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Reciprocal occupancy of BCL6 and STAT5 on Growth Hormone target genes: contrasting transcriptional outcomes and promoter-specific roles of p300 and HDAC3

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Abstract

Expression of the Growth Hormone (GH)-stimulated gene *Socs2* (Suppressor of Cytokine Signaling 2) is mediated by the transcription activator STAT5 (Signal Transducer and Activator of Transcription 5) and the transcription repressor BCL6 (B-Cell Lymphoma 6). ChIP-Sequencing identified *Cish* (Cytokine-Inducible SH2-containing protein) and *Bcl6* as having similar patterns of reciprocal occupancy by BCL6 and STAT5 in response to GH, though GH stimulates *Cish* and inhibits *Bcl6* expression. The co-activator p300 occupied *Socs2*, *Cish* and *Bcl6* promoters, and enhanced STAT5-mediated activation of *Socs2* and *Cish*. In contrast, on *Bcl6*, p300 functioned as a repressor and inhibited in conjunction with STAT5 or BCL6. The co-repressor HDAC3 (Histone deacetylase 3) inhibited the *Socs2*, *Cish* and *Bcl6* promoters in the presence of STAT5. Thus transcriptional outcomes on GH-regulated genes occupied by BCL6 and STAT5 are determined in a promoter-specific fashion by co-regulatory proteins which mediate the distinction between activating and repressive transcription factors.

Keywords

Socs2; Cish; Co-activator; Co-repressor; Adipocytes; ChIP-Seq

1. Introduction

The diverse actions of Growth Hormone (GH) are mediated by transcriptional programs which involve dynamic complexes containing activating and repressive transcription factors which bind DNA, and co-regulatory proteins which can modulate transcription through changes in chromatin (Kumar et al., 2004; Roeder, 2005; Rosenfeld et al., 2006). Well-studied examples of transcription factors for which transcription regulation involves

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Appendix: Supplementary material
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coordinated activator and repressor molecules are nuclear receptors such as estrogen or thyroid hormone receptors (Lonard and O'Malley, 2007; O'Malley et al., 2012; Perissi et al., 2004): In the absence of ligand, transcription of their target genes is repressed by association with co-repressor proteins such as SMRT (Silencing Mediator of Retinoic acid and Thyroid hormone receptor) or NCoR (Nuclear receptor Co-Repressor) in complex with the DNA-bound receptor. Upon ligand binding, the co-repressor is released and a co-activator such as p300 or CBP (Cyclic AMP response element Binding Protein) is recruited, mediating hormone-induced gene activation (Kumar et al., 2004; Perissi et al., 2004).

It is well-recognized that GH signaling to the nucleus involves STAT (Signal Transducers and Activators of Transcription) proteins, a family of transcription factors that mediate diverse cellular events in response to a large number of cytokines and growth factors, and that STAT5 plays a prominent role in mediating activation of GH-regulated genes (Herrington et al., 2000; Litterst et al., 2005; Waters et al., 2006). In contrast to STAT5 and other activating transcription factors such as C/EBP beta and CREB (Cui et al., 2008, 2011), repression of transcription in response to GH has been poorly understood. STAT5b has recently been implicated in the GH-mediated repression of IGF1 binding protein 1 transcription by indirectly impairing the actions of the FoxO1 transcription factor at the IGF1 binding protein 1 promoter (Ono et al., 2007). More recently, the transcription factor B-Cell Lymphoma 6 (BCL6) was found to repress potently both basal and GH-stimulated expression of Socs2 (Chen et al., 2009; Chia and Rotwein, 2010; Meyer et al., 2009). BCL6 is a transcriptional repressor, most extensively studied in immune regulation where it is a key participant in B cell differentiation (Basso and Dalla-Favera, 2010; Jardin et al., 2007; Schebesta et al., 2002; Staudt et al., 1999). Non-immune functions of BCL6 include protecting testicular germ cells from apoptosis (Kojima et al., 2001) and repressing proliferation of pancreatic β cells (Glauser and Schlegel, 2009; Karnik et al., 2007). BCL6 serves as a male-specific transcription factor mediating GH-regulated sexual dimorphism of gene expression in the liver (Meyer et al., 2009; Zhang et al., 2012). BCL6 as well as STAT5 have been reported to play oncogenic roles in lymphomas and breast cancer (Basso and Dalla-Favera, 2010; Tran et al., 2010), and a role for GH in breast cancer and other malignancies has also been proposed (Perry et al., 2008). Further, BCL6 and STATs have been reported to function as a transcription repressor–activator pair. Such a mechanism is likely since DNA binding motifs for BCL6 and STAT5 are very similar (Chang et al., 1996; Dent et al., 2002; Mascle et al., 2003; Seyfert et al., 1996) and BCL6 and STAT5 have potential to occupy the same DNA regulatory sequences.

The gene for Suppressor of Cytokine Signaling (*Socs*) 2 is exquisitely sensitive to negative regulation by BCL6 (Chen et al., 2009; Meyer et al., 2009) as well as to activation by STAT5 (Vidal et al., 2007). BCL6 and STAT5 bind reciprocally to a GH-regulated sequence in the *Socs2* promoter (Chen et al., 2009; Chia and Rotwein, 2010; Meyer et al., 2009): BCL6 occupies the *Socs2* promoter in the absence of GH, and also represses *Socs2* transcription (Chen et al., 2009). Upon GH treatment, occupancy of BCL6 decreases and STAT5 occupancy increases (Chen et al., 2009), resulting in potent activation of *Socs2* expression. Based in part on their reciprocal occupancy, BCL6 and STAT5 have been postulated to function as reciprocal regulators of transcription for some GH-regulated genes

(Chen et al., 2009; Conforto et al., 2012; Meyer et al., 2009), potentially contributing to GH responses.

In GH-regulated transcription complexes, negative regulation by BCL6 and positive regulation by STAT5 may also involve participation of co-regulators. BCL6 is well known to associate with co-repressors in immune cells, including histone deacetylases (HDACs), NCoR, SMRT (Bereshchenko et al., 2002; Dhordain et al., 1997, 1998; Huynh and Bardwell, 1998; Huynh et al., 2000), and the BCL6 co-repressor BCoR (Huynh et al., 2000). When functioning as a transcriptional activator, STAT5 interacts with co-activators such as p300, CBP and NCoA-1 (Nuclear receptor Co-Activator 1), which aid in driving STAT5-mediated transcription in response to cytokine stimulation (Chia and Rotwein, 2010; Kabotyanski et al., 2006; Litterst et al., 2003, 2005).

The reciprocal occupancy patterns and regulatory actions of BCL6 and STAT5 on *Socs2* gene expression suggest that their reciprocal regulation is integral to their function in response to GH, which promotes linear growth, induces insulin resistance and alters lipid metabolism (Ahmed and Farquharson, 2010; Albertsson-Wikland and Rosberg, 1988; Barbour et al., 2005; Cohen and LeRoith, 2012; Ikeda et al., 1998). For insight into GH-regulated transcriptional mechanisms mediated by this repressor–activator pair, the present studies used genome-wide chromatin immunoprecipitation (ChIP) and deep sequencing (ChIP-Sequencing) to identify BCL6 target genes and focus on GH-regulated genes for which BCL6 and STAT5 show reciprocal occupancy patterns. The contrasting transcriptional outcomes of these genes in response to GH prompted further investigation revealing changes in chromatin state and varying contributions of co-regulators p300 and HDAC3 in modulating the balance between repression and activation of genes regulated by BCL6 and STAT5 in response to GH.

2. Materials and methods

2.1. Materials

Murine 3T3-F442A preadipocytes were provided by H. Green (Harvard University) and M. Sonenberg (Sloan-Kettering, NY). The 293T human kidney cell line was provided by M. Lazar (University of Pennsylvania) and O. MacDougald (University of Michigan). Recombinant human GH (lot # AFP8990A) for cell culture studies was purchased from the National Hormone and Pituitary Program (Torrance, CA). Recombinant human GH (Lot# N70315) for animal experiments was from Genentech (San Francisco, CA), courtesy of Dr. R.K. Menon (University of Michigan). Insulin, dexamethasone, isobutylmethylxanthine, formaldehyde, Bradford reagent, sodium orthovanadate and SYBR green 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). Protease inhibitors leupeptin and aprotinin were purchased from Roche (Indianapolis, IN), and phenylmethylsulfonylfluoride (PMSF) from Mallinckrodt (St. Louis, MO).

2.2. Plasmids

For the *Socs2*, *Cish*, and *Bcl6* promoter-luciferase constructs, a region from 750 bp upstream to 250 bp downstream from the BCL6/STAT5 site identified by ChIP-Seq of each gene was PCR amplified from mouse genomic DNA and cloned into the multiple cloning region of pGL3-Basic (Promega, Madison, WI). Constructs were confirmed by sequencing at the University of Michigan DNA Sequencing Core. Cloning primer sequences and restriction enzyme sites are listed in Appendix: Supplementary Table S2. 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 His for Asp at residue 642 (CA-STAT5b) was generously provided by P. Rotwein (Oregon Health Science University) (Woelfle and Rotwein, 2004). The human p300 and HDAC3 expression constructs were generously provided by R. Kwok (University of Michigan) (Cesena et al., 2007a).

