

# **The Regulation of C/EBP $\beta$ Activity and Adipogenesis by the Smad3 MH1 Domain**

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## **DEDICATION**

To Mom, Dad and Colin, for the earth beneath my foundation.

## ABSTRACT

Retinoic Acid (RA) is a potent anti-adipogenic molecule. Recent experiments in our lab identified the transcription factor Smad3 as a novel RA target gene required for RA-mediated inhibition of adipogenesis. Smad3 was demonstrated to inhibit C/EBP $\beta$  DNA binding via an interaction between the Smad3-MH1 domain and C/EBP $\beta$ . The goal of this thesis was to evaluate the anti-adipogenic potential of the isolated Smad3 MH1 domain in the absence of RA treatment. Pooled 3T3-L1 preadipocyte stable cell lines expressing MH1 and empty vector controls were created and induced to differentiate. While MH1 protein expression was difficult to detect due to high protein lability, C/EBP $\beta$  DNA binding to its response element within the C/EBP $\alpha$  promoter was inhibited in MH1-expressing cells. Despite this, loss of C/EBP $\beta$  occupancy at the C/EBP $\alpha$  promoter did not result in changes in C/EBP $\alpha$  gene expression though adipogenesis was inhibited in these cultures. In contrast, PPAR $\gamma$  levels were reduced in MH1-expressing cells suggesting that regulation of PPAR $\gamma$  expression by the isolated MH1 domain drives the inhibition of adipogenesis in this model. The second goal of this thesis was to evaluate the regulation of Smad3 by RA. We determined that RA directly activates Smad3 transcription, which is not dependent on promoter demethylation. Furthermore, RA induces Smad3 nuclear accumulation in the absence of Smad3 phosphorylation.

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## LIST OF ABBREVIATIONS

Act-D	all-trans retinoic acid
ATRA	5-azacytidine
AZA	brown adipose tissue
BAT	bone morphogenetic protein
BMP	cyclic adenosine monophosphate
cAMP	CCAAT/enhancer binding protein
C/EBP	CCAAT/enhancer binding protein alpha
C/EBP $\alpha$	CCAAT/enhancer binding protein beta
C/EBP $\beta$	CCAAT/enhancer binding protein delta
C/EBP $\delta$	centre for disease control and prevention
CDC	chromatin immunoprecipitation
ChIP	cycloheximide
CHX	cAMP-response element binding
CREB	dulbecco's modified eagle medium
DMEM	electrophoretic mobility shift assay
EMSA	fluorescence activated cell sorting
FACS	glycogen synthase kinase 3 beta
GSK3 $\beta$	histone deacetylase 1
HDAC1	isobutylmethylxanthine
IBMX	mitogen activated protein kinase A
MAPK	MCE
actinomycin D	MEK1

MID	mitogen-activated protein kinase kinase 1
MSC	isobutylmethylxanthine-insulin-dexamethasone
NLS	mesenchymal stem cell
ORO	nuclear localisation sequence
PKA	oil red o
PPAR	protein kinase A
PPAR $\gamma$	peroxisome proliferator-activated receptor
RA	peroxisome proliferator-activated receptor gamma
RAR	retinoic acid
RARE	retinoic acid receptor
RTK	retinoic acid response element
RXR	receptor tyrosine kinase
SDS-PAGE	retinoid x receptor
Sp1	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SREBP	specificity protein 1
SVF	sterol receptor element binding protein
TGF $\beta$	stromal vascular fraction
TZD	transforming growth factor beta
VDR	thiazolidinedione
WAT	vitamin D receptor
mitotic clonal expansion	white adipose tissue

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## INTRODUCTION

From a teleological perspective, fat is believed to exist so that metazoans can survive during nourishment scarcities. However, in today's society energy-rich foods are plentiful and an increasingly sedentary lifestyle, and in some cases genetic predisposition, has led to the characterization of excessive fat as an epidemic called obesity. According to the Centers for Disease Control and Prevention (CDC) approximately 33.8% of the adults in the United States are obese (CDC, 2011). Worldwide, obesity levels are on the rise and with this, so are cardiovascular disease, type II diabetes, sleep apnea, stroke and a whole range of chronic diseases associated with excessive adipose tissue .

