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**FACULTÉ DES ÉTUDES SUPÉRIEURES
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**FACULTY OF GRADUATE AND
POSTDOCTORAL STUDIES**

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GRADE / DEGREE

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FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

**Investigating the Role and Regulation of Histone Deacetylase 1 (HDAC1) in Glucocorticoid-
Potentiated Preadipocyte Differentiation**

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**Investigating the role and regulation of histone deacetylase 1
(HDAC1) in glucocorticoid-potentiated preadipocyte
differentiation**

Claire Kuzmochka

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the degree of

Master's in Biochemistry

Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa
July, 2010

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ISBN: 978-0-494-73768-2
Our file *Notre référence*
ISBN: 978-0-494-73768-2

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Abstract

Corticosteroids promote central obesity in humans and enhance the efficiency of preadipocyte differentiation in culture. The glucocorticoid receptor (GR) stimulates preadipocyte differentiation, in part, by enhancing transcription of the adipogenic commitment factor *C/EBP α* . GR is hypothesized to enhance *C/ebp α* transcription through a mechanism that involves promoting the titration, and subsequent degradation, of the co-repressor histone deacetylase 1 (HDAC1) from the *C/EBP α* promoter.

The first section of this investigation demonstrates that HDAC1, and not HDAC2 is involved in suppressing GR-potentiated preadipocyte differentiation and that the ability of HDAC1 to inhibit the adipogenic program is dependent on its deacetylase activity. Furthermore, the results imply that additional inhibitors assist HDAC1 in actively suppressing *C/ebp α* transcription in the absence of corticosteroids, as well as indicating that HDAC1 likely has supplementary deacetylation targets at the *C/EBP α* promoter, in addition to K98,101 and 102 of *C/EBP β* . Finally, we demonstrate that D181A HDAC1 is a catalytically compromised, dominant negative mutant of HDAC1.

In the second section of this investigation we provide evidence that, contrary to published results, acetylation of HDAC1 at lysine residues 218, 220, 432, 438, 439 and 441 does not inactivate the deacetylase activity of the enzyme. However, we propose a new hypothesis which reconciles our results with the published findings: Alterations in the acetylation status of the six identified lysine residues of HDAC1 regulate the substrate specificity of the enzyme, rather than its catalytic activity.

Acknowledgements

First of all, I would like to thank my supervisor, Dr. Robert Haché, for welcoming me into his lab and for giving me the opportunity, the resources and the guidance to complete my graduate studies. It has truly been a beneficial learning experience.

I would also like to say a special thank you to Dr. Ella Atlas who played a critical role in guiding my Master's project and in helping me deal with all of the little bumps along the way. Thank you so much for your unending patience, support, confidence, guidance and encouragement.

I would like to thank Houssein Abdou-Salem whose input was not only critical to editing this manuscript, but who also spent countless hours over cups of coffee with me discussing experimental strategies, details of molecular models and providing me with invaluable advice on which experiment to tackle next. Thank you for taking the time to share your passion and your gift for science with me.

I would like to acknowledge the rest of the members of the Haché lab who created such a warm, helpful and supportive environment in which I could learn. Thank you so much for taking the time to teach and guide me. I feel so privileged to have met and worked with all of you.

I would also like to thank Mr. Luke Fera who willingly put aside the time to help me learn and understand statistics.

I would also like to thank my Thesis Advisory Committee members, Dr. Skerjanc and Dr. Ekker, who provided helpful and insightful feedback on my project. Thank you for sharing your expertise and taking the time to guide me on my path through graduate school.

I would also like to acknowledge The Heart and Stroke Foundation and CIHR who provided the funding that has supported my research for the last two years.

Last, but certainly not least, I would like to thank all of my family and friends who have walked with me during this experience. Your willingness to listen to me ramble about things you've never heard of, your unwavering support and your constant encouragement over the last two years has been invaluable.

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List of Abbreviations

181D-A HDAC1	histone deacetylase 1 with aspartic acid(D) residue 181 mutated to alanine (A)
6K-Q HDAC1	histone deacetylase 1 with lysine(K) residues 218, 220, 432, 438, 439 and 441 mutated to glutamine (Q)
6K-R HDAC1	histone deacetylase 1 with lysine residues(K) 218, 220, 432, 438, 439, and 441 mutated to arginine (R)
ATCC	american type culture collection
ACTH	adrenocorticotropic hormone
BSA	bovine serum albumin
cAMP	cyclic adenosine mono-phosphate
C/EBP	CCAAT/enhancer-binding protein
cDNA	complementary DNA
CIP	calf intestinal phosphatase
CMV	cytomegalovirus immediate-early promoter
CRF	corticotropin releasing factor
CS	calf serum
ddH ₂ O	double distilled water
Dex	dexamethasone
DMEM	dulbecco's modified eagle media
DNA	deoxyribonucleic acid
E.coli	<i>Escherichia coli</i>
ECL	enhanced chemiluminescence
FBS	fetal bovine serum
FL	full length

FWD	forward
GR	glucocorticoid receptor
GST	glutathione-s-transferase
HDAC	histone deacetylase
HDLP	histone deacetylase like protein
IF	immunofluorescence
IP	immunoprecipitation
LB	luria-bertani
MI	MIX + insulin
MID	MIX + insulin + dex
MIX	3-isobutyl-methylxanthine
MMTV	mouse mammary tumour virus
MW	molecular weight
NF- κ B	nuclear factor (NF)- κ B
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PID	p53 target protein in the deacetylase complexes
PPAR γ	peroxisome proliferator-activated receptor gamma
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard Error of the Mean
siRNA	small interference ribonucleic acid
SD	standard Deviation

TSA	trichostatin A
VPA	valproic acid
WT	wild type

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Introduction:

Glucocorticoid Hormones

Glucocorticoids are cholesterol-based hormones that are synthesized in the cortex of the adrenal gland in humans (Guyton and Hall, 2006). When an individual is exposed to an external or psychological stress it triggers the hypothalamus to release a hormone called the corticotropin releasing factor (CRF) which is transported to the anterior pituitary gland where it induces adrenocorticotrophic hormone (ACTH) release. ACTH then travels through the blood stream to the adrenal gland where it stimulates the adrenal gland to produce and release glucocorticoids (Guyton and Hall, 2006). The principal metabolic form of glucocorticoid is cortisol, in humans, and corticosterone in rats. The release of CRF, ACTH and cortisol is in part regulated by the circadian rhythm, where the highest level of blood cortisol peaks about one hour before waking up in the morning at a blood concentration of ~551nM, dips as low as ~138nM around midnight, and averages around ~300nM throughout the day (Guyton and Hall, 2006). However, if a stressful event introduces itself, then the glucocorticoid release pathway will be immediately activated and will cause the level of cortisol in the blood to increase within 4 minutes of stimulation (Chrousos, 1995 and Guyton and Hall, 2006).

Cortisol is involved in the regulation of a wide variety of physiological processes including: glucose, lipid and protein metabolism, successful memory consolidation, suppression of the inflammatory and immune responses, erythropoiesis, promotion of fetal lung development, increased bone turnover, programmed cell death, and the promotion of preadipocyte differentiation into mature adipocytes (Chrousos 1995, Gregoire *et al.*, 1998, Hirayama *et al.*, 2002, McGaugh and Roozendaal, 2002, Necela and Cidlowski, 2004,

Roozendaal, 2000, Smith and Sabry, 1983 and Torday, 1980). Cushing's syndrome is a pathological state in which the levels of cortisol in the blood become elevated for a prolonged period of time. In 85% of cases, Cushing's syndrome is caused by an increase in ACTH secretion, and when this increase in ACTH secretion is caused by a tumour in the pituitary gland, it is referred to as Cushing's disease (Andreoli *et al.*, 2007 and De Castro and Moreira, 2007). In the remaining 15% of cases, Cushing's syndrome is caused by increased secretion of cortisol into the bloodstream, independent of ACTH levels and is often caused by: adrenal adenomas, adrenal carcinomas, micronodular adrenal disease and autonomous macronodular adrenal disease (Andreoli *et al.*, 2007). Patients with Cushing's syndrome or disease present with symptoms that include but are not limited to: proximal muscle weakness due to enhanced protein catalysis and the inability to absorb sufficient amounts of glucose, sleep disturbances and mood swings, and the re-localization of fat stores from the extremities to the central regions of the body, referred to as central or visceral obesity (Andreoli *et al.*, 2007, Guyton and Hall, 2006, and Necela and Cidlowski, 2004).

Interestingly, these particular symptoms are mimicked in patients who undergo prolonged glucocorticoid-based treatments to deal with auto-immune and chronic inflammatory diseases such as rheumatoid arthritis, or to suppress the immune system following organ transplant (Pijl and Meinders, 1996, Wassenber *et al.*, 2005, and Wung *et al.*, 2008). The role that corticosteroids play in the accumulation of visceral fat is becoming an increased health concern as significant amounts of abdominal and organ fat have been linked to an increased risk of developing cardiovascular disease and have been shown to lower the overall health status of the individual (Lau *et al.*, 2007).

It has also been shown that adipose tissue contributes to the production of local levels of glucocorticoids through the action of the enzyme 11 β -hydroxysteroid dehydrogenase, which is responsible for converting the inactive form of glucocorticoid, cortisone, into cortisol. This discovery was made because hydroxysteroid dehydrogenase knockout mice were incapable of developing visceral obesity, even when treated with high levels of glucocorticoids for long periods of time (Masuzaki *et al.*, 2001, Seckl, *et al.*, 2004, and White *et al.*, 1995). These results suggest that once visceral fat is present it acts to promote its continued existence and is thus difficult to eliminate.

The Glucocorticoid Receptor physiology and mode of action

The release of cortisol and other cholesterol-based hormones is part of the mechanism utilized by higher organisms to respond to extracellular changes in the environment, such as stress. However, cortisol cannot induce changes in cellular behaviour alone. In order to transmit a signal to a cell and induce a response, cortisol must first bind to its intracellular receptor, the glucocorticoid receptor (GR).

The GR is a member of the nuclear receptor superfamily, along with the mineralocorticoid, progesterone and androgen receptors (Lu *et al.*, 2006 and Meijsing *et al.*, 2007). In the absence of hormone, GR is maintained in the cytoplasm of the cell through its association with a cytoplasmic multiprotein complex comprised of the chaperone proteins hsp90 and hsp70, phosphoprotein p23 and immunophilin p59 (Pratt and Toft, 1997). The interaction between GR and hsp90 maintains the receptor in a conformation that promotes maximum binding of ligand but also conceals the receptor's nuclear localization signal (NLS), so that it remains in the cytosol in the absence of hormone (Necela and Cidlowski,

2004). Upon binding of cortisol, the glucocorticoid receptor dissociates from the hsp90 protein complex, resulting in a conformational change that reveals the NLS of GR. Next, the receptor homodimerizes and is translocated to the nucleus where it regulates transcription of its target genes.

GR can influence gene transcription in four possible manners. First of all, GR can bind directly to the promoter of a gene, if the promoter region contains copies of a specific DNA binding domain referred to as a Glucocorticoid Response Element (GRE) defined as being; 5'-TGTACAxxxTCTTGT-3', where x can be any nucleotide, and the number of GREs and their location varies (Necela and Cidlowski, 2004). Once bound to the promoter, GR upregulates transcription by recruiting co-activator complexes containing histone acetyltransferases (HATs), such as p300, and other chromatin remodelling factors. The HATs will acetylate the lysine residues of the promoter's core histones and, with the help of the chromatin remodelling enzymes, will generate a more transcriptionally accessible promoter. The open state of the promoter and the DNA-bound co-activators will in turn attract the necessary basal machinery required for transcription of the targeted gene (Deroo and Archer and 2001, Jenkins *et al.*, 2001). Alternatively, ligand-bound GR can act as an inhibitor of transcription by competing out transcriptional co-activators in situations where the co-activator binding site overlaps with the GRE. This particular mechanism is observed at the promoter of the osteocalcin and prolactin genes (Morrison *et al.*, 1993, Sakai *et al.*, 1988). GR can also inhibit transcription by interacting with co-activator proteins which are bound to promoter, in close proximity to the GRE. The interaction between GR and the co-activators can inhibit them from enhancing transcription of the target gene. For instance, the

interaction between GR and activator protein-1 (AP-1) at the proliferin promoter prevents AP-1 from enhancing *proliferin* expression (Diamond *et al.*, 1990 and Drouin *et al.*, 1989).

The final mechanism through which GR can regulate transcription is a DNA-binding independent mechanism. In this situation GR does not interact directly with the promoter of the target gene, but rather, interacts with the co-factors responsible for regulating transcription of the target gene. Consequently, when GR regulates transcription through the DNA-binding independent mechanism it can either promote or suppress expression of its target genes, depending on the effects that GR exerts on the availability and the activity of the specific transcription factors with which it associates (Wiper-Bergeron *et al.*, 2003). For example, one of the most pharmacologically exploited functions of cortisol is its inhibitory effect on the inflammatory response (Barnes, 2006, Heizter *et al.*, 2007). The ability of glucocorticoids to impede inflammation occurs, in part, through the ability of GR to bind and sequester nuclear factor NF- κ B, which prevents the transcription factor from upregulating a whole array of pro-inflammatory genes (Tao *et al.*, 2001).

As previously mentioned glucocorticoids have the ability to enhance the efficiency of adipocyte differentiation *in vivo* and in culture (De Castro and Moreira, 2007, Gregoire *et al.*, 1998, Pijl and Meinders, 1996, Wassenber *et al.*, 2005, and Wung *et al.*, 2008). Recent evidence suggests that the mechanism through which GR enhances the adipogenic process involves upregulation of the adipogenic transcriptional cascade through a DNA-binding independent mechanism (Wiper-Bergeron *et al.*, 2003).

Preadipocyte Differentiation

Excess caloric energy is stored by higher organisms, such as humans and mice, as white adipose tissue (WAT). With the prevalence of obesity at epidemic levels in

industrialized countries and the high correlation between obesity and increased risk of diabetes, high blood pressure and heart disease, a significant amount of resources have been committed to deepen our understanding of the processes and factors involved in adipocyte formation (Gregoire *et al*, 1998, Lau *et al*, 2007).

Mature white adipocytes are differentiated from preadipocyte cells, which are multipotent fibroblast cells committed to the adipogenic pathway. Preadipocytes are derived from mesenchymal stem cells and although they can commit to become an adipocyte they still maintain the capacity to differentiate into other mesodermal cell types such as; osteoblasts, myocytes and chondrocytes (Cornelius *et al*, 1994 and Gregoire *et al.*, 1998). Recent investigations aimed at locating and recognizing preadipocytes *in-vivo* have identified undifferentiated adipocyte precursor cells which reside in the mural cell compartment of the adipose tissue vasculature. These cells, which have been termed adipocyte progenitor cells, can be successfully differentiated into mature adipocytes *in-vitro*, once they have been isolated and exposed to adipogenic stimuli (Rodeheffer *et al*, 2008 and Wei *et al*, 2008). In culture, both immortalized preadipocyte cell lines and primary preadipocytes will commence the differentiation program upon exposure to adipogenic stimuli which includes high caloric excess, phosphodiesterase inhibitors, insulin and glucocorticoids.

Although the ultimate goal is to delineate and comprehend the steps contributing to the development of human WAT, there are several limitations to using human primary preadipocytes such as: problems associated with retrieving large amounts of primary human preadipocytes, their short lifespan and their resistance to multiple molecular techniques. Thus, they are primarily used to confirm the findings discovered in an immortalized murine

cell line. The 3T3 L1 preadipocyte cell line is the model most widely used to study the molecular details of preadipocyte differentiation. The 3T3 L1 cell line was generated by immortalizing cells isolated from Day 17 Swiss Mouse embryos (Green & Kehinde, 1975; Green & Kehinde, 1976; Green & Meuth, 1974). 3T3 L1 preadipocyte cells are believed to reflect the earliest stage of preadipocyte commitment to adipogenesis. The accuracy with which the 3T3 preadipocytes parallel the physiological events that take place during WAT formation in mice was validated when subcutaneous injection of 3T3 F44A2 cells, another well established 3T3 preadipocyte cell line derived from the same Swiss Mouse embryos as the 3T3 L1 cells, into nude mice successfully resulted in the development of mature fat pads, which could not be distinguished from the mouse's own fat cells (Green and Kehinde, 1976 and 1979). Additionally, recent investigations have developed a method to differentiate 3T3 L1 cells *in vitro*, on a 3D polymeric scaffold so that the mature adipocytes interact and function more like *in vivo* fat tissue. (Fishbach *et al*, 2004). Implantation of the *in-vitro* generated fat tissue into nude mice resulted in further development and maintenance of the fat pads (Fishbach *et al*, 2004 and Weiser *et al*, 2008). By using these immortalized murine fibroblast and preadipocyte cell lines, in addition to human primary preadipocyte cells and animal models, to study and confirm details about the adipogenic process, research has successfully begun to elucidate the basic transcriptional and morphological events involved in the adipogenic process.

In order to differentiate 3T3 L1 preadipocyte cells, they must first be permitted to reach and be maintained at confluence for 48hrs, to ensure that growth arrest, which is a prerequisite for successful differentiation of both immortalized and human primary preadipocytes, has been achieved (Gregoire *et al.*, 1998). In culture, 3T3 L1 cells are

maintained in serum with 10% fetal bovine serum (FBS) and they are differentiated by incubating the cells with high levels of insulin, 3-isobutyl-1-methylxanthine (MIX), which is a phosphodiesterase inhibitor that increases intracellular levels of cyclic adenosine monophosphate (cAMP), and the synthetic glucocorticoid, dexamethasone, for 48hrs (Green & Kehinde, 1975; Rubin *et al*, 1978). Although glucocorticoids are not required for the differentiation process to occur, their presence during the initial 48hrs of differentiation accelerates the differentiation process, and maximizes the number of mature adipocytes that are generated (Gregoire *et al*, 1998 and Shugart & Umek, 1997).

In order for 3T3 L1 preadipocytes to differentiate into mature adipocytes, the cells must be reprogrammed to express the genes, produce the proteins, exhibit the morphology and perform the functions characteristic of a mature fat cell. In order for these fundamental changes to occur at the cellular level, global alterations in gene expression must first take place. Therefore, the preadipocyte differentiation process requires that the cells follow a specific series of alterations in gene transcription, which has been termed the adipogenic transcriptional cascade (Figure 1A).

The 3T3 L1 adipogenic transcriptional cascade

CCAAT/Enhancer Binding Proteins (C/EBPs)

There are six C/EBP proteins: C/EBP α , C/EBP β /LAP/NF-IL6, C/EBP δ /NF-IL6 β , C/EBP γ , C/EBP ϵ and C/EBP ζ /CHOP-10/gadd153 and they are all part of the basic leucine zipper (bzip) family of transcription factors. Of the six C/EBP family members, four of them, C/EBP α , C/EBP β , C/EBP δ and CHOP have been shown to play a role in the adipogenic transcriptional cascade ((Batchvarova *et al*, 1995, Cao *et al*, 1991, Yeh *et al*, 1995, and Wedel & Ziegler-Heitbrock, 1995). In order to regulate transcription, the C/EBP

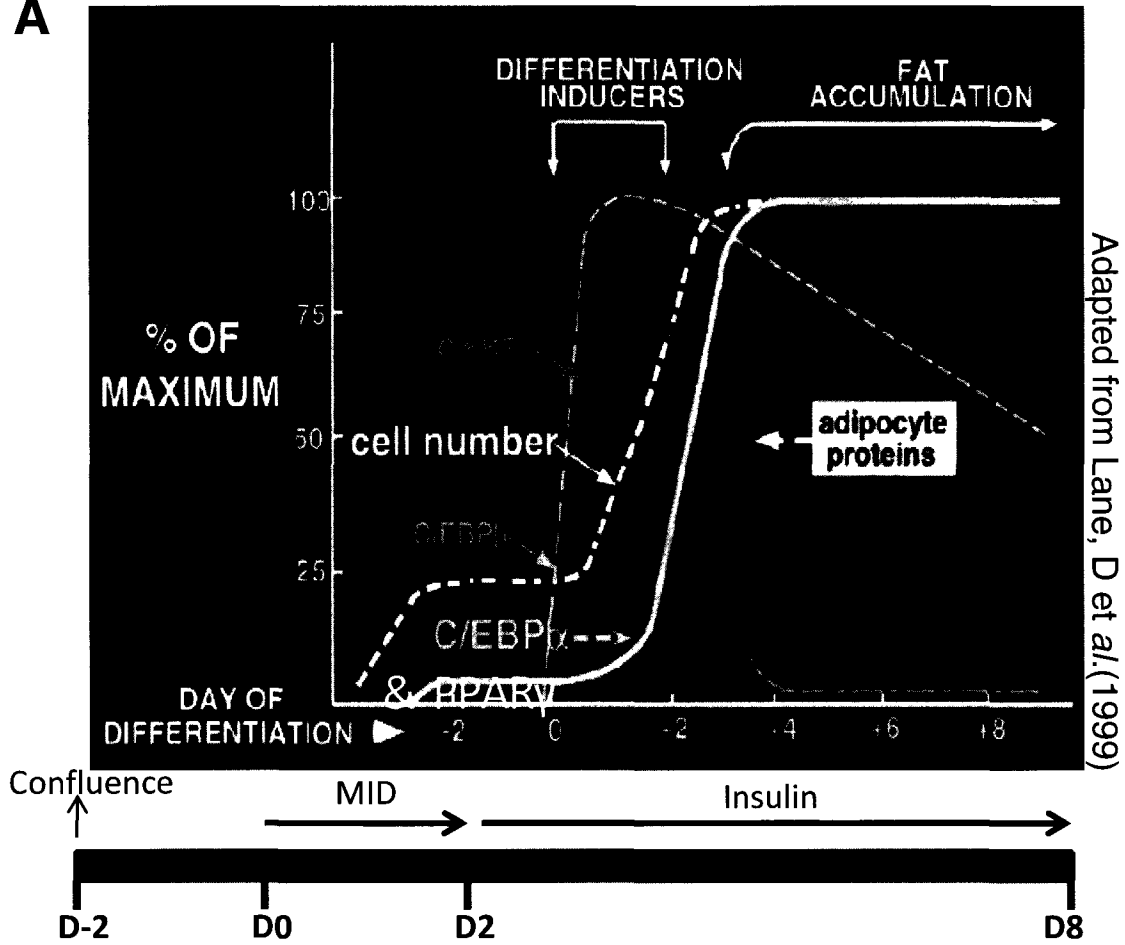
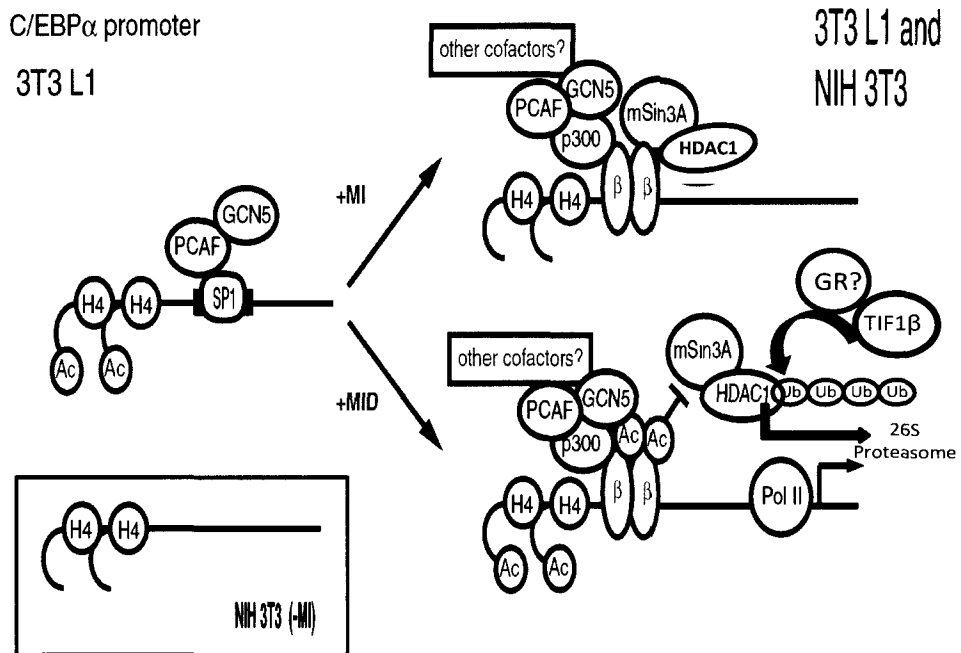
proteins homo- and hetero-dimerize before binding to C/EBP responsive elements within the promoter of their target genes (Landschulz *et al*,1989).

C/EBP β and C/EBP δ are two of the first transcription factors whose expression is increased during adipogenesis, and this occurs approximately 4hrs following the initiation of differentiation (Figure 1A). Once C/EBP β and C/EBP δ expression have been upregulated, they proceed to induce transcription of the master adipogenic regulators *C/ebp α* and *peroxisome proliferator-activated receptor gamma (ppar γ)*. However, elevated mRNA levels of both *C/ebp α* and *ppar γ* are not detectable until 48 to 72 hours following initiation of preadipocyte differentiation (Cao *et al*, 1991). C/EBP β and C/EBP δ are both involved in regulating the adipogenic transcriptional cascade and they are believed to complement each other's function, as deficiencies in preadipocyte differentiation are only observed upon silencing of both transcription factors (Tanaka *et al*, 1997). However, a preferential role for C/EBP β has been identified in the regulation of *C/ebp α* transcription, as ectopic expression of C/EBP β , but not C/EBP δ , is sufficient to enhance *C/ebp α* transcription and adipogenesis in the absence of adipogenic stimuli (Cao *et al*, 1991; Yeh *et al*,1995). Furthermore, ectopic expression of C/EBP β but not C/EBP δ , bestows NIH 3T3 fibroblast cells, which are derived from the same Day 17 Swiss mouse as 3T3 L1 but which are not committed to the adipogenic pathway, with the ability to differentiate into adipocytes following their exposure to the adipogenic cocktail (Wu *et al*, 1995; Yeh *et al*, 1995). The expression levels of both C/EBP β and C/EBP δ are maintained at elevated levels for the first 48hrs of the differentiation process, after which the levels of C/EBP δ decrease rapidly, while C/EBP β expression decreases slowly (Figure 1A and Cao *et al*, 1991). The decrease in C/EBP β and

Figure 1: The adipogenic transcriptional cascade and the molecular details of *C/ebpa* transcriptional regulation.

A) Temporal, schematic representation of the adipogenic transcriptional cascade that is responsible for directing the differentiation of preadipocytes into mature adipocytes (adapted from Lane, M.D. *et al*, 1999). Pre-mitotic cells, such as 3T3 L1 murine preadipocytes and NIH 3T3 murine fibroblasts, are held at confluence for two days before being stimulated to differentiate (defined as day zero). On Day 0, the cells are treated for 48hrs with an adipogenic cocktail comprised of the cyclic-AMP up-regulator, 3-isobutylmethylxanthine, insulin and dexamethasone, a synthetic corticosteroid. Within 4hrs of the induction of differentiation, transcription of *C/EBPβ* and *CEBPδ* is increased, and these two transcription factors proceed to induce expression of the adipogenic commitment factors *CEBPα* and *PPARγ*, by day 2 of differentiation. Transcriptional upregulation of *C/EBPα* and *PPARγ* coincide with a drop in *C/EBPβ* and *CEBPδ* expression, and with the termination of mitotic clonal expansion. Once *CEBPα* and *PPARγ* are expressed the cells are committed to the adipogenic pathway. The differentiating cells are subsequently maintained in complete media supplemented with insulin for an additional 6 days to promote lipid accumulation in the mature adipocytes.

B) Current model of *C/EBPβ* mediated *C/ebpa* transcription. The proposed mechanism of GR potentiated *C/ebpa* transcription suggests that in the absence of adipogenic stimuli (left) there is minimal *C/ebpa* expression in 3T3 L1 preadipocyte cells, in spite of the fact that the transcription factor Sp1 is present and the histone H4s are acetylated at the *C/EBPα* promoter, suggesting a transcriptionally accessible state. Alternatively, in NIH 3T3 cells (bottom left) the *C/EBPα* promoter is in a transcriptionally closed state in the absence of adipogenic stimuli. In both cell lines, treatment with MI (top, right) results in recruitment of *C/EBPβ* dimers to the *C/EBPα* promoter which become associated with both a co-activator complex, composed of GCN5 /PCAF and p300, and an mSin3A/HDAC1 co-repressor complex. In the absence of steroids the equilibrium favours the mSin3A/HDAC1 co-repressor complex over the co-activator complex, resulting in only minimal transcription of the *C/ebpa* gene. On the contrary, addition of dexamethasone to the adipogenic cocktail (bottom, right) results in the titration of the mSin3A/HDAC1 co-repressor complex from *C/EBPβ* and subsequent targeting of HDAC1 for 26S proteasomal degradation, mediated by TIF1β dependent ubiquitination of the deacetylase. Loss of the co-repressor complex from the *C/EBPα* promoter shifts the equilibrium towards the co-activator complex, thus promoting the acetylation of *C/EBPβ* and the histone H4s of the promoter and preventing re-association of the co-repressor complex. This in turn leads to the recruitment of the necessary transcriptional machinery and the subsequent upregulation in *C/ebpa* transcription.

A**B**

C/EBP δ expression coincides with the increase in cellular levels of C/EBP α and PPAR γ (Figure 1A)

The master adipogenic commitment factors; C/EBP α and PPAR γ

C/EBP α and PPAR γ are referred to as the adipogenic commitment factors because once their expression is induced the preadipocytes are committed to the differentiation process. Enhanced transcription of both *C/ebp α* and *ppar γ* is critical to the differentiation process as these two transcription factors are responsible for initiating transcription of a variety of differentiation dependent genes including; *leptin*, *GLUT4* and *ap2* (Hollenberg *et al*, 1997, Long & Pekala, 1996, Miller & Ntambi, 1996, Tontonoz *et al*, 1995, Tontonoz *et al*, 1994). Ectopic expression of either C/EBP α or PPAR γ is sufficient to drive preadipocyte differentiation in the absence of adipogenic stimuli in both 3T3 L1 preadipocytes and in the uncommitted NIH 3T3 fibroblasts (Freitag *et al*, 1994; Lin & Lane, 1994, Tontonoz *et al*, 1994b). Furthermore, loss of expression of either factor significantly disturbs the differentiation program, as cells unable to express PPAR γ were compromised in their ability to differentiate into mature adipocytes upon exposure to adipogenic stimuli, whereas cells unable to express C/EBP α successfully underwent differentiation but the mature adipocytes were insensitive to insulin (El-Jack *et al*, 1999, Rosen *et al*, 1999, Wu *et al*, 1999).

The exact timing and order that *C/ebp α* and *ppar γ* transcription are enhanced during the differentiation process is difficult to clarify because of the auto- and cross-regulation that occur between the two genes (Elberg *et al*, 2000; Legraverend *et al*, 1993; Wu *et al*, 1999). Genetic analysis has confirmed that the two transcription factors are involved in the same pathway, and the study has placed the initiation of *C/ebp α* transcription slightly before induction of *ppar γ* transcription (Rosen *et al.*, 2002). However, it should be noted that

expression of PPAR γ is required to sustain high expression levels of C/EBP α throughout the differentiation process (Zuo *et al.*, 2006).

It is also important to appreciate that the timing of each step in the transcriptional cascade is critical to proper differentiation of mature adipocytes. For example, growth-arrested preadipocytes must undergo at least one round of DNA replication and cell doubling, which is referred to as mitotic clonal expansion, before they can officially commit to the adipogenic differentiation program (Pairault and Green, 1979). This process of mitotic clonal expansions takes place during the first 48hrs of the differentiation process, therefore the expression of anti-mitotic factors such as C/EBP α must be inhibited until the cells are finished replicating, while expression of promitotic transcription factors, such as C/EBP β , must be maintained during the clonal expansion period and then their expression must be rapidly reduced in order for terminal differentiation of the cells to occur (Lane *et al.*, 1999 and Wiper-Bergeron *et al.*, 2003). The delay between the upregulation of C/EBP β and C/EBP δ expression, which occurs within 4 hrs of the initiation of differentiation, and C/EBP β - and C/EBP δ -mediated induction of *C/ebp α* and *ppar γ* transcription, which occurs within 48hrs post induction of differentiation, has been attributed, in part, to the inhibition of C/EBP β and C/EBP δ by CHOP-10. During the initial 12hrs of the adipogenic process CHOP binds and sequesters C/EBP β and C/EBP δ so that they cannot bind to DNA and regulate transcription. Approximately 12-16 hrs into the differentiation process CHOP is degraded, C/EBP β acquires its DNA binding ability and it can be detected at the C/EBP α promoter approximately 16-20hrs post-induction of differentiation (Morrison and Farmer, 1999 and Shao and Lazar, 1997). The inhibition of C/EBP β and C/EBP δ by CHOP-10 is thought to delay the expression of the anti-mitotic factor C/EBP α and thus ensures that clonal expansion can successfully occur before terminal differentiation commences (Umek *et al.*,

1991). Once C/EBP α expression is induced, it can then upregulate PPAR γ expression and the two adipogenic commitment factors can mediate proper transcriptional regulation of the rest of the adipogenic genes.

Interestingly, human preadipocytes do not undergo mitotic clonal expansion before commencing differentiation, suggesting that human preadipocytes represent a slightly later stage in the process of committing to adipogenesis. However, the order that C/EBP β , C/EBP δ , C/EBP α and PPAR γ are upregulated during the initiation of preadipocyte differentiation is very similar in human preadipocyte cells, as compared to 3T3 L1 mouse preadipocytes (Entenmann and Hauner, 1996, and Tomlinson *et al.*, 2006). These observations reinforce the biological relevance of using the 3T3 L1 model for studying preadipocyte differentiation.

**Our current model of transcriptional regulation of the C/EBP α
promoter in 3T3 L1 preadipocytes**

As previously mentioned, glucocorticoids are not required to induce 3T3 L1 preadipocyte differentiation but they have been shown to enhance the efficiency of the adipogenic process (Gregoire *et al.*, 1998, Shugart & Umek, 1997). Furthermore, the parallel between the ability of glucocorticoids to enhance 3T3 L1 differentiation and to promote the accumulation of central obesity observed in patients and mice models with either disease, or therapy induced hypercortisolemia (De Castro and Moreira, 2007, Gregoire *et al.*, 1998, Necela and Cidlowski, 2004, Pijl and Meinders, 1996, Wassenber *et al.*, 2005, and Wung *et al.*, 2008), has provided the physiological evidence to validate the importance of investigating the mechanism of GR-potentiated adipogenesis.

Research done in our laboratory has demonstrated that GR enhances initiation of the adipogenic cascade, in part, by upregulating transcription of *C/ebpa* (Wiper-Bergeron *et al.*, 2003). A study performed by Reichardt *et al.*, in 2000 demonstrated that mice expressing a mutant of GR with its DNA-binding domain (DBD) deleted, are normal weight and do not present any abnormalities in their ability to generate adipocytes. This discovery suggested that GR may enhance adipogenesis through a DBD-independent mechanism. This hypothesis was investigated and confirmed, as expression of a ligand binding domain (LBD) only GR mutant was able to increase both *C/EBPα* promoter-driven luciferase expression and 3T3 L1 preadipocyte differentiation in the presence of steroid (Wiper-Bergeron *et al.*, 2003).

Investigations into the regulation of the *C/EBPα* promoter in the presence of adipogenic stimuli revealed that the adipogenic transcription factor *C/EBPβ* is recruited to the promoter within 24 hrs of 3T3 L1 stimulation with MI/MID (Wiper-Bergeron *et al.*, 2003). In glucocorticoid-free conditions both a co-activator complex, composed of the histone acetyltransferases (HATs) p300/GCN5-PCAF, and a co-repressor complex, composed of the adaptor protein mSin3A and Histone Deacetylase 1 (HDAC1), were observed to associate with *C/EBPα* promoter bound-*C/EBPβ*. *C/EBPβ* is hypothesized to associate with the co-activator and the co-repressor complex in equilibrium. However, in the absence of hormone, the equilibrium is believed to favour the co-repressor complex resulting in minimal activation of *C/ebpa* transcription (Figure 1).

Based on microarray analysis of preadipocyte differentiation, HDAC1 was identified as a potential inhibitor of *C/EBPβ* (Soukas *et al.*, 2001). Overexpression of HDAC1 has been shown to suppress the ability of dexamethasone to induce *C/EBPα* promoter-driven luciferase expression and 3T3 L1 preadipocyte differentiation, while treatment of 3T3 L1

preadipocytes with the general HDAC inhibitors Trichostatin A (TSA) or Valproic Acid (VPA) replaced the ability of glucocorticoid treatment to induce preadipocyte differentiation (Wiper-Bergeron *et al.*, 2003). These results confirmed the hypothesis that HDAC1 is a suppressor of C/EBP β -mediated *C/ebp α* transcription.

Analysis of the interaction between HDAC1 and C/EBP β revealed that the proteins do not interact directly, but rather associate indirectly through the adaptor protein mSin3A (Wiper-Bergeron *et al.*, 2003). Furthermore, stimulation of cells with dexamethasone, in addition to MI, was discovered to promote the titration of mSin3A/HDAC1 away from C/EBP β and the successive degradation of HDAC1 by the 26S proteasome, within 24hrs of hormone treatment (Wiper-Bergeron *et al.*, 2003). GR-promoted dissociation of the co-repressor complex from C/EBP β and the subsequent degradation of HDAC1 are believed to shift the equilibrium in the favour of the co-activator complex, thus permitting GCN5-mediated acetylation of C/EBP β 's lysine residues 98, 101 and 102, which is a necessary event in the induction of C/EBP β transcriptional activity (Wiper-Bergeron *et al.*, 2003 and 2007).

Collectively, these findings have led us to hypothesize that GR enhances *C/ebp α* expression by stimulating the titration of the mSin3A/HDAC1 co-repressor complex away from C/EBP β and targeting HDAC1 for proteasomal degradation, thus promoting transcriptional activation of C/EBP β (Figure 1).

The following investigations were performed with the intention of confirming and further clarifying the role of HDAC1 in repressing the transcriptional activity of C/EBP β at the C/EBP α promoter. Furthermore, we hoped to gain some insight into the molecular mechanisms utilized by GR to control the adipogenic transcriptional cascade which is hypothesized to include regulation of HDAC1's inhibitory capability.

Histone Deacetylases (HDACs)

Histone deacetylases (HDACs) are an important family of transcription factors which assist in the regulation of gene expression by deacetylating the lysine residues of both histone and non-histone proteins (De Ruijter *et al.*, 2003 and Smith, 2007).

