake) (Figure 1.4). A subset of translocations, including those involving \textit{LMO2} and \textit{TAL1}, are thought to arise through this mechanism.
Figure 1.4. Illegitimate V(D)J recombination between TCRδ gene segment and LMO proto-oncogene locus bearing a functional RSS.

(A) A cryptic RSS, (dotted triangle) resembling a RSS is mis-targeted by the RAG. The V(D)J recombination proceeds in trans (B) and leads to a reciprocal chromosomal translocation generating 2 derivative chromosomes (C).

This illustration was modified from Marculescu R et al 2006.
In a second model, broken DNA ends introduced in a proto-oncogene are captured by a post cleavage RAG complex generated as an intermediate during normal V(D)J recombination. End joining in this scenario produces three joining products in various possible configurations. This is “type 2” translocation (repair failure) (Figure 1.5). Translocations involving TLX1 and the Bcl2 major breakpoint region are thought to occur by this mechanism (Marculescu et al., 2006; Lieber, Yu, & Raghavan, 2006).
Figure 1.5. "Type 2" translocation (repair failure) model.
(A) RAG binding, nicking, capture, synopsis and DSBs occur between a pair of TCR gene segments and distinct location at "fragile sites".
(B) Broken Ends are captured in the post-cleavage synaptic complex, leading to a synapse comprising the four V(D)J parts (Signal Ends, Coding Ends and the two Broken Ends).
(C) Illegitimate repair occurs using the NHEJ repair pathway.
(D) The two Broken Ends are joined with the two Coding Ends and generate a mixed joint at the derivative chromosome breakpoints.
This illustration was modified from Marculescu R et al 2006
To summarize, since a high proportion of lymphoid malignancies exhibit recurrent chromosomal translocations, resulting in inappropriate expression of transcription factors, including LMO2, TAL and TLX1, illegitimate V(D)J recombination may act as an initiating event in the development of these lymphoid malignancies. Therefore, mouse models defective in the V(D)J recombination pathway, provide a powerful tool to investigate early events contributing to leukemia.

1.5.4. Role of the NHEJ proteins in telomere stability

Telomeres, or the regions at the tips of chromosomes, have important functions including: 1) the protection of chromosomes from exonuclease attack, illegitimate recombination and degradation; 2) capping the chromosomes with TTAGGG repeats after spontaneous or induced breaks to prevent activation of DNA repair pathways; 3) positioning the chromosomes in the nucleus; 4) proper alignment of chromosomes for recombination during meiosis. In vertebrates, the DNA component of telomeres consists of tandem arrays of short repetitive TTAGGG sequences being oriented towards the end of the chromosome, ending in an essential 3’ single-stranded overhang that ranges in length from 50 to 400 nt (Moyzis et al., 1988; Huffman, Levene, Tesmer, Shay, & Wright, 2000). Telomeres provide a mechanism for cells to distinguish telomeric ends from the ends of random dsDNA breaks and protect the former from illegitimate end joining.

Telomeres gradually shorten in replicating cells due to end-processing therefore the maintenance of telomere length is a balance between loss of nucleotides of the telomere and re-addition to restore the telomere length. Telomere length is maintained in germ line cells by telomerase. However, most human somatic cells do not express sufficient telomerase activity to prevent telomere loss as the cells divide (Teixeira, Arneric, Sperisen, & Lingner, 2004). As a result, telomeres eventually shorten to the point where they initiate cell cycle arrest in G1, a state termed replicative senescence (Harley, Futcher, & Greider, 1990). Telomere shortening limits the number of somatic cells divisions, contributing not only to aging phenotypes, but also providing a tumour suppression mechanism.

Although telomerase is responsible for the addition of telomeric sequences with every cell cycle, other proteins also play important roles in the regulation and maintenance of telomere length and in the formation of a protective end-cap that prevents chromosome fusion. Many of these proteins are commonly associated with DNA repair. Thus, effective end-capping of
mammalian telomeres requires proteins of the NHEJ DNA repair pathway. For example, Ku70, Ku86 and the catalytic subunit DNA-PKcs are involved in the maintenance of telomeres. Mutations in any of these genes can lead to spontaneous chromosomal end-to-end fusions (Jeggo, Taccioli, & Jackson, 1995). It has been reported that the absence of DNA-PK results in end-to-end telomere fusions of uncapped telomeres, fusing not only chromosomes’ ends to each other, but also chromosomes’ ends to gamma radiation induced double-stranded DNA breaks (Bailey, Cornforth, Ullrich, & Goodwin, 2004a).

The role of the NHEJ protein DNA-PKcs in telomere fusions was first reported in primary mouse embryonic fibroblasts (MEFs) and primary kidney cells isolated from 6-8 month-old mice deficient in the DNA-PKcs. Cells from DNA-PKcs deficient (Prkdc<sup>Scid/Scid</sup>) mice accumulated a large number of telomere fusions, elevated levels of chromosome fragments and breaks, although telomere shortening was not reported (Gilley et al., 2001). These studies indicated that DNA-PKcs plays an important role in telomere capping thereby preventing end-to-end telomere fusions. Thus, DNA-PKcs and other proteins of the NHEJ machinery (Bailey & Goodwin, 2004; Goytisolo, Samper, Edmonson, Taccioli, & Blasco, 2001), participate in the capping of telomeres, but not in the maintenance of telomere length. Dysfunctional telomere capping was detected in human pancreatic carcinomas, osteosarcomas and epithelial cancers, and in murine tumours and tumour cell lines, indicating that telomere instability contributes to chromosome instability in various mouse and human malignancies (Gisselsson et al., 2001; Meeker et al., 2004). Mice tumours and tumour cell lines also typically display chromosomal rearrangements and extensive chromosome fusions (Chang, Khoo, & DePinho, 2001; Rudolph, Millard, Bosenberg, & DePinho, 2001).

Chromosome fusions and breaks may contribute to the loss of heterozygosity (LOH) of tumour suppressor genes. Preferential loss of telomere repeats from the ends of a particular chromosome harboring tumour suppressor genes or oncogenes may result either in their inactivation or activation due to chromosome translocation events and may also facilitate tumour progression. Collectively, telomere fusions and the associated chromosome/genomic instability promote tumour progression.
1.5.5. Mouse models for the study of human diseases

Mutant mice are conventionally generated by insertion mutagenesis, or by mutations that are introduced through homologous recombination in mouse embryonic stem cells (Copeland, Jenkins, 2010; Kool, Berns, 2009; Capecchi, 2005). Other methods have been developed to modify the genome by directly injecting DNA or mRNA of nucleases into the one-cell embryo to generate dsDNA break in the locus of interest, with subsequent error-prone NHEJ resulting in the mutant mice carrying a disrupted gene (Bogdanove, Voytas, 2011; Carroll et al., 2008; Urnov et al., 2010). In contrast to the above mentioned techniques targeting a single gene of interest, recently proposed CRISPR (clustered regulatory interspaced short palindromic repeat)/Cas system demonstrated the potential to edit up to five genes in mouse embryonic stem cells simultaneously (Wang, Yang, Shivalla et al., 2013). In this system, Cas proteins, CRISPR RNAs and trans-activating CRISPR RNAs can be programmed to produce sequence-specific dsDNA breaks via targeting and degrading foreign nucleic acids. In addition, some mutations may arise sporadically and represent particular interest due to targeting of genes potentially involved in malignant transformation. Therefore, mice carrying these mutations might be further used to generate double mutant mouse model systems by intercrossing of mice with a single mutation to verify collaborative genetic pathways in the cancerogenesis.

1.5.6. \textit{Prkdc}^{Scid/Scid} mice

In 1983, \textit{Prkdc}^{Scid/Scid} mice arose spontaneously from its parental strain CB17 and since then have been used as a model to understand the role of NHEJ in different cell types and how similar NHEJ deficiencies can result in human malignant disease (Bosma, Custer, & Bosma, 1983). These mice contain a nonsense mutation in the gene encoding the DNA-PK catalytic subunit (DNA PKcs) (\textit{Prkdc}).

This gene was originally mapped to chromosome 16 between the \textit{Ig\lambda} light chain locus and the mahoganoid (a recessive coat color marker) locus (Bosma & Carroll, 1991). The PRKDC protein is critical for the efficient recombination of the B- and T-cell receptors. Without this protein, B and T cell maturation is arrested at an early stage with B-lymphoid maturation arresting at the B220+CD19+ c-kit+CD43+CD25-IgM-IgD- pro-B cell stage in the bone marrow and thymocyte development arresting at the DN3 stage within the thymus (Hardy, Kemp, & Hayakawa, 1989).
Although Prkdc<sup>Scid/Scid</sup> mice are deficient in mature T and B lymphocytes, they contain low numbers of circulating immature lymphocytes and retain normal macrophage, granulocytes and natural killer (NK) cell functions. Bone marrow contains reduced numbers of developing B cells due to the block in B cell maturation and all lymphoid tissues are hypoplastic. Due to the lack of lymphoid cells, Peyer's patches and lymph nodes are tiny. In the spleen, lymphoid follicles and germinal centers are diminished with residual/rudimentary follicles containing only stromal cells, macrophages and large granular cells. The thymus is usually one tenth or less the normal size with poor corticomedullary delineation and disrupted stromal cell organization.

Although inactivation of the Prkdc gene affects early lymphocyte differentiation resulting in a severe deficiency of functional B and T cells, Prkdc<sup>Scid/Scid</sup> mice possess low levels of lymphoid cells with abnormal antigen receptor gene rearrangements. These leaky lymphoid development in Prkdc<sup>Scid/Scid</sup> mice is demonstrated by the ability of Prkdc<sup>Scid/Scid</sup> mice to reject allogeneic skin grafts, a T cell-dependent reaction (Bosma et al., 1988). Thus, it has been postulated either that productive rearrangements of antigen receptor genes occasionally occurs during lymphocyte development in Prkdc<sup>Scid/Scid</sup> mice or that recombinase activity may be normalized in some developing lymphocytes as a result of epigenetic variations at the Prkdc locus despite their impaired recombinase activity (Okazaki, Nishikawa, & Sakano, 1988).

Prkdc<sup>Scid/Scid</sup> mice spontaneously develop thymic lymphoma with low incidence and long latency. However, low-dose irradiation of Prkdc<sup>Scid/Scid</sup> mice dramatically increases the frequency and decreases the latency of thymic lymphomagenesis, but irradiation does not promote the development of other tumours (Williams et al., 2001). It has been suggested that the prevalence of chromosomal translocations involving antigen receptors in human lymphoid tumours results from the misjoining of RAG-1/2-induced dsDNA breaks in the Ig or TCR loci to random chromosomal dsDNA breaks adjacent to proto-oncogenes (Rabbitts, 1994). V(D)J misjoining events may give rise to the chromosomal translocations between antigen receptor genes and proto-oncogenes that are found in many lymphocytic leukemias and lymphomas and DNA-PKcs deficiency may increase such misjoining events in Prkdc<sup>Scid/Scid</sup> T-cell precursors.

Another possible mechanism of lymphoma initiation in Prkdc<sup>Scid/Scid</sup> mice is that V(D)J recombinase-dependent genetic alterations could also give rise to oncogenic mutations that do not involve antigen receptor genes. RAG-induced dsDNA breaks may be generated outside antigen receptor loci within “cryptic” recombination signal sequences at non-Ig or non-TCR loci thus providing oncogenic deletions (Cayuela, Gardie, & Sigaux, 1997). In addition, the ratio of cis-to-trans rearrangements in thymocytes from irradiated Prkdc<sup>Scid/Scid</sup> mice is approximately
5:1, whereas the ratio of cis-to-trans rearrangements in normal cells is 500 to 1,000:1 (Lista, Bertness, Guidos, Danska, & Kirsch, 1997). An increased ratio of cis-to-trans rearrangements in PrkdcScid/Scid mice also magnifies the risk of misjoining events involving oncogenes and Ig or TCR loci in lymphocytes.

Another possible mechanism of lymphoma development in PrkdcScid/Scid mice is genomic instability due to telomere fusions resulting from breakage/fusion/bridge (B/F/B) cycles and amplification of subtelomeric DNA or deregulated expression of tumour suppressor genes and oncogenes.

As a result of this propensity to spontaneously develop T-ALL, PrkdcScid/Scid mice offer an attractive model to investigate the possible collaborative role of unresolved double strand DNA breaks, telomere fusions and chromosomal translocations in the development of leukemia.

1.6. TLX1

1.6.1. TLX1 gene. Introduction

The TLX1 (also known as HOX11) gene is an example of a non-clustered class II homeodomain-containing gene whose deregulated expression is tightly connected with T-ALL. Initially detected in paediatric T-ALL, TLX1 was later shown to be an etiological cause of T-ALL initiation in both children and adults. TLX1 was found to contain a homeodomain DNA binding domain within its second exon and shares homology with the Drosophila 311 homeotic gene, which is involved in muscle development (Dube et al., 1991; Kennedy et al., 1991; Hatano, Roberts, Minden, Crist, & Korsmeyer, 1991; Dear & Rabbitts, 1994). The homeodomain of TLX1 exhibits more than 50% homology at the amino acid level with other HOX genes. TLX1 is located on chromosome 10, not chromosomes 2, 7, 12 or 17 where the class I homeodomain genes normally cluster. Therefore, in contrast to HOX clustered genes, TLX1 was designated as an orphan gene, although it is now correctly designated as a class II homeodomain containing transcription factor (Kennedy et al., 1991). In this chapter I will present a broad review of the normal function of TLX1 during embryogenesis and its role in leukemogenesis.
1.6.2. **TLX1 gene and T-ALL**

The t(10;14)(q24;q11) chromosome translocation was initially described in human T-ALL samples in 1986 by Dube *et al* (Dube, Raimondi, Pi, & Kalousek, 1986). A second group identified a variant translocation associated with T-ALL involving breakpoints on chromosome 10q24 and chromosome 7q35 (the t(7;10)(q35;q24) translocation) (Dube *et al*., 1991). It was later demonstrated that the breakpoints on chromosomes 14 and 7 involved the TCRα/δ locus located on chromosome 14 and the TCRβ gene locus on chromosome 7. Breakpoints on chromosome 10 were clustered within a 15-kb region and three groups identified the oncogene located at 10q24 to be the homeodomain-containing transcription factor HOX11 (Dube *et al*., 1991; Hatano, Roberts, Minden, Crist, & Korsmeyer, 1991; Kennedy *et al*., 1991). It was speculated that the reciprocal translocation resulting in aberrant activation of HOX11 is located at 10q24 locus, following juxtaposition with the TCRα gene locus in these T-ALLs. In a subsequent study, the translocation was shown to actually interrupt the δ chain gene, located within the α-chain locus in three patients (Kagan *et al*., 1987). HOX11 was later designated TLX1, in accordance with IUPAC nomenclature for the naming of homeodomain-containing proteins.

Translocations involving δ or β TCR loci and TLX1 were suggested to be the result of errors in normal TCR rearrangement. The TCR loci encodes the V(D)J DNA segments. RAG proteins, which initiate DNA rearrangement, recognize short signal sequence adjacent to coding segments. These sequences consist of heptamer and nonamer sequences separated by 12- or 23-nucleotide spacers. Cleavage occurs at the border between a signal sequence and a coding segment. Because breakpoints on chromosomes 10 and 14 are flanked by heptamers and nonamers which are normally recognized by RAG proteins, it was suggested that misrecognition by RAG complex initiates t(10;14)(q24;11) chromosomal translocations (Raghavan, Kirsch, & Lieber, 2001). However, flanking V(D)J chromosomal sequences were detected in only 50% of t(10;14)(q24;q11), suggesting other mechanisms are involved in these chromosomal translocations (Kagan *et al*., 1989).

A variant translocation, t(7;10)(q35;q24), juxtaposing the TLX1 coding sequence with the TCRβ locus has also been identified by another group in pediatric T-ALL (Kennedy *et al*., 1991). In addition, an inversion (10)(q24;p13) was reported in a single case of adult T-ALL. However, a single case report and additional complex chromosomal abnormalities including
translocations and deletions detected in this sample do not allow assessment of the implications of inversion (10)(q24;p13) in the pathogenesis of T-ALL (Itoyama et al., 1995).

The t(10;14)(q24;q11) and t(7;10)(q35;q24) translocations place the full and intact TLX1 gene under transcriptional regulatory control of the TCRδ and TCRβ locus, respectively, thereby resulting in the ectopic and inappropriate expression of TLX1 in thymocytes. However, expression of TLX1 was reported in pediatric T-ALL samples where no cytogenetically detectable abnormalities were detected. TLX1 activation in the absence of translocations involving chromosome 10q24 was initially detected by Northern blot and RT-PCR analysis in approximately 30% of T-ALL patients by Salvati et al. in 1995 (Salvati, Ranford, Ford, & Kees, 1995). Later, Affymetrix microarray and quantitative RT-PCR studies by Ferrando et al., detected aberrant expression of TLX1 in 50% of T-ALL patients in the absence of translocations (Ferrando et al., 2002). In addition, Kees et al., detected high levels of TLX1 expression in almost 20% of patients and low levels of TLX1 expression by quantitative RT-PCR in almost 30% of paediatric T-ALL samples (Watt et al., 2003). Combined, these data suggest other mechanisms in addition to chromosomal translocations are involved in the ectopic expression of TLX1 in T-ALL.

Several alternative mechanisms for the deregulation of TLX1 expression have been suggested. For example, demethylation of the TLX1 proximal promoter in two T-ALL samples with elevated TLX1 expression in the absence of translocations suggests that demethylation of the TLX1 promoter may be a precondition for alternative TLX1 gene activation (Watt, Kumar, & Kees, 2000). Mutations in the cis-regulatory sequences of the TLX1 may also initiate gene activation (Kees et al., 2003). In addition, aberrant expression of TLX1 in T-ALL may be a result of the loss of transcriptional suppressors that normally down-regulate TLX1 expression (Ferrando et al., 2004).

Phenotypically TLX1 positive human T-ALL samples are blocked at a premature CD4+CD8+ stage and, in the vast majority of cases, cells are TCRαβ positive and TCRγδ negative (Ferrando et al., 2002; Ferrando & Look, 2003; Asnafi et al., 2005). However, murine hemopoietic stem cells from fetal liver transduced with TLX1 retroviral vectors induced maturation arrest prior to the double positive CD4+CD8+ stage with thymocytes exhibiting a double negative CD4−CD8− phenotype indicating some degree of flexibility in the stages where differentiation block occurs (Owens, Hawley, Spain, Kerkel, & Hawley, 2006). Combined, these data suggest that TLX1 positive T-ALLs exhibit a differentiation block during the early stages of T-cell development.
1.6.3. *TLX1* and the TLX family of NK homeobox genes

*TLX1* belongs to a three member family of non-clustered class II homeodomain genes. Other members of this group include *TLX2* and *TLX3*, which are located on chromosomes 19 and 6, respectively (Wen, Tang, & Breitman, 1994). *TLX1*, *TLX2* and *TLX3* genes encode 330, 284 and 291 amino acid homeodomain proteins, respectively, and exhibit specific patterns of expression during embryonic development (Dear, Sanchez-Garcia, & Rabbitts, 1993; Logan, Millar, Bharadia, & Rouleau, 2002). All proteins encoded by the genes of the TLX family exhibit sequence similarity to *TLX1* both within and outside the homeodomain. Thus, murine *TLX2* and *TLX3* share 61.3% and 66.3% amino acid homology to *TLX1*, respectively (Dear, Sanchez-Garcia, & Rabbitts, 1993). The homeodomains of the TLX family belong to a distinct subclass of homeodomains possessing a threonine residue at amino acid position 247 within the third helix, rather than the more commonly found valine or isoleucine (Dube et al., 1991; Dear, Sanchez-Garcia, & Rabbitts, 1993). In addition, the *Drosophila* homologue of *TLX1*, *BarHl*, contains a threonine at position 47 within helix 3 (Kojima et al., 1991).

The presence of the threonine in the DNA-binding helix may modify target binding site recognition. For example, isoleucine in position 247 of the engrailed homeodomain-DNA complex contacts the fourth thymine of the TAAT core motif via a methyl-methyl interaction (Kissinger, Liu, Martin-Blanco, Kornberg, & Pabo, 1990). In contrast, the threonine in this position of the *TLX1* homeodomain forms a hydrogen bond with the amine side chain of a guanine nucleotides and thereby altering the *in vitro* DNA recognition motif of the *TLX1* homeodomain to TAAGTG (Tang & Breitman, 1995). The threonine residue has been shown to be an absolute requirement to regulate downstream target genes. For example, mutation of threonine 247, which changes the *TLX1* DNA target sequence from TAAC/TAAT to TAAT only, resulted in the inability of *TLX1* to activate *in vitro* expression of its downstream target *Aldh1*, an enzyme which catalyzes the oxidation of aldehydes to carboxylic acids (Owens et al., 2003). Moreover, the *Aldh1* gene was shown to be a target of *TLX1* exclusively in a threonine-247 dependent fashion in NIH-3T3 cells and transcriptional transactivation of *Aldh1* did not require the PBX interaction motif (PIM) (Masson, Greene, & Rabbitts, 1998). *Cyclin B1* represents another *TLX1* downstream target, with expression being regulated by phosphorylation of threonine-247. Data of our laboratory indicated that phosphorylation of threonine-247 residue plays a role in regulation of *cyclin B1* during G2/M progression. Substitution of threonine-247 with glutamic acid, which mimics a constitutively phosphorylated
TLX1, abrogates binding of TLX1 to the \textit{cyclin B1} promoter and decreases the levels of expression of \textit{cyclin B1}, which will be discussed in details in the TLX1 and the G2/M Checkpoint section (Chen et al., 2010).

In addition to the homeodomain, other highly conserved motifs have been identified in TLX family members. These domains include an acidic glutamine rich region at the C-amino terminus and a glycine rich region at the amino terminus. The role of these motifs was identified by the construction of TLX1 deletion mutants fused to the yeast GAL4 DNA binding domain (DBD) (Zhang, Shen, Ho, & Lu, 1996; Masson, Greene, & Rabbitts, 1998). These studies revealed that the N-terminal glycine/proline rich region, the C-terminal glutamine rich region and the homeodomain were all important for optimal transactivation of a reporter construct comprised of five GAL4 responsive elements.

Members of the TLX family also contain the five amino acid FPWME PBX interaction motif (PIM) which is required for the association with PBX1, a member of the TALE homeodomain superclass. This motif is related to the YIF-PWM-K/R pentapeptide motif of class I HOX proteins and is required for the formation of the TLX1/PBX multiprotein transcription complex (Phelan, Rambaldi, & Featherstone, 1995). Point mutational analysis revealed that the FPWME motif is an absolute requirement for \textit{in vitro} interaction of TLX1 with PBX proteins (Allen, Zhu, Hawley, & Hawley, 2000). Studies of truncated and mutated TLX1 proteins indicated that only the homeodomain and the N terminus were necessary for immortalization of hemopoietic precursors. Moreover, the PIM was shown to primarily play a role in \textit{TLX1} initiated immortalization of hemopoietic precursors (Owens et al., 2003). Thus, detailed examination of residues within the homeodomain demonstrated that mutation of threonine-247, previously reported to be indispensable for the activation of the \textit{Aldh1} did not affect \textit{TLX1} transforming activity. These data suggest a dual target specificity of the \textit{TLX1}: transactivation may be mediated \textit{via} threonine-247 dependent manner and the TLX1-PBX heterodimers mediated cellular immortalization.

More recent studies have detected other motifs within TLX1. Glutathione-S-transferase pull-down assays demonstrated that TLX1 interacts with Groucho/transducin-like Enhancer of split (Gro/TLE) proteins via an Engrailed homology 1 (Eh1-like) motif. This motif is located in the N terminal and includes amino acids 19 to 26. The full-length GST-TLX1 was capable of co-precipitating exogenous TLE1 from 293T cell extracts, but not a GST-TLX1 mutant containing a 119-amino acid NH2-terminal deletion. Moreover, NIH3T3 cells transduced with lentiviral vectors encoding this deletion mutant of the TLX1 protein resulted in reduced
expression of the TLX1 target, Aldh1a1, indicating that the Eh1-like motif is required for optimal induction of expression of the Aldh1a1 target gene (Riz et al., 2009).

Yeast two-hybrid interaction and co-IP studies revealed that the amino-terminal portion of the TLX1 protein (excluding the first 50 amino acids) was required for direct physical interactions of TLX1 with the cofactor proteins MEIS1 and MEIS2. This interaction required only one protein domain that was not a part of the homeodomain or the YPWMR motif of the TLX1 which is required for the interaction of TLX1 with PBX (Milech et al., 2010).

1.6.4. The role of the TLX1 in embryodevelopment

The first report outlining Tlx1 deficient mice (Tlx1−/−) generated by gene targeting was published in 1994 (Roberts, Shutter, & Korsmeyer, 1994). The expression of Tlx1 was assessed in normal control Tlx1+/− and heterozygous Tlx1+/− littermates by whole-mount in situ hybridization with an anti-Tlx1 antisense probe. Morphological features of developing organs, where expression of Tlx1 was detected, were compared among Tlx1+/+, Tlx1+/− and Tlx1−/− mice. Expression of Tlx1 was detected in first and second branchial arches, as well as in the motor nuclei that innervate them, in cranial nerves, trigeminal ganglia and in the abdominal portion of the splanchnic mesoderm. Tlx1−/− mice did not exhibit phenotypic defects other than the absence of a spleen and increased numbers of neutrophils and lymphocytes. The presence of Howell-Jolly bodies in erythrocytes was also detected in Tlx1−/− mice, but this condition is typical for asplenia. It was reported that development of the spleen in Tlx1−/− embryos was normal until embryonic day E11.5, followed by disorganization, decondensation and complete resorption of cells of the splanchnic mesoderm by day E13.5. Thus, ablation of a single gene resulted in the loss of the spleen, while other organs developed normally.

TUNNEL analysis was performed to gain insights into mechanisms of how Tlx1 deficiency contributed to the loss of the spleen in Tlx1−/− embryos. Comparison of control and Tlx1 deficient embryos at E13.5 did not reveal an increase in cell death within the site of normal spleen development. However, cells within the mesogastrium remained fused with the stomach. This finding supported the hypothesis that Tlx1 was required for the separation of cells of the splanchnic mesoderm from the stomach rather than to support cell viability. Based on the detection of a significantly larger stomach in Tlx1−/− mice relative to controls, it was suggested that, in the absence of Tlx1 initiated genetic programs, splanchnic mesoderm cells adopted a new fate. This hypothesis was in agreement with the increased stomach weight detected in Tlx1
embryos relative to those of $Tlx1^{+/+}$ and $Tlx1^{+/−}$ littermates (Roberts, Sonder, Lumsden, & Korsmeyer, 1995).

To further determine the function of $Tlx1$ in mice, Dear et al created $Tlx1$ deficient mice by incorporating the bacterial $lacZ$ gene into the first exon of $TLX1$ transcriptional unit. Production of β-galactosidase under the control of the endogenous $Tlx1$ promoter in heterozygous $Tlx1^{+/−}$ mice allowed detection of sites of $Tlx1$ expression. These studies confirmed the findings of Roberts et al and further demonstrated that expression of $Tlx1$ was also detected in the epithelial layer of the tongue, in the developing hindbrain along the pontine flexure and in the developing spinal cord (Dear et al., 1995). It was also noted that expression of $Tlx1$ in the developing spleen was spatially and temporally localized. Of note, these studies revealed that $TLX1$ expression was detected in the capsule and trabeculae, which combined formed the framework of the spleen until E18.5.

In agreement with previous reports, the spleen anlage was no longer detectable in $Tlx1^{−/−}$ embryos. To further define mechanisms involved in the elimination of splenic cells in $Tlx1^{−/−}$ embryos, a comparison of the numbers of apoptotic cells among $Tlx1^{+/+}$ and $Tlx1^{+/−}$ spleen cells was performed. A significant increase in cell death within the spleens of E12.5 $Tlx1^{−/−}$ mice relative to $Tlx1^{+/−}$ mice was reported. However, these results were in contrast to previous reports as they suggested the involvement of apoptotic pathways in elimination of the spleen anlage and indicated that $Tlx1$ played a role in the prevention of apoptosis in splenic precursor cells.

Alternatively, it was suggested that the defect in spleen development resulted from the inability of splenic precursors in $Tlx1^{−/−}$ mice to attract accessory cells or to secrete autostimulatory factors required for cell proliferation. To verify these possibilities, chimeric embryos, consisting of wild type and $Tlx1^{−/−}$ cells, were generated (Kanzler & Dear, 2001). It was reported that $Tlx1^{+/−}$ cells were unable to contribute to the spleen in the presence of wild-type cells and that $Tlx1^{−/−}$ cells were not detected in the developing spleen of examined chimeric embryos. These data indicated that during spleen development $Tlx1$ acted in a cell autonomous manner and that the block in spleen development in $Tlx1^{−/−}$ mice was due to neither the inability of the spleen anlage to attract cells nor the absence of autostimulatory factors normally secreted by $Tlx1$-expressing cells.

$Tlx1^{−/−}$ mice, in addition to providing data of the role of $Tlx1$ in embryogenesis, have also been used to identify downstream transcriptional targets of $TLX1$. Dear et al (Koehler, Franz, & Dear, 2000) reported deregulated expression of the Wilm’s tumour ($Wt1$) gene and the retinoic acid-synthesizing enzyme, aldehyde dehydrogenase 1 ($Aldh1$) during spleen development in
These studies found that day E10.5 Tlx1/- embryos had reduced expression of Wt1 mRNA and elevated expression of Aldh1 in the spleen anlage relative to control mice. Although Wt1 expression was reduced in the developing spleen of E12.5 Tlx1/- embryos, Tlx1 expression remained unaltered in Wt1/- mice, thereby indicating that Tlx1 regulated expression of Wt1, whereas Wt1 did not regulate Tlx1 expression. The ability of Tlx1 to regulate Wt1 expression was confirmed by using luciferase reporter assays in which Tlx1 was shown to transactivate the human WT1 promoter five-fold in a Tlx1-null fibroblast cell line relative to transfection of the WT1 promoter reporter construct alone. Since both Tlx1 and Wt1 deficient mice were asplenic, these studies indicated that both genes function in the development of the spleen and that Wt1 was a downstream target for Tlx1. Additional studies describing the expression of Wt1 in the developing spleen of day E10.5 control embryos reported that Wt1 expression was first detected twenty-four hours after induction of Tlx1 expression, indicating a spatial correlation of Wt1 and Tlx1 (Rackley et al., 1993).

In vitro and in vivo studies indicated that Aldh1 was a second transcription target of Tlx1 (Greene, Bahn, Masson, & Rabbitts, 1998). Northern blot analysis revealed elevated levels of Aldh1 in NIH-3T3 clones stably transfected with a TLX1 bearing expression vector. However, in the developing spleen, the presence of Aldh1 mRNA was inversely corresponded with Tlx1 expression. Thus, Aldh1 was absent in the spleen primordium in the presence of TLX1. Moreover, Aldh1 gene expression was not detected in the developing hindbrain of wild type embryos. In agreement with the above findings, Aldh1 expression was detected in the spleen anlage of E12.5 Tlx1/- embryos, suggesting negative regulation of Aldh1 expression by Tlx1 during embryogenesis. This paradox of activation of Aldh1 by Tlx1 in NIH-3T3 fibroblasts versus inactivation of Aldh1 expression in the spleen primordium in the absence of Tlx1 may be explained by the availability of cofactors required for Aldh1 expression under different in vitro and in vivo conditions.

1.6.5. The role of TLX1 in leukemogenesis

Several lines of evidences strongly support the potential of TLX1 to alter genetic programs regulating hemopoietic differentiation. The first data, confirming the ability of TLX1 to immortalize hemopoietic precursors, were obtained using in vivo experiments. In these studies, murine bone marrow cells transfected with a retrovirus bearing the TLX1 gene yielded cell lines
consisting of immature myelocytes (Hawley, Fong, Lu, & Hawley, 1994). In addition, constitutive expression of TLX1 in J2E murine erytholeukemic cells induced their block in differentiation and produced clones having a preponderance of less differentiated cells with increased adherence to plastic and a reduction in hemoglobin synthesis in response to erythropoietin (Zhang, Shen, Hawley, & Lu, 1999; Greene et al., 2002). In vivo experiments demonstrated the oncogenic potential of TLX1, in that transplantation of retrovirally transfected bone marrow cells overexpressing TLX1 into sublethally irradiated syngenic mice resulted in the development of hemopoietic malignancies in recipient mice. Two of nine recipients developed transplantable T cell malignancy after a long latency (Hawley et al., 1997).

The in vivo oncogenic potential of TLX1 in lymphoid cells was initially demonstrated in IgHμ-HOX11Tg mice (here after referred to as IgHμ-TLX1Tg). In these studies, expression of TLX1 was under the transcriptional control of the IgHμ promoter and enhancer thereby driving expression of the transgene throughout B cell development. These mice developed transplantable mature B cell lymphoma at an average age of 14 months with the primary site of lymphoma being the spleen with dissemination to distant organs (Hough et al., 1998). More recently, two transgenic mouse models with ectopic expression of TLX1 in immature thymocytes were developed. In the first model, the Lck proximal promoter was used to drive expression of TLX1 in T-cell progenitors with 92% of p56Lck-TLX1 transgenic mice developing T-ALL after an average 12 months (De et al., 2010). In the second model, a conditional doxycycline-regulated promoter was used to drive expression of TLX1 in immature thymocytes. Conditional doxycycline-regulated mice derived from different founders exhibited various penetrance to T-ALL (15%-60%) and developed disease after 5-7 months (Rakowski, Lehotzky, & Chiang, 2011).

Thus, these transgenic mouse models provide the strongest evidence that TLX1 acts as an oncogene if abnormally expressed in lymphocytes. Notably, the finding that all mice exhibited either T- or B cell malignancy with a delayed latency suggests that ectopic expression of TLX1 in the absence of additional mutations is insufficient to initiate malignant transformation.

