DYSREGULATION OF A NOVEL TYROSINE KINASE TARGET TYRO3 SIGNALING IN ACUTE MYELOID LEUKEMIA AND RELATED HEMATOLOGICAL MALIGNANCIES

By

Fatma Eryildiz

A THESIS

Presented to the Institute of Environmental Health,
Division of Environmental and Biomolecular Systems
and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Master of Science

August 2016

Division of Environmental & Biomolecular Systems

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Master's thesis of

Fatma Eryildiz

has been approved

Dr. Peter Zuber
Thesis Committee Chair

Dr. Jeffrey W. Tyner
Thesis Research Advisor

Dr. Anupriya Agarwal
Thesis Committee Member

Dr. Holly Simon
Thesis Committee Member

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List of Abbreviations

ABL1	Abelson murine leukemia viral oncogene encoded by ABL1 gene
AML	Acute Myeloid Leukemia
ATCC	American Type Culture Collection
AUC	Area under the curve
BCR-ABL	Fusion gene on chromosome 22 known as “The Philadelphia Chromosome”
cDNA	complementary DNA
CML	Chronic myeloid leukemia
CSF1R	Colony stimulating factor 1 receptor
DMSO	Dimethyl sulfoxide
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
FGFR	Fibroblast growth factor receptor
FLT3	Fms-like tyrosine kinase
FLT3-ITD	Fms-like tyrosine kinase with internal tandem duplication
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS6	Growth-arrest specific protein
GIST	Gastrointestinal stromal tumor
GM-CSF	Granulocyte macrophage colony-stimulating factor
HiDAC	High dose cytarabine
IC50	The half maximal inhibitory concentration
IL-6	Interleukin 6
JAK	Janus protein tyrosine kinase family
KIT	Gene name for KIT receptor tyrosine kinase
NRAS	Neuroblastoma RAS viral oncogene

NS	Non-specific or non-targeting siRNA
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PLK1	Polo-like kinase 1
PROS1	Protein S (Alpha)
PTMs	Post translational modifications
RNAi	RNA interference
RNA-SEQ	RNA whole transcriptome sequencing platform
RPMI	Roswell Park Memorial Institute Media
RTK	Receptor tyrosine kinase
RT-qPCR	Real-time quantitative PCR
SEM	Standard error of the mean
shRNA	short hairpin RNA
siRNA	small-interfering RNA
STAT5	Signal transducer and activator of transcription 5
TAM	A subfamily of receptor tyrosine kinases (Tyro3, Axl, Mertk)
TaqMAN	Hydrolysis probes used in quantitative PCR
TCGA	The Cancer Genome Atlas
TKI	Tyrosine kinase inhibitor
TNF alpha	Tumor necrosis factor 6
WHO	World Health Organization
WT	Wild-type

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude to my thesis advisor Dr. Jeffrey W. Tyner. His broad knowledge and critical thinking have been of great value for me. I am grateful for his guidance, advice, criticism, considerate attitude, encouragements and insight throughout the research. I really feel lucky that I was able to study under his supervision.

I want to thank Dr. Peter Zuber for accepting to be my thesis committee chair. I am grateful to him for standing up for me when I needed a new chair in the middle of my master's degree. I am also pleased for his comments and feedback during my research.

I would like to thank Dr. Holly Simon and Dr. Anupriya Agarwal for agreeing to be on my thesis committee and their valuable advices and helpful comments during preparation of this thesis.

I should also acknowledge Drs. James and Mei Whittaker. My first stop at OHSU was Whittaker lab and they were both very helpful for me to start both my OHSU and academic life. I admired Dr. Whittaker's attention to details in the experiments. I am thankful to him for being on my side while I take my first steps in my research career.

I am grateful to my lab-mates, all members of Tyner and Druker labs for their cooperation and creating scientific aura that helped me to grow a lot. Even though I am not able to write the names of every single member of this family, I should present my special thanks to Marilynn Chow for her excellent guidance and experimental helpfulness. Also Haijiao Zhang and Tamilla Nechiporuk were great helpers on my experimental designs. Without their help, neither this thesis nor my current practical knowledge

in the field would be possible. My thanks are also extended to all those who have helped me in my study and research, especially to our lab's fairy godmother Kara Johnson.

I must thank everyone else associated with Institute of Environmental Health, especially Nievita Bueno Watts and Vanessa Green for their great care and sincere helpfulness throughout my master's degree.

Lastly, I want to thank my family and friends. I want to send my sincerest gratitude to my father Tarik, who always supports me and my education abroad, thus all my success here is also his. I want to thank him for his endless emotional support. Additionally, my boyfriend who stood by me all the time, my biggest supporter against challenges in our daily lives with his advises. His joy in my good days and his propitiation in my bad ones give me strength when I am walking on my career path.

My friends from all around of the world always send me best wishes all the time in order not to make me feel alone. I especially appreciate my friend, Kirbee Johnston who also gave her valuable feedback during preparation of this thesis.

Finally, my master education was supported by Turkish Government under a merit scholarship program. Thus, I'd like to thank generosity of my home country that embraced me thru all my life including my time far from it.

Abstract

Acute myeloid leukemia (AML) is a form of blood cancer which mostly affects older people and undesirable outcomes of standard chemotherapy approaches are intolerable for this group of patients. Therefore, it is crucial to find more targeted and less toxic therapies for the treatment of AML.

Tyrosine kinases have been shown as outstanding targets for novel targeted cancer therapies by numerous studies. In order to determine novel tyrosine kinase targets as functional drivers of leukemogenesis, our lab employed a functional RNAi-assisted target identification assay. Using this method, we screened a significant number of AML patient specimens against a panel of siRNA which targets every individual tyrosine kinase and then we recorded the impact on cell viability.

Bioinformatics interpretation of screening data revealed significant sensitivity against silencing TYRO3 (8.16%) compared to other members of TAM family i.e. AXL (1.39%) and MERTK (0.35%) in our tested AML cases (n=576). Additionally, we validated TYRO3 tyrosine kinase sensitivity by individually silencing TYRO3 in several AML cell lines and observed decreased cell viability relative to non-specific siRNA in TYRO3 overexpressing cell lines. These both suggest that high expression of TYRO3 provides a cell survival advantage in AML.

We made further analysis seeking a correlation between TAM family receptor expression and TAM family targeting tyrosine kinase inhibitor sensitivity. According to our heat map analysis, we found that the expression of TYRO3 along with its ligands, GAS6 and PROS1 slightly correlate with drug sensitivity parameters. This finding also supports the hypothesis that TYRO3 plays a role in AML cell survival.

Finally, we found that TYRO3 and GAS6 mRNA levels both significantly increase in the TYRO3 sensitive patient group compared to the insensitive group in our validation experiments. Roughly 75% of TYRO3 sensitive AML patients have either TYRO3 or GAS6 mRNA increased by 2-3 fold (RNA-seq, n=82 and qPCR data, n=15). Besides, ligand stimulation on the TYRO3 dependent cell cultures following a serum starvation resulted in increased phospho-STAT5 protein level which is an important marker for tyrosine kinase activity and leukemia prognosis.

In summary, results of siRNA panel screening and subsequent validation experiments suggest that both the primary AML patient cells and the leukemia cell lines show a strong correlation between TYRO3 expression and sensitivity against TYRO3 mediated knockdown. Our RNA seq, qPCR, and immunoblotting results imply that TYRO3-GAS6 interaction is associated with leukemia cell survival in TYRO3 overexpressing cells. Therefore, this interaction is a promising target to induce leukemia cell death.

Chapter 1: Introduction

Acute myeloid leukemia (AML)

Acute Myeloid Leukemia (AML) is a form of blood cancer which is characterized by clonal expansion of abnormal dedifferentiated white blood cells (blasts) in the peripheral blood, bone marrow, and other tissues. AML is the deadliest and the most common form of acute leukemia among adults [1]. AML accounts for 1.2% of all types of cancer deaths in the United States [2]. An estimated 19,950 people will be diagnosed with AML in 2016, and 10,430 patients will die of the disease [3]. More than half of the patients are diagnosed at 65 years or older and a third of them are diagnosed at older than 75 years of age. With the advent of new therapeutic tools, AML is treatable in 35-40% of adult patients who are 60 years old and younger and in 5-15% of patients older than 60 years old [4, 5].

The classification of AML is critical to provide the unique form of treatment. For this purpose, a patient's bone marrow and peripheral blood are examined by light microscopy and flow cytometry to detect genetic abnormalities to diagnose AML and to identify the AML subtype causing disease. AML classification is based on World Health Organization (WHO) Classification of Tumors of Hematopoietic and Lymphoid Tissues which was last updated in 2008. According to AML classification by WHO, AML is categorized under some major categories: AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related AML, and AML not otherwise specified [6, 7, 8].

Furthermore, with the advent of next-generation sequencing, the mutational landscape of many cancers including AML has been revealed by studies of The Cancer Genome Atlas Research Work (TCGA) [9]. They have analyzed the genomes of 200 patients with AML using whole exome sequencing, RNA, microRNA sequencing and DNA methylation analysis. According to their studies, the most commonly mutated genes in AML are organized into functional categories: mutations in signaling genes (FLT3), mutations in myeloid transcription factors (RUNX1, NPM1) and transcription factor fusions by chromosomal rearrangements spliceosome-complex genes (SRSF2, SF3B1, U2AF1, and ZRSR2), genes involved in the epigenetic homeostasis of cells (ASXL1 and EZH2), cohesion complex gene mutations (STAG2 and RAD21), genes important for DNA methylation (DNMT3A, TET2, IDH1 and IDH2) and tumor suppressor genes (TP53) [9].

Also, each of these common mutations was analyzed in terms of their frequency and functional significance in the patient cohort. The frequent occurrence of mutations in receptor tyrosine kinase genes such as FLT3 and KIT has drawn the attention of researchers to develop effective tyrosine kinase inhibitors and eventually improve the outcomes of the AML patients [28].

Standard chemotherapy regimens for the treatment of AML

Treatment options for AML vary among patients based on the patient's age, cytogenetics, and prognostic factors [10]. Typically, AML treatments are designed for two group of patients based on their age: patients younger than 60 years and those 60 years and older.

AML is treated in two steps of chemotherapy depending on whether a patient is eligible for an intensive chemotherapy which is given in the first chemotherapy stage and is known as induction chemotherapy. The first chemotherapy stage starts with induction chemotherapy aiming to reduce the number of leukemic cells to an undetectable number, then if the initial therapy provides a complete remission to the patient then the patient continues to receive additional chemotherapy (consolidation or post-remission therapy) aiming to eradicate any residual leukemia cells as the second stage of chemotherapy. If post-remission therapy is not applied, almost all patients relapse AML [11].

The most common induction regimen is 7 + 3 because it includes cytarabine induction for seven consecutive days and anthracycline (generally daunorubicin) induction for following three consecutive days. Since this therapy has very toxic effects on patients such as tumor lysis syndrome, cardiac abnormalities, tissue necrosis, pancytopenia, nausea and vomiting, alopecia and death, elderly patients cannot tolerate these toxic effects of the therapy [8, 12, 13]. Thus, it is not recommended for patients older than 60 years old.

After initial chemotherapy, a bone marrow biopsy is applied to the patients to assess the presence of residual leukemia cells and if residual leukemia is detected, patients are treated with post remission chemotherapy to remove any residual leukemia cells. Post remission therapy includes high-dose cytarabine (HiDAC) for patients younger than 60 years old. While the younger patients yield a 4-year disease-free survival rate of 44%, 5% of the patients failed to survive due to treatment-related mortality. Unfortunately, the disease-free survival rate at 4 years decreases to 15% in the older patients [8, 12, 13].

To sum up, it is obvious that the toxicities based on all these less targeted chemotherapy regimens cannot be well tolerated by older adults.

Tyrosine kinases and targeted therapy implications for leukemia

Protein tyrosine kinases are a group of enzymes that transfer a phosphate group from ATP to tyrosine residues in acceptor polypeptides. So, these enzymes are collectively responsible for activation of downstream proteins by phosphorylation. They are coded by 90 TK and 43 TK-like genes. Their products regulate cellular proliferation, survival, differentiation, and cell motilization [14]. Thus, it has been shown that tyrosine kinases are associated with cancer pathogenesis and researchers have been targeting tyrosine kinases to find an efficient treatment for some cancer types.

Tyrosine kinase activity

Tyrosine kinases can be divided into two groups: receptor tyrosine kinases (RTKs) e.g. EGFR, PDGFR, FGFR and non-receptor tyrosine kinases (non-RTKs) e.g. SRC, ABL, FAK and Janus kinase.

The structural organization of the receptor tyrosine kinases consists of a single pass transmembrane hydrophobic helix with a specific ligand-binding extracellular domain and a catalytic intracellular kinase domain (ATP binding site) which is able to bind specific tyrosine residues in the acceptor peptides. Upon binding of a specific ligand to the extracellular domain causes a series of structural rearrangements in the RTK such as oligomerization of the receptor that leads to an increase in the enzymatic activity of the enzyme. It triggers a cascade of events through phosphorylation of intracellular proteins that transduce

the extracellular signal to the nucleus, causing changes in gene expression by activating multiple signaling pathways. Since their activity is strictly under control by the antagonizing effect of tyrosine kinase and tyrosine phosphatases, non-proliferating cells express a very low level of phosphorylated form of these proteins.

The non-receptor tyrosine kinases do not carry transmembrane domains and are found in the cytosol, the nucleus, and the inner surface of the plasma membrane. Non-RTKs are maintained in an inactive state by cellular inhibitor mechanisms and they are activated by a variety of intracellular signals through dissociation of inhibitors [14, 15].

