A novel transcriptional repressor-activator relationship in Growth Hormone-regulated gene expression

by

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Abstract

Growth Hormone (GH), a major regulator of normal growth and metabolism, regulates diverse physiological processes through regulation of specific target genes. A key activator of GH-regulated genes is Signal Transducer and Activator of Transcription (Stat) 5. However, mechanisms by which GH regulates transcriptional repression are poorly understood. A profile of GH-regulated genes in 3T3-F442A adipocytes identified the potent transcriptional repressor Bcl6 (B-cell lymphoma 6) as a novel GH-responsive molecule. Further, the gene for Socs2 (Suppressor Of Cytokine Signaling 2) is strongly inhibited by Bcl6, while Socs2 activation by GH is mediated by Stat5. Chromatin immunoprecipitation (ChIP) showed that endogenous Bcl6 occupies the Socs2 promoter in the absence of GH, and occupancy decreases with GH treatment, while Stat5 occupancy increases reciprocally.

To examine the reciprocal relationship between the repressor Bcl6 and the activator Stat5, we evaluated their genome-wide occupancy by ChIP-Sequencing in GH-responsive 3T3-F442A adipocytes, revealing over 3000 regions of occupancy for Bcl6 and over 900 for Stat5. Among these, gene ontology analysis of potential Bcl6 target genes identified genes involved in growth, differentiation, metabolism, and signaling.

Reciprocal occupancy of Bcl6 and Stat5 was observed not only on the Socs2 promoter, but also on the Cish (Cytokine-inducible SH2-containing protein) and Bcl6 gene promoters. However, Socs2 and Cish are stimulated by GH while Bcl6 is inhibited by GH. Analysis of transcription co-regulators suggests regulatory differences among these genes: p300 and Hdac3 (Histone deacetylase 3) occupied the Bcl6/Stat5 regulatory sequences of Socs2, Cish and Bcl6 constitutively. Promoter activation assays show that p300 co-activates Stat5-mediated induction of Socs2 and Cish expression, while Hdac3

functions as a co-repressor. In contrast, both p300 and Hdac3 may function as co-repressors when regulating Bcl6 expression.

This work provides insight into novel roles for Bcl6 in GH action, and mechanisms of transcriptional regulation involving reciprocal relationships by activating and repressing factors for genes in a major growth regulatory pathway.

Chapter 1

Introduction

Transcription and transcription factors: Activation and repression of gene expression

Transcription is the synthesis of RNA from DNA, and is the first step leading towards gene expression. Regulation of this process by various mechanisms is what allows cells to respond specifically to internal and environmental cues. The activation or repression of specific sets of target genes at the right time or in the right location allows the cells to adapt, counter, change, or develop as needed for the survival of the cell or the organism. Over the years, it has become clear that the important biological process of transcription is regulated at multiple levels, such as by the location of the gene on the chromosome (1, 2), the structure of the nearby chromatin (3), the availability and functional state of the transcription factors and their associated coregulatory proteins (4-6), to the structure, sequence, or export of the transcribed RNA sequence itself (7, 8). However, much of gene expression is controlled at the level of transcription initiation. For the process of transcription initiation, expression of a gene is mostly dependent on the cis-regulatory elements comprised of the regulatory DNA sequences of the gene and the trans-acting protein factors that interact with specific cis-acting elements to initiate or inhibit transcription.

Transcription factors are proteins that bind to specific DNA sequences to regulate transcription of a gene (9-11). These proteins contain one or more DNA binding domains

and a transactivating (transcription activation) domain. Transcription factors often also contain one or more regulatory domains, which can interact with other proteins to convey regulatory signals. DNA binding domains recognize and associate with specific DNA sequences, usually near the genes they regulate, and serve to locate the transcription factor in a position from which the activation domain can regulate transcription. Recent work has also shown that DNA sequences regulating gene transcription can be located quite far from the target gene, in the case of enhancer elements (12, 13), and in some cases can be located on a different chromosome altogether (14). Often, a gene will have multiple regulatory sequences for different transcription factors, and transcription of the gene depends on the net effects of several transcription factors and their associated proteins. Transcription activation domains interact with the transcriptional machinery, consisting of general transcription factors and RNA polymerase II (RNA pol II) (15), and stimulate the initiation and elongation steps of transcription (16). Transcription factors that activate expression of a gene are known as transcription activators, while transcription factors that serve to inhibit gene expression are transcription repressors.

Transcription factors can use various mechanisms to regulate expression of a target gene (17, 18). These mechanisms include stabilizing or inhibiting the binding of RNA pol II to DNA, and the recruitment of coregulatory protein factors to the transcriptional regulatory protein complex. Coregulatory proteins can aid in the activation or repression of transcription by modifying the activity of associated transcription factors, mediating the further recruitment of nuclear regulatory proteins, stabilizing or destabilizing associations with the general transcriptional machinery, or modifying the structure and accessibility of the surrounding chromatin.

The activity of transcription factors can also be regulated by multiple mechanisms. Some of these include the synthesis of the transcription factor, cellular localization of the transcription factor, and activation of the transcription factor by ligand binding, post-translational modification, or interaction with other transcription factors or coregulatory

proteins. The activity of many transcription factors is regulated by a combination of several mechanisms. For example, the activity of the growth hormone (GH)-regulated transcription factor C/EBPβ (CCAAT/enhancer binding protein beta) can be regulated by both activating and repressive phosphorylation events, protein acetylation, cellular localization, and interactions with C/EBPβ-associated nuclear proteins (19).

This thesis describes the novel growth hormone (GH)-regulated transcriptional repressor Bcl6 (B cell lymphoma 6), its reciprocal relationship with the GH-activated transcription factor Stat5, and the associated coregulatory proteins that interact with Bcl6 and Stat5 to regulate gene expression in response to GH. Through the work in this thesis, we have identified novel GH-responsive transcriptional complexes, and started to identify mechanisms by which these nuclear protein complexes participate in GH action to orchestrate the proper expression of several essential GH target genes. Teasing out potential downstream physiological effects of these newly-discovered alliances between known and newly-discovered GH regulatory factors in the nucleus using both cell culture and relevant animal models will add to our understanding of the molecular mechanisms regulating normal and aberrant growth and metabolism.

GH and the regulation of transcription

A useful system to study the many mechanisms of transcription and downstream effects of transcriptional regulation is the study of the regulation of growth hormone (GH) target genes. GH, a peptide hormone secreted by the pituitary gland, regulates a wide variety of physiological effects. In particular, GH has long been known as a major regulator of normal growth and carbohydrate, lipid, and protein metabolism (20, 21). In conjunction with insulin-like growth factor 1 (IGF1), GH promotes longitudinal growth by stimulating the proliferation and differentiation of osteoblasts and chondrocytes in long bone (22-25). GH also promotes a relative increase in lean body mass and decrease in

body lipid through its effects on targets such as the liver, muscle, and adipose tissue (26, 27). GH excess can result in diseases such as acromegaly or insulin resistance (26, 28, 29), while GH deficiency in children can result in growth failure and short stature, and GH deficiency in adults can result in a variety of non-specific problems, including truncal obesity and decrease in muscle mass (30, 31).

Many functions of GH occur through regulation of specific target genes. These responses are usually initiated by binding of GH to the GH receptor (32, 33). The GH receptor is a member of the cytokine receptor superfamily and is classified as a type I cytokine receptor. Other members include the prolactin receptor and leukemia inhibitory factor receptor. In the case of GH, a single molecule of GH binds to a GH receptor dimer and activates Jak2 (Janus kinase 2), a non-receptor tyrosine kinase that binds to the GH receptor. Jak2 phosphorylates itself and the cytoplasmic domain of the GH receptor to initiate downstream signaling (34). Signaling molecules, including signal transducers and activators of transcription (Stats), and proteins involved in the mitogen activated protein kinases (MAPKs) and phosphatidyl inositol 3' kinase (PI3K) signaling pathways, are among the messengers recruited to relay GH signals to the nucleus in order to modulate gene transcription (32, 33, 35, 36).

GH is secreted in a sex-dependent pulsatile manner throughout the day (37), and different genes are activated or repressed in time-dependent waves of expression (38). Regulation of gene expression by GH is often related to secretion patterns and hormone concentration, making genes that show changes in expression in response to GH (GH target genes) an ideal and versatile system for studying a wide variety of transcriptional regulatory networks and the downstream physiological effects of gene regulation. Regulation of gene transcription in response to GH occurs at multiple levels in the cell, including cellular localization of rate-limiting protein factors, post-translational modification of transcription factors and other nuclear proteins, chromatin modification,

and the formation, composition, and recruitment of transcriptional regulatory complexes (19).

Bcl6 as a newly recognized GH-regulated transcriptional repressor

Previous work on GH-mediated transcriptional regulation has mostly focused on mechanisms by which GH activates transcription. Therefore, it was of intense interest to identify mechanisms by which GH could inhibit or downregulate transcription, as proper balance between gene activation and repression is essential to the proper function of biological processes and survival of the cell. The identification of the eight twenty-one (ETO) protein as a potential C/EBPβ-interacting corepressor offered one of the first clues into potential mechanisms by which a GH-regulated transcription factor could interact with other proteins to downmodulate activity and achieve transcriptional repression (39, 40). The identification of Bcl6 (B cell lymphoma 6) as a GH-regulated transcriptional repressor and the study of how it regulates expression of GH target genes gave added insight into how GH mediates transcriptional repression and coordinates the activation and repression of a subset of GH-responsive genes (41) (Chapter 2).

To learn more about mechanisms by which GH regulates transcription, a gene expression profiling microarray analysis was carried out using GH-responsive 3T3-F442A adipocytes treated with GH for both short (30 min) and long time periods (4 h and 48 h). Analysis of the gene expression profiles revealed about 500 GH-regulated genes that were either activated or repressed in time-dependent waves of GH-regulated gene expression (38). Analysis of GH early response genes have revealed that many different regulatory mechanisms coordinate to regulate the transcription of genes in response to GH. Analysis of a subset of C/EBPβ-regulated genes among the GH early response genes revealed that even genes regulated by the same transcription factor are subjected to

further levels of transcriptional regulatory control to diversify responses mediated by the same transcription factor, and ensure specific regulation of gene expression (42).

As described in Chapter 2, further analysis of the profile of GH-regulated genes using the CRC (Chinese restaurant clustering) algorithm (43) grouped the genes in clusters based on the extent of expression change in response to GH regardless of the direction of gene expression. This analysis revealed a cluster of genes strongly regulated by GH at later time points (4 h and 48 h), with 48 h of GH treatment corresponding to a time point of chronic GH treatment where adipocytes show phenotypes of GH-induced insulin resistance (41). Among the genes in the cluster were known GH target genes, such as Igf1, Socs2 (suppressor of cytokine signaling 2), Cish (cytokine inducible SH2-containing protein), and Pik3r1 (phosphatidylinositol 3-kinase regulatory subunit alpha) (44-47). Pik3r1, in particular, has been implicated in GH-induced insulin resistance (47). This cluster of genes also included genes not previously recognized as GH target genes, such as Atf3, Klf5, and Bcl6. In particular, Bcl6 was found to be strongly repressed by GH, in contrast to the other genes in the cluster, and was the gene found to be the most highly repressed by GH in the entire profile. Strong repression of Bcl6 expression in response to GH was verified by quantitative real-time PCR experiments carried out in adipocytes and preadipocytes. Time course and dose response experiments showed that Bcl6 expression was repressed by GH concentrations as low as 5 ng/ml and as early as 30 minutes after treatment (41). As the Bcl6 gene codes for a transcriptional repressor, it was of interest as a gene candidate for further study. It was hypothesized that Bcl6 would be a new link in understanding how GH regulates, and especially how GH represses, the expression of its target genes. More recently, GH-regulated expression of Bcl6 has also been demonstrated in liver (48) and the C2C12 muscle cell line (49), and prolactin was shown to be a potent inhibitor of Bcl6 expression in human breast cancer cell lines (50). These results all suggest a broader role for Bcl6 outside of its previously known functions in the immune system, and hint, in particular, at novel roles for Bcl6 in GH physiology.

Bcl6: Master regulator of B cell differentiation

Bcl6 is a sequence-specific transcriptional repressor of the POZ/BTB (Pox virus and zinc finger/Bric-a-brac Tramtrack Broad complex)-zinc finger family (Figure 1.1) (51-54). The protein is localized to the nucleus and contains six Kruppel-type zinc fingers at the C- terminal and an N-terminal POZ-BTB domain. Each of the zinc fingers carries out specific interactions with either the target DNA or other Bcl6-associated proteins, with the four C-terminus zinc fingers required for DNA binding and recognition (55). Maximal repressive activity of Bcl6 requires the full POZ/BTB domain, as well as a repressive domain in the middle region of the protein (51). Bcl6 is known to associate with a variety of coregulatory proteins in order to carry out its repressive effects on transcription, and different coregulators are involved in modulating the transcription of different subsets of Bcl6 target genes. Examples of Bcl6-interacting corepressors include NCoR (nuclear receptor corepressor), SMRT (silencing mediator of retinoic acid and thyroid hormone receptor), various HDACs (histone deacetylases), ETO, CtBP (C-terminal binding protein), and the Bcl6-specific corepressor BCoR (Bcl6 corepressor) (56-65). Recently, the lysine acetyltransferase p300, best known for interacting with transcriptional activators as a coactivator molecule, was also shown to associate with Bcl6 (66). Acetylation of Bcl6 at a KKYK motif in the middle region of the protein inhibits Bcl6 activity by disrupting its ability to recruit HDACs and other corepressors, resulting in derepression of target genes inhibited by Bcl6 (66). Bcl6 is also phosphorylated at multiple sites by mitogen-activated protein kinases (MAPKs) (67). In particular, phosphorylation of Bcl6 at serines 333 and 343 by Erk (extracellular signal regulated kinase) 1 and Erk2 results in proteasome degradation of Bcl6 in B cells (67). Interestingly, a recent report suggests that Bcl6 may activate expression of the Oat1 (organic anion transporter 1) and Oat3 (organic anion transporter 3) genes in rat kidney proximal tubule cells (68). The mechanisms by which Bcl6 may be activating expression of these genes are still unclear.

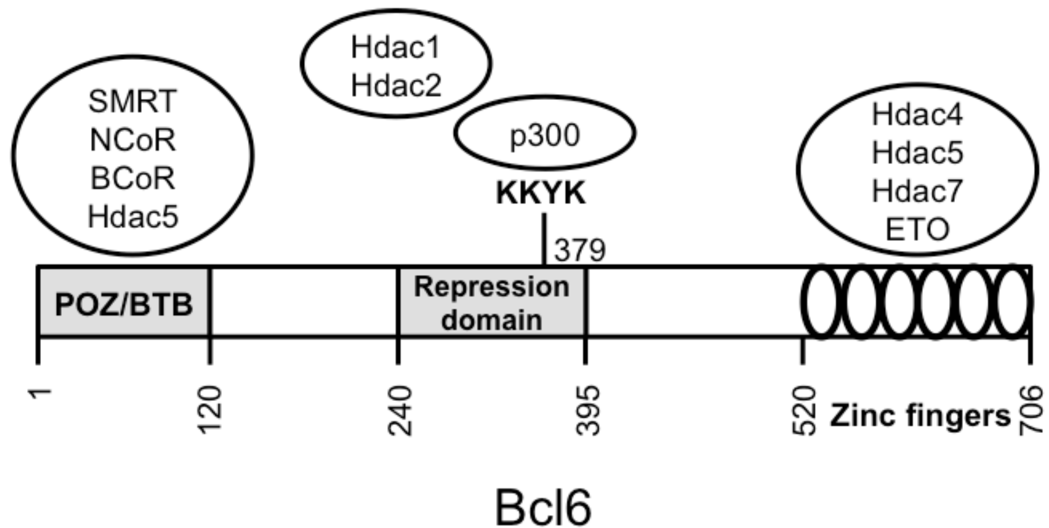

Figure 1.1

Schematic representation of Bcl6 domain structure. The Bcl6 protein contains an N-terminal POZ/BTB domain and a second repressive domain located in the middle of the protein (shaded boxes). The repressive domain in the middle region of the protein also contains the KKYK acetylation motif and PEST sequence phosphorylation motifs. Acetylation of Bcl6 inhibits its repressive activity by disrupting interaction with corepressive molecules. Phosphorylation of Bcl6 targets the protein for proteasomal degradation. Six Kruppel-type zinc fingers, represented by vertical ovals, are located at the C-terminus of the protein. Amino acid numbers are labeled as indicated, and Bcl6-interacting proteins are listed in ovals depicted above known or predicted interaction regions. Adapted from (52) and (56).

Bcl6 is best known for its functions in the immune system, particularly its role as a master regulator of B cell differentiation (53, 69-71). Expression of Bcl6 in the B cell lineage is regulated so that high levels of Bcl6 are only expressed in the germinal center. Germinal centers are specialized structures where B cells undergo class switch recombination and somatic hypermutation to produce a diverse array of antibodies. The high expression levels of Bcl6 in germinal centers functions to maintain the germinal center gene expression program and inhibit expression of genes involved in B cell activation, DNA damage response, or terminal differentiation. A ChIP (chromatin immunoprecipitation) on chip analysis to identify direct Bcl6 target genes in immune cells has also revealed that Bcl6 target genes are enriched for functions such as regulators of transcription, chromatin structure, protein ubiquitylation, cell cycle regulation, and DNA damage responses (72). As such, terminal differentiation of B cells cannot be initiated until expression of Bcl6 has been downregulated. Due to the anti-apoptotic functions of Bcl6 during B cell development, dysregulation of Bcl6 expression is a frequent cause of B cell lymphomas (53, 69).

New and novel functions of Bcl6 outside the immune system

Recent studies have shown that Bcl6 plays physiological roles outside of the immune system. Bcl6 has been identified as playing a role in protecting testicular germ cells from apoptosis (73, 74), repressing proliferation of pancreatic beta cells (75), and mediating sexual dimorphism in liver (48, 76). Bcl6 has also been shown to mediate inflammatory responses in the pancreas (77), and a role has been demonstrated for Bcl6 in regulating left-right patterning in Xenopus embryos by competing with the coactivator Mastermind-like 1 for the Notch1 intracellular domain, thereby restricting Notch-activated transcription to specific cell types (78). Roles for Bcl6 in muscle and bone differentiation have also been proposed (79), and its expression pattern during development suggests a potential role for Bcl6 in patterning visual and somatosensory pathways as well (80). The identification of Bcl6 as a GH target gene therefore suggests intriguing and novel

functions for Bcl6 in growth, metabolism, circadian rhythm, adipose differentiation, or adipose immune functions. As Bcl6 is a newly identified GH target gene, it was of interest to investigate whether or not Bcl6 was also a GH-regulated transcription repressor, and if it had GH-specific functions in adipocytes. These goals were the focus of Chapter 2 of this thesis. Identifying the physiological roles of Bcl6 in response to GH and the underlying transcriptional mechanisms by which Bcl6 target genes are regulated by GH may provide insight into how Bcl6 regulates the network of functions it has been found to participate in in other tissues. Of particular interest as a future project would be the identification of Bcl6 target genes in different tissues followed by analysis of similarities or differences in the subsets of genes targeted by Bcl6 in different cell types.

Stat5: GH-regulated transcription factor and major mediator of GH responses

Stat5 (signal transducer and activator of transcription 5) belongs to the Stat family of latent transcription factors, of which there are seven members (Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, Stat6) in mammalian cells. Stats are essential regulators of a subset of cytokine, growth factor, and hormone responses (81-84). It has been shown that Stat5a and Stat5b are crucial mediators for GH signaling to the nucleus (Figure 1.2) (85-88).

Stat5a and Stat5b are encoded by two separate genes, and the proteins share 91% identity in amino acid sequence (89). Knock out mouse studies have shown that the two proteins have both redundant and distinct functions (90-92). Stat5b has been shown to be the essential transducer of GH-stimulated Igf1 expression and a key mediator for GH-stimulated somatic growth and regulation of sexual dimorphism genes in the liver (85, 86, 88, 93). In this thesis, the function of Stat5 in regulation of the candidate target genes was examined without distinction between the isoforms, using antibodies that recognize both Stat5a and Stat5b; therefore, specific isoforms will only be referred to if pertinent.

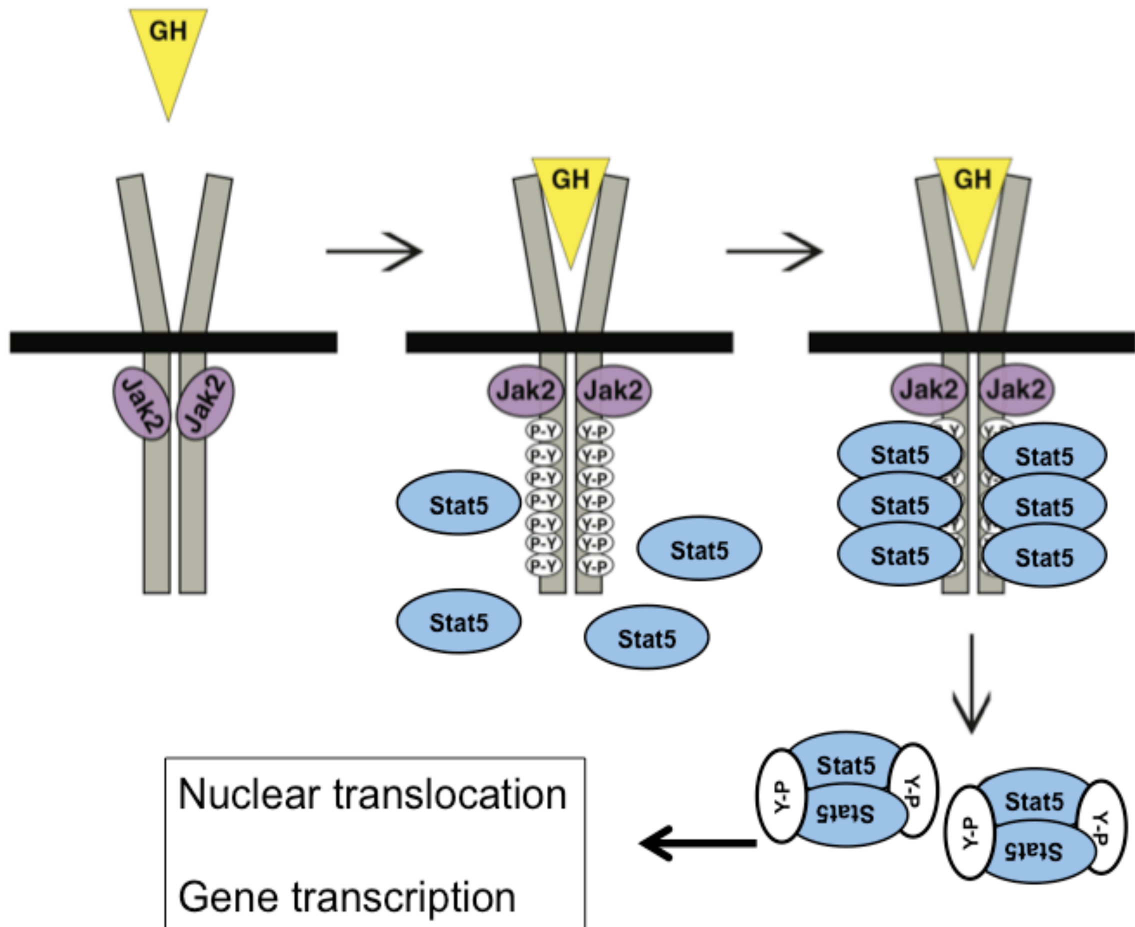

Figure 1.2

Activation of Stat5 by GH. (Left) The growth hormone receptor (GHR) is a constitutive dimer. A molecule of Jak2 is bound to the intracellular domain of each monomer. **(Middle)** Binding of GH to its receptor induces conformational changes in the GHR dimer, which stimulate the kinase activity of Jak2, leading to phosphorylation of tyrosine residues on Jak2, and phosphorylation of multiple tyrosine residues (Y-P) in the intracellular domain of the GHR. **(Right)** Many of the phospho-tyrosine sites on the GHR can bind Stat5, which is then phosphorylated on a single tyrosine residue by Jak2. This promotes the dissociation of Stat5 from the GHR, Stat5 dimerization, translocation to the nucleus, and activation of Stat5-regulated gene transcription. Adapted from (88).

In the absence of stimulus, Stat5 mostly resides in the cytoplasm as latent monomers. When a GH molecule binds to the GH receptor heterodimer, conformational changes in the receptor dimer are followed by recruitment, activation, and autophosphorylation of the receptor-associated tyrosine kinase, Jak2, and phosphorylation of multiple tyrosine residues in the intracellular domain of the GH receptor (85, 94, 95). Stat5 is recruited to the receptor through interactions between the Stat5 SH2 domain and specific receptor phosphotyrosine residues, and recruitment of Stat5 to the GH receptor induces the phosphorylation of Stat5 by Jak2 on a conserved carboxy-terminal tyrosine (Stat5a/b, Y694/Y699) (96-98). Phosphorylated Stat5 enables the formation of Stat5 dimers and dissociation of Stat5 from the GH receptor. Stat5 dimers translocate to and are retained in the nucleus, where they bind specific DNA sequences to regulate the expression of target genes (99, 100).

Stat5 is best known as a transcriptional activator, and is known to interact with a variety of coregulatory molecules, including p300, CBP (cyclic AMP response element binding protein binding protein), and NCoA1 (nuclear receptor coactivator 1) (101-103). Stat5 is also known to repress transcription, and has been shown to interact with corepressor molecules such as SMRT and HDACs (104-106). Paradoxically, other studies suggest that Stat5-dependent gene activation might require the recruitment and activity of HDACs as well (106, 107). Stat5b has been shown to play a critical role in inhibition of insulin-like growth factor binding protein 1 (Igfbp1) gene transcription by impairing the actions of the transcription factor FoxO1 (Forkhead box protein O1) on the Igfbp1 promoter (108). More recently, it has been postulated that Stat5 may form tetrameric complexes on target DNA when repressing transcription; these tetrameric Stat5 complexes interact with members of the Polycomb repressor family, which generates a repressive chromatin state inhibitory to transcription (109).

Reciprocal regulation of target gene transcription by Bcl6 and Stat5

Bcl6 binds to a DNA consensus similar to that for Stat5, with Bcl6 having a core motif of 5'-TTC CT(A/C) GAA-3', and Stat5 having a core motif of 5'-TTC C(A>T)G GAA-3' (51, 55, 110, 111). As Bcl6 and Stat5 share similar DNA binding motifs, it would be predicted that a subset of Bcl6 or Stat5 target genes could be dual-regulated by both transcription factors.

Examples of Bcl6 and Stat transcription factors both regulating a target gene using the same DNA regulatory region have been described previously. The consensus sequence for Stat transcription factors is highly similar among all family members, sharing the same core motif of 5'-TTC (N)2-4 GAA-3' (112). Despite the high structural conservation among Stat family members and the similarity in their DNA binding consensus sequences, each Stat member has its own distinct target genes and functions (81). Bcl6 and various Stat proteins have been shown to bind the same DNA sequences *in vitro* (113), and Bcl6 and Stat6 both regulate expression of the I epsilon promoter that controls immunoglobulin heavy chain class switching to IgE in B cells (114). The discovery of the reciprocal regulation of Socs2 by Bcl6 and Stat5 in response to GH (Chapter 2) was the first example of an *in vivo* system where a gene is regulated by both Bcl6 and Stat5 using the same DNA regulatory sequence. This was soon followed by other examples showing cooperation between Bcl6 and Stat5 in regulating a common target gene. Interplay between Bcl6 and Stat5 was identified in regulation of sex-specific genes in liver, with Bcl6 functioning to repress female-biased Stat5 target genes in male liver (48, 76). Common targets of Bcl6 and Stat5 in the liver showed enrichment for genes involved in lipid and drug metabolism, suggesting potential functional regulation of dual Bcl6 and Stat5 target genes outside the immune system (76). Bcl6 and Stat5 occupancy patterns across the genome in the presence and absence of GH have also been examined in adipocytes (Chapter 3). Analysis of Bcl6 occupancy patterns and gene expression data suggest that multiple genes strongly regulated by GH may also be Bcl6

target genes. Bcl6 has been found to occupy the Stat5 regulatory region of the Cish gene in liver (115), and results in this thesis suggest that Bcl6 and Stat5 regulate the expression of Cish and Bcl6 using the same DNA regulatory sequence in adipocytes as well (Chapter 4).

Recently, the Bcl6 ortholog in Drosophila has been described (116). Ken and Barbie (*ken*), the Drosophila ortholog of Bcl6, was shown to promote somatic stem cell renewal in the testis niche (74). Ken carries out some of its functions by functioning as a negative regulator of the Jak/Stat pathway, and recognizes a DNA consensus sequence that overlaps with the recognition sequence of the Drosophila Stat homolog Stat92E (116). Ken is thought to negatively regulate the Jak/Stat pathway by competing for a subset of Stat targets in vivo and repressing Stat-dependent expression of these genes, including *vvl* (ventral veins lacking) (116). Interestingly, Ken was not found to repress the expression of the Drosophila SOCS homolog, Socs36e (116). Socs36e is both a target and negative regulator of Jak/Stat signaling. It shows the closest homology to the mammalian Socs5, which has not been shown to be a Bcl6 target gene in mammalian cells (117). Nevertheless, it appears that dual regulation of target genes by Bcl6 and Stat5 is a transcriptional regulatory mechanism that has been conserved over evolution. Future work elucidating binding requirements for each transcription factor, and analysis of the subsets of genes regulated specifically by either Bcl6 or Stat5 compared to genes regulated by both factors in different organisms and different tissues, will shed light on the implications and functions of this conserved regulatory mechanism.

Transcriptional coactivator p300

Coregulatory proteins are protein factors that interact with transcription factors to activate or repress transcription (6, 118, 119). Coregulators do not directly bind to DNA, and often carry out their actions by modifying the activity of transcription factors or the

structure of the surrounding chromatin (118, 120, 121). Coregulators that enhance transcription are known as coactivators, while coregulators that function to repress transcription are known as corepressors. As expression of genes dually regulated by Bcl6 and Stat5 identified in Chapter 4 showed different responses to GH treatment, the role of several coregulatory proteins in mediating these different responses was also investigated in Chapter 4.

P300 was first identified as an adenovirus E1A binding protein (122). It is structurally and functionally similar to the CBP (CREB binding protein) coregulatory molecule. P300 is best known as a transcriptional coactivator, and is required for a wide array of cellular processes, including cell cycle progression and cellular differentiation (123-125). It contains an intrinsic lysine acetyltransferase activity, and known substrate proteins include histones and transcriptional regulatory proteins (126, 127). P300 is thought to regulate gene transcription by serving as an acetyltransferase and regulating the activity of transcription factors and the recruitment of RNA pol II, relaxing the chromatin structure to facilitate transcription, or functioning as a scaffold protein. A role for cytoplasmic p300 as an E4 ubiquitin ligase has also been described (128).

Activity of p300 itself can be regulated through a variety of post-translational modifications. Autoacetylation of p300 at multiple lysine residues appears to act as a switch to increase its acetyltransferase catalytic activity (129, 130). Phosphorylation of p300 by Akt promotes metabolic stability of p300 protein along with augmenting p300 acetyltransferase activity and its recruitment to target promoters (131-133). In contrast, phosphorylation of p300 by the MAP kinase p38 targets the protein for degradation by the proteasome (134). Ubiquitination of p300 is associated with the unphosphorylated form of p300, and targets the protein for degradation by the proteasome (135, 136), while sumoylation of p300 correlates with transcriptional repression (137).

Bcl6 and p300

P300 has been shown to bind and acetylate Bcl6 in immune cells. Acetylation of Bcl6 by p300 inhibits Bcl6 function by disrupting the ability of Bcl6 to recruit HDACs and other corepressive molecules (66). Bcl6 also regulates expression of p300 in immune cells. Bcl6 functions as a transcriptional repressor of p300 expression. The overexpression of Bcl6 and subsequent repression of p300 expression is thought to contribute to the development and maintenance of some B cell lymphomas due to loss of p300-mediated acetylation of p53 and Hsp90 (138). Supporting the importance of this inhibitory feedback loop between Bcl6 and p300 is the observation that 39% of diffuse large B-cell lymphoma and 41% of follicular lymphoma cases display genomic deletions and/or somatic mutations that remove or inactivate the lysine acetyltransferase coding domain of either p300 or CBP (139).

Stat5 and p300

The role of p300 as a coregulator of Stat proteins in various cell types has long been established (101, 103, 140-143). Recruitment of p300 by Stat1, Stat2, Stat3, Stat5, and Stat6 has all been demonstrated. Stat6 recruits p300 to carry out transcriptional activation of target genes in response to interleukin 4 stimulation (143), and p300 and CBP have been shown to bind to STAT5 and coactivate STAT5-mediated transactivation of β-casein expression (101). A role for p300 as a coactivator in Stat5b-mediated transcription of Igf1 in response to GH in liver has also been demonstrated (115). Stat5 squelching of limited pools of p300 has also been implicated in the Stat5b-mediated repression of nuclear factor kappa B (NF-kB) signaling in response to prolactin. This squelching demonstrates a mechanism by which Stat5 can indirectly function as a transcriptional repressor without binding to target gene DNA (144).

The histone deacetylase (HDAC) family and Hdac3

Potential roles for the coregulator Hdac3 in modulating Bcl6- and Stat5-mediated transcription of Socs2, Cish, and Bcl6 were also examined in Chapter 4. Hdac3 is a member of the histone deacetylase (HDAC) family of proteins. HDACs are a group of lysine deacetylases which catalyze the deacetylation of lysine residues on histones and other protein targets, including p53, CBP, and Stat1 (145-150). Removal of acetyl groups from histone tails by HDAC molecules typically correlates with condensed DNA structure and repression of transcription. Due to the key role HDACs play in regulating gene transcription, they have been implicated in a variety of biological pathways, including cell cycle, cellular differentiation, and signal transduction (121, 151, 152).

To date, eighteen HDAC family members have been identified in higher eukaryotes. They are separated into four classes based on homology to yeast histone deacetylases (152-156). Class I HDACs include Hdac1, 2, 3, and 8 and are related to yeast Rpd3 (reduced potassium deficiency 3). Class II HDACs are homologs of the yeast Hdac1 protein and this group is further divided into two subgroups based on sequence homology and domain organization. Class IIa HDACs include Hdac4, 5, 7, and 9. These molecules all contain a highly conserved C-terminal catalytic domain homologous to yeast Hda1, but their N-terminal domains bear no similarity to HDACs of other classes. Class IIb HDACs include Hdac6 and Hdac10. Hdac6 possesses an additional acetylase domain and a zinc finger motif, and participates in cytoplasmic functions such tubulin deacetylation and protein degradation, in addition to its function as a histone deacetylase. Class III HDACs are homologous to yeast Sir2 (silent information regulator 2) and are comprised of the seven silent information regulator proteins, or sirtuins (Sirt1-7). Hdac11 is a class IV HDAC due to its distinct structure, and shares homology with yeast Hos3 (high osmolarity sensitive protein 3). Class I, II, and IV comprise the classical family of zinc-dependent HDACs, while class III HDACs are nicotinamide adenine dinucleotide (NAD+)-dependent. Due to the diverse functions HDACs participate in and their roles in

transcriptional regulation, HDACs are key targets for drug development, and HDAC inhibitors are currently being explored as pharmaceutical agents for diseases ranging from neurodegeneration to cancer. Older HDAC inhibitors include the general class I and class II HDAC inhibitors trichostatin A and suberoylanilide hydroxamic acid (SAHA) (152). More recently, class selective and member specific inhibitory molecules have been developed for targeted inhibition of specific HDAC activity (154, 157, 158). Sirtuin activity has traditionally been inhibited by nicotinamide, although new small molecules have been developed for selective inhibition for use as pharmaceutical drugs (159, 160). Sirtuins have been proposed as therapeutic targets for type II diabetes due to the links between their enzymatic activity and the energy status of the cell (159, 161).

Hdac3 is a class I HDAC that is found in many tissues in the body, and is primarily localized to the nucleus, although it can also be detected in the cytoplasm and at the plasma membrane (162, 163). It has been found in complexes with corepressive molecules such as SMRT, NCoR, the Bcl6 corepressor BCoR, and other HDACs (155, 164-166). It regulates the expression of a variety of genes through its interaction with both histones and non-histone protein targets, and has been implicated in long term memory formation and NF-kB signaling (155, 167).

Hdac3 catalytic activity can be regulated by phosphorylation at serine 424 in the C-terminal domain, and phosphorylation at this site has been shown to increase enzymatic activity (168). Phosphorylation of this residue was not found to alter Hdac3 subcellular localization, and Hdac3 must be bound to the corepressors NCoR (nuclear receptor corepressor) or SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) to form an active enzyme complex (165, 169, 170). Hdac3 is the primary HDAC enzyme in NCoR/SMRT complexes; however, other HDAC molecules can be recruited to this complex in a transcription factor or promoter dependent-manner. In particular, the class IIa HDACs, Hdac4 and Hdac5, have been shown to interact with NCoR/SMRT at a site independent from the interaction site of Hdac3, and Hdacs 4, 5, 7, and 10 have been

shown to interact with Hdac3 but not Hdacs 1 and 2 (164, 165, 169, 171). It has been suggested that activity of several of these class II HDACs is dependent on their interactions with Hdac3 and NCoR/SMRT as part of a corepressor protein complex, and cellular localization of Hdac4 and Hdac5 is regulated by their phosphorylation state. Intriguingly, Hdac4, 5, and likely 7, are regulated through subcellular compartmentalization controlled by site-specific phosphorylation and binding of 14-3-3 proteins. The regulation of these HDACs is thus linked via 14-3-3 to cellular signaling networks (164), and suggests a mechanism by which GH signaling can regulate Hdac3-interacting transcription factors and their target genes. Future work to evaluate the contribution of other HDACs to Bcl6 and Stat5 target gene expression in response to GH will provide insight into this potential mechanism.

Hdac3 has been shown to mediate transcriptional repression in several ways (121, 151, 155). Deacetylation of histones results in a closed chromatin conformation that is inhibitory for transcription. Histone deacetylation also reduces recruitment of bromo-domain coactivators, such as p300 and CBP, to the chromatin. Hdac3 complexes can also interact with transcription factors to repress expression of a target gene. In some of these cases, Hdac3 repression is lost when binding of a ligand induces a conformational change in the complex, allowing disassociation of Hdac3 and recruitment and binding of a coactivator. Hdac3 histone deacetylation often occurs in concert with the activity of other repressive chromatin remodeling enzymes, such as methyltransferases, and Hdac3 is an essential component for the coordination and establishment of specific epigenetic profiles in the cell (172-174). Hdac3 has also been implicated in the maintenance of circadian rhythm and hepatic lipid homeostasis (175, 176), suggesting a role for Hdac3 in the regulation of metabolism and energy storage. A role for Hdacs 1, 2, and 3 as inhibitors of adipogenesis has also been described (177, 178).

Bcl6 and Hdac3

Hdac3 has been identified as a Bcl6 interacting protein, and has also been detected in complexes with the Bcl6 corepressor BCoR (59, 179). As Hdac3 is predicted to carry out many of its functions in association with NCoR or SMRT as part of a multi-protein complex (165), and Bcl6 in turn has been shown to recruit NCoR and SMRT when repressing transcription (63), it would not be unexpected for Hdac3 to play a role in regulating expression of Bcl6 and GH target genes in adipocytes as well. Hdac3 is recruited by Bcl6 to repress expression of DNA damage response genes such as ATR and TP53 (180, 181), and Hdac3 is also a drug target for B cell lymphoma therapy (152). More recently, Hdac3 was found to be recruited to a subset of dual Bcl6 and NF-kB regulatory regions in a Bcl6 dependent manner in macrophages to restrain inflammation and counter the activation of inflammatory genes by NF-kB and p300 in response to LPS stimulation (62). Bcl6/NCoR/SMRT complexes have also been implicated in repressing inflammation and preventing atherosclerosis (182). As hyperinflammation and atherosclerosis are both pathologies in which GH may play a role (183, 184), understanding the role of Bcl6 and Hdac3 in the regulation of GH target genes could shed insight on the pathogenesis of these diseases.

Stat5 and Hdac3

Studies on direct interactions between Stat5 and Hdac3 or the participation of Hdac3 in Stat5-mediated transcription are sparse. However, Stat5 is known to interact with other class I HDACs, and it has been demonstrated that HDAC activity may be required for Stat5-mediated induction of some target genes, including Cish (106, 107, 185). Stat5 has also been shown to interact with the Hdac3-interacting corepressor SMRT (170); this interaction may be a potential mechanism by which Stat5 mediates transcriptional repression, or a method by which a cell downregulates Stat5 activity (104).

Histone modifications and their role in transcriptional regulation

One of the potential mechanisms by which p300 and Hdac3 may be carrying out their regulatory effects on gene expression is through the post-translational modification of histones. Histones are small, basic proteins found in eukaryotic nuclei that package and order the DNA into subunits known as nucleosomes. Nucleosomes, which consist of about 146 base pairs of DNA wrapped around an octamer of core histone proteins, form the basic repeating unit of eukaryotic chromatin. Histones are one of the major components of chromatin, and histones, histone post-translational modifications, and the chromatin structure all play roles in gene regulation. Histones are grouped into five major families: H1/H5, H2A, H2B, H3, and H4. Histones H2A, H2B, H3, and H4 are known as the core histones, as two H2A/H2B dimers and an H3/H4 tetramer comprise the nucleosome core. Histones H1 and H5 are considered linker histones. The structure of histone proteins is highly evolutionarily conserved, and all histones have highly unstructured positively charged N-terminal domains known as histone tails where the bulk of histone post-translational modifications occur (186-190).

Histones have been postulated to regulate gene expression in several ways. First, histone density plays a role, as active genes tend to have less bound histone, while inactive genes are highly associated with histones. Secondly, post-translational modifications on histone tails are associated with both higher order chromatin structure, which is generated from folding and packaging of nucleosomes, and regulation of gene transcription. Alteration of chromatin structure and compaction can regulate the accessibility of a DNA sequence to transcriptional regulatory proteins. Histone modifications can also serve as binding sites for recruitment of transcription factors and transcriptional machinery, including other histone modification enzymes, which further remodel the chromatin to promote or inhibit transcription. More recently, specific histone modifications have come to be associated with particular gene regulatory regions, such as promoter elements or enhancers. It has been proposed that these histone modifications form a “histone code”, wherein “multiple

histone modifications, acting in a combinatorial or sequential fashion on one or multiple histone tails, specify unique downstream functions" (187, 191). Functional understanding of most histone modifications is currently still lacking, and most experimental data are focused on the functional consequences of specific prominent histone modifications with known catalyzing enzymes that can be experimentally manipulated.

