ABSTRACT

AUSTIN YOUNG SHULL
Super enhancer-associated molecular signatures reveal a dependency on immune and metabolic mechanisms in chronic lymphocytic leukemia
(Under the direction of HUIDONG SHI)

Chronic lymphocytic leukemia (CLL), characterized by the progressive and uncontrolled accumulation of CD19+ B cells, currently remains as an incurable malignancy despite recent advancements in treatment options. The difficulties of eliciting curative measures in CLL are partly driven by the adaptability of the transcriptional response in CLL cells. In this study, we sought to better understand the complexities of the CLL transcriptional profile by defining the large histone H3 lysine-27 acetylation regions known as “super enhancers” within B cells and determining which genes overexpressed in CLL overlapped with super enhancers. From this analysis, we identified 190 super enhancer-associated genes overexpressed in CLL and determined that many of the genes identified were either involved in immune signaling cascades (e.g. LCK, FCER2) or metabolic regulation (e.g. LSR, ENO2). These processes corresponded with our reverse phase protein array (RPPA) profile of CLL patients, which shows overexpression of immune signaling kinases (e.g. LCK) as well as alteration of metabolically sensitive translation regulators (e.g. 4E-BP1 phosphorylation). Additionally, we determined that CLL cells are apoptotically sensitive to dual PI3K/mTOR inhibition when compared to upstream B cell receptor pathway inhibition due to their differential effects on 4E-BP1 phosphorylation. Based on the derived information from our super enhancer expression signature, we then compared the effects of preferentially targeting super enhancers with either the BET bromodomain inhibitor
JQ1 or the cyclin dependent kinase-7 (CDK7) inhibitor THZ1. From this comparison, we saw that JQ1 could inhibit cell cycle progression in CLL cell lines as well as differentially disrupt transcription of genes involved in immune signaling. Contrastingly, we saw that THZ1 elicited a different response in CLL cells by inducing apoptosis and differentially downregulating genes involved in metabolism. The specific super enhancer-associated genes disrupted by the respective treatments further highlighted the dichotomy of JQ1 and THZ1-mediated effects, as JQ1 suppressed the B cell activation marker gene FCER2 whereas THZ1 suppressed the glycolytic enolase gene ENO2. Collectively, these results reveal that super enhancers play a role in mediating both immune signaling and metabolic expression signatures in CLL and that super enhancers can be differentially disrupted by BET bromodomain or CDK7 inhibition.

INDEX WORDS: Chronic lymphocytic leukemia, super enhancers, ChIPseq, RNAseq, RPPA, mRNA transcription, metabolism, immune signaling, BET bromodomain, CDK7
SUPER ENHANCER-ASSOCIATED MOLECULAR SIGNATURES REVEAL A DEPENDENCY ON IMMUNE AND METABOLIC MECHANISMS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Submitted to the Faculty of The Graduate School of Augusta University in partial fulfillment of the Requirements of the Degree of
(Doctor of Philosophy)

July
2016
SUPER ENHANCER-ASSOCIATED MOLECULAR SIGNATURES REVEAL A DEPENDENCY ON IMMUNE AND METABOLIC MECHANISMS IN CHRONIC LYMPHOCYTIC LEUKEMIA

This dissertation is submitted by Austin Young Shull and has been examined and approved by an appointed committee of the faculty of The Graduate School of Augusta University.

The signatures that appear below verify the fact that all required changes have been incorporated and that the dissertation has received final approval with reference to content, form, and accuracy of presentation.

This dissertation is therefore in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ACKNOWLEDGEMENTS

I offer my sincerest thanks to everyone who played a critical role in my development as a scientist during my tenure as a PhD student at Augusta University. I would first like to express my sincerest gratitude to my mentor Dr. Huidong Shi. I greatly appreciate the opportunity to train under his mentorship and learn in an academic environment that fostered a creative yet disciplined pursuit of knowledge. I also thank my PhD advisory committee members Drs. Gang Zhou, Jan van Riggelen, Darren Browning, and Kebin Liu for the guidance they provided. Their support throughout my progression as a PhD student has been immensely valuable. I also thank Dr. John Cowell for graciously taking the time to serve as a reader for my PhD dissertation committee.

I especially thank the members of Dr. Huidong Shi’s lab: Dr. Eun Joon Lee, Dr. Satish Kumar Noonepalle, Mr. Qimei Han, and Ms. Leah Pei. Each member has provided valued support with willingness and graciousness during my time at Augusta University. I am incredibly grateful to have been able to work with such encouraging colleagues. I also extend my sincerest gratitude toward the fellow members of the Georgia Cancer Center as well as fellow members of The Graduate School. Everyone associated with these respective institutions have made my training and development at Augusta University a cherished experience.

Most importantly, I want to thank my friends and family for providing welcomed encouragement during my time as a PhD student. I am especially grateful for my wife Libby. Her unconditional love, support, and encouragement have been and will continue to be the most humbling blessings provided to me on this earth.
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I. INTRODUCTION

A. STATEMENT OF PROBLEM

Chronic lymphocytic leukemia (CLL) is a malignant disease characterized by the progressive accumulation of CD19+ B cells that acquired the ability to overcome programmed cell death and oncogenically expand within the lymph node microenvironment. CLL is the most common leukemia in the western world, and though survival rates for CLL patients are typically longer than other types of leukemia patients (e.g. acute myeloid leukemia, acute lymphocytic leukemia), CLL currently remains incurable and creates a poor quality of life for many clinically affected by the disease. One of the major clinical issues contributing to CLL-related difficulties is the molecular heterogeneity present between CLL patients as well as heterogeneity within CLL cell populations. The dynamics regarding CLL cell heterogeneity can play a significant role in disease development due to CLL B cell subclones resistant to standard chemotherapy being able to preferentially expand and occupy the B-cell niche of a CLL patient, ultimately resulting in disease relapse.

In efforts to overcome the clinical nature of CLL from both a prognostic and therapeutic perspective, several large-scale biomarker studies using CLL patient samples have been employed to determine which molecular features provide both biological understanding of the malignancy as well as prognostic power in effectively treating this disease. These studies have mainly relied on gene expression profiling by microarray (Haslinger and others 2004) or next-generation RNA sequencing (Ferreira and others...
somatic mutation profiling by next-generation DNA sequencing (Quesada and others 2012; Schnaiter and others 2013), or differential DNA methylation analysis through either array (Cahill and others 2013) or DNA sequencing methodologies (Kulis and others 2012; Pei and others 2012). Such studies have created the opportunity to discover genetic and epigenetic marks as potential prognostic tools. Examples of these discovered prognostic markers include somatic mutations in genes like SF3B1, NOTCH1, and MYD88 (Cortese and others 2014; Schnaiter and others 2013) as well as differential gene expression and DNA methylation changes in genes like ZAP70 (Chantepie and others 2010; Wiestner and others 2003), LPL (Kaderi and others 2011), CRY1 (Eisele and others 2009; Hanoun and others 2012), and LDOC1 (Duzkale and others 2011). Along with discovering these single gene prognostic markers in CLL, genome-wide DNA methylation and gene expression studies have also exhibited genomic signatures that correspond with specific CLL subtypes, like IGVH mutation and CD38 expression status, and provided better insight into the molecular abnormalities of this disease (Ferreira and others 2014; Kulis and others 2012). Nevertheless, though the understanding of CLL pathobiology has been enhanced by these large-scale genomic efforts, much of the isolated information extracted from these type studies have yet to provide extensive clarity concerning the therapeutically vulnerable molecular programs in CLL.

By understanding this particular void, we sought to provide a comprehensive approach to characterizing the molecular programs in CLL. We hypothesized that an integrative signature comprised by the epigenetic, RNA, and protein landscapes in CLL will provide proper characterization of the molecular dependencies in CLL.
**Specific aim 1:** To perform chromatin immunoprecipitation sequencing (ChIPseq)-based histone H3 lysine-27 acetylation analysis, RNA sequencing-based transcriptome analysis, and reverse phase protein array (RPPA)-based expression analysis in CLL cells and integrate these findings into a common signature to determine whether certain molecular programs are differentially dysregulated within CLL patient B-cells.

Based on the general knowledge of how CLL B cells proliferate as well as from previous reports highlighting the relatively low occurrence of somatic mutation drivers in CLL, we realize CLL cells rely on specific transcription factor networks (e.g. NFAT, STAT3, NFKB) as well as certain translational machinery proteins (e.g. eIF4E) in a dysregulated fashion. With this understanding established, we hypothesized that **therapeutically targeting mRNA transcriptional and mRNA translational regulation can counteract CLL proliferative and survival mechanisms.**

**Specific aim 2:** To investigate the preclinical efficacy of disrupting mRNA transcription using the BET bromodomain inhibitor JQ1 and the cyclin-dependent kinase 7 inhibitor THZ1 as well as investigating the preclinical efficacy of disrupting mRNA translational activation using the dual phosphoinositide 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235.
B. REVIEW OF LITERATURE

Clinical overview of chronic lymphocytic leukemia

In 2016, approximately 18,960 new cases of CLL will be diagnosed within the United States, accompanied by 4,660 new deaths due to complications from CLL. The lifetime risk of developing CLL is approximately 1 in 200 and is more commonly presented during the later adult life stages. In fact, most CLL diagnoses typically occur between the ages of 65-74 years old and rarely occur in people under the age of 40. Furthermore, with the median age of diagnosis being approximately 72 years of age, the average age of death is approximately indistinguishable from the overall average age of death in the US at 79 years of age (SEER Stat Facts Sheets: Chronic Lymphocytic Leukemia seer.cancer.gov).

Nevertheless, the clinical concerns pertaining to CLL can be diluted when analyzing the diagnosed population as one homogenous cohort, for the clinical outcomes of CLL patients can be quite dynamic. For approximately one-third of patients diagnosed with CLL, no type of therapeutic intervention will ever be implemented against the indolent form of their disease, and these individuals will more than likely live a lifestyle relatively unhindered by CLL-based complications. Nevertheless, the other two-thirds of patients who acquire a more aggressive form of CLL will require some type of therapeutic intervention, and approximately half of the patients from this particular population will experience poor responses to common frontline chemotherapies, potentially dying from the disease within 2 to 3 years post relapse (Gribben 2010). These poor responses are either a consequence of initial resistance to the standard chemotherapy treatments or acquired resistance driven by subclonal selection of CLL cells refractory to
the frontline treatment (Landau and others 2013). Because of the heterogeneous outcomes that can arise from CLL patients, a great deal of effort has been placed in the past two decades into discovering molecular prognostic markers that correspond with clinical outcome and determining how the varying molecular characteristics of CLL subtypes play a role within the larger context of CLL progression.

Molecular characterization of CLL subtypes

Due of the heterogeneous directions in which CLL can clinically progress, molecular characterization has become increasingly important for determining the best mode of action for treating CLL patients. Such characterizations are used to provide prognostic power for clinicians that implement a “wait and watch” approach for CLL standard of care. By identifying the phenotypes of CLL cells during the earlier stages of presentation, clinicians are then able to predict with better certainty regarding which patients would benefit from chemotherapy at an earlier stage.

Chromosomal abnormalities

With nearly 80% of CLL patients demonstrating some presence of an event, one of the major features commonly analyzed in CLL patients is chromosomal abnormality. Some of the more common chromosomal abnormalities identified in CLL are 13q deletions, 11q deletions, 17p deletions, and trisomy 12, and each of these events provide insight into the molecular and clinical implication of the monitored malignancy. For example, 13q deletions, occurring in roughly 50% of CLL cases, are typically associated with the loss of certain microRNAs that are thought to play a regulatory role in B-cell receptor activation in CD19+ B cells. However, patients with 13q deletions tend to have better
outcomes than other CLL cases. On the other hand, 11q deletions, an event occurring in 20% of all CLL cases that leads to loss of the DNA repair gene ATM, tend to correspond with poorer prognosis as these cases are associated with an earlier age of diagnosis and worse overall outcomes. Trisomy 12 is also seen in approximately 20% of CLL patients, but its molecular and clinical implications are not quite clear. However, the chromosomal abnormality associated with the worst prognosis is the 17p deletion of the tumor suppressor gene TP53. Seen in approximately 10% of patients, CLL cases harboring this particular abnormality will typically progress rapidly, respond poorly to standard chemotherapy, and experience an overall shorter survival period than standard CLL cases (Gribben 2010).

**Gene mutation & expression status**

Mutation status and expression status of certain genes have also played a large role in delineating the prognostic outcomes of CLL patient populations. Certain point mutations have been identified as correlating with poor prognosis in CLL including TP53, NOTCH1, SF3B1, and MYD88 mutations (Cortese and others 2014; Schnaiter and others 2013). However, other than general TP53 alterations (deletions and mutations), no one particular gene is mutated in more than 10% of CLL cases (Landau and others 2013). Nevertheless, the nucleotide alteration most commonly analyzed in CLL is the hypermutation of the variable immunoglobulin heavy chain gene (i.e. IGVH). Classified as either “unmutated” or “mutated,” IGVH mutation status gives an understanding of the cellular origin for the particular CLL B-cell population, as IGVH unmutated CLL cells are believed to have originated from a naïve B-cell origin and IGVH mutated CLL cells are believed to originated from a memory B-cell origin (Kulis and others 2012).
IGVH status has profound implications on clinical outcome as patients with an IGVH mutated status have an average survival period of 25 years, whereas the survival period for patients with an IGVH unmutated status dramatically decreases to 8 years. Several prognostic expression markers generally correspond with IGVH mutation status. Two specific markers commonly analyzed within the clinic are CD38 and ZAP70 expression status. As certain clinics may not have the capability to perform proper sequencing on the IGHV gene, many rely on the differential expression of these two markers. High ZAP70 expression strongly correlates with the IGVH unmutated status and its associated poor prognosis, whereas CD38 expression is more variable between IGVH mutation status yet still corresponds with a worse prognosis in CLL patients (Gribben 2010).

**Treating CLL**

The discovery and implementation of these particular biomarkers have provided great advancement in determining the outlook of a disease that can have such a wide array of outcomes. However, though biomarker discovery has provided much aid in understanding prognosis of CLL progression, relatively little progress has been gained in understanding how to best target the molecular mechanisms that allow CLL cells to overcome apoptotic death and malignantly expand.

For several decades, the standard treatment option for CLL has been chemotherapy. The common chemotherapy regimens used in CLL treatment have been fludarabine (purine analog), cyclophosphamide (alkylating agent), chlorambucil (alkylating agent), or bendamustine (alkylating agent/purine analog). With this particular base of chemotherapy drugs and the development of monoclonal targeting antibodies,
combination chemo-immunotherapy eventually was employed, with the most popular combination used being fludarabine, cyclophosphamide, and the anti-CD20 antibody rituximab (i.e. FCR). These types of combinations typically demonstrate a positive response in patients at initial stages. However, the major issue that arises is acquired resistance to this standard of treatment, which leads to eventual relapse. As these particular treatments have a relatively non-specific mechanism of action, subclonal populations of CLL cells resistant to this type of therapy will preferentially expand over the cell populations sensitive to chemotherapy treatment. This action of subclonal selection will create much more aggressive forms of CLL and lead to shorter outcome for patients (Gribben 2010; Landau and others 2013).

