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Uncovering the role of HMG box protein TOX in T-cell Acute Lymphoblastic Leukemia

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Uncovering the role of HMG box protein TOX in T-cell Acute Lymphoblastic Leukemia (T-ALL)

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ABSTRACT

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of thymocytes. Although treatment outcome has improved, pediatric patients that relapse have less than a 30% chance of survival. Higher mortality rates associated with chemotherapeutic drug resistance have led to a greater need for the development of more effective compounds for the treatment of this disease. It is therefore important to identify new molecular pathways underlying T-ALL progression to identify potential targets. In this project, thymocyte selection-associated high mobility group box (TOX), a gene known to play crucial roles in T-cell differentiation, which is over-expressed in human T-ALL, was studied for modulating the pathogenesis of T-ALL. Through the utilization of transgenic zebrafish models, Tox was shown to act as a collaborating oncogene that increased disease aggression. Preliminary data suggest that Tox over-expression results in a HOX11 molecular signature characteristic of rapidly dividing cells, and likely induces different molecular pathways in the pathogenesis of T-ALL when compared to T-ALLs that express only Myc. Cell cycle analysis of human T-ALL cell lines revealed that TOX knockdown leads to a disturbance in cell cycle progression. Furthermore, Camptothecin drug sensitivity was observed in TOX knockdown cell lines. Uncovering the molecular pathways regulated by TOX may help identify novel drug targets for the treatment of T-ALL.
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BACKGROUND AND LITERATURE REVIEW

Hematopoiesis and Leukemogenesis

Hematopoietic stem cells, commonly known as HSCs, are able to form the many diverse blood cell types in vertebrates. HSCs are found in the bone marrow of adult mammals, and give rise to progenitor cells that eventually differentiate into mature blood cells (Orkin, 2000). There are two families of progenitor cells: myeloid and lymphoid, each dictating a route to a particular fate. Myeloid progenitor cells mature into red blood cells, platelets, granulocytes (neutrophils, basophils and eosinophils), and macrophages (Figure 1). Lymphoid progenitor cells differentiate into natural killer cells, T and B lymphocytes. Since mature blood cells are mainly short-lived and require replenishment, HSCs are capable of self-renewal, allowing the production of additional HSCs and multilineage progenitors (Orkin and Zon, 2008).

Figure 1: Hematopoietic stem cell (HSC) differentiation. HSCs reside in the bone marrow and yield progenitor cells capable of unilineage differentiation and the production of mature blood cells, such as red blood cells and lymphocytes (Domen et al., 2006).
Like other stem cells, HSCs have the ability to self-renew. Although not commonly found in differentiated blood lineages, self-renewal is reinitiated in leukemias (blood disorders). It is believed that the proliferative nature of leukemia arises from re-initiation of genetic programs underlying normal HSC development (Orkin and Zon, 2008). Genetic changes occur that alter the cells from their normal state, leading to uncontrolled leukemic growth and malignancy resulting in activation of self-renewal. As a result, the majority of signaling pathways and transcription processes associated with the maintenance of the hematopoietic system are involved in chromosomal aberrations, translocations or somatic mutations in leukemias (Orkin and Zon, 2008). For example, NOTCH1, a gene encoding a transmembrane receptor, is crucial for normal T-cell development. However, activating mutations of its extracellular or C terminal domains are associated with more than 50% of human T-cell acute lymphoblastic leukemia cases (Weng et al., 2004). Additionally, disturbance of transcription factors that regulate HSC fate and self-renewal, including the basic helix-loop-helix (bHLH) proteins (TAL1 and LYL1), GATA-factors (GATA1,2 and 3), the LIM-domain factors (LMO1/2), and the homeodomain protein members (HOX family) among many others are involved in a variety of lymphoid leukemias (Ferrando et al., 2004; Bresnick et al., 2010; Soulier et al., 2005).

The remarkable similarities between hematopoietic transcription factors and the initiation of malignancy provide entry points for understanding the mechanisms of self-renewal during oncogenesis. Identification of the common self renewal pathways between normal and malignant blood cells is crucial for the development of new treatments of blood disorders. Our lab focuses on identifying the mechanisms that lead to self renewal in T-cell Acute Lymphoblastic Leukemia (T-ALL).
T-cell Acute Lymphoblastic Leukemia (T-ALL)

**Brief overview of T-cell development and differentiation**

The first step in T-cell development takes place in the bone marrow (Figure 1) where HSCs give rise to lymphoid progenitor cells. These cells migrate from the bone marrow to accumulate in the thymus, which is the site of their maturation to T-lymphocytes (thymocytes) (Graux et al., 2006). In the thymus, the cells stay in close contact with thymic epithelial stromal cells, where Notch ligands provide the cells with growth factors. Upon pre-T-cell receptor (pre-TCR) expression, thymocytes lose their self-renewal capabilities and differentiate into double positive (DP) CD4⁺CD8⁺ cells (Bosselut, 2004).

The T-cell receptor (TCR) is a transmembrane heterodimer composed of α and β proteins. Each protein consists of a variable (V), diversity (D), joining (J) and constant (C) region (Graux et al., 2006). Through random V, D, J recombination, a diverse number of T cells are able to mature and survive (Tonegawa, 1983). Random recombination of the β chain allows for the expression of the pre-TCR, whereas recombination of the α chain allows for the expression of the mature αβTCR (Graux et al., 2006). Thymocytes that express the αβTCR are rescued from programmed cell death, and are able to mature further and undergo positive selection, where they are able to recognize foreign molecules and become bound to major histocompatibility complex (MHC) molecules (Bosselut, 2004). The cells can now exit the thymus as mature single positive (SP) CD4⁺ or CD8⁺ T-cells, where they are able to become components of the immune system as either T helper cells or cytotoxic T cells, respectively (Aifantis et al., 2008). A brief schematic of T-cell development and differentiation is portrayed below in Figure 2.
In contrast to normal development, thymocytes are arrested at early stages of differentiation in T-cell Acute Lymphoblastic Leukemia (T-ALL). In this case, the normal mechanisms that control T cell transformation, such as cell growth, proliferation, survival, and differentiation are disrupted. As a result, immature malignant thymocytes infiltrate the bone marrow and continue to self-renew until they overtake the blood of diseased patients (Uckun et al., 2012).

**T-ALL Disease Prevalence and Cure Rates**

T-cell Acute Lymphoblastic Leukemias account for approximately 15% of childhood and 25% of adult acute lymphoblastic leukemia (ALL) cases (Vlierberghe and Ferrando, 2012). It is more frequent in males than in females, and is often associated with a more unfavorable
prognosis than the more common B-cell lineage ALL (Aifantis et al., 2008). Patients usually exhibit infiltration of thymocytes in the bone marrow, high white blood cell counts, and involvement of the central nervous system when diagnosed. T-ALL can be treated using conventional chemotherapy with cure rates of up to 75% in children, and 50% in adults. However, patients with primary resistant or relapsed disease have a very poor prognosis with less than a 30% survival rate in the pediatric population (Vlierberghe and Ferrando, 2012). Understanding how and why certain tumor cells relapse and survive treatment is a key to overcoming the hurdle for treating this disease.

**Leukemia Propagating Potential and Clonal Evolution at Relapse**

Cancer self-renewal or leukemia-propagating potential is the process by which cancer cells are able to remake all the tumor cell types and reform malignancies following treatment. Cancer cells that survive treatment are termed leukemia-propagating cells or LPCs (Figure 3).

