6-19-2019

Divergent Transcriptional Regulation of Suppressors of Cytokine Signaling Genes in Adipocytes

Paula Mota de Sa
Louisiana State University and Agricultural and Mechanical College

Following this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations

Part of the Biochemistry Commons, Cell Biology Commons, Hormones, Hormone Substitutes, and Hormone Antagonists Commons, Molecular Biology Commons, Other Chemicals and Drugs Commons, and the Other Genetics and Genomics Commons

Recommended Citation
Mota de Sa, Paula, "Divergent Transcriptional Regulation of Suppressors of Cytokine Signaling Genes in Adipocytes" (2019). LSU Doctoral Dissertations. 4982.
https://digitalcommons.lsu.edu/gradschool_dissertations/4982

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Doctoral Dissertations by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
DIVERGENT TRANSCRIPTIONAL REGULATION OF SUPPRESSORS OF CYTOKINE SIGNALING GENES IN ADIPOCYTES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences

by

Paula Mota de Sa
B.A., University of Sao Paulo, 2013
August 2019
Acknowledgments

First and foremost, I would like to acknowledge my family for always supporting my education and the decisions I have made throughout my life; without them, I would not even be close to where I am today. I would also like to thank the Brazilian government for the scholarship opportunity, as it would have been much more difficult to pursue my studies abroad without this funding. I also would like to thank Jacqueline Stephens for being a fantastic mentor and for providing consistent support and guidance. There is no question that she played and will continue to play a significant role in the progress of my career. Lastly, I am very thankful to all my coworkers in the laboratory for all that we have shared, and for making my day-to-day lab experience a positive one.
Table of Contents

Acknowledgments .......................................................................................................................... ii

Abstract ........................................................................................................................................... v

Chapter 1. Literature Review .......................................................................................................... 1
  1.1. Adipocytes and adipose tissue ............................................................................................ 1
  1.2. Transcription factors ........................................................................................................ 4
  1.3. JAK-STAT signaling pathway ............................................................................................. 6
  1.4. Histone Deacetylases (HDACs) and HDAC inhibitors ..................................................... 8
  1.5. RNA polymerase II transcription ...................................................................................... 12
  1.6. Bromodomain and Extra-Terminal Domain (BET) proteins ......................................... 13

Chapter 2. Introduction ................................................................................................................... 15

Chapter 3. Material and Methods ................................................................................................ 23
  3.1 Cell culture .......................................................................................................................... 23
  3.2. Preparation of whole-cell extracts .................................................................................... 23
  3.3. Subcellular Fractionation .................................................................................................. 24
  3.4. Measurement of protein concentration ............................................................................. 24
  3.5. Gel electrophoresis and immunoblotting ......................................................................... 24
  3.6. Immunoprecipitation (IP) ................................................................................................ 25
  3.7. RT-qPCR .......................................................................................................................... 26
  3.8. Small interfering RNA (siRNA)-mediated knockdown .................................................... 26
  3.9. Chromatin immunoprecipitation (ChIP) .......................................................................... 27
  3.10. Statistical analysis ........................................................................................................... 28

Chapter 4. Results .......................................................................................................................... 31
  4.1. STAT5 acetylation levels are low in adipocytes ................................................................. 31
  4.2. GH-induction of STAT5 target genes ............................................................................... 38
  4.3. HDAC inhibitors increase Socs3 and decrease Cish expression ...................................... 38
  4.4. STAT5 and HDAC5 do not physically interact in adipocytes .......................................... 42
  4.5. HDACs KD do not recapitulate HDACi effects .................................................................. 45
  4.6. HDAC5i (LMK-235) does not affect mRNA stability ......................................................... 46
  4.7. BET inhibition has the same effects on Socs3 and Cish gene expression as pharmacological HDAC inhibition ..................................................................................... 46
  4.8. BRD2 and RNA Pol II binding to Socs3 and Cish correlate with changes in gene expression .................................................................................................................................. 51
  4.9. JQ1 disrupts HEXIM1 and Cyclin T1 interaction ............................................................... 60

Chapter 5. Discussion ..................................................................................................................... 66

Bibliography ..................................................................................................................................... 82
Abstract

The Janus Kinase - Signal Transducer and Activator of Transcription (JAK-STAT) signaling pathway transduces several signals crucial for development and homeostasis. Suppressors of cytokine signaling (SOCS) proteins control JAK-STAT signaling via a negative feedback loop. The transcription factor STAT5 is known to play a significant role in fat cell development and function, and several studies suggest that acetylation may affect STAT5 transcriptional activity. To test this hypothesis, we treated 3T3-L1 adipocytes with growth hormone (GH) to activate STAT5 in the presence or absence of histone deacetylase (HDAC) inhibitors. STAT5 acetylation levels were low in adipocytes and mostly unchanged by the inhibitors. Still, two STAT5 target genes from the SOCS family, Socs3 and Cish, were inversely regulated by general and specific HDAC inhibitors (Socs3 expression increased, while Cish levels decreased). Chromatin immunoprecipitation analyses revealed that changes in total and activated RNA polymerase II, but not STAT5A binding to Socs3 and Cish promoters highly correlated with changes in gene expression. Thus, we hypothesized that HDAC inhibitors were indirectly affecting another protein in the transcriptional complex. Members of the bromodomain and extra-terminal (BET) protein family bind acetylated histones and recruit transcription factors, thus playing a role in chromatin remodeling and transcription. Treatment with the BET inhibitor JQ1 produced the same divergent effects as HDAC inhibition on both Socs3 and Cish gene expression, as well as on RNA polymerase II binding. Moreover, BET proteins help drive productive elongation of mRNA by recruiting the positive transcription elongation factor (P-TEFb). We found that JQ1, but not the HDAC inhibitor LMK-235, could impact P-TEFb availability in a manner consistent with the Socs3 gene expression changes we observed. We propose a model in which GH-induced Cish transcription is dependent on the BET protein,
BRD2, and susceptible to inhibition by JQ1 (and indirectly by HDAC inhibitors), whereas \textit{Soxs3} mRNA elongation may involve recruitment of different factors, thus explaining the divergent effects of HDAC/BET inhibition on the two genes. Overall, our results demonstrate substantially different transcriptional regulation of \textit{Soxs3} and \textit{Cish}, suggesting distinct roles for these two related proteins in adipocytes.
Chapter 1. Literature Review

1.1. Adipocytes and adipose tissue

Adipocytes are specialized cells with three principal features. The most well-known is energy storage in the form of triglycerides. Second, they have the ability to respond to insulin. Third, they produce hormones that act on other tissues. All of these functions of adipocytes are important in the regulation of whole-body metabolism, and disruption of any of these roles can lead to metabolic dysfunction and type 2 diabetes. Regarding the storage of lipids in adipocytes, it may be intuitive to think that limiting fat accumulation would be beneficial; however, impairments in adipocyte development and lipid storage lead to ectopic fat accumulation in other tissues and to unhealthy metabolic states (reviewed in Gastaldelli 2011). The inability to respond to insulin normally, or insulin resistance, in adipocytes can cause systemic metabolic dysfunction involving many tissues (reviewed in Sethi and Vidal-Puig 2007). Finally, the endocrine functions of adipocytes are also crucial contributors to metabolic health. For example, leptin, a hormone produced exclusively in adipocytes, acts in the brain to suppress food intake; adiponectin, another fat-specific hormone, regulates whole-body insulin sensitivity.

Historically, adipocytes have been divided into two main types, white or brown adipocytes, possessing different morphology and functions. White adipocytes are characterized by a single large cytoplasmic lipid droplet that pushes the nucleus to the periphery of the cell; their central function is to store lipid. Brown adipocytes have multiple lipid droplets and a darker color because of higher mitochondrial content; they dissipate energy and produce heat in a process called non-shivering thermogenesis, which is facilitated by uncoupling protein 1 (UCP-1) (reviewed in Saely, Geiger, and Drexel 2012; reviewed in Jacqueline M Stephens 2012).
Rodents and other small mammals have a considerable amount of brown adipose tissue (BAT), while larger mammals generally lose their BAT in the early years of life. More recently, another type of fat cell has been described as a “beige” or “brite” adipocyte. Beige adipocytes are also thermogenic, but differently from brown adipocytes, they are located in white adipose tissue (WAT). The origin of beige adipocytes is controversial; some studies show they originate from unique precursors (Wu et al. 2012), while there is also evidence that they derive from white adipocytes (Cinti 2012). Unlike brown adipocytes, beige adipocytes are present throughout adulthood and are thought to be viable targets for anti-obesogenic therapies (Wu et al. 2012).

There are many adipose tissue (AT) depots with distinct anatomical locations and functions. Some researchers even support the idea that each fat depot should be considered a different tissue (reviewed in Kruglikov and Scherer 2016). In humans, visceral fat, upper body abdominal subcutaneous fat, and lower body subcutaneous fat constitute the three main adipose depots. In rodents, the most commonly used pre-clinical model to study human obesity, fat depots are differently distributed and great caution must be used in translating findings from rodents to humans (reviewed in Chusyd et al. 2016). Subcutaneous adipose tissue is considered a metabolic sink for storage of excess lipid that could be deleterious when present in circulation or in other non-adipose tissues. Visceral fat accumulates around internal organs and is commonly believed to be the predominant contributor to metabolic dysfunction (reviewed in Gastaldelli et al. 2002). Thus, diseases like diabetes, atherosclerosis, and hypertension are more prevalent in individuals with higher waist-to-hip ratios (indicative of higher amounts of visceral fat). Conversely, people with relatively higher levels of gluteofemoral body fat may be protected from obesity-associated diseases (reviewed in Manolopoulos, Karpe, and Frayn 2010).
Adipose tissue is not only composed of adipocytes. Other cell types present in AT include endothelial cells, preadipocytes, and immune cells such as macrophages, neutrophils, and lymphocytes. The combination of all non-adipocyte cell types in AT is called the stromovascular fraction (SVF). Obesity is associated with increased macrophage and T lymphocyte infiltration within adipose tissue (Weisberg et al. 2003; Kintscher et al. 2008), and there is substantial evidence that inflammation and decreased insulin sensitivity in AT contribute to whole-body insulin resistance (reviewed in Surmi and Hasty 2008).

In addition to being a source of classical endocrine factors, recent studies have shown that AT can produce and secrete microRNAs (miRNAs). These miRNAs are single-stranded noncoding RNAs of about 19-22 nucleotides in length that can negatively regulate translation in other tissues by decreasing target mRNA levels. The majority of adipose tissue-derived miRNAs is secreted in exosomes, which are extracellular vesicles (EVs) of about 50–200nm (Thomou et al. 2017). miRNAs play a role in the development and function of many cells, and computational calculations predict that a single miRNA regulates hundreds of different mRNAs (reviewed in Dumortier, Hinault, and Van Obberghen 2013). Variations in miRNA levels can be beneficial or detrimental. Increased levels of specific miRNAs have been correlated with several diseases such as cancer (Hata and Lieberman 2015), diabetes (Trajkovski et al. 2011) and cardiovascular disease (Caroli et al. 2013). Moreover, adipose-derived miRNAs decrease with age, and this process can be reversed by caloric restriction (Mori et al. 2012). Thus, miRNAs can be considered a new type of endocrine mediator that plays significant roles in the regulation of metabolism.

A variety of murine cell models have been used to study adipocyte development and function, including mesenchymal stem cells (MSCs) and preadipocyte lineages. Adipogenesis
can be divided into two main steps: commitment and terminal differentiation. MSCs are not committed to adipocyte lineage and therefore, depending on external stimuli can differentiate into numerous cells types such as myocytes, chondrocytes, and adipocytes. The most commonly used cell line from this category is C3H10T1/2 stem cells. Among the most commonly used preadipocyte cell lines are the 3T3-L1, 3T3-F422A, OP9, and 1246 cell lines. These types of cells are already committed to the adipocyte lineage and cannot differentiate into other cell types (reviewed in Ruiz-Ojeda et al. 2016). To better promote preadipocyte differentiation, researchers use specific hormonal cocktails for each of the different cell lines. The differentiation cocktails for 3T3-L1 and 3T3-F422A preadipocytes contain 3-isobutyl-1-methylxanthine (MIX), dexamethasone (DEX), and insulin. High levels of cyclic AMP (cAMP) are important for differentiation, and MIX promotes adipogenesis by inhibiting the enzyme that degrades cAMP. DEX, a glucocorticoid receptor agonist, induces transcription of genes necessary for adipogenesis. Lastly, insulin is an anabolic hormone which promotes lipid storage (Greenn and Meuth 1974; Green and Kehinde 1976). These various in vitro cell models are valuable tools to study adipocyte differentiation and alterations in lipid storage, insulin sensitivity, and adipokine secretion, which are the main functions of fat cells.

1.2. Transcription factors

Cells express selected genes in response to stimuli such as stress, hormones, cytokines, growth factors, and infections. In addition, cell type-specific gene expression regulation is what makes an adipocyte have a completely different form and function from a neuron, for example. Transcription factors play a major role in gene regulation; they bind to specific DNA response elements and can promote or repress gene expression. Transcription factors are categorized by the structural motifs in their DNA-binding domains. The most common motifs are helix-turn-
helix (HTH), helix-loop-helix (HLH), zinc finger (ZNF), or leucine zipper (reviewed in Papavassiliou 1995). The most thoroughly described motif is HTH, which contains two α-helices separated by a β-turn. Despite some variability, this motif is highly conserved throughout species. More recent studies have revealed that HTH domains are not only transcriptional regulators, but that they can also play a role in DNA repair, RNA metabolism, and even mediate protein-protein interactions (reviewed in Aravind et al. 2005). HLH transcription factors are crucial in several development pathways, such as neurogenesis, myogenesis, and sex determination. These proteins have two important regions, one comprising many basic residues that allow HLH to bind to DNA, and another containing mostly hydrophobic residues allowing HLH to form homodimers and/or heterodimers (reviewed in Murre et al. 1994). Zinc finger proteins are characterized by different combinations of cysteine and histidine residues coordinated by a zinc ion. Structural studies show that typical zinc-finger domains have two β-sheets and one α-helix. In addition to transcriptional regulation, ZFPs are involved in ubiquitin-mediated protein degradation, DNA repair, cell migration, among others (reviewed in Cassandri et al. 2017). Leucine zipper proteins contain four or five leucine residues spaced exactly seven residues apart and positioned on the outer portion of α-helices, permitting the interaction with another α-helix and providing a structural basis for dimerization. Several well-known transcription factors such as C/EBP, Fos, Jun and c-Myc are characterized by leucine zipper domains (reviewed in Struhl 1989; reviewed in Busch and Sassone-Corsi 1990).

In addition to DNA binding domains, transcription factors often have activation domains. Unlike DNA binding domains, which are very specific structures and bind to determinate DNA sequences, activation domains have more variable configurations. Early studies revealed that activation domains are composed of acidic residues that may act to form amphipathic α-helices.
However, not all activation domains are defined by acidic residues; some contain glutamine- and proline-rich motifs. Activation domains of transcription factors largely recruit other proteins to promote transcription. Moreover, these domains act synergistically. Therefore, two activation domains have stronger effects than either one acting separately (reviewed in Ptashne 1988; reviewed in Tasset et al. 1990).

1.3. JAK-STAT signaling pathway

The Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathway can be activated by a variety of growth factors and cytokines. The pathway transduces several signals critical for developmental and homeostatic processes, being critical to immune development, hematopoiesis, mammary gland development and adipogenesis. Typically, when a hormone or cytokine binds to its receptor, membrane-bound receptor proteins JAKs are brought together allowing transactivation by phosphorylation. Activated JAKs then phosphorylate STATs, permitting dimerization of STATs through their conserved SH2 domains. These activated STATs translocate to the nucleus and bind to response elements of targets genes, promoting or repressing their expression (reviewed in Rawlings, Rosler, and Harrison 2004). In mammals, there are four members in the JAK family (JAK1, JAK2, JAK3 and TYK2).

The STAT family, originally discovered in studies on IFNγ signaling (Shuai et al. 1992), is composed of 7 members: STATs 1, 2, 3, 4, 5A, 5B and 6. STAT proteins share six functional domains (Fig. 1.1): N-terminal, coiled-coil, DNA binding, linker, SH2 and transactivation domains. The N-terminal domain is important for dimerization with another STAT, while coiled-coil and transactivation domains interact with other proteins. The linker is involved in transcription activation. The SH2 domain is essential for receptor association (Lim and Cao 2006). The DNA binding domain recognizes palindromic DNA sequences, represented by
TTN5AA or TTN6AA, in the promoters of target genes (Decker and Kovarik 1999). In addition to being phosphorylated by JAKs, STATs are known to be regulated by other post-translational modifications including ubiquitination (T. K. Kim and Maniatis 1996), SUMOylation (Rogers, Horvath, and Matunis 2003; Ungureanu et al. 2005), methylation (Rho et al. 2001), and acetylation (Shankaranarayanan et al. 2001; R. Wang, Cherukuri, and Luo 2005).

