November 2023

Targeting BET Proteins Downregulates miR-33a To Promote Synergy with PIM Inhibitors in CMML

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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Date of Approval:
October 9, 2023

Keywords: PDX, GM-CSF, Biogenesis

ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor, Dr. Eric Padron. He has been instrumental in my development as a scientist, and I greatly appreciate all the effort he has put forth throughout my doctoral studies. I would like to thank my thesis committee: Dr. Conor Lynch, Dr. Andriy Marusyk, and Dr. Keiran Smalley whose feedback helped in finishing my main project and getting my results published. I would also like to thank Dr. Stephen Oh for taking time out of his schedule to serve as my external chair.

I would like to thank the members of the Padron lab both current and former. Their support over the years both technical, through help both learning and troubleshooting experiments, and personal has been greatly appreciated.

The Core Facilities at Moffitt, in particular the Flow Cytometer, Genomics, Tissue, and Microscopy cores, have been incredibly helpful. Without the knowledge and expertise of the people involved with these cores I would not have been able to complete all the research necessary to finish my project. Finally, I would like to thank the Dr. Ken Wright, Cathy Gaffney, Janet Opel, and Tiffany Ferrer and the Cancer Biology PhD program for all their help.

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ABSTRACT

Chronic Myelomonocytic Leukemia (CMML) is a rare myeloid malignancy with a dismal prognosis and no therapeutic options which are capable of altering the natural course of the disease. There remains a significant need for novel therapies that are able to meaningfully improve patient outcomes. In this study we explore the effectiveness of Bromodomain and Extra-Terminal domain protein inhibitor (BETi) combinations in CMML.

Preclinical studies in myeloid neoplasms have demonstrated efficacy of BETi. However, BETi demonstrate poor single agent activity in clinical trials. Several studies suggest that combinations with other anti-cancer inhibitors may enhance the efficacy of BETi. To nominate BETi combination therapies for myeloid neoplasms, we used a chemical screen with therapies currently in clinical cancer development and validated this screen using a panel of myeloid cell lines, heterotopic cell line models, and PDX models of disease.

We identified PIM inhibitors (PIMi) as therapeutically synergistic with BETi in myeloid leukemia models and show that PIM inhibition is able to overcome both single agent BETi and dual BETi/JAKi persistence. Mechanistically, we show that PIM kinase is increased after BETi treatment, and that PIM kinase upregulation is sufficient to induce persistence to BETi and sensitize cells to PIMi. Further, we demonstrate that miR-33a downregulation is the underlying mechanism driving PIM1 upregulation and its downregulation is likely due to global BETi dependent impairments in miRNA biogenesis. We also show that GM-CSF hypersensitivity, a hallmark of CMML, represents a molecular signature for sensitivity to combination therapy.

Inhibition of PIM kinases is a potential novel strategy for overcoming BETi persistence in myeloid neoplasms. Our data supports further clinical investigation of this combination.

CHAPTER ONE: CHRONIC MYELOMONOCYTIC LEUKEMIA

Disease History

In 1970 James Linman proposed the term Myelomonocytic Leukemia for a myeloproliferative disorder characterized by a high peripheral monocytosis and a bone marrow proliferation of immature myeloid cells[1]. In 1976, Chronic Myelomonocytic Leukemia (CMML) was preliminarily identified as a subtype of myelodysplastic syndromes (MDS) by the French-American-British (FAB) Co-Operative Group[2]. The FAB met again in 1982 and formally clinically and pathologically defined CMML as having absolute monocytosis (monocyte count > 1x109/L), an increase in bone marrow (BM) monocyte precursors, a blast cell percentage less than 5% in the peripheral blood (PB) and less than 30% of nucleated cells in the BM[3,4]. In 1994, due to the heterogeny in clinical presentation, the FAB reclassified CMML into two distinct categories: Myeloproliferative (CMML-MP) which was defined as having a white blood cell (WBC) count greater than $13x10^9$/L and dysplastic (CMML-MD) which was defined by a WBC less than $13x10^9$/L[5]. However, this FAB classification was not incorporated into the World Health Organization's (WHO) first CMML classification in 1999 due to the lack of consistent clinical and biological features in FAB's earlier classification[6]. The WHO classified CMML as a subtype of MDS and divided CMML into two groups based on the percentage of blasts in the BM and PB, CMML-1 (<5% blasts in PB and <10% blasts in BM) and CMML-2 (>5% blasts in PB and >10% blasts in BM) as this metric had prognostic value[6]. Finally, in 2016, the WHO revised the criteria for CMML and defined as a *bona fide* disease categorized under a new group of diseases termed Myelodysplastic/Myeloproliferative Neoplasms (MDS/MPNs). Specifically,

CMML was defined as: Persistent (at least 3 months) PB monocytosis (WBC ≥1 × 109/L, with monocytes accounting for ≥10% of the WBC count), BM dysplasia, and ≤20% blasts in BM and PB[7]. Additionally, with the discovery of molecular and clinical features to distinguish myeloproliferative from myelodysplastic[8-10] CMML was this time divided into three categories based on the blast percentage in the BM and PB: CMML-0 (<2% blasts in PB, <5% BM), CMML-1 (2-4% blasts in PB and 5-9% blasts in BM) and CMML-2 (5-19% blasts in PB and 10-19% blasts in BM)[7]. The most recent classification, determined by the International Consensus Classification of Myeloid Neoplasms and Acute Leukemias (ICC) in 2022, has removed CMML-0 after it was determined that its prognostic value was limited at best and classified CMML-1 as <5% blasts in PB, <10% in BM, and CMML-2 as 5%-19% blasts PB, 10%-19% in BM[11]. This new classification also identified a pre-CMML phenotype called clonal monocytosis of undetermined significance (CMUS). This involved persistent mild monocytosis (monocytes ≥ 10% and ≥ 0.5 × 109/L of the WBC), evidence of clonal hematopoiesis (CH) but indeterminate BM features that cannot be categorized as CMML and is associated with a higher risk of developing CMML[11].

Clinical Features and Mutational Profile

CMML is a rare disease (incidence rate of 0.4-1 per 100,000[12]) that primarily affects the elderly with 90% of cases being over 60 years of age and the median age of diagnosis between 73-75 years. It is more prevalent in males with a male:female ratio between 1.5-3:1[13,14]. It is characterized by monocytosis, specifically the expansion of the $CD14^{+}CD16^{-}$ classical monocyte subset, which have high levels of CCR2 and low levels of CX3CR1, to >94%[15]. This differentiates CMML from reactive monocytosis, which also leads to increased WBC and can occur from infection or acute and chronic inflammatory states[15]. Transformation to Acute

Myeloid Leukemia (AML) is also common in CMML patients, with the incidence between 15-30%[16,17]. Patients diagnosed with CMML-MD typically present with cytopenias, easy bruising and bleeding due to cytopenia, frequent infection, effort intolerance, and transfusion dependence[18,19]. CMML-MP patients can present with leukocytosis, hepatomegaly, splenomegaly, and symptoms associated with myeloproliferation: night sweats, bone pain, weight loss, cachexia and in some cases organ damage due to organomegaly[18,19]. Despite the diverse clinical presentation, the mutational burden in CMML is low (10-15 mutations per kilobase of coding DNA) compared to other cancers (100-1000 per kilobase)[20]. These mutations can be classified into a few distinct categories: epigenetic control of transcription (methylation: TET2, DNMT3A, IDH1, and IDH2; histone modification: EZH2, ASXL1, UTX)[21-23], spliceosome (SF3B1, SRSF2, U2AF1, ZRSR2, PRPF8)[14], cell signaling (JAK2, KRAS, NRAS, CBL, and PTPN11)[24-27], transcriptional regulators (RUNX1, SETBP1)[28,29] and rarely DNA damage response (TP53 and PHF6)[30]. The most common of these is TET2 (~60%) followed by SRSF2 (~50%), ASXL1 (~50%) and RAS pathway (~30%) mutations[19]. The exact genetic events that initiate CMML are likely heterogeneous, although TET2 mutations often establish the founding clone and manifest as antecedent clonal hematopoiesis. These mutations occur at the earliest stages of hematopoietic development in $CD34^{+}/CD38^{-}$ cells leading to early clonal dominance[31]. Secondary mutations may influence the development of an MDS or MPN phenotype, with ASXL1, DNMT3A, RUNX1, SETBP1, and SF3B1 being more associated with CMML-MD[32-34] and ASXL1, oncogenic RAS pathway mutations and JAK2 more associated with CMML-MP[32,33]. Cytogenetic abnormalities are seen in 20-30% of CMML patients[35]. The most common abnormalities are trisomy 8, loss of Y chromosome, abnormalities of chromosome 7, trisomy 21 and complex karyotypes[35].

Risk Stratification and Survival

There are multiple models of risk stratification for CMML that either rely solely on clinical parameters to stratify risk or combine clinical parameters with mutational analysis. The MD Anderson prognostic system (MDAPS), identified an HB (hemoglobin) level <12 g/dL, presence of PB immature myeloid cells (IMC), absolute lymphocyte count (ALC) >2.5 × 109/L, and ≥10% BM blasts as independent predictors for inferior OS[36]. The Dusseldorf Score (DS), which was based on MDAPS, found elevated lactate dehydrogenase, BM blast count >10%, male gender, HB <12 g/dL, and ALC >2.5 × 109/L were independently prognostic and categorized patients into 3 risk groups: Low (93 months), intermediate (26 months), and high (11 months)[37]. The CMML-specific prognostic scoring system (CPSS) found that FAB and WHO CMML-subtypes, red blood cell transfusion dependency, and the Spanish cytogenetic risk stratification system were prognostic and has 4 risk groups: low (72 months), intermediate-1 (31 months), intermediate-2 (13 months), and high (5 months)[38]. A study performed at the Mayo Clinic assessing clinical parameters in addition to ASXL1 mutation status found that HB <10 g/dL, platelet count <100 × 109/L, AMC >10 × 109/L, and circulating IMC were prognostic, however, ASXL1 mutation status was not[39]. This led to the development of the Mayo prognostic model with risk categories low (32 months), intermediate (18.5 months) and high (10 months)[39].

More recent models do take into account mutational status, thanks to increased availability of Next Generation Sequencing (NGS) data and a better biological understanding of the role of mutations in CMML outcomes. The GFM model also uses ASXL1 for its scoring system, although does not consider specific genetic variations and compared to the Mayo model does demonstrate an adverse prognostic impact with regard to ASXL1 mutations[40]. Additionally, WBC, age, anemia and platelets are used to stratify patients. GFM has 3 risk groups ranging

from 14.4 months to not reached[40]. The Mayo Molecular Model, a modification of the original Mayo model after an additional 417 CMML patients were analyzed, utilizes Hgb, AMC, peripheral immature myeloid cells, platelets and frameshift or nonsense ASXL1 mutation for its scoring system[41]. Patients are stratified into 4 risk groups with survival ranging from 16-97 months[41]. Finally, the CPSS was modified to include the mutations ASXL1, NRAS, SETBP1, and RUNX1 and additionally replaced FAB and WHO subtyping with BM blast percentage and WBC and renamed CPSS-mol[42]. This separated CMML patients into 4 risks groups similar to the Mayo molecular model with survival ranging from 18 months in the highest risk group to 144 months in the lowest with RUNX1 having the greatest genetic impact on risk[42]. Our lab is currently performing experiments as part of an international collaboration which aims to determine the optimal model for stratifying CMML patient risk.

Treatment Options and Outcomes

Outside of allogeneic stem cell transplant, no therapies have been demonstrated to modify the natural history of CMML. Therefore, the treatment strategy is symptom directed therapy. Treatment selection for CMML has been extrapolated from data in other related diseases. These therapies historically included: chemotherapy (etoposide, cytarabine, all-trans retinoic acid)[43,44], topoisomerase inhibitors (topotecan, 9-nitro-campothecin)[45,46] and lonafarnib (farnesyltransferase inhibitor)[47]. However, these therapies had low response rates and were associated with significant toxicities[19]. Today, patients in the lowest risk categories undergo careful monitoring of disease progression without treatment, so long as symptoms are manageable. For patients presenting with the MP-CMML subtype, cytoreductive agents such as hydroxyurea are used to help manage symptoms like splenomegaly. This can help prevent organ damage and these effects do have a modest effect on overall survival. Erythropoietin stimulating

agents are used for CMML-MD as these can alleviate symptoms like anemia[48]. CMML-MD is also treated with hypomethylating agents (HMA) such as 5-azacitidine, decitabine, and combination oral decitabine/cedazuridine (cytidine deaminase inhibitor). HMAs inhibit DNA methyltransferases, which can reduce genome-wide and sequence-specific methylation changes that contribute to neoplasia. HMA in CMML-MD patients can help restore normal hematopoiesis, which alleviates many of the symptoms associated with cytopenias, and marginally improves overall survival[49]. However, restoration of hematopoiesis is typically not durable, there is no impact on the mutational allele burden and patients that stop responding have rapid worsening of disease and frequent transformation to AML[49]. Despite many attempts, there are currently no reliable methods to predict response to HMA therapy[50,51]. Additionally, a large CMML-specific randomized clinical trial called the DACOTA study investigated the use of HMA in CMML-MP and found that HMA did not provide any advantage over hydroxyurea in this subtype[52].

Allogeneic hematopoietic cell transplantation (HCT) is the only therapy that can potentially be curative. However, only a small fraction of patients qualifies for HCT due to advanced age at diagnosis and other co-morbidities[53]. While this treatment is potentially curative, it is not without significant risks. Acute and chronic graft vs. host disease (GVHD), non-relapse mortality, and post-transplant disease relapse all contribute to the reduced effectiveness of this treatment[53]. As with HMA therapy, there are currently no reliable predictors of HCT success. There remains a significant clinical need for novel therapies for the treatment of this disease.

Current Experimental Therapies

Several therapies are under clinical investigation. In 2013 Padron et al. discovered that CMML cells were uniquely hypersensitive to GM-CSF, which controls signaling pathways

leading to the activation of STAT5[54]. Activation of this pathway is even further enhanced in the context of N and K-RAS, CBL and JAK2 mutations. Two clinical approaches tested on the results of this research: neutralizing GM-CSF levels through the use of an anti-GM-CSF antibody (Lenzilumab), or blocking downstream signaling with the use of a small molecule JAK inhibitor(Ruxolitinib). Lenzilumab has undergone a phase I trial and showed an overall response rate of 33% with minimal toxicity[55]. Ruxolitinib, has undergone a Phase I/II clinical trial, with a phase II extension currently ongoing, and shown clinical efficacy with an overall response rate of 38%, and is relatively well tolerated[56]. Since roughly 30% of patients with CMML have RAS pathway mutations, targeting this pathway could lead to significant clinical benefit. Tipifarnib, a farnesyltransferase inhibitor, demonstrated some clinical benefit in a small number of CMML patients in a larger study on MDS [57]. Unfortunately, as a single agent tipifarnib showed a suboptimal response in a recent phase II clinical trial, which has now been closed[58]. Inhibition of the RAS pathway can also be achieved via inhibition of downstream kinases such as MEK. Cobimetinib, a MEK1/2 inhibitor, is currently undergoing a phase II clinical trial to assess its efficacy in newly diagnosed or HMA-treated CMML patients with RAS pathway mutations (NCT04409639). For CMML-MP specifically, a recent pre-clinical study has shown there is a distinct gene expression signature associated with RAS pathway mutations that involves overexpression of PLK1[59]. Treatment with Volasertib, a PLK1 inhibitor which has already completed clinical trials in MDS and AML with a favorable safety profile, is currently being investigated in this genetic subtype[59]. Thus far, Bromo and Extra-Terminal Domain (BET) inhibitors have not been investigated in the context of CMML, but these compounds could potentially be powerful tools with the right combinations.

CHAPTER TWO: SCREENING OF BET INHIBITOR COMBINATIONS REVEALS BET/PIM INHIBITION AS A POTENTIAL THERAPY FOR THE TREATMENT OF CMML.

Note. Portions of this chapter have been adapted from previously published work in Letson and Padron, *Pharmacological Research*, 2019[60] and Letson et al., *Clinical Cancer Research*, 2023[61]

Introduction

Structure and Function of BET Proteins

The first mammalian BET proteins discovered were BRD2 and BRD4, which were both identified in 1996 [62,63]. BRD3 and BRDT were both identified in 1997[64,65]. BET proteins contain two tandem, N-terminal bromodomains (BD1 and BD2), which are responsible for recognizing acetylated lysine on histone tails and non-histone proteins, an extra-terminal (ET) domain, which recruits transcription factors, and in the case of BRD4 and BRDT an additional C-terminal domain (CTD), which interacts with positive transcription elongation factor b (P-TEFb). BRD2, 3, and 4 are ubiquitously expressed, while BRDT is expressed in both the testis and oocytes[64,66].

BRD4 and BRDT modulate transcription, after recognizing an acetylated lysine, through binding to P-TEFb via their CTD, the general initiation cofactor Mediator through an unknown mechanism, and phosphorylate RNA Pol II at serine 2 (Ser2) releasing paused transcription[67 68]. BRD4 can also interact with several other chromatin regulators through its ET domain, including the arginine demethylase JMJD6, lysine methyltransferase NSD3, and ATP-dependent nucleosome remodeling enzymes SWI/SNF and CHD4[69]. BRD4 deletion in mice is embryonic lethal, which demonstrates its importance in development.[70,71]. Conditional knockout of BRD4 in

mouse models has shown that it is crucial for myogenesis and adipogenesis, but dispensable for maintaining cell identity in differentiated cells[72]. Conditional knockout models also showed that BRD4 is essential for hematopoietic stem cell expansion and progenitor development, but plays a limited role in macrophage development and inflammatory response to LPS[73]. BRDT is required for maintaining the chromatin architecture of the sperm head, and chromatin remodeling during spermatogenesis[74]. It is also essential for germ cell differentiation, as it plays a role in reorganizing chromatin and facilitating histone eviction and replacement by transition proteins[68]. Finally, BRDT, specifically its BD1 domain, is required for mRNA splicing during spermatogenesis[75]

In contrast to BRD4 and BRDT, BRD2 and BRD3 do not require P-TEFb or Mediator to facilitate transcriptional elongation, and instead recognize hyperacetylated nucelosomes and remove nucleosomal barriers to transcription elongation by RNA Pol II[76]. BRD2 plays a key role in neuronal development through positive regulation of the E2F1 transcription factor, controlling neuronal differentiation, cell-cycle progression, and cell-cycle exit in neuroepithelial cells[77]. In mitogen-stimulated primary B cells, BRD2 directly binds the cyclin A promoter, increasing mRNA and protein levels while also advancing S-phase progression[78]. Experiments in HeLa cells uncovered a unique function of BRD2: the ability to affect the alternate splicing of multiple genes without affecting Pol II, although the mechanism for this regulation remains unknown[79]. Both BRD2 and BRD3 cooperatively play a role in erythropoiesis by directly interacting with the transcription factor GATA1 as depletion of BRD3 only effects transcription if BRD2 is also depleted[80]. Recently, BRD3 was shown to have an important role in the regulation of ribosomal RNA synthesis. BRD3 interacts with TCOF1, a critical regulator of ribosome biogenesis, within

the nucleolus and represses rRNA production[81]. Although no knockout of BRD3 has ever been tested, knockout of BRD2, like BRD4, is embryonic lethal[82].

Canonical Effects of BET Inhibition in Cancer.

BETi binding to any of the BET family of proteins blocks the binding of BD1 and BD2 to acetylated lysine and evicts BET proteins from chromatin, while the ET and CTD remain unaffected. This causes increased transcriptional pausing since HEXIM (Hexamethylene bis-acetamide-inducible protein), an inhibitor of p-TEFB, remains bound to RNA Pol II, inhibiting the elongation process, and results in a profound downregulation of genes. Genes targeted by BETi are typically associated with super-enhancers (SE), which are clusters of enhancers that are enriched for the transcriptional activation mark histone 3 lysine 27 acetylation. The most well characterized of theses SE genes impacted by BETi in cancer is downregulation of the MYC oncogene, which has been reported to be critical in hematologic malignancies and various solid tumors[83-89]. This downregulation is frequently accompanied by increases in the negative regulator of MYC, HEXIM1[84]. CDK6 is also frequently downregulated after BETi treatment in multiple cancer types, and this is most likely due to the upregulation of p21 that is often seen shortly after BETi treatment[90-92]. IL7R and BCL family members BCL2 and BCL-xL are also commonly downregulated[90,91,93-95]. In osteosarcoma and non-small cell lung cancer FOSL1 is the main driver of BETi effects, and not MYC[96,97]. In melanoma, neuroendocrine tumors, and eurothelial carcinoma SKP2 is downregulated by BETi and this is followed by induction of various CDK inhibitors, including p27 in melanoma and neuroendocrine, and p57KIP2 in eurothelial carcinoma[86,98,99].

With the advent of techniques such as Precision nuclear Run-On transcription coupled with deep sequencing (PRO-seq)[100], which provides both directional information and near

nucleotide resolution of the genome-wide positions of actively engaged RNA polymerases[101], and Thiol(SH)-linked alkylation for the metabolic sequencing of RNA (SLAM-seq)[102], which allows for the detection of recently synthesized RNA within the total RNA pool, it is now possible to rigorously interrogate the direct transcriptional effects of BET inhibition before any secondary effects occur (< 3 hours post inhibition)[103]. Zhao et al. treated human leukemia (Kasumi-1 cells) with JQ1 between 15 minutes and 1 hour, and showed that 1,905 genes had repressed gene body transcription. Gene ontology analysis showed that genes most robustly affected were stress response genes, although genes involving oxidative phosphorylation, RNA transport, Ribosome biogenesis, and mitochondrial diseases were also suppressed [101]. Transcription factor analysis unsurprisingly demonstrated that BET inhibitor-regulated genes were enriched for MYC targets, but also showed targets of E2F, IRF, NFMUE1, ELK1, and HIF1. Genes important for hematopoietic malignancies such as DNMT3A, BCL6, IKZF1, ATRX, ETV6, LMO2, CSF3R, PAX5, and TET2 were also downregulated at these early time points[101]. Muhar et al. also treated human leukemia cells (K562 and MV4-11) with varying doses of JQ1 for 30 minutes and assessed transcriptional changes. At higher doses they observed broad suppression of transcription. This global suppression of transcription was entirely dependent on inhibition of BRD4 as select degradation of BRD2 or BRD3 did not elicit the same transcriptional repression as BRD4 degradation or BET inhibition, indicating that, at least in human leukemia cells, the effects of BET inhibition is primarily mediated by BRD4[103]. Treating cells with a lower dose of JQ1 (200nM) affected only a small set of BETi-hypersensitive genes, many of which were associated with SEs[103].

Compared to very short-term treatment with BET inhibitors, sustained BET inhibition can result in cellular persistence, also known as non-genetic resistance, to BET inhibitors.