The B-cell receptor (BCR) pathway

With the apparent need of determining how to overcome chemotherapy resistance in patients and provide a more targeted approach in treating CLL, great efforts over the past decade have been placed into understanding the distinct molecular mechanisms of CLL as well as determining how to effectively target these distinct molecular mechanisms differentiating CLL cells from normal CD19+ B cells. Based on this scientific direction, one pathway known as the B-cell receptor (BCR) pathway revealed to be highly important in CLL pathogenesis. This particular pathway can control several different mechanisms concerning CLL activity. The receptor itself is composed of a membrane-bound immunoglobulin chain that is anchored by two transmembrane proteins, CD79a and CD79b. These two proteins form a heterodimer and contain an immunoreceptor tyrosine-based activation motif (ITAM) that bridges the extracellular signaling stimulus
with the intracellular signaling responses. With the ITAM protein domain activated, the LYN and SYK proteins are recruited to the intracellular domain and induce subsequent activation of the phosphoinositol-3 kinase delta isoform (PI3Kδ) and the Bruton’s tyrosine kinase (BTK) proteins. These two proteins play an integral role in activating several downstream intracellular pathways during activation of the B-cell receptor including the PI3K/AKT/mTOR, Phospholipase C gamma 2 (PLCγ2), nuclear factor of activated T-cells (NFAT), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB) cascades (Figure 1) (Burger 2012; Stevenson and others 2011).

**Targeting the BCR pathway in CLL**

Both the BTK and PI3Kδ proteins have become increasingly important targets when determining a means of disrupting CLL progression. The importance of these proteins became especially clear when Herman and colleagues demonstrated that both the PI3Kδ and BTK enzymes are overexpressed at the protein level in CLL patients. The increased enzymatic activity of these proteins corresponded with greater response to BCR activation and subsequent pro-tumorigenic signals in CLL. From this knowledge, Herman and colleagues implemented two chemical inhibitors effectively designed to respectively target the PI3Kδ and BTK proteins with the irreversible BTK inhibitor PCI-32765 (later referred as Ibrutinib) effectively disrupting BTK activation and CAL-101 (later referred as Idelalisib) effectively disrupting PI3Kδ activation (Herman and others 2011; Herman and others 2010). The development of these inhibitors provided a new avenue in effectively targeting CLL cells that are dependent upon BCR activation for cellular expansion. The promising effects of both Idelalisib and Ibrutinib carried forward
into successful clinical trials (Byrd and others 2013; Furman and others 2014), and both inhibitors received FDA approval in CLL patients with a refractory form of the disease.

Nevertheless, as both the BTK inhibitor Ibrutinib and the PI3Kδ inhibitor Idelalisib have provided a better outlook in CLL treatment, recent evidence suggests that CLL cells will also eventually become resistant to these particular single-agent inhibitors. Thus, based on these specific reports regarding BTK and PI3Kδ inhibitor resistance (Burger and others 2016; Cheng and others 2015) as well as the understood phenomenon of cancers cells developing resistance to single-agent kinase inhibitors (Fedorenko and others 2011; Sun and others 2014), more efforts have to be made in understanding the intricate network of molecular mechanisms cohesively working together to drive CLL progression.
Figure 1. Illustration of the B-cell receptor (BCR) pathway in CLL. Activation of the BCR pathway through antigen stimulation activates a branched network of pathways activated with CLL B cells. Several of the pathways regulated by BCR signaling include the AKT/mTOR cascade, PLCγ2 signaling, and subsequent activation of the NFAT and NKFB transcription factors. This diagram illustrates how BCR signaling can have an effect on both mRNA transcription and mRNA translation in CLL.
Transcription factors in CLL

CLL relies heavily on the dysregulated activation of transcription factors to provide expression of pro-survival and pro-proliferative transcripts. This understanding provides reasoning to understand how certain transcriptions factors are involved in CLL and how to best disrupt their transcriptional effects.

NFAT and NFKB

As mentioned earlier, NFKB and NFAT are two common transcription factors implemented in CLL pathogenesis. Both transcription factors are traditionally involved in the immune response and can induce expression of pro-survival genes including TRAF1 and FCER2 (CD23) (Carpentier and Beyaert 1999; Le Roy and others 2012; Wang and others 1998). The activation of these two transcription factors can be induced by BCR engagement in CLL cells, and the level of activation for these transcription factors can correlate with clinical outcome (Le Roy and others 2012). In particular, one gene microarray study comparing CLL cells from the peripheral blood with patient-matched CLL cells from the lymph node demonstrated an enrichment of upregulated NFKB target genes (Herishanu and others 2011). The activation of these transcription factors within the lymph node highlights that NFAT and NFKB play integral roles in driving CLL progression.

STAT3

Another transcription factor involved in the oncogenic functions of CLL is the signal transducer and activator of transcription 3 (STAT3) protein. Both IgM and interleukin-6 stimulation in CLL cells can phosphorylate STAT3 at the tyrosine-705 site. This phosphorylation event of STAT3 correlates with activation of known STAT3-mediated
transcripts such as *c-Myc* and *cyclin D1* and provides evidence for STAT3 driving CLL cell cycle progression (Rozovski and others 2014). Nevertheless, the serine 727-phosphorylation site, which is responsible for STAT3’s transcriptional activity, is consistently phosphorylated in CLL cells even under resting conditions (Hazan-Halevy and others 2010). This constitutive level of phosphorylation in STAT3 is thought to play a role in maintaining metabolic function. Specifically, Rozovski and colleagues demonstrated that STAT3 binds to the *LPL* gene promoter under resting conditions and upregulates the expression of lipoprotein lipase. Upregulation of lipoprotein lipase, in turn, facilitates free fatty acid metabolism in CLL cells and provides a means for CLL cells to maintain metabolic homeostasis (Rozovski and others 2015).

*HIF1-alpha*

Though fatty acid oxidation has been shown to be a major mechanism for maintaining proper metabolic function in CLL cells, reports also show that CLL cells can switch metabolic programming by activating the hypoxic-inducible factor 1-alpha (HIF1a) transcription factor. Results from Ghosh and colleagues demonstrate that HIF1a is highly enriched within the bone marrow of CLL patients when compared to healthy donors. Sustained levels of HIF1a in CLL cells are maintained by the loss of the von Hippel-Lindau protein, which is responsible HIF1-alpha protein degradation (Ghosh and others 2009). Later reports demonstrate that the stability of HIF1a protein levels allows for glycolytic induction in CLL cells when challenged with a hypoxic environment (Koczula and others 2016). Collectively, these reports suggest that CLL cells can properly adapt to the metabolic needs by utilizing oxidative phosphorylation when under resting conditions.
within the peripheral blood or to HIF1α-mediated glycolytic induction when under hypoxic conditions within the bone marrow microenvironment.

**Regulating mRNA transcription**

One key element central to all transcription factor-mediated gene expression is the proper recruitment and activation of RNA polymerase II. As activation of specific transcription factors is tightly regulated by an array of molecular mechanisms, RNA polymerase II is also tightly regulated by numerous cofactors that help mediate proper control of the larger RNA polymerase holoenzyme. One class of proteins that serves a role in the larger RNA polymerase complex is the bromodomain (BRD) proteins. These particular proteins are recruited to the enhancer and promoter region of genes to facilitate RNA polymerase II recruitment. This action is accomplished by the binding of bromodomains to acetylated histones proteins. Histones proteins are a part of the chromatin landscape in which genomic DNA is wrapped around and located. Proper access of DNA binding proteins to specified genomic regions is determined largely by the modification of histone proteins. One specific example of a histone modification playing a role in DNA accessibility is the addition of an acetyl group to Histone H3 at lysine 27 (H3K27Ac). This addition of an acetyl group reverses the electrostatic attraction between the positively charged histone protein and the negatively charged DNA, ultimately allowing transcriptional regulating proteins to physically access the overlapping genomic region. Thus, by the presence of H3K27 acetylation within the gene promoter and enhancer, bromodomains can directly bind to acetylated histones and enable recruitment of proteins involved in the RNA polymerase II complex. Such critical proteins recruited by
bromodomains include the Mediator protein, which provides anchoring for proteins within the polymerase complex and connects the looping cis-enhancer region to the promoter of the respective gene, as well as other cofactors responsible for direct RNA polymerase activation (Filippakopoulos and others 2010).

Nevertheless, for RNA polymerase II to have proper enzymatic activity, it must be phosphorylated within its carboxyl tail domain (CTD). The CTD of RNA polymerase II is comprised of 52 repeats of a 7-amino acid motif containing serine residues at positions 2, 5, and 7. These serine residues are phosphorylated in sequential order with serine-5 being phosphorylated during polymerase transcriptional initiation then serine-2 and serine-7 being phosphorylated during transcriptional elongation (Kuehner and others 2011). Interestingly, the proteins responsible for phosphorylating these serine residues in RNA polymerase II are a family of cyclin-dependent kinases (CDKs). Traditionally viewed as proteins involved in cell cycle progression, certain CDKs such as CDK7 and CDK9 play a critical role in activating RNA polymerase II-based transcription. Specifically CDK7 is responsible for enabling transcription initiation by phosphorylating RNAPII at serine-5, whereas P-TEFb/CDK9 is responsible for enabling transcription elongation by further phosphorylating RNAPII and relinquishing the transcriptional pausing negative elongation factor (NELF) protein from the RNAPII complex (Lim and Kaldis 2013). These measures concerning RNA polymerase regulation together with the mechanisms used to regulate specific transcription factor activation ultimately provide a heavily controlled process of mRNA transcription in eukaryotic cells (Figure 2).
Targeting mRNA transcription

One of the major hurdles in countering the pro-tumorigenic effects of transcription factors is that most transcription factors are not structured in ways that allow targeting through small molecule inhibition. Because of this caveat, the effects of gene expression through particular transcription factors have to be indirectly suppressed by targeting other components within the transcription factor axis. The best means of indirectly inhibiting transcription factor mediated gene expression have either relied on disrupting the signaling cascades that led to transcription factor activation or targeting the resulting effects of the transcription factor response. However, recent advances in drug discovery have enabled suppression of specific mRNA transcriptional programs in cancers by precisely targeting particular regulatory components of the RNA polymerase II complex (Figure 2). From this direction in therapeutic design, two classes of small molecule transcriptional inhibitors that demonstrate promising preclinical activity have been the BET bromodomain inhibitors (e.g. JQ1, OTX015) and the covalent CDK7 inhibitors (e.g. THZ1). The inhibitory effects of bromodomain inhibitors first gained recognition by their ability to therapeutically disrupt MYC oncogene transcription in several types of cancers. Several of these cancers sensitive to BRD inhibition included multiple myeloma (Loven and others 2013), diffuse large B cell lymphoma (Chapuy and others 2013), and acute leukemia (Coude and others 2015), all of which rely heavily on c-Myc to drive their oncogenic progression. These results were especially important due to the idea that a small molecule transcriptional inhibitor could effectively suppress the expression of c-Myc, a seemingly “undruggable” oncogenic transcription factor overexpressed in nearly 70% of all cancer types (Posternak and Cole 2016).
The results of oncogene transcription being suppressed by selective, small molecule bromodomain inhibitors were later followed by reports demonstrating the therapeutic effects of inhibiting the transcriptional initiator CDK7 using the covalent inhibitor THZ1. Kwiatkowski and colleagues first demonstrated that THZ1 at low nanomolar doses could effectively disrupt the transcription of genes highly expressed in T-cell acute lymphoblastic leukemia (T-ALL) cells with preferential downregulation of \( \text{RUNX1}, \text{TAL1}, \text{and GATA3} \) (Kwiatkowski and others 2014). Following reports further demonstrated the efficacy of THZ1 treatment in non-small cell lung cancer (NSCLC) by preferentially downregulating oncogenes such as \( \text{MYC} \) and \( \text{SOX2} \) (Christensen and others 2014). Interestingly, CDK7 inhibition was also able to exhibit subtype-specific therapeutic selectively in certain cancer types as THZ1 could preferentially inhibit MYCN-overexpressing neuroblastomas compared to MYCN-normal neuroblastomas (Chipumuro and others 2014) as well as preferentially inhibit triple-negative, basal-like breast cancer cells compared to luminal-like, estrogen-receptor positive breast cancer cells (Wang and others 2015). With the example established by these studies, not only is CDK7 a promising the therapeutic target, but could also shed light into which specific molecular programs drive the tumorigenic functions of specific cancer types.
Figure 2. Illustration of RNA polymerase II (RNAPII) regulation in eukaryotic cells.

This diagram demonstrates how histone acetylation at lysine-27 enables recruitment of the RNA polymerase complex through direct recruitment of bromodomain proteins like bromodomain 4 (BRD4). Other proteins in the complex are also recruited to perform their specific function such as CDK7 phosphorylating the RNAPII CTD at serine-5 to induce transcriptional initiation, Mediator serving as the anchoring protein for the RNAPII complex, and CDK9 causing phosphorylation of RNAPII at serine-2 and serine-7 to activate transcriptional elongation.
Discovery and inhibition of super enhancers

With the understanding that these small molecule inhibitors of transcription target relatively conserved components of the RNAPII complex, efforts were directed towards determining how these inhibitors preferentially target genes critical for tumorigenic functions in vastly different cancer types as well as providing proper rationale for pursuing transcriptional inhibition as a viable therapeutic in cancers. However, the preferential effects of these type inhibitors were made clear based on the seminal work of Loven and colleagues who demonstrated a phenomenon called “super enhancers” that occurs throughout different cell types (Figure 3). As briefly described earlier, gene promoters and enhancers serve as the genomic sequences that contain specific transcription factor binding sites (TFBS) and provide proper transcriptional machinery recruitment to genes designated for mRNA transcription. Characteristically, gene promoters exist within 1000-2000 base pairs (1-2kbp) of the transcriptional start site. However, enhancers can exist as far as 1,000,000 base pairs (1Mbp) from the transcriptional start site and serve a regulatory function in gene transcription through DNA looping. Based on the interaction between the histone code and genomic regulation, the understanding is that H3K27 acetylation corresponds with enhancer within the genome. Typically, the average span of an H3K27ac enhancer is approximately 1.5kbps. However, Loven and colleagues demonstrated that a small subset of enhancers occupied exponentially larger regions within the genome by spanning an average length of near 11.4kbps. These subsets of enhancers also corresponded with higher expression of the corresponding genes, as well as exponentially larger recruitment of transcriptional complex proteins, including Mediator and BRD4. The enhancers that exhibited these
particular characteristics were identified as super enhancers (Loven and others 2013). This report as well as subsequent reports demonstrated that super enhancers vary among different cancer types and that the super enhancer-related genes are preferentially sensitive to transcriptional inhibitors like JQ1 and THZ1 (Chapuy and others 2013; Kwiatkowski and others 2014). Thus, with the established understanding of how super enhancers operate within cancer as well as the therapeutic capabilities of transcriptional inhibitors like JQ1 and THZ1, it has become increasingly important to understand the context of super enhancers throughout other cancer types. Currently, most efforts have focused on studying the landscape of super enhancers in MYC-driven cancers. However, work is moving forward in understanding how super enhancers play a role in MYC-independent cancer growth (Jiang and others 2016). Thus, it is important to expand the context and roles of super enhancers in other cancers like chronic lymphocytic leukemia, which greatly relies on transcriptional dysregulation to drive its malignant state.
Figure 3. Summary of super enhancers and their sensitivity to transcription inhibitors. (A) provides an illustration of how a typical enhancer operates. Typical enhancers span approximately 1kb, and the recruitment of the RNAPII complex has a linear correlation with the respective gene expression. (B) provides an illustration of a super enhancer and how the wide spanning super enhancer exponentially increases the recruitment of the transcriptional complexes and subsequently increases gene expression exponentially. (C) demonstrates the therapeutic sensitivity super enhancers experience when treated with molecules like BET bromodomain and CDK7 inhibitors.
Regulating mRNA translation

