University of Cincinnati

Date: 2/22/2024

I, Jahnavi A Raval, hereby submit this original work as part of the requirements for the degree of Master of Science in Cancer and Cell Biology.

It is entitled:

Investigating co-mutational synergy in TET2 mutant leukemic transformation.

Student's name: **Jahnavi A Raval**

This work and its defense approved by:

Committee member: Andrew Volk, Ph.D.

Committee chair: Linde Miles, Ph.D.

Committee member: Erin Hertlein, Ph.D.

UNIVERSITY OF Cincinnati

48006

Investigating co-mutational synergy in *TET2* mutant leukemic transformation

A thesis submitted to the

Graduate College

of the University of Cincinnati

in partial fulfillment of the

requirements for the degree of

Master of Sciences

In the Department of Cancer and Cell Biology

of the College of Medicine

By

Jahnavi Raval

Bachelor's of Arts and Sciences , Troy University

July 2022

Committee Members:

Linde A. Miles, Ph.D. (Chair)

Erin K. Hertlei, Ph.D.

Andrew Volk, Ph.D.

Abstract

Acute myeloid leukemia (AML) is a rare hematological malignancy characterized by genetic and epigenetic aberrations in hematopoietic progenitor/stem cells (HPSCs). Molecular profiling studies of AML patients have suggested a sequential acquisition of co-occurring mutations during leukemogenesis, with mutations in epigenetic regulatory genes like *TET2* and *DNMT3A* often serving as the initiating events due to their high variant allele frequencies (VAF). Moreover, these initiating mutations exhibit a key role in clonal hematopoiesis (CH), a state marked by the expansion of dominant mutant HPSC clones without evident disease. Concurrent mutations, notably in genes such as *NPM1*, *NRAS*, and *FLT3*, are known to co-occur with epigenetic modifier mutations and are believed to manifest within a pre-existing mutant clone. While targeted therapies have been established for specific AML mutations like *FLT3* and *IDH1/2*, treating patients lacking these mutations or those who develop resistance remains a critical challenge. Recent strides in single-cell sequencing have revolutionized our comprehension of AML pathogenesis by unveiling the clonal architecture and mutation order, which is not possible through bulk sequencing approaches. Leveraging technologies such as single-cell targeted DNA sequencing, we and others have dissected genetic variations at the single-cell level, unraveling how specific combinations of mutations, such as *TET2/NPM1*, can synergistically propel leukemogenesis, resulting in the amplification of double mutant clones compared to single mutant clones. Furthermore, single-cell multi-omics investigations have delineated that the co-mutational context exerts a profound impact on the differentiation and immunophenotype of transformed cells. Investigating distinct genotypic states in AML aims to furnish invaluable insights into the intricate web of co-mutational synergy and the influence of mutation chronology on leukemia progression, ultimately directing the development of genotype-specific therapeutic strategies and furthering the era of precision medicine in AML management.

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Acknowledgement

I am filled with gratitude for the incredible individuals and institutions whose support and guidance have been instrumental to my work. Dr. Linde Miles' exceptional mentorship has been a beacon of inspiration and excellence. Her dedication to science, combined with a genuine commitment to nurturing my development as a researcher, has profoundly shaped my approach to scientific inquiry. I am deeply grateful for her unwavering support, insightful guidance, and the invaluable lessons I have learned under her tutelage. My heartfelt thanks for the constructive feedback and expert advice from my thesis committee members, Dr. Andrew Volk, and Dr. Erin Hertlein. Their thorough reviews, thoughtful questions, and encouraging words have significantly contributed to refining my research and broadening my perspective. Their contributions have been vital in enhancing the quality and depth of my thesis. The success of this research also owes much to the diligent assistance and collaboration of Robert Beal and Ashley Wooten, Research Assistants in our lab. Their expertise, dedication, and teamwork have been fundamental in navigating the challenges of our research endeavors. Robert's meticulous attention to experimental detail and Ashley's analytical skills have greatly complemented our work, making invaluable contributions to our project's success. A special acknowledgment goes to Dr. Bobby Bowman for his major contributions to the data analysis aspect of this research. His expertise in data interpretation and statistical analysis has been invaluable, providing clarity and depth to our findings. I extend my heartfelt gratitude to Cincinnati Children's Hospital for providing exceptional facilities and a supportive research environment. Last but certainly not least, I am profoundly thankful to the UC Cancer and Cell Biology Department for their constant support and encouragement. The department's commitment to fostering an environment of academic excellence and innovation has been a constant source of motivation, providing me with the opportunities and resources necessary for success.

Content

List of figures, table and illustration

Introduction

Acute Myeloid Leukemia (AML) stands as a formidable challenge in the realm of hematologic cancers, characterized by its aggressive nature and the broad diversity in its genetic and clinical presentation. According to the American Cancer Society's estimates for leukemia in the United States, AML demonstrates a significant impact with an anticipated 20,050 new diagnoses [Siegel RL et al, 2022]. The mortality associated with AML is substantial, with an estimated mortality rate of approximately 57.5% [Siegel RL et al, 2022]. The prevalence of AML predominantly in adults positions it as one of the more common types of leukemia within this demographic, despite it constituting only around 1% of all cancer cases emphasizing the disease's severity and the critical need for effective treatments and interventions. AML is not a singular disease but rather a compilation of disorders exhibiting diverse biological behaviors and outcomes, complicating both its treatment and prognosis. Recent observations suggest an emerging perspective that AML arises from the clonal proliferation of hematopoietic stem and progenitor cells. These cells acquire somatic mutations, leading to the disruption of normal blood cell development and function [Karantanou, C, 2022]. The discovery of specific genetic mutations and chromosomal abnormalities has profoundly influenced our understanding of AML's pathogenesis, leading to more precise diagnostic classifications and prognostic assessments.

The landmark discovery of identifying AML as a genetic disease paved the way for a deeper exploration of its genetic landscape [Grimwade, D., 2016]. In the context of AML, the 2-class hypothesis provides a comprehensive framework for understanding the development of the disease. This hypothesis suggests that the occurrence of AML involves the presence of at least one mutation from class 1, typically related to epigenetic regulators, and one from class 2, primarily associated with alterations in signaling pathways. This dual-hit model emphasizes the interaction

between disruptions in epigenetic processes and irregular signaling, both of which play crucial roles in the transformation of normal cells into leukemic cells [Conway et al, 2014].

Mutations in specific genes, such as *TET2*, *NPM1*, and *NRAS*, serve as examples of the convergence of these two classes of mutations and their importance in the progression of AML. *TET2* mutations, categorized under class 1, contribute to changes in DNA methylation patterns, leading to instability in epigenetic processes and abnormal expression of genes. Conversely, mutations in *NPM1* and *NRAS* fall into class 2, impacting signaling pathways essential for cell proliferation, survival, and differentiation. With advancements in sequencing technologies, researchers have unveiled a plethora of mutations that not only refine the diagnosis and prognosis of AML but also offer new avenues for targeted therapies. Among these, mutations in genes like *FLT3, NPM1, CEBPA, IDH1, IDH2, ASXL1, MLL, DNMT3A,* and *TET2* have been spotlighted for their roles in disease progression and their potential as therapeutic targets [Papaemmanuil et al, 2016] [Ley et al, 2013].

Recent insights into the clonal architecture of AML, facilitated by single-cell mutational profiling, have revealed a complex picture of clonal evolution and diversity [Morita K et al, 2020] [Miles LA, 2020]. This intricacy is further compounded by the presence of co-occurring mutations, particularly those affecting epigenetic regulators and signaling pathways, which synergize to drive the clonal expansion and dominance that characterize AML [Morita K et al, 2020] [Miles LA, 2020]. The integration of these genetic findings with functional protein expression profiling opens new doors for understanding AML's pathobiology and for developing personalized treatment strategies.

The evolving genetic landscape of AML, underscored by the significance of clonal hematopoiesis and the interplay of co-occurring mutations, underscores a pivotal shift in the approach to

managing this disease. It highlights the need for personalized therapeutic interventions that leverage our growing understanding of its genetic underpinnings. As we continue to unravel the complexities of AML, our strategies for diagnosis, risk stratification, and treatment are poised for transformation, heralding a new era of precision medicine in the battle against this challenging disease.

Chapter 1: Characterizing clonal evolution in myeloid malignancies.

Introduction

Clonal hematopoiesis (CH) is a condition marked by the predominance of blood cells originating from a single genetic clone, a phenomenon initially linked to blood cancers but later found in individuals without cancer, revealing its broader occurrence across the population [Bowman et al, 2018]. This discovery, propelled by advances in genome sequencing, showed that clonal expansions often result from mutations in specific genes involved in DNA methylation and chromatin regulation, and are more prevalent with aging. [Jaiswal, S., & Ebert, B. L., 2019]. Exploring the role of clonal fitness and its impact on disease evolution, especially in blood cancers, has become a vital area of medical research. Throughout life, cells accumulate genetic mutations due to various internal and external stresses, whether passive (i.e., harmless), deleterious (i.e., harmful), or occasionally beneficial, providing the mutated cell a competitive edge—known as clonal expansion. This process is pivotal in cancer development, where mutated cells typically outgrow healthy ones, potentially leading to cancer. Our ongoing research, alongside contributions from other labs, is unraveling how specific mutations enhance the fitness of cancer cells in leukemia, particularly examining how different mutations collaborate to expedite disease progression.