Figure 1.1. Functional domains of STAT proteins. N = N-terminal, CC = coiled-coil, DNA = DNA binding domain, LK = Linker domain, SH2 = SH2 domain, TA = transactivation domain.

The JAK-STAT signaling pathway is regulated by a class of proteins called suppressors of cytokine signaling (SOCS), which participate in a negative feedback loop to prevent sustained activation. SOCS proteins share a SH2 domain and a SOCS box at the C-terminus. The SH2 domain determines the target of each SOCS protein, and the SOCS box is known to associate with members of the ubiquitin ligase family, responsible for protein degradation (reviewed in Krebs and Hilton 2001; reviewed in Chikuma et al. 2017). SOCS proteins were first discovered by three independent groups (Naka et al. 1997; Endo et al. 1997; Starr et al. 1997), and a DNA database search revealed at least 20 proteins in mice and humans that share the SOCS box domain, but only proteins that contain both the SH2 and SOCS domains are named SOCS proteins (Hilton et al. 1998). The SOCS family is composed of SOCS1-7 and CISH, which was originally cloned in hematopoietic cell lines (Yoshimura et al. 1995). SOCS1, SOCS2, SOCS3 and CISH are the most thoroughly characterized proteins in the family. SOCSs are highly regulated at the transcriptional level by STATs; they are rapidly transcribed and translated, and they negatively regulate JAK-STAT pathway activation (reviewed in Trengove and Ward 2013).
1.4. Histone Deacetylases (HDACs) and HDAC inhibitors

The activity of an enzyme that catalyzes the removal of acetyl groups from lysine residues of proteins was first described in calf thymus extract (Inoue and Fujimoto 1969). Numerous studies followed that discovery, and since these enzymes were initially found to remove the acetyl groups from histones, they were called histone deacetylases (HDACs). Several years later, the first non-histone protein, p53, was described to be lysine acetylated (Gu and Roeder 1997). Thus, they are also called lysine deacetylases (KDACs). HDACs play important roles in histone modulation and in the regulation of gene expression. Currently, the HDAC superfamily is divided in four classes. Class I consists of HDAC1, 2, 3 and 8, which are homologous to the yeast deacetylase RPD3 protein. These HDACs are expressed ubiquitously and are localized mainly in the nucleus (reviewed in Taunton, Hassig, and Schreiber 1996). HDAC1 and 2 are highly homologous and have been described to be part of repressive complexes such as sin3, nucleosome remodeling and deacetylase (NuRD) and REST corepressor 1 (CoREST). HDAC3 is found in other repressive complexes, the nuclear receptor corepressor (N-CoR) and silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), while HDAC8 has not been shown to be involved in any repressor complex (reviewed in Haberland, Montgomery, and Olson 2009). Class II members are similar to yeast HDA1 protein. They are subdivided in Class IIa, which is comprised of HDAC4, 5, 7 and 9, and class IIb, which includes HDAC6 and 10. Class IIa HDACs have larger N-terminal portions and possess autonomous repressor activity (X. Zhou et al. 2000). Moreover, they have low catalytic activity and are commonly found in complexes with the class I HDACs (Fischle et al. 2002a; Jones et al. 2008). Class IIb is composed of HDACs 6 and 10 because they are evolutionarily closely related. However, HDAC6 is unique within the HDAC family since it is the only member possessing two
catalytic domains in tandem. Class IV is composed only of HDAC11, since its sequence similarity with other HDACs is limited. In addition, HDAC11 is not known to participate in any repressor complex, possibly suggesting a distinct role for this member (Reviewed in De Ruijter et al. 2003).

In contrast to classical HDACs, which are zinc-dependent, class III deacetylases are NAD$^+$-dependent. They are called silent information regulator two (Sirt) proteins or sirtuins and were first identified in studies on yeast that showed they were necessary for telomeric silencing effects (Pillus and Rine 1989; Gottschling et al. 1990). Some years later, sirtuins were demonstrated to be involved in the repair of double-strand DNA breaks, cell-cycle progression, and in the molecular mechanisms of ageing in several species (Mills, Sinclair, and Guarente 1999; Tissenbaum and Guarente 2001). In mammals, seven sirtuins have been described, and they are separated in classes I to IV. Even though they are highly conserved, sirtuins have diverse biological functions and unique binding partners and substrates (reviewed in North and Verdin 2004; reviewed in Haigis and Sinclair 2010).

HDAC activity is counterbalanced by another class of enzymes called histone acetyl transferases (HATs) or lysine acetyl transferases (KATs) that are responsible for adding acetyl groups to histones and non-histone proteins. Acetylation is an especially interesting modification because it connects cell metabolism to epigenetics, and altered HDAC and HAT activities have been associated with numerous types of cancer (R. J. Lin et al. 1998; L. Wang et al. 2011; Holmlund et al. 2013; Zhong et al. 2018). In fact, HDAC inhibitors were discovered empirically by screening for drugs that promoted tumor cell differentiation (Leder and Leder 1975); only later were HDACs revealed as their molecular targets. HDAC dysfunctions have also been connected with neurological diseases such as Alzheimer’s and Parkinson’s diseases (Guan et al.
Moreover, HDACs have been found to play a role in metabolic diseases. HDAC3 inhibition was shown to regulate PPARγ acetylation and activity, increasing glucose uptake in white adipose tissue and improving insulin sensitivity in diet-induced obese (DIO) mice (Jiang et al. 2014). In another study, HDAC9 expression was increased with DIO and impaired adipocyte differentiation. The deletion of HDAC9 resulted in improved insulin sensitivity and decreased weight gain and hepatic steatosis (Chatterjee et al. 2014). HDACs have also been described to repress the formation of type I myofibers in skeletal muscle, affecting insulin mediated glucose uptake (Potthoff et al. 2007).

The importance of HDACs in the control of gene expression and their involvement in diseases states has led to the discovery of natural and synthetic HDAC inhibitors. HDAC inhibitors can be classified in five groups: hydroxamic acids, cyclic peptides, aliphatic acids, benzamides and sirtuin inhibitors. The first natural hydroxamic acid found to inhibit HDACs was trichostatin A (TSA) (Yoshida et al. 1990). While TSA is mostly used in laboratory experiments because of its toxicity, vorinostat (suberoylanilide hydroxamic acid, SAHA) was the first hydroxamic acid inhibitor to be approved by the United States Food and Drug Administration (FDA) for the treatment of relapsed and refractory cutaneous T-cell lymphoma (CTCL) (Duvic et al. 2007). Later, two other inhibitors from this class, belinostat and panabiostat, were approved by the FDA for the treatment of peripheral T-cell lymphoma and multiple myeloma, respectively. Several other HDAC inhibitors such as resminostat, rocilinostat and practinostat are currently in clinical trials (reviewed in Eckschlager et al. 2017). The class of cyclic peptides is characterized by a complex structure; the most well-known is depsipeptide (romidepsin) which
was approved by the FDA to treat CTCL in 2009. The aliphatic acids such as valproic acid (VPA), butyric acid and phenylbutyric acid are relatively weak inhibitors. VPA is used for the treatment of epilepsy, bipolar disorders and migraines and is currently the subject of clinical trials for cancer, along with other aliphatic acids. Benzamides mostly inhibit class I HDACs; entinostat (MS-275-SNDX-275) and tacedinaline (CI994) are currently in clinical trials for a variety of cancers (reviewed in Kim and Bae 2011; reviewed in Eckschlager et al. 2017). Sirtuin inhibitors include the pan-inhibitor nicotinamide and the specific SIRT1 and SIRT2 inhibitors sirtinol, cambinol, and EX-527 (reviewed in Lavu et al. 2008).

The lack of specificity of most HDAC inhibitors is an obstacle in studies to assess HDAC function and efforts are ongoing to develop inhibitors that effectively target individual HDACs. Structurally, inhibitors of classical HDACs are characterized by a zinc binding group (ZBG), connected to a linker that mimics the lysine side chains of HDAC substrates. The chain is followed by a connecting unit (CU) that can be adapted to improve interactions and it is terminated by a functional “cap” group (Fig. 1.2.) (reviewed in Roche and Bertrand 2016). To date, the only selective HDAC inhibitor approved by the FDA is romidepsin, which strongly inhibits HDAC1 and 2. Other selective class I HDAC inhibitors such as entinostat (MS-275), tacedinaline (CI-994), and CHR-3996 are currently in clinical trials. Regarding class II inhibitors, the HDAC6 inhibitor Ricolinostat (ACY-1215) is in clinical trials, and thus far all selective class IIa inhibitors are in the preclinical phase (reviewed in Li and Seto 2016).

![Figure 1.2. Structure of Zn⁺-dependent HDAC inhibitors. Cap = Cap group, CU = Connecting unit, Linker = Hydrophobic linker, ZBG = Zinc-binding group.](image)
1.5. RNA polymerase II transcription

Transcription is divided into four main steps: transcription initiation, early elongation, productive elongation and termination. Transcription initiation involves dissociation of double-stranded DNA, the recruitment of RNA polymerase II to a promoter, and the recruitment of general transcription factors (GTFs). GTFs are required for the formation of the pre-initiation complex (PIC), which consists of TFIIB, TFIID (which includes the TATA-binding protein, TBP), TFIIE, TFIIF, TFIIH and Pol II. PIC drives basal transcription (reviewed in Nechaev and Adelman 2011), while activated transcription is dependent on additional signals that recruit specific transcription factors to response elements in gene promoters and/or enhancers.

The transition between initiation and early elongation involves the phosphorylation of the C-terminal domain (CTD) of RNA polymerase. The CTD contains a consensus sequence, YSPTSPS, that can be phosphorylated on several residues. When the polymerase binds to a promoter, it is phosphorylated at Serine-5 by CDK7, the catalytic subunit of TFIIH. This event facilitates polymerase promoter escape. The transition from promoter proximal paused RNA polymerase to productive elongation is initiated by the recruitment of positive transcription elongation factor b (P-TEFb), whose predominant form is composed of two catalytic CDK9 subunits and two regulatory Cyclin T1 subunits. P-TEFb phosphorylates Serine-2 residues of CTD, as well as the negative elongation factor (NELF) and the DRB sensitivity inducing factor (DSIF). After phosphorylation, NELF is displaced from Pol II and DSIF is converted to a positive elongation factor, allowing productive elongation to occur (reviewed in Nechaev and Adelman 2011; reviewed in Adelman and Lis 2012). The final step of termination can occur via different mechanisms. However, most Pol II mRNA transcripts use the canonical pathway of cleavage and polyadenylation machinery (reviewed in Nechaev and Adelman 2011).
The transition between early and productive elongation has emerged as a crucial step in the expression of most active genes that are highly regulated by P-TEFb. Studies have shown that P-TEFb availability is highly coordinated by interactions between positive and negative regulators. P-TEFb is inactive when associated with the small non-coding RNA 7SK and hexamethylene bisacetamide (HMBA) inducible protein 1 (HEXIM1). HEXIM1 is responsible for inhibiting the kinase activity of CDK9, while snRNA 7SK stabilizes the HEXIM1-P-TEFb interaction (He, Pezda, and Zhou 2006). Once dissociated from its inhibitory complex, P-TEFb can be recruited by other proteins. Among the positive regulators is the super elongation complex (SEC). SEC is composed of members from the AF4/FMR2 (AFF) family and members of the eleven-nineteen Lys-rich leukemia (ELL) family of RNA polymerase II (Pol II) elongation factors. Rapid transcriptional activation in the presence or absence of paused Pol II has been recognized as a SEC function (reviewed in Luo, Lin, and Shilatifard 2012). In addition to SEC, bromodomain and extra-terminal domain (BET) protein 4 (BRD4) is known to interact with P-TEFb via BRD4 bromodomains or a C-terminal P-TEFb interacting domain (PID) (Jang et al. 2005). Studies suggest that the bromodomains bind to acetylated Cyclin T1 while PID plays a role in the dissociation of P-TEFb from HEXIM1. In addition to P-TEFb, BET proteins can recruit transcription factors to gene enhancers and promoters and positively regulate gene expression (Schröder et al. 2012).

1.6. Bromodomain and Extra-Terminal Domain (BET) proteins

Bromodomain and Extra-Terminal Domain (BET) proteins are called readers because they bind to acetylated histones or other proteins and regulate chromatin remodeling and transcription elongation. In mammals, there are four members in the family, BRD2, BRD3 and BRD4, which are ubiquitously expressed, and the testis-specific BRDT. They are distinct from
other bromodomain-containing proteins (BCPs) because in addition to two conserved bromodomain motifs in the N-terminal region, they also possess in the C-terminal side an extra-terminal domain that facilitates additional protein-protein interactions (reviewed in Florence and Faller 2001). Both BRD2 and BRD4 have been shown to play critical roles in development. BRD2 null mice have neural tube defects and embryonic lethality (Gyuris et al. 2009), while BRD4 null mice embryos presented postimplantation lethality (Houzelstein et al. 2002). To date, no BRD3 KO mouse has been described. In the context of fat cells, BET inhibition has been reported to inhibit adipogenesis (Goupille et al. 2016).

Like HDAC inhibitors, BET inhibitors have been extensively investigated for the treatment of cancer and inflammatory diseases. In 2010, JQ1 was the first BET inhibitor to be described; it competitively binds to bromodomains and prevents BET proteins from binding to acetylated histones. Only (+)-JQ1 interacts with bromodomains, its stereoisomer (−)-JQ1 does not inhibit BET proteins. In that study, JQ1 promoted differentiation and growth arrest in squamous carcinoma (Filippakopoulos et al. 2010). In another study, the inhibitor I-BET exhibited anti-inflammatory properties in activated macrophages (Nicodeme et al. 2010). Currently, several BET inhibitors are in clinical trials for the treatment of a variety of cancers (reviewed in Stathis and Bertoni 2018).
Chapter 2. Introduction

Obesity, which is the consequence of an imbalance between caloric intake and energy expenditure, has nearly tripled worldwide since 1975. According to the World Health Organization (WHO), 39% of adults aged 18 years or older were overweight in 2016 and 13% were obese. The majority of the world’s population lives in countries where obesity kills more people than underweight (“World Health Organization; Obesity and Overweight rates” 2016). The increase of obesity has been followed by the development of obesity-associated health problems including type 2 diabetes mellitus (T2DM), cardiovascular diseases, stroke, fatty liver disease, and some types of cancers (Aronne 2002). The prevalence of diabetes worldwide among adults rose from 4.7% in 1980 to 8.5% in 2014 (“World Health Organization; Diabetes” 2014). In the US, about 9.4% of the population had diabetes in 2015, with T2DM accounting for 90 to 95% of all diabetes cases (for Disease Control 2017).

Although obesity contributes to the development of several diseases as cited above, this fact is not the complete story. Some normal weight individuals with low subcutaneous but high visceral fat mass are at higher risk for T2DM, while insulin sensitive healthy people with obesity are protected from metabolic syndrome (Klöting et al. 2010). Therefore, studying adipocyte physiology, metabolism and molecular mechanisms involved in metabolic disease states is critical to better understand the pathology of T2DM and develop new therapeutics to treat the disease.

The pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is known to induce insulin resistance by decreasing expression of several proteins important for the insulin signaling pathway, including glucose transporter type 4 (GLUT4), CCAAT/enhancer-binding protein-α (C/EBPα), and the insulin receptor (IR) (J M Stephens and Pekala 1991; Jacqueline M. Stephens,
Lee, and Pilch 1997). In 1993, it was shown that TNFα expression is elevated in adipose tissue in conditions of obesity and was associated with insulin resistance, or the failure to respond to normal concentrations of circulating insulin (Hotamisligil, Shargill, and Spiegelman 1993). Since then, other inflammation markers such as interleukin-6 (IL-6) (Bastard et al. 2002), C-reactive protein (Pradhan et al. 2001) and monocyte chemoattractant protein-1 (MCP-1) (Kanda et al. 2006) have been likely to insulin resistance in adipose tissue and to an increased risk of developing T2DM. In addition to pro-inflammatory cytokines such as TNF-α, IL-6 and MCP-1 that are largely produced in immune cells found in adipose tissue, there are endocrine hormones such as adiponectin and leptin that are produced and secreted from adipocytes.

Paradoxically, serum adiponectin levels are decreased with obesity and are positively associated with insulin sensitivity (Arita et al. 1999). Adiponectin’s insulin-sensitizing actions are accomplished by three different mechanisms. In skeletal muscle, adiponectin upregulates genes involved in fatty acid transport, including CD36 and acyl-coenzyme A oxidase. Also, this hormone activates peroxisome proliferator-activated receptor-α (PPARα) in skeletal muscle and liver, increasing fatty-acid utilization. Lastly, adiponectin activates adenosine monophosphate protein kinase (AMPK), stimulating glucose utilization and β-oxidation (reviewed in Yadav et al. 2013). Leptin was discovered in 1994 through positional cloning in ob/ob mice, an obesity mouse model resulting from a random mutation at Jackson Laboratory (Y. Zhang et al. 1994). These animals were extremely obese, hyperphagic, diabetic and infertile; they were found to be deficient in a circulating factor identified as leptin. Since leptin acts in the brain to suppress food intake, initially it was thought that leptin administration could be a promising therapy for obesity. However, leptin levels are directly correlated with body fat and obese humans develop leptin
resistance, which reveals the complexity of hormone regulation and metabolism (reviewed in Kelesidis et al. 2010).