As discussed above, tyrosine kinases can be constitutively activated by common mechanisms, namely gene mutation, chromosome translocation or overexpression. Their constitutive activation causes the growth stimulation which is conveyed to the nucleus without regulation.

One primary TK activation mechanism and probably the most investigated one is the fusion of a receptor or non-receptor TK with another protein as a result of chromosomal translocation. The new fusion protein causes constitutively active TK in the absence of ligand binding. The well-known example of this mechanism is BCR-ABL fusion gene, which is known as Philadelphia chromosome or Philadelphia translocation resulted by the reciprocal translocation of the genetic material between chromosomes 9 and 22. The product of this genetic abnormality is BCR-ABL fusion protein which contributes to TK constitutive activation [20].

Gene mutation is another mechanism which disrupts autoregulation of the tyrosine kinases. An example is mutations in Fms-like tyrosine kinase (FLT3) receptor in AML alter receptor signaling. It has been

shown that in AML, about 25–30% of patients harbor a constitutively active receptor tyrosine kinase FLT3 encoded by a FLT3 allele harboring internal tandem duplication (FLT3-ITD) mutation [16].

As a third mechanism, a TK can be dysregulated by an increased or aberrant expression of a receptor tyrosine kinase, its ligand or both. EGFR is overexpressed in many cancers, including breast as the most striking example [17, 18].

Lastly, differently from those mentioned mechanisms, a TK activity can increase due to a decrease in factors (tyrosine phosphatases or TK inhibitor proteins) limiting tyrosine kinase activity.

When the TKs' multiple level regulation taken into consideration, they appear as important agents for therapeutic uses in cancer treatment. They can be inhibited to interrupt their signaling pharmacologically by anti-TK drugs. Tyrosine kinase inhibitors as anti-TK drugs work to inhibit the catalytic activity of the protein tyrosine kinases but they do not inhibit protein kinases that phosphorylate serine or threonine residues. Example uses of such kinase inhibitors are discussed in the following section.

Kinase Inhibitors

The Success of Imatinib

Imatinib, one of the most successful and effective tyrosine kinase inhibitor (TKI), targets BCR-ABL tyrosine kinase which has been implicated as the direct cause of CML [14, 19]. What makes BCR-ABL an attractive target for TKI implication is that it has been found in 95% of patients with CML and also 5-10% of adults with acute leukemia who do not have a previously known CML history [20, 21]. It is also shown

that imatinib induces clinical outcomes of the patients who carry those mutations by suppressing the proliferation BCR-ABL-expressing cells in vitro and in vivo [20, 22]

Imatinib mesylate (Gleevec) targets several TKs including c-KIT and platelet-derived growth factor receptor (PDGFR) other than BCR-ABL. Similarly, it has been shown that imatinib is effective in some solid tumors such as gastrointestinal stromal tumors (GIST). Patients diagnosed with GIST frequently contain activating gene mutations in either KIT or platelet-derived growth factor A (PDGFRA). Since imatinib also inhibits the activity of the c-KIT and PDGFR kinases, it produces varying clinical responses in GIST similar to those in CML [23].

FLT3 Inhibitors

FLT3 is one of the major target RTK in AML which is encoded by the FLT3 allele with an activating mutation, the internal tandem duplication (FLT3-ITD). The presence of this mutation has been associated with poor disease-free and overall survival [16, 24, 25]. Several FLT3 inhibiting TKI has been developed and are being tested clinically. So far, these small-molecule inhibitors have provided a partial or transient response in the FLT3-ITD positive patients [16]. Thus, researchers are trying to understand the biology of FLT3 to find the ways to improve clinical outcomes of FLT3 inhibitors.

Purpose of this thesis

This chapter has described that targeted therapies of cancer and AML, in particular, differ from standard chemotherapy in several ways. For instance, targeted therapies act on a specific molecular target that

is critical for cancer cell survival in a group of patients or a cancer type, however, conventional therapies affect all rapidly dividing normal or cancerous cells. The targeted drugs are designed or chosen to interact with their target particularly, but medication used in standard therapies are empirically identified as they kill actively dividing cells, either cancerous or normal. Taking all of these facts into account, we can conclude that targeted cancer therapies are less toxic and tolerable compared to standard therapies [26]. Thus, defining new tyrosine kinase targets is critical for improving treatment strategies since aberrant tyrosine kinase activities are associated with the pathogenesis of leukemia and other cancers. Using the benefits of functional screening strategies for tyrosine kinase target identification, we endeavored to address some questions in the context of this thesis.

Chapter 2 will describe and assess the work we did to show how functional screening applies to the identification of a novel target and how these targets imply cancer pathogenesis. In order to do that, we have analyzed the screening data of a large patient cohort and investigated whether TYRO3, as a TAM family tyrosine kinase target, may be important for AML pathogenesis or not. Also, I detailed and confirmed my findings from patient data regarding how the loss of a dysregulated tyrosine kinase signaling is responded in terms of viability in immortalized leukemia cell lines.

Chapter 3 will continue to describe the bioinformatics data analysis of the patient cohort to show the correlation between TYRO3, its ligand or both overexpression and siRNA silencing or drug inhibition in patients diagnosed with AML and related hematological malignancies.

Finally, Chapter 4 will describe the determination of dysregulated TYRO3 signaling and how dysregulation maintains pro-survival signaling in leukemic cells. These will be discussed along with what is necessary for future TYRO3 signaling studies.

Chapter 2: TYRO3 as a novel tyrosine kinase target in AML

Biology of TYRO3

TYRO3, AXL, and MERTK are members of the TAM family of kinases, which share the same ligand, namely growth arrest-specific 6 (GAS6). GAS6 binding to these family members is shown to control cell survival and proliferation through the PI3K/AKT/mTOR or RAF/MEK/ERK1/2 pathways [29, 30]. Their aberrant expression has been described by previous studies in several cancer types [31] including AML [32-37], multiple myelomas [38] and melanoma [39-47].

Very few studies reported TYRO3 as a new therapeutic target while the other members of TAM family are dominating current literature. Especially, AXL is the most studied kinase of this family regarding its role in chemosensitivity and prognostic potential [16].

All three members of TAM family like all other receptor tyrosine kinases consist of an extracellular domain, a transmembrane domain, and a conserved intracellular kinase domain. In particular, they share a unique KWIAIES sequence in their conserved kinase domain. More specifically, two immunoglobulin-like (Ig) domains and two fibronectin type III (FNIII) domains comprise nearly the entire ectodomain of each family member (Figure 1). Also, phosphorylated tyrosine residues are shown, asterisks indicate autophosphorylation and known SH2 domain docking sites[48].

TYRO3, AXL, and MERTK genes represent a similar genomic structure that is between 3 kb and 5 kb, encoded by, respectively 20, 20 [49, 50, 51] and 19 exons [52-55]. Three different splice variants have

been identified for TYRO3 containing either exon 2A, exon 2B or exon 2C [56, 57, 58]. They encode a signal peptide which might be important for posttranslational processing, localization, and function of TYRO3.

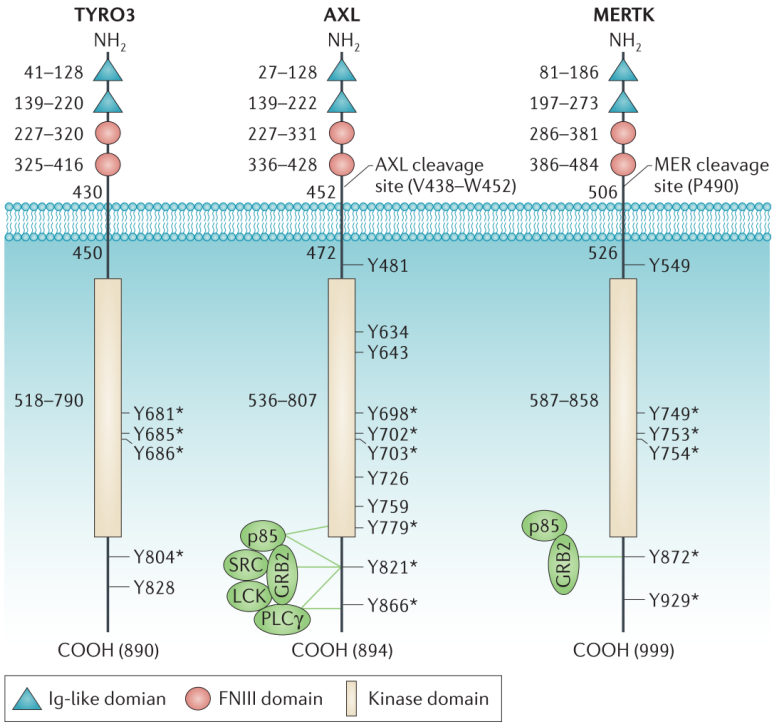

Figure 1. Schematic representation of TAM family receptor tyrosine kinases[1].

The genomic structures of TYRO3 and AXL are similar to each other more than to MERTK. But in terms of tyrosine kinase domain amino acid sequence, TYRO3 differs from the other two members of TAM family [59, 60, 62]. TYRO3 contains 890 amino acids and the molecular weight of TYRO3 is around 110

[1] Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] [31].

kDa as a result of posttranslational modifications (PTMs) such as phosphorylation, glycosylation or ubiquitination [63, 64] .

Ligands of TAM family

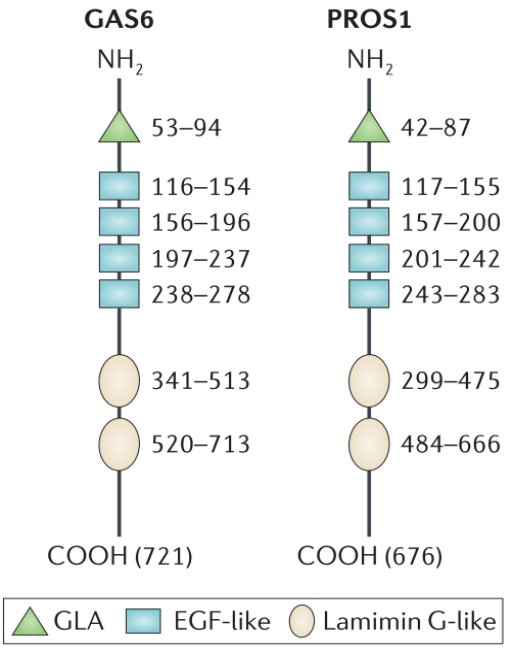

Figure 2. Structural representation of two best characterized ligands for TAM family[2].

The vitamin-K dependent protein GAS6 and the anticoagulant factor protein S1 (PROS1) are the known ligands of TAM family, although GAS6 binds and activates all three of the receptors with equal affinity

[2] Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] [31].

to TYRO3 and AXL and 3-10 fold lower affinity to MERTK [65, 66]. PROS1 only activates TYRO3 and MERTK. To the best of our knowledge, no known interaction between AXL and PROS1 is shown yet.

Both of the ligands share the same domain structure, but while PROS1 has thrombin cleavage sites, GAS6 does not contain these sites [67, 68]. For full activation of both, they need to carboxylate their glutamic acid residues which are located in the N-terminal domain in a vitamin K-dependent manner [69].

Activation of TYRO3

TAM RTKs can be activated conventionally or ligand independent. Conventional activation of RTKs requires a ligand/growth factor binding to the extracellular domain of the kinase, and subsequently ligand binding induces receptor dimerization and autophosphorylation its tyrosine residues within the cytoplasmic domain which create docking sites for other downstream regulatory proteins to bind and activate complex molecular interactions. Y681, Y685, Y686, and Y804 are the known auto phosphorylation sites for TYRO3 [31] as depicted in Figure 1.

In some cases, receptors can be activated without a ligand binding when they are overexpressed in a tissue type. They can form dimers on the cell surface in the absence of a ligand. Also, interestingly a heteromeric interaction is shown among the three TAM receptors using a xenograft mouse model. The study on $TYRO3^{-/-}$ and $MERTK^{-/-}$ mouse showed that AXL protein surface expression significantly decreased compared to WT. So, it might support the idea that the receptors are communicating with each other. [70].

In addition to these activation mechanisms, not exclusively TAM RTKs but in general all RTKs can be deactivated by several methods such as dephosphorylation of activated residues by phosphatases, receptor degradation or ligand dissociation [71]. Previous studies uncovered different forms of deactivation, including receptor solubilization and truncation in murine and human tissues [72-75]. However, it is important to bear in mind that the deactivation mechanism of this family needs further investigation.

Functions of TAM family

TAM family RTKs play a major role in engulfment and phagocytic clearance of apoptotic cells. Clearance of apoptotic cells is very important to maintain tissue homeostasis. In human body, more than 10^9 apoptotic cells are produced and removal of these cells are immediately carried out mainly by macrophages and to a lesser degree by dendritic cells. Macrophages express all three TAM receptors. The ligands GAS6 or PROS1 binds to these receptors, especially to MERTK or AXL, and this link might mediate macrophage recognition of apoptotic cells. This cleaning process is also crucial for resolution and termination of an inflammation [76, 77]. If one of these cleaners fail, it may prepare the environment to trigger or progress towards an autoimmune disease [78, 79].

TAM family RTKs also are important for feedback inhibition of the proinflammatory cytokine responses of the innate immune system to the pathogens. Dendritic cells use pattern recognition and Toll-like receptors to detect an antigen, virus particle or other pathogen-derived substance. Then, these receptors activate the proinflammatory cytokine responses such as tumor necrosis factor (TNF) α, interleukin (IL)-6 and type 1 interferons. These cytokines are important for the development of

inflammation response but their activation is supposed to be under control; otherwise it may lead chronic inflammation [80, 81].