The cellular complexity of the hematopoietic system, with its diverse cell types and regeneration capabilities, provides a unique lens to study how clonal expansion influences disease. Mutations in hematopoietic stem and progenitor cells can disrupt their normal growth and lead to an overgrowth of certain cell types, the condition also known as CH. This not only significantly increases the risk of developing leukemia but can also affect the body's other organs through the

malfunctioning of mutated blood cells, including cardiovascular diseases through enhanced inflammation, metabolic disorders like diabetes due to altered insulin sensitivity, and age-related conditions, reflecting its extensive influence beyond hematological diseases.

Delving into the genetics and epigenetics of clonal expansion, particularly in CH, reveals that the most common mutations affect genes controlling epigenetic states and gene splicing. These mutations can lead to changes in DNA methylation and histone modification, crucial for gene regulation. For example, mutations in *TET2* can increase DNA methylation, a common feature in blood cancers and CH [Cimmino et al, 2011]. Studies show how these mutations, and their interactions, can significantly influence blood stem cell behavior and disease progression.

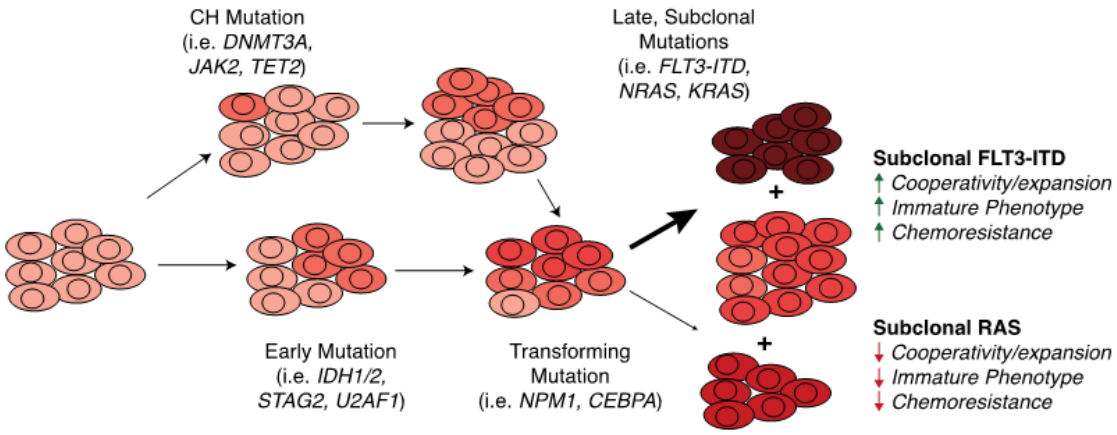


Figure 1: Representation of clonal hematopoiesis with sequential mutation acquisition based on previously determined Variant Allele Frequencies. Here, the epigenetic modifiers (*TET2; DNMT3A; IDH1/2*) imitates as early mutations and (*NPM1; RAS; FLT3*) are later acquired. Figure by Dr. Linde Miles.

Interestingly, the lack of specific CH mutations, which are frequently found in AML, points to a possible divergence in the pathogenesis of the disease. It also highlights the complexity of stem cell regulation and its implications for cancer development, as the interaction between different mutations can determine whether CH progresses to more severe conditions. In our exploration of CH, it is crucial to consider the ramifications of sequential mutations on disease progression. As depicted in Figure 1, it has been observed that early epigenetic modifiers like *DNMT3A, JAK2*, and *TET2* lay the groundwork for later genomic alterations as they initiate the stepwise acquisition of mutations [Shih et al, 2017]. The emergence of transforming mutations, such as *CEBPA* or *NPM1*, signifies a pivotal shift in the pathogenesis of leukemia. These mutations represent a critical juncture in leukemogenesis, as they contribute to the malignant transformation of hematopoietic cells. The mutations in *CEBPA*, a transcription factor critical for myeloid differentiation, and *NPM1*, which is involved in ribosome biogenesis and nuclear-cytoplasmic transport, underscore a mechanistic evolution in how leukemia cells proliferate and evade apoptosis. Their presence can markedly influence the disease's prognosis, treatment response, and overall clinical management strategies. However, the role of late, sub clonal mutations, particularly in genes such as *FLT3* and *RAS*, must not be overlooked. For instance, *FLT3-ITD* mutations foster increased cell proliferation and an immature phenotype, as well as contribute to chemoresistance [Dovey, 2017]. Similarly, mutations in *NRAS* are associated with enhanced cooperativity and expansion of leukemic clones, further exacerbating the disease. The contrast between *NRAS* and *FLT3* mutations highlights the complex interplay of genetic factors that can either drive CH towards full-blown leukemia or maintain a more indolent course. By integrating the data shown in Figure 1, we gain a more nuanced understanding of how specific downstream mutations contribute to the heterogeneity and aggressiveness of blood disorders. By understanding the genetic and epigenetic landscape of CH,

we gain valuable insights into blood diseases' origins and potential treatment targets. This research underscores the intricate balance of genetic factors that govern stem cell fate and disease transformation, offering clues to tackling these challenging conditions.

Materials and Methods

Transgenic Mouse Lines

C57Bl/6J Cre-inducible Tet2$^{-/-}$ (#017573), Nras^{G12D} (#008304) and Rosa26: TdTomato reporter (#007909) mice, tamoxifen inducible Rosa26: FlpO-ER (#018906) mice, and Flp-inducible *Npm1*^{cA} (#033164) mice were purchased from Jackson Laboratory. Mice were housed in specific pathogen-free animal facilities at the Cincinnati Children's Hospital Medical Center. Progressive breeders were utilized to generated compound allele mice. Genotyped mice were bred for offsprings to be *Tet2*$^{-/-}$, *Tet2*$^{-/-}$/*Npm1*^{cA} and *Tet2*$^{-/-}$/*Npm1*^{cA}/*Nras*^{G12D} transgenic mutant models. Every litter was genotyped, and data recorded on Transnetyx Genetic Monitoring services. Mice aged 6-10 weeks old were used for these studies. Mice were euthanized prior to bone marrow harvest. All methods and protocols were approved by the Animal Care and Committee of the Cincinnati Children's Hospital Medical Center.

BMEC (Bone Marrow Endothelial Cells) culture

Cryogenically stored vial of WT murine bone marrow endothelial cells is thawed at 37°C, pelleted, then resuspended in BMEC Media. Counted and cultured 1,000,000 cells in 13 mL media in a Fibronectin applied TC treated dishes (Corning) (incubated at 37°C with 5% CO_2). For cell splitting, 1mL/well Fibronectin is applied and left to adhere for 15-20 minutes at room temperature in a 12-well TC treated plate. Meanwhile, existing BMEC cells are harvested by rinsing with PBS, and then treating with Accutase. After incubation, cells are washed with BMEC media and collected in a falcon tube before centrifugation. The pellet is resuspended in fresh BMEC media, cell counted with AO/PI. 100,000 cells plated per well. BMECs were cultured 48-72 hours prior to HPSCs co-culture. (Jason Butler Laboratory, 2020)

Experimental approach layout

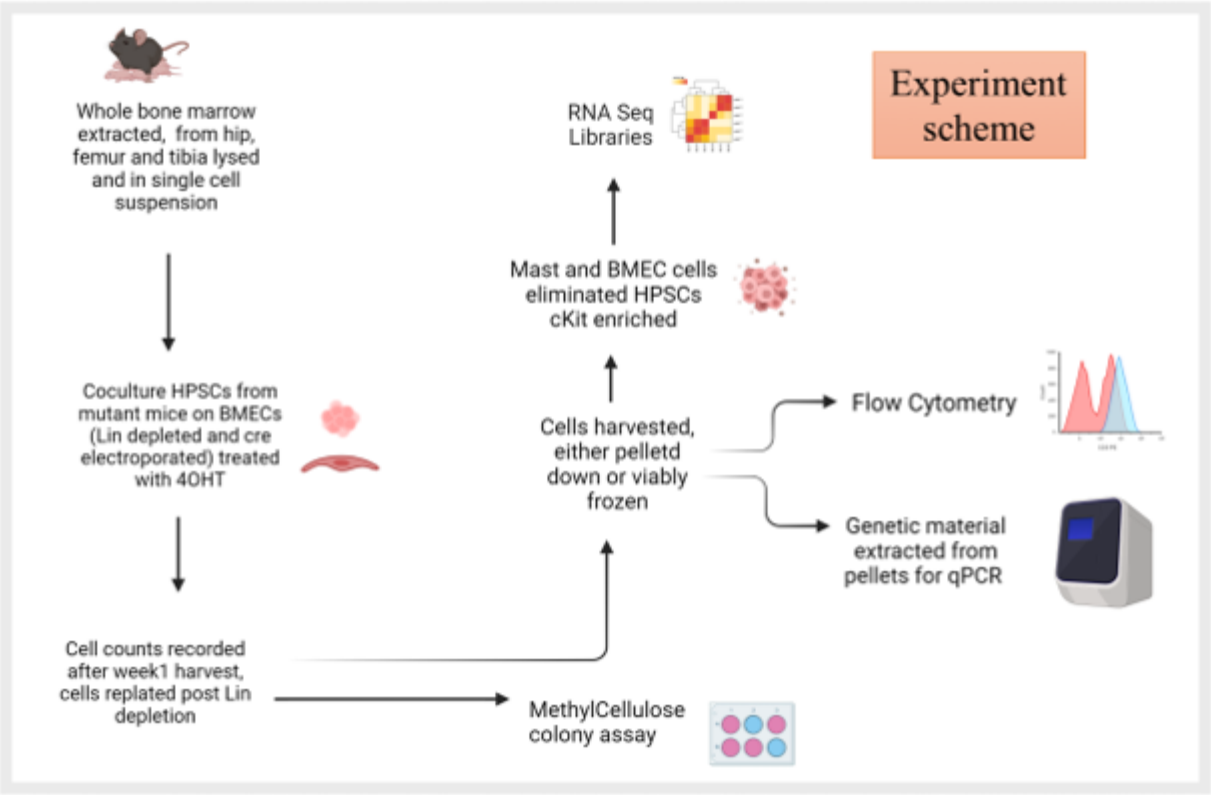


Illustration 1: The schematic representation of the entire experimental approach illustrating the flow of experiments from extraction of whole bone marrow cells, cell culture assay to the downstream experiments after week1 harvest of the cell culture; Colony forming/Methylcellulose assay and after the week 2 harvest, Flow cytometry, qPCR, and RNA Seq Libraries.