2.3. Cell culture

3T3-F442A preadipocytes in 15 cm plates 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. 2 days after confluence, cells were induced to differentiate by incubation in adipogenic medium (DMEM, 8% FBS, 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone, 2 μg/ml insulin). After 48 h, the medium was replaced with DMEM containing 8% FBS and 1 μg/ml insulin. 48 h later, medium was changed to DMEM with 8% FBS, to maintain cells until use in experiments. Prior to experiments with GH treatment, confluent 3T3-F442A preadipocytes or adipocytes 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 the times indicated, on day 7 following initiation of adipogenesis. 293T cells were cultured in DMEM with 8% calf serum.

2.4. In vivo *studies*

Tissues were obtained from mice treated chronically with GH or vehicle as follows: Mice used were control heterozygotes (+/−) in a colony crossed to generate targeted deficiency of BCL6 (Dent et al., 1997); for the responses measured, heterozygous mice were comparable to wild type controls (+/+). 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. Male mice were injected *intraperitoneally* with human GH (1.5 mg/kg BW) or with vehicle twice daily for 2.5 days, and were fasted for the final 16–18 h. Animals were euthanized between 1000 and 1200 h by CO_2 inhalation. Liver tissue was rapidly removed, weighed, frozen on dry ice and stored at −80 °C. Animal protocols were approved by the University Committee on Use and Care of Animals at the University of Michigan.

2.5. ChIP-Sequencing (ChIP-Seq)

To generate ChIP DNA libraries for high throughput 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 (Cui et al., 2005) using anti-BCL6 (N-3). ChIP DNA samples were pre-tested for quality by PCR using primers specific for the BCL6/STAT5 binding site in murine *Socs2* (Vidal et al., 2007), and multiple ChIP DNA samples immunoprecipitated (IP) with the same antibody under the same GH treatment conditions were pooled to obtain a sufficient amount of DNA (>10 ng) for generating ChIP-Seq libraries as recommended by the Illumina ChIP-Seq Library Preparation Protocol. ChIP-Seq 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-Seq libraries was validated by the University of Michigan DNA Sequencing Core using an Agilent Bioanalyzer and submitted for sequencing on the Illumina GAIIx sequencing platform. A DNA library was also prepared using input DNA from untreated adipocytes and sequenced in parallel as a background control. The ChIP-Seq experiment was independently performed twice.

Sequencing data obtained from the ChIP-Seq libraries were analyzed using the HPeak algorithm (http://www.sph.umich.edu/csg/qin/HPeak/), a hidden Markov model-based peak finding algorithm which allows for rigorous statistical inference (Qin et al., 2010). Distinct from other currently available algorithms, HPeak explicitly assumes probability distributions to model coverage profiles of DNA fragments. HPeak also provides the location of nearby genes and the genomic location of a peak. Occupancy profiles for Bcl6 on the mouse reference genome (mm9) were visualized using the UCSC Genome Browser (http://genome.ucsc.edu/). Data for replicate experiments were combined and peaks of occupancy with a peak height of 4 or greater, as determined by HPeak, were used in subsequent bioinformatic analyses.

To identify a subset of candidate peaks and related genes for experimental verification of BCL6 or STAT5 occupancy, ChIP-Seq peaks identified by HPeak (Qin et al., 2010) under each of the conditions (IP with antibody against BCL6, without or with GH for 48 h) were sorted by either peak height or *P*-value. The genes proximal to the top 200 ChIP-Seq peaks either upstream or downstream were prioritized according to previous studies reporting on them in conjunction with BCL6 or STAT5. Profiles of top peaks identified were selected using the ChIP-Seq data and the UCSC Genome Browser. Peak locations that showed signal across the various IP and treatment conditions were considered high priority for follow-up, as were locations that showed a single, distinct peak profile. Based on these multiple screens, a total of 35 ChIP-Seq peaks were selected for initial verification by standard ChIP-PCR of BCL6 and/or STAT5 binding in the presence or absence of GH. ChIP PCR primers were designed to flank the ChIP-Seq peaks identified by HPeak, 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).

2.6. Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously (Cui et al., 2005). Briefly, 3T3-F442A adipocytes or preadipocytes in 15 cm culture dishes were treated with 500 ng/ml GH for various times. Cells were washed twice in cold PBS and crosslinked in 1% formaldehye in PBS at room temperature for 15 min. 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. Sonication was carried out using a Misonix S-3000 sonicator eighteen times for 15 sec, with a 1 min pause between cycles, to achieve chromatin fragments of approximately 200 bp. IP was carried out with 4 μg of antibodies against BCL6 (N-3, Santa Cruz), STAT5 (C-17, Santa Cruz), STAT5a (L-20, Santa Cruz), STAT5b (Millipore, Billerica, MA), phosphorylated STAT5 (pY694) (71-6900, Invitrogen), p300 (N-15, Santa Cruz), HDAC3 (H-99, Santa Cruz), acetylated histone H3 (Millipore), acetylated histone H4 (Millipore), and H3K4me3 (Abcam, Cambridge, MA), as indicated. Samples incubated with an equivalent amount of normal rabbit immunoglobulin (IgG, Cell Signaling, Danvers, MA) served as negative controls. 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 (Cui et al., 2005), or by quantitative real time PCR (qPCR). PCR primers for agarose gel analysis of the BCL6/STAT5 site in the *Socs2* promoter have been described previously (Chen et al., 2009; Vidal et al., 2007). Primers for agarose gel analysis of the BCL6/STAT5 occupancy sites in the *Cish* and *Bcl6* promoters identified by ChIP-Seq were designed using NCBI Primer-BLAST (Appendix: Supplementary Table S2). For quantitative ChIP PCR experiments, qPCR was carried out as described (Huo et al., 2006) using ChIP DNA as the PCR template. Signals for each IP 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 ChIP qPCR were designed using NCBI Primer-BLAST (Appendix: Supplementary Table S2).

2.7. Transcription assays

293T cells were transfected by calcium phosphate co-precipitation (Cui et al., 2005) with 200 ng luciferase reporter construct and 100 ng of plasmid for mouse BCL6, CA-STAT5b, p300, or HDAC3 per well of a 12 well culture plate, alone or in combination as indicated. Each transfection was performed in duplicate in 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, lysed in 250 μl of 1× passive lysis buffer (Promega) and analyzed using luciferase reagents from Promega Dual Luciferase Kit. Luciferase in lysates was analyzed on a Biotek Synergy 2 microplate reader (Biotek, Winooski, VT) and normalized for protein concentration (Bradford assay). Luciferase activity is expressed as RLU (relative luciferase units) and normalized to basal values for the respective promoter construct, which was set as 1.0.

2.8. Statistics

Values from ChIP-qPCR and luciferase measurements were analyzed statistically using Student’s t-test with GraphPad Prism (La Jolla, CA) software.

3. Results

3.1. Reciprocal occupancy of BCL6 and STAT5 is regulated by GH

Reciprocal regulation of *Socs2* by GH, mediated by the transcription repressor BCL6 and the activator STAT5, derives from observations that occupancy of BCL6 and STAT5 on *Socs2* DNA is reciprocally regulated by GH. BCL6 occupancy on the STAT5 binding sequence in the *Socs2* promoter is evident in the absence of GH, and decreases progressively 30 min and 4 h after GH treatment of 3T3-F442A preadipocytes (Fig. 1A). As the occupancy of BCL6 decreases, the occupancy of STAT5 increases progressively, as detected by ChIP with a pan-STAT5 antibody, as reported previously (Chen et al., 2009). The pattern of decreasing BCL6 occupancy and increasing STAT5 occupancy with GH treatment is consistently observed in both 3T3-F442A adipocytes and preadipocytes (Chen et al., 2009). The increase in STAT5 occupancy with GH treatment reflects increased occupancy of both endogenous STAT5a and STAT5b on *Socs2* DNA in response to GH (Fig. 1A). The STAT5a and STAT5b isoforms share 91% amino acid identity (Grimley et al., 1999) and have both redundant and distinct functions (Adhikari et al., 2011; Hennighausen and Robinson, 2008; Teglund et al., 1998). Increased STAT5 occupancy on *Socs2* is accompanied by a GH-induced increase in the occupancy of tyrosyl phosphorylated STAT5 (Fig. 1B). The reciprocal occupancy of endogenous BCL6 and STAT5 on *Socs2* is detectable *in vivo* in liver of mice treated with GH (Fig. 1C; Meyer et al., 2009) and occupancy of endogenous BCL6 is also evident in mouse adipose tissue (not shown). For *Socs2*, functional consequences as well as occupancy of BCL6 and STAT5 are also reciprocal: BCL6 represses *Socs2* promoter activation in response to GH, while STAT5 activates it (Chen et al., 2009; Laz et al., 2009; Vidal et al., 2007). Thus, the highly sensitive reciprocal regulation of BCL6 and STAT5 serves as a paradigm to probe mechanisms for functional interactions of a regulated transcriptional repressor–activator pair by GH.