Obesity results from caloric excess being stored as lipid in specialized cells called adipocytes. There are two types of adipocytes found within the body: brown, found in brown adipose tissue (BAT) and white, found in white adipose tissue (WAT). Both adipocytes are capable of storing excess energy in the form of triglycerides. However, these two tissues have distinctly different functions. Brown adipose tissue (BAT), present only in significant amounts in the newborn, contributes to thermogenesis by uncoupling cellular respiration from the proton electrochemical gradient in mitochondria, using uncoupling protein-1 . It is interesting to note that higher amounts of BAT are typically associated with lean individuals . White adipose tissue (WAT), present in excess in obese individuals, acts as a storage depot for consumed calories (Park, Halperin et al. 2008) and has a virtually unlimited capacity to store fat. Adipocytes within WAT can hypertrophy to store excess triglycerides in response to caloric excess and new adipocytes can be created from precursor cells present in the WAT stroma and the bone marrow . As a result, it is not only important to understand the etiology of this disease, but also how it can be mitigated.

### **In vivo adipocyte precursors**

Adipose, muscle, cartilage, tendon and bone are known as mesenchymal connective tissues, which derive from the mesoderm (a germ layer of the gastrulating embryo) during embryogenesis. All of these tissues are able to remodel in the adult in response to use and environmental stress, and are associated with stem cell-like precursors which drive their growth and repair. The accepted model is that these tissues differentiate from a common mesenchymal stem cell (MSC) in response to environmental stimuli . The mesenchymal stem cell was first identified from guinea pig bone marrow as a fibroblast-like precursor cell in 1970 . These fibroblasts could differentiate into several tissue types, including adipose tissue; it is from MSCs that adipocyte precursors present in the stromal vascular fraction (SVF) of fat pads are believed to be derived and this fact has been suggested by studies performed in 1985, where precursors from the SVF were isolated, proliferated in culture and induced to differentiate into adipocytes .

The adipocyte precursor is poorly defined *in vivo*. Precursor cells isolated from the stromal vascular fraction of fat pads often yield an impure culture of fibroblasts for experimentation . However, there have been recent efforts to define a cell surface marker profile for these cells. Rodeheffer et al. recently isolated a population of cells from wild-type mouse adipose tissue through Fluorescence Activated Cell Sorting (FACS), using the cell surface markers CD29, CD34, Sca-1 and CD24. After sequentially isolating for each of these markers, they infused these cells into a lipodystrophic mouse model and showed that the putative adipocyte progenitors were capable of reconstituting fat and rescuing the diabetic phenotype of the mice . Interestingly, the transplant location within the lipodystrophic mouse was critical to the differentiation of these cells. If the progenitor population was injected intravenously then the cells were incapable of reconstituting the fat pads; however, if the putative preadipocytes were

injected into the fat pads of the lipodystrophic mouse, then they could assume their destined course of adipocyte differentiation . This information suggested that the preadipocyte environment must provide critical signals for progenitor differentiation.

### **Adipogenic regulatory factors**

#### **CCAAT/ Enhancer Binding Proteins (C/EBP)**

C/EBP factors are members of the CCAAT/Enhancer Binding Protein family of transcription factors present in several cell types, including hepatocytes, adipocytes and hematopoietic cells and are responsible for important systemic functions such as inflammation and metabolism .