Functional genomics

Functional genomics studies endeavor to answer the complex biological questions at the level of genes, RNA transcripts, and protein products. It starts by asking questions such as how the genes are regulated or how their expression changes when experiencing a disease. Several high throughput techniques are the workhorses for the functional genomics studies. Examples of these techniques include expression profiling microarrays, RNA sequencing, loss-of-function techniques and also bioinformatics tools to analyze data produced by these techniques to understand and discover the biologically meaningful patterns of how these interaction complexes are organized or disorganized. So that, we could provide a complete picture of how an organism's genome is transferred into meaningful data and then this data is processed to change or disrupt the function of the biological system in the RNA or protein level. In our research we utilized some of these high-throughput techniques to identify functional tyrosine kinase target genes.

Rapid target identification by functional siRNA screening

We have utilized an RNAi screen targeting tyrosine kinases to assess the contribution of a patient's kinome to the development of acute myeloid leukemia and related precursor neoplasms (Figure 3).

Using this tool, we have the opportunity to selectively reduce the expression of a specific tyrosine kinase genes (loss-of-function) and see the effects of this reduction on cell viability. This efficient method allows us to determine the potential targets behind the molecular vulnerabilities of cancerous cells [27].

In a siRNA panel, primary patient cells (10^5) are suspended in siPORT buffer (which contains the components that ensure enhanced delivery of intact siRNAs without degradation into cells) and incubated with 1 µM siRNA from a siRNA library individually targeting each member of the tyrosine kinome and pooled nonspecific siRNA controls. Cells are electroporated on a 96-well electroporation plate at 2220 V (equivalent of 300 V), 100 µsec, and 2 pulses. Then, cells are re-plated into culture media. 4 days later, cell viability and proliferation are determined by an MTS assay [27] (Figure 3).

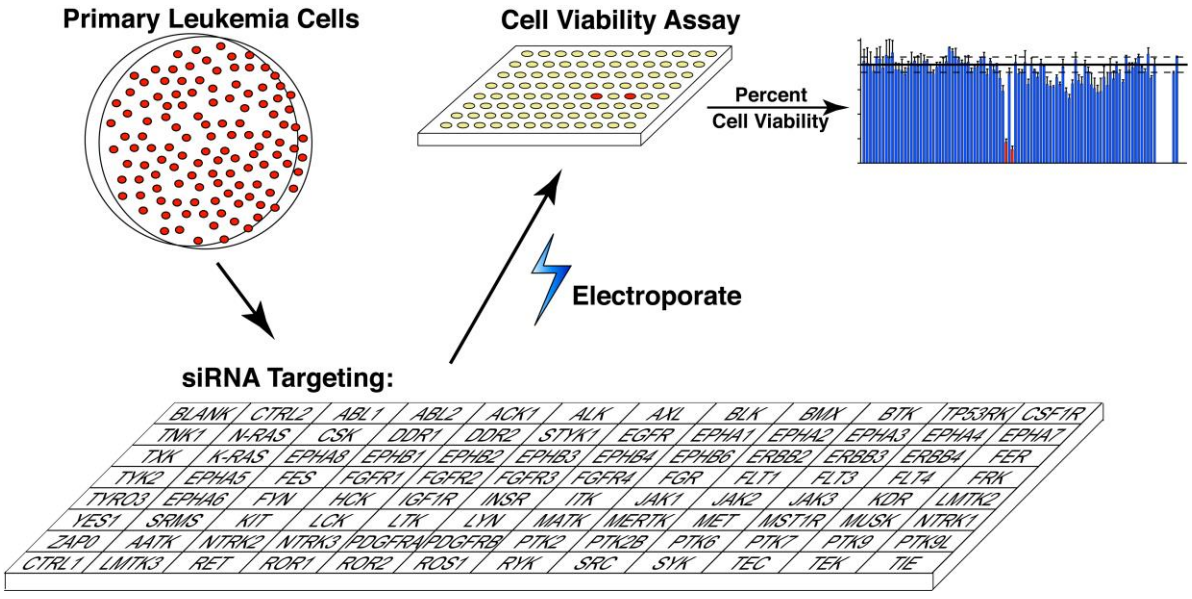

Figure 3. siRNA induced knockdown of the kinome in a 96-well plate on primary patient cells to assess the changes compare to non-targeting control siRNA effect on cell viability.

Electroporation is used as a method for introducing siRNAs into cells. It involves applying a short electrical pulse to induce transient cell membrane permeability and the formation of pores on the hydrophobic bilayer core of cell membrane allowing siRNAs diffuse across those microscopic pores.

Afterwards, MTS Cell Proliferation assay (cell viability assay) allows us to conduct a colorimetric quantification of formazan dye that absorbs at 490-500 nm and is produced by proliferating, viable cells. This serves as an indicator of the viability after electroporation of the cells in the presence of nonspecific siRNA, positive control siRNA and TYRO3 targeting siRNA.

For the analysis of viability scores that is taken from each siRNA screening, first all values are normalized to the median plate value. In the analysis phase, each individual kinase gets an average viability value and those are compared to the threshold which can be summarized as:

$$Average\ viability\ value^{3} - ZSCORE^{4} * standard\ deviation$$

The main validation criterion in this panel is whether a gene is a "HIT" or not for an individual patient. A gene is a "HIT" - or "TRUE" - if its viability is below the aforementioned threshold (the mean of cell viability across the whole plate minus 2*standard deviation), and vice versa it is a "FALSE" if it is above this threshold. We use this algorithm for the assessment of candidate target TK genes for an individual. Before moving further note that, selection of a statistically accurate ZSCORE value matters, because when the ZSCORE is very low most of the tyrosine kinase targets which are below the median value will

[3] Average viability value: the mean of all cell viability values across the whole library for each run.
[4] ZSCORE: number of standard deviations of all cell viability values across the whole library for each run.

be considered as a “HIT”. In contrast, when the ZSCORE is very high, a very low number of individual tyrosine kinase targets on the panel is able to stay below and considered as a “HIT”.

Being a “HIT” of a tyrosine kinase target for that individual patient sample run means that this target might have an importance for the contribution to leukemogenesis in that patient even though that kinase malignancy is not very common among the other patients. In other words, that target is important for signaling pathways required for growth and viability of malignant cells since loss of the protein product of that gene causes cells to lose their ability to proliferate, survive or undergone apoptosis.

Identification of TYRO3

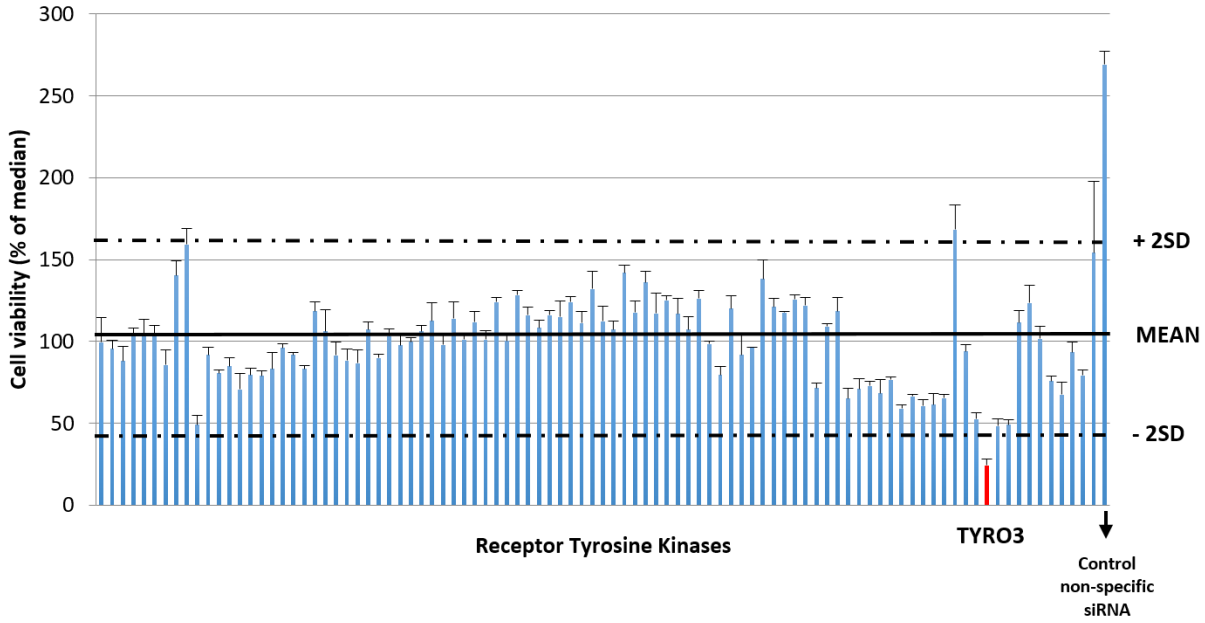

Figure 4. TYRO3 knockdown on patient 14-00578 results in significant decreased leukemia cell survival.

At an early stage of our research, we used Beat AML database to identify recurrent genetic abnormalities, exclusively in tyrosine kinases. Outcome of the namesake project led by Knight Cancer Institute of OHSU and Leukemia and Lymphoma Society (LLS), Beat AML is a relational database that brings functional genomics data including the ones needed for above analysis from AML patients together [83].

In our analysis of viability scores of a patient belongs to this cohort, we have taken the ZSCORE = 2 while it is a standard statistical threshold value. Then we calculated how much the cell viability score is away from the mean of all the plate as a factor of standard deviations and decided “HIT” values for that patient. In other words, we accept a value (of a kinase) as a “HIT” for a patient if the average viability value of a run is 2*standard deviation below of the median of the plate. Tyrosine kinome screening interpreted results of one single patient is shown in Figure 4.

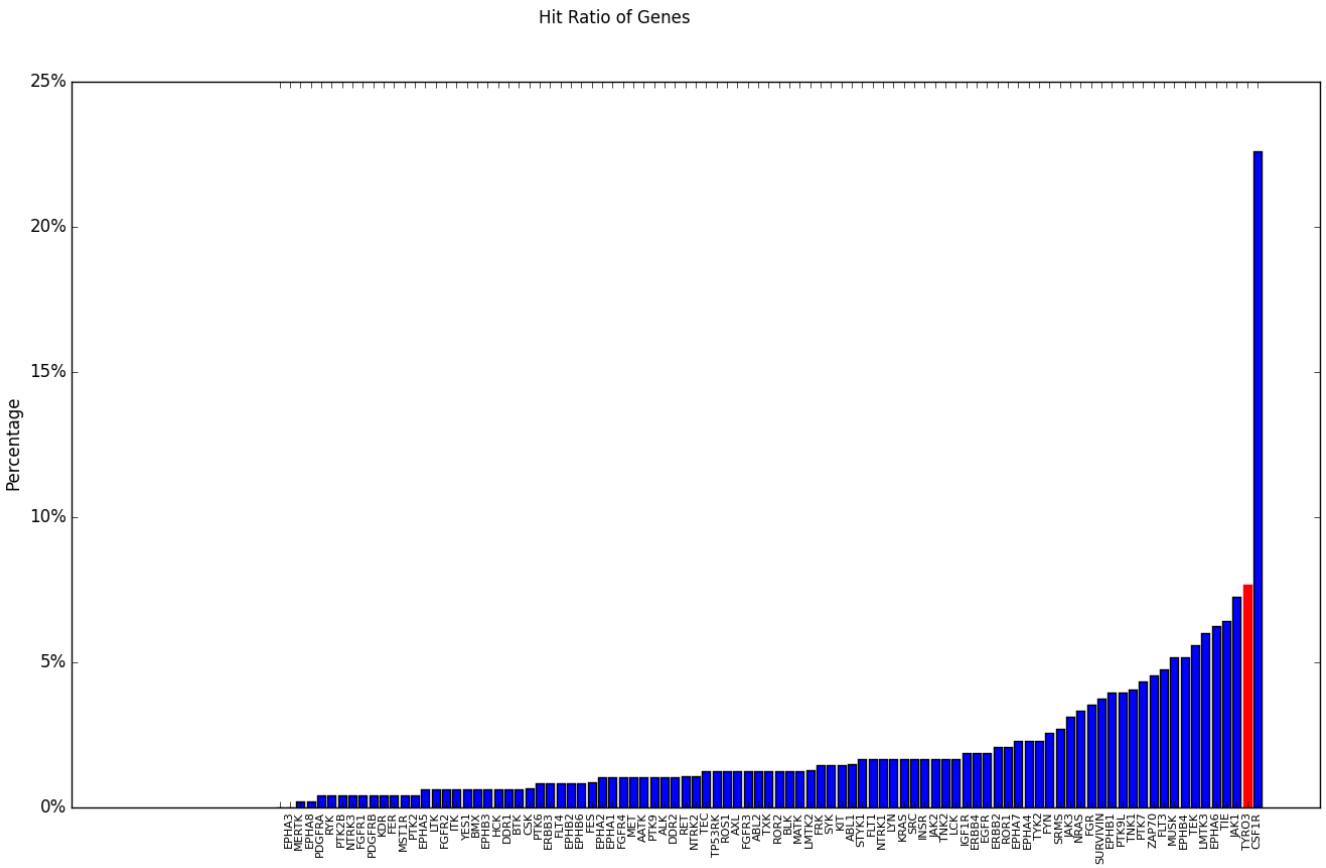

Figure 5. siRNA Screen Identifies TYRO3 as a functional target in AML samples.

