PROPOSED CHARACTERIZATION OF POSH FUNCTION IN HUMAN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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Master of Sciences

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by

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DEDICATION

This thesis is in dedication to the teacher who made the biggest difference, Lance Kupka. I have been able to pursue my love of science and education because you advocated for me first.

I would also like to give recognition to the College of Saint Scholastica's McNair Scholars program advisors. My pursuit of graduate education was made possible due to your mentorship. I cannot thank you enough.

Lastly, I would like to take the opportunity to thank my family and friends for their endless support on my educational journey. Without you, I would not have been able to pursue these opportunities. I extend a special thank you to my immediate family for all of the sacrifices you made to prioritize my education. And to Ryan for always believing in me.

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PROPOSED CHARACTERIZATION OF POSH FUNCTION IN HUMAN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

Breanna D. Cole

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ABSTRACT

T-cell Acute Lymphoblastic Leukemia (T-ALL) is an aggressive malignancy that is characterized by leukemic T-cells that can express reduced T-cell markers (such as CD5, CD4, or CD8) reflecting their transformation from an early developmental state. These transformed immature cells can proliferate uncontrollably crowding out the mature, non-transformed, normal T-cells. This significantly weakens the immune response and contribute to escape by this cancer from immune surveillance. As treatment strategies have advanced over time, the 5-year overall survival rate has increased to 85% event-free, with the best treatment resulting in the pediatric population. However, relapsed patients have severely limited options for treatment since their disease is often resistant to standard treatments, such as chemotherapy. *There is an unmet clinical need to find new molecular targets/immunotherapies to prevent relapse and improve overall long-term survival*. The focus on the present work is to look at human T-ALL signaling pathways and mutations to identify crucial points for developing more effective therapeutic strategies for personalized treatments.

In T-ALL, there can be several irregularities in MAPK signaling pathways and downstream products. An important question remains to be answered: is there a central

factor that joins many of the molecules participating in dysregulated signaling? Plausible candidates for such a role involve scaffolding proteins, which are known to play a large part in regulating MAPK signaling pathways. In this proposed work, we addressed the proposed POSH function in human T-ALL.

Most studies investigating POSH function in normal T-cells have focused primarily on the composition of the scaffolding complex and its downstream effects. In contrast, few studies have focused on POSH function in leukemic T-cells or expanded on the theme of identifying possible regulators of the formation of the scaffolding complex. The primary research questions that should be investigated are (1) what is the composition and function of the POSH scaffolding complex between different human T-ALL cell lines; (2) how do PTMs on POSH impact the formation of the scaffolding complex; and (3) what is the function of full-length POSH in human T-ALL and how does it differ between different types of human T-ALL? In order to answer these questions, we propose to utilize CRISPR-Cas technology to (A) develop mutant human T-ALL cell lines targeting phosphotyrosine-based modifications on POSH's SH3.3 domains; and (B) develop POSH-KO human T-ALL cell lines. Additionally, we proposed to conduct these experiments on HBP-ALL, Jurkat, and MOLT-14 cell lines on the basis of having different cell surface expression markers, different sensitivities to the Tat-POSH inhibitor, as well as similarities and differences in mutations.

We anticipate the proposed research in this document would mark the beginning of the much-needed investigation of whether POSH or other types of markers can be used in personalized therapies for treating human T-ALL.

CHAPTER 1

INTRODUCTION

1.1 T-cell Acute Lymphoblastic Leukemia (T-ALL)

T-ALL is an aggressive malignancy that is characterized by leukemic T-cells that can express reduced T-cell markers (such as CD5, CD4, or CD8) reflecting their transformation from an early developmental state. These transformed immature cells can proliferate uncontrollably in the bone marrow and blood crowding out the mature non-transformed, normal T-cells[1-2]. This process can significantly weaken the immune response and contribute to escape by this cancer from immune surveillance[1].

As treatment strategies have advanced over time, the 5-year overall survival rate has increased to 85% event-free, with the best treatment results in the pediatric population in which this rare cancer most often occurs[1]. However, relapsed patients have severely limited options for treatment since their disease is often resistant to standard glucocorticoids, radiotherapy, and chemotherapy[1]. Adult patients who have relapsed typically have a lower chance of survival compared to children[3-6]. Currently, the most promising treatment strategy for adults who have relapsed is an allogeneic transplant. However, the survival outcome is only 40%[6]. *There is a significant unmet clinical need to identify new molecular targets/immunotherapies to prevent relapse and improve overall long-term survival.* The focus of the present work is to look at human T-ALL signaling pathways and mutations to identify crucial points for developing more effective therapeutic strategies for personalized treatments.

1.2 Normal T-cell development & differentiation

Normal T-cell development occurs in a small organ in the lymphatic system called the thymus[7]. Here, T-cells develop mature T-cell markers (CD3+, CD4+, CD8+, etc.) and undergo T-cell receptor (TCR) rearrangement[8]. If CD4+ CD8+ double-positive (DP) TCR^{low} cells do not bind to MHC after undergoing TCR rearrangement over the course of several days, they will die. In order to leave the thymus and circulate throughout the body, DP thymocytes must first undergo positive selection (depiction of this process shown in **Figure 1**). During positive selection, DP cells will bind to cortical epithelial cells that express high levels of class I and II major histocompatibility complex (MHC) molecules. This results in the cells receiving signals that promote survival and further progression of development as the cells migrate to the medulla. The MHC the DP cell binds will determine the T-cell markers the mature cell will express. For example, DP cells that bind to MHC class I result in single-positive (SP) CD8+ cell surface expression, while DP cells that bind to MHC class II result in SP CD4+ cell surface expression.

Negative selection occurs primarily at the cortical-medullary junction and is mediated by medullary epithelial cells, dendritic cells, macrophages, and thymocytes. The primary goal of negative selection is to eliminate thymocytes through apoptosis if they possess high affinity to receptors for self. Cells that react moderately, and not too strongly, to the MHC/self-peptide complexes are allowed to exit into the periphery and finalize development[9-10].

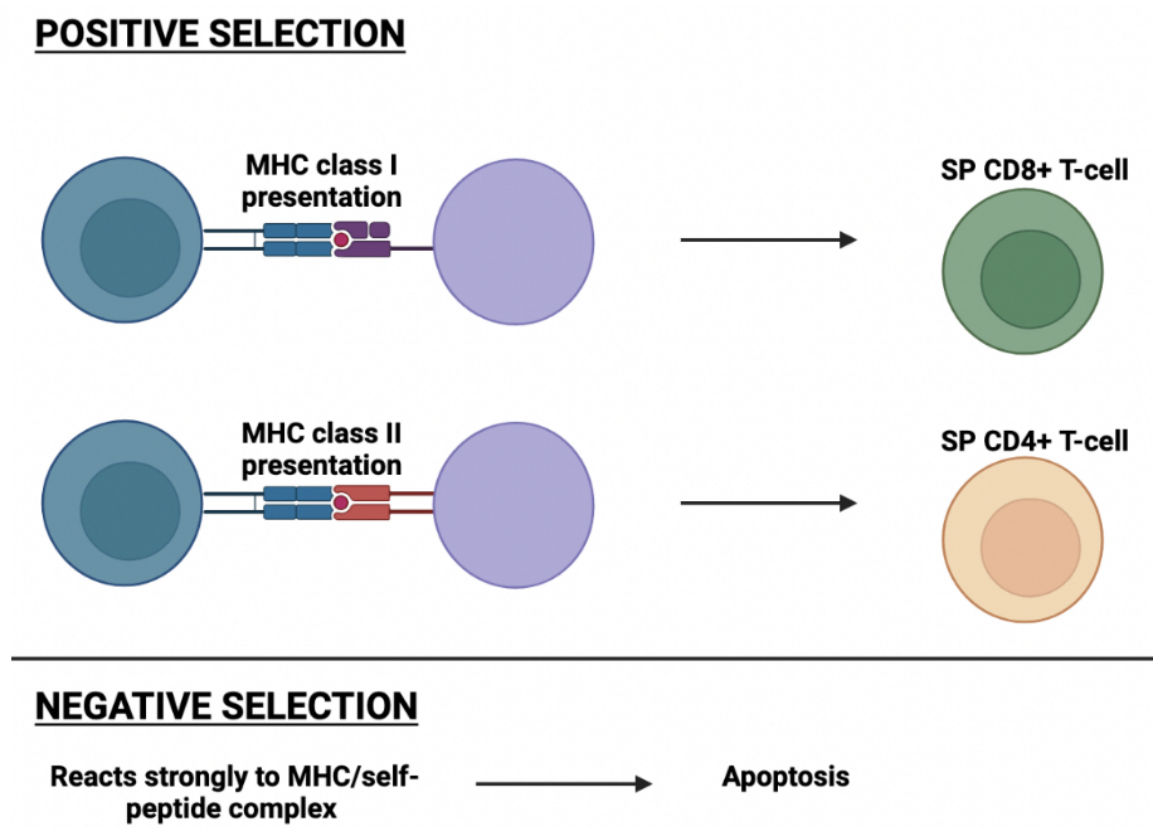

Figure 1. Normal T-cell development. DP cells will bind to cells presenting MHC. Binding to MHC class I results in CD8+ SP T-cells while binding to MHC class II results in CD4+ SP T-cells. Thymocytes that react too strongly to self peptide/MHC complexes undergo negative selection by apoptosis. Created with BioRender.

1.3 Signaling activity in human T-ALL

There are several signaling pathways that exhibit aberrant activation in human T-ALL. A study performed by Perbellini et al. aimed to functionally characterize the different signaling networks in human T-ALL and determine the differences between various cell lines. They accomplished this by utilizing phospho-specific flow cytometric techniques[11]. The pathways they decided to investigate displayed previously known

aberrant activity in human T-ALL such as Akt, ERK1/2, JNK, NFκB, etc. Their phospho-specific flow cytometric techniques allowed them to observe the heterogeneity of signaling activation between three different human T-ALL cell lines: Jurkat, CEM, and MOLT-4. This heterogeneity is an important consideration that supports the idea that personalized targeted T-ALL therapy is what is needed to address this significant unmet clinical need[11] depending on which signaling networks are active in each selected human T-ALL. Below is a discussion on the specifics of aberrant activation in human T-ALL.

