T-HELPER POLARIZATION: REGULATION BY NONCODING RNA AND SUPER-ENHANCERS

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To Cait, Mom, and Dad,

For never ending support and guidance

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LIST OF ABBREVIATIONS

ATAC-seq: Assay for Transposase accessible Chromatin Sequencing

BP: Base pairs

ChIP: Chromatin Immunoprecipitation

DRIP: DNA-RNA immunoprecipitation

ELISA: Enzyme Linked Immunosorbent assay

ESC: Embyronic Stem Cells

LncRNA: Long noncoding RNA

mRNA: messenger RNA

PAMP: Pathogen Associated Molecular Patterns

PBMC: Peripheral Blood Mononuclear Cells

PBS: phosphate buffered saline

PRC: Polycomb Repressive Complex

qPCR: Quantitative Polymerase Chain Reaction

RIP: RNA immunoprecipitation

RNA Pol II: RNA Polymerase II

siRNA: Short interfering RNA

STAT: Signal Transducer and Activator of Transcription

TBS: Tris buffered saline

TFH: T Follicular Helper

TH1/TH2/TH17: T Helper 1/2/17

TH2LCRR: T helper 2 Locus Control Region RNA

TSDR: Treg specific demethylated region

CHAPTER I

Introduction

The immune system is broadly separated into two branches, referred to as the innate and adaptive immune system. The innate immune system recognizes what are termed pathogen-associated molecular patterns or PAMPs and provides a rapid general response to infections occurring anywhere throughout the body. PAMPs are generally defined as chemical structures unique to classes of viruses and bacteria, such as lipopolysaccharide, double-stranded RNA, peptidoglycan, unmethylated CpG motifs, and peptidoglycans [1]. These structures are recognized by a series of sensors including toll-like receptors and other pattern recognition receptors present in eukaryotic organisms and stimulate a series of different signaling cascades resulting in expression of interferons and other cytokines that contribute to the control of viral and bacterial infections. This innate immune response is also a strong activator of the adaptive immune response [2].

The adaptive immune system is characterized by the ability to develop a specific immune response to a pathogen. The adaptive immune response is primarily comprised of lymphocytes, including antibody producing B cells and CD8+ and CD4+ T-cells [2–5]. Classically, T cell and B cell receptor genes undergo somatic gene rearrangement to create a clonal repertoire capable of recognizing any chemical structure expressed by invading pathogens. Both antibodies produced by B cells and T-cell receptors expressed by T-cells are highly specific for a given antigenic structure expressed by

pathogens and confer lifelong immunity to infection. Upon antigen recognition, lymphocytes massively proliferate and generate expanded clones with identical antigen receptors. Antigen-specific CD8+ T-cells recognize specific viral antigens and are cytotoxic for cells infected by specific viruses [3,6]. Antigen-specific CD4+ T-cells, or T helper cells, produce an array of signaling molecules called cytokines in response to infection that orchestrate the adaptive immune response [5].

Helper T-cell Polarization

Helper T-cells are also sub-divided into naïve, effector and memory T-cells [4]. Upon encounter with their specific antigen, naïve T-cells proliferate via IL-2 but do not express the cytokines critical for orchestrating the adaptive immune response [7]. The cytokine microenvironment during antigen encounter dictates T helper cell polarization. CD4+ helper T-cells differentiate into several classes of effector T-cells that are defined by the cytokines they produce and how they defend against infection [8]. Examples include effector T helper 1 (TH1) cells that produce IFN-γ, TH2 cells that produce IL-4, IL-5, and IL-13, TH17 cells that produce IL-17, T follicular helper (Tfh) cells that help B cells produce antibody against foreign pathogens, and T regulatory (Treg) cells which regulate immunity through anti-inflammatory IL-10 (Figure 1.1).

TH1 cells are defined by their production of the cytokine IFN-γ. IFN-γ plays a key role in controlling infection by both bacteria and viruses, but also acts as one of the first steps in TH1 cell polarization [9,10]. IFN-γ, initially produced by innate immune cells, binds to naïve T cells and induces signal transducer and activator of transcription (STAT) 1 expression [11]. STAT1 is part of a family of transcription factors that help regulate

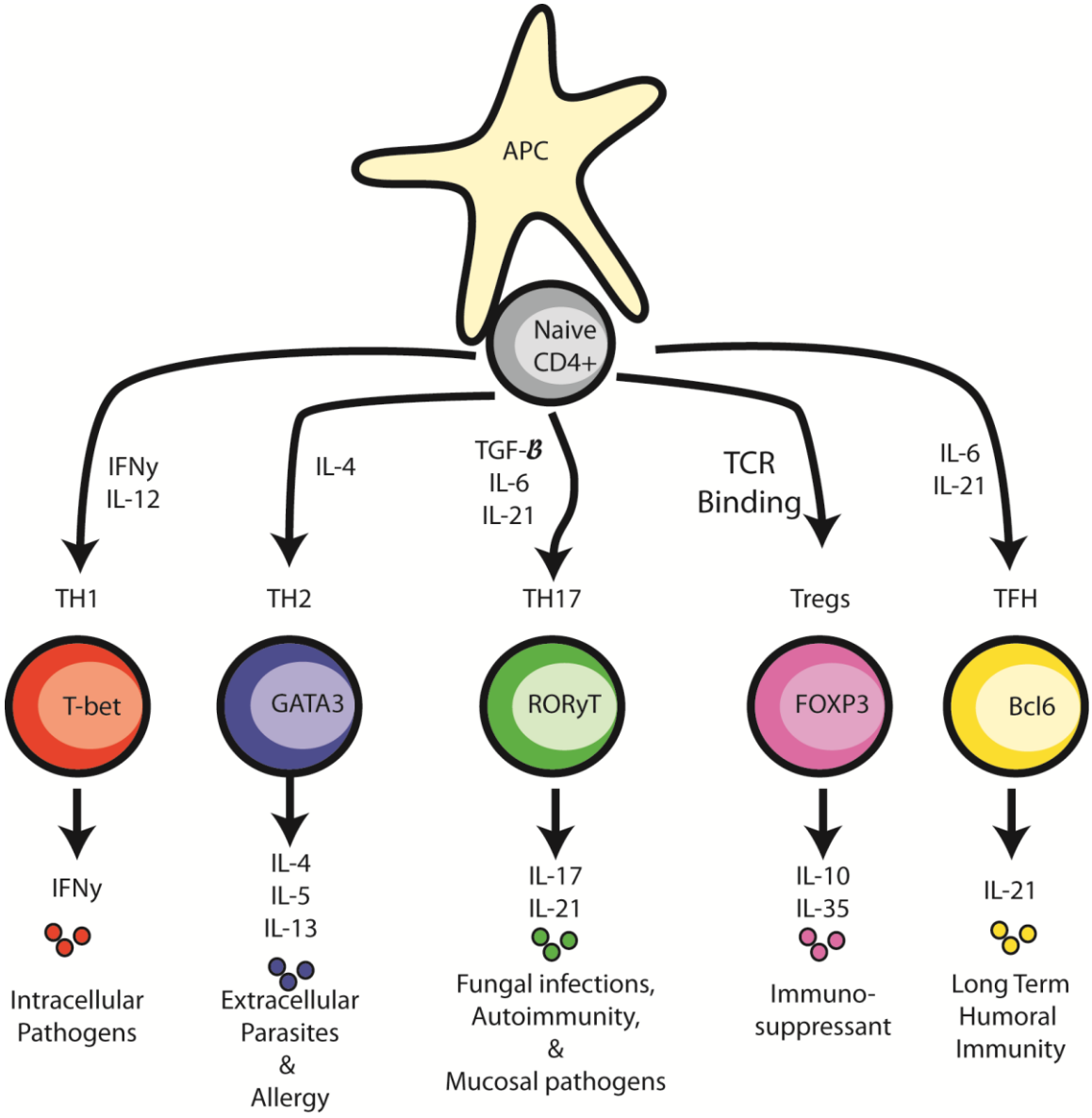

Figure 1-1. CD4+ T helper Cell Differentiation. Different subsets of T helper cells are polarized according to the soluble cytokines present during priming by an antigen-presenting cell (APC) (IL-12 and IFN-γ for TH1), a lineage defining transcription factor (T-bet for TH1), and a secreted cytokine to regulate a portion of the immune response (IFN-γ for TH1).

T helper cell polarization [12]. STAT1 expression activates the T-box transcription factor (T-bet) [13,14]. T-bet is referred to as the master transcriptional regulator of TH1 cells as it enhances expression of IFN-γ, creating a feed forward loop of IFN-γ and T-bet production [15,16]. T-bet deficient cells are unable to mount a TH1 response even in proper polarizing conditions [17]. T-bet also induces *IL12Rβ2*, one chain of the IL-12 receptor [13]. IL-12 binding leads to the expression of STAT4, another transcription factor necessary for IFN-γ production [18,19]. T-bet and STAT4 work in tandem to induce IFN-γ expression by rearranging chromatin structure throughout the *IFNG* locus resulting in activation of gene transcription [20–23]. IFN-γ expression is significantly reduced in STAT4 deficient cells [24]. The combined actions of the IFN-γ /STAT1/T-bet and the IL-12/STAT4 pathways lead to complete TH1 polarization.

TH2 cells are defined by the production of the cytokines IL-4, IL-5 and IL-13. Each of these cytokines have unique effects but are important for the immune response to helminth infection and play a role in allergy. Naïve T-cells polarize to TH2 cells by stimulation by IL-4, which induces the activation of STAT6 [25–27]. STAT6 directly binds and induces transcription of the *GATA3* gene, thought of as the master transcriptional regulator of TH2 polarization [28,29]. Gata3 directs TH2 polarization by binding to the TH2 cytokine gene locus, which contains the *IL4*, *IL5* and *IL13* genes [30]. TH2 differentiation and responses are almost entirely abrogated in Gata3 deficient cells [30–32]. However, Gata3 cannot regulate each cytokine gene effectively on its own and STAT6 activation is required for effective TH2 polarization [33]. Gata3 not only rearranges chromatin around

the TH2 cytokine locus, but also directly binds the *IL5* [34]and *IL13* promoters [35,36]. The combination of STAT6 and Gata3 is necessary for TH2 polarization.

TH17 cells produce a greater diversity of cytokines, including IL-17a, IL-17e, IL-17f, IL-21 and IL-22 [37,38]. IL-17 is a pro inflammatory signal involved in antifungal immunity at mucocutaneous surfaces. IL-17a and IL-17f are also involved in the pathogenesis of autoinflammatory and autoimmune disorders, including inflammatory bowel disease, psoriasis, and ankylosing spondylitis. TH17 cell polarization occurs in three transcriptional phases. T-cell activation in the presence of transforming growth factor-$\boldsymbol{\beta}$ (TGF-$\boldsymbol{\beta}$) and IL-6 drives the initial polarization of naive CD4+ T-cells to TH17 cells [39,40]. IL-6 activates STAT3, which helps induce expression of IL-21, and IL23R [41]. STAT3 expression induces ROR$\boldsymbol{\gamma}$t, referred to as the master transcriptional regulator of TH17 cells [42,43]. Once ROR$\boldsymbol{\gamma}$t is expressed, TH17 cells begin expressing hallmark cytokines like IL-17a and IL-17f. The second phase includes self-amplification by IL-21. Unlike TH1 and TH2 cells, the main TH17 cytokine does not amplify its differentiation. IL-21, which is produced by TH17 and innate immune cells, combines with TGF-$\boldsymbol{\beta}$ to amplify cell differentiation independent of IL-6 [44,45]. The final phase is IL-23 induced stabilization. IL23R expression occurs downstream of IL-6 and IL-21 and is further induced by IL-23 binding to its receptor [39,46]. IL-23 does not expand or drive differentiation but is required for effective TH17 cell maintenance.

Tfh cells are located in the follicular areas of lymphoid tissue and express B-cell promoting IL-21. The development of a Tfh from a naïve T-cell is slightly different from the other effector cells, as it must be trafficked to a germinal center. After T-cell receptor priming, naïve T-cells stimulated by IL-6 will express STAT3 [12,47]. STAT3, in the

absence of TGF-**β** binding will induce Bcl6, referred to as the master transcription factor, as well as IL-21 [47]. Bcl6 drives expression of CXCR5, the cell surface marker to allows Tfh cells to traffic to the B cell zone [48,49]. Once inside the B cell zone, Tfh cells will bind to B cells via ICOS and the T-cell receptor to form germinal centers [50]. Through prolonged interaction and varied cytokine secretion, Tfh cells contribute to class switching and memory formation by activated B cells [51].

The regulatory T cells, or Tregs, are a distinct subset of CD4+ T cells which suppress effector cells in the periphery at sites of inflammation, primarily regulating the other CD4+ T cell populations [52]. Tregs mediate immune responses by producing immunosuppressive cytokines like IL-10, IL-35 and TGF-**β**, inducing cytolysis via granzyme A/B and perforin, and disrupting T cell metabolic function by IL-2 deprivation [52]. Tregs can either form in the thymus, called tTregs, and emerge as a fully matured cell population or develop in the periphery, called pTregs, from CD4+ naïve T cells [53]. Tregs are characterized by constitutive expression of the transcription factor Forkhead box P3, FOXP3, as well as high expression of CD25 and low expression of CD127 [52]. During thymic selection, cells whose TCR bind with a moderate avidity to self-antigen, but still escape negative selection, will increase expression of CD25 and begin expressing FOXP3, referred to as the master transcriptional regulator of Tregs. Unlike other master transcription factors previously discussed, FOXP3 can be expressed at low levels in conventional inflammatory T cells without inducing the suppressive Treg functions [52]. Treg development requires FOXP3 expression along with a cell specific hypomethylation pattern within a region called the Treg specific demethylated region or

TSDR [54]. Only the combined expression of FOXP3 and epigenetic modification of the TSDR will induce Treg differentiation and immunosuppressive effects.

Master transcription factors drive T helper cell polarization by activating a cell specific gene profile. However, each transcription factor also represses cytokines and transcription factors related to alternative T helper cell fates. T-bet not only activates TH1 lineage specific genes but also inhibits expression of the cytokine IL-4 [22] and directly binds and sequesters Gata3 [55]. T-bet also blocks TH17 polarization by repressing ROR$\boldsymbol{\gamma}$t expression. Gata3 represses IFN-γ expression by repressing *STAT4* and *IL12Rβ2* mRNA transcription through chromatin remodeling [30,56]. TGF-$\boldsymbol{\beta}$1 suppresses the expression of T-bet and Gata3, inhibiting the naive CD4+ T-cell from adopting the TH1 or TH2 cell fates initially, but IL-23 can still induce IFN-γ expression later in development [57]. TH17 cells both *in-vitro* and *in-vivo* have shown the ability to produce IFN-γ and IL-17a after polarization. The conversion of IL-17 producers to IFN-γ producing cells is an important aspect in immunopathogenesis in disease models, and likely in autoimmune disease [58]. Tfh cells have been implicated in production of numerous other cytokines, including IFN-γ, IL-4, and IL-10 to regulate class switching in B cells [51].

In order to respond to the ever-evolving variety of pathogens, the human immune system must be heterogeneous and adaptable. The immune system must be able to mount the correct cellular response efficiently before an infection can irreparably damage tissue. Dynamic responses to extracellular signals allow a naïve T-cell to develop into the varied repertoire of effector T-cells described here, but our understanding of T-cell development is not complete. A better understanding of the

complex systems regulating TH cell development will contribute to our ability to activate the immune system to better fight infection as well as to better control the immune system when it is over-activated, for example, during autoimmune or other inflammatory diseases.

Long Noncoding RNA

Our understanding of gene regulation used to include the DNA sequence of a gene and the proteins bound within the gene region. The act of transcription produced an mRNA and was translated into a functional protein. The discovery of noncoding RNAs caused an immense shift in our understanding of RNA's role in molecular biology. A larger portion of the human genome produces noncoding RNAs than mRNA genes. Several functional RNAs have been discovered including transfer RNA (tRNA), ribosomal RNA (rRNA), and even more recently long noncoding RNA (lncRNA).

LncRNAs are >200bp in length (to distinguish them from smaller classes of RNAs, such as microRNAs) and possess little to no protein coding potential [59-61]. LncRNA genes are transcribed by RNA Pol II, mostly receive a 5' cap and a poly-A tail, and many contain introns that are spliced out like mRNA [62,63]. LncRNAs are typically riddled with stop codons and contain no verifiable reading frame, separating them from mRNA [64]. MicroRNAs and lncRNAs share some regulatory functions, but the processing and size of lncRNAs separate them from microRNAs. Functionally, many lncRNAs regulate expression of nearby protein-coding genes in the genome and therefore have been classified according to their genomic location relative to nearby protein-coding genes [60,63] (Figure 1.2). Four classes have emerged to define lncRNAs, including

antisense, intronic, intergenic, and divergent. Antisense lncRNAs are produced on the opposite strand of an mRNA gene, and in the opposite direction while sometimes overlapping in their transcriptional window. Intronic lncRNAs are produced almost entirely in the intron region of an mRNA gene. Intergenic lncRNAs are located between two mRNA genes, overlapping with neither gene and typically far from an mRNA gene. Finally, Divergent lncRNAs are produced on the opposite strand of an mRNA gene but are close enough they can share a promoter.

The function of lncRNA transcripts have become better understood in large part from studies that deplete the lncRNA without interfering with its gene locus. LncRNAs can bind proteins, RNA, and DNA, which endows them several regulatory functions [63-65]. Regulation by lncRNA transcripts involve 3 major actions: Sequestering, guiding, and scaffolding (Figure 1-3).

LncRNAs can bind proteins to sequester them from binding to their cognate DNA elements or to other proteins (Figure 1-3A). Often lncRNAs will contain a sequence motif like the DNA binding region in order to act as a decoy. For example, CTCF is a transcription factor that binds to promoter regions within multiple genes and is typically repressive [66]. *JPX*, a lncRNA transcribed from the X chromosome inactivation center will bind CTCF and impair the ability of CTCF to bind the *Xist* gene promoter resulting in stimulation of *Xist* transcription, which is required for X-chromosome inactivation in females [67]. Similarly, NF-YA is a transcription factor that activates genes that encode pro-apoptotic proteins in response to DNA damage. *PANDA* is a lncRNA that binds to the DNA-binding pocket of NF-YA thus sequestering NF-YA away from its cognate DNA binding enhancer elements [68]. Another example is *GAS5*, a lncRNA that binds the

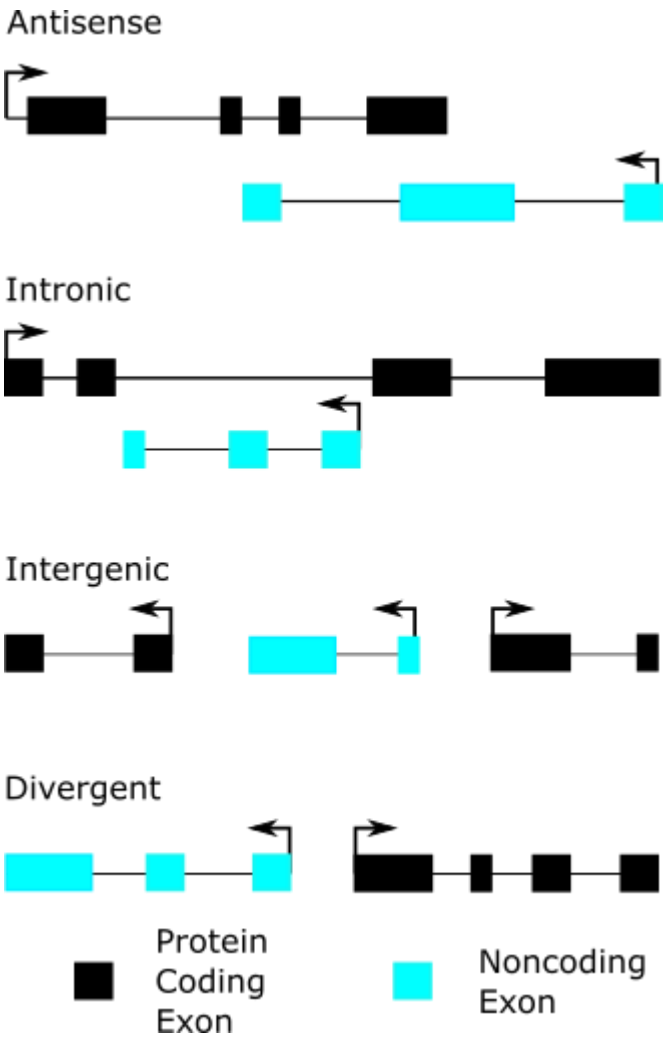

Figure 1-2 Anatomy of a LncRNA Loci. Antisense lncRNAs are transcribed from the opposite strand and overlap with its mRNA partner. Intronic lncRNAs initiate inside of an intron of a protein-coding gene. Intergenic lncRNAs are separate transcriptional units from protein-coding genes, occurring between two separate mRNAs irrelevant of distance. Divergent lncRNAs initiate on the opposite strand of its mRNA partner bidirectionally from a shared promoter region.

glucocorticoid receptors, thus preventing the glucocorticoid receptor from inducing transcription of its target genes [70]. Finally, lncRNAs not only act on proteins, as some have been shown to bind microRNAs and have been referred to as microRNA "sponges" [71]. MicroRNAs repress genes by binding mRNAs to either limit translation or degrade the mRNA via nuclease activity in the cytoplasm. LncRNAs bind to and sequester microRNAs through base pairing so they cannot bind to their target mRNAs [72].

