THE REGULATION OF PREADIPOCYTE DIFFERENTIATION BY GLUCOCORTICOIDS.

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The Regulation of Preadipocyte Differenciation by Glucocorticoids

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I dedicate this thesis to my grandmother, Joyce.

ABSTRACT

The adipogenic stimulus provided by glucocorticoids is most obvious in the central obesity of individuals with Cushing's syndrome. Glucocorticoids promote preadipocyte differentiation in culture, in part by enhancing the transcriptional potential of a key adipogenic factor, C/EBPβ. They do so by promoting proteasome dependent degradation of a pool of C/EBPβ-associated HDAC1, a transcriptional repressor protein. This potentiates C/EBPβ-mediated transcription of C/EBPα, a master regulator of differentiation. Analysis of the mechanisms by which HDAC1 is targeted for degradation has led to the identification of TIF1β as a cofactor of C/EBPβ-mediated transcription. TIF1β expression modulates the association of C/EBPβ with HDAC1 and promotes the ubiquitylation and proteasome dependent turnover of HDAC1 that appears to be required for the transactivation potential of TIF1β. Furthermore, TIF1β promotes 3T3 L1 preadipocyte differentiation through both HDAC1-dependent and independent mechanisms.

The elucidation of the molecular mechanisms by which glucocorticoids potentiate adipogenesis has been largely limited to murine model systems. In this work, I defined glucocorticoid requirements and their effects on the early transcriptional events that drive human primary preadipocyte differentiation. I identified striking similarities and some differences between the 3T3 L1 and human preadipocyte systems. Notably, accumulation of C/EBPβ was enhanced by glucocorticoids and C/EBPα was differentially expressed upon induction of differentiation in human preadipocytes. Furthermore, glucocorticoids provided a survival signal that was required for differentiating 3T3 L1 cells to progress through clonal expansion when cultured in chemically defined medium. Clonal expansion is a specific

property of the 3T3 L1 cell line that is not shared by human preadipocytes, which did not require glucocorticoids for survival of the initial stages of differentiation.

Lastly, I have shown that exposure of preadipocytes to glucocorticoids primes the cells with increased differentiation capacity. Microarray analysis of gene expression following pretreatment in human preadipocytes yielded factors that represent (1) previously unidentified, potential adipogenic targets of glucocorticoids and (2) potential mediators of this priming effect. Notably, pretreatment of human preadipocytes with physiological concentrations of steroid promoted increased insulin responsiveness. This response was specific to the human preadipocytes and was not observed in either the 3T3 L1 preadipocytes or human adipocytes.

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LIST OF ABBREVIATIONS

AF	activation function
aP2	adipocyte fatty acid binding protein
AR	androgen receptor
C/EBP	CCAAT enhancer binding protein
CS	calf serum
DBD	DNA binding domain
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
EDTA	ethylenediamine tetra-acetic acid
ER	estrogen receptor
ERR	estrogen-related receptor
FBS	fetal bovine serum
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HAT	histone acetyl transferase
HDAC	histone deacetylases
Hect	homology to E6AP-C-terminus
HP1	heterochromatin protein 1
HRE	hormone response element
HSD	hydroxysteroid dehydrogenase

Hsp	heat shock protein
IIF	indirect-immunofluorescence
InsR	insulin receptor
IP	Immunoprecipitation
IRS	insulin receptor substrate
LAP	liver activating protein
LBD	ligand binding domain
LIP	liver inhibitory protein
MCE	mitotic clonal expansion
Mdm2	murine double minute 2
MIX	3-isobutyl-1-methyl-xanthine
MMTV	mouse mammary tumour virus
MR	mineralocorticoid receptor
NHR	nuclear hormone receptor
NLS	nuclear localization sequence
NP-40	nonidet P40
NR	nuclear receptor
PBS	phosphate buffered saline
PHD	plant homeodomain
PI3K	phosphatidylinositol-3-kinase
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor

RAR	retinoic acid receptor
RING	really interesting new gene
RXR	retinoid X receptor
SC	Subcutaneous
SHR	steroid hormone receptor
TAG	Triacylglyceride
TIF	transcriptional intermediary factor
TR	thyroid hormone receptor
Ub	Ubiquitin
UPS	ubiquitin proteasome system
WAT	white adipose tissue

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INTRODUCTION

Glucocorticoid Physiology

Glucocorticoids are lipophilic steroid hormones. They are secreted by the adrenal cortex in response to adrenocorticotropic hormone (ACTH) stimulation under the tight control of the hypothalamus-pituitary-adrenal (HPA) axis (Chrousos, 1995). They are released both with circadian variation and in response to stress. Glucocorticoids are involved in the regulation of multiple physiological processes; they promote glucose homeostasis and protein, carbohydrate and lipid metabolism, they are potent anti-inflammatory agents, promote vasoconstriction, and regulate kidney function (Granner & Pilkis, 1990; Pilkis & Granner, 1992; Whitworth et al, 2000). In the brain, they are involved in hippocampal memory and long-term potentiation and they modulate behavior (Kim & Haller, 2007; McGaugh & Roozendaal, 2002; Roozendaal, 2000). Glucocorticoids are also important in both development and differentiation programs including lung maturation, regulation of bone turnover, erythropoeisis and promoting preadipocyte differentiation in white adipose tissue (Bauer et al, 1999; Gregoire et al, 1998; Hirayama et al, 2002; Smith & Sabry, 1983; Torday, 1980).

Naturally occurring glucocorticoids exist in two forms: the hormonally active steroids which are hydroxylated at position 11β (cortisol in humans and corticosterone in rodents), and the inactive 11β-keto metabolites (cortisone in humans and 11β-dehydrocorticosterone in rodents). The 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes regulate the local availability and abundance of active steroid. 11β-HSD1 is an NADPH-dependent oxo-reductase that is responsible for the conversion of the inactive metabolite to the hydroxylated

form. It is expressed in several glucocorticoid responsive organs including the liver, adipose tissue and the central nervous system. Knock-out studies in mice confirm that it is the major oxo-reductase in the body (Kotelevtsev et al, 1997). Conversely, 11β-HSD2 is an NAD^+-dependent dehydrogenase that is expressed in key mineralocorticoid responsive tissues, namely the kidney and placenta. In these tissues, 11β-HSD2 inactivates glucocorticoids to ensure the specificity of the mineralocorticoid receptor (MR) activity, which binds both mineralocorticoids (aldosterone) and active glucocorticoids with the same affinity (Funder, 1996).

The physiological effects of glucocorticoids are mediated at the cellular level by the glucocorticoid receptor (GR), a ligand-inducible transcription factor that regulates transcription of steroid-responsive genes. GR is expressed in all cell types with the highest level of mRNA expression in the lung, spleen, liver, and brain. The physiological importance of glucocorticoids is emphasized in the $GR^{-/-}$ mice which die shortly after birth due to pulmonary atelectasis and respiratory failure. These animals do not express liver gluconeogenic enzymes and have severely dysregulated glucose homeostasis. They have a large and disordered adrenal gland that is completely lacking the central medulla and they do not produce adrenaline. Furthermore, they have elevated circulating levels of ACTH and corticosterone due to impaired negative feedback control on the HPA axis (Cole et al, 1995).

Nuclear Receptor Superfamily

GR is a member of the nuclear receptor (NR) superfamily which defines a large family of ligand-dependent transcription factors that share an evolutionary history that is marked by responsiveness to lipophilic ligands. While many of them have known ligands,

including steroid hormones and products of lipid metabolism, there remain NRs for which ligands continue to be sought. NRs are believed to have arisen from a common ancestor (over 400 million years ago) and, to date, over 70 members of this family have been identified in both vertebrates and invertebrates (Nuclear Receptors Committee, 1999). These receptors have been classified into 6 subfamilies based on evolutionary analysis of their DNA binding domain (DBD) and ligand-binding domains (LBD). Within each subfamily, the members share a minimum of 80 - 90% identity in their DBDs and 40-60% identity in their LBDs (Laudet, 1997). Some nuclear receptors lack either a DBD or a LBD, and are grouped together as Class 0 receptors (Nuclear Receptors Committee, 1999).

The steroid hormone receptors, including GR, MR, progesterone receptor (PR), androgen receptor (AR), estrogen receptor (ER), along with the orphan estrogen-related receptors (ERRs), comprise the three groups of the Class III receptors. MR, GR, AR and PR comprise group IIIC and they share approximately 90% amino acid identity in their DBDs and > 50% amino acid identity in their LBDs (Laudet, 1997). The steroid receptors are unique to the NRs in that they bind as homodimers to cognate palindromic DNA hormone response elements (HREs). Whereas ER binds to the consensus sequence GGTCAnnnTGACC, the other SHRs recognize an imperfect palindromic hexameric repeat separated by three non-conserved nucleotides with the consensus sequence GGTACAnnnTGTTCT (Klein-Hitpass et al, 1988; Strahle et al, 1987). Class I receptors, including the thyroid hormone receptors (TRα,β), retinoic acid receptors (RARα,β,γ), vitamin D receptor (VDR) and peroxisome proliferator activated receptors (PPARα,β,γ), heterodimerize with the promiscuous binding partner, retinoid X receptor (RXRα,β,γ) (Class

II), and bind to direct repeats or inverted palindromes of the GGTACA half-site (Leid et al, 1992; Naar et al, 1991; Umesono et al, 1991).

The Glucocorticoid Receptor

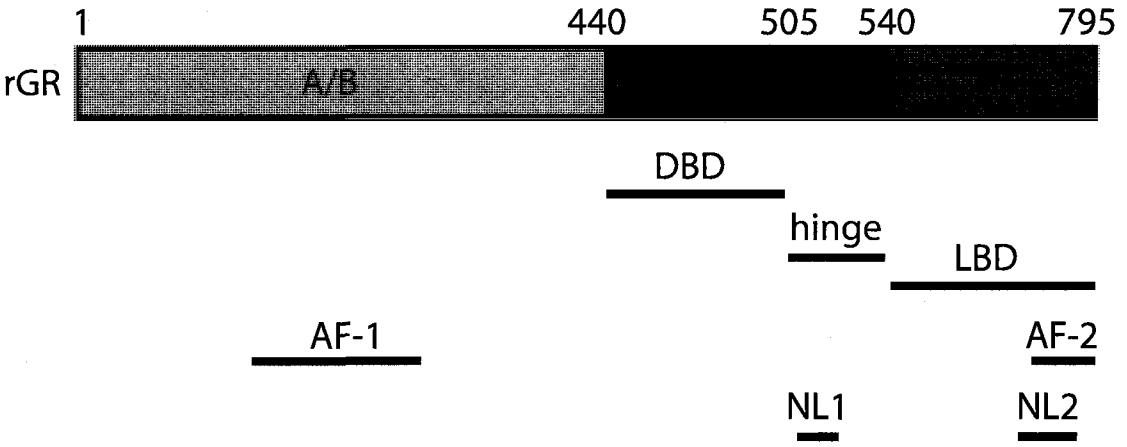
In 1985, human GR was the first full-length nuclear receptor to be cloned (Hollenberg et al, 1985). Two isoforms of GR representing splice variants are translated from one gene. Of these, GRα is the major cellular isoform of the receptor and contains 777 amino acids in human and 795 amino acids in rat. These two species of GRα share 83% identity. GRβ is encoded by 742 amino acids and differs from GRα in that the COOH-terminal 50 amino acids have been replaced with 15 non-homologous amino acids. Unlike GRα, GRβ lacks the ability to bind hormone and is constitutively localized to the nucleus. It has therefore been hypothesized to act as a dominant negative isoform that can modulate GRα function (Bamberger et al, 1995; Oakley et al, 1997). Furthermore, recent evidence suggests that multiple N-terminal splice variants exist; however, their function is largely unknown (Duma et al, 2006; Lu & Cidlowski, 2006).

Structure

GR is organized in a modular domain structure that is common to most NRs (Fig. 1). GR is comprised of an NH_2-terminal hypervariable domain (region A/B), a centrally located DBD within aa440-505 (region C), a highly flexible hinge region from aa511-539 (region D) and a COOH-terminal LBD within aa547-795 (region E) which confers hormone responsiveness. The receptor possesses two activation domains, AF-1 and AF-2 located at

Figure 1: Schematic presentation of the modular structure of the rat glucocorticoid receptor.

This structure is characteristic of members of the nuclear receptor superfamily. The highly variable N-terminal region (A/B) contains sequences comprising an activation function (AF-1). The zinc finger motif DNA binding domain (DBD) (region C) is encoded within amino acids 440-505. Region D (aa505-540) encodes the highly flexible hinge region followed by the ligand binding domain (LBD) that spans aa547-795 (region E) and contains the ligand-dependent AF-2 domain. GR contains two nuclear localization signals. NL1 is a classical NLS (aa513-515) which is masked in the native receptor and exposed following ligand binding. The less well characterized, conformation dependent, NL2 is located within the LBD and was recently shown to be contained within aa667-694.


1
440
505
540
795
rGR
A/B
DBD
hinge
LBD
AF-1
AF-2
NL1
NL2

the NH_2- and COOH- termini respectively. While AF-1 is constitutively active, AF-2 is exposed upon ligand binding due to a conformational change in the terminal helix of the LBD that is incurred upon ligand binding (Danielian et al, 1992; Moras & Gronemeyer, 1998).

The DBD is required for sequence specific DNA binding of NRs. It comprises nine conserved cysteine residues which are structural determinants for two zinc finger motifs. Each finger contains one zinc ion coordinated by four cysteine residues in a 2-cys-4 cross-bridge structure. The two fingers fold to form a compact structure with two α-helices at the core of the domain. The first helix is referred to as the recognition helix as it binds the major groove of the DNA and makes contact with specific base pairs within the recognition motif. Amino acids at the base of the first zinc finger, collectively referred to as the P-box, are required for discrimination of core recognition sequences (Mader et al, 1989). The second helix spans the carboxyl-terminus of the second zinc finger and forms at a right angle to the recognition helix. The D-box is encoded in the second helix within the second zinc finger (Luisi et al, 1991). Crystallization of the GR DBD bound to DNA revealed that the D-box is involved in DNA-dependent dimerization of the receptor and it is important in defining optimal spacing and alignment of the DNA recognition half-sites (Luisi et al, 1991).

The LBD of GR is comprised of 11 α-helices and 4 β-strands that fold into a 3-layer helical sandwich structure which contains a hydrophobic pocket for ligand binding (Bledsoe et al, 2002). Upon binding hormone, the LBD undergoes a conformation change in the terminal helix, which contains the AF-2; this structural change both stabilizes ligand binding and exposes protein-protein interaction interfaces required for coactivator binding (Danielian et al, 1992; Moras & Gronemeyer, 1998). Crystallization of the GR LBD bound to the

synthetic glucocorticoid dexamethasone (dex) and in complex with a coactivator peptide revealed novel features within this domain as compared to what had previously been shown for other receptors including ER. These include a novel dimerization interface, a second charge clamp that may be important for determining coactivator binding specificity and a distinct steroid binding pocket resulting from an additional side pocket that could contribute to ligand binding selectivity (Bledsoe et al, 2002).

Transcriptional Regulation by GR

In the absence of steroid, GR is localized in the cytoplasm in a transcriptionally inactive complex with heat shock proteins (hsp) and immunophilins including Hsp90, Hsp70, Hsp40 and p60 and p23 (Pratt, 1993; Pratt & Toft, 1997). Association with heat shock proteins is both required for hormone binding and masks the nuclear localization sequence (NLS) that is located in the hinge region (aa513–515) (Bresnick et al, 1989; Cadepond et al, 1991; Picard & Yamamoto, 1987; Sackey et al, 1996). Upon binding hormone, the Hsp-GR heterocomplex rapidly remodels, unmasking the NLS of GR, and the receptor translocates into the nucleus where it functions predominantly as a dimer to regulate transcription (Richter et al, 2007). Studies in our laboratory have shown that apart from DNA-dependent dimerization of the receptor that is dependent on the D-box within the DBD, GR contains distinct solution dimerization domains within the hinge region and within the LBD which was also revealed in the LBD crystal structure (Bledsoe et al, 2002; Savory et al, 2001).

GR is a prolific transcription factor that can either activate or repress transcription in a cell type- and promoter-specific context. The molecular mechanisms by which GR regulates transcription can be broadly classified into two categories. The first category

comprises the mechanisms by which GR is recruited to steroid responsive gene regulatory regions, either through direct DNA binding by the receptor or through physical association with DNA bound factors. The second category comprises the non-genomic actions of GR, in which the receptor influences target gene transcription either positively or negatively in the absence of recruitment to the steroid responsive gene regulatory regions.

Transcriptional Regulation Mediated by Direct Recruitment of GR to DNA

GRE-dependent Gene Activation: The classical mode of GR action is through the receptor binding to consensus glucocorticoid response elements (GREs) in gene regulatory regions. The classical GRE acts as an allosteric activator to direct cooperative binding of GR as homodimers through the D-box dependent dimerization motif, creating a high affinity binding site for the receptor (Luisi et al, 1991). Both 'simple GREs' and composite GREs have been described (Schoneveld et al, 2004).

Simple GREs exist in the absence of accessory transcription factor elements and have been identified in the serine/threonine protein kinase (SGK1), tyrosine hydroxylase and β2-adrenergic receptor gene promoters (Cornett et al, 1998; Hagerty et al, 2001; Itani et al, 2002). By contrast, for some genes, the ability of GR to induce transcription from a GRE is dependent on binding of accessory transcription factors to adjacent binding sites; these GREs are referred to as composite GREs and show greater inducibility by GR (Schoneveld et al, 2004). Composite GREs can lead to synergistic activation of transcription. On the mouse mammary tumour virus (MMTV) promoter, for example, transcriptional induction by both GR and Octamer transcription factors (Oct-1 and Oct-2) is much higher in transient reporter assays than the induction by either of these factors individually (Préfontaine et al, 1998). In

this context, it is hypothesized that these factors physically associate in solution, and high affinity binding by GR to the GRE brings Oct factors in close proximity to their cognate binding site, thus effectively increasing the local concentration of Oct and leading to increased transcriptional potential (Préfontaine et al, 1998). Due to variant cell-type dependent expression of certain transcription factors, composite GREs represent a mechanism to regulate cell-type dependent gene expression in the presence of steroid.

There is also evidence that GR can activate transcription from GRE half-sites. However, GR dependent transcription from GRE half-sites appears to require additional transcription factors as these elements have only been identified in the context of composite GREs (Segard-Maurel et al, 1996). GR binds to half-sites as monomers, although depending on the proximity of GRE half-sites, it can bind as a homodimer through the LBD dimerization domains independently of the D-box (Aumais et al, 1996).

NHRs, including ligand-bound GR, can directly recognize HREs that are packaged into chromatin when the HRE is appropriately exposed (Beato & Eisfeld, 1997). DNA-bound GR acts as a nucleating factor for the coordinated and ordered recruitment and alignment of transcriptional coregulatory complexes that are required for gene activation. In general, these factors act to re-structure chromatin and facilitate the assembly of the preinitiation complex as will be further discussed in more detail.

GRE-dependent Gene Repression: GR can negatively regulate transcription by binding to an atypical GRE termed a negative GRE (nGRE) which has a similar, but more variable consensus sequence (ATYACnnTnTGATCn) (Schoneveld et al, 2004). The proopiomelanocortin (POMC) gene is one example of a gene that is negatively regulated by GR through a nGRE (Drouin et al, 1993). Although the exact mechanism is unknown, it has

been proposed that GR, bound as a complex of a homodimer and monomer to the nGRE in the POMC promoter, antagonizes the actions of promoter-bound Nur77, an orphan receptor (Philips et al, 1997).

Similarly, composite GREs can physically occlude the binding of accessory transcription factors to their cognate sites within a promoter due to overlapping response elements. For example, GR represses the glycoprotein hormone α-subunit gene through antagonism of CREB bound to the cAMP response element (CRE) (Akerblom et al, 1988) and the osteocalcin gene by occluding the TATA box (Meyer et al, 1997).

Transcriptional Effects Mediated by Recruitment of GR to DNA through Protein-Protein Interactions

GR can also regulate gene transcription both positively and negatively in the absence of direct DNA binding. This can be mediated, in part, through binding to tethering GREs, in which GR physically interacts with DNA bound transcription factors to regulate gene transcription. In the case of the β-casein gene promoter, GR potentiates STAT5 mediated transcription through direct binding to DNA-bound STAT5 (Stocklin et al, 1996). It is hypothesized that the resultant increased transcription potential is mediated through the stronger activation domain of GR compared to that of STAT5.

Tethering GREs can also mediate gene repression. GR is recruited to AP-1 responsive promoters such as the human collagenase I gene through protein-protein interactions which results in a repressive transcriptional complex (Jonat et al, 1990; Yang-Yen et al, 1990).

Transcriptional Regulation Mediated by Indirect Mechanisms

GR also influences gene transcription through non-genomic mechanisms. In these cases, gene regulation occurs in the absence of GR recruitment to the gene regulatory regions. These mechanisms underlie the negative regulation of AP-1 and NF-κB dependent transcription by GR. Glucocorticoid-dependent repression of the inflammatory response is mediated by the negative regulation of AP-1 and NF-κB regulated genes. The mechanisms that constitute this antagonistic relationship are not completely understood and appear to be, in part, promoter specific.

Several mechanistic models have been proposed to account for GR function within this context. These include the direct interaction model, in which physical association between GR and AP-1 prevents binding of each of the factors to their respective response elements as has been observed for the pro-apoptotic granzyme B gene (Wargnier et al, 1998). Alternatively, there is evidence that GR competes with both AP-1 and NF-κB for binding to the coactivator CBP, which is present in the cells in limiting amounts (Kamei et al, 1996; Sheppard et al, 1998). However, this model has been challenged by the finding that GR-dependent repression of NF-κB and AP-1 occurs independently of the levels of CBP in the cell (De Bosscher et al, 2001; De Bosscher et al, 2000). Furthermore, other groups have shown that the potential of GR to repress NF-κB transactivation function is increased in the presence of CBP, suggesting that CBP could function as an integrator of GR-NF-κB cross-talk (McKay & Cidlowski, 2000). Lastly, GR has been shown to increase the expression of the I-κB, a negative regulator of NF-κB transactivation function, in some tissues (Auphan et al, 1995; Deroo & Archer, 2001); although others have suggested that this increase was not sufficient to repress NF-κB transcriptional potential as a GR mutant that could not induce I-

κB transcription retained its ability to repress NF-κB mediated transcription (Heck et al, 1997).

GR can also regulate transcription through non-genomic mechanisms that involve steroid-dependent modulation of transcriptional coregulatory factors. Studies performed by colleagues in this laboratory have shown that glucocorticoids potentiate C/EBPβ-mediated transcription in the absence of a physical association between GR and C/EBPβ from promoters that lacked a GRE. Furthermore, this effect is solely dependent on the LBD of the receptor (Boruk et al, 1998). Molecular analysis of this activation function revealed that in the absence of glucocorticoids, C/EBPβ associates with a subcomplex of the mSin3A-HDAC1 corepressor complex, thus repressing its activation potential (Wiper-Bergeron et al, 2003). Glucocorticoids promote the proteasome-dependent degradation of a subcellular pool of HDAC1 that specifically interact with C/EBPβ thus relieving its repressive effects and resulting in the up-regulation of C/EBPβ-mediated transcription. The effect was agonist dependent and could be recapitulated with the PR LBD, but not with RARα (Wiper-Bergeron et al, 2003). Further studies have shown that glucocorticoids also promote the acetylation of C/EBPβ by GCN5 within a lysine cluster from aa98-102 which is important for its ability to induce transcription. This acetylation event appears to act as a molecular switch for the transcriptional regulatory potential of C/EBPβ, as it disrupts the interaction of C/EBPβ with mSin3A and HDAC1 (Wiper-Bergeron et al, 2007).

The significance of gene regulation by GR in the absence of direct binding to GREs, came in 1998 with the generation of GR dimerization deficient mice ($GR^{dim/dim}$) (Reichardt et al, 1998). Cell transfection experiments had previously demonstrated that a point mutation in the D-box (A458T) effectively abolished the receptor's ability to bind DNA with high

affinity and transactivate GRE-dependent promoters while retaining its ability to repress AP-1 dependent collagenase transcription (Heck et al, 1994). As compared to homozygous GR-null mice which die shortly after birth, mice that carried the GR A458T mutation were viable despite significantly reduced inducibility of GRE-dependent genes such as the MMTV promoter in isolated fibroblasts. These mice were defective in the production of gluconeogenic enzymes (Reichardt et al, 1998). The viability of these mice suggested that non-genomic modes of GR action are important for development and that cooperative binding of GR to GREs is dispensable for survival. It is important to note, however, that the broad conclusion that dimerization mutations in the D-box inhibit all genomic effects of GR has been challenged by the finding that similar mutants retain their ability to bind to DNA and can up-regulate transcription from certain GRE containing promoters, perhaps through alternative dimerization interfaces in the LBD (Adams et al, 2003; Rogatsky et al, 2003).

Transcriptional Coregulatory Complexes

Eukaryotic genomic DNA is packaged into higher order chromatin structures. The basic unit of chromatin is the nucleosome, in which 146 bp of DNA is wrapped around a histone octamer that consists of two subunits each of histones H2A, H2B, H3 and H4 (Luger et al, 1997; Uberbacher & Bunick, 1989). Crystallization studies revealed that the amino-tails of histones are highly flexible and protrude from the nucleosome core (Luger et al, 1997). They are targets for posttranslational modification that regulates local chromatin structure and they are also believed to contribute to higher order chromatin structure by mediating intra-nucleosomal interactions (Berger, 2007; Uberbacher & Bunick, 1989).

Histone H1 is found in the linker region between nucleosomes and is thought to facilitate the generation of higher order chromatin structure such as chromatin fibers (Bustin et al, 2005).

Chromatin presents a structural impediment to transcriptional activation and serves as a target for the regulation of gene expression (Knezetic & Luse, 1986). DNA-bound nuclear hormone receptors (NHRs), as well as other sequence specific transcription factors, recruit coregulatory factors to modify chromatin structure (Beato & Eisfeld, 1997). This restructuring is required for recruitment of the basal transcriptional machinery and formation of the pre-initation complex. These factors are often components of larger heterocomplexes and can be defined by two classes: (1) ATP-dependent chromatin remodeling complexes that use energy from ATP hydrolysis to alter the position and/or stabilize nucleosomes in a non-covalent manner or (2) enzymatic factors that covalently modify histone tails. The latter complexes can be either stimulatory or repressive and possess components that include HATs and HDACs, methyltransferases and demethylases (HMT/LSD1), kinases and phosphatases, poly(ADP-ribose) polymerase (PARP), and ubiquitin and SUMO ligases (Rosenfeld et al, 2006).

In general, transcriptionally active promoters are associated with having increased H3K4 methylation, H3K9, H3K14 and H4K20 acetylation and H1b phosphorylation; whereas transcriptionally inactive promoters correlate with increased methylated H3K9, H3K27 and H4K20 (Rosenfeld et al, 2006). These modifications serve as epigenic markers for gene regulation. Histone modifications are also believed to serve as binding sites for additional transcriptional cofactors through their respective chromatin targeting domains (Jenuwein & Allis, 2001). For example, BROMO, PHD and chromo domains are present in several coregulatory factors. These domains have been shown to bind acetylated (BROMO) and trimethylated lysines (PHD, chromo) within histone tails (Bannister et al, 2001; Dhalluin

et al, 1999; Jacobs & Khorasanizadeh, 2002; Jacobson et al, 2000; Mellor, 2006; Nielsen et al, 2002; Owen et al, 2000). Together, regulated chromatin modification results in ordered recruitment of cofactors and progression through transcriptional activation.

Coactivator Complexes

Transcription activation by GR is dependent on its interaction with coactivator proteins (Lonard & O'Malley, 2006). Coactivators can be broadly categorized into three groups: the two aforementioned covalent and non-covalent chromatin targeting complexes, and components of the mediator complex. The mediator complex interacts with basal transcription machinery to assist in the formation of the pre-initiation complex. Coactivators are usually components of large multi-factor complexes. Gene activation can be regulated by the coordinated and tightly regulated activity of multiple coactivator complexes with distinct and diverse enzymatic activity.

Coactivators can interact with GR through the AF-1 and AF-2 activation domains (Aranda & Pascual, 2001; Kumar & Thompson, 2003). AF-1 function is not well characterized. It is active in the presence of both agonists and antagonists and is dependent on ligand binding and subsequent translocation of the receptor into the nucleus (Kumar & Thompson, 2003). The AF-2 domain, by contrast, is better characterized and is agonist-dependent. AF-2 is exposed as a result of a conformational change within the terminal helix of the LBD that is incurred upon binding hormone (Danielian et al, 1992; Moras & Gronemeyer, 1998). This conformational change reveals a conserved consensus motif, ΦΦXEΦΦ (where Φ is a hydrophobic residue) which folds into an amphipathic α-helical conformation with the two negatively charged residues being exposed. These residues

function with the signature motif within the COOH-terminal of helix 3 and 4 of the LBD to form a charge clamp (Moras & Gronemeyer, 1998; Nolte et al, 1998). This charge clamp makes physical contact with and stabilizes the interaction of an LxxLL motif encoded within coactivator proteins, which binds to a hydrophobic pocket at the base of the clamp (Heery et al, 1997).

Members of the p160 family of transcriptional coactivators serve as nucleating factors that direct the assembly of many of these complexes through direct LxxLL motif mediated interaction with liganded NHRs. To date, 5 members of the p160 family have been identified: NCoA-1 (nuclear receptor coactivator 1)/SRC-1 (steroid receptor coactivator 1), NCoA-2/GRIP-1 (GR interacting protein 1)/TIF2 (transcriptional intermediary factor 2), NCoA-3/p/CIP (p300/CBP interacting protein), NCoA-4/ARA70 and NCoA-6/ASC2 (Anzick et al, 1997; Onate et al, 1995; Torchia et al, 1997; Voegel et al, 1996). Despite SRC-1 and p/CIP having intrinsic HAT activity, the p160 proteins are believed to act primarily as molecular scaffolds that recruit larger coactivator complexes (Chen et al, 1997; Spencer et al, 1997). Two COOH-terminal activation domains facilitate the recruitment of the HATs CBP (CREB binding protein) and p300 (Hong et al, 1999; Kamei et al, 1996; Torchia et al, 1997; Voegel et al, 1998; Yao et al, 1996) and P/CAF (p300/CBP associated factor) and GCN5 (Chen et al, 1997; Korzus et al, 1998; Spencer et al, 1997) as well as the protein methyltransferase CARM1 (coactivator-associated arginine methyltransferase 1) respectively (Chen et al, 1999).

Acetylation of lysine residues within COOH-tails of histones H3 and H4 is correlated with a transcriptionally permissive promoter (Clayton et al, 2006). To date, there are four families of HAT enzymes that have been identified: the CBP/p300 family, GCN5-related N-acetyltransferases (GNATs) which include GCN5L and the closely related factor p/CAF, the

MYST (MOZ, YBF2, SAS2 and TIP60) family that include MOZ (monocytic leukemia zinc finger protein), HBO1 (HAT bound to ORC 1) and Tip60 (HIV Tat-interacting 60 kDa protein) and TAF250 (TBP associated factor 250) (Carrozza et al, 2003; Roth et al, 2001). These factors have distinct chromatin modifying actions and appear to be recruited to promoters with specific and coordinated timing to induce transcription. This was elegantly demonstrated by Metivier and colleagues who used chromatin immunoprecipitation analysis to study ERα dependent gene activation of the pS2 promoter. This study revealed the ordered, cyclic recruitment of p300, Tip60, GCN5, P/CAF, CBP and TAF250 to this promoter. Their presence at the promoter temporally correlated with substrate specific histone acetylation (Metivier et al, 2003).

CBP and p300 are recruited to liganded NHRs both through interactions with p160 family members as well as directly through their respective LxxLL motifs. Apart from their chromatin modifying function, they can also act as a bridge to facilitate the interaction between transcription factors and the basal transcription machinery as CBP has been shown to interact with TBP and TFIIB (Vo & Goodman, 2001). They can also recruit other coactivator molecules, such as P/CAF. P/CAF can also be recruited to NHRs through direct associations, as well as through p160 family members (Blanco et al, 1998; Korzus et al, 1998).

The extended capacity of coactivator complexes to differentially associate with each other and with DNA-bound transcription factors through unique and independent interaction interfaces highlights a mechanism by which transcriptional responses can be modulated, regulated and diversified depending on cellular-, promoter- and signal-specific cues. These

associations are tightly regulated by both protein expression and stability and post-translational modifications.

The role of the p160 family and HATs are not limited to genes regulated by NHRs. These factors are also recruited to promoters by other sequence specific transcription factors including Sp1 (Onate et al, 1995), NF-κB (Na et al, 1998; Sheppard et al, 1999; Werbajh et al, 2000), SRF (Kim et al, 1998a), AP-1 (Lee et al, 1998), CREB and STAT-1 (Korzus et al, 1998) and MyoD (Wu et al, 2005). The engagement of the same coactivators by GR and other transcription factors could contribute to mechanisms of transcriptional cross-talk between these factors, as they could compete for the same pool of coactivator molecules as has been proposed for CBP binding to both GR, AP-1 and NF-κB (Kamei et al, 1996; Sheppard et al, 1998).

Corepressor Complexes

Corepressor complexes bind to transcriptional activators and inhibit the formation of transcriptionally active complexes. This is mediated in a large part by maintaining the chromatin in a transcriptionally inert conformation. As such, histone deacetylases (HDACs) and methyltransferases are key effectors of transcriptional repression. Large, multi-factor corepressor complexes including SMRT (silencing mediator of retinoid and thyroid hormone receptor) and N-CoR (nuclear hormone receptor corepressor), mediate their repressive effects, in part, through recruitment of HDAC activity to promoter-bound transcription factors (Chen & Evans, 1995; Horlein et al, 1995; Li et al, 2000; Privalsky, 2004). HDAC-containing complexes are also recruited to promoters through protein-protein interactions

with DNA-bound transcription factors and through direct interactions with chromatin which is mediated by chromatin targeting proteins within the complexes.

Histone Deacetylases

Since the original cloning of HDAC1 in 1996 (Taunton et al, 1996), 18 mammalian HDACs have been identified. These have been classified based on their sequence homology to transcriptional repressors in yeast (Marmorstein, 2001). Class I HDACs, HDAC1, 2, 3 and 8 are homologues to the yeast protein Rpd3. They are nuclear proteins that are ubiquitously distributed across many cell types. By contrast, the Class II HDACs, including HDAC4, 5, 7 and 9 (Class IIa) and HDAC6 and 10 (Class IIb) are more closely related to the yeast HdaI protein. They are nuclear-cytoplasmic shuttling proteins and appear to have some tissue-specific distribution. HDAC11 appears to be a unique member of this family, showing homology to both ClassI and Class II HDACs (Gao et al, 2002). The Class III HDACs are most closely related to the yeast Sir2 protein (Imai et al, 2000). The sirtuin family, comprised of 7 members, is distinct from the Class I and II HDACs in that they require NAD+ as a cofactor, and they are localized to the cytoplasm (Afshar & Murnane, 1999). Their cellular function is largely unknown.

Class I HDACs, specifically HDAC1 and HDAC2 are components of larger multiprotein transcriptional repressor complexes, including the Sin3 (named for its core component mSin3A), NuRD (nucleosome remodeling and deacetylases complex) and CoREST (named for its core component CoREST) complexes (Ayer, 1999; Fleischer et al, 2003; You et al, 2001; Zhang et al, 1999). Apart from HDAC1 and HDAC2 these complexes each contain distinct components that could impart unique functions and cellular targets.

mSin3A exists in several heterocomplexes that can be biochemically fractionated from cells and may vary between cell types. The best described of the Sin3 complex consists of the HDAC1 and 2, mSin3A and its associated proteins SAP10, SAP30, SAP180, SAP130 and SAP45 and the histone binding proteins RbAp48 and RbAp46 (Fleischer et al, 2003). The Sin3 complex interacts with NCoR/SMRT through direct contact with the SAP30 component (Soderstrom et al, 1997). It has been implicated in transcriptional repression by NHRs, the Mad/Max heterodimer and p53 (Ayer et al, 1995; Murphy et al, 1999; Soderstrom et al, 1997).

C/EBPβ interacts with a Sin3 subcomplex that contains HDAC1 and mSin3A but lacks HDAC2 and the RbAp proteins. Glucocorticoids specifically target the HDAC1 that exists within this C/EBPβ associated complex for degradation by the 26S proteasome (Wiper-Bergeron et al, 2003).

There is a growing body of evidence that HDACs are intimately involved in context dependent transcriptional regulation by GR. HDAC activity has been shown to modulate GR-mediated transcription of the MMTV promoter (Li et al, 2002; Qiu et al, 2006), transcription from a simple promoter (Rocha et al, 2005), transcription of the granulocyte-macrophage colony-stimulating factor promoter (Ito et al, 2000) and the IL-5 gene (Ichijo et al, 2005). GR physically associates with both HDAC1 (Wiper-Bergeron et al, 2003) and HDAC2 (Ito et al, 2001; Ito et al, 2006) and indirectly with HDAC3 (Ichijo et al, 2005). Furthermore, its activity is indirectly affected by the actions of HDAC6 on Hsp90 (Kovacs et al, 2005; Murphy et al, 2005).

HDAC1

The importance of HDAC1 in mammalian development is evidenced by the lethality of the HDAC1$^{-/-}$ knockout mice due to severe proliferation defects (Lagger et al, 2002). Their apparent role in regulating cell cycle progression was further supported by the finding that HDAC1 was induced and promoted proliferation in response to interleukin-2 in murine T cells with maximal expression as cells progress through the G1/S boundary (Bartl et al, 1997). In addition to the aforementioned complexes, free HDAC1 has also been shown to inhibit myogenesis through interaction with MyoD (Mal et al, 2001), to inhibit cell cycle progression though association with retinoblastoma protein (Rb) (Brehm et al, 1998; Magnaghi-Jaulin et al, 1998) and to modulate NF-κB, Sp1 and YY1 regulated transcription (Ashburner et al, 2001). HDAC1 has also been shown to be recruited to AR occupied PSA elements where it represses transcription (Doetzlhofer et al, 1999; Gaughan et al, 2002; Yao et al, 2001). HDAC1 activity has been shown to be regulated by phosphorylation, sumoylation and ubiquitylation (David et al, 2002; Pflum et al, 2001).

Transcriptional Intermediary Factors

The transcriptional intermediary factors (TIFs) represent another family of coregulatory factors. TIF1, since renamed TIF1α, was originally identified as a cofactor that enhanced both RAR and RXR AF-2-mediated transcription in yeast and could interact with the LBDs of RAR, RXR and ER *in vitro* (Le Douarin et al, 1995). Since its discovery, TIF1β, TIF1δ and TIF1γ in mammals and *Bonus* in Drosophila have been identified (Beckstead et al, 2001; Khetchoumian et al, 2004; Le Douarin et al, 1996; Venturini et al, 1999). The TIF1 family is defined by the presence of an N-terminal self-assembling RBCC

tripartite domain comprised of a RING finger, 2 B-boxes and a Coiled-coil domain and a COOH-terminal PHD finger and BROMO domain unit (refer to Chapter I, Fig 5 for a schematic). While they share similar organization of domain structure, the overall amino acid sequence homology is low (31% between TIF1α and TIF1β).

Each of the family members has been reported to repress both activated and basal transcription when tethered to DNA (Beckstead et al, 2001; Friedman et al, 1996; Le Douarin et al, 1996; Venturini et al, 1999). This is largely mediated by epigenetic mechanisms that include histone modification and interaction with heterochromatin binding proteins. TIF1α interacts with and modulates NHR mediated transcription, including RAR, TR, VDR and ER through an LxxLL motif-dependent association with NHR AF-2 domains (Le Douarin et al, 1995). Similarly, *Bonus* interacts with, and silences the NHR βFTZ-F1 mediated transcription in Drosophila via its LxxLL motif (Beckstead et al, 2001).

TIF1β lacks the LxxLL motif and has been predominantly characterized as a potent transcriptional repressor protein for a large class of Krüppel-class C_2H_2 zinc-finger transcription factors that contain a Krüppel-associated box (KRAB) domain (Bellefroid et al, 1991; Friedman et al, 1996). There are between 300-700 genes that encode C_2H_2 Zn fingers in the human genome, approximately one-third of these have KRAB domains. The ability of TIF1β to repress these genes is dependent on (1) recruitment to the promoter through direct protein-protein interaction with the KRAB domain via its RBCC domain, (2) recruitment of deacetylase complexes including NuRD and N-CoR1 and the H3K9-methyltransferase SETDB1 via its C-terminal PHD and BROMO domains and (3) physical association with heterochromatin protein 1 (HP1) family members via a centrally located PxVxL HP1-

interaction motif (Friedman et al, 1996; Matsuda et al, 2001; Nielsen et al, 1999; Ryan et al, 1999; Schultz et al, 2001; Underhill et al, 2000).

Despite being a prolific transcriptional repressor, TIF1β can also activate transcription dependent on promoter context. Notably, TIF1β has been reported to up-regulate C/EBPβ and GR dependent transcription of the α-1 glycoprotein promoter establishing a link to both C/EBPβ and GR (Chang et al, 1998). It has also recently been shown to associate with the CArG box-binding factor A (CBF-A) on the fibroblast-specific protein-1 (FSP-1) promoter where it activates transcription and mediates early events in the epithelial-mesenchymal transition (EMT) (Venkov et al, 2007). However, the mechanisms underlying its activation potential are unknown. Furthermore, TIF1β is emerging as a regulatory factor in development and several differentiation systems. The TIF1β-null mouse is embryonic lethal, with arrested development prior to gastulation at approximately E5.5. The embryos had reduced cell number in the ectoderm, lacked mesoderm formation and showed altered morphology in their visceral endoderm (Cammas et al, 2000). TIF1β has been implicated in the differentiation of murine embryonic F9 model of early embryonic development and cellular differentiation as well as spermatogenesis (Cammas et al, 2004; Cammas et al, 2002; Weber et al, 2002).

Regulation of Transcription by the Ubiquitin-Proteasome System

Protein expression is governed by the balance between regulation of its induction and its turnover. The ubiquitin-proteasome system (UPS) regulates the degradation of proteins through the 26S proteasome which is comprised of the 20S core and 19S regulatory subunits (Pickart, 2001). The degradation of a protein through the 26S proteasome is a tightly

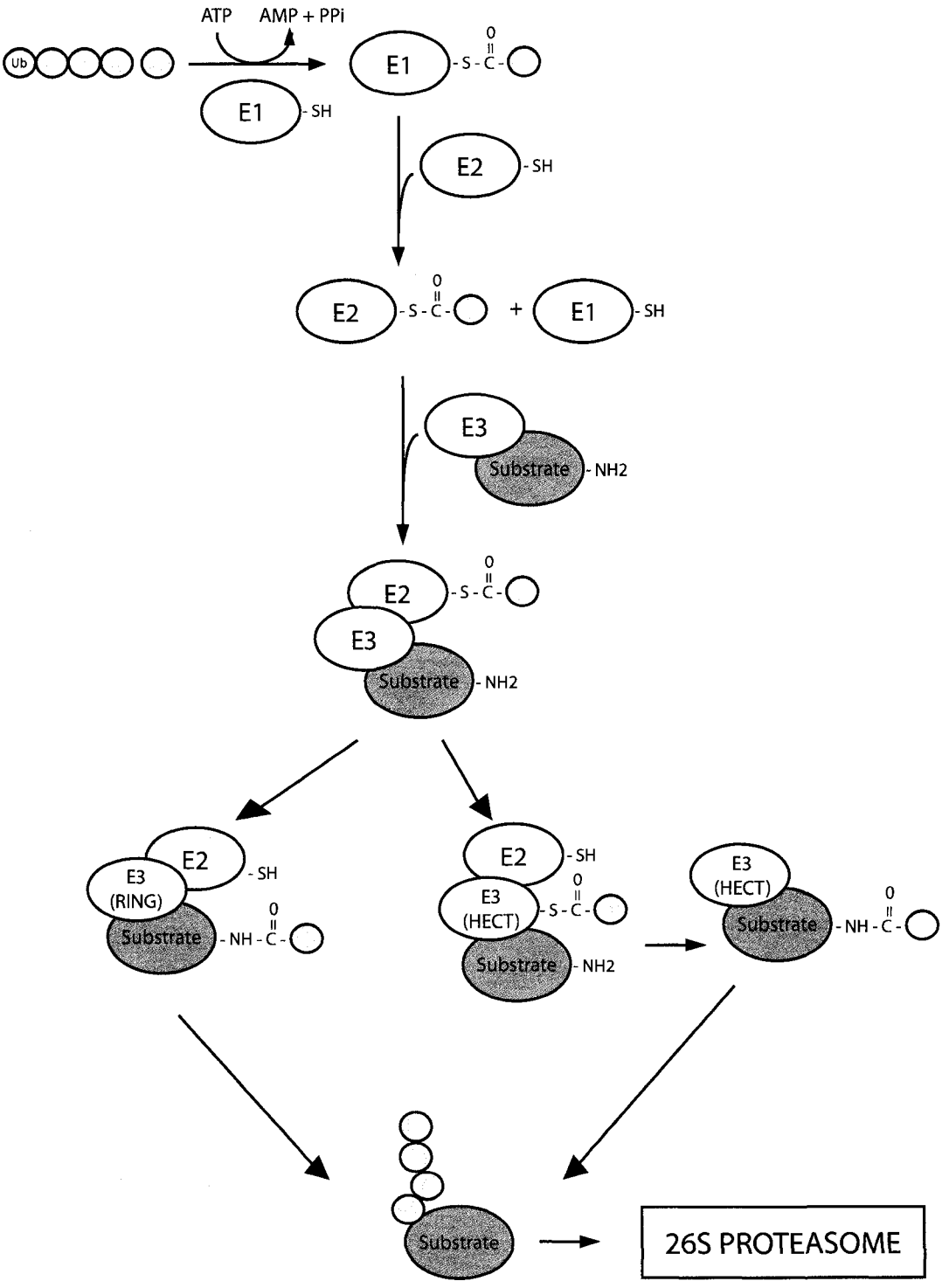
regulated multi-step process that requires the activation and sequential terminal transfer of a ubiquitin (Ub) moiety to the targeted substrate protein. Ub is a ubiquitously expressed, highly conserved protein of 76 amino acids (Pickart, 2001). Protein ubiquitylation serves many molecular roles, the primary of which is to target proteins for degradation. This function is mediated through the conjugation of Lys48-conjugated polyubiquitin chains with a minimum threshold of 4 moieties. Alternatively, lysine-monoubiquitylation has non-proteolytic functions including histone regulation, endocytosis, DNA repair and budding of retroviruses from the plasma membrane (Di Fiore et al, 2003). Moreover, monoubiquitylation of p53 has been shown to regulate its nuclear-cytoplasmic trafficking and target a subcellular pool to the mitrochondria in response to stress (Li et al, 2003; Marchenko et al, 2007).

Protein ubiquitylation is mediated by three distinct Ub conjugating enzymes (E1, E2 and E3) as is summarized in Figure 2. The first step involves the ATP-dependent activation and binding of Ub to the E1 Ub-activating enzyme through formation of a thiol ester linkage. The Ub moiety is then transferred to an active cysteine residue within the E2 Ub-conjugating enzyme. Approximately twenty E2 enzymes have been identified (Pickart, 2001). The terminal transfer of the Ub to an ε-amino group of a lysine residue on the substrate protein is mediated by E3 Ub ligases. The E3 ligases confer substrate specificity.

Currently, two classes of E3 ligases have been defined based on homology within their conserved enzymatic domains. These are the HECT (homology to E6AP C-terminus) domain and RING (really interesting new gene) finger domain containing E3 ligases. These two classes use distinct mechanisms to ubiquitylate their substrate proteins. HECT domain E3 ligases from a thiol ester bond between an internal cysteine residue within the active site

Figure 2: The Ubiquitin Proteasome System.

Ubiquitin (Ub) is initially translated as a polyubiquitin molecule from which ubiquitin monomers are cleaved by ubiquitin-C-terminal hydrolase enzymes. The COOH-terminal glycine residue of an ubiquitin monomer is first activated by E1 activating enzymes (E1) in an ATP-dependent process. This results in the formation of a thiol-ester bond between the Ub moiety and a cysteine residue within the E1 enzyme. The activated Ub is then transferred to a cysteine residue within the active site of E2 ubiquitin conjugating enzyme (E2) through a thiol-ester linkage. The terminal transfer of the Ub to the substrate protein is facilitated by E3 ligase enzymes (E3) which confer substrate specificity to this process. There are two classes of E3 ligases: HECT and RING domain E3. Ubiquitin is transferred from the E2 to a cysteine residue within HECT domain E3s from which the Ub is transferred to the substrate protein. By contrast, RING E3s mediate the transfer of the ubiquitin to the substrate protein directly from the E2 to the substrate in the absence of E3-Ub conjugate. In both cases, the Ub is ultimately bound to the substrate protein through an isopeptide linkage to an ε-amino group of a lysine residue in the targeted protein. The mechanism that underlies the addition of subsequent Ub moieties to the chain is less well understood. It is thought to be mediated by E3 enzymes and a class of E4 enzymes that are believed to conjugate Ub to substrate-bound multi-ubiquitin chains. Chain length can also be regulated by deubiquitylating enzymes. Upon reaching a threshold chain length of four Ub moieties, the substrate protein is degraded by the 26S proteasome. This figure has been adapted from A.M. Weissman (NR.MCB 2:169 2001)


ATP
AMP + PPi
Ub
E1
E1
SH
E2
SH
E2
S
C
O
E1
SH
E3
Substrate
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C
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Substrate
NH2
E3
(HECT)
Substrate
NH
C
O
Substrate
26S PROTEASOME

and the Ub, from which the Ub is transferred to the substrate protein (Huibregtse et al, 1995). RING finger domain E3 ligases do not appear to form a thiol ester intermediate with Ub and are therefore believed to facilitate the direct transfer of the Ub from the E2 enzyme to the substrate (Lorick et al, 1999). Furthermore, RING E3 ligases can act either independently, such as Mdm2 for example, or within the context of larger ubiquitin ligase complexes together with members of the cullin family (Pickart, 2001).

The regulation of polyubiquitin chain length and hence protein fate is a less well understood process. The identification of E4 enzymes that can conjugate Ub moieties onto the growing chain, as well as deubiquitylating enzymes suggests that this is a dynamically regulated and reversible process (Hanna et al, 2006; Hatakeyama & Nakayama, 2003; Koegl et al, 1999; Yao & Cohen, 2002). Further insight into this process has come recently with the report that substrate ubiquitylation can result from the transfer of preassembled polyubiquitin chains to the substrate protein (Li et al, 2007). Ultimately, polyubiquitylated substrates are degraded by the 26S proteasome. This is an ATP-dependent process and includes protein recognition, deubiquitylation, peptide unfolding and proteolysis within the 20S core subunit.

It is well established that the UPS contributes to the regulation of transcription. Proteasome activity is required for full activation potential of numerous transcriptional activators including NHRs, NF-κB, p53 and c-jun (Haupt et al, 1997; Kubbutat et al, 1997; Musti et al, 1997; Nawaz & O'Malley, 2004; Palombella et al, 1994). There is growing evidence that these functions are mediated by both proteolytic and non-proteolytic mechanisms and that the UPS contributes to multiple aspects of the transcription including initiation, elongation and termination (Collins & Tansey, 2006).

The proteolytic activity of the UPS is required for the targeted destruction of transcriptional activators, coregulatory complexes and basal transcription machinery, all of which have been correlated with active transcription (Muratani & Tansey, 2003). For transcriptional activators, transcriptional activation domains have been shown to overlap with destruction motifs resulting in an inverse relationship between protein activation function and protein stability and providing a mechanism for self-limiting control of transcription (Molinari et al, 1999; Salghetti et al, 2000).

Apart from regulation of transcription factor stability, the activation potential of these factors is also regulated by the UPS through degradation of coregulatory proteins. The discovery that the targeted degradation of C/EBPβ-associated HDAC1 is required for maximal transcriptional activation potential of C/EBPβ contributes to a growing body of evidence that the UPS can act as a molecular switch that governs the association of transcription factors with corepressors and coactivator complexes (Wiper-Bergeron et al, 2003). This is also true for NHRs, LIM homeodomain transcription factors, NF-κB and c-jun (Ostendorff et al, 2002; Perissi et al, 2004; Wiper-Bergeron et al, 2003). As such, components of the UPS including E2 and E3 ligases are emerging as transcriptional coactivators and as components of coregulatory complexes (Muratani & Tansey, 2003). For example, the E3 ligases Mdm2 and E6AP and the E2 enzyme UBCH7 have been shown to modulate NHR function, including that of GR (Garside et al, 2006; Nawaz et al, 1999a; Nawaz et al, 1999b; Sengupta & Wasylyk, 2001). The E3 ligase Skp2 is a coactivator of c-myc activity (von der Lehr et al, 2003). The prolific HATs p300 and most recently p/CAF have both been shown to have intrinsic E3 ligase activity that is responsible for the ubiquitylation of p53 and Hdm2 respectively (Grossman et al, 2003; Linares et al, 2007).

Furthermore, the deubiquitinating enzyme Ubp8 is a component of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) coactivator complex, and has been shown to control levels of H2B ubiquitylation (Daniel et al, 2004; Henry et al, 2003).

Preadipocyte Differentiation

Higher eukaryotes store excess caloric intake in the form of triacylgycerides (TAGs) in adipocytes in white adipose tissue (WAT). Adipocytes are specialized cells that contribute to whole body energy homeostasis by regulating the storage and mobilization of TAGs under conditions of caloric excess and restriction respectively. They also secrete factors involved in haemostasis, regulation of blood pressure, immune function and angiogenesis as well as leptin, a protein that is involved in satiety mechanisms (Kershaw & Flier, 2004).

Adipocyte precursor cells, preadipocytes, are committed fibroblasts derived from pluripotent mesenchymal progenitor cells that have the capacity to differentiate into chondrocytes, osteoblasts, myocytes and adipocytes given the appropriate metabolic cues (Gregoire et al, 1998). While the commitment process to the preadipocyte lineage is not well understood, the underlying core transcriptional response pathway that drives the differentiation of preadipocytes to mature adipocytes has been elucidated. Our understanding of the basic pathway has been largely derived from mechanistic studies performed in cell culture models of adipogenesis, cultures of primary preadipocytes and some of these pathways have been validated in transgenic mice. Different models may represent different stages of adipocyte development as the inductive stimulus required for differentiation vary somewhat.