Cellular persistence can develop in 3 different ways: drug tolerant persistor cells, unstable non-genetic resistance and stable non-genetic resistance. Drug tolerant persistor cells can evade drug treatment by reducing their growth and metabolism[104]. These cells occur in low frequency, are genetically identical to the rest of the tumor, and resensitize to drug treatment after withdrawal[105-107]. However, they can act as a reservoir to cancer cells and will eventually lead to the development of genetic mutations and true drug resistance[108]. Unstable non-genetic resistance can occur through a variety of adaptations including: reactivation of a targeted pathway[109,110], activation of a compensatory pathway[111,112], chromatin remodeling resulting in altered gene expression[113-115] and paracrine/autocrine cell communication[116-118]. Compared to drug tolerant persistors, these types of adaptations allow the cells to continue to proliferate, but similar to drug tolerant persistors withdrawal of drug will resensitize cells to drug treatment. Finally, Stable non-genetic resistance develops due to epigenetic changes that allow cells to remain resistant to drug treatment even after drug withdrawal[104]. In the case of BET inhibitors, a variety of unstable non-genetic resistance mechanisms are responsible for the development of cellular persistence, which are discussed in more detail below.

Type and Mechanism of Action of BET Inhibitors

There are two classes of BETi that include acetylated lysine mimetic and non-acetylated lysine mimetic[119]. Non-acetylated lysine mimetics bind the bromodomain (BD1 or BD2) within the acetylated lysine binding pocket but do not form the hydrogen bond that typically anchors acetylated lysine peptides. This interaction inhibits BET binding to chromatin through steric hindrance[119]. As the name suggests, acetylated lysine mimetics directly bind BDs through hydrogen bonding that mimics the binding of acetylated lysine. This mode of binding sets the inhibitor deeper into the binding pocket and competes with binding of acetylated lysine, thereby

blocking the interaction between BET proteins and acetylated lysine-containing proteins (Fig. 1) [119].

Inhibitors that act as acetylated lysine mimetics include: JQ1, IBET151, IBET762, OTX015, INCB054329, as well as many others [120-123]. A thorough review of the different classes of BET inhibitors as well as the scaffolds used in acetylated lysine mimetics was done by Filippakopoulos et al. 2014[119]. There have been two new developments in the acetylated lysine mimetic class. First, is the development of inhibitors for either BD1 or BD2 specifically[124,125]. These types of inhibitors will be crucial for dissecting the specific functions of individual BET protein family members. MT1 and biBET are bivalent and bind both BD1 and BD2 simultaneously, which significantly increases potency compared to typical monovalent mimetics (Fig. 2.1A) [126,127]. BET degraders, known as Proteolysis Targeted Chimeras (PROTACs), are another important class of compounds targeting BET proteins. These are designed to recruit BET proteins to E3 ubiquitin ligases, specifically, Cereblon (CRBN), or the von Hippel-Lindau protein (VHL), where they are then targeted for degradation by the proteasome (Fig. 2.1B)[128,129]. BET inhibitors currently undergoing clinical trials are described in Appendix A. Among the clinical trials that have already been completed, few have published results. The results that have been published have shown very few responses after treatment with BETi, with rare complete and partial responses[130-132]. Many clinical trials have been terminated due to either toxicity, failure to impact disease progression despite reaching a pharmacologically relevant dose, or unpredictable variability in pharmacokinetics[130,133-136]. In the case of PLX51107 despite reaching a therapeutic dose in phase 1 the study was still terminated by the company, citing a business decision[137].

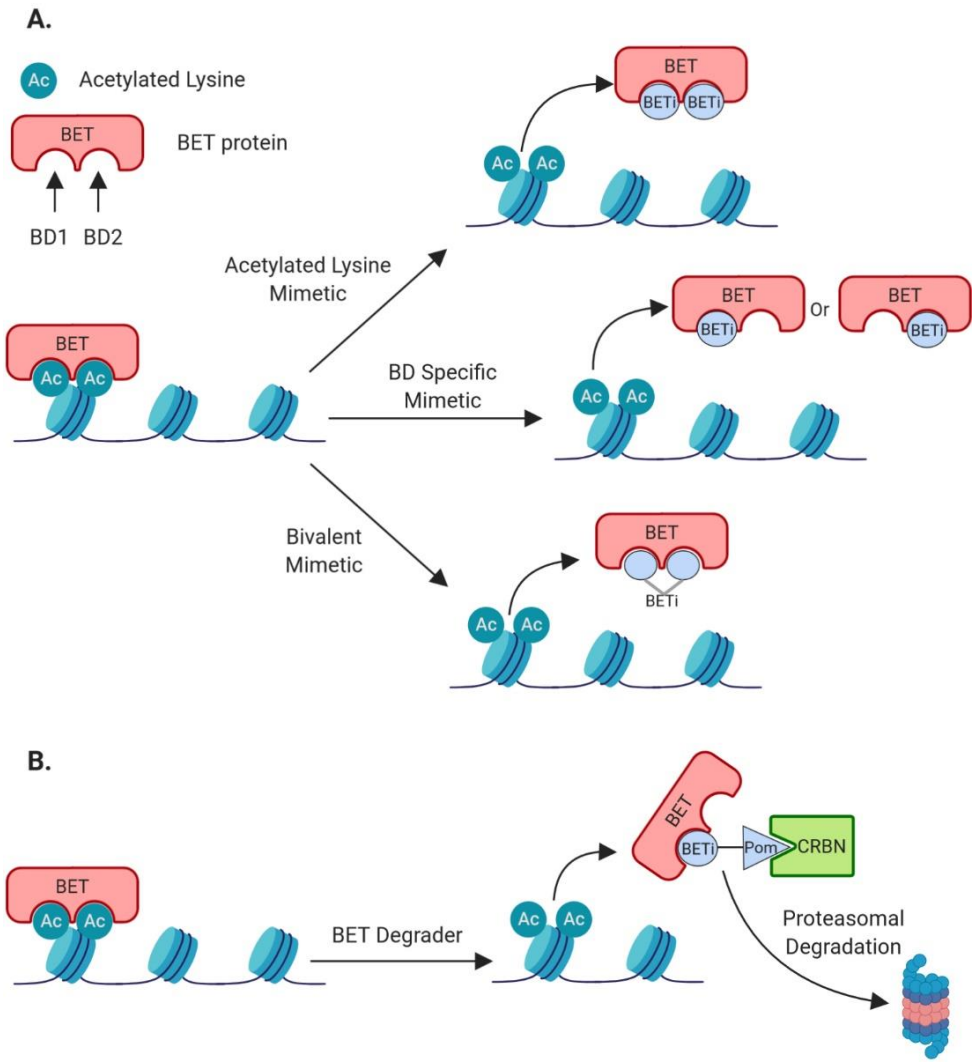

Figure 2.1: Mechanism of action of various BET inhibitors. (A) There are 3 classes of acetylated lysine mimetics. The first class (Top) binds either BD1 or BD2 indiscriminately. The second class (Middle) preferentially binds either BD1 or BD2. The last class (Bottom) binds both BDs simultaneously. In each case the result is a displacement of BET proteins from chromatin. (B) BET degraders are designed to tether BET proteins to Cereblon, which targets them for proteasomal degradation.

Non-canonical Effects of BET Inhibition in Cancer

As mentioned above, BETi's function by binding BD1 or BD2 and preventing recognition of acetylated lysine. However, a few studies have demonstrated that BETi does not

affect bromodomain-independent functions of BET proteins. This is particularly important because while JQ1 does not affect BRD4 expression at the mRNA level, at the protein level BRD4 expression is increased upon treatment with JQ1 in multiple different cancer types[94,128,138,139]. JQ1 treatment increases expression of both mRNA and protein levels of DUB3, a protein responsible for promoting BRD4 stabilization through deubiquitination, thereby increasing BRD4 protein levels[138]. JQ1 also reduces the interaction between SPOP, which triggers the ubiquitination and proteasomal degradation of target proteins mediated by RBX1-dependent recruitment of E2 ubiquitin-conjugating enzyme into the CRL complex, and BET proteins, prolonging their half-life[139]. Sustained treatment of triple negative breast cancer cells leads to a resistant phenotype that remains dependent on BRD4 for transcription and cell proliferation. BRD4 in these resistant cells is hyper-phosphorylated, which was attributed to a decrease in PP2A activity, and had a stronger association MED1 leading to a bromodomain independent chromatin recruitment mechanism[140]. BRD4 has been shown to directly bind both wild-type and mutant p53, but JQ1 does not impair this binding[141-143]. In OCI-AML3 cells harboring wild-type p53 it is theorized that JQ1 blocks BRD4 mediated recruitment of p53 to chromatin in response to DNA damage, and prevents the p53-dependent DNA damage response pathway[142]. In the context of mutant p53 in MYC overexpressing models of lymphoma, JQ1 was shown to directly affect p53 dependent genes[144]. In this case the p53-regulated genes Bbc3, Trp53inp1, Ddit4, and Gadd45a were up-regulated[144]. HDAC inhibitors also upregulate these p53 dependent genes, but downregulate genes that are distinct from BETi[144]. Combining the 2 compounds lead to a synergistic induction of apoptosis at much lower concentrations than either single treatment, which could potentially avoid toxicity[144]. C/EBPα and C/EBPβ have also been shown to bind to BRD4 in a bromodomain-independent manner, however, there has not been

further investigation into the consequences this may have on transcription[145]. A thorough interrogation of how the interaction landscape of BET proteins changes after BETi was recently published. This study observed that certain interactions remained constant after BETi, including CHD4 and JMJD6 which are both known to bind the ET domain of BRD4, and suggests a lysine acetylation independent interaction[81]. Surprisingly, other interactions were enhanced after BETi, and these included the MRN complex (RAD50, MRE11 and NBS1) and TP53, which may explain BETi effects on DNA damage[81]. Finally, a novel interaction was discovered between BET proteins and the ubiquitin ligase KBTBD8, although the implications for this interaction are currently unclear[81].

The role of BETi in reducing the DNA damage response (DDR) was described in 2013 [146]. JQ1 treatment increased ionizing-radiation-induced H2AX phosphorylation with JQ1 treatment in a number of different cancer types, including breast, prostate and particularly glioma[146]. This BETi dependent impact on the DDR was later found to be most pronounced in breast and ovarian cancer cells treated with PARP inhibitors. JQ1 impaired homologous recombination by decreasing the foci formation of BRCA1 and RAD51, and this sensitized cells to PARP inhibition. JQ1 also increased NHEJ proficiency, which may also play a role in sensitizing to PARP inhibition. Interestingly, no other protein in any of the DNA damage repair pathways was affected[147]. In contrast to breast and ovarian cancer, JQ1 treatment in leukemia and osteosarcoma cells actually increases RAD51 activity, as well as the canonical increase in HEXIM1. This increase causes replication fork slowing, and also blunts DNA damage response similar to breast and ovarian cancer[148]. In a model of prostate cancer DDR was also decreased after sustained BET inhibition due to an increase in activity of the PRC2 complex, further

illustrating the importance of BETi on DDR in cancer through distinct, cancer type specific mechanisms[149]

A recent study by Suzuki et al. made significant discoveries related to BETi's impact on the noncoding genome[150]. First, they found SEs associated with miRNAs in multiple different tissues[97]. Second, they found that JQ1 inhibited pri-miRNA processing of SE associated miRNAs through reduced DGCR8/Drosha recruitment to SE-miRNA loci, thus reducing miRNA levels (Fig. 2.2A). They suggested that this novel function of JQ1 may partially explain its effects on multiple cancer types, given that multiple miRNAs near SEs in cancer cells were also known to be oncogenic[150]. Other recent studies have implicated miRNA in BETi's effects on cancer. In a model of B-cell lymphoma it was demonstrated that the miR-17-92 cluster, a known negative regulator of BIM, was significantly downregulated after JQ1 treatment. This led to an increase in BIM mRNA and protein levels, and subsequent induction of apoptosis[151]. In AML, downregulation of this miRNA cluster was associated with an increase of the tumor suppressor TXNIP, which also lead to apoptosis[152]. Another set of miRNAs, miR-29C and miR-29B2, are downregulated in t(8;21) AML after JQ1 treatment and lead to an increase in MCL1 levels. Co-treatment with an MCL1 inhibitor led to cell death, whereas JQ1 treatment alone only triggered growth inhibition[101]. JQ1 treatment in colon cancer led to a decrease in miR-21 levels, an important regulator in colon cancer. Reduced miR-21 levels triggered a reduction in Wnt/β-catenin signaling, which resulted in apoptosis of the cells[153]. OTX015 inhibits a number of pathologically relevant miRNAs in DLBCL. MiRs 21, 92a, and 155 were all downregulated directly by OTX015. Unexpectedly, miR-96 was upregulated after OTX015 treatment, and this was an indirect effect caused by the downregulation of its negative regulator PRMT5[154]. OTX015 treatment in multiple myeloma leads to an increase in the expression of CRBN and

although this does not affect viability, it does greatly increase the sensitivity of myeloma cells to a class of compounds called immunomodulatory drugs (IMiDs). Analysis of a GEO dataset of multiple myeloma followed by confirmatory qPCR revealed that miR-205 negatively regulates CRBN, and OTX015 dramatically reduced the expression of miR-205[155]. Besides miRNA, one long non-coding RNA (lncRNA) has also been identified as a target of BETi. In glioblastoma multiforme (GBM) I-BET151 reduced levels of the lncRNA HOTAIR, which is often overexpressed in GBM and is crucial for tumor cell proliferation, while upregulating the GBM downregulated lncRNAs MEG3 and NEAT1 through a currently unknown mechanism (Fig. 2.2B)[156].

More evidence is mounting that, despite the BETi dependent induction of profound transcriptional downregulation, there are a small number of genes upregulated even before acquired resistance to BETi is observed. Both Muhar et al. and Zhao et al. identified a small number of upregulated genes in their analysis of BETi at very early time points (<3hr), which suggests that genetic upregulation is also a direct consequence of BETi[101,103]. Analyzing genes upregulated as a direct result of BETi could identify potential synergies before a resistant phenotype develops in the treatment of cancer. BET protein eviction from chromatin can lead to altered transcription factor binding and cause transcriptional upregulation independent of BET proteins. Indeed, although Zhao et al. had identified miR29C and miR29B2 as regulators of MCL1, BRD4 was bound to the MCL1 promoter prior to BETi. They speculated that BRD4 displacement could lead to MCL1 transactivation by other transcription factors[101]. This type of mechanism of upregulation was confirmed in MLL–AF9 leukemic stem cells generated to be

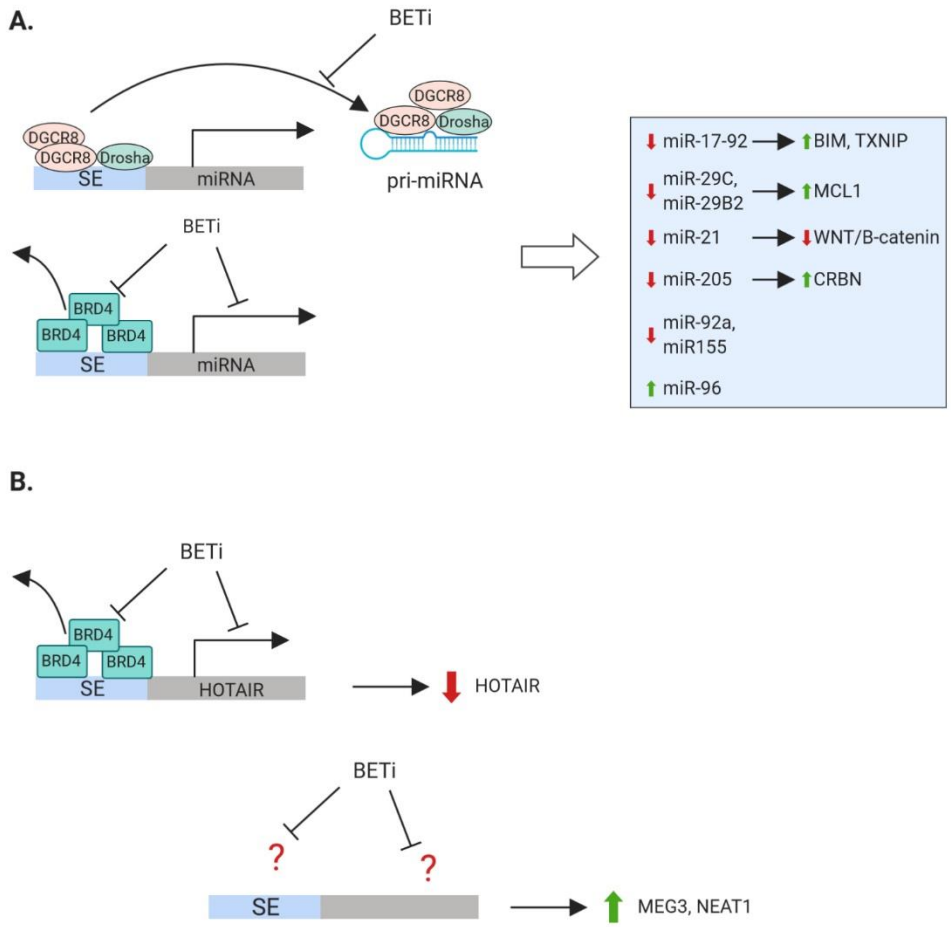

Figure 2.2: Mechanisms of BETi effects on ncRNA. (A) BETi regulation of miRNA through blocking Drosha and DGCR8 interaction with pri-miRNA or through displacement of BRD4 from miRNA super-enhancers. (B) BETi regulation of lncRNAs *HOTAIR, MEG3* and *NEAT1*.

resistant to the BETi I-BET. In the resistant cells, both the WNT/β-catenin and TGFβ pathways were upregulated. Notably, β-catenin was bound to chromatin in places where BRD4 was displaced, particularly at the regulatory elements of MYC, which maintained MYC expression and drove the resistant phenotype. Inhibition of the WNT/β-catenin pathway resensitized the cells to BETi[157].

Bidirectional kinome reprogramming may play a role in response and resistance to BETi. Ovarian cancer cells that acquired resistance to BETi after long term treatment were found to have upregulated pro-survival kinase networks to compensate[158]. Resistant cells showed increased levels or increased activating phosphorylation of one or multiple of the following receptor tyrosine kinases (RTKs): FGFR1–4, IGF1R, EGFR and PDGFRA. Some of the resistant cells showed decreased levels of RTKs, however, RNA-seq analysis showed that despite this decreased expression, increased ligand production led to increased signaling[158]. This led to increases in downstream RTK signaling, indicated by increased RAF, MEK, ERK2, JAK1 and increased phosphorylation of STAT3[158]. Interestingly, withdrawal of BETi from resistant cells almost completely reversed this compensatory signaling[158]. Certain RTKs were also upregulated after sustained BETi treatment in uveal melanoma cells. Uveal melanoma cells that acquired resistance to BETi showed increased levels of FGFR1, FGFR2, or FGFR1-4 depending on the cell line[159]. Increases in the ligand FGF were also seen after BETi, and addition of an FGFR inhibitor was able to resensitize resistant cells to BETi[159]. ERBB2-positive breast cancers treated with lapatinib, which targets ERBB2, become resistant through reactivation of ERBB2/ERBB3 and upregulation and increased signaling of multiple RTKs, including IGFR1, DDR1, MET, and FGFRs. SRC/FAK signaling and AKT reactivation are also BETi dependent mechanisms of resistance to lapatinib[160]. Co-treatment of resistant cells with JQ1 completely suppressed the upregulated kinases and targeted ERBB2 as well[160]. This suppression was solely due to JQ1's effect on BRD4, as inhibition of either BRD2 or BRD3 alone actually increased kinase transcription in response to lapatinib[160]. Models of metastatic breast cancer treated with PI3K inhibitors show similar RTK upregulation in resistant cells, but also show mTOR and MYC upregulation as mechanisms of resistance. Again, BETi treatment was able to suppress this

compensatory activation[161]. In head and neck squamous cell carcinoma, increased RTK signaling again mediated resistance, but an increased percentage of BRD4 positive cells was also found[162]. In this case BETi directly suppressed increased BRD4 signaling alongside suppressing increased HER3, MET and AXL signaling to overcome resistance[162]. Combining BET inhibitors with inhibitors targeting either ERBB2, PI3K, or anti-EGFR antibody was an effective way to prevent compensatory upregulation of RTKs. These non-canonical effects of BETi represent a unique opportunity to expand the clinical relevance of these compounds.

Materials and Methods

Cell Lines

U-937 (CVCL_0007), MV4-11 (CVCL_0064), SKM-1 (CVCL_0098), OCI-AML-3 (CVCL_1844), HEL (CVCL_0001), HL-60 (CVCL_0002), THP-1 (CVCL_0006) and ML-1 (CVCL_0436) cells were cultured in RPMI with 10% fetal bovine serum (FBS). KG-1 (CVCL_0374) cells were cultured in IMDM with 20% FBS. TF-1 (CVCL_0559) cells were cultured in RPMI with 10% FBS and 2ng/mL GM-CSF. M-07e (CVCL_2106) cells were cultured in IMDM with 10% FBS and 10 ng/mL GM-CSF. U-937, KG-1, TF-1, THP-1, HL-60, and MV-4-11 cells were obtained from ATCC. SKM-1, HEL, OCI-AML-3, and M07-e cells were obtained from DSMZ. ML-1 cells were obtained from ECACC. Cell lines were not authenticated, but were routinely tested for mycoplasma by HEK Blue Detection. Cell lines were thawed from cryopreservation and used in experiments for no longer than 3 months.

Patient Samples

Bone Marrow samples were obtained from 12 patients. Patient samples were collected after obtaining written informed consent. The use of human materials was approved by the Institutional Review Board of the Moffitt Cancer Center Scientific Review Committee and the

University of South Florida Institutional Review Board in accordance with the Declaration of Helsinki. Patient characteristics for patient samples used in PDX studies and Colony Forming Assays are shown in Supplemental Table S3.

Heterotopic Cell Line Models and CMML PDX

All animal studies were approved by the Moffitt Cancer Center Institutional Animal Care and Use Committee.

U-937, or SKM-1 P1-14 cells were resuspended in cold 0.9% sterile saline and mixed with Matrigel Matrix to a final protein concentration of 7mg/mL. $3x10^5$ U-937 or $1x10^6$ SKM-1 and SKM-1 P1-14 cells were injected into the right flank of NGS-S(RRID:IMSR_JAX:013062) mice (male, 20-30g, 10-30 weeks old) and allowed to reach between $100mm^3$ and $150mm^3$ before beginning treatment. Tumors were measured at least twice a week by caliper and tumor volume was calculated using the formula; Tumor volume = width × width × length × 0.52. Mice were randomized into 4 groups: vehicle, INCB057643, INCB053914 or Combination. INCB057643 was administered once a day at 10mg/kg, 5 days a week by oral gavage. INCB053914 was administered twice a day at 30mg/kg, 5 days a week by oral gavage. Both compounds were dissolved in 5% N,N-dimethylacetimide/95% 0.5% methylcellulose.