The C/EBP family of proteins are contain two critical regions for functionality: a N-terminal activation domain and a C-terminal DNA binding and heterodimerization domain referred to as the bzip domain . The “activation domain” confers transcriptional regulatory function to each of these proteins, which can range from activation to repression of target genes and is relatively non-conserved within the family with <20% sequence similarity between family members . In contrast, the bzip domain is highly conserved, with >90% sequence similarity amongst family members . It is composed of a region of basic amino acids allowing for interaction with DNA, followed by a leucine rich region referred to as the “leucine zipper,” allowing heterodimerization with other bzip containing transcription factors . The specificity of the C/EBPs interaction with the CCAAT box motif is determined by the amino acid sequence of the basic region, which is conserved amongst all C/EBPs, with the exception of C/EBP $\zeta$  (CHOP-10) which bears two proline substitutions in its basic region which abolishes DNA-binding to CCAAT box motifs . It is important to note that, though the C/EBPs consistently bind to CCAAT box motifs, it is not

uncommon that they interact with more divergent promoter elements such as cAMP response elements (CRE) .

C/EBP dimerization is a prerequisite for DNA binding to the CCAAT box motif. The most important shared characteristic of the C/EBP family is their non-polar leucine zipper motif, which allows for protein-protein interaction amongst family members. This motif consists of an alpha helical heptad repeat of four or five leucine residues, which, through non-polar interactions, form a coiled-coil structure with the leucine zipper of a neighbouring C/EBP transcription factor . The leucine zipper domain allows these proteins to both hetero/homodimerize with other bzip transcription factors to regulate transcription at target promoters .

The C/EBP family is composed of 5 members;  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  and  $\zeta$  which are all equally capable of intrafamilial heterodimerization by virtue of their leucine zippers . C/EBP $\alpha$  was the first member of the family to be identified by Dr. Steven McKnight's laboratory, with the subsequent chronological identification of additional C/EBPs resulting in the nomenclature used today . C/EBP  $\alpha/\beta/\delta$  have specific and well characterized roles in adipogenesis, which are summarized in numerous reviews .

### **C/EBP null mice**

To further study the role of C/EBPs in adipogenesis, knockout mice specific for C/EBP $\beta/\delta/\alpha$  were created. Tanaka et al. demonstrated that the C/EBP $\beta$  null mice had significantly inhibited adipogenesis in fat depots seen by Oil red O staining . Lipid droplet staining was sparse in the tissue compared to wild-type controls, in which almost every cell contained Oil red O stained multilocular lipid droplets. Consistent with this was the significantly

abrogated adipogenesis of mouse embryonic fibroblasts (MEFs) which were isolated from C/EBP $\beta$  null mice and induced to differentiate using a hormonal induction cocktail *in vitro* . In contrast, the Oil red O staining of fat pads from C/EBP $\delta$  null mice revealed only mild reduction of staining compared to wild-type, suggesting that C/EBP $\beta$  is of greater importance than C/EBP $\delta$  during early adipogenesis . To further complement this notion, *ex vivo* cultured MEFs from C/EBP $\delta$ -null animals had only slightly reduced differentiation compared to wild-type , thus demonstrating that C/EBP $\beta$ -mediated transcription is important for adipocyte commitment.

C/EBP $\alpha$  knockout mice had high post-natal lethality . As C/EBP $\alpha$  is required for liver gluconeogenesis, in its absence mice die soon after birth from an inability to synthesize hepatic glucose . Nevertheless, the isolation of C/EBP $\alpha$ -null MEFs was still possible . *Ex vivo* cultured C/EBP $\alpha$ -null MEF differentiation was significantly inhibited and adipogenesis could only be rescued by ectopic expression of Peroxisome Proliferator Activated Receptor gamma (PPAR $\gamma$ ), the master regulator of adipogenesis . Therefore, C/EBP $\alpha$  has a critical function during adipogenesis, as its absence results in insulin insensitivity and absence of white adipose tissue depots in null mice .