We repeat this process for all suitable patient data (n=576) in BeatAML database. Later, all gene target candidates are sorted by their “HIT” ratio of patients (Figure 5). In figure 5, x-axis shows the members of tyrosine kinome which are targeted in our siRNA screening panel and y-axis shows the hit number per kinase divided by the number of all patients (hit percentage). TYRO3 (8.16%) appears in the second order just after CSF1R even though only a small number of patient samples showed sensitivity to AXL(1.39%) and MERTK (0.35%). As seen in this graph, our siRNA screening panel reveals that TYRO3 might be more important than other members belonging to its family namely AXL and MERTK, even

though the current literature is dominated by them in the context of contribution to leukemogenesis. As a result, bioinformatics interpretation of rapid target identification by functional siRNA screening reveals TYRO3 as a potential novel tyrosine kinase target in AML.

Validation of TYRO3 kinase target sensitivity in cell lines

The same electroporation technique is used to introduce same siRNA molecules (Non-targeting siRNA, positive control siRNA, TYRO3 targeting siRNA) to different myeloid leukemia cell lines. In this way, we were able to determine an optimal electroporation parameter for each cell line since the electroporation conditions might slightly vary from a cell line to another cell line because of variation of physical properties in cell clones.

Cell line	***Electroporation Condition***	***# of the cells***
K562	250V, 4MS, 2x	2K
KO52	250V, 3.5MS, 2x	7.5K
U937	300V, 3.5MS, 2x	5K
MOLM14	250V, 4MS, 2x	5K
AML-5	250V, 4MS, 2x	5K

Table 1. Optimized electroporation conditions for several AML cell lines.

In the figures 6-10, x-axis shows several electroporation conditions and y-axis shows the cell viability values which are percentages of non-specific siRNA). K562 (Figure 6), U937 (Figure 7), KO52 (Figure 8),

MOLM14 (Figure 9), and AML-5 (Figure 10) cell lines (with the varying number per cell line/well) were electroporated using a square wave protocol with indicated voltages and pulse lengths with 2 pulses in the presence of non-specific (NS, indicated with gray color in the figures 6-10) and positive control (PLK1 for U937, KO52 and AML-5, ABL1 for K562 and FLT3 for MOLM14, indicated with yellow color in the figures 6-10). Selected positive controls for K562 and MOLM14 are their mutated oncogenes, respectively, ABL1 and FLT3. PLK1 (polo-like kinase 1), as another positive control for the rest of the cells, is used to disrupt controlled chromosome segregation and inhibition of mitosis. 20µl of electroporated cells were transferred to 90µl of culture media for counting viable cells by MTS assay at 96 hours in 6 replicates.

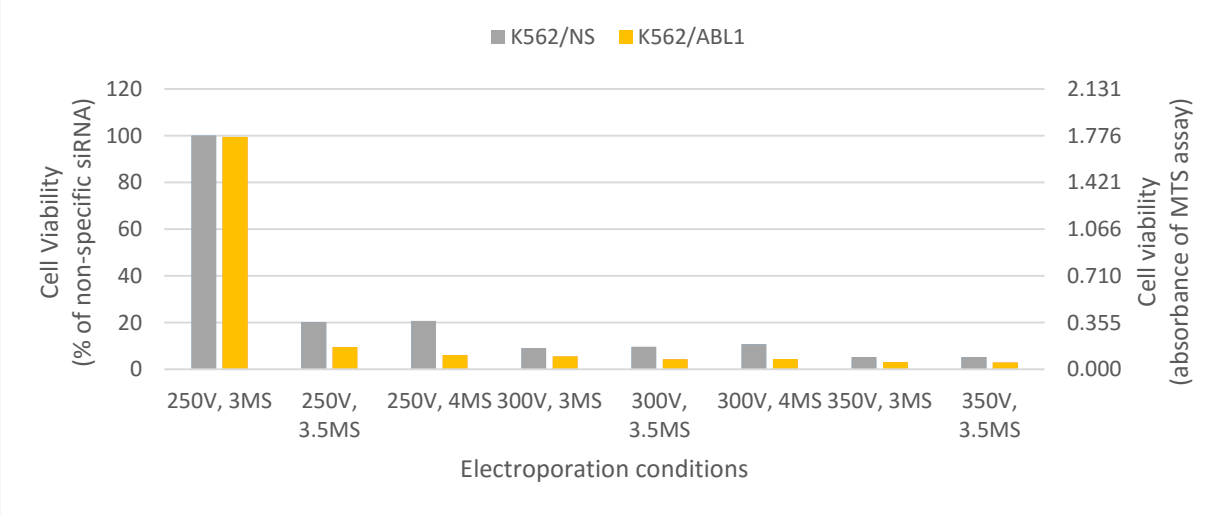

Figure 6. TYRO3 knockdown efficiency with different electroporation conditions in K562 cell line.

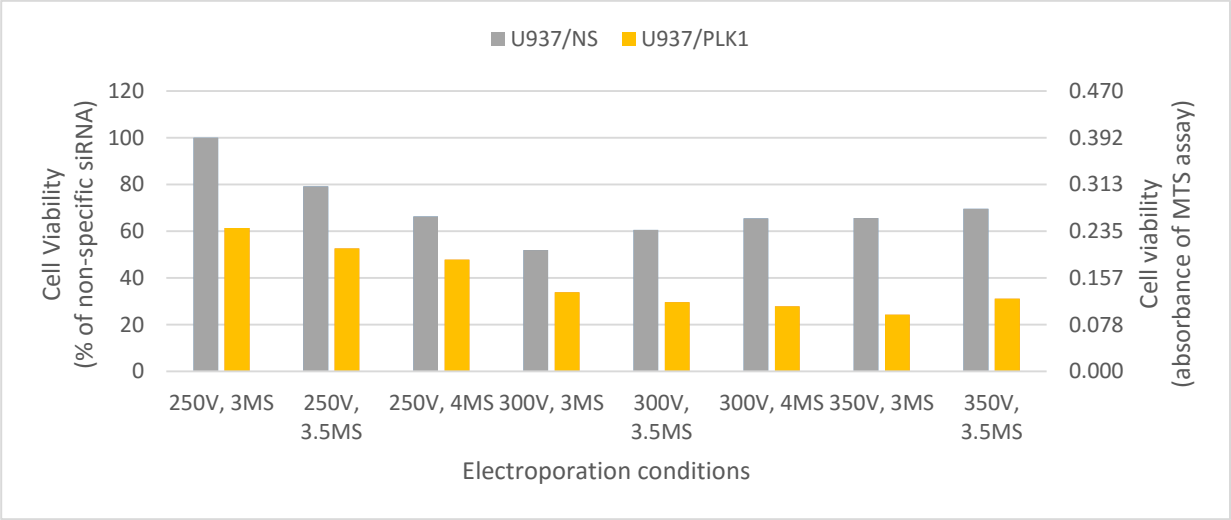

Figure 7. TYRO3 knockdown efficiency with different electroporation conditions in U937 cell line.

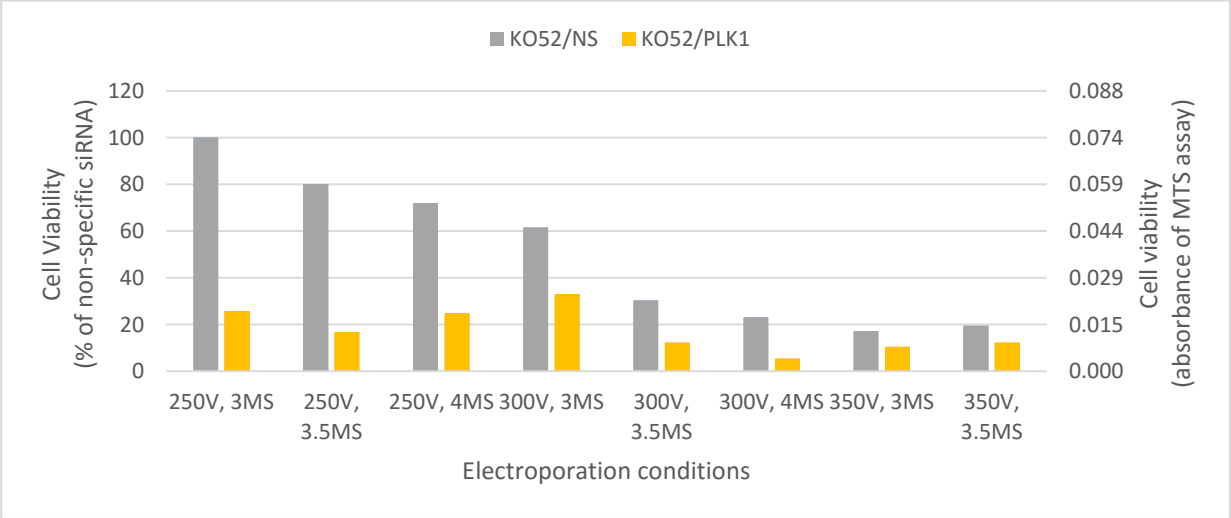

Figure 8. TYRO3 knockdown efficiency with different electroporation conditions in KO52 cell line.

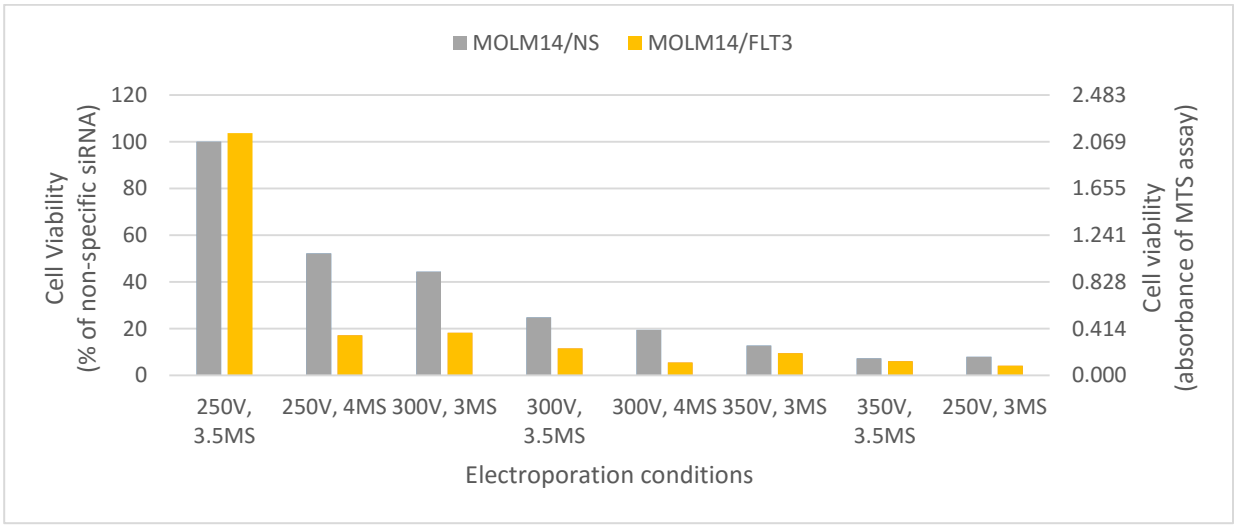

Figure 9. TYRO3 knockdown efficiency with different electroporation conditions in MOLM14 cell line.

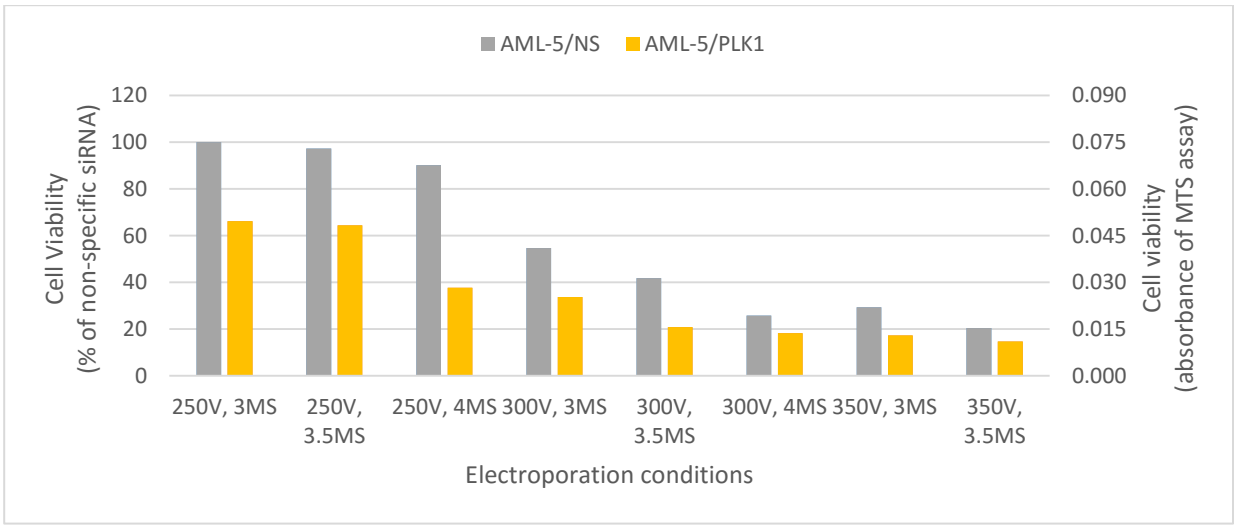

Figure 10. TYRO3 knockdown efficiency with different electroporation conditions in AML-5 cell line.

After we determined the optimal parameters for each cell line that will be used in this research, we tested them for TYRO3 kinase target sensitivity at that parameter. Optimized electroporation conditions for each cell line is listed in Table 1.