In order for the immune system to clear infections and tumors, signaling from T-cell activation and differentiation is critical[12]. Mitogen-Activated Protein Kinase (MAPK) signaling pathways, such as those involving c-Jun N-terminal Kinase (JNK), regulate processes such as activation, proliferation, differentiation, and death in T-cells[13,14]. JNK appears to play an oncogenic role in many cases of malignant lymphocytes[15-17]. Interestingly, inhibition of JNK activity in human T-ALL has been shown in some cases to lead to cell cycle arrest and apoptosis[18,19]. Knowing this, the question remains of how JNK may play an oncogenic role in human T-ALL. It is currently understood that in a highly studied human T-ALL cell line, Jurkat cells, there is high basal phosphorylation of JNK, but there is no increase when stimulating with H_2O_2, where kinase activation is favored[11,20]. However, there is an increase in JNK phosphorylation when stimulating the CEM cell line with H_2O_2. This indicates that JNK may have a divergent role contributing to human T-ALL progression that is dependent on the type of cell line[11].

Unregulated oncogenic activation of Ras signaling is a widespread event in human T-ALL[21,22]. Since the Ras/MAPK signaling pathway is important for cell survival,

differentiation, growth, etc., several studies have implemented trials where they inhibit Ras function through farnesyltransferase inhibitors (FTIs)[23,24] to determine if it leads to cell cycle arrest and apoptosis[25,26]. Alonso-Alonso et al. tested the effect of inhibiting Ras activity with the FTI tipifarnib[27,28] in 18 different human T-ALL cell lines. Although each of these human T-ALL cell lines have differences in mutations that contribute to signaling heterogeneity, the majority of these cell lines had a few similarities. For instance, most of the selected cell lines were sensitive to Ras inhibition, such as HBP-ALL, Jurkat, and MOLT-14. This suggests that there are other factors besides aberrant Ras/MAPK signaling contributing to the overall signaling heterogeneity in human T-ALL. When testing why some human T-ALL cell lines were more sensitive to tipifarnib treatment, Alonso-Alonso et al. observed that there was an increase in ERK activation in the tipifarnib-sensitive cell lines while tipifarnib-resistant cell lines had an increase in RelB expression[29]. These results allowed the authors to look at other signaling pathways in addition to Ras/MAPK signaling.

Another pathway that is severely dysregulated in human T-ALL is the Neurogenic locus notch homolog protein 1 (NOTCH1) signaling pathway, which is normally important for T-cell development[30]. In T-ALL, this dysregulated activity can be mainly attributed to activating mutations in the coding region of the NOTCH1 gene[31]. Such mutations were observed in over 60% of human T-ALL cases, while unmutated cases still showed upregulation of activated NOTCH1 expression. Aberrant activation of NOTCH signaling promotes leukemia by upregulating several pathways downstream such as the NFkB and P13K/Akt signaling pathways[32,33], inducing MYC transcription[34-36], which is important for anabolic cell growth[37].

The P13K/Akt signaling pathway is one of the most commonly dysregulated signaling pathways in human cancer overall, including T-ALL[38,39]. This signaling is important for the regulation of cell survival and proliferation, differentiation, apoptosis, etc. Aberrant activation of the P13K/Akt signaling pathway is often the result of inactivating mutations in the PTEN gene, which ablates its wild-type role as a negative regulator of the P13K/Akt signaling pathway[38,40]. Additionally, oncogenic NOTCH1 signaling and increased kinase signaling contribute to dysregulated P13K/Akt signaling[41]. Knowing this, many researchers have performed studies to identify new therapeutic strategies that inhibit this pathway.

NFkB signaling activity is involved in a variety of immune contexts in T-cells. Although mutations in NFkB gene are not common[41,42], researchers monitor this pathway since it can be activated and largely impacted by P13K/Akt and NOTCH1 signaling activity[43]. Oncogenic NOTCH1 signaling interacts with pathways that are important for the growth, survival, and proliferation of human T-ALL. An example of this is in relation to NOTCH1 signaling enhancing NFkB signaling. NOTCH1 accomplishes this by increasing NFkB expression and nuclear retention[44,45]. This activation can promote either p52 or RelB transcription via IKK complex activation[33]. Since NOTCH1 activation is upregulated in the majority of human T-ALL cases, many look at NFkB signaling activity to learn more about the progression of T-ALL. A study of primary human T-ALL samples showed high NFkB activity as well as a severe lack of growth of T-ALL after inhibiting NFkB activity[33]. Due to these findings, many researchers in this field believe that targeting NFkB also appears to be an attractive therapeutic strategy for T-ALL[46].

The ERK1 signaling pathway is another signaling pathway that has been identified to be upregulated in human T-ALL[11]. Interestingly, all of the cell lines Perbellini et al. tested for signaling activation in T-ALL displayed high basal phosphorylation of ERK1/2, with the Jurkat cell line being the highest. In H_2O_2-modulated conditions, ERK1/2 phosphorylation increased in the Jurkat cell line. It is important to note that the ERK1/2 phosphorylation observed in the Jurkat cell line is much higher than what is observed in normal T-cells. Also, it was noted that all of the human T-ALL cell lines that were tipifarnib-sensitive exhibited an increase in ERK activation status, leading to the hypothesis that Ras and ERK1/2 activation could be linked in these cancers[29]. This is interesting because it suggests a role between P13K/Akt and ERK signaling with human T-ALL function.

In summary, there are several signaling pathways that are highly dysregulated in human T-ALL that should be investigated in this proposed study. Further investigation allows for a better foundation for developing personalized therapeutic strategies in human T-ALL, which could provide help toward a currently unmet clinical need in the field.

1.4 Plenty of SH3s (POSH)

In T-ALL, there can be several irregularities in MAPK signaling pathways and downstream products. An important question remains to be answered: is there a central factor that joins many of the molecules participating in this dysregulated signaling? Plausible candidates for such a role could involve scaffolding proteins, which are known to play a large role in regulating MAPK signaling pathways[47,48]. A scaffolding protein provides docking sites for multiple substrates/products of a signaling cascade where

molecules can assemble together. This allows for the signaling activity to localize at specific sites and contributes to pathway regulation[49]. Specifically, the JNK signaling pathway has been demonstrated to be regulated by several scaffolding molecules such as the JNK-Interacting Protein 1 (JIP-1)[50], Plenty of SHs (POSH)[51], β-arrestin-2[52], Carma/Bcl10[53,54], etc. This document will address POSH function in human T-ALL.

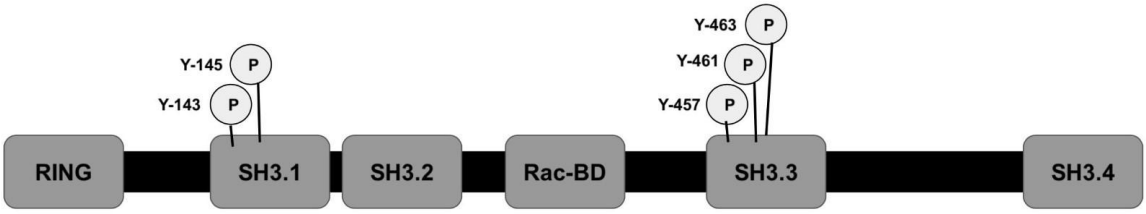

Figure 2. Plenty of SH3s functional domains. Depiction of Plenty of SH3s (POSH) functional domains. POSH has four SH3 domains, a non-canonical Rac binding domain (RBD) and an N-terminal RING finger domain. Additionally, this model shows the location of modifiable tyrosine residues on POSH's SH3.1 and SH3.3 domains.

POSH is a scaffolding protein that features four SH3 domains, a non-canonical Rac binding domain (RBD), and an N-terminal RING finger domain (depiction of POSH's functional domains is represented in **Figure 2**). Upon discovery, POSH was demonstrated to utilize GTPase, Rac1, for JNK and NFkB activation as well as contribute to apoptosis activity when overexpressed in fibroblasts[51]. Since then, the Daniels lab has performed extensive research characterizing the function of POSH in normal T-cells. For example, they have demonstrated that POSH and JIP-1 association induces TCR-mediated JNK activation in normal CD8+ T-cells. When this multiprotein complex was disrupted, defects in proliferation, decreased cytokine expression, and the inability to clear tumors was observed in these cells[55]. However, in normal CD4+ T-cells, POSH appears to function quite differently. For instance, disruption of the POSH scaffolding complex led to decreased survival and loss of Tak-1-mediated activation of JNK1 and JNK2[56].

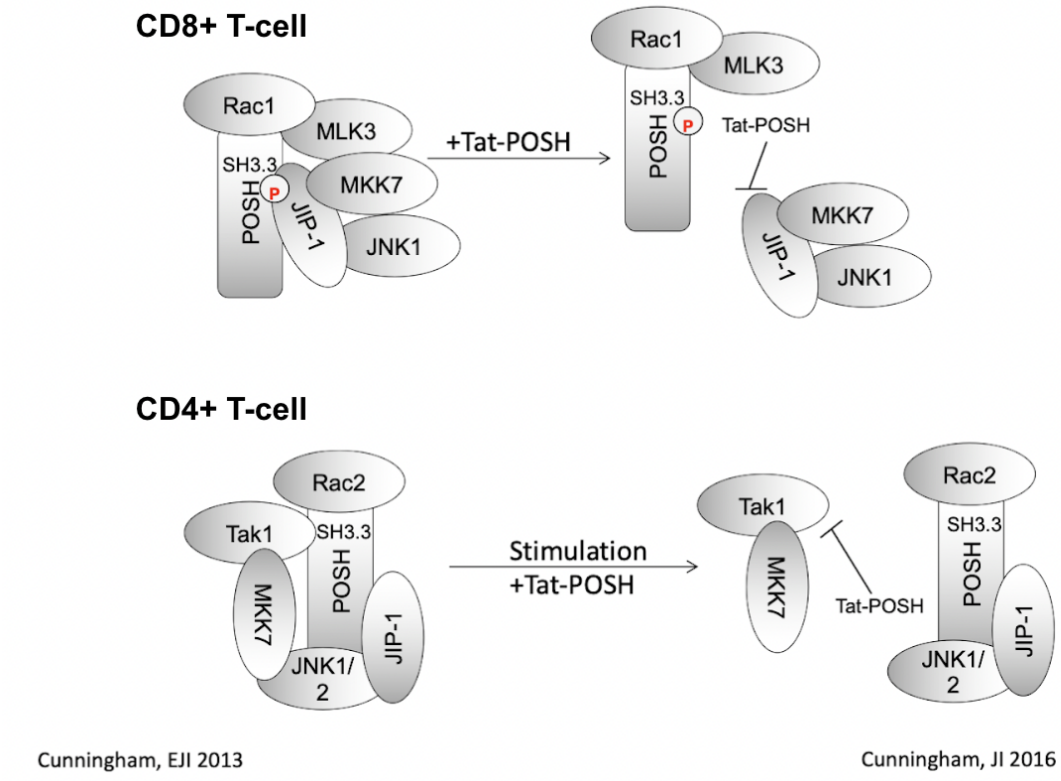

Figure 3. The POSH scaffolding complex looks different depending on the type of T-cell. Model depicting the arrangement of the POSH scaffolding complex between normal CD4+ and CD8+ T-cells. CD4+ and CD8+ T-cells were stimulated with anti-CD3 and anti-CD28 in the presence of IL-2. Subsequently, they were administered the Tat-POSH inhibitor and then lysed. Immunoprecipitation by flow cytometry (IP-FCM) was performed using carboxylate-modified latex (CML) beads coated with either anti-POSH (YY-5) or anti-JIP-1 (2J8) to determine the protein-to-protein interactions of the POSH scaffolding complex.