Cell culture assays

Murine whole bone marrow (WBM) was harvested from the hip, femur and tibia of the transgenic mice models. The WBM was subsequently depleted of mature cells using a Lineage marker depletion kit (EasySep™ Mouse Hematopoietic Progenitor Cell Isolation Kit; Catalog # 19856), electroporated with Cre mRNA (1mg/mL) (TriLink), and then plated at a density of 50,000

cells/well on BMECs (Bone marrow endothelial cells) in a 12 well TC-treated plate (Corning). Twenty-four hours post plating, cells were dosed with 4-hydroxytamoxifen (4OHT; 400nM) for 7 days. After 1 week in culture, cells were harvested, counted, depleted of cells expressing mature lineage markers including BMEC and mast cell markers (EasySep supplemented with Biolegend anti-mouseCD31, clone MEC 13.3 and anti-mouse FcƐRia, clone MAR-1), and re-plated on new BMECs for an additional 7 days. After the two-week total culture, cells from 3 wells/plate were counted and pelleted for RNA extraction. The cells harvested from the remaining wells were viably frozen in 90% FBS supplemented with 10% DMSO. Competitive studies were carried out using the same method with the exception of the $5.0x10^4$ cells/well consisting of a 1:1 ratio of wildtype (CD45.1) and mutant (*Tet2*$^{-/-}$, *Tet2*$^{-/-}$/*Npm1*^{cA} or *Tet2*$^{-/-}$/*Npm1*^{cA}/*Nras*^{G12D}).

Colony-forming assays

WBM was harvested from transgenic mice as above. Cells were electroporated with Cre and subsequently dosed with 4OHT for 1 week on BMECs as above. Post 7 days of culture, lineage negative cells were isolated from the BMEC culture, suspended in cytokine-containing methylcellulose-based media (MethoCult™ GF M3434), and plated ($1.5x10^4$/well) in duplicate wells of 6-well plates. Colony-forming units (CFUs) were counted using a Nikon (Eclipse Ts2). Cells were harvested from the MethoCult by washing off the semisolid media with PBS Buffer, spun and resuspended the cell pellet in 1mL of IMDM. Harvested cells counted and cultured again at 2,500 cells/well. Subsequent replatings were performed similarly every 7 days for a maximum of 8 weeks.

Gene expression with qPCR

The pelleted cells post each harvest were used to extract RNA (QIAwave RNA Mini Kit). Total RNA was then quantified and used as a template to generate cDNA using the Verso cDNA synthesis kit. Quantified cDNA was then used for real-time PCR with Power SYBR™ Green PCR Master Mix, primers LAM 248[1]/249[2] and LAM 248/250[3] following holding stage (50℃ for 2min, 95℃ for 10 min) and 40 cycles of (95℃ for 15 sec and 60℃ for 1 min). Furthermore, another qPCR with TaqMan Universal PCR master mix was performed with specific targeted genes and predesigned primers targeting *HoxA9, HoxB4, HoxA13* and *Meis1*. *Gapdh* was used as the reference gene. Each sample was measured in triplicate, and the threshold cycle (Ct) was determined for each gene. The ΔΔCt method was employed to evaluate gene expression changes.

Flow cytometry.

Cells post 2-week harvest were resuspended in FACS buffer and split equally to generate two samples from each well. Cells were pelleted and subsequently resuspended in 100uL of appropriate Pre-stain (Pre-stain A or B) and vortexed, after which the samples were stored at 4°C for 15-20 minutes (**Table 1**). Pre-stain A allows for the further resolution of HSCs within the LSK (Lin-Sca1+cKit+) gate. Pre-stain B resolves myeloid progenitors (MPs) and mature myeloid cells. Following this, 100uL of Stain (Stain A/B resolves Lineage negative populations, i.e. LSKs and LK/MPs) was added directly to each tube without removing the matching Pre-stain, and the tubes were vortexed again before being stored at 4°C for 15-20 minutes. After centrifugation, each pellet was resuspended in 200uL of DAPI (300 nM) stain solution. The tubes were vortexed and stored

[1] *Npm1* Universal Forward primer
[2] *Npm1* Reverse primer
[3] *Npm1c* Reverse primer

at 4°C until further use. The flow cytometric analysis was conducted on a BD LSR Fortessa maintained by the CCHMC Research Flow Cytometry Core shared facility. Results were then analyzed on Flowjo and plotted via GraphPad Prism.

Staining antibodies	
Pre stain A	CD11b - APC Cy7
	CD150 - BV605
	CD48 - PerCPCy5.5
	Gr1 - Alexa700
Pre stain B	CD16/32 (FcgR) - APC Cy7
	CD11b - BV605
	Gr1 - PerCPCy5.5
	CD34 - Alexa700
Stain A and B	Lineage cocktail - BV421
	Sca1 - PE Cy7
	CD117/cKIT - APC
	CD45.2 - FITC
	CD45.1 – PE
	Fc1εrA - Pac Blue

Table 1: List of antibodies for flow cytometry

Statistics

All data are represented as mean ± SEM. Comparisons between two samples were done using the unpaired *t* tests. Two-way ANOVA analyses followed by multiple unpaired t-tests were used for multiple group comparisons. Statistical analyses were performed with Graph Pad Prism 10 software. * P <0.1, **P<0.05, ***P<0.01, ns is non-significant.

Results

Characterizing the three distinct genotypes/genetic states: initiation (*Tet2*$^{-/-}$), pre-leukemia (*Tet2*$^{-/-}$; *Npm1*$^{cA/wt}$), and overt disease (*Tet2*$^{-/-}$; *Npm1*$^{cA/wt}$, *Nras*$^{G12D/wt}$).

The *HOX* genes, vital for embryonic development and hematopoiesis, are significantly linked to the onset of multiple hematologic malignancy sub-types when their expression is altered [Nagy, a., et.al. 2019]. Specifically, genome-wide analyses reveal overexpression of certain *HOXA* and *HOXB* loci, along with their co-factors, in AML with a normal karyotype [Spencer et al, 2015]. MEIS proteins are recognized as proto-oncogenes, collaborating with *HOX* genes in leukemia development. Studies have shown that HMP (*HOX, MEIS, PBX*) proteins can interact with PBX and HOX proteins to form complexes. While PBX-HOX complexes are known to attract histone deacetylases (HDACs) to suppress transcription, MEIS proteins oppose HDAC activity, thus promoting transcription initiation [Chang et al, 1997]. This dynamic interplay highlights the intricate mechanisms through which *MEIS* and *HOX* genes contribute to leukemia progression by modulating gene expression patterns.

To begin to characterize these specific genotypic states, we first wanted to look at the gene expression differences for important loci involved in stem cell and differentiation programs. We performed a qPCR with specific target genes. Notably, qPCR analysis in our mutant murine cells depicts a gradual upregulation of *HoxA13* (**Figure 2a**) and *HoxA9* (**Figure 2b**) with the addition of the secondary/downstream mutations (i.e. *Npm1/Nras*) aligning with the hypothesis of constructive interaction in the co-occurring mutations leading to disease progression. Surprisingly, an upregulation of *HoxA9* (**Figure 2b**) and *HoxB4* (**Figure 2d**) was observed in *Tet2* mutant clones of AML that could be a contributor to the blockage of differentiation and the proliferation of leukemic cells. Dysregulated expression of these Hox genes can alter the normal regulatory

pathways that control hematopoiesis, leading to the development and progression of AML. Our data (**Figure 2a-d**) aligns with the previous research that has established that the *Hox* gene family and *Meis1* are markedly overexpressed during the process of leukemogenesis [Nagy, a., et.al. 2019]. Understanding the composition and function of HMP complexes sheds light on the molecular mechanisms underlying AML pathogenesis and offers potential targets for therapeutic intervention.

Observation also highlighted a notable expression of the mutant *Npm1c* transcript in both double and triple mutant models, suggesting that *Npm1c* expression is not lost during disease progression or subsequent acquisition of concurrent mutations (**Figure 2e**).

