The Role And Molecular Mechanism Of Flt3 Over-Expression In A Murine Model Of Precursor-B Acute Lymphoblastic Leukemia

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Immunology and Collaborative Program in Developmental Biology
University of Toronto

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Abstract

Precursor B-cell acute lymphoblastic leukemia (pre-B ALL) is thought to arise from a dysregulated developmental process that results in abnormal survival, proliferation and dissemination of B-cell precursors. Our laboratory previously showed that $p53^{-/-}Rag2^{-/-}Prkdc^{scid/scid}$ triple mutant (TM) mice develop pre-B-ALL with 8-16 week latency. Strikingly, in most TM leukemic mice, leukemic blasts disseminate to the leptomeninges of the central nervous system (CNS) causing hydrocephalus, hind limb paresis and other signs of CNS pathology. Genome-wide expression profiling revealed that TM leukemic blasts over-express $FMS-like tyrosine kinase 3$ (Flt3) relative to normal B-cell progenitors. This finding was unexpected, since $Flt3$ is normally repressed by PAX5 upon B-cell commitment. CNS dissemination of leukemic blasts occurs frequently in poor prognosis pediatric and adult pre-B ALL patients, and therapies designed to target leukemic blasts in the CNS are associated with significant long-term
morbidities. Therefore, the overall goal of my thesis was to define mechanisms that drive CNS invasion of leukemic lymphoblasts using the TM mouse model of pre-B ALL.

Using several approaches, I demonstrate that TM B-ALLs from mice with CNS invasion over-express a truncated form of FLT3 that is constitutively active and promote ligand-independent growth and proliferation of TM leukemic cells. Furthermore, I show that this truncated form of FLT3 is encoded by chimeric Flt3 transcripts, in which the 5' exons of Flt3 that encode the extracellular ligand-binding region are replaced by endogenous murine leukemia virus (MuLV) long terminal repeat (LTR) elements. I show that LTR-Flt3 fusion genes arise prior to B-cell commitment from a complex multi-step genomic rearrangement process resulting in deletion of the Flt3 promoter and 5’exons encoding the ligand-binding domain, thus placing the Flt3 kinase domain under transcriptional control of endogenous retro-elements. Finally, I present evidence that these novel fusion genes serve as oncogenic drivers that promote aberrant survival and expansion of primitive hemato-lymphoid progenitors resembling normal lymphoid-biased B-cell progenitors. Taken together, my work has identified a novel mutational mechanism that drives over-expression of a ligand-independent constitutively active form of FLT3 in primitive B-cell progenitors, circumventing normal mechanisms that regulate their survival and proliferation to drive their leukemic transformation.
Acknowledgments

First and foremost, I would like to thank God and my family for helping me through this difficult and arduous journey. I would especially like to thank my sister Tasha Johnson for all her love and support throughout the years I have been away from home. I would also like to thank my mother Audrey Johnson for the countless hours she spent on the phone cheering me up; my brother Shawn Johnson for the all the times he made me laugh; and of course my best friend Jason Dupuis Mayer, who is like a brother to me, for his countless support and encouragement.

I would also like to thank my supervisors Dr. Cynthia Guidos and Dr. Jayne Danska for their countless support and encouragement. Thank you for sharing your depts of knowledge and for teaching me to think and write like a scientist. Thank you for being so kind with your time, advice, and ideas.

I would also like to thank my committee members Dr. Michael Ratcliffe, Dr. Alberto Martin and Dr. Brian Ciruna for your all your time, advice and support.

To soon to be Dr. Eniko Papp, I would like to thank you for all your help and advice and for making my years in Toronto memorable both inside and outside the lab.

To Dr. Philaretos Kousis, thank you for being such a great friend and kind person. I truly appreciate everything you have done for me over the years. God Bless~

To Dr. Peggy Wong, thank you so much for your countless words of advice and support and of course for all those late nights discussing papers and experiments.

To Ildiko Grandal and Steve Mortin-Toth, thank you for taking the time to teach me the molecular, biochemical and immunological skills I know today.

To Dr. Ioana Visan, thank you for being such a great friend and for all your encouraging words.

I would also like to thank all the other member of the lab, past and present, that made my stay in Toronto enjoyable with a special thanks to Amanda Sudworth, Dr. Deirdre Toomey, and Dr. Irina Matei for their kind support.

Last but not least, to all the great people I met in Toronto, with a special thanks to Valerie De Grandis and Kent Anjo for their countless support, and Dave Konkin for believing in the research we do.

This work was supported by a RESTRACOMP Fellowship from the Hospital for Sick Children Research Institute and a CIHR Canadian Graduate Scholarship Award.
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<tr>
<td>Mouse</td>
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<td>Human</td>
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**Abbreviation**

- **ALL**: Acute Lymphoblastic Leukemia
- **AML**: Acute Myeloid Leukemia
- **APC**: Allophycocyanin
- **BM**: Bone Marrow
- **bp**: base pair
- **CFSE**: Carboxyfluorescein Succinimidyl Ester
- **CLP**: Common Lymphoid Progenitor
- **CSF1R (c-FMS)**: Colony Stimulating Factor 1 Receptor
- **CNS**: Central Nervous System
- **DM**: Double Mutant ($p53^{-/-}Rag2^{+/-}Prkd^{Scid/Scid}$)
- **DNA-PK**: DNA-dependent Protein Kinase
- **DSB**: Double Stranded Break (in DNA)
- **EC**: Extracellular Probe corresponding to Flt3 exons 3-6
- **ERV**: Endogenous Retrovirus
- **FACS**: Fluorescent Activated Cell Sort
- **FL**: FLT3 Ligand
- **FLT3**: Fms-like tyrosine kinase 3
- **FMO**: Fluorescence Minus One
- **FSC**: Forward Scatter
- **FWD**: (Forward) Sense Primer
- **GL**: Germline
- **HBSS**: Hank’s Balanced Salt Solution
- **HSC**: Hematopoietic Stem Cell
- **IC**: Intracellular Probe corresponding to Flt3 exons 11-20
- **Ig**: Immunoglobulin
- **IgH**: Immunoglobulin Heavy Chain
- **IgL**: Immunoglobulin Light Chain
- **IL-6**: Interleukin-6
- **IL-7**: Interleukin-7
- **IL-7R**: IL-7 Receptor
- **IL-7R_{α}**: IL-7R α chain
- **ITD**: Internal Tandem Duplication
- **JM**: Juxtamembrane
- **KIT**: Steel Factor Receptor
- **λ_{s}**: component of the surrogate light chain
- **LBP**: Lymphoid-Biased Progenitor
- **Lin^{-}**: Lineage marker negative
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>LMPP</td>
<td>Lymphoid-Primed Multipotent Progenitor</td>
</tr>
<tr>
<td>LIC</td>
<td>Leukemia-Initiating Cell</td>
</tr>
<tr>
<td>LM-PCR</td>
<td>Ligation-mediated PCR</td>
</tr>
<tr>
<td>LSK</td>
<td>Lin- SCA1hi KIThi</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
</tr>
<tr>
<td>MLL</td>
<td>Mixed Lineage Leukemia</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotent Progenitor</td>
</tr>
<tr>
<td>MuLV</td>
<td>Murine Leukemia Virus</td>
</tr>
<tr>
<td>N-</td>
<td>Amino-</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NGF</td>
<td>N-Glycosylase F</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>p53</td>
<td>designation for TP53 tumor protein</td>
</tr>
<tr>
<td>PAX5</td>
<td>Paired Box Gene 5</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>Pre-BCR</td>
<td>Pre-B cell Receptor</td>
</tr>
<tr>
<td>Prkdc&lt;sup&gt;Scid/Scid&lt;/sup&gt;</td>
<td>Gene designation for the murine SCID mutation</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-Time PCR</td>
</tr>
<tr>
<td>R/U5</td>
<td>Redundancy region of the LTR</td>
</tr>
<tr>
<td>5′RACE</td>
<td>5′ Rapid Amplification of Complementary DNA Ends</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombinase Activating Gene</td>
</tr>
<tr>
<td>REV</td>
<td>(Reverse) Antisense Primer</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>SA</td>
<td>Splice Acceptor</td>
</tr>
<tr>
<td>SCA1</td>
<td>Stem Cell Antigen-1</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor (KIT Ligand)</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>SD</td>
<td>Splice Donor</td>
</tr>
<tr>
<td>SM</td>
<td>Staining Media</td>
</tr>
<tr>
<td>SPN</td>
<td>Spleen</td>
</tr>
<tr>
<td>TAP</td>
<td>Tobacco Acid Pyrophosphatase</td>
</tr>
<tr>
<td>TKD</td>
<td>Tyrosine Kinase Domain</td>
</tr>
<tr>
<td>TM</td>
<td>Triple Mutant (&lt;sup&gt;p53&lt;/sup&gt;−/− &lt;sup&gt;Rag2&lt;/sup&gt;−/− &lt;sup&gt;Prkdc&lt;/sup&gt;Scid/Scid)</td>
</tr>
<tr>
<td>U3</td>
<td>Unique 3′ region of the LTR</td>
</tr>
<tr>
<td>U5</td>
<td>Unique 5′ region of the LTR</td>
</tr>
<tr>
<td>UID</td>
<td>Unique Identifier</td>
</tr>
<tr>
<td>V(D)J</td>
<td>Variable, Diversity and Joining gene segments of Ig locus</td>
</tr>
<tr>
<td>VpreB</td>
<td>component of the surrogate light chain</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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<tr>
<td>w/v</td>
<td>weight/volume</td>
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Chapter 1

The focus of my thesis research was to characterize the role and molecular mechanism of Flt3 over-expression in the $p53^{+/}Rag2^{+/}Prkdc^{scid/scid}$ triple mutant TM murine model of precursor B-Acute lymphoblastic leukemia (ALL). In this Chapter, I review current knowledge of B-cell development, Flt3 expression, function and regulation, and its aberrant activation and expression in acute leukemias. I will also present an overview of B-ALL pathogenesis and murine models used to study leukemogenesis.

B-Cell Development

Early stages of B-cell development

B-cells arise from hematopoietic stem cells (HSC) that are able to self-renew and differentiate into all blood lineages (Fig. 1). HSCs are found in the LSK$^+$ cell population which consists of lineage negative (Lin$^-$) cells that express high levels of stem cell antigen-1 (SCA1) and steel factor receptor (KIT). Lineage markers used to identify Lin$^-$ cells typically include CD11b, Gr-1, B220, CD3 and Ter-119. The LSK$^+$ cell population contains long-term self-renewing HSCs, short-term self-renewing HSCs, and multipotent progenitors (MPP) that exhibit varying degrees of erythroid, myeloid, and lymphoid lineage potential without the ability to self-renew. A subset of MPPs referred to as lymphoid-primed multipotent progenitors (LMPPs) have granulocyte, macrophage, and B- and T-lymphoid potential but little megakaryocytic and erythroid potential. LMPPs differentiate into common lymphoid progenitors (CLPs) that have robust B-cell potential and only weak myeloid and T-lymphoid potential. Thus, CLPs are thought to represent a major source of B-cell precursors during hematopoiesis.
In the classical model of B-cell development, CLPs represent the lymphoid-restricted intermediate between HSCs and committed B-cell progenitors. However, two groups recently identified a novel lymphoid-biased progenitor (LBP) population in bone marrow (BM) from adult mice that has robust B-lineage potential\textsuperscript{15, 16}. The cell surface phenotype of LBPs is KIT\textsuperscript{−}SCA1\textsuperscript{hi} in contrast to CLPs which are KIT\textsuperscript{lo} and SCA1\textsuperscript{lo}\textsuperscript{15, 16}. LBPs generate B- and T-lymphoid cells when transferred into lethally or sublethally irradiated host and, unlike CLPs, do not exhibit residual myeloid potential\textsuperscript{15, 16}. However, LBPs are less efficient at generating B-cells when transplanted into sublethally irradiated hosts\textsuperscript{15, 16}, suggesting that the relative importance of LBPs as a lymphoid progenitor may be minor compared to CLPs. Nevertheless, LBPs are likely to be important B-cell progenitors when the production of CLPs is impaired. Consistent with this notion, Kit-deficient (Vickid) mice have very few CLPs but normal numbers of pre-pro-B and pro-B cells\textsuperscript{17}. Thus, LBPs may represent an alternative intermediate between HSCs and pre-pro-B cells in the B-cell development pathway. Taken together, these findings suggest that both CLPs and LBPs serve as early B-cell precursors for B-cell development.
Figure 1. Overview of B-cell Development. The rearrangement status of immunoglobulin (Ig) genes, the expression of signalling receptors and the sequential expression of various cell-surface and molecular markers are indicated for successive progenitor cell stages of B-lymphopoiesis. The current model of B-cell development proposes that the immediate precursors of pre-pro-B are CLPs. However, Snoeck\textsuperscript{16} and Allman’s\textsuperscript{18} group recently identified another B-cell precursor referred to as LBPs which are characterized by Lin\textsuperscript{-}Kit\textsuperscript{-}Sca\textsuperscript{-1}hiFlt3\textsuperscript{+}IL-7R\textsubscript{α}-surface phenotype. LBPs are thought to represent an alternative intermediate between HSCs and committed B-cells\textsuperscript{5,16}. 
**V(D)J Recombination During B-cell Development**

Genes encoding the immunoglobulin (Ig) heavy (H) and light (L) chain proteins are assembled during B-cell development by a somatic DNA recombination process known as V(D)J recombination. The *Igh* chain locus consists of V (variable), D (diversity), and J (joining) gene segments, whereas the *Igl* chain locus is comprised of V and J segments. Ig gene rearrangements are catalyzed by the V(D)J recombinase complex composed of recombinase activating gene (RAG)-1 and RAG-2 and components of the non-homologous end-joining (NHEJ) repair pathway including Ku70, Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Artemis, XRCC4, Ligase IV, and Cernunnos/XLF. V(D)J recombination is initiated when RAG-1 and RAG-2 recognize and cleave recombination signal sequences (RSS) that flank V, D and J segments. Subsequently, these site-specific double stranded DNA breaks (DSB) are repaired by non-homologous end-joining (NHEJ) with the insertion or deletion of nucleotides catalyzed by the lymphoid-specific enzyme terminal deoxynucleotidyl transferase (TdT).

NHEJ is an error-prone DNA repair pathway that seals DSBs without relying on a template. During V(D)J recombination NHEJ repair brings the ends of the broken DNA molecules together by forming a synaptic complex, consisting of two DNA ends, two Ku70/Ku80 heterodimers and two DNA-PKcs proteins. DNA-PK phosphorylates Artemis, which enhances the ability of Artemis to resolve NHEJ intermediates. XRCC4 and Cernunnos together with DNA-PK align the two DNA ends together to help recruit TdT, which contributes to junctional diversity by inserting random nucleotides to the DNA ends. DNA polymerase λ or μ also inserts additional nucleotides as needed to make both ends compatible for joining. Finally, Ligase IV mediates the rejoining of the DNA strands on opposite ends of the break.
The Ordered Nature of Ig Gene Rearrangement

As B-cells develop, antigen receptor genes are assembled from a tandem array of gene segments by V(D)J recombination, a somatic process in which DSBs are introduced at V, D and J gene segments and sequentially re-ligated to produce VDJ_H and VJ_L rearrangements at the Igh and IgL chain loci. Initially, the Igh and IgL loci are in germline configuration in HSC and early hematopoietic progenitors. As MPPs progressively differentiate into CLPs and pre-pro-B cells, RAG-1 and RAG-2 is expressed, which initiates D-J_H rearrangements at the Igh locus. After DJ_H rearrangement are completes, V_H genes become accessible to the V(D)J recombinase complex and VDJ_H recombination is initiated in early pro-B cells. Subsequently productive in-frame VDJ_H recombination in late pro-B cells results in cell surface expression of Igµ as part of the pre-B cell antigen receptor (pre-BCR), which marks the transition to the pre-B cell stage. Signalling through the pre-BCR promotes allelic exclusion at the Igh locus and induces clonal expansion of large pre-B cells and differentiation into small pre-B cells arrested in G1. Small pre-B cells re-activate V(D)J recombinase machinery to allow V(D)J recombination at the IgL chain loci, first at kappa (κ) and later at lambda (λ) if no in-frame VJ_L rearrangements are formed. Productive rearrangement of the VL and JL gene segments in the IgL locus results in the replacement of the pre-BCR surrogate light chain by Igκ or Igλ to form BCR-expressing immature B-cells.

Crucial Checkpoints in B-cell Development

The pre-BCR executes a crucial developmental checkpoint by promoting survival and expansion of B-cell precursors that have productively rearranged their Igh locus. Productive (in-frame) Igh-chain rearrangements allow expression of the pre-BCR complex comprised of membrane-bound IgH-chain protein µ associated with surrogate light chain proteins VpreB and
\( \lambda_5 \) as well as signalling components Ig\( \alpha \) and Ig\( \beta \). Pre-BCR signalling triggers clonal expansion and developmental transition from the pro-B to pre-B cell stage. As pro-B cells undergo pre-BCR-induced differentiation into large cycling pre-B cells, RAG-1 and RAG-2 are transiently downregulated to prevent further \( Igh \)-chain rearrangements. In murine B-cell development, interleukin-7 (IL-7) acts in concert with pre-BCR signalling to promote clonal expansion of large pre-B cells. Following a limited number of cell divisions, large pre-B cells stop cycling and differentiate into small resting pre-B cells. During this developmental transition VDJ rearrangement is reactivated to allow \( V_L \) to \( J_L \) rearrangements. Productive \( Igl \)-chain rearrangements are required for BCR expression, function, and differentiation of pre-B cells into \( IgM^+ \) immature B-cells. Subsequently, immature B-cells exit the BM and migrate to the spleen (SPN) where they can differentiate into peripheral mature B-cells.

**B-lineage Specification and Commitment**

B-lineage specification is regulated by extracellular factors such as FLT3 ligand (FL), stem cell factor (SCF) and IL-7 (Fig. 1), which in turn regulate the sequential action of different transcriptional regulators, including IKAROS, PU.1, E2A, EBF and PAX5. IKAROS and PU.1 are required for FLT3 expression. FLT3 signalling then acts in concert with PU.1 to induce expression of the IL-7 receptor (IL-7R), which then induces E2A expression. E2A and PU.1, induce EBF, which reinforces expression of E2A. E2A is also required for \( D_{H}J_{H} \) gene rearrangements and transcription from the \( IgH \) intronic enhancer. E2A together with EBF specifies the B-cell fate by driving expression of B-lineage specific genes in pro-B cells including \( mb-1, B29, Vpre-B \) and \( \lambda_5 \).

EBF also induces PAX5 expression, which reinforces the expression of B-lineage genes such as \( mb-1, CD19, \) and \( BLNK \), and promotes distal \( V_{H}D_{\text{JB}}J_{H} \) gene rearrangements. PAX5
activity is contingent upon EBF-dependent demethylation and chromatin remodeling to up-regulate \textit{mb-1}, and possibly other targets genes\textsuperscript{63}. Moreover, E2A and EBF are required to epigenetically prime and sequentially activate the \textit{Cd19} enhancer but in the absence of PAX5 cannot induce CD19 transcription\textsuperscript{64}. Thus, PAX5 is required to express the pan B-cell marker CD19.

PAX5 expression is needed for commitment to the B-cell fate and maintenance of B-lineage specific genes, such as \textit{CD19}, and to repress lineage-inappropriate genes including \textit{Flt3}, \textit{colony stimulating factor 1 receptor (Csf1r)}\textsuperscript{65}. Continuous PAX5 expression is required to maintain B-cell identity throughout B-cell development from the pro-B to the mature B-cell stage\textsuperscript{66-68}. \textit{Pax5} deficiency in mice arrests B-cell development at the pro-B cell stage\textsuperscript{68,69}. \textit{Pax5}\textsuperscript{−/−} pro-B cells exhibit extensive developmental plasticity and are able to give rise to T-lymphoid, myeloid, and erythroid cells in BM transplantation experiments\textsuperscript{68,69}. Moreover, conditional deletion of \textit{Pax5} in B-lineage cells results in mis-expression of lineage-inappropriate genes such as \textit{Flt3} and \textit{Notch1}\textsuperscript{67,69,70}. Conversely, restoration of \textit{Pax5} expression suppresses multi-lineage potential and promotes differentiation of \textit{Pax5}\textsuperscript{−/−} pro-B cells into mature B-cells\textsuperscript{69}.

The ability of \textit{Pax5}\textsuperscript{−/−} pro-B cells to differentiate into multiple hematopoietic lineages is similar to that of MPPs, which suggests that PAX5 represses genes needed to maintain an undifferentiated state\textsuperscript{71}. \textit{Flt3} is one of the genes associated with multipotency repressed by PAX5\textsuperscript{71,72}. FLT3 expression is usually extinguished early in B-lymphopoiesis at the pro-B cell stage concomitant with PAX5 and CD19 expression\textsuperscript{71,73,74}. The majority of pro-B cells are CD19\textsuperscript{+}FLT3\textsuperscript{−}, but a minor fraction of CD19\textsuperscript{+}FLT3\textsuperscript{low} and CD19\textsuperscript{+}FLT3\textsuperscript{+} pro-B cells are also detected in the BM of WT mice\textsuperscript{71}. In the absence of PAX5, CD19 expression is not induced and only CD19\textsuperscript{+}FLT3\textsuperscript{+} pro-B cells are detected, suggesting that B-cell progenitors progress from a FLT3\textsuperscript{−}CD19\textsuperscript{−} to CD19\textsuperscript{−}Flt3\textsuperscript{low} stage prior to B-cell commitment and PAX5 expression\textsuperscript{71}. Thus,
one of the key functions of PAX5 is to repress FLT3 in early lymphoid progenitors as progenitors undergo B-lineage specification.

**FLT3 Structure and Function**

FLT3 is a class III receptor tyrosine kinase (RTK) characterised by five Ig-like extracellular domains, a transmembrane domain, juxtamembrane domain (JM) and two intracellular tyrosine-kinase domains (TKD) linked by a kinase-insert domain (Fig. 2)\(^{75}\). Other members of the class III RTK family include platelet-derived growth factor receptor (PDGFR), c-KIT and CSF1R (also known as c-FMS)\(^{76}\). Signalling through the FLT3 and KIT receptors regulate survival, proliferation and differentiation of hematopoietic progenitors\(^{77}\), while CSF1R stimulates differentiation and proliferation of macrophages and monocytes\(^{78}\). In contrast, PDGFR is expressed at low levels on some hematopoietic cells but does not seem to play an important role in normal hematopoiesis\(^{77, 79, 80}\). Thus, signalling through FLT3 and other class III RTKs is important for differentiation of HSCs along the myeloid and B-cell pathway.

FLT3 activation requires ligand-induced dimerization of the receptor\(^{81-83}\). FL is a type I transmembrane protein that consists of an amino (N)-terminal signalling peptide, four extracellular helical domains, spacer and tether regions, a transmembrane domain, and a small cytoplasmic domain. FL is expressed by most tissues, including hematopoietic organs, prostate, ovary, kidney, lung, colon, small intestine, testis, heart and placenta, with the highest level of expression detected in peripheral-blood mononuclear cells\(^{84}\). FL can be tethered to the plasma membrane or proteolytically cleaved to produce a soluble homodimeric protein\(^{85-88}\). The biological relevance of these various isoforms are unknown but both the membrane-bound and soluble forms of FL can bind and activate the FLT3 receptor\(^{89}\).
Figure 2. Structure of Class III RTKs. Class III RTKs share a common topology, which consists of five Ig-like domains (IG1 to IG5), a transmembrane domain (TM), juxtamembrane domain (JM), an intracellular tyrosine-kinase domain (TKD) split in two parts by a flexible polypeptide hydrophobic linker, known as the kinase-insert domain (KID), and a carboxy terminal tail. Abbreviation: ECD, extracellular domain.

FLT3 is normally expressed in early hematopoietic progenitors and early B-cell precursors, but not in committed B-cells. Signalling through FLT3 is required for efficient production of CLPs and subsequent development of pro-B and pre-B cells in FL-deficient (Flt3L−/−) mice. Moreover, BM chimeras generated by mixing WT and Flt3−/− cells demonstrated that FLT3 is required for the generation of B-cells under competitive circumstances. Furthermore, in contrast to mice lacking only Flt3 or Il-7Rα, Flt3−/−Il-7Rα−/− double knock-out mice entirely lack B-cells. Similarly, early B-lymphopoiesis is more severely impaired in Flt3−/− KitW/Wv double mutant mice than in single mutants. Therefore, taken together, these findings suggest that FLT3 works in conjunction with other growth factor receptors including IL-7 and SCF to promote B-cell development.

Although targeted disruption of Flt3 only slightly decreases the size of the pro-B and pre-B cell compartments in steady-state BM, enforced expression of Flt3 significantly impairs B-lymphopoiesis by reducing the number of B-cell progenitors. However, enforced expression of Flt3 had no effects on T-cell development in the thymus, suggesting that inhibitory effects of sustained FLT3 signalling occurs after the CLP stage but before B-cell commitment. Similarly, Flt3ITD knock-in or transgenic mice have increased numbers of myeloid cells, CLPs and pre-pro-B cells, but decreased numbers of committed pro-B-cells, likely due to impaired pro-B cell
survival\textsuperscript{96}. Therefore, these studies suggest that failure to extinguish Flt3 expression upon PAX5-mediated B-cell commitment has profound inhibitory effects on developing B-cell progenitors.