Another example of lncRNA function is to act as guides to recruit chromatin modifying complexes to both gene loci and DNA regulatory elements (Figure 1-3B). Chromatin, or packaged DNA, is made up of histone protein octamers wrapped in DNA, together called nucleosomes. The histone proteins, including H2A, H2B, H3 and H4, contain flexible N-terminal tails that are modified to tighten or relax the binding between the histone proteins and bound DNA [73,74]. Patterns of histone modifications can be used to aid in the identification of active and inactive gene regions. Mono-, di-, and trimethylation of histone 3 lysine 9 (H3K9me1/2/3) and H3K27me1/2/3 are associated with transcriptional repression and inaccessible regions of DNA [75]. Acetylation and other methylation modifications, such as H3K27ac or H3K4me3, are associated with active and accessible enhancers or promoters. Histone modifications are added by chromatin modifying complexes such as acetyltransferases and methyltransferases. Certain chromatin modifying proteins, like WDR5 and PRC2, depend on lncRNAs to guide them to target loci [76]. WDR5 is a portion of the MLL methyltransferase that catalyzes formation of H3K4me1/2/3, a permissive chromatin mark found at active promoters and enhancers. WDR5 interacts with over 200 lncRNAs in Embryonic Stem Cells (ESC) and

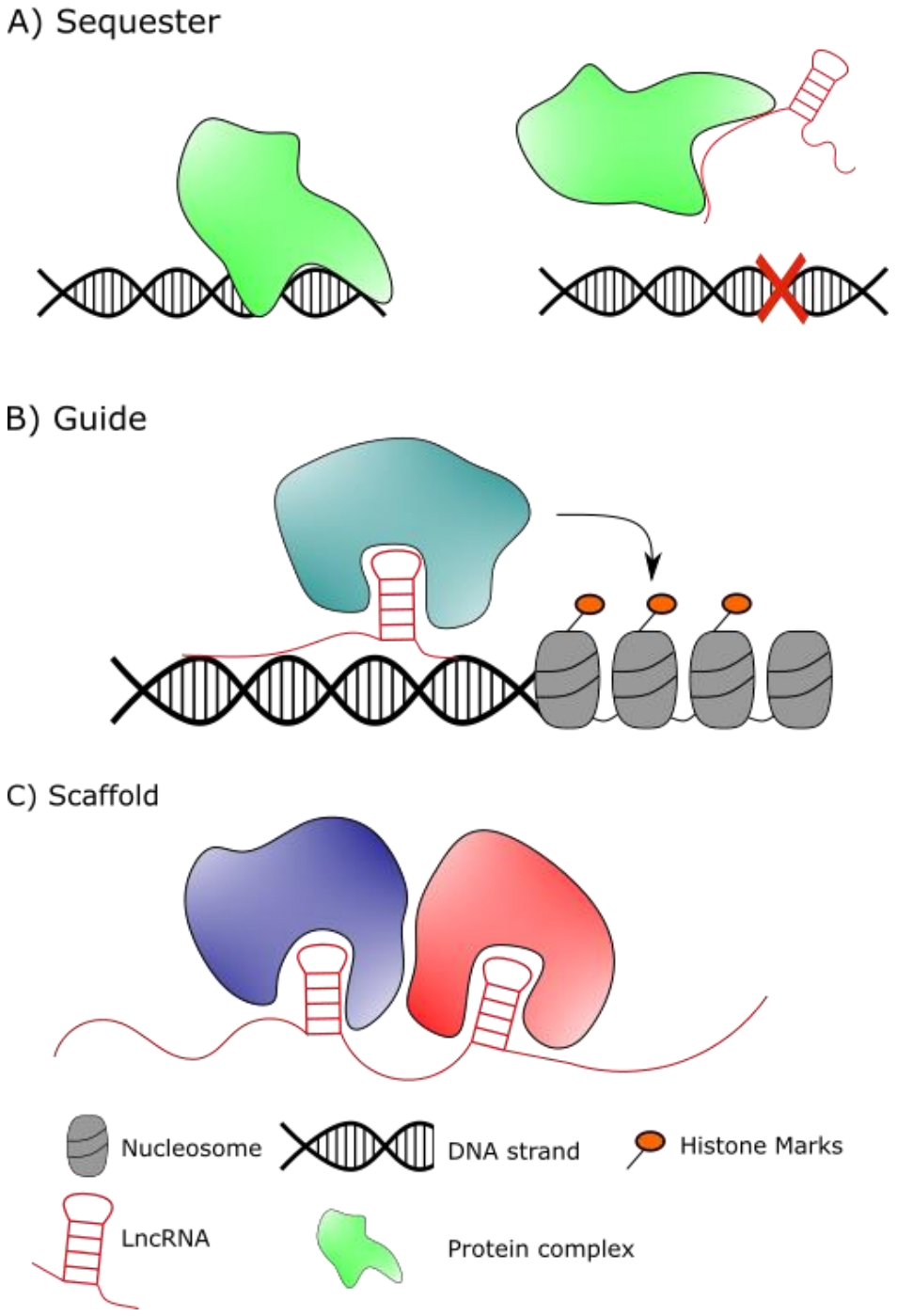

Figure 1-3. Long noncoding RNA (lncRNA) function. (a) LncRNAs sequester DNA binding proteins to repress function. (b) LncRNAs can act as guides to recruit chromatin modifying complexes to specific DNA regions. (c) LncRNAs may act as scaffolds to consolidate multiple protein complexes into a single unit.

has an identified RNA binding pocket [77]. When the RNA-binding pocket is mutated, WDR5 no longer accumulates on chromatin nor functions to sustain methylation patterns around active genes. The lncRNA, *HOTTIP*, binds WDR5 and helps recruit it to the *HOXA* gene to promote transcription [81]. Abrogation of *HOTTIP* lncRNA leads to decreased proliferation and apoptosis in ESCs [77,78]. The Polycomb Repressive Complex (PRC) is a methyltransferase responsible for silencing genes, contributing to heterochromatin formation by adding H3K27me3 marks when it is recruited to genomic loci [79]. In HeLa cells, ~20% of lncRNAs expressed in the cell are found to associate with the PRC2 complex [80]. *HOTAIR* is a lncRNA required for proper localization of PRC2 across the *HOXD* gene region [81]. *HOXD* genes are de-repressed when *HOTAIR* lncRNA expression is abrogated, resulting from an increase in H3K4me3 marks and increased RNA Pol II occupancy at the HOXD gene locus [82]. LncRNAs can bind and recruit either activating or repressive histone modifying complexes to regulate gene expression.

HOTAIR is also an example of a lncRNA with multiple functions, as it helps scaffold the PRC2 and the LSD1-CoRest complex [83] (Figure 1-3C). The LSD1-CoRest complex is a demethylase, removing methyl groups from histone H3, such as the permissive H3K4me3 mark. By combining the LSD1-CoRest complex with the PRC2 methyltransferase complex, *HOTAIR* lncRNA helps to form a complex that can simultaneously remove permissive marks and replace them with repressive chromatin marks to effectively silence a gene [83]. *ANRIL*, another lncRNA associated with the PRC, binds to subunits of both PRC1 and PRC2 to form a singular complex [84,85]. A reduction in *ANRIL* transcripts significantly suppresses cell proliferation and leads to

apoptosis in osteosarcoma cell lines. As further studies continue, the unique versatility presented by lncRNAs will surely identify new functions of this diverse array of RNAs.

LncRNAs not only regulate transcription through the functions of their RNA transcripts but can also regulate neighboring genes through the act of lncRNA gene transcription or by impacting RNA splicing. *BLUSTR* is a divergent lncRNA located next to the mRNA gene *SFMBT2* [86]. *SFMBT2* expression is repressed when *BLUSTR* transcription is disrupted but is not impacted by deletion of entire exons downstream of the *BLUSTR* start site. *UPPERHAND* (*UPH*) is another divergent lncRNA which regulates an mRNA gene partner independent of a specific sequence [87]. *UPH* shares a promoter region with *HAND2 and* disrupting transcription by introducing a premature poly(A) signal impaired *HAND2* expression. *HAND2* was unaffected by reducing *UPH* transcripts via antisense oligonucleotides or inserting new sequences into the *UPH* gene locus. Cis-acting gene regulation by transcription or splicing is not unique to only lncRNAs, as mRNA pairs have shown similar regulatory action.

Recently, a few lncRNAs have been implicated in the formation of R-Loops [88]. An R-loop is the formation of a DNA-RNA-DNA triplex (Figure 1-4). R-loops occur in G-C rich regions and may occur more often following negative supercoiling from transcription. R-loops span anywhere from 100-2000 bp (base pairs) and can form within their own gene locus or binding to similar sequences distant from their origin [89,90]. R-loops can be both activating or repressive, depending on where they form within a gene [91,92]. R-loops formed around promoters or transcription start sites tend to increase adjacent gene transcription and are associated with hyperacetylation. R-loops formed around a transcriptional termination site are typically repressive by virtue of formation of

repressive histone methylation marks that may result in displacement of RNA pol II. Several lncRNAs have been found to form R-loops that either activate or repress gene transcription. *VIM-AS1* is an antisense lncRNA, which forms an R-loop to activate the *Vimentin* gene [93]. *KHPS1* lncRNA also forms an R-loop to activate *SPHK1*, a proto-oncogene related to renal cancer [94]. *MEG3* is another lncRNA, which forms an R-loop, but it represses factors that regulate the cytokine TGF-**β** in macrophages [95]. The study of R-loop formation by lncRNAs is still in its infancy but may shed light on new functions or contribute further insight into the ways that lncRNAs can guide multiple binding partners to DNA.

LncRNAs can perform a significant role in cell development and lineage commitment in the human body. LncRNAs are expressed in a more cell type specific manner than mRNAs [96]. Cell type specific expression has implicated numerous lncRNAs in regulating and maintaining cell identity. *Xist*, one of the first regulatory noncoding RNAs ever identified, plays a key role in X chromosome dosage compensation during early embryogenesis [97-99]. *Xist* RNA transcripts coat the surface of one X chromosome in somatic cells to recruit protein complexes to repress transcription across the X chromosome, which is sustained throughout the entire lifetime of somatic cells [99] .Another lncRNA, Braveheart (*BVHRT*), is necessary for effective development nascent mesoderm to a cardiac fate [100]. *BVHRT* forms a stem loop structure to recruit chromatin modifying complexes and is necessary for transcription of MesP1. Cardiomyocytes cannot reach terminal cell fate when *BVHRT* is abrogated. Mutations and dysregulation of these lncRNAs have been implicated in disease. Loss of *Xist* results in re-expression of genes on the X-chromosome[101-102].

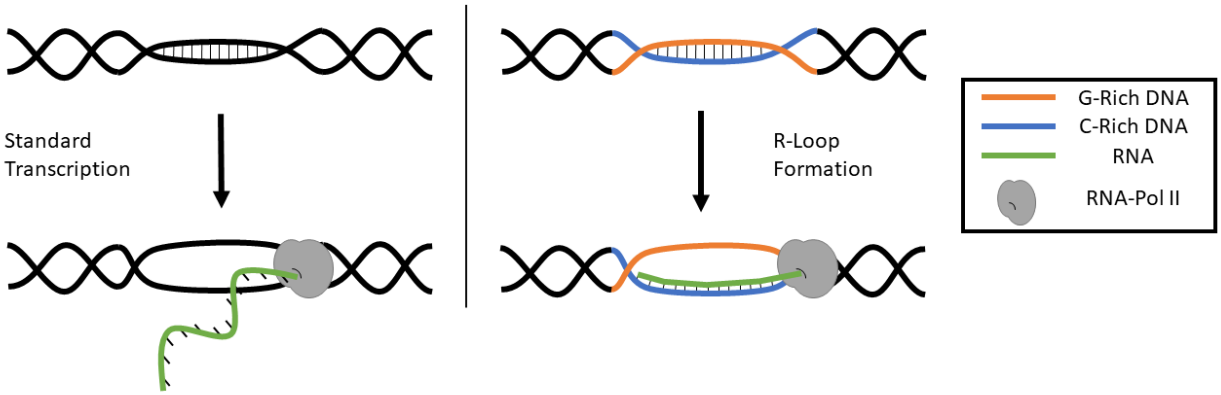

Figure 1-4. Model of R-Loop Formation. Following standard transcription, an RNA is normally trafficked away from its gene locus to be further processed as shown on the left. Sometimes, typically in a G-C rich region, it stays bound within its own locus forming a DNA:RNA hybrid, also known as an R-Loop as shown on the right.

The overexpression of X-linked genes is prevalent in female lymphocytes from lupus patients and in mouse models of autoimmunity [102]. Multiple single nucleotide polymorphisms (SNPs) located within *BVHRT* have been implicated in an increased susceptibility to myocardial infarction.

Thousands of unique lncRNAs have been identified expressed by CD4+ T-cells at different stages of development and differentiation thanks to RNA sequencing efforts. The cellular diversity of the immune system is matched by its lncRNA diversity. *IFNG-AS1*, an antisense lncRNA located in the *IFNG* gene locus, is expressed by NK cells, CD8+, and CD4+ T-cells [103]. *IFNG-AS1* is induced by STAT4 and T-bet dependent pathways and interacts with T-bet to stimulate *IFNG* transcription during early TH1 differentiation [104,105]. Like previous examples, *IFNG-AS1* also interacts with WDR5 to recruit and modify chromatin modifications in the *IFNG* gene locus. *Linc-MAF-4* has also

been identified in TH1 cells [115]. *MAF*, the mRNA partner, is expressed in polarized TH2 cells and promotes expression of TH2 cytokine genes. *Linc-MAF-4* recruits the repressive LSD1-EZH2 complex to repress the *MAF* gene in TH1 polarized cells [116]. *Linc-Maf-4* knockdown in non-polarized T-cells leads to Gata3 and IL-4 expression. Exogenous *linc-MAF-4* transcripts repress TH2 cytokine gene expression, demonstrating a key role for this lncRNA in TH1 polarization.

Locus control regions or LCRs are genomic enhancers that stimulate expression of linked genes that are members of a gene family, such as the globin genes or the growth hormone genes. The *IL4, IL5*, and *IL13* genes are also syntenic in vertebrate genomes and considered a gene family. In mice, expression of TH2 cytokine genes is dependent upon a genomic enhancer region termed the TH2 locus control region of TH2 LCR. The TH2 LCR functions by producing a lncRNA, termed the *TH2LCRR* [117]. *TH2LCRR* has at least 4 unique isoforms, but each shares a central conserved nucleotide sequence. The *TH2LCRR* binds and guides WDR5 to the TH2 cytokine locus resulting in addition of permissive H3K4 methylation marks to stimulate transcription. Depletion of all *TH2LCRR* isoforms substantially reduces expression of IL-4, IL-5, and IL-13, but abrogation of individual isoforms by RNA interference does not effectively deplete TH2 cytokine expression. These results suggest that a common function of LCRs may be to transcribe a lncRNA that regulates expression of each member of a linked gene family, such as the TH2 cytokine gene family.

Initial studies in TH17 cells have identified *lncDDIT4*, a lncRNA related to DNA-damage inducible transcript 4 (DDIT4) [109]. DDIT4 is a cytoplasmic protein, which inhibits mTORC1 activity. *LncDDIT4* lncRNA knockdown results in reduced expression of

DDIT4 and increased expression of IL-17. Taken together, these results suggest that induction of the lncRNA, *lncDDIT4*, leads to increased expression of the DDIT4 protein that, in turn, inhibits the mTOR pathway resulting in decreased function of effector TH17 cells. Hundreds of additional lncRNAs have been identified that are preferentially expressed by TH1, TH2, and TH17 lineages but studies of individual functions of these lncRNAs are still limited.

In the past decade, the use of next-generation sequencing has changed our perception that most of the human genome is composed of insignificant “junk” to an appreciation of the broad array of regulatory RNAs transcribed by vertebrate genomes LncRNAs represent one such1 class of novel regulators of gene transcription and cell differentiation. The cell-type specific nature of lncRNAs makes them ideal biomarkers for evaluating cell populations and disease states in autoimmune disorders and cancer. Although numerous lncRNAs have been identified, functional studies have proceeded more slowly. Evaluation of molecular and cellular functions of individual lncRNAs, including their binding partners, downstream effects, and secondary structure will provide greater insights into the role of lncRNAs in cell development.

Super-Enhancers

Cell development and fate is dictated by variation in the expression of genes. Our understanding of the activation and repression of gene transcription has evolved immensely to include the histone code, transcription factor complexes, and functional RNAs. As our knowledge of new regulatory factors has grown, so has our understanding of conserved regulatory regions within the DNA sequence. The

interactions between enhancers and promoters represent some of the earliest studies of gene regulation and continue today.

Enhancers are DNA elements that increase the transcriptional output of a target gene. Enhancers regulate a gene by helping to recruit the protein complexes necessary for efficient transcription to the promoter region [110]. Enhancers can regulate genes independent of orientation or distance to the target gene, even targeting genes on other chromosomes [111-113]. Distal enhancers can reach and interact with their target gene through DNA looping to facilitate the binding of transcription factors and RNA polymerase II (RNA Pol II) to the target gene promoter [114,115]. Active enhancers can be identified by their lack of nucleosomes, leaving exposed DNA available for binding by transcription factors [73,74]. Nucleosomes are the central subunit of packaged DNA and include 8 histone proteins, made up of two tetramers, and 147 bp of DNA. When DNA is packaged within nucleosomes, it is much less accessible for protein binding, limiting transcription and replication.

Enhancers can be identified by multiple methods. DNase-1 is an endonuclease, which cleaves regions of open accessible DNA that lack nucleosomes. Active enhancers are hypersensitive to DNAse-1 cleavage thanks to exposed transcription factor binding sites [116]. DNA sequencing technology is used to identify and map enhancers via DNase1. The histone proteins flanking enhancer regions provide further evidence thanks to unique protein modifications [73]. The histones flanking active enhancer regions are typically modified with H3K27ac and H3K4me1, while inactive enhancers are bound by nucleosomes often modified with repressive methylation marks. More recently, a new technique called ATAC-seq (Assay for Transposase

accessible Chromatin Sequencing) has developed to measure exposed chromatin [110]. ATAC-seq uses a Tn5 transposase to simultaneously fragment and tag portions of DNA free of nucleosomes, which are then amplified and sequenced. The advantage of ATAC-seq versus the DNase hypersensitivity method is the increased nucleotide resolution and efficiency of measurement.

Although a single enhancer can activate the expression of a gene, high levels of cell type specific expression of genes are typically associated with enhancer rich regions of the genome. Genome regions particularly rich with multiple enhancers are termed super-enhancers [117,118]. A super-enhancer is a combined group of enhancers typically within ~12.5kb of each other enriched with a higher volume of transcriptional coactivators, like Mediator (Med1), P300, and BRD4, when compared to typical enhancers [118,119] (Figure 1-5). P300 is an acetyltransferase which adds acetyl groups to surrounding histones. BRD4 and Med1 bind to those acetyl groups and recruit RNA Pol II to stimulate transcription [75,120,121]. H3K27ac and H3K4me2/3 are similarly more concentrated around super-enhancers than typical enhancers [117-119]. Super-enhancers tend to span larger regions than a typical enhancer, but total length of a super-enhancer region is variable and can range from 10 kb to 100 kb while the average length of a typical enhancer is about 1 kb. The defining features of a super-enhancer are the exceptionally high enrichment of transcriptional activators and chromatin marks, as identified by chromatin immunoprecipitation and whole genome DNA sequencing, ChIP-seq (Chromatin Immunoprecipitation sequencing), and exceptionally high transcription of the target gene.

Super-enhancers can direct and maintain cell identity by regulating lineage-defining genes during cell development [122,123]. Initially studied in ESCs, Klf4 and Esrrb are transcription factors which are necessary to maintain pluripotency [117]. Klf4 and Esrrb are both regulated by an associated super-enhancer region. Additionally, most super-enhancers identified in ESCs bind both transcription factors. However, super-enhancers are not limited to pluripotent ESCs. Transcription factors Foxo1 and Ebf1 are needed for B cells to develop beyond common lymphoid progenitors [117,124]. Both transcription factors are regulated by super-enhancers, which are further enriched in pro B cells. In CD4+ T-cells, the master transcription factors T-bet, Gata3, RORγt, and Bcl6 are each regulated by a predicted super-enhancer in their respective lineage [123]. Additionally, these transcription factors preferentially bind within super-enhancer regions linked to their respective target cytokine genes [123,125]. Most lineage defining genes of CD4+ T-cells, including transcription factors, cytokines, and cytokine receptors, have a predicted super-enhancer associated with their transcription during polarization.