In an attempt to identify mutations that collaborate with deregulated TLX1 expression in the development of lymphoma, our laboratory undertook retroviral insertional mutagenesis studies. We used the murine AIDS (mAIDS) virus to specifically infect premalignant B cells of IgHμ-TLX1Tg mice. Acceleration of the malignant phenotype was used as an indication that the virus had integrated in the vicinity of a gene that, when deregulated, collaborated with TLX1 in the development of lymphoma. One mAIDS viral insertion site identified in these studies was
the *Ubr1* gene locus. *Ubr1* is an E3 ubiquitin ligase located on mouse chromosome 2. *Ubr1* protein is a component of the N-end rule pathway which recognizes and targets protein substrates possessing a degradation signal for ubiquitin dependent proteolysis (Ditzel et al., 2003). *Ubr1*−/− mice are phenotypically normal with the only subtle defects in muscle protein degradation (Kwon et al., 2002). Studies crossing *Ubr1*−/− mice with *IgHμ-TLX1*<sup>Tg</sup> mice indicated that *IgHμ-TLX1*<sup>Tg</sup> /*Ubr1*−/− double mutant mice exhibited a statistically significant acceleration in the onset of mature, large B-cell lymphoma relative to control *IgHμ-TLX1*<sup>Tg</sup> /*Ubr1*<sup>+/−</sup> littermates (Chen, Kwon, Lim, Dube, & Hough, 2006). Micronucleus assays revealed an increased presence of micronuclei in *IgHμ-TLX1*<sup>Tg</sup> /*Ubr1*−/− B lymphocyte cultures relative to *IgHμ-TLX1*<sup>Tg</sup> /*Ubr1*<sup>+/−</sup> cultures. Furthermore, the propensity of *IgHμ-TLX1*<sup>Tg</sup> /*Ubr1*−/− B lymphocyte for chromosome missegregation was confirmed by the karyotyping of primary B lymphocyte cultures which revealed an increased incidence of hypodiploid cells. Therefore, these studies provided the first direct evidence that chromosome missegregation accelerated the progression of lymphoma initiated by *TLX1* in B lymphocytes.

**1.6.6. Mechanism of ectopic expression of *TLX1* in leukemogenesis**

The mechanisms of *TLX1* mediated transformation still remain to be fully elucidated. Early studies from Korsmeyer’s laboratory reported that TLX1 interacted, via a region (amino acids 149–190) upstream of the homeodomain, with the catalytic subunit of the serine threonine phosphatases PP2A and PP1 (Kawabe, Muslin, & Korsmeyer, 1997). These studies demonstrated that *TLX1* inhibited the activities of PP1 and PP2A phosphatases at the G1/S cell cycle checkpoint in Jurkat T cell lines leading to speculation that loss of PP1 and PP2A activity promoted progression of cells through the G1/S check point (Riz & Hawley, 2005). Moreover, PP1 plays a role in the G1/S transition through the cell cycle by direct dephosphorylation of the retinoblastoma (RB) protein (Yan & Mumby, 1999). In addition, elevated levels of expression of *E2F*, *c-Myc*, and the *cAMP-responsive element binding protein* (*CREBP*), all of which are involved in the regulation of the G1/S cell cycle checkpoint, were detected in *TLX1*-expressing Jurkat cells. Therefore, it was speculated that inappropriate expression of *TLX1* in Jurkat T cells might accelerate passage of cells through the G1/S by hyperphosphorylation of the RB protein and deregulation of the G1/S effectors.
More recently, a second potential mechanism of TLX1 mediated malignant transformation was proposed by Chen et al (Chen et al., 2006). The murine mAIDS proviral insertion mutagenesis studies identified 17 TLX1 collaborative genes with seven of these being involved in the regulation of the mitotic spindle checkpoint. Cytokinesis-block micronucleus assay detected increased micronuclei formation in \( \text{IgH}_\mu\text{-TLX}1^{Tg} \) B lymphocyte cultures relative to control cultures treated with colchicine indicating aberrant chromosomes segregation. Abnormal regulation of the spindle checkpoint in \( \text{IgH}_\mu\text{-TLX}1^{Tg} \) B lymphocyte cultures was confirmed by quantification of B cells in G1, S and G2/M phases of the cell cycle and by BrdU immunolabelling of cycling cells. Thus, \( \text{IgH}_\mu\text{-TLX}1^{Tg} \) B lymphocytes exhibited a shortened mitotic arrest after colchicine treatment relative to control B lymphocyte cultures and the number of BrdU positive B cells was increased. Combined, these studies showed that elevated expression of TLX1 in B cells deregulated the spindle checkpoint ultimately leading to a heightened predisposition for the development of aneuploidy, thereby accelerating lymphomagenesis. Moreover, \( \text{IgH}_\mu\text{-TLX}1^{Tg} \) B cell cultures showed elevated expression of the mitotic effector molecules, cyclin A, cyclin B1, cdc20 and aberrant bypass of spindle checkpoint arrest (Chen et al., 2006). Collectively these data indicate that inappropriate expression of TLX1 in B cells mediates malignant transformation via deregulation of mitotic checkpoints and activation of effectors molecules. These findings are in agreement with recently published data indicating that TLX1 downregulates expression of CHEK1, a gene which is required for checkpoint mediated cell cycle arrest in response to DNA damage thereby contributing to the deregulation of the mitotic checkpoint in nontransformed preleukemic thymocytes in \( p56^{Lck}\)-TLX1 transgenic mice (De et al., 2010).

1.6.7. Murine models expressing TLX1 in cells of a lymphoid compartment

Early data demonstrating the potential of TLX1 to initiate malignant transformation have come from experiments performed \textit{in vitro} utilizing myeloid and erythroid cell lines (Hawley, Fong, Lu, & Hawley, 1994; Greene et al., 2002). Later studies using relevant mouse models confirmed the oncogenic potential of TLX1 and greatly facilitated the elucidation of the molecular mechanisms of TLX1 in the initiation of lymphoid malignancies. The first description of a TLX1 transgenic mouse model was published in a short abstract presented at the American Society of
Hematology in 1992 by Hatano et al, although a complete description of these mice was never published. This preliminary report indicated that 30% of the LCK-TLX1 transgenic mice expressing the transgene in thymocytes, developed T cell lymphoma.

Our laboratory attempted to generate TLX1-transgenic mice by placing the human TLX1 cDNA under the transcriptional control of the proximal LCK promoter to drive high levels of transgene expression in the early stages of thymocyte development. Although numerous attempts were made to generate the LCK-TLX1 transgenic model, these were not successful leading to speculation that embryonic expression of TLX1 was lethal. Additional studies were undertaken to develop transgenic mice using the immunoglobulin heavy chain (IgHμ) promoter and enhancer to drive high levels of the human TLX1 cDNA in B cells. In contrast to LCK-TLX1 mice, IgHμ-TLX1Tg mice were viable and expressed the transgene throughout all stages of B cell development. Approximately 50% of these mice developed B cell lymphoma by 16 months of age and all mice died by 20 months. The primary site of lymphoma was the spleen with frequent dissemination to the thymus, lymph nodes, lungs, liver and kidneys. Lymphoma cells did not disseminate to the bone marrow. All lymphomas were clonal and exhibited a mature IgM+IgD+ phenotype. Moreover, transplantation of tumour cells into PrkdcScid/Scid mice resulted in the development of B cell lymphoma in recipient mice. Although, expression of TLX1 in human B-ALL disease has not been reported, this in vivo model represented the first physiologically relevant model of TLX1-associated disease initiated in lymphoid cells (Hough et al., 1998).

Ferrando et al, successfully generated p56Lck-TLX1 transgenic mice (De et al., 2010). These mice constitutively expressed TLX1 in thymocytes under the control of the Lck promoter. Overall, 92% of p56Lck-TLX1 transgenic mice developed clonal thymic tumours with a CD4+CD8+ double positive immunophenotype cells with tumour cells disseminating to distant organs. Half of the tumour samples revealed significant heterogeneity showing two or more immunophenotypically unique cell populations. Premalignant thymocytes at the DN1 stage exhibit downregulated expression of CHEK1, a key regulator of the mitotic spindle checkpoint, which was also shown to be consistently downregulated in human TLX1-initiated leukemias. Moreover, similar to human TLX1-initiated leukemias, p56Lck-TLX1 transgenic tumours developed secondary cooperating mutations of Notch1, Pten and Bcl11b in tumour samples, supporting the relevance of the deregulated pathways in p56Lck-TLX1 transgenic tumours to those in human T-ALLs (De et al., 2010). However, offspring of only two of twelve founder transgenic mice constitutively expressing TLX1 in thymocytes developed T-ALL. It was speculated that the low penetrance of disease in successive generations may be related to the
negative consequences of expression of TLX1 in hemopoietic stem cells and more mature progenitors.

A third TLX1 transgenic mouse model using the conditional doxycycline-inducible promoter to drive TLX1 was also reported (Rakowski, Lehotzky, & Chiang, 2011). In this model, expression of TLX1 was placed under the control of an inducible Lck-promoter in order to limit conditional expression to the T-lineage cells. This inducible system required two transgenes. The first transgene expressed the tetracycline transactivator (TTA) from the proximal Lck promoter resulting in expression of TTA in early thymocytes. The second transgene expressed the full-length human TLX1 cDNA under the control of the tet operon. Backcrossing mice to generate double transgenic mice resulted in the restricted expression of TLX1 in immature thymocytes after the addition of doxycycline. These mice exhibited transplantable CD4+CD8+ T-ALL after a median 210 days with a penetrance of 60%. Comparable to the human scenario, 67% of tumours harbored Notch1 activation.

Notably, all three murine models showing deregulated expression of TLX1 in lymphocytes developed lymphoid malignancies after a long latency. This suggests that additional genetic mutations are required to initiate the full-blown disease. Identification of these secondary mutations represents an important next step in understanding the multistep mechanism of TLX1 initiation of T-ALL.

1.7. The role of TLX1 in the cell cycle

Mounting evidence indicates that one of the major mechanisms in TLX1-induced leukemia is its ability to alter normal passage of a cell through the cell cycle. These alterations arise as a result of the interaction of TLX1 with key players of the cell cycle control system. In this section I will provide a brief overview of the cell cycle and its checkpoints. Data supporting a role for TLX1 in the modulation of multiple cell cycle processes will be discussed.

1.7.1. The cell cycle and the cell cycle checkpoints

All proliferating cells pass through the cell cycle. The cell cycle can be defined as an orderly sequence of phases whose main function is to duplicate DNA in the chromosomes and then to segregate the copies into two daughter cells. The cell cycle is divided into two main phases: the interphase and the mitotic phase. The interphase, which is defined as the non-mitotic phase, is
subdivided into three distinct phases: the Gap1 (G1) phase, the synthesis (S) phase and the Gap2 (G2) phase. DNA duplication takes place during S phase, which requires 10-12 hours. However, most cells require much more time to grow and double the proteins and organelles that they require to replicate their DNA and to divide. Therefore, the G1 phase precedes S phase to ensure adequate component synthesis, favorable external conditions and extracellular signals from other cells. If conditions are unfavorable, cells delay passage through the G1 phase or may be directed to a G0 state until conditions to grow and to divide are favorable. During the final G2 phase, the cells ensure that DNA replication and all preparations have been completed properly and that cells are ready to enter mitosis.

To ensure completion of the previous cell cycle phase and that daughter cells received undamaged DNA during mitosis a control system arrests the cell cycle at specific checkpoints. Checkpoints exist at the border between G1 and S (G1/S) phases, between G2 and M (G2/M) phases and at the metaphase to anaphase transition.

The G1/S checkpoint allow cell to pass into S phase only if genomic DNA is undamaged. Arrest at this point prevents replication of damaged DNA. If the quality of DNA is unsatisfactory, the cell is delayed at this phase or directed to G0 for an extended period. The G2/M checkpoint prevents cell entry into mitosis if DNA replication is not complete. Therefore, cells that fail to replicate all their chromosomes do not enter mitosis. In addition, arrest in G2 allows double strand DNA breaks to be repaired before mitosis, otherwise the broken portion of chromosomes will not be properly segregated during anaphase. A third checkpoint, the spindle assembly checkpoint, represents a control mechanism at the metaphase-to-anaphase transition during mitosis. The function of this checkpoint is to ensure that metaphase chromosomes are properly attached to the spindle apparatus and aligned at the metaphase plate before migration of sister chromatid to opposite poles of the cell. As the checkpoints provide cellular responses to DNA damage, their alterations allow propagation of cells with inappropriate replication of mutated DNA and accumulating genomic abnormalities, thereby increasing the risk of cancer development.

1.7.2. The G1/S checkpoint

The G1 to S transition is regulated by two converging pathways comprising activation of cyclins, cyclin dependent kinases and phosphorylation of proteins of the retinoblastoma family. The first pathway relies on the expression and phosphorylation of a number of the D-type
cyclins, cyclin-dependent kinases (Cdks) and members of the retinoblastoma gene family (Rb, p107 and p130) (Lew, Dulic, & Reed, 1991; Xiong, Connolly, Futcher, & Beach, 1991; Brown, Phillips, & Gallie, 1999). The second pathway involves the c-Myc gene which stimulates the expression of cyclin E and Cdc25a (Vlach, Hennecke, Alevizopoulos, Conti, & Amati, 1996). Both pathways are mediated by the activation of the p53 tumour suppressor which activates cell cycle inhibitory proteins and the CDC25 family of phosphatases. The G1/S checkpoint prevents entry of cells into S phase if damaged DNA is present.

The first pathway regulating the G1 to S transition is initiated when activated cyclin D interacts with Cdk4, Cdk6 and Cdk2. These Cyclin D-Cdk complexes phosphorylate the retinoblastoma protein (pRb). When hypophosphorylated, pRb binds and represses the activity of the E2F family of transcription factors resulting in inhibition of downstream target genes required for entry into S phase (Nevins, 1998). In contrast, the inactivating phosphorylation of Rb by cyclin dependent kinases results in the release of the E2F transcription factor which then induces expression of genes that mediate S phase entry and facilitate DNA replication (Giacinti & Giordano, 2006). Thus, overexpression of cyclin D1 as a result of amplification of chromosome 11q13 was detected in such adult cancers as squamous cell carcinomas, esophageal carcinomas and breast carcinomas (Sherr, 1996). Moreover, transgenic mice which overexpressed cyclin D1 in mammary epithelial cells exhibited mammary carcinoma (Wang et al., 1994). Loss-of-function mutations of Rb have been identified in breast carcinomas, lung carcinomas and osteosarcomas (Hunter, 1997; Weinberg, 1995).

The second pathway regulating the G1 to S transition involves the c-MYC gene, which activates expression of cyclin E and Cdc25a genes. Mutations of the c-MYC gene are the among the most common mutations detected in human cancers (Henriksson & Luscher, 1996). Acting as a transcription factor, c-Myc was proposed to activate Cdc25a phosphatase (Galaktionov, Chen, & Beach, 1996). Cdc25a dephosphorylates and activates cyclin E/Cdk2 and cyclin A/Cdk2 complexes that promote the G1/S transition (Blomberg & Hoffmann, 1999).

Cell cycle inhibitory proteins negatively regulate both pathways promoting entry of cells into S phase. These proteins belong to INK4 and Kip/Cip groups (Sherr & Roberts, 1995). Four INK4 family proteins (p15INK4B, p16INK4A, p18INK4C and p19INK4D) bind to and inactivate cyclin dependent kinases and thus function as tumour suppressors. Inactivation of CDK prevents Rb phosphorylation leading to cell cycle arrest at the G1/S checkpoint. Loss of INK4 has been detected in various types of cancer (Sherr & McCormick, 2002; Krimpenfort et al., 2007). In addition, mice deficient for Ink4a developed melanoma (Krimpenfort, Quon, Mooi, Loonstra, &
Berns, 2001). The Kip/Cip family includes three members, p27Kip1, p57Kip2 and p21Cip1, which inhibit Cdk2 and Cdk4 activity preventing entry into the S phase (Blain, Montalvo, & Massague, 1997). Inactivation of Kip/Cip proteins has been associated with carcinogenesis (Bottini et al., 2009; Borriello et al., 2011; Li, Ji, Liu, Li, & Zhou, 2012).

Inactivation of the INK4 locus often results in inactivation of another gene product from an alternative reading frame that overlaps sequences encoding p16INK4A (Quelle, Zindy, Ashmun, & Sherr, 1995). This gene encodes the p14ARF protein that is required for activation of p53 (Kamijo et al., 1997). p53 induces expression of target genes which inhibit pRb phosphorylation thereby preventing G1/S progression (Brugarolas et al., 1999). The critical role of p53 in preventing entry of cells with DNA damage into S phase was confirmed by its deregulated expression in human tumours (Ozbun & Butel, 1995).

1.7.3. TLX1 and the G1/S checkpoint

The first data indicating that TLX1 may influence G1/S cell progression were published in 1993. Zhang et al reported that endogenous TLX1 RNA and protein levels were increased in Jurkat T cells at the G1/S phase and downregulated toward the G2/M checkpoint (Zhang, Gong, Minden, & Lu, 1993). Using oligonucleotide microarray analysis, Ferrando et al found that high levels of TLX1 were correlated with increased expression of the c-MYC proto-oncogene in paediatric T-ALL samples (Ferrando et al., 2002). Moreover, Korsmeyer et al detected that TLX1 bound PP1/PP2A phosphatases, resulting in abrogation of G2/M cell cycle arrest (will be discussed in the next section) (Kawabe, Muslin, & Korsmeyer, 1997). Based on these findings and the fact that PP1 phosphatases also function at the G1/S checkpoint, the role of TLX1 in G1/S progression was first hypothesized.

To further elucidate pathways deregulated by abnormal TLX1 expression, Riz et al performed a high throughput analysis of gene expression profiles of KP3 and SIL cell lines established from patients with T-ALL expressing high levels of TLX1 (Dube et al., 1991; Hatano, Roberts, Minden, Crist, & Korsmeyer, 1991). Their expression profiles were compared with profiles of Jurkat T cells transfected with TLX1 and with control Jurkat T cells transfected with the MSCVHyg retrovirus (Riz & Hawley, 2005). Numerous differentially expressed genes were detected. Some of the identified genes belonged to the E2F and c-Myc initiated transcriptional networks, which are critical for G1/S progression.
Previous experiments using truncated TLX1 proteins suggested that the direct interaction of the TLX1 homeodomain and the regulatory regions of target genes was a prerequisite for the oncogenic potential of TLX1 (Owens et al., 2003). In opposition to this, new data of genome wide expression profiling suggested the possibility that transcriptional deregulation of TLX1 target genes involved in G1/S progression was caused by homeodomain interaction independent mechanism. Thus, Berndt et al reported that PP1 dephosphorylates Rb during G1 progression (Berndt, Dohadwala, & Liu, 1997). They proposed that G1/S deregulation might be achieved by the TLX1-PP1-Rb-E2F/MYC axis. In this model it was suggested that TLX1 associated with PP1 and repressed its activity. When repressed, PP1 was unable to dephosphorylate Rb. In its hyperphosphorylated state, Rb was unable to repress the activity of the E2F and therefore PP1 dependent G1 arrest was abrogated. Activation of c-MYC resulted in dephosphorylation and activation of cyclin E/Cdk2 and cyclin A/Cdk2 complexes that promoted G1/S cell transition. Thus, TLX1 may activate E2F downstream target genes indirectly via interaction with PP1 and c-MYC activation (Figure 1.6).

1.7.4. The G2/M checkpoint

The G2/M checkpoint prevents cells with damaged or incompletely replicated DNA to enter into mitosis. Transition of cells through G2/M is initiated by activation of the Cdc2 kinase/cyclin type B complex (Cdc2/cyclin B1), also known as the maturation promoting factor (MPF) which is only active at the G2/M checkpoint. During the G2 phase, retention of MPF in the cytoplasm is mediated by the exportin protein, CRM1 (Hagting, Karlsson, Clute, Jackman, & Pines, 1998). As cells approach the G2/M checkpoint, binding of exportin to MPF is blocked, which allows the Cdc2/cyclin B1 complex to accumulate in the nucleus (Yang et al., 1998). To be active and to induce entry into mitosis, the Cdc2/cyclin B1 complex must also be dephosphorylated at threonine 14 and at tyrosine 15 by Cdc25 phosphatases (Porter & Donoghue, 2003). The loss of inhibitory phosphorylation causes activation of MPF, which in turn activates proteins required for chromatin condensation and mitotic spindle dynamics required for entry into mitosis (Doree, Labbe, & Picard, 1989).

In the case of DNA damage, entry into mitosis is prevented by two mechanisms and both of them alter the activity of MPF. The first mechanism relies on inactivated phosphorylation of Cdc2 at the Thr14 and Tyr15 residues by Wee1 and Myt1 kinases whose inactivation causes acceleration of the cell cycle (Mueller & Leise, III, 2005). The second mechanism is achieved
by the protein kinases Chk1 and Chk2. When DNA is damaged or unreplicated, these kinases
become activated and phosphorylate the Cdc25 phosphatase. Phosphorylation of Cdc25 inhibits
its activity and promotes its sequestration outside the nucleus and therefore prevents it from
activating the Cdc2/cyclin B complex (Sanchez et al., 1997; Zeng et al., 1998).

In addition to inactivation of MPF by kinases, other proteins act at the G2/M checkpoint
to facilitate cell cycle arrest and allow time to repair damaged DNA or induce apoptosis. For
example, BRCA1 is localized to sites of damaged DNA in a large BRCA1-associated genome
surveillance complex (BASC). This complex contains proteins that repair double-strand DNA
breaks and ATM/ATP kinases that activate CHK1/CHK2 kinases (Wang et al., 2000;
Chaturvedi et al., 1999). Another related protein is p53, which senses damaged DNA during G2
and activates genes involved in apoptosis (Vogelstein, Lane, & Levine, 2000). Also, p53
activates p21/waf1 which blocks activating phosphorylation of CDC2 thus inducing G2 arrest
(Smits et al., 2000). Another downstream target of p53, the GADD45, is also implicated in
G2/M arrest. It has been shown that GADD45 binds CDC2 and dissociates it from cyclin B1,
thus transforming MPF into an inactive complex thereby maintaining cell cycle arrest (Zhan et
al., 1999). A binding partner for p53, the 53BP1 protein, acts as an adapter to bring proteins and
enzymes together at loci of DNA damage. For example, 53BP1 was detected in one
BRCA1/CHK1 complex and its reduction with siRNA resulted in decreased levels of p53 and a
reduction in the phosphorylation of CHK1 in irradiated cells (Wang, Matsuoka, Carpenter, &
Elledge, 2002).

1.7.5. **TLX1 and the G2/M checkpoint**

The first data describing how deregulated expression of **TLX1** interferes with normal G2/M cell
cycle progression were obtained by Kawabe in experiments with Xenopus oocytes and Jurkat T
cells transfected with **TLX1** (Kawabe, Muslin, & Korsmeyer, 1997). To identify that the TLX1
motif responsible for interaction with the serine threonine phosphatases PP2A and PP1, deletion
mutants of the TLX1 protein were generated and protein associations tested. It was shown that
the TLX1 amino terminal, between amino acids 149-190, bound to the catalytic subunits of
PP2A and PP1, and that this association prevented activation of MPF at G2/M. To assess the
consequences of **TLX1** - PP2A and PP1 interactions, Xenopus oocytes were injected with
recombinant TLX1 and TLX2 proteins and rates of germinal vesicle breakdown (GVBD), which
normally occurs at M phase, were compared. Normally TLX2 proteins do not interact with
PP2A and PP1. It was noticed, that oocytes injected with TLX1 exhibited a higher rate of GVBD relative to those injected with TLX2. These data indicated that TLX1 suppressed phosphatase activity that allowed oocytes to enter into M phase faster. In the next experiment, Jurkat T cells were transfected with a vector expressing $\text{TLX1}$ and cells were exposed to $\gamma$ irradiation, thus introducing double strand DNA breaks. Cells lacking expression of TLX1 accumulated at G2/M checkpoint, whereas cells expressing TLX1 did not undergo a G2/M arrest. These studies indicated that interactions between TLX1 and the PP2A and PP1 phosphatases resulted in abrogation of G2/M cell cycle checkpoint (Figure 1.6).
Figure 1.6. Possible cell cycle target genes of the TLX1.
Simplified scheme of signaling pathways which regulate cell cycle checkpoint progression and may be malfunctioned by the TLX1 overexpression. The G1/S transition is regulated PP1-Rb-E2F/MYC axis. TLX1 may activate E2F downstream target genes indirectly via interaction with PP1 and c-MYC activation. TLX1 targets PP1 that prevents Rb dephosphorylation and Rb unable to repress activity of the E2F. TLX1 may activate c-MYC that resulted in activation of cyclin E/Cdk2 and cyclin A/Cdk2 complexes that promoted G1/S cell transition. TLX1 bind to the catalytic subunits of PP2A and PP1 and that this association prevents activation of MPF at G2/M. TLX1 mediates expression of cyclin B1 which as a part of MPF regulates G2/M transition.
The ability of TLX1 to bind serine-threonine phosphatases at G2/M was confirmed in our laboratory (Chen et al., 2010). Thus, TLX1-3T3 cells treated with colchicine or nocodazole exhibited the presence of an additional, low mobility band on immunoblots. A linear correlation between the low mobility band with the percentage of cells arrested at G2/M and the presence of the radiolabeled 35S-γATP TLX1 isoform indicated that this additional band represented a phosphorylated form of TLX1. These studies suggested that TLX1 was phosphorylated during mitosis. FLAG-tagged TLX1 truncated mutants were also generated in our laboratory to detect the region of TLX1 that was phosphorylated during the G2/M portion of the cell cycle. These studies indicated that the region between amino acids 187-260 was phosphorylated. Taking into account the presence within this region of the Thr247 residue, which is a distinctive feature of the TLX family of homeodomain proteins, point mutants where Thr247 was substituted with an alanine (T247A) were generated. Phosphorylation of the T247A mutant was not detected after treatment with nocodazole suggesting that Thr247 was the amino acid that underwent G2/M cell cycle specific phosphorylation. In addition, recombinant PP1 catalytic subunit added with nocodazole to TLX1-3T3 cultures eliminated phosphorylated low mobility TLX1 band indicating that PP1 phosphatase plays a role in regulating the TLX1 phosphorylation state. Altogether these data indicated that TLX1 was phosphorylated at Thr247 at G2/M and this phosphorylation regulates activity of TLX1 in a cell cycle specific manner.

Given Thr247 is a defining feature of the TLX family, we speculated that phosphorylation of this amino acid might affect expression of downstream target genes. Data obtained in our laboratory indicated that cyclin B1, a critical player in the MPF, was a putative target for TLX1 (Chen, Kwon, Lim, Dube, & Hough, 2006). Chen et al demonstrated, using chromatin immunoprecipitation assays, that a region more than 4 kb upstream of the cyclin B1 start site bound TLX1. The next step was to investigate the effect of phosphorylation on cyclin B1 activation. To accomplish this, mutants that substituted Thr247 with alanine (T247A) or with glutamic acid (Thr247E) to mimic constitutive phosphorylation, were generated. Western immunoblot analysis detected elevated levels of cyclin B1 expression in NIH 3T3 cells in the presence of TLX1 and decreased levels in the presence of the Thr247E mutant. EMSA and ChIP analyses detected a decreased association of T247E mutants with the cyclin B1 promoter, whereas similar association with cyclin B1 promoter of the T247A mutant and wild type TLX1 was noted. Taken together these data indicated that TLX1 mediated the regulation of cyclin B1, a critical player in G2/M progression, via the Thr247 residue, in a phosphorylation dependent manner.
1.7.6. Spindle assembly checkpoint

The spindle assembly checkpoint (SAC) controls proper attachment of chromosomes to spindle microtubules and ensures accurate chromosome segregation during mitosis at the metaphase-anaphase transition. The SAC machinery includes such well characterized members as BUB1, BUBR1, Mps1 kinases and MAD1/MAD2 proteins (Musacchio & Salmon, 2007). CHK1, along with the recently identified kinases, PRP4 and TAO1, have also been proposed to participate in the SAC (Zachos et al., 2007; Hubner et al., 2010). Together these checkpoint proteins form the mitotic checkpoint complex (MCC). The MCC negatively regulates the ubiquitin ligase anaphase-promoting complex (APC) by its binding to CDC20 which is required for activation of APC via MAD2 (Yu, 2002). When activated, the APC ubiquitinates cyclin B1, which is a critical factor for activation of CDK1, a kinase required for exit from mitosis.

Another APC target is securin, an inhibitor of the protease separase, required to cleave the cohesion complex that holds chromatids together during the early stages of mitosis (Harper, Burton, & Solomon, 2002). Combined, the ultimate goal of this complex is to prevent separation of sister chromatids and activation of the master mitotic kinases until signals detecting equal tension from properly attached kinetochores to the spindle poles by microtubules of all metaphase chromosomes are received (Uchida et al., 2009). Sister chromatids are held together by cohesion complexes which should be cleaved to complete anaphase (Peters, 2006). As soon as the signal confirming satisfied spindle assembling is received, the MCC is degraded. Degradation of the MCC is initiated when the p31comet inhibitor binds to the MAD1-MAD2 complex and to CDC20 (Yang et al., 2007). This binding results in the phosphorylation of MAD2 and its sequestration away from the MCC along with CDC20 ubiquitination that together disassembles the APC inhibitory complex (Kim et al., 2010; Reddy, Rape, Margansky, & Kirschner, 2007). This results in activation of separase which cleaves the cohesion ring thus initiating the separation of sister chromatids that trigger anaphase. Degradation of cyclin B1 at this stage initiates cytokinesis and promotes exit from mitosis (Echard & O'Farrell, 2003). (Figure 1.7)
Figure 1.7. **TLX1 and the spindle assembly downstream target genes.**

During prometaphase unattached kinetochores catalyze the formation of the mitotic checkpoint complex (MCC). This complex is composed of Bub1, BubR1, Bub3, Mps1, Mad1/Mad2, Cdc20, Chk1, Prp4, Tao1 and inhibits APC/C. Once kinetochores properly attached, the p31comet inhibitor binds to the MAD1-MAD2 complex, phosphorylates and sequesters its away from the MCC. Sequestration of the MAD1-MAD2 complex along with CDC20 ubiquitination disassembles the APC inhibitory complex that results in activation of separase and degradation of Cyclin B1. Combined these result in cleavage of the cohesion ring, separation of sister chromatids and mitotic exit. Tlx1 overexpression may interfere with normal chromatid separation by interaction with pericentromeric satellite 2 DNA sequences which are involved in sister chromatid cohesion and by upregulation of Cyclin B1. This illustration was modified from Lara-Gonzalez, Westhorpe and Taylor, 2012.
1.7.7. *TLX1* and the spindle assembly checkpoint

The first data suggesting a role of *TLX1* in the regulation of chromosome segregation and spindle assembly check point function were reported by Heidary *et al* (Heidari, Rice, Phillips, Kees, & Greene, 2006). Using the whole genome PCR approach and chromatin immunoprecipitation assays, they showed that TLX1 proteins directly interact with pericentromeric satellite 2 DNA sequences, a component involved in cohesion of sister chromatids and regulation of kinetochore formation. In addition, Chen *et al* shown that overexpression of the *TLX1* modulated expression of *cyclin B1* and was associated with accelerated passage through the G2/M phase of the cell cycle (Chen *et al.*, 2010) (Figure 1.7).

Our laboratory first provided convincing evidence that abnormal expression of *TLX1* altered normal chromosomal segregation in premalignant B cells and in mature B cell lymphomas. Thus, B cells overexpressing *TLX1* and deficient in the ubiquitin protein ligase, Ubr1, were prone to a hypoploid karyotype. In addition, micronucleus assays detected an increased presence of micronuclei in *IgHμ-TLX1Tg/Ubr1−/−* primary B lymphocyte cultures (Chen, Kwon, Lim, Dube, & Hough, 2006). Moreover, lymphomas that developed in *IgHμ-TLX1Tg* mice exhibited an abnormal karyotype due to deregulated expression of mitotic regulators and increased development of micronuclei, which was confirmed by microarray analysis, qPT-PCR and micronucleus assays (Chen *et al.*, 2006). Ferrando *et al* also reported aneuploidy in *TLX1* overexpressing T-ALL samples and deregulated mitotic genes in *TLX1* overexpressing premalignant thymocytes detected by karyotyping and GSEA analyses (De *et al.*, 2010). Combined these studies indicate that deregulated expression of *TLX1* contributes to the development of T and B cell malignancies by deregulation of chromosome segregation. Taken together, these data indicate that deregulation of cell cycle progression is probably a predominant mechanism of TLX1-initiated malignancies.

1.8. Experimental rational and hypothesis

Numerous data indicate that *TLX1*-associated malignancies require the accumulation of additional mutations for initiation and progression of disease (Hough *et al.*, 1998c; Chen, Kwon, Lim, Dube, & Hough, 2006; De *et al.*, 2010; Rakowski, Lehotzky, & Chiang, 2011). Also, mice transplanted with *TLX1*-ectopically expressing bone marrow progenitors developed malignancy only after 7-12 months (Hawley *et al.*, 1997). Moreover, all mouse models
overexpressing TLX1, either in B cells or T cells developed disease with delayed latency varying in average from six to fourteen months (Hough et al., 1998; De et al., 2010; Rakowski, Lehotzky, & Chiang, 2011).

An additional mutation that has been shown to be associated with the progression of TLX1-initiated disease includes inactivation of the Ubr1 gene, the E3 ubiquitin ligase family member, which resulted in a moderate, but statistically significant acceleration of lymphoma in IgHμ-TLX1Tg mice (Chen, Kwon, Lim, Dube, & Hough, 2006). Additional studies have shown that expression of the transcription factor Notch1 and tumour suppressor genes Bcl11b, Phf6 and Pten were dysregulated in the thymic tumours developed by p56Lck-TLX1 mice indicating that these genetic alterations were acquired during T-ALL progression (De et al., 2010). In agreement with these results, increased expression of the Notch1 was also detected in the thymic tumours developed by inducible Lck-TLX1 transgenic mice (Rakowski, Lehotzky, & Chiang, 2011). Combined, these findings demonstrate the potential of dysregulated TLX1 to initiate the premalignant phenotype but that additional mutations are required to convert premalignant cells to malignant cells. However, clearer understanding of the molecular mechanisms that collaborate in the pathogenesis of TLX1-induced disease is required. Therefore new approaches were proposed to more comprehensively identify the network of TLX1 collaborating genes and the population of cells that are the targets for the TLX1-driven malignant transformation.