**Acute Lymphoblastic Leukemia**

Hematopoiesis progresses through stepwise differentiation of HSC to generate a hierarchy of progenitor populations with progressively restricted developmental potentials, which leads to the production of all blood lineages. Within this developmental scheme leukemias can be viewed as aberrant hematopoietic processes. Acute leukemias consist of a diverse group of mainly precursor-stage lymphoid and myeloid cell malignancies. ALLs are neoplasms of T-cell or B-cell lymphoid progenitors\textsuperscript{97-99}. About 70-90\% of ALL cases belong to the B-cell lineage, which are subdivided into different subtypes\textsuperscript{97, 100} including E2A-rearranged (often E2A-PBX1), BCR-ABL, TEL-AML1, Mixed lineage leukemia (MLL)-rearranged, hypodiploid or hyperdiploid karyotypes (Table 1)\textsuperscript{101-103}. Prognostically favorable subtypes include TEL-AML1, E2A-PBX1 and hyperdiploid ALL. Conversely, BCR-ABL and MLL-rearranged B-ALL have poor prognosis\textsuperscript{103}. Although improved protocols for diagnosis, risk stratification, and treatment have increased survival, lethal relapses often accompanied by dissemination of leukemic blasts to the CNS still occur in 20\% of childhood and 40-60\% of adult ALL patients\textsuperscript{104, 105}. The lack of markers predictive of relapse and CNS dissemination reflects our lack of knowledge underlying the molecular mechanisms of leukemogenesis.
Table 1. ALL subtype frequency and prognosis implication

<table>
<thead>
<tr>
<th>ALL Subtypes</th>
<th>Frequency of Cases (%)</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TEL-AML1</strong></td>
<td>20-25</td>
<td>Favorable</td>
</tr>
<tr>
<td>Hyperdiploid (&gt;50 chromosome)</td>
<td>25</td>
<td>Favorable</td>
</tr>
<tr>
<td><strong>E2A-rearranged ALL</strong> (often E2A-PBX1)</td>
<td>5</td>
<td>Favorable</td>
</tr>
<tr>
<td><strong>BCR-ABL1</strong></td>
<td>5</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>MLL-rearranged ALL</td>
<td>5</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>T-lineage (T-ALL)</td>
<td>15</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>B-Lineage Other (BCR-ABL1-like)</td>
<td>25 (15)</td>
<td>Intermediate (Unfavorable)</td>
</tr>
</tbody>
</table>

*Precursor B-ALL arises from defects in B-cell development*

Precursor B-ALL is caused by genetic lesions that block B-cell development and drive aberrant survival and proliferation of B-cell precursors. Human B-ALL lymphoblasts are often blocked early in B-cell development and the most common forms have the following surface immunophenotype: surface Ig<sup>-</sup>, CD10<sup>+</sup>, CD24<sup>+</sup>, cytoplasmic CD79<sup>+</sup>, and terminal deoxynucleotidyl transferase (TdT)<sup>+</sup>, with variable surface expression of lymphoid markers CD20, CD22, CD45 and myeloid markers CD13 and CD33<sup>100</sup>. B-ALL blasts have frequent genetic alterations in genes encoding the principal regulators of B-cell development (e.g., *PAX5*, *IKZF1*, and *EBF1*), lymphoid signalling (*BTLA, CD200, BLNK, VPREB1*), transcription factors (*ETV6, ERG*), cell cycle regulators (*CDKN2A, CDKN2B, RB1*), and tumor suppressors (*ATM, PTEN*)<sup>100</sup>. Altogether, these observations suggest that precursor B-cell leukemogenesis results from the acquisition of genetic lesions that sustain cell growth and proliferation with direct disruption of pathways controlling B-cell development and differentiation.
**FLT3 Expression in Acute Leukemias**

Constitutive FLT3 activation confers a strong proliferative and survival advantage to hematopoietic cells and is frequently involved in the development of hematological malignancies\(^{106, 107}\). High levels of wild-type (WT) FLT3 were reported in 70-90% of acute myeloid leukemia (AML) patient samples and a large proportion of B-ALL leukemic blasts\(^{108-112}\) including those with *MLL* rearrangements\(^{113, 114}\). Interestingly, constitutive phosphorylation of FLT3 was detected in B-ALL patient samples with high levels of WT FLT3\(^{115-117}\), suggesting that FLT3 over-expression is sufficient to activate the receptor in a ligand-independent manner. Importantly, treating leukemic blasts from *MLL*-rearranged B-ALLs with FLT3 inhibitors resulted in a pronounced cytotoxic and apoptotic response, suggesting these leukemic blasts are highly dependent on FLT3 signalling for survival\(^{116, 118}\). Altogether, these observations suggest that FLT3 over-expression in AML and ALL promotes ligand-independent growth of leukemic blasts.

Common FLT3 mutations include point mutations in the activation loop of the second TKD and internal tandem duplication (ITD) of the JM domain\(^{89, 107}\). FLT3-ITD mutations result from variable in frame amino acid duplications in the JM domain that disrupt the auto-inhibited structure of the inactive kinase to promote FLT3 activation\(^{81}\). FLT3-TKD mutations usually involve point mutations or micro-deletions in the activation loop of the kinase domain\(^{119}\). FLT3-ITD mutations occur in 17-20% of *de novo* adult AML, 5-17% of childhood AML, and 3% of B-ALL\(^{120-126}\) but rarely in *MLL*-rearranged leukemias\(^{125, 127}\). In contrast, FLT3-TKD mutations occur in 7% of *de novo* adult AML and 2.8% of ALL cases. Thus, activating FLT3 mutations occur in both AML and ALL.

FLT3-ITD and FLT3-TKD activate several signal transduction pathways. WT FLT3 and FLT3-TKD activate RAS/RAF/MEK/ERK\(^{128, 129}\) and PI3K/AKT\(^{129-131}\) signal transduction
pathways (Fig. 3). More specifically, FL-dependent activation of WT FLT3 results in the phosphorylation of the following signalling components: SHC, SHP-2, SHIP, GRB2 and SRC family kinases\textsuperscript{129, 132}. The p85 subunit of PI3K does not directly bind FLT3 but forms a complex with GRB2, CBL, CBLB, SHP-2 and SHIP in BaF3 hematopoietic progenitor cells stably transfected with WT FLT3\textsuperscript{131, 133, 134}. Although FLT3 does not have Janus kinase (JAK) binding sites, WT FLT3 has been proposed to induce STAT5 activation because \textit{Stat5a}\textsuperscript{-/-} progenitor cells did not proliferate in response to FL\textsuperscript{135}. Therefore, JAK-independent activation of STAT5a is required for some of the proliferative effects of ligand-dependent FLT3 activation. Nonetheless, tyrosine phosphorylation or DNA-binding of STAT5 following WT FLT3 stimulation with FL was not detected\textsuperscript{136, 137}. In contrast, FLT3-ITD induces high levels of ligand-independent STAT5 activation\textsuperscript{89, 136-138} but only weak ERK1/2 and AKT activation\textsuperscript{137}. Therefore, WT FLT3 and FLT3-ITD exhibit distinct signalling properties.
Figure 3. FLT3 and FLT3-ITD signal transduction pathways. Schematic shows signalling pathways activated by WT and FLT3-ITD to induce cell proliferation and survival.

**Hallmarks of Leukemogenesis**

Tumorigenesis is a multistep process in which normal cells evolve progressively to a neoplastic state by accumulating mutations that enable aberrant cell survival, growth and metastatic dissemination\(^{139, 140}\). These allow tumor cells to abnormally sustain proliferative signalling and cell growth, evade growth suppressors, resist cell death, enable replicative immortality, induce a block in differentiation, and activate tissue invasion and metastasis\(^{139, 140}\). The succession of genetic lesions that confer one type of growth advantage over another progressively leads to the conversion of normal cells into tumor cells\(^{139, 140}\). More specifically, acquisition of oncogenic
driver mutations transforms normal cells into cancer cells. Therefore, the genomic instability of cancer genomes, unlimited proliferative capacity, tumor heterogeneity, various morphologies, metabolic differences, and the interplay of tumor cells with the microenvironment contribute to the development and progression of leukemia.

**Clonal evolution versus leukemia-initiating cell model of leukemogenesis**

ALL is a heterogeneous disease with regard to the genetic lesions that cause the leukemia and the tumorigenic capacity of the individual cells that make up the tumor. Two models have been proposed to explain tumor heterogeneity: clonal evolution and the leukemia-initiating cell (LIC) model. The clonal evolution model posits that over time tumor cells will acquire various combinations of mutations and through genetic drift and stepwise natural selection for the fittest, the most aggressive cells will drive tumor progression\(^\text{141}\). Tumor initiation takes place in the cell-of-origin, which is the cell that has acquired the necessary driver mutations that provide a selective growth advantage\(^\text{142}\). As the tumor progresses, genetic instability and uncontrolled proliferation allow the accumulation of additional mutations that endow some clones with new characteristics such as resistance to apoptosis and self-renewal\(^\text{139, 140}\). Throughout this process, the tumor cells can become invasive and cause metastasis or become resistant to therapies and cause recurrence\(^\text{141, 143-147}\).

Conversely, the leukemia-initiating cell (LIC) model posits that tumor initiation, progression, and recurrence are driven by small subpopulations of tumorigenic cells with stem cell-like properties\(^\text{148, 149}\). Just as normal stem cells differentiate into phenotypically diverse progeny with limited proliferative potential, LIC undergo epigenetic changes analogous to normal cell differentiation to form phenotypically diverse tumor cells that compose the bulk of the tumor\(^\text{150}\). John Dick and colleagues, first identified LICs in AML by showing that rare cells
within the CD34^+CD38^- subset of AML patient BM or blood could regenerate the diversity and phenotype of the original leukemia when xeno-transplanted into irradiated immunodeficient recipient mice, whereas more differentiated CD34^+CD38^+ cells could not\textsuperscript{151, 152}. Notably, when CD34^+CD38^- and CD34^+CD38^+ cells were isolated from the BM of recipient mice and transferred into secondary hosts, only CD34^+CD38^- cells could regenerate the leukemia, suggesting that long-term self-renewal capacity resides exclusively in the CD34^+CD38^- fraction\textsuperscript{151}. These studies revealed that AML is organized as a hierarchy\textsuperscript{151, 152}, and strongly suggested that intra-tumoral heterogeneity could be explained by the LIC model, at least for AML.

While studies by many groups have confirmed the hierarchical organization of AML\textsuperscript{151-155}, there is no consensus on whether B-ALLs are also organized as a hierarchy\textsuperscript{156-161}. Cox \textit{et al.} showed that CD19^+CD34^+ cells from standard-risk B-ALL samples could transfer leukemia to immunodeficient recipient mice (Fig. 4A)\textsuperscript{158}. In contrast, other groups demonstrated that only CD19^+ cells from both standard and high-risk B-ALL could generate leukemia in xeno-transplant studies (Fig. 4B)\textsuperscript{156, 161}. However, LeViseur \textit{et al.} found that in standard-risk B-ALL samples, only CD19^+ cells contained LIC, whereas in CD19^+CD34^+, CD19^+CD34^+, and CD19^+CD34^- cells from high-risk B-ALL samples all contained LICs (Fig. 4C). In summary, the nature of the LIC and the steepness of hierarchical organization appear to be quite variable.
Figure 4. Subsets containing LICs in human B-ALL. A) Cox et al. study in standard risk B-ALL\textsuperscript{158}. B) Castor et al. and Colbaleda et al. study in standard and high-risk B-ALL\textsuperscript{156,157}. C) Le Viseur et al. study in high-risk B-ALL\textsuperscript{160}. Subsets containing LICs in different studies are shown in the red box.

Mouse Models of B-cell Leukemias

Animal models that accurately recapitulate the molecular pathogenesis of the disease have greatly improved our understanding of B-cell leukemogenesis, because they provide a tool to identify oncogenes and genetic lesions that cooperate with known mutations to promote leukemogenesis\textsuperscript{162}. The oncogenic contribution of various types of genetic lesions in promoting leukemia has been studied using a variety of genetically engineered mouse strains, such as $E\mu$-
Myc transgenics, which express c-Myc under the control of Igh enhancers and typically develop precursor B-ALL with a 6-9 month latency\textsuperscript{163, 164}. c-Myc is found mutated or transcriptionally deregulated in many human lymphoid malignancies\textsuperscript{165}, but the long latency of Myc-induced leukemogenesis suggests that dysregulated expression of c-Myc alone is insufficient to transform B-cell precursors. Therefore, E\textsubscript{\textmu}-Myc transgenic mice have been widely used to identify mutations that co-operate with Myc to promote B-cell leukemogenesis.

**Targeted disruption of apoptotic pathways accelerate leukemogenesis**

The onset of c-Myc-induced leukemogenesis is greatly accelerated by mutations that disrupt apoptotic pathways. Over-expression of BCL-2 or BCL-X\textsubscript{L}\textsuperscript{166} and targeted disruption of the p19\textsuperscript{ARF}-MDM2-p53 pathway\textsuperscript{167, 168} accelerate leukemogenesis in E\textsubscript{\textmu}-Myc transgenics, suggesting that enforced expression of c-Myc induces apoptosis. Indeed, ectopic expression of c-Myc in quiescent cells induces p19\textsuperscript{ARF} expression, which stabilizes p53 and promotes cell cycle arrest and/or apoptosis\textsuperscript{163-165}. Consistent with these observations, retroviral insertional mutagenesis screens performed by infecting newborn mice with Moloney murine leukemia virus (MuLV) revealed that c-Myc is frequently mutated in p53\textsuperscript{-/-} and p19\textsuperscript{ARF,-/-} mice\textsuperscript{169}. Moreover, Bmi-1, a repressor of the Cdkn2a locus encoding p19\textsuperscript{ARF} and p16 INK4a, is often over-expressed by proviral insertion in E\textsubscript{\textmu}-Myc transgenics infected with MuLV\textsuperscript{168}. Therefore, two different genetic approaches demonstrated the importance of inactivating the p19\textsuperscript{ARF}-MDM2-p53 tumor suppressor pathway during Myc-induced B-cell leukemogenesis.

**DNA damage response pathways and Leukemogenesis**

DNA damage response pathways are often dysregulated in cancer, resulting in the accumulation of genetic lesions including deletions, amplifications and point mutations affecting single genes and structural rearrangement involving multiple genes\textsuperscript{170}. Unrepaired DSBs are
mutagenic. V(D)J-specific DSB accumulate in NHEJ-deficient B-cell precursors and activate the p53-dependent DNA damage response\textsuperscript{171, 172}. Multiple rounds of DNA breakage and rejoining compromise genomic stability in lymphocyte precursors\textsuperscript{173, 174}. V(D)J-specific DSB in p53-deficient SCID lymphocytes result in disseminated pro-B cell leukemia\textsuperscript{173, 175, 176}. Therefore, p53-deficiency in mice lacking essential NHEJ genes such as \textit{Ku80}, \textit{XRCC4} and \textit{Ligase IV} causes lethal early B-cell leukemia\textsuperscript{172, 177, 178}. Collectively, these data reveal that DNA damage checkpoints prevent oncogenic transformation in lymphocyte precursors when DSB repair is compromised.

Misrepair of DSBs promotes tumorigenesis by activating proto-oncogenes or inactivating tumor suppressor genes, resulting in growth dysregulation and neoplastic transformation\textsuperscript{179}. Normally, developing lymphocytes are dependent on the production of a functional BCR for survival and proliferation\textsuperscript{33}. In the absence of a functional BCR, there is a strong selection for translocation partners that confer a growth or survival advantage to pro-B cells\textsuperscript{180}. Aberrant joining of antigen receptor gene segments to random DSBs often creates oncogenic translocations\textsuperscript{181}. We and others have shown that early B-cell leukemias that develop in \( p53^{-/-} \) \( Ku80^{-/-} \), \( p53^{-/-} XRCC4^{-/-} \) and \( p53^{-/-} SCID \) mice frequently harbor translocations involving the \textit{Igh} locus and the \textit{c-Myc} proto-oncogene, resulting in dysregulated c-MYC expression\textsuperscript{172, 177, 178}. Notably, our laboratory showed that RAG-1/2-dependent DSBs were required to induce \textit{Igh}/c-\textit{Myc} translocations in \( p53^{-/-} Prkdc^{scid/scid} \) double mutant (DM) mice, since \( Rag-2^{-/-} p53^{-/-} Prkdc^{scid/scid} \) TM mice develop B-cell leukemias lacking \textit{Igh}/c-\textit{Myc} translocations\textsuperscript{172}. Therefore, RAG-1/2-induced DSBs contribute to genomic instability and leukemogenesis in mice deficient in both \( p53 \) and \( Prkdc \) or other NHEJ components.

In addition to \( p53^{-/-} Prkdc^{scid/scid} \) DM mice, our laboratory has generated \( Rag-2^{-/-} p53^{-/-} Prkdc^{scid/scid} \) TM mice that develop precursor B-ALL\textsuperscript{172}. Ultimately, both DM and TM mice
develop CD19+ precursor B-ALL with a 8-16 week latency\textsuperscript{172, 173}. Unexpectedly, about 75\% of TM leukemias have a unique capacity to disseminate to the CNS, providing a novel mouse model to study the molecular mechanisms that drive B-cell leukemogenesis with this morbid clinical complication. Although \textit{Rag-2}\textsuperscript{-/-} \textit{Prkdc}\textsuperscript{scid/scid} TM mice lack V(D)J recombinase activity, global genomic instability conferred by the loss of p53 and DNA-PK is sufficient to transform TM pro-B cells\textsuperscript{172}. RAG-independent genomic rearrangements involving endogenous \textit{Murine leukemia virus (MuLV)} retroviral elements and \textit{Flt3} create an oncogenic form of FLT3 that drives B-cell leukemias with the ability to invade the CNS in TM mice. Therefore, I will provide a brief overview of MuLV and its role in leukemogenesis.

**MuLV and Leukemogenesis**

MuLV is a slow transforming retrovirus that consists of an encapsidated dimer of single stranded RNA enclosed in a capsid, which is in turn enclosed in a lipid bilayer envelope\textsuperscript{182}. Retroviruses are unique because they transform their genetic material from RNA to DNA, which is integrated into the host genome as a provirus\textsuperscript{183}. Transcription of the provirus is required to translate and process the virion proteins needed to replicate the viral genome. Slow transforming retroviruses induce mono- or oligo-clonal tumors by de-regulating gene expression through integration of the provirus into the host genome\textsuperscript{183, 184}.

Transcriptional control elements present in the MuLV genome has the potential to dysregulate cellular gene expression by enhancing or disrupting normal transcription through enhancer-trap and gene-trap mechanisms\textsuperscript{184}. An enhancer-trap involves fusing a weak promoter or enhancers to an open reading frame encoding a protein. Given that the activity of enhancers is not restricted to the most proximal promoter but can act over large distances\textsuperscript{185}, the integration of MuLV promoters and enhancers upstream or within the 5’ untranslated regions (UTR) has the
potential to activate or increase gene expression using a mechanism similar to enhancer-trap insertional mutagenesis. MuLV insertions can also occur in introns or non-coding exons and mutate genes through gene-trapping\textsuperscript{183}. Consequently, read-through of the transcriptional machinery from the MuLV proviral promoter into cellular genes results in the expression of chimeric transcripts\textsuperscript{183}. In these chimeric transcripts, the MuLV proviral splice donors (SD) are spliced to exons or cryptic splice acceptors (SA) in introns of cellular genes\textsuperscript{183}. Therefore, elements in the MuLV provirus that regulate transcription of the viral genome can enhance or disrupt normal transcription of cellular genes and thus induce oncogenic mutations.

**MuLV Provirus Structure**

Mutation of cellular genes by retroviral insertions is mediated by proviral elements that drive and regulate MuLV transcription. A typical replication-competent MuLV provirus varies between 7-11 kb in size and consists of coding sequences for *gag*, *pol* and *env* genes flanked by two LTRs (Fig. 3)\textsuperscript{183}. Each LTR is composed of a unique 3’ (U3) and unique 5’ (U5) region separated by a redundancy (R) region. The *U3* region contains enhancers and a promoter. The *R* region contains the start and termination sites for transcription\textsuperscript{182} and is followed by the *U5* region that contains a T-rich site\textsuperscript{186}. The primer binding site (PBS), 3’ of the LTR, is used to initiate reverse transcription of the MuLV RNA genome into DNA. 3’ of the PBS is the major MuLV SD used to generate *Env* mRNA.

The *U3* promoter consists of sequences involved in the recruitment of the basal transcriptional machinery such as a TATA-box and GC-rich sequences\textsuperscript{187}. The enhancer region contains binding sites for transcription factors such as Ets\textsuperscript{188}, NF1\textsuperscript{189} and CBF\textsuperscript{190} family members. Efficient viral replication is restricted to cellular environments that provide the various transcription factors required for high levels of viral transcription. The transcription factor sites
present in the enhancer region govern MuLV tissue tropism. For example, Moloney MuLV preferentially causes T-cell lymphoma and B-cell leukemias\textsuperscript{183} whereas Graffi-type MuLV typically causes myeloid leukemias\textsuperscript{191, 192}. Therefore, the proviral elements present in the LTR that regulate retroviral transcription and SD sites normally used for splicing of subgenomic sized viral transcripts have the potential to alter cellular gene transcription cell-type specific manner.

**Figure 5. MuLV provirus structure.** Schematic of a representative replication-competent provirus. Location of 5’ and 3’ LTRs, \textit{gag}, \textit{pol} and \textit{env} genes, primer binding site (PBS), and splice donor (SD) sites are shown.

**Endogenous retrovirus distribution in the mouse genome**

Endogenous retroviruses (ERV) are derived from ancient retroviral infections that became permanently integrated into the host genome and inherited in a Mendelian fashion\textsuperscript{193}. ERVs and other retrovirus-like elements are widely distributed and make up approximately 10\% of the mouse genome\textsuperscript{193-195}. Common inbred mouse strains can be grouped according to the number of ecotropic proviruses detected in their genome. The genomic DNA from virus negative strains does not hybridize to retrovirus-specific \textit{env} northern blot probes and include 129, NZB, and CBA/H mice\textsuperscript{196, 197}. Low-virus inbred strains contain a single genome-length provirus, and include BALB/c and (C57BL/6) B6 mice, which have a complete MuLV proviral genome found
on chromosome 5 and 8, respectively. Lastly, high-virus strains contain multiple complete MuLV proviral genomes located on several different chromosomes. Some high-virus strains, such as AKR, are viremic at birth and express several endogenous MuLV that eventually cause leukemia through the activation of proto-oncogenes such as c-Myc. Therefore, the presence of endogenous MuLV predisposes certain mouse strains to develop leukemia.

**Effects of ERV elements on gene expression**

Although most ERVs are unable to replicate their genome, chemical treatment, UV irradiation, DNA transfection, recombination and trans-complementation can induce activation of ERVs in vivo, allowing MuLV replication, re-infection and mobilization to different sites in the host genome. Endogenous MuLV proviruses are subject to recombination with other retroviral-like elements or cellular sequences in the host genome. For example, aberrant repair of DSBs caused by ionizing radiation leads to thymic lymphomas in CB-17 and STS mice due to aberrant recombination of ERVs with cellular proto-oncogenes such as Notch1. It is the aberrant repair of DSBs and integration of MuLV LTR promoters, enhancers or polyadenylation sites in vicinity of host genes that de-regulates gene expression to promote activation of proto-oncogenes or inactivation tumor suppressors in mice treated with ionizing radiation. Therefore, I propose global genomic instability conferred by loss of p53-regulated DNA damage checkpoints combined with disrupted NHEJ-mediated telomere maintenance and DSB repair favor genomic rearrangements involving endogenous MuLV elements and the Flt3 locus to activate an oncogenic form FLT3 to drive leukemogenesis in TM B-ALLs that disseminate to the CNS.
Summary of Thesis Chapters

The focus of my graduate research was to characterize the role and molecular mechanism of Flt3 over-expression in the TM murine model of precursor B-ALL. Chapter 2 provides the details and protocols used for all experiments. Chapter 3 examines the expression and function of FLT3 and other class III RTKs in TM leukemias and identifies a novel mutant form of FLT3. Chapter 4 investigates the nature and molecular mechanism of Flt3 over-expression in TM leukemias. Chapter 5 determines whether TM leukemias are organized as a hierarchy and whether the genetic alterations that create mutant FLT3 occur prior to B-cell commitment and PAX5 expression. Finally, Chapter 6 discusses the implications of the findings presented in Chapters 3-5 on our understanding of human B-ALL.
Chapter 2
Material & Methods

Mice

Mice with mutations in DNA damage surveillance and NHEJ repair pathways were used to identify dysregulated pathways of B-cell development that are subverted to give rise to precursor B-ALL. The methods by which the mutant mice were derived and genotyped are described below. All mice were bred and housed in pathogen-free conditions at the Hospital for Sick Children Animal facility and the Toronto Center for Phenogenomics.

\( p53^{-/-} Prkdc^{scid/scid} \) and \( p53^{-/-} Rag2^{-/-} Prkdc^{scid/scid} \)

\( p53^{-/-} \) 129/Sv and \( Prkdc^{scid/scid} \) ICR parental strains were bred to generate \( p53^{-/-} Prkdc^{scid/scid} \) (DM) mice and their \( p53^{+/-} Prkdc^{scid/scid} \) littermates as previously described\(^{173} \). \( Rag2^{-/-} Prkdc^{scid/scid} \) mice were generated from \( Rag2^{-/-} \) (129/Sv and B6) and \( Prkdc^{scid/scid} \) (CB-17) progeny. \( Rag2^{-/-} Prkdc^{scid/scid} \) mice were bred with \( p53^{+/-} Prkdc^{scid/scid} \) mice to generate \( Rag2^{-/-} p53^{+/-} Prkdc^{scid/scid} \) mice as previously described\(^ {173} \). \( p53^{-/-} Rag2^{-/-} Prkdc^{scid/scid} \) (TM) mice were generated by intercrossing \( Rag2^{-/-} p53^{+/-} Prkdc^{scid/scid} \) mice or by mating \( Rag2^{-/-} p53^{+/-} Prkdc^{scid/scid} \) males with \( Rag2^{-/-} p53^{+/-} Prkdc^{scid/scid} \) females as previously described\(^ {172} \). Mice were genotyped by PCR amplification of tail DNA using the following primers: \#629, 5W1-5’ (5’- GTG TTT CAT TAG TTC CCC ACC TTG AC - 3’); \#630, 3W1-3’ (5’- AGA GCA AGA ATA AGT CA G AAG CCG -3’); \#176, 5N-5’ (5’- GTG GGA GGG ACA AAA GTT CGA GGC C -3’); and \#178, 3N2-3’ (5’- TTT ACG GAG CCC TGG CGC TCG ATG T -3’) at a final concentration of 0.2 µM each. Primer numbers refer to unique identifiers (UID) used in our laboratory. Taq PCR Master Mix solution (Qiagen) containing Taq DNA Polymerase, PCR Buffer, and 200 µM of each dNTP, and 1.5 mM MgCl\(_2\) was used for genotyping. PCR reactions were multiplexed and run using the
following cycling conditions: 94°C for 15’ followed by 32 cycles of 30” at 94 °C, 90” at 63°C, and 90” at 72°C; and a final extension at 72°C for 10’ on the Engine Tetrad (MJ Research, now BioRad) or a Gene Amp® PCR system 9700 (Applied Biosystems) thermal cycler. PCR products were separated on a 2% gel to reveal the 120 bp knock-out band (Neo) and the 335bp WT band.

**Tail DNA Isolation**

Crude tail genomic DNA was isolated by digesting 3-5 mm of mouse tail overnight in Tissue Digestion Solution B (Autogen) with 1:40 dilution of 1 mg/mL proteinase K stock (Invitrogen) at 55°C. Digested tails were centrifuged at room temperature (RT) for 5’ at 10,000 rpm. 10 µL of crude tail DNA supernatant was diluted in 90 µL of water and incubated at 95°C for 15’ to inactivate proteinase K. 1 µL of diluted DNA was used for genotyping in a final 10 µL PCR reaction volume.

**Cell Isolation**

SPN and lymph nodes (LN) from leukemic and control mice were dissected. Single cell suspensions were prepared by mincing tissues with scissors and disrupting cells by passing them through a sterile 70 µm nylon mesh cell strainer with Staining Media [Hank’s Balanced Salt Solution (HBSS), 2% fetal bovine serum (FBS), 10 mM HEPES (pH 7.2)] using a 3 mL syringe. BM single cell suspensions were made by flushing tibias and femurs with SM using a 27G ½ needle. Cells were disaggregated by flushing them in and out a 3 mL syringe 3-4 times. Cell suspensions were filtered through a 70 µm nylon mesh cell strainer. BM, LN and SPN cells were pelleted by centrifugation at 400xg for 5’. Red blood cells were removed from BM, LN and WT SPN cells by resuspending the pellet in 1.5 mL (BM, LN and WT SPN) or 4 mL (enlarged SPN) of Geys lysis solution (Stock A: 0.65 M NH4Cl; 0.025 M KCl; 4.2 mM Na2HPO4•7H2O; 0.9 mM
KH₂PO₄; 0.03 M glucose, Stock B: 0.02 M MgCl₂•6H₂O; 0.01 M MgSO₄•7H₂O; 0.02 M CaCl₂, 
Stock C: 0.27 M NaHCO₃, 1X Gey’s Solution: 20 parts Stock A; 5 parts Stock B, 5 parts Stock 
C; 70 parts sterile H₂O) on ice for 5’. Cells were washed by adding 5-10 volumes of SM and then 
pelleted by centrifugation at 400xg for 5’ at 4°C. Cells were resuspended in SM and stained with 
0.05% Trypan Blue (w/v) with 2 mM NaN₃ in PBS and counted using a hemocytometer.