Super-enhancers have been increasingly analyzed for their role in transcription and cell development, but we lack a complete understanding of functional differences between super-enhancers and typical enhancers. Super-enhancers are differentiated from typical enhancers by the concentration of transcription factors bound to the enhancer region, total levels of chromatin marks at the enhancer region, and the total length of the enhancer region as defined by Whyte et al [118,119]. These predicted super-enhancers tend to be located in the genome next to highly transcribed, lineage-specific genes. However, super-enhancers have not been shown to induce transcription more effectively than a typical enhancer, *in-vitro*, a key definition of an enhancer. Additional

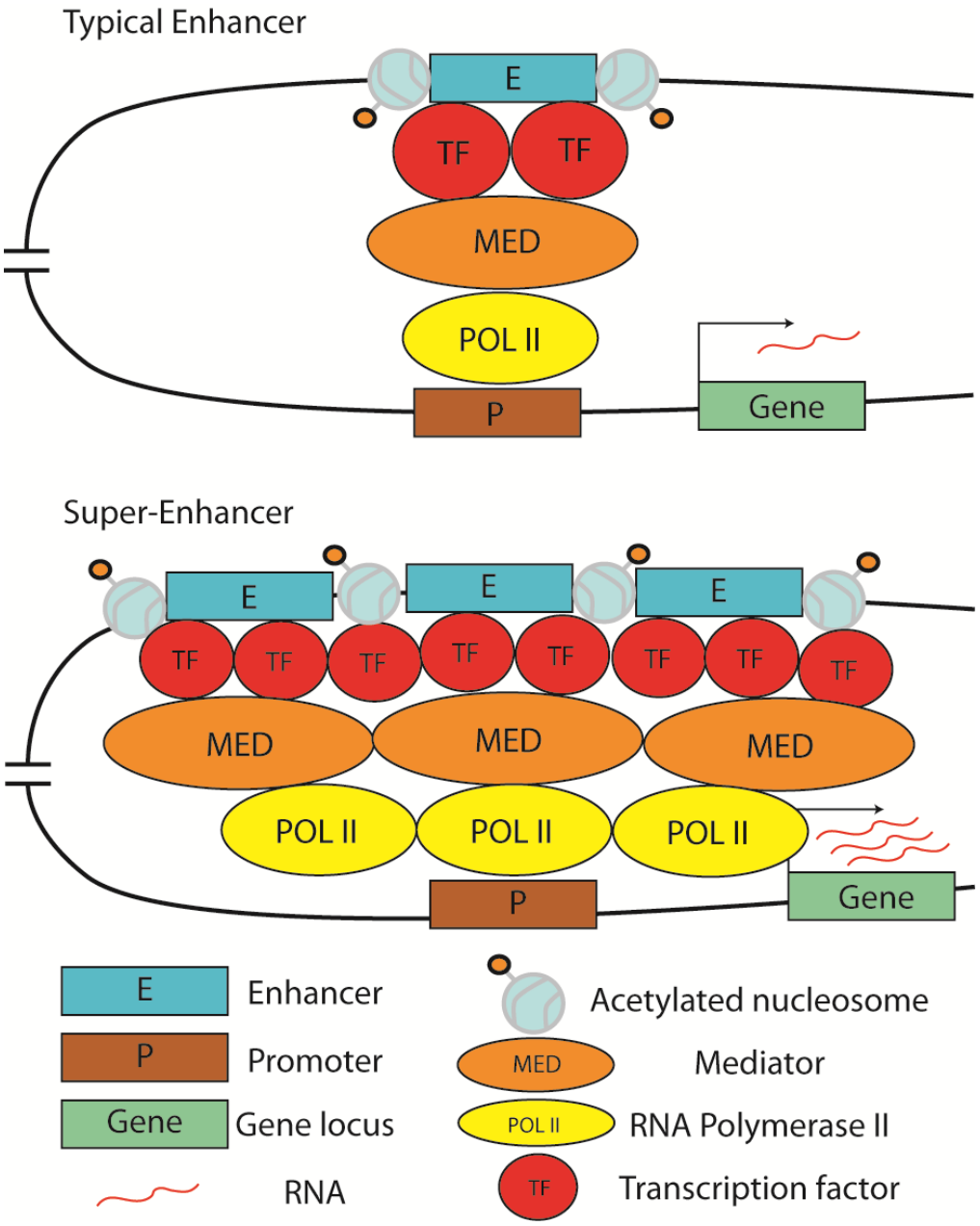

Figure 1-5. Typical and Super-Enhancer Regulation. Transcription factor binding to enhancers results in recruitment of the Mediator complex, helping to attract the RNA Pol II complex to a gene specific promoter. Super-enhancers increase the concentration of factors, increasing transcription rate of the target gene locus.

research evaluating the unique functions of super-enhancers will allow for a more uniform definition separating super-enhancers from typical enhancers.

Summary

The following three chapters will examine the contributions of unique cell specific epigenetic factors to the expression of T-helper cell specific genes. These studies evaluate regulation primarily in T-helper polarized cells but extend to include memory T cells and NK cells. The first chapter examines the role of a divergent lncRNA, *GATA3-AS1*, in the expression of Gata3 and the polarization of TH2 cells. *GATA3-AS1* is necessary for effective TH2 polarization and regulates Gata3 expression by recruiting a chromatin modifying complex to remodel the *GATA3* gene locus. The second chapter examines the methods for evaluating R-Loop formation within cells using the DRIP (DNA-RNA immunoprecipitation) assay with the S9.6 antibody. The final chapter demonstrates the necessary role of a super-enhancer in IFN-γ expression amongst multiple cell lineages. IFN-γ expression is reduced in all cells following treatment with the bromodomain inhibitor, JQ1, but expression is recovered after removing the treatment from TH1 cells. Our results suggest that bromodomain inhibitors may disrupt the function of both innate and adaptive arms of the immune response. Bromodomain inhibitors are being developed for treatment of certain malignancies as well as inflammatory disease and our findings suggest that a consequence of bromodomain inhibitors as treatments for disease may be to inhibit the normal immune response.

CHAPTER II

DIVERGENT LNCRNA GATA3-AS1 REGULATES GATA3 TRANSCRIPTION IN T-HELPER 2 CELLS

Abstract

Long non-coding RNAs (lncRNAs) possess a diverse array of regulatory functions including activation and silencing of gene transcription, regulation of splicing, and coordinating epigenetic modifications. *GATA3-AS1* is a divergent lncRNA gene neighboring *GATA3*. Gata3 is considered the master regulator of TH2 lineage commitment enabling TH2 effector cells to efficiently transcribe genes encoding cytokines IL-4, IL-5, and IL-13. Here, we show that the *GATA3-AS1* lncRNA is selectively expressed under TH2 polarizing conditions and is necessary for efficient transcription of *GATA3*, *IL5*, and *IL13* genes, while being sufficient to induce *GATA3* expression. *GATA3-AS1* is required for formation of permissive chromatin marks, H3K27 acetylation and H3K4 di/tri-methylation, at the *GATA3-AS1-GATA3* locus. Further, *GATA3-AS1* binds components of the MLL methyltransferase and forms a DNA-RNA hybrid (R-loop) thus tethering the MLL methyltransferase to the gene locus. Our results indicate a novel regulatory function for a divergent lncRNA and provide new insight into the function of lncRNAs in T helper cell differentiation.

Introduction

Long noncoding RNAs (lncRNAs) represent a new class of regulatory molecules impacting a vast array of biological functions. LncRNAs are defined as >200 nucleotides in length but possess little if any protein-coding potential [63]. LncRNA genes are oftentimes named in reference to their neighboring protein-coding genes in the genome [126,127]. Divergent lncRNAs represent one such class and divergent lncRNA gene transcriptional start sites are juxtaposed to their adjacent mRNA gene transcriptional start sites and may impact transcription of this mRNA by various mechanisms. Previous studies have implied that divergent lncRNAs may have no true function but transcription of the divergent lncRNA may make the gene locus more accessible or alternatively, compete for mRNA gene promoters or proximal enhancers [126,128,129].LncRNAs may be localized to the cytoplasm or nucleus. One common mechanism by which lncRNAs act is to recruit histone modifying machinery to gene loci and activate or repress transcription of target mRNA gene loci [103]. However, how lncRNAs find their target gene loci, which can be in cis or trans, is less well understood. One mechanism that has been described is via formation of DNA-RNA hybrids. An example is the lncRNA, *VIM-AS1*, which modifies its neighboring *VIM* gene by forming an R-loop [93]. An R-loop can form via G-Rich RNA hybridization to a DNA sequence, forming an RNA: DNA hybrid.

GATA3-AS1 represents one divergent lncRNA and shares a promoter region with *GATA3*. The Gata3 transcription factor is considered the master transcriptional regulator of T Helper 2 (TH2) lineage commitment [130] and is required for induction of *IL4*, *IL5*, and *IL13*, genes required for expression of sentinel TH2 cytokines [131]. *GATA3-AS1* levels

are elevated in human TH2 cells compared to other T helper cell subsets [117]. Expression of *GATA3-AS1* is also increased in response to allergen stimulation in patients with allergy or asthma suggesting *GATA3-AS1* may contribute to disease pathogenesis [132].

In this study, we show *GATA3-AS1* is necessary for efficient transcription of Gata3 as well as *IL5* and *IL13* genes. *GATA3-AS1* both binds to the MLL H3K4 methyltransferase and forms an R-Loop within its own locus to facilitate chromatin remodeling within the *GATA3-GATA3-AS1* locus.

Materials and Methods

Cell Culture, RNA Isolation and Quantitative RT-PCR. Human peripheral blood mononuclear cells (PBMC) were cultured under TH0, TH1, TH2, and TH17 polarizing conditions as previously described [117]. Cultures were harvested after 5 (TH1, TH2) or 7 days (TH17). Cultures were also re-stimulated with anti-CD3 for 2 days for analysis of effector cells (TH1-E, etc.). Total RNA isolation, cDNA synthesis using poly-A selection and analysis by quantitative PCR (qPCR) was performed as previously described [117]. Expression levels of target transcripts were normalized to levels of *GAPDH* using the formula 2(*GAPDH* Ct-target gene Ct). Primer Pairs used in qPCR reactions are listed in Supplementary Table 1. The study was approved by the institutional review board at Vanderbilt University Medical Center. Written informed consent was obtained at the time of blood sample collection.

Cell Fractionation Assay. Human PBMC were incubated to produce TH2 primary and effector populations. Cytoplasmic and nuclear fractions were isolated using

a PARIS kit (AM1921, ThermoFisher). RNA from each fraction was isolated as described above.

RNAi Transfections. Human PBMC were incubated for a total of 5 days under TH2-polarizing conditions. Cells were transfected after 2d of culture with Lipofectamine RNAiMax (Life Technologies) using either an inventoried Silencer Select negative control siRNA or custom designed Silencer Select siRNA for *GATA3-AS1* (DesignID: AD0IWKB and AD1RUQJ), or *GATA3* (DesignID: AD6RNGV amd AD5IPAN) per supplied protocols. Cells were harvested after 5 days and used for either RNA analysis via qPCR, ChIP analysis, ELISA, and Western Blot

Enzyme Linked Immunosorbent Assay (ELISA). Elisa assays were performed according to instructions provided by the kits to analyze IL-4 (555194,BD Biosci), IL-5 (555202, BD Biosci), IL-13 (88-7439-88, Invitrogen), and IFN-γ (555142, BD Biosci) proteins. Cultures were performed as described under RNAi transfections. Cultures were harvested and analyzed by ELISA.

Western Blot. Cells were lysed with RIPA buffer supplemented with protease inhibitors (cOmplete Mini, Roche) and phosphatase inhibitors (PhosStop inhibitor cocktail, Roche). Protein concentration of each sample was determined by Pierce BCA Protein Assay kit. Lysates were subjected to SDS/PAGE followed by blotting with the indicated antibodies. Signal was detected using the IR-dye conjugated secondary antibodies and the Odyssey scanner (Li-cor Biosciences). Antibodies against the following proteins were used: GATA3 (#199428, Abcam) and β-Actin (#47778, Santa Cruz).

***In-Vitro* Transcription.** Full length *GATA3-AS1* was generated by PCR amplification, agarose gel purified using a QIAquick gel extraction kit (28704, QIAGEN) and cloned into a TOPO-TA dual promoter transcription vector (K462001, ThermoFishter). Clone identify was verified by digestion of plasmids with Spe1 (R0133S, NEB) and Not1 (R0189S, NEB), and DNA sequencing via GENEWIZ. *GATA3-AS1* transcripts were produced via the T7 promoter using the maxiscript T7 transcription kit (AM1312, ThermoFisher). Full length transcripts were transfected into TH0 cells at day 2, at concentrations of 0.5 uM and 0.1 uM similar to RNAi transfections.

Chromatin immunoprecipitation (ChIP). ChIP procedures were as previously described [117] using an anti-H3K4me2/3 (ab6000, Abcam), anti-H3K27ac (ab4729, Abcam), or anti-mouse IgG (sc-2025, SantaCruz Biotech). DNA was isolated from beads via phenol chloroform extraction and purified using QiaQuick PCR purification kits. Isolated chromatin was analyzed using SYBR-Green qPCR (Applied Biosystems). Values were expressed as fraction of total input from chromatin samples.

RNA-immunoprecipitation (RIP). RIP assays were performed as described previously [117]. Briefly, TH2 primary cultures were harvested, lysed, and chromatin sheared by sonication followed by incubation with an isotype IgG control antibody (sc2025, SantaCruz Biotech), anti-WDR5 (ab56919, Abcam), or anti-p300 (ab14984, Abcam) overnight at 4°C. Protein A/G beads (sc2003, SantaCruz Biotech) were added to lysates and incubated at 4°C for an additional 4 hours. Beads were pelleted, supernatants harvested, and beads were washed and suspended in Tri-Reagent. RNA was isolated and analyzed via qRT-PCR as described above.

DNA-RNA Hybrid Immunoprecipitation (DRIP). DRIP assays were performed as described [133] using the track 17 in development of protocol. Briefly, TH2 primary cultures were harvested, lysed, and chromatin sonicated at 2x10 min (medium, Diagenode Bioruptor) to yield an average chromatin size of ~500 bp. Samples were treated with proteinase K at 65°C overnight. Total nucleic acid was isolated by phenol chloroform extraction. Six micrograms of nucleic acid was rotated overnight with S9.6 antibody followed by incubation with protein A/G beads (SantaCruz Biotech) for 4 hours. Beads were subsequently washed 4 times followed by isolation via Tri-Reagent for RNA or phenol chloroform extraction for DNA. Samples were analyzed by qPCR as described above.

Results

Selective expression of *GATA3-AS1* by TH2 populations. Genes encoding *GATA3-AS1* and *GATA3* are adjacent to each other on human chromosome 10 (Figure 2-1A). *GATA3* is transcribed in the sense orientation and *GATA3-AS1*, the antisense orientation. Transcriptional start sites for *GATA3* and *GATA3-AS1* are separated by ~1200 bp. Thus, *GATA3* and *GATA3-AS1* belong to the general class of divergent lncRNA/mRNA pairs. At least six *GATA3-AS1* splice variants have been identified that utilize four major exons. Whole genome RNA-sequencing confirmed multiple regions were transcribed in primary (upper panel) and effector (lower panel) TH2 cultures (Figure 2-1B). Each exon and intron region of *GATA3-AS1* was evaluated in TH2 primary cells with targeted primer pairs to analyze total expression at these sites.

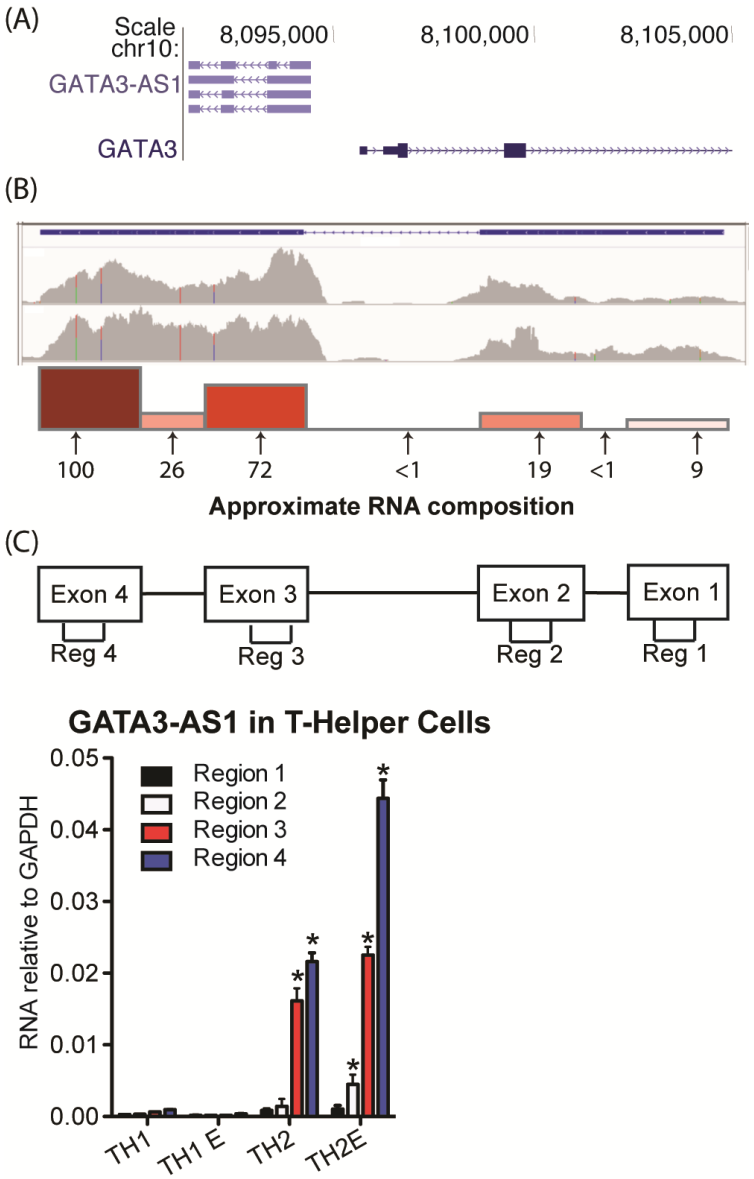

Figure 2-1. *GATA3-AS1* is expressed in TH2 cells. (A) Genomic locations of human *GATA3-AS1* known isoforms and GATA3 with directions of transcription in sense (>) and antisense orientations (<) from UCSC genome browser (hg19 build). (B) Whole genome RNA-seq tracks of *GATA3-AS1* expression in primary (upper) and effector (lower) TH2 cells, Y-axis is in FPKM. Approximate composition of *GATA3-AS1* transcripts in TH2 primary cells was verified by PCR. Arrows indicate approximate genomic locations of seven PCR primer pairs used for the analysis. Numbers below the arrows represent relative transcript amounts at each primer location, and are expressed relative to the highest expressed region. Also depicted as a bar graph above the arrows. (C) PCR primer pair locations used to measure exons 1-4 designated as regions 1-4, respectively. Expression levels of *GATA3-AS1* regions 1–4 in TH1, TH1-E, TH2, and TH2-E, subsets. Results are expressed relative to levels of GAPDH (n = 3), Statistical significance vs. TH1 effector cells was determined by Students T-test. *P < 0.05.

GATA3-AS1 transcripts contained the four predicted exons, as well as the most downstream intron (Figure 2-1B). The first and second predicted introns were not present. We decided to examine the four predicted exons, designated regions 1-4, to determine their expression levels in T helper cell cultures. We found that regions 1-4 were selectively expressed in primary and effector TH2 cultures relative to primary and effector TH1 cultures (Figure 2-1C). Regions 3 and 4 exhibited higher expression levels than regions 1 and 2 consistent with our RNA-sequencing results. We subsequently searched for TH2-specific noncoding RNAs in the mouse genome around Gata3. We examined published RNA-seq data [134] and identified three lncRNA genes in the vicinity of Gata3 selectively expressed in TH2 cultures relative to TH1 and TH17 cultures. These were 103 kb, 290 kb, and 336 kb, respectively, 3' of Gata3, named lincR-Gata3-3'S-336K (S=transcribed in sense direction relative to Gata3), lincR-Gata3-3'S-290K, and lincR-Gata3-3'. Given their genomic distances from Gata3, these did not fall into the general divergent lncRNA class.