Thus far 18 mammalian HDACs have been identified and classified (Brunmeir *et al.*, 2009). These 18 HDACs have been subdivided into four different families, based on their sequence similarity (Gregoretta *et al.*, 2004). Class I HDACs are most similar to the yeast deacetylase protein Rpd3, and this class includes HDAC1, HDAC2, HDAC3 and HDAC8. Class II HDACs are more similar to the yeast Hda1 protein, and is composed of HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9 and HDAC10. HDAC11 was given its own family, Class IV, because it is so different from the other HDACs that have been characterized. Class III HDACs are the Sir2-NAD⁺ dependent deacetylases, which will not be discussed in detail (Brunmeir *et al.*, 2009 and De-Ruijter *et al.*, 2003). The only other major functional characteristic that differentiates the Class I from the Class II HDACs is that Class I HDACs are mainly localized to the nucleus, whereas the Class II HDACs travel between the cytoplasm and the nucleus (De-Ruijeter *et al.*, 2003).

Classically HDACs are regarded as inhibitors of transcription because the first cellular function identified for HDACs was their ability to deacetylate the lysine residues of the N-terminal tails of core histones (Hassig *et al.*, 1998). The acetylation status of histones has been shown to regulate the availability of DNA for transcription, as non-acetylated histones carry a slightly positive charge which attracts the DNA and causes it to coil more tightly around them, thus decreasing the access of transcription factors to the DNA. On the other hand, acetylated histones possess a global negative charge which repels the DNA and

causes it to uncoil from the histones and become more open and available to transcriptional activation (Allfrey *et al.*, 1964, Allfrey, 1996 and Vidali *et al.*, 1968).

HDACs are known to play essential regulatory roles in many important cellular and physiological processes including: the differentiation of new muscle and fat cells, the regulation of programmed cell death and fetal development (Brunmeir *et al.*, 2009, De Ruijter *et al.*, 2003, Glozak *et al.*, 2005, Mal *et al.*, 2001, Marks *et al.*, 2000, Sartorelli *et al.*, 1999 and Wiper-Bergeron *et al.*, 2003). However, over the last decade a significant shift in the perception of both HDAC function and physiological importance has occurred. First of all, HDACs have been shown to be capable of regulating cellular functions through the deacetylation of non histone proteins such as p53, MyoD and YY1 (Glozak *et al.*, Smith, 2007). For example, acetylation of p53 is critical to its ability to inhibit the cell cycle and induce apoptosis (Gu and Roeder, 1997, Liu *et al.*, 1999) and the deacetylation of p53 by HDAC1 inactivates p53's transcriptional ability and permits the cell to re-enter the cell cycle and continue dividing. A dysregulation of HDAC1 resulting in inactivation of p53 is believed to contribute to tumour progression in cancer patients, which is one of the reasons why HDAC inhibitors are currently being tested as anti-cancer agents (Juan *et al.*, 2000 Lin *et al.*, 2006, Luo *et al.*, 2000 and Vigushin *et al.*, 2002).

Interestingly, it has also been demonstrated that the presence of HDAC1 is actually required to activate transcription of some genes such as the mouse mammary tumour virus (MMTV) promoter and *c-jun*. In these systems loss of HDAC expression or inhibition of its deacetylase activity results in transcriptional silencing of the genes (Qiu *et al.*, 2006 and Thomson *et al.*, 2001). These examples highlight the complexity of the role of HDACs within the cell, and they demonstrate that understanding how the specificity and activity of

HDACs are regulated will be essential in elucidating the molecular details of the various cellular roles of HDACs.

The Structure of HDAC

Determining the structure of a protein is often extremely helpful in developing an understanding of which specific domains are responsible for the various cellular functions of the protein of interest. Currently, only the crystal structures of the Histone Deacetylase Like Protein (HDLP) from *Aquifex aeolicus* and human HDAC8 have been determined. Interestingly, after the structure of HDLP was deduced, that information was used to create Homology Models of the structures of the various class I human HDACs 1,2,3 and 8 (Wang *et al.*, 2005). Fairly soon after the homology study was performed the crystal structure for HDAC8 was elucidated (Somoza *et al.* 2004, and Vannini, *et al.*, 2004) and it was very similar to the predicted structure from the Homology Model study. This finding implies that the models generated by Wang *et al.* (2005) are similar to the actual structures of the HDACs and supports the validity of the findings from the HDAC homology modelling study.

The crystal structures of HDLP and HDAC8 and the models produced for HDACs1-2-3 and 8 have provided us with a significant amount of information that has helped us to begin understanding the structural components of the HDACs that are responsible for the enzymes deacetylase activity. We now know that the catalytic domain of HDACs is organized into a catalytic channel, which contains the active site of the HDAC enzyme at the bottom, and which branches off on the side to form a fairly long side pocket (Finnin *et al.*, 1999, Somoza *et al.* 2004, Vannini, *et al.*, 2004 and Wang *et al.*, 2005). This side pocket has been predicted to house the acetyl group following its removal from a lysine residue (Figure 2B) (Wang *et al.*, 2005). Furthermore, using these models it was determined that the catalytic site at the bottom of the catalytic channel is composed of a Zn^{2+} ion that is

Figure 2: The sequence alignment and basic X-Ray crystal structure of class I Histone Deacetylases. (Pictures are adapted from Wang, *et al*, 2005)

A) Sequence alignment of Histone Deacetylase Like Protein (HDLP) from *A. aeolicus* and class I human HDACs 1, 2, 3 and 8. To date, the major structural components of the class I HDACs have been identified as; the core nuclear localization domain, the charge relay system, the catalytic pocket, the Zn²⁺ binding residues and the 14Å pocket. These structural features of the class I HDACs, which convey functional properties to the enzymes, were identified through the analysis of the X-ray crystal structures of HDLP and HDAC8 and by the comparison of homology models of HDACs 1, 2, 3 and 8 to these established deacetylase protein structures. Subsequent sequence alignment of the class I HDACs with HDLP have identified completely or conservatively substituted amino acids across the class I HDAC family, and the importance of a large number of these specific amino acid residues to the various structural components of the class 1 HDACs have been confirmed through a multitude of mutational studies. The specific amino acids which have been recognized as being essential to a particular structural feature of the class I HDACs are identified by the various coloured dots.

B) A CPK model of HDLP representing the surface conformation of HDLP when the general HDAC inhibitor Trichostatin A is bound to the catalytic channel of the enzyme. The 11Å catalytic channel and 14Å pocket of HDLP are shown here and these structural features were also identified in HDAC8. Importantly, the catalytic site of class 1 HDACs, which is comprised of the charge relay system and the Zn²⁺ binding domain, has been determined to reside at the bottom of the 11Å channel, while the 14Å internal pocket is hypothesized to accommodate the acetate group, following its removal from a lysine residue.

coordinated by two aspartic acid residues and one histidine residue, which are indicated on the sequence of HDACs in Figure 2A. Moreover, five amino acids, two histidines, two aspartic acids, and one tyrosine, were identified as being part of a charge relay system that is critical to the catalytic activity of the HDACs, as these amino acids are believed to mediate the transfer of the electrons required for deacetylation to occur (Figure 1A). The importance of the Zn^{2+} coordinating amino acids and of the charge relay system residues was confirmed by site-directed mutagenesis studies performed on both HDAC1 and HDAC8. For these experiments, HDAC1 or HDAC8 was mutated at one of the Zn^{2+} coordinating amino acids, or one of the charge relay system amino acids, and each mutation independently produced a catalytically compromised HDAC1 or 8 mutant (Buggy, *et al*, 2000, Finnin, *et al*, 1999, Somoza, *et al*, 2004 and Vannini, *et al*, 2004). It is also important to note that the catalytic domain of the HDAC proteins is highly conserved across both isoforms and homologues. For example there is 35.2% similarity between HDLP and HDAC1, and the amino acids involved in Zn^{2+} coordination and charge relay system are conserved between various deacetylase enzymes (Figure 2B) (Wang *et al.*, 2005). This suggests that all deacetylase enzymes utilize the same deacetylation mechanism and has led to the hypothesis that the specificity and function of HDACs must be regulated through differences in the C-terminal domains of the HDAC isoforms. The C-terminal domain of HDACs is the most diversified segment between the different HDAC isoforms and has been previously hypothesized to regulate the specificity of the individual HDAC isoforms for their substrates (De-Ruijter *et al.*, 2003 and Luo *et al*, 2000).

Interestingly, a recent study conducted by Qiu *et al.* (2006), demonstrated that the deacetylase activity of GR-associated HDAC1 is regulated by changes in its own acetylation

status at lysine residues 218,220,432,438,439 and 441. This discovery implies that post-translational modifications could be involved in regulating the specificity and enzymatic activity of HDACs.

The Physiological Importance of understanding HDACs

As previously mentioned, HDACs are involved in the regulation of a large number of extremely important cellular processes such as differentiation, development and apoptosis. However, the dysregulation of HDACs has also been implicated in a large number of life-threatening and debilitating diseases. As previously described, the dysregulation of HDAC1 can lead to deacetylation and inactivation of p53, which in turn results in uncontrolled mitosis and contributes to the malignant phenotype of cancer (Juan *et al.*, 2000 and Luo *et al.*, 2000). Furthermore, HDAC2 and HDAC3 have been identified as playing a role in inhibiting the expression of the HIV-1 long terminal repeat, which contributes to the ability of the HIV virus to remain dormant and escape viral therapies (Keedy *et al.*, 2009). Therefore, HDAC inhibitors are being tested as both anti-cancer and anti-HIV drugs, in addition to being investigated for use in the treatment of inflammatory diseases, such as rheumatoid arthritis, asthma, cardiovascular disease and neurological diseases (Halili *et al.*, 2009).

One problem with using HDAC inhibitors as a form of disease therapy is that neither isoform nor class-specific HDAC inhibitors are currently available. Due to their lack of specificity and the involvement of HDACs in a wide variety of cellular processes, general HDAC inhibitors, such as valproic acid (VPA), can cause some unpleasant and unplanned side effects (Davis *et al.*, 1994). Therefore, if HDAC inhibitors are going to be used for widespread medical use, it will be essential to develop specific HDAC inhibitors that can act

on a single HDAC isoform or class so that side effects of the treatment are minimized. In order to generate specific HDAC inhibitors, we first need to understand how the specificity and function of each HDAC isoform is regulated.

Consequently, our investigation is focused on examining the role and the regulation of HDAC1 during the process of GR-potentiated enhancement in C/EBP β mediated *C/ebp α* transcription. This will provide insight into the molecular details of GR-potentiated adipogenesis and supply information about the regulation of HDAC1's specificity and catalytic activity.

Materials and Methods

Plasmid Constructs

pcDNA3.1(-)-Flag, WT HDAC1- HA-Flag, D181A HDAC1- HA-Flag, 6K-R HDAC1-HA-Flag and 6K-Q HDAC1-HA-Flag : The DNA constructs for the full length (FL) human HDAC1 (WT HDAC1), HDAC1 with aspartic acid 181 mutated to alanine (D181A HDAC1), HDAC1 with lysine residues 218, 220, 432, 438, 439 and 441 mutated to arginine (6K-R HDAC1), and HDAC1 with lysine residues 218, 220, 432, 438, 439 and 441 mutated to glutamine (6K-Q HDAC1) were all cloned in the pcDNA3.1 (-) vector from Invitrogen (Burlington, ON). All of the coding sequences for the WT and mutant HDAC1s are directly followed by the coding sequences for HA and Flag tags. The Flag-pcDNA3.1 (-) and WT HDAC1-HA-Flag pcDNA3.1 constructs were already available in Dr. Haché's laboratory, with the coding sequencing for WT-FL-human HDAC1 inserted between the XbaI and BamHI restriction sites, in the multiple cloning region of the vector, and the HA-Flag coding sequences inserted between the BamHI and HindIII restriction sites of the multiple cloning region. The mutant HDAC1s were created by performing site-directed mutagenesis on the pcDNA3.1 (-) WT HDAC1-HA-Flag construct and the process is described in detail in the site-directed mutagenesis section. Expression of the constructs is driven by the cytomegalovirus immediate-early (CMV) promoter and the vector contains both ampicillin and neomycin resistant genes to allow for bacterial antibiotic selection and generation of stable mammalian cell lines, respectively.

Site-Directed Mutagenesis of HDAC1 to generate the 6K-R and 6K-Q HDAC1 mutants:

Mutation of lysine(K) residues 218, 220, 432, 438, 439 and 441 of HDAC1 to either arginine(R) or glutamine (Q) to produce 6K-R HDAC1 or the 6K-Q HDAC1 mutant was

performed in 2 steps using the protocol from the Quick Change Kit® (Stratagene, La Jolla, CA). The first step mutated lysine residues 218 and 220, and the second step mutated lysine residues 432, 438, 439 and 441.

For the first step, for mutation of K 218 and 220 to Q the primers used were:

Forward: 5' CTGGCGAAGGCGAGTATTATG 3'

Reverse: 3' GACCGCTTCCGCTCATAATAC 5'.

For mutation of K 218 and 220 to R the primers used were:

Forward: 5' CTGGCAGAGGCAGGTATTAG 3'

Reverse: 3' GACCGTCTCCGTCCATAATC 5'.

For the second step, for mutation of K 432, 438, 439 and 441 to Q, the primers used were:

Forward 5' CCGCGAGAACTCTTCCAACCTTCGAAGAAGCCGAGAGAG 3'

Reverse: 3' GGCGCTCTTGAGAAGGTTGAAGCTTCTTCGGCTCTCTC 5'

For mutation of K 432, 438, 439, 441 to R the primers used were:

Forward 5' CCGCAGGAACTCTTCCAACCTTCAGAAGAGCCAGGAGAG 3'

Reverse 3' GGCGTCCTTGAGAAGGTTGAAGTCTTCTTCGGTCCTCTC 5'.

For the first step of site-directed mutagenesis (mutation of K 218 and 220 to Q or R) reaction mixtures of a final volume of 50µL were prepared in PCR tubes: 4µL of 2.5 mM DNTPs, 5µL of 10X Pfu Turbo Buffer, 125ng of forward primer, 125ng of reverse primers, 25ng of the template DNA, 1.25 µL of *Pfu Turbo* DNA polymerase (Stratagene, La Jolla, CA), and volume was made up to 50µL with ddH₂O. The reactions were then set up to undergo the following cycles in a PCR machine: 2min. at 95°C for 1 cycle, then 30 sec at 95°C, 57°C for 1min. and 68°C for 14 min., repeated 25 times, followed by incubation of the reactions at 16°C overnight. After the site-direct mutagenesis was completed, 10µL of each reaction was run on a 1% agarose gel and ethidium bromide stained DNA was viewed under UV light to

verify the presence of a band corresponding to the correct molecular weight of ~7 Kb. The remaining 40 μ L of the reaction mixture was then digested for 1hr, at 37°C, with 1.5 μ L of Dpn1 to remove the template DNA. Following digestion of the original DNA, 1 μ L of each of the site-directed mutagenesis reaction mixtures was transformed into DH5 α cells via electroporation. Following a 40 min incubation of the electroporated E.coli DH5 α cells, in a 37°C bacterial shaker, 80 μ L of the electroporation mixture was plated onto Luria-Bertani (LB)-Agar plates, supplemented with 100 μ g/mL ampicillin. Plates were grown upside down, overnight in a 37°C bacterial incubator.

The following day, 5 colonies were picked for each construct (K-R and K-Q HDAC1), and each colony was grown overnight, in a 37°C bacterial shaker, at 225rpm in 5mL of LB supplemented with 100 μ g/mL of ampicillin. The following morning, DNA was extracted from each colony using the QIAprep Spin Miniprep Kit® (Qiagen, Mississauga, ON) and 5 μ L of the DNA obtained from each clone, along with the original WT HDAC1 pcDNA3.1 DNA was digested with the restriction enzymes XbaI and NdeI, both from New England Biolabs (Mississauga, ON), for 1hr at 37°C, in a final reaction volume of 20 μ L, to verify the identity of the DNA. The restriction digest reactions were then run on a 1% agarose gel, and the ethidium bromide stained fragments were viewed under UV light. Positive clones, which were identified by the presence of both a 1.5Kb and 5.5 Kb DNA fragment, were sent for sequencing to have the mutations verified via forward, reverse and internal sequencing. Forward sequencing was performed with primers for the T7 primer site, reverse sequencing using the BGH primer site (both promoters are integrated into the pcDNA3.1(-) vector) and internal sequencing was performed using a custom fwd primer (5'CTAAAGTATCACCAGAGG3') designed to bind to base pairs 493-510 of HDAC1s coding sequence, to begin sequencing just before the first two mutation sites, aa 218 and 220

(bp 652-654 and 658-660). A single clone that was in frame and had lysine residues 218 and 220 mutated to arginine (K-R) and a single clone that was in frame and had lysine residues 218 and 220 mutated to glutamine (K-Q), as verified via sequencing, were used to perform the second step of the site-directed mutagenesis to induce the next four K-R or K-Q mutations using the primers described above to obtain the 6K-Q and 6K-R 218-220 432, 438, 439 and 441 HDAC1 mutants. The rest of the procedure, including verification of positive clones and of successful mutations, was performed for step two using the same procedures and parameters as described for step one.

pLXSN C/EBP β , WT HDAC1- HA-Flag, D181A HDAC1- HA-Flag, K-R HDAC1-HA-Flag and 6K-Q HDAC1-HA-Flag constructs:

In order to create stable cell lines, the proteins of interest were expressed using a retroviral strategy with the pLXSN vector . Flag, WT HDAC1-HA-Flag, D181A HDAC1-HA-Flag, 6K-R HDAC1-HA-Flag, 6K-Q HDAC1-HA-Flag and WT, full length, mouse C/EBP β were expressed in the pLXSN vector. The pLXSN vector contains both ampicillin and neomycin resistant genes to allow for bacterial antibiotic selection and generation of stable mammalian cell lines, respectively. The pLXSN-C/EBP β construct was available in Dr. Haché's laboratory. On the other hand, the WT and HDAC1-HA-Flag mutants were sub-cloned from the pcDNA3.1(-) vector into the pLXSN vector as described below.

Cloning of HDAC1-HA-Flag constructs into the pLXSN vector:

All of the HDAC1-HA-Flag constructs were sub-cloned from pcDNA3.1 (-) (invitrogen, Burlington, ON) into pLXSN (Clontech, Mississauga, ON). The HDAC1-HA-Flag constructs were excised from pcDNA3.1(-) (1 μ g of each construct) by using the restriction

enzyme PmeI (New England Biolabs, Mississauga, ON), and pLXSN (2 μ g) was linearized by digestion with HpaI (New England Biolabs, Mississauga, ON), as per the manufacturer's instructions. The digested DNA samples were run on a 1% agarose gel and the fragments corresponding to the HDAC1-HA-Flag fragments (~1.5Kb) and the fragment corresponding to pLXSN (~5.4 Kb) were cut out from the gel and purified using the QIAquick gel extraction kit® (QIAGEN, Valenci, CA), as per the manufacturer's instructions. To minimize vector re-ligation, the purified and linearized pLXSN vector was incubated with 2 μ L of Calf Intestinal Phosphatase (CIP) (New England Biolabs, Mississauga, ON), for 1hr at 37°C, in a final volume of 40 μ L, to remove the phosphate groups from the ends of the linearized vector. The amount of purified linearized pLXSN and excised HDAC1 (WT, D181A, 6K-R or 6K-Q) insert retrieved was quantified by running 2 μ L of the purified constructs on a 1% agarose gel, and comparing the intensity of the bands to that of the ladder. Ligation reactions were performed using 100ng of the purified, dephosphorylated and linearized pLXSN vector and each of the HDAC1 fragments at a ratio of 1:6 (plasmid to insert) and 1 μ L of T4 ligase (Invitrogen, Golden, CO), in a final volume of 25 μ L, overnight at 16°C. As a negative control, 100ng of the linearized, dephosphorylated pLXSN vector alone was prepared and incubated O/N in the same reaction conditions.

The following day, 2 μ L from each of the reaction mixture was transformed via electroporation into electrocompetent E.coli DH5 α cells purchased from invitrogen (Invitrogen, Golden, CO). Following a 40 minute incubation of the electroporated DH5 α cells in 1mL of SOC media (10g Bacto-tryptone, 2.5g bacto-yeast, 0.25g of NaCl, 20mL of 1M glucose, dissolved/diluted in 475mL of ddH₂O), at 37°C, 200 μ L of each transformation was plated onto a separate LB-Agar plate (10g of NaCl, 10g of Bacto-Tryptone or peptone, 5g of Bacto-yeast extract, 15g Agar all diluted to a final volume of 1L and adjusted to a pH

of 7.5), supplemented with ampicillin (100 µg/mL), under sterile conditions. The LB-Agar plates were incubated in a 37°C bacterial incubator overnight. The next day, 4 colonies from each of the HDAC1 constructs were picked and each colony was grown in 5mL of LB media supplemented with 100 µg/mL of ampicillin, overnight at 37°C, in a bacteria shaker, at 225 rpm.

Plasmid DNA from 1mL of each culture was purified using the QIAprep Spin Miniprep Kit® (Qiagen, Mississauga, ON), as per the manufacturer's instructions. To screen for both the presence and the orientation of the HDAC1 inserts in the pLXSN vector, the DNA from the minipreps was digested for 1hr with the restriction enzyme NdeI (New England Biolabs, Mississauga, ON), at 37°C, for 1hr, in a final volume of 20µL. The digests were resolved on a 1% agarose gel, containing ethidium bromide, at 100V. Positive clones, with the inserts in the correct orientation, were verified via sequencing, using a forward pLXSN primer and the custom designed, forward, internal HDAC1 primer whose sequence is provided in the Site-Directed Mutagenesis section. The positive and sequence verified clones were further amplified and the DNA was extracted using the QIAprep spin Maxi Kit® (Qiagen, Mississauga, ON) as per the manufacturer's instructions.

pTL2-GR: the full length, wild type, rat glucocorticoid receptor (GR) was expressed using the pTL2 vector, and was already available in Dr. Haché's laboratory. GR was expressed in Cos-7 cells for co-immunoprecipitation experiments and in NIH 3T3 cells for reporter gene dual luciferase transcription assays

pMSV WT and K98-102R C/EBPβ mutant : WT mouse C/EBPβ and K98-102R mutant mouse C/EBPβ, were expressed using the pMSV vector. These constructs were used in

reporter gene dual luciferase transcription assays in NIH 3T3 cells and were already available in Dr. Haché's laboratory.

pRL-CMV: the *Renilla* luciferase expression construct under the control of the *Cytomegalovirus* (CMV) promoter (Promega, Madison, WI, USA) was expressed in the PRL vector and this construct was already available in Dr. Haché's laboratory.

Cx14/12 LUC: This construct was already available in the laboratory and contains a 382bp fragment of the mouse *C/EBPα* promoter (+7 to -355) was cloned into the pxp1 reporter vector between the *KpnI* and *HindIII* (3332) sites, directly preceding the coding sequence for the *Firefly*-luciferase expression construct.

Cell Culture

NIH 3T3 (ATCC#:CRL-1658, American Type Culture Collection, Manassas, VA) and 3T3 L1 cells (ATCC#: CL-173, American Type Culture Collection, Manassas, VA) were maintained at 37°C in 10% CO₂. NIH 3T3 cells, fibroblast cells harvested from day 17 Swiss mice embryos, were maintained in Dulbecco's Modified Eagle Media (DMEM) (GIBCO-Invitrogen, Burlington, ON) containing 4.5g/L glucose, supplemented with penicillin, streptomycin and 10% Calf Serum (CS) (HyClone, Logan, UT, USA) and 3T3 L1 cells were maintained in DMEM containing 1.0 g/L glucose, supplemented with 10% CS (HyClone, Logan, UT, USA).

Cos-7 (ATCC CRL-1651) and Phoenix, amphi viral packaging cells (from G. Nolan, American Type Culture Collection, Manassas, VA), were maintained at 37°C, in 5% CO₂, in DMEM with 4.5 g/L glucose (high glucose) and supplemented with penicillin, streptomycin and 10% Fetal Bovine Serum (HyClone, Logan, UT, USA).

Transient Transfection of cells using FuGENE 6® Transfection Reagent

Throughout this project, the FuGENE 6® transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN) was used to perform all transient transfections with the exception of the siRNA experiments. In all cases, 3µL of FuGENE was used for every 1µg of DNA being transfected. The total volume of FuGENE needed was added directly to the total volume of serum-free media that was required (300µL/plate for 10 cm plates, and 100µL/well for 6 well plates), while minimizing interaction between the FuGENE reagent and the sides of the tube as much as possible. The solution was then mixed by inversion and was left to incubate at room temperature for 5 min. In the meantime, the DNA was prepared in a separate tube and once the incubation time was over the FuGENE+media mixture was added directly to the DNA, and the solution was mixed by inversion. The FuGENE and DNA were allowed to incubate at room temperature for 15 minutes, before adding the proper amount of the solution directly to the media in which the cells were being maintained (10mL for a 10 cm dish and 2 mL for a 6 well plate). FuGENE is non-toxic to cells so the media was changed within 24-48hrs of the transfection, depending on the conditions of the experiment being performed.

Generation of Stable Cell lines

Generation of retroviral viruses

Approximately $1.5-2 \times 10^6$ of phoenix-ampho cells (American Type Culture Collection, Manassas, VA) were seeded in a 60mm culture dish and one plate of phoenix cells was seeded per desired virus. When the cells had reached 70-80% confluency, they were transfected with pLXSN vector (Clontech, Mississauga, ON) containing the DNA coding sequence of interest.

The phoenix cells were transfected with 1µg of the pLXSN-DNA construct, containing the gene of interest to be incorporated into the virus, using FUGENE 6® Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN), as previously described. The following morning, the medium on the phoenix cells were replaced with 4mL of fresh DMEM +10% FBS with no antibiotics, and 24hrs later (2 days after the initial transfection) the first round of virus was harvested. The virus was removed from each dish containing the phoenix cells by aspirating the media into a 5mL syringe, through an 18 gauge needle. The needle was then immediately discarded and replaced with a filter of pore size 0.45 µm. The media containing the virus was passed through this filter, into a 15mL falcon tube. If the cells to be infected were not yet ready, the viruses were stored at -80°C.

A fresh 4mL of phoenix media was added to each of the dishes containing the phoenix cells, and the cells were incubated overnight (O/N) at 37°C, in 5% CO₂, so that a second round of virus could be harvested using the same procedure.

Retroviral infection of cells.

The cells to be infected (3T3 L1, NIH 3T3 or Cos-7) with the viruses were seeded in a 10 cm plate and upon reaching 50-70% confluence, were infected with the viruses containing supernatant harvested from the phoenix cells. Immediately prior to the addition of the viral-containing supernatants, the supernatant was diluted by half with fresh media suitable for each cell line and supplemented with 4 µg/ml polybrene. Once the viral solution was prepared, the medium on the cells to be infected were aspirated and were replaced with the 6mL per dish of media, virus and polybrene and the cells were incubated at 37°C, at their respective CO₂ levels, overnight to allow the infection to take place. The following morning 4mL of fresh media, was added to each plate to ensure the cells had the proper nutrients. At

the end of the day the virus-containing media was removed and replaced with 10mL of fresh complete media.

The cells were split once they reached 80-90% confluency, and were seeded into 15 cm dishes with the proper antibiotic selection, G418 (GIBCO-Invitrogen, Golden, CO) at a final concentration of 400-500 $\mu\text{g}/\text{mL}$, for the selection of the successfully infected cells. A plate of uninfected cells was selected along with the infected cells to ensure that the selection process was successful in eliminating any uninfected cells. Infected cells were selected for 8-11 days in G418, and selection only ceased when the plate of uninfected cells died. Successful expression of the desired protein was verified via western blot analysis, which is described in detail in a later section. Stable cell lines that were produced for this project include 3T3 L1 and Cos-7 cells expressing one of the HDAC1-HA-Flag constructs (WT, D181A, 6K-R, or 6K-Q) and NIH 3T3 expressing WT C/EBP β

siRNA mediated knockdown of HDAC1 and HDAC2 in NIH 3T3 cells and HDAC1 in 3T3 L1 cells

NIH 3T3 cells stably expressing WT C/EBP β , or 3T3 L1 cells, were seeded into 6-well plates ($1.5\text{-}2 \times 10^5$ cells/well) and were grown to 70% confluence before being transfected with siRNA SMARTpool (4 oligos) for mouse HDAC1 or HDAC2 (Dharmacon-Thermo Scientific, Chicago, IL). For HDAC1 siRNA knock down in NIH 3T3s 200pmol/well and for 3T3 L1 cells 400pmol/well of HDAC1 SMARTpool-siRNA was diluted in serum and antibiotic free media to a final volume of 185 μL /well; whereas 300pmol/well of HDAC2 SMARTpool-siRNA, also in a final volume of 185 μL was used for knockdown of HDAC2 in NIH 3T3 cells. Oligofectamine® reagent (Invitrogen, Burlington, ON) at a concentration of 3 μL /100pmol of siRNA, was diluted in serum and antibiotic-free

media to a final volume of 15 μ L/well. The diluted Oligofectamine solution was then mixed with the siRNA solution and left to incubate at room temperature for 20 min. Following incubation, 200 μ L of the Oligofectamine/siRNA/media mixture was added to each well, which had already contained 800 μ L of fresh antibiotic-free media. The cells were incubated for 4hrs, to allow for sufficient transfection of the siRNA. After 4hrs, the siRNA-Oligofectamine mixture was replaced with 2mL of fresh media.

The efficiency of siRNA knocked down was assessed by performing western blot analysis of HDAC1 or HDAC2 in the whole cell extracts of the transfected cells 48hrs following siRNA transfection, using antibodies against HDAC1 (Affinity BioReagents, Golden, CO) or HDAC2 (Santa Cruz Biotechnology, Santa Cruz, CA). Expression levels of HDAC1 and HDAC2 were quantified by performing densitometry with the program ImageQuant[®], and values were normalized to actin expression levels, which was immunoblotted using the actin H-300 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The cells were then used, 24hrs after siRNA transfection, in differentiation experiments as described below.

In each of the siRNA experiments, a parallel number of dishes of cells were transfected with scrambled siRNA (5'ACUACCGUUGUUAUAGGUGUU3') (Thermo Scientific-Dharmacon, Chicago, IL). The scrambled siRNA was transfected into the cells using the same protocol as described above and the amount of scrambled siRNA transfected was equal to the amount of HDAC1 or HDAC2 siRNA transfected into the cells. The cells infected with the scrambled siRNA were differentiated alongside the HDAC1 and HDAC2 siRNA treated cells as a negative control for siRNA transfection. Additionally, the levels of HDAC1 and HDAC2 in the scrambled siRNA treated cells were compared to the levels of

the proteins in cells that were not transfected with siRNA, to ensure that the scrambled sequence did not have any effects on HDAC1 or HDAC2 expression.

Differentiation of NIH 3T3 and 3T3 L1 cells

Differentiation of NIH 3T3 or 3T3 L1 cells is normally initiated two days after cells have reached confluence, other than in the siRNA experiments, where differentiation was initiated 48hrs after siRNA transfection and 24hrs after cells had reached confluence, to coordinate maximum HDAC1 or 2 knockdown with the time frame in which glucocorticoids influence preadipocyte differentiation. In both situations, the day on which differentiation was initiated is referred to as Day 0. To initiate differentiation, cells were treated with 100nM insulin, 500 μ M MIX (MI), or 100nM insulin, 500 μ M MIX and 250nM dex (MID), for 48hrs. For the remainder of the differentiation period, the cells were treated with media supplemented with 100nM insulin.

The effects of the conditions on the ability of dexamethasone to enhance preadipocyte differentiation was first examined by quantitative polymerase chain reaction (qPCR) of *C/ebp α* and *ppar γ* on Day 2 of differentiation for NIH 3T3 cells and day 3 for 3T3 L1 cells. Furthermore, NIH 3T3 cells were harvested on Day 4 of differentiation and 3T3 L1 cells were harvested on Day 7 and differentiation efficiency was visually examined by using Oil Red O to stain the neutral lipids of mature adipocytes and quantitatively examined via western blot analysis of known adipogenic markers using antibodies adipsin P-16, C/EBP α 14-AA and PPAR γ H-100 from Santa Cruz Biotechnology (Santa Cruz, CA). All western blots were quantified using densitometry through the program ImageQuant® and the values were normalized to actin protein expression levels, which was immunoblotted using the actin H-300 antibody (Santa-Cruz Biotechnology, Santa Cruz, CA).

Quantitative Polymerase Chain Reaction (qPCR)

RNA extraction

RNA was extracted from NIH 3T3 cells on day 2 of differentiation and from 3T3 L1 cells on day 3 of differentiation using the Qiagen RNeasy Mini Kit® (Qiagen, Mississauga, Ontario, Canada), as per the manufacturer's instructions. The RNA was kept at -80°C overnight. The following day, the concentration of RNA in the samples was calculated by performing spectroscopy at 260nm. For each sample, the volume corresponding to 5µg of RNA was obtained and was converted into cDNA using the following protocol.

For each sample, the volume of harvested RNA equivalent to 5µg was added to 2.5µL of 10X DNase1 buffer, 0.5µL of DNase, made up to a final volume of 25µL with diethylpyrocarbonate (DEPC) treated water and incubated at 37°C for 10 min. Then, 2.5µL of 50mM EDTA, pH 7.5, was added to each sample and the reactions were incubated at 65°C for 10 min., after which the samples were chilled on ice. Next, 22µL of each reaction mixture was added to 2µL of Oligo(dT) (Invitrogen, Carlsbad, CA) and 2µL of 10mM dNTP, and the solutions were incubated for 5min. at 70°C, chilled on ice and centrifuged for 10 seconds. Afterwards, 8µL of 5X First-Strand buffer, 4µL of 0.1M DTT and 0.2µL RNasin were added to each reaction and the samples were then incubated at 42°C for 2 min. Following the 2 min. incubation, 1µL of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) was added to each reaction mixture, and the reactions were placed back at 42°C for 50 min, followed by a 15 min incubation at 70°C. The samples were then chilled at 4°C and stored long term at -20°C to be used for quantitative PCR.

qPCR

Reaction mixtures for quantitative PCR were prepared in 96 well plates or 8 strip PCR tubes, to a final volume of 25 μ L which included: 12.5 μ L of SYBR green PCR Master Mix (Applied Biosystems INC, Forster City, CA), 0.3 μ M (final) of both the forward and reverse primers, 2 μ L of ddH₂O (the blank), standard, or sample, in some cases (for *C/EBP α*) 1 μ L of DMSO was also added and the remainder of the volume is made up with ddH₂O. Standards containing 1pg, 0.1pg, 0.01 pg, 0.001 pg and 0.0001 pg of the amplified DNA fragment were run at the same time as the experimental samples, and a standard curve of log [DNA] was generated by the computer program. The quantity of each cDNA in experimental sample was determined by the computer program by plotting the results for each sample on the standard curve. qPCR was performed for *C/ebp α* (Forward: 5'TGGACAAGAACAGCAAGCG3', Reverse primer: 5'CCATGGCCTTGACCAAGGAG3'), *ppary* (Forward: GAA ACT CTG GGA GAT TCT CC, Reverse: GCT GGA GAA ATC AAC TGT GG), and β -actin (Forward: GACTTCGAGCAAGAGATGGC and Reverse: CCAGACAGCACTGTTGGC). *C/ebp α* and *ppary* values were normalized to β -actin values. qPCR experiments were run on the 7500 Real Time PCR System light cycler®, and the results were quantified using the 7500 System SDS software®.

Oil Red O Staining

3T3 L1 cells at Day 7 of differentiation, or NIH 3T3 cells at Day 4 of differentiation, were washed gently with 2mL of 1X phosphate buffered saline (PBS)/well (in a 6 well dish), before being fixed for 0.5-1hr in 500 μ L of 10% Formalin (or 4% Formaldehyde). After 0.5-1hr the formalin was removed and was rinsed off by washing cells twice with 2mL of 1X

PBS/well, and 1mL of Oil Red O stain (Sigma-Aldrich) (3.5g/500mL of propylene glycol) was added to each well. Cells were incubated in stain for 2hrs-overnight, on a nutator, after which the Oil Red O was removed (and stored for reuse), and each well was washed 3 times with 1mL of ddH₂O. To prevent drying, 1 mL of ddH₂O was added to each well, and if stained cells were being stored, 1-2 drops of 10% Formalin (or 4% Formaldehyde) was added to each well, and plates were stored at 4°C until pictures were taken. The number of Oil Red O stained cells was visually assessed and was recorded by taking pictures on a phase contrast light microscope at 3X magnification. The results of the Oil Red O stain provide a visual representation of the amount of mature adipocytes that were produced during the differentiation process as the stain causes the accumulated lipid droplets of mature adipocytes to turn red. The amount of adipocyte differentiation between the MI and MID samples, and between the various conditions was visually assessed by the author, and the conclusion as to the amount of differentiation that occurred as a result of the Oil Red O stain was confirmed by the results of the qPCR experiments performed for the expression of *c/ebpα* and *ppary*, and by performing western blot analysis of the adipocyte markers; PPAR γ , C/EBP α and Adipsin, as described below. Furthermore, the trends observed in the Oil Red O stains were confirmed by performing multiple, independent differentiation experiments.

Western Blot Analysis

Whole Cell Lysate preparation for western blot analysis

Cells that were harvested for the sole purpose of performing western blot analysis to examine protein expression levels were subjected to the following procedure; media was aspirated from the cells and dishes were washed three times with 1X PBS (6mL for 10cm plates and 2mL/well for 6 well dishes). After the final wash was aspirated, cold 1X PBS

(1mL for 10cm plate and 300 μ L/well for a 6 well dish) was added to the plates, cells were scraped into the cold PBS, were transferred into Eppendorf tubes and kept on ice until all samples had been harvested. The cells were pelleted via centrifugation for 2min. at 4000 X g, at 4°C, the PBS was aspirated and the cells were re-suspended in either 200 μ L (10cm plate) or 50 μ L (6 well dish) whole cell lysis buffer (50mM Tris pH 7.4, 0.5% NP40, 150mM NaCl, 5mM EDTA, 1mM DTT and 1X complete protease inhibitor cocktail® (Roche Diagnostics Corporation, Indianapolis, IN)). Samples were incubated on ice for 10-20 min. to allow cells to swell, and were then sonicated for 10 seconds each, at power level 1 with the Branson Sonifier 450 sonicator. Next, the samples were centrifuged at 10000 X g, for 20min. to pellet cellular debris, and the protein concentration of each sample was determined via standard Bradford Assay using 0.06, 0.125, 0.25, 0.5, 1 and 2 μ g (final amounts in a total volume of 500 μ L) of bovine serum albumin to create the standard curve. For Western blot analysis 25-50 μ g of protein was used.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to standard Laemmli method. Proteins were denatured through the addition of SDS loading buffer (62.5mM Tris pH6.8, 10% Glycerol, 2% sodium dodecyl sulphate (SDS), 0.05% bromophenol blue, 355mM 2-mercaptoethanol) and boiling of the samples at 95°C for 7min. The samples were then spun at 10000 X g for 1 min., loaded into a mini 10-12% protein gel, and were separated according to the desired resolution which was determined by the resolution of 10 μ L of the protein ladder (Dual colour Precision Plus Protein Ladder, Bio Rad, Toronto, ON). Gels used for western blot were transferred onto polyvinylidene fluoride (PVDF) membranes for 1hr at 100V or O/N at 20V, according to the standard protocol.