As previously mentioned, one of the functions of adipocytes is energy storage in the form of triglycerides. The equilibrium between adipose tissue lipolysis and lipogenesis is an important factor for metabolic health. Obesity is associated with increased basal lipolysis in AT and a resulting elevation in circulating free fatty acids (FFA). Higher FFA levels are accompanied by a decrease in whole-body glucose utilization (reviewed in Randle 1998) and by impaired beta cell function and insulin secretion (Oprescu et al. 2007).

Expansion of adipose tissue occurs through both increases in cell size (hypertrophy) and increases in cell numbers (hyperplasia). Since hyperplasia results from cell proliferation and mature adipocytes do not divide, preadipocyte differentiation, or adipogenesis, is required for adipose tissue hyperplasia. Adipogenesis is controlled by several factors from hormones to cytokines and signaling cascades. One of the most studied areas of adipogenesis is the transcriptional control of this highly elaborate cellular process. The nuclear receptor peroxisome proliferator activated receptor gamma \((\text{PPAR}_\gamma)\) is the major regulator of adipogenesis. Moreover, members of the CCAAT/enhancer-binding proteins (C/EBPs) family also play a pivotal role in adipocyte differentiation (reviewed in de Sá et al. 2017).

In addition to PPAR\(\gamma\) and C/EBPs, the STAT transcription factors are important for adipocyte development and function. STATs regulate gene expression in a tissue-specific manner. In fat cells, STATs 1, 3, 5A, 5B and 6 are expressed. STATs 1, 3, 5A and 5B are regulated during adipocyte differentiation while STAT6 levels stay constant (J M Stephens, Morrison, and Pilch 1996). Of the STATs expressed in adipocytes, STAT5 plays a prominent role in adipocyte differentiation and function, and it has been the focus of the Stephens
laboratory. When STAT5 was originally discovered as a regulator of the β-casein gene, it was called mammary gland factor (MGF) (Schmitt-Ney et al. 1991). STAT5A and 5B derived from a duplication event and therefore are highly homologous; most differences are in the C-terminal region in which STAT5A is a little longer (Yaming Wang and Levy 2012).

The importance of STAT5 for adipocyte differentiation has been demonstrated in several ways. In one study, ectopic expression STAT5A was sufficient to promote adipogenesis in BALB/c and NIH-3T3 non-precursor cells, and STAT5B enhanced STAT5A-induced adipogenesis (Floyd and Stephens 2003). Moreover, constitutively active STAT5A was able to substitute for GH-dependent differentiation of 3T3-F442A preadipocytes (Shang and Waters 2003). In vivo studies demonstrated that mice lacking only STAT5A or 5B had abnormal adipose tissue and mice lacking both STAT5A and 5B were more severely affected (Teglund et al. 1998). Since these are global knockouts, however, the effects of STAT5 deletion might be due to developmental issues and do not explore the role of STAT5 in adipocytes. More recently, Kaltenecker and colleagues have generated a knockout (KO) mouse that lacks STAT5 only in mature adipocytes. These mice presented increased insulin sensitivity and adiposity as well as decreased lipolysis rate (Kaltenecker et al. 2017). In our own studies, adipose-specific STAT5 KO mice are also more insulin sensitive, and they also exhibited higher adiposity compared to control mice. However, we saw no effects of the KO on lipolysis (RICHARD et al. 2018). Although it is largely speculated that mice with reduced growth hormone signaling are obese due to a loss of lipolysis, further studies are needed to confirm these speculations.

In addition to the generation of adipose-specific STAT5 KO mice, the identification of novel STAT5 interacting proteins is another approach to try and understand the functions of STAT5 proteins in adipocytes. To conduct this analysis, STAT5 was immunoprecipitated from
mature 3T3-L1 adipocytes and the precipitate was analyzed by mass spectrometry. Surprisingly, pyruvate dehydrogenase complex (PDC) was found to interact with STAT5 in the adipocyte nucleus (Richard, Hang, and Stephens 2017). PDC is normally found in the mitochondrial matrix where it catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA (reviewed in Wieland 1983). The presence of PDC in the nucleus is a recent observation. In a study in cancer cells, PDC was able to produce acetyl-CoA in the nucleus and modulate histone acetylation (Sutendra et al. 2014). Another study in prostate cancer cells, have shown that nuclear PDC was responsible for controlling the expression of sterol regulatory element-binding transcription factor (SREBF)-target genes by mediating histone acetylation (Chen et al. 2018). Thus, the interaction between STAT5 and PDC in the nucleus of mature 3T3-L1 adipocytes led us to hypothesize that PDC could be generating acetyl-CoA for acetylation of STAT5 or histones, or otherwise regulating the function of other proteins in the STAT5 transcriptional complex.

Currently, the role of acetylation in STAT5 transcriptional activity is still unclear. The majority of studies have established a positive relationship between STAT5 acetylation and its ability to regulate transcription. In one study, acetylation of the prolactin receptor (PRLR) affected its dimerization and phosphorylation as well as the subsequent activation of STAT5 by acetylation (Ma et al. 2010). In another study, STAT5A SUMOylation inhibited acetylation through competing with the lysine 696 and decreased STAT5A transactivation activity (Van Nguyen et al. 2012). In addition, prolactin-mediated transcriptional activity of STAT5A and 5B was enhanced by their interaction with the HAT protein p300/CBP (CAAT-binding protein) (Pfitzner et al. 1998). However, other studies indicate that HDAC inhibitors, which increase acetylation levels in the cell, prevent induction of STAT5 target genes (Rascle, Johnston, and
Amati 2003). In addition, mutations on multiple STAT5 lysine residues did not alter STAT5 transcriptional activity (Pinz et al. 2015a).

At the outset, the objective of this project was to investigate the role of acetylation in STAT5 transcriptional activity in mature 3T3-L1 adipocytes. To explore this topic, we chose two main approaches. The first was to assess the modulation of STAT5 acetylation by HDAC inhibition, and the second was to analyze the effects of HDAC inhibitors on the expression of STAT5 target genes. Our initial experiments determined that STAT5 acetylation was barely detectable in mature 3T3-L1 adipocytes, and in experiments where STAT5 acetylation was detected, it was only modestly regulated by HDAC inhibitors. We had planned to knock down the wild-type STAT5 and knock in STAT5 mutated at different lysine residues, then analyze STAT transcriptional activity by STAT5 promoter element luciferase assay. This would have determined whether acetylation of one specific residue was responsible for STAT transactivation and whether regulation of STAT5 target genes would be affected by these mutations. This first part of the project was not performed because we concluded that STAT5 acetylation levels in mature 3T3-L1 adipocytes were very low and thus unlikely to play a major role in STAT5 transcriptional activity.

In fat cells, STAT5 is activated by growth hormone (GH) or prolactin (PRL) (Zvonicek et al. 2003; Fleenor, Arumugam, and Freemark 2006). Thus, our second approach consisted of treating mature 3T3-L1 adipocytes with GH and examining the expression of some genes that are activated by STAT5, such as insulin growth factor 1 (IGF-1) and insulin-like growth factor-binding protein-3 (IGFBP-3) (Woelfle and Rotwein 2004; Hochberg et al. 2015), as well as members of the suppressors of cytokine signaling (SOCS). We also investigated the regulation of B-Cell Lymphoma 6 (BCL6) gene, which is inhibited by STAT5 (G. Lin et al. 2014). In
addition, adipocytes were treated with HDAC inhibitors to modulate acetylation levels in the cell. The results demonstrated that HDAC inhibitors greatly affected the expression of two members of the SOCS family. The inhibitors decreased STAT5-mediated upregulation of *Cish*, while induction of *Socs3* was substantially increased. This opposite modulation has been reported previously in separate experimental contexts (Pinz et al. 2015b; Xiong et al. 2012), but this divergent regulation of STAT5 target genes by HDAC inhibitors in the same cells under the same conditions is a novel finding.

These effects on *Cish* and *Socs3* gene expression were not recapitulated when HDACs were knocked down using siRNA. Therefore, we hypothesized that the function of other proteins were being affected by HDAC inhibition, possibly members of the BET protein family. Indeed, inhibition of BET proteins by JQ1 resulted in the same divergent regulation of *Socs3* and *Cish* as HDAC inhibition. In fact, studies demonstrate that HDAC and BET inhibitors induce and repress similar genes (Bhadury et al. 2014). In our studies, chromatin immunoprecipitation (ChIP) analysis demonstrated that changes in STAT5A binding to the promoter region of *Socs3* and *Cish* did not necessarily correlate with changes in gene expression. However, differences in binding of the BET protein BRD2 to the transcription start sites and binding of RNA polymerase II to the coding region strongly correlated with changes in gene expression.

While the inhibition of BET proteins can be readily associated with reduced transcriptional elongation and consequently decreased gene expression; increased gene expression by BET inhibition is more challenging to understand. Notably, studies have shown that HIV-1 gene transcription is dependent on P-TEFb (Mancebo et al. 1997) and that HDAC (Bartholomeeusen et al. 2013) or BET inhibition (Bartholomeeusen et al. 2012a) can increase HIV-1 gene expression. This upregulation results from a secondary effect of HDAC and BET
inhibitors to release P-TEFb from its inhibitory complex so that it is more available for transcription elongation.

BET and HDAC inhibitors that have been reported to release P-TEFb from its inhibitory complex also increase the expression of its negative regulator HEXIM1 (Bartholomeeusen et al. 2012b, 2013). In our study, the BET inhibitor JQ1 transiently decreased the association between Cyclin T1 and HEXIM1 and induced the expression of HEXIM1. These results suggest that the HDAC and BET inhibition have different mechanisms of action on adipocyte SOCS regulation, but results in similar outcomes on gene expression. Moreover, although they are members of the same family, SOCS3 and CISH likely have very distinct functions, at least in the context of fat cells. This project underscores the complexity of studying the transcriptional control of genes by epigenetic modulators.
Chapter 3. Material and Methods

3.1 Cell culture

Murine 3T3-L1 preadipocytes were grown in Dulbecco’s Modified Eagle’s Media (DMEM; from Sigma-Aldrich, St. Louis, MO) with 10% bovine calf serum (Calf). Two days after reaching confluence, cells were induced to differentiate using a standard MDI induction cocktail of 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1.7 μM insulin in DMEM containing 10% characterized fetal bovine serum (FBS). HyClone Calf and FBS were purchased from Thermo Scientific or GE Healthcare Life Sciences (Logan, UT). The medium was changed every 48 – 72 h during growth, differentiation, and maintenance. When specified, cells were serum deprived with DMEM containing 0.3% bovine serum albumin (BSA; Cat #: A6003, Sigma-Aldrich, St. Louis, MO). Otherwise, cells were serum deprived by changing the media to DMEM containing 1% Calf for 16 to 24 h before treatment with the inhibitors. Trichostatin A (TSA), nicotinamide (NAM), sodium butyrate and (+)-JQ1 were purchased from Sigma-Aldrich (St. Louis, MO). The inhibitors CI994 (HDAC1), RGFP966 (HDAC3), LMK-235 (HDAC5) and CAY10603 (HDAC6) were purchased from Selleckchem (Houston, TX). Recombinant murine GH (mGH) was purchased from Dr. A.F. Parlow at the National Hormone and Peptide Program (NHPP; Torrance, CA).

3.2. Preparation of whole-cell extracts

Cell monolayers were rinsed once with phosphate buffered saline (PBS), then scraped into non-denaturing IP buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% IGEPAL CA-630, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, 1 mM 1,10-phenanthroline), and a phosphatase inhibitor (0.2 mM sodium...
vanadate). The cell suspension was subjected to a freeze/thaw cycle at -80°C, passed through a 20G needle five times, and clarified by centrifugation at 13,000 x g for 10 min at 4°C.

3.3. Subcellular Fractionation

Mature adipocytes from eight 10-cm culture plates were scraped into 1 mL NHB buffer per plate. NHB is comprised of 20 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, and the same protease and phosphatase inhibitors used for IP buffer. After adding 10% NP-40 to the cell suspension using the formula: volume of cell lysate x 0.15/9.85 (0.015% final concentration of NP-40), it was homogenized on ice using 16 strokes in a Dounce homogenizer. The extract was centrifuged at 2500 rpm in a Beckman GS-6KR centrifuge with a swinging bucket rotor for 5 min at 4°C. Subsequently, the supernatant containing the cytosolic fraction was transferred to a fresh tube. The nuclear pellet was washed once with half of the initial volume of NHB buffer and re-centrifuged at 4°C for 5 min at 2500 rpm in the Beckman GS-6KR centrifuge. The supernatant was discarded, while the nuclear pellet was resuspended in IP buffer and incubated on ice for 0.5 – 1 h. To break open the nuclei, the nuclear extract was passed through a 20G needle four times; the extract was then clarified by centrifuging at 13,000 x g for 10 min at 4°C.

3.4. Measurement of protein concentration

Protein content of cell and tissue extracts was quantified using the Bicinchoninic acid (BCA) assay kit from Sigma-Aldrich (Cat #: BCA1) according to manufacturer’s instructions.

3.5. Gel electrophoresis and immunoblotting

Cell extracts (25-120 μg total protein) were separated on 6, 7.5 or 10% sodium dodecyl sulfate (SDS) polyacrylamide (PA) gels (acrylamide, Cat #: EC-890, from National Diagnostics, Atlanta, GA) and transferred to nitrocellulose membranes (Cat #: 162-0115, BioRad, Hercules,
CA) as previously described (Richard et al. 2013). Results were visualized with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) and enhanced chemiluminescence (Pierce/Thermo Scientific) as previously described (Richard et al. 2013) or IRDye-conjugated secondary antibodies (Licor Biosciences, Lincoln, NE) and scanned with the Odyssey infrared scanner (Licor Biosciences) (Fuller et al. 2014).

3.6. Immunoprecipitation (IP)

Cell extracts (230-500 μg total protein) were incubated with specific antibodies overnight on a mini-tube rotator at 4°C. Protein A-conjugated agarose (IPA300 Protein A Resin; Cat #: 10-2003-02) from Repligen or Protein A/G Plus-agarose (Cat #: sc-2003) beads from Santa Cruz Biotechnology (Dallas, TX) were added to the antibody-epitope mixture. The conjugation reaction continued for an additional 1 – 2 h at 4°C with rotation. Next, the beads were pelleted by centrifugation at 13,000 rpm for 3 min at 4°C. The supernatant was removed by aspiration and the beads were washed 3 times with ice cold 1X IP buffer with protease inhibitors. Between each wash, the beads were pelleted by centrifugation at 12,000 rpm for 1 min at 4°C and the supernatant was removed by aspiration. After the final wash, the IP antibody and immunoprecipitated proteins were eluted from the bead resin into 2X SDS loading buffer (LB) by boiling the samples for 10 min at 100°C. Samples were flicked multiple times during the heat step to ensure efficient elution. The samples were briefly centrifuged and the supernatants analyzed by SDS-PAGE and immunoblotting. A mock sample containing a pool of equal concentration of all samples and no IP antibody was used as a negative control to ensure there was no unspecific binding of the beads to protein samples.
3.7. RT-qPCR

3T3-L1 adipocyte monolayers were harvested in RLT lysis buffer for total RNA purification using the RNeasy mini kit from Qiagen (Hilden, Germany). RNA concentrations were quantified by spectrophotometry. cDNA was synthesized by reverse transcription (RT) using 10 µl of RNA and 10 µl of RT master mix following the protocol of High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The following mouse (m) genes were examined by RT-qPCR: mCyclophilin a, mNoNo, mSoCs3, mCish, mBcl6, mIgf1, mSoCs2, mIgfbp3, mHexim1, using Takara SYBR premix (Takara Bio USA, Mountain View, CA) and primers purchased from Integrated DNA Technologies (IDT, Skokie, IL). Primers 1 and 2 sequences for each gene are shown in Table 3.2. Assay wells contained 4 ng cDNA in a reaction volume of 10 µl (3 µl of cDNA at 1.33 ng/µl, plus 7 µl of master mix). Analysis was performed using Applied Biosystems 7900HT Fast Real-Time PCR System and SDS 2.4 software (Applied Biosystems, Foster City, CA). Cyclophilin A and NoNo were used as endogenous controls.