Differences in POSH function depending on the type of normal T-cell also resulted in different compositions of the POSH scaffolding complex (model depiction of POSH's scaffolding complexes represented in **Figure 3**). In normal CD8+ T-cells, the

POSH scaffolding complex contains Rac1, MLK3, JIP-1, MKK7, and JNK1. When performing Tat-POSH-based competitive inhibition of the POSH SH3.3 domain, this resulted in the loss of JIP-1 which suggested the arrangement of the POSH scaffolding complex in normal CD8+ T-cells displayed in **Figure 3**. However, in normal CD4+ T-cells, the POSH scaffolding complex contains Rac2, Tak-1, MKK7, JIP-1, JNK1, and JNK2. When performing competitive inhibition of POSH's SH3.3 domain with the Tat-POSH inhibitor, Tak-1 was lost resulting in the suggested arrangement of the POSH scaffolding complex in normal CD4+ T-cells (**Figure 3**).

As a result of these observations, the Daniels lab asked another question: what is causing POSH's divergent function depending on the type of normal T-cell?

1.5 POSH's post-translational modifications (PTMs) and involvement in cancer

The Daniels lab hypothesized that POSH exhibits divergent function depending on the normal type of T-cell due to differential PTMs. The rationale for this hypothesis is in reference to a study where Akt-based phosphorylation on POSH's RBD prevented Rac from binding[57,58] which contributed to changes in the composition of the scaffolding complex. Additionally, there are five tyrosine residues within the POSH/JIP-1 binding sites[59] that can be subjected to phosphorylation (specific residues represented in **Figure 2**).

To test this hypothesis, normal CD4+ and CD8+ T-cells were lysed and precipitated with anti-POSH and anti-phosphotyrosine (4G10) antibodies (Abs). Interestingly, a significant increase in phosphotyrosine-based modifications on POSH

was observed in normal CD8+ T-cells that were not observed in normal CD4+ T-cells. This indicates that the PTMs of the tyrosine residues identified in the POSH/JIP-1 binding sites contributed to some of the differences in POSH function between different types of normal T-cells[56]. For example, PTMs on POSH in normal CD8+ T-cells could contribute to higher binding affinity of JIP-1 to POSH's SH3.3 domain resulting in JNK1 activation. However, the absence of PTMs on POSH in normal CD4+ T-cells could have resulted in Tak-1 having a higher binding affinity which contributed to JNK1 and JNK2 activation.

In addition to phosphotyrosine-based modifications on the functional domains of POSH, POSH also has phosphoserine-based modifications on the RBD (serine residue 304) through Akt phosphorylation[59]. This phosphorylation on POSH prevented activated Rac to bind to the RBD which identified a mechanism where Akt promotes cell survival and regulates apoptotic activity[59]. Given this nature, this residue would also be of interest to investigate how these PTMs contribute to POSH function in human T-ALL.

In addition to POSH's demonstrated functional properties in normal T-cells, POSH has been shown to be involved in other types of cancer. For example, in breast cancer cells, phosphorylation on POSH has been demonstrated to be involved in regulating apoptosis through its association with Akt[58]. In prostate cancer, POSH has been shown to be upregulated as well as interact with E3 ubiquitin ligase, Siah2, to reduce caspase activity in response to death ligands[60-63].

Considering everything that has been established about POSH's function in regard to normal T-cells, PTMs, and cancer, the Daniels lab wanted to determine how POSH functions in leukemic T-cells.

1.6 POSH impacts the survival of human T-ALL

The Daniels lab has previously demonstrated that POSH is important for T-cell signaling, survival, and effector function through its interaction with the JNK signaling pathway[55,56] thus impacting the composition of the scaffolding complex. Differences in the composition of the scaffolding complex correlate with different levels of phosphotyrosine-based modifications on POSH's SH3 domains in normal T-cells (**Figures 2 & 3**). Since the composition of the POSH scaffolding complex is largely influenced by signaling cascade members associating with POSH's SH3 domains, the Daniels lab investigated the functional consequence of competitive inhibition on POSH's SH3.3 domain in regard to human T-ALL survival. To accomplish this, they used their novel Tat-POSH inhibitor via HIV Tat protein domain that carries an identical sequence to POSH SH3.3 (**Figure 4**). Various human T-ALL cell lines were treated with the Tat-POSH inhibitor or Tat-Scrambled (as a control). This resulted in the

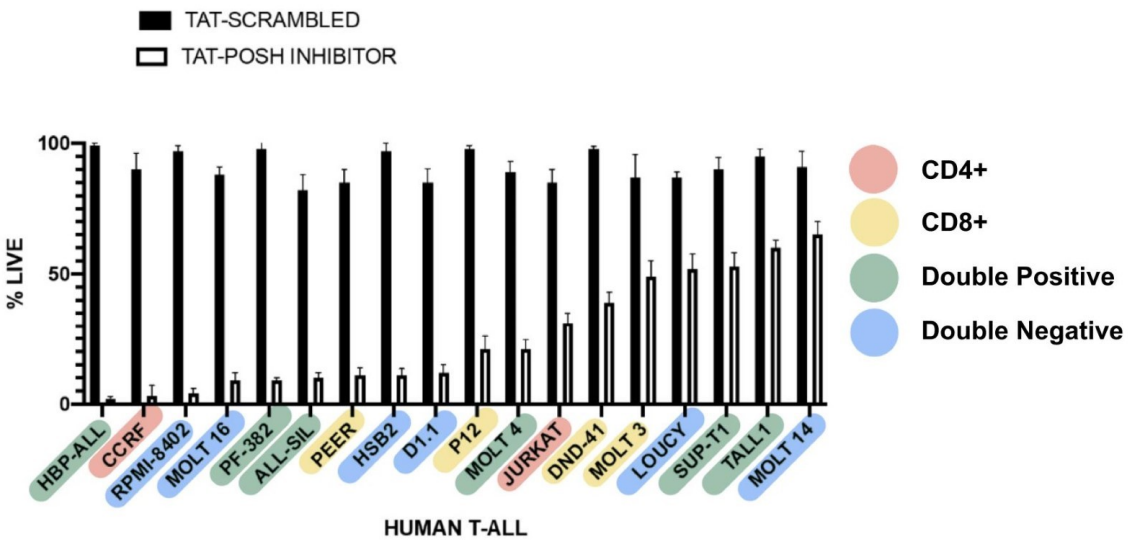

Figure 4. Competitive inhibition of POSH's SH3.3 domain significantly decreases human T-ALL survival. The Tat-POSH inhibitor was administered to various human T-ALL cell lines. The percentage of the live Tat-POSH-treated T-ALL cells compared to Tat-Scrambled-treated (control) T-ALL cells was calculated. Cell surface expression markers (such as CD4 and CD8) are also notated through color.

Tat-POSH-treated human T-ALL cell lines having a similar overall outcome–*competitive inhibition of POSH's SH3.3 domain significantly decreases human T-ALL survival*. However, each Tat-POSH-treated human T-ALL cell line was differently sensitive to competitive inhibition of POSH's SH3.3 domain. Surprisingly, these differences did not correlate with different cell surface expression markers (eg. CD4+ or CD8+). Although it is formally possible that these observations could be due to failure of an effective dose of the Tat-POSH inhibitor to penetrate the T-ALL cell lines, we feel this is unlikely. When performing the same experiment on various human B-ALL cell lines assessed by flow cytometry, the levels of detected fluorescently labeled Tat-POSH were clearly positive and comparable between each cell line (data not shown).

Overall, the data presented here led to the following questions: (1) why are there differences in sensitivity to competitive inhibition of POSH's SH3.3 domain between the human T-ALL cell lines; and (2) are there differences in the composition and function of the POSH scaffolding complex between different human T-ALLs?

1.7 Pilot study

One of the ways I propose to test the functional characteristics of POSH between different human T-ALL cell lines is through the development of POSH mutant and POSH knockout (KO) cell lines. I propose to implement this through CRISPR-Cas technology. To accomplish this, a lentivirus for vesicular stomatitis virus (VSV-G) to deliver the construct to the target human T-ALL cells should be used. To ensure the system would successfully infect a wide variety of cell types, various human cell lines were transduced with this lentivirus. As shown in **Figure 5** via GFP expression, the selected cell lines

were successfully targeted, one of which is a leukemic cell line. Additionally, the same experiment was performed on Jurkat cells and they also became GFP+ (data not shown). Given this data, I am confident this type of methodology can be used to develop POSH mutant T-ALL cell lines.

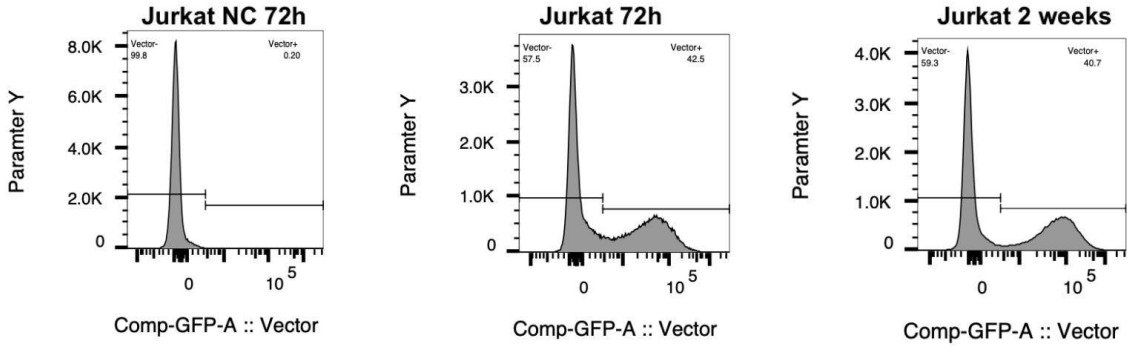

Figure 6. Pilot study performed in Jurkat cells. HEK293FT cells were transfected with the parent vector carrying the POSH-KO guide sequence, an envelope plasmid, and a packaging plasmid. The parent vector features a GFP reporter gene for selection. After 48 hours, the media of the producer cells containing the virus is collected and administered to Jurkat T-ALL cells. GFP expression is represented on the x-axis. Left: Jurkat Negative Control (no vector); Middle: Jurkat 72 hours post-transduction; Right: Jurkat 2 weeks post-transduction.