![Figure 3: A simple schematic of leukemia-propagating potential in T-ALL. The leukemia propagating cell (LPC) is able to survive treatment and self-renew to remake the tumor population.](image)

Primary T-ALL arises from the expansion of transformed T-cell clones, however the mechanism underlying relapsed T-ALL is largely unknown. Recent studies have shown clonal relationships between primary and relapsed leukemias. It is believed that relapsed T-ALLs develop from a rare LPC clone that is able to survive treatment and remake a more aggressive and treatment-resistant tumor population (Blackburn et al., 2012). The relapse clone has been
identified to be present at low levels during diagnosis, and acquires genomic mutations that contribute to its growth and survival (Mullighan et al., 2008). Additional studies were able to recapitulate the process of relapse in patients in immunodeficient mice by showing that the development of human leukemia in mice results from an oncogenic selection and expansion of a pre-existing leukemic subclone (Clappier et al., 2011). However, the molecular mechanisms associated with relapse clones remain poorly understood.

**T-ALL Subtypes**

Due to the complexity of thymocyte development, multiple transcription factors play key roles in regulating the stages of T-cell differentiation. Chromosomal translocations involving transcription factors have long been associated with unique subtypes of leukemia (Gilbertson, 2011). Various studies in T-ALL patient cases have identified chromosomal translocations of major transcription factors found at different stages of thymocyte differentiation, leading to aberrant T-cell expression and oncogenic activity. Those transcription factors include the basic helix-loop-helix (bHLH) family members such as TAL1, TAL2, LYL1; the LMO genes (LMO1 and LMO2); and the homeobox genes HOX11 and HOX11L2 (Ferrando and Look, 2003). Studies using microarray gene expression analysis in T-cell leukemic lymphoblasts have identified at least five main different molecular sub-types that involve the activation of these transcription factors: (1) HOX11 (TLX1), (2) HOX11L2 (TLX3), (3) TAL1 and LMO1/2 (4) LYL1 and LMO2, and (5) MLL/ENL (Ferrando and Look, 2003). Each of these T-ALL subtypes is blocked at different stages of thymocyte differentiation, becoming a unique molecular signature of T-ALL. TAL1 and LMO1/2 expressing leukemias account for almost 60% of childhood T-ALL cases and have the worst prognosis; whereas HOX11-positive lymphoblasts
have a better prognosis when treated with modern chemotherapy (Ferrando and Look, 2003). Figure 4 shows the major T-ALL subtypes and their prevalence in childhood and adult cases.

Although the five main subtypes of T-ALL utilize different molecular pathways, gain of function of Notch is shared among all subtypes. Studies by Weng et al. (2006) have shown that Notch acts through the direct activation of Myc. In fact, Myc and Notch are central regulators of this disease (Palomero et al., 2006).

**Figure 4: Distribution of the 5 different transcription factor oncogenes in pediatric vs. adult T-ALL cases. TAL1 is most prevalent in both childhood and adult cases, while HOX11 is more dominant in adult cases vs. pediatric. The LMO1 and LMO2 transcription factors are expressed most frequently in TAL1 or LYL1 - positive cases (Ferrando and Look, 2003).**

**Zebrfish as a Model for T-ALL**

**Advantages of Using Zebrfish as a Model System for Cancer**

Hematopoiesis serves as the central paradigm for understanding HSC biology and roles in aging, disease, and oncogenesis (Orkin and Zon, 2008). Because the process of hematopoiesis is conserved throughout vertebrate evolution, animal models have shed new light onto the molecular mechanisms underlying human hematopoiesis (Orkin and Zon, 2008). Specifically, the zebrafish (*Danio rerio*), has evolved as a great tool for the study of blood development and
tumor malignancy (Langenau et al., 2003). The many attributes of the zebrafish represent clear advantages that are particularly useful in the analysis of hematopoietic development. First, its externally fertilized, transparent embryos allow for easy visualization and manipulation of all its developmental stages from one-cell stage into adulthood (Martin et al., 2011). Second, each female can produce approximately 200 eggs per week and can mate weekly, allowing for a large clutch size. This is particularly helpful because it makes large-scale screening possible (Ransom et al., 1996). Furthermore, their small size allows for ease of maintenance (thousands of animals can be raised in a relatively small space). Most importantly, due to its optical clarity, the observation and the analysis of tumor formation and development are facilitated (Langenau et al., 2003).

Other than its ease of manipulation, the zebrafish has become a powerful tool for performing unbiased forward genetic screens (Driever and Fishman, 1996), allowing for the discovery of pathways involved in development (Martin et al., 2011), regeneration, and cancer (Chen and Zon, 2009). The large numbers of animals needed for a mutagenesis screen makes them extremely expensive for any current vertebrate model organism, except for the zebrafish, which, due to its fecundity, is a perfect animal model for genetic screens. For example, one screen has identified many informative mutations that have been linked with hematopoietic defects, which gave insight into a multitude of genes important for vertebrate blood development (Driever and Fishman, 1996).

**Myc-induced Transgenic zebrafish Model of T-ALL**

The zebrafish Myc-induced T-ALL is a powerful model for the study of disease mechanisms and cancer self renewal. T-ALL can be induced in zebrafish by expressing the mouse eMyc gene under the control of the T-cell specific rag2 promoter (Langenau et al., 2003).
Leukemic cells expressing Myc fused to a fluorescent reporter arise in the thymus, and can be observed by 21 days post injection. The leukemia then spreads into the gills and disseminates into skeletal muscle and abdominal organs (Figure 5; Langenau et al., 2003). Cellular analysis of Myc-induced T-ALL shows an expression of the transcription factors TAL1 and LMO2, factors found in the most common and treatment-resistant subtype of pediatric T-ALL (Langenau et al., 2005).

T-ALLs can be induced by the delivery of linearized transgenes directly into one-cell stage embryos. 5-10% of mosaic transgenic animals develop leukemia by 50 days of life, and up to a thousand embryos can be injected daily.

![Image](200x278 to 413x468)

Figure 5: Myc-induced Transgenic Zebrafish model of T-ALL. External features of leukemic zrag2:cmMyc mosaic fish. Wild-type fish (A and B) and Leukemic fish (C and D). In Leukemic fish, lymphocytes are infiltrating the nose (N), eyes (E), and fins (F). A,C: Lateral view; B,D: dorsal view (from Langenau et al., 2003).

The Myc-induced leukemias express T-cell specific markers, such as CD3, CD4, CD8, Lck and rag1/2, but do not express the B-cell specific marker, IgM (Langenau et al., 2003). Furthermore, zebrafish Myc-induced leukemias are oligoclonal, representing heterogeneity within the tumors that greatly complements human disease (Smith et al., 2010). Millions of zebrafish T-ALL cells
can be extracted from individual zebrafish, to assess the proliferation, apoptosis, and cancer self renewal processes (some of the main hallmarks of cancer). The creation of syngeneic (genetically identical) zebrafish strains has facilitated limiting dilution cell transplantation experiments, which can directly assess self-renewal potential in Myc-induced T-ALL (Migireuv and Revskoy, 2006; Smith et al., 2010). Overall, the zebrafish Myc-induced model of T-ALL provides a unique platform for the study of T-ALL and identification of conserved molecular and genetic mechanisms that drive human disease.

**Previous Work: Transgenic Screen to Uncover Relapse Associated Oncogenes in T-ALL**

The Myc-induced T-ALL model can be used to assess collaborating genetic events. A gene of interest can be injected along with Myc and its capability to accelerate the time to tumor onset and progression can be assessed. Through genomic copy number abnormalities and microarray gene expression profiling, four studies have identified amplified and/or over expressed genes in both relapsed human and mouse T-ALL (Maser et al., 2007; Mullighan et al., 2008; Winter et al., 2007; Remke et al., 2009). All of the amplified regions are associated with T-ALL relapse. However, many of genes have yet to be assessed for their ability to increase disease progression and to drive relapse. Our lab has specifically selected 43 genes that were identified in more than one study, and exert roles in T-cell differentiation and development. Figure 6a below shows a subset of genes that were selected by our lab in a screen to assess their ability to collaborate with Myc to decrease the time to leukemia onset.