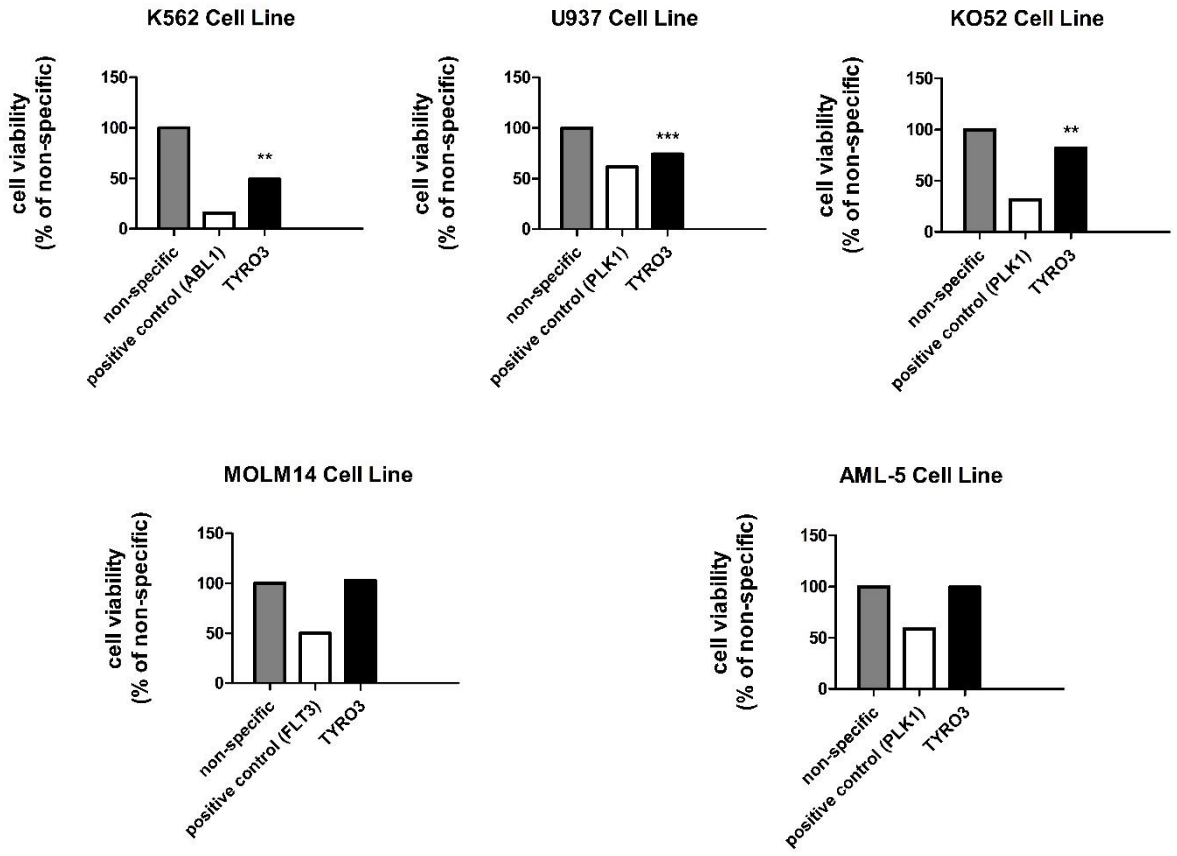

Figure 11. siRNA mediated knockdown of TYRO3 in several AML cell lines.

Each cell line was electroporated in the presence of non-specific, positive control (PLK1 for U937, KO52 and AML-5, ABL1 for K562 and FLT3 for MOLM14) additionally TYRO3-targeting siRNA and plated in culture media. After 4 days, cells were subjected to an MTS assay to measure cell viability. Figure 11

shows that while some of the cell lines were showing high degree of sensitivity (K562, U937, KO52) to TYRO3 silencing, some of the cell lines were resistant (MOLM-14, AML-5) to TYRO3 silencing.

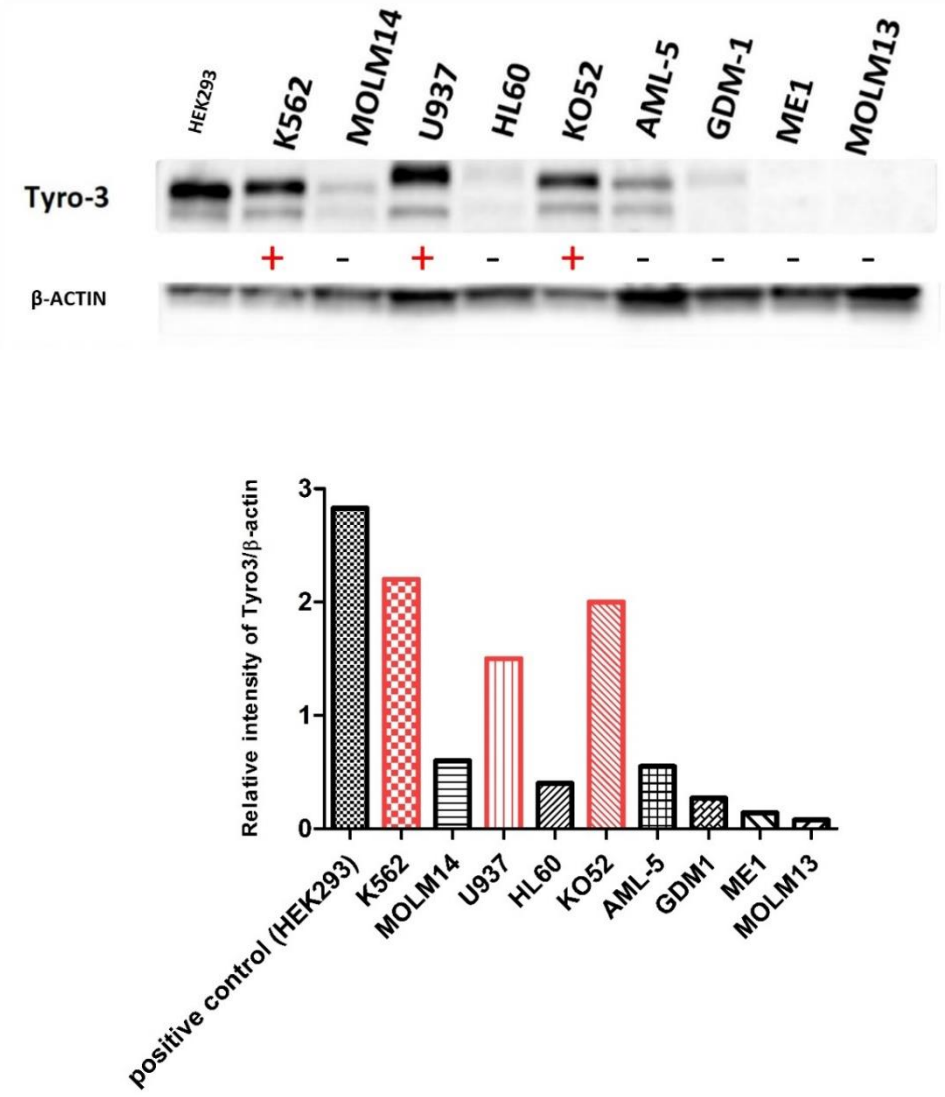

Figure 12. Immunoblot analysis of TYRO3 expression in several AML cell lines.

Given that TYRO3 is required for those cell lines to maintain cell viability, we hypothesized that there is a correlation between TYRO3 protein expression levels in cell lines and their TYRO3 siRNA sensitivity.

We further subjected whole cell lysates to immunoblot analysis using TYRO3 specific antibodies and normalized the results to control protein expression. Data shown in Figure 12 are relative intensity levels of TYRO3 in several AML cell lines. TYRO3 positive cell lines are marked by red positive sign and others are marked with black negative signs. HEK293, the positive control for TYRO3 expression is unmarked.

According to the immunoblotting results, in a strong correlation with TYRO3 knockdown sensitivity data, while K562, U937, KO52 cell lines were the ones which have relatively high level of TYRO3 expression, MOLM14, and AML-5 cell lines were the ones which do not show high expression of TYRO3.

Sensitivity to TAM family inhibitor UNC2025A

In last two decades, several therapeutically useful kinase inhibitors have been approved by the FDA along with a large number of other compounds and many of them are in the pre-clinical stages and waiting to be approved. This renders small molecule kinase inhibitor development as an area of surging interest in human kinome studies. Although there are many examples of successful kinase inhibitor development, we were in need of a new TKI targeting TYRO3 efficiently. I believe more studies on TYRO3 will make it an established, well-defined target and will also make it easier to develop a potential TKI.

In our study, we used a MERTK/FLT3 dual inhibitor UNC2025A, which also inhibits TYRO3. We have assessed in vitro cell viability sensitivity dynamics of UNC2025A in both cell cultures and in our patient cohort.

IC50 (Inhibitory Concentration) and Area under the Curve values are frequently used in pharmacology and important parameters to show how effective a drug is. The term IC50 refers to the concentration

of an inhibitor which will reduce cell viability by 50%. AUC can be defined as the area under the fitted dose response curve. So, the lower IC50 and AUC refers to a more effective drug/inhibitor.

As a further step of confirmation TYRO3 expression and siRNA sensitivity correlation, we wanted to see whether these AML cell lines show a similar pattern of TYRO3-targeting small molecule kinase inhibitor sensitivity. In order to show that, five different AML cell lines are treated with different drug concentrations, 5 x 10^3 cells/well with 4 replicates 72 hours; subsequently cell viability was assessed by an MTS cell viability assay (Figure 13) as described in the Methods (Chapter 4, siRNA and kinase inhibitors). K562, U937, and KO52 (cell lines which are both sensitive/dependent to TYRO3 knockdown and have TYRO3 high expression), as happens in the siRNA sensitivity assay, showed different degrees of sensitivity to UNC2025A. MOLM14 (which is neither a high TYRO3 expressing or TYRO3 sensitive/dependent cell line) also showed a high degree of sensitivity to UNC2025A inhibition as we already expected. MOLM14 AML cell line, which has been previously defined as an FLT3 - a commonly mutated oncogene - internal tandem duplication (+) cell line [85] and UNC2025A as a MERTK/FLT3 dual inhibitor is very effective on this cell line targeting the main leading responsible oncogene in the cancer pathogenesis. All the other cell lines which are used in this research are previously shown as FLT3 ITD or D835 (a point mutation which results with constitutive activation of the receptor FLT3) negative [85]. Later, we have proposed to use a negative cell line AML-5, which is neither a high TYRO3 expressing nor TYRO3 sensitive/dependent cell line. AML-5, which is also FLT3 (-), did not display a high degree of drug sensitivity so it was an ideal negative control for the understanding of TYRO3 dysregulation.

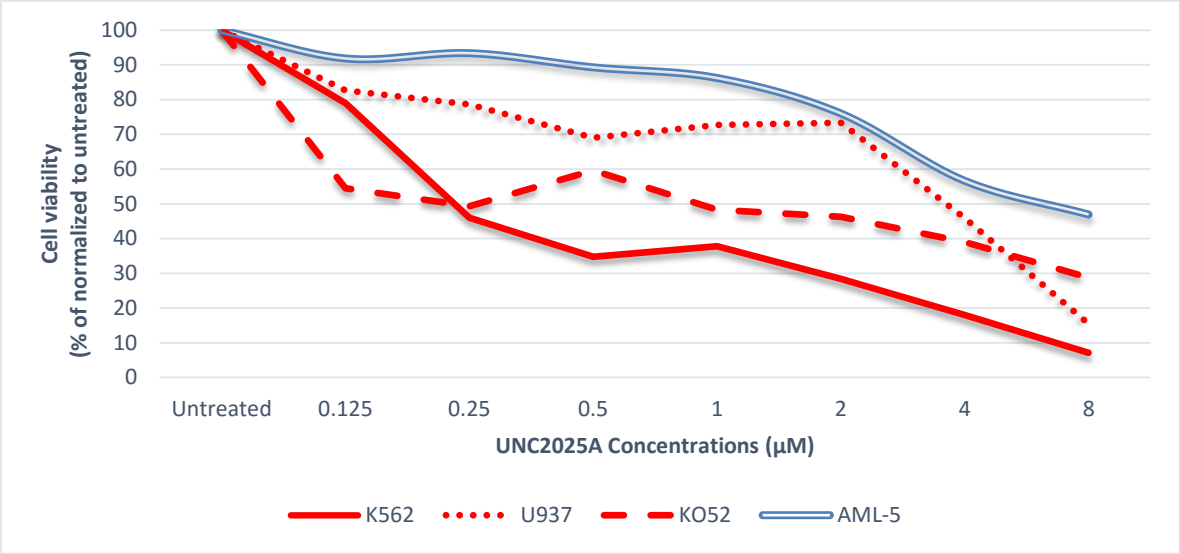

Figure 13. TYRO3 inhibitor (UNC2025A) response in each cell line.

Cell line	K562	U937	KO52	AML-5
IC50 of UNC2025A (µM)	0.197537	2.935364	0.13613	3.178082

Table 2. IC50 values of UNC2025A for the cell lines.

Ligand/Receptor Double Knockdown

We tested to see whether the double knockdown of both ligand and TYRO3 may have a more striking impact on cell viability as proposed previously [27]. For this purpose, we have used both GAS6 targeting and TYRO3 targeting siRNA in addition to non-specific and positive control (PLK1) siRNA. Data for KO52 and U937 cell lines are shown in Figure 14 and Figure 15 respectively.