LncRNAs may function in the nucleus or cytoplasm [135]. Therefore, we asked if *GATA3-AS1* existed primarily in nuclear or cytoplasmic compartments of TH2 cells. We isolated nuclear and cytoplasmic fractions, isolated total RNA from each fraction and evaluated *GATA3-AS1* levels by PCR. *HPRT* was used as a cytoplasmic specific control mRNA, while *VIM-AS1* represented our nuclear specific control [93]. We found that *GATA3-AS1* was located primarily within the nuclear fraction (Figure 2-2A). We compared the kinetics of induction of *GATA3-AS1* under TH2 polarizing conditions to induction of *GATA3, IL4, IL5,* and *IL13* (Figure 2-2B, 2-2C). We found a continual

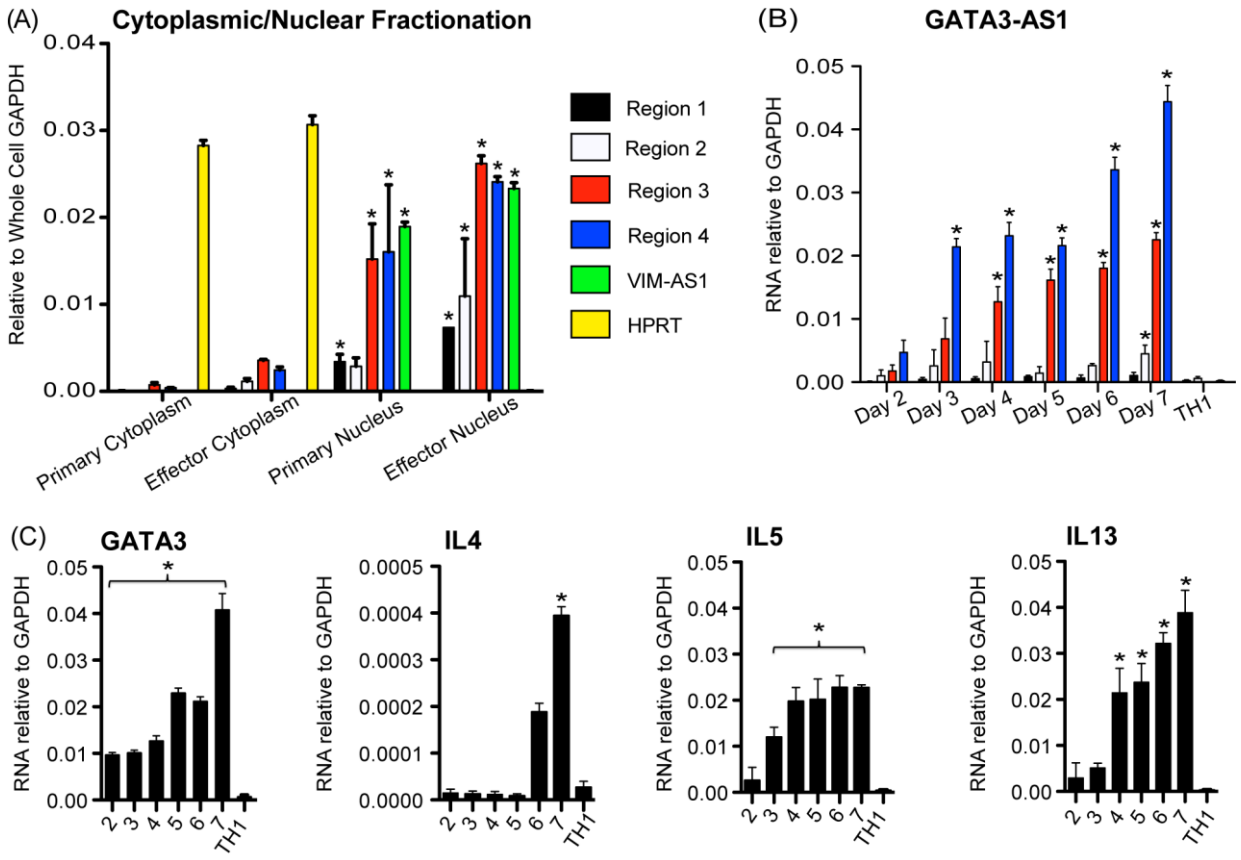

Figure 2-2. *GATA3-AS1* is localized in the nucleus and expression increases during TH2 cell polarization. (A) Cellular and nuclear fractions of primary and effector TH2 populations analyzed via qPCR. Values represent ΔΔCT vs. whole cell *GAPDH*. *VIM-AS1* lncRNA is a nuclear control, while *HPRT* mRNA represents a cytoplasm specific control. Statistical significance vs. relative cytoplasmic fraction was determined by Students T-test (n = 3). *P < 0.05 (B) Total PBMCs were cultured under TH1 or TH2 polarizing conditions, and RNA was isolated on consecutive days. Mean ± S.D. gene transcripts of *GATA3-AS1* were quantified via qPCR and normalized to *GAPDH*. (C) Similar analyses were completed for *GATA3, IL4, IL5, and IL13*. Statistical significance vs. TH1 effector cells on day 7 was determined by Students T-test (n = 3). *P < 0.05.

increase in expression *of GATA3, IL4, IL5,* and *IL13* over time. *GATA3-AS1* displayed a similar increase in transcript levels as a function of time after stimulation. Thus, *GATA3-AS1* induction paralleled induction of genes known to mark the TH2 differentiation pathway.

Depletion of *GATA3-AS1* disrupts induction of *GATA3, IL13* and *IL5* genes.

To further explore possible functions of *GATA3-AS1*, we designed an siRNA to target the most highly expressed region of the lncRNA, region 4. Total human PBMCs were cultured under TH2 conditions and the *GATA3-AS1* specific siRNA was transfected into cells on day 2. A scrambled siRNA with nonspecific target was used as a negative control. Total RNA was isolated on day 5 when cells reached primary stage. We found that *GATA3-AS1* regions 2, 3, and 4 were significantly reduced after transfection compared to transfection with the scrambled control siRNA (Figure 2-3A). Transfection with the *GATA3-AS1* specific siRNA also led to a marked reduction in *GATA3, IL13*, and *IL5* mRNAs. GATA3-AS1 reduction similarly reduced protein levels of IL-5, IL-13, (Figure 2-3B) and Gata3 (Figure 2-3C). IFN-γ production was not significantly impacted by transfection in TH1 cells, showing minimal off target effects. This demonstrates the *GATA3-AS1* transcript is required for effective induction of major TH2 genes. In contrast, *IL4* showed increased transcript levels following the lncRNA knockdown. This is consistent with previous studies showing that knockdown of *GATA3* may induce compensatory effects resulting from increased STAT6 binding to the *IL4* locus [31]. Alternatively, more complete depletion of *GATA3* may be required to abrogate *IL4* induction in this model system. Taken together, these results demonstrate that *GATA3-*

AS1 plays an important role in *GATA3* induction during initial primary TH2 differentiation. Our interpretation is that depletion of *GATA3-AS1* results in loss of

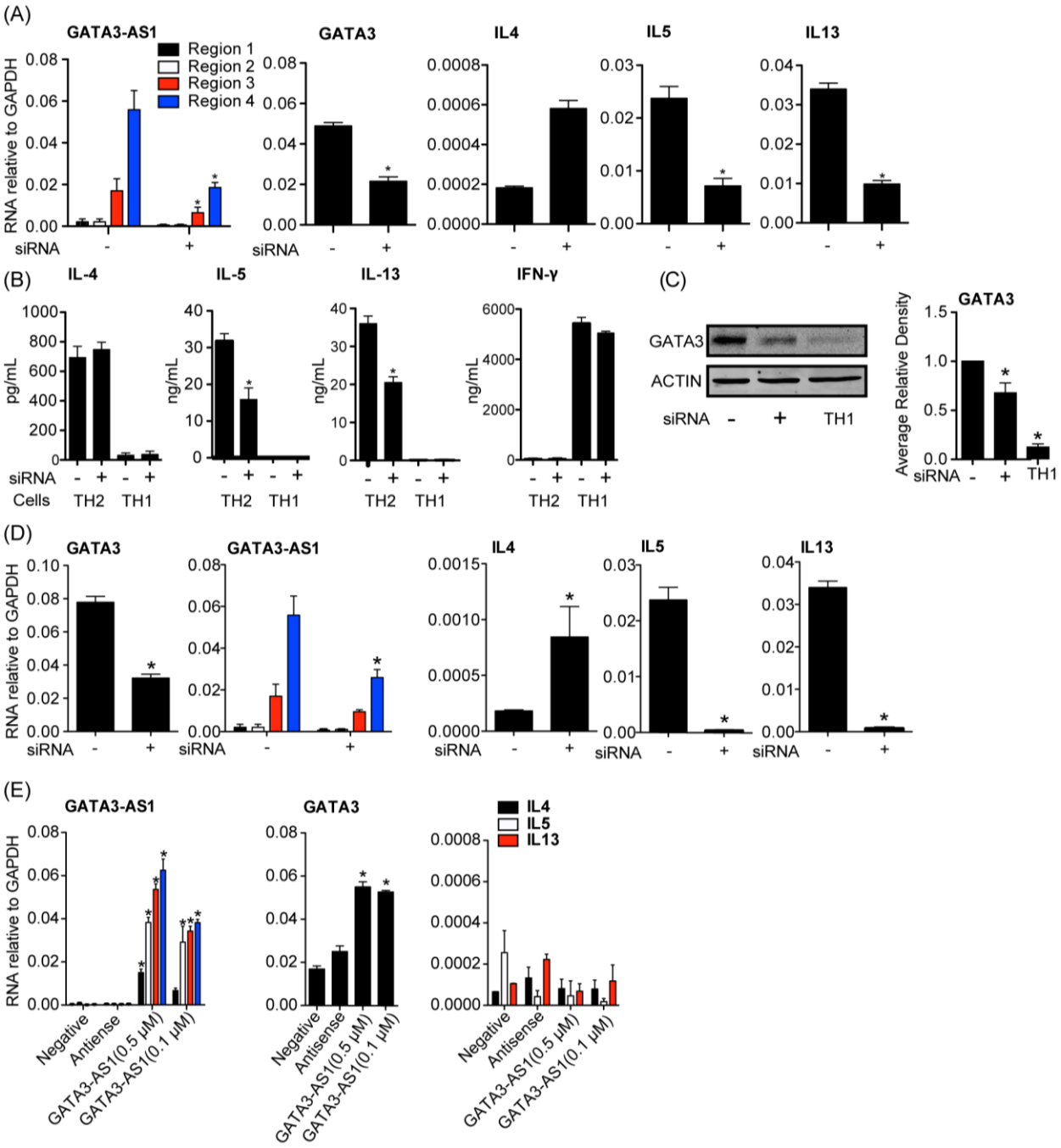

Figure 2-3. *GATA3-AS1* and *GATA3* form a necessary feed forward loop regulating TH2 polarization. (Previous Page) (A) PBMCs were cultured under TH2 polarizing conditions for 2 days and transfected with a GATA3-AS1 specific siRNA (+) or scrambled siRNA (–). GATA3-AS1, GATA3, IL4, IL5, and IL13 transcripts were determined by qPCR on day 5 and results expressed relative to GAPDH. Statistical significance was determined using Students T-test by comparing GATA3-AS1 siRNA knockdown to scrambled control knockdown (n = 3). *P < 0.05 (B) PBMCs were cultured under TH2 or TH1 polarizing conditions and transfected with GATA3-AS1 specific siRNA (+) or scrambled siRNA (–). Protein was analyzed by ELISA (for IL-4, IL-5, and IL-13, n = 3,) or (C) Western Blot (GATA3, Densitometry). Statistics were calculated using a Paired T-test compared negative siRNA controls. *P < 0.05. (D) Similar to A, but an siRNA specific to GATA3 was transfected on day 2. Results and statistics similar to A, but (n = 4). (E) PBMCs were cultured under TH0 conditions for 2 days, and transfected with GATA3-AS1 transcripts produced from Topo-TA *in-vitro* transcription vector. Cells were transfected with a scrambled siRNA (negative), the antisense of GATA3-AS1(antisense), GATA3-AS1 at 500 μM (AS1 High), and GATA3-AS1 at 100 μM (AS1 Low). Analysis and statistics completed similarly to A and B, (n = 5).

induction of *GATA3* under TH2 differentiation conditions leading to reduced IL-13 and IL-5 expression.

Despite expanding literature on lncRNAs, impact of the mRNA partner on transcription of a divergent lncRNA is not well understood. Gata3 induction creates a positive feedback loop, by binding to its own promoter region [136,137]. Because of the shared promoter region, Gata3 protein binding may impact the expression of *GATA3-AS1* during polarization. To test this hypothesis, we decreased *GATA3* levels via siRNA knockdown to evaluate its impact on the expression of *GATA3-AS1* (Figure 2-3D). *GATA3-AS1* showed a significant reduction following *GATA3* depletion, indicating

GATA3 is also required for effective *GATA3-AS1* expression. Thus, *GATA3-AS1* is necessary for effective induction of *GATA3*, while *GATA3* subsequently increases *GATA3-AS1* transcript levels creating a kind of feed-forward loop.

Above results demonstrated that siRNA-mediated knockdown of *GATA3-AS1* reduced *GATA3* induction under TH2 polarizing conditions. Therefore, we asked if elevated *GATA3-AS1* was sufficient to induce *GATA3* expression. We used a Topo-TA cloning vector to produce full length transcripts of *GATA3-AS1* and verified the product via sequencing. We transfected *GATA3-AS1* into TH0 cells at two different concentrations (0.5 and 0.1 μM) and analyzed *GATA3* and genes encoding TH2 cytokines. We found *GATA3* transcript levels were significantly higher following transfection of *GATA3-AS1*, but this transfection did not induce genes encoding TH2 cytokines (Figure 2-3E). Thus, *GATA3-AS1* is sufficient to increase *GATA3* in non-polarized T-cells, further demonstrating that regulation *GATA3* via *GATA3-AS1* is dependent on the RNA transcript, not on the act of its transcription.

***GATA3-AS1* binds and recruits MLL methyltransferase via WDR5.** Several lncRNAs localized in the nucleus can modify transcription of target genes by impacting the state of chromatin in the region [138]. This can be achieved by facilitating binding of large histone modifying complexes to chromatin and/or direct interaction with DNA [83]. To investigate the effect of *GATA3-AS1* on chromatin marks, we performed the knockdown of *GATA3-AS1* as described above and isolated chromatin from TH2 primary cells on day 5. We processed chromatin for ChIP assays and performed immunoprecipitations with antibodies specific for either H3K27ac or H3K4me2/3 marks. We designed a series of PCR primer pairs to interrogate genomic regions across

GATA3-AS1 and *GATA3* genes (Figure 2-4A). Following knockdown of *GATA3-AS1* via siRNA transfection, we found significantly decreased H3K27ac and H3K4me2/3 activating marks across *GATA3-AS1* and *GATA3* genomic regions including the shared promoter (Chip 3-5), *GATA3-AS1* introns and *GATA3* introns (Figure 2-4B, 2-4C). The variation in chromatin marks was not present in chip primers 9 and 10, located after the second exon of the *GATA3* gene. These results indicate that *GATA3-AS1* is required for adequate addition of H3K27ac and H3K42/3me marks to both its own gene locus, the shared promoter region and the *GATA3* locus.

Previous studies have demonstrated that one mechanism that lncRNAs can alter the epigenetic code is by binding to chromatin modifying complexes to facilitate their recruitment to target genomic loci [139]. An example is the MLL H3K4-methyltransferase complex of which WDR5 is an essential component [140]. To investigate this possible interaction, we performed RNA immunoprecipitations using antibodies specific for WDR5. We isolated RNA from the immunoprecipitates and analyzed recovery of *GATA3-AS1* transcripts via qPCR. We found that a significantly higher portion of *GATA3-AS1* regions 3 and 4 were immunoprecipitated with antibodies to the WDR5 protein than the pan-hnRNP negative control antibody or an isotype control (Figure 2-4D). We performed a similar immunoprecipitation using antibodies to p300. p300 is one chromatin modifying enzyme responsible for formation of H3K27ac marks [141]. Unlike the MLL methyltransferase, there was no detectable interaction between *GATA3-AS1* and the p300 complex in TH2 cells (Figure 2-4E). These results demonstrate *GATA3-AS1* binds the MLL H3K4-methyltransferase complex via interactions involving primarily region 4.

***GATA3-AS1* forms an R-Loop.** An R-loop is the formation of a DNA:RNA:DNA triplex, in which an RNA binds within its own gene region, instead of being released

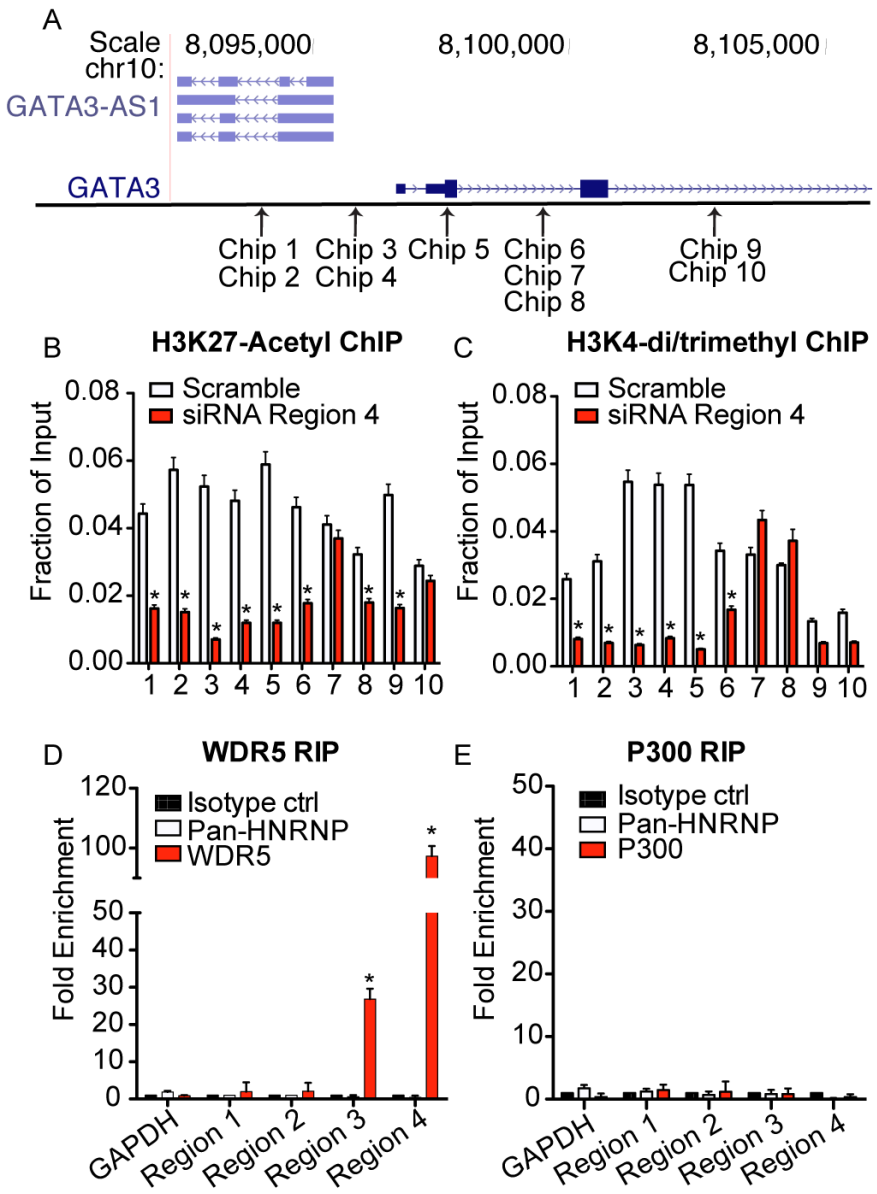

Figure 2-4. Expression of *GATA3-AS1* changes chromatin marks throughout the *GATA3-AS1-GATA3* gene locus. TH2 cultures were transfected with an siRNA specific for *GATA3-AS1* region 4 or a scrambled control siRNA on day 2 as described in Figure 2-3. Cultures were harvested and processed for ChIP assays on day 5. Results are expressed as fraction of input, mean ± S.D. (n = 3). *P < 0.05. (A) Approximate genomic positions of PCR primers used for ChIP assays. (B) ChIP assays for H3K27ac. (C) ChIP asays for H3K4me3. (D) WDR5 was immunoprecipitated from TH2 effector whole cell lysates. RNA was isolated from immunoprecipitated WDR5, and GATA3-AS1 regions 1–4 determined by qPCR. Results are expressed as fold enrichment relative to isotype control, mean ± S.D. (n = 3). *P < 0.05. (E) As in (D), except an antibody to p300 was employed for RNA immunoprecipitation.

following polymerase activity as normally occurs. R-loops, when formed at the transcriptional start site may produce an open gene region to promote transcription [142]. R-Loops typically form within a region of high G-C skew. In a recent study [93], *VIM-AS1* was found to form an R-loop within the VIM gene region. Using the recently developed R-loop database [143], we searched the *GATA3-AS1* and *GATA3* genomic locus and identified the intron between regions 2-3 of *GATA3-AS1* as having a high probability of forming an R-Loop because of the high relative G-C skew throughout the region (Figure 2-5A). We predicted that formation of an R-Loop may help tether the lncRNA to this region, and to test this hypothesis, we performed a DNA-RNA immunoprecipitation assay (DRIP). The DRIP assay follows a standard ChIP assay protocol, but uses the S9.6 antibody, specific for RNA-DNA hybrid structures [133]. The DRIP assay allows for analysis of both the bound RNA transcript, as well as the region of DNA to which it has bound. The RNA typically stays localized to its own gene locus, but the formation of the R-Loop at the transcription start site or the termination site indicates different regulatory functions. We completed the DRIP and isolated RNA samples from immunoprecipitates and found that all four regions of the *GATA3-AS1* lncRNA were enriched in the immunoprecipitates (Figure 2-5B). Regions 1, 2, and 3 were significantly enriched compared to the GAPDH control similar in magnitude to the *VIM-AS1* positive control. We performed a similar immunoprecipitation but isolated the DNA fraction via phenol chloroform extraction. We detected R-Loop formation in an RNase H dependent manner located within the G-C rich central intron of GATA3-AS1 (Figure 2-5C). These results, taken together, indicate that *GATA3-AS1* binds the DNA region within the central intron

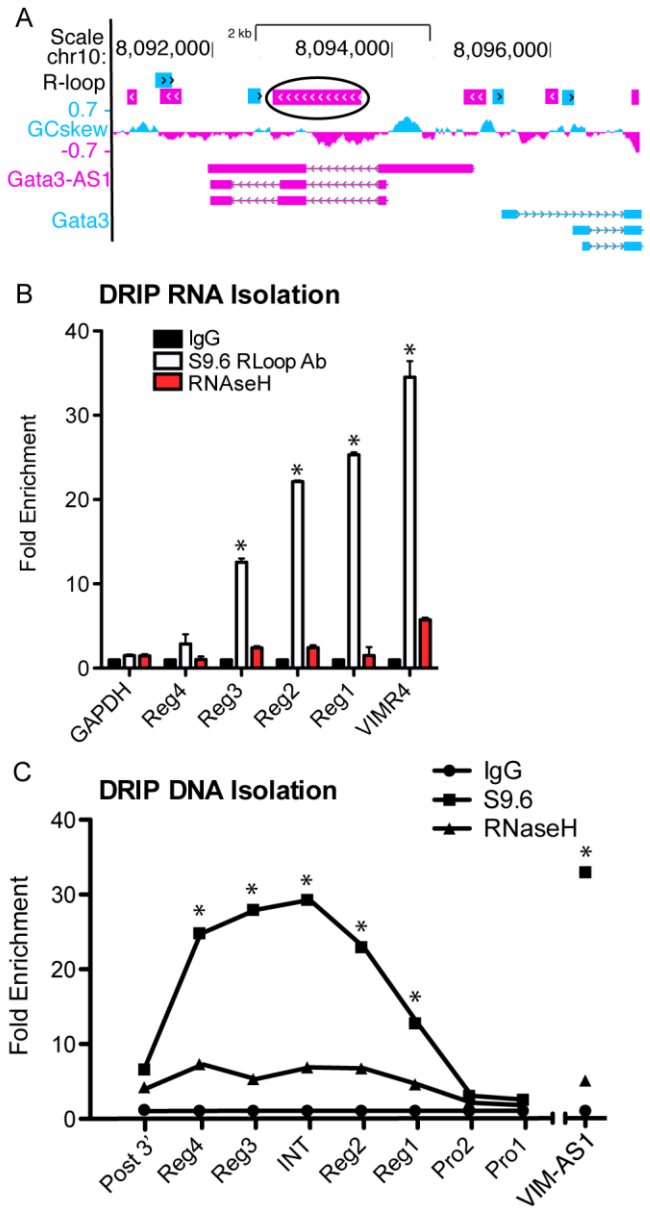

Figure 2-5. *GATA3-AS1* RNA forms an R-Loop with the central intron of *GATA3-AS1*. (A) Predictive R-Loop formations according to the R-Loop database. The largest and most likely R-Loop is circled, located between regions 2 and 3 of *GATA3-AS1*. (B) DRIP assay completed with RNA isolation. Results are expressed as fold enrichment relative to the RNase H negative control. Chromatin was treated with RNase to remove R-Loops as an additional control, mean ± S.D. (n = 3). *P < 0.05. (C) DRIP assay similar to (B), but DNA was isolated from the R-Loop immunoprecipitates. PRO1 and 2 represent the shared promoter region, INT is the central intron of GATA3-AS1, and Post3' is past the GATA3-AS1transcriptional end site. Statistical evaluations were performed using Students t-test vs. Rnase H negative control. All data represent mean± S.D. as fraction of input (n = 4). *P < 0.05.

of *GATA3-AS1* and could represent a mechanism by which the divergent lncRNA is able to recruit chromatin modifying complexes to the *GATA3* gene locus.