Of the 23 genes listed below, seven genes have been identified to collaborate with Myc and accelerate time to tumor onset in our zebrafish model. Those include genes linked to hematopoiesis (TOX and IL7R), proliferation (DVL1 and HIF1a), apoptosis (SET), and self-renewal (BMI1). Figure 6b below summarizes the results. My project focused on TOX, a gene
shown to accelerate the Myc-induced onset of T-ALL (p = 0.007), and an HMG box protein known to play crucial roles in T-cell differentiation.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Known Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IKZF</td>
<td>hematopoietic development</td>
</tr>
<tr>
<td>MEF2c</td>
<td>involved in apoptosis</td>
</tr>
<tr>
<td>MSI2</td>
<td>associated with stem cells</td>
</tr>
<tr>
<td>HHEX1</td>
<td>hematopoietic differentiation</td>
</tr>
<tr>
<td>TTYH2</td>
<td>chloride ion channel</td>
</tr>
<tr>
<td>RORC</td>
<td>involved in normal hematopoiesis</td>
</tr>
<tr>
<td>AurKAIp1</td>
<td>associated with proliferation</td>
</tr>
<tr>
<td>HOXA9</td>
<td>function unknown</td>
</tr>
<tr>
<td>PRKCA</td>
<td>involved in cell cycle, cell adhesion</td>
</tr>
<tr>
<td>GTR</td>
<td>T-cell development, apoptosis</td>
</tr>
<tr>
<td>ABL1</td>
<td>cell differentiation, proliferation, proliferation</td>
</tr>
<tr>
<td>VDZ1</td>
<td>regulates hematopoiesi</td>
</tr>
<tr>
<td>HOXA10</td>
<td>hematopoietic function</td>
</tr>
<tr>
<td>ERG</td>
<td>oncogene, regulates proliferation and development</td>
</tr>
<tr>
<td>GRAP2</td>
<td>chloride ion channel</td>
</tr>
<tr>
<td>FNPB1</td>
<td>associated with endocytosis</td>
</tr>
<tr>
<td>Notch</td>
<td>involved in normal hematopoiesis</td>
</tr>
<tr>
<td>BMI1</td>
<td>differentiation, apoptosis</td>
</tr>
<tr>
<td>IL7R</td>
<td>T-cell development, apoptosis</td>
</tr>
<tr>
<td>HIF1a</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TOX</td>
<td>regulates T-cell development</td>
</tr>
<tr>
<td>SET</td>
<td>involved in apoptosis</td>
</tr>
<tr>
<td>DVL1</td>
<td>regulates proliferation, development</td>
</tr>
</tbody>
</table>

**Figure 6: Identification of relapse-associated oncogenes that collaborate with Myc to accelerate time to leukemia onset in zebrafish.** (a) Prioritized list of genes associated with relapsed human T-ALL. (b) Kaplan Meir Analysis: red line shows tumor onset curves in Myc expressing animals. Black lines are genes that do not collaborate. Blue denotes time to tumor onset in oncogenes that collaborate with Myc to accelerate leukemia onset. P-values noted are shown in parenthesis. N>10 (Langenau lab, unpublished).

**Thymocyte selection associated high mobility group box (TOX)**

**Known Structure and Function**

Tox encodes a nuclear protein of the high-mobility group (HMG) family of DNA binding proteins (Wilkinson et al., 2002). HMG box proteins all share a DNA-binding domain, and are divided into two groups. The first group includes transcription factors, with a single HMG box motif that binds DNA in a sequence-specific manner. On the other hand, the second group has multiple HMG box motifs, and bind DNA in a sequence-independent manner (Wilkinson et al.,
HMG box proteins are well known for the regulation of DNA-dependent processes, such as transcription, replication, and DNA strand repair, all of which require the bending and unwinding of chromatin structure (Bianchi and Beltrame, 1998). As a result, they contain DNA-binding domains that allow them to bind the DNA and modify its structure (O'Flaherty and Kaye, 2003). *Tox* (thymocyte selection-associated HMG-box gene) is part of the HMG box family proteins. The TOX protein is 526 amino acids long with a nuclear localization signal (NLS domain) and a single centrally located HMG box (O'Flaherty and Kaye, 2003). Although TOX expression is likely tightly regulated to allow for proper T-cell differentiation, similar to sequence-specific HMG box transcription factors, it also shares many factors of sequence-independent HMG box DNA binding proteins (Wilkinson et al., 2002). Therefore, it remains unknown whether TOX functions specifically or un-specifically to regulate gene expression during thymic selection.

**Role in T-cell Development and Differentiation**

TOX is transiently upregulated during β-selection and positive selection in developing thymocytes (Aliahmad et al., 2004). Transgenic approaches in mice have been utilized in order to define the role of Tox in T-cell development. During positive selection, TCR signaling causes the downregulation of the CD4 and CD8 double positive (DP) thymocytes leading to a double dull (DD) phenotype (CD4<sup>lo</sup>CD8<sup>lo</sup>) followed by re-expression of CD4 in order to yield the CD4<sup>+</sup>CD8<sup>lo</sup> cells, which serve as precursors to the single positive (SP) cell population (Aliahmad et al., 2008). TOX loss of function and gain of function experiments have shown that it is induced by TCR signals early in positive selection, first in the DD cells and at the CD4<sup>+</sup>CD8<sup>lo</sup> stage (Aliahmad et al., 2012).
In the absence of TOX, the DD to CD4<sup>+</sup>CD8<sup>lo</sup> transition is severely inhibited, causing an arrest in the development of CD4 SP T cells but an increase in the development of the CD8 SP population (Aliahmad and Kaye, 2008). However, the CD8 SP population failed to mature or exit the thymus (Aliahmad and Kaye, 2008). These results strongly indicate that Tox is required during the early positive selection stages, and that the transitional CD4<sup>lo</sup>CD8<sup>lo</sup> and CD4<sup>+</sup>CD8<sup>lo</sup> thymocyte populations is key in the pathway towards positive selection and maturation of the thymocytes. Additional analyses have also shown TOX upregulation during αβ-selection due to pre-TCR signaling (Aliahmad et al., 2012). Figure 7 below summarizes TOX expression during T-cell development and differentiation.

Figure 7: Tox expression in T-cell differentiation. DN: Double Negative, DP: Double Positive, DD: Double Dull, SP: Single Positive thymocytes. Tox expression is increased in early thymocyte precursors by pre-TCR activation and in the initial activation and differentiation period during the double dull stage but down-regulated during the final maturation stage. The triangle represents increasing Tox expression from the DD to DP transition.
Many studies have analyzed TOX function in T-cell development, and it seems to be tightly regulated in the thymus in a stage-specific manner (Aliahmad et al., 2012). However, not much is known about its role in T-ALL and disease progression. In this study, we utilize the zebrafish T-ALL model to better understand the function of Tox in T-ALL.
PROJECT PURPOSE

Our laboratory has developed a novel transgenic zebrafish screen to identify potential collaborators in T-ALL pathogenesis and aggression. This approach identified collaborating oncogenes by selecting from a panel of genes present from recurrent genomic amplifications in human relapsed T-ALL. Of the 23 genes examined thus far in our screen, 7 genes (BMI1, SET, IL7R, DVL1, HIF1α, Notch and TOX) have been identified as collaborators of disease progression. My project focused on identifying a role for TOX in regulating T-ALL growth. TOX is a gene known to play crucial roles in T-cell development and differentiation; however, its role in T-ALL remains unknown. My project aimed to achieve the following goals:

- Confirm that TOX collaborates with Myc and Notch to accelerate the time to tumor onset and enhance leukemia progression
- Assess if TOX alone is sufficient to induce T-ALL
- Assess whether TOX changes the molecular subtype of T-ALL by affecting the expression of the known T-ALL oncogenic transcription factors
- Determine whether cell cycle and apoptosis are affected by TOX
- Determine the effect of TOX loss of function on leukemia onset and progression
- Extend studies to human cell lines in order to identify the cellular phenotype of TOX
- Examine if TOX expression alters the sensitivity/resistance of chemotherapeutic drugs (ex. Camptothecin)

Uncovering the role of TOX may lead to the identification of new pathways involved in the pathogenesis of T-ALL.
MATERIALS AND METHODS

Zebrafish lines and husbandry

Zebrafish were raised according to standard procedures adapted from the zebrafish book (Westerfield, 2000). Microinjected zebrafish embryos were generated by crossing adult TU/AB wildtype couples. The crosses between adult TU/AB fish were performed once a week. Each couple was put together overnight on appropriate mating boxes, and the embryos were collected and maintained in fish water, which was changed daily. Dead and deformed embryos at 24 hours post fertilization (hpf) and 3 days post fertilization (dpf) were sorted. Wildtype AB lines were used for transplantation experiments and TALEN injections.

Generation of Mosaic Transgenic zebrafish that develop T-ALL

Gateway cloning of constructs for injection

All genes for injection were amplified by PCR and cloned into an entry vector using the pENTR Directional TOPO Cloning kit (Invitrogen). The entry clones were selected on Kanamycin plates, the DNA was then verified by sequencing at the Massachusetts General Hospital sequencing core with the following universal primers: T3, T7, rag2 promoter, M13-forward and M13-reverse. LR reactions were performed using LR Clonase II (Invitrogen) to combine the entry clone containing the gene of interest with the destination vector containing the T-cell specific promoter, \textit{rag2}. After sequencing verification, 10 µg of each plasmid was linearized by restriction digestion at 37°C for two hours. The \textit{rag2-ztox}, \textit{rag2-mouse-cMyc} and \textit{rag2-mCherry} plasmids were linearized with XhoI, and the \textit{rag2-z-notch^{NICD}} plasmid was linearized with NotI. 200 ng of linearized product was verified by a 0.8% TAE agarose gel,
while the rest was purified on a column using the PCR Purification kit (Qiagen) and eluted in EB buffer (Qiagen).

**A screen to identify collaborating T-ALL oncogenes**

One thousand one-cell stage embryos were injected daily with 40 ng/µl of each plasmid. The injection mix for the control fish consisted of 40ng/µl of *rag2:*m-Cherry (as the fluorescent reporter) with 40 ng/µl of *rag2:* mouse-cMyc (*m-cMyc*) or *rag2:*z-notch1aICD (zebrafish *notch1a*). For the experimental injections, one group contained all three constructs (the mix consisted of an additional 40 ng/µl of *rag2:*ztox (zebrafish tox) or *rag2:*hTOX (human TOX)). The second experimental group contained 40 ng/µl of *rag2:*m-Cherry with 40 ng/µl of *rag2:*ztox. Time to tumor onset was assessed using Kaplan Meier curves. Co-injection of the transgenes leads to co-expression in the developing tumor. Figure 8 summarizes the co-injection strategy used.

![Diagram](image)

**Figure 8:** Generation of m-Cherry labeled Myc-induced and Notch-induced transgenic zebrafish T-ALL by a novel co-injection approach to identify genes involved in T-ALL onset. The control groups were used to identify normal time to leukemia onset. Raw data was analyzed using Kaplan Meier analysis with sample size (n) of ten per gene.
Screening for T-ALL using a Fluorescent Microscope

At 21 days post fertilization, the zebrafish larvae were analyzed for fluorescent reporter expression in the thymus using an epifluorescence microscope at 20X magnification, using a 535/35 excitation wavelength and a 610/40 emission filter to detect m-Cherry fluorescence. Animals with m-Cherry positive thymi were monitored for disease onset every 7 days.

From daily injections of a thousand embryos, approximately 5% of the fish were m-Cherry positive. Stage 0 fish have m-Cherry positive cells contained within the thymus; in stage 1, the cells locally disseminate from the thymus indicative of thymic hyperplasia; stage 2 fish show m-Cherry positive fish infiltrating the head and spreading through the peritoneal cavity; finally, stage 3 fish represent leukemic fish with half of the fish m-Cherry positive and has been previously shown to correlate with infiltration into the marrow: the hallmark of leukemia (Blackburn et al., 2012). Figure 9 below portrays the four different stages used to define T-ALL aggression during screening.

Figure 9: Staging of T-ALLs. (A) Stage 0, m-Cherry⁺ thymus, no tumor formation. (B) Stage 1, hyperplasia, cells are disseminated from the thymus, (C) Stage 2, Lymphoma, cells spread towards the head, (D) Stage 3, leukemia, half the fish is m-cherry+.
T-ALL Cell Transplantation

T-ALL fish were sacrificed according to the IACUC procedure. The cells were extracted in 0.9 x phosphate buffered saline (PBS) and 5% fetal bovine serum (FBS), strained through a 40µm filter (BD Biosciences). Cells were centrifuged at 2000rpm, for 10min, cell pellets were re-suspended in 0.9 x PBS + 5% FBS and counted using a hemocytometer. One-million un-sorted cells were transplanted by intra-peritoneal injection into six- to nine- month old irradiated AB or TU/AB strain recipients. Fish were then monitored for T-ALL growth 10 days post-transplantation, every 7 days.

Human T-ALL Cell Culture Maintenance

The following human T-ALL cell lines were used: KOPTK1, DND41, RPMI8402, PEER, MOLT13, CCRF-CEM, P12-ICHIKAWA, KARPAS-45, SUPT-7, SUPT-13, HPB-ALL, JURKAT, Loucy, MOLT16, ALL-SIL and MOLT4. All cell lines were maintained by Riadh Lobbardi (Langenau lab, Molecular Pathology Unit). Molt4 Tox knockdown (shRNA) cell lines were created and provided by Riadh Lobbardi, who had training in the BL2+ biosafety level.

Cytospins (Leukemia Cell Morphology) and Histological Analysis

1 x10^5 T-ALL cells were cytospun onto slides and air dried for 5 minutes. Slides were then run through a series of washing and staining steps: 100% May Grunwald (4 min); 4% Giemsa (4 min); dH2O (briefly). The slides were dried and mounted with a 24 x 60mm cover slip. Histological analysis was also performed on whole zebrafish heads and sections stained by Hematoxylin-Eosin (H & E Staining) in the Molecular Pathology unit at Massachusetts General Hospital. All slides were stored at room temperature. Images from cytopsin and histology slides were acquired using a brightfield microscope with a Olympus D72 camera and the scale was set in the Olympus software.
Cell Cycle and Apoptosis analysis

Unsorted cells were isolated from leukemic fish and human T-ALL cells and analyzed for DNA synthesis using the Click IT EDU Alexa Fluor 647 protocol (Invitrogen). Cells were pulsed with EdU for two hours and then fixed with 4% paraformaldehyde before Alexa Fluor conjugation. DAPI or Propidium Iodide was used as a counter-stain to exclude dead cells. Unsorted cells were also stained with Annexin V conjugated to Alexa Fluor 647 in the presence of DAPI according to the manufacturer's protocol to quantify apoptotic cells. All analysis were performed using the fluorescence activated cell sorter, FACSariaII machine (BD Biosciences).