For CMML PDX experiments, frozen BMMCs were first thawed and treated with DNAse I for 15 minutes to create a single cell suspension. Cells were washed once and resuspended in 0.9% sterile saline and injected via tail vein into NSG-S mice (male or female, 20-30g, 10-30 weeks old) sub-lethally irradiated the day prior. At least 2 million cells were injected into each mouse and treatment started between 2-3 weeks after injection. Mice were randomized into the same groups as the heterotopic cell line models. Treatment lasted 2 weeks and all mice were

euthanized shortly after the end of treatment. The spleen, peripheral blood and femurs were taken for analysis. One femur and a portion of the spleen were fixed in formalin and used for IHC. Another portion of the spleen, peripheral blood and bone marrow were further processed by creating a single cell suspension and lysing red blood cells with ACK lysis buffer. Cells were then washed with PBS and stained with zombie violet viability dye (Biolegend Cat#: 423114) before fixation in 1.6% formaldehyde and storage at 4°C.

Viability Assays

For the drug screen, cells were plated with compounds in 384 well plates and viability was assessed after 72hrs using Cell-Titre Glo (Promega) according to the manufacturer's instructions. For all other viability assays, cells were plated with compounds in 96 well plates and viability was assessed after 72hrs using CCK8 following the manufacturer's instructions. synergy was calculated using Zero Interaction Potential (ZIP) via SynergyFinder[163]

Persistent Cell Lines

U-937 and TF-1 cells were grown in medium containing 500nM INCB054329 and SKM-1 cells were grown in medium containing 300nM INCB054329. Persistence was determined by significantly increased IC_{50} by CCK8 and steady growth in medium containing INCB054329. Double persistent cell lines were generated by taking BETi persistent cell lines and treating with 1μM Ruxolitinib until cells demonstrated consistent growth under this condition.

Colony Forming Assays

Frozen BMMCs were thawed and prepared in a similar manner to PDX experiments. Cells were then resuspended in IMDM + 2% FBS at a concentration of 200,000 cells per mL. 300μL of cell suspension and 3μL of each compound were added to 3mL Methocult 4034 (StemCell Cat#: 04034) and mixed by vortexing for 1 minute. 1.1 mL of cell mixture was plated in StemCell

smart dishes, incubated for 14 days and read on StemVision (Stem Cell Technologies) for the final colony count.

Results

Preliminary Screening Identifies BETi and PIMi as Synergistic.

To nominate synergistic BETi combinations for the treatment of CMML. We utilized an in-house targeted chemical screen of 300 compounds which are FDA approved or in clinical cancer development[164]. U-937 and SKM-1 cells, both human monocytic leukemia cell liens, were incubated with the IC_{20} (U-937:155nM, SKM-1: 30nM) of INCB054329 and two doses (0.5μM and 2.5μM) of each library compound. Cell viability was evaluated 72hrs post-treatment using CellTitre-Glo. Combinations with a drug - base/drug + base ratio greater than 2 were chosen for further consideration as previously described[164]. As expected, known synergies with JAK, HDAC, CDK, MEK, and PI3K inhibitors were found supporting the validity of our chemical screen to identify clinically relevant BETi combinations[161,165-173]. After previously published interactions were filtered out (Appendix B), the only combination with a drug - base/drug + base ratio greater than 2 was with SGI-1776, a pan-PIM kinase inhibitor (Fig. 2.3A). PIM kinases are a family of serine/threonine kinases which are primarily known for their anti-apoptotic properties, although they have also been shown to increase cellular proliferation and are known to interact with the *MYC* oncogene. Their characteristics will be further discussed in **Chapter 3**. To validate therapeutic synergy between these BET and PIM inhibitors, we repeated the experiment in three human myeloid cell lines (U-937, TF1 and SKM-1) with 7 doses of INCB054329 and either pan-PIM inhibitors SGI-1776 or INCB053914. In all lines, and in both PIM inhibitors SGI-1776 or INCB053914. In all lines, and in both BET/PIM inhibitor combinations, *in vitro* synergy was observed consistent with our initial compound screen (Fig.

2.4B). AZD1208, another PIM inhibitor included in the screen, did not achieve the threshold. However, given that the only compound that met our threshold was a PIM inhibitor we performed formal synergy analysis and were able to demonstrate that AZD1208 was indeed synergistic with BET inhibitors similarly to that observed with INCB054329 and SGI-1776 (Fig. 2.3B). Importantly, synergy was evident in the low dose PIMi chemical screen and most models when testing low doses of both BETi and PIMi. We decided to further profile PIM inhibitors as well as a select few of the other top hits in our screen using patient sample colony forming assays (CFAs) to determine whether PIM inhibitors would remain the most effective combination. Bone marrow mononuclear cells from CMML patients were plated with a single dose of both INCB054329 and each different test compound either as a single agent or in combination. Compared to AKT, JAK and MEK inhibition, PIM inhibition remained the most potent BET inhibitor combination (Fig. 2.4A and B).

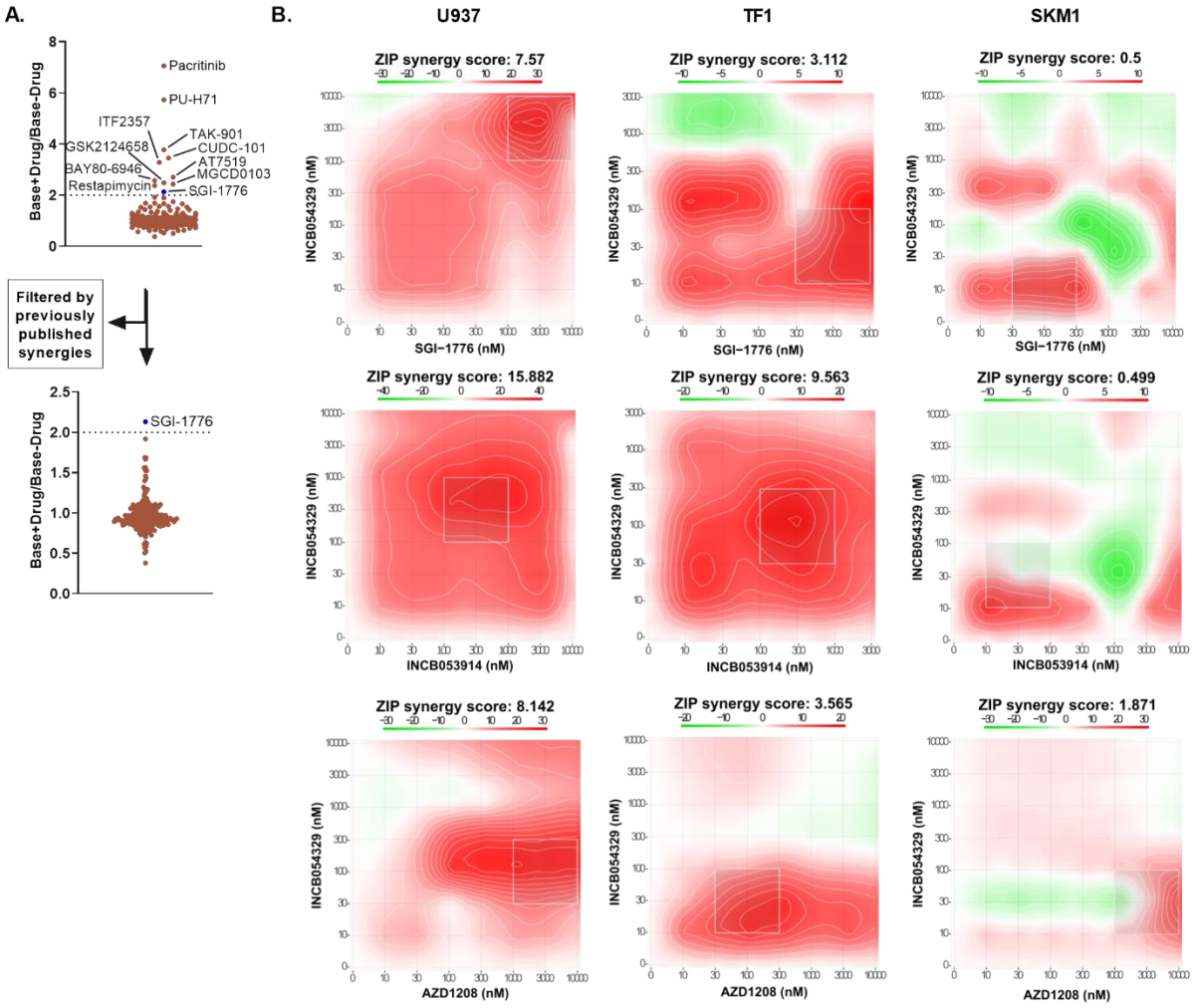

Figure 2.3: Drug screen demonstrates BETi/PIMi synergy. (A)Results of compound screen performed in U-937 and SKM-1 cells. Top: Ratio of base drug +/- experimental compounds for all targets. Bottom: Targets filtered by previously published research. (B) ZIP synergy plots generated by SynergyFinder of U-937, SKM-1 and TF1 cell lines; red indicates synergy, green indicates antagonism. Cell lines were treated with 7 increasing doses of both BETi and PIMi for 72hrs.

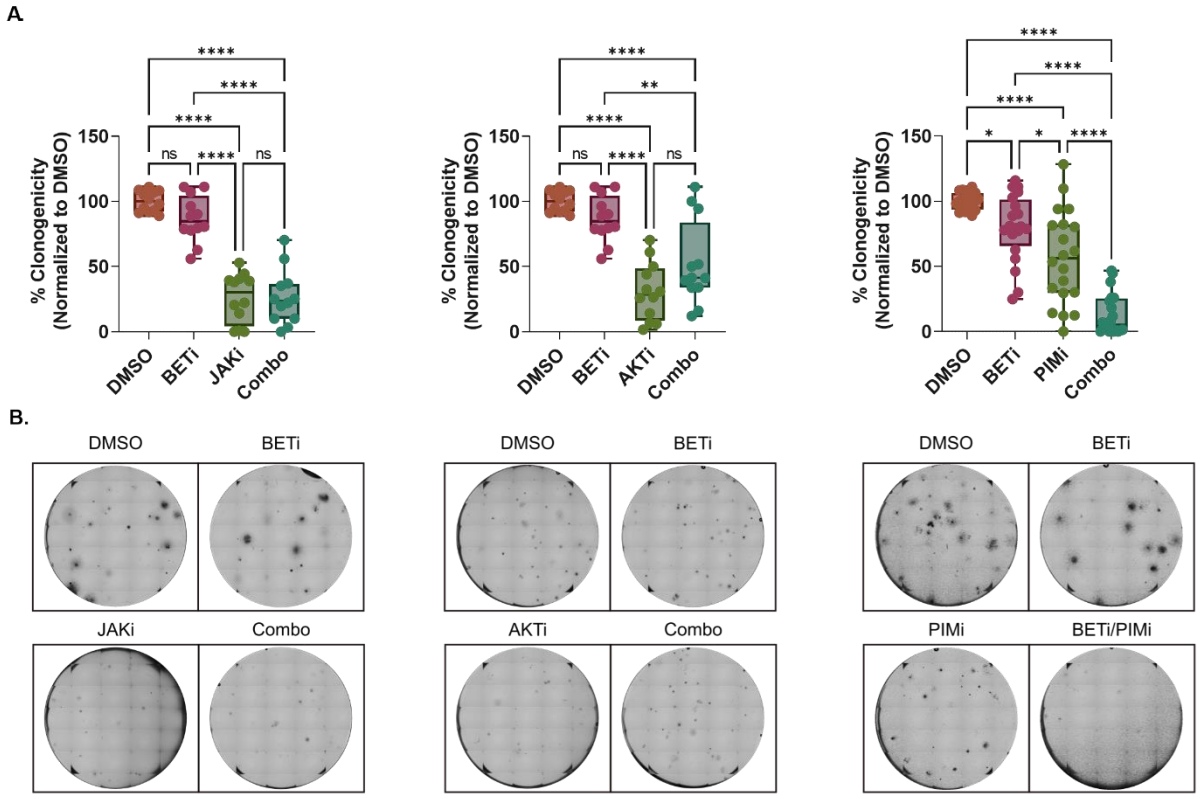

Figure 2.4: Patient Sample CFAs. (A) CFA data from 6 (JAKi and AKTi) or 10 (PIMi) patient samples treated with a single dose or combination of each compound. (B) Representative images from data collected in A.

Persistent Cell Lines are Particularly Sensitive to PIM Inhibition

Rapid persistence to BETi has been shown to occur through various mechanisms in both leukemia and solid tumors which contributes to its limited single-agent clinical efficacy[157,158,174-176]. To determine whether PIMi could overcome persistence to BETi, we generated 3 BET persistent human leukemia cell lines. We then compared the IC_{50} of PIMi to that of the parental cell lines tested. Persistence was achieved by daily treatment of cell lines with 500nM INCB054329 or 300nM INCB054329 for SKM-1 cells (Fig. 2.5A). These cells were chronically exposed to INCB054329 to maintain persistence. Consistent with the notion that these cells demonstrate BET inhibitor persistence, drug withdrawal for 1 week led to resensitization to BETi

(Fig. 2.5B). At 60 days, all three cell lines demonstrated an increase in PIMi sensitivity compared to their parental counterparts, particularly in the human monocytic leukemia cell line U-937 (Fig. 2.5A). Persistence to BETi/JAKi combination therapy has also been reported in the related myeloproliferative diseases (MPNs) with promising clinical efficacy. Since JAKi/PIMi combinations have been tested in AML and MPN clinical trials, we tested whether PIM inhibition may be able to overcome BETi/JAKi persistence. We generated BETi/JAKi persistent U937 cells by chronically treating U937 54329P cells with ruxolitinib

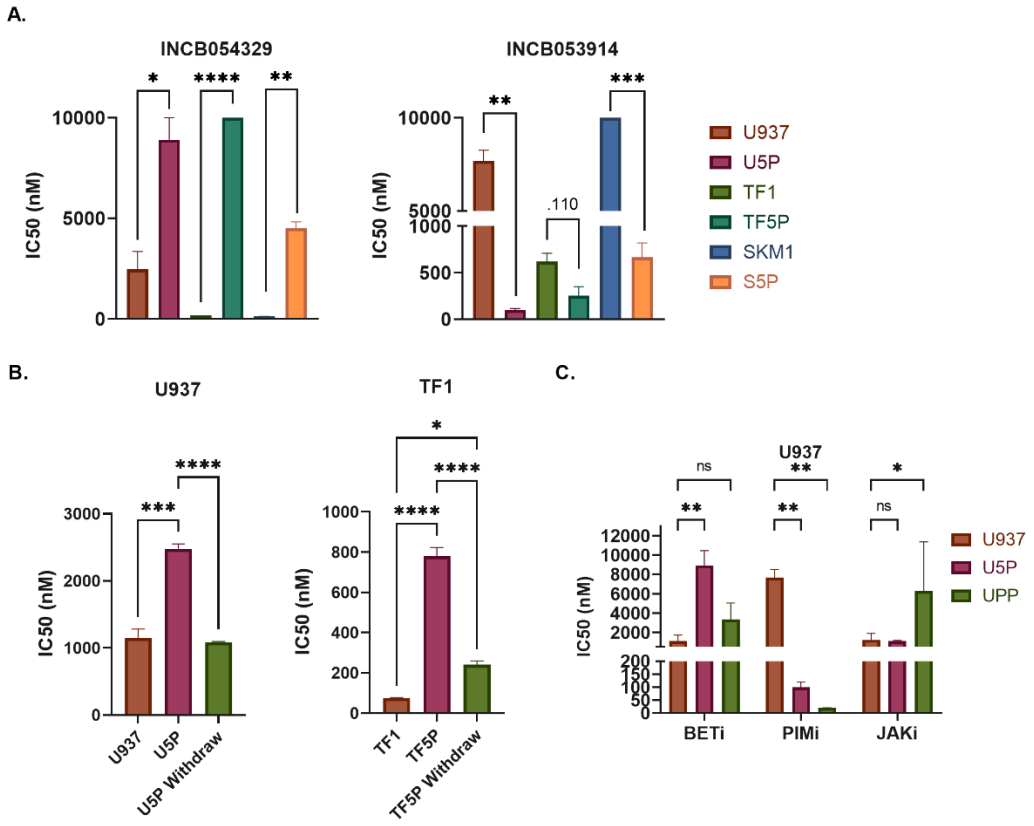

Figure 2.5: PIMi can overcome BETi persistence. (A) IC_{50} of parental cell lines and their persistent counterparts treated with either BETi(INCB054329) or PIMi(INCB053914) for 72hrs. (B) IC50 values of parental and persistence cell lines treated with INCB054329 for 72hrs. U5P and TF5P are persistent and withdraw indicates cells were grown without INCB054329 for 1 week. (C) IC50 values of parental single persistent (U5P) and double persistent (UPP) U937 cells treated with either BETi, PIMi or JAKi for 72hrs.

until these cells proliferated at 1μM. Although IC50 curves were not significantly different in the double persistent lines, they were able to grow under constant 500nM INCB054329 and 1μM Ruxolitinib (Fig 2.5C). These double persistent U937 cells were significantly more sensitive to PIM inhibition than either wild type U937 cells or BETi persistent U937 cells by CCK8 (Fig. 2.5C).

In vivo Cell Line Models Replicate Synergy Seen in vitro

To determine whether the observed *in vitro* synergy was present *in vivo*, heterotopic tumors were established in NSG-S mice[177] with either U-937 (n=10/group) or SKM-1 cells (n=10/group). After tumors reached between 100 and 150mm^3, drug treatment was started with 10 mpk (milligrams per kilogram) INCB057643 and 30 mpk INCB053914 via oral gavage either as single agent or in combination and continued for 2 weeks, with tumor measurements occurring twice per week and at endpoint. These experiments identified a statistically significant decrease in tumor volume utilizing both cell line models with combination treatment suggesting that this combination strategy may be effective *in vivo* (Fig. 2.6A).

PDX Models of CMML Demonstrate BET/PIM Synergy

To determine whether combination therapy was a viable therapeutic approach *in vivo*, we generated CMML patient derived xenografts (PDX) as previously described (Table 1) [178]. After engraftment was established in each model, mice were randomized (3-5 mice per group) and treated with BET inhibitor, PIM inhibitor, or the combination for 2 weeks using the same doses as heterotopic cell line xenograft experiments (Fig. 2.6B). Initially, mice treated with the maximum tolerated dose of BETi and PIMi rapidly lost weight and had unacceptable toxicity

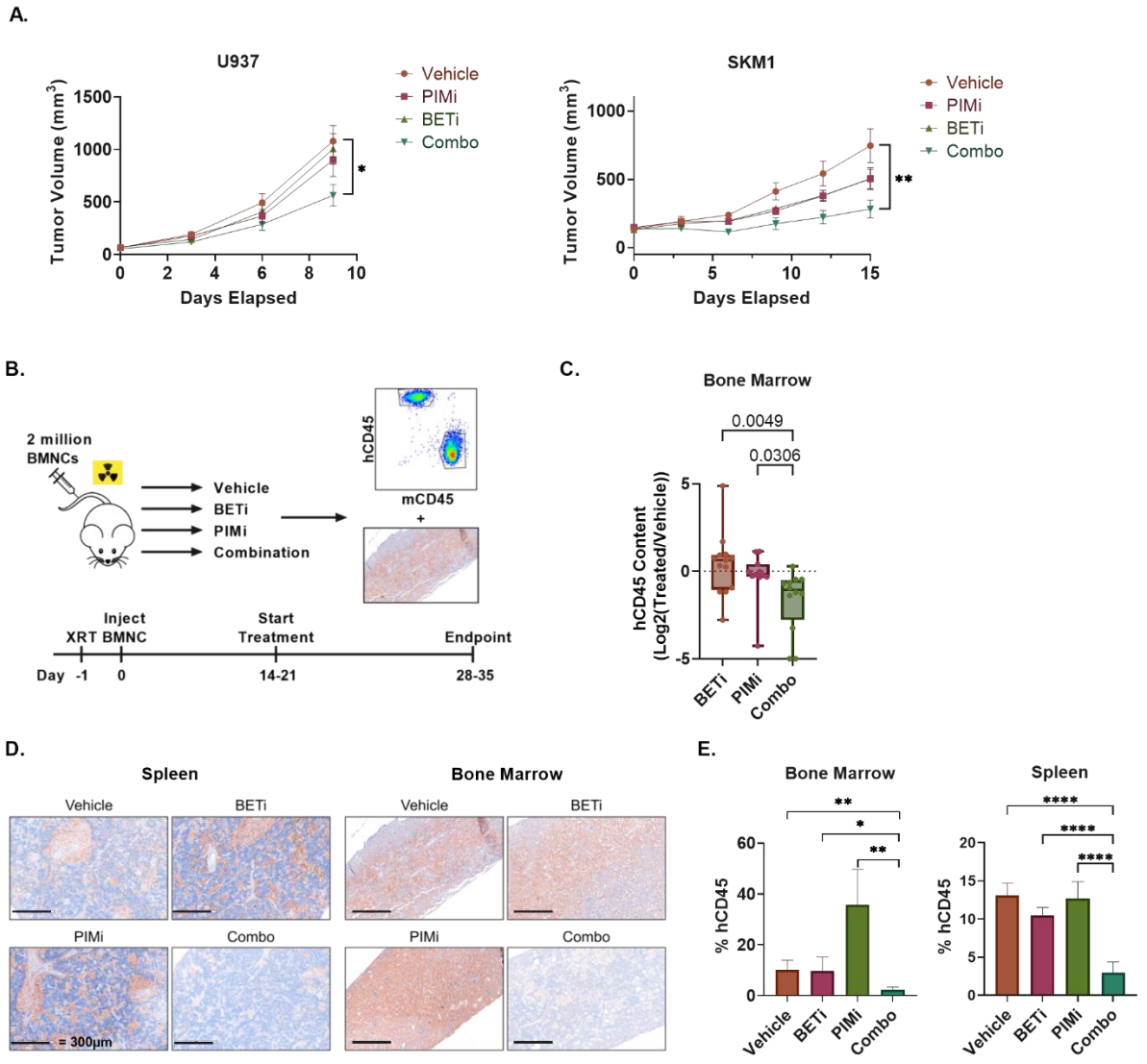

Figure 2.6: *In vivo* models of disease replicate synergy seen *in vitro*. (A) Tumor size calculations of mice subcutaneously injected with either U-937 or SKM-1 cells and treated with BETi(INCB057643), PIMi(INCB053914) or combo (U-937: Vehicle and BET n=10, PIMi n=8 Combo n=9; SKM-1: Vehicle, BETi, PIMi n=9, Combo n=8). Mice were treated for 2 weeks unless ulceration occurred. (B) Graphical representation of PDX experiment timeline. (C) Flow cytometry analysis of hCD45 in bone marrow of mice from 4 PDX experiments with 4 unique patients. Mice were treated with either BETi(INCB057643), PIMi(INCB053914) or combination. Significance determined using Kruskal-Wallis. (D) Representative images of bone marrow and spleen slides stained with hCD45. (E) Quantification of hCD45 in bone marrow and spleen IHC slides from PDX experiments.

