Murine Tbk1 is Required by FLT3+ Leukemia Stem Cells Yet Dispensable in Homeostatic Hematopoiesis

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LOYOLA UNIVERSITY CHICAGO

MURINE TBK1 IS REQUIRED BY FLT3+ LEUKEMIA STEM CELLS YET DISPENSABLE IN HOMEOSTATIC HEMATOPOIESIS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN CELLULAR AND MOLECULAR ONCOLOGY

BY
AUSTIN PATRICK RUNDE
CHICAGO, IL
AUGUST 2023
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Dedicated to my greatest source of inspiration—my family.
It's not whether you get knocked down; it's whether you get up.

Vince Lombardi
Acute myeloid leukemia (AML) is a devastating disease that carries a dismal prognosis. Despite typically being responsive to frontline chemotherapy, AML often returns in the form of a treatment-resistant disease that is fatal without a hematopoietic stem cell transplant (HSCT). While curative, HSCT donors are difficult to find and the transplant process itself exposes patients to chemotherapy and/or radiation. Additionally, a significant portion of AML patients present with refractory disease, failing to respond to frontline chemotherapy altogether.

While targeted therapies are available for select patient populations, these drugs currently serve only to augment chemotherapy are still inferior to standard-of-care regimens; thus, the search to find the “Gleevec® for AML” continues.

Our research is an effort to find a targeted therapy for AML that is effective in all patient populations regardless of mutation status, immunophenotype, and patient fitness. Our goal is to discover a therapy that can serve to augment or replace cytotoxic chemotherapy, sparing patients from the harsh effects of cytotoxic therapy while still helping them achieve and maintain remission.
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LIST OF ABBREVIATIONS

129S5  129S5/SvEvBrd mouse background
4GFS  the Zhang lab’s proprietary cocktail of IL-3, IL-6, GM-CSF, and SCF for the culturing of mouse HSPCs
4-OHT 4-hydroxytamoxifen/afimoxifene, a principal metabolite of tamoxifen
7+3 induction phase of AML frontline chemotherapy (7-day delivery of Ara-C + 3-day delivery of daunorubicin/idarubicin)
aa  amino acid(s)
AAALAC  Association for Assessment and Accreditation of Laboratory Animal Care
Ab  antibody
ACSL1  acyl-CoA synthetase long-chain family member 1
AEL  acute erythroid leukemia
AF9  protein AF-9/mixed-lineage leukemia; translocated to, 3 (MLLT3)/MLLT3 Super Elongation Complex Subunit/YEATS3
ALLFTD  ARTFL LEFFTDS Longitudinal Frontotemporal Lobar Degeneration/ NCT04363684
ALS  amyotrophic lateral sclerosis
AML  acute myeloid leukemia/acute myelogenous leukemia; acute non-lymphocytic leukemia (ANLL)
AMML  acute myelomonocytic leukemia
AMML Eo  acute myelomonocytic leukemia with eosinophilia
AMKL  acute megakaryoblastic leukemia
AMoL  acute monoblastic/monocytic leukemia
AML-MRC  AML with myelodysplasia-related changes
AML, NOS  AML, not-otherwise-specified
AMX  amlexanox/Amlx/Aphthasol®/AA-67
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>ANLL</td>
<td>acute non-lymphocytic leukemia (see “AML”)</td>
</tr>
<tr>
<td>APL</td>
<td>acute promyelocytic leukemia (PML-RARA⁺ AML)</td>
</tr>
<tr>
<td>APRIL</td>
<td>A proliferation-inducing ligand/TNFSF13</td>
</tr>
<tr>
<td>Ara-C</td>
<td>cytarabine/cytosine arabinoside/1-β-D-Arabinofuranosylcytosine/Cytosar-U®</td>
</tr>
<tr>
<td>Ara-CTP</td>
<td>cytarabine triphosphate/ara-Cytidine-5'-triphosphate</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia-telangiectasia mutated/ATM serine/threonine kinase</td>
</tr>
<tr>
<td>ATRA</td>
<td>all-trans retinoic acid/tretinoin/Vesanoid®</td>
</tr>
<tr>
<td>AZI2</td>
<td>5-azacytidine-induced protein 2/NAP1</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6 mouse background</td>
</tr>
<tr>
<td>BET</td>
<td>Bromodomain and Extra-Terminal motif</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BPDCN</td>
<td>blastic plasmacytoid dendritic cell neoplasm</td>
</tr>
<tr>
<td>CAC</td>
<td>citric acid cycle/Krebs cycle/tricarboxylic acid cycle (TCA)</td>
</tr>
<tr>
<td>CCD</td>
<td>coiled-coil domain</td>
</tr>
<tr>
<td>CCL</td>
<td>CC-motif chemokine ligand</td>
</tr>
<tr>
<td>ccRCC</td>
<td>clear-cell renal cell carcinoma</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CD11b</td>
<td>macrophage-1 antigen (Mac-1)/integrin alpha M (ITGAM)</td>
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<tr>
<td>CD115</td>
<td>proto-oncogene feline McDonough sarcoma (c-FMS)/colony stimulating factor 1 receptor (CSF1R)</td>
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<td>CD117</td>
<td>proto-oncogene c-KIT (c-KIT)/stem cell factor receptor (SCFR)</td>
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<td>signaling lymphocytic activation molecule 1 (SLAMF1)/SLAM</td>
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<td>CD15s</td>
<td>sialyl-LewisX (sLeX)/SSEA-1</td>
</tr>
<tr>
<td>CD62E</td>
<td>E-selectin</td>
</tr>
<tr>
<td>CEP170</td>
<td>Centrosomal protein 170kDa</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CFU-E</td>
<td>erythroid CFU</td>
</tr>
<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
</tr>
<tr>
<td>CTx</td>
<td>chemotherapy</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC-motif chemokine ligand</td>
</tr>
<tr>
<td>DA</td>
<td>daunorubicin + Ara-C</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate/diethyl decarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNO</td>
<td>daunorubicin/Cerubidine®</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sulfate sodium</td>
</tr>
<tr>
<td>E</td>
<td>glutamate/glutamic acid</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ELN</td>
<td>European LeukemiaNet</td>
</tr>
<tr>
<td>EMD</td>
<td>extramedullary disease</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>EthBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>eWAT</td>
<td>epididymal white adipose tissue</td>
</tr>
<tr>
<td>FAB</td>
<td>French-American-British</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FLAG-IDA</td>
<td>fludarabine + high-dose Ara-C + G-CSF + idarubicin</td>
</tr>
<tr>
<td>FLAMSA</td>
<td>fludarabine + amsacrine + high-dose Ara-C + G-CSF</td>
</tr>
<tr>
<td>FTD</td>
<td>frontotemporal dementia</td>
</tr>
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<td>granulocyte colony-stimulating factor/CSF3/filgrastim (E. coli-recombinant G-CSF [Neupogen®])</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
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</tr>
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<td>GMP</td>
<td>granulocyte-monocyte progenitor</td>
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</table>
H&E  hematoxylin and eosin
H3K4  lysine 4 of histone H3
H3K4me3  trimethylation on lysine 4 of histone H3
Hb  hemoglobin
HFD  high-fat diet
HPC  hematopoietic progenitor cell
HSC  hematopoietic stem cell
HSCT  hematopoietic stem cell transplant
HSE  herpes simplex virus-1 encephalitis
HSV-1  herpes simplex virus-1
HSPC  hematopoietic stem/progenitor cell/c-Kit+ mouse bone marrow cells
HTLV-1  human T-lymphotropic virus type 1
IACUC  Institutional Animal Care and Use Committee
IAV  influenza A virus
IF  immunofluorescence
IFN  interferon
IFN-I  type-I interferon
IHC  immunohistochemistry
IKK  inhibitor of nuclear factor-kB kinase
IKKε  IKK family member, subunit epsilon/IKKi/IKBKE
IL  interleukin
IRF  interferon regulatory factor
ITD  internal tandem duplication
JAX  The Jackson Laboratory
K  lysine
KC  keratinocyte chemoattractant/keratinocyte-derived chemokine/CXCL1
KD  kinase domain
KRAS  proto-oncogene Kirsten rat sarcoma virus
Lin  lineage-associated surface antigens (B220, CD3, CD11b, Gr1, TER119, and CD41, unless otherwise specified)
LMPP  lympho-myeloid-primed progenitor (Flt3+CD150−LSK)
LPS  bacterial lipopolysaccharides
LSC  leukemia stem cell
LK  Lin−c-Kit+ mouse bone marrow cell
LSK  Lin−Sca1+c-Kit+ mouse bone marrow cell
LUMC  Loyola University Medical Center
M-CSF  monocyte colony-stimulating factor/CSF1
mAb  monoclonal antibody
MAVS  mitochondrial antiviral-signaling protein
MCE  MedChemExpress
MDS  myelodysplastic syndrome
MDSC  myeloid-derived suppressor cell
MEP  myelo-erythroid progenitor
MkP  megakaryocyte progenitor
ML-DS  myeloid proliferations related to Down syndrome
MLKL  Mixed lineage kinase domain like pseudokinase
MLL  Mixed-lineage leukemia 1 (MLL1)/histone-lysine N-methyltransferase 2A (KMT2A)
MLL-AF9+  harboring the MLL-AF9 fusion gene
MMB  momelotinib/CYT387
MLL-r  MLL1-rearranged
moDC:  monocyte-derived dendritic cell(s)
MPP  multipotent progenitor cell
MPN  myeloproliferative neoplasm
MPO  myeloperoxidase
MRD  minimal residual disease
MS  myeloid sarcoma (chloroma)
mtDNA  mitochondrial DNA
MYD88  myeloid differentiation primary response 88
NaOAc  sodium acetate
NASH  non-alcoholic steatohepatitis
NCAM  neural cell adhesion molecule/CD56
NCCN  National Comprehensive Cancer Network
NDP52  nuclear dot protein 52
NSCLC  non-small cell lung cancer
NTG  normal-tension glaucoma
NuMA  Nuclear mitotic apparatus protein 1
OPTN  optineurin
OS:  overall survival
PAS  periodic acid-Schiff
PBS  phosphate-buffered saline
PCR  polymerase chain reaction
PD-L1  programmed cell death-ligand 1
Plts  platelets
pUbN  polyubiquitination
PROTAC  proteolysis-targeting chimera
PTM  post-translational modification
PINK1  PTEN-induced kinase 1
PRR  pattern recognition receptor
qPCR  quantitative PCR
R/R AML  relapsed/refractory AML
RANTES  regulated on activation, normal T cell expressed and secreted/CCL5
RBC  red blood cell(s)/erythrocyte(s)
RIPK1/3  Receptor-interacting serine/threonine-protein kinase 1/3
RLR  RIG-I-like receptor
RNA-seq  RNA sequencing  
ROS  reactive oxygen species  
RT-PCR  reverse-transcriptase PCR  
RXB  ruxolitinib  
S  serine  
sAML  secondary AML  
SC  single cell(s)  
SCF  stem cell factor/c-Kit ligand/steel factor  
SDD  scaffold dimerization domain/coiled-coil domain 1 [CCD1]  
shRNA  small hairpin RNA  
SINTBAD  similar to NAP1 TBK1 adaptor/TBK1-binding protein 1 (TBKBP1)  
SM-FeSV  feline sarcoma virus-McDonough strain  
SPF  specific pathogen-free  
SQSTM1  sequestosome 1/p62  
STING  stimulator of interferon genes  
TACI  Transmembrane activator and CAML interactor/TNFRSF13B  
TAE  tris base-acetic acid-EDTA  
T  threonine  
t-AML  therapy-related AML  
TANK  TRAF family member-associated NF-kappa B activator  
TAPE  TBK1-associated protein in endolysosomes/CC2D1A/Freud-1/Aki-1  
TBK1  TANK-binding kinase 1/NF-κB-activating kinase (NAK)/TRAF2-associated kinase (T2K)/Encephalopathy, Acute, Infection-Induced 8 (IIAE8)/Frontotemporal dementia and/or amyotrophic lateral sclerosis 4 (FTDALS4)  
TBK1L  zebrafish isoform of TBK1 that lacks the KD and ULD  
TBK1s  human/mouse isoform of TBK1 that lacks the KD  
TCGA  The Cancer Genome Atlas  
Th17  IL-17+ helper T cells
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>TICAM-1</td>
<td>TIR domain containing adaptor molecule 1/TIR-domain-containing adapter-inducing interferon-β (TRIF)</td>
</tr>
<tr>
<td>TKD</td>
<td>tyrosine kinase domain mutation</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TME</td>
<td>tumor microenvironment</td>
</tr>
<tr>
<td>TNC</td>
<td>total nucleated cell(s)</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>ULD</td>
<td>ubiquitin-like domain</td>
</tr>
<tr>
<td>ULK1</td>
<td>Unc-51-like autophagy activating kinase 1</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VP3</td>
<td>viral capsid protein 3</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cell(s)/leukocyte(s)</td>
</tr>
<tr>
<td>WBM</td>
<td>whole bone marrow</td>
</tr>
<tr>
<td>WBMT</td>
<td>WBM transplant</td>
</tr>
<tr>
<td>WGCNA</td>
<td>weighted gene co-expression network analysis</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type/+</td>
</tr>
<tr>
<td>XFG</td>
<td>exfoliation glaucoma</td>
</tr>
<tr>
<td>XRT</td>
<td>radiotherapy</td>
</tr>
<tr>
<td>Y</td>
<td>tyrosine</td>
</tr>
<tr>
<td>YBX1</td>
<td>Y-box-binding protein 1/YB-1</td>
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ABSTRACT

The TANK-binding kinase 1 (TBK1) is a cytoplasmic, serine/threonine kinase and a critical activator of the innate immune system. Reports have implicated TBK1 as a promoter of multiple cancer types. Acute myeloid leukemia (AML) is a cancer arising from the hematopoietic stem/progenitor cells (HSPCs). Although ~70% of AML patients achieve complete remission with chemotherapy, at least half will relapse due to the failure to eradicate leukemia stem cells (LSCs). As our previous data indicate the TLR-TAK1/TICAM-1 axes regulate LSCs, we suspect TBK1 also regulates LSCs. To study TBK1 in AML, we generated tamoxifen-inducible, \textit{Tbk1}\textsuperscript{NULL} mice and \textit{Tbk1}\textsuperscript{NULL} \textit{MLL-AF9}\textsuperscript{+} mouse HSPCs. We found that \textit{Tbk1} deletion ablates the c-Kit\textsuperscript{+}Flt3\textsuperscript{+} subset of \textit{MLL-AF9}\textsuperscript{+} HSPCs without perturbing homeostatic hematopoiesis; the loss of \textit{Tbk1} also increases the expression of c-Fms/CD115 on \textit{MLL-AF9}\textsuperscript{+} HSPCs. Our study suggests that TBK1 blockade can selectively and effectively target FLT3\textsuperscript{+} LSCs in AML treatment.
CHAPTER 1

TARGETING THE LEUKEMIA STEM CELL: AN UNMET CLINICAL NEED

Introduction

Acute myeloid/myelogenous leukemia (AML), sometimes called acute non-lymphocytic leukemia (ANLL), is a cancer of the blood-forming (hematopoietic) tissues that reside in the bone marrow (BM) [1, 2]. AML typically occurs in patients aged 65 years or older and necessitates treatment with cytotoxic CTx [3-5]. AML usually responds to frontline CTx, but up to 40% of newly-diagnosed patients present with refractory disease (primary resistance) or cannot tolerate the treatment required to achieved complete remission (CR) [6]. While 60-70% of patients achieve complete remission CR, around half of these patients relapse (acquired/secondary resistance) within 3 years from diagnosis; relapsed AML is often CTx-resistant and necessitates a hematopoietic stem cell (HSC) transplant (HSCT) [4]. Overall, AML still has a dismal long-term prognosis even for patients who initially respond to treatment [7, 8]. Thus, treatments that offer all patients a better chance at achieving sustained CR are urgently needed.
Non-cytotoxic (targeted) therapies for AML are available for patients with specific genetic abnormalities [9, 10]. For example, **PML-RARA**\(^+\) AML can be virtually cured with the use of tretinoin (all-trans retinoic acid [ATRA]/**Vesanoid\(^\circledR\)**) and arsenic trioxide (As\(_2\)O\(_3\)/**Trisenox\(^\circledR\)**), as these agents rapidly and selectively induce the differentiation of **PML-RARA**\(^+\) cells and cause degradation of the PML-RAR\(\alpha\) oncoprotein [11, 12].

Patients harboring mutations in the FMS-like tyrosine kinase 3 (**FLT3**) or isocitrate dehydrogenase 1/2 (**IDH1/2**) genes may be treated with drugs that specifically inhibit the mutated FLT3 receptor (CD135) or IDH1/2 enzyme(s) [10, 13]. Agents targeted against hedgehog signaling (glasdegib [**Daurismo\(^\circledR\)**]) and BCL-2 (venetoclax [**Venclexta\(^\circledR\)**]) are approved for combination use with low-dose CTx in patients who cannot tolerate the frontline regimen [14-19]; while these drugs may be most effective in patients who display overactive hedgehog signaling and/or high BCL-2 expression, pathways both exploited by LSCs, there is not yet a method to determine these parameters clinically. And yet, despite targeting a pathway critical to LSCs, **Daurismo\(^\circledR\)** failed to extend overall survival (OS) in newly-diagnosed AML patients when added to standard daunorubicin + Ara-C CTx (NCT03416179) [20, 21].

Experimental therapies like uproleselan, an E-selectin antagonist, and revumenib, a menin inhibitor for patients with **MLL1** rearrangements or **NPM1c** mutations, have shown promise in clinical trials [22, 23]. However, most targeted therapies for AML (aside from **Vesanoid\(^\circledR\)** + **Trisenox\(^\circledR\)** for **PML-RARA**\(^+\) AML) are unable to induce CR alone and must be used in combination with standard or low-dose CTx [16-18]. While targeted therapies can augment cytotoxic CTx to improve response rates, drug
resistance is still a concern [24-27]. Patients treated with any agent—targeted or cytotoxic—can develop resistance and relapse.

Leukemia stem cells (LSCs) AML are responsible for acquired resistance and relapse in AML, and likely even contribute to primary resistance [2, 28]. While it is established that targeting LSCs is the key to preventing relapse, no therapies exist that effectively target LSCs. As such, we seek to discover novel molecular targets that will allow clinicians to target LSCs directly. We believe that TBK1 is critical to the function of LSCs such that TBK1 blockade will sensitize LSCs to cytotoxic CTx, increasing the odds of achieving and maintaining CR in AML.

**Aim 1: Determine if Tbk1 is Required for Murine Hematopoiesis**

Blocking the action of TBK1 can be done with small molecules that have been characterized *in vivo* and some of which are orally-bioavailable, such as GSK8612 [29]. However, as there is not yet a way to deliver such drugs to AML cells specifically, TBK1 inhibition occurs systemically. While TBK1 has been studied in cell-specific settings, such as in B cells and dendritic cells, its function in hematopoiesis is unknown and must be determined before TBK1 blockade is pursued clinically [29-31]. To this end, we will use a whole-body (global [via the Rosa26 promoter]), *Tbk1*-knockout (*Tbk1*^null^[Tbk1^−/−]) mouse model to determine if the loss of *Tbk1* imparts untoward effects on hematopoiesis.
Aim 2: Determine if \textit{Tbk1} Blockade Could be a Means of Anti-AML Therapy

Based on previous data from our lab, the established & emerging roles of TBK1 in cell physiology, and recent reports examining TBK1 in AML, we anticipate TBK1 is a molecular target for the basis of AML treatment [29, 32-36]. To this end, we will utilize \textit{in vitro} and \textit{in vivo} approaches in the \textit{MLL-AF9}+ setting to determine if Tbk1 blockade holds any promise as a means to treat AML.
CHAPTER 2
LITERATURE REVIEW AND PROJECT RATIONALE

Structure of TBK1

The TANK-binding kinase 1 (TBK1; NF-κB-activating kinase [NAK]/TRAF2-associated kinase [T2K]/IIAE8/FTDALS4) harbors four domains: an N-terminal kinase domain (KD [9-300aa]), a ubiquitin-like domain (ULD [305-383aa]), a scaffold-dimerization domain (SDD/coiled-coil domain 1 [CCD1]; 407-657aa), and a second coiled-coil domain at the C-terminus (CCD2; 659-713aa) [29, 37] (Figure 1, adapted from Runde, et al.) [29]. The KD harbors the catalytic domain, containing the K38, D135, and S172 residues required for kinase activity; the ULD regulates kinase activity and binding of TBK1 to substrates, such as IRF3/7 [38]; the SDD, harboring a leucine zipper (LZ) and helix-loop-helix (HLH), allows for homodimerization [39, 40]; CCD2 enables interaction of TBK1 with adaptors, such as TBK1-binding protein 1 (TBKBP1; similar to NAP1 TBK1 adaptor [SINTBAD]), TRIF, and OPTN [41, 42].
Human/mouse TBK1 is highly homologous to the inhibitor of nuclear factor-κB kinase (IKK) subunit epsilon (IKKε; IKKi/encoded by IKBKE), sharing 46-64% homology in protein sequence [43-45]. TBK1 and IKKε have such a high degree of homology that they can be inhibited by the same molecules, such as amlexanox (AMX), momelotinib (MMB), BAY985, and BX795 [34, 46-52]. The similarity between the two has led many to investigate whether the kinases are wholly redundant or possess unique functions, and it appears that the kinases are not functionally identical [49, 53, 54]. For example, global deletion of Tbk1 is embryonic-lethal, whereas global Ikbke\textsuperscript{NULL} mice are viable but hypersensitive to respiratory viruses [55]. Thus, while TBK1 and IKKε have overlapping functions, such as mediating IFN-β production, we believe that they each have tissue- and disease-specific functions and are not entirely redundant.

Figure 1. Schematic depicting the TBK1 Protein and its Domains (adapted from Runde, et al. and created with PowerPoint\textsuperscript{®}). TBK1 contains four prototypical domains, from the N- to C-terminus: a KD (9-300aa), a ULD (305-383aa), an SDD/CCD1 (407-657aa), and a CCD2 (659-713aa). The KD harbors the catalytic site (including the S172 necessary for kinase activity); the ULD regulates kinase activity and facilitates binding of TBK1 to its substrates; the SDD/CCD1 (harboring an LZ and HLH) allows for homodimerization; the CCD2 enables interaction of TBK1 with adaptors like SINTBAD and OPTN.
Genetic and Protein-level Regulation of TBK1

TBK1 is a serine/threonine kinase that is a critical activator of the vertebrate innate immune system [56, 57]. TBK1 is 729 amino acids (aa) in length and has a molecular weight of ~84 kDa [58-60]. TBK1 is highly conserved between humans (UniProt: Q9UHD2) and mice (UniProt: Q9WUN2), displaying 94.1% homology in protein sequence [44, 45]. TBK1 is encoded by the TBK1 gene. In humans, TBK1 contains 22 exons and resides on chromosome 12q14.2; in mice, Tbk1 contains 21 exons and resides on chromosome 10D2 [61]. RNA sequencing (RNA-seq) data (PRJEB4337) has revealed that, in addition to innate immune cells, TBK1 is expressed significantly in nearly all human tissues [59, 62]. Notably, TBK1 expression is highest in the testis and BM, and lowest in the pancreas.

The transcriptional regulation of TBK1 has not been well-studied, but splice variants have been reported. In humans and mice, Deng, et al. identified TBK1s, a truncated isoform that lacks exons 3-6 [63, 64]. While TBK1s is without a KD, it can compete with the full-length TBK1 for the binding of RIG-I and mitochondrial antiviral-signaling protein (MAVS), thereby inhibiting activation of the downstream pathway and hampering interferon-β (IFN-β) production amid viral infection. In zebrafish, Zhang, et al. reported TBK1-like (TBK1L), an isoform that lacks both the KD and ULD [65]. Like TBK1s, TBK1L can disrupt the activation of the RLR-MAVS axis during Carp sprivivirus infection. While JASPAR predicts TBK1 to be a target gene of at least 111 transcription factors, including: ARNT, BRCA1, CEBPB, ESR1/2, FOXO3, GATA1/2/3, HLF, HOXA5,
MECOM, MYB, MYC, NFIL3, PAX2/4/5/6, PBX1, RUNX1/2, SOX2/5/9/10/17, SP1, SRY, STAT1/3, TP53/63, ZFX, relatively little is known about the genetic regulation of TBK1 [66, 67]. However, its regulation at the protein-level has been thoroughly investigated.

At rest, TBK1 exists as a homodimer in the cytoplasm [68, 69]. TBK1 is activated by membrane-bound and cytosolic pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), which initiate the innate immune response. Activation of PRRs leads to the production of inflammatory cytokines, such as TNF, IL-6, IL-1β, and IL-12, as well as the production of type-I interferon (IFN-I) [70]. TBK1 is activated by the following PRRs: TLR3/4/7/8/9, cyclic GMP-AMP synthase (cGAS), DAI, DDX41, AIM2, MDA5, LGP2, and RIG-I [56]. In addition to PRRs, TBK1 can be induced by the stimulator of interferon genes (STING), tumor necrosis factor (TNF) family of ligands and receptors (e.g., APRIL and TACI [TNFRSF13B]), other kinases (e.g., IKKβ), 5-azacytidine-induced protein 2 (AZI2; NAP1), cytokines (e.g., IL-1, CXCL4, GM-CSF) and even the oncogenic KRASG12 GT Pase [29, 30, 71-77].

The activation and function of TBK1 involves a multi-step process of post-translational modifications (PTMs) at various residues spanning the protein [29, 69, 78]. PTMs govern both the enzymatic activity of TBK1 and its interaction with adaptor proteins (Table 1).
Table 1. Select Post-translational Modifications of TBK1 and Effect on Protein Function, if Applicable (Adapted from Runde, et al.) [29].

<table>
<thead>
<tr>
<th>PTM</th>
<th>Site</th>
<th>Mediators</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylation</td>
<td>Y354; Y394</td>
<td>Lck/Hck/Fgr</td>
<td>Inhibitory</td>
<td>[79]</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>S510</td>
<td>AKT1</td>
<td>Inhibitory</td>
<td>[80]</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>Y179</td>
<td>SRC</td>
<td>Activating</td>
<td>[81]</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>S716</td>
<td>PKCθ</td>
<td>Activating</td>
<td>[82]</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>S172</td>
<td>TBK1 (trans-autophosphorylation); ULK1; IKKβ; PINK1/PARKIN; GSK3β—can be reversed by PPM1B</td>
<td>Activating (rate-limiting, enables kinase activity)</td>
<td>[69, 76, 83-86]</td>
</tr>
<tr>
<td>K27-linked pUbn</td>
<td>K344</td>
<td>NEDD4</td>
<td>Activating (enables association with NDP52)</td>
<td>[87]</td>
</tr>
<tr>
<td>K33-linked pUbn</td>
<td>K670</td>
<td>E3 ligase is unknown—can be reversed by USP38/NLRP4</td>
<td>Activating</td>
<td>[88, 89]</td>
</tr>
<tr>
<td>K48-linked pUbn</td>
<td>K670</td>
<td>DTX4/TRIP</td>
<td>Inhibitory</td>
<td>[88-90]</td>
</tr>
<tr>
<td>K48-linked pUbn</td>
<td>K251; K372</td>
<td>Siglec1/TRIM27</td>
<td>Inhibitory</td>
<td>[91]</td>
</tr>
<tr>
<td>K63-linked pUbn</td>
<td>K30; K401</td>
<td>MIB2; TRAF2/3/6; NRDP1; RNF128 E3 ligases—can be</td>
<td>Activating (precedes S172)</td>
<td>[40, 58, 68, 92, 93]</td>
</tr>
<tr>
<td>Ubiquitination</td>
<td>K69; K154; K372</td>
<td>Activating</td>
<td>[94]</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
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<td>------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>Acetylation</td>
<td>K241; K692; additionally, K30, K154, K236, K251, K607, K646, &amp; K691 are proposed</td>
<td>Acetyltransferases are unknown—can be reversed by HDAC3/4/9</td>
<td>Variable (HDAC3 and HDAC9/DNMT3A promote TBK1 activity, whereas HDAC4 inhibits it)</td>
<td>[95-97]</td>
</tr>
<tr>
<td>PTM</td>
<td>Target</td>
<td>PTM Type</td>
<td>PTM Function</td>
<td>Reference</td>
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<td>-------------------</td>
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<td>------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SUMOylation</td>
<td>K694</td>
<td>SUMO1/2/3 (trans-FSUMOylation)—can be blocked by Gam1</td>
<td>Activating (enables association with AZI2, SINTBAD, and TANK)</td>
<td>[98]</td>
</tr>
<tr>
<td>S-nitrosylation</td>
<td>C423</td>
<td>Spontaneous—can be reversed by GSNOR</td>
<td></td>
<td>[99]</td>
</tr>
<tr>
<td>Hydroxylation</td>
<td>P48</td>
<td>EGLN1</td>
<td>Inhibitory (TBK1 becomes substrates for pVHL and PPM1B)</td>
<td>[100]</td>
</tr>
</tbody>
</table>

By determining which adaptors TBK1 can associate with, PTMs also govern the subcellular localization of TBK1, further regulating its function. For example, localization to the Golgi will allow interaction with STING and optineurin (OPTN), localization to the centromere will allow interaction with nuclear mitotic apparatus protein 1 (NuMA) and centrosomal protein 170kDa (CEP170), and localization to the mitochondria will allow interaction with PINK1/PARKIN, p62/sequestosome 1 (SQSTM1), and acyl-CoA synthetase long-chain family member 1 (ACSL1) [101-108]. Distinct domains/motifs spanning TBK1 allow interaction with a wide array of adaptor proteins.
Function of TBK1

TBK1 is best known for being a master activator of the innate immune system, mediating IFN-I production in response to viral/bacterial infection or, in the case of TLR7/8, stimulation by an imidazoquinolone such as imiquimod [Aldara®] (Figure 2, adapted from Runde, et al.) [29, 109]. Upon PRR stimulation, a cascade involving adaptor proteins will facilitate the activation of TBK1 [29]. Common TBK1 adaptors include: TIR domain containing adaptor molecule 1 (TICAM-1; TIR-domain-containing adapter-inducing interferon-β [TRIF]), mitochondrial antiviral-signaling protein (MAVS), myeloid differentiation primary response 88 (MYD88), TBK1-associated protein in endolysosomes (TAPE; CC2D1A/Freud-1/Aki-1), TRAF family member-associated NF-kappa-B activator (TANK), and nuclear dot protein 52 (NDP52) [29, 32, 110-113].
Activated TBK1 initiates the production of IFN-I, like IFN-α and IFN-β, by phosphorylating transcription factors of the interferon regulatory factor (IRF) family.