In support of the previously mentioned data, a pilot study was performed on Jurkat cells using validated guide sequences obtained from the GeCKO library[64]. For a POSH-KO to ensure the human T-ALL cell lines will survive long enough to perform the outlined functional *in vitro* assays (specific details on the methodology are outlined in **Chapter 4, Figure 7**). The parent vector used (lentiCRISPRv2+GFP) features a GFP reporter gene as a selectable marker for delivery. GFP expression was sought to observe the delivery and survival via LIVE/DEAD Aqua stain at 72-hour and 2-week time points. As shown in **Figure 6**, the targeted Jurkat cells remained viable and GFP+ over the time course (LIVE/DEAD Aqua stain data not shown). Given the results of the pilot study, I

am confident that it is possible to perform the outlined methodology in **Chapter 4** to develop POSH mutant and KO T-ALL cell lines.

1.8 Research question

The vast majority of studies investigating POSH function in normal T-cells have focused primarily on determining the composition of the scaffolding complex and its downstream effects. In contrast, few studies have focused on POSH function in leukemic T-cells or expanded on the theme of identifying possible regulators of the formation of the scaffolding complex. The primary research questions that I propose should be investigated are (1) what is the composition and function of the POSH scaffolding complex between different human T-ALL cell lines; (2) how do PTMs on POSH impact the formation of the scaffolding complex; and (3) what is the function of full-length POSH in human T-ALL and how does it differ between each type of cell line? In order to answer these questions, I propose to utilize CRISPR-Cas technology to (A) develop mutant human T-ALL cell lines targeting phosphotyrosine-based modifications on POSH's SH3.3 domain; and (B) develop POSH-KO human T-ALL cell lines.

I propose to conduct the outlined experiments on HBP-ALL (CD3+CD4+CD8+), Jurkat (CD3+CD4+CD8-), and MOLT-14 (CD3+CD4-CD8-) cell lines. These cell lines have been selected to represent the different levels of sensitivity to competitive inhibition of POSH's SH3.3 domain via Tat-POSH. HBP-ALL was the most sensitive, Jurkat was moderately sensitive, and MOLT-14 was the least sensitive. As shown in **Figure 4**, differences in sensitivity to the Tat-POSH inhibitor did not correlate with cell surface expression markers such as CD4 or CD8. This is an important observation because it

eliminates the possibility that different types of human T-ALL (notated by cell surface expression markers) are the reason some T-ALL cell lines are more sensitive to treatment with the Tat-POSH inhibitor than others.

It is worth noting that the selected human T-ALL cell lines have similarities and differences in mutations (**Table 1**). For example, MOLT-14 and HBP-ALL share a mutation in the same residue of the TP53 gene (important for cell cycle regulation and tumor suppression) which was previously shown to be involved in tumorigenesis in hematologic malignancies such as lymphocytic leukemias[65]. Although these cell lines are mutated on the same residue, the outcome is different with MOLT-14 having an Arg273His TP53 mutation and HBP-ALL having an Arg273Cys TP53 mutation.

Each of the selected human T-ALL cell lines has one mutation in the F-box and WD repeat domain containing 7 (FBXW7) gene which is a common tumor suppressor in human cancers[66]. Interestingly, MOLT-14 and HBP-ALL have the same Arg465His FBXW7 mutation while the Jurkat cell line has an Arg505Cys mutation. Since MOLT-14 and HBP-ALL are on opposite ends of the spectrum for sensitivity to treatment with the Tat-POSH inhibitor, the Arg465His FBXW7 mutation is unlikely to impact this phenomenon.

In addition to being a tumor suppressor for several human cancers, FBXW7 is a negative regulator of NOTCH1, which is involved in normal T-cell development and differentiation[67]. HBP-ALL has two mutations on the NOTCH1 gene (Leu1574Pro & Asp2442fs*39) and Jurkat has one (Arg162His). These cell lines are more sensitive to treatment with the Tat-POSH inhibitor than the MOLT-14 cell line, which has no mutations in the NOTCH1 gene. This indicates that these mutations in NOTCH1 might

be involved in human T-ALL survival when POSH's SH3.3 domain is competitively inhibited.

Lastly, an important factor to consider is that HBP-ALL and Jurkats are the only selected human T-ALL cell lines with a mutation in the PTEN gene[68]. This might be a reason why these cell lines are more sensitive to competitive inhibition of POSH's SH3.3 domain because PTEN is a tumor suppressor gene that antagonizes the P13K-Akt signaling pathway (which is important for cell proliferation and survival). When the PTEN gene is mutated, the P13K-Akt signaling pathway increases its activity which could cause unregulated G1/S phase cell cycle progression[69].

Gene	HBP-ALL	Jurkat	MOLT-14
FBXW7	Arg465His (unspecified)	Arg505Cys (het)	Arg465His (het)
HRAS	Ala134Ser (unspecified)	0	0
NOTCH1	Leu1574Pro (het) Asp2442fs*39 (het)	Arg1627His (het)	0
PTEN	Arg159Ser (unspecified)	0	0
TP53	Cys124Ter (hom) Arg273Cys (unspecified)	Arg196Ter (het)	Arg213Ter (het) Arg273His (het)
WT1	Ser121Ter (unspecified)	0	0
BAX	0	Glu41Glyfs*33 (het) Glu41Argfs*19 (het)	0
INPP5D	0	Gln345Ter (het) c.1097+1065__1097+1112del47 (het)	0
MSH2	0	Arg711Ter (hom)	0
MSH6	0	Phe1088Serfs*2 (hom)	0
NRAS	0	0	Gly12Asp (het)

Table 1. Mutations found in selected human T-ALL cell lines. Represented are the number and type of mutations found in each of the selected human T-ALL cell lines (HBP-ALL, Jurkat, MOLT-14). **Het** abbreviation stands for heterozygous for the mutation. **Hom** abbreviation stands for homozygous for the mutation. This information was accessed at Cellosaurus through the Swiss Institute of Bioinformatics.

Overall, HBP-ALL, Jurkat, and MOLT-14 cell lines have been selected on the basis of having different cell surface expression markers, different sensitivities to the Tat-POSH inhibitor, as well as similarities and differences in mutations. This will allow for the chance to determine if any of those factors are also involved in the role POSH

serves in human T-ALL. It is anticipated that this proposed study will allow researchers to further understand the biological relevance of the potential findings.

CHAPTER 2

THE COMPOSITION OF THE POSH SCAFFOLDING COMPLEX IN HUMAN T-ALL

2.1 Gap, hypothesis, & rationale

Knowing the composition of the POSH scaffolding complex is vital for understanding the mechanism by which POSH activates downstream signaling pathways. The composition of the POSH scaffolding complex has been determined in various types of cells including normal T-cells. However, it has yet to be determined what the scaffolding complex looks like in various types of human T-ALL cell lines where signaling activity is severely dysregulated. An important question remains to be answered: how does the POSH scaffolding complex assemble and what is its function in human T-ALL?

I hypothesize that the POSH scaffolding complex will differ depending on the type of human T-ALL cell line. Additionally, POSH is expected to continue to associate with signaling cascade members such as a JNK, an MKK, a Rac, JIP-1, Tak-1, or Akt proteins because POSH has previously been shown to interact with these signaling cascade members in various types of other cells including normal T-cells[55,56]. It is expected that the composition of the POSH scaffolding complex will be different depending on the type of human T-ALL cell line because several studies have determined that different signaling pathways are activated depending on the type of human T-ALL

cell lines[11]. Addititionally, the composition of the POSH scaffolding complex assembles differently depending on which type of normal T-cell[55,56].

2.2 Proposed methodology

2.2.A Co-immunoprecipitation by flow cytometry (IP-FCM)

In order to determine the composition of the POSH scaffolding complex between different types of human T-ALL cell lines, each selected cell line will be treated with Tat-POSH or Tat-Control and then subsequently lysed. To pull down the POSH scaffolding complex, carboxylate-modified latex (CML) beads coated with C-terminus-binding POSH E1 monoclonal antibody (mAb) will be used. When the pulldown is complete, samples will be incubated with fluorochrome-conjugated Abs probing for signaling cascade proteins that are known to be candidates to form complexes with POSH. These will include but are not limited to JNK1, JNK2, MKK4, MKK7, MLK3, JIP-1, Tak-1, Akt, MEKK1, Rac-1, Rac-2, etc. To ensure there are similar amounts of POSH in each co-IP assay, POSH mAb will be used as a probe in parallel. Each antigen will be normalized to the detected level of POSH. To determine the background binding of the mAbs to the CML beads, proteins that are known not to be in complex with POSH (such as ERK1) will be probed.

Table 2. Chapter 2 experimental conditions.

Negative Control	Experimental Condition
Tat-Control-treated human T-ALL	Tat-POSH-treated human T-ALL

2.2.B Functional analysis of the POSH scaffolding complex in human T-ALL

I propose to perform various functional *in vitro* assays after treatment with Tat-POSH or Tat-Control to characterize the impact that the composition of the POSH scaffolding complex has on human T-ALL signaling activity and function. To determine how the composition of the POSH scaffolding complex influences proliferation in each selected human T-ALL cell line, CFSE labeling will be performed. The progress of proliferation will be monitored by observing the dilution of the dye in the membrane of the dividing T-ALL cells. To determine how the composition of the POSH scaffolding complex contributes to the activation of various signaling pathways in T-ALL, immunoblots will be performed to determine the levels of phosphorylated JNK1/2 (p-JNK1/2), p-p38, p-NFκB, p-MKK7, p-Akt, and p-ERK1. As an additional indicator of signaling activation, various transcription factor activity assays will be performed to determine the levels of activated c-Jun, NFκB p52 (regulation of cell proliferation), and NFκB Rel[70]. To determine how the composition of the POSH scaffolding complex contribute to apoptosis activity in each selected human T-ALL cell line, immunoblot analyses will be performed to determine the levels of pro-apoptotic (Nim, Puma, Noxa) and anti-apoptotic (Mcl-2, Bcl2) markers. Additionally, the amount of caspase 3/7 will be measured. Since cell cycle entry is highly dysregulated in human T-ALL, it would be insightful to determine if this is correlated with certain compositions of the POSH scaffolding complex. To determine this, the percentage of Ki67+ T-ALL cells will be detected using flow cytometric techniques. Experimental conditions are outlined in **Table 2**.