3.8. Small interfering RNA (siRNA)-mediated knockdown

3T3-L1 mature adipocytes (5-7 days post-MDI) were trypsinized from 6-well plates and re-plated in 12-well plates at a density of 5.8 x 10^5 cells/cm^2 in antibiotic-free medium (10% FBS/DMEM). Adipocytes were transfected using the protocol from Dharmacon with DharmaFECT Duo reagent (Dharmacon; Cat #: T-2010-03) and 50 nM of non-targeting (Cat #: D-001810-10-50), Hdac4 (Cat #: L-043626-00-0005), Hdac5 (Cat #: L-062182-00-0005), Hdac6 (L-043456-02-0005) siRNA or a combination of two at a time in OptiMEM reduced serum medium (Thermo Fisher; Cat #: 31985088). Twenty-four hours later, siRNA-containing media was removed and replaced with antibiotic-free 10% FBS/DMEM. After 24-28 hours, the media
was replaced by 1% CALF antibiotic-free. On day 2 post-transfection, cells were treated with vehicle or 5 nM GH for 1 hour. Adipocytes were harvested for RNA in RLT buffer (Qiagen) to assess knockdown efficiency and effects on STAT5 target gene expression. For knockdown experiments, *Nono* was used as the housekeeping gene.

3.9. Chromatin immunoprecipitation (ChIP)

The medium on mature 3T3-L1 adipocytes (7 – 15 days post-MDI) was replaced with 1% CALF for 16 – 24 h prior to treatment with LMK-235 for 2 hrs, JQ1 for 30 mins, or DMSO control. Next, the cells were treated with vehicle or GH for 30 min. Six or seven 10-cm plates per treatment group were used (~4 x 10^7 cells). ChIP experiments were performed using the SimpleChIP® Kit (Magnetic Beads) from Cell Signaling Technology (Cat #: 9003S; Danvers, MA) according to the manufacturer’s instructions. To crosslink DNA and interacting proteins, cells were incubated at room temperature with 1% formaldehyde (Cat #: 252549) from Sigma-Aldrich for 10 min. The crosslinking reaction was stopped by the addition of glycine provided in the kit. The medium was aspirated from adherent adipocytes, and plates were rinsed 2 times with ice-cold PBS. Cells from each plate were scraped in 1ml ice-cold PBS + 1000 μM PMSF (phenylmethane sulfonyl fluoride). Cell suspensions from each treatment were combined in a 15-ml conical tube and centrifuged as directed. Nuclei preparation, chromatin digestion, and ChIP assays were performed as indicated in the manufacturer’s instructions. For each ChIP, 8-12 μg of digested, cross-linked chromatin were incubated with the following amounts of antibodies (Table 3.1), as recommended by the manufacturer: rabbit anti-STAT5A (2 μg), rabbit anti-acetyl histone H3 (5 μg), rabbit anti-acetyl-histone H4 (1 μg), mouse anti-RNA polymerase II (1 μg), rabbit anti-phosphoserine2 RNA Pol II (4 μg), rabbit anti-BRD2 (1:50 dilution), non-immune IgG control antibody rabbit IgG from ChIP kit (2 μg), or mouse IgG from RNA pol II (1 μg). After
protein digestion and DNA purification using the columns provided by the kit, input and immunoprecipitated DNA samples were quantitated using the real-time quantitative PCR (qPCR) in a total volume of 10 μl containing 8 μl reaction master mix and 2 μl DNA (~100 μg/ml), using an Applied Biosystems 7900HT Fast Real-Time PCR System. We used the qPCR amplification program specified in the kit instructions with the exception that the initial denaturation step was performed for 10 min at 95°C. We also added a dissociation curve step at the end of the reaction. Primers used to amplify different regions of Cish and Socs3 genes are described in table 3.3. A standard curve prepared with only 2% input samples and 5-fold serial dilutions was used to access amplification efficiency. Immunoprecipitation efficiency was calculated using the percent input approach and the following equation: Percent input = 2% \times 2^{(C[T]_{2\%\text{ Input Sample}} - C[T]_{\text{IP Sample}})}; where C[T] = threshold cycle of the PCR reaction.

3.10. Statistical analysis

Data were plotted as mean ± standard error (S.E.). The qPCR data were analyzed using either one-way ANOVA or two-way ANOVA. For ChIP experiments, data were analyzed using two-way ANOVA with Bonferroni’s post hoc test. Differences with p < 0.05 were considered statistically significant.
Table 3.1. List of antibodies used for IPs, ChIPs and Western blots.

<table>
<thead>
<tr>
<th>Antibody against</th>
<th>Cat number/Company</th>
<th>Host Species/Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-Histone H3</td>
<td>#06-599; Millipore Sigma</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>Acetyl-Histone H4</td>
<td>#06-866; Millipore Sigma</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>Acetylated-Lysine</td>
<td>#9441; Cell Signaling</td>
<td>Rabbit monoclonal</td>
</tr>
<tr>
<td>BRD2 (D89B4)</td>
<td>#5848; Cell Signaling</td>
<td>Rabbit monoclonal</td>
</tr>
<tr>
<td>BRD4</td>
<td>#PA5-41550; ThermoFisher Scientific</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>Cyclin T1</td>
<td>#ab184703; Abcam</td>
<td>Rabbit monoclonal</td>
</tr>
<tr>
<td>HDAC4 (D8T3Q)</td>
<td>#15164; Cell Signaling</td>
<td>Rabbit monoclonal</td>
</tr>
<tr>
<td>HDAC5 (D1J7V)</td>
<td>#20458; Cell Signaling</td>
<td>Rabbit monoclonal</td>
</tr>
<tr>
<td>HDAC6 (D21B10)</td>
<td>#7612; Cell Signaling</td>
<td>Rabbit monoclonal</td>
</tr>
<tr>
<td>HEXIM1</td>
<td>#ab25388; Abcam</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>Histone H3</td>
<td>#9715; Cell Signaling</td>
<td>Rabbit monoclonal</td>
</tr>
<tr>
<td>Mouse IgG – Isotype control</td>
<td>#ab46540; Abcam</td>
<td>Mouse monoclonal</td>
</tr>
<tr>
<td>Rabbit IgG – Isotype control</td>
<td>#2729; Cell Signaling</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>RNA polymerase II CTD repeat YSPTSPS (pS2)</td>
<td>ChIP Grade ab5095; Abcam</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>RNA Polymerase II</td>
<td>#17-620; Millipore Sigma</td>
<td>Mouse monoclonal</td>
</tr>
<tr>
<td>STAT3(124H6)</td>
<td>#9139; Cell Signaling</td>
<td>Mouse monoclonal</td>
</tr>
<tr>
<td>STAT5A</td>
<td>E289; ab213219; Abcam</td>
<td>Rabbit monoclonal</td>
</tr>
</tbody>
</table>
Table 3.2. List of primers used for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer 1</th>
<th>Primer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abhd5 (Cgi-58)</td>
<td>5'-GAGAGAACATCAGCGGTCCATA-3'</td>
<td>5'-CCCACATCTACATCACACACCTT-3'</td>
</tr>
<tr>
<td>Bcl6</td>
<td>5'-AGTACATCGGTCGGCTGGCGAGAGAAG-3'</td>
<td>5'-CAGAGATGTCCTCCATACCTG-3'</td>
</tr>
<tr>
<td>Cish</td>
<td>5'-CCGCCCATTGGCTCCA-3'</td>
<td>5'-GCTCCCTTCTCTTTCCATCC-3'</td>
</tr>
<tr>
<td>Cyclophilin a (Ppia)</td>
<td>5'-GCCACAGCGGTCAATAGCTAAGGCAC-3'</td>
<td>5'-TGCAAACAGCTCGAAGAGAGACGC-3'</td>
</tr>
<tr>
<td>Hdac4</td>
<td>5'-CGAGTGTCACCTCATCACCATA-3'</td>
<td>5'-GCCAAATCTCTCTCAACAGACA-3'</td>
</tr>
<tr>
<td>Hdac5</td>
<td>5'-GGATCGTTGAGAATGCTGTC-3'</td>
<td>5'-CTTCAACTCTGGTGAGGCAGCCTAC-3'</td>
</tr>
<tr>
<td>Hdac6</td>
<td>5'-GGATGGAGAAATAAAGAGCATGTCAGCC-3'</td>
<td>5'-AGAAGCACCGCATTTAGAG-3'</td>
</tr>
<tr>
<td>Hexim1</td>
<td>5'-CGACGCTCAAACTAGCAACTGTA-3'</td>
<td>5'-CTCGATTGCCCACCTACTGTC-3'</td>
</tr>
<tr>
<td>Igf1</td>
<td>5'-ATGCTCTCAGGTGCTGTGTGC-3'</td>
<td>5'-AGTACATCCAGTTCTTCCACAG-3'</td>
</tr>
<tr>
<td>Igfp3</td>
<td>5'-CATCTCAAGTTCTCTACTGC-3'</td>
<td>5'-CCATACTTGGTCACACAGCA-3'</td>
</tr>
<tr>
<td>Lpl</td>
<td>5'-CCACCGCCGGCTAGTGTTCCAG-3'</td>
<td>5'-AATCTCTTTGCCTGGTCATTGC-3'</td>
</tr>
<tr>
<td>Nono</td>
<td>5'-CATCATCAGCATCACCACCA-3'</td>
<td>5'-TCTTCAGGATCTAGTCAAGCC-3'</td>
</tr>
<tr>
<td>Nrip1</td>
<td>5'-CTCGCCAACTCTTGGACCA-3'</td>
<td>5'-CTTCCCTTCCACATAGCAGA-3'</td>
</tr>
<tr>
<td>Socs2</td>
<td>5'-TCTGGGGACTGCCTTTACCAAC-3'</td>
<td>5'-CTTCCGACGTTCTTCCACATAGC-3'</td>
</tr>
<tr>
<td>Socs3</td>
<td>5'-GGAAATTTGGCTGTGGTGTA-3'</td>
<td>5'-GAGATTTGCCTGCGAGCTA-3'</td>
</tr>
</tbody>
</table>

Table 3.3. List of primers used for ChIP-qPCR.

<table>
<thead>
<tr>
<th>Gene Region</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cish SBS</td>
<td>5'-CGTCCAGCCATACGATTGGGT-3'</td>
<td>5'-CAGGCGTCTAGTGCTTTGGA-3'</td>
</tr>
<tr>
<td>Cish TSS</td>
<td>5'-GTCGCCACAGGCAATCGCTTTCAGCTCC-3'</td>
<td>5'-TGCCAGGGGTGCGAAGGCTCAGG-3'</td>
</tr>
<tr>
<td>Cish Coding</td>
<td>5'-TACCCCTCCAATCTGACTGAG-3'</td>
<td>5'-TTCCCTCCAGATGTGACTGTTG-3'</td>
</tr>
<tr>
<td>Socs3 SBS</td>
<td>5'-GCACAGCCCTCTTCAGTGACAGG-3'</td>
<td>5'-GTATTTACCGCCAGCTACCC-3'</td>
</tr>
<tr>
<td>Socs3 TSS</td>
<td>5'-CGTGACTGCTGGCGGGTAAATAC-3'</td>
<td>5'-GGAGAGACAGCGTCTTGACGAG-3'</td>
</tr>
<tr>
<td>Socs3 Coding</td>
<td>5'-CATTTGAAGGAGAGACAGATGAG-3'</td>
<td>5'-CATAGGAGAGACAAAGACAGAC-3'</td>
</tr>
</tbody>
</table>
Chapter 4. Results

4.1. STAT5 acetylation levels are low in adipocytes

In order to examine modulation of STAT5 acetylation, 3T3-L1 mature adipocytes were pre-treated for 6 hours with HDAC inhibitors Trichostatin A (TSA), Nicotinamide (NAM) and Sodium Butyrate (NaBu) at 5 µM, 20 mM and 10 mM, respectively, then treated with vehicle or 5 nM GH for 20 minutes to induce STAT5 phosphorylation and activation. In Fig. 4.1, whole-cell extracts were collected, 400 µg of total protein was immunoprecipitated with the pan-acetyl lysine antibody, and samples were analyzed by Western blot using the STAT5A antibody overnight. As shown on the left side of Fig. 4.1, a modest amount of acetylated STAT5 was detected. In the vehicle conditions there was a minor increase in the band intensity with the presence of HDAC inhibitors, but IgG control band was also lower. In addition, no alterations were observed in the GH conditions. On the right side of the figure, direct Western blot samples detection shows a high-intensity band corresponding to STAT5A, confirming the high expression levels of STAT5A in adipocytes.

In the next experiment, 3T3-L1 mature adipocytes were pre-treated with the same HDAC inhibitors, then with vehicle or GH for 20 minutes. We then performed the experiment in the reverse configuration. Whole-cell extracts were immunoprecipitated with STAT5A antibody, and samples were analyzed by Western blot using the pan-acetyl lysine antibody overnight. On the left side of Fig. 4.2 (immunoprecipitated samples), we observed a band of equivalent size to STAT5 (approximately 92 kDa). Surprisingly, however, STAT5 acetylation was modestly decreased by HDAC inhibitors in the presence of GH. We also observed a decrease in band intensity in the vehicle + inhibitors condition versus vehicle only; however, the IgG band is also
weaker, suggesting that some of the beads may have been lost. On the right side, the direct Western blot for pan-acetyl lysine could not detect a band of equivalent molecular weight as STAT5, even with 100 μg of total protein loaded. Moreover, the inhibitors did not produce the expected increase in global protein acetylation, but rather seemed to specifically enhance the intensities of a small number of bands, mostly at high molecular weights. For this reason, we examined Histone H3 acetylation to verify the efficacy of the inhibitors. Western blot analysis was performed using 100 μg of whole-cell extracts. As shown in Fig. 4.3, acetyl-histone H3 was increased by HDAC inhibition, while total H3 levels were unchanged. We also tested whether serum deprivation the day prior to treatment affected these outcomes. We confirmed that the inhibitors increased histone H3 acetylation levels and determined that serum deprivation enhanced this effect.

Figure 4.1. STAT5 acetylation was not altered by pan-HDAC inhibitors. 3T3-L1 mature adipocytes were serum-deprived overnight. The following day, cells were pre-treated for 6 hours (figure caption cont’d.)
with HDAC inhibitors Trichostatin A (TSA) and Nicotinamide (NAM) at 5 µM and 20 mM respectively, then treated with 5 nM GH or vehicle (NaHCO₃) for 20 minutes. Whole-cell extracts were isolated, and 400µg of lysates were incubated overnight with pan-acetyl lysine antibody. STAT5A antibody was used for Western blot analysis. This experiment was independently repeated on a separate batch of adipocytes.

Figure 4.2. STAT5 acetylation was modestly decreased by pan-HDAC inhibitors. 3T3-L1 mature adipocytes were serum deprived overnight. The next day, cells were pre-treated for 6 hours with HDAC inhibitors Trichostatin A (TSA), Nitotinamide (NAM), and Sodium Butyrate (NaBu) at 5 µM, 20 mM, and 10 mM, respectively, then treated with 5 nM GH or vehicle (NaHCO₃) for 20 minutes. Whole-cell extracts were isolated, and 400µg of lysates were incubated overnight with STAT5A antibody. Pan-acetyl-lysine antibody was used for Western blot analysis. This experiment was independently repeated on a separate batch of adipocytes.
Figure 4.3. HDAC inhibitors increase Histone H3 acetylation. Mature 3T3-L1 adipocytes were serum-deprived overnight or kept in media containing 10% bovine serum. Adipocytes were pre-treated for 6 hours with HDAC inhibitors Trichostatin A (TSA), Nitotinamide (NAM) and Sodium Bytarate (NaBu) at 5 µM, 20 mM and 10 mM, respectively. Then, the NaHCO₃ vehicle was added to cells that were harvested 20 minutes later. Western blot analysis was performed with 100 µg of cell lysates using acetylated histone H3 and total histone H3 antibodies. This experiment was independently repeated on a separate batch of adipocytes.

In gene expression experiments described later in this Chapter, HDAC inhibitors that were more specific to individual HDACs could recapitulate the effects of TSA on STAT5 target genes. Therefore, I repeated the experiments presented in Figs. 4.1 and 4.2, this time pre-treating adipocytes with 10 µM of the specific HDAC5 inhibitor (LMK-235) for 3 hours, then with vehicle or GH for 1 hour. In addition, there was a possibility that modulation of STAT5 acetylation would be better detected in isolated nuclear and cytosolic fractions. Immunoprecipitation was performed using 230 µg of total protein and pan-acetyl lysine antibody. The resulting blot was probed with the STAT5A antibody. As shown on the left side of Fig. 4.4, levels of acetylated STAT5 in the nuclear fractions were enhanced in the presence of GH, while band intensities in the cytosolic samples were considerably lower and were unchanged with GH treatment. Although LMK-235 did not alter acetylation levels in nuclear or cytosolic fractions, either in vehicle or GH conditions, the use of cellular fractions allowed us to
detect higher intensity bands for acetylated STAT5 than we did in whole-cell extracts with the same antibody combination (Fig. 4.1). This experiment also determined that acetylated STAT5 is mostly present in the nucleus of adipocytes with GH treatment.