Western Blot Analysis and Protein detection

After the proteins were transferred to PVDF membranes, the membranes were blocked for 1hr or O/N (at 4°C) in 5% non-fat milk, diluted in Phosphate Buffered Saline with 0.1% Tween-20 (PBST). After the membranes were blocked, they were incubated on a nutator for 2hrs at room temperature, or at 4°C, O/N, on a rotating wheel, in primary antibody, diluted in 5% non-fat milk (dilutions varied depending on the antibody, and the manufacturer). After incubation with the primary antibody, membranes were washed three times, for 10 min., in 1X PBST, and then horseradish peroxidase(HRP) linked-secondary antibody diluted in 5% non-fat milk at the corresponding dilution; 1:10000 for anti-rabbit, anti-goat and 1:20000 for anti-mouse, was added to the membranes for 1hr, at room temperature, on a shaker. Anti-rabbit and mouse secondary antibodies are from GE Healthcare, (Buckinghamshire, UK), while the anti-goat secondary antibody is from Santa Cruz Biotechnology Incorporated (Santa Cruz, CA) . After an hour, the secondary antibody was removed, and membranes were washed three times, for 10 minutes in 1X PBST. After the third wash, the membrane was incubated for 1 minute in enhanced chemiluminescence (ECL) (Perkin Elmer, Shelton, CT, USA) solution. The membranes were exposed to Kodak BioMax autoradiography film. Protein expression levels were quantified by performing densitometry on the protein bands of interest using the program ImageQuant®.

The same membranes were probed for multiple proteins by stripping off antibodies and ECL in between different immunoblots. Membranes were stripped by incubating them in 3mL re-blot plus (Millipore, Bellirica, MA) diluted in 22 mL of ddH₂O, for 25 min, and re-blocking the membranes in 5% non-fat milk, before adding the next primary antibody.

Co-immunoprecipitation Experiments in Cos-7 cells

Conditions, transfection and hormone treatment of Cos-7 cells

Cos-7 cells (5×10^5) were seeded onto 10cm plates, in phenol red-free complete high glucose DMEM (GIBCO, Invitrogen, Burlington, ON), supplemented with 10% Charcoal-stripped Fetal Bovine Serum (FBS) (HyClone, Logan, UT, USA), penicillin, streptomycin, sodium pyruvate (110mg/L), and maintained at 37°C and 5% CO₂. Cells were grown to 70% confluence before being transfected with 2µg of WT HDAC1-HA-Flag alone (as a negative control) or with 2 µg of pcDNA3.1-Flag, or a mutant HDAC1-HA-Flag construct (WT, D181A, 6K-R or 6K-Q), in addition to 1µg of pTLr GR and 500ng of PMSV-WT C/EBPβ. Transfection was carried out with the use of FuGENE 6® transfection reagent (Roche, Diagnostics Corporation, Indianapolis, IN) as previously described.

Two days following transfection, the Cos-7 cells were incubated for 4hrs in 10mL/plate of phenol red-free media + 10% charcoal-stripped FBS supplemented with 100nM dex (+dex condition), or the equivalent volume of ethanol (-dex). The cells were then washed and harvested as described in the whole cell lysate preparation section for western blots. After the cells were pelleted and the PBS was aspirated, the cells were re-suspended in 200 µL (10 cm plate) of immunoprecipitation lysis buffer (20mM HEPES pH 7.6, 20% Glycerol, 1.5 mM MgCl₂, 0.5 M NaCl, 0.2M EDTA, 0.1% Triton-X 100, 1mM DTT and 1X Protease Inhibitor Cocktail® (Roche Diagnostics, Indianapolis, IN)) and samples were incubated on ice for 30 min. to lyse cells. The samples were then centrifuged for 10 min., at 13 000 X g, at 4°C, to pellet any cellular debris. The protein concentration of the supernatant was determined via standard Bradford Assay as previously described.

Immunoprecipitation

For immunoprecipitation of the HDAC1-HA-Flag constructs, 500 μ g-1 mg of total protein was used, and 5% of the volume of the whole cell extracts used for the immunoprecipitation was put aside to be run on a gel, alongside the immunoprecipitation samples to correct for protein expression levels. The volume of the whole cell extracts used for the immunoprecipitation was decreased from 500 mM NaCl (which was used during the cell lysis) to 150mM NaCl through dilution of the sample with immunoprecipitation dilution buffer, which is the same as the immunoprecipitation lysis buffer but without any NaCl. The Flag-HDAC1 constructs were immunoprecipitated from the Cos-7 whole cell extracts by adding 30 μ L of 50% Red-Anti-Flag M2 Affinity Gel beads (Sigma Aldrich, St. Louis, MO) suspended in immunoprecipitation dilution buffer, to the samples, and incubating the reaction mixtures at 4°C, on a rotating wheel for 2hrs, or overnight. After the incubation, the beads were pelleted by centrifuging the samples for 2 min. at 4°C, at 3500 X g. The immunoprecipitation buffer was aspirated from the samples, the beads were washed and re-suspended by flicking in 1mL of immunoprecipitation-wash buffer (which is the same as the immunoprecipitation-lysis buffer, but with no glycerol and with 150mM NaCl, and were centrifuged again at 3500 X g, for 2 min. at 4°C. The wash process was repeated 3-4 times per sample. After the final wash and spin, the buffer was aspirated and the beads were re-suspended in 15 μ L of 6X SDS-PAGE buffer. At this point, 6X SDS-PAGE buffer was also added to the 5% inputs, and all of the samples were boiled and migrated on 10% SDS polyacrylamide gels (one gel for the 5% inputs, and one for the immunoprecipitation samples) and transferred to PVDF membranes as previously described.

Co-Immunoprecipitation detection

The 5% input and immunoprecipitation membranes were probed for the transfected HDAC1-HA-Flag constructs (WT, D181A, 6K-R or 6K-Q) and for endogenous HDAC1 using, anti HA (1:400, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and anti-HDAC1 (1:2500, Affinity Bioreagents, Golden, Co) antibodies, respectively. Both the tagged-HDAC1 constructs and the endogenous HDAC1 constructs were examined via western blot analysis to determine the efficiency of the immunoprecipitation, and to examine expression levels of the transfected HDAC1 constructs relative to the endogenous protein levels.

Both membranes were stripped, as previously described, and re-probed with antibodies against transcription factors known to be involved in the regulation of *C/ebpa* transcription. The co-factors investigated include; mSin3A (AK-11, Santa Cruz) and C/EBP β (C-19, Santa Cruz) and GR (1:1000, Affinity Bioreagents, Golden, CO). The efficiency with which the different HDAC1 constructs co-immunoprecipitated the above transcription factors was quantified by performing densitometry on the western blots results with the program ImageQuant[®]. Densitometry values for the co-immunoprecipitated transcription factors were normalized to the amount of HDAC1-HA-Flag construct with which they were co-immunoprecipitated.

Immunofluorescence

To investigate the localization of the different HDAC1 mutants, 1×10^5 3T3 L1 cells stably expressing pLXSN-Flag, pLXSN-WT HDAC1-HA-Flag, pLXSN-D181A HDAC1-HA-Flag, pLXSN-6K-R HDAC1-HA-Flag, or pLXSN-6K-Q HDAC1-HA-Flag were plated onto coverslips, which had been sterilized by soaking them in ethanol, flaming them and then placing them in six well dishes with 2mL of media. The following day, after the cells had

adhered to the coverslips, they were treated with either 1 μ M dexamethasone, or the equivalent volume of ethanol diluted in DMEM containing 1 μ g/L glucose and supplemented with 10% CS, for 4hrs. Following steroid or ethanol treatment, cells were washed 2 times with 2mL of 1X PBS and were fixed in 1ml/well, of 10% formalin (EM Sciences, Gibbstown, NJ, USA) for 30 min at room temperature. Then the cells were permeabilized through incubation for 30 min. at room temperature in 1mL of 0.5% Triton-X (EM Sciences, Gibbstown, NJ, USA) diluted in PBS. Next the cells were blocked for 1 hour at room temperature with 1mL of 5% Bovine Serum Albumin (Sigma-Aldrich, Oakville ON) , diluted in PBS. Primary antibodies Anti-FLAG[®] M2 (#3165, Sigma-Aldrich, Oakville ON) and Anti-HDAC1 (Affinity BioReagents, Golden, CO), were diluted to 1:500 and 1:1000, respectively, to a final volume of 500 μ L per well in PBST, and were added to cells for 1hr, at room temperature on nutator. After an hour the primary antibodies were removed and cells were washed 3 times for 5min. in 2mL of 1X PBST, following which the secondary antibodies, Alexa Fluor 540, donkey anti-mouse and Alexa Fluor 5488 donkey anti-rabbit (both from Invitrogen, Burlington, ON) were diluted in PBST to a final concentration of 1:500, and 500 μ L per well of the secondary antibody solution was added to cells for 30 min., at room temperature, on a nutator.

Cells were then washed 3 times for 5 min. with 2mL of 1X PBST, after which the coverslips were placed face down on top of a drop of Vectashield mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA, USA, 94010). The coverslips were sealed along the edges with nail-polish, left to dry at room temperature for 20 minutes, and then either analyzed using fluorescence microscopy (Zeiss-Axiovert, 200M microscope) or wrapped in tin foil and stored at -20 $^{\circ}$ C until analysis. Cells were visualized using a 63X Oil Objective lens and pictures were taken using the multi-channel acquisition option, so that

overlapping pictures of DAPI (blue), HDAC1 (red) and Flag-HDAC1 constructs (Green) could be obtained.

Monitoring dex-induced proteasomal degradation of pcDNA3.1 HDAC1-HA-Flag constructs in Cos-7 cells

Cos-7 cells (5×10^5 cells) virally transduced to express pLXSN alone, WT HDAC1-HA-Flag, 6K-R HDAC1-HA-Flag or 6K-Q HDAC1-HA-Flag, were plated into 10 cm dishes, with phenol red-free DMEM supplemented with 10% charcoal-stripped FBS (GIBCO-Invitrogen, Burlington, ON). Cells were grown to 70% confluence, upon which they were transfected with $1 \mu\text{g}$ of pTLr-GR and 500ng of pMSV-C/EBP β , using the FuGENE 6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN) as previously described. The following day the cells were treated with 250nM dex alone, the equivalent volume of ethanol alone, 250nM dex + 1 μM MG132, or ethanol + 1 μM MG132 for 24hrs. Then, the cells were harvested, lysed and prepared for western blot analysis, as described. The protein concentrations of the samples were quantified using a standard Bradford assay and approximately, 40 μg of protein, resuspended in SDS loading dye was loaded onto a 10% SDS polyacrylamide gel and separated by electrophoresis. The proteins were then transferred to a PVDF membrane and degradation of the HA-Flag tagged HDAC1 proteins was investigated by western blot analysis using the primary HA antibody (1:400, Santa Cruz Biotechnologies, Santa Cruz, CA) and the primary HDAC1 antibody (1:2500, Affinity Bioreagents, Golden, CO).

C/EBP α promoter-driven dual luciferase reporter assays

NIH 3T3 cells (8.5×10^4 cells/well) were seeded into 6 well dishes and maintained in phenol-red-free media, supplemented with 10% charcoal-stripped FBS, penicillin and

streptomycin. The following day the cells were transfected with the indicated combination of 50ng of the CMV promoter-*Renilla* Luciferase reporter construct, 250ng of the C/EBP α promoter-*Firefly* Luciferase reporter construct, 100ng of pTLrGR, 50ng of pMSV C/EBP β WT or K98-102R, 150ng of one of the pcDNA3.1 HDAC1-HA-Flag constructs (WT, D181A, 6K-R or 6K-Q) and the amount of DNA being transfected was kept constant for every well by topping it up with Flag-pcDNA3.1 plasmid. The DNA was transfected into the NIH 3T3 cells using FUGENE 6® transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN).

The following day the cells were treated with 1 μ M dex, or the equivalent volume of ethanol diluted in phenol red-free media supplemented with charcoal-stripped serum. After the 24hr treatment the media was aspirated, the cells were washed 2 times with 2mL of 1XPBS/well and the cells were lysed for 20 min., on a nutator, at room temperature (RT) with 300 μ L/well of 1X Passive Lysis Buffer (Promega Corporation, Madison, WI). After the cells were successfully lysed, 20 μ L from each well was added into a 96 well plate, and the luciferase activity, both *Renilla* and *Firefly*, for each condition was assessed using the Dual Luciferase Report Assay Kit (Promega Corporations, Madison, WI) following the manufacturer's protocol in a luminometer (LUMIstar from BMG Labtech, Nepean, ON, Canada).

C/EBP α *Firefly* luciferase values were normalized to the *Renilla* luciferase luminescence values, to correct for transfection efficiency. The values shown in the figures are the average of three independent experiments, each performed in triplicate.

Colorimetric Deacetylase Activity Assay

Harvesting Nuclear Extracts from Cos-7 cells stably expressing HDAC1-HA-Flag constructs

Cos-7 cells stably expressing HDAC1-HA-Flag constructs (WT, D181A, 6K-R or 6K-Q) were generated using the strategy with the pLXSN vector, as previously described. In order to harvest nuclear extracts from these cells to be used to immunoprecipitate the HDAC1-HA-Flags for the deacetylase assay, 2 x 15cm plates of each stable Cos-7 line were seeded and maintained in 25 mL/plate of phenol red-free DMEM (GIBCO-Invitrogen, Burlington, ON) supplemented with 10% charcoal-stripped FBS, penicillin and streptomycin (HyClone, Logan, UT, USA). As a negative control, 2x15cm plates of uninfected Cos-7 cells were seeded, allowed to grow to 70% confluence, and were transfected with 4 μ g of Flag-pcDNA3.1 DNA, using the FuGENE 6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN).

Once the cells reached confluence, they were treated for 4hrs with 100nm dex or the equivalent volume of ethanol diluted in phenol red-free media. After the hormone treatment was complete, the media was aspirated and each plate was washed twice with 10mL of 1XPBS. Next, 3mL of cold 1XPBS was added to each plate, cells were scraped, transferred into a 10mL Falcon tube and kept on ice until all of the samples were ready. The cells were pelleted by centrifuging the tubes for 5 min, at 4°C and 750 X g. The supernatant was then carefully aspirated and the cells were re-suspended in 5mL of cell lysis buffer (10mM HEPES, pH 7.6, 15mM KCl, 2mM EDTA, 0.50 mM spermidine, 0.15 mM spermine, 0.5% NP-40, 1.0 mM DTT, and 1X Complete Protease Inhibitor Cocktail). The re-suspended cells were then incubated on ice for 5 min to lyse the cell membranes.

After the incubation the suspension was underlaid with 0.5 mL of Cell Lysis Buffer containing 30% sucrose (cell lysis buffer with 0.88mM sucrose added) and the suspensions were centrifuged for 10 min, at 1500 X g, at 4°C. Next, the supernatant was carefully aspirated, and the sides of the tube were carefully dried with a kimwipe so as not to disturb the pellet. The pellet was then re-suspended in 150µL of Nuclear Lysis Buffer (10mM HEPES, pH 7.6, 100mM KCl, 0.1 mM EDTA, 10% Glycerol, 3mM MgCl₂, 1.0 mM DTT and 1X Complete Protease Inhibitor Cocktail). The concentration of KCl in the nuclear lysis buffer was increased from 100mM to 550mM, to lyse the nuclei. The lysis samples were incubated on ice for 30 min, with occasional mixing by inversion, to allow for nuclear lysis, after which they were centrifuged at 10000 X g, for 20min, at 4°C to pellet the chromatin. The supernatant was immediately separated into two Eppendorf tubes containing 75-80µL of nuclear extract each. The nuclear extracts were either immediately used in an immunoprecipitation, or were stored at -80°C until they were needed.

Immunoprecipitation of Flag and HDAC1-HA-Flag constructs from Cos-7 cells

A single tube of Cos-7 nuclear extract (75-80µL) for each construct (Flag, WT HDAC1-HA-Flag, D181A HDAC1-HA-Flag, 6K-R HDAC1-HA-Flag and 6K-Q HDAC1-HA-Flag) was used to immunoprecipitate the various HDAC1 constructs. The same immunoprecipitation protocol previously described was used, except that the immunoprecipitation was performed using KCl (150mM), instead of NaCl, the samples were incubated on the wheel at 4°C for 2hrs, instead of O/N, and the beads were washed 4 times with wash buffer, to minimize the amount of contaminants, before being used for the deacetylase activity assay. After the final wash and spin, the buffer was aspirated and the

beads with Flag, or HDAC1-HA-Flag (WT, D181A, 6K-R or 6K-Q) attached to them were used immediately for the colorimetric deacetylase assay.

Colorimetric Deacetylase Activity Assay

This assay was performed with the use of the HDAC Colorimetric Assay/Drug Discovery Kit* - AK-501-A Color de Lys® Assay System (BIOMOL-Enzo Life Sciences International, Inc., Plymouth Meeting, PA). The beads with Flag, or HDAC1-HA-Flag (WT, D181A, 6K-R or 6K-Q) attached to them were re-suspended in 80µL of 0.5 mM Color-De Lys® substrate. Immediately, 30µL of the beads+substrate was removed and added to an eppendorf tube containing 20µL of 5µM TSA diluted in Assay buffer, to stop the deacetylase reaction before it could start. This procedure was repeated for each HDAC1 construct, including the Flag negative control, and these samples were defined as 0' time points, to correct for any background activity.

Another 30µL of the beads + substrate/sample was removed from the original tube, added to 20µL of assay buffer and was incubated for 1hr, at 37°C, in a shaker, along with the 0' time point samples. As a positive control, 5µL of Hela Nuclear extracts (provided in the assay kit) was added to 20µL of assay buffer and 25µL of 0.5mM substrate, and was also incubated for 1hr, at 37°C, in the shaker, along with a sample containing 25µL of assay buffer + 25µL of 0.5mM substrate, which was later used as the blank.

From the remaining beads+substrate solution, a 10µL aliquot was removed, 2µL of 6X SDS-loading buffer was added, and the samples migrated on a 10% SDS-PAGE gel. The proteins were then transferred to a PVDF membrane, as previously described, and probed using the primary anti-HA antibody (1:400, Santa Cruz Biotechnologies, Santa Cruz, CA), to correct the activity for the amount of HDAC1-HA-Flag construct that was

immunoprecipitated in the different samples and to examine any differences in the amount of HDAC2 co-immunoprecipitated with the various HDAC1 constructs. The amount of the HDAC1-HA-Flag construct immunoprecipitated was quantified by performing densitometry on the western blot results for the HA-tag, using the program ImageQuant®.

After an hour, all of the samples were removed from the 37°C shaker, and transferred to a 96 well plate. To the 0' time point samples, 50µL of 1X Color de Lys developer was added, while to the blank, the positive Hela-nuclear extract control, and to the 1hr samples, 50µL of 1X Color De Lys developer containing 2uM TSA was added to stop the reaction. The 96 well plate was incubated at 37°C for 10-15min, with the dish covered so that no evaporation occurred, to allow the developer to react with any deacetylated substrate. Then, the absorbance of each well at 405nm was immediately read. The absorbance of each of the 1hr samples was corrected for the absorbance of the blank and for the absorbance of the beads alone and/or the absorbance of their corresponding 0' time point (whichever A_{405nm} value was higher).

Densitometry of the western blot for the HA-tag, also allowed the A_{405nm} values for the 1hr time samples to be corrected for differences in the amount of HDAC1-HA-Flag (WT, D181A, 6K-R or 6K-Q) immunoprecipitated for each construct and in both the + or – Dex conditions, as previously described. While densitometry performed on the Western blot results for HDAC2 confirmed that the phenotypes observed for the various HDAC1 constructs were due to alterations in the catalytic activity of the HDAC1 mutants and not due to alterations in the amount of HDAC2 that was co-immunoprecipitated with the enzymes.

Chapter 1: Investigating the role of HDAC1 and its catalytic activity in C/EBP β mediated *C/ebp α* transcription

Rationale: Induction of the adipogenic transcriptional cascade involves C/EBP β and C/EBP δ mediated transcriptional upregulation of the master adipogenic factors C/EBP α and PPAR γ (Lin and Lane, 1994, Darlington, *et al.*, 1998, Rosen, *et al.*, 1999.). Glucocorticoids play a variety of roles which combine to amplify the efficiency of adipogenesis (Wu *et al.*, 1996, Smas, *et al.*, 1999 and Zuo, *et al.*, 2006, Pantoja, *et al.*, 2008) and our lab has focused on elucidating the molecular details of glucocorticoid-potentiated *C/ebp α* transcription. We have previously demonstrated that ligand-bound GR upregulates C/EBP β mediated *C/ebp α* transcription by enhancing the transcriptional activity of C/EBP β through the titration of the mSin3A/HDAC1 co-repressor complex away from the C/EBP α promoter, and targeting of HDAC1 for 26S proteasomal degradation by TIF1 β (Figure 1) (Wiper-Bergeron, *et al.*, 2003, Tomlinson J, unpublished).

These earlier studies identified HDAC1 as an inhibitor of glucocorticoid-potentiated *C/ebp α* transcription, as over-expression of HDAC1 inhibited both C/EBP α promoter-driven reporter expression and dex-potentiated preadipocyte differentiation, while inhibition of HDACs with the general histone deacetylase inhibitors TSA, VPA (Wiper-Bergeron, *et al.*, 2003) and sodium butyrate (Toscani, *et al.*, 1990) enhanced and replaced the ability of steroids to potentiate the adipogenic process. However, due to the general nature of the pharmaceutical inhibitors which were used in these earlier studies we cannot conclusively state that the deacetylase activity of HDAC1 is required for inhibiting C/EBP β mediated *C/ebp α* transcription. Furthermore the molecular mechanism through which the

mSin3A/HDAC1 co-repressor complex inhibits the ability of C/EBP β to induce *C/ebp α* transcription has yet to be fully characterized.

Based on these earlier results it was hypothesized that HDAC1 is a critical inhibitor of C/EBP β mediated *C/ebp α* transcription and that glucocorticoids enhanced *C/ebp α* transcription through a mechanism which involves the inactivation of the deacetylase activity of HDAC1 and/or its titration from C/EBP β bound to the C/EBP α promoter. In order to investigate this hypothesis we designed two main objectives:

- 1) To clarify the importance of HDAC1 in the regulation of *C/ebp α* transcription in the presence of adipogenic stimuli we sought to validate that HDAC1 is the key deacetylase involved in modulating *C/ebp α* transcription.
- 2) In order to elucidate the molecular details of how HDAC1 inhibits C/EBP β mediated *C/ebp α* transcription we investigated whether the deacetylase activity of HDAC1 is important to its inhibitory role in C/EBP β mediated *C/ebp α* transcription.

Results

Knockdown of HDAC1 with small interference ribonucleic acids (siRNA) enhances the ability of dexamethasone to potentiate adipogenesis of NIH 3T3 murine fibroblast cells

The earlier studies which identified HDAC1 as an inhibitor of dex-induced preadipocyte differentiation demonstrated that inhibition of HDACs through treatment of 3T3 L1 cells for 4hrs with the general HDAC1 inhibitors Trichostatin-A(TSA) or Valproic Acid (VPA) replaced the ability of steroid treatment to upregulate the adipogenic differentiation process (Wiper-Bergeron, 2003). However, these studies did not inhibit HDAC1 exclusively, as specific HDAC inhibitors remain unavailable (Bieliauskas, *et al.*, 2008). Therefore, in order to validate our model and verify that HDAC1 is the deacetylase responsible for regulating the transcriptional activity of the C/EBP α promoter, we used small interference RNA (siRNA) to knockdown murine HDAC1 protein expression in NIH 3T3 fibroblasts and 3T3 L1 murine preadipocyte cells during the first 48hrs of preadipocyte differentiation. This allowed us to examine the effects of decreased HDAC1 expression on the ability of GR to enhance *C/ebp α* transcription and preadipocyte differentiation, and to determine whether decreased HDAC1 expression mirrored the ability of TSA or VPA treatment to replace the ability of dex to enhance the adipogenic process.

NIH 3T3 murine fibroblast cells expressing WT C/EBP β by viral transduction were transiently transfected with of 200pmol of a 4-oligo smartpool of HDAC1 siRNA or scrambled (control) siRNA. The NIH 3T3 uncommitted fibroblast cell line was used to optimize the siRNA experiments as they are much easier to transiently transfect than 3T3 L1 murine preadipocyte cells and their differentiation process is much faster than that of the 3T3 L1s due to C/EBP β overexpression. Consequently, use of NIH 3T3 cells allowed us to quickly assess whether loss of HDAC1 expression affected adipogenesis.

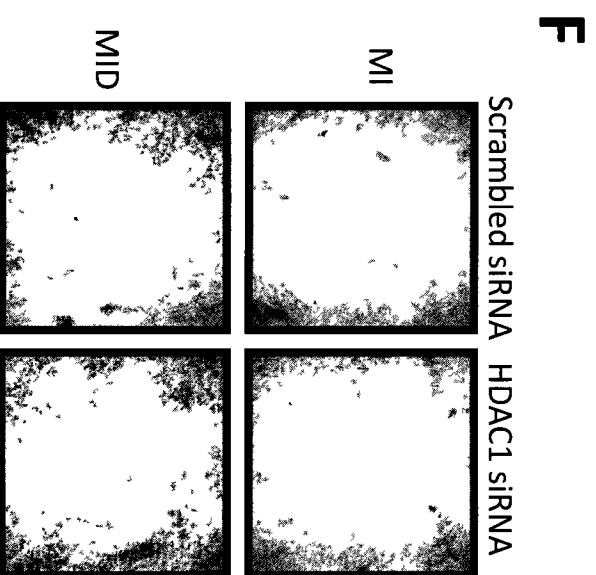
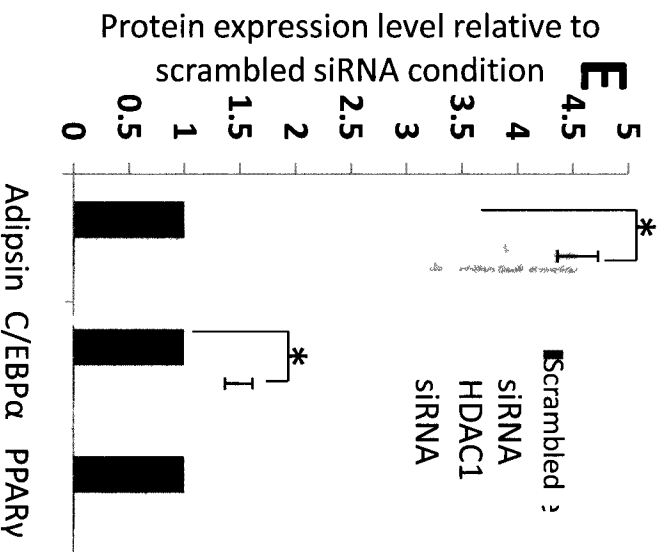
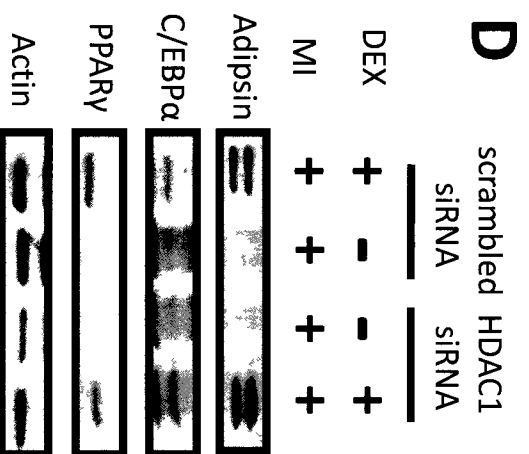
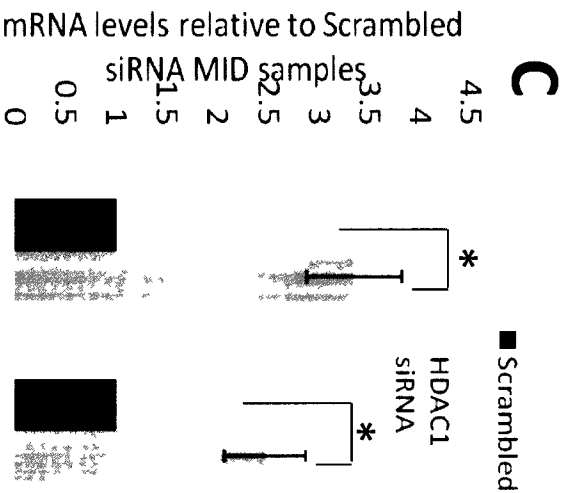
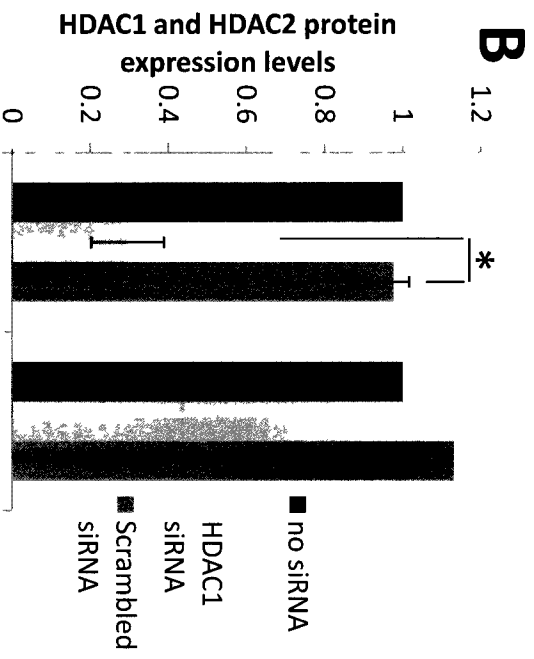
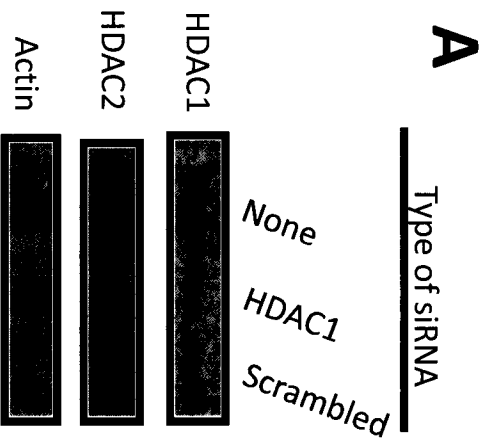
The efficiency of the siRNA knockdown of HDAC1 was examined by western blot analysis of HDAC1, 48hrs after siRNA transfection (Figure 3A). When the blot was quantified by densitometry (Figure 3B) the data revealed that the HDAC1 siRNA successfully reduced the HDAC1 protein expression level to approximately 40% of the HDAC1 protein level in both the un-transfected and the scrambled siRNA treated cells (Figure 3A, and B). Western blot analysis of HDAC1 expression also confirmed that treatment of NIH 3T3 cells with scrambled siRNA did not affect HDAC1 expression levels, as compared to an un-transfected control (Figure 3A and B). Furthermore, western blot analysis of HDAC2 confirmed that neither the HDAC1 smart-pool siRNA nor the scrambled siRNA have any effect on HDAC2 protein expression levels (Figure 3A and B).

NIH 3T3 cells transfected with either HDAC1 or scrambled siRNA were induced to differentiate 48hrs after the initial siRNA transfection, with MIX and insulin in the absence (MI) or presence of dex (MID). This time point was chosen so that the maximal knockdown of HDAC1 would coincide with the initial 48hrs of preadipocyte differentiation, which has been identified as the time frame during which glucocorticoids potentiate the adipogenic process (Green and Kehinde, 1975 and Tomlinson *et al.*, 2006).

To investigate whether knockdown of HDAC1 affects the ability of dex to induce transcriptional upregulation of the adipogenic commitment factors *C/EBP α* and *PPAR γ* , quantitative PCR (qPCR) was first performed for the two adipogenic factors on samples harvested 48hrs after the initiation of differentiation. The qPCR results demonstrated that knockdown of HDAC1 with siRNA, and treatment of the cells with MID resulted in a 3 fold increase in both *C/ebp α* and *ppar γ* transcription, as compared to the scrambled siRNA-MID

Figure 3: siRNA knockdown of HDAC1 in NIH 3T3 cells increases the efficiency of preadipocyte differentiation in a dex-dependent manner

A) Western blot analysis of HDAC1, HDAC2 and actin, 48hrs following transfection of C/EBP β expressing NIH 3T3 cells with either no siRNA, scrambled siRNA or HDAC1 siRNA. B) Quantification of HDAC1 and HDAC2 protein expression levels from A. (values are normalized to actin expression, n=3 repeats, \pm SD). C) Quantitative PCR (qPCR) was performed for *C/ebpa* and *ppary*. RNA was harvested from the NIH 3T3 cells transfected with scrambled or HDAC1 siRNA, 48hrs following initiation of differentiation with MI or MID. Only the qPCR values for the MID samples are shown, as the values for the MI samples were too low to be accurately quantified. qPCR values are presented relative to the level in the scrambled MID sample and were normalized to β -actin values. (n=2X duplicates, \pm SEM). D) Western blot analysis of the adipocyte markers: adipisin, C/EBP α and PPAR γ in siRNA-treated NIH 3T3 cells on Day 4 of differentiation. Two days after NIH 3T3 cells were transfected with the siRNA, they were stimulated to differentiate with either MI (MIX, insulin) or MID (MI and dex) for 48hrs, followed by 2 days of incubation in media with just insulin. Three independent experiments were performed. E) Quantification of D. All protein expression levels were quantified relative to the results of the NIH 3T3 cells treated with scrambled siRNA and MID (n=3, \pm SD). F) The efficiency of NIH 3T3 differentiation was also evaluated by staining the neutral lipids with Oil Red O stain, to identify mature adipocytes. The experiment was also performed in triplicate. A student t-test was performed in all data where *p<0.05.



control samples (Figure 3C). Effect of the knockdown on differentiation induced by MI alone could not be assessed because the qPCR values for *C/ebpa* and *ppary* were so low that they could not be accurately quantified by the qPCR machine..

Next, the effect of HDAC1 knockdown on preadipocyte differentiation was assessed on day 4 of the differentiation process. Western blot analysis of; C/EBP α and PPAR γ (Figure 3D) in addition to adipsin, which is an adipocyte marker, was performed. Quantification of the immunoblot results revealed that adipsin protein expression levels were upregulated by 4.5 fold, C/EBP α protein expression levels were upregulated by 2 fold and PPAR γ protein expression levels were upregulated by 60% in the HDAC1 siRNA transfected NIH 3T3 cells treated with MID, as compared to the scramble siRNA control MID cells (Figure 3D and E). It is important to note that upregulation of the early adipogenic markers is observed only in the presence of dexamethasone for both the scrambled and HDAC1 siRNA treated cells, similar to the trends from the qPCR reactions (Figure 3C).

Subsequently, we assessed the efficiency of differentiation by performing Oil red O staining of the neutral lipids which accumulate in mature adipocytes on Day 4 of differentiation (Figure 3F). More Oil red O staining is observed in the HDAC1 siRNA MID treated NIH 3T3 cells compared to the scrambled siRNA MID treated cells, as seen in Figure 3F. Again, the increase observed in preadipocyte differentiation in the cells treated with HDAC1 siRNA was unexpectedly observed only in cells treated with steroid. These results demonstrate that knockdown of HDAC1 increases the ability of corticosteroid treatment to potentiate both *C/ebpa* and *ppary* transcription and preadipocyte differentiation in a dexamethasone-dependent manner only.

Knockdown of HDAC1 with siRNA in 3T3 L1 preadipocytes enhances GR-mediated *C/ebpa* transcription and preadipocyte differentiation

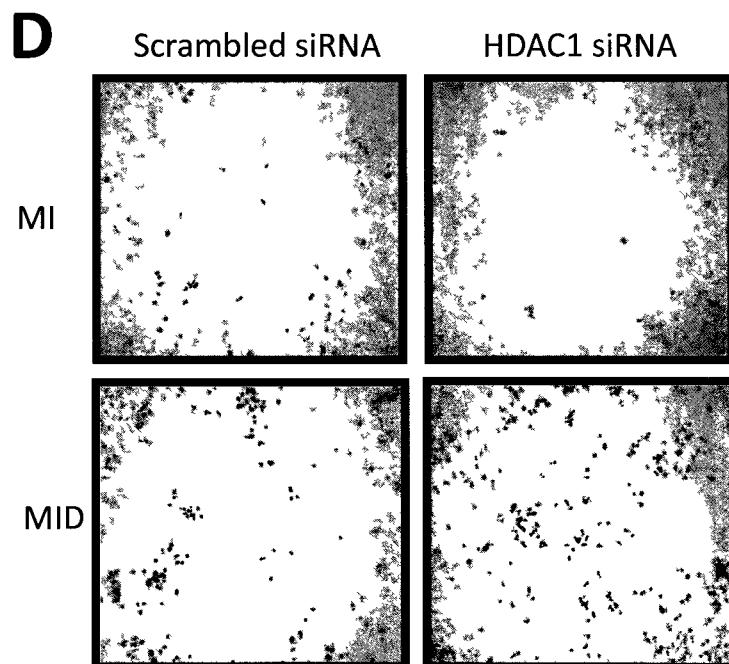
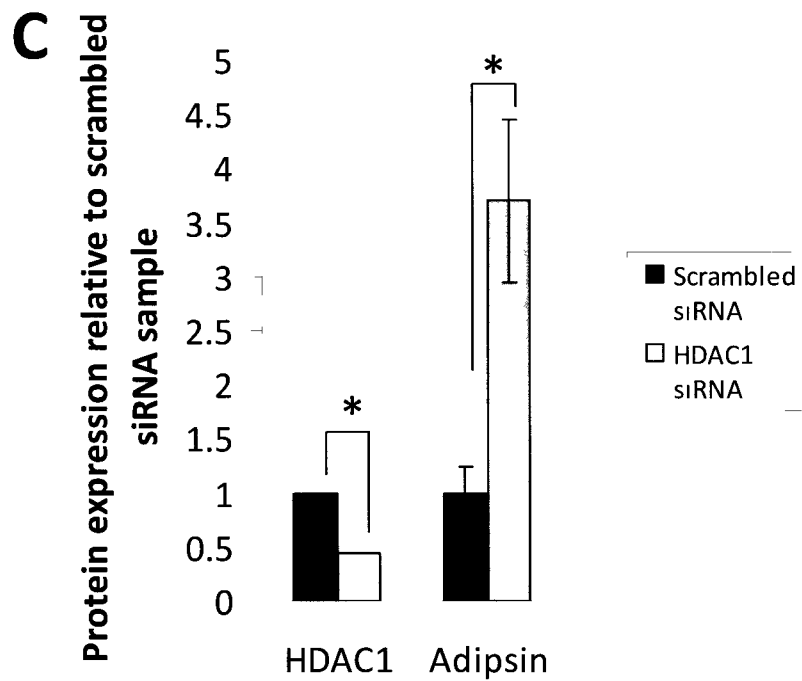
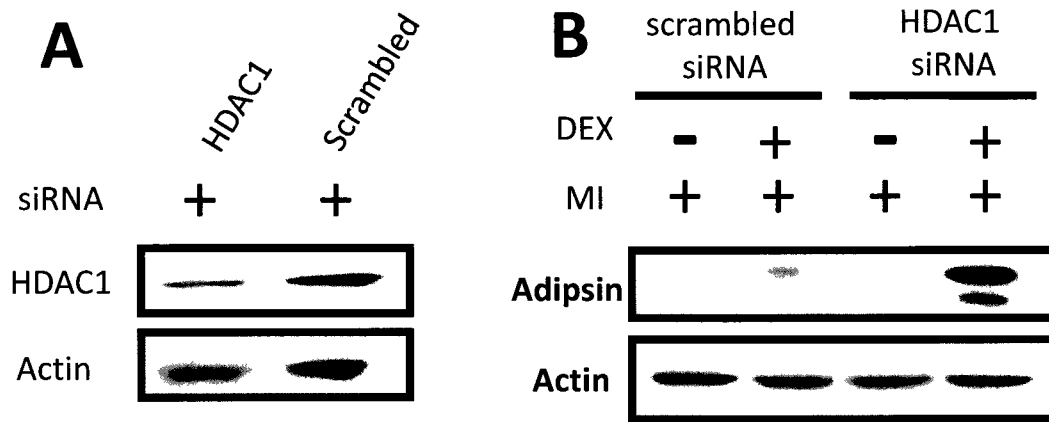
In order to extend the results of the siRNA knockdown in the multipotent NIH 3T3 fibroblasts the experiment was repeated in 3T3 L1 murine preadipocyte cells. The 3T3 L1 cell line is a widely used and accepted cell culture model of preadipocyte differentiation, derived from the same Day 17 Swiss mice embryos as the NIH 3T3s (ATCC).