Discussion

We show that the lncRNA, *GATA3-AS1*, is selectively expressed under TH2 differentiation conditions, is primarily localized to the nucleus and is necessary for efficient expression of *GATA3* during TH2 lineage commitment and expression of *IL5* and *IL13*, genes encoding cytokines critical for TH2 cell function. Further, *GATA3-AS1* lncRNA is sufficient to induce *GATA3* expression further indicating this divergent lncRNA has biologic function, though it cannot induce total TH2 polarization independent of other factors such as c-Maf or STAT6. *GATA3-AS1* is necessary for chromatin remodeling at the *GATA3-AS1-GATA3* locus. Chromatin modifications are accomplished by the ability of *GATA3-AS1* RNA transcripts to specifically bind the MLL H3K4-methyltransferase complex and tether it to the *GATA3-AS1-GATA3* locus. *GATA3-AS1* also forms a DNA-RNA hybrid within the *GATA3/GATA3-AS1* locus, termed an R loop suggesting one mechanism by which *GATA3-AS1* may recruit the MLL H3K4-methyltransferase complex to the locus.

In addition to *GATA3-AS1*, lncRNAs are involved in additional aspects of TH cell differentiation and function. For example, the lncRNA, *TH2LCRR*, is selectively transcribed from the TH2 locus control region (LCR) in developing TH2 cells, binds the MLL H3K4-methyltransferase complex, is necessary for chromatin remodeling at *IL4*, *IL5* and *IL13* genomic loci and induction *of IL4, IL5,* and *IL13* genes in response to TH2 lineage commitment [117]. It is not known if TH2LCRR forms a DNA-RNA hybrid as

described here but this seems possible. The lncRNA, *LincR-Ccr2-5'AS*, is induced under TH2 polarizing conditions and regulates induction of genes encoding chemokine receptors, including Ccr2, as well as TH2 cell migration, in vivo [134]. In contrast to *GATA3-AS1* and *TH2LCRR*, its mechanism of action is not known but this lncRNA does not seem to contribute to chromatin remodeling at these gene loci. *IFNG-AS1* (*Tmevpg1, NeST*) is critical for *IFNG* expression during TH1 lineage commitment, by T effector memory cells, and *in-vivo* [103-105,144]. The lncRNA, *Rmrp*, plays an important role in TH17 lineage commitment by regulating interactions between *Ror-γt* and its RNA helicase DEAD-box protein 5 (DDX5) binding partner [145]. Thus, lncRNAs regulate many facets of TH cell differentiation and function, and *GATA3-AS1* significantly contributes to TH2 development.

GATA3-AS1 can be considered one member of the class of divergent lncRNAs in which the transcriptional start site of the lncRNA gene is very close (~100-1000 bp) to the transcriptional start site of the adjacent mRNA gene. In this case, it has not been clear if the lncRNA actually has a function. It has been proposed that these divergent lncRNA/mRNA pairs may share common promoters or proximal enhancers and that competition may impact gene expression rather than the RNA that is produced from the divergent lncRNA gene. Alternatively, the very act of transcription of the lncRNA gene may facilitate chromatin remodeling to alter transcription of the neighboring mRNA gene. Our results are consistent with the interpretation that the *GATA3-AS1* lncRNA, as opposed to these other proposed mechanisms, is necessary for the adequate transcription of *GATA3*. Importantly, the *GATA3-AS1* transcript alone is sufficient to induce an increase in *GATA3* expression. These results are unique among divergent

lncRNAs and show the noncoding RNA molecule as an essential feature of its regulatory capacity. Despite the increase in *GATA3* following *GATA3-AS1* transfection, *GATA3-AS1* transcripts alone could not induce TH2 polarization. Other factors, like STAT6 and c-MAF, also involved in TH2 polarization, may also be necessary.

Formation of RNA-DNA hybrids or R-loops are common, and R-loops are prevalent along chromosomes impacting many cellular processes [142]. Similarly, one mechanism by which lncRNAs are thought to act is by recruiting histone modifying enzymes to target gene loci. However, except in a few instances [146], it has not been entirely clear how these lncRNA-enzyme complexes find their target gene loci. The formation of an R-Loop by *GATA3-AS1* may allow the effective recruitment of chromatin modifying enzymes to the *GATA3-AS1-GATA3* locus, but further experiments will be necessary to demonstrate this relationship. R-loop formation may represent a general mechanism employed by lncRNAs to find their target gene loci or may be unique to divergent lncRNA/mRNA gene pairs that exist in close proximity in the genome.

CHAPTER III

IMMUNOPRECIPITATION OF DNA-RNA HYBRIDS USING THE S9.6 ANTIBODY

Abstract

Formation of DNA:RNA hybrids or R-loops contribute to numerous biologic processes. Development of the S9.6 antibody makes analysis of R-Loops (DNA:RNA hybrids) possible through immunoprecipitation. Here we describe the isolation of DNA:RNA hybrid structures through the use of the S9.6 antibody. Using this protocol, both the DNA and RNA binding partners of the R-loop can be analyzed via qPCR, whole genome sequencing or other methods.

Introduction

DNA:RNA hybrids (or R-loops) are important regulators of transcription and impact processing at initiation, elongation, and termination [90,91,93,133,147,148]. R-loops are triple stranded nucleic acid structures including a DNA:RNA hybrid strand and one displaced DNA strand [149]. R–loops can regulate transcription by blocking transcriptional silencing by preventing binding of DNA methyltransferases and subsequent DNA methylation of CpG islands [89], induce transcriptional termination by RNA polymerase pausing [150], and repress transcription by promoter occlusion4. Ranging in size from 50-2000 bp, R-loops are abundant around promoters and modify transcription to both induce and repress impacted genes [151]. Antisense and divergent long noncoding RNAs can also form R-loops to regulate transcription of neighboring mRNA genes [93,152]. R-

loops can also cause replicative stress [153], genome instability [151], and chromatin alterations that may be associated with cancer [150,154]. Effective analysis of R-loop formation and location is critical to understand transcriptional dynamics.

R-loops were previously studied using a variety of techniques including mobility shift assays, in situ hybridization, and native bisulfite modification. Analysis of R-loops greatly expanded following the development of the of the S9.6 antibody that specifically recognizes these DNA:RNA hybrids [155]. The S9.6 antibody binds to DNA:RNA hybrids in a sequence independent manner, making immunoprecipitation of the R-loop (DNA:RNA immunoprecipitation or DRIP) a standard detection method [156]. The DRIP technique, first introduced in 2010, made analysis of R-loops more accessible and comprehensive through qPCR analysis and whole genome sequencing [92]. The DRIP technique does have limitations. The S9.6 antibody binds double stranded RNA, though not as effectively as it binds DNA:RNA hybrids [91]. Additionally, formaldehyde crosslinking can increase the number of false positives detected [157]. Despite this, the DRIP assay is still by far the most effective tool to analyze R-loop locations and size.

Here we describe an immunoprecipitation procedure to analyze both the DNA and RNA fractions involved in R-Loop formation (Figure 3-1). In this protocol, cells are fixed with formaldehyde, lysed, and sonicated similar to a standard ChIP assay. R-loops are immunoprecipitated using the S9.6 antibody, followed by extraction of either the RNA or DNA fraction from the immunoprecipitate. DNA or RNA fractions can be analyzed via qPCR or sequencing to provide effective analysis of R-loop formation around a single gene or throughout the entire genome.

Materials and Methods

*Carry out all methods with samples on ice unless otherwise specified

Fixation

1. Using no more than 10 million cells, add formaldehyde directly to the media, dropwise to a final concentration of 0.75% of total volume, and rotate for 10 minutes. (Example: a 3 ml culture would receive 60.8 µl of formaldehyde).
2. Add glycine to a final concentration of 125 mM to the media, and rotate again for 5 minutes at room temperature.
3. Isolate cells by centrifuging at 4°C and 300 x g for 10 minutes.
4. Pour off the supernatant from the sample.
5. Wash once in ice cold PBS at an equivalent volume to the previous media volume.
6. Repeat step 3 and remove as much PBS as possible without disturbing the cell pellet (See Note 3).

Nuclei Isolation

Materials Used

- Hypotonic solution: 20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl210% NP-40
- R-Loop Digestion Buffer: 100 mM NaCl, 10 mM Tris pH 8, 25 mM EDTA pH 8, 0.5% SDS, 0.2 mg/ml Proteinase K (add just prior to use) (See Note 1)
- FA Lysis Buffer: 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin (See Note 2)

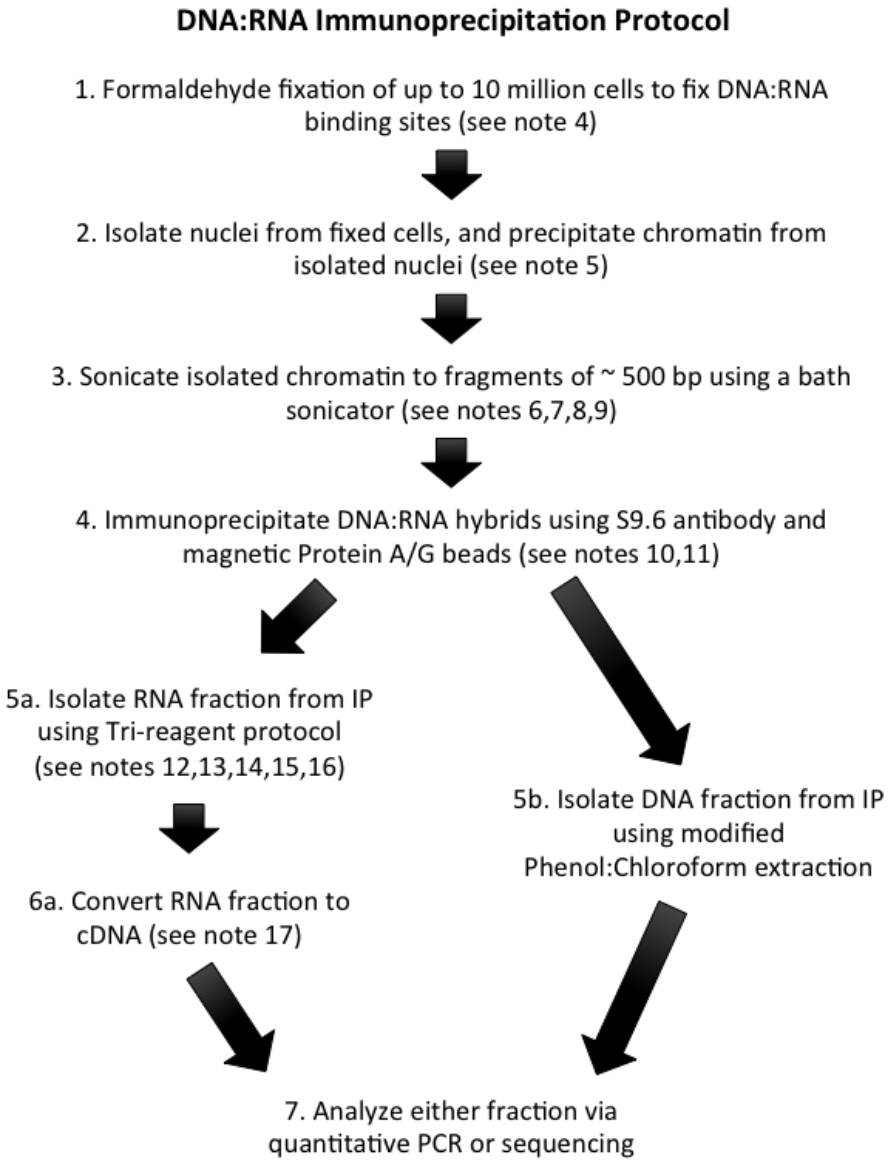

Figure 3-1. DNA:RNA immunoprecipitation protocol flowchart. Each step is uniform until after the S9.6 antibody immunoprecipitation, in which the DNA and RNA fractions are processed separately so both can be isolated from the immunoprecipitated.

1. Resuspend cell pellet in 500 µl of Hypotonic Solution and pipette up and down to mix.
2. Incubate for 15 minutes.
3. Add 25 µl of 10% NP40 detergent and vortex on highest setting for 10 seconds.
4. Centrifuge at 4°C at 720 x g for 10 minutes.
5. A pellet should be visible at the bottom of the tube containing cell nuclei. Remove the supernatant from the tube without disturbing the pellet.
6. Resuspend pellet in 1 ml of R-Loop Digestion Buffer and leave overnight in a 65°C water bath (approx. 12-16 hours). Remove samples from the water bath, and immediately place on ice.
7. Add 500 µl of 25:24:1 Phenol:Chloroform:Isoamyl Alcohol to the sample, pipetting up and down to mix effectively.
8. Centrifuge sample at 4°C at maximum speed (~21,000 x g) for 10 minutes.
9. Isolate the top aqueous layer, which will contain the nucleic acids from the sample.
10. Combine aqueous layer with ¼ volume of 9M sodium acetate (if aqueous layer is 500 µl, add 125 µl of sodium acetate).
11. Add equivalent volume of ice-cold isopropanol to the sample (if combined volume from step 5 is 625 µl, add 625 µl of isopropanol).
12. Add 3 µg of glycogen and invert tube 5 times to effectively mix. (See Note 4).
13. Place on ice for 10 minutes.
14. Centrifuge sample at 4°C and ~21,000 x g for 10 minutes.
15. Pour off supernatant and resuspend in 1 ml of 70% ethanol, followed by another centrifugation at 4°C and ~21,000 x g for 10 minutes.
16. Pour off supernatant and resuspend the sample in 500 µl of FA Lysis Buffer

Sonication

Materials used:

- Diagenode Bioruptor Sonicator
- New England Biolabs 100 bp DNA ladder
- New England Biolabs 6x loading dye

1. Nucleic acids should be effectively resuspended to dissolve into the FA Lysis Buffer before sonication. Sonication should be completed to form an average fragment size of ~500 bp of DNA (See Note 5).
2. Sonication in a Diagenode Bioruptor can be completed in 2 sessions of 15 minutes each, at the medium setting with on and off pulses of 30 seconds (See Note 6).
3. Prepare a 1.5% agarose gel to analyze the size of DNA fragments of your sonicated nucleic acid.
4. Load nucleic acid into the gel with a 6x loading dye and a separate 100 bp DNA ladder to determine fragment size.
5. Electrophorese at 100 volts until the ladder reaches 2/3 of the way down the gel.
6. Analyzed gel should indicate an average fragment size of ~500 bases, this is ideal to identify R-Loop formation.
7. Prior to beginning immunoprecipitation, reserve 25 µl of chromatin from each sample to use as an input control, or around 5% of the total sample. Store this chromatin for use in final analysis (See Note 7).
8. Each sample will be analyzed using 3 reactions: A non-specific antibody control (IgG), a sample with the S9.6 antibody that has been treated by RNaseH (negative control) and a sample with the S9.6 antibody (experimental).

9. From your sonicated chromatin, isolate 40 µg of nucleic acid for each of these three reactions (See Note 8).
10. To your negative control, add 5 µl of RNase H. To all 3 samples, add the salt solution for the RNase H reaction. Incubate all three samples at 37°C on a heat block for 1 hour. RNase can be heat inactivated at 65°C for 20 minutes.
11. To each sample, add FA lysis buffer to raise the total volume to 500 µl.

S9.6 Immunoprecipitation

Materials used

- Anti-Mouse IgG antibody
- S9.6 DNA-RNA Hybrid antibody
- Pierce Protein A/G Magnetic Beads
- DynaMag2 magnetic tube rack
- Bead Wash Buffer: TBS containing 0.05% Tween 20

1. Add 10 µg of anti-mouse IgG to the isotype control, and 10 µg of S9.6 antibody to the negative and experimental samples.
2. For each sample including controls, prepare 25 µl of Pierce Protein A/G magnetic beads. Wash with 25 µl of bead wash buffer.
3. Place tubes on a Dynamag 2 magnetic tube rack (or equivalent magnet) to isolate beads, then remove and discard supernatant while saving the beads (See Note 9).
4. Repeat wash with 500 µl of Bead wash buffer, and isolate beads on magnetic rack.
5. Resuspend beads in 25 µl of FA lysis buffer.
6. Add beads to each sample and rotate at 4°C for 4 hours.

7. After 4-hour rotation, place each tube on the magnetic rack, and pipette away the supernatant, saving for future use (See Note 10).
8. Remove tube from magnetic rack, and softly pipette 500 µl of wash buffer over the beads containing the immunoprecipitate. Without vortexing, mix beads to wash.
9. Place tubes back on magnetic rack and remove the wash buffer without disturbing the bead pellet on the side of the tube.
10. Repeat steps 7 and 8 two more times.
11. Wash 1x with 500 µl of ice-cold PBS.
12. If you plan to isolate the RNA fraction, proceed to RNA Extraction. If you plan to isolate the DNA fraction, proceed to DNA Extraction.

RNA Extraction

Materials used

- Tri-Reagent (RNA/DNA/Protein) Isolation reagent
- QIAGEN RNeasy Minelute Cleanup Kit
- Tris-EDTA Buffer: 1M Tris pH 8, 0.5M EDTA pH 8, H2O

1. Resuspend beads in 1 ml of TRI-Reagent, and freeze the sample at -80°C to extract RNA from beads (See Note 11)
2. Thaw sample completely before moving on to the rest of the RNA isolation, usually ~15 minutes at room temperature (See Note 12).
3. Add 200 µl of chloroform, followed by vigorous vortexing and mixing by inversion. Vortex multiple times, during which two layers will begin to form.
4. Samples should incubate at room temperature for 10 minutes, after which the two phases should be clearly visible.

5. Centrifuge at ~21,000 x g for 10 minutes at 4°C.
6. Transfer the aqueous phase to new eppendorf tubes and add 500 µl of ice cold isopropanol and 2 µl of glycogen to each tube (See Note 13).
7. Mix by inversion and incubate at room temperature for 10 minutes.
8. Centrifuge at 21,000 x g for 10 minutes at 4°C. Following this spin, a pellet should be visible at the bottom of the tube.
9. Pour off supernatant from each tube and wash with 1 ml ice-cold 75% ethanol.
10. Centrifuge at 21,000 x g for 10 minutes at 4°C.
11. Pour off supernatant and let sample air dry for 10 minutes to remove excess ethanol.
12. Resuspend in 100 µl of Tris-EDTA Buffer.
13. Using the RNeasy MinElute Cleanup Kit, add 350 µl of Buffer RLT to each sample.
14. Add 250 µl of 100% ethanol to each sample and mix by pipetting up and down.
15. Transfer each sample to a Minelute column, and centrifuge at 13,500 x g for 30 seconds. Discard the flow thru following centrifugation.
16. Add 500 µl of Buffer RPE to the Minelute column, and again centrifuge at 13,500 x g for 30 seconds. Again, discard the flow thru.
17. Add 500 µl of 80% ethanol to each column, and centrifuge for 2 minutes at 13.500 x g. Discard the flow thru.
18. Centrifuge the dry column with the cap open at 13,500 x g for 5 minutes.
19. Move to a new eppendorf tube for collection and dispose of the previous collection tube.