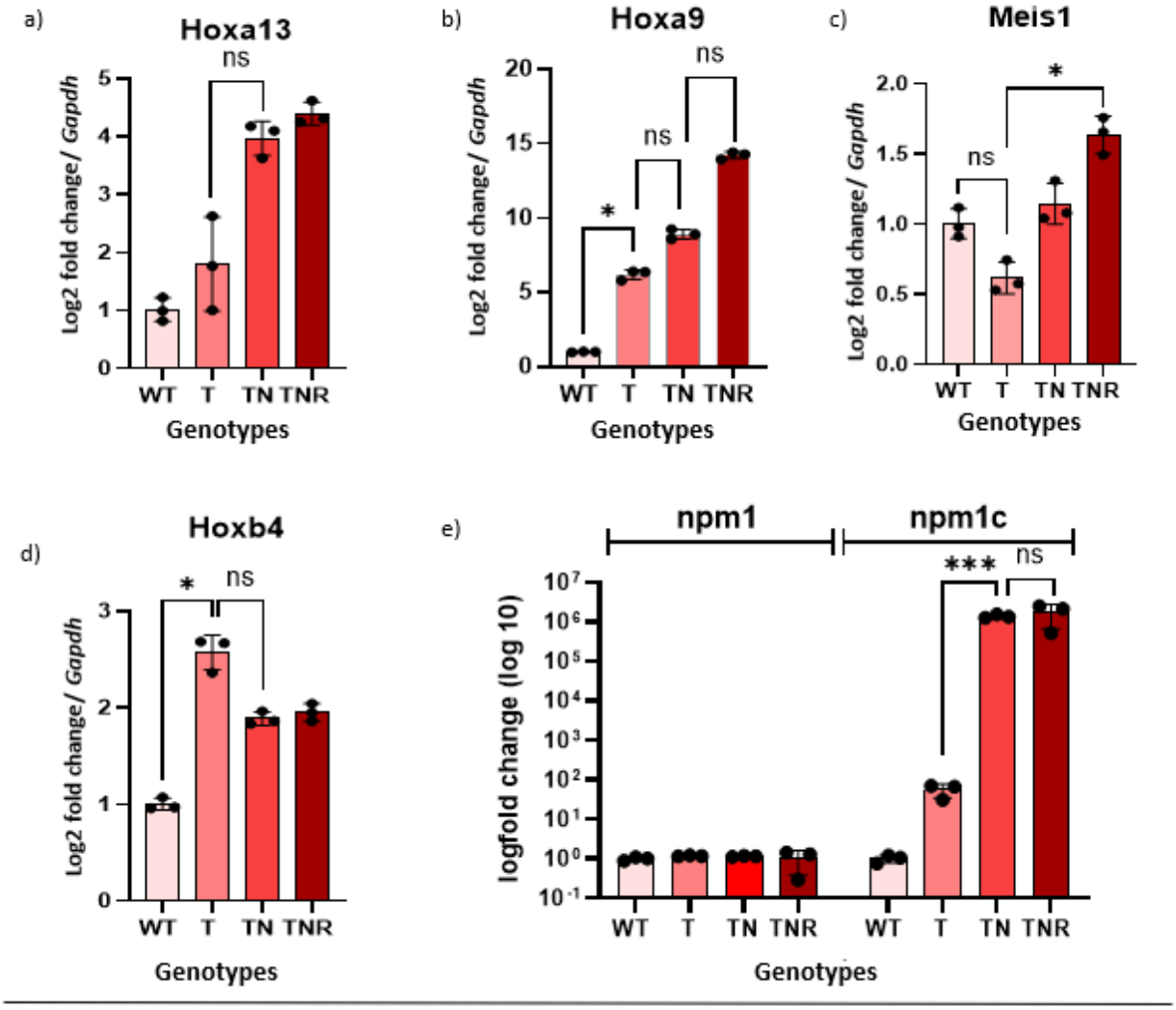


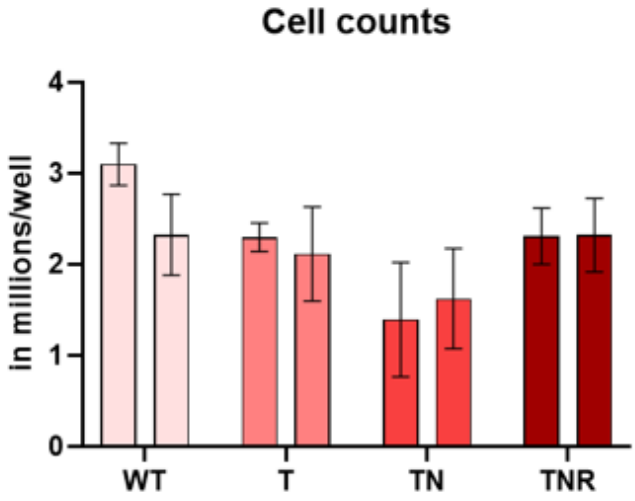
Figure 2: 11 qPCR gene expression analysis based on log fold change. Log fold change in targeted genes a) Hoxa13, b) Hoxa9, c) Meis1 d) Hoxb4 across the four genotypes with y axis representing log fold changes and the genotypes listed on x axis. e) the first set of bars represents the wildtype npm1 expression and the later set is the mutant npm1 expression among all four genotypes. Here, WT- $Tet2^{wt/wt}$; $Npm1^{wt/wt}$, $Nras^{wt/wt}$, T-$Tet2^{-/-}$; $Npm1^{wt/wt}$, $Nras^{wt/wt}$, TN- $Tet2^{-/-}$; $Npm1^{cA/wt,}$ $Nras^{wt/wt}$ and TNR- $Tet2^{-/-}$; $Npm1^{cA/wt}$, $Nras^{G12D/wt}$ - *p < 0.1, **p < 0.05, and "ns" denoting not significant.

In the bone marrow microenvironment, hematopoietic stem cells (HSCs) are situated proximal to the vasculature, indicating a potential requirement for direct cellular interaction in the preservation of indigenous stem cell populations [Kunisaki et al, 2013]. The BMEC-Akt1 variant exhibits the ability to maintain authentic long-term hematopoietic stem cells (LT-HSCs) within an ex vivo environment; this delineated co-culture methodology providing a robust platform for discovering and evaluating growth factors that regulate the quiescence, self-renewal, and proliferation of LT-HPSCs [Poulus et al, 2015]. To compare fitness of mutant cells to WT counterparts and evaluate if co-mutational synergy contributes to clonal fitness, we plated equal number of HPSCs extracted from each genotype, individually, on bone marrow endothelial cells (BMECs), which allow for the expansion of mutant cells and flux through myelopoietic differentiation while sustaining a pool of viable HPSCs. Cells were cultured for 2 weeks total with cell counts taken at the end of week 1 and 2. Even with non-significant differences in observation, it is plausible that the double and triple mutant clones have a higher growth at week 2 harvest as compared to respective week 1, which is not the case for wildtype and single mutant indicating potential increased cellular fitness in stem like non-differentiating cells (**Figure 3a**). We also observed a decreased cell count in the double mutant clone by 30% as compared to the single and triple mutant clones post the two-week harvest (**Figure 3a**). This finding is potentially indicative of variations in differentiation status, apoptotic rates, cell cycle dynamics, or microenvironmental influences.

Normal hematopoietic differentiation has been shown to initiate from within a specialized group of cells recognized as long-term repopulating hematopoietic stem cells (LT-HPSCs) [Orkin and Zon, 2008]. These stem cells undergo a distinctive asymmetric division, giving rise to two distinct cell types; One cell maintains its LT-HPSC identity, preserving the stem cell pool, while the other follows a trajectory towards ultimately culminating in a fully differentiated mature blood cell

[Orkin and Zon, 2008]. The capability of cells to self-renew is typically thought to be restricted to the hematopoietic stem cell pool. However, this ability is hijacked in mutant leukemia blasts and is thought to be due to the consequences of specific mutations commonly seen in AML. To directly assess the HPSC self-renewing capacity, the methylcellulose colony-forming assay is employed as a rapid *in vitro* method [Orkin and Zon, 2008]. We performed replating assays with cells harvested from the BMEC co-cultures for up to 8 weeks. A substantial increase in colony formation and replating potential in both double and triple mutants was observed as compared to wildtype and single mutant cells, indicating the presence of a dominant stem cell-enriched population, thus highlighting a significant elevation in self-renewing capacity, particularly in the *Tet2*$^{-/-}$; *Npm1*$^{cA/wt}$ and *Tet2*$^{-/-}$*; Npm1*$^{cA/wt}$*, Nras*$^{G12D/wt}$ groups (**Figure 3b**). Thereafter, the harvested cell counts data indicated higher cell counts in wildtype and single mutant as compared to double and triple mutant, which is contrary to the colony forming assays. At the same time, both the assays indicate higher stem like self-renewing and regeneration capacities of double and triple mutant based on their replating numbers.

a)



b)

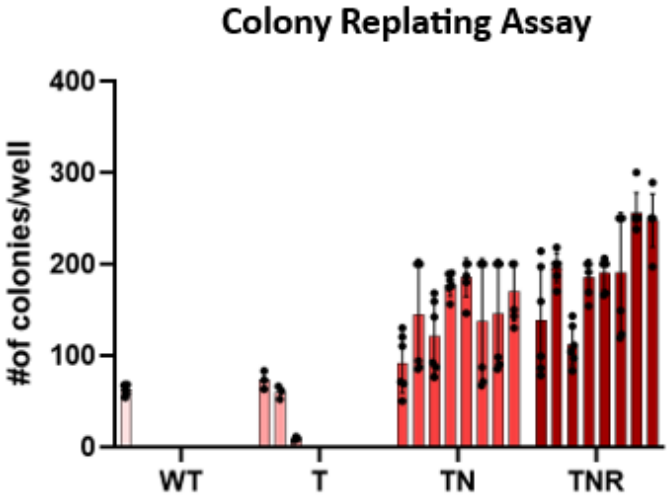


Figure 3: Cell proliferation and fitness assay. a) average cells count after week 1 and week 2 harvest. b) Colony formation assay; an average of colony counts per genotype, represented by each bar at a 7-day time point

The use of multiparameter flow cytometry for immunophenotypic analysis in AML is a potent method for accurately distinguishing between lymphoid and myeloid lineages. This approach is essential in contemporary diagnosis and the effective management of the disease. In mouse models which express transgenes and/or marker genes like enhanced green fluorescent protein (EGFP) in hematopoietic cells, flow cytometry allows for the straightforward isolation of leukemic cells [Dohner, H et. al 2017]. Following the observation of a potential increase in self-renewing capacity among genotypes with concurrent mutations after replatings, in cell proliferation and fitness assays, we hypothesized the possible emergence of immature cell populations within these specific genotypes. To investigate this, we performed flow cytometry analysis of hematopoietic cells harvested from 2-week BMEC co-cultures to understand the immunophenotypic distribution of the cells carrying respective mutations. We observed a significant increase in Lineage negative cell populations observed in double mutant and triple mutant (**Figure 4a**). This is contrary to the previous results where double mutant cells have a relative stunted growth as compared to other genotypes (**Figure 3a**). However, integrating these findings strongly indicates the potential immature cell accumulation for the double mutant cells concomitant with lower cell replication and expansion.