In humans, TLX1-induced T-ALL is characterized by a block in T-cell development at the early cortical CD4+CD8+ Double Positive stage. Tumour cells also showed an increased propensity to chromosomal abnormalities (Ferrando et al., 2002; Ferrando & Look, 2003; De et al., 2010; De & Ferrando, 2011). Taking into account tumour phenotype and the delayed time for TLX1-induced leukemia initiation, we speculated that thymocytes accumulated additional mutations during Double Negative stages of T cell development or during more immature stage designated as the Early Thymocyte Progenitor (ETP). Therefore, we hypothesized that unresolved double strand DNA breaks resulting from defective non homologous end joining (NHEJ) double strand DNA repair might act as additional genetic mutations and contribute to T-ALL progression in TLX1 overexpressed thymocytes. Specifically, by generation of the double mutant IgHμ-TLX1TgPrkdcScid/Scid mouse model system we predicted that TLX1 overexpression in DNA-PK deficient thymocytes would:

1) accelerate the onset of hemopoietic malignancies in IgHμ-TLX1TgPrkdcScid/Scid mice
2) elicit new downstream target genes that synergize with ectopic TLXI expression in the cellular transformation process

Three **Specific Aims** of this study were proposed to comprehensively test this hypothesis:

(1) Determine whether dysregulated expression of TLXI in immature thymocytes lacking expression of DNA-PK accelerated the development of T-ALL by facilitating the survival of cells with genomic instability using both *in vitro* cell culture systems and *in vivo* animal models.

(2) Identify target cells susceptible to genetic mutations responsible for the initiation of leukemia and to define genes that, when mutated, dysregulated genetic pathways eventually resulting in malignancy.

(3) Determine: a) whether dysregulated expression of TLXI in myeloid progenitors lacking expression of DNA-PK accelerated the development of AML via deregulation of apoptotic and proliferative pathways; b) general molecular mechanisms of action of the TLXI oncogene in the initiation and progression of hemopoietic malignancies.

### 1.9. Thesis overview

The following thesis presents my findings addressing the above mentioned Specific Aims to identify genes and molecular mechanisms which functionally collaborate with TLXI within a certain cell fraction in initiating malignancies.

In Chapter 3, I described the T-ALL phenotype of *IgHμ-TLXI*TgPrkdc<sup>Scid/Scid</sup> mice, the results of microarray analysis and functional *in vitro* and *in vivo* assays performed with the premalignant thymocytes of *IgHμ-TLXI*Tg<sup>Prkdc<sup>Scid/Scid</sup> mice. Ectopic expression of TLXI and loss of DNA-PK accelerated both T-ALL and AML in *IgHμ-TLXI*Tg<sup>Prkdc<sup>Scid/Scid</sup> mice. The chapter also details a number of possible TLX1 target genes and molecular mechanisms responsible for the initiation of T-ALL as a result of TLXI overexpression in DNA-PK-deficient thymocytes. I also identified secondary mutations which may play a supportive role in the pathogenesis of TLXI-initiated T-ALL.
Chapter 4 is focused on the identification of T-ALL-ICs in \( \text{IgH} \mu-TLX1^{Tg}\Prkdc_{\text{Scid/Scid}} \) mice. Using transplantation assays, subfractions of flow sorted T-ALL cells from \( \text{IgH} \mu-TLX1^{Tg}\Prkdc_{\text{Scid/Scid}} \) mice were injected into secondary and tertiary sublethally irradiated \( \Prkdc_{\text{Scid/Scid}} \) recipients. These studies showed that both CD4\(^+\)CD8\(^-\) and CD4\(^-\)CD8\(^+\) subpopulations were able to induce T-ALL in recipient mice. Furthermore, subfractionation of DN cells on the basis of CD44 and CD25 indicated that all DN-like subfractions were able to initiate T-ALL in recipient mice. However, sorting on the basis of c-kit expression resulted in the identification of a subpopulation of tumour cells that was enriched in T-ALL-ICs. Thus, the Early Thymocyte Progenitors fraction of thymocytes, characterized by expression of ckit\(^+\)CD44\(^+\)CD25\(^-\)CD4\(^-\)CD8\(^-\), may represent the origin of the T-ALL-IC but the subsequent accumulation of mutations in both ETP and their progeny has resulted in a large array of phenotypically different subpopulations of tumour cells with the ability to initiate T-ALL in recipient mice.

In Chapter 5 I investigated in greater details the AML phenotype developing in \( \text{IgH} \mu-TLX1^{Tg}\Prkdc_{\text{Scid/Scid}} \) mice and studied the molecular mechanisms dysregulated in myeloid progenitors. Data obtained in series of \textit{in vitro} and \textit{in vivo} functional assays with myelocytes allowed me to demonstrate that molecular mechanisms responsible for the development of AML in \( \text{IgH} \mu-TLX1^{Tg}\Prkdc_{\text{Scid/Scid}} \) mice are similar to those dysregulated by ectopic expression of \( TLX1 \) in thymocytes deficient in the DNA-PK. Indeed, decreased apoptosis and increased proliferation were detected both in premalignant myeloid progenitors and thymocytes of \( \text{IgH} \mu-TLX1^{Tg}\Prkdc_{\text{Scid/Scid}} \) mice relative to \( \Prkdc_{\text{Scid/Scid}} \) control littermates. Data presented in this chapter indicated that the abnormal expression of \( TLX1 \) manifests its oncogenic function in thymocytes and myeloid cells although the early stages of B cell development were not affected.

Finally, in Chapter 6 I summarized the work presented in this dissertation and proposed future experiments that might be undertaken to better understand the role of the \( TLX1 \) gene in leukemia progression and to identify the subpopulation of AML-initiating cells in \( \text{IgH} \mu-TLX1^{Tg}\Prkdc_{\text{Scid/Scid}} \) mice.
Chapter 2

Materials and Methods
2.1. Animal procedures

2.1.1. Construction of IgHμ-TLX1Tg and IgHμ-TLX1TgPrkdcScid/Scid mice

Mice were bred and maintained under pathogen-free conditions and monitored daily for signs of illness. Mice were euthanized and necropsied when moribund. All manipulations were performed under Canadian Animal Care protocols and approved by the Sunnybrook Research Animal Care Committee (Permit number: 12-005).

Double mutant IgHμ-TLX1TgPrkdcScid/Scid mice were generated by mating previously described IgHμ-TLX1Tg mice (original name IgHμ-HOX11 transgenic mice (Hough et al., 1998; Rosic-Kablar, Chan, Reis, Dube, & Hough, 2000), bred onto the CD1 genetic background, with CB17 ICR- PrkdcScid/Scid mice bred onto an ICR genetic background (Charles River Laboratories (Wilmington, MA, USA). IgHμ-TLX1TgPrkdcScid/Scid and PrkdcScid/Scid mice were mated and progeny were genotyped and used to generate cohorts.

The presence of the IgHμ-TLX1 transgene was revealed by PCR amplification of a 287-bp fragment using 5′-AACCGCAGATACACAAAGGA-3′ forward and 5′-TGGGCCAGGCTCTTCTGGAA-3′ reverse primers. Mice homozygous for the Prkdc mutation (PrkdcScid/Scid) were detected by flow cytometry analysis of peripheral blood stained with pan B-cell specific (B220 clone RA3-6B2, 1:200 dilution) and pan T-cell specific (CD3 clone 145-2C11, dilution 1:100) antibodies. Peripheral blood (100µl) was collected from the punctured tail vein by pipetting with 100 µL tip, mixed with 10µl of Heparin in the Eppendorf tube and washed in D-PBS containing 0.5% BSA (FACS buffer). 2.0 ml of 1X Red Blood Cell Lysis Buffer was added to tube. The detailed protocol for the red blood cell lysis is available from Biolegend http://www.biolegend.com/media_assets/support. Mice were maintained in pathogen-free conditions and monitored daily for the onset of leukemia. All manipulations were performed in accordance with Canadian Animal Care protocols and approved by the Sunnybrook Research Animal Care Committee.

2.1.2. Transplantation of tumour cells into PrkdcScid/Scid recipients

Six to seven weeks old-recipient PrkdcScid/Scid female mice bred onto a Balb/c genetic background (Jackson Laboratories) were used as transplantation recipients. This strain was chosen as they are
less susceptible to the development of hematopoietic malignancies as compared with \textit{Prkdc}^{Scid/Scid} (ICR) mice. Recipients received sublethally (300 rads) total body irradiation 24 hours prior to cell injection (Shepherd Mark 1 $^{137}$ cesium gamma sours; J. L. Shepherd & Associates, Ca, USA). Drinking water was supplemented with Baytril (100 mg/L; Bayer Inc., ON, Canada). Tumours were harvested aseptically using blunt dissections, sampled for histology and flow cytometry and processed to single cell suspensions. Tumours were mechanistically dissociated by mincing, passaged through a 40 µM sterile screen and resuspended in D-PBS containing 0.5% BSA. Red blood cells in the splenic tumours were eliminated by Red Blood Cell Lysis Buffer according standard protocol [http://www.biolegend.com/media_assets/support](http://www.biolegend.com/media_assets/support), washed twice, counted using 0.4% trypan blue and diluted to desired concentration. Purified tumour cell subpopulations were obtained after bulk tumour depletion of lineage-restricted cells using biotin-labeled antibodies specific for mouse antigens Gr1, CD11b, Ter119 and F4/80 (BD Pharmingen). Biotin-conjugated antibodies were reacted with streptavidin magnetic beads and lineage positive cells depleted using the MACS column sorter according to manufacturer’s instructions (Miltenyi Biotech, CA USA). Lineage negative (lin$^-\$) tumour cells were sorted for ckit$^+$CD44$^+$CD25$^+$CD4$^-$CD8$^-$Lin$^-$, ckit$^-$CD44$^+$CD25$^+\$CD4$^-$CD8$^-$Lin$^-$, ckit$^+$CD44$^+$CD25$^+\$CD4$^+$CD8$^+$Lin$^-$, ckit$^+$CD44$^+$CD25$^+\$CD8$^+$Lin$^-$ and ckit$^+$CD44$^+$CD25$^+$CD8$^+$Lin$^-$ on a FACS ARIA flow sorter with II FACS DIVA software (BD Biosciences). To provide easier access to a \textit{Prkdc}^{Scid/Scid} lateral vein, the mouse was warmed with a heat lamp prior to injection, isoflurane-anesthetized and the desired concentration of tumour cells diluted in 150 µl of FACS buffer was transplanted in the tail vein with a 30G needle. Mice were further monitored for signs of illness and moribund mice were sacrificed and thymus, spleen and bone marrow were harvested and sampled for histology and FACS analysis.

2.1.3. Statistics

Disease-free survival during an observation period was calculated from the date of transplantation to the date when mice became terminally ill using the Kaplan-Meier estimator. Column comparison analysis was performed using the logrank test with the GraphPad Prism 4 software. All tests with \( p < 0.05 \) were accepted as statistically significant. The LIC frequency was determined using Poisson distribution statistics and the L-Calc Version 1.1 software program providing 95% confidence intervals and t-test calculations to compare tumour-initiating
2.2. Cell culture procedure

2.2.1. Co-culture of thymocytes on OP9-DL1 stromal cells

GFP-expressing OP9-DL1 cells (kindly provided by Dr. Juan Carlos Zúñiga-Pflücker) were cultured in 6-well dishes (BD Falcon) to 80% confluence in αMEM (Gibco) medium containing 20% FBS (Hyclone) and 100 U/ml Penicillin/Streptomycin (Gibco). Cultures were incubated in a humidified atmosphere at 37°C with 5% CO₂. Fetal livers were dissected from E13.5 embryos and dispersed to single cell suspensions in HBSS supplemented with 10% FBS by passage through a 40 μm nylon cell strainer (BD Falcon). Ten percent of cells were used for genotyping with the remaining cells depleted of lineage-restricted cells using biotin-labeled antibodies specific for mouse lineage specific antigens (B220, Gr-1, Mac-1, Ter119 and F4/80; BD Pharmingen). Lineage-positive cells were bound to Miltenyi Biotec streptavidin magnetic beads and depleted using a MACS column according to manufacturer’s instructions (Miltenyi Biotech, Auburn, CA USA). Lineage negative (Lin⁻) hematopoietic stem cells (HSC) were sorted for expression of the CD117/c-kit (APC) and Sca-1 (PE) antigens on a FACS ARIA flow sorter using II FACS DIVA software (BD Biosciences). C-kit⁺Sca⁻Lin⁻ cells (10³) were resuspended in 3 ml of complete DMEM medium supplemented with murine Flt-3 Ligand (5 μg/ml), IL-7 (5 μg/ml) and SCF (10 μg/ml; all from R&D Systems, Minneapolis MN, USA) and plated onto an OP9-DL1 feeder layer. Cells were harvested on day-7, passed through a 70 μm cell strainer (BD Falcon), stained with CD44 (PE) and CD25 (APC) antibodies and thymocyte subpopulations sorted as follows: CD44⁺CD25⁻ (DN1), CD44⁺CD25⁺ (DN2), CD44⁻CD25⁺ (DN3) and CD44⁻CD25⁺. OP9-DL1 cells were distinguished from thymocytes based on GFP expression.

2.2.2. Methylcellulose culture (Colony Forming Unit assays)

Methylcellulose culture medium (MethoCult® GF M3434) was prepared by StemCell Technologies (Vancouver, BC, Canada). The medium contained 1% methylcellulose in Iscove’s Modified Dulbecco’s Medium (IMDM), 1% bovine serum albumin, 30% fetal bovine serum (FBS), 2mM L-glutamine, murine IL-3 (5 ng/ml), IL-6 (5 ng/ml) and SCF (10
ng/ml), erythropoietin (5 ng/ml). 1.5x10^5 and 3x10^5 bone marrow cells or 3x10^5 and 3x10^6 spleen cells were mixed with 4.0 ml of MethoCult and 1 ml of the MethoCult cell mixture was plated into culture dishes. Colonies were incubated in moisturized chambers (BD Falcon) for eight days in a humidified atmosphere at 37°C with 5% CO₂.

All dishes were scored for the number of colony per dish. Colonies were then harvested, MethoCult washed out with HBSS supplemented with 10% FBS and the total number of cells per dish was determined. The average number of cells per colony was calculated by dividing a total number of cells per dish by the counted number of colonies.

### 2.3. Molecular Biology Procedures

#### 2.3.1. Primers

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<td><strong>Quantitative Reverse Transcriptase-PCR (qRT-PCR) primers</strong></td>
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### 2.3.2. Reverse Transcriptase PCR (RT-PCR)

Genomic DNA was purified using DNAzol (Invitrogen) and total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA, USA). Total RNA was amplified with MessageAmp™ II aRNA kit (Invitrogen) according to the manufacturer specifications. The *IgHμ-TLX1* transgene was detected with the following primers: h*TLX1*-1560F (5'-TGGGCATCTATGGGAGAGTG-3') and h*TLX1*-1799R (5'-CTGGATTGGGCTGGGATGT-3'). The following PCR conditions were used: initial denaturation at 95°C for 3 minutes; 34 cycles of denaturation (95°C) for 30 seconds, annealing (55°C) for 30 seconds, extension (72°C) for 30 seconds and a final cycle of 7 minutes at 72°C. All reactions were carried out in a 25 µl volume containing 50-100 ng genomic DNA for PCR or 10-50 ng total RNA for reverse transcribed cDNA, and 0.2 µmol of each primer, 10x buffer, 1.5 U AmpliTaq DNA Polymerase and 2.5 µM MgCl₂ (Invitrogen) using the PCR C1000 Thermal Cycler (BioRad). The identity of PCR products was verified by

<table>
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<th>Primer Reverse</th>
<th>Temperature</th>
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DNA sequencing (The Center for Applied Genomics, The Hospital for Sick Children, Toronto, ON).

**2.3.3. Quantitative Reverse Transcriptase PCR (qRT-PCR)**

First-strand cDNA synthesis was performed using the SuperScript III First Strand cDNA reverse transcriptase kit and random primers (Invitrogen). qRT-PCR was performed with the QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer’s protocol with 1 µl of the reverse transcription reaction product (from 10-50 ng total RNA) amplified for 39 cycles with an ABI PRISM 7000 Applied Biosystems Sequence Detection System and analyzed with ABI Prism 7000 SDS software (Applied Biosystems, Carlsbad, CA, USA).

**2.4. Gross tissue and cell phenotype analysis**

**2.4.1. Sample storage**

Thymi, spleens, bone marrow and tumours from healthy and moribund *IgHμ-TLX1Tg Prkdc<sup>Scid/Scid</sup>* and *Prkdc<sup>Scid/Scid</sup>* mice were harvested and aliquoted for DNA, RNA, histology, chromosome and flow cytometry analysis. Cells were frozen in fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) containing 10% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA).

**2.4.2. Histology and Immunohistochemistry**

Organs isolated from moribund *IgHμ-TLX1Tg Prkdc<sup>Scid/Scid</sup>* and *Prkdc<sup>Scid/Scid</sup>* mice as well as healthy *IgHμ-TLX1Tg Prkdc<sup>Scid/Scid</sup>* mice were fixed in 4% PFA overnight, paraffin embedded, sectioned and stained with hematoxylin-eosin (Sigma, St. Louis, MO, USA). A certified pathologist performed all morphological interpretations of tissue sections. Tumours were also cryopreserved in O.C.T. compound (Tissue-Tek; Fisher Scientific, Waltham, MA, USA) for cryosectioning and 5 µm sections were stained with purified rat anti-mouse Thy1.2 (clone53-2.1; BD Pharmingen). Antibody binding was visualized using an anti-rat IgG-HRP (Santa Cruz Biotechnology) and Peroxidase Substrate Kit DAB (Vector Laboratories, Inc.). Sections were counterstained with hematoxylin 1:5 (Fluka).
2.4.3. Flow cytometry

Single cell suspensions of murine thymi, spleens and bone marrow were prepared in D-PBS containing 0.5% BSA (FACS buffer). Fc receptor blocking was performed by preincubation with an unconjugated anti-Fcγ antibody (clone 2.4G2; 1mg/ml 1:500 for thymocytes, 1:200 for splenocytes and bone marrow cells) for 10 minutes on ice. 10^6 cells were then stained with cocktails of fluorochrome-conjugated antibodies appropriately diluted in FACS buffer for 30 minutes at 4°C then washed twice in 5 ml of FACS buffer. Fluorescein isothiocyanate (FITC), R-phycoerythrin (PE) or allophycocyanin (APC)-conjugated antibodies and dilutions used were: CD25-APC (Clone PC61.5 dilution 1:300), CD44-PE (Clone IM7 1:200), CD4-PE-Cy7 (Clone RM4-5 dilution 1:200), CD8-APC-Cy7 (Clone: 53-6.7 1:200), CD3-PerCP-Cy5 (Clone: 145-2C11 dilution 1:100), TCR-β-Alexa Fluor 700 (Clone: H57-597 1:100), c-kit-APC (CD117, Clone 2B8 dilution 1:800), Sca1-PE (Clone: E13-161 dilution 1:100) (all from BD Pharmingen, Franklin Lakes, NJ, USA), Thy1.2-PE (Clone 30-H12 1:400), TCR-αβ-FITC (Clone H57 1:200), B220-PE (Clone RA3-6B2 1:200), Gr-1-FITC (Clone RB6-8C5 1:100), Mac-1-APC (Clone M1/70 1:200), CD4-APC (Clone GK1 1:800), CD8-FITC (Clone 53-6.7.2 1:400) (all from the Sunnybrook Core Hybridoma Facility, Toronto, ON, Canada). Cells were resuspended in 300 μl FACS buffer containing 1μg/ml PI (Invitrogen, Carlsbad, CA, USA) for dead cell exclusion. Samples were analyzed on a FACS Calibur cytometer using CellQuest Pro software (BD Biosciences).

2.5. Functional analysis

2.5.1. Single cell preparation of bone marrow, thymi and spleens samples

Femurs were crushed with a pestle and then dispersed to single cells by gentle pipetting through a 1000 μl pipette tip. Spleens and thymi were harvested and dispersed to single cell suspensions in HBSS supplemented with 10% FBS by passage through a 40 μm nylon cell strainer (BD Falcon).
2.5.2. Calculation of the absolute number of cells

Total bone marrow and spleen cellularity was calculated by counting cells stained with trypan blue (Invitrogen) in a counting chamber using light microscopy. Cells that did not take up trypan blue were considered viable. Absolute thymocyte numbers were determined by multiplying the percentage of DN1, DN2 and DN3 cells (as determined by flow cytometry based on expression of CD44 and CD25) by the total number of thymocytes per thymus as determined by microscopic cell counting.

2.5.3. Cell death analysis

Thymocytes, bone marrow and spleen cells isolated from healthy, age and sex matched IgHµ-TLX1TgPrkdcScid/Scid and PrkdcScid/Scid littermates were resuspended in 500 μl Annexin V binding buffer and incubated with 2 μg/ml Annexin V-Cy5 antibody (BioVision, CA) with 1.0 μg/ml PI for 5 minutes at RT in the dark. Cells were analyzed using FACS Calibur (BD Biosciences) and CellQuest software.

2.5.4. In vivo bone marrow, thymocyte and spleen cells proliferation assay

Healthy IgHµ-TLX1TgPrkdcScid/Scid and age and sex matched PrkdcScid/Scid littermates were injected intraperitoneally with 150 μl of 10 mg/ml BrdU in sterile 1X Dulbecco’s phosphate-buffered saline (D-PBS) (BD Pharmingen). Mice were sacrificed 2 and 7 hours post injection, thymi, spleen and bone marrow were harvested and single cell suspensions prepared in D-PBS containing 2% FBS (Gibco). Cells were fixed in 80% ethanol for 30 minutes and washed in 1X D-PBS for 5 minutes. Cells were then treated with RNase (Invitrogen) and 1.5 M HCl for 30 minutes. 5x10⁵ - 10⁶ pretreated cells were stained with an anti-BrdU mouse monoclonal antibodies (1:100, cat# 4828-30-06, Trevigen, Gaithesburg, MD) for 30 minutes at 4°C, washed in 5 ml of 1X D-PBS and stained with a FITC-conjugated anti-mouse secondary antibody (1:500, cat# 554060, BD Pharmingen). After three washes, cells were resuspended in 300 μl 1X D-PBS and analyzed on FACS Calibur cytometer using CellQuest Pro software.
2.6. Cell cycle analysis

2.6.1. Bromodeoxyuridine (BrdU) labeling and bypass of the spindle cell cycle checkpoint

To induce mitotic arrest, day-4 IgH\(\mu\)-TLX1\(^{Ts}\)Prkdc\(^{Scid/Scid}\) and Prkdc\(^{Scid/Scid}\) c-kit\(^+\)Sca\(^+\)Lin\(^-\) HSC-derived T cell precursors grown on the OP9-DL1 co-culture were treated with 100 ng/ml colchicine overnight. BrdU was added to a final concentration of 20 \(\mu\)M and incubated at 37\(^\circ\)C for 2 hours. T-lineage cells were collected by centrifugation at 1,300 rpm for 5 minutes and fixed in 80\% ethanol, followed by DNA denaturation in 2 N HCl for 1 hour at 37\(^\circ\)C. Cells were pretreated by incubation with 1X D-PBS supplemented with 2\% BSA for 1 hour at 37\(^\circ\)C. Immunostaining was performed using an anti-BrdU antibody (Trevigen, Gaithersburg, MD) at a dilution of 1:100 for 2 hours at RT, followed by staining with a FITC-conjugated anti-mouse antibody (BD Pharmingen) at a dilution of 1:500 for 1 hour at RT. Counterstaining for detection of nuclei was performed with VECTASHIELD DAPI mounting medium (Vector Laboratories, Inc. Burlingame, CA). Slides were evaluated using fluorescence microscopy (Axiovert 200M, Zeizz, GmbH, Germany). In all, 20 random fields were scored for the frequency of BrdU positive cells.

2.6.2. Cell cycle analysis

Day-4 IgH\(\mu\)-TLX1\(^{Ts}\)Prkdc\(^{Scid/Scid}\) and Prkdc\(^{Scid/Scid}\) HSC-derived thymocytes, co-cultured in the OP9-DL1 system, were subjected to a double-thymidine block, treated with 100 ng/ml colchicine then harvested at 8 hour intervals, stained with PI and analyzed by flow cytometry for DNA content as previously described (Muehlbauer & Schuler, 2005). For each sample, 10,000 events were acquired using a FACS Calibur and cell cycle analysis was performed using FlowJo software (BD Biosciences).
2.7. Cytogenetic Procedures

2.7.1. Karyotyping (prepared by Dr. Mary Ann George (The Centre for Applied Genomics, MaRS Centre))

Primary leukemic cells were dissected from lymphoid tissues of moribund \( IgH\mu-TLXI^{Tg}Prkdc^{Scid/Scid} \) mice and immediately processed to single cell suspensions. Fresh tumour samples were placed in RPMI 1640 medium (Wisent; Montreal, Quebec, Canada) containing 10% FBS. Cells were arrested in metaphase by treatment with colcemid (0.03μg/mL; Invitrogen). Metaphase chromosomes were prepared according to standard cytogenetic protocols, using hypotonic 0.075M KCl and Carnoy’s fixative (Padilla-Nash, Wu, Just, Ried, & Thestrup-Pedersen, 2007). Slides were aged overnight at 55°C then G-banded using 4X USP Pancreatin (diluted to 0.4X) followed by staining with Leishmann/Giemsa stain. 20 metaphase spreads were analyzed and imaged with a CCD camera (VDS Vosskühler GmbH) using an Olympus microscope set in bright-field mode with a 100X/N.A. 1.40 oil immersion objective. Co-ordinates were recorded for metaphase relocation following Spectral karyotyping (SKY). Prior to SKY, slides were destained with Carnoy’s fixative, rehydrated with an ethanol series, pretreated with 2X SSC at 37°C, then post-fixed with 1% formaldehyde/PBS/MgCl\(_2\) and washed in PBS. SkyPaint\textsuperscript{TM} probes were used according to the manufacturer’s instructions (Applied Spectral Imaging, Carlsbad, CA). Metaphases were viewed with an Olympus BX61 microscope (Olympus, Center Valley, USA) equipped with a SpectraCube SD300 (Applied Spectral Imaging, Migdal HaEmek, Israel). SKY images were analyzed using SkyView Version 2.1.1 (Applied Spectral Imaging).

2.7.2. Ploidy analysis

Numerical abnormalities in chromosome numbers of mitotic cells were estimated by calculating the proportion of aneuploid mitotic thymocytes and tumour cells relative to cells displaying a normal karyotype. 40 and 100 random metaphases of thymocytes and tumour cells, respectively, were photographed and chromosomes were enumerated using light microscopy (LEICA DM LB2, Bannockburn, IL, USA). Metaphase chromosome spreads were prepared using standard Geimsa staining techniques described in Cytogenetic analysis of tumours section (Padilla-Nash, Wu, Just, Ried, & Thestrup-Pedersen, 2007).
2.8. Gene Expression Profiling (performed by Dr. Yanzhen Zheng, Dr. M. Hough Laboratory)

2.8.1. RNA isolation and high-density oligonucleotide microarray screening

Double mutant $IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}$ and control $Prkdc^{Scid/Scid}$ mice were sacrificed at 6-8 weeks of age. DN1, DN2, DN3 and CD44$^-CD25^-$ subpopulations were sorted based on expression of CD44 and CD25. To minimize sample variability caused by individual differences among animals, individual thymocyte subpopulations from 2-5 sex matched mice were pooled (total $10^4$-$10^5$ cells). The pooled cell pellets were homogenized in TRIzol reagent (Invitrogen) and stored at $-80^\circ C$ for total RNA isolation. Total RNA was isolated and purified with RNeasy kits (Qiagen) according to the manufacturer’s instructions. $TLX1$ genotypes were confirmed prior to microarray experiments at the TCAG Microarray Facility. The detailed protocol for the sample preparation and microarray processing is available from Affymetrix http://www.affymetrix.com/support.

2.8.2. Gene Set Enrichment Analyses (GSEA), data analysis, Bioinformatics and statistical analysis

Affymetrix Expression Console was used for the probe set summarization, with normalization using MAS5.0 statistical algorithm. The “present” (P) or “absent” (A), “marginal” (M) calls were generated per Affymetrix protocol using the default settings. GeneSpring 11.0 software (Silicon Genetics, Inc. Redwood City, CA) was used for data transforming, filtering, statistical analysis, clustering, and visualization. CEL files were imported to GeneSpring GX11.0 and data were normalized to the mean of all samples. Quality controls for samples: internal controls show 3’/5’ ratio <3; hybridization controls show well gradient; the signals of sample replicates show well correlation coefficient around 1; principal component analysis (PCA) scores; and whole sample clustering on conditions show the same stages cluster more together. Quality controls for probe sets: filter probe sets on expression: raw data $\geq$ 20 percentile at least 1 out of 8 samples; filter on flags Present or Marginal at least 1 of the 8 samples. Statistical Analysis: two samples T Test unpaired with asymptotic p-value $P \leq 0.05$. 1.4X fold change cutoff.
Gene annotation queries were conducted through Affymetrix NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx/) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) were used for the gene annotation enrichment analysis (http://david.abcc.ncifcrf.gov/).

Gene Set Enrichment Analysis (GSEA) was conducted using GSEA software: http://www.broadinstitute.org/gsea/index.jsp. Data sets were from Affymetrix Expression Console, $IgH\mu$-TLX1$^\text{Tg}$Prkdc$^{\text{Scid/Scid}}$ DN1 vs. Prkdc$^{\text{Scid/Scid}}$ DN1; $IgH\mu$-TLX1$^\text{Tg}$Prkdc$^{\text{Scid/Scid}}$ DN2 vs. Prkdc$^{\text{Scid/Scid}}$ DN2; $IgH\mu$-TLX1$^\text{Tg}$Prkdc$^{\text{Scid/Scid}}$ DN3 vs. Prkdc$^{\text{Scid/Scid}}$ DN3. Data files were collapsed to gene symbols when doing analysis. Gene sets were from the GSEA web Molecular Signatures Database (MSigDB) version 3.0, mainly C2, the curated gene sets and C5, the GO gene sets. Analysis was done using 1,000 gene set permutations and signal-to-noise ratio metric.

**Accession code.** Microarray data are available in the Gene Expression Omnibus (GEO) with accession numbers GSE47421.
Chapter 3

Ectopic expression of *TLX1* in hematopoietic progenitors accelerates malignancies in *TLX1* transgenic mice deficient in DNA-PK

This chapter is a modified version of the following publication:

Konstantin Krutikov, Yanzhen Zheng, Alden Chesney, Xiaoyong Huang, Andrea K. Vaags, Valentina Evdokimova, Edwin Chen and Margaret R. Hough (2013) Ectopic Expression of *TLX1* in Hematopoietic Progenitors Accelerates Malignancies in *TLX1* Transgenic Mice Deficient in DNA-PK. *PLOS ONE.*

Paper was submitted and returned for minor modifications. Paper currently is in the process of preparation for resubmittion.
3.1. Attribution of data

I have performed all the work described in this chapter except for histological specimens prepared at the Department of Pathology in the Sunnybrook Research Institute; RNA isolation, high-density oligonucleotide microarray screening, data analysis, bioinformatics and statistical analysis were performed by Dr. Yanzhen Zheng; Spectral karyotype (SKY) analysis was performed by Dr. Mary Ann George (The Centre for Applied Genomics, MaRS Centre).
3.2. Abstract

The noncluster homeodomain containing gene, \textit{HOX11/TLX1} (\textit{TLX1}) is detected at the breakpoint of the t(10;14)(q24;q11) chromosome translocation in patients with T cell Acute Lymphoblastic leukemia (T-ALL). This translocation results in the inappropriate expression of \textit{TLX1} in T cells. The oncogenic potential of \textit{TLX1} was demonstrated in \textit{IgH\textsubscript{\mu}-TLXI\textsuperscript{Tg}} mice, which developed mature B cell lymphoma after a long latency period suggesting the requirement of additional mutations to initiate malignancy. To determine whether dysregulation of genes involved in the DNA damage response contributed to tumour progression, we crossed \textit{IgH\textsubscript{\mu}-TLXI\textsuperscript{Tg}} mice with \textit{Prkdc\textsuperscript{Scid/Scid}} mice. \textit{IgH\textsubscript{\mu}-TLXI\textsuperscript{Tg}} \textit{Prkdc\textsuperscript{Scid/Scid}} mice rapidly developed T-ALL. Expression analysis of \textit{IgH\textsubscript{\mu}-TLXI\textsuperscript{Tg}} \textit{Prkdc\textsuperscript{Scid/Scid}} thymocytes revealed dysregulated expression of cell cycle, apoptotic, mitotic spindle and anaphase-promoting complex genes in double negative (DN) 2 and DN3 stage thymocytes. Moreover, DN1, DN2 and DN3 \textit{TLX1}-expressing thymocytes showed downregulated expression of ribosomal and mitochondria ribosomal protein genes. Proto-oncogenes \textit{c-myc} and \textit{c-myb} were also dysregulated in thymocytes. Hypoploidy in \textit{IgH\textsubscript{\mu}-TLXI\textsuperscript{Tg}} \textit{Prkdc\textsuperscript{Scid/Scid}} thymocytes and tumour cells confirmed abnormal function of the spindle checkpoint. Finally, \textit{IgH\textsubscript{\mu}-TLXI\textsuperscript{Tg}} \textit{Prkdc\textsuperscript{Scid/Scid}} thymocytes exhibited a proliferative advantage and decreased apoptosis. These findings indicate that ectopic expression of \textit{TLX1} in \textit{Prkdc\textsuperscript{Scid/Scid}} thymocytes dysregulated proliferation, apoptosis and the spindle checkpoint and linked to a possible new oncogenic mechanism by altering ribosomal protein synthesis.
3.3. Introduction

The homeodomain containing gene, *TLX1*, belongs to the nonclustered subclass of homeodomain transcription factors. *TLX1* was initially identified at the breakpoint of the t(10;14)(q24;q11) reciprocal chromosome translocation in patients with T cell Acute Lymphoblastic leukemia (T-ALL) (Dube et al., 1991; Hatano, Roberts, Minden, Crist, & Korsmeyer, 1991b; Kennedy et al., 1991). This translocation places the entire *TLX1* coding region under the transcriptional control of the T cell receptor δ (*TCRδ*) promoter resulting in inappropriate expression of *TLX1* in T cells, and is found in ~5% of pediatric T-ALL and 30% of adult T-ALL cases (Ferrando et al., 2002). *TLX1*-initiated T-ALL exhibits a block at the stage of β-selection. This maturation arrest might be due to down regulation of the *BCL11B* tumour suppressor gene (Ikawa et al., 2010) or as a result of recruitment of TLX1 by the transcription factor ETS1 to the enhancer of the TCR-α locus leading to repression of *Va-Jα* rearrangements (Dadi et al., 2012). Normally, *TLX1* is not expressed in adult tissues with the exception of the liver (Hatano, Roberts, Minden, Crist, & Korsmeyer, 1991), but is critical for the development of the spleen during embryogenesis (Roberts, Shutter, & Korsmeyer, 1994; Roberts, Sonder, Lumsden, & Korsmeyer, 1995; Dear et al., 1995).