Real-time reverse transcription-PCR

RNA was extracted from unsorted zebrafish leukemic cells and human cell lines using an RNeasy Mini kit with on-column DNase treatment (Qiagen). Total RNA was reverse transcribed using superscript III and quantitative real-time PCR was performed using Power Syber Green Master mix (Applied Biosciences). Zebrafish and human data were normalized to β-actin 2 and GAPDH expression respectively. The calculation of the fold change was processed using the $2^{-\Delta\Delta Ct}$ method. Quantitative analysis were performed in triplicate and repeated twice.

Western Protein Analysis

Protein expression was measured by Western Blot analysis. Proteins were extracted from T-ALL human cell lines using RIPA Buffer and protein concentrations were measured using the Bradford assay with Coomassie Blue Reagent. Proteins obtained from cell lysates were stored in 1x laemmli buffer, ran on a 4-15% SDS page gel and transferred onto PVDF membranes, which were blocked with 5% dry milk powder dissolved in Tris-buffered-saline buffer (TBS) containing 1% Tween 20. Human TOX antibody (eBioscience) was used at a dilution of 1:1000. GAPDH antibody (Cell Signaling) was used at a dilution of 1:3000. The rat and rabbit secondary
antibodies were used at a dilution of 1:10,000 and 1:2500, respectively. The western blot revelation (luminol Santa Cruz) was performed 5 minutes at room temperature and the membrane was exposed in a dark room to autoradiography film for 10 seconds to 5 minutes.

**Camptothecin Drug Treatment**

Camptothecin (CPT) was diluted in DMSO to achieve a final concentration of 10nM, 100nM, 1µM, 10µM and 100µM. DMSO was used as the control. The Molt4 T-ALL human cell line was assessed for the drug treatments. Analysis was performed in triplicate. After 24 hours of treatment, the plate was analyzed using Titer Glo Reagent (VWR), which measures the amount of viable cells in each well based on ATP activity.

**Transcription Activator-Like Effector Nucleases (TALENs)**

*ztetox* TALENs were provided to me by Finola Moore in the Langenau Lab (Massachusetts General Hospital Molecular Pathology Unit). TALEN DNA was linearized by PmeI (NEB) and purified using the MinElute PCR Purification kit (Qiagen). 1µg of purified linearized TALEN DNA was transcribed according to the mMMessage mMachine T7 ULTRA kit (Ambion). RNA was precipitated by 3M Sodium acetate, pH 5.0 and extracted by phenol/chloroform. The RNA pellet was resuspended in 40µl RNAse-free injection water and the concentration was adjusted to 200 ng/µl. 125 pg of RNA of each of the two TALEN arms was injected into one-cell stage zebrafish embryos.

Microinjected zebrafish embryos were raised to 1 day post fertilization (dpf). Genomic DNA was extracted from 12 embryos with lysis buffer containing: 10 mM Tris, 1mM EDTA, 200 mM NaCl, 0.5% SDS and 100 µg/ml proteinase K. Tubes were incubated for at least two hours at 50°C and the proteinase K was inactivated at 95°C for 10 minutes. Genomic DNA was purified using phenol/chloroform extraction and resuspended with 40 µl of TE buffer. PCR was
then performed using specific primers (ztox-forward primer sequence: CACCATGGACGT
GAGATTTTATCCA, ztox-reverse primer sequence: TCAGGTCAGGTACAGGGCTTTATC) that span the ztox gene. Genomic PCR products were purified and mutation rate was determined using the T7E1 endonuclease (NEB). PCR products were heated in order to dissociate the strands and then allowed to slowly re-anneal, creating PCR products where the wildtype strand have annealed to a mutant strand. Endonuclease T7E1 will cleave PCR fragments at these sites of mismatch, resulting in shorter PCR fragments that are visualized by gel electrophoresis. High mutation rate was determined by the intensity of the shorter bands. Uninjected control embryos were compared to injected embryos. Figure 10 below summarizes the methodology used to assess somatic mutation rates of TALENs.
Figure 10: Illustration of the methodology used to assess somatic mutation rate of TALENs. Each TALEN arm binds to a half-site with the dimeric fokI nuclease domains cleaving the DNA within the intervening sequence. DNA mutations are introduced when double stranded DNA breaks anneal by error-prone non-homologous end joining (NHEJ), creating insertion and/or deletion mutations (indels). Successful mutagenesis with the T7E1 Endonuclease results in smaller bands as shown in the mutagenized (Mut) lanes compared to the control (Cont) lanes. (Moore et al., 2012).
RESULTS

Tox collaborates with Myc and Notch to accelerate zebrafish T-ALL progression

Previous studies have documented a role for TOX in regulating cell survival, growth, and early thymocyte development and differentiation (See Background). Studies showing an over-expression of TOX in human T-ALL samples has provided a segue for the study of this gene in the pathogenesis of T-ALL. However, the mechanistic role of TOX in regulating T-ALL remains to be elucidated. The first question addressed in this project was to determine whether TOX collaborates with Myc to accelerate the time to tumor onset, independently verifying our earlier results from the screen. Using the Myc-induced transgenic zebrafish that accurately recapitulates the most aggressive and treatment resistant subtype of T-ALL, a screen that measures collaborating events in T-ALL was performed. Specifically, linearized DNA constructs were co-injected into one-cell stage embryos, and a portion of the resulting mosaic animals integrated transgenes as concatamers in their genome, leading to transgene co-expression within thymocytes and leukemic growth. To assess if Tox collaborates with Myc to accelerate the time to tumor onset, one cell stage TU/AB strain embryos were microinjected with linearized plasmids (1) rag2:m-cMyc + rag2:mCherry, (2) rag2:m-cMyc + rag2:ztox + rag2:mCherry, (3) rag2:ztox + rag2:mCherry.

Mosaic animals were assessed for the development of mCherry-positive thymocytes at 21 days post injection (dpi) and subsequently followed for time to T-ALL onset. Transgenic animals that co-expressed Myc and ztox exhibited a significant accelerated time to leukemia onset compared to zebrafish expressing Myc alone (28 ± 7.0 days, compared with 35 ± 14.0 days, p=0.02, Figure 11a). In addition to accelerating time to tumor onset, TOX also resulted in a more highly penetrated disease, where 60% of Myc and ztox co-expressing fish gave rise to leukemia
by day 28 compared to 10% of positive Myc fish (n=10, p=0.02; Figure 11a). The ztox gene was also injected alone to assess its ability to be oncogenic by itself. In this experimental group, thymic hyperplasia was observed by day 28 post injection; however, no tumor onset was observed in these fish (Figure 1a, blue curve). As a result, the Tox gene is not oncogenic by itself, and must collaborate with Myc to accelerate time to tumor onset.

Since Myc and Notch are central regulators of T-ALL progression, and both exert non-overlapping roles in regulating T-ALL pathways, we sought to utilize the Notch-induced zebrafish T-ALL model to assess if Tox could also collaborate with Notch to accelerate time to tumor onset independently of Myc. The following experimental groups were compared: (1) rag2: zNotch1a^{NICD} + rag2:mCherry, (2) rag2: zNotch1a^{NICD} + rag2:ztox + rag2:mCherry, (3) rag2: zNotch1a^{NICD} + rag2:ztox + rag2:mCherry.

Figure 11: Tox collaborates with Myc and Notch to promote T-ALL progression in mosaic transgenic zebrafish. (a) Kaplan-Meier analysis of disease progression in Myc and (b) Notch mosaic transgenic zebrafish. Fish were scored for leukemia onset. The total number of animals used (n) for each experimental group is 10 animals and the P-value comparing Myc/Notch transgenic fish to Myc/Notch + ztox expressing fish is shown.
(3) *rag2:ztox + rag2:mCherry*. My results show that Notch collaborates with Tox to significantly accelerate time to leukemia onset compared to Notch zebrafish alone (p=0.03, n=10). As shown in Figure 11b, 50% of Tox-Notch positive animals developed leukemia by 42 dpi, compared to Notch injected fish, which normally develop leukemia within seven months. Thus, the results indicate that Tox collaborates with both Myc and Notch independently to accelerate time to tumor onset. Subsequent analysis focused exclusively on T-ALL that developed in the Myc-induced transgenic model.