3T3 L1 cells are the most widely studied cell culture model for adipogenesis, and are believed to model the early stages of preadipocyte differentiation. This immortalized cell line was clonally isolated from Swiss 3T3 cells derived from disaggregated 17- to 19-day mouse embryos (Green & Kehinde, 1975; Green & Kehinde, 1976; Green & Meuth, 1974). The accuracy of this cell line to reflect the development of fat pads *in vivo* has been validated by studies showing that injection of 3T3 L1 preadipocytes into nude mice results in the development of fat pads that are histologically identical to the natural fat (Green & Kehinde, 1975). Differentiation of 3T3 L1 cells in culture is induced with insulin, 3-isobutyl-1-methyl-xanthine (MIX) which is a phosphodiesterase inhibitor that is thought to increase intracellular cAMP, and the synthetic glucocorticoid dexamethasone (dex) in the presence of 10% fetal bovine serum (FBS) (Green & Kehinde, 1975; Rubin et al, 1978). Glucocorticoids are required for the initial 48 h of differentiation for maximal adipogenesis (Rubin et al, 1978).

The absolute requirement of glucocorticoids for preadipocyte differentiation in culture varies between culture models. In cell culture models such as 3T3 L1 cells, glucocorticoids are not strictly required for differentiation but increase the efficiency with which preadipocytes differentiate (Gregoire et al, 1998). In general, they are believed to lower the threshold to commitment to allow a greater number of cells within the culture to differentiate (Shugart & Umek, 1997). Conversely, glucocorticoids appear to be required for the differentiation of primary preadipocytes derived from a number of mammals, including human, mouse and rabbit preadipocytes (Gregoire et al, 1998). In vivo, there is a direct correlation between glucocorticoids and adiposity as will be discussed in more detail below.

Transcriptional Regulation of Adipogenesis

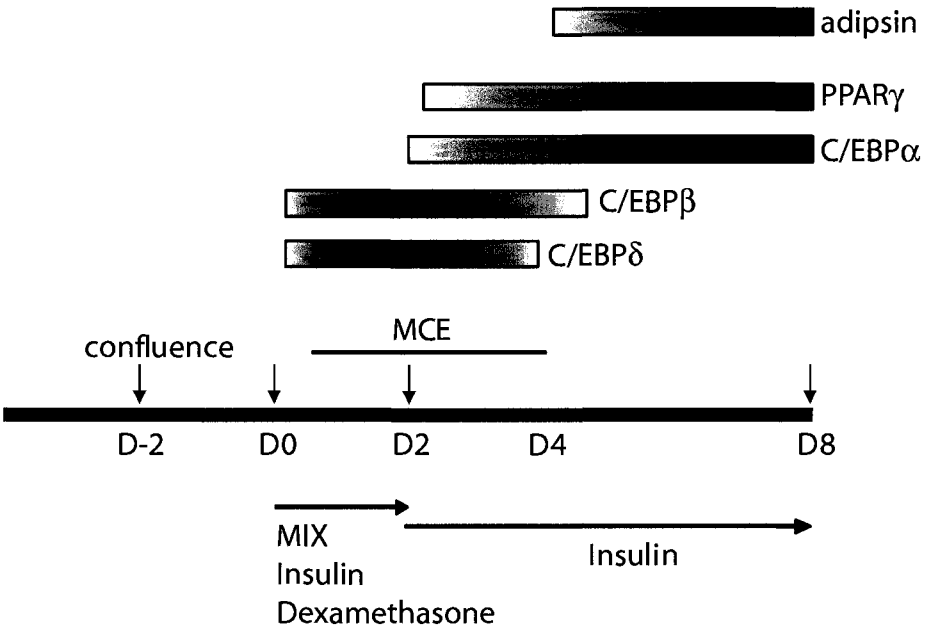
In general, adipogenesis occurs in two stages. During the determination phase, the pluripotent stem cells commit to the adipocyte lineage. These committed cells are referred to as preadipocytes. As previously stated, little is known about the process of commitment. The preadipocyte then undergoes terminal differentiation. During this process, the preadipocytes take on characteristics of mature adipocytes and acquire the required machinery for lipid synthesis and transport, insulin sensitivity and the secretion of adipocyte specific factors. Terminal differentiation is driven by a core transcriptional response pathway consisting of members of the C/EBP family of transcription factors and the nuclear receptor PPARγ. This pathway is summarized in Figure 3.

CCAAT/ Enhancer Family Members

There are six known C/EBP family members: C/EBPα, C/EBPβ/LAP/NF-IL6, C/EBPδ/NF-IL6β, C/EBPγ, C/EBPε and C/EBPζ/CHOP-10/gadd153 (Wedel & Ziegler-Heitbrock, 1995). The C/EBP proteins belong to a larger family of basic leucine zipper (bZip) transcription factors that are characterized by a COOH-terminal leucine zipper dimerization domain and a basic DBD (Landschulz et al, 1989). They vary in their ability to activate transcription. C/EBP proteins bind C/EBP response elements as both homo- and heterodimers thus diversifying their transcriptional regulatory potential (Landschulz et al, 1989). C/EBPα, C/EBPβ, C/EBPδ and C/EBPζ/CHOP-10 have all been shown to be involved in preadipocyte differentiation (Batchvarova et al, 1995; Cao et al, 1991; Yeh et al, 1995). C/EBPδ and C/EBPβ are induced early during differentiation in response to dex and

Figure 3: Differentiation of 3T3 L1 preadipocytes is driven by C/EBP transcription factors and PPARγ.

Differentiation of 3T3 L1 preadipocytes is a 10-day process that is initiated as the cells reach confluence (Day -2). Two-days post confluence (Day 0), growth arrested cells are induced with a hormonal cocktail consisting of isomethylbutylxanthine (MIX), insulin and the synthetic glucocorticoid dexamethasone. 48 h later (Day 2) and every two days thereafter, the media is replaced with media containing insulin alone until Day 8. C/EBPδ and C/EBPβ are induced rapidly in response to stimulation with dex and MIX respectively with maximal expression by 24 h and decline thereafter. Upon stimulation the preadipocytes synchronously re-enter the cell cycle and undergo 2 to 3 rounds of mitosis, which is often referred to as mitotic clonal expansion (MCE). Following MCE, the cells terminally exit the cell cycle. This coincides with C/EBPδ and C/EBPβ dependent induction of C/EBPα transcription as C/EBPα is antimitotic. C/EBPα induces PPARγ expression. Their expression is maximal by day 4 and remains elevated throughout terminal differentiation due to both auto- and cross- transcriptional regulatory mechanisms. C/EBPα and PPARγ, master regulators of adipogenesis, are responsible for the transcription of many adipocyte specific genes, including adipsin.


adipsin
PPARγ
C/EBPα
C/EBPβ
C/EBPδ
confluence
MCE
D-2
D0
D2
D4
D8
MIX
Insulin
Dexamethasone
Insulin

MIX respectively. They in turn, initiate transcription of C/EBPα, a master transcriptional regulator of terminal differentiation (Cao et al, 1991).

Apart from preadipocyte differentiation, C/EBPβ is an important regulatory factor for liver development and regeneration, development of ovaries and the uterus and for development of the immune system (Nagy et al, 1994; Poli, 1998; Sterneck et al, 1997; Sundfeldt et al, 1999; Takiguchi, 1998; Xie et al, 2004). C/EBPβ exists in three isoforms resultant from alternative use of internal translational start codons. The full-length isoform, also referred to as Liver Activating Protein (LAP), contains all three NH_2-terminal activation domains and is transcriptionally active. The second isoform lacks the NH_2-terminal 21 amino acids, but retains all three activation domains. By contrast, the third isoform, also referred to as Liver Inhibitory Protein (LIP), is transcriptionally inactive as it lacks the activation domains. LIP can act as a dominant negative isoform as it retains its dimerization domain, thus can decrease the transactivation potential of the transcriptionally active isoforms through dimerization. The ability of C/EBPβ to regulate transcription can be modulated by altering the relative levels of the LAP and LIP isoforms in the cells. Expression of LAP, but not LIP is sufficient to drive differentiation of C/EBPβ$^{-/-}$ MEFs (Tang et al, 2003a).

The expression of C/EBPβ, but not C/EBPδ is sufficient to promote adipogenesis in response to hormonal induction in NIH 3T3 fibroblasts (Wu et al, 1995; Yeh et al, 1995). Furthermore, exogenous expression of C/EBPβ in 3T3 L1 cells induces C/EBPα expression and stimulates adipogenesis in the absence of hormonal cues (Cao et al, 1991; Yeh et al, 1995). While C/EBPδ is considered to be less adipogenic than C/EBPβ, it appears to complement C/EBPβ function, as defects in adipogenesis are only observed upon genetic

ablation of both proteins (Tanaka et al, 1997). In culture, C/EBPβ and C/EBPδ are induced within 4 h of treatment with MIX and glucocorticoids respectively (Cao et al, 1991). Their protein expression is maximal by 24 h and remains elevated through 48 h at which point C/EBPβ expression steadily declines, whereas C/EBPδ expression declines more rapidly (Cao et al, 1991). These factors are responsible for initiating the transcriptional up-regulation of C/EBPα and PPARγ, which are the master regulators of differentiation.

C/EBPα and PPARγ are responsible for the induction of many adipocyte specific genes including aP2, GLUT4 and leptin (Hollenberg et al, 1997; Long & Pekala, 1996; Miller & Ntambi, 1996; Tontonoz et al, 1995; Tontonoz et al, 1994a). C/EBPα is responsible for conferring insulin sensitivity to the adipocyte (El-Jack et al, 1999; Wu et al, 1999). Both C/EBPα and PPARγ are necessary and sufficient for preadipocyte differentiation. Genetic knockout of either of these factors results in severe defects in WAT development, whereas ectopic expression of either is sufficient to drive adipogenesis of 3T3 L1 in the absence of hormonal inducers and to induce the differentiation of non-committed fibroblasts such as NIH 3T3 cells to the adipocyte lineage (Freytag et al, 1994; Lin & Lane, 1994; Rosen et al, 1999; Tontonoz et al, 1994b). Defining the mechanism for the temporal regulation of C/EBPα and PPARγ expression is complicated due to both auto- and cross-regulatory mechanisms of induction between these factors (Elberg et al, 2000; Legraverend et al, 1993; Wu et al, 1999). Genetic studies have established that they act in the same pathway with C/EBPα acting upstream of PPARγ (Rosen et al, 2002).

Despite the early induction of C/EBPβ and C/EBPδ in differentiating 3T3 L1 cells, C/EBPα and PPARγ are not detectable until approximately 24-48 h post-stimulation. This lag time has been proposed to allow cells to progress through clonal expansion due to the

anti-mitotic properties of C/EBPα (Umek et al, 1991). As such, their transcriptional potential is tightly regulated to ensure proper temporal induction of C/EBPα. Upon induction, C/EBPβ and C/EBPδ DNA binding ability is inhibited by heterodimerization with CHOP-10, a dominant-negative C/EBP family member that cannot bind DNA due to the presence of two proline residues within its DBD (Ron & Habener, 1992; Tang & Lane, 2000). Approximately 12-16 h into differentiation CHOP-10 is down-regulated, and C/EBPβ and C/EBPδ acquire DNA binding ability. Upon acquiring DNA binding activity, they transiently localize to centromeric regions through binding to multiple consensus C/EBP response elements in the heterochromatic DNA. They are also able to bind DNA response elements on target gene promoters (Tang & Lane, 1999). The acquisition of DNA binding ability coincides with the synchronous re-entry of the cells into the cell cycle at the G1-S transition. Mitotic clonal expansion is a prerequisite of terminal differentiation of 3T3 L1 cells, and C/EBPβ has been shown to be required for this process (Tang et al, 2003a; Tang et al, 2003b; Zhang et al, 2004).

The transcriptional activity of C/EBPβ during differentiation is further regulated by posttranslational modifications, including phosphorylation (Park et al, 2004; Tang et al, 2005) and acetylation (Cesena et al, 2007; Wiper-Bergeron et al, 2007; Wiper-Bergeron et al, 2003) and by association with the mSin3-HDAC1 corepressor sub-complex (Wiper-Bergeron et al, 2003). Additionally, the transcriptional potential of C/EBPβ and C/EBPδ during early preadipocyte differentiation has been shown to be antagonized by ETO/MTG8, GATA2/3 transcription factors and delta-interacting protein A (DIPA). Like CHOP-10, the expression of these factors is high in preadipocytes and is down-regulated as differentiation progresses (Bezy et al, 2005; Rochford et al, 2004; Tong et al, 2000; Tong et al, 2005).

Human Primary Preadipocytes

The present molecular understanding of the factors and pathways that drive adipogenesis has largely been limited to studies using tissue culture model systems, with the 3T3 L1 model being pivotal to this knowledge base. Whether or not these pathways are conserved in the context of human preadipocytes remains to be determined due largely to the previous unavailability of cell culture systems. Human primary preadipocytes can be isolated from the stromal vascular cells of adipose tissue from both subcutaneous and omental (visceral) adipose tissue depots from patients undergoing abdominal surgery (Hauner et al, 1987). Advances in the ability to differentiate these cells in culture have provided the means to study how the mechanisms that drive 3T3 L1 differentiation relate to human preadipocytes.

Although many adipogenic factors such as the requirement for insulin and glucocorticoids and the appearance of C/EBPs and PPARγ are similar in both murine and human primary systems, there are some key differences (Harp et al, 2001; Hauner et al, 1989): (1) The kinetics of differentiation of human preadipocytes are longer than those for the mouse cells. Confluent human preadipocytes are stimulated for 4 days with MIX, insulin, dex and a PPARγ agonist and require 14 days to terminally differentiate (Adams et al, 1997; Hauner et al, 1989); (2) Human primary preadipocytes differentiate in the absence of post-confluence mitosis in response to stimulation with the hormonal cocktail (Bell et al, 2000; Entenmann & Hauner, 1996); (3) Finally, the requirements for glucocorticoids and the PPARγ agonists appear to be more absolute in serum-free differentiation protocols for primary preadipocytes than for certain established cell lines (Hauner et al, 1989). The

differentiation of human primary preadipocytes will be discussed in greater detail in Chapter II (page 104).

Glucocorticoids, Visceral Obesity and the Metabolic Syndrome

Glucocorticoids promote adipogenesis *in vivo*. This is most obvious in patients with Cushing's syndrome, a condition of prolonged hypercortisolemia, who develop central (visceral) obesity (Boscaro et al, 2001). Weight gain is also a side-effect of prolonged immunosuppressive glucocorticoid therapies (Pijl & Meinders, 1996). Naturally occurring polymorphisms in GR that render it more responsive to dex correlate with increased WAT and decreased lean body mass; conversely, polymorphisms that decrease the receptor's responsiveness to dex correlate with decreased WAT and increased lean body mass (Di Blasio et al, 2003; Dobson et al, 2001). Furthermore, weight loss that follows adrenalectomy in animals is prevented by glucocorticoid replacement (Freedman et al, 1986; Sainsbury et al, 2001).

When present in excess, the effects of glucocorticoids on adipose tissue can lead to the development of visceral obesity, also referred to as intra-abdominal obesity. Obesity is a growing epidemic in North America. The 2004 Canadian Community Health Survey reported 59.2% of Canadian adults (14.1 million) aged 18 years and older are overweight (body mass index (BMI) $\geq$ 25 kg/m^2) and obese (BMI $\geq$ 30 kg/m^2), up from the 13.8% obesity rate reported in 1978/79. Perhaps more alarming is the increase in the prevalence of obesity in children and adolescents. The same study reports that 26% of Canadians aged 2-17 (~1.6 million) are overweight and obese. This is up from 15% in 1978/79 (Shields, 2005; Tjepkema, 2005). The impact of obesity on both Canadian health and the economy is

significant. In 1997, the direct costs of obesity in Canada were estimated to be more than $1.8 billion or 2.4% of the total Health Care budget. The three largest contributors were hypertension, type II diabetes and coronary artery disease (Birmingham et al, 1999).

Visceral obesity, hypertension, type II diabetes, insulin resistance, impaired glucose tolerance, and dyslipidaemia are metabolic risk factors that collectively define a condition referred to as the metabolic syndrome. They are associated with increased susceptibility to develop atherosclerotic coronary heart disease and morbidity (Brotman & Girod, 2002). There is increasing evidence that impaired glucocorticoid signalling contributes to the development of these adverse metabolic conditions (Brotman & Girod, 2002; Masuzaki et al, 2001; Peeke & Chrousos, 1995). The relationship between glucocorticoid signalling and metabolic syndrome is most evident in patients with Cushing's syndrome which manifest the same pathophysiology that is associated with the metabolic syndrome (Boscaro et al, 2001).

Glucocorticoid excess promotes visceral obesity. The increased glucocorticoid responsiveness of visceral adipose tissue as compared to subcutaneous depots is thought to be due to a combination of increased GR expression and more importantly, 11βHSD1 expression and activity in this tissue (Bujalska et al, 1997; Joyner et al, 2000; Rebuffe-Scrive et al, 1990). This is supported by the finding that an individual with both Cushing's syndrome and impaired cortisone to cortisol conversion, lacked visceral obesity (Jamieson et al, 1999). Visceral obesity, in turn, can lead to insulin resistance and perpetuate metabolic syndrome as was demonstrated by improved hepatic insulin sensitivity and decreased glucose production following surgical removal of visceral fat pads in obese Sprague-Dawley rats (Gabriely et al, 2002).

Both obesity and BMI positively correlate with increased cortisol urinary excretion (Dunkelman et al, 1964; Fraser et al, 1999); however, non-Cushinoid individuals with idiopathic obesity and the metabolic syndrome exhibit only mild perturbations in the hypothalamic-pituitary-adrenal axis and have normal circulating levels of steroid (Glass et al, 1981). Growing evidence suggest that the local production of glucocorticoids by the action of 11βHSD1 enzymes contributes to the pathogenesis of visceral obesity and the metabolic syndrome.

11βHSD1-null mice resisted weight gain when fed a high fat diet, exhibited an attenuated gluconeogenic response in response to fasting, in part through down-regulation of hepatic gluconeogenetic enzymes such as PEPCK and Glucose-6-Phosphatase, had improved glucose tolerance, increased hepatic insulin sensitivity, and increased induction for lipogenic enzymes in the liver (Kotelevtsev et al, 1997; Morton et al, 2001; Morton et al, 2004). Conversely, mice with targeted over-expression of 11βHDS1 in adipose tissue developed visceral obesity due to hypertrophy of mesenterial fat depots and developed metabolic syndrome with systemic insulin resistance (Masuzaki et al, 2001; Masuzaki et al, 2003). Hepatic-overexpression of 11βHSD1 produced mice with dyslipidaemia, hypertension and insulin resistance without obesity (Paterson et al, 2004). Finally, mice in which glucocorticoid signalling was attenuated specifically in adipose tissue by targeted transgenic expression of 11βHSD2, which metabolizes active glucocorticoids, resulted in mice that were resistant to obesity when fed a high fat diet (Kershaw et al, 2005). Collectively, these transgenic models establish glucocorticoids as key mediators of the pathophysiology that contributes to the development of visceral obesity and the metabolic syndrome.

Experimental Rationale

Glucocorticoids contribute to multiple aspects of adipose tissue function including its localization, differentiation and function. Dysregulation of glucocorticoid signalling can contribute to disease states including the development of visceral obesity and the metabolic syndrome. These conditions correlate with increased morbidity. I hypothesize that glucocorticoids contribute to metabolic disease by making an important contribution to the establishment of a permissive environment that facilitates the commitment of a preadipocyte to differentiate.

The adipogenic stimulus provided by glucocorticoids is complex and has multiple cellular targets that ultimately converge to facilitate preadipocyte differentiation; these pathways are only beginning to be elucidated. Furthermore, our mechanistic understanding of these pathways has largely been limited to studies performed in murine cell culture models. The contribution of glucocorticoids to human preadipocyte differentiation has not been elucidated at a molecular level.

My thesis project was driven by three main objectives:

(1) To define the molecular mechanism by which glucocorticoids enhance C/EBPβ-mediated transcription by promoting the 26S proteasome-dependent degradation of HDAC1. Specifically, I sought to identify a relevant E3 ligase that could ubiquitylate HDAC1.

(2) To assess the role of glucocorticoids in promoting differentiation of human primary preadipocyte differentiation. To do so, I first had to establish the human primary preadipocyte differentiation system in the laboratory and define the temporal requirements for glucocorticoids. I sought to assess the contribution of glucocorticoids to

the early, known transcriptional events that drive differentiation, namely the expression of C/EBP factors and PPARγ.

(3) Preadipocytes are continuously exposed to low levels of glucocorticoids as a result of both circulating levels of steroid and local steroid production within adipose tissue through the activity of 11βHSD1 enzymes. The impact of this on the differentiation capacity of these cells has never been assessed. I sought to assess what effect constitutive and acute glucocorticoid treatment of both human primary and murine 3T3 L1 preadipocytes prior to the initiation of differentiation had on the subsequent differentiation potential of these two models.

MATERIALS AND METHODS

DNA Plasmids

All expression plasmids and transcription reporter plasmids are listed in tables in Appendix A. Table 1 comprises plasmids that were acquired by other researchers and Table 2 lists expression constructs that were cloned either by me or a colleague in the Haché group as indicated. Basic molecular biology techniques were used to clone plasmids. In general, plasmids were cloned by restriction enzyme digest mediated excision of desired fragments from host plasmid and re-ligation into compatible restriction enzyme sites in desired vectors. Where appropriate, the digested vector was treated for 30 min at 37°C with calf intestinal alkaline phosphatase (CIAP). Vector and insert were incubated at a molar ratio of 1:3 to 1:5 with T4 DNA ligase overnight at 16°C. All restriction enzymes, CIAP and T4 DNA ligase were purchased from New England Biolabs (Pickering, ON). The ligation reaction was transformed into *Esherichia coli* DH5α bacteria by electroporation and plated onto Luria Broth (LB) agar plates containing 100μg/ml ampicillin. Single colonies were picked the next day and positive clones were screened by restriction digest. Positive clones were verified by DNA sequencing which was performed externally by StemCore (Ottawa, ON).

Bacterial Culture and Plasmid Purification

All expression plasmids were transformed into competent *E. coli* DH5α strain by electroporation. Luciferase reporter constructs were transformed into competent *E. coli* RB404 strain (*dam*$^-$ *dcm*$^-$) to avoid the generation of cryptic glucocorticoid response elements due to methylation of BamH I sites (Truss et al, 1992). Transformed bacteria were grown overnight as described above. Single colonies were picked the next day and used to

inoculate 3ml culture of LB containing 100μg/ml ampicillin which was grown overnight (for small scale plasmid preparation) or over the day (for large scale plasmid preparation).

Small scale plasmid preparations were performed using standard alkaline lysis protocols (Sambrook, 1989). Following ethanol precipitation, plasmid DNA was resuspended in 50μL TE buffer (10mM Tris-HCl pH 7.9, 1mM ethylenediamine tetra-acetic acid (EDTA)) in the presence of RNAse A (0.2mg/ml). Alternatively, plasmid DNA was purified using the QIAprep® Spin Miniprep Kit (Qiagen, Mississauga, ON) as per manufacturer's protocol.

For large scale plasmid purification, the 5ml LB culture was used to inoculate 0.5-1L culture of LB with ampicillin, which was grown overnight at 37°C. For all constructs utilized for transfection experiments, plasmid DNA was harvested using the alkaline lysis protocol (Sambrook, 1989). Isolated DNA was subjected to double cesium chloride gradient centrifugation in the presence of 200μg/ml ethidium bromide (EtBr) to visualize the DNA. EtBr was extracted using salt-saturated isopropanol, and DNA was dialyzed twice against 4L TE buffer at 4°C for a minimum of 4 h. For all other plasmids, plasmid DNA was purified using the Qiagen® Plasmid Maxi Kit exactly as per manufacturer's protocol.

Tissue Culture

Cell Maintenance

Cos7 cells (ATCC #CRl-1651) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Burlington, ON) supplemented with non-essential amino acids (Invitrogen, Burlington, ON), L-methionine (Sigma, Oakville, ON), penicillin (100U/ml) (Sigma, Oakville, ON), streptomycin (100mg/ml) (Invitrogen, Burlington, ON) and 10%

FBS (Wisent, St-Bruno, QC). NIH 3T3 cells (ATCC #CRL-1658) were grown in DMEM supplemented with non-essential amino acids, L-methionine, penicillin, streptomycin and 10% calf serum (CS) (Invitrogen, Burlington, ON). Phoenix Ampho cells (ATCC #SD3443, with the permission of Dr. G. Nolan, Standford University) were grown in DMEM supplemented with penicillin, streptomycin and 10% FBS (Invitrogen, Burlington, ON). 3T3 L1 cells (ATCC #CL-173) were grown in low glucose DMEM (1.5g glucose/L) supplemented with 10% CS. Human primary preadipocytes (Zen-Bio Inc., Research Triangle Park, NC) were maintained in low glucose DMEM supplemented with penicillin, streptomycin, nystatin (50U/ml) (Sigma, Oakville, ON) and 20% CS. Cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2 (Cos7, NIH 3T3, Phoenix, human primary preadipocytes) or 10% CO_2 (3T3 L1).

Transfection of Plasmid DNA

Cos7 and NIH 3T3 cells were seeded a day prior to transfection at a density of approximately $4.5x10^5$ cells per 60mm tissue culture dish. Cos7 cells were transfected using Lipofectamine reagent (Invitrogen, Burlington, ON). Equal amounts of plasmid DNA was diluted to final volume of 100μl in OptiMem (Invitrogen, Burlington, ON). For reporter assays, I used 200ng reporter, 25-100ng C/EBPβ, 100ng GR_{505C}, and 0.1-1.0μg TIF1β (or Mdm2, E6AP). Each condition was performed in duplicate. Amounts of DNA used were standardized across conditions using respective empty expression vectors and sheared salmon sperm DNA. Diluted DNA was mixed with 10μl Lipofectamine pre-diluted with 90μl OptiMEM. DNA-Lipofectamine mixtures (200μL total) were complexed for 45 min at room temperature. Complexes were dropped onto cells that had been washed twice with

phosphate buffered saline (PBS) and the media replaced with 2ml OptiMEM. 16-20 h later, transfection was stopped by addition of 2ml phenol red-free DMEM supplemented with non-essential amino acids, penicillin, streptomycin and 20% lot-matched charcoal stripped FBS (SFBS) (Wisent, St-Bruno, QC). Media was replaced with same phenol red-free DMEM (10% SFBS) 24 h following transfection. Where indicated, cells were treated with 1μM dex (Steraloids, Newport, RI) or 1μM MG132 (Sigma, Oakville, ON) for 20 h. For all other experiments, the amount of plasmid DNA transfected is indicated in parenthesis in the figure legends.

NIH 3T3 were transfected using Fugene 6 transfection reagent (Roche Applied Science, Laval, QC). Plasmid DNA was diluted in OptiMEM and complexed with Fugene reagent in a 3:1 ratio (mg DNA: ml Fugene) in a final concentration of 1μgDNA/0.1ml OptiMEM. Mixture was left at room temperature for a minimum of 20 min. Complexes were dropped onto 2ml DMEM supplemented with penicillin, streptomycin and 10% CS. Media was replaced 20 h later. Reporter assays were performed as described for Cos7 cells. Amounts of plasmids used in other experiments are indicated in the figure legends. Cos7 cells were transfected with Fugene (using the same protocol) for the experiments to assess TIF1β-dependent ubiquitylation of HDAC1 (Chapter 1, Fig 5).

Reporter Gene Expression Analysis using Luciferase Assay

Transfected cells were rinsed twice with PBS and harvested in 400μl 1x Reporter Lysis Buffer (Promega, Madison, WI). Extracts were centrifuged briefly to pellet cellular debris, and supernatants transferred to a new tube. 20μl of the cellular extract was pipetted into a 12 x 75mm borosilicate glass tube. To this extract, 100μl of Luciferase Assay Reagent

(Promega, Madison, WI) was added using the auto-inject device of the Monolight 2010 Luminometer (Applied Luminescence Laboratory). The luminescence generated from this reaction was measured for 10s (as relative luciferase units). Light intensity is proportional to luciferase concentration in the range of 10^{-16} M to 10^{-8} M. Relative luciferase units were corrected for protein content using a Bradford Assay (Bio-Rad, Hercules, CA) as per manufacturer's protocol, using bovine serum albumin (BSA) to generate the standard curve. Data represents average fold induction from a minimum of three independent experiments, each done in duplicate ± standard error of the mean (SEM).

Immunoprecipitation and Western Analysis

Cell Culture and Treatment with Dex and MG132

Where appropriate, cells were transfected as described above and harvested on the 2nd day post-transfection. Where indicated, cells were treated for with 1μM dex and/or 1μM MG132 or vehicle for the indicated amount of time immediately prior to harvesting.

Preparation of Whole Cell Extracts

Cells were rinsed twice with cold PBS and scraped in 500μl-1ml PBS using a rubber policeman. Cells were pelleted by centrifugation at 6 000 X g for 5 min at 4°C. For Western analysis, the cell pellet was lysed in 100-300μl IPH buffer (50mM Tris pH 7.4, 150mM NaCl, 0.5% Nonindet-P40 (NP-40), 5mM EDTA and fresh 2mM DTT and protease inhibitor cocktail (Roche, Laval, QC)). The cells were kept on ice for 10 min following which the lysates were sonicated for 10s at 30% duty cycle (Branson Sonifier 450) and cleared by centrifugation for 5 min at 13 000 X g. For immunoprecipitation experiments, the cells were

lysed in 300μl whole cell lysis (WCL) buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 0.5% NP-40, 10% glycerol, 2mM DTT (fresh) and protease inhibitor cocktail). Cells were incubated on a rotating wheel for 25-40 min at 4°C then cleared by centrifugation for 5 min at 13 000 X g. In both cases, the supernatants were transferred to a new tube and the concentration determined using a Bradford Assay. For straight Western analysis, equal amounts of protein (30-50μg) were resolved by SDS-PAGE.

Co-immunoprecipitation Assays

Cells (100mm dish) were lysed in 300μl WCL buffer. Between 0.5-1mg of whole cell lysate was used for co-immunoprecipitation. The final concentration of lysate was standardized between all conditions in a given experiment with whole cell lysis buffer. The extracts were immunoprecipitated at an ideal final concentration of 1mg protein/ml in a final concentration of 0.1-0.2% NP-40 by addition of IP dilution buffer (whole cell lysis buffer with no NP-40). Extracts were incubated with 1-5μg primary antibody on the rotating wheel for 1 h or overnight. Thereafter, 30μl Protein A Sepharose beads were added for 1 h at 4°C. The immunoprecipitates were washed a minimum of 3 times with 1ml cold WCL buffer. The stringency of the wash was modified by changing the concentration of NP-40 and NaCl in the wash buffer. For washing, the precipitates were rocked gently and spun at 4 000 X g for 2 min. Upon the final wash, the supernatant was completely aspirated and 6xSDS buffer was added to the beads. These samples were resolved by SDS-PAGE.

Preparation of Protein A Sepharose Beads

Protein A conjugated sepharose beads (Sigma, Oakville, ON) were hydrated in distilled water overnight on a rotating wheel at 4°C. The beads were then pelleted by centrifugation for 20 min at 2500rpm in a Beckman Coulter Allegra 6R centrifuge. Hydrated beads were blocked with 50mg/ml BSA in IP buffer (50mM Tris pH 7.4, 150mM NaCl, 5mM EDTA, 0.05% NP-40 and 0.02% NaN_3) by rotating overnight at 4°C. The blocking solution was removed and the beads were stored at 4°C as a 50% slurry in IP buffer.

In vivo 6xHis-tagged Ubiquitin Conjugation Assay

Assay was performed as described (Leng et al, 2003). Cos7 and NIH 3T3 cells (100mm dish) were transiently transfected with 2μg His-Ub (pMT107), 0.25μg pCDNA3.1.HA-HDAC1, 0.5μg pSG5puro.Flag-TIF1β and 1.25μg sheared salmon sperm DNA using Fugene 6 as described previously. 24 h following transfection, cells were treated for 20 h with 1μM MG132. Cells were harvested and immunoprecipitated essentially as described above, with the following modifications. Cell pellets were resuspended in cold RIPA buffer (20mM Tris pH 7.4, 5mM EDTA, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.025% SDS, protease inhibitor cocktail, 40μM MG132) and incubated on ice for 10 min, then sonicated twice for 10s at 35% duty cycle. Lysates were cleared by centrifugation at 13 000 X g for 10 min. 0.5mg cellular extract was immunoprecipitated at a final concentration of 1mg/ml in a final buffer containing 20mM Tris pH 7.4, 5mM EDTA, 150mM NaCl, 0.5% NP-40, 0.05% sodium deoxycholate, 0.0125% SDS, protease inhibitor cocktail and 40μM MG132. HA-tagged HDAC1 was immunoprecipitated using 5μg anti-HA(12CA5) (Roche, Laval, QC) antibody for either 2 h or overnight at 4°C. Following the

addition of beads as previously described, the immunoprecipitate was washed 4 times with 1ml of RIPA buffer. The proteins were then resolved by SDS-PAGE and His-tagged ubiquitylated HDAC1 visualized by Western analysis using an anti-6xHIS antibody.

SDS-Polyacrylamide Gel Electrophoresis

Protein samples (straight Western and immunoprecipitation) were denatured by boiling at 95°C for 5 min in SDS loading buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate (SDS), 0.05% bromophenol blue, 355mM 2-mercaptoethanol) and centrifuged for 1 minute at 13 000 X g. Extracts were resolved on 8-15% acrylamide gels with a 1 cm 4% stacking gel using a Mini-PROTEAN II electrophoresis system (Bio-Rad, Hercules, CA). Protein was then transferred to PVDF membrane (Bio-Rad, Hercules, CA) using a Mini-Trans-Blot apparatus (Bio-RA, Hercules, CA) following standard procedures.

Western Analysis

The PVDF membrane was rinsed briefly in PBS containing 0.5% Tween-20 (PBS-T), then blocked for 30 min in 5% milk powder in PBS-T (with the exception of the PY20 anti-phospho tyrosine antibody which was blocked in 3% BSA in PBS-T). The membrane was incubated with primary antibody in blocking solution for either 2 h at room temperature or overnight on a rotating wheel at 4°C. Primary antibodies used are listed in Appendix A, Table 4 with the supplier information and concentrations at which they were used. Following incubation with primary antibody, membranes were washed three times in PBS-T for 10 min then incubated with horseradish peroxidase conjugated secondary antibody (anti-rabbit Fab fragments 1:50 000, anti-mouse 1:50 000 (Amersham, Baie D'Urfe, QC), anti-goat 1:10 000, Santa Cruz, Santa Cruz, CA) for 30 min to 1 h at room temperature.

Membranes were then washed three times in PBS-T for 10 min. Signals were detected by chemiluminescence (Western Lightning, Perkin Elmer, Woodbridge, ON). For serial reprobing, membranes were stripped for 20 min at room temperature using 1x Re-Blot Plus Mild Solution (Chemicon International, Temecula, CA), rinsed once with PBS-T then reprobed following above conditions.

Where indicated, signal intensities of Western blots were quantified using ImageQuant software. All experiments are a representative of a minimum of three independent experiments. For studies using the human primary preadipocytes (Chapters 2, 3), experiments were repeated with preadipocytes derived from 3 to 4 individual donors, and a pool of five donor samples as indicated in the figure legends. Where indicated, data was presented as average protein induction ± standard deviation (SD). Where applicable, statistical significance was assessed using two-tailed, paired Student's t-Tests.

Retroviral Infection of 3T3 L1 and NIH 3T3 cells

Cells were infected with pLXSN-based (Clontech) replication incompetent retroviruses generated in Phoenix Ampho packaging cells. Phoenix cells were seeded at a density of 2.5 x 10^6 cells in a 60mm tissue culture dish 24 h prior to transfection. Immediately prior to transfection, the media was changed to regular Phoenix media containing 25μM chloroquine (Sigma, Oakville, ON). Cells were transfected by calcium chloride precipitation. 10μg DNA was diluted with sterile pyrogen-free water to a final volume of 438μl and mixed with 62μl $CaCl_2$. To this mixture, 500μl 2x HBS (50mM HEPES pH 7.05, 10mM KCl, 12mM Dextrose, 280mM NaCl, 1.5mM Na_2HPO_4) was added, mixed by pipetting and immediately dropped onto cells. Fresh media was added

approximately 16 h later and again approximately 8 h thereafter. 48 h following the transfection, the virus-containing supernatant was collected and passed through a 0.45μM syringe filter. Virus was either used immediately or stored at -80°C.

3T3 L1 and NIH 3T3 cells were infected at approximately 50% confluence in 100mm tissue culture dishes. The cells were incubated overnight with a total of 6ml of virus, regular media and 4μg/μl polybrene (Sigma, Oakville, ON). The next morning, the media was supplemented with an additional 4ml of fresh media, and the media was completely changed 24 h following infection. Beginning 48 h post-infection, 3T3 L1 and NIH 3T3 cells were selected for 7-10 days in the presence of 400μg/ml and 300μg/ml G418 (Invitrogen, Burlington, ON) respectively to ensure expression of desired factor in all cells.

Preadipocyte Differentiation

Differentiation of 3T3 L1 preadipocytes and NIH 3T3 fibroblasts

3T3 L1 preadipocytes were maintained in low glucose DMEM with 10% CS. Serum lots were screened to maximize the efficiency of dex-dependent differentiation. 3T3 L1 cells were differentiated either in the presence and absence of serum as indicated. For differentiation in the presence of serum: two days post-confluent cells were stimulated (Day 0) with 3T3 L1 media containing 100nM insulin (Sigma, Oakville, ON), 0.5mM 3-isobutyl-1-methyl-xanthine (MIX) (Sigma, Oakville, ON) and 250nM dex as indicated for 48 h. Thereafter, the media was replaced every two days with media containing 100nM insulin. Cells were differentiated for 7-8 days. In general, 3T3 L1 cells were differentiated in Falcon brand 6-well dishes using 3ml media/well. For immunofluorescence experiments, cells were differentiated in Falcon 12-well dishes using 1ml media/well.

An NIH 3T3 cell line that stably expressed C/EBPβ was generated by retroviral infection. Cells were differentiated under serum-containing conditions as described above.

For differentiation experiments in serum-free media, at two days post-confluence (Day 0), the media was replaced with serum-free DMEM-Ham's F12 (1:1, vol/vol) media supplemented with 33μM biotin, 17μM pantothenate, 10μg/ml transferrin, 0.2nM triiodothyronine, and 0.25mg/ml fetuin (Sigma, Oakville, ON). Differentiation was induced by treatment with 100nM insulin, 0.5mM MIX and 250nM dex as indicated for 48 h. Thereafter, media was replaced every 2 days with serum-free medium containing 100nM insulin for 5 to 6 days.

Differentiation of human primary preadipocytes

Cryopreserved, subcutaneous human primary preadipocytes from female donors with a normal BMI (22.5±0.2 kg/m2) were purchased from Zen-Bio Inc. Preadipocytes were thawed at 37°C, resuspended in 10ml culture media and spun for 5 min at 2000rpm in a Beckman Coulter Allegra 6R centrifuge with no break. One vial of cells was seeded into three Nunc T75 flasks. Upon reaching 85-90% confluency, preadipocytes were pooled and expanded (1:2) once prior to seeding into wells for differentiation. Media was replaced every two days. For experiments in which preadipocytes from multiple donors were pooled, the cells were pooled immediately upon thawing.

For differentiation, human preadipocytes were seeded at a density of approximately 50,000 cells/well in Nunc 12-well culture dishes. Upon reaching confluence (Day 0), the media was replaced with serum-free DMEM-Ham's F12 (1:1, vol/vol) media supplemented with 33μM biotin, 17μM pantothenate, 10μg/ml transferrin, 0.2nM triiodothyronine,

100U/ml penicillin, 100mg/ml streptomycin, and 50U/ml nystatin (Sigma, Oakville, ON). To stimulate differentiation, the cells were treated with 100nM insulin, 0.5mM MIX from day 0 to day 4, 1μM dex for the initial 48 h and 5μM troglitazone from day 2 to day 4. On day 4 and every 3-4 days thereafter, the media was replaced with serum-free media containing 100nM insulin until day 8, after which the media was not replaced.

Exposure of Preadipocytes to Glucocorticoids Prior to Differentiation

For the pretreatment experiments (Chapter 3) 3T3 L1 and human primary preadipocytes were stimulated in two ways. For 1nM dex/proliferation pretreatment, both human primary and 3T3 L1 were grown exactly as described above with the exception that they were cultured in growth media containing 1nM dex or vehicle for 7 to 10 days prior to initiating differentiation. For human preadipocytes, dex was added to the media following the first split. Dex remained in the culture media until inducing differentiation (Day 0). For the pharmacological concentration of glucocorticoid for 48 h pretreatment, both 3T3 L1 and human primary preadipocytes were grown to confluence as described above. Upon reaching confluence, human preadipocytes were stimulated with 1μM dex or vehicle in growth media containing 3% CS for 48 h. Similarly, 3T3 L1 preadipocytes were stimulated with 250nM dex in complete media (10% CS) for 48 h with the exception of RT-PCR based analysis of insulin signalling components in 3T3 L1 preadipocytes (Chapter 3, Fig 6C, 9B) in which the cells were stimulated with 1μM dex in 3%CS to mimic the conditions used for the human preadipoctyes. Thereafter (Day 0), differentiation was induced as described above.

For all differentiation experiments, Day 0 was defined as the time point at which preadipocytes were stimulated with induction cocktail.

Oil Red O Staining of Neutral Lipid Content in Mature Adipocytes

To assess neutral lipid content, mature adipocytes were washed twice with PBS and fixed with 2ml 10% formalin in PBS for 1 h. Cells were then washed once with PBS and stained with 2ml Oil Red O (3.5g in 500ml propylene glycol) for 2 h then washed with distilled water (Schwarz, 1997). Photomicrographs of Oil Red O stained cells were taken with a Leica MZ125 microscope and represent approximately 85% of a well in a 12-well dish. Where applicable (Chapter 2), images were quantified for relative Oil Red O staining using Image J software.

siRNA-Mediated Gene Knock-Down

siRNA oligonucleotides targeted against GFP and TIF1β were purchased from Dharmacon. TIF1β oligos were purchased as a SMARTpool mixture of oligonucleotides that target four distinct sequences within TIF1β mRNA. The GFP oligo was a 23mer targeted against the sequence 5'-AAGACCCGCGCCGAGGUGAAGUU-3'. NIH 3T3/C/EBPβ^{+} cells were seeded in 6-well dishes and transfected one day prior to reaching confluence using Oligofectamine® transfection reagent (Invitrogen, Burlington, ON). Immediately prior to transfection, the cells were washed twice with PBS and the media was replaced with 800μl serum-free media with no antibiotics. 100pmol siRNA oligos were diluted in 185μl serum-free media then mixed with 3μl Oligofectamine diluted in 15μl with serum-free media. Reaction was incubated for 20 minutes at room temperature then dropped onto cells. Media was replaced with complete media 4 h later. Cells were then grown to confluence and induced to differentiate as previously described.

Chromatin Immunoprecipitation Analysis of Promoter Occupancy

Chromatin immunoprecipitations were performed essentially as described (Yahata et al, 2001). 3T3 L1 preadipocytes that were retrovirally infected with control vector (pLXSN) or TIF1β were induced to differentiate under serum-free conditions with MIX and insulin or vehicle as indicated for 24 h. Cells were then washed twice in serum-free media and fixed with 1% formaldehyde at room temperature for 10 min. Cells were harvested then serially washed in PBS, buffer I (0.25% Triton X-100, 10mM EDTA, 0.5mM EGTA and 10mM Hepes, pH 6.5) and buffer II (200mM NaCl, 1mM EDTA, 0.5mM EGTA, 10mM HEPES pH6.5) and the cell pellets were resuspended and sonicated in sonication buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.0, 1mM DTT, protease inhibitor cocktail). Lysates were cleared by centrifugation at 13 000 X g for 10 min at 4°C and the supernatants were transferred to a new tube. They were diluted 1:20 in dilution buffer (1% TritonX-100, 150mM NaCl, 2mM EDTA, 50mM Tris pH 8.0, 1mM DTT and protease inhibitor cocktail). Lysates were then incubated with 2μg of antibody overnight at 4°C. Antibodies used were anti-Gal4DBD (non-specific control), C/EBPβ C-19, HDAC1 C-19, RNA polII N-20 (all from Santa Cruz Biotech, Santa Cruz, CA), TIF1β (ABR, Golden, CO), and acetyl-H4 (Upstate Biotechnology, Charlottesville, VI). The next morning, immune-complexes were precipitated using Protein A beads, with 2μg of sheared salmon sperm DNA. The precipitates were sequentially washed for 10 min each at 4°C in TSEI (0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris pH8.0, 150 mM NaCl), TSE II (0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris pH8.0, 500 mM NaCl), buffer III (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris pH 8.0) and twice in TE. Samples were then extracted 3 times in 100μl of 1% SDS, 0.1 M $NaHCO_3$. Cross-linking

was reversed by heating the eluates at 65°C overnight. DNA fragments were purified using the QIAquick® PCR purification kit. (Qiagen, Mississauga, ON) and amplified by PCR using the following primers for positions within the murine C/EBPα promoter: -334 and –118 (5'-TAGTGTTGGCTGGAAGTGGGTGACTTAG AGGC-3', 5'TTCTCCTGTGACTTTCCAAGGCGGTGAGTG-3'), -108 and +17 (5'-TAAGACC CAGCAGGCACCAT CCTACTG-3', 5'-AGTTAGAGTTCTCCCGGCATGGCGAG-3'). Results shown are representative of a minimum of 3 independent experiments.

Protein Visualization by Indirect Immunofluorescence

3T3 L1 cells were seeded onto poly-L-lysine (Sigma, Oakville, ON) coated glass coverslips within a well of a 6-well dish and differentiated as previously described. Upon harvesting, the coverslip was gently rinsed once with PBS and the cells were fixed by incubation with 1ml 4% paraformaldehyde in PBS for 20-30 min in the dark at 4°C. Coverslips were then washed with PBS and the cells permeabilized by incubation with 1ml 0.5% TritonX-100 in PBS for 30 min at room temperature. The coverslips were washed again in PBS and incubated with a blocking solution of 5% BSA in PBS for a minimum of 1 h at room temperature. Thereafter, the cells were incubated with primary antibody in PBS overnight with gentle rocking at 4°C. The primary antibodies used are listed in Appendix A with the supplier information and working concentrations. The next morning, the coverslips were washed 3 times for 2 min each with PBS and incubated with fluorophore-conjugated secondary antibodies diluted 1:500 in PBS for 45 min at room temperature. Secondary antibodies used were: Alexa 488-conjugated donkey anti-mouse, Alexa 594-conjugated donkey anti-mouse and Alexa 488-conjugated donkey anti-rabbit (Molecular Probes, Eugene, OR). Cells were then washed 3 times with PBS for 2 min each, mounted onto glass

slides using Vectashield mounting solution with DAPI stain (Vector Laboratories Inc., Burlingane, CA). Images were acquired using either a Nikon Eclipse TE300 microscope or a Bio-Rad MRC 1024 confocal microscope as stated in the figure legends. Where indicated, confocal images were processed and merged using ImageJ software.

Real-Time PCR Analysis of mRNA Expression

Total RNA was extracted using an RNeasy® RNA Isolation Kit (Qiagen, Mississauga, ON) exactly as described by the manufacturer. 2-5μg total RNA was DNase1 decontaminated by incubation with 5U DNase1 (Amersham, Piscataway, NJ) (in 40mM Tris pH 7.5, 6mM $MgCl_2$ in total volume of 25μl) and was incubated at 37°C for 10 min. Reaction was stopped by addition of 5mM EDTA pH 7.5 and incubation at 65°C for 10 min then chilled on ice. First-Strand cDNA synthesis was performed using Oligo dT primers (Invitrogen, Burlington, ON) and SuperScript II Reverse Transcriptase (Invitrogen, Burlington, ON) as per manufacturer's protocol. In brief, Oligo dT primers (500μg/ml) were first annealed to total RNA in the presence of 0.8M dNTP (total 26μl) by incubation for 5 min at 70°C then chilled on ice. This mixture was then incubated with 0.01M DTT and RNAsin in 1X First-Strand Buffer (Invitrogen, Burlington, ON) and incubated for 2 min at 42°C following which 1μl SuperScript II was added. The reaction proceeded for 50 min at 42°C followed by 15 min at 70°C and cooled at 4°C. For real-time reactions, 2-4 μl of cDNA was amplified using a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) using a Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Reactions were prepared in 25μl with 0.3μM (final) of each primer pair. The cDNA was amplified using the following PCR program: 95°C for 10 min and 40 cycles of 95°C for 15s,

gene specific annealing temperature for 30s, 72°C for 35s. This was followed by 1 cycle of the dissociation program: 95°C for 15s, 60°C for 1 min and 95°C for 15s. Primer pairs and respective annealing temperatures for each gene target are listed in Appendix A, Table 4. Reactions were standardized against G3PDH (Gorzelniak et al, 2001). Data represents relative mRNA abundance from a minimum of three experiments each done in duplicate. For experiments using human primary preadipocytes, experiments were performed in a minimum of three individual donors and in some cases a pool of 5 donor samples as indicated. Data is shown ± SEM. Statistical significance was determined using two-tailed, paired Student's t-Tests.

Effect of Actinomycin D, Cycloheximide and MG132 on the Expression of C/EBPβ

Human primary preadipocytes were treated with differentiation cocktails as described above in the presence of one of 10μg/ml actinomycin D (Sigma, Oakville, ON), 20μM cycloheximide (Sigma, Oakville, ON), 1μM MG132 or vehicle for the times indicated in individual experiments. For cycloheximide treatments, cells were pulsed for 15 min with 20μM cycloheximide prior to stimulation with differentiation cocktail. Cells were treated for 4 or 8 h with cocktail then harvested and processed by Western analysis and quantified as described above.

Analysis of DNA Synthesis by ^{3}H-Thymidine Incorporation

Both human preadipocytes and 3T3 L1 cells were induced to differentiate with complete induction cocktail (MIX, insulin and dex) as described above. Cells were pulsed with 2μCi ^{3}H-thymidine/well (human/12-well dish) (Amersham, Piscataway, NJ) or 4μCi

3H-thymidine/well (3T3 L1/6-well dish) for 12 h, then washed once with ice cold PBS and incubated with ice cold 5% TCA 2 x 15 min at room temperature. Wells were washed with PBS and extracts scraped in 0.5N NaOH/0.5% SDS. Thymidine incorporation was measured by measuring total DPM in scintillation counter (Beckman Coulter LS6500). Data is represented averages of two (human) or three (3T3 L1) independent experiments each done in duplicate ± SEM. Statistical significance was determined using two-tailed, paired Student's t-Tests.

Analysis of Cell Death by In Situ TUNEL Assay

Human and murine cells were seeded onto poly-L-lysine coated coverslips and induced to differentiate for 48 h under serum-free conditions. Assay was performed using a Fluourescein based In Situ Cell Death Detection Kit (Roche, Laval, QC) as per manufacturer's instructions. In brief, cells were fixed with 4% paraformaldehyde for 30 min at room temperature, rinsed in PBS and permeabilized with 0.1% TritonX-100 in 0.1% sodium citrate for 2 min on ice. DNA ends were then labeled with fluorescein-dUTP using terminal transferase for 1 h at 37°C, rinsed with PBS and analyzed by fluorescence microscopy using a Nikon Eclipse TE300 microscope. For positive controls, fixed cells were treated with 50U-100U DNAse I for 10 min prior to labeling of DNA ends with fluorescein-dUTP.

Microarray Analysis of Gene Expression Profiles in Human Primary Preadipocytes

Experimental Design

Human subcutaneous primary preadipocytes from 5 female donors (average BMI 22.5±0.2kg/m^2) were combined prior to the initial seeding. Preadipocytes were seeded into

15 Nunc T75 flasks. At this point, the flasks were divided into 3 groups of 5 flasks and each group was treated independently from the others. These defined the replicates for microarray analysis. The 5 flasks from one group were split into 10 flasks. Of these, 3 flasks were immediately treated with 1nM dex for the 1nM dex/proliferative pretreatment. The 7 remaining flasks were left untreated. All 10 flasks were grown to approximately 90% confluence then split into 12 well dishes at a density of 50,000 cells/well. The 1nM dex pretreated cells were continuously cultured with 1nM dex until reaching confluency. Untreated cells were maintained as the control. The remaining 12 well dishes were grown to confluence then treated with or without 1μM dex for 48 h in the presence of 3% CS.

Sample Preparation

On Day 0 (Day 0 was the time point at which differentiation would have otherwise been induced for each pretreatment respectively), total RNA was harvested using the Qiagen RNeasy Kit® exactly as described under the RT-PCR methodology. RNA from 3 wells was pooled and quantified for microarray analysis. Additionally, RNA from 3 wells was pooled for future RT-PCR based validation of microarray results. This was stored at -80°C. Whole cell lysates were also prepared at Day 0 for future Western analysis of identified factors. In the remaining wells, differentiation was induced with MIX and insulin, or MIX, insulin and dex. From these, some were used for terminal differentiation to confirm that the pretreatment had worked prior to sending the samples for microarray analysis. Other wells were harvested for future Western analysis and the cell pellets were stored at -80°C.

Microarray Analysis

Three control and three dex treated RNA samples were analyzed by StemCore (formally the Ottawa Genome Centre). 50ng of total RNA was amplified by StemCore. Samples were hybridized to the Affymetrix Human Genome U133 Plus 2.0 GeneChip Array. This microarray contains 54 675 probesets. StemCore provided us with raw data and quality control values. Based on quality control analysis of the 1μM dex/48H pretreatment arrays, it was determined that the data from only two control and two dex treated arrays would be used for further analysis of gene expression profiles. For the 1nM dex/proliferation pretreatment, all three arrays for both control and treated conditions were used for subsequent analysis.

