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Title: Computational and functional analysis of growth hormone-regulated genes identifies the transcriptional repressor Bcl6 as a participant in GH-regulated transcription

Short title: Analysis of GH-regulated gene profile identifies Bcl6

Precis: An integrative computational strategy and functional studies reveal the transcriptional repressor Bcl6 as a novel participant in GH-regulated gene expression in adipocytes.

Authors: Yili Chen[1]*, Grace Lin[2]*, Jeffrey S. Huo[2], Deborah Barney[3], Zhenni Wang[3], Tamara Livshiz[3], David J.States[1,4], Zhaohui S. Qin [1,5], Jessica Schwartz[1,2,3]

Affiliations: [1]Bioinformatics Program, [2]Program in Cellular and Molecular Biology, Departments of [3]Molecular & Integrative Physiology, [4]Human Genetics, and [5]Biostatistics, University of Michigan, Ann Arbor, MI

Corresponding author:
Jessica Schwartz, Ph.D.
Dept of Molecular and Integrative Physiology
University of Michigan
Ann Arbor, MI 48109-5622
Tel: 734-647-2124
Fax: 734-647-9523
Email: jeschwar@umich.edu

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Abstract

For insight into transcriptional mechanisms mediating physiological responses to Growth Hormone (GH), data mining was performed on a profile of GH-regulated genes induced or inhibited at different times in highly responsive 3T3-F442A adipocytes. Gene Set Enrichment Analysis (GSEA) indicated that GH-regulated genes are enriched in pathways including phosphoinositide (PI) and insulin signaling, and suggested that SOCS2 and PI3 Kinase regulatory subunit p85α (Pik3r1) are important targets. Model-based Chinese Restaurant Clustering (CRC) identified a group of genes highly regulated by GH at times consistent with its key physiological actions. This cluster included IGF-1, PI3K p85α, SOCS2 and CIS. It also contains the most strongly repressed gene in the profile, B-cell lymphoma 6 (Bcl6), a transcriptional repressor. Quantitative real-time PCR verified the strong decrease in Bcl6 mRNA following GH treatment, and induction of the other genes in the cluster. Transcriptional network analysis of the genes implicated Signal Transducer and Activator of Transcription (Stat) 5 as hub regulating the most responsive genes, Igf1, Socs2, Cish and Bcl6. Transcriptional activation analysis demonstrated that Bcl6 inhibits SOCS2-luciferase and blunts its stimulation by GH. Occupancy of endogenous Bcl6 on SOCS2 DNA decreased after GH treatment, while occupancy of Stat5 increased concomitantly. Thus, GH-mediated inhibition of Bcl6 expression may reverse the repression of SOCS2 and facilitate SOCS2 activation by GH. Together these analyses identify Bcl6 as a participant in GH-regulated gene expression, and suggest an interplay between the repressor Bcl6 and the activator Stat5 in regulating genes which contribute to GH responses.

Introduction

Sequencing of the human genome has provided new tools for addressing long-standing questions regarding transcription regulatory events. While growth hormone (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 Jak2 (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 Insulin-like Growth Factor 1 (IGF1), c-*fos*, Spi2.1 and Cyp2 and Cyp3 family members (3-10). 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 they relate to physiological responses to GH.

GH is a major promoter of post-natal 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 (11-13). The signaling molecule phosphoinositide 3' kinase (PI3K) has been implicated in GH-induced insulin resistance (14, 15), and the gene encoding its regulatory subunit p85 alpha (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 datasets from 3T3-F442A adipocytes treated with GH for 48 hr, a time when GH induces insulin resistance, or for 4 hr 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 hr) (16). The present analysis utilized the gene datasets 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 CRC algorithm identified the same genes in a cluster of genes highly responsive to GH at 48 hr; 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 Stat5, 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.