TBK1 most often phosphorylates and activates IRF3 (S386/S398/T404) and IRF7 (S425/S426/S437/S438), as well as IRF5 (S158/S309) in certain contexts. In response to *Pneumoviridae* but not LPS, TBK1 stimulates the activation of IRF1 by phosphorylating p65/RELA (S536), promoting IFN-β production; IFN-β production itself...
stimulates IRF1 activity in an autocrine manner via IFNAR1/2-STAT1 [68, 114-119]. Recent reports have also shown that zebrafish, but not human, TBK1 phosphorylates IRF6—whether mouse Tbk1 interacts with IRF6 has not been investigated [115, 116]. TBK1 can also act on behalf of the innate immune system in an IRF-independent manner, antagonizing various Picornaviridae infections by serving as an E3 ligase against viral capsid protein 3 (VP3) [120]. Recently, TBK1 has been implicated in the adaptive immune system as well: Lee, et al. demonstrated that B-cell Tbk1 is required for germinal center formation in mice, and Xiao, et al. reported a role for TBK1 in the crosstalk between T cells and dendritic cells [31, 121].

Non-immune functions of TBK1 have also been identified. For example, TBK1 regulates metabolism by inhibiting the insulin receptor (IR) via phosphorylation on S994 and promotes the activity of the estrogen receptor α (ERα; ESR1) via phosphorylation on S305 [122, 123]. TBK1 regulates cell survival by inhibiting the TNF-α-RIPK1/3-MLKL axis and modulating the production of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF; colony-stimulating factor 2 [CSF2]), TNF-α, IL6/8, CCL2/20/26, CCL5 (Regulated on Activation, Normal T cell Expressed and Secreted [RANTES]), and CXCL1 (keratinocyte chemoattractant [KC]/keratinocyte-derived chemokine) [102, 124-129]. TBK1 is also a master regulator of PINK1/PARKIN-driven mitophagy. Mitophagy is a quality control process critical to the health of virtually all cells. In this process, damaged and/or redundant mitochondria are degraded by the autophagosome [102, 124, 130, 131].
At designated mitochondria, PINK1/PARKIN recruits TBK1 and mediates its activation. The interaction between TBK1 and PINK1/PARKIN allows TBK1 to recruit and stabilize the receptors required for mitophagy, such as OPTN, NDP52, and p62/SQSTM1. The interaction between TBK1 and PINK1/PARKIN doubles as a “fail-safe”, forcing G2/M arrest if a cell attempts to divide during mitophagy: upon activation by PINK1/PARKIN, TBK1 is prevented from localizing to the centromere and thus cannot activate NuMA/CEP170 to enable cell division [106, 107, 124].

Mitophagy is a means by which cells can control levels of reactive oxygen species (ROS). As mitochondria emit ROS from normal metabolism and after having sustained damage, mitophagy can be utilized to reduce the cell’s ROS burden [132]. ROS can lead to cancer as they increase the risk of incurring mutations from oxidative DNA damage [131, 133]. As mitophagy is an effective mechanism by which ROS can be reduced, it is crucial to the viability of stem cells, as ROS can cause them to differentiate and forfeit their tissue-renewal capabilities [131, 132, 134-138]

While mitophagy serves to prevent cancer, it can be exploited by cancer cells to adopt a more aggressive phenotype and resist CTx [133, 139-144]. In fact, chemotherapies are noted to exert their anticancer effects at least in part by increasing mitochondrial ROS production and dysfunction [145]. Targeting mitochondrial metabolism (e.g., citric acid cycle [CAC]) may be a promising anticancer strategy, as the CAC inhibitor devimistat (CPI-613®; Cornerstone Pharmaceuticals, Inc.) has made it to clinical trials for pancreatic cancer (NCT03504423) and R/R AML (NCT01768897)
As such, while not explored in the scope of this thesis, targeting TBK1 to block mitophagy could be a means of anticancer treatment.

Furthermore, kinase-independent/non-enzymatic functions of TBK1 have been reported. For example, TBK1 promotes β-oxidation by sequestering ACSL1 at the mitochondria, preventing its translocation to the endoplasmic reticulum, and induces the degradation of IKKe by preventing it from binding TANK [104, 122, 123, 149]. Thus, TBK1 is not merely an activator of the immune system but rather is a highly versatile regulator of cell physiology.

The functions of TBK1 have been studied in knockout-mouse models. Many tissue-specific, Tb1-knockout mice have been developed (Table 2, adapted from Runde, et al.) [29]. Global Tb1Null models present a challenge, as the germline loss of Tb1 is embryonic-lethal in C57BL/6 (B6) mice [150]. Global Tb1Null B6 mice die of liver failure near embryonic day 14.5 due to RIPK1/3-MLKL-driven cell death, as the loss of Tb1 allows necroptosis to occur amid basal levels of TNF-α [151]. However, germline Tb1Null 129S5/SvEvBrd (129S5) mice are viable (although born at a reduced Mendelian frequency) as they are deficient in Tnfr2 [127]. B6 mice can only be used as global Tb1Null models if they also lack either Tnfa or Tnfr1, or if Tb1-deletion is controlled temporally such as with the Rosa26-CreERT2 or Mx1-Cre system [152]. Deletion of exon 2 has been the most common Tb1-knockout strategy.
Table 2. Tissue Deletion Specificity, *Tbk1* Deletion Strategy, and Phenotypes of *Tbk1*<sup>NULL</sup> Mouse Models (Adapted from Runde, et al.) [29].

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Deletion strategy</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6-germline <em>Tbk1</em>&lt;sup&gt;NULL&lt;/sup&gt;</td>
<td>ES cell-targeting: loss of most of exon 1, all of intron 1, and all of exon 2</td>
<td>Embryonic lethality; death near E14.5 due to liver degeneration.</td>
<td>[150]</td>
</tr>
<tr>
<td>129S5-germline <em>Tbk1</em>&lt;sup&gt;NULL&lt;/sup&gt;</td>
<td><em>Prm1</em>-Cre: exon 2 (<em>Tbk1</em> deleted in parental sperm)</td>
<td>Reduced IFN-β/RANTES secretion and IRF3 activity in macrophages, increased circulating monocytes, inflammatory skin changes, increased serum levels of TNFα/GM-CSF/IL-6/CXCL1, and increased sensitivity to LPS. <em>Increased</em> insulin-sensitivity on high-fat diet (HFD) [153].</td>
<td>[127]</td>
</tr>
<tr>
<td>B6-global <em>Tbk1</em>&lt;sup&gt;NULL&lt;/sup&gt;</td>
<td><em>Rosa26</em>-CreER</td>
<td>Increased number of splenic memory CD4&lt;sup&gt;+&lt;/sup&gt; T cells (CD44&lt;sup&gt;hi&lt;/sup&gt;CD62L&lt;sup&gt;lo&lt;/sup&gt;) at the expense of naive CD4&lt;sup&gt;+&lt;/sup&gt; T cells (CD44&lt;sup&gt;lo&lt;/sup&gt;CD62L&lt;sup&gt;hi&lt;/sup&gt;); no changes in CD4&lt;sup&gt;+&lt;/sup&gt;/CD8&lt;sup&gt;+&lt;/sup&gt;-thymocyte populations.</td>
<td>[152]</td>
</tr>
<tr>
<td>Neuron-<strong>Tbk1</strong>null</td>
<td><em>Nestin-Cre</em></td>
<td>Cognitive and locomotor deficits consistent with an ALS/FTD-like pathology.</td>
<td>[154]</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------</td>
<td>--------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Adipocyte-<strong>Tbk1</strong>null</td>
<td><em>Adiponectin-Cre</em></td>
<td>On HFD: adipose tissue inflammation, decreased insulin-sensitivity, reduction in HFD-induced obesity, and increased AMPK activity.</td>
<td>[85]</td>
</tr>
<tr>
<td><strong>Myeloid-Tbk1</strong>null (neutrophils retain <strong>Tbk1</strong>)</td>
<td><em>LyzM-Cre</em></td>
<td>Increased survival due to decreased immune response in influenza A virus (IAV) infection model. Increased progression of ALS-like pathology in mouse model of ALS [155]. Mice developed spontaneous hypertrophy of epididymal white adipose tissue (eWAT) and increased M1 macrophage infiltration, and mice on HFD developed NASH-like pathology and increased sensitivity to DSS-induced colitis [77].</td>
<td>[156]</td>
</tr>
<tr>
<td><strong>T cell-Tbk1</strong>null</td>
<td><em>CD4-Cre</em></td>
<td>Increased activation of AKT-mTORC1 axis leading to impaired T-cell egress from</td>
<td>[152]</td>
</tr>
<tr>
<td>Cell Type</td>
<td>Cre Line</td>
<td>Effect</td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>----------</td>
<td>------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>B cell- <strong>Tbk1</strong>&lt;sup&gt;NULL&lt;/sup&gt;</td>
<td><strong>CD19-Cre</strong></td>
<td>Unrestrained production of IgA and accumulation in the kidneys, leading to development of an IgA nephropathy-like pathology, and increased NF-κB activation. [30]</td>
<td></td>
</tr>
<tr>
<td>Dendritic cell- <strong>Tbk1</strong>&lt;sup&gt;NULL&lt;/sup&gt;</td>
<td><strong>CD11c-Cre</strong></td>
<td>Upregulation of costimulatory molecules imparting better T-cell priming abilities of dendritic cells, development of autoimmune-like pathology, enhanced antitumor immunity, increased IFNAR activity. [31]</td>
<td></td>
</tr>
<tr>
<td>Intestine epithelia- <strong>Tbk1</strong>&lt;sup&gt;NULL&lt;/sup&gt;</td>
<td><strong>Villin-Cre</strong></td>
<td>Increased production of MT1, increased number of Th17 cells in lamina propria, increased production of IL-1β by macrophages, increase in both number and size of intestinal tumors. [157]</td>
<td></td>
</tr>
<tr>
<td>Hepatocyte- <strong>Tbk1</strong>&lt;sup&gt;NULL&lt;/sup&gt;</td>
<td><strong>Albumin-Cre</strong></td>
<td>Increased lipid accumulation in liver, reduced β-oxidation, and reduction in</td>
<td></td>
</tr>
</tbody>
</table>
TBK1 Mutations in Disease

As TBK1 regulates a remarkable array of cellular processes, alterations in TBK1 function have been implicated in several disease states. In fact, over 100 mutations in TBK1 have been associated with neurological, immunological, ophthalmological, and hematological pathologies (Table 3) [29, 40, 158-164].

In amyotrophic lateral sclerosis (ALS)/frontotemporal dementia (FTD), loss of TBK1 function is believed to compromise mitophagy, contributing directly to neuronal dysfunction and degeneration [158, 165]. In fact, TBK1 mutations are so strongly associated with ALS/FTD that the ALLFTD cohort study (NCT04363684) lists “TBK1 gene mutation” on the inclusion criteria [166].

In herpes simplex virus-1 (HSV-1) encephalitis (HSE), loss of TBK1 function hampers the innate immune response to HSV-1 infection [160, 167]. Without TBK1, TLR3 signal transduction is impeded and IFN production is insufficient; as a result, HSV-1 can replicate excessively, leading to HSE in both pediatric and adult patients.

Similarly, in severe COVID-19, loss of TBK1 function blunts TLR3 signal transduction and the production of IFN-I, allowing undue replication of SARS-CoV-2 and worsened infection [168]. Conversely, in a single pediatric patient with COVID-19, co-existing TBK1 and TNFRSF13B (TACI) mutations are believed to have caused the overzealous, fatal immune response to SARS-CoV-2 [169].
Table 3. Select *TBK1* Mutations, Associated Pathology, Effect on Protein (Mechanism), and Pathway Implicated, if Applicable.

<table>
<thead>
<tr>
<th>Aberration</th>
<th>Pathology</th>
<th>Mechanism [170]</th>
<th>Pathway affected</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>D50A</td>
<td>HSE</td>
<td>Decreased protein stability (hypomorphic)</td>
<td>Defective TLR3 signaling</td>
<td>[160]</td>
</tr>
<tr>
<td>G159A</td>
<td>HSE</td>
<td>Kinase-dead (amorphic)</td>
<td>Defective TLR3 signaling</td>
<td>[160]</td>
</tr>
<tr>
<td>Duplication/ Triplication</td>
<td>NTG</td>
<td>Increased gene dosage (hypermorphic)</td>
<td>Hyperactive autophagy in retinal ganglion cells</td>
<td>[161, 162, 171, 172]</td>
</tr>
<tr>
<td>Duplication</td>
<td>XFG</td>
<td>Increased gene dosage (hypermorphic)</td>
<td>?</td>
<td>[173]</td>
</tr>
<tr>
<td>E653fs; L688Rfs*14</td>
<td>ALS/FTD</td>
<td>Impaired interaction with OPTN (hypomorphic)</td>
<td>?</td>
<td>[174]</td>
</tr>
<tr>
<td>E696K</td>
<td>ALS/FTD</td>
<td>Impaired interaction with OPTN (hypomorphic)</td>
<td>Defective mitophagy</td>
<td>[175, 176]</td>
</tr>
<tr>
<td>R357Q; I450K; M559R; Q565P</td>
<td>ALS/FTD</td>
<td>Ablated homodimerization (amorphic)</td>
<td>Defective mitophagy?</td>
<td>[176]</td>
</tr>
<tr>
<td>G217R; M559R</td>
<td>ALS/FTD</td>
<td>Kinase-dead (amorphic)</td>
<td>Defective mitophagy?</td>
<td>[176]</td>
</tr>
<tr>
<td>Mutation</td>
<td>Syndrome</td>
<td>Phenotype Description</td>
<td>Pathway/Function</td>
<td>Reference(s)</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------</td>
<td>---------------------------------------------------------------------------------------</td>
<td>-------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>R47H</td>
<td>ALS/FTD</td>
<td>Near total loss of kinase activity (hypomorphic)</td>
<td>Defective mitophagy?</td>
<td>[176]</td>
</tr>
<tr>
<td>Q565P</td>
<td>ALS/FTD</td>
<td>Reduced kinase activity (hypomorphic)</td>
<td>Reduced p62, IRF3, and NF-κB phosphorylation?</td>
<td>[177, 178]</td>
</tr>
<tr>
<td>E643del</td>
<td>ALS/FTD</td>
<td>Reduced protein levels (hypomorphic)</td>
<td>Defective autophagy?</td>
<td>[179]</td>
</tr>
<tr>
<td>T79del</td>
<td>ALS/FTD</td>
<td>Reduced protein levels, possibly reduced kinase function (hypomorphic)</td>
<td>Defective autophagy?</td>
<td>[180]</td>
</tr>
<tr>
<td>N489I; 3' UTR (2 distinct substitutions); N63S (also found in ALS/FTD [180])</td>
<td>Multiple myeloma</td>
<td>?</td>
<td>?</td>
<td>Multiple Myeloma CoMMpass Study (TCGA) [181]</td>
</tr>
<tr>
<td>S206N</td>
<td>Acute myeloid leukemia</td>
<td>?</td>
<td>?</td>
<td>TCGA AML Dataset [182]</td>
</tr>
<tr>
<td>Skipping of exon 16</td>
<td>COVID-19, fatal</td>
<td>Truncation leading to partial loss of Autoimmunity complicated</td>
<td>?</td>
<td>[169]</td>
</tr>
<tr>
<td>SDD and ablation of CCD2 (hypomorphic)?</td>
<td>by accompanying loss-of-function mutation in TNFRSF13B?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>------------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(homozygous; predicted to yield truncated/R574Sfs*11 isoform)</td>
<td>F24S; R308* (heterozygous with WT)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COVID-19, severe (hypomorphic)</td>
<td>Impaired TLR3 signaling/type I IFN production?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>?</td>
<td>[168]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Role of TBK1 in Cancer**

In addition to these pathologies, TBK1 is becoming increasingly implicated in the pathogenesis of cancer [29, 72, 122, 182-192]. Specifically, TBK1 contributes to cancer by promoting cell proliferation and blunting antitumor immune responses. However, other mechanisms are becoming evident.

TBK1 has come to light as a promoter of several cancer types and predictor of metastasis risk and thus, a possible molecular target for the basis of cancer treatment. Recent reports have suggested that TBK1 promotes prostate cancer [183, 193, 194], thyroid cancer [195], head/neck squamous cell carcinoma [196, 197], non-small cell lung cancer (NSCLC) [72, 101, 186, 198, 199], diffuse large B-cell lymphoma (DLBCL) [126, 200-202], AML [33, 34], cervical cancer [203], multiple myeloma/plasma cell leukemia [204], ccRCC [100], and pancreatic cancer [185]. In ER\(^+\) breast cancer, while it is established that TBK1 stabilizes the ER\(\alpha\), the role of this interaction in disease...
pathogenesis is not yet clear: TBK1 can facilitate tamoxifen-resistance by activating the Erα yet, the loss of TBK1 promotes invasion/migration following Erα destabilization [122, 205]. However, in HER2+ breast (and colorectal) cancer, TBK1 promotes disease progression [80, 206, 207].

While TBK1 mutations are not yet routinely reported in cancer, 138 mutations have been identified in different cancer types across 127 patients with The Cancer Genome Atlas (TCGA) [208]. Notably, almost ¼ of the patients harboring TBK1 mutations had endometrial cancer (30 out of 127).

TBK1 can promote cancer in a cell-intrinsic manner by modulating internal signaling pathways. TBK1 can drive cell division through its interaction with NuMA/CEP170 at the centrosome and by facilitating activation of the AXL/AKT3, AXL/KRAS\textsuperscript{G12}/RALB, STAT3, PI3K/AKT/mTOR1, MYC, p62, and NF-κB/BCL-X\textsubscript{L} pathways. [34, 74, 108, 150, 209].

TBK1 has been observed to exploit STAT3 across multiple cancer types, albeit through distinct mechanisms. In dendritic cells (DCs), activation of STAT3 decreases the expression of costimulatory molecules on DCs, abrogating their ability to stimulate T cells [31]. In DLBCL cells, activation of STAT3 induces production of protumor cytokines, such as IL-10 and CCL3/4 [200]. In human T-lymphotropic virus 1 (HTLV-1)-transformed T cells, TBK1 is co-opted by Tax to induce activation of STAT3 and maintain the survival of HTLV-1+ cells [202]. However, while TBK1 can be exploited by cancer cells to drive their survival and proliferation, reliance on TBK1 also sensitizes certain cells to TBK1 inhibition.
In cancer cells with certain mutations, such as KRAS$^{G12}$-mutated NSCLC and VHL$^{NULL}$ ccRCC, TBK1 becomes hyperactivated [72, 100, 210]. While such hyperactivation enhances cell proliferation, it simultaneously imparts a vulnerability such that KRAS-mutated and VHL$^{NULL}$ cells are killed by TBK1 inhibition [72, 81, 211]. TBK1 blockade may even sensitize such tumors to treatment and, in the case of NSCLC, may augment KRAS$^{G12C}$ inhibitors like sotorasib (Lumakras®) and adagrasib (Krazati®) [212, 213]. In mouse models of KRAS$^{G12D}$-mutated lung cancer, addition of JQ1, a Bromodomain and Extra-Terminal motif (BET) protein inhibitor, re-sensitized cancer cells to combination TBK1/MEK inhibition by blocking IGF1 and YAP1 signaling [214].

Additionally, AML cell lines that display high MYC activity, such as the MOLM14, OCIAML5, HL-60, and KASUMI-1, and NRAS-mutated melanoma cells are also sensitive to pharmacologic TBK1 inhibition [34, 72, 100, 210, 214]. Despite largely being a promoter of cancer, TBK1 acts as a tumor suppressor in specific intestinal cells.

It was discovered that TBK1 has tissue-specific, tumor-suppressive roles when a Villin-specific deletion of Tbk1 led to accelerated formation of intestinal polyps in Apc$^{Min/+}$ mice [157]. The loss of Tbk1 in villin$^+$ cells allows for the increased production of metallothionein 1 (MT1) in the lamina propria, promoting increased IL-1β production from resident macrophages. IL-1β stimulates the proliferation of inflammatory Th17 cells which produce cytokines such as IL-17 and IL-22; thus, loss of Tbk1 allows for the expansion of Th17 cells and ensuing inflammation from Th17-secreted IL-17 and IL-22. Mechanistically, Tbk1 restrains MT1 production by inhibiting NF-κB in villin$^+$ intestinal epithelia.
TBK1 can also promote cancer by modulating the tumor microenvironment (TME) and the behavior of T cells locally and distantly. Within tumor cells, TBK1 induces the production of immunosuppressive cytokines, such as IL-6 and RANTES, which act in autocrine and paracrine fashions to facilitate tumor growth [190, 209, 215]. Additionally, TBK1 stimulates tumor cells to upregulate expression of PD-L1, an immune checkpoint molecule that protects them from destruction by antitumor T cells [82, 203, 216, 217]. Locally, TBK1 can act within tumor-infiltrating CD4\(^+\) and CD8\(^+\) T cells to restrain the production of antitumor cytokines, such as IL-2 and IFN-\(\gamma\) [217]. Outside the TME, TBK1 acts within dendritic cells to hamper their ability to prime T cells [82, 190, 216, 217]. As a proof-of-concept, pharmacologic inhibition of TBK1 has been proven to slow tumor growth and enhance the effectiveness of immune checkpoint blockade, providing evidence that TBK1 blockade has anticancer efficacy [217].

**TBK1 Inhibitors in Cancer Treatment**

Several small-molecule inhibitors and proteolysis-targeting chimeras (PROTACs) against TBK1 have been characterized and tested in an array of *in vitro* and *in vivo* cancer models (*Table 4*, adapted from Runde, *et al.* [29]). AMX and MMB have even made it to clinical trials for the treatment of metabolic disorders and myelofibrosis/lung cancer, respectively [50, 218, 219]. TBK1 inhibitors may be TBK1-selective or have additional activity against IKK\(\varepsilon\); IKK\(\varepsilon\)-selective inhibitors have not been characterized. Additionally, some TBK1 inhibitors also block the action of other proteins. Many TBK1 inhibitors show good druglikeness and appear to be promising candidates for the
We hypothesize that combination use with immune checkpoint blockade and/or cytotoxic CTx would be of the greatest benefit.

Table 4. List of TBK1 Inhibitors (Adapted from Runde, et al.) [29].

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Target(s) Inhibited</th>
<th>Clinical Trial (if Applicable)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amlexanox (AMX; Aphthasol®/Amlin/AA-673)</td>
<td>TBK1/IKKε</td>
<td>NCT01842282; NCT01975935</td>
<td>[50, 85, 104, 153, 183, 204, 220-224]</td>
</tr>
<tr>
<td>AZ13102909/AZ909</td>
<td>TBK1</td>
<td>-</td>
<td>[225]</td>
</tr>
<tr>
<td>BAY985</td>
<td>TBK1/IKKε</td>
<td>-</td>
<td>[51]</td>
</tr>
<tr>
<td>BX795</td>
<td>TBK1/IKKε; Aurora B; NUAK1; PDK1</td>
<td>-</td>
<td>[52, 226, 227]</td>
</tr>
<tr>
<td>Compound I</td>
<td>TBK1/IKKε</td>
<td>-</td>
<td>[216]</td>
</tr>
<tr>
<td>Compound II</td>
<td>TBK1/IKKε</td>
<td>-</td>
<td>[192, 228]</td>
</tr>
<tr>
<td>DMX14</td>
<td>TBK1/IKKε</td>
<td>-</td>
<td>[229]</td>
</tr>
<tr>
<td>GSK8612</td>
<td>TBK1</td>
<td>-</td>
<td>[33, 230]</td>
</tr>
<tr>
<td>MDK10496</td>
<td>TBK1/IKKε</td>
<td>-</td>
<td>[187]</td>
</tr>
<tr>
<td>Compound</td>
<td>Targets</td>
<td>Clinical Trials</td>
<td>References</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>----------------------------------------------</td>
<td>----------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Momelotinib (MMB)/CYT387/GS03 87</td>
<td>TBK1/IKKe; ACVR1; ALK2; JAK1/2</td>
<td>NCT02101021; NCT02206763; NCT02258607; NCT04173494/MOMENTUM; NCT01969838/SIMPLIFY1; NCT02101268</td>
<td>[34, 218, 219, 231-235]</td>
</tr>
<tr>
<td>MPI0485520</td>
<td>TBK1/IKKe</td>
<td>-</td>
<td>[236]</td>
</tr>
<tr>
<td>MRT67307</td>
<td>TBK1/IKKe</td>
<td>-</td>
<td>[84]</td>
</tr>
<tr>
<td>MRT68601</td>
<td>TBK1</td>
<td>-</td>
<td>[52]</td>
</tr>
<tr>
<td>UNC6587 (Cereblon PROTAC)</td>
<td>TBK1</td>
<td>-</td>
<td>[100]</td>
</tr>
<tr>
<td>15a</td>
<td>TBK1/IKKe; Aurora A; GSK3β</td>
<td>-</td>
<td>[237]</td>
</tr>
<tr>
<td>200A</td>
<td>TBK1/IKKe</td>
<td>-</td>
<td>[189]</td>
</tr>
<tr>
<td>3i (VHL PROTAC)</td>
<td>TBK1</td>
<td>-</td>
<td>[238]</td>
</tr>
</tbody>
</table>

**Overview of AML**

In AML, a clone of cancerous myeloid cells expands in the BM [1, 2, 239]. AML cells consume resources as they proliferate, hampering the function of normal HSPCs and eventually spilling into the bloodstream. A hematologic neoplasm is evident when a high blast percentage in the BM or blood is detected, and AML is diagnosed when ~20% of cells in the BM or peripheral blood (PB) are myeloid blasts; the normal blast
percentage in the BM is less than 5% and close to 0% in the blood [240]. AML cells can extravasate from the vasculature to enter the spleen, destroying its normal architecture, and AML cells can also infiltrate extramedullary sites such as the kidneys, skin and gums (leukemia cutis), and spine, brain, abdomen, and mouth where they can even form solid tumors (chloroma/myeloid sarcoma [MS]) [1, 241, 242]. As normal hematopoiesis is increasingly disturbed, AML patients experience fatigue (due to anemia), infection (due to leukopenia [chiefly, neutropenia]), and bruising (due to thrombocytopenia). As AML cells are inert in terms of infection-fighting abilities, AML patients are at high risk of infection despite presenting with high white blood cell counts, sometimes exceeding 100 K/μL [239, 243, 244]. AML progresses rapidly and an AML burden of $10^{12}$ cells is fatal [245].

While a rare cancer overall, accounting for ~1% of all cancer cases in the US, AML is the most common type of leukemia [9]. In the US in 2023, it is estimated that 20,380 people will be newly-diagnosed with AML and around 11,310 people will die from the disease [3, 4]. Risk factors for AML include: male sex (~34% increase in risk), age, tobacco exposure, ionizing radiation exposure, previous CTx treatment (namely, anthracyclines, alkylating agents, and epipodophyllotoxin derivatives [etoposide/teniposide]), some genetic disorders (e.g., Fanconi anemia, Down syndrome [specifically, the M7 subtype], and Li-Fraumeni syndrome), and myelodysplastic syndrome (MDS; 1/3rd of MDS patients will develop AML) [246-249]. Benzene exposure is a unique risk factor for AML: following hepatic metabolism, benzene metabolites are
transported to the BM and undergo further oxidation by myeloperoxidase (MPO), yielding a mutagenic benzoquinone metabolite in myeloid cells [250, 251].

Prognosis is strongly and inversely associated with age, mostly because older patients often have comorbidities that preclude the use of standard CTx [7, 8, 19, 252, 253]. As well, older patients are more likely to present with drug resistance and unfavorable cytogenetics, specifically with alterations to chromosomes 5, 7, and 17 [252]. Aside from PML-RARA\textsuperscript{+} AML, the 5-year survival rates are 69% and 27% for patients below and above age 20, respectively [9, 254].

**Subtypes of AML**

AML is not one cancer, but a complex, highly heterogenous group of myeloid neoplasms [255]. Several AML subtypes exist, each with unique clinical presentations and molecular classifications (immunophenotypes) [256]. As such, AML can be classified by the World Health Organization (WHO) into any one of seven subtypes [241, 257, 258]:

- AML with recurrent genetic abnormalities (e.g., MLL1-AF9\textsuperscript{+}, PML-RARA\textsuperscript{+}, RUNX1-ETO\textsuperscript{+}, NPM1-mutated, CEBPA-mutated [biallelic], ETC.)