Microarray Data Analysis and Statistical Significance

The raw data was processed and analyzed by Dr. Alan Mears at the University of Ottawa Eye Institute. The raw data contained intensity values that were associated with given probes. These values were then transformed into expression values, a process referred to as normalization. The data was first analyzed using Affymetrix MICROARRAY SUITE v5.0 (MAS5) to establish present and absent calls for each probeset. The data was normalized and the signal intensities were calculated using two independent algorithms: Robust MultiChip Average (RMA) and GC-RMA (Hero, 2004; Yoshida et al, 2004). Both software packages are from the R project (http://www.r-project.org). Based on these values, the average fold change (AFC) (+dex relative to –dex) of each probeset was calculated for both RMA and GC-RMA based analysis respectively. For each of these, the statistical significance was determined for probesets with a minimum AFC of 1.5.

Statistical validation was determined using the FDRCI statistical method (False Discovery Rate Confidence Interval) (Benjamini et al, 2001; Benjamini, 1995; Reiner et al, 2003; Yoshida et al, 2004). This is a two-step procedure that generated FDRCI-derived P-values for the specified minimum fold change (1.5). For more detailed information about FDRCI, please refer to reference (Yoshida et al, 2004). FDRCI generates a ranking of the list of probesets according to increasing FDRCI significance level having the minimum fold change of 1.5. The higher the ranking, the greater the probability that the AFC determined is real. All probesets with a P-value of >1 were reported.

Therefore, for a given probeset two AFC and FDRCI derived P- values were generated based on two independent algorithms (RMA and GC-RMA). For my own analysis, I included only those probesets that had a statistically relevant AFC of 1.5 by both methods. For simplicity, I reported only the values generated by RMA analysis within the results section of Chapter 3.

Insulin Signalling Assay

Human Primary Preadipocytes

Human preadipocytes were grown to confluence in a 12-well dish. At confluence, cells were treated with dex or vehicle for 48 h in growth media with 3% CS. Cells were then stimulated for 5 min or 1 h with 100nM insulin, 0.5mM MIX and 100nM insulin (MI) or MI and 1μM dex (MID) or vehicle in serum-free media as described under differentiation conditions. Media was aspirated and cells were lysed directly in the well by addition of 100μl cold IPH buffer containing 10% glycerol, 1mM sodium orthovanadate (Na_3VO_4), 5mM sodium pyrophosphatate (NaPPi) and 50mM NaF. Cells were scraped using a rubber

policeman and immediately sonicated for 10s at 30% duty cycle. The cellular extracts were cleared by centrifugation at 13 000 X g for 10 min. From the ~100μl sample, 50μl was removed and immediately denatured by boiling in SDS lysis buffer to prevent phosphatase activity. From the remaining lysate, Bradford assays were performed using standard protocols. 30μg of each sample was resolved by SDS-PAGE on an 8% polyacrylamide gel and Western analysis was performed as described.

For each experiment, this assay was repeated in a minimum of 2 separate donor derived preadipocytes and a pool containing 5 donor preadipocytes.

Human Adipocytes

To assess the effect of glucocorticoids on insulin sensitivity in human adipocytes, the primary preadipocytes were differentiated using an alternative protocol that yields higher differentiation efficiency than observed with the previously described protocol. This was done in collaboration with Dr. Alexander Sorisky who has developed this protocol (Artemenko et al, 2005). Subcutaneous primary preadipocytes were plated at a density of 3 x 10^4 cells/cm^2 in DMEM supplemented with 10% FBS (Invitrogen, Burlington, ON) and antibiotics. The following day (confluence), the cells were induced to differentiate in DMEM supplemented with 10% FBS, antibiotics, 0.85μM insulin, 100μM indomethacin, 0.5μM dex and 0.25mM MIX. Thereafter, the media was not replaced until day 12, at which point differentiated adipocytes were placed in DMEM supplemented with 10% FBS and antibiotics for 2 days, then treated with or without 1μM dex for 48 h. Two individual donor cells and a pool of 5 donor preadipocytes were differentiated respectively. Following treatment with dex, the insulin signalling assay was performed exactly as described above. RNA samples were also harvested for RT-PCR analysis.

CHAPTER I: THE IDENTIFICATION AND CHARACTERIZATION OF TRANSCRIPTIONAL INTERMEDIARY FACTOR 1β AS AN ADIPOGENIC FACTOR

CONTRIBUTION OF COLLABORATORS

Dongmei Wu performed the chromatin immunoprecipitation assays presented in Figure 8A.

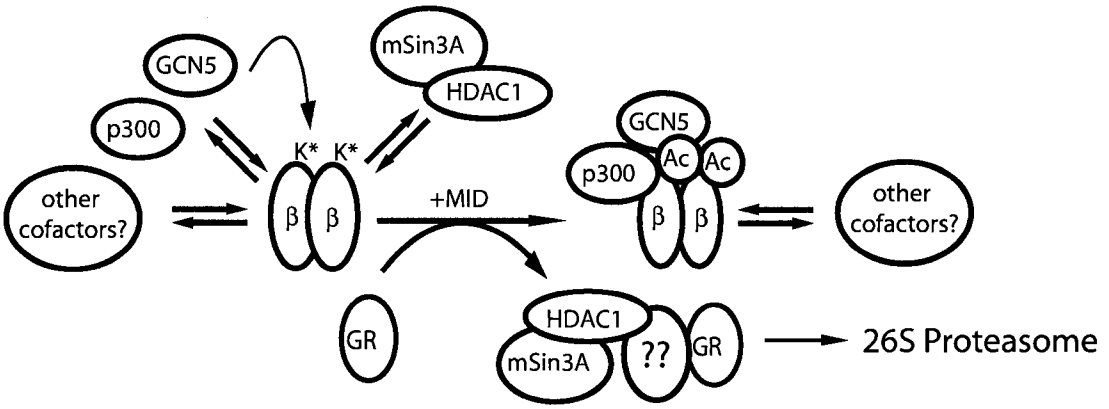
INTRODUCTION

GR potentiates the transcriptional regulatory activity of C/EBPβ by a recently identified mechanism that is dependent solely on the LBD of the receptor and that occurs in the absence of a detectable physical association between GR and C/EBPβ (Boruk et al, 1998; Wiper-Bergeron et al, 2003). Studies in our laboratory have shown that this is mediated by: (1) targeting of a C/EBPβ–associated transcriptional repressor, histone deacetylase 1 (HDAC1), for degradation by the 26S proteasome and (2) stimulating GCN5-mediated acetylation of C/EBPβ within a lysine cluster from K98-K102 that is required for its maximal transcriptional activity (as summarized in Fig.1). Furthermore, these effects appear to be integral to the ability of glucocorticoids to potentiate 3T3 L1 preadipocyte differentiation (Wiper-Bergeron et al, 2007; Wiper-Bergeron et al, 2003).

Specifically, glucocorticoids target a subcellular pool of HDAC1 that is present in a variant of the mSin3A complex and represents approximately 36% of HDAC1 in preadipocytes. This population specifically co-fractionates with C/EBPβ in the absence of

Figure 1: Glucocorticoids enhance the transcriptional potential of C/EBPβ by promoting its acetylation within a lysine cluster at K98-K102.

C/EBPβ is found in a dynamic complex with histone acetyltransferases and histone deacetylases. Steroid treatment induces the degradation of C/EBPβ-associated HDAC1 by the 26S proteasome, allowing GCN5 mediated acetylation of C/EBPβ within a lysine cluster from K98-K102. The relevant E3 ligase that functions with the glucocorticoid receptor to target HDAC1 for degradation remains to be identified.


GCN5
p300
other cofactors?
mSin3A
HDAC1
K* K*
β β
+MID
GR
GCN5
p300
Ac
Ac
β β
other cofactors?
HDAC1
mSin3A
??
GR
26S Proteasome

steroid (Wiper-Bergeron et al, 2003). In the present study, I sought to identify E3 ligases that might act in concert with GR to stimulate the turnover of HDAC1 that is linked to the coactivation of C/EBPα transcription by C/EBPβ. I therefore surveyed three candidate E3 ligases that had been functionally linked to GR and/or C/EBPβ including Murine double minute 2 (Mdm2), E6-Associated Protein (E6-AP) and TIF1β (KAP-1, KRIP-1, TRIM28) for their ability to modulate the HDAC1 that interacts with C/EBPβ to repress the activation of C/EBPα transcription (Chang et al, 1998; Honda et al, 1997; Huibregtse et al, 1993a; Kinyamu & Archer, 2003; Nakamura et al, 2000; Pan & Chen, 2003; Sengupta & Wasylyk, 2001).

E6-AP is a HECT domain containing E3 ligase and a coactivator of PR, GR, AR and ER mediated transcription (Huibregtse et al, 1995; Nawaz et al, 1999b). It was originally identified as the factor that mediated the interaction of human papillomavirus-type and 18 E6 proteins with the tumour suppressor p53 and it could promote the ubiquitylation and proteasome dependent degradation of p53 (Hatakeyama et al, 1997; Huibregtse et al, 1993b; Scheffner et al, 1993). The carboxy 350 amino acids constitute the HECT domain for which the HECT class of E3 ligases was defined (homologous to the E6-AP carboxy terminus).

Mdm2, a RING domain containing E3 ligase has been shown to regulate GR stability. In response to stress, the human homologue of Mdm2, Hdm2, forms a trimeric complex with liganded GR and p53; this promotes their translocation to the cytoplasm and Hdm2-dependent ubiquitylation and turnover of GR and p53 (Sengupta & Wasylyk, 2001). Similarly, other studies have shown that activation of ER promotes the expression of Mdm2 in MCF-7 cells, which in turn promotes the degradation of GR (Kinyamu & Archer, 2003).

Lastly, TIF1β was identified as a potential E3 ligase due to the presence of an amino terminal RING finger within the RBCC domain. Nrdp1, Efp, ARD1 and RFP2 are all RBCC domain containing proteins that have been shown to possess E3 ligase activity (Diamonti et al, 2002; Sanchez et al, 1998; Urano et al, 2002; Vichi et al, 2005). As was discussed in more detail in the general introduction (page 22), TIF1β was shown to coactivate both GR and C/EBPβ-mediated transcription of the α1-glycoprotein promoter, which was dependent on functional GR and C/EBP response elements within the promoter, as well as to potentiate GR-mediated transcription of the MMTV promoter (Chang et al, 1998).

I hypothesized that an HDAC1-specific E3 ligase would transactivate C/EBPβ-mediated transcription and that it would potentiate 3T3 L1 preadipocyte differentiation. My results provide evidence that TIF1β potentiates C/EBPβ-mediated transcription by targeting HDAC1 for degradation by the 26S proteasome. Furthermore, TIF1β enhances 3T3 L1 preadipocyte differentiation, and acts in part to regulate C/EBPα expression.

RESULTS

TIF1β enhances C/EBPβ-mediated transcription

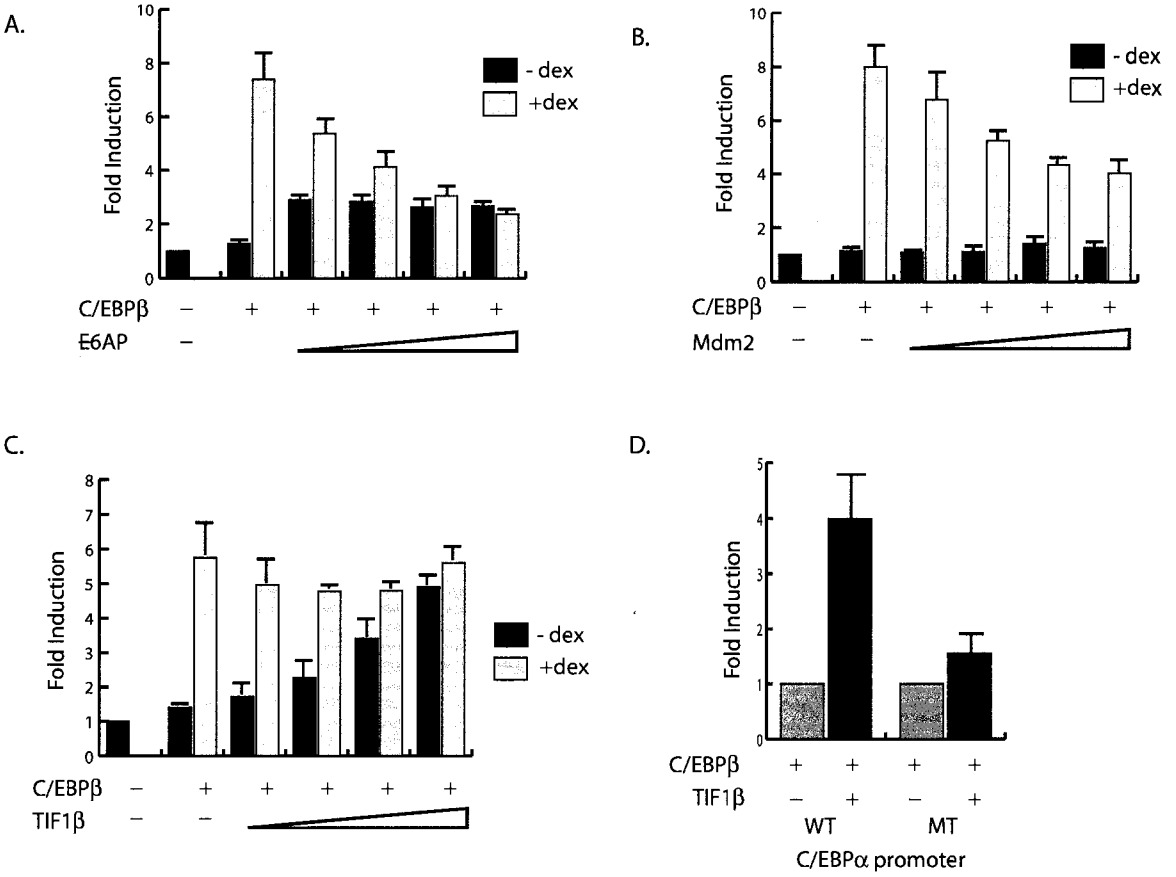
The ligand binding domain of GR is sufficient to potentiate C/EBPβ-mediated transcription and to enhance the turnover of a specific C/EBPβ associated pool of HDAC1 by the 26S proteasome. These results suggested a functional link between the GR_{LBD} and an HDAC1-specific E3 ligase that relieved repression of the C/EBPα promoter. In Cos7 kidney fibroblasts, ectopically expressed C/EBPβ resulted in a minimal induction of transcription of a C/EBPα reporter gene, whereas co-expression of the GR_{LBD} enhanced C/EBPα transcription 4- to 7- fold in the presence of the synthetic glucocorticoid dexamethasone (dex) (Fig. 2A).

Co-expression of E6AP, previously reported as a coactivator of GR, resulted in a modest 2-fold enhancement of C/EBPα transcription in the presence of C/EBPβ, but also abrogated the LBD-mediated enhancement of C/EBPα transcription (Fig. 2A). Similarly, the C/EBPα promoter was refractory to Mdm2 in the presence of C/EBPβ and absence of dex and Mdm2 abrogated the effect of dex (Fig. 2B). This could be due to decreased GR function in the presence of ectopic Mdm2, as Mdm2 has been shown to promote the degradation of GR in other cell types (Sengupta & Wasylyk, 2001). Together, this suggested little, if any, role of E6AP and Mdm2 in C/EBPα transcription and a negative role on the effects of glucocorticoids in this process.

By contrast, co-expression of C/EBPβ with TIF1β strongly enhanced C/EBPβ-dependent transcription of C/EBPα up to 5-fold to the level of induction previously achieved with GR (Fig. 2C). Moreover, in the presence of ectopic TIF1β, GR_{LBD} was ineffective in

Figure 2: TIF1β potentiates C/EBPβ-mediated transcription of C/EBPα.

(A) Effect of E6AP, **(B)** Mdm2, and **(C)** TIF1β on fold-induction of C/EBPβ and GR_{505C}-facilitated transcription from the C/EBPα promoter (-355/+7) in Cos7 cells. Cells were transfected with increasing amounts of E6AP, Mdm2 or TIF1β (0.1–1.0μg) as described in the Materials and Methods and were treated with vehicle (black bars) or 1μM dex (grey bars) for 20 h. Relative luciferase units were corrected for total protein levels. Data represents average fold induction relative to basal transcription ± SEM, where n=3. **(D)** The ability of TIF1β to potentiate transcription from the C/EBPα promoter is dependent on the C/EBPβ binding site in the C/EBPα promoter. Relative luciferase activity was measured in Cos7 cells transfected with expression plasmids for C/EBPβ, TIF1β (0.5μg), and either a wild-type (wt) C/EBPα promoter or a promoter with a mutant C/EBP response element (mt) as indicated. Data represents average fold induction relative to C/EBPβ alone ± SEM, n=3.


A.
Fold Induction
- dex
+dex
C/EBPβ
E6AP
B.
Fold Induction
- dex
+dex
C/EBPβ
Mdm2
C.
Fold Induction
- dex
+dex
C/EBPβ
TIF1β
D.
Fold Induction
C/EBPβ
TIF1β
WT
MT
C/EBPα promoter

eliciting a further enhancement of C/EBPα transcription. This suggested that either TIF1β also directly reduced the potency of GR or that TIF1β enhanced C/EBPα transcription by acting in the same or a closely related pathway to GR. The inability of TIF1β to stimulate transcription of a C/EBPα reporter that contains a deletion of the C/EBP response element linked the effect of TIF1β to C/EBPβ-dependent transcription (Fig. 2D). Taken together, this identifies TIF1β as a potential co-regulator of C/EBPβ-mediated transcription on the C/EBPα promoter.

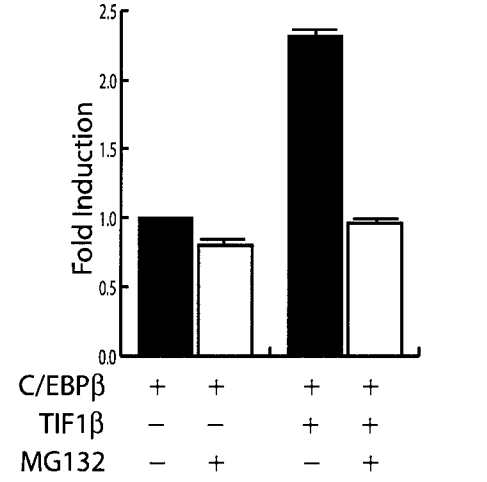
TIF1β promotes 26S proteasome dependent degradation of HDAC1

One of the features of the potentiation of C/EBPβ-mediated induction of C/EBPα transcription by glucocorticoids was that treatment with the proteasome inhibitor MG132 blocked glucocorticoid-mediated enhancement of transcription and prevented steroid-dependent down-regulation of HDAC1 (Wiper-Bergeron et al, 2003). Similarly, the increase of C/EBPα transcription by TIF1β was lost in the presence of MG132 (Fig. 3A), suggesting that the ability of TIF1β to potentiate C/EBPβ-mediated transcription is dependent on a functional 26S proteasome. Furthermore, Western analysis reveals that TIF1β (Fig. 3B), but not Mdm2 (Fig. 3C) expression, significantly reduced endogenous HDAC1 levels (0.65 ± 0.11-fold) and that this effect was prevented by treatment with MG132. Moreover, similar to the effect of dex, ectopic expression of TIF1β prevented the association of HDAC1 with C/EBPβ as assessed by a co-immunoprecipitation assay (Fig. 3D).

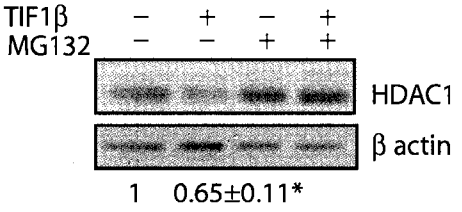
Figure 3: TIF1β promotes 26S proteasome-dependent degradation of HDAC1.

(A) MG132 treatment blocks the ability of TIF1β to induce transcription of the C/EBPα promoter. Cos7 cells were transfected as indicated and treated with either vehicle (black bars) or 1μM MG132 for 20 h. Relative luciferase units were corrected by total protein levels. Data represents average fold-induction ± SEM, where n=3. **(B)** HDAC1 protein levels are decreased in the presence of ectopic TIF1β (1.0μg) but not **(C)** Mdm2 (0.5-1.0μg) as assessed by Western analysis. Where indicated, cells were treated for 16 h with 1μM MG132. Western images represent results obtained from 3 independent experiments. Relative HDAC1 expression was quantified by densitometry using ImageQuant® software and is indicated ± SD ($*p \leq 0.05$). **(D)** C/EBPβ was immunoprecipitated from Cos7 whole cell lysates in the presence or absence of exogenous TIF1β. For co-immunoprecipitation experiments, Cos7 cells were transfected with 0.5μg pMSV-C/EBPβ and 2.0μg pRSV-TIF1β. Result is representative of a three independent experiments.

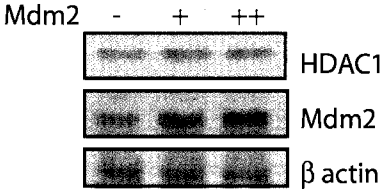
A.


2.5
2.0
1.5
1.0
0.5
0.0
Fold Induction
C/EBPβ + + + +
TIF1β − − + +
MG132 − + − +

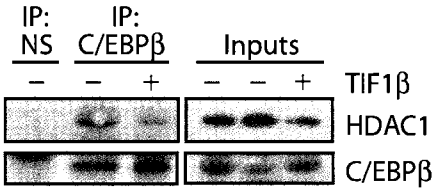
B.


TIF1β − + − +
MG132 − − + +
HDAC1
β actin
1 0.65±0.11*

C.


Mdm2 - + ++
HDAC1
Mdm2
β actin

D.


IP: NS
IP: C/EBPβ
Inputs
− − + − − +
TIF1β
HDAC1
C/EBPβ

TIF1β interacts with HDAC1 and GR, but not with C/EBPβ

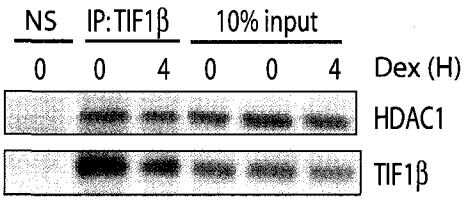
GR and TIF1β were phenotypically similar with respect to their effects on HDAC1 stability and modulation of C/EBPβ mediated transcription suggesting a convergence of action of these two factors. To further explore whether GR and TIF1β acted in concert on HDAC1 and C/EBPβ, I explored the potential for a physical interaction between these factors. TIF1β co-immunoprecipitated with endogenous HDAC1 independently of the presence of dex, thus establishing a direct connection between TIF1β and HDAC1 (Fig. 4A). Conversely, while previous reports have suggested that TIF1β physically interacts with C/EBPβ through the RBCC domain of TIF1β (Chang et al, 1998), under the conditions of this study, TIF1β co-immunoprecipitation with C/EBPβ occurred solely under conditions where TIF1β was significantly over-expressed (Fig. 4B). Because the required level of expression was greater than those used in both the transient transfection reporter assays and those employed to see effects on HDAC1 stability (Fig. 2C, 3B), a stable association between TIF1β and C/EBPβ did not appear to be required for TIF1β to potentiate C/EBPβ mediated transcription and to target HDAC1. This was reminiscent of the similar observation that the effect of GR on C/EBPβ is exerted in the absence of a physical interaction between these two proteins (Boruk et al, 1998; Wiper-Bergeron et al, 2003).

GR co-immunoprecipitated with TIF1β and this interaction was dependent on dex (Fig. 4C). Similarly, TIF1β and GR were detected in HDAC1 immunoprecipitates following 4 h dex treatment (Fig. 4D), which suggests that these proteins can exist in a multimeric complex. While consistent with a direct regulatory relationship between GR and TIF1β in targeting HDAC1, these results remain preliminary and do not exclude the possibility that

Figure 4: TIF1β associates with both HDAC1 and GR.

(A) Endogenous HDAC1 co-immunoprecipitates with ectopic TIF1β (1.0μg pRSV-TIF1β) in the presence or absence of dex. **(B)** TIF1β only immunoprecipitates with C/EBPβ under conditions where it is significantly over-expressed. **(C)** Ectopic GR (1.0μg) co-immunoprecipitates with Flag-TIF1β (1.0μg). Cells were treated with 1μM dex for 4 and 24 h where indicated. **(D)** Ectopic TIF1β and GR co-immunoprecipitate with endogenous HDAC1 following 4 h dex treatment. Data represents results obtained from 3 independent experiments.

A.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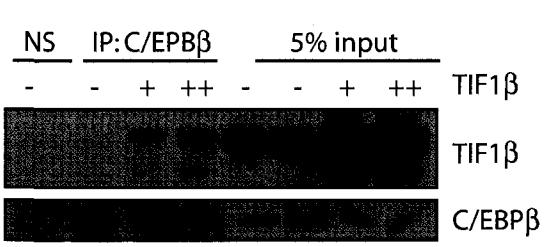
Dex (H)
HDAC1
TIF1β

B.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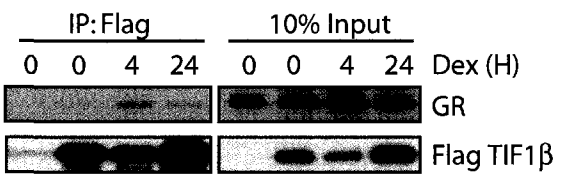
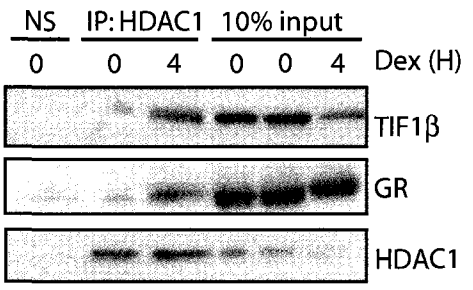
IP:C/EPBβ
5% input
- - + ++ - - + ++
TIF1β
TIF1β
C/EBPβ

C.


IP:Flag
10% Input
0 0 4 24 0 0 4 24
Dex (H)
GR
Flag TIF1β

D.


NS
IP:HDAC1
10% input
0 0 4 0 0 4
Dex (H)
TIF1β
GR
HDAC1

these two factors work independently through parallel pathways. Further studies are required to address this question. One key experiment would be to determine if knock-down of TIF1β expression blocks the ability of steroid treatment to abrogate the interaction between C/EBPβ and HDAC1 and to promote the turnover of HDAC1.

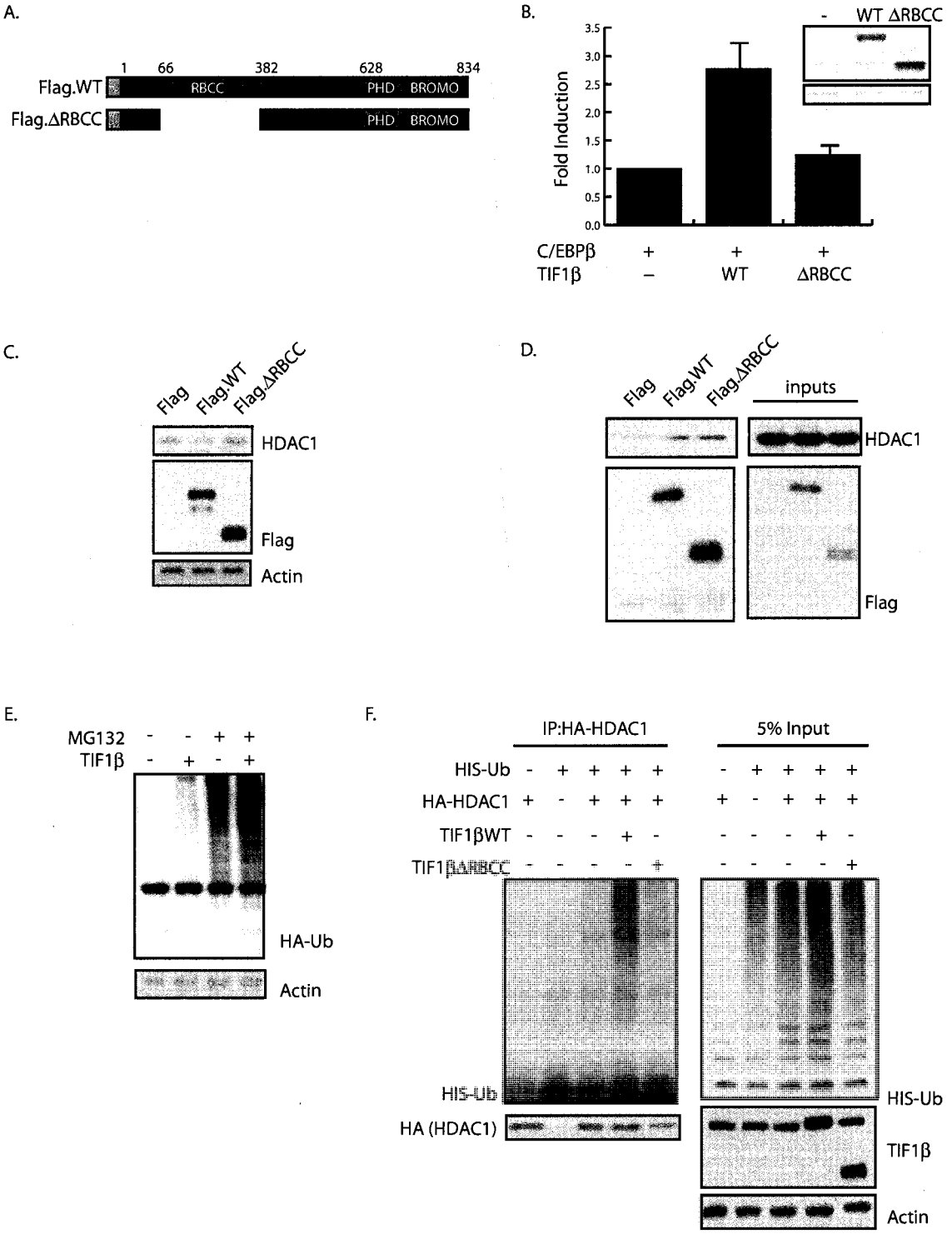
TIF1β promotes polyubiquitylation of HDAC1 in an RBCC domain-dependent manner

Proteasome mediated protein degradation is largely dependent on the poly-ubiquitylation of substrate proteins by E3 ligases. One prominent class of E3 ligases are RING finger domain containing proteins. The RING domain in TIF1β suggested it as a candidate E3 ligase for HDAC1 turnover. Deletion of the RING-containing RBCC domain from TIF1β (Δ80-383) (Fig. 5A) abrogated its ability to potentiate C/EBPβ-induced C/EBPα transcription (Fig. 5B). This loss in activation potential correlated with the inability of TIF1βΔRBCC expression to reduce HDAC1 levels (Fig. 5C). Interestingly, however, TIF1βΔRBCC retained its ability to interact with HDAC1 (Fig. 5D).

I next assessed whether TIF1β could function as an E3 ligase to promote ubiquitylation of substrate proteins. Ectopic expression of TIF1β in Cos7 cells increased total cellular incorporation of co-expressed HA-ubiquitin (Ub) both in the presence and absence of MG132 (Fig. 5E). To test if TIF1β could specifically promote ubiquitylation of HDAC1 in Cos7 cells, I co-expressed HIS-ubiquitin conjugates with ectopic HA-HDAC1 and TIF1β. Immunoprecipitation of HA-HDAC1 from lysates that were treated for 20 h with MG132 reveal increased ubiquitylation of HDAC1 in cells that ectopically express WT, but

Figure 5: The RBCC domain of TIF1β is required for both its transcriptional regulatory potential and the ubiquitylation of HDAC1.

(**A**) Schema of Flag-tagged WT TIF1β (Flag.WT) and RBCC domain deletion mutant (Flag.ΔRBCC). (**B**) The ability of TIF1β to up-regulate C/EBPβ mediated transcription from the C/EBPα promoter requires the RBCC domain. Data represent average fold-induction of luciferase activity relative to Flag alone ± SEM where n=3. Expression levels of the TIF1β constructs were verified by Western analysis of whole cell lysates using an antibody against the Flag tag (inset). (**C**) Western analysis of HDAC1 protein levels in cells transfected with vector alone (Flag), or TIF1βΔRBCC (0.5μg). (**D**) HDAC1 associates with the ΔRBCC mutant. Cos7 cells were transfected with 1.0μg of the indicated Flag.TIF1β plasmids. TIF1β was immuno-precipitated from Cos7 whole cell extracts using an antibody against the Flag tag. (**E**) Western analysis of total ubiquitylation in whole cell lysates that were co-transfected with expression plasmids for HA-Ubiquitin (HA-Ub) (0.5μg) and empty vector or TIF1β (1.0μg). Cells were treated with 1μM MG132 for 16 h where indicated. (**F**) Immunoprecipitated HDAC1 is ubiquitylated when purified from cells that express WT but not the ΔRBCC mutant. Cos7 cells were co-transfected with expression plasmids for His_6-Ub, HA-HDAC1 and Flag-TIF1β_{WT} or Flag-TIF1$\beta_{\Delta RBCC}$ as indicated. HDAC1 was immunoprecipitated from whole cell lysates following treatment with 1μM MG132 for 16 h. HDAC1 ubiquitylation was detected by immunoblotting immunoprecipitates with antibody against the HIS tag (left panel). Western analysis of input whole cells lysates (right panel) reveal increased total ubiquitylation in cells that express WT TIF1β but not ΔRBCC mutant. All panels represent data that is representative of results obtained from a minimum of 3 independent experiments.


A.
1
66
382
628
834
Flag.WT
RBCC
PHD
BROMO
Flag.ΔRBCC
PHD
BROMO
B.
-
WT
ΔRBCC
Fold Induction
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C/EBPβ
+
+
+
TIF1β
–
WT
ΔRBCC
C.
Flag
Flag.WT
Flag.ΔRBCC
HDAC1
Flag
Actin
D.
Flag
Flag.WT
Flag.ΔRBCC
inputs
HDAC1
Flag
E.
MG132
TIF1β
HA-Ub
Actin
F.
IP:HA-HDAC1
5% Input
HIS-Ub
HA-HDAC1
TIF1βWT
TIF1βΔRBCC
HIS-Ub
HA (HDAC1)
HIS-Ub
TIF1β
Actin

not ΔRBCC TIF1β (Fig. 5F). Furthermore, the ability of TIF1β to promote total cellular ubiquitylation as observed in Fig. 5E, was lost upon deletion of the RBCC domain (Fig. 5F, inputs). Taken together, these results present TIF1β as an E3 ligase that can act specifically on HDAC1.

TIF1β potentiates differentiation of murine 3T3 L1 preadipocytes

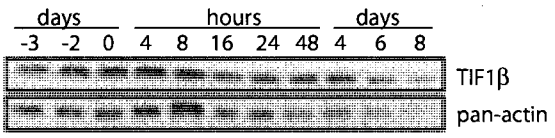
Differentiation of 3T3 L1 preadipocytes is driven by a coordinated and tightly regulated transcription cascade consisting of members of the C/EBP family and PPARγ. C/EBPδ and C/EBPβ are induced early upon stimulation of differentiation and they in turn regulate the expression of C/EBPα and PPARγ. Glucocorticoids promote adipogenesis, in part, by potentiating C/EBPβ mediated transcription of C/EBPα through targeting C/EBPβ-associated HDAC1 for degradation and stimulating GCN5-dependent acetylation of C/EBPβ. My data to date has linked the ability of TIF1β to enhance C/EBPβ mediated transcription of C/EBPα to its effect on HDAC1 turnover in Cos7. Notably, its transactivation potential was abrogated upon deletion of the C/EBPβ response element in this promoter (Fig. 2D). I therefore hypothesized that TIF1β, like GR, may be an adipogenic factor.

Western analysis reveals that TIF1β was endogenously expressed in 3T3 L1 cells and that its expression appeared to be stable throughout differentiation, where Day -2 is the day at which preadipocytes reach confluence and Day 0 is the day at which preadipocytes are stimulated to differentiate (Fig. 6A). Retroviral mediated over-expression of TIF1β enhanced differentiation of 3T3 L1 preadipocytes as assessed by Oil Red O staining of neutral lipids in mature adipocytes (Fig. 6B) and Western analysis of the late adipogenic

Figure 6: TIF1β promotes differentiation of 3T3 L1 preadipocytes.

(A) Western analysis of endogenous TIF1β expression throughout the course of differentiation of 3T3 L1 cells where day -2 represents cells reaching confluence. Differentiation was induced at day 0 with complete differentiation cocktail that included MIX, insulin and dex (MID). Preadipocytes were differentiated in the presence of serum. **(B)** Oil Red O staining of neutral lipids in day 8 adipocytes in control (pLXSN) and TIF1β-infected (pLXSN-TIF1βWT) 3T3 L1 preadipocytes that were either not stimulated (-MI) or induced with MI or MID and differentiated for 8 days as described in the Materials and Methods. The expression level of TIF1β is indicated in Figure 8B. **(C)** Western analysis of the late adipogenic marker adipsin expression in Day 8 adipocytes. **(D)** NIH 3T3 cells that stably express ectopic C/EBPβ were transfected with siRNA oligos targeted against either GFP (siCtrl) or TIF1β (siTIF1β). TIF1β expression compared to control siGFP cells during the initial 48 h of stimulation with MID was confirmed by Western analysis where HIAP2 serves as a loading control (upper panels). Western analysis of adipsin expression in day 8 adipocytes that were either not stimulated (-MI) or induced with a MI or MID containing differentiation cocktail (lower panels). Cells were differentiated in the presence of serum.

A.


days
hours
days
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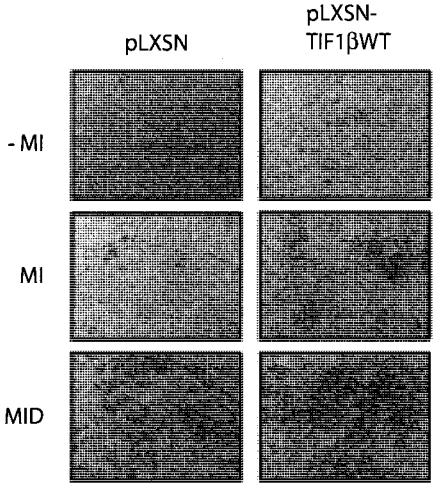
TIF1β
pan-actin

B.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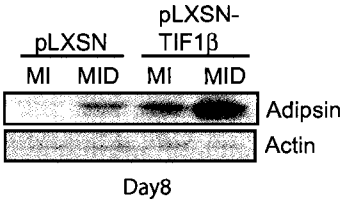
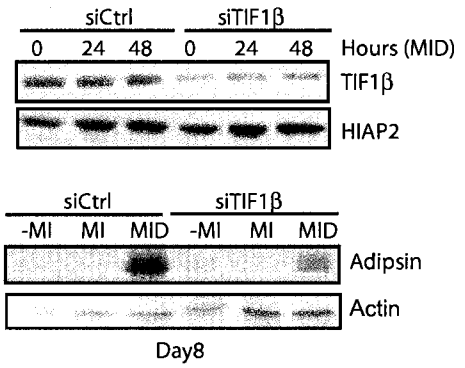
pLXSN-
TIF1βWT
- MI
MI
MID

C.


pLXSN
pLXSN-
TIF1β
MI MID MI MID
Adipsin
Actin
Day8

D.


siCtrl
siTIF1β
0 24 48 0 24 48
Hours (MID)
TIF1β
HIAP2
siCtrl
siTIF1β
-MI MI MID -MI MI MID
Adipsin
Actin
Day8

marker adipsin (Fig. 6C). Importantly, TIF1β increased differentiation when cells were stimulated in the absence of dex (MI) and appeared to be additive (Fig. 6B) or synergistic (Fig. 6C) with dex (MID). This suggested that TIF1β may partially replace the requirement for dex by acting in a parallel or the same pathway as GR but that it also may function independently of GR to promote differentiation.

NIH 3T3 cells are uncommitted fibroblasts derived from the same lineage as the 3T3 L1 preadipocytes and can be differentiated into adipocytes under the same conditions as 3T3 L1 cells given ectopic expression of C/EBPβ (Yeh et al, 1995). To establish the requirement for TIF1β in preadipocyte differentiation, we assessed the differentiation potential of NIH 3T3 cells that stably express C/EPPβ through retroviral infection, in which TIF1β expression was transiently knocked down using siRNA oligos (Fig. 6D, upper panel). Importantly, transfection of these cells with the siRNA oligos resulted in some cell death and dramatically reduced the overall level of differentiation independently of the siRNA target. Knockdown of TIF1β decreased MID stimulated adipogenesis as assessed by Western analysis of adipsin expression in mature adipocytes (Fig. 6D, lower panel). Taken together, these results establish TIF1β as an adipogenic factor.

TIF1β transactivated C/EBPβ dependent transcription of C/EBPα in Cos7 cells by a mechanism that was not synergistic or additive to the effects of GR on this promoter. By contrast, ectopic expression of TIF1β in 3T3 L1 preadipocytes acted synergistically with GR to promote differentiation (Fig. 6C). This indicated that the actions of TIF1β in preadipocytes may be more complex than what had been observed in Cos7 cells. Indeed, there were significant differences in the regulation of the C/EBPα promoter by TIF1β in the NIH 3T3 fibroblasts compared to what was observed in Cos7 cells. TIF1β expression

enhanced transcription of the C/EBPα promoter (Fig. 7A). This effect was strong (16-fold induction) and it occurred independently of dex treatment; however, unlike Cos7 cells, TIF1β acted additively with dex to induce transcription. Furthermore, the ability of TIF1β to stimulate transcription occurred independently of a functional C/EBP response element in the C/EBPα promoter (Fig. 7B). Similar to Cos7 cells, the activation potential of TIF1β was dependent on the RBCC domain (Fig. 7C). Preliminary results suggest that ectopic expression of TIF1β can promote polyubiquitylation of HDAC1 in NIH 3T3 cells, and this requires the RBCC domain (data not shown). Taken together, these results suggest that the mechanism of TIF1β-dependent coactivation of the C/EBPα promoter is sensitive to cell type and may be affected either directly or indirectly, by (an) auxiliary cell-type specific factor(s). The C/EBPβ-independent regulation of this promoter may be independent of its ability to promote ubiquitylation of HDAC1.

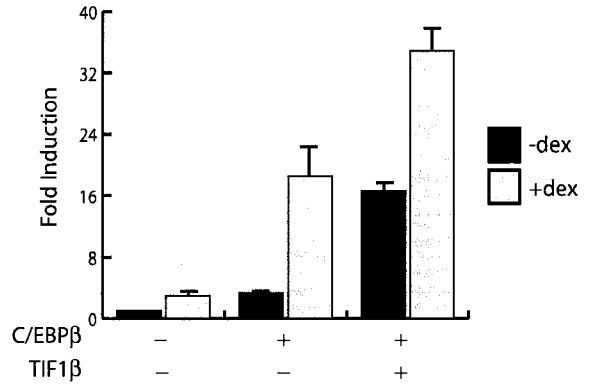
TIF1β acts during the early phase of adipogenesis to induce expression of C/EBPα

TIF1β expression promotes adipogenesis and it acts as a transactivator of C/EBPα transcription in reporter gene assays. My results in Cos7 cells suggest that the former is mediated in part through targeted degradation of HDAC1, a process that Wiper-Bergeron and colleagues demonstrated was required for induction of C/EBPα expression and differentiation of 3T3 L1 preadipocytes (Wiper-Bergeron et al, 2003). Using a chromatin immunoprecipitation (ChIP) assay, in which proteins (transcription factors, modified histones) are detected on a gene promoter *in vivo*, they demonstrated that inclusion of glucocorticoids in the differentiation cocktail of 3T3 L1 preadipocytes abrogated the recruitment of HDAC1 to the endogenous C/EBPα promoter. This correlated with a dex-

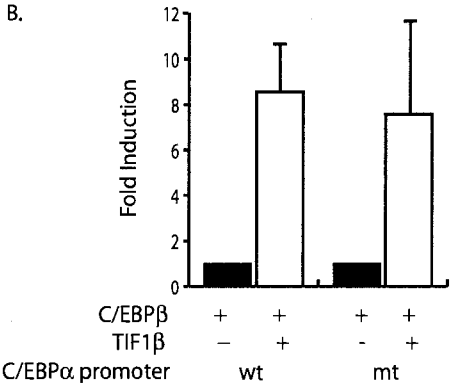
Figure 7: TIF1β transcriptional activity is sensitive to cell-type specific factors.

(A) TIF1β and glucocorticoids act synergistically to induce transcription from the C/EBPα promoter in NIH 3T3 cells. Transcription assay was performed as described in Fig. 2. **(B)** TIF1β-dependent induction of C/EBPα in NIH 3T3 cells occurs independently of C/EBPβ. Transcription assays using the wildtype (wt) or mutant (mt) C/EBPα promoter were performed as described in Fig 2D. **(C)** The TIF1βΔRBCC mutant does not potentiate C/EBPβ mediated transcription from the C/EBPα promoter in NIH 3T3 cells. Data represents average fold induction ± SEM, where n=3.

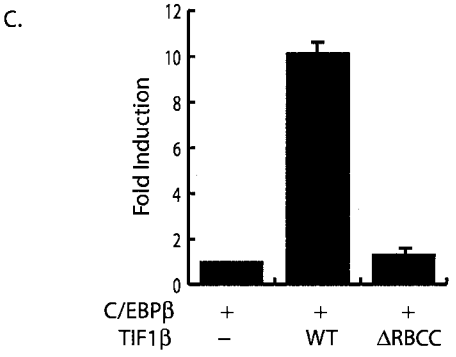
A.


40
32
24
16
8
0
Fold Induction
-dex
+dex
C/EBPβ – + +
TIF1β – – +

B.


12
10
8
6
4
2
0
Fold Induction
C/EBPβ + + + +
TIF1β – + - +
C/EBPα promoter wt mt

C.


12
10
8
6
4
2
0
Fold Induction
C/EBPβ + + +
TIF1β – WT ΔRBCC

dependent increase in histone H4 acetylation and recruitment of RNA polymerase II to the C/EBPα promoter (Wiper-Bergeron et al, 2003).

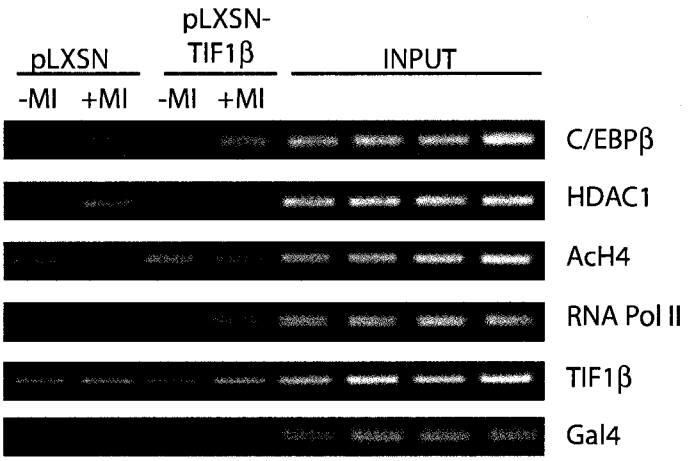
Ectopic TIF1β expression in 3T3 L1 cells resulted in increased differentiation when glucocorticoids were absent from the inductive cocktail. I therefore sought to determine if ectopic TIF1β affected recruitment of HDAC1 to the endogenous C/EBPα promoter in preadipocytes stimulated for 24 h with MIX and insulin (MI). ChIP analysis revealed MI dependent recruitment of C/EBPβ to the C/EBPα promoter in both control (pLXSN) cells and cells that have been retrovirally transduced with TIF1β (pLXSN-TIF1β) (Fig. 8A). However, while HDAC1 was recruited to the promoter following stimulation with MI in control pLXSN-infected cells, it was not detected on the promoter when TIF1β was overexpressed. The pattern of HDAC1 recruitment inversely correlated with levels of histone H4 acetylation and recruitment of RNA pol II. Taken together with the previous report that inclusion of dex in the inductive cocktail also abrogated HDAC1 recruitment to the promoter, these results suggest that TIF1β and GR function appear to converge in 3T3 L1 cells to abrogate HDAC1 recruitment to- and activation of the C/EBPα promoter as defined by increased histone H4 acetylation and recruitment of RNA pol II.

Somewhat unexpectedly, ChIP analysis revealed that TIF1β was constitutively associated with the C/EBPα promoter (Fig. 8A). The presence of TIF1β on the C/EBPα promoter in the unstimulated state (-MI) was not anticipated for its role as an activator of this promoter. However, this finding is consistent with its well-characterized role as a repressor protein and suggests that TIF1β could be involved in repression of the C/EBPα promoter in the unstimulated preadipocyte. Furthermore, in the TIF1β-infected cells, TIF1β occupancy of the C/EBPα promoter did not discernibly increase. Under these conditions, and in

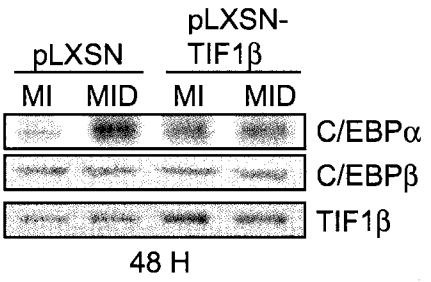
Figure 8: TIF1β regulates C/EBPα expression during early adipogenesis.

(A) Chromatin immunoprecipitation analysis of C/EBPβ, HDAC1, acetylated histone H4 (AcH4), RNA polymerase II (RNA Pol II) and TIF1β occupancy at the C/EBPα promoter in control (pLXSN) and TIF1β-infected (pLXSN-TIF1β) 3T3 L1 cells treated for 24 h in the absence (-MI) or presence of MIX and insulin (+MI) under serum-free conditions. Input represents between 1-3%. **(B)** Western analysis of C/EBPα, C/EPBβ and TIF1β expression in preadipocytes that have been stimulated with MIX and insulin (MI) or MIX, insulin and dex (MID) for 48 h in control (pLXSN) or TIF1β-infected (pLXSN-TIF1β) 3T3 L1 cells. **(C)** Western analysis of C/EBPα and C/EBPβ expression in control and siRNA-mediated TIF1β-knockdown NIH 3T3/C/EBPβ^{+} fibroblasts (as described in Fig6D) 48 h following stimulation with MI or MID. The efficiency of TIF1β knockdown during the initial 48 h is shown in Figure 6D. All data is representative of results obtained from a minimum of three independent experiments.

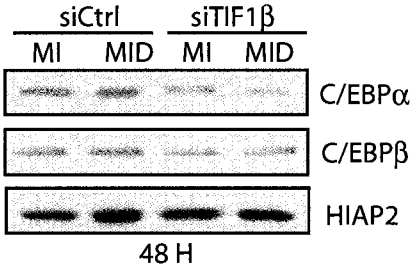
A.


pLXSN
pLXSN-TIF1β
INPUT
-MI
+MI
-MI
+MI
C/EBPβ
HDAC1
AcH4
RNA Pol II
TIF1β
Gal4

B.


pLXSN
pLXSN-TIF1β
MI
MID
MI
MID
C/EBPα
C/EBPβ
TIF1β
48 H

C.


siCtrl
siTIF1β
MI
MID
MI
MID
C/EBPα
C/EBPβ
HIAP2
48 H

response to MI, C/EBPβ was recruited to the promoter in the absence of HDAC1. This is consistent with an effect of TIF1β on the C/EBPβ-HDAC1 interaction that occurs elsewhere in the nucleus, and not on the C/EBPα promoter itself. This is further consistent with the previous findings that GR is not detectable on the C/EBPα promoter (Wiper-Bergeron et al, 2003).

Taken together, the results obtained from the ChIP analysis imply that the regulation of C/EBPα expression by TIF1β in 3T3 L1 preadipocytes is more complex than originally anticipated and that TIF1β has other roles in its regulation that are independent of its effects on HDAC1.

The apparent TIF1β-dependent de-repression of this promoter following stimulation with MI resulted in an increase in C/EBPα expression (Fig. 8B). Conversely, C/EBPα expression was reduced in NIH 3T3/C/EBPβ^{+} fibroblasts in which TIF1β expression had been knocked-down by siRNA (Fig. 8C).

Regulation of 3T3 L1 differentiation by TIF1β is mediated through multiple mechanisms

The modulation of HDAC1 occupancy on the C/EBPα promoter and the up-regulation of C/EBPα expression observed in the preadipocyte model was consistent with my previous findings that TIF1β transactivated C/EBPα transcription in reporter gene assays in both Cos7 and NIH 3T3 cells. However, the constitutive presence of TIF1β at the C/EBPα promoter suggested that the mechanism through which the over-expressed TIF1β acted was likely to be complex. In both Cos7 and NIH 3T3 cells, TIF1β-dependent regulation of C/EBPα transcription was dependent on the RBCC domain. I therefore tested the ability of the

TIF1βΔRBCC mutant to potentiate differentiation of 3T3 L1 preadipocytes. Contrary to what might be expected, the ability of ectopic TIF1β expression to promote differentiation occurred independently of the RBCC domain. Indeed, preadipocytes that expressed the TIF1βΔRBCC mutant differentiated more efficiently than cells expressing WT TIF1β as assessed by Oil Red O staining of day 8 adipocytes (Fig. 9A), and Western analysis of adipsin (Fig. 9B). Additionally, increased ectopic TIF1β-dependent expression of C/EBPα expression occurred independently of the RBCC domain both in the absence (MI) and presence of dex (MID) (Fig. 9C). This suggests that the involvement of TIF1β in 3T3 L1 preadipocyte differentiation is multi-potential and may be mediated through by several molecular mechanisms, at least one of which can compensate for the loss of TIF1β dependent regulation of HDAC1.

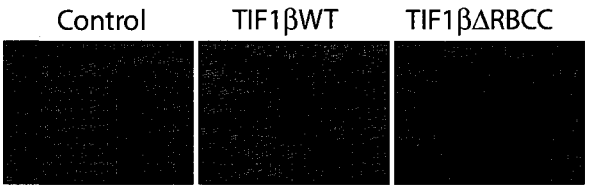
TIF1β and C/EBPβ co-localize to nuclear speckles during early differentiation

It has previously been shown that TIF1β localizes to distinct, heterochromatin-rich, nuclear speckles in mouse embryonic F9 cells and that this localization is required for their differentiation. This association is mediated through an interaction with members of the heterochromatin protein family (HP1α,β,γ) (Cammas et al, 2004; Cammas et al, 2002; Ryan et al, 1999; Sripathy et al, 2006). This phenotype resembles the transient localization of C/EBPβ with heterochromatic foci in 3T3 L1 preadipocytes (Tang & Lane, 1999). I therefore hypothesized that regulation of adipogenesis by TIF1β could involve a dynamic interaction with HP1α at heterochromatin-rich nuclear foci.