As the regulatory cofactors of the RNAPII holoenzyme complex efficiently control activation of mRNA transcription, specific regulatory proteins involved with the ribosomal complex efficiently control activation of mRNA translation. One specific class of proteins responsible for regulating translational activation in eukaryotic cells is the eukaryotic translation initiation factor (eIF) family of proteins. The regulatory pathway involving these proteins begins when the eIF4E translation initiator protein binds to the methyl-7-guanosine triphosphate (m\textsuperscript{7}GTP) cap of processed mRNA. The binding of eIF4E to mRNA is critical for the initial recruitment and binding of other translational machinery proteins such as the eIF4G scaffolding protein, the eIF4A RNA helicase, and the mRNA decoding 40S ribosomal subunit. However, this complex of proteins, known as the eIF4F complex, is refrained from forming and activating mRNA translation when the inhibitory 4E-binding protein (4E-BPs) is directly bound to eIF4E. Nonetheless, 4E-BPs will dissociate from eIF4E when phosphorylated by mTOR, allowing for the eIF4F complex to form and ultimately mRNA translation to continue forward. The kinase activity of mTOR is also responsible for phosphorylating and activating the ribosomal S6 kinases (S6Ks), which in turn will increase the RNA helicase activity of eIF4A and phosphorylate the S6 component of the 40S ribosomal subunit (Ma and Blenis 2009). Thus, with the action of several translational machinery proteins dependent upon mTOR-mediated phosphorylation, it is understood that mTOR plays a master regulatory role in governing the translation of mRNA into proteins within eukaryotic cells (Figure 4).
Figure 4. Illustration representing mTOR/eIF4F-mediated regulation of mRNA translation. The diagram shows that when the mTOR complex 1 (mTORC1) is inactivated, 4E-BP is able to bind to the mRNA-coupled eIF4E protein and inhibit the ability of the eIF4F translation complex to be formed. Yet, when activated, mTORC1 will phosphorylate 4E-BP and cause its dissociation from eIF4E. This dissociation of 4E-BP allows for formation of the translation complex with eIF4G, eIF3, eIF4A, the eIF2 ternary complex, and the 40S ribosomal subunit. The mTORC1 complex is also capable of phosphorylating S6K, which in turn, increases translation activity.
Deregulated mRNA translation in CLL

Recent reports have highlighted the importance of mRNA translation in propagating and maintaining the tumorigenic characteristics of CLL. One report by Willimott and colleagues highlighted the importance of mRNA translation in CLL by demonstrating that CLL cells could be sensitized to the BH3-mimetic ABT-737 when treated with the cap-dependent translation inhibitor 4EGI-1. This induced susceptibility, mediated by 4EGI-1’s ability to suppress the protein expression of BCL2L1 and BCL2A1, underscores the importance of CLL cells maintaining the translation of critical anti-apoptotic proteins (Willimott and others 2013). Another study by Yeomans and colleagues demonstrated the concept that CLL promotes mRNA translational activity to enhance tumorigenic functions by revealing increased translational activity in CLL cells when stimulated with BCR pathway inducing anti-IgM. This amplified translational activity in CLL cells was accompanied by increased expression of the eukaryotic imitator proteins eIF4A and eIF4G1 as well as by increased protein translation of the mRNA-encoding MYC. These results ultimately highlighted the integral function of mRNA translation in promoting CLL cell expansion (Yeomans and others 2016). With this understanding regarding the dynamics of mRNA translation in CLL activation as well as the knowledge that expression of crucial CLL drivers like BTK is controlled at the protein level rather than the mRNA level (Herman and others 2011), it has become increasingly apparent that mRNA translation and the resulting protein landscape should be considered when investigating the molecular mechanisms of CLL pathogenesis.
II. MATERIALS AND METHODS

Obtainment & isolation of CLL patient samples

Whole blood samples from the peripheral blood of CLL patients were obtained from the Georgia Cancer Center Tumor Bank at Augusta University and North Shore-LIJ Health System in compliance with the Institutional Review Boards from the respective institutions. Whole blood samples from healthy donors were purchased from a local blood bank. Once obtained, CD19+ B-cells were selected from whole blood by using the RosetteSep™ B-cell isolation kit (STEMCELL Technologies, Vancouver, Canada), and isolated by Ficoll density centrifugation using the Ficoll-Paque PLUS® reagent from GE Healthcare Life Sciences (Little Chalfont, United Kingdom). Peripheral blood mononuclear cell (PBMC) populations were also isolated from patient samples using Ficoll-density centrifugation.

Cell culture conditions and reagents

Isolated primary CD19+ B-cells, PBMCs, and the MEC1 and MEC2 CLL cell lines were cultured in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (HyClone Laboratories, Logan, Utah, USA) at 37°C in a 5% CO₂ incubator. The HS-5 bone marrow stromal cell line and the Huh7.5 hepatocarcinoma cell line were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), high glucose supplemented with 10% FBS and 1% P/S.
For hypoxia induction experiments, cells were cultured in a hypoxic chamber incubator that maintained oxygen percentage levels at 1% compared to the approximate 22% oxygen levels in ambient air. Media used for hypoxia experiments was pre-incubated in the hypoxia incubator for at least 2 hours before adding to cells in hypoxia. Cells were cultured in hypoxia for approximately 12-16 hours.

The small molecule inhibitors used for these studies were purchased from the following retailers: Ibrutinib, Idelalisib, NVP-BEZ235 (Selleckchem, Houston, Texas, USA), JQ1, THZ1 (Cayman Chemical, Ann Arbor, Michigan, USA). All inhibitors were solubilized in DMSO. Rabbit Ant-Human IgM IMMUNOBEAD® reagent was purchased from Irvine Scientific (Santa Ana, California, USA).

**Chromatin immunoprecipitation (ChIP) sequencing preparation & analysis**

ChIP was performed in MEC1 cells using the rabbit polyclonal anti-acetyl-Histone H3 (Lys27) antibody (EMD Millipore, Darmstadt, Germany). Briefly, 2X10^7 viable (>90%) MEC1 cells were cross-linked with 1% formaldehyde for 5 minutes at room temperature followed by the addition of 0.125M glycine for 5 minutes to quench the cross-linking process. Cells were then lysed in cell lysis buffer (all subsequent buffers used contain protease inhibitors) on ice for 15min followed by centrifugation in order to pellet the intact nuclei. The nuclear pellet was then lysed in 200ul SDS-Nuclear lysis buffer and incubated on ice for 30 minutes with intermittent vortexing. Sonication was then performed with a Bioruptor sonicator (Diagenode) set at HIGH power with 30sec ON/OFF cycles for 40 cycles in order to obtain 200-500bp DNA fragments. Ten percent of the sonicated chromatin supernatant was removed for later INPUT applications, and
the remaining supernatant was then split into two equal volumes samples with 10µg of the H3K27Ac antibody added to one sample and 10ug of a rabbit IgG control antibody added to the second sample. The samples were then placed in a rotator and incubated at 4°C for 16 hours. A slurry of Protein A/G magnetic beads were blocked with 2% BSA and incubated with chromatin-antibody complexes for 2hr. Subsequent washes were performed for 5 minutes at 4°C with low salt buffer, high salt buffer, lithium chloride (LiCl) wash buffers and two washes of TE buffer. After eluting the DNA from the antibody-coupled magnetic beads, crosslinking reversal and phenol-chloroform-based DNA isolation were performed. Purity and selectivity of the ChIP isolation was validated by polymerase chain reaction. After verification, DNA from the INPUT and DNA from the H3K27Ac sample was then used to create sequencing libraries using the KAPA Hyper Prep Kit for Illumina (Kapa Biosystems, Wilmington, MA) according to the manufacturer’s protocol. ChIPseq libraries were sequenced on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) with average obtainment of 20-40 million reads per library.

Reads from the sequenced ChIP libraries were aligned, and INPUT-subtracted H3K27Ac peaks were generated using the Model-based Analysis for ChIPseq (MACS) software package to determine the location of active enhancers regions (Zhang and others 2008). The MACS-generated H3K27Ac ChIPseq enhancer peaks were then analyzed using an in-house script to determine the presence and location of “super enhancers.” Super enhancers were defined by ranking the ChIPseq signal of isolated enhancers, as well as the concatenated joining of enhancers that resided within a 12.5kb window of each other. The ranked enhancer signals were then graphed on a XY scatter plot based
on the H3K27Ac enhancer signal rank (X) and the H3K27Ac peak signal (Y). The ranked graph reveals a curved line within the data that contains a point of inflection in the ChIPseq signals. The slope of the tangent line was then calculated for the curve, with the identified inflection point in the curve defining the threshold between “super enhancer” and “typical enhancer.” Enhancer peaks above the inflection point are then identified as “super enhancers,” and enhancer peaks below the inflection point are identified as “typical enhancers” (Pott and Lieb 2015). The bedGraphToBigWig command was used to generate wiggle files from the ChIPseq BED files in order to visualize the genomic location of enhancers within the UCSC genome browser. Publically available H3K27Ac ChIPseq data for normal CD19+ B cell (retrieved from the Epigenomics Road Map and the immortalized B cell line GM12878 (retrieved from the Encyclopedia of DNA elements-ENCODE database) were also used for super enhancer analysis.

RNA sequencing preparation & analysis

Sequencing libraries for stranded mRNA were prepared using the KAPA Stranded mRNA-Seq Kit for Illumina platforms (Kapa Biosystems, Wilmington, MA) following manufacturer’s instructions. Briefly, 2μg of total RNA was depleted of ribosomal RNA using supplied mRNA capture magnetic beads. Eluted mRNA was fragmented to a size of 300-400bp by incubating the mRNA at 90°C for 6 minutes followed by 1st strand and 2nd strand cDNA synthesis. AMPure XP beads (Beckman Coulter, Brea, CA, USA) were then used to purify the synthesized products when required. An A-tailing enzymatic reaction was then performed on the cDNA product followed by ligation of Illumina TruSeq sequencing adapters containing a unique index for each library. The libraries
were then analyzed by the Agilent 2100 Bioanalyzer system in order to determine size and quality of the prepared library. The prepared libraries were pooled together in a final of concentration of 10nM and sequenced on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). Thirty to sixty million 100bp single-end reads were obtained from each sequencing run.

TopHat 2.0.8b (Trapnell and others 2009) was used for mapping sequenced reads to the human genome (hg19). For RNAseq reads of clinical samples, FeatureCounts 1.4.6 (Liao and others 2013) computed the number of reads mapped to each gene and transcript, followed by DESeq 1.10.1 (Anders and Huber 2010) for differential expression analysis. For RNAseq reads of CLL cell lines treated with transcription inhibitors, Cufflinks 2.2.0 was used to calculate the normalized number of RNA fragments per kilobase per million reads followed by Cuffdif 2.2.0 to determine differential expression analysis (Pollier and others 2013). BedTools 2.16.2 (Quinlan and Hall 2010) and bedGraphToBigWig generated wiggle files in order visualize RNAseq reads on UCSC genome browser tracks.

**Reverse phase protein array (RPPA) preparation & analysis**

Isolated CD19+ B cells from 18 CLL patients and 6 healthy donors were lysed in RIPA buffer supplemented with proteasome inhibitor and phosphatase inhibitor (Pierce, Rockford, IL, USA). The lysates were then prepared to provide 1-1.5mg/ml of total protein for RPPA construction. RPPA construction of the primary B-cell lysates was then performed as previously described by the RPPA Core Facility at The University of Texas MD Anderson Cancer Center (Tibes and others 2006). Figure 5 illustrates the
methodology behind RPPA-mediated expression profiling. Briefly, protein lysates are printed on nitrocellulose-coated slides aligned in a spot array fashion. Slides are then blocked with blocking buffer to prevent non-specific antibody binding followed by incubation of the primary antibody. An HRP-streptavidin-coupled secondary antibody is then used to detect the primary antibody, and the signal of the protein lysates are detected by chemiluminescence. One antibody is used for each nitrocellulose slide containing the entire panel of CLL patient and healthy donor samples. For this particular study, 118 total and 49 phospho-specific antibodies were used and are listed in Table 1. The signals from the nitrocellulose slides corresponding with the utilized antibodies are then normalized for differential protein expression and protein phosphorylation analysis.
Figure 5. Illustration of RPPA preparation process for CLL study. Lysates were spotted in a serial dilution on nitrocellulose-coated slide to determine optimal signaling for antibody-based protein detection. The intensity of each signaled was measured and normalized to the overall background signal of the array. The protein array process has many similarities to the preparation used for gene microarrays.
Table 1. List of antibodies used in RPPA study

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<th>Description</th>
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**Bioinformatic characterization analysis**

Hierarchical clustering analysis and heat maps for RPPA and RNAseq expression data were generated using the Partek Genomics Suite (Partek Inc., St. Louis, Missouri, USA). Annotation of genes associated with enhancer regions from H3K27Ac ChIPseq data were generated using an in-house script powered by the GenePattern genomics platform from the Broad Institute (Reich and others 2006). Gene ontology analysis for RNAseq and ChIPseq data were generated using the MSigDB suite in the Gene Set Enrichment Analysis platform (Liberzon and others 2015) as well as using the PANTHER Classification System (Mi and Thomas 2009). Gene ontology analysis for RPPA data was generated using the Ingenuity Pathway Analysis (IPA) platform provided by Qiagen Bioinformatics (Hilden, Germany). Venn diagram-based overlap between genes associated with super enhancers, overexpression in clinical RNAseq data, and transcription inhibitor sensitivity in CLL cell line RNAseq were generated using the open source web platform Biovenn (Hulsen and others 2008).

**RNA extraction & quantitative reverse transcriptase PCR**

RNA from Primary CLL cells, primary normal B-cells, MEC1, and MEC2 cells were isolated using a Trizol-based extraction kit (Qiagen, Hilden, Germany). Isolated RNA
was then converted to cDNA by reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, California, USA). Both random hexamers and oligo(dT) primers were combined within the primer mix to ensure efficient transcript coverage during 3' to 5' cDNA synthesis. Quantitative RT-PCR (qPCR) was performed using Power SYBR® Green Master Mix (Invitrogen Life Technologies) and qRT-PCR and ran on a CFX96 C1000 Real-Time PCR thermal cycler (Bio-Rad, Hercules, California, USA). ACTIN or 18S were used as housekeeping genes when required. All qRT-PCR reactions were performed in triplicate. Primer sequences for qRT-PCR are listed in Table 2.