- AML with myelodysplasia-related changes (AML-MRC)

- Therapy-related AML (t-AML)

- AML, not otherwise specified (AML, NOS)

- Myeloid sarcoma (noted by Burns in 1811 [259]; described as “chloroma” in 1853 by King, as the tumors appeared green due to the MPO of the myeloid cells [242, 260])
• Myeloid proliferations related to Down syndrome (e.g., transient abnormal myelopoiesis and myeloid leukemia associated with Down syndrome)
• Blastic plasmacytoid dendritic cell neoplasm (BPDCN)

AML can arise de novo, that is, in patients who have not been previously treated with CTx/XRT nor have a history of MDS/myeloproliferative neoplasms (MPN) [261]. In contrast, AML can arise secondarily (sAML), resulting from the progression of MDS/MPN or previous CTx/XRT treatment. Patients with de novo AML, AML-MRC, and t-AML each present with distinctive mutations and cytogenetics [262].

In cases of AML, NOS, the French-American-British (FAB) classification system can be used to aid diagnosis based on cell morphology [239, 256, 263]. While the FAB system has largely been replaced by more sensitive techniques, like flow cytometry and genome sequencing, it is still used to complement molecular methods. The FAB classification consists of nine major subtypes, some of which have additional subclassifications based on differentiation status [264]:

• M0 (undifferentiated acute myeloblastic leukemia)
• M1 (acute myeloblastic leukemia with minimal maturation)
• M2 (acute myeloblastic leukemia with maturation)
• M3 (acute promyelocytic leukemia [APL]/PML-RARA+ AML)
• M4 (acute myelomonocytic leukemia [AMML])
• M4eos (acute myelomonocytic leukemia with eosinophilia [AMML Eo])
• M5 (acute monoblastic/monocytic leukemia [AMoL])
  o M5a (monocytic without differentiation [acute monoblastic leukemia])
- M5b (monocytic with differentiation [acute monocytic leukemia])
  - M6 (acute erythroid leukemia [AEL])
  - M7 (acute megakaryoblastic leukemia [AMKL])

While not listed under the FAB classification, acute basophilic leukemia (ABL) and acute panmyelosis with myelofibrosis (APMF) are recognized by the WHO as rare subtypes of AML, NOS [258, 265].

**Pathophysiology of AML**

AML encompasses a diverse group of cancers that arise when a hematopoietic stem/progenitor cell (HSPC) incurs an AML-initiating mutation, such as rearrangement of *mixed-lineage leukemia 1* (*MLL1*; hereafter referred to as *MLL*), biallelic loss of *CEBPA*, or *FLT3* mutation [28, 266]. An excellent report by Chopra, *et al.* posits that AML can arise through one of two mechanisms: either an HSC incurs an AML-initiating mutation that enables rapid proliferation, or a myeloid progenitor gains aberrant self-renewal capacity [28, 267]. In both cases, a myeloid cell with a survival advantage and self-renewal capacity results.

The t(9; 11)(p22; q23) translocation is an AML-driving event that produces the *MLL-AF9* oncogene and is present in nearly 1/3rd of AML patients [268]. *MLL-AF9* encodes MLL-AF9, an abnormal transcription factor wherein the N-terminus of MLL is fused to the C-terminus of AF9 [269, 270]. Normally, the MLL epigenetic modifier (*KMT2A* on chromosome 11) facilitates the expression of development- and hematopoiesis-related genes, such as *HOXA9* and *MEIS1* (whose ectopic expression alone can cause leukemia) [271]. MLL is a histone methyltransferase, an epigenetic
“writer”, that installs methyl groups on K4 of histone H3 (H3K4); namely, MLL trimethylates H3K4 (H3K4me3) [271, 272]. However, upon fusion with AF9 (MLLT3 on chromosome 9), MLL loses its ability to correctly regulate target genes and becomes a potent driver of high-risk, acute leukemias [269, 273]. The MLL-AF9 translocation is found in both pediatric and adult AML, is associated with cases of t-AML (specifically after treatment with etoposide [Vepesid®]/teniposide [Vumon®]), and imparts an increased risk of relapse [247, 270, 271, 274-276].

FLT3 mutations, such as the internal tandem duplication (ITD) and tyrosine kinase domain (TKD) mutations, are also common drivers of AML [277-282]. Mutations in FLT3 are detected in 25-30% of all AML cases; the FLT3-ITD accounts for approximately 83% of FLT3 mutations in AML [281, 283]. These mutations cause the FLT3 receptor tyrosine kinase to become constitutively active, leading to the aberrant growth/survival of afflicted cells. Although, it is reported that even unmutated FLT3 (FLT3WT) is overexpressed in almost all cases of AML [277, 279, 280] and can facilitate leukemogenesis in the presence of certain driver mutations. For example, FLT3WT is exploited in MLL-AF9+ AML to promote disease progression through the FLT3WT-JAK3/STAT3 axis [276-281, 284-286]. Expectedly, MLL-AF9+ AML is worsened by FLT3 mutations [276, 287].

Despite harboring the same driver mutations, the cells comprising the AML liquid tumor are heterogenous, with some being more or less differentiated than their counterparts [2, 267, 288, 289]. The more-differentiated, most abundant AML cells are referred to as “blasts” and, while being responsible for AML symptoms, they are not
believed to possess leukemogenic abilities; if transplanted, AML blasts are unable to reconstitute the disease. The less-differentiated, less abundant AML cells that initiate and maintain the disease are the LSCs; if transplanted, LSCs will reconstitute the disease.

**Conventional Treatment of AML**

Untreated AML constitutes a medical emergency [253, 290]. Aside from the M3 subtype, the frontline CTx regimen for newly-diagnosed/previous-untreated AML typically employs cytotoxic CTx and consists of two phases [10, 257, 291]. First, in the “7+3” or “induction” phase, cytarabine (Ara-C [Cytosar-U®]) is given in combination with daunorubicin (DNO [Cerubidine®]), sometimes abbreviated as the “DA” protocol. Occasionally, idarubicin (Idamycin®) will be used instead of DNO. Then, in the “consolidation” phase, Ara-C is dose-escalated and used alone [292-295]. The 7+3 phase of frontline CTx is primarily used as “cytoreduction”, a means of rapidly clearing AML blast cells from the body to help restore normal hematopoiesis and alleviate symptoms, whereas consolidation is used to prevent relapse, ideally killing any remaining LSCs [10, 293, 296-298]. While there is not currently an established treatment regimen for relapsed/refractory AML (R/R AML), a combination of fludarabine, high-dose Ara-C, G-CSF, and idarubicin (FLAG-IDA protocol) yields CR in approximately half of R/R AM patients CR [4, 299]. Additionally, amsacrine in combination with fludarabine, high-dose Ara-C, and G-CSF (FLAMSA protocol) can be used to prepare eligible R/R AML patients for an allogenic HSCT [300].
Mechanistically, Ara-C is a prodrug of cytarabine triphosphate (Ara-CTP), a nucleoside analog that causes chain termination and S-phase arrest upon incorporation into DNA [294, 301-303]. DNO is an anthracycline-type drug that exerts antineoplastic effects through multiple mechanisms. Chiefly, DNO blocks the relegation action of topoisomerase II during DNA replication, which leads to the accumulation of DNA double-stranded breaks and activation of the ATM/p53-mediated DNA damage response [144, 304-306]. Additionally, DNO damages DNA via intercalation, which causes histone eviction, and causes ROS formation upon reaction with O$_2$. While Ara-C and DNO are effective, AML cells possess mechanisms to counter their effects. AML cells can resist Ara-C by expressing SAMHD1, a hydrolase that degrades Ara-CTP, the active metabolite of Ara-C [26, 27]. AML cells can overexpress ABC transporters, such as P-glycoprotein and ABCG2, to efflux intracellular DNO [307].

Cytogenetics, whole-genome sequencing, and immunophenotyping are becoming increasingly valued in the diagnosis, risk stratification, and treatment planning of AML [308]. AML risk-stratification by genetics is indicated by 2023 National Comprehensive Cancer Network (NCCN) and 2022 European LeukemiaNet (ELN) guidelines; for example, *MLL-AF9*+ AML carries a better prognosis versus *TP53*-mutated AML [309, 310]. Although, despite vast knowledge of the genetics and pathophysiology of AML, precision medicine and targeted therapies are lacking. Cytotoxic CTx remains the standard-of-care for most AML patients despite their typically advanced age and frailty.
Targeted Therapies for AML

Targeted therapies are available but currently limited to patients harboring specific genetic signatures [14, 295]. Inhibitors specific to mutated FLT3 (midostaurin [Rydapt®] & gilteritinib [Xospata®]) and IDH1/2 (ivosidenib [Tibsovo®]/enasidenib [Idhifa®]) are available, and an antibody-drug conjugate can be used for patients with CD33+ AML cells (gemtuzumab ozogamicin [Mylotarg®]).

Recently, following promising results from the AUGMENT-101 clinical trial (NCT04065399), revumenib (SNDX-5613, Syndax) displayed efficacy in acute leukemia patients harboring MLL rearrangements (MLL-r [e.g., MLL-AF9 or MLL-AF6]) or NPM1 mutations that cause NPM1 to translocate to the cytoplasm (NPM1c) [22, 311]. Revumenib disrupts the interaction between MLL and menin (MEN1), thereby preventing MLL from inducing transcription of leukemia-promoting genes like HOXA9, PBX3, and MEIS1.

Expectedly, MEN1 mutations have been reported in patients who progress on revumenib monotherapy, highlighting the necessity of combination regimens even with targeted agents. However, combination/targeted therapy requires the discovery of feasible targets and development of molecules [24]. Recent research has suggested that various components of TLR signaling, including TICAM-1 and RIPK1/3, are feasible molecular targets for the treatment of MLL-AF9+ AML. While targeted therapies applicable to a larger cohort of AML patients are emerging, such as E-selectin antagonists (uproleselan [phase III: NCT03616470]; GlycoMimetics, Inc.) and p53-restoring agents (eprenetapopt/APR-246; Aprea Therapeutics, Inc.), all cases of non-
M3 AML still necessitate cytotoxic CTx [23, 312]. Thus, there is dire need for the discovery of novel molecular targets [19, 23, 312].

**Targeting TLR Signaling to Treat AML**

TLRs are expressed on many cell types, including normal HSPCs and AML cells. In normal hematopoiesis, HSPCs express varying levels of TLR2/4/7/8/9 [313]. Activation of TLRs causes HSPCs to produce inflammatory cytokines and skews them towards myeloid fate (myelopoiesis) at the cost of lymphoid fate (lymphopoiesis). Stimulation of TLR7/8 on human CD34+ HSCs causes them to produce IL-1β, IL-6, IL-8, TNF-α, and GM-SCF, and further promotes their differentiation into macrophages and monocyte-derived dendritic cells (moDCs) [314]. In addition, stimulation of TLR7 on common myeloid progenitors (CMPs) promotes their fate as monocyte/macrophages, and this effect is enhanced in by IFN-1. Activation of TLR2/4 promotes cycling and myeloid differentiation of murine Lin⁻Sca1⁺c-Kit⁺ cells (LSK cells [LSKs]), mediated largely through MYD88-dependent signaling. The effect of TLR stimulation on HSPCs may be illustrated by the "left shift" (bandemia) observed clinically in patients with severe infections [315]. Bandemia indicates emergency granulopoiesis and increased G-CSF production, likely mediated at least in part by TLR activation on HSPCs [316, 317].

TLRs are also expressed differentially on AML cells. Specifically, TLR2/4/7/8 are expressed highly, TLR1/5/9/10 to a lesser degree, and TLR3 expression is undetectable. Relevant to our study, the AML subtypes commonly driven by MLL-AF9 (M4 and M5) express markedly higher levels of TLR4 and TLR7 [274, 318].
The role of TLR signaling in AML appears somewhat pleotropic, however. While AML cells have been thoroughly demonstrated to rely on TLR signaling for their survival and leukemogenicity, TLR agonism has been shown to be an effective anti-AML strategy in mouse models [319-321]. While TLR2 and TLR4 mRNA levels are reported to inversely correlate with OS of AML patients, selective TLR stimulation has been shown to possess antileukemic effects, specifically in MLL-AF9+ AML models [322, 323]. While TLR1 is required for the survival of MLL-AF9+ mouse HSPCs, TLR1/2 agonism reduces leukemogenicity of transplanted MLL-AF9+ cells by promoting their differentiation [324]. Moreover, TLR4/7 stimulation induces increased expression of CD54, CD80, and CD86 on AML M4/M5 cells, enhancing their susceptibility to T cell-mediated clearance [325].

Our group and others have demonstrated that blockade of TLR signaling, genetic and pharmacologic, has anti-AML effects in vitro and in vivo. Specifically, our lab has demonstrated that blocking TLR and TLR-related signaling hampers the leukemogenicity of MLL-AF9+ cells in human cell lines and mouse models.

Volk, et al. demonstrated the necessity of TNF signaling in MLL-AF9+ mouse cells and human AML cell lines [35]. It was determined that while normal HSPCs do not require TNF for their survival, several human AML cell lines (HL-60, ML2, NB4, and THP-1) rely on autocrine TNF, which activates their JNK-AP1 and NF-κB axes. Notably, not only does TNF promote the survival of AML cells also proves toxic to local HSPCs. Thus, AML cells may “weaponize” TNF as a strategy to overtake the BM. Blocking JNK
or NF-kB signaling delays leukemogenesis in a mouse model of \textit{MLL-AF9}\textsuperscript{+} AML, supporting the targeting of TNF/JNK/NF-kB signaling as a means of AML treatment.

Xin, \textit{et al.} showed that \textit{MLL-AF9}\textsuperscript{+} cells rely on RIPK1/RIPK3, serine/threonine kinases that mediate TNF- and IFN-I-induced cell death, for their survival [36, 326]. It was determined that pharmacologic blockade of RIPK1/3 sensitizes AML cells to IFN-\(\gamma\)-induced differentiation, leading to a reduction in AML cell growth and clonogenicity. In addition, the loss of either kinase alone significantly delays leukemogenesis in an \textit{MLL-AF9}\textsuperscript{+} AML mouse model, and this benefit is strongly augmented by the addition of IFN-\(\gamma\). These data provide further support for the targeting of TNF- and RIPK1/3-associated cellular machinery as anti-AML therapy.

Most recently, Cannova determined that \textit{MLL-AF9}\textsuperscript{+} mouse LSCs (c-Kit\textsuperscript{+}CD11b\textsuperscript{lo}) rely on MYD88 and TICAM-1 for leukemogenesis [32]. Following TLR stimulation, TICAM-1 recruits TBK1 and is phosphorylated by TBK1 on its S210, S212, and T214 residues [29, 327]. An activated TICAM-1 will recruit IRF3 to facilitate its phosphorylation by TBK1. TBK1 is an integral component of TLR signaling, as TBK1 is required for the TICAM-1-mediated activation of IRF3 [328]. Subsequent studies suggested that TBK1 is a feasible target for AML treatment, as pharmacologic inhibition with BX795 hampers the clonogenicity of \textit{MLL-AF9}\textsuperscript{+} mouse HSPCs; however, shRNA knockdown of \textit{Tbk1} in the same cells failed to recapitulate the effects seen with BX795. It is possible that the knockdown of \textit{Tbk1} was insufficient, but it is also possible that the other targets of BX795 (IKK\(\varepsilon\), Aurora B, NUAK1, and PDK1) are involved in the clonogenicity of these cells. Regardless, \textit{Tbk1} warrants further investigation [32].
These data together strongly demonstrate that TLR signaling is critical to the survival and leukemogenicity of AML LSCs. However, while preliminary data support the targeting of TBK1—a transducer of TLR3/4/7/8/9 and TICAM-1 signaling—for AML treatment, the role of TBK1 has yet to be determined [29, 320].

**TBK1 as a Molecular Target for AML Treatment**

Considering previous data and being a critical component of TLR signaling, we hypothesize that TBK1 is a molecular target that can be employed for AML treatment. Specifically, just as targeting TICAM-1 did, targeting TBK1 may antagonize the p27\textsuperscript{Kip1}-quiescent AML cells that are CTx-resistant and responsible for relapse: LSCs (c-Kit\textsuperscript{+}CD11b\textsuperscript{+}). In addition to Cannova’s preliminary data, recent reports provide additional evidence that TBK1 blockade has anti-AML effects.

In 2018, Liu, et al. demonstrated that human AML cells that rely on the YBX1-MYC axis for their survival (MOLM14, OCIAML5, HL-60, and KASUMI-1) also require TBK1 and/or IKK\epsilon; although, it was not determined if just one or both kinases are required for survival [34]. This finding was recapitulated in a mouse xenograft model using MOLM-14 cells, which are *MLL-AF9*\textsuperscript{+} and *FLT3-ITD*\textsuperscript{+} [34, 282]. MMB, a TBK1/IKK\epsilon inhibitor, dose-dependently reduced the leukemia burden and spleen sizes of xenografted mice. While MMB also inhibits JAK1/2, ACVR1, and ALK2, its antileukemic effects were determined to be specific to TBK1/IKK\epsilon, at least in MOLM-14 cells, independent of JAK1/2, ACVR1, or ALK2 blockade.
Mechanistically, MMB exerts its effects by dampening YBX1 phosphorylation (S102), causing a dose-dependent reduction in MYC protein levels [34]. Although, TBK1 and IKKe are not anticipated to directly interact with YBX1, but rather facilitate its interaction with an unidentified kinase; the authors ruled-out the involvement of AKT. While MMB also reduces the clonogenicity of AML cells more significantly than healthy CD34+ HSCs, the control cells were not unaffected; thus, the authors cautioned that prolonged blockade of TBK1/IKKe could be harmful to normal hematopoiesis.

Additionally, the authors mined cDNA microarray datasets from 2004 (GSE1159) and 2009 (GSE13159). As such, while IKBKE expression (204549_at) is slightly/positively correlated (p = 0.04) with the OS of AML patients, TBK1 expression (218520_at) has no bearing (p-value unlisted) [34, 329-331]. However, Liu, et al. only reported on the 2004 dataset when investigating TBK1. In contrast, BloodSpot mined the 2009 dataset when they reported that TBK1 expression is strongly/negatively correlated (p = 0.00227) with OS of AML patients while IKBKE expression is not predictive (p = 0.327) [332-336] (Figure 3). Thus, further research regarding TBK1 and IKBKE expression in AML is required, with RNA-seq now being the gold-standard for determining the prognostic value of gene expression.
In 2021, Chen, et al. demonstrated that pharmacologic TBK1 blockade sensitizes human AML cells to CTx [33]. Specifically, treatment with GSK8612, a TBK1-selective inhibitor, sensitizes human AML cell lines (HL-60 and KASUMI-1) and patient samples to 100nM DNO. Notably, the \( MLL-AF9 \) THP-1 cell line was not sensitized by GSK8612, possibly due to a decreased expression of TBK1 compared to HL-60 and KASUMI-1 cells. Mechanistically, inhibiting TBK1 dampens activation of CDK2, promoting G0/G1 arrest. It was determined that TBK1 activates CDK2 through AKT1/2, such that pharmacologic inhibition of AKT1/2 sensitized cells to DNO similarly to GSK8612. Specifically, TBK1 or AKT1/2 blockade augments the apoptosis-inducing ability of DNO.

Figure 3. TBK1 mRNA Expression is Negatively Correlated with AML Prognosis, based on Microarray Data. Kaplan-Meier curves generated by BloodSpot based on median expression of (A) TBK1 or (B) IKBKE mRNA in human AML patients. Based on cDNA microarray data from 2009, TBK1 mRNA level is significantly/negatively correlated \((p = 0.00227)\) with probability of OS whereas IKBKE mRNA is not correlated \((p = 0.327)\). Above-median expression of TBK1 mRNA portends decreased probability of OS in AML.
The authors also found that TBK1 protein is significantly increased in the BM of AML patients compared to healthy controls. Notably, R/R AML patients displayed significantly higher levels of TBK1 compared to AML patients who achieved CR; this finding could suggest that TBK1 contributes to treatment-resistance in AML.

It was determined that the upregulation of inflammation-associated genes drives resistance to FLT3 inhibitors in FLT3-ITD+ AML cells [277, 337]. Specifically, MV4-11 cells are observed to upregulate inflammatory gene sets as they develop resistance to quizartinib (GSE116432). Although, treatment with the glucocorticoid dexamethasone resensitizes both MV4-11 cells and FLT3-ITD+ patient samples/xenograft models to quizartinib. Mechanistically, dexamethasone induces increased expression of the proapoptotic BIM and degradation of the antiapoptotic MCL-1, leading to increased cell death in FLT3-ITD+ but not FLT3WT human AML cells.

Subsequently, after mining the RNA-seq data from Gebru, et al., Wooten’s group utilized an in silico approach (weighted gene co-expression network analysis [WGCNA]) to identify possible therapeutic targets to counter drug-resistance in FLT3-ITD+ AML [283, 337]. The authors posited that TBK1 upregulates a set of genes in response to dexamethasone in quizartinib-resistant, MV4-11 cells. Specifically, genes hypothesized to be regulated by TBK1 are associated with extracellular matrix remodeling and collagen synthesis. While the significance of this finding is unknown, it suggests that TBK1 functions in opposition to dexamethasone in quizartinib-resistant, FLT3-ITD+ AML cells. Furthermore, midostaurin (Rydapt®)—a staurosporine derivative used in the treatment of FLT3-mutated AML—was recently demonstrated to inhibit TBK1 with an
IC$_{50}$ of 9 nM [338, 339]. While the significance of this finding is unknown, TBK1 blockade may contribute to the antileukemic activity of midostaurin (Rydapt$^{	ext{®}}$).

While these reports support the targeting of TBK1 as a means of AML treatment, the role of TBK1 in normal hematopoiesis has yet to be determined. For TBK1 blockade to be an effective anti-AML strategy, any untoward effects on normal hematopoiesis must be minimal and, ideally, TBK1 inhibition should preferentially afflict AML cells.
CHAPTER 3

AIM 1: DETERMINE IF TBK1 IS REQUIRED FOR MURINE HEMATOPOIESIS

Results: Global Tbk1 Deletion in Murine Hematopoiesis

TBK1 mRNA Expression Levels Appear Somewhat Consistent Across the Human and Murine Hematopoietic Systems

The ideal CTx would selectively kill cancer cells while sparing the normal, healthy counterparts. Most conventional chemotherapies show some degree of selectivity as they preferentially afflict the most rapidly dividing cells, which includes but is not limited to cancer cells [340]. Cells of the gastrointestinal mucosa, hair follicle, and especially hematopoietic system also divide rapidly, hence the side effects characteristic of cytotoxic CTx (e.g., diarrhea, non-androgenic alopecia, and myelosuppression, respectively). Similar to antibiotics, which target the 70S ribosome or other proteins unique to bacteria, selectivity can be conferred to anticancer drugs by targeting a gene/protein unique to or overexpressed by cancer cells; excellent examples of this are the targeting of BCR-ABL1 in CML or HER2 (trastuzumab [Herceptin®]) in HER2+ breast cancer [341, 342]. While TBK1 seems to be a promising target for AML treatment, we sought to determine if targeting TBK1 may selectively afflict AML cells.
To this end, we examined RNA-seq datasets catalogued on Haemosphere/Haemopedia to investigate TBK1 expression across the human (GSE115736) and murine (GSE116177) hematopoietic systems [343]. We believe that increased expression of TBK1 would suggest an increased reliance on its protein product, thus making cells with high TBK1 expression more sensitive to TBK1 inhibition.

From the RNA-seq data examined, TBK1 appears to be expressed at similar levels across cells of the human (Figure 4) and murine (Figure 5) hematopoietic systems, aside from some select populations where it is expressed at relatively lower levels, such as reticulocytes (likely at least partially due to enucleation), NK cells, and plasma/plasmacytoid dendritic cells. However, this conclusion was based on a visual inspection of log2(tpm+1)-plotted expression levels and can only be interpreted as such; obviously, a statistical comparison would be more reliable.
Figure 4. TBK1 mRNA Expression Level is Somewhat Consistent Across the Human Hematopoietic System. The RNA-seq data catalogued by Haemosphere (GSE115736 [Haemopedia-Human-RNASeq]) from human hematopoietic cells was log2(tpm+1)-plotted and visually inspected. Between the select cell types depicted therein, TBK1 mRNA level appears roughly consistent (aside from decreased levels in plasmacytoid dendritic cells and CD4^+ T cells). NOTE: graph has been broken (black bars) between 0 and 4 to allow for better visualization.
Development of a Tamoxifen-inducible, Global Tbk1-knockout Mouse

To study the role of Tbk1 in normal and malignant hematopoiesis, we generated tamoxifen-inducible, global Tbk1-knockout (Tbk1\textsuperscript{NULL}) mice. All mice were maintained on the B6 background.

The Tbk1-deletion strategy employed was adapted from Marchlik, et al. and produced by Lexicon Pharmaceuticals (Lexicon; Figure 6A) [127, 344]. The deletion strategy involved flanking exon 2 of Tbk1 with loxP sites (floxing; Tbk1\textsuperscript{fx}). Floxed-homozygous (Tbk1\textsuperscript{fx/fx}) mice were crossed with Rosa26-CreER\textsuperscript{T2+} mice until Tbk1\textsuperscript{fx/fx};Rosa26-CreER\textsuperscript{T2+} mice were generated (Figures 6B and 6C). Tbk1\textsuperscript{fx/fx} mice NOT harboring Rosa26-CreER\textsuperscript{T2} were used as “wild-type” (WT) controls to control for tamoxifen exposure. In addition, Tbk1\textsuperscript{+/fx};Rosa26-CreER\textsuperscript{T2+} mice were bred as floxed-heterozygous controls; however, time did not allow for analysis of these mice within the scope of this thesis.
Tbk1 Deletion is Complete in the Bone Marrow of a 6-week-old Mouse

Under basal conditions, the CreER^T2 recombinase resides in the cytoplasm, inactive, sequestered by heat-shock protein 90 (Hsp90) [345, 346]. Upon exposure to 4-hydroxytamoxifen (4-OHT), a principal metabolite of tamoxifen, CreER^T2 is released from Hsp90 and allowed to enter the nucleus for its recombinase activity. Notably, especially for in vitro deletion, CreER^T2 is unresponsive to tamoxifen and requires 4-OHT for activation. As tamoxifen is metabolized to 4-OHT, intraperitoneal delivery of tamoxifen is acceptable as it will undergo hepatic metabolism following movement across the peritoneal mesothelium and absorption into the bloodstream [347].

Following a regimen indicated by Zhong, et al., mice were injected at 6-8 weeks of age with 100mg/kg tamoxifen for 4 days consecutively (Figure 7A; hereafter, referred
to as “tamoxifen regimen”) [348]. Deletion was checked via PCR of genomic DNA (gDNA).

In 6-week-old mice, Tbk1 is deleted efficiently in c-Kit+ and c-Kit− BM cells (Figure 7B) by 12-days post-tamoxifen regimen; tail and other hematopoietic tissues were not analyzed. All mice in our study underwent the tamoxifen regimen between 6 and 8 weeks of age, unless otherwise specified. As such, it was anticipated that the deletion would be complete in subsequent mice, and mice were not individually assessed for deletion until later in the study. As the BM gives rise to the entire hematopoietic system, it was presumed that a complete deletion of Tbk1 in the BM was sufficient for our analyses. As turnover of the murine hematopoietic system occurs approximately every 16 weeks, a “washout” period of 3-6 months was allowed between the tamoxifen regimen and analysis, to allow for complete regeneration of the—now Tbk1NULL—hematopoietic system. It was believed that deletion of Tbk1 need only occur in HSPCs to achieve a Tbk1-deficient hematopoietic system, so Tbk1-deletion was not routinely checked in following mice.
To determine if Tbk1 is required to produce mature hematopoietic cells in the BM and PB, as well as erythroid progenitors in the BM, BM and PB were harvested from Tbk1NULL mice at approximately 3-5-months post-deletion. Mice were age-matched (5-6
months old) and compared to Tbk1
fx/fx controls that underwent the same tamoxifen regimen; sex-matching was not performed.

The harvested tissues were analyzed via FACS, and relevant populations measured as percentage of total nucleated cells (TNCs) in the PB (PB-TNCs; Figure 8A) or BM (BM-TNCs; Figure 8B); doublets eliminated by gating on single cells (SC). B cells (B220*), T cells (CD3*), monocytes (CD11b*Gr1*), granulocytes (Gr1*), megakaryocytes (CD41*), and erythroid progenitors (Ter119*) were analyzed. Representative FACS gating strategies are shown (Figures 9A and 9B). Statistics could not be performed to the low number of mice analyzed (n = 2 of each). While several other batches of mice were analyzed, technical issues with staining and sample preparation precluded them from our analysis.
Figure 8. Tbk1 Deletion Does Not Affect Levels of Mature Blood Cells nor Erythroid Progenitors in the PB or BM. To determine if the loss of Tbk1 affects homeostatic hematopoiesis, the levels of mature cells in the PB and BM, as well as erythroid progenitors and megakaryocytes in the BM, were analyzed via FACS. The data were quantified, though statistical analyses could not be performed due to insufficient numbers of mice (n = 2 Tbk1WT, 2 Tbk1NULL). (A) PB-TNCs. (B) BM-TNCs.
Notwithstanding the low sample size, it appears that *Tbk1* is not required for the production of mature cells in the BM or PB, nor is *Tbk1* required for the function of erythroid progenitors.

**Tbk1**\(^\text{NULL}\) Mice Display a Significant Increase in the Myeloid-Biased MPP3 Population of HSPCs

To determine if *Tbk1* is required for the function of select HSPCs, BM was harvested from *Tbk1*\(^\text{NULL}\) mice at approximately 3-5-months post-deletion. Mice were age-matched (5-6 months old) and compared to *Tbk1*\(^{fx/fx}\) controls that underwent the same tamoxifen regimen; sex-matching was not performed. The harvested tissues were

Figure 9. Representative Gating Strategies for FACS Analysis of Mature Blood Cells, Erythroid Precursors, and Megakaryocytes. After gating on TNCs and eliminating doublets (SC gating), B cells (B220\(^+\)), T cells (CD3\(^+\)), monocytes (CD11b\(^+\)Gr1\(^-\)), granulocytes (Gr1\(^+\)), megakaryocytes (CD41\(^+\)), and erythroid progenitors (Ter119\(^+\)) were analyzed. (A) PB-TNCs and (B) BM-TNCs.
analyzed via FACS, and relevant populations measured as percentage of BM-TNCs.