Additionally, we observed differences in specific subpopulations[4] of cells within the lineage-negative cell compartments across the genotypes. Specifically, we found an increased percentage of LSKs cells in the double mutant compared to single, suggesting an expansion of a population that includes HPSCs and MPPs, which have previously been associated with leukemogenesis

[4] LSKs: Lin-Sca1+cKit+ (Hematopoietic stem and progenitor cells), MPPs: Multipotent progenitors, MPs: Myeloid progenitors, FcGR+ MPs: Fc gamma receptor positive myeloid progenitors and Lin-CD11b+: Lineage negative, CD11b positive cells

[Kumar et al, 2008]. Similarly, the increased percentage of MP in the triple mutant suggested an increase in myeloid lineage differentiation, a phenomenon often observed in myeloid leukemias (**Figure 4b**). The changes in cell populations among the genotypes reflected the underlying genetic alterations driving leukemic transformation and progression. For instance, an increased proportion of immature progenitors and alterations in specific lineage pathways were indicative of the disease stage and aggressiveness, as well as potential targets for therapeutic intervention. The data implied that the genotypes in question had distinct impacts on hematopoietic cell populations, which could correlate with their role in leukemic progression and provided insights into the disease mechanism.

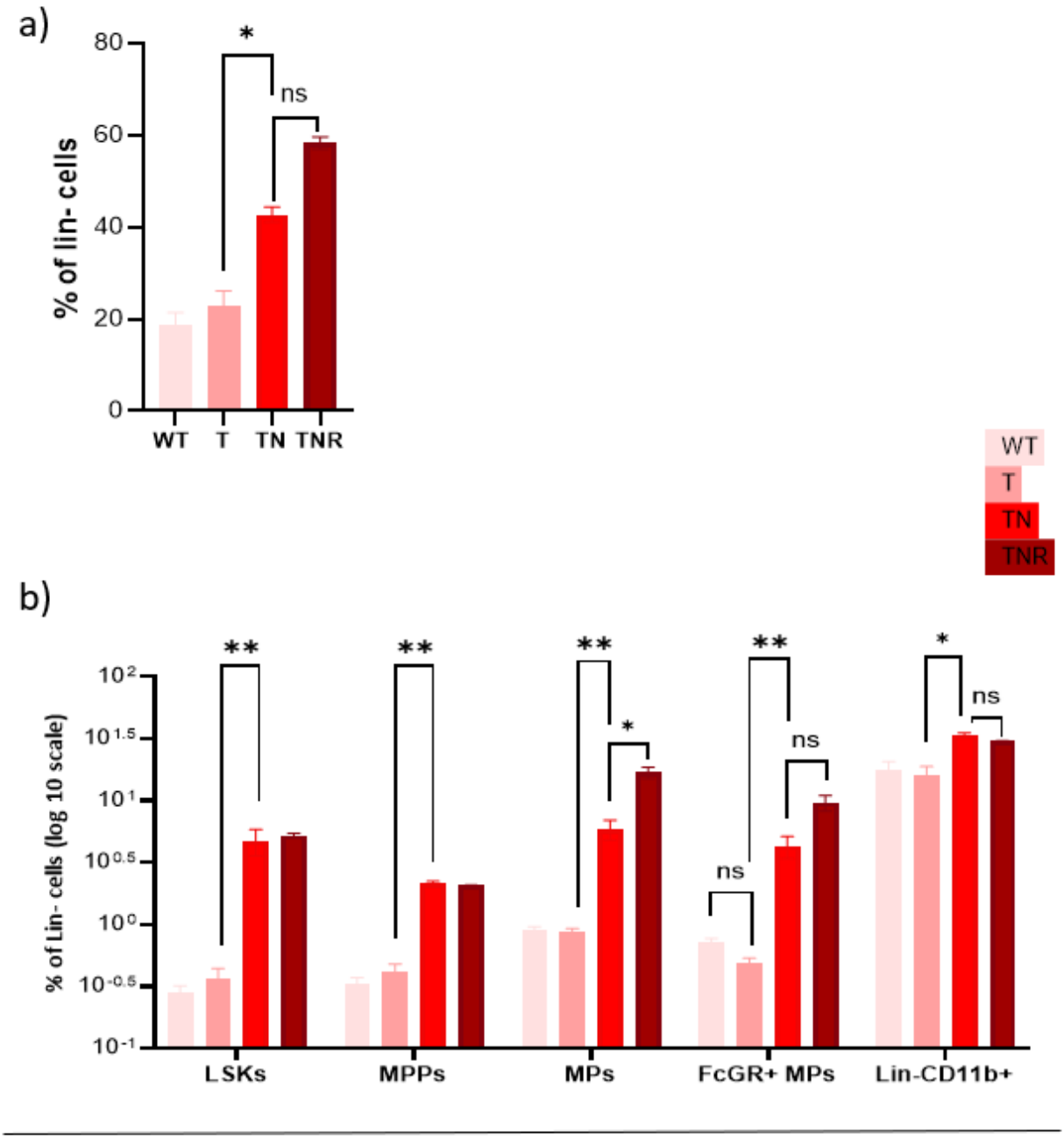


Figure 4: Immunophenotyping of individual genotype obtained via flow cytometry. a) Lineage negative cell population out of the total live cells within individual genotypes. b) Different cell populations within the Lineage negative cocktail of respective genotypes. *$p < 0.1$, **$p < 0.05$, and "ns" denoting not significant.

Competitive analysis to evaluate the clonal fitness of different genetic states of AML progression.

The optimal evaluation of the mutants' clonal fitness occurs in a competitive environment, where their survival and dominance are challenged by another genotype. To test this, mutant cells were cultured in a 1:1 ratio with the wildtype to assess both fitness and the impact of mutants. We hypothesized that the quantitation of the proportion of wildtype versus mutant cells in a competitive environment allows for us to compare cellular compartments and overall clonal fitness. The 2-week harvest cell counts even under competetive setting aligns with our previous cell count data (**Figure 3a**), highlighting increased cell fitness and survival of double and triple mutant at week2 compared to respective week1, which is not the case for single mutant (**Figure 5a**).

TET2 loss-of-function mutations distinctively affect hematopoiesis by promoting hypermethylation, thereby leading to an expansion of LSKs and a reduction in 5-hydroxymethylcytosine levels, in contrast to the hypomethylation observed with *DNMT3A* mutations. This alteration in methylation patterns implicates *TET2* mutations in the intricate regulation of HPSCs function, pointing towards a multifaceted mechanism that disrupts the balance of self-renewal and differentiation [Bowman et al, 2018]. Parallely in our competetive study, we observed *Tet2*-only clones displayed pronounced dominance while observing LSKs, MPs and overall lineage negative cocktail as compared to the double and triple mutant clones (**Figure 5b-d**).

A non-significant escalation in clonal fitness was also observed in triple mutant as compared to the double among the lineage negative population (**Figure 5c**). This hierarchy of clonal fitness, particularly evident in LSK and overall lineage-negative populations assessed underscoring the

critical role of *Tet2* mutations in driving clonal expansion and fitness within the leukemic landscape.