**Immunomagnetic Bead Selection**

Single cell suspensions were enriched for CD19⁺ B-cells by immunomagnetic bead seperation. 
Cells were labeled by incubating 1-2x10⁷ cells with CD19 immunomagnetic beads (10 µL/1x10⁷ 
cells, Miltenyi Biotec) for 30’ at 4°C, and washed by adding 10 volumes of SM, and pelleted by 
centrifugation at 400xg, 4°C for 5’. The pellet was resuspended in SM (1x10⁸ cells/500 µL), 
filtered through a nylon mesh into 15 mL tubes and processed by magnetic bead separation on an 
AutoMACS (Miltenyi Biotec) instrument using POSSEL-S (positive selection, sensitive mode) 
according to the manufacturer’s instructions. Viable cell yields were determined by trypan blue 
exclusion. To determine the efficiency of the CD19 positive selection, pre- and post-enrichment 
cells from both fractions were stained with CD19-APC or CD19-PE Cy7 and analyzed by flow 
cytometry.

**Cell Culture**

*Primary ex vivo leukemic cells:* Single cell suspensions were prepared from BM and SPN 
isolated from leukemic TM and DM mice and grown in OPTI-MEM media supplemented with 
10% FBS, 5.5 x 10⁻⁵ M β-mercaptoethanol, 2 mM L-glutamine and 1% penicillin/streptomycin.

*5637 Bladder Carcinoma:* 5637 bladder carcinoma cells were grown in α-MEM supplemented 
with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin until the cells were 70% 
confluent. Conditioned media was prepared by removing media from confluent cells and filtering
it through 0.2 µm syringe filter discs or 0.2 µm filter unit (Diamed Lab Supplies). 5637 bladder carcinoma cell lines were obtained from Dr. Mark Minden (Princess Margareth Hospital, Ontario Cancer Institute).

**OCI-AML.5:** OCI-AML-5 (M4) AML cell line expressing WT FLT3. OCI-AML.5 cells were grown in α-MEM (Gibco) supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin and 10% conditioned media from the 5637 bladder carcinoma or 2ng/mL recombinant human IL-3, human FL, and human G-CSF. OCI-AML.5 cell lines were obtained from Dr. Mark Minden (Princess Margareth Hospital, Ontario Cancer Institute).

**MV4-11:** MV4-11 is a biphenotypic B myelomonocytic leukemia cell line expressing FLT3-ITD. MV4-11 cells (ATCC# CRL-9591) were grown in Iscove's Modified Dulbecco's Media supplemented with 10% FBS.

**WEHI-3:** WEHI-3 cells were grown in RPMI 1640 supplemented with 10% FBS and 0.05 mM β-mercaptoethanol. WEHI-3 conditioned media was prepared as follows: 2x10^5-2x10^6 viable cells were resuspended at a density of 1x10^6 cells/mL in RPMI 1640 without serum and grown for 3 days. WEHI-3 culture supernatant was harvested and filtered using a 0.2 µm filter apparatus to use as conditioned media for BaF3 cells.

**BaF3:** BaF3 cells were grown in BaF3 media (RPMI 1640 supplemented with 10% FBS 10ng/mL recombinant mouse IL-3 or 10% conditioned media from WEHI-3 cells).

**BaF3/FLT3:** We obtained a MigR1-Flt3 vector from Dr. Stephen L. Nutt (Walter and Eliza Hall Institute of Medical Research) containing full-length murine Flt3 cDNA upstream of an IRES-GFP cassette. To generate BaF3 cells expressing murine WT Flt3, Dr. Paul Kowalski transiently transfected 293T cells with MigR1-Flt3 obtained from Dr. Stephen Nutt (Walter Eliza Hall Institute of Medical Research, Division Molecular Immunology)^71, MSCV gag-pol packaging plasmid, and pMD2 VSVG envelope. 48 hours post-infection the supernatant from infected 293T
cells was filtered through a 0.45 µm filter. 1 mL of transfected supernatant was added to 5x10^6 BaF3 cells and spun at 1,000xg for 1 hour. Media was replaced and cells left in the incubator at 37°C to recover for 3 days, after which GFP^+ cells were sorted and grown in BaF3 media.

**EBF6^-/-**: EBF6^-/- cells were obtained from Dr. Kay Medina (Mayo Clinic, Department of Immunology, College of Medicine) and grown in EBF6^-/- media (OPTI-MEM supplemented with 5% FBS, 0.1% β-mercaptoethanol, 2 mM L-glutamine, 1% penicillin/streptomycin, 10 ng/mL recombinant murine IL-7 and 10ng/mL recombinant human FL). EBF6^-/- cells were grown on lethally irradiated OP9 stromal cells.

**OP9**: OP9 stromal cells were maintained in OP9 media (α-MEM supplemented with 15% FBS). When OP9 stromal cell culture reached 70% confluency, the OP9 cells were detached by incubating the cells at 37°C for 4 mL of 0.25% Trypsin-EDTA. Cells were disaggregated by rinsing the flask with 10 mL of OP9 media. Cells were pelleted by centrifugation at 300xg for 5’ at RT, resuspended at 1x10^6 cells/mL in α-MEM, and exposed to 2,000 rads. Irradiated cells were plated at 500,000 to 750,000 cells per T-25 flask containing 5 mL of EBF6^-/- media. Flasks were incubated overnight for cells to attach to the plate. 1:10 dilution of confluent EBF6^-/- cells was added to irradiated OP9 cells.

**Histology**

Sterna were dissected and fixed for 1.5 hours in 3 mL of Fixing Solution: 0.2% Glutaraldehyde, 1.5% Formaldehyde, 5 mM EGTA pH 7.3, 2 mM MgCl₂ and PBS. The sternum was washed 3X in 3 mL Washing Buffer (2mM MgCl₂, 0.01% Deoxycholate, 0.02% Nonidet-P40, and PBS) for 15’. Sternum was stored in PBS at 4°C until samples were taken to the Hospital for Sick Children Pathology Department (Toronto) to be embedded in paraffin and sectioned onto slides.
Wright-Giemsa and Hematoxylin and Eosin staining were performed on sternum sections by the Hospital for Sick Children Pathology Department.

**Immunohistochemistry**

TM leukemic cells were thawed in StemSpan (Stem Cell Technology) supplemented with 10% FBS and 1X penicillin/streptomycin and left to recover for 2 hours at 37°C. 1x10^6 leukemic TM SPN cells were injected into 8 week-old subletally irradiated (650 cGy) immunodeficient CD45.1 congenic B6 Rag2−/− mice. At first signs of hind limb paralysis, recipient mice were sacrificed and the tibias and femurs were dissected and fixed in 10% formalin before being thoroughly rinsed and decalcified in 14% EDTA dissolved in PBS (pH 7.5). Fresh solution was added each day until decalcification was completed. 5 μm thick paraffin sections were cut for FLT3 immunohistochemistry. Sections were deparaffinized by incubating slides in xylene 3X for 5’ each. Sections were then hydrated by washing slides 2X 10’ each in 100% ethanol, 2X 10’ each in 95% ethanol, followed by 2X 5’ each in distilled Milli-Q H₂O. Slides were stored in PBS for at least 5’ prior to antigen unmasking. Slides were placed in boiling Antigen Unmasking Solution (1mM EDTA pH 8.0) for 2’ before turning down the heat to incubate the slides at sub-boiling temperatures for an additional 15’. Slides were left to cool at RT for 45’, washed 3X 5’ each in Mill-Q H₂O, and incubated for 10’ in 1% H₂O₂. Slides were washed 3X 5’ each in Milli-Q H₂O and left in PBS for at least 5’ prior to blocking sections for 1 hour with 200-400 μL Blocking Solution (PBS supplemented with 10% normal goat serum). Blocking solution was removed by rinsing sections with PBS prior to incubation with 200-400 μL Staining Solution [anti-FLT3 rabbit monoclonal antibody (clone 8F2, Cell signalling Technologies) diluted 1:100 in PBS with 2% normal goat serum] overnight at 4°C. Sections were washed 3X 5’ each with PBS prior to incubation with biotinylated goat-anti-rabbit secondary (Vector Laboratories).
diluted 1:100 in PBS with 2% normal goat serum for 1 hour at RT. FLT3 antibody binding was revealed using VectaStain Elite ABC standard kit (Vector Laboratories). ABC reagent was prepared at least 30’ prior to use. Sections were rinsed 3X 5’ each in PBS and incubated with ABC reagent solution for 30’. Sections were rinsed 3X 5’ each in PBS prior to adding 200-400 µL DAB substrate. When sections turned brown the slides were rinsed with PBS before immersing in Milli-Q H₂O and counterstained in 100% Meyer’s hematoxylin (Sigma Aldrich) for 5’. Sections were rinsed 3X 5’ each in tap water and dehydrated by drying slides for 2-3 days at RT. Coverslips were mounted and slides imaged using a Zeiss light microscope, a spot camera and spot advanced software (Spectra Services).

Flow Cytometry

Antibodies

Fluorochrome-conjugated antibodies recognizing various murine cell surface proteins were used to characterize leukemic and control cells by flow cytometry. Antibodies were specific for the following proteins (clones in brackets): CD3ε (145-2C11), CD4 (RM4-5), CD8α (YTS-169.4, 53-6.7), CD11b (M1/70), CD11c (HL3), CD16/32 (2.4G2), CD19 (ID3), CD22 (Cy34.1), CD43 (S7), CD45R (B220, RA3 6B2), CD45.1 (A20), CD45.2 (104), CD115/c-FMS (AFS98), CD117/c-KIT (2B8), CD127/IL-7Rα (A7R34), CD135/FLT3 (A2F10), TCRβ (H57-597), Sca-1 (E13-161.7), Gr-1 (RB6-8C5) and Ly76 (TER-119). All antibodies were purchased from BD Bioscience, eBioscience or Sunnybrook and Women’s Hospital Research Institute Antibody Core Facility. Secondary reagents used to detect biotin-conjugated antibodies were streptavidin-phycoerythrin (PE)-Texas Red or streptavidin-allophycocyanin (APC). All antibodies were titrated prior to use and used at saturating concentrations. Isotype controls for IgG2a, rat IgG2b,
and Armenian hamster IgG were purchased from BD Biosciences and used at 1-2 µg/mL staining solution.

**Staining Protocol**

Single cell suspensions were prepared from BM and SPN and the number of viable cells were determined by trypan blue exclusion. Cells were pelleted by spinning at 400xg for 5’ and 1-2x10⁶ cells were stained on ice for 30’ in 50 µL of 1° Antibody Stain [staining media (SM) with saturating amounts (determined by previous titrations) of biotin or fluorochrome-conjugated antibodies]. Cells were washed by adding 5-10 volumes SM and underlayed with 2-5 volumes of FBS followed by centrifugation at 400xg for 5’ at 4°C. Fluorescence minus one (FMO) controls were also performed for some antibody conjugates (FLT3, c-KIT, and c-FMS). In primary stains that had a biotin-conjugated antibody, a second stain was performed with 50 µL of 2° Antibody Stain (SM with saturating amounts of streptavidin-fluorochrome conjugated antibodies). FMO controls for biotin-conjugated antibodies were performed by omitting the biotin-conjugated antibody in the 1° Antibody Stain. Prior to FACS analysis, cells were washed as mentioned above and resuspended in 500 µL SM containing 1 µg/mL of propidium iodide (PI) or 1 µg/mL Sytox Blue (Invitrogen). Cell suspensions were filtered through 85 µm Nitex mesh (Sefar America) into round bottom tubes. FACS analysis of cell surface markers was performed on a FACSCalibur equipped with a 15 mW blue 488 nm laser and 20 mW red diode 635 nm laser with CellQuest software (Beckton Dickinson) or on a LSRII (Beckton Dickinson) equipped with a 100 mW blue 488 nm laser, a 20 mW red diode 633 nm laser, a 25 mW violet 407 nm laser, and a 20 mW UV 355 nm laser with BD FACSDiva software (BD Bioscience). Data files were uploaded into FlowJo (Tree Star) for analysis. Dead cells were excluded from analysis based on low forward scatter (FSC) and high PI or Sytox Blue staining.
**Compensation Controls:** For most experiments, anti-mouse, anti-rat or anti-hamster Ig, κ and negative control compensation particles (BD Bioscience) were stained with fluorochrome-conjugated antibody needed for compensation. Positive and negative compensation particles were labeled by adding one drop of each (positive and negative) compensation particles set in 100 µL SM with saturating amounts of fluorochrome-labeled antibody followed by incubation at RT for 45’. The compensation particles were then washed with 2 mL SM and pelleted by centrifugation for 5’ at 400xg. The supernatant was aspirated and compensation particles were resuspended in 500 µL SM. Approximately, 20,000 events were collected at 200-300 events/second for compensation in FlowJo. Otherwise, cells were stained in 50 µL SM with saturating amounts of the fluorochrome-conjugated antibody needed for compensation.

**Cell Sorting:** BM cells were then stained and resuspended at a final concentration of 30x10^6 cells/mL in 2 µL PI, filtered through 85µm nylon mesh into sterile polypropylene round bottom tubes and sorted on a MoFlo (Dako Cytomation) or FACS Aria (BD Biosciences) instrument.

**CFSE labeling:** Single cell suspensions (2x10^6 cell/mL) were resuspended in HBSS with 5% FBS (v/v) and incubated with 5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 5’ at RT, and quenched by adding an equal volume of FBS. Cells were pelleted by centrifugation for 5’ at 400xg and washed 2X in HBSS with 5% FBS (v/v) prior to plating at 200,000 cells/well in a 24 well plate. Where indicated, cytokines [murine recombinant IL-7 at 10 ng/mL, FL at 100 ng/mL and/or SCF at 100 ng/mL (Peprotech)] were added prior to incubation at 37°C for 2 or 3 days. Cells were washed with 3 mL HBSS per well prior to immunofluorescence and FACS analysis.
FLT3 Inhibition Assays

$^3$H-Thymidine incorporation Assay: Cells were plated at a density of 100,000 cells/well in 96-well plates. Threefold serial dilution of FLT3 inhibitor AGL 2043 (Calbiochem) was prepared in cell culture media. Treatments were performed in triplicate. Cells were incubated with the inhibitor for 48 hours at 37°C. 1 μCi [methyl-H$^3$] thymidine was added to wells for the last 6 hours of incubation. Cells were harvested using an Inotech cell harvester (Inotech Biosystems International) as per manufacturer’s instructions.

CellTiter-Blue® Viability Assay: The effects of AGL 2043 on survival was assessed by performing a CellTiter-Blue® viability assay (Promega) according to the manufacturer’s protocol. Briefly, 20 μl/well of CellTiter-Blue® reagent was added to each well for the last 3 hours of incubation with the inhibitor. The reaction was stopped and stabilized by adding 100 μL 3% SDS (v/v). Fluorescence (560/590 nm excitation/emission) was recorded within 24 hours. Fluorescence of blank control was subtracted from raw fluorescence for treated and untreated samples before calculating the % Survival (Treated/Untreated).

RNA Isolation & Quantitative Real-Time PCR

RNA extraction: Total RNA was isolated from BM, SPN and LN using TRIZOL® (Invitrogen). Cells were washed once with HBSS, centrifuged at 400xg for 5’ at 4°C, and resuspended in 0.5-1x10$^7$ cells/50 μL HBSS. Cells were lysed with 1 mL TRIZOL® reagent per 0.5-1x10$^7$ cells by flicking tube back and forth and incubated for 5’ at RT. 0.3 mL of chloroform was added per 1 mL TRIZOL® reagent for initial homogenization and centrifuged at 12,000xg for 15’ at 4°C. The aqueous phase was transferred to a fresh tube with an equal volume of pre-mixed 5:1 acid phenol:chloroform. Tubes were vortexed and centrifuged at 16,000xg for 10’ at 4°C. The aqueous phase was transferred to a new tube and an equal volume of pre-mixed 1:1 isoamyl
alcohol: chloroform was added to samples and centrifuged at 16,000xg for 10’ at 4°C. RNA was precipitated with 1 mL isopropanol per 1 mL TRIZOL® reagent initially used, stored overnight at -20°C, and centrifuged at 16,000xg for 30’ at 4°C. The RNA pellet was washed with 1 mL 80% Ethanol and centrifuged at 7,500xg for 10’ at 4°C. The pellet was air-dried for no more than 10’ and dissolved in RNase-Free water (Invitrogen), and purified using QIAGEN RNeasy® Mini Kit (Qiagen) as per manufacturer’s protocol. RNA concentration was measured by spectrophotometric analysis. A$_{260}$/A$_{280}$ ratio of 2.0 was expected for pure RNA. RNA integrity was confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies) prior to experimental use.

**cDNA synthesis:** 100-500 ng of RNA was reversed transcribed in 20 µL cDNA synthesis solution [1X TaqMan RT Buffer, 5.5 mM MgCl$_2$, 500 µM of each dNTP (A, C, G, T), 2.5 µM Oligo d(T)$_{16}$ or Random Hexamer, 0.4 U/µL RNAse inhibitor, and 1.25 U/µL MultiScribe Reverse Transcriptase (Applied Biosystems)] using the following thermal cycling conditions: 25°C for 10’, 42°C for 1 hour, and 95°C for 5’ on a Gene Amp® PCR system 9700 machine. To check if the RNA was contaminated with genomic DNA, 1 µL of reverse transcribed product was amplified with β-Actin primers #67, 5’- TGG GTC AGA AGG ACT CCT ATG - 3’ (forward), and #68, 5’- CAG GCA GCT CAT AGC TCT TCT- 3’ (reverse), at a final concentration of 0.2 µM each with Taq PCR Master Mix solution (Qiagen) using the following thermal cycling conditions: 94°C for 15’; 37 cycles of 30” at 94°C, 55°C for 90”, and 90” at 72°C; and a final extension at 72°C for 10’ on the Engine Tetrad or a Gene Amp® PCR system 9700 machine. PCR products were run on a 2% gel. The expected cDNA PCR product size for β-Actin is 590 bp.

**Quantitative Real-Time PCR:** Quantitative real-time PCR (qRT-PCR) was carried out using SYBR® Green PCR Master Mix (Applied Biosystems) with an ABI Prism® 7900HT Sequence
Detector (Applied Biosystems). Flt3 primers used were #1051, 5’- CAT CCT GAG CTC CAG CT- 3’ (sense), and #1052, 5’- GCC GTA GGA CCA GAC GTC AC- 3’ (antisense), at a final concentration of 100 nM. β-Actin control primers used were #453, 5’- AGA GGG AAA TCG TGC GTG AC -3’ (sense), and #454, 5’- CAA TAG TGA CCT GGC CGT -3’ (antisense), at a final concentration of 100 nM. Reactions were performed in triplicate. 25 µL reaction volumes were prepared in optical 96-well plates as follows: 12.5 µL SYBR® Green 2X mix, 0.35 µL of 10 µM stock sense and antisense primer, 7 µL H2O and 5 µL cDNA, corresponding to 1-2 ng RNA. Cycling conditions were: 50°C for 2’, 95°C for 10’ followed by 40 cycles at 95°C for 15” and 59°C for 1’. Standard curves were generated from 10^1 to 10^6 copies of Flt3 PCR fragments (see below) and β-Actin plasmid #2670 (Lab UID, Table 2) measured in the same plate as unknowns for absolute quantification of transcripts. Data was collected and analyzed using SDS 2.1 program (Applied Biosystems). The number of templates in each sample was extrapolated from standard curves. Normalized expression value of Flt3 transcript was calculated by dividing the number of Flt3 templates by the number of β-Actin templates.

Table 2: Plasmids used to generate probes and standard curves

<table>
<thead>
<tr>
<th>Gene</th>
<th>Plasmid Name</th>
<th>Vector Name</th>
<th>Plasmid #</th>
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</thead>
<tbody>
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<td>Flt3 ATCC 10959073</td>
<td>pCR-Blunt II_TOPO</td>
<td>4380</td>
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<tr>
<td>β-Actin</td>
<td>Murine β actin IMAGE clone</td>
<td>pCR4-TOPO</td>
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<td>5200</td>
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<tr>
<td>Kit</td>
<td>BC075716 - IRAV141F2</td>
<td>pCMV.SPORT6</td>
<td>5210</td>
</tr>
</tbody>
</table>

Legend: Plasmid number refers to lab UID. Vector name corresponds to the vector in which the cDNA product was cloned. Plasmids were used to create standard curves for qRT-PCR and to generate Flt3 and β-Actin northern blot probes.

Generation of PCR fragments: Flt3 PCR fragments were generated by setting up 6-8 PCR reactions using forward primer #1051 (5’- CAT CCT GAG CTC CAG CT – 3’) and reverse primer #1052 (5’- GCC GTA GGA CCA GAC GTC AC- 3’) on WT BM cDNA. 5-10 µL of the
reaction was run on a 2% gel and the 121 bp product was extracted and purified using the QIAquick® PCR Purification Kit (Qiagen). The DNA concentration was measured by spectrophotometric analysis and used to calculate the copy number. $10^9$ to $10^7$ copies serial dilutions were prepared and stored at -20°C.

**Western Blotting**

Cells were washed in 1 mM Na$_3$VO$_4$ in HBSS and lysed with 10 mL NP-40 lysis buffer (10 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 150 mM NaCl, 1% NP-40) supplemented with 1X Halt Phosphatase inhibitor cocktail (Thermo Scientific) and 1 tablet of Complete Mini Protease Cocktail Tablets (Roche Diagnostics) to prepare post-nuclear extract. Bio-Rad DC Protein Assay was performed to quantify protein (see below). 5 µg of protein was loaded onto an 8% non-reducing sodium dodecylsulfate (SDS)-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were incubated with rabbit anti-FLT3 (8F2) antibody in blocking buffer [5% BSA (w/v)] overnight at 4°C. Prior to incubation with goat anti-rabbit HRP-conjugated secondary antibody, membranes were washed 3X 15’ each at RT in blocking buffer. Membranes were also incubated with an anti-β-ACTIN antibody (AC-40, Sigma) for 1 hour at RT as a loading control. Immunoblots were developed by enhanced chemiluminescence as per manufacturer’s instructions.

**Bio-Rad DC Protein Assay:** 25µL solution A+S [20 µL of Solution S (Bio-Rad) mixed with 1 mL of Solution A (Bio-Rad)] was added to 5 µL post-nuclear extract or bovine serum albumin (BSA) standards (0 mg/mL to 2 mg/mL). 200 µL of Solution B (Bio-Rad) was added to each sample and incubated at RT for 15’. Absorbance was measured at 750 nm using a spectrophotometer within the hour and absorbance of the BSA standards versus concentrations
were plot and used to determine the extinction coefficient to calculate the concentrations of the unknown samples.

**N-Glycosylase F Treatment:** 2 U N-Glycosylase F (Roche Diagnostics) was added to 20 µg of protein in NP-40 lysis buffer supplemented with 0.5% SDS and 1% β-mercaptoethanol prior to overnight incubation at 37°C.

### Immunoprecipitation

Post-nuclear extracts were prepared using NP-40 Lysis Buffer. μMACS Miltenyi colloidal super-paramagnetic MicroBeads conjugated to protein A was used for FLT3 immunoprecipitation according to manufacturer’s instructions. Briefly, 50 µL of μMACS Protein A MicroBeads (Miltenyi Biotec) was added to 500 µg of protein in 1 mL NP-40 Lysis Buffer for 30’ on ice and passed through µ columns (Miltenyi Biotec) placed in the μMACS Separator (Miltenyi Biotec). The flow-through was collected in an eppendorf tube as Pre-Cleared Lysate. 10 µg of polyclonal rabbit anti-FLT3 cytoplasmic domain (Upstate, Cat# 06-647) and 100 µL μMACS Protein A MicroBeads was added to Pre-Cleared Lysate for 1 hour on ice and passed through a µ column placed in the μMACS Separator. The flow-through was collected and stored at -80°C as a control and the µ column was washed 4X with 250 µL of NP-40 Lysis Buffer and once with 100 µL Low Salt Buffer (20 mM Tris HCL pH 7.5). 20 µL of pre-heated (95°C for 5’) SDS Elution Buffer (50 mM Tris HCL pH 6.8, 50 mM DTT, 1% SDS, 0.005% bromophenol blue, 10% glycerol) was added to µ column for 5’ to prepare for sample elution. Samples were eluted with 50 µL pre-heated (95°C, 5’) SDS Elution buffer. Subsequently, samples were run on a 10% non-reducing SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Bio-Rad). Membranes were immunoblotted with anti-FLT3 (8F2) antibody overnight at 4°C followed by incubation with goat anti-rabbit HRP-conjugated secondary antibody for 1 hour at RT in
blocking buffer. For FLT3 phospho-tyrosine detection, membranes were incubated with monoclonal mouse pTyr-100 antibody (Cell signalling Technologies) overnight in blocking buffer at 4°C followed by incubation with goat anti-mouse IgG HRP-conjugated secondary antibody for 1 hour at RT (Santa Cruz). Immunoblots were developed by enhanced chemiluminescence as per manufacturer’s instructions. Membranes were stripped with Restore Plus Western Blot Stripping Buffer (Thermo Scientific) according to manufacturer’s protocol prior to incubating membranes with anti-FLT3 (8F2) antibody for FLT3 phospho-tyrosine detection.

**Northern Blotting**

10 µg total RNA was denatured at 65°C for 10’, electrophoresed on a 1% formaldehyde agarose gel in MOPS Liquid Concentrate (200 mM MOPS, 50 mM MOPS Sodium Acetate, 10 mM EDTA, 0.1% DEPC, pH 7.0, EMD Chemicals) at 50-55 volts for 16-18 hours, and transferred overnight onto a Zeta-Probe® GT Genomic Tested Blotting Membrane (Bio-Rad Laboratories) by upward capillary transfer in 20X SSC (Invitrogen Inc). RNA was cross-linked to the membrane by 1200 µJoules/cm² of ultraviolet irradiation in a Stratalinker (Stratagene). Membranes were incubated in Pre-Hybridization Solution [50% formamide, 6X SSPE (Invitrogen), 2X Denhardt’s Reagent, 0.5% SDS and 100 ng/mL of salmon sperm DNA] for 2 hours at 42°C. 25-100 ng of Flt3 DNA probe (see below) was labeled with ≈ 50µCi dCT³²P and Random Primers DNA labeling system (Invitrogen) according to manufacturer’s protocol. Hybridization was performed at 42°C for 18 hours. Membranes were washed 2X 10’ each in Wash Solution 1 (1X SSC, 0.1% SDS) at RT and 2X 5’ each in Wash Solution 2 (0.2X SSC, 0.1% SDS) at 65°C. Membranes were striped by washing 2X 20’ each in Stripping Buffer (0.1X...
SSC, 0.5% SDS) at 95°C. Membranes were exposed to film for 24 hours to check stripping efficiency before re-hybridizing with a second Flt3 or β-Actin probe.

**Flt3 and Actin Probes:** 7 µg of plasmid containing full-length murine Flt3 cDNA (#4380, Table 1) was digested with 25 U BglII and 60 U BamH1 in NEBuffer 3 (New England Biolabs) supplemented with 100 µg/mL BSA overnight at 37°C to make the Flt3 EC probe (569 bp). 7 µg of Flt3 plasmid (#4380, Table 2) was digested with 25 U BglII and 25 U HincII in NE Buffer 3 supplemented with 100 µg/mL BSA overnight at 37°C to make the Flt3 IC probe (709 bp). 5ug of plasmid containing murine β-Actin cDNA (#4765, Table 2) was digested with 30 U XbaI and 30 U EcoRV in NEBuffer 2 overnight at 37°C to make Actin probe (648 bp). Reactions were terminated by incubating samples at 65°C for 20’. cDNA probes were seperated in a 2% gel and extracted using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer’s protocol.