Materials and Methods

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

deposited in GEO (GSE2120). The data were submitted to the following computational analyses:

Gene Set Enrichment Analysis. To identify pathways in which GH-regulated genes are enriched, GSEA (17) v1.0 Java desktop application (http://www.broad.mit.edu/gsea/) was downloaded from Broad Institute of MIT and Harvard. 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 (18-20). 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 (RMA) (21) 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 Chinese Restaurant Clustering (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 from http://www.sph.umich.edu/csg/qin/CRC. The CRC algorithm automatically infers the number of clusters while assigning 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 hr (cluster D), except for Iigp 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 hr (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 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 (TSS) and +100 bp downstream of last TSS 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): 5'-CGTGGCACAGACTTGGTGTTT-3' (sense) and 5'-GGCAGTGCTGGTGGATCC-3' (antisense). Data were normalized to GAPDH, 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) and H4IIE rat hepatoma cells (from J. Messina) were analyzed by immunoblotting with anti-Bcl6 (C-19) (1:500, Santa Cruz) as described (27). Lysates from Ramos B-cells (from R. Kwok and W. Dunnick)

served as positive controls as they typically express Bcl6. The proteins were visualized using IRDye 800-coupled antirabbit IgG (1:12000) on an Odyssey infrared scanning system. Molecular weight was estimated using protein molecular weight standards from Invitrogen.

Transcriptional activation. 293T cells (28) were transfected by calcium phosphate co-precipitation with a SOCS2-luc plasmid in which the intronic GH-responsive sequence of human SOCS2 drives TK-luciferase (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-beta-galactosidase (beta-gal) for normalization. Eighteen hr later, cells were deprived of serum by replacing it with 1% bovine serum albumin, incubated with human GH (500 ng/ml=22 nM, Lilly) for 24-26 hr, and used to measure luciferase activity as described previously (32). Data were analyzed by ANOVA with Bonferroni correction for multiple comparisons, using Prism.

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), anti-acetylated histone H4 (AcH4, Upstate) or normal rabbit IgG (Santa Cruz). The anti-Stat5 antibody used recognizes both Stats 5a 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 (34). Quantification of the ChIP data was performed using BioRad Multi-Analyst software.

Results

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 which were stimulated in time-dependent patterns following GH treatment in 3T3-F442A adipocytes, as well as 287 genes which were repressed at the same times, as reported previously (16).

Gene set enrichment analysis (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, where 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 dataset of GH-regulated genes in 3T3-F442A adipocytes. Since 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, JAK-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 which was relatively highly ranked was Toll-Like Receptor signaling; gene sets which 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 hr.

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 dataset 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 "Chinese restaurant" process (Chinese Restaurant Cluster) (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 which were defined by CRC analysis (Fig 1, left), Cluster D was selected for initial analysis because it contains genes showing the greatest fold changes 4-48 hr after GH (Fig 1, right), 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 Insulin-like Growth Factor (IGF) 1 (3, 4, 37), and signaling molecules in the SOCS family, SOCS2 and CISH (29, 38), as well as PI3K p85α (15). Note that 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 3 (ATF3) and Kruppel-Like Factor 5 (KLF5); signaling molecules Repressor of G-Protein Signaling 16 (RGS16) and Interferon-inducible p47 GTPase (IiGP1); the cytokine Interleukin-6 (IL-6); and Growth Arrest and DNA Damage Inducible (GADD) 45 gamma. These genes were induced by GH in adipocytes, and all except IL-6 showed highest levels of induction at 4 and 48 hr. 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%.

The changes in expression of most of the genes in this cluster, including the dramatic decrease in Bcl6 expression, were confirmed by quantitative real-time PCR (qt-pcr) of RNA from GH-treated 3T3-F442A adipocytes (Fig 2). The overall changes in mRNA for each gene correspond with microarray findings at 4 or 48 hr: Expression of SOCS2 and CISH mRNA was the most highly stimulated by GH (50-60 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 3) was built for genes in this cluster and their known interacting transcription factors, using the GeneGo database (St Joseph, MI) 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 which 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 IGF-1 (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 (39-41). The coordination via Stat5 of these four genes, as revealed in the network, suggests that the genes may be co-regulated by mechanisms involving Stat5 in response to GH. Genes for KLF5, ATF3 and IL-6 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 promoter regions of genes in Cluster D (Table 1). Conserved sites for Stats and for Bcl6, as well as for 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 CREB family of transcription factors, are also regulated by GH (44, 45). Bcl6 and KLFs/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 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 hr, in both 3T3-F442A adipocytes and preadipocytes (Supplementary Fig 1) Further, the endogenous Bcl6 protein was detectable in 3T3-F442A preadipocytes (Fig 4, inset); Bcl6 was also detected in H4IIE rat hepatoma cells.