**Characterization of Myc induced transgenic T-ALL zebrafish**

T-ALL is typically characterized by arrest of malignant thymocytes at early stages of development that can frequently be assessed by size and morphology differences and infiltration into the blood stream and bone marrow (Vlierberghe and Ferrando, 2012). In order to observe if there is a difference in lymphoblast morphology between *Myc* and *Myc + Tox* injected fish, May-Grunwald Giemsa staining was performed. I observed that all tissues of the diseased fish, irrespective of whether the T-ALL was induced by Myc or Myc + Tox, have progressive infiltration of lymphoblasts (dark purple staining) into extrathymic structures when compared with wild-type fish (as observed by the arrowheads in Figure 12a). I also assessed lymphoblast morphology. Normal T-lymphocytes were obtained from wild-type kidney marrow, the site of hematopoiesis in zebrafish, and are largely characterized by lymphocytes spread throughout the sample and mixed with RBCs, macrophages and many different blood lineages. By contrast, T-ALL tissues were comprised of approximately 90% blasts, were organized in clumps, and were similar in size and morphology irrespective of genotype (Figure 12b).

In order to analyze the gene expression patterns in transformed lymphoblasts, quantitative real time PCR was performed on one fish from each experimental group: (1) Tu/AB wild-type,
(2) rag2:m-cMyc + rag2:mCherry and (3) rag2:m-cMyc + rag2:hTOX (human Tox) + rag2:mCherry. The human form of the TOX gene was injected here to more closely recapitulate human disease. First, transgene incorporation was measured (Figure 13a). Myc- and Myc + Tox-induced leukemias expressed almost equal levels of m-cMyc. The TOX gene was expressed in very high levels in the Myc + hTOX injected fish compared to Myc alone, as expected. This result verifies the expression of the transgenes and the equal mouse Myc expression levels, excluding the possibility that variation in Myc transgene levels correlates with T-ALL progression. Then, to confirm that the leukemias are of T-cell origin, the expression of the T-cell specific markers cd3, cd4, cd8, lck, rag2, and the B-cell specific marker, IgM were assessed.

\[
\begin{align*}
\text{Wild type} & \quad \text{Myc} & \quad \text{Myc+ Tox} \\
5X & \quad \text{image} & \quad \text{image} & \quad \text{image} \\
20X & \quad \text{image} & \quad \text{image} & \quad \text{image} \\
40X & \quad \text{image} & \quad \text{image} & \quad \text{image}
\end{align*}
\]

*Figure 12: Microscopy images of mosaic transgenic zebrafish with Myc and Myc + Tox-induced T-ALL. (a) Hematoxylin-Eosin stained sections of head sections from adult zebrafish (b) May-Grunwald Giemsa stained cytospun kidney marrow cells from wild-type and mosaic transgenic zebrafish from each genotype. Scale bar, 1mm.*
Each of the leukemias expressed high levels of T-cell specific markers but not IgM, confirming the derivation of these leukemias from cells of the T lymphoid lineage and not the B-cell lineage (Figure 13b). Interestingly, the Myc + hTOX transgenic animals expressed significantly higher levels of zcd8 and the T-cell specific gene, zlec compared to Myc alone transgenic animals, a characteristic of Tox that is also seen in Tox transgenic mice (See Background, Aliahmad et al., 2012).

Figure 13: Quantitative real-time reverse transcription-PCR analysis of mosaic transgenic zebrafish with Myc and Myc+Tox induced T-ALL. Relative expression of the (a) injected constructs (b) T-cell-specific genes cd3, cd4, cd8, Lck, rag2 (normalized to whole blood), and the B-cell gene IgM(normalized to whole kidney marrow) (c) T-ALL specific oncogenes scl, lmo2 and (d) hox11 in normal TU/AB thymocytes, and mCherry-positive T-ALL cells from mosaic transgenic animals.
We further investigated if TOX expression alters the developmental stages of T-cell arrest - reflected in the expression of characteristic T-ALL oncogenes. Expression of the T-ALL oncogenic transcription factors such as \textit{SCL}, \textit{LMO2} and \textit{HOX11} are well known to affect T-ALL progression and identify distinct molecular subtypes of disease (See Background). Specifically, Myc-induced transgenic zebrafish are known to express both \textit{SCL} and \textit{LMO2}, mimicking a specific molecular subtype of T-ALL. To determine whether the collaborating oncogene, Tox, alters the T-ALL subtype and induces the expression of other T-ALL oncogenes, the expression levels of the three most common transcription factors, \textit{SCL}, \textit{LMO2} and \textit{HOX11} were tested by real-time RT-PCR across the transgenic animals. As shown in Figure 13c, Myc tumors show an increase in the expression levels of \textit{Scl} and \textit{Lmo2}, whereas Myc + hTOX tumors do not show any expression of these transcription factors. By contrast, Myc + hTOX tumors show a significantly higher expression of \textit{Hox11} compared to Myc alone transgenic fish (Figure 13d). As a result, Tox appears to be changing the molecular signature of T-ALLs by acting to modulate different molecular pathways, suggesting that thymocytes might be arrested at an earlier stage of development.

**Effect of Tox activity on T-ALL proliferation or apoptosis**

To understand the effect of Tox in T-ALL progression, T-ALL zebrafish were analyzed for potential effects on cell cycle and apoptosis. However, due to an insufficient number of primary T-ALL zebrafish, tumors were transplanted into irradiated AB recipients and allowed to grow. Cell cycle (EdU staining) and apoptosis (Annexin V labeling) were performed on three tumors from each of the following experimental groups: (1) \textit{rag2:m-cMyc + rag2:mCherry} and (2) \textit{rag2:m-cMyc + rag2:hTOX + rag2:mCherry} transplanted fish. EdU cell cycle analysis indicated that transplanted \textit{Myc + hTOX} tumors show an increase in the percentage of cells in S-
phase compared to Myc transplanted leukemias (3.0 ± 0.75 cells compared to 0.6 ± 0.22 cells, P = 0.0002; Figure 14a). Additionally, AnnexinV staining revealed that Myc + hTOX transplanted T-ALL cells were less apoptotic than Myc-induced leukemias (20.8 ± 9.9 cells compared to 53.9 ± 5.4 cells, P = 0.002; Figure 14b). This data suggests that Tox expression increases the aggression of the tumors and alters both the proliferation and apoptosis rates in fully transformed T-ALL cells. However, since these are transplanted tumors, they might have already undergone epigenetic modifications that would affect the result obtained. Future experiments will assess a role for TOX in regulating proliferation and apoptosis in primary leukemias.

**Figure 14:** Myc + Tox-induced T-ALL shows an increase of aggressiveness in transplanted tumors. (a) EdU staining to quantify the percentage of cells in S phase of transplanted T-ALL transgenic strains. (b) AnnexinV staining of apoptotic cells of transplanted T-ALL transgenic strains. Each point represents one sample. ***P = 0.0002 and **P=0.002, Myc compared with Myc + Tox expressing transplanted T-ALL. N=3 for each experimental group.