Figure 4.4. STAT5 acetylation is not affected by HDAC5 inhibitor (LMK-235). Mature 3T3-L1 adipocytes were serum-deprived overnight. The next day, adipocytes were pre-treated for 3 hours with 10 µM of HDAC5 inhibitor LMK-235, then with 5 nM GH or vehicle (NaHCO₃) for 1 hour. Nuclear and cytosolic extracts were isolated, and 230 µg of lysates were incubated overnight with pan-acetyl lysine antibody. Western blot analysis was performed using STAT5A antibody. This experiment was independently repeated on a separate batch of adipocytes.

In order to verify the efficacy of LMK-235, we examined if Histone H3 acetylation was affected by this inhibitor. Adipocytes were pre-treated for 3 hours with increasing doses of LMK-235, and V or GH was added for one more hour before the cells were harvested. Western blot analysis shown in Fig. 4.5 demonstrates a dose-dependent increase in Histone H3 acetylation by LMK-235, confirming that LMK-235 modulates acetylation in 3T3-L1 adipocytes. ERK1/2 was used as a loading control.
Figure 4.5. HDAC5i (LMK-235) increases Histone H3 acetylation. Mature 3T3-L1 adipocytes were serum-deprived overnight. Adipocytes were pre-treated with increasing doses of LMK-235 for 3 hours. Then, vehicle (NaHCO₃) or GH was added to cells 1 hour prior to harvest. Western blot analysis was performed with 50 µg of whole-cell extracts using acetylated histone H3 and ERK1/2 antibodies. This experiment was independently repeated on a separate batch of adipocytes.

To corroborate our observations on GH-induced acetylation of nuclear STAT5, we repeated cell treatments with the same experimental conditions as in Fig. 4.4, and then used the reverse configuration of antibodies. Nuclear and cytosolic fractions were isolated. Immunoprecipitation was performed using 260 µg of total protein and STAT5A antibody overnight. The resulting blot was probed with the pan-acetyl lysine antibody. As shown on the left side of Fig. 4.6, low intensity bands corresponding to acetylated STAT5 were detected in the nuclear but not cytosolic fractions. Direct Western blot analysis shown on the right side of the figure demonstrated that LMK-235 had few effects on cytosolic proteins, except for enhancing the intensity of a single band similar in size to IgG, both in the presence and absence of GH. Thus, this experiment confirmed the findings from Fig 4.4, in which STAT5 acetylation levels are higher in the nucleus than in the cytoplasm of adipocytes. However, band intensities were considerably lower and LMK-235 modestly increased STAT5 acetylation in the nuclear samples.
Figure 4.6. Nuclear STAT5 acetylation is modestly increased by HDAC5 inhibitor (LMK-235). Mature 3T3-L1 adipocytes were serum-deprived overnight. The next day, adipocytes were pre-treated for 3 hours with 10 μM of HDAC5 inhibitor LMK-235, then with 5 nM GH or vehicle (NaHCO₃) for 1 hour. Nuclear and cytosolic extracts were isolated, and 260 μg of lysates were incubated overnight with STAT5A antibody. Western blot analysis was performed using pan-acetyl lysine antibody. This experiment was independently repeated on a separate batch of adipocytes.

Given the robust difference in the nuclear band intensity under these conditions (Fig. 4.6), compared to Fig. 4.4, we cannot exclude the possibility that a highly acetylated protein interacting with STAT5 is being precipitated by the pan-acetyl lysine antibody, and contributing to a higher intensity band when the membrane is probed for STAT5A. In this case, we would not be detecting strictly STAT5 acetylation. Another possibility is that in Fig. 4.6, STAT5A antibody does not pull down acetylated protein very well. As expected, the direct Western of the cytosolic fractions (30 μg protein) shown on the right side of Figure 4.4 reveals very high expression of STAT5A in adipocytes. We conclude that STAT5 acetylation levels in mature 3T3-L1
adipocytes are low. In addition, STAT5 acetylation is most likely not accounting for the changes in GH-mediated induction of STAT5 target genes since HDAC inhibitors had minimal effects on STAT5 acetylation, despite their ability to modulate GH-induced STAT5 target gene expression.

4.2. GH-induction of STAT5 target genes

Since STAT5 is a transcription factor, we examined the expression of known STAT5 target genes at several time points (0.5 to 48 hours) after a single acute dose of GH. Of the genes tested, \( \text{Socs3} \) and \( \text{Cish} \) were the most acutely upregulated. \( \text{Socs3} \) levels were elevated at 30 minutes and peaked at 1 hour, and returned to near basal levels at 2 hours. \( \text{Cish} \) expression was upregulated at 1 hour and remained elevated after 48 hours. As previously reported (Walker, Nelson, and Frank 2007), \( \text{Bcl6} \) was downregulated by GH treatment at all time points. \( \text{Igf1} \) and \( \text{Soes2} \) were upregulated at later time points than \( \text{Cish} \) and \( \text{Socs3} \). \( \text{Igf1} \) levels were significantly higher between 4 and 48 hours. \( \text{Soes2} \) was upregulated at 2 hours, and levels started to decline after 12 hours. \( \text{Igfbp3} \) was significantly upregulated only after 12 hours and levels continued to be elevated even at the 48 hour time point (Fig. 4.7).

4.3. HDAC inhibitors increase \( \text{Socs3} \) and decrease \( \text{Cish} \) expression

Given that \( \text{Socs3} \) and \( \text{Cish} \) were the STAT5 target genes most acutely regulated by GH, we chose to focus on these two genes in the following experiments. In adipocytes treated with general HDAC inhibitors, TSA or NaBu, the ability of GH to induce \( \text{Socs3} \) was increased and the ability of GH to induce \( \text{Cish} \) gene expression was decreased under the same conditions (Fig. 4.8). Notably, the use of NAM, an inhibitor of SIRT deacetylases, had no effect on GH-induced \( \text{Cish} \) expression and only a minor effect on \( \text{Socs3} \) expression. When all three inhibitors were combined, we observed an additive effect on \( \text{Cish} \) suppression, while \( \text{Socs3} \) upregulation was attenuated, compared with TSA or NaBu alone.
Figure 4.7. Regulation of STAT5 target genes by GH. Mature 3T3-L1 adipocytes were treated with 5 nM GH and harvested at indicated time points. Relative gene expression of Socs3, Cish, Bcl6, Igfbp3, Igf1 and Socs2 were quantified by RT-qPCR using Cyclophilin a as the reference gene. Each data point consists of three replicates. This experiment was replicated at least three times in different batches of cells. Data are presented as means ± S.E. (n=3 per treatment). All time points are compared to time 0. Statistical significance was determined using one-way ANOVA. *P<0.05, **P<0.005, ***P<0.001, ****P<0.0001.
Figure 4.8. Inhibition of classical HDACs, but not SIRTs, substantially increases the expression of \textit{Socs3} and decreases the expression of \textit{Cish}. Fully differentiated 3T3-L1 adipocytes were serum-deprived overnight and then pre-treated for 5 hours with 5 µM Trichostatin A (TSA), 20 mM Nicotinamide (NAM), and/or 10 mM Sodium Butyrate. Next, 5 nM GH or vehicle (NaHCO$_3$) were added 1 hour before cells were harvested. Gene expression levels were quantified by RT-qPCR, \textit{Cyclophilin a} was used as the reference gene. Data are presented as means ± S.E. (n=3 per treatment). This experiment was replicated at least three times in different batches of cells. Treatments were normalized to vehicle condition in the absence of inhibitor and all GH conditions were compared to GH control. Statistical significance was determined using one-way ANOVA. *P<0.05, **P<0.005, ***P<0.001, ****P<0.0001.

Since TSA and NaBu target several different HDACs, we replicated the previous experiments using more specific inhibitors. Adipocytes were pretreated with inhibitors of HDACs 1, 3, 5 or 6 for 5 hours, then exposed to GH for 1 hour. As shown in Fig. 4.9, inhibition of either HDAC5 or 6, but not HDAC1 or 3, produced similar effects to those observed with TSA and NaBu.

Of all inhibitors tested, the HDAC5 (LMK-235) and HDAC6 (CAY10603) inhibitors showed the most potent effects on the GH-induced upregulation of \textit{Socs3} and downregulation of \textit{Cish} expression. Due to technical difficulties with CAY10603, we performed most of our subsequent studies using only the HDAC5 inhibitor. To further validate the effects of LMK-235 on \textit{Socs3} and \textit{Cish} expression, adipocytes were pre-treated with LMK-235 for various times (30 minutes to 5 hours) and with GH for one hour prior to harvesting for gene expression analyses.
As shown in Fig. 4.10A, the effect of LMK-235 is acute, as a 30-minute pretreatment (2 μM) was sufficient to downregulate Cish to a similar level as longer pretreatment times. For Socs3, increased pretreatment times resulted in higher levels of GH-induced expression. In addition to the time course experiment, we tested a variety of doses of this inhibitor. As shown in Fig. 4.10B, a lower dose of LMK-235 (50 nM) was sufficient to reduce GH-induced Cish expression, while GH-induced Socs3 expression was highly increased at 500 nM. Hence, a ten-fold higher dose of LMK-235 was required for a similar upregulation of Socs3 than for downregulation of Cish expression.

**Figure 4.9.** Pharmacological inhibition of HDAC5 or 6, but not HDAC1 or 3, increases the expression of Socs3 and decreases the expression of Cish. Fully differentiated 3T3-L1 adipocytes were serum-deprived with 0.3% BSA DMEM overnight, then pre-treated for 5 hours with the following specific HDAC inhibitors: 2.5 μM CI994 (HDAC1), 10 μM RGFP966 (HDAC3), 10 μM LMK-235 (HDAC5) and 15 μM CAY10603 (HDAC6). Next, 5 nM GH or an equivalent volume of vehicle (NaHCO₃) were added 1 hour before the cells were harvested. Gene expression levels were quantified by RT-qPCR and normalized for Cyclophilin a expression. Data are presented as means ± S.E. (n=3 per treatment). This experiment was replicated three times in different batches of cells. Treatments were normalized to vehicle condition in the absence of inhibitor. Statistical significance was determined comparing all GH conditions to GH control using one-way ANOVA. * p < 0.05 and **** p < 0.0001.
Figure 4.10. The effect of LMK-235 (HDAC5i) on gene expression is acute, and lower doses suppress *Cish* expression than enhance *Socs3*. (A) Mature adipocytes were pretreated with 2 μM LMK-235 for 0.5, 2 or 5 hours. Vehicle or 5 nM of GH were added, and cells were harvested 1 hour later. (B) Cells were pretreated for 2 hours with increasing doses of LMK-235. Vehicle or 5 nM GH were added 1 hour before cells were harvested. Gene expression levels were quantified by RT-qPCR and normalized to *Cyclophilin a* expression. Data are presented as means ± S.E. (n=3 per treatment). This experiment was replicated three times in different batches of cells. Treatments were normalized to vehicle condition in the absence of inhibitor. Statistical significance was determined using two-way ANOVA. * p < 0.05, *** p < 0.001, **** p < 0.0001.

4.4. STAT5 and HDAC5 do not physically interact in adipocytes

The robust effect of the HDAC inhibitor LMK-235 on STAT5 target genes led us to hypothesize that HDAC5 was involved in the STAT5 transcriptional complex and possibly being affected by GH. To test this hypothesis, we performed immunoprecipitation experiments. Mature
adipocytes were treated with vehicle or GH for either 20 minutes or 1 hour. Whole-cell extracts were incubated with STAT5A antibody overnight and Western blot analysis was performed using HDAC5 antibody. As shown on the left side of Fig. 4.11, HDAC5 and STAT5 do not physically interact in 3T3-L1 adipocytes under the conditions we examined. HDAC5 Western blot control is shown on the right side of the figure.

**Figure 4.11.** STAT5 and HDAC5 do not physically interact in 3T3-L1 adipocytes. 3T3-L1 mature adipocytes were serum-deprived overnight then treated with vehicle (NaHCO₃) or 5 nM GH for 20 minutes or 1 hour. Whole-cell extracts were isolated, and 400µg of lysates were incubated overnight with STAT5A antibody. Next, HDAC5 antibody was used for Western blot analysis. This experiment was independently repeated on a separate batch of adipocytes.

HDACs possess a nuclear localization signal (NLS) and nuclear export sequence (NES) (McKinsey, Zhang, and Olson 2001; reviewed in Yang and Grégoire 2005) that allow these proteins to shift between the nucleus and cytoplasm of the cell. Given STAT5 and HDAC5 can be present in the cytosol and nucleus of the cell, I next tested if any interaction could be detected in nuclear extracts. Fig. 4.12 shows no physical interaction even in nuclear extracts. In Western
blot analysis, an increase in HDAC5 in the nucleus was observed after 1 hour GH treatment. This observation suggest that HDAC5, like STAT5 migrates to the adipocyte nucleus upon GH treatment, and while I could not detect an interaction between the two proteins, the possibility that they interact transiently upon GH treatment cannot be ruled out. Another possibility is that HDAC5 participates in the GH-signaling pathway independently of STAT5. In addition, as described later in this chapter, because the HDAC6 inhibitor (CAY10603) also had profound effects on STAT5 target genes, we performed immunoprecipitation experiments to detect a possible interaction between HDAC6 and STAT5. However, the HDAC6 antibody did not perform well, and no interaction was detected (data not shown).

**Figure 4.12.** STAT5 and HDAC5 do not physically interact in the adipocyte nucleus. 3T3-L1 mature adipocytes were serum-deprived overnight, and then treated with vehicle (NaHCO3) or GH (figure caption cont’d.)
nM GH for 20 minutes or 1 hour. Nuclear extracts were isolated and 400 µg of lysates were incubated overnight with STAT5A antibody. HDAC5 antibody was used for western blot analysis. This experiment was independently repeated on a separate batch of adipocytes.

4.5. HDACs KD do not recapitulate HDACi effects

In addition to the pharmacological approach, we performed siRNA-mediated knockdown (KD) of the HDACs targeted by LMK-235. LMK-235 specificity was described by Marek and colleagues (Marek et al. 2013), and it is shown in table 4.1. HDAC5 IC50 is 4.2 nM, HDAC4 IC50 is 11.9 nM and HDAC6 IC50 is 56 nM. Since these three HDACs were the most affected by LMK-325, we knocked them down, individually or in combination, and examined the effects on Socs3 and Cish gene expression. The top section of Fig. 4.13 shows Hdac4, Hdac5 and Hdac6 gene expression levels, confirming KD efficiency varying between 50-70%. However, Socs3 and Cish expression were largely unaffected by the knockdowns. HDAC5 protein levels were also examined, confirming reduced HDAC5 protein levels in KD samples compared to non-targeting control (data not shown).

Table 4.1. LMK-235 specificity to histone deacetylases (HDACs).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC5</td>
<td>4.2 nM</td>
</tr>
<tr>
<td>HDAC4</td>
<td>11.9 nM</td>
</tr>
<tr>
<td>HDAC6</td>
<td>56 nM</td>
</tr>
<tr>
<td>HDAC1</td>
<td>320 nM</td>
</tr>
<tr>
<td>HDAC11</td>
<td>850 nM</td>
</tr>
<tr>
<td>HDAC2</td>
<td>880 nM</td>
</tr>
<tr>
<td>HDAC8</td>
<td>1,280 nM</td>
</tr>
</tbody>
</table>

The lack of effect on Socs3 and Cish gene expression with HDAC4, 5 and 6 knockdowns could be explained by incomplete elimination of HDACs or by compensation through enhancement of another HDAC when one of them was reduced. The results also raise the possibility that the effects of HDAC inhibitors on STAT5 target gene expression were not due
the inhibition of HDACs’ enzymatic activity, but rather to other effects on a protein/proteins in
the STAT5 transcriptional complex.

4.6. HDAC5i (LMK-235) does not affect mRNA stability

To determine if LMK-235 was affecting \textit{Socs3} or \textit{Cish} mRNA stability and potentially
accounting for the observed effects on gene expression, we treated adipocytes with 5 \( \mu \text{g/ml} \)
actinomycin D in the presence or absence of LMK-235. Cells were harvested at different time
points (15 to 180 minutes) and relative gene expression was measured. Statistical analysis using
two-way ANOVA demonstrated that LMK-235 had no impact on \textit{Socs3} or \textit{Cish} mRNA stability
(Fig. 4.14), suggesting that the observed effects of the inhibitor were likely due to alterations in
gene transcription.

4.7. BET inhibition has the same effects on \textit{Socs3} and \textit{Cish} gene expression as
pharmacological HDAC inhibition

Since HDAC KDs did not recapitulate the effects of HDAC inhibitors, it is possible that
the inhibitors influence STAT-mediated transcription by interfering with the functions of other
proteins. BET proteins were plausible candidates for this, because they are sensitive to
modulation of acetylation. To investigate whether BET proteins were involved in our observed
changes in \textit{Socs3} and \textit{Cish} gene expression, I acquired the inhibitor thienotriazolodiazepine, JQ1,
which competitively binds to bromodomains of BET proteins and prevents their binding to
acetylated proteins, including histone tails. As shown in Fig. 4.15A, as was the case with LMK-
235, JQ1 caused dose-dependent downregulation of \textit{Cish} and upregulation of \textit{Socs3}. In contrast
to LMK-235, however, the effects of JQ1 decreased with longer pretreatment times, especially
for \textit{Socs3} (Fig. 4.15B). Although not significant, in vehicle condition LMK-235 trends to
increase *Cish* expression (Fig 4.10), while JQ1 trends to decreased *Cish*, even in the absence of GH (Fig. 4.15).