20. Add 18 µl of ultrapure water directly to the column. Let the column sit at room temperature for 5 minutes, then centrifuge at 13,500 x g for 1 minute (See Note 14).
21. Discard the column and proceed to cDNA conversion (See Note 15).

cDNA conversion

Materials used:

- Invitrogen Superscript 3 First Strand Synthesis System
- QIAGEN Qiaquick PCR purification kit

1. Place 18 µl of RNA from previous step to a strip cap tube for use in a thermal cycler. Combine with 1 µl of dNTPs, and 1 µl of Oligo(dt) if your RNA of interest is polyadenylated, or 1 µl random hexamers if it is not or you don't know.
2. Incubate at 65°C in a thermal cycler for 5 minutes. Then place on ice, or at 0°C for at least 1 minute.
3. To each sample, add 4 µl of 10x RT buffer, 8 µl of 25 mM Magnesium Chloride, 4 µl of 1 M DTT, 2 µl of RNaseOUT, and 2 µl of Superscript III RT (See Note 16).

a. If using Oligo dT, go directly to step 5. If using random hexamer, samples should be incubated at 25°C for 10 minutes before proceeding

4. Incubate at 50°C for 50 minutes, followed by 85°C for 5 minutes.
5. Add 1 µl of RNase H to each sample and incubate at 37°C for 20 minutes.
6. Using the QIAquick PCR purification kit, add 105 µl of Buffer PB to each reaction.
7. Place the mixture of sample and buffer to a QIAquick column and centrifuge at 13,500 x g for 1 minute. Discard the flow thru.
8. Add 750 µl of Buffer PE to each column, and centrifuge at 13,500 x g for 1 minute. Discard the flow thru.

9. Centrifuge the dry column with cap open at 13,500 x g for 1 minute.
10. Place the column in a fresh eppendorf tube for collection and add 50 µl of Buffer EB to the center of the column.
11. Centrifuge at 13,500 x g for 2 minutes.
12. Proceed to quantitative PCR or other method of analysis.

DNA Extraction

Materials used:

- Elution Buffer: 100 mM NaHCO3, 1% SDS

1. Add 120 µl of elution buffer to the beads and rotate for 15 minutes at room temperature.
2. The antibody/DNA complex is now present in the supernatant and separate from the beads. Place tubes on the magnetic rack, and isolate supernatant in a new eppendorf tube.
3. Add 5 µl of proteinase K to each tube and allow at least 4 hours (or overnight) of incubation in a 65°C water bath.
4. The following morning, add 500 µl of phenol chloroform and vortex vigorously.
5. Centrifuge for 5 minutes at 4°C at 21,000 x g.
6. Remove the top aqueous layer and move to a new eppendorf tube.
7. Add 125 µl (or 1/4th of total volume of aqueous layer) of 9M sodium acetate.
8. Add 625 µl (or combined volume of sample and sodium acetate) of 100% isopropanol to each sample.
9. Add 2 µl of glycogen to each sample.
10. Vortex vigorously and place on ice for 10 minutes.

11. Centrifuge for 10 minutes at 4°C at 21,000 x g.

12. Pour off supernatant and let the pellet air dry.

13. Resuspend in 50 µl Ultrapure water and proceed to qPCR evaluation.

Quantitative PCR (qPCR) evaluation

- ABI Quantstudio 3 or equivalent qPCR platform
- ABI PowerUP SYBR Green Reaction Mix
- PCR primers for each unique target region

1. Dilute samples uniformly to the volume necessary for your PCR reactions using 5 µl of sample per reaction (50 µl provides 10 reactions, so if you plan to run 20 reactions, dilute all samples to 100 µl total).

2. Each 25 µl reaction should include:

   a. 12.5 µl of SYBR Green reaction mix

   b. 6.25 µl of Ultrapure water

   c. 1.25 µl of PCR primers at 10 µM concentrations)

   d. 5 µl of sample per reaction

3. Each sample will run at the following temperatures and cycles to complete the qPCR.

   a. 2 minutes at 50.0°C

   b. 10 minutes at 95.0°C

   c. 40 cycles repeating

      i. 15 seconds at 95.0°C

ii. 60 seconds at 60.0°C

4. Change qPCR settings according to your reagents and qPCR instrument, but these settings are applicable to the ABI qPCR system.

Notes

1. Proteinase K activity can degrade over time, so prepare the R-Loop digestion buffer in large quantities without the proteinase K included. Before using the digestion buffer, combine the premade buffer with the proteinase K for the most effective digestion.
2. Cell Signaling provides a 10x cell lysis buffer that we used for our FA lysis buffer that works great, as opposed to preparing all the reagents. Combine this with the New England Biolabs Proteinase K, which is the total buffer used in these reactions.
3. Excess PBS left in can really affect the function of the NP40 detergent, so pour off the supernatant PBS first, and then pull as much off as possible with a pipette to limit how much is left in the sample.
4. This isolation should contain a large amount of nucleic acid, so a visible pellet should be present. If not, attempt the isolation again from the original sample.
5. It is never going to be perfect 500 bp fragments, but keeping your fragments almost entirely below the 1200-1500 bp range will significantly reduce your background and the number of false positives you will find. Hybrid DNA:RNA formations are almost always larger than 50 bases, so if your fragments are mostly around 500 bp, you will have a pretty good sample to work with for the immunoprecipitation.

6. You absolutely must use a bath sonicator for this step. Using a needle sonicator results in a significant decrease in the overall yield recovered. You can even just use a cocktail of endonucleases for sonication, but a bath sonicator works most effectively and efficiently. The Bioruptor works great at the settings presented, but this step does take optimizing, because every sonicator is a little bit different.

7. The input is necessary for the final calculation for the qPCR determinations, just like a standard ChIP experiment. If used for the RNA fraction, it needs to be incorporated during RNA isolation immediately starting at step 6.13. If used for the DNA determination, it can be incorporated at step 8.3. Otherwise, treat as you would any input for a standard ChIP assay.

8. Another step that could need optimizing: For most ChIP experiments, 25 µg works well. With immune cells, I scaled up to 40 µg, and this produced an effective qPCR measurement. If you end up with too much background, or not enough signal, possibly scaling up or down the initial chromatin per reaction may help.

9. Pierce Protein A/G magnetic beads work great for this assay, though other brands of magnetic A/G beads may also be effective. Agarose beads did not allow for a clean enough immunoprecipitation and resulted in too high background Ct values.

10. Saving the supernatant is not incredibly important, but it can help you verify when the experiment failed. If the supernatant nor your experimental sample test positive for your gene of interest, then something went wrong before this step. If it is present in the supernatant, but not your experimental, then it is most likely negative for DNA:RNA hybrid formation.

11. Tri-Reagent, or another alternative, Trizol, are both available to complete the RNA extraction. No matter which you use, RNA extraction utilizing guanidinium-thiocyanate-phenol-chloroform will provide the most efficient and accurate results for this protocol.

12. I would advise freeze/thawing 3 times before beginning the experiment, as additional freeze thaws could help increase RNA yields by a small amount. At this point you are working with a small total amount of nucleic acid, so every bit should be preserved as much as possible.

13. A dyed glycogen, like Glycoblue, makes this step much easier. Unlike the previous isolation, this step does not have much material present in comparison. So, using the dyed glycogen makes it easier to see if there is any nucleic acid present.

14. The instructions for the Minelute kit do not include the incubation step with ultrapure water on top of the column, but we have found that soaking the column really helps maximize RNA recovery.

15. This is a good time to quantitate your RNA, possibly using a nanodrop in order to test if sample is present or not. It will never be a very high concentration, but this is a good step to make sure whether anything has gone wrong in your previous steps.

16. It is by far easier to prepare a master mix for these reagents as opposed to pipetting them all individually. I would advise combining before adding to the samples.

CHAPTER IV

BROMODOMAIN INHIBITOR JQ1 REVERSIBLY BLOCKS IFN-γ PRODUCTION

Abstract

As a class, 'BET' inhibitors disrupt binding of bromodomain and extra-terminal motif (BET) proteins, BRD2, BRD3, BRD4 and BRDT, to acetylated histones preventing recruitment of RNA polymerase 2 to enhancers and promoters, especially super-enhancers, to inhibit gene transcription. As such, BET inhibitors may be useful therapeutics for treatment of cancer and inflammatory disease. For example, the small molecule BET inhibitor, JQ1, selectively represses *MYC*, an important oncogene regulated by a super-enhancer. IFN-γ, a critical cytokine for both innate and adaptive immune responses, is also regulated by a super-enhancer. Here, we show that JQ1 represses IFN-γ expression in TH1 polarized cultures, CD4+ memory T-cells, and NK cells. JQ1 treatment does not reduce activating chromatin marks at the *IFNG* locus but displaces RNA polymerase II from the locus. Further, IFN-γ expression recovers in polarized TH1 cultures following removal of JQ1. Our results show that JQ1 abrogates IFN-γ expression, but repression is reversible. Thus, BET inhibitors may disrupt the normal functions of the innate and adaptive immune response.

Introduction

Bromodomain and extraterminal domain (BET) proteins are a family of transcriptional mediators, which assist in the recruitment of RNA Polymerase II (RNA pol II) to enhancers and promoters [121,158]. This family of proteins consists of BRD2, BRD3, BRD4, and BRDT. These proteins bind histone acetylated lysine residues via two highly conserved amino-terminal bromodomains [159]. BRD4 has been extensively studied for its role in transcriptional initiation and elongation [120,121,158-161]. BRD4 interacts with both the mediator complex, and the positive elongation factor B (P-TEFb) at enhancers and promoter regions, respectively [121,162]. BRD4 is expressed in almost all human tissues, and its role in transcription has made it a primary target for possible cancer therapies [163,164].

JQ1 is a bromodomain inhibitor, which selectively binds to the amino-terminal twin bromodomains of BET proteins [165]. JQ1 treatment displaces BRD4, inhibiting its ability to read acetylated lysine residues [166]. As a result, JQ1 selectively represses the *MYC* oncogene [167] in a variety of cancer cell lines and animal models of cancer, including acute myeloid leukemia [164], Burkitt's lymphoma [168], and multiple myeloma [167]. JQ1 represses *MYC* expression by interrupting the Mediator-BRD4 complexes located in its super-enhancer region [166,169]. A super-enhancer is a cluster of enhancers within close proximity that are densely populated by transcription factors, active histone marks, and co-activators [118,119]. Super-enhancers are thought to regulate genes that encode proteins that define cell identity as well as proteins that contribute to human disease, including cancers and inflammatory disease [123,170]. In fact, BET inhibitors, such as JQ1 show efficacy in pre-clinical models of cancer as well as autoimmune disease [164,167,168,171–174] .

Despite its potential as a cancer treatment, JQ1 inhibitors repress the expression of multiple genes, not only oncogenes [184]. For example, JQ1 treatment abrogates expression of IFNG by memory T-cells [176]. Interferon gamma (IFN-γ) is a cytokine that plays a critical role in both innate and adaptive immunity against viral and bacterial infections. IFN-γ is expressed by effector CD4+ (TH1) and CD8+ (TC1) T-cells, memory CD4+ and CD8+ T-cells, as well as natural killer (NK) cells and natural killer T (NKT) cells [176]. Another BET inhibitor, I-BET 762, was found to repress IFN-γ expression by TH1 cells during development [172].

Although BET inhibitors have shown efficacy in a variety of pre-clinical models of malignancy, we do not have a complete understanding of its impact on immune cells, nor how long any immunosuppressive effects that exist may last. Here, we sought to evaluate the ability of JQ1 to inhibit production of IFN-γ by TH1 polarized cultures, CD4+ memory T-cells, and NK cells. Our results demonstrate that JQ1 significantly reduces IFN-γ expression in all 3 cells types up to 5 days following treatment. JQ1 does not alter levels of activating H3K27 acetylation (H3K27ac) chromatin marks at the IFNG gene locus but displaces RNA pol II from the *IFNG* locus. Finally, inhibition of IFN-γ expression by JQ1 is not irreversible as ability of TH1 polarized cultures to produce IFN-γ is recovered after removal of JQ1.

Materials and Methods

Cell isolations and culture. TH1 Polarized PBMC Cultures: Total Human PBMCs were isolated from healthy control subjects with no chronic or acute conditions using Ficoll-Hypaque centrifugation. All subjects included in the study were of Caucasian

descent between ages 25-32. PBMCs (10^6 cells/ml) were stimulated with plate bound anti-CD3 (OKT3,CRL-8001, American Type Tissue Collection, ATCC), soluble mouse anti-human CD28 (1 □g/ml; 555725; BD Biosciences) and IL-12 (10 ng/ ml, BD Biosciences) without addition of IL-2 or anti-cytokine neutralizing antibodies essentially as previously described [83,141]. PBMCs were cultured in RPMI 1640 media (11875093, ThermoFisher) supplemented with 10% fetal bovine serum, penicillin-streptomycin and L-glutamine at 37ºC in 5% CO_2 in air. As outlined in Figure 1A, cells were treated with JQ1 for varied periods of time, followed by a re-stimulation with anti-CD3 for 48 hours.

CD4+ Memory T-cells: Single cell suspensions were prepared from human spleen. CD4+ memory T-cells were purified by negative selection (Stemcell, 19157). CD4+ memory cells (10^6 cells/ml) were stimulated with anti-CD3 for 24 hours as described in Figure 2A. Cells were treated with JQ1 for varied periods of time, and re-stimulated with fresh plate bound anti-CD3 for 48 hours. Natural Killer cells: NK cells were activated and expanded from human PBMCs using the NK cell activation and expansion kit (Miltenyi Biotec, 130-094-483) for up to a period of 21 days. NK cells were plated in 3 mL cultures at 10^6 cells/ml, and treated with JQ1 as described in Figure 3A. After treatment with JQ1, NK cells were stimulated with IL-12 (10 ng/mL: 554613, BD Pharmingen) and IL-18 (10 ng/mL: 4179-25, Biovision) for 48 hours. JQ1 (SML1524-5MG, Sigma Aldrich) was dissolved in DMSO at a final concentration of 10 mM and diluted into complete medium for addition to cell cultures. The study was approved by the institutional review board at Vanderbilt University Medical Center. Written informed consent was obtained at the time of blood sample collection. Spleen cells were obtained from Tennessee Donor Services under approved protocols with informed consent.

Quantitative Real-Time PCR. Total RNA isolation, cDNA synthesis using poly-A selection and analysis by qPCR were performed essentially as previously described [177]. All expression levels were normalized to GAPDH using the formula $2^{(\text{GAPDH Ct-target gene Ct})}$. Primer pairs used in analysis are provided in supplemental figures. Housekeeping genes were evaluated by a different calculation in Figure 4B, to evaluate reference gene quality, using the formula (1/target gene Ct)/(1/target gene Ct at 0 nM treatment). These assays specifically were normalized to total cDNA concentration of 2 ng/μL.

MTT-cell Proliferation Assay. MTT assays were performed using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (G4001, Promega). Absorbances were determined using an EMax plus Microplate Reader at 570 nm. Cell survival was calculated by (absorbance of treatment/absorbance at 0 nM).

Chromatin Immunoprecipitation (ChIP). ChIP procedures were as previously described using anti-H3K27ac (ab4729, Abcam) anti-H3K27me (AB6002, Abcam), anti-RNA Polymerase II (AB817, Abcam), or anti-mouse IgG (SC2357, Santa Cruz) antibodies [117]. DNA was isolated using Pierce Protein A/G magnetic beads (88802, ThermoFisher) via phenol chloroform extraction. Isolated chromatin was analyzed using SYBR-Green qPCR (Applied Biosystems).

Enzyme Linked Immunosorbent Assay (ELISA). ELISA was performed according to instructions provided by the manufacturer to analyze IFN-γ protein (BD Bioscience, 555142).

Statistics. JQ1 treatments and the corresponding qPCR or ELISA analyses were evaluated using a 1-way ANOVA test with Dunnett's Multiple comparison test for each

concentration comparison. ChIP analyses were expressed as fraction of input and evaluated using an unpaired t-test with Welch's correction. Unless otherwise stated, *=P<0.05 and data are represented as mean ± S.D.

Results

JQ1 represses IFN-γ expression by TH1 cells, memory T-cells and NK cells.

To determine the impact of JQ1 on IFNG expression by TH1 polarized PBMC cultures, we treated cells at multiple time points of cell culture. PBMCs were stimulated under TH1 polarizing conditions and treated with 50, 150, and 500 nM final concentrations of JQ1 at different times during the polarization process, harvested and restimulated with anti-CD3 (Figure 4-1A). *IFNG* transcripts were significantly reduced in cells treated for 24 or 48 hours with either 150 or 500 nM final concentrations of JQ1 (Figure 4-1B). *IFNG* mRNA was also reduced in PBMC treated under TH1 polarizing conditions for 4 or 5 days prior to JQ1 treatment (Figure 4-1C). Further, we increased the duration of JQ1 treatment to 3, 4, and 5 days to see if cells would recover IFN-γ expression (Figure 4-1D). In each of these treatments, *IFNG* mRNA was significantly decreased at all concentrations of JQ1 treatment. Total RNA isolated from cells in culture did not change according to the JQ1 concentration indicating that JQ1 treatment did not have a significant impact on total levels of cellular RNA in the different cultures (Figure 4-1E). These results indicate that JQ1 treatment significantly inhibited IFNG mRNA expression by TH1 polarized PBMC cultures.

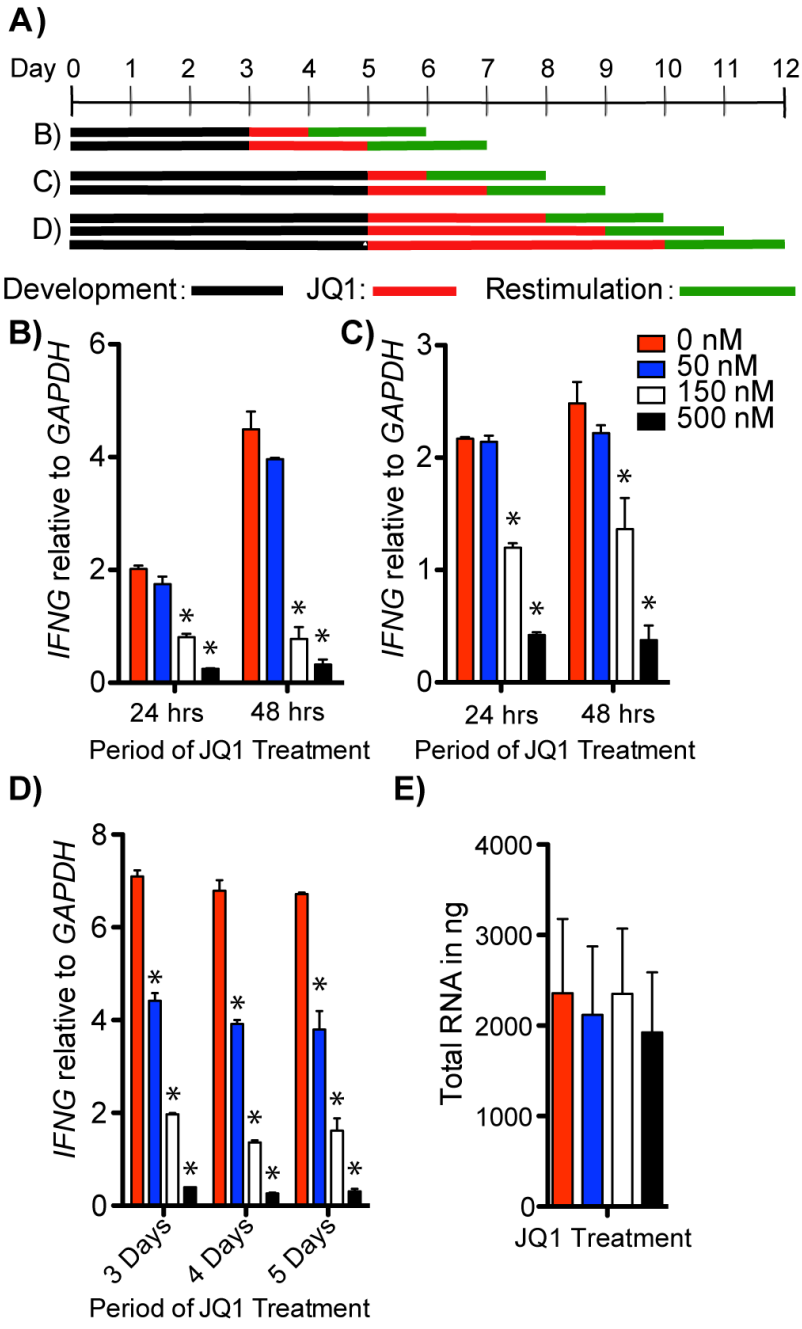

Figure 4-1. *IFNG* RNA transcripts are significantly reduced in TH1 polarized cultures by the BET inhibitor JQ1. (A) Experimental design; ▬: period of stimulation with anti-CD3, anti-CD28, IL-12, ▬: period of JQ1 treatment, ▬: period of restimulation with anti-CD3. (B-D) Y-axes are levels of *IFNG* mRNA relative to *GAPDH* mRNA, X-axes are treatment times with JQ1, N=4 each. (E) Average total RNA isolated from B, C and D cultures at each concentration of JQ1, N=12. *=P<0.05

We previously demonstrated that acute exposure of CD4+ memory T-cells to JQ1 prevented induction of IFN-γ in response to anti-CD3 stimulation [178]. To expand upon these studies, we stimulated CD4+ T memory cells for 24 hours with anti-CD3 to induce *IFNG* mRNA expression, treated cells with varying amounts of JQ1 for varying periods of time, and then re-stimulated cultures with anti-CD3 (Figure 4-2A). *IFNG* mRNA expression in memory cells treated with JQ1 for 24 and 48 hours was significantly reduced at 150 and 500 nM concentrations of JQ1 (Figure 4-2B). Similarly, when treated for 3, 4, or 5 days, *IFNG* mRNA expression was significantly reduced in CD4+ memory T-cells (Figure 4-2C). Total RNA isolated from memory cell cultures was significantly reduced in longer term cultures at 500 nM concentrations, which could indicate an impact on cell viability or total RNA expression or both (Figure 4-2D). Despite this, *IFNG* mRNA expression was significantly reduced at 150 nM concentrations of JQ1 in CD4+ memory T-cells and we found no significant loss of total RNA yield in these cultures. These data indicate that JQ1 treatment reduces *IFNG* mRNA in CD4+ memory T-cells, like TH1 polarized PBMC cultures.