**Tox TALEN mutagenesis in AB zebrafish**

Tox gain-of-function experiments in transgenic zebrafish have shown that TOX collaborates with Myc to alter the molecular signature, enhances time to tumor onset, and is
involved in increased aggression of the tumors likely by increasing proliferation and decreasing apoptosis levels. These experiments suggest TOX is sufficient to induce T-ALL aggression, but does not assess if it is required for T-ALL onset. To address this question, TALEN technology was utilized in order to create Tox knockout mutant zebrafish. First, each arm of the \( \text{ztox} \) TALEN was designed and transcribed into RNA (See Methods). Addition of a poly-A-tail was necessary for the stabilization of the RNA and can be visualized by gel electrophoresis as seen in Figure 16a. The increase in the size of the transcript, confirms poly-A-tail addition in both arms. The \( \text{ztox} \) TALEN left and right arms were then microinjected in one-cell stage AB embryos. To determine if mutagenesis of the TALENs was successful, genomic DNA from un-injected AB embryos and TALEN injected AB embryos was extracted from 12 embryos and a PCR corresponding to the mutagenized region was performed. If Tox mutagenesis worked, the T7E1 enzyme will cleave PCR products at sites of mismatch, resulting in shorter PCR fragments that can be visualized by gel (See Methods). As shown in Figure 16b, T7E1 treatment of the \( \text{ztox} \) TALEN injected embryos resulted in shorter 200bp fragments compared to no bands in the AB control T7E1 digested embryos, confirming successful mutagenesis of the TALENs. As a result, the remaining injected embryos were allowed to grow into adult fish in order to be able to establish founders with somatic mutations of the \( \text{ztox} \) gene. Future experiments will assess if Myc can transform TOX-deficient T cells.

**Investigation of Tox's cellular phenotype in T-ALL human cell lines**

To more closely investigate the role of Tox in T-ALL progression, we chose to extend our studies into human T-ALL cell lines. To verify the Tox phenotype in zebrafish, TOX expression was analyzed for its ability to have a similar effect in human disease. To determine
Extracted genomic DNA from 12 embryos.

Rest of embryos continue to grow into adult fish to establish founders.
which T-ALL cell lines express TOX, RNA and protein expression of TOX was investigated. As expected, TOX was expressed in 6 T-ALL human cell lines tested by q-PCR, and 12 of 16 by Western blot analysis (Figure 17). Since Tox was already verified as being over-expressed in microarrays of human T-ALL, it was not surprising to see its over-expression in human T-ALL cell lines. To understand the cellular phenotype of Tox in human T-ALL cell lines, we investigated if knockdown of Tox exerts effects on cell cycle and proliferation. MOLT4 shRNA stable knockdown cell lines were obtained and TOX knockdown was verified by western blot analysis (Figure 18a). Tox protein expression was reduced by 80% in sh1, and 70% in sh2, compared to the scramble shRNA (Figure 18a). Additionally, TOX knockdown was shown to

![Figure 17: Tox over-expression in human T-ALL cell lines. (a) real time quantitative RT-PCR showing relative expression of hTOX in 6 different T-ALL cell lines. (b) Western Blot showing protein expression of hTOX in 16 different T-ALL cell lines.](#)
decrease cell viability compared to wild-type MOLT4 cell line and scramble shRNA (Figure 18b). EdU cell cycle analysis was then performed on the knockdown cell lines and compared to the scramble shRNA. Interestingly, two different phenotypes were observed between the two different knockdowns (Figure 19). In sh1, a decrease in the number of proliferating cells (in S phase) was observed compared to the scramble, with no increase in the number of dividing cells.

**Figure 18**: Tox knockdown in Molt4 cell lines. (a) Protein expression by Western Blot portraying loss of Tox protein activity in the knockdown cell lines (TOX sh1 and TOX sh2). (b) Cell Viability of Tox knockdowns measured by Titer glo.
(in G2/M phase). Leading to the result that Tox loss leads to a decrease in proliferation. However, in sh2, cells appear to be proliferating (as observed in the increase in S phase compared to scramble) but not dividing. This shows that Tox knockdown leads to a S phase block, where cells are arrested in S phase and are not able to continue dividing. Due to conflicting results, it is hard to fully understand how TOX alters the cell cycle. However, different Tox knockdown levels might affect Tox's phenotype on the cell cycle. As a result, this experiment needs to be repeated on additional knockdowns and cell lines.

![Bar chart showing cell cycle distribution](image)

*Figure 19: EdU cell cycle analysis of Molt4 Tox knockdown cell lines (sh1 and sh2) compared to the scramble shRNA (scl) cell line.*

**Camptothecin drug treatment leads to sensitivity in Tox knockdown cell lines**

Recent mass spectrometry data from our laboratory suggests that Tox interacts with Ku70 and Ku80, two proteins that are involved in DNA damage repair mechanisms (Wang et al., 2006;
Reynolds et al., 2012). If TOX is involved in DNA damage, it would be interesting to assess if there is any correlation between the knockdown of TOX and the chemotherapeutic drug, Camptothecin. Camptothecin has been used as anticancer therapy for more than 15 years (Tomicic and Kaina, 2013) and acts by inhibiting DNA Topoisomerase I (TOPI), leading to the formation of bulky DNA-TOPI complexes that inhibit DNA replication and transcription. DNA repair mechanisms tend to resolve the TOPI structure that is covalently bonded to DNA (Christodoulou et al., 2012). As a result, the sensitivity or resistance to Camptothecin-based therapy (CPT) is based on the extent of DNA repair mechanisms (Tomicic and Kaina, 2013). If Tox expression in T-ALL is involved in DNA damage repair mechanisms where it acts by inhibiting the formation of bulky DNA-TOPI complexes, then knockdown of TOX will lead to increased drug sensitivity. MOLT4 stable knockdown cell lines were treated with five different doses of Camptothecin, and cell viability was measured 24 hours post treatment. Interestingly, Tox knockdown was found to exert similar effects to the scramble shRNA except at one specific concentration (100 nM) where sensitivity was observed (Figure 20). Thus, TOX imparts an elevated resistance to CPT, suggesting that TOX modulates DNA repair.
Figure 20: Effect of Camptothecin drug treatment on Tox knockdown cell line. Molt4 sh1 and sh2 knockdown cell lines were compared to scramble shRNA at 5 different drug concentrations. The circle represents the concentration at which Tox shows sensitivity to the drug (100 nM).
DISCUSSION

The HMG-box protein Tox has been implicated in the regulation of thymocyte selection, development and lineage commitment. In previous studies, genomic copy number abnormalities and microarray gene expression profiling identified TOX as over-expressed and amplified in a subset of human T-ALL. Although TOX function in thymocyte development is clearly understood, its role in T-ALL remains unknown. Here, we utilized a model of Myc-induced T-ALL in transgenic zebrafish to assess the molecular mechanisms underlying TOX expression in T-ALL.

Tox collaborates with Myc and Notch to increase T-ALL aggression

As part of a zebrafish genetic screen, our laboratory sought to identify if Tox over-expression in the Myc-induced model of T-ALL would lead to an increase in tumor aggression. As shown in Figure 11a, Tox greatly enhances the time to tumor onset in collaboration with Myc, but fails to induce leukemia when injected alone. This data suggests that Tox is not oncogenic by itself but must collaborate with Myc to increase T-ALL aggression. Next, the Notch-induced zebrafish model of T-ALL was also assessed for collaborating genetic events. Normally, Notch induces leukemia within seven months post injection. The data in Figure 11b show that Tox collaborates with Notch to induce leukemia within only 50 days post injection. Therefore, Tox collaborates with both Myc and Notch independently to accelerate time to tumor onset and increase tumor progression.