3.2. Socs2, Cish *and* Bcl6 *genes are reciprocally regulated by BCL6 and STAT5*

BCL6 occupancy throughout the genome was examined using high-throughput ChIP-Seq to identify candidate BCL6 target genes. GH-responsive 3T3-F442A adipocytes were treated with GH for 0 or 48 h, a time when *Socs2* expression is strongly induced by GH (Chen et al., 2009; Huo et al., 2006). ChIP was carried out using antibodies against BCL6, and ChIP-Seq libraries were prepared from the resulting ChIP DNA, which were subjected to deep sequencing. Sequencing data were analyzed using the HPeak algorithm (Qin et al., 2010) which identified over 3000 potential binding sites for BCL6 across the genome (Lin G and Schwartz J, manuscript in preparation). Candidate peaks of BCL6 occupancy for follow-up were identified based on peak heights, peak significance, and peak profiles, as well as on the reported association of nearby genes with either BCL6 or STAT5; the gene or genes closest to a peak were predicted to be the most likely targets for transcriptional regulation. Based on these initial screens, 35 candidate BCL6 occupied loci (Appendix: Supplementary Table S1) were processed for initial verification by standard ChIP-PCR for BCL6 and STAT5, using primers specific for the genomic regions of ChIP-Seq signal for each of the 35 loci, on samples from 3T3-F442A adipocytes treated without or with GH. Among these, three showed reciprocal occupancy of BCL6 and STAT5 in response to GH. One was *Socs2*: Examination of the BCL6 ChIP-Seq peak profiles associated with the *Socs2* locus showed a

single BCL6 peak near the *Socs2* transcription start site (TSS) (Fig. 2A, top panel); the peak height could be substantially reduced in cells treated with GH. The GH-regulated BCL6 occupancy on *Socs2* detected by ChIP-Seq is consistent with prior ChIP observations of BCL6 occupancy on a functional STAT5 motif on *Socs2*, which overlaps with this ChIP-Seq locus (Chen et al., 2009; Chia and Rotwein, 2010; Meyer et al., 2009; Vidal et al., 2007).

In addition, two other genomic regions of BCL6 occupancy in ChIP-Seq were found to show clear reciprocal occupancy by BCL6 and STAT5 in response to GH: One region was identified as a ChIP-Seq peak of BCL6 signal near the TSS of the *Cish* (Cytokine-Inducible SH2-containing protein) gene (Fig. 2A, middle panel). The second region was a peak of BCL6 near the TSS of the *Bcl6* gene itself (Fig. 2A, bottom panel). For *Cish*, a BCL6 peak was not detected after GH; for the Bcl6 locus, the peak height of BCL6 occupancy was reduced 60% after GH. Not only were the BCL6 peaks located in the proximal promoter region of each of these three genes (Fig. 2A), but they also corresponded with BCL6 or STAT motifs predicted by HPeak. Peaks tested for other candidate genes in the initial screen were primarily at genomic locations such as introns or intergenic regions (Appendix: Supplementary Table S1). Reciprocal occupancy of BCL6 and STAT5 was confirmed by standard ChIP in 3T3-F442A adipocytes treated with or without GH for 48 h. For both the *Cish* and the *Bcl6* loci, occupancy of BCL6 was higher without GH and decreased after GH treatment (Fig. 2B). Conversely, STAT5 occupancy was lower in the absence of GH and higher with GH treatment (Fig. 2B) (Chen et al., 2009). The similarity in binding patterns among the three genes may be related to similarity in the BCL6 and STAT5 consensus DNA binding motifs (Baron et al., 1995; Hartatik et al., 2001; Ihle, 1996); for convenience, they are referred to here collectively as “BCL6/STAT5 regulatory region” in the proximal promoters of these genes (Fig. 5A).

Despite the similarity in BCL6 and STAT5 binding, the transcriptional responses to GH of the three genes do not coincide: GH *stimulates* the expression of *Cish* and *Socs2*, but GH potently *inhibits* the expression of *Bcl6* mRNA (Fig. 2C). It is not surprising that *Socs2* and *Cish* show similar binding and responses to GH, since the *Cish* gene product is a member of the SOCS family proteins (Croker et al., 2008; Kile et al., 2002): SOCS2 and CIS proteins suppress GH and cytokine signaling. Further, *Cish* is a STAT5 target gene (Matsumoto et al., 1997) which also binds BCL6 (Chia and Rotwein, 2010). On the other hand, for the *Bcl6* gene, GH treatment and BCL6 itself repress transcription (auto-repression) (Chen et al., 2009; Mendez et al., 2008; Pasqualucci et al., 2003). STAT5 binding has been recognized to be cell type specific (Kang et al., 2013), and STAT5 has been reported either to activate or repress *Bcl6* transcription depending on cell type (Diehl et al., 2008; Icardi et al., 2012; Karnik et al., 2007; Scheeren et al., 2005; Tran et al., 2010; Walker et al., 2007). The discrepancy between the shared reciprocal occupancy patterns of BCL6 and STAT5 in response to GH for *Socs2*, *Cish* and *Bcl6*, and their contrasting transcriptional outcomes in response to GH, where *Bcl6* is inhibited while *Socs2* and *Cish* are potently stimulated, was examined further to assess whether distinct mechanisms involving BCL6 and STAT5 might link transcription factor binding and transcriptional outcomes of the GH-regulated genes.

3.3. GH induces histone acetylation at the promoters of Socs2 *and* Cish, *but not* Bcl6

Transcription generally proceeds when chromatin is in an open conformation, which can be assessed by histone acetylation. To examine whether changes in the chromatin state of the respective BCL6/STAT5 occupied regions were different among *Socs2*, *Cish* and *Bcl6*, histone modifications were analyzed in the presence and absence of GH. Adipocytes were treated with GH for 24 h (Fig. 3A), as *Socs2* and *Cish* expressions are strongly induced and *Bcl6* expression is strongly inhibited with chronic GH (Fig. 2C) (Chen et al., 2009). ChIP was performed using antibodies against acetylated histone H3 (AcH3), acetylated histone H4 (AcH4), or trimethylated histone H3 lysine 4 (H3K4me3), general markers of open and accessible chromatin. Each of these histone marks was detectable on *Socs2*, *Cish* and *Bcl6* in the absence of GH (Fig. 3), suggestive of active gene transcription (Berger, 2002; Grunstein, 1997; Ruthenburg et al., 2007; Strahl and Allis, 2000). On both *Socs2* and *Cish*, levels of AcH4 increased significantly in response to GH (Fig. 3A), consistent with GH increasing active transcription of these genes. In contrast, AcH4 levels on *Bcl6* barely changed with GH treatment. The significance of the higher basal levels of AcH3 and AcH4 on *Bcl6* is not clear. Strong signal for H3K4me3 was detected with and without GH at the BCL6/STAT5 regulatory regions of *Socs2*, *Cish*, and *Bcl6*, consistent with accessible chromatin and active promoter regions for all three genes.

Adipocytes were also treated with GH for 30 min (Fig. 3B), to examine changes in chromatin state during an earlier time of GH treatment. Timing of GH responses in adipocytes has long been of particular interest since acute (e.g. 30 min) and chronic (4 h or longer) metabolic responses to GH differ (Goodman, 1968), and pulsatile GH secretion patterns can govern its regulation of gene expression (Tannenbaum et al., 2001). Further, adipocyte gene expression is time-dependent, showing circadian rhythmicity (Wang and Lazar, 2008). Nevertheless, the histone modifications after 30 min GH (Fig. 3B) were generally comparable to those after 24 h, although AcH3 was significantly increased by GH on *Socs2* at 30 min. On both *Socs2* and *Cish*, levels of AcH4 increased significantly in response to GH, while AcH4 levels on *Bcl6* barely changed with GH treatment. Strong signal for H3K4me3 was detected with and without GH at the BCL6/STAT5 regulatory regions of *Socs2*, *Cish*, and *Bcl6*. Overall, these findings suggest that stimulation of *Socs2* and *Cish* by GH relative to its inhibition of *Bcl6* may reflect differences among the genes in the state and changes in chromatin at the level of regulation of H4 acetylation.

3.4. Occupancy of p300 and HDAC3 does not distinguish Bcl6 *from* Socs2 *and* Cish

To examine whether co-regulatory proteins associated with BCL6 and/or STAT5 might distinguish *Socs2* and *Cish* from *Bcl6*, recruitment of representative co-regulators, the co-activator p300 and the co-repressor HDAC3, to the three genes was compared by ChIP. p300 is a ubiquitous lysine acetyltransferase which in addition to acetylating histones, is known to associate with and acetylate numerous transcription factors (Gingras et al., 1999) including both BCL6 and STAT5 (Bereshchenko et al., 2002; Paulson et al., 1999). p300 is an effective co-activator in adipocytes (Erickson et al., 2001) and is regulated by GH (Cui et al., 2005, 2011). The role of p300 as a co-activator of STAT5 has been well described (Litterst et al., 2005; Paulson et al., 1999; Pfitzner et al., 1998). The acetylation status of STAT5, as modulated by p300 and HDACs, is a critical determinant of its activity (Icardi et

al., 2012; Rascle et al., 2003). The HDACs have often been implicated in transcriptional repression (Fischle et al., 2001; Icardi et al., 2012; Narlikar et al., 2002), and multiple Class I and Class II HDACs associate with BCL6 (Jardin et al., 2007). HDAC3 is a Class I HDAC which plays a prominent role in regulation of genes mediating adipocyte metabolism and adipogenesis (Alenghat et al., 2008; Feng et al., 2011). Further, HDAC3 regulates a subset of BCL6 target genes involved in the innate immune response (Barish et al., 2010). Accordingly, p300 and HDAC3 were examined as representative co-activator and co-repressor respectively, to examine if they also participate in reciprocal regulation of transcription by BCL6 and STAT5.