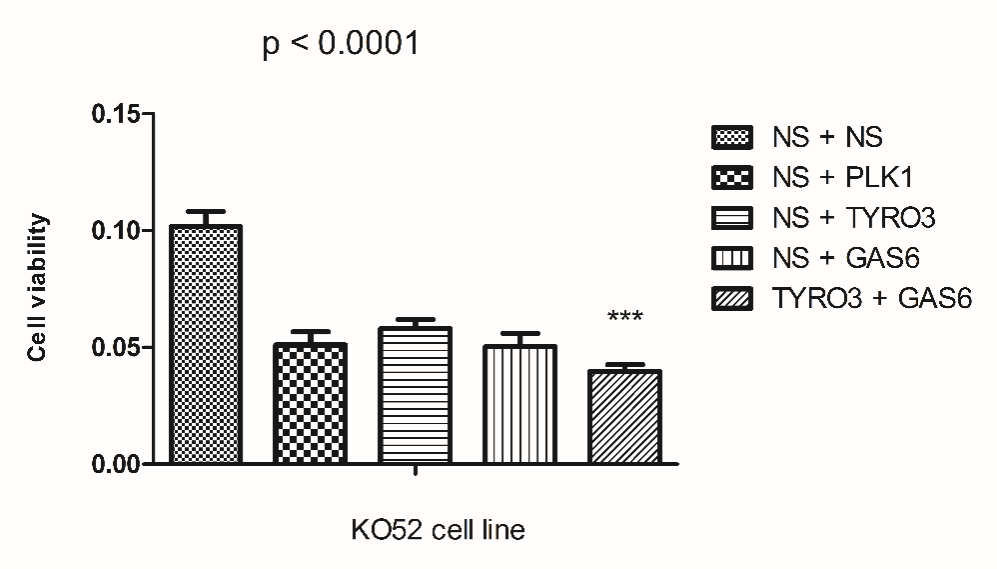

Figure 14. Double knockdown of KO52 cell line.

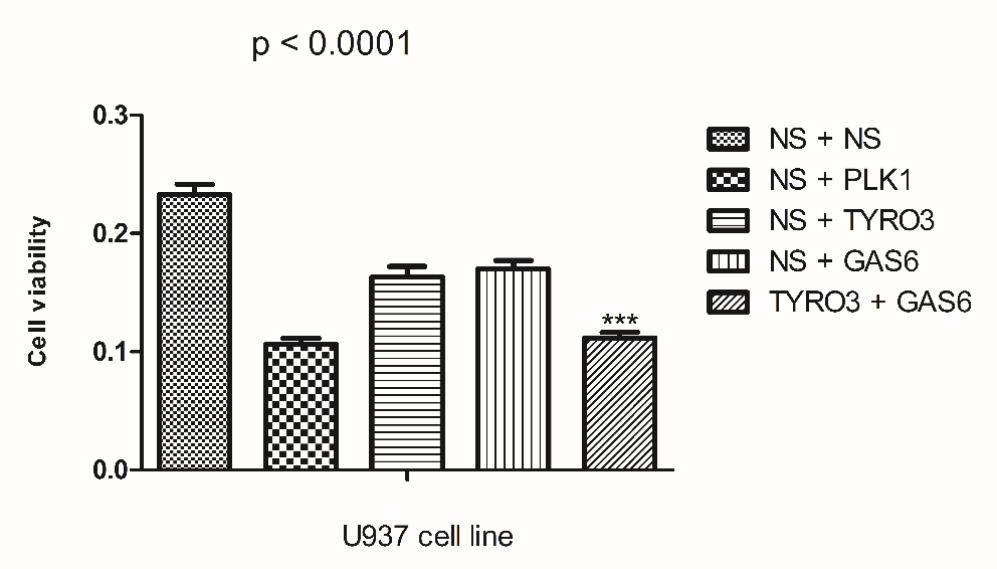

Figure 15. Double knockdown of U937 cell line.

Summary

TYRO3, as one of the members of TAM family receptor tyrosine kinases, is defined as a potential therapeutic target in several cancer types. Also, shRNA-mediated knockdown of TAM members AXL and MERTK has been associated with the promotion of apoptosis and slow down of proliferation [84] in cell cultures. However, there have not been established studies which assess TYRO3 as a potential tyrosine kinase target in acute myeloid leukemia.

In our study, we have shown that TYRO3 activation causes that the cells to gain a leukemogenic potential. We have used an siRNA screening panel which has been established previously [27] on a group of patient samples to validate the potential kinase targets from an individual's tyrosine kinome. Bioinformatics interpretation of siRNA kinome screen identified TYRO3 as one of the top targetable kinases among receptor tyrosine kinases (RTKs) in as much as more than 8% of patient samples are sensitive to knockdown of TYRO3 (n=576).

Given that TYRO3 has a role in the AML potential for patient samples, we hypothesized to reach the same results using cell cultures. Some of the cell lines were significantly more sensitive than the others to siRNA-mediated knockdown of TYRO3. Likewise, several lines showed different expression levels of TYRO3 and there was a positive correlation in sensitivity and expression level.

Abundance of TYRO3 expression, somehow, provides cells the means to resist activating mechanisms of apoptosis by sending survival signals to their micro-environment which will eventually lead them to repress apoptosis or proliferation promoting pathways. So, this is the reason why we were successful to suppress the proliferation of TYRO3-dependent cell cultures by an RNA interference mechanism.

In addition to the previous display of the TYRO3 siRNA-mediated sensitivity in TYRO3 overexpressing cell lines, we also showed that this sensitivity may be elevated with an additional knockdown of the ligand GAS6. With the proof that comes from the double knockdown in cell lines, we have shown that TYRO3 is not effective on the cells to continue proliferation, unaccompanied. So, TYRO3 sensitive cell lines may be sensitized more efficiently by the addition of ligand targeting siRNA. When GAS6 upregulation is taken into account, usage of both ligand and receptor siRNA-mediated knockdown, more effectively, breaks the signal transduction and effect on the cell viability are more dramatic.

Chapter 3: In vitro drug sensitivity prediction based on expression of TAM receptors

Heat map analysis of in vitro drug sensitivity across AML primary patient samples

Inhibitory Concentration (IC50) and Area under the Curve (AUC) values are frequently used in pharmacology and they are important parameters to show how effective a drug is. The term IC50 refers to the concentration of an inhibitor which will reduce cell viability by 50%. AUC can be defined as the area under the fitted dose response curve. Thus, the lower IC50 and AUC more effective drug/inhibitor is.

Our lab has previously screened over 1000 primary patient specimens against a panel of single-agent small-molecule inhibitors including UNC2025A. The impact of the drug on the cell viability was assessed by a viability assay on day 3 using a tetrazolium reagent (MTS assay).

In order to demonstrate the UNC2025A sensitivity in our TYRO3 overexpressing patients, we have collected IC50 and Area under the Curve (AUC) values from our patient cohort and used RNA seq data for gene expression of TYRO3, AXL, MERTK, GAS6 and PROS1. We created a heat map analysis which will allow us to see the anti-correlation between drug parameters (IC50 and AUC) and transcript/RNA abundances of this family members and their ligands. In this case, an anti-correlation between a drug's IC50 value and TYRO3 expression refers to its effectiveness on a TYRO3 overexpressing patient.

Respectively, expression degree of each member of TAM receptor kinase family and each ligand of this family are added to heat map analysis. In addition to that, we sorted the patients into 2 groups by whether they have an FLT3 internal tandem duplication (FLT3 ITD) or a mutation (D835) which leads to the same oncogene activation or not. For the analysis, we have used 2 different correlation assessment values, Pearson correlation and Spearman correlation, for each member expression in 2 different patient cohort which is separated according to their FLT3 ITD status as either positive or negative. All the FLT3 (+) patients have FLT3 ITD with or without D835 mutation except one patient with only D835 positivity. Heat map analysis of the correlation in FLT3 (-) and FLT3 (+) graphs are shown in Figure 17 and Figure 18.

In Figure 17 and Figure 18, each row depicts the expression levels of a member of this family (TYRO3, AXL, and MERTK) and their ligands (GAS6, PROS1) except the last bar (named as IC50 of UNC2025A) which shows IC50 value of this drug per patient. Patients are recorded with the numbers as indicated in the bottom of the figures 17 and 18. In addition to that, an average mRNA expression value of all TAM receptors and their ligand GAS6 and a max value of GAS6-PROS1-TYRO3 which belongs to one individual patient are computed during this correlative heat map analysis. All seven squares in each line of the same row belongs to one individual's relative transcript abundances of a member among the other patients in the same group. All the patients in Figure 17 have been validated as FLT3 (-), while the patients in Figure 18 have been validated as FLT3 (+). While red squares represent the highest expression, blue squares represent the lowest expression among the others. The color scale is depicted in each figure itself.

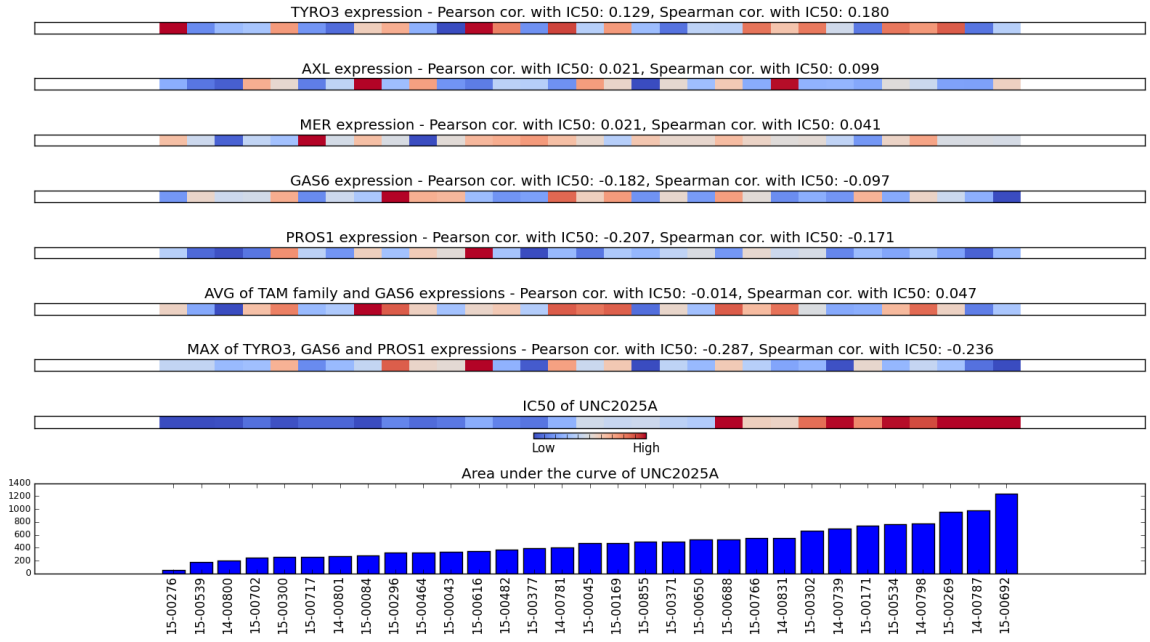

Figure 16. Heat map of correlation between drug parameters and TAM family expression in FLT3 negative patients.

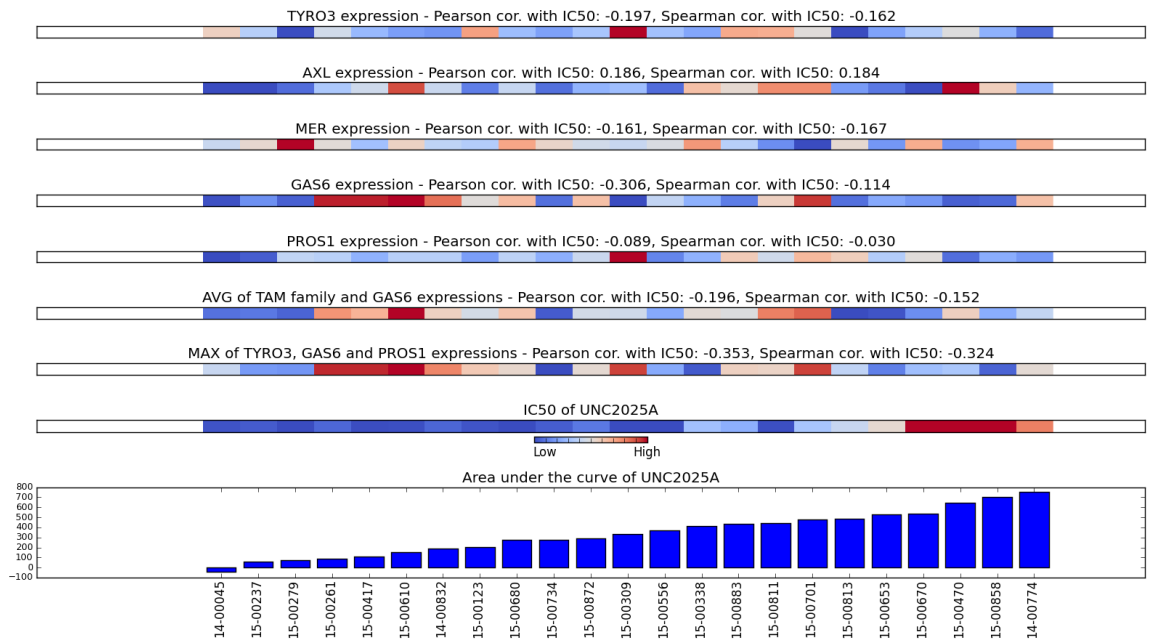

Figure 17. Heat map of correlation between drug parameters and TAM family expression in FLT3 positive patients.

In the Figure 17, MAX value represents a slight anti-correlation between in vitro drug sensitivity and expression of TYRO3, PROS1 and GAS6. However, we could not observe a strong anti-correlation between TYRO3 or other receptors' expression and drug sensitivity parameters. Interestingly, there is a higher anti-correlation between IC50 and GAS6 expression in the Figure 18 in which IC50 and MAX correlation shows a significant sensitivity degree as well for that cohort.