Histone modifications participate in processes such as regulation of transcription, DNA damage, and cell division. Most of the well-studied histone modifications have been associated with regulation of transcription. Acetylation of histones H3 and H4, trimethylation of histone H3 lysine 4 (H3K4me3), and trimethylation of histone H3 lysine 36 (H3K36me3) in particular, are associated with active transcription. Aceytlation of histone tails by lysine acetylases neutralizes the positive charge on histone tails, reducing the interactions between histones and negatively charged DNA. This results in a more relaxed chromatin structure, which is more accessible to the transcriptional machinery. Similarly, deacetylation of histones H3 and H4 by lysine deacetylases such as HDACs correlate with a more condensed and inaccessible chromatin state, and is associated with transcriptional repression. H3K4me3 is associated with the promoters of active genes, although the precise function of this histone modification at the promoter region is unclear (192-194). H3K36me3 is associated with the gene body of actively transcribed genes. In yeast, the methyltransferase Set2 (SET domain containing 2) associates with elongating RNA pol II and deposits H3K36me3 marks on histones as the gene is transcribed. H3K36me3 marks then recruit the Rpd3 histone deacetylase complex to remove acetyl marks from surrounding histones to repress spurious transcription (195-197).

Histone modifications strongly correlative with transcriptional repression include trimethylated histone H3 lysine 27 (H3K27me3) and trimethylated histone H3 lysine 9 (H3K9me3). H3K27me3 is deposited by PRC2 (polycomb repressive complex 2), and is a clear mark of transcriptionally silent chromatin (198, 199). H3K27me3 is further

recognized by other repressive chromatin remodeling factors, which aid chromatin compaction in the genomic vicinity. H3K9me3 is catalyzed by the histone-lysine N-methyltransferase SUV39H1 in humans (200, 201). It is a well-characterized marker of heterochromatin and strongly associated with gene repression. Similar to H3K27me3, H3K9me3 also serves as a binding site for further recruitment of repressive chromatin remodeling proteins.

Summary and aims

As a major regulator of normal growth and metabolism, GH carries out many of its diverse physiological functions by the specific and coordinated expression and repression of a multitude of target genes (19). One of the mechanisms by which GH regulates the expression of these target genes is the regulation of GH-responsive transcription factors. Much of the work on how GH regulates transcription has focused on how GH induces gene expression through the activity of GH-regulated transcription factors such as Stat5. As a careful balance of gene activation and repression needs to be maintained to ensure proper function of biological processes, the goal of this thesis was to investigate mechanisms by which GH could repress or downregulate gene expression in order to fill in this gap in understanding.

In Chapter 2, the identification of the transcription repressor Bcl6 as a novel GH target gene (41) opened up new avenues for understanding mechanisms by which GH could inhibit gene expression. As Bcl6 is a novel GH target gene, it was of interest to investigate whether or not Bcl6 was also a novel GH-regulated transcription repressor, and if it had GH-specific functions in adipocytes. In Chapter 3, high-throughput sequencing techniques and genome-wide bioinformatics analyses were utilized to identify candidate Bcl6 target genes in adipocytes. Intriguingly, the GH-regulated transcription factors Bcl6 and Stat5 share a similar DNA binding consensus sequence, suggesting that

they may function as reciprocal regulators of GH-mediated gene transcription. Chapter 4 describes the identification and characterization of GH target genes regulated by both Bcl6 and Stat5 using the same DNA regulatory region using genome-wide Bcl6 and Stat5 occupancy profiles generated in Chapter 3. The molecular mechanisms involved in regulation of dual Bcl6 and Stat5 target genes in response to GH was also investigated, with a focus on the functional contributions of the coregulators p300 and Hdac3 on Bcl6- and Stat5-mediated transcriptional regulation of these dual Bcl6/Stat5 target genes. Chapter 5 discusses a proposed integrated model of transcription regulation for dual Bcl6 and Stat5 target genes in response to GH, and speculates on other GH-regulated transcription factors and nuclear proteins that may also participate in this process. Together, these studies describe a novel repressor-activator relationship in GH-regulated gene transcription, and provide insight into molecular mechanisms by which target genes can be differentially regulated by the same transcription factors in response to the same environmental stimulus.

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Chapter 2

Identification of Bcl6 as a novel GH-regulated transcriptional repressor: Bcl6 and Stat5 reciprocally regulate Socs2 gene expression*

Introduction

Sequencing of the human genome has provided new tools for addressing long-standing questions regarding transcription regulatory events. Whereas GH has long been recognized as a key regulator of growth and metabolism, new questions continually arise as to how it modulates cellular function. We have made considerable progress in understanding how GH signals through the GH receptor and its associated tyrosine kinase Janus kinase-2 (1, 2) and have achieved some knowledge of how GH regulates gene transcription, through analysis of well-studied GH target genes such as those encoding Igf1, C-fos, Spi2.1, Cyp2, and Cyp3 family members (3, 4, 5, 6, 7, 8, 9, 10). However, to understand the diverse and complex events that are modulated by GH, it is informative to gain a global perspective of how GH regulates target genes and how GH target genes relate to physiological responses to GH.

GH is a major promoter of postnatal longitudinal growth. In addition, GH antagonizes insulin action as part of the coordinated mechanisms by which multiple hormones maintain metabolic homeostasis. In conditions of chronic GH excess, development of insulin resistance and diabetes have long been recognized (11, 12, 13). The signaling

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Chen Y*, Lin G*, Huo JS, Barney D, Wang Z, Livshiz T, States DJ, Qin ZS, Schwartz J. (2009) Computational and functional analysis of growth hormone (GH)-regulated genes identifies the transcriptional repressor B-cell lymphoma 6 (Bcl6) as a participant in GH-regulated transcription. Endocrinology150:3645-3654. *Co-first authors.

molecule phosphoinositide 3′ kinase (PI3K) has been implicated in GH-induced insulin resistance (14, 15), and the gene encoding its regulatory subunit p85 α (PI3K p85α) is induced by GH in adipocytes (15, 16). Adipose tissue is one of the most sensitive target tissues for GH and plays a central role in its metabolic actions. In the present study, a profile of GH-regulated genes obtained using GH-treated adipocytes (16) was subjected to deep data mining to gain insight into genes involved in the physiological responses to GH. Through an integrative series of computational analyses, B cell lymphoma 6 (Bcl6), a transcriptional repressor, was identified as a new participant in GH-regulated gene transcription. Functional studies support the role of Bcl6 in transcriptional repression of GH-regulated genes.

The gene profile used for analysis was obtained from microarray data sets from 3T3-F442A adipocytes treated with GH for 48 h, a time when GH induces insulin resistance, or for 4 h or 30 min for insight into triggering events. Genes were induced or inhibited by GH in time-dependent waves associated with biological processes regulated by GH. Expression of genes involved in transcription regulation was induced at 30 min, and expression of genes associated with lipid and cholesterol biosynthesis was inhibited at later times tested (48 h) (16). The present analysis used the gene data sets to assess physiological pathways in which the GH-regulated genes are enriched and to identify key regulatory genes, with results pinpointing the genes for suppressor of cytokine signaling 2 (Socs2) and PI3K p85a. Clustering using the model-based Chinese Restaurant Clustering (CRC) algorithm identified the same genes in a cluster of genes highly responsive to GH at 48 h; the cluster also included the gene for Bcl6, a transcriptional repressor, which was potently inhibited by GH. Transcriptional network analysis predicted relationships among the GH-regulated genes and showed expression of several of them, including Bcl6, to be linked to signal transducer and activator of transcription (Stat) 5, an established GH-regulated transcription factor. The computational predictions led to measurements suggesting inverse roles of Bcl6 and Stat5 in regulating the Socs2 gene. Together these analyses identify the transcriptional repressor Bcl6 as a new transcriptional mediator in GH action and suggest that Bcl6 may play general roles in responses to GH and other regulators of metabolism and growth.

Results

Gene Set Enrichment Analysis (GSEA) predicts GH-regulated pathways

For insight into mechanisms by which gene transcription mediates diverse responses to GH, a profile of genes regulated by GH was subjected to deep data mining. The profile identified 225 genes that were stimulated in time-dependent patterns after GH treatment in 3T3-F442A adipocytes as well as 287 genes that were repressed at the same times, as reported previously (16).

GSEA was used to identify pathways that showed overall gene expression changes with GH treatment. GSEA discerns changes between two biological states in the expression levels of sets of genes selected *a priori* in transcriptional profiling experiments. This method has the advantage of evaluating the overall effect of a gene set rather than any single gene, a feature important to studies of complex physiological responses such as growth or metabolic regulation, in which small changes in a set of genes in a pathway might have a larger impact than a greater change in a single gene (17). GSEA was performed on the entire microarray data set of GH-regulated genes in 3T3-F442A adipocytes. Because earlier analysis of the gene profile (16) revealed GH-regulated waves of gene transcription over time, the gene expression data for each time point were analyzed separately. The outcome yielded ranked gene sets at each time, evaluated through their normalized enrichment scores (NES), which indicate the extent that genes within the set are enriched in the study samples. Combining NES score rankings for each pathway at all time points indicated that the top five highly enriched gene sets regulated by GH include (in order) cell growth and death, insulin signaling, apoptosis, Janus kinase-Stat signaling and phosphoinositide signaling. These gene sets are consistent with reported biological processes and physiological functions regulated by GH (2, 12, 13, 35). Another gene set that was relatively highly ranked was Toll-like receptor signaling; gene sets that were inhibited include pathways for tyrosine metabolism and glutathione metabolism. Because the original samples for microarray were obtained under conditions associated with GH-induced insulin resistance, the pathway for insulin signaling was

examined further. Interestingly, within this pathway, the genes encoding Socs2 and PI3K p85α were the most highly induced after GH treatment for 48 h.

Cluster analysis singles out Bcl6 as a novel gene inhibited by GH

The goal of clustering gene expression profiles is to group genes that display related expression patterns. Hierarchical clustering (36) of the data set of GH-regulated genes studied here had revealed clusters of genes sharing similar temporal patterns of response to GH (16). In the present analysis, a recently developed, model-based clustering approach based on the CRC algorithm (22) was applied to cluster the genes regulated by GH (16). CRC is able to recognize complex correlation patterns such as time-shifted and/or inverted patterns and group them into the same cluster and thus reveals functionally related genes not detected by most other clustering approaches. Inverted patterns are as important as positively correlated patterns because they show activities and regulatory functions of repressive transcription regulators.

Of the 12 clusters that were defined by CRC analysis (Fig. 2.1, left panel), Cluster D was selected for initial analysis because it contains genes showing the greatest fold changes 4--48 h after GH (Fig. 2.1, right panel), consistent with delayed timing of GH responses such as longitudinal growth and insulin resistance (12, 13, 35). The genes include well-studied GH target genes encoding Igf1 (3, 4, 37) and signaling molecules in the SOCS family, Socs2 and Cish (29, 38), as well as PI3K p85α (15). The genes for Socs2 and PI3K p85α were also highlighted in the GSEA pathway analysis. Additional genes stimulated by GH in this paradigm encode the transcription factors activating transcription factor (ATF) 3 and Kruppel-like factor (KLF) 5; signaling molecules repressor of G-protein signaling 16 and interferon-inducible p47 GTPase; the cytokine IL6; and growth arrest and DNA damage inducible-45γ. These genes were induced by GH in adipocytes, and all except IL6 showed highest levels of induction at 4 and 48 h. Importantly, one other gene in the cluster stood out as being the most highly repressed by GH: the gene encoding the transcriptional repressor Bcl6 was inhibited by GH by almost 75%.

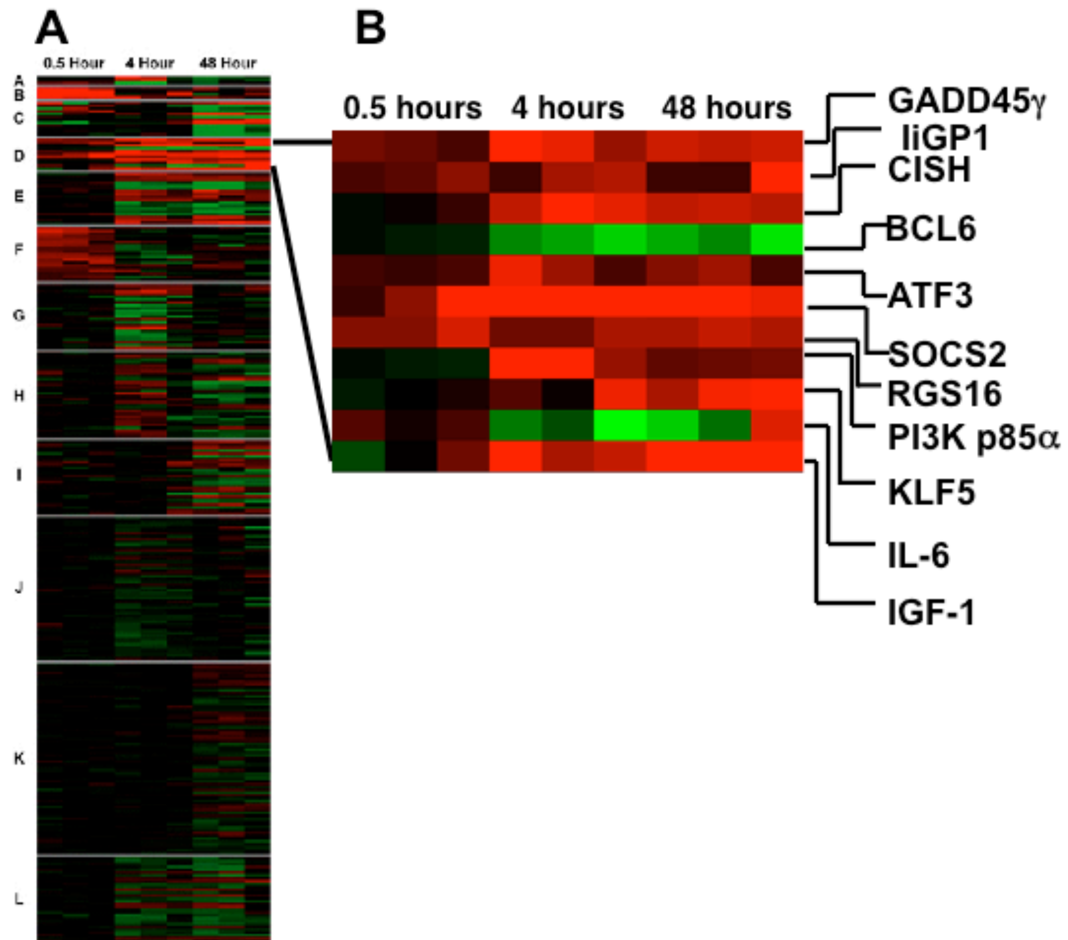


Figure 2.1

CRC analysis of GH-regulated genes clusters coregulated genes. A, Heat map shows results of CRC cluster analysis of a data set of GH-regulated genes. Among the 12 defined clusters, Cluster D contains genes showing greatest differential regulation by GH at 4 and 48 h. Red indicates increase; green indicates decrease. B, Expanded heat map of Cluster D itemizing products of the individual genes. (Data by Y Chen, JS Huo, ZS Qin.)

The expression patterns in response to GH of most of the genes in this cluster, including the dramatic decrease in Bcl6 expression, were confirmed by quantitative real-time PCR of RNA from GH-treated 3T3-F442A adipocytes (Fig. 2.2). The overall changes in mRNA for each gene correspond with microarray findings at 4 or 48 h: Expression of Socs2 and Cish mRNA was the most highly stimulated by GH (50- to 60-fold). Bcl6 expression was inhibited almost 80%. These findings identify the gene encoding the transcriptional repressor Bcl6 as a novel target of GH and suggest that regulation of transcriptional repression plays a role in GH-regulated gene expression.

Transcriptional network analysis highlights Stat 5-regulated genes, including the repressor Bcl6

For an unbiased view of transcriptional relationships among the GH-regulated genes in Cluster D, a transcriptional regulation network (Fig. 2.3) was built for genes in this cluster and their known interacting transcription factors, using the GeneGo database (St. Joseph, MO) with MetaCore software from GeneGo. The network relates the genes submitted with transcription factors that regulate them, as reported in the literature; for genes submitted that encode transcription factors, the target genes that they regulate are also linked. The analysis revealed links for regulation of seven of the genes in the cluster. Prominently, Stat5 emerged as a hub linking four of the genes: Stat5 has been shown to activate expression of the genes for Socs2, Cish, and Igf1 (4, 29, 38). Bcl6, the only gene in the cluster that was repressed by GH, was also linked to Stat5. Several reports have shown Stat5 to be a regulator of Bcl6 expression, either as an inducer or a repressor, depending on cell type (39, 40, 41). The coordination via Stat5 of these four genes, as revealed in the network, suggests that the genes may be coregulated by mechanisms involving Stat5 in response to GH. Genes for KLF5, ATF3, and IL6 appear from the network analysis to be transcriptionally regulated by mechanisms not directly involving Stat5.

Because the genes in Cluster D had similar expression patterns in response to GH over time, it was speculated that they might be regulated by similar transcriptional mechanisms. The binding sites for five transcription factor families were searched in the

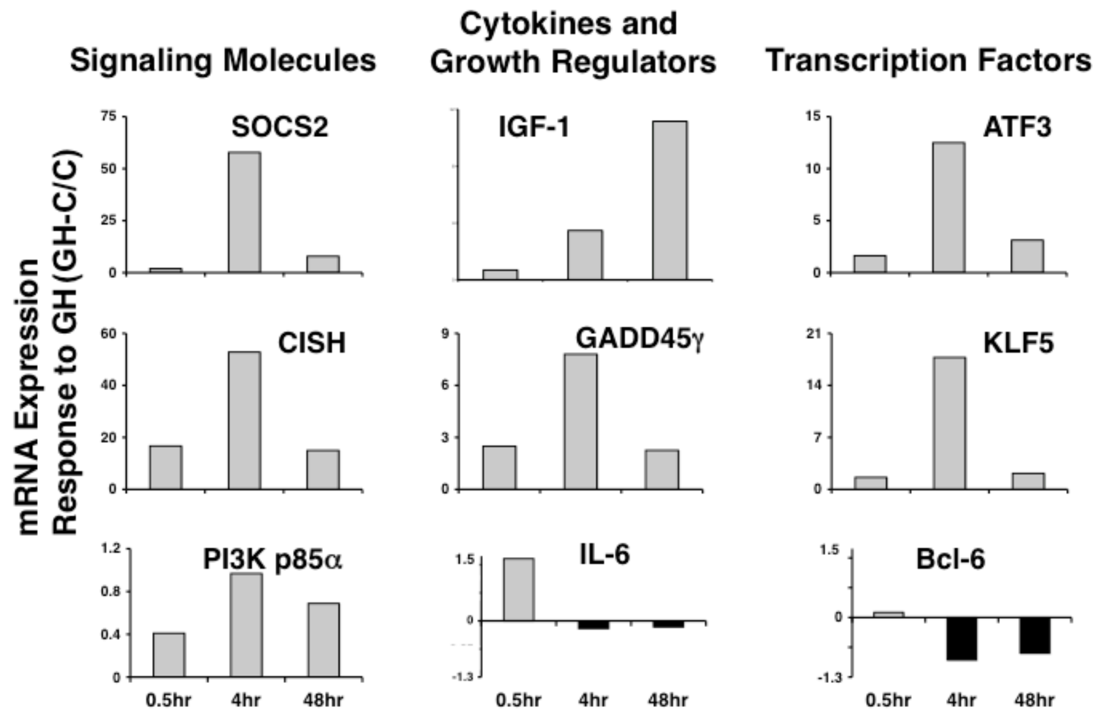


Figure 2.2

Expression of mRNA for representative genes in Cluster D. 3T3-F442A adipocytes were treated with GH for the indicated times; RNA was prepared and analyzed by quantitative real-time PCR. Responses to GH are expressed as (GH-C)/C. Gray bars indicate activation of gene expression in response to GH, black bars indicate repression. Data are representative of two to eight independent experiments. The GH-responsive genes are grouped vertically in general categories related to function. (Data by D Barney, JS Huo.)

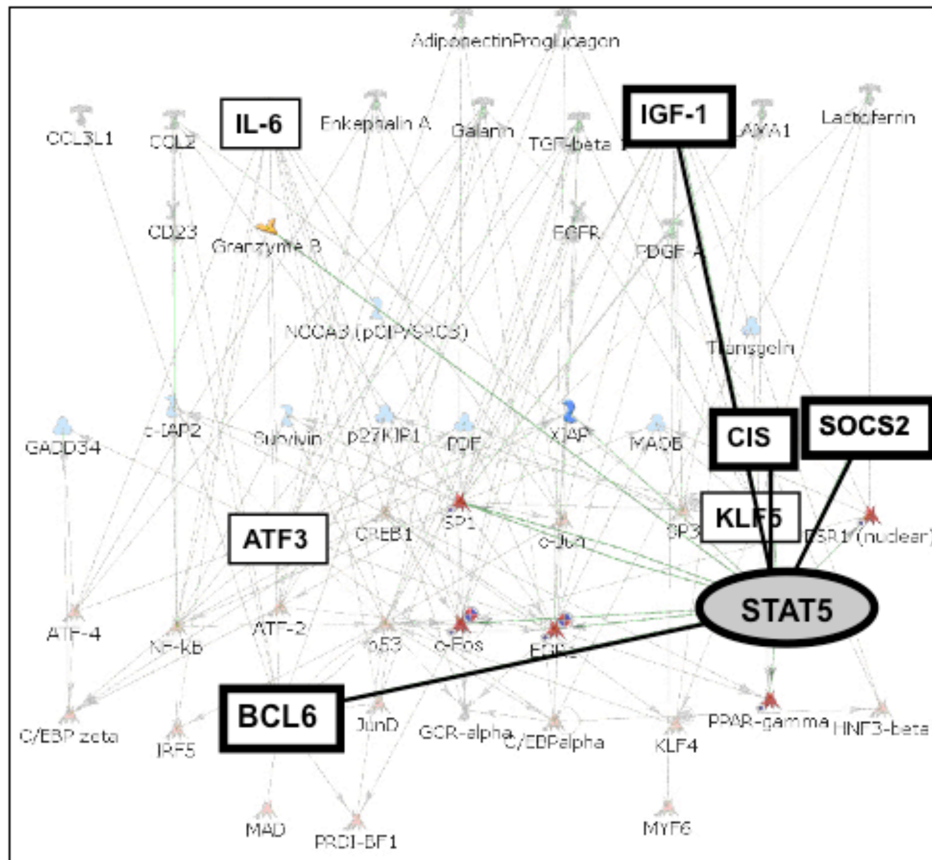


Figure 2.3

Network analysis using GeneGo MetaCore identifies coregulated GH target genes coordinated by Stat 5. Boxes indicate genes submitted; oval designates transcription factor hub. (Data by Y Chen.)

promoter regions of genes in Cluster D (Table 2.1). Conserved sites for Stats and for Bcl6, as well as for CCAAT/enhancer binding protein (C/EBP), KLF/SP1, and ATF family factors, were predicted in almost every gene in the cluster. Stats and C/EBP family proteins are reported mediators of GH-regulated gene transcription (42, 43); ATFs, members of the cAMP response element-binding protein family of transcription factors, are also regulated by GH (44, 45). Bcl6 and KLF/SP1 factors appear to be novel transcriptional regulators of the GH target genes containing these binding sites.

The transcriptional repressor Bcl6 mediates expression of GH target genes

The relationships between Bcl6 and Stat5 suggested by network and promoter analyses raise the possibility that these two transcription factors may be coordinated in regulating the expression of some of the other genes in this cluster. Socs2 expression is not only highly stimulated by GH, but Socs2 protein participates in GH signaling (46, 47). When the most highly stimulated (Socs2) and most highly repressed (Bcl6) genes in the present analysis were compared, the lowest GH concentrations (5 ng/ml) inhibited Bcl6 mRNA and induced Socs2 expression, suggesting an inverse relationship that is highly responsive to GH (Fig. 2.4). Consistent with this, in multiple experiments, inhibition of Bcl6 and induction of Socs2 expression by GH followed a similar time course, with little or no responses at 30 min and substantial responses at 4 or 48 h, in both 3T3-F442A adipocytes and preadipocytes (Fig. 2.5). Furthermore, the endogenous Bcl6 protein was detectable in 3T3-F442A preadipocytes; Bcl6 was also detected in H4IIE rat hepatoma cells (Fig. 2.4, inset).

The inverse regulation of Bcl6 and Socs2 by GH was evaluated further because Socs2 is an established Stat5 target gene. The human Socs2 gene contains a GH-inducible region in the promoter, which contains two GH-responsive Stat5 binding sites (29) (Fig. 2.6). This sequence, fused upstream of thymidine kinase-luc (termed Socs2-luc), was transfected into 293T cells along with a construct encoding GH receptor. Treatment with GH for 24 h stimulated Socs2-luc as expected (Fig. 2.7). However, when Bcl6 was expressed, the activity of Socs2-luc was strongly inhibited (Fig. 2.7, white bars),

Gene	Stat Sites	Bcl6 Sites	Cebp Sites	Klf Sites	Atf Sites
Atf3	**Y** (R)	N	**Y** (H,M,C,R)	**Y** (H,M,C,R)	**Y** (H,M,C,R)
Klf5	Y (H,P,M,R)	**Y** (B,R)	**Y** (H,P,M,C,R)	**Y** (M,P,H,R,C)	**Y** (H,P,M,C,R)
Socs2	**Y** (B,R)	**Y** (B,R)	**Y** (-)	**Y** (B,R)	**Y** (B,R)
Cish	**Y** (H,P,M,C,B,R)	**Y** (H,P,M,C,B,R)	**Y** (H, P)	**Y** (H,P,M,C,B,R)	**Y** (M,R,B)
Pik3r1	**Y** (P, M)	**Y** (H,P,C)	**Y** (-)	**Y** (-)	N
Gadd45γ	**Y** (-)	**Y** (-)	N	**Y** (H,P,M,C,B,R)	N
Igf1	Y (H,M,C,B,R)	**Y** (R)	**Y** (H,M,C,B)	**Y** (-)	**Y** (H,M,C,B)
Iigp1	**Y** (-)	**Y** (-)	**Y** (-)	**Y** (-)	N
Rgs16	**Y** (-)	**Y** (R)	**Y** (-)	**Y** (H,P,R)	N
Bcl6	**Y** (H,P,M,C,B,R)	**Y** (H,P,M,C,B,R)	**Y** (H,P,M,C,R)	**Y** (H,P,M,C,R)	**Y** (-)
Il6	**Y** (M, B)	N	**Y** (H,P,M,C,R,B)	**Y** (H,P,M,C,R,B)	**Y** (H,P,M,C,R,B)

Table 2.1

Predicted transcription factor binding sites in GH-regulated genes. Promoters of murine genes in cluster D were analyzed using Genomatix tools to predict binding sites for the indicated transcription factors. KLF sites were searched as SP1 sites; ATF sites were searched as CREB family sites. Letters indicate that conserved sites are found in orthologs of corresponding species as follows: H: *Homo sapiens*; P: *Pan troglodytes*; M: *Macaca mulatta*; C: *Canis familiaris*; B: *Bos Taurus*; R: *Rattus norvegicus*. Outcomes are expressed as Y = sites were found; Y(-) = sites were found in mouse genes, but the sites were not conserved in any orthologs; N = no sites were found. (Data by Y Chen.)

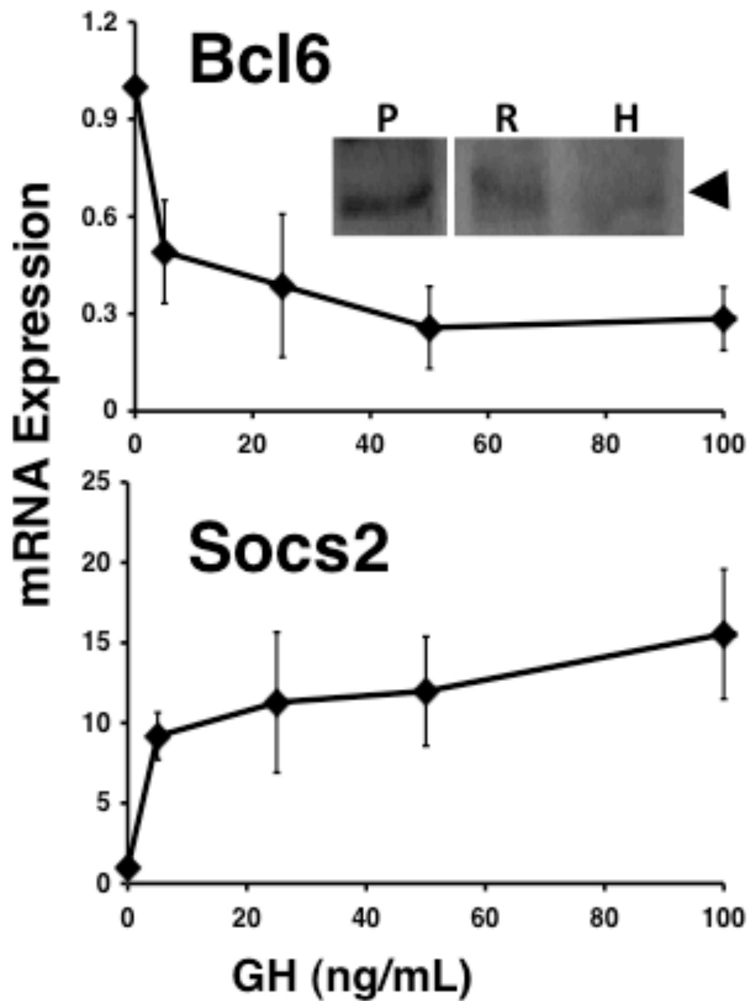


Figure 2.4

Inverse regulation of Bcl6 and Socs2 mRNA in response to GH. Dose response: Comparison of Bcl6 (top) and Socs2 (bottom) mRNA in 3T3-F442A preadipocytes after 48 h incubation with varying concentrations of GH. Data are expressed as fold change due to GH over control (set to 1). Each point shows mean ± SE for three independent experiments. (Data by T Livshiz.) Inset, Bcl6 protein is detected in 3T3-F442A preadipocytes and hepatoma cells. Immunoblotting with anti-Bcl6 demonstrates the presence of Bcl6 protein (arrowhead) in lysates of 3T3-F442A preadipocytes (lane P) and H4IIE hepatoma cells (lane H). Lane R shows Ramos B cells for reference. Lysates used in immunoblotting were from untreated cells for all cell types.

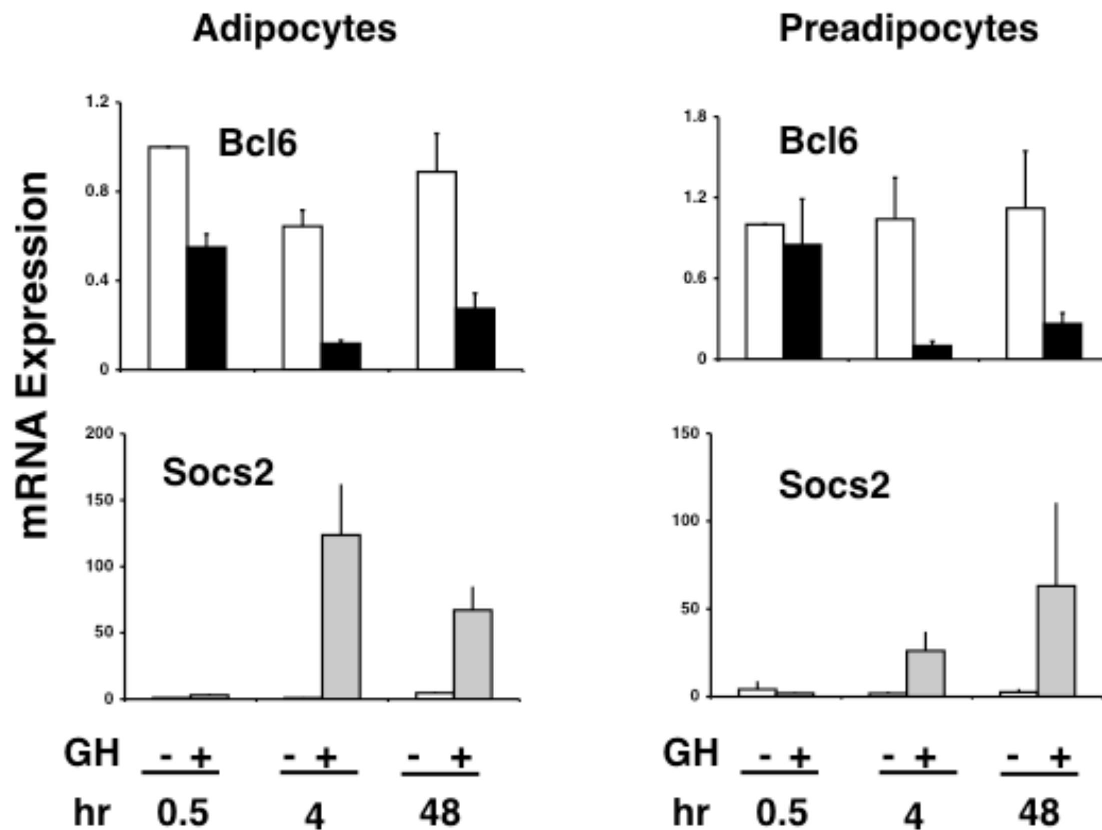
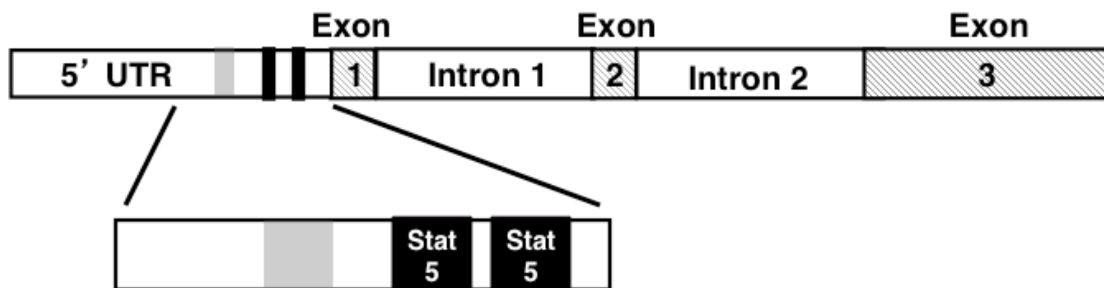


Figure 2.5

Inverse regulation of Bcl6 and Socs2 mRNA in response to GH. 3T3-F442A adipocytes or preadipocytes were incubated without (open bars) or with (filled bars) GH for the times indicated. RNA was prepared and analyzed by quantitative real-time PCR using primers for Bcl6 (top) or Socs2 (bottom), as for Fig. 4. Data are expressed as fold change relative to control (set to 1). Each bar shows mean ± SE (n=5 independent experiments) for adipocytes, mean ± range (n=2) for preadipocytes. Responses to GH in adipocytes were statistically significant ($p < 0.01$) for Bcl6 at 0.5, 4 and 48 hr, and for Socs2 ($p < 0.001$) at 4 hr. (Data by D Barney.)



Stat5 binding consensus: `TTCC(A>T)GGAA`

SOCS2 promoter: `TTCCTGGAA`

Bcl6 binding consensus: `TTCCT(A/C)GAA`

Figure 2.6

Schematic of the human SOCS2 gene. The GH responsive region of SOCS2 is located in the promoter region of the gene. This sequence contains two GH-responsive Stat5 sites (labeled black boxes) and an enhancer box (E-box) site (gray box). Shown below are the DNA binding consensus sequences for Bcl6 and Stat5, as well as the Stat5 consensus sequences found in the SOCS2 promoter. Schematic not to scale. Modified from (29).

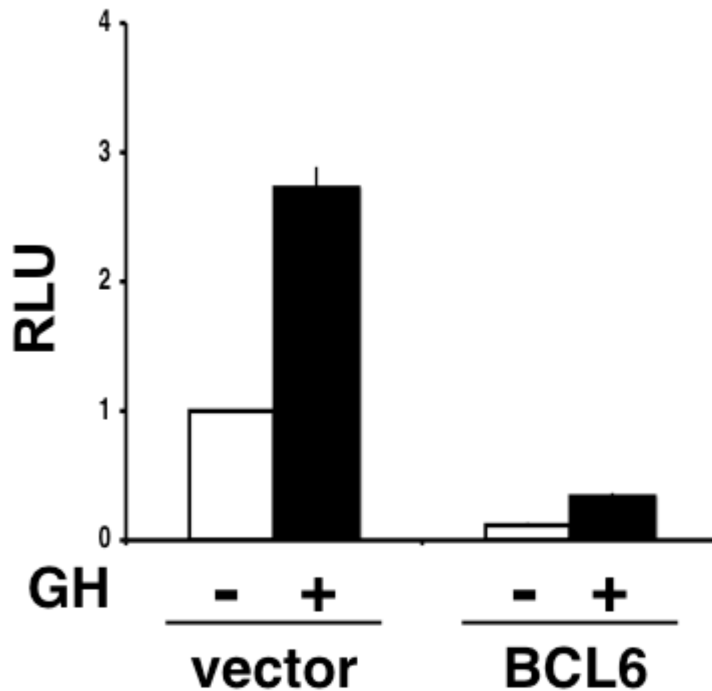


Figure 2.7

Bcl6 mediates expression of GH target genes: Bcl6 inhibits expression of Socs2-luc. A plasmid containing the GH-responsive sequence of the Socs2 gene upstream of luciferase (Socs2-luc), and plasmids for the GH receptor and β-galactosidase were transfected into 293T cells in the absence or presence of an expression plasmid for Bcl6. Cells were treated without (white bars) or with GH (black bars) for 24 h, and luciferase activity was measured, expressed as RLU (relative luciferase units). Bars, mean ± se for observations in six independent experiments. The expression of Socs2-luc in the presence of Bcl6 was significantly ($P < 0.01$) less than in the absence of Bcl6 (compare white bars). The response to GH (compare white and black bars) was significant ($P < 0.01$) in the absence of Bcl6 but was not significant in the presence of Bcl6. Socs2-luc in the presence of GH (compare black bars) in the absence of Bcl6 was significantly ($P < 0.01$) greater than in the presence of Bcl6. (Data by ZN Wang.)

consistent with the role of Bcl6 as a transcriptional repressor. In the presence of Bcl6, GH failed to elicit a significant stimulation of Socs2-luc over the repressed basal activity. Consistent with inverse regulation by GH, Socs2-luc is inhibited by Bcl6, whereas GH, via Stat5, activates it, as expected (29).

Endogenous Bcl6 and Stat5 also show an inverse relationship in occupying endogenous Socs2 DNA, as demonstrated by chromatin immunoprecipitation (ChIP). Because Socs2 is regulated by Bcl6 as well as Stat5, the occupancy of these two factors on the Socs2 GH-responsive sequence was examined *in vivo* by ChIP (Fig. 2.8). Occupancy of endogenous Bcl6 on Socs2 was evident in the absence of GH and decreased progressively after GH treatment; Bcl6 occupancy was almost undetectable 48 h after GH. For Stat5, occupancy on Socs2 was negligible or absent without GH treatment (time 0), but Stat5 occupancy increased after GH. Thus, the occupancy of Bcl6 decreased as that of Stat5 increased. Occupancy of acetylated histone H4 is a positive control consistent with transcriptional activation of Socs2; no occupancy was detected with IgG or beads alone. Together these findings indicate that endogenous Bcl6 occupies the Socs2 gene *in vivo*. The occupancy of Bcl6 on DNA encompassing the GH-responsive sequence of Socs2 decreases with GH treatment, whereas occupancy of Stat5 increases with GH. The inverse relationship between occupancy of Bcl6 and Stat5 on Socs2 DNA is consistent with their respective abilities to repress and activate Socs2.