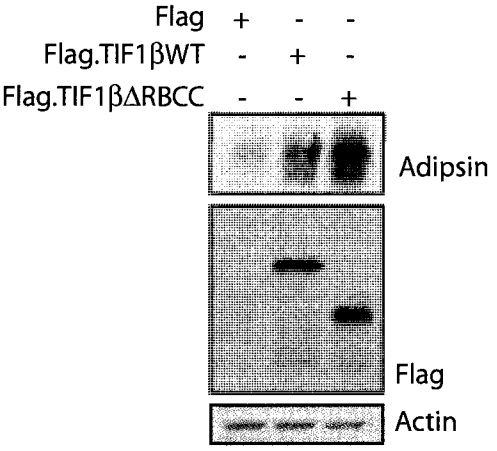
Figure 9: The differentiation potential of TIF1β is regulated by its RBCC domain.

3T3 L1 cells were retrovirally transduced with empty Flag plasmid (Control), Flag-TIF1βWT or Flag-TIF1βΔRBCC expression plasmids. Cells lines were differentiated with MID under serum-free conditions as described in Materials and Methods. **(A)** Oil Red O staining of neutral lipids in mature adipocytes differentiated in response to MID treatment. **(B)** Western analysis of adipsin and Flag expression in day 8 adipocytes. **(C)** Western analysis of C/EBPα and C/EBPβ expression 48 h post-simulation with either MI or MID as indicated. Data represents results obtained from a minimum of three individual repeats.

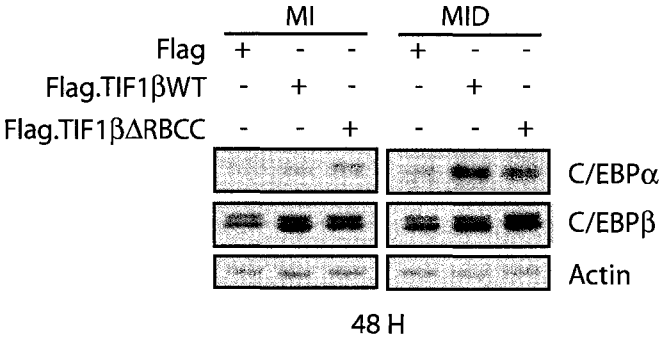
A.


Control
TIF1βWT
TIF1βΔRBCC
Day 8

B.


Flag + - -
Flag.TIF1βWT - + -
Flag.TIF1βΔRBCC - - +
Adipsin
Flag
Actin
Day 8

C.


MI
MID
Flag + - - + - -
Flag.TIF1βWT - + - - + -
Flag.TIF1βΔRBCC - - + - - +
C/EBPα
C/EBPβ
Actin
48 H

Indirect immunofluorescence (IIF) of Flag-tagged WT TIF1β revealed diffuse nuclear staining in non-stimulated, post-confluent 3T3 L1 cells; the Flag epitope allowed me to detect TIF1β as the TIF1β antibody used in Western was insufficient for IIF assays. Upon stimulation with MID, however, TIF1β redistributed to prominent DAPI-rich foci within 4 h and remained associated with these foci through 48 h (Fig. 10A). Similarly, and as previously described, the association of C/EBPβ with these foci is transient during the initial 48 h of differentiation (Fig. 10B) (Tang & Lane, 1999) whereas HP1α localizes constitutively with these punctate foci (Fig. 10C). Co-immunofluorescence studies reveal that TIF1β, HP1α and C/EBPβ spatially co-localize at these heterochromatin-rich foci 24 h post-stimulation with MID (Fig.10D). Interestingly, whereas C/EBPβ remained associated with the DNA as cells progressed through mitosis as has been previously shown (Tang & Lane, 1999), TIF1β is excluded from the DNA during this process (Fig.10E). Thus, while TIF1β and C/EBPβ appear to act in close spatial juxtaposition, these results are consistent with the lack of a direct interaction between these factors as previously evidenced through their inability to co-immunoprecipitate (Fig 4B).

To investigate whether the targeting of TIF1β to these foci was required for differentiation, I generated a series of transduced 3T3 L1 cell lines each of which expressed a TIF1β mutant including: (1) the previously described ΔRBCC mutant, (2) a ΔC-terminus mutant that lacked the PHD and BROMO domains (Δ564C) and (3) a point mutant in which the interaction with HP1 proteins had been abolished (V488L490/AA) (Cammas et al, 2004) (Fig. 11A). I assessed the nuclear localization of these factors 24 h following stimulation with MID (Fig. 11B). As anticipated, the interaction of TIF1β with HP1 proteins was indispensible in its ability to co-localize with the punctuate DAPI staining or C/EBPβ.

Figure 10: TIF1β is recruited to heterochromatic foci with C/EBPβ and HP1α during early differentiation of 3T3 L1 preadipocytes.

(A) Visualization of Flag-TIF1βWT in retrovirally transduced 3T3 L1 preadipocytes at day 0 and 4 h, 24 h and 48 h post induction with MID differentiated under serum-free conditions. TIF1β was detected by indirect immunofluorescence using a primary antibody for the Flag epitope (upper panels) and compared to foci observed with DAPI staining of the same cells (lower panels). Images were acquired using a Nikon Eclipse TE300 microscope. **(B)** Detection of C/EBPβ and **(C)** HP1α localization as described in (A). **(D)** Overlay of TIF1β and C/EBPβ fluorescence signals (upper panels) and HP1α and C/EBPβ fluorescence signals (lower panels) 24 h post-stimulation with MID in Flag-TIF1βWT expressing 3T3 L1 preadipocytes. Images were acquired using a Bio-Rad MRC 1024 confocal microscope. Confocal images were processed and overlaid using ImageJ software. **(E)** C/EBPβ and TIF1β staining in cells undergoing various stages of mitosis. Data represents results obtained in a minimum of three independent experiments.

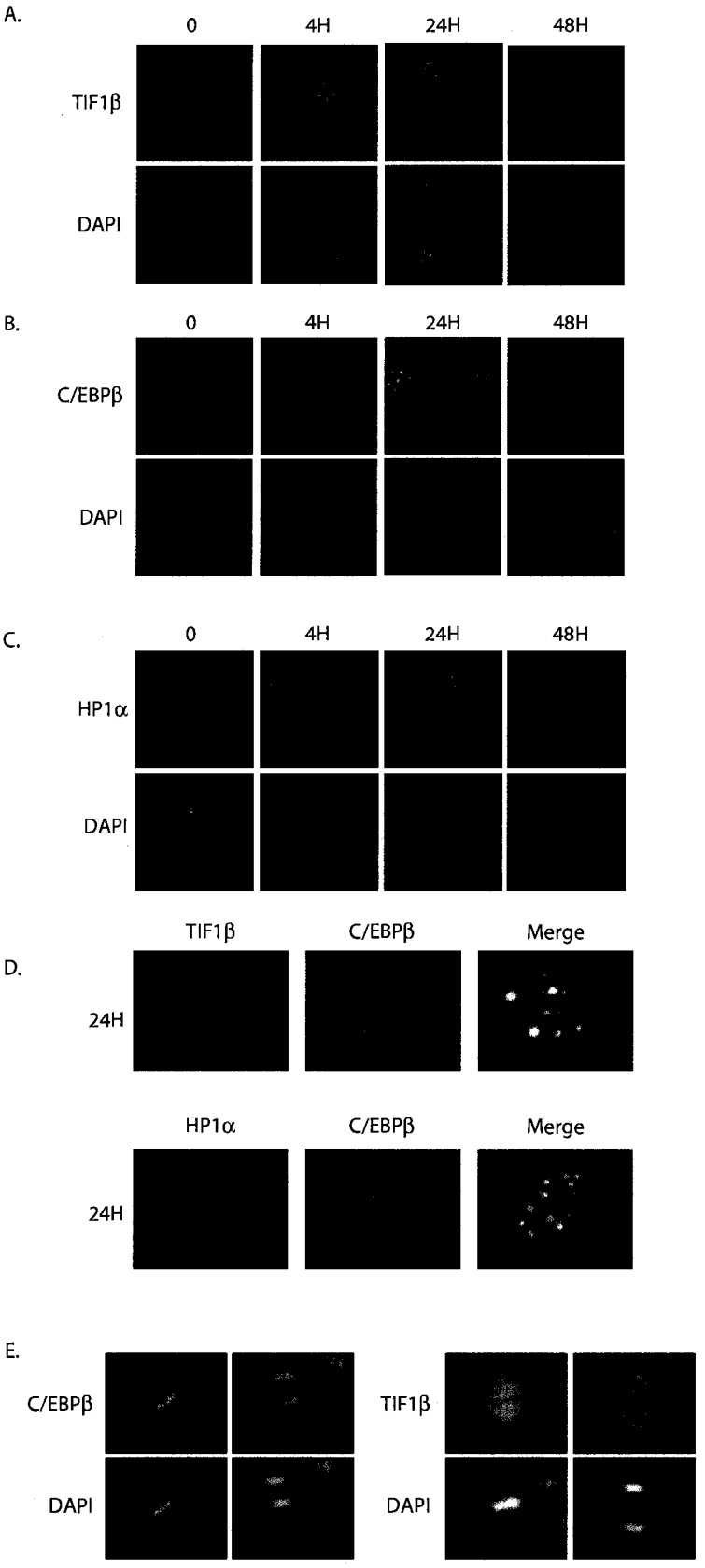
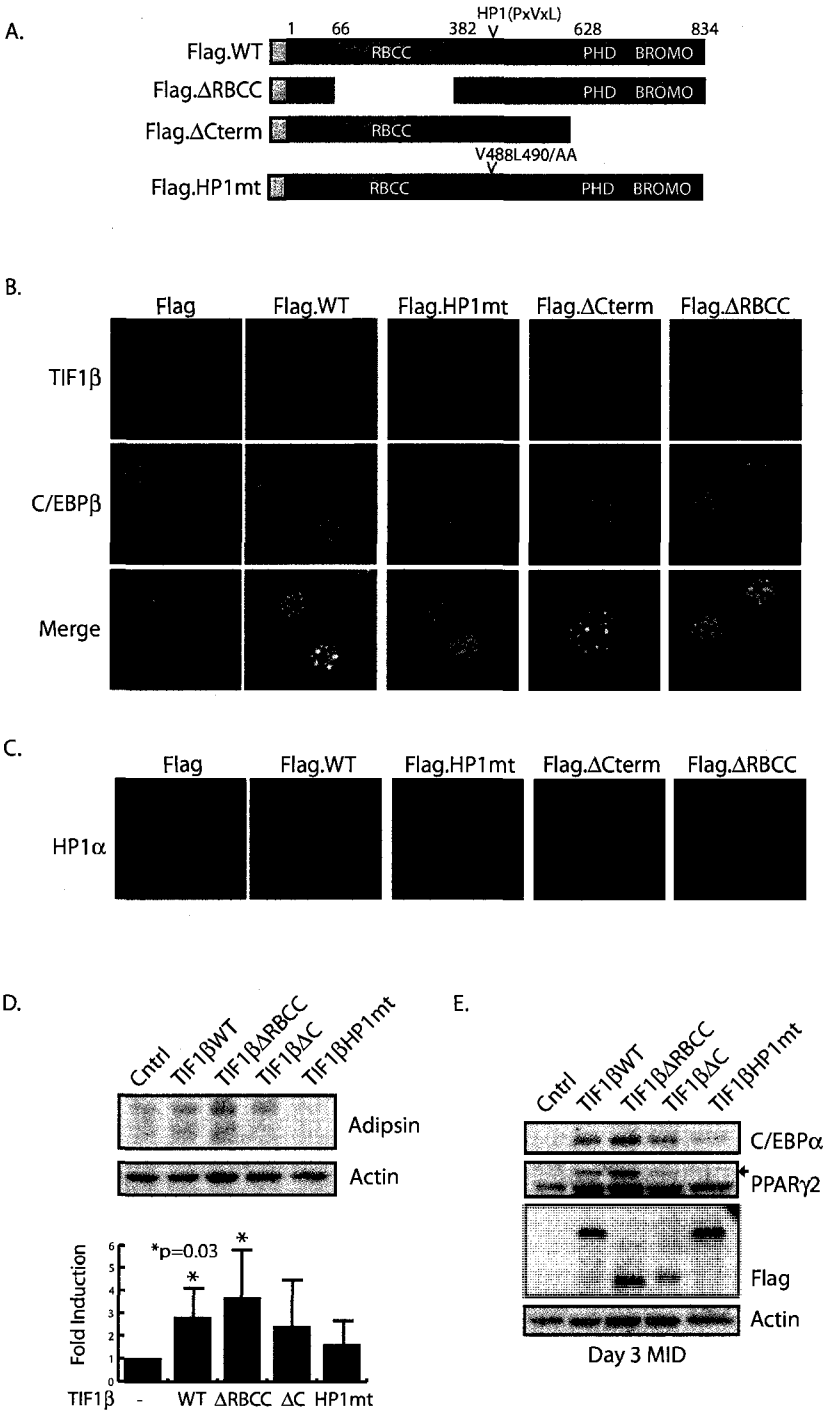

A.
0
4H
24H
48H
TIF1β
DAPI
B.
0
4H
24H
48H
C/EBPβ
DAPI
C.
0
4H
24H
48H
HP1α
DAPI
D.
TIF1β
C/EBPβ
Merge
24H
HP1α
C/EBPβ
Merge
24H
E.
C/EBPβ
DAPI
TIF1β
DAPI

Figure 11: The association of TIF1β with HP1α is required for differentiation of 3T3 L1 preadipocytes.

(A) Schema of the Flag-TIF1β constructs used to generate 3T3 L1 cell lines. **(B)** Indirect-immunofluorescence detection of TIF1β (using Flag antibody) and C/EBPβ 24 h post-induction with MID under serum-free conditions. **(C)** HP1α localization 24 h post-MID stimulation in each of the TIF1β isoform expressing cell lines. **(D)** Western analysis of adipsin expression (upper panels) in mature, day 8 adipocytes stimulated with MID under serum-free differentiation conditions. Expression was quantified using ImageQuant software and plotted as average fold induction ± SD (lower panel). **(E)** Western analysis of C/EBPα and PPARγ expression 3 days post-induction with complete cocktail in serum-free media.


A.
HP1(PxVxL)
1
66
382
628
834
Flag.WT
RBCC
PHD
BROMO
Flag.ΔRBCC
PHD
BROMO
Flag.ΔCterm
RBCC
V488L490/AA
Flag.HP1mt
RBCC
PHD
BROMO
B.
Flag
Flag.WT
Flag.HP1mt
Flag.ΔCterm
Flag.ΔRBCC
TIF1β
C/EBPβ
Merge
C.
Flag
Flag.WT
Flag.HP1mt
Flag.ΔCterm
Flag.ΔRBCC
HP1α
D.
Cntrl
TIF1βWT
TIF1βΔRBCC
TIF1βΔC
TIF1βHP1mt
Adipsin
Actin
Fold Induction
*p=0.03
TIF1β - WT ΔRBCC ΔC HP1mt
E.
Cntrl
TIF1βWT
TIF1βΔRBCC
TIF1βΔC
TIF1βHP1mt
C/EBPα
PPARγ2
Flag
Actin
Day 3 MID

Furthermore, the RBCC domain but not the PHD and BROMO domains were required for TIF1β for its localization to nuclear speckles. The localization of HP1α and C/EBPβ occurred independently of the TIF1β isoform expressed (Fig. 11B, C). This provides further evidence that the interaction between TIF1β and C/EBPβ is not direct and suggests that TIF1β is not required for C/EBPβ speckling; however, it is important to note that these cells express endogenous TIF1β.

The interaction of TIF1β with HP1α appeared to be required for differentiation as the 3T3 L1 cells that expressed the HP1-interaction mutant had decreased differentiation capacity as compared to WT-expressing preadipocytes as measured by decreased adipsin expression (Fig. 11D) and this correlated with decreased induction of both C/EBPα and PPARγ expression at day 3 of differentiation compared to the WT TIF1β (Fig. 11E). However, the adipogenic potential of ectopic TIF1β does not strictly correlate with its localization to nuclear foci. The terminal PHD and BROMO domains, which are not required for foci localization, are required for enhanced TIF1β-dependent differentiation as there is no significant difference between the adipsin levels of these cells as compared to the control cells (Fig. 11D lower panels). Surprisingly, this correlated with an increase in C/EBPα and PPARγ expression (Fig. 11E). By contrast, the RBCC domain, which is required for nuclear speckling is not required for differentiation. As previously demonstrated (Fig. 9), the ΔRBCC mutant expressing preadipocytes differentiated to a greater extent than WT expressing cells as assessed by adipsin expression (Fig. 11D). Moreover, expression of the ΔRBCC mutant resulted in up-regulation of C/EBPα and PPARγ (Fig. 11E).

DISCUSSION

TIF1β potentiates C/EBPβ mediated transcription

I have identified TIF1β as a coregulator of C/EBPβ function. TIF1β promotes HDAC1 poly-ubiquitylation and subsequent targeting to the 26S proteasome thereby modulating the association between C/EBPβ and HDAC1. TIF1β has been largely characterized as a potent transcriptional repressor protein that is recruited to target promoters through the RBCC-dependent association with KRAB domain containing zinc-finger transcription factors. My finding that TIF1β potentiates C/EBPβ mediated transcription contributes to growing evidence that transcriptional regulation by TIF1β is bi-functional (Chang et al, 1998; Venkov et al, 2007) and is the first report of mechanistic insight into its activation function.

The mechanism driving the regulatory potential of TIF1β for C/EBPα expression is complex. This is made evident by the differences of the requirement for the RBCC domain in transient reporter assays compared to the endogenous protein induction in 3T3 L1 preadipocytes (Fig 5B, 7, 9C). Furthermore, the COOH-terminal PHD and BROMO domains, as well as an interaction with HP1 proteins, all appear to differentially affect its ability to induce C/EPBα expression during early differentiation (Fig 11E). The constitutive presence of TIF1β on the endogenous C/EBPα promoter in preadipocytes suggests added complexity (8A). Together, the data suggests that multiple regulatory mechanisms might be involved in TIF1β-dependent modulation of C/EBPα expression. The data in Cos7 cells supports a model whereby TIF1β acts, in part, by targeting HDAC1 to the 26S proteasome and prevents the interaction of HDAC1 with C/EBPβ, in a manner that is similar that of the

GR_{LBD}. Modulation of HDAC1 by TIF1β appears to require the RBCC domain. It remains to be determined, however, if TIF1β is directly implicated in the GR dependent turnover of HDAC1, or if TIF1β functions in a parallel pathway to degrade HDAC1. The data suggest that TIF1β, GR and HDAC1 factors can exist in equilibrium, and shifting the equilibrium towards accessible TIF1β, for example through ectopic expression of TIF1β, is sufficient to overcome the dependence on glucocorticoids. I detect an HDAC1 and TIF1β interaction that is independent of glucocorticoid treatment (Fig. 4A). However, the existence of a multimeric complex between GR, TIF1β and HDAC1 following 4 h dex treatment (Fig. 4D), suggests that there is potential for GR and TIF1β to function in the same pathway to target HDAC1 to the proteasome. Two possibilities are that the incorporation of GR into the TIF1β-HDAC1 complex may either (A) enhance the association of TIF1β with HDAC1 and/or (B) enhance the activity of TIF1β towards HDAC1. Further studies are required to discriminate between these two possibilities.

While I provide evidence that TIF1β can promote the ubiquitylation of HDAC1, further analysis is required to conclude that it is a *bona fide* E3 ligase with HDAC1 as a substrate. Furthermore, it is possible that TIF1β-dependent targeting of HDAC1 to the 26S proteasome requires additional unidentified cofactors. It is noteworthy that both TIF1β and HDAC1 have been identified as cofactors in Mdm2 dependent ubiquitylation and degradation of p53 (Ito et al, 2002; Wang et al, 2005). The human homologue of Mdm2, Hdm2 can also form a complex with GR and p53 that leads to the degradation of both GR and p53 under hypoxic conditions (Sengupta & Wasylyk, 2001). Given that TIF1β, HDAC1 and Mdm2 and possibly GR, can form a multimeric complex that contains E3 ligase activity, it is possible that HDAC1 can also get ubiquitylated within the multimeric complex.

Additionally, conditions under which the multimeric complex forms, could represent a context in which TIF1β functions independently of GR to ubiquitylate HDAC1.

Glucocorticoids regulate both HDAC1 function and stability through posttranslational modifications

Our laboratory has previously demonstrated that a specific fraction of total cellular HDAC1 is targeted for degradation in response to glucocorticoids (Wiper-Bergeron et al, 2003). Similarly, studies by Qui *et al.* suggest that a distinct subpopulation of HDAC1 is acetylated by p300 in response to glucocorticoid treatment, which attenuates its enzymatic activity (Qiu et al, 2006). Acetylation of lysine residues of a protein can have several outcomes. It can alter its enzymatic activity, DNA binding properties, subcellular localization and protein-protein interactions (Kouzarides, 2000; Sterner & Berger, 2000). It can also protect a protein from degradation by blocking potential ubiquitylation sites as has been demonstrated for p53 (Ito et al, 2002). The acetylation of HDAC1 by p300 in response to steroids has been mapped to 6 lysine residues within its C-terminus. Independent studies have mapped HDAC1 ubiquitylation to within this region (David et al, 2002). I propose that glucocorticoid dependent regulation of HDAC1 by acetylation and ubiquitylation is a dynamically regulated process. The equilibrium between acetylation and ubiquitylation would be governed by the dynamic interaction with different cofactors, for example, GR-associated TIF1β or p300. Accordingly, TIF1β has been shown to potentiate GR-induced transcription on the MMTV promoter (Chang et al, 1998). Whether posttranslational modification of HDAC1 in response to steroid occurs on the same lysine residues, and if

acetylation is a prerequisite for ubiquitylation or if they are independent events remains to be determined.

Overall, the implication that the induction of direct transcriptional targets of GR are also regulated by modulation of HDAC1 activity in response to glucocorticoids, also implies by extrapolation that the role of TIF1β in this capacity extends beyond the non-genomic targets of the receptor that include C/EBPβ function. As such, TIF1β may play an important regulatory role in other systems which are regulated both independently by glucocorticoids and those in which GR acts in concert with C/EBPβ, including, but not limited to, immune system function, myogenesis, osteogenesis, liver regeneration and memory consolidation (Granner & Pilkis, 1990; Hirayama et al, 2002; Kim & Haller, 2007; Lloberas et al, 1998; McGaugh & Roozendaal, 2002; Nerlov et al, 1998; Nishio et al, 1993; Roozendaal, 2000).

TIF1β regulates the early phase of 3T3 L1 preadipocyte differentiation

TIF1β has been implicated in the differentiation of the mouse embryonic F9 cell model of early embryonic development and cellular differentiation, and spermatogenesis and most recently, epithelial mesenchymal transition (Cammas et al, 2004; Cammas et al, 2002; Weber et al, 2002; Venkov et al, 2007). Our results provide the first evidence that TIF1β potentiates preadipocyte differentiation, and that it acts, at least in part, during the early phase of differentiation to regulate expression of C/EBPα. Both over-expression of TIF1β in 3T3 L1 preadipocytes and siRNA mediated knockdown of TIF1β in NIH 3T3 cells affected C/EBPα expression during early differentiation in a way that positively correlated with overall differentiation of the respective models (Fig. 6).

Regulation of C/EBPα induction by TIF1β however, appears to be mediated at several levels and may involve both repressive and activating functions. ChIP analysis 24 h following stimulation with MI alone revealed C/EBPβ recruitment that occurred independently of TIF1β. Ectopic TIF1β-expression, however, abrogated the recruitment of HDAC1 to the C/EBPα promoter and promoted increased acetylation of histone H4 and increased recruitment of RNA pol II (Fig. 8A). This correlated with increased expression of C/EBPα protein (Fig. 8B). While the evidence suggests that TIF1β can promote ubiquitylation and turnover of HDAC1 in an RBCC-dependent manner, and that this facilitates the potentiation of C/EBPβ mediated transcription in both Cos7 and NIH 3T3 cells (Fig. 5, 7C), the TIF1βΔRBCC mutant potentiated differentiation as efficiently as the WT protein (Fig. 9). This correlated with increased C/EBPα expression 48 h following stimulation with both MI and MID.

The timing of the induction of C/EBPα is critical to progression through clonal expansion due to its anti-mitotic properties (Umek et al, 1991). The presence of endogenous TIF1β at the C/EBPα promoter in the unstimulated preadipocytes suggested that it could also act as a repressor of this promoter in the basal state. This is consistent with its well-characterterized role as a transcriptional repressor protein.

While the process of C/EBPα gene activation has been extensively studied, the repression of this gene is less well characterized. There are reports that AP-2α binds to a negative regulatory element in the C/EBPα promoter in the unstimulated state (Jiang et al, 1998). The binding of AP-2α physically occludes binding of Sp3 (or Sp1) transcription activating factors to their respective regulatory elements in the promoter. Upon stimulation with hormonal cocktail, AP-2α expression is down-regulated and Sp3 (or Sp1) binds to the

promoter (Jiang & Lane, 2000). It is possible that TIF1β is recruited to the unstimulated C/EBPα promoter through protein-protein interaction with AP-2α, an AP-2α-associated factor or an unidentified DNA-bound factor. To determine if TIF1β contributes to AP-2α dependent repression one could test whether knock-down of endogenous TIF1β affects the ability of AP-2α to repress the promoter.

Alternatively, TIF1β may be recruited to this promoter independently of a DNA-bound factor. It could be targeted to the chromatin through its BROMO domain, which can bind acetylated histones (Dhalluin et al, 1999; Owen et al, 2000). The C/EBPα promoter is enriched in histone H4 acetylation in its basal state as demonstrated in the ChIP analysis (Fig. 8A). These could serve as a docking substrate to recruit TIF1β.

Histone modification at promoters regulate transcriptional competency of that gene. For example, methylation of H3K9, H3K27 and H4K20 are often associated with a repressed promoter, whereas H3K4, H3K14 and H4K20 acetylation correlate with a transcriptionally active promoter (Rosenfeld et al, 2006). There is evidence that TIF1β mediates its repressive role, in part, through recruitment of a K9-specific methyltransferase, SETBD1 through its COOH-terminal PHD and BROMO domains (Schultz et al, 2002). This is consistent with the findings that TIF1β can induce "local heterochromatin microenvironments" at transcriptionally silenced promoters. These are marked by increased H3K9 methylation and recruitment of HP1 proteins (Sripathy et al, 2006). TIF1β also associates with the NCoR-1 and Mi2α corepressor complexes (Schultz et al, 2001; Underhill et al, 2000). It is possible that TIF1β contributes to basal C/EBPα silencing by mediating enzymatic modification of histones.

To assess the role of TIF1β as a repressor of the C/EBPα promoter in the absence of hormonal stimulation (-MI), one could perform a more detailed analysis of histone modification at the C/EBPα promoter to include the aforementioned methylation events and a more detailed profile of histone acetylation. If TIF1β does contribute to maintaining the unstimulated C/EBPα promoter in a repressed state, then a decreased expression of TIF1β (siRNA) may lead to decreased methylation and increased acetylation of the promoter in the absence of MI.

The increase of C/EBPα expression in the presence of the TIF1βΔRBCC mutant could be explained within this repressive context. It is possible that the mutant can displace the WT protein on the C/EBPα promoter, but that it lacks the repressive function. This would lead to de-repression of the basal promoter and increased C/EBPα expression independent of C/EBPβ being recruited to the promoter (-MI state). The presence of the TIF1βΔRBCC mutant at the promoter could be verified by ChIP analysis. If this mutant is present at the promoter, then one could further evaluate changes in histone modifications at the promoter as discussed previously.

An alternative explanation for the increase in C/EBPα expression in the presence of the TIF1βΔRBCC mutant is that TIF1β could be acting to repress an unknown factor that positively regulates C/EBPα transcription either directly or indirectly. TIF1β is a transcriptional repressor protein for a large class of zinc-finger transcription factors (Friedman et al, 1996). This function is dependent on the RBCC domain as this domain facilitates both KRAB-domain mediated targeting to the repressed promoter as well as TIF1β oligomerization. Therefore, over-expression of this mutant could result in the de-repression of this unidentified factor as was discussed for the C/EBPα promoter. In the presence of

TIF1βΔRBCC, increased expression of this factor could compensate for the lack of enhanced TIF1β-dependent ubiquitylation of HDAC1 in the absence of the RBCC domain. Of particular relevance, TIF1β expression also correlates with increased PPARγ expression 24 h post-stimulation in 3T3 L1 cells (Fig. 11E).

The relative induction of C/EBPα and PPARγ expression is complicated by both auto- and cross-regulatory mechanisms due to the presence of both PPAR and C/EBP response elements in their respective promoters (Elberg et al, 2000; Legraverend et al, 1993; Wu et al, 1999). Genetic evidence suggests that they are induced in a single pathway and that C/EBPα is required for PPARγ expression (Rosen et al, 2002). Therefore, increased PPARγ expression upon ectopic expression of TIF1β may simply reflect increased C/EBPα abundance. Alternatively, PPARγ may independently be regulated by TIF1β, and in the presence of ectopic TIF1β there is a shift in the equilibrium of C/EBPα and PPARγ expression, leading to increased PPARγ expression which in turn increases C/EBPα expression.

TIF1β is associated with heterochromatic foci during early phases of differentiation

The differentiation potential of TIF1β was dependent on its association with HP1 proteins and its subsequent HP1-dependent recruitment to foci in centromeric heterochromatin. Expression of the TIF1β HP1-interaction mutant in preadipocytes, which did not colocalize with HP1α and C/EBPβ at DAPI-rich foci (Fig. 11B), did not further potentiate differentiation in response to stimulation with MID (Fig. 11D).

The role of TIF1β at these foci could be several-fold. Heterochromatin is largely considered transcriptionally silenced DNA that is defined by an enrichment of HP1 proteins, hypoacetylated nucleosomes and increased tri-methylation of lysine 9 of histone H3 (H3K9) (Grewal & Jia, 2007). HP1 proteins bind to sites of H3K9 methylation and they, in turn, recruit K9 methyltransferases, such as Suv39h1 and Suv39h2 in mouse, creating a feed-forward mechanism for silencing (Hediger & Gasser, 2006). Furthermore, HP1 proteins can dimerize which contributes to local DNA compaction and generation of higher order chromatin structure (Brasher et al, 2000). TIF1β is recruited to heterochromatin through association with HP1α (Cammas et al, 2002; Nielsen et al, 1999; Ryan et al, 1999). TIF1β itself, interacts with a K9-specific methyltransferase, SETDB1 as well as chromatin remodeling complexes that possess histone deacetylation activities (Schultz et al, 2002; Underhill et al, 2000). As such, it is possible that TIF1β participates in the maintenance of heterochromatin structure in the 3T3 L1 cells.

Interestingly, recent reports have shown that TIF1β is required for global chromatin decondensation in response to DNA damage. Chromatin decondensation is initiated by ATM-dependent phosphorylation of TIF1β at Ser824 following factor co-localization at sites of DNA breaks (Ziv et al, 2006). While we do not detect Ser824 phosphorylation by Western analysis or indirect immunofluoresence studies throughout the course of 3T3 L1 differentiation (data not shown), it is enticing, though speculative, to consider that TIF1β could mediate a similar function in regulating global chromatin structure as these cells progress through two rounds of mitosis prior to terminal differentiation.

Alternatively, but not exclusively, the localization of TIF1β at these sites could be integral to its role as a transcriptional repressor and could be required for the silencing of

KRAB domain transcription factor regulated genes that are repressed by TIF1β. There is growing evidence that nuclear organization and compartmentalization and association of genetic loci with transcriptionally active or inactive regions is an important transcriptional regulatory mechanism (Francastel et al, 2000). The association of genetic loci with these regions can be facilitated by association with specific transcription factors. For example, the lymphocyte specific transcription factor, Ikaros, has been shown to distribute in distinct heterochromatin-containing foci with HP1 proteins and co-localize specifically with transcriptionally inactive genetic loci (Brown et al, 1997). Additionally, the repression function of two KRAB domain containing zinc finger transcriptional repressors, KRAZ1 and KRAZ2 are dependent on their ability to be recruited to heterochromatic loci through association with TIF1β (Matsuda et al, 2001). A similar mechanism could underlie TIF1β-dependent silencing of non-adipocyte genes, which is a critical aspect of terminal differentiation, albeit one that is not well understood.

TIF1β dependent regulation of C/EBPα expression in 3T3 L1 preadipocytes.

While I have postulated that TIF1β could act as both a repressor and activator of the C/EBPα locus, I suggest that this is mediated through two distinct populations of TIF1β in the cell. The population that mediates basal repression appears to be constitutively associated with the promoter as evidenced by the ChIP assay. The suggestion that this is mediated by two different populations of TIF1β is based on the following findings: (A) ChIP analysis of the C/EBPα promoter reveals histone H4 acetylation and RNA pol II occupancy in the basal state ((Wiper-Bergeron et al, 2003), Fig 8A). This represents a repressed, but 'poised' promoter and not likely a promoter that is being repressed through association with

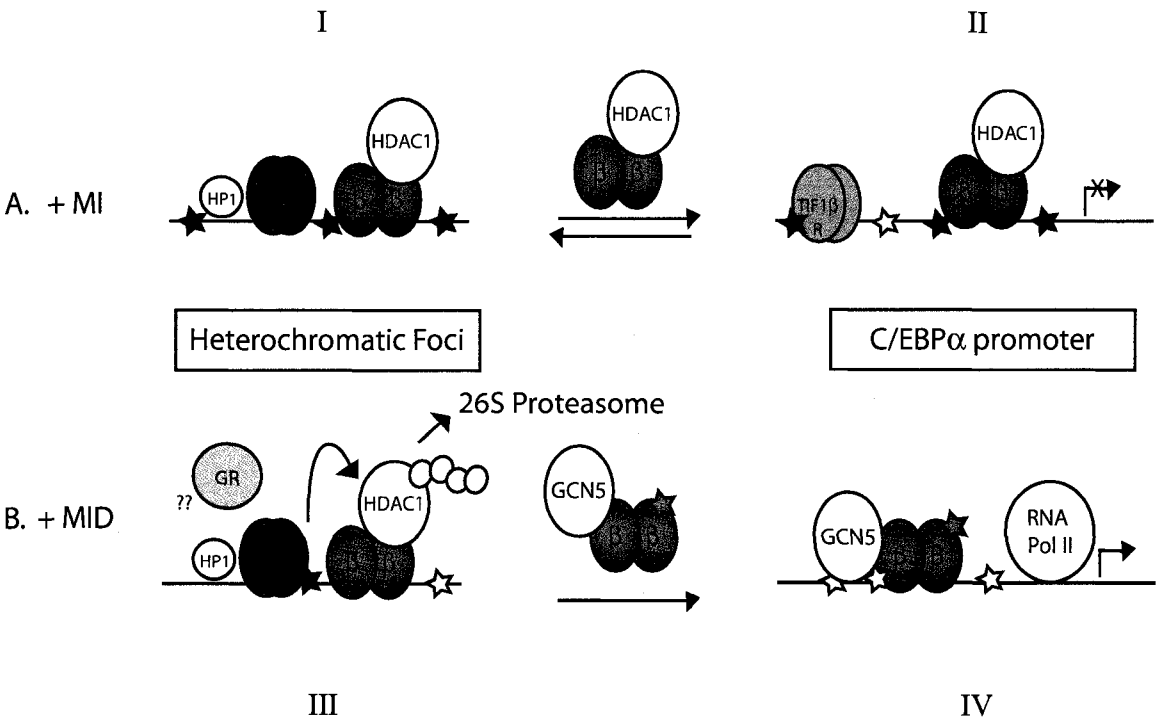
heterochromatin as previously discussed. It therefore seems unlikely that the TIF1β detected on this promoter in the basal state (-MI) is associated with heterochromatin. (B) Over-expression of TIF1β in these cells abrogates the recruitment of HDAC1 to the promoter independently of a change in TIF1β occupancy at that promoter (Fig. 8A). Furthermore, I do not detect a physical association between TIF1β and C/EBPβ. This is consistent with our previous model that the turnover of HDAC1 by GR occurs elsewhere in the nucleus and not on the C/EBPα promoter (Wiper-Bergeron et al, 2003).

It is possible, that the activation of the C/EBPα locus by TIF1β is mediated by the population of TIF1β that colocalizes to heterochromatin with C/EBPβ. Indeed, the decreased differentiation potential of the TIF1βHP1mt correlated with a decreased induction of C/EBPα protein (Fig. 11D, E). The heterochromatic foci are enriched in certain factors including TIF1β, C/EBPβ and HP1α and as such they are readily visualized by indirect immunofluorescence. In parallel studies, IIF of HDAC1 localization during early preadipocyte differentiation revealed consistent, pan-nuclear staining of the deacetylases (data not shown). This suggests that HDAC1 could be recruited to these heterochromatic foci. It is possible that this is dependent on C/EBPβ. I hypothesize that the population of HDAC1 that associates with C/EBPβ is marked for degradation as a consequence of the enriched co-localization of these, and possibly yet to be identified factors at the nuclear foci. I propose that these factors are in equilibrium at these foci and that their association with the foci is dynamic. As such, the degradation of HDAC1 liberates C/EBPβ which can then induce transcription of the C/EBPα gene. Our previous studies suggest that the full activation potential of C/EBPβ correlates with increased GCN5-dependent acetylation of C/EBPβ within a lysine cluster at K98-K102. This model has been summarized in Figure 12.

Figure 12: Proposed model by which TIF1β potentiates C/EBPβ mediated transcription in stimulated 3T3 L1 preadipocytes.

(A) In the absence of glucocorticoids (+ MI), C/EBPβ is associated with an mSin3A-HDAC1 subcomplex (mSin3A is not shown). **(I)** Upon acquiring DNA binding capacity, C/EBPβ associates with heterochromatic foci. TIF1β and HP1α co-localize at these foci. In this model, it is suggested that HDAC1 is recruited to heterochromatin with C/EBPβ. Heterochromatin is marked by increased histone H3 hypoacetylation and H3K9 trimethylation (★). **(II)** C/EBPβ can also bind to the C/EBPα promoter; however its transcriptional potential is repressed due to its association with HDAC1. Although repressed, this promoter is poised, as indicated by detectable histone H4 acetylation (☆). A distinct population of TIF1β (repressor = R) is present on the promoter and may contribute to maintaining this locus in a repressed state.

(B) (III) In the presence of glucocorticoids (+ MID), HDAC1 is targeted for degradation through the 26S proteasome. In this model, I suggest that the ubiquitylation of HDAC1 occurs as a result of its association with C/EBPβ in the presence of enriched TIF1β (activator = A) at these foci. I suggest that glucocorticoids provide a stimulus that engages TIF1β activity and results in the marking of HDAC1 for degradation. The possible means by which glucocorticoids do so is discussed in the text. It remains to be determined if GR physically associates with these factors at the foci. **(IV)** Loss of HDAC1 leads to accumulation of acetylated C/EBPβ (AcK98-K102) (★), which induces transcription of the C/EBPα gene. Active gene transcription correlates with increased histone H4 acetylation and recruitment of RNA polymerase II to the promoter.


I
II
A. + MI
HDAC1
HP1
HDAC1
HDAC1
TF1β
R
Heterochromatic Foci
C/EBPα promoter
26S Proteasome
B. + MID
GR
??
HP1
HDAC1
GCN5
GCN5
RNA
Pol II
III
IV

The ubiquitylation promoting enzymatic activity of TIF1β appears to be inducible. Glucocorticoids could serve as its inducer in this system. The pivotal role of glucocorticoids is highlighted by our previous findings in which, in the absence of exogenous TIF1β expression, inclusion of glucocorticoids in the inductive cocktail is required for C/EBPα promoter activation as defined by loss of HDAC1 occupancy, increased histone H4 acetylation and recruitment of RNA pol II (Wiper-Bergeron et al, 2003). The role of GR in stimulating TIF1β-dependent ubiquitylation of HDAC1 could be: (1) GR could drive the accumulation of TIF1β at these foci through unknown mechanisms; (2) GR could increase the E3 ligase activity of the protein, through an unknown mechanism; (3) GR could enhance the association of TIF1β with HDAC1 through formation of a heterocomplex; (4) GR could regulate factors that prevent TIF1β from ubiquitylating HDAC1. For example, GR could titrate out negative factors from these foci. Further studies are required to differentiate between these possibilities.

To begin to dissect the molecular mechanisms and to validate this model, further insight into the components of these foci, their interactions at the foci and the control of their temporal recruitment and exit to these foci is warranted. Fluorescence microscopy-based techniques provide a powerful tool to begin addressing these questions. A new fluorescent protein fragment complementation assay to detect protein-protein interactions in living cells has recently been described by MacDonald and Micknick (MacDonald et al, 2006). In this assay, two synthetic fragments of the Venus fluorescent protein are fused to two proteins of interest. These fusion proteins are co-expressed in cells. Physical association of the two proteins results in reconstitution of the Venus fluorophore which can be detected in live cells by fluorescence microscopy. This technique is amenable to measuring both spatial and

temporal recruitment/association of proteins. It is presently being established in the Haché laboratory for independent studies. I propose that this system could be used to determine the association of TIF1β, HDAC1, C/EBPβ, HP1α and GR in differentiating preadipocytes with a specific interest in the association of these proteins at heterochromatic foci. Using this technique, one could assess physical association of these factors at the foci, the inter-dependence of cofactors in these associations (ie knockdown of endogenous factor/ does dex affect the association between TIF1β and HDAC1?) and the temporal recruitment of these proteins to the foci and how this is affected by treatment with glucocorticoids.

A limitation of this assay is that the complementation of the fluorophore results in an irreversible reaction, which leads to 'trapping' associated proteins. Therefore, to assess if these factors are dynamically associating with foci, another method would be required. Fluorescence Recovery After Photobleaching (FRAP) analysis using green fluorescent protein (GFP) fusion proteins of factors of interest would allow one to measure the exchange rate of these factors with the foci (Carrigan et al, 2007; Walther et al, 2005). One could then determine if the kinetics are altered in the presence of steroids or any of the factors as discussed above.

TIF1β dependent regulation of 3T3 L1 differentiation.

Homozygous knockout of the TIF1β gene results in embryonic lethality. It is therefore not surprising to find it involved in several aspects of nuclear metabolism. The complexity of the mechanisms by which TIF1β regulates both overall differentiation as well as the induction of C/EBPα specifically, is reflected in the findings that there is no obvious correlation between localization to foci, induction of C/EBPα expression and differentiation

capacity between each of the WT, ΔRBCC, ΔC-terminus and HP1-interaction mutants. A major limiting factor in the interpretation of these results is the presence of endogenous TIF1β and that its function can depend on homo-oligomerization of the protein (Peng et al, 2000). Repeating these experiments in a TIF1β-null background would provide great insight into the mechanisms of action of each of these mutants and to delimit the potential interdependency of these roles. A key experiment in defining this interdependency would be to assess TIF1β, HDAC1, RNA pol II and histone modifications at the C/EBPα promoter in stimulated preadipocytes that exclusively expressed the WT or mutant isoforms of TIF1β.

In summary, I have provided evidence that TIF1β is a novel regulator of the early events in 3T3 L1 preadipocyte differentiation, including the induction of the master regulator, C/EBPα. I have also demonstrated that TIF1β potentiates C/EBPβ transcriptional activity. I propose that this is mediated, at least in part, by modulating the association of C/EBPβ with the repressor protein HDAC1 and promoting turnover of HDAC1 by the 26S proteasome. I hypothesize that this is required for the remodeling of the C/EBPα promoter observed in the 3T3 L1 cells. However, this regulation may not be limited to this function, as the interaction with the HP1 proteins and the repressive function conferred by the RBCC domain also appear to affect C/EBPα expression. Further experimentation would be required to determine if these seemingly distinct functionalities of TIF1β have either a direct or an indirect impact on C/EBPα expression. It would also be interesting to better define its role as an E3 ligase and assess whether this activity is required for its role in other systems, such as DNA repair, regulation of p53 stability, differentiation systems, localization to heterochromatic foci and the regulation of transcription.

CHAPTER II: MODULATION OF EARLY HUMAN PREADIPOCYTE DIFFERENTIATION BY GLUCOCORTICOIDS

This chapter constitutes a published manuscript:

Tomlinson J.J, A. Boudreau, D. Wu, E. Atlas and R.J.G. Haché. (2006) Modulation of Early Human Preadipocyte Differentiation by Glucocorticoids. *Endocrinology.* **147**(11):5284.

The manuscript was written in close collaboration with Dr. R.J.G. Haché.

CONTRIBUTION OF AUTHORS

Both D. Wu and A. Boudreau contributed technical assistance to the data presented in this manuscript. I was involved in the technical aspects of all of the experiments presented with the exception of the real-time PCR based analysis of mRNA expression presented in Figure 3, which was performed by A. Boudreau and D. Wu.

I contributed to the compiling and presentation of the data in each of the figures. I performed the quantification of Oil Red O staining and protein expression (Figure 1 and 2) and performed the statistical analysis of the data throughout the manuscript.

Dr. E. Atlas provided an intellectual contribution to research presented herein and also critically reviewed the manuscript during its preparation.

*Reprinting of this manuscript in its submitted version was done with permission from the Endocrine Society.

INTRODUCTION

Glucocorticoids provide a potent stimulus for adipogenesis. The manifestation of this stimulus is most obvious in the truncal obesity of patients with Cushing's syndrome, which is a direct consequence of prolonged hypercortisolemia (Boscaro et al, 2001). Further, there are many similarities between the clinical features of non-Cushinoid obese and hypercortisolemic patients, including central fat accumulation, elevated blood pressure, insulin resistance with impaired glucose tolerance and dyslipidemia (Bjorntorp & Rosmond, 2000). The relationship between glucocorticoids and adipogenesis is complex. Circulating glucocorticoids contribute to white adipose tissue development which in turn is supplemented by locally produced steroid, by mature white adipose tissue, as a result of the action of 11 β-hydroxysteroid dehydrogenase type 1 (11β-HSD1). In animal models, over-expression of 11β-HSD1 in mature adipocytes has provided a transgenic model for visceral obesity (Masuzaki et al, 2001). Conversely, 11β-HSD1 knockout mice are resistant to visceral fat accumulation (Morton et al, 2001).

Although the physiological relevance of glucocorticoids in promoting adipogenesis is well accepted, definition of the specific stimulatory role of glucocorticoids in the transcriptional cascade leading to preadipocyte differentiation has been largely limited to studies with established rodent preadipocyte cell lines such as murine 3T3 L1 cells (Green & Meuth, 1974; Gregoire, 2001; Gregoire et al, 1998). Adipogenic stimulation of post-confluent 3T3 L1 cells cultured in the presence of calf serum, initiates a 48 h period of clonal expansion and induction of a cascade of transcription factors beginning with C/EBPβ and C/EBPδ. Commitment to differentiate is reached within 48 h of induction upon expression of C/EBPα and PPARγ (Gregoire et al, 1998).

In these murine cells, glucocorticoid treatment is stimulatory only during the initial 48 h clonal expansion phase (Rubin et al, 1978). Glucocorticoid stimulation has been ascribed to direct stimulation of C/EBPδ transcription (Cao et al, 1991), repression of the anti-adipogenic factor preadipocyte factor 1 (Pref-1) (Smas et al, 1999), and the depletion of a specific subcellular pool of HDAC1 that represses the activation of C/EBPα transcription by C/EBPβ (Wiper-Bergeron et al, 2003).

Significant differences between primary human preadipocytes and the 3T3 L1 murine model are that the human cells differentiate directly in the absence of clonal expansion and differentiation is accomplished in serum-free medium. In this instance, early reports have suggested a requirement for glucocorticoids during the entire 14-day course of differentiation. Further, differentiation is also dependent on a 4 day course of treatment with the PPARγ agonist troglitazone that is dispensable for murine preadipocytes (Adams et al, 1997; Hauner et al, 1989).

In the present study, we have performed a detailed analysis of the contribution of glucocorticoids to the differentiation of primary human preadipocytes. Specific features of the differentiation were compared to the effects of steroid on the differentiation of murine 3T3 L1 cells stimulated to differentiate in chemically defined, serum-free medium. We report that the temporal effects of steroid are conserved between human and murine systems despite the absence of clonal expansion in human preadipocytes prior to terminal differentiation and that glucocorticoid and PPARγ agonist treatment act sequentially to promote differentiation. Significantly, in addition to the induction of C/EBPδ and the expected effects on C/EBPα and PPARγ, steroid treatment also modulated the rapid initial accumulation of C/EBPβ. Remarkably, glucocorticoid treatment was required for murine

cells to survive the initial clonal expansion phase of differentiation in chemically defined serum-free conditions.

RESULTS

Dexamethasone and troglitazone sequentially stimulate the differentiation of primary human preadipocytes

To assess the temporal contribution of glucocorticoid and troglitazone stimulation to the differentiation of human preadipocytes, we titrated the conditions required for optimal differentiation of subcutaneous human preadipocytes derived from donors with normal BMI (22.5 ± 0.2 kg/m^2). Cells were induced to differentiate upon reaching confluence (day 0), as summarized in Fig 1A.

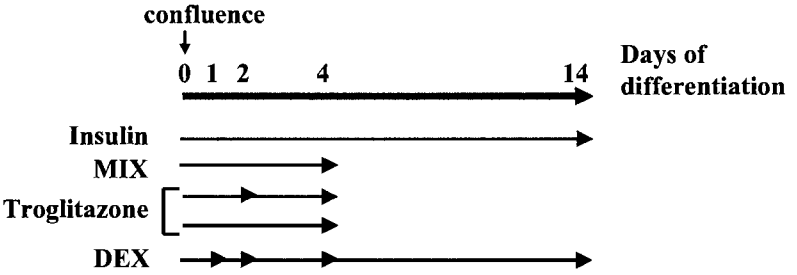
Dex treatment (10^{-6}M) provided a potent stimulus for differentiation, with its addition to the insulin/MIX/troglitazone differentiation cocktail inducing a marked increase in the levels of the adipogenic marker aP2 (adipocyte fatty acid binding protein) in the mature adipocytes at day 14. Lipid accumulation was also enhanced 2-3 fold by dex treatment as revealed by the increased staining intensity of neutral lipids in the mature adipocytes by Oil Red O (Fig 1B). The stimulatory effect of dex was exerted early during the differentiation, with only 24 h of treatment being sufficient to provide significant increases in aP2 expression and Oil Red O staining at day 14. Similar to previous studies with the murine 3T3 L1 model (Rubin et al, 1978), inclusion of dex with the adipogenic cocktail for the first 48 h of stimulation of primary human preadipocytes provided the optimal stimulatory effect, with steroid treatment for longer periods providing no additional benefit.

Examination of the optimal timing for the combination of dex and troglitazone treatment revealed a sequential benefit of the two agonists on primary human preadipocytes (Fig 1C). Little differentiation was observed when both dex and troglitazone were left out of

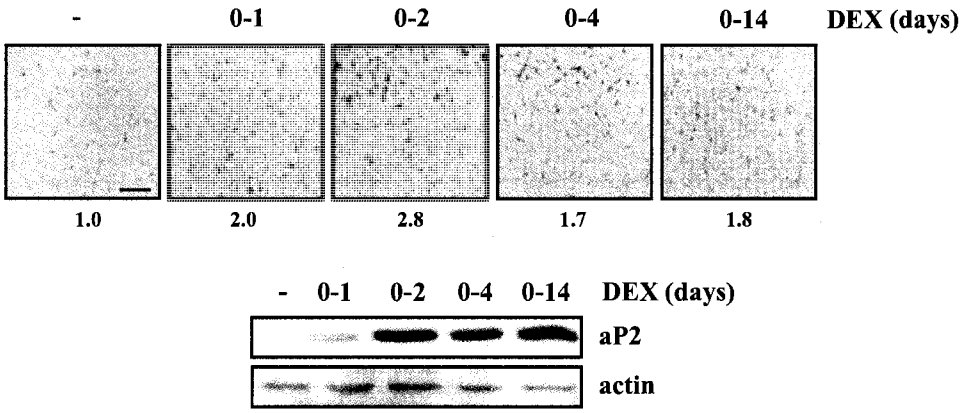
Figure 1. Transient glucocorticoid and troglitazone treatments act sequentially to stimulate the differentiation of human preadipocytes.

(**A**) Schema of the timing of the insulin (100 nM), MIX (0.5 mM), troglitazone (5 μM) and dex (1 μM) treatments employed for the experiments shown in panels B and C through the 14 d course of differentiation of primary human preadipocytes cultured in chemically defined serum-free medium. Arrowheads denote the length of individual treatment times. (**B**) The upper panel shows photomicrographs of Oil Red 0 stained mature adipocytes14 days subsequent to the induction of differentiation by treatment with insulin, MIX and troglitazone for days 0-4 and dex as indicated. The scale bar represents 1mm. Numbers below the panels represent quantification of the amount of staining relative to the untreated condition as determined using Image J software. The lower panel shows corresponding Western analysis of aP2 and actin protein levels in duplicates of the upper. (**C**) Analysis of lipid accumulation and aP2 expression as in (B) for cells induced to differentiation with insulin and MIX for days 0-4, and dex and troglitazone as indicated (days). aP2 expression was quantified using ImageQuant software and is represented below the blot as relative aP2 induction.