2.2.C Determine PTMs on POSH's functional domains between the selected human T-ALL cell lines

In normal T-cells, the composition of the POSH scaffolding complex assembled differently and correlated with differential phosphotyrosine-based modifications on POSH's SH3.1 and SH3.3 domains in normal CD8+ T-cells. It would be interesting to determine if there are differences between each selected human T-ALL cell line that correlate with these types of modifications as well. To answer this question, each selected human T-ALL cell line will be harvested, lysed, and then given a phospho-POSH mAb to perform a subsequent immunoblot. This methodology will allow for the ability to see general differences in phosphotyrosine-based modifications on POSH between each of the selected human T-ALL cell lines (if any exist). A significant limitiation of this technique is that it will not determine the exact locations of these modifications. To overcome this limitation, mass spectrometry should be performed.

2.3 Expected results

2.3.A General

The data that will be collected will provide information on how the POSH scaffolding complex is formed between different human T-ALL cell lines. Generally, it is expected that POSH will associate with a JNK, a Rac, an MKK, JIP-1, Tak-1, or Akt proteins since these proteins have associated with POSH in normal T-cells[55,56]. As justification for this idea, there is aberrant MAPK/ERK and P13K/Akt signaling in human T-ALL[11]. Additionally, I hypothesize that each selected human T-ALL cell line may have different mechanisms of signaling activation that will be demonstrated through

different compositions of the POSH scaffolding complex. I further hypothesize that cell lines with a similar composition of the POSH scaffolding complex may have similar results in the functional *in vitro* assays described in the methodology.

If the composition of the POSH scaffolding complex leads to the activation of signaling pathways that promote proliferation, there should be an observed decrease in the dilution of the dye (meaning greater dye retention due to less mitosis) in the membrane of the dividing human T-ALL cells treated with Tat-POSH compared to the Tat-Control-treated samples when performing CFSE labeling. To determine which signaling cascade pathways are activated through the formation of the POSH scaffolding complex, there might be decreased protein expression or signaling-activated protein in the performed immunoblots in the Tat-POSH-treated samples compared to the Tat-Control-treated samples. The same logic will be applied to the results of the transcription factor activity assays. To determine if the composition of the POSH scaffolding complex is activating pathways important for regulating apoptosis activity, there should be an observed decrease in the expression or pro-apoptotic and/or anti-apoptotic markers as well as a decrease in the detected caspase 3/7 in the Tat-POSH-treated samples compared to the Tat-Control-treated samples. Lastly, if the composition of the POSH scaffolding complex is important for activating pathways regulating cell cycle entry, then there might be a decrease in the percentage of Ki67+ cells in the Tat-POSH-treated samples compared to the Tat-Control-treated samples.

Phosphotyrosine-based modifications of POSH appear to correlate with differences in the composition of the POSH scaffolding complex and downstream signaling activation in normal T-cells[55,56]. Due to this observation, I hypothesize that there

will be differences in the level of phosphotyrosine-based modifications on POSH's functional domains between each selected human T-ALL cell line.

2.3.B HBP-ALL

As described above, the HBP-ALL cell line has several mutations that may help anticipate what might be observed in the composition of the POSH scaffolding complex as well as the results of the functional *in vitro* assays outlined in the *2.2 Proposed methodology* section. Perhaps the most notable mutation is the Arg159Ser mutation on the PTEN gene, which is a negative regulator of the P13K/Akt signaling pathway. Inactivating mutations on the PTEN gene in human T-ALL have been demonstrated to contribute to aberrant P13K/Akt signaling[38,39]. This leads me to hypothesize that HBP-ALL will have the highest P13K/Akt signaling activity compared to the other selected human T-ALL cell lines since HBP-ALL has an inactivating mutation on PTEN and was more sensitive to competitive inhibition of POSH's SH3.3 domain. If this hypothesis is true, it should be evident in decreased detected p-Akt in the Tat-POSH-treated HBP-ALL samples.

Interestingly, oncogenic NOTCH1 signaling and increased kinase signaling also contribute to aberrant P13K/Akt signaling[41]. HBP-ALL has two mutations in the NOTCH1 gene: Leu1574Pro and Asp2442fs*39 (more than the other selected human T-ALL cell lines). In addition to mutations in the NOTCH1 gene, there is also an Arg465His mutation in the FBXW7 gene, which is a negative regulator of NOTCH1 activity. Oncogenic NOTCH1 signaling also contributes to direct NFκB activation. There are several mechanisms of NFκB activation. It is expected that there will be a decrease in NFκB RelB activity[29]. In support of this, a study looking at transcription factor activity in

100 blood cell cancer cell lines showed that HBP-ALL also had lower RelB activity[71]. However, RelA had significantly higher activity in HBP-ALL indicating alternative NFκB activity[71]. Another trait of tipifarnib-sensitive human T-ALL cell lines, such as HBP-ALL, is higher activation of ERK1 signaling[29]. Since POSH hasn't been known to interact with ERK-1 in other cells, there should be no change in the detected level of p-ERK1 between the Tat-Control- and Tat-POSH-treated HBP-ALL samples.

Regarding the composition of the POSH scaffolding complex in HBP-ALL, I hypothesize that because HBP-ALL is CD3+CD4+CD8+, there will be JNK1 activation through JIP-1 binding because normal CD8+ T-cells had JIP-1-dependent activation of JNK1[55,56]. I anticipate that the composition of the POSH scaffolding complex in human T-ALL will activate pathways that are important for proliferation, cell survival, cell cycle entry, etc. Additionally, because there is CD8+ cell surface expression on HBP-ALL, it is hypothesized that HBP-ALL may have phosphotyrosine-based modifications on POSH's functional domains because this occurred in normal CD8+ T-cells[56].

Overall, the HBP-ALL cell line offers a unique opportunity to examine the impact that inactivating mutations on PTEN, NOTCH1, FBXW7, as well as high Tat-POSH inhibitor sensitivity has on the function of POSH within this cell line.

2.3.C Jurkat

The Jurkat cell line is CD3+CD4+CD8- and is moderately sensitive to treatment with the Tat-POSH inhibitor. In regard to PTMs, it is hypothesized that the Jurkat cell line may have little to none phosphotyrosine-based modifications on POSH's functional domains because of the CD4+ cell surface expression on this cell line. Due to this, it is hypothesized that POSH may participate in Tak-1-dependent activation of JNK1/2

because this occurs in normal CD4+ T-cells[56]. On the same basis, it is possible that the composition of the POSH scaffolding complex in Jurkat will activate pathways that are important for cell survival, but not have an impact on cell cycle entry[56]. This might be demonstrated through the composition of the POSH scaffolding complex as well as observing no changes in Ki67+ T-ALL cells between Tat-POSH- and Tat-Control-treated samples. In support for the JNK signaling pathway activation, it has been demonstrated by phospho-specific flow cytometric techniques that the Jurkat cell line has higher basal phosphorylation on JNK than in normal T-cells[11].

The Jurkat cell line has a few mutations that may give insight into which signaling pathways are to be activated through POSH's scaffolding function. The most notable mutations are the Arg505Cys mutation on the FBXW7 gene and the Arg1672His mutation on the NOTCH1 gene. These mutations indicate that there will be aberrant NOTCH1 signaling which would potentially contribute to an increase in P13K/Akt and NFκB signaling activation. In support of this, the Jurkat cell line has higher basal phosphorylation on Akt[11]. Jurkats are also tipifarnib-sensitive which means that NFκB activity will not be through RelB[29] and it is unlikely that NFκB activity will be through RelA since this was downregulated[71] in Jurkats. Even so, due to the aberrant signaling of NOTCH1, it is still expected that there will be NFκB activity. It is possible that this may be through other mechanisms such as p52. Lastly, because of tipifarnib sensitivity, Jurkats are also expected to have relatively high amounts of detected p-ERK1 in the signaling activation immunoblots, indicating high ERK1 signaling activity. However, since POSH has not been known to interact with ERK1 in other types of cells, there

should be no difference in the amount of detected p-ERK1 between the Tat-Control- and Tat-POSH-treated samples.

2.3.D MOLT-14

The MOLT-14 cell line is CD3+CD4-CD8- and the least sensitive to treatment from the Tat-POSH inhibitor. Unfortunately, this cell line also has the least amount of preliminary studies performed on it making it difficult to predict how the POSH scaffolding complex will assemble contributing to signaling activation and functions. Even so, I hypothesize that MOLT-14 will have few (if any) phosphotyrosine-based modifications on POSH's functional domains due to no CD8+ cell surface expression. It is possible that POSH will activate the JNK signaling pathway through Tak-1 (like CD4+ T-cells) or through another mechanism that has yet to be determined.

Although there have not been a lot of preliminary studies performed on MOLT-14, mutations may give insight as to what signaling activity may occur in this cell line. For example, MOLT-14 has an Arg465His mutation on the FBXW7 gene but no mutation in the NOTCH1 gene. This indicates that perhaps there will be some increase in NFκB activity, but not as much as the Jurkat or HBP-ALL cell lines. MOLT-14 is also tipifarnib-sensitive[29], so it is expected that if there is NFκB activity, it might be activated through a different mechanism than RelB such as p52. It is also possible that MOLT-14 may have the lowest detected NFκB activity because of these mutations. MOLT-14 should have relatively high ERK1 activity due to the tipifarnib sensitivity[29] but there should be no differences in p-ERK1 activation between Tat-Control- and Tat-POSH-treated samples since this protein has not previously associated with POSH.

Since MOLT-14 is the least sensitive to treatment with the Tat-POSH inhibitor, I anticipate that POSH in the MOLT-14 cell line may prioritize activating pathways that are not related to cell survival, proliferation, etc. This may be demonstrated by MOLT-14 having the lowest activation of pathways such as Akt as well as no significant differences in the CFSE labeling between Tat-POSH- or Tat-Control-treated MOLT-14 samples.