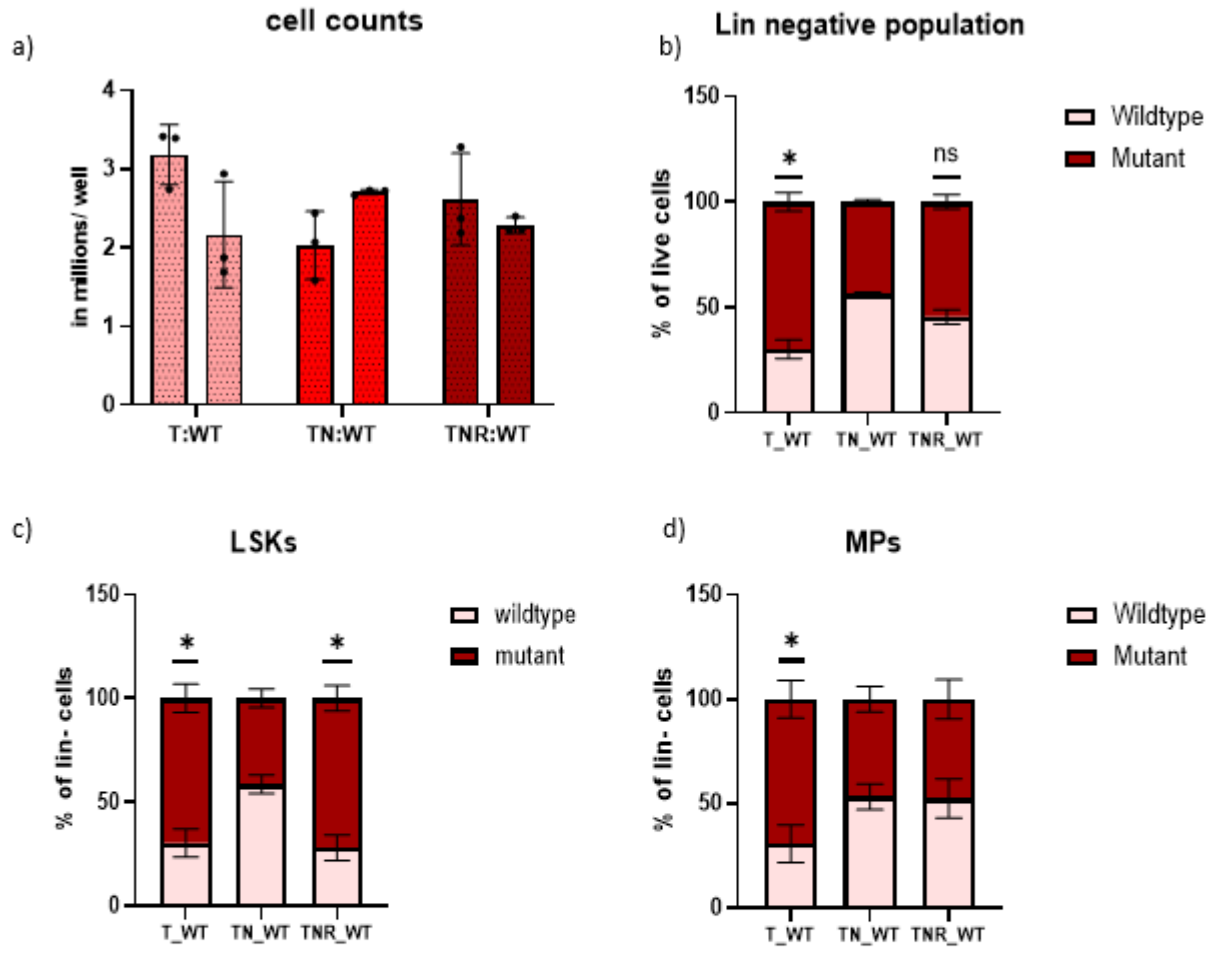


Figure 5 Competitive Study analysis: Individual cell population growth distribution among the co-culture and the differences among the three combinations. Here, T_WT is *Tet2*$^{-/-}$; *Npm1*$^{wt/wt}$, *Nras*$^{wt/wt}$: *Tet2*$^{wt/wt}$; *Npm1*$^{wt/wt}$, *Nras*$^{wt/wt}$, TN_WT *is Tet2*$^{-/-}$; *Npm1*$^{cA/wt,}$ *Nras*$^{wt/wt}$: *Tet2*$^{wt/wt}$; *Npm1*$^{wt/wt}$, *Nras*$^{wt/wt}$ and TNR_WT is *Tet2*$^{-/-}$; *Npm1*$^{cA/wt}$, *Nras*$^{G12D/wt}$: *Tet2*$^{wt/wt}$; *Npm1*$^{wt/wt}$, *Nras*$^{wt/wt}$, co-cultured in a 1:1 ratio. a) The average harvested cell counts after 7-day culture. b) The Lin negative population distribution out of all the live cells, characterized further into individual c) LSKs and d) Myeloid Progenitor populations. *p < 0.1, **p < 0.05, and "ns" denoting not significant.

Discussion

The research delves into the intricate landscape of CH and its implications for the development and progression of AML. By scrutinizing distinct genotypes—T, TN, and TNR—and examining the synergy among co-occurring mutations, particularly focusing on *Tet2* as a pivotal clonal dominance factor, we contribute to a nuanced understanding of AML pathogenesis. CH has emerged as a significant precursor to hematologic malignancies, including AML. Leveraging advancements in genome sequencing, we have unveiled the prevalence of clonal expansions driven by mutations in genes regulating DNA methylation and chromatin organization. These mutations, often associated with aging, underscore the intricate interplay between genetic anomalies and disease progression [Bowman et al, 2018]. Our findings underscore the pivotal role of *Tet2* mutations in shaping clonal fitness and driving leukemic evolution. *Tet2* loss-of-function mutations disrupt hematopoiesis by promoting hypermethylation, altering DNA methylation patterns crucial for HPSC function leading to an expansion of LSKs, myeloid Progenitors and other stem cell-like populations. The dominance of *Tet2*-only clones in competitive environments highlights the substantial impact of *Tet2* mutations on clonal expansion within the leukemic landscape. Furthermore, our study elucidates the dynamic interplay between mutations in *Hox, Meis1* and co-occurring downstream mutations, such as *Npm1* and *Nras*, in AML progression. Dysregulated expression of *Hox* genes and *Meis*1 contributes to altered gene expression patterns, fostering leukemogenesis and disease progression [Nagy, a., et.al. 2019]. The gradual upregulation of *Hoxa13* and *Hoxa9*, particularly in the presence of secondary mutations, suggests a constructive interaction driving disease progression.

Our observation regarding clonal fitness underscores the critical role of *Tet2* mutations in driving clonal expansion and fitness, with *Tet2*-only mutants exhibiting superior fitness compared to the

other genotypes. particularly evident in LSKs and overall lineage-negative populations aligning with previous research. Surprisingly, the latter noticed increasing clonal fitness with the addition of Nras mutation, in certain populations, can depict elevation in CH with disease progression. Our multiparameter flow cytometry analysis reveals distinct alterations in cell populations across genotypes, reflecting the underlying genetic alterations driving leukemic transformation and progression. These observations offer valuable insights into potential mechanistic approaches while target selections for further investigation. The study underscores the pathogenetic complexities of AML through a co-occurrent mutational model, focusing on the impact of *Tet2, Npm1,* and *Nras* mutation combinations. This approach aligns with the literature that conceptualizes AML as a disease characterized by the stepwise accumulation of genetic alterations, which synergistically contribute to leukemogenesis. A limitation of our study is the experimental challenge in replicating the precise sequential acquisition of mutations as it occurs in vivo, which restricts our ability to definitively establish a hierarchy among these mutations. This is a significant gap, considering the current understanding that the order in which mutations occur can profoundly influence disease phenotype, progression, and response to therapy.

In the final analysis, our study sheds light on the interplay between distinct genotypes, co-occurring mutations, and clonal fitness in AML progression. Understanding these dynamics is pivotal for the development of targeted therapies aimed at disrupting leukemic progression and improving patient outcomes. However, further research is required to unravel the intricacies of AML pathogenesis.

Chapter 2: Gene regulatory networks in *Tet2* mutant transformation

Introduction

AML is a hematologic malignancy characterized by the rapid proliferation of immature myeloid cells. Despite initial responsiveness to chemotherapy, the vast majority of patients, approximately 75%, experience a relapse and succumb to the disease within five years following their diagnosis [Dovey et al, 2017]. The pursuit of understanding AML pathogenesis and evolution has extended beyond the realm of genetically resistant clones to encompass non-genetic factors contributing to the disease's functional heterogeneity. Such interest is fueled by observations that AML cell diversity partially mirrors the complexity of myeloid development, wherein normal hematopoietic stem cells (HPSCs) differentiate into a variety of mature blood cell types through a cascade of lineage-committed progenitor states. This differentiation process, well-documented by recent advancements in single-cell RNA sequencing (scRNA-seq), is aberrantly paralleled in AML, where both primitive leukemia stem cells (LSCs) and differentiated cells can coexist, each playing distinct roles in disease progression and therapy resistance [Petti et al, 2019].

RNA-seq emerges as a pivotal tool in dissecting the heterogeneity of malignant and stromal cell populations within AML, offering insights into the nuances of stemness, developmental hierarchies, and tumor-immune interactions. Yet, the challenge of distinguishing malignant from normal cells amidst AML's intricate differentiation hierarchies necessitates a methodological evolution towards combined single-cell transcriptional and genetic profiling. Within this scientific endeavor, our research focuses on *TET2*-mutant leukemogenesis, exploring the transformation from normal hematopoiesis to overt leukemia through a series of genetic alterations. By establishing *ex vivo* co-cultures that simulate different stages of leukemic evolution (initiation by

Tet2 mutation, progression to pre-leukemia with additional *Npm1* mutation, and culmination in overt disease with further *Nras* mutation), we aim to dissect the synergistic impact of these mutations on gene regulatory networks and signaling pathways. Through RNA sequencing of hematopoietic stem cells derived from these co-cultures, we intend to delineate the transcriptional shifts accompanying each mutational milestone, thereby shedding light on the pivotal biological pathways altered in sequence-dependent manners. Subsequent investigations will focus on validating these gene networks, probing the expression changes, and signaling pathways critical for in vivo leukemic transformation. By employing Bulk RNA-seq analysis across the mutation spectrum, we aspire to uncover differentially active signaling pathways, paving the way to identify novel therapeutic targets tailored to specific genetic backgrounds of AML.