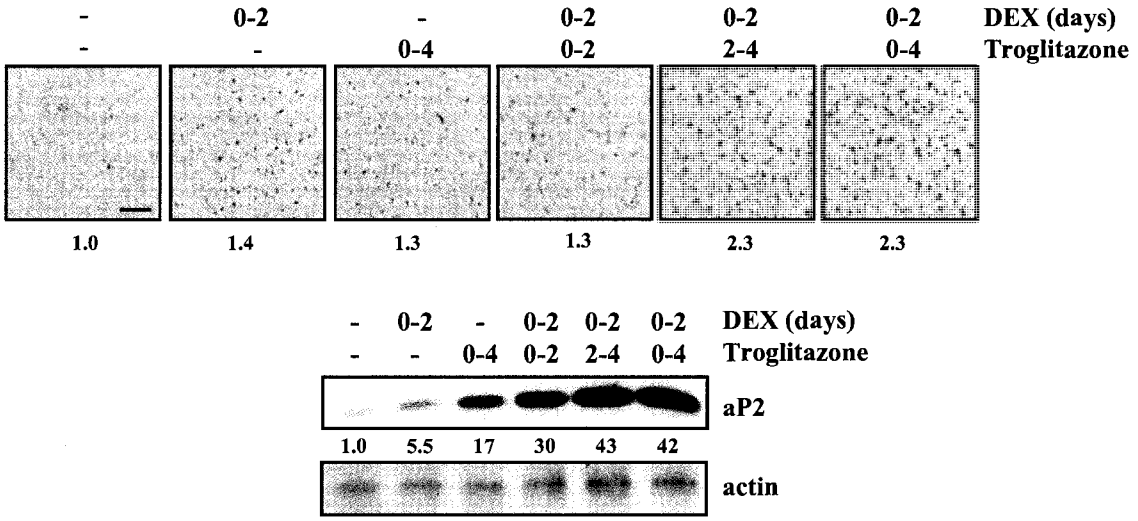
A.


confluence
0 1 2 4 14
Days of differentiation
Insulin
MIX
Troglitazone
DEX

B.


- 0-1 0-2 0-4 0-14 DEX (days)
1.0 2.0 2.8 1.7 1.8
- 0-1 0-2 0-4 0-14 DEX (days)
aP2
actin

C.


- 0-2 - 0-2 0-2 0-2 DEX (days)
- - 0-4 0-2 2-4 0-4 Troglitazone
1.0 1.4 1.3 1.3 2.3 2.3
- 0-2 - 0-2 0-2 0-2 DEX (days)
- - 0-4 0-2 2-4 0-4 Troglitazone
aP2
1.0 5.5 17 30 43 42
actin

the differentiation cocktail, with detection of an aP2 signal requiring prolonged Western blot exposure. Treatment with dex for 48 h or troglitazone for 4 d independently stimulated differentiation, with troglitazone being more effective than dex in eliciting aP2 expression (17 fold compared to 5.5 fold increase). However, the two treatments were most effective in combination, with dex treatment for days 0-2 followed by troglitazone treatment from days 2-4 providing for optimal expression of aP2 and lipid accumulation. Differentiation was not further enhanced by including troglitazone in the cocktail for all 4 days, showing that steroid and troglitazone are contributing independently and sequentially to differentiation.

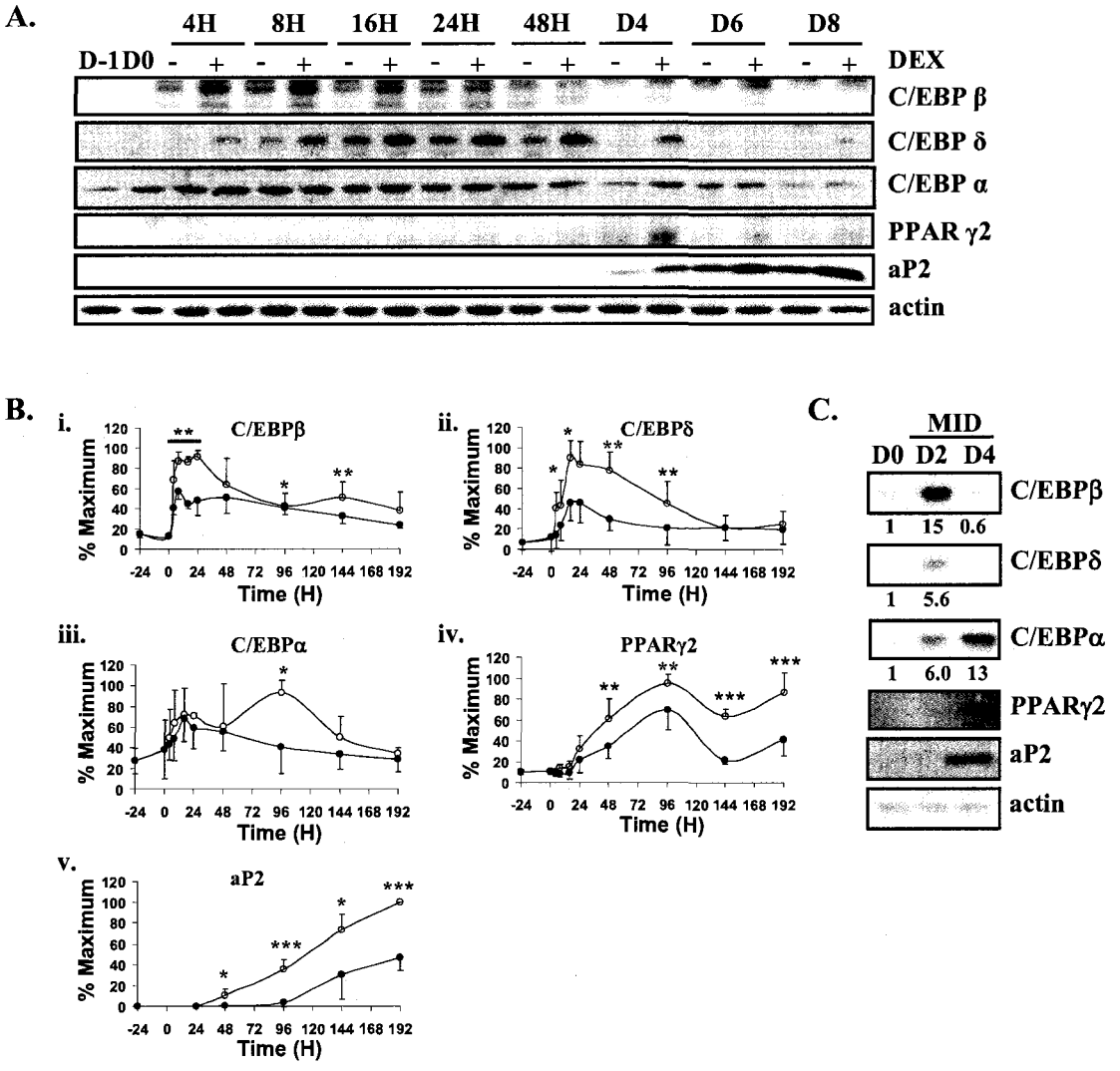
Dex treatment enhances the early accumulation of C/EBPβ

Western analysis of C/EBPβ, C/EBPδ, C/EBPα, PPARγ and aP2 accumulation in primary human preadipocytes (Fig 2), revealed both similarities and striking differences to the responses seen previously in murine cells. C/EBPβ levels were induced in the human cells within 4 h of treatment with adipogenic cocktail lacking dex (Fig 2A,B), consistent with results from 3T3 L1 cells in which C/EBPβ is also induced immediately (Cao et al, 1991; Yeh et al, 1995). However, while in murine cells the induction of C/EBPβ is dex-independent (Cao et al, 1991; Yeh et al, 1995), in the human cells, the early accumulation of C/EBPβ was reproducibly enhanced 2-2.5 fold by dex treatment during the first 24 h. This steroid-mediated enhancement of C/EBPβ accumulation was reproduced over a series of 5 independent trials including a total of 9 different donor samples.

The increase in C/EBPβ due to dex treatment was transient, with C/EBPβ levels declining in dex-treated cells to the level observed in MIX/insulin-treated cells by 48 h. (Fig

Figure 2. Glucocorticoid treatment stimulates accumulation of adipogenic factors.

(A) Western analysis of protein expression during the course of human preadipocyte differentiation. Preadipocytes were harvested 24 h prior to treatment (D-1) and at the times indicated during treatment with insulin (day 0-14), MIX, and troglitazone (day 0-4) in the presence or absence of 10^{-6} M dex (day 0-2). **(B)** Graphical presentation of the protein expression profiles as a function of percentage of maximal protein expression. MIT (•) and MITD (○) +/- SD. n = 3 to 5 and was comprised of up to 4 individual experiments with different cells derived from different donors and one pool of cells derived from another 5 donor samples. Students paired T-tests were performed for each time point to compare MIT and MITD expression levels. *$p \leq 0.10$, ** $p \leq 0.05$, *** $p \leq 0.01$. **(C)** Western analysis of adipogenic factors expressed at indicated times in 3T3 L1 cells differentiated under serum-free conditions as described in the materials and methods. Signals were quantified as described in the methods and represent relative induction.


A.
4H
8H
16H
24H
48H
D4
D6
D8
D-1D0 - + - + - + - + - + - + - + - +
DEX
C/EBP β
C/EBP δ
C/EBP α
PPAR γ2
aP2
actin
B.
i.
C/EBPβ
ii.
C/EBPδ
iii.
C/EBPα
iv.
PPARγ2
v.
aP2
% Maximum
Time (H)
-24 0 24 48 72 96 120 144 168 192
C.
MID
D0 D2 D4
C/EBPβ
1 15 0.6
C/EBPδ
1 5.6
C/EBPα
1 6.0 13
PPARγ2
aP2
actin

2B, panel i). C/EBPβ levels remained readily detectable thereafter from days 2-8. By contrast, a limited analysis of the response of 3T3 L1 cells to adipogenic stimulus in serum-free chemically defined medium showed a more dramatic decline in C/EBPβ levels than reported previously in the presence of serum (Cao et al, 1991; Yeh et al, 1995), with expression declining to near undetectable levels by day 4 (Fig 2C).

The induction of C/EBPδ in primary human preadipocytes closely paralleled its induction in murine cells (Cao et al, 1991; Yeh et al, 1995). Accumulation was rapid, dex dependent and sustained through 96 h (Fig 2A,B, panel ii).

The detection of C/EBPα protein expression in pre-confluent cells (Day –1) and in cells as they reach confluence (Day 0) contrasted with previous reports in human preadipocytes in which its expression was only detected subsequent to an adipogenic stimulus (Halvorsen et al, 2001). However, it is consistent with results from murine preadipocytes where modest levels of C/EBPα expression prior to differentiation is thought to be linked to the growth arrest of confluent cultures (Altiok et al, 1997; Shao & Lazar, 1997; Umek et al, 1991).

C/EBPα expression in the primary human preadipocytes appeared to be bi-phasic. There was a modest, but reproducible 1.5-2 fold dex-independent induction in response to the differentiation stimulus within the first 4 h of treatment that was sustained for 48 h. By contrast, in murine cells the basal level of C/EBPα is rapidly suppressed upon exposure to adipogenic cocktail and only becomes re-induced between 24-48 h into differentiation (Fig 2C). When dex was included in the adipogenic cocktail, C/EBPα accumulation was enhanced (Fig 2B, panel iii) and was maintained at maximal levels to day 4, before declining thereafter to the levels seen in MIX/insulin treated cells (Fig 2B, panel iii).

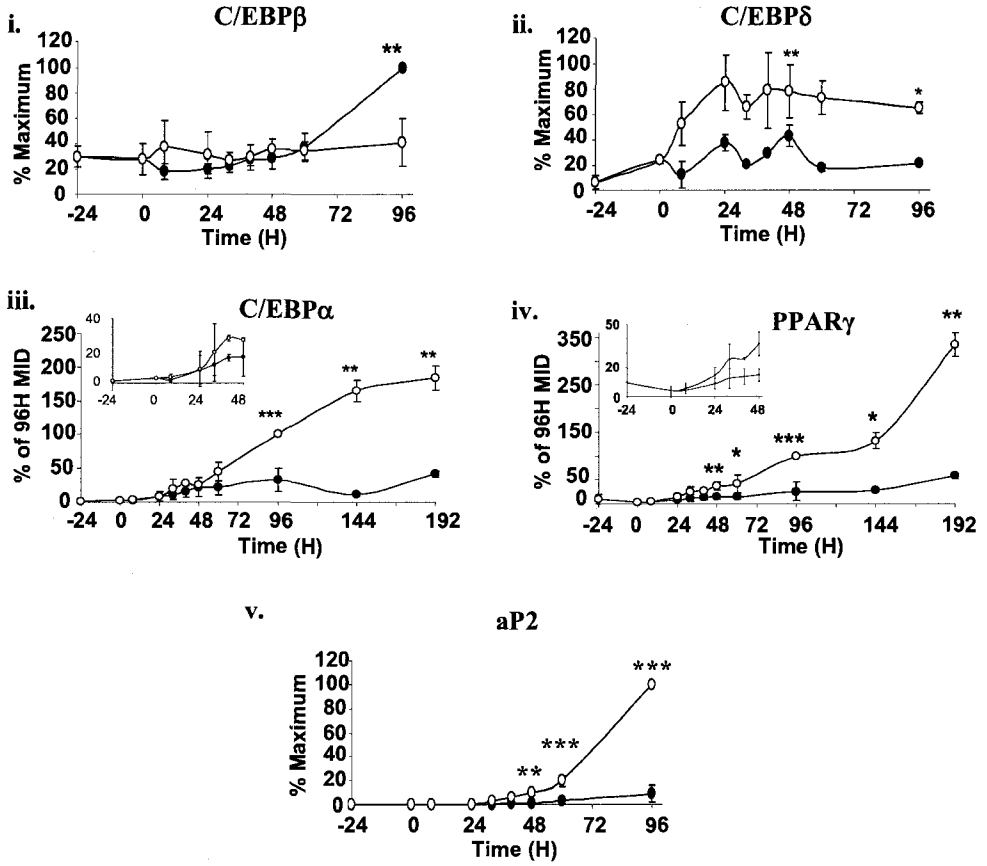
By contrast to C/EBPα, PPARγ expression was first detected at 48 h, peaked at 96 h and was enhanced throughout the course of its expression by the initial presence of dex in the differentiation cocktail (Fig 2B, panel iv). This profile parallels what has been observed in 3T3 L1 cells differentiated in both the presence and absence of serum (Shao & Lazar, 1997; Staiger et al, 2002).

Lastly, upon MIX/insulin dex treatment, aP2 was detected by day two and accumulated essentially linearly through day 8 (Fig 2B, panel v). When dex was omitted from the differentiation cocktail aP2 accumulation was decreased and aP2 expression reached our limit of detection at day 4. This parallels the effect of dex seen on the induction of aP2 in murine systems such as 3T3 L1 cells (Bernlohr et al, 1985).

To determine whether the changes in the protein levels of adipogenic factors observed correlated directly with differences in mRNA level, we performed Real-Time PCR analysis of their expression in parallel with the Western analysis (Fig 3). Interestingly, the rapid accumulation of C/EBPβ protein in our primary human preadipocyte cultures was not accompanied by the dramatic cAMP (MIX)-dependent increase in C/EBPβ mRNA that has been reported for murine cell lines (Cao et al, 1991; Yeh et al, 1995). Indeed, there was no significant difference between the C/EBPβ levels at 24 h, regardless of the treatment (panel i). Curiously, 4 days into the treatment of the human preadipocytes with insulin, MIX and troglitazone, conditions under which only modest preadipocyte differentiation was observed (Fig 1), there was a consistent and reproducible 3-4 fold spike in C/EBPβ mRNA. This induction was not observed upon the inclusion of dex from day 0 to 2 of differentiation and did not appear to be translated into an increase in C/EBPβ protein levels (Fig 2A, B). Additional mRNA analysis indicated that this change in C/EBPβ mRNA levels, while

Figure 3. Effects of glucocorticoid treatment on mRNA induction during preadipocyte differentiation.

RNA was extracted from primary human preadipocytes 24 h prior to treatment (-24H) and at the indicated times during treatment with insulin (day 0-14), MIX and troglitazone (day 0-4) in the absence (•) or presence (○) of 10^{-6}M dex (day 0-2). Each expression profile has been standardized against control G3PDH mRNA levels. Data is presented as the average of the percentage of maximal mRNA levels for each gene over a minimum of 3 repeats each done in duplicate performed with cells derived from different donor samples, except for C/EBPα and PPARγ mRNA, where data is presented as percentage of 96 H MID. The insets in C/EBPα and PPARγ graphs highlight the scale for data points between –24 H and 48 H. Data is plotted +/- SEM. Students paired T-tests were performed for each time point to compare MIT with MITD. *$p \leq 0.10$, ** $p \leq 0.05$, *** $p \leq 0.01$.


i.
C/EBPβ
% Maximum
Time (H)
ii.
C/EBPδ
% Maximum
Time (H)
iii.
C/EBPα
% of 96H MID
Time (H)
iv.
PPARγ
% of 96H MID
Time (H)
v.
aP2
% Maximum
Time (H)

reproducible, also was transient and returned to baseline levels by day 5 of treatment (data not shown). By contrast, induction of C/EBPδ mRNA occurred similarly to the induction of C/EBPδ protein, with an early response that was strongly enhanced by dex and which was sustained for four days (panel ii).

Prior to differentiation, mRNAs for C/EBPα and PPARγ were expressed at very low levels (panels iii, iv). The early 4 h induction of C/EBPα observed at the protein level (Fig 2A, B) was not reflected by detectable induction of mRNA synthesis, suggesting that the early up-regulation of C/EBPα expression that was unique to the primary human preadipocytes is regulated through non-transcriptional mechanisms. Induction of both C/EBPα and PPARγ mRNAs was detectable by 24 h and their levels continued to increase thereafter. Induction was also stimulated by early dex treatment, with both mRNAs being 5 fold higher at day 4 in the presence of dex. Interestingly while C/EBPα protein levels declined after day 4 and PPARγ levels had reached their peak, the mRNA levels for both factors continued their strong dex-dependent increase through day 8 of differentiation.

Lastly, aP2 mRNA expression paralleled protein accumulation in both the timing of its appearance and in the enhancement of expression by dex.

Early accumulation of C/EBPβ is dependent on C/EBPβ transcription

While C/EBPβ protein levels in the primary human preadipocytes increased within 4 h of MIX/insulin treatment and were further enhanced by the addition of dex, C/EBPβ mRNA levels were not significantly different from basal levels at 8 and 24 h following stimulation (Fig 3 panel i). This suggested that the early C/EBPβ protein accumulation in primary human preadipocytes was mediated through a mechanism other than the

transcriptional regulation described for 3T3 L1 cells. Therefore, we examined the early accumulation of C/EBPβ in greater detail (Fig 4).

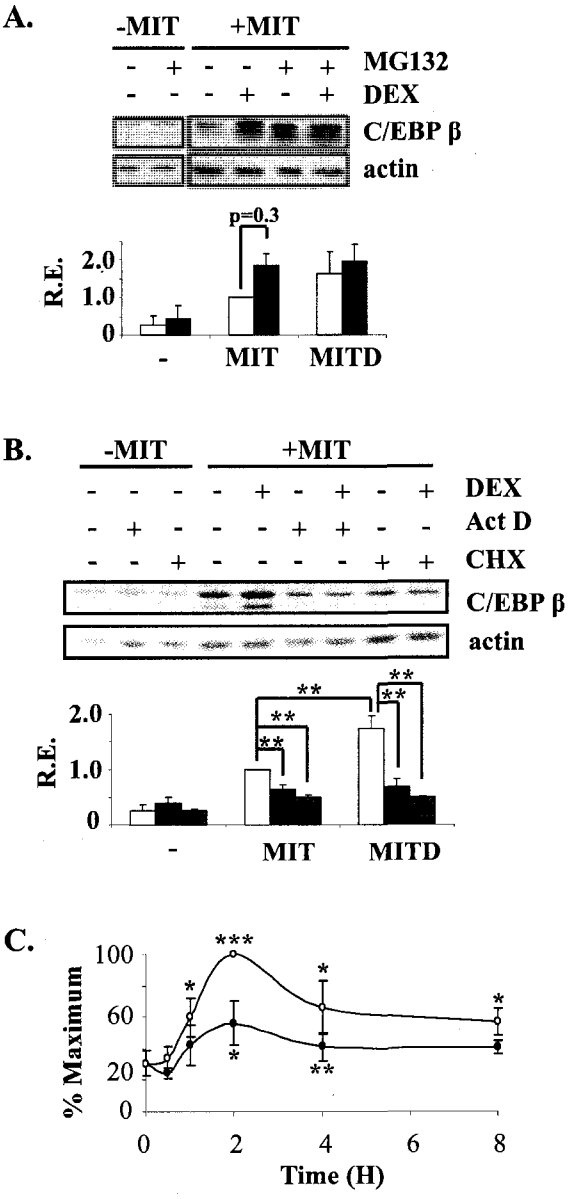
In the first instance, we tested the effect of the proteasome inhibitor MG132 on the accumulation of C/EBPβ at 4 h following adipogenic stimulation (Fig 4A). In untreated cells and cells treated with MIX/insulin/dex for 4h, inclusion of MG132 in the culture had no effect on the accumulation of C/EBPβ. Similarly, although addition of MG132 to the treatment with MIX/insulin alone appeared to have a slight effect on C/EBPβ levels, it did not reach statistical significance over the course of the 3 repetitions performed on individual patient samples. This indicated that protein stabilization through inhibition of 26S proteasome-mediated degradation was unlikely to account for the upregulation of C/EBPβ expression mediated by dex. A similar lack of stabilization was observed at 8 h post stimulation, although MG132 treatment was observed to stabilize the glucocorticoid receptor (GR) over this time period (data not shown).

By contrast, addition of the protein synthesis inhibitor cycloheximide significantly inhibited the induction of C/EBPβ by MIX/insulin and MIX/insulin/dex at both 4 h (Fig 4B) and 8h (data not shown), demonstrating that the induction of C/EBPβ depended on ongoing C/EBPβ translation. Addition of actinomycin D, which inhibits the initiation of new transcription, also prevented the induction of C/EBPβ protein, demonstrating a requirement for new transcription subsequent to the adipogenic stimulus.

To determine whether the adipogenic stimuli induced a rapid pulse of transcription not apparent in our initial mRNA analyses, we refined our RT-PCR analysis to examine mRNA induction between 0 and 8 h post-stimulation (Fig 4C). This revealed a rapid 4-fold induction of C/EBPβ mRNA by MIX/insulin/dex treatment that peaked at 2 h ($p=0.005$).

Figure 4. Enhancement of C/EBPβ expression by glucocorticoid is mediated through transcription.

(A) Western analysis of C/EBPβ expression following 4 h stimulation in the presence or absence of insulin, MIX and troglitazone (MIT), dex and 1μM MG132 as indicated. Lower: Average fold induction relative to the MIT condition ± SEM from 3 independent experiments performed in duplicate with cells derived from different donors. Open bars: no treatment, black bars: MG132 treatment. p-value was determined using Student's paired t-Test. **(B)** Upper: Western analysis of C/EBPβ expression following 4 h stimulation as described in (A) with 10μg/ml actinomycin D or 20μg/ml cycloheximide (CHX) as indicated. Lower: Average fold induction as described in (A). Open bars: no treatment, black bars: Act D treatment, grey bars: CHX treatment. **(C)** Real-time PCR analysis of C/EBPβ mRNA levels during early differentiation. Cells were induced to differentiate in either the absence (•) or presence (○) of 10^{-6}M dex. Data plotted as average mRNA abundance as percentage of maximum +/- SEM where n = 3. Significance of the difference of each data point was assessed relative to day 0 as described in (A). *$p \leq 0.10$, ** $p \leq 0.05$.


A.
-MIT
+MIT
MG132
DEX
C/EBP β
actin
p=0.3
R.E.
2.0
1.0
0
-
MIT
MITD
B.
-MIT
+MIT
DEX
Act D
CHX
C/EBP β
actin
**
R.E.
2.0
1.0
0
-
MIT
MITD
C.
***
100
60
20
0
% Maximum
0
2
4
6
8
Time (H)

Although induction receded rapidly thereafter, C/EBPβ mRNA levels were still modestly elevated at 8 h (p=0.1). Treatment with MIX/insulin alone induced a modest 2-fold induction of C/EBPβ mRNA at 2 h (p=0.1), and C/EBPβ mRNA returned to baseline by 4 h. Notably the addition of dex to the MIX/insulin treatment provided for a significant elevation of C/EBPβ mRNA beyond the effect of MIX/insulin alone from 4 h (p=0.05) through 8 h (p=0.1). These results indicate that the induction of C/EPBβ transcription, though brief, is required for the accumulation of C/EPBβ by MIX/insulin and its further stimulation by dex.

Dexamathasone communicates a survival signal to murine preadipocytes

Previous data have suggested that human preadipocytes in defined medium differentiate directly in response to stimulus (Bell et al, 2000; Entenmann & Hauner, 1996). By contrast, murine preadipocytes differentiated in the presence of serum undergo one to two rounds of cell division in progressing to a commitment point beyond which they are irrevocably committed to differentiation (Gregoire et al, 1998). To determine whether this difference reflected the difference in culture conditions, we monitored ^{3}H-thymidine incorporation into primary human preadipocytes and 3T3 L1 cells over the first 4 days following the stimulation of differentiation in defined medium (Fig 5). Further, as dex treatment is known to be anti-mitotic in other systems (Rogatsky et al, 1997; Sanchez et al, 1993; Webster et al, 1991) and to retard the proliferation in undifferentiated primary preadipocytes (Halvorsen et al, 2001), we specifically monitored the effect of dex treatment in this assay.

Consistent with the initial reports, the stimulation of newly confluent primary human preadipocytes with differentiation cocktail led to an immediate cessation of DNA synthesis

(Fig 5A). Moreover, this effect was independent of the inclusion of dex in the differentiation cocktail. By contrast, for 3T3 L1 cells, addition of differentiation cocktail in the presence of dex stimulated DNA replication that initiated between 12 and 24 h post-treatment and reinitiated between 60-72 h, consistent with two rounds of cell division over that period (Fig 5B,C). Thus clonal expansion of 3T3 L1 murine preadipocytes appears to be an inherent step in the differentiation pathway that is distinct from the behaviour of primary human preadipocytes and does not simply reflect a serum response. Notably, in the presence of serum, two rounds of replication, reflected by two well-distinguished peaks of ^{3}H-thymidine incorporation, were detected in the 3T3 L1 cells, whereas under serum-free conditions replication progressed in an apparently less coordinated manner, with thymidine incorporation seen through the period from 12-72 h.

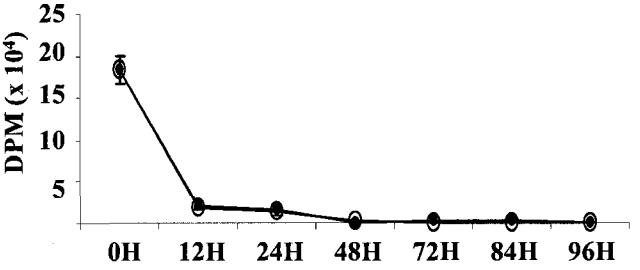
Unexpectedly however, treatment of post–confluent, serum-free 3T3 L1 cells with insulin and MIX in the absence of dex restricted DNA replication to 12-24 h following the onset of treatment, with an almost complete cessation of replication thereafter (Fig 5B). While this could indicate that dex contributes directly to DNA replication of 3T3 L1 cells during the clonal expansion phase, microscopic tracking of the cells over the period of the experiment revealed massive lifting of the 3T3 L1 cells from the plate so that few cells remained in culture at 96 h (data not shown).

Microscopic comparison of mature primary human adipocytes at day 14 and 3T3 L1 cells at day 7 respectively, differentiated under serum-free conditions in complete cocktail, revealed that while the human cultures remained confluent and featured a mixture of lipid-laden and undifferentiated cells, the 3T3 L1 cells were sub-confluent and consisted almost entirely of lipid laden cells (Fig 6A). The sparseness of the 3T3 L1 cells was a specific consequence of the absence of serum as cells differentiated in the presence of serum

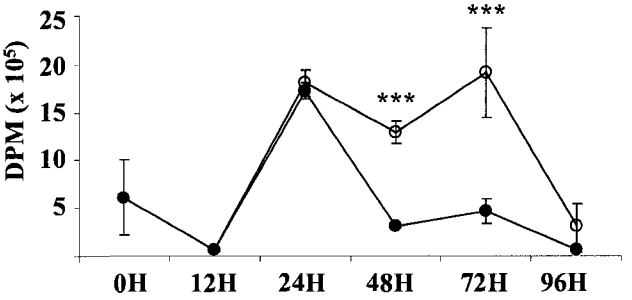
Figure 5. Dex is required for 3T3 L1 cells to complete clonal expansion in the absence of serum.

(A) ^{3}H-thymidine incorporation into primary human preadipocytes over 96 h following the stimulation of differentiation by treatment with insulin, MIX and troglitazone in the presence (○) or absence (●) of 10^{-6}M dex (day 0-2). Approximately $2.5x10^{5}$ cells were pulsed with ^{3}H-thymidine for 12 hours prior to harvesting. (B-C) ^{3}H-thymidine incorporation into 3T3 L1 cells over 96 H following the stimulation of differentiation of 2 day post-confluent cells by treatment with insulin and MIX in the presence (○) or absence (●) of 0.25 μM dex (day 0-2) differentiated in the absence **(B)** or presence **(C)** of serum. There were approximately $8x10^{5}$ cells upon reaching confluence at D0. Data is plotted as DPM values +/- SEM for three independent experiments performed in duplicate. Student's paired t-Tests were performed for each time point in (A) and (B). *** $p \leq 0.01$.

A.


DPM (x 10⁴)
25
20
15
10
5
0H
12H
24H
48H
72H
84H
96H

B.


DPM (x 10⁵)
25
20
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10
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***
***
0H
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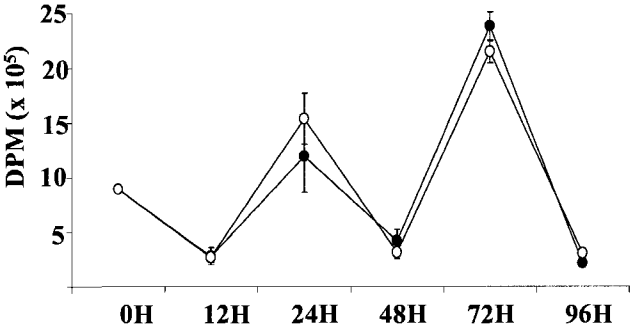
24H
48H
72H
96H

C.


DPM (x 10⁵)
25
20
15
10
5
0H
12H
24H
48H
72H
96H

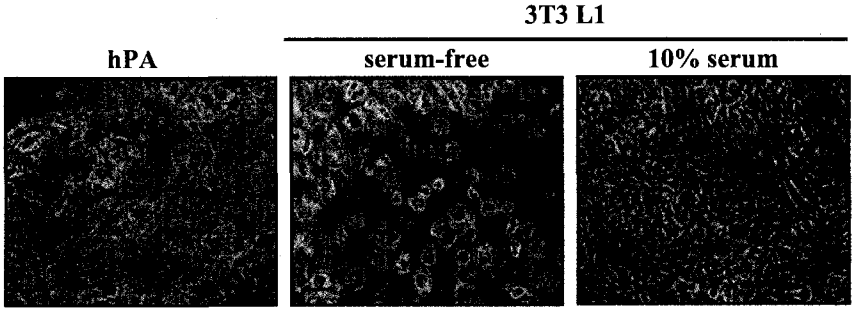
remained confluent and consisted of a mixture of lipid-laden and undifferentiated cells (Fig 6A). Phase contrast photomicrographs taken at day 0 in Fig 6B confirm that the cells were confluent upon induction of differentiation.

These results suggested that dex treatment of 3T3 L1 preadipocytes stimulated in the absence of serum provided a survival signal for cells that committed to differentiate. TUNEL analysis revealed that in the absence of dex, treatment of 3T3 L1 cells with insulin and MIX in the absence of serum resulted in massive cell death within 48 h (Fig 6B). Cell death was strongly reduced but not completely eliminated by the inclusion of dex in the cocktail. By contrast, little evidence of cell death was observed in primary human preadipocytes regardless of the inclusion of dex in the treatment. These results reveal a fundamental difference in the role of dex in the differentiation of primary human preadipocytes and murine 3T3 L1 cells.

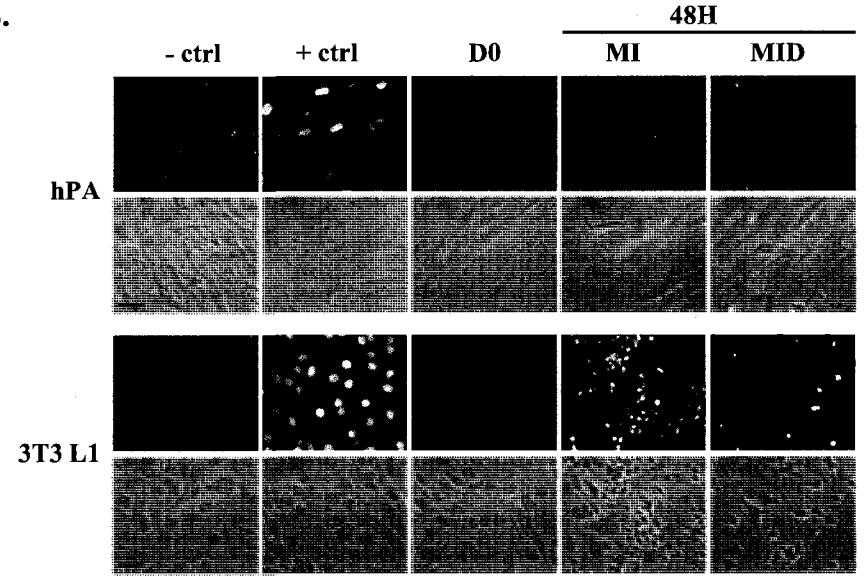
Figure 6. Dex provides a signal that allows committed 3T3 L1 cells to survive to terminally differentiate.

(A) Phase contrast photomicrographs of Oil-Red-O stained human primary cells (hPA) at day 14 of differentiation and 3T3 L1 cells differentiated in the absence (serum-free) or presence of 10% serum at day 7 as indicated. **(B)** In Situ TUNEL assay analysis of cell death (top) and phase contrast images (bottom) in confluent human primary (D0) (upper 2 panels) and 2 day post-confluent murine 3T3 L1 preadipocytes (D0) (lower 2 panels), and cells differentiated in the absence of serum 48 h following treatment with either MIX and insulin alone (MI) or with dex (MID). The negative control (- ctrl) represents assay conditions lacking terminal transferase while the positive control (+ ctrl) represents cells that have been treated with DNase I prior to end-labeling. Scale bar represents 40μm.

A.


3T3 L1
hPA
serum-free
10% serum

B.


48H
- ctrl
+ ctrl
D0
MI
MID
hPA
3T3 L1

DISCUSSION

Previous studies have indicated that C/EBP family members and PPARγ are upregulated during human primary preadipocyte differentiation (Halvorsen et al, 2001; Harp et al, 2001; Urs et al, 2004). However, this is the first report that provides a detailed examination of the expression profiles of these factors during early differentiation and is also the first to specifically examine the impact of glucocorticoids on this process. Titration of dex treatment indicated that the action of glucocorticoids are restricted to the first 48 h of differentiation and are reflected thereafter by increases in transcription factor and adipogenic marker levels. Further, the primary effect of steroid appears to be to facilitate the decision for the preadipocyte to differentiate, rather than to enhance differentiation within individual cells, as dex treatment resulted in more Oil-Red-O positive cells.

A first notable difference in the profile of factor expression in primary human preadipocytes as compared to what is known in the 3T3 L1 model, was an immediate upregulation of C/EBPα that was accomplished within 4 h of exposure to insulin and MIX. By contrast in murine cells, the basal level of C/EBPα detected in confluent preadipocytes is abolished upon stimulation with differentiation cocktail. This has been shown to be a specific requirement for clonal expansion, given the anti-mitotic properties of C/EBPα (Shao & Lazar, 1997; Umek et al, 1991). Thus, the immediate induction of C/EBPα may contribute to that lack of clonal expansion in human preadipocytes. It also could however, reflect that the human preadipocytes have completed clonal expansion prior to isolation from donors thereby allowing for the direct induction of a factor that contributes to commitment in response to adipogenic stimulation. Most interestingly, the early induction in C/EBPα

protein levels occurred without an obvious early increase in its mRNA, suggesting a mechanism of control of C/EBPα that may be unique to human cells.

Subsequently, between days 2-4, C/EBPα and PPARγ were induced in a manner that correlates with induction of their mRNAs and which was enhanced by the inclusion of steroid for the first 48 h of stimulation. Thus steroid treatment shows a memory effect, with early short term treatment translating into accentuated downstream effects on secondary targets following the withdrawal of steroid. Interestingly, following day 4, the pattern of C/EBPα and PPARγ accumulation diverged, with PPARγ protein levels remaining elevated in concert with a continuing induction of PPARγ mRNA, while C/EBPα levels declined from their peak at day 4, despite the continued elevation of C/EBPα mRNA. This difference may reflect a predominant role of PPARγ over C/EBPα in the mature adipocyte. It also reinforces a future need to investigate non-transcriptional mechanisms that may control C/EBPα expression that appear to occur specifically in the primary human preadipocyte.

The early induction and temporal expression profile of C/EBPβ in 3T3 L1 cells has been suggested to reflect a requirement for C/EBPβ for mitotic clonal expansion (Gregoire et al, 1998; Tang et al, 2003a; Zhang et al, 2004). In the human preadipocytes, the early induction of C/EBPβ protein was remarkably similar to that in 3T3 L1 cells despite a lack of clonal expansion. This suggests that the C/EBPβ profile is an inherent characteristic of the early transcriptional cascade that drives differentiation and that its induction reflects more than a requirement for clonal expansion.

In murine cells C/EBPβ induction is dependent on MIX treatment alone, is mediated prominently through the induction of transcription, and is independent of glucocorticoid treatment (Cao et al, 1991; Yeh et al, 1995). However, the induction of C/EBPβ in primary

human preadipocytes was strongly dependent on dex and involved only a modest and shorter term induction of transcription. Investigation of additional potential mechanisms for regulating C/EBPβ excluded interference with the targeting of C/EBPβ to the 26S proteasome and demonstrated a requirement for transcription. While the actinomycin D results highlight a need for new transcription for the induction of C/EBPβ, given the low initial levels of C/EBPβ mRNA, they do not exclude direct effects on the RNA or on the efficiency of translation. Indeed, the interaction of RNA binding proteins, such as CUG repeat binding protein 1, with C/EBP mRNAs has been reported in other systems as a regulatory mechanism for their expression (Timchenko et al, 2005; Timchenko et al, 1999).

In chemically defined differentiation media, 3T3 L1 cells required dex to progress through the second of two rounds of post-confluence mitosis that occurred between 48 and 96 h (Fig 5). While it has been previously shown that this still occurs in 3T3 L1 cells differentiated in the absence of serum, this is the first evidence that glucocorticoids are required for this process (Schmidt et al, 1990). Furthermore, we demonstrated by TUNEL staining that in the absence of dex, there was extensive cell death at the onset of the second round of mitosis at 48 h (Fig 6). In the presence of glucocorticoids, the majority of cells that survived were committed adipocytes. While we cannot exclude the possibility that committed cells also died, we suggest that glucocorticoids are required for the survival of committed preadipocytes, as complete cell death was observed by day 8 in the absence of steroid, conditions under which there was no terminal differentiation.

Glucocorticoids have primarily been characterized as anti-mitotic and pro-apoptotic stimuli, although there are also reports of GR-mediated survival signals in other cell systems (Mendoza-Milla et al, 2005; Mikosz et al, 2001; Moran et al, 2000). The survival signal

contributed by dex is a novel function for GR in the regulation of 3T3 L1 adipogenesis. This signal may be linked to supporting progression through mitotic clonal expansion, as the human preadipocytes, which do not undergo clonal expansion, did not die in the absence of dex when differentiated under similar chemically defined conditions. The effect of dex persisted beyond its presence in the culture as the second round of mitosis was initiated following dex withdrawal at 48 h. We note that one potential candidate mediator of this signal may be the growth arrest-specific gene product Gas6, a direct immediate-early target of GR in differentiating 3T3 L1 cells which is required for establishing dex-dependent post-mitotic growth arrest (Shugart & Umek, 1997). Gas6 has also been shown to promote the survival of oligodendrocytes (Shankar et al, 2003).

In summary, our study demonstrates that while glucocorticoids appear to promote the decision of a preadipocyte to enter the differentiation pathway, the specific effects of the steroid appear to be mediated at multiple levels and to exert distinct effects on primary human preadipocytes from immortalized murine cells that emphasize the need to focus future studies on the human cell system. While our study focused on pharmacological doses of steroid and preadipocytes derived from subcutaneous tissue from female donors, both regional and gender differences in GR distribution have been described. The elevation of GR levels in male visceral preadipocytes correlates with their predisposition to visceral adipose tissue (Joyner et al, 2000). It will be therefore important to determine the extent to which these differences in GR expression affect the potential of the steroid to stimulate adipogenesis.

CHAPTER III: GLUCOCORTICOIDS PRIME PREADIPOCYTES FOR DIFFERENTIATION.

CONTRIBUTION OF COLLABORATORS

Dongmei Wu and Adele Boudreau both contributed technical assistance to the data presented in this chapter. Dongmei Wu performed the RT-PCR based analysis of mRNA expression (Tables 3, 7 and Figures 6, 8, 9).

Dr. Alan Mears at the University of Ottawa Eye Institute processed the raw microarray data for both the 1μM/48H and 1nM/proliferative pretreatment conditions.

Dr. Alexander Sorisky provided us with primary antibodies for components of the insulin signalling pathway and primary preadipocyte lots for analysis of insulin sensitivity in mature adipocytes (Figure 8). Dr. AnneMarie Gagnon differentiated the human primary preadipocytes into mature adipocytes for Figure 8. She provided us with the stimulated and control cells, with which I performed the insulin signalling assay (Figure 8C) and D. Wu extracted mRNA for RT-PCR analysis (Figure 8B).

INTRODUCTION

Glucocorticoids regulate multiple aspects of adipose tissue physiology including its distribution, differentiation and function. In excess, they promote visceral obesity and contribute to the development of metabolic syndrome (Peeke & Chrousos, 1995). As discussed in the general introduction (page 36), there is growing evidence in both rodent and human correlative studies as well as in murine genetic models that the activity of 11βHSD1

is a key component to the development of the metabolic syndrome and visceral obesity. The mechanistic detail by which altered glucocorticoid signalling contributes to the development of these adverse metabolic conditions remains to be determined.

Preadipocytes are continuously exposed to glucocorticoids *in situ* due to both steroid present in the circulatory system as well as adipose tissue specific 11βHSD1 activity. While the effects of glucocorticoids during differentiation are well studied, the effect of exposure of preadipocytes to glucocorticoids prior to differentiation is unknown. I hypothesized that this would increase their differentiation potential.

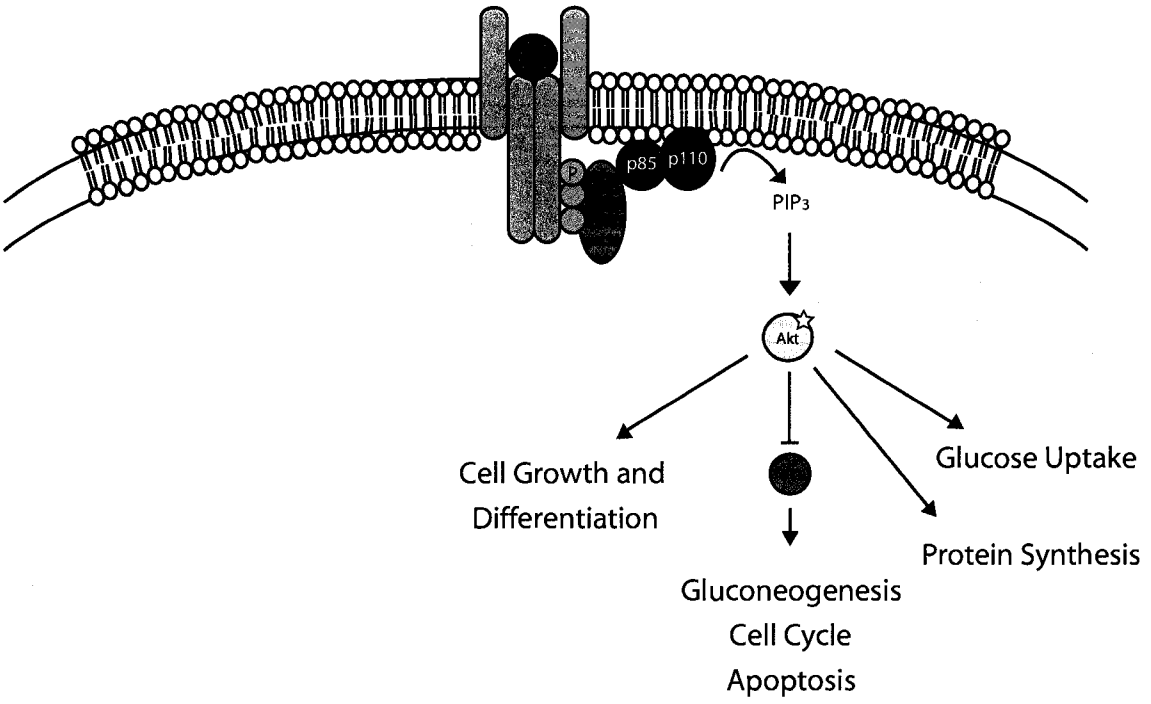
To assess this question, we mimicked the continuous exposure of preadipocytes to physiological concentrations of steroid by incubating the cells with a continuous low-dose concentration (1nM) of dex in the growth medium for 7-10 days during the proliferative phase leading up to confluence and the induction of differentiation. In parallel studies, we treated confluent preadipocytes with a pharmacological concentration of dex for 48 hours prior to the initiation of differentiation. We found that pretreatment with glucocorticoids increased the differentiation potential of both human primary preadipocytes and murine 3T3 L1 preadipocytes; however these cell types were differentially sensitive to steroid. Specifically, both cells types differentiated to a greater extent when pretreated with a pharmacological concentration of dex for 48 h prior to stimulating differentiation. By contrast, only the human cells were sensitive to continuous culture in 1nM dex during proliferation, resulting in higher differentiation potential of these cells compared to control preadipocytes. Microarray analysis performed subsequent to both pretreatment conditions identified candidate genes whose altered expression could mediate these effects as well as previously unidentified targets of glucocorticoid action in preadipocytes.

Of particular interest, we identified that glucocorticoids sensitize human primary preadipocytes to insulin, a proadipogenic hormone that is critical to the differentiation of preadipocytes (Gregoire et al, 1998; Rubin et al, 1978). Insulin binding to the insulin receptor stimulates a tyrosine phosphorylation signalling cascade; ligand binding to the insulin receptor (InsR), a receptor tyrosine kinase, leads to auto-phosphorylation of tyrosine residues within the intracellular domains of the InsR itself. Phosphorylated tyrosine residues serve as docking sites for the insulin receptor substrate effector proteins (IRSs). Tyrosine phosphorylation of IRSs by the InsR leads to IRS binding to and the activation of phosphatidylinositol-3-kinase (PI3K). Akt kinase, a major insulin effector protein, is activated downstream of PI3K. Ultimately, this signalling cascade is involved in the regulation of glucose metabolism and cell growth and differentiation as summarized in Figure 1 and reviewed by Taniguchi and Emanuelli (Taniguchi et al, 2006).

In the present study, we demonstrate that exposure of human preadipocytes to physiological concentration of glucocorticoids led to the up-regulation of key components of the insulin signalling pathway at both the mRNA level and protein level, including the InsR, IRS1, IRS2 and PI3K catalytic subunit (p85α) which correlated with increased insulin signalling. This effect was specific to the human primary preadipocytes and was not observed in mature adipocytes.

Figure 1: Simplified schema of the insulin signalling pathway highlighting PI3K dependent activation of Akt.

Insulin binding to the insulin receptor stimulates a tyrosine phosphorylation signalling cascade. Ligand binding to the insulin receptor (InsR), leads to auto-phosphorylation of tyrosine residues within the intracellular domains of the InsR. These serve as docking sites for the insulin receptor substrate effector proteins (IRSs). Tyrosine phosphorylation of IRSs by the InsR leads to IRS binding to and the activation of phosphatidyl-3-kinase (PI3K) which results in the accumulation of PIP_3. This leads to the activation of several serine/threonine kinases including PDK-1 and -2 (not shown). PDKs phosphorylate Akt kinase on Thr308 and Ser473, resulting in its activation. Activated Akt kinase promotes several insulin-sensitive processes including glucose uptake, protein synthesis and cell growth and differentiation programs. It negatively regulates downstream targets of FoxO transcription factors by inhibiting their transcriptional activity. These targets include gluconeogenesis, cell cycle progression and apoptosis.


p85
p110
PIP3
Akt
Cell Growth and
Differentiation
Glucose Uptake
Protein Synthesis
Gluconeogenesis
Cell Cycle
Apoptosis

RESULTS

Exposure of confluent preadipocytes to glucocorticoids prior to inducing differentiation increases their differentiation potential.

Confluent human primary preadipocytes are induced to differentiate at Day 0 by stimulation with MIX, insulin and dex (MID) for 48 h (Fig 2A panel *i*). Thereafter they are stimulated for 48 h with MIX, insulin and the PPAR agonist, troglitazone, followed by insulin alone. Similarly, post-confluent murine 3T3 L1 preadipocytes are stimulated to differentiate by induction with MID for 48 h followed by continuous culture with insulin alone (Fig 2B panel *iv*).

For our initial analysis, we assessed the differentiation of human primary preadipocytes and 3T3 L1 cells that had been incubated with 1μM and 250nM dex respectively for 48 h upon reaching confluence (Fig 2 panels *ii* and *v*). Differentiation was subsequently induced (Day 0) with either MIX and insulin alone (MI) or MID from 0-48 h. Dex pretreated preadipocytes stimulated with both MI and MID differentiated to a greater extent than control cells as assessed by Oil Red O staining of neutral lipids in mature adipocytes as well as Western analysis of the late adipogenic markers, aP2 and adipsin in the human and murine adipocytes respectively (Fig 3A, B). The additive nature of the sequential steroid treatments (compare ± pretreament + MID) suggested that glucocorticoids may be acting through distinct pathways during these two stimulatory phases; however, there appeared to be some overlapping function as pretreated preadipocytes stimulated with MI differentiated to a much greater extent than control cells (compare ± pretreament + MI).

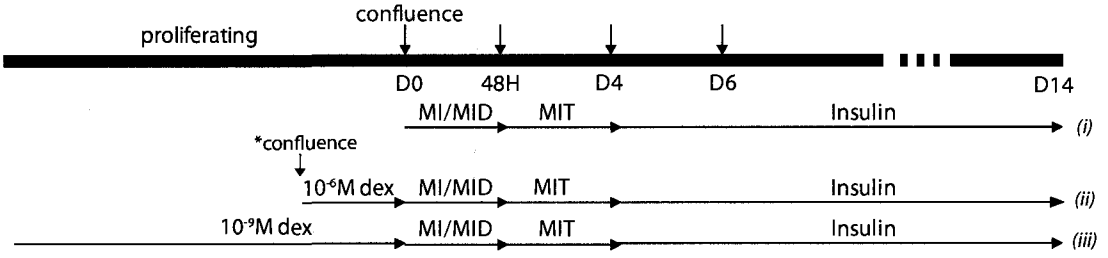
To seek potential mediators of this stimulatory effect, we performed Western analysis on known adipogenic targets of GR to assess their expression profile as the cells approached

Figure 2: Summary of the analysis of modulation of glucocorticoid treatment in differentiation of human primary and murine 3T3 L1 preadipocytes.

(A) Human primary preadipocytes ***(i)*** were grown to confluence (D0) and stimulated for 48 h with 0.5mM MIX, 100nM insulin (MI) in the presence or absence of 1μM dex (MID) in chemically defined medium, followed by 48 h with MIX, insulin and 5μM troglitazone (MIT). Thereafter, the cells were maintained with insulin until Day 14. For 1μM dex pretreatment conditions ***(ii)***, preadipocytes were grown to confluence and held for 48 h in 1μM dex or vehicle in 3% serum then stimulated as described in above. For 1nM dex pretreatment conditions ***(iii)***, cells were maintained in growth medium supplemented with 1nM dex or vehicle for 7-10 days until reaching confluence. Differentiation was induced at Day 0 under standard conditions.

(B) For differentiation of 3T3 L1 preadipocytes, *(iv)* 2 days post-confluent (D0) cells were stimulated with 0.5mM MIX, 100nM insulin (MI) in the presence or absence of 250nM dex (MID) for 48 h in media containing 10% FBS (unless otherwise indicated). Thereafter, the cells were re-fed with media containing insulin every two days and differentiated for 8 days. Upon induction of differentiation, 3T3 L1 cells re-enter the cell cycle and undergo 2-3 rounds of mitotic clonal expansion (MCE) upon which they growth arrest and terminally differentiate. For the 250nM pretreatment conditions (v), cells were incubated with 250nM dex in 3% serum for 48 h upon reaching confluence (D-2). Differentiation was then stimulated (D0) as described above ***(iv)***. ***(vi)*** 3T3 L1 cells were incubated with 1nM dex for 7-10 days through the proliferative growth phase until Day 0. Differentiation was induced with standard inductive cocktail. For each differentiation condition described, Day 0 is defined as the day on which differentiation was induced with differentiation cocktail.