Transgenic mice have played a central role in defining the molecular mechanisms of *TLX1*-initiated hemopoietic malignancies. Transgenic mice expressing *TLX1* in the B-cell compartment by placing the *TLX1* gene under the control of the immunoglobulin heavy chain (IgH) promoter and enhancer sequence developed mature marginal zone B-cell lymphomas after an extended latency (Hough et al., 1998; Rosic-Kablar, Chan, Reis, Dube, & Hough, 2000). Surprisingly, although *IgHμ-TLX1* transgenic mice expressed elevated levels of *TLX1* in thymocytes during the early stages of development, no cases of T-ALL were detected in these mice. More recently, *p56Lck-TLX1* and inducible *Lck-TLX1* transgenic mice with T-cell specific *TLX1* expression were reported (De et al., 2010; Rakowski, Lehotzky, & Chiang, 2011). *TLX1* expression induced a block in thymocyte development at the DN2 stage and, similar to human *TLX1*-initiated T-ALL, *TLX1* transgenic mice developed cortical stage tumours with a heterogeneous CD4 and CD8 immunophenotype. Moreover, *TLX1*-initiated T-ALL acquired activating *NOTCH1* mutations (Rakowski, Lehotzky, & Chiang, 2011). Furthermore, Riz et al. reported that NOTCH1 and TLX1 coregulated transcription in T-ALL cells (Zweier-Renn et al., 2010). These findings are consistent with reports that more than 50% of T-ALL patients carry
*NOTCH1* activating mutations raising the possibility that *NOTCH1* dysregulation is an indispensible condition for *TLX1* driven T-ALL (Weng et al., 2004).

The association of TLX1 with aberrant cell cycle checkpoints has been known for many years. In the first such report, TLX1 was shown to interact with protein phosphatases PP2A and PP1 to disrupt a G2/M cell cycle checkpoint (Kawabe, Muslin, & Korsmeyer, 1997). Subsequently, high throughput comparison of gene expression profiles of two cell lines established from patients with TLX1-positive T-ALLs revealed modulated expression of numerous genes associated with G1/S progression (Riz & Hawley, 2005). More recently, multiple groups have reported that ectopic expression of *TLX1* results in dysregulation of the spindle checkpoint (Chen, Kwon, Lim, Dube, & Hough, 2006d; Chen et al., 2006), attributable, in part, to down-regulated expression of the mitotic checkpoint regulator, *Chek1* (De et al., 2010).

In this report, we hypothesize that accumulation of DNA damage such as unresolved double strand (DS) DNA break resulting from defective nonhomologous end joining (NHEJ) DS DNA repair might also contribute to T-ALL progression in thymocytes expressing *TLX1*.

The catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) plays a critical role in NHEJ pathway. It is also critical for V(D)J recombination, a process which relies on NHEJ to promote immune system diversity at the Ig or TCR loci. Mice containing a germline inactivating mutation in *Prkdc* (the gene encoding DNA-PKcs) are viable but have severe combined immunodeficiency due to their V(D)J recombination defects. B cell development in these *Prkdc<sup>Scid/Scid</sup>* mice is arrested at the B220<sup>+</sup>CD19<sup>+</sup>IgM<sup>-</sup> stage, whereas thymocyte development is arrested at the DN3 stage. T cell progenitors in the *Prkdc<sup>Scid/Scid</sup>* express Thy1.2 and IL-2R, but lack CD3, TCRαβ, and TCRγδ (Hardy, Kemp, & Hayakawa, 1989). Moreover, DNA-PK plays an important role in the the NHEJ DNA repair response, and its deficiency in mice contributes to genomic instability (Bailey, Cornforth, Ullrich, & Goodwin, 2004; Gilley et al., 2001; Shang et al., 2010). Furthermore, as mature IgM/IgD cells do not develop in *Prkdc<sup>Scid/Scid</sup>* mice and this is the target cell of *TLX1*-initiated lymphoma in *IgHμ-TLX1<sup>Tg</sup>* mice, we speculated that in the absence of lymphomagenesis, loss of DNA-PK in thymocyte progenitors expressing *TLX1* could lead to the development of T-ALL. Therefore, in addition to investigating the possibility that a lack of DNA-PK facilitates cancer progression, these double mutant *IgHμ-TLX1<sup>Tg</sup>* *Prkdc<sup>Scid/Scid</sup>* mice also had the potential to yield a model with an increased propensity for the development of T-ALL.
Herein, we created double mutant $IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}$ mice, and show that these mice exhibit statistically significant accelerated onset of T-ALL relative to $Prkdc^{Scid/Scid}$ mice. This implicates the absence of DNA-PK as a cooperating event for $TLX1$ initiated malignancy. Moreover, we identify numerous genetic pathways that are perturbed in association with $TLX1$ overexpression, including those involved in chromosome segregation, cell cycle checkpoints, apoptosis, ribosome, mitochondrial ribosome and lymphocyte development.

3.4. Results

3.4.1. Similar premalignant phenotypes in young

$IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}$ and $Prkdc^{Scid/Scid}$ mice

To determine whether dysregulation of a DNA repair pathway collaborated with ectopic expression of $TLX1$ in disease progression, we crossed $IgH\mu-TLX1^{Tg}$ mice with CB17 ICR-$Prkdc^{scid}$ mice (referred to as $Prkdc^{Scid/Scid}$ mice). We used the CB17 ICR-$Prkdc^{scid}$ mouse strain as these mice differ from CB17 Balb/c-$Prkdc^{scid}$ mice in that they are prone to the spontaneous development of T-ALL and sporadically develop AML and nonthymic tumours (Imada, 2003; Custer, Bosma, & Bosma, 1985; Huang, Westmoreland, Jain, & Fukumura, 2011).

The thymi of double mutant $IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}$ mice were significantly reduced in cellularity as compared to wild type mice, but were increased relative to those of $Prkdc^{Scid/Scid}$ mice (Table 3.1). Spleens of $IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}$ mice were also reduced in cellularity as compared to wild type mice, but were increased as compared to spleens of $Prkdc^{Scid/Scid}$ littermates (Table 3.2). Due to the lack of lymphoid cells, Peyer's patches and lymph nodes of $IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}$ and $Prkdc^{Scid/Scid}$ mice were small and difficult to detect (Custer, Bosma, & Bosma, 1985). Histologically, thymi of $IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}$ and $Prkdc^{Scid/Scid}$ mice lacked cortico-medullary delineation and consisted of predominantly epithelial cells and immature thymocytes. In the spleen, lymphoid follicles and germinal centers were devoid of lymphoid cells and populated with fibroblasts and plasma cells. Bone marrow histology appeared normal.
3.4.2. Expression of the TLX1 transgene in thymocytes of

\( \text{IgH} \mu - \text{TLX1}^{Tg} \text{Prkdc}^{\text{Scid/Scid}} \) mice

The TLX1 transgene in the double mutants was driven by the IgH\( \mu \) promoter. Although the activity of the IgH\( \mu \) promoter is typically restricted to B cells (Gerlinger et al., 1986; Reik et al., 1987), leaky expression in thymocytes and myeloid progenitors has been reported (Bergman, Rice, Grosschedl, & Baltimore, 1984; Wasylyk & Wasylyk, 1986). Therefore, to quantitate leaky TLX1 expression during T lymphopoiesis driven by the IgH\( \mu \) promoter, thymocytes were derived from wild type, Prkdc\( ^{\text{Scid/Scid}} \) and IgH\( \mu - \text{TLX1}^{Tg} \text{Prkdc}^{\text{Scid/Scid}} \) mice by co-culture of murine fetal liver cells on OP9-DL1 stromal cells and TLX1 expression was analyzed by RT-PCR. Weak TLX1 transgene expression was detected in DN1, DN2 and DN3 thymocytes and in a small fraction of CD44\(^-\)CD25\(^-\) cells. Expression was down regulated as cells progressed from DN1 to the DN3 stage and was significantly decreased in CD44\(^-\)CD25\(^-\) cells, consistent with declining activity of the IgH\( \mu \) promoter during thymocyte maturation as previously reported (Bergman, Rice, Grosschedl, & Baltimore, 1984; Wasylyk & Wasylyk, 1986). Expression of TLX1 was not detected in DN1, DN2, DN3 or CD44\(^-\)CD25\(^-\) thymocytes derived from Prkdc\( ^{\text{Scid/Scid}} \) or wild type mice indicating that the TLX1 detected originated from the IgH\( \mu - \text{TLX1} \) transgene and not the endogenous mouse Tlx1 gene (Figure 3.1A, B). To confirm that the TLX1 transgene was also expressed in vivo, quantitative RT-PCR analysis of thymocytes obtained from six week old IgH\( \mu - \text{TLX1}^{Tg} \text{Prkdc}^{\text{Scid/Scid}} \) and Prkdc\( ^{\text{Scid/Scid}} \) age- and sex-matched littermates was performed. Expression of the TLX1 transgene was detected in DN1, DN2, DN3 and CD44\(^-\)CD25\(^-\) thymocytes from IgH\( \mu - \text{TLX1}^{Tg} \text{Prkdc}^{\text{Scid/Scid}} \) mice. Of note, the level of expression of the IgH\( \mu - \text{TLX1} \) transgene in thymocytes was significantly lower than that detected in purified B220\(^+\) splenocytes from IgH\( \mu - \text{TLX1}^{Tg} \) mice (Figure 3.1C, D).
Figure 3.1. Expression of TLX1 in IgHμ-TLX1Tg Prkdc<sup>Scid/Scid</sup> premalignant thymocytes.

A. Thymocytes were harvested on day-7 from the OP9-DL1 co-culture system and flow sorted into DN1, DN2, DN3 and CD44-CD25- fractions on the basis of CD25 and CD44 expression. Purified B220+ splenocytes isolated from wild type and IgHμ-TLX1Tg mice were used as negative and positive controls, respectively. B. RT-PCR analysis showing expression of the TLX1 transgene in immature thymocytes grown in the OP9-DL1 co-culture system established from hematopoietic stem cells obtained from IgHμ-TLX1Tg Prkdc<sup>Scid/Scid</sup> and wild type (WT) mice. TLX1 expression was not detected in immature thymocytes derived from Prkdc<sup>Scid/Scid</sup> and WT mice. C. Total thymocytes were obtained from thymi of six week old IgHμ-TLX1Tg Prkdc<sup>Scid/Scid</sup> and Prkdc<sup>Scid/Scid</sup> mice and DN1, DN2, DN3 and CD44-CD25- cells were separated by flow cytometry based upon CD44 and CD25 expression. D. qRT-PCR analysis showing expression of the TLX1 transgene in DN1, DN2, DN3 and CD44-CD25- thymocyte fractions flow sorted from the thymi of IgHμ-TLX1Tg Prkdc<sup>Scid/Scid</sup> and Prkdc<sup>Scid/Scid</sup> mice. B220+ splenocytes from IgHμ-TLX1Tg healthy mice were used as a positive control whereas thymocytes from Prkdc<sup>Scid/Scid</sup> mice were used as a negative control. Expression of the TLX1 transgene in the thymi of IgHμ-TLX1Tg Prkdc<sup>Scid/Scid</sup> (black) and Prkdc<sup>Scid/Scid</sup> (pink) mice is shown.
3.4.3. Accelerated leukemogenesis in $\text{IgH}^\mu$-TLX$^1\text{TgPrkdc}^{\text{Scid/Scid}}$ mice

To determine whether ectopic expression of TLX1 collaborated with DNA-PK deficiency in the pathogenesis of hemopoietic malignancies, a cohort of 60 $\text{IgH}^\mu$-TLX$^1\text{TgPrkdc}^{\text{Scid/Scid}}$ mice and 50 age and sex matched $\text{Prkdc}^{\text{Scid/Scid}}$ mice were followed for 24 months. At the end of this period, all mice had developed a malignancy with histological features and flow cytometric profiles consistent with either T-ALL or acute myeloid leukemia (AML) (Figure 3.2A). However, $\text{IgH}^\mu$-TLX$^1\text{TgPrkdc}^{\text{Scid/Scid}}$ mice succumbed to disease at earlier time points relative to $\text{Prkdc}^{\text{Scid/Scid}}$ littermates. The median survival of the complete cohort of $\text{IgH}^\mu$-TLX$^1\text{TgPrkdc}^{\text{Scid/Scid}}$ and $\text{Prkdc}^{\text{Scid/Scid}}$ mice, irrespective of disease diagnosis, was 6.6 and 10.25 months, respectively (Figure 3.2D). The one-year survival probability estimate of $\text{IgH}^\mu$-TLX$^1\text{TgPrkdc}^{\text{Scid/Scid}}$ mice was also decreased relative to those of $\text{Prkdc}^{\text{Scid/Scid}}$ littermates developing either T-ALL or AML (Figure 3.2E).

Fifty nine percent of $\text{IgH}^\mu$-TLX$^1\text{TgPrkdc}^{\text{Scid/Scid}}$ mice and 54% of $\text{Prkdc}^{\text{Scid/Scid}}$ mice developed an immature T-cell. The median survival of $\text{IgH}^\mu$-TLX$^1\text{TgPrkdc}^{\text{Scid/Scid}}$ mice with T-ALL was 6.5, whereas $\text{Prkdc}^{\text{Scid/Scid}}$ mice exhibited a protracted latency with a median survival of 8.25 (Figure. 3.2B, C).
Figure 3.2. **TLX1 accelerates the development of T-ALL and AML in IgHu-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mice relative to Prkdc<sup>Scid/Scid</sup> mice.** Cohorts of mice were monitored for signs of disease for 25 months. A diagnosis of AML or T-ALL was made based on histological examination of bone marrow, spleen and thymic tissues. A. Disease free survival of Prkdc<sup>Scid/Scid</sup> and IgHu-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mice during the observation period, \(p<0.0001\). B. T-ALL free survival of Prkdc<sup>Scid/Scid</sup> and IgHu-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mice, \(p=0.003\). C. AML free survival of Prkdc<sup>Scid/Scid</sup> and IgHu-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mice, \(p<0.0001\). D. Median survival of the complete cohort of IgHu-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> and Prkdc<sup>Scid/Scid</sup> mice developing T-ALL or AML. Column labeled as T-ALL/AML corresponds to Figure 1A and shows the median survival for IgHu-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> or Prkdc<sup>Scid/Scid</sup> mice from cohorts developing either T-ALL or AML. The columns labeled T-ALL or AML correspond to Figure 1B and 1C and indicate median survival of IgHu-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> or Prkdc<sup>Scid/Scid</sup> mice from cohorts developing T-ALL or AML respectively. E. A one-year Kaplan-Meier survival probability estimate of the complete cohort of IgHu-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> and Prkdc<sup>Scid/Scid</sup> mice developing T-ALL or AML.
3.4.4. Characterization of T-ALL in \( \text{IgH}\mu\cdot\text{TLX1}\text{TgPrkdScid/Scid} \) mice

Histological examination of tissues of \( \text{PrkdScid/Scid} \) and \( \text{IgH}\mu\cdot\text{TLX1}\text{TgPrkdScid/Scid} \) mice exhibiting T-ALL indicated that thymic involvement in 100% of animals, while splenic and bone marrow infiltration of tumour cells were detected in the 67% and 52% of mice, respectively. This suggests that the thymus was the primary site for disease initiation. The architecture of the thymus was disrupted with indistinguishable cortico-medullary delineation. The spleens had disrupted follicles, with patchy areas infiltrated with a large, relatively uniform population of lymphocytes having polylobulated pleomorphic nuclei with diffuse, loose, speckled, open chromatin and prominent nucleoli (Figure 3.3A).

Immunohistological analysis was performed using frozen sections of thymi and spleens isolated from age- and sex-matched healthy and terminal \( \text{IgH}\mu\cdot\text{TLX1}\text{TgPrkdScid/Scid} \) mice. Staining of sections with the pan T-cell antibody, Thy1.2, revealed numerous Thy1.2\(^+\) cells in the subcortical area of the thymi while Thy1.2\(^+\) cells were absent in the medullar area of thymi and were not detected in the spleens of healthy mice. In contrast, a homogeneous population of Thy1.2\(^+\) tumour cells was detected throughout the thymi and spleens of terminal mice (Figure 3.3B).

Flow cytometry analysis revealed heterogeneity in the tumour phenotype with respect to expression of CD4 and CD8 (Figure 3.3C) with tumour cells isolated from the thymus, spleen and bone marrow expressing levels of both CD4 and CD8 or only CD4\(^{lo}\) with decreased levels of expression of CD44 and CD25. Other tumours consisted of CD44 and CD25 expressing cells with profiles typical of DN1, DN2 and DN3 cells or only DN3 thymocytes. Tumour cells did not expressed αβ- or γδ- T cell receptors, indicative of an immature thymocyte phenotype (Figure 3.3C).
Figure 3.3. **TLX1-induced T-ALL in IgHµ-TLX1\(^{Tg}\)Prkdc\(^{Scid/Scid}\) mice.**

A. Hematoxylin and eosin staining of tissues isolated from healthy Prkdc\(^{Scid/Scid}\) and IgHµ-TLX1\(^{Tg}\)Prkdc\(^{Scid/Scid}\) (left and middle panels correspondingly) mice and mice diagnosed with T-ALL (right panel) revealed disrupted architecture in the thymus and spleen of mice with T-ALL (right top and middle panels). Thymi, spleen and the bone marrow were packed with leukemic cells in moribund IgHµ-TLX1\(^{Tg}\)Prkdc\(^{Scid/Scid}\) mice (right panel). Thymi, spleens and bone marrow of control Prkdc\(^{Scid/Scid}\) and IgHµ-TLX1\(^{Tg}\)Prkdc\(^{Scid/Scid}\) mice (left and middle panels) were not affected. Magnification x40 (overview) and x100 (insert). Scale bars, 10 µm.

B. Immunohistochemical analysis of a thymi and spleen of a healthy IgHµ-TLX1\(^{Tg}\)Prkdc\(^{Scid/Scid}\) mouse (top row) and a moribund IgHµ-TLX1\(^{Tg}\)Prkdc\(^{Scid/Scid}\) mouse (bottom row) stained with an anti-Thy1.2 antibody. Cells reacting with the Thy1.2 antibody were not detected in the medullar area of the thymus or in the spleen of the healthy IgHµ-TLX1\(^{Tg}\)Prkdc\(^{Scid/Scid}\) mouse. The thymus and spleen of moribund mouse were disorganized and infiltrated with Thy1.2 positive lymphoma cells. Magnification x20. Scale bars, 10 µm.

C. Thymi, spleens and bone marrow of healthy (left side) and moribund (right side) IgHµ-TLX1\(^{Tg}\)Prkdc\(^{Scid/Scid}\) mice were examined by flow cytometry using CD44, CD25, CD4, CD8, CD3, TCR\(\alpha\beta\), TCR\(\gamma\delta\) and Thy1.2 antibodies. Analysis revealed the abnormal presence of cells expressing low levels of CD4\(^+\)CD8\(^+\). Immature CD4\(^+\)CD25\(^+\) thymocytes were detected in spleens and bone marrow of moribund IgHµ-TLX1\(^{Tg}\)Prkdc\(^{Scid/Scid}\) mice, whereas these cells were absent in the hematopoietic tissues of healthy IgHµ-TLX1\(^{Tg}\)Prkdc\(^{Scid/Scid}\) mice. Increased numbers of immature Mac-1\(^{hi}\)Gr-1\(^{lo}\), Mac-1\(^{hi}\)Gr-1\(^{int}\) and Mac-1\(^{hi}\)Gr-1\(^{lo}\) myelocytes were not detected in moribund IgHµ-TLX1\(^{Tg}\)Prkdc\(^{Scid/Scid}\) mice relative to healthy IgHµ-TLX1\(^{Tg}\)Prkdc\(^{Scid/Scid}\) mice.
The majority (60%) of thymic tumours had a mixed phenotype expressing low-levels of CD4 and CD8 along with a combination of CD44 and CD25 (Figure 3.4).

**Figure 3.4. Immunophenotype of TLX1-induced T-ALL.**
Immunophenotype distribution showing heterogeneous expression of CD44, CD25, CD4 and CD8 in IgH\(\mu\)-TLX1\(\tilde{\alpha}\)Prkd\(\tilde{\alpha}\)SCID\(\tilde{\alpha}\)SCID tumours. Representative flow diagrams showing heterogeneous expression of CD4 and CD8 in TLX1-initiated leukemias are shown.
Forty one percent of $IgH\mu$-TLX1$^Tg$ Prkdc$^{Scid/Scid}$ mice developed AML, with a median survival of 7.0 months and 46% of Prkdc$^{Scid/Scid}$ control mice developed AML with a median survival of 13.11 months (Figure 3.2C, D). In these cases, histological analysis of bone marrow and spleens from terminally ill mice revealed the presence of leukemic cells with a primitive myeloid phenotype (Figure 3.5A). Histological analysis indicated the absence of tumour cells in the thymus while flow cytometry analysis using CD44 and CD25 antibodies indicating that thymi contained a normal profile of immature DN thymocytes typical for mice with the Prkdc$^{Scid/Scid}$ mutation. Moreover, thymocytes did not express TCRβ, CD3, CD4 and CD8 receptors and thus, had not progressed beyond the DN3 stage of development. Flow cytometric analysis of bone marrow and spleens cells stained with Gr-1 and Mac-1 antibodies revealed an increased proportion of Mac-1$^{lo}$Gr-1$^{lo}$, Mac-1$^{hi}$Gr-1$^{int}$ and Mac-1$^{hi}$Gr-1$^{lo}$ cells in $IgH\mu$-TLX1$^Tg$ Prkdc$^{Scid/Scid}$ mice relative to Prkdc$^{Scid/Scid}$ mice (Figure 3.5B), supporting a diagnosis of AML (Jaiswal et al., 2003; Khandanpour et al., 2012).
Figure 3.5. TLX1-induced AML in IgHμ-TLX1TgPrkdcScid/Scid mice.

A. Hematoxylin and eosin staining of tissues isolated from control PrkdcScid/Scid (left panels), healthy IgHμ-TLX1TgPrkdcScid/Scid (middle panels) and tissues from IgHμ-TLX1TgPrkdcScid/Scid mice diagnosed with AML (right panels). H&E staining revealed increased numbers of cells with a myeloblastic phenotype in the spleen and bone marrow of IgHμ-TLX1TgPrkdcScid/Scid mice (middle and bottom panels on the right side). Thymi, spleens and bone marrow of control PrkdcScid/Scid (left) and IgHμ-TLX1TgPrkdcScid/Scid mice (middle panel) and thymi of moribund IgHμ-TLX1TgPrkdcScid/Scid mice were not affected (top right panel). Magnification x40 (overview) and x100 (insert). Scale bars, 10 µm. B. Thymi, spleens and bone marrow of healthy (left panels) and moribund (right panels) IgHμ-TLX1TgPrkdcScid/Scid mice were examined by flow cytometry using CD44, CD25, CD4, CD8, CD3, TCRαβ, TCRγδ and Thy1.2 antibodies, specific for T cells, and Gr-1 and Mac-1 antibodies, specific for myeloid cells. IgHμ-TLX1TgPrkdcScid/Scid mice lacked CD4+CD8+ DP and CD4+ and CD8+ SP thymocytes as expected. Immature DP and mature SP T cells were also not detected in the spleens and bone marrow of either sick or healthy IgHμ-TLX1TgPrkdcScid/Scid mice. Gr-1 and Mac-1 staining revealed an increased proportion of immature myeloid cells in the spleens and bone marrow of moribund mice relative to the spleen and bone marrow of healthy mice.
Figure 3.5.
3.4.5. Dysregulated expression of genes involved in cell cycle, apoptosis, ribosomal proteins and genes involved in oxidative phosphorylation in premalignant thymocytes

To identify molecular pathways dysregulated in the earliest stages of TLX1-induced transformation, we used Affymetrix GeneChip microarrays to compare gene expression profiles of premalignant DN1, DN2 and DN3 thymocytes from age and sex matched IgHμ-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} and control Prkdc\textsuperscript{Scid/Scid} mice. We identified 103 TLX1-associated differentially expressed genes (DEGs) in DN1 thymocytes, (89 up regulated, 14 genes down regulated), 151 DEGs in DN2 thymocytes (139 up regulated, 12 genes down regulated) and 522 DEGs in DN3 thymocytes (495 up regulated, 27 genes down regulated) (Figure 3.6A-C). Dysregulated expression of genes involved in cell cycle regulation and apoptosis pathways were most prominent in DN2 and DN3 thymocyte subpopulations with 4, 12 and 28 cell cycle genes dysregulated in DN1, DN2 and DN3 stage thymocytes, respectively. We next applied gene set enrichment analysis (GSEA) to our gene expression profiles and discovered up-regulation of genes encoding components of the spindle complex in DN2 thymocytes from IgHμ-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mice (NES= 2.21, q-value= 0.02) (Figure 3.6D). Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis also confirmed that genes regulating mitosis and the cell cycle, particularly genes involved in chromosome segregation, were highly enriched in DN3 thymocytes isolated from IgHμ-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mice. Specifically, we noted upregulation of genes in the Anaphase-Promoting Complex (APC), including Anapc1, Anapc5, Cdc23 and the Cohesin complex, including Smc1a, Rad21 and Stag1.

GSEA analysis also revealed dysregulated expression of genes involved in apoptosis in thymocytes isolated from IgHμ-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} relative to Prkdc\textsuperscript{Scid/Scid} mice. In particular, there were 10, 15 and 48 apoptosis genes dysregulated in IgHμ-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} DN1, DN2 and DN3 thymocytes subpopulations, respectively. In DN3 thymocytes, many anti-apoptotic genes were up regulated, including Prnp, Stambp, Men1, Dhcr24, Aars, Akt1, Bre, Rasa1, Adora2a, Api5, Dnajc5, Stat5a, Tnfaip3, and Gsk3b, while some important apoptosis-inducing genes were down regulated, including Bax, Stk17b and Elmo1. DAVID analysis showed apoptosis pathway genes Casp3, Dffb and Apaf1 (all upregulated), Bax (downregulated) and survival genes IKK-β, PIK3CD, PRKACB and Akt/PKB (all upregulated) were highly enriched in IgHμ-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} thymocytes (enrichment fold: 3; p-value: 0.016). Overall, there was
an increasing fold change in the DEGs common to DN1, DN2 and DN3, indicating that the consequence of \textit{TLX1} expression on target gene expression was more profound as the thymocytes matured from the DN1 stage through to the DN3 stage.
Figure 3.6. Heat map of the top ranking differentially expressed genes in flow sorted premalignant thymocytes.

A-C. Gene expression heat map of the top ranking differentially expressed genes in DN1 (A), DN2 (B) and DN3 (C) flow-sorted thymocytes from IgH\mu-\textit{TLX1}\textsuperscript{+}Prkd\textsuperscript{Scid/Scid} and Prkd\textsuperscript{Scid/Scid} mice. D. GSEA revealed high enrichment of mitotic spindle and tubular formation gene sets in IgH\mu-\textit{TLX1}\textsuperscript{+}Prkd\textsuperscript{Scid/Scid} DN2 thymocytes, which appear to comprise 75% of the presented genes. Red and blue indicate higher and lower expression, respectively. The first four columns (\textit{TLX1}\textsuperscript{+}) show IgH\mu-\textit{TLX1}\textsuperscript{+}Prkd\textsuperscript{Scid/Scid} DN1 (A), DN2 (B, D) and DN3 (C) thymocytes. The last four columns (\textit{TLX1}\textsuperscript{+}) represent DN1 (A), DN2 (B, D) and DN3 (C) thymocytes from Prkd\textsuperscript{Scid/Scid} mice.
GSEA analysis also detected down-regulated expression of ribosomal genes and genes involved in oxidative phosphorylation in thymocytes isolated from \(IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}\) relative to \(Prkdc^{Scid/Scid}\) mice. Ribosomal gene sets were highly enriched in TLX1-downregulated genes in the DN1, DN2 and DN3 compartments of \(IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}\) mice (NES: -2.71; FDR q-value: 0.0); (Supplementary Figure S 3.1A-B). Specifically, genes encoding ribosomal proteins comprised 86%, 92% and 94% of DEGs in TLX1-expressing DN1, DN2 and DN3 thymocytes, respectively. These targets included \(RPS20, RPS7, RPL11, RPS18, RPS15A, RPL14, RPL35, MRP24\) and \(RPL13\).

Finally, decreased expression of genes required for oxidative phosphorylation was also observed for DN1 and DN3 thymocytes (NES=1.81, q-value=0) (Supplementary Figure S 3.1C-D). The majority of these down-regulated genes were localized to DN1 and DN3 \(IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}\) thymocytes.

We next validated dysregulated expression of a subset of these DEGs in thymocytes from \(IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}\) and \(Prkdc^{Scid/Scid}\) control mice by qRT-PCR (Figure 3.7). Analysis of DN1, DN2 and DN3 thymocytes confirmed elevated expression of \(Aurka, Bub1\) and \(Anaps5\) in \(TLX1\)-expressing thymocytes. Furthermore, we demonstrate increased expression of the apoptosis genes, \(Brca1\) and \(Birc5\) in DN2 thymocytes and decreased expression of \(Chek1\) in DN1 thymocytes from \(IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}\) mice. Finally, expression of additional genes involved in cell cycle regulation known to be associated with human T-ALL initiation and progression were also assessed. qRT-PCR analysis confirmed upregulated expression of the cell cycle regulators, \(cyclin B1\) and \(cyclin A\) and upregulated expression of the oncogenes, \(c\text{-}myc\) and \(c\text{-}myb\), in \(IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid}\) thymocytes relative to \(Prkdc^{Scid/Scid}\) thymocytes (Figure 3.7). Combined, these results suggest that \(TLX1\) in collaboration with loss of DNA-PK dysregulates the expression of genes involved in the regulation of the cell cycle, the spindle checkpoint, chromosome segregation and apoptosis pathways.
Supplementary Figure 3.1. GSEA Gene Sets for Ribosome and Oxidative Phosphorylation in $\text{IgH}^+\text{TLX1}^+\text{Prkdc}^{\text{Scid/Scid}}$ and $\text{Prkdc}^{\text{Scid/Scid}}$ premalignant thymocytes. Enrichment plots showing downregulation of the Ribosome Gene Sets in DN1-DN3 thymocytes from $\text{IgH}^+\text{TLX1}^+\text{Prkdc}^{\text{Scid/Scid}}$ mice. B. Heat map showing gene expression of the ribosomal gene set. Red and blue indicate higher and lower expression, respectively. C. Enrichment plots showing downregulation of the Oxidative Phosphorylation Gene Sets in DN1 and DN3 thymocytes from $\text{IgH}^+\text{TLX1}^+\text{Prkdc}^{\text{Scid/Scid}}$ compared to $\text{Prkdc}^{\text{Scid/Scid}}$ mice. D. Heat map showing gene expression of the Oxidative Phosphorylation Gene Set. Red and blue indicate higher and lower expression, respectively.
Dysregulated expression of a subset of genes in thymocyte subpopulations from $IgH_{\mu}$-TLXI$^{Tg}$Prkdc$^{Scid/Scid}$ and Prkdc$^{Scid/Scid}$ control mice was confirmed by qRT-PCR. Analysis of DN1, DN2 and DN3 thymocytes confirmed differential expression of Aurka, Bub1 and Anaps5, all of which are involved in chromosome segregation. Furthermore, we detected increased expression of the apoptosis genes, Brca1 and Birc5 in DN2 thymocytes and decreased expression of Chek1 in DN1 thymocytes from $IgH_{\mu}$-TLXI$^{Tg}$Prkdc$^{Scid/Scid}$ mice (Figure 3.7).