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Immunoblot analysis

The following antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA): β-Actin (Cat# SC-47778). The following antibodies were purchased from Cell Signaling Technologies (Danvers, Massachusetts, USA): Lck (Cat# 2752S), Phospho-Akt Ser473 (Cat# 4060S), Phospho-Akt Thr308 (Cat# 4056S), Akt (Cat# 9272S), mTOR (Cat# 2972S), Phospho-p70 S6 Kinase Thr389 (Cat# 9234S), p70 S6 Kinase (Cat# 2708S), Phospho-4E-BP1 Ser65 (Cat# 9451S), 4E-BP1 (Cat# 9452S), and eIF4G (Cat# 2498S). The following antibodies were purchased from Bethyl Laboratories (Montgomery, Texas, USA): RNA polymerase II (Cat# A300-653A), Phospho-RNA polymerase II Ser2 (Cat# A304-407A), and Phospho-RNA polymerase II Ser5 (Cat# A304-408A). The following antibodies were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA): SF2/ASF antibody (Cat# 32-4500). The goat anti-rabbit IgG (H+L) Dylight 800 secondary antibody (Cat # 35571) and the goat anti-mouse IgG (H+L) Dylight 680 secondary antibody (Cat # 35518) were also purchased from Thermo Fisher Scientific.

To briefly describe, cells were harvested and lysed in 1X RIPA buffer (Pierce, Rockford, IL) containing protease and phosphatase inhibitor cocktails (Roche). Forty micrograms of total protein, determined by bicinechonic acid (BCA) assays, was run on a 4-20% SDS-PAGE acrylamide-bisacrylamide gel (Bio-Rad Laboratories, Hercules, California, USA) and transferred onto an Immobilon-FL PVDF membrane (EMD Millipore, Darmstadt, Germany). The membranes were then incubated with respective primary antibodies at 4°C overnight at 1:1000 dilution in 5% non-fat dry milk solubilized in phosphate buffer saline with 0.1% Tween-20. Dylight secondary antibodies coupled
with fluorophores were then used at 1:10,000 dilutions in 5% non-fat dry milk to
determine the relative levels of the probed protein. The fluorescent signal was detected
using an Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, Nebraska, USA). Detection of β-Actin was used as a loading control.

**Presto Blue® cell viability assays**

To determine the effects on CLL cell viability when treating with varying concentrations
of the utilized small molecular inhibitors, CLL cells were cultured with dose-dependent
concentrations of each inhibitor in 200 microliters of RPMI-1640 medium (25,000
cells/well of a 96-well dish for MEC1 and MEC2 cells; 500,000 cells/well of a 96-well
dish for primary CLL cells). Cells treated with the designated concentration of the
inhibitors were cultured under 5% CO₂ incubation for a 72-hour period for inhibitory-
concentration 50 (IC₅₀) assays or at varying time points for time-dependent growth curve
assays. Each treatment condition was performed in triplicate. Two hours before each
measured time point, 20 microliters of the Presto Blue® viability reagent (Thermo Fisher
Scientific, Waltham, Massachusetts, USA) was added to each experimental well of the
96-well plates and incubated in the CO₂ incubator. Presto Blue® is a relatively nontoxic
resazurin-based colorimetric/fluorometric dye that measures metabolic respiration of live
cells in order to infer the relative levels of cell viability. After the two hour incubation,
the 96-well plates containing the Presto Blue® reagent were measured for fluorescent
intensity on a Molecular Devices Spectramax M Series Multi-Mode Microplate Reader
(Sunnyvale, California, USA). Relative cell growth was then determined by normalizing
the relative fluorescence readings to a paired zero-time point measurement.
**Annexin V/DAPI apoptosis analysis**

Apoptotic death of CLL cells was assessed by detecting the percentage of annexin V-positive/DAPI-positive cells between different inhibitor treatment conditions. Cells were first washed with cold PBS and then blocked with staining buffer (PBS plus 2% fetal bovine serum (FBS) and 0.09% sodium azide). Cells were again washed with cold PBS and then stained with an Annexin V-FITC antibody (Biolegend, San Diego, CA, USA) for 30 minutes at 4°C. Cells were then washed again in PBS, pelleted, and suspended in 500 microliters of staining buffer containing the DAPI staining dye (Sigma-Aldrich, St. Louis, MO, USA). The percentage of Annexin V and DAPI+ cells was then assessed by flow cytometry using an LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). FlowJo software (Ashland, Oregon, USA) was used to analyze the flow cytometry results.

**Cell cycle analysis**

To assess cell cycle progression, CLL cell lines MEC1 and MEC2 were starved of 10% FBS in RPMI-1640 media overnight in order to synchronize cell cycle phases within the cultures. After starving overnight, cells were resuspended in complete media and treated with designated concentrations of inhibitors for either 24 or 72 hours. The cells were then harvested, fixed in 2% paraformaldehyde, and stained with DAPI. Cell cycle progression then assessed by flow cytometry using an LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using FlowJo (Ashland, Oregon,
USA) to determine the relative percentage of cells that were in the G₁, S, or G₂M phases of cell cycle.

**L-lactate production assay**

L-Lactate production was measured using the Glycolysis Cell-Based Assay Kit (Cat# 600450, Cayman Chemical, Ann Arbor, Michigan, USA) based on the manufacturer’s protocol. For MEC1 and MEC2 cells, 500,000 cells were treated dose-dependently with THZ1 in one milliliter of RPM-1640 medium supplemented with 1% FBS for 6 and 24 hours. For primary CLL cells, 10,000,00 cells were cultured in three milliliters of RPMI-1640/1% FBS media under normoxic or hypoxic conditions for 16 hours with or without 50nM THZ1. At the designated time points, cells were pelleted and 10 microliters of the media supernatant was used for the colorimetric-based assay. Colorimetric readings for each treatment condition were performed in duplicate, and lactate production from the readings was calculated based on a standard curve.

**2-NBDG glucose uptake assay**

Five hundred thousand MEC1 and MEC2 cells treated with 50nM THZ1 were cultured in 1mL DMEM media, low glucose containing 100 micrograms of 2-NBDG, a deoxy-glucose analog fluorescently active when incorporated into cells (Cayman Chemical. After two hours of 37°C/5% CO₂ incubation, treated MEC1 and MEC2 cells were resuspended in 100ul PBS, and fluorescence was measured on a Cytation multi-mode plate reader (BioTek Instruments Inc. Winooski, Vermont, USA).
Flow cytometry detection of immunological markers

Cell surface CD23, CD19, PD-1 and intracellular IL-10 expression was measured in MEC1 and MEC2 cells by flow cytometry. Antibodies used for analysis are anti-human CD279 (PD-1)-APC Clone: J105 (Cat#17-2799-42, eBiosciences, San Diego, California, USA), anti-human CD23-FITC Clone: EBVCS-5 (Cat# 38505, Biolegend, San Diego, California, USA), anti-human CD19-APC Clone: HIB19 (Cat# 302211, Biolegend), anti-human IL-10-APC Clone: JES3-19F1 (Cat# 506806, Biolegend), and Mouse IgG2b, K Isotype control PE (Cat# 12-4732-41, eBiosciences). Cells were resuspended in staining buffer containing PBS pH 7.4, 2% FBS, and 0.09% sodium azide. Alternatively, cells were also permeabilized with methanol for purposes of staining intracellular IL-10 expression. Cells were then blocked with staining buffer for 10 minutes at 4°C followed by centrifugation and resuspension in 50µL staining buffer. Five microliters of the respective antibodies were then added to cells and incubated at 4°C for 30 minutes. The cells were then prepped for flow cytometry analysis by washing and resuspending cells in 500µL staining buffer. Flow cytometry was performed on a LSRII flow cytometer, and analysis was conducted using FlowJo.

Statistical analysis

Statistical analysis, excluding sequencing and protein array data, was performed using Graphpad Prism 6.0 (Graphpad Software, La Jolla, California, USA). Either Student’s t-test or ANOVA was applied when determining statistical significance.
III. RESULTS

Super enhancers (SEs) are present in CLL & normal B cells

To establish a general understanding of the chromatin landscape residing within CLL and normal B cells, we performed H3K27Ac ChIPseq in the MEC1 CLL cell line and compared the results to the H3K27Ac ChIPseq profiles from the immortalized B cell line GM12878 as well as a normal primary CD19+ B cell (data extracted from ENCODE and Epigenome Road Map, respectively). Overall, more H3K27Ac enhancer peaks were called in GM12878 and the CD19+ B cell than in MEC1, though this outcome could be partially due to the differences in sequencing read depth between the three B cell subtypes analyzed. However, based on inspection of the quantitative H3K27Ac enrichment signals and visual comparison of UCSC genome tracks, the enhancer landscape within the three B cell subtypes shared a great deal of homology.

To gain further insight into dynamics of the enhancer landscape in B cells, we performed super enhancer analysis on the ChIPseq results of the three B cells and identified the super enhancers present within the landscape of each H3K27Ac enhancer profile. As expected from previous results from other cell and tissue types, only a small subset of enhancers were identified as super enhancers in each of the B cell subtypes (approximately 4% of all called H3K2Ac enhancers) with GM12878 containing 741 super enhancers, MEC1 containing 374 super enhancers, and the CD19+ B cell containing 523 super enhancers (Figure 6).

To then determine both the unique and common super enhancers that exist between the 3 super enhancer profiles, we gene annotated all of the identified super
enhancer regions and created a Venn diagram comparing the overlap of super enhancer-associated genes in MEC1, GM12878, and the CD19+ B cell. As demonstrated in Figure 7, over 400 genes are associated with super enhancers in all 3 B cell subtypes. Many of the super enhancers found in all three ChIPseq profiles correspond with genes that are typically associated with classic B-cell characteristics and function including CD27, CD79A, PLCG2, and BLNK (Avery and others 2005; Woyach and others 2012). However, the super enhancer profiles do demonstrate notable differences between one another as the CD19+ B cell and GM12878 share a great number of super enhancer genes (347) not seen in the CLL cell line MEC1 as well as each cell type having their own distinct subset of super enhancer genes (MEC1=124; GM12878=786; CD19+ B cell=550) (Figure 7). Noteworthy super enhancers that are not common in all 3 super enhancer profiles include FCER2 (CD23), LCK, PTPRC (CD45), RXRA, and NFATC1. Nevertheless, it is important to note that many of the identified super enhancers that do not overlap in all 3 ChIPseq profiles were right under the threshold of being deemed as a super enhancer. For example LCK, deemed a super enhancer gene in GM12878, is associated with the 561st ranked enhancer in MEC1, which is relatively close to the super enhancer threshold created at 374th ranked enhancer in MEC1. Thus, as mentioned earlier, the differences between super enhancers in the 3 B cell subtypes may be due to the variability in sequencing read coverage.
Figure 6. Super enhancer identification from histone H3 lysine-27 acetylation ChIPseq in B cells.

Based on the XY plots comparing the overall H3K27ac enrichment signal and the enrichment signal rank of identified H3K27Ac enhancers, less than 5% of enhancers are identified as super enhancers (SEs) with GM12878 containing 741 SEs, MEC1 containing 374 SEs, and CD19+ B-cell containing 524 super SEs.
Figure 7. Comparison of super enhancers demonstrates a common overlap between all B cell types analyzed.

(A) Venn diagram reveals the overlap of SE genes occurring between GM12878, MEC1, and CD19+ B-cell. (B) Examples of super enhancers discovered within the three B-cell H3K27Ac ChIPseq profiles include super enhancers in CBX7, APOBEC3G, ENO2, SEPT9 and FGR.
**Common gene signatures of SE-associated genes exist in all CLL & normal B cells**

We next wanted to determine the particular gene ontology pathways enriched in each of the super enhancer gene subsets from MEC1, GM12878, and the CD19+ B cell. Based on investigation using the MSigDB gene hallmark sets, we see that the pathways significantly enriched in each of the super enhancer subsets are fairly common. As demonstrated in Figure 8A, we see that the top enriched pathway in all three super enhancer profiles is TNFA_SIGNALING_VIA_NFKB. This result is not incredibly surprising as it is well established that B cells rely on the immune-related NF-kappa B signaling for growth and proliferation (Herishanu and others 2011). We also observe other pathways related to immune regulation being enriched in the B cell super enhancer profiles including INTERFERON_GAMMA_RESPONSE, INFLAMMATORY RESPONSE, and ALLOGRAFT_REJECTION. These enriched pathways, however, are somewhat expected as B cells rely on immune-related stimulation to proliferate, specifically CLL cells who hijack these similar mechanisms to oncogenically expand.

However, it is also interesting to note that many of the pathways that overlap between the 3 B cell super enhancer profiles share certain molecular properties. Specifically, with the identification of pathways such as MTORC1_SIGNALING, FATTY_ACID_METABOLISM, HYPOXIA, CHOLESTEROL_HOMEOSTASIS, and OXIDATIVE_PHOSPHORYLATION being significantly enriched in super enhancer gene subsets, we recognized that genes involved in metabolic functions comprise a significant portion of super enhancer genes in B cells, suggesting that metabolic regulation plays a critical role in B cell maintenance and growth. This importance of metabolic regulation in B cells is reaffirmed when determining the percent distribution of
genes involve in general gene ontology (GO) biological processes. As Figure 8B shows, metabolic processes constitutes the largest percentage of biological processes in the super enhancer gene subsets identified by PANTHER, with over 25 percent of SE genes demonstrating some association with metabolism in each of the super enhancer profiles. Thus, pathway characterization of the SE genes demonstrates an integral connection between the enhancer landscape and metabolic regulation in B cells.
Figure 8. Ontology analysis of genes associated with SEs in MEC1, GM12878, and CD19+ B cell H3K27Ac ChIPseq.

(A) Pathway analysis for genes associated with MEC1, GM12878, and CD19+ B-cell was performed using the MSigDB database from gene set enrichment analysis (GSEA). Pathways shown for those significantly enriched in each cell type (FDR p-value < 0.05) and ranked based on what on significance. (B) Gene ontology analysis from PANTHER shows the percent distribution of SE genes involved in specific biological processes with metabolic processes comprising the largest percentage.
A subset of SE-associated genes are overexpressed in CLL patients

To understand how the super enhancer landscape corresponds with the gene expression patterns in B cells, we performed RNAseq on B cells from 47 CLL patient samples as well as 5 healthy donors and analyzed the transcript levels of genes associated with super enhancers. Based on established understanding from previous reports in other tissue types, we wanted to determine whether super enhancer-associated genes correlate with higher expression in B cells. As shown in Figure 9, the average transcript levels of genes associated with super enhancers are significantly higher than genes not associated with super enhancers in primary B cell samples, an observation consistent among all three B cell enhancer profiles (Loven and others 2013; Whyte and others 2013).