Materials and methods

Library formation

The viably frozen samples of each genotype post week 2 BMEC co-culture harvest were "mock Lineage depleted", i.e. without the isolation cocktail, with additional antibodies to remove excess of macrophages and BMECs. The cells were then cKIT enriched (with EasySep™ Mouse CD117 (cKIT) Positive Selection Kit Catalog # 18757). Purified cells ($1.0x10^5$) from three replicates of each genotype (WT, T, TN, TNR) were stored at -20℃ in 300uL of Trizol. RNA was purified from Trizol samples using the Direct-zolTM RNA Microprep (R2062). RNA was subsequently quantified with High Sensitivity Screen Tape on the Aligent TapeStation. RNA was then used as a template for rt-PCR to generate cDNA with SmartScribe (Takara Bio). For Reverse transcriptase, 1x cycle (42℃ 90min, 95℃ 1 min); 10x cycle for PCR amplification, 10xcycles of exponential phase (98℃ 10sec, 65℃ 30sec, 68℃ 3min), and linear phase (72℃ 10 min, 4℃ for infinity). We then quantified cDNA via High Sensitivity D5000 ScreenTape on the Bioanalyzer. Libraries were generated with the Aligent Nextera XT DNA library preparation kit, quantified on Qubit, normalized, and pooled and then subsequently sent for sequencing on a NovaSeq 6000 using Genewiz.

RNA-seq Data Analysis

FASTQ files generated from the RNA sequencing were de-multiplexed through a java script generated from Takara. FASTQs were then mapped, and transcript counts were quantified using STAR (genome version mm10 and transcript version gencode M13) [Dobin & Gingeras, 2015]. DESeq2 was used to perform RNA-sequencing analysis once counts were obtained from STAR [Love et al, 2013]. Gene set enrichment analysis was performed via the fgsea package using gene sets obtained from the msigdbr package. The gsva package was used to analyze single sample gene

set enrichment. Figures were generated using tidy heatmaps and ggplot2 packages. Original RNA-sequencing analysis was performed by Dr. Robert Bowman (University of Pennsylvania).

The comparative gene expression datasheets were analyzed in R Studio (Posit Software) to filter out dysregulated genes. These filters to set out the genes were based on the importance of the respective genes in Hallmark pathways (gene data set downloaded from GSEA) and the potential driving capacity of leukemogenesis.

Results

To identify differentially expressed genes and gene networks as co-mutations were added, we performed bulk RNA-sequencing on *WT, Tet2, Tet2/Npm1, and Tet2/Npm1/Nras* mutant HPSCs. Principal Component Analysis (PCA) is a statistical method used to analyze and visualize patterns in high-dimensional data by reducing its dimensionality, graphically representing genotypic similarities. It was first performed by Dr. Bobby Bowman on the resulting RNA sequencing data to explore the genetic variance among three different genotypes associated. The total PCA variance here accounts for 67% of the variability present in the gene expression profiles based on the presence *Npm1c* among the *WT, Tet2, Tet2/Npm1,* and *Tet2/Npm1/Nras* mutant HPSCs. The PCA results suggest that while there is considerable genetic variance among the different genotypes studied, the first two principal components, represent the most significant sources of variability in the data, with PC1 accounting for *Tet2* mutation and PC2 accounting for *Npm1* mutation. The closeness of the double mutant (TN) and triple mutant (TNR) genotypes on the PCA plot indicates that their gene expression profiles are relatively similar, implying that the transition from pre-leukemic to overt disease state may not involve major changes in gene expression captured by these principal components. However, the distinct positioning of the initiation genotype (T) suggests significant genetic differences at the onset of AML compared to the other disease states (**Figure 6**).

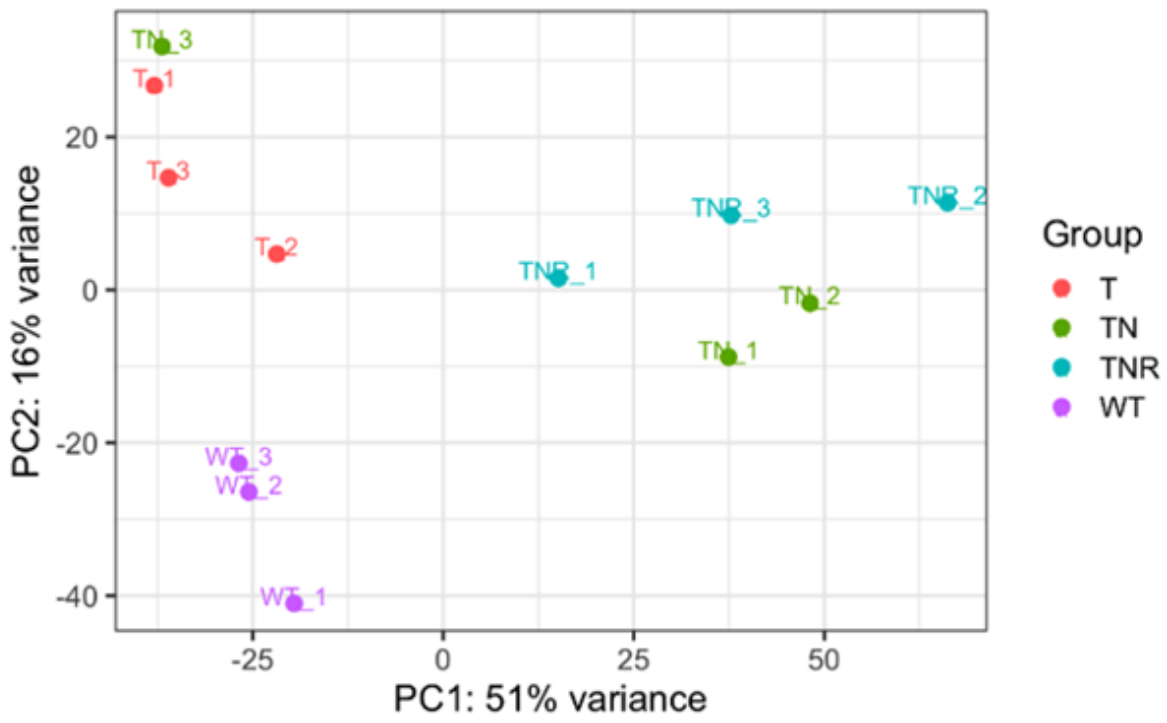


Figure 6: Principal Component Analysis of single cell RNA Sequenced data [Analyzed by Dr. Bobby Bowman]

The Normalized Enrichment Score (NES) serves as a pivotal metric in the analysis, especially when evaluating the functional impact of gene set enrichment across different genotypic states [Subramanian et al, 2005]. While interpreting, it is crucial to recognize that NES accounts for the relative ranking of genes within a predefined set, offering a nuanced view of how specific mutations might alter cellular processes integral to the disease pathophysiology. **Figure 7**, the graph illustrates the comparative NES of hallmark pathways within the context of AML featuring distinct genotypic states (T, TN, TNR) normalized to WT. These values signify the metabolic pathway efficiency based on the respective gene set involved, which provides an insight into the underlying drivers of the mutations observed. For instance, the significant reduction or near-complete abrogation of crucial immune and inflammatory pathways in the TNR genotype, as highlighted (**Figure 7**), suggests a profound impact of *Nras* mutations on the cellular immune response and inflammation processes that potentially facilitates a more aggressive disease phenotype.

The observed trend of increasing NES from single to double mutant states, followed by a reversal or complete loss from double to triple mutant states (**Figure 7**), illustrates a complex interaction between genetic alterations and their functional consequences. This pattern may reflect a compensatory mechanism, or a saturation point in the leukemic cells' ability to manipulate these pathways for survival and proliferation. In the transition to the triple mutant state, the addition of the *RAS* mutation could introduce a level of genomic instability or cellular stress that overwhelms the cells' ability to further exploit these pathways for leukemogenic advantage, thereby leading to a diminished or altered NES. Therefore, even though the data from PCA analysis suggests higher similarities between double and triple mutants, the NES comparison highlights the underlying loss

of functions of vital hallmark pathways, thus indicating increased disease aggression in triple mutants.