To compare the participation of p300 or HDAC3 in the regulation of *Socs2*, *Cish* and *Bcl6*, 3T3-F442A adipocytes were treated with GH for 24 h or 30 min (Fig. 4). ChIP was performed for p300 and for HDAC3, as well as for BCL6 and STAT5. Here, the ChIP samples were analyzed by qPCR for quantitative analysis. The ChIP-qPCR trends agree with the prior ChIP findings that for *Socs2*, *Cish* and *Bcl6* genes, BCL6 occupancy is consistently higher in the absence of GH and decreases with GH treatment (Fig. 4). Conversely, occupancy of STAT5 is lower in the absence of GH, and increases upon GH treatment, consistent with the highly reproducible reciprocal changes in occupancy of BCL6 and STAT5 in response to GH determined previously by ChIP-PCR.

In the same experiments, p300 occupancy was detected on both *Socs2* and *Cish* in the basal state. Occupancy of p300 was also evident on *Bcl6*, suggesting that the absence of p300 does not distinguish *Bcl6* from *Socs2* and *Cish*. Occupancy of HDAC3 was detected on *Socs2*, *Cish*, and *Bcl6* in the basal state. In general, the occupancy signals of HDAC3, as well as those of other HDACs tested (HDAC1, HDAC2, HDAC5, data not shown), and of p300, were lower than basal signals for BCL6 or STAT5 under the conditions of these experiments. Nevertheless, the detectable occupancy of HDAC3 on *Socs2* and *Cish* as well as *Bcl6* suggests that the presence vs absence of HDAC3 does not itself account for differences between repression and activation of these genes.

Although transcriptional responses to GH distinguish repression of *Bcl6* from activation of *Socs2* and *Cish*, the occupancy patterns of p300 or HDAC3 were changed minimally if at all by GH. Changes in p300 occupancy were not detected on any of the three genes after 24 h GH (Fig. 4A). Treatment with GH for 30 min increased the occupancy of the p300 on *Socs2* (Fig. 4B), consistent with GH-stimulated *Socs2* expression, though a comparable increase in p300 occupancy after GH was not detected on *Cish*, nor was a decrease of the co-activator observed on *Bcl6* (Fig. 4B). Chronic exposure to GH did not alter the pattern: Thus, the differences in transcriptional responses to GH among *Socs2*, *Cish* and *Bcl6* do not appear to reflect differences in occupancy of p300 in response to GH. The occupancy of HDAC3 also did not appear to be regulated by GH, since HDAC3 was not detectably altered by GH treatment when promoters of *Socs2*, *Cish* or *Bcl6* were evaluated. Comparable HDAC3 occupancy with or without GH was observed after 24 h or 30 min GH (Fig. 4) for all three genes. Among other co-repressors that have been reported to associate with *Bcl6*, occupancy of NCoR, SMRT and mSin3A was detected in the basal state on *Socs2* and *Cish* as well as on *Bcl6*. In initial trials, their patterns of occupancy were not different among the three genes, and were not reproducibly altered by GH (data not shown). These observations

suggest that both the co-activator p300 and the co-repressor HDAC3 occupy the promoters of *Socs2, Cish* and *Bcl6*, but that differences in basal occupancy of p300 and HDAC3, or changes in their occupancy in response to GH, do not coincide with differences in their transcriptional responses.

3.5. Activation of Socs2 *and* Cish *promoters by STAT5 is enhanced by p300*

Since p300 and HDAC3 occupancy was detected at the BCL6/STAT5 regulatory regions of the *Socs2, Cish*, and *Bcl6* genes, and since these co-regulators function in adipocytes and are regulated by GH, the functional contributions of p300 and HDAC3 on BCL6- or STAT5-mediated regulation of *Socs2, Cish*, and *Bcl6* transcription were examined. BCL6/STAT5-occupied sequences for the *Socs2, Cish*, and *Bcl6* promoters were fused upstream of the luciferase gene (Fig. 5A). The promoter constructs contained sequences spanning approximately 750 bp upstream and 250 bp downstream of the BCL6/STAT5 site identified by ChIP-Seq for each gene (Fig. 5A). To assess functional contributions of p300, activation of the three reporter constructs was evaluated in context of the expression of p300 alone, and p300 when co-expressed with BCL6 or with constitutively active STAT5b (CA-STAT5b). CA-STAT5b was used both to pinpoint the contributions of STAT5b on the regulation of the promoters of each of the three genes and as a representative of the downstream consequence of GH treatment, although overexpression of CA-STAT5b in these studies may not be directly reflective of physiological conditions *in vivo*.

For *Socs2*-luc (Fig. 5B, top), p300 alone did not alter *Socs2*-luc expression greatly under the conditions tested, compared to basal levels. BCL6 expression potently inhibited the *Socs2* reporter gene as observed previously with a similar *Socs2*-luc construct (Chen et al., 2009). In the presence of BCL6, promoter activity was slightly greater with addition of p300 than with BCL6 alone, but *Socs2*-luc expression remained lower than basal *Socs2*-luc or with p300 alone, reinforcing the strong inhibition of the *Socs2* promoter by BCL6. The addition of CA-STAT5b alone did not substantially elevate *Socs2*-luc above basal levels (Fig. 5B top). In support of CA-STAT5 as activator, the addition of p300 in the presence of CA-STAT5b doubled the activation of the promoter compared to CA-STAT5b alone or to basal *Socs2*-luc levels. A general trend toward activation was observed with p300 and STAT5 over STAT5 alone, though the increase was not significant. Regulation of *Cish*-luc (Fig. 5B, middle) by p300 was similar to the pattern for *Socs2*-luc: p300 alone did not appear to alter *Cish*-luc expression compared to basal levels. BCL6 alone potently and significantly inhibited *Cish*-luc expression demonstrating that BCL6 functions as a repressor for *Cish*. The inhibition by BCL6 was maintained despite co-expression of p300. CA-STAT5b alone did not activate the *Cish*-luc promoter above basal levels, though the expression of p300 in conjunction with CA-STAT5b doubled *Cish*-luc activation above levels with CA-STAT5b alone, with p300 alone, or with basal *Cish*-luc, consistent with the ability of p300 to enhance STAT5 activation of *Socs2* and *Cish*.

3.6. p300 functions as a co-repressor on the Bcl6 *promoter*

Regulation of *Bcl6*-luc in the presence of p300 (Fig. 5B, bottom) contrasted with the changes observed for *Socs2*-luc and *Cish*-luc. The expression of p300 alone modestly reduced *Bcl6*-luc expression compared to basal levels under the conditions of these

experiments. The expression of BCL6 alone strongly and significantly repressed *Bcl6*-luc promoter activation compared to basal conditions, as it did for *Socs2* and *Cish*. However, for *Bcl6*-luc, the addition of p300 in combination with BCL6 protein repressed *Bcl6*-luc expression further, significantly below the repression exerted when BCL6 alone was expressed. Thus, p300 appears to exacerbate the repression by BCL6 on the *Bcl6* promoter.

The *Bcl6* promoter was also distinctly different from *Socs2* and *Cish* promoters when p300 was co-expressed in combination with CA-STAT5b. Expression of CA-STAT5b alone did not alter *Bcl6*-luc expression relative to basal values (Fig. 5B, bottom), as observed for *Socs2* and *Cish* promoters under the conditions of these experiments. Strikingly, however, p300 significantly inhibited *Bcl6*-luc expression in the presence of CA-STAT5b. Not only did p300 decrease *Bcl6*-luc, but this change was in sharp contrast to the apparent increases exerted by p300 with CA-STAT5b on *Socs2* and *Cish*. These findings suggest that the Bcl6 promoter may be 'primed' for repression, such that p300 functions as a co-repressor and/or recruits other factors in the presence of STAT5 or BCL6 and renders STAT5b inhibitory on the *Bcl6* promoter rather than activating. The relative changes elicited in the presence of p300 are consistent with the transcriptional outcomes for the three genes: increases for *Socs2* and *Cish* and decreases with *Bcl6*.

3.7. HDAC3 serves as a co-repressor for STAT5 on Socs2 *and* Cish *as well as* Bcl6

HDAC3 alone was expressed under conditions in which it did not reduce basal promoter activation for *Socs2* or *Cish* (Fig. 6, top and middle). In the context of the potent repression elicited by expression of BCL6 alone, the addition of HDAC3 did not repress expression of *Socs2*-luc or *Cish*-luc further. However, in the presence of CA-STAT5b which alone did not alter promoter activation, the addition of HDAC3 significantly inhibited both the *Socs2* and *Cish* promoters, compared to either HDAC3 alone or CA-STAT5b alone. These observations are consistent with co-repression and/or recruitment of other factors by HDAC3 to STAT5b on *Socs2* and *Cish*. Co-expression of HDAC3 significantly inhibited *Bcl6*-luc when expressed in combination with BCL6 protein (Fig. 6, bottom) or with STAT5.

Thus, the *Bcl6* promoter is distinguished from *Socs2* and *Cish* in being inhibited by HDAC3 in the presence of BCL6, and strikingly, in being inhibited by p300 in the presence of BCL6 or STAT5. Together these findings show that BCL6 is a potent inhibitor of three GH-regulated genes which share reciprocal regulation of BCL6 and STAT5 occupancy in response to GH. However, for *Bcl6*, a gene repressed rather than stimulated by GH, p300 is repressive rather than activating, and renders STAT5b inhibitory.