The inverse regulation of Bcl6 and SOCS2 by GH was evaluated further, since SOCS2 is an established Stat5 target gene. The human SOCS2 gene contains a GH-inducible region in the first intron which contains two GH-responsive Stat5 binding sites (34). This sequence, fused upstream of TK-luciferase (termed SOCS2-luc), was transfected into 293T cells along with DNA for GH receptor. Treatment with GH for 24 hr stimulated SOCS2-luc as expected (Fig 5A). However, when Bcl6 was expressed, the activity of SOCS2-luc was strongly inhibited (Fig 5A, open bars), 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, while Stat5 activates it, as expected (34) (data not shown).

Endogenous Bcl6 and Stat5 also show an inverse relationship in occupying endogenous SOCS2 DNA, as demonstrated by chromatin immunoprecipitation (ChIP). Since SOCS2 is regulated by Bcl6 as well as by Stat5, the occupancy of these two factors on the SOCS2 GH-responsive sequence was examined *in vivo* by CHIP (Fig 5B). Occupancy of endogenous Bcl6 on SOCS2 was evident in the absence of GH and decreased progressively after GH treatment; Bcl6 occupancy was almost undetectable 48h after GH. For Stat5, occupancy on SOCS2 was negligible or absent without GH treatment (time 0), but Stat5 occupancy increased following GH. Thus, the occupancy of Bcl6 decreased as that of Stat5 increased (Supplementary Fig 2). 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, while 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, where it is crucial to understand how genes cooperate to carry out related series of reactions. Despite the fundamental advantages offered by high-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 utilized 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 since 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 while 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 POZ/BTB 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 co-repressors such as Smrt, NCoR and BCoR, as well as histone deacetylases (HDACs) (50-53), and it associates with chromatin remodeling complexes and other repressors (54-56). 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 FoxO1, and HNFs 3 beta, 4 alpha and 6 (9, 57-59). It remains to be determined whether Bcl6, and associated transcription regulatory factors, interact in complexes to regulate GH target genes.

Since 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, since Bcl6 occupies SOCS2 DNA within a GH-responsive sequence (34), and its occupancy decreases upon GH treatment. Further, 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 following 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 which 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 following 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 Erks 1/2 and acetylation to activate transcription factors such as C/EBP beta (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, since in a subset of germinal center cells, Stat5 increased expression of Bcl6 (41), while 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). Further, Prolactin, which activates Stat5, induces Bcl6 in pancreatic beta cells (39); Bcl6 in turn represses Menin expression, which allows the beta-cell proliferation of adaptive islet growth, as in pregnancy or obesity (39). However, a preliminary 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 and Schwartz, data not shown). These observations raise possibilities for identifying previously unrecognized functions of Bcl6 in metabolic regulation. Bcl6 -/- mice are reported to show growth retardation (66-68), 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, since Stat5 has been amply demonstrated to participate in regulation of genes implicated in metabolic regulation and adipogenesis (70-73). 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 GO categories, correspond with established physiological responses to GH (16). GSEA identified 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 (75). 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.

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Table 1

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 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.

Figure Legends:

Figure 1. CRC analysis of GH-regulated genes clusters co-regulated genes. A. Heat map shows results of CRC cluster analysis of a dataset of GH-regulated genes. Among the 12 defined clusters, Cluster D contains genes showing greatest differential regulation by GH at 4 and 48 hr. Red indicates increase; green indicates decrease. B. Expanded heatmap of Cluster D itemizing products of the individual genes.