Expression of additional genes involved in cell cycle regulation and human T-ALL initiation and progression were also assessed. qRT-PCR analysis confirmed upregulated expression of the cell cycle regulators, cyclinB1 and cyclin A and upregulated expression of the oncogenes, c-myc and c-myb, in $IgH_{\mu}$-TLXI$^{Tg}$Prkdc$^{Scid/Scid}$ thymocytes relative to Prkdc$^{Scid/Scid}$ thymocytes (Figure 3.7). Combined, these results suggest that TLX1 in collaboration with loss of DNA-PK dysregulates the expression of genes involved in the regulation of the cell cycle, the G2/M checkpoint, chromosome segregation, apoptosis, protein synthesis and oxidative phosphorylation.
Figure 3.7. qRT-PCR analysis of genes in premalignant thymocytes of Prkdc<sup>Scid/Scid</sup> and IgHu-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mice.  

qRT-PCR analysis of selected genes whose protein products are involved in chromosome segregation (Chek1, Aurka and Bub1), cell cycle progression (Cyclin A, Cyclin B1, Anapc5, c-myc and c-myb) and apoptosis (Birc5, Brca1). Red bars are DN1, brown are DN2 and green are DN3 cells. The black histograms show analysis of total IgHu-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> thymi. Samples are presented as pairs with the first bar representing the level of expression of cells isolated from Prkdc<sup>Scid/Scid</sup> mice and the second from IgHu-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mice. Data were normalized relative to β-actin.
3.4.6. Dysregulated gene expression in tumour cells of 

\(IgH_\mu-TLX1^{Tg}Prkdc^{Scid/Scid}\) mice

Next, we wanted to test whether the T-ALL-like disease seen in the \(IgH_\mu-TLX1^{Tg}Prkdc^{Scid/Scid}\) mice were molecularly similar to human T-ALL. To do this, we assessed the expression of several genes which had previously been shown to be associated with the progression of human T-ALL such as \textit{NOTCH1}, \textit{PTEN} and \textit{BCL11B} (De et al., 2010; Weng et al., 2004; Palomero et al., 2007; Gutierrez et al., 2011; Gutierrez et al., 2011) in ten tumours isolated from \(IgH_\mu-TLX1^{Tg}Prkdc^{Scid/Scid}\) and \(Prkdc^{Scid/Scid}\) mice. Consistent with gene expression patterns in human T-ALLs, these analyses revealed a statistically significant (p < 0.05) reduction in the expression of \textit{Bcl11b} and \textit{Pten} in premalignant thymocytes with expression being further diminished in all T-ALL arising in \(IgH_\mu-TLX1^{Tg}Prkdc^{Scid/Scid}\) mice relative to total thymocytes and tumours isolated from \(Prkdc^{Scid/Scid}\) mice (Figure 3.8). Furthermore, elevated expression of \textit{Notch1} was detected in six of ten tumours isolated from \(IgH_\mu-TLX1^{Tg}Prkdc^{Scid/Scid}\) mice relative to \(Prkdc^{Scid/Scid}\) (p < 0.05). These data, combined with the microarray analysis, indicated that the process of clonal tumour evolution in \(IgH_\mu-TLX1^{Tg}Prkdc^{Scid/Scid}\) mice resulted in a similar gene expression profile as were similar to those reported for \textit{TLX1}-initiated human T-ALL.
Figure 3.8. qRT-PCR analysis of thymic tumours derived from Prkdc<sup>Scid/Scid</sup> and Ig<sup>H</sup>μ-<sup>TLX1</sup>T<sup>g</sup>Prkdc<sup>Scid/Scid</sup> mice.

qRT-PCR analysis of expression of Bcl11b, Pten and Notch1 in tumours isolated from Prkdc<sup>Scid/Scid</sup> and Ig<sup>H</sup>μ-<sup>TLX1</sup>T<sup>g</sup>Prkdc<sup>Scid/Scid</sup> mice. Control samples included RNA obtained from the thymi of premalignant Ig<sup>H</sup>μ-<sup>TLX1</sup>T<sup>g</sup>Prkdc<sup>Scid/Scid</sup> and Prkdc<sup>Scid/Scid</sup> mice. Ten independent tumours isolated from Prkdc<sup>Scid/Scid</sup> and Ig<sup>H</sup>μ-<sup>TLX1</sup>T<sup>g</sup>Prkdc<sup>Scid/Scid</sup> mice are represented by green and brown boxes, respectively. The pink and black histograms show expression levels of genes in total thymi from Prkdc<sup>Scid/Scid</sup> and Ig<sup>H</sup>μ-<sup>TLX1</sup>T<sup>g</sup>Prkdc<sup>Scid/Scid</sup> mice, respectively. Data were normalized relative to β-actin. Each bar represents one tumour sample.
3.4.7. Increased cellularity of thymi from $IgH\mu$-$TLX1^{Tg}Prkdc^{Scid/Scid}$ mice

To explore the effects of $TLX1$ expression on thymus cellularity, assessments of absolute numbers, apoptosis and proliferation of thymocytes were performed using thymi isolated from premalignant $IgH\mu$-$TLX1^{Tg}Prkdc^{Scid/Scid}$ and $Prkdc^{Scid/Scid}$ mice. Cell counts revealed a statistically significant increase in the absolute number of thymocytes isolated from thymi of $IgH\mu$-$TLX1^{Tg}Prkdc^{Scid/Scid}$ mice relative to $Prkdc^{Scid/Scid}$ control littermates (Figure 3.9A). Furthermore, a statistically significant increase in the numbers was noted for individual DN subpopulations (Figure 3.9B). Cell viability, as assessed by Annexin V and Propidium Iodide (PI) staining (Figure 3.9C), revealed a statistically significant increase in the proportion of viable thymocytes and a reduction in apoptotic and dead thymocytes obtained from $IgH\mu$-$TLX1^{Tg}Prkdc^{Scid/Scid}$ thymi relative to $Prkdc^{Scid/Scid}$ controls (Figure 3.9D). Moreover, the proportion of proliferating thymocytes from $IgH\mu$-$TLX1^{Tg}Prkdc^{Scid/Scid}$ mice was also significantly increased, as established by $in$ $vivo$ BrdU incorporation (Figure 3.9E, F). Combined, these data suggested that the increased cellularity of thymi of premalignant $IgH\mu$-$TLX1^{Tg}Prkdc^{Scid/Scid}$ mice resulted from decreased cell death and increased proliferation of immature DNA-PK-deficient $TLX1$-expressing thymocytes.
Figure 3.9. Expression of TLX1 in IgHμ-TLX1Tg^{Prkdc}Scid/Scid premalignant thymocytes increases cell viability and provides a proliferative advantage.

A. Absolute cell numbers of thymocytes isolated from six week old IgHμ-TLX1Tg^{Prkdc}Scid/Scid and Prkdc^{Scid/Scid} littermates (p < 0.0001), n=20. B. Thymocytes obtained from 20, six weeks old IgHμ-TLX1Tg^{Prkdc}Scid/Scid and Prkdc^{Scid/Scid} littermates were flow sorted based on expression of CD44 and CD25. Absolute numbers of DN thymocytes were calculated using percentages obtained after flow sorting (p < 0.0001). C. Flow cytometric analysis of apoptosis in thymi obtained from three, six week old IgHμ-TLX1Tg^{Prkdc}Scid/Scid and Prkdc^{Scid/Scid} littermates. Cells were stained with Annexin V and PI. The lower left quadrants of each panel contain viable cells, which exclude PI and are negative for FITC-Annexin V binding. The upper right quadrants contain dead cells, which are positive for Annexin V binding and PI uptake. The lower right quadrants represent the early apoptotic cells, which are Annexin V positive and PI negative. D. Percentages of viable, apoptotic and dead thymocytes in thymi of IgHμ-TLX1Tg^{Prkdc}Scid/Scid and Prkdc^{Scid/Scid} littermates, as determined by flow cytometry with Annexin V and PI staining. Error bars represent SD (p < 0.0001). E. Histogram showing premalignant immature thymocytes obtained from control Prkdc^{Scid/Scid} (left side) and IgHμ-TLX1Tg^{Prkdc}Scid/Scid (right side) mice, 2 hours (top row) and 7 hours (bottom row) after intraperitoneal injection with 10 µM BrdU. Staining with an anti-BrdU antibody revealed increased percentages of BrdU incorporating cells in IgHμ-TLX1Tg^{Prkdc}Scid/Scid mice relative to Prkdc^{Scid/Scid} mice at both time points. F. The percentage of proliferating cells in thymi of IgHμ-TLX1Tg^{Prkdc}Scid/Scid and Prkdc^{Scid/Scid} littermates as determined by flow cytometric analysis after staining thymocytes with an anti-BrdU antibody and PI. Error bars represent SD, n=3, (p < 0.0001). Statistically significant differences between compared samples are indicated by asterisks.
3.4.8. Aneuploidy in premalignant thymocytes and thymic tumours

Our previous studies revealed that overexpression of TLX1 in B cells is associated with dysregulated chromosome segregation (Chen et al., 2006). To determine whether dysregulated chromosome segregation is also associated with ectopic expression of TLX1 in T cells, we compared chromosome numbers in metaphase spreads prepared from thymocytes and tumour cells obtained from premalignant and terminally ill IgHμ-TLX1TgPrkdScid/Scid and PrkdScid/Scid mice. Premalignant thymocytes were analyzed directly after isolation of thymi and after in vitro culture in the OP9-DL1 co-culture system. This analysis indicated that premalignant thymocytes and tumour cells of IgHμ-TLX1TgPrkdScid/Scid mice had a statistically significant increase in the numbers of aneuploid cells relative to PrkdScid/Scid samples (Figure 3.10A, B).

Spectral karyotype (SKY) analysis of three tumours isolated from IgHμ-TLX1TgPrkdScid/Scid mice confirmed these results. In addition, these studies revealed a consistent loss of chromosome 12 and gain of 17 in the cells of two independently arising tumours (Figure 3.10C). Moreover, two of three tumours showed an unbalanced trisomy 15 with tumour cells containing two normal chromosomes 15 and a Robertsonian translocation consisting of chromosome 5 and chromosome 15, replacing one chromosome 5. Breakpoints in both the 5 and 15 chromosomes were located within the centromeric region (Figure 3.10D).
Figure 3.10. Chromosome analysis of premalignant thymocytes and tumours from Prkdc<sup>Scid/Scid</sup> and IgH<sub>μ</sub>-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mice.

A. Representative micrographs of Giemsa stained diploid (top left), hyperdiploid (top right), and hypoploid (bottom) chromosome spreads from IgH<sub>μ</sub>-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mice. B. Ploidy assessment of cultured thymocytes and thymocytes thymic tumours obtained from premalignant and moribund Prkdc<sup>Scid/Scid</sup> and IgH<sub>μ</sub>-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mice, respectively. For each of three samples, 40 to 100 metaphase spreads were analyzed. The percentage of aneuploid spreads relative to the total number of analyzed spreads was determined. Statistically significant differences (p <0.05) are indicated by asterisks. C. Spectral karyotyping analysis of a hypoploid tumour isolated from an IgH<sub>μ</sub>-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mouse. Loss of chromosome 12 and gain of chromosome 17 was found in 10% and 5% of analyzed cells, respectively. The karyotype is indicated below. D. An abnormal, unbalanced trisomy 15 karyotype with a Robertsonian translocation resulting in a derivative chromosome consisting of chromosome 5 and chromosome 15 that replaced one chromosome 5 is shown. In addition, two normal copies of chromosome 15 are present. Breakpoints are within the centromeric region of both chromosomes. The karyotype is indicated below.
3.4.9. Dysregulation of the spindle cell cycle checkpoint

We speculated that the abnormal karyotypes detected in thymocytes of \( \text{IgH} \mu \text{-TLX1}^{\text{Tg}} \text{Prkdc}^{\text{Scid/Scid}} \) mice were due to dysregulation of the spindle cell cycle checkpoint. To address this possibility, we compared the frequency at which premalignant thymocytes from \( \text{IgH} \mu \text{-TLX1}^{\text{Tg}} \text{Prkdc}^{\text{Scid/Scid}} \) and \( \text{Prkdc}^{\text{Scid/Scid}} \) mice bypassed the spindle cell cycle checkpoint. HSCs isolated from fetal livers of day E13.5 embryos were co-cultured with OP9-DL1 cells and HSCs were allowed to mature to early stage thymocytes for four days. Cells were then arrested with colchicine, a mitotic spindle poison, and the proportion of cells in G1, S or G2/M phase of the cycle determined at eight-hour intervals by analysis of DNA content. In two independently performed experiments, \( \text{IgH} \mu \text{-TLX1}^{\text{Tg}} \text{Prkdc}^{\text{Scid/Scid}} \) thymocytes showed a reduced proportion of thymocytes in G2/M at all-time points assessed, relative to \( \text{Prkdc}^{\text{Scid/Scid}} \) thymocytes, suggesting an increased ability to bypass this checkpoint (Figure 3.11A). To further verify the ability of \( \text{IgH} \mu \text{-TLX1}^{\text{Tg}} \text{Prkdc}^{\text{Scid/Scid}} \) premalignant thymocytes to bypass the spindle cell cycle checkpoint, cells were subjected to colchicine-induced spindle checkpoint arrest and then cultured in the presence of BrdU. Incorporation of BrdU into the nucleus was used as an indicator of cell division. These analyses indicated a statistically significant increase in BrdU positive thymocytes for \( \text{IgH} \mu \text{-TLX1}^{\text{Tg}} \text{Prkdc}^{\text{Scid/Scid}} \) mice relative to thymocytes from \( \text{Prkdc}^{\text{Scid/Scid}} \) mice (30% ± 4.6 versus 16.3% ± 5.1; \( p<0.05 \)) (Figure 3.11B). Taken together, these results indicated that the aneuploidy observed in \( \text{IgH} \mu \text{-TLX1}^{\text{Tg}} \text{Prkdc}^{\text{Scid/Scid}} \) thymocytes resulted from dysregulated control of the spindle checkpoint in \( \text{TLX1} \) overexpressing cells.
### Figure 3.11. Aberrant checkpoint regulation in thymocytes of *IgHμ-TLX1Tg Prkdc<sup>Scid/Scid</sup> mice*.

Percentage of thymocytes in S and G2/M phases of the cell cycle was determined by DNA content analysis. A greater proportion of *Prkdc<sup>Scid/Scid</sup>* thymocytes accumulated in the G2/M phase of the cell cycle after mitotic arrest with colchicine relative to *IgHμ-TLX1Tg Prkdc<sup>Scid/Scid</sup>* thymocytes. B. Aberrant spindle checkpoint regulation in *IgHμ-TLX1Tg Prkdc<sup>Scid/Scid</sup>* and *Prkdc<sup>Scid/Scid</sup>* T-lymphocyte cultures. Cycling T lymphocytes following colchicine-induced arrest were detected by BrdU immunolabeling. White arrows on the merged images indicate cycling thymocytes. For scoring of BrdU-positive cells, 20 random fields were counted. Statistically significant differences (p < 0.05) are indicated by asterisks.
3.5. Discussion

The first transgenic mouse model confirming the oncogenic potential of \textit{TLX1} was reported by Hough \textit{et al} (Hough et al., 1998). These mice expressed elevated levels of \textit{TLX1} throughout all stages of B cell development, during the early stages of thymocyte development and in myeloid progenitors. A premalignant stage involving mature B cells was detected as early as eight weeks of age. Most mice died of a mature B cell lymphoma although \textasciitilde{}20\% died of myeloid hyperplasia. The average age of death was 14 months. More recently, a mouse model was described in which ectopic \textit{TLX1} expression was restricted to the T cell compartment. These mice develop a T-ALL-like disease with a disease latency of 35 weeks (De et al., 2010). In both reports, there is an extended latency prior to the development of malignant disease, suggesting that ectopic expression of \textit{TLX1} by itself was insufficient to initiate malignancy and that additional mutations were required for premalignant cells to progress to full malignancy.

In this report, we shed light on an additional process, namely the loss of the DNA repair enzyme DNA-PK, which can collaborate with \textit{TLX1} in neoplastic transformation. \textit{IgH\mu-TLX1}^{Tg}Prkd\textsubscript{c}Scid/Scid mice rapidly died of T-ALL or AML at a significantly reduced latency compared to Prkd\textsubscript{c}Scid/Scid control mice. That a proportion of mice developed myeloid malignancies is consistent with our previous report that expression of \textit{TLX1} in myeloid progenitors predisposed mice to myeloid hyperplasia (Hough et al., 1998). Furthermore, these studies indicated that the dysregulated expression of \textit{TLX1} did not influence disease phenotype, but rather, accelerated malignant transformation of not only T-ALL, but also AML in \textit{IgH\mu-TLX1}^{Tg}Prkd\textsubscript{c}Scid/Scid mice (Doetschman, 2009).

Gene expression studies suggest that the T-cell malignancies which arise in \textit{IgH\mu-TLX1}^{Tg}Prkd\textsubscript{c}Scid/Scid bear multiple phenotypic similarities with human \textit{TLX1}-overexpressing T-ALL and a recently described \textit{LCK-TLX1} model of T-ALL: (1) Malignant thymocytes from \textit{IgH\mu-TLX1}^{Tg}Prkd\textsubscript{c}Scid/Scid mice exhibit dysregulated expression of \textit{Pten}, \textit{Notch1} and \textit{Bcl11b}, genes which are frequently mutated in human T-ALL and in other \textit{TLX1} transgenic mouse models (De et al., 2010; Gutierrez et al., 2011a; Palomero et al., 2007; Weng et al., 2004). (2) Expression of \textit{Chek1}, an important regulator of the spindle cell cycle checkpoint, was diminished in thymocytes of both \textit{IgH\mu-TLX1}^{Tg}Prkd\textsubscript{c}Scid/Scid, \textit{p56Lck-TLX1} mice and in human T-ALL. Consequently, thymocytes from both mouse models demonstrate spindle checkpoint dysfunction and a predisposition towards aneuoploidization. Interestingly, our results differ from that of De Keermaecker \textit{et al}. in indicating increased expression of spindle checkpoint
factors Aurka and Bub1. This discrepancy may be linked to the different levels of TLX1 expression in mouse models, differences in the genetic backgrounds of the mice and a gene expression modulatory effect of DNA-PK deficiency. It is interesting to note that for several of these spindle checkpoint proteins, both increased and decreased expression has been correlated with checkpoint dysfunction, as has been seen for various (Yang et al., 2012; Ricke, Jeganathan, & van Deursen, 2011). (3) Gene set enrichment analysis revealed downregulation of ribosomal proteins in all premalignant thymocyte subpopulations in IgHμ-TLX1TgPrkdcScid/Scid mice. This observation is particularly interesting in light of recently identified loss-of-function mutations in the ribosomal protein genes RPL5 and RPL10 in 9.8% of pediatric T-ALLs (De et al., 2013). Moreover, decreased expression of RPL22 has been observed in 10% of all T-ALLs (Rao et al., 2012), and Rpl22 haploinsufficiency in MyrAkt2 transgenic mice are prone to develop T-ALL. Of note, significant downregulation of both Rpl5 and Rpl22 could also be detected in IgHμ-TLX1TgPrkdcScid/Scid premalignant thymocytes. To our knowledge, a similar effect on ribosomal protein expression has not been observed in other mouse models of TLX1-induced malignancies.

Cumulatively, this suggests that many of the biological processes driving human T-ALL are preserved in IgHμ-TLX1TgPrkdcScid/Scid mice. These mice therefore may be a useful model for studying some of the events associated with TLX1-associated leukemogenesis, particularly the role of ribosomal protein dysfunction in T-ALL.

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<th>Wild type (10^9) n=10</th>
<th>PrkdcScid/Scid (10^9) n=10</th>
<th>IgHμ-TLX1TgPrkdcScid/Scid (10^9) n=10</th>
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<td>1.51 ± 0.6</td>
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<th>PrkdcScid/Scid (10^7) n=10</th>
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<td>1.22 ± 0.6</td>
<td>0.813 ± 0.280</td>
<td>1.026 ± 0.32</td>
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Table 3.3. List of primers

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<th>R Primer Sequence</th>
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<td>5' - CTGGATTGGGCTGGGATGT-3'</td>
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<td>5' - TCAACAGAGGTCGTCATGCT-3'</td>
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<tr>
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Chapter 4

T-ALL initiating cells are present at high frequency in all leukemia subpopulations of $IgH_\mu$-$TLX1^{Tg} Prkdc^{Scid/Scid}$ mice
4.1. Attribution of data

I have performed all the work described in this chapter except for histological specimens prepared at the Department of Pathology in the Sunnybrook Research Institute; transplantations of tumour cells were performed by Dr. Margaret Hough; sorting of tumour cells was performed by G. Knowles and A. Khandani.
4.2. Abstract

Despite significant advances in treatment, T-ALL is still considered a high-risk disease. Leukemia initiating cells (L-ICs) are responsible for the initiation and propagation of leukemia and likely contribute to disease relapse after therapy. \textit{IgH}^{\mu}-\textit{TLX1}^{Tg}\textit{Prkdc}^{Scid/Scid} mice express \textit{TLX1} succumb to T-cell acute lymphoblastic leukemia (T-ALL) at an average age of 6.5 months. Using allogeneic transplantation of flow-sorted \textit{IgH}^{\mu}-\textit{TLX1}^{Tg}\textit{Prkdc}^{Scid/Scid} leukemic cells into \textit{Prkdc}^{Scid/Scid} recipient mice, we determined that T-ALL-ICs originated within the thymus, were present in all tumour subfractions and were enriched within the CD44^-/c-kit^+ subpopulation. Future studies will determine whether therapeutic targeting of this subpopulation is effective in reducing disease relapse.
4.3. Introduction

Tumour heterogeneity is a common feature of most malignancies with tumours consisting of distinct subpopulations of cells with definable characteristics. In attempting to explain tumour heterogeneity, the CSC model proposes that a hierarchy of cells exists within a tumour that recapitulates normal, steady-state cellular development. Cancer stem cells (CSCs) are a rare subfraction of tumour cells that are capable of self-renewal, proliferation and differentiation into the various cell subpopulations that comprise the tumour. Although it has been established that many human hemopoietic malignancies, including AML (Bonnet & Dick, 1997), CML (Wang et al., 1998), pediatric B-cell ALL (Cox et al., 2004; Castor et al., 2005) and T-ALL (Cox et al., 2007; Gerby et al., 2011; Chiu, Jiang, & Dick, 2010) support a hierarchical CSC model, recent work has shown that CSCs may not be as rare as first believed and that tumour heterogeneity is the result of random or stochastic influences that alter the behavior of individual cells within the tumour. This scenario is consistent with the clonal evolution model for the generation of tumour heterogeneity.

The presence, identity and function of CSCs are defined by transplantation assays that demonstrate the cancer initiating potential of purified subpopulations of tumour cells. In the case of human cancers, the xenotransplantation model in which candidate subfractions of tumour cells are assessed for their ability to establish long-term serial engraftment in immunocompromised recipient animals is used to demonstrate the existence of tumourigenic and nontumourigenic subpopulations of cells and to quantitate the frequency of CSCs in the tumour. However, recent studies have shown that assay conditions can significantly affect the engraftment of transplanted cells. Limitations of the xenogeneic microenvironment to provide growth factors and survival signals to support cancer initiating cell (CIC) engraftment in addition to residual host immune function may all serve to underestimate the frequency of cancer initiating cells. For example, the frequency of human T-ALL initiating cells (T-ALL-IC) as assessed after transplantation into NOD/SCID (NS) mice, was reduced when compared with studies of human AML or B-ALL xenografts (Cox et al., 2007; Rehe et al., 2013; Jamieson et al., 2004; Le et al., 2008). However, frequencies increased after intrafemoral injection into NOD/scid/IL2Rynull (NSG) recipient mice that had been pretreated with an anti-CD122 antibody (Chiu, Jiang, & Dick, 2010). The concept that xenotransplantation underestimates the frequency of CICs was further supported in syngeneic transplantation studies using Eμ-myc, Eμ-N-ras and the PU.1 deficient transgenic mouse models. These mouse models, derived using genes
implicated in analogous human tumours, develop relatively homogeneous malignancies after the accumulation of additional genetic mutations. Transplantation of graded numbers of tumour cells into syngeneic recipients revealed that injection of as few as ten cells was sufficient to initiate malignancies (Kelly, Dakic, Adams, Nutt, & Strasser, 2007). Moreover, transplantation of subpopulations expressing stem cell antigens did not enrich for CICs.

The clinical relevance of the CSC has not been clearly established. There is wide speculation that in order to eliminate cancer cells and to prevent relapse, efficient targeting of the CSC is essential. The development and testing of novel therapeutic strategies targeting CSC will require the identification of CSC-specific markers, the isolation and characterization of CSCs.

**TLX1** is a member of an evolutionary conserved family of non-clustered homeobox genes that is expressed during embryogenesis and involved in spleen and neuron development (Roberts, Shutter, & Korsmeyer, 1994; Qian, Shirasawa, Chen, Cheng, & Ma, 2002). **TLX1** expression defines a distinct subtype of T-ALL characterized by a block in maturation at the early cortical stage of thymocyte development. Although intensive chemotherapy has significantly improved the outcomes for patients with TLX1-initiated T-ALL, relapses still occur and therefore new therapeutic approaches are required. The origin of the **TLX1**-initiated subtype of T-ALL initiating cell (T-ALL-IC) is unclear. Identification of such cells is of potential clinical significance as it may help to design specific targeting therapeutics thereby improving outcomes.

To address this question and better define mechanisms of **TLX1**-mediated transformation, we previously reported generation of *IgHμ-TLX1* mice which developed a transplantable **IgM****IgD** mature B-cell lymphoma at an average age of 14 months, with the primary site of lymphoma development being the spleen. **TLX1** was also capable of transforming T cells, as demonstrated in two mouse models (De et al., 2010; Rakowski, Lehotzky, & Chiang, 2011). Because all aforementioned models exhibited a prolonged latency, it was proposed that **TLX1** expression alone may not be sufficient to induce full blown T-ALL.

In this study, to identify mutations that collaborate with **TLX1** to trigger T-ALL, we generated *IgHμ-TLX1* mice expressing low levels of **TLX1** in early thymocyte progenitors and deficient in the DNA-dependent protein kinase, **DNA-PK**. *IgHμ-TLX1Prkdc* mice rapidly developed the disease, indicating that loss of **DNA-PK** combined with ectopic expression of **TLX1** in thymocyte progenitors inevitably leads to T-ALL.
Interestingly, about 46% of IgH\textsubscript{\textmu}-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mice also developed AML, suggesting that multiple hemopoietic subpopulations could be susceptible to TLX1-induced transformation leading to phenotypically distinct malignancies, such as T-ALL and AML.

We hypothesized that Early Thymocyte Progenitors (ETP) represent subpopulation with the most enrichment of cells responsible for onset of malignancy. These cells were defined as T-ALL Initiating Cell (T-ALL-IC) and could initiate thymic tumours from transformed ETP in IgH\textsubscript{\textmu}-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mice. Serial transplantations of flow cytometry-sorted tumour cells allowed us to identify CD44\textsuperscript{+} and c-kit-positive early thymocyte progenitors which persist in the thymus throughout postnatal period and puberty as cells susceptible for TLX1-mediated transformation. Moreover, we found that these cells appear to be highly enriched among the T-ALL-initiating cell populations, and their frequency strictly correlates with leukemia latency and penetrance in recipient mice. To our knowledge, this is the first study to identify and characterize TLX1-susceptible cells responsible for leukemia development. As such, it provides a clinically-relevant mouse model and identifies target cells for therapeutic intervention.
4.4. Results

4.4.1. Leukemia initiating cells reside within the thymus of

\(Ig\mu-TLX1^{Tg}\) \(Prkdc^{Scid/Scid}\) mice

Similar to \(Prkdc^{Scid/Scid}\) mice, T cell maturation in generated double mutant \(Ig\mu-TLX1^{Tg}\) \(Prkdc^{Scid/Scid}\) mice was arrested at an immature Double Negative (DN) stage with thymi consisting of DN1 (CD44^+CD25^-), DN2 (CD44^+CD25^+) and DN3 (CD44^-CD25^+) subpopulations but lacking CD4^+CD8^+ Double Positive (DP) cells (Figure 4.1A). The thymic architecture of \(Prkdc^{Scid/Scid}\) and \(Ig\mu-TLX1^{Tg}\) \(Prkdc^{Scid/Scid}\) mice was disrupted with no evidence of the cortical region being detected. On the IRC genetic background, both \(Ig\mu-TLX1^{Tg}\) \(Prkdc^{Scid/Scid}\) and control \(Prkdc^{Scid/Scid}\) mice were prone to the development of T-ALL with tumour cells consisting of CD44^+CD25^, CD44^+CD25^+, CD44^-CD25^, CD4^+ and CD8^+ cells but lacked surface CD3 and TCR\(\alpha/\beta\) expression (Figure 4.1B).
Figure 4.1. Phenotype of healthy and moribund mice deficient in DNA-PK. 
A. H&E and flow cytometry of healthy Prkdc<sup>Scid/Scid</sup> and IgH<sub>μ</sub>-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mice. B. Disrupted architecture in the thymus and bone marrow. Black arrows indicate tumour cells. Abnormal presence of immature thymocytes in the bone marrow and CD<sup>4</sup><sup>-</sup>CD<sup>8</sup><sup>-</sup> cells in the thymus.
To determine if the T-ALL-IC resided in the bone marrow or in the thymus we injected cohorts of sublethally irradiated Prkdc<sup>Scid</sup>/Scid (Balb/c) recipients with either bone marrow or thymic tumour cells. Donor IgH<sub>μ</sub>-TLX1<sup>Tg</sup>Prkdc<sup>Scid</sup>/Scid mice were selected based on the presence of tumour cells expressing CD44, CD25, CD4, and CD8 in the thymus and bone marrow samples with no evidence of tumour cell infiltration, as revealed by histopathology and flow cytometry (Figure 4.1B). Recipient mice were injected with either intact 10<sup>7</sup> bone marrow cells or 10<sup>7</sup> thymic tumour cells (Figure 4.2A). To control for spontaneous T-ALL development in recipient mice, Prkdc<sup>Scid</sup>/Scid mice were irradiated and then injected with FACS Buffer. Each cohort consisted of six recipient mice were monitored for 16 months post injection for signs of disease.

Remarkably, all recipients transplanted with thymic tumour cells died within five to six weeks following injection (Figure 4.2B). Histopathological analysis showed that tumour cells engrafted the spleens and bone marrow of the recipient mice, while thymi retained a normal architecture for Prkdc<sup>Scid</sup>/Scid mice (Figure 4.2C). Flow cytometric analysis of cells isolated from the bone marrow and spleen of recipient mice revealed that the bone marrow and spleens were comprised of CD44<sup>+</sup>CD25<sup>−</sup>, CD44<sup>+</sup>CD25<sup>+</sup> and CD44<sup>+</sup>CD25<sup>+</sup> cells (Figure 4.2C). Moreover, between 60 and 85 percent of the cells coexpressed CD4 and CD8 suggesting that the IgH<sub>μ</sub>-TLX1<sup>Tg</sup>Prkdc<sup>Scid</sup>/Scid tumour cells had engrafted the spleens and bone marrow of recipient mice (Figure 4.2D).

In contrast to recipients injected with thymic tumour cells, three of the six Prkdc<sup>Scid</sup>/Scid recipients injected with bone marrow cells died after a prolonged latency of the disease (from 22 to 30 weeks post-transplantation), while the remaining three recipients remained healthy for the duration of the observation period. Of the three moribund bone marrow recipients, one showed evidence of myeloid hyperplasia in the bone marrow with no evidence of T-ALL while the remaining two bone marrow recipients exhibited T-ALL with the thymus being the site of tumour development. One of five irradiated control Prkdc<sup>Scid</sup>/Scid mice exhibited T-ALL disease with the same prolonged latency and thymic involvement while the remaining four mice were healthy. Given both the extended latency for disease development in recipients of IgH<sub>μ</sub>-TLX1<sup>Tg</sup>Prkdc<sup>Scid</sup>/Scid bone marrow cells and the involvement of the thymus in tumour development in these animals, it is likely that these leukemias arose spontaneously after transformation of Prkdc<sup>Scid</sup>/Scid cells and were not derived from donor IgH<sub>μ</sub>-TLX1<sup>Tg</sup>Prkdc<sup>Scid</sup>/Scid bone marrow cells. Furthermore, the finding that control recipient mice injected with FACS
Buffer and recipients injected with $Ig\mu-TLX1^{Tg}Prkdc^{Scid/Scid}$ bone marrow cells developed T-ALL originating in the thymus with an extended latency further supports this conclusion. Combined, the above findings suggested that the T-ALL L-ICs resided in the thymus of $Ig\mu-TLX1^{Tg}Prkdc^{Scid/Scid}$ mice.
Figure 4.2. Transplantation of cells isolated from the thymus and bone marrow of a moribund IgHμ-TLX17Prkdcscid/scid mouse into irradiated Prkdcscid/scid recipients. (A) T-ALL in the thymus and normal bone marrow. Black arrows indicate tumour cells. (B) Kaplan–Meier survival curves for transplanted recipients, (p < 0.001). (C) Similar T-ALL phenotype in recipients as the donor cells. (D) Tumour cells coexpressed CD4+CD8+.
4.4.2. All CD44/CD25 thymic tumour subpopulations were capable of inducing T-ALL in \( Prkdc^{Scid/Scid} \) recipient mice

To precisely identify and characterize T-ALL-ICs, thymic subpopulations sorted from \( IgH\mu-TLX1^{Tg} Prkdc^{Scid/Scid} \) moribund mice were injected into sublethally irradiated recipients. We transplanted four flow-sorted tumour subpopulations: CD44\(^+\)CD25\(^-\) (F-1), CD44\(^+\)CD25\(^+\) (F-2), CD44\(^-\)CD25\(^+\) (F-3) and CD44\(^-\)CD25\(^-\) (F-4) (Figure 4.3A). Control mice were injected with bone marrow cells isolated from 8-week old \( IgH\mu-TLX1^{Tg} Prkdc^{Scid/Scid} \) mice or FACS Buffer. All recipient mice injected with thymic tumour cells died of T-ALL within five to six weeks of injection (Figure 4.3B). Flow cytometric analyses of tumour cells isolated from the bone marrow and spleens of recipient mice revealed that they expressed a similar CD44\(^+\)CD25\(^+\)CD4\(^+\)CD8\(^+\) phenotype as the donor tumour cells (Figure 4.3C). Although tumour cells were detected in both the spleens and bone marrow, they were not detected in the thymi of recipient mice. Control mice and recipients injected with bone marrow cells remained healthy throughout the observation period. These findings suggested that T-ALL-ICs resided in the thymus and were present in all subfractions sorted on the basis of CD44 and CD25 expression. In an attempt to further enrich for T-ALL-ICs, we sorted cells based on c-kit, CD4 and CD8 expression.
Figure 4.3. All subpopulations of IgHμ-TLX1TgPrkdc<sup>Scid/Scid</sup> thymic tumour cells initiated T-ALL in recipients. A. Thymus of donor mouse. Black arrows indicate tumour cells. Tumour cells were sorted based on the CD44 and CD25 expression (brown squares). B. Kaplan–Meier survival curves of thymic tumour cells recipients and control mice. C. All tumour cells subpopulations expressed CD4<sup>+</sup>CD8<sup>+</sup>.
4.4.3. Sorting of the c-kit⁺CD44⁺ tumour subpopulation enriched for the T-ALL-IC