By establishing that B cell super enhancers overall correspond with higher gene expression in B cells, we then wanted to determine whether certain super enhancer genes are significantly overexpressed in CLL patient CD19+ B cells compared to normal CD19+ B cells from healthy donors. Depicted in Figure 10A, we performed an overlapping comparison of genes significantly overexpressed in 47 CLL patient RNAseq samples (Student’s t-test p-value<0.05) and genes associated with super enhancers in each of the 3 enhancer profiles. Based on this analysis, we observed that 74 MEC1 super enhancer genes, 139 GM12878 super enhancer genes, and 108 CD19+ B cell super enhancer genes were significantly overexpressed in CLL patients, and supervised hierarchical clustering analysis of these 3 CLL super enhancer expression profiles could separate the super enhancer expression profile of CLL patients from healthy donors (Figure 10A).
Because the B cell super enhancer profiles share many of the same genomic properties, we combined the super enhancer expression profiles and created a consolidated 190 gene CLL super enhancer expression profile (Figure 10B). We then ranked the average transcriptional abundance for each of the 190 CLL super enhancer genes and discovered that immune-regulatory genes like FCER2, CD27, and LCK as well as genes related to metabolic regulation like RXRA, FGR, and ENO2 have high transcriptional abundance in CLL B cells (Figure 10C). The enrichment of biological processes in the clinically overexpressed CLL super enhancer genes are represented by PANTHER analysis which demonstrates that cell-communication signaling and primary metabolic processes are the defining functional components of the super enhancer genes overexpressed in CLL (Figure 10D).
Figure 9. RNAseq transcript levels of SE genes compared to non-SE genes in primary B cells.

Box-and-whisker plots represent the average RNAseq read counts from 52 CLL and healthy B cell RNAseq profiles for genes associated with SEs and genes not mapped to SE regions. In each of the 3 SE profiles from MEC1, GM12878, and the C19+ B cell, SE genes are correspond with significantly higher expression than other genes throughout the mRNA transcriptome.
Figure 10. RNAseq analysis in CLL patients reveals overexpression of a subset of SE genes overexpressed in CLL.

(A) Venn diagrams and corresponding heatmaps demonstrate the overlap between SE associated transcripts in MEC1, GM12878, and CD19+ B cell and transcripts that are significantly overexpressed (t-test p-value < 0.05) in CLL patients (CLL patients = 47 vs. healthy donors = 5).  

(B) Among the 76 upregulated MEC1 SE genes, 139 upregulated GM12878 SE genes, and 108 upregulated SE genes, 190 unique SE genes are cumulatively upregulated in CLL patients compared to healthy B cells.  

(C) Gene rank graph of overexpressed CLL genes RNAseq count from the 47 analyzed CLL RNAseq profiles.  

(D) PANTHER histogram of processes significantly enriched in the 190 SE-associated genes.
**RPPA analysis demonstrates an underlying protein signature in CLL patients**

Based on the integration of information gathered from the super enhancer profile of B cells as well as the corresponding RNAseq expression profile from CLL patients, we then wanted to investigate whether the CLL expression signature at the protein level reflects patterns seen at the gene regulatory level. Thus, to gain a better understanding of the expression profile of CLL at the protein level, we collected 18 CLL patient and 6 healthy CD19+ B-cell lysates for RPPA analysis. The CLL lysates collected for this study are comprised of samples containing varying IGVH mutation, CD38 expression, FISH, ZAP70 expression, treatment, and Rai stage status in order to determine whether these clinical parameters demonstrate any large-scale protein signatures. From the collected array data, we gained an initial understanding of how the clinical features overlapped with the corresponding molecular signature determined by non-supervised hierarchical clustering. Based on the clustered dendrogram and the heatmap patterns, there seems to be a clear separation in molecular profiles between CLL patients and healthy donors samples, demonstrating an overall transformation at the protein level between the CLL and normal phenotype. However, the contrast between CLL patient subtypes is less stark as there seems to be no clear separation within the unsupervised hierarchical clustering patterns of the clinical subfeatures ascribed to the patient samples. Thus, unsupervised clustering would suggest that an underlying protein signature that distinguishes itself from normal CD19+ B-cells is common among CLL patient samples (Figure 11).

By further investigating the RPPA intensities of the antibody probes, we were able to determine through correlation comparison that eIF4G, SYK, RBM15, and GAB2 protein expression is higher in CLL than in healthy donor B-cells, whereas Annexin I and
cleaved caspase 7 is lower in CLL compared to healthy donors (Figure 12A). In fact, based on log-fold/rank comparison of all probes represented in the RPPA panel, the mRNA scaffolding protein eIF4G is the most upregulated protein in our CLL cohort, whereas the pro-apoptotic protein events such as Annexin I expression and cleaved-caspase 7 are the most downregulated events in CLL (Figure 12B). These extreme protein expression changes are examples that highlight the aberrant changes within the CLL protein signature as well as provide insight into specific mechanisms altered in CLL.
Figure 11. Unsupervised hierarchical in RPPA samples clustering separates CLL and normal B cells.

Non-supervised hierarchical clustering of normalized RPPA probe intensities using Euclidean distance. Heatmap demonstrates the separation of the CLL protein landscape and normal B-cell landscape without any statistical separation. Based on the clinical parameters, the altered protein expression is generally ubiquitous among CLL patients.
Figure 12. Comparative analysis of RPPA protein intensities in CLL and normal B cells samples.

(A) Scatter plot comparing the average intensity of all 167 probes in CLL versus the average probe intensity in healthy B-cells. (B) Rank ordering plot based on the transformed (log2) fold ratio between CLL expression and normal B-cell expression. Rank ordering plot demonstrates the contrast between eIF4G upregulation, diminished cleaved caspase-7, and Annexin I downregulation from other probes in the RPPA dataset.
RPPA reveals alteration in proteins involved in mTOR-mediated translational control

To systematically investigate the specific protein events that significantly correspond with the CLL phenotype, we further analyzed the protein arrays using ANOVA to determine which antibodies had significant differential probe intensities between CLL and normal B-cells samples. Based on this analysis, we determined that 58 distinct antibody probe intensities separate the CLL phenotype from the normal B-cell phenotype (Fold change > 1.25 or < -1.25, FDR p-value<0.05) with 38 probes being upregulated and 20 being downregulated in CLL. The differentially altered proteins and phosphoproteins identified include proteins that have previously been associated with CLL, specifically the spleen tyrosines kinase (SYK) (Hoellenriegel and others 2012), the LCK proto-oncogene (Talab and others 2013), and the Notch-1 signaling activator (Rosati and others 2009). Paradoxically, we also identified the pro-apoptotic proteins BIM and BAK to be upregulated in our RPPA dataset. Nevertheless, the protein profile demonstrated by RPPA analysis helps provide a larger frame of reference for the molecular pathology of CLL as BIM overexpression in CLL commonly correlates with the overexpression of its direct anti-apoptotic antagonist BCL2, thus counteracting pro-apoptotic function of BIM (Del Gaizo Moore and others 2007). A similar counteraction also occurs for the pro-apoptotic functions of BAK due to the fact that its pro-apoptotic binding partner BAX is significantly downregulated in CLL patients (Pohland and others 2006). By identifying protein expression patterns of BIM, BCL2, BAK, and BAX as well as identifying the inhibitory phosphorylation of pro-apoptotic BAD at serine 112 (Hayakawa and others
RPPA analysis helps provide a comprehensive illustration of the molecular characteristics in CLL.

Supervised hierarchical analysis using ANOVA further revealed differential expression of several noteworthy proteins, including BRAF, STAT5A, and the Wnt pathway-related protein DVL3. Nonetheless, many of the proteins differentially altered in our array dataset are specifically involved in the AKT/mTOR signaling pathway. This signaling cascade is highly important in several types of cancers due to the fact that AKT and mTOR regulate numerous tumorigenic events including cell proliferation, apoptotic resistance, autophagy (Annovazzi and others 2009), cell cycle progression (Rassidakis and others 2005), nutrient-dependent growth (Whiteman and others 2002), and protein translation initiation (Gingras and others 1998). Interestingly, the expression of total AKT and total mTOR were both significantly upregulated in CLL compared to healthy donor B cells. These two events observed in our CLL samples are specifically notable, as upregulation of other kinases at the protein level have previously been identified in CLL including the critical PI3Kδ and BTK kinase proteins (Herman and others 2011; Herman and others 2010). We also identified overexpression of scaffolding proteins that are traditionally involved in the AKT/mTOR pathway including IRS1, RICTOR, and GAB2, which have been implicated as oncogenic contributors in other cancer types (Bocanegra and others 2010; McDonald and others 2008; Porter and others 2013). With overexpression of these AKT/mTOR-related signaling proteins, we also observed higher phosphoprotein levels for PDK1 at serine 241, 4E-BP1 at serine 65 and threonine 70, p70S6K at threonine 389, BAD at serine 112, and PRAS40 at threonine 246 in CLL samples as compared to normal donors (Figure 13A-B).
To help determine the specific molecular mechanisms that are altered in CLL, we interrogated the total proteins from the differentially expressed probes in the RPPA dataset and observed overrepresentation of signaling events regulating the PI3K/AKT pathway, with pathways specifically involved in the mRNA translation machinery of eukaryotic cells. Overrepresentation of these pathways biologically corresponds with the phosphorylation of the translation regulator 4E-BP1 at serine 65 and overexpression of eIF4G, AKT, mTOR, and SF2/ASF, a splicing protein recently implicated as a mTOR-dependent translational regulator (Karni and others 2008; Michlewski and others 2008). The upregulation of these protein alterations therefore highlights the possibility of mRNA translation as a tumorigenic mechanism in CLL (Figure 14).
Figure 13. Hierarchical clustering analysis reveals preferential enrichment of mTOR-related proteins upregulated in CLL.

(A) Supervised hierarchical clustering analysis of RPPA results using an ANOVA FDR p-value threshold lower than 0.05 and a fold difference threshold of greater than 1.25 and less than -1.25. Based on this threshold, 58 probes were differentially altered in CLL with 38 probes being upregulated (along red bar) and 20 probes being downregulated (along blue bar). (B) Ontology analysis of the differently expressed probes using Ingenuity Pathway Analysis (IPA).
Figure 14. Western blot validation of proteins involved in the regulation of mRNA translation.

Western blot panel shows that total AKT, mTOR, SF2/ASF, and eIF4G are upregulated at the protein level, and 4E-BP1 is hyperphosphorylated at serine-65. The upregulation of these events indicate a reliance on mRNA translation in CLL based on the role these proteins serve as well as the observation that these proteins are not overexpressed at the gene transcript level (data not shown).
mTOR-dependent 4E-BP1 phosphorylation is uncoupled from AKT activation

We then compared the distinct phosphorylation events of the AKT/mTOR pathway to reveal the overall phosphorylation patterns of AKT/mTOR-related substrates in CLL. By normalizing the phosphoprotein levels to the internal levels of the corresponding total protein, we observed 4E-BP1 at serine 65 had higher phosphorylation levels in CLL compared to healthy donors. However, the high phosphorylation at this site did not correspond with other AKT/mTOR related phosphorylation sites, including AKT threonine 308, AKT serine 473, p70S6K threonine 389, and mTOR serine 2448, which were all relatively hypophosphorylated. These phosphorylation patterns are quite interesting due to the fact that 4E-BP1 phosphorylation typically is dependent upon AKT phosphorylation (Gingras and others 1998) (Figure 15A-B).

Because these B cells were obtained from the peripheral blood and significant phosphorylation of AKT typically occurs through microenvironment interactions in the lymph node, we wanted to determine how AKT activation compares with 4E-BP1 serine 65 phosphorylation during ex vivo stimulation. To accomplish this feat, we performed a side-by-side activation of CLL B-cells cells and healthy donor B-cells with 10ug/ml anti-IgM for 1, 2, and 4 hours to determine the precise AKT threonine 308/4E-BP1 serine 65 phosphorylation pattern in CLL. Based on our results, AKT phosphorylation was higher in both CLL and normal B-cells during IgM stimulation. However, where healthy donors demonstrated increased phosphorylation of 4E-BP1 during IgM stimulation, CLL cells demonstrated consistently high and unchanging levels of 4E-BP1 phosphorylation, regardless of AKT activation. Based on the results, it appears 4E-BP1 serine 65 phosphorylation in normal B cells is dependent upon AKT phosphorylation, whereas 4E-
BP1 sustains a constant phosphorylated state independent of AKT activation in CLL B cells. This result would suggest that a disconnect takes place between AKT activation and 4E-BP1 phosphorylation during CLL transformation (Figure 15C).
Figure 15. 4E-BP1 phosphorylation is maintained independently of Akt activation in CLL cells.

(A) Normalized phosphorylation fold changes of AKT/mTOR substrates in CLL samples from RPPA (error bars=95% CI). (B) Paired comparison of CLL samples demonstrating the disconnection between AKT T308 phosphorylation and 4E-BP1 serine 65 phosphorylation. (C) IgM activation of CLL sample 8816 and normal donor SC30. 4E-BP1 seems to be constitutively phosphorylated in CLL regardless of AKT pT308 activation, whereas the phosphorylation of 4E-BP1 in normal B-cells is dependent upon IgM-mediated AKT activation.
The dual PI3K/mTOR inhibitor NVP-BEZ235 causes greater apoptosis than BCR pathway inhibitors by disrupting 4E-BP1 & eIF4G

With the gained understanding of 4E-BP1 phosphorylation and eIF4G overexpression in CLL, we wanted to test whether targeting the effects of these proteins would affect CLL viability and survival. With this rationale in mind, we compared the inhibitory capability of the BTK inhibitor Ibrutinib and the PI3Kδ inhibitor Idelalisib against the NVP-BEZ235, a dual PI3K/mTOR kinase inhibitor that has demonstrated ability to attenuate both 4E-BP1 phosphorylation and eIF4G expression in AML (Chapuis and others 2010).

To compare these three inhibitors, we first performed an IC50 proliferation assay for each of the three inhibitors in the MEC1 CLL cell line. From this experiment, we determined NVP-BEZ235 to have a lower IC50 (1.482nM) than the other two inhibitors in MEC1 (Figure 16A). We then co-cultured 15 CLL patient B-cells with Huh7.5 cells, an adenocarcinoma cell line that provides a protective microenvironment for CLL viability and treated the primary cells with each inhibitor for 48 hours at their maximum effective doses of 10uM. Based on Annexin V/DAPI measurements, NVP-BEZ235 was able to significantly lower cell viability compared to Ibrutinib and Idelalisib. This lowered viability correlated with a potent induction of apoptosis in CLL cells as NVP-BEZ235 caused a significant increase in Annexin V+/DAPI+ cell death compared with side-by-side treatment of Ibrutinib and Idelalisib (Figure 16B).

We then wanted to determine if any differential 4E-BP1 and eIF4G alterations occurred when treating CLL cells with the three inhibitors. To observe any differential effects, we co-cultured CLL cells with HS-5 stromal cells and performed a side-by-side treatment with Ibrutinib, Idelalisib, and NVP-BEZ235. After 6 hours, we did observe a
significant difference in 4E-BP1 phosphorylation levels when comparing the three treatments as NVP-BEZ235 significantly diminished 4E-BP1 serine 65 phosphorylation compared to Ibrutinib and Idelalisib, both of which caused little change in 4E-BP1 phosphorylation (Figure 17).

Interestingly, this decrease in 4E-BP1 phosphorylation also correlated with greater cleavage of the eIF4G protein. This event previously characterized in immortalized lymphoma cell lines treated with chemotherapy, currently has not been characterized in clinical CLL samples. However, by treating CLL patient B cells with the 3 inhibitors, we observed the lowest levels of the 220kDa eIF4G expression with NVP-BEZ235 treatment, while simultaneously observing increased detection of the protein fragments that correspond in size with the previously identified C-terminal fragment of apoptotic cleavage of eIF4G (C-FAG) (Bushell and others 2000). This observation implies that the mRNA translation is ultimately disrupted during apoptotic induction due to the enhanced apoptotic cleaving of the eIF4G translation initiator protein. Finally, we collectively compared the semi-quantitative 4E-BP1 phosphorylation and eIF4G cleavage levels with the Annexin V+/DAPI+ cells in each treatment and determined that greater apoptotic induction corresponds with greater 4E-BP1 dephosphorylation and eIF4G cleavage, suggesting that attenuating the protein events that upregulate mTOR-dependent translational mechanisms can impede cell survival in CLL (Figure 17).
Figure 16. The dual PI3K/mTOR inhibitor NVP-BEZ235 induces apoptosis in CLL cells.