Representative FACS gating strategy is shown (Figure 10D). All murine HSPCs are Lin-c-Kit+ (LK cells [LKS]), but only some are also positive for Sca1+ (LSK cells [LKSs]). Thus, all HSPCs reside within the LK compartment of the BM, which includes both LKS and LKSs.

I observed that the loss of Tbk1 does not affect the size of the LK compartment as percentage of BM-TNCs (Figure 10A). However, deletion of Tbk1 does lead to an
increase in the number of LSKs within the LK compartment, though not at the cost of LK cells \((p = 0.037; \text{Figure 10A})\). To determine the nature of this increase, the LSK subset was examined further using Flt3 and CD150.

I found that the loss of \(Tbk1\) leads to an increase in the number of Flt3\(^{-}\) LSKs \((p = 0.005; \text{Figure 10B})\). Following this observation, Flt3\(^{-}\) HSPCs were examined using CD150 and CD48, wherein it was revealed that the MPP3 (Flt3\(^{-}\)CD150\(^{-}\)CD48\(^{+}\) LSK), population was increased significantly \((p = 0.003; \text{Figure 10C})\). In addition, while not significant, a trend towards increased \((p = 0.160; \text{Figure 10C})\) MPP2 was observed.

From these data, it was determined that the increase in LSK cells in \(Tbk1^{\text{NULL}}\) mice was most likely due to an increase in the MPP3 population. This suggests that \(Tbk1\) promotes the differentiation of HSPCs, such that loss of \(Tbk1\) causes an accumulation of HSPCs at the MPP3 stage or increase in the size of the MPP3 compartment, and possibly also the MPP2 stage.

**\(Tbk1^{\text{NULL}}\) Mice Do Not Display Changes in Levels of Specific Myeloid Progenitors**

To determine if \(Tbk1\) is required for the function of specific myeloid progenitors, BM was harvested from \(Tbk1^{\text{NULL}}\) mice at approximately 3-5-months post-deletion. Mice were age-matched (5-6 months old) and compared to \(Tbk1^{fx/fx}\) controls that underwent the same tamoxifen regimen; sex-matching was not performed. The harvested tissues were analyzed via FACS, and relevant populations measured as percentage of BM-TNCs. Representative FACS gating strategy is shown (\text{Figure 11D}).
No significant changes were observed in granulocyte/monocyte progenitors (GMP/CFU-GM), common myeloid progenitors (CMP/CFU-GEMM), differentiated precursors (DP) (Figure 1A), megakaryocyte progenitors (MkP [Lin−c-Kit+CD41+CD150+]; Figure 1B), pre-colony-forming units-erythroid (Pre CFU-E), colony-forming units-erythroid (CFU-E), pre-megakaryocyte-erythroid progenitors (Pre-MegE), or pre-myeloid-erythroid progenitors (Pre-MEP) (Figure 1C) populations in Tbk1null mice. These data indicate that Tbk1 does not regulate the aforementioned myeloid progenitors.
Figure 11. Deletion of Tbk1 Does Not Cause Significant Changes in Myeloid Progenitor Populations. To determine if the loss of Tbk1 affects myeloid progenitors, the levels of myeloid progenitors in the BM were analyzed via FACS. The data were quantified and unpaired t-test used for all statistical analyses (n = 3 Tbk1WT, 3 Tbk1NULL). (A) Non-MKP HSPCs. (B) MKPs (Lin-c-Kit+CD41+CD150+). (C) CD16/32-CD27- Non-MKPs. (D) Representative gating strategy for FACS analysis. After gating on TNCs and eliminating doublets (SC gating), the myeloid progenitor subsets were analyzed.

**Tbk1NULL Mice Display a Significant Increase in Circulating Neutrophils**

To determine if Tbk1NULL mice displayed abnormalities in blood-cell counts, PB was isolated via cardiac puncture (post-mortem) and analyzed using HemaVet®. PB herein was isolated from Tbk1NULL mice approximately 6-months post-deletion, making them ~7-8 months of age. Mice were age-matched and compared to Tbk1fx/fix controls that underwent the same tamoxifen regimen; sex-matching was not performed.
*Tbk1*^NULL^ mice displayed a trend towards increased (p = 0.067) total white blood cells (WBCs)/leukocytes (Figure 12A) and a significant increase (p = 0.008) in neutrophils (Figure 12B); no changes were observed in red blood cells (RBCs), hemoglobin (Hb), platelets (Plts) (Figure 12A) or other leukocytes (Figure 12B). These data indicate that *Tbk1* negatively regulates the production of neutrophils and seems to, overall, restrain WBC production. In contrast to Marchlik, *et al.*, no differences were observed in circulating monocytes (p = 0.136), however, differences in mouse background (129S5/B6) and *Tbk1*-deletion strategy (germline/tamoxifen-inducible) confound comparisons between our model and Marchlik, *et al.*’s [127].
Given the alterations observed in leukocyte counts, it was hypothesized that there may be an increase in the mass of primary (thymus) and/or secondary (spleen) lymphoid tissue in Tbk1^NULL mice. Spleen and thymus herein were isolated from Tbk1^NULL mice approximately 6-months post-deletion, making them ~7-8 months of age. Mice were age-matched and compared to Tbk1^fx/fx controls that underwent the same tamoxifen regimen; sex-matching was not performed. No significant differences in

\textbf{Tbk1^NULL Mice Do Not Display Significant Changes in Spleen nor Thymus Mass}

Figure 12. Tbk1^NULL Mice Display Increased Circulating Neutrophils. To determine if Tbk1 deletion affects hematologic parameters, PB from mice 6-months-post-deletion was analyzed using HemaVet\textsuperscript{®} hematology analyzer. The data were quantified and unpaired t-test used for all statistical analyses (n = 3 Tbk1^WT, 4 Tbk1^NULL). (A) WBCs (p = 0.067), RBCs, Hb, and Plts. (B) WBC differential: monocytes, neutrophils (p = 0.008), eosinophils, basophils, and lymphocytes.
spleen or thymus mass were observed, but a trend towards increased ($p = 0.099$) spleen mass was noted (Figure 13) Regardless, these data suggest that the loss of $Tbk1$ does not significantly affect the mass of primary or secondary lymphoid tissue.

![Figure 13. Tbk1\textsuperscript{NULL} Mice Do Not Display Significant Changes in Spleen nor Thymus Mass. To determine if $Tbk1$ deletion affects the size of secondary (spleen) and primary (thymus) lymphoid tissue, organs from mice 6-months-post-deletion were harvested and weighed. The masses were quantified and unpaired t-test used for both statistical analyses ($n = 3 \quad Tbk1^{WT}$, 4 $Tbk1^{NULL}$). Significant differences were not observed in spleen nor thymus mass between mice.](image)

$Tbk1^{NULL}$ Mice Display Histologic Abnormalities in the Spleen and Kidney

To further characterize the phenotype of $Tbk1^{NULL}$ mice, histologic analysis was performed on several tissues. In addition, while tissues were harvested and prepped from many mice, the images shown have been selected to account for sex-matching
(male), age-matching (~8 months old), and post-tamoxifen regimen-matching (~6 months post-injection) mice (n = 1).

As the hematopoietic phenotype of \textit{Tbk1}^{\text{NULL}} mice is of primary interest, thymus and spleen were analyzed, as well as the liver due to its propensity to reflect hematologic abnormalities such as hemolytic anemia, disseminated intravascular coagulation (DIC), and neoplasms, including AML [349]. Kidney tissue was analyzed additionally, following Jin, \textit{et al.}'s observation of an IgA nephropathy-like pathology in mice with \textit{Tbk1}^{\text{NULL}} B cells [30]. Of note, kidneys were isolated from a different pair of 8-month-old, male mice that were ~4 months-post-tamoxifen. The Department of Pathology & Laboratory Medicine at Loyola University Medical Center (LUMC) performed tissue embedding/cutting/staining and assisted with analysis.

The thymus isolated from a \textit{Tbk1}^{\text{NULL}} mouse appeared largely normal (\textbf{Figure 14B}), although an increased amount of red blood cells and possible dendritic cells were noted compared to the thymus of the control mouse (\textbf{Figure 14A}). However, these findings could be benign and thus cannot yet be ruled as pathologic.
Figure 14. Thymus of Tbk1\textsuperscript{NULL} Mouse Appears Grossly Normal. To determine if \textit{Tbk1} deletion affects thymic architecture, histologic analysis was performed. Thymi were isolated from mice, H&E-stained, and morphology was inspected. Yellow arrows indicate possible dendritic cells. (A) \textit{Tbk1}\textsuperscript{WT} mouse (n = 1). (B) \textit{Tbk1}\textsuperscript{NULL} mouse (n = 1). Objective magnification is indicated with scale bars.
The spleen isolated from a $Tbk1^{\text{NULL}}$ mouse (Figure 15B) appeared to have poorly developed and less abundant germinal centers compared to $Tbk1^{\text{WT}}$ mice (Figure 15A). However, the disrupted architecture may be due to aggressive harvesting/preparation of spleen tissue. While it is the opinion of the author that the altered spleen morphology is due to $Tbk1$ deletion, this requires further validation.

Figure 15. Spleen of $Tbk1^{\text{NULL}}$ Mouse Appears to Have Maldeveloped Germinal Centers. To determine if $Tbk1$ deletion affects splenic architecture, histologic analysis was performed. Spleens were isolated from mice, H&E-stained, and morphology was inspected. (A) $Tbk1^{\text{WT}}$ mouse (n = 1). (B) $Tbk1^{\text{NULL}}$ mouse (n = 1). Objective magnification is indicated with scale bars.
As loss of B cell-intrinsic \textit{Tbk1} leads to uncontrolled IgA production and nephropathy in the mouse, kidney tissue from a \textit{Tbk1}^{\text{NULL}} mouse was examined. While immunohistochemistry (IHC) or immunofluorescence (IF) is required to confirm IgA deposition, periodic acid-Schiff (PAS) staining can be used to detect proliferation of mesangial cells in the glomerulus (mesangial hypercellularity). While not specific to IgA nephropathy, mesangial hypercellularity can occur in response to IgA deposition and can thus provide a basis for pursuing IHC/IF [350, 351]. As such, anti-IgA IHC/IF was withheld pending the PAS staining results.

PAS staining revealed the kidneys to be grossly normal. Though, in consultation with LUMC, it was noted that there appears to be a slight increase in the number of mesangial cells of \textit{Tbk1}^{\text{NULL}} mice. However, the increase is subtle and has not been quantified, and did not constitute mesangial hypercellularity. However, these data mandate further investigation to assess IgA deposition in the kidneys of \textit{Tbk1}^{\text{NULL}} mice. (Figure 16B).
It appeared that the livers of Tbk1\textsuperscript{NULL} mice contained a significant number of mononuclear infiltrates, particularly around the vasculature; however, some control mice also displayed similar infiltration, which may be a result of the tamoxifen regimen (Figure 17). However, it was observed Tbk1\textsuperscript{NULL} mice seemed to have more infiltrates, though these were not quantified. Additionally, 3 Tbk1\textsuperscript{NULL} mice displayed hemosiderin-
containing "pigment-laden" macrophages in the liver [352]. However, sufficient samples were not available to account for sex- and age-matching, so a conclusion cannot be made. Further examination of the Tbk1\textsuperscript{NULL} liver is recommended.

Figure 17. Liver of Tbk1\textsuperscript{NULL} Mouse may have Mononuclear Cell Infiltration. To determine if Tbk1 deletion affects liver architecture, histologic analysis was performed. Livers were isolated from mice, H&E-stained, and morphology was inspected, which revealed mononuclear cell infiltrates in both groups, with a possible increase in Tbk1\textsuperscript{NULL} mice, though this requires quantification. Yellow arrow indicates suspected pigment-laden macrophage. (A) Tbk1\textsuperscript{WT} mouse (n = 1). (B) Tbk1\textsuperscript{NULL} mouse, (n = 1). Objective magnification is indicated with scale bars.
Results: Competitive Transplantation of \( Tbk1^{\text{NULL}} \) Whole Bone Marrow

Loss of \( Tbk1 \) in Whole Bone Marrow Does Not Affect Engraftment Ability of HSPCs

The physiology and fate of cells are governed by both cell-*intrinsic* and *extrinsic* factors. For example, in the BM, the self-renewal capacity of HSPCs is largely maintained by sustained activation of c-KIT, a receptor tyrosine kinase found on the surface of HSPCs [353]. BM stromal cells express transmembrane and soluble c-KIT ligand (stem cell factor [SCF], KIT-ligand [KL], and steel factor) to nourish and maintain the stemness of local HSPCs. Without continuous the activation of c-KIT, which typically occurs via direct contact with stromal cells, HSPCs lose their self-renewal and hematopoietic abilities [354].

Thus, the BM provides an excellent example of how cell-intrinsic (c-KIT on HSPCs) and cell-extrinsic (SCF on stromal cells) factors regulate physiology. Similarly, TBK1 can act to promote cancer in tumor cell-intrinsic and -extrinsic roles. For example, TBK1 acts within the KRAS\(^{G12}\)-mutated NSCLC cell to activate cell survival pathways. Additionally, TBK1 can act within TME-infiltrating T cells to negate the production of antitumor cytokines, acting to promote cancer in a tumor cell-extrinsic role.

To determine if \( Tbk1 \) has BM-intrinsic functions, whole BM (WBM) transplantation (WBMT) was employed. Initially, two WBMT experiments were planned: a non-competitive (replacement with donor \( Tbk1^{\text{NULL}} \) WBM) and competitive (replacement with
WB M consisting of half $Tbk1^{\text{WT}}$ donor WBM and half wild-type support WBM. Unfortunately, too many mice were lost to busulfan ($Busulfex^\text{®}$) conditioning prior to transplant, so only competitive transplantation could be performed ($n = 11$ [$Tbk1^{\text{WT}} = 5$ & $Tbk1^{\text{NULL}} = 6$]).

To this end, $Tbk1^{\text{WT}}$ and $Tbk1^{\text{NULL}}$ WBM were isolated from CD45.2$^+$ (donor $[Ptprc^{b}]$) mice. The isolated WBM were then mixed in an approximate 1/1 ratio ($1.0 \times 10^6$ WBM cells total [$5.0 \times 10^5$ of each]) with WBM isolated from a wild-type, CD45.1$^+$ (support $[Ptprc^{a}]$) mouse (Figure 18). Cell mixtures were then delivered in 200uL 1X PBS to lethally-conditioned, CD45.1$^+$ recipient (host) mice and FACS analysis was performed on PB (cheek bleed) at 2 and 5 months post-WBMT. Mice were sacrificed at 8 months post-WBMT, and PB and BM was analyzed. Mice were age-matched (~6 weeks old) and sex-matched (male); mice received WBM cells in an approximate 1/1 combination of either [$Tbk1^{\text{WT}}$ donor + WT support] OR [$Tbk1^{\text{NULL}}$ donor + WT support].
To determine if \textit{Tbk1} regulates the engraftment of transplanted WBM cells, the amount of donor (CD45.2$^+$ [CD45.1$^-$]) cells in the PB was analyzed via FACS as percentage of PB-TNCs (Figure 19). Differences were not observed in the level of circulating donor cells, which suggests that \textit{Tbk1} does not influence the engraftment ability of transplanted WBM. Representative FACS gating strategy is shown (Figure 20).

Figure 18. Schematic Depicting Competitive WBMT Protocol using Tbk1$^\text{NULL}$ WBM and Downstream Analyses. To determine if \textit{Tbk1} regulates the engraftment of transplanted WBM cells and if \textit{Tbk1} has WBM-intrinsic functions, competitive WBMT was performed (n = 5 \textit{Tbk1}WT, 6 \textit{Tbk1}NULL); created with BioRender.com.
Figure 19. Loss of Tbk1 in WBM Does Not Influence Engraftment of HSPCs in Competitive Transplantation. To determine if Tbk1 governs the engraftment ability of transplanted HSPCs, the percentage of donor cells (CD45.2⁺ [CD45.1⁻]) in the PB was checked via FACS at 2, 5, and 8 months-post-WBMT. The data were quantified and unpaired t-test used for all statistical analyses (between Tbk1WT [n = 5] and Tbk1NULL [n = 6] mice at each timepoint). Percentage of donor (CD45.2⁺ [CD45.1⁻]) cells of PB-TNCs. NOTE: as CD45.1⁺ support cells were used in WBMT, CD45.2⁺ cells cannot display 100% engraftment (i.e., remaining 30-40% of PB-TNCs are CD45.1⁺).
Loss of Tbk1 in Whole Bone Marrow Does Not Affect Lymphopoiesis

To determine if Tbk1 has lineage-specific effects, lymphocytes were measured similarly. The loss of Tbk1 has no effect on the reconstitution of circulating B (CD45.2+B220+; Figure 21A) and T cells (CD45.2+CD3+; Figure 21B), which suggests that WBM-intrinsic Tbk1 does not regulate the production of circulating lymphocytes. Representative FACS gating strategy is shown (Figure 20).
Loss of Tbk1 in WBM Transiently Hampers Granulopoiesis but Not Monopoiesis

To determine if Tbk1 has lineage-specific effects, myeloid cells were measured similarly. The loss of Tbk1 has no effect on the reconstitution of circulating monocytes (CD45.2+CD11b+Gr1-; Figure 22A), but temporarily impedes granulopoiesis (CD45.2+Gr1+; Figure 22B). Specifically, while both groups of mice displayed a transient decrease in granulocytes between 2 and 5 months post-WBMT, Tbk1\textsuperscript{NULL} mice displayed a more significant decrease (p = 0.038) at 5 months. Although, by 8 months post-WBMT, all mice displayed comparable recovery of granulocyte counts following WBM transplant. These data indicate that while the loss of Tbk1 in WBM can delay...
count recovery of granulocytes, this temporary delay resolves with time. Representative FACS gating strategy is shown (Figure 20).

Figure 22. Loss of Tbk1 in WBM Transiently Hampers Granulopoiesis but Not Monopoiesis in Competitive Transplantation. To determine if Tbk1 governs the ability of transplanted WBM to produce myeloid cells, the percentage of donor granulocytes (CD45.2⁺Gr1⁺) and monocytes (CD45.2⁺CD11b⁺Gr1⁻) cells in the PB was checked via FACS at 2, 5, and 8 months-post-WBMT. The data were quantified and unpaired t-test used for all statistical analyses (between Tbk1WT [n = 5] and Tbk1NULL [n = 6] mice at each timepoint). (A) Granulocytes (p = 0.038 at 5 months post-WBMT) displayed a significant but transient delay in reconstitution in Tbk1NULL WBMT. (B) Monocytes.

Loss of Tbk1 in Whole Bone Marrow Does Not Affect Recovery of Mature Blood Cells nor Erythroid Progenitors

It was not known how the loss of Tbk1 in WBM affects the BM itself. To this end, BM was harvested and analyzed via FACS; relevant populations measured as percentage of BM-TNCs. In terms of lymphocytes, myeloid cells, erythroid progenitors,
and megakaryocytes in the BM, the loss of Tbk1 in WBM had no effect on these populations (Figure 23A). Representative FACS gating strategy is shown (Figure 23B).

Figure 23. Loss of Tbk1 in WBM Does Not Affect Reconstitution of Mature Cells nor Erythroid Progenitors in the Bone Marrow. To determine if the loss of Tbk1 affects homeostatic hematopoiesis, the levels of mature cells in the PB and BM, as well as erythroid progenitors and megakaryocytes in the BM, were analyzed via FACS at 8-months-post-WBMT. The data were quantified and unpaired t-test used for all statistical analyses (n = 5 Tbk1WT, 6 Tbk1NULL mice). (A) Donor cells as percentage of BM-TNCs. (B) Representative gating strategy for FACS analysis. After gating on TNCs and eliminating doublets (SC gating), donor B cells (CD45.2+B220+), donor T cells (CD45.2+CD3+), donor monocytes (CD45.2+CD11b+Gr1+), and donor granulocytes (CD45.2+Gr1+) were analyzed.

Loss of Tbk1 in Whole Bone Marrow Does Not Significantly Affect HSPCs

HSPCs (Figure 24A-B) were also analyzed as percentage of BM-TNCs, and representative FACS gating strategies are shown (Figures 23C). Differences in HSPC populations were not significantly different (CD45.2/CD45.1 sorting was not available for
this analysis). While a trend may exist towards increased HSPCs in $Tbk1^{NULL}$ mice, any difference herein was not significant.

![Graph and images showing data analysis and gating strategies](image)

**Figure 24.** $Tbk1$ Deletion in WBM Does Not Significantly Affect the Reconstitution of HSPCs. To determine if the loss of $Tbk1$ affects HSPCs, the levels of HSPCs in the BM were analyzed via FACS at 8-months-post-WBMT. The data were quantified and unpaired t-test used for all statistical analyses ($n = 5$ $Tbk1^{WT}$, 6 $Tbk1^{NULL}$). (A) HSPC compartment (LKs + LSKs). (B) MPP4/LMPP and Flt3$^-$ LSKs (HSC and MPP1-MPP3). (C) Representative gating strategy for FACS analysis. After gating on TNCs and eliminating doublets (SC gating), the HSC and MPP1-MPP4 subsets were analyzed.

**Loss of $Tbk1$ in Whole Bone Marrow Does Not Significantly Affect Specific Myeloid Progenitors**

Additional myeloid progenitors were also analyzed as percentage of BM-TNCs, and representative FACS gating strategies are shown (**Figures 25D**). It was determined
that, similar to global deletion, the loss of *Tbk1* in WBM did not impart significant changes in any of the assayed myeloid progenitors (GMP, DP, CMP, MkP [Lin–c-Kit+CD41+CD150*], CFU-E, Pre-CFU-E, Pre-MegE, and Pre-MEP; Figure 25A-25C). However, a trend towards increased Pre-CFU-E (CD41+CD16/32-CD27- CD150*Endoglin*) and Pre-MegE (CD41+CD16/32-CD27-CD150*Endoglin*) HPCs was observed in *Tbk1*null mice (Figure 25C). Despite not reaching significance, the trend observed herein indicates the WBM-intrinsic role of *Tbk1* in regulating myeloid progenitor populations warrants further investigation. CD45.2/CD45.1 sorting was not available for this analysis.
Loss of \textit{Tbk1} in Whole Bone Marrow Causes Increased Monocytes

To determine if the loss of \textit{Tbk1} in WBM causes hematologic abnormalities, PB was isolated via cardiac puncture (post-mortem) and analyzed using \textit{HemaVet$^\text{TM}$}. At 8 months post-WBMT, \textit{Tbk1$^{\text{NULL}}$} recipients displayed a trend towards increased ($p = 0.051$) total WBCs, lymphocytes ($p = 0.062$), and neutrophils ($p = 0.097$) (\textbf{Figure 26A}), and a significant increase ($p = 0.021$) in monocytes (\textbf{Figure 26B}); no changes were observed in red blood cells (RBCs), hemoglobin (Hb), platelets (Plts) (\textbf{Figure 26A}) or
neutrophils, eosinophils, basophils, nor lymphocytes. Spleen and thymus mass were not analyzed.

**Figure 26.** Loss of Tbk1 in WBM Causes Increased Monocytes and a Trend Towards Increased Lymphocytes and WBCs. To determine if Tbk1 deletion in WBM affects hematologic parameters, PB from mice 8-months-post-WBMT was analyzed using *HemaVet®* hematology analyzer. The data were quantified and unpaired t-test used for all statistical analyses (n = 3 Tbk1 WT, 6 Tbk1 NULL). (A) WBCs (p = 0.051), RBCs, Hb, and Plts. (B) WBC differential: monocytes (p = 0.021), neutrophils (p = 0.097), eosinophils, basophils, and lymphocytes (p = 0.062).

**Tbk1 Deletion is Revealed to be Incomplete at 4 and 7 Months-post-tamoxifen**

The deletion of Tbk1 was previously confirmed in a 6-week-old mouse, 12 days after the tamoxifen regimen; PCR was used to confirm a complete deletion of Tbk1 in c-Kit⁺ and c-Kit⁻ BM cells (**Figure 7B**). As the BM gives rise to the entire hematopoietic system, it was presumed that complete deletion of Tbk1 in the BM was sufficient for our
purposes. Thus, PCR to confirm deletion in subsequent mice was not routine, and mice were presumed to be $Tbk1^{\text{null}}$ following results from the “test” mouse.

However, upon re-evaluation, it was decided that PCR should be used to confirm $Tbk1$ status of all analyzed mice. Much to our surprise, PCR indicated that the deletion of $Tbk1$ was not complete in hematopoietic tissues and the tails of mice that had undergone the tamoxifen regimen both 4 (Figure 27A) and 7 (data not shown) months prior. $Tbk1^{\text{fx}}$ alleles were clearly evident in the spleen (S), thymus (Thy), and PB of two age-matched (+/- 2 weeks) $Tbk1^{\text{fx/fx};\text{Rosa26-CreERT2+}}$ mice that underwent the same tamoxifen regimen as the initial “test” mouse. More surprising is the fact that the WBM of both sampled mice still displayed a near-complete deletion of $Tbk1$, while the PB displayed a clearly incomplete deletion; as the BM produces cells that move directly to the PB, this result cannot be explained at this time. It may be that either the hematopoietic system had not yet fully turned over, that a small fraction of $Tbk1$-retaining HSPCs (“escapers”) were able to repopulate sufficiently, and/or that other cell types, such as epithelia, are responsible for the observed heterozygosity. False positivity was ruled-out, however, as the negative control resulted as expected. It is not known whether the cells harboring the $Tbk1^{\text{fx}}$ allele are $Tbk1^{\text{null/fx}}$ or $Tbk1^{\text{fx/fx}}$.

The deletion was initially assessed only in the BM of a 6-week-old male mouse (Figure 7B). As such, the deletion was re-assessed using a second “test” mouse (12-week-old female), this time with S, Thy, PB, WBM, and tail included in PCR; BM was not separated based on c-Kit status. Importantly, the only mouse available was a 12-week-old female, in contrast to the 6-week-old male used in the initial test. Tissues were
harvested 11 days post-tamoxifen regimen, PCR was performed, and it was learned that while deletion in the thymus appeared complete, $Tbk1^{lx}$ alleles were detected in tail, WBM, PB, and S (Figure 27B). Non-specific amplification (false positive) of the PCR results was ruled-out, as the negative control (ddH$_2$O) resulted as expected. Interestingly, the deletion efficiency observed in the PB herein was comparable to that of the WBM (Figure 27B), in stark contrast to the deletion in PB in the mice 4-months post-tamoxifen regimen (Figure 27A). These data are perplexing and cannot be explained at this time, aside from supporting the notion that $Tbk1$ confers a survival advantage to mouse cells.
Should mice need to be injected past 8 weeks of age, intensification of the regimen is recommended. As well, it is not known if sex affects the tamoxifen-mediated induction of CreER\textsuperscript{T2}. A larger dosage and/or longer duration of tamoxifen may be necessary, especially as mice increase in age and mass. Yu, et al. employed the Rosa26-CreER system and induced deletion with 2mg IP tamoxifen for 4 consecutive days [152]. However, the authors only provided evidence that the deletion was relatively complete in thymic T cells 2 weeks after injection, and—to our knowledge—did not specify the mass nor age of the injected mice.

**Aim 1 Conclusion:** *Tbk1* is Dispensable in Short-term, Homeostatic Hematopoiesis

The genetic loss of *Tbk1* in the adult mouse does not lead to overt, untoward effects on the hematopoietic system nor animal overall—*no mice died from anything other than suspected tamoxifen toxicity*, which was deemed cause-of-death for mice that died during the regimen or within 7 days following the last dose and do not display
a clear alternative cause (mice that die from tamoxifen toxicity often display yellow-pale livers and/or intestinal hemorrhage [unpublished observations, Zhang lab]). While \textit{Tbk1} restrains leukocyte production and has roles in regulating the MPP3 population in the BM, \textit{Tbk1} regulates the production of mature blood cells, including platelets and erythrocytes, only minimally, at least in a short-term timeframe (3-6 months in B6 mice). However, as our mice are housed in specific pathogen-free (SPF) facilities, and as we did not challenge them with pathogen or vaccine, the effect of global \textit{Tbk1}-knockout on the immune system is unknown and requires exploration. While the loss of \textit{Tbk1} may cause a differentiation block, causing the accumulation of HSPCs at the MPP3 stage, hematologic abnormalities suggestive of blocked differentiation (\textit{e.g.}, anemia, thrombocytopenia, leukopenia, ETC.) were not observed. However, these abnormalities may only become apparent after more time (6+ months) has elapsed following \textit{Tbk1} deletion. Nonetheless, in terms of life-sustaining (homeostatic) hematopoiesis, \textit{Tbk1} appears to be dispensable for at least 6 months, suggesting short-term blockade of \textit{Tbk1} is safe. Although, the long-term effect of \textit{Tbk1} deletion on hematopoiesis, immunity, and homeostasis overall requires investigation.
AIM 2: DETERMINE IF TBK1 BLOCKADE COULD BE A MEANS OF ANTI-AML THERAPY

Results

MLL-AF9 Induces Increased Expression of Tbk1 mRNA

As it was determined that Tbk1 minimally regulates homeostatic hematopoiesis in the adult mouse, we sought to determine if Tbk1 blockade may be a useful strategy in the treatment of AML. To this end, we elected to study Tbk1 in MLL-AF9+ cells, as this is an aggressive, clinically relevant model of AML. The MLL-AF9 oncogene drives high-risk leukemias in humans, specifically in pediatric patients and cases of t-AML, and aggressive disease in mouse models [247, 270, 271, 274-276]. Our lab has previously generated an MLL1-AF9-containing plasmid using the MSCV-puro backbone (MSCV-puro-MLL-AF9). Transfection of 293T cells and transduction of mouse HSPCs is depicted (Figure 28)
Forty-eight hours after transduction, to allow for production of the puromycin N-acetyltransferase (pac), cells were treated with puromycin to select for \textit{MSCV-puro-MLL-AF9}+ cells. Following puromycin selection, RNA was isolated from \textit{MLL-AF9}+ HSPCs. Then, all isolated RNA (wild-type \textit{WTc-Kit}+ and \textit{MLL-AF9}+ HSPCs) was reverse-transcribed into cDNA and subjected to multiplex, quantitative PCR (qPCR). This entire procedure was performed twice, separately (n =2 [biological duplicate]).
While this study was not sufficiently powered, it appears that \textit{MLL-AF9}\textsuperscript{+} mouse HSPCs express greater levels of \textit{Tbk1} mRNA (Figure 29). While these data may indicate that \textit{MLL-AF9}\textsuperscript{+} HSPCs rely on \textit{Tbk1} more than wild-type counterparts, ideally making \textit{MLL-AF9}\textsuperscript{+} cells more sensitive to \textit{Tbk1} inhibition, the increased \textit{Tbk1} mRNA may simply be an artifact of the faster cycling rate of leukemic cells.