3T3 L1 murine preadipocyte cells were transiently transfected with 400 pmol of the HDAC1 smart-pool siRNA or 400 pmol of scrambled siRNA, as a negative control. Western blot analysis of HDAC1 in the whole cell lysates of 3T3 L1 cells harvested 48hrs after transfection of the siRNA, (Figure 4A) and quantification by densitometry of the HDAC1 protein expression levels (Figure 4C) confirms that the HDAC1 siRNA successfully decreased HDAC1 protein expression to approximately 45% of the scrambled siRNA protein level.

The 3T3 L1 preadipocyte cells transfected with HDAC1 or scrambled siRNA were induced to differentiate 48hrs after siRNA transfection, with MIX and Insulin (MI), or MIX, Insulin and dexamethasone (MID) for 48hrs. The cells were then maintained for the remaining 5 days of the differentiation process in media supplemented with just insulin. On Day 7 of differentiation the cells were harvested and western blot analysis was performed on the adipogenic marker; adipsin (Figure 4B), and densitometry was used to quantify the protein expression level (Figure 4C). 3T3 L1 cells treated with MI exhibited little to no expression of adipsin, while 3T3 L1 cells transfected with HDAC1 siRNA and treated with MID, demonstrated a significant 3.7 fold increase in adipsin protein levels (Figure 4C). Additionally the HDAC1 siRNA-MID cells displayed more differentiation than their scrambled siRNA MID treated counterparts, as assessed by Oil red O staining (Figure

Figure 4: siRNA knockdown of HDAC1 in 3T3 L1 preadipocytes increases the efficiency of preadipocyte differentiation in a dex-dependent manner

A) Western blot analysis of HDAC1 expression, relative to the actin loading control, 48hrs after 3T3 L1s were transfected with 400pmol of HDAC1 or scrambled siRNA. (n=2) B) Western blot analysis of the adipocyte marker adipsin on Day 7 of differentiation. Two days after 3T3 L1 cells were transfected with scrambled or HDAC1 siRNA the cells were stimulated to differentiate with either MI or MID for 48hrs, followed by 5 days of incubation in media with insulin. (n=2) C) Quantification of western blot analysis of HDAC1 in A, and of western blot analysis of adipsin in B. Densitometry values were normalized to actin protein expression level, and protein expression levels are expressed relative to the Scrambled MID condition. (n=2 \pm SD, A student t-test was performed in all data where *p< 0.05) D) Oil red O staining of the neutral lipids of mature adipocytes, on Day 7 of 3T3 L1 differentiation of the scrambled or HDAC1 siRNA treated cells, treated with MI or MID. (n=2)



4E). It is also important to note that, in agreement with the results from the HDAC1 siRNA experiments in NIH 3T3 cells, knockdown of HDAC1 with siRNA only enhances preadipocyte differentiation in the presence of dex (Figure 4B and D).

Overall, the results of the siRNA mediated knockdown of HDAC1 in 3T3 L1s concur with the results of the same experiment in NIH 3T3s. Taken together, the results of the siRNA knockdown of HDAC1 in NIH 3T3 and 3T3 L1 cells support the theory that HDAC1 is an inhibitor of C/EBP β mediated *C/ebpa* transcription, since a decrease in HDAC1 expression further increases the ability of steroid treatment to potentiate the adipogenic process. However, the results also suggest that the mechanism through which glucocorticoids upregulate *C/ebpa* transcription is more complex than simply promoting the titration HDAC1 from the promoter, and targeting the co-repressor for degradation, as decreased HDAC1 expression was unable to replace the effects of glucocorticoid treatment on adipogenesis.

Knockdown of HDAC2, by siRNA, has no significant effect on dex-potentiated differentiation of NIH 3T3 WT C/EBP β positive cells

HDAC1 and HDAC2 share 82% sequence identity and are often found together in co-repressor complexes (De Ruijter, *et al*, 2003). Previous studies in our laboratory have demonstrated that HDAC1, but not HDAC2 or HDAC4, co-immunoprecipitates with C/EBP β (Wiper-Bergeron, *et al*, 2003). However, the co-immunoprecipitation experiment demonstrating that HDAC2 is not part of the HDAC1/C/EBP β complex was only performed in Cos-7 cells. In order to verify that HDAC2 is not involved in regulating *C/ebpa* transcription and preadipocyte differentiation, NIH 3T3 cells expressing WT C/EBP β by viral transduction were transiently transfected with 300pmol of a 4-oligo smartpool of

HDAC2 siRNA or with scrambled control siRNA. Western blot analysis of HDAC2 (Figure 5A) and quantification of the immunoblots (Figure 5C), performed on whole cell extracts harvested 48hrs after the transfection of the siRNA, confirmed that the HDAC2 siRNA effectively reduced HDAC2 protein expression by approximately 40%, as compared to the scrambled siRNA cells and the untransfected cells. Importantly, western blot analysis of HDAC2 and HDAC1 confirmed that the scrambled siRNA had no effect on either HDAC2 or HDAC1 expression (Figure 5A and Figure 5C), which successfully validates the use of the scrambled siRNA as a negative control. Western blot analysis of HDAC1 expression was also performed to ensure that the HDAC2 siRNA was not affecting the expression levels of HDAC1, as confirmed in figure 5A and 5C.

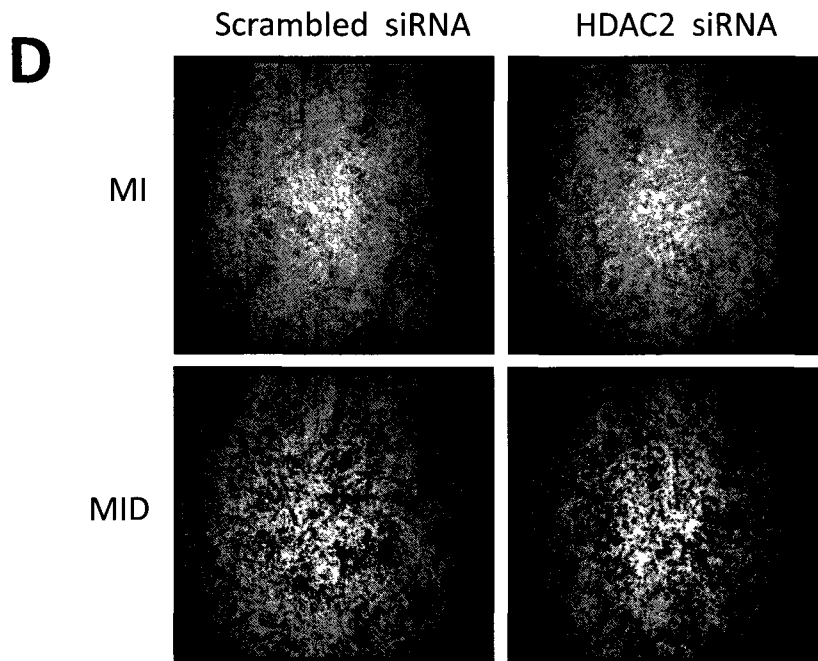
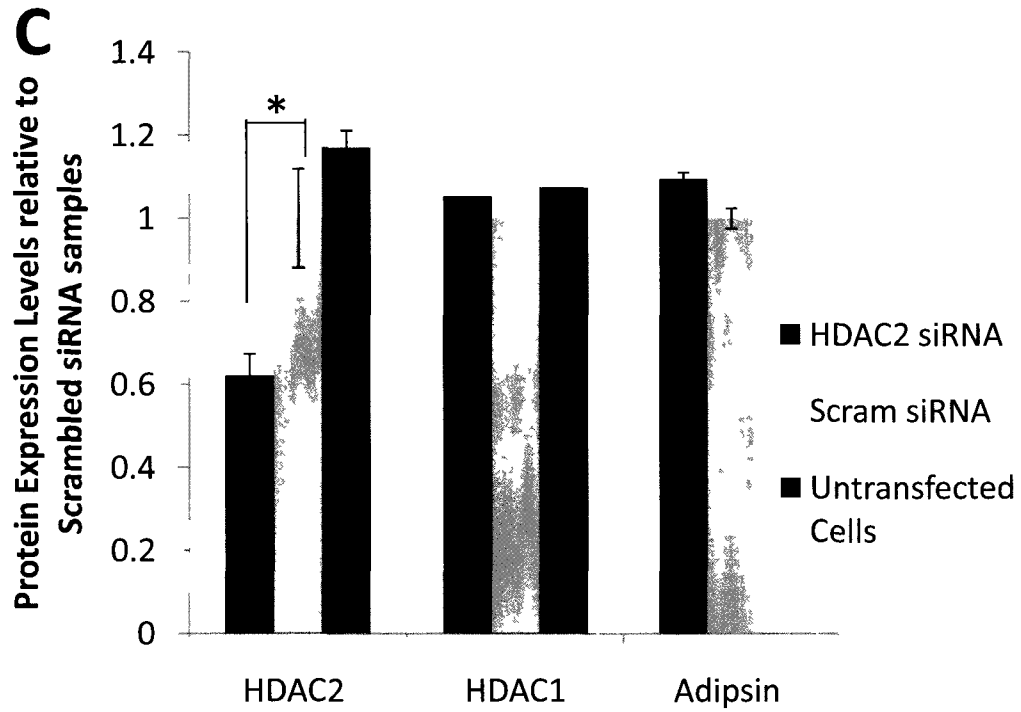
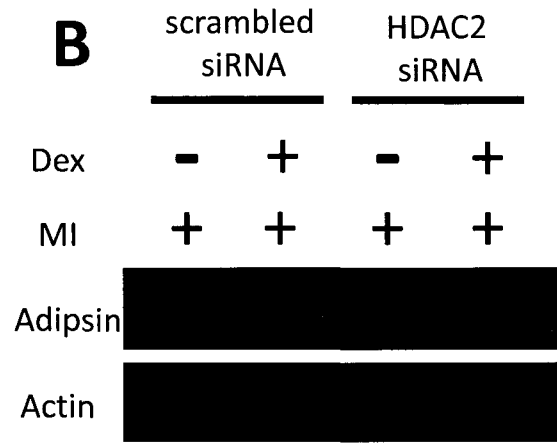
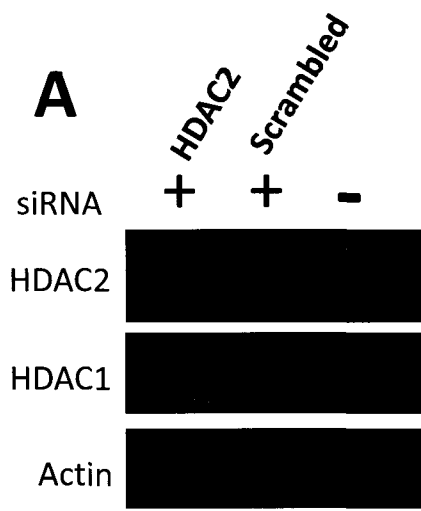
Approximately 48hrs following the transfection of the cells with the siRNA, the cells were induced to differentiate for 48hrs with MIX and insulin, in the absence (MI) or presence of dex (MID). Analysis of the efficiency of differentiation on day 4 revealed no observable differences in the expression level of the adipocyte marker, adiponin, between the HDAC2 and scrambled siRNA treated cells (Figure 5B and C), nor in the lipid accumulation of the mature adipocytes visualized by Oil red O staining (Figure 5D). The results of this experiment support previous findings indicating that HDAC2 is not involved in regulating *C/ebpa* transcription, preadipocyte differentiation, or in the ability of glucocorticoids to potentiate this process (Wiper-Bergeron *et al.*, 2003).

Mutation of aspartic acid residue 181 to alanine (D181A) in HDAC1 results in significant loss of deacetylase activity

Up to this point the results support the hypothesis that HDAC1 is an inhibitor of glucocorticoid-potentiated *C/ebpa* transcription and preadipocyte differentiation, while

Figure 5: siRNA knockdown of HDAC2 in NIH 3T3 cells does not affect dex-potentiated differentiation

A) Transfection of NIH 3T3 cells stably expressing WT C/EBP β with HDAC2 siRNA reduces HDAC2 expression (lane 1) compared to HDAC2 protein expression levels in cells treated with scrambled siRNA (lane 2), or cells treated with no siRNA (lane 3), 48 hrs following siRNA transfection. (n=2). B) Two days following transfection of C/EBP β positive NIH 3T3s with siRNA, the cells were stimulated to differentiate with MI or MID for 48hrs, proceeded by incubation in media supplemented with insulin for an additional 48 hrs. The efficiency of the cells to differentiate into mature adipocytes was assessed on Day 4 of differentiation via western blot analysis of the adipocyte marker, adipsin (n=2). C) Quantification of HDAC1, HDAC2 and Adipsin protein expression levels. Quantification of HDAC2 and HDAC1 protein expression levels 48hrs following siRNA transfection of NIH 3T3s. Protein expression levels were normalized to actin protein levels and the expression of HDAC1 and HDAC2 were quantified relative to their expression levels in scrambled siRNA transfected cells. (n=2, \pm SD). A student t-test was performed where *p<0.05. D) Oil Red O staining of mature adipocytes on day 4 of differentiation of HDAC2 or scrambled siRNA treated NIH 3T3 cells.



HDAC2 is not involved in these processes. However, it remains unclear whether or not the role of *C/ebpa* transcription is dependent on the enzymatic activity or a structural element of HDAC1. In order to investigate whether or not the deacetylase activity of HDAC1 is essential to its inhibitory role in preadipocyte differentiation, the construction of an HDAC1 mutant which lacks deacetylase activity was undertaken.

The catalytic core of HDACs is highly conserved across isoforms and across species (Finnin, *et al.*, 1999, De Ruijter, *et al.*, 2003). Structural studies have identified several conserved amino acids which comprise the charge relay system that is responsible for the deacetylase activity of HDACs (Hassig *et al.*, 1998, Kadosh *et al.*, 1998, Somoza, *et al.*, 2004, Finnin *et al.*, 1999, Vannini, *et al.* 2004, and Vanommeslaeghe, *et al.*, 2005). In HDAC1 these amino acids are: histidine residues 138 and 139, aspartic acid residues 176 and 181, and tyrosine residue 304 (Wang *et al.*, 2005). Mutational analyses performed by other groups have demonstrated that substitutions at these amino acids compromise the ability of HDAC1 to deacetylate its substrates (Hassig *et al.*, 1998, Kadosh *et al.*, 1998, and Finnin *et al.*, 1999 and Buggy *et al.*, 2000). Therefore, an HDAC1 mutant was constructed in which lysine residue 181 was mutated to alanine, D181A HDAC1, which was predicted to compromise HDAC1's deacetylase activity.

To test the deacetylase activity of the HDAC1 constructs, WT HDAC1-HA-Flag, D181A HDAC1-HA-Flag, or Flag-pcDNA3.1, as a negative control, were transiently transfected into Cos-7 cells. The Flag tagged WT HDAC1 or D181A HDAC1 were immunoprecipitated from the nuclear extracts of cells, which had first been treated for 4hrs with 100 nM dex, or the equivalent dilution of ethanol, with sepharose beads linked to the anti-Flag antibody. Published results by Qiu *et al.*, demonstrated that steroid treatment inactivates GR-bound HDAC1 at the MMTV promoter, within 2 hrs, by promoting the

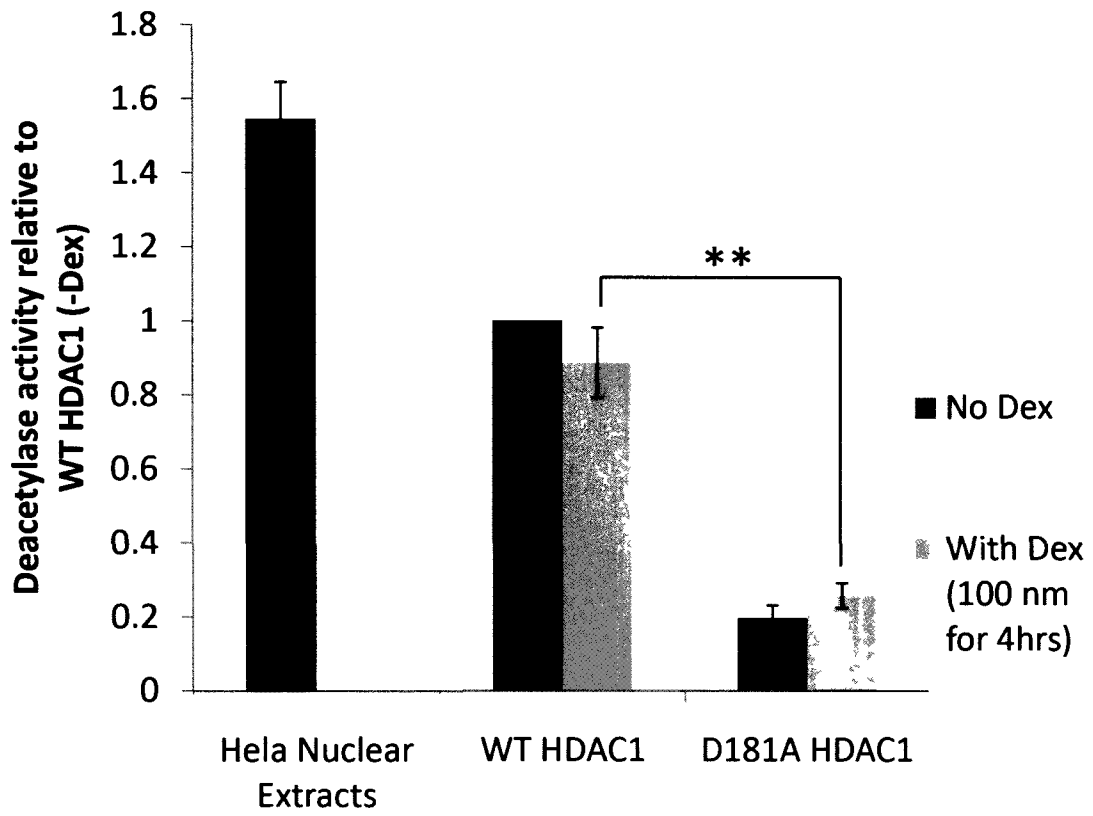
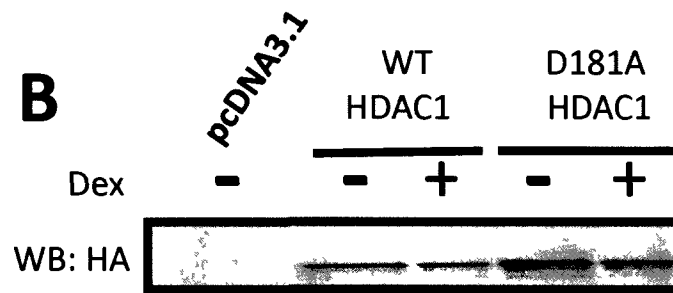
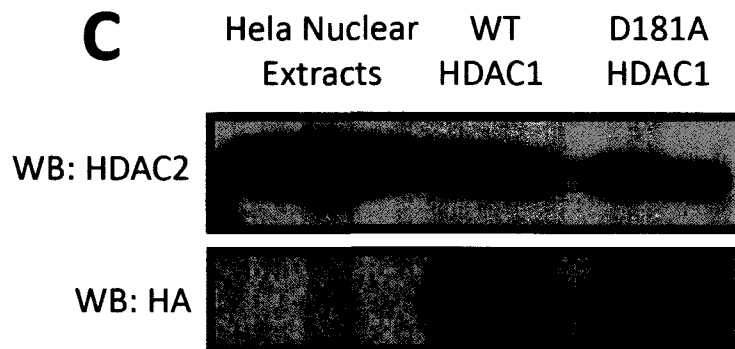
acetylation of a select number of lysine residues within the HDAC1 protein. Therefore, we chose to examine the activity of both WT and D181A HDAC1 in both the presence and absence of steroid in order to begin investigating whether glucocorticoid treatment influenced the catalytic activity of HDAC1 in other systems, and to determine whether or not mutation of aspartic acid 181 to alanine affected the catalytic activity of HDAC1, as predicted.

In addition, since HDAC1 and HDAC2 are often found in the same co-repressor complexes and are known to co-immunoprecipitate together (De-Ruijeter, 2002, Hassig, *et al.*, 1998 and Wiper-Bergeron, *et al.*, 2003), we sought to confirm that any differences observed in the deacetylase activity of the WT versus the D181A HDAC1 mutant are due to changes in the enzymatic activity of the HDAC1 constructs and not to alterations in the ability of the HDAC1 to interact with HDAC2. To do so, WB analysis of HDAC2 was performed on the immunoprecipitate samples of the HDAC1 constructs (Figure 6C). The amount of HDAC2 co-immunoprecipitated was quantified using densitometry and, once normalized to the amount of HDAC1-HA-Flag construct that was successfully immunoprecipitated, it was confirmed that no variation in the ability of HDAC1 to interact with HDAC2 was caused by the D181A mutation in HDAC1 (Figure 6C). Therefore, we can confidently infer that any differences that are observed in the deacetylase activity of WT versus the D181A HDAC1 mutant are due to differences in the ability of the HDAC1 construct to deacetylate its targets and not due to differences in the specific activity of HDAC2.

The results of the colorimetric deacetylation assay demonstrate significant deacetylase activity for the HeLa nuclear extracts, indicating the assay is successful in detecting deacetylase activity (Figure 6A). The WT HDAC1 demonstrated significant

Figure 6: Mutation of aspartic acid 181 to alanine in HDAC1 results in significant loss of deacetylase activity.

A) Results of the colorimetric HDAC1 deacetylase assay using the Fleur De Lys HDAC Colorimetric Assay/Drug Discovery Kit*. The deacetylase activity is represented as relative to the activity of WT HDAC1 in the absence of dex. In each experiment the deacetylase activity of the WT and D181A HDAC1 were corrected for the activity of the Flag-beads alone or of the 0' time point samples, depending on which condition produced higher background. The activity readings were also corrected for the amount of HDAC1 protein immunoprecipitated, presented in B. (n=3Xduplicates, \pm SEM, where **= $p < 0.01$, which represents a student t-test) B) Western blot analysis of the WT HDAC1-HA-Flag and D181A HDAC1-HA-Flag constructs used for the HDAC1 colorimetric deacetylase assay, and used to normalize the activity levels. C) The amount of HDAC2 that was co-immunoprecipitated with the HDAC1-HA-Flag constructs was examined to ensure that the differences in the deacetylase activity of the various HDAC1 constructs are due to alterations in the enzymatic activity of HDAC1 and not due to differences in the ability of the HDAC1 mutants to interact with HDAC2. The amount of HDAC2 that was co-immunoprecipitated with WT HDAC1-HA-Flag was set as 1. WT:1 D181A:1.003.

A**B****C**

deacetylase activity while the D181A mutant HDAC1, on the other hand, exhibited only 25% of the deacetylase activity compared to that of the WT HDAC1. The minimal deacetylase activity of the D181A HDAC1 mutant could be due to the associated HDAC2, among other possible HDACs, since its association was not affected, or it could be due to residual catalytic activity remaining in the mutant protein itself. The deacetylase activity of neither the D181A nor WT HDAC1 was affected by 4hrs of dexamethasone treatment (Figure 6A). The absence of a steroid-dependent effect on the deacetylase activity of WT or D181A HDAC1 suggests that the length of steroid treatment needs to be optimized in order to conclusively determine whether or not treatment with dex has a regulatory effect on the enzymatic activity of HDAC1. Alternatively it may also indicate that regulation of the deacetylation activity of HDAC1 is a system specific mechanism that may not be detectable using a generalized assay, such as the one utilized in this investigation. Ultimately, additional investigation is needed to further explore and understand the mechanisms that regulate HDAC1 deacetylase activity. Finally, these results confirm the hypothesis that mutation of aspartic acid residue 181 of HDAC1 to alanine impedes the deacetylase activity of the enzyme.

The deacetylase activity of HDAC1 is essential to its ability to inhibit dex-induced *C/ebpα* transcription

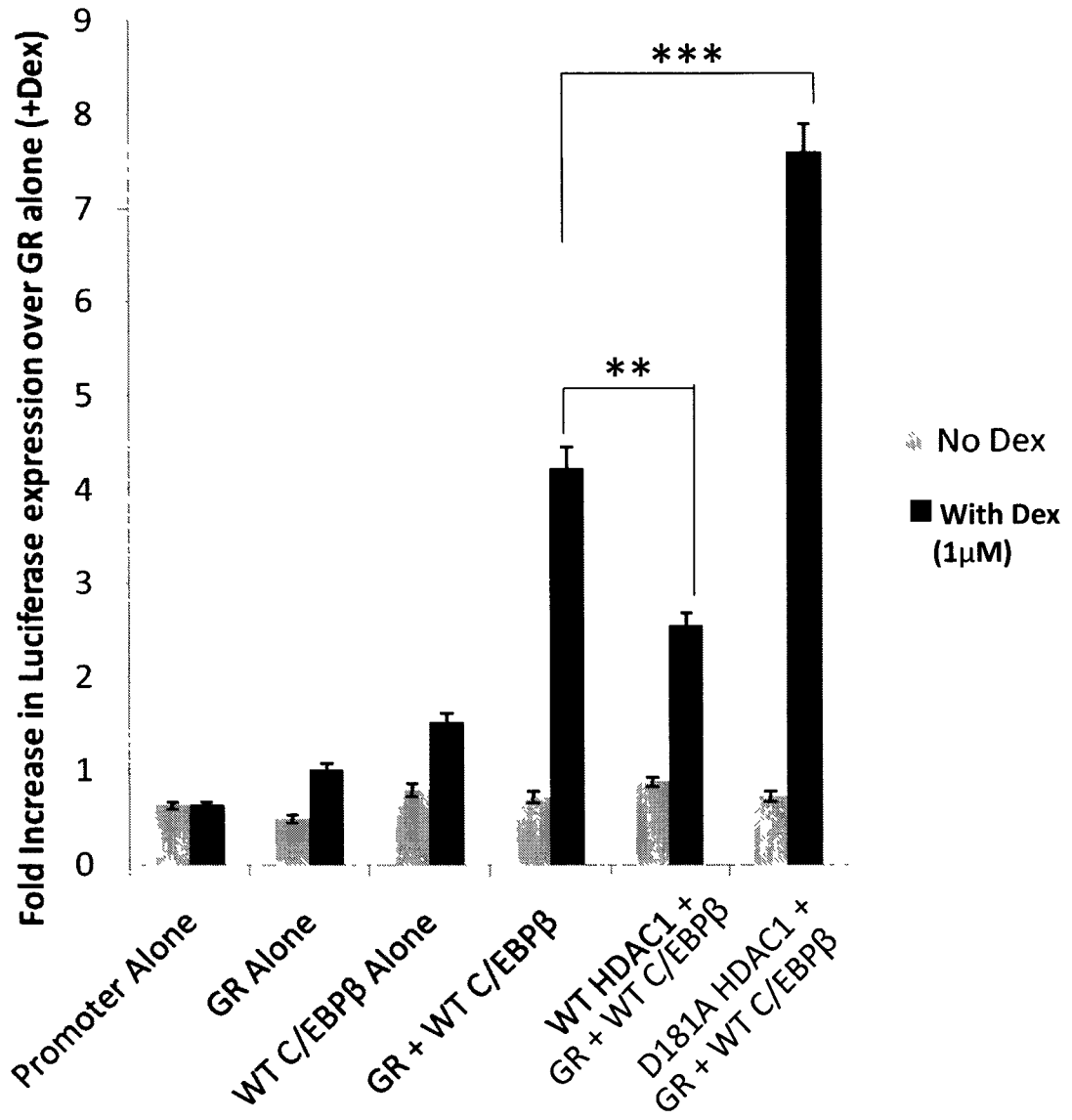
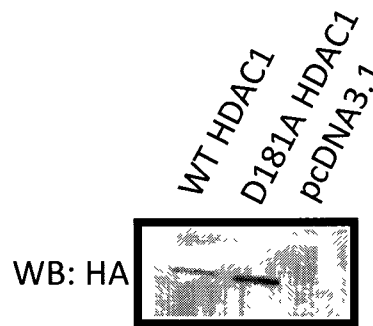
Since the colorimetric deacetylase assay confirmed that the D181A HDAC1 mutant has reduced deacetylase activity, it was used to investigate whether HDAC1s deacetylase activity is required for its inhibitory role at the *C/EBPα* promoter. NIH 3T3 cells were transiently transfected with *C/EBPα* promoter-*Firefly* luciferase and CMV promoter-*Renilla* luciferase, in addition to the indicated combination of GR, *C/EBPβ*, pcDNA3.1, WT OR

D181A HDAC1. Cells were treated with 1 μ M dexamethasone or the equivalent dilution of ethanol for 24hrs, after which the luminescent values for both the *Firefly* and *Renilla* luciferase proteins were read. The results of the luciferase reporter assay confirm, as has been previously established in our laboratory (Wiper-Bergeron, *et al.*, 2003), that upon exogenous WT C/EBP β and GR expression, dex is able to increase the amount of C/EBP α promoter-driven luciferase expression, by approximately 4 fold, compared to the cells transfected with GR alone (Figure 7A). When WT HDAC1 was expressed with GR and C/EBP β the dex effect was reduced from 4 to 2.5 fold (Figure 7A). On the other hand, cells transfected with D181A HDAC1 displayed an 8 fold upregulation in C/EBP α promoter-driven luciferase, or double the dex effect (Figure 7A). Interestingly, there is no observable increase in C/EBP α promoter-driven luciferase expression in cells transfected with D181A HDAC1, GR and C/EBP β , in the absence of steroid (Figure 7A). The successful expression of 150 ng of WT and D181A HDAC1 in the NIH 3T3 cells was verified by western blot analysis of the HA tag (Figure 7B).

These results support the theory that the deacetylase activity of HDAC1 is essential to its ability to inhibit *C/ebpa* transcription. However, the fact that this increase is dependent on steroid treatment coincides with the results of the HDAC1 siRNA experiments in NIH 3T3 and 3T3 L1 cells. Together these results support the hypothesis that the ability of glucocorticoids to upregulate *C/ebpa* transcription and adipocyte differentiation involves a complicated mechanism that is not solely dependent on the inactivation and/or the titration of HDAC1 from the *C/ebpa* promoter because neither interfering with HDAC1 deacetylase activity nor decreasing HDAC1 protein levels by over 50% was sufficient to enhance *C/ebpa* transcription alone. Furthermore, expression of the catalytically compromised D181A

Figure 7: The deacetylase activity of HDAC1 is essential to its ability to repress dex-induced transcriptional upregulation at the C/EBP α promoter.

A) NIH 3T3 cells were transiently transfected with the indicated combination of GR, C/EBP β , WT HDAC1 or D181A HDAC1, in addition to C/EBP α promoter-*Firefly* luciferase reporter and CMV-*Renilla* luciferase reporter constructs. C/EBP α promoter-driven luciferase expression was measured in the presence and absence of dex for each condition using a luminometer. *Firefly* luciferase expression is quantified as the fold increase relative to GR alone (+dex) and all *Firefly* luciferase values were normalized to CMV promoter-driven *Renilla* luciferase expression to correct for transfection efficiency. (n=3X triplicates \pm SEM). A student t-test was performed in all data where ** p<0.01 and *** p<0.001. B) Western blot analysis of HA, demonstrating expression levels of 150ng of WT HDAC1-HA-Flag and D181A HDAC1-HA-Flag, which were transiently transfected into NIH 3T3 cells for the dual luciferase reporter assays.

A**B**

HDAC1 not only fails to inhibit dex-induced luciferase expression, but rather doubles steroid induced C/EBP α promoter activity relative to the GR+C/EBP β condition, suggesting that D181A HDAC1 behaves as a dominant negative HDAC1 mutant.

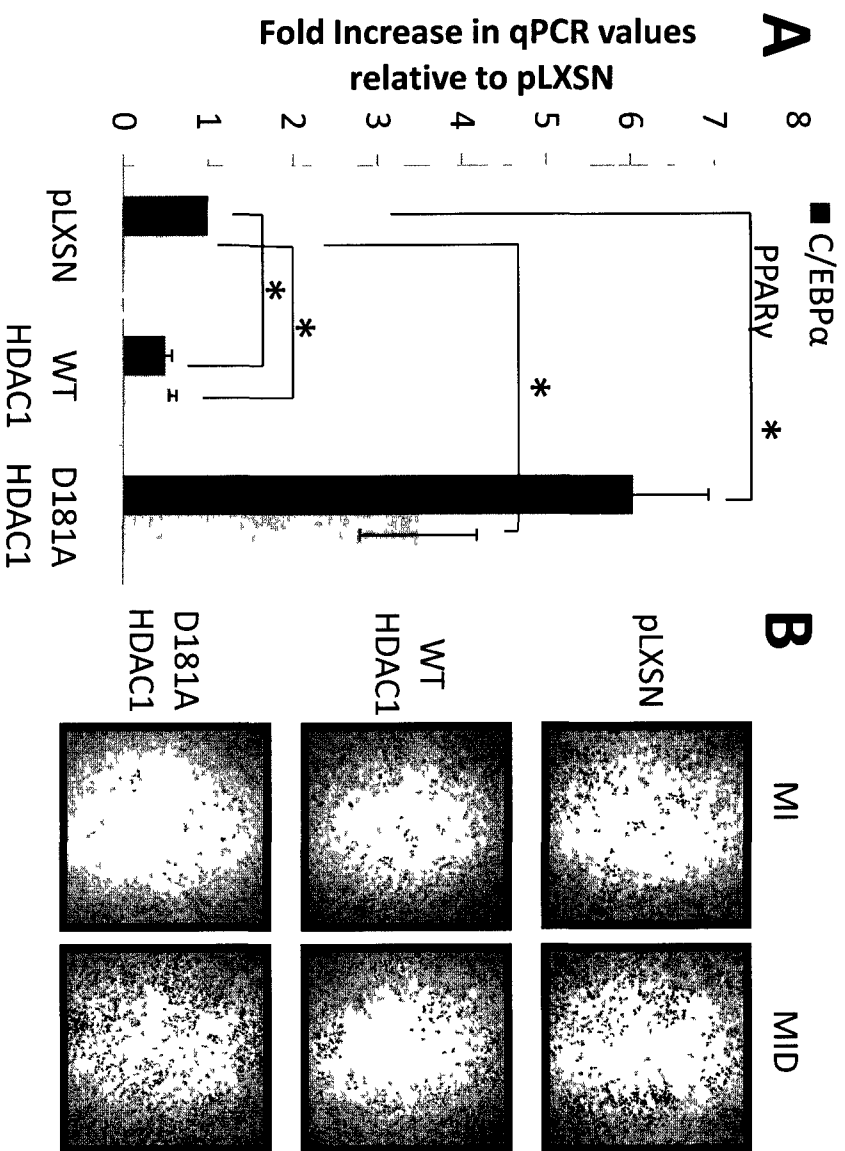
HDAC1 deacetylase activity is essential to its ability to suppress dexamethasone potentiated preadipocyte differentiation

Subsequently, we investigated whether expression of D181A HDAC1 in differentiating 3T3 L1 preadipocytes would produce the same dex-dependent phenotype as it did in the C/EBP α promoter-driven reporter assay. 3T3 L1 murine preadipocyte cells were virally transduced to express either pLXSN (negative control), WT HDAC1-HA-Flag or D181A HDAC1-HA-Flag. The stable cells were then induced to differentiate with MIX and insulin (MI) or MIX, insulin and dex (MID) for 48hrs, followed by five-six days of incubation in insulin supplemented media.

In order to investigate the effect of WT versus D181A HDAC1 expression on endogenous *C/ebp α* transcription, we first harvested RNA 72hrs after the induction of differentiation, converted it into cDNA, and used the cDNA to perform quantitative PCR for the master adipogenic transcription factors C/EBP α and PPAR γ . Extremely low levels of both *C/ebp α* and *ppar γ* mRNA were detected in all of the MI conditions. However, the mRNA levels of the adipogenic transcription factors in the MI conditions were so low that accurate quantification was not possible and so these values are not presented in Figure 8A. On the other hand, significant differences are seen in the MID conditions (Figure 8A) as 3T3 L1 cells stably expression WT HDAC1 and treated with MID display a 50% reduction in *C/ebp α* and *ppar γ* expression as compared to the pLXSN MID samples. In contrast, the cells expressing the D181A HDAC1 mutant demonstrated a 6 fold and a 3.5 fold increase in *C/ebp α* and *ppar γ* transcription, respectively, relative to the pLXSN MID control.

Figure 8: HDAC1's deacetylase activity is essential to its ability to suppress GR-potentiated preadipocyte differentiation as the catalytically compromised D181A HDAC1 enhances the ability of dex to potentiate 3T3 L1 murine preadipocyte differentiation

A) Quantitative PCR for the master adipogenic factors *C/ebpα* and *pparγ*, 72hrs after 3T3 L1 cells stably expressing pLXSN, pLXSN WT HDAC1 or pLXSN D181A HDAC1 have been induced to differentiate with either MI or MID. Only the qPCR values for the MID conditions are presented and the values are quantified relative to the expression levels in the pLXSN MID sample and they were normalized relative to β -actin expression levels. (n=3Xduplicates \pm SEM). A student t-test was performed for all data and * = $p < 0.05$. B) Oil Red O staining of 3T3 L1 cells stably expressing pLXSN, pLXSN-WT HDAC1-HA-Flag or pLXSN-D181A HDAC1-HA-Flag on day 7 of the differentiation process (n=3). C) Western blot analysis of adipocyte markers; adipisin, *C/EBPα* and *PPARγ* on day 7 of differentiation of 3T3 L1 cells stably expressing pLXSN, pLXSN WT HDAC1 or pLXSN D181A HDAC1 as shown (n=3). D) Quantification of the protein expression of the adipocyte markers in 3T3 L1 cells presented in C, normalized to actin expression levels. Protein expression levels are expressed as relative to the pLXSN MID samples, and only the MID protein expression levels are presented (n=3 \pm SEM). A student t-test was performed for all data comparing them to the pLXSN MID condition, where * $p < 0.05$, ** $p < 0.01$.