Since c-kit is known to be expressed in hemopoietic stem cells and early thymocyte progenitors (Shortman & Wu, 1996; Godfrey, Zlotnik, & Suda, 1992), we used this surface antigen as a selection marker to enrich for T-ALL-ICs. Tumour subpopulations were sorted on the basis of CD44, CD25, CD4, CD8 and c-kit expression and included: c-kit⁺CD44⁺CD25⁻CD4⁻CD8⁻ (F1/c-kit⁺), c-kit⁺CD44⁺CD25⁻CD4⁺CD8⁻ (F1/c-kit⁻), c-kit⁻CD44⁺CD25⁻CD4⁻CD8⁻ (F2/c-kit⁺), c-kit⁻CD44⁺CD25⁻CD4⁺CD8⁻ (F2/c-kit⁻), c-kit⁻CD44⁺CD25⁺CD4⁻CD8⁻ (F3/c-kit⁻) and c-kit⁻CD44⁻CD25⁺CD4⁺CD8⁻ (F4/c-kit⁻). Equal numbers of sorted cells were then transplanted into sublethally irradiated recipients. It should be noted that c-kit⁻CD44⁻CD25⁻CD4⁻CD8⁻ and c-kit⁺CD44⁻CD25⁻CD4⁻CD8⁻ cells were not detected and thus were not included in the analysis. Cohorts, each consisting of six recipient mice, were monitored for 200 days after transplantations for signs of disease. These studies indicated that all tumour subpopulations were able to initiate leukemia with subpopulation engraftment always being detected in the spleen and bone marrow in recipient mice (Table 4.1). There was a correlation between the number of cells injected and the latency and frequency of disease development (Figure 4A-F). Most significantly, F1/c-kit⁺ and F2/c-kit⁺ tumour cells were more potent inducers of disease relative to F3/c-kit⁻ and F4/c-kit⁻ cells. These data shown a correlation with cell dose derived from IgHμ-TLX1TgPrkdcScid/Scid primary tumour and both tumour initiation and disease latency. As few as 500 F1/c-kit⁺ and F2/c-kit⁺ tumour cells were sufficient to induce T-ALL in recipient mice, while 10³ to 10⁴ cells lacking c-kit expression were required to initiate disease in recipients (Figure 4.4, compare panels A and C with B and D). Among the c-kit⁻ subpopulations, only F3 cells consistently showed a higher penetrance and earlier onset of T-ALL in recipients. Furthermore, the F4 cells showed the lowest penetrance and initiated T-ALL only after transplantation of 10⁴ to 10⁵ cells (Figure 4.4B, E-F, see also Table 4.1). Interestingly, regardless of the injected tumour subfraction, all recipient mice developed T-ALL tumours containing a subpopulation of c-kit positive cells (Figure 4.5A-F). Of further note, F1/c-kit⁺-induced tumours exhibited the highest percentage of CD44⁺CD25⁺c-kit⁺ cells in the spleen and bone marrow (Figure 4.5G), and this also correlated with higher penetrance and earlier onset of T-ALL developed in recipients (Figure 4.4, Table 4.1). Tumours derived after injection of F3 cells also showed a higher percentage of c-kit⁺CD44⁺ cells as compared with recipients of either F1/c-kit⁺ for F2/c-kit⁺.
cells. In contrast, the F4/c-kit^−-injected cohort exhibited the lowest proportion of CD44^+CD25^- c-kit^+ cells, both in the spleen and bone marrow, along with the latest onset of disease and lowest penetrance. Limiting dilution analysis found that L-IC frequency in c-kit^+ cell fractions is higher than in c-kit^- fractions (Table 4.2). Combined, these data strongly suggested that T-ALL-ICs were enriched within subfractions of tumour cells expression the c-kit antigen.
Figure 4.4. Kaplan-Meyer survival curves of Prkdc<sup>Scid/Scid</sup> recipient mice transplanted with sorted IgHu-TLX1<sup>TgPrkdc<sup>Scid/Scid</sup></sup> tumour cells. A-F. Mice were injected with F1/c-kit<sup>+</sup>, F1/c-kit<sup>-</sup>, F2/c-kit<sup>+</sup>, F2/c-kit<sup>-</sup>, F3/c-kit<sup>+</sup> and F4/c-kit<sup>-</sup> flow sorted tumour cells, respectively. N indicates the number of injected mice. C-kit<sup>+</sup> cells fractions recipients exhibited early onset and high penetrance of disease.
Figure 4.5. Early onset of T-ALL in Prkdc<sup>Scid</sup>/Scid recipients is correlated with increase proportion of tumour cells expressed the c-kit. A-F. Flow cytometry of samples isolated from recipients transplanted with flow-sorted F1/c-kit<sup>-</sup>, F1/c-kit<sup>+</sup>, F2/c-kit<sup>-</sup>, F2/c-kit<sup>+</sup>, F3/c-kit and F4/c-kit<sup>-</sup> tumour cells, respectively. Four recipients from each cohort were analyzed. G. Difference in the mean of c-kit<sup>-</sup>CD44<sup>+</sup> cells in recipients (*p<0.05).
### 4.4.4. Self-renewal of T-ALL-ICs in secondary and tertiary Prkdc<sup>Scid/Scid</sup> recipient mice

To provide functional evidence for the presence of self-renewing L-ICs within the bulk tumours, we performed serial transplantations of unfractionated primary tumour cells into secondary and tertiary Prkdc<sup>Scid/Scid</sup> recipients. CD44<sup>+</sup>CD25<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> tumour cells were isolated from the thymus of a primary IgH<sub>μ</sub>-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mouse (Figure 4.6A) and 10<sup>7</sup> cells were injected into six secondary recipient mice. Control mice were injected with 10<sup>7</sup> bone marrow cells isolated from 8-week old IgH<sub>μ</sub>-TLX1<sup>Tg</sup>Prkdc<sup>Scid/Scid</sup> mice or FACS Buffer. Secondary recipients developed leukemia six weeks after injections with the tumour cells being detected in the spleens and bone marrow (Figure 4.6B), while control mice and recipients injected with bone marrow cells remained healthy throughout the observation period (Figure 4.6C). 10<sup>7</sup> tumour cells isolated from the spleens of secondary recipients were transplanted in six tertiary recipients with all recipients succumbing to leukemia after four weeks. Similar to secondary recipients, thymi were not affected and transplanted tumour cells were detected in the spleen and bone marrow of tertiary recipients with engrafted cells being composed of CD44<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup> (Figure 4.6B) cells suggesting clonal T lymphopoiesis in the spleens and marrow of recipient mice. Remarkably, tertiary recipients exhibited significantly accelerated T-ALL development and died within three to four weeks of transplantation relative to secondary recipients transplanted with the same number of tumour cells (Figure 4.6C). The T-ALL latency was reduced from 33.5 days in the secondary recipient cohort to 27 days in the tertiary recipients (Figure 4.6D).
Figure 4.6. Acceleration of T-ALL in secondary and tertiary Prkdc<sup>Scid/Scid</sup> recipients. A. T-ALL in the thymus of donor mouse. B. Normal thymus and T-ALL in spleens and bone marrow recipients. Kaplan–Meier survival curves of cohorts of recipients. Difference (p=0.0015) between recipient curves. D. Difference in the mean T-ALL latency (*p=0.035).
Limiting dilution analysis was used to identify the frequency of T-ALL-ICs within the bulk tumour population and in tertiary recipients. A range of tumour cell doses \(10^7, 10^6, 10^5, 10^4, 10^3, 10^2\) derived from primary \(IgH\mu-TLX1^TgPrkdc^{Scid/Scid}\) mice (Figure 4.7A) was injected into secondary recipients and mice were monitored for 16 weeks for the development of disease. Engrafted tumour cells \(10^6, 10^5, 10^4, 10^3, 10^2\) obtained from the spleen of secondary recipients were then transplanted into tertiary recipients. As expected, the time required for T-ALL development was correlated with the number of tumour cells injected. In secondary recipients, the average latency period for the development of leukemia was 41 days with terminal leukemia occurring after injection of \(10^4\) or more tumour cells (Figure. 4.7B). In tertiary recipients, the minimal number of cells required to induce leukemia was \(10^2\) with leukemia developing after an average of 21 days (Figure 4.7C). In addition, the T-ALL latency was reduced in \(10^4, 10^5\) and \(10^6\) tertiary recipient groups relative to those for the secondary recipient cohorts (Figure 4.7D). Combined, these findings suggested that T-ALL-ICs underwent both self-renewal and expansion of the numbers after transplantation in recipient mice.

T-ALL cells were detected in the spleen and bone marrow of all secondary and tertiary recipients and appeared to exhibit similar flow cytometry profiles (Figure 4.8A, B). Importantly, the proportion of \(c\text{-kit}^+\text{CD44}^+\) cells in the bone marrow and spleens of tertiary recipients increased relative to secondary recipient mice suggesting expansion of this subpopulation of cells after transplantation (Figure 4.8C, D). Since \(IgH\mu-TLX1^TgPrkdc^{Scid/Scid}\) tumour cells consistently engrafted the bone marrow and spleens of secondary and tertiary \(Prkdc^{Scid/Scid}\) recipient mice, we used limiting dilution analysis to determine the frequency of T-ALL-ICs in primary tumour and recipients. L-Calc software was used to determine that the frequency of T-ALL-ICs as being 1 in 4,910 secondary tumour cells and 1 in 689 tertiary tumour cells (Table 4.3).
Figure 4.7. Tertiary recipients had accelerated onset of leukemia in all groups relative to secondary recipients. A. T-ALL in the thymus of donor mouse. B. Kaplan–Meier survival curves of primary thymic tumour cells recipients. C. Kaplan–Meier survival curves of tertiary recipients injected with tumour cells isolated from secondary recipients. D. Difference in the mean T-ALL latency (*p<0.05).
Figure 4.8. T-ALL phenotype in secondary and tertiary Prkdc<sup>Scid/Scid</sup> recipients.

A. H&E of organs isolated from moribund recipients. Black arrows indicate tumour cells.
B. T-ALL cells in the spleen the bone marrow in recipients.
C. C-kit<sup>−</sup>CD44<sup>+</sup> expression on tumour cells. D. Mean percentages of c-kit<sup>−</sup>CD44<sup>+</sup> cells between secondary and tertiary recipients (p < 0.01).
4.5. Discussion

Cancer is a slowly progressing disease that requires time to develop. During this time different alterations in genes responsible for genetic stability arise sequentially in the target cell and cause cascade of mutations throughout the genome while cell matures (Loeb, 2001). Some of mutations will acquire a selective advantage eventually transforming normal cell into cancer initiating cell. Similar to general pathogenesis of cancer, leukemia is initiated when a target cell accumulated enough mutations, the cooperative effect of which were sufficient to acquire cell to divide, invade and metastasize (Vogelstein et al., 1988). Currently, the dominant model of leukemia indicates that L-IC likely reside within the HSC compartment, where self-renewal is physiologically activated, as has been shown for AML by Dick et al (Bonnet & Dick, 1997). However, generation of transgenic mice for myeloid leukemia using an hMPR-8 promoter, which targets expression of the transgene to progenitors restricted to the myeloid lineage that are not self-renewing, resulted in the development of AML in Fas deficient hMPR8-bcl-2 transgenic mice (Lagasse & Weissman, 1994; Traver, Akashi, Weissman, & Lagasse, 1998). Furthermore, transplantation of primary B-ALL patient cells into immunodeficient NOD/SCID mice provided evidence that L-IC may reside among more mature B cell progenitors with a CD34+CD19+ phenotype (Le et al., 2008). Moreover, transplantation of sorted pediatric T-ALL into the NOD/SCID mouse model detected that L-IC may reside among cells with different stages of maturation, but more immature cells were more highly enriched with disease potential (Cox et al., 2007). Overall these data suggest that cooperative effect of acquired mutations defines the stage of maturation where normally developing cell will be transformed into L-IC and more mature cells residing outside of the bone marrow may be a target for malignant transformation.

Previous works indicated that early thymocyte progenitors (ETP) are lymphoid lineage restricted, reside within the thymus and have a potential to differentiate to T cells, Natural Killers and B cells (Kondo, Scherer, King, Manz, & Weissman, 2001; Bhandoola, von, Petrie, & Zuniga-Pfucker, 2007). Other studies indicate that thymic ETP, in addition also possess dendritic, and myeloid potential, as shown by the growth of Mac1+ cells in OP9 cultures initiated from cells with c-kit+CD25 Lin- and c-kit+CD25+Lin- phenotypes (Allman et al., 2003; Min, Montecino-Rodriguez, & Dorshkind, 2004; Bhandoola, von, Petrie, & Zuniga-Pfucker, 2007; Bell & Bhandoola, 2008; Luc et al., 2012). Only a rare subset of ETP as was shown has the potential to give rise to B cells (Benz & Bleul, 2005; Rolink, Massa, Balciunaite, & Ceredig, 2005).
2007). In addition, several reports indicated that leukemic transformation effects occurred in a cell which is committed to the T lineage, but still retains myeloid potential (Plasschaert, Kamps, Vellenga, de Vries, & de Bont, 2004; Asnafi et al., 2003). \( IgH\mu-TLX1^{Tg}Prkc_{Scid/Scid} \) mice developed T-ALL disease in the thymus and AML disease in the bone marrow and spleen. Development of T-ALL in the thymus as a primary site in \( IgH\mu-TLX1^{Tg}Prkc_{Scid/Scid} \) mice indicated that T-ALL-IC most probably resided in the thymus among lymphoid restricted progenitors. However, 45% of \( IgH\mu-TLX1^{Tg}Prkc_{Scid/Scid} \) double mutant transgenic mice unexpectedly developed AML disease in addition to the anticipated T-ALL (paper is under review). The significant percentage of AML cases could not be explained by sporadically evoked mutations, the random effects of which might alter the phenotype of the disease, but rather that mutations occur in progenitors that still have the potential to differentiate into both, lymphoid and myeloid cells and the net result of these mutations will define phenotype of malignancy. These findings highlighted additional questions regarding the cell of origin for malignant transformation as well as the organ site where mutations accumulate to initiate malignancy in \( IgH\mu-TLX1^{Tg}Prkc_{Scid/Scid} \) mice. Thus, detection of T-ALL and AML diseases in \( IgH\mu-TLX1^{Tg}Prkc_{Scid/Scid} \) mice indicated the presence of several different developmentally restricted cell populations or cells with multipotent potential to accumulate mutations, which may finally drive cell to T-ALL or AML fate. For example, ectopic expression of \( TLX1 \) in myeloid multipotent progenitors could provide the initial transforming event, but then depending on the additional mutations accumulating in the target cell, progenitors could be directed down either the myeloid or T cell lineages. Alternatively, DN1 thymocytes with the potential to mature into not only thymocytes, but also B, myeloid and dendritic cells could be the target and again, depending on the nature of the addition mutations acquired, AML or T-ALL would develop. Also, only myeloid progenitors with additional mutations give rise to AML and only thymocyte progenitors which accumulate mutations give rise to T-ALL (Supplementary Figure 4.1).
Supplementary Figure 4.1. A model depicting T-ALL initiation in IgHμ-TLX1TsPrkdcScid/Scid mice. T-ALL arises within the DN2-DN3 thymocytes (red dashed circle). Mutations arise in the HSC and MPP (black dashed oval) or in DN1 thymocytes (black dashed circle) with the potential to initiate AML or T-ALL. TLX1 expression is indicated in red.
In the present study, we provided data which indicate that progenitor cells in \(\text{IgH}\mu\text{-TLXI}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}}\) mice accumulate cooperative mutations and initiate T-ALL at the developmental stage of the most immature thymocytes following seeding of the thymus. Serial transplantation of thymic tumours and intact bone marrow of moribund \(\text{IgH}\mu\text{-TLXI}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}}\) mice into sublethally irradiated \(\text{Prkdc}^{\text{Scid/Scid}}\) recipients indicated that progenitors that accumulate T-ALL initiating mutations likely reside in the thymus. The low penetrance of disease in recipient mice transplanted with bone marrow cells, the similar latency of tumour development in irradiated control \(\text{Prkdc}^{\text{Scid/Scid}}\) mice and in \(\text{Prkdc}^{\text{Scid/Scid}}\) mice injected with \(\text{IgH}\mu\text{-TLXI}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}}\) bone marrow cells, and that both irradiated controls and mice injected with bone marrow cells showed development of thymic tumours allowed us to speculate that \(\text{Prkdc}^{\text{Scid/Scid}}\) bone marrow recipient mice developed disease as a combined result of DNA-PK deficiency and irradiation, rather than as a result of T-ALL-IC residing in the bone marrow. Furthermore, if the T-ALL-IC originated in the bone marrow we would anticipate that the latency period for leukemia development would be similar to that seen in the mice injected with thymic tumour cells.

Transplantation of flow sorted T-ALLs into sublethally irradiated \(\text{Prkdc}^{\text{Scid/Scid}}\) recipients and limiting dilution analysis allowed us to determine that T-ALL-ICs reside in fractions with upregulated c-kit expression in CD44\(^+\)Lin\(^-\) and CD44\(^+\)CD25\(^+\)Lin\(^-\) populations and have a greater leukemia initiating potential than tumour cell fractions where c-kit was not expressed. The importance of upregulated c-kit in T-ALL-IC was also shown in transplantation of T-ALL from \(\text{Pten}\) deficient mice into irradiated severe combined immune deficient mice (Guo et al., 2008). Importantly, the \(\text{TLXI}\) transgene expression was detected at higher levels in the immature progenitors, DN1 and DN2 subpopulations in premalignant thymocytes in \(\text{IgH}\mu\text{-TLXI}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}}\) mice indicating its unambiguous role in T-ALL initiation. The model of leukemia initiation in \(\text{IgH}\mu\text{-TLXI}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}}\) mice that emerges from these cumulative data is that immature progenitors enter the thymus before malignant transformation or with some genetic alterations, which insufficient to initiate diseases by themselves. Abnormal expression of \(\text{TLXI}\) allows cells to acquire T-ALL initiating mutations, including the ability to self-renew, via cooperative effect of deregulated apoptotic, proliferation genes and genes involved in the regulation of the cell cycle.

Due to the absence of AML donors we were unable to perform transplantation of AML cells into recipients and define the site where AML-L-IC resides. Transplantation of frozen
AML samples didn’t initiate leukemia in recipients that may be explained by the increased sensitivity of L-IC to low temperature. However, absence of myeloid leukemia in all recipients ruled out possibility that tumour cells have a propensity to initiate AML and AML-L-ICs disseminate from the thymus into the peripheral circulation and engraft the bone marrow. In addition, a significantly decreased time of disease onset and increased penetrance among subsequent Prkdc<sup>Scid/Scid</sup> recipients indicated that the frequency of T-ALL-IC was increased in tumours developed by each new generation of mice. Importantly to notice, that even though AML-L-ICs were presented in the thymus at low frequency, their numbers should be expanded upon transplantation similarly to T-ALL-ICs, but none of secondary recipients developed AML. These data confirm that thymus is the site where immature thymocytes acquire only those mutations which initiate T-ALL. Collectively, the data we presented here suggest that AML appears unlikely to have arisen in a cell that already seeded the thymus.

It remains unclear, why all Prkdc<sup>Scid/Scid</sup> recipients of bulk tumours and sorted tumour cell populations exhibited T-ALL in the spleen and bone marrow while the thymus was never affected even if disease developed after a long latency. One explanation may be that the majority of transplanted T-ALL-ICs first seed the bone marrow and spleen, which provide an optimal microenvironment for their fast expansion. This may prompt substitution of normal bone marrow cells with tumour cells which becomes lethal before the much fewer numbers of L-IC expand in the thymus to the levels detectable by flow cytometry.
Table 4.1. T-ALL engraftment in $Prkdc^{Scid/Scid}$ recipients

Engraftment was considered as positive if recipient developed T-ALL as detected by H&E and flow cytometric analysis. Limiting dilution analysis using L-Calc software was used to detect L-IC frequency.

<table>
<thead>
<tr>
<th>Transplanted T-ALL cell fraction</th>
<th>T-ALL dose</th>
<th>N of engrafted/total N of injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1/c-kit$^+$</td>
<td>$5 \times 10^2$</td>
<td>6/8</td>
</tr>
<tr>
<td>F1/c-kit$^+$</td>
<td>$10^4$</td>
<td>8/8</td>
</tr>
<tr>
<td>F1/c-kit$^+$</td>
<td>$10^4$</td>
<td>8/8</td>
</tr>
<tr>
<td>F1/c-kit$^-$</td>
<td>$5 \times 10^2$</td>
<td>0/5</td>
</tr>
<tr>
<td>F1/c-kit$^-$</td>
<td>$10^4$</td>
<td>0/5</td>
</tr>
<tr>
<td>F1/c-kit$^-$</td>
<td>$10^4$</td>
<td>2/5</td>
</tr>
<tr>
<td>F1/c-kit$^-$</td>
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<td>1/5</td>
</tr>
<tr>
<td>F4/c-kit$^-$</td>
<td>$10^3$</td>
<td>3/5</td>
</tr>
</tbody>
</table>
Table 4.2. Frequency of leukemia initiating cells in transplanted flow sorted tumour cells

Limiting dilution analysis using L-Calc software found that the maximum L-IC frequency is in F1/c-kit⁺ and F2/c-kit⁺ cell fractions (1 in 397 and 1 in 812 cells, respectively).

<table>
<thead>
<tr>
<th>Frequency of L-IC in cell suspension</th>
<th>95% confidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-kit⁺CD44⁺CD25⁻ CD4⁺CD8⁻ (F1/c-kit⁺)</td>
<td>1/397 (1/544-1/290)</td>
</tr>
<tr>
<td>C-kit⁻CD44⁺CD25⁻ CD4⁺CD8⁻ (F1/c-kit⁻)</td>
<td>1/59,694 (1/97,705-1/36,470)</td>
</tr>
<tr>
<td>C-kit⁺CD44⁺CD25⁺CD4⁻ CD8⁻ (F2/c-kit⁺)</td>
<td>1/812 (1/1,251-1/527)</td>
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<tr>
<td>C-kit⁻CD44⁺CD25⁺CD4⁻ CD8⁻ (F2/c-kit⁻)</td>
<td>1/31,115 (1/52,101-1/18,582)</td>
</tr>
<tr>
<td>C-kit⁺CD44⁻CD25⁺CD4⁻ CD8⁻ (F3/c-kit⁺)</td>
<td>1/4,940 (1/7,950-1/3,069)</td>
</tr>
<tr>
<td>C-kit⁻CD44⁻CD25⁻ CD4⁺CD8⁺ (F4/c-kit⁻)</td>
<td>1/93,916 (1/159,373-1/55,343)</td>
</tr>
</tbody>
</table>
Table 4.3. Frequency of leukemia Initiating cells in bulk tumour cells in secondary and tertiary recipients

Limiting dilution analysis using L-Calc software found that L-IC frequency in secondary recipients lower (1 in 4,910 cells) than in tertiary recipients (1 in 689 cells).

<table>
<thead>
<tr>
<th></th>
<th>Frequency of L-IC in cell suspension</th>
<th>95% confidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary recipients</td>
<td>1/4,910</td>
<td>(1/7,861-1/3,066)</td>
</tr>
<tr>
<td>Tertiary recipients</td>
<td>1/689</td>
<td>(1/1,048-1/453)</td>
</tr>
</tbody>
</table>
Chapter 5

TLX1-facilitated development of acute myeloid leukemia in

IgHμ-TLX1^{Tg} Prkδ^{Scid/Scid} mice
5.1. Attribution of data

I performed all the work described in this chapter except for histological specimens prepared at the Department of Pathology in the Sunnybrook Research Institute.
5.2. Abstract

The non-cluster homeobox gene, *HOX11/TLX1 (TLX1)* acts as an oncogene when abnormally expressed in thymocytes and B cells. However, little is known about the oncogenic potential of enforced expression of *TLX1* in myelocytes. *IgHμ-TLX1*<sup>Tg</sup>*Prkdc<sup>Scid/Scid</sup>* mice were initially generated to better understand *TLX1* deregulated genetic pathways in premalignant thymocytes. However, 45% of *IgHμ-TLX1*<sup>Tg</sup>*Prkdc<sup>Scid/Scid</sup>* mice developed Acute Myeloid Leukemia (AML) indicating that *TLX1* enhanced the oncogenic potential in myeloid cells. We established that *TLX1*- induced AML was more dependent on the CD1 genetic background in the *IgHμ-TLX1*<sup>Tg</sup>*Prkdc<sup>Scid/Scid</sup>* mice. To identify molecular mechanisms deregulated by abnormal expression of *TLX1* in cells of the myeloid compartment we performed functional *in vitro* and *in vivo* assays with myeloid progenitors from premalignant *IgHμ-TLX1*<sup>Tg</sup>*Prkdc<sup>Scid/Scid</sup>* mice. Our data showed that overexpression of *TLX1* in *IgHμ-TLX1*<sup>Tg</sup>*Prkdc<sup>Scid/Scid</sup>* myeloid progenitors decreased apoptosis and provided them with a proliferative advantage relative to myeloid progenitors from *Prkdc<sup>Scid/Scid</sup>* littermates. Similar mechanisms were shown to operate in *TLX1* overexpressing T cells deficient in the *DNA-PK*. Thus, the *IgHμ-TLX1*<sup>Tg</sup>*Prkdc<sup>Scid/Scid</sup>* mouse model provides the first direct evidence *in vivo* that ectopic expression of *TLX1* in myeloid progenitors facilitates the development of AML.
5.3. Introduction

Homeobox genes are critical players in cell proliferation and differentiation and their deregulated expression has been shown to contribute to hemopoietic malignancies (Owens & Hawley, 2002). *TLX1* is a non-cluster homeobox gene encoding an oncogenic transcription factor, which is frequently overexpressed and activated in T cell Acute Lymphoblastic leukemia (T-ALL) (Dube et al., 1991; Hatano, Roberts, Minden, Crist, & Korsmeyer, 1991b; Kennedy et al., 1991; Ferrando et al., 2002). *TLX1* was shown to be activated in T-ALL samples obtained from both pediatric and adult patients and believed to be one of initiating events in T cell malignant transformation.

The *in vivo* oncogenic potential of *TLX1* in B cells was initially demonstrated in *IgHμ-HOX11*^Tg^ mice (Hough et al., 1998). Potential of *TLX1* to initiate T-ALL in mouse models was reported by two independent research groups (De et al., 2010; Rakowski, Lehotzky, & Chiang, 2011). Ectopic expression of *TLX1* could also contribute to other types of hemopoietic malignancies. For example, Hawley et al. showed that retroviral infection of hemopoietic stem cells and multipotent progenitors resulted in their immortalization and their subsequent differentiation into erythroid or myeloid-like cells (Keller, Wall, Fong, Hawley, & Hawley, 1998; Greene et al., 2002; Hawley, Fong, Lu, & Hawley, 1994). In line with in vitro results, ectopic expression of *TLX1* in mouse bone marrow caused differentiation arrest and myeloid cell immortalization (Hawley et al., 1997). Moreover, a low proportion of *IgHμ-TLX1*^Tg^ mice developed myeloid hyperplasia although myeloid leukemia was not detected. Altogether, these data demonstrated the oncogenic potential of *TLX1* in different cell types including myeloid and lymphoid cells.

Given the finding of Korsmeyer et al. showing that *TLX1* enabled cells with double strand DNA breaks (DS-DNA breaks) to bypass the G2/M cell cycle check point, (Kawabe, Muslin, & Korsmeyer, 1997) we generated *IgHμ-TLX1*^Tg^*Prkdc*^Scid/Scid^ double mutant transgenic mice with rapidly developed leukemia with a median age of disease onset of 6.6 months with 100% penetrance. Approximately half of the *IgHμ-TLX1*^Tg^*Prkdc*^Scid/Scid^ mice developed T-ALL (described in Chapter 3) while the remaining mice developed a hemopoietic disease with histological features and flow cytometry profiles consistent with acute myeloid leukemia (AML).

To identify molecular mechanisms that contributed to *TLX1*-induced transformation, we performed functional assays using cells obtained from premalignant *IgHμ-TLX1*^Tg^*Prkdc*^Scid/Scid^ mice. Our data indicated that ectopic *TLX1* expression contributed to enhanced proliferation and
increased survival of myeloid progenitors, which may potentially be responsible for development of AML. In particular, we found that TLX1 overexpression increased total bone marrow and spleen cellularity and improved viability of spleen and bone marrow cells derived from IgHμ-TLX1TgPrkdcScid/Scid mice. In addition, expression of TLX1 increased the frequency of myeloid progenitors in premalignant IgHμ-TLX1TgPrkdcScid/Scid mice, as revealed by flow cytometric analysis and verified by colony forming unit assays (CFU). We established that colonies derived from myeloid progenitors obtained from the bone marrow or spleen of premalignant IgHμ-TLX1TgPrkdcScid/Scid mice were larger and contained more cells than those from PrkdcScid/Scid mice. Increased proliferation was also confirmed in vivo using BrdU labeling assays which revealed an increased proportion of BrdU-positive cells in the bone marrow and spleens of IgHμ-TLX1TgPrkdcScid/Scid mice relative to PrkdcScid/Scid littermates. Moreover, considering that similar effects of increased proliferation and survival were also observed in TLX1-overexpressing T cells, we conclude that this could be a common mechanism operating in different IgHμ-TLX1TgPrkdcScid/Scid cell types.

5.4. Results

5.4.1. IgHμ-TLX1TgPrkdcScid/Scid mice exhibit accelerated development of AML

To identify genes that collaborated with TLX1 in progression of hemopoietic malignancies, we generated IgHμ-TLX1TgPrkdcScid/Scid double mutant mice. This was achieved by mating IgHμ-TLX1Tg mice with either CBySmn.CB17-Prkdcscid/J Balb/c or CB17 ICR-Prkdcscid mice (referred to as PrkdcScid/Scid mice). Approximately 50% of IgHμ-TLX1TgPrkdcScid/Scid mice developed T-ALL (see Chapter 3) with the remaining mice developing a disease with histological features and flow cytometry profiles consistent with AML. As was the case with T-ALL (Chapter 3, Figure 3.2), TLX1 accelerated AML in IgHμ-TLX1TgPrkdcScid/Scid mice (Figure 5.1A). IgHμ-TLX1TgPrkdcScid/Scid mice developed AML more rapidly than PrkdcScid/Scid littermates with the median survival of IgHμ-TLX1TgPrkdcScid/Scid mice developing AML being 7.0 months, whereas PrkdcScid/Scid mice exhibited delayed onset of diseases with a median survival 14.0 months (Figure 5.1B). The one-year survival probability estimate of IgHμ-TLX1TgPrkdcScid/Scid mice was also decreased relative to those of PrkdcScid/Scid littermates developing AML (Figure 5.1C).
Figure 5.1. Two-year survival analysis of Prkdc<sup>Scid/Scid</sup> and IgH<sub>μ</sub>-TLX1<sup>tg</sup>Prkdc<sup>Scid/Scid</sup> mice. Kaplan–Meier plot survival curves of Prkdc<sup>Scid/Scid</sup> and IgH<sub>μ</sub>-TLX1<sup>tg</sup>Prkdc<sup>Scid/Scid</sup> mice that developed Acute Myeloid Leukemia (p<0.0001). B. Median survival of IgH<sub>μ</sub>-TLX1<sup>tg</sup>Prkdc<sup>Scid/Scid</sup> and Prkdc<sup>Scid/Scid</sup> mice developing AML. The columns correspond to Figure 5.1A and show the median and standard deviation of the survival for IgH<sub>μ</sub>-TLX1<sup>tg</sup>Prkdc<sup>Scid/Scid</sup> or Prkdc<sup>Scid/Scid</sup> mice developing AML (p<0.0001). C. A one-year Kaplan-Meier survival probability estimate of IgH<sub>μ</sub>-TLX1<sup>tg</sup>Prkdc<sup>Scid/Scid</sup> and Prkdc<sup>Scid/Scid</sup> mice developing AML.
Previous studies (Chen et al., 2006) indicated that mice bred on the CD1/IRC genetic background developed leukemia more rapidly than mice bred on other genetic backgrounds. We therefore maintained the IgHμ-TLX1TgPrkdcScid/Scid mice on the IRC background by crossing IgHμ-TLX1TgPrkdcScid/Scid mice with CB17 ICR- PrkdcScid (PrkdcScid/Scid) mice. Interestingly, we noticed that after several backcrosses, a higher proportion of mice developed T-ALL and fewer mice developed AML that was more prominent in the IgHμ-TLX1TgPrkdcScid/Scid mice bred on the mixed CD1/Balb/c genetic background. Eventually, all IgHμ-TLX1TgPrkdcScid/Scid and PrkdcScid/Scid mice developed only T-ALL, while the AML phenotype for unclear reasons was lost. Additional studies are needed to resolve the contribution of the genetic background on the development of hemopoietic malignancies in IgHμ-TLX1Tg mice. Studies described below were conducted using IgHμ-TLX1TgPrkdcScid/Scid that had been backcrossed fewer than 10 generations.

Histological examination of thymus, spleen and bone marrow tissues from IgHμ-TLX1TgPrkdcScid/Scid and PrkdcScid/Scid mice diagnosed with AML showed that the thymus was not involved in the malignant process. Thymus architecture was typical for DNA-PK-deficient mice, including lack of a distinct cortex and parenchyma with no discrete differences between the cortical and medullar zones. The medulla zone was depleted due to the absence of double positive thymocytes. In contrast to the thymus, spleens of mice affected with AML were significantly enlarged (Table 5.1) with disrupted follicules and expansion of leukemic cells. Leukemic cells were presented with a primitive myeloid phenotype and were characterized by a large oval nucleus composed of fine non aggregated nuclear chromatin with a few distinct nucleoli. Bone marrow exhibited hypercellularity (Table 5.2) and was infiltrated with blast cells (Figure 5.2A).
Figure 5.2. Development of AML in IgH\(\mu\)-TLX1\(\text{Tg}\)Prkdc\(\text{Scid}/\text{Scid}\) mice.
A. Hematoxylin and eosin staining of hematopoietic tissues isolated from premalignant Prkdc\(\text{Scid}/\text{Scid}\) and IgH\(\mu\)-TLX1\(\text{Tg}\)Prkdc\(\text{Scid}/\text{Scid}\) mice (left and middle columns correspondingly) and IgH\(\mu\)-TLX1\(\text{Tg}\)Prkdc\(\text{Scid}/\text{Scid}\) mice diagnosed with AML (right column). The spleen and bone marrow of IgH\(\mu\)-TLX1\(\text{Tg}\)Prkdc\(\text{Scid}/\text{Scid}\) mice are packed with cells displaying a myeloblastic phenotype (middle and bottom panels in the right column). Magnifications x40 (overview) and x100 (insert). White arrows indicate representative AML cells. Scale bars: 10 \(\mu\)m. B. Thymi, spleens and bone marrow of healthy (left side) and moribund (right side) IgH\(\mu\)-TLX1\(\text{Tg}\)Prkdc\(\text{Scid}/\text{Scid}\) mice were examined by flow cytometry using cell surface antigens to distinguish double negative thymocyte subpopulations (CD44, CD25, CD4, CD8) as well as Gr-1 and Mac-1 which are specific for myeloid cells.
Flow cytometric analysis of thymocytes from \(IgH\mu-TLXI^TgPrkd^Scid/Scid\) mice that developed AML confirmed that the thymi were not involved in the malignant process. Staining with CD44 and CD25 antibodies revealed that thymi contained a normal profile of immature DN thymocytes typical for mice with the \(Prkd^Scid/Scid\) mutation. The thymocytes did not express TCR\(\beta\), CD3, CD4 and CD8 antigens and thus, had not progressed beyond the DN3 stage of development. By contrast, staining of spleens and the bone marrow of terminally ill \(IgH\mu-TLXI^TgPrkd^Scid/Scid\) mice with Gr-1 and Mac-1 antibodies revealed an increased proportion of Mac-1\(^{lo}\)Gr-1\(^{lo}\), Mac-1\(^{hi}\)Gr-1\(^{int}\) and Mac-1\(^{hi}\)Gr-1\(^{lo}\) cells (Jaiswal et al., 2003; Khandanpour et al., 2012) suggesting in increase in the proportion of immature myeloid cells (Figure 5.2B).