Next, I examined the expression of STAT5 target genes in a GH time course, comparing DMSO control, LMK-235 or JQ1 alone, or a combination of both. Adipocytes were pretreated for 30 minutes with JQ1 (1 µM) and/or for 2 hours with LMK-235 (1 µM). As shown in Fig. 4.16, inhibitor pretreatments (Time 0) did not profoundly affect the expression of most STAT5 target genes; however, *Bcl6* was modestly affected in the absence of GH. When adipocytes were treated with GH (0.5 to 6 hours), the inhibitors produced an additive effect on gene expression. *Socs3* upregulation was even higher in the presence of LMK-235 and JQ1, while *Cish* suppression was even greater in the presence of both inhibitors. The effects seemed to also be additive for *Igf1*, which showed a suppression pattern similar to that of *Cish*. For *Socs2*, LMK-235 alone upregulated gene expression at 6 h, while JQ1 alone or in combination with LMK-235 suppressed its expression.
Figure 4.13. HDAC4, 5, or 6 knockdowns did not recapitulate the effects of HDAC inhibitors. Fully differentiated 3T3-L1 adipocytes were transfected with 50 nM non-targeting (NT) siRNA or HDAC4, 5, or 6 siRNA, or a combination of 2 at a time. siRNA-containing media was removed 24 hours later and replaced with 10% FBS. After 48 hours, FBS was replaced by 1% CALF. The following day, (figure caption cont’d.)
cells were treated with vehicle (10 μM NaHCO3) or 5 nM murine growth hormone (GH) for 1 hour. RT-qPCR analysis was performed using Nono as the reference gene. Knockdown of Hdac4, 5, and/or 6 was confirmed by examining their gene expression levels. Expression levels of the target genes Socs3 and Cish were also analyzed. Data are presented as means ± S.E. (n=3 per treatment). This experiment was replicated twice in different batches of cells. Treatments were normalized to vehicle condition in the absence of inhibitor. Statistical significance was determined comparing all GH conditions to GH control using one-way ANOVA. * p < 0.05, ** p < 0.005, *** p < 0.001, **** p < 0.0001.

**Figure 4.14.** LMK-235 has no effect on Socs3 or Cish mRNA stability. Fully differentiated adipocytes were treated with 2 μM LMK-235 followed by 5 μg/ml actinomycin D. Cells were harvested at the indicated time points. Gene expression levels were quantified by RT-qPCR, Cyclophilin a was used as the reference gene. Data are presented as means ± S.E. (n=3 per treatment). This experiment was replicated at least three times in different batches of cells. Statistical significance was calculated using two-way ANOVA, there was no difference between treatments.
Figure 4.15. JQ1 upregulates Socs3 and downregulates Cish in a similar manner as LMK-235. (A) Mature 3T3-L1 adipocytes were pretreated for 30 minutes with JQ1 at different concentrations. Vehicle or 5 nM of GH were added 1 hour before the cells were harvested. (B) Mature adipocytes were pretreated with 2 μM JQ1 for indicated times, and vehicle or 5 nM of GH were added 1 hour before the cells were harvested. Gene expression levels were quantified by RT-qPCR. Cyclophilin a was used as the reference gene. Data are presented as means ± S.E. (n=3 per treatment). This experiment was replicated three times in different batches of cells. Treatments were normalized to vehicle condition in the absence of inhibitor. Statistical significance was determined using two-way ANOVA. *P<0.05, **P<0.005, ***P<0.001, ****P<0.0001.
4.8. BRD2 and RNA Pol II binding to *Socs3* and *Cish* correlate with changes in gene expression

To better investigate DNA-protein interactions in a site-specific manner, we performed chromatin immunoprecipitation (ChIP) analysis. Fig. 4.17A represents the schematics of the three target regions that were amplified by qPCR, one region containing STAT binding sites, another containing the transcription start site (TSS), and the last one within the coding region of *Cish*. Fig. 4.17B corresponds to the equivalent regions of *Socs3*. As shown in Figures 4.17C and 4.17D, STAT5A binding to STAT binding sites increased with GH treatment, and this increase was even greater in the presence of LMK-235 at both *Socs3* and *Cish* promoters. JQ1 slightly increased STAT5A binding in the vehicle/basal condition and had no significant effects in the GH condition. The similarities in STAT5A binding to the *Socs3* or *Cish* promoters suggest that the opposite effects on gene expression by HDAC or BET inhibition are not due to significant differences in STAT5A binding.

Since STAT3 can also be activated in adipocytes and *Socs3* is a well-known STAT3 target gene (L. Zhang et al. 2006), I also examined its binding to *Cish* and *Socs3* promoters. As shown in Fig. 4.17E no significant changes with GH or inhibitors were detected on the *Cish* STAT binding site. At *Socs3*, only JQ1 substantially increased STAT3 binding. Moreover, GH treatment seems to decrease STAT3 binding, perhaps permitting more STAT5 binding (Fig. 4.17F).
Figure 4.16. Effects of LMK-235 and JQ1 on STAT5 target genes are greatest when combined. Mature adipocytes were pre-treated for 2 hours with 1 μM LMK-235, for 30 min with 1 μM JQ1 or a combination of both. DMSO was used as a control. Subsequently, the cells were treated with 5 nM GH and harvested at the indicated time points. Relative gene expression of Socs3, Cish (figure caption cont’d.)
Socs2, Igf1 and Bcl6 were quantified by RT-qPCR using Cyclophilin α as the reference gene. Data are presented as means ± S.E. (n=3 per treatment). This experiment was replicated two times in different batches of cells.

Next, we assessed changes in BET protein binding to Cish and Socs3 promoters. The binding of BRD4, the most thoroughly characterized BET protein, was lower than that of BRD2, and binding levels were comparable to IgG control (Fig. 4.18A-D). In addition, results with BRD4 were inconsistent. In one experiment, BRD4 appeared to be recruited to the Cish TSS by GH and its binding was decreased by JQ1 (Fig. 4.18A). However, when this experiment was repeated, these effects of GH and JQ1 on BRD4 binding to the Cish promoter were not observed (Fig. 4.18C). BRD2, on the other hand, was consistently recruited by GH to the Cish TSS in three independent experiments. These results are in accordance with previous studies that revealed BRD2 as the BET protein involved in STAT5 transcriptional activity (Liu et al. 2014a; Pinz et al. 2015a). The recruitment of BRD2 to the Cish promoter was attenuated by LMK-235 in the GH condition and by JQ1 in both conditions (Fig. 4.18E). Conversely, neither BRD2 nor BRD4 were recruited to Socs3 TSS by GH. However, the presence of LMK-235 increased BRD2 binding in both vehicle and GH conditions (Fig. 4.18F). These results suggest that BRD2 is necessary for GH activation of Cish but not Socs3.

As expected, total RNA polymerase II binding to Cish and Socs3 TSS was increased by GH treatment. In the presence of LMK-235, RNA Pol II binding to Cish TSS was decreased only in the GH condition (Fig. 4.19A), which is consistent with the gene expression pattern observed with LMK-235 (Fig. 4.10). Moreover, LMK-235 enhanced RNA Pol II binding to the Socs3 TSS in both the V and GH conditions. JQ1 increased Pol II binding to the Socs3 TSS only in the vehicle condition and slightly decreased its binding in the presence of GH (Fig. 4.19B). At the same time, JQ1 decreased Pol II binding to the Cish TSS in both V and GH conditions, which is
also consistent with the observed changes in gene expression, since JQ1 always decreases *Cish* expression in the presence or absence of GH (Fig. 4.15). The binding of Pol II phosphorylated at Ser2, a marker of RNA elongation, to the coding regions of *Socs3* and *Cish* was increased with GH. While association of this activated Pol II to the coding region of *Cish* was suppressed by the inhibitors (Fig. 4.19C), the increase was even greater for *Socs3* when HDACs or BETs were inhibited (Fig. 4.19D). These results indicate that HDAC or BET inhibitors decrease RNA Pol II recruitment to the *Cish* promoter and increase *Socs3* transcription elongation.

Because BET proteins bind to acetylated histones, we assessed acetylated H3 and H4 levels at the TSS and coding regions of both *Cish* and *Socs3*. For acetyl-histone H3, the inhibitors had very similar effects in *Cish* and *Socs3*. However, GH produced a significant increase in binding only at the *Cish* TSS. Interestingly, in both genes, LMK-235 reduced acetylated H3 binding at the TSS while enhancing binding in the coding region. Compared to acetylated H3, acetyl-histone H4 levels differed more between *Cish* and *Socs3*. Yet, we saw the same increase with GH and reduction with LMK-235 at the *Cish* TSS. Moreover, acetylation levels at the *Cish* TSS were much higher for all conditions, when compared to *Socs3*. These findings support the hypothesis that *Cish* transcription depends on BRD2 and therefore higher acetylation levels at TSS. LMK-235 decreases acetylation levels at the *Cish* TSS while increasing them in the coding region. These shifts result in displacement of BRD2 from the TSS.
Figure 4.17. Changes in STAT5 and 3 binding to Socs3 and Cish promoters do not correlate with changes in gene expression. Schematics of Cish (A) and Socs3 (B) regions that were amplified by qPCR, including STAT binding site, transcription start site (TSS) and coding region of the genes. (C-F). Mature adipocytes were pre-treated with 2 μM LMK-235 for 2 hours, or 2 μM JQ1 for 30 minutes. DMSO was used as a control. Next, cells were treated with vehicle (V; NaHCO₃) or 5 nM GH for 35 min. Chromatin was cross-linked using formaldehyde, and nuclear extracts were subjected to ChIP using STAT5A and STAT3 antibodies. The amount of bound DNA was measured by qPCR at the STAT binding site and percent input was calculated. This experiment was replicated three times in different batches of cells. Statistical significance was calculated using two-way ANOVA with Bonferroni’s post hoc test. * denotes significance between DMSO and inhibitor treatments within either the V or GH condition. # denotes significance between V and GH for each control and inhibitor treatment. * p < 0.05, ** p < 0.005, *** p < 0.001, **** p < 0.0001; * p < 0.05, ### p < 0.005, #### p < 0.001, ##### p < 0.0001.
Figure 4.18. BRD2 is necessary for GH induction of *Cish* but not *Socs3*. Mature adipocytes were pre-treated with 2 μM LMK-235 for 2 hours, or 2 μM JQ1 for 30 minutes. DMSO was used as a control. Next, cells were treated with vehicle (V; NaHCO₃) or 5 nM GH for 35 min. Chromatin was cross-linked using formaldehyde, and nuclear extracts were subjected to ChIP using BRD2 and BRD4 antibodies. The amount of bound DNA was measured by qPCR at the TSS and percent input was calculated. This experiment was replicated three times in different batches of cells. Statistical significance was calculated using two-way ANOVA with Bonferroni’s post hoc test. * denotes significance between DMSO and inhibitor treatments within either the V or GH condition. # denotes significance between V and GH for each control and inhibitor treatment. * p < 0.05, ** p < 0.005, *** p < 0.001, **** p < 0.0001; # p < 0.05, ### p < 0.005, #### p < 0.001, ##### p < 0.0001.
Figure 4.19. Changes in RNA Polymerase II binding to *Socs3* and *Cish* reflect the fluctuations in gene expression. Mature adipocytes were pre-treated with 2 μM LMK-235 for 2 hours, or 2 μM JQ1 for 30 minutes. DMSO was used as a control. Next, cells were treated with vehicle (V; NaHCO₃) or 5 nM GH for 35 min. Chromatin was cross-linked using formaldehyde, and nuclear extracts were subjected to ChIP using RNA Pol II and RNA Pol IIpS² antibodies. The amount of bound DNA was measured by qPCR and percent input was calculated. This experiment was replicated three times in different batches of cells. Statistical significance was calculated using two-way ANOVA with Bonferroni’s post hoc test. * denotes significance between DMSO and inhibitor treatments within either the V or GH condition. # denotes significance between V and GH for each control and inhibitor treatment. * p < 0.05, ** p < 0.005, *** p < 0.001, **** p < 0.0001; # p <0.05, ### p <0.005, #### p <0.001.
Figure 4.20. Effects of inhibitors on Histone H3 acetylation were very similar for SoCs3 and Cish. Mature adipocytes were pre-treated with DMSO for 2 hours, 2 μM LMK-235 for 2 hours, or 2 μM JQ1 for 30 minutes. Next, cells were treated with vehicle (V; NaHCO₃) or 5 nM GH for 35 min. Chromatin was cross-linked using formaldehyde, and nuclear extracts were subjected to ChIP using anti-acetyl histone H3. The amount of bound DNA to SoCs3 and Cish STAT binding site, transcription start site (TSS), and coding region was measured by qPCR and percent input was calculated. This experiment was replicated three times in different batches of cells. (figure caption cont’d.)
Statistical significance was calculated using two-way ANOVA with Bonferroni’s post hoc test. * denotes significance between DMSO and inhibitor treatments within either the V or GH condition. # denotes significance between V and GH for each control and inhibitor treatment. * p < 0.05, ** p < 0.005, *** p < 0.001, **** p < 0.0001; # p < 0.05, ### p < 0.005, #### p < 0.001, ##### p < 0.0001.

**Figure 4.21.** Acetyl Histone 4 levels were higher at the TSS of Cish than of Socs3. On the day (figure caption cont’d.)
before the experiment, mature adipocytes were fed with 1% CALF. On the day of the experiment, cells were pre-treated with DMSO for 2 hours, 2 μM LMK-235 for 2 hours, or 2 μM JQ1 for 30 minutes. Next, cells were treated with vehicle (V; NaHCO₃) or 5 nM GH for 35 min. Chromatin was cross-linked using formaldehyde, and nuclear extracts were subjected to ChIP using anti-acetyl histone H4. The amounts of bound DNA to Socs3 and Cish STAT binding sites, transcription start sites and coding regions were measured by qPCR and percent input was calculated. This experiment was replicated three times in different batches of cells. Statistical significance was calculated using two-way ANOVA with Bonferroni’s post hoc test. * denotes significance between DMSO and inhibitor treatments within either the V or GH condition. # denotes significance between V and GH for each control and inhibitor treatment. * p < 0.05, ** p < 0.005, *** p < 0.001, **** p < 0.0001; # p <0.05, ### p <0.005, #### p <0.001.

Rabbit IgG antibody was used as the negative control on ChIP experiments. IgG binding levels at Socs3 and Cish STAT binding site, transcription start site (TSS) and coding regions ranged between 0.02 and 0.1% input (Fig. 4.22).

4.9. JQ1 disrupts HEXIM1 and Cyclin T1 interaction

Displacement of BRD2 from the Cish promoter upon HDAC or BET inhibition explains why GH induction of Cish was being negatively affected by these inhibitors. The upregulation of Socs3 by HDAC inhibition is probably due an increase in chromatin accessibility. On the other hand, the upregulation of Socs3 by JQ1 is more challenging to explain because BET proteins are positively correlated with transcription. Extensive work done in HIV transcription has shed light on the mechanism by which JQ1 could upregulate some genes. It is possible that the effects of JQ1 on Socs3 may not involve BET proteins at all. As previously described here, JQ1 and other inhibitors were reported to affect the interaction between the positive elongation factor-b (P-TEFb) and its negative regulator HEXIM1, leaving P-TEFb more available for transcription elongation. As a consequence, HEXIM1 is upregulated at the mRNA and protein levels to restore the equilibrium between active and inactive P-TEFb.
Figure 4.22. Rabbit IgG levels bound to the three amplified region of Cish and Socs3. On the day before the experiment, mature adipocytes were fed with 1% CALF. On the day of the experiment, cells were pre-treated with DMSO for 2 hours, 2 μM LMK-235 for 2 hours, or 2 μM JQ1 for 30 minutes. Next, cells were treated with vehicle (V; NaHCO₃) or 5 nM GH for 35 min. Chromatin was cross-linked using formaldehyde, and nuclear extracts were subjected to ChIP using rabbit IgG antibody. The amounts of bound DNA to Socs3 and Cish STAT binding sites, transcription start sites and coding regions were measured by qPCR and percent input was (figure caption cont’d.)
calculated. This experiment was replicated three times in different batches of cells. Statistical significance was calculated using two-way ANOVA with Bonferroni’s post hoc test. * denotes significance between DMSO and inhibitor treatments within either the V or GH condition. # denotes significance between V and GH for each control and inhibitor treatment. * p < 0.05, ** p < 0.005, *** p < 0.001, **** p < 0.0001; # p < 0.05, ### p < 0.005, #### p < 0.001.