A. **Human Primary Preadipocytes**



B. **Murine 3T3 L1 Preadipoctyes**

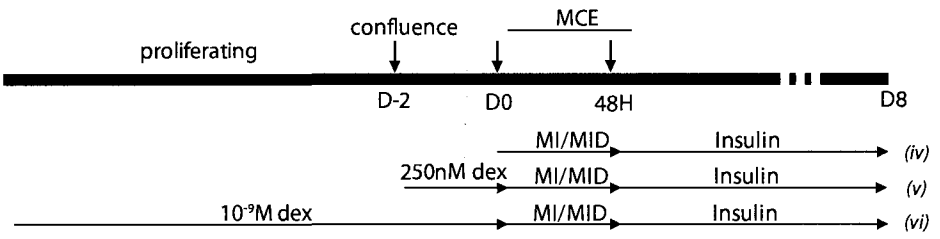
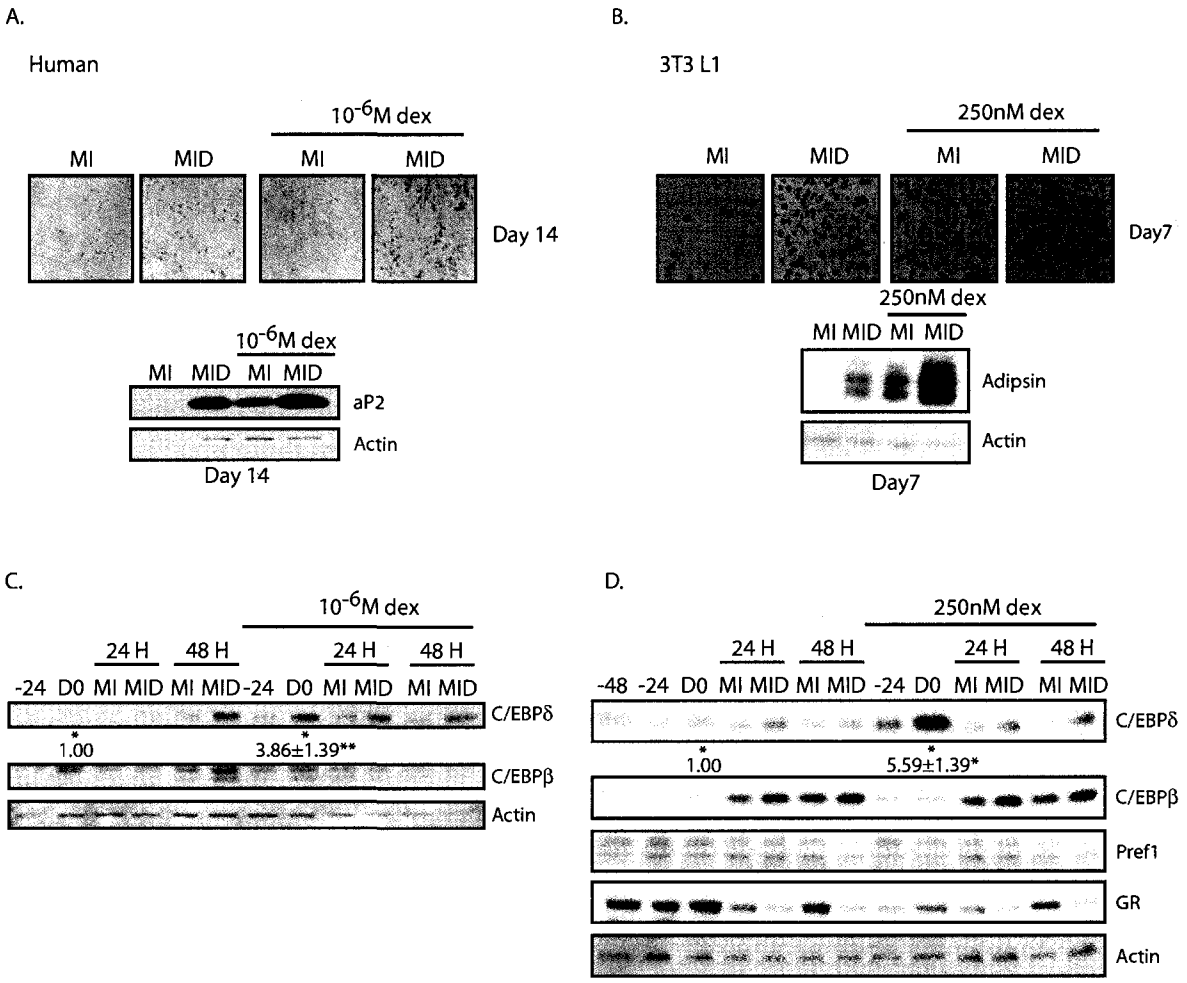


Figure 3: Exposure of confluent human primary and 3T3 L1 preadipocytes to glucocorticoids for 48 h increases their differentiation potential.

Preadipocytes were differentiated as described in Fig 2 panels *ii* and *v* respectively. **(A)** Photomicrographs of Oil Red O staining of neutral lipids (upper panel) and Western analysis of aP2 expression (lower panel) in whole cell extracts derived from Day 14 human adipocytes. **(B)** Photomicrographs of Oil Red O stained adipocytes (upper panel) and Western analysis of adipsin expression (lower panel) in pretreated and control Day 7 3T3 L1 adipocytes. **(C)** Western analysis of C/EBPδ and C/EBPβ expression in pretreated and control human primary preadipocyte whole cell lysates at indicated time points where -24 represents 24 h prior to the onset of differentiation (D0). Where indicated with an (*), C/EBPδ induction was quantified by densitometry using ImageQuant software. Expression is represented as average fold induction relative to D0 ± SD, where n = minimum of 3 independent experiments. **p=0.15. Statistical significance was determined using Students paired T-tests in Microsoft Excel. **(D)** Western analysis of C/EBPδ, C/EBPβ, Pref-1 and GR expression in 3T3 L1 whole cell lysates as described in **(C)** where *p=0.05 for average induction of C/EBPδ expression. All data is representative of a minimum of 3 independent experiments. For differentiation of human primary preadipocytes, experiments were performed with preadipocytes derived from a minimum of 2 separate donor samples and 1 pool of 5 donor samples.


A.
Human
10-6M dex
MI
MID
MI
MID
Day 14
10-6M dex
MI MID MI MID
aP2
Actin
Day 14
B.
3T3 L1
250nM dex
MI
MID
MI
MID
Day7
250nM dex
MI MID MI MID
Adipsin
Actin
Day7
C.
10-6M dex
24 H
48 H
24 H
48 H
-24 D0 MI MID MI MID -24 D0 MI MID MI MID
C/EBPδ
1.00
3.86±1.39**
C/EBPβ
Actin
D.
250nM dex
24 H
48 H
24 H
48 H
-48 -24 D0 MI MID MI MID -24 D0 MI MID MI MID
C/EBPδ
1.00
5.59±1.39*
C/EBPβ
Pref1
GR
Actin

confluence (-24 h) and through the initial 48 h of differentiation in the presence and absence of pretreatment. C/EBPδ was up-regulated following 48 h of dex in both the human (3.86±2.47-fold, p=0.15) and murine (5.59±1.39-fold, p=0.05) cells (Fig 3C, D). In the human cells, C/EBPδ expression remained elevated compared to control cells through the initial 24 h of differentiation and by 48 h its expression was similar to that in the control cells at the same time point. Similarly, while there was a dramatic induction of C/EBPδ expression at -24 h and Day 0 in the 3T3 L1 cells, the high level of expression was not sustained through the initial 48 h of differentiation and its expression normalized by 24 h. Conversely, there was no change in C/EBPβ expression in response to pretreatment with high concentration of dex in both the human and murine preadipocytes (Fig 3C, D).

To evaluate the impact of early induction of C/EBPδ expression on subsequent differentiation potential, we performed both retroviral infection based over-expression of C/EBPδ to mimic dex dependent induction at Day 0, and employed a transient siRNA approach to blunt C/EBPδ induction at Day 0 in a context that was permissive to induction by 24 h MID in 3T3 L1 preadipocytes. While these studies suggested that increased C/EBPδ expression at Day 0 does increase the differentiation potential of 3T3 L1 cells, both of these models retained their sensitivity to the pretreatment with steroid, suggesting that other factors were involved (D.W. data not shown)

Preadipocyte factor 1 (Pref-1) is a glucocorticoid responsive, anti-adipogenic factor whose expression must be down-regulated for differentiation to progress (Smas et al, 1999). Under standard differentiation conditions, Pref-1 expression decreased in 3T3 L1 cells in response to glucocorticoid treatment by 48 h MID stimulation (Fig 3D). When these cells were pretreated with steroid, Pref-1 expression was less than in control cells at Day 0 and

through the initial 48 h. By 48 h MID, its expression was decreased to the same extent as the control cells. Interestingly, expression at 48 h in the absence of dex (MI) was also lower than control cells at that same time point, which could contribute to the increased differentiation observed in the pretreated, MI stimulated cells compared to the non-pretreated cells (Fig 3B, compare ± pretreatment + MI).

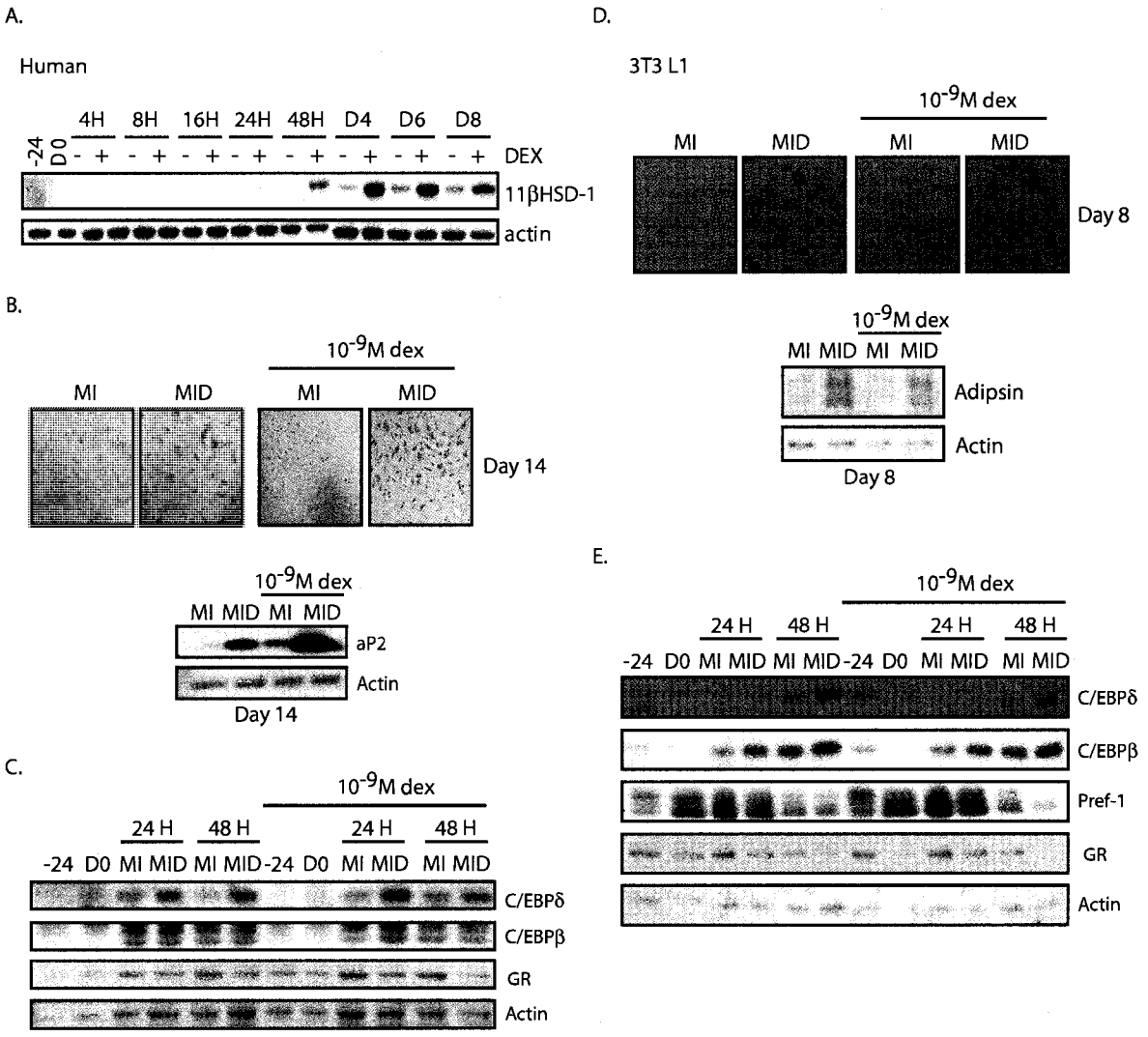
Further analysis of GR expression in 3T3 L1 cells revealed lower expression of the receptor in cells that had been pre-stimulated with steroid (Fig 3D, compare -24 h and Day 0 ± pretreatment). This is consistent with ligand-dependent down-regulation of the receptor that has previously been reported. Interestingly, while GR expression was decreased at Day 0 in pretreated cells compared to control preadipocytes, these cells retained responsiveness to steroid as determined by increased dex-dependent differentiation following pretreatment (Fig 3B, compare pretreated, MI and MID). Thus, GR levels did not appear to be suppressed below levels that mediated the adipogenic effect of dex.

Human primary preadipocytes and murine 3T3 L1 preadipocytes differ in their sensitivity to glucocorticoids.

Human subcutaneous preadipocytes up-regulate 11βHSD1 expression as they mature into adipocytes (Fig 4A and (Bujalska et al, 1997)), thus promoting local production of active steroid within the adipose tissue. To determine if continuous exposure of preadipocytes to physiological concentrations of glucocorticoids could affect their potential to differentiate, we cultured both proliferating human primary and 3T3 L1 preadipocytes in growth media with 1nM dex continuously through to confluence (human) or 2 days post-

Figure 4: Continuous culture of human primary preadipocytes in the presence of 1nM dex increases their differentiation potential.

(A) Western analysis of 11βHSD-1 expression profile during the time course of human preadipocyte differentiation. Preadipocytes were induced to differentiate with MI (- dex) or MID (+ dex) under differentiation conditions described in Fig 2, panel *i*. Whole cell lysates were harvested at the indicated times, where -24 represents 24 h prior to the stimulation with inductive cocktail (D0). **(B)** Human primary preadipocytes were grown in the continuous presence of 1nM dex or vehicle upon seeding through reaching confluence (Day 0) then induced to differentiate in the absence (MI) or presence (MID) of dex as described in Fig 2A, panel *iii*. Photo-micrographs of Oil Red O staining of neutral lipids (upper panel) and Western analysis of aP2 expression (lower panel) in whole cell extracts derived from pretreated and control Day 14 human adipocytes. **(C)** Western analysis of C/EBPδ, C/EBPβ and GR expression in pretreated and control human primary preadipocytes. Cells were harvested and whole cell extracts were prepared at the indicated time points as described in Material and Methods. **(D)** 3T3 L1 preadipocytes were grown in the continuous culture of dex for 7-10 days and induced to differentiate as described in Fig 2B, panel *iv*. Photomicrographs of Oil Red O stained Day 8 adipocytes (upper panel) and Western analysis of adipsin expression (lower panel) in pretreated and control Day 8 3T3 L1 adipocytes. **(E)** Western analysis of C/EBPδ, C/EBPβ, Pref-1 and GR expression in pretreated and control 3T3 L1 preadipocytes harvested at indicated time points. All data is representative of a minimum of 3 independent experiments. For differentiation of human primary preadipocytes, experiments were performed with preadipocytes derived from a minimum of 2 separate donor samples and 1 pool of 5 donor samples.


A.
Human
4H 8H 16H 24H 48H D4 D6 D8
-24 D0 - + - + - + - + - + - + - + - + DEX
11βHSD-1
actin
B.
10-9M dex
MI MID MI MID
Day 14
10-9M dex
MI MID MI MID
aP2
Actin
Day 14
C.
10-9M dex
24 H 48 H 24 H 48 H
-24 D0 MI MID MI MID -24 D0 MI MID MI MID
C/EBPδ
C/EBPβ
GR
Actin
D.
3T3 L1
10-9M dex
MI MID MI MID
Day 8
10-9M dex
MI MID MI MID
Adipsin
Actin
Day 8
E.
10-9M dex
24 H 48 H 24 H 48 H
-24 D0 MI MID MI MID -24 D0 MI MID MI MID
C/EBPδ
C/EBPβ
Pref-1
GR
Actin

confluence (3T3 L1) at which point differentiation was induced under standard differentiation conditions (Day 0) (Fig 2A panel *iii*, B panel *vi*).

Human preadipocytes were sensitive to pretreatment with 1nM dex during their proliferative phase. Cells exposed to 1nM dex had increased differentiation potential compared to control cells as assessed by increased Oil Red O staining of neutral lipids and increased aP2 expression in Day 14 adipocytes (Fig 4B). aP2 expression revealed that the pretreatment resulted in the greatest increase in differentiation when preadipocytes were subsequently induced in the presence of dex (compare ± pretreatment, MID), though there was a modest effect of pretreatment on subsequent dex independent differentiation (compare ± pretreatment, MI).

Western analysis of C/EBPδ, C/EBPβ and GR expression in human preadipocytes as they converge on confluence and during the early phase of differentiation, demonstrated that the expression of these factors was not affected by the pretreatment conditions (Fig 4C).

By contrast to human preadipocytes, 3T3 L1 preadipocytes were not responsive to 1nM dex inclusion during proliferation with respect to subsequent differentiation as assessed by Oil Red O staining and adipsin expression (Fig 4D). Furthermore, C/EBPδ, C/EBPβ, Pref-1 and GR expression profiles were unaffected by pretreatment with 1nM dex (Fig 4E).

Both human primary and 3T3 L1 preadipocytes had increased differentiation potential following 48 h stimulation with a high dose of dex upon reaching confluence. Furthermore, human primary preadipocytes were sensitive to continuous culture in 1nM dex. These results suggested that glucocorticoids primed the cells with increased differentiation capacity. To identify mediators of these priming effects in human preadipocytes, we performed microarray analysis to identify genes whose expression was altered in response to

each of the pretreatment conditions compared to non-pretreated cells. As summarized in Figure 5, subcutaneous preadipocytes from 5 individual donors (average BMI of 22.5 ± 0.2 kg/m^2) were pooled and divided into two experimental conditions. The first group (Fig 5A (i)) was pretreated with either 1μM dex or left untreated for 48 h upon reaching confluence (1μM dex/48H pretreatment). The second group (Fig 5A (ii)) was grown in the presence or absence of 1nM dex in growth media upon seeding through reaching confluence at Day 0 (1nM dex/proliferation pretreatment). RNA was harvested at the time point in which cells would have otherwise been stimulated for differentiation (Day 0) and used for microarray analysis using Affymetrix human genome U133 Plus 2.0 GeneChip arrays. This was performed by StemCore. Following each pretreatment condition, preadipocytes were subsequently differentiated to confirm that the pretreatment promoted increased differentiation in these samples (Fig 5B, C).

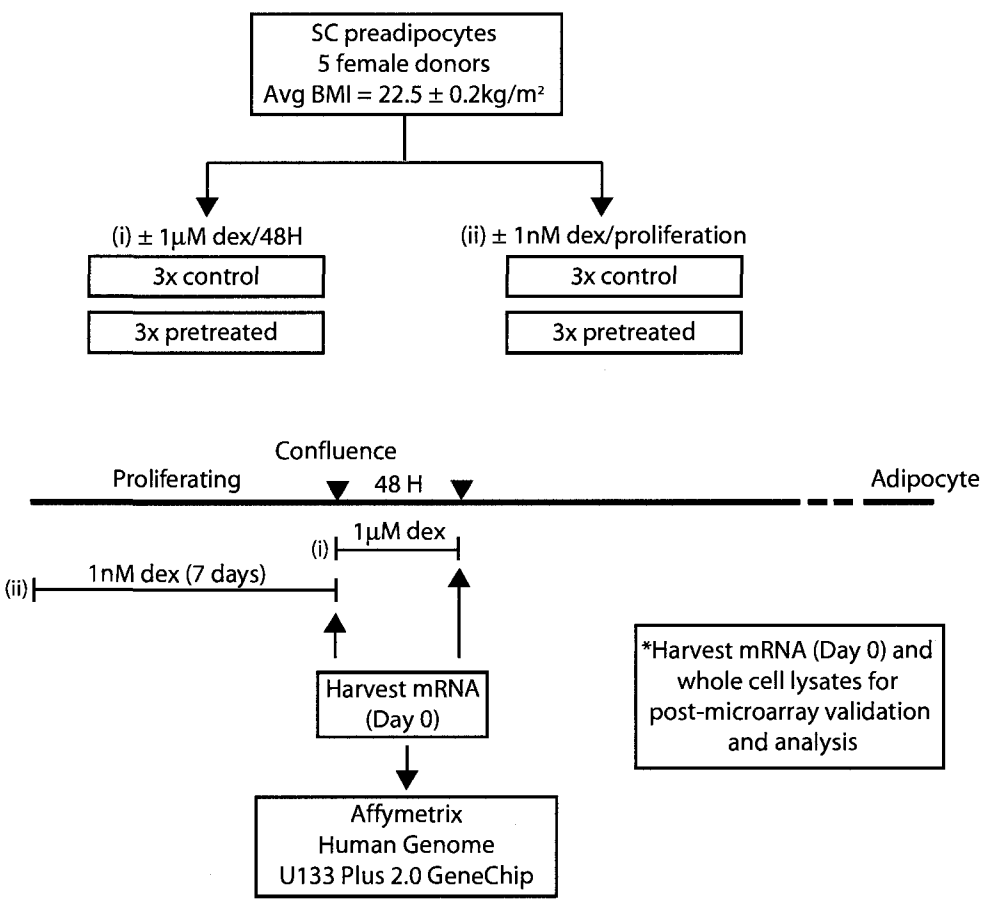
1μM dex/48H pretreatment results in substantial changes in gene expression.

The raw microarray data was stringently processed to identify 'true positives' and establish statistical significance. This was performed in collaboration with Dr. Alan Mears at the University of Ottawa Eye Institute. In the first step, data was analyzed using the MAS5 algorithm to determine absent/present calls for the probesets. Based on MAS5 analysis, any probeset that was considered 'absent' in each of the replicates of both experimental conditions, as well as those probesets that were not calculated as being 'present' on any of the GeneChips, were excluded. From the initial 54 675 probe sets on the GeneChip, of which 62 are Affymetrix control probes, 40 220 probesets (or ~74%) were excluded based on the absent/present call. The signal quantification and normalization for the remaining

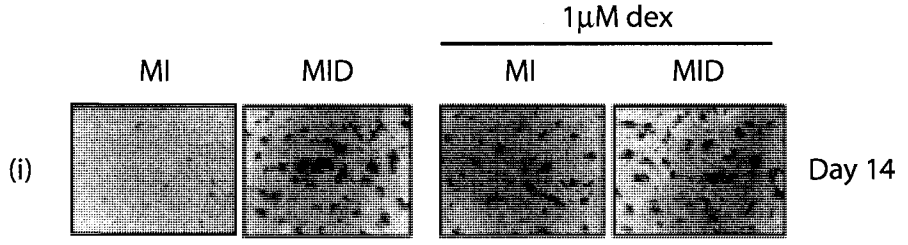
Figure 5: Microarray analysis of gene expression patterns following pretreatment with glucocorticoids.

(A) Outline of experimental design for preparation of microarray samples. Experimental conditions are described in detail in the Material and Methods. Primary subcutaneous preadipocytes isolated from adipose tissue of 5 female donors were pooled into one culture. The cells were then divided into 2 groups: (i) was used for 1μM dex/48H and control pretreatment in triplicate and (ii) for 1nM dex/proliferation and control pretreatments in triplicate. Cells were cultured and stimulated as described and RNA was harvested at the respective (i) and (ii) Day 0. RNA and whole cell lysate samples were also harvested at Day 0 and stored at -80°C for future validation of microarray results and future Western analysis. Microarray analysis was performed using Affymetrix Human Genome U133 Plus 2.0 GeneChip Arrays. Raw data was processed as described in the Materials and Methods. Preadipocytes from each triplicate of both 1μM dex/48H **(B)** and 1nM dex/proliferation **(C)** pretreatment conditions were differentiated for 14 days and stained with Oil Red O to confirm pretreatment efficiency prior to performing microarray analysis. Photomicrographs of one representative of the triplicates for each pretreatment are shown.

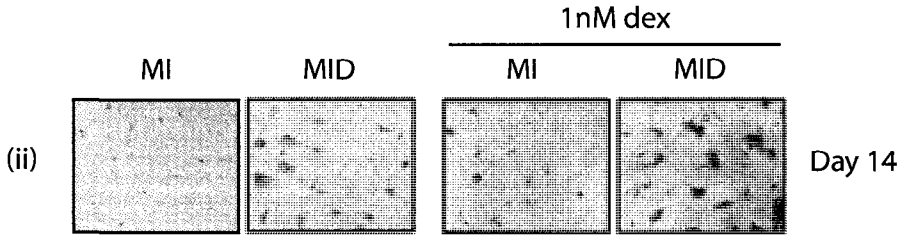
A.


SC preadipocytes
5 female donors
Avg BMI = 22.5 ± 0.2kg/m²
(i) ± 1μM dex/48H
3x control
3x pretreated
(ii) ± 1nM dex/proliferation
3x control
3x pretreated
Confluence
Proliferating
48 H
Adipocyte
(i) 1μM dex
(ii) 1nM dex (7 days)
Harvest mRNA
(Day 0)
*Harvest mRNA (Day 0) and whole cell lysates for post-microarray validation and analysis
Affymetrix
Human Genome
U133 Plus 2.0 GeneChip

B.


1μM dex
MI
MID
MI
MID
(i)
Day 14

C.


1nM dex
MI
MID
MI
MID
(ii)
Day 14

probesets were then generated from the raw GeneChip data using two independent reliable algorithms: RMA (Robust MultiChip Average) and GC-RMA (Irizarry et al, 2003; Yoshida et al, 2004). From this data, average fold change (AFC) values were calculated for the RMA and GC-RMA analysis respectively (+ dex relative to – dex). The statistical validation of probesets with a minimum of 1.5-fold AFC was analyzed using FDRCI (False Discovery Rate Confidence Interval) (Hero, 2004; Yoshida et al, 2004). This generated FDRCI-derived P-values. Any probeset with a P-value of 1 was discarded. A minimum AFC of 1.5-fold was chosen for the following reasons. C/EBPδ is a known transcriptional target of GR and we had shown C/EBPδ protein to be induced by dex in this system ((Cao et al, 1991), Fig 3C). It therefore served as an internal control in our microarray analysis; its mRNA was induced 1.641-fold in response to dex by RMA-based analysis. Given this fold induction and knowing that microarrays tend to quantitatively underestimate the expression fold-change, we established a cut-off of 1.5-fold change in gene expression for statistical analysis.

Based on these criteria, microarray analysis of gene expression following 1μM/48H pretreatment revealed a significant fold change of ≥1.5-fold in 677 probe sets based on both the RMA and GC-RMA analysis. Of these, 483 probesets were induced in response to glucocorticoid pretreatment and 239 probesets were decreased. These represented gene products that are involved in multiple aspects of cellular function including transcriptional regulators, regulators of the cell cycle and apoptosis, components of the cytoskeleton, factors involved in cell mobility and adhesion, skeletal development, and signalling pathways including Wnt, growth factor and insulin signalling components. A complete table of microarray results can be found in Appendix B. For simplicity, the AFC values derived from RMA analysis is reported herein and in Appendix B.

Genes that showed the greatest fold change following pretreatment with 1μM dex:

Of the 677 probe sets that were differentially expressed in response to dex, 12 genes were induced and 2 genes were repressed ≥ 5-fold (Table 1). These diverse gene products have been implicated in the regulation of metabolic processes (ADH1B, GPX3, PDK4, GLUL), transcription (GPX3, DK2P586A0522) and cell cycle (ADAMTS1), and they comprise components of signal transduction pathways (DKK1, ERRFI1), Golgi apparatus (GALNTL2) and cytoskeleton (ADAMST1, POSTN), a protease inhibitor (SERPINE 2) and a oxidative stress response (GPX3, SEPP1).

Glucocorticoid Sensitive Transcriptional Regulatory Factors:

My main research interest has been the transcriptional regulation of adipogenesis and specifically, the effects of glucocorticoids on the early transcriptional events that drive differentiation. Table 2 comprises a list of transcription factors whose expression was differentially regulated 1.5-fold or greater at Day 0 in response to glucocorticoid treatment. In summary, 47 genes previously been shown to be involved in the regulation of transcription were induced and 17 were repressed. For a subset of these genes, namely GPX3, BTG1, HIPK2, FOXO1A, LMO3, ELL2, FOXO3A, KLF9, TTRAP, C/EBPδ, KLF6, KLF15, CREB3L1 and JUN, the fold change in mRNA abundance was validated by quantitative real-time PCR (qRT-PCR) analysis of mRNA samples that were harvested coincident with the microarray samples (Table 3).

Table 1: Glucocorticoid responsive genes with an average fold change (AFC) ≥ 5 fold following pretreatment with 1μM dex for 48 h in human primary preadipocytes. AFC was determined using RMA algorithms and all genes reached statistical significance as determined by FDR-CI as described in Materials and Methods.

Unigene (avadis)	Gene Symbol	Gene Name	AFC	Function
Hs.4	ADH1B	alcohol dehydrogenase IB (class I), beta polypeptide	29.972	Metabolism (alcohol)
Hs.386793	GPX3	glutathione peroxidase 3 (plasma)	18.622	Metabolism (anti-oxidative)
Hs.558328	FKBP5	FK506 binding protein 5	10.182	Protein Folding
Hs.411308	GALNTL2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	7.815	Golgi component
Hs.8364	PDK4	pyruvate dehydrogenase kinase, isoenzyme 4	7.514	Metabolism (glucose)
Hs.567339	C20orf118	Chromosome 20 open reading frame 118	7.232	
Hs.40499	DKK1	dickkopf homolog 1 (Xenopus laevis)	7.121	Wnt signalling antagonist
Hs.518525	GLUL	glutamate-ammonia ligase (glutamine synthetase)	6.958	Glutamine biosynthesis, Nitrogen metabolism
Hs.11169	ERRFI1	ERBB receptor feedback inhibitor 1	6.487	Stress response, Rho GTPase activator
Hs.534115	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	6.177	Cell proliferation (negative), EM component
Hs.288771	DKFZP-586A0522	DKFZP586A0522 protein	5.927	S-adenosylmethionine-dependent methyl-transferase activity
Hs.275775	SEPP1	selenoprotein P, plasma, 1	5.152	Oxidative stress response
Hs.136348	POSTN	periostin, osteoblast specific factor	-6.028	Skeletal Development (EM)
Hs.38449	SERPINE2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	-8.096	Serine protease inhibitor, Development and cell differentiation

Table 2: Glucocorticoid responsive transcriptional regulatory factors with average fold change (AFC) ≥ 1.5 fold in confluent human primary preadipocytes exposed to 1μM dex for 48 h. AFC was determined using RMA algorithm and statistical significance was analyzed using FDR-CI. Factors highlighted in bold were validated by qRT-PCR (see Table 3).

Unigene (avadis)	Gene Symbol	Gene Name	AFC
Hs.386793	**GPX3**	**glutathione peroxidase 3 (plasma)**	18.622
Hs.255935	**BTG1**	**B-cell translocation gene 1, anti-proliferative**	4.008
Hs.194329	TCEAL4	transcription elongation factor A (SII)-like 4	3.696
Hs.397465	**HIPK2**	**Homeodomain interacting protein kinase 2**	3.690
Hs.370666	**FOXO1A**	**forkhead box O1A (rhabdomyosarcoma)**	3.186
Hs.504908	**LMO3**	**LIM domain only 3 (rhombotin-like 2)**	3.054
Hs.468490	LHCGR	Luteinizing hormone/choriogonadotropin receptor	2.739
Hs.166017	MITF	microphthalmia-associated transcription factor	2.590
Hs.79334	NFIL3	nuclear factor, interleukin 3 regulated	2.425
Hs.192221	**ELL2**	**elongation factor, RNA polymerase II, 2**	2.373
Hs.220950	**FOXO3A**	**forkhead box O3A**	2.227
Hs.12420	PHF17	PHD finger protein 17	2.157
Hs.360174	SNAI2	snail homolog 2 (Drosophila)	2.149
Hs.522074	TSC22D3	TSC22 domain family, member 3	2.137
Hs.503093	ZFP36L2	zinc finger protein 36, C3H type-like 2	1.996
Hs.519445	NR2F1	Nuclear receptor subfamily 2, group F, member 1	1.935
Hs.150557	**KLF9**	**Kruppel-like factor 9**	1.909
Hs.84928	NFYB	nuclear transcription factor Y, beta	1.909
Hs.95243	TCEAL1	transcription elongation factor A (SII)-like 1	1.897
Hs.136398	ZCCHC6	zinc finger, CCHC domain containing 6	1.816
Hs.192221	ELL2	elongation factor, RNA polymerase II, 2	1.793
Hs.403010	**TTRAP**	**TRAF and TNF receptor associated protein**	1.762
Hs.473317	PCMTD2	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 2	1.753
Hs.76884	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	1.728

Hs.487046	SOD2	superoxide dismutase 2, mitochondrial	1.713
Hs.288773	ZNF294	zinc finger protein 294	1.708
Hs.478588	BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51)	1.708
Hs.196102	RB1CC1	RB1-inducible coiled-coil 1	1.699
Hs.311776	TCEAL3	transcription elongation factor A (SII)-like 3	1.698
Hs.126550	VPS4B	vacuolar protein sorting 4B (yeast)	1.656
Hs.440829	**CEBPD**	**CCAAT/enhancer binding protein (C/EBP), delta**	1.641
Hs.134859	MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	1.627
Hs.506829	LASS6	LAG1 longevity assurance homolog 6 (S. cerevisiae)	1.581
Hs.513609	RBL2	retinoblastoma-like 2 (p130)	1.576
Hs.326387	MORF4L2	mortality factor 4 like 2	1.576
Hs.171426	NCOA7	nuclear receptor coactivator 7	1.574
Hs.446678	NCOA2	Nuclear receptor coactivator 2	1.573
Hs.4055	**KLF6**	**Kruppel-like factor 6**	1.565
Hs.166017	MITF	microphthalmia-associated transcription factor	1.565
Hs.272215	**KLF15**	**Kruppel-like factor 15**	1.551
Hs.434286	CHES1	checkpoint suppressor 1	1.551
Hs.347991	NR2F2	nuclear receptor subfamily 2, group F, member 2	1.548
Hs.434953	HMGB2	high-mobility group box 2	1.543
Hs.435535	ZNF395	zinc finger protein 395	1.541
Hs.155396	NFE2L2	nuclear factor (erythroid-derived 2)-like 2	1.540
Hs.478589		Hypothetical LOC389185	1.517
Hs.135406	CEBPZ	CCAAT/enhancer binding protein zeta	1.516
Hs.5308	UBA52	ubiquitin A-52 residue ribosomal protein fusion product 1	-1.556
Hs.190977	ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	-1.583
Hs.486993	TULP4	tubby like protein 4	-1.625
Hs.530930	ZNF423	zinc finger protein 423	-1.642
Hs.431498	FOXP1	forkhead box P1	-1.645
Hs.58756	PER2	period homolog 2 (Drosophila)	-1.676

Hs.405961	**CREB3L1**	**cAMP responsive element binding protein 3-like 1**	-1.720
Hs.149261	RUNX1	runt-related transcription factor 1	-1.935
Hs.443687	FHL2	four and a half LIM domains 2	-2.043
Hs.516826	TRIB3	tribbles homolog 3 (Drosophila)	-2.185
Hs.282326	DSCR1	Down syndrome critical region gene 1	-2.294
Hs.491745	TCEA1	transcription elongation factor A (SII), 1	-2.343
Hs.190977	ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	-2.627
Hs.326035	EGR1	Early growth response 1	-2.907
Hs.525704	**JUN**	**v-jun sarcoma virus 17 oncogene homolog (avian)**	-2.926
Hs.515162	CALR	calreticulin	-3.949
Hs.136348	POSTN	periostin, osteoblast specific factor	-6.028

Table 3. Quantitative RT-PCR based validation of average fold change (AFC) in mRNA levels of selected transcriptional regulatory factor genes. Factors were identified by microarray analysis of human preadipocytes following 48 h pretreatment with 1μM glucocorticoid as compared to untreated cells.

Gene	AFC Microarray	Fold Change qRT-PCR	Previously Identified Effect on Adipogenesis
Upregulated			
BTG1	4.008	4.62	
C/EBPδ	1.641	2.67	Stimulatory (Cao et al, 1991; Gregoire et al, 1998)
ELL2	2.373	2.74	
FOXO1A	3.186	7.85 ± 1.01*	Inhibitory (CA isoform) (Nakae et al, 2003)
FOXO3A	2.227	3.12 ± 0.54*	
GPX3	18.622	72.1	
HIPK2	3.690	6.74	
KLF6	1.551	2.99	Stimulatory (Li et al, 2005)
KLF9	1.909	3.43	
KLF15	1.551	25.0	Stimulatory (Mori et al, 2005)
LMO3	3.054	18.8 ± 5.43*	
TTRAP	1.762	2.0	
Downregulated			
CREB3L1	-1.720	- 1.95	
JUN	-2.926	-1.8	

*average of 3 experiments done in replicate ± SEM. Other values represent analysis from one replicate using either duplicate RNA samples from the microarray or 3T3 L1 cells as indicated. For these samples, RT-PCR analysis was done in duplicate, where error between duplicates was between 0.1 and 1.6%

1μM dex/48H pretreatment increases the insulin sensitivity of human primary preadipocytes

One unexpected finding of the microarray analysis was the steroid dependent enrichment in components of the insulin signalling pathway, as this contrasted the predominant insulin resistance promoting function of glucocorticoids (Table 4). Specifically, there was a dex dependent induction of the insulin receptor (InsR) (+3.8 fold), insulin receptor substrate 1 and 2 (IRS1, IRS2) (+1.6 fold and +1.9 fold respectively) and phosphoinositide-3-kinase, regulatory subunit 1 (PI3K p85α) (+3.8 fold). Each of these factors were validated by qRT-PCR analysis (Fig 6A). Furthermore, the dex dependent increase in mRNA correlated with increased protein expression at Day 0 (Fig 6B). PI3K is comprised of both a regulatory (p85) and catalytic (p100) subunit. Notably, while p85α expression was increased both at the mRNA and protein level, we did not observe a concomitant increase in PI3K catalytic subunit, p110α, expression (Fig 6B).

In light of these findings in the human preadipocytes, 3T3 L1 preadipocytes gave an unexpected result in which 1μM/48H pretreatment resulted in a non-significant decrease in InsR (0.98 ± 0.59-fold) and IRS1 (0.68 ± 0.42-fold) mRNA and a non-significant increase in IRS2 (1.30 ± 0.63-fold) mRNA measured by qRT-PCR (Fig 6C).

Having shown increased expression of components of the insulin signalling pathway in the human preadipocytes, we next sought to determine if this rendered pretreated cells more sensitive to subsequent insulin stimulation. Indeed, we observed steroid dependent increased insulin pathway signal transduction as assessed by Western analysis of protein tyrosine phosphorylation (P-Tyr) in response to 5 minute stimulation with insulin (insulin,

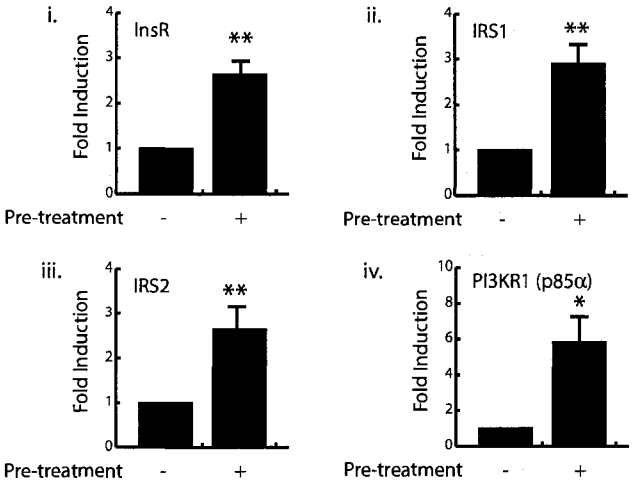
Table 4: Components of the insulin signalling cascade are up-regulated in response to 1μM dex for 48 h in confluent human primary preadipocytes. Average fold change (AFC) was determined using RMA algorithms. Statistical validation was calculated using FDRCI.

Unigene (avadis)	Gene Symbol	Gene Name	AFC
Hs.132225	PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	3.779
Hs.442344	IRS2	insulin receptor substrate 2	1.904
Hs.465744	INSR	insulin receptor	1.593
Hs.471508	IRS1	insulin receptor substrate 1	1.585

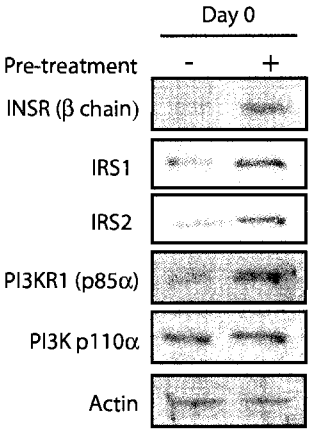
Figure 6: Glucocorticoids increase key components of the insulin signalling pathway in human primary preadipocytes.

(**A**) Real-time PCR analysis of average fold induction in mRNA abundance at Day 0 of the insulin receptor (InsR), insulin receptor substrate 1 (IRS1), IRS2 and the regulatory domain of PI3K (PI3KR1 or p85α) in 1μM dex/48H pretreated (+) compared to control (-) human preadipocytes. For analysis, n=3, where samples were derived from two independent repeats using the pooled culture of preadipocytes from 5 donors, and one individual donor sample. Each reaction was performed in duplicate. Data is plotted ± SEM; *$p \leq 0.05$, **$p \leq 0.01$ as determined using a Student's paired T-test. (**B**) Western analysis of protein expression at Day 0 in control (-) and pretreated (+) cells. Average fold induction ± SD was calculated from Western analysis by densitometry using ImageQuant software; $n \geq 3$, including preadipocyte samples from a minimum of one individual donor and one pooled culture. P-value was determined as described in (A). (**C**) RT-PCR analysis of InsR, IRS1 and IRS2 expression following 250nM dex/48H pretreatment in 3T3 L1 cells. Each reaction was performed in duplicate. Data represents average fold induction ± SEM where n=3. Fold changes did not reach statistical significance using a Student's paired T-test.

A.

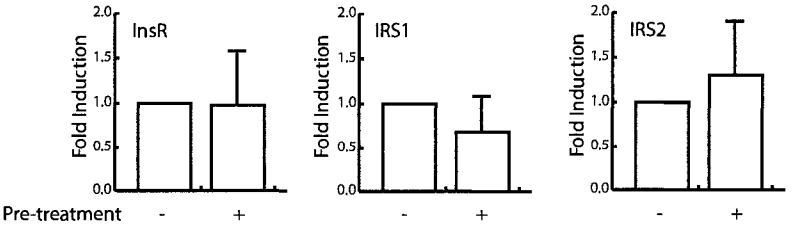


B.



	Average Fold Induction ± SD	P Value
INSR (β chain)	1.92 ± 0.90	0.03
IRS1	2.32 ± 0.62	0.02
IRS2	2.60 ± 1.06	0.06
PI3KR1 (p85α)	3.12 ± 0.40	0.16
PI3K p110α	0.61 ± 0.29	0.22

C.



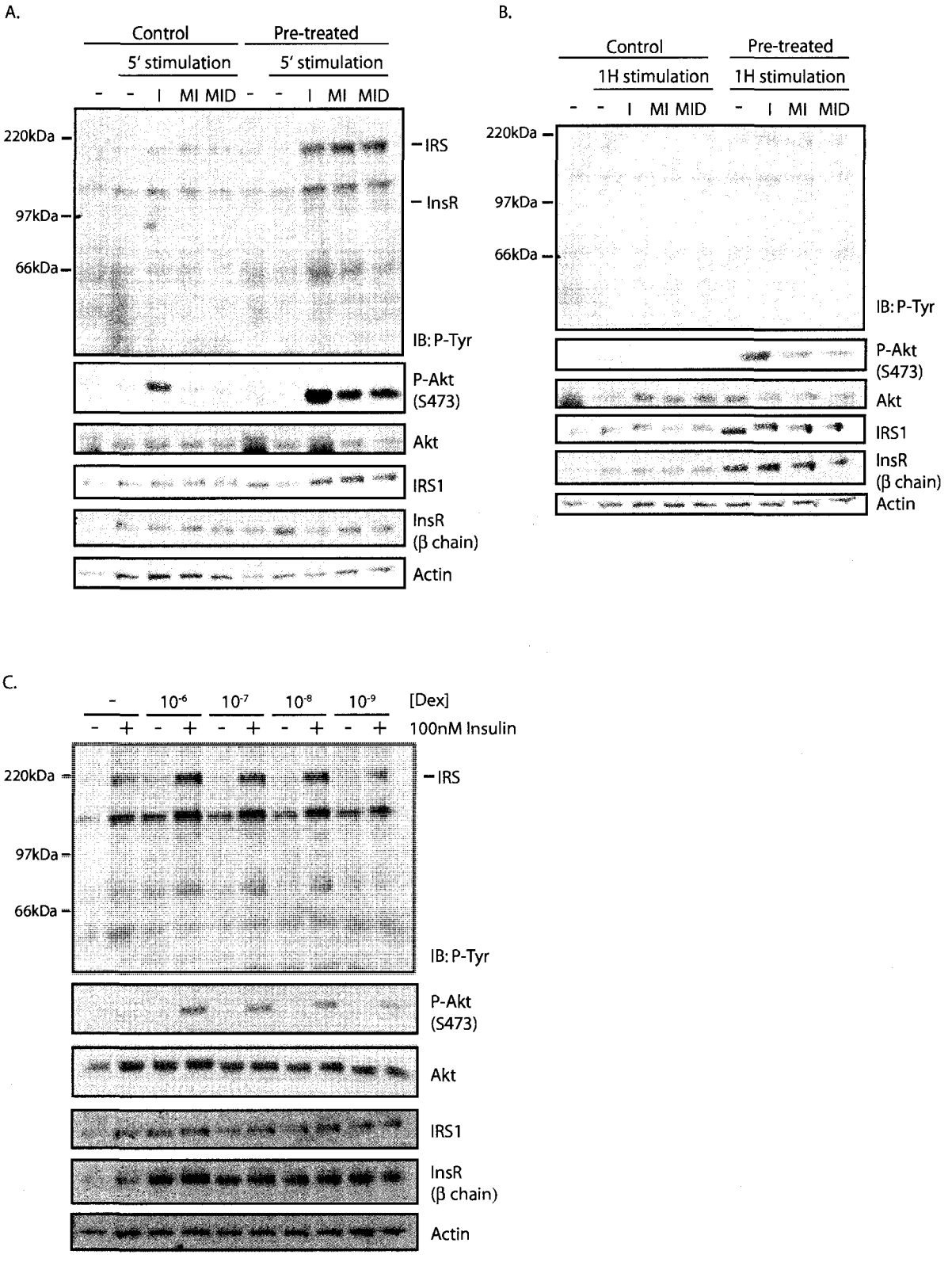
MI or MID) (Fig 7A). Specifically, there was a strong induction of P-Tyr signal that overlaid with the anti-IRS1 signal (~185kDa) and a faint P-Tyr substrate around 97kDa that overlaid with anti-InsR signal. The increase in P-Tyr correlated with increased activation of the downstream effector Akt, as assessed by Western analysis of Akt-Ser473 phosphorylation. Phosphorylation was maximal following stimulation with insulin alone, whereas there was no detectable difference when stimulated with MI or MID, suggesting that MIX negatively regulates Akt-Ser473 phosphorylation. By 1 h of stimulation, the levels of P-Tyr had been attenuated; however, there was sustained activation of Akt (phospho-Ser473) that was higher in pretreated cells as compared to control preadipocytes (Fig 7B).

To determine if this response was sensitive to physiological concentrations of hormone, we titrated the concentration of dex during the 48 h pretreatment of preadipocytes and assessed their response to insulin stimulation (Fig 7C). Exposure of human preadipocytes to 10^{-8}M, but not 10^{-9}M hormone was sufficient to increase insulin sensitivity as assessed by both increased P-Tyr and activation of Akt in response to stimulation with 100nM insulin for 5 minutes. Taken together, these results suggest that exposure of adipose tissue to physiological concentrations of glucocorticoids could contribute to promoting adipogenesis by sensitizing preadipocytes to insulin.

Glucocorticoid dependent up-regulation of the insulin signalling pathway in preadipocytes is particularly interesting as glucocorticoids have been reported to predominantly antagonize the actions of insulin in target tissues including adipose tissue, and when present in excess, glucocorticoids can promote insulin resistance. While the exact underlying mechanisms for this action are undefined, there are reports that glucocorticoids attenuate insulin signalling by decreasing IRS1 expression in primary cultured rat adipocytes, 3T3 L1 and 3T3 F442A adipocytes and omental human primary adipoctyes

Figure 7: Pretreatment of human preadipocytes with physiological concentrations of glucocorticoids results in increased insulin sensitivity.

(A) Control and 1μM dex/48H pretreated human preadipocytes (Day 0), were either non-stimulated (-) or stimulated with 100nM insulin (I), 0.5mM MIX and 100nM insulin (MI) or MI and 1μM dex (MID) in serum-free, chemically defined media for 5 minutes or **(B)** 1 H, upon which cells were immediately processed for Western analysis. Western analysis of total cellular phosphorylated tyrosine residues (P-Tyr) (top immunoblot) revealed major bands that overlaid with IRS signal and minor bands that overlaid with InsR signal which are indicated in panel A. Immunoblots were serially reprobed with antibodies against phospho-Akt (phosphorylated-S473), total cellular Akt, IRS1 and the InsR (β–chain). **(C)** Confluent preadipocytes were pretreated as previously described, but with exposure to varying concentrations of dex (10^{-6}M to 10^{-9}M). Level of insulin signalling was assessed as in (A) following 5 minute stimulation with 100nM insulin (+) in serum-free media, compared to non-stimulated control cells (-). All panels are representative of a minimum of 3 independent experiments including a minimum of 1 repeat using preadipocytes from a pool of 5 donor samples, and 2 individual donor samples.


A.
Control
Pre-treated
5' stimulation
5' stimulation
- - I MI MID - - I MI MID
220kDa
97kDa
66kDa
IRS
InsR
IB: P-Tyr
P-Akt (S473)
Akt
IRS1
InsR (β chain)
Actin
B.
Control
Pre-treated
1H stimulation
1H stimulation
- - I MI MID - I MI MID
220kDa
97kDa
66kDa
IB: P-Tyr
P-Akt (S473)
Akt
IRS1
InsR (β chain)
Actin
C.
- 10-6 10-7 10-8 10-9 [Dex]
- + - + - + - + - + 100nM Insulin
220kDa
97kDa
66kDa
IRS
IB: P-Tyr
P-Akt (S473)
Akt
IRS1
InsR (β chain)
Actin

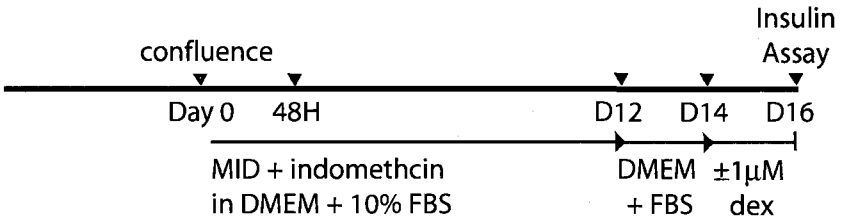
(Buren et al, 2002; Lundgren et al, 2004; Turnbow et al, 1994). To determine if glucocorticoid induced insulin sensitization was specific to the human primary preadipocytes we compared the responsiveness of lipid-laden human adipocytes to insulin following 48 h incubation with 1μM dex (Fig 8A). InsR was slightly, but significantly up-regulated (+1.43 ± 0.18, $p = 0.05$), there was no statistically significant change in IRS1 expression (+1.29 ± 0.29, $p = 0.22$) and while IRS2 mRNA was induced +2.63 ± 1.18-fold, this also did not reach statistical significance over three repeats ($p = 0.19$) (Fig 8B). The minimal induction of InsR mRNA was insufficient to confer insulin sensitivity as specific assessment of insulin signalling revealed that glucocorticoids did not affect mature adipocytes' responsiveness to insulin (Fig 8C).

Our results highlight that the effects of glucocorticoids on InsR and IRS expression are cell type specific. This suggested that the transcriptional effects of glucocorticoids were dependent on either cell-type specific auxilliary factors and/or were mediated indirectly through a factor(s) whose expression is differentially regulated by GR within the different cellular contexts. The Forkhead box 1A (FoxO1A) transcription factor has recently been demonstrated to up-regulate both the InsR gene and IRS2 through sequence specific binding to a consensus motif in their respective promoters (Ide et al, 2004; Puig et al, 2003; Puig & Tjian, 2005). Both FoxO1A and the related family member FoxO3A, which bind the same consensus DNA sequences, were induced in response to 1μM dex/48H pretreatment in the microarray (+3.186 and +2.227 respectively, Table 3). We therefore assessed the expression profiles of both FoxO1A and FoxO3A in response to 1μM dex/48H treatment within the three adipogenic contexts to determine if they correlated with induction of InsR and/or the

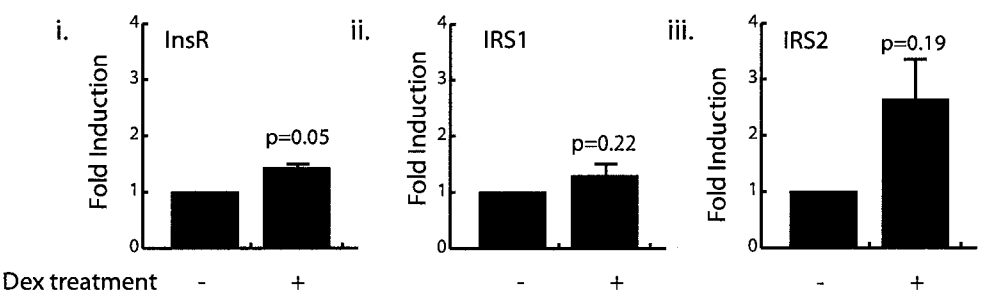
Figure 8: Glucocorticoids do not increase insulin sensitivity in human adipocytes.

(A) Human primary subcutaneous preadipocytes were differentiated using a modified differentiation protocol to maximize differentiation efficiency as described in detail in the Materials and Methods. Cells were differentiated in complete serum and in the continuous presence of 0.5μM dex for 11-12 days. Dex was withdrawn for 48h prior to the stimulation of Day 14 (D14) adipocytes with 1μM dex for 48h. **(B)** RT-PCR analysis of InsR, IRS1 and IRS2 mRNA expression in D16 adipocytes following 48 h treatment with vehicle (-) or dex (+). Each reaction was performed in duplicate. Data represents average fold induction ± SEM, where n=3 (1 pooled sample and 2 individual donor samples). P values were determined using Student's paired *T*-tests. **(C)** Western analysis of the activated insulin signalling pathway as described in Fig 7, including pan-phosphorylated tyrosine containing proteins (P-Tyr), phospho-Akt (P-Akt, S473), total Akt, IRS1 and InsR. Control and dex treated adipocytes were stimulated with vehicle (-) or 100nM insulin (+) for 5 minutes prior to harvesting. Results are representative of three repeats as described in (B).