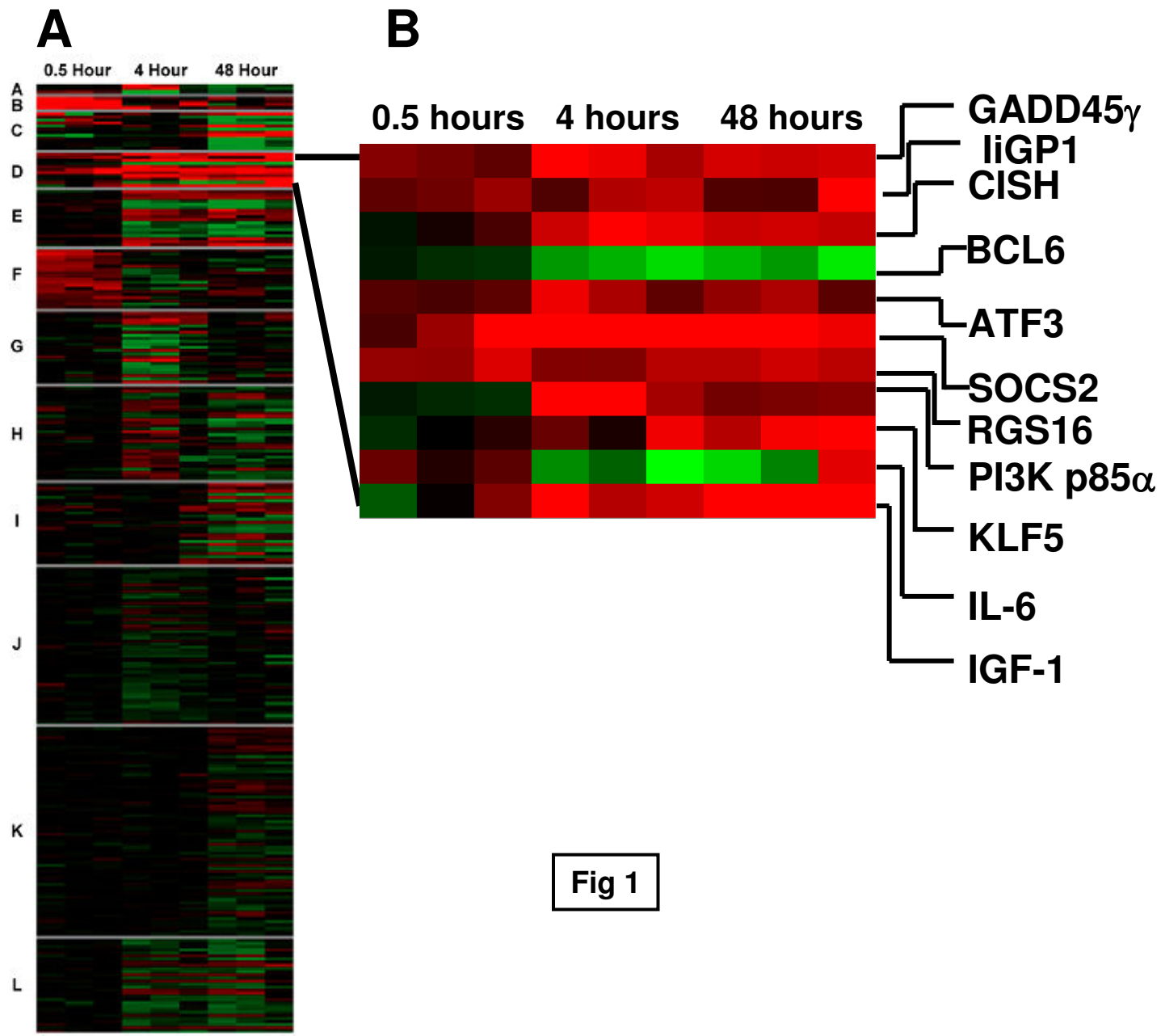
Figure 2. Expression of mRNA for representative genes in Cluster D. 3T3-F442A adipocytes were treated with GH for the indicated times; RNA was prepared and analyzed by qt-pcr. Responses to GH are expressed as (GH-C)/C. Data are representative of 2-8 independent experiments. The GH-responsive genes are grouped vertically in general categories related to function.

Figure 3. Network analysis using GeneGo MetaCore^{TM} identifies co-regulated GH target genes coordinated by Stat 5. Boxes indicate genes submitted; oval designates transcription factor hub.