RNA seq and qPCR TYRO3/GAS6 Expression Correlation with siRNA sensitivity

We choose to further examine the TYRO3 siRNA sensitivity screening results from our same patient cohort to confirm the fact that elevated TYRO3 expression correlates with siRNA mediated TYRO3 sensitivity. In this scenario, we also added GAS6 ligand expression for comparison. Although TYRO3 expression is very low in some patients from TYRO3 sensitive group of patients, we realized that GAS6 expression level is relatively higher among the other patients. So, we have aimed to see a significant difference in either GAS6 or TYRO3 expression level between sensitive and insensitive group of patients. In order to do that, we have separated patients into 2 group according to their siRNA screening panel results. TYRO3 insensitive patients give a FALSE against TYRO3 knockdown, and TYRO3 sensitive patients give a TRUE i.e. "HIT", against TYRO3 knockdown.

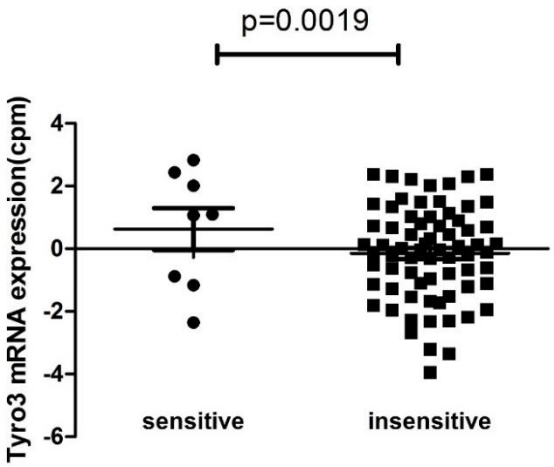

Figure 18. TYRO3 mRNA expression and TYRO3 siRNA sensitivity correlation by RNAseq.

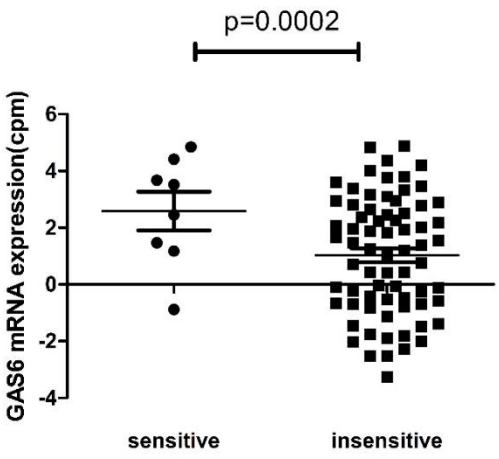

Figure 19. GAS6 mRNA expression and TYRO3 siRNA sensitivity correlation by RNAseq.

Figure 19 and Figure 20 display a comparison of mRNA transcript levels of TYRO3 and GAS6, separately, in TYRO3 sensitive and insensitive group of patients (x-axis, n = 82) according to previously described siRNA screening panel. Y-axis shows the counts per million of TYRO3 and GAS6 mRNA by RNA seq. A significant level of decrease in both TYRO3 and GAS6 transcript levels by RNA seq in insensitive group is observed.

For further confirmation, we have done the same analysis for quantitative PCR results belonging to a smaller group of patients randomly selected from the same cohort (Figure 21 and 22). Likewise RNA seq comparison, we separated patients into two groups as TYRO3 sensitive and insensitive (x-axis). This time y-axis shows 2e-ΔΔCT values for mRNA abundances of TYRO3 and GAS6. As seen in Figure 21 and 22, we have observed a significant decrease in the expression levels of both TYRO3 and GAS6 (n=15) in accordance with previous results.

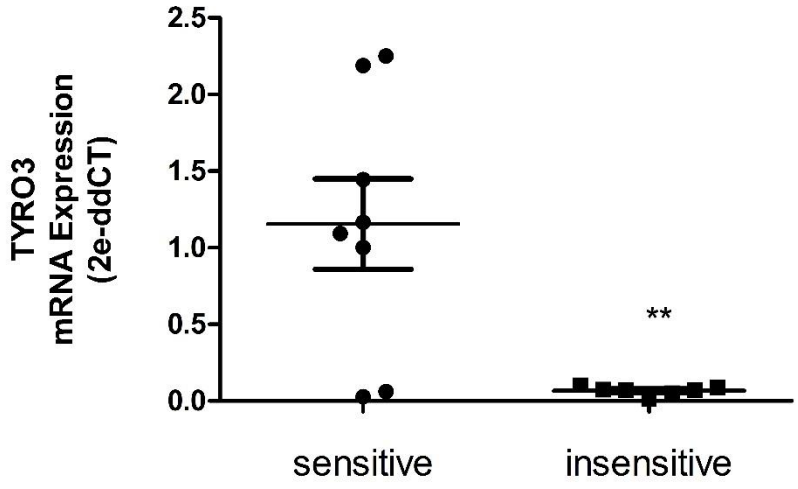

Figure 20. TYRO3 mRNA expression and TYRO3 siRNA sensitivity correlation by qPCR.

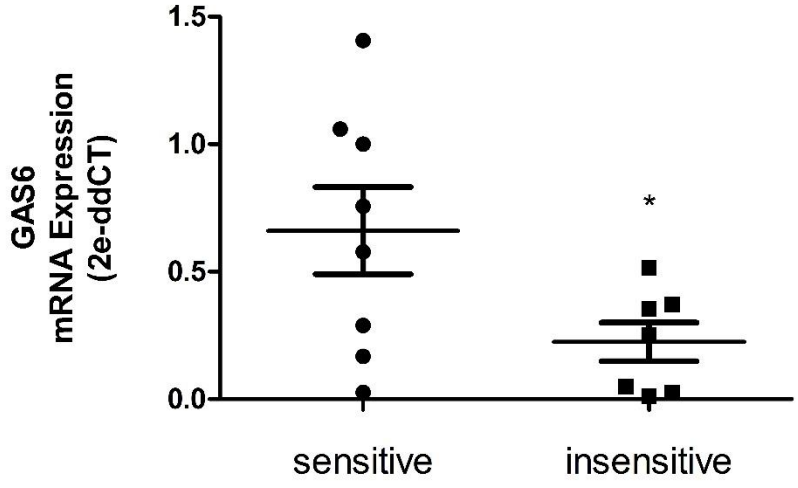

Figure 21. GAS6 mRNA expression and TYRO3 siRNA sensitivity correlation by qPCR.

So far, we have shown that an increased protein expression in either GAS6 or TYRO3 is correlated with not only TYRO3 targeting siRNA sensitivity but also TYRO3 targeting small molecule kinase inhibitor

sensitivity in both TYRO3 siRNA sensitive in vitro cultures and primary patient specimens. It has been confirmed that most of the TYRO3 sensitive patients show elevation trend in TYRO3 and GAS6 expression levels compared to the levels in insensitive group by quantitative PCR. Also, it has been noted that mRNA level expression of TYRO3 differs more distinctly between two groups compared to mRNA level expressions of GAS6.

Summary and Discussion

The results presented here are aiming to show the facts that small molecule kinase inhibitor sensitivity correlates with either TYRO3 receptor or TYRO3 ligand mRNA transcript levels in *in vitro* primary patient specimens by heat-map analysis and TYRO3 knockdown sensitivity correlates with both TYRO3 receptor and TYRO3 ligand mRNA transcript levels in *in vitro* primary patient specimens by RNA seq and qPCR.

We generated a heat map to show the correlation between UNC2025A sensitivity parameters IC50 and AUC, and elevated mRNA transcript level of TYRO3 and its ligand GAS6. Since the kinase inhibitor UNC2025A is a multi-targeted inhibitor, including FLT3, we needed to limit this analysis to FLT3 (-) patients to eliminate the expected correlation caused by FLT3 targeting effect of UNC2025A (MERTK inhibitor, the drug). When the maximum level of TYRO3, GAS6 or PROS1 taken for each individual patient, we observed that drug response of that patient shows a modest correlation with this maximum value. Interestingly, the observed correlation of high expression/high drug response in FLT3 ITD positive patients is higher. Even though it still remains questionable, there might be a crosstalk between GAS6/TYRO3 interaction and FLT3 oncogene activation.

Lastly, we have shown that both GAS6 and TYRO3 mRNA transcript levels are increased in sensitive group of patients compared to insensitive group.

Chapter 4: TYRO3 Dysregulation

Based on the findings mentioned in Chapter 3, we hypothesized that the ligand overexpression may result with prominent activation of TYRO3 followed by homodimerization of the receptor to transmit the extracellular signal to the cells so that it initiates cascade phosphorylation of downstream proteins which are required for growth and viability of malignant cells.

Downstream signaling of TYRO3 is activated by ligand stimulation and inhibited by TAM inhibitor

To examine the receptor activation of TYRO3 and its effects on downstream signaling events, we starved TYRO3 sensitive cells 4 hours in serum-free media and at the end of 4 hours stimulated with recombinant GAS6 for 0, 5, 10, 15, 30 minutes with a constant concentration of 200 µg/ml to have more pronounced effects on downstream signaling. Later, ligand treated cells washed with PBS, lysed and assessed the phosphorylation of STAT5, AKT and ERK in these cell lysates by SDS PAGE using antibodies against these proteins. Immunoblotting results are shown in Figure 23 and 24.

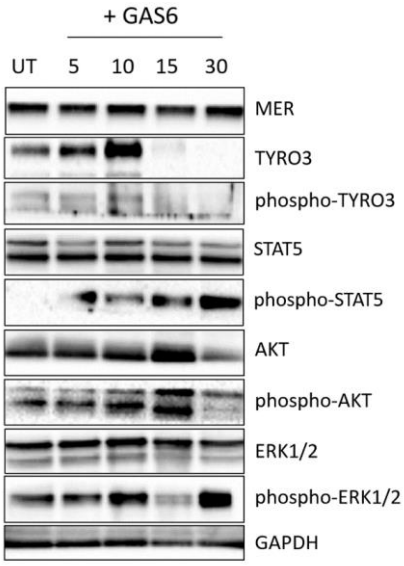

Figure 22. GAS6 stimulation in TYRO3 positive U937 cell line.

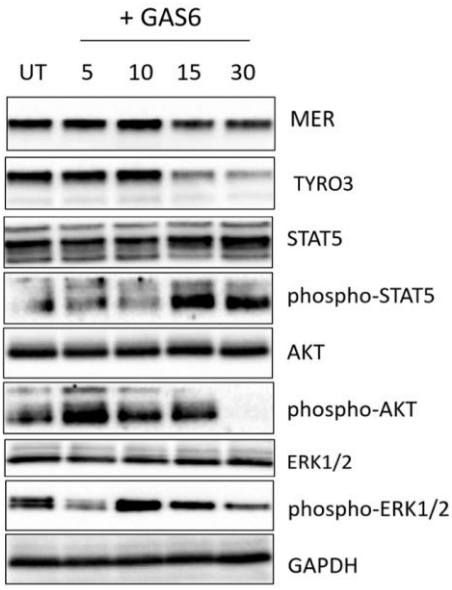

Figure 23. GAS6 stimulation in TYRO3 positive KO52 cell line.

In addition, we sought to determine whether the drug (UNC2025A) could inhibit the enhanced signal generation upon ligand stimulation. We treated serum-starved (as described above) TYRO3 sensitive cells with two different drug concentration for 1 hour, subsequently added the recombinant GAS6 to the media for 15 minutes in a constant concentration. Afterward, we assessed the phosphorylation of above mentioned proteins to see the effects of GAS6 stimulation on downstream signaling in the setting of UNC2025A treatment. Immunoblotting results are shown in Figure 25 and 26.

In both positive cell lines, STAT5 phosphorylation is induced after ligand addition to the media. TYRO3 receptor level decreased which might be due to receptor degradation after ligand binding while MERTK receptor did not show a change at all. Even though there is a slight increase in phosphorylation of AKT and ERK, they did not show consistent results as we saw in the phosphorylation of STAT5 (Figure 23 and 24).

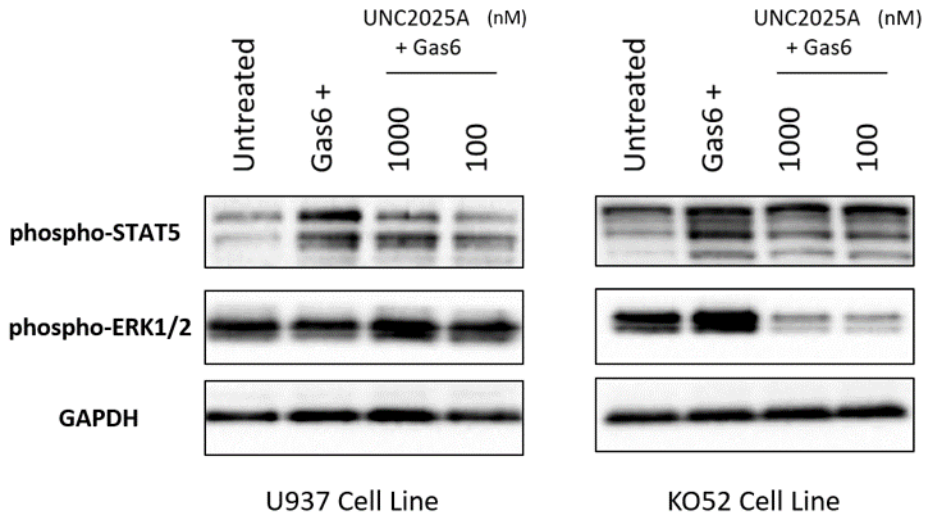

Figure 24. Drug inhibition in TYRO3 positive U937 and KO52 cell line.