2.4 Alternative outcomes

The Daniels lab has successfully determined the composition of the POSH scaffolding complex in normal T-cells[55,56] so it is unlikely that there will be issues in performing the outlined methodology in this chapter. However, it is possible that it will be difficult to properly detect the composition of the POSH scaffolding complex through IP-FCM. If this occurs, I suggest performing a co-IP assay through immunoblotting techniques. It is also possible that the protein-antibody complexes will not stay intact depending on where the Ab binds. To circumvent this, there are other POSH mAbs that can be utilized. If there are difficulties with detecting POSH or other proteins known to be in complex with POSH due to their epitopes being hidden within the scaffolding complex, several antibodies probing for the same protein will be administered in a cocktail.

A significant potential problem is there might not be any differences between the Tat-POSH- and Tat-Control-treated conditions while performing the outlined functional *in vitro* assays. An alternative hypothesis is that the POSH scaffolding complex is formed by other means than the POSH SH3.3 domain. To test this hypothesis, other Tat-POSH inhibitors will be developed to target the other SH3 domains to get a more complete

understanding of the formation of the POSH scaffolding complex in human T-ALL. Additionally, it is possible there will not be significant differences between the selected human T-ALL cell lines. If this occurs, there is a diverse repertoire of human T-ALL cell lines featured in **Figure 4** that can be used for the outlined methodology to answer the experimental question of this chapter.

Lastly, it is possible there might not be differences in the phosphorylation-based modifications on POSH's functional domains between each selected human T-ALL cell line, but there are differences in the results of the functional *in vitro* assays. If this is evident, an alternative hypothesis is that other factors are contributing to the differences in the functional *in vitro* assays such as phosphoserine-based modifications[58].

2.5 Rigor & reproducibility

Each experiment will be replicated in triplicate to ensure the results of the outlined functional *in vitro* assays in this chapter are accurate. To determine the statistical significance between the Tat-POSH- and Tat-Control-treated conditions between two selected human T-ALL cell lines, a two-way Student's t-test will be performed. Some results may be normalized to the Tat-Control-treated conditions. To determine the differences between all selected human T-ALL cell lines tested together, a two-way ANOVA test followed by a Tukey post-hoc test will be performed. All statistical analyses will be performed using Prism GraphPad and I will consider a p-value less than 0.05 to be statistically significant.

CHAPTER 3

PHOSPHOTYROSINE-BASED MODIFICATIONS ON POSH

3.1 Gap, hypothesis, & rationale

In human T-ALL, empirical evidence is lacking that would indicate what is regulating the composition of the POSH scaffolding complex in human T-ALL. Interestingly, in **Figure 4**, each of the human T-ALL cell lines that were subjected to competitive inhibition of POSH's SH3.3 domain were differently sensitive. This indicates that POSH might provide a different function in each of these cell lines by activating different pathways. I hypothesize that phosphotyrosine-based modifications on POSH's SH3.3 domain regulate the composition of the POSH scaffolding complex by determining which signaling cascade members bind to POSH, contributing to the downstream signaling pathway that becomes activated. As a basis for this hypothesis, previous studies have demonstrated that phosphorylation-based modifications on POSH impact downstream signaling pathway activation in non-T-ALL cells. For example, phosphorylation by Akt on the RBD of POSH prevents Rac from binding to POSH[50,51] thus contributing to changes in the composition of the scaffolding complex. Another study has identified five tyrosine residues on POSH's SH3.1 and SH3.3 domains that can be subjected to phosphorylation within the POSH/JIP-1 binding sites[50,51]. Additionally, the Daniels lab has demonstrated that the composition of the POSH scaffolding complex is different depending on the normal T-cell type. This correlated with differential

phosphotyrosine-based modifications on POSH that were specific to normal CD8+ T-cells[48].

3.2 Proposed methodology

3.2.A Prime editing

To understand the role of phosphotyrosine-based modifications on the composition of the POSH scaffolding complex and processes occurring in human T-ALL, residues Y-457, Y-461, and Y-463 on POSH's SH3.3 domain will be replaced with either phenylalanine or histidine residues through CRISPR prime editing (PE). PE is an editing technique that is used to edit sequences without making a double-stranded break (DSB). Rather than using Cas9 for editing, PE uses Cas9 nickase fused to a reverse transcriptase so it does not generate DSBs[72].

To design the PE experiment, decisions on the pegRNA design, PE system, selection of PE architecture and installation of silent mutations will be made through the guidance of Doman et. al[73]. The mode of delivery that I propose is a lentiviral vector as it would be similar to what was performed in the pilot study (**Figure 6**). Plasmids for this proposed experiment should include a plasmid carrying the prime editor, an epegRNA cloning vector, an sgRNA cloning vector, and other plasmids for the sgRNA preparation. Additionally, it would be important to tell the difference in the successfully targeted T-ALL cells between the modified POSH protein and WT POSH protein. In order to do this, I suggest selecting a lentiviral vector with a FLAG-TAG on it.

To achieve efficient delivery of the PE materials, the plasmids will be transfected into HEK293FT producer cells. The cell culture media containing the virus will be

collected 48 hours post-transfection and then subsequently delivered to the target human T-ALL cell lines. The successfully targeted cells will undergo selection and then undergo single-cell expansion. After single-cell expansion, the genomic DNA will be isolated. Sanger sequencing and a Tracking of Indels by DEcomposition (TIDE) analysis will be performed for each of the isolates to determine if there was a successful modification to the genomic DNA. To confirm the genetic modifications at the protein level, an immunoblot probing for POSH and phosphotyrosine-based modifications (4G10) will be performed. To determine if homozygous-modified human T-ALL cells are dying (if POSH is essential for survival in some of the selected human T-ALL cell lines), the genomic DNA will be isolated 24 hours post-transduction, and TIDE analysis will be performed and compared with the single-isolates data to determine the differences in populations.

3.2.B Functional analysis of POSH-mutant T-ALL cell lines

The same functional *in vitro* assays outlined in **Chapter 2** will be performed to determine the impact that phosphotyrosine-based modifications on POSH's SH3.3 domain have on POSH function in human T-ALL. The experimental conditions are outlined in **Table 3**.

Table 3. Chapter 3 experimental conditions.

Negative Control	Experimental Condition
Tat-Control-treated human T-ALL	Tat-POSH-treated human T-ALL
Tat-Control-treated POSH-modified (Phe) human T-ALL	Tat-POSH-treated POSH-modified (Phe) human T-ALL
Tat-Control-treated POSH-modified (His) human T-ALL	Tat-POSH-treated POSH-modified (His) human T-ALL

3.3 Expected results

3.3.A General

The data that will be collected will provide information on how phosphotyrosine-based modifications on POSH regulate the composition of the POSH scaffolding complex and signaling activity in human T-ALL. It is expected that the successfully targeted human T-ALL cell lines will either be heterozygous or homozygous for the mutation. The human T-ALL cell lines that are homozygous for the mutation should not be capable of producing the specifically targeted phosphotyrosine-based modifications. This will provide the opportunity to determine the impact of specific phosphotyrosine-based modifications being absent on POSH's functional domains. The human T-ALL cell lines that are heterozygous for the mutation may have decreased phosphotyrosine-based modifications compared to the WT human T-ALL cell lines. These cell lines may also provide information on the effect of decreasing gene dose for a specific phosphorylatable residue. If both heterozygous and homozygous cells are available, both will be used for the outlined experiments in this chapter.

If phosphotyrosine-based modifications on POSH's SH3.3 domain contribute to changes in its binding affinity for certain client proteins, it is expected that there should be differences demonstrated in the composition of the POSH scaffolding complex in the POSH-modified human T-ALL cell lines compared to the POSH-WT human T-ALL cell lines. Differences in the composition of the POSH scaffolding complex should exhibit differences in the results of the functional *in vitro* assays between POSH-modified and POSH-WT human T-ALL cell lines.

I hypothesize decreased JIP-1 binding in the POSH-modified human T-ALL cell lines since higher levels of phosphotyrosine-based modifications on POSH's SH3.3 domain correlated with JIP-1 binding to POSH in normal T-cells[56]. Additionally, it is hypothesized that POSH-modified human T-ALL cell lines with a similar composition of the POSH scaffolding complex may have similarities in the results of the functional *in vitro* assays outlined in this chapter. If this does not occur, this indicates that phosphotyrosine-based modifications on POSH's SH3.3 domain is not regulating the composition of the POSH scaffolding complex in human T-ALL. Another possibility is that there are other types of PTMs on POSH's functional domains that are contributing to the composition and downstream signaling pathway activation or that perhaps another molecule is involved in this process.

3.2.B HBP-ALL

Due to what was observed by Cunningham et al. 2016, I hypothesize that the POSH-mutant HBP-ALL cell lines may have the most changes in regard to POSH function, composition of the scaffolding complex, etc. The HBP-ALL cell line is CD4+CD8+ and due to the CD8+ cell surface expression, I hypothesize that this cell line may have high amounts of PTMs on the modifiable tyrosine residues[55,56]. It is predicted that by disrupting the phosphotyrosine-based modifications on POSH SH3.3, there might be an observed loss of JIP-1-dependent activation of JNK1 and instead see Tak-1-dependent activation of JNK1 and JNK2.

3.3.C Jurkat & MOLT-14

As described in **Chapter 2**, the Jurkat and MOLT-14 cell lines are predicted to have little to no phosphotyrosine-based modifications on POSH's functional domains due

to lack of CD8+ cell surface expression and observations made in normal T-cells[55,56]. Given this prediction, there might be little to no differences between the POSH-mutant and POSH-WT Jurkat/MOLT-14 cell lines. Specifically, if the hypothesis is correct, there might be no change in the composition of the POSH scaffolding complex in the POSH mutant Jurkat/MOLT-14 cell lines. No changes in the composition of the POSH scaffolding complex could also lead to no changes in signaling activation that would be evident by no changes in the results of the functional *in vitro* assays.

It is important to note is that this chapter is specifically examining phosphotyrosine-based modifications on POSH's SH3.3 domain. This would not determine the impact that other types of PTMs on POSH's functional domains could have in human T-ALL. More analyses would be needed to address this latter point.