Figure 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 hr 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 3 independent experiments. Inset: BCL6 protein is detected in 3T3-F442A preadipocytes and hepatoma cells. Immunoblotting with anti-Bcl6 demonstrates the presence of Bcl6 protein in lysates of 3T3-F442A preadipocytes (lane P) and H4IIE hepatoma cells (lane H). Lane R shows Ramos B-cells for reference. Tick mark on right shows migration of 80 kD molecular weight marker.

Figure 5. Bcl6 mediates expression of GH target genes. A. 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 beta-gal were transfected into 293T cells, in the absence or presence of an expression plasmid for Bcl6. Cells were treated without (open bars) or with GH (black bars) for 24 hr and luciferase activity was measured, expressed as RLU (relative luciferase units). Bars represent 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 open bars). The response to GH (compare open 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. B. 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, 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.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GADD45γ
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KLF5
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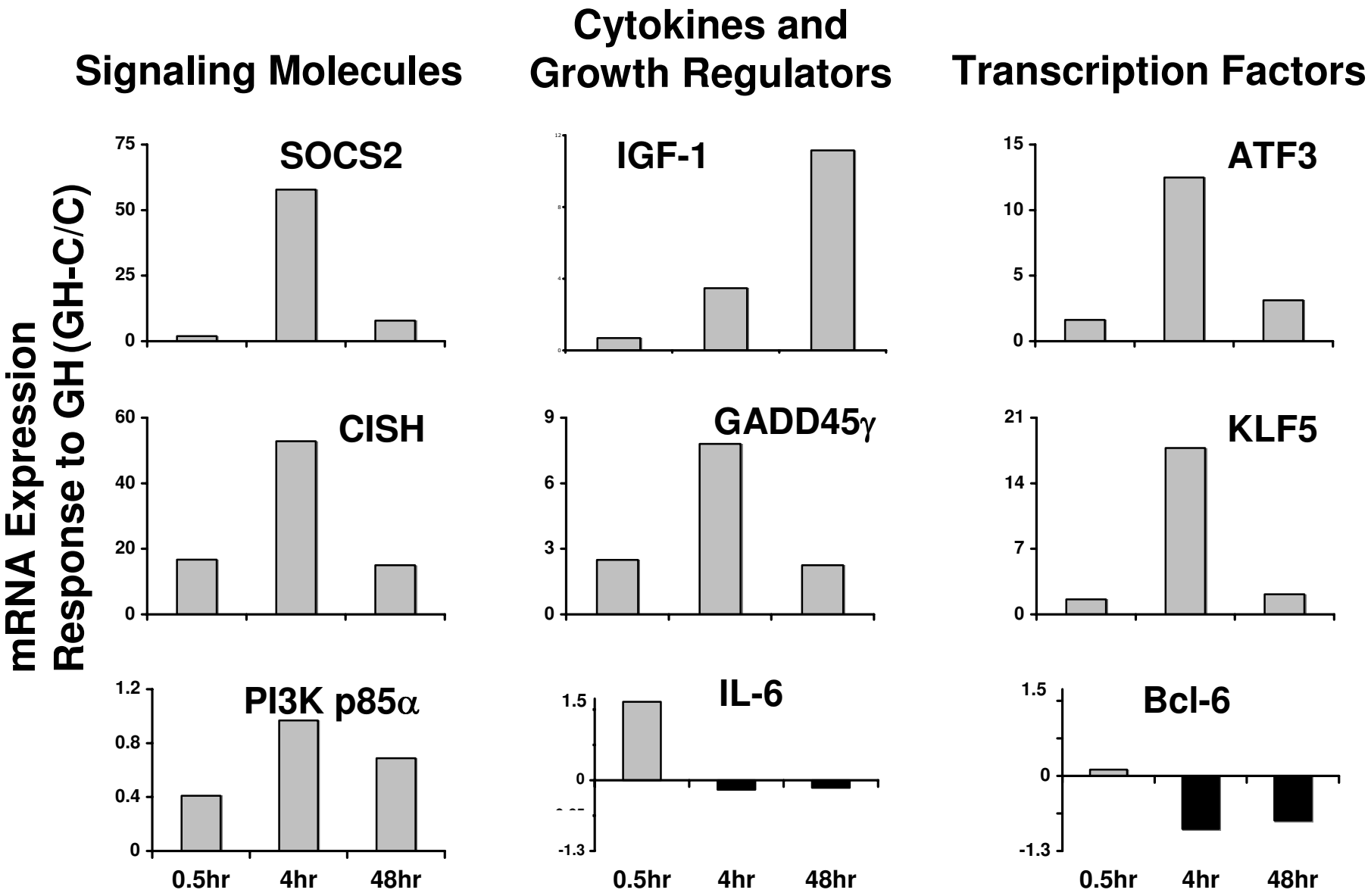
IGF-1