Next, in order to investigate whether the capacity of D181A HDAC1 to enhance the ability of dex to induce *C/ebpα* and *ppary* transcription translates into amplification of preadipocyte differentiation we harvested mature 3T3 L1 adipocytes expressing pLXSN, WT HDAC1-HA-Flag or D181A HDAC1-HA-Flag on Day 7 of differentiation to visually assess the efficiency of differentiation by staining the lipid droplets with Oil red O (Figure 8B). Minimal differentiation was observed in the 3T3 L1 cells under the MI conditions which made it difficult to accurately visually assess whether there were any differences between the cells expressing WT, D181A HDAC1 or pLXSN MI conditions (Figure 8B). However, when dex was added to the MI adipogenic cocktail the 3T3 L1-pLXSN control displayed an increase in Oil red O stained mature adipocytes (Figure 8B). Furthermore, 3T3 L1s expressing WT HDAC1 demonstrated less Oil red O staining when compared to the pLXSN-3T3 L1s (Figure 8B) and cells expressing the D181A HDAC1 mutant exhibited more differentiated mature adipocytes compared to the 3T3 L1 pLXSN control (Figure 8B).

The efficiency of differentiation of the virally transduced 3T3 L1 cells was also assessed by performing western blot analysis for the adipocyte markers; adipsin, C/EBPα and PPARγ on Day 7 of differentiation (Figure 8C). Densitometry was used to quantify the protein expression levels for each of the markers and the values were normalized to actin and shown in Figure 8D. 3T3 L1 cells expressing pLXSN, WT HDAC1-HA-Flag or D181A HDAC1-HA-Flag which were treated with MI showed no expression of adipsin, a final adipocyte marker, in the western blot analysis (Figure 8C). Interestingly, both PPARγ and C/EBPα expression levels were increased in D181A HDAC1 expressing 3T3 L1s in the MI conditions. However, this upregulation did not translate into an increased expression of the adipogenic marker, adipsin or increased differentiation in the MI alone condition and therefore was not quantified, but this observation is addressed in the discussion. Viral

transduction of 3T3 L1 cells with WT HDAC1 decreased the expression levels of adipisin and C/EBP α by 60-65% and PPAR γ by 25% relative to the pLXSN 3T3 L1 control cells, on day 7 following MID treatment (Figure 8C and D). Alternatively, 3T3 L1s stably expressing the D181A HDAC1 mutant exhibited a 75% increase in the protein expression levels of adipisin, C/EBP α and PPAR γ , relative to pLXSN 3T3 L1s (Figure 8C and D).

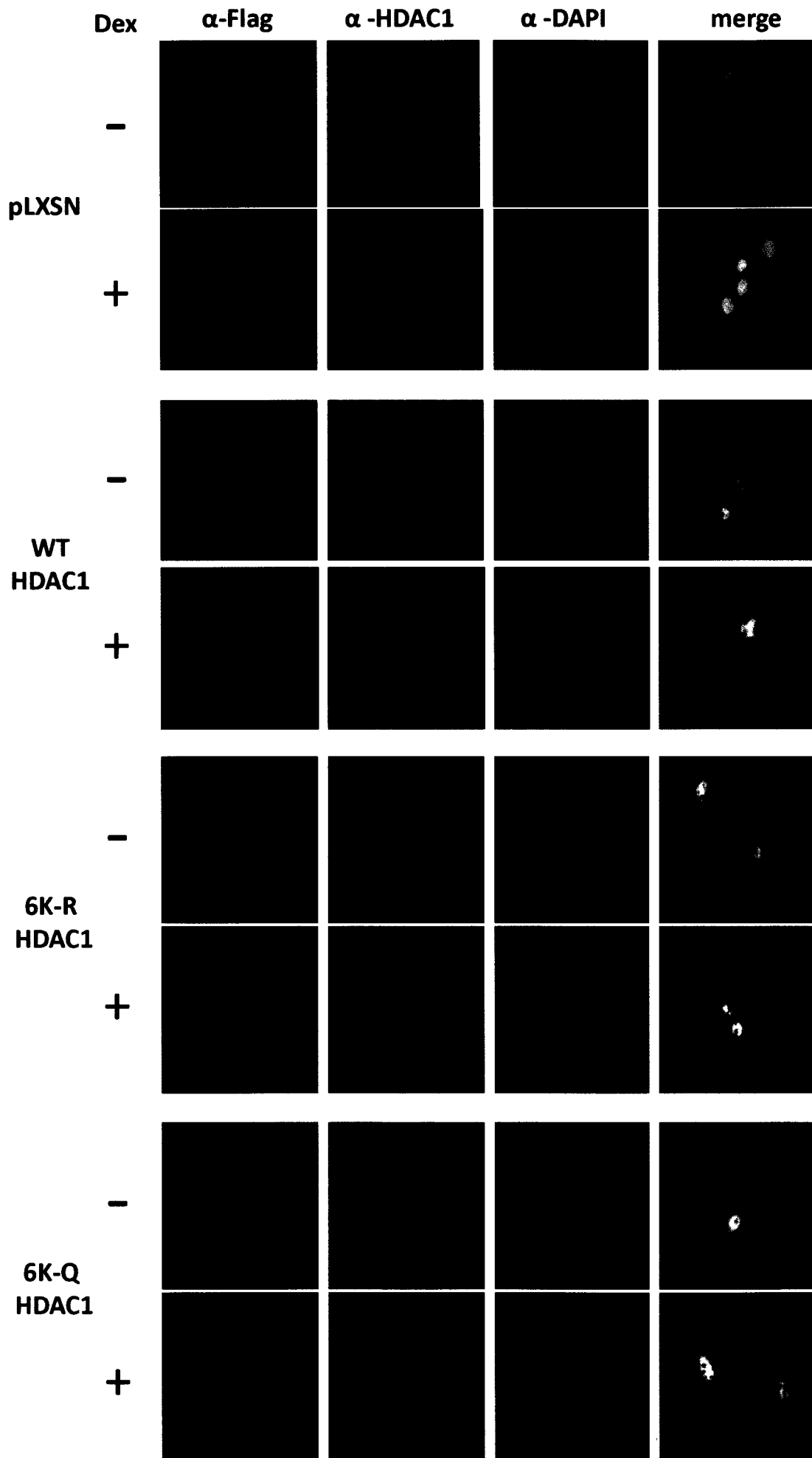
Thus far, the results of the experiment confirm that HDAC1 is an inhibitor of dex-potentiated *C/ebp α* transcription and preadipocyte differentiation and suggests that the deacetylase activity of HDAC1 is essential to this inhibitory role. Furthermore, the results support the assumption that D181A HDAC1 is a dominant negative HDAC1 mutant since expression of the catalytically compromised mutant not only fails to inhibit dex potentiated *C/ebp α* transcription (Figure 7) and preadipocyte differentiation (Figure 8), but it further enhances the ability of corticosteroids to stimulate the adipogenic process..

D181A HDAC1 is properly localized to the nucleus

To ensure that the phenotype observed for the D181A HDAC1 mutant is due to loss of its deacetylase activity and not to cellular mislocalization of the protein as a result of the mutation, immunofluorescence was used to examine the localization of the D181A HDAC1-HA-Flag and WT HDAC1-HA-Flag, which is expected to be exclusively located in the nucleus (Johnson, 2002). 3T3 L1 cells virally transduced to express pLXSN, WT HDAC1-HA-Flag or D181A HDAC1-HA-Flag, were treated for 4hrs with 1 μ M Dex, or the equivalent dilution of ethanol, before immunofluorescence was performed with a primary antibody against the Flag tag fused to the virally transduced HDAC1 proteins (green) (first column of panels on the left), and an antibody against the endogenous HDAC1 (red) (second column of pannels from the left) (Figure 9). The nuclei were stained with DAPI (third column of panels from the left). In the pLXSN-Flag control cells, cytosolic staining was observed with the

Figure 9: Mutation of aspartic acid 181 to alanine (D-A) in HDAC1 does not result in mislocalization of the HDAC1 protein.

3T3 L1 cells stably expressing pLXSN-Flag, pLXSN WT HDAC1-HA-Flag or pLXSN D181A HDAC1-HA-Flag were plated onto coverslips and treated for 24hrs with either ethanol or 1 μ M dex. Immunofluorescence was then performed on the cells for the Flag tag (green), HDAC1 (Red) and DAPI (to stain the nucleus blue). Two independent experiments were performed, using a 63X Oil objective lens.



anti-Flag antibody (Figure 9), however in cells expressing HA-Flag-WT or D181A HDAC1, the Flag antibody is clearly localized to the nucleus, similar to the endogenous HDAC1 (Figure 9). Furthermore, the nuclear localization of both the endogenous and virally transduced HDAC1s is unaffected by dexamethasone treatment. These results confirm that the phenotype observed upon ectopic expression of D181A HDAC1 is due to the loss of its deacetylase activity and not to mislocalization of the protein within the cells.

Mutation of amino acid 181 from aspartic acid to alanine (D-A) in HDAC1 affects the ability of the enzyme to interact with co-transcription factors known to be involved in *C/ebpa* transcriptional regulation; mSin3A, C/EBP β and GR

Previous studies in our laboratory have demonstrated that in the absence of steroid C/EBP β interacts with the mSin3A/HDAC1 co-repressor complex and that this interaction is upset following 24hrs of steroid treatment (Wiper-Bergeron, *et al.*, 2003). Moreover, GR has been shown to interact with the mSin3A/HDAC1 complex and this interaction increases in a dex-dependent manner. These results, in combination with other findings, led to our hypothesis that GR enhances *C/ebpa* transcription by titrating the mSin3A/HDAC1 co-repressor complex from C/EBP β and promoting HDAC1 for 26S proteasomal degradation, thus allowing for transcriptional activation of C/EBP α promoter-bound C/EBP β .

In order to investigate whether or not mutation of aspartic acid 181 to alanine in HDAC1 affects its ability to interact with other known regulators of *C/ebpa* transcription co-immunoprecipitation experiments were carried out in Green African Monkey kidney cells (Cos-7 cells). The cells were transiently transfected with the indicated pcDNA3.1 WT or D181A HDAC1-HA-Flag, in addition to GR and C/EBP β . The cells were then treated for 4hrs with 100nm dex or the vehicle, after which the cells were harvested and Flag-pcDNA3.1, WT HDAC1-HA-Flag or D181A HDAC1-HA-Flag were immunoprecipitated

from the whole cell extracts. Visualization and quantification of the immunoblots revealed that WT HDAC1 efficiently interacted with GR, mSin3A and C/EBP β , as previously demonstrated (Wiper-Bergeron, *et al.*, 2003). Furthermore, treatment with dex, for 4hrs increased the ability of WT HDAC1 to interact with both mSin3A and C/EBP β by two fold, whereas it had no affect on the ability of the deacetylase to bind to GR (Figure 10 A and B). Visual analysis and quantification of the interactions between D181A HDAC1 and the three transcription factors revealed that the mutant deacetylase interacts with mSin3A and C/EBP β twice as efficiently as WT HDAC1, in the presence of dex, and treatment with dex enhanced these interactions as compared to the minus dex conditions (Figure 10A and B). On the other hand D181A HDAC1 appears to interact approximately 30% less efficiently with GR than WT HDAC1, and this interaction is unaffected by steroid treatment (Figure 10A and B).

Expression of the catalytically compromised D181A HDAC1 mutant restores the ability of dex to upregulate *C/ebpa* expression in the presence of the transcriptionally compromised K98-102R C/EBP β acetylation mutant

Earlier studies designed to begin elucidating the mechanism through which the transcription factor C/EBP β regulates transcription of its target genes have determined that acetylation of C/EBP β increases its transcriptional activity (Wiper-Bergeron, *et al.*, 2007, Cesena, *et al.*, 2007, and Cesena, *et al.*, 2008). More specifically, acetylation of C/EBP β at lysine residues 98, 101 and 102 has been shown to be necessary for C/EBP β to be capable of maximally activating dex-potentiated *C/ebpa* transcription (Wiper-Bergeron, *et al.*, 2007). Mutation of lysine residues 98,101 and 102 of C/EBP β to arginine, inhibits GR-potentiated titration of HDAC1 from the C/EBP α promoter, suggesting that deacetylation of C/EBP β 's 98,101 and 102

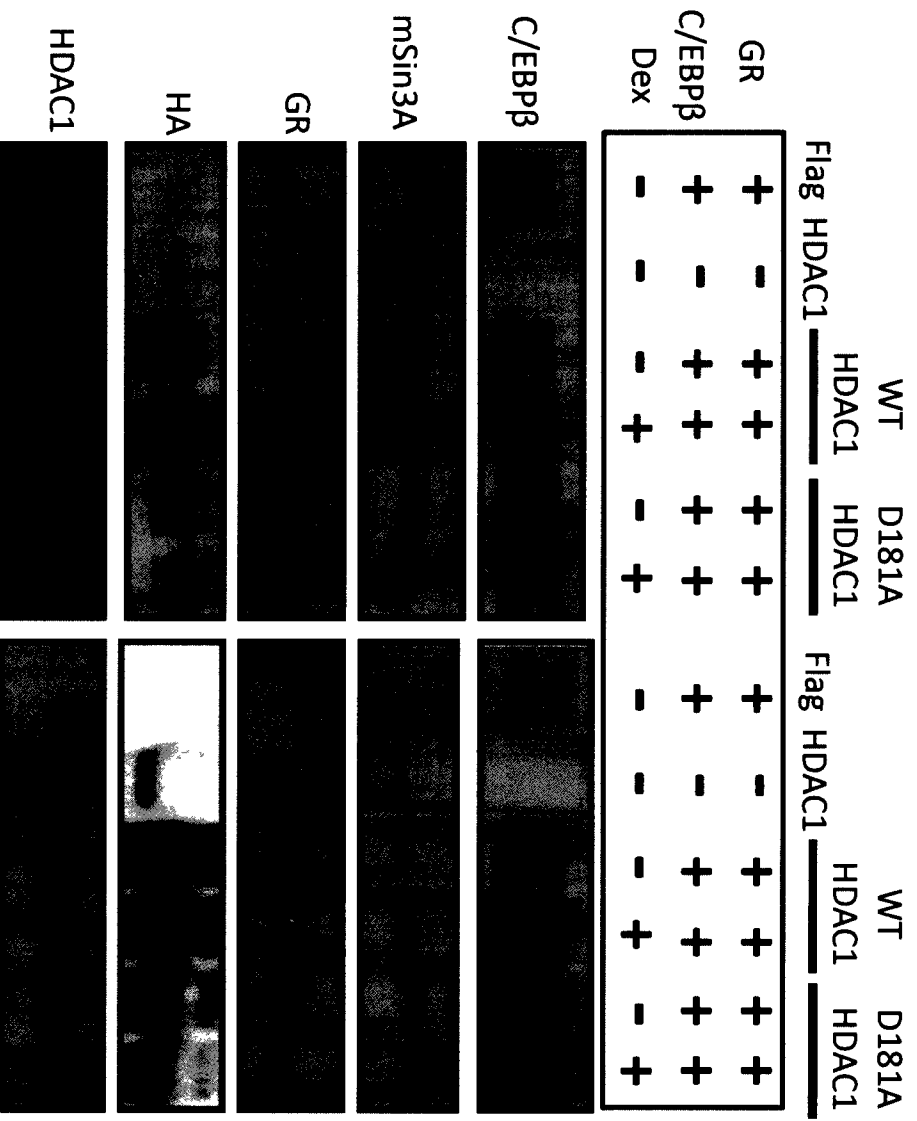
Figure 10: Mutation of amino acid 181 from aspartic acid to alanine (D-A) in HDAC1 affects the ability of the enzyme to interact with co-transcription factors known to be involved in *C/ebpa* transcriptional regulation; mSin3A, C/EBP β and GR.

A) Co-immunoprecipitation of known *C/ebpa* transcriptional regulators, with WT or D181A HDAC1. Cos-7 cells were transiently transfected with Flag-pcDNA3.1, WT HDAC1-HA-Flag or D181A HDAC1-HA-Flag, in addition to GR, and C/EBP β as indicated. Two days later the cells were treated for 4hrs with 100nM dex and were then lysed. The Flag tagged proteins were subsequently immunoprecipitated from the whole cell lysates. Immunoblot analysis of transcription factors known to be involved in the regulation of *C/ebpa* transcription was performed: mSin3A, GR and C/EBP β . (n=3) B) Quantification of the amount of the transcription factor co-immunoprecipitated with WT or D181A HDAC1 was done with the program Image J and values were normalized to the amount of immunoprecipitated HDAC1-HA-Flag construct. The amount of co-immunoprecipitated transcription factor is represented as relative to WT HDAC1 (+dex). (n=3 \pm SEM). A student t-test was performed, where * p<0.05.

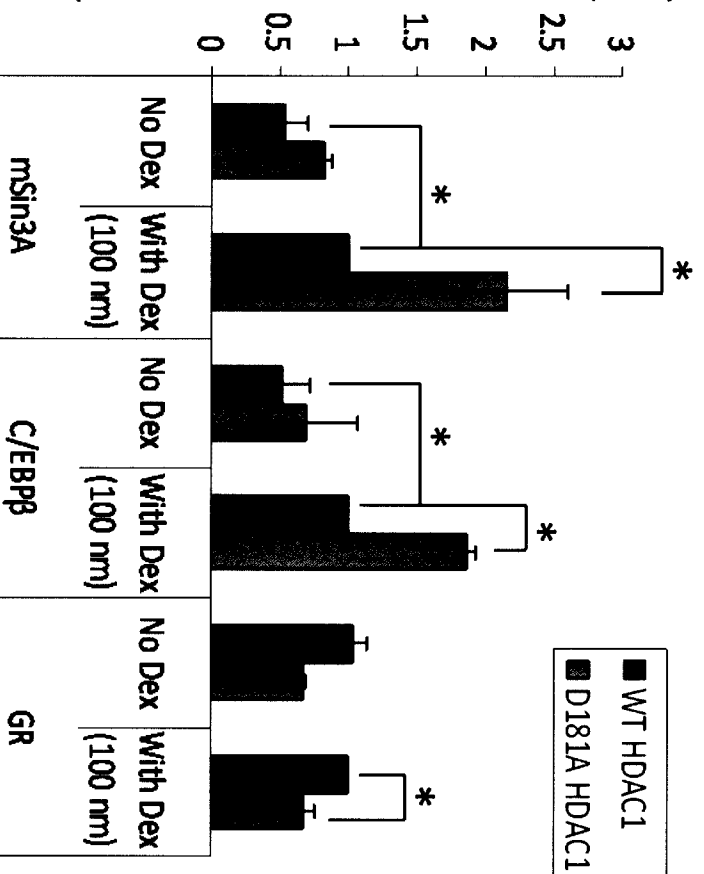
A

5% Inputs

IP: Flag

**B**

Quantification of Co-immunoprecipitation experiments relative to WT HDAC1 (-dex)



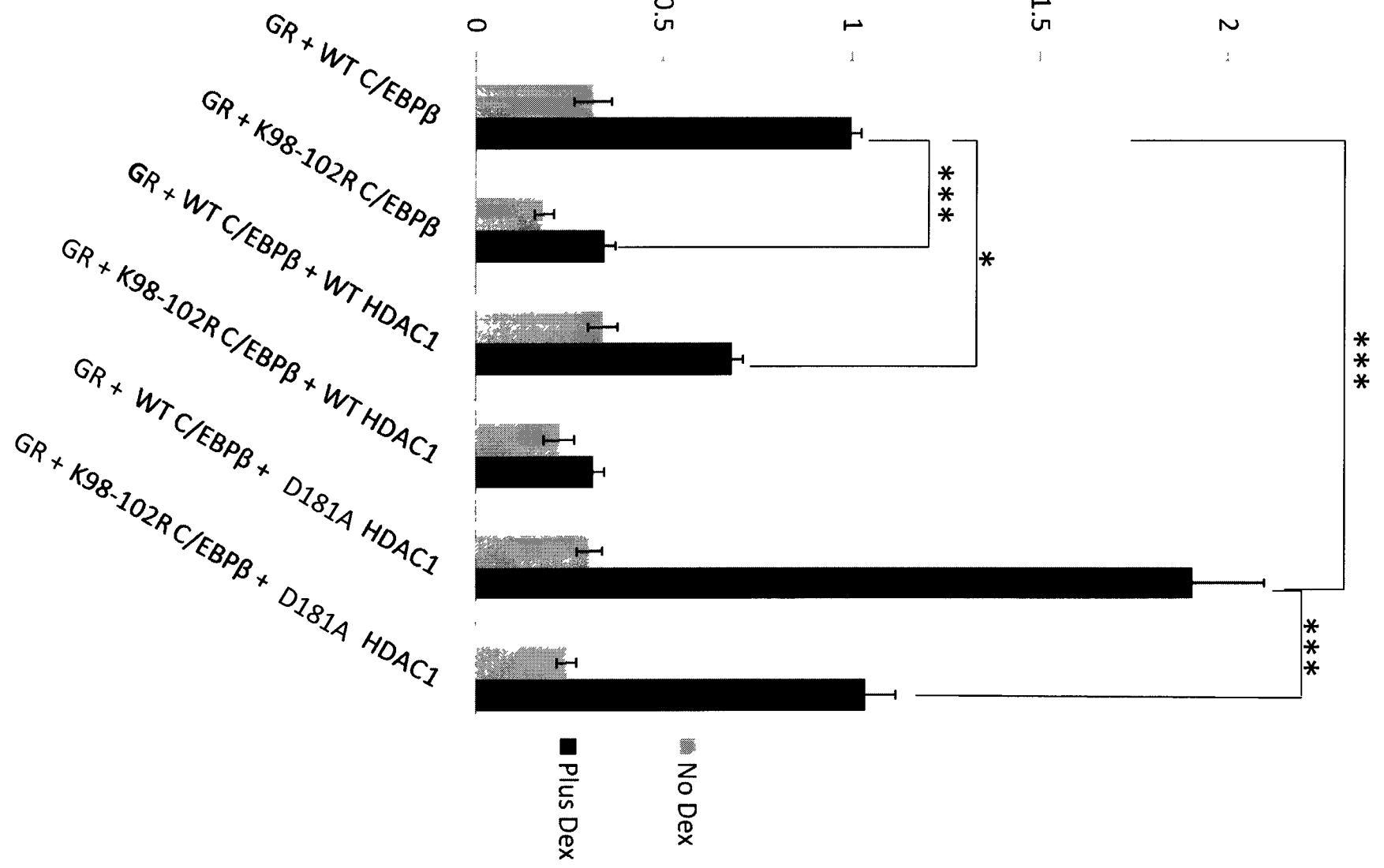
lysine residues may be a key step in the ability of HDAC1 to remain at the *C/EBP α promoter* and inhibit transcription in the absence of glucocorticoids (Wiper-Bergeron, *et al.*, 2007).

To gain some insight into the deacetylation targets of HDAC1 at the *C/EBP α promoter* we assessed the effect of WT and D181A HDAC1 on dex-potentiated *C/EBP α promoter-driven Firefly luciferase* expression in the presence of WT *C/EBP β* or the transcriptionally compromised K98-102R *C/EBP β* acetylation mutant. If deacetylation of lysine residues 98,101 and 102 are HDAC1's only deacetylation targets at the *C/EBP α promoter* then expression of the deacetylase compromised D181A HDAC1 mutant would not be expected to have any effect on the inability of the transcriptionally compromised K98-102R *C/EBP β* mutant to mediate GR induced *C/EBP α promoter-driven luciferase* expression.

To test this, NIH 3T3 cells were transiently co-transfected with *C/EBP α promoter-Firefly luciferase*, *CMV-Renilla luciferase*, GR and WT or K98-102R *C/EBP β* , in addition to pcDNA3.1, WT HDAC1 or D181A HDAC1. The cells were treated for 24hrs with 1uM dex and harvested to measure the *Firefly* and *Renilla* luciferase luminescent values. Treatment of NIH 3T3 cells transiently transfected with WT *C/EBP β* and GR, with dex resulted in successful induction of *C/EBP α promoter-driven-luciferase* expression and the luciferase expression of all other conditions was quantified relative to this induction (Figure 11). Co-transfection of the K98-102R *C/EBP β* mutant, in the place of WT *C/EBP β* , with GR resulted in a 70% decrease in the ability of steroid treatment to induce luciferase expression, as previously established (Figure 11) (Wiper-Bergeron, *et al.*, 2007). When WT-HDAC1 was co-transfected along side WT *C/EBP β* the dex effect dropped by approximately 40%, as shown in figure 11. Interestingly, co-transfection of WT HDAC1 did not enhance the ability of K98-102R *C/EBP β* mutant to suppress

Figure 11: Expression of the enzymatically compromised D181A HDAC1 mutant restores the ability of dex to upregulate transcription at the C/EBP α promoter in the presence of the transcriptionally compromised K98-102R C/EBP β acetylation mutant. A C/EBP α promoter based transcription assay was performed in NIH 3T3 cells to test K98-102R C/EBP β transcriptional activity in the presence of D181A HDAC1. Statistical analysis of the experiment was performed using the program SPSS Statistics 17.0, to perform an ANOVA (Analysis of Variance). The p values were calculated by performing an ANOVA on the results, followed by a Dunnett test to calculate the p-values between the dex effect and the samples with WT and D181A HDAC1 +GR+WT C/EBP β , and a Bonferroni test to calculate the p-values between each WT C/EBP β and K98-102R C/EBP β condition. (n=3Xtriplicates \pm SEM) *p<0.05, **p<0.01, ***p<0.001

Fold Increase in Luciferase Expression over the Dex Effect



the dex effect (figure 11). As previously observed in Figure 7, co-transfection of D181A HDAC1 with WT C/EBP β and GR increased the induction of C/EBP α promoter-driven luciferase expression and effectively doubled the dex effect (Figure 11). More importantly, co-transfection of D181A HDAC1 with K98-102R C/EBP β restored the ability of GR to induce C/EBP α promoter-driven luciferase expression to WT C/EBP β + GR levels (Figure 11). However, although C/EBP α promoter activity is present this is a 50% decrease in luciferase expression compared to when the D181A HDAC1 mutant is co-transfected with WT C/EBP β .

The observation that co-transfection of the catalytically compromised HDAC1 mutant alongside the transcriptionally compromised K98-102R C/EBP β mutant, restored the ability of GR to potentiate luciferase expression driven by the CEBP α promoter suggests that HDAC1 has additional deacetylase targets at the C/EBP α promoter besides lysine residues 98,101 and 102 of C/EBP β . Additional experimentation is needed to verify that the lysine residues 98,101 and 102 of C/EBP β are deacetylation targets of HDAC1 and to identify other potential targets of HDAC1 at the C/EBP α promoter.

Discussion

Glucocorticoids enhance the efficiency of adipogenesis during the first 48hrs of the differentiation process, in part, by upregulating transcription of the adipogenic commitment factor *C/ebpa* (Darlington *et al*, 1998, Mandrup and Lane, 1997, Rosen *et al*, 2002 and Wu, *et al*, 1999). Previous studies in our laboratory have identified that HDAC1 is a repressor of C/EBP β mediated *C/ebpa* transcription and that steroid treatment promotes the titration of the mSin3A/HDAC1 co-repressor complex from C/EBP β through a mechanism that includes targeting of HDAC1 for 26S proteasomal degradation (Lane, *et al*, 1999, Wiper-Bergeron *et al*, 2003 and Wiper-Bergeron *et al*, 2007). Therefore, in order to gain additional insight into the molecular details of GR-potentiated preadipocyte differentiation through *C/ebpa* transcription, I decided to investigate the specific role of HDAC1 in the regulation of *C/ebpa* transcription.

HDAC1 but not HDAC2 is involved in the inhibition of C/EBP β -mediated *C/ebpa* transcription

Previous studies performed in our laboratory clearly established that HDAC1 is an inhibitor of the adipogenic process as over-expression of HDAC1 abrogated GR's ability to potentiate both *C/ebpa* transcription and preadipocyte differentiation while treatment with the general HDAC inhibitors TSA or VPA replaced the ability of dex to enhance adipogenesis (Wiper-Bergeron *et al*, 2003). However, given the general nature of the inhibitors used in this study it remains uncertain whether or not HDAC1 possesses the specific deacetylase activity responsible for inhibiting the transcriptional activity of C/EBP β . By transiently knocking down HDAC1 expression with siRNA, during the initial 48hr window during which glucocorticoids influence the adipogenic cascade we demonstrated that a 60% reduction in the level of HDAC1 significantly increased the ability of GR to

potentiate both the transcription of the adipogenic commitment factors, *C/ebpa* and *ppary* (Figure 3B) and preadipocyte differentiation in both NIH 3T3 and 3T3 L1 cell lines (Figures 3 and 4). Since a reduction in HDAC1 expression enhanced the efficiency with which glucocorticoids can upregulate the adipogenic transcriptional cascade, these results support the hypothesis that HDAC1 is an inhibitor of GR-potentiated adipogenesis.

However, the ability of siRNA knockdown of HDAC1 to enhance the adipogenic process was only observed in the steroid conditions, and not in cells treated with MIX and insulin alone (Figure 3 and 4). This observation implies that a 60% reduction in cellular HDAC1 expression is insufficient to replace the potentiating affects of dexamethasone on the adipogenic process. These results may indicate that a decrease of 60% in the levels of HDAC1 protein expression is insufficient to titrate all of the HDAC1 enzymes from C/EBP β , thus allowing for inhibition of *C/ebpa* transcription in the absence of steroid.

To investigate the effect of reduced HDAC1 expression on the interaction between HDAC1 and C/EBP β co-immunoprecipitations could be performed to compare the amount of HDAC1 that interacts with C/EBP β in the scrambled versus HDAC1 siRNA treated cells. Additionally, chromatin immunoprecipitation (ChIP) experiments on the C/EBP α promoter could be conducted to examine the relative quantity of HDAC1 that is recruited to the promoter in MI conditions in cells treated with HDAC1 versus scrambled siRNA.

If a 60% reduction in HDAC1 expression proves to be insufficient to significantly reduce the amount of HDAC1 bound to C/EBP β at the C/EBP α promoter, then additional studies could be undertaken to increase the efficiency of HDAC1 knockdown. Either the experimental conditions utilized to knockdown HDAC1 with the smart pool siRNA in the

above experiments could be optimized to further increase the efficiency of HDAC1 knockdown, or an alternative method, such as the use of retroviral siRNA to create a stable cell line with HDAC1 knocked down, could be considered.

Alternatively, the failure of the siRNA mediated 60% reduction in HDAC1 expression to induce adipogenesis in the absence of glucocorticoid could also indicate that a more complex mechanism involving multiple proteins in addition to mSin3A and HDAC1, is responsible for suppressing *C/ebpa* transcription in the absence of GR. If this is the case, then reduction of HDAC1 expression alone would likely be insufficient to induce *C/ebpa* transcription, as other inhibitory mechanisms could compensate. This possibility will be discussed further in a subsequent section.

Additionally, it was confirmed that HDAC2 does not play a role in the regulation of *C/ebpa* transcription, as siRNA knockdown of HDAC2 produced no phenotype in NIH 3T3 differentiation experiments, relative to the scrambled siRNA control (Figure 5).

Investigating the possibility that HDAC2 was involved in the regulation of *C/ebpa* transcription was of particular importance because HDAC1 and HDAC2 have both been shown to be associated with the mSin3A co-repressor complex, although HDAC2 does not interact with C/EBP β in Cos-7 cells (Wiper-Bergeron *et al.*, 2003). Furthermore, deacetylation of GR through its association with HDAC2 has been shown to be required for GR to be able to associate with the NF- κ B complex and prevent the complex from initiating transcription of the genes responsible for the chronic inflammatory response (De-Ruijter *et al.*, 2002, Hassig *et al.*, 1998 and Ito, K., *et al.*, 2003). Given the importance of HDAC2 in the ability of GR to inhibit the chronic inflammatory response in a DNA-independent manner, and the fact that GR also indirectly enhances *C/ebpa* transcription, it was reasonable

to hypothesize that HDAC2 may play a role in regulating dex-induced *C/ebpa* transcription. All together, these results confirm that HDAC1, but not HDAC2, is involved in regulating C/EBP β mediated *C/ebpa* transcription.

Aspartic Acid 181 is critical to the HDAC1s catalytic activity

In order to investigate the importance of HDAC1 enzymatic activity to suppression of *C/ebpa* transcription, we generated a human HDAC1 mutant with aspartic acid residue 181 mutated to alanine (D181A HDAC1). We predicted that D181A HDAC1 would be catalytically inactive based on the fact that aspartic acid 181 in HDAC1 is one of the five highly conserved amino acids which have been identified as comprising the charge relay system that is responsible for conferring the deacetylase activity to the HDAC family of enzymes (Figure 2A) (Finnin, *et al*, 1999, Somoza, *et al*, 2004 and Vannini, *et al*, 2004). Mutational studies performed by mutating other residues within the charge relay system in either HDAC1, HDAC8 or the Histone Deacetylase Like Protein (HDLP), have all produced inactive deacetylase mutants, providing additional support to our hypothesis that the D181A HDAC1 mutant will be catalytically compromised (Hassig. *et al*, 1998, Kadosh *et al.*, 1998, Finnin, *et al*, 1999, and Vanommelaeghe, *et al*, 2005). The deacetylase activity of the D181A HDAC1 mutant was assessed using a commercially available colorimetric histone deacetylase activity assay kit which revealed that the D181A HDAC1 mutant exhibits only about 25% of the deacetylase activity of the WT HDAC1 enzyme (Figure 6A). We propose that the remaining 25% deacetylase activity observed for the D181A HDAC1 mutant is either due to the residual deacetylase activity of the D181A HDAC1 mutant, or it could be the result of the presence of HDAC2 that co-immunoprecipitated with our HA-Flag tagged

HDAC1 constructs (Figure 6c). HDAC2 is known to co-immunoprecipitate with HDAC1 as they are often found together in co-repressor complexes, and it is extremely difficult to immunoprecipitate one without co-immunoprecipitating at least a small fraction of the other (Hassig, *et al*, 1998 and Wiper-Bergeron, *et al.*, 2003). Notably, western blots for HDAC2 were performed on the immunoprecipitated HA-Flag pcDNA3.1, WT and D181A HDAC1 and confirmed that no variations in the amount of HDAC2 co-immunoprecipitated between the HDAC1 constructs (Figure 6C). Therefore, as predicted mutation of aspartic acid 181 to alanine in HDAC1 generated a catalytically compromised HDAC1 mutant which confirms that aspartic acid 181 plays a critical role in the HDAC1 catalytic function.

We plan to validate the results of the colorimetric deacetylase activity assay by performing an *in vitro* deacetylation assay using a variety of known HDAC1 C¹⁴-acetylated substrates, such as histones, MyoD and p53. This experiment is described in detail further along in the discussion, however it will be important to minimize or completely obstruct the co-immunoprecipitation of HDAC2 with the HDAC1-HA-Flag constructs in order to properly assess the differences in the deacetylase activity of the different HDAC1 constructs. Therefore, during the preparation of our HA-Flag WT and HDAC1 constructs for the *in-vitro* deacetylation assays, we could minimize the amount of HDAC2 that is co-immunoprecipitated simply by increasing the KCl concentration that is used during the immunoprecipitation. Alternatively, to entirely prevent HDAC2 from co-immunoprecipitating with HDAC1 in these *in-vitro* assays we could generate our HDAC1-HA-Flag constructs in Sf-19 insect cells, as described by Qiu *et al.* (2006), as these cells do not express HDAC2 but they do perform the post-translational modifications that are required for HDAC1 to be catalytically active. The disadvantage to optimizing the KCl

concentration during the immunoprecipitation and in producing the HDAC1 constructs in Sf-19 cells is that the ability of HDAC1 to interact with mammalian co-factors could be affected as high salt concentrations are known to inhibit protein-protein interactions and because the Sf-19 insect cells do not express mammalian cofactors. The reason that it is important for HDAC1 to be able interact with other potentially important mammalian co-factors is that experimental evidence suggests that the specificity of HDAC1 for specific substrates is regulated through its association with specific co-factors (De-Ruijter *et al*, 2003). For example, p53 cannot bind to purified baculovirus-produced HDAC1 as it requires the p53 target protein in the deacetylase complexes (PID) to mediate the interaction (Luo *et al.*, 2000). Therefore, regardless of which method we chose to purify our HDAC1-HA-Flag constructs, we will have to take care to ensure that the ability of HDAC1 to deacetylate all substrates is maintained. This can be done, by testing the deacetylase activity of just purified WT-HDAC1-HA-Flag from the nuclear extracts or from Sf-19 cells, on each of the substrates, before examining the deacetylase activity of the mutants. This will be a time consuming and yet important process.

HDAC1 plays an inhibitory role in GR-potentiated adipogenesis that is dependent on the its enzymatic activity

Co-expression of the D181A catalytically compromised HDAC1 mutant during C/EBP α promoter-driven luciferase reporter assays and in differentiating 3T3 L1 murine preadipocytes improved the ability of corticosteroid treatment to enhance both the expression of luciferase driven by the C/EBP α promoter (Figure 7) and the efficiency of 3T3 L1 differentiation into mature adipocytes (Figure 8). On the other hand, expression of WT HDAC1 decreased the effectiveness of steroid treatment to upregulate both C/EBP α

promoter-driven luciferase expression and preadipocyte differentiation, as expected (Figures 7 and 8). If the deacetylase activity of HDAC1 is important to its inhibitory role at the C/EBP α promoter, then we would expect the catalytically compromised D181A HDAC1 mutant to be unable to inhibit the adipogenic process. However, in these experiments the catalytically compromised D181A HDAC1 has not only lost its ability to inhibit dexamethasone-potentiated *C/ebp α* transcription and preadipocyte differentiation, but expression of the enzymatically compromised deacetylase actually amplifies the ability of GR to upregulate the adipogenic program (Figures 7 and 8). The ability of the D181A HDAC1 to enhance GR-potentiated adipogenesis mimicks the enhanced differentiation observed upon siRNA knockdown of HDAC1 (Figure 3 and 4). These results imply that the loss of the deacetylase activity of the HDAC1 is equivalent to loss of expression of the enzyme which strongly supports our hypothesis that the deacetylase activity of HDAC1 is required for its role in the regulation of C/EBP β mediated *C/ebp α* transcription and demonstrates that D181A HDAC1 acts as a dominant negative HDAC1 mutant.