3.4 Alternative outcomes

It is possible there will not be significant differences between the POSH-modified and POSH-WT human T-ALL cell lines. Such a result could be due to a couple of possibilities: (1) phosphotyrosine-based modifications on POSH do not regulate these functions in human T-ALL; and/or (2) there is more POSH-WT protein present than the POSH-modified protein, such as could be possible in a heterozygous modified cell line. If phosphotyrosine-based modifications on POSH are not regulating these functions in human T-ALL, an alternative hypothesis is that phosphoserine-based modifications on POSH (through the RBD) are contributing to these functions. To test this hypothesis, serine 304[57,58] will be mutated and replaced with a cysteine residue utilizing the same methodology outlined in this chapter. To test the functional characteristics of this

mutation, the same functional *in vitro* assays outlined in this chapter will be performed. If there is more endogenous POSH-WT protein present than the POSH-modified protein, then a tyrosine-kinase inhibitor can be administered to inhibit phosphorylation on those modifiable residues in the POSH-WT protein, and perhaps some insights could be gained despite the generalizable lack of site-specific targeting of this latter strategy. The same functional *in vitro* assays should be performed as described in this chapter.

3.5 Rigor & reproducibility

Each experiment will be replicated in triplicate to ensure the results of the outlined functional *in vitro* assays in this chapter are accurate. To determine the statistical significance between the POSH-mutant and POSH-WT conditions between each selected human T-ALL cell line, a two-way Student's t-test will be performed. Some results may be normalized to the POSH-WT conditions. To determine the differences between many selected human T-ALL cell lines together, a two-way ANOVA test followed by a Tukey post-hoc test will be performed. All statistical analyses will be performed using Prism GraphPad and I will consider a p-value less than 0.05 to be statistically significant.

CHAPTER 4

DETERMINING THE FUNCTION OF POSH IN HUMAN T-ALL VIA CRISPR POSH-KO

4.1 Gap, hypothesis, & rationale

The main question to be addressed in this chapter is: what is the function of full-length POSH protein in human T-ALL? The Daniels lab has begun to characterize the function of POSH in human T-ALL cell lines through competitive inhibition of POSH's SH3.3 domain (**Figure 4**). However, POSH's other functional domains are still active and remain open for potential molecular interactions when assembling the scaffolding complex. This means that POSH's full function through all of its functional domains has not yet been characterized in human T-ALL. I propose that this should be accomplished by knocking out POSH protein expression through CRISPR-Cas9 technology and validated guide sequences obtained from the Addgene GeCKO library[64].

I hypothesize that the POSH scaffolding complex contributes to the hyperactivation of MAPK signaling pathways in human T-ALL. MAPK signaling pathways, such as the JNK signaling pathway, have higher basal phosphorylation in human T-ALL when compared with normal T-cells (observed in Jurkat cell line)[11]. Scaffolding proteins, such as POSH, have been shown to regulate these signaling pathways[47,48,51] thus POSH could be a central factor contributing to this aberrant signaling activity in human T-ALL. In addition, competitively inhibiting POSH's SH3.3 domain resulted in a significant decrease in the survival of the human T-ALL cell lines that were

tested (**Figure 4**). However, some human T-ALL cell lines were less sensitive suggesting that POSH's scaffolding functions mediated by its SH3.3 domain may be essential for some cell lines, but not for all. Progressing toward the proposed studies, a POSH-KO sgRNA was delivered in Jurkat cells which remained viable and GFP+ two weeks post-transduction (**Figure 6**), allowing enough time for functional *in vitro* assays to be performed to characterize POSH's function.

4.2 Proposed methodology

4.2.A Development of POSH-KO human T-ALL cell lines

Table 4. Validated guide sequences for POSH-KO.

Sequence
TTCATAGTTGTATAATGCTT
TTGACTTCCCCATGGTACCA
GCC TTC CTG CAG CCC AGT TT
CTC TTC GGA TCC CAG TCA GA
GGGCGGGGTCACAATTAACC
GTGGTACTTATATGAACCTG

POSH-KO T-ALL cell lines will be developed through lentiviral-mediated CRISPR-Cas9 technology. Validated guide sequences obtained from the GeCKO library[64] (**Table 4**) will be cloned into pLentiCRISPRv2+GFP (Addgene plasmid #82416)[74]. This plasmid features a GFP reporter gene for selection to validate the delivery of the construct. For virus production, psPAX2 (Addgene plasmid #12260)[75] and VSV-G (Addgene plasmid #14888)[76] will be used to develop a lentivirus pseudotyped for VSV-G in HEK293FT producer cells. The cell culture media containing the produced virus will be collected 48 hours post-transfection and used to transduce the selected human T-ALL cell lines. After transduction, GFP expression and survival by LIVE/DEAD Aqua stain will be monitored at 24-, 48-. 72-, and 96-hour time points via flow cytometry.

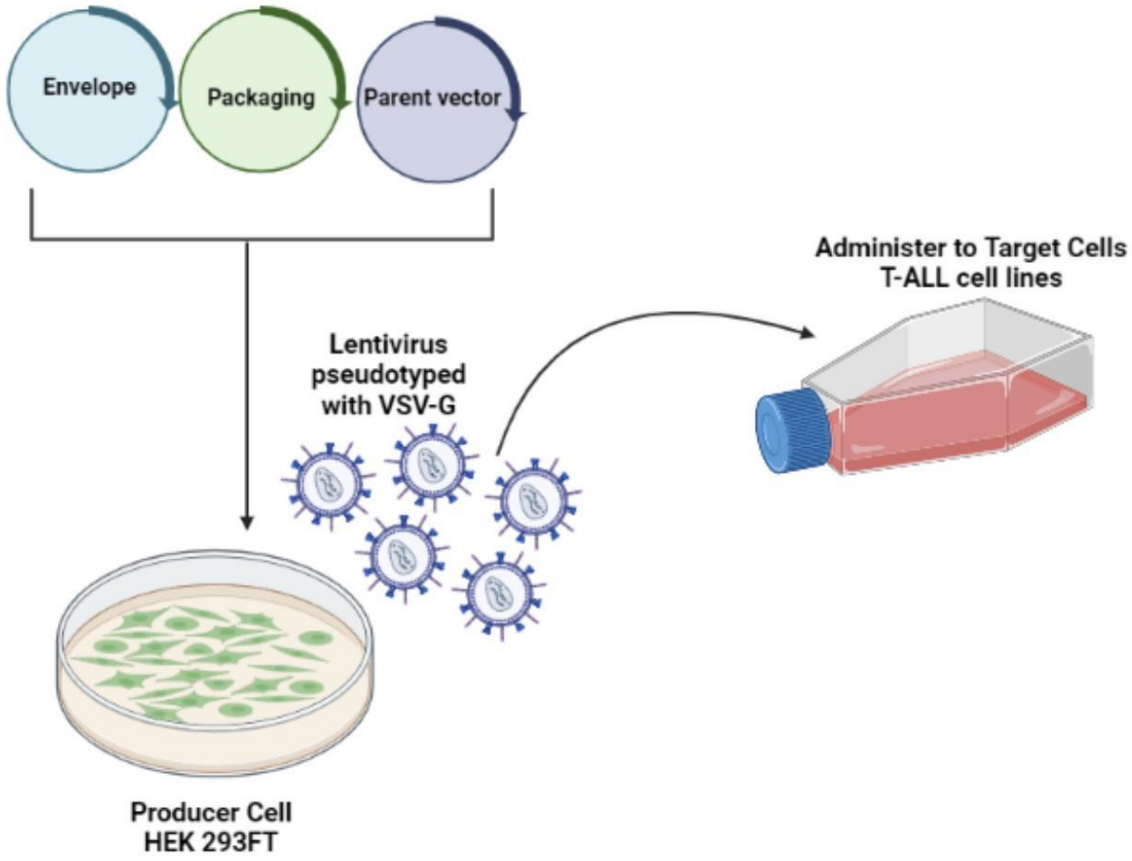

Figure 7. Depiction of delivery of the construct to target human T-ALL cells. HEK293FT cells are transfected with the parent vector carrying the POSH-KO guide sequence, an envelope plasmid, and a packaging plasmid. After 48 hours, the media of the producer cells containing the virus is collected and administered to target T-ALL cells. Created with BioRender.

4.2.B Validation of delivery

Delivery of the construct will be validated by flow cytometry and fluorescent microscopy through GFP expression. GFP+ cells will undergo fluorescence-activated cell sorting in a 96-well plate to undergo single-cell isolation followed by *in vitro* expansion. After single-cell expansion, the genomic DNA will be isolated. Sanger sequencing and

TIDE analysis will be performed for each of the isolates to determine if there was a successful modification of the genomic DNA. To confirm the genetic modifications at the protein level, an immunoblot probing for POSH will be performed. To determine if homozygous-modified human T-ALL cells are dying (if POSH is essential for survival in some of the selected T-ALL cell lines), the genomic DNA will be isolated 24 hours post-transduction, and TIDE analysis will be performed and compared with the single-isolates data to determine the differences in populations.

4.2.C Functional analysis of POSH-KO in human T-ALL cell lines

The same functional *in vitro* assays outlined in **Chapter 2** will be performed to determine the impact POSH-KO has on human T-ALL function and downstream signaling activation. To determine if it is POSH's scaffolding function that contributes to these functions in human T-ALL, the results of the functional *in vitro* assays will be compared with POSH-WT T-ALL cell lines treated with the Tat-POSH inhibitor (treatment with Tat-Control as a control). This is an important control because it will provide a direct comparison between a complete POSH protein expression KO compared to only inhibiting POSH's SH3.3 domain while the other functional domains remain unaffected. If POSH's SH3.3 domain is contributing to the vast majority of these functions, it should be demonstrated by having the results of the functional *in vitro* assays between the POSH-KO and Tat-POSH-treated human T-ALL cell lines being similar.

To further determine the results of the functional *in vitro* assays are due to POSH not being able to activate certain signaling pathways, the results will be compared to POSH-WT human T-ALL cell lines with inhibitors for proteins known to associate with

POSH to form its scaffolding complex (for example, inhibiting JNK signaling activity). Experimental conditions are shown in **Table 5**.

Table 5. Chapter 5 experimental conditions.

Negative Control	Experimental Condition
POSH-WT human T-ALL	POSH-KO human T-ALL
Tat-Control-treated POSH-WT human T-ALL	Tat-POSH-treated POSH-WT T-ALL (to compare with POSH-KO results)

4.3 Expected results

4.3.A General

The data that will be collected in this chapter will provide information on how POSH functions in human T-ALL. For validation of the delivery of the construct, it is expected to see GFP+ T-ALL cell lines that are heterozygous and homozygous for the mutation. To characterize the phenotype of each population, the outlined methodology in this chapter will be performed. This is advantageous because it will allow researchers to determine how decreased (heterozygous) and no (homozygous) POSH protein expression impacts human T-ALL signaling function.