Fig 1

Fig 2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Cytokines and Growth Regulators
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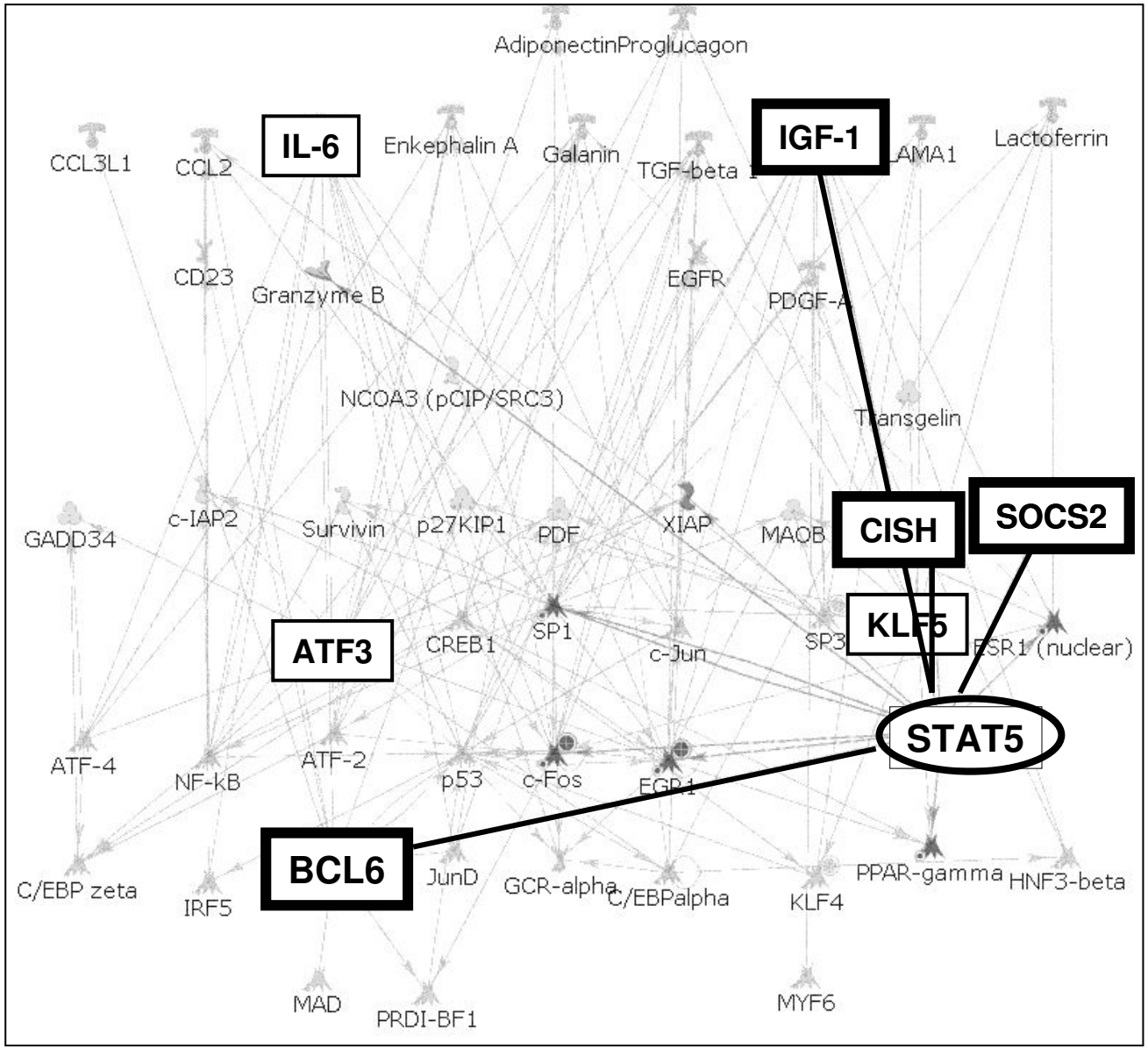
SOCS2
IGF-1
ATF3
CISH
GADD45γ
KLF5
PI3K p85α
IL-6
Bcl-6
0.5hr
4hr
48hr