Discussion

An integrative computational strategy reveals novel aspects of GH-regulated transcription

High throughput technologies such as DNA microarrays provide a global view of the expression patterns of all genes in the genome. This is important for analyzing complex biological processes such as those regulated by GH, including longitudinal growth or insulin resistance, in which it is crucial to understand how genes cooperate to carry out related series of reactions. Despite the fundamental advantages offered by high-

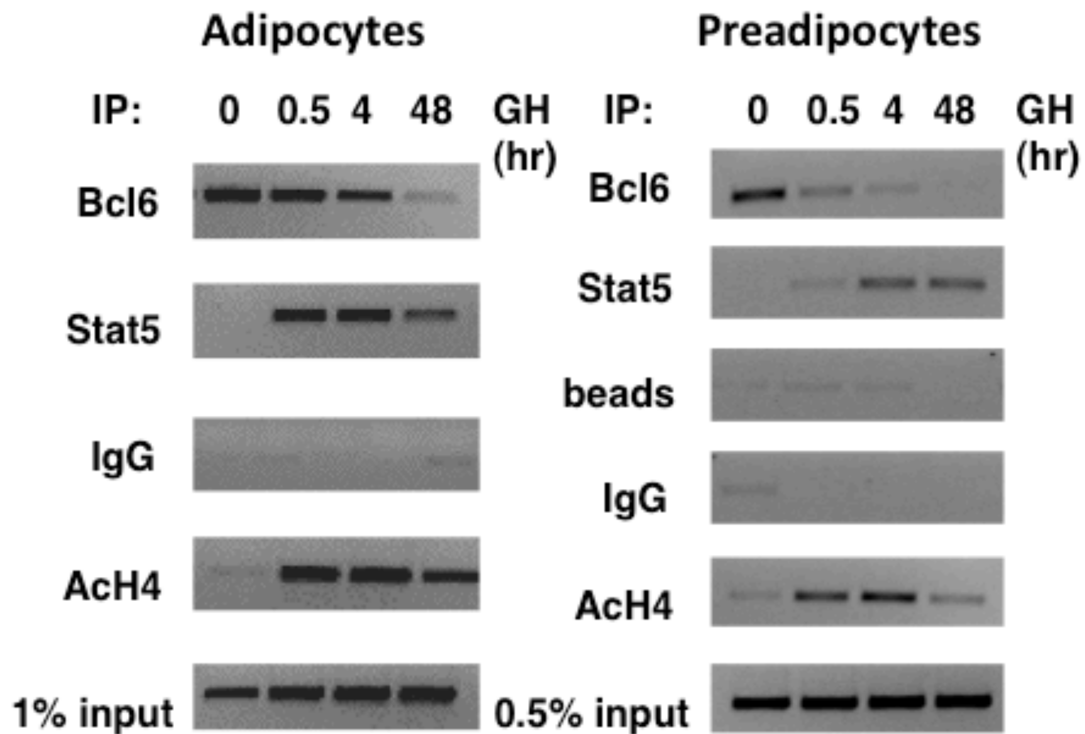


Figure 2.8

Bcl6 and Stat5 occupy Socs2 inversely in response to GH. 3T3-F442A adipocytes or preadipocytes were treated with GH for the indicated times and nuclei were analyzed by ChIP, using antibodies against Bcl6, Stat5, or acetylated histone 4 (AcH4), with primers for the GH-responsive sequence in the Socs2 gene. Samples treated with IgG or no antibody served as controls; 1% or 0.5% input is shown as indicated. Data are representative of over seven independent experiments. Comparable Bcl6 occupancy was seen when immunoprecipitations for Bcl6 were carried out using a different antibody against Bcl6 (Bcl6 C-19, Santa Cruz) (data not shown). IP, Immunoprecipitation.

throughput technologies, a key challenge is how to extract biological insights effectively from massive and noisy data. Powerful computational and statistical data mining tools have been developed and are able to translate the raw and noisy data into new scientific knowledge when applied appropriately. The computational tools used, when applied independently, provide pieces of evidence from different perspectives. Here an integrative computational data mining strategy was used; the combined approaches allow interpretation in the face of limitations inherent in each computational tool. This study demonstrates the effectiveness of a strategy using an array of computational tools to bring fresh insights and provide new clues for understanding complex biological processes regulated by hormones.

Through deep data mining, this study used several distinct computational approaches which reinforced each other to identify Bcl6 as a new player in GH-regulated transcription, and suggest its interplay with Stat5. GSEA focused on functionally important GH target genes, which facilitated identifying a cluster of interest. CRC clustering singled out Bcl6 because it identified Bcl6 among the genes most highly regulated by GH at 4-48 h, despite the fact that Bcl6 was the one gene that was highly repressed, whereas other genes in the cluster were stimulated. Construction of a transcriptional network highlighted the role of Stat5 by linking Bcl6 with Stat5 as well as Socs2 with Stat5, suggesting Socs2 as a possible target gene of Bcl6. Promoter analysis predicted that some GH target genes contain both Stat5 and Bcl6 sites. Thus, the combination of high throughput technologies, integrative use of data mining tools, and statistical analysis, applied to questions in understanding GH transcription and its physiological actions have provided an efficient strategy to improve our understanding of mechanisms of transcriptional repression and their interface with transcriptional activation in the context of GH action.

The transcriptional repressor Bcl6 participates in GH-regulated transcription

This work implicates Bcl6 in GH-regulated transcription and demonstrates that Bcl6 serves as a potent repressor. The transcriptional repression properties of Bcl6 are well

documented (48), based on its established roles in immune function. Bcl6 is a member of the poxvirus and zinc finger/broad-complex, tramtrack and bric-a-brac zinc finger family of proteins. Translocations involving Bcl6 occur frequently in B cell lymphomas. Bcl6 is known for its essential role in B cell differentiation in germinal centers (48, 49). Transcriptional repression by Bcl6 involves a variety of mechanisms: it recruits corepressors such as silencing mediator of retinoid and thyroid receptors (SMRT), nuclear receptor corepressor (NCoR), and Bcl6 interacting corepressor (BCoR) as well as histone deacetylases (50, 51, 52, 53), and it associates with chromatin remodeling complexes and other repressors (54, 55, 56). A potent transcriptional repressor such as Bcl6 has not previously been implicated in GH-regulated transcription. Previous investigation of gene repression by GH showed that Stat5 was modulated by other transcription regulatory molecules such as Forkhead box O1 (FoxO1), and hepatocyte nuclear factor (HNF)-3β, -4α, and -6 (9, 57, 58, 59). It remains to be determined whether Bcl6, and associated transcription regulatory factors, interact in complexes to regulate GH target genes.

Because Bcl6 expression is dramatically inhibited by GH, the consequence of such reversal of repression likely contributes to activation of genes in response to GH. Socs2 is an excellent candidate because Bcl6 occupies Socs2 DNA within a GH-responsive sequence (29), and its occupancy decreases on GH treatment. Furthermore, expression of Bcl6 inhibited Socs2-luc under basal conditions; a small residual increase with GH was not statistically significant. Together these findings are consistent with Socs2 being a new gene target of Bcl6. It can be speculated that in response to GH, Bcl6 functions in coordination with Stat5, which increases occupancy on Socs2 DNA in a pattern reciprocal to the decrease in occupancy of Bcl6 after GH treatment. The consequence of both the increase in Stat5 and the decrease in Bcl6 is to increase expression of Socs2 in response to GH. Thus, Bcl6 and Stat5 may participate in an inverse transcriptional repressor/activator relationship to facilitate expression of Socs2 and perhaps other GH-regulated genes. It is relevant in this regard that the consensus DNA sequences that bind Bcl6 and Stat5 are remarkably similar (60, 61). It is also recognized that reduced availability of Bcl6 protein through its degradation or inactivation may contribute to its

reduced occupancy after GH; Erk-mediated phosphorylation and acetylation of Bcl6 have been reported to result in its degradation or inactivation (62, 63). GH is known to induce phosphorylation via Erk 1/2 and acetylation to activate transcription factors such as C/EBPβ (28, 32) and may similarly modulate other transcription regulatory proteins in the nucleus. Overall, Bcl6 appears to be a highly responsive new player in GH-regulated transcription.

Several studies have implicated Stats, particularly Stat5, in the regulation of Bcl6 gene expression. This regulation may be cell type specific because in a subset of germinal center cells, Stat5 increased expression of Bcl6 (41), whereas in B-lymphoma and other hematopoietic cell lines, Stat5 inhibited expression of Bcl6 (40). Interestingly, Stat3 activation is coordinated with Bcl6 inhibition in regulating Blimp1 in plasma cell differentiation during B cell maturation (64). Furthermore, prolactin, which activates Stat5, induces Bcl6 in pancreatic β-cells (39); Bcl6 in turn represses Menin expression, which allows the β-cell proliferation of adaptive islet growth, as in pregnancy or obesity (39). Another report indicates that prolactin inhibits Bcl6 expression in breast cancer cell lines (65), suggesting that regulation of Bcl6 may vary with cell type. Further analysis of the role(s) of Bcl6 in repressing GH-regulated gene expression will provide new mechanistic insights into GH action.

Contributions of Bcl6 to physiological responses to GH

A novel observation in this study is that the transcriptional repressor Bcl6 is expressed and tightly regulated in adipocytes. Bcl6 is also detected in hepatoma cells, consistent with preliminary findings of Bcl6 mRNA in mouse liver (LaPensee, C., and J. Schwartz, unpublished data). These observations raise possibilities for identifying previously unrecognized functions of Bcl6 in metabolic regulation. Bcl6−/− mice are reported to show growth retardation (66, 67, 68), but their metabolic status has not been described. In a gene profile of human muscle biopsy samples before and after euglycemic insulin clamp studies, the gene for Bcl6 was reported to be repressed by insulin (69). A metabolic role of Bcl6 is further supported in this study by the observed coordination of

the functions of Bcl6 with those of Stat5 in regulating Socs2 because Stat5 has been amply demonstrated to participate in regulation of genes implicated in metabolic regulation and adipogenesis (70, 71, 72, 73). In this regard, it is of note that the original microarray experiments that generated the gene profile analyzed in this study were performed using adipocytes under conditions in which GH can induce insulin resistance (16, 74). The functions of many of the GH-regulated genes, identified by gene ontology categories, correspond with established physiological responses to GH (16). GSEA identified the insulin signaling pathway as one of the major pathways regulated by GH, implicating Socs2 and PI3K p85α. Suggestions that Socs2 contributes to insulin resistance are also reported (34). The present findings that Bcl6 is expressed in adipocytes and highly regulated by GH, which can induce insulin resistance, open new directions for analyzing mechanisms involving Bcl6 and transcriptional repression in regulation of metabolism in conditions such as obesity and diabetes.

Together, these studies demonstrate how systems biology approaches involving integrative computational strategies can lead to identification of Bcl6 as a new participant in GH-regulated gene transcription, incorporating a potent and tightly regulated repressor as part of its regulatory mechanisms. Bcl6 is of additional interest because of its potential interplay with Stat5 in regulating target genes; such interactions may have broad relevance in metabolic and growth regulation and in the spectrum of cytokine actions that contribute to immune regulation and overall growth in normal and pathological conditions.

Materials and Methods

Computational analysis

This study analyzes a microarray data set with 561 probe sets showing significant differential expression signals in 3T3-F442A adipocytes treated with GH (500 ng/ml) for 30 min, 4 h, or 48 h (16). Microarray data, based on U74A chip (Affymetrix, Santa Clara,

CA), have been deposited in Gene Expression Omnibus (GSE2120). The data were submitted to the following computational analyses.

Gene set enrichment analysis (GSEA)

To identify pathways in which GH-regulated genes are enriched, GSEA (17) version 1.0 Java desktop application (http://www.broad.mit. edu/gsea/) was downloaded from Broad Institute of Massachusetts Institute of Technology and Harvard (Boston, MA). The analyses were performed separately on the microarray data at each of the three time points. The 175 gene sets used in the analysis were built from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (18, 19, 20). Each gene set is comprised of all of the genes either in one murine KEGG pathway or in multiple murine KEGG pathways belonging to the same category documented by KEGG (http://www.genome.jp/kegg/pathway. html). For example, the cell growth and death category includes the pathways for cell cycle, apoptosis, and p53 signaling. The microarray data were normalized using robust multichip average (21) and then analyzed using GSEA, with the maximum gene set size of 500 and the minimum gene set size of 25; the other parameters were set as default. Outcomes were evaluated as normalized enrichment scores (NES).

Gene expression profile clustering

To examine patterns among highly regulated genes, the CRC algorithm 1.0 (22), a model-based algorithm capable of clustering genes displaying complex correlations (e.g. time shifted and/or inverted), was downloaded (http://www.sph.umich.edu/csg/qin/CRC). The CRC algorithm automatically infers the number of clusters and assigns cluster membership. CRC also ranks predicted clusters and their members. The settings for CRC clustering were selected to allow clustering of inverted, but not time-shifted correlations, due to the limited number of time points.

Transcriptional network analysis

To evaluate transcriptional relationships among genes highly responsive to GH, a transcriptional network was built using the GeneGo MetaCore database and tools for the cluster of genes (murine), which were strongly regulated by GH at 4 and 48 h (cluster D), except for interferon-inducible p47 GTPase, which was not available in the GeneGo database.

Promoter analysis

Conserved transcription factor binding sites of interest were predicted by scanning the promoters of the genes in the cluster showing a similar pattern of responses to GH at 4 and 48 h (cluster D), using Genomatix tools. The orthologs for six species (Homo sapiens, Pan troglodytes, Macaca mulatta, Canis familiaris, Bos Taurus, Rattus norvegicus) of the analyzed mouse genes were retrieved from the Genomatix (Munich, Germany) genome annotation system ElDorado. The Genomatix optimized promoter length was used when available; otherwise a default region of −500 bp upstream of first transcription start site and +100 bp downstream of last transcription start site was used. The promoter sequences of orthologs were aligned with Genomatix DiAlign and the binding sites were predicted with Genomatix MatInspector. The associated motif matrices of Stat5 and Bcl6 contained in Genomatix MatBase were used for query.

Physiological analyses

Gene expression

Murine 3T3-F442A adipocytes or preadipocytes were incubated with GH for various times. RNA was isolated and analyzed by quantitative real-time PCR as described previously (16). Published primer sequences were used for Atf3, Gadd45γ, Gapdh (16); Socs2 (23); Cish, Bcl6 (24); KLF5 (25), and Igf1 (26). The primers for Pik3r1 were designed using Primer Express software (Applied Biosystems, Foster City, CA): 5’-

CGTGGCACAGACTTGGTGTTT-3’ (sense) and 5’-GGCAGTGCTGGTGGATCC-3’ (antisense). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase, which was constant between experimental groups, and responses to GH are expressed either as (GH-control)/control, or as fold change, where control = 1.

Immunoblotting

Lysates of 3T3-F442A preadipocytes (from H. Green; Harvard University, Boston, MA) and H4IIE rat hepatoma cells (from J. Messina; University of Alabama, Birmingham, AL) were analyzed by immunoblotting with anti-Bcl6 (C-19) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) as described (27). Lysates from Ramos B cells [from R. Kwok (University of Michigan, Ann Arbor, MI) and W. Dunnick (University of Michigan, Ann Arbor, MI) served as positive controls because they typically express Bcl6. The proteins were visualized using IRDye 800-coupled antirabbit IgG (1:12000; Rockland Immunochemicals, Gilbertsville, PA) on an Odyssey infrared scanning system (LI-COR Biosciences, Lincoln, NE). Molecular weight was estimated using protein molecular weight standards from Invitrogen (Carlsbad, CA).

Transcriptional activation

293T cells (28) were transfected by calcium phosphate coprecipitation with a Socs2-luciferase (luc) plasmid in which the GH-responsive sequence of the human Socs2 promoter drives thymidine kinase-luc (29), in the presence of the plasmids CMV-human Bcl6 (30) or pcDNA3. Cells were additionally transfected with plasmids for rat GH receptor (31) and RSV-β-galactosidase for normalization. Eighteen hours later, cells were deprived of serum by replacing it with 1% BSA, incubated with human GH (500 ng/ml = 22 nm; Eli Lilly, Indianapolis, IN)) for 24--26 h and used to measure luciferase activity as described previously (32). Data were analyzed by ANOVA with Bonferroni correction for multiple comparisons, using Prism (GraphPad, La Jolla, CA).

Chromatin Immunoprecipitation (ChIP)

3T3-F442A adipocytes or preadipocytes were treated with GH (500 ng/ml) for the indicated times. Lysates of cross-linked cells (input) were used for ChIP as described (33) using anti-Bcl6 (N-3), anti-Stat5 (C-17) (Santa Cruz), antiacetylated histone H4 (AcH4, Millipore, Billerica, MA), or normal rabbit IgG (Santa Cruz). The anti-Stat5 antibody used recognizes both Stat5a and 5b. The term Stat5 is used herein to refer to both Stat5a and Stat5b, unless a distinction between them is pertinent. Primers targeting the binding site for Stat5 in murine Socs2 were based on published sequences (29).

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Chapter 3

Identification of novel Bcl6 target genes and potential roles for Bcl6 in GH signaling by high throughput ChIP-Sequencing

Introduction

Bcl6 is a potent transcriptional repressor best known for its role in the immune system, where it functions as a key regulator of Bcl6 differentiation (1-4). Bcl6 expression is high in germinal centers as developing B cells undergo somatic hypermutation and class switch recombination, and Bcl6 expression must be turned off for B cells to exit the germinal center stage and undergo terminal differentiation. Mechanisms by which dysregulation of Bcl6 results in pathologies of the immune system, such as B or T cell lymphoma, have long been a target of study.

A microarray analysis carried out in cultured 3T3-F442A adipocytes revealed Bcl6 to be the gene most strongly inhibited in response to GH at later time points (4 h and 48 h), when adipocytes show phenotypes consistent with insulin resistance (5, 6). This suggested a novel role for Bcl6 in GH function, and possibly in the pathogenesis of diseases such as insulin resistance and diabetes. Bcl6 expression had not previously been described in adipocytes, and it was unknown what physiological relevance Bcl6 might have in GH and adipocyte specific functions such as regulation of metabolism or adipocyte differentiation. As demonstrated in Chapter 2, further experiments showed that not only is Bcl6 a novel GH-regulated target gene, the Bcl6 protein itself also functions as a transcriptional repressor of the GH target gene Socs2, further suggesting that Bcl6 is a mediator in GH-regulated transcription and GH signaling (6).

To gain insight into the roles that Bcl6 may play in GH action, a high throughput sequencing approach was utilized to identify potential Bcl6 target genes across the genome. ChIP-Sequencing, also known as ChIP-Seq, is a high throughput sequencing technique where chromatin immunoprecipitation (ChIP) is combined with massively parallel DNA sequencing to identify DNA-protein interactions across the genome (7, 8). To map global binding sites for Bcl6 and Stat5 in the presence or absence of GH, ChIP was carried out in highly GH-responsive 3T3-F442A adipocytes treated with GH for 0 or 48 hours. Antibody against Bcl6 or Stat5 was used to immunoprecipitate chromatin fragments associated with these transcription factors, and the ChIP DNA was used to generate a sequencing library comprised of Bcl6 or Stat5 target sites in vivo. The ChIP-Sequencing libraries were then sequenced, and analysis of the sequence output with mouse whole-genome sequence databases yielded regions where Bcl6 or Stat5 signal is enriched on a genomic sequence in either the presence or absence of GH. These genomic regions of enriched ChIP-Sequencing signal for Bcl6 and Stat5 represent potential Bcl6 or Stat5 binding sites *in vivo*, and the genes closest to the putative binding sites are genes with a high likelihood of being regulated by either Bcl6 or Stat5. While the development of the ChIP-Sequencing technique is fairly recent, this versatile approach has already been used to characterize genome-wide binding sites for several key transcription factors and the corresponding chromatin landscape for important biological processes such as growth factor response (9, 10), differentiation of adipocytes and other cells (11), and embryonic development (12, 13). Results from such studies have also provided much insight on transcriptome evolution (14-16), epigenetics (13, 17, 18), and differences in transcription regulatory networks between cell types (19).

Analysis of the Bcl6 and Stat5 ChIP-Sequencing results provided us with maps of potential Bcl6 and Stat5 binding sites in adipocytes in either the presence or absence of GH. From these data, we plan to identify novel Bcl6 target genes. Of particular interest would be the identification and verification of novel GH-regulated Bcl6 target genes, as this would greatly expand our knowledge of how GH represses transcription through Bcl6, and increase our understanding of novel functions for Bcl6 outside of the immune system. As Bcl6 has more recently been described to participate in protecting testicular

germ cells from apoptosis (20), repressing proliferation of pancreatic β cells (21), and mediating sexual dimorphism of gene expression in the liver (22, 23), the ChIP-Sequencing results will be invaluable for comparison with other available or future ChIP-Sequencing datasets, and allow us to computationally assess similarities and differences in Bcl6 or GH action in different tissues, under different treatment conditions, or in different species. Similarly, the Stat5 ChIP-Sequencing profiles could provide us with novel Stat5 target genes, and comparison of Stat5 ChIP-Sequencing profiles from different cell types would provide insight into cell type specific functions of Stat5.

Concurrently, comparison of the Bcl6 and Stat5 occupancy profiles in the presence or absence of GH would also help identify GH target genes reciprocally regulated by Bcl6 and Stat5 in response to GH, similar to what is seen for Socs2 (Chapter 2). This analysis would shed light on whether or not the mechanism of reciprocal regulation of transcription by an activator-repressor pair using the same DNA regulatory region is operant for other GH target genes. Analysis of the downstream biological effects or transcriptional regulatory analysis of genes found to be reciprocally regulated by Bcl6 and Stat5 could provide insight on the types of genes likely to be regulated by this mechanism, and may provide clues as to how such a regulatory mechanism evolved. Interestingly, Ken and Barbie, the Drosophila homolog of Bcl6, also appears to repress expression of a subset of Jak/Stat target genes in the fly (24), suggesting that cooperation between Bcl6 and Stat5 in regulating target genes is an ancient mechanism that has been maintained over the course of evolutionary history. Similarly, identification of genes regulated by both Bcl6 and Stat5, but not necessarily in the same reciprocal manner as Socs2, would help us better understand the opposing roles of Bcl6 and Stat5 in GH response, and the variety of mechanisms used by different genes to modulate transcriptional responses regulated by these two transcription factors.

Results

Bcl6 expression is inhibited by insulin and EGF as well as GH in 3T3-F442A preadipocytes, suggesting a broad role for Bcl6 in adipocyte biology

Previous data showed that Bcl6 is a novel GH target gene in 3T3-F442A adipocytes and preadipocytes, and treatment of cells with GH strongly inhibited Bcl6 mRNA expression at concentrations as low as 5 ng/ml (6). To learn more about mechanisms by which Bcl6 gene expression is regulated in preadipocytes, and identify potential roles for Bcl6 in adipocyte biology, 3T3-F442A preadipocytes were treated with varying concentrations of epidermal growth factor (EGF) or insulin (Figure 3.1) to test for potential regulation of Bcl6 expression under different treatment conditions. Cells were treated with the various factors for 24 h, a time point at which Bcl6 expression is strongly inhibited by GH (6). EGF has been found to play a dual role in proliferation and differentiation of 3T3-L1 preadipocytes to adipocytes, with low concentrations (<1 nM) promoting adipogenesis, while higher concentrations appear to inhibit adipogenesis (25). Insulin and GH are required factors for 3T3-F442A adipocyte differentiation (26-29), and chronic GH treatment of 3T3-F442A adipocytes results in an insulin resistant phenotype in the cells (30, 31). Results showed that mRNA expression of Bcl6 is inhibited by EGF and insulin in 3T3-F442A preadipocytes even at the lowest treatment concentrations, and expression remains repressed to a similar extent with increasing treatment concentrations, suggesting that expression of Bcl6 is strongly regulated by other growth factors and hormones important in adipocyte biology.

Bcl6 is a novel transcriptional repressor of Socs2 gene expression in an animal model

Previous results showed that in addition to being a novel GH-regulated gene, translated Bcl6 protein also functioned as a transcriptional repressor on the GH target gene Socs2 (6). Luciferase reporter gene assays showed that the presence of Bcl6 inhibited expression of a human Socs2-luciferase reporter gene and blunted activation of Socs2-

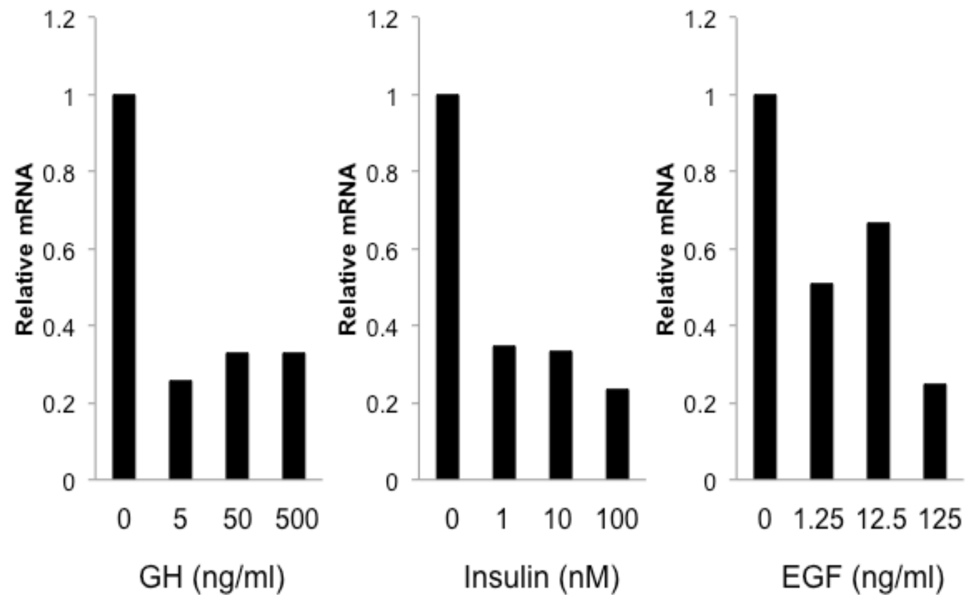

Figure 3.1

Multiple growth factors and hormones regulate Bcl6 mRNA expression in preadipocytes. 3T3-F442A preadipocytes were treated with the indicated concentrations of GH, insulin, or epidermal growth factor (EGF) for 24 h. RNA was harvested and Bcl6 mRNA expression was measured by quantitative real-time PCR. mRNA expression was normalized to Bcl6 expression in untreated cells for each experiment, set as 1.0. Results are from a single experiment with each treatment condition performed once.

luciferase by GH (Figure 2.7). ChIP data showed occupancy of endogenous Bcl6 and Stat5 on Socs2 DNA in both preadipocytes and adipocytes, with occupancy of Bcl6 high in the absence of GH and decreasing after GH treatment. The occupancy pattern of Stat5 on the Socs2 gene was reciprocal to that of Bcl6, with little to no occupancy of Stat5 in the absence of GH and strong and rapid recruitment of Stat5 to the Socs2 promoter upon GH treatment (Figure 2.8).

To verify whether inhibition of Socs2 expression by Bcl6 also occurs in an *in vivo* physiological context, Socs2 mRNA expression was measured in the livers of wild type mice or mice with Bcl6 deficiency (Bcl6 knock out mice) (32) (Figure 3.2). As Bcl6 knock out mice have little to no adipose tissue, another GH-responsive target organ, liver, was used to examine consequences of Bcl6 deficiency in mice. Results showed that basal Socs2 mRNA expression was greatly elevated in Bcl6 knock out mouse liver compared to wild type, consistent with endogenous Bcl6 repressing Socs2 expression in an animal model as well as in cultured 3T3-F442A cells. As the mice had not been subjected to any treatment, these results also suggest that the loss of Bcl6 repression is sufficient to induce some level of Socs2 gene expression above the average basal levels found in wild type mouse liver.

ChIP assays carried out using livers from male wild type (Figure 3.3A, lanes 1 and 2) and Bcl6 knock out mice (Figure 3.3A, lanes 3 and 4) showed that there was occupancy of Bcl6 on Socs2 DNA in wild type mice but no occupancy of Bcl6 on Socs2 DNA in Bcl6 knock out mice, as expected. In the absence of Bcl6, Stat5 and phosphorylated Stat5 occupy Socs2 DNA (Figure 3.3A, lanes 3 and 4), suggesting that the recruitment of activated Stat5 to the Socs2 promoter is not dependent on the prior occupancy of Bcl6. Furthermore, in ChIP carried out on livers from male Bcl6 heterozygous mice treated with GH (Figure 3.3B, lanes 2 and 3) or vehicle (Figure 3.3B, lane 1), occupancy of Bcl6 on Socs2 DNA was lower in the samples from the GH-treated mice compared to the untreated mouse. In contrast, occupancy of Stat5 and phosphorylated Stat5 was higher in samples from GH-treated mice compared to the untreated sample (Figure 3.3B), a pattern that had previously been observed in cultured adipocytes (Figure 2.8). These results

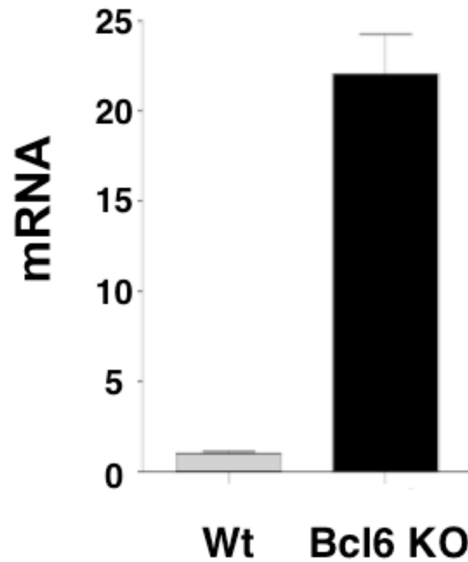

Figure 3.2

Bcl6 represses Socs2 transcription *in vivo*. Endogenous Socs2 mRNA expression was measured by quantitative real-time PCR in livers of male wild type (Wt, gray bar) or Bcl6 knock out mice (Bcl6 KO, black bar). mRNA expression, calculated as fold-change for Bcl6 KO compared to WT, is shown as the mean + SE for 4 mice of each genotype. Socs2 mRNA was significantly increased ($p<0.05$) in Bcl6 knock out compared to wild type. (Data by C LaPensee, in collaboration with AL Dent, Indiana Univ.)

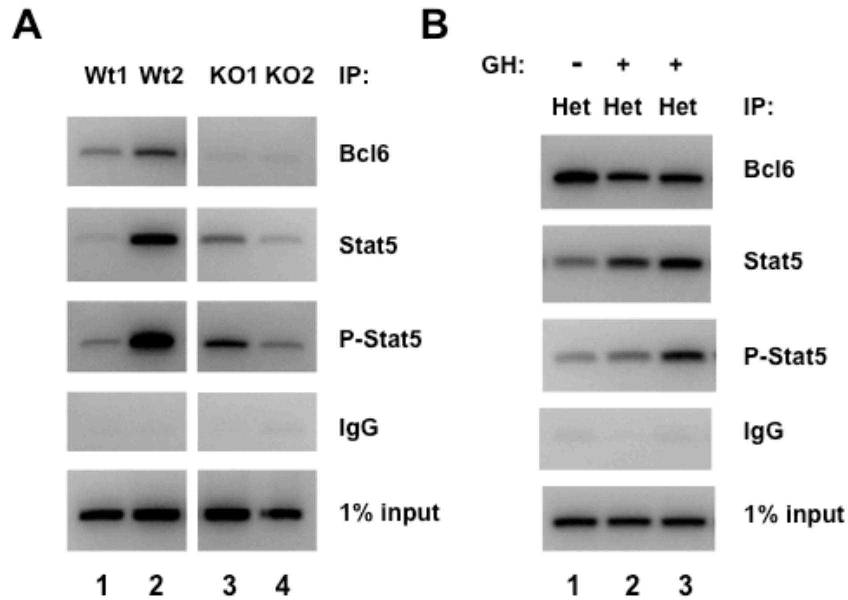

Figure 3.3

Bcl6 and Stat5 occupy Socs2 DNA *in vivo*. A. ChIP was carried out on livers of male wild type (Wt, n=2) or Bcl6 knock out (KO, n=2) mice. Immunoprecipitation was carried out using antibody against Bcl6, Stat5, or phosphorylated Stat5 (P-Stat5), and samples were analyzed with primers for the Bcl6/Stat5 binding site in the Socs2 gene. Samples immunoprecipitated with IgG served as negative controls; 1% input is shown as indicated. **B.** ChIP was carried out on livers of male Bcl6 +/- mice (Het) treated with (n=2) or without (n=1) GH as indicated. Immunoprecipitation was carried out using antibody against Bcl6, Stat5, or phosphorylated Stat5 (P-Stat5), and samples were analyzed with primers for the Bcl6/Stat5 binding site in the Socs2 gene. Samples immunoprecipitated with IgG served as negative controls; 1% input is shown as indicated.

suggest that reciprocal occupancy of Bcl6 and Stat5 on Socs2 DNA occurs in a physiological animal model as well as in cell culture systems, and supports the hypothesis that Bcl6 plays important and as yet unknown roles in GH biology.

High throughput ChIP-Sequencing identifies Bcl6 and Stat5 occupancy across the genome

As cell culture and animal models both support biological roles for Bcl6 in GH action and in non-canonical Bcl6 target tissues outside of the immune system, we decided to use a high throughput sequencing approach to identify novel Bcl6 target genes to learn more about the functions of Bcl6 in GH signaling and actions. ChIP-Sequencing is a technique that combines the specificity of ChIP for identifying DNA-bound transcription factors with massively parallel sequencing to provide highly sensitive, genome-wide identification of DNA associated with transcription regulatory proteins (7, 8).

3T3-F442A adipocytes were treated with or without GH for 48 hours and ChIP was carried out using antibody against Bcl6 or Stat5. ChIP-Sequencing libraries were prepared from the resulting ChIP DNA using the Illumina ChIP-Seq sample prep kit, and samples were sequenced by the UM Sequencing Core using a Solexa/Illumina Genome Analyzer (Figure 3.4). Input genomic DNA from untreated adipocytes was sequenced as a normalization control to identify enriched regions of sequencing signal across the genome. As there was difficulty in obtaining sufficient starting material, ChIP-Sequencing libraries for immunoprecipitation background controls from samples immunoprecipitated with IgG were not generated. A total of two separate ChIP-Sequencing runs were carried out, and the sequence read data from both runs were combined to form a single dataset that was used in subsequent analyses. The ChIP-Sequencing dataset was analyzed with the H-Peak algorithm (33) to identify locations of Bcl6 or Stat5 occupancy across the mouse genome. H-Peak identified over 3000 potential binding sites for Bcl6 and at least 900 for Stat5 across the genome (Table 3.1). In particular, the number of potential locations of Bcl6 occupancy across the genome

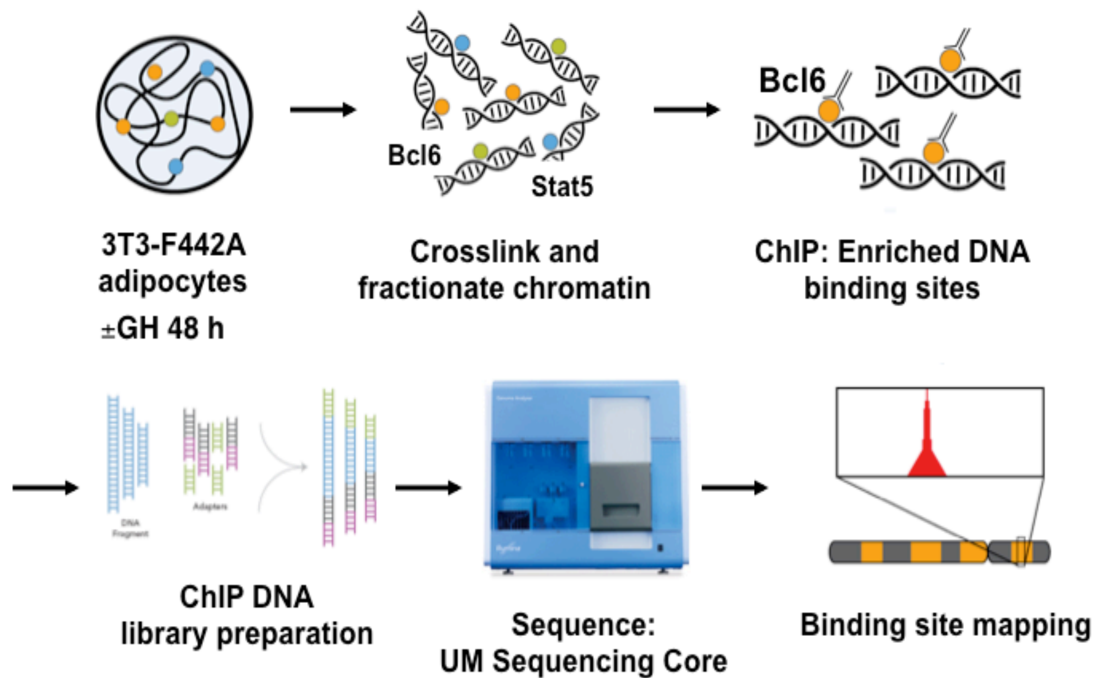

Figure 3.4

Schematic of ChIP-Sequencing experiment design. 3T3-F442A adipocytes were treated with GH for 0 or 48 h. Immunoprecipitation was carried out using antibodies against Bcl6 or Stat5. ChIP samples were made into ChIP-Sequencing libraries using the Illumina ChIP-Seq sample prep kit, and Solexa sequencing was carried out by the University of Michigan Sequencing Core. A library sample of input genomic DNA was used as a background control. ChIP-Sequencing results were analyzed by the H-Peak algorithm to identify enriched peaks of Bcl6 or Stat5 signal across the mouse genome (H-Peak analysis by ZS Qin.)

GH (time)	IP (antibody)	Regions of occupancy (normalized to input)	Motifs
0 h	Bcl6	3777	Stat, Bcl6
48 h	Bcl6	1549	Stat, Bcl6
0 h	Stat5	1329	(Stat)
48 h	Stat5	937	Stat

Table 3.1

Summary and analysis of ChIP-Sequencing results. ChIP-Sequencing was performed using 3T3-F442A adipocytes treated with GH (48 h) or without GH (0 h). Immunoprecipitation was carried out using antibodies against Bcl6 or Stat5 as indicated. Samples were analyzed as shown in Figure 3.4. ChIP-Sequencing results were analyzed using the H-Peak algorithm and results from 2 independent ChIP-Sequencing experiments were combined for bioinformatic analysis. Shown are the number of regions enriched for Bcl6 or Stat5 occupancy across the genome as identified by H-Peak under different GH treatment conditions. The Bcl6 and Stat binding consensus motifs were found to be enriched and highly ranked in the output sequences generated by ChIP-Sequencing. The Stat binding consensus motif was less highly ranked in the 0 h GH treatment Stat5 immunoprecipitation sequencing results compared to the other conditions, and is indicated in parentheses. (Analysis by ZS Qin.)

decreased in response to GH treatment, consistent with the repression of Bcl6 expression upon GH treatment.

In both ChIP-Sequencing runs, a peak of Bcl6 signal was mapped near the promoter of the Socs2 gene, consistent with previous ChIP results (Figure 3.5). Motif analysis carried out on the sequencing data showed Bcl6 and Stat5 motifs were highly ranked among the sequences for the 0 hr GH Bcl6 immunoprecipitation data and the 48 hr GH Bcl6 immunoprecipitation data (Table 3.1). Stat motifs were found to be highly ranked in 48 hr GH Stat5 immunoprecipitation data, and were listed among common transcription factor binding motifs identified in the 0 hr GH Stat5 immunoprecipitation data, consistent with experiment design and expected results (Table 3.1).

Bioinformatic analysis of ChIP-Sequencing results identifies potential novel Bcl6 target genes

A goal of the ChIP-Sequencing approach was to obtain information on novel Bcl6 target genes and novel GH-regulated Bcl6 target genes. Comparisons of the genomic occupancy profiles between the Bcl6 immunoprecipitation data and the Stat5 immunoprecipitation data would also identify genes that are potentially regulated by both Bcl6 and Stat5, similar to what had previously been seen for Socs2 (Figure 2.7, Figure 2.8). Gene profiles of the known Bcl6 occupancy site on the Socs2 gene and motif analysis of the sequencing data supported that the ChIP-Sequencing results were biologically reliable (Table 3.1, Figure 3.5). Therefore, the ChIP-Sequencing datasets were subjected to bioinformatics analyses to mine for potential functions of Bcl6 in GH action, and mechanisms by which regulation of Bcl6 target genes in response to GH occurs. Novel Bcl6 target genes of particular interest would be genes involved in GH signaling pathways, GH-regulated metabolic pathways, and adipocyte differentiation, as these would be examples of previously unknown non-immune functions of Bcl6 in GH action.

To identify genes that were likely to be regulated by Bcl6, the 0 h GH Bcl6 immunoprecipitation dataset was analyzed as Bcl6 occupancy across the genome was

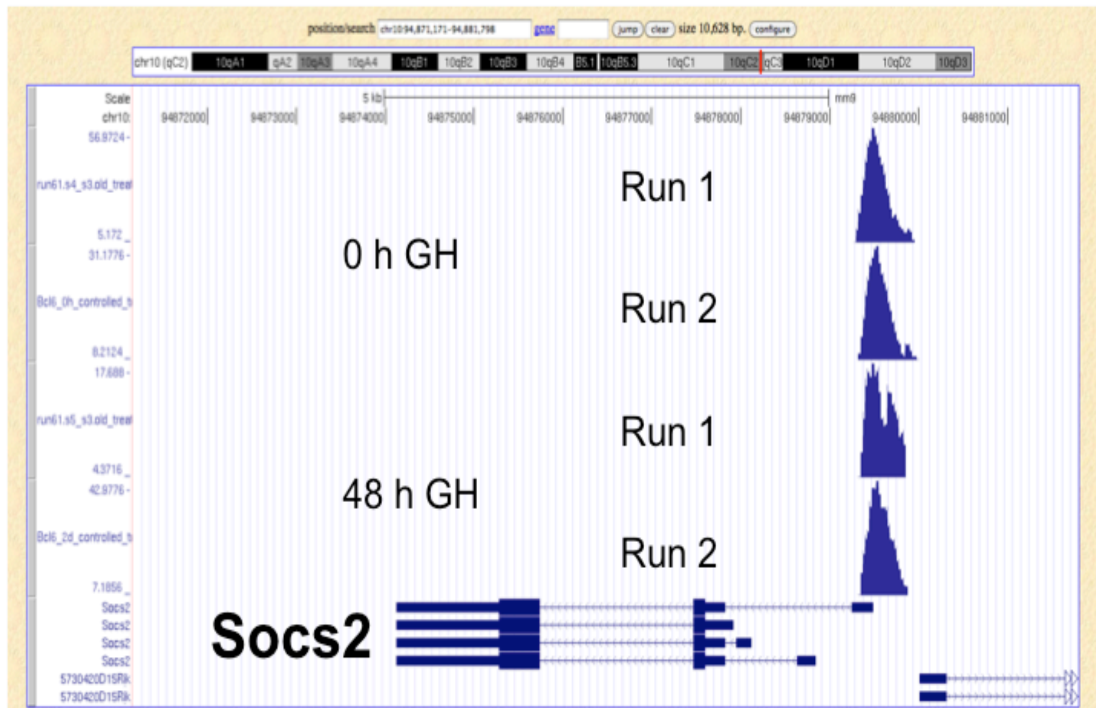

Figure 3.5

Distribution of Bcl6 signal on Socs2. Two separate ChIP-Sequencing runs were carried out. A peak of Bcl6 occupancy was identified at the transcription start site of the Socs2 gene in both runs in the presence and absence of GH, corresponding with previous ChIP results (Figure 2.8). ChIP-Sequencing peak profiles were visualized using the UCSC Genome Browser.

predicted to be higher in the absence of GH based on previous observations (6). Genes that contained a peak of Bcl6 binding identified by ChIP-Sequencing and H-Peak analysis located within 10 kilobases of the transcription start site were considered top candidate Bcl6 target genes. These genes were subjected to Gene Ontology analysis to evaluate biological functions of potential novel Bcl6 target genes. Results showed that high percentages of candidate Bcl6 target genes are involved in cellular processes such as growth, differentiation, metabolism, and signaling (Figure 3.6). Further experimental evaluation of whether or not these genes are bona fide Bcl6 target genes and the transcriptional mechanisms by which expression of these genes is regulated by GH and Bcl6 will greatly increase our knowledge of transcriptional mechanisms in general and further our understanding of the novel roles of Bcl6 in cellular physiology. It would also be interesting to compare this list of potential Bcl6 target genes with known Bcl6 target genes in immune cells (34-36), as this would provide insight into similarities and differences in the types of genes regulated by Bcl6 in different tissues.