In the results of the functional *in vitro* assays, it is hypothesized that there will be a decrease in the activation of certain signaling pathways that POSH is involved in, such as the JNK signaling pathway. The rationale for this anticipation is in reference to where it was demonstrated that POSH is important for regulating JNK activation in lymphocytes[55,56] and there is high basal phosphorylation on JNK in human T-ALL[11]. It is

also hypothesized that there will be a decrease in Akt and Tak-1 signaling since these are previously known to associate with POSH[55,56].

If POSH expression is important for the activation of signaling pathways important for promoting proliferation in human T-ALL, there should be an observed decrease in the dilution of the dye in the membrane of dividing POSH-KO T-ALL cells when performing CFSE labeling. To determine which pathways POSH expression is important for activation, there should be decreased protein expression or PTM level in the signaling activation immunoblots in the POSH-KO T-ALL cells compared to the POSH-WT conditions. The same logic will be applied to the transcription factor activity assays as another way of determining the outcome of signaling activation. If POSH expression is important for activating signaling pathways important for regulating apoptosis activity, there should be decreased expression of the pro- and/or anti-apoptotic markers as well as a decrease in the detected caspase 3/7 in the POSH-KO T-ALL cells vs. POSH-WT conditions. If POSH expression is important for activating pathways that regulate cell cycle entry in human T-ALL, it is hypothesized that there may be a decrease in the percentage of Ki67+ cells in the POSH-KO cells compared to the POSH-WT cells.

4.3.B HBP-ALL

As shown in **Figure 4**, HBP-ALL was the most sensitive to competitive inhibition of POSH's SH3.3 domain via the Tat-POSH inhibitor. With this in mind, it is anticipated that there may be difficulty developing POSH-KO HBP-ALL cell lines. To combat this, an alternative strategy is described in *4.4 Alternative outcomes*. If it is possible to develop POSH-KO HBP-ALL cell lines that survive long enough to perform the outlined functional *in vitro* assays, I hypothesize decreased cell cycle entry and proliferation, and

decreased Akt, JNK, and NFκB signaling activity. Additionally, it is hypothesized that there will be increased pro-apoptotic activity and caspase activation. Previous observations on signaling activity in the HBP-ALL cell line support these hypotheses[29,41].

Additionally, because HBP-ALL was highly sensitive to competitive inhibition of POSH's SH3.3 domain, it is possible there will not be HBP-ALL cell lines that are homozygous for the mutation if POSH is essential for survival in this cell line and only heterozygous HBP-ALL cell lines can be developed. The same functional *in vitro* assays can still be performed, but the alternative strategies as described in *4.4 Potential problems & alternative strategies* should also be considered.

4.3.C Jurkat

As shown in **Figure 4**, the Jurkat cell line was moderately sensitive to competitive inhibition of POSH's SH3.3 domain. In the pilot study (**Figure 6**), Jurkats were used to develop POSH-KO T-ALL cell lines to determine if they will survive long enough to perform the outlined methodology on them. Given the results of the pilot study, I am confident that the outlined methodology in this chapter will be able to be performed. Additionally, I hypothesize that because the Jurkat cell line was less sensitive to competitive inhibition of POSH's SH3.3 domain, there will be the formation of both heterozygous and homozygous POSH-KO Jurkat cell lines.

Since it was previously hypothesized that the Jurkat cell line will have a similar composition of the POSH scaffolding complex as normal CD4+ T-cells, it is further hypothesized that there may be a decrease in Tak-1-dependent activation of JNK1 and JNK2. Tak-1 signaling is important for NFκB activation and proliferation[78]. If I correctly hypothesized the composition of the POSH scaffolding complex for the Jurkat cell line,

this should be evident in decreased proliferation (via CFSE labeling), and a decrease in NFκB p52 activation via signaling activity immunoblots and transcription factor activity assays. Additionally, Jurkats have higher basal phosphorylation on JNK and Akt[41], and POSH is known to associate with these signaling pathways, so POSH-KO Jurkat cells should have decreased JNK and Akt signaling activation. Lastly, since the Jurkat cell line was tipifarnib-sensitive, POSH-KO Jurkat cell lines are hypothesized to have no changes in ERK1 signaling activation since POSH does not interact with ERK1.

5.3.D MOLT-14

As shown in **Figure 4**, the MOLT-14 cell line was the least sensitive to competitive inhibition of POSH's SH3.3 domain. This indicates that POSH function within this cell line may not prioritize activation of signaling pathways important for survival, proliferation, and apoptosis regulation. If this hypothesis is correct, there may be little to no changes observed in the activation of Akt, NFκB p52, and the level of pro-apoptotic and/or anti-apoptotic markers. Additionally, there may be no differences in CFSE labeling. Since the MOLT-14 cell line is tipifarnib-sensitive, it is hypothesized that POSH-KO MOLT-14 cell lines will not have a change in ERK-1 signaling activation since POSH has not previously interacted with this molecule. Lastly, if POSH expression is not present, there would be disruption of the POSH scaffolding complex and therefore there should be a decrease in JNK signaling activation.

4.4 Alternative outcomes

Given the results of the pilot study, I am confident that the outlined methodology in this chapter can be performed, at least for some of our selected target cell lines. Even

so, there might be difficulties detecting signaling cascade proteins known to associate with POSH. If this occurs, several Abs probing for the same proteins will be administered in a cocktail. Additionally, it is possible that there may not be significant differences between the POSH-WT and POSH-KO T-ALL cell lines in the results of the functional *in vitro* assays. An alternative hypothesis is that there is compensation for POSH function being provided by a homolog called POSH2 (SH3RF3)[79]. To test this hypothesis, POSH2-KO human T-ALL cell lines will be developed using similar methodology and validated guide sequences from the GeCKO library[64]. Multiplex CRISPR assembly should be performed to develop double POSH-POSH2-KO human T-ALL cell lines to test the full extent of the compensation being provided by POSH-2[80].

It is a possibility that there will be significant differences in the results of the functional *in vitro* assays between POSH-KO and Tat-POSH-treated WT T-ALL cell lines. If this occurs, it is likely that POSH's scaffolding function through its SH3.3 domain is not contributing to these functions. To test this hypothesis, the results of the functional *in vitro* assays of the POSH-KO T-ALL cell lines will be compared with POSH-WT T-ALL cell lines treated with Tat-POSH inhibitors that competitively inhibit POSH's other SH3 domains.

Lastly, if POSH is essential for function for some of the selected human T-ALL cell lines, they might not survive long enough to perform the functional *in vitro* assays to characterize function. It is also possible that specific cell lines will not be homozygous for the POSH-KO. To circumvent this, CRISPR-activate (pLV-hUbC-VP64 dCas9 VP64-T2A-GFP Addgene plasmid #59791)[81] and CRISPR-repress (pLenti-dCas9-KRAB-blast Addgene plasmid #89567)[82] techniques can be performed to

characterize the function of POSH in human T-ALL by either increasing or interfering with the transcription of POSH respectively. This is advantageous because these techniques involving dCas9 do not permanently modify the genomic DNA since dCas9 can bind to but not cleave the target DNA[83]. After validating the delivery of the construct, the same functional *in vitro* assays outlined in this chapter could be performed.

4.5 Rigor & reproducibility

Each experiment will be replicated in triplicate to ensure the results of the outlined functional *in vitro* assays in this chapter are accurate. To determine the statistical significance between the POSH-KO and POSH-WT conditions between each selected human T-ALL cell line, a two-way Student's t-test will be performed. Some results may be normalized to the POSH-WT conditions. To determine the differences between multiple selected human T-ALL cell lines together, a two-way ANOVA test followed by a Tukey post-hoc test will be performed. All statistical analyses will be performed using Prism GraphPad and we will consider a p-value less than 0.05 to be statistically significant.

CHAPTER 5

POTENTIAL IMPACT OF PROPOSED RESEARCH

To date, the vast majority of studies investigating POSH function in normal T-cells have focused primarily on the composition of the scaffolding complex and its downstream effects. In contrast, few studies have focused on POSH function in leukemic T-cells or expanded on possibly unique combinations of members of the formation of the scaffolding complex. With the use of the Daniels lab's novel Tat-POSH inhibitor and CRISPR-Cas techniques, results from experiments in this proposal will provide new insights on several levels: (1) the composition and function of the POSH scaffolding complex between different types of human T-ALL cell lines; (2) the extent to which phosphotyrosine-based modifications on POSH's SH3.3 domain regulate the composition of the POSH scaffolding complex in human T-ALL; and (3) how important full-length POSH expression is for human T-ALL survival, proliferation, signaling activation, apoptosis, cell cycle entry, etc.

I anticipate that this proposed research will provide a foundational basis for future studies involving primary human T-ALL samples that will allow us to further understand the biological relevance of our potential findings. However, there are several steps that need to be completed in order to reach that future point in the field.

The nature of this proposed research would be performed primarily in an *in vitro* model–human T-ALL cell lines. The potential results and conclusions made from this proposed research would provide the necessary details in order to design *in vivo* experiments with mice. In regard to developing POSH-KO mice, there are already

validated guide sequences provided by the Addgene GeCKO library[64] that can be utilized to develop a construct, and Daniels lab is well into POSH-KO mouse work. I propose to perform similar experiments to those proposed here, using a fully developed POSH-KO mouse model to further characterize POSH function when it can be applied to T-ALL development and therapeutics.

Once *in vivo* experiments are performed and analyzed, this would potentially provide a foundational basis for similar experiments involving the collection of the primary human T-ALL samples. The benefit of performing these proposed experiments on primary human T-ALL samples is that it might provide diverse profiles of T-ALL disease state thus contributing to the information needed to identify markers within the human T-ALL signaling pathways that can be used for personalized therapies. For example, it is expected that with the diverse types of human T-ALL, there would potentially be different molecular targets. Identifying these potential molecular targets would open up the possibility of performing more studies determining the impact of targeting the identified molecules in the various types of human T-ALL.

In summary, the proposed research in this document would mark the beginning of the much-needed investigation of determining if POSH or other types of markers can be used in personalized therapies for treatment of T-ALL. Although there is a significant amount of research that would need to be conducted to reach that future point in the field, beginning with the proposed research in this document would be an excellent start.

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