**Mapping Flt3 Truncation**

100 ng total RNA was reverse transcribed using SuperScript® VILO cDNA Synthesis Kit (Invitrogen) according to manufacturer’s protocol. Relative qRT-PCR was performed using oligonucleotide primers specific to exon pairs spanning Flt3 (Table 3) or β-Actin (endogenous control) at a final concentration of 100 nM. 25 µL reactions were prepared in an optical 96-well plate as follows: 12.5 µL SYBR® Green 2X mix, 0.25 µL 10 µM stock sense and antisense primers, 7 µL H2O and 5 µL cDNA corresponding to 1-2 ng total RNA. Reactions were performed in triplicate. PCR cycling conditions were: 50°C for 2’, 95°C for 10’ followed by 40 cycles at 95°C for 15” and 59°C for 1”. Data were collected on an ABI Prism® 7900HT Sequence Detector and analyzed using SDS 2.1 program. Relative threshold cycle values of Flt3 transcripts was measured and normalized to β-Actin taking into account the primer pair
efficiency (E) according to the Pfaffl mathematical model\textsuperscript{207}. The Relative Expression Ratio was calculated as shown below, where the control used was EBF6\textsuperscript{-/-} cDNA:

\[
\text{Relative Expression Ratio} = \frac{(E_{\text{Flt3 Exon}})^{\Delta Ct \text{ Flt3 Exon (Control-Sample)}}}{(E_{\text{Actin}})^{\Delta Ct \text{ Actin (Control-Sample)}}}
\]

The efficiency of the primer pairs was determined by measuring the slope \((R^2 \geq 0.98)\) of the standard curve generated from a serial dilution of \(10^1 - 10^7\) copies of Flt3 (#4380, Table 3) or \(\beta\)-actin plasmid (#4765, Table 2) on an ABI Prism\textsuperscript{®} 7900HT Sequence Detector and calculated as follows:

\[
E = 10^{(-1/\text{Slope})} - 1
\]

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Sense Primer Sequence</th>
<th>Antisense Primer Sequence</th>
<th>Standard Curve Slope</th>
<th>(R^2)</th>
<th>Efficiency (E)</th>
<th>% Efficiency (%E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flt3</td>
<td>TTCCCTGCTTCGGTCTTTTA</td>
<td>CTCGTTCTGCTGACGTTCA</td>
<td>-4.06</td>
<td>0.99</td>
<td>0.76</td>
<td>76</td>
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<tr>
<td>Exon 4-5</td>
<td>AAGCGCCCACTACACAGTA</td>
<td>TCCCTGGTTTCTCCTCCCT</td>
<td>-3.49</td>
<td>0.99</td>
<td>0.93</td>
<td>93</td>
</tr>
<tr>
<td>Exon 6-7</td>
<td>GCTGGGCTAGAAGATGGCATG</td>
<td>TCCGCCCTTTGCCAGATGA</td>
<td>-3.64</td>
<td>0.99</td>
<td>0.88</td>
<td>88</td>
</tr>
<tr>
<td>Exon 7-8</td>
<td>CGGAAGAGGACGACCAAAG</td>
<td>GCCAAGGAATCGGAAATCAT</td>
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<td>Exon 9-10</td>
<td>GTAAAGAGAGAGAGCTGAG</td>
<td>GAACTGGGCTCATCTTTTTT</td>
<td>-3.68</td>
<td>0.99</td>
<td>0.87</td>
<td>87</td>
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<tr>
<td>Exon 11-12</td>
<td>GCTGCTGGCTGACGACGAGCTG</td>
<td>GCCACCCCTGGGCTCATC</td>
<td>-3.54</td>
<td>0.99</td>
<td>0.92</td>
<td>92</td>
</tr>
<tr>
<td>Exon 13-14</td>
<td>GCCCTGGGCTGCATGCTATG</td>
<td>GCCGGTCATTGGGACGAC</td>
<td>-3.50</td>
<td>0.99</td>
<td>0.93</td>
<td>93</td>
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<tr>
<td>Exon 16-17</td>
<td>GCTGCTGCTGACGACGAGCTG</td>
<td>TGGAGGGTCTGGGACGCCG</td>
<td>-3.80</td>
<td>0.99</td>
<td>0.83</td>
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<tr>
<td>Exon 23-24</td>
<td>ATCCCTCCCCCAACCTCGAGTC</td>
<td>GATGGATGTCTGGGACGCCG</td>
<td>-3.81</td>
<td>0.98</td>
<td>0.83</td>
<td>83</td>
</tr>
<tr>
<td>(\beta)-Actin</td>
<td>AGAGGGAAATCGTCGCTGAC</td>
<td>CAATAGTGGCCTGGGCCG</td>
<td>-3.65</td>
<td>0.99</td>
<td>0.88</td>
<td>88</td>
</tr>
</tbody>
</table>

Legend: The standard curve slope and \(R^2\) values were measured by running a serial dilution of \(10^1 - 10^7\) copies of Flt3 plasmid (#4380, lab UID) or \(\beta\)-Actin cDNA (#4765, lab UID) using SYBR\textsuperscript{®} Green PCR Master Mix on an ABI Prism\textsuperscript{®} 7900HT Sequence Detector. Efficiency for primer pair binding to target was calculated according to Pfaff’s Mathematical model\textsuperscript{207}.

**5’ Rapid Amplification of Complementary DNA Ends (5’RACE)**

10 µg of total RNA was isolated with TRIZOL\textsuperscript{®} and subjected to polyA\textsuperscript{+} selection using MicroPoly(A) Purist kit (Ambion) according to manufacturer’s protocol. 5’RACE was performed using FirstChoice RLM-RACE kit (Ambion) with the following modifications to the manufacturer’s protocol. 250 ng of polyA\textsuperscript{+} RNA in 7 µL H\textsubscript{2}O was treated with 2 µL Tobacco
Acid Pyrophosphatase (TAP) in TAP Buffer (10 µL final volume) for 1 hour at 37°C. 2 µL of TAP-treated polyA⁺ RNA was ligated to 1 µL 5’RACE Adaptor with 5 U T4 RNA Ligase in RNA Ligase Buffer (10 µL final volume) for 1 hour at 37°C. 2 µL ligated-RNA (or minus TAP control) was reverse transcribed using 4 µL dNTP mix, 2 µL Random Decamers, 1 µL RNase Inhibitor, and 1 µL M-MLV Reverse Transcriptase in RT Buffer (20 µL final volume) in a thermal cycler at 42°C. Products were amplified using a nested PCR approach with 5’RACE Adaptor and Flt3 gene specific primers (Table 4) using Expand Long PCR Template kit (Roche Diagnostics). 2 µL of outer product was used for the nested PCR reaction. PCR cycling conditions for the outer and nested PCR reaction were: 94°C for 3’; 37 cycles at 94°C for 15”, 57°C for 30”, 68°C for 5’; and a final extension at 68°C for 7’.

**PCR-based Detection of Chimeric Transcripts**

500 ng of total RNA was reverse transcribed into cDNA. 2 µL of cDNA was amplified using a primers listed in Table 4 at 0.2 µM using Taq PCR Master Mix (Qiagen) solution containing Taq DNA Polymerase, PCR Buffer, and 200 µM each dNTP, and 1.5 mM MgCl₂, and the following cycling conditions: 94°C for 15’; 40 cycles of 30” at 94°C, 90” at 50°C and 2’ at 72°C; and a final extension at 72°C for 10’. Products were seperated on a 2% gel, purified using QIAquick® Gel Extraction Kit, and cloned into pDrive Vector using QIAGEN® PCR Cloning Plus Kit according to manufacturer’s protocol. Clones were sent for sequencing at The Centre for Applied Genomics (Hospital for Sick Children, Toronto) using T7 (5’- GTA ATA CGA CTC ACT ATA G - 3’) and SP6 (5’- CAT TTA GGT GAC ACT ATA G - 3’) promoter primers. All sequence alignments were done using MacOSX MegAlign 5.51 (DNASTAR).
**Table 4: Oligonucleotide primers**

<table>
<thead>
<tr>
<th>Primer UID</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5’RACE</strong></td>
<td></td>
</tr>
<tr>
<td>5’RACE Adaptor</td>
<td>-</td>
</tr>
<tr>
<td>Outer Adaptor Primer-FWD</td>
<td>-</td>
</tr>
<tr>
<td>Outer Flt3 Exon 14-REV</td>
<td>3011</td>
</tr>
<tr>
<td>Control Flt3 Exon 10-FWD</td>
<td>3013</td>
</tr>
<tr>
<td>Inner Adaptor Primer-FWD</td>
<td>-</td>
</tr>
<tr>
<td>Inner Flt3 Exon 12/13-REV</td>
<td>3012</td>
</tr>
<tr>
<td><strong>PCR-based detection of chimeric transcripts</strong></td>
<td></td>
</tr>
<tr>
<td>ERV U5-FWD</td>
<td>3014</td>
</tr>
<tr>
<td>Outer Flt3 Exon 12-REV</td>
<td>3015</td>
</tr>
<tr>
<td>Inner Flt3 Exon 11-REV</td>
<td>3016</td>
</tr>
<tr>
<td><strong>PCR-based detection of genomic rearrangements</strong></td>
<td></td>
</tr>
<tr>
<td>ERV MuLV-FWD</td>
<td>3017</td>
</tr>
<tr>
<td>Outer Flt3 intron10-REV</td>
<td>3018</td>
</tr>
<tr>
<td>Inner Flt3 exon10-REV</td>
<td>3019</td>
</tr>
<tr>
<td><strong>LM-PCR</strong></td>
<td></td>
</tr>
<tr>
<td>GWA+ (Adaptor)</td>
<td>3020</td>
</tr>
<tr>
<td>GWAB- (Adaptor)</td>
<td>3021</td>
</tr>
<tr>
<td>Outer LM Adaptor Primer-FWD</td>
<td>3022</td>
</tr>
<tr>
<td>Outer LM Flt3 Specific-REV</td>
<td>3023</td>
</tr>
<tr>
<td>Inner LM Adaptor Primer-FWD</td>
<td>3024</td>
</tr>
<tr>
<td>Inner LM MuLV Specific-REV</td>
<td>3025</td>
</tr>
<tr>
<td><strong>Sequencing Primers</strong></td>
<td></td>
</tr>
<tr>
<td>T7 Promoter Primer-FWD</td>
<td>3026</td>
</tr>
<tr>
<td>SP6 Promoter Primer-REV</td>
<td>3027</td>
</tr>
<tr>
<td>M13 (-40)-FWD</td>
<td>3028</td>
</tr>
<tr>
<td>M13-REV</td>
<td>3029</td>
</tr>
</tbody>
</table>

**Legend:** Sense (FWD) and antisense (REV) oligonucleotide primer numbers refer to lab UID. Endogenous retrovirus (ERV) specific primers were designed based on 5’RACE sequencing (U5) and NCBI BLAST (U3, GeneBank accession #U43202.1) based on genomic DNA sequence obtained from TM B-ALL 4593 results. Ligation-mediated PCR (LM-PCR) primers were designed using genomic DNA sequences from leukemic cells. LM-PCR adaptors and adaptor primers sequences were obtained from Genome Walker Universal Kit (Clontech) manual. GWAB- adaptor is 5’phosphorylated (5Phos) and 3’ amino modified (3AmMO) to block extension of the 3’ end of the adaptor-ligated genomic fragments. pDrive vector (Qiagen primers were obtained from QIAGEN® PCR Cloning Plus Kit manual.

**PCR-based Detection of Genomic Rearrangements**

Genomic DNA was amplified using the Expand Long Template PCR System (Roche Diagnostics) in Expand Long Template Buffer#2 with the sense primer *ERV MuLV*-FWD (5’-
AGA CTG AGT CGC CCG GGT ACC-3’) and antisense primer Outer Flt3 intron 10-REV (5’-TGT GGC CTT TGT GGG CAC TGC A -3’). The PCR cycling conditions were: 93°C for 3’; 37 cycles of 93°C for 15”, 65°C for 30”, and 68°C for 5’; and a final extension at 68°C for 10’. 2 µL of the outer PCR reaction was used for the inner PCR reaction using Expand Long Template PCR System (Roche Diagnostics) in Buffer#2, the sense primer ERV MuLV-FWD (5’-AGA CTG AGT CGC CCG GGT ACC-3’), antisense primer Inner Flt3 exon10-REV (5’- AAC TGG GCG TCA TCA TTT TCT GCA -3’) and the same PCR cycling conditions as above.

**Ligation-mediated PCR**

250-700 ng of genomic DNA was digested with 15 U of DraI, EcoRV, PvuII, StuI, Smal and SspI (New England Biolabs, 40 µL final volume) according to conditions listed in Table 5. After 16-hour digestion, 360 µL dH₂O was added to each tube for a final volume of 400 µL before adding 400 µL of neutral Phenol/Chloroform (v/v). Tubes were vortexed briefly and centrifuged at 12,000 rpm for 5’ at 4°C. Aqueous phase was transferred to a new tube and 1 µL glycogen. 0.1 volume 3M sodium acetate and 2.5 volumes 100% Ethanol was added to precipitate digested DNA overnight at -20°C. Subsequently, tubes were centrifuged at 12,000 rpm for 30’ at 4°C, supernatant was aspirated, and 1 mL of cold 70% Ethanol was added to pellet. Tubes were centrifuged at 12,000 rpm for 10’ at 4°C, supernatant aspirated and pellet left to air-dry 10’ at RT. DNA was resuspended in 9 µL Nuclease-free H₂O (Ambion). 1 µL LM-PCR Adaptors (30 µM stock made from 30 µL of 100 uM GWA⁺ + 30 µL of 100 µM GWAB⁻ + 40 µL dH₂O, see Table 4 for sequences) was ligated to 9 µL purified digested DNA in a 15 µL final volume with 1 U T4 DNA Ligase (Invitrogen) in T4 DNA Ligase Buffer overnight at 16°C. Reactions were stopped by incubating samples at 70°C for 5’. 25 µL of Nuclease-free H₂O was added to each tube. Expand Long Template PCR System (Buffer#2), the sense primer Outer LM Adaptor
Primer-FWD (5’- GTA ATA CGA CTC ACT ATA GGG C -3’), and antisense primer Outer LM Flt3 Specific-REV (5’- AAG CAT GGC GCT TAC AAG AGC AGT TTC -3’) was used to amplify products by touchdown PCR. The PCR cycling conditions for the outer PCR reaction were: 95°C for 3’; 7 cycles at 95°C for 30”, 68°C for 12’; 32 cycles of 95°C for 30”, 66°C for 12’; a final extension at 68°C for 12’; and hold at 16°C. 2 µL of the outer reaction was used for the nested PCR reaction using Expand Long Template PCR System (Buffer#2), the Inner LM Adaptor Primer-FWD (5’- ACT ATA GGG CAC GCG TGG T-3’) and the antisense Inner LM MuLV Specific-REV primer (5’- CTC TGA GGA GAC CCT CCC AAG GAT CAG-3’). The PCR cycling conditions for the inner PCR reaction were: 95°C for 3’; 5 cycles of 95°C for 30”, 68°C for 12’; 20 cycles of 95°C for 30”, 66°C for 12’; a final extension at 68°C for 12’; and hold at 16°C. Products were seperated on a 1% agarose gel, purified using QIAquick® Gel Extraction Kit and cloned using QIAGEN® PCR Cloning Plus Kit. Clones were sent for Sanger sequencing at The Centre for Applied Genomics (Hospital for Sick Children, Toronto).

Table 5: Restriction enzymes used for genomic DNA digestion

<table>
<thead>
<tr>
<th>Tube</th>
<th>Restriction Enzyme</th>
<th>NEBuffer</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL1</td>
<td>Dral</td>
<td>4</td>
<td>37°C, 16 hours</td>
</tr>
<tr>
<td>DL2</td>
<td>EcoRV</td>
<td>3+BSA</td>
<td>37°C, 16 hours</td>
</tr>
<tr>
<td>DL3</td>
<td>PvuII</td>
<td>3+BSA</td>
<td>37°C, 16 hours</td>
</tr>
<tr>
<td>DL4</td>
<td>Stul</td>
<td>4</td>
<td>37°C, 16 hours</td>
</tr>
<tr>
<td>DL5</td>
<td>SmaI</td>
<td>4</td>
<td>25°C, 16 hours</td>
</tr>
<tr>
<td>DL6</td>
<td>SspI</td>
<td>4</td>
<td>37°C, 16 hours</td>
</tr>
</tbody>
</table>

Legend: Genomic DNA was digested in 15 U of restriction enzyme in NEBuffer 4 or NEBuffer 3 (New England Biolabs) supplemented with 100 µg/ml BSA where listed.
Chapter 3

Identification of a Truncated Form of FLT3 in B-Cell Leukemias that Disseminate to the CNS

Radia M. Johnson\textsuperscript{1,2}, Eniko Papp\textsuperscript{3,4}, Ildiko Grandal\textsuperscript{4}, Paul Kowalski\textsuperscript{2}, Jayne S. Danska\textsuperscript{1,3,4}, Cynthia J. Guidos\textsuperscript{1,2}

\textsuperscript{1}Department of Immunology, University of Toronto; \textsuperscript{2}Program in Developmental & Stem Cell Biology, The Hospital for Sick Children Research Institute; \textsuperscript{3}Department of Medical Biophysics, University of Toronto; \textsuperscript{4}Program in Genetics & Genome Biology, The Hospital for Sick Children Research Institute.

Author contributions: R.M.J performed all experiments and analyzed all data with the following exceptions. I.G. performed the qRT-PCR experiments in Fig. 1A and 1C. P.K. performed transfection in Fig. 2. E.P. prepared the samples for histology Fig. 3C.
ABSTRACT

To investigate molecular mechanisms underlying B-cell leukemogenesis, our laboratory has generated and characterized $Rag-2^{-/-}p53^{-/-}Prkdc^{scid/scid}$ TM mice. These mice develop precursor B-ALL with high incidence and short latency. Strikingly in $\approx 75\%$ of TM mice the leukemia disseminated to the CNS, which is a characteristic of many poor prognosis pediatric and adult B-ALL cases. Previous work from our laboratory showed that TM leukemias over-express $Flt3$ in contrast to normal B-cell progenitors. Normally, $Flt3$ is repressed early in B-cell development co-incident with the onset of CD19 expression. Therefore, we sought to investigate how aberrant FLT3 expression in CD19$^+$ precursor B-ALLs contributes to leukemogenesis. In this chapter, we show that TM B-ALLs from mice with leukemia dissemination to the CNS over-express a truncated form of FLT3.
INTRODUCTION

Precursor B-ALL is caused by clonal proliferation, accumulation, and tissue infiltration of neoplastic B-lymphocyte progenitors\textsuperscript{98, 208}. B-ALL is the most common childhood malignancy, with overall cure rates above 80\%\textsuperscript{99, 209}. However, children who relapse have dismal outcomes, and fewer than 50\% of adult B-ALL patients are cured\textsuperscript{99, 209, 210}. Lethal B-ALL relapses are often caused by dissemination of leukemic blasts to the CNS where they are protected from systemically administered chemotherapeutic agents\textsuperscript{211-213}. To decrease the incidence of CNS relapse, up to 40\% of pediatric B-ALL patients receive intrathecal methotrexate or cranial irradiation, but these treatments frequently cause significant CNS pathologies, such as cognitive delay, endocrine failure, and treatment related brain tumors\textsuperscript{214-216}. Therefore, there is an urgent need to define molecular mechanisms that drive B-cell leukemogenesis in order to design novel therapeutics to improve B-ALL treatment and reduce dissemination to the CNS.

Chromosomal translocations and copy number alterations in genes encoding key regulators of hematopoiesis and B-cell development play important roles in the pathogenesis of B-ALL. In some cases, these genomic aberrations arise by aberrant targeting or repair of DNA breaks generated by the \textit{RAG} endonuclease that initiates \textit{V(D)J} recombination during lymphocyte development\textsuperscript{217-219}. Among the most common mutations in pediatric B-ALL are copy number alterations that decrease expression or function of \textit{PAX5}\textsuperscript{220}. This transcription factor induces B-cell commitment by repressing hematopoietic progenitor and alternative lineage programs while concomitantly inducing B-cell specific genes such as \textit{Cd19}\textsuperscript{65}. A key target of PAX5 repression in early B-cell progenitors is \textit{Flt3}, a class III receptor tyrosine kinase that promotes survival and proliferation of multipotent hematopoietic progenitors upon binding of FL. Thus, upon B-cell commitment and expression of CD19, B-cell progenitors become insensitive to the growth promoting effects of FL that is ubiquitously expressed\textsuperscript{89}. 
Two types of mutation have been shown to promote FL-independent activation of FLT3 in human leukemias. Most commonly, internal tandem duplication of the JM region (FLT3-ITD) allows FL-independent dimerization and activation. Less frequently, point mutations in the TKD promote FL-independent FLT3 activation of the FLT3 receptor. Such FLT3 mutations are found in up to 30% of acute myeloid leukemia cases, but are rare in B-ALL. However, expression of either WT or mutant FLT3 is very frequent in CD19+ lymphoblasts from B-ALL patient samples harboring rearrangements in the MLL epigenetic regulator. This genetic subtype is the most common form of B-ALL arising in infants, and is associated with high CNS relapse rates and poor overall survival. However, FLT3 signalling has not been directly linked with CNS dissemination of B-ALL.

We and others have studied molecular mechanisms of B-cell leukemogenesis by generating mice lacking both p53 and Pkrdc or other non-homologous DNA end-joining (NHEJ) components. These mice rapidly develop lethal B-ALL with high incidence, due to global genomic instability conferred by loss of p53-regulated DNA damage checkpoints combined with disrupted NHEJ-mediated telomere maintenance and double strand DNA break repair. In particular, RAG-induced DNA breaks in the telomeric Igh locus, which arise in B-cell progenitors, undergo complex translocations and amplifications of the telomeric Igh locus due to repeated cycles of telomere bridge-breakage fusion. Interestingly, loss of RAG function does not prevent B-cell leukemogenesis in p53 and Pkrdc DM mice, but profoundly alters the cytogenetic profiles and clinical course of disease. Most notably, TM B-cell leukemias have a unique capacity to disseminate to the CNS and cause CNS pathologies similar to those seen in patients with B-ALL, providing a novel mouse model to study mechanisms that drive this morbid clinical complication.
To identify dysregulated pathways that contribute to B-cell leukemogenesis, our laboratory performed genome-wide expression profiling studies and found the gene that most significantly distinguished TM B-ALLs from normal B-cell progenitors and DM B-ALLs was \textit{Flt3} (R. Gladdy, J. Danska, C. Guidos, \textit{unpublished observation}). Therefore, to understand how aberrant \textit{Flt3} over-expression contributes to B-ALL pathogenesis, we sought to investigate the expression and function of FLT3 in TM leukemias. Here we report that TM B-ALLs from mice with CNS signs over-express a truncated form of FLT3 that is constitutively active and promotes ligand-independent growth of TM leukemic cells. Thus, the data presented in this chapter suggest that over-expression of this mutant form of FLT3 might be an oncogenic driver of precursor-B ALL in TM mice.
RESULTS

TM B-ALLs from mice with CNS signs over-express Flt3 transcript

To confirm that TM leukemic cells ectopically express FLT3, we first evaluated relative abundance of Flt3 transcripts in a cohort of DM and TM B-ALLs using qRT-PCR. We found TM B-ALLs from mice with CNS signs expressed 10 to 1,000 fold higher levels of Flt3 mRNA than TM B-ALLs arising in mice without CNS signs or DM mice (Fig. 6). In contrast, c-Kit and c-Fms transcript abundance was not statistically different between TM B-ALLs from mice with CNS signs and DM B-ALLs (Fig. 6). Interestingly, despite their high levels of Flt3 transcripts, TM B-ALL samples showed variable surface FLT3 expression (Fig. 7A). To investigate this discrepancy, we concurrently examined Flt3 mRNA and surface expression in an independent cohort of TM and DM B-ALLs. Consistent with our initial findings, we detected low levels of Flt3 mRNA in TM and DM B-ALLs from mice lacking CNS signs, in accordance with the low levels of surface FLT3 (Fig. 7B). However, despite high levels of Flt3 transcripts, most CD19+ cells did not stain with an antibody directed against the extracellular region of FLT3, suggesting that these TM B-ALLs express a FLT3 variant lacking most or the entire extracellular domain. Therefore, TM B-ALLs from mice with CNS signs aberrantly over-express Flt3 transcript despite variable levels of surface FLT3 detectable by flow cytometry.
Figure 6. Over-expression of Flt3 but not c-Fms and c-Kit mRNA in leukemic TM cells. qRT-PCR analysis of Flt3, c-Fms and c-Kit expression in BM cells isolated from leukemic TM and DM mice. mRNA levels were normalized to \(\beta\)-Actin to derive Relative Expression values. The difference between the two groups for Flt3 was statistically significant (*** indicates p value < 0.0001) by 1-way ANOVA Kruskal-Wallis test.
Figure 7. Leukemic B-cells from TM mice over-express Flt3 mRNA despite variable surface FLT3 expression. A) Flow cytometric analysis of FLT3 expression on primary ex vivo BM cells from leukemic TM and DM mice. Two-parameter 5% probability plots depict FLT3 versus CD19 expression gated on live single cells (left panel). Quadrant markers were set using FMO controls. Scatter plot depicts the ratio of FLT3/FMO mean fluorescence intensity (MFI) gated on CD19+ cells (right panel). B) Lack of correlation between FLT3 surface expression and transcript levels for 11 samples from leukemic TM mice with CNS signs, 3 DM mice and 1 TM mouse without CNS signs (indicated by *). Controls for Flt3 mRNA expression included cultured pro-B cells, sorted pre-B cells, and the Raw 267 myeloid cell line.
As a model to study FLT3 expression and function, we infected the interleukin-3 (IL-3)-dependent hematopoietic progenitor BaF3 cell line with a MigR1-Flt3 retrovirus construct obtained from Dr. Stephen L. Nutt, Walter and Eliza Hall Institute of Medical Research and sorted the GFP-expressing cells to generate the BaF3/FLT3 cell line. The BaF3/Flt3 transcript contains an internal ribosome entry site (IRES) and GFP open reading frame (Fig. 8A). FLT3 expression in BaF3/FLT3 cells was validated by flow cytometry (Fig. 8B). To test FLT3 function in Ba/F3 cells, I cultured BaF3 and BaF3/FLT3 cells with different doses of FL and measured proliferation. As a control, I also cultured both cell lines with IL-3 and measured proliferation. In contrast to BaF3 cells, BaF3/FLT3 cells proliferated in a dose-dependent manner to FL (Fig. 8C). However, BaF3/FLT3 cells proliferated much more extensively in the presence of IL-3 compared to FL, suggesting that FLT3 activation induces a much weaker proliferative response than IL-3 (Fig. 8D). Therefore, WT FLT3 activation does not confer cell autonomous growth to IL-3-independent BaF3 cells.
Figure 8. Validation of FLT3 expression and function in BaF3/FLT3 cells. A) Schematic of the 6.0 kb BaF3/Flt3 transcript expressed in BaF3/FLT3 cell line. B) Flow cytometric analysis of FLT3 and GFP expression on BaF3 cells. C) Validation of FLT3 function in BaF3/FLT3 cells. Cells were plated in a 96-well plate at 100,000 cells/well with different concentrations of recombinant mouse FL and proliferation was measured 48 hours later by [methyl-\(^{3}H\)] thymidine incorporation. Measurements were made in triplicate. Data shown are representative of 3 independent experiments. D) FL and IL-3-induced proliferation in BaF3 and BaF3/FLT3 cells. Cells were incubated with or without 10 ng/mL murine recombinant IL-3 or 100 ng/mL murine recombinant FL and proliferation was measured 48 hours later by [methyl-\(^{3}H\)] thymidine incorporation. Measurements were made in triplicate and data shown are representative of 3 independent experiments.
To determine whether the discrepancy between surface and transcript levels of FLT3 in TM B-ALLs was due to expression of a mutant form of FLT3, we examined protein expression by western blot with an antibody directed against the intracellular region of FLT3. As expected, WT FLT3 expressed in BaF3 progenitor cells exhibited fully-glycosylated (160 kDa) and immature partially-glycosylated (130 kDa) isoforms\textsuperscript{110, 231} (Fig. 9). In contrast, lower apparent molecular weight (MW) truncated forms of FLT3 (trFLT3) were detected in 19/19 TM B-ALLs from mice with CNS signs but not in 5/5 TM B-ALLs from mice lacking CNS signs (Fig. 9). In some samples, we also detected faint higher MW forms of FLT3 (Fig. 9). Immunoprecipitation with an anti-FLT3 antibody directed against the cytoplasmic domain followed by western blot analysis with anti-FLT3 (8F2) confirmed that higher MW bands for FLT3 in addition to trFLT3 were present in TM B-ALLs with a high frequency of CD19\textsuperscript{+}FLT3\textsuperscript{+} (Fig. 10). Interestingly, these higher MW bands seemed to include bands that were distinct from WT FLT3 (Fig. 10), suggesting that these TM B-ALLs may express higher molecular forms of mutant FLT3. In summary, these data demonstrate that TM B-ALLs from mice with CNS signs aberrantly express a truncated form of FLT3.
Figure 9. TM SPN cells isolated from leukemic TM mice express a truncated form of FLT3. A) Western blot analysis of FLT3 (top panel) and β-ACTIN (bottom panel) expression in TM B-ALLs isolated from mice with (+) or without (-) CNS signs as compared to BaF3 and BaF3/FLT3 cells. Data shown are representative of 19 TM B-ALLs from mice with CNS signs and 5 TM B-ALLs from mice without CNS signs.