To identify genes that are likely to regulated by Bcl6 in response to GH, the list of genes that contained a peak of Bcl6 binding identified by ChIP-Sequencing within 10 kilobases of the transcription start site in the absence of GH was analyzed in conjunction with a list of genes found to be strongly regulated by GH ($p<0.001$) at 48 h in our adipocyte microarray (6, 37). A list of genes that were identified by both ChIP-Sequencing and the microarray was obtained, and these genes are expected to be the top candidates regulated by Bcl6 in response to GH (Table 3.2). Many of these genes are known to be physiologically relevant in GH responses, but most of them are not currently known to be regulated by Bcl6. Further experimental verification of GH and Bcl6-mediated regulation of these genes will provide novel information on the roles of Bcl6 in GH action and the mechanisms by which GH regulates transcription of these genes.

Discussion

The role of Bcl6 as a transcriptional repressor that functions as a master regulator of the B cell differentiation program has long been known (1-4); however, little is known of

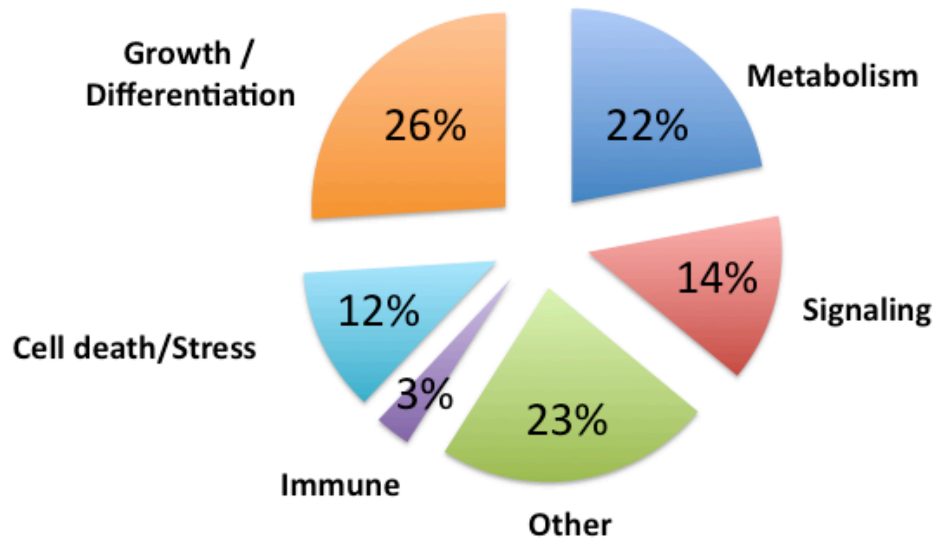

Figure 3.6

Gene Ontology analysis of Bcl6 occupied genes at 0 h GH treatment shows a high percentage of genes associated with GH function. Genes containing a peak of Bcl6 binding identified by ChIP-Sequencing located within 10 kilobases of the transcription start site in the absence of GH were subjected to Gene Ontology analysis to evaluate biological functions of potential novel Bcl6 target genes. Results showed that a high percentage of candidate Bcl6 target genes are involved in cellular processes such as growth, differentiation, metabolism, and signaling. (Analysis by RC McEachin, Rajasree Menon.)

Activated genes	Full gene name	Bcl6 peak location
Socs2	Suppressor of cytokine signaling 2	TSS
Rgs16	Regulator of G-protein signaling 16	Intergenic
Igfals	Insulin-like growth factor binding protein, acid labile subunit	Intergenic
Agt	Angiotensinogen	Intergenic, intron, exon
Cish	Cytokine inducible SH2-containing protein	TSS
St3gal4	ST3 beta-galactoside alpha-2,3-sialyltransferase 4	5'UTR
Igf2	Insulin-like growth factor 2	Intergenic
Ly6c1	Lymphocyte antigen 6 complex, locus C1	Intron
Cp	Ceruloplasmin	Intergenic
Bnip3	BCL2/adenovirus E1B interacting protein 3	Intergenic, intron
Gbp6	Guanylate binding protein 6	Intergenic
Itgb1bp1	Integrin beta 1 binding protein 1	5'UTR
Npc2	Niemann Pick type C2	Intron
Fbxo45	F-box protein 45	Intergenic

Repressed genes	Full gene name	Bcl6 peak location
Bcl6	B-cell leukemia/lymphoma 6	TSS
Fasn	Fatty acid synthase	Intron, exon, 5'UTR
Myc	Myelocytomatosis oncogene	Intergenic
Sdpr	Serum deprivation response	Intergenic, 5'UTR
Idh1	Isocitrate dehydrogenase 1 (NADP+), soluble	5'UTR
Marcks	Myristoylated alanine rich protein kinase C substrate	Intergenic, intron
Rgs2	Regulator of G-protein signaling 2	Intergenic
Scd2	Stearoyl-Coenzyme A desaturase 2	Intron
Scd1	Stearoyl-Coenzyme A desaturase 1	Intergenic, intron
Il1rl1	Interleukin 1 receptor-like 1	Exon
Cav1	Caveolin 1	Intergenic, intron
Anxa2	Annexin A1	Intergenic

Table 3.2

Combined analysis of ChIP-Sequencing and microarray data yields list of potential Bcl6- and GH-regulated genes in 3T3-F442A adipocytes. To identify genes that are likely to regulated by Bcl6 in response to GH, a list of genes that contained a peak of Bcl6 binding identified by ChIP-Sequencing located within 10 kilobases of the transcription start site in the absence of GH was analyzed in conjunction with a list of genes found to be strongly regulated by GH ($p<0.001$) at 48 h in the adipocyte microarray. Shown are lists of genes obtained from the combined analysis grouped by direction of gene expression change in response to GH, and ranked by significance of response to GH in the adipocyte microarray. TSS, transcription start site; 5'UTR, 5' untranslated region. (Analysis by Rajasree Menon.)

Bcl6 function and the identity of its target genes outside this context. We recently demonstrated a role for Bcl6 in GH action in adipocytes (6). Expression of Bcl6 is strongly regulated by GH, suggesting a role for Bcl6 in GH response (6, 22, 38). The Bcl6 protein in turn was found to function as a transcriptional repressor of the GH target gene Socs2 (6). Further experiments found that Bcl6 expression was inhibited by EGF and insulin, a growth factor and a hormone respectively, that also have important functions in cellular proliferation and adipocyte differentiation (25, 29), supporting a biological role for Bcl6 outside of the immune system. ChIP and qPCR experiments carried out using mouse liver tissue further suggest that Bcl6 regulates, and most likely represses, expression of GH target genes in a physiological model as well as in cell culture systems.

High throughput ChIP-Sequencing provides insight on genome-wide Bcl6 and Stat5 occupancy

High throughput ChIP-Sequencing using ChIP samples from 3T3-F442A adipocytes treated with or without GH for 48 h and immunoprecipitated with antibody against either Bcl6 or Stat5 was used to identify Bcl6 and Stat5 occupancy across the genome in the presence or absence of chronic GH treatment. Results yielded over 3700 potential Bcl6 occupancy sites in the absence of GH, and the number of sites where Bcl6 was potentially occupying the genome decreased to about 1500 upon GH treatment. This is consistent with our previous observations that GH inhibits Bcl6 mRNA expression, which would likely decrease Bcl6 protein levels, leading to less Bcl6 occupancy. Another possibility for the decrease in Bcl6 occupancy upon GH treatment is that activation of GH signaling may also result in post-translational modification of the Bcl6 protein, such as phosphorylation through extracellular-signal-related kinases (ERKs) (39) or acetylation by acetylases such as p300 (40). GH is known to cause post-translational modifications of many transcription factors, and thus regulate their actions (41). A relevant example is the tyrosyl phosphorylation and activation of Stat5 in response to GH treatment (42, 43). Similarly, GH treatment can induce post-translational modification of the transcription factor C/EBPβ (44-47). Phosphorylation of murine C/EBPβ by Erk 1/2 at Thr-188 and

acetylation at Lys-39 by p300 both appear to facilitate the GH-dependent transcriptional activation activity of C/EBPβ at early response genes, such as c-Fos. Phosphorylation and acetylation of Bcl6 have been shown previously (39, 40); both of these post-translational modifications target Bcl6 for proteasomal degradation and would contribute to a loss of Bcl6 occupancy across the genome.

However, we observed around 1300 potential occupancy sites for Stat5 in the absence of GH, and only around 900 potential occupancy sites after GH treatment. As GH induces Stat5 phosphorylation, dimerization, and recruitment to the nucleus where activated Stat5 can then bind to the regulatory regions of its target genes (42, 43), we did not expect to see a decrease in the number of Stat5-occupied regions after GH treatment. Surprisingly, Stat5 occupancy peaks were not detected at several known Stat5 occupancy sites even in the GH-treated genomic profiles, including the GH-responsive Stat5 site in the Socs2 promoter (6, 48, 49). Examination of the sequences obtained from the Stat5 sequencing runs showed that many of the sequences in the Stat5 data consisted of overamplified sequences that may be artifacts that occurred during PCR amplification of the ChIP-Sequencing library. As there appeared to be variability in the quality of the ChIP-Sequencing libraries prepared from the different treatment and immunoprecipitation conditions, in addition to possible differences in the sequencing efficiency for each library, the approach used for further analysis of the ChIP-Sequencing results was to concentrate efforts on analyzing a particular dataset of interest, and to use comparisons with data from other treatment conditions as supporting, but not decisive, information for identifying follow-up gene candidates.

We focused on the 0 h GH Bcl6 immunoprecipitation samples to identify potential novel Bcl6 target genes and potential novel GH-regulated Bcl6 target genes. Gene ontology analysis was performed for about 1500 genes that contained a peak of Bcl6 binding identified by ChIP-Sequencing, located within 10 kilobases of the transcription start site in the absence of GH, to identify biological processes that candidate Bcl6 target genes could be involved in. Results showed that a large number of candidate Bcl6 target genes were involved in processes such as metabolism (21%), signaling (10%), and growth and

differentiation (31%), while a relatively small number of candidate Bcl6 target genes were associated with biological processes more canonically associated with Bcl6 function in the immune system, such as immune function (4%) and cell stress and cell death (10%).

Bcl6 occupancy is detected at widespread locations across the genome

As the ChIP-Sequencing experiment was carried out in cultured adipocytes, where genes related to metabolic or differentiation processes are likely to be actively expressed, it is possible that Bcl6 occupancy was detected at some of these sites due to the open chromatin structure near these genes facilitating Bcl6 occupancy of consensus binding sequences or low affinity sequences available in these genomic regions, and that not all of the Bcl6 occupancy sites detected are actively involved in transcriptional regulation of nearby genes. Many of the Bcl6-occupied regions identified by ChIP-Sequencing did not show significant peak height as determined by H-Peak or a distinct peak profile when visualized using the UCSC Genome Browser, further suggesting that Bcl6 may not be regulating transcription at all of the occupancy sites detected. It should also be noted that a large percentage of the potential binding sites detected for Bcl6 fall in intronic (48%) or intergenic (31%) regions of genes, with transcription start site (TSS, 3%), 5'UTR (12%), 3'UTR (2%), and exon (5%) sequences making up the remainder, similar to the widespread binding patterns observed for Stat1 (9), GATA1 (50), or MyoD (51).

A current hypothesis on the function of widespread binding for certain transcription factors is that the weaker binding sites serve as a reservoir for the transcription factors and this acts as a buffering system for maintaining optimal transcription factor availability levels to fine-tune occupancy of factors at bona fide regulatory regions (52). Expression and function of Bcl6 is regulated at multiple levels, from transcription to translation (53-55), and both overexpression and depletion of Bcl6 can be deleterious to cell survival (20, 56-60). This suggests that Bcl6 plays important roles in cell physiology, and the proper function of this system relies on the careful control, specific balance, and rapid response of Bcl6 levels in the cell to external stimuli. It would not be surprising if

Bcl6 availability at regulatory DNA bindings sites, and thus Bcl6 action and response to stimuli, could be further modulated by sequestration of the protein at non-regulatory regions of the genome.

Another hypothesis is that transcription factors with widespread genome occupancy, as observed for Bcl6, carry out other functions when they occupy regions of the chromatin not directly associated with regional gene transcription. For example, the transcription factor CTCF is now recognized to have a broad role in nuclear and chromatin organization (61, 62). Similarly, MyoD binding induces changes in chromatin structure, and genomic regions occupied by MyoD are enriched with histone marks associated with active or accessible chromatin (51). Bcl6 is known to interact with many coregulatory proteins associated with chromatin remodeling (35, 63, 64). Therefore, it is possible that Bcl6 is associating either directly or indirectly with the chromatin at some of these potential binding sites and functioning as part of a chromatin remodeling complex, and not as a transcription factor. In either case, it is likely that Bcl6 is not directly regulating expression of target genes at all of the potential binding sites identified through ChIP-Sequencing.

Bioinformatic analyses of ChIP-Sequencing and gene expression data suggest novel roles for Bcl6 in regulation of GH physiology

However, occupancy peaks with strong peak height and distinct peak profiles were detected on multiple genes, including Socs2, suggesting that at least some of these Bcl6 occupancy sites are involved in direct regulation of nearby genes. Further experiments to examine if transcription of any of the Bcl6 candidate genes are regulated by Bcl6 will identify novel Bcl6 target genes and potentially provide information that can be used to better correlate ChIP-Sequencing data with biological regulation of a nearby gene by a candidate transcription factor in our future studies. At the moment, the ChIP-Sequencing data suggest that what is known about the role of Bcl6 in cellular and physiological processes may be just the surface of the potential multitude of functions in which Bcl6 might participate.

The list of genes that contained a peak of Bcl6 binding identified by ChIP-Sequencing located within 10 kilobases of the transcription start site at 0 h GH treatment was also analyzed in conjunction with a list of genes found to be strongly regulated by GH ($p<0.001$) at 48 h in the adipocyte microarray. A list of 12 genes that were repressed by GH and 15 genes that were activated by GH were detected in both the ChIP-Sequencing results and the GH-regulated gene profile and are considered the gene candidates most likely to be regulated by Bcl6 in response to GH. Among the 27 genes that appeared in this list, several are very interesting candidates to follow up: Fasn (fatty acid synthase), Scd1 (stearoyl-Coenzyme A desaturase 1), Scd2 (stearoyl-Coenzyme A desaturase 2), Igfals (insulin-like growth factor binding protein acid labile subunit), Igf2 (insulin-like growth factor 2), Rgs2 (regulator of G protein signaling 2), Rgs16 (regulator of G protein signaling 16), Socs2, and Cish (cytokine inducible SH2-containing protein). All have known roles in GH signaling, adipocyte differentiation, and/or lipid synthesis and metabolism (65-70), while angiotensinogen (encoded by the Agt gene) is a hormone precursor known to be synthesized by adipose tissue (71). Several of the genes in the list also have predicted Bcl6 occupancy sites in classical regulatory regions such as the 5'UTR or proximal promoter region (identified by H-Peak as TSS, transcription start site) of the gene. It would be expected that Bcl6 is likely to participate in regulation of those genes. Current plans are to verify GH regulation of these genes by qPCR and Bcl6 regulation of these genes using reporter gene constructs or by examining the mRNA expression of these genes in Bcl6 knock out mouse tissues compared to wild type. Occupancy of Bcl6 on the predicted genomic locations will also be experimentally verified using ChIP, and changes in Bcl6 occupancy at sites in response to GH examined.

Of note is that several of the genes were predicted by ChIP-Sequencing to have multiple Bcl6 binding sites in different locations of the gene; for example, ChIP-Sequencing detected Bcl6 occupancy signal in multiple introns, exons, and intergenically for Agt. Of these regions, locations showing a peak height above 10 as determined by H-Peak, along with distinct peak profiles, would be top candidate peaks for follow-up verification using

ChIP. Of great interest would be identifying which regions provide the most regulatory input towards transcription of Agt and the underlying mechanisms involved.

Conclusions and future directions

In conclusion, the ChIP-Sequencing results have provided us with a wealth of exciting directions to pursue in regards to learning more about GH biology, Bcl6 function, and mechanisms of transcription. However, as a last point of interest, it is also worth mentioning that little is known about the mechanisms by which GH regulates transcription of the Bcl6 gene itself, or the effects of GH treatment on function of the Bcl6 protein. It is known that Bcl6 autoregulates its own transcription by occupying Bcl6 binding sites in the upstream region of the Bcl6 gene and inhibiting gene expression (54). It is also known that Stat5 regulates Bcl6 gene expression, although the direction of regulation and the mechanisms involved seem to differ depending on cell type and sequence context (72-75). As Stat5 is a well known GH-regulated transcription factor, and Bcl6 expression is strongly inhibited by GH, it would be interesting to see if Bcl6 and Stat5 regulate expression of Bcl6 in response to GH similarly or differently from their known mechanisms of action in other cell types.

As the downstream effects of Bcl6 expression also seem to differ between cell types, with Bcl6 overexpression promoting cell survival in B cells but possibly promoting cell death in preadipocytes (C LaPensee unpublished data), investigating the results of this differential transcriptional regulation of Bcl6 in adipocytes compared to other cell types would also be interesting. A preliminary experiment where 3T3-F442A preadipocytes were treated with various signaling disruptors (U0126, MEK inhibitor; wortmannin, phosphoinositide-3-kinase inhibitor; forskolin, adenylyl cyclase activator) showed that treatment of cells with these disruptors did not inhibit the GH-induced repression of Bcl6 mRNA expression, suggesting that these GH-regulated signaling pathways are not involved in modulating expression of the Bcl6 gene. As the Jak/Stat inhibitor AG490 was unable to sufficiently reduce Jak/Stat activation in response to GH in 3T3-F442A preadipocytes, we were unable to test the effect of this signaling pathway on regulation of

Bcl6 using this inhibitor. Further experiments using different GH-responsive cell lines or reporter gene assays could shed light on the mechanisms by which Bcl6 expression is regulated in response to GH and how these mechanisms may be similar or different to how Bcl6 is regulated in other cell lines or in response to other growth factors.

Materials and Methods

Bcl6-deficient mice

The generation of mice with targeted deficiency of Bcl6 has been described previously (32). Animals were euthanized between 1000 and 1200 h by CO_2 inhalation and cervical dislocation. In some of the present studies, tissues were obtained courtesy of Dr. A. L. Dent, from a colony of Bcl6 knock out (KO) mice housed at the Indiana University School of Medicine. Bcl6 heterozygous (+/-) male and female mice from the Indiana colony were used to establish a breeding colony at the University of Michigan to generate Bcl6 knock out mice. Pups were genotyped by PCR analysis of tail DNA as previously described (76). Mice were housed on a 12-h light, 12-h dark cycle (lights on at 0600 h), with food and water available ad libitum unless otherwise specified. Liver tissue was rapidly removed, weighed, frozen on dry ice and stored at -80°C. For the analysis of GH responses in vivo, Bcl6 +/- male mice were injected IP with human GH (1.5mg/kg BW) or with vehicle twice daily for 2.5 days, and were fasted for the final 16-18 hr. Animal protocols were approved by the Indiana University School of Medicine Animal Use and Care Committee or the University Committee on Use and Care of Animals at the University of Michigan. All parameters were comparable when mice from Indiana and Michigan colonies were compared.

Materials

Stocks of murine 3T3-F442A preadipocytes were provided by H. Green (Harvard Univ) and M. Sonenberg (Sloan-Kettering, NY). Recombinant human GH (Lot# N70315) for animal experiments was from Genentech (So. San Fancisco CA), and for cell culture

studies was a gift from Lilly (Indianapolis, IN). Insulin, dexamethasone, and isobutylmethylxanthine were from Sigma (St. Louis, MO). Epidermal growth factor was from Cell Signaling Technology (Danvers, MA). Culture media, L-glutamine, and antibiotic-antimycotic were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) and calf serum (CS) were from Atlanta Biologicals (Lawrenceville, GA). Bovine serum albumin (BSA, Fatty acid-free, cat. # 62150101) was purchased from Proliant (Ankeny, IA). TRIzol® Reagent was purchased from Invitrogen, and Taqman Reverse Transcription Kit from Applied Biosystems (Carlsbad, CA). Protease inhibitors leupeptin and aprotinin were purchased from Roche (Indianapolis, IN), and phenylmethylsulfonylfluoride (PMSF) from Mallinckrodt (St Louis, MO). Sodium orthovanadate and SYBR green were purchased from Sigma (St. Louis, MO).

Cell Culture

3T3-F442A preadipocytes were differentiated into adipocytes as follows: Preadipocytes were grown to confluence in Dulbecco's Modified Eagle Medium, 1% L-glutamine, 1% antibiotic-antimycotic (DMEM) containing 8% calf serum. 48 h later, cells were induced to differentiate by exposure to adipogenic medium (DMEM, 8% FBS, 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone, 2 μg/ml insulin). After 48 h, medium was replaced with DMEM containing 8% FBS and 1 μg/ml insulin. 48 h later, medium was changed to DMEM with 8% FBS, in which cells were maintained until use in experiments. Prior to all experiments with GH, insulin, or EGF treatment, 3T3-F442A cells were deprived of serum for 16-18 h in DMEM containing 1% BSA instead of serum. Cells were then treated without or with human GH (500 ng/ml = 22 nM) for 48 h on day 7 following initiation of adipogenesis.

Quantitative Real-time PCR (qPCR)

Murine 3T3-F442A preadipocytes were incubated with GH, insulin, or epidermal growth factor for 24 h. RNA was isolated and analyzed by quantitative real-time PCR (qPCR) as described previously (37). Primers for Bcl6 have been described previously (77), and data

were normalized to glyceraldehyde-3-phosphate dehydrogenase expression levels (37). For mouse liver experiments, qPCR was performed on total RNA isolated from mouse liver tissues. The Socs2 qPCR primer sequences were based on published studies (78). Gene expression was normalized to RPLP0 (Ribosomal protein, large P0) expression levels, and is expressed as fold-change compared to control group, where the control is set to1.0. All qPCR data were analyzed using the 2ΔΔcT method (79). Values from analysis of wild type and Bcl6 knock out mice were compared statistically using Student's t-test.

Chromatin Immunoprecipitation (ChIP)

ChIP was carried out as described for cells (6, 80). The following modifications were made for analysis of liver: Frozen mouse liver (~200 mg) was thawed, minced in PBS and incubated in 1% formaldehyde in PBS for 15 min at room temperature before centrifugation for 2 min at 1500 rpm. The pellet was suspended in ChIP SDS lysis buffer (80) and sonicated using a Misonix S-3000 sonicator eighteen times for 15 seconds, with a 1 minute pause between cycles to achieve approximately 200 base pair fragments. For each immunoprecipitation, 100 µg of liver protein was incubated overnight at 4 °C with 4 µg of anti-Bcl6 antibody (N-3, Santa Cruz), anti-Stat5 antibody (C-17, Santa Cruz), or phosphorylated Stat5 antibody (cat. #71-6900, Zymed). Samples incubated with an equivalent amount of normal rabbit IgG (Santa Cruz) served as a negative control. 1% input was used to indicate the relative amount of each sample used for individual ChIP analysis. ChIP samples were analyzed by PCR using primers targeting the reported Bcl6/Stat5 binding site in the murine Socs2 gene (6, 48). PCR products were separated on 2% agarose gels and stained with ethidium bromide.

Generation of ChIP DNA libraries for high throughput ChIP-Sequencing

3T3-F442A adipocytes cultured in 15 cm culture plates were treated with GH (500 ng/ml) for 0 or 48 h (40 plates for each treatment condition). Lysates of cross-linked cells (input) were used for ChIP as described (80) using anti-Bcl6 (N-3) and anti-Stat5 (C-17)

(Santa Cruz). ChIP DNA samples were pre-tested for quality by PCR using primers specific for the Bcl6/Stat5 binding site in murine Socs2 (48), and multiple ChIP DNA samples immunoprecipitated with the same antibody under the same GH treatment conditions were pooled to obtain a sufficient amount of DNA (>10 ng) for generating ChIP-Sequencing libraries as recommended by the Illumina ChIP-Sequencing Library Preparation Protocol. ChIP-Sequencing libraries were generated by ligation of adapter sequences and PCR amplification of chromatin fragments using the Illumina ChIP-Seq Sample Prep Kit following the manufacturer's protocol. The quality of the ChIP-Sequencing libraries was then validated by the University of Michigan Sequencing Core using an Agilent Bioanalyzer and submitted to the University of Michigan Sequencing Core for high throughput sequencing on the Illumina GAIIx sequencing platform. A DNA library was also prepared using input DNA from untreated adipocytes and sequenced for use as a background control. A background control for immunoprecipitation artifacts carried out using ChIP-Sequencing libraries generated from ChIP samples immunoprecipitated with normal rabbit IgG was not carried out due to difficulties in obtaining sufficient starting material.

Analysis of ChIP-Sequencing output

Identification of genome-wide occupancy of Bcl6 and Stat5 by H-Peak

Sequencing data obtained from the ChIP-Sequencing libraries was analyzed using the H-Peak algorithm (http://www.sph.umich.edu/csg/qin/HPeak/), a hidden Markov model-based peak finding algorithm which allows for rigorous statistical inference (33). Distinct from other currently available algorithms, H-Peak explicitly assumes probability distributions to model coverage profiles of DNA fragments. H-Peak also provides the location of nearby genes and the genomic location of a peak. Occupancy profiles for Bcl6 or Stat5 on the mouse genome were visualized using the UCSC Genome Browser (http://genome.ucsc.edu/). Peaks of occupancy with a peak height of 4 or greater, as determined by H-Peak, were used in subsequent bioinformatic analyses.

Gene Ontology analysis of ChIP-Sequencing dataset from Bcl6 immunoprecipitation sample in the absence of GH

A list of genes that contained a peak of Bcl6 signal within 10 kilobases of the transcriptional start site without GH treatment was generated from the 0 h GH treatment Bcl6 immunoprecipitation ChIP-Sequencing data. This list of genes was then subjected to Gene Ontology (http://www.geneontology.org/) analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) using the standard stringency settings.

Computational analysis of combined ChIP-Sequencing dataset from Bcl6 immunoprecipitation samples in the absence of GH and microarray results

A microarray data set with 561 probe sets showing significant differential expression signals in 3T3-F442A adipocytes treated with GH (500 ng/ml) for 48 h has been described previously (37) and updated as of Nov./Dec. 2011. A list of genes that showed significant differential expression in response to GH at 48 h under stringent selection criteria ($p<0.001$) in the microarray was compared with the list of genes that contained a peak of Bcl6 signal within 10 kilobases of the transcriptional start site without GH treatment as identified from the 0 h GH treatment Bcl6 immunoprecipitation ChIP-Sequencing data. Genes that appeared in both lists were considered top candidates for biological verification of Bcl6- and GH-mediated transcriptional regulation.

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Chapter 4

Mechanisms of GH-mediated reciprocal regulation of target gene expression by Bcl6 and Stat5

Introduction

To identify novel Bcl6 target genes and better understand the role of Bcl6 in GH physiology, high throughput ChIP-Sequencing was carried out to identify potential Bcl6 occupancy sites across the genome. ChIP-Sequencing results yielded several thousand potential Bcl6 occupancy sites in 3T3-F442A adipocytes in the absence of GH, when Bcl6 occupancy is expected to be highest and occupancy of target genes regulated by Bcl6 is more likely to be detected (1). Combined analysis of ChIP-Sequencing and microarray results from GH-treated adipocytes also provided a list of genes likely to be regulated by Bcl6 in response to GH. Included in this list of genes are many well known GH target genes or genes that would logically participate in GH action. Identifying the role that Bcl6 plays in regulation of these genes would greatly expand our understanding of both GH-mediated transcriptional repression and mechanisms by which GH carries out its physiological actions.

In addition to identifying novel Bcl6 target genes, another goal of the ChIP-Sequencing was to identify genes that might be regulated by both Bcl6 and Stat5, with a focus on genes that are regulated by both Bcl6 and Stat5 in response to GH using the same regulatory DNA sequence. As shown previously, the GH target gene Socs2 is reciprocally regulated by Bcl6 and Stat5 in response to GH, with Bcl6 playing a repressive role and Stat5 playing an activating role (1). Bcl6 and Stat5 show similar DNA binding consensus sequences (2-5), and appear to occupy the same DNA regulatory region in the Socs2 promoter under different treatment conditions to regulate the changes in Socs2

transcription (1). This mechanism of regulation by Bcl6 and Stat5 for Socs2 is one of the first examples of a reciprocal transcriptional activator-repressor relationship in GH-mediated gene transcription. Therefore, the goal was to identify other genes regulated by both Bcl6 and Stat5 to examine if the mechanism of reciprocal regulation by these two transcription factors is common to other GH target genes, or if this mechanism is unique to regulation of Socs2. Furthermore, analysis of other reciprocally regulated gene candidates could yield insight into mechanisms by which GH regulates the actions of the Bcl6/Stat5 activator-repressor switch.

Experimental validation of 35 ChIP-Sequencing peaks identified Cish (cytokine-inducible SH2-containing protein) and Bcl6 as two other gene candidates that may be reciprocally regulated by Bcl6 and Stat5 in response to GH, in addition to the known example of Socs2. Occupancy patterns of Bcl6 and Stat5 on all three genes was similar, with Bcl6 occupancy high in the absence of GH and decreasing progressively after GH treatment, while occupancy of Stat5 was low in the absence of GH and increased with treatment. However, as Socs2 and Cish expression is induced by GH while Bcl6 expression is repressed (1), the effects that Bcl6 and Stat5 have on the transcriptional state of these genes is likely different, despite the similar occupancy patterns.

At the molecular level, transcription is regulated by both the recruitment of specific transcription factors to their respective target genes and the recruitment of coregulatory proteins, which modulate transcription but may not directly bind the target DNA (6-8). Coregulators often associate with target DNA through their interaction with transcription factors. The nuclear proteins present in a transcription regulatory complex work in concert to direct the transcriptional output of a gene. Coregulatory molecules that have an activating effect on transcription of a target gene are generally known as coactivators, while coregulators that inhibit gene transcription are called corepressors. However, recent studies have shown that proteins canonically known as coactivators, such as p300, can also have repressive roles on transcription (9), and corepressors such as histone deacetylases (HDACs) are involved in activating the expression of some genes (10, 11).

Bcl6 is well known to associate with corepressors when repressing transcription of target genes in immune cells. Examples include histone deacetylases, NCoR (nuclear receptor corepressor), and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) (12-16). Similarly, when functioning as a transcriptional activator, Stat5 is known to interact with many coactivating molecules, such as p300, CBP (cyclic AMP response element binding protein binding protein) and NCoA-1 (nuclear receptor coactivator 1), which aid in driving Stat5 target gene transcription in response to cytokine stimulation (17-20). So far, little is known about the role of corepressors in GH-mediated transcription or mechanisms by which Bcl6 regulates GH target genes.

As shown below, ChIP was carried out to identify candidate coregulatory molecules involved in Bcl6- or Stat5-mediated transcription of Socs2, Cish, or Bcl6 and evaluate how these coregulators relate to transcriptional regulation. The coregulatory molecules p300 and Hdac3 were found to consistently occupy the promoters of Socs2, Cish, and Bcl6 in both the presence and absence of GH. Reporter gene assays carried out using regulatory sequences of Socs2, Cish, and Bcl6 fused to the firefly luciferase gene, in conjunction with overexpression constructs for Bcl6, Stat5, p300, and Hdac3, showed that p300 and Hdac3 participate in the regulation of Socs2, Cish, and Bcl6 expression by Bcl6 and/or Stat5. Results suggest that the effects of any transcription factor-coregulator combination on transcription of the reporter gene were different for each gene regulatory sequence tested, with regulation of the Socs2 and Cish promoters, which are both activated by GH, being more similar to each other compared to regulation of the Bcl6 promoter, which is inhibited by GH. In the case of Socs2-luciferase and Cish-luciferase, the coregulatory molecules tested appeared to exert their effects mainly through their interactions with Stat5, with p300 in particular providing a coactivating effect on Stat5-mediated transcription. In the case of Bcl6-luciferase, p300 and Hdac3 appeared to interact with both Bcl6 and Stat5, with the results of these interactions mostly favoring transcriptional repression. This is a relatively unique situation, especially for p300. These results highlight the various different and intricate input signals involved in regulation of each GH target gene even by the same transcription factors, resulting in distinct downstream transcriptional outputs.

The Bcl6/Stat5 target genes identified through ChIP-Sequencing have provided a novel system to study the roles of coactivators and corepressors in GH-mediated transcription, and results from these experiments have already identified several coregulators associated with Bcl6 in response to GH stimulus. Further study will provide novel insight into similarities and differences between mechanisms of Bcl6 action in GH-responsive metabolic tissues and in immune tissues, as well as further our understanding of how the many protein components in a transcriptional regulatory complex function together to drive or repress transcription of a target gene.

Results

ChIP-Sequencing identifies Cish and Bcl6 as candidate genes reciprocally regulated by Bcl6 and Stat5

ChIP-Sequencing results were expected to provide information on novel Bcl6 target genes, novel GH-regulated Bcl6 target genes, and genes potentially regulated by both Bcl6 and Stat5. To identify gene candidates reciprocally regulated by Bcl6 and Stat5 similar to what is seen for regulation of Socs2 expression (1), follow-up candidate peaks identified through ChIP-Sequencing were chosen based on literature searches for genes associated with Bcl6 or Stat5, peak height values, peak p values, or peak profiles generated from ChIP-Sequencing analysis, with the gene or genes located closest to a peak predicted to be the most likely targets for transcriptional regulation (Figure 4.1). 35 candidate Bcl6 and/or Stat5 occupancy peaks identified through ChIP-Sequencing were analyzed using ChIP and primers specific for the genomic regions of ChIP-Sequencing signal (Table 4.1).

Of the 35 candidate occupancy regions tested by ChIP, two regions (Figure 4.2) showed clear reciprocal occupancy of Bcl6 and Stat5 in response to 48 hours of GH treatment in adipocytes (Figure 4.3). The first region was a ChIP-Sequencing peak of Bcl6 signal identified in the transcription start site of the Cish (cytokine-inducible SH2-containing

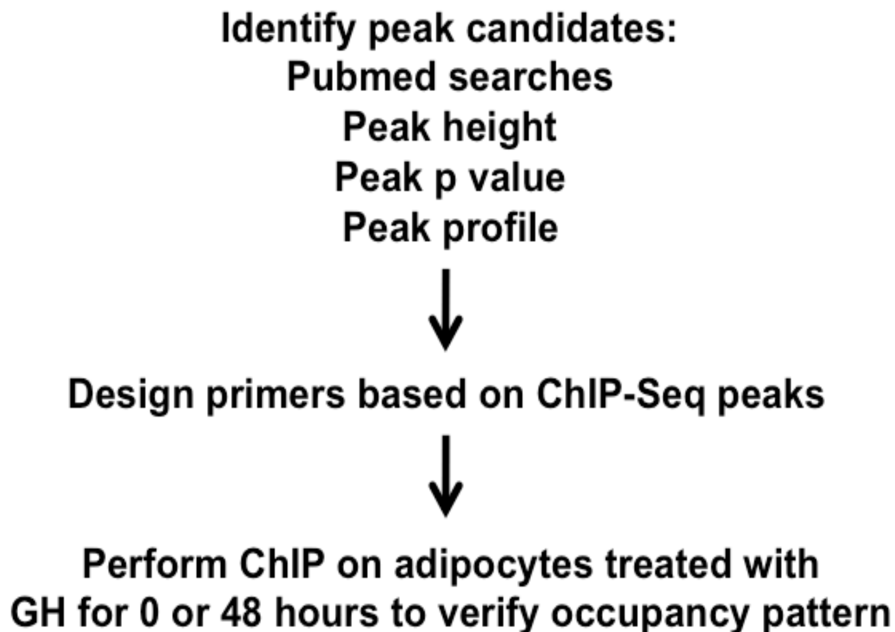


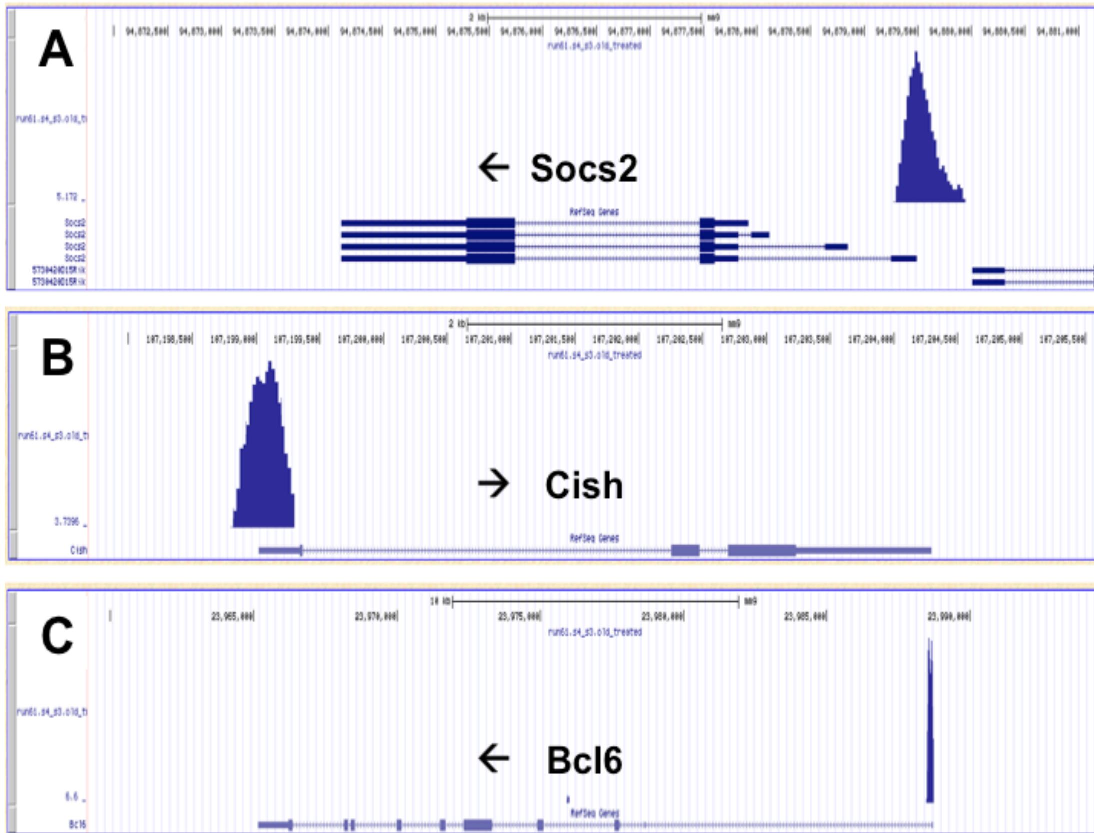
Figure 4.1

Schematic for selection of follow up peaks of Bcl6 and/or Stat5 occupancy from ChIP-Sequencing output data. To find genes reciprocally regulated by Bcl6 and Stat5 in response to GH, sequences on the genome that have the potential to be bound by both Bcl6 and Stat5, and sequences where occupancy of Bcl6 and Stat5 changed in response to GH would be of particular interest. ChIP-Sequencing data for each of the four immunoprecipitation conditions were sorted by peak height and peak p value, and the highest or most statistically significant peaks were given priority as candidates. The names of genes closest to these candidate peaks were also subjected to a Pubmed literature search, and genes that have previously been published to have biological relationships with either Bcl6 or Stat5 were also given priority. UCSC Genome Browser (http://genome.ucsc.edu/) profiles were also generated for peaks of interest, and peaks that showed defined peak enrichment profiles were also prioritized for experimental verification. ChIP primers were designed to flank the peaks identified by ChIP-Sequencing that were selected for further testing.

Transcription	Signaling	Enzyme	Transport	Other
Bcl6	Agt	Lars2	Sec24c	Abt1
Cebpb	Cish	Psmc6	Slc7a15	Asb18
Hist1h4a	Cxcr7	Ptprr		Ctnna3
Med16	Ghr	Srxn1		Dnajc12
Nr0b1	Npy5r			Fam181b
Zfp322a	Traf5			Gm853
				Iqca
				Isoc2b
				Plin4
				Ppp1r10
				Rd3
				Serac1
				Serinc2
				Serpinf1
				Synpo2l
				Tchp
				Tomm34

Table 4.1

35 ChIP-Sequencing peaks were tested for reciprocal occupancy of Bcl6 and Stat5 in response to GH using ChIP. Shown is a list of genes closest to the ChIP-Sequencing peaks tested for reciprocal occupancy of Bcl6 and Stat5 in response to GH using ChIP samples generated from 3T3-F442A adipocytes treated with GH for 0 or 48 hours and immunoprecipitated with antibody against Bcl6 or Stat5. Genes are grouped in general categories by a documented function.



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Gene	Chromosome	Coverage	Height	Location
Socs2	Chr10:94879276-94880050	775	88	TSS
Cish	Chr9:107198726-107199400	675	61	TSS
Bcl6	Chr16:23988401-23988850	450	28	TSS

Figure 4.2

Distribution of Bcl6 signal on Socs2, Cish, and Bcl6. Peaks of Bcl6 occupancy were identified at the transcription start sites of Cish and Bcl6, in addition to the previously identified site of Bcl6 occupancy at the Socs2 transcriptional start site. Shown are the UCSC Genome Browser profiles for Bcl6 occupancy in the absence of GH for **A.** Socs2, **B.** Cish, and **C.** Bcl6. Arrows to the left of gene names indicate direction of transcription, from 5' to 3'. **D.** H-Peak output results for the Bcl6 occupancy sites for Socs2, Cish, and Bcl6 from the combined ChIP-Sequencing run data. Shown are the peak chromosomal locations, peak coverage, peak height, and peak location in the respective genes. TSS: transcription start site.