It is also important to mention that stable expression of the D181A HDAC1 mutant in 3T3 L1 cells resulted in a dex-independent increase in the protein expression levels of both C/EBP α and PPAR γ on Day 7 of differentiation (Figure 8C). However, the increase in the expression of two adipocyte markers did not correspond to an increase in the development of mature adipocytes in the MI+D181A HDAC1 3T3 L1 preadipocytes, as the cells did not positively stain with Oil Red O, nor were there detectable levels of adiponectin expression in the Western blot performed on Day 7 (Figure 8).. Furthermore, qPCR results indicated that mRNA levels of *C/ebp α* and *ppar γ* were so low that they were undetectable 72 hrs following the initiation of differentiation. Therefore, the increased expression of C/EBP α and PPAR γ

observed in the 3T3 L1 cells expressing D181A HDAC1 and treated with MI is likely due to the effect of D181A HDAC1 on the expression level of a regulator of *C/ebpa* or *ppary* expression, or a gene downstream from *C/ebpa* and *ppary*, which subsequently leads an increase in the expression of the two adipogenic transcription factors. Alternatively, it may simply take the D181A HDAC1 mutant longer to enhance expression of *C/ebpa* to a detectable level without the assistance of GR to help relieve the inhibitors at the *C/EBPα* promoter. Whether or not the delayed increase in *C/EBPα* and *PPARγ* expression translates into enhanced preadipocyte differentiation in the MI treated, D181A 3T3 L1 cells could be assessed by allowing all of the MI treated 3T3 L1 cells to differentiate for a longer period of time before assessing the efficiency of differentiation.

Inclusively, these results support our current model which features the role of HDAC1 as a suppressor of *C/EBPβ* mediated *C/ebpa* transcription and they demonstrate that the deacetylase activity of HDAC1 is essential to the ability of the deacetylase to inhibit the adipogenic process, thus successfully fulfilling both of our objectives. Furthermore, our results also suggest that the D181A HDAC1 can act as a dominant D181A HDAC1 mutant, which could be a useful tool for further clarifying the specific role of HDAC1 in adipogenesis and other physiological processes.

HDAC1 is not the only inhibitor responsible for restraining *C/ebpa* transcription in the absence of GR

The capability of D181A HDAC1 to enhance both *C/EBPα* promoter-driven luciferase expression and preadipocyte differentiation is dependent on dexamethasone treatment of the cells, as no enhancement in luciferase expression or 3T3 L1 differentiation was observed in cells expressing D181A HDAC1 in the minus dex conditions (Figure 7 and

8). Additionally, the same phenotype was observed in the HDAC1 siRNA experiments (Figures 3 and 4) where an increase in differentiation was only detected in the HDAC1 siRNA treated cells incubated with steroid. Based on our current understanding of the regulation of C/EBP β mediated *C/ebp α* transcription these results were unexpected.

Founded on earlier work performed in our laboratory, we have developed a model for the regulation of *C/ebp α* transcription in the presence of adipogenic stimuli which is illustrated in Figure 1B. In this model, HDAC1 has been identified as the active inhibitor of *C/ebp α* transcription and the ability of GR to enhance *C/ebp α* transcription is believed to involve inactivation and/or titration of HDAC1 from C/EBP β (Wiper-Bergeron *et al*, 2003). Thus far in this investigation I have confirmed that HDAC1 is a suppressor of C/EBP β -mediated *C/ebp α* transcription and have demonstrated that the deacetylase activity of HDAC1 is essential to the ability of the enzyme to suppress *C/ebp α* transcription. Based on these results and on our previous understanding of *C/ebp α* transcriptional regulation we hypothesized that loss of HDAC1 expression or inactivation of its catalytic activity would replace the ability of glucocorticoids to enhance the adipogenic process. However, the lack of both C/EBP α promoter activity and preadipocyte differentiation in the steroid free conditions when HDAC1 expression was reduced by over half (Figures 3 and 4), or when the catalytically compromised D181A HDAC1 mutant was ectopically expressed (Figures 7 and 8) implies that HDAC1 is not the only inhibitor responsible for obstructing C/EBP β -mediated *C/ebp α* transcription, in the absence of GR.

One possible explanation for the discrepancies between the expected and the experimental results of the siRNA HDAC1 differentiation experiments and the D181A HDAC1 mutant is that the repression of C/EBP β mediated *C/ebp α* transcription is not solely

dependent on HDAC1 and its deacetylase activity. The co-repressor complex is composed of two proteins; mSin3A and HDAC1, however, in the HDAC1 siRNA and D181A HDAC1 experiments only one component of the co-repressor complex, HDAC1, is knocked down or is functionally compromised. In these circumstances mSin3A, whose over-expression has been shown to potentiate the ability of HDAC1 to inhibit transcription of the human luteinizing hormone receptor gene (Zhang and Dufau, 2002), may still be capable of binding to C/EBP β at the *C/ebp α* promoter. This interaction between mSin3A and C/EBP β in steroid-free conditions could be inhibiting the ability of the PCAF/GCN5/p300 co-activator complex to bind C/EBP β and activate *C/ebp α* transcription, as the binding site for the co-repressor and the coactivator complexes on C/EBP β have been recently discovered to overlap (Salem, H.S, unpublished).

Furthermore, if mSin3A is capable of binding to C/EBP β and impeding activation of the *C/ebp α* promoter, then this could also help clarify why expression of the catalytically compromised D181A HDAC1 mutant further increases the magnitude of GR stimulated adipogenesis. HDAC1 has been hypothesized to inhibit *C/ebp α* transcription in the absence of GR by maintaining both C/EBP β and the histones of the C/EBP α promoter in a deacetylated and thus transcriptionally inactive state (Figure 1B). Consequently, it was proposed that GR promotes the titration of mSin3A/HDAC1 from C/EBP β , and targets HDAC1 for degradation in order to allow for the hyperacetylation of C/EBP β and of the C/EBP α promoter that is required for initiation of *C/ebp α* transcription to take place. If, as we propose above, mSin3A also plays a role in inhibiting *C/ebp α* transcription by blocking the binding site of the coactivator complex on C/EBP β , then when HDAC1 expression is decreased or when a catalytically inactive HDAC1 (D181A) is recruited to the C/EBP α

promoter, mSin3A could still inhibit *C/ebpα* transcription, but the decrease in the quantity or the activity of the associated HDAC1 could allow for the accumulation of acetylated C/EBPβ and histones at the promoter. Consequently, when dex is added to the system and GR promotes the titration of mSin3A away from the C/EBPα promoter, this could allow for faster and more efficient upregulation of *C/ebpα* transcription since both C/EBPβ and the promoter histones would already be in their transcriptionally active states (Wiper-Bergeron *et al*, 2003 and 2006).

To investigate whether or not mSin3A can bind to C/EBPβ at the C/EBPα promoter in the absence of HDAC1 chromatin-histone immunoprecipitation (CHIP) experiments should be performed for the *C/ebpα* promoter, from immunoprecipitations of mSin3A in WT C/EBPβ positive NIH 3T3 cells treated with HDAC1 or scrambled siRNA. These cells should be treated with either MI or MID for 24hrs, which is the established time point at which the mSin3A/HDAC1 co-repressor complex is titrated away from the C/EBPα promoter (Wiper-Bergeron, *et al*, 2003). This is a well established protocol within our laboratory and could be completed with reasonable ease. An alternative possibility is that there are other, currently unidentified co-repressors involved in inhibiting C/EBPβ mediated *C/ebpα* transcription.

HDAC1 is responsible for curbing the efficiency with which GR potentiates the adipogenic process

As previously mentioned, 3T3 L1 cells with a 60% reduction in HDAC1 expression or expressing the enzymatically compromised D181A HDAC1 differentiated much more efficiently than control cells (Figure 8). Furthermore, expression of D181A HDAC1 enhanced the ability of dex treatment to induce C/EBPα promoter-driven luciferase

expression by two fold, relative to the control (Figure 7). These results suggest that instead of simply inhibiting C/EBP β -mediated *C/ebp α* transcription in the absence of GR, as previously hypothesized, HDAC1 and its deacetylase activity are required to restrain the magnitude with which GR can enhance preadipocyte differentiation. HDAC1 is hypothesized to suppress *C/ebp α* transcription by deacetylating both C/EBP β and the C/EBP α promoter histones. It is possible, as described above, that the recruitment of the enzymatically compromised, dominant negative, D181A HDAC1 protein results in premature acetylation of C/EBP β and the C/EBP α promoter histones which could prime the promoter, and allow it to respond more efficiently to steroid treatment.

Alternatively, perhaps a regulatory mechanism is in place in which the mSin3A/HDAC1 co-repressor complex cycles from the C/EBP α promoter, thus allowing for periods of enhanced transcriptional activation when the co-repressor complex is titrated away from the promoter, followed by decreased periods of transcription upon the return of the co-repressor complex to the promoter. This hypothesis would help to explain our results as cycling of a catalytically compromised HDAC1 back to the promoter would not allow for the deacetylation required to inactivate transcription, resulting in increased transcription, while siRNA mediated knockdown of HDAC1 could have decreased expression of the enzymes to the point where not enough HDAC1 was present for cyclic regulation of *C/ebp α* transcription to occur. Importantly, GR is known to cycle to and from the GRE sites in the promoters of its target genes, such as the MMTV promoter (Qiu *et al.*, 2006). Therefore, perhaps GR indirectly regulates transcription through similar cyclic mechanism that involves regulating the availability of co-factors rather than directly binding to DNA.

To date, we have only established that the mSin3A/HDAC1 co-repressor complex is titrated from the C/EBP α promoter 24hrs following MID treatment. However, a ChIP time course has never been completed to investigate whether or not the mSin3A/HDAC1 complex cycles back to the C/EBP α promoter. Furthermore ChIPs could also be performed to examine the acetylation status of the predicted substrates of HDAC1 at the C/EBP α promoter, the histone H4s and C/EBP β , to see if their acetylation status is altered in a cyclic pattern during the first 48hrs of the differentiation process, and whether that pattern changes upon expression of D181A HDAC1.

It is also important to mention that the D181A HDAC1 mutant interacts two fold more efficiently with both mSin3A and C/EBP β , and less 30% less efficiently with GR than WT HDAC1. Although the importance of these findings is not currently fully understood, the observation that the D181A HDAC1 interacts more efficiently with co-transcription factors suggests that this HDAC1 mutant may spend more time at the C/EBP α promoter, than its WT counterpart. The results of the co-immunoprecipitation experiment should be confirmed in an adipocyte system by performing the same immunoprecipitation experiments in 3T3 L1 preadipocytes. Furthermore, a time course ChIP, beginning at 16hrs, when C/EBP β is known to be bound to the C/EBP α promoter, and going until 48hrs would allow us to determine whether D181A HDAC1 associates more efficiently with the promoter, in addition to giving us more specific information about the regulation of WT HDAC1 at the C/EBP α promoter, as already suggested.

In combination, these results suggest that our current model of C/EBP β regulated *C/ebp α* transcription is incomplete. These findings imply that the role of HDAC1 at the C/EBP α promoter during preadipocyte differentiation is dependent on its enzymatic activity

and involves restraining the magnitude of GR-induced adipogenesis, rather than plainly inhibiting *C/ebpa* transcription in steroid-free conditions.

These results also suggest that additional repressive mechanisms, besides just deacetylation of the C/EBP α promoter and of C/EBP β by HDAC1, are responsible for inhibiting transcription of *C/ebpa* in the absence of glucocorticoids and that the molecular details of GR-mediated alleviation of *C/ebpa* transcriptional inhibition involves more than just promoting the titration of HDAC1 from the C/EBP α promoter and targeting of the deacetylase for proteasomal degradation.

Lysine residues 98, 101 and 102 of C/EBP β are not the only acetylation targets at the C/EBP α promoter whose acetylation status is important in the regulation of GR-potentiated *C/ebpa* transcription

Acetylation of lysine residues within C/EBP β is a well-known mechanism of post-translationally controlling the transcriptional activity of this transcription factor (Cesena, *et al*, 2007 and 2008, Wiper-Bergeron *et al* 2007 and Xu. M *et al*, 2003). Previous studies have identified that acetylation of lysine residues 98,101 and 102 of C/EBP β by GCN5/PCAF is required for C/EBP β to become transcriptionally active and induce *C/ebpa* transcription following dexamethasone treatment (Wiper-Bergeron *et al*, 2006). Moreover, we have also demonstrated that expression of a transcriptionally compromised C/EBP β acetylation mutant in which lysine residues 98,101 and 102 are mutated to arginine (K98-102R C/EBP β) inhibits the ability of GR to upregulate *C/ebpa* transcription and preadipocyte differentiation, in addition to impeding the dissociation of HDAC1 from C/EBP β (Wiper-Bergeron *et al*. 2007). However, when we co-expressed the K98-102R transcriptionally compromised C/EBP β acetylation mutant with the D181A HDAC1 catalytically compromised mutant in a

C/EBP α promoter-driven reporter assay, the ability of K98-102R C/EBP β mutant to upregulate C/EBP α promoter-driven transcription, in the presence of GR, was restored to the level expected when WT C/EBP β and GR are co-expressed(Figure 11). These results emphasize the importance of the acetylation of lysine residues 98, 101 and 102 for maximal transcriptional activation of C/EBP β because even though the catalytically compromised D181A HDAC1 increased the transcriptional activity of the compromised K98-102R C/EBP β , these levels were only half of those induced with D181A HDAC1 and WT C/EBP β . The observation that the K98-102R C/EBP β mutant only diminished the dex + D181A HDAC1 induced luciferase expression by half suggests that there are likely to be additional deacetylation target at the C/EBP α promoter, besides lysine residues 98,101 and 102 of C/EBP β , that must be acetylated in order for maximal transcriptional activation of *C/ebp α* transcription to occur.

Before experimentation is performed to identify additional deacetylation targets of HDAC1 at the C/EBP α promoter we should first confirm in differentiation assays that co-expression of D181A HDAC1 with the transcriptionally compromised K98-102R C/EBP β can rescue dex-potentiated adipogenesis. In this experiment stable NIH 3T3 cells retrovirally transduced to express the Flag-tag, WT HDAC1 or D181A HDAC1 should be transiently transfected with WT or K98-102R C/EBP β at one day post confluence and treated the following with MI or MID for 48hrs and then differentiated for an additional two-three days in media supplemented with insulin. Transient transfection can be used, because this will ensure that the maximum level of WT or K98-102R C/EBP β expression occurs during the 48hr window in the adipogenic process during which glucocorticoids exert their enhancing

affects (Tomlinson *et al.*, 2006). The use of NIH 3T3 cells is favorable as they only express very low levels of endogenous C/EBP β and they respond well to transfection techniques.

Once the ability of the D181A HDA1 mutant to rescue the ability of the transcriptionally compromised C/EBP β mutant to enhance preadipocyte differentiation in the presence of dex is confirmed, we should begin investigating possible deacetylation targets of HDAC1 at the *C/ebpa* promoter. In addition to lysine residues 98,101 and 102 of C/EBP β four other lysine residues K39, 117, 215 and 216 of C/EBP β have been identified as deacetylation substrates of HDAC1 in *in-vitro* deacetylation assays (Cesena *et al.*, 2006 and 2008 and Xu, M. *et al.*, 2003). The K39R C/EBP β mutant exhibited a 70% reduction in its ability to mediate transcriptional activation of a C/EBP α promoter-driven reporter construct, compared to WT C/EBP β (Cesena *et al.*, 2006), while the two other mutants, K117R and K215/216R C/EBP β mutants only demonstrated a 25-40% reduction in their ability to mediate transcriptional activation of the C/EBP α promoter-driven reporter construct (Cesena *et al.*, 2006). These findings imply that regulation of the acetylation status of K39 is particularly influential in controlling transcriptional activity of C/EBP β . To investigate whether K39 of C/EBP β is important to the ability of the transcription factor to induce *C/ebpa* transcription a K39R C/EBP β point mutant should be generated and the effect of its expression on C/EBP α promoter-driven luciferase expression and NIH 3T3 differentiation should be assessed.

If expression of the K39R C/EBP β mutant compromised both C/EBP α expression and NIH 3T3 cell differentiation then it would be interesting to generate a K39, 98-102R C/EBP β mutant and assess whether this mutant abrogates the ability of dex to initiate the adipogenic transcriptional cascade to a fuller extent than the K98-102R C/EBP β mutant, and

whether co-expression of the D181A HDAC1 mutant would be able to rescue the ability of the K39, 98-102R C/EBP β mutant to initiate *C/ebp α* transcription in the presence of steroid. These experiments would provide insight into whether K39, 98-102 of C/EBP β are the only deacetylation targets of HDAC1 at the C/EBP α promoter. If acetylation of K39 is established not to be an important deacetylation target of HDAC1 than the phenotypes produced by K117R or K215/216R C/EBP β mutants in C/EBP α promoter-driven luciferase assays and NIH 3T3 differentiation experiments could be assessed.

It is also a possibility that HDAC1 has more than one additional deacetylation substrate at the C/EBP α promoter. A decrease, but not a complete elimination, of the ability of D181A HDAC1 to rescue the transcriptional capacity of one of the C/EBP β acetylation mutants in the C/EBP α promoter-driven luciferase assay and in the NIH 3T3 differentiation assays would indicate that multiple additional deacetylase targets of HDAC1 are present at the C/EBP α promoter. Another potential C/EBP α promoter bound HDAC1 deacetylase target are the histones of the C/EBP α promoter. To investigate whether expression of the D181A HDAC1 results in enhanced histone acetylation, compared to WT HDAC1, ChIP experiments could be performed for the C/EBP α promoter in differentiating 3T3 L1 cells treated with MI or MID, and the amount of acetylated-H4 associated with the promoter could be investigated. The results of this experiment would assist in determining the deacetylation targets of HDAC1 at the C/EBP α promoter and they would also help to clarify whether or not expression D181A HDAC1 further enhances the ability of GR to promoter *C/ebp α* transcription by permitting hyperacetylation of the C/EBP α promoter in the absence of steroids, as hypothesized earlier.

Experimental Limitations

One weakness of our experimental approach is that we knocked-down or enzymatically compromised HDAC1 which is a ubiquitously expressed protein, known to be involved in regulating transcription of a multitude of genes (De-Ruijeter, *et al*, 2002). Although several studies have demonstrated that the expression levels of only a very small fraction of genes are affected by inhibition of HDACs by general HDAC inhibitors (Glaser *et al*, 2003, Moreira, J.M. *et al*, 2003, Mudduluru, J.J. *et al*, 2004, Reid, G. *et al*, 2005 and Van Lint *et al*, 1996) it is still possible that a gene coding for a regulator of *C/ebpa* or *ppary* transcription is affected by knockdown of HDAC1 expression or over-expression of the catalytically compromised D181A HDAC1, which could in turn be contributing to the observed phenotypes.

An additional limitation of our study is that we only investigate whether or not HDAC1 and HDAC2, play a role in the regulation of C/EBP β mediated *C/ebpa* transcription. It remains a possibility that additional HDACs are involved in regulating *C/ebpa* transcription, particularly since HDACs rarely regulate transcription alone and because the results of this investigation strongly suggest that additional inhibitory mechanism are in place to impede significant *C/ebpa* transcription in the absence of GR (De-Ruijeter *et al*, 2002). One possibility is that HDAC3 could be involved in regulating *C/ebpa* transcription. Both HDAC1 and HDAC3 are involved in the regulation of PPAR γ expression (Zuo *et al*, 2005 and Lai *et al*, 2008), expression of C/EBP α is responsible for the induction of *ppary* transcription in the adipogenic transcriptional cascade and expression of PPAR γ has been determined to be responsible for maintaining high levels of *C/ebpa* transcription during adipogenesis (Zuo *et al*, 2006). Therefore, given the amount of cross-regulation between

C/ebpa and *ppary* it is possible that similar regulator mechanisms are in place to control transcription of both genes. Consequently it would be interesting to assess if overexpression or inhibition/inactivation of HDAC3 affects *C/ebpa* transcription or preadipocyte differentiation, in both the absence and presence of glucocorticoids.

In spite of these restrictions, this investigation has produced some interesting and important information about the role of HDAC1 in the regulation of C/EBP β mediated *C/ebpa* transcription and the adipogenic process, and the importance of its deacetylase activity to these functions.

Conclusion

In summary, we have successfully created a catalytically compromised, dominant negative mutant of HDAC1 which can be used to investigate the importance of HDAC1s deacetylase activity in a variety of systems. The results of our investigation into the specific role of HDAC1 at the C/EBP α promoter support the previous observations that HDAC1, but not HDAC2, plays a suppressive role in the regulation of *C/ebp α* transcription during adipogenesis, and that this role is dependent on the deacetylase activity of the enzyme. Our results also suggest that the deacetylase activity of HDAC1 may be important in moderating the efficiency with which GR is able to enhance *C/ebp α* transcription and potentiate preadipocyte differentiation. Furthermore, these findings suggest that our current model of *C/ebp α* transcriptional regulation is incomplete and that supplementary inhibitory mechanisms, in addition to the mSin3A/HDAC1 complex exist to impede *C/ebp α* transcription in the absence of hormone. Finally, our results suggest that the acetylation status of C/EBP β s 98, 101 and 102 lysine residues are not the only deacetylation targets at the C/EBP α promoter whose acetylation status is involved in regulating *C/ebp α* transcription.

Chapter 2: Investigating whether the regulation of the acetylation status of HDAC1 at lysine residues 218, 220, 432, 438, 439 and 441 is involved in modifying HDAC1's inhibitory role at the C/EBP α promoter during glucocorticoid-potentiated preadipocyte differentiation

Rationale:

In 2006, Qiu *et al.*, demonstrated that GR and HDAC1 interact at the MMTV promoter to regulate its transcription, and that following glucocorticoid treatment the HDAC1 associated with GR becomes increasingly acetylated at lysine residues 218, 220, 432, 438, 439 and 441 by p300. Furthermore, Qiu *et al.*, demonstrate that acetylation of these six specific lysine residues inactivated the ability of HDAC1 to deacetylate histone H4s. They subsequently generated two HDAC1 acetylation mutants that had opposite deacetylase activities; 6K-R HDAC1 had the six previously mentioned lysine residues mutated to arginine, which cannot be acetylated, thus creating a constitutively active HDAC1 mutant, whereas the 6K-Q HDAC1 mutant has the same 6 lysine residues mutated to glutamine, which mimics acetylation and resulted in an inactive HDAC1 protein (Qiu *et al.*, 2006). The result would predict that alterations in the acetylation status of HDAC1 at lysine residues 218, 220, 432, 438, 439 and 441 could affect the ability of GR to potentiate C/EBP α expression by regulating the titration and/or inactivation of HDAC1.

Alterations in the acetylation status of HDAC1 at the C/EBP α promoter could regulate HDAC1's deacetylase activity, as published by Qiu *et al.*, (2006), and/or regulate its stability by either inhibiting or facilitating ubiquitylation of the protein and thus play a role in regulating its proteosomal degradation (Jeong, *et al.*, 2002, Giandomenico *et al.*, 2003 and Grönroos, 2002).

OBJECTIVE:

- 1) I intend to use the 6K-R and 6K-Q HDAC1 mutants published by Qiu, *et al.* (2006) to confirm the importance of HDAC1s deacetylase activity to its inhibitory role in the regulation of C/EBP β mediated *C/ebpa* transcription and preadipocyte differentiation. In the process, I also plan to assess whether glucocorticoid treatment promotes changes in the acetylation status of HDAC1 at the six lysine residues identified by Qiu, *et al.* (2006) and determine whether the regulation of the acetylation status of HDAC1 plays a role in regulating the activity and/or stability of C/EBP β associated HDAC1.

Hypothesis:

During the initiation of glucocorticoid-potentiated *C/ebpa* transcription, GR regulates the activity and/or the stability of C/EBP β -associated HDAC1, by controlling the acetylation status of lysine residues 218, 220, 432, 438, 439 and 441, as identified by Qiu *et al.* (2006).

Results

HDAC1 acetylation mutants: 6K-R HDAC1 and 6K-Q HDAC1

We generated the 6K-R and 6K-Q HDAC1 mutants described by Qiu *et al.*, (2006) using two-step site-directed mutagenesis on the wild type human HDAC1 construct (Figure 12). The first two lysine residues, amino acids 218 and 220, are within the highly conserved, catalytic core of the HDAC1 protein. The second set of lysine residues at amino acids 432, 438, 439 and 441, are found in the highly variable C-terminal region of the protein and are believed to be a part of the nuclear localization sequence (NLS) of the protein (Figure 12) (De-Ruijter *et al.*, 2002 and Taplick *et al.*, 2001). We verified that the correct mutations had been inserted, and that the sequence was in frame by performing sequencing on the constructs with forward, reverse and internal primers, as described in the Material and Methods section.

Both 6K-R and 6K-Q exhibit deacetylase activity in the colorimetric deacetylase activity assay

According to Qiu *et al.* (2006) the 6K-R HDAC1 mutant is constitutively active, whereas the 6K-Q HDAC1 mutant is catalytically inactive. To verify that the deacetylase activity of the 6K-R and 6K-Q HDAC1 mutants corresponded with the published results, a colorimetric deacetylase activity assay was performed. The HDAC1 variants or the Flag negative control were immunoprecipitated with Flag affinity beads from the nuclear extracts of virally transduced Cos-7 cells stably expressing the indicated Flag, or HDAC1-HA-Flag construct (WT, D181A, 6K-R or 6K-Q), which had been treated with 100nM Dex or the equivalent volume of ethanol, as the vehicle control, for 4hrs prior to being harvested.

Figure 12: HDAC1 acetylation mutants: 6K-R HDAC1 and 6K-Q HDAC1

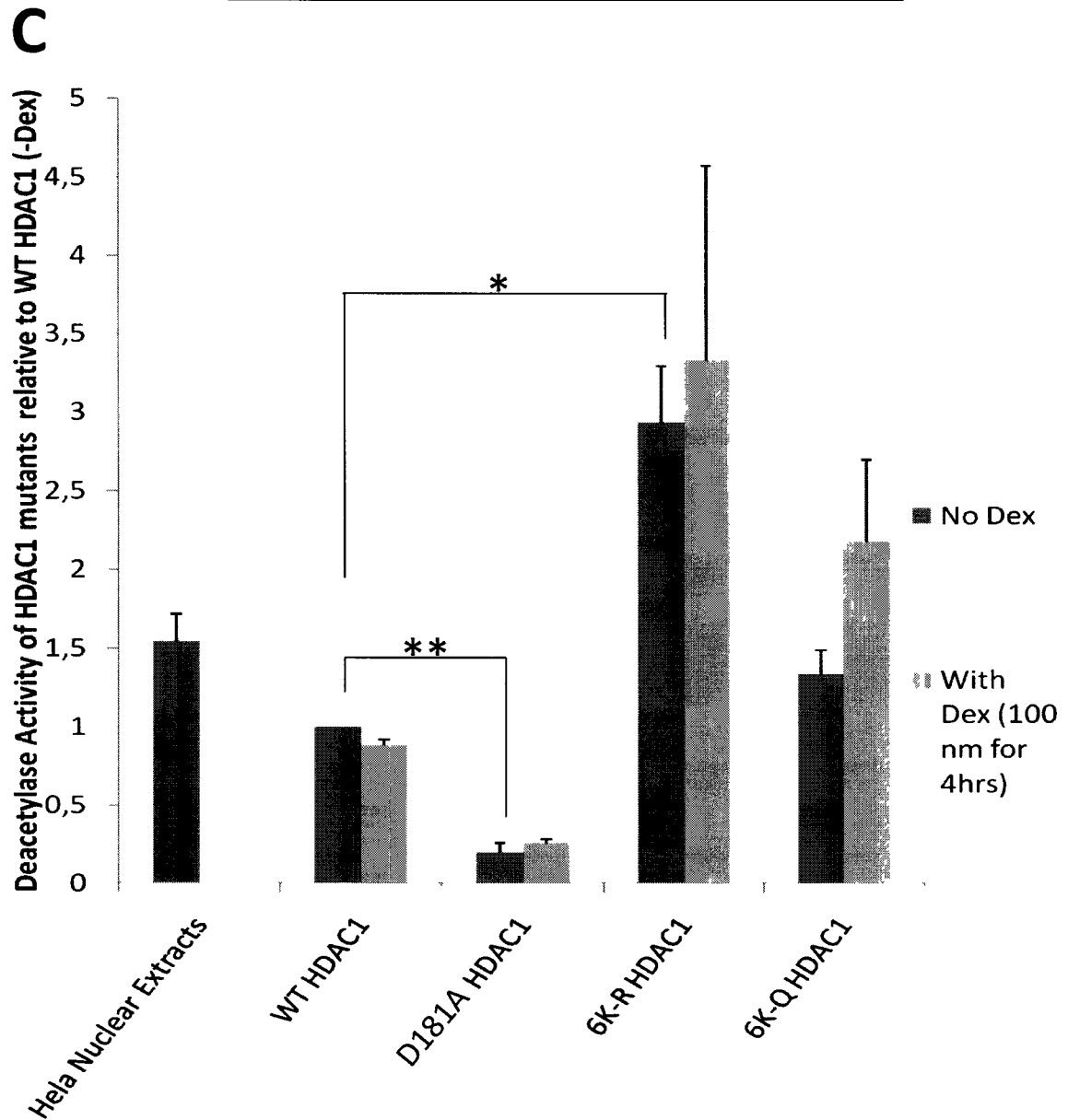
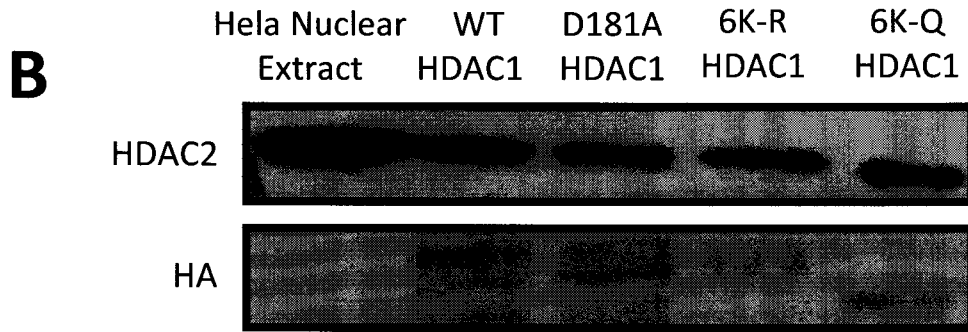
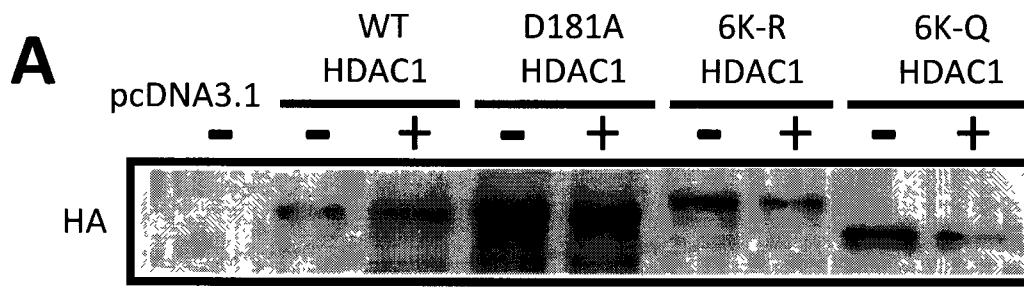
Two step site-directed mutagenesis was used to mutate lysine (K) residues 218, 220, 432, 438, 439, 441 of HDAC1-HA-Flag pcDNA3.1 to either arginine (R) or glutamine (Q). The 218 and 220 mutations are within the catalytic core of HDAC1, whereas the 432, 438, 439 and 441 mutations are in the C-terminal region of the protein, within a potential nuclear localization sequence. Mutations were verified by sequencing with a forward, reverse and internal primer for the HDAC1 sequence.

Immunoprecipitation of the HDAC1 variants in the presence and the absence of dex treatment allowed us to investigate whether hormone treatment induced any changes in the deacetylase activity of the various HDAC1 constructs. The beads, with the HDAC1-HA-Flag constructs attached, were resuspended in assay buffer and 10uL/condition was removed to perform western blot analysis of HA to verify the expression and the amount of the HDAC1-HA-Flag construct immunoprecipitated for each condition. This allowed us to normalize the deacetylase activity results to the amount of enzyme present in the assay (Figure 13A). An additional 10uL was removed from the hormone treated conditions and western blot analysis of HDAC2 was performed, as in Figure 6, which confirmed that variations in the amount of HDAC2 that co-immunoprecipitated with the HDAC1 constructs were not contributing to any differences observed in the deacetylase activity assay (Figure 13B) (De-Ruijeter *et al.*, 2002 ,Hassig *et al.*, 1998 and Wiper-Bergeron *et al.*, 2003).

All of the deacetylase activity values were expressed relative to WT HDAC1 (-dex) (Figure 13C), which exhibited significant deacetylase activity both in the presence and absence of dex, whereas the D181A catalytically compromised HDAC1 mutant demonstrated a significant reduction in its deacetylase activity (~70%), relative to WT HDAC1 (Figure 13C), as previously demonstrated in Figure 6. The 6K-R HDAC1 mutant, which is published as being constitutively active, exhibited 2.5-3 fold more deacetylase activity than WT HDAC1, while the 6K-Q HDAC1 mutant, which is published as being an inactive mutant of HDAC1 (Qiu, *et al.*, 2006), demonstrated less deacetylase activity than the 6K-R HDAC1 mutant but more enzymatic activity than the WT HDAC1 in this assay (Figure 13C). For all of the HDAC1 constructs, little difference was observed in their deacetylase activity in the presence versus the absence of dex (Figure 13C).

Figure 13: 6K-R and 6K-Q HDAC1 mutants have increased or similar deacetylase activity compared to WT HDAC1.

A) Western blot analysis of the HA tag, to demonstrate successful immunoprecipitation of the HDAC1-HA-Flag, constructs (WT, D181A, 6K-R or 6K-Q). HDAC1-HA-Flag constructs were immunoprecipitated with Flag-affinity beads from the nuclear extracts of Cos-7 cells which had been retrovirally infected with the HDAC1-HA-Flag constructs and treated with 100nm dex or ethanol for 4hrs prior to lysis (n=3). B) Western blot Analysis of the HDAC2 that co-immunoprecipitated with HDAC1-HA-Flag constructs. The amount of HDAC2 that co-immunoprecipitated with the HDAC1-HA Flag constructs was quantified to ensure that the differences in the deacetylase activity of the various HDAC1 constructs are due to alterations in the enzymatic activity of HDAC1 and not due to differences in the amount of HDAC2 that was co-immunoprecipitated. Densitometry was performed on the HDAC2 and HA blots, the amount of HDAC2 was normalized to the amount of HDAC1-HA construct co-immunoprecipitated, and the amount of HDAC2 co-immunoprecipitated for WT HDAC1-HA-Flag was set as 1. For the HDAC1 constructs the amount of HDAC2 co-immunoprecipitated relative to WT HDAC1 is: WT:1 D181A:1.003, 6K-R :0.9334, 6K-Q: 0.9677. C) Results of the colorimetric HDAC1 deacetylase assay using the Fleur De Lys HDAC Colorimetric Assay/Drug Discovery Kit*. The deacetylase activity of the mutant HDAC1s is represented relative to the activity of WT HDAC1 in the absence of dex (n=3 ± SEM). A student t-test was performed, where *p<0.05, **p<0.01.



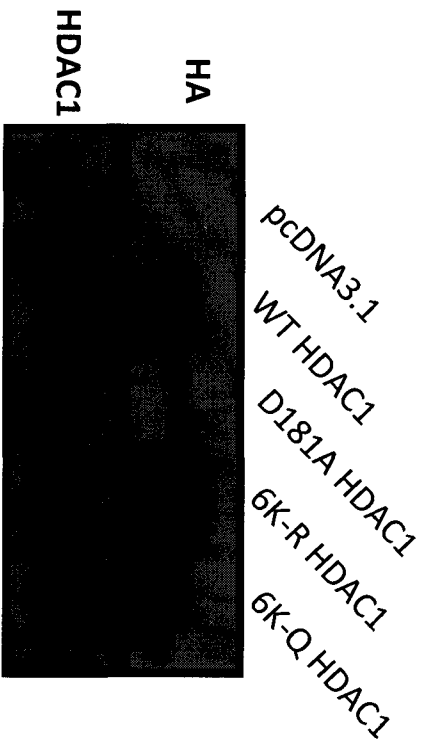
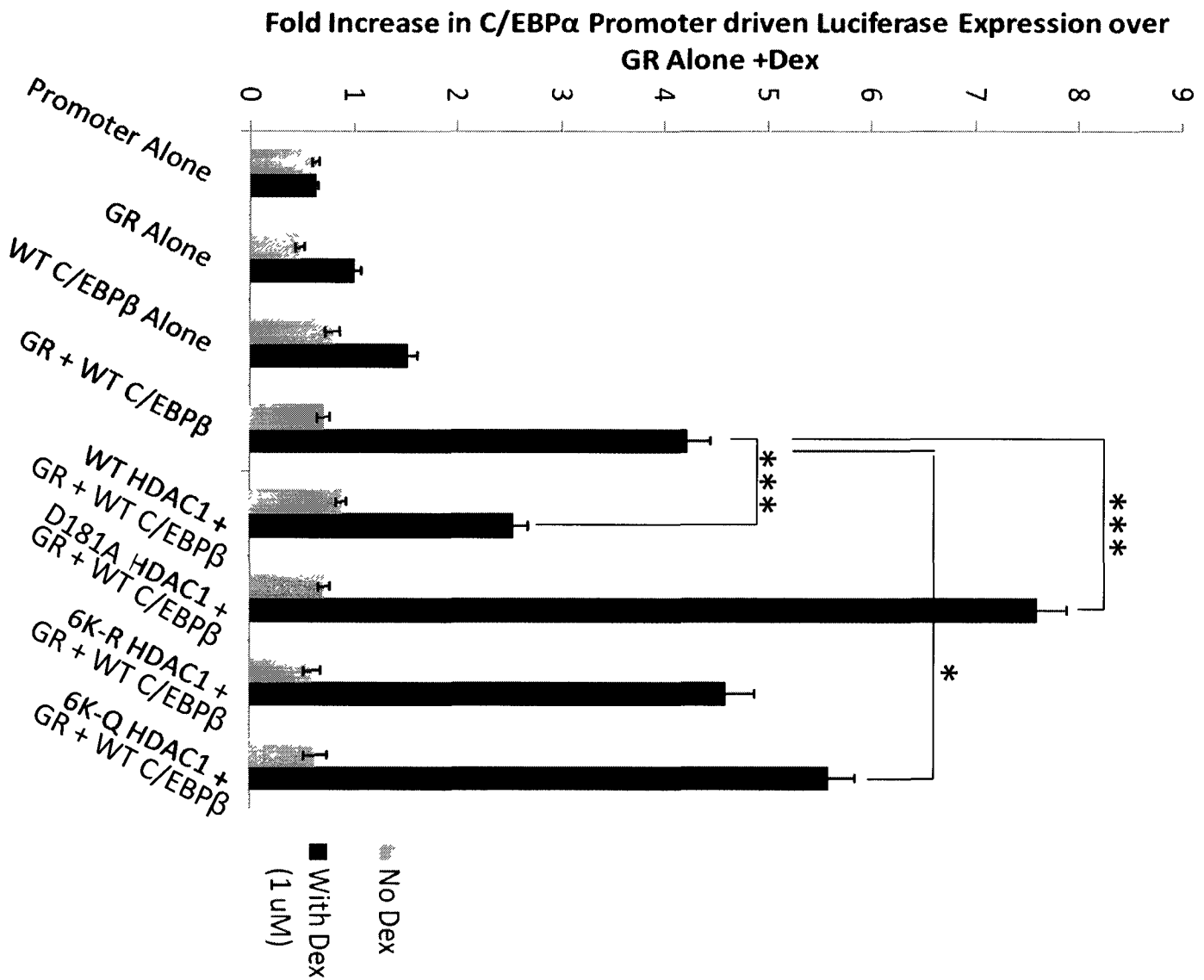
The results for the 6K-Q HDAC1 mutant do not coincide with the predicted outcome based on the results of Qiu, *et al.*, (2006), as 6K-Q HDAC1 clearly exhibits deacetylase activity in the colorimetric deacetylase activity assay (Figure 13C), while Qiu *et al.* (2006) published that the 6K-Q HDAC1 is catalytically inactive. On the other hand, the colorimetric deacetylase assay results for the 6K-R HDAC1 mutant do correlate with the published results stating that it is an enzymatically active HDAC1 mutant (Qiu *et al.*, 2006), as in Figure 13C 6K-R HDAC1 clearly demonstrates significantly more deacetylase activity than WT HDAC1.