\textit{Tbk1} May Confer a Survival Advantage to \textit{MLL-AF9}\textsuperscript{+} HSPCs

To further study \textit{Tbk1} in \textit{MLL-AF9}\textsuperscript{+} cells, \textit{Tbk1}\textsuperscript{NULL} \textit{MLL-AF9}\textsuperscript{+} cells were desired. To this end, \textit{Tbk1}\textsuperscript{fx/fx};\textit{Rosa26-CreER}\textsuperscript{T2+} \textit{MLL-AF9}\textsuperscript{+} cells were treated with 200nM 4-
OHT, to induce deletion of *Tbk1* in vitro. However, at least in this protocol, it was learned thereafter that 200nM 4-OHT is insufficient to induce complete deletion of *Tbk1*. Specifically, if CreER\textsuperscript{T2} is incompletely induced, escapers are enriched for via plating in *MethoCult\textsuperscript{TM} M3434 methylcellulose* (M3434) (**Figure 30**). Single-colony PCR would be required to determine whether escapers are *Tbk1\textsuperscript{NULL/fx}* or *Tbk1\textsuperscript{fx/fx}*. It should be addressed that two bands are visualized in the PCR positive control shown in **Figure 30**—this is due to an untreated, floxed-heterozygous (*Tbk1\textsuperscript{+/fx}* gDNA template being used. As the *Tbk1\textsuperscript{+}* and *Tbk1\textsuperscript{NULL}* bands differ by only 9bp (332bp and 323bp, respectively), untreated *Tbk1\textsuperscript{+/fx}* gDNA can easily be mistaken for *Tbk1\textsuperscript{NULL/fx}* gDNA.

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**Figure 30.** *Tbk1*-retaining Cells are Enriched for in M3434 Culture. Results from 3% agarose gel electrophoresis/*Tbk1* PCR. Lexicon primers #18, #23, and #24 were used to determine *Tbk1* status. gDNA templates from *Tbk1\textsuperscript{fx/fx};Rosa26-CreERT2+ MLL-AF9* cells treated with 200nM 4-OHT or vehicle in 1\textsuperscript{st} M3434 and washed into liquid media after 6 days (Vehicle & 4-OHT). Following re-plating in M3434 without 4-OHT or vehicle (2\textsuperscript{nd} M3434; pVehicle & p4-OHT) and re-sampling of DNA, PCR indicates re-emergence of the *Tbk1\textsuperscript{fx}* allele. Untreated *Tbk1\textsuperscript{+/fx}* gDNA (positive) and ddH\textsubscript{2}O (negative) controls are shown. Vertical spacing between lanes indicates image cropping.
Following recommendations from other investigators, 3,000 \( Tbk1^{fx/fx};Rosa26\text{-}CreER^{T2+} MLL\text{-}AF9^{+} \) cells were plated in M3434 media (1\(^{st}\) M3434) for colony forming unit (CFU) analysis with either 200nM 4-OHT (4-OHT) or EtOH-PBS (vehicle) (Figure 31A). After 6 days, cells were washed and transferred to liquid media for 48 hours, allowing for \textit{in vitro} expansion and DNA sampling for \( Tbk1 \) PCR; subsequent PCR suggested the deletion of \( Tbk1 \) was virtually complete (Figure 31B).

After 48 hours in liquid media, 3,000 cells were isolated from each liquid culture and plated again in M3434 (2\(^{nd}\) M3434), without the addition of 4-OHT (p4-OHT) or vehicle (pVehicle) (Figure 31D). After 6 days, cells were washed and transferred to liquid media for 48 hours, to allow for similar \textit{in vitro} expansion and DNA sampling for \( Tbk1 \) PCR. Herein, subsequent PCR herein indicated a re-emergence of the \( Tbk1^{fx} \) allele in the cells previously believed to be \( Tbk1^{NULL} \) (Figures 31E). Considering this finding, DNA was re-sampled from the cells that had been washed from the 1\(^{st}\) M3434 which, at this point, had been maintained in liquid media for 2-3 weeks; subsequent \( Tbk1 \) PCR indicated that these cells now displayed a similar re-emergence of the \( Tbk1^{fx} \) allele (Figure 31C).

Notably, in cells that had been maintained in liquid media, the \( Tbk1^{fx} \) banding was of significantly lesser intensity compared to the \( Tbk1^{NULL} \) banding (Figure 31C); this contrasted with the banding intensities observed in cells following the 2\(^{nd}\) M3434 (Figure 31E). This indicates that M3434 exerts a stronger selection pressure than liquid media in favor of \( Tbk1 \)-retaining cells. As M3434 more strongly selects for LSCs than liquid media, wherein most \( MLL\text{-}AF9^{+} \) cells are blasts, we believe these data suggest
that *Tbk1* is more critical in *MLL-AF9*+ LSCs compared to blast counterparts [355]. Deletion of *Tbk1* would be expected to most significantly afflict the cells that *Tbk1* is most required by, which we believe are *MLL-AF9*+ LSCs.

Figure 31. *Tbk1*<sup>fx</sup> Allele Reappears in *MLL-AF9* Cells Previously Determined to be *Tbk1*<sup>NULL</sup>. Schematic depicting initial attempt to delete *Tbk1* *in vitro*; created with BioRender.com. Lexicon primers #18, #23, and #24 were used to determine *Tbk1* status in all PCRs. Untreated *Tbk1*<sup>+/fx</sup> gDNA (positive) and ddH<sub>2</sub>O (negative) controls are shown. Vertical spacing between lanes indicates image cropping. (A) 3,000 *Tbk1*<sup>fx/fx</sup>; Rosa26-CreER<sup>T2+</sup> *MLL-AF9* cells are plated in M3434 with either 200nM 4-OHT or vehicle. (B) DNA sampled from cells washed into liquid media from initial M3434 plating and cultured for 48 hours; results from 3% agarose gel electrophoresis/ *Tbk1* PCR. (C) Cells from initial M3434 plating were cultured for 2-3 weeks in liquid media and PCR was performed again; results from 3% agarose gel electrophoresis/ *Tbk1* PCR. (D) After 48-hour culturing in liquid media, cells were plated in a 2<sup>nd</sup> M3434 assay without added 4-OHT or vehicle, then transferred to liquid media similarly after 6 days. DNA was sampled for PCR after cells had been in liquid media for 48 hours. (E) Results from 3% agarose gel electrophoresis/ *Tbk1* PCR.
These data indicate that if induction of CreER<sup>T2</sup> is incomplete, Tbk<sup>1fx</sup> escapers will re-emerge after culturing in M3434 or liquid media; thus, the in vitro deletion protocol requires optimization. Moreover, these data suggest that Tbk1 confers a survival advantage to MLL-AF9<sup>+</sup> cells, although the mechanism is not yet known.

**In Vitro Treatment with 1uM 4-OHT for 4 Days Yields Complete Deletion of Tbk1**

It was determined that 200nM 4-OHT was insufficient to induce complete deletion of Tbk1 in MLL-AF9<sup>+</sup> cells. To this end, the in vitro deletion protocol was intensified, with both the dosage of 4-OHT and duration of treatment escalated.

*<sup>Tbk1</sup>fx/fx;Rosa26-CreER<sup>T2+</sup> MLL-AF9<sup>+</sup>* and *Tbk1<sup>fx/fx</sup> MLL-AF9<sup>+</sup>* control cells were treated with 1uM 4-OHT in liquid media for 4 days. After 48 hours, cells were passaged, media was swapped, and 4-OHT re-added again for a final concentration of 1uM. Following 4 days of 1uM 4-OHT treatment, cells were transferred to fresh, liquid media (without 4-OHT) and DNA was sampled 48 hours later for Tbk1 PCR. Similar to the initial deletion attempt, PCR herein suggested the deletion was also complete.

However, to confirm that Tbk1 was completely deleted, two, serial M3434 platings were conducted; DNA was sampled for Tbk1 PCR after cells were transferred to liquid media following the 2<sup>nd</sup> plating. PCR subsequently confirmed that Tbk1 was completely deleted with the intensified regimen (**Figure 32A and 32B**). Notably, serial M3434 platings were performed twice, in independent experiments, confirming the intensified regimen induces complete deletion of Tbk1. Furthermore, western blot, performed by Allan Youmaran, BS of our group, confirms the loss of total Tbk1 protein in *Tbk1<sup>NULL</sup> MLL-AF9<sup>+</sup>* cells and (**Figure 32B**).
Based on Morphology, \textit{Tbk1}^{\text{NULL}} \textit{MLL-AF9}^{+} \text{HSPCs} \text{Seem to be More Differentiated}

Cell morphology is used to aid in the diagnosis and classification of AML, especially in cases of AML, NOS [239, 256, 263]. Lobularity and size of the nucleus are strong indicators of differentiation in normal and malignant myeloid cells: cells with with

![](image)

Figure 32. Treatment with 1\mu M 4-OHT for 4 Days in Liquid Media Yields Complete Deletion of Tbk1. To determine if the intensified 4-OHT regimen could induce complete deletion of \textit{Tbk1}, PCR and western blot were used to confirm deletion following 2 consecutive M3434 assays. (A) Results from 3\% agarose gel electrophoresis/\textit{Tbk1} PCR. gDNA templates are from \textit{Tbk1}^{\text{fx/fx}} (Cre\text{ERT2}^\text{-} \text{control cells}) and \textit{Tbk1}^{\text{fx/fx}};\text{Rosa26-Cre\text{ERT2}^\text{+} (Cre\text{ERT2}^\text{+} \text{cells})} \textit{MLL-AF9}^{+} \text{cells} treated with 1\mu M 4-OHT for 4 days (ran in duplicate). Left-most lane contains 1kB DNA ladder, Lexicon primers \#18, \#23, and \#24 were used to determine \textit{Tbk1} status, untreated \textit{Tbk1}^{\text{fx/fx}} gDNA (positive) and ddH\text{2O} (negative) controls are shown. Image is representative of two, independent experiments. (B) Western blot depicting total \textit{Tbk1} protein expression in \textit{Tbk1}^{\text{NULL}} \textit{MLL-AF9}^{+} \text{cells} and \textit{Tbk1}^{\text{WT}} counterparts, histone H3 used as loading control; performed by Allan Youmaran, BS of our group. Horizontal spacing between lanes indicates image cropping.
larger, rounder nuclei are typically less differentiated than those with smaller, more lobular/kidney-shaped nuclei [356, 357]. Clinically, and not specific to AML, cancer cells that are less differentiated are almost invariably more aggressive than differentiated counterparts [358, 359]. As such, inducing the differentiation of AML cells is an extremely effective therapeutic strategy, such in cases of IDH1/2-mutated or PML-RARA+ AML [10, 13, 291]. However, while morphology can aid in the diagnosis of AML, morphology alone cannot provide an accurate diagnosis or determination of disease aggressiveness.

To determine if Tbk1 regulates cell morphology, and possibly differentiation, H&E staining was performed on Tbk1WT MLL-AF9+ (Figure 33A) and Tbk1NULL counterparts (Figure 33B) that had been cultured in liquid media.
As the AML liquid tumor is highly heterogeneous, cells will differ in their differentiation status, and differentiation must be considered generally, as opposed to an all-encompassing determination of all cells. With this, based on morphology alone, it appears that the \( \text{Tbk1}^{\text{NULL}} \) culture is generally more differentiated than the \( \text{Tbk1}^{\text{WT}} \) culture. The loss of \( \text{Tbk1} \) seems to increase the number of differentiated cells within the...
MLL-AF9+ culture, but this observation was not quantified as it was in a report by Somervaille, et al. [360]. Clinically, ~500 cells will be assessed and quantified, and the differentiation status of the disease will be determined as a percentage of differentiated cells/total cells assessed (per Jiwang Zhang, MD/PhD). While morphology can only be used to support more definitive measures, such as flow cytometry, it appears that Tbk1 regulates the morphology of MLL-AF9+ cells, which provides rationale for confirming if Tbk1 regulates these cells’ differentiation. Although, these observations in AML cells are consistent with a PDAC model wherein Tbk1^NULL tumor cells are more differentiated than Tbk1^WT counterparts [185].

**Tbk1^NULL MLL-AF9+ HSPCs Proliferate Faster**

Observations by lab personnel suggested that there is a difference in the growth rates of Tbk1^WT MLL-AF9+ cells and Tbk1^NULL counterparts. Specifically, Tbk1^NULL MLL-AF9+ cells seem to proliferate faster, thereby exhausting culture media more quickly than Tbk1^WT counterparts. To further interrogate these observations, growth rate/curve analysis was performed.

To this end, 10,000 cells of each type were seeded (4 replicates); cells were counted every 24 hours via trypan blue-exclusion. While there was initially no difference in the growth rate of the cells, Tbk1^NULL MLL-AF9+ cells show a significantly increased (p < 0.0001) growth rate at 96 hours post-seeding (Figure 34A). The raw cell count was log-transformed and plotted (Figure 34B) to further analyze the data, as it was surprising to see the difference in growth rate only apparent after several days.

However, based on observations and reports from other labs, it is estimated that only
15-25% of $MLL$-$AF9^+$ cells in bulk culture are LSCs, with the remaining being blast cells [360]. As $Tbk1^{\text{NULL}}$ $MLL$-$AF9^+$ cells differ in growth rates only after several days, this could suggest that $Tbk1$ regulates a less-abundant, LSC population of $MLL$-$AF9^+$ cell in culture, at least more so than it does the more-populous blast cells, though this interpretation requires further validation.

Following results of the growth curve analysis, cell cycle analysis was performed. To assess the cells in the conditions they were most often maintained in, cells were simply isolated from bulk, media-replete culture conditions (not seeded specifically for cell cycle analysis). Time and resources only allowed for a single analysis, so the results are interpreted cautiously ($n = 1$).

However, the results from cell cycle analysis (Dean-Jett-Fox algorithm; Figure 34C and 34D) support both our observations and the results of the growth curve analysis. Specifically, $Tbk1^{\text{NULL}}$ $MLL$-$AF9^+$ cells were more likely to exist in S or G2/M phase than $Tbk1^{\text{WT}}$ cells at any given time, suggesting an increase in cycling rate.
Figure 34. Tbk1\textsuperscript{NULL} MLL-AF9\textsuperscript{+} Cells Proliferate Faster than Tbk1\textsuperscript{WT} Counterparts. To quantify the observation that Tbk1 deletion caused MLL-AF9\textsuperscript{+} cells to grow faster, growth rate/curve analysis and cell cycle analysis (using PI) were performed. (A) 10,000 cells were plated (3 replicates) and counted daily via trypan blue-exclusion. Cultures were homogenized and half volume discarded, then fresh media + growth factor added at 48 and 72 hours to prevent cell exhaustion. Raw cell counts plotted as [total cells x 10\textsuperscript{4}]. Unpaired t-test used for all statistical analyses (between Tbk1\textsuperscript{WT} and Tbk1\textsuperscript{NULL} cells at each timepoint; p < 0.0001 at 96 hours-post-seeding). (B) Log transformation of [total cells x 10\textsuperscript{4}] from Figure 33A to assess the rate-of-change in cell growth. Unpaired t-test used for all statistical analyses (between Tbk1\textsuperscript{WT} and Tbk1\textsuperscript{NULL} cells at each timepoint; p = 0.040 and 0.004 at 72 and 96 hours-post-seeding, respectively). (C) Representative histograms of cell cycle analyses and use of Dean-Jett-Fox algorithm for analysis. (D) Quantification of FACS data (n = 1).
**Tbk**null **MLL-AF9**+ HSPCs Show a Trend Toward Reduced Clonogenicity

Given the alterations in cell morphology and cycle rate, we further sought to investigate the functional characteristics of Tbknull **MLL-AF9**+ cells. To this end, colony-forming unit (CFU)/clonogenic ability (clonogenicity) assays, an established surrogate measure for stemness and leukemogenicity, were performed [355].

In 2 independent experiments, cells were plated in M3434 for 7 days, manually counted and washed, then cultured in liquid media overnight and replated in M3434 again before manual counting again after 7 days. Results from the initial experiments cannot be interpreted accurately, as the positive control (Tbk1**WT** MLL-AF9+) did not result as expected (Figure 35). Specifically, MLL-AF9+ cells are expected to maintain clonogenicity over several platings; ours did not and, instead, decreased significantly (p = 0.0012 and 0.0043) in the second plating of each experiment, mostly invalidating the results. However, while both cell types had reduced clonogenicity in each 2nd plating, Tbknull cells seemed to form fewer colonies, though this requires confirmation with a positive control (Tbk1**WT** MLL-AF9+) that results correctly.
As such, subsequent experiments by Wei Wei of our group indicated that \( Tbk1^{\text{NULL}} \) MLL-AF9\(^+\) cells do show significantly decreased clonogenicity compared to \( Tbk1^{\text{WT}} \) counterparts; this suggests that \( Tbk1 \) does in fact promote the clonogenicity of MLL-AF9\(^+\) cells (data not shown).

Figure 35. \( Tbk1^{\text{NULL}} \) MLL-AF9\(^+\) Cells Show a Trend Toward Reduced Clonogenicity. To determine if \( Tbk1 \) influences the clonogenicity of \( MLL-AF9^+ \) cells, 3,000 \( MLL-AF9^+ \) cells either \( Tbk1^{\text{NULL}} \) or \( Tbk1^{\text{WT}} \) were isolated from liquid media, transferred to M3434, and aliquoted (\( n = 2 \), with 3 replicates each). Colonies were manually counted at 7-days-post-seeding and washed, then cultured overnight and replated similarly in a 2\(^{\text{nd}}\) CFU assay. Two-way ANOVA was used for each analysis (comparing means +/- standard deviation), as it was desired to know (1) how \( Tbk1^{\text{WT}} \) and \( Tbk1^{\text{NULL}} \) groups compared to each other at both timepoints (inter-group comparison) as well as (2) how the groups compared to themselves across both timepoints (intra-group comparison).
Tbk1\textsuperscript{NULL} MLL-AF9\textsuperscript{+} HSPCs are Sensitized to Serum Starvation

Serum starvation is a widely employed laboratory technique used to study autophagy, apoptosis, and to synchronize cells at G0/G1 [361]. As a type of nutrient stress, mammalian cells will activate autophagy/mitophagy and p27\textsuperscript{kip1}-quiescence in response to serum starvation [362]. As Tbk1 is a critical regulator of mitophagy, we sought to determine if Tbk1\textsuperscript{NULL} MLL-AF9\textsuperscript{+} cells showed any alterations in their sensitivity to serum starvation [363].

To this end, Tbk1\textsuperscript{NULL} MLL-AF9\textsuperscript{+} cells were transduced with the MSCV-YFP retrovirus and yellow fluorescent protein (YFP)-expressing (YFP\textsuperscript{+}) cells were selected for via FACS. Following selection, Tbk1\textsuperscript{NULL} MLL-AF9\textsuperscript{+} YFP\textsuperscript{+} and Tbk1\textsuperscript{WT} MLL-AF9\textsuperscript{+} cells were mixed in an approximate 1/1 ratio (which was ultimately ~70% Tbk1\textsuperscript{NULL}). 6.0 x 10\textsuperscript{4} cells were seeded (3 replicates) in RPMI-1640 with (control/serum-replete conditions [Serum\textsuperscript{+}]) or without 10% FBS (starved/serum-depleted conditions [Serum\textsuperscript{-}]). 24 hours later, YFP-positivity was checked via FACS and analyzed as percentage of TNCs. With this method, any change in the YFP\textsuperscript{+} percentage indicates a net effect on Tbk1\textsuperscript{NULL} cells.

It was observed that the percent of TNCs that were YFP\textsuperscript{+} decreased significantly (p < 0.0001) in serum-starved conditions compared to control, serum-replete conditions (Figure 36A). This finding suggests that Tbk1\textsuperscript{NULL} MLL-AF9\textsuperscript{+} cells are significantly more sensitive to this type of nutrient stress than Tbk1\textsuperscript{WT} counterparts. However, this analysis cannot determine whether the change in YFP-positivity was due to increased cell death,
decreased proliferation, or a combination of both in \( \text{Tbk1}^{\text{NULL}} \) \( \text{MLL-AF9}^+ \) cells. Further analysis of the data revealed that YFP-positivity did not significantly increase in control conditions (Figure 36B).

Figure 36. \( \text{Tbk1}^{\text{NULL}} \) \( \text{MLL-AF9}^+ \) Cells are Sensitized to 24-hour Serum Starvation. To determine if \( \text{Tbk1} \) regulates the survival of \( \text{MLL-AF9}^+ \) cells amid nutrient stress, serum starvation was performed on a co-culture of \( \text{Tbk1}^{\text{WT}} \) and \( \text{Tbk1}^{\text{NULL}} \) YFP+ cells (n = 1, with 3 replicates). The \( \text{Tbk1}^{\text{NULL}} \) percentage of the co-culture was assessed via FACS (as YFP-positivity) before and after 24-hour serum starvation. (A) YFP-positivity is significantly decreased (p < 0.0001) compared to serum-replete control conditions following 24-hour serum starvation; unpaired t-test used for statistical analysis. (B) YFP-positivity did not significantly increase over 24 hours in control conditions; two-way ANOVA used for statistical analysis (comparing means +/- standard deviation; inter- and intra-group comparisons). (C) Representative gating strategies for FACS analysis.

This finding indicates that without nutrient stress, \( \text{Tbk1}^{\text{WT}} \) \( \text{MLL-AF9}^+ \) cells do not outcompete \( \text{Tbk1}^{\text{NULL}} \) counterparts in liquid media within 24 hours; moreover, any survival deficit caused by the loss of \( \text{Tbk1} \) is exacerbated by nutrient stress. However, it
is still unknown how the loss of Tbk1 sensitizes MLL-AF9+ cells to serum starvation. Representative gating strategy is shown (Figure 36C); gating on TNC and SC is representative of that used in all FACS analyses of MLL-AF9+ cells.

**c-Kit*Flt3+ MLL-AF9+ HSPCs are Resistant to DNO but Not Ara-C.**

A 2013 study by our lab group demonstrated that mouse MLL-AF9+ LSCs (c-Kit*CD11blo [least differentiated]) are resistant to DNO and Ara-C, compared to the MLL-AF9+ mitotic (c-Kit*CD11bhi [mid-differentiation]) and post-mitotic (c-Kit*CD11bhi [most differentiated]) blast cells [271]. It was determined MLL-AF9+ LSCs are p27Kip1-quiescent and therefore less sensitive to the cytotoxic effects of CTx. In the same study, it was shown that expression of p27Kip1 is induced by activation of c-Kit and Flt3; however, it was not determined whether c-Kit*Flt3+ MLL-AF9+ cells are similarly resistant to CTx. As such, we sought to determine if c-Kit*Flt3+ MLL-AF9+ cells are also drug-resistant. To this end, 5.0 x 10^4 Tbk1WT MLL-AF9+ and Tbk1NULL counterparts (genetic control) were treated for 24 hours with varying dosages of either DNO (20-100nM) or Ara-C (0.5-30uM). The number of c-Kit*Flt3+ cells were then measured as percentage of MLL-AF9+ TNCs, and representative FACS gating strategies are shown (Figure 37C; FACS plots from Tbk1WT groups are shown to illustrate Tbk1-dependent shift in cell populations). Cells were seeded with 3 replicates.

It was observed that Tbk1WT c-Kit*Flt3+ MLL-AF9+ cells are resistant to DNO but NOT Ara-C, as only treatment with DNO caused a significant enrichment for c-Kit*Flt3+ cells (Figure 37A and 37B).
Moreover, a striking reduction in the amount of c-Kit+$^{+}$Flt3$^{+}$ cells was observed in the ddH$_2$O-treated control $Tbk1^{\text{NULL}}$ groups. Specifically, the c-Kit+$^{+}$Flt3$^{+}$ population was significantly reduced following the loss of $Tbk1$ in $MLL$-$AF9^{+}$ cells across all Ara-C treatment groups (but only certain DNO treatment groups, which is attributed to
culturing variabilities). Oddly, it was noted that 100nM DNO causes an enrichment in 
$Tbk1^{\text{NULL}}$ c-Kit$^+$Flt3$^+$ population, for which no explanation is yet available; it could be that 
the π-conjugated, aromatic structure of DNO (which causes its bright red color) may 
confound FACS analysis when high doses such as 100nM are used.

Additionally, in relation to the study by Zhang, et al., it was desired to know if 
several of the involved genes are of prognostic significance in AML. Microarray data 
from BloodSpot (GSE13159) were examined to determine if median gene expression 
was correlated with probability of OS (Table 5; encoded protein listed in parenthesis if 
naming differs significantly from gene name) [332, 335, 336].

Table 5. Select Genes, P-value, Correlation with Probability of OS in AML (if 
Applicable), and Microarray Probe Used [332, 335, 336].

<table>
<thead>
<tr>
<th>Gene</th>
<th>p-value</th>
<th>Correlation</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>$CDKN1B$ (p27$^{\text{Kip1}}$)</td>
<td>0.837</td>
<td>None</td>
<td>209112_at</td>
</tr>
<tr>
<td>$FLT3$</td>
<td>0.040</td>
<td>Negative</td>
<td>206674_at</td>
</tr>
<tr>
<td>$FLT3LG$ (FLT3 ligand)</td>
<td>0.184</td>
<td>None</td>
<td>206980_s_at</td>
</tr>
<tr>
<td></td>
<td>0.073</td>
<td>None (trend toward negative)</td>
<td>210607_at</td>
</tr>
<tr>
<td>$KIT$</td>
<td>0.058</td>
<td>None (trend toward positive)</td>
<td>205051_s_at</td>
</tr>
<tr>
<td>$KITLG$ (SCF)</td>
<td>0.3</td>
<td>None</td>
<td>207029</td>
</tr>
<tr>
<td></td>
<td>0.779</td>
<td>None</td>
<td>211124_s_at</td>
</tr>
<tr>
<td></td>
<td>0.704</td>
<td>None</td>
<td>226534_at</td>
</tr>
<tr>
<td>$ITGAM$ (CD11b)</td>
<td>0.78</td>
<td>None</td>
<td>205785_at</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>Negative</td>
<td>205786_s_at</td>
</tr>
</tbody>
</table>
Unexpectedly, most genes did not correlate with probability of OS. Even more surprising was the finding that \textit{ITGAM} mRNA levels either did not correlate or even predicted worse survival, depending on the probe used. As \textit{ITGAM} encodes a marker positively associated with differentiation (CD11b), it was anticipated that increased \textit{ITGAM} expression would almost invariably portend a higher probability of OS, as more differentiated cancers tend to be less aggressive [358, 359]. Equally surprising was the finding that, while not significant, \textit{KIT} expression trended towards a positive correlation with OS. In contrast to \textit{ITGAM}, \textit{KIT} encodes a surface receptor negatively associated with differentiation. Thus, it was hypothesized that \textit{KIT} correlation would be opposite that of \textit{ITGAM}.

While microarray data is valuable, it is limited by the fact that mRNA levels do not inherently indicate protein expression. In fact, mRNA levels can differ greatly from actual protein expression, such as is the case with basal expression of \textit{TP53} mRNA compared to p53 protein [364]. These studies illustrate the importance of functional studies when investigating the role of a gene/protein in predicting prognosis.

\textit{Tbk1 Regulates MLL-AF9$^+$ LSCs}

Based on the findings in the previous experiment, we sought to confirm that \textit{Tbk1} indeed maintains the c-Kit$^+$Flt3$^+$ pool of \textit{MLL-AF9}$^+$ cells. Additionally, we sought to determine if \textit{Tbk1} regulates the c-Kit$^+$CD11b$^{lo}$ subset previously identified by our lab (Zhang, \textit{et al.}) to be resistant to DNO and Ara-C [271]. To this end, \textit{\sim}1 million \textit{Tbk1$^{NULL}$ MLL-AF9}$^+$ and \textit{Tbk1$^{WT}$ counterparts were isolated from maintenance in liquid media and analyzed for c-Kit, Flt3, and CD11b expression via FACS. The size of each population
was measured as percentage of TNCs. Four samples were taken from each liquid culture (4 replicates), so statistics could not be performed.