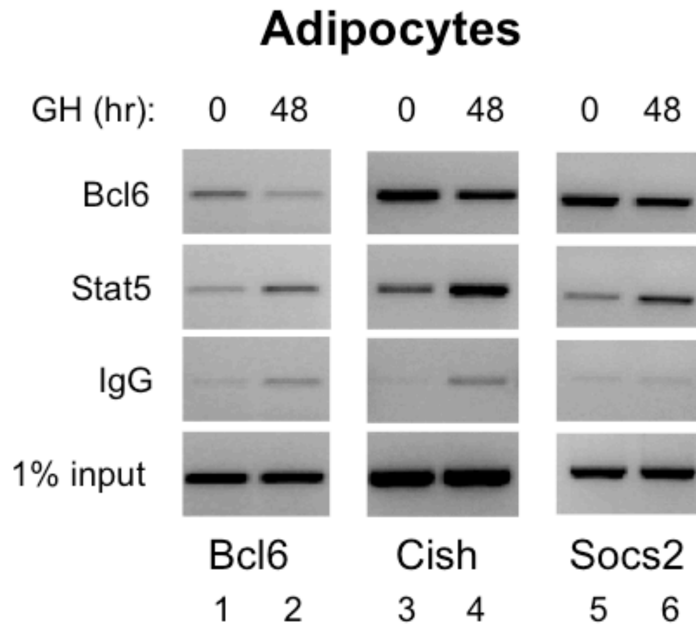


Figure 4.3

The Socs2, Cish, and Bcl6 regulatory regions identified by ChIP-Sequencing show reciprocal occupancy of Bcl6 and Stat5 in response to GH in adipocytes. 3T3-F442A adipocytes were treated with GH for 0 or 48 h and ChIP was carried out using antibodies against Bcl6 and Stat5. ChIP DNA was probed with primers specific to the Bcl6 occupancy sites predicted by ChIP-Sequencing (peaks) on the Cish (lanes 3 and 4) and Bcl6 (lanes 1 and 2) genes. PCR using primers specific to the known reciprocal Bcl6/Stat5 site in the Socs2 promoter was carried out as a positive control (lanes 1 and 2). 1% input was used as an internal control and samples treated with IgG were used as negative controls. Of the 35 ChIP-Sequencing peaks tested (Table 4.1), predicted occupancy sites for Bcl6 which were identified at the Cish and Bcl6 transcriptional start sites showed reciprocal occupancy of Bcl6 and Stat5 in response to GH, in addition to the previously identified site at the Socs2 promoter.

protein) gene (Figure 4.2, B), and the second region was a peak of Bcl6 signal identified in the transcription start site of the Bcl6 gene (Figure 4.2, C). The verification by ChIP of reciprocal Bcl6 and Stat5 occupancy suggested that expression of Cish and Bcl6 mRNA in response to GH is reciprocally regulated by Bcl6 and Stat5. Cish is a member of the SOCS (suppressor of cytokine signaling) family proteins (21, 22), of which Socs2 is also a member, and is a known Stat5 target gene (23). Occupancy of Bcl6 has been reported previously at the Cish promoter in liver (17). Bcl6 is known to inhibit the expression of its own mRNA (24-26), and Stat5 has been reported to have either an activating or repressive effect on Bcl6 transcription depending on cell type (27-31). However, this is the first evidence suggesting that Bcl6 and Stat5 may work together to regulate the expression of either Cish or Bcl6 in response to GH in adipocytes using dual Bcl6/Stat5 regulatory regions in the proximal promoters of these genes.

In the case of both the Cish ChIP-Sequencing peak and the Bcl6 ChIP-Sequencing peak, occupancy of Bcl6 was high without GH treatment, and occupancy of Bcl6 decreased with GH. Stat5 occupancy was low in the absence of GH and increased with GH treatment, similar to what had previously been seen for Socs2 (Figure 4.3). In the case of the three reciprocally regulated gene candidates identified so far, the location of the Bcl6 and Stat5 reciprocal occupancy ChIP-Sequencing peaks were all located in the transcription start site (TSS)/proximal promoter of the gene (Figure 4.2), while peaks tested on other genes (Table 4.1) were located at other genomic locations such as introns or intergenic regions, suggesting that the location of the regulatory region may be a possible common feature of Bcl6/Stat5 reciprocal regulation sites.

Mechanisms by which Bcl6 and Stat5 regulate Socs2, Cish, and Bcl6 expression are likely to be different

As shown previously, Cish mRNA expression increases with GH treatment in a pattern similar to that seen for Socs2, with Socs2 and Cish being the genes most highly induced by GH treatment at later time points, such as 24 and 48 h (Figure 2.2). However, Bcl6 expression is strongly inhibited by GH treatment (Figure 2.2). This suggests that while the

Bcl6 and Stat5 occupancy patterns of the regulatory regions of the three genes are similar, the mechanisms of action of Bcl6 and Stat5, and the effects that Bcl6 and Stat5 have on transcription of these genes, is likely different. Furthermore, the occupancy patterns of Bcl6 and Stat5 in response to GH on Socs2 and Cish in both preadipocytes and adipocytes were very similar (Figure 4.4). Bcl6 occupancy on Socs2 and Cish was 3-6 times higher in the absence of GH compared to the presence of GH, and Stat5 occupancy increased by 2-5 fold upon GH treatment. These results correlated with the reciprocal occupancy of Bcl6 and Stat5 on the Socs2 gene in response to GH in both preadipocytes and adipocytes observed previously (Figure 2.8).

In contrast, the occupancy pattern of Bcl6 and Stat5 on the regulatory region of the Bcl6 gene was different between preadipocytes and adipocytes. Bcl6 occupancy signal on the Bcl6 promoter was higher in adipocytes in the absence of GH than in the presence of GH, but in preadipocytes Bcl6 occupancy was low in the absence of GH and did not decrease with GH treatment. In adipocytes, Stat5 occupancy was low in the absence of GH and increased 4 fold with GH. Stat5 occupancy in preadipocytes in the absence of GH appeared to be higher than Stat5 occupancy in the absence of GH in adipocytes. However, Stat5 occupancy in preadipocytes only showed a slight increase (less than 1.5 fold) upon GH treatment. Occupancy patterns for phosphorylated Stat5 showed the same trends as occupancy of Stat5 for all conditions, consistent with activated Stat5 mediating transcriptional regulation. Quantification of occupancy evaluated by qPCR corresponded with previous observations evaluated by resolving PCR products on agarose gels (1), supporting the results.

Overall, ChIP results for the Bcl6 gene in adipocytes show a much more distinct reciprocal pattern of occupancy between Bcl6 and Stat5 in response to GH compared to preadipocytes (Figure 4.4). The different occupancy patterns in adipocytes and preadipocytes for Bcl6 and Stat5 in response to GH on the Bcl6 gene, compared to the similar pattern of reciprocal occupancy on the Socs2 and Cish genes for Bcl6 and Stat5 in both adipocytes and preadipocytes, would also suggest that there are differences in the mechanisms by which Bcl6 and Stat5 regulate the expression of Socs2 and Cish compared

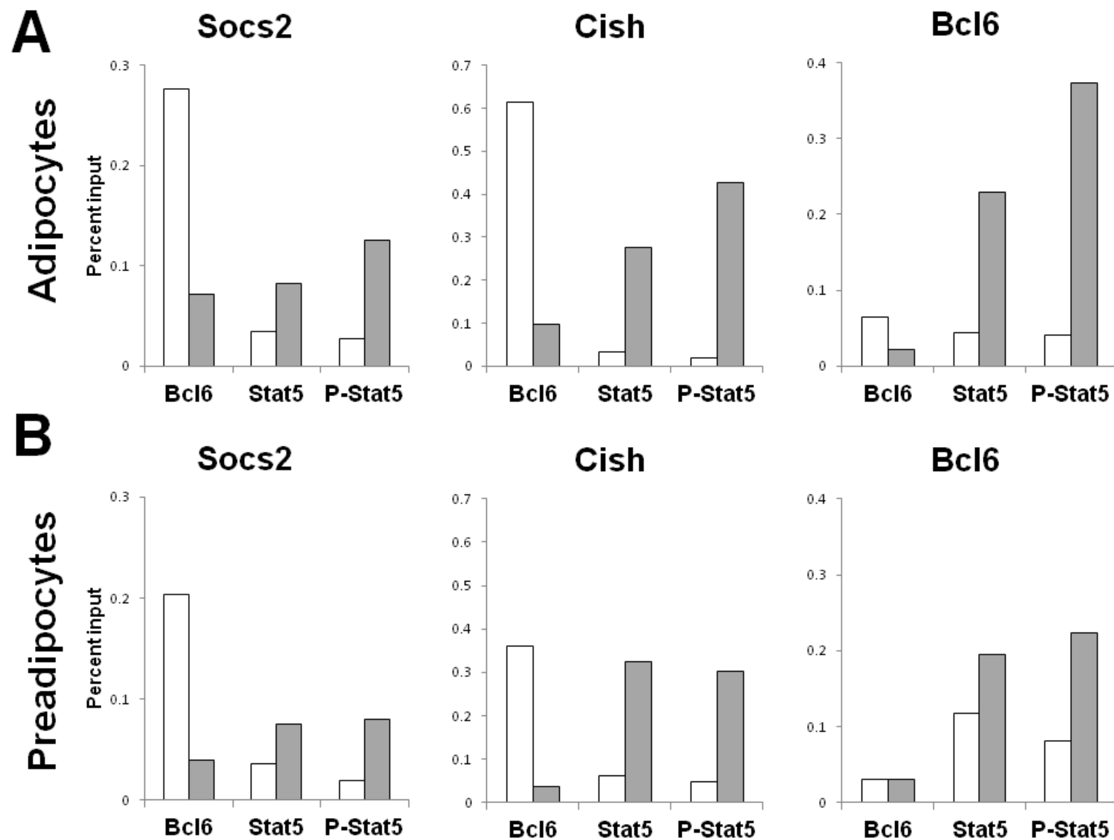


Figure 4.4

Occupancy patterns for Bcl6 and Stat5 in response to GH differ in adipocytes and preadipocytes on the Bcl6 promoter, but not on the Socs2 or Cish promoters. 3T3-F442A adipocytes (**A**) or preadipocytes (**B**) were treated without (white bars) or with GH (gray bars) for 24 h. ChIP was carried out using antibodies against Bcl6, Stat5, or phosphorylated Stat5 (P-Stat5). ChIP samples were probed with primers specific to the Bcl6/Stat5 occupancy sites identified by ChIP-Sequencing and quantified using quantitative real-time PCR. Immunoprecipitation samples were normalized to amplification signal from the input samples for each respective treatment condition. Socs2 and Cish show similar occupancy patterns in both adipocytes and preadipocytes for Bcl6 and Stat5 in response to GH. However, for the Bcl6 promoter, reciprocal occupancy of Bcl6 and Stat5 in response to GH is more marked in adipocytes than preadipocytes. Results are representative of 4 experiments.

to Bcl6, and that these mechanisms may even be different for the same gene under various conditions.

Changes in histone modification profiles on the reciprocally occupied regions of Socs2, Cish, and Bcl6 correlate with changes in gene expression in response to GH

To identify differences in transcriptional regulation by Bcl6 and Stat5 on the candidate reciprocally regulated genes Socs2, Cish, and Bcl6, ChIP was carried out for several well-studied histone modifications to examine the chromatin state of the respective Bcl6/Stat5 reciprocally occupied regions in the presence and absence of GH. Adipocytes were treated with GH acutely for 30 min to identify changes in chromatin state during early responses after treatment, when the transcriptional state of the gene is being established in response to hormone stimulus (Figure 4.5, A). ChIP was carried out using antibodies against acetylated histone H3 (AcH3), acetylated histone H4 (AcH4), and trimethylated histone H3 lysine 4 (H3K4me3), all general markers of open and accessible chromatin, and suggestive of active gene transcription (32-35). ChIP results (Figure 4.5, A) show that levels of AcH3 and AcH4 increase two to five fold on the Socs2 and Cish regulatory regions in response to GH, while AcH3 and AcH4 levels increase by less than two fold on the Bcl6 regulatory region. The presence of AcH3 and AcH4 at the Socs2, Cish, and Bcl6 promoters is consistent with active transcription of these target genes in response to GH. Further, the marked enrichment of AcH3 and AcH4 on Socs2 and Cish compared to their lesser changes in enrichment observed for Bcl6 is consistent with the contrast between strong induction of Socs2 and Cish in response to GH and inhibition of Bcl6 expression by GH. The slight increase in levels of AcH3 and AcH4 on Bcl6 in response to GH suggests that Bcl6 is a highly regulated and responsive gene and that the chromatin remains accessible to the various transcriptional regulatory factors that are recruited to it by different stimuli, enabling the cell to respond to the extracellular environment. Similarly, strong signal for H3K4me3 is detected at all three regulatory regions in both the presence and absence of GH, once again consistent with active transcription and regulation of Socs2, Cish, and Bcl6.

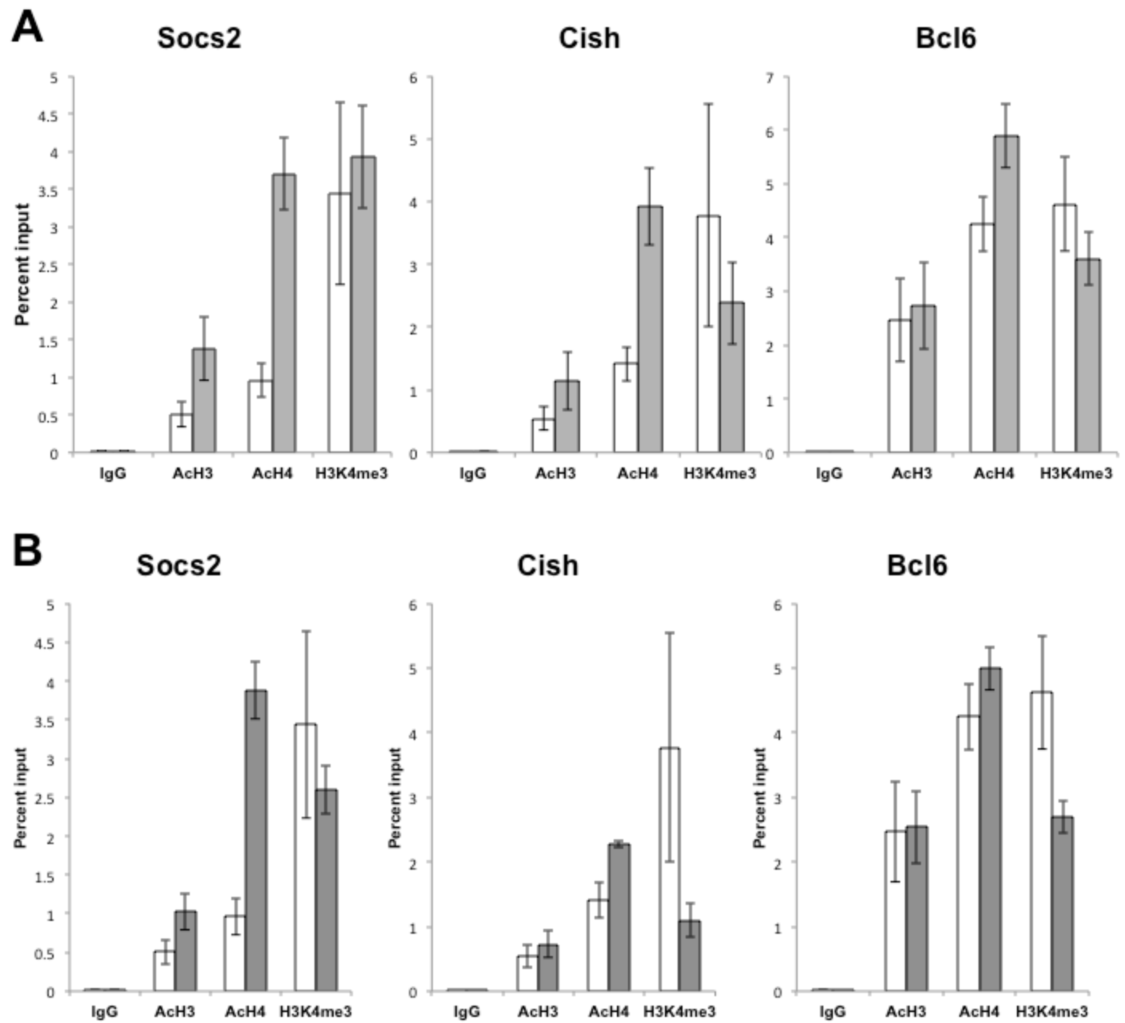


Figure 4.5

Enrichment of AcH3 and AcH4, histone marks associated with transcription activation, increases rapidly and levels remain elevated in response to GH on the Socs2 and Cish, but not the Bcl6 promoter. 3T3-F442A adipocytes were treated with (**A**) GH for 0 (white bars) or 30 min (gray bars) or (**B**) GH for 0 (white bars) or 24 h (gray bars). ChIP was carried out using antibodies against AcH3, AcH4, or H3K4me3. Enrichment of these activating histone marks at the Bcl6/Stat5 sites identified by ChIP-Sequencing in the Socs2, Cish, and Bcl6 promoters was analyzed using quantitative real-time PCR. Signal from ChIP DNA was normalized to signal from input samples of respective treatment conditions. Samples treated with IgG were used as negative controls. Results shown are the mean and standard error from 4 separate experiments for (**A**) and (**B**). Responses to GH were statistically significant ($p < 0.05$) for AcH3 at 0.5 hr and AcH4 at 0.5 and 24 h on Socs2, and for AcH4 at 0.5 and 24 h on Cish.

Levels of AcH3, AcH4, and H3K4me3 on the regulatory regions of Socs2, Cish, and Bcl6 were also examined at the later time of 24 hours after GH treatment when Socs2 and Cish are known to be strongly induced and Bcl6 expression is strongly inhibited (Figure 4.5, B). ChIP results (Figure 4.5, B) in adipocytes show that at 24 h AcH4 levels tend to still be elevated by GH for the Bcl6/Stat5 occupied region in all three genes; however, the relative level of enrichment in response to GH was greater for Socs2 and Cish (about 2 to 3 fold) than the negligible changes on Bcl6, consistent with Socs2 and Cish expression being induced and Bcl6 expression being repressed at 24 h of GH treatment. In contrast to AcH4, AcH3 levels on the Bcl6/Stat5 regulatory region for all three genes were modestly elevated if at all compared to AcH3 levels in the absence of GH. H3K4me3 levels tended to be repressed by GH for the genomic regions examined, perhaps indicative of a mechanism for modulating the transcriptional state of these genes to trend towards repression as the GH stimulus subsides.

ChIP was also carried out for several histone marks associated with repressed or inactive chromatin, such as trimethylated histone H3 lysine 9 (H3K9me3) (36-38) and trimethylated histone H3 lysine 27 (H3K27me3) (39, 40). Signals for these repressive chromatin marks at the Bcl6/Stat5 regulatory regions of Socs2, Cish, and Bcl6 were nearly undetectable (data not shown), consistent with the active transcription and regulation of these genes in response to cellular stimuli such as GH.

The coregulatory proteins p300 and Hdac3 occupy the Bcl6/Stat5 regulatory region of Socs2, Cish, and Bcl6 in the presence and absence of GH

An important mechanism for transcriptional regulation is the recruitment of coregulatory proteins to transcription factor-DNA complexes. To evaluate if histone deacetylases (HDACs), ubiquitous coregulators best known for functioning as corepressor molecules (41), participated in regulation of Socs2 or Bcl6 expression, preadipocytes were treated with trichostatin A (TSA), a general class I and II HDAC inhibitor, and changes in Socs2 and Bcl6 transcript levels measured by qPCR (Figure 4.6). In a preliminary experiment, induction of Socs2 mRNA expression by GH appeared to be inhibited in cells treated

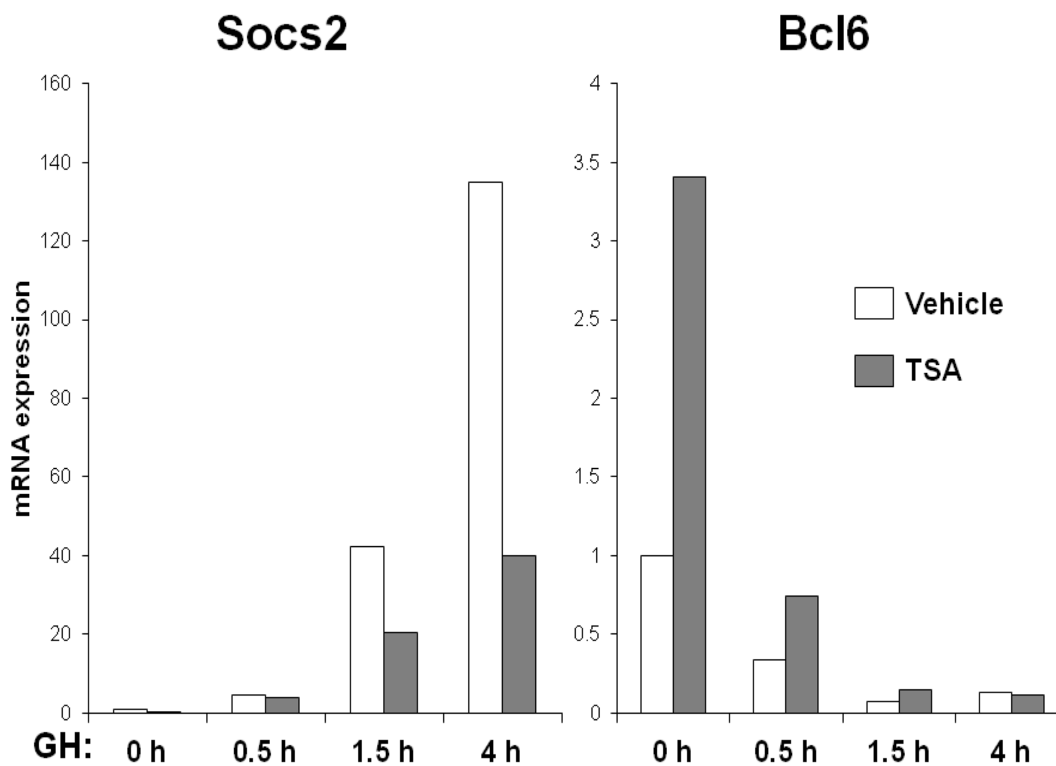


Figure 4.6

Histone deacetylases participate in regulation of Socs2 and Bcl6 mRNA expression. 3T3-F442A preadipocytes were pretreated with DMSO vehicle (white bars) or 1 μM trichostatin A (TSA, gray bars) for 18 h before treatment with GH for the indicated times. Socs2 and Bcl6 mRNA expression was analyzed using quantitative real-time PCR. mRNA expression was normalized to Socs2 (left) or Bcl6 (right) expression in vehicle treated cells at 0 h GH treatment, set as 1.0. Results are from 1 experiment.

with TSA, suggesting that HDAC activity potentiates Socs2 activation in response to GH. Conversely, basal Bcl6 levels were elevated in TSA-treated cells, although the repression of Bcl6 expression in response to GH appeared to be generally similar in treated and untreated cells, suggesting that HDACs play a role in regulating Bcl6 transcript levels, but not necessarily GH-mediated repression of Bcl6 expression. Together, these results suggest that one or several HDAC molecules play a role in Socs2 and Bcl6 transcription, and that the identity of the HDAC family member or members involved or the mechanism of HDAC action on transcription of the two genes appears to be different.

To identify if specific coregulatory proteins are associated with Bcl6 and Stat5 at the Socs2, Cish, and Bcl6 regulatory regions, ChIP was carried out using antibodies against p300 and Hdac3. P300 is a lysine acetyltransferase known to interact with several classes of transcription factors (42), including Bcl6 and Stat5 (12, 43). P300 is widely expressed and participates in multiple cellular events, including metabolism and adipocyte differentiation, and has been shown to participate in regulation of GH target genes (17, 44-47). Hdac3 is an ubiquitously expressed class I HDAC that has been implicated in lipid and energy metabolism and adipogenesis (48-50), and has also been identified as an important cofactor in regulating a subset of Bcl6 target genes involved in the innate immune response (51).

To examine occupancy of p300 and Hdac3 on the Bcl6/Stat5 regulatory regions of the Socs2, Cish, and Bcl6 genes, adipocytes were treated with GH for 30 min, an early time point when the transcriptional state of the gene in response to GH is likely being established (Figure 4.7, A). For all three genes, Bcl6 occupancy was higher in the absence of GH, and Bcl6 occupancy decreased with GH treatment. Occupancy of Stat5 was lower in the absence of GH, and increased upon GH treatment. These results matched the highly responsive reciprocal changes in occupancy of Bcl6 and Stat5 in response to GH previously observed on the Socs2, Cish, and Bcl6 genes (Figure 4.3 and 4.4). ChIP results show that p300 occupancy is detected on Socs2, Cish, and Bcl6 in the absence of GH. Occupancy of p300 on Socs2 increased in response to GH. Occupancy of p300 on Cish and Bcl6 appeared to be similar in both the absence and presence of GH.

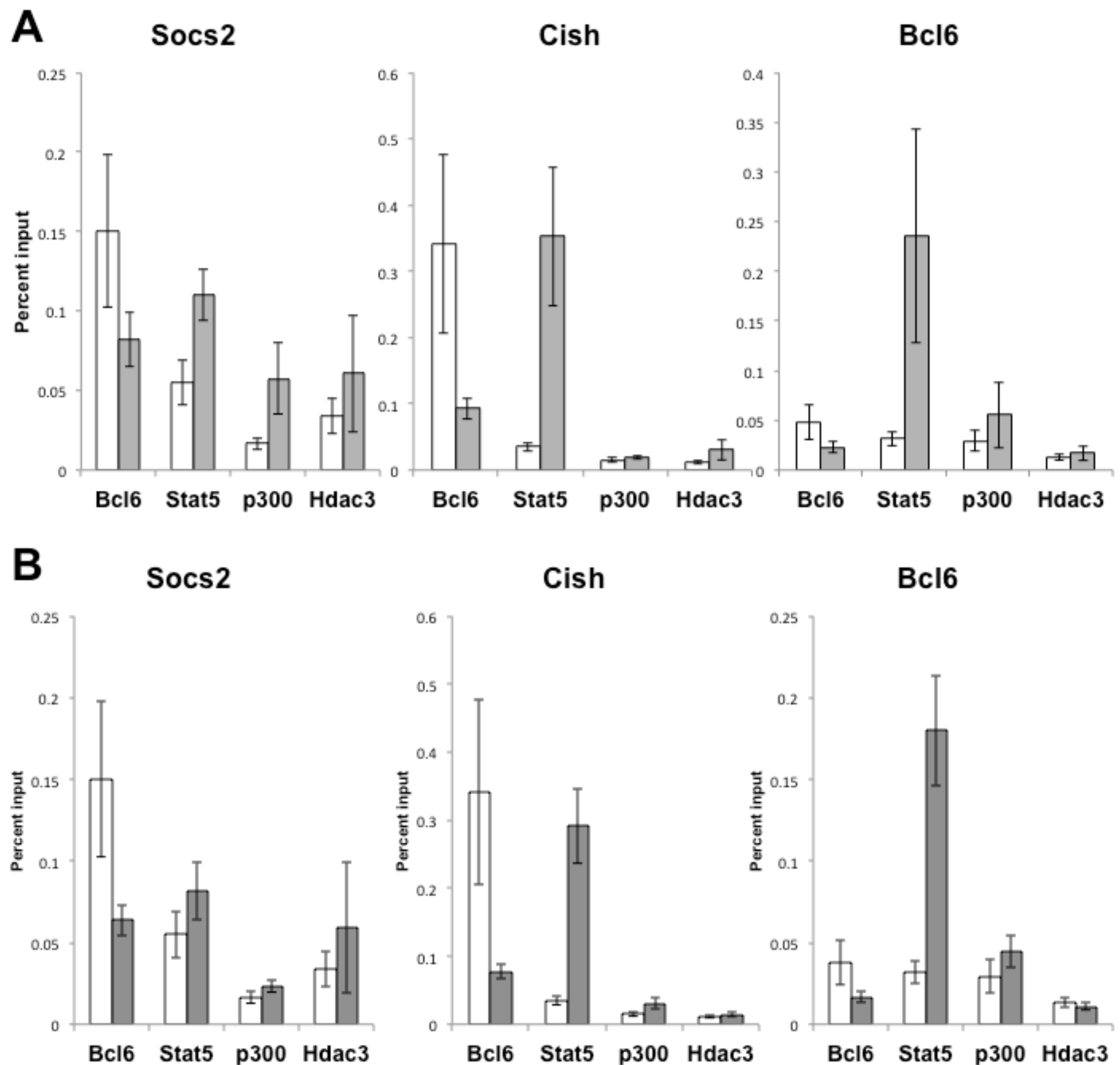


Figure 4.7

ChIP for candidate coregulator molecules identifies p300 and Hdac3 as potential factors playing a role in Bcl6/Stat5-mediated transcription of Socs2, Cish, and Bcl6 in response to GH. 3T3-F442A adipocytes were treated with (**A**) GH for 0 (white bars) or 30 min (gray bars) or (**B**) GH for 0 (white bars) or 24 h (gray bars). ChIP was carried out using antibodies against Bcl6, Stat5, p300, and Hdac3. ChIP DNA was analyzed using quantitative real-time PCR and primers specific to the Bcl6/Stat5 sites predicted by ChIP-Sequencing in the Socs2, Cish, and Bcl6 gene promoters. Signals from ChIP samples were normalized to signal from input DNA samples from respective treatment conditions. Results shown are the mean and standard error for 3 to 7 independent experiments for (**A**) and (**B**). Results were combined and graphed together as Bcl6 and Stat5 occupancy and changes in occupancy in response to GH for each gene in all experiments were comparable. Responses to GH were statistically significant ($p < 0.05$) for Stat5 at 0.5 and 24 hr on Cish, and for Stat5 at 24 h on Bcl6.

Occupancy of Hdac3 was also detected on Socs2, Cish, and Bcl6 in the absence of GH. In response to GH, the occupancy of Hdac3 trended toward a slight increase upon treatment. In general, the occupancy signal of Hdac3, as well as that of other HDACs tested (Hdac1, Hdac2, Hdac5, data not shown), appeared to be relatively low under the conditions of these experiments, and changes in occupancy to be of small magnitude, suggesting that active recruitment of HDACs to Socs2, Cish, and Bcl6 by Bcl6 or Stat5 is not a major mechanism in regulating transcription of these genes. Alternatively, the presence or absence of Bcl6 or Stat5 may be a major determinant for the transcriptional output of these genes, and HDACs may be present at constant low levels on all three genes. Thus, specific interactions of HDACs with the recruited transcription factors can be considered to fine-tune and modulate transcript levels. Similarly, the signal for p300 occupancy was relatively low on all three genes under the conditions of these experiments, suggesting that the transcription factor itself at these regulatory regions is a main determinant for transcriptional output, and p300 is a secondary determinant to better regulate and control the outcome of gene activation or repression. These relationships are discussed further in Chapter 5.

ChIP for p300 and Hdac3 was also carried out on adipocytes treated with GH for 24 hours, a later time point of treatment when Socs2 and Cish expression are greatly elevated and Bcl6 expression strongly repressed by GH (Figure 4.7, B). For all three genes, Bcl6 occupancy was higher in the absence of GH, and decreased with GH treatment while occupancy of Stat5 was lower in the absence of GH, and increased upon GH treatment, correlating with the reciprocal changes in occupancy of Bcl6 and Stat5 in response to GH previously observed (Figure 4.3 and 4.4). P300 occupancy was detected on all three genes in the absence of GH, and occupancy increased slightly on all three genes even with prolonged GH treatment. Occupancy of Hdac3 was detected at all three genes in the presence and absence of GH, but the changes in occupancy pattern in response to GH were variable. A possible slight increase in Hdac3 occupancy in response to GH was seen for Socs2, while Hdac3 occupancy levels remained about the same in the presence and absence of GH for Cish. Occupancy of Hdac3 on Bcl6 decreased slightly with GH treatment on Bcl6. Overall, Hdac3 and p300 occupancy signals were detectable

but relatively low under the conditions of these experiments, consistent with the extent of occupancy and changes observed in p300 and Hdac3 occupancy levels seen at 30 min of GH treatment.

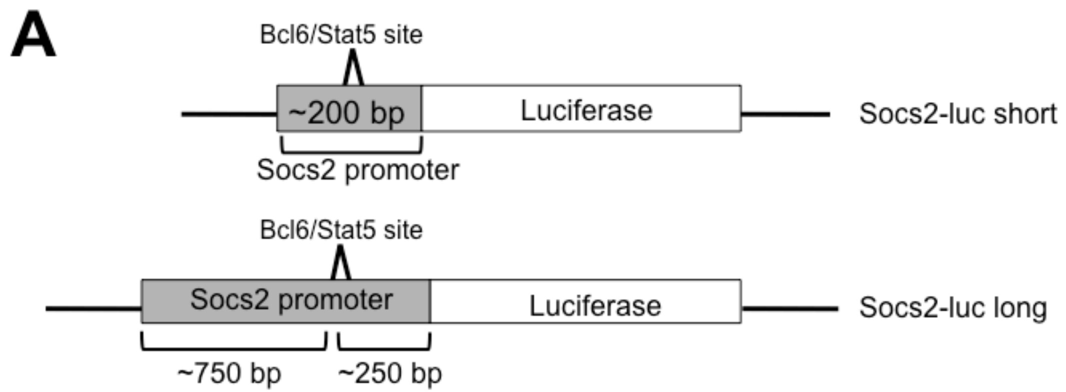
Socs2, Cish, and Bcl6 are differentially regulated by p300 and Hdac3

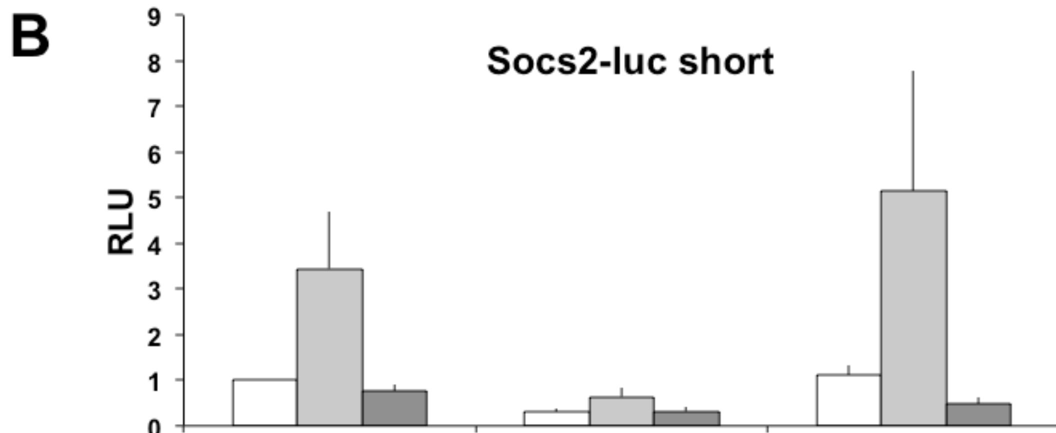
As p300 and Hdac3 occupancy were detected at the Bcl6/Stat5 regulatory regions of the Socs2, Cish, and Bcl6 genes in both the presence and absence of GH, but occupancy of these coregulatory molecules did not change markedly in response to GH treatment, it was of interest to evaluate the functional contributions of p300 and Hdac3 on Bcl6- or Stat5-mediated regulation of Socs2, Cish, and Bcl6 transcription. To evaluate the regulatory effects of p300 and Hdac3 on Bcl6- or Stat5-mediated regulation of Socs2, Cish, or Bcl6 expression, reporter gene assays were carried out. Reporter gene constructs in which the sequences for the Socs2, Cish, and Bcl6 promoters were fused upstream of the gene coding for firefly luciferase were generated for the three genes. For each promoter, a short and a long promoter construct were generated. The short promoter constructs (Figures 4.8-4.10, A, top schematics) each contained a 200 bp fragment with the Bcl6/Stat5 site identified by ChIP-Sequencing for the respective gene near the center of the fragment. In each case this approximately corresponds to the transcription start site. The long promoter constructs (Figures 4.8-4.10, A, bottom schematics) contained more of the native genomic sequence context, including about 750 bp upstream and 250 bp downstream of the Bcl6/Stat5 site for each gene. All constructs were tested for their responses to the presence of Bcl6, constitutively active Stat5b, p300, or Hdac3 individually, and Bcl6 or constitutively active Stat5 in combination with either p300 or Hdac3. Constitutively active Stat5b (CA-Stat5b) was used as both a representation of GH treatment and to pinpoint the contributions of Stat5b on the regulation of the promoter of each of the three genes.

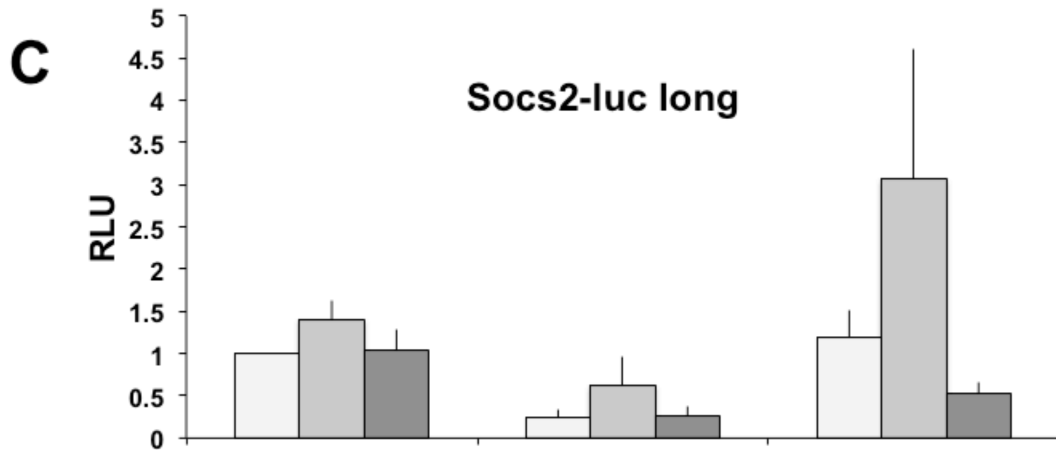
In the case of Socs2-luc short (Figure 4.8, B), p300 had an activating effect on basal Socs2-luc short expression (Figure 4.8, B, lanes 1, 2), while Hdac3 had a slightly repressive effect on basal Socs2-luc short expression (Figure 4.8, B, lanes 1, 3). Bcl6 inhibited Socs2 promoter activation (Figure 4.8, B, lanes 1, 4), correlating with previous

Figure 4.8

P300 and Hdac3 can modulate Stat5-mediated transcription of Socs2-luc short and Socs2-luc long. A. Schematic of the Socs2-luc short and Socs2-luc long constructs. A 200 bp fragment surrounding the Bcl6/Stat5 occupancy region (mouse chromosome 10: 94879550-94879558) in the Socs2 promoter was cloned upstream of luciferase and the construct designated as Socs2-luc short (top). A DNA fragment consisting of the genomic sequences about 750 bp upstream and about 250 downstream of the Bcl6/Stat5 occupancy region (mouse chromosome 10: 94879550-94879558) in the Socs2 promoter was cloned upstream of luciferase and the construct designated as Socs2-luc long (bottom). Transcription start site of the Socs2 gene: mouse chromosome 10: 94879491 (from NCBI Reference Sequences). **B.** Socs2-luc short was transfected into 293T cells in the presence or absence of Bcl6, constitutively active Stat5b (CA-Stat5b), p300, and Hdac3 alone or in combination as indicated. Luciferase activity is expressed as RLU (relative luciferase units) and normalized to basal Socs2-luc short values (lane 1), set as 1.0. Bars represent means and error bars show + standard errors from 7 to 10 independent observations. Results of all experiments were combined and graphed together, as fold changes of response over basal luciferase levels between overlapping conditions were comparable between experiments. Comparisons between lane 1 and lane 4, lane 1 and lane 6, lane 1 and lane 9, and lane 7 and lane 9 reached statistical significance ($p<0.05$). **C.** Socs2-luc long was transfected into 293T cells in the presence or absence of Bcl6, constitutively active Stat5b (CA-Stat5b), p300, and Hdac3 alone or in combination as indicated. Luciferase activity is expressed as RLU (relative luciferase units) and normalized to basal Socs2-luc long values (lane 1), set as 1.0. Bars represent means and error bars show + standard errors from 3 independent experiments. Comparisons between lane 1 and lane 4 reached statistical significance ($p<0.05$).







	1	2	3	4	5	6	7	8	9
Bcl6	-	-	-	+	+	+	-	-	-
CA-Stat5b	-	-	-	-	-	-	+	+	+
p300	-	+	-	-	+	-	-	+	-
Hdac3	-	-	+	-	-	+	-	-	+

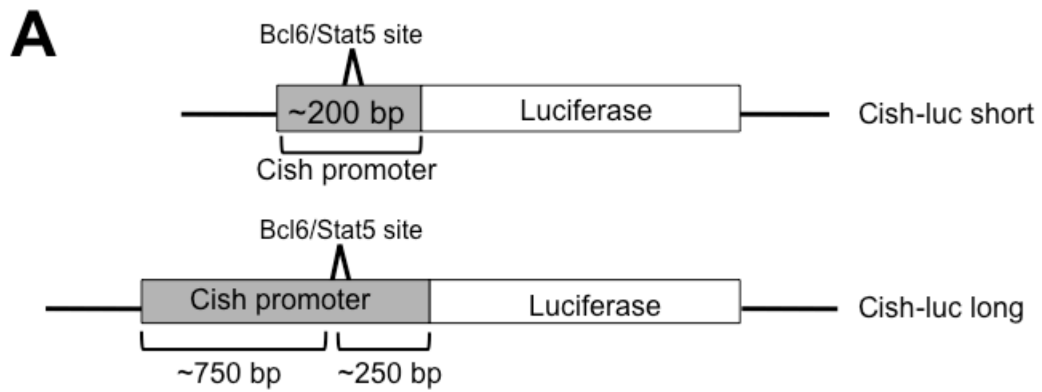
results (1). In the presence of Bcl6, neither p300 (Figure 4.8, B, lane 5) nor Hdac3 (Figure 4.8, B, lane 6) appeared to markedly alter reporter gene expression compared to the effects of Bcl6 alone (Figure 4.8, B, lane 4) on Socs2-luc short, although p300 may have a derepressive effect on Bcl6-mediated inhibition of Socs2-luc short expression (Figure 4.8, B, lanes 4, 5). The addition of CA-Stat5b alone did not significantly activate reporter gene expression over basal Socs2-luc short expression levels (Figure 4.8, B, lanes 1, 7). However, in the presence of CA-Stat5b, the presence of p300 activated reporter gene expression (Figure 4.8, B, lanes 7, 8), even further than p300 alone activated basal Socs2-luc levels (Figure 4.8, B, lanes 2, 8). In the presence of CA-Stat5b, the addition of Hdac3 inhibited reporter gene expression when compared to CA-Stat5b alone (Figure 4.8, B, lanes 7, 9), or Hdac3 alone (Figure 4.8, B, lanes 3, 9).