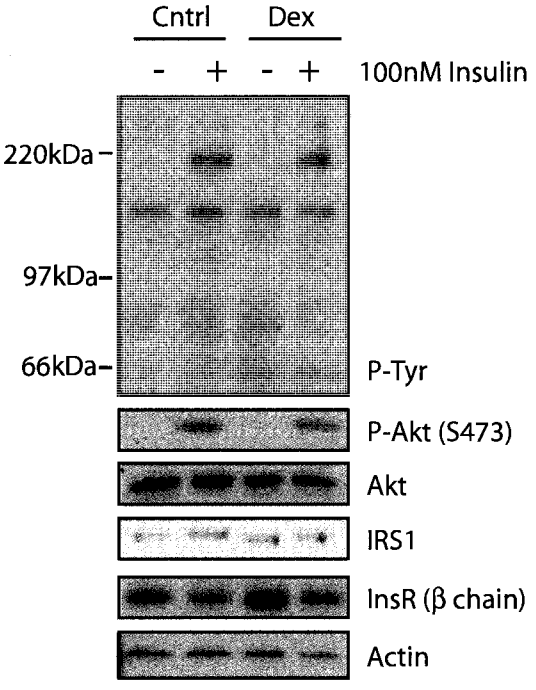
A.


confluence
Insulin
Assay
Day 0
48H
D12
D14
D16
MID + indomethcin
in DMEM + 10% FBS
DMEM
+ FBS
±1μM
dex

B.


i.
InsR
Fold Induction
p=0.05
ii.
IRS1
p=0.22
iii.
IRS2
p=0.19
Dex treatment
-
+

C.


Cntrl
Dex
- + - +
100nM Insulin
220kDa
97kDa
66kDa
P-Tyr
P-Akt (S473)
Akt
IRS1
InsR (β chain)
Actin

IRSs (Fig 9). FoxO1A mRNA was induced 7.85 ± 1.01-fold, and its protein expression increased 1.92 ± 0.70-fold in pretreated, Day 0 human primary preadipocytes (Fig 9A). Similarly, FoxO3A mRNA was induced 3.12 ± 0.54-fold and its protein expression increased 3.08 ± 0.68-fold in Day 0 preadipocytes. By contrast, FoxO1A mRNA was induced 1.90 ± 0.23-fold in pretreated 3T3 L1 preadipocytes and there was no significant change in FoxO3A expression (1.34 ± 0.44-fold) (Fig 9B). These results suggest that differential responsiveness of FoxO1A and FoxO3A, to glucocorticoids might account for the difference in glucocorticoid effect on human primary and 3T3 L1 preadipocytes. However, both FoxO1A and FoxO3A were induced in the human adipocytes, albeit not as strongly as in the human preadipocytes (4.32 ± 0.90 and 1.86 ± 0.15 respectively) (Fig 9C). The induction of FoxO1A and FoxO3A in human preadipocytes suggests the potential of a more complex relationship between the expression of these factors and their activity.

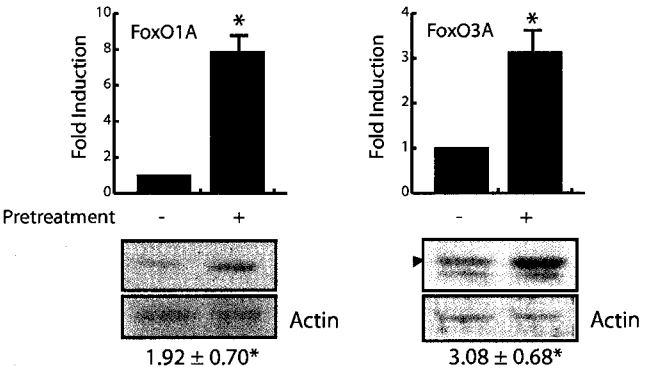
Exposure of human primary preadipocytes to 1nM dex during proliferation leads to minimal alterations in gene expression profiles at Day 0

Continuous culture of human primary preadipocytes in the presence of 1nM dex for 7 days, which strongly potentiated differentiation, nonetheless had only a minimal effect on the gene expression profiles of human primary cells as they reached confluence (Day 0). Microarray analysis was performed as was described previously. Based on both RMA and GC-RMA analysis, we identified 7 up-regulated and 9 repressed genes whose mRNA abundance was significantly altered ≥1.5-fold based on FDR-CI analysis, in response to exposure to glucocorticoids (Table 5). These gene products were associated with metabolic processes (CPM, ADH1B, MAOA, CD36 and MMP1), regulation of transcription

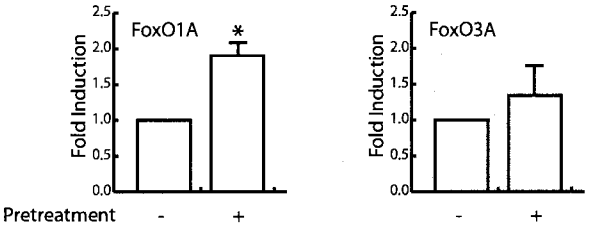
Figure 9: FoxO transcription factors are differentially regulated by glucocorticoids.

(A) (upper panels) Average fold change of FoxO1A and FoxO3A mRNA expression following 1μM dex/48H pretreatment in confluent human preadipocytes as quantified by RT-PCR performed in duplicate. Data is represented ± SEM, where n= a minimum of 3 as described in Fig 6. (lower panel) Western analysis of FoxO protein expression in control and pretreated preadipocytes at Day 0. Signal intensities were quantified by densitometry using ImageQuant software and used to calculate average fold induction by dex compared to control ± SD where $n \geq 3$ (* $p \leq 0.05$). **(B)** Average fold change in FoxO mRNA expression in response to 250nM dex/48H pretreatment in 3T3 L1 preadipocytes. Each reaction was performed in duplicate. Data represents average fold induction ± SEM where n=3 (*$p \leq 0.05$). **(C)** Average fold change in FoxO mRNA expression ± SEM in response to 1μM dex/48H treatment in human adipocytes (* $p \leq 0.05$). RNA samples used for analysis are the same as described in Fig 8.

A. Human Preadipocyte: 1μM dex/48H



B. 3T3 L1 Preadipocyte: 1μM dex/48H



C. Human Adipocyte: 1μM dex/48H

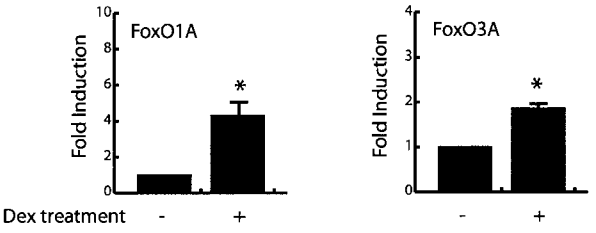


Table 5: Average fold change (AFC) in gene expression in confluent (Day 0) human primary preadipocytes. Preadipocytes were grown in the continuous presence of 1nM dex for 7 days until reaching confluence upon which RNA was harvested as described in Fig 5. Microarray data was processed as described in Table 2 and in the Materials and Methods. The induction of LMO3 (in bold) was validated by RT-PCR (Table 7).

Unigene (avadis)	Gene Symbol	Gene Name	AFC	Function
Hs.484551	CPM	carboxypeptidase M	2.102	Metabolism (aromatic compounds), Immune response
Hs.4	ADH1B	alcohol dehydrogenase IB (class I), beta polypeptide	1.710	Metabolism (alcohol)
Hs.183109	MAOA	monoamine oxidase A	1.631	Metabolism (catacholamines), neurotransmitters catabolism
Hs.1584	COMP	cartilage oligomeric matrix protein	1.618	Skeletal Development (EM)
Hs.522074	TSC22D3	TSC22 domain family, member 3	1.589	Transcription Factor
Hs.21422	NRCAM	neuronal cell adhesion molecule	1.580	Cell adhesion, Neuronal cell migration
Hs.504908	**LMO3**	**LIM domain only 3 (rhombotin-like 2)**	1.521	Transcription Factor
Hs.106015	DDEF1	development and differentiation enhancing factor 1	-1.548	Regulator of GTPase activity
Hs.159226	HAS2	hyaluronan synthase 2	-1.552	PM component, glcosyl-transferase activity
Hs.35861	RIS1	Ras-induced senescence 1	-1.562	Unknown
Hs.553486	BDKRB1	bradykinin receptor B1	-1.595	Immune Response
Hs.336046	IL13RA2	interleukin 13 receptor, alpha 2	-1.657	Immune Response
Hs.164021	CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	-1.721	RNA-dependent DNA replication, Inflammatory response
Hs.438231	TFPI2	tissue factor pathway inhibitor 2	-1.877	Blood coagulation, endopeptidase inhibitor
Hs.120949	CD36	CD36 antigen (collagen type I receptor, thrombospondin receptor)	-1.958	Lipid Metabolism
Hs.83169	MMP1	matrix metallopeptidase 1 (interstitial collagenase)	-2.587	EM – collagen catabolism, peptidoglycan metabolism

(TSC22D3, LMO3), components of the extracellular matrix (COMP, MMP1) and plasma membrane (HAS2), factors involved in the immune response (BDKRB1, IL13RA2, CXCL6) and cell adhesion (NRCAM), as well as a signalling molecule (DDEF1) and an endopeptidase inhibitor (TFPI2).

LMO3 is up-regulated in response to both pretreatment conditions.

Of the 16 genes identified to be affected by prolonged exposure to 1nM glucocorticoids, 8 of these were also regulated in response to the 1μM/48H pretreatment (Table 6). Of particular interest, LIM domain only 3 (LMO3), a transcriptional adaptor protein was conserved between the two pretreatment conditions (Foroni et al, 1992). We further analyzed LMO3 expression by qRT-PCR in response to glucocorticoid pretreatment in 3T3 L1 cells (summarized in Table 7). Interestingly, LMO3 was induced by both pretreatment conditions in human preadipocytes, it was not induced in 3T3 L1 cells. It is important to note that the mRNA levels of LMO3 were at the threshold of detection in 3T3 L1 cells and were significantly lower than those detected in human primary preadipocytes. The ability to measure its induction in 3T3 L1 cells was limited and these results would require further validation. It suggested, however, that LMO3 may be a factor whose relevance is specific to human primary preadipocytes. Taken together with it being identified in both microarrays, LMO3 is an interesting factor to investigate further.

Table 6: Average fold change (AFC) in gene expression in response to glucocorticoids of factors identified to be differentially expressed as a result of both 1μM/48H and 1nM/proliferation pretreatment conditions in human primary preadipocytes. LMO3 (in bold) was validated by RT-PCR in both human primary and 3T3 L1 preadipocytes (Table 7).

Gene Symbol	Gene Name	1μM/48H (AFC)	1nM/prolif. (AFC)	Function
CPM	carboxypeptidase M	2.389	2.102	Metabolism (aromatic compounds), Immune response
ADH1B	alcohol dehydrogenase IB (class I), beta polypeptide	29.972	1.710	Metabolism (alcohol)
MAOA	monoamine oxidase A	2.598	1.631	Metabolism (cata-cholamines), neuro-transmitters catabolism
TSC22D3	TSC22 domain family, member 3	2.317	1.589	Transcription Factor
NRCAM	neuronal cell adhesion molecule	1.758	1.580	Cell adhesion, Neuronal cell migration
LMO3	**LIM domain only 3 (rhombotin-like 2)**	**3.054**	**1.521**	**Transcription Factor**
IL13RA2	interleukin 13 receptor, alpha 2	-2.324	-1.657	Immune Response
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	-2.367	-2.587	EM – collagen catabolism, peptidoglycan metabolism

Table 7. Fold Change in LM03 expression following both 1μM/48H and 1nM/proliferation pretreatment conditions in both human primary preadipocytes and murine 3T3 L1 cells. Fold change was measured by quantitative RT-PCR.

Gene	IμM dex/48H Human	IμM dex/48H 3T3 L1	1nM dex/prolif. Human	1nM dex/prolif. 3T3 L1
LMO3	18.8 ± 5.43*	0.67 ± 0.93*	2.04	-2.36

*average of 3 experiments done in replicate ± SEM including samples derived from five pooled donors and two individual donors. Other values represent analysis from one replicate using either duplicate RNA samples that were harvested coincident with the microarray samples, or RNA from 3T3 L1 cells as indicated. RT-PCR analysis was done in duplicate, where error between duplicates was between 0.1 and 3.7%.

DISCUSSION

Glucocorticoids Prime Preadipocytes for Differentiation

Glucocorticoids potentiate preadipocyte differentiation that is stimulated by insulin and cAMP. In addition to the stimulatory role of glucocorticoids in differentiation of preadipocytes in culture, our results suggest that glucocorticoids may also have a stimulatory role in priming preadipocytes for differentiation (Gregoire et al, 1998; Hauner et al, 1989; Hauner et al, 1987; Tomlinson et al, 2006). This may be an important means by which glucocorticoids promote adipogenesis *in vivo* as adipose tissue locally produce active steroid through the enzymatic activity of 11βHSD1, thereby providing an environment *in situ* where preadipocytes may be continuously exposed to active steroid. This may also be relevant to the mechanisms by which conditions of hypercortisolemia and increased 11βHSD1 activity promote the development of visceral obesity and the pathophysiology of the metabolic syndrome in diseased states (Peeke & Chrousos, 1995; Stewart & Tomlinson, 2002; Wolf, 2002).

Both human primary and 3T3 L1 preadipocytes had increased differentiation potential when stimulated for 48 h with a pharmacological concentration of dex prior to inducing differentiation (Fig 3). Microarray analysis of genes whose expression is altered in glucocorticoid stimulated preadipocytes immediately prior to the induction of differentiation (Day 0) has provided us with candidate factors that (1) represent previously unknown targets of glucocorticoid action in preadipocytes and (2) that may mediate the stimulatory potential of glucocorticoids under these pretreatment conditions (Appendix B).

Microarray Analysis of Human Primary Preadipocytes Following 1μM dex/48H Pretreatment.

Differentiation of human preadipocytes in culture is a coordinated process that includes growth arrest, which is associated with reorganization of the extracellular matrix, followed by terminal differentiation defined by transcriptional regulation of genes that are required for adipocyte function. Microarray analysis of human preadipocytes following pretreatment has identified glucocorticoid regulated genes that are related to each of these processes. We observe changes in genes that encode transcriptional regulators, cell cycle regulators, structural factors, factors involved in cell motility and adhesion, signalling molecules and metabolic enzymes. This implies that multiple mechanisms underlie the priming effects of glucocorticoids. Genes with greater than a 5-fold change in expression (Table 2) include the known GR target metabolic enzymes ADH1B (+29.972) and GPX3 (+18.622) which have never been shown to be involved in adipogenesis. ADAMTS1, a metalloproteinase-disintegrin with antiproliferative properties, is induced (+6.177-fold) by dex. ADAMTS1 knockout mice show adipose tissue malformation (Shindo et al, 2000). DKK1, a Wnt signalling antagonist, is up-regulated (+7.121) by dex; DKK1 has recently been shown to be secreted by human preadipocytes and to stimulate adipogenesis (Christodoulides et al, 2006). These authors show that while endogenous DKK1 is not detectable in murine primary preadipocytes and 3T3 L1 cells, exogenous expression in 3T3 L1 cells stimulates differentiation. The lack of detectable endogenous DKK1 in 3T3 L1 cells suggests that this factor may be particularly relevant to human preadipocyte differentiation. Similarly, Wnt signalling has been shown to prevent preadipocyte differentiation in 3T3 L1 cells by inhibiting C/EBPα and PPARγ induction. By contrast, disruption of the Wnt

pathway results in spontaneous differentiation (Bennett et al, 2002; Ross et al, 2000). Finally, pretreatment resulted in the down-regulation of the osteoblast specific factor POSTN, a secreted cell adhesion molecule of relatively unknown function (Horiuchi et al, 1999).

My primary research interest is to delimit the transcriptional regulation of events that drive early preadipocyte differentiation as they relate to glucocorticoid function. As such, I sought transcriptional regulatory factors within the microarray that could mediate the effects of steroid with respect to the aforementioned processes that define adipogenesis.

The initial analysis of known glucocorticoid targets revealed pretreatment dependent induction of C/EBPδ expression at Day 0 in both human and murine preadipocytes (Fig. 2C, D). We therefore used it as a positive control for the microarray analysis. C/EBPδ mRNA was induced 1.641 within the microarray. We identified 47 up-regulated and 17 down-regulated transcriptional regulatory factors at Day 0 (Table 3) and validated 14 of these by qRT-PCR (Table 4). These represented both known and potentially newly identified adipogenic factors that could mediate the effects of glucocorticoids. Of particular interest were the following factors:

BTG-1: B-cell translocation gene 1 was induced 4.6-fold in response to steroid pretreatment. It is an anti-proliferative protein that may contribute to prerequisite growth arrest at the onset of adipogenesis (Rouault et al, 1992). It has been shown to be involved in both erythropoeisis and myogenesis (Busson et al, 2005; Kolbus et al, 2003). During early erythroid differentiation, BTG-1 transcription is induced by FoxO3A, upon which BTG-1 functionally interacts with the protein arginine methyltransferase (PRMT1), the activity of which is required for erythropoeisis to progress (Bakker et al, 2004; Bakker et al, 2007;

Kolbus et al, 2003; Lin et al, 1996). FoxO3A expression is induced by dex in human preadipocytes (Table 4) and may mediate dex dependent induction of BTG-1 in preadipocytes as is observed in erythropoeisis. Furthermore, BTG-1 contains a nuclear receptor interaction motif (LxxLL) and has been shown to functionally interact with ERα (Prevot et al, 2001). Therefore, BTG-1 may function with nuclear receptors in preadipocytes including GR and/or PPARγ to regulate differentiation.

HIPK2: Homeodomain-interacting protein kinase 2 is a member of the HIPK family of nuclear threonine/serine kinases (HIPK1, 2 and 3) (Kim et al, 1998b). HIPK2 functions as a transcriptional regulatory factor by interacting with and phosphorylating key transcription factors including homeodomain proteins, p53, CtBP1 and Myb1 (D'Orazi et al, 2002; Hofmann et al, 2002; Kanei-Ishii et al, 2004; Zhang et al, 2005). Phosphorylation of homeodomain transcription factors represses their transactivation potential (Kim et al, 1998b). Furthermore, HIPK2 has recently been shown to bind to and modulate the HAT activity of p300 (Aikawa et al, 2006). HIPK2 may represent a prolific regulatory factor that could be mechanistically relevant to the regulation of adipogenesis and a downstream mediator of glucocorticoid action.

KLF family members: We identified the induction of three members of the Kruppel-like family of zinc finger transcription factors: KLF6 (1.551-fold), KLF9 (1.909-fold) and KLF15 (1.551-fold). Both KLF6 and KLF15 along with KLF5 have previously been identified as proadipogenic factors (Li et al, 2005; Mori et al, 2005; Oishi et al, 2005). Of particular relevance to glucocorticoid signalling, KLF6 functions with HDAC3 to down-regulate Pref-1 expression and promote differentiation of 3T3 L1 cells. Glucocorticoids promote adipogenesis, in part by down-regulating Pref-1 transcription; however the underlying

mechanism is unknown (Smas et al, 1999). Should KLF6 expression be similarly regulated in 3T3 L1, dex dependent induction of KLF6 could mediate the down-regulation of Pref-1 observed in these cells (Fig 3D) and in turn contribute to the increased differentiation of MI stimulated 3T3 L1 preadipocytes following pretreatment (Fig 3B). The role of Pref-1 has been characterized in murine models; however, little is known about its role and regulation in human primary preadipocytes. Further experiments are required to determine if it is involved in the regulation of human preadipocyte differentiation. Importantly, Pref-1 was not identified as being significantly repressed in our microarray analysis.

C/EBPs: Apart from C/EBPδ induction, glucocorticoids promoted the induction of C/EBPζ (CHOP-10) (1.516-fold). C/EPBζ negatively regulates the activation potential of C/EBPs by inhibiting their DNA binding ability (Ron & Habener, 1992). Upon being induced in differentiating 3T3 L1 cells, C/EBPβ and C/EBPδ activities are repressed through dimerization with C/EBPζ. C/EBPζ must be down-regulated for 3T3 L1 differentiation, thus contributing to acquired transcriptional potential of C/EBPβ and C/EBPδ and subsequent induction of C/EBPα (Tang & Lane, 2000). The involvement and role of C/EBPζ in human primary preadipocyte differentiation has never been assessed. Our previous studies demonstrate that within 4 h of stimulation with MID, there is an accumulation of C/EBPδ, C/EBPβ and C/EBPα proteins in human primary preadipocytes; all three are potential targets of the inhibitory actions of elevated C/EBPζ. With respect to the mechanisms underlying the stimulatory effect of pretreatment, it is possible that dex-dependent induction of C/EBPζ functionally antagonizes the concurrent induction of C/EPBδ that we also observe. Further analysis is required to validate this induction by RT-PCR and to determine if this has any

affect on its expression at the protein level. If it is validated, this is the first indication that C/EBPζ is regulated by GR.

Microarray Analysis of Human Primary Preadipocytes Following 1nM dex/proliferation Pretreatment.

Human, but not 3T3 L1 preadipocytes were sensitive to continuous culture in 1nM dex. This highlights the importance and relevance of working with human primary preadipocyte cell models to study glucocorticoid action. These two models appear to have differential sensitivity to glucocorticoids and to be regulated by both overlapping (for example dex dependent up-regulation of C/EBPδ) and distinct (dex-dependent insulin sensitization) proadipogenic mechanisms. Microarray analysis of gene expression in preadipocytes poised at the onset of differentiation following continuous culture in 1nM dex, revealed substantial differences as compared to 1μM dex/48H with respect to both the number of genes and magnitude of altered expression. Expression of 16 genes was affected by culture in 1nM dex, and the greatest fold changes were less than 3-fold (CPM (+2.102) and MMP1 (-2.587)) (Table 6). Of these genes, 8 were present in both microarray analyses (Table 7).

LMO3 was up-regulated by dex in response to both 1μM dex/48H and 1nM dex/proliferation pretreatment conditions (+18.8- and +2.04-fold respectively) in human preadipocytes. LMO3 is member of a family of 4 LIM-only adaptor proteins (LMO1-4) that lack intrinsic DNA binding ability (Bach, 2000). The family is defined by the presence of two tandem conserved cysteine-rich zinc finger motifs (LIM domain) that serve as docking sites for molecular scaffolds (Bach, 2000). The LMO proteins are involved in early

embryogenesis during which they are required for neural tube development. LMO3 acts as an oncogene in human neuroblastoma; evidence suggests that this may be mediated through interaction with the neuronal transcription factor HEN2 (Aoyama et al, 2005). With the exception of this report, the function of LMO3 in adult tissue and its transcriptional regulation are unknown.

Ongoing analysis in our group by D. Wu has positivity correlated ectopic expression of LMO3 with enhanced differentiation potential of both human primary and 3T3 L1 preadipocytes. qRT-PCR based temporal profiling of LMO3 expression upon dex treatment reveals that it is induced within 4 h of stimulation and is maximal by 48 h. The early induction of LMO3 suggests that it could be induced directly by GR. Experiments are ongoing to determine if (1) this factor is required for adipogenesis, (2) LMO3 is a direct GR target gene and (3) this factor can mediate the stimulatory effect of pre-exposure to steroids.

Glucocorticoids Increase Insulin Sensitivity in Human Subcutaneous Preadipocytes

Exposure of human subcutaneous preadipocytes, but not 3T3 L1 preadipocytes, to dex for 48 h prior to inducing differentiation led to up-regulation of key components of the insulin signalling cascade including InsR, IRS1, IRS2 and p85α both at the mRNA and protein levels (Fig 6). This corresponded to increased insulin sensitivity as observed by increased total cellular tyrosine phosphorylation and activated Akt in response to stimulation with insulin in these cells (Fig 7). Furthermore, insulin sensitization was conferred in response to physiological concentrations of steroid pretreatment (Fig 7C). Given the stimulatory role of insulin in promoting preadipocyte differentiation in culture and *in vivo*, this could represent a mechanism by which glucocorticoids prime human preadipocytes for

differentiation in culture, and could be physiologically relevant to promoting adipogenesis *in situ*, due to the local production of active glucocorticoids by adipose tissue (Cinti et al, 1998; Rubin et al, 1977).

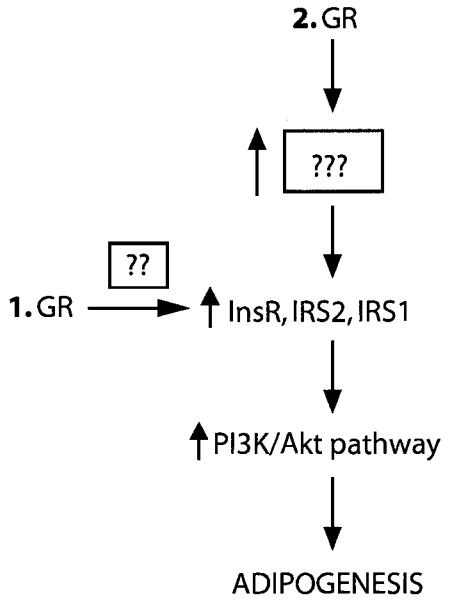
Theoretically, glucocorticoids may have a dual stimulatory effect in increasing adipocyte number through the differential regulation of insulin sensitivity in preadipocytes and adipocytes. Adipose tissue responds to caloric excess by either increasing adipocyte number through the recruitment and differentiation of preadipocytes (hyperplasia) and/or increasing their lipid accumulation (hypertrophy). Adipocyte hypertrophy occurs in obesity. Hypertrophy is thought to contribute to adipose tissue dysfunction and has been proposed to increase the risk of cardiovascular disease due to the secretion of pro-inflammatory and pro-atherogenic adipokines (Heilbronn et al, 2004). In response to chronic caloric excess, glucocorticoids may therefore provide a protective stimulus by limiting adipocyte hypertrophy (as glucocorticoids promote insulin resistance in adipocytes), thereby maintaining healthy adipocytes, while promoting the formation of new adipocytes to accommodate the increased energy intake. The preadipocytes are rendered more sensitive to insulin by glucocorticoids, thereby promoting their differentiation.

Human subcutaneous preadipocytes represent a cellular milieu that is permissive to dex dependent up-regulation of insulin signalling by transcriptional mechanisms. To this end, GR could be acting either directly or indirectly to induce transcription of the InsR, IRS1 and IRS2 genes as summarized in Figure 10. However, due to the opposing effects of GR on InsR, IRS1 and IRS2 induction both in these studies (compare human preadipocyte to human adipocytes and 3T3 L1 preadipocytes), and in previous studies reported in muscle, liver and adipocytes models in culture (Buren et al, 2002; Lundgren et al, 2004; Saad et al, 1995; Saad et al, 1993), it is unlikely that GR acts both directly and independently to up-regulate these

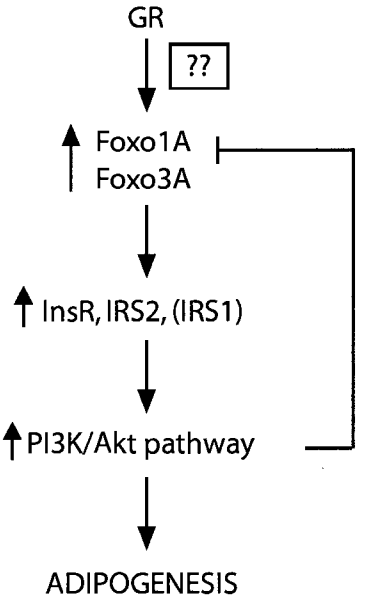
Figure 10: Glucocorticoid dependent up-regulation of insulin signalling components is cell type specific.

(A) Schema of the possible mechanisms for GR dependent transcription of InsR, IRS1 and/or IRS2. (1) Regulation may either be direct and require cell-type specific auxiliary factors and/or (2) indirect through cell-type dependent induction of intermediary transcription factors. These in turn would up-regulate InsR, IRS1 and/or IRS2 transcription. Up-regulation of these components results in increased insulin sensitivity, and in the context of pretreatment of the preadipocyte, this may lead to increased differentiation potential. **(B)** FoxO1A and/or FoxO3A represent potential mediators of GR dependent upregulation of the insulin signalling components. GR, with potential cell-type specific auxiliary factors, induce FoxO1A and FoxO3A expression. These in turn induce InsR and IRS gene transcription. Upon induction of differentiation, the PI3K/Akt pathway is activated which could create a negative feedback loop, by blocking the transcriptional potential of the FoxO proteins. The net result is increased insulin sensitization by dex in the human preadipocytes.

A.


2. GR
???
??
1. GR
InsR, IRS2, IRS1
PI3K/Akt pathway
ADIPOGENESIS

B.


GR
??
Foxo1A
Foxo3A
InsR, IRS2, (IRS1)
PI3K/Akt pathway
ADIPOGENESIS

genes. Indeed, ongoing studies by D. Wu in the laboratory have recently shown that both INSR and IRS1 expression are induced only within 24 to 48 h dex stimulation in the confluent human primary preadipocytes, suggesting that they are not direct targets of GR. Instead, there may be cell type specific auxiliary factors that are required for GR mediated transcription of these genes. Whether or not these factors are direct transcriptional targets of GR and/or act with GR to up-regulate transcription remains to be determined.

Each of the InsR, IRS1 and IRS2 expression is regulated by C/EBP transcription factors (Araki et al, 1995; Foti et al, 2003; Matsuda et al, 1997; Yamamoto et al, 2002). While it is possible that dex-dependent up-regulation of C/EBPδ could mediate induction of these promoters in human preadipocytes, this appears to be insufficient as C/EBPδ was induced in the absence of increased InsR, IRS1 and IRS2 in response to dex in 3T3 L1 preadipocytes.

I hypothesize that the insulin sensitizing effects of dex are transmitted through the FoxO transcription factors. FoxO1A and FoxO3A were both induced following 1μM/48H pretreatment (Table 4) and confirmed by qRT-PCR to be induced 7.85 ± 1.01 and 3.12 ± 0.54-fold respectively. By contrast, they were only induced 1.90 ± 0.21 and 1.34 ± 0.44-fold at the mRNA level respectively in 3T3 L1 preadipocytes in which the insulin signalling pathway is insensitive to dex, thus making them attractive candidate mediators of this effect, and suggesting that FoxO3A may be the more relevant factor.

Forkhead box O transcription factors serve as master switches for key cellular processes including proliferation and cell cycle, cell survival and apoptosis, differentiation, and oxidative stress resistance. In this regard, they are important mediators of insulin and growth factor action and are downstream targets of the activated PI3K/Akt pathway. In the

absence of growth hormone and insulin stimulation, FoxOs bind to sequence specific consensus motifs within numerous genes including those involved in metabolism (such as PEPCK, G6Pase, IGFBP-1 and PGC-1α (Barthel et al, 2005; Guo et al, 1999; Nakae et al, 2001; Schmoll et al, 2000; Yeagley et al, 2001)), cell cycle regulation (cyclin dependent kinase inhibitors $p21^{CIP1}$ and $p27^{Kip1}$ (Seoane et al, 2004; Stahl et al, 2002)) and regulation of cell death (the proapoptotic gene BIM (Dijkers et al, 2002; Dijkers et al, 2000; Gilley et al, 2003; Stahl et al, 2002; Sunters et al, 2003)). Their transcriptional potential is highly regulated by both phosphorylation and acetylation; most notably, FoxOs are phosphorylated by activated Akt downstream of insulin and growth factor signalling, which results in their nuclear exclusion and attenuated transcriptional activity (Van Der Heide et al, 2004).

Studies in *Drosophila* and mammalian cells have reported that FoxO1A (dFoxO1 in *Drosophila*) functions as an insulin sensor through FoxO1A dependent expression of the InsR and IRS2 by binding to response elements in their respective promoters (Puig et al, 2003; Puig & Tjian, 2005). Under conditions of limited nutrient availability, InsR and IRS2 are up-regulated by FoxO1A. Upon insulin stimulation, Akt kinase phosphorylates FoxO1A thus attenuating its activity by causing its nuclear exclusion. This creates a negative feedback loop that controls insulin sensitivity at the transcriptional level. The regulation of IRS2 expression by FoxO1A has independently been reported in hepatic cells by Ide and colleagues (Ide et al, 2004). I propose that upon GR-dependent induction, FoxO1A and/or FoxO3A are functioning in a similar manner to sensitize the human preadipocytes to insulin (Fig. 10B). If this is true, then ectopic expression of a constitutively active isoform of the FoxOs, in which the inactivating-Akt phosphorylation site has been mutated (Brunet et al, 1999; Nakae et al, 2001), should confer insulin sensitivity in the absence of dex to human

primary preadipocytes. Theoretically, it may also transmit insulin sensitivity to the otherwise unresponsive 3T3 L1 preadipocytes. Conversely, a dominant negative isoform, which binds DNA but lacks the COOH-terminal transactivation domain (Nakae et al, 2003), should inhibit dex-dependent up-regulation of InsR and IRS2 in the human preadipocytes. These studies are currently underway in the laboratory using a lentiviral based gene delivery system.

If FoxOs are responsible for the up-regulation of the InsR and IRS2 genes, then several important questions remain to be answered including (A) what happens to their cellular localization upon induction of differentiation, which can be assessed using immunofluorescence, and how does this affect overall differentiation? (B) What is the relative contribution of FoxO1A versus FoxO3A in this pathway? This should be addressed in the ongoing experiments in which the respective constitutively active isoforms are ectopically expressed in the preadipocytes. (C) How are FoxO1A and/or FoxO3A regulated by GR? Preliminary experiments in the laboratory suggest that the FoxOs are induced within 4 h of stimulation with dex (D. Wu data not shown). This suggests that their expression could be a direct GR target. This could be confirmed by ChIP assays on the FoxO promoters. However, the expression of FoxO3A appears to be more complex, as it is not induced by dex in 3T3 L1 cells and may require differentially expressed auxillary factor(s). If this hypothesis is correct, then the presence of such factor(s) could confer glucocorticoid-dependent insulin sensitivity to a given cell type.

Ultimately, the identification of mediators of GR dependent regulation of the InsR and IRS genes may provide insight into how glucocorticoids promote insulin resistance. In our study, dex had no effect on insulin signalling activity in the mature adipocytes despite slightly enhancing InsR mRNA expression (Fig 8B, C). This is consistent with reports in

which human primary adipocytes isolated from subcutaneous adipose tissue depots were insensitive to dex dependent insulin resistance in culture; by contrast, adipocytes isolated from omental depots had decreased IRS1 expression in response to dex that correlated with decreased insulin sensitivity (Lundgren et al, 2004). In our studies, dex induced a 4.32 ± 0.90 and 1.86 ± 0.15-fold induction in FoxO1A and FoxO3A mRNA levels respectively in human adipocytes (Fig 9C). Should the FoxOs be involved in dex dependent up-regulation of this pathway in the preadipocyte, it would be interesting to further characterize the protein expression and transcriptional activation potential of the FoxOs in the context of the mature adipocyte as compared to the preadipocyte. Analysis of protein localization may provide insight into their differential activity in these two cellular contexts. Furthermore, it would be enlightening to assess whether lentiviral-based delivery of a constitutively active isoform of either FoxO1A or FoxO3A in mature adipocyte can confer insulin sensitivity.

GENERAL DISCUSSION

Glucocorticoids strongly promote preadipocyte differentiation in culture and *in vivo*. The work presented in this thesis highlights the complex nature of glucocorticoid action in preadipocytes as they mediate multiple aspects of adipogenesis through both direct and indirect mechanisms. Chapter II highlights the role of glucocorticoids in promoting the core C/EBP-PPARγ transcriptional cascade that drives adipogenesis. While it was previously known that GR directly regulates transcription of the C/EBPδ gene, we have shown that it is also involved in the early accumulation of C/EBPβ in human preadipocytes. This appears to be mediated through transcriptional and possibly translational mechanisms. Furthermore, PPARγ and C/EBPα expression in human preadipocytes is also dependent on the presence of dex in the induction cocktail. This is most likely mediated indirectly by GR through C/EBPδ and C/EBPβ. Chapter I, taken together with previous reports from our laboratory, highlights that GR also contributes to adipogenesis by potentiating the transcriptional activity of C/EBPβ to enhance transcription of the C/EBPα gene (Chapter I, (Wiper-Bergeron et al, 2007; Wiper-Bergeron et al, 2003)). I have identified TIF1β as a cofactor in this regulatory pathway. Lastly, Chapter III provides evidence that glucocorticoids may contribute to priming preadipocytes for differentiation *in situ*. Microarray analysis performed in Chapter III also highlights that glucocorticoids contribute to adipogenesis at multiple levels through the regulation of the expression of factors that underlie all aspects of this process including transcriptional regulators, signalling pathways, components of the cytoskeleton and metabolic enzymes.

The relevance of studying glucocorticoid action in human primary preadipocytes

Murine 3T3 L1 preadipocytes are believed to reflect the earliest committed state of preadipocyte differentiation available, they have a relative ease of handling, a strong differentiation response and are amenable to molecular biological analysis. For these reasons, they represent the most widely used model system to study adipogenesis and most of the molecular understanding of the mechanisms of preadipocyte differentiation has been elucidated in these cells. However, 3T3 L1 and other cell culture models may not exactly reflect what happens in human preadipocytes. By contrast, human primary preadipocytes are the most relevant accessible model for preadipocyte differentiation while at the same time they are the least well understood.

Our analysis of the early events that drive human preadipocyte differentiation and their regulation by glucocorticoids and comparing these findings to what is observed in the 3T3 L1 cells has revealed some key differences in both the regulation of human primary preadipocyte and 3T3 L1 differentiation and the effects of glucocorticoids on these processes. Specifically, we observed an earlier induction of C/EBPα accumulation that occurred in the absence of increased mRNA abundance and dex-dependent transcriptional regulation of C/EBPβ expression; both of which appear to be restricted to the human preadipocytes. Alternatively, glucocorticoids provide a survival signal in the absence of serum that is required for 3T3 L1 cells to progress through clonal expansion. By contrast, human preadipocytes do not undergo post-induction mitosis, nor do they undergo apoptosis in the absence of serum or dex (Chapter II) (Bell et al, 2000; Entenmann & Hauner, 1996). I have also provided evidence that human primary and murine 3T3 L1 preadipocytes are differentially responsive to the priming effects of pretreatment with glucocorticoids.

Notably, 3T3 L1 preadipocytes are refractive to the stimulatory effects of continuous culture in 1nM dex during proliferation.

Furthermore, both human primary and 3T3 L1 preadipocytes had greater differentiation potential when pretreated with pharmacological concentrations of steroid for 48 h prior to the induction of differentiation. However, the responses of these two cell types to glucocorticoids differed, as indicated by their differential sensitization to insulin and differential expression of LMO3 and FoxO3A (Chapter III). Taken together, these results highlight the relevance of defining the role of glucocorticoids in human primary preadipocytes and apart from glucocorticoid action, the need to evaluate and validate the mechanisms that have been defined in other culture models.

As such, it would be particularly relevant to my studies to assess the role of TIF1β in human primary preadipocyte differentiation. The findings presented in Chapter I suggest that TIF1β may have a versatile and complex role in the 3T3 L1 differentiation. These include the transcriptional regulation of C/EBPα expression, the transcriptional repression of as of yet unknown genes, and possibly a role in the maintenance of heterochromatin through localization to heterochromatic foci during early differentiation. Further studies are required to determine if these functions are mutually exclusive of one another, or if in fact they represent a unified pathway that intricately regulate preadipocyte differentiation. These activities also need to be validated in human primary preadipocytes.

The work presented here is the first indication that TIF1β and HP1α co-localize with C/EBPβ at heterochromatic foci in 3T3 L1 cells. Furthermore, the association between TIF1β and HP1α appears to be required for efficient differentiation of these cells. Future studies are warranted to identify other proteins that localize to these foci and the regulation

of the recruitment and exit of foci-associated factors from these loci. These studies may provide insight into the function of these foci in differentiation. In 3T3 L1 cells, the recruitment of C/EBPβ to these foci has been attributed to the regulated timing and control of clonal expansion (Tang & Lane, 1999). By contrast, human primary preadipocytes differentiate directly in response to stimuli in the absence of clonal expansion. If TIF1β is similarly recruited to nuclear heterochromatic foci in human preadipocytes, these cells may therefore represent a model that could allow one to dissect the function of TIF1β– and possibly HP1α and C/EBPβ-enriched foci that is independent of clonal expansion.

Omental and subcutaneous adipose tissue are differentially sensitive to glucocorticoids

Glucocorticoids also differentially regulate adipose tissue depots *in situ*. Omental, or visceral, adipose tissue appears to be more responsive to glucocorticoids due both to the increased expression of GR as well as increased local expression and activity of the 11βHSD1 enzyme (Bujalska et al, 1997; Pedersen et al, 1994). In diseased states, glucocorticoids predominantly promote visceral obesity and contribute to the development of the metabolic syndrome. The particular health risk contributed by central obesity was recently recognized by the first ever Canadian Clinical Practice Guidelines on the Management and Prevention of Obesity in Adults and Children that recommended that the waist circumference of all adult Canadians be measured along with the standard weight and height measurements during physicals, to establish a risk profile for cardiovascular disease and overall health status (Lau et al, 2007).

To date, we have restricted our analysis of the effects of glucocorticoids to subcutaneous depot-derived preadipocytes. However, the evidence of depot-specific

differences in glucocorticoid responses suggests that molecular analysis of the effects of glucocorticoids on the early events that drive omental differentiation is therefore warranted. In particular, 11βHSD1 expression and activity is higher in omental adipose tissue and therefore our work presented in Chapter III may be most relevant to the effects of glucocorticoids on omental preadipocyte differentiation (Bujalska et al, 1997). Given that glucocorticoids promote insulin resistance in cultures of adipocytes derived from omental but not subcutaneous adipose tissue and that this is through down-regulation of IRS1 expression (Lundgren et al, 2004), it would be particularly interesting to evaluate insulin sensitivity and the transcriptional regulation of InsR and IRS genes in response to glucocorticoids in omental preadipocytes. Differential regulation of this pathway by glucocorticoids in the adipocytes and/or preadipocytes from these depots may provide a human-specific platform to evaluate the contribution of the FoxOs to the up-regulation of this pathway or alternatively to identify cell-type specific factors that mediate the effects of glucocorticoids. The identification of these factors could also be relevant to other tissues such as muscle and liver in which glucocorticoids have been shown to promote insulin resistance.

The mineralocorticoid receptor – a mediator of glucocorticoid function in preadipocytes?

MR is a NHR that is closely related to GR. It regulates electrolyte balance and blood pressure by regulating trans-epithelial sodium transport through tight epithelia (Funder, 2005). It is expressed in epithelial tissue including distal parts of the nephron, colon, sweat and salivary glands and the placenta. It has also been reported to be expressed in non-epithelial tissues including cardiovascular tissue, in the central nervous system and recently in adipose tissue. MR is a high affinity receptor for cortisol (human) and corticosterone

(rodent) as well as for aldosterone. Furthermore, the relative affinity of MR for glucocorticoids is greater than 10-fold higher than that of GR (Arriza et al, 1987). Glucocorticoids circulate 100- to 1000-fold higher than the concentration of aldosterone. The specificity of MR activity is regulated through tissue-specific expression of 11βHSD2 dehydrogenase that inactivates glucocorticoids in aldosterone responsive tissues such as the kidney and the placenta.

Previous studies have reported that MR plays a role in brown adipose tissue development, and pharmacological studies suggest that aldosterone may promote adipogenesis (Hauner et al, 1989; Penfornis et al, 2000; Rondinone et al, 1993; Viengchareun et al, 2001; Zennaro et al, 1998). Other studies have shown that human adipose tissue secretes an unknown adipokine that stimulates aldosterone synthesis and secretion from adrenal cells (Ehrhart-Bornstein et al, 2003). This is postulated to explain the relative hyperaldosteronism that is often observed in obese subjects. Furthermore, there are reports of increased prevalence of metabolic syndrome in patients with primary aldosteronism (Fallo et al, 2006). These findings suggest that aldosterone and MR play a role in adipose tissue biology.

NHR receptor profiling in 3T3 L1 cells revealed that MR is expressed in a bi-phasic pattern that overlaps the temporal expression pattern of GR, although its mRNA abundance is lower than that of GR (Fu et al, 2005). Both factors are expressed at the onset of differentiation then immediately decline to their lowest expression by 4 h of stimulation with MID. Their expression then reemerges as the cells convert to mature adipocytes. In primary cells derived from mouse adipose tissue, both MR and GR expression are higher in the adipocyte compared to the preadipocyte. This highlights the importance of

glucocorticoids and perhaps mineralocorticoids to adipocyte function in addition to the role of GR in promoting adipogenesis. The aforementioned effects of aldosterone on adipose tissue are mediated by MR. However, the expression of MR, in the absence of adipose tissue 11βHSD2 activity (Bujalska et al, 1997), suggests that MR could contribute to the effects of glucocorticoids on adipose tissue function and differentiation. Recent studies by Caprio and colleagues have shown that MR promotes 3T3 L1 and 3T3 F442A differentiation in response to aldosterone and appears to account for some aspects of glucocorticoid action as MID stimulated differentiation was not completely blocked when GR expression was knocked down using siRNA (Caprio et al, 2007).

In light of these new findings and in the context of our molecular understanding of the mechanistic action of glucocorticoids in both human primary and 3T3 L1 adipogenesis, it would be interesting to evaluate the relative contribution of GR and MR to the effects of glucocorticoids on preadipocyte differentiation. For example, Wiper-Bergeron et al. demonstrated that the PR LBD, but not RAR could replace the GR LBD in promoting 3T3 L1 differentiation and the turnover of HDAC1 (Wiper-Bergeron et al, 2003). Can the MR LBD function in a similar manner? Does aldosterone exposure similarly sensitize preadipocytes to subsequent differentiation? What is the relative expression of GR and MR in human primary preadipocytes? These are just some of the questions that could be addressed.

Moving forward in our understanding of glucocorticoid function

The work presented here provides a platform for several potentially interesting projects to further define the mechanisms by which glucocorticoids promote preadipocyte

differentiation. The microarray analysis alone has provided a plethora of interesting, previously unidentified targets of dex-stimulated differentiation. For example, how do the KLF transcription factors, BTG-1 and HIPK2 function in both human primary and 3T3 L1 preadipocytes and are they direct transcriptional targets of GR? Ongoing studies in our lab show that LMO3 promotes adipogenesis. How is this mediated? With what factors is it interacting? How is its expression regulated? And finally, apart from their potential role in dex-dependent insulin sensitization, the FoxO transcription factors may have other significant roles in adipogenesis including contributing to growth arrest and the up-regulation of adipocyte specific metabolic enzymes. A more detailed analysis of their expression and the regulation of their transcriptional potential in human primary preadipocytes in non-pretreated cells could be enlightening.

Aside from the specific role of glucocorticoids in promoting adipogenesis, other findings in this thesis have shown that the adipogenesis model may be a system to study effects of glucocorticoid action that might not be physiologically restricted to adipose tissue. Specifically, glucocorticoids provide a survival signal in the absence of serum factors in 3T3 L1 cells and they regulate insulin sensitivity in a cell-type dependent manner.

CONCLUDING REMARKS

Together, the data presented in this thesis contributes to our understanding of the molecular action of glucocorticoids in adipogenesis and the co-factors that contribute to GR action in this system. While my work has led to advances in this understanding, a great deal remains to be done. The contribution of adipose tissue specific 11βHSD activity and the corresponding increase in local glucocorticoid production is emerging as an important factor

in the development of obesity, insulin resistance, hypertension and the metabolic syndrome. Understanding how glucocorticoids act to promote adipogenesis can lead to therapeutic regimes that could combat these complex metabolic disorders. Already, selective 11βHSD inhibitors are being developed as therapeutics for this reason (Alberts et al, 2002; Tomlinson et al, 2007). This is of particular relevance and urgency to the health of Canadians as obesity rates are at epidemic proportions and the prevalence of obesity in Canadian youth is rising.

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APPENDICES

APPENDIX A: Supplemental Materials and Methods

Table 1: DNA plasmids acquired from other researchers.

Plasmid Name	Type of Plasmid	Description	Source	Reference
6RGR	Mammalian Expression	Encodes full length rat glucocorticoid receptor.	Dr. K. Yamamoto	(Pearce & Yamamoto, 1993)
pCDNA3.1-HDAC1	Mammalian Expression	Encodes full length human HDAC1 cDNA with a COOH-terminal HA and FLAG tag.	Dr. J. Gimble	(Clarke et al, 1997)
pCX14/12	Luciferase Reporter	Contains the -350 to +7 murine C/EBPα promoter driving expression of luciferase gene.	Dr. P. Antonsen	(Legraverend et al, 1993)
pCXLDE	Luciferase Reporter	Point mutations of pCX14/12 construct disrupting the C/EBP response element.	Dr. N. Wiper-Bergeron	(Wiper-Bergeron et al, 2003)
pCR3.1 E6AP	Mammalian Expression	Encodes full length human E6APcDNA	Dr. B. O'Malley	(Nawaz et al, 1999b)
pGEX2T-TIF1β	Bacterial Expression	Encodes mTIF1β (aa14-837) with an N-terminal GST tag.	Dr. S. Lee	(Chang et al, 1998)
pHA-Ubiquitin (MT 123)	Mammalian Expression	Plasmid contains cassette containing 8 copies of ubiquitin cDNA each with NH_2-termianl HA tag. Cassette is driven by CMV promoter/enhancer region.	Dr. D. Bohmann	(Treier et al, 1994)
pHIS$_6$-Ubiquitin (MT107)	Mammalian Expression	Plasmid contains cassette containing 8 copies of ubiquitin cDNA each with NH_2-terminal HIS$_6$ tag. Cassette is driven by CMV promoter/enhancer region.	Dr. D. Bohmann	(Treier et al, 1994)

pLXSN	Retroviral Cloning Vector	Insert under the control of the Moloney Murine Leukemia virus 3' LTR	Purchased from Clontech	
pLXSN-C/EBPβ	Retroviral Infection	Used to produce retrovirus containing full length murine C/EBPβ	Dr. N. Wiper-Bergeron	(Wiper-Bergeron et al, 2003)
pMSV-C/EBPβ	Mammalian Expression	Encodes full length murine C/EBPβ cDNA.	Dr. S.K McKnight	(Cao et al, 1991)
pRC-MDM2	Mammalian Expression	Encodes full length murine MDM2 cDNA.	Dr. B. McKay	
pRSV-β-Gal	Mammalian Expression	Expresses the yeast LacZ gene, β-galactosidase (β-gal) from the Rous Sarcoma Virus (RSV) promoter.	M. Walker (UCSF, CA)	
pRSV-TIF1βfl	Mammalian Expression	Encodes full length murine TIF1β cDNA.	Dr. S. Lee	(Chang et al, 1998)
pSG5puroFlagNT- mTIF1β	Mammalian Expression	Encodes full length murine TIF1β with an N-terminal Flag tag.	Dr. P. Chambon	(Cammas et al, 2004)
pSG5puroFlagNT-mTIF1βV488A/L489A	Mammalian Expression	Encodes full length murine TIF1β with site directed mutagenesis of HP1-interaction domain.	Dr. P. Chambon	(Cammas et al, 2004)
$pTL_2myc\text{-}GR_{505C}$	Mammalian Expression	Encodes rGR C-terminal ligand binding domain (aa505-795)	Dr. N. Wiper-Bergeron	(Wiper-Bergeron et al, 2003)

Table 2: Cloning strategies for the generation of expression plasmids. Plasmids were cloned using restriction enzyme (R.E.) digestion and subcloning based strategies as indicated. More detail is provided in the Materials and Methods.

Plasmid Name	Cloning Strategy	Description
pLXSN-TIF1β	R.E./Subcloning	Excised full length TIF1β from pRSV-TIF1β in two steps. First it as digested with Hind III and the generated overhang was filled in using Klenow (NEB). The linearized vector was then digested with EcoR I. The 2.5kb fragment was gel purified and cloned into pLXSN in Hpa I/EcoR I sites.
pLXSN-Flag	Anneal Oligo/ R.E Digest	Generated oligomers that encoded Flag tag, XhoI and SmaI sites and generated 5' EcoR I and 3' BamH I overhangs when annealed (this oligomer was designed to conserve spacing and sequence between NH_2-terminal Flag tag and TIF1β within pSG5puroFlagNT- mTIF1β. Upper: 5' AA TTC GGA TCT CC ACC ATG GAC TAC AAA GAC GAT GAC GAT AAA CTC GAG GGT TCC CCC GGG G. Lower: 5' GATC CCC CGG GGG AAC CCT CGA GTT TAT CGT CAT CGT CTT TT AGT CCA TG TGG AGA TCC G. Oligos were annealed by heating at 68°C for 10 min followed by a gradual cooling to room temperature. Annealed oligo was ligated into pLXSN in EcoR I/BamHI sites. *Cloned by D. Wu.
pLXSN-Flag TIF1β	R. E./Subcloning	Full length TIF1β was excised from pSG5puroFlagNT- mTIF1β with Bgl II. TIF1β/Bgl II fragment was gel purified and ligated into pLXSN-Flag in BamH I site. Orientation of insert was verified by digestion with XhoI and positive clones were further verified by sequencing.
pLXSN-Flag TIF1βΔRBCC	R. E./Subcloning	TIF1βΔRBCC was excised from pSG5puroFlagNT- TIF1βΔRBCC with Bgl II. TIF1βΔRBCC /Bgl II fragment was gel purified and ligated into pLXSN-Flag in BamH I site. Orientation of insert was verified by digestion with XhoI/Kpn I and positive clones were further verified by sequencing.
pLXSN-Flag TIF1βΔC	R. E./Subcloning	TIF1βΔC was excised from pSG5puroFlagNT- TIF1βΔC with Bgl II. TIF1βΔRBCC /Bgl II fragment was gel purified and ligated into pLXSN-Flag in BamH I site. Orientation of insert was verified by digestion with XhoI and positive clones were further verified by sequencing.
pLXSN-Flag TIF1βHP1mt	R. E./Subcloning	Full length TIF1βHP1mt was excised from pSG5puroFlagNT- TIF1βHP1mt with Bgl II. TIF1β/Bgl II fragment was gel purified and ligated into pLXSN-Flag in BamH I site. Orientation of insert was verified by digestion with XhoI and positive clones were further verified by sequencing.
pRSV- TIF1βΔRBCC	R. E. Digest/ Religation	Digested pRSV-TIF1β with BamH I (cuts twice within TIF1β cDNA to excise aa80-383). Gel purified 6.4kb fragment and re-ligated.

pSG5-Flag-TIF1βΔRBCC	R.E./Subcloning	Excised TIF1β BamH I/Kpn I fragment from pRSV-TIF1βΔRBCC in a 2-step process. First digested with Kpn I (cuts at bp 2210 within TIF1β). Then performed a partial digest with BamH I (cut at bp 231 within TIF1β). Gel purified the BamH I/Kpn I (bp 231-2210) fragment. For preparation of vector, digested pSG5puroFlagNT- mTIF1β with BamH I and Kpn I (unique enzyme sites within TIF1β as indicated above). Gel purified ~7.5kb fragment. Ligated TIF1β BamH I/Kpn I (bp 231-2210) fragment into pSG5puroFlagNT- mTIF1β BamHI/Kpn1 backbone.
pSG5-Flag-TIF1 βΔC	R.E. Digest	Digested pSG5-Flag-TIF1β with Sac I (cuts twice within TIF1β cDNA to excise aa564-834). Gel purified ~7.5kb fragment and re-ligated.