It was observed that the loss of \textit{Tbk1} seemed to shift \textit{MLL-AF9}\textsuperscript{+} cells from the c-Kit\textsuperscript{+}CD11b\textsuperscript{lo} to the c-Kit\textsuperscript{+}CD11b\textsuperscript{hi} immunophenotype (Figure 38A). Strikingly, it was found that Tbk1 deletion almost completely ablates the c-Kit\textsuperscript{+}Flt3\textsuperscript{+} population (Figure 38B). However, with \( n = 1 \), further validation of these findings are necessary. Though, if true, these observations would be consistent with previous data, such as increased growth rate and morphology changes, that suggest \textit{Tbk1\textsuperscript{NULL} MLL-AF9}\textsuperscript{+} cells are more differentiated than \textit{Tbk1\textsuperscript{WT}} counterparts. Representative FACS gating strategies are shown (Figure 38C).
c-Kit$^+$Flt3$^+$ MLL-AF9$^+$ Cells are Promoted by AMX

Deletion of Tbk1 in MLL-AF9$^+$ cells reduces the size of the c-Kit$^+$Flt3$^+$ and c-Kit$^+$CD11b$^{lo}$ LSC pools, which suggests that Tbk1 is a target for the basis of AML treatment. However, as this is a genetic model, a means of clinical translation still has yet to be determined. To this end, we sought to determine if treatment with AMX, a TBK1/IKK$\epsilon$ inhibitor, can elicit effects similar to genetic ablation of Tbk1. and analyzed its effect on the MLL-AF9$^+$ LSC pools. Representative FACS gating strategy is shown (Figure 41).
To this end, $5.0 \times 10^4$ Tbk1$^{WT}$ MLL-AF9$^+$ and Tbk1$^{NULL}$ counterparts (genetic control) were treated for 24 hours with varying dosages of AMX (50-300uM), and the size of each population was measured as percentage of TNCs. Much to our surprise, it was found that high-dose (300uM) AMX causes an increase in the size of the c-Kit$^+$Flt3$^+$ population (Figure 39A); in terms of the c-Kit$^+$CD11b$^{lo}$ population, AMX had no effect (Figure 39B). These data suggest that AMX would be contraindicated in MLL-AF9$^+$ AML patients and that pharmacologic blockade of TBK1/IKKε causes a compensatory upregulation of c-Kit$^+$Flt3$^+$ population in Tbk1$^{WT}$ MLL-AF9$^+$ cells. While the involvement of IKKε is not yet known, Tbk1 certainly regulates the c-Kit$^+$Flt3$^+$ population as no significant changes were seen with AMX treatment in any Tbk1$^{NULL}$ cells (Figure 39). While IKKε regulation of the c-Kit$^+$Flt3$^+$ population of MLL-AF9$^+$ cells cannot yet be ruled out, Tbk1 must be ruled-in.
GSK8612 has Variable Effects on MLL-AF9+ LSCs

Following the results of the AMX treatment, we sought to determine if pharmacologic blockade of Tbk1 alone can mirror those following genetic ablation. While we wish to determine how pharmacologic IKKε blockade affects these cells, IKKε-selective inhibitors are not yet available. To this end, 5.0 x 10^4 Tbk1WT MLL-AF9+ and Tbk1NULL counterparts (genetic control) were treated for 24 hours with varying dosages (1-4uM) of GSK8612, a bromine-containing small molecule that strongly blocks the action of Tbk1 only. The size of each population was measured as percentage of TNCs, and representative FACS gating strategy is shown (Figure 41).

In contrast to AMX, it was observed that 2uM and 4uM GSK8612 dose-dependently decreases the size of the c-Kit+CD11blow pool of MLL-AF9+ cells (Figure 40B). Strangely, 1uM and 2uM GSK8612 causes an increase in the size of the c-Kit+Flt3+ pool; this increase, however, is lost with 4um GSK8612 treatment.

Figure 39. c-Kit+Flt3+ MLL-AF9+ Cells are Increased by High-dose AMX. To determine if AMX alters the size of the c-Kit+Flt3+ and c-Kit+CD11blow subsets of MLL-AF9+ cells, cells were treated with increasing dosages of AMX for 24 hours, and the size of the c-Kit+Flt3+ and c-Kit+CD11blow compartments were determined as percentage of TNCs via FACS (n = 1, with 3 replicates). Cells were isolated/washed and stained following drug treatment, analyzed via FACS; the data were quantified, and statistical analyses performed using two-way ANOVA (comparing means +/- standard deviation; inter- and intra-group comparisons). (A) Size of the c-Kit+Flt3+ population is shown. (B) Size of the c-Kit+CD11blow population is shown.
Unfortunately, these data do not provide clear information on the effect of GSK8612 on MLL-AF9+ cells; while low-dose GSK8612 seems it could exacerbate AML, high-dose GSK8612 may have the opposite effect. However, much is still unknown and these data require further interpretation and validation. While AMX and GSK8612 do not seem to have significant off-target effects (as Tbk1^NULL cells were largely unaffected by both), it is possible that off-target effects in the presence of functional Tbk1 exist and thus, complicate the clinical translation of Tbk1 deletion.

Figure 40. c-Kit^+Flt3^+ MLL-AF9^+ Cells are Increased with Low-dose GSK8612 and c-Kit^+CD11b^lo Cells are Decreased with GSK8612 in a Dose-dependent Manner. To determine if GSK8612 alters the size of the c-Kit^+Flt3^+ and c-Kit^+CD11b^lo subsets of MLL-AF9^+ cells, cells were treated with increasing dosages of GSK8612 for 24 hours, and the size of the c-Kit^+Flt3^+ and c-Kit^+CD11b^lo compartments were determined as percentage of TNCs via FACS (n = 1, with 3 replicates). Cells were isolated/washed and stained following drug treatment, analyzed via FACS; the data were quantified, and statistical analyses performed using two-way ANOVA (comparing means +/- standard deviation; inter- and intra-group comparisons). (A) Size of the c-Kit^+Flt3^+ population is shown. (B) Size of the c-Kit^+CD11b^lo population is shown.
Loss of *Tbk1* Causes Increased Expression of c-Fms on *MLL-AF9* HSPCs

The c-FMS proto-oncogene protein (c-FMS; CD115/CSF1R/macrophage-colony stimulating (M-CSF) receptor [M-CSFR]), is a receptor tyrosine kinase that binds M-CSF and IL-34; c-FMS is the human analog of the *v-Fms* gene of the feline sarcoma virus-McDonough strain (SM-FeSV) [365, 366]. c-FMS is required for normal development of the mammalian embryo, skeleton, brain, and hematopoietic system. Specifically, c-FMS...
is a differentiation marker that is expressed increasingly with differentiation towards the monocyte/macrophage fate [367]. In the AML setting, however, c-FMS\textsuperscript{WT} is overexpressed, especially cases that are inv(16)\textsuperscript{+} or of the M5 subtype; moreover, mutations in CSFR1 drive AML and other myeloid neoplasms [368-370]. Clinically, c-FMS is a target of the multi-kinase inhibitor PLX3397 (pexidartinib [Turalio\textsuperscript{®}, Plexxikon/Daiichi Sankyo]).

Mechanistically, as found in an \textit{MLL-AF10}\textsuperscript{+} AML mouse model, PU.1 is co-opted by MLL-AF10 to induce expression of c-Fms [371]. c-Fms\textsuperscript{hi} \textit{MLL-AF10}\textsuperscript{+} cells were found to possess greater leukemogenicity compared to c-Fms\textsuperscript{lo} counterparts, indicating that the upregulation of c-Fms potentiates AML progression, despite being a marker of differentiation. As c-FMS is expressed on human AML cells and is being investigated as a therapeutic target in R/R \textit{FLT3-ITD}\textsuperscript{+} AML (NCT01349049 [pexidartinib (Turalio\textsuperscript{®})]), we sought to determine whether c-Fms expression is altered by Tbk1 blockade in our \textit{MLL-AF9}\textsuperscript{+} model [372].

To this end, \textit{Tbk1}\textsuperscript{WT} \textit{MLL-AF9}\textsuperscript{+} and \textit{Tbk1}\textsuperscript{NULL} counterparts (genetic control) were treated for 24 hours with varying dosages of AMX (50-300uM) and GSK8612 (1-4uM), or DMSO as vehicle control, and the amount of c-Fms\textsuperscript{hi} cells was measured as percentage of TNCs; representative gating strategy for FACS analysis is shown (\textbf{Figure 42}).

Firstly, it was noticed that deletion of \textit{Tbk1} alone causes a dramatic increase in c-Fms expression on \textit{MLL-AF9}\textsuperscript{+} cells (\textbf{Figure 42}). Specifically, in both DMSO-treated groups, it was observed that the c-Fms\textsuperscript{hi} population was significantly larger in the
*Tbk1*^NULL^ cells compared to *Tbk1*^WT^ cells—this finding is depicted in Figure 43 and quantified in Figure 44A and 44B). Notably, these data indicate that *Tbk1* is a negative regulator of c-Fms expression in *MLL-AF9*^+^ cells.

**Figure 42.** *Tbk1* Negatively Regulates c-Fms Expression on *MLL-AF9*^+^ Cells. To determine if the loss of *Tbk1* affects c-Fms expression, c-Fms expression was compared via FACS between DMSO-treated *Tbk1*^WT^ and *Tbk1*^NULL^ (n = 2, with 3 replicates each). FACS data were analyzed using FSC-H/c-Fms (CD115) gating and a significant right-shift was observed in *Tbk1*^NULL^ cells, indicating increased c-Fms expression.

**c-Fms Upregulation Occurs Independently of c-Kit Status on *Tbk1*^NULL^ *MLL-AF9*^+^ HSPCs**

It was observed that the loss of *Tbk1* causes increased c-Fms expression on *MLL-AF9*^+^ cells. However, it was not known whether the c-Fms^hi^ population was
comprised mostly of either c-Kit⁺ or c-Kit⁻ cells, or if it contained a relatively equal mixture of both. Thus, we sought to determine the c-Kit profile of the c-Fms⁺ population.

To this end, the data from Figure 42 were re-examined, and cells were separated by c-Kit and c-Fms (Figure 43B). It was determined that the increased c-Fms expression, while somewhat more apparent in c-Kit⁺ cells, was mostly independent of c-Kit status (Figure 43A). Thus, c-Fms appears to be increased on both c-Kit⁺ MLL-AF9⁺ cells and c-Kit⁻ counterparts following Tbk1 deletion.

**Figure 43.** Increased c-Fms Expression Following Tbk1 Deletion Occurs in Both c-Kit⁺ and c-Kit⁻ MLL-AF9⁺ Cells. To determine if the increased c-Fms expression correlated with c-Kit expression, c-Kit/c-Fms gating was used to analyze the FACS data collected from the DMSO-treated cells of the AMX and GSK8612 experiments. Unpaired t-test was used for both statistical analyses (n = 2, with 3 replicates each). (A) Quantification of FACS data, indicating significantly increased expression of c-Fms on c-Kit⁺ cells and a strong trend towards increased c-Fms expression on c-Kit⁻ cells. (B) Representative FACS gating strategies depict increased c-Fms expression in Tbk1⁰ cells, relatively independent of c-Kit status, though the increase in c-Kit⁻ cells did not reach significance in the second biological replicate.
Tbk1 and IKKε May Act Oppositely in Regulating c-Fms expression on $\textit{MLL-AF9}^+$ HSPCs

While deletion of $\textit{Tbk1}$ causes significantly increased expression of c-Fms on $\textit{MLL-AF9}^+$ cells, the effect of pharmacologic blockade is not as clear. Interestingly, 24-hour treatment AMX and GSK8612 appears to elicit opposing effects, which suggests that IKKε is involved in regulating c-Fms expression. In fact, Tbk1 and IKKε seem to oppose the function of one another in regulating c-Fms expression: while Tbk1 restricts c-Fms levels, these data suggest IKKε promotes it.

Treatment with AMX has largely no effect on $\textit{Tbk1}^{\text{WT}}$ cells, whereas AMX causes a dose-dependent reduction in c-Fms expression in $\textit{Tbk1}^{\text{NULL}}$ cells (Figure 4A). As AMX inhibits both TBK1 and IKKε, this suggests that IKKε is involved in governing c-Fms expression on $\textit{MLL-AF9}^+$ cells.

Treatment with GSK8612 has the opposite effect that AMX does and affects both $\textit{Tbk1}^{\text{WT}}$ and $\textit{Tbk1}^{\text{NULL}}$ cells (Figure 4B). As GSK8612 inhibits only Tbk1, the increase seen in the $\textit{Tbk1}^{\text{NULL}}$ group likely represents off-target effects, as only high-dose GSK8612 elicited any change. However, in the $\textit{Tbk1}^{\text{WT}} \textit{MLL-AF9}^+$ cells, GSK8612 dose-dependently increased the size of the c-Fms$^{\text{hi}}$ population, with even low-dose (1uM) having an effect ($p = 0.015$).
Figure 44. TBK1 and IKKε May Act in Opposition in Regulating c-Fms Expression. To determine if pharmacologic Tbk1 blockade can recapitulate the increased c-Fms expression seen with genetic loss of \( TBK1 \), \( MLL-AF9 \) cells were treated for 24 hours with increasing dosages of either AMX or GSK8612 (\( n = 1 \) for each, with 3 replicates each). Following treatment, cells were isolated/washed, and analyzed via FACS. The data were quantified and two-way ANOVA used for statistical analyses (comparing means +/- standard deviation; inter- and intra-group comparisons). Treatment with AMX has an effect only in \( TBK1^{\text{NULL}} \) cells, where it dose-dependently decreases c-Fms expression, which suggests IKKε involvement; in contrast, GSK8612 causes an effect only in \( TBK1^{\text{WT}} \) cells, where it dose-dependently increases c-Fms expression (FSC-H/c-Fms used to separate cells). (A) AMX treatment. (B) GSK8612 treatment. (C) AMX-treated \( TBK1^{\text{NULL}} \) cells, dosages indicated. (D) GSK8612-treated \( TBK1^{\text{WT}} \) cells, dosages indicated.
**GSK8612 Promotes the c-Kit+Flt3+c-Fms\(^{hi}\) Population of MLL-AF9\(^{+}\) HSPCs**

It was determined that Tbk1 negatively regulates c-Fms expression on MLL-AF9\(^{+}\) cells; specifically, Tbk1 strongly regulates c-Fms expression on c-Kit\(^{+}\) cells and seems to do so roughly equally in c-Kit\(^{-}\) counterparts, although the latter requires further validation (Figure 43A and 43B).

In conjunction with the observation that Tbk1 is required for the maintenance of c-Kit\(^{-}\)Flt3\(^{+}\) MLL-AF9\(^{+}\) cells, we sought to determine how Tbk1 regulates the c-Kit\(^{+}\)Flt3\(^{+}\) subset of c-Fms\(^{hi}\) MLL-AF9\(^{+}\) cells. To this end, FACS data from the AMX (Figure 45A) and GSK8612 (Figure 45B) treatments were analyzed; the size of the c-Kit\(^{+}\)Flt3\(^{+}\)c-Fms\(^{hi}\) population of MLL-AF9\(^{+}\) cells determined as percentage of TNCs.

It was learned that the loss of Tbk1 alone did not significantly affect the size of the c-Kit\(^{-}\)Flt3\(^{+}\)c-Fms\(^{hi}\) population of MLL-AF9\(^{+}\) cells. However, treatment of Tbk1\(^{WT}\) MLL-AF9\(^{+}\) cells with GSK8612 caused a significant increase in the amount of c-Kit\(^{+}\)Flt3\(^{+}\)c-Fms\(^{hi}\) cells with all doses of GSK8612 (1-4uM). Whether GSK8612 dose-dependently increases c-Fms expression on c-Kit\(^{+}\)Flt3\(^{+}\) cells is not clear.

These data indicate that 1-4uM GSK8612 causes an undesirable effect in MLL-AF9\(^{+}\) cells. However, we believe that the increase in the c-Kit\(^{+}\)Flt3\(^{+}\)c-Fms\(^{hi}\) population is not directly due to Tbk1 blockade, but rather is a result of the killing of the more abundant Flt3\(^{-}\) cells, which are more sensitive to CTx; this enrichment of Flt3\(^{+}\) cells was also seen with DNO treatment (Figure 37). Doses above 4uM GSK8612 may be required to kill Flt3\(^{+}\) MLL-AF9\(^{+}\) cells.
Figure 45. GSK8612 Upregulates the c-Kit$^+$Flt3$^+$c-Fms$^{hi}$ Population of MLL-AF9$^+$ Cells. To determine if pharmacologic Tbk1 blockade can recapitulate the increased c-Fms expression seen with genetic loss of Tbk1, MLL-AF9$^+$ cells were treated for 24 hours with increasing dosages of either AMX or GSK8612 (n = 1 of each, with 3 replicates each). Following treatment, cells were isolated/washed, and analyzed via FACS. The data were quantified, and two-way ANOVA used for statistical analyses (comparing means +/- standard deviation; inter- and intra-group comparisons). Treatment with AMX has an effect only in Tbk1$^{\text{NULL}}$ cells, where it dose-dependently decreases c-Fms expression, which suggests IKK$\varepsilon$ involvement; in contrast, GSK8612 causes an effect only in Tbk1$^{\text{WT}}$ cells, where it dose-dependently increases c-Fms expression (FSC-H/c-Fms used to separate cells). (A) AMX treatment. (B) GSK8612 treatment. (C) GSK8612-treated Tbk1$^{\text{WT}}$ cells, dosages indicated.
Loss of *Tbk1* in Transplanted *MLL-AF9*+ HSPCs Causes Chloroma in B6 Mice

Our data suggest that Tbk1 blockade could be a means of antagonizing *MLL-AF9*+ LSCs. We believe that targeting LSCs is required to maintain CR in AML but remains a clinically challenging endeavor. While our data suggest that Tbk1 blockade would be beneficial in the treatment of AML, the *in vivo* significance of our findings had not been determined.

As the cells of the AML liquid tumor are highly heterogeneous, compromised of LSCs and blast cells, an *in vivo* model that investigated the role of *Tbk1* in both cell types was required (Figure 46). To this end, a model was developed where mice would be given *Tbk1*\(^ {fx/tx}\);*Rosa26*-CreER\(^ {T2+}\) *MLL-AF9*+ cells and would undergo the tamoxifen regimen at two different timepoints.
As LSCs are responsible for disease initiation (leukemogenesis), Tbk1 would be deleted in one group of mice shortly after delivery of MLL-AF9+ cells, just after the transplanted cells were expected to have engrafted (Figure 47). At this stage, we believe that most AML cells are LSCs and thus, seek to determine if deletion of Tbk1 at this stage can delay symptom onset (leukemogenesis model/model A). As blast cells are responsible for symptoms (disease burden), Tbk1 would be deleted in another group of mice shortly before AML symptoms were expected to develop (Figure 47). At this stage, we believe blast cells are most abundant and thus, seek to determine if deletion of Tbk1 at this stage can extend OS (burden model/model B).
To this end, a syngeneic mouse model was used wherein 3.0 x 10^6 MLL-AF9+ cells were delivered to 8-week-old, CD45.2+/wild-type male mice that had undergone sublethal, 20mg/kg IP busulfan (Busulfex®) conditioning 48 hours prior. Four groups of mice were used: 2 groups that received Tbk1fx/fix;Rosa26-CreER\textsuperscript{T2+} cells (n = 8) and 2 groups that received Tbk1fx/fix control cells (n = 7). In model A, half of the mice underwent the tamoxifen regimen ~2 weeks post-transplant, shortly after the transplanted cells were expected to have engrafted (n = 4 CreER\textsuperscript{T2+} + 4 control). In model B, the remaining mice (n = 4 CreER\textsuperscript{T2+} + 3 control) underwent the tamoxifen regimen ~80 days post-transplant, when AML symptoms were expected to develop. Unfortunately, 1 CreER\textsuperscript{T2+} and 1 control mouse from model A died from suspected tamoxifen toxicity, and 1 CreER\textsuperscript{T2+} mouse from model A had to be euthanized due to injury.

Figure 47. Schematic Depicting Conditioning and Transplant Protocol for our In Vivo Model of MLL-AF9+ AML; created with BioRender.com.
Nearing 3 months post-transplant, on the same day, 2 of the 3 mice remaining in the control group of model A were found dead. The timing of death and severe ascites found in both mice during necropsy suggested AML, but a lack of splenomegaly in both mice negated a post-mortem diagnosis of AML (tissue morbidity/decomposition precluded FACS, blood smear, and HemaVet® analyses). The remaining mouse in the control group of model A displayed no signs of disease and was sacrificed.

Several days later, it was observed that 1 of the 2 mice remaining in the CreER^{T2+} group of model A (hereafter, referred to as the “Tbk1^{NULL}_A mouse”) displayed difficulty moving. Upon further examination, it was revealed that the mouse’s mobility was likely limited due to the presence of a large, cumbersome mass in the pelvic cavity; ulceration through the ventral skin, near/atop the bladder, was evident. The mouse was euthanized, and necropsy revealed a white, encapsulated tumor situated at the ventral aspect of the sacrum. In addition, the spleen was noted to be pale and severely enlarged (splenomegaly), and the BM was also observed to be pale upon flushing. The sacral tumor, BM, spleen, liver, and PB were harvested for FACS analysis and/or H&E staining. Following previous analyses, FACS staining included c-Kit, CD11b, c-Fms, and Flt3. The remaining mouse in the CreER^{T2+} group of model A displayed no signs of disease and was sacrificed.

Approximately two weeks later, a mouse in the control group of model B (n = 4; hereafter, referred to as the “Tbk1^{WT}_B mouse”) was observed to be lethargic, kyphotic, and hypothermic after several days of the tamoxifen regimen. The mouse was euthanized due to suspected tamoxifen toxicity, but necropsy revealed a pale,
moderately enlarged spleen, so hematopoietic tissues were collected and analyzed to
determine if the animal had AML. The BM of the $Tbk1^{WT_B}$ mouse was also abnormally
pale and, compared to the $Tbk1^{NULL_A}$ mouse, the liver was appreciably darker and
redder in color. BM, spleen, PB, and liver were harvested for FACS analysis and H&E
staining; however, only FSC-A/SSC-A could be assessed from FACS analysis due to
technical difficulties (Figure 48A). The remaining 3 mice in the control group of model B
displayed no signs of disease and were sacrificed.

Based on forward scatter-area (FSC-A) and side scatter-area (SSC-A) alone,
excessive myeloid infiltration consistent with AML was observed in the BM, spleen, and
PB of both mice (Figure 48A-B).
In the Tbk1\textsubscript{NULL} mouse, antibody staining revealed flow cytometry profiles consistent with AML (Figure 49). Notably, 0.3\% of BM-TNCs were c-Kit\textsuperscript{+}Flt3\textsuperscript{+} (LMPP-like cells; red arrow in Figure 49), in sharp contrast to the 3-5\% expected in mice with MLL-AF9\textsuperscript{+} AML (per Jiwang Zhang, MD/PhD). This finding confirms that Tbk1 maintains the c-Kit\textsuperscript{+}Flt3\textsuperscript{+} population of MLL-AF9\textsuperscript{+} AML cells. c-Kit\textsuperscript{+}/CD11b	extsuperscript{lo/hi} and c-Kit\textsuperscript{+}/c-Fms	extsuperscript{-}/lo/hi populations were analyzed, but conclusions could not be made given the lack of FACS
data from mice with $Tbk1^{WT}$ $MLL$-$AF9^{+}$ AML. Moreover, the biological significance of increased c-Fms expression following $Tbk1$ deletion in $MLL$-$AF9^{+}$ cells is not yet known. Notwithstanding, these data are shown to serve as the first-known FACS profiles of $Tbk1^{NULL}$ $MLL$-$AF9^{+}$ cells in adult, male B6 mice.
H&E staining was performed on the isolated hematopoietic tissues from both mice. Regarding the sacral tumor (Figure 51), histology was deemed consistent with
published reports of IHC-verified chloroma/MS (Figures 50) [373]. However, IHC/IF for CD68-KP1 (expressed in nearly all cases of chloroma), MPO, and/or c-Kit would be required to confirm the diagnosis [374]. While rare, chloroma development can occur in AML (specifically in M2/4/5 subtypes) as blasts cells proliferate at extramedullary sites, perhaps homing to neural cell adhesion molecule (NCAM; CD56) [242, 375]. Two case studies have reported RUNX1-ETO+ pediatric AML patients who displayed sacral chloromas and abdominal chloromas have been reported in children with MLL1-rearranged leukemia [376-378].
Figure 50. Monocytic Chloroma of the Gallbladder by Alexiev, et al. To support the diagnosis of chloroma in our $Tbk1^{\text{NULL}}$ mouse, an H&E-stained, 100X-magnified section of a CD43$^{+}$CD68$^{+}$ monocytic chloroma of the gallbladder published by Alexiev, et al is shown for comparison. OpenAccess article reproduced with permission under BioMed Central Ltd.'s Creative Commons Attribution License.
Figure 51. Tbk1\textsuperscript{NULL}\textsubscript{A} Mouse Developed a Likely Chloroma, Secondary to MLL-AF9\textsuperscript{+} AML. The sacral/pelvic mass was isolated from our \textit{Tbk1\textsuperscript{NULL}\textsubscript{A}} mouse (n = 1) and fixed in zinc formalin then stored in 70\% EtOH. After fixation, the mass was H&E-stained and histologic analysis was performed. While IHC for MPO, c-Kit, and/or CD68 is needed to confirm a diagnosis of chloroma, the H&E staining of our isolated tissue is consistent with published reports of IHC-confirmed (CD43\textsuperscript{+}CD68\textsuperscript{+}) chloroma (Alexiev, \textit{et al.}). Objective magnification indicated with scale bars. (A) 4X-magnified section showing epidermis (top) with subcutaneous infiltration of AML blasts. (B-C) 4X-magnified sections showing the extent of AML-cell infiltration within the connective tissue of the integument. (D) 10X-magnified section showing normal adjacent epidermis (top)/subcutaneous tissue. (E-F) 20X-magnified sections of Figure B and C, respectively [black squares], further displaying AML-cell infiltration of the integument. (G) 10X-magnified section showing epidermis (top) and subcutaneous infiltration of AML cells, for comparison with Figure D; yellow arrow indicates AML cells.
Further supporting diagnoses of AML, myeloblasts were observed in the PB (Figure 52), livers (Figure 53), and spleens (Figure 54) of both mice. Notably, loss of Tbk1 did not prevent infiltration of AML cells in the livers and spleens of both mice. While the Tbk1\textsuperscript{NULL}_A mouse seemed to display less AML burden in the liver, this may simply reflect a difference in disease progression, unrelated to Tbk1 deletion. Furthermore, normal spleen and follicular architecture was lost in both mice due to infiltration of AML cells, and while fewer blast cells were visualized in the PB smear from the Tbk1\textsuperscript{NULL}_A mouse, HemaVet\textsuperscript{®} analysis nor quantification were performed to confirm the WBC count.
Figure 52. Tbk1 Deletion in AML Cells May Cause Decreased Blasts in the PB of Mice with MLL-AF9+ AML. AML is characterized by the infiltration of malignant blasts in the bloodstream, and PB smear is used clinically to diagnose AML. To confirm the diagnosis of AML and to determine if Tbk1 deletion caused a change in the frequency of AML blasts in the bloodstream, PB was isolated from mice with suspected AML, methanol-fixed and H&E-stained (Hema 3® kit, Fisher). Following H&E staining, histologic analysis was performed. Tbk1 deletion may cause a reduced frequency of MLL-AF9+ cells in the bloodstream, though the observations were not quantified. Yellow arrows indicate suspected myeloblasts. (A) Tbk1WT (n = 1). (B) Tbk1NULL (n = 1). Objective magnification indicated with scale bars.
Figure 53. Loss of Tbk1 Does Not Prevent Hepatic Infiltration by AML Cells. As the liver is commonly infiltrated by AML cells, livers of mice with suspected AML were harvested. Following H&E staining, histologic analysis was performed to determine if Tbk1 deletion prevents MLL-AF9+ cells from infiltrating the liver. Tbk1 deletion does not prevent liver infiltration by MLL-AF9+ cells. (A) Tbk1^WT^B (n = 1). (B) Tbk1^NULL^A (n = 1). Objective magnification indicated with scale bars.
Around 100 days-post-transplant (~20 days-post-tamoxifen), 1 of the 2 mice remaining in the \textit{Tbk1}^{\text{NULL}} group of model B was found dead, and a cause of death could not be determined due to tissue morbidity/decomposition. Approximately 3 weeks later (~40 days-post-tamoxifen), the remaining mouse in the \textit{Tbk1}^{\text{NULL}} group of model B (hereafter, referred to as the "\textit{Tbk1}^{\text{NULL}}_B \text{ mouse}") began to display slight difficulty moving. Upon further examination, a small, ulcerated lesion—reminiscent of the \textit{Tbk1}^{\text{NULL}}_A mouse—was discovered on the ventral aspect of the mouse in the left
inguinal region (Figure 55). The $Tbk1^{\text{NULL}_B}$ mouse was euthanized, and necropsy revealed a white, encapsulated tumor situated within the pelvic cavity, similar though significantly smaller than the mass previously isolated from the $Tbk1^{\text{NULL}_A}$ mouse. In addition, a pale and severely enlarged spleen (splenomegaly) was evident, and the BM was observed to be pale upon flushing. While the isolated lesion is strongly suspected to be an $MLL$-$AF9^+$ chloroma, H&E staining is currently pending and IHC/IF would still be needed to confirm. The sacral tumor, liver, BM, spleen, kidney, liver, and PB were harvested for FACS analysis and/or H&E staining. Following previous analyses, FACS staining included c-Kit, c-Fms, CD11b, and Flt3, though time did not allow for the incorporation of these data in this thesis. Photos of the $Tbk1^{\text{NULL}_B}$ mouse (taken by Rohit Thalla, BS) are included and represent the type of lesion also seen in the $Tbk1^{\text{NULL}_A}$ mouse (Figure 55).
Strikingly, despite severe splenomegaly and actively progressing solid tumors, neither the $Tbk1^{NULL_A}$ nor $Tbk1^{NULL_B}$ mouse displayed the characteristic symptoms of murine AML (e.g., lethargy, kyphosis, and hind-limb immobility), whereas the $Tbk1^{WT_B}$ mouse did. While the significance of these observations is unknown, they may suggest that even though $Tbk1$ deletion does not prevent the development of AML, loss of $Tbk1$ may lessen the disease severity and extend OS, as mice with $MLL-AF9^+$ AML usually deteriorate rapidly, often within days of exhibiting symptoms [379, 380]. Regardless, our in vivo study is not sufficiently powered to draw conclusions. Notwithstanding, we observed that the mice given $Tbk1^{NULL}$ $MLL-AF9^+$ cells who showed evidence of AML ($Tbk1^{NULL_A}$ and $Tbk1^{NULL_B}$) did so in the form of extramedullary disease (EMD), specifically as what are likely pelvic/abdominal chloromas; in contrast, the control mouse that developed AML ($Tbk1^{WT_B}$) displayed the characteristic symptoms of murine AML, showed no signs of EMD/chloroma, and was moribund.