Figure 10. TM leukemic cells with detectable surface FLT3 express trFLT3 and several higher MW forms. Post-nuclear extracts from BaF3/FLT3 cells or SPN from leukemic TM mice were immunoprecipitated with an anti-FLT3 antibody directed against the cytoplasmic domain and immunoblotted with anti-FLT3 antibody (8F2, top panel). Two-parameter 5% probability plots depict FLT3 versus CD19 expression gated on live cells (bottom panel). Quadrant markers were set using FMO controls. Data shown are representative of 4 leukemic TM mice with CNS signs.
Since FLT3 requires N-glycosylation for proper trafficking from the endoplasmic reticulum through the Golgi to the cell surface, we sought to determine whether trFLT3 proteins were N-glycosylated by treating extracts with N-glycosylase F (NGF). As expected, NGF treatment collapsed the fully-glycosylated 160 kDa and partially-glycosylated 130 kDa isoforms of FLT3 into a single band of 110 kDa (Fig. 11A). NGF treatment also reduced the apparent MW of trFLT3 in TM B-ALL samples, suggesting that this mutant form of FLT3 was not retained in the endoplasmic reticulum (Fig. 11A). Finally, immunohistochemistry experiments demonstrated that trFLT3 localized to the plasma membrane of TM B-ALL blasts in the BM (Fig. 11B). Collectively, these data suggest TM B-ALLs express an N-glycosylated truncated form of FLT3 that is transported to the plasma membrane.

Figure 11. TM leukemic cells express a truncated membrane-associated form of FLT3. A) NGF sensitivity of trFLT3 protein in BaF3/FLT3 cells or BM from leukemic TM mice. Protein extracts were treated with NGF (+) or vehicle (-) prior to separation by SDS-PAGE and immunoblotting for FLT3 and ß-ACTIN. B) Immunohistochemical staining of FLT3 expression on sections of femur from leukemic TM mice. Slides were counterstained with 100% Meyer’s hematoxylin. The slide was photographed at 40X magnification. Arrows indicate brown anti-FLT3 staining on leukemic blasts (turquoise) and lack of staining on normal lymphocytes (yellow). Data shown are representative of 3 TM B-ALLs from mice with CNS signs.
TM B-ALLs from mice with CNS signs depend on FLT3 activity for proliferation & survival

To establish whether TM B-ALLs from mice with CNS signs depend on FLT3 kinase activity for survival and proliferation, we treated primary ex vivo TM leukemic cells with the ATP-competitive FLT3 inhibitor AGL 2043. However, proliferation of TM leukemic cells was not enhanced by FL addition (Fig. 12A), suggesting that FLT3 activity may be ligand-independent in TM leukemias. In normal cells, FL binding is required to induce auto-phosphorylation of tyrosine residues in the FLT3 kinase domain. However, immunoprecipitation studies revealed that trFLT3 was tyrosine-phosphorylated in unstimulated primary ex vivo TM leukemic cells, suggesting that trFLT3 is constitutively activated in TM B-ALLs (Fig. 12B). Consistent with these findings, we found that AGL 2043 significantly impaired FL-independent proliferation of CD19+ cells isolated from leukemic TM mice with CNS signs (Fig. 13A, left panel). Since, AGL 2043 also impaired proliferation of FLT3-ITD-dependent MV4-11 cells with a similar IC50 (Fig. 13A, right panel), these data suggest that proliferation of TM B-ALLs depends on a ligand-independent constitutively active form of FLT3. Furthermore, AGL 2043 impaired the survival of primary ex vivo TM B-ALLs from mice with CNS signs in a dose-dependent manner (Fig. 13B, left panel). In contrast, the inhibitor had no effect on the survival of DM and TM B-ALLs from mice lacking CNS signs (Fig. 13B, right panel). Therefore, TM B-ALLs that invade the CNS depend on ligand-independent activation of trFLT3 for survival and proliferation.
Figure 12. FLT3 is tyrosine-phosphorylated in TM leukemic cells. A) Effects of FL on the proliferation of TM leukemic cells. Primary ex vivo BM cells were cultured with 100 ng/mL FL for 48 hours. Proliferation was measured in triplicate by [methyl-H³] thymidine incorporation. Results are shown for 2 TM B-ALLs from mice with CNS signs. B) Post-nuclear extracts from unstimulated primary ex vivo SPN from leukemic TM mice were immunoprecipitated with an anti-FLT3 antibody directed against the cytoplasmic domain followed by immunoblot analysis with p-Tyr-100 antibody, then stripped and re-probed with anti-FLT3 (8F2) antibody.

Figure 13. TM leukemic cells depend on FLT3 activity for survival and proliferation. A) Effects of AGL 2043 inhibitor on CD19⁺ TM leukemic (left panel) and FLT3-ITD-dependent MV4-11 (right panel) cell proliferation. CD19⁺ cells were separated by immunomagnetic bead separation from leukemic TM SPN and cultured in the absence of FL. Proliferation was measured in triplicate by [methyl-H³] thymidine incorporation. Graphs depict normalized proliferation (AGL 2043/vehicle) +/- standard error from the mean (SEM, error bars) from triplicate cultures for each sample. Data shown are representative of 3 independent experiments for MV4-11 cells and 9 TM B-ALLs from mice with CNS signs. B) Effects of AGL 2043 inhibitor on survival of B-ALL cells. Primary ex vivo BM and/or SPN from leukemic TM mice cells were cultured with AGL 2043 for 48 hours with 10 ng/mL IL-7, 100 ng/mL FL and 100 ng/mL SCF. Cell viability was assessed using the CellTiter-Blue® assay. Graphs show normalized survival (treated/untreated) relative to vehicle control for 3 TM B-ALLs from mice with CNS signs (left panel) versus 3 DM B-ALLs and 2 TM B-ALLs from mice without CNS signs (right panel).
DISCUSSION

In this chapter, I showed that TM B-ALLs from mice with CNS signs over-expressed Flt3 transcript relative to DM B-ALLs and normal B-cell precursors, but expressed variable levels of surface FLT3. Immunoblot studies revealed that TM B-ALLs expressed a truncated form of FLT3 that absolutely correlates with the ability of leukemic blasts to disseminate to the CNS. Although we could detect higher MW forms of FLT3 in some TM B-ALLs, the vast majority exclusively expressed trFLT3, which was tyrosine phosphorylated in the absence of FL stimulation. Our data further demonstrate that trFLT3 lacks a region of the extracellular domain. Hence, the loss or disruption of the ligand-binding region of FLT3 may be responsible for constitutive activation of trFLT3. However, no mutations were found by sequencing the juxta-membrane and cytoplasmic regions of Flt3 from TM leukemias (L. Nutter, M. Kamani, J. Danska, C. Guidos, unpublished observations). Therefore, I suggest that trFLT3 is encoded by a novel kind of Flt3 mutation that is distinct from the ITD and TKD mutations found in human B-ALL and AML. FLT3 over-expression in human B-ALL induces constitutive activation of the receptor in the absence of FL\textsuperscript{118}. Therefore, it is possible that over-expression of trFLT3 may promote FL-independent homodimerization of the receptor to drive leukemogenesis in TM B-ALLs.

I showed that the FLT3 inhibitor AGL 2043 could inhibit proliferation of TM B-ALLs from mice with CNS signs. In contrast, TM and DM B-ALLs from mice lacking CNS signs did not depend on FLT3 activity for survival. The pattern of B-ALL sensitivity correlated with differential expression of Flt3 but not c-Fms or c-Kit mRNA levels. Therefore, although AGL 2043 could potentially inhibit c-KIT and c-FMS with the same IC\textsubscript{50}\textsuperscript{234}, trFLT3 was the only class III RTK differentially expressed between the B-ALLs from mice with and without CNS signs. Hence, these data suggest that TM B-ALLs from mice with CNS signs depend on a ligand-
independent constitutively active form of FLT3. In accordance with these findings, we found that the proliferation of TM leukemic cells was not enhanced by the addition of FL, suggesting that trFLT3 is insensitive to FL. Collectively, these data suggest that TM B-ALLs express and depend on a ligand-independent active form of FLT3 for survival and proliferation.

Ligand-independent trFLT3 mutants may serve as an oncogenic driver in TM B-ALLs. The multistep model of leukemogenesis proposes that hematopoietic progenitors and/or B-cell precursors undergo clonal transformation through the acquisition of chromosomal rearrangements and/or gene mutations that confer a proliferative and/or survival advantage associated with a block in differentiation\textsuperscript{180}. Genomic instability conferred by the loss of p53-regulated DNA damage checkpoints, disrupted NHEJ-mediated telomere maintenance and DSB repair have been proposed to facilitate leukemogenesis by accelerating the rate at which premalignant clones accumulate oncogenic driver mutations\textsuperscript{139, 140}. Interestingly, activating FLT3 mutations have already been shown to be oncogenic drivers in human AML\textsuperscript{235}. Hence, we propose that the genomic instability caused by the loss of p53 and NHEJ-deficiency in TM mice enable trFLT3 mutants to be selected for as an oncogenic driver in TM mice because trFLT3 can confer a selective proliferation advantage to committed B-cell progenitors.

Ectopic expression of FLT3 in CD19\textsuperscript{+} progenitor cells may also be important in human B-ALL. Genome-wide analysis of genetic alterations in pediatric B-ALL revealed that PAX5 was the gene most frequently targeted by somatic mutation\textsuperscript{220}. These PAX5 mutations generated hypomorphic alleles or reduced PAX5 levels. Thus, these mutations are predicted to alter PAX5 DNA-binding or transcriptional activity\textsuperscript{220}. B-ALL patients with PAX5 translocations produce PAX5 fusion proteins that are thought to act as dominant-negatives by binding PAX5 transcriptional targets without recruiting the necessary co-factors to regulate transcription\textsuperscript{220}. Thus, PAX5 genetic lesions may contribute to B-ALL pathogenesis by promoting misexpression
of lineage-inappropriate genes such as FLT3. However, Pax5 and most PAX5 gene targets were not differentially expressed between TM B-ALLs and normal pro-B cells (R. Gladdy, J. Danska, C. Guidos, unpublished observations), suggesting that PAX5 levels and activity are normal in TM B-ALLs. Therefore, these findings suggest that impaired PAX5 function is not responsible for ectopic expression of trFLT3 in TM B-ALLs.

In conclusion, my findings strongly suggest that constitutive activation of trFLT3 promotes B-cell leukemogenesis in TM mice by conferring ligand-independent growth to CD19+ B-cell progenitors. I speculate that impaired PAX5 function might explain the aberrant co-expression of surface FLT3 and CD19 observed in a subset of TM B-ALLs that express higher MW forms of FLT3, but this remains to be tested. Nonetheless, the majority of TM B-ALLs exclusively expressed trFLT3 and had normal PAX5 levels and function, suggesting that expression of trFLT3 is not subject to PAX5-mediated repression. Altogether, our findings suggest that aberrant over-expression and constitutive activation of trFLT3 contributes to B-cell leukemogenesis in TM mice with CNS signs by conferring ligand-independent growth to B-cell progenitors.
Chapter 4

Genomic Rearrangements Create Novel Ligand-Independent Fli3 Alleles That Drive B-Cell Leukemia with CNS Invasion

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Author contributions: R.M.J performed all experiments and analyzed all data with the following exceptions: E.P. and I.G provided DNA for Fig. 5 and 7. P. K. provided advice on sequencing.
**ABSTRACT**

TM B-ALLs from mice with CNS dissemination of leukemic blasts over-express a constitutively active form of trFLT3 that confers ligand-independent growth to TM leukemic cells. However, the molecular basis of FLT3 truncation and mechanism of ectopic over-expression in committed CD19⁺ leukemic cells was not determined in my previous study. Using a variety of molecular approaches, in this chapter I demonstrate that TM B-ALLs express short Flt3 transcripts in which the 5' exons encoding the extracellular ligand-binding region are replaced by endogenous MuLV LTR elements. These LTR-Flt3 fusion transcripts arise from genomic rearrangements that insert endogenous MuLV retroviral elements into Flt3 intron 9, driving ectopic transcription of exons 10-24. As a result, these genomic rearrangements permit aberrant over-expression of trFlt3 in committed CD19⁺ leukemic cells by removing the Flt3 promoter, thereby circumventing PAX5 repression.
**INTRODUCTION**

Data presented in Chapter 3 showed that TM B-ALLs from mice with CNS signs aberrantly express high levels of Flt3 transcript compared to DM B-ALLs and normal B-cell progenitors. Normally, PAX5 represses Flt3 expression by interfering with transcription factor-binding at the promoter\(^{236}\). De-repression of Flt3 and hence ectopic expression of FLT3 on CD19\(^+\) progenitor cells may be an important step in human precursor-B ALL pathogenesis. Genome-wide analysis of genetic alterations in pediatric B-ALL revealed PAX5 was the gene most frequently targeted by somatic mutation\(^{220}\). These Pax5 mutations generated hypomorphic alleles or reduced PAX5 levels. These mutations are predicted to alter PAX5 DNA-binding or transcriptional regulatory function\(^{220}\). Similarly, precursor-B ALL patients with PAX5 translocations produced PAX5 fusion proteins that act as a dominant-negative by retaining the ability to bind PAX5 transcriptional targets without recruiting the necessary co-factors to regulate transcription\(^{220}\).

Thus, these PAX5 genetic lesions may contribute to pre-B ALL pathogenesis by promoting misexpression of lineage-inappropriate genes such as FLT3. Therefore, ectopic Flt3 expression in TM B-ALLs could have resulted from impaired PAX5 expression or function. However, we could not find evidence for reduced Pax5 levels in TM B-ALLs relative to normal pro-B and pre-B cells in our gene expression profiling studies (Gladdy, R., Danska, J., Guidos, C., unpublished observations). Thus, the molecular mechanism of ectopic Flt3 expression in TM B-ALLs remained to be determined.

Anton Berns and colleagues have demonstrated that insertional mutagenesis with infectious MuLV can promote aberrant Flt3 expression to accelerate lymphoid leukemogenesis in \(p19^{ARF}\^-/\^-\) and \(p53^-\^-\) mice\(^{169}\). Interestingly, this group identified two different common insertion sites (CIS) near or within the Flt3 locus in lymphoid tumors. The first was upstream of Flt3 and
predicted to act as an enhancer to promote aberrant transcription of WT FLT3. The second CIS was located in intron 9 and generated an N-terminally truncated form of FLT3. Thus, transcriptional control elements within infectious MuLV retroviruses can cause ectopic expression of full-length or truncated Flt3 by enhancer-trap or gene-trap mechanisms, respectively.

The leukemogenic capacity of endogenous MuLV proviruses is also well-recognized\textsuperscript{183}. Partial retroviral genomes and non-replication-competent or non-infectious MuLV is found throughout the mouse genome\textsuperscript{237}. The distribution of MuLV proviruses is variable between different mouse strains. Depending on the number of complete MuLV proviruses, common inbred stains can be grouped into low-virus or high-virus strains\textsuperscript{201}. For example, Balb/c mice are a low-virus strain because they only have one full-length copy of the MuLV genome located on chromosome 5\textsuperscript{201}. In contrast, high-virus strains, such as AKR, have multiple copies of the MuLV genome at different chromosomal locations\textsuperscript{201}. Even if most ERVs are defective, endogenous proviruses can contribute to the replication of other retroviral elements or to related exogenous viruses by complementation or recombination\textsuperscript{182, 201}. Indeed, AKR strains have a high incidence of developing spontaneous lymphomas that are associated with the activation of endogenous MuLV proviruses through recombination of endogenous MuLVs\textsuperscript{200}. In low-virus mouse strains, irradiation or DNA damaging chemicals can induce rearrangements of endogenous partial MuLV genomes that result in activation of proto-oncogenes or inactivation of tumor suppressors\textsuperscript{204, 238-240}. Since MuLV LTRs contain transcriptional control elements that are highly active in hematopoietic and lymphoid lineages\textsuperscript{187}, genomic rearrangements induced by exogenous DNA damage that involve MuLV proviral elements and host genes can potentially lead to leukemia.
In this chapter, I describe experiments that allowed me to identify the molecular mechanism responsible for trFLT3 over-expression in leukemic TM mice. I discovered that in TM leukemias, the 5’exons of Flt3 were replaced by endogenous MuLV LTR sequences. Strikingly, I only detected LTR-Flt3 fusion transcripts in TM B-ALLs from mice with CNS disseminated leukemic blasts, suggesting that trFLT3 signalling may be involved in this poorly understood pathologic process. Moreover, I showed that in some cases, chimeric LTR-Flt3 transcripts arise from genomic rearrangements that insert an endogenous MuLV LTR into intron 9, suggesting that ectopic expression of trFlt3 in CD19+ leukemic blasts occurs via a MuLV gene-trap mechanism.
**RESULTS**

**TM B-ALLs express a short Flt3 transcript lacking exons 1-9**

To characterize Flt3 transcripts that encode trFL3 in TM B-ALLs, we performed Northern blot analyses of total RNA using an extracellular (EC) probe corresponding to exons 3-6, and an intracellular (IC) probe corresponding to exons 13-20 of WT Flt3 (Fig. 14A, top panel). The EC and IC probe recognized sequences that encode a part of the ligand-binding region and kinase domain of FLT3, respectively. We also ran total RNA from EBF6−/− cells, a Flt3-expressing multipotent hematopoietic progenitor cell line established from Ebf1−/− mice, as a positive control for full-length (3.5 kb) WT Flt3, which consists of 24 exons. As expected, the 3.5 kb Flt3 transcript expressed by EBF6−/− cells was detected with both probes (Fig. 14A, bottom panels). In contrast, all TM B-ALLs analyzed expressed short 2.4 kb transcripts (denoted as trFlt3) that could only be detected with the IC probe (Fig. 14A, bottom right panel). The absence of N-terminal Flt3 exons from trFlt3 was validated by semi-quantitative RT-PCR studies, since Flt3 could be amplified from TM B-ALL samples with primer pairs spanning exons 10-14 or 11-17 but not with primers spanning exons 1-14, and only sometimes with primers spanning exons 3-6 (Fig. 14B).

To more precisely determine which exons were present in trFlt3 transcripts, we evaluated the relative abundance of specific Flt3 exons using a ratiometric qRT-PCR assay. In all 20 TM B-ALLs evaluated, high levels of trFlt3 mRNA could only be detected with primers pairs spanning exons 3’ of exon 10 (Fig. 14C), suggesting that trFlt3 transcripts lacked exons 1-9 that encode the first three Ig-like domains. This portion of the extracellular region has been shown to be important for ligand binding in Flt3-related class III RTKs. Taken together, these data reveal that trFlt3 transcripts expressed by TM B-ALLs lack exons 1-9 and are thus likely to encode a ligand-independent form of FLT3.
Figure 14. TM leukemic cells express trFlt3 transcripts lacking exons 1-9. A) Northern blot analysis of Flt3 mRNA in TM B-ALLs. Schematic of probes used for Northern blotting is shown (top panel). The EC and IC probes corresponded to Flt3 exons 3-6 and 11-20, respectively. The first lane contained total RNA from EBF6-/- cells. Lanes 1-6: total RNA from BM (lane 1) and SPN (lanes 2-6) from TM B-ALL 2857 (1,2), 2853 (3), 3080 (4), 2366 (5), and 4593 (6), respectively. B) RT-PCR analysis of Flt3 transcripts in TM B-ALLs. Total RNA was isolated from TM B-ALLs and BaF3/FLT3 cells and subjected to RT-PCR with primers flanking exons 1-14 (lane 1), exons 10-14 (lane 2), exons 3-9 (lane 3), and exons 10-17 (lane 4). Results are shown for 5 TM B-ALLs from mice with CNS signs and are representative of 3 independent experiments. C) Ratiometric qRT-PCR analysis of Flt3 in TM B-ALLs. Relative Flt3 abundance in a cohort of TM B-ALLs were measured using a series of primer pairs that span the indicated adjacent Flt3 exons. cDNA from TM B-ALLs cells was amplified using a series of primer pairs that span the indicated adjacent exons. Each measurement was made in triplicate and threshold cycle values for each Flt3 amplicon were normalized to β-Actin threshold values for each sample to obtain relative expression ratios. Results are shown for 20 TM B-ALLs from mice with CNS signs.
**TM B-ALLs over-express a chimeric LTR-Flt3 transcript**

To determine the transcriptional start site for *trFlt3*, we performed 5’RACE on poly(A)-selected mRNA using nested reverse primers anchored in *Flt3* exon 12/13 and 14 (Fig. 15A). Unexpectedly, sequencing of the cloned 5’RACE products revealed that all 4 TM B-ALL samples analyzed expressed chimeric *Flt3* transcripts containing endogenous *MuLV* sequences fused in-frame to *Flt3* exon 10 (Fig. 15B). In all cases, *Flt3* exons 1-9 were replaced by the Redundancy (*R*) and Unique 5’ (*U5*) regions of the *MuLV* Long Terminal Repeat (*LTR*), and the transcriptional start sites were located in the *R* region of the *LTR*. Thus, these 5’ RACE studies revealed that TM B-ALLs express chimeric transcripts containing *MuLV LTR* sequences fused in-frame to *Flt3* exon 10.

Sequence analysis identified 2 distinct types of U5/exon 10 junctions in chimeric *LTR-Flt3* transcripts detected in the 5’ RACE studies. In Type 1 chimeric transcripts, represented by TM B-ALL 2366, the *LTR-Flt3* transcripts contained only the 5’ part of the U5 region fused to *Flt3* exons 10, whereas the Type 2 chimeric transcripts (found in the 3 other samples) contained the full *R/U5 LTR* sequence (Fig. 15B). Interestingly, the Type 2 transcripts also contained a 7 bp ‘ACTATAT’ sequence of unknown origin in between *R/U5* and *Flt3* exon 10. My observation that there were 2 different types of *U5/exon 10* junctions suggested that the *LTR-Flt3* chimeric transcripts might originate from several distinct types of genomic rearrangements.

I also cloned a second 5’ RACE product from TM B-ALL 6530 in which exons 2 and 3 of the *malate dehydrogenase 2* (*Mdh2*) gene were fused in reverse transcription orientation to *Flt3* exon 12 (Fig. 15C). *Mdh2* is located on the positive strand of chromosome 5 at 136.25 Mb in G2, whereas *Flt3* is located on the negative strand at 148.14 Mb in G3. Thus, an inversion or complex genomic rearrangement likely produced this *Mdh2-Flt3* fusion transcript. Alternative end joining repair pathways such as microhomology-mediated end joining, which rely on 2-25
bp microhomologous sequences to repair DSBs, are often associated with chromosomal abnormalities including deletions, translocations and inversions\textsuperscript{243-247}. Interestingly, the TCTGGTC sequence found at the junction of the fusion transcripts was also represented in the genomic sequence for Flt3 and Mdh2 (Fig. 15C), suggesting that it provided a region of microhomology that mediated alternative-pathways of end-joining DSB repair to create the Mdh2-Flt3 fusion gene.

\textit{LTR-Flt3} transcripts lacked Flt3 exons 1-9 and \textit{Mdh2-Flt3} transcripts lacked Flt3 exons 1-11. Thus, both fusion transcripts lacked the normal translation initiation site for Flt3. Therefore, we used \textit{in silico} tools to identify potential initiator methionine residues in Flt3 exons 10-12. The first in-frame ATG with an adequate Kozak sequence was in Flt3 exon 10 and corresponded to methionine residue M431 (NP_034359.2). Two additional in-frame ATG codons in exon 12 (M431 and M504) could also potentially be used to translate chimeric \textit{LTR-Flt3} or \textit{Mdh2-Flt3} transcripts. The sizes of trFLT3 proteins in the TM B-ALLs (\approx 70-75 kDa, Fig. 9, Chapter 3) roughly corresponded to those predicted for these translation products using the ExPASy Compute pI/Mw bioinformatics tool (http://web.expasy.org/compute_pi/). Therefore, methionine residues present in either exon 10 or 12 could be used to initiate translation of trFLT3 from \textit{LTR-Flt3} and \textit{Mdh2-Flt3} fusion transcripts.
Figure 15. TM BM cells from leukemic mice express fusion transcripts composed of an endogenous MalV LTR and Flt3.

A) 5’ RACE product for TM B-ALL 2366. Poly(A)-selected RNA isolated from BM from leukemic TM mice was used to perform 5’RACE with nested forward primers in the adapter sequence and nested reverse primers in Flt3 exon 12/13, respectively (top panel). Lanes 1 to 4 correspond to BaF3/FLT3 (1), unligated adapter control without Tobacco Acid Pyrophosphatase (2), H2O control (3), and TM B-ALL 2366 (4), respectively. 5’ RACE products were cloned and sequenced to reveal the transcription start site of trFlt3 from TM sample 2366 (right panel). The sequence is color-coded as follows: adapter (gray), LTR redundancy (green) and unique 5’ regions (red), and Flt3 exons (purple). B) Schematic of 5’ RACE products detected in TM B-ALLs. The 7 bp ACTATAT insertion is shown in black. Data are shown for 4 TM B-ALLs from mice with CNS signs.