### 5.4.2. Leaky expression of the \(TLX1\) transgene in HSCs and more mature progenitors

Activity of the \(IgH\) promoter and enhancer is predominantly restricted to B cells (Gerlinger et al., 1986; Reik et al., 1987), although leaky expression has been reported in thymocytes and fibroblastic myeloid progenitors (Bergman, Rice, Grosschedl, & Baltimore, 1984; Wasylyk & Wasylyk, 1986). To determine whether the \(IgH\) promoter and enhancer directed expression of the \(IgH\mu-TLXI\) transgene in myeloid cells, we isolated hemopoietic stem cells (HSC) and multipotent progenitors and analysed the subfractions from \(IgH\mu-TLXI^TgPrkd^Scid/Scid\) mice for \(TLX1\) expression. To this end, bone marrow cells were flow-sorted based on their expression profiles, namely c-kit\(^{+}\)Sca1\(^{+}\)Lin\(^{-}\) (HSCs and multipotent progenitors - MPP), c-kit\(^{-}\)Sca1\(^{-}\)Lin\(^{-}\) (common myeloid progenitors (CMP)) and c-kit\(^{-}\)Sca1\(^{-}\)Lin\(^{-}\) containing megakaryocyte/erythrocyte (MEP), granulocyte/macrophages (GMP) and lineage restricted progenitors (Seita & Weissman, 2010), and the extracted RNA samples were subjected to RT-PCR and quantitative PCR analysis (Figure 5.3A). As seen in Figure 5.3B, RT-PCR analysis revealed that \(TLX1\) was expressed at low levels in hemopoietic stem cells and multipotent progenitors obtained from healthy \(IgH\mu-TLXI^TgPrkd^Scid/Scid\) mice. These findings were confirmed by quantitative RT-PCR on flowsorted bone marrow cells obtained from healthy \(IgH\mu-TLXI^TgPrkd^Scid/Scid\) mice. Expression of the \(TLX1\) transgene was identified in all bone marrow cell fractions obtained from \(IgH\mu-TLXI^TgPrkd^Scid/Scid\) mice, although the expression levels were significantly lower than those detected in purified B220\(^{+}\) splenocytes from \(TLX1\) transgenic mice (Figure 5.3C). Therefore, \(TLX1\) was expressed in immature thymocytes, HSCs and multipotent progenitors.
Figure 5.3. Analysis of TLX1 transgene expression in hemopoietic stem cells and more mature progenitors. A. Bone marrow from PrkdcScid/Scid and IgHμ-TLX1Tg/PrkdcScid/Scid 6-8 weeks littermates were lineage depleted using CD45R, Gr-1, CD11b, Ter119 and F4/80 cells and hemopoietic stem cells and progenitors were isolated by FACS based on expression of c-kit and Sca1. Sorting gates for c-kit Sca1-lin, c-kit Sca1-lin and c-kit Sca1-lin cells representing HSCs and more mature progenitors are indicated with grey squares. In this study, HSCs and MPP are defined as c-kit Sca1-lin, CMP and CLP are c-kit Sca1-lin and MEP, GMP and lineage restricted progenitors are defined as c-kit Sca1-lin cells. B. Reverse transcription-PCR analysis showing expression of the TLX1 transgene in bone marrow of IgHμ-TLX1Tg/PrkdcScid/Scid and PrkdcScid/Scid mice flow sorted to c-kit Sca1-lin, c-kit Sca1-lin and c-kit Sca1-lin cell subpopulations. Purified B220+ splenocytes of wild type and of IgHμ-TLX1Tg mice were used as negative and positive controls, respectively. C. Quantitative reverse transcription-PCR analysis showing elevated levels of expression of the TLX1 transgene in the flow sorted IgHμ-TLX1Tg/PrkdcScid/Scid bone marrow subpopulations. Purified B220+ splenocytes isolated from IgHμ-TLX1Tg mice were used as a positive control. Expression of the TLX1 transgene in the whole thymi in IgHμ-TLX1Tg/PrkdcScid/Scid (black) and PrkdcScid/Scid (pink) mice were included for comparison of expression levels in other hemopoietic subpopulations. Data normalized relative to β-actin.
5.4.3. Enhanced viability of bone marrow and spleen cells from 
\(IgH\mu-TLX1^{\text{Tg}}Prkdc^{\text{Scid/Scid}}\) mice relative to \(Prkdc^{\text{Scid/Scid}}\) controls

Previous studies indicated that expression of \(TLX1\) in \(DNA-PK\)-deficient immature thymocytes was associated with increased proliferation and enhanced survival of thymocytes of premalignant \(IgH\mu-TLX1^{\text{Tg}}Prkdc^{\text{Scid/Scid}}\) mice as compared to \(Prkdc^{\text{Scid/Scid}}\) littermates (see Chapter 3, Figure 3.9). To determine whether this was also the case in myeloid cells, the absolute numbers and viability of bone marrow and spleen cells were determined. As seen in Figure 5.4A, gross autopsy of spleens from premalignant \(IgH\mu-TLX1^{\text{Tg}}Prkdc^{\text{Scid/Scid}}\) mice revealed an increase in weight when compared to spleens from \(Prkdc^{\text{Scid/Scid}}\) control littermates. Furthermore, there was a statistically significant (p < 0.0001) increase in the absolute number of cells in spleen and the bone marrow in \(IgH\mu-TLX1^{\text{Tg}}Prkdc^{\text{Scid/Scid}}\) mice relative to \(Prkdc^{\text{Scid/Scid}}\) controls (Figure 5.4B,C and Table 5.3 and Table 5.4). Annexin V and Propidium Iodide staining of bone marrow and spleen revealed a significant reduction in the percentages of apoptotic and dead cells in all three examined \(IgH\mu-TLX1^{\text{Tg}}Prkdc^{\text{Scid/Scid}}\) mice relative to those obtained from three \(Prkdc^{\text{Scid/Scid}}\) premalignant littermates (Figure 5.4D). These data are consistent with the notion that inhibition of apoptosis may be at least partially responsible for the increased cellularity observed in the thymus, spleen and the bone marrow of \(IgH\mu-TLX1^{\text{Tg}}Prkdc^{\text{Scid/Scid}}\) mice (Figure 5.4E).
Figure 5.4. Increased cell numbers and viability of bone marrow and spleen samples isolated from premalignant \( \text{IgH}\mu\text{-TLX1}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) mice relative to \( \text{Prkdc}^{\text{Scid/Scid}} \) mice. A. Spleen weight of randomly selected ten six-week old \( \text{IgH}\mu\text{-TLX1}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) and ten \( \text{Prkdc}^{\text{Scid/Scid}} \) littermates (p < 0.0001). An asterisk indicates a statistically significant difference between samples. B. Absolute spleen cell numbers of ten six-week old \( \text{IgH}\mu\text{-TLX1}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) and ten \( \text{Prkdc}^{\text{Scid/Scid}} \) littermates (p < 0.0001). Statistically significant difference between compared samples is indicated by asterisk. C. Absolute number of cells in the bone marrow of ten six-week old \( \text{IgH}\mu\text{-TLX1}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) and ten \( \text{Prkdc}^{\text{Scid/Scid}} \) littermates (p < 0.0001). Statistically significant difference between compared samples is indicated by asterisk. D. Flow cytometry analyses of cells stained with Annexin V and PI to assess apoptosis of bone marrow and spleen samples obtained from six week old \( \text{IgH}\mu\text{-TLX1}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) and \( \text{Prkdc}^{\text{Scid/Scid}} \) littermates. The lower left quadrants of each panel show the proportion of viable cells. The upper right quadrants contain necrotic cells, while the lower right quadrants show the proportion of apoptotic cells. These experiments were repeated from samples obtained from three \( \text{IgH}\mu\text{-TLX1}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) and three \( \text{Prkdc}^{\text{Scid/Scid}} \) littermates with similar results being noted. E. The absolute cell numbers of viable, apoptotic and necrotic cells in bone marrow and spleens obtained from \( \text{IgH}\mu\text{-TLX1}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) and \( \text{Prkdc}^{\text{Scid/Scid}} \) littermates determined by flow cytometry with Annexin V and PI staining. Error bars represent SD (p < 0.0001).
5.4.4. Assessment of the frequency of myeloid progenitors in \( \text{IgH}^\mu-\text{TLX1}^Tg \text{Prkdc}^{\text{Scid/Scid}} \) and \( \text{Prkdc}^{\text{Scid/Scid}} \) mice

To determine whether the increased cellularity of spleens and marrow of \( \text{IgH}^\mu-\text{TLX1}^Tg \text{Prkdc}^{\text{Scid/Scid}} \) resulted from an increased number of myeloid progenitors, we used flow cytometry and CFU assays to determine the frequency of myeloid progenitors in bone marrow and spleen samples. Bone marrow and spleen cells were obtained from ten healthy 6-8 week old \( \text{IgH}^\mu-\text{TLX1}^Tg \text{Prkdc}^{\text{Scid/Scid}} \) and ten \( \text{Prkdc}^{\text{Scid/Scid}} \) mice and analyzed by flow cytometry using Gr-1 and Mac-1 antibodies. Cells expressing low levels of Mac-1 and Gr-1 have previously been determined to be immature myeloid cells (Cuenco, Nucifora, & Ren, 2000; Jaiswal et al., 2003). As seen in Figure 5A, bone marrow and spleens of \( \text{IgH}^\mu-\text{TLX1}^Tg \text{Prkdc}^{\text{Scid/Scid}} \) mice contained an increased percentage of immature myeloid cells relative to those found in \( \text{Prkdc}^{\text{Scid/Scid}} \) littermates. Taking into consideration the enlarged spleens seen in \( \text{IgH}^\mu-\text{TLX1}^Tg \text{Prkdc}^{\text{Scid/Scid}} \) mice, the absolute number of Mac-1\(^{hi}\)Gr-1\(^{lo}\) cells was counted. Statistical analysis of flow cytometry data showed a significant increase of the absolute number of immature myelocytes in the spleen of \( \text{IgH}^\mu-\text{TLX1}^Tg \text{Prkdc}^{\text{Scid/Scid}} \) mice relative to \( \text{Prkdc}^{\text{Scid/Scid}} \) littermates, while the difference between immature myelocytes derived from bone marrow was not statistically significant (2.15±0.23 \( \times 10^6 \)) in \( \text{IgH}^\mu-\text{TLX1}^Tg \text{Prkdc}^{\text{Scid/Scid}} \) mice and 1.33±0.14 \( \times 10^6 \) in \( \text{Prkdc}^{\text{Scid/Scid}} \) mice) (Figure 5B). These results, therefore, indicate that myeloid progenitors are specifically enriched in the spleens of TLX transgenic animals.

To verify these findings, we performed colony forming unit assays (CFU) by plating \( 10^5 \) bone marrow and \( 10^5 \) cells in methylcellulose. These studies confirmed a statistically significant increase in CFU frequency when spleen cells from \( \text{IgH}^\mu-\text{TLX1}^Tg \text{Prkdc}^{\text{Scid/Scid}} \) mice were compared to those from the control littermates (p <0.05, Figure 5C). These data showed that \( \text{TLX1} \) increase frequency of myeloid progenitors in the spleen of \( \text{IgH}^\mu-\text{TLX1}^Tg \text{Prkdc}^{\text{Scid/Scid}} \) mice.

Based on our results indicating that \( \text{TLX1} \) expression in immature DNA-PK-deficient thymocytes provided them with a proliferative advantage both in vivo and in vitro (Chapter 3), we hypothesized that expression of the \( \text{IgH}^\mu-\text{TLX1} \) transgene in myeloid progenitors may also promote their proliferation. Indeed, we found that colonies derived from spleen cells of \( \text{IgH}^\mu-\text{TLX1}^Tg \text{Prkdc}^{\text{Scid/Scid}} \) mice were larger (Figure 5D) and consisted of greater numbers of cells compared to those of \( \text{Prkdc}^{\text{Scid/Scid}} \) mice (Figure 5E). A difference, if any, in size and numbers of colonies formed from the bone marrow was negligible. These data suggest that ectopic
expression of *TLX1* provided a proliferative advantage for myeloid progenitors residing in spleens.
Figure 5.5. Increased absolute numbers of immature myelocytes and the numbers of colony forming units in the bone marrow and spleens obtained from premalignant IgHμ-TLX1TgPrkdcScid/Scid mice relative to PrkdcScid/Scid controls. A. Bone marrow and spleen cells were obtained from ten healthy six-week old IgHμ-TLX1TgPrkdcScid/Scid and ten PrkdcScid/Scid littermates and reacted with Gr-1 and Mac-1 antibodies specific for myeloid cells. Flow cytometric analysis revealed an increased percentage of immature myelocytes in the spleens of IgHμ-TLX1TgPrkdcScid/Scid mice. B. Absolute cell numbers of immature myelocytes in the bone marrow and spleens of IgHμ-TLX1TgPrkdcScid/Scid and PrkdcScid/Scid littermates was accessed by flow cytometry with Gr-1 and Mac-1 antibodies. Increased numbers of Mac-1hi and Gr-1lo cells were found in the spleens obtained from IgHμ-TLX1TgPrkdcScid/Scid mice relative to PrkdcScid/Scid littermates. Statistically significant differences between compared samples are indicated by asterisks (n=10). C. CFU assays to assess the frequency of myeloid progenitors were performed using cells obtained from three healthy five to six weeks old IgHμ-TLX1TgPrkdcScid/Scid and three PrkdcScid/Scid littermates. Colonies were counted eight days after plating. The assays were performed in triplicates and repeated twice. A statistically significant differences in the total number of colonies derived from spleens (p = 0.043) and bone marrow (p = 0.0015) is indicated by asterisks. D. CFU assays were established using bone marrow and spleen cells obtained from three healthy five weeks old IgHμ-TLX1TgPrkdcScid/Scid and three PrkdcScid/Scid littermates. Colonies were counted eight days after plating in Methylcellulose. Colonies grown from the cells obtained from spleens of IgHμ-TLX1TgPrkdcScid/Scid mice appeared larger than those established from the cells obtained from PrkdcScid/Scid mice. E. Colonies were counted and harvested eight days after plating. The average number of cells per colony was calculated by dividing the total number of cell per plate by the number of colonies counted per dish. Colonies derived after plating splenocytes of IgHμ-TLX1TgPrkdcScid/Scid mice contained a statistically significantly increase in the number of cells relative to colonies derived from the spleens of PrkdcScid/Scid mice. Statistically significant differences between compared samples are indicated by asterisks.
5.4.5. BrdU assays for the analysis of proliferation

Bone marrow cells and splenocytes from premalignant control $Prkdc^{Scid/Scid}$ and $IgH\mu$-TLX1$^TgPrkdc^{Scid/Scid}$ littermates were then analyzed for the presence of proliferating cells using an in vivo BrdU labeling technique (Figure 5.6A). Statistical analysis of three mice per group at 2 hours and 7 hours after intraperitoneal injection with BrdU revealed a significant increase in the numbers of BrdU-positive proliferating bone marrow cells and splenocytes, correspondingly, derived from $IgH\mu$-TLX1$^TgPrkdc^{Scid/Scid}$ mice relative to their control littermates in both time courses (Figure 5.6B).

Overall, our data demonstrated that the increased total cellularity in the bone marrow and spleens in premalignant $IgH\mu$-TLX1$^TgPrkdc^{Scid/Scid}$ mice was due to enhanced splenocyte and bone marrow cells viability, increased number of myeloid CFU and elevated splenocytes proliferation.
Figure 5.6. *IgHμ-TLX1 Tg Prkdc<sup>Scid/Scid</sup>* premalignant bone marrow and spleen possess a proliferative advantage *in vitro* and *in vivo*.

A. *In vivo* BrdU assays to assess proliferation of bone marrow and spleen cells obtained from control *Prkdc<sup>Scid/Scid</sup>* (left side) and *IgHμ-TLX1 Tg Prkdc<sup>Scid/Scid</sup>* (right side) mice, 2 hours (top row) and 7 hours (bottom row) after intraperitoneal injection with 10 μM BrdU. B. The percentage of proliferating cells isolated from the bone marrow and spleens of three healthy five weeks old *IgHμ-TLX1 Tg Prkdc<sup>Scid/Scid</sup>* and *Prkdc<sup>Scid/Scid</sup>* littermates was accessed by flow cytometry using a FITC-anti-BrdU antibody and PI staining. Error bars represent SD, p < 0.0001. Statistically significant differences between compared samples are indicated by asterisks.
5.4.6. Expression of the \textit{IgH\(\mu\)-TLX1} transgene in the spleen and bone marrow of moribund \(IgH\(\mu\)-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid}\) mice.

We sought to determine whether the TLX1 transgene was expressed in the bone marrow and spleens of animals that developed AML. To this end, spleens and bone marrow of six moribund \(IgH\(\mu\)-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid}\) mice were analyzed by histology and flow cytometry to confirm their disease phenotype (Figure 5.7A). Quantitative RT-PCR analysis detected a significant (2 to 5 fold) variability in TLX1 expression levels in spleens and bone marrow specimens, which was comparable to those detected in premalignant \(IgH\(\mu\)-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid}\) thymocytes (Figure 5.7B). RT-PCR amplification human TLX1 transgene was confirmed by sequencing (Figure 5.7C). DNA sequencing confirmed that the amplified cDNA fragments were the \(IgH\(\mu\)-TLX1\) transgene and complete homology of the expressed transgene in tumours obtained from \(IgH\(\mu\)-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid}\) mice to the human TLX1 gene. These data indicated that TLX1 was expressed in immature myelocytes and its expression levels were not organ- or tumour-stage-specific.
Figure 5.7. Analysis of TLX1 transgene expression in the spleen and the bone marrow of IgHμ-TLX1TgPrkdcScid/Scid mice diagnosed with AML. Quantitative RT-PCR analysis was performed with bone marrow and spleens of six IgHμ-TLX1TgPrkdcScid/Scid mice (304, 489, 505, 609, 682, 697) that developed AML at the age from three to six months. The TLX1 transgene was expressed at levels comparable to those detected in premalignant thymocytes. As a positive control, premalignant thymocytes of IgHμ-TLX1TgPrkdcScid/Scid mouse and purified B220+ cells from spleen of IgHμ-TLX1Tg mouse which express TLX1 transgene were used. Thymocytes from premalignant PrkdcScid/Scid mouse were used a negative control.
5.4.7. Influence of genetic background to the disease phenotype in $IgH_\mu$-TLX1$^{Tg}$Prkdc$^{Scid/Scid}$ mice

Almost half of the $IgH_\mu$-TLX1$^{Tg}$Prkdc$^{Scid/Scid}$ mice developed AML, whereas another half developed T-ALL (Chapter 3). We attempted to establish a link between the sex and genetic background of examined mice and the disease phenotype. We determined that sex was not a predisposing factor for AML development in $IgH_\mu$-TLX1$^{Tg}$Prkdc$^{Scid/Scid}$ mice (Supplementary Figure 5.1 A, B). It is important to note that the genetic background of each generation was similar, since we maintained the mouse colony by crossing only “brother-sister” offsprings. We found that Prkdc$^{Scid/Scid}$ (Balb/c/CD1), Prkdc$^{Scid/Scid}$ (CD1) and $IgH_\mu$-TLX1$^{Tg}$Prkdc$^{Scid/Scid}$ (CD1) mice developed AML or T-ALL with similar frequency (Supplementary Figure 5.1 C, D), while $IgH_\mu$-TLX1$^{Tg}$Prkdc$^{Scid/Scid}$ mice bred onto Balb/c/CD1 exhibited significant propensity to T-ALL development (Supplementary Figure 5.1 C). These results indicate that depending on the genetic background, TLX1 expression may promote T-ALL (in Balb/c/CD1 mice) or both T-ALL and AML (in CD1/IRC mice). Moreover, $IgH_\mu$-TLX1$^{Tg}$Prkdc$^{Scid/Scid}$ mice continuously bred with Balb/c developed neither T-ALL nor AML and remained healthy for up to the two-year observation period, indicating that CD1/IRC genetic background is crucial for leukemia development in $IgH_\mu$-TLX1$^{Tg}$Prkdc$^{Scid/Scid}$ mice.
Supplementary Figure 5.1. Genetic background and sex did not influence the phenotype of disease developed by IgHμ-TLX1TgPrkdcscid/scid mice.

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<th>Sex/Phenotype</th>
<th>AML</th>
<th>T-ALL</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>male %</td>
<td>44.5</td>
<td>55.5</td>
<td>100</td>
</tr>
<tr>
<td>female %</td>
<td>47.3</td>
<td>52.7</td>
<td>100</td>
</tr>
</tbody>
</table>

Statistical analysis of cohort groups of IgHμ-TLX1TgPrkdcscid/scid and Prkdcscid/scid mice indicated that: A. There was no statistically significant difference in the phenotype of disease developed by IgHμ-TLX1TgPrkdcscid/scid males and females (p=0.28). B. Kaplan–Meier plot survival curves of male and female IgHμ-TLX1TgPrkdcscid/scid (blue and red lines, respectively) mice. Survival curves for male and female IgHμ-TLX1TgPrkdcscid/scid mice indicating no significant differences in the onset of AML development (p=0.2). C. There was no significant difference in the phenotype of disease exhibited by Prkdcscid/scid mice bred on either the Balb/c/CD1 or CD1 genetic backgrounds (p=0.48). D. There was no significant difference in the predisposition for the development of AML or T-ALL between IgHμ-TLX1TgPrkdcscid/scid CD1 mice (p=0.24). There is a difference in the percentage of T-ALL and AML phenotype developed by IgHμ-TLX1TgPrkdcscid/scid Balb/c/CD1 (p<0.05).
5.5. Discussion

Expression of the TLX1 transgene in multiple hemopoietic cell types, including immature B cells, immature thymocytes, HSC and more mature progenitors allowed us to study its effect in lymphoid and myeloid cells. Interestingly, B cell malignancy has never been detected in IgHμ-TLX1TgPrkdcScid/Scid mice, despite the observed TLX1 expression in immature B cells. This is in contrast to the IgHμ-TLX1Tg and IgHμ-TLX1Tg/Ubr1−/− mice which were reported to develop mature B cell lymphoma (Hough et al., 1998; Chen, Kwon, Lim, Dube, & Hough, 2006). Utilization of our IgHμ-TLX1TgPrkdcScid/Scid mouse model therefore allowed us to circumvent the development of B cell lymphoma and allowed for the development and analysis of T-ALL and AML. Therefore, our IgHμ-TLX1TgPrkdcScid/Scid mouse model provides a meaningful tool for investigating TLX1-triggered effects in various cell types.

In this study, we established that loss of DNA-PK contributes to AML development caused by ectopic expression of TLX1 in myeloid progenitors, although the role of DNA-PK deficiency in AML initiation remains unclear. DNA-PK is not only involved in the DS DNA breaks ligation but also in telomere capping. DNA-PK-deficient cells are thus more susceptible to telomere fusions which, in turn, may lead to deregulated expression of multiple genes located within the telomeres region, including tumour suppressors and oncogenes (Bailey & Goodwin, 2004; Goytisolo, Samper, Edmonson, Taccioli, & Blasco, 2001). This may underlay the ability of TLX1 to disrupt proper mitotic checkpoints, the phenomenon that allows malignant cells to survive and to accumulate mutations, as we observed in thymocytes (Chapter 3) and in immature myelocytes (Chapter 5). Similar to premalignant thymocytes (Chapter 3), loss of DNA-PK may also contribute to disregulated expression of genes whose protein products are involved in tubular formation and cell cycle progression in IgHμ-TLX1TgPrkdcScid/Scid myeloid progenitors, given a known role for DNA-PK in microtubule formation, chromosome segregation and cell cycle progression (Lee et al., 2011).

Analysis of IgHμ-TLX1TgPrkdcScid/Scid mice revealed that double mutant mice rapidly died of AML. The median 14-months survival of PrkdcScid/Scid mice was reduced to 7.0 months in the IgHμ-TLX1TgPrkdcScid/Scid mouse cohort. The bone marrow and spleens of AML-diseased IgHμ-TLX1TgPrkdcScid/Scid mice were infiltrated with primitive cells expressing Mac-1loGr-1lo, Mac-1hiGr-1int and Mac-1hiGr-1lo, indicating that TLX1 acts as an oncogene in cells of different origin. It should also be noted that simultaneous development of T-ALL and AML was observed in only
Supplementary Figure 5.2. Mixed T-ALL and AML diseases phenotype developed by \textit{IgH}μ-\textit{TLX1}\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mouse.

A. Hematoxylin and eosin staining of thymus, spleen and femur obtained from \textit{IgH}μ-\textit{TLX1}\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} with T-ALL and AML. H&E staining revealed disrupted architecture in the thymus, which was packed with leukemic cells (top panel). In the spleen and the bone marrow of \textit{IgH}μ-\textit{TLX1}\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mouse cells with a myeloblastic phenotype were detected (middle and bottom panels). Magnifications x40 (overview) and x100 (insert). Black arrows indicate T-ALL cells. White arrows indicate AML cells. Scale bars: 10 μm.

B. Thymus, spleen and bone marrow of moribund \textit{IgH}μ-\textit{TLX1}\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mouse were examined by flow cytometry using T cell markers: CD44, CD25, CD4, CD8 and Gr-1 and Mac-1 markers specific for myeloid cells. T-ALL flow cytometry profile (top row) was detected in the thymus of the sick \textit{IgH}μ-\textit{TLX1}\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mouse along with an increased proportion of immature Gr-1"Mac-1"myeloid cells in the spleen and the bone marrow (middle and bottom rows).
one moribund $IgH\mu$-TLXI$^{Tg}$Prkdc$^{Scid/Scid}$ mouse (Supplementary Figure 5.2 A, B), suggesting that initiation of different types of leukemia in response to TLXI is mutually exclusive.

Also, our results showed that similar to T-ALL, TLXI accelerated AML development while not influencing the disease phenotype. We established that similar to premalignant thymocytes (Chapter 3, Figure 3.9), $IgH\mu$-TLXI$^{Tg}$Prkdc$^{Scid/Scid}$ myeloid progenitors possessed enhanced viability and proliferation relative to those from Prkdc$^{Scid/Scid}$ mice. This is in contrast to previous studies using $IgH\mu$-TLXI$^{Tg}$ and $IgH\mu$-TLXI$^{Tg}$ /Ubr1$^{Tg}$ mice, which did not detect any effect on apoptosis or proliferation in premalignant B cells (Hough et al., 1998; Chen, Kwon, Lim, Dube, & Hough, 2006). In the $p56^{Lck}$-TLXI mouse model, TLXI overexpression in premalignant thymocytes enhanced apoptosis while not affecting proliferation (De et al., 2010). Importantly, enhanced proliferation and reduced apoptosis were also found to operate in premalignant myelocytes and myeloid progenitors obtained from healthy $IgH\mu$-TLXI$^{Tg}$Prkdc$^{Scid/Scid}$ mice indicating that this effect only seen in the absence of DNA-PK. These results are also indicative of the universal TLXI-driven mechanism which operates in various cell types and is directed to induce proliferation and to inhibit apoptosis in the absence of DNA-PK.

Dysregulated TLXI expression in cells of the myeloid lineage has never been reported in humans, potentially due to the absence of the V(D)J recombination in myeloid cells and the other mechanism to activate TLXI in the absence of translocations may not work in human myeloid cells. However, studies in transgenic mice where TLXI is artificially expressed support our findings and show that TLXI affects maturation and proliferation of hemopoietic cells. Therefore, our $IgH\mu$-TLXI$^{Tg}$Prkdc$^{Scid/Scid}$ mouse model provides the first in vivo evidence that TLXI overexpression in HSC and myeloid progenitors induces oncogenic transformation.

Interestingly, by monitoring the offsprings of the original cohort, we noticed that the first generation of $IgH\mu$-TLXI$^{Tg}$Prkdc$^{Scid/Scid}$ and Prkdc$^{Scid/Scid}$ mice exhibited higher AML penetrance than the second generation. In the third generation, AML cases were sporadic and, eventually, the AML phenotype was completely lost. All following generations of $IgH\mu$-TLXI$^{Tg}$Prkdc$^{Scid/Scid}$ and Prkdc$^{Scid/Scid}$ mice developed T-ALL exclusively.

We also established that AML or T-ALL development in $IgH\mu$-TLXI$^{Tg}$Prkdc$^{Scid/Scid}$ is dependent on the genetic background, in agreement with other studies showing that the disease phenotype in genetically engineered mice is often influenced by strain-specific modifier genes (Montagutelli, 2000; Doetschman, 2009; Kearney, 2011). Moreover, $IgH\mu$-TLXI$^{Tg}$ mice with the CD1 or C57BL/6 background developed marginal zone lymphoma or follicular lymphoma,
respectively, with the latter one showing longer latency period (Chen et al., 2006). In this study, we established that $IgH\mu$-$TLX1TgPrkdc^{Scid/Scid}$ (Balb/c/CD1) mice developed predominantly T-ALL, whereas $Prkdc^{Scid/Scid}$ (Balb/c/CD1), $Prkdc^{Scid/Scid}$ (CD1) and $IgH\mu$-$TLX1TgPrkdc^{Scid/Scid}$ (CD1) developed T-ALL or AML almost equally (Supplementary Figure 5.1 C, D). Of further note, $IgH\mu$-$TLX1TgPrkdc^{Scid/Scid}$ (Balb/c) mice did not develop any disease during the entire 2-year observation period. Overall, these data suggest that the CD1 genetic background induces leukemia development in $IgH\mu$-$TLX1TgPrkdc^{Scid/Scid}$ mice and the Balb/c background is more likely to act as modifier towards T-ALL. It remains to be studied why the AML phenotype was eventually lost in all mice cohorts and did not re-occur in multiple generations of mice during the 2-year observation period.
Table 5.1. Total number of cells in the spleen in arbitrary selected wild type, \( IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid} \) premalignant and malignant mice

<table>
<thead>
<tr>
<th>Wild type ((10^8))</th>
<th>( IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid} )(10(^7))</th>
<th>( IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid} )(10(^7)) AML</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.22 ± 0.6</td>
<td>1.026 ± 0.32</td>
<td>3.81±0.46</td>
</tr>
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</table>

Table 5.2. Total number of cells in the bone marrow in arbitrary selected wild type, \( IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid} \) premalignant and malignant mice

<table>
<thead>
<tr>
<th>Wild type ((10^7))</th>
<th>( IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid} )(10(^7))</th>
<th>( IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid} )(10(^7)) AML</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.94±0.17</td>
<td>1.79±0.14</td>
<td>2.19±0.11</td>
</tr>
</tbody>
</table>

Table 5.3. Total number of cells in the spleen in arbitrary selected premalignant mice

<table>
<thead>
<tr>
<th>Wild type ((10^8))</th>
<th>( Prkdc^{Scid/Scid} )(10(^7))</th>
<th>( IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid} )(10(^7))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.22 ± 0.6</td>
<td>0.813 ± 0.280</td>
<td>1.026 ± 0.32</td>
</tr>
</tbody>
</table>

Table 5.4. Total number of cells in the bone marrow in arbitrary selected premalignant mice

<table>
<thead>
<tr>
<th>Wild type ((10^7))</th>
<th>( Prkdc^{Scid/Scid} )(10(^7))</th>
<th>( IgH\mu-TLX1^{Tg}Prkdc^{Scid/Scid} )(10(^7))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.94±0.17</td>
<td>1.58±0.15</td>
<td>1.79±0.14</td>
</tr>
</tbody>
</table>
Chapter 6

Discussion and Future Directions
6.1. Summary

Since first reports describing the discovery of the TLX1 gene (also known as HOX11) focused efforts have been made to characterize downstream molecular mechanisms deregulated as a result of the ectopic expression of this transcription factor. The first data were obtained from the in vitro experimental systems and high throughput microarray analysis of human tumour samples. While recognizing the importance of these data for our understanding TLX1-induced regulatory mechanisms, it is also worth mentioning the obvious disadvantages of these approaches.

The major limitation of the in vitro approach is that it is based upon pure cell lines grown under artificial cell culture conditions that do not faithfully recapitulate in situ cell micro-environments and the complexity of the entire organism. Not surprisingly, in vitro and in vivo studies sometime come to opposite conclusions. For instance, Aldh1, a downstream transcription target of Tlx1 (Greene, Bahn, Masson, & Rabbitts, 1998), was found to be elevated in NIH-3T3 cells transfected with TLX1. However Aldh1 mRNA was not detected in the presence of Tlx1 in the spleen primordium and in the developing hindbrain of wild type embryos. Moreover, Aldh1 expression was detected in the developing spleen of E12.5 Tlx1−/− embryos, supporting the negative effect of TLX1 on its expression. Therefore, in vitro data need to be carefully analyzed and verified by in vivo studies.

High-throughput microarray analysis of human tumour samples is another common approach that is widely used to identify TLX1-affected genes. Considering cellular heterogeneity within a tumour specimen and accumulation of additional gene alterations during tumour progression, this approach cannot distinguish between primarily and secondary affects (Russo, Zegar, & Giordano, 2003). For example, microarray analysis of human TLX1-positive T-ALL samples performed by Ferrando et al. revealed multiple deregulated genes, but it is not possible to determine whether these genes were directly affected by TLX1 or deregulated expression of these genes may have been occurred during tumour progression. In addition, these altered expression profiles could simply reflect the developmental block observed in TLX1+ human tumour samples (Ferrando et al., 2002). Therefore, while these findings can be helpful for the development of predictions of disease progression and choosing treatment options, they cannot be used for identifying primary transcription targets and pathways responsible for initiation and progression of TLX1-induced T-cell malignancy (Ferrando & Look, 2003).
The research described in this thesis was designed to combine data from in vitro and in vivo experiments, in addition to data from gene expression profiling of premalignant cells and tumour specimens derived from our novel $IgH\mu$-TLX1$^{Ts}$Prkdc$^{Scid/Scid}$ mouse model. This mouse model was obtained by crossing $IgH\mu$-TLX1$^{Ts}$ (previously designated $IgH\mu$-HOX11$^{Ts}$ mice) (Hough et al., 1998) and Prkdc$^{Scid/Scid}$ mice. Analysis of $IgH\mu$-TLX1$^{Ts}$Prkdc$^{Scid/Scid}$ mice revealed that thymocyte maturation was arrested at the Double Negative 3 (DN3) stage of development. Although there are several mouse models overexpressing TLX1 transgene in immature thymocytes (De et al., 2010; Rakowski, Lehotzky, & Chiang, 2011), our model is unique and advantageous since TLX1 expression under the control of the “leaky” $IgH\mu$ promoter is presented not only in immature T-cell thymocytes where T-ALL initiating events occur but also in hemopoietic stem cells, multipotent progenitors and throughout B cell development allowing for the investigation of the TLX1 oncogenic potential in a variety cell types.