To investigate if inhibitors were affecting the interaction between the regulatory subunit of P-TEFb (Cyclin T1) and HEXIM1, adipocytes were treated with LMK-235 or JQ1 for 2, 6, or 24 hours. Whole-cell lysates were immunoprecipitated with Cyclin T1 antibody, and Western blot analysis was performed in the following day. In Fig. 4.23, the fold-change values for BRD2 and HEXIM1 are indicated under the blot images. For the 2-hour JQ1 treatment, the interaction between Cyclin T1 and HEXIM1 was reduced, while interaction with BRD2 was increased. As the interaction with HEXIM1 increased over time, the interaction with BRD2 decreased. With LMK-235 treatment, the interaction between Cyclin T1 and HEXIM1 was not reduced. However, the interaction with BRD2 decreased over time, probably because LMK-235 treatment decreased BRD2 expression as shown later in Fig. 4.25.

**Figure 4.23.** JQ1 transiently reduces HEXIM1-Cyclin T1 interaction. Mature 3T3-L1 adipocytes were treated with 2 μM JQ1 or 2 μM LMK-235 for the indicated times. Control (CTL) cells were (figure caption cont’d.)
untreated. Whole-cell extracts were collected in IP buffer and 300 μg of total protein was incubated with anti-Cyclin T1 antibody overnight. Interacting HEXIM1 and BRD2 were detected by Western blot. The mock sample was composed of a pool of all samples without any antibody. Band intensities were quantified and Cyclin T1 was used as a loading control to calculate fold-change values, which are shown below the BRD2 and HEXIM1 immunoblots.

I also examined whether the HDAC inhibitors or JQ1 that upregulated Socs3 and downregulated Cish had any effects on Hexim1 expression. A time course experiment showed that only JQ1 significantly upregulated Hexim1, while LMK-235 and CAY10603 (HDAC6i) had no impact on Hexim1 gene expression. The upregulation by JQ1 is noticeable at 2 hour, peaks at 6 hours, and begins to wane at 24 hours, while remaining significantly higher than in DMSO control or HDAC inhibition conditions. A combination of JQ1 with LMK-235 did not further upregulate Hexim1 (Fig. 4.24). As previously shown, LMK-235, CAY10603, and JQ1 increased GH induction of Socs3 while decreasing Cish induction.

Subsequently, I measured HEXIM1 protein levels at the same time points and observed a progressive accumulation of HEXIM1 when the cells were treated with JQ1 but not LMK-235. LMK-235 modestly decreased HEXIM1 protein levels at 24 hours treatment. Unexpectedly, JQ1 and LMK-235 had profound and opposite effects on BRD2 protein levels. While JQ1 upregulated BRD2, LMK-235 drastically decreased BRD2 protein levels. Quantification of BRD2 and HEXIM1 was performed using the regulatory part of P-TEFb, (Cyclin T1) as a control; results are expressed as fold-change over control condition and shown below the Western blot images (Fig. 4.25).
Figure 4.24. JQ1 upregulates *Hexim1*. Fully differentiated adipocytes were treated with 2 μM LMK-235, 10 μM CAY10603, or 2μM JQ1, or a combination of LMK-235 and JQ1. Next, 5 nM GH was added and cells were harvested at the indicated time points. Gene expression levels were quantified by RT-qPCR, using *Nono* as the reference gene. Data are presented as means ± S.E. (n=3 per treatment). This experiment was replicated twice in different batches of cells. Statistical significance was calculated using two-way ANOVA with Bonferroni’s post hoc test. * denotes significance between DMSO and inhibitor treatments. # denotes significance between time points. * p < 0.05, ** p < 0.005, *** p < 0.001, **** p < 0.0001; # p < 0.05, ## p < 0.005, ### p < 0.001, #### p < 0.0001.
Figure 4.25. JQ1 upregulates HEXIM1 and BRD2, while LMK-235 robustly downregulates BRD2 expression. Mature 3T3-L1 adipocytes were treated with 2 μM JQ1 or 2 μM LMK-235 for the indicated times. Control (CTL) cells were untreated. Whole-cell extracts were collected in IP buffer and 25 μg of protein was used for gel electrophoresis. HEXIM1, BRD2, and Cyclin T1 protein levels were detected by Western blot. Band intensities were quantified and Cyclin T1 was used as a loading control to calculate fold-change. Statistical significance was calculated using one-way ANOVA. * denotes significance between CTL and respective treatments.
Chapter 5. Discussion

Our previous work demonstrated that STAT5 interacts with the pyruvate dehydrogenase complex (PDC) in the adipocyte nucleus in a GH-dependent manner (Richard, Hang, and Stephens 2017). This interaction led us to hypothesize that PDC could be generating acetyl-CoA for acetylation of STAT5 or histones, or affecting the function of other proteins in the STAT5 transcriptional complex. It is important to point out that we had not measured the production or fate of acetyl-CoA derived specifically from PDC, therefore, the GH-mediated STAT5-PDC interaction we observed only provided the suggestion that acetylation may be important for STAT5 transcriptional activity in adipocytes. The initial focus of this research was to effectively measure STAT5 acetylation in mature 3T3-L1 adipocytes. The role of acetylation in STAT5 transcriptional activity is controversial in the literature. STAT5B acetylation was detected by mass spectrometry at K359, K694 and K701 (corresponding to K359, K689 and K696 of STAT5A) by Ma et al, who found that mutations of K359 and K694 reduced prolactin-induced STAT5 activity measured by luciferase assay. In addition, mutations of K694 or K701, which are close to the SH2 domain, impaired dimerization of STAT5B, suggesting a positive role of acetylation in STAT5 downstream transcriptional activity (Ma et al. 2010). In another study, STAT5A SUMOylation competed with acetylation at K696 and decreased STAT5A transcriptional activity (Van Nguyen et al. 2012).

Conversely, studies using HDAC inhibitors have shown that increasing acetylation levels in the cell suppresses STAT-dependent gene activation. In macrophages, the HDAC inhibitor TSA repressed STAT5-mediated transcription, but had no effects on STAT5 phosphorylation or binding activity (Sebastián et al. 2008). Similarly, activation of STAT5 target genes in the IL-3-dependent pro-B cell line Ba/F3 was blocked by HDAC inhibitors, while mutations in STAT5
lysine residues, had no effects on STAT5 transcription activity (Rascle, Johnston, and Amati 2003; Pinz et al. 2015). Comparable results were observed in the activation of genes dependent on STATs 1 or 2 (Nusinzon and Horvath 2003; Sakamoto, Potla, and Larner 2004) and STAT3 (Catania et al. 2006). However, a study performed in human colorectal cancer cells demonstrated that TSA induced the expression of two STAT3 target genes, SOCS1 and SOCS3 (Xiong et al. 2012).

STAT5 is highly expressed in mature 3T3-L1 adipocytes and in adipocytes in vivo. As shown in Fig. 4.1, 75 μg of total protein is sufficient to observe robust STAT5A expression in mature 3T3-L1 adipocytes. However, when 400 μg of total protein was immunoprecipitated with pan-acetyl lysine antibody and the membrane was probed for STAT5A (Fig. 4.1) or the inverse configuration of antibodies (Fig. 4.2) under the same experimental conditions, only low-intensity bands corresponding to STAT5A size are observed. In addition, pan-HDAC inhibitors had little effects on STAT5 acetylation. Western blot analysis examining histone H3 acetylation confirmed the efficacy of inhibitors (Fig. 4.3). When adipocytes were treated with HDAC5 inhibitor (LMK-235) and nuclear and cytosolic fractions were isolated, higher intensity bands were observed in the nuclear fractions treated with GH. However, LMK-235 had no effects on STAT5 acetylation (Fig 4.4). Western blot analysis probing for acetyl-histone H3 demonstrated that LMK-235 increased histone acetylation in a dose-dependent manner (Fig. 4.5). The reverse configuration of antibodies employed in figure 4.4, detected far lighter bands in the nuclear fractions (Fig 4.6). A possible explanation is that highly acetylated protein/proteins interact with STAT5 in the nucleus upon GH activation, and this complex is immunoprecipitated by pan-acetyl lysine antibody, contributing to the high-intensity bands observed when the membrane is probed for STAT5A (Fig. 4.4). When samples are immunoprecipitated with STAT5A antibody, these acetylated
proteins are not enriched in the STAT5A complex, explaining the lower intensity bands observed in figure 4.6. Thus, we believe this antibody configuration is a more faithful representation of STAT5 acetylation levels in 3T3-L1 adipocytes, which were very low, compared to the expression of total STAT5A protein.

In addition, the HDAC inhibitors TSA, NaBu, NAM or LMK-235 had little or no impact on STAT5 acetylation (Figs. 4.1, 4.2, 4.4 and 4.6), even though they substantially increased the acetylation levels of a small number of bands, mostly at high molecular weights (Fig. 4.2) and Histone H3 (Figs. 4.3 and 4.5) in adipocytes. It is important to point out that the methods utilized in this work measured changes in total STAT5 acetylation and we cannot exclude the possibility that acetylation of a single STAT5 lysine residue could be affected by HDAC inhibitors and escape detection by our methods. We also carry out immunoprecipitation experiments with an acetylated-STAT5B antibody, however, the antibody did not perform well (data not shown) and we were unable to find a better acetylated-STAT5 antibody. To specifically assess the contribution of each lysine residue, we would have to use more specific antibodies or perform mutations on each potential lysine residue that could be acetylated in STAT5. However, as previous studies mentioned above, mutations in lysine residues had no impact on STAT5 activation measured by luciferase assay or on the induction of STAT5 target genes (Pinz et al. 2015). Based on these studies and our own observations, we concluded that the effects of HDAC inhibitors on STAT5 target genes are largely independent of STAT5 acetylation. Thus, we decided to not further investigate STAT5 acetylation in adipocytes.

STAT5 can be activated by GH or PRL in adipocytes (Zvonic et al. 2003; Fleenor, Arumugam, and Freemark 2006). Thus, to validate the induction or repression of known STAT5 target genes, a GH time course was performed. Our results were consistent with published data
from other groups. Indeed, \textit{Soxs3} has been reported to be rapidly and transiently upregulated by GH in 3T3-F222A fibroblasts, in which levels peaked after 30 min GH exposure and returned to basal levels after 2 hours. In the same study, \textit{Soxs1} was activated to a lesser extent but had a very similar induction pattern to that of \textit{Soxs3} (Adams et al. 1998). As shown in Fig. 4.7, we observed the same acute and transient induction of \textit{Soxs3} by GH in 3T3-L1 adipocytes (\textit{Soxs1} induction was not measured in our study). Adams \textit{et al.} also found that \textit{Soxs2} expression was only induced at later time points, starting at 2 hours and peaking at 4 hours treatment, while maximal induction of \textit{Cish} was observed at 1 hour GH-treatment, with sustained activation at later time points (Adams et al. 1998). Very similar induction patterns of Socs genes were observed in rat hepatocytes treated with GH (Tollet-Egnell et al. 1999). As shown in Figure 4.7, our analysis of GH-induced gene expression changes in adipocytes therefore closely mirrors observations from other cell types.

STAT5 is thought to mediate the growth-promoting effects of GH in the liver through upregulation of IGF-1. A study in hepatocytes showed STAT5 binding to a distal DNA region and induction of \textit{Igf1} expression with 8 hours GH treatment (Y. Wang and Jiang 2005). In adipocytes, a 4-hour exposure to GH upregulated \textit{Igf1} (Fleenor, Arumugam, and Freemark 2006). Our study confirms \textit{Igf1} induction by GH in 3T3-L1 adipocytes at 4 hours, with sustained high levels until the last time point at 48 hours. Induction of \textit{Igfbp3} by GH has been described in rat beta-cells (De et al. 1995) and in the adipose tissue of acromegaly patients (Hochberg et al. 2015). In the current study, expression levels in adipocytes were significantly increased at 12 hours, and even more elevated after 48 hours (Fig. 4.7). Since IGFBP-3 is an IGF carrier in the circulation and \textit{Igf1} expression was increased by GH, it is reasonable that \textit{Igfbp3} is upregulated at later time points compared to \textit{Igf1}. Despite GH induction, we did not find evidence to show
that \textit{Igfbp3} is a STAT5 target gene. Thus, \textit{Igfbp3} might be induced by another GH-induced signaling pathway that does not involve STAT5. Additionally, contrary to genes that are positively regulated by GH-STAT5 signaling, \textit{Bcl6} has been reported to be negatively regulated by STAT5 in 3T3-F442A adipocytes (G. Lin et al. 2014) and in B-lym phoma cells (Walker, Nelson, and Frank 2007). Our results confirm a rapid and sustained repression of \textit{bcl6} expression by GH in 3T3-L1 adipocytes (Fig. 4.7).

\textit{Socs3} and \textit{Cish} were the STAT5 target genes most acutely upregulated by GH treatment, and treatment of adipocytes with some HDAC inhibitors had profound and opposite effects on GH induction of \textit{Socs3} and \textit{Cish}. Among the inhibitors tested, the general HDAC inhibitors, TSA and NaBu (Fig. 4.8) and the more specific inhibitors LMK-235 (HDAC5i) and CAY10603 (HDAC6i) (Fig. 4.9) were able to modulate the ability of GH to induce \textit{Socs3} and \textit{Cish} expression. Surprisingly, treatment of adipocytes with 20 mM of the SIRT inhibitor NAM had no effects on GH-induction of \textit{Cish} and minimal effects on \textit{Socs3} expression, despite the fact that SIRT1 had been reported to interact with STAT5 and that 10 mM of NAM was shown to affect GH induction of \textit{Igf1} in hepatocytes (Yamamoto et al. 2013). Moreover, the HDAC3 inhibitor (RGFP966) had no effects on \textit{Socs3} and \textit{Cish} gene expression even though STAT5 was previously shown to interact with HDAC3 in Ba/F3 cells (Nanou et al. 2017).

The potent opposite effects of HDAC5i (LMK-235) on \textit{Socs3} and \textit{Cish} gene expression (Fig. 4.10), led us to consider a possible physical interaction between STAT5 and HDAC5. However, immunoprecipitation experiments failed to detect any interaction between STAT5 and HDAC5 (Figs. 4.11 and 4.12) or between STAT5 and HDAC6 (data not shown). However, 1 hour GH treatment increased HDAC5 translocation to the adipocyte nucleus (Fig. 4.12). It has been known for several years that phosphorylation is the principal mechanism regulating nucleo-
cytoplasmic shutting of class IIa HDACs (McKinsey, Zhang, and Olson 2001; Kao et al. 2001). Nuclear export of HDAC5 was shown to involve calcium/calmodulin-dependent kinase (CaMK)-mediated phosphorylation at Ser\textsuperscript{259} and Ser\textsuperscript{498} (Jia-YiWei et al. 2015; McKinsey, Zhang, and Olson 2001), while nuclear retention is mediated by cAMP-activated protein kinase A (PKA) phosphorylation at Ser\textsuperscript{280} (Ha et al. 2010). Moreover, a study demonstrated that hormone-dependent regulation of HDAC4 interferes with its cellular localization (B. Wang et al. 2011). Given that GH triggers other pathways besides JAK-STAT signaling (reviewed in Chia 2014), it is possible that HDAC5 translocation to the adipocyte nucleus is mediated by a different GH signaling pathway that does not involve a physical interaction with STAT5.

In addition to inhibiting HDAC5, LMK-235 can also affect other HDACs (Table 4.1), particularly the other class IIa member, HDAC4, and the class IIb member HDAC6. A possible role for HDAC6 in regulating STAT5-mediated transcription is supported by the effects of the HDAC6 inhibitor (CAY10603) on Socs3 and Cish gene expression (Fig. 4.9). HDAC4 is very similar to HDAC5, and we could not obtain a more selective HDAC4 inhibitor. As an alternative to pharmacological inhibition, we used siRNA-mediated knockdown to individually downregulate HDAC4, HDAC5 or HDAC6. However, this approach did not recapitulate the effects of inhibitors on STAT5-mediated transcription, even when two HDACs were knocked down in combination (Fig. 4.13). These observations could be due to residual levels of protein following siRNA-knockdown or may result from compensation by other HDACs when one or two of them are knocked down. Another explanation is that enzymatic inhibition of HDACs and depletion by siRNA knockdown might generate different cellular effects, as suggested by the results of a study comparing HDACi treatment and HDAC class I knockdowns showing less than 4% overlap among the genes altered by these two modes of inhibition (Dejligbjerg et al. 2008).
Thus, HDAC inhibitors can have alternative effects that are independent from inhibition of HDAC enzymatic activity. In neuroblastoma cell lines, the class I HDAC inhibitor sodium valproate (VPA) promoted cell death by down regulation of survivin, an anti-apoptotic protein, through activation of the AKT pathway, while HDAC knockdown had no effects on survival (Shah et al. 2013). This concept is reinforced by evidence that Class IIa HDACs, which include HDACs 4 and 5, have weak catalytic activity and are dependent on complexes that include other HDACs, such as HDAC3 (Fischle et al. 2002). Additionally, Pinz et al demonstrated that only the HDAC inhibitors that induce histone hyperacetylation influenced STAT5-mediated transcription. In that study, the inhibitors that had no effects on gene expression, MGCD0103 and MS-275 (Entinostat), were benzamides (Pinz et al. 2015). In the current study LMK-235 induced histone hyperacetylation (Fig. 4.5), but we have not tested the other inhibitors individually. Curiously, the inhibitors that had the most profound effects on STAT5 target genes (TSA, LMK-235 and CAY10603) are all hydroxamic acids, while CI994 (HDAC1i) and RGFP966 (HDAC3i) are benzamides. Although we have not determined that the difference between these two classes of inhibitors in our experiments are due to histone hyperacetylation, the results suggest that hydroxamic acids and benzamides have different modes of action and that only hydroxamic acids may influence STAT5-mediated transcription.