B. 5’ RACE PRODUCTS:

**Found in TM B-ALLs:**

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C. Schematic of Mdh2-Flt3 transcripts detected in TM B-ALL 6530. Mdh2-003 and Flt3 sequence overlap is shown in bold purple and shaded in blue.
To screen a larger cohort of TM B-ALL samples for *MuLV LTR-Flt3* fusion transcripts, we performed RT-PCR using a 5’ primer anchored in the *LTR* coupled with a 3’ primer anchored in *Flt3* exon 12. All TM B-ALL samples analyzed in this screen (18 in total) expressed *LTR-Flt3* fusion transcripts, but 2 different product sizes were detected (Fig. 16A). Sequencing of cloned products revealed that this size heterogeneity was due to 2 different types of *U5-Flt3* exon 10 junctions. The first corresponded to the Type 2 transcripts detected by 5’ RACE and had ‘ACTATAT’ insertions, but in one case only the last 4 ‘ATAT’ nucleotides were present, likely due to deletion (Fig. 16B). We also detected a third type of *LTR-Flt3* exon 10 junction (designated as Type 3) which contained *MuLV* proviral sequence 3’ of the *LTR* up to the endogenous *MuLV* SD (Fig. 16B). Interestingly, there was a single-nucleotide polymorphism in the *MuLV* sequence 5’ of the *LTR* between the 11 TM B-ALLs samples with Type 3 transcripts, suggesting that the proviral MuLV sequences may have come from at least two different sources. Type 1 transcripts similar to those identified using 5’RACE were also detected in 2 TM B-ALL samples by RT-PCR. These samples (TM 4593, 6973, 2366) also contained Type 2 or Type 3 transcripts, suggesting that Type 1 transcripts may result from alternative splicing of Type 2 or Type 3 transcripts. Taken together, these data suggest that *trFlt3* transcripts arise from genomic rearrangements that juxtapose endogenous *MuLV LTR* transcriptional control elements to *Flt3* exons 10-24.
Figure 16. Additional LTR-Flt3 fusion transcripts observed in BM and/or SPN from leukemic TM mice. A) RT-PCR screen for LTR-Flt3 transcripts in BM and/or SPN from leukemic TM mice using primers anchored in R/U5 and Flt3 exon 12. Lanes 1-12: cDNA from BM (lanes 2, 3, 5, 7, 9, 11) and SPN (lanes 1, 4, 6, 8, 10, 12) from TM B-ALL 2366 (1), 4593 (2), 9962 (3, 4), 9970 (5, 6), 6530 (7), 6973 (8), 4413 (9, 10), and 3786 (11, 12). Results shown are representative of 18 TM B-ALLs analyzed in total. B) Schematics depict the Type 2 (middle, 7/18 samples) and Type 3 (bottom, 11/18 samples) RT-PCR products. The sequence is color-coded as follows: U5 (red), provirus sequences (black) and the proviral PBS and SD sequences (highlighted in gray), and Flt3 exon 10 (purple). Nucleotide insertions at the U5/exon 10 junction of Type 2 sequences are shown in bold (blue and black), a single-nucleotide polymorphism was also observed in the U5 region of the Type 2 LTR-Flt3 transcript detected in TM 4593 (underlined red), and the single-nucleotide polymorphism in Type 3 sequences is underlined.
TM B-ALLs display genomic DNA rearrangements involving an endogenous MuLV LTR and the Flt3 locus

To identify genomic rearrangements involving endogenous MuLV retroviral elements and the Flt3 locus as well as the potential source of the ‘ACTATAT’ insertions, I screened genomic DNA from TM B-ALL samples using a 5’ R/U5 primer coupled with nested 3’ primers anchored in Flt3 exon 10 and intron 10. LTR-Flt3 amplicons were not obtained from TM B-ALLs with Type 3 fusion transcripts, suggesting that the endogenous MuLV sequence was inserted too far upstream of Flt3 exon 10 to be detected by this PCR strategy. In contrast, I readily amplified LTR-Flt3 fusion genes from TM B-ALLs with Type 2 transcripts, and sequence analysis of cloned PCR products from 2 different B-ALL samples revealed the presence of MuLV LTR sequences inserted into the same location of Flt3 intron 9, immediately upstream of an ‘ACTATAT’ sequence (Fig. 17A, B). Thus, intron 9 is likely the origin of these nucleotides in Type 2 transcripts. However, both samples exhibited distinct large interstitial deletions both upstream and downstream of the intron 9 insertion site, indicating that they arose from different and likely complex multi-step genomic LTR-Flt3 rearrangements.
A. LTR-Flt3 fusion gene in TM B-ALL 4593 (5/7)

Figure 17. LTR-Flt3 fusions detected in genomic DNA isolated from leukemic TM BM. Schematic of the WT Flt locus on chromosome 5 above the schematic for the LTR-Flt3 fusion genes detected by PCR of genomic DNA is depicted for TM B-ALL 4593 (A) and 6530 (B). Δ indicates sequences deleted from fusion genes. Positions shown refer to location in the mouse genome assembly NCBI37/mm9 chromosome 5. MuLV U5 (red), Flt3 intron 9 (black) and exon 10 (purple) sequence are shown. ACTATAT 7bp insertion (underlined) observed in Type 2 LTR-Flt3 transcripts correspond to intron 9 sequence located at Chr5:148,169,628-148,175,201. The fraction of clones harboring the indicated sequence indicated in brackets for each sample.
The ‘ACTATAT’ sequence is immediately followed by a consensus splice donor (SD) sequence (GTPurineAGT)\textsuperscript{248} in intron 9 (Fig. 17A), suggesting that it may function as a cryptic SD to allow splicing of the R-U5 LTR region into Flt3 exon 10 (Fig. 18). Indeed, Type 2 LTR-Flt3 transcripts contain some or all of the ‘ACTATAT’ sequence from intron 9, revealing that transcription of the fusion genes reads through the end of U5 into intron 9. In contrast, Type 3 transcripts contain the primer binding site (PBS) and SD downstream of U5 in the MuLV provirus. Although I could not identify LTR-Flt3 fusion genes in samples with Type 3 transcripts, I infer that there is strong selection for cells that retain the MuLV proviral SD to allow splicing of the R/U5 LTR region into Flt3 exon 10 (Fig. 18). Collectively, these studies suggest that LTR-Flt3 fusion genes arise from a complex multi-step genomic rearrangement process during leukemogenesis in TM mice, but there is strong selection for progenitors with LTR-Flt3 fusion genes that can generate R/U5-Flt3 exon 10-24 chimeric transcripts by splicing events that utilize either cryptic intron 9 (Type 2) or proviral (Type 3) SD sequences. Alternatively, a cryptic SD within U5 can also be used in both cases. However since the ‘ACTATAT’ was the only intron 9 sequence retained in TM B-ALL 6530 (Fig. 17B), it seems likely that in this case chimeric LTR-Flt3 transcripts would be generated without splicing by reading through transcription from U5 to Flt3 exon 10.
Figure 18. Proposed model for the generation of LTR-Flt3 transcripts in TM leukemic cells. Schematic of Type 1, Type 2 and Type 3 LTR-Flt3 transcripts relative to proposed genomic locus. Type 1 and 2 transcripts use a cryptic SD in Flt3 intron 9 and U5, respectively. Type 3 transcripts are generated by splicing at the MuLV proviral SD to the SA of Flt3 exon 10.
I also detected several other complex rearrangements in which endogenous *MuLV* sequences were inverted and juxtaposed, and which sometimes contained intergenic regions derived from other chromosomes (Fig. 19). Interestingly, I could detect rearrangements involving *R/U5* and other regions of the *MuLV* provirus or rearrangements involving intergenic DNA in TM B-ALL 4593 in which I could also detect *LTR-Flt3* gene fusions. I could also detect recurrent genomic rearrangement involving intergenic DNA fused to *MuLV* provirus in TM B-ALLs 1684, 1666 and 3786. Together, these findings reveal that endogenous retro-elements display considerable genomic instability in hematopoietic progenitors from TM mice, likely due to compromised DNA repair and DNA damage responses. Furthermore, our observations strongly suggest that rearrangements that insert *MuLV LTR* elements into *Flt3* intron 9 create *LTR-Flt3* fusion genes that encode an oncogenic trFLT3 protein in TM B-cell progenitors.
Figure 19. Other complex genomic rearrangements involving endogenous MuLV detected in genomic DNA isolated from leukemic TM BM. A) Schematic of other DNA amplicons detected in TM B-ALLs. Regions in gray correspond to intergenic DNA from several potential chromosomal locations in the mouse genome. The accession for the top BLAST result is shown for the endogenous MuLV sequence detected in each sample (Accession# J01998.1, nt 16-577; FJ544576.1, nt 2,586-3,542). The fraction of clones detected with each sequence is shown in ( ) under the TM B-ALL mouse ID. Results are shown for 4 TM B-ALLs from mice with CNS signs.
To identify genomic sequences upstream of the R/U5 in LTR-Flt3 fusions, we performed LM-PCR on genomic DNA from 3 TM B-ALLs by digesting DNA with restriction enzymes followed by ligation to an adaptor sequence. Subsequently, we performed a nested PCR with 5’ primers anchored in the adaptor and 3’ primers anchored in intron 10 and the endogenous MuLV R/U5 LTR sequence. The products were cloned and sequenced to reveal sequences 5’of R/U5 in the Flt3 locus. As expected, we detected U3 sequence upstream of the MuLV LTR region in Flt3 intron 9, which corresponded to nucleotides 176-720 of the endogenous Murine leukemia virus envelope glycoprotein gene (Accession # AF490352.1, Fig. 20). This sequence was mapped using the NCBI BLAST search tool to several locations in the mouse genome (Table 6), including 5qA3 and 5qB3, which are centromeric to Flt3 (5qG3). Therefore, the MuLV provirus sequence used to create the LTR-Flt3 fusions could have come from several possible locations including chromosome 5. Collectively, these data suggest that complex rearrangements involving chromosome 5, and potentially other chromosomes, place an endogenous MuLV LTR in Flt3 intron 9 to drive over-expression of trFLT3.
Figure 20. U3 region detected upstream LTR-Flt3 rearranged DNA in leukemic TM cells. A) Schematic representation of primers (↔) used for LM-PCR. PCR products were cloned and sequenced to reveal the sequence 5' of R/U5, which corresponded to endogenous MuLV U3 sequence (accession# AF490352.1, nt 176-720). The same sequence was obtained in 3 independent TM B-ALLs (TM 4593, 1666 and 1684).

Table 6. Potential MuLV LTR Locations in B6 mice

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Legend. LM-PCR inner reaction products were cloned and sequenced to reveal several potential locations in the B6 mouse genome. Table shows Top 10 matched locations using UCSC BLAT algorithm (assembly NCBI37/mm9). Results shown were obtained for all 3 TM B-ALLs (TM 4593, TM 1666 and 1684).
DISCUSSION

The data presented in this chapter revealed that TM B-ALLs express several types of LTR-Flt3 fusion transcripts derived from genomic rearrangements involving an endogenous MuLV provirus and the Flt3 locus. These LTR-Flt3 transcripts lacked exons 1-9 of full-length Flt3, which encodes the N-terminal Ig-like domains of the ligand-binding region of FLT3. Therefore, these data suggest that there is a strong selection for genomic rearrangements that drive ectopic expression of Flt3 exons 10-24 in TM B-ALLs that disseminate to the CNS. Loss of the N-terminal Ig-like domains of full-length FLT3 is likely to profoundly alter the protein conformation and promote constitutive FL-independent activation of the receptor.

The mechanism of ligand-induced activation of KIT, a FLT3-related class III RTK, was elucidated by protein crystallography before and after ligand stimulation. Yuzawa et al. showed that SCF binds the first 3 N-terminal Ig-like domains (IG1-IG3) to cause receptor dimerization through lateral interactions between the 4th and 5th Ig-like domains in neighboring KIT proteins. SCF-binding is primarily responsible for inducing conformational changes in the 4th and 5th N-terminal Ig-like domain (IG4-IG5) to allow receptor homodimerization. In addition, most activating oncogenic mutations in c-KIT occur in IG5. These mutations are thought to enhance the homotypic interactions between neighboring IG5 domains. Thus, these findings suggest that receptor activation is highly dependent on lateral interactions between the membrane-proximal regions of IG4 and IG5 in neighboring KIT molecules. Given the structural homology between c-KIT and FLT3, we propose that the loss of exons 1-9 changes the conformation of the receptor and enables lateral IG4-IG4 and IG5-IG5 interactions between adjacent trFLT3 molecules in the absence of FL. Hence, genomic rearrangements that drive transcription of exons 10-24 may be selected for in TM B-ALLs because they remove the ligand-
binding domains of full-length FLT3 thus enabling IG4 and IG5 domain interactions with neighboring trFLT3 molecules and inducing FL-independent activation of the receptor.

Given that loss of the extracellular ligand-binding region of FLT3 is thought to facilitate constitutive activation of the receptor, we propose that trFLT3 is selected for in TM B-ALLs to enable leukemic cells to sustain chronic proliferation. During leukemogenesis, incipient leukemic cells must acquire traits that enable them to evade growth suppressors, resist cell death, enable replicative immortality, block differentiation, and activate tissue invasion/metastasis\(^{139}\)\(^{140}\). The acquisition of these traits is facilitated in TM pro-B cells by the loss of p53-regulated DNA damage checkpoints combined with disrupted NHEJ-mediated telomere maintenance and DSB repair in TM mice\(^{172}\)\(^{173}\). In contrast to DM B-ALLs that are driven by recurrent \(Igh/c\)-Myc translocations\(^{172}\), \(Rag2\)-deficiency no longer permits targeted DSBs to allow accumulation of \(Igh/c\)-Myc translocations in TM pro-B cells\(^{172}\). Instead, misrepair of randomly generated DSBs through activation of alternative-end joining repair pathways, such as microhomology-mediated end joining repair, promotes chromosomal abnormalities including deletions, translocations, inversions, and other genomic rearrangements\(^{243-247}\). Furthermore, loss of telomeric DNA due to defects in NHEJ-mediated telomere maintenance promotes amplification and deletion of chromosomal segments and other genomic rearrangements\(^{172}\)\(^{177}\)\(^{178}\). In mice, the \(Flt3\) locus is found in the telomeric region of chromosome 5. Thus, loss of telomeric DNA and repair with chromosomal segments containing \(MuLV\) proviral elements has the potential to create gene fusions encoding an oncogenic form of \(Flt3\). Together, these findings suggest that defects in genome maintenance and repair allow generation of genetic alterations that can potentially create gene fusions that favor neoplastic transformation.

\(MuLV\ LTR\) promoters and/or enhancers can promote transcription of host genes or further enhance transcription from a native promoter by acting as an enhancer when rearranged near a
transcribed gene\textsuperscript{184, 205}. Moreover, \textit{MuLV LTRs} contain transcriptional control elements that are highly active in hematopoietic and lymphoid lineages\textsuperscript{187}. Therefore, the promoter and enhancers present in the \textit{U3} region of the \textit{MuLV LTR} upstream of \textit{Flt3} exon 10 drive expression of trFLT3. However, \textit{Mdhd2-Flt3} transcripts also encode trFLT3. The \textit{Mdhd2} promoter is active in hematopoietic cells, but it seems likely that the \textit{Mdhd2} promoter is lost in the genomic rearrangement that generates \textit{Mdhd2-Flt3} transcripts because exons 3 and 4 of \textit{Mdhd2} are inverted. Therefore, these findings suggest that an alternative promoter may be used to drive \textit{Mdhd2-Flt3} transcription.

Normally, PAX5 represses \textit{Flt3} transcription at B-cell commitment by interfering with transcription factor binding at the promoter\textsuperscript{236}. However, TM B-ALLs circumvent PAX5-mediated repression by using \textit{MuLV LTR} transcriptional regulatory elements to drive expression of tr\textit{Flt3}, suggesting that genomic rearrangements creating \textit{LTR-Flt3} transcripts may occur prior to B-cell commitment. Interestingly, Moloney \textit{MuLV} insertional mutagenesis screens revealed that \textit{Flt3} was exclusively mutated in \textit{p53}\textsuperscript{-/-} and \textit{p19\textsuperscript{ARF}}\textsuperscript{-/-} lymphomas but not in WT mice\textsuperscript{169}, suggesting that disruption of the \textit{p19\textsuperscript{ARF}}-MDM2-p53 tumor suppressor pathway is required to select for tr\textit{FLT3} mutants. Holmes \textit{et al.} reported ectopic expression of FLT3 in HSCs impairs the generation of CD19\textsuperscript{+} committed pro-B cells\textsuperscript{71}. Congruent with these findings, Li \textit{et al.} showed that B-cell development is impaired in \textit{Flt3\textsuperscript{ITD}} knock-in mice because constitutive FLT3-ITD signalling in uncommitted (B220\textsuperscript{+}CD19\textsuperscript{-}) B-cell progenitors leads to apoptosis\textsuperscript{96}. Hence, loss of the \textit{p19\textsuperscript{ARF}}-MDM2-p53 tumor suppressor pathway may be required to allow CD19\textsuperscript{+} cells to be generated despite constitutive tr\textit{FLT3} activation prior to B-cell commitment.

In summary, we observed strong selection for genomic rearrangements that replace exons 1-9 encoding the ligand-binding region of FLT3 with endogenous \textit{MuLV LTR} elements in TM B-ALL that disseminate to the CNS. As a result, TM leukemic cells expressed high levels of \textit{LTR-}
*Flt3* fusion transcripts encoding a truncated form of FLT3 lacking the FL-binding domain. Given that trFLT3 is constitutively active and promotes the survival and proliferation of TM leukemic cells, *MuLV LTR-Flt3* genomic rearrangements may be selected for in TM B-ALLs to provide the cells with a selective growth advantage to drive leukemogenesis. Collectively, the data presented in this chapter suggest that RAG-independent rearrangements involving endogenous *MuLV* proviral elements and the *Flt3* locus create *LTR-Flt3* fusion transcripts that encode a constitutively active trFLT3 protein to drive B-cell leukemias that disseminate to the CNS.
Chapter 5

Chimeric MuLV LTR-\textit{Flt3} transcripts appear before B-cell commitment and drive lymphoid progenitor transformation in TM mice

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Author contributions: R.J performed all experiments and analyzed all data with the following exceptions. E.P. performed the IV injections for Fig. 5 and 6. E.P. and R.J. jointly performed the sorts and FACS analyses for Figs. 5 and 6.
**ABSTRACT**

Given that Flt3 expression is normally repressed upon B-cell commitment and CD19 expression, we sought to determine whether the genomic rearrangements creating LTR-Flt3 transcripts are selected for prior to B-cell commitment. Interestingly, both CD19⁺ and CD19⁻ cells from leukemic TM mice displayed aberrant growth factor-independent proliferation that was abrogated by FLT3 inhibition *in vitro*, suggesting that both CD19⁺ and CD19⁻ cells depend on trFLT3 for proliferation. Further characterization of the CD19⁻ fraction of leukemic TM BM revealed significant alterations in the abundance of different lymphoid-restricted progenitors, suggesting that trFLT3 signalling prior to B-cell commitment alters the early stages of B-cell development. Transplantation studies revealed that CD19⁻ progenitors and CD19⁺ cells from leukemic TM BM transferred the leukemia to recipient mice, suggesting that leukemia-initiating cells (LIC) are present prior to B-cell commitment and CD19 expression. Importantly, RT-PCR analysis revealed that LTR-Flt3 transcripts were expressed in committed CD19⁺ leukemic blasts and Lin⁻CD19⁻ progenitors isolated from leukemic TM mice, suggesting that the genomic rearrangements that give rise to LTR-Flt3 transcripts occur prior to B-cell commitment and PAX5 expression. Finally, we found that the CD19⁻ fraction of leukemic TM BM contain multiple clones expressing different LTR-Flt3 transcripts. In contrast, the CD19⁺ fraction of TM leukemic cells predominantly expressed one type of LTR-Flt3 transcript, suggesting that ongoing genomic rearrangements and genetic diversification through clonal evolution promotes B-ALL leukemogenesis in TM mice.
**INTRODUCTION**

All blood cells arise from HSCs capable of self-renewal and differentiation into distinct myeloid and lymphoid lineages. The current model of hematopoiesis proposes that the first and decisive lineage commitment step of adult HSCs results in early separation of myelopoiesis and lymphopoiesis\(^{149}\). Commitment to the B-cell fate occurs through successive loss of erythroid, megakaryocytic, and myeloid potential to generate lymphoid-biased progenitors that will eventually give rise to CD19\(^+\) B-cells. More specifically, LMPPs that are distinguished from other MPPs by FLT3\(^{hi}\) and IL-7R\(\alpha^+\) surface expression give rise to CLPs (Lin\(^-\) KIT\(^+\)SCA1\(^lo\)FLT3\(^+\)), which generate uncommitted pre-pro-B (B220\(^-\)CD19\(^-)\) cells that differentiate into committed pro-B (B220\(^+\)CD19\(^+)\) cells. Although CLPs exhibit some residual myeloid potential under certain culture conditions\(^{251,252}\), they predominantly give rise to lymphoid cells\(^{252}\) and are generally accepted as major intermediates between LMPPs and CD19\(^+\) pro-B-cells\(^{253,254}\). In addition to CLPs, LBPs (Lin\(^-\)KIT\(^-\)SCA-1\(^hi\)FLT3\(^+)\) were identified as an alternative lymphoid progenitor\(^{15,16}\). In contrast to CLPs, LBPs do not have latent myeloid potential but generate B-cells less efficiently *in vitro* and *in vivo\(^{15,16}\). Nevertheless, these findings suggest that CD19\(^+\) pro-B-cells are potentially generated from at least 2 distinct lymphoid-biased progenitors.

B-cell specification and development is regulated by extracellular factors such as FL, SCF and IL-7\(^{56-59}\), which in turn regulate the sequential action of different transcriptional regulators including IKAROS, PU.1, E2A, EBF and PAX5. FLT3 signalling within a subset of MPP cells is thought to initiate B-cell specification by promoting IL-7R expression\(^{59}\). FLT3 and IL-7R signalling are necessary for CLP production and subsequent development of pro-B and pre-B cells\(^{90,91}\). IL-7 is also critical in regulating EBF expression to promote B-cell specification at the CLP and pre-pro-B cell stage during B-cell development\(^{255}\). More specifically, Kikuchi *et*
*al.* showed that IL-7 stimulation is necessary before the pre-pro-B cell stage to maintain abundant levels of EBF and B-cell potential\textsuperscript{255}. Although IL-7 cooperates with FL and SCF to induce pro-B cell differentiation *in vivo*,\textsuperscript{256, 257} IL-7 stimulation is sufficient to promote differentiation of CLPs into B220\(^+\)CD19\(^+\) pro-B cells after 4 days of culture\textsuperscript{15}. In contrast, LBPs absolutely require FL and IL-7 stimulation to generate B220\(^+\)CD19\(^+\) B-lineage cells *in vitro*\textsuperscript{15}. Therefore, CLP and LBPs require different cytokine combinations to generate B-cells.

During normal B-cell development, PAX5 is upregulated upon B-cell commitment and induces *Cd19* expression while repressing *Flt3* as well as genes required for myeloid (*c-fms*) and T-cell (*Notch1*) development\textsuperscript{65}. Thus, most pro-B-cells express *Cd19* but not *Flt3*. In addition to B-cell specification, FLT3 and IL-7 signalling are important for proliferation of early B-cell progenitors\textsuperscript{258-260}. *FL\(^+\)* mice have reduced numbers of LMPPs and CLPs in adult BM\textsuperscript{91, 261}. In contrast, *IL-7\(^{-}\)* mice have normal numbers of LMPPs but reduced numbers of CLPs\textsuperscript{261, 262}, suggesting that FL and IL-7 are important for the production of early lymphoid progenitors. IL-7 is required for the proliferation of committed pro-B and pre-B cells *in vitro*\textsuperscript{263}. However, cells expressing the pre-BCR have a significant advantage to proliferate in low levels of IL-7\textsuperscript{263, 264}, suggesting that IL-7 stimulation may be required for the proliferation of pro-B cells prior to pre-BCR expression. *IL-7\(^{-}\)* mice are blocked at the pro-B cell stage similar to *Rag2\(^{-}\)* mice that are unable to form the pre-BCR\textsuperscript{53, 265}, suggesting that IL-7 is required for clonal expansion of pro-B cells prior to pre-BCR expression and transition to the pre-B cell stage. TM pro-B cells lack *Rag2* and, consequently, cannot express a pre-BCR to promote clonal expansion of committed pro-B cells and transition to the pre-B cell stage. Data presented in Chapter 3 and 4 showed that TM B-ALLs express *LTR-Flt3* transcripts encoding a FL-independent active form of FLT3. Taken together, these findings suggest that in the absence of a pre-BCR the generation of MuLV
**LTR-Flt3** transcripts in leukemic TM mice would maintain FLT3 signalling and promote clonal expansion of committed pro-B cells.

Studies I presented in Chapter 4 provide evidence that MuLV transcriptional control elements drive ectopic expression of trFLT3, thus preventing repression of FLT3 expression and signalling by PAX5 at the onset of B-cell commitment. Through this mechanism, ectopic FLT3 signalling in TM pro-B cells harboring *MuLV-Flt3* rearrangements likely substitutes for pre-BCR signalling to promote their survival and proliferation. However, my prior study did not determine whether **LTR-Flt3** genomic rearrangements are selected for prior to or after PAX5 expression and B-cell commitment. Therefore, I sought to determine the cell-of-origin of the **LTR-Flt3** rearrangements in TM B-ALLs.

The studies presented in this chapter revealed that the **LTR-Flt3** rearrangements occur prior to CD19 expression, suggesting that trFLT3 signalling begins prior to B-cell commitment and PAX5 expression. Indeed, CD19⁻ as well as CD19⁺ cells from leukemic mice depended on FLT3 activity for proliferation. We also observed an expansion of LBPcs coupled with a loss of CLPs, suggesting that expression of trFLT3 impairs the early phases of B-lymphopoiesis. Interestingly, LICs were present in both CD19⁺ blast and CD19⁻ cell populations from leukemic TM mice, suggesting that LICs arise prior to B-cell commitment. Collectively, these findings suggest that genomic rearrangements that create **LTR-Flt3** transcripts are selected for prior to B-cell commitment and PAX5 expression to transform lymphoid progenitors and drive B-ALL in TM mice with CNS signs.
RESULTS

CD19- cells depend on FLT3 activity for growth factor-independent growth in TM B-ALLs

In Chapter 3, I showed that ligand-independent FLT3 signalling promotes survival and proliferation of CD19+ cells from leukemic TM mice. To determine whether progenitors prior to B-cell commitment also proliferate in the absence of exogenous growth factors, I labeled primary ex vivo BM cells from normal and leukemic TM or DM mice with CFSE and cultured them with or without SCF, FL and IL-7. On day 2, flow cytometry was used to quantify CFSE levels in CD19+ and CD19- cells in each culture. CFSE signal dilution suggested that, some CD19- cells from DM B-ALLs divided in response to exogenous growth factors, but a subset of CD19- cells extensively divided in the absence of these added growth factors (Fig. 21A). In contrast, CFSE signal dilution suggested that CD19- cells from TM B-ALLs were similar in the presence and absence of growth factors, suggesting that a subset of CD19- cells exhibit growth factor-independent proliferation (Fig. 21A). Hence, a subset of CD19- cells from leukemic TM mice were capable of extensive division in the absence of exogenous growth factors.