Table 1: Patient characteristics for CFA and PDX experiments.

Samples used in PDX							
ID	AGE	SEX	WHO	FAB	MAYO	Blast%	Mutations
RX-1-001	unknown	unknown	CMML-0	MPN-CMML	2	1	SRSFF2 (c.43T>G, p.S15A; 50.8%), NRAS (c.35G>A, p.G12D; 32.7%), TET2 (c.5037C>A, p.Y1679*; 48.8%), TET2 (c.2305delC, p. Q769Sfs*44, 39%)
3-F-004	67	Male	CMML-2	MD-CMML	2	16	NRAS (p.G12S), SF3B1 (p.K666N), TET2 (p.R123fs and p.H839fs)
3-G-004	60	Female	CMML-0	MP-CMML	2	1	TET2, CBL, SRSF2, SETBP1
KB-10-005	75	Female	unknown	unknown	unknown	unknown	NRAS, ASXL1, TET2, ZRSR2

Samples used in CFAs							
2-C-001	71	Male	CMML-1	MPN-CMML	unknown	5	TET2, SRSF2, NRAS
2-J-001	57	female	unknown	unknown	unknown	unknown	KRAS, NOTCH1
2-V-001	76	Male	CMML-0	MP-CMML	3	5.5	TET2(p.G1703* C.5107G>T, p.N1823Kfs*23), CBL(p.P417R), SRSF2(p.P95L)
3-D-001	71	Male	CMML-0	MP-CMML	unknown	unknown	CEBPA, EZH2, NRAS, STAG2, TET2
3-E-002	54	Male	CMML-1	MP-CMML	3	5	SRSF2(p.P95H), TET2(p.Q734Rfs*17, c.2198del)
3-G-001	60	Female	CMML-0	MPN-CMML	2	1	TET2, CBL, SRSF2, SETBP1
2-D-001	82	Male	CMML-2	MP-CMML	2	5	NRAS, SRSF2, SETBP1, ASXL1
3-F-001	67	Male	CMML-2	MD-CMML	2	15	TET2, NRAS, SF3B1
1-A-001	49	female	CMML-0	MD-CMML	3	1.2	CEBPA, KRAS
2-U-001	56	Female	CMML-0	MPN-CMML	3	4	from 8 months prior: TET2 (c.5735A>G, p.H1912R), TET2 (c.2491C>T, p. Q831*), SETBP1 (c. 1879C>T, p. R627C), NRAS (c. 35G>T, p. G12V)

(data not shown). However, given that *in vitro* synergy optimally occurred at lower doses of both inhibitors PDX experiments were repeated with low dose BET and PIM inhibition. After 2 weeks of treatment, all mice were simultaneously euthanized in order to compare engraftment, consistent with other preclinical myeloid studies on drug efficacy[179,180]. Mice treated with either low-dose INCB057643 or INCB053914 alone showed a variable response to treatment, similar to *in vitro* experiments, while the combination was consistently able to reduce leukemic engraftment as evidenced by a reduced percentage of human CD45+ cells in the bone marrow [179-181] by both flow (Fig. 2.6C) (BETi vs Combo mean rank diff. = 13.49. PIMi vs Combo mean rank diff. = 11.30) and IHC (Fig. 2.6D and E).

Discussion

Despite advances in the molecular pathobiology and genetics of myeloid malignancies, no targeted therapeutics have demonstrated an impact on overall survival or augment natural history. This is especially evident in CMML where there are no CMML-specific approved therapies and the vast majority of patients will succumb to disease within 5 years [19]. To address this therapeutic gap, we utilized a targeted chemical screen and identified BETi and PIMi as a synergistic combination in preclinical models *in vitro* and *in vivo*. It is consistent with the notion that BET inhibitor and kinase inhibitor combination therapy may be an attractive therapeutic strategy in hematologic malignancies[182-186]. Our broader chemical screen identified numerous potential BET inhibitor combinations, including many previously published interactions, supporting the validity of our results. Further profiling of top interactions revealed that AKT inhibitor MK2206 was antagonistic in patient sample CFAs. AKT inhibitors have been studied in combination with BET inhibitors and demonstrated synergy in preclinical models in many different cancers. The AKT/PI3K and PIM kinase pathways overlap significantly in terms of

downstream targets[187], so it's unclear why there would be such a dramatic difference in synergistic potential within our CFA experiments. Certainly, disease specific characteristics and downstream targets unique to PIM or AKT/PI3K could contribute to these differences, and would require further investigation to determine the exact cause. Similarly, a combination of BET/JAK inhibition has been shown to be effective in preclinical models of AML, but was not synergistic in our CFA models. Although there is research to support a BET/MEK inhibitor combination in secondary AML, we were unable to properly assess the efficacy of MEK inhibitors in our CFA model[188]. The single agent dose of MEK inhibitor used in CFA experiments was too potent and prevented the formation of any colonies in all samples. We decided to focus on the novel combination with PIM inhibitors as this combination performed the best in secondary testing with a small subset of compounds in CFAs.

Heterotopic mouse models of disease utilizing U937 and SKM1 cells also demonstrated synergy between BETi and PIMi. Consistent with the heterogeneity of response to single agent BETi in clinical trial, single agent BET inhibition was also minimally effective in SKM1 cells and completely ineffective in U937 cells. Additionally, we utilized our CMML PDX models, which recapitulate many features of the human condition [178], to credential combination BETi/PIMi therapy in a randomized murine clinical trial and identified statistically superior repression of human leukemia engraftment with combination therapy across all models.

Cellular persistence poses a significant challenge in single agent BET inhibitor treatment. We generated 3 BETi persistent cell lines and found that all 3 cell lines were much more sensitive to PIMi demonstrating that treatment with PIMi could overcome cellular persistence to BETi. Additionally, we determined our chronically BETi treated cells were persistent and not resistant. After 2 weeks of drug withdrawal, U937 and TF-1 persistent cells returned to their

parental BETi sensitivities, consistent with previous reports of drug withdrawal in chronic BETi treated cells. This has some interesting implications for clinical therapy. In our mouse models we delivered the BETi/PIMi combination simultaneously and saw significant reduction in tumor size/ engraftment, but there are studies in AML involving a BETi/JAKi combination that suggests a staggered approach may still provide full clinical benefit while reducing toxicity and this could apply to BETi/PIMi as well. Further investigation of PIM levels after BETi withdrawal would be necessary to determine whether a staggered approach would be feasible.

Clinical trials in AML have identified a subset of patients that relapse after combination BET/JAK treatment. To investigate the potential of PIM inhibitors to overcome this, we generated U937 cells which were persistent to both BETi and JAKi. These cells displayed reduced growth kinetics, but were able to survive and proliferate under both 500nM BETi and 1uM JAKi. These cells were dramatically more sensitive to PIM inhibition than parental cells and additionally were significantly more sensitive than single BETi persistent cells. While this data could simply represent the cell's inability to survive the increased selective pressure of 3 separate chemical compounds, given the magnitude of difference in sensitivity and the knowledge that each one of these compounds is known to affect the JAK/STAT pathway it is far more likely that the JAK/STAT pathway is absolutely crucial for the survival of myeloid leukemia cells. Significantly more research is required before moving forward, but PIM inhibitors should be considered for further clinical study in patients who have failed BET/JAK combination therapy. Additionally, significant care would need to be taken in the way this treatment is administered as these compounds have overlapping toxicities. Our data provides strong evidence to clinically test low dose BET and PIM inhibition in CMML, which we predict

would be most responsive to this combination based on its hypersensitivity to GM-CSF[54,178], which will be discussed further in **Chapter 3**.

CHAPTER THREE: BET INHIBITOR INDUCED PIM UPREGULATION MEDIATES PERSISTENCE TO BET INHIBITION

Note. Portions of this chapter have been adapted from previously published work in Letson et al., *Clinical Cancer Research*, 2023[61]

Introduction

Characteristics of PIM Kinase Family Members

Proviral Integration site for Moloney murine leukemia virus (PIM) kinases were first discovered while studying murine leukemia virus (MuLV)-induced lymphoma[189]. PIM1 was first identified as a small chromosomal region with frequent proviral insertions that were associated with the transcriptional activation of the Pim1 gene[189,190]. PIM2 and PIM3 were discovered upon further insertional mutagenesis in Pim1 deficient transgenic mice and Pim1/Pim2 deficient transgenic mice respectively[191,192]. PIM proteins are serine/threonine kinases located on different chromosomes and share 60% sequence similarity between the isoforms[193,194]. While PIM3 only has 1 transcriptional start site which codes a single protein, PIM1 and PIM2 have multiple transcriptional start sites. PIM1 has two, coding a 44kda (PIM1-L), which localizes primarily in the cytoplasm, and 34kDa (PIM1-S) which localizes primarily in the nucleus[195,196]. PIM1-L has an additional N-terminus that can anchor it to the cell membrane, and PXXP domain, which can interact with SRC homology 3 domains[196]. PIM2 has three isoforms that include a 40kDa, 38kDa and 34kDa protein, although very little is known about how these variants may differ in functionality. It has been suggested that the 34kDa variant is more associated with antiapoptotic activity than the 38kDa and 40kDa forms[197]. While all PIM isoforms are ubiquitously expressed,

there are some tissue specific differences: PIM1 is highly expressed in hematopoietic cells, gastric, head and neck, and prostate tumors[198,199]; PIM2 is highly expressed in lymphoid and brain tissues[200]; PIM3 is highly expressed in breast, kidney and brain tissues[201]. Despite differences in tissues specificity and protein isoforms, multiple studies have demonstrated functional redundancy between PIM kinase isoforms[202,203]. Because of this redundancy, it is believed that PIM kinases can compensate for the loss of a single PIM kinase.

Regulation of PIM Kinases

PIM kinases also have a very short half-life (~5min) and their transcriptional and post-transcriptional regulation is tightly controlled[187]. PIM kinases are unique compared to other kinases in that they lack any regulatory domains and their activity is correlated to protein expression[204]. As such, they are considered constitutively active, with regulation occurring entirely by either transcription or protein stability. At the transcriptional level, PIM kinases are regulated largely through JAK/STAT pathway activation[205]. Cytokine and growth factor signaling at the receptor leads to JAK phosphorylation, subsequent STAT phosphorylation and dimerization which leads to transcription of STAT targets. STAT1, STAT3 and STAT5 specifically are the primary transcription factors that regulate PIM expression[206,207]. Interestingly PIMs can in turn regulate SOCS, a negative regulator of STATs, leading to a negative feedback loop[208]. In addition to STATs, NF-κB, through CD40 stimulation, and KLF5, in response to DNA damage can also regulate the expression of PIM kinases[209,210]. PIM kinase transcripts contain a long GC-rich region in their 5'-UTRs, which results in a weak transcript that requires cap-dependent translation[211]. Overexpression of EIF4E, a cap-binding factor, was shown to greatly enhance the expression of PIM kinases through increased cap-dependent translation[212]. A number of MicroRNAs (miRNA) have been discovered that can negatively regulate PIM

transcription through 3'-UTR mediated degradation of mRNA[213-216]. At the protein level, PIM kinases are regulated through ubiquitination and proteasomal degradation. The specific E3-ligase complexes that mediate PIM degradation are still unknown, however, PIM protein stability can be altered by the chaperone proteins HSP70 and HSP90. Binding of the Beta subunit of HSP90 stabilizes PIM proteins[217], whereas HSP70 binds ubiquitinated PIM proteins and promotes their degradation[218]. Hypoxic conditions also prevent PIM proteasomal degradation through an HSP90 dependent manner[219]. PIM kinases have also been shown to interact with PP2A, which results in the dephosphorylation and subsequent ubiquitination and proteasomal degradation of PIM proteins[220,221].

PIM Kinases in Cancer

PIM kinases are considered weak oncogenes. Overexpression of PIM kinases in T- and B-cells resulted in lymphomas, but with very low incidence rate and a high latency period[222]. In prostate cancer cell lines, PIM overexpression was unable to transform benign cells[223]. However, overexpression in prostate cancer cell lines representative of later stages of the disease significantly enhanced the tumorigenic potential of these cells[224]. Thus, overexpression of PIM kinases is typically seen in later stages of disease and is frequently associated with significantly worse outcomes. PIM kinases have a substantial number of downstream targets that may explain why their overexpression can enhance the pathogenicity of a variety of cancers.

PIM kinases directly phosphorylate the cell cycle regulatory proteins p21waf1 and p27kip. PIM1 specifically phosphorylates p21waf1, stabilizing the protein and leading to its accumulation in the cytoplasm and increased cell proliferation[225,226]. p27kip, which is responsible for halting cell cycle progression at the G1 phase, is regulated at both the transcriptional and translational level by PIM kinases. Transcriptionally, PIM kinases

phosphorylate and inactivate FoxO1a and FoxO3a, two transcription factors that drive p27kip expression[227]. Translationally, PIM kinases can directly phosphorylate p27kip at Thr157, which promotes its interaction with 14–3–3 proteins and proteasomal degradation in the cytoplasm[228]. PIM kinases also directly phosphorylate SKP2, the E3 ligase associated with p27, at Thr417, stabilizing SKP2 and enhancing the proteasomal degradation of p27[229]. PIM kinases can further promote cell cycle progression through phosphorylation and activation of CDC25A (G1-S phase) and CDC25C (G2-M phase) as well as inhibition of the CDC25 inhibitory protein cTak-1[199,230,231]. Proliferation through increased cell cycle progression requires increased protein synthesis, which PIM kinases can contribute to as well. PIM kinases phosphorylate PRAS40, an inhibitory subunit of the mTORC1 complex, increasing mTOR-mediated phosphorylation of 4EBP1 and enhance the expression of EIF4E and B[232]. Overexpression of PIM kinases is also sufficient to maintain phosphorylation of 4EBP1 and increase translation independent of the mTORC1 complex[233].

The most well-known and well-studied functions of PIM kinases are their anti-apoptotic and cell survival functions. PIM kinases directly phosphorylate Bad at Ser112, disrupting its associating with Bcl-2 and Bcl-XL, promoting its binding to 14-3-3 and increasing its retention in the cytosol, resulting in an anti-apoptotic effect[234]. ASK1 signaling through the MAPK pathway can promote stress-induced cell death, but phosphorylation by PIM kinases completely inactivates this pathway, promoting cell survival[235]. Recent studies have demonstrated PIM kinases to be important for regulating cellular ROS levels. They can reduce ROS levels through upregulation of NRF2, which translocates into the nucleus and activates a transcriptional program that upregulates antioxidant molecules[236]. Overexpression of PIM1 or PIM2 increases mitochondrial membrane potential and basal PIM levels regulate the level and activity of DRP1,

which promotes mitochondrial fission[197,237]. These effects on mitochondria contribute to both reduced ROS levels and regulate apoptosis.

PIM Kinases in Drug Resistance

Many of the above-mentioned properties of PIM kinases in cancer can serve as resistance mechanisms in the context of a variety of drug treatments for cancer. There are, however, specific mechanisms related to drug resistance that PIM kinases are known to effect. Overexpression of PIM kinases protects cancer cells from a wide variety of chemotherapies, such as taxane- and platinum-based therapies[238]. This is likely due to PIM kinase regulation of multiple drug transporters. PIM kinases promote the expression of multi-drug resistance associated ATP-binding cassette (ABC) proteins, which act as efflux pumps for multiple chemotherapies[239]. The G-subfamily ABC transporter (ABCG2) is directly phosphorylated by PIM1, which promotes its multimerization and translocation to the cell surface[240]. PIM kinases also regulates the expression of a multidrug transporter associated with therapeutic resistance, p-glycoprotein (Pgp). Phosphorylation by PIM1 protects it from proteasomal degradation as well as enhances its glycosylation and translocation to the cell surface[241].

PIM kinase downstream targets share considerable overlap with PI3k/AKT/mTOR pathway downstream targets, leading to overexpression of PIM kinases being a significant mechanism of resistance for therapies involving this pathway. In prostate cancer cells, treatment with the pan-AKT inhibitor GSK690693 lead to the increase of PIM1 along with several other kinases[242]. Treatment with either inhibitor or siRNA against PIM1 prevented the increase of both AKT and the other RTKs[242]. An in vitro screen in breast cancer cells for genes that conferred resistance to PI3K inhibitors revealed both PIM1 and PIM3 to be highly associated with PI3K inhibitor resistance, and PIM1 expression was predictive of response to PI3K inhibitors in breast

cancer cell lines[243]. Even dual inhibitors of this pathway can be affected by PIM expression. Lung cancer cells treated with a dual PI3K/mTOR inhibitor also showed an increase in PIM1 expression, and a PIM inhibitor was able to resensitize these cell lines to PI3k/mTOR inhibitors[244]. Triple inhibitors, targeting PIM/PI3K/mTOR have been developed, and show considerably more efficacy in a variety of cell lines compared to either single agent alone[245].

Despite not sharing downstream targets as in the case of PI3k/AKT/mTOR inhibitors, PIM kinases do promote resistance to a variety of other tyrosine kinase inhibitors (TKIs). MET inhibitors are frequently used to treat lung and gastric cancer with MET amplification. In cell lines chronically treated with MET inhibitors, PIM1 and PIM3 are both overexpressed and PIM1 overexpression in parental cell lines was sufficient to induce de novo MET inhibitor resistance [246]. MET amplification is also seen in the context of EGFR-TKI resistance in lung cancer, and again, PIM1 and PIM3 are overexpressed after MET inhibitor treatment and contribute to resistance[247]. PIM1 overexpression also mediated resistance to ALK inhibitors in anaplastic large cell lymphoma cell lines[248]. In AML, FLT3 internal tandem duplication (ITD) is a frequently seen mutation that can be treated using FLT3 inhibitors[249]However, these patients eventually become resistant due to further mutations within the ITD. PIM inhibitors have been used to overcome this resistance and although PIM inhibitors alone have no effect, these inhibitors can resensitize cells to FLT3 inhibition[249,250].

PIM Kinase Inhibitors

The first generation of PIM inhibitors preferentially targeted PIM1, with significantly less potency towards the other isoforms[251]. Once it was determined that PIM kinases can be compensatory to one another and to fully inhibit downstream PIM targets required the inhibition of all 3 isoforms, the second generation of PIM inhibitors were developed to target all

isoforms[251]. SGI1776 is the only first-generation inhibitor that went into clinical trial. However, the phase I trial was terminated due to cardiac toxicity and lack of efficacy[251]. AZD1208 was one of the first second-generation inhibitors to move into clinical trial. Multiple phase I studies were performed in advanced solid tumors and advanced hematologic malignancies. Unfortunately, none of those phase I studies demonstrated sufficient clinical responses and similar to SGI1776, AZD1208 was associated with adverse events leading to its termination[252]. LGH447 and INCB053914 are the two most recently developed second-generation inhibitors, both of which have considerable more potency than AZD1208. INCB053914 has been in 3 phase I clinical trials in advanced hematologic malignancies where it was favorably tolerated, successfully inhibited PIM activity and had few adverse events, which could be treated with additional therapy[253]. PIM447 has undergone 5 phase I clinical trials in advanced hematologic disease and, similar to INCB053914, was both well tolerated and had few adverse events. PIM447 has even shown some clinical efficacy in a limited relapse/refractory multiple myeloma study, with a partial response rate of 8.9% and a clinical benefit rate of 25.3%[254]. Similar to BET inhibitors, as single agents PIM inhibitors are not particularly effective, however, given the properties of PIM kinases these inhibitors could prove to be more effective in combination.

Materials and Methods

All animal studies were approved by the Moffitt Cancer Center Institutional Animal Care and Use Committee.

SKM-1 or SKM-1 P1-14 cells were resuspended in cold 0.9% sterile saline and mixed with Matrigel Matrix to a final protein concentration of 7mg/mL.$1x10^6$ SKM-1 and SKM-1 P1-14 cells were injected into the right flank of NGS-S(RRID:IMSR_JAX:013062) mice (male, 20-30g, 10-30 weeks old) and allowed to reach between $100mm^3$ and $150mm^3$ before beginning

treatment. Tumors were measured at least twice a week by caliper and tumor volume was calculated using the formula; Tumor volume = width × width × length × 0.52. Mice were randomized into 4 groups: vehicle, INCB057643, INCB053914 or Combination. INCB057643 was administered once a day at 10mg/kg, 5 days a week by oral gavage. INCB053914 was administered twice a day at 30mg/kg, 5 days a week by oral gavage. Both compounds were dissolved in 5% N,N-dimethylacetimide/95% 0.5% methylcellulose.

For CMML PDX experiments, frozen BMMCs were first thawed and treated with DNAse I for 15 minutes to create a single cell suspension. Cells were washed once and resuspended in 0.9% sterile saline and injected via tail vein into NSG-S mice (male or female, 20-30g, 10-30 weeks old) sub-lethally irradiated the day prior. At least 2 million cells were injected into each mouse and treatment started between 2-3 weeks after injection. Mice were randomized into the same groups as the heterotopic cell line models. Treatment lasted 2 weeks and all mice were euthanized shortly after the end of treatment. The spleen, peripheral blood and femurs were taken for analysis. One femur and a portion of the spleen were fixed in formalin and used for IHC.

Viability Assays

For the drug screen, cells were plated with compounds in 384 well plates and viability was assessed after 72hrs using Cell-Titre Glo (Promega) according to the manufacturer's instructions. For all other viability assays, cells were plated with compounds in 96 well plates and viability was assessed after 72hrs using CCK8 following the manufacturer's instructions. synergy was calculated using Zero Interaction Potential (ZIP) via SynergyFinder[163]

Persistent Cell Lines

U-937 and TF-1 cells were grown in medium containing 500nM INCB054329 and SKM-1 cells were grown in medium containing 300nM INCB054329. Persistence was determined by significantly increased IC_{50} by CCK8 and steady growth in medium containing INCB054329.

Colony Forming Assays

Frozen BMMCs were thawed and prepared in a similar manner to PDX experiments. Cells were then resuspended in IMDM + 2% FBS at a concentration of 200,000 cells per mL. $300\mu L$ of cell suspension and 3µL of each compound were added to 3mL Methocult 4034 (StemCell Cat#: 04034) and mixed by vortexing for 1 minute. 1.1 mL of cell mixture was plated in StemCell smart dishes, incubated for 14 days and read on StemVision (Stem Cell Technologies) for the final colony count.

Western Blotting

All cells were lysed using RIPA lysis buffer and protein quantified using BCA. SDS–polyacrylamide gel electrophoresis was performed using 7.5, 10 or 12.5% bis-tris gels and protein was transferred to PVDF membranes using a wet transfer system (Bio-Rad). Membranes were blocked with 5% milk in TBS-T and incubated overnight with primary antibody in either milk or BSA at manufacturer recommended concentrations. Blots were washed multiple times in TBS-T before addition of HRP-conjugated secondary antibody diluted in 5% milk and incubated for 1hr at room temperature. Antibodies used: BRD2(RRID:AB_10835146), BRD4(RRID:AB_2687578), FLAG-TAG(RRID:AB_2572291), PIM1(RRID:AB_2799461), PIM2(RRID:AB_2163921), PIM3(RRID:AB_1904094), BRD3(RRID:AB_1907250), Actin(RRID:AB_476744), Vinculin(RRID:AB_477629).