Neither the 6K-R nor the 6K-Q HDAC1 mutant significantly affects GR-mediated upregulation of transcription at the C/EBP α promoter

To investigate the phenotypes of the 6K-R and 6K-Q HDAC1 mutants a transcription assay was performed in NIH 3T3 cells, as described previously, and a western blot analysis of the HA tag was also performed to confirm the expression of the HDAC1-HA-Flag mutants (Figure 14A). The results of the dual luciferase transcription assay revealed the predicted increase in C/EBP α promoter-driven luciferase expression upon treatment of the cells expressing WT C/EBP β and GR with dex (14B). The predicted decrease in the dex effect upon ectopic expression of WT HDAC1, and the expected two fold dex effect observed upon ectopic expression of the D181A catalytically compromised HDAC1 mutant, were also observed (Figure 14B), which suggests that the C/EBP α promoter-reporter assay was working as expected. However, neither the 6K-R nor the 6K-Q HDAC1 mutants produced the predicted results. The 6K-R HDAC1 mutant is declared by Qiu, *et al.*, (2006) to be constitutively active and the results of the colorimetric deacetylase assay support this statement (Figure 13C). However, WT HDAC1 has been shown in previous studies by our laboratory and by another group (Wiper-Bergeron, 2003, Yoo *et al.*, 2006) to play an

Figure 14: The 6K-R HDAC1 mutant does not affect the ability of GR to induce upregulation of C/EBP α promoter-driven luciferase expression, while the 6K-Q mutant causes a slight increase in GR-mediated C/EBP α promoter-driven transcription.

A) Immunoblot HA demonstrating the expression of 150ng of WT, D181A, 6K-R or 6K-Q HDAC-HA-F1ag in NIH 3T3 cells. B) A C/EBP α promoter-driven transcription assay was performed in NIH 3T3 cells, as previously described, to assess the 6K-R and 6K-Q HDAC1 ability to promote GR dependent C/EBP α promoter activity (n=3X triplicates \pm SEM). An ANOVA test, followed by a Bonferonni test were performed to determine the statistical significance of the results *p<0.05 and ***p<0.001.

A**B**

inhibitory role in corticosteroid potentiated *C/ebpa* transcription and preadipocyte differentiation. The results of section one confirm the importance of HDAC1 and of its deacetylase activity in the modulation of corticosteroid mediated induction of the adipogenic transcriptional cascade. Therefore, based on what is understood about the inhibitory role of HDAC1 at the *C/EBPα* promoter we would expect the 6K-R catalytically enhanced HDAC1 mutant to inhibit dex-potentiated *C/ebpa* transcription more efficiently, or at the very least, to the same extent as WT HDAC1. However, expression of 6K-R HDAC1 has no observable effect on dex-induced *C/EBPα* promoter-driven luciferase expression, as seen in Figure 14B. On the other hand, we would expect the 6K-Q mutant HDAC1 to produce a similar phenotype to the D181A mutant HDAC1, if it is indeed inactive as published (Qiu, *et al.*, 2006), or for it to suppress dex-induced *C/EBPα* promoter-driven luciferase expression to the same extent as WT HDAC1, if it is active as shown in the colorimetric deacetylase assay (Figure 13B). Unexpectedly, neither of these phenotypes is produced upon ectopic expression of the 6K-Q mutant in the NIH 3T3 cells; only a moderate 20% increase in the dex effect is observed, when compared to the condition with *C/EBPβ* + GR+ dex condition (Figure 14B).

The 6K-R and 6K-Q mutants cannot rescue the ability of dex to upregulate *C/ebpa* transcription in the presence of the transcriptionally inactive K98-102R *C/EBPβ* mutant

In the hopes of revealing more about the 6K-R and the 6K-Q HDAC1 acetylation mutants and their phenotypes we tested the effect of co-expression of the transcriptionally compromised K98-102R *C/EBPβ* mutant with 6K-R or 6K-Q HDAC1, on transcriptional activation of *C/EBPα* promoter-driven *Firefly* luciferase expression.

In the presence of WT C/EBP β all HDAC1 constructs behaved as shown in figure 14. However, when the various conditions were repeated in the presence of the K98-102R C/EBP β acetylation mutant, the ability of GR to induce C/EBP α promoter-driven luciferase expression was decreased by 70% relative to the corresponding WT C/EBP β condition (Figure 15).

Furthermore, co-transfection of neither WT, 6K-R nor 6K-Q HDAC1 restored the ability of dexamethasone to induce luciferase expression in the presence of the K98-102 C/EBP β mutant. However, as observed in Figure 10, when the D181A HDAC1 mutant was expressed alongside the K98-102R C/EBP β mutant the capacity of steroid treatment was restored to the level of the dex effect (Figure 15).

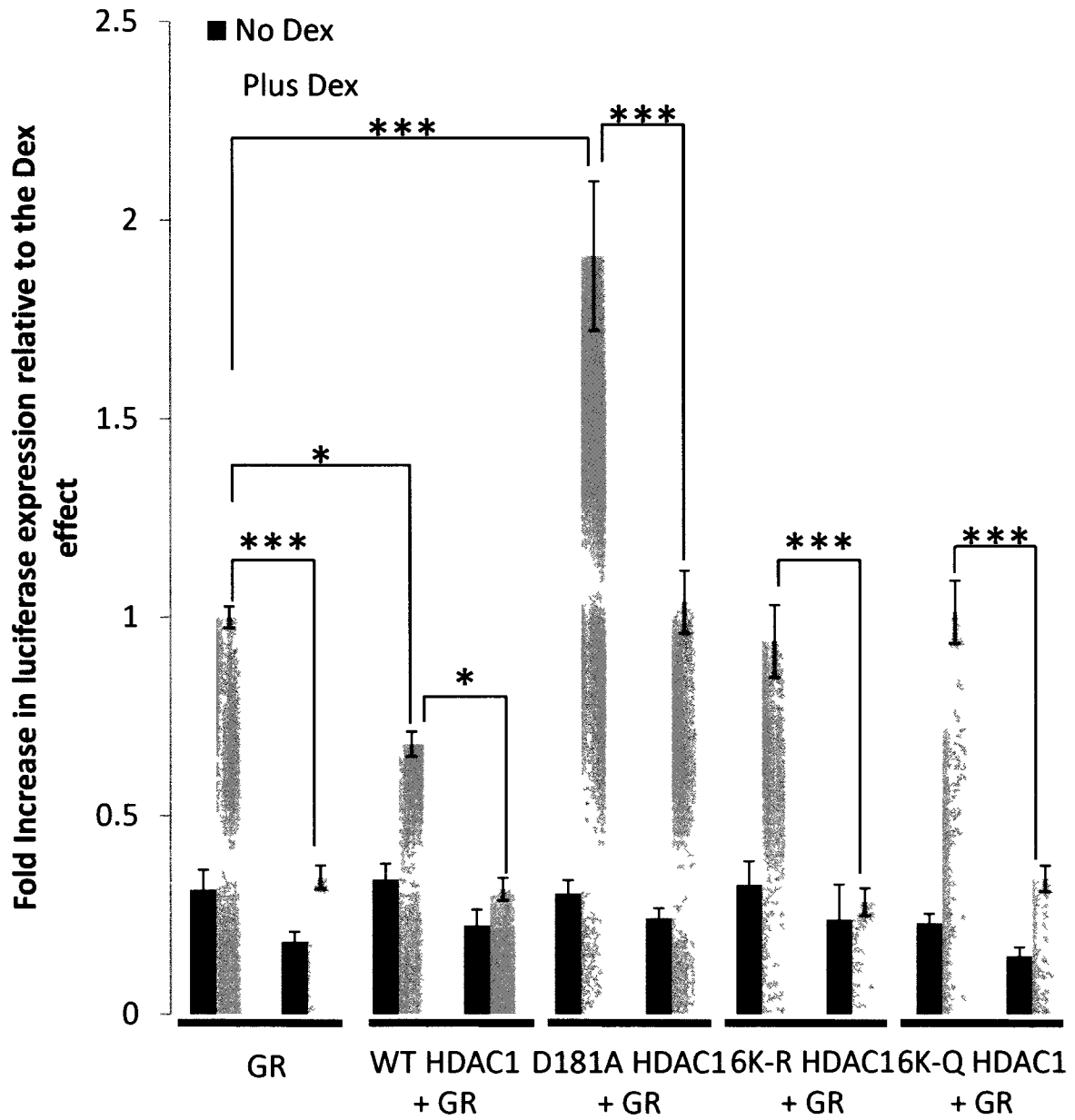
These results do not correlate with the expected phenotype of the 6K-Q HDAC1, as an inactive HDAC1 mutant would be expected to produce results similar to those of the D181A catalytically compromised HDAC1 mutant, while a constitutively active, such as the 6K-R HDAC1, would be expected to further decrease the ability of steroid treatment to induce luciferase expression. However, in this experiment both the 6K-R and the 6K-Q HDAC1 constructs produced the same phenotype as WT HDAC1, upon co-expression with the K98-102R C/EBP β mutant.

Both the 6K-R and the 6K-Q HDAC1 mutants enhance the ability of dexamethasone to potentiate preadipocyte differentiation

In order to determine whether the results of the C/EBP α promoter-driven reporter assays are relevant to adipocyte differentiation, the effects of the 6K-R and 6K-Q HDAC1 on 3T3 L1 murine preadipocyte differentiation were investigated. 3T3 L1 murine preadipocyte cells were virally transduced to express the different HDAC1 constructs and were

Figure 15: The 6K-R and 6K-Q HDAC1 mutants cannot rescue the ability of GR to upregulate C/EBP α promoter-driven transcription in the presence of the transcriptionally compromised K98-102R C/EBP β mutant.

A C/EBP α promoter-driven transcription assay was performed in NIH 3T3 cells, as previously described, to assess the capacity of 6K-R and 6K-Q HDAC1 to rescue the ability of the transcriptionally compromised K98-102R C/EBP β mutant to enhance C/EBP α promoter-driven luciferase expression. (n=3X triplicates \pm SEM). The p values were calculated by performing an ANOVA on the results, followed by a Dunnett test to calculate the p-values between the Dex effect and the samples with WT or one of the mutant HDAC1s+GR+WT C/EBP β , and a Bonferroni test to calculate the p-values between each WT C/EBP β and K98-102R C/EBP β condition. *p<0.05, ***p<0.001



WT C/EBPβ	+	-	+	-	+	-	+	-	+	-
K98-102R C/EBPβ	-	+	-	+	-	+	-	+	-	+

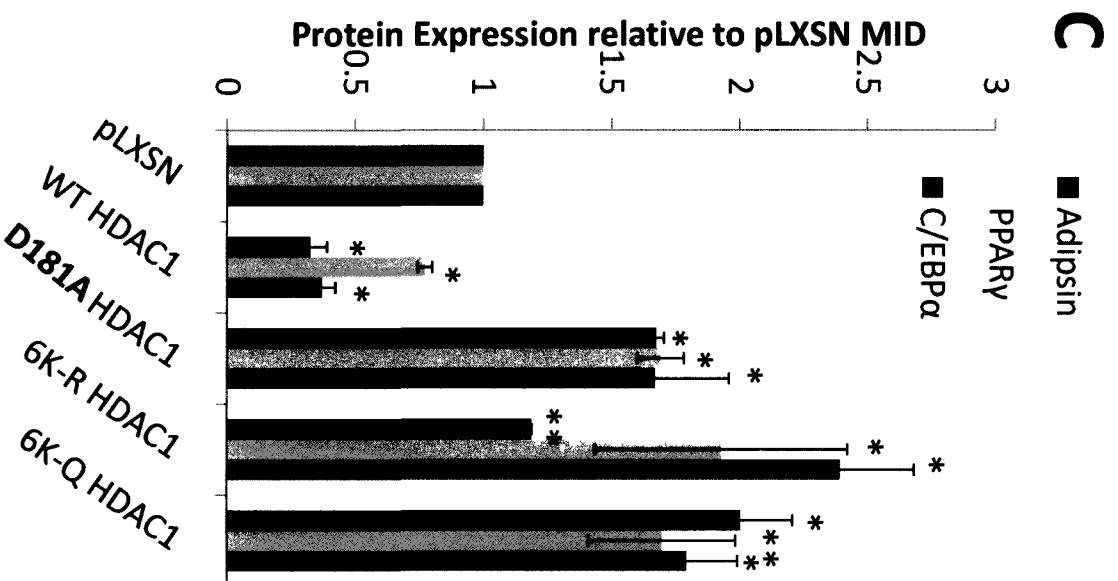
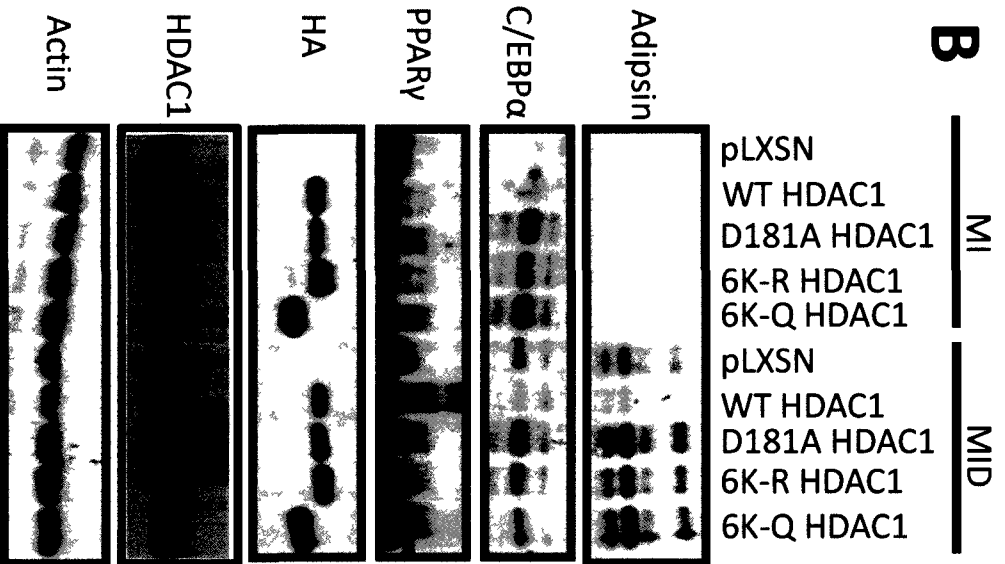
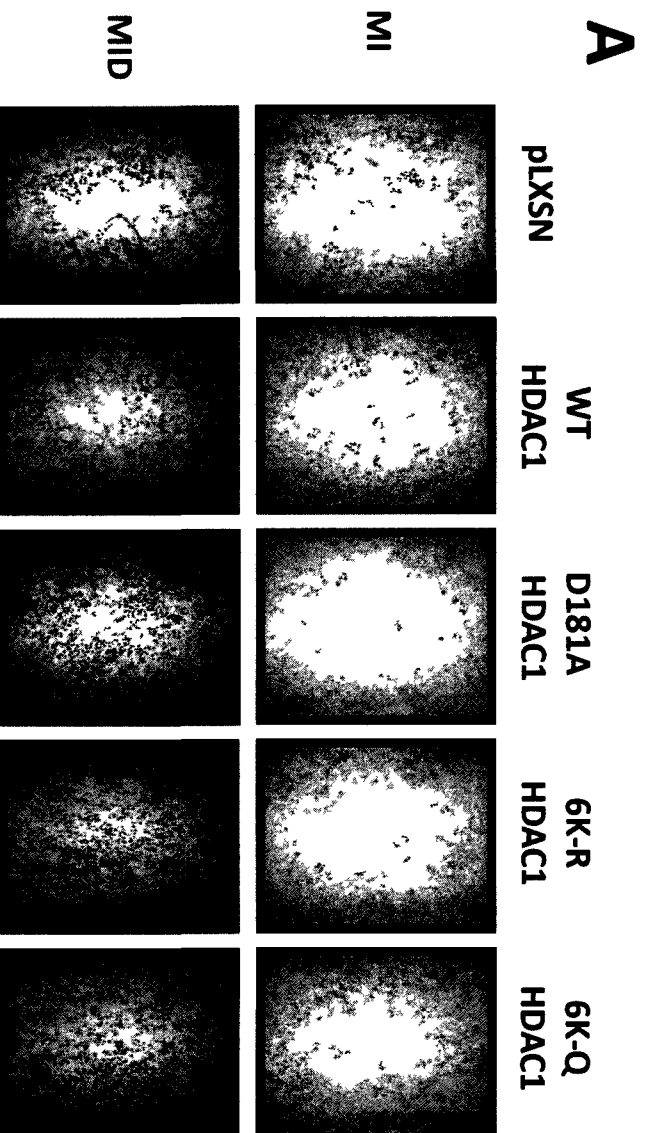
subsequently differentiated, as previously described. On Day 7 of differentiation, the cells were harvested and the efficiency of differentiation in the 3T3 L1 cells expressing the different HDAC1 constructs was determined by Oil red O staining of mature adipocytes and western blot analysis of the adipocyte markers; adipsin, C/EBP α and PPAR γ .

Visual examination of the Oil Red O staining revealed that for all of the conditions very little differentiation was observed with the MI treatment alone (Figure 16A), however an increase in Oil red O staining was observed in the pLXSN 3T3 L1 control cells treated with MID (Figure 16A). Expression of WT HDAC1 in 3T3 L1 preadipocytes significantly inhibited the ability of GR to potentiate the differentiation of 3T3 L1 preadipocytes into mature adipocytes, as revealed by the decreased Oil red O staining in the WT HDAC1 cells compared to the pLXSN control cells (Figure 16A). Conversely, 3T3 L1 cells virally transduced to express D181A, 6K-R or 6K-Q HDAC1 mutants all demonstrated a considerable increase in Oil red O staining, compared to MID treated control cells (Figure 16A).

The results of the Oil red O staining were validated by western blot analysis of the adipocyte markers adipsin, C/EBP α and PPAR γ (Figure 16B). The immunoblots were quantified using densitometry, normalized to actin expression and are expressed in graph format in Figure 16C. Immunoblot analysis of adipsin, a late marker for adipocytes, confirmed the Oil red O results, as no adipsin is visible in the MI samples, indicating that minimal differentiation occurred. On the contrary, a higher amount of adipsin is visible in the pLXSN MID lane (Figure 16B). The level of adipsin expression is reduced by approximately 60% (Figure 16B, C) upon expression of WT HDAC1, while 3T3 L1 cells

Figure 16: Both the 6K-R and the 6K-Q HDAC1 mutants enhance the ability of Dex to potentiate preadipocyte differentiation.

A) Oil Red O staining of the neutral lipids of mature adipocytes on day 8 of differentiation. 3T3 L1 preadipocytes, stably expressing the indicated pLXSN constructs were induced to differentiate into mature adipocytes via treatment with either MI or MID (n=3). B) Western blot analysis of the adipocyte markers adiponin, C/EBP α and PPAR γ , on Day 8 of 3T3 L1 differentiation are shown (n=3). C) Quantification of the protein expression levels of the adipocyte markers immunoblotted in B normalized to actin are shown (n=3 \pm SEM). Only the expression levels of the adipocyte markers for samples treated with MID are shown. The p-values were calculated by performing a student t-test between the indicated sample and pLXSN MID, where *p<0.05 and **p<0.01.



expressing D181A HDAC1, 6K-R HDAC1, or 6K-Q HDAC1 exhibit increased adipsin levels of 1.7, 1.2 and 2 fold over the pLXSN control, respectively (Figure 16C).

Furthermore, the trends observed in C/EBP α and PPAR γ protein expression in the hormone treated 3T3 L1 cells follow the same trends observed for adipsin. Significant expression of both adipocyte markers was observed in the pLXSN MID 3T3 L1s (Figure 16B), while 3T3 L1 cells retrovirally transduced to express WT HDAC1 exhibit reduced protein expression levels of 0.6 for C/EBP α and 0.2 for PPAR γ relative to the pLXSN control cells (Figure 16B and C). On the contrary, the protein expression levels of both C/EBP α and PPAR γ are upregulated in 3T3 L1s expressing D181A by 1.7 fold. Similarly, cells expressing 6K-R or 6K-Q HDAC1 showed a 2 fold and a 1.7 fold increase of both markers, respectively, relative to the control cells (Figure 16 B and C). Interestingly, even though minimal differentiation was observed in all of the MI conditions, expression of C/EBP α and PPAR γ was detected in the western blots of the 3T3 L1 cells expressing the D181A, 6K-R and 6K-Q HDAC1 mutants.

Together, the results of the Oil red O staining and the western blot analysis of the adipocyte markers; adipsin, C/EBP α and PPAR γ demonstrate that both the 6K-R and 6K-Q HDAC1 mutants enhanced of the ability of dex to potentiate preadipocyte differentiation. These results conflict with the results reported in the first section of this thesis in which we have shown that HDAC1 inhibits C/EBP α transcription and preadipocyte differentiation through a mechanism that requires its deacetylase activity. Therefore, to observe the ability of the 6K-R HDAC1 mutant which exhibited 2.3-3 times more catalytic activity than WT HDAC1 in the *in-vitro* deacetylase activity assay, to enhance the ability of dex to upregulate preadipocyte differentiation, is puzzling. Furthermore, although ectopic expression of the

6K-Q HDAC1 would be expected to enhance dex-potentiated preadipocyte differentiation based on the published catalytically inactive phenotype of the mutant (Qiu, *et al.*, 2006), this result is contradictory to the phenotypes produced by the 6K-Q HDAC1 mutant in the other experiments within this particular investigation.

Mutation of HDAC1's lysine residues 218, 220, 432, 4328, 439 and 441 to arginine or glutamine does not affect the localization of the HDAC1 proteins to the nucleus

All of the experiments performed by Qiu, *et al* (2006) to assess the activity of the 6K-R and 6K-Q HDAC1 mutants were performed in *in-vitro* systems, whereas our experiments were conducted in cellular systems. One possibility that could account for the unexpected results produced by the 6K-R and the 6K-Q HDAC1 mutants in our experiments is that the mutations result in an alteration in the subcellular localization of the protein. Therefore, in order to verify that the phenotypes produced by the 6K-R and 6K-Q HDAC1 mutants in this investigation are due to alterations in their function as deacetylase enzymes and not to mislocalization of the protein to somewhere outside the nucleus, immunofluorescence was used to perform a localization study. Verifying the localization of the 6K-R and 6K-Q HDAC1 mutants is of particular importance as the nuclear localization sequence (NLS) of mouse HDAC1 has been mapped to residues 438-482, with the core motif including amino acids 438-445, and the mouse HDAC1 aligns perfectly with the human HDAC1 construct in this region (De-Ruijeter *et al*, 2002 and Taplick *et al*, 2001). Therefore, it is possible that the mutations at lysine residues 438,439 and 441 disrupt the nuclear localization of the 6K-R and 6K-Q HDAC1 mutants, rather than affect their deacetylase activity.

The 3T3 L1 cells expressing only Flag-pLXSN demonstrated diffuse cytoplasmic staining for the Flag-protein and localized fluorescence for HDAC1 in the nucleus, which overlapped with the DAPI stain, confirming that endogenous HDAC1 is localized to the nucleus and that this localization is unaffected by corticosteroid treatment (Figure 17). For the virally transduced HDAC1-HA-Flag constructs(WT, 6K-R and 6K-Q) the Flag-immunofluorescence displayed clear localization of the Flag-tagged, virally transduced HDAC1 proteins to the nucleus and confirmed that, similar to the endogenous HDAC1 protein, the nuclear localization of the virally transduced Flag-HDAC1 constructs is unaffected by treatment of the cells with dex (Figure 17).

The results of the localization study confirm that mutation of lysine residues 218, 220, 432, 438, 439 and 441 to arginine or glutamine does not interfere with proper nuclear localization of the protein.

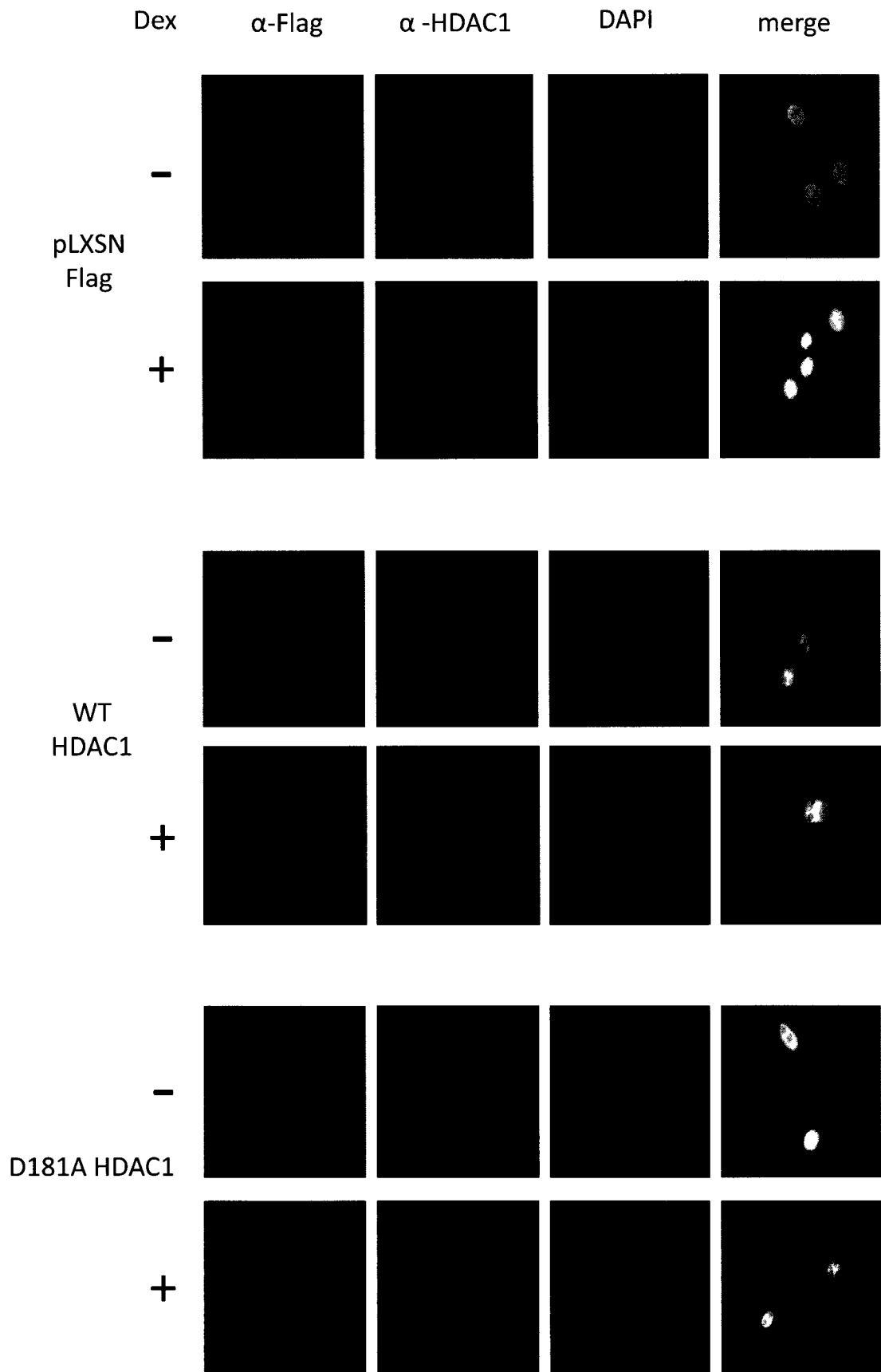
6K-R and 6K-Q substitutions do not grossly affect dex-induced HDAC1 degradation

Next we proceeded to determine if mutation of HDAC1 at lysine residues 218,220, 432, 438, 439 and 441 affects dex-induced targeting of HDAC1 to the 26S proteasome for degradation.

Earlier studies in our laboratory revealed that within 24hrs of steroid treatment the HDAC1 co-repressor complex is titrated from the C/EBP α promoter and is targeted for degradation by the 26S proteasome via ubiquitylation of HDAC1 by the E3 ligase TIF1 β (Wiper-Bergeron, *et al.*, 2003 and Tomlinson., J., unpublished results). Importantly, because degradation of HDAC1 does not occur at detectable levels prior to 24hrs following initiation of differentiation, the HDAC1 constructs that were immunoprecipitated in our

Figure 17: Mutation of HDAC1's lysine residues 218, 220, 432, 438, 439 and 441 to arginine or glutamine does not affect the localization of the Flag-tagged HDAC1 proteins to the nucleus.

3T3 L1 cells stably expressing pLXSN or one of the pLXSN-HDAC1-HA-Flag constructs (WT, 6K-R or 6K-Q) were plated onto coverslips and treated for 24hrs with 1uM Dex or ethanol. Immunofluorescence was then performed on the cells for the Flag tag (green), HDAC1 (Red) and DAPI (to stain the nucleus blue). Two independent experiments were performed, but representative pictures from a single experiment are shown.



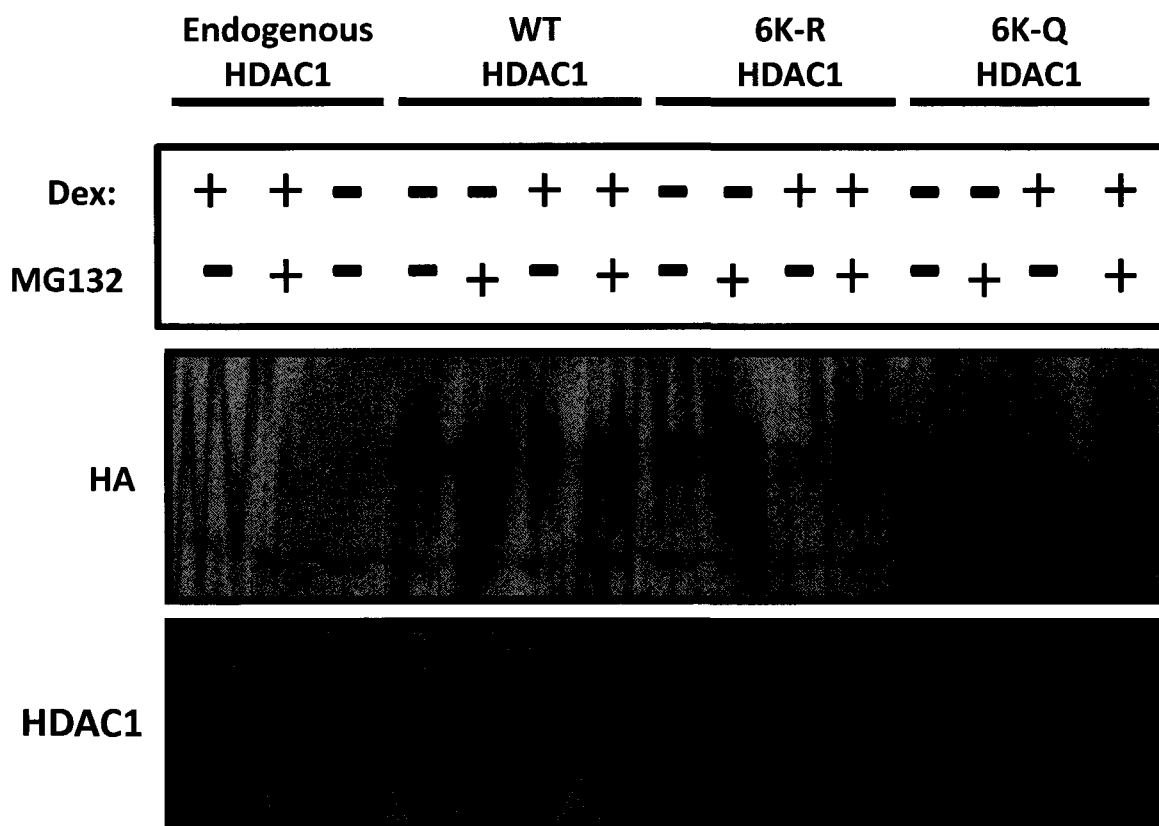
deacetylation assays and co-immunoprecipitation experiments were not compromised by this phenomenon, as they were only incubated with Dex for 4hrs.

We were interested in assessing whether or not the mutations inserted into HDAC1 to create the 6K-R and 6K-Q HDAC1 mutants had any effect on the ability of dex to target the mutant HDAC1 proteins for 26S proteasomal degradation. We were particularly interested in verifying proper dex-induced degradation of the 6K-R and 6K-Q HDAC1 mutants since recent studies have identified a role for acetylation in regulating the ability of a protein to be ubiquitylated and degraded (Jeong, *et al.*, 2002, Grönroos, *et al.* 2002, Giandomenico, *et al.*, 2003, and , *et al.*, 2004).

To do so, Cos-7 cells virally transduced to express pLXSN, WT HDAC1-HA-Flag, 6K-R HDAC1-HA-Flag or 6K-Q HDAC1-HA Flag were transiently transfected with GR and WT C/EBP β . The cells were treated for 20hrs with ethanol (vehicle), ethanol and 1 μ M of the 26S proteasome inhibitor MG132, 100nm dex, or 100nm dex and 1 μ M MG132. Following the treatment, the expression levels of endogenous and ectopically expressed HDAC1-HA Flag proteins were assessed by western blot analysis of HA and HDAC1. Treatment of cells with dex has been shown to decrease the protein levels of both endogenous and ectopically expressed HDAC1 compared to cells treated with the vehicle. Moreover, treatment of the cells with MG132 concomitantly with dex treatment has been shown to inhibit this decrease in HDAC1 expression (Wiper-Bergeron, *et al.*, 2003). Identical results were reproduced, as treatment of Cos-7 cells with dex resulted in a noticeable decrease in endogenous and ectopically expressed WT HDAC1 protein levels, as compared to the vehicle (ethanol) treated cells (Figure 18). More importantly, simultaneous treatment of the cells with MG132 and dex blocked the degradation of both endogenous and transfected WT HDAC1 (Figure 18). Assessment of the ability of dex to induce degradation

Figure 18: 6K-R and 6K-Q substitutions do not grossly affect dex-induced HDAC1 degradation.

Cos-7 cells were transiently transfected with 2ug of Flag-pcDNA3.1 or one of the HDAC1-HA-Flag constructs (WT, 6K-R or 6K-Q) and 1ug of GR. One day later the transfected cells were treated for 20hrs with the indicated combination of 1μM MG132 and/or 100nM dex or the equivalent volume of ethanol. The cells were subsequently harvested and Western blot for HA was performed to examine the degradation state of the HDAC1-HA-Flag constructs following hormone treatment. Western blot analysis was also performed for HDAC1 to examine the expression of both the endogenous and transiently transfected HDAC1 forms under the various treatment conditions. Two independent experiments were performed, although the pictures from a single representative experiment are presented.



of the transiently transfected 6K-R and 6K-Q HDAC1 mutants by western blot analysis of the HA tag, demonstrated that dex treatment leads to decreased expression of 6K-R and 6K-Q HDAC1 (Figure 18). Furthermore, the dex-induced degradation of the 6K-R and 6K-Q HDAC1 mutants is inhibited by treatment of the cells with MG132 in addition to dex (Figure 18). Therefore, the 6K-R and 6K-Q HDAC1 mutants are degraded by the 26S proteasome in the same manner as WT and endogenous HDAC1 and no particular construct appears to be degraded more efficiently than another.

These results suggest that the acetylation status of HDAC1 at lysine residues 218, 220, 432, 438, 439 and 441 are not involved in dex-induced targeting of HDAC1 for proteasomal degradation, or in the regulation of HDAC1 stability, although further investigation is needed to quantify the amount and rate of WT and mutant HDAC1 proteasomal degradation.

Mutation of lysine residues 218, 220, 432, 438, 439 and 441 on HDAC1 to arginine or glutamine affects the ability of HDAC1 to interact with other co-factors known to be involved in *C/ebpα* transcriptional regulation

Finally, we proceeded to investigate if the 6K-R or 6K-Q mutations in HDAC1 would affect its ability to associate with recognized HDAC1 interactors and co-regulators of *C/ebpα* transcription. Cos-7 cells were transiently transfected with GR, C/EBP β and one of the HDAC1 constructs, treated for 4hrs with 100nm dex, or the equivalent dilution of ethanol and were then harvested for lysis. The HDAC1-HA-Flag constructs were immunoprecipitated from the whole cell lysates and western blot analysis was performed for GR and the adaptor protein mSin3A in order to examine whether mutant HDAC1 variants were compromised in their ability to bind these known HDAC1 interactors and C/EBP α transcriptional regulators.