4. Discussion

4.1. Reciprocal occupancy of gene promoters by BCL6 and STAT5 mediates diverse transcriptional outcomes in response to GH

BCL6 is known as a transcription repressor, and STAT5 is named for its general role as a transcription activator (Basso and Dalla-Favera, 2010; Cesena et al., 2007b; Herrington et al., 2000; Schebesta et al., 2002; Staudt et al., 1999; Waters et al., 2006). This study

demonstrates that BCL6 and STAT5 show reciprocal occupancy patterns in response to GH on multiple genes: BCL6 occupies the proximal promoters of GH target genes *Socs2, Cish* and *Bcl6* in the absence of GH and decreases after GH treatment, while STAT5 occupancy is low in the absence of GH and increases after treatment. However, the similarity in their occupancy patterns does not lead to similar transcriptional outcomes, since *Socs2* and *Cish* expressions are stimulated by GH while *Bcl6* expression is inhibited. Differences in transcriptional response are specific for each promoter, and depend not only on the transcription factor occupancy but also on interactions with co-regulatory proteins.

Despite differences in transcriptional responses to GH, the occupied promoter sequences for *Socs2, Cish* and *Bcl6* have several features in common. They contain the highly similar binding motifs shared by STAT5 and BCL6 and the site of BCL6/STAT5 occupancy is close to the TSS on each gene. ChIP-Seq showed that only 3% of all BCL6 peaks identified throughout the genome lie at the TSS, in contrast to approximately 80% in intronic or intergenic sequences; the remainder are located in exons or 3′UTR (Lin G and Schwartz J, manuscript in preparation). This is in general agreement with a report that most STAT sites reside outside of traditional promoters (Nelson et al., 2004).

The reciprocal relationship of BCL6 and STAT5 on DNA may reflect a variety of scenarios: The GH-induced decrease in BCL6 binding may be secondary to the potent inhibition of *Bcl6* transcription by GH and resulting decrease in availability of BCL6 protein for binding. One can postulate that GH-activated STAT5 displaces BCL6 on a shared BCL6/STAT5 motif; STAT5 has recently been reported to outcompete STAT3 in occupying the *Bcl6* promoter (Walker et al., 2013). In this regard, two predicted BCL6 and/or STAT5 binding sites are present within the BCL6/STAT5 occupancy regions identified by ChIP-Seq, for *Socs2*, for *Cish* and for *Bcl6* (Fig. 5A). Thus, BCL6 and STAT5 may bind to different motifs within the occupied region. It is currently unclear which occupancy site or sites BCL6 and STAT5 occupy in these regulatory regions, and to what extent each of these sites is functional. Flanking sequences also impact the transcriptional outcome for the *Bcl6* promoter (Ramachandrareddy et al., 2010; Walker et al., 2007). Although thousands of STAT sites were identified throughout the genome by ChIP-Seq in fibroblasts, the majority of STAT sites were reported to be ‘opportunistic’ while only 40% were associated with transcriptional responses (Zhu et al., 2012). Which among the abundant Bcl6 sites across the genome are functional remain to be determined.

BCL6 and STAT5 occupancy appear to be commonly detected on the *Socs2, Cish*, and *Bcl6* promoters in cell types known to be responsive to STAT5 regulation, such as liver, adipocytes and immune cells (Appendix: Supplementary Table S3). Furthermore, the chromatin structure surrounding the *Socs2, Cish*, and *Bcl6* promoters suggest that the promoters of these genes are potentially accessible to the transcriptional machinery in multiple cell types, based on association of these three genes with H3K9Ac, H3K27Ac, and possibly H3K4Me3 (Appendix: Supplementary Table S3). This suggests that reciprocal regulation of *Socs2, Cish* and *Bcl6* by BCL6 and STAT5 in response to external stimuli may occur in multiple cells types, although the precise molecular mechanisms and co-regulatory molecules involved are likely to differ.

Post-translational modifications to BCL6 (Bereshchenko et al., 2002; Moriyama et al., 1997; Niu et al., 1998) and STAT5 (Cesena et al., 2007b; Herrington et al., 2000) such as phosphorylation and acetylation induced by signaling molecules activated by GH could also influence the association of co-activators or co-repressors with BCL6 and STAT5 and the recruitment of these complexes to specific genes (Icardi et al., 2012; Mendez et al., 2008). Recent studies have shown that Rac1 signaling induces a BCL6/STAT5 transcriptional switch on cell-cycle-associated target gene promoters (Barros et al., 2012). The reciprocal relationship between BCL6 and STAT5 occupancy may also contribute to regulation by other cytokines such as prolactin, a key factor involved in roles of BCL6 and STAT5 in normal breast development and oncogenic breast cancer growth (Tran et al., 2010). In light of the variety of factors which influence STAT5 and BCL6 binding, it remains to be determined whether or how other loci occupied by BCL6 have functional consequences, and the extent to which signaling pathways and cell type impact the binding at BCL6/STAT5 sites and the transcriptional outcomes.

4.2. Transcriptional responses to BCL6 and to STAT5 are modulated by co-regulatory proteins p300 and HDAC3

On the promoters of *Socs2* and *Cish*, which are stimulated by GH, BCL6 serves as a repressor when it is expressed alone. A GH-induced decrease in *Bcl6* mRNA and consequent loss of BCL6 repression of *Socs2* and *Cish* are consistent with the increased expression of these two genes in response to GH. Though p300 often serves as a co-activator, the potent repression of *Socs2* and *Cish* by BCL6 persists even when p300 is co-expressed. Interaction of p300 with BCL6 protein results in acetylation of BCL6 at a KKYK motif, leading to disruption of BCL6 interactions with co-repressive molecules and loss of BCL6 inhibition on some target genes (Bereshchenko et al., 2002). On *Socs2*, the slight increase in promoter activity observed with co-expression of p300 and BCL6 relative to BCL6 alone suggests that for the *Socs2* promoter p300 may be sufficient to diminish the BCL6-induced repression slightly, consistent with loss of BCL6 repressive activity upon acetylation (Bereshchenko et al., 2002) in conjunction with p300. On the *Cish* promoter, p300 did not alter BCL6-induced repression when p300 was co-expressed with BCL6, suggesting promoter-specific differences in sensitivity to repressive and activating factors. Additionally, *Socs2* was more responsive than *Cish* to GH-stimulated acetylation of H3 at 30 min GH, while the acetylation of H4 was equivalently stimulated by GH on *Socs2* and *Cish*; in contrast, on *Bcl6*, histone acetylation was not regulated by GH.

For *Socs2* and *Cish*, in contrast to the relative imperviousness of BCL6 to co-regulation by p300 when they were expressed together, STAT5 activation increased only when it was co-expressed with p300, but not alone. The addition of p300 with STAT5, relative to STAT5 alone, doubled the activation of the *Socs2* and *Cish* promoters, a trend consistent with co-activation, though the increase was modest compared to the magnitude of p300 co-activation of some other genes (Chia and Rotwein, 2010; Pfitzner et al., 1998). These findings are in general agreement with *in vivo* observations in which, on *Socs2* and *Cish*, p300 did not serve as an effective co-activator for STAT5b in response to GH (Chia and Rotwein, 2010; Chia et al., 2010); it was suggested that other undefined co-activators or acetylases serve this function.

Co-repressor proteins also occupy the *Socs2* and *Cish* promoters. Deacetylase activity has been associated with both BCL6 and STAT5 (Bereshchenko et al., 2002; Dhordain et al., 1997; Icardi et al., 2012; Rascle et al., 2003). In fact, deacetylase activity is reported to be required for STAT5 to enable RNA Pol II recruitment (Rascle et al., 2003; Sebastián et al., 2008). For BCL6, its reported interactions with multiple HDAC proteins are important for the repression by BCL6 (Bereshchenko et al., 2002; Cotto et al., 2010; Dhordain et al., 1997). In particular, HDAC3 associates with BCL6 in repressing a subset of NF-κB target genes in bone marrow derived macrophages (Barish et al., 2010). The present results suggest that HDAC3 functions as a co-repressor for STAT5 but not for BCL6 on the *Socs2* and *Cish* promoters. Multiple HDACs may be involved in regulating transcription of *Socs2* and *Cish* by STAT5 at different steps of the transcriptional response.

4.3. The Bcl6 *promoter is repressed by p300*

Repression of the *Bcl6* promoter by p300 clearly distinguishes *Bcl6* from *Socs2* and *Cish* promoters, whose activity is increased by p300 in the presence of STAT5. Co-expression of p300 with BCL6 inhibited the *Bcl6* promoter significantly below the level with BCL6 expression alone, consistent with p300 serving as a co-repressor for BCL6 on this promoter. While p300 has acetylase activity, the repression by p300 is likely not related to acetylation of BCL6, as acetylation of BCL6 disrupts its interactions with co-repressors, resulting in a loss of BCL6-mediated repression (Bereshchenko et al., 2002). Other functions of p300 such as serving as a scaffold to recruit other interacting proteins may contribute to its repressive properties. It is likely that p300 exerts repression by interacting or coordinating with other nuclear factors that associate with BCL6, such as HDACs. Repression of *Bcl6*, *Socs2* and *Cish* by HDAC3 is in agreement with well-accepted co-repressor roles of HDACs (Cunliffe, 2008; Fischle et al., 2001; Narlikar et al., 2002). These findings extend observations that HDAC3 occupies multiple immune-related genes within the BCL6 and NF-κB cistromes (Barish et al., 2010). Further, HDAC1 interacts with the POZ domain of BCL6, and HDAC inhibitors reduce BCL6-mediated repression (Dhordain et al., 1998). In repressing *Bcl6*, other co-repressor proteins likely work in conjunction with p300 and/or HDACs. Among these, NCoR and SMRT, which are reported to enhance the enzymatic function of HDAC3 (You et al., 2013), partner with HDACs in the repression of adipogenic genes (Alenghat et al., 2008), and the NCoR cistrome overlaps with the BCL6 cistrome in regulating atherogenic genes in bone marrow (Barish et al., 2012). Other co-repressor candidates include CtBP, a co-repressor in autorepression of *Bcl6* (Mendez et al., 2008), and the BCL6 co-repressor BCoR (Huynh et al., 2000). A hybrid mechanism involving two independent repressive complexes associated with BCL6, one involving HDAC3 and p300, has recently been reported (Hatzi et al., 2013). Interactions among BCL6 and co-regulators are thought to play a role in the pathogenesis of conditions such as B-cell lymphoma (Cerchietti et al., 2010; Pasqualucci et al., 2011).