Given that the lack of KIT surface expression distinguishes LBPs from other hematolymphoid progenitors including HSCs, MPPs, LMPPs and CLPs, I sought to examine KIT expression on the subset of CD19- cells that extensively divide in TM B-ALLs (Fig. 21B). Interestingly, the subset of CD19- cells from TM B-ALL 8901 that extensively divided were KIT-, suggesting that the subset of CD19- cells that extensively divided did not include KIT+ lympho-myeloid progenitors. Therefore, these data suggest that TM B-ALLs contain a subset of CD19’KIT- cells that exhibit growth factor-independent proliferation.
Figure 21. Growth factor-independent proliferation of CD19 cells isolated from leukemic TM BM. A) Effects of growth factors on CD19 cell proliferation from TM and DM B-ALLs were evaluated by CFSE dilution. CFSE-labelled cells were cultured with or without 10 ng/mL IL-7, 100 ng/mL FL, and 100 ng/mL SCF for 2 days. Day 2 CFSE profiles are shown for CD19+ (green) and CD19- (shaded gray) cells from CB-17 BM (left panel), DM B-ALL 8101 (middle panel) and TM B-ALL 8901 (right panel). The gates show the frequency of CD19- dividing cells. Data shown are representative of 4 TM B-ALLs from mice with CNS signs and 2 DM B-ALLs without CNS signs. B) Histograms show CFSE levels of CD19-KIT- (left panel) and CD19-KIT+ (right panel) relative to CD19+ leukemic cells from TM 8901 cultured in media only or supplemented with IL-7, FL, and SCF. Day 2 CFSE profiles are shown for CD19+ (green) and CD19- (shaded gray). The gates show the frequency of CD19+ dividing cells.
To confirm that CD19<sup>-</sup> cells from TM B-ALLs extensively divide, I also measured proliferation of purified CD19<sup>-</sup> and CD19<sup>+</sup> cells from leukemic TM BM by [methyl-H<sup>3</sup>] thymidine incorporation. Interestingly, the CD19<sup>-</sup> fraction proliferated as much as the CD19<sup>+</sup> fraction in most TM B-ALL samples (Fig. 22A). However, the number of viable cells recovered was low from some TM B-ALLs (Table 7), suggesting that a significant proportion of CD19<sup>-</sup> cells did not survive in culture. Therefore, although the cells were extensively dividing in some samples, the low viability suggests that only a minor subset of CD19<sup>-</sup> cells could proliferate in the absence of exogenous growth factors in vitro. Altogether, these data suggest that both CD19<sup>+</sup> and CD19<sup>-</sup> cells from leukemic TM mice exhibit growth-factor independent proliferation.

Table 7: Fold change in viable cell recovery for CD19<sup>+</sup> and CD19<sup>-</sup> cells from leukemic TM mice cultured 2 days in the absence of exogenous growth factors

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Fold change in viable cell recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD19&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>TM 2436</td>
<td>3.2</td>
</tr>
<tr>
<td>TM 4211</td>
<td>0.3</td>
</tr>
<tr>
<td>TM 4213</td>
<td>N/A</td>
</tr>
<tr>
<td>TM 4551</td>
<td>0.5</td>
</tr>
<tr>
<td>TM 4624</td>
<td>0.4</td>
</tr>
<tr>
<td>TM 4545</td>
<td>4.6</td>
</tr>
<tr>
<td>TM 4766</td>
<td>N/A</td>
</tr>
<tr>
<td>TM 5224</td>
<td>0.2</td>
</tr>
<tr>
<td>TM 5118</td>
<td>0.2</td>
</tr>
<tr>
<td>TM 5242</td>
<td>0.5</td>
</tr>
<tr>
<td>TM 1279</td>
<td>N/A</td>
</tr>
<tr>
<td>TM 671</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Legend: CD19<sup>-</sup> cells from leukemic TM mice were prepared as described for Fig. 22 and cultured in the absence of exogenous growth factors. The fold change of viable cell recoveries were calculated by dividing the number of viable cells (based on trypan blue exclusion) on Day 2 by the number of cells plated on Day 0. N/A means not available because the cells recovered were not counted on Day 2.
Figure 22. Proliferation of CD19\(^+\) cells isolated from leukemic TM BM depend on FLT3 activity in a ligand-independent manner. A) Proliferation of CD19\(^-\) and CD19\(^+\) leukemic cells in the absence of exogenous growth factors. Proliferation was measured by [methyl-H\(^3\)] thymidine incorporation and disintegrations per minute (DPM) are shown. B) Effects of AGL 2043 inhibitor on CD19\(^-\) and CD19\(^+\) cells from leukemic TM BM. CD19\(^-\) and CD19\(^+\) cells were enriched by immunomagnetic bead separation and cultured in the absence of FL with 10 µM AGL 2043. Proliferation was measured in triplicate by [methyl-H\(^3\)] thymidine incorporation. Graphs depict normalized proliferation (AGL 2043/vehicle) +/- SEM (error bars) from triplicate cultures for each sample. Results are shown for 10 TM B-ALLs from mice with CNS signs.
To determine whether CD19\(^{-}\) cells from TM leukemic mice depend on ligand-independent FLT3 activity for proliferation, we cultured CD19\(^{+}\) and CD19\(^{-}\) cells from TM B-ALLs with 10 \(\mu\)M AGL 2043 in the absence of FL or other exogenous growth factors and measured proliferation by [methyl-\(^{3}\)H] thymidine incorporation. Strikingly, AGL 2043 attenuated proliferation of CD19\(^{-}\) cells from leukemic TM BM by >50\% or more in 6/10 TM leukemias evaluated, suggesting that proliferation of some cells in the CD19\(^{-}\) fraction of leukemic BM was FLT3-dependent (Fig. 22B). However, CD19\(^{+}\) cells were generally more AGL 2043-sensitive than CD19\(^{-}\) cells, suggesting that a subset of CD19\(^{-}\) TM leukemic cells were resistant to FLT3 inhibition. Taken together, these data demonstrate that both CD19\(^{+}\) and CD19\(^{-}\) cells from leukemic TM BM exhibited aberrant growth factor-independent proliferation that was driven by ligand-independent FLT3 signalling, suggesting that trFLT3 may be expressed prior to B-cell commitment.

To determine whether trFLT3 is expressed prior to B-commitment and CD19 expression, I initially sorted CD19\(^{-}\) and CD19\(^{+}\) cells. To exclude myeloid cells from the CD19\(^{-}\) subset, I included CD11b as a marker and sorted CD19\(^{-}\)CD11b\(^{-}\). Indeed, I detected several sizes of \(LTR-Flt3\) transcripts in CD19\(^{-}\), CD11b\(^{+}\) and CD19\(^{+}\) cells sorted from leukemic TM BM (Fig. 23). The expected size of the Type 1, Type 2 and Type 3 \(LTR-Flt3\) transcripts are 230, 280 and 400 bp, respectively. Interestingly, I could detect multiple bands corresponding to the various \(LTR-Flt3\) transcripts, suggesting that each population sorted contained multiple clones expressing different types of \(LTR-Flt3\) transcripts (Fig. 23). However, the \(LTR-Flt3\) transcripts detected in myeloid CD11b\(^{+}\) cells may reflect contamination from the CD19\(^{+}\) blast population, because the sort purity of the CD11b\(^{+}\) cells was lower than other subsets (Table 8). Importantly, I could detect these \(LTR-Flt3\) transcripts in CD19\(^{-}\)FLT3\(^{+}\) and CD19\(^{-}\)FLT3\(^{-}\) cells from TM B-ALL 3039 (Fig. 23), suggesting that trFLT3 is expressed prior to CD19 expression.
Figure 23. LTR-Flt3 transcripts are expressed in CD19+ cell populations from leukemic TM mice. CD19+CD11b−, CD19+CD11b+ and CD19− cells were sorted from leukemic TM BM from mouse #4593 and #6973. CD19+Flt3−, CD19+Flt3+, CD11b− and CD19− cells were sorted from leukemic TM BM from mouse #3039. RT-PCR screen for LTR-Flt3 transcripts in TM B-ALLs as described in Fig. 16, Chapter 4. Results shown are representative of the 3 TM B-ALLs analyzed in total.

Table 8: Sort Purity and number of cells recovered from each TM B-ALL subset

<table>
<thead>
<tr>
<th></th>
<th>TM 4593</th>
<th>TM 3039</th>
<th>TM 6973</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD19</strong></td>
<td>100%</td>
<td>100%</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>(5.4x10⁶)</td>
<td>(2.6x10⁶)</td>
<td>(558 099)</td>
</tr>
<tr>
<td><strong>CD19</strong></td>
<td>100%</td>
<td>-</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td>(3 500)</td>
<td>-</td>
<td>(45 109)</td>
</tr>
<tr>
<td><strong>CD19+Flt3</strong></td>
<td>-</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>(10 478)</td>
<td>-</td>
</tr>
<tr>
<td><strong>CD19+Flt3</strong></td>
<td>-</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>(46 149)</td>
<td>-</td>
</tr>
<tr>
<td><strong>CD11b</strong></td>
<td>92%</td>
<td>92%</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>(130 000)</td>
<td>(617 545)</td>
<td>(108 535)</td>
</tr>
</tbody>
</table>

**Legend:** The post-sort purity for each subset is shown. The number of cells recovered post-sort is also shown in ().
CD19<sup>−</sup> cells from TM leukemias can aberrantly differentiate into CD19<sup>+</sup> lymphoblasts

Normally during B-cell development, CD19<sup>−</sup> cells require IL-7 to generate CD19<sup>+</sup> cells in vitro<sup>15</sup>. To examine whether CD19<sup>−</sup> cells from TM B-ALLs could generate CD19<sup>+</sup> leukemic blasts, we assessed the ability of CD19<sup>−</sup> cells isolated from leukemic TM mice to generate CD19<sup>+</sup> leukemic blasts in media only. Interestingly, CD19<sup>−</sup> cells from leukemic TM mice generated CD19<sup>+</sup> cells that resembled CD19<sup>+</sup> leukemic blasts in the absence of exogenous growth factors (Fig. 24). However, we only observed growth factor-independent differentiation of CD19<sup>−</sup> cells into CD19<sup>+</sup> leukemic blasts from leukemias in which the CD19<sup>−</sup> cells exhibited robust cell proliferation (2/8 cases, Table 9). Therefore, these findings suggest that CD19<sup>−</sup> cells from some TM B-ALLs have the capacity to aberrantly differentiate into CD19<sup>+</sup> leukemic blasts in the absence of growth factors required for B-cell development in vitro.

Figure 24. CD19<sup>−</sup> cells from leukemic TM BM can aberrantly differentiate into CD19<sup>+</sup> blasts in vitro. CD19<sup>+</sup> and CD19<sup>−</sup> cells were immunomagnetically separated from leukemic BM (TM 2436, 4551) and SPN (TM 4551) and cultured 48 hours in media. Two-parameter probability plots (5% with outliers) show CD19 versus FSC for both fractions at Day 0 (left) and Day 2 (right) for TM B-ALL 2436 and 4551. Results are shown for 2/8 TM B-ALLs from mice with CNS signs.
Table 9. Summary of leukemic TM mice with CD19⁻ cells that can differentiate into CD19⁺ blasts

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>CD19⁻ CD19⁺ blasts</th>
<th>CD19⁻ Proliferation (DPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM 2049</td>
<td>NO</td>
<td>N/A</td>
</tr>
<tr>
<td>TM 2436</td>
<td>YES</td>
<td>113097</td>
</tr>
<tr>
<td>TM 4211</td>
<td>NO</td>
<td>3380</td>
</tr>
<tr>
<td>TM 4551</td>
<td>YES</td>
<td>85898</td>
</tr>
<tr>
<td>TM 5242</td>
<td>NO</td>
<td>420</td>
</tr>
<tr>
<td>TM 4545</td>
<td>NO</td>
<td>15689</td>
</tr>
<tr>
<td>TM 5118</td>
<td>NO</td>
<td>6284</td>
</tr>
<tr>
<td>TM 5224</td>
<td>NO</td>
<td>1236</td>
</tr>
</tbody>
</table>

Legend: CD19⁻ cells from leukemic TM mice were prepared as described for Fig. 24 and cultured in the absence of exogenous growth factors. On Day 2, proliferation was measured in triplicates by [methyl-3H] thymidine incorporation. N/A means not available because proliferation was not assessed on Day 2.

TM B-ALLs display abnormalities in the lymphoid progenitor compartments

My observation that chimeric LTR-Flt3 transcripts can be detected in CD19⁻ cells from leukemic TM mice, combined with my finding that proliferation of CD19⁻ cells was attenuated by AGL 2043, suggested that trFLT3 is expressed and active prior to B-cell commitment. To identify potential effects of ligand-independent FLT3 signalling on progression through the pre-commitment stages of B-cell development, I designed 8-color and 10-color staining panels to quantify the abundance and/or phenotype of hemato-lymphoid progenitor subsets among the CD19⁻ fraction of these mice. The traditional strategy for identifying rare subsets of cells lacking mature lineage markers (ie. Lin⁻ cells) uses a “dump” channel containing many antibodies (conjugated with the same fluorochrome) and then gating on the “dump-negative” cells for further sub-fractionation with other markers (Fig. 25A). However, the brightness and proportion of cells staining with each antibody in the dump channel vary widely, presenting significant challenges for objectively identifying the Lin⁻ fraction¹². Therefore I used a sequential gating
strategy to identify and further subdivide the Lin\(^{-}\) fraction. First, I used a “mini-dump” Pacific Blue channel to exclude dead cells (stained with Sytox Blue) as well as myelo-erythroid cells (expressing TER-119, Gr-1, CD11c, CD11b) and T-cells (expressing CD3). Next, I excluded doublets according to their FSC versus SSC profile. Then, I excluded cells expressing CD19, a marker of B-cell commitment, and B220 (a marker of pre-pro-B cells) from the dump-negative fraction single cells. In the example shown, the final dump/CD19/B220-negative fraction represented 1.7% of total BM in B6 mice versus 1.1% in a leukemic TM mouse.
Figure 25. Serial gating strategy used to detect Lin⁻ progenitors by flow cytometry. A) Traditional strategy used for identifying Lin⁻ cells uses a “dump” channel containing many antibodies (conjugated with the same fluorochrome) against mature lineage markers. Lin⁻ fraction is shown in red boxes. B) Lin⁻ cells were gated on as shown for WT B6 (top panel) and leukemic TM 8811 (bottom panel) BM cells. Frequency (%) of parent (i.e. previous) gate is shown. Abbreviation: PB, Pacific Blue.
My panels also included antibodies specific for SCA1, KIT, FLT3 and IL-7R (all linked to different fluorochromes) so that I could evaluate the numbers of several primitive hematolymphoid progenitor subsets within the dump/CD19/B220-negative fraction in B6 mice as compared to leukemic TM mice (Fig. 26A). These included Lin\(^{-}\) SCA1\(^{+}\) KIT\(^{+}\) (LSK) cells containing HSCs and MPPs, CLPs and LBPs (Table 10)\(^{15,16}\). Importantly, the CLP subset in WT B6 mice expressed low levels of KIT (Fig. 26B) as previously reported\(^{12}\). Similarly, the CLPs detected in leukemic TM mice expressed low levels of KIT (Fig. 26B). There was no significant difference in the frequency or number of LSK cells in BM of leukemic TM mice compared to B6 mice (Fig. 26C). In contrast, both the frequency and number of CLPs were significantly decreased and LBPs were significantly increased in leukemic TM mice (Fig. 26C). These data demonstrate that during B-cell leukemogenesis in TM mice, the canonical CLP pathway of early B-cell development is significantly impaired, whereas the alternative LBP pathway of B-cell development is greatly enhanced. Therefore, I suggest that the alternative LBP B-cell development pathway is subverted to give rise to B-ALL in TM mice.

**Table 10. Primitive hematopoietic progenitor subset cell surface markers**

<table>
<thead>
<tr>
<th>Subset</th>
<th>Cell Surface Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSK</td>
<td>Lin(^{-}) SCA1(^{+}) KIT(^{+})</td>
</tr>
<tr>
<td>HSC</td>
<td>LSK(^{+}) FLT3(^{-})</td>
</tr>
<tr>
<td>MPP</td>
<td>LSK(^{+}) FLT3(^{+}) IL-7R(^{-})</td>
</tr>
<tr>
<td>LMPP</td>
<td>LSK(^{+}) FLT3(^{hi}) IL-7R(^{+})</td>
</tr>
<tr>
<td>CLP</td>
<td>Lin(^{-}) SCA1(^{lo}) KIT(^{+}) FLT3(^{hi}) IL-7R(^{hi})</td>
</tr>
<tr>
<td>LBP</td>
<td>Lin(^{-}) SCA1(^{hi}) KIT(^{-}) FLT3(^{+}) IL-7R(^{-})</td>
</tr>
</tbody>
</table>

Abbreviations: hi, high.
Figure 26. Quantification of hematopoietic progenitors in the BM of leukemic mice. A) Flow cytometric analysis of KIT versus SCA1 distribution of viable Lin− singlets in BM of a B6 (left panel) and leukemic TM (right panel) mouse (#8811). Two-parameter probability plots (5% with outliers) show KIT vs Sca1 profiles of Lin− fraction as defined using the strategy shown in Fig. 25. LSK cells (red), CLPs (blue), and LBPs (purple) are shown in bold gates for each mouse. The frequency of gated SCA1lo (CLP) and KIT−SCA1hi (LBP) populations are shown. Data shown are representative of 6 B6 and 6 leukemic TM mice. B) Levels of KIT surface expression on CLP (blue) and LSK+ (black) cell population in WT B6 (top panel) and leukemic TM 8811 (bottom panel) BM. C) Frequency and absolute number analysis of progenitor subsets. Scatter plot summary of the frequency (top panel) and absolute number (bottom panel) of LSK+ cells, CLP and LBP cell populations in leukemic TM (♦) and C57BL/6 BM (■). ** indicates p value <0.01 by Mann-Whitney t test. Results are shown for 6 WT B6 and 6 leukemic TM mice.
**CD19** and CD19**\(^{+}\)** cells from leukemic TM mice can recapitulate the leukemia in immunodeficient mice

Given the presence of chimeric *LTR-Flt3* transcripts as well as the expansion of cells with an LBP-like phenotype in leukemic TM BM, we asked whether we could detect LICs prior to B-cell commitment and CD19 expression. Since normal lymphoid progenitors express FLT3, we also fractionated the CD19**\(^{-}\)** population into FLT3**\(^{+}\)** and FLT3**\(^{-}\)** subsets in some experiments. In all experiments, the CD19**\(^{-}\)** subset was also Lin**\(^{-}\)** (Fig. 27), but for simplicity will be referred to as CD19**\(^{-}\)**. We evaluated the CD19**\(^{+}\)** (also Lin**\(^{-}\)**) and CD11b**\(^{+}\)** BM fractions for LIC function (Table 11). We evaluated the capacity of each subset (all from CD45.2**\(^{+}\)** leukemic TM mice) to generate CD19**\(^{+}\)** leukemic blasts after intravenous transfer to immunodeficient B6.CD45.1 *Rag2**\(^{-}\)/-** hosts (Fig. 27). At least 3 mice were injected per population. An equal number of total CD19**\(^{-}\)** cells from B6.CD45.1/2 mice were co-injected with each sorted subset as a control for engraftment, since they contain HSCs and MPPs. Recipient mice were sacrificed at the first signs of leukemia, which included domed head, labored breathing, enlarged lymph nodes and hind limb paralysis/paresis. Alternatively, where indicated recipients were time sacrificed at 9 or 13 weeks post-transplant.

**Table 11. Progenitor subsets sorted for adoptive transfer experiments**

<table>
<thead>
<tr>
<th>Sorted Subset</th>
<th>Progenitor populations included</th>
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</thead>
<tbody>
<tr>
<td>CD19**(^{-})** FLT3**(^{-})**</td>
<td>HSC</td>
</tr>
<tr>
<td>CD19**(^{-})** FLT3**(^{+})**</td>
<td>MPP, LMPP, CLP, LBP</td>
</tr>
<tr>
<td>CD19**(^{+})**</td>
<td>Committed B-cells</td>
</tr>
<tr>
<td>CD11b**(^{+})**</td>
<td>Myeloid cells</td>
</tr>
</tbody>
</table>
Figure 27. Schematic of adoptive transfer experiment. CD19⁺, CD11b⁺, CD19¹Flt3⁺ and CD19⁻Flt3⁻ BM cells were sorted from leukemic TM donor mice and 10,000 cells from each subset were injected into sublethally irradiated (650 cGy) CD45.1⁺ hosts. Lineage markers included Gr-1, CD11c, CD3 and Ter-119. B220 was removed from the Lin cocktail so as not to exclude pre-pro-B cells from the Lin⁻ sorted subsets. 10,000 CD45.1/2⁻ Lin⁻ BM cells from B6.CD45.1/2 mice were co-injected into each recipient to ensure the IV injections were successful. Flow cytometric profile and gates used to sort CD19⁺, CD11b⁺, CD19¹Flt3⁺ and CD19⁻Flt3⁻ BM cells are shown for TM donor 7981. The experiment was repeated with 3 independent TM B-ALL donors, 7981, 9514, and 1381.
Recipients of CD11b+ cells sorted from 3 different leukemic TM donors lacked clinical signs of leukemia 13 weeks post-transplant, and no donor-derived cells were detected in the BM of recipient mice (Fig. 28A). Importantly, however, progeny of the co-injected normal B6 donor subset were detected in each recipient, demonstrating that the experiment worked technically (Fig. 29). Thus, although rare CD11b+ cells from leukemic TM mice may have expressed chimeric LTR-Flt3 transcripts (Fig. 23), they did not engraft or cause leukemia (Table 12). In contrast, recipients of CD19+ cells from all 3 leukemic TM donors showed clinical signs of leukemia by 3 weeks post-transplant and had to be euthanized (Table 12). In all cases, we detected CD19+ donor-derived leukemic blasts in the recipients’ BM (Fig. 28B). Importantly, I detected trFLT3 in the SPN of one of these recipients (Fig. 28C). Collectively, these experiments demonstrate that CD19+ but not myeloid CD11b+ cells from TM leukemic mice contain LICs.

**Table 12: Frequency of recipient mice that developed B-ALL after adoptive transfer of the indicated subsets from leukemic TM donors.**

<table>
<thead>
<tr>
<th>TM Donor</th>
<th>CD19+</th>
<th>CD19-FLT3-</th>
<th>CD19-FLT3+</th>
<th>CD11b+</th>
</tr>
</thead>
<tbody>
<tr>
<td>7981</td>
<td>3/3</td>
<td>1/3</td>
<td>2/4</td>
<td>0/3</td>
</tr>
<tr>
<td>9514</td>
<td>4/4</td>
<td>0/4</td>
<td>2/3</td>
<td>0/4</td>
</tr>
<tr>
<td>1381</td>
<td>3/3</td>
<td>1/4</td>
<td>4/5</td>
<td>0/3</td>
</tr>
</tbody>
</table>

**Legend:** Recipient mice were sacrificed at the first signs of leukemia, which included domed head, labored breathing, enlarged lymph nodes and hind limb paralysis/paresis. Alternatively, where indicated recipients were time sacrificed at 9 or 13 weeks post-transplant. Recipients were considered leukemic if the mice had > 30% CD19+ lymphoblasts in the BM or showed clinical signs of leukemia. None were technical failures because we could detect CD45.1/2+ control cells in all recipients shown in Table 12.
Figure 28. CD19<sup>+</sup> but not CD11b<sup>+</sup> cells from leukemic TM BM can initiate leukemia in recipient mice. A) Flow cytometric analysis of donor versus host-derived cells 13 weeks after transferring CD11b<sup>+</sup> cells (A) or CD19<sup>+</sup> cells (B) from leukemic TM donors. A) CD45.1 versus CD45.2 distributions are shown for 3 recipients of CD11b<sup>+</sup> cells from TM donor 1381. B) CD45.1 versus CD45.2 profiles of total live singlets (left panel), and CD19 versus FSC profiles of live CD45.1<sup>-</sup>CD45.2<sup>+</sup> donor-derived cells (right panel) are shown for recipient 9293 3 weeks after injecting CD19<sup>+</sup> cells from TM donor 9514. Data shown are representative of all recipients of CD11b<sup>+</sup> and CD19<sup>+</sup> cells from 3 different TM donors (7981, 9514, and 1381). C) FLT3 immunoprecipitation of SPN cells from recipient 9293 of CD19<sup>+</sup> cells from TM donor 9514 was performed as described in Figure 9. As a positive control FLT3 was also immunoprecipitated from BaF3/FLT3 cells as well as primary TM B-ALLs 1191 and 1021.
Figure 29. Distribution of CD45.2+ donor-derived cells from leukemic TM mice relative to host-derived and control CD45.1/2+ cells. Bar graphs show percentage of total viable BM singlets derived from the leukemic TM donor-derived (CD45.2+), control B6 donor (CD45.1/2+) and host (CD45.1+) cells detected 3 weeks after transplantation of CD19+ and 9-13 weeks after transplantation of CD19-FLT3- or CD19-FLT3+ subsets from TM donors 7981, 9514, and 1381. Recipients lacking CD45.1/2+ cells derived from the control B6 donor were excluded from the analysis as technical failures.
As expected, CD19\(^-\) FLT3\(^-\) cells, which contain HSCs and MPPs, from all 3 leukemic TM donors engrafted recipient mice at 9-13 weeks post-transplant (Fig. 29). However, most recipients were not leukemic (Table 12). We also detected engraftment of CD19\(^-\) FLT3\(^+\) cells from all 3 leukemic TM donors (Fig. 29). In some cases, most donor progeny were CD19\(^-\) cells (Fig. 30A) and most recipients were not leukemic, likely indicating that they were derived from normal HSCs and MPPs. However, in 8/12 CD19\(^-\) FLT3\(^+\) recipients, >30% of the CD45.2\(^+\) cells were CD19\(^+\) blasts (Fig. 30A and Table 12). In contrast, CD19\(^+\) blasts comprised >30% of the CD45.2\(^+\) cells in only 2/11 CD19\(^-\) FLT3\(^-\) recipients (Fig. 30B and Table 12). Nonetheless, recipients of CD19\(^-\) FLT3\(^-\) cells occasionally developed full-blown leukemia with hind-limb paralysis and enlarged SPN at 4 weeks post-transplant (Fig. 30B, recipient #7784 of donor 7981).

Altogether, these data suggest that LICs are present in the CD19\(^-\) fraction of BM from leukemic TM mice, but are most abundant in the CD19\(^-\) FLT3\(^+\) fraction. Importantly, recipients of CD19\(^-\) FLT3\(^+\) cells developed leukemic CD19\(^+\) blasts at 12-13 weeks post-transplant, whereas recipients of CD19\(^+\) cells developed full-blown leukemia by 3 weeks post-transplant. Collectively, these data suggest that LICs are present in both FLT3\(^-\) and FLT3\(^+\) subsets prior to B-cell commitment and CD19 expression. However, since CD19\(^-\) FLT3\(^+\) cells induce leukemia with a longer latency than CD19\(^+\) cells, LICs in the CD19\(^-\) FLT3\(^+\) subset appear to be rare and may require additional mutations to induce leukemia.
A. **TM Donor Subset:**

Figure 30. **CD19**^{+} **cells from leukemic TM mice contain LICs.** A) Flow cytometric analysis of CD19^{+} recipient 1467 (top panel) and CD19^{−} recipient 1400 (middle panel) post-transplant. CD45.1 versus CD45.2 distribution (left) and CD19 versus FSC profile of CD45.1^{−}CD45.2^{+} donor-derived cells (right) are shown for recipients. Recipient #1467 and #1400 from TM donor 1381 were time sacrificed at 13 weeks and recipient #7784 from TM donor 7981 was sacrificed because the mouse developed full-blown leukemia with CNS signs at 4 weeks post-transplant. B) CD19^{−} (blue) and CD19^{+} (red) cells among CD45.2^{+} progeny after CD19^{+} and CD19^{−} adoptive transfer. Recipient mice with leukemic CD19^{−} blasts exhibiting high FSC are denoted by asterisk (*). Results are shown for 9 CD19^{+} and 12 CD19^{−} recipient mice from TM donors 1381, 7981 and 9514. Above the bars is the % CD45.2^{+} donor-derived cells detected in each recipient.
**LTR-Flt3 transcripts arise prior to B-cell commitment**

In Chapter 4 I showed that TM B-ALLs express 3 types of LTR-Flt3 fusion transcripts. Briefly, Type 2 LTR-Flt3 transcripts are generated from genomic rearrangements that insert MuLV LTR sequences into Flt3 intron 9, a process that is accompanied by large interstitial deletions of intron 9 as well as proviral sequences 5’ of the LTR U5 region (Fig. 17, Chapter 4). In contrast, Type 3 LTR-Flt3 transcripts are generated from distinct types of LTR-Flt3 fusion genes that retain the PBS and SD of the MuLV provirus (Fig. 18, Chapter 4). Since Type 1 and Type 2 fusion transcripts are derived from distinct genomic rearrangement events, they can be used as clonal markers. Therefore, we evaluated the relative abundance of Type 1 versus Type 2 LTR-Flt3 fusion transcripts (using the RT-PCR strategy described for Fig. 16, Chapter 4) in CD19\(^{-}\) and CD19\(^{+}\) cells from each leukemic TM mouse in order to gain insight into clonal evolution during B-cell leukemogenesis in TM mice.