Figure 55. $Tbk1^{NULL_B}$ Mouse Developed a Likely Chloroma, Secondary to $MLL-AF9^+$ AML. Representative image of the ventral aspect of a 9-month-old, 20-30g male mouse that was given $3.0 \times 10^6$ $Tbk1^{fx/fx},Rosa26$-CreER$^{T2+}$ $MLL-AF9^+$ HSPCs ~120 days prior and underwent tamoxifen regimen ~40 days prior to euthanasia and photographing. Images depicts a lesion identified in the left inguinal region that is representative of a similar but larger lesion observed 1-2 months earlier in the $Tbk1^{NULL_A}$ mouse. The identified lesions are believed to be due to integumentary destruction by infiltrating $Tbk1^{NULL}$ $MLL-AF9^+$ AML cells. Blue square depicts a close-up image of the lesion. Photos taken by Rohit Thalla, BS of our group.
One confounding variable is the fact that the $Tbk1^{\text{NULL}}_A$ and $Tbk1^{\text{WT}}_B$ mice underwent the tamoxifen regimen several weeks apart, and it is not known if tamoxifen influences the development of EMD in $MLL-AF9^+$ AML; though, the $Tbk1^{\text{NULL}}_B$ and $Tbk1^{\text{WT}}_B$ mice underwent the tamoxifen regimen at the same time, so these mice can be compared appropriately. As such, considering the insufficient sample size of our study, it seems that $Tbk1$ deletion in $MLL-AF9^+$ cells induces the formation of EMD and extends OS in B6 mice, though this interpretation must be met with caution due to our insufficient sample size and these data must be replicated.

**Aim 2 Conclusion:** $Tbk1$ is Required by c-Kit$^+$Flt3$^+$ $MLL-AF9^+$ HSPCs and May Prevent Chloroma in $MLL-AF9^+$ AML

Deletion of $Tbk1$ causes a near-total ablation of the c-Kit$^+$Flt3$^+$ subset of $MLL-AF9^+$ cells; this is expected to be desirable in terms of eradicating LSCs. However, loss of $Tbk1$ simultaneously causes an increase in c-Fms expression on $MLL-AF9^+$ cells, which may be less desirable in terms of treating AML, though the *in vivo* significance of this finding is unknown. As well, deletion of $Tbk1$ in $MLL-AF9^+$ AML cells appears to cause blasts to extravasate from the PB and form a chloroma at the sacrum. While chloroma-formation is of unknown significance, and only occurred in a single mouse, it could suggest that $Tbk1^{\text{NULL}}$ $MLL-AF9^+$ AML cells are more differentiated and consequently more responsive to CTx. While typically responsive to DA or other anthracycline- or Ara-C-based CTx regimens, chloromas are largely of indeterminate prognostic significance [381, 382]. However, chloromas portend a poorer prognosis when detected in patients with $RUNX1$-$ETO^+$ AML [383, 384]. Nonetheless, it appears
that deletion of $Tbk1$ causes $MLL$-$AF9^+$ cells to cause EMD in the form of chloroma, an uncommon presentation of AML, which warrants further investigation.
CHAPTER 5
DISCUSSION

Summary

Our study herein primarily sought to determine whether TBK1 should continue to be pursued as a molecular target for the basis of AML therapy. However, the role of TBK1 in normal hematopoiesis also required elucidation, as a treatment modality should not be pursued if the possible untoward effects have not been explored thoroughly.

In terms of life-sustaining/homeostatic hematopoiesis (*i.e.*, production of WBCs, RBCs, Hb, and Plts) in the adult B6 mouse, *Tbk1* plays little role. This was determined simply by the observation that no *Tbk1* null mice died from anything other than what was attributed to tamoxifen toxicity—that is, any *Tbk1* null mice that died did so during the tamoxifen regimen or within 1 week of its completion. However, the deletion of *Tbk1* is not without effect on hematopoiesis: it was observed that the global loss of *Tbk1* causes a significant increase in the number of circulating neutrophils and the size of the MPP3 population of HSPCs.
It is unknown whether the increase in the MPP3 population is a true differentiation block, which would be expected to eventually lead to decreased output of mature hematopoietic cells (e.g., WBCs, RBCs/Hb, and platelets) over time; however, overt hematologic abnormalities were not detected in $Tbk1^{\text{NULL}}$ mice for up to 6 months following deletion, so the altered size of the MPP3 compartment may simply be a benign increase.

Additionally, the loss of $Tbk1$ seemed to cause increased mononuclear cell infiltration in the liver, reminiscent of Marchlik, et al.’s 129S5 global $Tbk1^{\text{NULL}}$ model wherein mononuclear and granulomatous infiltrates were seen in various tissues (e.g., lungs, liver, kidney, eyes, spleen, and skin) and increased circulating monocytes were noted [127]. However, in our WBM-intrinsic deletion of $Tbk1$, increased circulating monocytes were noted. Additionally, proliferation of the mesangial cells in the kidney of a $Tbk1^{\text{NULL}}$ mouse was noted, which could indicate some renal pathology underway. Thus, global deletion of $Tbk1$ is not entirely benign regarding its effect on hematopoiesis. However, it must be noted that, at least in the oncologic/AML setting, most treatments are of finite duration. Thus, TBK1 blockade as means of anticancer treatment would likely be transient, so the finding that genetic deletion was tolerable by mice suggests that temporary, pharmacologic blockade of TBK1 would be tolerable in humans. To this notion, hematological adverse events were not observed with oral AMX up to 50mg TID for 10 weeks (NCT01842282) [50].
Future Directions

In terms of experimental design, several optimizations would be made. Firstly, mice would be sex-matched in all studies. While mice were age-matched in comparisons and sex-matched in the WBM deletion of *Tbk1*, mice were not sex-matched in the global *Tbk1*\textsuperscript{NULL} model. It is not known how sex may affect the *Tbk1*\textsuperscript{NULL} phenotype, but it is known that male mice have been observed to display a different LSK/LK cell ratio (unpublished observations, Zhang lab) and male humans have an approximately a 1/3\textsuperscript{rd}-increased lifetime risk of AML. Thus, sex should be controlled for in all studies.

Secondly, while most studies utilizing the Cre-\textit{loxP} system use floxed;Cre\textsuperscript{-} mice as controls, it is my opinion that non-floxed;Cre\textsuperscript{+} mice should also be used, following a recent study by Rossi, \textit{et al} [385]. The authors determined that that 75mg/kg IP tamoxifen on postnatal days 9, 10, and 11 causes myelotoxicity in non-floxed;*Rosa26-CreER\textsuperscript{T2+}* B6 mice. Notably, control mice did not display the severe reaction to tamoxifen seen in *Rosa26-CreER\textsuperscript{T2+}* mice. This suggests that CreER\textsuperscript{T2} may act promiscuously, at least in neonatal mice, recombining gDNA at “cryptic” or “pseudo-\textit{loxP}” sites when no alleles are floxed. Additionally, most groups (including ours) seem to use floxed;*Rosa26-CreER\textsuperscript{T2-}* controls and do not employ non-floxed;*Rosa26-CreER\textsuperscript{T2+}* mice [152, 385]. Thus, it is my opinion that inclusion of non-floxed;*Rosa26-CreER\textsuperscript{T2+}* mice is optimal.
While some of our data herein is of unknown significance, such as the increased c-Fms expression imparted by Tbk1 deletion and drug treatment, we believe that TBK1 blockade warrants further exploration as a means of anti-AML treatment. Our belief is supported by the data in this thesis, those of Cannova, 2018, and those from a Map3k7/Tak1\textsuperscript{NULL} mouse model (unpublished observations, Zhang lab) [32]. In fact, specifically following the observation that Tbk1 deletion ablates the c-Kit\textsuperscript{+}Flt3\textsuperscript{+} population of MLL-AF9\textsuperscript{+} cells, it is our recommendation that RNA-seq be performed on Tbk1\textsuperscript{WT} MLL-AF9\textsuperscript{+} cells and Tbk1\textsuperscript{NULL} counterparts to determine what genes are differentially expressed. We hypothesize that Tbk1\textsuperscript{NULL} MLL-AF9\textsuperscript{+} cells will show decreased expression of AML-associated genes, such as MEIS1 and HOXA9.

Mechanistically, we believe that the role TBK1 in regulating mitophagy is of particular interest in targeting the LSC.

In terms of future models, we recommend that GSK8612 be tested for its anti-AML abilities. While it is unlikely GSK8612 alone would suffice as an effective CTx, GSK8612 should still be tested as monotherapy, though we hypothesize it is better suited as an augmentation agent for standard DA. As such, we propose an experimental setup wherein mice with Tbk1\textsuperscript{WT} MLL-AF9\textsuperscript{+} AML be treated with ether [DMSO +/- DA] or [GSK8612 +/- DA], to determine if GSK8612 can (1) act as monotherapy and/or (2) enhance the efficacy of standard DA, ideally allowing for a dose-reduction of DA without sacrificing efficacy. Moreover, given the increased c-Fms expression on Tbk1\textsuperscript{NULL} MLL-AF9\textsuperscript{+} cells, the anti-AML efficacy of c-Fms blockade warrants investigation. For example, c-Fms\textsuperscript{hi} MLL-AF9\textsuperscript{+} cells may be more sensitive to
pexidartinib (Turalio®); however, the biological significance of increased c-Fms expression in $Tbk1^{\text{NULL}} MLL$-$AF9^+$ cells is not yet known. An experimental setup wherein mice with $Tbk1^{\text{WT}} MLL$-$AF9^+$ AML be treated with ether [DMSO +/- DA] or [pexidartinib + GSK8612 +/- DA] would be informative (GSK8612 is suggested herein to pharmacologically increase c-Fms expression on $Tbk1^{\text{WT}} MLL$-$AF9^+$ cells [Figure 44B]).

Secondly, we recommend that an anti-mitochondrial agent be tested in combination with GSK8612 to determine whether this drug combination has greater anti-AML effects than either agent alone; while devimistat (CAC inhibitor) has been proposed, it has been suggested that electron transport chain (ETC) blockade be pursued alternatively if antagonizing mitochondrial metabolism is desired. Additionally, it would also be of interest to determine if GSK8612 displays synergy with revumenib in the $MLL$-$AF9^+$ setting.

Thirdly, we recommend that $Tbk1$ deletion in $MLL$-$AF9^+$ AML be revisited. Specifically, we envision a repeat experiment wherein mice receive either $Tbk1^{\text{NULL}} MLL$-$AF9^+$ cells or $Tbk1^{\text{WT}} MLL$-$AF9^+$ cells. As EMD in the form of chloroma—an uncommon presentation of AML—was seen in a mouse given $Tbk1^{\text{NULL}} MLL$-$AF9^+$ cells, we believe it should be determined whether this was due to $Tbk1$ deletion in the AML cells or occurred simply by chance. As the mechanisms that govern chloroma-formation are still somewhat poorly-understood, it would be of interest to know how $Tbk1$ restricts this presentation of EMD in $MLL$-$AF9^+$ AML [384].
Fourthly, E-selection (CD62E), which harbors sialyl-LewisX (sLeX), has been implicated as a contributor to the chemoresistance of AML LSCs [386, 387]. LSCs produce cytokines to induce upregulation of CD62E on vascular endothelia. Engagement between LSCs and endothelial cells via CD62E promotes LSC survival by stimulating the NF-κB/BCL-2 pathway within LSCs (see uproleselan by GlycoMimetics, Inc.) [388, 389].

It is the hope of all those involved in this project that this information will improve understanding of MLL-AF9+ LSC physiology. Specifically, we hope that our research regarding TBK1 will contribute to making AML treatment more tolerable and more effective, thereby allowing CR to be achieved (and maintained) more readily.

Closing

Using cDNA microarray analysis, our lab has previously determined that patients with MLL-r leukemia express high levels TLR- and TNF-related genes including TLR2, TLR3, TLR4, CSF1R, TNFA, IL1B, and MYD88 [35, 36, 390]. The increased expression of TLRs, TNF-related receptors, and associated machinery likely leads to increased activation of TBK1, as TBK1 resides downstream of these receptors/pathways [32, 56]. Thus, we expect MLL-AF9+ cells to display increased TBK1 activity. Furthermore, it is established that CDKN1B (p27Kip1) is a target gene of MLL-r, including MLL-AF9, and is upregulated in AML cells, specifically in c-Kit+CD11blo MLL-AF9+ cells, as shown by our lab [271, 391, 392]. Mechanistically, p27Kip1 is co-opted by MLL-r to maintain quiescence, which confers resistance to chemotherapies like DNO and Ara-C [271]. The loss of Tbk1 in our mouse model is observed to afflict the cells previously identified by
Zhang, et al. to be p27Kip1-quiescent; as such, we suspect Tbk1 promotes the stability of p27Kip1 in these cells, though by a mechanism not yet known.

As TBK1 is a critical regulator of mitophagy, and increased TBK1 activation is hyperactivates mitophagy, we expect MLL-AF9+ cells to display increased mitophagic flux [100, 102, 393]. As mitophagy is exploited by cancer cells to adopt an aggressive, CTx-resistant phenotype, an upregulation of mitophagy is likely at least partially responsible for the chemoresistance and aggressiveness observed in MLL-AF9+ AML cells [133, 139-145].

In conclusion, we believe that MLL-AF9 co-opts the TLR/TNF-TBK1 axis to upregulate mitophagy, allowing MLL-AF9+ cells to maintain a quiescent, CTx-resistant, c-Kit*Flt3* (LMPP-like) phenotype (Figure 56), as I found that Tbk1 is required for the survival of c-Kit*Flt3* MLL-AF9+ AML cells. It has been established that TAK1 restricts apoptosis/necroptosis induced by inflammatory cytokines by simultaneously blocking RIPK1-mediated cell death and promoting NF-κB/JNK-mediated expression of pro-survival genes, such as BCL2—Cannova found that Tak1 is required for the survival of c-Kit*Flt3* (GMP-like) MLL-AF9+ LSCs [32, 394-397].
I hypothesize that, due to the upregulated TLR/TNF signaling induced by MLL-AF9, the TBK1-mitophagy axis is upregulated and LMPP-like/c-Kit+Flt3+ phenotype can be maintained in *MLL-AF9*+ LSCs.
CHAPTER 6

METHODS

Mice

Mice were housed in accordance with the National Institutes of Health’s Guidelines for the Care and Use of Animals in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-certified, specific pathogen-free animal facility in the Cardinal Bernardin Cancer Center at LUMC [398]. Mice were exposed to 12-hour light/dark cycle and housed in microisolator cages/laminar flow system [399]. All procedures were performed in accordance with an Institutional Animal Care and Use Committee (IACUC)-approved protocol from Loyola University Chicago (IACUC protocol: 2020010).

Generation of $Tbk1^{fx/fx} \cdot Rosa26-CreER^{T2+}$ Mice

All mice were maintained on B6 background. $Tbk1$-knockout strategy involved flanking exon 2 of $Tbk1$ with loxP sites (indicated previously by Marchlik, et al.) [127]. Loss of exon 2 ablates the kinase activity of Tbk1, as exon 2 encodes the translation-initiating methionine and a G-Q-G-A-T-A amino acid sequence that coordinates ATP and Mg$^{2+}$ in the kinase domain [127]. $Tbk1^{fx/fx}$ mice were purchased from Lexicon
Pharmaceuticals (The Woodlands, TX, USA). Casp8\(\text{fx/fx}\);Rosa26-CreER\(^{T2+}\) (Casp8\(^{NULL}\)) mice were previously available to our lab, and as Rosa26-CreER\(^{T2}\) was desired for this model, Casp8\(^{NULL}\) mice were crossed with Tbk1\(^{fx/fx}\) mice until Tbk1\(^{fx/fx};\)Rosa26-CreER\(^{T2+}\) progeny were attained; extensive backcrossing was required to eliminate the Casp8\(^{fx}\) allele. Rosa26-CreER\(^{T2+}\) mice (stock #: 008463) were previously purchased from Jackson Labs (JAX; Bar Harbor, ME, USA) [400]. All genotypes and gene deletion were confirmed via PCR using gDNA. CD45.1\(^+\) recipient (stock #: 002014) and CD45.2\(^+\) donor mice (stock #: 000664) used in transplantation studies were also purchased from JAX [401, 402].

Tbk1-knockout strategy, adapted from Marchlik, et al. and produced by Lexicon [127, 344]
**In vivo Deletion of Tbk1**

200mg/mL stock of tamoxifen (B5965, ApexBio) was prepared by mixing tamoxifen in 100% ethanol (EtOH; 2701, Decon Labs). The 200mg/mL tamoxifen stock was then diluted 10-fold with corn oil (901414, MP Biomedicals) to achieve a working solution of 20mg/mL tamoxifen. The solution was vortexed vigorously, wrapped in foil to protect from light, and placed in 37°C, 250RPM-shaking incubator for ~24 hours. After incubation, the solution was kept protected from light and stored in 4°C for up to 6 days. To induce CreERT2-mediated deletion of Tbk1, mice were weighed and tamoxifen was delivered intraperitoneally (IP) at a dosage of 100mg/kg for 4 consecutive days [348].

Example dose calculation (extra days and volume included, to account for spillage):

(8 mice · 5 days · 200uL) = 8mL of 20mg/mL tamoxifen working solution required;

→ 8mL / 10 = 0.8mL EtOH · 200mg tamoxifen = 160mg tamoxifen in 800uL EtOH;

→ 8mL – 0.8mL = 7.2mL corn oil added to tamoxifen-EtOH mixture to achieve 10-fold dilution.

**Isolation of CD117/c-Kit+ Mouse BM Cells (HSPCs)**

CD117/c-Kit+ mouse BM cells (hematopoietic stem/progenitors [HSPCs]) were isolated from the total BM eluate using EasySep™ Mouse CD117 (c-Kit) Positive Selection Kit (18757, STEMCELL Technologies), in accordance with the manufacturer’s protocol. HSPCs were isolated and cultured overnight in standard culture conditions with 8-10% 4GFS (see *Ex Vivo Culture of Mouse HSPCs*).
**Mouse Pre-transplant Conditioning Regimen**

CD45.1\(^+\) recipient mice undergoing competitive Tbk1\(^\text{NULL}\) WBMT were lethally conditioned (myeloablation) with 1 dose of 40mg/kg IP busulfan (71288-116-11, Meitheal Pharmaceuticals; 1,4-butanediol dimethanesulfonate/Busulfex\(^\text{®}\), Otsuka America Pharmaceutical, Inc.) 48 hours before tail-vein delivery of \(1.0 \times 10^6\) WBM cells in 200uL 1X PBS [403]. NOTE: it is our recommendation 2 doses of 20mg/kg IP busulfan (delivered 24 hours apart) be used instead of a single, 40mg/kg dose—subsequent studies from our lab have suggested that the halved regimen (40mg/kg cumulative) is as effective but better tolerated. CD45.2\(^+\)/wild-type recipient mice undergoing adaptive MLL-AF9\(^+\) HSPC transplant (Figure 47/In vivo AML Mouse Model) were sublethally conditioned with 1 dose of 20mg/kg IP busulfan (non-myeloablation) 48 hours before tail-vein delivery of \(3.0 \times 10^6\) MLL-AF9\(^+\) cells (NO support cells delivered) [404, 405].

**Mouse Post-transplant Antibiotic Prophylaxis**

Enrofloxacin (E0786, TCI America; Baytril\(^\text{®}\), Elanco) was added to mouse drinking water to prevent opportunistic infection during count recovery following busulfan conditioning. Every other day, 5mL of 16mg/mL enrofloxacin was added to a full, 450mL water bottle.

<table>
<thead>
<tr>
<th>16mg/mL Enrofloxacin</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>Enrofloxacin</td>
<td>0.8g</td>
</tr>
<tr>
<td>ddH(_2)O</td>
<td>50mL</td>
</tr>
</tbody>
</table>
**In Vivo AML Mouse Model**

Following non-myeloablative/sublethal conditioning, $3.0 \times 10^6$ *MLL-AF9* HSPCs were delivered in 200uL 1X PBS via tail-vein injection to conditioned, CD45.2+/wild-type, 8-week-old male mice. HSPCs were either *Tbk1*^{fx/fx};*Rosa26-CreER^{T2+} (*Tbk1^{NULL}) or *Tbk1*^{fx/fx} (control) cells. Tamoxifen was then delivered to half of the *Tbk1^{NULL} & control mice at ~2 weeks post-transplant (model A/leukemogenesis) and the other half of *Tbk1^{NULL} & control mice at ~12 weeks post-transplant (model B/progression).

**Harvest of Mouse BM**

Mice were euthanized with CO₂ and subsequent cervical dislocation. Following euthanasia, femur(s) and tibia(s) were harvested; following harvest of leg bones, marrow was flushed with 1X PBS (10X PBS Buffer pH 7.4; AM9625, Thermo-Fisher) using a 1mL syringe. Marrow clumps were dissociated with 1mL syringe, then marrow was filtered into 50mL conical tube(s) using 40uM filter.

**Harvest of Mouse PB**

Within 1-3 minutes from cervical dislocation, PB was collected by using a 1mL syringe to pierce the heart at the apex and extract blood. Blood was deposited into lavender collection tube(s) (20.1278.100, Sarstedt). Prior to heart puncture, syringes were washed with 0.5M, pH 8.0 ethylenediamine tetra-acetic acid (EDTA; BP2482, Fisher) to coat the barrel, and 20uL EDTA was added to each lavender tube(s).
Agarose Gel Electrophoresis

Desired amount of agarose (15510-019, Life Technologies) was transferred to a glass flask and 1X TAE buffer swirled in. Mixture was microwaved for 1-3 minutes on high until agarose was fully dissolved. Mixture was allowed to cool for 2 minutes before 3uL of 10mg/mL ethidium bromide (EthBr) was added per 50mL 1X TAE buffer. After mixing in EthBr, gel was poured and allowed to cool for at least 45 minutes before loading samples and running. 4uL GeneRuler DNA Ladder Mix, ready to use (SM0333, Fisher Scientific) was used. 120V for ~1 hour.

<table>
<thead>
<tr>
<th>50X TAE Buffer Stock</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>Tris-Base (BP152-1, Fisher)</td>
<td>242g</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>57.1mL</td>
</tr>
<tr>
<td>0.5M, pH 8.0 EDTA (BP2482, Fisher)</td>
<td>100mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>1L</td>
</tr>
</tbody>
</table>

1X TAE buffer working solution was made by diluting 0.3L 50X TAE buffer stock in 14.7L MilliQ ddH₂O.

Bacterial Transformation and Plasmid Isolation

~50uL DH5a chemically-competent E. coli (New England Biolabs) were thawed on ice in a microcentrifuge tube(s). Upon thawing, 2uL of either MSCV-puro-MLL-AF9 (MLL1-AF9 was cloned into the MSCV-puro backbone [68469, Addgene] in-house) or Gag/Pol plasmid (14887, Addgene) was added to tube(s). The mixture was incubated for 30 minutes on ice, then heat-shocked at 42°C for 30 seconds, then 200uL Miller’s
Luria Broth (LB) Agar (MBPE-3060, GrowCells.com) was added to tube(s) and tube(s) transferred to a 37°C 250RPM-shaker for 1 hour. After shaking, 50uL of the mixture was plated on LB-Ampicillin+ plates and incubated in 37°C/5%CO₂/100% humidity overnight. The next day, colonies were selected for use with either PureYield™ Plasmid Midiprep System or PureYield™ Plasmid Maxiprep System (A2492/A2392, Promega) in accordance with manufacturer's protocol.

*MLL1-AF9* cloned into the MSCV-puro backbone (in-house).

**Creation of 293T Transfection Reagent**

Opti-MEM™ (31985062, Thermo-Fisher) was added to 5mL polystyrene tube(s) in the biosafety hood. While vortexing, Gag/Pol plasmid was added to tube(s), then DNA-of-interest (e.g., MSCV-puro-MLL-AF9 or MSCV-YFP), and then PEI MAX® (NC1038561, Fisher Scientific) was added. NOTE: addition of reagents should follow in
the order as described. After addition of all reagents, tube(s) capped and allowed to incubated at RT in the hood for 15 minutes.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>DNA-of-Interest</td>
<td>6ug</td>
</tr>
<tr>
<td>Gag/Pol</td>
<td>6ug</td>
</tr>
<tr>
<td>1ug/uL PEI MAX®</td>
<td>50ug</td>
</tr>
<tr>
<td>Opti-MEM™</td>
<td>450uL</td>
</tr>
</tbody>
</table>

**293T Transfection and Collection of Retroviral Supernatant**

12 hours before transfection, 3.0 x 10^6 293T cells were plated on a 10cm^2 tissue culture dish in 8mL DMEM-High Glucose media (SH30022.01, HyClone/GE) with 10% FBS and 1%P/S, then incubated in 37°C/5%CO_2/100% humidity. After incubation, transfection reagent was added dropwise to 293T cells, carefully, down the side of the tissue culture plate, as not to disturb the 293T cells. 293T cells were incubated for 18 hours before DMEM media was exchanged; after media was swapped, cells were incubated for 48 hours before the DMEM media (now harboring the retroviral supernatant) was collected. Retroviral supernatant was aliquoted to cryotube(s), snap-frozen (allowed to sit on dry ice for 30 minutes), then transferred to storage in -80°C.

**Retroviral Transduction (Spinduction/Spinoculation) of Mouse HSPCs**

1.0 x 10^5 mouse HSPCs were transferred to 5mL polystyrene tube(s) and centrifuged at 1800RPM for 5 minutes. Supernatant was discarded and replaced with 3mL of 293T-produced retroviral supernatant (e.g., MSCV-puro-MLL-AF9). 4uL of
4ug/uL polybrene (TR-1003, Sigma) was then added. The mixture was vortexed vigorously before being allowed to incubate in the biosafety hood at RT for 10 minutes. After incubation, mixture was centrifuged at 32°C at 2200RPM for 4 hours. After centrifugation, supernatant was discarded and cells were resuspended in usual culture conditions at 37°C/5%CO₂/100% humidity. Puromycin selection was initiated 48 hours later (allowing for transcription/translation of _pac_), wherein 1mg/mL puromycin (ant-pr-1, Invivogen) was added to the cells for a final concentration of 1X. The same protocol was used for transduction with _MSCV-YFP_; although, selection of YFP⁺ HSPCs was performed 48 hours later via FACS (FACSAria III, BD Biosciences).

**Ex Vivo Culture of Mouse HSPCs**

Following c-Kit⁺ selection and _MSCV-puro-MLL-AF9_ transduction, mouse HSPCs were cultured in RPMI-1640 with L-glutamine (SH30027.01, Cytiva) with 10% FBS and 1% Penicillin-Streptomycin (30-002-CI, Corning). Our proprietary mixture of IL-3, IL-6, SCF, and GM-CSF (4GFS; generated in-house) was added for a final concentration of 3-10% of the total media volume, based on experimental requirements; for simply maintaining cultures, 3.75% was determined empirically to be appropriate. Cells were incubated at 37°C/5%CO₂/100% humidity. Copper(II) sulfate pentahydrate (C8027, Sigma-Aldrich) was added to incubator water for a final concentration of 3% (60g in 2L of ddH₂O). Cell cultures were maintained in 3mL RPMI-1640/3.75% 4GFS in one well of a 6-well plate; _in vitro_ FACS/growth curve analyses were conducted using 2mL RPMI-1640/3.75% 4GFS in one well of a 12-well plate.
**In Vitro Knockout of Tbk1**

1mg/mL 4-hydroxytamoxifen (4-OHT [afimoxifene]; H7904, Sigma-Aldrich) was added to healthy, media-replete cultures of both *Tbk1*^fx/fx*;Rosa26-CreER^{T2+} MLL-AF9*+ and *Tbk1*^fx/fx MLL-AF9*+ (control) mouse HSPCs for a final concentration of 1uM. Cells were incubated at 37°C/5%CO₂/100% humidity for 48 hours. Cells were then isolated and centrifuged at 1800RPM for 5 minutes; after pelleting, media was discarded and cells were resuspended in fresh RPMI-1640/8% 4GFS and re-plated. 1mg/mL 4-OHT was then re-added for a final concentration of 1uM. Cells were incubated for an additional 48 hours before being transferred to fresh RPMI-1640/3.75% 4GFS. PCR-genotyping and downstream analyses were performed 48 hours later.

**Growth Curve Analysis**

Adapted from Fisher Scientific/Mather, *et al.* [406]. 5.0 x 10^4 MLL-AF9*+ mouse HSPCs were plated in 2.175mL (2.1mL RPMI-1640/3.75% 4GFS) on a 12-well plate. Cells incubated at 37°C/5%CO₂/100% humidity and counted via trypan blue-exclusion every 24 hours. At 48 and 72 hours post-seeding, cultures were homogenized and 1.5mL was removed from each well, then 1.5mL RPMI-1640/3.75% 4GFS re-added (to prevent overgrowth/media exhaustion). The same volume of media was removed from each well, which would ensure that any difference in cell numbers was maintained between *Tbk1*^WT and *Tbk1*^NULL cells.