(A) Dose-dependent treatment analysis of Ibrutinib, Idelalisib, and NVP-BEZ235 in the MEC1 CLL cell line to compare the IC50 for each inhibitor. (B) Annexin V/DAPI apoptotic assay comparing 10uM Ibrutinib, 10uM Idelalisib, and 10uM NVP-BEZ235 treatment in CLL cells co-cultured with Huh7.5 cells (n=15). Based on the results, NVP-BEZ235 was able to cause more apoptotic death during the 48hr period.
Figure 17. NVP-BEZ235 causes differential effects on 4E-BP1 phosphorylation and eIF4G cleavage compared to BTK and PI3Kδ inhibition.

At 6 hours, 10uM NVP-BEZ235 causes greater dephosphorylation of 4E-BP1 as well as greater cleavage of eIF4G in HS-5 co-cultured CLL cells compared to 10uM Ibrutinib and 10uM Idelalisib. Decreasing 4E-BP1 phosphorylation and increasing eIF4G cleavage correspond with higher apoptotic death in primary CLL samples (errors bars=S.E.M.).
**BET bromodomain inhibitor JQ1 disrupts CLL cell line proliferation via cell cycle arrest**

With an established understanding of the super enhancer-mediated transcription landscape and the corresponding findings from our RPPA expression profile, we then wanted to understand how newly developed transcription inhibitors that demonstrate preferential activity towards super enhancers affect the molecular profile of CLL cells. To study this particular direction in therapeutic targeting for CLL, we tested the therapeutic response of the BET bromodomain inhibitor JQ1 in the MEC1 and MEC2 CLL cell lines. We observed that JQ1 has an IC$_{50}$ value of 0.4979uM and 0.168uM in MEC1 and MEC2, respectively (Figure 18A) and can suppress proliferation of MEC1 and MEC2 in a time-dependent and dose-dependent manner (Figure 18B). This suppression in cell proliferation appears to be mediated by G$_1$-cell cycle arrest as demonstrated by DAPI-cell cycle analysis after 72 hours (Figure 18C). However, the cell cycle arrest appears does not correspond with cell apoptosis in these cell lines as a negligible increase in Annexin V/DAPI+ cells occurs in JQ1-treated MEC1 and MEC2 cells after 72 hours (Figure 18D). These results would indicate that JQ1 may preferentially disrupt the transcription of genes responsible for driving CLL proliferation compared to genes responsible for maintaining CLL survival.
Figure 18. JQ1 disrupts MEC1 and MEC2 cell proliferation.

(A) JQ1 has an IC50 below 1μM in both CLL cell lines MEC1 & MEC2. (B) JQ1 inhibits MEC1 and MEC2 proliferation in a dose-dependent and time dependent manner. (C-D) Cell cycle and Annexin V/DAPI analysis demonstrates that THZ1 disrupts cell-cycle progression while causing little apoptotic induction in MEC1 & MEC2.
Transcriptional profile reveals that JQ1 effects mRNA transcription of immune regulatory genes

To determine how the transcriptional landscape is altered in CLL cells by bromodomain inhibition, we treated MEC1 and MEC2 cells with 1uM JQ1 and performed RNAseq-based expression analysis at isolated time points over a 24-hour period. Unsupervised hierarchical clustering of the JQ1-treated cell lines displays a time-dependent pattern of transcriptional downregulation for a subset of genes (Figure 19A). Based on the global expression pattern in the RNAseq profile, we then wanted to determine the specific subset of genes sensitive to JQ1 treatment in MEC1 and MEC2. We defined JQ1-sensitive transcripts by filtering transcripts that had a fragments-per-kilobase-per-million value difference (ΔFPKM) greater than 5 when subtracting each time point from the reference 0-hour time point. These transcripts were then additionally filtered by selecting transcripts with a fold difference lower than 0.5 when comparing the 24-hour time point to the 0-hour time point. This particular method of filtering was applied in order to determine transcripts that are: (a) abundantly transcribed, (b) consistently downregulated over time, and (c) greatly diminished by bromodomain inhibitor treatment.

Based on the described method of filtering, we identified 209 genes as JQ1-sensitive in MEC1 (approximately 1.5% of live transcripts in MEC1) and 537 genes as JQ1-sensitive in MEC2 (approximately 3.7% of live transcripts in MEC2). MSigDB Gene Hallmark analysis of these JQ1-sensitive transcripts reveals that a significant representation of the downregulated transcripts in both MEC1 and MEC2 are involved in immune signaling mechanisms including TNFA_SIGNALING_VIA_NFKB, INFLAMMATORY_RESPONSE, and IL2_STAT5_SIGNALING (Figure 19B). A
ranking of the JQ1-sensitive transcripts based on fold ratios reveals that several of the more JQ1-sensitive transcripts have some role in immune regulatory functions including \textit{LCK}, \textit{PDCD1}, \textit{FCER2}, and \textit{CXCR4} in MEC1 and \textit{NFKB1}, \textit{CCR7}, \textit{FCER2}, and \textit{NOTCH1} in MEC2 (Figure 19C). We verified several of the identified transcripts suppressed by JQ1 such as seeing protein downregulation of LCK in MEC1 cells (Figure 20A) as well as downregulation of \textit{LCK}, \textit{RXRA}, \textit{PDE4A}, and \textit{BTK} in primary patient samples (Figure 20B). We also validated the downregulation of PD-1 (protein encoded by \textit{PDCD1}) in MEC1 cells after treated with 1uM JQ1 for 72 hours. This result is quite intriguing based on previous reports that show that regulatory B cells can increase levels of the cytokine IL-10 by activating PD-1 with the PDL1 ligand (Xiao and others 2016). To see if we could both mimic this result in MEC1 cells as well as suppress the PD-1/PDL1-mediated production of IL-10 through JQ1, we stimulated MEC1 cells with 10ug/mL of a PDL1 ligand and treated the stimulated cells with 1uM JQ1. We observed that not only did IL-10 production increase with PD-1 stimulation, but also production of IL-10 was suppressed when MEC1 cells were cultured with both PDL1 and 1uM JQ1 for 72 hours (Figure 20C). These results demonstrate the pleiotropic effects of JQ1 on CLL cells.
Figure 19. RNAseq expression profile of MEC1 and MEC2 treated over time with 1uM JQ1.

(A) Unsupervised heat map of expression changes occurring in MEC1 and MEC2 when treated time-dependently with 1uM JQ1. (B) MSigDB pathway enrichment of genes downregulated by THZ1 treatment (downregulation=$\Delta$FPKM > 5 at each time point; fold change < 0.5 after 24 hours) (C) Rank plot of genes downregulated by 1uM JQ1 in MEC1 and MEC2.
Figure 20. The effects of JQ1 treatment are validated at mRNA and protein level. (A) Western blot validates that the protein expression of LCK is decreased by 1uM JQ1 treatment after 72 hours. (B) qRT-PCR validates the downregulation of JQ1-sensitive transcripts in primary CLL patient samples (n=3). (C) PD-1, a JQ1 sensitive transcript, is downregulated at the protein level by 1uM JQ1 after 72 hours. This result is coupled with the observation that PDL1/PD-1 engagement that upregulates IL-10 production is suppressed by 1uM JQ1 after 72 hours.
**JQ1 suppresses expression of clinically relevant CLL genes**

By establishing an expression profile for MEC1 and MEC2 cells treated with JQ1, we then wanted to determine which of the JQ1-sensitive transcripts are overexpressed in CLL patients. To determine the clinical connection of JQ1-sensitive transcripts, we constructed a Venn diagram revealing the MEC1 JQ1-sensitive transcripts and MEC2 JQ1-sensitive transcripts that overlapped with genes significantly overexpressed in our CLL patient RNAseq profile. Figure 20 reveals that of the 209 JQ1-sensitive transcripts in MEC1 and 537 JQ1-sensitive transcripts in MEC2, 23 transcripts from MEC1 and 38 transcripts from MEC2 are significantly overexpressed in CLL patients. As depicted by the RNAseq profile from Figure 19, many of the clinically significant genes deemed sensitive to JQ1 are related to immune function including *LCK*, *FAIM3*, and *PDCD1* from MEC1 and *NFAT5*, *CCR7*, *TRAFl* in MEC2 (Figure 20A).

Nevertheless, from the 23 transcripts in MEC1 and 38 in MEC2, *FCER2* is surprisingly the only clinically significant transcript that overlaps between both cell lines (Figure 21A). We validated this downregulation of CD23 at the protein level in both cell lines by flow cytometry (Figure 21B). This particular observation is interesting to note due to fact that CD23 expression is considered both an indicator of B cell activation as well as a canonical cell surface marker that differentially defines the presence of CLL progression in the peripheral blood (Erlanson and others 1998; Gibson and others 1989). It is also interesting to note that MEC1 and MEC2, which were derived at different stages from the same CLL patient (MEC1 at initial presentation and MEC2 at relapse) (Rasul and others 2014; Stacchini and others 1999), have different gene expression identities and respond differently to bromodomain inhibition at the transcriptional level. Such a result...
has potentially profound impact concerning the disruption of CLL progression based on the idea that CLL cells can heterogeneously utilize different transcriptional responses to enable CLL proliferation. Therefore, bromodomain inhibition through JQ1 could unbiasedly disrupt different subsets of transcriptional targets that are critical for specific subclones of CLL cell populations.
Figure 21. Genes overexpressed in CLL patients are suppressed by JQ1.

(A) Venn diagram showing overlap between genes downregulated by JQ1 and genes significantly overexpressed in CLL patients. MEC1 and MEC2 have noticeably different targets downregulated by JQ1, with FCER2 (CD23) being the only common transcript downregulated in both cell lines. Many of the targets, however, are involved in immune regulatory functions. (B) Flow cytometry demonstrating the loss of CD23 expression in both MEC1 and MEC2 when treated with JQ1 for 24 hours.
CDK7 inhibitor THZ1 disrupts CLL viability by inducing apoptosis

Based on the inhibitory effects elicited by bromodomain inhibition in CLL cells, we then wanted to determine whether the covalent CDK7 inhibitor THZ1 could demonstrate a similar inhibitory pattern. We first performed a cell viability experiment comparing the IC\textsubscript{50} values of THZ1 in MEC1 and MEC2 cells against CR8 and flavopiridol, two other cyclin-dependent kinase-based transcriptional inhibitors previously studied in CLL cells (Chen and others 2005; Cosimo and others 2013). By calculating the IC\textsubscript{50} values for the analyzed CDK inhibitors, we observed that THZ1 had a noticeably lower IC\textsubscript{50} in both MEC1 and MEC2 (7.23nM and 7.35, respectively) when compared to flavopiridol and CR8 (Figure 22A). This effective dose at lower nanomolar concentrations is also reflective in cell proliferation assays, which demonstrate the time and dose dependent effects of THZ1 in MEC1 and MEC2 (Figure 22B).

To determine the means in which cell proliferation is suppressed in MEC1 and MEC2 cells, we performed cell cycle analysis and observed that sub-G\textsubscript{1} cell cycle arrest occurs dose-dependently in THZ1-treated CLL cell lines after 24 hours. This result in cell cycle arrest was coupled with a significantly large increase in Annexin V+/DAPI+ cells in THZ1-treated MEC1 and MEC2 cells after 24 hours, indicating a large induction of cell apoptosis (Figure 22C). This induction of apoptosis in THZ1-treated MEC1 and MEC2 differ from JQ1-treated MEC1 and MEC2, which undergo G\textsubscript{1}-cell cycle arrest without inducing apoptosis.

We then wanted to understand whether reduction in cell viability of MEC1 and MEC2 cells are reflected in primary CLL samples. Using a resazurin-based assay, we assessed the viability of CLL patients PBMCs (n=7) treated \textit{in vitro} with increasing
concentrations of THZ1 and observed a dose-dependent reduction in cell viability after 24 hours (Figure 23A). It also appears that CLL PBMCs are significantly more sensitive to THZ1 compared to healthy donor PBMCs (n=3) when treated with the same concentrations for 48 hours (Figure 23B). The reduction in cell viability was further characterized by a significant increase of apoptosis in CLL B-cells treated with THZ1 in vitro for 24 hours (Figure 23C). It is also interesting to note that THZ1 could also overcome the protective effects of HS-5 stromal cell co-culture and significantly reduce viability of CLL B cells co-cultured in this particular microenvironment for 24 hours when compared to DMSO and fludarabine-treated CLL B cells (Figure 23D).

In terms of validating whether THZ1 is directly affecting the activation of RNA polymerase II in CLL cells, we treated MEC1, MEC2, and two primary CLL patient B cell samples with 50nM and 100nM THZ1 at 6 and 24-hour time points to detect the phosphorylation levels of RNA polymerase II using Western blot. Depicted in Figure 24, we saw that THZ1 caused a striking reduction in both RNAPII Ser2 and Ser5 phosphorylation in each of the 4 samples analyzed at the specified time points. This sharp reduction in RNAPII phosphorylation ultimately indicates RNAPII activity as a primary molecular mechanism targeted by THZ1 in CLL cells.
Figure 22. CDK7 disrupts MEC1 and MEC2 cell growth.

(A) Compared to other known cyclin-dependent transcriptional inhibitors, THZ1 demonstrates greater sensitivity in CLL cell lines MEC1 & MEC2. (B) THZ1 inhibits MEC1 and MEC2 proliferation in dose-dependent and time dependent manner. (C) Cell cycle and Annexin V/DAPI analysis demonstrates that THZ1 disrupts cell-cycle progression and induces apoptosis in MEC1 & MEC2.
Figure 23. Primary CLL samples are sensitive to THZ1 treatment *ex vivo*.

(A) THZ1 reduces cell viability in primary CLL PBMCs (n=7) in a dose-dependent manner after 24 hours.  (B) After 48 hours, CLL PBMCs (n=7) demonstrate greater sensitivity to THZ1 than healthy donor PBMCs (n=3).  (C) THZ1 induces apoptosis in primary CLL cells (n=9).  (D) 50nM THZ1 induces higher levels of apoptosis in CLL cells co-cultured in HS-5 stromal cells compared to 2.5uM of fludarabine (Fluda).
Figure 24. RNA polymerase II phosphorylation is disrupted by THZ1 in vitro.