As reported in Chapter 3, the absence of DNA-PK was a significant contributing factor in TLX1-initiated T-ALL. We established that TLX1 overexpression decreased apoptosis and provided DNA-PK-deficient thymocytes with a proliferative advantage as was detected both in vitro and in vivo experiments. The enhanced proliferation could serve to increase the pool of progenitor cells susceptible to transformation by accumulating additional mutations while the inability to respond to apoptotic inducing signals would promote the survival of cells that had accumulated additional mutations that promote the malignant transformation of premalignant T cells.

We also described deregulation in the expression of genes involved in the regulation of the spindle assembly cell cycle checkpoint which could cause genomic instability by facilitating the survival of cells that had undergone chromosomal missegregation events leading to aneuploidy and contributing to leukemia progression. Chromosome missegregation is likely a mechanism promoting progression of TLX1-expressing premalignant cells to the malignant phenotype since aneuploid karyotypes were detected in vitro cultured thymocytes and thymocytes from premalignant $IgH\mu$-TLX1$^{Ts}$Prkdc$^{Scid/Scid}$ mice. Moreover, the deregulated expression of genes involved in the regulation of the spindle assembly cell cycle checkpoint in $IgH\mu$-TLX1$^{Ts}$Prkdc$^{Scid/Scid}$ thymocytes, including Chek1, Aurka, Bub1, Prc1, TTK, Tubg1, TPX2 and Cenpf, further supports the hypothesis that chromosome missegregation contributes to TLX1-initiated T-ALL progression. Moreover, these genes have previously been reported to be deregulated in various cancers including T-ALL (De et al., 2010; Sinha et al., 2011; Feik et al., 2009; Ricke & van Deursen, 2011; Ricke, Jeganathan, & van Deursen, 2011; Shimo et al., 2007;
Salvatore et al., 2007; Martin, Patrick, Bissell, & Fournier, 2008; Grover et al., 2012; O’Brien et al., 2007; Ohshima et al., 2000). Other genes which we detected in premalignant IgHμ-TLXI1TgPrkdcScid/Scid thymocytes and which might facilitate leukemia progression are the tumour suppressor gene Phf6, the antiapoptotic genes, Brca1, Birc5 and genes involved in the regulation of mitosis, including the anaphase-promoting complex (APC), cyclinB1 and cyclinB2 (Van et al., 2010; Friedenson, 2007; Bhojwani et al., 2006; Hamamura et al., 2007; Kanavaros et al., 2001).

Gene expression profiling revealed that ribosomal proteins (RPs) were down-regulated in the DN1, DN2 and DN3 in IgHμ-TLXI1TgPrkdcScid/Scid premalignant thymocyte subpopulations. The expression of mitochondrial ribosomal genes was also diminished in IgHμ-TLXI1TgPrkdcScid/Scid DN1 and DN3 thymocytes. Since abnormal expression of ribosomal proteins and mitochondrial ribosomal genes has been reported in a variety of solid tumours and leukemias these genes might serve as candidates in studies aimed at identifying diagnostic or prognostic markers for T-ALL (Shanmugam, McBrayer, & Rosen, 2009; Sugapriya, Shanthi, & Sachdanandam, 2008).

Chapter 4 presents our studies on the existence of T-ALL-initiating cells (T-ALL-ICs) in IgHμ-TLXI1TgPrkdcScid/Scid mice. By serial transplantation of premalignant bone marrow, bulk T-ALL cells and flow sorted T-ALL cells derived from moribund IgHμ-TLXI1TgPrkdcScid/Scid mice into secondary and tertiary sublethally irradiated PrkdcScid/Scid recipients, we were able to demonstrate the presence of self-renewing T-ALL-ICs. We established that T-ALL-ICs originated in the thymus and could be detected in all thymocyte subfractions. We also established that a fraction of flow-sorted c-kit+CD44+ tumour cells (ETP) was enriched for T-ALL initiating cells and the frequency of T-ALL-ICs was highest in the c-kit+CD44+CD25-CD4-CD8- subpopulation relative to other flow-sorted cell fractions. Although not directly assessed, we would speculate that the same mechanisms of enhanced proliferation, reduced susceptibility to apoptosis inducing signals and deregulation of the spindle assembly cell cycle checkpoint contributed to the accumulation of mutations within T-ALL-ICs thereby contributing to the earlier onset of leukemia observed in secondary and tertiary recipients.

In chapter 5, we reported the expression of TLXI in myeloid progenitors and described the AML phenotype that developed in 41% of IgHμ-TLXI1TgPrkdcScid/Scid and 44% of PrkdcScid/Scid mice. Functional assays performed with immature myelocytes derived from young IgHμ-TLXI1TgPrkdcScid/Scid mice revealed deregulated molecular mechanisms similar to those
detected in premalignant thymocytes. Specifically, similar to immature thymocytes, reduced apoptosis and increased proliferation were detected in premalignant $\text{IgH}_\mu$-TLX1$^{\text{Tg}}$Prkdc$^{\text{Scid/Scid}}$ myeloid progenitors compared to those derived from Prkdc$^{\text{Scid/Scid}}$ littermates. An important observation coming from the cohort groups described in Chapters 3 and 5 is that TLX1 does not appear to affect disease phenotype, but rather accelerates the onset of leukemia development, indicating that T-ALL and AML development in the $\text{IgH}_\mu$-TLX1$^{\text{Tg}}$Prkdc$^{\text{Scid/Scid}}$ mouse model could be the result of cooperative effects of deregulated spindle checkpoint, reduced apoptosis and increased proliferation which are induced by TLX1 in a cell type-independent manner.

In this Discussion Chapter, I will elaborate on the relevance of our findings to human T-ALL, the overall significance of our findings with particular attention to the role of chromosomal instability, deregulated apoptosis and cell-cycle progression in the pathophysiology of TLX1-initiated malignancy. Among other potentially affected pathways, I will discuss the significance of expression of genes encoding ribosomal proteins and reduced expression of mitochondrial ribosomal genes which we detected in DN1-DN3 premalignant thymocytes by Affimexrix GeneChip microarray. Finally, future experiments will be discussed.

6.2. Clinical relevance of the $\text{IgH}_\mu$-TLX1$^{\text{Tg}}$Prkdc$^{\text{Scid/Scid}}$ mouse model to human T-ALL

The mouse model that we created and described in this thesis closely recapitulates the features observed in TLX1-associated human T-ALL. A significant degree of aneuploidy and similarities in deregulated gene expression profiles are both suggestive of common genetic programs perturbed during T-ALL initiation in both our mouse model and human TLX1-initiated T-ALL. This allows for the reliable extension of our findings to the pathophysiology of human TLX1-induced T cell malignancies. We propose that ectopic TLX1 expression triggers deregulated cell division, resulting in aneuploidy, which was detected in both premalignant thymocytes and thymic tumours developing in $\text{IgH}_\mu$-TLX1$^{\text{Tg}}$Prkdc$^{\text{Scid/Scid}}$ mice as compared to Prkdc$^{\text{Scid/Scid}}$ littermates. In line with these findings, aberrant TLX1 expression resulting from the chromosomal rearrangement of the 10q24 locus in human T-ALL specimens was also shown to be associated with the highest percentage of chromosomal abnormalities relative to those initiated by other chromosomal abnormalities. For instance, aneuploidy was detected in 29% of patients with TLX1-initiated T-ALL, relative to 18% of those affected by T-ALL initiated by
deregulated expression of TLX3, 11% by LMO2 and 12% by LMO1 (see Mitelman database of chromosome aberrations in cancer http://cgap.nci.nih.gov/Chromosomes/Mitelman; Table 6.1 and Appendix 1). Aberrant TLX1 expression appears to be associated with a common set of gene mutations in both TLX1 transgenic mouse models and human T-ALL. For instance, activating mutations of NOTCH1 were detected in 50 - 60% of human T-ALLs (Weng et al., 2004; Sulis et al., 2008) and Notch1 overexpression was identified in six out of ten IgHμ-TLX1TgPrkdcScid/Scid thymic tumours. Pten was found to be downregulated in 20% of human T-ALLs (Palomero et al., 2007; Maser et al., 2007) and seven out of ten IgHμ-TLX1TgPrkdcScid/Scid tumours. Likewise, the tumour suppressor gene, BCL11b, was found to be mutated or deleted in 16% of human T-ALL cases and in six out of ten tumour samples isolated from IgHμ-TLX1TgPrkdcScid/Scid (De et al., 2010). Moreover, premalignant IgHμ-TLX1TgPrkdcScid/Scid thymocytes exhibited upregulated expression of anti-apoptotic and proliferation-associated genes, including Brcal, Birc5, the Anaphase Promoting Complex gene Anapc5, as well as c-myc, c-myb and cyclins A, B1 and B2. Deregulated expression of these genes has also been reported in human T-ALL (Friedenson, 2007; Bhojwani et al., 2006; Hamamura et al., 2007; Kanavaros et al., 2001; Flotho et al., 2007; Van et al., 2010; Beck et al., 1995; Dudley, Wang, & Sun, 2009). Finally, IgHμ-TLX1TgPrkdcScid/Scid premalignant thymocytes exhibited downregulated expression of Chek1, a key regulator of the mitotic spindle checkpoint, and whose expression was significantly reduced in human T-ALL (De et al., 2010). Combined, these findings indicate that the IgHμ-TLX1TgPrkdcScid/Scid mouse model is a valuable tool to study early mechanisms driving human T-ALL and to explore treatment options.

The generation of the transgenic mouse model expressing TLX1 in thymocytes was challenging. Early attempts to generate LCK-TLX1Tg mice in our laboratory were unsuccessful and this was initially thought to be due to a dosage-dependent lethal effect of TLX1 on the developing embryo. However, we subsequently were able to generate IgHμ-TLX1Tg mice that expressed TLX1 in an array of hemopoietic cells, including thymocytes and throughout all stages of B cell development. These mice were viable and fertile, suggesting that other factors prevented the generation of LCK-TLX1Tg mice in our hands. It is interesting to note that although IgHμ-TLX1Tg mice expressed TLX1 in DN thymocytes, they did not develop T-ALL.

More recently, two independent research groups developed LCK-TLX1 transgenic mice using two different approaches. In 2010, Ferrando et al. reported the generation of p56Lck-TLX1 transgenic mice in which the expression of TLX1 in immature thymocytes was directed by the
LCK promoter (De et al., 2010). Similar to \textit{IgH\(\mu\)-TLX1\(^{Tg}\)Prkdc\textsuperscript{Scid/Scid}} mice, \(p56^{Lck}\)-TLX1 transgenic mice exhibited deregulation of the mitotic regulator gene, \textit{Chek1}, in DN1 thymocytes and developed T-ALL. Tumour cells were primarily immature CD4\(^+\)CD8\(^+\) thymocytes with aneuploidy and deregulated expression of \textit{Notch1}, \textit{Bcl11b} and \textit{Pten}. A second approach was undertaken by Rakowski \textit{et al.} in 2011. This research group generated a conditional doxycycline-regulated TLX1 transgenic mouse model (Rakowski, Lehotzky, & Chiang, 2011) in which expression of a tetracycline transactivator (TTA) in immature thymocytes was driven by the proximal LCK promoter, while TLX1 expression was under the control of the tet operon. Similar to \textit{IgH\(\mu\)-TLX1\(^{Tg}\)Prkdc\textsuperscript{Scid/Scid}} and \(p56^{Lck}\)-TLX1 mice, conditional doxycycline-regulated TLX1 transgenic mice developed T-ALL comprised of immature CD4\(^+\)CD8\(^+\) populations with \textit{Notch1} activation. Thus, ectopic expression of TLX1, in the absence of loss of DNA-PK is sufficient to induce T-ALL in mice. The lack of T-ALL development in \textit{IgH\(\mu\)-TLX1\(^{Tg}\)} mice can be explained by TLX1 dosage-dependent effects of transgene expression as TLX1 expression was high in premalignant thymocytes and tumours derived from \(p56^{Lck}\)-TLX1 and in doxycycline-regulated TLX1 transgenic mouse models (De et al., 2010; Rakowski, Lehotzky, & Chiang, 2011), while TLX1 expression in the premalignant thymocytes of \textit{IgH\(\mu\)-TLX1\(^{Tg}\)Prkdc\textsuperscript{Scid/Scid}} mice was low.

In spite of similarities in leukemic phenotypes and gene expression profiles, there is substantial evidence that \textit{IgH\(\mu\)-TLX1\(^{Tg}\)Prkdc\textsuperscript{Scid/Scid}} mice may differ from the other two aforementioned TLX1 transgenic mouse models. For instance, \textit{IgH\(\mu\)-TLX1\(^{Tg}\)Prkdc\textsuperscript{Scid/Scid}} mice developed both T-ALL and AML, and exhibited higher penetrance and earlier onset of disease. This is in contrast to \(p56^{Lck}\)-TLX1 and doxycycline-regulated TLX1 transgenic mouse models which developed only T-ALL. The development of AML in \textit{IgH\(\mu\)-TLX1\(^{Tg}\)Prkdc\textsuperscript{Scid/Scid}} mice is likely due to ectopic expression of TLX1 in myeloid progenitors whereas TLX1 expression in \(p56^{Lck}\)-TLX1 and doxycycline-regulated TLX1 transgenic mice is restricted to T-lineage cells. The increased disease penetrance and reduced latency is likely the result of the collaboration of deregulated genetic pathways resulting from ectopic TLX1 expression and loss of DNA-PK. Furthermore, aneuploidy was detected in premalignant thymocytes, from as early as eight weeks \textit{IgH\(\mu\)-TLX1\(^{Tg}\)Prkdc\textsuperscript{Scid/Scid}} mice indicating that numerical chromosomal abnormalities were not acquired during leukemia progression but rather represented early TLX1-related T-ALL initiating events. The aneuploidy is likely due to the deregulated expression of genes involved in the regulation of the mitotic checkpoint of premalignant \textit{IgH\(\mu\)-TLX1\(^{Tg}\)Prkdc\textsuperscript{Scid/Scid}} thymocytes.
Also, in contrast to the \( \text{IgH}\mu-\text{TLXI}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) mouse model, premalignant \( p56^{\text{Lck}}-\text{TLXI} \) thymocytes exhibited increased rates of apoptosis but normal proliferation (De et al., 2010). Thus, the absence of DNA-PK in \( \text{IgH}\mu-\text{TLXI}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) mice collaborates with ectopic expression of \( \text{TLXI} \) in thymocytes to deregulate both apoptosis and proliferation.

One interesting finding of our studies was that while \( \text{TLXI} \) is expressed in the premalignant thymocytes of \( \text{IgH}\mu-\text{TLXI}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) mice, transgene expression was not detected in most \( \text{IgH}\mu-\text{TLXI}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) tumour cells. This finding indicated that although ectopic expression of \( \text{TLXI} \) initiates T-ALL, its continuous expression is not necessary for tumour progression. Although, it can be argued that residual levels of \( \text{TLXI} \) persist in T-ALL-ICs, and that the survival of these cells is sufficient for tumour progression, it is also likely that the acquisition of mutations and chromosome abnormalities have mediated the activation of alternative pathways allowing \( \text{TLXI} \)-initiated tumour cells to persist in the absence of \( \text{TLXI} \) expression. These findings are in agreement with studies in doxycyclin-regulated \( \text{TLXI} \) transgenic mice where loss of \( \text{TLXI} \) expression in tumour cells did not result in remission of T-ALL.

### 6.3. \( \text{TLXI} \)-induced events in the T-cell malignant transformation and proposed experiments

The data presented in this thesis suggest that ectopic \( \text{TLXI} \) expression and loss of DNA-PK collaborate to contribute to mitotic dysfunction and the activation of antiapoptotic and proliferation pathways and that these mechanisms are primarily responsible for the development of T-ALL and AML in \( \text{IgH}\mu-\text{TLXI}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) mice (Figure 6.1). Below, I discuss these findings and their importance in more detail.

Aneuploidy was consistently detected in tumours of \( \text{IgH}\mu-\text{TLXI}^{\text{Tg}} \), \( \text{IgH}\mu-\text{TLXI}^{\text{Tg}}\text{Prkdc}^{\text{Scid/Scid}} \) and \( p56^{\text{Lck}}-\text{TLXI} \) transgenic mouse models, suggesting a close relationship between ectopic \( \text{TLXI} \) overexpression and defects in cell cycle checkpoints (Chen, Kwon, Lim, Dube, & Hough, 2006; Chen et al., 2006a; De et al., 2010). Most probably, these numerical chromosomal alterations were caused by cell cycle or spindle assembly checkpoints malfunctions. Indeed, microarray analysis and qRT-PCR revealed abnormal expression of multiple genes whose protein products are involved in checkpoints regulation. This includes \( \text{Chek1}, \text{Aurka}, \text{Bub1}, \text{Prc1}, \text{TTK}, \text{Tubg1}, \text{TPX2}, \text{Cenpf}, \text{Anaps5} \) and the Cohesin complex.
(Chapter 3). Taking into account that the aforementioned genes are also dysregulated in various other types of cancer (De et al., 2010; Sinha et al., 2011; Feik et al., 2009; Ricke & van Deursen, 2011; Ricke, Jeganathan, & van Deursen, 2011; Shimo et al., 2007; Salvatore et al., 2007; Martin, Patrick, Bissell, & Fournier, 2008; Grover et al., 2012; O’Brien et al., 2007; Ohshima et al., 2000), our data suggest that their deregulated expression, induced by ectopic TLX1 expression, results in the production of proteins that promote aneuploidy and induce T-ALL development. The observed abnormal activation of cell cycle-associated genes, such as those encoding cyclins A, B1 and B2, could also facilitate malfunction of the spindle assembly checkpoint in TLX1-expressing premalignant thymocytes, thereby facilitating tumour initiation. Interestingly, cyclins A, B1 and B2 are frequently dysregulated in human leukemias and their increased levels of expression were also detected in IgHμ-TLX1TgPrkdcScid/Scid premalignant thymocytes. Chen et al. shown that a TLX1-T47E mutant mimicking a constitutively activated TLX1 was incapable of upregulating CyclinB1, but was still able to induce numerical chromosomal alterations in TLX1-transfected NIH-3T3 fibroblasts (Chen et al., 2010). It would therefore be interesting to investigate whether the T47E mutation affects the expression of other cyclin-coding genes shown to be activated by TLX1. This could be tested by transfecting flow-sorted HSCs derived from PrkdcScid/Scid mice with the plasmid encoding the TLX1-T47E mutant. These cells along with those derived from IgHμ-TLX1TgPrkdcScid/Scid and PrkdcScid/Scid mice would be grown on the layer of OP9-DL1 cells and the expression levels of cyclins B1, B2 and A tested by qRT-PCR. The expectation is that if regulation of these genes is mediated in a Thr47-independent manner, there would be no differences in levels of expression of the cyclins in TLX1-T47E, PrkdcScid/Scid and IgHμ-TLX1TgPrkdcScid/Scid thymocytes. This would be further studied by cell cycle analysis and Giemsa staining of TLX1-T47EPrkdcScid/Scid, IgHμ-TLX1TgPrkdcScid/Scid and PrkdcScid/Scid non-sorted thymocytes. Mitotic bypass would be monitored in colchicine-treated cells. If the T47E-TLX1 mutant were capable of promoting mitotic bypass, there would be no differences in the DNA content and aneuploidy of TLX1-T47EPrkdcScid/Scid and IgHμ-TLX1TgPrkdcScid/Scid thymocytes. These assays would allow us to determine whether phosphorylation of the Thr47 residue affects cyclins A, B1 and B2 expression and contributes to chromosomal instability observed in IgHμ-TLX1TgPrkdcScid/Scid premalignant thymocytes.

Deregulated apoptosis in premalignant IgHμ-TLX1TgPrkdcScid/Scid thymocytes may represent an additional event facilitating T-ALL initiation, as it is one of the established hallmarks of tumour development (Hanahan & Weinberg, 2011). Indeed, by GSEA and qRT-
PCR analysis of IgHμ-TLXI^TgPrkdc^Scid/Scid premalignant thymocytes, we detected dysregulated expression of numerous genes involved in apoptosis including Bcl11b and Brca1 (Chapter 3). By flow-sorting immature T-cells derived from thymi of premalignant IgHμ-TLXI^TgPrkdc^Scid/Scid and Prkdc^Scid/Scid mice, we detected a small fraction of CD44^−CD25^− cells similar to DN4-stage thymocytes (Chapter 3). Interestingly, although the percentage of CD44^−CD25^− cells was generally low (3–4% of the total population), it was two to three times higher in IgHμ-TLXI^TgPrkdc^Scid/Scid thymocytes compared to those from Prkdc^Scid/Scid mice. This may be indicative of the direct involvement of TLXI in propagation of the CD44^−CD25^− cells, by inducing proliferation or inhibiting apoptosis in this particular cell population. Future studies could be initiated to test these possibilities by comparing rates of proliferation and apoptosis in CD44^−CD25^− cells derived from premalignant IgHμ-TLXI^TgPrkdc^Scid/Scid and Prkdc^Scid/Scid littermates by PI/Annexin V-based flow-cytometry and BrdU incorporation assays. In addition, expression levels of potential TLXI targets identified by gene expression profiling, such as Bcl11b, Brca1 and Birk5 could be analyzed by qRT-PCR and Western blotting. If differential expression of these proteins in CD44^−CD25^− populations derived from IgHμ-TLXI^TgPrkdc^Scid/Scid and Prkdc^Scid/Scid were confirmed, then additional studies could be initiated to knock-down expression of corresponding genes using siRNA approaches and to test whether reduced expression of these genes affects proliferation or apoptosis in these cells when compared to scrambled control siRNAs.

Deregulated expression of downstream target genes may be detected by using the Comparative Genomic Hybridization (CGH) or array CGH (aCGH) technique (Pinkel, Albertson, 2005). This approach would allow us not only to verify deregulated genes, but also to detect how the malfunction of genes involved in proliferation and apoptosis promotes genomic instability in premalignant thymocytes. Further comparison of genomic instability between premalignant thymocytes and already existent data obtained by SKY analysis from IgHμ-TLXI^TgPrkdc^Scid/Scid T-ALL samples would also allow us to distinguish chromosome aberrations arising during early stages of malignant transformation from those acquired en route during leukemia progression.

As the Brca1 gene product interacts with components of DNA repair and regulates expression of genes that are involved in the DNA repair pathway (Deng & Wang, 2003), future strategies involving the development of IgHμ-TLXI^TgPrkdc^Scid/Scid Brca1^−/− triple transgenic mice could be used to further address the role of DNA repair in TLXI-induced malignancies. The
proposed experiments would allow us to verify the microarray gene expression data and to clarify mechanisms underlying the observed anti-apoptotic and pro-proliferating functions of \textit{TLX1}.

The role of downregulated ribosomal proteins in the T-ALL initiation and progression still needs to be elucidated. Recently, decreased levels of expression of ribosomal proteins \textit{RPL5, RPL10} and \textit{Rpl22} have been reported in human T cell acute lymphoblastic leukemias (De et al, 2013; Rao et al, 2012). Therefore, it would be of interest to verify whether ribosomal proteins are also deregulated in CD44\textsuperscript{-}CD25\textsuperscript{+} populations designated as “early malignant state” derived from \textit{IgH\textsubscript{\mu}-TLX1\textsuperscript{Tg}Prkd\textsuperscript{Scid/Scid}} mice relative to \textit{Prkd\textsuperscript{Scid/Scid}} mice. Downregulated expression of ribosomal proteins in DN1-DN3 premalignant thymocytes has previously been shown (Chapter 3). Detection of the same ribosomal protein genes inactivated in CD44\textsuperscript{-}CD25\textsuperscript{+} populations and in DN1-DN3 premalignant thymocytes by microarray and verification by qRT-PCR would allow us to confirm that \textit{TLX1} abnormal expression in premalignant thymocytes deficient in the DNA-PK has disturbed a principally new genetic pathway from premalignant to early malignant T-cells. This would enhance our understanding of the general picture of deregulated genetic pathways and eventually provide options for developing therapeutic strategies directed at preventing T-ALL progression.
Figure 6.1. **TLX1-deregulated molecular pathways in IgHu-TLX1^{Tg}Prkdc^{Scid/Scid} premalignant thymocytes.** Activating mutations of TLX1 result in its inappropriate expression in IgHu-TLX1^{Tg}Prkdc^{Scid/Scid} premalignant thymocytes. Aberrant expression of TLX1 leads to downregulation (blue box and arrows) and upregulation (red box and arrows) of a number of genes which promote aneuploidy and affect proliferation and viability of IgHu-TLX1^{Tg}Prkdc^{Scid/Scid} premalignant thymocytes. Secondary mutations, including Notch1, Pten, and Bcl11b are acquired by transformed IgHu-TLX1^{Tg}Prkdc^{Scid/Scid} thymocytes during tumour progression.
6.4. **TLX1-initiated malignant transformation of immature progenitors in IgHµ-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mice**

*IgHµ-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mice* developed two different types of malignancies, most likely initiated in cells of different origin. T-ALL was a prevalent form of leukemia developed by 56% of monitored *IgHµ-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mice*, with the thymus being identified as a primary organ involved in malignant transformation (Chapter 3). We proposed that, most likely, T-ALL initiates in thymus-residing immature thymocyte progenitors (Chapter 4). The other 44% of monitored mice developed AML, with the thymus and bone marrow and spleen being infiltrated with leukemic cells (Chapter 5). The origin of AML-ICs cells *IgHµ-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mice* has not been elucidated. We believe that identification of these cells is of crucial importance as this would allow us to establish whether T-ALL and AML arise in the same progenitor subpopulation and the disease phenotype is determined by acquisition of mutations that activate alternative genetic pathways. Alternatively, if T-ALL- and AML-initiating cells are from unique hemopoietic subpopulations, the disease would be pre-determined, and, in this case, acquired mutations may affect the onset and the pace of disease progression but not the disease phenotype (Figure 6.2). This important issue could be addressed in a series of proposed experiments, as outlined below.

To identify AML-initiating cells, we would perform transplantation assays using tumour cells from *IgHµ-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mice* that develop AML (Chapters 3 and 5). Tumour cells would be obtained from the bone marrow of a terminally ill *IgHµ-TLX1\textsuperscript{Tg}Prkdc\textsuperscript{Scid/Scid} mouse*. Different cell populations, including hemopoietic stem cells (HSC), multipotent progenitors (MPPC), common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) would be purified by flow-sorting and injected into femur or lateral tail vein of sublethally irradiated *Prkdc\textsuperscript{Scid/Scid} mice*. Cell fractions would be sorted as follow c-kit\textsuperscript{+}Sca1\textsuperscript{+}Flk2\textsuperscript{−}Slamf1\textsuperscript{+}Lin\textsuperscript{−} (HSC), c-kit\textsuperscript{+}Sca1\textsuperscript{+}Flk2\textsuperscript{−}Slamf1\textsuperscript{−}Lin\textsuperscript{−} (MPP), Flk2\textsuperscript{−}IL7Ra\textsuperscript{+}CD27\textsuperscript{+}Lin\textsuperscript{−} (CLP) and c-kit\textsuperscript{+}Sca1\textsuperscript{−}/lowCD34\textsuperscript{+}FcgR\textsuperscript{low}Lin\textsuperscript{−} (CMP) (Seita & Weissman, 2010). To ensure identification of the subpopulation of cells most enriched for AML-ICs, we would use limiting dilution analysis by injecting cohorts of recipient mice with between 10 to 10\textsuperscript{5} cells and monitoring survival over an extended time period. Sublethally irradiated *Prkdc\textsuperscript{Scid/Scid} mice* will be injected with PBS and will be used as a negative control. The cell fraction capable of initiating AML with the highest penetrance and the shortest latency period would identify the cell subpopulation containing
AML-initiating cells. These experiments will allow us to identify AML-IC which could then be used as drug targets in the development of more efficient therapeutic strategies.
Figure 6.2. Detection of L-IC in IgHμ-TLX1 Tg Prkdc<sup>Scid/Scid</sup> mice.
Leukemia cells would be obtained from the bone marrow of AML-affected IgHμ-TLX1 Tg Prkdc<sup>Scid/Scid</sup> mice and HSC, MPP, CLP and CMP subpopulation isolated by flow sorting. Sublethally irradiated Prkdc<sup>Scid/Scid</sup> mice will be injected with PBS and will be used as a negative control.
6.5. Final thoughts

The overall aim of this thesis was to elucidate the molecular mechanisms of TLX1-driven tumorogenesis and to detect the origin of leukemia-initiating cells. We established that immature thymocytes were the cells of origin for TLX1-induced T-ALL. TLX1 expression appears to be associated with numerical chromosomal abnormalities, via abrogation of the spindle checkpoint, tubular formation machinery and genes involved in inhibition of apoptosis and induction of proliferation, providing insights into the molecular mechanisms underlying leukemia development. Similar changes induced by TLX1 in cells of myeloid and mature B cell (Chen et al., 2006) origins suggest the possibility of common mechanisms that can be activated by TLX1 in various cell types and, therefore, our findings have a broader applicability. In addition, similarities in phenotype and genomic profiles of TLX1-induced T-ALL of IgHμ-TLX1TgPrkdcScid/Scid mice to human TLX1-induced T-ALL makes the IgHμ-TLX1TgPrkdcScid/Scid mouse model clinically relevant, providing a useful tool for exploration of therapeutic treatment options.
Table 6.1. Cytogenetic analysis and frequency of aneuploidy in leukemias

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Disease</th>
<th>Total Cases</th>
<th>% Hypodiploid (Cases)</th>
<th>% Hyperdiploid (Cases)</th>
<th>% Aneudiploid (Cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLX1</td>
<td>10q24</td>
<td>T-ALL</td>
<td>48</td>
<td>8% (4)</td>
<td>21% (10)</td>
<td>29% (14)</td>
</tr>
<tr>
<td>TLX3</td>
<td>5q35</td>
<td>T-ALL</td>
<td>11</td>
<td>0% (0)</td>
<td>18% (2)</td>
<td>18% (2)</td>
</tr>
<tr>
<td>LMO2</td>
<td>11p13</td>
<td>T-ALL</td>
<td>82</td>
<td>3% (3)</td>
<td>8% (7)</td>
<td>11% (10)</td>
</tr>
<tr>
<td>LMO1</td>
<td>11p15</td>
<td>T-ALL</td>
<td>29</td>
<td>3% (1)</td>
<td>9% (3)</td>
<td>12% (4)</td>
</tr>
<tr>
<td>TAL1</td>
<td>1p32</td>
<td>T-ALL</td>
<td>38</td>
<td>0% (0)</td>
<td>2% (1)</td>
<td>2% (1)</td>
</tr>
<tr>
<td>TAL2</td>
<td>7q34</td>
<td>T-ALL</td>
<td>39</td>
<td>5% (2)</td>
<td>13% (5)</td>
<td>18% (7)</td>
</tr>
<tr>
<td>TCR-MYC</td>
<td>8q24</td>
<td>T-ALL</td>
<td>76</td>
<td>4% (3)</td>
<td>4% (3)</td>
<td>22% (17)</td>
</tr>
</tbody>
</table>

Appendix 1. Catalogue of 10q24-associated cytogenetic abnormalities obtained from the Mitelman database of chromosome aberrations in cancer

**Rearrangements Involving 10q24**

45,X,-Y,t(10;14)(q24;q11)
46,XY,add(3)(p21),t(10;14)(q24;q11),-12,add(12)(p1?),+mar,inc
46,XX,t(10;14)(q24;q11)
46,XY,t(10;14)(q24;q11)
46,XY,t(10;14)(q24;q11)
46,XY,t(10;14)(q24;q11),del(22)(q13)
46,XY,der(4)t(4;17)(q31;q21),t(10;14)(q24;q11),-17,+mar/45,XY,der(4)x2,t (10;14),-17
46,XX,t(10;14)(q24;q11)
46,XY,t(10;14)(q24;q11)
47,XY,t(10;14)(q24;q11),+12,del(12)(p?)x2
45,X,-Y,del(9)(p13),t(10;14)(q24;q11),del(12)(p11p12)
46,XY,t(10;14)(q24;q11)
46,XX,t(10;14)(q24;q11)
46,XX,del(9)(p22),t(10;14)(q24;q11)
46,XY,t(10;14)(q24;q11)
46,XX,del(6)(p11),t(10;14)(q24;q11)
46,XY,t(10;14)(q24;q11)
46,XY,inv(1)(p11q12)/46,idem,t(10;14)(q24;q11),del(12)(p11p12)
46,XY,del(6)(q15q23),del(6)(q24),t(10;14)(q24;q11)
46,XY,t(10;14)(q24;q11)
45,XX,dic(9;12)(p11;p12),t(10;14)(q24;q11),-16,+mar
46,XY,t(10;14)(q24;q11)
46,XY,t(10;14)(q24;q11)
46,XY,t(10;14)(q24;q11),-14,+mar
47,XY,+8,t(10;14)(q24;q11)/47,idem,del(7)(q22)
47,XY,del(6)(q13q25),+8,t(10;14)(q24;q11)
46,XY,t(5;7)(p13;q36),t(10;14)(q24;q11)/46,idem,del(13)(q14q32)
48,XY,+7,+9,t(10;14)(q24;q11)
46,XX,t(10;14)(q24;q11)
46,XY,t(9;20)(p21;q12),t(10;14)(q24;q11),del(12)(p12)
45,X,-Y,t(10;14)(q24;q11)
47,XY,t(10;14)(q24;q11),+20
46,XX,t(10;14)(q24;q11)
46,XY,t(10;14)(q24;q11)
46,XY,t(10;14)(q24;q11)
42-48,XY,t(3;11)(p12;p15),t(7;10)(q34;q24),t(8;10)(q21;q?24),+11,+12
47,XY,t(5;14),del(6)(q12),t(7;10)(q34;q24),+8
46,XY,t(7;10)(q34;q24)
47,XY,t(7;10)(q34;q24),+8,del(9)(p21)
46,XY,t(7;10)(q34;q24)
46,XY,t(7;10)(q34;q24)
46,XY,t(7;10)(q34;q24)
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