Table 3: Primary antibodies used for Western analysis, indirect-immunofluoresence (IIF) and immunoprecipitation (IP). Antibodies recognized either human (H), murine (M) or both species. For IP, primary antibodies were used at a concentration of 2-5μg antibody/500μg cellular extract as described in the Materials and Methods.

Primary Antibody	Species	Supplier	Western Analysis	IIF	IP
11βHSD1	H	Cayman Chemical Co.	1:500		
Actin (H-300)	H/M	Santa Cruz Biotechnology	1:400		
Adipsin	M	Santa Cruz Biotechnology	1:400		
Akt (C-20)	H/M	Santa Cruz Biotechnology	1:400		
P-Akt S473	H/M	Cell Signaling Technology	1:1000		
aP2/FABP4	H	Cayman Chemical Co.	1:1000		
C/EBPα (14AA)	H/M	Santa Cruz Biotechnology	1:400		
C/EBPβ (C-19)	H	Santa Cruz Biotechnology	1:400	1:500	Yes
C/EBPδ (C-22)	H	Santa Cruz Biotechnology	1:400		
E6AP (PA3-843)	H	Affinity Bioreagents	1:1000		
Flag M2	-	Sigma Aldrich	1:1000-1:5000	1:1000	Yes
FOXO1A (PA1-17036)	H/M	Affinity Bioreagents	1:500		
FOXO3A (PA1-17028)	H/M	Affinity Bioreagents	1:500		
Gal4DBD (RK 5C1)	Yeast	Santa Cruz Bioreagents			Yes
GR (MA1-510)	H/M	Affinity Bioreagents	1:1000		Yes
HA (12CA5)	-	Roche	1:1000		Yes
HDAC1 (PA1-860)	H/M	Affinity Bioreagents	1:1000-1:3000		Yes
HIAP2	H/M	R & D	1:1000		
6xHIS	-	BD Bioscience	1:1000-1:5000		
HP1α clone 15.19s2	M	Upstate Biotechnology	1:1000	1:1000	
INSR β (MA1-22006)	H/M	Affinity Bioreagents	1:1000		
IRS1 (06-248)	H/M	Upstate Biotechnology	1:1000		
IRS2 (06-506)	H/M	Upstate Biotechnology	1:1000		
Mdm2 (SMP14)	H/M	Santa Cruz Biotechnology	1:400		
PI3K p85α (Z-8)	H	Santa Cruz Biotechnology	1:400		
PI3K p110β (H-239)	H	Santa Cruz Biotechnology	1:400		
Phosphotyrosine-PY20*	H/M	BD Transduction Laboratories	1:1000		
PPARγ (H100)	H	Santa Cruz Biotechnology	1:400		
PREF-1	M	Chemicon International	1:2500		
TIF1β (PA1-852)	H/M	Affinity Bioreagents	1:1000-1:3000		Yes

*blocked PY20 in 3% BSA in PBS-T

Table 4: Primers used for quantitative real-time PCR analysis and primer-specific PCR conditions. Primers are targeted against human (H) or mouse (M) gene sequences. Where applicable, the accession number represents species from which sequences were used to design primers. Sequences are listed 5' to 3'. The temperature (T_{anneal}) used for the annealing step of the PCR reaction is listed in °C. Where indicated (*) PCR reaction mixture contained 5% DMSO.

Gene	Species	Accession No.	Forward Primer	Reverse Primer	T_{anneal} (°C)
aP2 (FABP4)	H	BT006809	catcagtgtgaatggggatg	gtggaagtgacgcctttcat	58
BTG1	H	NM_001731	ctgttcaggcttctcccaag	tcgttctgcccaagagaagt	55
C/EBPα	H	Y11525	tggacaagaacagcaacgag	ccatggccttgaccaaggag	58*
C/EBPβ	H	BC005132	agaagaccgtggacaagca	gcttgaacaagttccgcagg	59
C/EBPδ	H	BC094715	agaagttggtggagctgtcg	ggtatgggtcgttgctgagt	59*
CREB3L1	H	NM_052854	ttgaagagagtccggaggaa	attctccagggtctccacct	55
ELL2	H	NM_012081	tcgaccttcaatccagttcc	taaatcccaggcaattgagc	55*
FOXO1A	H/M	NM_002015	aagagcgtgccctacttcaa	ctgttgttgtccatggatgc	55
FOXO3A	H/M	NM_001455	accaattctaacgccagcac	caggtcgtccatgaggtttt	55
G3PDH	H/M	AY340484	accacagtccatgccatcac	tccaccaccctgttgctgta	60
GPX3	H	NM_002084	catcccatgtccaccatgta	tgcctggcagtacacagaac	55
HIPK2	H	NM_022740	agggagcgacatgttggtag	aaaggggttttgctctggtt	55
HIPK2	M	AF208292	atccatgctgacctcaaacc	accacatgtcaattgcctca	55*
INSR	H/M	NM_022740	atgccttggacaaccagaac	gtcttcagggcaatgtcgtt	55
IRS1	H	NM_05544	caagaccatcagcttcgtga	agagtcatccacctgcatcc	60
IRS1	M	NM_010570	tcctatcccgaagagggtct	attggggtccactctctgtg	55*
IRS2	H	NM_003749	ggcttccagaatggtctcaa	aagtcaatgctggcgtaggt	60*
IRS2	M	AF090738	gtagttcaggtcgcctctgc	cagctattgggaccaccact	55*
JUN	H	NM_002228	acagagcatgaccctgaacc	ccgttgctggactggattat	55
KLF6	H	NM_001300	gagccctgctatgtttcagc	aaagttcctcggagctgtca	55
KLF9	H	NM_001206	acagtggctgtgggaaagtc	aactgcttttccccagtgtg	55
KLF15	H	NM_014079	acacaagagccacctccatc	ctgggcaaccttgacattct	58*
LMO3	H	NM_018640	caaagcccctgaattgttgt	cagcactctgttggagtgga	55
LMO3	M	BC057086	tgaggactgcctgaagtgtg	aagctgacaggcaaagcagt	55*
PPARγ	H	BT007281	cataaagtccttcccgctga	gggctccataaagtcaccaa	55
PIK3R1	H/M	NM_181523	ctgcctcctaaaccaccaaa	taccaaaaaggtcccgtctg	55
TTRAP	H	NM_016614	ggcggcaggaagatggagtt	ctccaccggaggctcgaagt	60

APPENDIX B: Supplemental Data

Table 5: Glucocorticoid responsive genes following pretreatment with 1μM dex for 48 h in human primary preadipocytes. Average fold change (AFC) was determined using RMA algorithms and all genes reached statistical significance as determined by FDR-CI as described in Materials and Methods. Genes are listed in alphabetical order.

Unigene (Avadis)	Gene Symbol	Gene Title	AFC
Hs.58351	ABCA8	ATP-binding cassette, sub-family A (ABC1), member 8	1.843
Hs.477015	ABI3BP	ABI gene family, member 3 (NESH) binding protein	-1.835
Hs.438236	ABLIM1	actin binding LIM protein 1	1.866
Hs.234898	ACACB	acetyl-Coenzyme A carboxylase beta	1.634
Hs.406678	ACSL1	acyl-CoA synthetase long-chain family member 1	3.864
Hs.471461	ACSL3	acyl-CoA synthetase long-chain family member 3	-1.589
Hs.386283	ADAM12	ADAM metallopeptidase domain 12 (meltrin alpha)	-4.719
Hs.534115	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	6.177
Hs.534221	ADAMTS15	ADAM metallopeptidase with thrombospondin type 1 motif, 15	1.991
Hs.58324	ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5 (aggrecanase-2)	4.562
Hs.3416	ADFP	adipose differentiation-related protein	1.644
Hs.4	ADH1B	alcohol dehydrogenase IB (class I), beta polypeptide	29.972
Hs.477887	AGTR1	angiotensin II receptor, type 1	2.992
Hs.371240	AKAP12	A kinase (PRKA) anchor protein (gravin) 12	1.558
Hs.259461	PALM2-AKAP2	A kinase (PRKA) anchor protein 2 /// PALM2-AKAP2 protein	1.523
Hs.558319	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	2.230
	AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	1.571
Hs.150693	ALCAM	activated leukocyte cell adhesion molecule	2.476
Hs.42572	ALDH1L2	aldehyde dehydrogenase 1 family, member L2	-2.052
Hs.293970	ALDH6A1	Aldehyde dehydrogenase 6 family, member A1	2.048
Hs.152774	ALS2CR3	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 3	1.791
Hs.121520	AMIGO2	adhesion molecule with Ig-like domain 2	1.571
Hs.426312	AMOTL2	angiomotin like 2	1.592
Hs.369675	ANGPT1	angiopoietin 1	2.109
Hs.555903	ANGPTL1	angiopoietin-like 1	1.733
Hs.105016	ANKRD13C	ankyrin repeat domain 13C	1.529
Hs.493272	ANKRD15	ankyrin repeat domain 15	1.640
Hs.335239	ANKRD28	ankyrin repeat domain 28	1.868
Hs.165859	ANTXR1	anthrax toxin receptor 1	2.451
Hs.162963	ANTXR2	anthrax toxin receptor 2	3.659
Hs.406238	AOX1	aldehyde oxidase 1	3.390
Hs.401954	ARG99	ARG99 protein	1.873
Hs.516790	ARHGEF2	rho/rac guanine nucleotide exchange factor (GEF) 2	-1.731

Hs.518060	ARL6IP5	ADP-ribosylation-like factor 6 interacting protein 5	1.953
Hs.9728	ARMCX1	armadillo repeat containing, X-linked 1	1.512
Hs.489207	ASNS	asparagine synthetase	-1.613
Hs.435655	ASPN	asporin (LRR class 1)	-3.153
Hs.291196	ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	1.974
Hs.272011	B4GALT1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	-2.724
Hs.438993	BCAT1	branched chain aminotransferase 1, cytosolic	-1.672
Hs.478588	BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51) /// B-cell CLL/lymphoma 6 (zinc finger protein 51)	1.708
Hs.334370	BEX1	brain expressed, X-linked 1	2.490
Hs.503704	BIRC2	baculoviral IAP repeat-containing 2	1.583
Hs.558490	BTBD15	BTB (POZ) domain containing 15	1.690
Hs.255935	BTG1	B-cell translocation gene 1, anti-proliferative	4.008
Hs.93675	C10orf10	chromosome 10 open reading frame 10	1.616
Hs.124673	C18orf4	chromosome 18 open reading frame 4	2.219
Hs.497159	C1orf21	chromosome 1 open reading frame 21	1.817
Hs.518662	C1orf24	chromosome 1 open reading frame 24	-2.210
Hs.518662	C1orf24	chromosome 1 open reading frame 24	-1.789
Hs.259412	C1orf63	chromosome 1 open reading frame 63	1.567
Hs.567339	C20orf118	Chromosome 20 open reading frame 118	15.809
Hs.283869	C20orf121	chromosome 20 open reading frame 121	1.613
	C20orf19	chromosome 20 open reading frame 19	1.972
Hs.483067	C5orf13	Chromosome 5 open reading frame 13	-1.674
Hs.429608	C5orf18	chromosome 5 open reading frame 18	1.531
Hs.126409	C6orf105	chromosome 6 open reading frame 105	-2.139
Hs.109798	C6orf48	chromosome 6 open reading frame 48	-2.011
Hs.519930	C6orf62	chromosome 6 open reading frame 62	-1.588
Hs.292737	C9orf47	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 3	1.718
Hs.210995	CA12	carbonic anhydrase XII	-1.581
Hs.515162	CALR	calreticulin	-3.949
Hs.274873	CARS	cysteinyl-tRNA synthetase	-1.908
Hs.430589	CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	1.893
Hs.34333	CCBE1	collagen and calcium binding EGF domains 1	1.512
Hs.13291	CCNG2	Cyclin G2	1.670
Hs.399891	CD109	CD109 antigen (Gov platelet alloantigens)	1.587
Hs.504641	CD163	CD163 antigen	2.035
Hs.130014	CD302	CD302 antigen	1.937
Hs.369574	CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3	1.509
Hs.22065	CDC42SE1	CDC42 small effector 1	-1.961
Hs.22065	CDC42SE1	CDC42 small effector 1	-1.589
Hs.464829	CDH2	cadherin 2, type 1, N-cadherin (neuronal)	-2.972
Hs.121549	CDIPT	CDP-diacylglycerol--inositol 3-phosphatidyltransferase (phosphatidylinositol synthase)	1.993
Hs.106070	CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	2.160

Hs.494700	CDW92	Solute carrier family 44, member 1	2.545
Hs.440829	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	1.641
Hs.135406	CEBPZ	CCAAT/enhancer binding protein zeta	1.516
Hs.363396	CFH	complement factor H	1.879
Hs.180141	CFL2	cofilin 2 (muscle)	1.514
Hs.390736	CFLAR	CASP8 and FADD-like apoptosis regulator	1.566
Hs.47357	CH25H	cholesterol 25-hydroxylase	-2.570
Hs.434286	CHES1	checkpoint suppressor 1	1.551
Hs.382202	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	-1.775
Hs.496587	CHRDL1	chordin-like 1	1.990
Hs.560596	chromosome 18 open reading frame 50	Chromosome 18 open reading frame 50	1.537
Hs.74368	CKAP4	cytoskeleton-associated protein 4	-1.771
Hs.380627	CKLFSF6	chemokine-like factor superfamily 6	1.508
Hs.85201	CLEC2B	C-type lectin domain family 2, member B	1.519
Hs.436657	CLU	clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	-2.006
Hs.445890	CNIH4	cornichon homolog 4 (Drosophila)	1.549
Hs.523446	COL11A1	collagen, type XI, alpha 1	3.189
Hs.211933	COL13A1	collagen, type XIII, alpha 1	-1.665
Hs.409034	COL15A1	collagen, type XV, alpha 1	-2.175
Hs.172928	COL1A1	collagen, type I, alpha 1	-3.040
Hs.443625	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	-1.790
Hs.17441	COL4A1	collagen, type IV, alpha 1	1.601
Hs.270437	COL4A3BP	Collagen, type IV, alpha 3 (Goodpasture antigen) binding protein	1.514
Hs.210283	COL5A1	collagen, type V, alpha 1	-1.849
Hs.134830	COL8A1	Collagen, type VIII, alpha 1	1.920
Hs.464422	COLEC12	collectin sub-family member 12 /// collectin sub-family member 12	-1.669
Hs.558458	COPS8	COP9 constitutive photomorphogenic homolog subunit 8 (Arabidopsis)	1.847
Hs.96530	COX11	COX11 homolog, cytochrome c oxidase assembly protein (yeast) /// COX11 homolog, cytochrome c oxidase assembly protein (yeast)	1.879
Hs.522699	COX7B	cytochrome c oxidase subunit VIIb	-1.619
Hs.484551	CPM	carboxypeptidase M	2.358
Hs.405961	CREB3L1	cAMP responsive element binding protein 3-like 1	-1.720
Hs.332847	CRIM1	Cysteine rich transmembrane BMP regulator 1 (chordin-like)	1.757
Hs.513779	CRISPLD2	cysteine-rich secretory protein LCCL domain containing 2	3.061
Hs.443681	CSPG2	chondroitin sulfate proteoglycan 2 (versican)	-2.540
Hs.208597	CTBP1	C-terminal binding protein 1	-1.658
Hs.309288	CUGBP2	CUG triplet repeat, RNA binding protein 2	2.273
Hs.102914	CUL4B	cullin 4B	1.617
Hs.512181	CXorf33	chromosome X open reading frame 33	1.648
Hs.465413	CYB5	cytochrome b-5	1.607
Hs.221941	CYBRD1	cytochrome b reductase 1	2.081
Hs.154654	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	1.507

Hs.48950	DACT1	dapper, antagonist of beta-catenin, homolog 1 (Xenopus laevis)	-2.040
Hs.369761	DAZAP2	DAZ associated protein 2	1.501
Hs.156316	DCN	Decorin	2.458
Hs.535739	DKFZp434L142	hypothetical protein DKFZp434L142	2.431
Hs.328458	DKFZP434P211	POM121-like protein	1.691
Hs.288771	DKFZP586A0522	DKFZP586A0522 protein	5.927
Hs.40499	DKK1	dickkopf homolog 1 (Xenopus laevis)	7.121
Hs.380282	DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	1.579
Hs.195403	DOCK5	Dedicator of cytokinesis 5	2.200
Hs.473133	DOK5	docking protein 5	-2.065
Hs.80552	DPT	Dermatopontin	2.375
Hs.335034	DPYD	dihydropyrimidine dehydrogenase	1.846
Hs.282326	DSCR1	Down syndrome critical region gene 1	-2.294
Hs.304192	DSTN	destrin (actin depolymerizing factor)	1.552
Hs.171695	DUSP1	dual specificity phosphatase 1	2.236
Hs.308048	EBF	Early B-cell factor	2.494
Hs.486410	ECHDC1	enoyl Coenzyme A hydratase domain containing 1	1.896
Hs.126667	EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	2.165
Hs.82002	EDNRB	endothelin receptor type B	2.834
Hs.333388	EEF1D	Eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)	-1.606
Hs.302754	EFCBP1	EF-hand calcium binding protein 1	1.503
Hs.326035	EGR1	Early growth response 1	-2.907
Hs.271667	EHBP1	EH domain binding protein 1	1.542
Hs.478553	EIF4A2	eukaryotic translation initiation factor 4A, isoform 2	1.657
Hs.522995	EIF4EBP2	Eukaryotic translation initiation factor 4E binding protein 2	1.700
Hs.534314	EIF5A	eukaryotic translation initiation factor 5A	-1.509
Hs.192221	ELL2	elongation factor, RNA polymerase II, 2	2.373
Hs.520189	ELOVL5	ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	1.716
Hs.104925	ENC1	ectodermal-neural cortex (with BTB-like domain)	-1.870
Hs.190977	ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	-2.627
Hs.497788	EPRS	glutamyl-prolyl-tRNA synthetase	-1.797
Hs.26139	EPS8	epidermal growth factor receptor pathway substrate 8	2.111
Hs.519346	ERBB2IP	erbb2 interacting protein	1.547
Hs.11169	ERRFI1	ERBB receptor feedback inhibitor 1	6.487
Hs.461187	EXOSC6	Exosome component 6	-1.721
Hs.492618	EXT1	exostoses (multiple) 1	-2.080
Hs.434053	FAM3C	family with sequence similarity 3, member C	1.632
Hs.10784	FAM46A	family with sequence similarity 46, member A	-1.584
Hs.481371	FAT	FAT tumor suppressor homolog 1 (Drosophila)	-1.654
Hs.519294	FBN2	fibrillin 2 (congenital contractural arachnodactyly)	2.373
Hs.508284	FBXL3	F-box and leucine-rich repeat protein 3	1.556
Hs.403933	FBXO32	F-box protein 32	1.672

Hs.362733	FEM1B	fem-1 homolog b (C. elegans)	2.016
Hs.284244	FGF2	fibroblast growth factor 2 (basic)	-1.644
Hs.443687	FHL2	four and a half LIM domains 2	-2.043
Hs.558328	FKBP5	FK506 binding protein 5	10.182
Hs.103934	FKBP9	FK506 binding protein 9, 63 kDa	-1.888
Hs.387057	FLJ13710	hypothetical protein FLJ13710	-2.038
Hs.118183	FLJ22833	hypothetical protein FLJ22833	1.640
Hs.29692	FLJ36031	Hypothetical protein FLJ36031	1.862
Hs.289044	FLJ37927	CDC20-like protein	-1.679
Hs.506309	FLJ46688	FLJ46688 protein	1.555
Hs.149566	FMNL2	formin-like 2	1.496
Hs.520525	FNDC1	fibronectin type III domain containing 1	-2.673
Hs.370666	FOXO1A	forkhead box O1A (rhabdomyosarcoma)	3.186
Hs.220950	FOXO3A	forkhead box O3A	2.227
Hs.431498	FOXP1	forkhead box P1	-1.645
Hs.369448	FRAS1	Fraser syndrome 1	2.541
Hs.558804	FTH1	ferritin, heavy polypeptide 1	-1.713
Hs.558330	FTL	ferritin, light polypeptide	-1.731
Hs.74050	FVT1	Follicular lymphoma variant translocation 1	1.578
Hs.173859	FZD7	frizzled homolog 7 (Drosophila)	-1.631
Hs.524250	GABARAPL1 /// GABARAPL3	GABA(A) receptor-associated protein like 1 /// GABA(A) receptors associated protein like 3	1.944
Hs.47099	GALNT12	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12 (GalNAc-T12)	-2.170
Hs.411308	GALNTL2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	7.815
Hs.544577	GAPDH	glyceraldehyde-3-phosphate dehydrogenase	-1.571
Hs.404321	GARS	glycyl-tRNA synthetase	-2.570
Hs.65029	GAS1	growth arrest-specific 1	1.798
Hs.436062	GBE1	glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV)	1.695
Hs.521568	GCNT1	glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase)	1.621
Hs.78619	GGH	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	1.557
Hs.125180	GHR	growth hormone receptor	2.306
Hs.28988	GLRX	glutaredoxin (thioltransferase)	1.823
Hs.518525	GLUL	glutamate-ammonia ligase (glutamine synthetase)	6.958
Hs.495710	GPM6B	glycoprotein M6B	1.956
Hs.148685	GPRC5B	G protein-coupled receptor, family C, group 5, member B	2.281
Hs.386793	GPX3	glutathione peroxidase 3 (plasma)	18.622
Hs.386793	GPX3	glutathione peroxidase 3 (plasma)	6.427
Hs.40098	GREM1	gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	-1.810
Hs.524625	GRK5	G protein-coupled receptor kinase 5	1.662
Hs.556040	H19	H19, imprinted maternally expressed untranslated mRNA	-2.531
Hs.449291	HBLD2	HESB like domain containing 2	1.677
Hs.294133	HEBP1	heme binding protein 1	1.626
Hs.197644	HECA	headcase homolog (Drosophila)	1.653

Hs.7917	HIGD1A	HIG1 domain family, member 1A	1.709
Hs.397465	HIPK2	Homeodomain interacting protein kinase 2	3.690
Hs.201918	HIPK3	Homeodomain interacting protein kinase 3	1.729
Hs.58877	HMCN1	hemicentin 1	-1.846
Hs.434953	HMGB2	high-mobility group box 2	1.543
Hs.77558	HMGN3	high mobility group nucleosomal binding domain 3	1.647
Hs.42151	HNMT	histamine N-methyltransferase	2.147
Hs.504352	HNT	neurotrimin	-1.664
Hs.48384	HS3ST3B1	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	2.210
Hs.432648	HSPA2	heat shock 70kDa protein 2	2.682
Hs.522394	HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	-1.807
Hs.513743	HSRG1	MON1 homolog B (yeast)	1.501
Hs.90753	HTATIP2	HIV-1 Tat interactive protein 2, 30kDa	1.603
Hs.445403	IARS	isoleucine-tRNA synthetase	-2.444
Hs.76884	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	1.728
Hs.76095	IER3	immediate early response 3	-4.694
Hs.450230	IGFBP3	insulin-like growth factor binding protein 3	-1.852
Hs.369982	IGFBP5	insulin-like growth factor binding protein 5	-1.631
Hs.336046	IL13RA2	interleukin 13 receptor, alpha 2	-2.324
Hs.557403	IL1R1	interleukin 1 receptor, type I	2.116
Hs.28792	INHBA	Inhibin, beta A (activin A, activin AB alpha polypeptide)	-2.758
Hs.523360	INPP5A	inositol polyphosphate-5-phosphatase, 40kDa	1.587
Hs.7089	INSIG2	insulin induced gene 2	1.535
Hs.465744	INSR	insulin receptor	1.593
Hs.418133	IPLA2(GAMMA)	intracellular membrane-associated calcium-independent phospholipase A2 gamma	1.536
Hs.133294	IQGAP3	IQ motif containing GTPase activating protein 3	-2.196
Hs.471508	IRS1	insulin receptor substrate 1	1.585
Hs.442344	IRS2	insulin receptor substrate 2	1.904
Hs.508597	ITGBL1	integrin, beta-like 1 (with EGF-like repeat domains)	1.674
Hs.525704	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	-2.926
Hs.334017	K-ALPHA-1	tubulin, alpha, ubiquitous	-2.011
Hs.144795	KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	-2.414
Hs.524731	KCTD10	potassium channel tetramerisation domain containing 10	1.546
Hs.520210	KDELR2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	-1.783
Hs.554798	KDELR3	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	-1.630
Hs.151220	KIAA0992	palladin	2.498
Hs.1048	KITLG	KIT ligand	1.766
Hs.272215	KLF15	Kruppel-like factor 15	1.551
Hs.4055	KLF6	Kruppel-like factor 6	1.565
Hs.150557	KLF9	Kruppel-like factor 9	1.909
Hs.509264	KLHDC2	kelch domain containing 2	1.744
Hs.505104	KLHDC5	kelch domain containing 5	1.918

Hs.200841	LAMA2	laminin, alpha 2 (merosin, congenital muscular dystrophy)	2.253
Hs.489646	LAMB1	laminin, beta 1	1.924
Hs.497039	LAMC1	laminin, gamma 1 (formerly LAMB2)	1.534
Hs.496684	LAMP2	lysosomal-associated membrane protein 2	1.679
Hs.506829	LASS6	LAG1 longevity assurance homolog 6 (S. cerevisiae)	1.581
Hs.205865	LDLRAD3	low density lipoprotein receptor class A domain containing 3	1.643
Hs.23581	LEPR	Leptin receptor	2.184
Hs.445351	LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1)	-1.821
Hs.502176	LGR4	leucine-rich repeat-containing G protein-coupled receptor 4	-1.707
Hs.468490	LHCGR	Luteinizing hormone/choriogonadotropin receptor	2.739
Hs.507798	LHFP	lipoma HMGIC fusion partner	1.672
Hs.469593	LIMS1	LIM and senescent cell antigen-like domains 1	-1.885
Hs.475353	LMCD1	LIM and cysteine-rich domains 1	2.529
Hs.504908	LMO3	LIM domain only 3 (rhombotin-like 2)	3.054
Hs.438385	LOC145853	hypothetical LOC145853	2.039
	LOC149448	hypothetical protein LOC149448	1.506
Hs.259046	LOC152078	hypothetical protein BC010062	1.602
Hs.205952	LOC201895	Hypothetical protein LOC201895	1.557
Hs.283378	LOC253981	hypothetical protein LOC253981	1.938
	LOC284262	hypothetical protein LOC284262	1.520
Hs.32478	LOC387758	similar to RIKEN cDNA 1110018M03	1.583
Hs.534794	LOC401151	hypothetical gene supported by BC062741	2.669
Hs.136247	LOC401494	similar to RIKEN 4933428I03	1.589
Hs.102267	LOX	lysyl oxidase	1.608
Hs.116479	LOXL2	lysyl oxidase-like 2	-2.123
Hs.288467	LRRC15	leucine rich repeat containing 15	-2.135
Hs.555920	LTB4DH	leukotriene B4 12-hydroxydehydrogenase	1.614
Hs.49787	LTBP1	latent transforming growth factor beta binding protein 1	2.311
Hs.512776	LTBP2	latent transforming growth factor beta binding protein 2	-1.737
Hs.406475	LUM	Lumican	-1.697
Hs.134859	MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	1.627
Hs.5258	MAGED1	melanoma antigen family D, 1	-1.684
Hs.183109	MAOA	monoamine oxidase A	2.598
Hs.335079	MAP1B	microtubule-associated protein 1B	1.509
Hs.368281	MAP2	Microtubule-associated protein 2	1.540
Hs.390428	MAP3K4	Mitogen-activated protein kinase kinase kinase 4	-3.026
Hs.519909	MARCKS	Myristoylated alanine-rich protein kinase C substrate	-2.118
Hs.355867	MARS	methionine-tRNA synthetase	-1.925
Hs.510402	MCP	membrane cofactor protein (CD46, trophoblast-lymphocyte cross-reactive antigen)	1.656
Hs.368934	MGC40157	hypothetical protein MGC40157	-2.104
Hs.560915	MGC4677	hypothetical protein MGC4677	-1.961
	MGC5618	hypothetical protein MGC5618	1.863
Hs.389700	MGST1	microsomal glutathione S-transferase 1	1.921

Hs.501928	MICAL2	microtubule associated monoxygenase, calponin and LIM domain containing 2	-3.566
Hs.166017	MITF	microphthalmia-associated transcription factor	2.590
Hs.463483	MMD	monocyte to macrophage differentiation-associated	4.655
Hs.83169	MMP1	matrix metallopeptidase 1 (interstitial collagenase)	-2.367
Hs.375129	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	-1.958
Hs.326387	MORF4L2	mortality factor 4 like 2	1.576
Hs.339024	MSRB3	methionine sulfoxide reductase B3	1.514
Hs.502773	MTCBP-1	membrane-type 1 matrix metalloproteinase cytoplasmic tail binding protein-1	1.616
Hs.268698	MTHFD1L	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	-2.209
Hs.469030	MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	-1.648
Hs.498187	MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	1.781
Hs.336994	MTSS1	metastasis suppressor 1	1.927
Hs.369422	MXRA5	matrix-remodelling associated 5	-3.302
Hs.439620	MYO1B	myosin IB	-1.847
Hs.351851	NANOS1	nanos homolog 1 (Drosophila)	1.537
Hs.524599	NAP1L1	nucleosome assembly protein 1-like 1	1.712
Hs.306322	NAV3	neuron navigator 3	1.602
Hs.277721	NBR1	neighbor of BRCA1 gene 1	1.497
Hs.446678	NCOA2	Nuclear receptor coactivator 2	1.573
Hs.171426	NCOA7	nuclear receptor coactivator 7	1.574
Hs.514920	NDP52	nuclear domain 10 protein	1.843
Hs.5025	NEBL	nebulette	1.992
Hs.37982	NEDD9	neural precursor cell expressed, developmentally down-regulated 9	2.787
Hs.146542	NEGR1	neuronal growth regulator 1	1.722
Hs.24119	NEK7	NIMA (never in mitosis gene a)-related kinase 7	-1.829
Hs.22370	NEXN	nexilin (F actin binding protein)	2.855
Hs.155396	NFE2L2	nuclear factor (erythroid-derived 2)-like 2	1.540
Hs.79334	NFIL3	nuclear factor, interleukin 3 regulated	2.425
Hs.84928	NFYB	nuclear transcription factor Y, beta	1.909
Hs.448588	NGFRAP1	nerve growth factor receptor (TNFRSF16) associated protein 1	1.726
Hs.356624	NID1	nidogen 1	4.491
Hs.369840	NID2	nidogen 2 (osteonidogen)	2.270
Hs.551539	NME7	non-metastatic cells 7, protein expressed in (nucleoside-diphosphate kinase)	1.853
Hs.458607	NOPE	likely ortholog of mouse neighbor of Punc E11	-1.684
Hs.519445	NR2F1	Nuclear receptor subfamily 2, group F, member 1	1.935
Hs.347991	NR2F2	nuclear receptor subfamily 2, group F, member 2	1.548
Hs.21422	NRCAM	neuronal cell adhesion molecule	1.758
Hs.128686	NUCB2	nucleobindin 2	-1.650
Hs.213061	NUCKS	Nuclear casein kinase and cyclin-dependent kinase substrate 1	-1.686
Hs.213061	NUCKS1	nuclear casein kinase and cyclin-dependent kinase substrate 1	-2.139
Hs.475103	NUP50	nucleoporin 50kDa	1.498
Hs.155915	ODZ2	odz, odd Oz/ten-m homolog 2 (Drosophila)	-1.582

Hs.430849	OSBPL8	oxysterol binding protein-like 8	-2.250
Hs.500047	P4HA1	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I	-1.942
Hs.519568	P4HA2	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide II	-1.556
Hs.464336	P4HB	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase-associated 1)	-1.691
Hs.259461	PALM2-AKAP2	PALM2-AKAP2 protein	1.659
Hs.494928	PAPPA	pregnancy-associated plasma protein A, pappalysin 1	-1.834
Hs.308480	PCMTD1	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 1	1.598
Hs.567281	PCYOX1	prenylcysteine oxidase 1	1.990
Hs.50823	PDCD6	programmed cell death 6	1.625
Hs.352298	PDGFD	platelet derived growth factor D	2.415
Hs.212102	PDIA6	protein disulfide isomerase family A, member 6	-1.780
Hs.8364	PDK4	pyruvate dehydrogenase kinase, isoenzyme 4	7.514
Hs.368525	PDLIM1	PDZ and LIM domain 1 (elfin)	1.900
Hs.480311	PDLIM5	PDZ and LIM domain 5	1.591
Hs.58756	PER2	period homolog 2 (Drosophila)	-1.676
Hs.520421	PERP	PERP, TP53 apoptosis effector	2.081
Hs.102471	PHACTR2	phosphatase and actin regulator 2	1.537
Hs.12420	PHF17	PHD finger protein 17	2.157
Hs.477114	PHLDB2	pleckstrin homology-like domain, family B, member 2	1.536
Hs.132225	PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	3.779
Hs.477866	PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	-1.951
Hs.477869	PLSCR4	phospholipid scramblase 4	1.859
Hs.372031	PMP22	peripheral myelin protein 22	1.707
Hs.503709	PORIMIN	pro-oncosis receptor inducing membrane injury gene	2.091
Hs.136348	POSTN	periostin, osteoblast specific factor	-6.028
Hs.192233	PPL	periplakin	1.843
Hs.416769	PPM1B	protein phosphatase 1B (formerly 2C), magnesium-dependent, beta isoform	1.869
Hs.303090	PPP1R3C	protein phosphatase 1, regulatory (inhibitor) subunit 3C	-1.841
Hs.1908	PRG1	proteoglycan 1, secretory granule	2.958
Hs.64016	PROS1	protein S (alpha)	1.996
Hs.25338	PRSS23	protease, serine, 23	-1.855
Hs.494261	PSAT1	phosphoserine aminotransferase 1	-4.818
Hs.89545	PSMB4	proteasome (prosome, macropain) subunit, beta type, 4	-1.730
Hs.494538	PTCH	patched homolog (Drosophila)	2.098
Hs.201978	PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	1.732
Hs.470477	PTP4A2	protein tyrosine phosphatase type IVA, member 2	-1.551
Hs.506852	PTPN11	protein tyrosine phosphatase, non-receptor type 11 (Noonan syndrome 1)	1.978
Hs.148340	PTPRG	protein tyrosine phosphatase, receptor type, G	1.684
Hs.49774	PTPRM	protein tyrosine phosphatase, receptor type, M	1.748
Hs.127657	PTX3	pentraxin-related gene, rapidly induced by IL-1 beta	1.833
Hs.173656	RAB11FIP2	RAB11 family interacting protein 2 (class I)	1.738

Hs.99528	RAB31	RAB31, member RAS oncogene family	1.697
Hs.99528	RAB31	RAB31, member RAS oncogene family	1.646
Hs.98910	RAFTLIN	raft-linking protein	1.734
Hs.446680	RAI2	retinoic acid induced 2	1.505
Hs.6906	RALA	v-ral simian leukemia viral oncogene homolog A (ras related)	-1.901
Hs.508480	RAP2A	RAP2A, member of RAS oncogene family	2.174
Hs.98643	RAP2B	RAP2B, member of RAS oncogene family	-2.024
Hs.196102	RB1CC1	RB1-inducible coiled-coil 1	1.699
Hs.513609	RBL2	retinoblastoma-like 2 (p130)	1.576
Hs.470412	RBMS1	RNA binding motif, single stranded interacting protein 1	1.645
Hs.334587	RBPMS	RNA binding protein with multiple splicing	1.522
Hs.288880	RDH14	retinol dehydrogenase 14 (all-trans and 9-cis)	1.543
Hs.388918	RECK	reversion-inducing-cysteine-rich protein with kazal motifs	1.620
Hs.232021	REV3L	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)	4.777
Hs.7527	REXO2	REX2, RNA exonuclease 2 homolog (S. cerevisiae)	-1.896
Hs.507866	RGC32	response gene to complement 32	1.790
Hs.526902	RGMB	RGM domain family, member B	-1.688
Hs.386726	RGS4	regulator of G-protein signalling 4	-4.223
Hs.445030	RHOBTB3	Rho-related BTB domain containing 3	4.152
Hs.549125	RHOQ	Ras homolog gene family, member Q	1.710
Hs.512381	RHOQ /// LOC284988	ras homolog gene family, member Q /// similar to ARHQ protein	1.735
Hs.472270	RIN2	Ras and Rab interactor 2	1.681
Hs.283749	RNASE4	ribonuclease, RNase A family, 4	2.303
Hs.469199	RNF103	ring finger protein 103	1.651
Hs.58617	ROCK2	Rho-associated, coiled-coil containing protein kinase 2	1.849
Hs.410817	RPL13	ribosomal protein L13	-1.546
Hs.558383	RPL18A	ribosomal protein L18a	-1.872
Hs.406300	RPL23	ribosomal protein L23	-2.325
Hs.547172	RPL24	ribosomal protein L24	-1.643
Hs.523463	RPL27A /// LOC389435	ribosomal protein L27a	-2.140
Hs.356371	RPL28	ribosomal protein L28	-1.699
Hs.556047	RPL36A	ribosomal protein L36a	-1.642
Hs.433701	RPL37A	Ribosomal protein L37a	-1.970
Hs.380953	RPL38	Ribosomal protein L38	-1.863
Hs.406620	RPS10	Ribosomal protein S10	-1.595
Hs.433529	RPS11	ribosomal protein S11	-1.684
Hs.397609	RPS16	ribosomal protein S16	-1.948
Hs.512057	RPS20	ribosomal protein S20	-1.938
Hs.226390	RRM2	ribonucleotide reductase M2 polypeptide	-1.731
Hs.135254	RSPO3	R-spondin 3 homolog (Xenopus laevis)	-1.570
Hs.149261	RUNX1	runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	-1.935
Hs.472630	SAMHD1	SAM domain and HD domain 1	2.209

Hs.193133	SASH1	SAM and SH3 domain containing 1	1.753
Hs.28491	SAT	spermidine/spermine N1-acetyltransferase	3.485
Hs.459321	SATL1	Spermidine/spermine N1-acetyl transferase-like 1	2.333
Hs.558396	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	-2.412
Hs.379191	SCD5	stearoyl-CoA desaturase 5	2.256
Hs.488282	SEC61G	Sec61 gamma subunit	-1.773
Hs.535917	SEP10	Septin 10	3.070
Hs.275775	SEPP1	selenoprotein P, plasma, 1	5.152
Hs.154706	SERAC1	serine active site containing 1	-1.616
Hs.530412	SERBP1	SERPINE1 mRNA binding protein 1	1.664
Hs.146668	SERINC1	serine incorporator 1	1.835
Hs.414795	SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-2.421
Hs.38449	SERPINE2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	-8.096
Hs.241579	SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	-1.734
Hs.436687	SET	SET translocation (myeloid leukemia-associated)	-1.775
Hs.481022	SFRP2	secreted frizzled-related protein 2	-2.443
Hs.105700	SFRP4	secreted frizzled-related protein 4	-1.838
Hs.68714	SFRS1	splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor) /// splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	-1.959
Hs.479693	SFRS11	splicing factor, arginine/serine-rich 11	1.806
Hs.309090	SFRS7	splicing factor, arginine/serine-rich 7, 35kDa	-1.699
Hs.296323	SGK	serum/glucocorticoid regulated kinase	-1.893
Hs.156540	SGNE1	secretory granule, neuroendocrine protein 1 (7B2 protein)	-1.778
Hs.519018	SH3D19	SH3 domain protein D19	2.064
Hs.75069	SHMT2	serine hydroxymethyltransferase 2 (mitochondrial)	-1.757
Hs.410977	SIDT2	SID1 transmembrane family, member 2	1.616
Hs.75231	SLC16A1	solute carrier family 16 (monocarboxylic acid transporters), member 1	-1.682
Hs.30246	SLC19A2	solute carrier family 19 (thiamine transporter), member 2	1.761
Hs.187946	SLC20A1	solute carrier family 20 (phosphate transporter), member 1	-1.806
Hs.132553	SLC25A24	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 24	1.496
Hs.292509	SLC35F5	solute carrier family 35, member F5	1.887
Hs.491232	SLC39A14	solute carrier family 39 (zinc transporter), member 14	-1.606
Hs.529285	SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	4.420
Hs.494700	SLC44A1	solute carrier family 44, member 1	2.364
Hs.14846	SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	-2.260
Hs.390594	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	-1.872
Hs.22891	SLC7A8	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	2.528
Hs.331268	SMILE	SMILE protein	1.819
Hs.487200	SMOC2	SPARC related modular calcium binding 2	2.659
Hs.486357	SMPDL3A	sphingomyelin phosphodiesterase, acid-like 3A	2.100
Hs.515011	SMURF2	SMAD specific E3 ubiquitin protein ligase 2	-1.761

Hs.127406	SMYD3	SET and MYND domain containing 3	-1.704
Hs.432755	SNAG1	Sorting nexin associated golgi protein 1	1.614
Hs.360174	SNAI2	snail homolog 2 (Drosophila)	2.149
Hs.511149	SNAP23	synaptosomal-associated protein, 23kDa	1.646
Hs.461117	SNTB2	Syntrophin, beta 2 (dystrophin-associated protein A1, 59kDa, basic component 2)	1.498
Hs.468426	SOCS5	suppressor of cytokine signaling 5	-1.777
Hs.487046	SOD2	superoxide dismutase 2, mitochondrial	1.713
Hs.111779	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	-1.809
Hs.440414	SPG20	spastic paraplegia 20, spartin (Troyer syndrome)	1.706
Hs.445818	SPON1	spondin 1, extracellular matrix protein	-2.196
Hs.12152	SRPRB	signal recognition particle receptor, B subunit	-1.653
Hs.15154	SRPX	sushi-repeat-containing protein, X-linked	1.665
Hs.496710	STAG2	stromal antigen 2	1.553
Hs.233160	STC2	stanniocalcin 2	2.642
Hs.88297	STK17B	Serine/threonine kinase 17b (apoptosis-inducing)	2.017
Hs.521651	STMN2	stathmin-like 2	1.752
Hs.253903	STOM	stomatin	2.255
Hs.520383	STX7	Syntaxin 7	1.563
Hs.153026	SWAP70	SWAP-70 protein	1.608
Hs.153026	SWAP70	SWAP-70 protein	1.593
Hs.480615	SYNPO2	Synaptopodin 2	1.953
Hs.279245	TACC1	transforming, acidic coiled-coil containing protein 1	1.826
Hs.210891	TBC1D4	TBC1 domain family, member 4	1.615
Hs.491745	TCEA1	transcription elongation factor A (SII), 1	-2.343
Hs.95243	TCEAL1	transcription elongation factor A (SII)-like 1	1.897
Hs.311776	TCEAL3	transcription elongation factor A (SII)-like 3	1.698
Hs.194329	TCEAL4	transcription elongation factor A (SII)-like 4	3.696
Hs.272168	TDE1	tumor differentially expressed 1	1.520
Hs.516578	TFPI	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	2.318
Hs.82028	TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	1.549
Hs.482390	TGFBR3	Transforming growth factor, beta receptor III (betaglycan, 300kDa)	2.319
Hs.371147	THBS2	thrombospondin 2	-1.778
Hs.149991	THOC2	THO complex 2	1.533
Hs.134643	THY1	Thy-1 cell surface antigen	-1.701
Hs.522632	TIMP1	TIMP metallopeptidase inhibitor 1	-1.768
Hs.104839	TIMP2	TIMP metallopeptidase inhibitor 2	1.598
Hs.297324	TIMP3	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	-1.583
Hs.50382	TJP2	tight junction protein 2 (zona occludens 2)	2.106
Hs.276876	TM2D1	TM2 domain containing 1	1.575
Hs.279929	TMED9	transmembrane emp24 protein transport domain containing 9	-1.680
Hs.126598	TMEM45A	transmembrane protein 45A	1.663
Hs.556805	TMEM64	transmembrane protein 64	2.464

Hs.446574	TMSB10	thymosin, beta 10	-2.412
Hs.522584	TMSB4X /// TMSL3	thymosin, beta 4, X-linked /// thymosin-like 3	-1.929
Hs.143250	TNC	tenascin C (hexabrachion)	-1.681
Hs.437322	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	-2.171
Hs.133892	TPM1	tropomyosin 1 (alpha)	-1.912
Hs.133892	TPM1	tropomyosin 1 (alpha)	-1.770
Hs.516826	TRIB3	tribbles homolog 3 (Drosophila)	-2.185
Hs.522074	TSC22D3	TSC22 domain family, member 3	2.137
Hs.368214	TTC3	tetratricopeptide repeat domain 3	-1.733
Hs.403010	TTRAP	TRAF and TNF receptor associated protein	1.762
Hs.524395	TUBA3	tubulin, alpha 3	-1.970
Hs.436035	TUBA6	tubulin alpha 6	-1.800
Hs.512712	TUBB2	tubulin, beta 2	-1.646
Hs.486993	TULP4	tubby like protein 4	-1.625
Hs.514685	TWSG1	twisted gastrulation homolog 1 (Drosophila)	1.626
Hs.337766	TXNRD1	thioredoxin reductase 1	2.536
Hs.5308	UBA52	ubiquitin A-52 residue ribosomal protein fusion product 1 /	-1.556
Hs.518731	UCHL1	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	-2.041
Hs.127310	UHMK1	U2AF homology motif (UHM) kinase 1	1.605
Hs.477128	URB	steroid sensitive gene 1	-1.647
Hs.42400	USP12	ubiquitin specific peptidase 12	1.629
Hs.480597	USP33	ubiquitin specific peptidase 33	1.848
Hs.431081	USP53	ubiquitin specific peptidase 53	2.789
Hs.73793	VEGF	vascular endothelial growth factor	-3.598
Hs.435215	VEGFC	vascular endothelial growth factor C	1.705
Hs.533317	VIM	vimentin	-1.624
Hs.126550	VPS4B	vacuolar protein sorting 4B (yeast)	1.656
Hs.497599	WARS	tryptophanyl-tRNA synthetase	-1.955
Hs.82318	WASF3	WAS protein family, member 3	1.743
Hs.533287	WBP5	WW domain binding protein 5	1.769
Hs.478095	WDR56	WD repeat domain 56	-1.800
Hs.478095	WDR56	WD repeat domain 56	-1.732
Hs.492974	WISP1	WNT1 inducible signaling pathway protein 1	2.355
Hs.85951	XPOT	Exportin, tRNA (nuclear export receptor for tRNAs)	-2.257
Hs.213264	YARS	tyrosyl-tRNA synthetase	-1.737
Hs.406096	ZA20D2	zinc finger, A20 domain containing 2	1.576
Hs.136398	ZCCHC6	zinc finger, CCHC domain containing 6	1.816
Hs.503093	ZFP36L2	zinc finger protein 36, C3H type-like 2	1.996
Hs.288773	ZNF294	zinc finger protein 294	1.708
Hs.435535	ZNF395	zinc finger protein 395	1.541
Hs.530930	ZNF423	zinc finger protein 423	-1.642
Hs.558505	ZRANB1	zinc finger, RAN-binding domain containing 1	1.777
Hs.536218		Transcribed locus	10.481

Hs.61596	Transcribed locus	3.507
Hs.432336	Transcribed locus, weakly similar to NP_060190.1 signal-transducing adaptor protein-2; brk kinase substrate [Homo sapiens]	1.823
Hs.130526	Transcribed locus	-3.409
Hs.507676	Similar to hypothetical protein LOC231503	3.235
Hs.28625	Transcribed locus	-1.821
Hs.433995	CDNA FLJ26120 fis, clone SYN00419	1.737
Hs.49943	Transcribed locus, moderately similar to XP_517655.1 PREDICTED: similar to KIAA0825 protein [Pan troglodytes]	1.920
Hs.559602	Transcribed locus	1.975
		2.090
Hs.175569	Transcribed locus	1.850
Hs.128753	Full-length cDNA clone CS0DD009YB17 of Neuroblastoma Cot 50-normalized of Homo sapiens (human)	1.650
Hs.379253	CDNA FLJ39164 fis, clone OCBBF2002656	2.080
Hs.445588	Transcribed locus	1.865
		1.645
		-1.725
Hs.355655	CDNA FLJ36584 fis, clone TRACH2013450 /// MRNA; cDNA DKFZp564A222 (from clone DKFZp564A222)	1.636
Hs.478589	Hypothetical LOC389185	1.517
Hs.94499	Transcribed locus	-1.784
Hs.29802	Transcribed locus	-2.131
Hs.27621	CDNA FLJ12815 fis, clone NT2RP2002546	-1.626
Hs.536218	Transcribed locus	1.631
		1.918
Hs.146050	Transcribed locus	1.650
Hs.99472	MRNA; cDNA DKFZp564O0862 (from clone DKFZp564O0862)	1.507
		-1.565
Hs.379253	CDNA FLJ39164 fis, clone OCBBF2002656	1.569
Hs.532596	CDNA: FLJ23566 fis, clone LNG10880	1.834
		-1.733
Hs.257786	Transcribed locus	1.662
Hs.547494	Transcribed locus, weakly similar to NP_055301.1 neuronal thread protein AD7c-NTP [Homo sapiens]	1.580
Hs.530762	Transcribed locus	1.657
Hs.301296	CDNA: FLJ23131 fis, clone LNG08502	1.504
Hs.123119	CDNA FLJ37828 fis, clone BRSSN2006575	1.562
Hs.547618	Transcribed locus	-1.520
Hs.559452	MRNA full length insert cDNA clone EUROIMAGE 1509279	1.658
Hs.24359	CDNA FLJ11174 fis, clone PLACE1007367	1.537
Hs.61426	Mesenchymal stem cell protein DSC96	1.500

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Publications:

Bertinato J, J.J. Tomlinson, C. Schild-Poulter and R.J.G. Haché. (2003) *Evidence implicating Ku antigen as a structural factor in RNA polymerase II-mediated transcription.* *Gene* **302**:53-64

Tomlinson J.J, A. Boudreau, D. Wu, E. Atlas and R.J.G. Haché. (2006) *Modulation of Early Human Preadipocyte Differentiation by Glucocorticoids.* Endocrinology **147**(11):5284-5293

Wiper-Bergeron N, H. Abdou Salem, J.J. Tomlinson, D. Wu and R.J.G. Haché. *Glucocorticoid mediated preadipocyte differentiation is mediated through acetylation of C/EBPβ by GCN5.* Proc Natl Acad Sci USA **104**(8):2703-2708

Abstracts and Posters:

J.J. Tomlinson, D. Wu, A. Boudreau and R.J.G. Haché. *A Role for Glucocorticoids in Priming Preadipocytes for Differentiation.* EMBO, September 2005.

J.J. Tomlinson, D. Wu, and R.J.G. Haché. *TIF1β functions as a coregulator of the glucocorticoid receptor in the stimulation of preadipocyte differentiation.* EMBO, September 2005.

J.J. Tomlinson, D. Wu, and R.J.G. Haché. The Identification of *TIF1β as a regulator of preadipocyte differentiation.* University of Ottawa, April 2005.

J.J. Tomlinson, D. Wu and R.J.G. Haché. *Glucocorticoids Potentiate Preadipocyte Differentiation through LBD-mediated Downregulation of an HDAC1-Containing Complex.* Keystone Symposia, March 2004.

J.J. Tomlinson, Y.A. Lefebvre, D. Wu, L. Pope, R.J.G. Haché, N. Wiper-Bergeron. *Glucocorticoids Potentiate Preadipocyte Differentiation through LBD-mediated Downregulation of an HDAC1-Containing Complex.* 19th International Congress of Biochemistry and Molecular Biology, October 2003.

J.J. Tomlinson, N. Wiper-Bergeron and R.J.G. Haché. *Glucocorticoid Regulated Turnover of Histone Deacetylase 1.* University of Ottawa, April 2003.

J.J. Tomlinson, D. Rhodda, C. Schild-Poulter and R.J.G. Haché. *DNA-Dependent Protein Kinase Mediated Repression of Mouse Mammary Tumour Virus Expression.* University of Ottawa, April 2000.

S. Soubeyrand, J. Tomlinson, W. Giffin and R.J.G. Haché. *Characterization of Two New DNA Activators of the DNA-dependent Protein Kinase.* 18th International Congress of Biochemistry and Molecular Biology, July 2000.

Scholarships and Awards:

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Ontario Graduate Studentship: 2003-2004
NSERC (PGSA): 2001-2003
University of Ottawa National Excellence Award: 2001-2004
Ontario Graduate Scholarships in Science and Technology: 2000-2001
Strategic Areas of Development Award: 2000-2001
University of Ottawa Admissions Scholarship: 2000-2001
NSERC Undergraduate Student Research Award: 2000
University of Ottawa Merit Scholarship: 1998-2000
Dean's Honour List: University of Ottawa: 1996-1998
Loeb Research Institute Summer Internship: 1997-1999
University of Ottawa Entrance Scholarship: 1996-1997

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References are available upon request.