The relative regulatory effects of Bcl6, Stat5, p300, and Hdac3 on Cish-luc short reporter gene activation (Figure 4.9, B) appeared to be similar to those observed for Socs2-luc short (Figure 4.8, B). The presence of p300 appeared to slightly activate Cish-luc expression compared to basal levels (Figure 4.9, B, lanes 1, 2). The addition of Hdac3 appeared to have a slight repressive effect on basal reporter gene expression (Figure 4.9, B, lanes 1, 3). Bcl6 inhibited Cish-luc short reporter gene expression (Figure 4.9, B, lanes 1, 4). In the presence of Bcl6, neither p300 (Figure 4.9, B, lane 5) nor Hdac3 (Figure 4.9, B, lane 6) appeared to greatly alter reporter gene expression compared to the effects of Bcl6 alone (Figure 4.9, B, lane 4), suggesting that Bcl6 functions as a strong repressor of Cish transcription and this potent repression does not require the actions of p300 or Hdac3.

The addition of CA-Stat5b alone did not appear to activate reporter gene expression above basal levels (Figure 4.9, B, lanes 1, 7). However, in the presence of CA-Stat5b, the addition of p300 further activated reporter gene expression compared to the effects of CA-Stat5b alone (Figure 4.9, B, lanes 7, 8) or p300 alone (Figure 4.9, B, lanes 2, 8). In the presence of CA-Stat5b, the addition of Hdac3 had a repressive effect on reporter gene expression compared to basal Cish-luc short expression levels (Figure 4.9, B, lanes 1, 9),

Figure 4.9

P300 and Hdac3 can modulate Stat5-mediated transcription of Cish-luc short and Cish-luc long. A. Schematic of the Cish-luc short and Cish-luc long constructs. A 200 bp fragment surrounding the Bcl6/Stat5 occupancy region (mouse chromosome 9: 107198991-107199010) in the Cish promoter was cloned upstream of luciferase and the construct designated as Cish-luc short (top). A DNA fragment consisting of the genomic sequences about 750 bp upstream and about 250 downstream of the Bcl6/Stat5 occupancy region (mouse chromosome 9: 107198991-107199010) in the Cish promoter was cloned upstream of luciferase and the construct designated as Cish-luc long (bottom). Transcription start site of the Cish gene: mouse chromosome 9: 107199020 (from NCBI Reference Sequences). **B.** Cish-luc short was transfected into 293T cells in the presence or absence of Bcl6, constitutively active Stat5b (CA-Stat5b), p300, and Hdac3 alone or in combination as indicated. Luciferase activity is expressed as RLU (relative luciferase units) and normalized to basal Cish-luc short values (lane 1), set as 1.0. Bars represent means and error bars show + standard errors from 3 independent experiments. Comparisons between lane 1 and lane 4 reached statistical significance ($p<0.05$). **C.** Cish-luc long was transfected into 293T cells in the presence or absence of Bcl6, constitutively active Stat5b (CA-Stat5b), p300, and Hdac3 alone or in combination as indicated. Luciferase activity is expressed as RLU (relative luciferase units) and normalized to basal Cish-luc long values (lane 1), set as 1.0. Bars represent means and error bars show + standard errors from 3 independent experiments. Comparisons between lane 1 and lane 9 reached statistical significance ($p<0.05$).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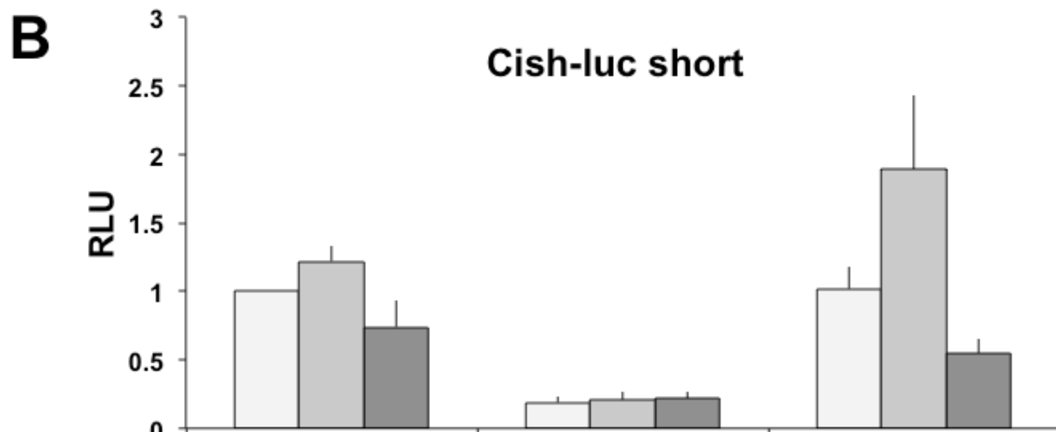
Luciferase
Cish-luc short
Cish promoter
Bcl6/Stat5 site
Cish promoter
Luciferase
Cish-luc long
~750 bp
~250 bp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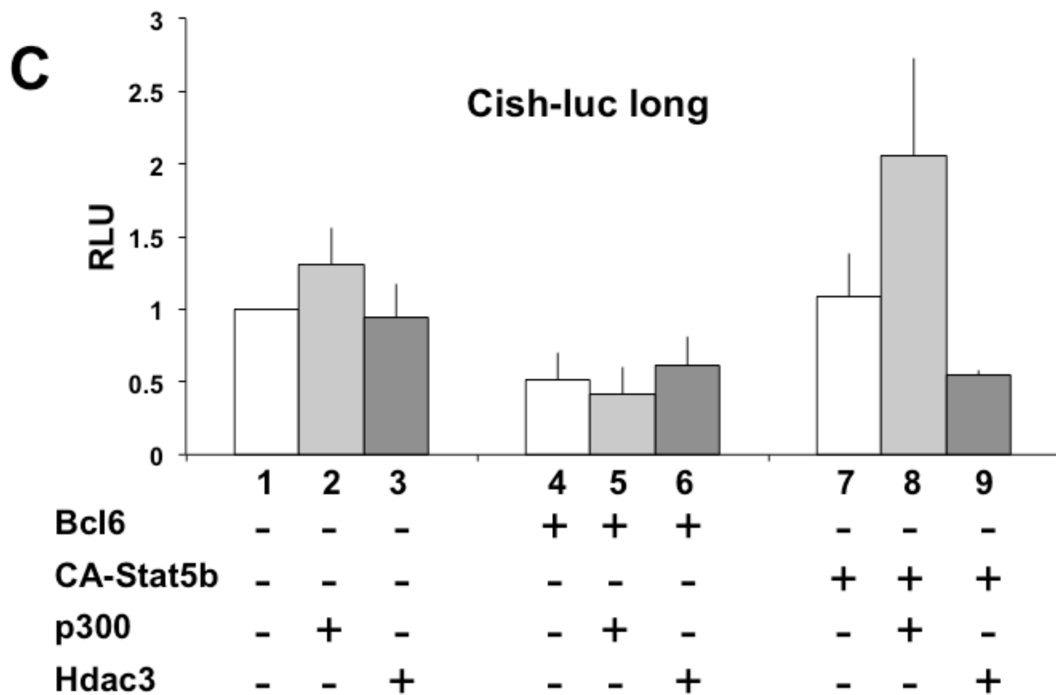
Cish-luc short
RLU
3
2.5
2
1.5
1
0.5
0


C
Cish-luc long
RLU
3
2.5
2
1.5
1
0.5
0
1 2 3 4 5 6 7 8 9
Bcl6 - - - + + + - - -
CA-Stat5b - - - - - - + + +
p300 - + - - + - - + -
Hdac3 - - + - - + - - +

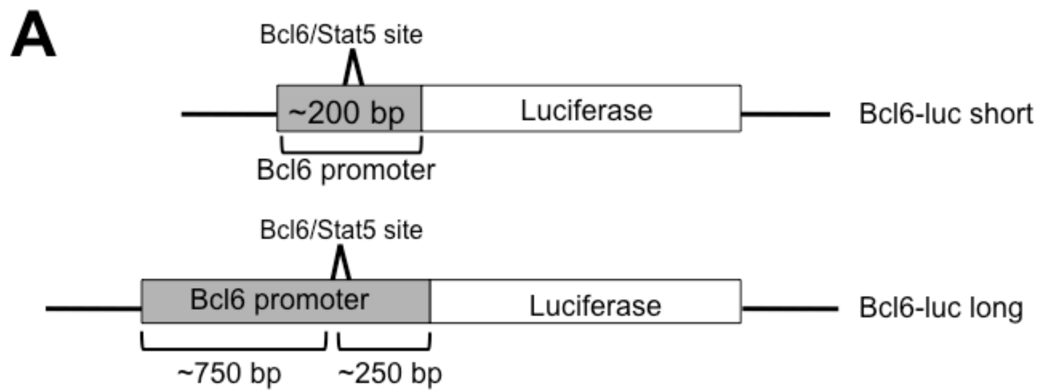
Cish-luc short in the presence of CA- Stat5b alone (Figure 4.9, B, lanes 7, 9) or Hdac3 alone (Figure 4.9, B, lanes 3, 9).

Regulation of the Bcl6/Stat5 regulatory sequence of Bcl6-luc short (Figure 4.10, B) appeared to be very different from regulation of Socs2-luc short (Figure 4.8, B) or Cish-luc short (Figure 4.9, B). Interestingly, both p300 (Figure 4.10, B, lane 2) and Hdac3 (Figure 4.10, B, lane 3) appeared to slightly elevate Bcl6-luc short expression compared to basal levels (Figure 4.10, B, lane 1). Bcl6 inhibited Bcl6-luc short expression (Figure 4.10, B, lanes 1, 4) as predicted, since Bcl6 is known to repress its own transcription (24). In the presence of Bcl6, neither p300 nor Hdac3 appeared to greatly alter reporter gene expression compared to the effects of Bcl6 alone (Figure 4.10, B, lanes 4, 5, 6), similar to what was seen for Bcl6-mediated inhibition of Socs2-luc short and Cish-luc short expression. The potential lack of coregulator involvement in Bcl6-mediated repression of the Socs2, Cish, and Bcl6 short promoter constructs suggests that Bcl6 functions as a potent repressor on all three short promoter constructs, and the functional effects of p300 and Hdac3 on the short luciferase constructs are primarily carried out through their interactions with Stat5 upon Stat5 recruitment to the promoter.

Surprisingly, the presence of CA-Stat5b alone was sufficient to markedly induce expression of Bcl6-luc short over basal levels (Figure 4.10, B, lanes 1, 7). This corresponds with previous results shown for the corresponding region of the human Bcl6 gene (27). Strikingly, the addition of p300 (Figure 4.10, B, lane 8) as well as Hdac3 (Figure 4.10, B, lane 9) in the presence of CA-Stat5b resulted in repression of Bcl6-luc short expression compared to the presence of CA-Stat5b alone (Figure 4.10, B, lane 7). In the case of Hdac3, reporter gene expression was reduced to basal levels (Figure 4.10, B, lanes 1, 9). These intriguing observations suggest that Stat5 may function as a repressor of Bcl6 expression *in vivo* with the aid of associated coregulatory proteins.

Figure 4.10

P300 as well as Hdac3 appear to have repressive effects on Stat5-mediated regulation of Bcl6-luc short and Bcl6-luc long. A. Schematic of the Bcl6-luc short and Bcl6-luc long constructs. A 200 bp fragment surrounding the Bcl6/Stat5 occupancy region (mouse chromosome 16: 23988676-23988725) in the Bcl6 promoter was cloned upstream of luciferase and the construct designated as Bcl6-luc short (top). A DNA fragment consisting of the genomic sequences about 750 bp upstream and about 250 downstream of the Bcl6/Stat5 occupancy region (mouse chromosome 16: 23988676-23988725) in the Bcl6 promoter was cloned upstream of luciferase and the construct designated as Bcl6-luc long (bottom). Transcription start site of the Bcl6 gene: mouse chromosome 16: 23965138 (from NCBI Reference Sequences). **B.** Bcl6-luc short was transfected into 293T cells in the presence or absence of Bcl6, constitutively active Stat5b (CA-Stat5b), p300, and Hdac3 alone or in combination as indicated. Luciferase activity is expressed as RLU (relative luciferase units) and normalized to basal Bcl6-luc short values (lane 1), set as 1.0. Shown are combined preliminary results from 2 experiments. Results were combined and graphed together for ease of comparison, as fold changes of response over basal luciferase levels between overlapping conditions were comparable between experiments. Overlapping conditions are graphed as the mean fold change values over basal from 2 experiments. **C.** Bcl6-luc long was transfected into 293T cells in the presence or absence of Bcl6, constitutively active Stat5b (CA-Stat5b), p300, and Hdac3 alone or in combination as indicated. Luciferase activity is expressed as RLU (relative luciferase units) and normalized to basal Bcl6-luc long values (lane 1), set as 1.0. Bars represent means and error bars show + standard errors from 3 independent experiments. Comparisons between lane 1 and lane 4, lane 1 and lane 5, lane 1 and lane 6, and lane 7 and lane 9 reached statistical significance ($p<0.05$).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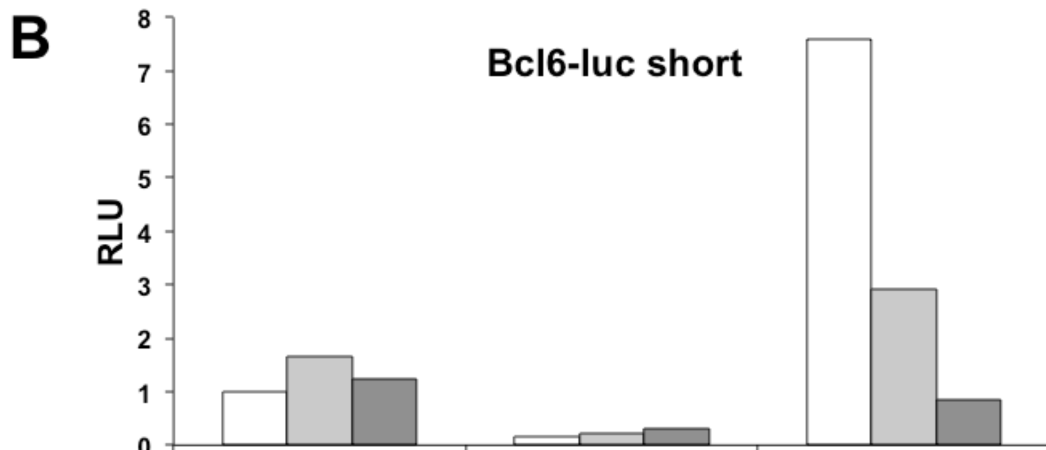
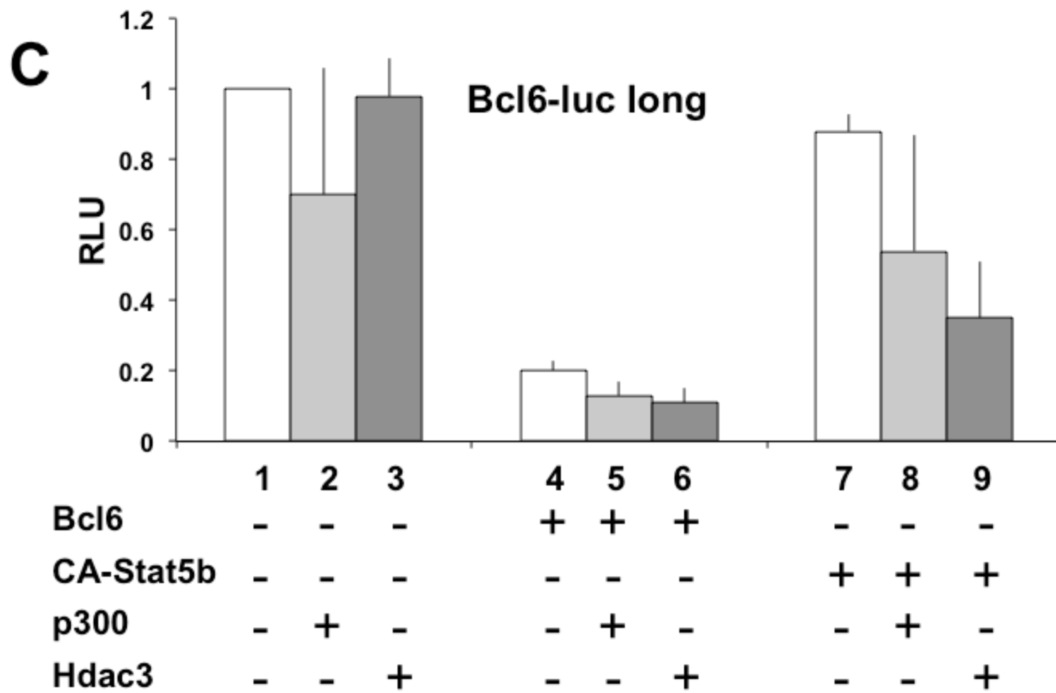
Luciferase
Bcl6-luc short
Bcl6 promoter
Bcl6/Stat5 site
Bcl6 promoter
Luciferase
Bcl6-luc long
~750 bp
~250 bp


B
Bcl6-luc short
RLU
8
7
6
5
4
3
2
1
0


C
Bcl6-luc long
RLU
1.2
1
0.8
0.6
0.4
0.2
0
1 2 3 4 5 6 7 8 9
Bcl6 - - - + + + - - -
CA-Stat5b - - - - - - + + +
p300 - + - - + - - + -
Hdac3 - - + - - + - - +

Regulation of Bcl6 expression is more dependent on promoter sequence context than Socs2 and Cish

To investigate if the genomic sequence context surrounding the Bcl6/Stat5 regulatory regions in the Socs2, Cish, and Bcl6 promoters had an effect on Bcl6- or Stat5-mediated transcriptional regulation of target gene expression, the regulatory effects of p300 and Hdac3 on Bcl6- or Stat5-mediated regulation of the long luciferase constructs were tested. For the Socs2-luc long construct (Figure 4.8, C), relative changes were similar to those seen for the Socs2-luc short construct (Figure 4.8, B). Interestingly, p300 and Hdac3 did not appear to greatly alter Socs2-luc long expression levels compared to basal reporter gene levels when added alone (Figure 4.8, C, lanes 1, 2, 3). Bcl6 inhibited reporter gene expression (Figure 4.8, C, lanes1, 4). In the presence of Bcl6, p300 (Figure 4.8, C, lane 5) may have a derepressive effect on Bcl6-mediated inhibition of Socs2-luc long expression when compared to Bcl6 alone (Figure 4.8, C, lane 4). However, Socs2-luc long expression levels in the presence of both Bcl6 and p300 (Figure 4.8, C, lane 5) are lower than in the presence of p300 alone (Figure 4.8, C, lane 2), once again demonstrating the strong inhibitory effects of Bcl6 on the Socs2 promoter. The addition of Bcl6 and Hdac3 together (Figure 4.8, C, lane 6) did not appear to greatly alter reporter gene expression compared to conditions with only Bcl6 added (Figure 4.8, C, lane 4).

The addition of CA-Stat5b alone did not activate Socs2-luc long above basal levels (Figure 4.8, C, lanes 1, 7). However, in the presence of CA-Stat5b, the addition of p300 further activated the promoter (Figure 4.8, C, lane 8) compared to CA-Stat5b alone (Figure 4.8, C, lane 7) or basal levels (Figure 4.8, C, lane 1). In the presence of CA-Stat5b, the addition of Hdac3 (Figure 4.8, C, lane 9) inhibited the promoter compared to Hdac3 alone (Figure 4.8, C, lane 3) or CA-Stat5b alone (Figure 4.8, C, lane 7).

Regulation of Cish-luc long by Bcl6, Stat5, p300, and Hdac3 (Figure 4.9, C) for the most part resembled observations for Cish-luc short (Figure 4.9, B). P300 appeared to have a slightly activating effect on basal Cish-luc long expression (Figure 4.9, C, lanes 1, 2) while Hdac3 had a slight repressive effect compared to basal (Figure 4.9, C, lanes 1, 3).

Bcl6 inhibited Cish-luc long expression (Figure 4.9, C, lanes 1, 4). However, in contrast to regulation of the three short promoter constructs and Socs2-luc long, where p300 appeared to either have potential derepressive effects or no marked effects at all on Bcl6-mediated inhibition of reporter gene expression, results for regulation of Cish-luc long show slight inhibitory effects for Bcl6 and p300 together compared to Bcl6 alone (Figure 4.9, C, lanes 4, 5) as well as relative to p300 alone (Figure 4.9, C, lane 2). Instead, Hdac3 appeared to have a slight derepressive effect on Bcl6-mediated inhibition of Cish-luc long expression (Figure 4.9, C, lanes 4, 6). However, in general, the presence of p300 or Hdac3 in addition to Bcl6 (Figure 4.9, C, lanes 5, 6) did not appear to greatly alter the effects of Bcl6-mediated repression of Cish-luc long expression compared to Bcl6 alone (Figure 4.9, C, lane 4).

CA-Stat5b did not greatly activate the Cish-luc long promoter above basal values (Figure 4.9, C, bars 1, 7). The presence of p300 in conjunction with CA-Stat5b (Figure 4.9, C, lane 8) further activated reporter gene expression compared to Cish-luc long in the presence of CA-Stat5b alone (Figure 4.9, C, lane 7), basal Cish-luc long expression levels (Figure 4.9, C, lane 1), or p300 alone (Figure 4.9, C, lane 2). In the presence of CA-Stat5b, the addition of Hdac3 (Figure 4.9, C, lane 9) further inhibited reporter gene expression compared to CA-Stat5b alone (Figure 4.9, C, lane 7) or Hdac3 alone (Figure 4.9, C, lane 3).

In light of the inhibition of Bcl6 mRNA expression by GH, relative to activation of Socs2 and Cish mRNA expression by GH, it is of great interest that regulation of Bcl6-luc long by Bcl6, Stat5, p300, and Hdac3 (Figure 4.10, C) appeared to be very different from the changes observed for regulation of Socs2-luc long and Cish-luc long (Figures 4.8, C and 4.9, C). The presence of p300 (Figure 4.10, C, lane 2) and Hdac3 (Figure 4.10, C, lane 3) did not appear to greatly alter Bcl6-luc long expression compared to basal levels (Figure 4.10, C, lane 1). The addition of Bcl6 strongly repressed Bcl6-luc long reporter gene expression compared to basal levels (Figure 4.10, C, lanes 1, 4). Interestingly, the addition of either p300 (Figure 4.10, C, lanes 4, 5) or Hdac3 (Figure 4.10, C, lanes 4, 6) in combination with Bcl6 appeared to further repress reporter gene expression to a slight, but

noticeable extent, compared to the repression seen in the presence of Bcl6 alone (Figure 4.10, C, lane 4).

In contrast to what was observed for Bcl6-luc short, the addition of CA-Stat5b did not activate Bcl6-luc long expression (Figure 4.10, C, lanes 1, 7). The presence of CA-Stat5b together with Hdac3 (Figure 4.10, C, lane 9) still resulted in further inhibition of Bcl6-luc long expression compared to Bcl6-luc long expression levels with CA-Stat5b alone (Figure 4.10, C, lane 7) or Hdac3 alone (Figure 4.10, C, lane 3). Similar to observations for Bcl6-luc short, p300 appeared to have the same repressive effect on Stat5-mediated activation of reporter gene expression (Figure 4.10, C, lanes 7, 8), once again suggesting that Stat5 might function as a transcriptional repressor of Bcl6 expression *in vivo* with the aid of coregulatory proteins.

Regulation of the Bcl6 promoter by Bcl6, Stat5, p300, and Hdac3 appears to be distinct from regulation of the Socs2 and Cish promoters. Interestingly, regulation of Bcl6-luc long (Figure 4.10, C) and Bcl6-luc short (Figure 4.10, B) also appeared to be different, demonstrating the complex layers of Bcl6 transcriptional regulation. These distinctive results for the Bcl6 promoter are consistent with a trend for Bcl6 transcription to be highly regulated by many intricate input signals, and correlate with the repression of Bcl6 expression upon GH treatment as compared to activation of Socs2 and Cish.

Discussion

Bcl6 and Stat5 reciprocally occupy regulatory regions of the Socs2, Cish, and Bcl6 genes

Socs2 expression is reciprocally regulated by Bcl6 and Stat5 in response to GH, with Bcl6 acting as a repressor in the absence of GH, and Stat5 functioning as an activator upon treatment, as shown in Chapter 2. High throughput ChIP-Sequencing allowed us to characterize the occupancy profiles of Bcl6 and Stat5 across the genome in the presence and absence of GH, as shown in Chapter 3. To identify other genes on which Bcl6 and

Stat5 share a DNA regulatory sequence, similar to Socs2, candidate ChIP-Sequencing peaks were selected and tested by ChIP for Bcl6 occupancy, Stat5 occupancy, and changes in Bcl6 and Stat5 occupancy in response to GH.

It was predicted that gene candidates would be identified that were reciprocally regulated in a manner similar to Socs2, as Stat5 is best known as a transcriptional activator, and is a well characterized GH-regulated transcription factor (52-56). Bcl6 is also best known as a transcriptional repressor; currently, in most documented examples where Bcl6 directly regulates expression of a target gene, it has functioned as a repressor (2, 57, 58). However, both Bcl6 and Stat5 associate with other nuclear protein factors, and the activity of these associated proteins on transcriptional regulatory complexes containing Bcl6 or Stat5, could also lead to loss of Bcl6 repression on target gene expression, or conversely, Stat5-mediated downregulation of transcription. Stat5 has been postulated to repress Bcl6 expression in immune cells (27), and is known to repress germline transcription from the Igk (immunoglobulin kappa chain complex) locus (59). A recent study proposed a role for Bcl6 in activating expression of Oat (organic anion transporter) 1 and Oat3 expression in rat kidney proximal tubule cells, which may be a rare example of Bcl6 functioning as a transcription activator (60). If such examples appeared among our candidate genes, they would provide exciting model systems to characterize the less well-studied mechanisms of Stat5-mediated repression or Bcl6-mediated activation.

Another potential possibility is that Bcl6 and Stat5 may work together to regulate expression of a target gene, but in a context where occupancy of these factors would not be reciprocal in response to GH. So far, no studies report that Bcl6 and Stat5 interact in a protein complex, and co-immunoprecipitation experiments using overexpressed Bcl6 and Stat5 together in 293T cells did not show interaction between the two factors (data not shown). However, it is possible that Bcl6 and Stat5 could both be occupying the same regulatory sequence on a gene using tandem or closely spaced consensus sequences, since Stat5 sites often occur in pairs or clusters (61, 62). If examples of this type of regulatory mechanism were identified and verified using the ChIP-Sequencing data, computational characterization of the occupancy sequences and comparison with other Bcl6 and Stat5

occupancy sequences would provide insight into potential sequence requirements for either Bcl6 or Stat5 occupancy. Experimental analysis of the binding kinetics and factor requirements for Bcl6 and Stat5 association with these genomic sequences would also provide endogenous models with which to examine the specificity requirements for a DNA sequence to serve as a Bcl6 binding site, a Stat5 binding site, or a dual Bcl6/Stat5 site.

Currently, the three reciprocally regulated gene candidates identified all contain a Bcl6/Stat5 regulatory site in the proximal promoter of the gene (designated as transcription start site by H-Peak). The occupancy patterns of Bcl6 and Stat5 at regulatory regions identified by ChIP-Sequencing for all three genes are also similar to each other. Bcl6 occupancy is high without GH and decreases with treatment, while the occupancy of Stat5 is low in the absence of GH and increases with GH treatment. Genomatix analysis of the Bcl6/Stat5 occupancy region for predicted Bcl6 or Stat5 binding sites revealed two potential occupancy sequences for Bcl6 and/or Stat5 on the Cish and Bcl6 genes (Figure 4.11). Currently it is unknown which sequences Bcl6 and Stat5 occupy, or if Bcl6 and Stat5 occupy the same binding motif on the Cish and Bcl6 promoters. Alternatively, Bcl6 and Stat5 could be occupying the Socs2, Cish, and Bcl6 promoters at as yet unidentified motifs.

The Bcl6/Stat5 occupancy regions for Socs2, Cish, and Bcl6 are located near the transcriptional start sites of the respective genes

Prior to experimental verification of the selected ChIP-Sequencing peaks, it was predicted that in addition to identifying regulatory sites in genomic sequences commonly associated with regulation of gene transcription, such as promoters, examples might also be discovered where Bcl6 and Stat5 regulate expression of target genes using genomic regulatory regions not commonly associated with direct regional regulation of transcription. Stat5, in particular, has been shown to regulate gene expression using intronic binding sites (61-63). Peaks with strong peak height or significant p values were predicted to be more likely to represent actual binding and regulatory events *in vivo*.

Stat5 binding consensus: TTC C(A>T)G GAA

Bcl6 binding consensus: TTC CT(A/C) GAA

Socs2: TTC CAG GAA

Cish: TTC CTG GAA TTC TTG GAA

Bcl6: TTC CTA GAA TTC TAA GAA

Figure 4.11

Predicted Bcl6/Stat5 binding motifs in the Socs2, Cish, and Bcl6 promoters as identified by ChIP-Sequencing. Bcl6 and Stat5 share a highly similar DNA binding consensus sequence; shown above are the Stat5 and Bcl6 binding consensus motifs for reference. Listed below are the Genomatix predicted Bcl6 or Stat5 binding sequences (boxed) identified in the region of Bcl6 occupancy detected on the mouse Socs2, Cish, or Bcl6 genes by ChIP-Sequencing. Location for Socs2 motif: chromosome 10: 94879550-94879558. Location for Cish motis: (Left) Chromosome 9: 107198991-107198999, (Right) Chromosome 9: 107199002-107199010. Location for Bcl6 motifs: (Left) Chromosome 16: 23988676-23988684, (Right) Chromosome 16: 23988717-23988725. At present it is unknown which motif Bcl6 and/or Stat5 occupies on the Cish and Bcl6 promoters, if Bcl6 and/or Stat5 can occupy both sequences, or if Bcl6 and/or Stat5 occupy the Cish and Bcl6 promoters at an as yet unidentified binding motif. Adapted from (3) and (56). (Genomatix analysis by ZS Qin.)

Among the 35 candidate ChIP-Sequencing peaks tested, many were located in upstream intergenic regions, introns, or downstream regulatory regions. However, of the peaks tested, the only regions that showed significant occupancy of both Bcl6 and Stat5 enriched over an IgG negative control, and the only occupancy regions that also showed reciprocal occupancy of Bcl6 and Stat5 in response to GH, were located near the transcription start sites of the Socs2, Cish, and Bcl6 genes.

Many transcription factors are thought to primarily regulate transcription from target gene promoters. For example, ChIP-Sequencing experiments have shown that Rap1 binding in yeast and PHA-4 binding in C. elegans show strong correlation of target gene expression when these transcription factors are bound at gene promoters, and binding of these factors at the promoter region of target genes was strongly enriched over binding at other genomic locations (64, 65). However, our ChIP-Sequencing results revealed widespread Bcl6 and Stat5 binding across the genome with a large number of sites showing only modest ChIP-Sequencing signal. Transcription factor occupancy of either Bcl6 or Stat5 was also not preferentially enriched on promoter regions in our ChIP-Sequencing results (Table 4.2). As discussed in Chapter 3, this widespread binding may be a mechanism by which the level of a transcription factor is buffered in the cell, with lower affinity binding sites serving as a sequestration mechanism (66). Another possibility is that these transcription factors may be participating in different functions at these secondary or weaker sites, such as chromatin remodeling or maintenance of nuclear architecture, and not functioning as canonical transcription factors (66). Widespread binding of transcription factors in genomic locations outside of gene promoters has also been observed in other ChIP-Sequencing studies for Stat5 using mouse embryonic fibroblasts (MEFs) from wild type or Stat5a knock out mice treated without or with GH for 45 minutes (67). Interestingly, of the genes identified in the MEFs to have Stat5 occupancy at promoter or upstream regions correlating with a strong Stat binding motif at the occupancy region, the only genes shown to be highly regulated by Stat5 and responsive to GH were Socs2, Cish, Bcl6, and the SOCS family member Socs3. Bcl6 occupancy was not detected on Socs3 in our ChIP-Sequencing results, and our profile of GH-regulated genes showed that Socs3 was transiently induced by GH at the earliest time after treatment

Immunoprecipitation:	Bcl6		Stat5	
GH treatment:	0 h	48 h	0 h	48 h
Total Peak count:	3777	1549	1329	937
3'UTR (%)	1.1	1.2	0.7	1.5
5'UTR (%	7.1	7.4	4.2	7.5
Exon (%)	2.7	3.0	1.2	1.6
Intergenic (%)	58.3	59.9	68.7	64.8
Intron (%)	29.1	25.6	24.9	24.5
TSS region (%)	1.7	2.9	0.2	0.3

Table 4.2

Analysis of Bcl6 and Stat5 occupancy peak locations in ChIP-Sequencing results. (Data analysis by Rajasree Menon.)

(30 min). As Socs2, Cish, and Bcl6 were some of the genes most strongly regulated by GH at later times in our gene profile (1), regulation of these genes by Bcl6 and Stat5 may be an indicator of highly GH responsive genes. As the Stat5 ChIP-Sequencing study in MEFs excluded Stat5 sites that were not in promoter or upstream regions, and selected for Stat5 occupancy sites with a strong Stat binding consensus sequence (67), it remains to be determined if other Stat5 occupancy sites detected by ChIP-Sequencing in MEFs regulate expression of target genes.

In light of the widespread binding of Bcl6 and Stat5 detected across the genome in our ChIP-Sequencing results, it is striking that Bcl6 and Stat5 were found to occupy the transcription start sites of all three of the reciprocally regulated gene candidates. Socs2, Cish, and Bcl6 were also clustered together in the group of genes found to be most strongly regulated by GH at later time points according to CRC analysis of the microarray of GH-treated adipocytes (1). It is currently unknown if there are other examples of reciprocally occupied Bcl6 and Stat5 sites in the genome; however, the current data lead to the postulation that reciprocal regulation of a GH target gene by Bcl6 and Stat5 is a mechanism used for regulating transcription of highly responsive GH target genes, and that the Bcl6/Stat5 regulatory region for such genes trend toward being located in the proximal promoter. Proximity to transcription start sites correlating with gene activation has also been demonstrated for PPARγ and C/EBPα, two key transcription factors involved in regulating genes mediating the adipogenic differentiation program (68). The location of the Bcl6/Stat5 regulatory region in Socs2, Cish, and Bcl6 also suggests that Bcl6 and Stat5 may tend to function as transcription factors when located relatively near a gene promoter and perhaps function differently as chromatin-associated proteins when occupying other genomic regions. Further bioinformatic mining of ChIP-Sequencing and gene expression data, and experimental verification of predicted Bcl6/Stat5 regulatory regions, may identify additional reciprocally regulated gene candidates and provide information on functional or structural relationships among these Bcl6- and Stat5-regulated genes.

Conversely, novel candidate Bcl6 target genes identified by ChIP-Sequencing may be more likely to be direct Bcl6 targets when the predicted Bcl6 binding site is located closer to the gene promoter. Proximity has guided our approach towards analyzing and prioritizing candidate Bcl6 target genes identified through ChIP-Sequencing: Genes that contained a Bcl6 occupancy peak within 10 kb of the gene promoter in the absence of GH were selected for functional bioinformatics analyses. Similarly, of the genes identified as top candidates for being GH-regulated Bcl6 target genes by comparing the ChIP-Sequencing and microarray data, genes with predicted Bcl6 binding sites in upstream promoter, 5' UTR regions, or within the first intron are considered top priority candidates for experimental validation and further exploration.

Transcription of Socs2, Cish, and Bcl6 is differentially regulated by Bcl6 and Stat5

The occupancy patterns for Bcl6 and Stat5 on Socs2, Cish, and Bcl6 were similar for all three genes. Occupancy of Bcl6 was high in the absence of GH and decreased with GH treatment, while occupancy of Stat5 was low in the absence of GH and increased with GH. However, Bcl6 stands out, since Socs2 and Cish mRNA expression is induced by GH while Bcl6 expression is inhibited (1), suggesting that the transcriptional consequences mediated by Bcl6 and Stat5 on expression of the three genes is different. To better characterize the transcriptional state of these genes in response to GH, and in the presence or absence of Bcl6 and Stat5, ChIP was carried out to probe for changes in the association of histone modification marks at the Bcl6/Stat5 regulatory site. Histone marks associated with active transcription and open chromatin structure were examined at an early treatment time after GH treatment (30 min) when the reciprocal switch of Bcl6 and Stat5 is already known to occur, based on previous observations (Figure 2.8).

ChIP revealed a marked increase in AcH3 and AcH4 on the Socs2 and Cish promoters in response to GH, corresponding to induction of Socs2 and Cish mRNA expression by GH, in keeping with AcH3 and AcH4 being histone marks associated with active transcription and accessible chromatin structure (32, 34, 69, 70). These results were similar to previous results demonstrating an increase in AcH3 and AcH4 at the promoters of Socs2 and Cish

in response to GH treatment (60 min) in rat liver (17). In contrast, on the Bcl6 promoter, AcH3 and AcH4 were highly enriched over background signal; however, the extent of enrichment did not significantly increase in response to GH, correlating with Bcl6 repression by GH. H3K4me3, another histone mark associated with active transcription (32, 71), was found to be strongly enriched at all three promoters. These results suggest that Socs2, Cish, and Bcl6 are located in open chromatin and these genes are poised to be actively transcribed and regulated.

Histone marks commonly associated with heterochromatin and untranscribed chromatin were not found to be enriched at the Socs2, Cish, and Bcl6 promoters, suggesting that the regulatory regions of these genes need to remain accessible to allow rapid modulation of transcription in response to stimuli. It is likely that the repression of Bcl6 expression in response to GH is regulated by the binding of protein factors at inhibitory regulatory regions in the gene, and it is speculated that restructuring of the chromatin state of the gene plays a lesser role than repressor binding in controlling transcriptional output. Some of the most likely candidates for inhibitory factors repressing Bcl6 expression in response to GH are Bcl6 and Stat5 themselves, as Bcl6 is well known as a transcriptional repressor, and Stat5 has been found to repress Bcl6 expression in response to cytokine treatment in immune cells (27, 57).

Beyond Socs2: Bcl6 and Stat5 regulate expression of Cish and Bcl6 in response to GH in adipocytes

After the identification of Socs2 as a novel Bcl6 target gene, and characterization of reciprocal regulation of Socs2 expression by Bcl6 and Stat5 in response to GH, it was hypothesized that Bcl6 would likely regulate the expression of other GH target genes in adipocytes, and the mechanism of reciprocal regulation of transcription by Bcl6 and Stat5 would apply to other Bcl6 or Stat5 target genes beyond Socs2. As reported in Chapter 3, high throughput ChIP-Sequencing techniques (72-74) and genome-wide bioinformatic analyses (75) were applied to identify both novel Bcl6 target genes in adipocytes and other GH target genes reciprocally regulated by Bcl6 and Stat5. Among the candidate

Bcl6 target genes identified using this approach, Cish and the Bcl6 gene itself were shown to be regulated by Bcl6 using promoter construct reporter gene assays (Figure 4.9, 4.10, lanes 1 and 4), and ChIP has shown reciprocal occupancy of Bcl6 and Stat5 on the same DNA regulatory region of these genes (Figure 4.3).

Cish is a known GH and Stat5 target gene, and Bcl6 occupancy at the Cish promoter has been demonstrated previously (17). However, this is the first example where Bcl6 inhibition of the Cish promoter has been demonstrated.

Bcl6 is known to repress its own transcription using upstream Bcl6 binding sites (24-26). It has also been shown that Bcl6 expression is regulated by Stat5, with Stat5 acting as either an activator or repressor depending on cell type and promoter sequence context (27-31). The functional promoter analyses in this chapter are the first examples showing Bcl6 and Stat5 functioning to regulate Bcl6 expression using the same regulatory region. However, it should be noted that there are two predicted Bcl6 and/or Stat5 binding sites in the Bcl6/Stat5 occupancy regions identified by ChIP-Sequencing for the Cish and Bcl6 genes (Figure 4.11). It is currently unknown which occupancy site or sites Bcl6 and Stat5 occupy in these regulatory regions. However, the occupancy pattern of Bcl6 and Stat5 on this region in response to GH is reciprocal in adipocytes, suggesting that Bcl6 plays the major regulatory role in the absence of GH, and Stat5 occupancy of the regulatory region upon GH treatment mediates Cish and Bcl6 expression in response to GH.

The complex relationship of Stat5-mediated transcriptional regulation of Bcl6 expression

In preadipocytes and adipocytes, GH, insulin, and EGF strongly inhibit Bcl6 gene expression (Figure 2.1). Bcl6 promoter assays support that downregulation of Bcl6 transcription may be mediated at least partially by Stat5 in conjunction with associated coregulatory molecules such as Hdac3, suggesting that Stat5 might function as a transcriptional repressor for Bcl6 and inhibit Bcl6 expression (Figure 4.10). GH also strongly inhibits Bcl6 expression in liver (76) and muscle (77) as well as adipose tissue

(C LaPensee, unpublished data). However, the relationship between Stat5 and the regulation of Bcl6 expression appears to be extremely complex and to depend heavily on cell-specific or possibly stimulation specific or chromatin specific context.