RNA Extraction

Total RNA from cultured cells was extracted using either Quick-RNA Miniprep (Zymo Research) for gene expression or miRNeasy/miRNeasy advanced (Qiagen) for miRNA detection.

q-RT-PCR

RNA was converted into cDNA using iScript® Reverse Transcription Supermix for RTqPCR (Bio-Rad). qRT-PCR reactions were performed for in triplicate using probes designed and ordered from IDT (PIM1, PIM2, TBP). Primers: PIM1 forward: GCTTCGGCTCGGTCTACTCA, reverse: AGTGCCATTAGGCAGCTCTC; PIM2 forward: GGACACCGCCTCACAGATCG, reverse: TGACTGAGTCTGACAAGGGGG; TBP forward: CACGAACCACGGCACTGATT, reverse: TTTTCTTGCTGCCAGTCTGGAC.

Electroporation

The Neon Transfection System(ThermoFisher Scientific) with 100µL tips was used to deliver siRNA or miRNA mimics. Cells were first washed with PBS and resuspended in R buffer at a concentration of $5x10^7$ cells per mL. siRNA was added to a final concentration of 5µM, mixed thoroughly and cells were electroporated with the following settings: 1400V, 10 pulse width, 3 pulses. Cells were then added to 10mL RPMI with 10% FBS and incubated for 72 (siRNA) hours before collection for qPCR and western blotting.

ChIP-PCR

U-937, SKM-1, or MV411 cells were serum starved overnight. The next day, cells were stimulated with 10ng/mL GM-CSF for 15mins and immediately fixed with 1% formaldehyde for 10mins. Formaldehyde was quenched with glycine and cells were washed 2X with cold PBS before being snap frozen on dry ice and stored at -80ºC. Fixed cells were then prepared using the SimpleChIP Magnetic Bead Kit (Cell Signaling Technologies) according to manufacturer's recommendations. DNA was sheared using a Qsonica Q800R3 with the following settings: 50%

amplitude, 30sec pulse, 5min shearing time. DNA was sheared 5min, spun down, and sheared an additional 5min. Antibodies for STAT5(RRID:AB_2737403), RNA PolII(RRID:AB_2732926), and IGG Negative Control (Included with kit) were incubated overnight before continuing with the protocol according to manufacturer's recommendations. Primers for PIM1 promoter and enhancer were custom designed and ordered from IDT. PIM1 Enhancer forward: CTTCAACTGCTGTGCTGGTTC, Reverse: CGGGGTTAGTAGTGCAAGGC; PIM1 Promoter forward: GCAGCATCTGGCATCACAAC, Reverse: AAAGCACCAGCGAATCCTGA.

RNA-seq and GSEA

U-937 cells were treated with DMSO, JQ1 and INCB054329 for 24hrs in quadruplicate. Persistent cells treated with INCB054329 were also included in quadruplicate. RNA was extracted and screened for quality on an Agilent BioAnalyzer(RRID:SCR_019715). The samples were then processed for RNA-sequencing using the Nugen Universal RNA-seq kit(NuGEN). Briefly, 100 ng of RNA was used to generate cDNA and a strand-specific library following the manufacturer's protocol. Quality control steps including BioAnalyzer library assessment and quantitative RT-PCR for library quantification were performed. Two libraries failed QC and were excluded. The libraries were then sequenced the Illumina NextSeq 500 v2 sequencer with two high-output 75-base paired-end runs in order to generate approximately 25 to 30 million read pairs per sample. Sequencing data was mapped to hg38 using STAR "Spliced Transcripts Alignment to a Reference"[255]. Raw data was cleaned by removing any genes with less than 10 reads or present in less than half of the samples before running differential analysis through DESeq2(RRID:SCR_015687)[256]. Normalized counts were run through GSEA 4.1.0(RRID:SCR_003199) with default parameters except permutation type, which was set to gene_set[257].

Transduction of Cells with PIM1

SKM-1 cells were transduced with a Flag-Tagged, 34kDa isoform of PIM1 in a pCDH-CMV-MCS-EF1α-GreenPuro Cloning and Expression Lentivector(System Biosciences) via the Spinfection method. Briefly, cells were resuspended in Opti-MEM and plated into 6-well plates along with fresh virus, Lipofectamine-2000 and polybrene. Cells were centrifuged for 90mins at 2200rpm in a 37°C centrifuge, and incubated at 37°C for 1 hour, after which 1 mL of normal growth medium was added and cells were incubated overnight. Cells were then centrifuged and resuspended in normal growth medium. After 1 week, cells were single cell sorted for GFP positivity. Single cell clones were profiled for successful transduction by western blotting for Flag-tag.

Statistical Analysis

Statistical analyses and graphical representations were performed using GraphPad Prism 9.0 (RRID:SCR_002798). For comparisons involving only 2 sets of data, unpaired t-tests were used. For data involving more than 2 comparison, one-way ANOVA was used. For experiments involving tumor size measurements over time, or the SKM-1 co-culture experiment, we first measured the area under the curve (AUC) of each individual sample to get as single data point describing that sample over time. Then, depending on the groups being compared, either unpaired t-test or one-way ANOVA was used to test significance.

Results

Neither MYC nor SRC Family Kinase Upregulation Contribute to BETi Persistence.

Reactivation of MYC was one of the first major mechanisms of persistence to BET inhibition to be characterized. To explore this possibility, we profiled MYC protein levels in our persistent cells lines by western blot to determine whether MYC reactivation could explain the

persistence to BET inhibition seen in persistent cell lines. In U937 cells, MYC levels were initially decreased following short-term exposure to BETi, but recovered to normal levels in persistent cell lines consistent with previously published reports (Fig. 3.1A). TF-1 cells, on the other hand, displayed high MYC levels that remained unchanged in both short-term and chronically treated cells, indicating that MYC reactivation could not completely explain BET inhibitor persistence in our models (Fig. 3.1A). Since RTKs can play a significant role in persistence to BETi, we utilized an array-based platform to quickly profile the phosphorylation levels of 28 RTKs and 11 important signaling nodes in short-term and chronically treated cells. While there were small changes in phosphorylation levels in U937 cells, namely a decrease in AKT(ser473)(Fig. 3.1B), in TF-1 cells SRC family kinase(SFK) phosphorylation levels were initially decreased in short term treated cells, but dramatically increased in the chronically treated cells (Fig. 3.1C). In a larger screen of the phospho-proteome using phospho-tyrosine pulldown (pTyr), SFK Y416 phosphate was the top ranked hit (Fig. 3.1D). Given these results, we profiled two SRC inhibitors: Saracatinib and Dasatinib. Saracatinib was antagonistic in both U937 and SKM1 cells, where Dasatinib was mildly synergistic in TF-1 cells and antagonistic in U937 cells (Fig. 3.2A). In persistent cells, treatment with dasatanib and saracatinib produced similar results: both compounds were ineffective at reducing the viability of persistent cells (Fig. 3.2B). Since saracatinib is a more specific inhibitor of SRC with fewer off target effects, we decided to profile its effectiveness in patient sample CFAs. Once again, saracatinib was not synergistic in any of the samples tested (Fig. 3.2C and D).

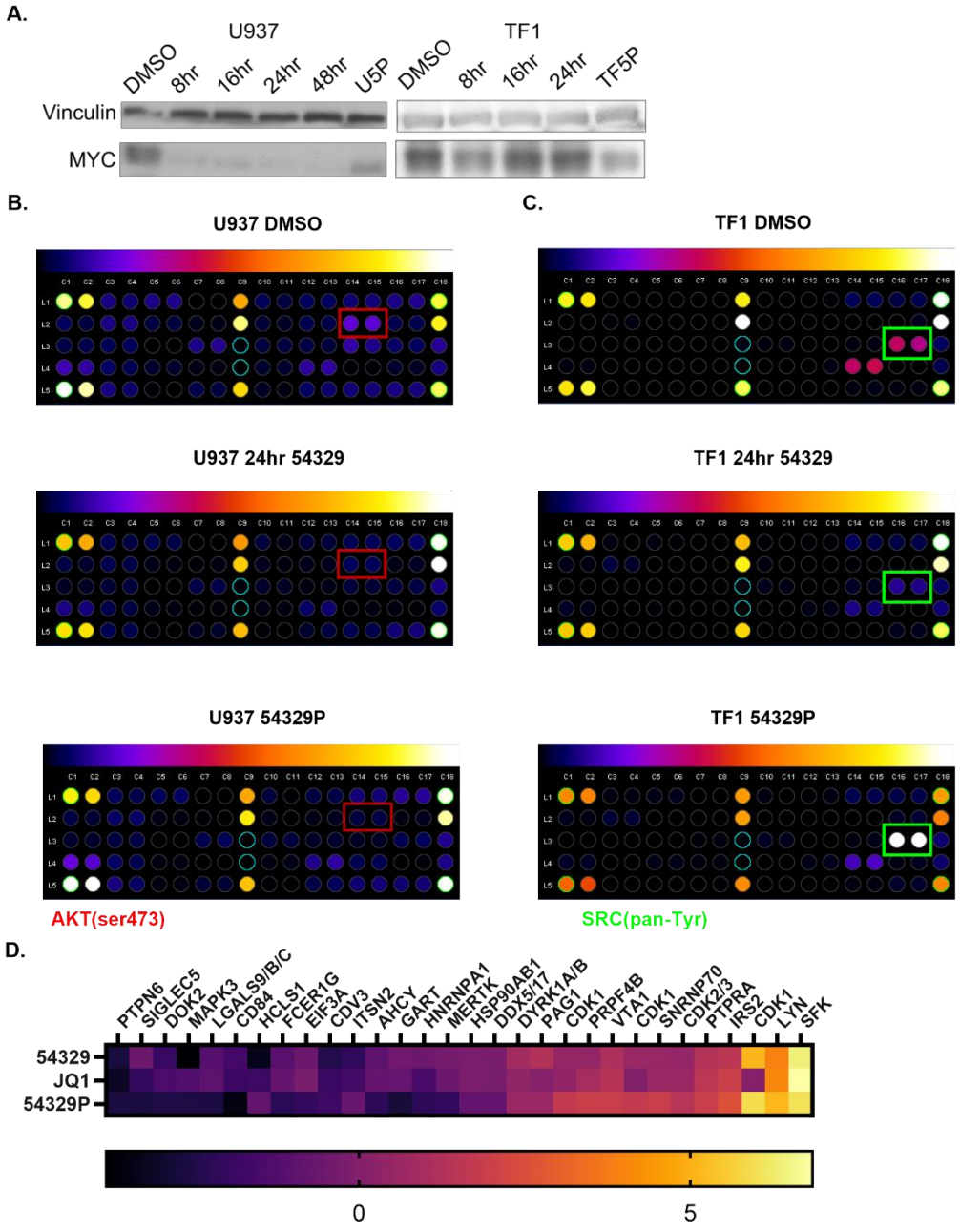

Figure 3.1: MYC and pSFK in persistent cell lines. (A) Western blot of MYC levels in U937 and TF-1 cells treated with BETi. (B) Normalized phospho-RTK Arrays performed on U937 and (C) TF-1 Cells treated with BETi for 24hrs or chronically treated. (D) Heatmap of significant hits for pTyr proteomics experiments in U937 cells treated with BETi.

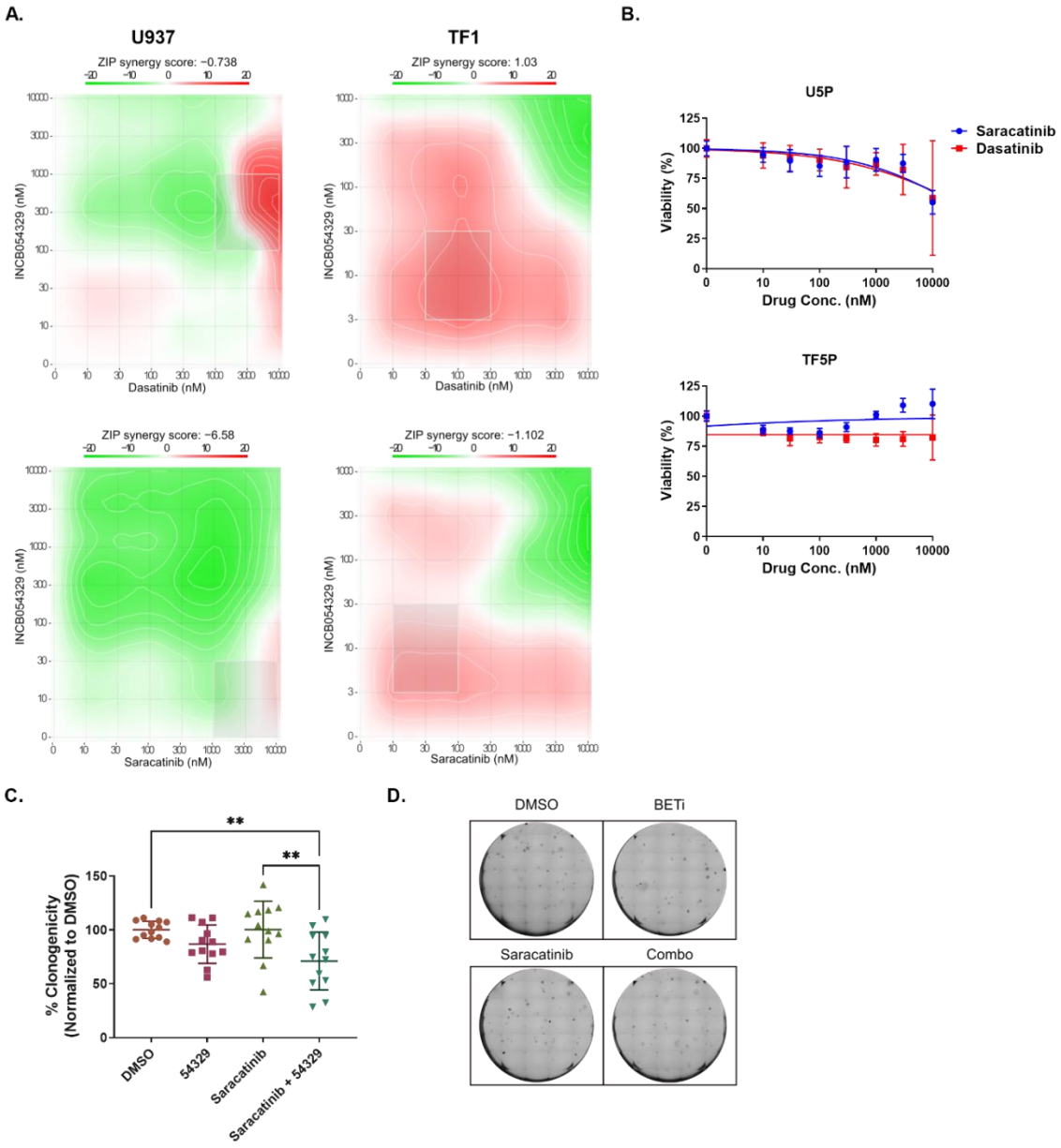

Figure 3.2: SFK inhibitors are not synergistic with BETi. (A) ZIP Synergy analysis of U937 and TF-1 cells treated with BETi and SFKi for 72hrs. Red indicates synergy, green indicates antagonism. (B) Viability of U937 and TF-1 54329 persistence cell lines treated with SFKi for 72hrs. (C) Colony counts of CMML patient samples treated with BETi, SFKi, and combination for 2 weeks. (D) Representative image of patient sample colonies associated with data in C.

PIM kinases are upregulated in response to BETi in a subset of leukemia cell lines and correlate to PIMi sensitivity.

PIM inhibitors were the most synergistic with BET inhibitors in our initial screening and we decided to further profile PIM kinases to determine whether they could be responsible for persistence to BET inhibition. Assessing PIM kinase protein and RNA expression of cells treated with BETi after 24 hrs revealed a significant increase in expression of PIM kinases. This increase was highest in BETi persistent cells where significant increases in PIM1 and PIM2 were observed (Fig. 3.3A-B). Notably, PIM kinases have been previously implicated in drug resistance in AML[258-260]. Further, time course studies demonstrated that PIM mRNA upregulation occurs as early as 8hrs (Fig. 3.3C). Because PIM1 was expressed in both short-term treated and persistent cell lines, we prioritized this PIM isoform for further investigation in subsequent experiments. Differential gene expression analysis of RNA-seq data from U-937 cells identified that PIM1 was among the top 20 upregulated genes in BETi treated cells compared to DMSO control (Enrichment score = -3.298) and that a gene set previously reported to be enriched in PIM overexpressing myeloid cells was also upregulated in our BETi treated cells (Fig. 3.3D and E)[261]. We next confirmed the increased PIM levels after BETi in multiple myeloid leukemia cell lines. Four of nine cell lines demonstrated increased PIM kinase protein levels at 24 hours (Fig. 3.4A). While PIM upregulation was heterogeneous, the BETi dependent increases in PIM levels correlated to increased synergy with BETi and PIMi *in vitro*

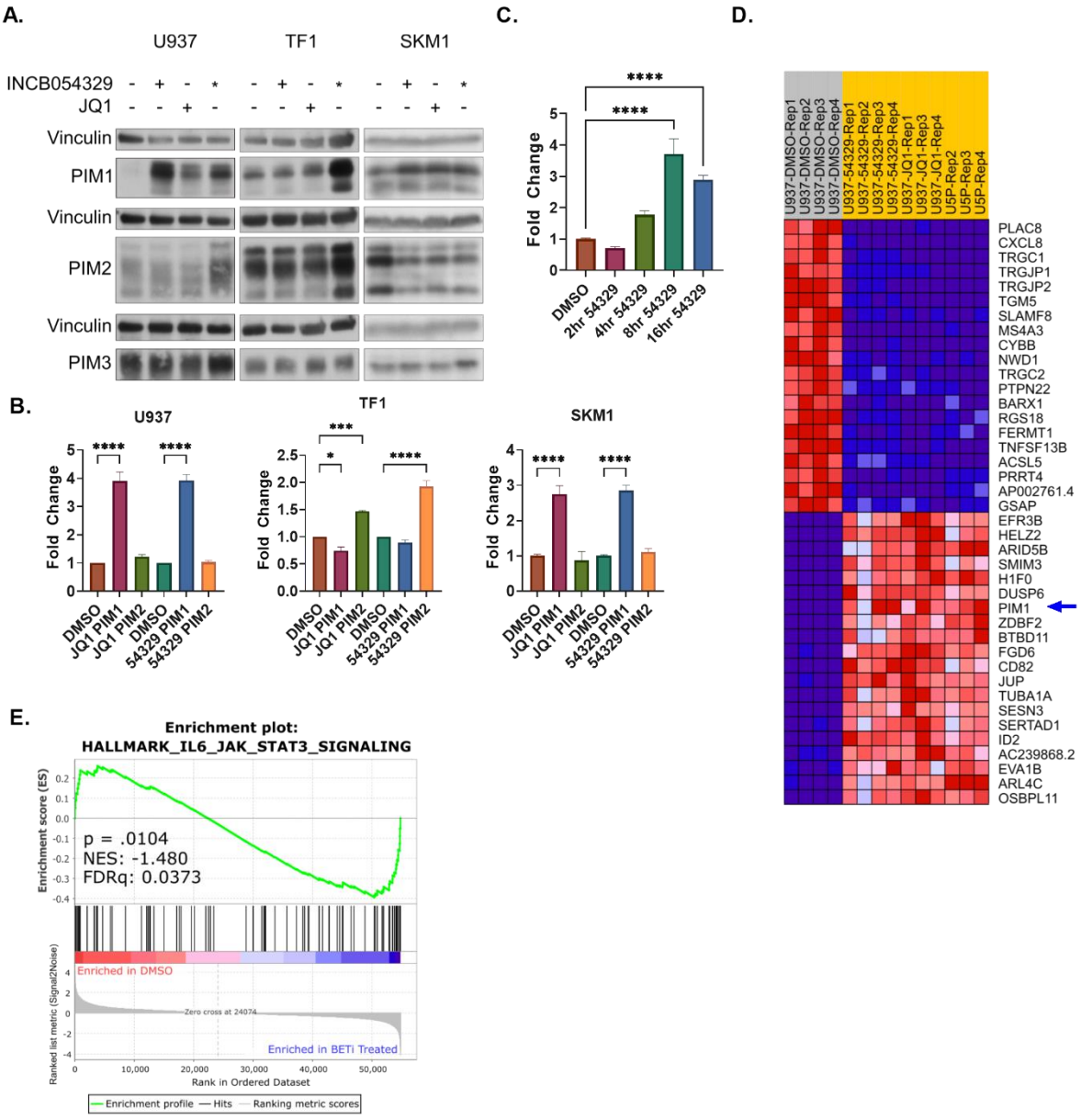

Figure 3.3: PIM kinases are upregulated after BETi treatment. (A) Western blot of cells lines treated with BETi for 24hrs. + indicates treatment, * indicates persistent cell lines. (B) qPCR of PIM1 and PIM2 levels in U937, TF-1, and SKM1 cells treated with BETi for 24hrs. (C) qPCR of PIM1 levels in U937 cells treated with BETi for 2-16hrs. (D) RNA-seq analysis of BETi treated (500nM) U-937 cells showing the top 20 up and downregulated genes. U5P indicates the persistent cell line. Red = upregulated, Blue = downregulated. (E) GSEA enrichment plot corresponding to RNA-seq data in D.

(Fig. 3.4B and C). Since current inhibitors in clinical development are pan-BET inhibitors, including those tested here, we sought to investigate which BET proteins were most associated with PIM upregulation. We individually genetically depleted BRD2, 3 and 4 in U-937 and SKM-1 cells and found that only BRD4 knockdown resulted in significant upregulation of PIM1 levels (Fig. 3.4D and E) consistent with the known expression of BRD4 in the hematopoietic compartment[94,262]. Collectively, these data suggest that BET inhibition leads to increased PIM expression in a subset of cell lines that is associated with synergy between BET and PIM inhibitors.