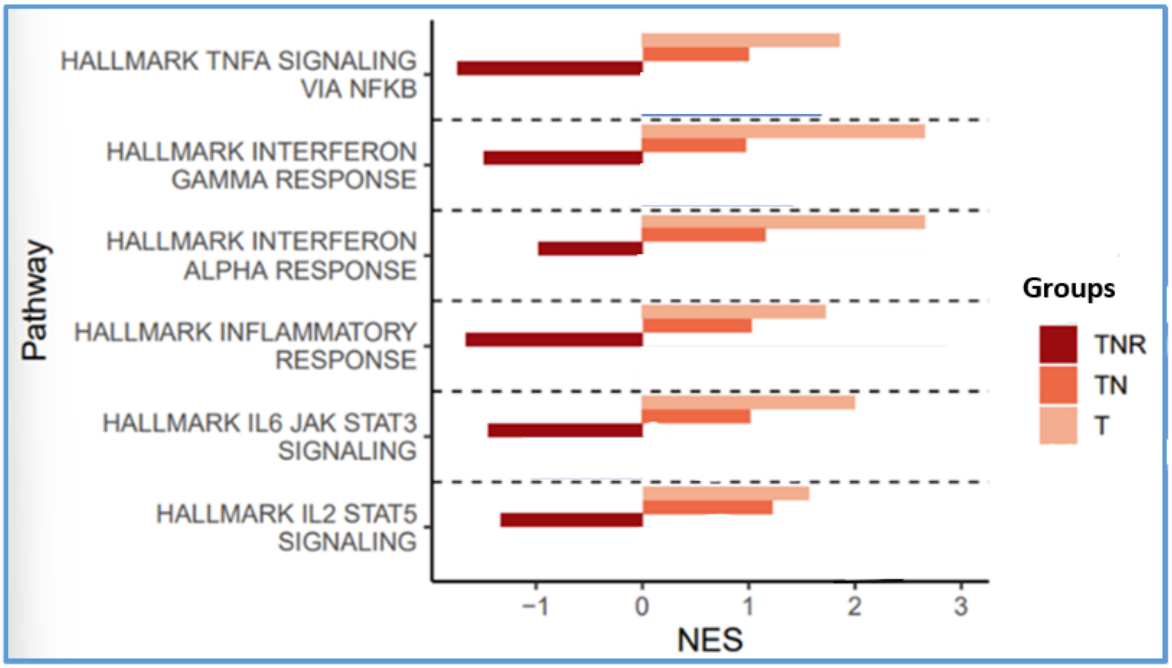


Figure 7: Normalized Enrichment Score (NES) of various hallmark gene sets. [Analyzed by Dr. Bobby Bowman]

To undertake a comprehensive investigation and elucidate the distinct roles of individual genes an extensive analysis of gene expression data was performed. This exploration of gene dysregulation associated with mutational acquisition involved an in-depth examination of downstream mutations. The importance of these genes was evaluated based on their roles within hallmark pathways, transcriptional regulation mechanisms, or signaling cascades that could serve as potential therapeutic targets to monitor alterations in disease progression subsequent to gain or loss of function of the selected targets. Further exploration of this data involved the analysis and cataloging of genes associated with downstream mutations based on their potential role in the process of transformation. For example, *S100a8* and *S100a9* genes, pivotal in inflammatory pathways and immune cell recruitment, exhibited decreased expression during *Npm1c* acquisition and nearly complete loss of function after *Nras* acquisition, correlating with inhibition of tumor growth and metastasis as observed in various other cancer types like colorectal and breast cancer. Although *DAPP1*'s direct involvement in these pathways is unclear, its role in upstream or parallel signaling pathways could impact mTOR signaling and subsequently influence oncogenic processes (**Figure 8**). Another point of interest, *LMO2* and its dysregulation or aberrant expression has been implicated in several types of leukemia, including T-cell acute lymphoblastic leukemia (T-ALL), where it can contribute to the transformation of normal blood cells into cancerous cells [Rahman et al, 2017].

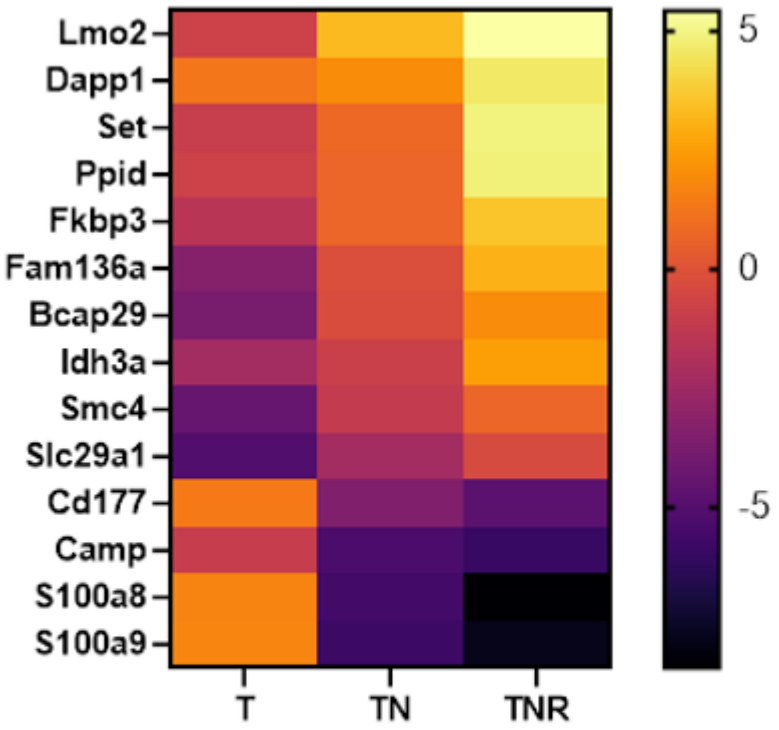


Figure 8: Gene Expression Alterations through Disease Progression.

Discussion

Understanding AML remains a critical challenge in cancer research due to its aggressive nature and high relapse rates despite initial treatment success. In our pursuit to unravel the complexities of AML, we delve into the interplay of genetic mutations and their impact on disease progression. AML, like many cancers, exhibits a diverse landscape of cell types, including both primitive LSCs and more differentiated cells, each influencing the course of the disease and its response to therapy. To better understand this intricate dynamic, we utilized bulk RNA sequencing technology, which offers a window into the genetic diversity of AML cell populations. By dissecting these populations, we aim to uncover the genetic and molecular mechanisms driving disease evolution and therapeutic resistance. Our focus lies particularly on the role of *TET2* mutations in leukemogenesis, exploring how additional mutations, such as *NPM1* and *NRAS*, interact to shape the disease phenotype. Through ex vivo co-culture experiments mimicking different stages of leukemic evolution, we observed distinct transcriptional shifts accompanying each mutational milestone. Notably, our findings suggest that the transition from pre-leukemic to overt disease states may not involve drastic changes in gene expression profiles, as indicated by the PCA. On the contrary the complete loss of function of several hallmark pathways were observed in the NES while progressing from pre-leukemic to overt disease. A proteomic analysis test via Mass spectrometry can potentially provide a broader range of information.

The initiation genotype (T) appears genetically distinct from later disease states, highlighting significant genetic divergence at the onset of AML. Further analysis focused on identifying differentially expressed genes and gene networks associated with co-mutations. Our investigation revealed genes which play critical roles in tumorigenesis and immune regulation, during leukemogenesis. The aberrant expression of those genes underscores the complexity of leukemia

pathogenesis and highlights the need for comprehensive molecular profiling to guide targeted therapies. To sum up, our study underscores the importance of integrating genomic and transcriptomic analyses to unravel the intricate mechanisms driving AML progression by elucidating the molecular underpinnings of disease evolution.

Conclusion

Our investigation into AML has unveiled intricate genetic architectures dictating disease evolution. AML's heterogeneity, spanning primitive leukemia stem cells to differentiated entities, poses formidable therapeutic challenges. Our inquiry delved deeply into the genetic milieu of AML, notably spotlighting Tet2 mutations as pivotal in leukemogenesis. Through meticulous dissection of distinct genotypes and elucidation of co-occurring mutations like *Npm1* and *Nras*, we gleaned insights into the mechanistic underpinnings of disease progression.

A revelation from our endeavor lies in *Tet2* mutations' central role in shaping clonal fitness and propelling leukemic expansion. *Tet2* loss-of-function perturbs hematopoietic homeostasis, engendering aberrant DNA methylation dynamics and fostering the proliferation of select cellular cohorts pivotal in AML pathogenesis. Dynamic crosstalk among mutations in *HOX* genes, *MEIS* proteins, and ancillary genetic anomalies, delineating a complex molecular framework orchestrating AML's trajectory. The incremental upregulation of specific gene cascades, especially in the presence of secondary mutations, intimates collaborative molecular programs driving disease progression. Additionally, our findings shed light on the progressive upregulation of specific gene pathways, particularly in the presence of secondary mutations. This phenomenon suggests the existence of collaborative molecular programs that fuel the relentless advancement of AML.

Moreover, our investigations extend beyond the molecular level to uncover distinctive changes in cellular populations across different genotypes. These insights not only deepen our understanding of AML pathogenesis but also offer valuable clues for identifying potential therapeutic targets. Our focus remains on providing novel mechanistic insights into disease biology and progression. These

insights pave the way for future research aimed at translating our findings into clinically relevant strategies for combating AML and improving patient outcomes.

Our findings resonate with the prevailing 2-class hypothesis in AML research, where class 1 mutations (e.g., in signaling pathways like *NRAS*) and class 2 mutations (e.g., in epigenetic regulators like *TET2*) interact to drive leukemogenesis. The inclusion of *Npm1* mutations complements this framework, reinforcing the notion that disruptions in both cellular signaling and epigenetic regulation are pivotal for AML progression. This is in line with existing literature, which has identified mutations in *TET2, NPM1,* and *NRAS* not only as common in AML but also as influential in disease progression and therapeutic outcomes.

The next logical steps in this research trajectory would involve addressing the limitation of sequential mutation induction through advanced genetic engineering techniques, such as CRISPR/Cas9, to model the natural evolution of AML more accurately. Additionally, expanding the study to include a broader array of mutations could offer deeper insights into the complex molecular interplay at play in AML pathogenesis. This would ideally be complemented by longitudinal studies tracking clonal evolution in patients over time, thereby enriching our understanding of AML's dynamic genetic landscape. Furthermore, integrating functional genomics with proteomic analyses could elucidate the downstream effects of these mutations, offering novel therapeutic targets. Ultimately, this research aims to pave the way for personalized medicine approaches in AML treatment, tailored to the specific mutational profiles and biological characteristics of individual patients' leukemia.

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