We next evaluated effects of JQ1 treatment on NK cells. NK cells were treated with JQ1 for varying periods of time at 50, 150, and 500 nM final concentration and stimulated with IL-12 and IL-18 (Figure 4-3A). Like TH1 cells, *IFNG* expression was significantly reduced in NK cells treated with JQ1 (Figure 4-3B). *IFNG* mRNA was similarly reduced when treated for 3-5 days at 150 and 500 nM concentrations of JQ1 (Figure 4-3C). Total RNA isolated from NK cells did not significantly change according to JQ1 treatment, indicating cell viability and total cellular RNA yield were not affected by the JQ1 treatments (Figure 4-3D). These results indicate that IFN-γ expression was significantly reduced in

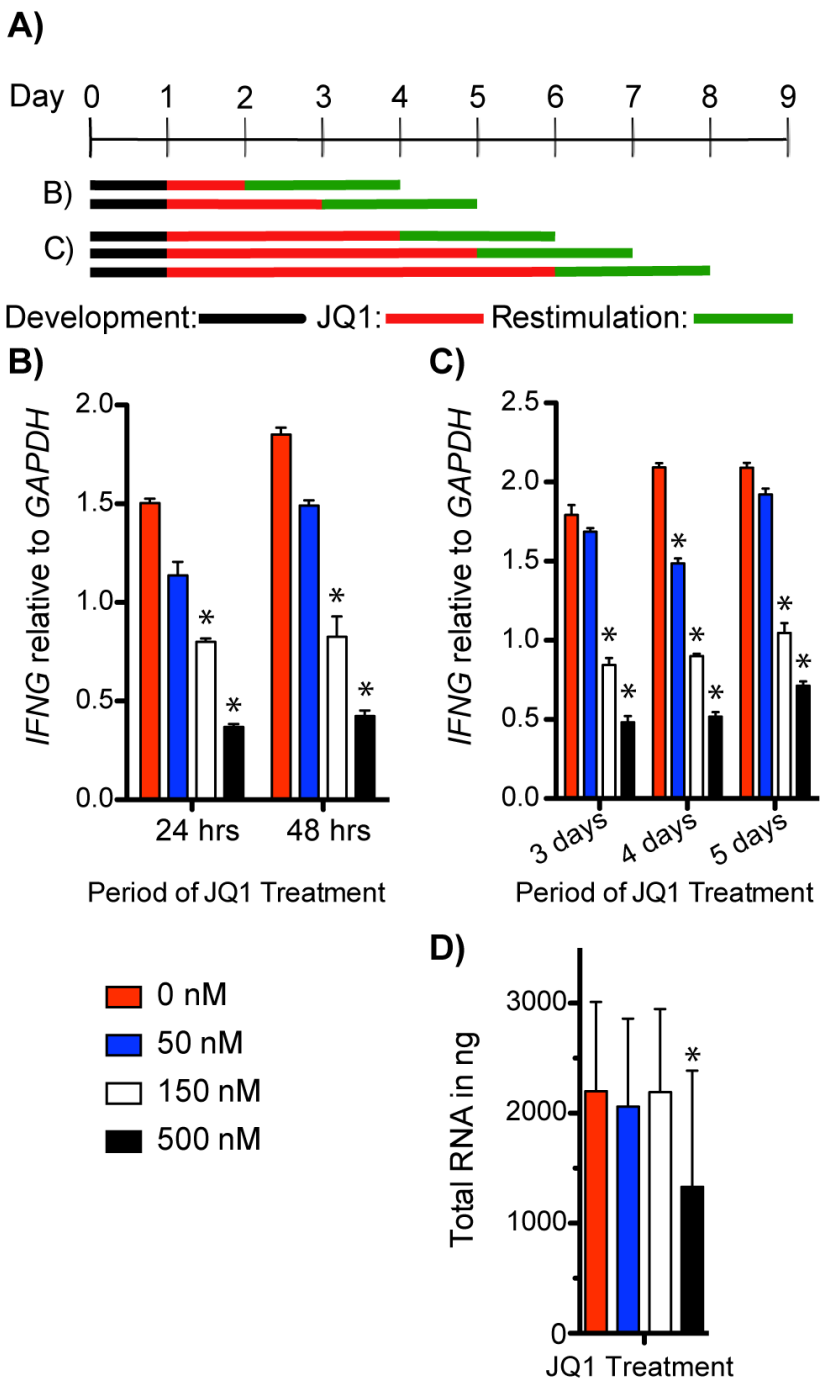

Figure 4-2. *IFNG* mRNA expression is significantly reduced in CD4+ T memory cells following JQ1 treatment. (A) Experiment design; ▬: period of stimulation with anti-CD3, ▬: period of JQ1 treatment, ▬: period of restimulation with anti-CD3. (B, C) Y-axes are levels of *IFNG* mRNA relative to *GAPDH* mRNA, X-axes are treatment times with JQ1, N=4. D) Average total RNA isolated from (B & C) samples at each concentration of JQ1 treatment N=6. *=P<0.05

NK cells following JQ1 treatment, similar to TH1 polarized PBMC cultures and memory CD4+ T-cells.

JQ1 effects on cell viability. We used the 'MTT assay' to determine if culture with JQ1 affected viability of the differenT-cell types. We found no loss of viability in TH1 polarized PBMC, NK, or CD4+ memory T-cell cultures after treatment with concentrations of JQ1 that significantly diminished IFNG expression (Figure 4-4A). As a second control experiment, we determined if culture with JQ1 affected expression levels of standard 'housekeeping' genes, *GAPDH*, *HPRT* and *ACTB*. We found that culture with JQ1 did not affect expression levels of *GAPDH* and *HPRT* but reduced levels of *ACTB* by ~25% in TH1 polarized PBMC cultures (Figure 4-4B). We also evaluated effects of culture with JQ1 on other genes that encode proteins critical for differentiation and function of TH1, NK, and CD4+ memory T-cells, *STAT4*, *TBX21* (T-bet), *IL12RB1* and *IL12RB2* [20]. We found that culture with JQ1 did not affect expression of *STAT4* and *TBX21* but did cause a reduction of *IL12RB1* and *IL12RB2* expression levels (Figure 4-4C). Inhibition of expression of *IL12RB1* and *IL12RB2* by JQ1 was similar in magnitude to inhibition of expression of *IFNG*. We also examined expression of genes that encode proteins participating in the biologic activity of bromodomain-containing proteins, including *MED1*, part of the mediator complex, *HEXIM1*, part of the P-*TEFb* complex, and *POLR2A*, part of the RNA polymerase 2 complex [20]. We found that culture with JQ1 did not alter expression levels of these genes (Figure 4-4D). Thus, under conditions where culture with JQ1 resulted in a marked reduction in IFNG expression levels, changes in viability, expression of 'housekeeping' genes, of *STAT4* and *TBX21*, and of *MED1*, *HEXIM1* and

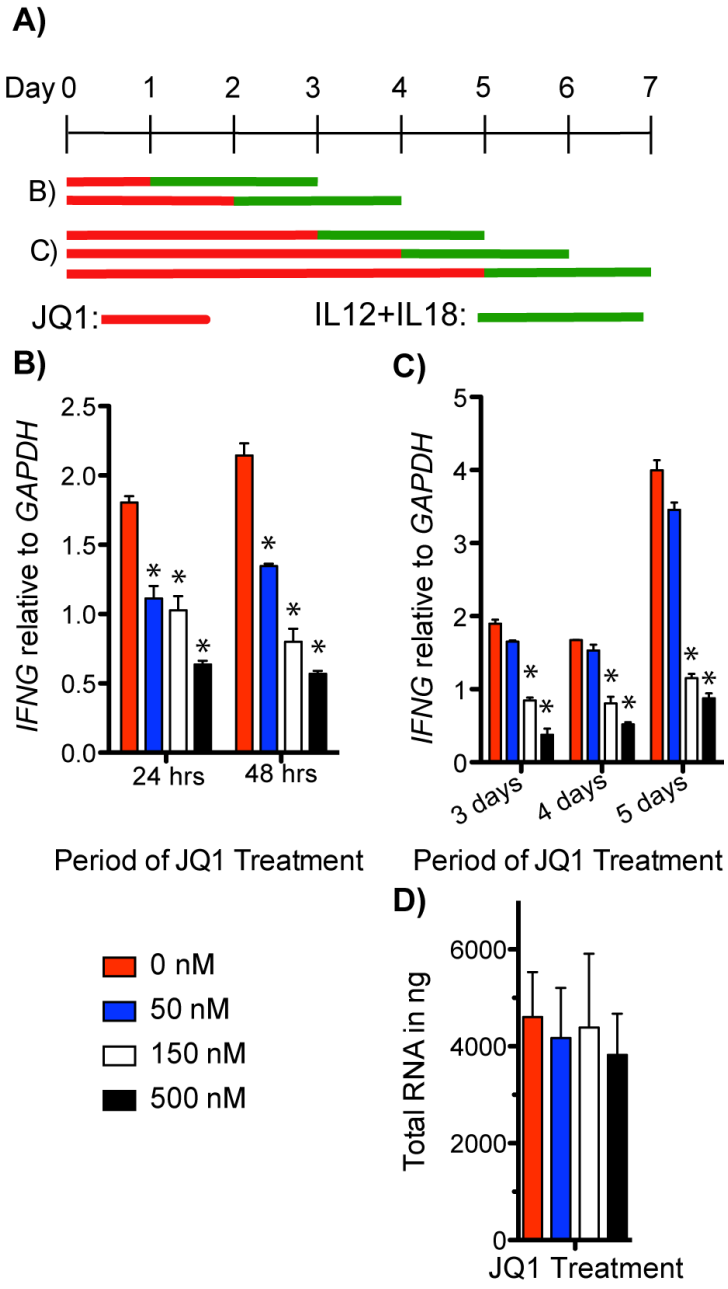

Figure 4-3. Induction of NK cell *IFNG* mRNA expression is reduced after JQ1 treatment. (A) Experimental design; ▬: period of JQ1 treatment, ▬: period of stimulation with IL-12 and IL-18. (B, C) Y-axes are levels of *IFNG* mRNA relative to *GAPDH* mRNA, X-axes are treatment times with JQ1, N=4. (D) Average total RNA isolated from samples from B and C at each concentration of JQ1, N=8. *=P<0.05

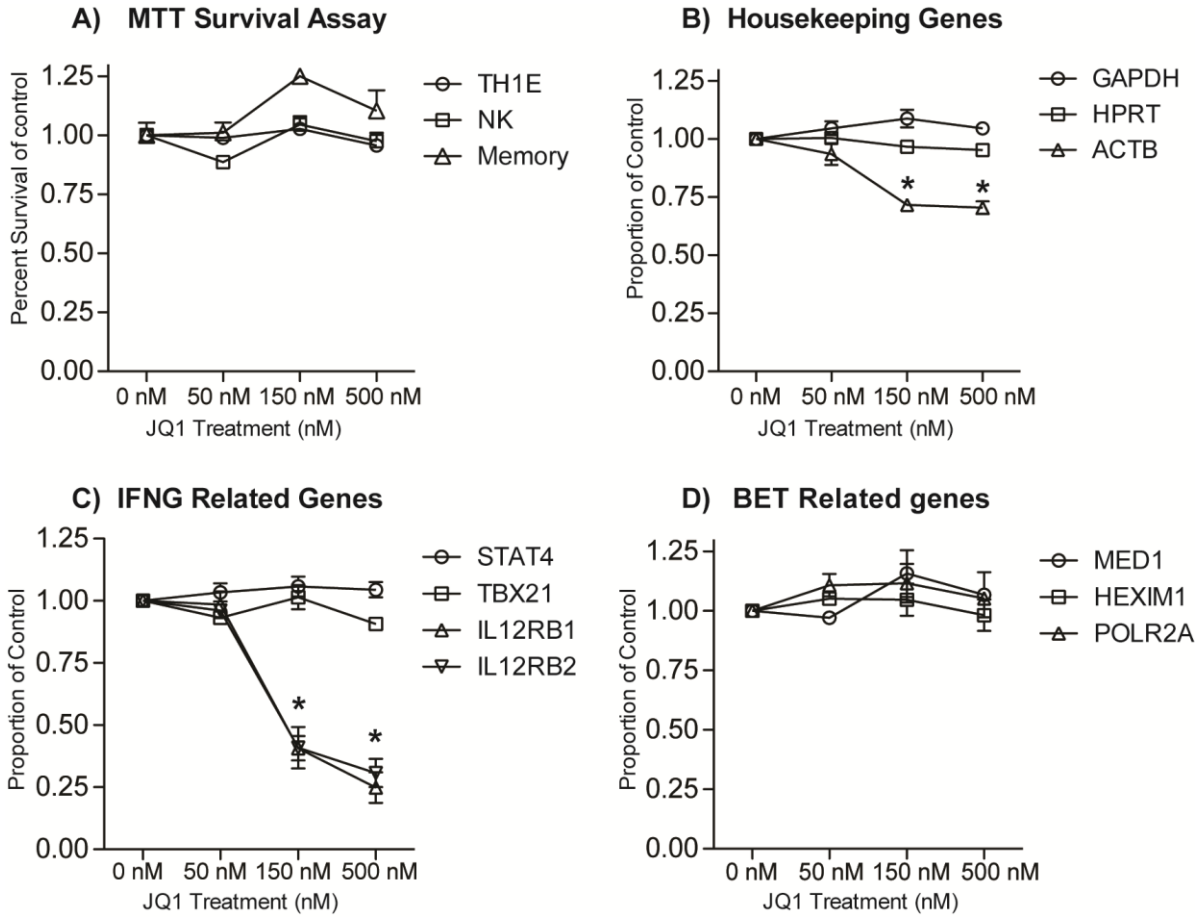

Figure 4-4. JQ1 treatment is not cytotoxic to TH1 polarized PBMC cultures but does repress other genes besides *IFNG*. (A). Cell cultures were treated with the indicated concentrations of JQ1. Viability was determined after 48 hours using the MTT assay. Results are expressed as percent of the no treatment control, N=4 (B) TH1 polarized PBMC cultures were treated with JQ1 for 48-hour JQ1. RNA was isolated and analyzed by qPCR. Reactions were standardized to 2 ng/μL of cDNA and calculated relative to 0 nM control. N=3 (C-D) As in B, but qPCR results were calculated relative to GAPDH.

POLR2A were not observed. However, genes that encode the IL-12 receptor beta subunits were equally sensitive to culture with JQ1 as was *IFNG*.

JQ1 abrogates RNA Pol II binding to the *IFNG* locus. We next sought to investigate epigenetic changes throughout the IFNG locus and how chromatin marks may be modified by JQ1 treatment. The *IFNG* gene locus has a large network of enhancers similar to a super-enhancer (Figure 4-5A) [117,123,178,179]. These regions are marked by H3K27ac, which make the region more accessible to binding transcription factors and Pol II [119,180]. We cultivated TH1 polarizing PBMC cultures for 5 days, treated with 150 and 300 nM final concentrations JQ1 for 24 hours and isolated chromatin for ChIP assays. We evaluated regions of the IFNG locus previously shown to be highly enriched for H3K27ac marks and recruitment of RNA Pol II 24. We found that JQ1 treatment did not significantly change the levels of H3K27ac marks throughout the *IFNG* locus (Figure 4-5B). We also analyzed H3K27me3 marks, indicators of an inactive enhancer [181], and found that chromatin within the *IFNG* locus showed no increase in repressive H3K27me3 marks following JQ1 treatment (Figure 4-5C). We similarly performed ChIP assays for RNA Pol II throughout the IFNG locus. JQ1 treatment caused a significant decrease in the binding of RNA Pol II both upstream and downstream of the *IFNG* gene (Figure 4-5D). Therefore, JQ1 effectively displaced bound RNA Pol II from the *IFNG* locus but did not change levels of either H3K27ac or H3K27me epigenetic marks at the *IFNG* locus.

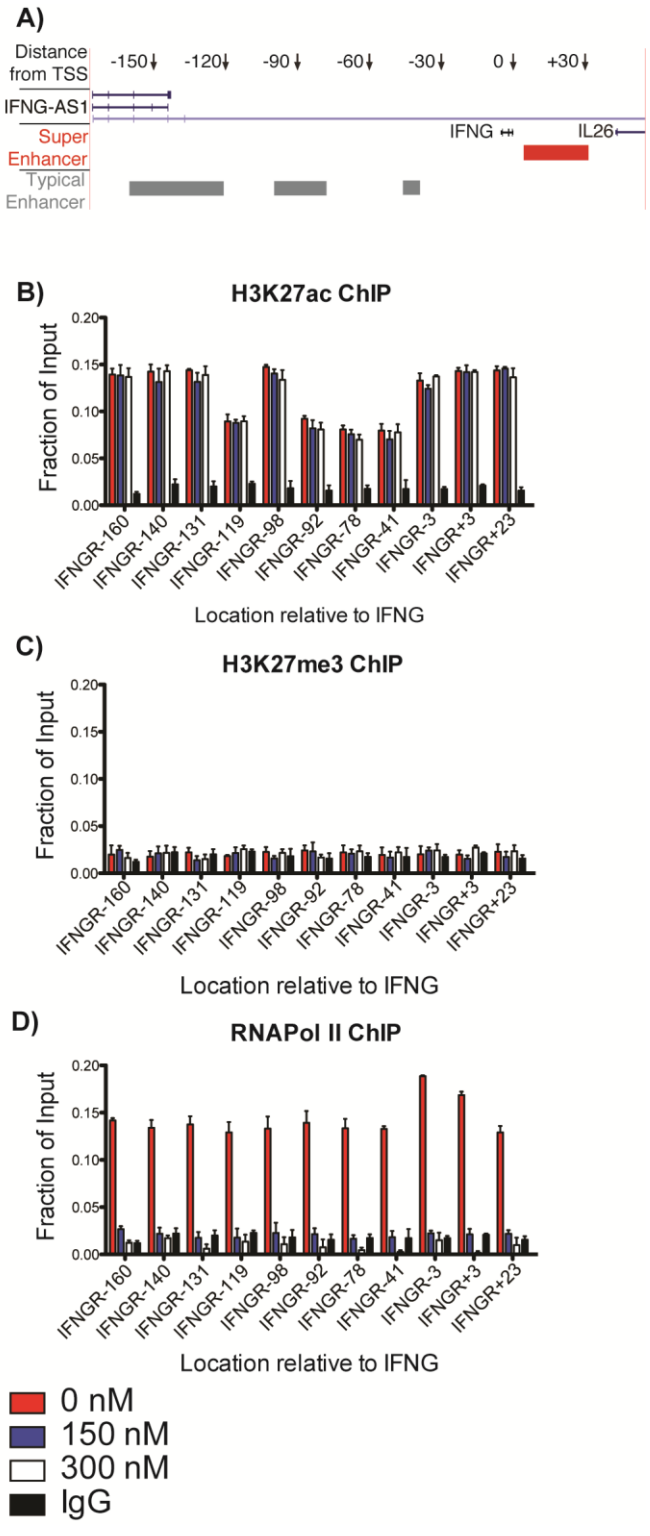

Figure 4-5. JQ1 treatment does not alter H3K27ac or H3K27me marks but abrogates RNA pol2 binding at the *IFNG* locus. (A) Schematic of predicted enhancer locations around *IFNG* locus. Numbers and arrows represent points distance in Kb from transcription start site of *IFNG* gene. Predicted super-enhancers in red (▬) and typical enhancers in grey (▬), according to [20,108,114,169]. (B) TH1 cells were cultured as in Figure 1C. ChIP-qPCR assays were performed to measure H3K27ac levels at the *IFNG* locus. Positions, X-axis, are relative to the *IFNG* transcription start site (e.g., IFNGR-160 = 160Kb downstream of TSS), Y-axis is fraction of input DNA, N=3. Each region evaluated for H3K27ac was significantly higher than IgG control but did not vary according to JQ1 concentration. (C) as in A, but ChIP-qPCR assays were performed to measure H3K27me levels, N=3. No H3K9me3 ChIP result was significantly different from the IgG control. (D) as in A, but ChIP-qPCR assays were performed to measure RNA pol II recruitment, N=3. RNA pol II ChIP 0 nM controls were significantly different from IgG controls at each location. Similarly, RNA pol II ChIP 0 nM controls were significantly different from JQ1 treatments at every location.