PIM1 Overexpression is Sufficient to Induce Persistence to BETi and Sensitivity to PIMi

We next sought to determine whether increases in PIM1 alone could drive persistence to BET inhibition as well as contribute to the observed synergy seen *in vitro*. To test this, single cell PIM overexpressing SKM-1 clones were derived by transducing a GFP expressing lentiviral vector encoding PIM1 (Fig. 3.5Ai and ii). All four SKM-1 clones engineered to overexpress PIM1 were both persistent to BETi (Fig. 3.5Bi), and significantly more sensitive to PIMi (Fig. 3.5Bii). Moreover, PIM1 levels correlated with persistence to BET inhibition (R^2=0.9925, p=.0037), indicating that PIM1 overexpression is sufficient for BET inhibitor persistence and sensitization to PIM inhibition *in vitro* (Fig. 3.5Ci). Of note, although all PIM1 overexpressing clones were more sensitive to PIM inhibition, there was no correlation between levels of PIM1 expression and PIMi sensitivity (Fig. 3.5Cii). Additionally, we performed *in vitro* competition assay by co-culturing SKM-1 cells with two isogenic PIM1 overexpressing clones in the

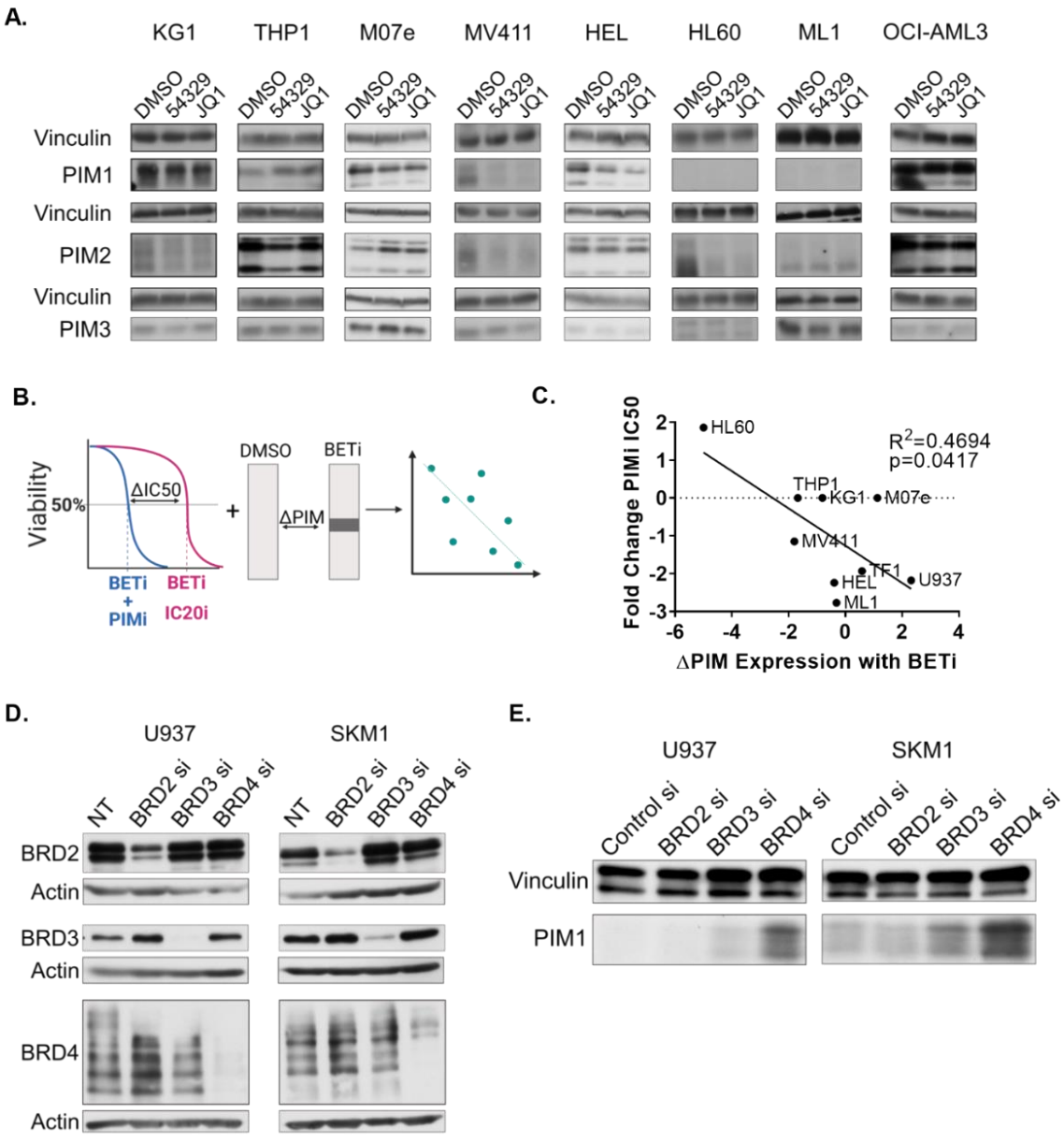

Figure 3.4: PIM upregulation is not universal. (A) Western blot of PIM kinases in cell lines treated with BETi for 24hrs. (B) Graphic detailing the method for generating the data in Fig. 3.4C. Figure created in Biorender. (C) Correlation plot of PIMi IC50 and PIM kinase changes of cells treated with BETi for 24hrs. (D) Western blot of BET family proteins in cells treated with siRNA against each individual BET protein. (E) Western blot of PIM1 in cells treated with siRNA against BET proteins.

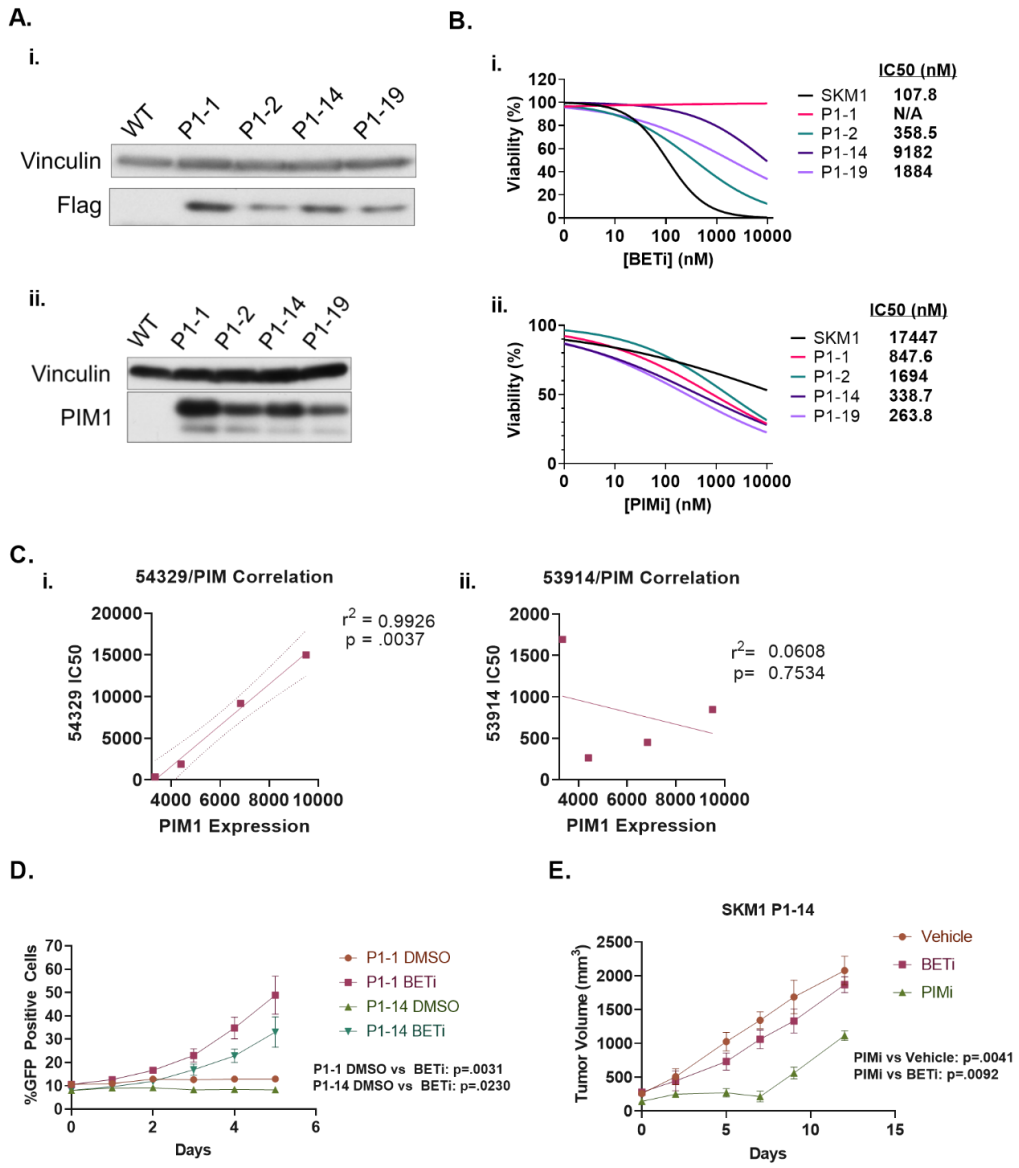

Figure 3.5: PIM1 overexpression is sufficient to induce BETi persistence. (A)Western blot of SKM-1 cells transduced with Flag-Tagged PIM1. Flag(i), PIM1(ii). (B) IC_{50} curves of SKM-1 cells treated with BETi (INCB054329)(i) and PIMi(INCB053914)(ii). Cells were incubated with drug for 72hrs. (C) Correlation of PIM1 expression and BETi IC_{50} for WT and PIM1 overexpressing SKM-1 cells. (D) Competition assay with SKM-1 P1-1 and SKM-1 P1-14 cells cultured with WT cells at a 1:10 ratio and treated with BETi for 5 days. Flow cytometry was used to determine GFP positivity (E) Tumor volumes of mice with subcutaneously implanted SKM-1 P1-14 cells treated with PIMi. N=4 mice per group, PIMi N=3.

presence BETi or vehicle control. After 5 days of treatment with BETi, there was a statistically significant increase in PIM overexpressing isogenic cells indicating that PIM1 overexpressing cells were selected in the presence of their parental counterparts (Fig. 3.5D). To determine whether PIM1 overexpression leads to BET inhibitor persistence and PIM sensitivity *in vivo,* heterotopic SKM-1 xenograft models were generated of P1-14 SKM-1 PIM overexpressing clones and isogenic controls. As in the above *in vivo* experiments flank tumors were allowed to grow until 100-150 mm^3 and treatment was initiated for two weeks. These experiments demonstrated statistically significant decreases in tumor volume in PIM over expressing SKM-1 clones after PIM inhibition compared to parental cells suggesting that PIM overexpression is sufficient for PIM inhibitor sensitivity *in vivo* (Fig. 3.5E).

Upregulation of the GM-CSF/STAT5 Axis is Associated with Sensitivity to Combination Therapy

The GM-CSF/STAT5 axis is the canonical upstream signal required for PIM transcription of all isoforms [203,263-265]. Given the above mechanism of synergy with respect to PIM upregulation and our previous studies demonstrating that GM-CSF hypersensitivity is a universal feature of the monocytic leukemia CMML, we hypothesized that the subset of monocytic leukemia cell lines which upregulated PIM after BET inhibition could be identified *a priori* by their respective pre-treatment GM-CSF sensitivity. We posited that leukemia cells, which were responsive to GM-CSF stimulation at low doses would be primed to upregulate PIM upon BETi treatment. To explore this possibility, we measured pSTAT5 in the presence or absence of low dose (0.1ng/ml) GM-CSF. Consistent with our hypothesis, cells that exhibited BET dependent PIM protein upregulation demonstrated greater pSTAT5 activation after only 0.1ng/mL of GM-CSF stimulation compared to cells that did not upregulate PIM kinase

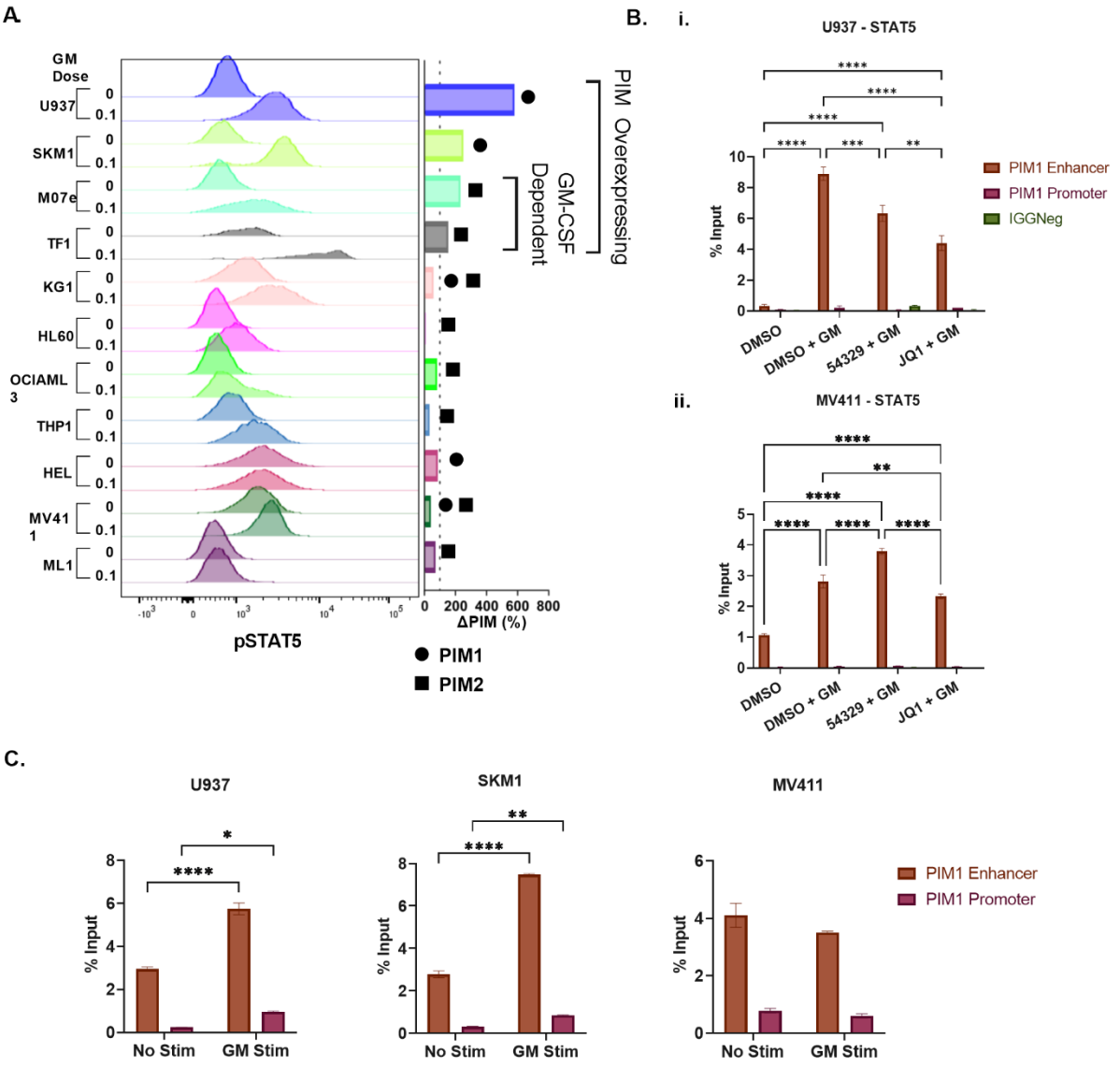

Figure 3.6: PIM upregulation is marked by GM-CSF hypersensitivity. (A) Flow cytometry analysis of pSTAT5 levels after stimulation with 0.1ng/mL GM-CSF in 11 myeloid cells lines with corresponding changes in PIM protein levels after treatment with a BETi. Different PIM protein are denoted by a black circle (PIM1) or black square (PIM2) above each bar. (B) ChIP-PCR of STAT5 levels in U-937 and MV-4-11 cells at the PIM1 promoter and enhancer after stimulation with GM-CSF(10ng/mL) and treatment with BETi (U937 500nM, MV-4-11 100nM). (C) ChIP-PCR of RNA PolII at the PIM1 promoter and enhancer in U-937, SKM-1 and MV-4-11 cells after stimulation with 10ng/mL GM-CSF).

(Fig. 3.6A). In the context of *PIM1* specifically, pSTAT5 enrichment was accompanied by STAT5 occupation at the *PIM1* downstream enhancer in PIM1 upregulating leukemia cells at a far greater magnitude compared to those cell lines that did not upregulate PIM1 after BETi (Fig. 3.6B). Additionally, GM-CSF stimulation led to enrichment of RNA PolII at both the *PIM1* enhancer and promoter in *PIM1* upregulating cell lines but not in a leukemia cell line that did not upregulate PIM1 (Fig. 3.6C). Collectively our data suggests that GM-CSF hypersensitive myeloid leukemia cells are transcriptionally primed at the *PIM1* loci, upregulate PIM kinases after BETi, and are associated with synergy to BET and PIM inhibition.

CMML is a rare hematologic malignancy classified as a Myelodysplastic/Myeloproliferative overlap syndrome by the World Health Organization[7]. Clinically and pathologically, this disease is characterized by bone marrow dysplasia, peripheral monocytosis, cytopenias, and a propensity for transformation to Acute Myeloid Leukemia, all of which contribute to a poor overall survival[7]. Molecularly, CMML is hallmarked by GM-CSF hypersensitivity in a mutational and subtype independent manner [54,266]. To determine whether this molecular feature was associated with transcriptionally primed PIM, we leveraged our previously published multi-omic epigenetic dataset of 16 CMML patients that enabled us to probe chromatin accessibility and histone marks at the PIM1 loci [267]. Both when viewed in aggregate (Fig. 3.7A) or as individual patients (Data not shown) the PIM1 promoter and enhancer demonstrated epigenetic marks consistent with transcriptional activity supporting the notion that CMML may represent a subtype of leukemia enriched for sensitivity to BET and PIM inhibition. Finally, we profiled PIM expression in our PDX models to determine whether the postulated mechanism of synergy occurred in primary patient samples. Immunohistochemistry

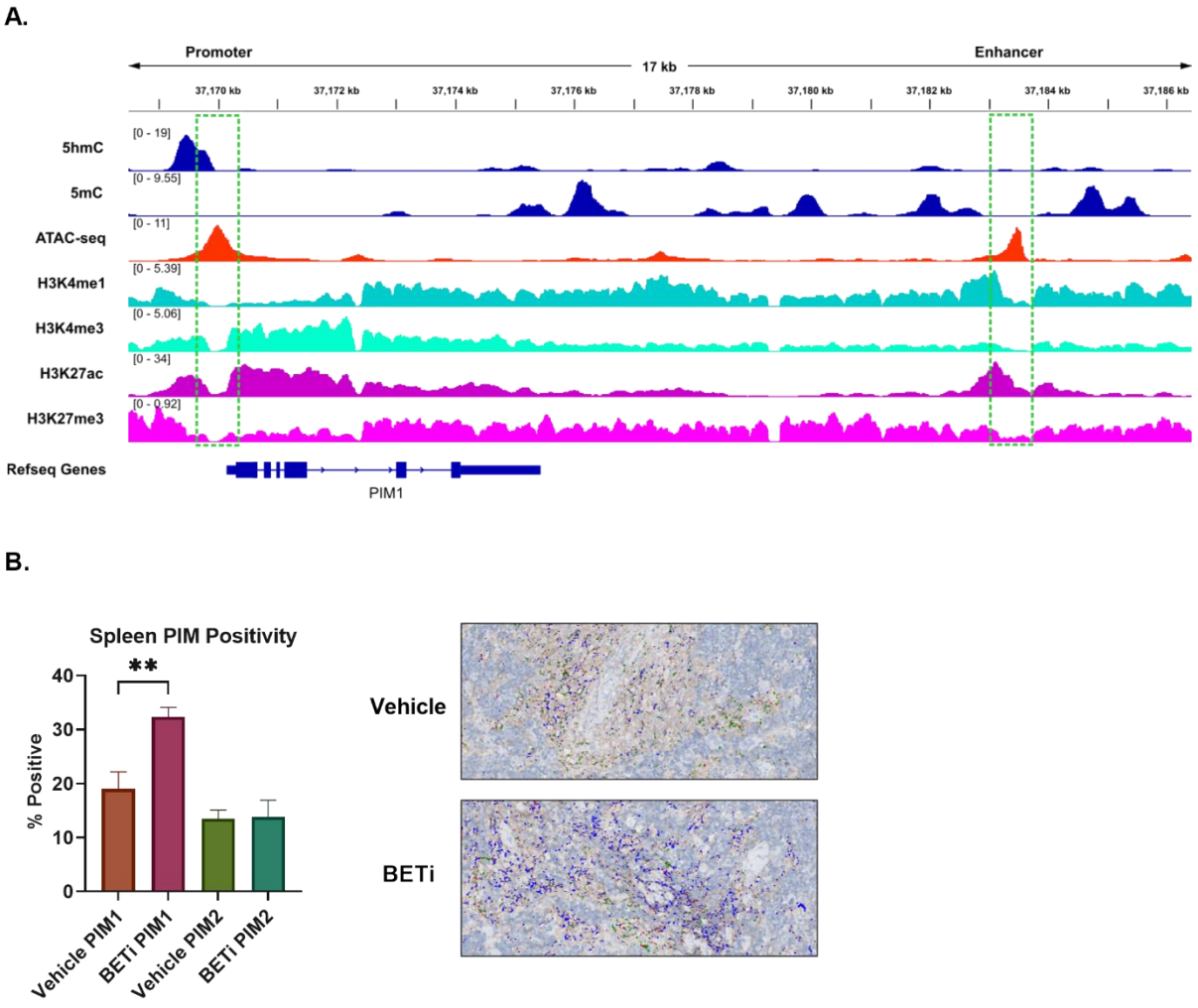

Figure 3.7: PIM1 is upregulated in patient samples treated with BETi. (A) ChIP-seq data from 16 unique CMML patients at the PIM1 locus. (B) Left: Representative image of a PDX spleen stained with hCD45, PIM1 and PIM2. Slides were stained with individual markers and overlaid using a computational program described in methods. Blue color represents area of hCD45 and PIM1 colocalization. Right: Quantification of the colocalization of hCD45/PIM1 and hCD45/PIM2 in spleen samples taken from PDX experiments.

(IHC) was performed on spleen sections using rabbit anti-PIM1 and anti-PIM2. Our anti-PIM1 and anti-PIM2 antibodies cross-react with human and mouse tissues. To overcome this limitation we computationally overlaid PIM IHC with that of human CD45 to quantitate human specific

PIM expression(see methods)(Fig. 3.7B). This analysis demonstrated that PIM upregulation occurred after BET inhibitor treatment *in vivo* in primary samples.

Discussion

Our initial focus was on characterizing our persistent cell lines to identify targetable vulnerabilities that could be exploited with combination therapy. Although there have been many mechanisms of persistence to BET inhibition proposed in various different cancers, MYC upregulation after sustained BETi treatment has been well characterized and occurs in hematological malignancies. We profiled this phenomenon in our persistent cell line models and found that while U937 persistent cells did seem to follow the trend of short-term downregulation followed by long-term upregulation, TF-1 persistent cells had consistently high MYC levels in both short-term and long-term treated cells indicating that while reactivation may contribute to persistence, it is likely not the major mechanism. While PIM kinases are known to interact with and stabilize MYC, leading to increased MYC levels, increased PIM expression is likely not leading to the increased MYC levels seen in U937 cells as PIM levels increase well before MYC levels[268,269].