THZ1 is capable of disrupting the phosphorylation of the carboxyl terminal domain (CTD) of RNA polymerase II in CLL cell lines and primary CLL cells. THZ1 inhibits CDK7 and subsequently suppresses phosphorylation of RNAPII CTD Ser5, which is the first phosphorylation step in RNAPII processing. RNAPII CRD Ser2 loss of phosphorylation is an indirect consequence of disrupting CDK7-mediated Ser5 phosphorylation.
**THZ1 disrupts expression of genes regulating metabolic processes**

Based on the in vitro effects of THZ1 CLL survival in both CLL cell lines and primary CLL B cells, we then wanted to determine the specific expression signatures that are disrupted in CLL cells when treated with THZ1. To investigate the suppressive of THZ1 at the transcriptional level, we conducted a time course RNAseq expression comparison of both MEC1 and MEC2 cells treated with 50nM THZ1 over a 12-hour period. As seen in Figure 25A, unsupervised heatmaps of all active transcripts in both MEC1 and MEC2 demonstrate a subset of gene transcripts being increasingly downregulated over the 12-hour period. As conducted with the JQ1 RNAseq treatment experiments in Figure 19, THZ1-sensitive transcripts were determined by filtering transcripts that had a ΔFPKM greater than 5 at each time point when subtracting from the 0-hour reference. These transcripts were then additionally filtered by selecting transcripts with a fold difference lower than 0.5 when comparing the 24-hour time point to the 0-hour reference. Based upon this filtering method, we determined that 728 transcripts were sensitive to THZ1 in MEC1 (approximately of 5.2% of active MEC1 transcripts), and 647 transcripts were sensitive to THZ1 in MEC2 (approximately 4.5% of active MEC2 transcripts).

In contrast to pathways significantly enriched in JQ1-sensitive transcripts from Figure 19B, many of the pathways significantly enriched in THZ1-sensitive transcripts are specifically involved in metabolic regulation. Some of these pathways from the MSigDB Gene Hallmarks enriched in the THZ1-sensitive transcripts included HYPOXIA, MTORC1_SIGNALING, FATTY_ACID_METABOLISM, GLYCOLYSIS, and OXIDATIVE_PHOSPHORYLATION (Figure 25B). In particular, when analyzing the entire THZ1-expression signature for both MEC1 and MEC2 at 8 hours using Gene
Set Enrichment Analysis (GSEA), we discovered that two of the most significantly
enriched gene sets involve hypoxia induction and HIF1a activation gene expression
signature (Figure 25C) (Elvidge and others 2006).

Several of the metabolic transcripts sensitive to THZ1 treatment were validated
using quantitative RT-PCR. Examples of these transcripts include glycolytic enzymes
such as SLC2A1, SLC2A3, HK2, ENO2, and PKFKB4 as well as glycine-serine
metabolism enzymes such as PHGDH and PSAT1 (Figure 26A). Also, to test whether the
downregulation of these genes were just a general consequence of CDK inhibitor
treatment, we compared the transcript levels of THZ1-sensitive metabolism genes against
cells treated with varying levels of flavopiridol and observed that the analyzed transcript
were more affected in THZ1 treated cells than in flavopiridol treated cells (Figure 26B).
Finally, with mTORC1 signaling being both a major MSigDB Gene Hallmark pathway
disrupted in THZ1-treated cells and an enriched pathway in our RPPA profile, we wanted
to determine whether mTOR phosphorylation events could be diminished by THZ1. As
Figure 26C shows, mTORC1 substrates 4E-BP1 and p70S6K were hypophosphorylated
when treated with increasing concentrations of THZ1 over time. Along with mTORC1-
dependent phosphorylation events being decreased, we also observed that the
metabolically sensitive AKT Ser473 phosphorylation had essentially been fully repressed
by THZ1 treatment in both MEC1 and MEC2. The culmination of these results indicates
that transcriptional repression of THZ1-sensitive transcripts disrupts several core
molecular mechanisms that sustain the metabolic processes in CLL cells.
Figure 25. RNAseq expression analysis reveals disruption of metabolic-related expression signatures in THZ1-treated MEC1 and MEC2.

(A) Unsupervised heat map of expression changes occurring in MEC1 and MEC2 when treated time-dependently with 50nM THZ1. (B) MSigDB pathway enrichment of genes downregulated by THZ1 treatment (downregulation=ΔFPKM > 5 at each time point; fold change < .5 after 12 hours) (C) Gene Set Enrichment Analysis (GSEA) of RNAseq treatment profile after 8 hours. THZ1 treatment significantly correlates with disruption of HIF1A/hypoxic response.
Figure 26. THZ1 disrupts glycolysis gene expression and Akt/mTOR-dependent signaling events.

(A) Quantitative RT-PCR validation of genes affected by THZ1 in RNAseq profile of MEC1 and MEC2. (B) Quantitate RT-PCR comparison of THZ1 and Flavopiridol (FP) treatment in MEC1 shows specific sensitivity of genes affected by THZ1 after 6 hours. (C) Western blot showing that THZ1 downregulated metabolically related AKT/mTOR phosphorylation events including 4E-BP1 Ser65, p70S6K Thr389, and AKT Ser473 phosphorylation.
THZ1 renders CLL cells incapable of inducing glycolysis

Based on the transcriptional and signaling events THZ1 disrupts in CLL cells, we then wanted to determine whether certain phenotypic outcomes would match with the corresponding molecular targets. Based on observations in the THZ1-treated CLL cell lines, we know that several genes suppressed by THZ1 are involved in glycolytic metabolism. Thus, we wanted to determine whether CDK7 inhibition could ultimately suppress the glycolytic response by measuring glucose uptake and lactate production in THZ1-treated cells. As shown in Figure 27A, we tested the effects of THZ1 on glucose uptake in MEC1 and MEC2 cells by incorporating the glucose analog 2-[(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) into media containing low amounts of glucose. 2-NBDG is an effective dye for measuring relative levels of glucose uptake due to the fact that cells will fluoresce when incorporating 2-NBDG into the cell from the surrounding environment. We treated MEC1 and MEC2 cells for 2 hours with 50nM THZ1, measured the relative levels of 2-NBDG within the cells, and observed that THZ1 was capable of repressing glucose uptake within a relative short time frame. This repression in glucose uptake also corresponded with repression in glycolytic output as production of the end product of glycolysis, L-lactate, is obstructed by THZ1 treatment. Illustrated in Figure 27B, production of L-lactate is marginally stunted in a dose dependent manner after 6 hours in both MEC1 and MEC2. However, this obstruction of L-lactate production by dose-dependent concentrations of THZ1 is greatly amplified when measured after 24 hours of treatment, suggesting that THZ1 can immediately and sustainably block glycolytic production in CLL cell lines.
Based on the ability of THZ1 to disrupt glycolytic production in CLL cell lines, we then wanted to determine whether THZ1 could disrupt glycolytic metabolism in primary CLL cells. Established by previous reports concerning metabolism in CLL, we understand that CLL cells are metabolically plastic in their abilities to provide energy for growth and survival (Jitschin and others 2015). One of the means in which CLL cells demonstrate their metabolic plasticity is by their readied ability to upregulate glycolysis-based metabolism when under hypoxic conditions (Koczula and others 2016). Therefore, in line with this established mechanism in primary CLL cells, we wanted to determine whether THZ1 could disrupt hypoxia-mediated glycolytic induction. We tested the effects of THZ1 in CLL cells under hypoxic conditions by culturing DMSO or 50nM THZ1-treated CLL B cells (n=3) either under normoxic or hypoxic (1% O₂) conditions for 16 hours and measured the levels of L-lactate for each condition. As shown in Figure 28A, we see that while no accumulation of L-lactate was detected in either the DMSO or THZ1 treated cells when under normoxic conditions, significant reduction in L-lactate production occurred in primary CLL cells treated with THZ1 under hypoxic conditions compared to the patient matched DMSO-treated primary CLL cells. This inhibition of hypoxia-driven glycolytic induction is coupled with the halted induction of glycolytic enzyme gene expression when primary cells were treated with 50nM THZ1 for 12 hours under hypoxic conditions (Figure 28B).
Figure 27. THZ1 inhibits lactate production and glucose uptake in MEC1 and MEC2.

(A) 2-NBDG incorporation shows that after 2 hours, THZ1 can mitigate glucose uptake in both MEC1 and MEC2 compared to non-treated cells. (B) L-lactate is marginally lower at 6 hours in MEC1 and MEC cells dose-dependently treated with THZ1. The levels of L-lactate become more markedly suppressed in THZ1-treated MEC1 and MEC2 when measured at 24 hours.
Figure 28. THZ1 inhibits glycolytic induction of primary CLL B cells cultured under hypoxia.

(A) L-lactate production assay shows that 50nM THZ1 can markedly disrupt lactate production in primary CLL cells (n=3) when under hypoxic conditions for 16 hours. (B) Quantitative RT-PCR shows expression of hypoxia-inducible genes in primary CLL cells are suppressed when treated with 50nM THZ1 for 12 hours.
**THZ1 disrupts expression of clinically relevant genes associated with metabolism**

By establishing a signature of THZ1-sensitive transcripts from MEC1 and MEC2 cells as well as determining the functional consequences of THZ1 treatment in CLL cells, we then wanted to determine the specific THZ1-sensitive transcripts overexpressed in CLL patients. To determine the clinical connection of THZ1-sensitive transcripts, we constructed a Venn diagram for both MEC1 THZ1-sensitive transcripts and MEC2 THZ1-sensitive transcripts that overlapped with genes deemed significantly overexpressed from our CLL patient RNAseq profiles. Figure 28 shows that 34 of the 728 JQ1-sensitive transcripts in MEC1 and the 53 of the 647 THZ1-sensitive transcripts in MEC2 are significantly overexpressed in CLL patients. Many of the clinically overexpressed genes deemed sensitive to THZ1 are related to metabolic function. In fact, as demonstrated, we see that “metabolic processes” comprises the largest percentage of ontology classes within the clinically associated THZ1-sensitive transcripts based on PANTHER characterization (25.8% for MEC1 and 23.4% for MEC2) (Figure 29).

Examples of these transcripts involved in metabolism include glycolytic related transcripts such as the *PKFP* phosphofructokinase-plate isoform and the *EN02* enolase-gamma isoform. There are also clinically related THZ1 transcripts involved with lipid metabolism like the cholesterol esterification enzyme *LCAT* and the acyl-CoA dehydrogenase enzyme *ACAD11*. Nevertheless, from the 34 transcripts in MEC1 and 53 in MEC2, two of the three the clinically significant transcripts that overlap between both cell lines are involved in regulating glucose levels (*PP1R3E* and *ENO2*), a result that further highlights the foundational importance of maintaining metabolism in CLL.
Figure 29. THZ1 disrupts a subset of genes overexpressed in CLL patients.

(A) Venn diagram showing overlap between genes downregulated by THZ1 and genes significantly overexpressed in CLL patients. MEC1 and MEC2 have different targets downregulated by THZ1, though ENO2, PPP1R3E, and IZUMO4 transcript levels are downregulated in both cell lines. Based on percent distribution of gene ontology using PANTHER, many of the targets downregulated by THZ1 are involved in metabolic processes.
**JQ1 & THZ1 disrupt different sets of CLL SE-associated genes**

Through our comprehensive process of defining the B cell super enhancers associated with gene overexpression in CLL, the transcriptional targets sensitive to bromodomain inhibition by JQ1, and the transcriptional targets sensitive to CDK7 inhibition by THZ1, we finally wanted to determine which transcripts disrupted by JQ1 and THZ1 reflect the clinically overexpressed CLL transcripts driven by B cell super enhancers. To determine this particular subset of super enhancer-mediated transcripts sensitive to JQ1 and THZ1, we performed a Venn diagram comparison of the 190 identified CLL super enhancer genes and determined which of these transcripts are downregulated by either THZ1 or JQ1 within the tested MEC1 and MEC2 cells. Figure 30 demonstrates that out of the 190 CLL super enhancer genes, 11 transcripts in MEC1 and 11 transcripts in MEC2 were downregulated by JQ1. Transcripts identified in this overlap include the proto-oncogene *LCK*, the retinoid-x-receptor *RXRA*, and the histone deacetylase *HDAC7* from MEC1 as well as the NF-kappaB cofactor *TRAF1*, the polo-like kinase *PLK3*, and the pro-tumorigenic microRNA host gene *MIR155HG* in MEC2. For the CLL super enhancer genes sensitive to THZ1, 14 transcripts were downregulated in MEC1 and 16 downregulated in MEC2. These identified transcripts include the Polycomb Repressive Complex 1 (PRC1) protein *CBX7*, the Wnt pathway ligand *WNT10A*, and the cytidine deaminase *APOBEC3G* in MEC1 as well as the oncogenic associated non-coding RNA *MALAT1*, the memory B cell marker *CD27*, and the lipoprotein receptor *LSR* in MEC2. Interestingly, we identified only two overlapping CLL super enhancer targets effectively suppressed by both JQ1 and THZ1, which were the highly expressed Fc fragment of the IgM receptor gene *FAIM3* in MEC1 and the highly expressed chemokine receptor gene...
Nevertheless, from the 22 JQ1-sensitive and 30 THZ1-sensitive CLL super enhancer transcripts divided among MEC1 and MEC2, we observed that the underlying super enhancer gene suppressed by JQ1 in both MEC1 and MEC2 was the CD23 gene $\text{FCER2}$, whereas the two underlying super enhancer genes suppressed by THZ1 in both MEC1 and MEC2 was the glycolytic enolase gene $\text{ENO2}$ and the IZUMO family gene $\text{IZUMO4}$. The discovery that these sensitive-transcripts in both MEC1 and MEC2 are differentially disrupted when comparing the two treatments is quite intriguing based on the phenotypic responses of the respective cell lines. When treating JQ1, we see that both MEC1 and MEC2 can no longer progress through the cell cycle and proliferate but are not necessarily directed towards apoptotic death. This phenotypic output of JQ1 treatment biologically corresponds with the loss of CD23 induction, which is a surrogate marker for CLL cell activation. However, when treating with THZ1, we see that both MEC1 and MEC2 undergo apoptosis, and this apoptosis is coupled with the loss of metabolic mechanisms critical for maintaining CLL cell survival. Thus, taken together, this comparative analysis suggests the notion that different super enhancer genes can be heterogeneously disrupted by specific transcriptional inhibitors, and this differential targeting can be contextually based on both the underlying expression profile of the CLL cell subtype and the specific transcriptional inhibitor implemented for treatment.
**Figure 30. JQ1 and THZ1 targets different subsets of SEs in CLL**

Venn diagram showing overlap between the JQ1 and THZ1 targets clinically overexpressed in CLL and the CLL-specific SE genes. Based on the overlapping results, THZ1 and JQ1 downregulate different CLL SE genes, and the specific targets that each transcription inhibitor downregulates reflects the different phenotype of the treated cells. For example, JQ1 disrupts the NFAT-activated FCER2 super enhancer and suppresses cell proliferation, whereas THZ1 disrupts the ENO2 super enhancer and shuts down metabolic functions.
IV. DISCUSSION

Based on the integrative interpretation of events discovered from our H3K27Ac enhancer profile within B cells, RNAseq expression profile of CLL patient samples, RPPA protein profile from CLL patient samples, and RNAseq expression signature of CLL cells treated with transcriptional inhibitors JQ1 and THZ1, we have been able to elucidate the molecular mechanisms mediated by super enhancer-associated oncogenes in CLL. From our comparative analysis, we demonstrate that the identified mechanisms are generally involved with either immune signaling or metabolic-related programs.