Fig 3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Granzyme B
EGFR
PDGF-A
NCOA3 (pCIP/SRC3)
Transgelin
GADD34
c-IAP2
Survivin
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PDF
XIAP
MAOB
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STAT5
ATF-4
NF-kB
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c-Fos
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BCL6
C/EBP zeta
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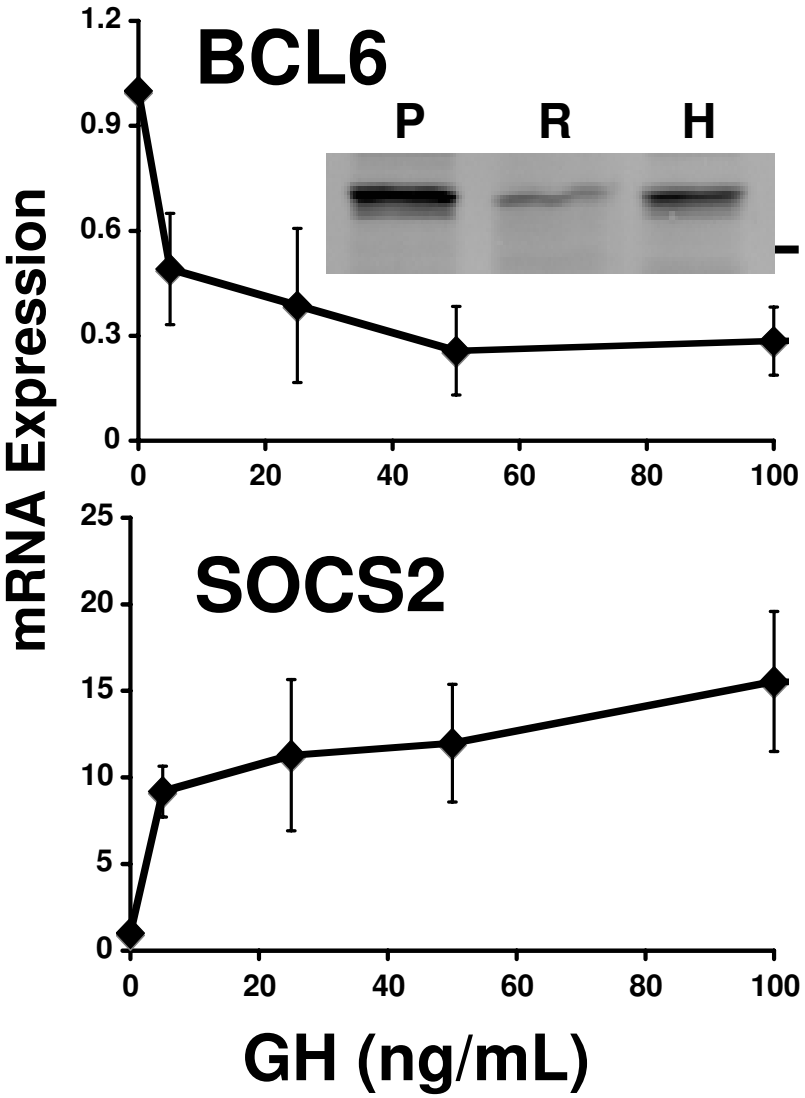
Fig 4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P
R
H
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0.9
0.6
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20
40
60
80
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20
40
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80
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GH (ng/mL)