We then refocused on profiling RTKs after data published in ovarian cancer suggested long-term BET inhibitor treatment resulted in kinome reprogramming. Both a small array based RTK profiling and a larger screen utilizing pTyr phosphoproteomics revealed pSFK levels to be significantly increased after BET inhibition. Elevated pSFKs have been shown to contribute both to worse prognosis and resistance to therapy in a variety of different cancers, including hematologic malignancies[270]. In our models, however, elevated SFK levels did not appear to have any effect on BET inhibitor persistence. Using two different SFK inhibitors, Saracatinib and Dasatinib, we showed that there was little or no synergy with BET inhibitors in cell lines or

patient sample CFAs. Additionally, the viability of BET inhibitor persistent cell lines was not affected by SFKi treatment. Future studies would be required to determine what is causing increased SFK phosphorylation levels and what impact this increase is having on BETi treated cells.

Last, our study identified that PIM protein and RNA levels were paradoxically upregulated after BET inhibitor treatment in multiple cell lines, including all persistent cell lines, and that PIM1 upregulation was necessary for sensitivity to PIM inhibition. Although no correlation was observed between PIM1 protein levels and PIMi sensitivity, all PIM1 overexpressing cells were significantly more sensitive to PIM inhibition than their parental control perhaps due to a threshold effect that was met in all of our PIM1 overexpression cell lines. That PIM kinase upregulation was sufficient to induce this phenotype, without upstream activation, is consistent with its known mechanism of phosphorylation. Unlike many serine threonine kinases which require a secondary phosphorylation event in order to become active, PIM kinases are constitutively active after translation[204]. While we fully investigated BET inhibitor dependent PIM1 upregulation, which was increased in all BET inhibitor persistent cells generated and our PDX models, our data provide a mechanism of synergy likely consistent in the context of other PIM isoforms given their overlapping function and known role in chemotherapy resistance[187]. Further experiments utilizing PIM2 and PIM3 overexpressing cells would be necessary to fully answer this question. One remaining question, which could have significant impact on how BET/PIM combination therapy is delivered, is how long PIM kinase upregulation is sustained once BETi is withdrawn. A recent study in DLBCL has implicated upregulated PIM3 as a potential resistance mechanism to CDK9 inhibition, which is known to phenocopy BET inhibition. This study observed that 24hr after CDK9i withdrawal PIM3 was significantly

upregulated, suggesting that PIM levels can remain elevated after drug withdrawal. Additionally, further proteomic experiments would be needed to determine how PIM kinases are leading to persistence. Their effect on apoptotic proteins would be an obvious answer, but there are quite a few downstream processes affected by PIM kinases which could lead to the development of a persistent phenotype.

Finally, we identified GM-CSF hypersensitivity as a hallmark for PIM upregulation after BET inhibition. Determining which patients will benefit most from a drug combination is crucial for a successful clinical trial. We noticed that out of the 4 cell lines which upregulated PIM kinases after BET inhibition, 2 of them were dependent on GM-CSF for survival and proliferation. We profiled all cell lines for GM-CSF hypersensitivity by measuring pSTAT5 levels after GM-CSF stimulation and found that only cells that upregulated PIM kinases after BET inhibition demonstrated GM-CSF hypersensitivity. Given CMML is characterized by GM-CSF hypersensitivity it is highly likely this combination will be particularly effective, and this could potentially apply more broadly to any myeloid malignancy that demonstrates GM-CSF hypersensitivity.

CHAPTER FOUR: BET INHIBITORS REGULATE PIM1 KINASE THROUGH DOWNREGULATION OF MIR-33A

Note. Portions of this chapter have been adapted from previously published work in Letson et al., *Clinical Cancer Research*, 2023[61]

Introduction

MiRNA Structure and Function

MicroRNAs (miRNA) are a class of small non-coding RNAs between 14-21 nucleotides in length. Originally discovered in 2000, the newest release of miRbase (v22.1, 2019) has annotated 2654 mature miRNA sequences[271]. miRNAs function as epigenetic regulators of the genome. Specifically, they bind within the 3' untranslated region of target mRNA sequences with imperfect complementarity and either repress translation directly (less common) or promote mRNA deadenylation leading to mRNA decay (primary mechanism)[272]. Because of imperfect complementarity, individual miRNA can have many different mRNA targets and as such, miRNAs play a role in a wide variety of biological functions including: development, metabolism, cell differentiation, organogenesis an apoptosis. Dysregulation of such important epigenetic regulators also has consequences in disease, in particular cancer, where altered levels of miRNA are common.

MiRNA Biogenesis

Mature miRNA sequences can either be intergenic, or are located with introns and exons within coding genes. Intergenic miRNA have their own promoter and are transcribed independently, while miRNA within a coding gene typically share promoters with their host

genes[273]. miRNA biogenesis starts with transcription by RNA polymerase II (Pol II) which results in pri-RNA, a large RNA containing at least 1 hairpin structure and a 5` cap[274,275]. Pri-RNA are then processed by the microprocessor complex consisting of Drosha (an RNAse III enzyme that functions as a double stranded RNA endoribonuclease) and DiGeorge syndrome critical region gene 8 (DGCR8, a double stranded RNA binding protein) in the nucleus to form pre-miRNA[276,277]. Pre-miRNA are exported into the cytoplasm by XPO5 and Ras-related nuclear protein (RAN) where the final steps of the processing take place[278,279]. Once in the cytoplasm Dicer cleaves pre-miRNA into double-stranded RNA (dsRNA), which is then loaded into argonaute (AGO) via ATP-dependent chaperone proteins[280-282]. AGO proteins promote the assembly of the RNA-induced silencing complex (RISC), which causes the passenger strand of the dsRNA to be ejected (leaving the mature miRNA) and mediates recognition of the target mRNA[282]. Depending on how the dsRNA is loaded into AGO, the passenger strand can be selected instead meaning a single pre-miRNA can lead to two different miRNA depending on strand selection[282]. miRNA can also be processed independently of Drosha and DGCR8. Mirtrons, or miRNA who's pre-mirna encompass and entire intron, rely on pre-mirna splicing as opposed to processing by Drosha and DGCR8[283,284]. The pre-mirna of a mirtron is linearized by the debranching enzyme DRB1 which can then be exported by XPO5 and follows the same cleavage by dicer and subsequent steps as the canonical miRNA biogenesis pathway[285].

MiRNA Regulation

Transcriptional regulation of miRNA occurs in a similar manner to mRNA, where transcription factor binding at enhancers and promoters can influence levels of miRNA. MiRNA within a coding gene are typically co-regulated with the gene where they reside, while intergenic miRNA are independently regulated[286-289]. MiRNA interaction with transcription factors can also

result in feedback loops, where miRNA can enhance or inhibitor their own transcription[272]. Like mRNA, methylation also plays a significant role in transcriptional regulation of miRNA. Hypermethylation of miRNA promoters results in downregulation of those miRNA, while treatment with hypomethylating agents can reverse hypermethylation phenotypes and increase miRNA expression[290].

Post-transcriptionally, miRNA expression can be regulated in a variety of different ways. Changes in expression or chemical modifications of the proteins involved in miRNA biogenesis can impact miRNA expression. SRSF3 promotes miRNA processing by the microprocessor through recruitment of DROSHA to the basal junction[291]. Microprocessor activity can also be enhanced by binding of a heme molecule, which promotes the interaction of DGCR8 with the pri-miRNA hairpin[292,293]. The elements of microprocessor can also regulate each other and the ratio of DGCR8 to drosha is critical in maintaining appropriate miRNA processing. DGCR8 stabilizes drosha by interacting with its middle domain, whereas drosha can cleave hairpins present in DGCR8 mRNA leading to degradation[294]. High DGCR8 levels can inhibit drosha processing activity causing decreases in miRNA levels[294]. RNA editing enzymes called Adenosine deaminases acting on RNA (ADAR), which edit adenosine residues into inosine in dsRNA, can edit pri-miRNA and prevent further processing by either DICER or drosha depending on the circumstances leading to pri-miRNA degradation[295,296]. ADAR2 in particular can block Drosha-mediated miRNA processing independent of its RNA editing activity by simply binding to the pri-miRNA[297].

MiRNA Decay

MiRNA molecules are generally considered to be very stable with some transcripts having half-lives of hours or even days, although there are exceptions such as miRNA involved

in the cell cycle which rapidly decay as the cell undergoes mitosis[298]. MiRNA involved in the cell cycle typically have a 3' sequence motif that destabilizes the transcript, causing increased turnover[298]. Uridine or Adeonsine addition to the 3' end of miRNA can also induce degradation. In humans and mice GLD-2 polyadenylation of a select set of miRNA, which can lead to either degradation or reduced associated with AGO2 and 3 depending on the cellular context[298]. TUT4/7 uridylate miRNAs in humans and the fate of uridylated miRNA is context dependent similar to adenylation. Mono-uridylation can lead to more efficient miRNA processing, whereas poly-uridylation leads to either degradation or reduced target recognition[299]. Finally, semi-perfect complementarity between miRNA and target mRNA surprisingly results in miRNA degradation instead of mRNA degradation and has been termed target mediated miRNA decay(TDMD). This interaction involves pairing of the mRNA to both 5' and 3' ends of the miRNA, which leads to a conformational change in the RISC complex, poly-ubiquitination and subsequent degradation of AGO catalyzed by ZSWIM8[300]. This exposes the miRNA to cytoplasmic nucleases leading to its degradation as well. Given their wide array of downstream targets, dysregulation of miRNA can lead to unintended consequences in gene regulation.

Materials and Methods

RNA Extraction

Total RNA from cultured cells was extracted using either Quick-RNA Miniprep (Zymo Research) for gene expression or miRNeasy/miRNeasy advanced (Qiagen) for miRNA detection.

q-RT-PCR

RNA was converted into cDNA using iScript® Reverse Transcription Supermix for RTqPCR (Bio-Rad). qRT-PCR reactions were performed for in triplicate using off the shelf TaqMan assays (SREBF2, pri-33a, pre-33a) (ThermoFisher Scientific). For miRNA, cDNA was generated

using the TaqMan Advanced miRNA cDNA Synthesis Kit. miRNA qRT-PCR reactions were also performed in triplicate using off the shelf TaqMan Advanced miRNA Assays for miR-33a, miR-33b, miR-16, and miR-26b and miR-93(Endogenous Control miR)(ThermoFisher Scientific).

Electroporation

The Neon Transfection System(ThermoFisher Scientific) with 100µL tips was used to deliver siRNA or miRNA mimics. Cells were first washed with PBS and resuspended in R buffer at a concentration of $5x10^7$ cells per mL. siRNA or miRNA mimics were added to a final concentration of 5µM, mixed thoroughly and cells were electroporated with the following settings: 1400V, 10 pulse width, 3 pulses. Cells were then added to 10mL RPMI with 10% FBS and incubated for either 48 (miRNA mimics) or 72 (siRNA) hours before collection for qPCR and western blotting. For experiments with miRNA mimics, INCB054329 was added 24hrs after electroporation.

miRNA Array

RNA was extracted from U-937 cells treated with DMSO, INBC054329 or JQ1 for 24hrs using miRNeasy Advanced kit (Qiagen). Thermo GeneChip miRNA 4.0 arrays were processed and hybridized according to the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA). Briefly, 500ng of RNA was processed using the FlashTag Biotin HSR RNA Labeling Kit and following poly-adenylation and ligation of a biotinylated RNA tag, the product was hybridized to GeneChip miRNA 4.0 arrays at 48C for 16 hours at 60 RPM using the GeneChip Hybridization Oven 645. The hybridized miRNA arrays were then washed and stained using the GeneChip Fluidics Station 450, followed by scanning on the Thermo GeneChip Scanner 3000 7G. Data

were reviewed for quality control and analysis was performed using the GeneChip Transcriptome Analysis Console v4.0.

Statistical Analysis

Statistical analyses and graphical representations were performed using GraphPad Prism 9.0 (RRID:SCR_002798). For comparisons involving only 2 sets of data, unpaired t-tests were used. For data involving more than 2 comparison, one-way ANOVA was used. For experiments involving tumor size measurements over time, or the SKM-1 co-culture experiment, we first measured the area under the curve (AUC) of each individual sample to get as single data point describing that sample over time. Then, depending on the groups being compared, either unpaired t-test or one-way ANOVA was used to test significance.

Results

BETi decreases miR-33a expression leading to increased PIM1 levels

BETi exert profound effects on the transcriptome but are generally thought to *downregulate* transcriptional activity[103,301]. Therefore, to resolve the paradoxical increase in PIM levels after treatment we first explored BET inhibitor dependent miRNA depletion hypothesizing that depletion of miRNAs that target PIM may lead to the observed increases in PIM levels. BET inhibitors can augment miRNAs via inhibition of miRNA biogenesis at super enhancer regions and/or via direct transcriptional repression of precursor RNA species[101,150]. We treated both U-937 and SKM-1 cells with either an Argonaute RISC Catalytic Component 2 (AGO2) inhibitor (Acriflavin) or a Dicer inhibitor (Poly-l-lysine), two central components of miRNA biogenesis, and measured protein PIM1 levels. Indeed, treatment with either AGO or Dicer inhibitors was sufficient to increase PIM1 levels across both cell lines suggesting that inhibition of miRNA biogenesis can recapitulate BET inhibitor induced PIM1 upregulation (Fig. 4.1A). To narrow

down putative repressed miRNAs that may be negative regulators of PIM1, we used the computational approach outlined in Figure 4.1B. Briefly, miRNAs were identified by cross referencing putative PIM1 binding miRNA from the microRNA Data Integration Portal (miRDIP), miRNA with super enhancers from Suzuki et al. and published PIM1 interacting miRNA[213,216,302-304]. This led to the identification of 4 putative miRNAs whose expression was evaluated after BET inhibitor treatment. Of these, miR-33a was the only miRNA with a significant time dependent decrease after treatment with two BETi (Fig. 4.1C). Although this reduction in miR-33a was modest, previous literature supports modest changes in miRNA leading to large impacts on gene expression of its targets[151]. This was consistent with GSEA analysis of whole transcriptome RNA-sequencing performed in U-937 cells that demonstrated increased expression of genes targeted by miR-33a(Fig. 4.1D). To determine if miR-33a depletion was necessary for BET dependent PIM upregulation, we electroporated a miR-33a mimic into both U-937 and SKM-1 cells treated with either BETi or DMSO for 24hrs and collected pellets for both RNA and protein after 48hrs (Fig. 4E). Evaluation of PIM1 protein levels demonstrated that cells with miR-33a overexpression were protected from BETi dependent PIM upregulation (Fig. 4.1F). These data suggest that reduced levels of miR-33a after BET inhibition leads to an increase in PIM1 expression.

BETi does not directly impact miR-33a transcription or its miRNA precursors

To explore whether BETi directly and specifically impact miR-33a we profiled transcript levels of *SREBF2* after BET inhibition as miR-33a is intronically located between exons 19 and 20 of this gene (Fig. 4.2A). This analysis demonstrated a transient increase in *SREBF2* transcript expression after 4hr treatment suggesting that BET inhibitors do not directly impact primary

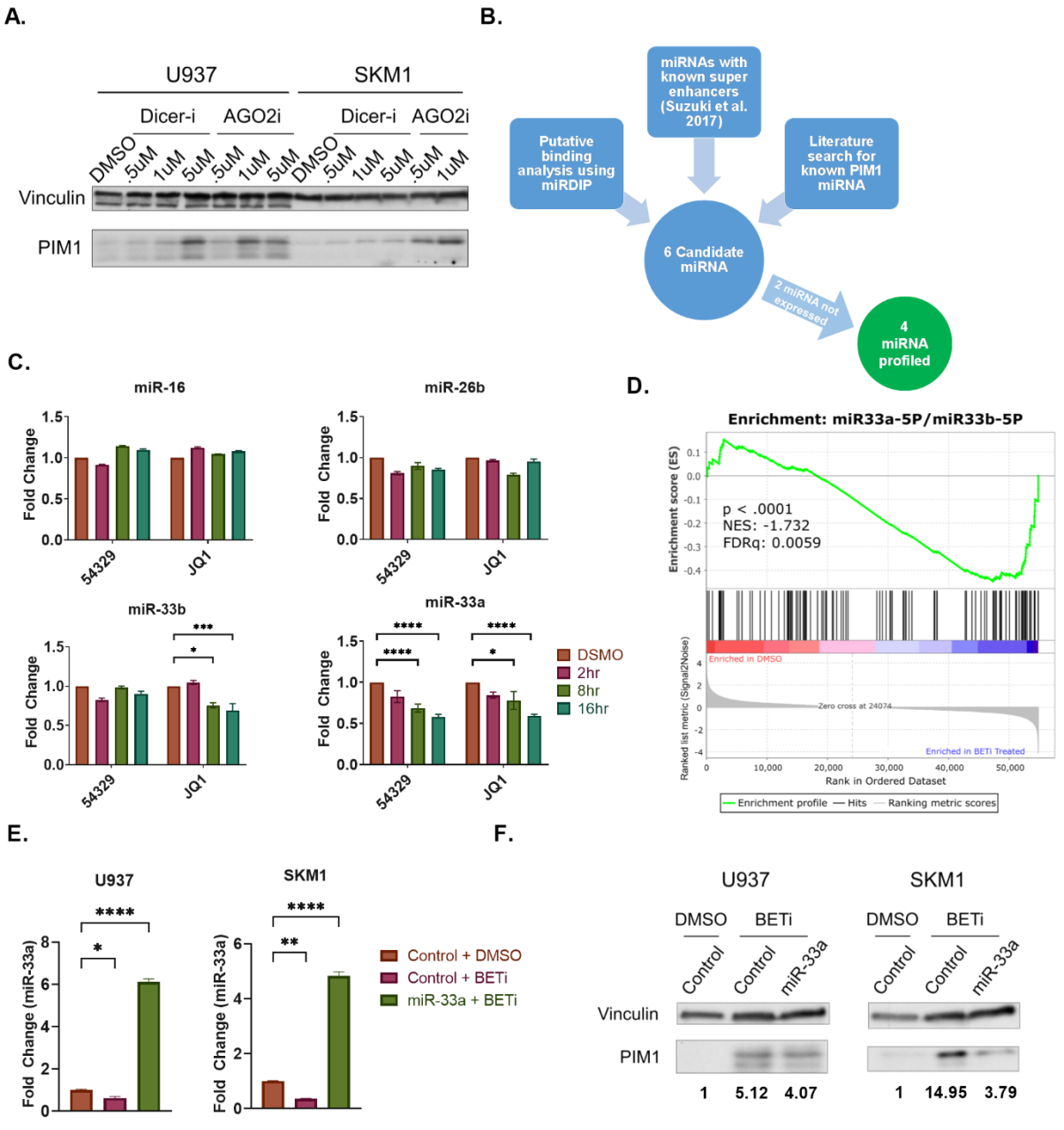

Figure 4.1: BETi downregulation of miR-33a leads to increased PIM1 expression. (A) Western blots of PIM1 in cells treated with AGO2 or Dicer inhibitors. (B) Flow chart of process for selecting miRNAs for further analysis. (C) qPCR of 4 candidate miRNAs in SKM-1 cells treated with BETi 500nM U937, 100nM SKM1) for 2-16hrs. (D) GSEA enrichment plot for miR-33a/miR-33b targets in U-937 cells treated with BETifor 24hrs. (E) qPCR of cells treated with both miR-33a mimic and BETi(500nM U937, 100nM SKM1). (F) Western blot of cells treated with miRNA mimic and BETi(500nM U937, 100nM SKM1).

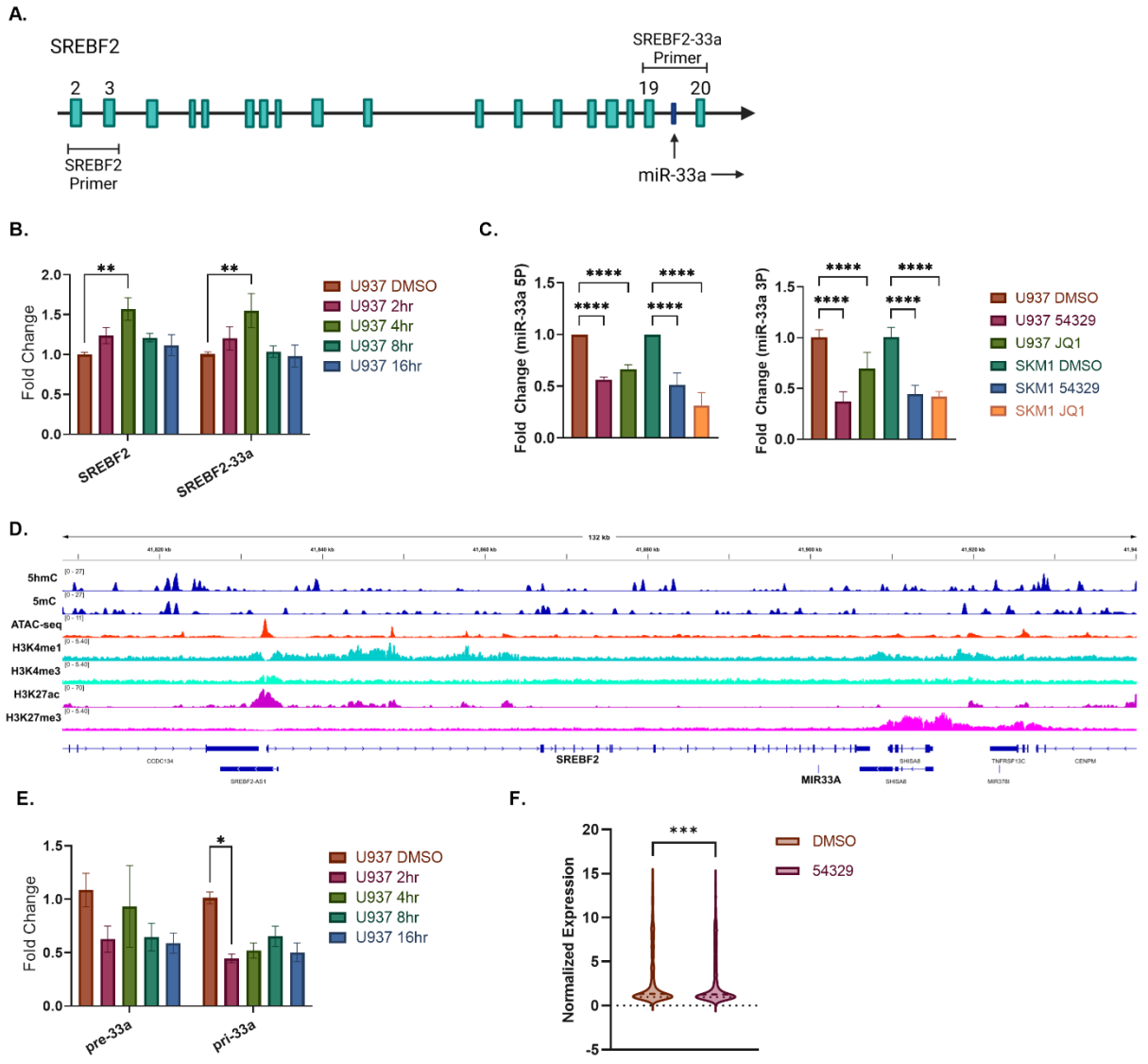

Figure 4.2: BETi does not directly regulate miR-33a. (A) Schematic representation of miR-33a location within *SREBF2* and location of primers used in B. Figure created in Biorender. (B) qPCR of *SREBF2* levels in U-937 cells treated with BETi for 2-16hrs. (C) qPCR of miR-33a-5p (Left) and miR-33a-3p (Right) levels in U-937 and SKM-1 cells treated with BETi for 24hrs. (D) ChIP-seq data from 16 unique CMML patients at the *SREBF2* locus, generated from the same dataset used in Figure 5D. (E) qPCR of pre-miR-33a and pri-miR-33a levels in U-937 cells treated with BETi from 2-16hrs. (F) Levels of miRNA expression in U-937 cells treated with INBC054329 for 24hrs obtained from the Affymetrix GeneChip miRNA Array 4.0.

miR-33a transcription in leukemia cells (Fig. 4.2B). This was observed both with primers probing the intronic region between exons 19 and 20 as well as primers measuring total *SREBF2* (Fig. 4.2C). Importantly, no other promoters were identified in our CMML ChIP-seq data near *SREBF2* that would transcribe miRNA-33a independently (Fig. 4.2D). While the rapid turnover of miRNA precursor species precludes precise measurements of their relative abundances after treatment, we attempted to profile the range of miR-33a precursors after BET inhibitor treatment at different time points. Indeed, mature miRNA isoforms (i.e. 3p and 5p) were consistently depleted upon BET inhibitor treatment, but pre-miR-33a did not significantly decrease congruent with the postulated role of BET inhibitor repression of miRNA biogenesis[150](Fig. 4.2C and E). Moreover, broad miRNA expression profiling in U-937 cells demonstrated a global statistical down regulation of miRNAs in 2 replicates suggesting that miR-33a downregulation may occur through impairment of miRNA biogenesis (Fig. 4.2F).