We first looked to understand the precise landscape of H3K27ac-based enhancers within the CLL cell line MEC1, the normal GM12878 cell line, and the normal primary CD19+ B cell. Based on our analysis, we were intrigued to discover that the enhancer profile between the three B cell subtypes were relatively similar due to the fact the phenotypic presence of these B cells subtypes are quite different. These similarities were even further highlighted when discovering similar super enhancers between these three analyzed enhancer profiles as well as identifying similar ontology pathways among the super enhancer associated genes from the three enhancer profiles. These similarities between the enhancer profiles were further understood based on the fact that super enhancer genes from the separate profiles demonstrated higher levels of transcription in primary B cells compared to genes not associated with super enhancers. With MEC1 being an EBV+ immortalized CLL cell line, GM12878 being an EBV+ normal B cell
line, and the CD19+ B cell being a normal primary cell, it is interesting to note that B cells maintain an underlying B cell enhancer profile irrespective of specific transformation events such as EBV or CLL-driven conversions.

As we further analyzed the enhancer profiles by investigating the specific genes associated with super enhancers, we do identify genes that have been associated with classical B cell and CLL functions. Examples of these genes include the anti-apoptotic gene BCL2, the B cell receptor related genes PLCG2 and CD79A, and the memory B cell marker CD27. Interestingly, one gene not identified with a super enhancer in any of the 3 B cell subtypes was the MYC oncogene. Typically, MYC is a common gene associated with super enhancers in many cancer types including multiple myeloma and diffuse-large B cell lymphoma (Chapuy and others 2013; Loven and others 2013). However, though MYC has previously been identified as a factor in CLL progression (Krysov and others 2012; Yeomans and others 2016), CLL does not exhibit the same MYC oncogene addiction characteristics when compared to other cancer types.

Furthermore, unlike certain cancers, CLL cells are not defined by clearly identifiable oncogenes that drive their tumorigenic characteristics. Thus, based on the heterogeneity of genes that can be overexpressed in CLL patients, we wanted to identify super-enhancer associated genes that are clinically overexpressed among 47 CLL patient samples when compared to 5 healthy donor samples. From this comparison, we identified genes that have been previously defined as CLL drivers including LCK (Talab and others 2013) and BCL2 (Masood and others 2012) as well as other identified cancer genes that could potentially serve in promoting CLL tumorigenesis such as the glycolytic enzyme ENO2 (Fujita and others 1987; Selga and others 2008) and the chromobox
homolog CBX7 (Klauke and others 2015). In total, we identified 190 genes overexpressed in CLL that are associated with the defined B cell super enhancers. The interesting caveat for these results is that the super enhancers associated with these recognized CLL oncogenic factors are relatively ubiquitous within the all three B cell super enhancer profiles. This occurrence would suggest that some additional alteration within the identified super enhancers help drive the differential expression of super enhancer genes overexpressed in CLL. One possible explanation, as previously demonstrated by Mansour and colleagues in T-ALL cells, could be the addition of non-coding somatic point mutations within the super enhancers genomic sequence that by consequence incorporate a novel transcription factor-binding site (Mansour and others 2014). This novel transcription factor-binding site would then ultimately cause greater transcriptional output for the corresponding gene. Such occurrences would suggest that a number of alterations within super enhancers could change the dynamics of how certain genes are differentially expressed in cancer. Regardless of what causes differential expression of this subset of super enhancer genes in CLL, we observed that the overwhelming biological processes associated with these genes either involve cell-communication signaling (i.e. immune signaling) or metabolic processes. This result culminated with previously published reports and MSigDB hallmark analysis of the super enhancer profiles suggests that CLL cells greatly rely on metabolic regulation and immune-related signaling to maintain cell survival and promote proliferation.

We furthered our understanding of the molecular consequences of CLL-based super enhancers by conducting an RPPA analysis for primary CLL samples and provide a comprehensive view of specific protein alterations that take place in CLL. We were able
to determine from our selected samples that CLL contains a common protein expression signature that is seemingly more consistent among the CLL subtypes that can be characterized by different genetic abnormalities. This common protein signature coincides with previous results demonstrating general overexpression of BCR-related signaling proteins, like BTK, regardless of a patient’s clinical subtype (Herman and others 2011; Herman and others 2010).

We then analyzed the RPPA dataset to determine the precise proteins significantly altered between CLL and healthy B cells. We revealed upregulation of proteins that are commonly associated with CLL pathogenesis, including apoptotic resistance (e.g. BCL2 upregulation, BAX downregulation, cleaved caspase-7 downregulation) and PI3K/AKT/mTOR pathway proteins (e.g. AKT, mTOR, IRS1, GAB2, p70S6K) (Del Gaizo Moore and others 2007; King and others 1998; Li and others 2011; Scarfo and Ghia 2013). Within the realm of the AKT/mTOR pathway, we specifically determined that proteins involved in mRNA translational regulation were being upregulated in CLL patient samples. These aberrant events regarding protein translation include overexpression of the translation initiator eIF4G and phosphorylation of the cap-dependent translation regulator 4E-BP1, an event directly regulated by the metabolically sensitive mTOR pathway. This biological event of translational activation also coincides with the occurrence of metabolic-related genes being enriched in super enhancers. This occurrence is interesting to note due to the connection metabolic activity and activation of protein translation machinery. Additionally, the intriguing caveat of this translational machinery upregulation was its uncoupling from upstream AKT activation. Though total AKT and mTOR are upregulated in our RPPA dataset, the PI3K/AKT-related
phosphorylation activity for these proteins appears to be negligible. This type of result is expected as the RPPA CLL patient samples were collected from the peripheral blood and are not in their active state, which occurs in the lymph node. However, 4E-BP1 serine 65 phosphorylation, an event that typically is regulated by the cascading phosphorylation of the PI3K/AKT/mTOR pathway (Gingras and others 1998), was hyperphosphorylated regardless of AKT activation as demonstrated by IgM stimulation comparisons. This result would suggest that 4E-BP1 phosphorylation, a typical indicator of translational activation, is potentially needed to help maintain survival of dormant CLL cells in the peripheral blood. This disconnect between AKT activation and 4E-BP1 activation provided rationale for investigating the effectiveness of simultaneously targeting both upstream and downstream of the PI3K/AKT/mTOR pathway in CLL. We examined this concept by determining the inhibitory capability of the dual PI3K/mTOR inhibitor NVP-BEZ235. From our results, we saw that 4E-BP1 and eIF4G were significantly altered in CLL cells treated with NVP-BEZ235 when compared to Ibrutinib and Idelalisib, two drugs that target upstream BCR-related effectors involved in PI3K/AKT activation (Burger 2012; Robak and Robak 2013). The inhibitory results of NVP-BEZ235 in CLL correspond with the effectiveness of NVP-BEZ235 treatment in other AKT/mTOR-driven cancer types and suggests that mTOR activity, which can be controlled through several independent factors including metabolic sensing, must be considered when considering the molecular dependencies in CLL cells.

With the continuity of molecular patterns discovered between super enhancer-associated and RPPA-associated expression signatures, we then wanted to determine the molecular effects of transcriptional inhibitors that demonstrate preferential activity
toward super enhancers in CLL cells. Based on this effort, we tested the preclinical activity of the bromodomain inhibitor JQ1 and the cyclin-dependent kinase-7 (CDK7) inhibitor THZ1. Interestingly, though they were designed to disrupt two different proteins that are involved in the sample RNA polymerase II machinery complex, we observed that the two inhibitors produced differing responses within our treated MEC1 and MEC2 CLL cell lines. In the case of bromodomain inhibition, we saw that JQ1 could cause G1-cell cycle arrest in both MEC1 and MEC2 without causing apoptosis, whereas CDK7 inhibition by THZ1 treatment caused substantial induction of apoptosis in the two cell lines. These differences of inhibitory effects were further separated when comparing the RNAseq expression profiles of MEC1 and MEC2 cells treated with the respective inhibitors. By defining the transcripts sensitive to JQ1 and THZ1 within MEC1 and MEC2, we determined that JQ1 and THZ1 preferentially disrupt the transcription of different gene sets. Specifically, we see that a considerable portion of genes suppressed by JQ1 are involved in immune signaling cascades including TRAF1, LCK, and FCER2. On the other hand, we see that THZ1 predominantly disrupts transcription of genes that are involved in regulating metabolic functions within the cell. These metabolic-related genes include both lipid metabolism genes like LSR, ACAD11, LPIN2, and LCAT as well as glycolysis genes like ENO2, PP1R3E, and PFKP. This ability to disrupt genes implicated in both lipid metabolism and glycolytic metabolism is particularly important to point out based on previous reports that demonstrate the ability of CLL cells to metabolically adapt between oxidative phosphorylation and HIF1α-mediated glycolysis based on their residing microenvironment conditions (Koczula and others 2016; Rozovski and others 2015; Sharma and others 2014). Additionally, disrupting transcription of
genes involved in metabolic activation is coupled with loss of mTOR-dependent phosphorylation events like 4E-BP1 phosphorylation, which aligns with the observation that 4E-BP1 phosphorylation corresponds with survival in CLL cells.

Our concluding analysis comparing the super enhancer associated CLL oncogenes with transcripts sensitive to JQ1 and THZ1 further connects the differential transcriptional effects with their respective phenotypic outcomes. By this comparison, we observed that the FCER2 super enhancer, which corresponds with the B cell activation marker CD23, is the underlying super enhancer disrupted by JQ1 in both cell lines, and the ENO2 super enhancer, which corresponds with the committal glycolytic enzyme enolase-2, is one of the underlying super enhancer disrupted by THZ1 in both cell lines.

Based on our integrative analysis connecting the super-enhancer expression landscape with the molecular phenotype of CLL cells, we see that CLL utilizes this altered transcriptional profile to provide advantageous ability in promoting proliferation through extracellular immune signaling mechanisms like the BCR signaling pathway and NFkB activation cascade. As well, we also see that CLL differentially express genes involved in metabolic mechanisms to maintain cell survival throughout its engagement with numerous environments (e.g. bone marrow, peripheral blood, lymph node). This sustained metabolic signature is also reflected at the protein level by the persistent presence of mTOR-dependent 4E-BP1 phosphorylation. Nevertheless, though these overall mechanisms seemed to be maintained in all CLL cells, RNAseq-based treatment studies in the primary CLL cell line MEC1 and the relapsed CLL cell line MEC2 with the super enhancer inhibitors JQ1 and THZ1 show that different CLL subtypes can rely more
heavily on different transcripts within the same molecular programs. Thus, it may be important to consider transcriptional inhibitors like THZ1 and JQ1 as potential therapeutic options in a disease like CLL. With the ability for CLL to subclonally expand by treatment-induced tumor evolution, therapeutic targeting of transcription could be utilized in disrupting CLL progression by the unbiased suppression of oncogenes heterogeneously overexpressed among the varying CLL subclonal populations.
V. SUMMARY

Chronic lymphocytic leukemia (CLL) is a malignant disease of CD19+ B cells that resist apoptosis and oncogenically proliferate within the lymph node. The major hurdle in treating CLL is the ability for CLL cell populations to subclonally expand after initial therapy and eventually cause a relapsed and more advanced form of the disease (Landau and others 2013). This ability to subclonally expand is a consequence of CLL cells utilizing heterogeneous measures to activate the needed mechanisms for survival and growth. Because CLL cells have altered transcriptional profiles when compared to normal B cells, we sought to analyze the histone H3K27Ac enhancer landscape in B cells and determine which overexpressed transcripts in CLL correspond with the newly defined phenomenon associated with oncogene transcription known as “super enhancers” (Loven and others 2013). From this characterization, we then sought to determine whether transcriptional inhibitors that preferentially suppress super-enhancer-associated oncogene expression could serve as a therapeutic potential in CLL.

We first characterized the enhancer landscape in the immortalized CLL cell line MEC1, the immortalized normal B cell line GM12878, and a CD19+ primary B cell sample in order to identify super enhancers in each B cell type. We identified 741 super enhancers in GM12878, 374 super enhancers in MEC1, and 523 super enhancers in the CD19+ B cell sample. Though variation did exist, the super enhancer landscape was largely similar between the 3 B cell types. We then identified the genes that annotated
with the B cell super enhancers and determined that genes correspond among super enhancers were enriched in pathways that mediate immune signaling (e.g. TNFA_SIGNALING_VIA_NFKB) as well as pathways that mediate metabolic regulation (e.g. MTORC1_SIGNALING). We then compared the super-associated genes with genes overexpressed in CLL and identified genes 190 genes overexpressed in CLL that have an association with a B cell super enhancer. Immune receptor signaling and metabolic regulation were further characterized as important factors in CLL based on this derived gene subset.

We then performed RPPA analysis comparing CLL patient B cells with healthy donor B cells to determine if the CLL protein landscape demonstrated any parallels with the defined CLL super enhancer signature. From our results, we observed that one of the most deregulated events at the protein level involved mTOR-dependent mRNA translation regulators. Specifically, the translation initiator eIF4G was overexpressed and the translation inhibitor 4E-BP1 was hyperphosphorylated in CLL, suggesting a sustained activation of the mRNA translation complex. Interestingly, the sustained 4E-BP1 hyperphosphorylation was uncoupled from AKT activation, suggesting that mTOR mediates 4E-BP1 phosphorylation through an AKT independent mechanism such as metabolic sensing (Sharma and others 2014). We also saw that the dual PI3K/mTOR inhibitor NVP-BEZ235 causes greater apoptosis in primary CLL cells and differential dephosphorylation of 4E-BP1 when compared to the B cell receptor signaling inhibitors Ibrutinib and Idelalisib. These results collectively demonstrate an AKT-autonomous dependence on the metabolically sensitive mTOR kinase in CLL as well as demonstrate
continuity with the metabolic expression signatures identified in the CLL super enhancer associated gene subsets.

By identifying the molecular signatures associated with super enhancer-associated CLL oncogenes, we then analyzed the effects of super enhancer targeting transcriptional inhibitors JQ1 and THZ1. The BET bromodomain inhibitor JQ1 caused cell cycle arrest in both the primary CLL cell line MEC1 and the patient-matched relapsed cell line MEC2 (Rasul and others 2014; Stacchini and others 1999) without inducing apoptosis. Based on RNAseq expression analysis, this arrest in cell cycle correlated with the downregulation of CLL oncogenes implemented in immune signaling cascades, many of which were super enhancer genes (e.g. FCER2, LCK, TRAF1). Interestingly, the CLL oncogenes downregulated by JQ1 in MEC1 and MEC2 were different among the two cell lines with super enhancer-associated gene FCER2 being the only overlapping JQ1-sensitive transcript. In contrast, the CDK7 inhibitor THZ1 elicited a different molecular response in MEC1 and MEC2 by being greatly inducing apoptosis in the CLL cell lines. This activation of apoptosis also occurs in cultured primary CLL cells treated with THZ1. Apoptotic induction corresponded with downregulation of genes involved in metabolic regulation with several being super enhancer-associated genes (e.g. ENO2, SEPT9, LSR).

Taken together, these results collectively suggest that CLL cells rely on super-enhancer mediated oncogene expression to maintain metabolic stability and promote immune receptor signaling activation. Based on this described mechanism, transcription inhibitors like JQ1 and THZ1, which can preferentially disrupt specific molecular signatures heterogeneously present among CLL cell populations, may serve a promising therapeutic option against the clonal and subclonal expansion of CLL.
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