**Serum Starvation**

*Tbk1*^NULL YFP*+ MLL-AF9*+ and *Tbk1*^fx/fx (YFP*) MLL-AF9*+ cells were isolated from bulk cultures and mixed in ~1/1 ratio; subsequent FACS analysis revealed mixture was
closer to ~70% YFP+ (Tbk1\textsuperscript{NULL}). 6.0 x 10\textsuperscript{4} cells from the mixture were seeded in 3mL RPMI-1640 (with or without 10% FBS)/5% 4GFS on a 6-well plate (3 replicates) and incubated at 37°C/5%CO\textsubscript{2}/100% humidity. 24 hours later, YFP-positivity was checked via FACS.

**Clonogenicity Assay**

3.0 x 10\textsuperscript{4} MLL-AF9\textsuperscript{+} mouse HSPCs were isolated from bulk culture and plated (3 replicates, ~1 x 10\textsuperscript{4} cells per well) in MethoCult\textsuperscript{TM} GF M3434 (03434, STEMCELL Technologies) in accordance with manufacturer’s protocol. Cells were incubated at 37°C/5%CO\textsubscript{2}/100% humidity for 6-7 days, depending on experimental requirements.

**70% EtOH Fixation and Cell Cycle Analysis**

Protocol provided by Bert Ladd. 1.0 x 10\textsuperscript{6} cultured MLL-AF9\textsuperscript{+} mouse HSPCs were transferred to 15mL conical tube(s) and pelleted via centrifugation at 1800RPM for 5 minutes. Media was discarded and 1mL of 70% EtOH was added dropwise while vortexing gently; the EtOH-fixed cells were then stored at -20°C for 7 days. On the day of analysis, a mastermix of 1X PBS, 100ug/mL propidium iodide (PI; P1304MP, Invitrogen), and 100ug/mL RNase A (EN0531, ThermoFisher), and was created. EtOH-fixed cells were then re-pelleted via centrifugation at 2500RPM for 5 minutes and EtOH was discarded. 1 x 10\textsuperscript{6} cells were resuspended in 1mL mastermix, vortexed vigorously, then aliquotted to 5mL polystyrene tube(s). Samples were incubated at 4°C for 30-60 minutes before FACS analysis.
Polymerase Chain Reaction (PCR)

PCR was used to determine the *Tbk1*, *Casp8*, and *Rosa26-CreER<sub>T2</sub>* status of mice and cultured cells. Reactions employed 10mM dNTP Mix (18427013, Life Technologies) and reagents from Promega: 5X Green GoTaq<sup>®</sup> Flexi Buffer (M891A), GoTaq<sup>®</sup> Flexi DNA Polymerase (M829B), 25mM MgCl<sub>2</sub> (A351H).

To assess *loxp* sites, PCRs were setup using the following parameters:

<table>
<thead>
<tr>
<th><strong>Tbk1/Casp8 (loxp assessment)</strong></th>
<th><strong>Ratio</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>13.075</td>
</tr>
<tr>
<td>5X Green GoTaq&lt;sup&gt;®&lt;/sup&gt; Flexi Buffer</td>
<td>5</td>
</tr>
<tr>
<td>25mM MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2.6</td>
</tr>
<tr>
<td>10mM dNTP</td>
<td>0.2</td>
</tr>
<tr>
<td>10uM 5’ Primer (<em>J1091/J0875</em>)</td>
<td>1</td>
</tr>
<tr>
<td>10uM 3’ Primer (<em>J1092/0876</em>)</td>
<td>1</td>
</tr>
<tr>
<td>GoTaq&lt;sup&gt;®&lt;/sup&gt; Flexi DNA Polymerase</td>
<td>0.125</td>
</tr>
<tr>
<td>Template DNA</td>
<td>2</td>
</tr>
</tbody>
</table>

To assess CreER<sup>T2</sup>-mediated deletion of *Tbk1*, PCRs were setup using the following parameters:

<table>
<thead>
<tr>
<th><strong>Tbk1 (post-tamoxifen/4-OHT)</strong></th>
<th><strong>Ratio</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>13.075</td>
</tr>
<tr>
<td>5X Green GoTaq&lt;sup&gt;®&lt;/sup&gt; Flexi Buffer</td>
<td>5</td>
</tr>
<tr>
<td>25mM MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Ratio</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>14.075</td>
</tr>
<tr>
<td>5X Green GoTaq® Flexi Buffer</td>
<td>5</td>
</tr>
<tr>
<td>25mM MgCl₂</td>
<td>2.6</td>
</tr>
<tr>
<td>10mM dNTP</td>
<td>0.2</td>
</tr>
<tr>
<td>10uM 5’ Primer (J0807)</td>
<td>0.5</td>
</tr>
<tr>
<td>10uM 3’ Primer (J0808)</td>
<td>0.5</td>
</tr>
<tr>
<td>10uM 5’ Control Primer (J0001)</td>
<td>0.2</td>
</tr>
<tr>
<td>10uM 3’ Control Primer (J0002)</td>
<td>0.2</td>
</tr>
<tr>
<td>GoTaq® Flexi DNA Polymerase</td>
<td>0.125</td>
</tr>
<tr>
<td>Template DNA</td>
<td>2</td>
</tr>
</tbody>
</table>

To determine *Rosa26-CreERᵀ²* status, PCR reactions were setup using the following parameters:
Primers ordered from Integrated DNA Technologies (IDT).

**Tbk1** (sequences indicated by Lexicon Pharmaceuticals and purchased from IDT)

<table>
<thead>
<tr>
<th>Lexicon # (IDT ID)</th>
<th>Sequence</th>
<th>Band Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>#18 (J1091)</td>
<td>5’-GCCAGATATCCCTGTAACCTAC-3’</td>
<td>332bp (<strong>Tbk1</strong>&lt;sup&gt;WT&lt;/sup&gt;)</td>
</tr>
<tr>
<td>#24 (J1092)</td>
<td>5’-CAGATGGTTGGAGGTGCTGAT-3’</td>
<td>419bp (<strong>Tbk1</strong>&lt;sup&gt;fx&lt;/sup&gt;)</td>
</tr>
<tr>
<td>#23 (J1093)</td>
<td>5’-CCTACAGCTGCAGACGCCCTCAT-3’</td>
<td>775bp (<strong>Tbk1</strong>&lt;sup&gt;PGK-Neo&lt;/sup&gt;); 323bp (<strong>Tbk1</strong>&lt;sup&gt;NULL&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

**Rosa26-CreER<sup>T2</sup>**

<table>
<thead>
<tr>
<th>IDT ID</th>
<th>Sequence</th>
<th>Band Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>J0807</td>
<td>5’-CGGAGATCATGCAAGCTGGT-3’</td>
<td>~600bp (<strong>Rosa26-CreER&lt;sup&gt;T2+&lt;/sup&gt;</strong>)</td>
</tr>
<tr>
<td>J0808</td>
<td>5’-TCTCCACCATGCCCTTCA-3’</td>
<td>~600bp (<strong>Rosa26-CreER&lt;sup&gt;T2+&lt;/sup&gt;</strong>)</td>
</tr>
</tbody>
</table>

**Il2** (reaction control for use with Rosa26-CreER<sup>T2</sup> PCR-genotyping [murine interleukin 2])

<table>
<thead>
<tr>
<th>IDT Name</th>
<th>Sequence</th>
<th>Band Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>J0001</td>
<td>5’-CTAGGCCACAGATTGAAAGATCT-3’</td>
<td>324bp (<strong>Il2</strong>&lt;sup&gt;WT&lt;/sup&gt;)</td>
</tr>
<tr>
<td>J0002</td>
<td>5’-GTAGGTGGAATTCTAGCATCATCC-3’</td>
<td>324bp (<strong>Il2</strong>&lt;sup&gt;WT&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>
**Mouse Tcrb rearrangement** (Provided graciously by Dr. Thorsten Feyerabend & Dr. Hans-Reimer Rodewald [407, 408])

<table>
<thead>
<tr>
<th>IDT Name</th>
<th>Sequence</th>
<th>Band Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>J1112 [Dβ1:Jβ1-F]</td>
<td>5'-GGGGTAGACCTATGGGAGGG-3'</td>
<td>[408]</td>
</tr>
<tr>
<td>J1113 [Dβ1:Jβ1-R]</td>
<td>5'-CCAAGACCATGGTCATCCAAAC-3'</td>
<td></td>
</tr>
<tr>
<td>J1114 [Dβ2:Jβ2-F]</td>
<td>5'-GTAGGCACCTGTGGGGAAGAAACT-3'</td>
<td>[407]</td>
</tr>
<tr>
<td>J1115 [Dβ2:Jβ2.7-R]</td>
<td>5'-TGAGAGCTGTCTCTACTATCGATT-3'</td>
<td></td>
</tr>
</tbody>
</table>

**PCR-genotyping thermocycle:**

<table>
<thead>
<tr>
<th>PCR Thermocycle</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>5 minutes</td>
<td></td>
</tr>
<tr>
<td>94°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>65°C</td>
<td>45 seconds</td>
<td>10 cycles</td>
</tr>
<tr>
<td>72°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>94°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>60°C</td>
<td>45 seconds</td>
<td>30 cycles</td>
</tr>
<tr>
<td>72°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>5 minutes</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>
RNA Isolation

Isolated cells were pelleted in microcentrifuge tube(s) via centrifugation at 1800RPM for 5 minutes and resuspended in 1mL TRIzol™ (15596026, Invitrogen). Tube(s) centrifuged at 1800RPM for 5 minutes and the supernatant was transferred to separate microcentrifuge tube(s). 20uL of 5M acetic acid was added to the supernatant, then mixture was inverted, vortexed, and allowed to rest, undisturbed, at RT for 5 minutes. 100uL of 1-bromo-3-chloropropane (B9673, Sigma) was then added and mixture was vortexed again, vigorously, for 15 seconds before being allowed to rest at RT again for an additional 5 minutes. Mixture was then centrifuged in at 4°C at 13,000RPM for 15 minutes. After centrifugation, the aqueous phase was carefully extracted, with care taken not to agitate the organic phase. The extracted solution was transferred to separate microcentrifuge tube(s), then 500uL isopropanol (A451-4, Fisher) was added. Mixture was vortexed vigorously, then 1uL glycogen (G1767-1VL, Sigma-Aldrich) was added and mixture was transferred to -20°C for ~18 hours. Mixture was then allowed to warm at RT for 10 minutes before centrifugation at 13,000RPM for 10 minutes. Supernatant was discarded, then 250uL diethyl pyrocarbonate (DEPC)-treated water was added followed by 750uL ice-cold 100% EtOH (75% EtOH final concentration). Mixture was vortexed before being centrifuged again at 13,000RPM for 5 minutes. Supernatant was discarded and pelleted RNA was allowed to air-dry at RT for 5 minutes. 30uL DEPC-treated ddH₂O was added and tube(s) incubated in 65°C-dry bath for 30 minutes before being transferred to -80°C.
DNase I Treatment and Reverse Transcriptase PCR (RT-PCR)

High-Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems) was used for cDNA synthesis from isolated RNA.

DNase I mastermix was setup:

<table>
<thead>
<tr>
<th>DNase I Treatment</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>2ug</td>
</tr>
<tr>
<td>10X DNase I Buffer</td>
<td>1uL</td>
</tr>
<tr>
<td>DNase I (1U/uL)</td>
<td>1uL</td>
</tr>
<tr>
<td>DEPC-treated ddH$_2$O</td>
<td>[Bring volume to 10uL]</td>
</tr>
</tbody>
</table>

DNase I thermocycle:

<table>
<thead>
<tr>
<th>DNase I Thermocycle</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Add 1uL 50mM EDTA</td>
<td>-</td>
</tr>
<tr>
<td>65°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>4°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

Following DNase I treatment, RT-PCR mastermix was setup:

<table>
<thead>
<tr>
<th>RT-PCR Mixture</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>2 ug [up to 10uL]</td>
</tr>
<tr>
<td>10X RT Buffer</td>
<td>2uL</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.8uL</td>
</tr>
</tbody>
</table>
### 10X RT Random Primers
- 2uL

### Reverse Transcriptase
- 1uL

### ddH2O
- Bring volume to 20uL

---

**RT-PCR thermocycle:**

<table>
<thead>
<tr>
<th>RT-PCR Thermocycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>10 minutes</td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>120 minutes</td>
<td></td>
</tr>
<tr>
<td>85°C</td>
<td>8 seconds</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

### Quantitative PCR (qPCR)

qPCR reactions were setup using the following parameters:

<table>
<thead>
<tr>
<th>qPCR Mixture</th>
<th>Ratio</th>
<th>Example: x7 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA (WT&lt;sup&gt;C-Kit&lt;sup&gt;+ or MLL-AF9&lt;sup&gt;+)</td>
<td>4uL</td>
<td>28uL</td>
</tr>
<tr>
<td>2X Buffer</td>
<td>14uL</td>
<td>98uL</td>
</tr>
<tr>
<td>Control Gene probe (ActB [VIC]; Mm.PT.39a.22214843.g, Integrated DNA Technologies)</td>
<td>1uL</td>
<td>7uL</td>
</tr>
<tr>
<td>Gene-of-Interest probe (Tbk1 [FAM]; Mm00451150_m1, Thermo-Fisher)</td>
<td>1uL</td>
<td>7uL</td>
</tr>
<tr>
<td>Reaction Volume</td>
<td>20uL</td>
<td>-</td>
</tr>
<tr>
<td>qPCR Thermocycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>95°C</td>
<td>3 minutes</td>
<td>Hold</td>
</tr>
<tr>
<td>95°C</td>
<td>5 seconds</td>
<td>40 cycles</td>
</tr>
<tr>
<td>60°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

*TaqMan®* reagents and comparative $C_T$ ($\Delta\Delta C_T$) method used for multiplex qPCR. *QuantStudio™ Real-Time PCR Software* (Applied Biosystems) and *Microsoft Excel®* (Microsoft) were used to perform $2^{-\Delta\Delta C_T}$ data analysis.

**NOTE:** to use qPCR to check for *Tbk1* deletion in our mouse model, a *TaqMan®* probe that encompasses exon 2 is required. As probe Mm00451150_m1 includes exons 3-5, it is not suitable for assessment of *Tbk1* exon 2 excision, and thus may yield a false negative result when attempting to confirm *Tbk1* deletion.

**Isolation of gDNA from Mouse Tails**

At 2-3 weeks of age, ~3mm of the distal tail was biopsied from desired mice and kept in microcentrifuge tube(s). NOTE: the ossification of tail vertebrae necessitates anesthesia for tail biopsies performed on mice greater than 3 weeks of age [409]. 500uL of 1X tail lysis buffer and 7uL of 20mg/mL proteinase K were added to tube(s) and tube(s) vortexed vigorously, then placed in 65°C water bath for ~18 hours. Tube(s) vortexed vigorously for at least 10 seconds and tail digestion was assessed; if incomplete digestion, an additional 3uL of 20mg/mL proteinase K was added and
tube(s) returned to 65°C water bath for an additional ~3 hours. Once tail digestion was confirmed, tube(s) centrifuged at 13,000RPM for 10 minutes and the supernatant was collected, then transferred to fresh microcentrifuge tube(s). 500uL isopropanol was added to the supernatant and tube(s) centrifuged similarly. After centrifugation, supernatant was discarded and 800uL ice-cold 70% EtOH was added to the tube(s). Tube(s) then centrifuged at 13,000RPM for 5 minutes. Supernatant was discarded and tube(s) inverted and placed on paper towel, allowed to air-dry at RT for 20 minutes. Once EtOH had evaporated, 200uL ddH₂O was added to each tube and tube(s) vortexed, then placed in 37°C water bath for 30 minutes. Following incubation in water bath, DNA was quantitated or stored at -20°C until quantitation; following quantitation, DNA was stored in -20°C. NOTE: Precedent suggests that the total amount of template gDNA (with an OD260/280 ratio near 1.8) should be at least 10ng but not exceed 130ng for a 25uL reaction [410]. My recommendation is to quantitate DNA and dilute accordingly, prior to performing PCR; in the unlikely event the quantitation reveals the template gDNA to be too dilute, see “DNA Precipitation from H₂O” and attempt to concentrate gDNA before obtaining a new tail biopsy.

10X tail lysis buffer stock requires the following reagents:

<table>
<thead>
<tr>
<th>100mM Tris-HCl, pH 8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>5mM EDTA</td>
</tr>
<tr>
<td>0.2% SDS (L3771-1KG, Sigma)</td>
</tr>
<tr>
<td>200mM NaCl (S271, Fisher)</td>
</tr>
</tbody>
</table>
NOTE: Tris-HCl, pH 8.5 was prepared using Tris Base (BP152-1, Fisher) and Hydrochloric Acid (HCl; A144-212, Fisher).

Example preparation:

<table>
<thead>
<tr>
<th>10X Tail Lysis Buffer</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris-HCl, pH 8.5</td>
<td>20mL</td>
</tr>
<tr>
<td>0.5M EDTA (BP2482, Fisher)</td>
<td>2mL</td>
</tr>
<tr>
<td>20% SDS</td>
<td>2mL</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>8mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>168mL</td>
</tr>
</tbody>
</table>

DNA Quantitation

DNA was quantitated using NanoDrop (Thermo-Fisher); 2uL ddH₂O used as blank and 2uL DNA used for quantitation.

DNA Precipitation from H₂O

Adapted from Qiagen [411]. 3M sodium acetate (NaOAc) was added to DNA stock at 0.1X the volume of water, then ice-cold 100% EtOH was added at 2.5X the (initial) volume of water. Mixture was vortexed vigorously placed in -20°C for at least 3 hours. After cooling, mixture was centrifuged at 13,000RPM at 4°C for 15 minutes. Supernatant was then discarded and microcentrifuge tube(s) filled maximally with ice-cold 70% EtOH, then centrifuged similarly. Supernatant was discarded and tube(s) inverted and placed on paper towel, allowed to air-dry at RT for 20 minutes. After EtOH
had evaporated, 10uL ddH2O was added, tube(s) vortexed, then placed in 37°C water bath for 30 minutes. After water bath, tube(s) stored in -20°C. NOTE: 5M NaCl can be used in place of 3M NaOAc.

**Statistical Analyses**

Quantification of results and statistical analyses performed using *GraphPad Prism* 9.3.1 [412]. Unpaired (Student’s/independent) t-test used when comparing 1 independent variable across 2 unrelated groups; paired t-test not applicable for our analyses. Two-way ANOVA used when comparing 2+ independent variables across 2 different groups assumed to have equal variances for (e.g., comparing *Tbk1*<sup>WT</sup> and *Tbk1*<sup>NULL</sup> cells both treated with 4 different drug dosages).

**Cell Counting**

After mixing in 1/1 ratio with trypan blue, cells were counted using the T10 Automated Cell Counter (1450001, BioRad) and accompanying dual-chamber counting slides (1450011, BioRad).

**Western blot/Immunoblot**

Performed by Allan Youmaran, BS.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Catalog #, Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PBS pH 7.4, RNase-free</td>
<td>AM9625, Ambion</td>
</tr>
<tr>
<td>10X Cell Lysis Buffer</td>
<td>9803, Cell Signaling Technology</td>
</tr>
<tr>
<td>(contains phosphatase</td>
<td></td>
</tr>
<tr>
<td>Inhibitors; 5mL in 45mL ddH2O for 1X working solution</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>cOmplete™ ULTRA Tablets, Mini, EDTA-free, EASYpack Protease Inhibitor Cocktail (1 tablet per 50mL 1X Cell Lysis Buffer)</td>
<td>05892791001, Roche</td>
</tr>
<tr>
<td>40% Acrylamide/Bis Solution 29:1</td>
<td>HC2040, Fisher</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>L3771-1KG, Sigma</td>
</tr>
<tr>
<td>10% ammonium persulfate (APS)</td>
<td>A3678-25G, Sigma (in ddH2O)</td>
</tr>
<tr>
<td>N,N,N',N'-Tetramethylethylenediamine (TEMED)</td>
<td>T9281, Sigma-Aldrich</td>
</tr>
<tr>
<td>Product Name</td>
<td>Catalog Number</td>
</tr>
<tr>
<td>------------------------------------------------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Bicinchoninic acid (BCA)/Smith assay</td>
<td>23225</td>
</tr>
<tr>
<td>Loading dye</td>
<td></td>
</tr>
<tr>
<td>10X electrophoresis/running buffer, pH 8.3</td>
<td>30.3g Tris Base + 144.0g glycine (M103-5KG, Amresco) + 10.0g SDS + 1L ddH₂O</td>
</tr>
<tr>
<td>Trans-Blot Turbo 5X Transfer Buffer</td>
<td>10026938</td>
</tr>
<tr>
<td>NaCl</td>
<td>S271</td>
</tr>
<tr>
<td>Tris base</td>
<td>BP152</td>
</tr>
<tr>
<td>Tween-20</td>
<td>2287</td>
</tr>
<tr>
<td>Trans-Blot Turbo RTA Midi 0.2 µm Nitrocellulose Transfer Kit</td>
<td>1704271</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>A7906</td>
</tr>
<tr>
<td>TBK1/NAK (E8I3G) Rabbit mAb</td>
<td>38066</td>
</tr>
</tbody>
</table>
Anti-rabbit IgG, horseradish peroxidase (HRP)-linked Antibody  
7074P2, Cell Signaling Technologies

WesternBright Quantum Kit  
K-12042-D10, Advansta

PageRuler Plus Prestained Protein Ladder, 250 uL  
26619, Thermo

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**Hematology Analysis of Mouse PB**

Assistance provided by Nick Achille. *HemaVet*® 950FS (Drew Scientific, Inc.) was used for analysis of mouse PB.

**Photographs**

Assistance provided by Dr. Xianzhong Ding and Dr. Mitch Denning. Photos were taken using Revolve R4 microscope (ECHO/Bico) and Olympus BX41 microscope/Olympus DP21 camera with cellSens software.

**Histology**

Assistance provided by Lourdcy Pazhampally, Dr. Maria Picken, and Dr. Xianzhong Ding. Isolated tissue(s) were kept in conical tube(s) filled with 10% zinc formalin (313-095, Fisher Healthcare) and placed on gyrating rocker for 2-3 days, until delivery to the LUMC Department of Pathology & Laboratory Medicine.
Sectioning/cutting, embedding, and H&E staining was performed by Lourdcy Pazhampally. Samples transferred to 70% EtOH after zinc formalin-fixation for long-term storage.

**Cytocentrifuge**

45μL was sampled from cell culture and “spun” onto glass slides using the Epredia™ Cytospin™ 4 Cytocentrifuge (A78300003, Thermo-Fisher).

**Hema 3® Kit**

After cytocentrifugation, cells were fixed and stained using the Hema 3® kit (Fisher). Following fixation and both staining steps, slides were briefly submerged in RT ddH$_2$O to rinse.

<table>
<thead>
<tr>
<th>Order of Use</th>
<th>Reagent</th>
<th>Duration</th>
<th>Serial #</th>
</tr>
</thead>
<tbody>
<tr>
<td>1$^{st}$</td>
<td>Fixative</td>
<td>30 seconds</td>
<td>122-929</td>
</tr>
<tr>
<td>2$^{nd}$</td>
<td>Solution I</td>
<td>30 seconds</td>
<td>122-937</td>
</tr>
<tr>
<td>3$^{rd}$</td>
<td>Solution II</td>
<td>15 seconds</td>
<td>122-952</td>
</tr>
</tbody>
</table>
Figures

Where applicable, figures/schematics created with GraphPad Prism (version 9.3.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com) and/or BioRender.com (BioRender.com Basic Plan is suitable for publication of this thesis on ProQuest®, confirmed with Mayet of BioRender.com on 05/23/2023) and/or Microsoft PowerPoint® [412, 413].

Drug Treatments

For in vitro drug treatments (DNO, Ara-C, AMX, and GSK8612), 5.0 x 10^4 MLL-AF9^+ cells were seeded using 100uL liquid media (RPMI-1640/10% FBS/1% Penicillin-Streptomycin) into each well of a 12-well plate. Then, 75uL of 4GFS and an additional 2mL liquid media were added, bringing the total volume in each well to 2.175mL. After seeding, cells were incubated for 24 hours before drug treatment. The \([C_1V_1 = C_2V_2]\) equation was used to calculate the volume of drug to add for the desired final concentration; cells were treated for 24 hours before being collected, washed in 1X PBS, and prepped for FACS analysis.

Drugs

Daunorubicin (HY-13062A [Cerubidine®]) and Ara-C (HY-13605 [Cytosar-U®]) were purchased from MedChemExpress (MCE) and resuspended in ddH₂O. Amlexanox (HY-B0713) and GSK8612 (HY-111941) were also purchased from MCE but required resuspension in dimethyl sulfoxide (DMSO). Busulfan (71288-116-11, Meitheal
Pharmaceuticals \([Busulfex^\text{®}])\) was provided *graciously* by Dr. Patrick Hagan and LUMC [414].

<table>
<thead>
<tr>
<th>Structure of daunorubicin (MCE)</th>
<th><img src="image1" alt="Structure of daunorubicin" /></th>
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<tbody>
<tr>
<td>Structure of Ara-C (MCE)</td>
<td><img src="image2" alt="Structure of Ara-C" /></td>
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<td>Structure of amlexanox (MCE)</td>
<td><img src="image3" alt="Structure of amlexanox" /></td>
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<td>Structure of GSK8612 (MCE)</td>
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We thank Dr. Rolf Brekken (UT Southwestern Medical Center) for graciously providing details regarding Compound II.

**Lysis of RBCs/Hemolysis**

<table>
<thead>
<tr>
<th>10X Hemolytic Buffer Stock</th>
<th>Ratio</th>
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<tr>
<td>NH₄Cl</td>
<td>83g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>10g</td>
</tr>
<tr>
<td>0.5M, pH 8.0 EDTA BP2482, Fisher)</td>
<td>0.37g</td>
</tr>
<tr>
<td>MilliQ ddH₂O</td>
<td>1L</td>
</tr>
</tbody>
</table>

1X working solution created by diluting 100mL 10X stock in 900mL MilliQ ddH₂O.

**Fluorescence-activated Cell Sorting (FACS [Flow Cytometry]) Analyses**

Assistance provided by Pat Simms/Corbin Pomykata/Bert Ladd [FACS core], Ryan Mack/Kanak Joshi/Wei Wei [Zhang lab], and Lola Badmus/Janani Prakash/Nick Achille [Zeleznik-Le lab]. FACS analyses were conducted using LSRFortessa™ (BD Biosciences) the flow cytometry data were analyzed and gating depicted using FlowJo™ v10.8 Software (BD Life Sciences) [415]. Antibody cocktails were created using 1X PBS in the following concentrations and stored using amber microcentrifuge tube(s):
[0.5uL Ab · (#samples + 1)]

[50uL 1X PBS · (#samples + 1)]

Example calculation is:

5 samples to be stained = 3uL desired Ab(s) in 300uL 1X PBS.

With room lights off, cells-to-be-stained were transferred to 5mL polystyrene tube(s) and centrifuged at 1800RPM for 5 minutes. After centrifugation, tube(s) inverted and supernatant allowed to drain; for blood samples, supernatant was vacuumed- or pipetted-off, taking care to leave 100-200uL of supernatant remaining. Tube(s) then flicked to resuspend cells in the 100-200uL residual supernatant. 50uL of desired Ab cocktail was then added to desired samples, tube(s) flicked again and transferred to 4°C for 30-60 minutes for staining. After staining, cells were washed twice with ~3mL 1X PBS via similar centrifugation and brought to FACS core at the Cardinal Bernardin Cancer Center for analysis.

Compensations were created using the following concentrations: 1 drop UltraComp eBeads™ Compensation Beads (01-2222-42, Invitrogen) per 3mL 1X PBS, aliquotting 200uL of the mixture to desired 5mL polystyrene tube(s):

1 drop beads + [200 · (#fluorophores + 1)]uL 1X PBS

Example calculation is:

5 fluorophores in panel = 1 drop beads in 1.2mL 1X PBS
→ aliquot 200uL to each 5mL polystyrene tube(s), including unstained control (6 tube(s) total).
Then, 0.5μL corresponding Ab was added to the 200μL bead-1X PBS mixture aliquotted to each tube(s); tube(s) protected from light and stored in 4°C until analysis.

All antibodies listed below are reactive against mouse antigens (anti-mouse).

<table>
<thead>
<tr>
<th>Antigen-Fluorophore</th>
<th>Manufacturer</th>
<th>Catalog #</th>
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**MilliQ ddH₂O Water**

Molecular biology-grade double distilled water (ddH₂O) was acquired using the MilliQ Direct Water Purification System (MilliQ; ZR0Q008WW, Sigma-Aldrich).

**Open Researcher and Contributor ID (ORCID) iD**

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REFERENCE LIST


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VITA

The author, Austin Runde, was born in Aurora, IL on September 15, 1998 to Dan and Janet Runde. He attended Loyola University Chicago where he earned a Bachelor of Science, *cum laude*, in Molecular Biology in May of 2021. After graduation, Runde matriculated into the Loyola University Chicago Stritch School of Medicine master's degree (MS) program and began his graduate education in the Cellular and Molecular Oncology (CMO) program under the mentorship of Dr. Jiwang Zhang.

Runde's thesis work has served to support the Zhang lab’s application for an R01 grant.

After completion of his graduate studies, Runde will matriculate into the Loyola University Chicago Stritch School of Medicine’s Doctor of Medicine (MD) program, continuing to pursue his dream of becoming a pediatric hematologist-oncologist.