Stat5 inhibits Bcl6 expression in T follicular helper cells (78), and was found to negatively regulate Bcl6 expression in interleukin 7-dependent B cell precursors (79). Stat5a was also shown to inhibit Bcl6 mRNA expression in several breast cancer cell lines in response to prolactin stimulation (28). Erythropoietin, an activator of Stat5, is likely to repress Bcl6 expression in erythroblasts in a Stat5-dependent manner (80), and activation of Stat5 downregulates Bcl6 expression in B-lymphoma cells and other hematopoietic cell lines (27). However, other reports have indicated that Stat5 induces Bcl6 expression in primary human B cells (30, 31). Stat5 also appears to activate Bcl6 expression in pancreatic beta cells in response to prolactin (29). It has been proposed, and demonstrated using reporter gene assays, that the effect of activated Stat5 on Bcl6 gene expression depends highly on the specific promoter sequence context included (27). These assays (27) (Chapter 4) have also demonstrated that there are likely multiple transcription factor binding sites and regulatory regions dispersed throughout the Bcl6 gene, such that the integrated input from transcriptional complexes occupying the various regulatory regions under any condition in a specific cell type determines the final transcriptional outcome. Therefore, although Stat5 appears to inhibit Bcl6 expression in most of the examples studied so far, it is also possible that Stat5 acts as an activator of Bcl6 expression in specific cells types under specific conditions and chromatin structures.

One possible explanation for the different regulatory effects of Stat5 on Bcl6 gene expression in different cell types may be differences in expression level or tissue specificity of various coregulatory molecules. Many coregulatory molecules are known to be rate-limiting or have tissue-specific distribution (41, 81).

Another possibility for varied responses of the Bcl6 promoter to Stat5 is that the chromatin structure of the Bcl6 gene may vary in different cell types, and Stat5 may have access to different sets of occupancy sites in different tissues. This hypothesis is

supported by the observations of the differences between Stat5-mediated transcriptional regulation of the Bcl6-luc short (Figure 4.10, B) and Bcl6-luc long (Figure 4.10, C) constructs. The presence of constitutively active Stat5b alone was sufficient to induce Bcl6-luc short expression over basal levels, similar to what had been shown previously for the homologous sequence from the human Bcl6 gene (27). This is in contrast to what was seen for Stat5-mediated regulation of Socs2-luc short and Cish-luc short, where CA-Stat5b alone did not appear to be sufficient to activate reporter gene expression. However, in the case of Bcl6-luc long, the addition of CA-Stat5b did not induce reporter gene expression above basal levels, suggesting that there are inhibitory sequences present in the longer Bcl6 promoter construct that were absent in the shorter one. These inhibitory sequences may be differentially accessible in different cell types. This is supported by observations from promoter luciferase experiments carried out using the homologous long promoter sequence from the human Bcl6 gene (27). When a longer sequence of the human Bcl6 gene was included in the promoter construct, CA-Stat5 repressed reporter gene expression (27), suggesting that Stat5 regulation of the Bcl6 promoter is different between human and mouse, likely partially due to sequence or chromatin context.

A third possibility is that the amount of Stat5 varies in different cells types, resulting in different occupancy patterns of Stat5 on the Bcl6 gene in different tissues, and potentially leading to the formation of different Stat5 transcriptional regulatory complexes at multiple locations in the Bcl6 gene. A recent study has shown that sporadic Stat5 occupancy at DNA sequences with a Stat binding motif increased in cells overexpressing Stat5 compared to cells expressing endogenous levels of Stat5 (67). However, the increased Stat5 binding did not correlate with increased expression of nearby genes or confer GH-responsiveness to these genes.

Future work to understand the similarities and differences in how Stat5 regulates Bcl6 gene expression in response to different growth factors and in different cell types, and the potential cooperative or conflicting effects of Bcl6 and Stat5 on Bcl6 gene expression,

will help us understand more about how Bcl6 is regulated, its potential physiological functions, and regulation of gene transcription in general.

Potential differential regulatory mechanisms for Socs2, Cish, and Bcl6 expression: The role of coregulators in Bcl6/Stat5-mediated transcriptional regulation

One of the potential mechanisms by which Bcl6 and Stat5 may be carrying out differential regulatory effects on Socs2, Cish, and Bcl6 expression is the recruitment of coregulatory proteins to the transcriptional regulatory complex. Coregulatory proteins are protein factors that interact with transcription factors to activate or repress transcription (6, 7, 82). Coregulators do not directly bind to the DNA, and often carry out their actions by modifying the activity of transcription factors or the structure of the surrounding chromatin (6, 8, 69). Coregulators that enhance transcription are known as coactivators, while coregulators that function to repress transcription are known as corepressors. In the past, molecules such as p300 and CBP have been considered coactivators, due to their lysine acetyltransferase activity and ability to acetylate histone tails, resulting in a relaxed chromatin structure conducive to transcription. Molecules such as histone deacetylases (HDACs) have traditionally been considered corepressors due to their lysine deacetylase activity, and the ability to deacetylate histone tails, resulting in a compacted and inaccessible chromatin state (32-34, 69, 70). However, p300/CBP and HDACs can target other substrates besides histones (83, 84), and more recently, examples of p300 repressing gene expression (9) and HDAC molecules participating in transcriptional activation have been described (10, 11).

Both Bcl6 and Stat5 are reported to interact with many coregulatory proteins in various transcriptional complexes to mediate their effects on expression of a target gene (19, 24, 58, 85). Therefore, it was considered likely that coregulatory molecules would participate in the reciprocal regulation of Socs2 expression by Bcl6 and Stat5 in response to GH. It was also considered likely that either the same, or a different subset of coregulators would be involved in regulation of Cish and Bcl6 expression by Bcl6 and Stat5.

Previous work characterizing the roles of corepressors in Bcl6-mediated transcriptional regulation focused on the role of Bcl6 in the immune system (16, 24, 58, 86). While the role of Bcl6 in adipocytes and mechanisms by which it regulates GH target genes is still unclear, several of the characterized Bcl6 coregulators, such as p300, CBP, Hdac1, and Hdac3 are known to be present in adipocytes and play roles in regulation of GH target genes by other transcription factors such as Stat5 and C/EBPβ (19, 47, 48, 85, 87). P300, in particular, has been shown to acetylate Bcl6 at a KKYK motif in the middle region of the protein (Figure 1.1). Acetylation of Bcl6 leads to the disruption of Bcl6 interactions with certain corepressive molecules, resulting in loss of Bcl6 inhibition on some target genes (12). The role of p300 as a coactivator of Stat and Stat5 target genes has been well described (20, 43).

In the quest to evaluate whether candidate coregulator molecules could be mediating the differential regulatory effects of Bcl6 and Stat5 on Socs2, Cish, and Bcl6 expression, the activities of two coregulators, p300 and Hdac3, were examined in more detail by promoter activation assays and occupancy on target genes by ChIP.

P300 in regulation of Bcl6- or Stat5-mediated transcription of Socs2, Cish, or Bcl6

Bcl6 and p300 in regulation of Socs2 and Cish

ChIP detected p300 occupancy at the Socs2, Cish, and Bcl6 promoters in 3T3-F442A adipocytes. Occupancy appeared to increase in response to GH for all three genes, but the increase was most detectable on the Socs2 promoter. Results from promoter construct reporter gene assays carried out in 293T cells suggest that in the case of Socs2 and Cish, p300 does not appear to greatly alter Bcl6-mediated inhibition of reporter gene expression, although p300 may have a derepressive effect on Bcl6-mediated repression of the Socs2 promoter. Bcl6 appears to function as a potent transcription repressor of Socs2 and Cish expression, and Bcl6 may be inhibiting Socs2 and Cish expression by associating with other as yet unidentified coregulatory molecules to repress gene transcription. Socs2 and Cish promoter activation assays would suggest that p300-

mediated inhibition of Bcl6 repression is likely not a mechanism by which Socs2 and Cish expression is highly induced by GH. However, significantly elevated Socs2 mRNA levels were observed in livers of mice deficient in Bcl6 (Bcl6 knock out mice) compared to livers of wild type mice, suggesting that loss of Bcl6 occupancy may result in significant derepression of Socs2 expression and activation of transcription *in vivo*. Due to the similarity in regulation of Socs2 and Cish by Bcl6 and Stat5, a similar mechanism may be involved in regulation of Cish transcription *in vivo*. As informative as it is to analyze promoter function under controlled experimental conditions, differences have been observed between regulation of gene expression in reporter gene assays compared to regulation of endogenous genes by transcription factors and associated coregulators where the activity of synthetic promoter fragments in response to p300/CBP did not match the activity observed for endogenous genes in MEFs (88). These differences may be due to differences in chromatin structure between the promoter constructs and the endogenous gene promoter, or due to different levels of protein factors between cell types.

Stat5 and p300 in regulation of Socs2 and Cish

For the Socs2 and Cish promoters, p300 provided a coactivating effect on Stat5-mediated transcription of reporter gene expression on both the short and long promoter constructs, consistent with p300 functioning as a coactivator for Stat5, and activated Stat5 inducing Socs2 and Cish expression in response to GH (1). Interestingly, CA-Stat5b alone did not appear to be sufficient to activate Socs2 or Cish reporter gene expression above basal levels, and in some experiments, a slight inhibitory effect was seen. This would correspond with previous results seen for activation of Cish expression by Stat5 in response to Il3 stimulation; constitutively active Stat5 alone was not sufficient to activate Cish expression, and the presence or activity of other factors was necessary to achieve Stat5-mediated transcriptional activation of Cish expression (10). Previous promoter activation assays for Socs2-luc demonstrated that treatment with GH results in induction of Socs2-luc expression (Figure 2.7) (1, 89). As it appears that p300, in addition to activated Stat5, is necessary for Stat5-mediated activation of Socs2 and Cish expression,

GH treatment may result in the recruitment or activation of p300 activity, thereby stimulating Stat5-mediated activation of Socs2 and Cish.

Strikingly, in the case of the Socs2-luc short promoter construct, the addition of p300 alone greatly activated basal reporter gene expression, without addition of CA-Stat5b. This may be due to the high basal levels of Stat5 and phosphorylated Stat5 in 293T cells (A Taylor, G Lin, unpublished data). It is likely that the activating effects of overexpressed p300 on basal Socs2-luc short reporter gene expression is at least partially due to the activity of endogenous Stat5 present in 293T cells. The activation of Socs2-luc short in the presence of p300 and endogenous Stat5 would be consistent with the activation of the Socs2 promoter seen when both CA-Stat5b and p300 are transfected into the cells. The hypothesis that endogenous Stat5 can activate reporter gene expression above basal values when p300 is transfected into the cells is supported by preliminary experiments where the activation of basal Socs2-luc short expression by the addition of p300 was inhibited in cells treated with the Jak/Stat inhibitor AG490 (data not shown). Due to the high levels of endogenous Stat5 in the 293T cell line, future experiments characterizing the effects of Stat5 and its associated coregulatory molecules on Socs2, Cish, and Bcl6 expression should likely be carried out in a different cell line or focus on observing the effects of Stat5 depletion on gene expression. Examining the regulatory effects of p300 on Socs2 expression by depletion of Stat5 may also help clarify if the derepressive effect of p300 on Bcl6-mediated inhibition of the Socs2 promoter is due to potential competitive effects of endogenous Stat5 on the Socs2 promoter constructs. Future experiments where levels of Bcl6 and Stat5 are titrated and the binding kinetics of Bcl6 and Stat5 are determined will also help in understanding the interplay between Bcl6 and Stat5 on determining gene expression.

P300 in Bcl6- and Stat5- mediated regulation of Bcl6 gene expression: Bcl6-luc short

In the case of Bcl6, when the short promoter fragment of the Bcl6 gene was used to drive reporter gene expression, preliminary results showed that p300 did not appear to greatly alter Bcl6-mediated repression of reporter gene expression, similar to what was seen for

Socs2 and Cish. In contrast to the Socs2 and Cish short promoter constructs, CA-Stat5b alone was sufficient to induce Bcl6-luc short expression, and p300 appeared to have an inhibitory effect on Stat5-mediated transcription of Bcl6 reporter gene expression.

One possible explanation for the difference in ability of CA-Stat5b to activate reporter gene expression from the Socs2, Cish, and Bcl6 short promoter constructs is that a putative potentiating factor necessary for activation of Stat5 activity is present on the Bcl6 promoter and absent on the Socs2 and Cish promoters. Another possibility is that there are different subsets of genes that can be activated by different post-translationally modified forms of Stat5. For example, in addition to its tyrosyl phosphorylation, Stat5 can be phosphorylated at different serine residues. Different phosphorylated forms of Stat5 may then result in the recruitment or dissociation of various protein factors to the transcriptional machinery.

Transcription factor complex specific interactions between p300 and the hypothetical Stat5 potentiating factor or different post-translationally modified forms of Stat5 could also explain the repressive activities of p300 on Stat5-mediated activation of Bcl6-luc short expression compared to the coactivation effects of p300 on Stat5-mediated induction of Socs2 and Cish short promoter construct expression. At the moment it is unknown if these preliminary results are representative of Stat5 and p300 regulatory interaction on the short Bcl6 promoter construct. If these results are consistent, they could be a rare example of Stat5 functioning as a transcriptional repressor and p300 functioning as a corepressor, likely using novel, and as yet unknown, mechanisms. One possible mechanism may be that p300 is regulating or interacting with other Stat5-associated proteins to regulate the net activity of the transcriptional complex, rather than p300 functioning as a histone acetylase to relax chromatin structure in order to promote transcription.

P300 in Bcl6- and Stat5- mediated regulation of Bcl6 gene expression: Bcl6-luc long

Interestingly, when a longer segment of the Bcl6 promoter was used in the reporter gene construct to evaluate sequence context effects on transcriptional regulation, results indicated that p300 appeared to have a corepressive effect on Bcl6-mediated transcriptional repression of reporter gene expression. One possibility for this observation is again that p300 is acting as a scaffold protein to recruit other factors to Bcl6 to repress transcription, and is not functioning to acetylate Bcl6 and inhibit Bcl6 repressive activity, or acetylate histones to relax the surrounding chromatin structure and promote transcription. Similar to what was seen for Stat5-mediated transcription of the Bcl6 short promoter construct, p300 appeared to have a repressive effect on Stat5-mediated reporter gene expression of the Bcl6 long promoter construct. This suggests that p300 could potentially function as a Stat5-associated corepressor when regulating Bcl6 expression *in vivo*. The differences in transcriptional regulation by Bcl6, Stat5, and p300 between the Bcl6 short promoter construct and Bcl6 long promoter construct also suggest that there are multiple regulatory regions in close proximity on the Bcl6 promoter, and the sum effects of various input signals determine the final level of Bcl6 transcription. This is in contrast to regulation of Socs2 and Cish promoter construct expression, where the regulatory effects of Bcl6, Stat5, and p300 appear to be generally similar for both the long and short promoter constructs, suggesting that the major Bcl6, Stat5, and p300 regulatory regions for the Socs2 and Cish long promoter constructs are located within the sequence used in the short promoter constructs.

P300 is structurally and functionally similar to CBP, and the two proteins belong to the same family of transcriptional coregulators (90-92). It is predicted that regulation of CBP activity is similar to that for p300. As many of the signaling pathways regulating p300 activity are activated in response to GH, it would be of interest for future work to involve pinpointing the molecular mechanisms by which p300 interacts with Bcl6 or Stat5 in response to GH to regulate target genes, and elucidating the effects of GH signaling on post-translational modification and function of these proteins and their interactions. Similarly, it would be of interest to see if CBP participates in regulation of Socs2, Cish,

and Bcl6 expression, or if p300 and CBP target different subsets of GH or Bcl6/Stat5 reciprocally occupied target genes.

Hdac3 in regulation of Bcl6- or Stat5-mediated transcription of Socs2, Cish, or Bcl6

Bcl6 has been shown to interact with Hdac3, and more recently, a high throughput sequencing study has shown that Bcl6 and Hdac3 occupancy correspond on a subset of Bcl6 target genes in immune cells (16, 51, 58, 93). Many of these genes are related to inflammatory responses, and LPS treatment induced the recruitment of p300 to these regulatory regions resulting in increased histone acetylation. Another high throughput sequencing study has hinted at a role for Hdac3 in the regulation of lipid metabolism genes in liver in response to circadian rhythm (50). The role of Hdac3 in Stat5-mediated transcriptional regulation has not been well studied; however, two other class I HDACs, Hdac1 and Hdac2, have both been shown to be important in Stat5-regulated gene transcription and adipogenesis (85, 87, 94). Another report has shown evidence that activation of Stat5 at certain target genes may be dependent on the activity of one or more HDACs by an as yet unidentified mechanism (10), further supporting an important role for HDACs in Stat5-regulated gene transcription. In this chapter, ChIP probing for histone modifications also showed an increase in activating histone mark enrichment at the Socs2 and Cish promoters with GH. In addition, qPCR experiments carried out on 3T3-F442A preadipocytes treated with the class I and class II HDAC inhibitor trichostatin A showed that induction of Socs2 mRNA by GH was inhibited in the presence of HDAC inhibitor, suggesting that HDACs may factor in GH stimulation of Socs2. On the other hand, Bcl6 mRNA levels were elevated in cells treated with trichostatin A. These results support roles for HDACs and other coregulatory or chromatin remodeling factors in Socs2, Cish, and Bcl6 transcription in adipocytes.

Bcl6 and Hdac3 in regulation of Socs2 and Cish

ChIP detected occupancy of Hdac3 at the Socs2, Cish, and Bcl6 promoters in 3T3-F442A adipocytes. Occupancy of Hdac3 generally appeared constitutive and did not change

markedly with GH treatment for all three genes. Results using promoter reporter gene assays in 293T cells indicate that for the Socs2 and Cish promoter constructs, Hdac3 did not appear to greatly alter Bcl6-mediated inhibition of reporter gene expression.

Stat5 and Hdac3 in regulation of Socs2 and Cish

Hdac3 appears to have a repressive effect on Stat5-mediated transcription of Socs2 and Cish reporter gene expression. The repressive effects of Hdac3 on Stat5-mediated transcription from the Socs2 and Cish promoters may be indicative of an extra layer of regulatory control involved in modulating Socs2 and Cish expression *in vivo*, where transcription is induced to a certain extent upon GH treatment, but mechanisms must be in place to prevent overexpression or be prepared to downregulate transcription levels as necessary.

Repression of Stat5-mediated activation of Socs2 and Cish promoter construct expression is consistent with the role of Hdac3 as a corepressor, and may be a mechanism by which the cell can fine tune Socs2 and Cish expression levels or downregulate transcription in response to further stimuli or loss of GH. However, GH induction of Socs2 mRNA levels was inhibited in cells that had been treated with trichostatin A, a class I and class II HDAC inhibitor, which would have indicated that HDAC activity was needed for activation of Socs2 expression in response to GH and Stat5, consistent with previous results seen for Cish where HDAC activity was needed for activation of Cish expression by Stat5 (10, 95). One possibility for the conflicting results between the inhibitor treatment experiment and promoter activation assays may be that these experiments are measuring different steps in the Stat5-mediated transcriptional activation process. Endogenous HDACs present in 293T cells may have already primed the overexpressed CA-Stat5b for transcriptional activation upon p300 recruitment, while the presence of additional Hdac3 resulted in downregulation of reporter gene expression. Another possibility is that the conditions of the reporter gene assay are not fully reflective of the events that occur for endogenous Socs2 expression in adipocytes. If so, the presence of other environmental factors and genomic or chromatin context cues would be expected to

give different results. As results for the Socs2 long promoter construct and the Cish promoter constructs suggest that a similar mechanism of regulation by Bcl6, Stat5, and Hdac3 is seen for both the short and long promoter fragments, this would suggest that either all significant regulatory regions for Bcl6, Stat5, and Hdac3 are already present in the short promoter fragments of Socs2 and Cish, and differences in HDAC regulation of Stat5-mediated Socs2 and Cish expression between promoter constructs and adipocytes are due to chromatin context or cell specific factor availability, or that an alternative regulatory region for Hdac3 activity on Socs2 and Cish expression in adipocytes lies outside the fragments tested in the long promoter constructs.

Hdac3 in Bcl6- and Stat5- mediated regulation of Bcl6 gene expression

For the Bcl6 promoter, the effects of Hdac3 on Bcl6- or Stat5-mediated transcription of Bcl6 reporter gene expression varied greatly from what was seen for Socs2 and Cish, and was dependent on the length of the Bcl6 promoter sequence included in the construct. Hdac3 did not appear to greatly alter Bcl6-mediated inhibition of Bcl6-luc short expression, but did appear to have a repressive effect on Stat5-mediated activation of Bcl6-luc short expression. When the long Bcl6 promoter construct was used, Hdac3 appeared to have inhibitory effects on both Bcl6- and Stat5-mediated transcription of reporter gene expression, suggesting that multiple types of protein interactions are occurring at the Bcl6 promoter, likely in a DNA context-dependent manner.

Nearly all combinations of transcription factors and coregulators that were tested had the potential to be inhibitory for either Bcl6-luc short or Bcl6-luc long expression under the conditions tested, differentiating regulation of Bcl6 expression from regulation of Socs2 or Cish transcription. The differences in regulation of Bcl6-luc short and Bcl6-luc long expression by Bcl6, Stat5, p300, and Hdac3 also suggest that the Bcl6 promoter contains multiple cis regulatory regions near the Bcl6/Stat5occupancy site identified by ChIP-Sequencing. *In vivo*, several or all of these regulatory regions and the associated proteins may work in concert, with the net effects of the factors present on the Bcl6 gene under any condition determining the transcriptional output of the gene. It has already been shown

that Bcl6 is one of the most variably expressed genes in liver (96), and previous studies have shown that Bcl6 is highly regulated and transcription is under the complex control of many regulatory regions and nuclear proteins (24, 27, 57, 58). Our results also suggest that Bcl6 expression is highly regulated in adipocytes in response to GH and other growth factors and hormones. Further characterization of Bcl6 regulatory sequences and the associated protein factors will shed more light on the complex transcriptional regulatory titration involved in precisely regulating Bcl6 expression levels. Preliminary overexpression or siRNA-mediated knock down experiments carried out in preadipocytes suggest that altering the levels of Bcl6 expression in either direction is deleterious for the cell (C LaPensee, G Lin, data not shown). It will be interesting to see if varying levels of Bcl6 protein in adipocytes or other metabolic or GH target tissues using knock out animals or an inducible expression system may also result in differences in downstream gene activation or repression.

Occupancy patterns of p300 and Hdac3 at the Socs2, Cish, and Bcl6 promoters suggest that Bcl6 and Stat5 occupancy are a major determinant of transcriptional output for Socs2, Cish, and Bcl6 expression

Occupancy signal for p300 and Hdac3 by ChIP was weak for all three promoters compared to Bcl6 and Stat5 occupancy signal under the experimental conditions used. One possibility for the low ChIP signal is that the antibodies for coregulatory proteins are not very efficient. This problem could potentially be circumvented by overexpressing a protein tagged version of the coregulator at endogenous levels and could possibly be combined with depletion of endogenous coregulator by RNAi to limit off target effects. In this manner, immunoprecipitations could be carried out using antibody against the protein tag, which would be likely to give more efficient immunoprecipitation and provide stronger and cleaner signals. In a study carried out in rat liver, p300 occupancy signal at the Socs2 and Cish promoters was also low compared to occupancy of Stat5b, correlating with the results seen in adipocytes (17). However, p300 occupancy signal in the rat livers was highly enriched at the Igf1 promoter, suggesting that p300 occupancy at the Socs2 and Cish promoters may indeed be lower than other p300-occupied regions in the genome.

Regardless, even with relatively low signal, the presence of both p300 and Hdac3 were reproducibly detected at the Socs2, Cish, and Bcl6 promoters, suggesting these signals are biologically relevant. A similar situation has been observed previously in the recruitment of p300 and C/EBPβ to the c-fos promoter in response to GH (47). ChIP occupancy signal for p300 was low compared to signal for C/EBPβ; however, sequential chromatin immunoprecipitation assays demonstrated reliable recruitment of p300 to the c-fos promoter in association with C/EBPβ, and promoter activation assays confirmed a functional role for p300 in regulation of c-fos gene expression.

The constitutive occupancy of p300 and Hdac3 detected at the Socs2, Cish, and Bcl6 promoters, and the relatively small changes in coregulator occupancy in response to GH treatment compared to the occupancy changes seen for Bcl6 and Stat5 at the Bcl6/Stat5 regulatory region suggest that corecruitment or secondary recruitment of coregulators to the target regulatory sequence upon Bcl6 or Stat5 binding in response to signaling stimulus is not a major regulatory mechanism for coregulator action at these genes. The occupancy patterns observed by ChIP suggest that the occupancy of Bcl6 or Stat5 at the promoters of these genes are the main determinants of the direction of gene expression, and p300 and Hdac3 may be constitutively present at low levels to modulate and fine tune changes in gene expression based on the identity of the transcription factor present, or in conjunction with the transcription factor present and other transcriptional regulatory proteins in proximity. This model is further discussed in Chapter 5. Such a model would be opposite of a scenario where activation of target gene transcription is dependent on the recruitment of a rate-limiting coregulatory protein to the transcription factor complex, or where the direction of target gene transcription is dependent on the identity of the recruited coregulator, such as in the case of nuclear receptors and their coregulators (7, 81, 82, 97, 98).

Conclusions and future directions

Analysis and experimental verification of ChIP-Sequencing results led to the identification of Cish and Bcl6 as genes reciprocally occupied by Bcl6 and Stat5 in response to GH, in

addition to Socs2. The occupancy patterns for Bcl6 and Stat5 on the regulatory region identified in the gene promoters were similar, with Bcl6 occupancy high without GH and decreasing with treatment, while Stat5 occupancy was low without GH and increased with treatment. Socs2 and Cish expression are induced by GH and Bcl6 expression is repressed (1), and this was reflected in different patterns of changes in enrichment of histone marks associated with active transcription in response to GH at the regulatory regions of the three genes. ChIP also showed constitutive occupancy of p300 and Hdac3 at the regulatory regions of all three genes, suggesting that the changes in the presence or absence of Bcl6 or Stat5 is a major switch that regulates activation or repression of transcription for Socs2, Cish, and Bcl6, and p300 and Hdac3 may be fine tuning the transcriptional output based on their interactions with either Bcl6 or Stat5. Promoter activation assays showed that association of p300 or Hdac3 with Stat5 has marked effects on transcription from the Socs2 and Cish promoters, with p300 functioning as a coactivator for Stat5 and Hdac3 as a corepressor. On the other hand, regulation of the Bcl6 promoter was different, with p300 and Hdac3 exerting corepressive effects in the presence of Stat5 for the short promoter construct, and p300 and Hdac3 having corepressive effects in the presence of both Bcl6 and Stat5 for the longer Bcl6 promoter construct. These results provide important mechanistic insight into the molecular interactions mediating regulation of target gene expression by reciprocal occupancy of Bcl6 and Stat5 in response to GH.

Experiments using promoter reporter construct variants such as Bcl6/Stat5 regulatory site mutants or longer/shorter promoter constructs will help to better characterize the contributions of the DNA sequence to the Bcl6/Stat5 reciprocal regulatory mechanism of Socs2, Cish, and Bcl6 transcription. Manipulating the levels or activity of endogenous Bcl6, Stat5, p300, and Hdac3 in adipocytes, such as by depletion alone or in combination with RNAi or treatment with small molecule inhibitors, will help clarify issues surrounding the precise sequence of events and mechanisms of action by which these protein factors interact with the target gene DNA and each other to influence transcription. Examination of potential GH-mediated post-translational modifications on Bcl6, Stat5, p300, and Hdac3, and the effects these post-translational modifications have on

transcription factor or coregulator activity in mediating Socs2, Cish, and Bcl6 transcription will better characterize the role of GH signaling in mediating transcription by Bcl6/Stat5 in a reciprocal manner. Identification of other nuclear proteins that interact with Bcl6, Stat5, p300 or Hdac3, and investigating if these proteins also play a role in regulating Socs2, Cish, and Bcl6 expression in response to GH by modulating transcription factor or coregulator activity, or by modifying the surrounding chromatin, will help to better characterize the transcriptional regulatory complexes that Bcl6 and Stat5 form in response to GH on the Socs2, Cish, and Bcl6 promoters, and further our understanding of mechanisms by which GH regulates gene expression.

Materials and Methods

Identification of candidate ChIP-Sequencing peaks

To identify a subset of candidate peaks and related genes for experimental verification of Bcl6 or Stat5 occupancy, ChIP-Sequencing peaks identified by the H-Peak software (75) under each of the four conditions (immunoprecipitation with antibody against Bcl6 or Stat5, each without or with GH for 48 h) were sorted by either peak height or peak p value, and the top 200 proximal genes either upstream or downstream of the ChIP-Sequencing peak were submitted to a Pubmed literature search to find previous studies on Bcl6 or Stat5 in conjunction with these proximal genes. Genes that appeared in Pubmed abstracts along with Bcl6 or Stat5 were given more priority. Genome browser profiles of top H-Peak identified peaks were generated using the ChIP-Sequencing data and the USCS Genome Browser (http://genome.ucsc.edu/). Peak locations that showed signal across the various immunoprecipitation and treatment conditions were highly prioritized for follow up verification, as were locations that showed a single, distinct peak profile. A total of 35 ChIP-Sequencing peaks were selected for experimental verification of Bcl6 and/or Stat5 binding in the presence or absence of GH. ChIP primers were designed to flank the ChIP-Sequencing peaks identified by H-Peak, with the final PCR product

around 200 bp in length. Primers were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome).

Materials

Murine 3T3-F442A preadipocytes were provided by H. Green (Harvard Univ) and M. Sonenberg (Sloan-Kettering, NY). The 293T human kidney cell line was provided by M. Lazar (Univ Pennsylvania) and O. MacDougald (Univ Michigan). Recombinant human GH for cell culture studies was purchased from the National Hormone and Pituitary Program (Torrance, CA). Insulin, dexamethasone, isobutylmethylxanthine, formaldehyde, and trichostatin A (TSA) were from Sigma (St. Louis, MO). Culture media, L-glutamine, and antibiotic-antimycotic were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) and calf serum (CS) were from Atlanta Biologicals (Lawrenceville, GA). Bovine serum albumin (BSA, Fatty acid-free, cat. # 62150101) was purchased from Proliant (Ankeny, IA). TRIzol® Reagent was purchased from Invitrogen, and Taqman Reverse Transcription Kit from Applied Biosystems (Carlsbad, CA). Protease inhibitors leupeptin and aprotinin were purchased from Roche (Indianapolis, IN), and phenylmethylsulfonylfluoride (PMSF) from Mallinckrodt (St Louis, MO). Sodium orthovanadate and SYBR green were purchased from Sigma (St. Louis, MO).

Plasmids

The expression construct for mouse Bcl6 in pCMV-SPORT6.1 was purchased from Open Biosystems (Lafayette, CO). The constitutively active rat Stat5b construct containing a point mutation of histidine for asparagine at residue 642 was generously provided by P. Rotwein (Oregon Health and Science Univ) (99). The p300 and Hdac3 expression constructs were generously provided by R. Kwok (Univ Michigan). For the Socs2, Cish, and Bcl6 short promoter luciferase constructs, an approximately 200 bp fragment with the Bcl6/Stat5 site predicted by ChIP-Sequencing in the center was PCR amplified from mouse genomic DNA and cloned into the multiple cloning region of pGL3-Basic

(Promega, Madison, WI). For the Socs2, Cish, and Bcl6 long promoter luciferase constructs, a region from about 750 bp upstream to 250 bp downstream from the ChIP-Sequencing predicted Bcl6/Stat5 site of each gene was PCR amplified from mouse genomic DNA and cloned into the multiple cloning region of pGL3-Basic. Cloning primer sequences and restriction enzyme sites are as follows:

Short promoter constructs:

Socs2-luc short forward: 5'-CC GAG CAC CTC GAG GTG CAG GGT C-3' (XhoI)
Socs2-luc short reverse: 5'-GAG GCG GCG AGA TCT TGG CAA GAG-3' (BglII)

Cish-luc short forward: 5'-CCC GCC CCA CTC GAG CAC GTC AG-3' (XhoI)
Cish-luc short reverse: 5'-CAT CTT CCT AGA TCT GCG GGC TTT G-3' (BglII)

Bcl6-luc short forward: 5'-GGG AGG GCG CTC GAG GAG CGC GCC-3' (XhoI)
Bcl6-luc short reverse: 5'-GGA GCG GCA AGA TCT GCG GCG GCG G-3' (BglII)

Long promoter constructs:

Socs2-luc long forward: 5'-GA CAA ACG CTC GAG GCC TGT GAC-3' (XhoI)
Socs2-luc long reverse: 5'-CA AGC TTC AGA TCT CCG GGC ACA AG-3' (BglII)

Cish-luc long forward: 5'-CT GAG CTC CAA GGT ACC CTG AAT TC-3' (KpnI)
Cish-luc long reverse: 5'-CCC TGT ACG CAA AGG ACC ATG TCC C-3' (XhoI)

Bcl6-luc long forward: 5'-GCA TCC CAA GGT ACC ATC CCC AAA G-3' (KpnI)
Bcl6-luc long reverse: 5'-CGA AAA GCT AGA TCT TAC AGT GGG-3' (BglII)

Cell Culture

3T3-F442A preadipocytes were differentiated into adipocytes as follows. Preadipocytes were grown to confluence in Dulbecco's Modified Eagle Medium containing 1% L-glutamine, and 1% antibiotic-antimycotic (DMEM) and 8% calf serum. 48 h later, cells were induced to differentiate by exposure to adipogenic medium (DMEM, 8% FBS, 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone, 2 μg/ml insulin). After 48 h, medium was replaced with DMEM containing 8% FBS and 1 μg/ml insulin. 48 h later,

medium was changed to DMEM with 8% FBS, in which cells were maintained until use in experiments. Prior to all experiments with GH treatment, 3T3-F442A cells were deprived of serum for 16-18 h in DMEM containing 1% BSA instead of serum. Cells were then treated without or with human GH (500 ng/ml = 22 nM) for 48 h on day 7 following initiation of adipogenesis. 293T cells were cultured in DMEM and 8% calf serum.

Quantitative Real-time PCR (qPCR)

Murine 3T3-F442A preadipocytes in 10 cm plates were serum deprived and incubated with 1 μM trichostatin A overnight before treatment with GH. Equivalent volume of DMSO vehicle was used for controls. RNA was isolated and analyzed by quantitative real-time PCR (qPCR) as described previously (100). Primers for Socs2 and Bcl6 have been described previously (96, 101), and data were normalized to glyceraldehyde-3-phosphate dehydrogenase (100) using the 2ΔΔcT method (102) and expressed as fold-change compared to control untreated cells, where the control is set to1. For quantitative ChIP PCR experiments, qPCR was carried out as described (100) using ChIP DNA as the PCR template. Signals for each immunoprecipitation condition were normalized to the signal for input ChIP DNA for each of the respective treatment conditions. Input DNA amplification signal was consistent between all experiments. Primer sequences for quantitative ChIP PCR were designed using NCBI Primer-BLAST and sequences are as follows:

Socs2 forward: 5’- GGC CTA AAG GTT CCC TCC TA-3’
Socs2 reverse: 5’- AGC CAA TGC CTA TTA AGC CA-3’
Cish forward: 5’- TAG ACG CCT GCA CCC CCG TT-3’
Cish reverse: 5’- GGG GCC AGG CGC CTC CTA AT-3’
Bcl6 forward: 5’- CTT CGC TGT AGC AAA GCT CG-3’
Bcl6 reverse: 5’- GAA GAA TTA GCC CCA GAC CC-3’

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously (47). Briefly, 3T3-F442A adipocytes or preadipocytes in 15 cm culture dishes were treated with 500 ng/ml GH for various times. Cells were washed 2X in cold PBS and crosslinked in 1% formaldehye in PBS at room temperature for 15 minutes. Sonication was carried out using a Misonix S-3000 sonicator eighteen times for 15 seconds, with a 1 minute pause between cycles, to achieve chromatin fragments of approximately 200 bp. Immunoprecipitation was carried out using antibodies against Bcl6 (N-3) (Santa Cruz), Stat5 (C-17) (Santa Cruz), phosphorylated Stat5 antibody (Zymed, Grand Island, NY), p300 (N-15) (Santa Cruz), Hdac3 (H-99) (Santa Cruz), acetylated histone H3 (Millipore, Billerica, MA), acetylated histone H4 (Millipore), and H3K4me3 (Abcam, Cambridge, MA). Samples incubated with equivalent amount of normal rabbit IgG (Cell Signaling, Danvers, MA) served as a negative control. 1% input was used to indicate the relative amount of each sample used for individual ChIP analysis.

ChIP samples were analyzed either by PCR followed by separation of products on 2% agarose gels and staining with ethidium bromide (47), or by quantitative real time PCR. Primers for agarose gel analysis of the Bcl6/Stat5 site in the Socs2 promoter have been described previously (1, 89). Primers for agarose gel analysis of the Bcl6/Stat5 occupancy sites in the Cish and Bcl6 promoters identified by ChIP-Sequencing were designed using NCBI Primer-BLAST, and sequences are as follows:
Cish forward: 5'- GCT GGG ACG CAG CGG ACA AA-3'
Cish reverse: 5'- GTC CCA GAG ACA ACG GCG GC-3'
Bcl6 forward: 5'-CGC TCG CCG CCC TAT AGT GC-3'
Bcl6 reverse: 5'- GAG CGG GCG GTT CCA TCG G-3'

Reporter gene assays

293T cells were transfected by calcium phosphate coprecipitation (47) with 200 ng luciferase reporter construct and 100 ng of mouse Bcl6, constitutively active rat Stat5b,

p300, or Hdac3 expression construct per well of a 12 well culture plate alone or in combination as indicated. Each transfection condition was done in duplicate for each experiment. DNA transfection amounts were normalized across transfections using pcDNA3 (Invitrogen). After 24 h incubation at 37 °C, cells were washed twice in cold PBS and lysed in 250 µl of 1X passive lysis buffer (Promega Dual Luciferase Kit, Promega). Luciferase reagents were from the Promega Dual Luciferase Kit. 10 µl of cell lysate from each transfection was read in duplicate on a 96 well opaque white plate using a Biotek Synergy 2 microplate reader (Biotek, Winooski, VT). Luciferase readings were normalized to respective lysate protein concentrations measured by Bradford assay.

Statistics

Values from analysis of transcription factor occupancy, coregulator occupancy, or histone modification signal in adipocytes treated without or with GH for various times were compared statistically using Student's t-test. Basal luciferase values for each reporter gene construct were compared to various transfection conditions using Student's t-test. Each transcription factor transfection condition was also compared to the transcription factor together with either p300 or Hdac3 using Student's t-test for each reporter construct. All analyses were carried out using GraphPad Prism (La Jolla, CA) software.

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Chapter 5

Conclusions and Future Directions

Growth hormone is a peptide hormone secreted by the pituitary gland that regulates diverse physiological processes including statural growth and lipid, protein, and carbohydrate metabolism (1-5). Many functions of GH are believed to occur through regulation of specific target genes. While much is known about how GH regulates transcription of its target genes, research on GH-regulated gene transcription has focused on mechanisms involved in transcriptional activation. However, transcriptional repression and silencing are equally important but more poorly understood aspects of gene expression. The goal of this thesis was to explore factors involved in GH-mediated transcriptional repression and thus better understand GH action and transcriptional repression in general.

Bcl6 is a newly identified GH-regulated transcriptional repressor

In Chapter 2, analysis of a microarray profile of 3T3-F442A adipocytes treated with GH for various times identified Bcl6 as the gene most strongly repressed by GH at later time points (4h and 48 h) that coincide with GH-induced insulin resistance (6). The Bcl6 gene encodes a transcriptional repressor best known for its function in the immune system as a master regulator of B cell differentiation (7). At the time, expression of Bcl6 had not previously been identified in adipocytes, and there was little known about mechanisms by which GH could repress transcription of its target genes.

To learn more about roles Bcl6 might have in regulation of GH target genes, bioinformatic network analysis was carried out on a subset of genes from the microarray gene profile

found to be strongly regulated under GH conditions that repress Bcl6 expression (8). The network suggested an interaction between Bcl6 and Signal Transducer and Activator of Transcription (Stat) 5, a transcription factor well known to be regulated by GH and to mediate GH-regulated gene transcription (9-11). Quantitative real-time PCR experiments showed that the GH- and Stat5-mediated stimulation of Socs2 (Suppressor of Cytokine Signaling 2) mRNA and Bcl6 mRNA inhibition were reciprocal with each other in response to increasing duration or concentrations of GH, suggesting that Bcl6 and Stat5 may reciprocally regulate the expression of some GH target genes (8).

Bcl6 and Stat5 reciprocally regulate Socs2 gene expression in response to GH

Expression of Bcl6 was also found to inhibit expression of a Socs2-luciferase reporter driven by the GH-responsive Stat5 sequence in the Socs2 promoter, and to blunt the GH-induced increase in Socs2-luciferase expression in 293T cells co-expressing the GH receptor. Chromatin immunoprecipitation (ChIP) experiments further showed a reciprocal relationship between endogenous Bcl6 and Stat5 on the Socs2 promoter: occupancy by Bcl6 is high in untreated cells and decreases progressively after GH treatment, while occupancy by Stat5 increases with GH (8). Bcl6 has also been postulated to function reciprocally with Stat5 in the regulation of target gene expression in immune cells (12, 13). These observations served as the first example that reciprocal regulation of target gene expression by Bcl6 and Stat5 may be GH dependent and function as part of a general repressor/activator regulatory mechanism of gene transcription. Based on our findings, it was hypothesized that Bcl6 is a GH-regulated transcriptional repressor with novel roles in GH-mediated transcriptional repression, and that Bcl6 and Stat5 function as reciprocal regulators of other GH-regulated genes.