On the *Bcl6* promoter, STAT5b is rendered repressive by both p300 and HDAC3. Based on deacetylase inhibitor studies, HDACs have been found to be required for recruitment of the basal transcription machinery to STAT5 target genes (Rascle et al., 2003; Sebastián et al., 2008); thus HDACs appear to participate in STAT5-mediated transcriptional activation. In its canonical role as a transcriptional co-repressor, as observed here, HDAC3 is likely to

recruit additional co-repressors, many of which are known to interact with STAT5. Among these are FoxO1 and FoxO3 (Fernandez de Mattos et al., 2004; Ono et al., 2007), KRAB (Krebs et al., 2012) and Sin3a (Icardi et al., 2012), as well as NCoR/SMRT with HDAC3 (Fischle et al., 2002; Guenther et al., 2001). Such mechanisms involving co-repressors such as HDACs mediate STAT5 serving as a repressive transcription factor, for target genes in lymphocyte development (Heltemes-Harris and Farrar, 2012) and for genes such as IRF1 and other NF-κB regulated genes (Luo and Yu-Lee, 2000). STAT5 has also been shown to interact with the histone H3Lys27 methylase Ezh2, thus initiating the formation of repressive chromatin (Mandal et al., 2011). Similarly, this study documents that STAT5b is repressive when co-expressed with HDAC3 for *Socs2, Cish* and for *Bcl6*.

The role of p300 in STAT5-mediated repression of the *Bcl6* promoter is non-traditional, since p300 has long been known to be recruited to STATs for transactivation (Paulson et al., 1999). The diverse roles of p300 as co-activator and co-repressor reflect the promoter-specific properties of co-regulators in determining transcriptional outcome (Luo and Yu-Lee, 2000). The ability of p300 to recruit or serve as a scaffold for other co-regulators is also a contributor in determining transcriptional outcome. For example, p300 recruits HDAC6 to the STAT5 regulated sequence in *Spi2.1* to mediate negative regulation of GH-STAT5b-mediated transcription, which is dependent on activation of RhoA/ROCK (Ling and Lobie, 2004). Such versatility of co-regulator function on different promoters is reflected in recent demonstration that genes associated with NF-κB-driven inflammatory responses and tissue remodeling were highly enriched in the BCL6 and the overlapping NCoR/SMRT cistromes (Barish et al., 2012).

Taken together, the present findings demonstrate critical roles of a pair of transcription factors which exhibit reciprocal occupancy on multiple genes in response to GH, but which mediate contrasting transcriptional outcomes in response to GH dependent on the target gene. The occupancy of BCL6 appears to be a consistent determinant for repression of target genes, while occupancy of STAT5, which is often activating for GH-regulated genes, leads to repression for the *Bcl6* promoter. The transcriptional outcomes associated with the similar occupancy pattern for BCL6 and STAT5 on the different target genes are in turn determined by co-regulatory proteins associated with BCL6 and/or STAT5. HDAC3 consistently mediated transcriptional repression in conjunction with STAT5 or BCL6 on all three of the genes studied. On the other hand, p300 enhanced activation of GH-stimulated *Socs2* and *Cish* genes, but p300 mediated repression of *Bcl6*, which is inhibited by GH. Molecular interactions of p300 and HDAC3 with the transcription factors BCL6 and STAT5, or other associated regulatory proteins may be critical determinants in transcriptional outcome, and in response to signaling by GH and other regulators implicated in normal and oncogenic growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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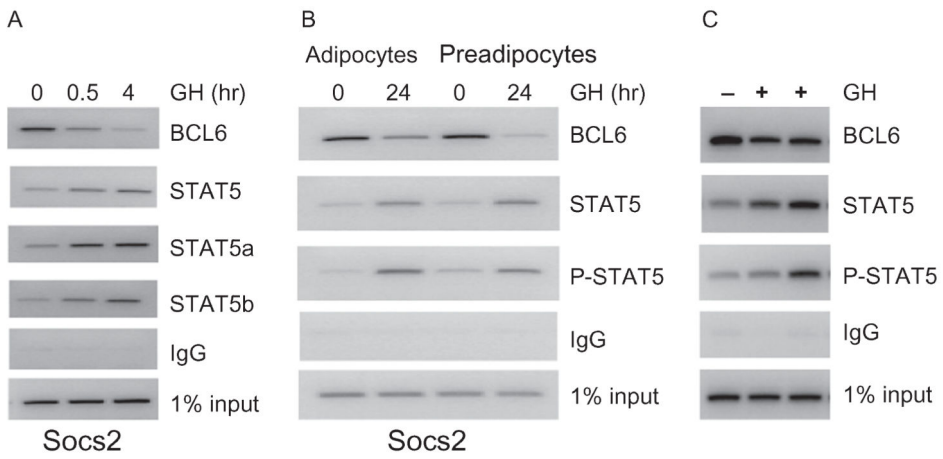

Fig. 1.

BCL6 and STAT5 occupy *Socs2* DNA reciprocally in response to GH. (A) 3T3-F442A preadipocytes were treated with GH for 0, 0.5 or 48 h and ChIP was carried out using antibodies against BCL6, STAT5, STAT5a or STAT5b. ChIP DNA was probed with primers specific to the STAT5 occupancy sequence on the *Socs2* promoter. IgG served as a negative control and 1% input was used as an internal control, in this and subsequent figures. Data are representative of at least two experiments. (B) 3T3-F442A preadipocytes and adipocytes were treated with GH for 0 and 24 h and ChIP was carried out using antibodies against BCL6, STAT5, and phosphorylated STAT5 (P-STAT5). ChIP DNA was probed with primers specific to the STAT5 occupancy sequence on the *Socs2* promoter. (C) Male littermate mice were treated ip with GH (1.5 mg/kg in 48 h) (+) or vehicle (−). Liver was used for ChIP analysis using antibodies against BCL6, STAT5 or P-STAT5 and probed for *Socs2* as above.

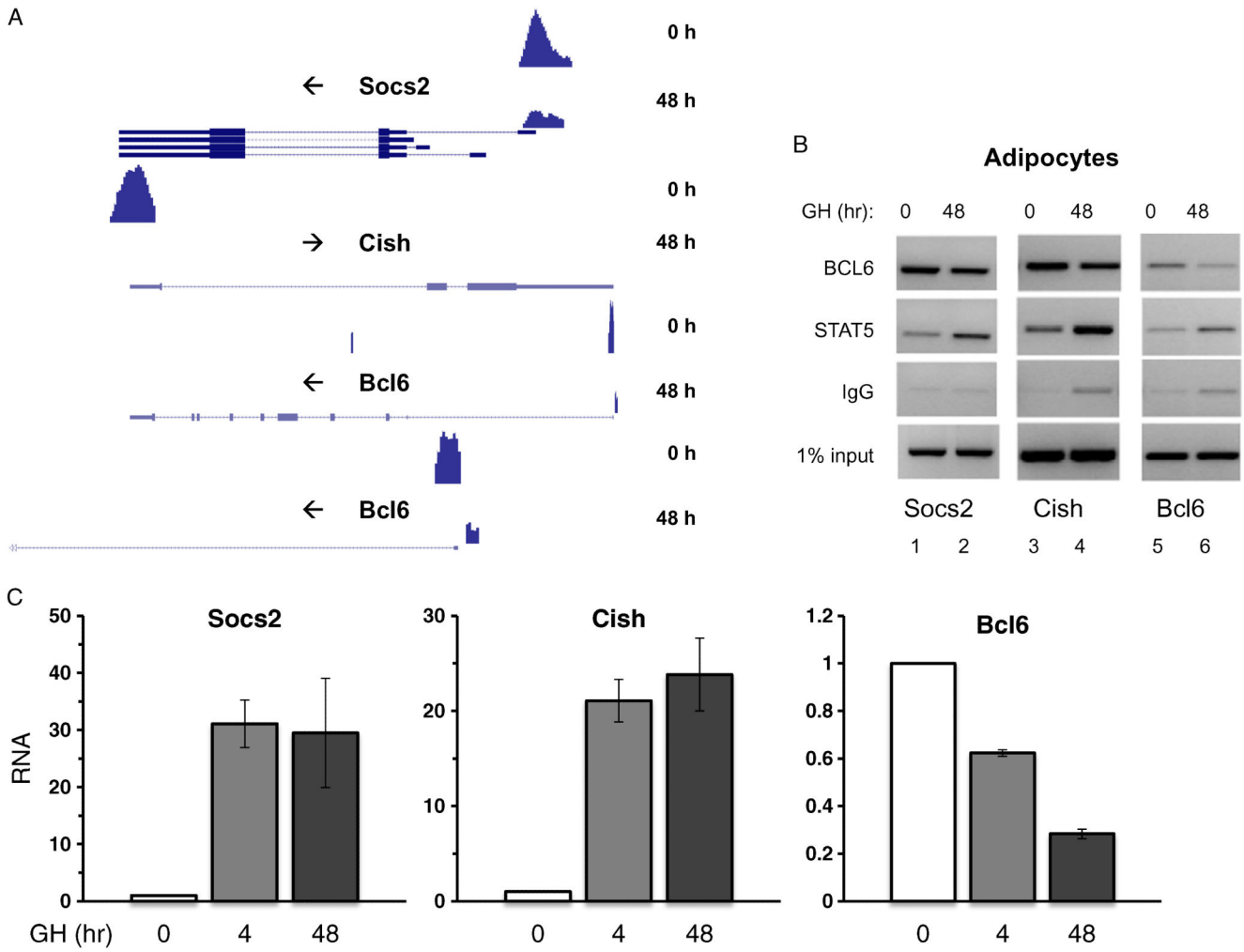

Fig. 2.