Fig 5A

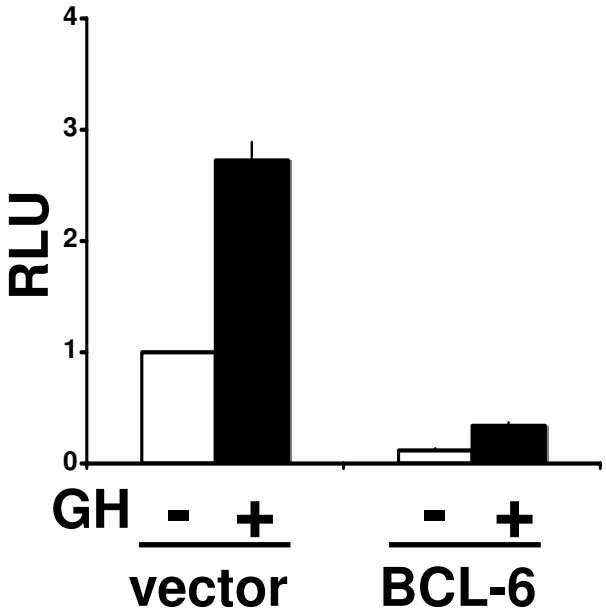
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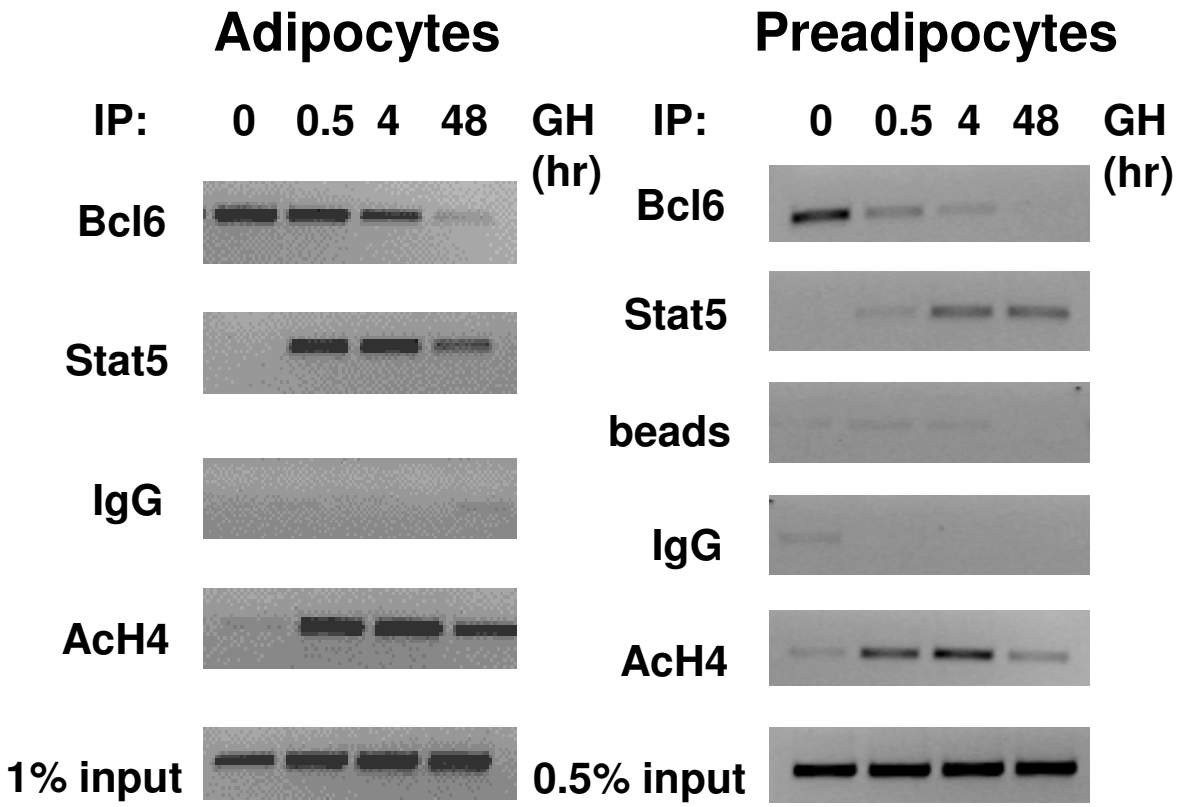
Fig 5B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Stat5
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