HDACs are called co-repressors since they remove acetyl groups from histones, thereby making the chromatin more condensed and less accessible to transcription. Consequently, HDAC inhibition is expected to increase transcription (reviewed in Berger 2007). Yet, more recent studies demonstrate that HDACs can also be found in active genes and may promote transcription elongation (Z. Wang et al. 2009; Greer et al. 2015a). In our study, the upregulation of Soes3 by HDAC inhibition is consistent with the concept that chromatin is more accessible to
transcription. However, the downregulation of *Cish* expression by HDAC inhibition is more perplexing. Since the HDAC inhibitors TSA and NaBu were previously reported to affect mRNA stability (Krishnan et al. 2010), we also investigated this possibility using the HDAC5 inhibitor in the presence of actinomycin D. Our results demonstrated that LMK-235 had no effect on the mRNA stability of either *Cish* or *Socs3* (Fig. 4.14).

In our study, the observed effects of HDAC inhibitors on gene expression are more likely due to interference with other protein function than to inhibition of HDAC enzymatic activity. Bromodomain and extra-terminal (BET) proteins bind to acetylated histones, recruit transcription factors, and promote chromatin remodeling and transcription elongation (reviewed in Taniguchi 2016). Given the possible role of histone acetylation on STAT5-mediated transcription and the fact that HDAC inhibitors have been reported to block mRNA elongation, (Greer et al. 2015a), we hypothesized that these BET proteins could be mediating the effects of HDAC inhibitors in our experiments. In order to investigate the involvement of BET proteins in STAT5-mediated transcription, mature adipocytes were treated with the BET inhibitor JQ1, and *Cish* and *Socs3* gene expression was analyzed (Fig. 4.15). The effects of JQ1 on *Cish* expression were strikingly similar to those we had observed with LMK-235, suggesting that LMK-235 may indirectly modulate BET protein function and, consequently, *Cish* expression. Surprisingly, JQ1 also affected *Socs3* expression in a manner similar to LMK-235; that is, JQ1 further enhanced GH-induced *Socs3* expression. This was unexpected, since BET proteins are positively associated with gene transcription, and BET inhibition would be expected to repress transcription. In addition to the same pattern of induction and repression, the combination treatment with LMK-235 and JQ1 had additive effects (Fig. 4.16). These results are consistent with previous reports.
that HDAC and BET inhibitors induce and repress similar genes and produce similar biological effects (Bhadury et al. 2014).

Chromatin immunoprecipitation (ChIP) was used to investigate the involvement of BET proteins as well as STAT5 and RNA polymerase recruitment to \textit{Socs3} and \textit{Cish} promoters. Although HDAC and BET inhibition had divergent effects on the expression of \textit{Cish} versus \textit{Socs3}, STAT5 binding patterns were similar for both genes, indicating that differences in STAT5 binding were not driving the changes in gene expression (Fig. 4.17C). Because \textit{Socs3} has been reported to be a STAT3 target gene in the interleukin-6 (IL-6) signal transduction pathway (L. Zhang et al. 2006), we investigated whether the binding of STAT3 to \textit{Socs3} and \textit{Cish} promoters correlated with the changes in gene expression. However, binding of STAT3 to the \textit{Cish} promoter was low, and no significant changes with inhibitors were detected (Fig. 4.17E). In the \textit{Socs3} promoter, GH treatment decreased STAT3 binding (Fig. 4.17F). These results suggest that \textit{Socs3} is not a STAT3 target gene in the GH signaling pathway in adipocytes.

In addition to the binding of STATs, we also examined the role and binding of BET proteins. BRD4 binding levels to \textit{Socs3} and \textit{Cish} promoters were comparable to IgG negative (Fig. 4.23) control and generally unaffected by treatments (Figs. 4.18A-D). BRD2, however, showed higher levels of binding than BRD4 and was recruited to the \textit{Cish} promoter by GH. This binding was inhibited by either LMK-235 or JQ1, suggesting that GH-induction of \textit{Cish} is dependent on BRD2 and that inhibitors displace BRD2 from the \textit{Cish} TSS (Fig. 4.18E). Consistent with this observation, the involvement of BRD2 in STAT5-mediated transcription has been previously reported in human leukemia cells and Ba/F3 B cell line (Liu et al. 2014; Pinz et al. 2015). BRD2 was not further recruited to the \textit{Socs3} promoter by GH, providing a possible explanation for the divergent effects of HDAC and BET inhibition on \textit{Cish} versus \textit{Socs3} gene
expression (Fig. 4.18F). In addition, acetylation levels at Cish TSS (Figs. 4.20C and 4.21C) were about two-fold higher than Socs3 TSS (Figs. 4.20D and 4.21D), and concomitant with decreased BRD2 binding to Cish TSS by LMK-235, histone acetylation levels were unexpectedly reduced in the same region and increased at the coding region (Figs. 4.20E and 4.21E). Although we could not detect increased BRD2 binding to the coding regions (data not shown), these observations further support the hypothesis that BRD2, which is required for the GH induction of Cish, is being displaced from the TSS by LMK-235, thus affecting GH-induced gene expression. In agreement with this hypothesis, a genome-wide approach demonstrated that HDAC inhibitors shifted BRD4 binding from acetylated promoters and enhancers to newly acetylated sites in gene bodies and intergenic regions (Greer et al. 2015). In addition, we also found that the HDAC inhibitor LMK-235 substantially decreased BRD2 protein levels (Fig. 4.25). Therefore, not only is BRD2 being displaced from the Cish promoter, but its availability in the cell is also lower. This is a novel observation. In fact, a study performed in HeLa cells showed that HDAC inhibitors caused selective depletion of bromodomain containing proteins, with the exception of BET proteins that were upregulated (Mackmull et al. 2015).

BRD2 has been reported to interact with components of the transcriptional machinery, including RNA Polymerase II, serving as a scaffold protein (Peng et al. 2007; LeRoy, Rickards, and Flint 2008). When RNA polymerase binding was analyzed, GH-mediated recruitment of Pol II to the Cish promoter was decreased by LMK-235 or JQ1 (Fig. 4.19A). Whether the reduced levels of activated Pol II binding to the coding region of Cish (Fig. 4.19C) are completely explained by decreased Pol II recruitment or further effects on mRNA elongation, needs further investigation. LMK-235 increased Pol II recruitment to the Socs3 promoter in either V or GH conditions, JQ1 had no effects on GH recruitment of Pol II to Socs3 TSS compared to DMSO.
control (Fig. 4.19B). Therefore, the upregulation of *Socs3* by JQ1 was not explained by increased Pol II recruitment. However, the binding of Pol II phosphorylated at Ser\(^2\) to the coding region of *Socs3* was highly increased by JQ1 (Fig. 4.19D). Since this is a well-known marker of elongation (reviewed in Bowman and Kelly 2014), these data suggest that JQ1 is positively impacting *Socs3* mRNA elongation.

The upregulation of *Socs3* by JQ1 was initially challenging to explain. However, extensive research performed in the HIV field has shed light on how the inhibition of proteins that promote transcription elongation could upregulate some genes. In fact, the phenomenon does not necessarily involve BET proteins but an off target effect of JQ1 (Bartholomeeusen et al. 2012b) and other BET inhibitors (Lu et al. 2016) that results in the release of elongation factor P-TEFb from its inhibitory complex. HEXIM1 acts to inhibit P-TEFb when they are both associated with 7SK snRNA (Michels et al. 2004). In our experiments, the decreased association between Cyclin T1 (the regulatory subunit of P-TEFb) and HEXIM1 following JQ1 exposure, coupled with the increased association between BRD2 and Cyclin T1 (Fig. 4.23), as well as the upregulation of HEXIM1 (Figs. 4.24 and 4.25) support the hypothesis that JQ1 interferes with the binding of P-TEFb to its inhibitory complex. Disruption of the P-TEFb inhibitory complex shifts the equilibrium to free P-TEFb form and causes the cell to recognize that HEXIM1 levels are insufficient. Activation of cellular feedback mechanisms upregulate HEXIM1 expression to reassemble the 7SK snRNA inhibitory complex. Most studies have focused on BRD4 interactions with the P-TEFb complex via its Cyclin T1 subunit. Moreover, only BRD4 possesses a C-terminal segment called P-TEFb interacting domain (PID) that can actively dissociate P-TEFb from HEXIM1. However, depletion of that domain does not completely eliminate the association between BRD4 and CyclinT1 (Schröder et al. 2012; Taniguchi 2016),
indicating that another region of BET proteins may facilitate this interaction. Our study confirms that BRD2 can also be present in a protein complex with Cyclin T1 (Fig. 4.23). Whether this is a direct interaction or mediated by other proteins will require further investigation.

While more studies will be necessary to better explain the divergent transcriptional regulation of $Socs3$ and $Cish$, we have created a hypothetical model (Fig. 5.1), showing that GH upregulation of $Cish$ is dependent on BRD2 recruitment to the promoter, which leads to recruitment of RNA polymerase II and P-TEFb for transcription elongation. When JQ1 is present, it displaces BRD2 from the $Cish$ promoter, thus decreasing its gene expression. GH upregulation of $Socs3$, however, is likely dependent on other factors. Since P-TEFb when not bound to its inhibitory complex, is reported to associate with either BRD4 or the super elongation complex (SEC) (Smith, Lin, and Shilatifard 2011; reviewed in Q. Zhou, Li, and Price 2012), we hypothesize that SEC is responsible for recruiting P-TEFb to the $Socs3$ promoter. When JQ1 is present, it disrupts the interaction between P-TEFb and HEXIM1, allowing more P-TEFb to be available for recruitment by SEC, which could further increase GH-induced $Socs3$ expression (Fig. 5.1).

To confirm the model proposed, genome-wide approaches could be used to investigate how BRD2 distribution in the chromatin is affected by HDAC and BET inhibitors. In addition, to gain a more complete picture of proteins involved in the GH-induced transcription regulation of $Socs3$ and $Cish$, methods targeting chromatin in a site-specific manner would be ideal. Several techniques have been described in which a specific part of the genome is isolated and associated proteins are analyzed. Reverse ChIP analysis, for example, is one such method. In another method performed in *Saccharomyces cerevisiae*, a specific area of the chromosome is targeted via site-specific recombination in tandem with affinity purification (Hamperl et al. 2014).
Moreover, methods performed with CRISPR technology use the CRISPR associated protein 9 (Cas9) and guide RNA (gRNA) to directly purify a discrete section of the chromatin, and then the associated proteins are identified by mass spectrometry (Waldrip et al. 2014; Tsui et al. 2018). This type of technique could confirm if any components of the SEC protein complex are present at the \textit{Socs3} promoter, and could identify differentially recruited transcription factors, chromatin remodeling factors and histone markers.

SOCS3 and CISH are members of the family of suppressors of cytokine signaling. All members of this family possess a conserved Src homology 2 (SH2) domain and a C-terminal SOCS box. The SH2 domain inhibits the signaling pathway via direct or competitive binding inhibition to phosphorylated intermediates while the SOCS box is associated with ubiquitination machinery targeting proteins to the proteasome for degradation (Babon et al. 2009). Studies comparing SOCS proteins have revealed different mechanisms of inhibition among the family members. SOCS3 and SOCS1 possess a kinase inhibitory region (Sasaki et al. 1999) that is responsible for Janus tyrosine kinase (JAK) inhibition. In addition, SOCS3 can bind simultaneously to JAK and cytokine receptors revealing higher levels of specificity (Babon et al. 2012; Kershaw et al. 2013). On the other hand, CISH does not interact with JAKs. The binding of CISH to more distal tyrosine residues of GHR leads to direct competition with STAT5 binding sites. Alternatively, CISH is involved in a proteasome-dependent mechanism that may induce GHR internalization and destruction of GHR-JAK2-CISH complexes (Ram and Waxman 1999; Landsman and Waxman 2005). Differences in the activation patterns of \textit{Socs3} and \textit{Cish} have been described (Adams et al. 1998; Tollet-Egnell et al. 1999) and we have confirmed these differences in our studies.
The fast and acute upregulation of \textit{Socs3} represents a classical feed-back loop. \textit{Cish}, however, is upregulated later and its expression level remains highly elevated even after 48 hours of GH stimulation. The roles of SOCS3 and CISH as suppressors of JAK-STAT signaling in adipocytes could be further investigated by introducing mutations at residues important for the interactions between SOCS proteins and GH receptor or JAKs. Alternatively, siRNA-mediated knockdowns of SOCS3 or CISH could be used to examine STAT5 phosphorylation and direct effects of this signaling pathway.

Thus, despite similar protein structures and roles in inhibiting cytokine signaling pathways, differences in gene activation patterns and divergent responses to HDAC and BET inhibition demonstrate that these very closely related proteins might have different functions, at least in the context of cultured murine adipocytes. This project underscores the complexity of epigenetic modulation, as the same inhibitors have such profound and opposite effects on these very closely related proteins. Since several HDAC and BET inhibitors are currently in clinical trials and some of their effects described here are not due to direct enzymatic inhibition of their protein targets. Our research highlights the implications of possible off-target effects of these pharmacological inhibitors and the importance of thoroughly investigating their full effects.
Figure 5.1. Model for potential differences between *Socs3* and *Cish* gene expression. Under normal conditions, when 3T3-L1 adipocytes are stimulated with GH, the JAK-STAT signaling pathway is activated. STAT5 dimerizes and binds to STAT binding sites within the *Cish* and *Socs3* promoters. For the *Cish* promoter, BRD2 is recruited by GH treatment. Then, BRD2 binds acetylated (figure caption cont’d.)
histones and recruits RNA polymerase II and the positive elongation factor (P-TEFb). P-TEFb phosphorylates Ser2 on the RNA pol II CTD to promote elongation. When the cells are pre-treated with JQ1 before GH stimulation, BRD2 is displaced from chromatin. Therefore, RNA polymerase and P-TEFb recruitment to Cish promoter is decreased. At the Soc3 promoter, GH treatment stimulates the recruitment of P-TEFb by the super elongation complex (SEC). P-TEFb then phosphorylates RNA Pol II and productive elongation occurs. When the cells are pre-treated with JQ1, P-TEFb is released from its inhibitory complex and then becomes more available to further promote Soc3 elongation in the presence of GH.
Bibliography


Bartholomeeusen, Koen, Koh Fujinaga, Yanhui Xiang, and B Matija Peterlin. 2013. “Histone Deacetylase Inhibitors (HDACIs) That Release the Positive Transcription Elongation Factor b (P-TEFb) from Its Inhibitory Complex Also Activate HIV Transcription*.” https://doi.org/10.1074/jbc.M113.464834.


Chatterjee, Tapan K, Joshua E Basford, Ellen Knoll, Wilson S Tong, Victor Blanco, Andra L Blomkalns, Steven Rudich, Alex B Lentsch, David Y Hui, and Neal L Weintraub. 2014.


Fuller, Scott, Allison J. Richard, David M. Ribnicky, Robbie Beyl, Randall Mynatt, and


Michels, Annemieke A, Alessandro Fraldi, Qintong Li, Todd E Adamson, FranÇ Ois Bonnet, Van Trung Nguyen, Stanley C Sedore, et al. 2004. “Binding of the 7SK SnRNA Turns the HEXIM1 Protein into a P-TEFb (CDK9/Cyclin T) Inhibitor.” The EMBO Journal 23:
2608–19. https://doi.org/10.1038/sj.emboj.7600275.


Wang, Yaming, and David E. Levy. 2012. “Comparative Evolutionary Genomics of the STAT


https://doi.org/10.1016/S0006-291X(03)00179-7.
Vita

Paula Mota de Sá was born in Sao Paulo, Brazil. She received her bachelor’s degree in biological sciences from University of Sao Paulo (USP), where she became especially interested in the biomedical field. In 2013, she was selected for an exchange program in the United States to study English for one semester at Dillard University in New Orleans, Louisiana. Moreover, she worked in an immunology research laboratory and studied biological science for one year at Howard University in Washington, DC. During this exchange program, she also worked as a summer intern in the cancer field at Amgen, in Thousand Oaks, California, then returned to Brazil and worked with recombinant proteins for about one year at the University of Sao Paulo. In August 2015, she moved back to the US to pursue her PhD in Biochemistry at Louisiana State University (LSU), where she worked in the fields of obesity and type 2 diabetes.