BCL6 and STAT5 show reciprocal occupancy at transcription start sites of *Socs2*, *Cish*, and *Bcl6*. (A) Peaks of BCL6 occupancy determined by ChIP-Seq were identified by HPeak at the transcription start sites (TSS) of *Socs2*, *Cish* and *Bcl6*. Shown are the UCSC Genome Browser profiles for BCL6 occupancy in 3T3-F442A adipocytes treated with GH for 0 or 48 h. Y axis scales were set at 60 for *Socs2*, 35 for *Cish*, and 20 for *Bcl6* as determined by maximum peak height in the absence of GH as calculated by HPeak. Top panel: *Socs2*. TSS: mouse chromosome (chr) 10: 94879491. Peak location 0 h GH: chr10: 94879276-94880050; 48 h GH: chr10: 94879326-94879975. Predicted BCL6/STAT consensus sites: chr10: 94879539-94879547 and chr10: 94879550-94879558. Second panel: *Cish*. TSS: chr9: 107199020. Peak location 0 h GH: chr9: 107198726-107199400; 48 h GH: chr9: 107198751-107199150. Predicted BCL6/STAT consensus sites: chr9: 107198991-107198999 and chr9: 107199002-107199010. Third panel: *Bcl6*. TSS: chr16: 23988698. Peak location 0 h GH: chr16: 23988401-23988850; 48 h GH: chr16: 23988776-23988900. Predicted BCL6/STAT consensus sites: chr16: 23988676-23988684 and chr16: 23988717-23988725. Bottom panel: Peak profile at *Bcl6* promoter region with expanded scale to visualize peaks in detail. Arrows to the left of gene names indicate

direction of transcription, from 5′ to 3′. Gene structure diagrammed at bottom of each profile. (B) 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 corresponding to ChIP-Seq (peaks) on the *Socs2* (lanes 1 and 2), *Cish* (lanes 3 and 4) and *Bcl6* (lanes 5 and 6) genes. (C) 3T3-F442A adipocytes were treated with GH for 0, 4 or 48 h. RNA was prepared and analyzed by qPCR using primers for Socs2, Cish and Bcl6. Bars show mean + SE of triplicates in an experiment which was repeated at least 3 times.

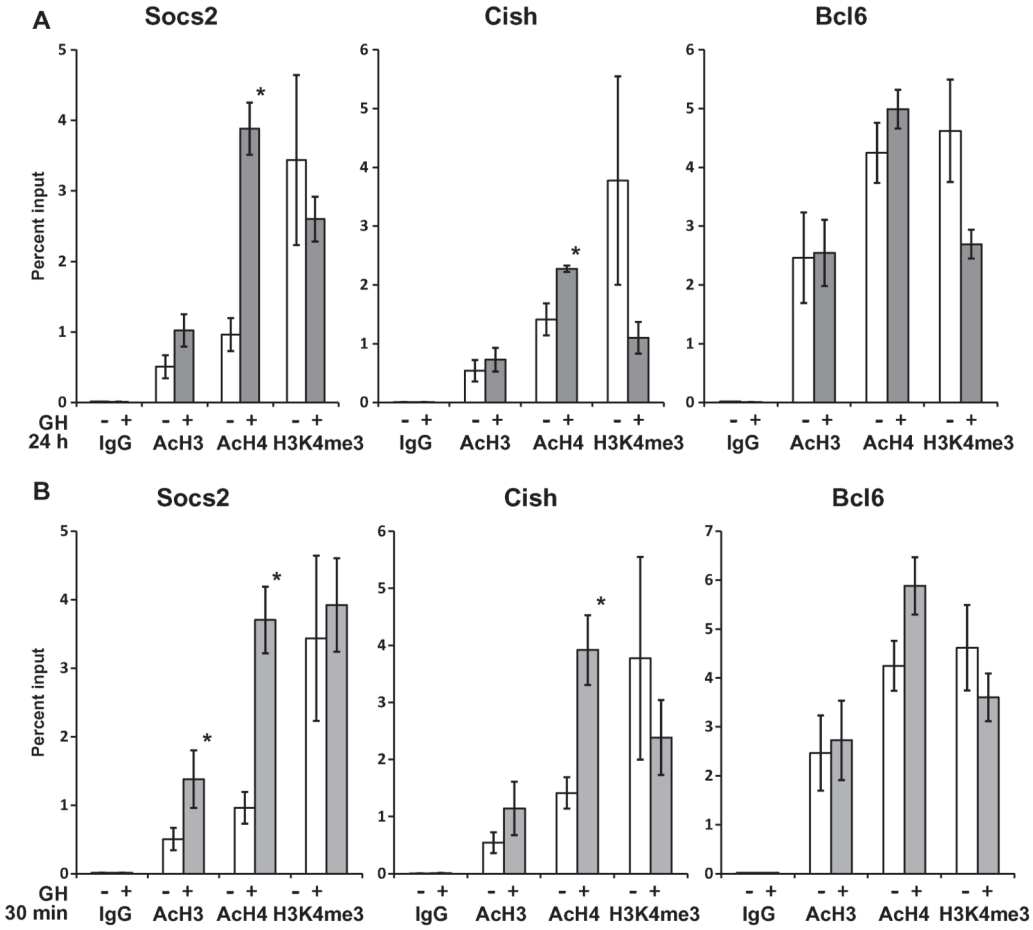

Fig. 3.

Enrichment of AcH3 and AcH4 increases in response to GH on *Socs2* and *Cish*, but not *Bcl6*. 3T3-F442A adipocytes were treated without (open bars) or with GH (gray bars) for (A) 24 h or (B) 30 min. ChIP was performed using antibodies against AcH3, AcH4, or H3K4me3. QPCR was used to analyze enrichment of these activating histone marks at the BCL6/STAT5 sites on *Socs2*, *Cish*, and *Bcl6* promoter sequences. Signal from ChIP DNA was normalized to input samples for respective treatment conditions. IgG was used as negative control. Each bar shows the mean ± SE from 4 independent experiments. Asterisks (*) indicate responses to GH that are statistically significant ($P < 0.05$).

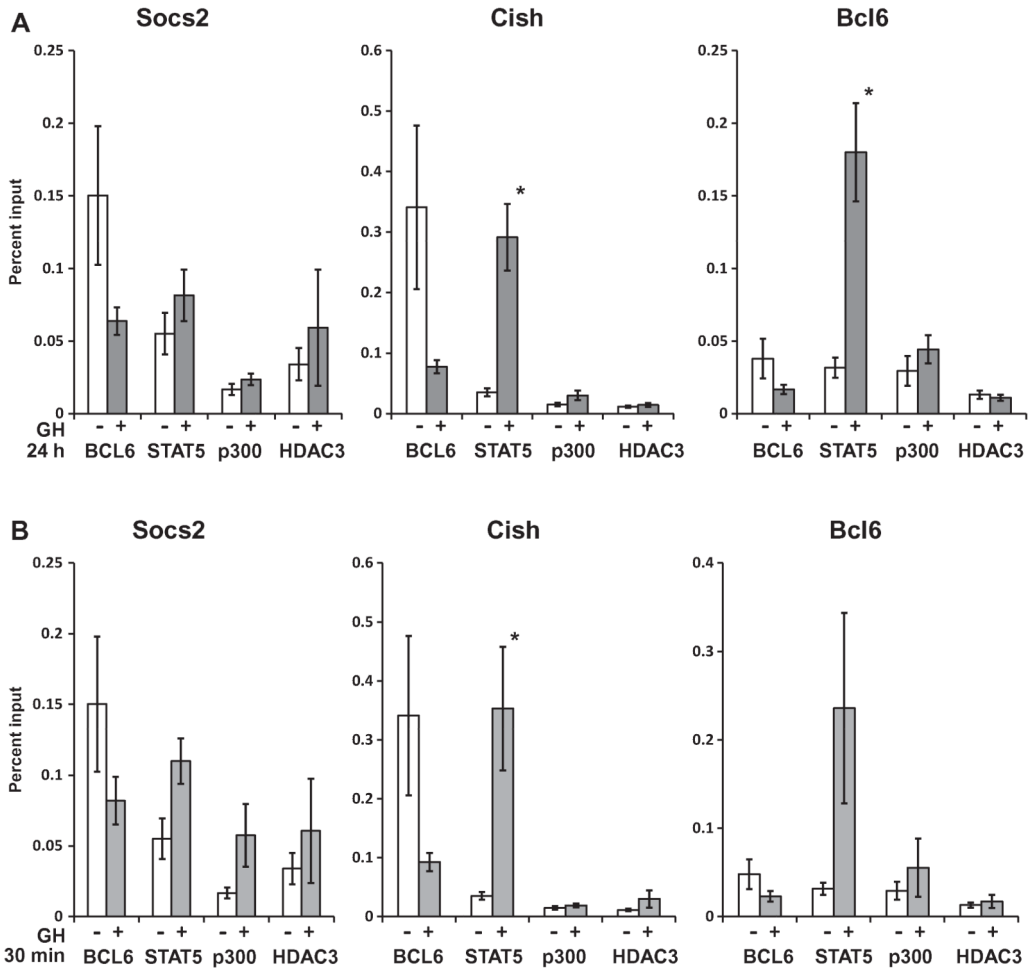

Fig. 4.