Discussion

Given the profound downregulation of transcriptional activity and the paradoxical increase in PIM kinase RNA in our leukemia models, we hypothesized BET inhibitors may downregulate a post-transcriptional repressor of PIM. Indeed, general miRNA repression using AGO2 and dicer inhibitors resulted in increased levels of PIM1 kinase. More specifically, miR-33a, a known regulator of PIM kinase, was downregulated and necessary for the observed BETi dependent PIM upregulation[213,305,306]. Further, our data strongly suggests that BETi dependent impairments in miRNA biogenesis, and not unique transcriptional repression of mir-33a precursors, underlies the mechanism of miR-33a downregulation making this proposed combination therapy mechanistically novel.

As in Chapter 3, we focused solely on PIM1 and profiled its regulatory miRNAs to determine their relevance in its regulation after BET inhibition. PIM2 and PIM3 are both regulated by different sets of miRNA than PIM1. Although we did not observe increases in PIM3 in any of our cell lines, miRNA profiling of the PIM2 upregulating cell lines M07e and TF-1 would be necessary to determine whether this mechanism of regulation is shared across PIM isoforms. Additionally, in the case of U937 cells, miR-33a mimic experiments were unable to fully rescue PIM upregulation indicating there could be secondary mechanism contributing to increased PIM1 levels. It is possible there are additional downregulated miRNA that were missed or filtered out during screening that contribute along with miR-33a to downregulate PIM1. This is supported by the fact that although miR-33b was not consistently downregulated in our initial screen, our GSEA signature includes both miR-33a and miR-33b targets. Profiling miR-33b levels more closely and conducting mimic experiments might implicate this miRNA in addition to miR-33a in U937 cells. Alternatively, protein stability or protein turnover could be affected in a way that increases PIM levels in concert with derepression from lowered miR-33a.

CHAPTER FIVE: RESULTS AND FUTURE DIRECTIONS

Summary of Results

We performed a large, clinically relevant compound screen and discovered synergy between BET and PIM inhibitors. We validated this combination in a panel of 3 myeloid cell lines and 3 different PIM inhibitors to confirm synergy, as well as 10 unique patient BMMCs using CFAs and in both experiments the BETi and PIMi combination was significantly more effective than single agent. Other combinations such as BETi and JAKi or BETi and AKTi did not replicate synergy in either the full synergy or CFA experiments. Since long term treatment with BETi typically leads to persistence, we generated 3 BETi persistent cell lines, determined by increased BETi IC50. When treated with PIMi, these BETi persistent cells had significantly reduced viability compared to their parental counterparts, indicating that PIMi was able to overcome BETi persistence. We next performed *in vivo* cell line experiments using heterotopic models of myeloid cell lines. In both cell lines tested, the combination treatment was significantly more effective than either single agent alone. Finally, we utilized our CMML PDX model, which recapitulates many of the features of the actual disease, and profiled this combination in 4 unique patient samples. In all 4 samples, combination treatment was significantly more effective at reducing the bone marrow engraftment of CMML BMMCs by both flow cytometry and IHC, demonstrating that BETi/PIMi combination treatment may be effective at treating CMML.

Next, we wanted to determine the mechanism behind this synergy. Because we were using a PIM inhibitor, we decided to profile this family of kinases directly. We treated the same

3 myeloid cell lines we used to determine synergy with BETi for 24hrs and checked PIM protein and RNA levels by western blot and qPCR respectively. In all 3 cell lines and all persistent cell lines, at least 1 PIM kinase was upregulated at both the RNA and protein level. Since PIM1 was upregulated in all persistent cell lines we prioritized this isoform for further profiling. RNA-seq analysis of U937 cells treated with BETi for either 24 hours or chronically revealed PIM1 to be one of the Top 20 upregulated genes in BETi treated cells. While this upregulation was not present in all cell lines tested, there was a significant correlation between PIM upregulation and reduced PIM inhibitor IC50 after BETi. Given the unique properties of PIM kinases we decided to overexpress PIM1 in SKM1 cells to determine the effect on BETi and PIMi sensitivity. Cells overexpressing PIM1 were significantly more resistant to BET inhibition, indicating that PIM1 overexpression was sufficient to induce BET inhibitor resistance. Heterotopic models of PIM1 overexpressing SKM1 cells also demonstrated increased resistance to BETi and sensitivity to PIMi *in vivo*. PIM1 overexpressing cells were also able to outcompete wildtype cells in co-culture when treated with BETi further implicating PIM kinases in BETi resistance. Since increases in PIM kinase were not universal, we next explored whether we could predict which cells would upregulate PIM after BETi. Given that two of the four cell lines which overexpressed PIM were dependent on GM-CSF for growth we hypothesized that GM-CSF hypersensitivity may play a role in PIM expression after BETi. Flow cytometry analysis of pSTAT5 levels of cells treated with low dose GM-CSF revealed that only those cells which had a significant increase in pSTAT5 levels after GM-CSF stimulation, indicating hypersensitivity, upregulated a PIM kinase isoform after BETi. This was confirmed by assessing chromatin occupancy of pSTAT5 and RNA PolII at the PIM1 enhancer and promoter. Only in cells with increased PIM1 expression after BETi was there increased occupancy of both pSTAT5 and RNA PolII at the

promoter and enhancer of PIM1. Most importantly, when we analyzed IHC from our PDX models for PIM1 expression, we found that BETi treatment did indeed increase PIM1 levels, indicating this phenomenon was present in actual patient samples.

BET inhibition typically results in broad downregulation of transcription, so upregulation of PIM after BETi was surprising and we decided to investigate how BETi might lead to PIM upregulation. We hypothesized that BET inhibition may be downregulating inhibitory miRNA related to PIM, and profiled miRNA levels after BETi. As a proof of concept, we treated cells with two broad miRNA inhibitory agents, a DICER inhibitor and AGO2 inhibitor. Treatment with both compounds in U937 and SKM1 cells lead to increased PIM1 levels, demonstrating inhibition of miRNA could lead to increases in PIM. We narrowed down the list of potential PIM1 associated miRNA through the use of binding predictions and previous literature to four: miR-16, 26b, 33a and 33b. MiR-33a was the only miRNA that was downregulated in a time dependent manner in both BETi treatments. We further profiled miR-33a's importance in PIM1 regulation by electroporating a miR-33a mimic into U937 and SKM1 cells. When treated with BETi, miR-33a mimic partially or fully prevented increased PIM1 levels in U937 and SKM1 cells respectively. We also wanted to explore how exactly BETi was altering miR-33a levels. Since miR-33a is an intronic miRNA located in the gene *SREBF2*, we first checked the levels of *SREBF2* after BETi treatment. *SREBF2* levels were not affected by BETi treatment in either U937 or SKM1 cells, indicating BETi was not reducing miR-33a levels through repression of its host gene. We next looked at the levels of both the pri and pre-miRNA forms of miR-33a to see if BETi was inhibiting Drosha and hindering miRNA maturation. Both pre- and pri-33a were uniformly down, indicating BETi was not acting on Drosha. We then looked at the levels of the 3p form of miR-33a to assess if strand switching could be playing a role in decreased miR-33a-

5p levels. Again, both forms were uniformly downregulated, indicating strand switching was not a factor. Finally, we looked at global miRNA levels using a miRNA Array and found a small but significant global reducing in miRNA, indicating that BETi was globally inhibiting miRNA biogenesis in some fashion.

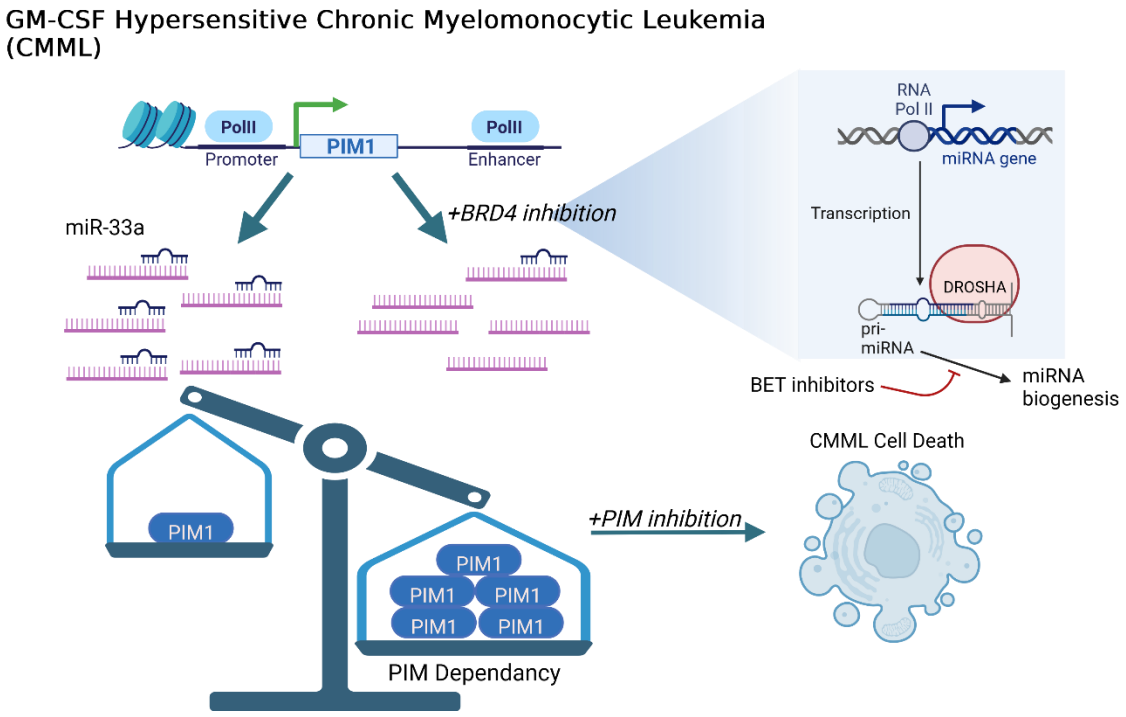

Figure 5.1: Graphical Representation of Results. In GM-CSF hypersensitive cells, miR-33a is expressed and keeps PIM1 levels low. With the addition of a BETi, miR-33a levels are reduced through a blockade of miRNA biogenesis and lead to increased PIM1 levels, dependence on PIM signaling and increased sensitivity to PIM inhibition.

In summary, in GM-CSF hypersensitive cells, BET inhibitors downregulate miR-33a, an important regulator of PIM1, leading to increases in PIM1 RNA and protein levels. This increase in PIM levels leads to BETi persistence, which can be countered using PIMi leading to cell death (Fig. 5.1).

Future Directions

Our goal is to use this research as preclinical rational for a clinical trial. There are, however, experiments which would help inform this future clinical trial trial. First, our research does not deeply explore on the potential toxicity associated with these compounds. While we did not note overt toxicity over the two-week dosing period in our *in vivo* experiments, longer treatment of mice, especially non-transplanted mice, would help elucidate potential toxicity associated with long-term treatment. Dose escalation in mice would also help uncover potentially dose limiting toxicities before clinical trial, although this may not fully translate into humans. Treatment of normal HSPCs in the *in vitro* setting, such as CFAs, would also help to understand potential toxicity. Exploring dosing strategies is another potential solution to toxicity and efficacy. Given that BET inhibitor persistence can be temporary, a cycling of drugs, such as initial treatment with BETi along followed by either single PIMi or BETi/PIMi combination, rather than up-front combination may help limit toxicities and allow for a longer duration of treatment.

BET/JAK combination therapy is currently undergoing clinical trials in hematologic malignancies, and these trials have identified patients who fail this combination. We generated BET/JAK double persistent U937 cells and found that PIM inhibition was able to overcome this persistence, indicating this could be a clinically relevant triple combination. Generation of more double persistent cell lines and treatment of these cell lines with a PIM inhibitor would be necessary to prove a dependence on PIM signaling in this context. Treating CFAs of BMMCs from patients who have failed BET/JAK treatment with PIM inhibitor would be another important pre-clinical experiment to validate this combination. As with the BET/PIM combination, profiling toxicity would be incredibly important when considering a triple

combination. Treatment of non-cancerous cells *in vitro* and non-transplanted mice *in vivo* with the triple combination would be necessary to determine if an up-front triple combination is even feasible given overlapping toxicities.

We identified GM-CSF hypersensitivity as a hallmark of PIM upregulation in CMML. While this may be specific for CMML, it's possible that cytokine sensitivity in general plays a role in PIM expression after BETi. There are many different cytokines that activate the JAK/STAT pathway many of which are also implicated in myeloid malignancies as well as other cancers. IL-6 in particular has been implicated in many different cancers including MDS, AML, Breast and Lung adenocarcinomas[307,308]. Profiling of cytokine sensitivity in other cancers may identify these cancers as susceptible to BETi/PIMi combination treatment. Additionally, finding a better biomarker would be incredible useful in translation to the clinic. Omics profiling of our cell lines comparing those that upregulate PIM vs those that do not may elucidate a clinically relevant biomarker. RNA-seq or proteomic analysis could identify a gene or gene signature that is specific to cells which upregulate PIM and are sensitive to combination treatment. Profiling of relevant cellular receptors through flow cytometry or potentially CyTOF could identify a unique receptor or receptor signature marking sensitive cells. Clinically, a multi-omic approach in patients who responded to BET/PIM combination treatment would also be useful in finding a biomarker and additionally, we could utilize clinical parameters (Clinical Blood Count with Differential) and profile the plasma of responding patients for cytokines and other metabolites that correlate with response.

Lastly, while we investigated PIM upregulation as a mechanism of persistence to BETi, other cell lines we profiled did not upregulate PIM. Generation of more persistent cell lines, specifically in cell lines where PIM was not upregulated after short term BETi treatment would

help elucidate whether PIM upregulation is a consistent feature of chronic BETi treatment of myeloid cell lines, or whether other mechanisms contribute to persistence.

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APPENDIX A: BET INHIBITOR CLINICAL TRIALS.

Inhibitor	NCT Number	Phase	Conditions	Status
ABBV-075	NCT02391480	I	Breast Cancer, NSCLC , AML, MM, Prostate Cancer, SCLC, NHL	Completed
BAY 1238097	NCT02369029	I	Neoplasms	Terminated
BI 894999	NCT02516553	I	Solid tumors, NHL	Completed
BMS-986158	NCT03936465	I	Solid Tumor, Lymphoma, Brain Tumor, Pediatric Cancers	Recruiting
	NCT02419417	I/II	Advanced Tumors	Completed
CC-90010	NCT03220347	I	NHL, Neoplasms	Active, Not recruiting
	NCT02158858	I/II	MF, Acute Myelocitic Leukemia, MDS/MPN Neoplasm, MDS	Active, not recruiting
	NCT02157636	I	MM	Completed
	NCT01949883	I	Lymphoma	Completed
FT-1101	NCT02543879	I	AML, Acute Myelogenous Leukemia, MDS, NHL	Completed
GS-5829	NCT02392611	I	DLBCL, T-cell lymphoma, solid tumors	Completed
	NCT02983604	I/II	Breast Cancer	Terminated
	NCT02607228	I/II	Metastatic CRPC	Terminated
I-BET-151	NCT02630251	I	Cancer	Terminated
I-BET-762	NCT01943851	II	Neoplasms	Completed (dose limiting toxicities identified)
	NCT01587703	I	Carcinoma, Midline	Completed
INCB054329	NCT02431260	I/II	Solid Tumors and Hematologic Malignancy	Terminated

INCB057643	NCT02959437	I/II	Solid Tumors, Advanced Malignancies, Metastatic Cancer	Terminated
	NCT02711137	I/II	Solid Tumors	Terminated
OTX015	NCT02698189	I	AML, DLBCL	Terminated
	NCT02698176	I	NMC, TNBC, NSCLC, CRPC	Terminated
	NCT02296476	II	GBM	Terminated
	NCT02259114	I	NMC, TNBC, NSCLC, CRPC, PDAC	Completed
	NCT01713582	I	AML, DLBCL, ALL, MM	Completed
PLX51107	NCT02683395	I	Solid tumors, lymphoma, AML, MDS	Terminated
TEN-010	NCT03068351	I	MM	Completed
	NCT02308761	I	AML, MDS	Completed (No longer considering monotherapy)
	NCT01987362	I	Solid Tumors, Advanced Solid Tumors	Completed
ZEN003694	NCT03901469	II	TNBC	Recruiting
	NCT02711956	I/II	Metastatic CRPC	Completed
	NCT02705469	I	Metastatic CRPC	Completed

APPENDIX B: REFERENCES USED FOR COMPOUND FILTERING

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APPENDIX C: INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

APPROVALS

USF UNIVERSITY OF SOUTH FLORIDA

RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO:	Eric Padron, M.D.
FROM:	Farah Moulvi, MSPH, IACUC Coordinator Institutional Animal Care & Use Committee Research Integrity & Compliance
DATE:	11/20/2017
PROJECT TITLE:	Investigating the therapeutic potential of combination bromodomain inhibitor and PIM kinase inhibitor in CMML
FUNDING SOURCE:	Incyte Corporation
IACUC PROTOCOL #:	R IS00004320
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC **APPROVED** your request to use the following animals in your **protocol for a one-year period beginning 11/20/2017:**

Mouse: NOD-scid IL2Rgnull-3/GM/SF (NSGS)	820
(6-24 weeks, either M/F)	

Please take note of the following:

- **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system.** After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

- **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

- **All costs invoiced to a grant account must be allocable to the purpose of the grant.** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

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UNIVERSITY OF SOUTH FLORIDA
USF RESEARCH & INNOVATION

RESEARCH INTEGRITY & COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO:	Eric Padron, M.D.
FROM:	Farah Moulvi, MSPH, IACUC Coordinator Institutional Animal Care & Use Committee Research Integrity & Compliance
DATE:	10/29/2020
PROJECT TITLE:	Validating the efficacy of BET bromodomain and PIM kinase inhibitor combination in mouse models of CMML.
FUNDING SOURCE:	Non-Profit (Private Foundations, H. Lee Moffitt Cancer Center, etc.), For Profit (Industry Sponsored) or Other Incyte Corporation
IACUC PROTOCOL #:	R IS00008509
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC **APPROVED** your request to use the following animals in your **protocol for a one-year period beginning 10/29/2020:**

Mouse: NOD-scid IL2Rgnull-3/GM/SF (NSGS) (6-24

408

weeks, either M/F)

Please take note of the following:

- **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system.** After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

- **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

- **All costs invoiced to a grant account must be allocable to the purpose of the grant.** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
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APPENDIX D: INSTITUTION REVIEW BOARD APPROVAL

6100 Merriweather Dr., Suite 600
Columbia, MD 21044
410-884-2900

CONTINUING REVIEW APPROVAL

CR00475827

DATE:	2 Aug 2023
TO:	Eric Padron, M.D.
PROTOCOL:	Incyte Corporation - MCC 19727, A Sequential Two-Stage Dose Escalation Study to Evaluate the Safety and Efficacy of Ruxolitinib for the treatment of Chronic Myelomonocytic Leukemia (CMML): A Phase 2 Expansion. (Pro00030294)
CONTINUING REVIEW APPROVAL DATE:	28 Jul 2023– **Via Full Board Review, IRB #00000971**
EXPIRATION DATE:	28 Jul 2024

Thank you for providing the information required for Advarra IRB to conduct continuing review of the protocol and your site.

In addition to the information you provided, the IRB reviewed the current protocol referenced above, the Consent Form(s) for the study, and other supporting information.

The IRB approved continuation of the above referenced protocol. The IRB determined there were no changes required to the current Consent Form(s).

If the study is expected to last beyond the approval period, you must request and receive re-approval prior to the expiration date noted above. A report to the Board on the status of this study is due prior to the expiration date or at the time the study closes, whichever is earlier. It is recommended that you submit status reports at least 4 weeks prior to your expiration date to avoid any additional fees or lapses in approval.

Approved investigators and sites are required to submit to Advarra for review, and await a response prior to implementing, any amendments or changes in the protocol; advertisements or recruitment materials ("study-related materials"); investigators; or sites (primary and additional).

Approved investigators and sites are required to notify Advarra of the following reportable events, including, but not limited to: unanticipated problems involving risks to subjects or others; unanticipated adverse device effects; protocol violations that may affect the subjects' rights, safety, or well-being and/or the completeness, accuracy and reliability of the study data; subject death; suspension of enrollment; or termination of the study.

Please review the IRB Handbook located in the "Reference Materials" section of the Advarra CIRBI™ Platform (www.cirbi.net). A copy of the most recent IRB roster is also available.

Thank you for continuing to use Advarra IRB to provide oversight for your research project.

APPENDIX E: COPYRIGHT PERMISSIONS OF PREVIOUSLY PUBLISHED MATERIAL

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