Drug inhibition assay in TYRO3 positive cell lines, U937 and KO52, confirms that phospho-STAT5 protein expression is increased in both TYRO3 positive cell lines and two different drug concentration slightly attenuated the increased phospho-STAT5 expression due to GAS6 stimulation. Also, this data shows that phospho-ERK signaling is not affected by GAS6 stimulation or drug inhibition in U937 cell line. In contrast, KO52 cell line demonstrate that GAS6 stimulation is effective on increased phospho-ERK protein expression and the drug, UNC2025A, is effective to inhibit this GAS6-mediated increased phospho-ERK expression.

Drug inhibition assay in MOLM14 negative cell line, interestingly, shows that phospho-STAT5 and phospho-ERK expression significantly increased upon GAS6 stimulation and drug effectively inhibited the enhanced signal (Figure 25). It might be due to a possible crosstalk mechanism between receptors

FLT3 and TYRO3, even though MOLM14 cell line has very low TYRO3 expression compared to positive cell lines.

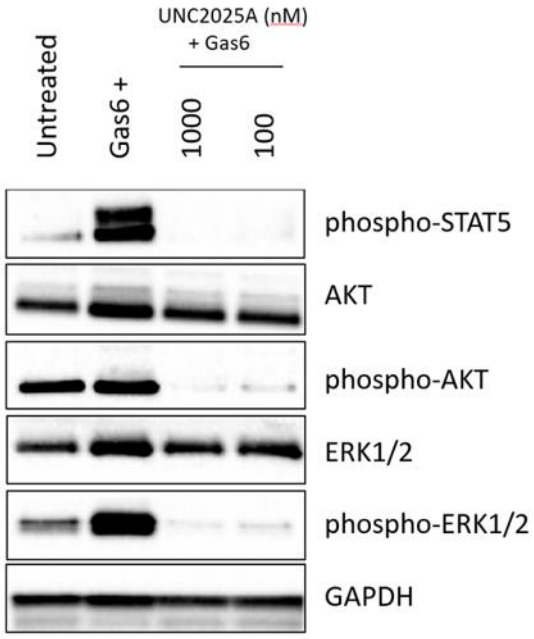

Figure 25. Drug inhibition in TYRO3 negative MOLM14 cell line.

Methods and materials

Cell Cultures

K562, U937, HeLa and HEK293 cells were obtained from American Type Culture Collection (Manassas, VA). KO52 and SKNO-1 cells are obtained from the JRCB Cell Bank. AML-5, HL-60, GDM-1, ME-1, MOLM13 and MOLM14 cells are obtained from DSMZ (German Cell Line Bank). K562, U937, MOLM14,

KO52 cells were cultured in RPMI medium supplemented with 10% FBS, (Atlanta Biologicals), penicillin/streptomycin (Invitrogen), and fungizone at 37°C in 5% CO2. AML-5 cells were cultured with 20% FBS (Atlanta Biologicals), penicillin/streptomycin (Invitrogen), and fungizone, in addition to that 2% GM-CSF was added to the culture media every 3 days.

For ligand stimulation, cells were serum starved 4-6 hours in RPMI supplemented with l-glutamine, penicillin/streptomycin and fungizone (serum-free starvation media). After starvation, cells were stimulated for 0, 5, 10, 15, 30 minutes with 200 ng/mL recombinant growth arrest specific 6 Gas6 protein (R&D Systems) and cell lysates were subjected to immunoblot analysis as described in "Immunoblotting". For kinase inhibition combined with ligand stimulation, leukemia cells (5x106 /mL) were cultured with UNC2025 or DMSO equivalent to 1000nM, 10uM UNC2025 for one hour, subsequently either ligand Gas6 or Pros1 was added to the media with a time range mentioned above. Cell lysates were prepared and several signaling proteins were detected by immunoblot using specific antibodies listed in Table 3.

Antibody Name	*Concentration*	*Company*	*Catalog #*	*Secondary Antibody*
TYRO3	1:1000	Cell Signaling	#5585	Rabbit, 1:5000
p-TYRO3	1:500	Abnova	#PAB29209	Rabbit, 1:5000
AXL	1:1000	Cell Signaling	#8661	Rabbit, 1:5000
p-AXL	1:500	Cell Signaling	#5724	Rabbit, 1:5000
MERTK	1:1000	Cell Signaling	#4319	Rabbit, 1:5000
Total STAT5	1:1000	Cell Signaling	#9363	Rabbit, 1:5000
p-STAT5	1:1000	Cell Signaling	#9351	Rabbit, 1:5000
Total AKT	1:1000	Cell Signaling	#9272	Rabbit, 1:5000
p-AKT	1:1000	Cell Signaling	#9271	Rabbit, 1:5000
Total ERK1/2	1:1000	Cell Signaling	#9102	Rabbit, 1:5000
p-ERK1/2	1:1000	Cell Signaling	#9101	Rabbit, 1:5000
4G10	1:500	Millipore	#05-321	Mouse, 1:5000
GAPDH	1:5000	Thermo Fisher	#AM4300	Mouse, 1:5000
B-ACTIN	1:5000	Millipore	#MAB1501	Mouse, 1:5000
B-TUBULIN	1:5000	Millipore	#05-661	Mouse, 1:5000

Table 3. List of antibodies used in this research.

siRNA and kinase inhibitors

All siRNAs and reagents were purchased from Thermo Fisher Scientific Dharmacon RNAi and reconstituted in 1x siRNA Buffer for a final concentration of 40 µM. UNC2025A was purchased from Meryx, Incorporation and diluted at 1:1000 in DMSO.

All patient specimens were obtained after informed consent with IRB approval at Oregon Health & Science University, Stanford University, University of Utah, UT-Southwestern and University of Colorado-Denver. Primary patient cells were cultured for 72 hours in 384-well plates with graded concentrations of UNC2025 and several other kinase inhibitors or vehicle and relative numbers of viable cells were determined. IC50 values were calculated by non-linear regression.

For siRNA screening panel, patient specimens (10^5) were suspended in siPORT buffer and incubated with 1 M siRNA from an siRNA library individually targeting each member of the tyrosine kinase family and single and pooled nonspecific siRNA controls. Cells were electroporated on a 96-well electroporation plate at 2220 V (equivalent of 300 V), 100 sec, 2 pulses. Cells were re plated into culture media and, for determination of cell viability and proliferation, cells were subjected to the CellTiter 96 AQueous One solution cell-proliferation assay (MTS; Promega). All values were normalized to the mean of the plate. Values represent percent mean (normalized to nonspecific control wells) plus or minus SEM (n = 576).

Immunoblotting

For immunoprecipitation, lysates were incubated overnight with antibody 4G10 (EMD Biosciences). Immune complexes were precipitated with protein A-sepharose beads (Amersham Biosciences,

Piscataway, NJ), washed 3 times in lysis buffer. For both immunoblotting and immunoprecipitation protocols, cells were washed in phosphate-buffered saline and lysed in 1 × cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with tyrosine phosphatase inhibitor cocktail-2 (Sigma-Aldrich, St. Louis, MO), PMSF and complete protease inhibitor tablet (Roche, Indianapolis, IN). I have used 50 µg protein lysate typically for an Immunoblot technique and 500 µg for an Immunoprecipitation. Sample buffer, 1/3 of the protein lysate, was added onto the protein lysate and boiled at 95°C for 10 minutes. Samples are then loaded into a Criterion 4-15% polyacrylamide gel, and run at 0.05 A/gel for 1 hour. Once the gels are run, the gel-resolved proteins are transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) at either 25V for 18 hours or 100V for 2 hours. PVDF membranes are blocked in 5% BSA for 1 hour and blotted in primary antibody overnight. A list of primary antibodies used in this study is shown in Table 3. Secondary antibodies include mouse, rabbit, and goat, and are typically used at 1:5000 for 45 minutes. Blots are developed with standard Western blotting detection reagents such as ECL from Amersham. Blots are visualized with the Bio-Rad Chemi-Doc system (Image Lab software from Bio-Rad).

Confirmation of RNAi silencing

For confirmation of efficient knockdown in U937 and KO52 cells, cells transfected as previously indicated pulses and whole cell lysates (see “Immunoblotting”) were harvested after 72 hours using standard procedures and probed with TYRO3 specific antibodies.

Real time RT-PCR

RNA was isolated from both commercial cell lines and patients using the RNeasy Mini kit (Qiagen). RNA was transcribed to cDNA in a 20µl reaction using 60 µmol/L oligodt primers, 0.5 mmol/L dNTPs, 100 units RNaseOUT, 5 mmol/L DTT (DTT: dithiothreitol), 1× First Strand buffer, and 500 units SuperScript III reverse transcriptase following manufacturer's instructions (Invitrogen by Life Technologies, Grand Island, NY). Subsequently, cDNA quantified in a 20µl reaction including FAM-labeled hydrolysis probes specific to TYRO3, GAS6 and GAPDH using via real-time RT-PCR following the TaqMan Gene Expression Assay protocol (Life Technologies). Human probes: GAPDH assay Hs02758991_g1, TYRO3 assay Hs00170723_m1, GAS6 assay Hs01090305_m1. Cycling conditions on a Light-Cycler 480 instrument (Roche) included 10 min at 95°C followed by 50 cycles of 95°C for 10 sec and 60°C for 20 sec. Samples were plated in triplicate, and every assay included a water control.

By this method, we were able to show the changes in gene expression, after normalized to a housekeeper gene, such as GAPDH as I have used in this study, and relative to the expression of a comparison gene or an untreated sample. Expression results were analyzed using the comparative CT method ($2^{-\Delta\Delta CT}$ method). ΔCT is the difference between the threshold cycles for the target and the housekeeping gene, and ΔCR is the difference between the threshold cycles for an untreated sample and the housekeeping gene. In this study, we have compared the levels of gene expression between two groups rather than using one reference gene or untreated sample.

Statistical methods

Graphical and statistical data were generated using either Microsoft Excel, Python package Matplotlib version 2.7 or GraphPad Prism (GraphPad Software, La Jolla, CA). For cell viability assays, a Student's t test was carried out for each siRNA treatment compared with non-specific siRNA. The P values for the t tests are indicated by asterisks: *, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$; ***, $P < 0.001$.

Summary

Abnormal regulation of tyrosine kinases has been shown in several cancer types previously and they are mentioned by numerous studies as targets for making improvements in the treatment of several hematological malignancies. Defining kinase vulnerabilities is a critical step for the development of novel kinase inhibitors that block dysregulated tyrosine kinase signaling for not only leukemia treatment but also for the treatment of other solid cancers.

One of the most exciting targeted therapy tools in use for cancer treatment more than two decades is small molecule kinase inhibitors specifically designed to inhibit kinases which function in a variety of cellular processes.

In this research, we showed how a novel tyrosine kinase target can be identified by functional siRNA screening and implicated in acute myeloid leukemia. By this functional screening, we found that TYRO3 protein expression contributes to leukemia cell survival. In addition to that, we have confirmed that both TYRO3 and its ligand, GAS6, are significantly elevated in TYRO3 sensitive AML patients by RNA seq

and qPCR which implies that TYRO3 and GAS6 interaction is important for the pathogenesis of AML. Further, ligand stimulation results with increased STAT5 activity, which might be important for TYRO3-sensitive leukemic blasts to gain this leukemic phenotype. So we propose that TYRO3-GAS6 interaction might be an alternative mechanism of STAT5 activation in AML blasts.

In order to determine whether a TYRO3 inhibitor would successfully inhibit TYRO3-mediated STAT5 phosphorylation, we tried to attenuate TYRO3 mediated p-STAT5 signaling. Both of the TYRO3 positive cell lines responded to inhibitor by decreasing phospho-STAT5 protein expression.

We also expanded our TYRO3 research to include TYRO3 drug response on TYRO3 dependent primary patient cells and utilize a heat map analysis that allows us to correlate drug sensitivity and mRNA transcript abundances of TYRO3, GAS6, and PROS1. When we assessed two different group of patients, FLT3 (+) and FLT3 (-), the heat map analysis showed a compelling result that while there is a slight correlation between TYRO3 expression and drug sensitivity parameters in FLT3 (-) group of patients, this correlation is stronger in FLT3 (+) patient group. Additionally, TYRO3 negative cell line MOLM14 (expressing FLT3 ITD) responds to GAS6 stimulation which may suggest that there is a hypothetical crosstalk between TYRO3/GAS6 interaction and FLT3 oncogene with direct or indirect interaction and a resulting STAT5 activity. Although we did not further analyze this hypothesis, previous publications [16, 33] refer to this crosstalk mechanism too.

Future Directions

It is crucial to observe phosphorylation of TYRO3 in the TYRO3 dependent cell lines to prove that increased phospho-STAT5 levels are mediated by activated TYRO3. The phospho-TYRO3 antibody that we used in this research was not very effective in allowing us to observe the change in phospho-TYRO3 levels. An alternative we are considering for that purpose is using tagged antibodies.

Also, phospho-STAT5 level interestingly increased upon GAS6 treatment in a TYRO3 negative cell line likewise we saw in TYRO3 positive cell lines as mentioned previously in this chapter. In addition to that, phospho-ERK is increased upon GAS6 treatment. It may suggest that a possible crosstalk between TYRO3 and FLT3 in mediating cell survival message. The further experimental investigations are needed to confirm potential FLT3-TYRO3 crosstalk. For example, the same drug inhibition assay described in Chapter 4, would be designed to test that p-STAT5 and p-ERK levels increased due to crosstalk between FLT3 and TYRO3 by using siRNA-mediated AXL and MER silenced MOLM14 cell line. Another way would be culturing MOLM14 cells with TYRO3 inhibitor in the presence and absence of GAS6 and counting cells regularly to show whether there is a growth advantage due to GAS6 addition.

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