Importantly, I detected LTR-Flt3 transcripts in CD19\(^{-}\) and CD19\(^{+}\) cells isolated from leukemic TM mice (Fig. 31A), consistent with the presence of LICs in the CD19\(^{-}\) fraction. In cells from leukemic TM mouse #4593, I detected only Type 1 transcripts in the CD19\(^{-}\) subset but additionally detected Type 2 transcripts in CD19\(^{+}\) fraction (Fig. 31A). However, since Type 1 transcripts can potentially arise by alternative splicing from either Type 2 or Type 3 transcripts, this finding cannot be interpreted as evidence of clonal evolution. I also detected LTR-Flt3 transcripts in CD19\(^{-}\) FLT3\(^{+}\) and CD19\(^{-}\) FLT3\(^{-}\) cells from leukemic TM mouse #3039 (Fig. 31B), consistent with the presence of LICs in both fractions. My analysis of these subsets from leukemic TM #6973 was more informative, since Type 2 transcripts were not detected in the CD19\(^{-}\) CD11b\(^{-}\) fraction but was predominant in CD19\(^{+}\) cells (Fig. 31A). These data suggest that a clone with LTR-Flt3 rearrangements generating Type 2 transcripts was rare in the CD19\(^{-}\) fraction but evolved to become predominant among leukemic CD19\(^{+}\) blasts. Alternatively, ongoing Flt3
rearrangements created a new clone expressing Type 2 \( LTR-Flt3 \) transcripts after CD19 expression.

Interestingly, I also detected Type 1 and Type 3 \( LTR-Flt3 \) transcripts in both the CD19\(^{-}\) FLT3\(^{-}\) and CD19\(^{-}\) FLT3\(^{+}\) subsets from leukemic TM mouse #3039 (Fig. 31B), suggesting that multiple clones were present prior to B-cell commitment and PAX5 expression in this TM mouse. However, only the clone expressing the Type 3 \( LTR-Flt3 \) transcript was detected among CD19\(^{+}\) cells (Fig. 31B). Collectively, these data strongly suggest that \( LTR-Flt3 \) gene fusions first arise prior to B-cell commitment in primitive hemato-lymphoid TM progenitors, but only some cells become fully transformed to generate leukemic CD19\(^{+}\) blasts that predominate in lymphoid tissues and disseminate to the CNS.
Figure 31. Chimeric LTR-Flt3 transcripts are detected in Lin-CD19 progenitors from leukemic TM mice. Schematic illustrating the types of LTR-Flt3 transcripts detected in each sorted subset. A) CD19-CD11b+ and CD19+ cells were sorted for TM B-ALL 4591 and 6973. B) Lin-FLT3, Lin-FLT3 and CD19+ cells were sorted from TM B-ALL 3039 as described in Fig. 27. RT-PCR was performed on total RNA as described in Fig. 16 in Chapter 4. Amplified products were cloned and sequenced. The frequency of clones corresponding to each type of LTR-Flt3 transcript is shown in brackets ( ) as a fraction of the total number of clones sequenced.
DISCUSSION

Data presented in Chapters 3 and 4 showed that CD19+ leukemic blasts from TM mice ectopically express a constitutively active trFLT3 protein that is encoded by chimeric LTR-Flt3 transcripts. The expression of trFLT3 is driven by MuLV LTR transcriptional control elements. Consequently, expression of trFLT3 is not likely subject to PAX5 repression at B-cell commitment in leukemic TM mice. However, it was not clear from our previous work whether LTR-Flt3 fusion transcripts were expressed prior to B-cell commitment and PAX5 expression. Here we report that LTR-Flt3 transcripts are detected in CD19−CD11b− cells, suggesting that genomic rearrangements that give rise to LTR-Flt3 transcripts occur prior to B-cell commitment and PAX5 expression. Similarly, we detected LTR-Flt3 transcripts in Lin−FLT3− cells, which includes HSCs, as well as Lin−FLT3+ cells that are enriched for progenitors with lymphoid potential including LMPPs, CLPs and LBPs. Collectively, suggesting that trFLT3 is expressed in hemato-lymphoid progenitors.

Normally ectopic expression of WT FLT3 prior to B-cell commitment and PAX5 expression impairs B-cell development by reducing the number of committed pro-B cells71. Consistent with these observations, Flt3ITD knock-in mice have increased numbers of LSK+ cells, CLPs, and uncommitted pre-proB cells (B220+CD19−) but decreased numbers of committed proB cells (B220−CD19+), pre-B and mature B-cells96, 266. Furthermore, late pro-B cells exhibit increased apoptosis from Flt3ITD knock-in relative to littermates expressing WT Flt3. These data suggest that expression of FLT3-ITD prior to B-cell commitment promotes expansion of hemato-lymphoid progenitors and early B-cell precursors while impairing the survival of committed pro-B cells96. In contrast to Flt3ITD knock-in mice, I observed normal numbers of LSK+ cells and significantly reduced numbers of CLPs in the BM of leukemic TM mice compared to WT mice, suggesting that the survival or CLP development was impaired in leukemic TM mice. However,
we did observe increased numbers of LBPs in the BM of leukemic TM mice compared to WT mice, suggesting that the expression of trFLT3 prior to B-cell commitment promotes expansion of LBPs. Taken together, these data suggest that constitutive FL-independent activation of trFLT3 prior to B-cell commitment and PAX5 expression promotes expansion of lymphoid-restricted LBPs while impairing the development and/or survival of CLPs in leukemic TM mice.

LBPs are thought to be mostly quiescent in steady-state BM\(^9\). However, LBPs can generate B-cells following intravenous transplant into sublethally irradiated hosts\(^{15, 16}\), suggesting that lymphopenic conditions favor expansion and differentiation of LBPs into B-cells. LBPs also generate small numbers of B-cells in culture but require co-stimulation with IL-7 and FL\(^{15}\). Our CFSE studies revealed that a subset of CD19\(^-\)KIT\(^-\) cells, which potentially include LBPs, extensively divided in the absence of growth factors in vitro, suggesting that expression of trFLT3 prior to B-cell commitment might promote the proliferation of LBPs. Consistent with this notion, CD19\(^-\) and CD19\(^+\) cells from leukemic TM cases displayed FL-independent proliferation that was sensitive to FLT3 inhibition, suggesting that trFLT3 promotes the proliferation of both subsets. Furthermore, we observed aberrant growth factor-independent differentiation of CD19\(^-\) cells into CD19\(^+\) blasts from a subset of TM mice, suggesting that trFLT3 signalling is sufficient to induce differentiation of malignant B-cell precursors into CD19\(^+\) blasts. Altogether, these data suggest that expression of trFLT3 prior to B-cell commitment and PAX5 expression induces ligand-independent growth and aberrant differentiation of CD19\(^-\) B-cell precursors, and potentially LBPs, into CD19\(^+\) leukemic blasts.

Given that CD19\(^-\) B-cell precursors from leukemic TM mice can aberrantly differentiate into CD19\(^+\) blasts, we determined whether we could detect LICs in both populations. In contrast to other reports that suggest that LICs for B-ALL are exclusively found in the CD19\(^-\) or CD19\(^+\) cell population in human B-ALL\(^{156, 158-160}\), we detected LICs in Lin\(^-\) progenitors and committed
CD19+ blasts isolated from leukemic TM mice, suggesting that both subsets contain cells with tumorigenic potential. Furthermore, LICs were enriched in the CD19-FLT3+ cells relative to the CD19-FLT3- population, suggesting that the CD19+ leukemic blasts were maintained by a subset of CD19-FLT3+ cells. However, the CD19-FLT3+ cells required 13 weeks to initiate leukemia in recipients, whereas CD19+ cells caused full-blown leukemia in recipient mice 3 weeks post-transplant. Interestingly, LeViseur et al. showed that both CD19- and CD19+ cells isolated from high-risk B-ALL patient samples were able to regenerate the full heterogeneity of the initial leukemia (CD19- and CD19+ cells) with the same latency, suggesting that the LICs exhibit phenotypic plasticity or de-differentiation. In contrast, the CD19+ cells from leukemic TM mice did not generate CD19- cells in recipients, suggesting that the LICs present in the CD19+ blast population do not exhibit phenotypic plasticity or de-differentiate into CD19- leukemic cells. Altogether, these findings suggest that TM B-ALLs are organized as a hierarchy in which LICs that are found in the hemato-lymphoid progenitor compartment give rise to CD19+ blasts with tumorigenic capacity.

Interestingly, although LTR-Flt3 transcripts were detected in CD19-FLT3+ cells from leukemic TM mice, CD19-FLT3+ cells took longer to initiate leukemia in recipient mice than CD19+ cells, suggesting that the expression of trFLT3 prior to B-cell commitment and PAX5 expression is not sufficient to cause full-blown leukemia in recipient mice. Although constitutive activation of FLT3 in Lin- progenitors is predicted to increase the proliferation of hemato-lymphoid progenitors266-268, additional mutations may be required to allow these progenitors to aberrantly differentiate into CD19+ blasts. We propose that the increased proliferation of Lin- FLT3+ cells and the global genomic instability conferred by the loss of p53 and Prkdc-deficiency would allow the Lin’FLT3+ cells to accumulate additional mutations and through clonal evolution select for the most fit and aggressive clones to drive leukemogenesis in recipient mice.
The fact that we could also detect different types of \textit{LTR-Flt3} transcripts in CD19$^-$CD11b$^-$ and Lin$^-$ progenitors in TM leukemias that were absent from the CD19$^+$ blast population suggests that multiple clones expressing different \textit{LTR-Flt3} fusions exist prior to B-cell commitment. Furthermore, these findings suggest that selection and ongoing genomic rearrangements in the CD19$^+$ blast population causes the emergence of a dominant CD19$^+$ cell population expressing one type of \textit{LTR-Flt3} transcript. Altogether, these findings suggest that hemato-lymphoid progenitors undergo clonal evolution to generate clones that acquire the ability to differentiate into CD19$^+$ blasts to drive B-ALL in TM mice.

In summary, the data presented in this chapter suggest that the genomic rearrangements that create \textit{LTR-Flt3} transcripts occur prior to B-cell commitment and PAX5 expression to induce ligand-independent growth of CD19$^-$ and CD19$^+$ cells. Furthermore, we observed expansion of LBPs associated with a significant loss of CLPs, suggesting that an alternative pathway of B-cell differentiation is subverted to give rise to precursor B-ALL in TM mice. Finally, we show that TM B-ALLs are organized as a hierarchy in which LICs are found in a subset of Lin$^-$ progenitors and CD19$^+$ blasts. Overall, our data suggest that expression of trFLT3 prior to B-cell commitment and PAX5 expression promotes leukemogenesis by allowing hemato-lymphoid progenitors to aberrantly proliferate and differentiate into CD19$^+$ blasts.
Chapter 6
Thesis Discussion

Overview
The aim of my thesis research was to determine the role and molecular mechanism of Flt3 over-expression in TM B-ALLs and to determine whether the genetic alterations causing Flt3 over-expression occurred prior to B-cell commitment. The studies presented in Chapter 3 identified a novel FLT3 mutation that results in ligand-independent activation of the receptor. Furthermore, the survival and proliferation of TM leukemic cells was dependent on FLT3 activity, suggesting that trFLT3 signalling promotes malignant cell growth of TM B-ALLs. Molecular studies presented in Chapter 4 revealed that TM leukemias expressed aberrant trFlt3 transcripts in which the 5’ exons are replaced by endogenous MuLV LTR elements, suggesting that over-expression of trFlt3 is driven by MuLV transcriptional control elements. Finally, data presented in Chapter 5 revealed that the LTR-Flt3 transcripts occur prior to B-cell commitment and PAX5 expression, suggesting that trFLT3 expression in primitive hemato-lymphoid progenitors subverts early pathways of B-cell development to promote B-cell leukemogenesis in TM mice. Altogether, the studies presented in my thesis identified a novel mutational mechanism that drives over-expression of a constitutively active mutant form of FLT3 in primitive hemato-lymphoid progenitors, ultimately leading to leukemic transformation of TM pro-B cells.

TM Model of B-cell Leukemogenesis
Leukemias arise from normal cells that progressively evolve into neoplastic cells through the acquisition of traits that enable them to sustain proliferative signalling, evade growth suppressors, resist cell death, enable replicative immortality, induce a block in differentiation, and activate invasion and metastasis\textsuperscript{139, 140}. Genomic instability conferred by the loss of p53-
regulated DNA damage checkpoints combined with disrupted NHEJ-mediated telomere maintenance and DSB repair in TM mice enables the acquisition of these traits in hematopoietic cells. Data presented in Chapter 5 suggest that genomic rearrangements involving the Flt3 locus and endogenous MuLV retroviral elements are selected for prior to B-cell commitment to provide Lin⁻CD19⁺ progenitors with the ability to proliferate in the absence of exogenous growth factors (Fig. 32). Furthermore, expression of LTR-Flt3 transcripts prior to B-cell commitment allows B-cell precursors to aberrantly differentiate into CD19⁺ cells that ectopically express trFLT3 despite the presence of PAX5. Ectopic expression of trFLT3 promotes constitutive ligand-independent activation of the receptor and uncontrolled proliferation of these cells, leading to the accumulation of CD19⁺ blasts in the BM, and eventually dissemination to the spleen and CNS. In summary, our studies demonstrate that trFLT3 is an oncogenic driver in TM B-ALLs from mice with CNS signs.
Figure 32. TM Model of B-cell Leukemogenesis. Schematic shows aberrant differentiation of Lin-CD19- cells expressing LTR-Flt3 chimeric transcripts into the dominant CD19+ blast population. Defects in DSB repair facilitate the acquisition of these genomic rearrangements in Lin-CD19- progenitors. MuLV LTR-Flt3 gene fusions encode a truncated form of FLT3 that is constitutively active in the absence of FL. Expression of trFLT3 prior to B-cell commitment and PAX5 expression subverts normal pathways of B-cell development to give rise to CD19+ leukemic blasts. Red, blue and green circles represent different malignant clones with LTR-Flt3 fusion transcripts.
The Role of Aberrant FLT3 Activity in Human B-ALL

Despite the paucity of FLT3 mutations in ALL, FLT3 expression has been detected in a large proportion of B-ALL lymphoblasts\textsuperscript{108, 109, 112}. The highest levels of FLT3 was reported in MLL-rearranged and hyperdiploid ALL\textsuperscript{113, 114, 116, 117}. Consistent with these findings, FLT3 over-expression was also detected in leukemia and lymphoma cell lines\textsuperscript{269, 270}. FLT3 is rendered constitutively active through activating mutations in the JM and TKD, autocrine secretion of FL, or self-activation induced by FLT3 over-expression\textsuperscript{112, 271}. Interestingly, leukemic lymphoblasts isolated from B-ALL patients with high levels of WT FLT3 were highly dependent on FLT3 signalling for survival\textsuperscript{116}, suggesting that aberrant FLT3 activation contributes to leukemogenesis by promoting the survival of leukemic blasts.

FLT3 signalling may promote leukemogenesis through aberrant activation of the RAS/MAPK pathway\textsuperscript{89, 137}. In fact, activating FLT3 mutations are often mutually exclusive with \textit{NRAS/KRAS2/PTPN11} mutations in B-ALL\textsuperscript{272-274} and AML\textsuperscript{275-277}, suggesting that these mutations mediate their oncogenic effects through aberrant activation of a common RTK/RAS signalling pathway. Interestingly, a recent genome-wide study revealed that the genes most frequently mutated in high-risk ALL include \textit{NRAS, KRAS, PAX5, JAK2, FLT3, PTPN11}, and \textit{TP53}\textsuperscript{278}. Therefore, impaired PAX5 activity, aberrant RAS/MAPK and/or FLT3 signalling may contribute to B-cell leukemogenesis. Strikingly, we also observed RAS/MAPK pathway activation in BaF3 cells expressing trFLT3 (Papp, E., Danska, J., Guidos, C., \textit{in preparation}), suggesting that trFLT3 activates the RAS/MAPK signalling pathway (Fig. 33). Taken together, these findings suggest that FLT3 over-expression in human B-ALL might promote leukemogenesis through aberrant RAS/MAPK signalling.
Aberrant FLT3 activation is also thought to promote leukemogenesis through JAK-independent constitutive STAT5 activation\textsuperscript{137, 279, 280}. Expression of FLT3-ITD in BaF3 and 32D cells induces robust STAT5 activation\textsuperscript{137, 281}. Aberrant STAT5 activation has also been reported downstream FLT3-ITD signalling in AML\textsuperscript{136, 137} and FLT3-TKD signalling in hyperdiploid B-ALL and MLL-rearranged leukemias\textsuperscript{119, 282}. Constitutive STAT5 activation is thought to promote leukemogenesis by stimulating cell proliferation and preventing apoptosis by upregulating genes encoding cell cycle regulators and apoptosis inhibitors such as \textit{bcl-x(L)}, \textit{Mcl-1}, \textit{cyclins D1/D2}, and \textit{c-Myc}\textsuperscript{283, 284}. Similar to FLT3-ITD, trFLT3 induced robust levels of FL-independent
phospho-STAT5 in BaF3 cells (Papp, E., Danska, J., Guidos, C., *in preparation*). Furthermore, primary TM B-ALL blasts expressed very high basal levels of phospho-STAT5 that were sensitive to FLT3 inhibition (Papp, E., Danska, J., Guidos, C., *in preparation*). Thus, trFLT3 induces FL-independent activation of STAT5 in TM B-ALLs. Therefore, aberrant trFLT3 signalling also promotes leukemogenesis through constitutive STAT5 activation.

Constitutive STAT5 activation has been reported in a variety of hematological malignancies including AML, chronic myeloid leukemia and ALL; and is thought to play a causal role in leukemic transformation. Moreover, constitutive STAT5 activation has been shown to be essential for the initiation of myelo- and lymphoproliferative neoplasms induced by FLT3-ITD, BCR-ABL, and TEL-JAK2 translocations. Recently, Heltemes-Harris *et al.* demonstrated that Ebf1 and Pax5 haploinsufficiency synergized with a constitutively active form of STAT5 to rapidly induce B-cell leukemias in transgenic mice. Thus, these findings suggest that targeted disruption of genes encoding the principal regulators of B-cell development collaborate with aberrant STAT5 activation to promote leukemogenesis. Interestingly, we also observed constitutive STAT5 activation in primary *ex vivo* leukemic blasts (Papp, E., Danska, J., Guidos, C., *in preparation*), suggesting that trFLT3-dependent STAT5 activation might collaborate with the block in differentiation caused by the loss of Rag2 in TM mice to promote B-cell leukemogenesis.

Constitutive STAT5 activation is also thought to promote leukemogenesis by enhancing self-renewal. Genome-wide expression studies on AML and MLL patient samples revealed that LICs express genes associated with self-renewal. FLT3-ITD-dependent STAT5 activation has been shown to enhance self-renewal and alter normal differentiation of human CD34+ cord blood cells. Thus, mutations resulting in STAT5 activation may promote leukemogenesis by enabling committed precursor cells to self-renew. Given that trFLT3 induces
robust levels of phospho-STAT5, I propose that trFLT3 promotes leukemogenesis by enabling TM leukemic cells to self-renew. Data presented in Chapter 5 showed that LICs and LTR-Flt3 transcripts could be detected in both Lin\(^{-}\)CD19\(^{-}\) progenitors and CD19\(^{+}\) blasts, suggesting that trFLT3 might promote self-renewal of both subsets to drive leukemogenesis. Therefore, constitutive STAT5 activation induced by aberrant FLT3 signalling might drive B-cell leukemogenesis by promoting self-renewal of lymphoid-restricted progenitors and CD19\(^{+}\) blasts in TM B-ALLs.

**The LIC Population is Heterogeneous in Human B-ALL**

Data presented in Chapter 5 showed that LICs were present in both Lin\(^{-}\)CD19\(^{-}\) progenitors and committed CD19\(^{+}\) blasts isolated from leukemic TM mice. However, the LICs detected in the CD19\(^{+}\) subset did not exhibit developmental plasticity or de-differentiate into CD19\(^{-}\) leukemic cells. Moreover, LTR-Flt3 transcripts were detected in both Lin\(^{-}\)CD19\(^{-}\) and CD19\(^{+}\) populations that also contained LICs, suggesting that expression of trFLT3 prior to B-cell commitment confers tumorigenic capacity to both populations. Therefore, our findings suggest that TM B-ALLs are organised as a hierarchy because the LTR-Flt3 fusions occurs prior to B-cell commitment and PAX5 expression. Given that human B-ALLs comprise of multiple cytogenetically distinct subtypes characterised by different genetic lesions and potential oncogenic drivers, it is reasonable to expect that the identity of the LIC population will vary between patient samples. Consequently, the search for LICs in B-ALL studies might have produced conflicting results because of the different types of genetic abnormalities underlying each leukemia studied\(^{156-160}\).
Activating FLT3 Mutations Collaborate with Inactivation of the p53 Tumor Suppressor Pathway to Promote Lymphoid Leukemias

Activating FLT3 mutations have been identified in lymphoid and myeloid acute leukemias\textsuperscript{120-126} and have been shown to drive myeloproliferative neoplasms and lymphoid and myeloid leukemias in mice\textsuperscript{137, 266-268, 282, 302}. Flt3\textsuperscript{ITD} knock-in mice that have an 18 bp ITD mutation inserted into the genomic DNA sequence coding the JM domain of murine Flt3\textsuperscript{268}, have increased numbers of hematopoietic cells, including HSCs, LMPP, CLPs, CMPs GMPs, and myeloid cells, and significantly reduced numbers of committed B-cell progenitors, suggesting that the inhibitory effects of FLT3-ITD signalling on B-cell development occurs after the CLP stage\textsuperscript{96, 266-268, 303}. More specifically, Li \textit{et al.} showed that FLT3-ITD signalling induced apoptosis of pro-B cells in Flt3\textsuperscript{ITD} knock-in mice due to increased DSBs at J\textsubscript{H} recombination signals in early pro-B cells, suggesting that ongoing D-J\textsubscript{H} rearrangements were more abundant in Flt3\textsuperscript{ITD} knock-in mice compared to WT littermate mice\textsuperscript{96}. During B-cell development, FLT3-dependent RAS activation is thought to regulate IL-7 signalling by inducing IL-7R\textsubscript{α}-chain expression and/or through additional effects on IL-7R-dependent STAT5 activation\textsuperscript{304}. Together FLT3 and IL-7R signalling is thought to induce E2A and/or EBF expression\textsuperscript{59}, which would increase D-J\textsubscript{H} rearrangements\textsuperscript{305}. Thus, constitutive FLT3 signalling might impair B-cell development through increased VDJ-induced DSB caused by enhanced E2A and/or EBF activity. Altogether, these findings suggest that constitutive FLT3 signalling might promote myeloproliferative neoplasms and AML at the expense of lymphoid leukemias because these mutations impair the survival of pro-B cells.

Consistent with this idea, TP53 mutations rarely occur in AML\textsuperscript{306, 307} and have not been detected with activating FLT3 mutations\textsuperscript{308}, suggesting that oncogenic FLT3 possibly favors the development of myeloid leukemias in the absence of TP53 mutations. In contrast, components of
the p53 pathway that are critical for cell cycle progression and apoptosis such as p14\textsuperscript{ARF} (p19\textsuperscript{ARF} in mice) are frequently mutated in ALL\textsuperscript{278,309,310}. Data presented in Chapter 4 together with data published by Anton Berns’ group\textsuperscript{169}, suggest that disruption of the p53-MDM2-p19\textsuperscript{ARF} tumor suppressor pathway allows trFLT3 mutations to be selected for during leukemogenesis. Activating FLT3 mutations including trFLT3 might need to collaborate with the inactivation of the p53 tumor suppressor pathway to drive B-cell leukemias because constitutive FLT3 signalling prior to PAX5 expression impairs the early stages of B-cell development\textsuperscript{71,96}. In contrast, activating FLT3 mutations that arise in committed B-cell progenitors would not require inactivation of the p53-MDM2-p19\textsuperscript{ARF} tumor suppressor pathway because ectopic expression of FLT3 does not impair the survival and proliferation of committed CD19\textsuperscript{+} B-cells\textsuperscript{71}. Altogether, these findings suggest that activating \textit{FLT3} mutations that occur prior to B-cell commitment and PAX5 expression collaborate with the disruption of the p53-MDM2-p19\textsuperscript{ARF} tumor suppressor pathway to drive B-cell leukemogenesis.

\textit{Aberrant Flt3 signalling in B-cell Leukemias with CNS Invasion}

Although \textit{FLT3\textsuperscript{ITD}} knock-in mice predominantly develop myeloproliferative neoplasms\textsuperscript{266-268,303}, Lee \textit{et al.} reported that one of the transgenic mice expressing human \textit{FLT3\textsuperscript{ITD}} developed immature B-cell leukemia with hind limb paralysis\textsuperscript{302}, suggesting that FLT3-ITD-expressing CD19\textsuperscript{+} leukemic blasts had the capacity to invade the CNS. Thus, these findings, together with data presented in Chapter 3, suggest that constitutive FLT3 signalling favor dissemination of leukemic lymphoblasts to the CNS. The blood-brain barrier normally prevents lymphocytes from entering the CNS, which is an immune privileged site\textsuperscript{311}. However, inflammation associated with brain trauma, infection, or autoimmunity allow lymphocytes to breach the blood barrier\textsuperscript{312,313}. Nuclear factor-kappa B (NF-\kappa B) regulates many genes involved in inflammation including
Interleukin-1, Interleukin-6 (IL-6), TNF-α, RANKL. Interestingly, NF-κB is constitutively activated in a large proportion of AML blasts. Furthermore, Birkenkamp et al. showed that enforced NF-κB activation is mediated by RAS/MAPK and PI3K/AKT pathways. Given that both these pathways are activated downstream FLT3 activation, Takahashi et al. tested the ability of BaF3 cells expressing WT FLT3 to activate an NF-κB responsive reporter and target genes and showed that persistent FLT3 signalling induced NF-κB pathway activation and IL-6 expression. Collectively, these findings suggest that constitutive FLT3 signalling helps create a pro-inflammatory environment to enable leukemic blasts to infiltrate the CNS. Therefore, it is possible that trFLT3 also activates the NF-κB pathway to create a pro-inflammatory environment allowing TM leukemic cells to invade the CNS.

**Summary**

Aberrant FLT3 activation is thought to play an important role in the development of certain types of leukemia and lymphoma. Using the TM model of leukemogenesis, I identified a novel activating FLT3 mutation that drives B-cell leukemias by promoting the growth and proliferation of leukemic cells. Furthermore, I showed that the genomic rearrangements that give rise to this FL-independent active form of FLT3 occur prior to B-cell commitment and PAX5 expression. Although constitutive FLT3 signalling impairs B-cell differentiation by inducing apoptosis of committed pro-B cells, TM leukemic cells evade this block in development due to their inability to activate the p53 DNA damage checkpoint. Lastly, my studies suggest that aberrant FLT3 activation might be important to allow leukemic blasts to invade the CNS. In conclusion, my studies provide a rational for treating poor prognosis and relapse B-ALL patients with FLT3 inhibitors.
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