ChIP identifies p300 and HDAC3 on *Socs2*, *Cish*, and *Bcl6*. 3T3-F442A adipocytes were treated without (open bars) or with GH (gray bars) for (A) 24 h or (B) 30 min. ChIP was performed with antibodies against BCL6, STAT5, p300, or HDAC3. ChIP DNA was analyzed by qPCR with primers for the BCL6/STAT5 sites on *Socs2*, *Cish*, and *Bcl6*. Signals were normalized to input DNA from respective treatment conditions. Each bar shows the mean ± SE for 7 (BCL6 and STAT5) or 3 (p300 and HDAC3) independent experiments. Asterisks (*) indicate responses to GH that are statistically significant ($P < 0.05$).

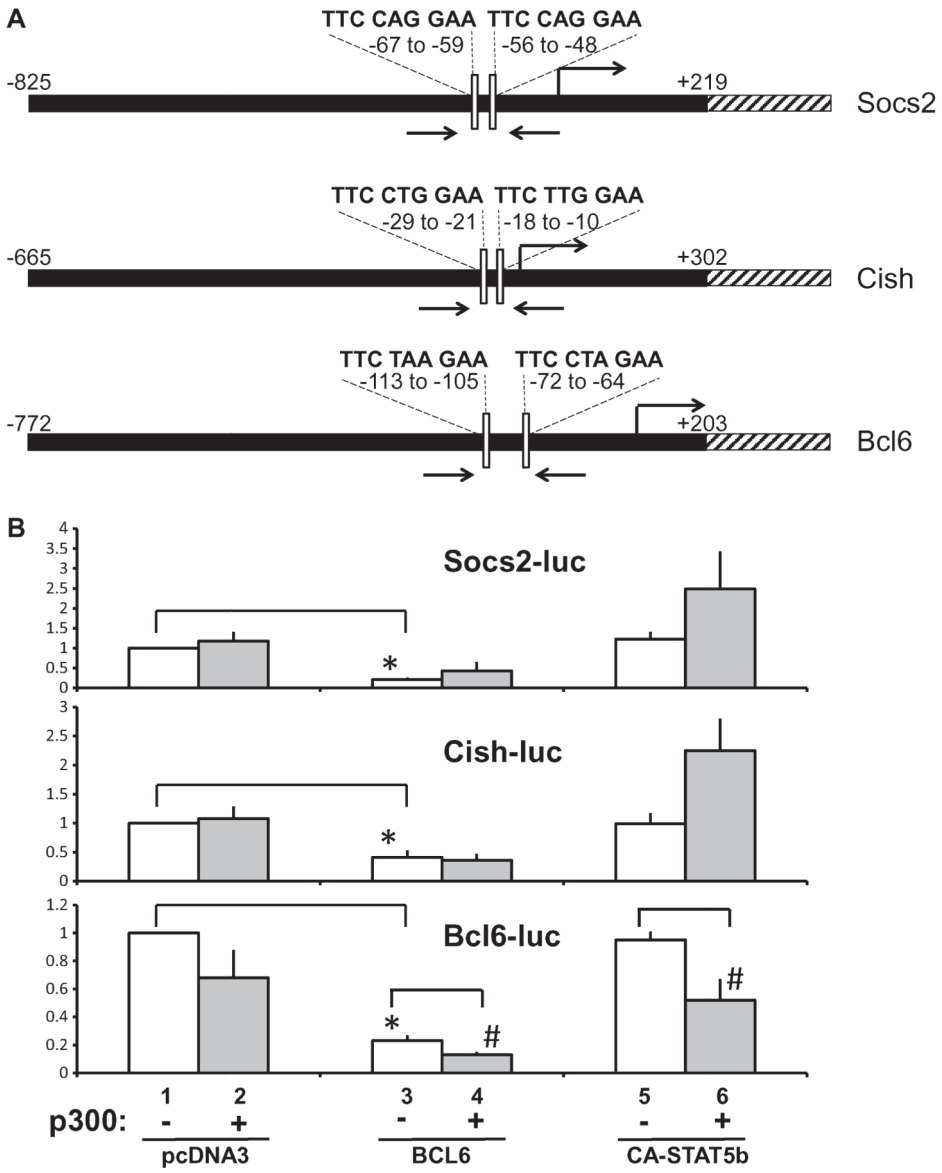

Fig. 5.

p300 represses transcription of the *Bcl6* promoter. (A) Diagram of *Socs2*, *Cish* and *Bcl6* genes showing relationship of genomic promoter sequences (black) driving luciferase (hatched) in reporter plasmids, predicted BCL6/STAT sites within occupied sequence (white bars), and location of ChIP primers (arrows). Predicted BCL6/STAT motifs are indicated above motif locations. Chromosomal locations are indicated in the legend of Fig. 2A. Diagrams are not to scale. (B) Plasmids for *Socs2*-luc (top), *Cish*-luc (middle) or *Bcl6*-luc (bottom) were transfected into 293T cells in the absence (bars 1–2) or presence of BCL6 (bars 3–4), or constitutively active STAT5b (CA-STAT5b) (bars 5–6). Plasmid for p300 (gray bars 2, 4, 6) or empty vector (pcDNA3, open bars 1, 3, 5) was co-expressed as indicated. Bars represent means + SE for 5 (*Socs2*, *Cish*) or 6 (*Bcl6*) independent experiments. Asterisks (*) indicate that BCL6 (bar 3) significantly ($P < 0.05$) represses

expression compared to basal luciferase (bar 1). Hatch signs (#) show responses to p300 that are significantly ($P < 0.05$) different from the respective pair in the absence of p300.

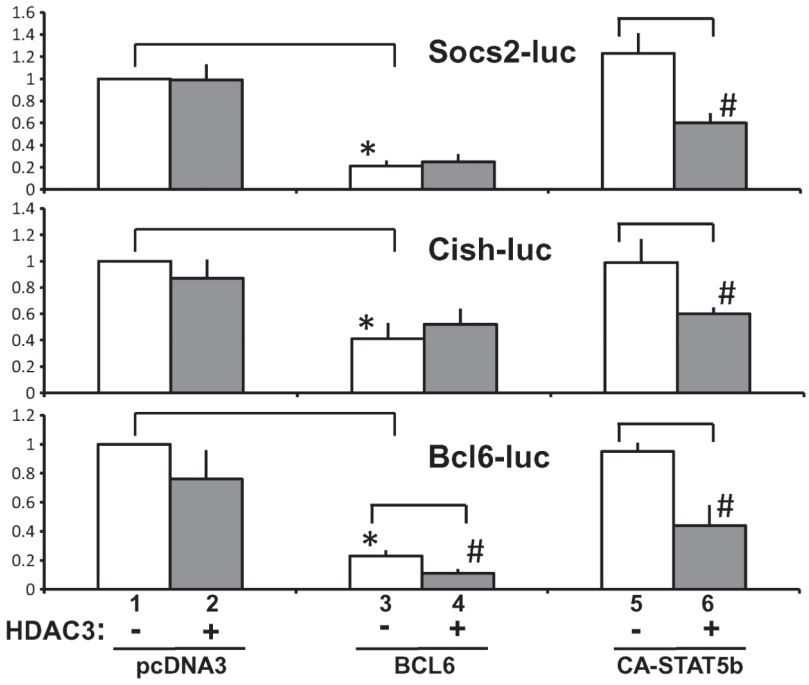

Fig. 6.

HDAC3 co-represses STAT5b on *Socs2*, *Cish* and *Bcl6*. Plasmids for *Socs2-luc* (top), *Cish-luc* (middle) or *Bcl6-luc* (bottom) were transfected into 293T cells in the absence (bars 1–2) or presence of BCL6 (bars 3–4), or constitutively active STAT5b (CA-STAT5b) (bars 5–6). Plasmid for HDAC3 (gray bars 2, 4, 6) or empty vector (pcDNA3, open bars 1, 3, 5) was co-expressed as indicated. Bars represent means + SE for 5 (*Socs2*, *Cish*) or 6 (*Bcl6*) independent experiments. Asterisks (*) indicate that BCL6 significantly ($P < 0.05$) repressed expression compared to basal luciferase (bar 1). Hatch signs (#) show responses to HDAC3 that are significantly ($P < 0.05$) lower than respective pair in the absence of HDAC3.