Tox expression correlates with a high HOX11 molecular signature

Since Tox appears to be utilizing a specific mechanism to increase T-ALL aggression, we further characterized the Myc + Tox induced tumors to assess whether Tox alters the molecular subtype of T-ALL. First, high expression of the T-cell specific markers (cd3, cd4, lck, rag2) and
not the B-cell specific marker (IgM) was observed in both Myc and Myc + TOX tumors, verifying that the tumors are T-cell in origin (Figure 13b). Interestingly, a significant increase in the cd8 population in the Myc + Tox tumors was observed compared to Myc-induced leukemias. This phenotype correlates well with what is observed in mice, where over-expression of Tox leads to an increase in the CD8+ population (Aliahmad et al., 2012). However, this phenotype needs to be explored further in order to identify whether the increase in cd8 is in the DP, DD or SP stages of T-cell development. This could be performed by FACS of the tumor population with specific markers targeting the different stages of CD8 T cell development. The tools and protocols for proceeding to FACS experiments on zebrafish cells are currently in development in the laboratory.

The expression of three transcription factors (SCL, LMO2 and HOX11), known to induce two different subtypes of T-ALL, were examined. Myc-induced T-ALL expresses both SCL and LMO2, mimicking a specific molecular subtype of T-ALL (Langenau et al., 2003). Our hypothesis was that Tox expression would alter the T-ALL subtype and induce expression of different oncogenic transcription factors. In fact, Scl and Lmo2 expression were not observed in T-ALLs that express both Myc and TOX, whereas Myc alone expressing tumors expressed high levels of both of these genes (Figure 13c). Instead, HOX11 was highly expressed in the Myc + TOX tumors when compared to those that express Myc alone, verifying that Tox might act through a HOX11 molecular signature rather than the expected SCL and LMO2 signature observed in Myc-induced leukemias.

HOX11 is expressed in the early cortical stages of T-cell development after the αβ TCR rearrangements and formation of the DP T-cell population (Figure 21). This correlates well with Tox's expression in T-cell development, where it is highly expressed in DP thymocytes (Figure
On the other hand, SCL (TAL1) expression appears in the late cortical stages of T-cell development (Figure 21). We can therefore speculate that Tox expression in T-ALL leads to an earlier block in T-cell development. However, due to an insufficient number of primary tumors from each experimental group, this experiment will need to be repeated with more leukemic zebrafish in order to verify the observed phenotype. Furthermore, it will be important to correlate the expression obtained through qRT-PCR with protein expression using Western Blot analysis. High protein expression of HOX11 in Myc + Tox induced leukemias vs. Myc tumors will validate the phenotype observed.

Over-expression of Tox correlates with increased proliferation and decreased apoptosis

To further investigate how TOX regulates tumor aggression, it was crucial to assess the difference in proliferation and apoptosis between T-ALLs that express Myc alone versus those that express both Myc + TOX. My data suggest that Tox expression increases the number of proliferative cells and decreases the number of apoptotic cells. As a result, Tox expression
increases the aggression of the tumors by altering both the proliferation and apoptosis rates in fully transformed T-ALL cells. It will be important to repeat these experiments in primary T-ALL to confirm this role in primary leukemias.

**Loss of Function Experimental plans**

Tox gain-of-function experiments in zebrafish provided us with some insight into the mechanisms by which Tox acts to increase aggression in the Myc-induced model of T-ALL. However, Tox loss-of-function has yet to be assessed for modulating T-ALL growth and progression. To address this question, I have utilized the TALEN knockout technology in hopes of creating Tox-deficient zebrafish. If Tox mutant zebrafish are not embryonic lethal, our ultimate goal is to address the effect of Tox loss of function on the Myc leukemia onset. Since it takes four months to establish founders for heterozygous Tox mutants and an additional four months to generate homozygous null animals, I have been unable to perform the experiment outlined below due to time constraints (Figure 22). However, based on what we observed in the gain-of-function model, we expect to see a decrease in time to tumor onset in the Tox mutant zebrafish. Figure 22 below summarizes the expected results from this experiment. However, in the case where time to tumor onset is not altered or where the mutants are embryonic lethal, additional experiments to perform include in situ hybridization or ISH for HSC markers, such as RUNX1 and Myb in order to assess if Tox loss of function affects HSC development.

**Assessment of the Cellular Phenotype of Tox in human MOLT4 T-ALL cell lines**

To verify that Tox exerts similar effects in human disease, we extended our studies to human T-ALL cell lines. The MOLT4 cell line was chosen in particular due to its high expression of Tox (Figure 17). Tox was first knocked down in the MOLT4 cell line and its effect
on cell viability was assessed. Results show a significant decrease in cell viability compared to the scramble (Figure 18b). Our next question was to address the cause of this phenotype. Cell cycle analysis revealed two different phenotypes corresponding to the two different knock down cell lines used (Figure 19). In sh1, results show that Tox reduction lead to a decrease in proliferation. By contrast, knockdown with sh2 resulted in S phase arrest and subsequent failure to divide. Due to these conflicting data, my results are inconclusive with respect to what phase of cell cycle analysis revealed two different phenotypes corresponding to the two different knock down

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Figure 22: Tox loss-of-function experimental plans. After establishing Tox mutant founders and heterozygous fish, a cross between Tox heterozygous mutants will be performed and embryos will be injected with the rag2-mouse cMyc transgene. Time to tumor onset will be assessed using Kaplan Meier analysis. Our prediction is that Tox homozygous mutant zebrafish will develop leukemia at a much slower rate than the heterozygous or wildtype fish.

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the cell cycle is affected by TOX knockdown. This could be due to different knock down levels between the two different cell lines (sh1 and sh2). Therefore, this experiment needs to be verified with additional Tox knockdowns and additional cell lines. Future experiments that analyze the effect of Tox on cell cycle progression should be focused only on analyzing the S phase of the cell cycle by synchronization of all the cells at this specific phase. This will allow for easier interpretation between the different knock down cell lines. Additionally, it would be interesting to assess if high expression of the cell cycle proteins are present in sh2, where we observe a block of the cells in S phase.

**Effect of Camptothecin Drug treatment on Tox knockdown cell lines**

Camptothecin (CPT) is a chemotherapeutic drug known to act through the inhibition of DNA Topoisomerase I leading to a block in DNA replication. Sensitivity or resistance to CPT therapy depends on the extent of DNA damage repair mechanisms (Tomicic and Kaina, 2013). Recent laboratory data suggested an interaction of TOX with Ku70 and Ku80, two proteins known to play a role in DNA damage repair mechanisms. If TOX is involved in DNA damage repair, our hypothesis was that a knockdown of TOX would lead to sensitivity to CPT treatment. However, the results obtained show a striking similarity between the knockdown cell lines and the scramble control, except at one concentration (100 nM), where Tox knockdown correlates with CPT sensitivity (Figure 20). In order to investigate this further, drug treatment with this specific concentration at different time intervals will need to be assessed. Moreover, it will be crucial to assess the effect of using a combination of drugs (ex. Dexamethasone + Camptothecin) on the knockdown of TOX. Furthermore, to validate the results, it will be important to address if the opposite effect (resistance to CPT) is observed when Tox is over-expressed in our in vivo zebrafish model of T-ALL.
Future Directions

Although this work has established the importance of TOX in the development and progression of T-ALL, it remains unclear what the exact function of TOX is in T-ALL. My finding that TOX converts Myc expressing T-cells into leukemias that have a HOX11 molecular signature opens new opportunities to study how Tox regulates differentiation arrest in the context of Myc and Notch. This will allow us to better understand the molecular pathways utilized by TOX and be able to have specific targets for drug treatments. Furthermore, since it remains unknown whether Tox is a transcription factor, our lab is interested in answering this question. This can be accomplished using ChIP sequencing technology. In this regard, we would be able to identify whether Tox binds in a specific-sequence manner or acts independently. If Tox acts in a sequence-specific manner, we would be able to identify the specific targets it regulates and whether it is positive or negative regulation. After further characterizing the role of TOX in T-ALL, our ultimate goal is to have specific drug targets for the treatment of the human T-ALL disease.
BIBLIOGRAPHY