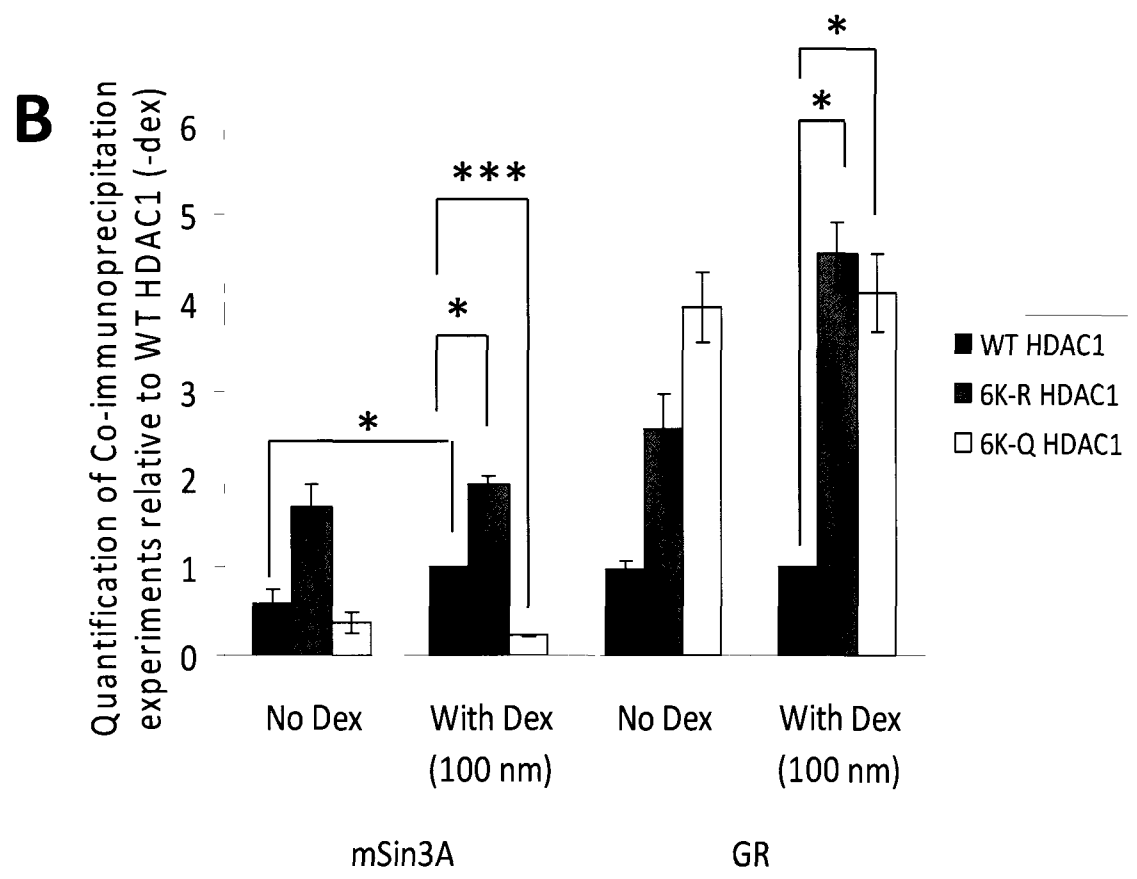
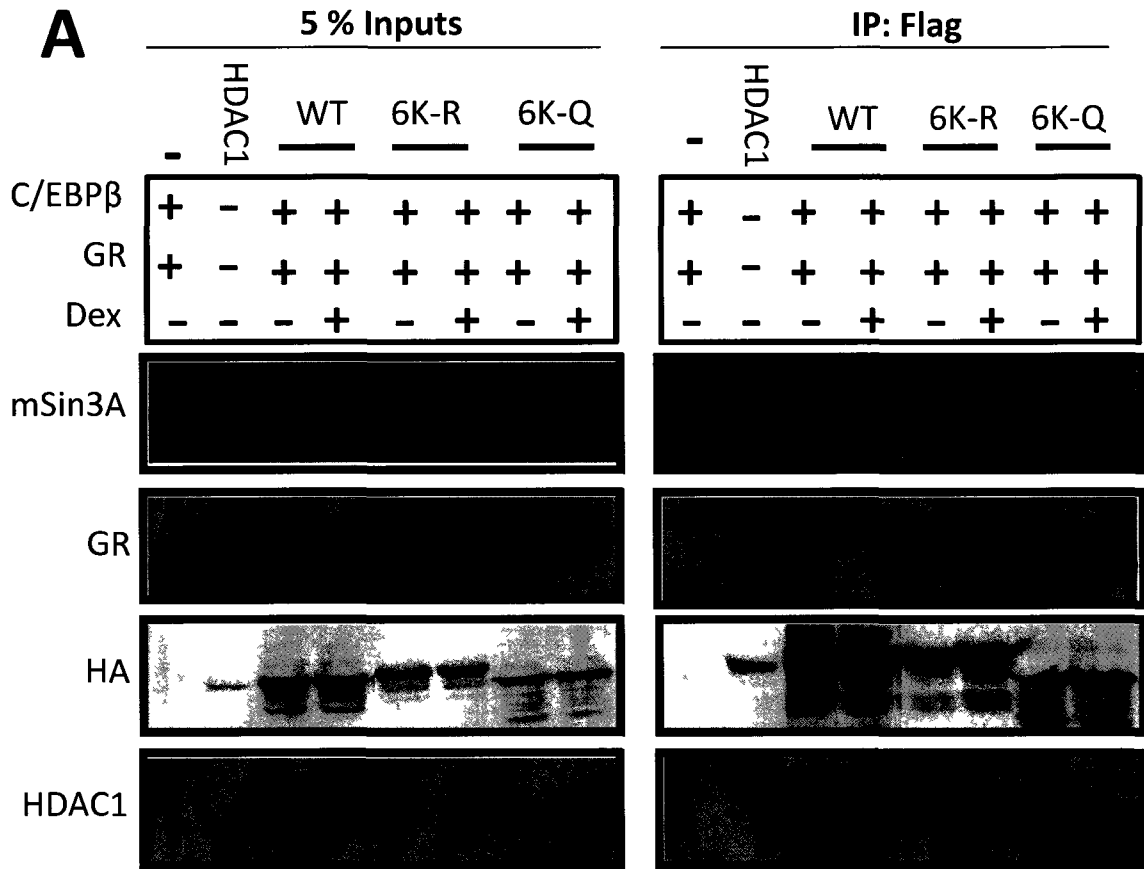
Consistent with the results in Figure 10, the co-repressor mSin3A was successfully co-immunoprecipitated with WT HDAC1 and treatment with steroid for 4hrs, which is before significant degradation of HDAC1 is observed, increased the efficiency of this interaction by 2 fold (Figure 19A and B)) (Wiper-Bergeron *et al*, 2003). Western blot analysis of mSin3A also revealed that the 6K-R HDAC1 mutant interacts two fold more efficiently with the co-repressor than WT HDAC1 does, while the ability of the 6K-Q HDAC1 mutant to associate with mSin3A appears to be compromised, as it only binds about 30% of the amount of mSin3A compared to WT HDAC1 (Figure 19A and B). Furthermore, neither the interaction between 6K-R nor 6K-Q HDAC1 with mSin3A is affected by treatment with dex (Figure 19A and B).

Western blot analysis of GR demonstrated that the receptor is efficiently co-immunoprecipitated with WT HDAC1, and that this interaction is unaffected by dexamethasone treatment (Figure 19 A and B). Following 4hrs of steroid treatment both the 6K-R and the 6K-Q HDAC1 mutants interact approximately 4 fold more efficiently with GR than WT HDAC1. (Figure 19A and B). While steroid treatment does not affect the ability of the 6K-Q HDAC1 mutant to interact with the steroid receptor, dex treatment does increase the efficiency of the 6K-R HDAC1-GR interaction (Figure 19A and B).

In summary, while mutation of lysine residues 218, 220, 432, 438, 439 and 441 to arginine increased the efficiency of HDAC1 to interact with mSin3A, mutation of the same residues to glutamine significantly interrupts the ability of HDAC1 to interact with its co-repressor. However, mutation of the six lysine residues to arginine or glutamine increased the efficiency of the HDAC1 mutants interaction with GR (Figure 19B).

Figure 19: Mutation of lysine residues 218, 220, 432, 438, 439 and 441 on HDAC1 to arginine or glutamine affects the ability of HDAC1 to interact with co-regulators of *C/ebpa* transcription

A) Cos-7 cells were transiently transfected with the HDAC1-HA-Flag constructs (WT, 6K-R or 6K-Q), in addition to GR. Two days later the transfected cells were treated for 4hrs with 100nm of dex or the equivalent volume of ethanol. The HDAC1-HA-Flag constructs (WT, 6K-R or 6K-Q) were subsequently immunoprecipitated using Flag-affinity beads and the ability of the WT and mutant HDAC1s to interact with other transcription factors known to be involved in the regulation of *C/ebpa* transcription, such as GR and mSin3A, was assessed by western blot analysis (n=3). The blots shown were modified so that the Co-immunoprecipitation results for the D181A HDAC1 mutant could be removed and allow for a better comparison between WT HDAC1 and the 6K-R or 6K-Q HDAC1 mutant. B) Quantification of the amount of GR and mSin3A co-immunoprecipitated with WT, 6K-R or 6K-Q HDAC1. The amount of GR or mSin3A that was co-immunoprecipitated with the various HDAC1-HA-Flag constructs was quantified by performing densitometry with Image J. The densitometry results were normalized to the amount of HDAC1-HA-Flag with which the co-factor was co-immunoprecipitated (n=3 ± SEM). P-values were calculated by performing a student t-test between the results for WT HDAC1 and the results of the indicated HDAC1 mutant. *p < 0.05, **p<0.01.



Given the fact that both the 6K-R and 6K-Q HDAC1 mutants interact more efficiently with GR it is possible that the 6K-R and 6K-Q mutants are titrated away from the C/EBP α promoter by GR at a faster rate than WT HDAC1. However, additional experimentation including both a time course chromatin histone immunoprecipitation (ChIP) experiment and a time course examining the rate of degradation of the WT and mutant HDAC1s needs to be performed to validate this theory.

Discussion

The first section of this thesis confirmed that HDAC1 plays an important role in moderating the intensity with which GR upregulates *C/ebpa* transcription and demonstrated that this suppressive role is dependent on the deacetylase activity of the enzyme. However, we were also interested in understanding the molecular details of how GR promotes the titration of the mSin3A/HDAC1 co-repressor complex from the *C/ebpa* promoter, and targets HDAC1 for proteasomal degradation (Wiper-Bergeron *et al.*, 2003).

A paper by Qiu, *et al.*, published in 2006, revealed that during corticosteroid induced upregulation of transcription at the MMTV promoter, GR is associated with HDAC1 and that glucocorticoid treatment leads to progressive, p300 mediated acetylation of GR-associated HDAC1 at lysine residues 218,220,432,438,439, and 441. Furthermore, they showed that acetylation of HDAC1 at these six lysine residues inactivates the deacetylase activity of the enzyme and they proceeded to generate two HDAC1 mutants with opposing deacetylase activities; one mutant which mimics the acetylated protein, 6K-Q HDAC1, and was shown to be catalytically inactive, and another mutant which cannot be acetylated, 6K-R HDAC1, and was demonstrated to be constitutively active (Qiu, *et al.*, 2006).

We planned to investigate the phenotypes produced by the 6K-R and 6K-Q HDAC1 mutants in an adipogenic system for two major reasons. The first was to confirm that the deacetylase activity of HDAC1 is important to its regulatory role at the *C/EBPα* promoter by using the HDAC1 acetylation mutants with their opposing acetylation activity. Our second motive was to use the 6K-R and 6K-Q acetylation mutants to examine whether GR altered the activity and/or targeted *C/EBPβ* associated HDAC1 for proteasomal degradation by

regulating the acetylation status of the six lysine residues identified by Qiu *et al.*, (2006). Acetylation of proteins such as Smad7, p21 and HIF-1 α has been shown to promote the targeting of the protein for 26S proteasomal degradation, in some cases, and obstruct targeting of the protein for degradation, in other situations (Chen *et al.*, 2004, Giandomenico *et al.*, 2003, Gronroos *et al.*, 2002 and Jeong *et al.*, 2002). Therefore, the acetylation status of HDAC1 could play a role in regulating the stability of the protein during GR-mediated initiation of adipogenesis.

The phenotypes produced by the 6K-R and 6K-Q HDAC1 acetylation mutants in the adipogenic system suggest a role for post-translational acetylation of HDAC1 in the regulation of the substrate specificity rather than the catalytic activity of the enzyme

Our initial plan was to begin experimentation with the 6K-R and 6K-Q HDAC1 mutants by ectopically expressing the acetylation mutants in C/EBP α promoter-driven *Firefly* luciferase reporter assays and in differentiating 3T3 L1 preadipocytes. Since we established in Chapter 1, with the catalytically compromised D181A HDAC1 mutant, that the deacetylase activity of HDAC1 is essential to its ability to moderate GR-potentiated *C/ebpa* transcription we believed that expression of the constitutively active (6K-R) and inactive (6K-Q) HDAC1 mutants in these systems would allow us to 1) confirm the importance of HDAC1s deacetylase activity in GR-potentiated adipogenesis using different HDAC1 mutants, and 2) would provide some insight into whether alterations in the acetylation status of lysine residues 218, 220, 432, 438, 439 and 441 play a role in regulating HDAC1s deacetylase activity or stability during adipogenesis.

However, after the 6K-R HDAC1 and the 6K-Q HDAC1 mutants were generated, we performed a commercially available colorimetric deacetylase assay to verify that the

deacetylase activities of the mutants correlated with their published phenotypes.

Unexpectedly, the 6K-Q HDAC1 mutant, which is published as being the inactive mutant, exhibited slightly more deacetylase activity than the WT HDAC1, while the 6K-R HDAC1 mutant was significantly more catalytically active than the WT HDAC1 (Figure 13) (Qiu *et al.*, 2006).

Even though the activity of the 6K-Q HDAC1 mutant did not correlate with its published phenotype (Qiu *et al.*, 2006) we continued on to examine the phenotypes produced by ectopically expressing either the 6K-Q or the 6K-R HDAC1 mutant in a C/EBP α promoter-driven reporter assay and in differentiating 3T3 L1 preadipocytes. Neither the 6K-R nor the 6K-Q HDAC1 mutant caused detectable alterations in the ability of dexamethasone to potentiate C/EBP α promoter-driven luciferase expression (Figure 14), while viral transduction of either of the HDAC1 acetylation mutants enhanced the ability of GR to upregulate 3T3 L1 preadipocyte differentiation (Figure 16). These results contradict both the published phenotypes of the 6K-R and the 6K-Q HDAC1 mutants, which are supposed to be constitutively active and inactive, respectively, and our understanding that HDAC1 plays a suppressive, deacetylase activity dependent role in GR-potentiated adipogenesis.

If the 6K-R and 6K-Q had opposing catalytic activities, as described by Qiu *et al.* (2006), then we would expect the constitutively active 6K-R HDAC1 to inhibit GR-potentiated C/ebp α transcription and preadipocyte differentiation to at least the same extent, if not more effectively, than WT HDAC1. On the other hand, we would expect the inactive 6K-Q HDAC1 mutant to either have no effect or to enhance the ability of corticosteroid treatment to upregulate C/ebp α transcription and 3T3 L1 differentiation, similar to the phenotypes produced with the D181A HDAC1 mutant. If both the 6K-R and 6K-Q are

catalytically active, as suggested by our colorimetric deacetylation assay, then we would predict that both HDAC1 acetylation mutants would inhibit GR enhanced *C/ebpα* transcription and 3T3 L1 preadipocyte differentiation with the same or increased efficiency as WT HDAC1.

The observation that the 6K-R and 6K-Q HDAC1 mutants both failed to significantly affect *C/EBPα* promoter-driven *Firefly* luciferase expression in the presence of dex (Figure 14), while expression of either 6K-R or 6K-Q HDAC1 enhanced the efficiency of dex-induced 3T3 L1 differentiation (Figure 16) suggests that they do not exhibit differential catalytic activities in our system. Furthermore, these observations suggest that the 6K-R and 6K-Q HDAC1 mutants enhance the efficiency of 3T3 L1 differentiation downstream of the *C/ebpα* gene, since neither HDAC1 mutant appears to have an effect on *C/EBPα* promoter-driven luciferase expression (Figure 14), whereas expression of either mutant is capable of enhancing preadipocyte differentiation in a dex-dependent manner.

These results are particularly counterintuitive because Hager's group very convincingly demonstrated in an *in vitro* deacetylase assay that the 6K-Q HDAC1 mutant could not deacetylate Histone H4, while the 6K-R HDAC1 mutant could. Hager's group also confirmed this result by revealing that tethering of the 6K-R or WT HDAC1 to the Gal4-promoter inhibited transcriptional activation of the promoter and expression of the luciferase reporter gene, while tethering of the 6K-Q HDAC1 to the promoter of the same reporter construct was not sufficient to inhibit luciferase expression (Qiu *et al*, 2006). Therefore, given the inconsistency of our results and those produced by Hager's group we propose a new hypothesis for the importance of the acetylation status of HDAC1's lysine residues 218, 220, 432, 438, 439 and 441, which reconciles our results with those of Qiu *et*

al., (2006): We hypothesize that the acetylation status of lysine residues 218, 220, 432, 438, 439 and 441 is responsible for regulating the substrate specificity of HDAC1, rather than the deacetylase activity of the enzyme.

Justification of our new hypothesis

Each HDAC is responsible for controlling the rate of transcription for a specific spectrum of genes, inspite of how much sequence similarity the classes and the isoforms within a specific class, share. It has been established that in certain cases specific co-factors are required in order for HDACs to be able to be able to deacetylate their targets (De Ruijeter *et al.*, 2003, Luo *et al.*, 2000, and Riester *et al.*, 2007). For example, the PID protein, which stands for p53 target protein in the deacetylase complexes and which is identical to the human metastasis-associated protein 2 (MTA2), has been shown to mediate the interaction between HDAC1 and p53, thus allowing for the deacetylation of p53 by HDAC1 and inhibition of p53's ability to halt the cell cycle (Luo *et al.*, 2000). Upregulation of HDAC1 mediated inhibition of p53 is hypothesized to be involved in the development and progression of cancer (Luo *et al.*, 2000).

The discovery that co-factors are essential for HDAC mediated deacetylation of certain substrates lead to the hypothesis that the specificity of HDACs may be regulated by the binding of exclusive co-factors, although significant evidence to support this hypothesis is still needed (De Ruijeter *et al.*, 2003, Luo *et al.*, 2000, and Riester *et al.*, 2007). The results presented in Chapter 2 better support the hypothesis that the regulation of the acetylation status of lysine residues 218, 220, 432, 4328, 439 and 441 controls the substrate specificity of HDAC1 by altering the ability of the enzyme to interact with essential co-

transcription factors, rather than regulating HDAC1s enzymatic activity as proposed by Qiu *et al.*, (2006).

Quantification of co-immunoprecipitation experiments revealed that the ability of 6K-R and 6K-Q HDAC1 to interact with transcription factors known to be involved in the regulation of *C/ebpα* transcription and believed to be involved in modulating the role of HDAC1 during GR-potentiated *C/ebpα* transcription, is affected by the mutations. Both mutants interact approximately 4 fold more efficiently with GR than WT HDAC1, which is important because GR has been hypothesized to enhance *C/ebpα* transcription by titrating HDAC1 from the promoter and targeting the enzyme for 26S proteasomal degradation (Figure 19). Consequently, enhanced interaction between GR and 6K-R and 6K-Q HDAC1 could indicate an increased rate of titration and/or degradation of the HDAC1 mutants, which may not have been detected by the simple experiment that we conducted (Figure 18). Furthermore, both mutants demonstrated changes in their ability to bind the co-repressor mSin3A which is the protein responsible for mediating the interaction between HDAC1 and C/EBPβ (Figure 19). Interestingly the mutants displayed opposing changes in their ability to interact with mSin3A as 6K-R HDAC1 exhibited enhanced mSin3A binding, while 6K-Q HDAC1 displayed compromised mSin3A binding. These results not only demonstrate that changes in the acetylation status of HDAC1 cause alterations in the ability of the enzyme to interact with other transcription factors, which supports our new hypothesis, but it all suggests that regulation of the acetylation status of lysine residues 218, 220, 432, 438, 439 and 441 may be involved in altering the ability of HDAC1 to interact with mSin3A. Reproduction of this result in co-immunoprecipitation experiments performed in 3T3 L1

preadipocytes would indicate that further investigation into the role that post-translational acetylation of HDAC1 plays in controlling the HDAC1-mSin3A interaction should occur.

The inability of two HDAC1 mutants, which are known to be catalytically active, to have any affect on *C/EBPα* promoter-driven transcription, a process in which HDAC1 has been established to play a suppressive role, suggests that perhaps the 6K-R HDAC1 and 6K-Q HDAC1 mutants are compromised in their ability to bind to the *C/EBPα* promoter. If mutation of the six lysine residues compromises the ability of the HDAC mutants to bind to certain co-factors then 6K-R and 6K-Q HDAC1 may be compromised in their ability to bind to the specific co-factors required to regulate *C/ebpα* transcription such as *C/EBPβ*, or another currently unidentified co-regulator of *C/ebpα* transcription. If the HDAC1 mutants could not interact with the specific co-factors required for them to be targeted to the *C/EBPα* promoter than their expression would not have an affect on *C/ebpα* transcription, and endogenous WT HDAC1 would be the only HDAC1 present at the promoter. However, both mutants enhanced the efficiency with which dex treatment potentiated 3T3 L1 differentiation (Figure 16). Together these results suggest that the 6K-R and 6K-Q HDAC1 mutants may not be targeted to the *C/EBPα* promoter but they might maintain the ability to bind to co-factors involved in regulating the transcription of other genes in the adipogenic transcriptional cascade, thus resulting in enhanced preadipocyte differentiation downstream from the *C/ebpα* gene.

To determine if the 6K-R and 6K-Q HDAC1 mutants are efficiently recruited to the *C/ebpα* promoter, a ChIP should be performed in 3T3 L1 cells stably expressing either WT, 6K-R or 6K-Q HDAC1. Co-immunoprecipitation experiments could be conducted to investigate whether the ability of 6K-R and 6K-Q HDAC1 to interact with *C/EBPβ* is also

influenced by the mutations. Furthermore, immunoprecipitation of Flag tagged HDAC1 WT and mutant constructs followed by affinity chromatography and then exposure of the identified proteins to a GST-C/EBP β affinity column could be used to identify other co-factors that may be involved in regulating the interaction between HDAC1 and C/EBP β . This is the method that was utilized by Luo *et al.*, (2006) to identify the co-factor PID that mediates the interaction between HDAC1 and p53. .

It is important to note that of the six mutated lysine residues in the 6K-R and 6K-Q HDAC1 mutants three of them, lysine residues 438,439 and 441, are within the core signal (aa. 438-445) of mouse-HDAC1's nuclear localization (438-482) (Taplick *et al*, 2001). Furthermore, amino acids 438-445 are conserved between human and mouse HDAC1 and the amino acid sequence of human and mouse HDAC1 is identical in length (Wang *et al*, 2005). This observation suggests that amino acids 438-482 could also act as the NLS for human HDAC1 and implies that mutation of lysine residues 438-439 and 441 in human HDAC1 could result in problems with the cellular localization of the 6K-R and 6K-Q HDAC1 mutants. We confirmed, using immunofluorescence, that both the 6K-R and the 6K-Q HDAC1 mutants are primarily localized to the nucleus of 3T3 L1 cells (Figure 17). However, as mentioned, it remains possible that the induced mutations compromise the ability of the 6K-R and 6K-Q HDAC1 to recognize, and bind to C/EBP β , or another currently unidentified co-regulator of *C/ebpa* transcription, and would therefore be unable to replace the effect of endogenous HDAC1 on *C/ebpa* transcription.

Experimental investigation of our new hypothesis

In order to begin to investigate whether or not our new hypothesis is valid I propose to perform an *in-vitro* deacetylation assay on a multitude of different HDAC1 substrates including; Histones, p53, MyoD, C/EBP β (WT and K98-102R). The proteins will first be *in-vitro* acetylated with the appropriate HAT ; p300 or PCAF, after which the acetylated proteins will be purified and incubated with immunoprecipitated WT, D181A, 6K-R or 6K-Q HDAC1 (either from SF-19 insect cells, or Cos-7 cells). The level of deacetylation of the substrate in each condition could be monitored by visualization using PhosphoImager analysis, or by radioactivity count.

We have both a positive control, WT HDAC1, to ensure that the assay conditions support deacetylase activity, and a negative control, D181A HDAC1 which we have demonstrated is catalytically inactive. We are currently in the process of optimizing the deacetylation assay using histones as the substrate, because these are the most widely used and accepted HDAC substrate, they are efficiently acetylated by both PCAF and p300, and it takes ten times less histones than non-histone proteins (1ug versus 10ug) to obtain an observable acetylation level on film (De-Ruijter *et al.*, 2002, Glozak *et al.*, 2005, Hassig *et al.*, 1998, Riester *et al.*, 2007 and Smith, C.L. 2007). Furthermore, beginning with histones as the deacetylase substrate will allow us to confirm that the D181A HDAC1 is in fact a catalytically inactive mutant and to first examine whether the 6K-R and 6K-Q HDAC1 mutants exhibit their published activity phenotypes, as constitutively active and inactive mutants, before continuing on with the rest of the HDAC1 substrates (Qiu *et al.*, 2006).

For the non-histone proteins we have chosen four. 1) MyoD, which is essential to the initiation of muscle differentiation and which is transcriptionally activated by PCAF mediated acetylation and is inactivated through its deacetylation by HDAC1 (Glozak *et al.*, 2005, Mal *et al.*, 2001, Sartorelli *et al.*, 1999). 2) p53 which is an important cell cycle regulator whose transcriptional activity is stimulated by its acetylation by p300 and is attenuated by HDAC1 mediated deacetylation (Juan *et al.*, 2000 and Luo *et al.*, 2000). Importantly, the dysregulation of the transcription of p53, by HDAC1 can contribute to cancer and is one of the many reasons why HDAC inhibitors are now being tested in clinical trials as potential anti-cancer drugs. (Juan *et al.*, 2000 and Luo *et al.*, 2000). 3 and 4) Both WT and K98-102R C/EBP β so that we can provide a direct proof on whether WT C/EBP β can be deacetylated by HDAC1 at lysine residues 98,101 and 102, and whether other lysine residues in C/EBP β can be deacetylated by HDAC1.

Conclusion

The 6K-R and 6K-Q HDAC1 acetylation mutants were generated by Qui *et al*, 2006, and were demonstrated to be constitutively active and inactive, respectively. In our investigation neither the 6K-R nor the 6K-Q HDAC1 mutant exhibited the expected results with regards to their deacetylase activity or the phenotypes that they produced in our adipogenesis system. However, the conflicting results between our experiment and the published results of Hager's group lead to the development of the hypothesis that controlling the acetylation status of lysine residues 218,220, 432, 438, 439 and 441 of HDAC1 may regulate the substrate specificity of the deacetylase enzyme, rather than the deacetylase activity of the protein. The results of this investigation could potentially provide important and novel insight into the mechanism through which the specificity of the HDAC family of enzymes is regulated.

General Discussion

The biological relevance of studying glucocorticoid-potentiated adipogenesis and the role of HDAC1 in this process

Glucocorticoids are known to potentiate the differentiation of new fat cells, *in vivo* and in cell culture, and to promote the accumulation of visceral fat tissue in humans (Castro and Moreira, 2007, Peeke and Chrousos, 1995, and Reichard *et al.*, 2000). Glucocorticoids upregulate the adipogenic process, in part, by enhancing transcription of one of the master adipogenic factors, C/EBP α (Wiper-Bergeron *et al.*, 2003). Therefore in the first section of this investigation we sought to delineate the specific role played by HDAC1 at the C/EBP α promoter, during the first 48hrs of GR stimulated differentiation.

By investigating and understanding the molecular mechanism through which GR enhances the efficiency of preadipocyte differentiation we will gain a better understanding of how both Cushing's syndrome and prolonged corticosteroid based therapies induce central obesity in patients (Peeke and Chrousos, 1995, Pijl and Meinders, 1996, Wassenber *et al.*, 2005 and Wung *et al.*, 2008). Understanding the process of corticosteroid induced adipogenesis is of particular importance for a variety of reasons which include: how frequently glucocorticoids based therapies are prescribed as anti-inflammatory and immunosuppressive agents, the recent link identified between the accumulation of visceral fat and the increased risk in the development of heart disease and the decrease in overall health that coincides with central weight gain (Lau *et al.*, 2007). Therefore, although corticosteroid based drugs do treat the symptoms of inflammation and help prevent organ rejection, the increased central obesity that results as a side effect of these drugs predisposes these individuals to other diseases, and can decrease their overall health status. Thus, if we

could elucidate the molecular mechanism utilized by GR to enhance the efficiency of preadipocyte differentiation, then we might be able to either create a corticosteroid based drug which does not cause central obesity, or we may be able to generate a method of preventing this particular side effect of glucocorticoid therapy. Furthermore, increasing our knowledge of the basic molecular details which leads to the formation of new fat cells will also add to our comprehension of the processes involved in the development of obesity.

Previous studies in our lab have identified a suppressive role for HDAC1 in the regulation of *C/ebpα* transcription and they have lead us to hypothesize that GR upregulates C/EBPβ-mediated *C/ebpα* transcription by promoting the titration of the mSin3A/HDAC1 co-repressor complex away from C/EBPβ, and targeting HDAC1 for 26S proteasomal degradation. The studies conducted in Chapter 1 indicate that HDAC1 has a suppressive effect on GR-potentiated *C/ebpα* expression which is dependent on the deacetylase activity of the enzyme. Furthermore, the results suggest that the key role of HDAC1 at the C/EBPα promoter involves curbing the intensity with which GR enhances transcription of the adipogenic factor, rather than just inhibiting transcriptional activation of C/EBPβ in the absence of steroid, as previously hypothesized. These results are promising as they illuminate the complexity of both the inhibitory mechanism responsible for preventing significant *C/ebpα* transcription in the absence of steroid, and the molecular steps undertaken by GR to upregulate the adipogenic transcriptional cascade, in the presence of steroid. It will subsequently be important to identify the additional co-inhibitors responsible for restraining C/EBPβ-mediated *C/ebpα* transcription, in the absence of corticosteroids, as described in detail in Chapter 1.

What's more, individuals suffering with depression or epilepsy who were treated with the general HDAC inhibitor Valproic Acid (VPA) to regulate their disease, reported increases in visceral fat accumulation, similar to the side effects observed during prolonged corticosteroid treatment (Davis *et al.*, 1994). When Wiper-Bergern *et al.* (2003) demonstrated that treatment of preadipocyte cells in culture with VPA or TSA replaced the ability of GR to induce transcriptional activation of the *C/EBP α* promoter and enhanced preadipocyte differentiation, it was proposed that the general HDAC inhibitors activated the adipogenic transcriptional cascade by replacing the ability of GR to obstruct HDAC1. However, our recent results indicate that this hypothesis is incorrect because neither a reduction in HDAC1 expression, nor inhibition of HDAC1's deacetylase activity was sufficient to substitute GRs ability to upregulate the adipogenic process. Therefore, the ability of the general HDAC inhibitors to induce adipocyte differentiation in the absence of steroid likely involves the upregulation of genes downstream of *C/ebp α* which are essential to the adipogenic cascade. Investigating whether additional HDACs, such as HDAC3, are involved in the regulation of *C/ebp α* expression and identification of the HDACs responsible for regulating the transcription of other important adipogenic factors, such as PPAR γ , will be essential to understanding the molecular details of adipogenesis and the side effects of VPA treatment.

Investigating the function, structure and regulation of Histone Deacetylases (HDACs)

In addition to clarifying the molecular details of glucocorticoid-potentiated adipogenesis, our investigation also revealed some valuable information about the regulation

of HDAC1's substrate specificity and clarified details about the structural features of the protein which confer the enzyme its deacetylase activity.

HDACs are important cellular enzymes which are known to be involved in regulating a multitude of essential developmental and life sustaining processes through their ability to both directly and indirectly control the rate of transcription by deacetylating the lysine residues of both histone and non-histone proteins (Brunmeir *et al.*, 2009, De Ruijter *et al.*, 2003, Glozak *et al.*, 2005, Mal *et al.*, 2001, Marks *et al.*, 2000, Sartorelli *et al.*, 1999 and Wiper-Bergeron *et al.*, 2003).

In addition to being important in normal cellular function and development the dysregulation of several HDACs has been shown to play influential roles in several diseases. For example, the dysregulation of HDACs has been linked to the development and progression of certain types of cancers (Juan, *et al.*, 2000, Luo *et al.*, 2000, Marks *et al.*, 2000), and the use of HDAC inhibitors as anticancer agents has made it to the Phase I and II clinical trials (Lin *et al.*, 2006, Marks *et al.*, 2000, Vigushin and Coombes, 2002). Furthermore, the possibility of using specific HDAC Class I inhibitors to disrupt dormant HIV-1 infection is also being explored, as HDAC 2 and 3 have been shown to be involved in maintaining the virus in a dormant, and thus undetectable and untreatable state (Keedy *et al.*, 2009). Unfortunately, there are currently no specific HDAC inhibitors available which allow us to inhibit individual or a particular class of HDACs (De-Ruijter, 2003 and Bieliauskas and Pflum, 2008). However, investigating and gaining an understanding of the relationship between the structural components of HDACs and their catalytic activity and identifying the mechanisms through which the specificity of HDACs is regulated will provide critical

information which can be used to assist in the development of isoform and class specific deacetylase inhibitors.

In the first chapter of this investigation a point mutant of HDAC1 with aspartic acid residue 181 mutated to alanine, D181A HDAC1, was generated. This mutant demonstrated severely compromised deacetylase activity, in a colorimetric deacetylation activity assay (Figure 5), suggesting that, as predicted, the D181A HDAC1 mutant was catalytically inactive. Over the last decade, significant progress has been made in elucidating the structural features of HDACs and in beginning to correlate those features with functional aspects of the enzymes. Our increased knowledge of the general structure of HDACs comes from the X-ray structures of Histone Deacetylase Like Protein (HDLP) and class I HDAC8 (Finnin *et al.*, 1999, Somoza *et al.*, 2004 and Vannini *et al.*, 2004). These structures, coupled with homology modeling techniques indicate that the general structure of HDACs is that the N-terminal end folds to form a ~12 Å^o-deep narrow catalytic pocket, with a tube-like internal cavity of ~14Å^o, which branches off from the catalytic pocket (Wang *et al.*, 2005). The residues comprising the 12Å^o deep catalytic pocket are extremely highly conserved across both isoforms and homologues of HDACs. At the bottom of this catalytic pocket lies the active site of the HDAC enzyme, where two aspartic acid and one histidine residue coordinate the Zn²⁺ ion that is critical to the deacetylase activity of the enzyme (Finnin *et al.*, 1999 and Vanommeslaeghe *et al.*, 2005). An additional five amino acids, two histidine, two aspartic acids and one tyrosine residue, have been determined to encompass the charge relay system that is required to transfer the electrons which permit HDACs to deacetylate their target lysine residue (Finnin *et al.*, 1999, Somoza *et al.*, 2004, Vannini *et al.*, 2004 and Vanommeslaeghe *et al.*, 2005). In HDAC1, aspartic acid residue 181 corresponds to the

second aspartic acid residue within the charge relay system, and since mutants produced by other groups where other residues within the charge relay system have been mutated have been shown to be catalytically inactive, it was predicted that the D181A HDAC1 mutant would also be catalytically compromised (Hassig *et al.*, 1998, Kadosh *et al.*, 1998, Finnin *et al.*, 1999 and Vanommeslaeghe *et al.*, 2005). The results of our colorimetric deacetylase assay confirm that the D181A HDAC1 is catalytically inactive, and this observation is supported by the phenotype produced by the D181A HDAC1 mutant in the adipogenic system. Not only does the D181A HDAC1 mutant provide us with a catalytically inactive mutant of HDAC1 but it also confirms the importance of aspartic acid 181 to the catalytic activity of the enzyme, which in turn helps to validate the proposed models of the HDAC active site (Finnin *et al.*, 1999, Somoza *et al.*, 2004 and Vannini *et al.*, 2004),

Furthermore, in Chapter 2 of this investigation we attempted to determine whether the regulation of the acetylation status of lysine residues 218, 220, 432, 438, 439 and 441 is involved in controlling the activity or stability of HDAC1 during the adipogenic process, as acetylation of these six lysine residues has been shown to inactivate HDAC1's ability to deacetylate Histone H4 *in-vitro* (Qiu *et al.*, 2006). However, the differential activity mutants produced by Qiu *et al.* (2006), 6K-R and 6K-Q, did not produce the expected results in either the colorimetric deacetylase assay, the C/EBP α promoter *Firefly* luciferase assays nor in the 3T3 L1 preadipocyte differentiation experiments (Figures 13, 14, 15 and 16). These discrepancies lead us to hypothesize that the acetylation status of the six lysine residues identified by Qiu *et al.*, (2006), regulates the specificity rather than the catalytic activity of HDAC1. Future experiments are required to test this hypothesis by examining the ability of the WT, D181A, 6K-R and 6K-Q HDAC1 constructs to deacetylate a variety of known

HDAC1 substrates. It is important to acknowledge that very little is known about how the specificity of HDACs is regulated, although it has been strongly suggested that the C-terminal region of the protein, which is the most variable region, is likely involved, which is where four of the six acetylation mutations were inserted in the 6K-R and the 6K-Q HDAC1 mutants (De Ruijter *et al.*, 2003 and Riester *et al.*, 2007). The theory that the substrate specificity of HDACs is regulated by the co-factors with which the enzyme interacts is a new and emerging hypothesis. Our prediction that the acetylation status of lysine residues 218, 220, 432, 438, 438 and 441 of HDAC1 is involved in regulating the HDAC1s substrate specificity is original and further investigation into this hypothesis could uncover novel information about how the activity and specificity of a ubiquitous and essential family of enzymes is regulated.

Furthermore, uncovering new information about how the specificity and activity of HDACs are regulated is essential to the aspirations and attempts to begin generating class, isoform or perhaps even substrate specific HDAC inhibitors. These types of inhibitors would be extremely beneficial as they would allow us to effectively treat various HDAC related diseases, from developmental problems to particular types of cancer, while significantly minimizing the side effects of the treatment.

Concluding remarks

The results of this investigation have clarified the role of HDAC1 in the regulation of the expression of the master adipogenic commitment factor *C/ebpa*, during glucocorticoid-potentiated adipogenesis. Our investigation has confirmed that HDAC1 and not HDAC2 is involved in regulating *C/ebpa* transcription, we have demonstrated that the deacetylase activity of HDAC1 is essential to its suppressive role at the *C/ebpa* transcription and we have provided evidence suggesting that HDAC1 is not the sole inhibitor of *C/ebpa* transcription. These discoveries indicate that the regulation of C/EBP β mediated *C/ebpa* transcription is much more complex than previously known. These results have allowed us to refine our molecular model of GR induced adipogenesis and have lead to the planning and development of future investigations which will only further clarify our understanding of these mechanisms. Furthermore, our study has resulted in the development of a enzymatically inactive mutant of HDAC1, D181A HDAC1, which behave as a dominant negative HDAC1 mutant in the adipogenic system. The dominant negative D181A HDAC1 construct could be used by us and by other groups to investigate both the role of HDAC1 deacetylase activity in a variety of cellular functions, and to begin scrutinizing and elucidating more information about the relationship between the molecular structure of HDAC1 and its functional roles.

Additionally our investigation has lead us to propose a novel mechanism in which the acetylation status of HDAC1 at lysine residues 218, 220, 432, 438, 439 and 441 is involved in regulating the specificity of the enzyme for its substrates, perhaps by affecting the ability of HDAC1 to associate with specific co-factors, rather than the enzymes deacetylase activity, as was previously published (Qiu *et al.*, 2006).

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Education:

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- Currently a Masters candidate in Biochemistry
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References are available upon request.