TH1 polarized cultures recover their ability to produce IFN-γ after removing JQ1. JQ1's half-life is only 0.9 hours after intravenous injection, or 1.4 hours when administered orally [169]. However, the half-life in tissue culture is not well understood, and we wanted to determine if cells could recover their functions when JQ1 was removed from culture. We treated TH1 polarized PBMC cultures for 24 or 48 hours with JQ1 on day 5

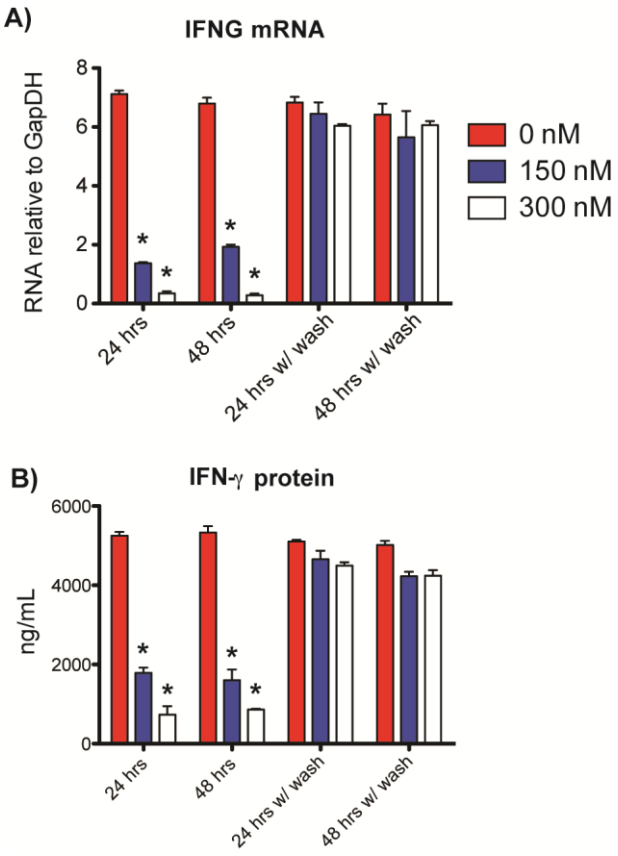

Figure 4-6. *IFNG* expression recovers following JQ1 removal. (A). After 5 days. TH1 cultures were treated with JQ1 at 150 and 300 nM final concentrations for 24 or 48 hours. Cells were either restimulated with anti-CD3 for 48 hours, or washed with new media lacking JQ1 and restimulated with anti-CD3 for 48 hours. RNA was isolated and *IFNG* analyzed by qPCR and normalized to *GAPDH*, N =3. (B) As in A except culture fluids were harvested and IFN-γ levels determined by ELISA, N=3.

of development, similar to Figure 1C. Following treatment, cells were either washed and plated with fresh media lacking JQ1 or cultures were continued in the presence of JQ1. We found that *IFNG* mRNA transcripts recovered to pre-treatment levels in TH1 polarized cultures after being washed and re-plated in fresh media (Figure 6A). We completed a similar experiment but analyzed IFN-γ protein by ELISA. Similarly, IFN-γ was reduced in cultures treated with JQ1 (Figure 6B), but IFN-γ production also recovered following a wash and re-plating with fresh media, similar to the mRNA results. These results indicate that *IFNG* mRNA and protein levels are reduced following JQ1 treatment but recover to pre-treatment levels following removal of JQ1.

Discussion

At nanomolar concentrations, the BET inhibitor, JQ1, inhibits expression of *IFNG* mRNA and IFN-γ protein by TH1 polarized cultures, memory CD4+ T-cells, and NK cells. Under these conditions, JQ1 does not interfere with presence of extensive activating H3K27ac marks across the *IFNG* locus nor does JQ1 induce formation of repressive H3K27me marks across the locus. Rather, JQ1 treatment results in almost complete loss of RNA Pol II recruitment across the *IFNG* locus. Further, effects of JQ1 are reversible and removal of JQ1 by media replacement results in complete recovery of *IFNG* mRNA and IFN-γ protein expression by effector TH1 cells. Our results are consistent with a model whereby JQ1 inhibition of IFNG expression by TH1 polarized cultures, memory CD4+ T-cells and NK cells results from almost complete loss of RNA Pol II recruitment across the IFNG locus. Further, removal of JQ1 allows BET proteins to rebind to the locus

and re-establish RNA Pol II recruitment across the IFNG locus resulting in efficient IFNG expression.

BET inhibitors disrupt function of both typical-enhancers and super-enhancers [169,182-184]. The general view is that functions of super-enhancers and genes driven by super-enhancers are more sensitive to effects of BET inhibitors than typical enhancers [169]. The IFNG locus is composed of two large enhancers, each spanning >30 kb, and these have been designated super-enhancers in different studies [113,119]. Almost complete inhibition of *IFNG* expression is achieved at nanomolar concentrations of JQ1. MYC and downstream c-MYC functions and expression, which require function of a nearby super-enhancer, are also inhibited at similar nanomolar concentrations of JQ1 [168,169,174]. Thus, *IFNG* most likely also falls into the class of genes requiring super-enhancers for their expression that also exhibit high sensitivity to BET inhibitors, such as JQ1.

IFN-γ plays a critical role in the adaptive immune response to control infection by intracellular pathogens, including bacteria and viruses, during both initial effector responses and memory responses to infection, as well as malignant transformation and growth [166,175-178]. Major sources of IFN-γ include NK/NKT-cells and T-cells. When NK/NKT-cells immigrate to the periphery, activating epigenetic markings at the *IFNG* locus already exist and these cells are fully capable of producing IFN-γ in response a variety of extracellular stimuli [177,185,186]. In contrast, once in the periphery, naïve T-cells have to endure additional developmental programs to produce the required activating epigenetic markings at the *IFNG* locus to allow efficient IFN-γ production in response to stimulation by antigen [187-189]. Thus, it might be expected that treatment with BET

inhibitors, such as JQ1*, in-vivo*, may significantly impair both innate and adaptive arms of immunity that play critical roles controlling infection by intracellular pathogens.

BET inhibitors function by displacing BET proteins from acetylated lysine motifs, but do not directly reverse the chromatin marks [167,190,191]. The repression of *IFNG* in TH1 polarizing cultures match this model of regulation, as indicated by a continued presence of H3K27ac marks, lack of formation of repressive H3K27me3 marks and displacement of RNAPol II from the *IFNG* locus following JQ1 treatment. However, *IFNG* expression recovered after removing JQ1 from the cultures at both concentrations. These results indicate that immunosuppressive effects of BET inhibitors, like JQ1, may be reversible.

Certain BET inhibitors have shown very good efficacy in various pre-clinical models of cancer and inflammatory disease [164,167,168,171-174]. It seems likely that BET inhibitors will therefore move forward to actual human clinical studies to treat various malignancies as well as inflammatory diseases. Our results suggest that BET inhibitors may significantly impair both innate and adaptive arms of the immune response, but these effects are reversible. The repression of *IFNG* by JQ1 treatment is observed in the major IFN-γ producing cell types. It remains to be determined if inhibition of the immune response by BET inhibitors will limit their therapeutic usefulness.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

The immune system is one of the diverse cell systems in human body. In order to respond to constantly evolving pathogens, the cells of the immune system must present an equally dynamic response. Diverse cell lineages require a complex regulatory network to activate and repress the appropriate genes. The exact mechanisms controlling gene regulation and cell identity in many immune cells are still unknown. The work presented attempts to understand some of the mechanisms regulating T-helper cell polarization, and how similar mechanisms may regulate the diverse cells of the immune system. The goal of these experiments was to understand the impact of epigenetic factors on the expression of T-helper cell specific gene expression and their role in polarization.

In the second chapter we demonstrated *GATA3-AS1*, the divergent lncRNA partner of *GATA3*, was necessary for effective Gata3 expression and subsequent TH2 polarization. Gata3 regulates cytokine expression by modifying the chromatin landscape in each of the TH2 specific cytokines but binds the promoters of *IL5* and *IL13* [33]. In previous work, IL-5 and IL-13 were reduced following Gata3 knockdown in established TH2 cells, but IL-4 was mostly unchanged [31]. These results indicate *GATA3-AS1* primarily acts by regulating the *GATA3 locus* and not the cytokine genes directly. The *TH2LCRR* lncRNA is similarly required for the effective transcription of the hallmark TH2 cytokines but acts by modifying the chromatin landscape within the TH2 cytokine gene

locus [117]. *GATA3-AS1* is also sufficient to activate Gata3 expression within TH0 cells but cannot induce TH2 polarization. TH2 cell development requires other cofactors, like STAT6, so Gata3 alone cannot activate the downstream cytokine expression [31]. *GATA3* transcription in response to exogenous *GATA3-AS1* indicates a function for the lncRNA transcript, as opposed to the act of transcription.

In our next set of experiments, we demonstrated two molecular interactions for *GATA3-AS1*: the association with WDR5 and the formation of an R-loop. LncRNAs like *GATA3-AS1* are necessary to recruit WDR5 or other chromatin modifying complexes to modify its own gene locus, as well as the *GATA3* gene locus. The R-loop formed by *GATA3-AS1* occurs within its own gene locus and may act to anchor *GATA3-AS1* to the *GATA3-AS1-GATA3* locus prior to interacting with WDR5. Both molecular interactions led us to a combined model of regulation (Figure 5-1). The model presented matches previous reports of the known interactions between WDR5 and the lncRNA HOTTIP [77], but additional experiments are necessary to demonstrate a direct interaction between GATA3-AS1 and WDR5. An alternative model might be that the GATA3-AS1 transcript may stimulate the catalytic activity of MLL instead of recruiting the complex to chromatin. Initial transcription and R-loop formation may allow a more receptive histone conformation and increase methyltransferase activity. Additionally, the R-loop formed by GATA3-AS1 may be the binding target of WDR5. GADD45A is an epigenetic reader which binds specifically to R-loops to recruit the demethylase TET1 [192]. GADD45A recognizes the R-loop formation and openly accessible chromatin but cannot directly bind to the lncRNA alone.

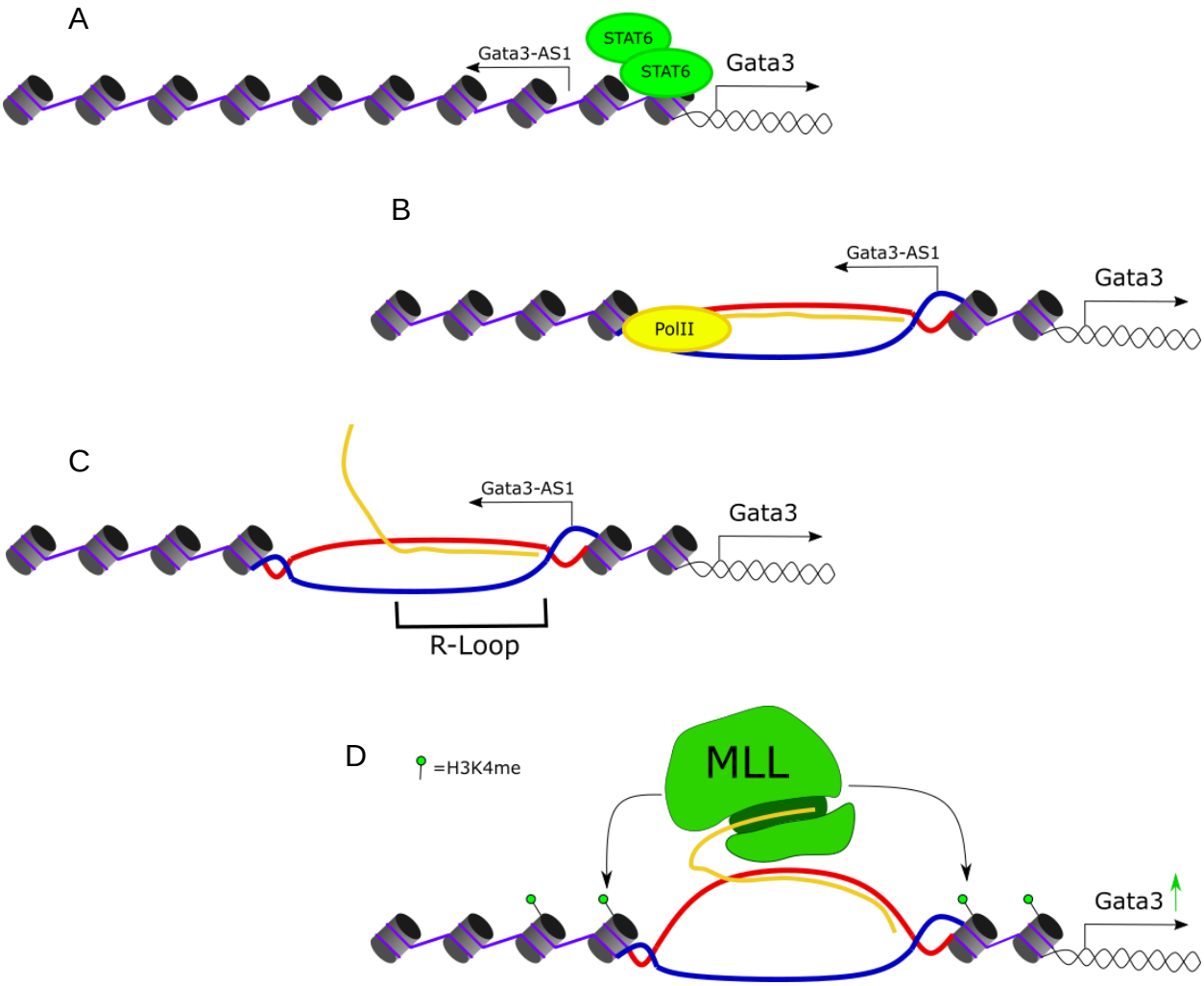

Figure 5-1. MLL complex Recruitment by *GATA3-AS1*. Proposed model by which GATA3-AS1 recruits the MLL methyltransferase to modify chromatin surrounding the GATA3-AS1 and GATA3 gene locus. A) Initial expression of *GATA3-AS1* and *GATA3* begins by STAT6 binding to the shared promoter region. B) RNA Pol II transcribes *GATA3-AS1*. C) *GATA3-AS1* forms an R-Loop within the 5' and central region of its gene locus. D) *GATA3-AS1* binds and recruits the MLL protein complex via WDR5 to apply H3K4me3 marks throughout the gene locus and make both gene loci more accessible.

In the third chapter, we describe an optimized assay for the isolation and evaluation of R-loops. The DRIP (DNA:RNA immunoprecipitation) assay is based on the development of the S9.6 antibody which specifically targets R-Loops. Reliable assays for evaluation of R-loops are increasingly important as more evidence connects lncRNAs and R-loop formation. The optimized assay described in this chapter is a significant contribution to the functional analysis of R-loops.

In the fourth chapter, we demonstrate that IFN-γ expression in multiple cell types is inhibited by disruption of super-enhancer function via BRD4 displacement. JQ1 treatment, the bromodomain inhibitor, did not disrupt most housekeeping genes or genes that encode proteins required for transcriptional function, but does disrupt expression of multiple TH1 lineage specific genes that play important roles in IFN-γ production. Resting NK cells are capable of IFN-γ expression hours after stimulation, while naive T cells must undergo extensive chromatin rearrangement to make the *IFNG* gene locus more permissive for transcription [176]. Effector memory T cells retain many of these epigenetic modifications to more rapidly respond to stimulus and initiate IFN-γ production. JQ1 treatment disrupted IFN-γ expression in each of these cell types, suggesting a shared mechanism of regulation. We conclude that the *IFNG* locus is regulated in each of these cell types by a common super-enhancer due to shared sensitivity to JQ1.

In subsequent experiments, we found bromodomain inhibition by JQ1 does not modify the chromatin landscape around *IFNG* in TH1 cells and *IFNG* repression is reversible. The *IFNG* gene locus did not lose permissive H3K27ac, nor gain repressive H3K27me following treatment with JQ1. BRD4 binds to RNA Pol II and recruits RNA Pol

II to enhancers, which was displaced following JQ1 inhibition [121]. When JQ1 treatment is removed by washing cells in fresh media, IFN-γ expression recovers within 48 hours. Therefore, we conclude the immunosuppressive effects of JQ1 are not permanent in TH1 cells.

Dual function of divergent lncRNAs

Initial research of lncRNAs has focused on which cell types express these unique transcripts, and most have been accomplished by RNA sequencing. The functions of individual lncRNA transcripts are still mostly not understood, as our classifications of lncRNAs are based on how close they are located to mRNA genes. Divergent lncRNAs make up a significant portion of annotated lncRNAs in the human genome. Our study of *GATA3-AS1* has identified two molecular interactions that are not unique by themselves but are novel for a single divergent transcript. LncRNAs are known to bind WDR5, amongst many other chromatin modifying complexes [77]. A few others form R-Loops within their own gene locus [93-95]. The combined interaction, indicating a DNA/RNA/protein complex could provide insight into a conserved mechanism for divergent lncRNAs. The shared promoter region between a divergent lncRNA gene and its mRNA gene partner are typically < 1kb. Abrogation of *GATA3-AS1* transcript levels by siRNA removes permissive acetylation marks from both its own gene region, and the 5' portion of the *GATA3* mRNA gene. The R-loop formation within *GATA3-AS1* and WDR5 binding could allow efficient recruitment of the MLL methyltransferase and other chromatin complexes to both genes. A similar mechanism could be possible for RNAs binding to the repressive PRC2 complex, which also has numerous lncRNA binding

partners [85]. Despite known interactions between lncRNAs and their protein partners, the capacity to attract those complexes to specific regions in the genome is still not well understood. R-loop formation could represent the anchoring mechanism for targeted protein recruitment. Future studies of protein bound lncRNAs should incorporate R-loop detection as a possible mechanism for anchoring a given regulatory lncRNA to its target gene locus.

Unlike mRNAs, whose triplet codon structure defines the protein product that will eventually be formed, lncRNAs still lack identified motifs that represent functional regions. Recent work on *Braveheart* [193] and *Hottip* [82] have begun to identify specific nucleotide sequences within a lncRNA that contribute to their function of binding chromatin modifying complexes. The broad array of lncRNAs that bind to complexes like WDR5 and PRC2 do not have a large consistent sequence motif but could share a common secondary structure to interact with these protein complexes. The interface between lncRNAs and their binding partners could be the key to understand how so many variable lncRNAs could share few conserved binding partners.

Immune cell fate is dependent on lncRNAs

LncRNAs have recently been described as “the master regulators of cell fate.” Unique lncRNAs in distinct T-cell types, like *IFNG-AS1, GATA3-AS1,* and *TH2LCRR*, are necessary for T-cell polarization and effector cytokine expression [117]. Lineage-specific lncRNAs have been identified in each T-cell subset including some that may regulate major regulatory factors in TH17 and Tfh cells. *GATA3-AS1* is over-expressed in PBMCs from patients with seasonal allergic rhinitis [132], and our work demonstrated

that the transcript alone can induce the expression of *GATA3,* and reduction in *GATA3-AS1* reduces IL-5 expression, a primary cytokine involved in allergic asthma. *GATA3-AS1* could be targeted to reduce allergic inflammation directly within the TH2 cells. CD4+ T-cells play important roles in numerous autoimmune disorders including rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus, and each of these T-cell lineages express unique lncRNAs which could represent potential therapeutic targets to modulate immune responses and treat autoimmunity [194].

JQ1 represses cytokines and receptors, but not master transcription factors

Super-enhancers primarily associate with lineage defining genes in developing cells [117,118]. In hematopoietic cells, 26% of single nucleotide polymorphisms (SNPs) associated with rheumatoid arthritis were located within predicted super-enhancers, along with over 30% associated with multiple sclerosis and type 1 diabetes [123]. JQ1 and other bromodomain inhibitors have strong anti-inflammatory properties and exhibit encouraging effects *in-vitro* and in and in mouse models of rheumatic disease [171-173]. Bromodomain inhibition by JQ1 reduces expression of IFN-γ in multiple cell lineages and the cytokine receptors *IL12RB1* and *IL12RB2* in TH1 cells. However, the primary transcription factors in TH1 cells, T-bet and Stat4, are not reduced by JQ1 treatment. Each of these genes has a related super-enhancer according to predictive models and chip-seq experiments [123]. This represents a disconnection between the predicted model of super-enhancer regulation and genes repressed by bromodomain inhibitors. Key regulatory nodes in CD4+ T-cells, like the TH2LCR, are located around cytokine and cytokine receptor genes, representing possible variations in super-enhancers which

may be more sensitive to bromodomain inhibition. Analysis of the full transcriptome in JQ1 treated TH1, TH2, and TH17 cells will provide a delineation between BET inhibitor sensitive and resistant genes. Identification of BET-inhibition sensitive genes may represent a strategy to identify critical immune-response genes and, as such, may help identify genes and their protein or lncRNA products that may represent attractive targets for the treatment of immune-mediated diseases, such as autoimmune and allergic diseases, as well as provide new insights into how super-enhancers may regulate their target genes.

LIST OF PUBLICATIONS

1. Gibbons, H. R., Shaginurova, G., Kim, L. C., Chapman, N., Spurlock, C. F., & Aune, T. M. Divergent lncRNA GATA3-AS1 regulates GATA3 transcription in T-Helper 2 cells. *Frontiers in immunology*, *9*, 2512 (2018).

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