High throughput ChIP-Sequencing reveals Bcl6 and Stat5 occupancy patterns across the genome

In Chapter 3, further investigation of the role Bcl6 plays in GH-mediated transcriptional responses was examined. Socs2 mRNA expression was found to be strongly upregulated

in livers of mice deficient in Bcl6 (14) compared to wild type, suggesting that endogenous Bcl6 functions as a repressor of Socs2 transcription in an animal model as well as cultured cells. The *in vivo* and *in vitro* findings support the hypothesis that Bcl6 plays a role in physiological GH functions such as metabolism and adipose differentiation. ChIP carried out using livers of non-Bcl6 deficient mice treated with or without GH showed reciprocal occupancy of Bcl6 and Stat5 on the Socs2 promoter in response to GH, similar to what had been seen in cultured adipocytes, once again suggesting that endogenous Bcl6 plays an important physiological role in GH action *in vivo*. Bcl6 mRNA expression was also found to be inhibited in preadipocytes by insulin and EGF treatment, suggesting wider roles for Bcl6 in adipose tissue functions outside of GH signaling. Therefore, to identify Bcl6 target genes and further understand roles Bcl6 might be playing in GH action, high throughput ChIP-Sequencing was carried out to identify potential Bcl6 and Stat5 target genes across the genome.

ChIP-Sequencing was carried out using 3T3-F442A adipocytes treated with GH for 0 or 48 hours to match the microarray time points where expression of Bcl6 was highest (0 h) and strongly inhibited (48 h) (8). As chronic GH treatment in adipocytes results in an insulin resistant phenotype (4, 15), it was also reasoned that by comparing results from the two treatment conditions, we would be able to identify Bcl6 and/or Stat5 target genes potentially involved in the pathogenesis of diabetes and metabolic syndrome. ChIP was carried out using antibodies against Bcl6 or Stat5, and ChIP-Sequencing libraries prepared from the ChIP DNA samples were sent to the University of Michigan Sequencing Core for high throughput sequencing. ChIP-Sequencing results were analyzed using the H-Peak algorithm, and results showed over 3000 regions of occupancy for Bcl6 and at least 900 for Stat5 on each of the genomic profiles. The number of occupancy regions for Bcl6 decreased in the GH-treated genomic profile, consistent with GH-mediated repression of Bcl6 expression. A Bcl6-enriched signal peak was observed near the GH-regulated sequence of the Socs2 promoter, consistent with previous ChIP results (8, 16). Motif analysis of ChIP-Sequencing results also showed that sequences occupied by Bcl6 matched strongly with predicted Bcl6 motifs, and sequences occupied by Stat5 matched strongly with predicted Stat motifs.

Combined analysis of the GH gene profile and ChIP-Sequencing datasets identify candidate GH-regulated Bcl6 target genes

Gene ontology analysis of genes predicted to likely be regulated by Bcl6 showed that over half of these genes functioned in biological processes related to GH action such as growth and differentiation, metabolism, or signaling. Comparison of genes from the ChIP-Sequencing analysis and previous microarray results to identify genes likely to be regulated by both GH and Bcl6 generated a list of candidate target genes. Several of these genes are known GH target genes or well known to participate in GH and adipocyte biology. Among these genes, Igfals (insulin-like growth factor binding protein acid labile subunit), Igf2 (insulin-like growth factor 2), Fasn (fatty acid synthase), Scd1 (stearoyl-Coenzyme A desaturase 1), Scd2 (stearoyl-Coenzyme A desaturase 2), Agt (angiotensinogen), Rgs16 (regulator of G-protein signaling 16), and Il1rl1 (interleukin 1 receptor-like 1) would be of particular interest as follow up candidates for further investigation due to their known biological functions in adipose tissue and liver (17-22), or in the case of Il1rl1, as previously described Bcl6 target genes in the immune system (23).

Examination of the mechanisms by which Bcl6 and GH coordinate to regulate expression of these potential target genes will expand understanding of GH physiology and transcriptional regulation, and could potentially lead to development of new therapies and drug targets for diseases such as hypertension and diabetes. For example, characterization of mice deficient in Bcl6 found that the animals exhibited multiple features of dysregulated lipid metabolism. Bcl6 deficiency was accompanied by decreased hepatic expression of Stearoyl-CoA desaturase 1 (Scd1) and Fatty acid synthase (Fasn), which encode lipogenic enzymes (C LaPensee, manuscript submitted). Both of these genes were also identified as potential GH- and Bcl6-regulated target genes by ChIP-Sequencing and microarray analysis, lending credibility to the other candidate genes identified, and suggesting that analysis of these other candidate genes in Bcl6 deficient mice could provide insight into the effects that transcriptional dysregulation of this potential gene

network has on growth and metabolism. Similarly, expression of angiotensinogen (Agt), which was also identified as a potential GH and Bcl6 target gene, was found to be elevated in livers of mice deficient in Bcl6 compared to wild type mice. Agt expression was induced by GH treatment in adipocytes, and Bcl6 occupancy was detected on the Agt gene. The regulation of Agt by GH and Bcl6 could provide insight into the development of hypertension and cardiovascular disease in metabolic syndrome and insulin resistance (22). Development of compounds that target Bcl6 activity, or the Stat5 and the Stat5-mediated regulation of Bcl6 expression in response to GH or other growth factors could lead to new pharmaceutical agents in the treatment of these diseases.

The Socs2, Cish, and Bcl6 gene promoters are reciprocally occupied by Bcl6 and Stat5 in response to GH

Another goal of the ChIP-Sequencing experiment was to identify genes that were regulated by both Bcl6 and Stat5 in order to better understand how this transcriptional activator-repressor pair could function together to regulate expression of GH target genes. Therefore, Chapter 4 of this thesis focused on identifying other gene candidates regulated by both Bcl6 and Stat5 in response to GH, and elucidating possible mechanisms by which Bcl6 and Stat5 participate in modulating expression of these target genes.

Genomic regions identified by ChIP-Sequencing showing significant signal for Bcl6 or Stat5 were selected as candidate sequences to test for occupancy of these transcription factors in response to GH in adipocytes. After experimental verification, a region in the Cish promoter and a region in the Bcl6 promoter identified by ChIP-Sequencing were both found to be reciprocally occupied by Bcl6 and Stat5 in response to GH. In addition to Socs2 (8), analysis and verification of the ChIP-Sequencing results has currently yielded these three gene candidates for study of mechanisms for reciprocal regulation of transcription by Bcl6 and Stat5. As Socs2 and Cish expression are induced by GH while Bcl6 expression is strongly repressed (8), Socs2, Cish and Bcl6 were used as experimental systems to test for similarities and differences in the manner by which Bcl6 and Stat5 transcriptionally regulate the expression of different target genes in response to GH.

Socs2, Cish, and Bcl6 are differentially regulated in response to GH

To this end, histone modifications associated with the promoter regions of Socs2, Cish, and Bcl6 were probed for changes in response to GH treatment or Bcl6/Stat5 occupancy to provide general supporting information on the transcriptional states of the target genes and potential actions of coregulatory proteins. Histone marks associated with active gene transcription increased rapidly in response to GH on the Socs2 and Cish promoters, consistent with the strong induction of these genes with GH treatment (8). Histone marks associated with active transcription were present at the Bcl6 promoter; however, enrichment of these histone modifications was not strongly induced by GH, consistent with Bcl6 expression being repressed by GH treatment (8, 24, 25) and suggesting that Bcl6 is a highly responsive gene that needs to remain accessible to the transcriptional machinery in order to respond quickly to changes in the cellular environment.

P300 and Hdac3 constitutively occupy the Socs2, Cish, and Bcl6 promoters

As Bcl6 and Stat5 are both known to interact with many coregulatory proteins to regulate target gene transcription (7, 10, 26-29), the effects of the coregulatory proteins p300 and Hdac3 on transcription of Socs2, Cish, and Bcl6 were also examined by ChIP and promoter activation assays. ChIP results showed strong and reproducible reciprocal changes in Bcl6 and Stat5 occupancy on all three gene promoters in adipocytes. However, occupancy signal and changes in recruitment of p300 and Hdac3 were small for all three genes respective to signal observed for Bcl6 or Stat5 under the experimental conditions used, with only Socs2 showing a more marked increase in p300 recruitment in response to GH. The findings so far support a putative mechanism in which the presence or absence of Bcl6 or Stat5 at the gene promoters are main determinants for the transcriptional state of the gene (Figure 5.1). Coregulatory proteins appear to be present at relatively low but constant levels, and the effects they exert on transcriptional output, either through their effects on Bcl6 and Stat5, the surrounding chromatin, or other nuclear protein factors, are likely to depend on the identity of the transcription factor they are interacting with.

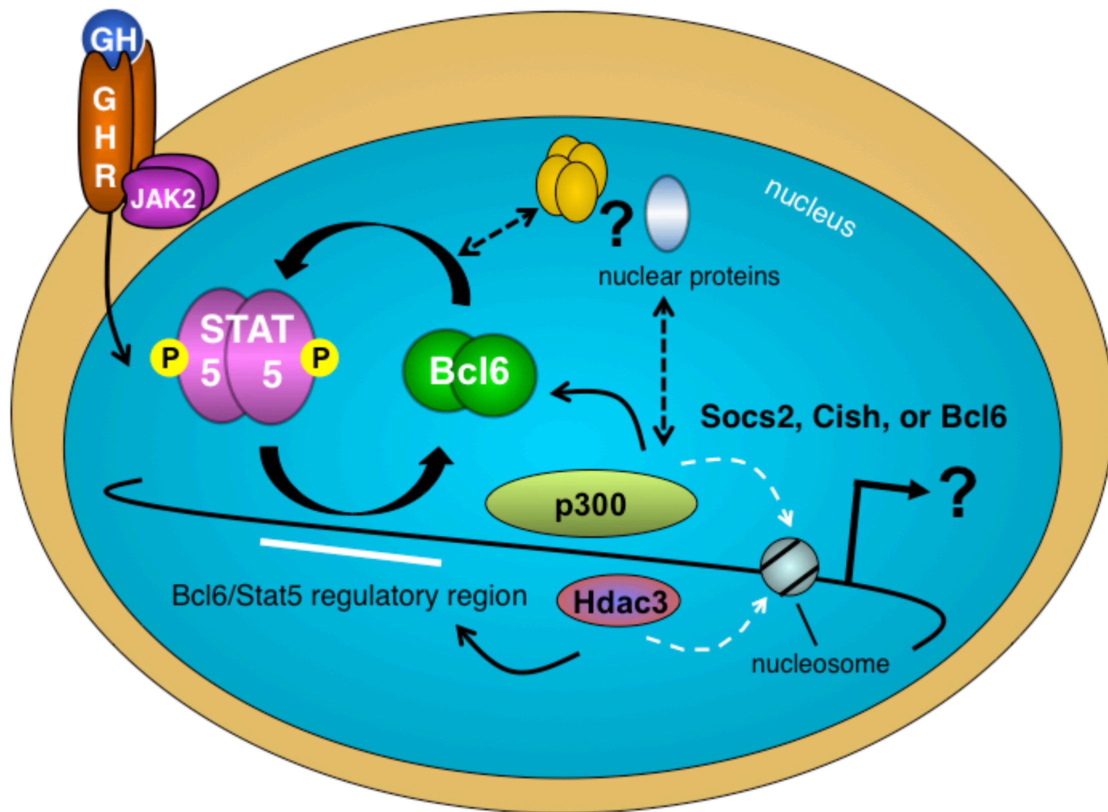

Figure 5.1

Proposed model of Bcl6- and Stat5-mediated transcriptional regulation of Socs2, Cish, and Bcl6 expression in response to GH. The Socs2, Cish, and Bcl6 genes contain a transcriptional regulatory region in the promoter that is occupied by Bcl6 in the absence of GH and activated by Stat5 in the presence of GH. The presence of Bcl6 or Stat5 determines the transcriptional output of each gene in response to GH. The coregulatory proteins p300 and Hdac3, and perhaps other nuclear proteins, are present in the vicinity of the Bcl6/Stat5 regulatory region in both the presence and absence of GH, and interact with either Bcl6 or Stat5 depending on which transcription factor is present. P300 and Hdac3 may be modulating transcription of Socs2, Cish, and Bcl6 through their enzymatic activities on Bcl6, Stat5, other nuclear proteins, or through remodeling of the chromatin structure in the promoter region. The coregulators may also be functioning as scaffold proteins for the recruitment of other nuclear transcriptional regulatory proteins, Bcl6, or Stat5 to form a multi-protein transcriptional regulatory complex. The complex and differential interactions between Bcl6, Stat5, coregulators, and the surrounding chromatin in response to GH coordinate to regulate differential Bcl6- and Stat5-mediated transcriptional outcomes for Socs2, Cish, and Bcl6 expression in response to GH. Solid arrows indicate either known relationships, or interactions or relationships that were tested or demonstrated in this thesis. Dotted arrows indicate potential interactions.

Proposed model for regulation of Socs2, Cish, and Bcl6 expression in response to GH by Bcl6 and Stat5

Bcl6 functions as a potent repressor of Socs2 and Cish transcription

In the proposed model, Bcl6 and Stat5 reciprocally occupy the Bcl6/Stat5 regulatory region of the Socs2, Cish and Bcl6 promoters in response to GH. Bcl6 occupancy is high in the absence of GH, and based on results from Socs2, Cish, and Bcl6 promoter activation assays, Bcl6 functions as a repressor when regulating expression of all three genes (Figure 5.2). The coregulators p300 and Hdac3 are present in the vicinity of the Bcl6/Stat5 regulatory region of the Socs2, Cish, and Bcl6 genes in the absence of GH (Figure 5.2). Results from promoter activation assays suggest that p300 may be interacting with Bcl6 on the Socs2 promoter to relieve Bcl6-mediated inhibition of Socs2 expression. Potential mechanisms by which p300 may be relieving Bcl6-mediated repression include p300-mediated acetylation of Bcl6, resulting in disruption of Bcl6 interaction with corepressors (30), or p300-mediated acetylation of histones near the Bcl6/Stat5 regulatory region, resulting in a more accessible chromatin state or the recruitment of activating protein factors (31). Another possibility is that p300 is functioning as a scaffold protein in a transcriptional regulatory complex, and recruits other nuclear proteins to the Bcl6 transcriptional regulatory complex that mediate derepression of Bcl6-mediated inhibition of Socs2 expression.

For regulation of the Cish promoter, results from promoter activation assays suggest that the presence of p300 does not greatly alter Bcl6-mediated inhibition of Cish expression. However, p300 may still be an essential structural component of the transcriptional regulatory complexes that form in association with Bcl6 at the Cish promoter.

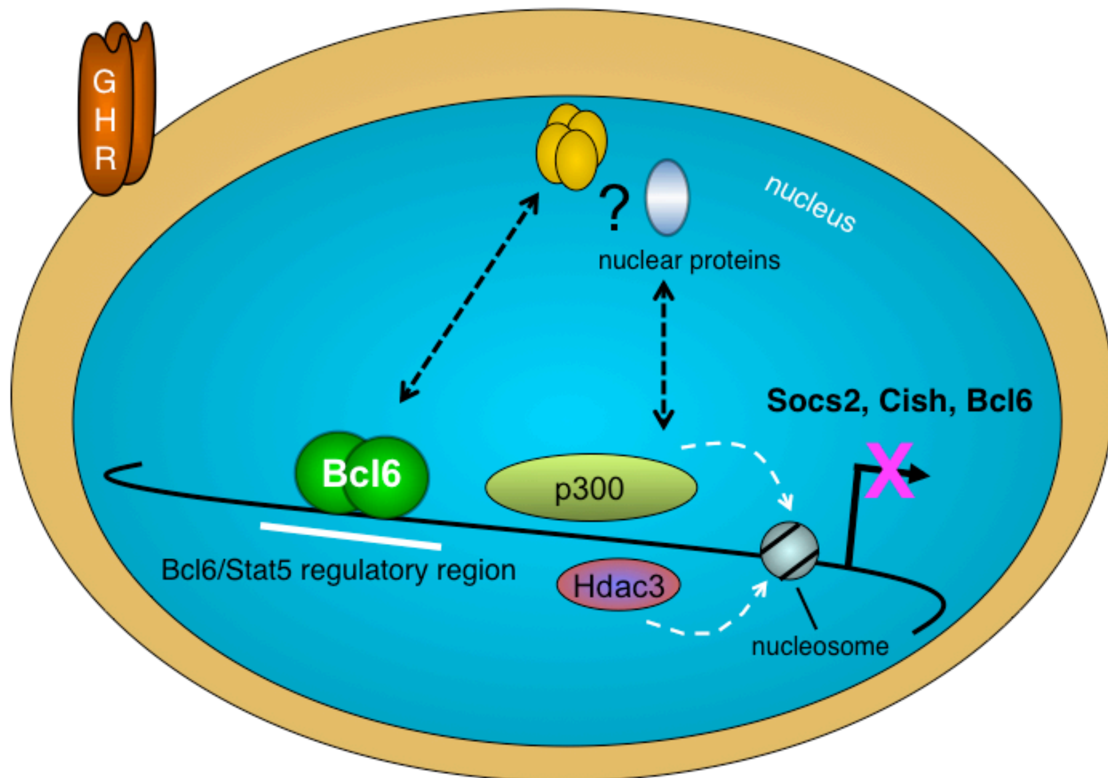

Figure 5.2

Model: Bcl6 represses Socs2, Cish, and Bcl6 transcription in the absence of GH. Bcl6 occupies the Bcl6/Stat5 regulatory regions of the Socs2, Cish, and Bcl6 promoters in the absence of GH. Bcl6 functions as a transcriptional repressor of Socs2, Cish, and Bcl6 expression. The coregulatory proteins p300 and Hdac3, and perhaps other nuclear proteins, are present in the vicinity of the Bcl6/Stat5 regulatory region. P300 and Hdac3 do not appear to greatly modulate Bcl6-mediated repression of Socs2, Cish, and Bcl6 expression. However, p300 and Hdac3 may be interacting with other nuclear proteins to regulate transcription, or have alternate regulatory functions such as remodeling the nearby chromatin structure. Dotted arrows indicate potential interactions.

Bcl6 inhibits the Bcl6 promoter: p300 may function as a Bcl6 corepressor in regulation of Bcl6 expression

Importantly, results from promoter activation assays for the Bcl6 promoter suggest that p300 may have a corepressive effect on Bcl6-mediated repression of Bcl6 transcription, either through interactions of p300 with other nuclear proteins, or due to an as yet unidentified activity of p300 on Bcl6 function. The corepressive effect of p300 on Bcl6-mediated inhibition of Bcl6 expression also appears to depend on the accessibility of different regulatory regions to transcription factor or coregulator occupancy in the Bcl6 promoter. The presence of Hdac3 did not appear to greatly alter Bcl6-mediated inhibition of Socs2 and Cish promoter construct expression. Hdac3 appeared to have a corepressive effect when associated with Bcl6 at the Bcl6 promoter. The corepressive effects of both p300 and Hdac3 on Bcl6-mediated inhibition of Bcl6 transcription suggest that Bcl6 mRNA levels in adipocytes need to be tightly controlled and restricted to low levels, and overexpression of Bcl6 may be deleterious to the cell.

Stat5, in conjunction with p300, activates Socs2 and Cish transcription in response to GH

Upon GH treatment, Bcl6 occupancy at the Bcl6/Stat5 regulatory regions decreases for all three genes, either due to GH inhibiting Bcl6 expression, GH decreasing Bcl6 occupancy, or both. At the same time, Stat5 occupancy increases, and Socs2 and Cish expression are induced, and Bcl6 expression is repressed, suggesting that Stat5 functions as an activator for Socs2 and Cish (Figure 5.3), and a repressor for Bcl6 expression in adipocytes (Figure 5.4). Promoter activation assays support this hypothesis; association of Stat5 with p300 at the Socs2 and Cish promoters appears to induce gene transcription (Figure 5.3).

Stat5 may function as a repressor of Bcl6 gene expression

Interestingly, based on our findings so far, the effects of Stat5 on Bcl6 promoter activation differed depending on the DNA sequence context. Stat5 activated Bcl6 reporter

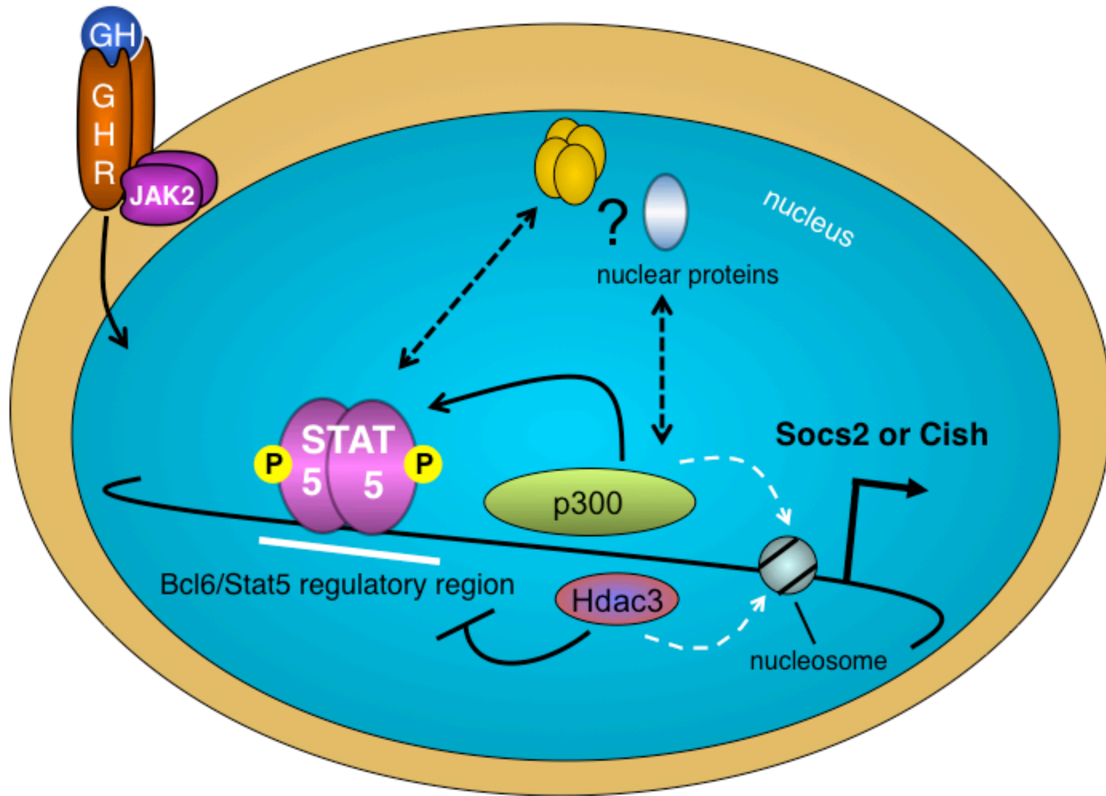

Figure 5.3

Model: Stat5 induces Socs2 and Cish expression upon GH treatment. The Bcl6/Stat5 regulatory regions of the Socs2 and Cish promoters are occupied by activated Stat5 in the presence of GH. The coregulatory proteins p300 and Hdac3, and perhaps other nuclear proteins, are present in the vicinity of the Bcl6/Stat5 regulatory region. P300 functions as a coactivator for Stat5-mediated induction of Socs2 and Cish transcription in response to GH. Hdac3 has an inhibitory effect on Stat5-mediated transcription of Socs2 and Cish. The corepressive effect of Hdac3 on Stat5-mediated Socs2 and Cish transcription could function as a mechanism to fine tune transcript levels or downregulate transcription. P300 and Hdac3 may also be interacting with other nuclear proteins to regulate transcription, or have alternate regulatory functions such as remodeling the nearby chromatin structure. Solid arrows indicate known interactions or relationships; dotted arrows indicate potential interactions.

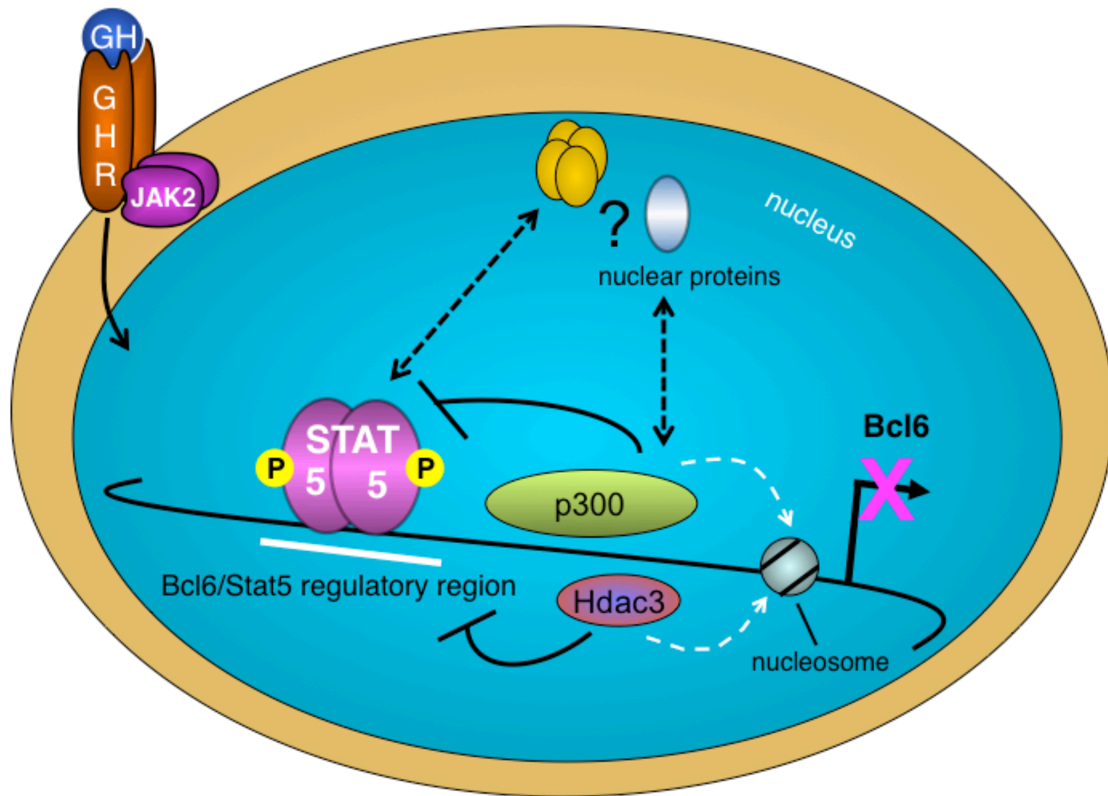

Figure 5.4

Model: Stat5 may function as a repressor of Bcl6 transcription in the presence of GH. The Bcl6/Stat5 regulatory region of the Bcl6 promoter is occupied by activated Stat5 in the presence of GH. The coregulatory proteins p300 and Hdac3, and perhaps other nuclear proteins, are present in the vicinity of the Bcl6/Stat5 regulatory region. Both p300 and Hdac3 appear to function as corepressors of Stat5-mediated transcription of Bcl6. Stat5, in conjunction with p300 and Hdac3, may be functioning as a repressor of Bcl6 expression *in vivo*. P300 and Hdac3 may also be interacting with other nuclear proteins to regulate transcription, or have alternate regulatory functions such as remodeling the nearby chromatin structure. Solid arrows indicate known interactions or relationships; dotted arrows indicate potential interactions.

gene expression when a short Bcl6 promoter fragment, consisting of about 200 base pairs with the Bcl6/Stat5 occupancy region identified by ChIP-Sequencing in the center, was used in the Bcl6 promoter construct. Stat5 did not have an activating effect on Bcl6 reporter gene expression when a longer fragment of the Bcl6 promoter was used, and appeared to have a slight inhibitory effect. As endogenous Bcl6 expression is repressed by GH in adipocytes (8), it is likely that Stat5 is functioning as a repressor of Bcl6 expression in adipocytes in the context of the native gene (Figure 5.4). Surprisingly, interaction of p300, which is typically a coactivator, with Stat5 on the Bcl6 promoter resulted in inhibition of Stat5-mediated Bcl6 reporter gene expression for both the short and long Bcl6 promoter constructs. This suggests that p300 corepression of Stat5-mediated transcriptional repression may contribute to a mechanism by which Bcl6 expression is inhibited by Stat5 *in vivo*.

Hdac3 functions as a corepressor of Stat5-mediated transcription of Socs2, Cish, and Bcl6

Hdac3 appeared to have a corepressive effect on Stat5-mediated transcription of Socs2 and Cish, either due to interactions between Hdac3 and Stat5, Hdac3 and other nuclear proteins, or Hdac3 and the surrounding nucleosomes (Figure 5.3). The constitutive presence of Hdac3 at the Socs2 and Cish promoters, and the corepressive effects of Hdac3 on Stat5-mediated transcriptional activation of Socs2 and Cish expression may be indicative of a repressive regulatory mechanism that functions to prevent Socs2 and Cish overexpression, or as a mechanism to downregulate Socs2 and Cish expression after activation. As the Socs2 and Cish proteins are potent signaling inhibitors, it is logical that Socs2 and Cish mRNA levels would be tightly regulated. Another possibility is that p300 and Hdac3 constitutively occupy the Socs2, Cish, and Bcl6 promoters in order to constantly remodel the surrounding chromatin and prime the gene for multiple cycles of transcription. Various studies have shown that lysine acetyltransferases and lysine deacetylases are both targeted to transcribed regions of active genes marked by phosphorylated RNA pol II, suggesting that the dynamic cycle of histone acetylation and deacetylation are both needed to prime genes for further activation (31-33). Hdac3

appears to have corepressive effects on Stat5-mediated transcriptional regulation of Bcl6 gene expression (Figure 5.4), with the combined results of Stat5 and Hdac3 resulting in inhibition of Bcl6 reporter gene expression compared to basal levels or lower, once again suggesting that Bcl6 expression needs to be tightly regulated, and low levels of Bcl6 expression are sufficient to carry out Bcl6 cellular functions in adipocytes.

Future experiments will better characterize reciprocal regulation of Socs2, Cish, and Bcl6 expression by Bcl6 and Stat5 in response to GH

Future experiments that manipulate the levels or activity of Bcl6, Stat5, p300 and Hdac3 in cells followed by evaluation of Socs2, Cish, or Bcl6 transcript levels will help to better characterize the mechanisms and protein interactions involved in regulation of Socs2, Cish, and Bcl6 transcription. Of particular interest would be depletion of Bcl6 or Stat5 in cells, followed by rescue with mutant forms of Bcl6 or Stat5 deficient in DNA binding activity or protein interaction with p300 or Hdac3. Similar experiments in which p300 and Hdac3 levels are depleted and rescued with mutant p300 and Hdac3 lacking catalytic activity would evaluate whether the catalytic activities of p300 and Hdac3 are crucial to regulation of Socs2, Cish, and Bcl6 expression, or if p300 and Hdac3 may be primarily functioning as scaffold proteins for Bcl6, Stat5, or other proteins, similar to the CSL (CBF1/Suppressor of Hairless/Lag1) DNA-binding protein, which functions as a constitutively present tether at the Hes1 (hairy and enchancer of split-1) promoter for both corepressor and coactivator complexes (34, 35). ChIP experiments showing Stat5 occupancy in liver from mice deficient in Bcl6 have demonstrated that Stat5 occupancy of the Socs2 promoter is not dependent on the previous occupancy of Bcl6, and co-immunoprecipitation experiments have not detected protein-protein interactions between Bcl6 and Stat5 under experimental conditions currently tested. Therefore, it would also be interesting to observe if the presence of p300 or Hdac3 at the Socs2, Cish, or Bcl6 promoters is required for the recruitment of or switch in occupancy between Bcl6 and Stat5 in response to GH, similar to the coregulator FOG1 (friend of GATA 1), which facilitates the transcription factor switch from GATA2 to GATA1 at the GATA2 promoter (36).

Identifying potential post-translational modifications on Bcl6, Stat5, p300 or Hdac3 in response to activation of GH signaling pathways, and the downstream effects these post-translational modifications have on protein-protein interactions, transcriptional regulatory complex recruitment, and chromatin modification would also be of interest. For example, phosphorylation of murine C/EBPβ at T188 in response to GH signaling is known to recruit p300 to C/EBPβ, resulting in and activation of c-fos transcription (37). Similarly, the identification of other proteins involved in the Bcl6- or Stat5-mediated regulation of Socs2, Cish, or Bcl6 expression in response to GH, and how these proteins would function in the context of a Bcl6 or Stat5 transcriptional regulatory complex in association with p300 or Hdac3 would provide insight into the molecular mechanisms of gene regulation.

Characterization of the multiple regulatory elements that appear to be present on the Bcl6 promoter would provide insight into how multiple regulatory regions and their associated nuclear proteins coordinate to recruit RNA pol II and/or the basal transcriptional machinery to the gene promoter or remodel the surrounding chromatin to promote or inhibit transcription. Genomatix analysis of the Socs2, Cish, and Bcl6 promoters also revealed potential binding sites for other transcription factors, including the well-characterized GH-regulated transcription factor C/EBPβ (8). The Bcl6 corepressor ETO has been shown to interact with C/EBPβ in preadipocytes and adipocytes and play a role in adipogenesis (38, 39). This suggests another mechanism by which GH can repress gene transcription. Potential interactions between Bcl6, ETO, and C/EBPβ also suggest that Bcl6 could function as a cofactor instead of a direct transcription factor when regulating expression of GH target genes, similar to what is seen when Bcl6 represses the expression of CDKN1A (cyclin dependent kinase inhibitor 1A, p21) in germinal centers through its interaction with the transcriptional activator Miz1 (Myc-interacting zinc finger protein 1) (40). Results from future experiments examining the expanded mechanisms of Bcl6 and Stat5 action in regulation of GH target genes will provide insight into basic mechanisms of transcription, reciprocal regulation of transcription by a transcriptional activator and a transcriptional repressor, and mechanisms by which GH represses gene transcription.

Further considerations on the complexity of transcriptional regulation: Chromatin acetylation, transcription, and adaptability of gene regulation

Proposed mechanisms of p300 and Hdac3 action on reciprocal regulation of Socs2, Cish, and Bcl6 expression by Bcl6 and Stat5

P300 and Hdac3 have been identified in these studies as coregulatory factors that participate in Bcl6- and Stat5-mediated regulation of Socs2, Cish, and Bcl6 expression in response to GH (Figure 5.1). One mechanism by which these coregulators may be influencing the activity of Bcl6 or Stat5 may be through their enzymatic activities on Bcl6 or Stat5. Acetylation is known to play a role in regulating the activity of both Bcl6 and Stat5. Acetylation of Bcl6 inhibits the ability of Bcl6 to recruit some corepressors and inhibit transcription (30), and a lysine deacetylase activity is necessary for Stat5 transcriptional activity on some target genes, including Cish (41). Another possibility is that p300 and Hdac3 are serving as scaffold proteins in multi-protein transcription complexes, and it is the recruitment and interaction of other proteins to this complex that mediate the regulatory effects p300 and Hdac3 have on Bcl6, Stat5, and gene expression. Co-immunoprecipitation experiments suggest that Bcl6 and Stat5 do not reside in the same protein complex when regulating expression of Socs2, Cish, and Bcl6. However, it appears that p300 and Hdac3 occupy the promoters of these genes both in the absence and presence of GH and may interact with both Bcl6 and Stat5 when the transcription factors are recruited. Therefore, Bcl6 and Stat5 may be the changing components in a promoter-associated transcriptional regulatory complex where p300 and Hdac3 act selectively depending on the identity of the transcription factor occupying the promoter (Figure 5.1). A third possibility is that p300 and Hdac3, as a lysine acetyltransferase and lysine deacetylase, respectively, may function at the promoters of these Bcl6/Stat5 target genes to modify the chromatin state of these genes as dynamic cycle of histone acetylation and deacetylation may be needed to prime genes for further activation (31-33).

P300 and Hdac3 may function to regulation histone acetylation at the Socs2, Cish, and Bcl6 promoters

ChIP for general activating histone marks showed that acetylated histone H3 and acetylated histone H4 signal increased from 2 to 5 fold on the Socs2 and Cish promoters in response to GH in adipocytes, correlating with induction of gene expression upon treatment. Acetylation of histones is indicative of open chromatin, which is conducive to active transcription (31), and the actions of p300 on the Socs2 and Cish promoters may be to acetylate histones and remodel the surrounding chromatin. Enrichment of acetylated histones near the Bcl6 promoter increased as well, but to a much lesser extent, suggesting that the chromatin needs to remain accessible to the transcriptional machinery, as changes in the transcription level of Bcl6 may need to be modulated at any time. Similarly, the role of Hdac3 may be to inhibit or balance the extent of histone acetylation at the Bcl6 promoter, and may also function to downregulate Socs2 and Cish expression at later time points, or upon the loss of GH signal, by compressing the chromatin structure to render it temporarily inaccessible or less available for transcription factor binding.

While the enrichment patterns of acetylated histone marks supports potential roles for p300 and Hdac3 in regulating transcription by remodeling the chromatin at these gene promoters, recent studies suggest that the relationship between the chromatin structure and gene transcription is even more complex than previously thought. Using mutant mouse fibroblasts with both p300 and CBP knocked out, it was demonstrated that p300/CBP are chiefly responsible for global acetylation of histone H3, and the p300/CBP knock out cells exhibited strongly attenuated histone H4 acetylation at CREB target genes in response to cyclic-AMP. However, transcription at these genes was not uniformly inhibited, and results suggested that the recruitment of p300/CBP to a genomic location, and the associated histone acetylation marks being present at this site, do not absolutely correlate as a requirement of gene activation. Target gene context, such as the DNA sequence, appeared to influence the extent to which p300 and CBP were necessary for transcription at target genes. Transcription in the absence of p300/CBP correlated with genes having more cyclic AMP response elements, more bound CREB (cyclic AMP

response element binding protein) transcription factor, and more CRTC2 (cAMP-response element binding protein-regulated transcription coactivator 2, a non-histone acetyltransferase CREB coactivator). The results suggested that genes with a greater local concentration and diversity of coregulators had more resilient-inducible expression, and regulation of these genes was less dependent on the presence of any one cofactor or cofactor-induced chromatin remodeling (42, 43).

The Bcl6 promoter likely contains multiple regulatory sequences to coordinate complex transcriptional regulatory input factors

As p300 and Hdac3 appear to interact with both Bcl6 and Stat5 on the Bcl6 promoter, and differences are observed in coregulator and transcription factor interactions when Bcl6 promoter sequences located adjacent to or more distal from the Bcl6/Stat5 occupancy site are tested in promoter activation assays (Chapter 4, Figure 4.10), there appear to be multiple regulatory sequences in the Bcl6 promoter and multiple coregulators providing transcriptional input. It is appealing to hypothesize that Bcl6 transcription is being regulated by multiple cofactors and nucleoprotein interactions using a variety of highly adaptive mechanisms. Promoter reporter gene assays suggest that p300 and Hdac3 do not greatly alter Bcl6-mediated repression of Socs2 and Cish expression, and full induction of Socs2 and Cish transcription in response to GH is dependent on the presence of both activated Stat5 and p300, suggesting that regulation of Socs2 and Cish transcription is highly dependent on Bcl6, Stat5, and p300, and loss of any one factor would cause a larger perturbance to gene expression levels of Socs2 and Cish, compared to Bcl6. This is supported by the observation that basal Socs2 mRNA levels increase in livers of Bcl6 knock out mice (Figure 3.2), but a corresponding increase in Stat5 occupancy was not observed at the Socs2 promoter (Figure 3.3A).

During initial screens to identify potential coregulator candidates involved in the mechanism of reciprocally mediated Bcl6/Stat5 transcription, the occupancy of several other corepressor molecules reported to interact with Bcl6 in immune cells, such as CtBP (44) and the Bcl6 specific corepressor BCoR (45), were also probed for by ChIP in

adipocytes (data not shown). In particular, the Bcl6 corepressor ETO (46) had been shown previously to interact with C/EBPβ in adipocytes and preadipocytes, and participate in adipogenesis (39). However, occupancy signals for all of the corepressors tested were extremely low and subsequent experiments focused on characterizing p300 and Hdac3 action on Bcl6- or Stat5-mediated transcription. Concerns with antibody quality aside, these results would once again support the hypothesis that occupancy of Bcl6 and Stat5, rather than coregulators, are major determinants of transcriptional output for Socs2, Cish, and Bcl6, and the regulatory input provided by coregulators is likely for the purposes of fine-tuning expression levels. However, of the three genes tested, ChIP signals for several of the known Bcl6 corepressors were higher for the Bcl6/Stat5 regulatory region of the Bcl6 promoter compared to Socs2 and Cish, suggesting that these coregulators may also play a role in regulation of Bcl6 expression in adipocytes, and regulation of Bcl6 expression may be fine tuned by the input of multiple coregulatory molecules. If regulation of Bcl6 transcription is indeed influenced by input from multiple coregulatory molecules in addition to p300 and Hdac3, this could be another difference in mechanisms by which Bcl6 and Stat5 are regulating Bcl6 expression in response to GH, in contrast to the regulation of Socs2 and Cish, which could be more dependent on p300, Hdac3, and chromatin acetylation than Bcl6.

On a last note, Bcl6 and Stat5 are both transcription factors strongly regulated in response to GH signaling. It is interesting that of the current genes known to be regulated by both Bcl6 and Stat5 in response to GH using a Bcl6/Stat5 dual regulatory region, Socs2 and Cish are negative regulators of cytokine and GH signaling (47, 48), while Bcl6 is predicted to be a major repressor of GH target genes (8, 24, 49). Speculation would infer that genes strongly regulated by both Bcl6 and Stat5 in response to GH may be genes that enhance or suppress the GH signal in the cell and provide feedback on the status of hormone stimulation. Analysis of high throughput ChIP-Sequencing results and GH microarray data will provide more candidate Bcl6 target genes and candidate Bcl6/Stat5 reciprocally regulated genes to help us further understand this repressor-activator relationship in GH gene transcription and GH function.

Conclusions

In summary, through the work in this thesis, we have expanded our understanding of mechanisms by which GH can repress transcription of target genes, identified regulatory roles that the novel GH-regulated transcriptional repressor Bcl6 might be playing in GH action, and characterized the molecular mechanisms underlying differential transcriptional regulation of GH target genes by reciprocal occupancy of Bcl6 and Stat5. Future work characterizing novel Bcl6 target genes in GH action and the mechanism of reciprocal Bcl6- and Stat5-mediated transcriptional regulation will continue to provide insight into GH physiology and mechanisms of transcriptional regulation.

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