### **Peroxisome Proliferator Activated Receptors (PPAR)**

The Peroxisome Proliferator-Activated Receptors (PPAR) are a family of ligand-activated nuclear receptors known as metabolic sensors. They are structurally similar to other nuclear receptors having the typical modular structure: variable N-terminal domain, centrally located DNA-binding domain and C-terminal ligand binding domain containing a ligand-dependent activation function . PPARs were first identified in the 1980s, by Isseman and Green as a nuclear receptor which was both activated by, and confined to, tissues in mice that responded to

chemicals capable of inducing peroxisome proliferation (clofibrate, herbicides, plasticizers) . It was only later that these nuclear receptors were fully cloned and characterized from *Xenopus laevis* oocytes . Though their many of endogenous ligands remain elusive, PPARs are capable of responding to various lipid signals and other molecular cues allowing for a great diversity of function, hence the reason why they are referred to as “metabolic sensors” . Among the biological and physiological effects of PPAR action are cell survival, proliferation and differentiation, development, homeostasis, cancer/inflammation, and aging .

To date, three lipid-activated PPARs (PPAR $\alpha$ / $\beta$ / $\delta$ / $\gamma$ ) have been discovered and characterized to have function in several tissues . All three PPARs bind direct repeats of the half-site AGGTCA as heterodimers with the retinoid X receptor (RXR) and in the presence of ligand, they promote the recruitment of transcriptional co-regulators which eventually recruit RNA polymerase II to the target genes promoter . PPAR $\alpha$  and  $\beta$ / $\delta$  (hereafter referred to as PPAR $\beta$ ) appear to control functions opposite that of PPAR $\gamma$ . PPAR $\alpha$  and  $\beta$  upregulate enzymes required for fatty acid uptake, fatty acid  $\beta$ -oxidation, ketogenesis and mitochondrial respiration (Toblin 2006). PPAR $\alpha$ -null mice are unable to regulate fatty acid metabolism during periods of starvation and become hypoketotic and hypoglycemic . Similarly, transgenic mice over-expressing an overly active PPAR $\beta$  fusion protein with VP16 have increased mitochondrial respiration and are capable of high levels of fatty acid  $\beta$ -oxidation, allowing them to run on treadmills for significantly extended periods of time compared to controls . While PPAR $\alpha$  and  $\beta$  stimulate processes involved in lipid utilization, PPAR $\gamma$  promotes lipid storage and adipocyte differentiation . PPAR $\gamma$  has two isoforms, PPAR $\gamma$ 1 and PPAR $\gamma$ 2, generated through alternative promoters and differential splicing, giving PPAR $\gamma$ 2 an additional 30 amino acids in its N-terminus and rendering it somewhat more effective at activating the transcription of a PPAR $\gamma$

reporter construct than PPAR $\gamma$ 1 . Although PPAR $\gamma$ 1 is able to drive the adipogenic process, PPAR $\gamma$ 2 is expressed almost exclusively within adipocytes and its deficiency leads to impaired adipogenic development and insulin sensitivity . It is for these reasons that PPAR $\gamma$  is a target for the treatment of type II diabetes through the use of Thiazolidinediones (TZDs), which are a class of clinical ligands of PPAR $\gamma$  that have the common side effect of adipose weight gain . Through PPAR $\gamma$ , these drugs increase the amount of adipocytes capable of responding to insulin, thereby allowing better control of blood sugar .

### **The Adipogenesis Program**

*In vitro*, the modelling of adipogenesis can be achieved through the use of several cell lines, notably 3T3-L1 preadipocytes, which were isolated from disaggregated Swiss day 17 mouse embryos . The 3T3 mouse fibroblast cell line was established in 1963 by Todaro and Green, but was further characterized into the 3T3-L1 and 3T3-F442A preadipocyte cell lines . 3T3-L1 cells are already committed to the adipocyte lineage and can be chemically induced to differentiate efficiently *in vitro* by a hormonal cocktail of isobutylmethylxanthine (IBMX, a cAMP phosphodiesterase inhibitor), insulin (peptide hormone) and dexamethasone (a synthetic glucocorticoid), collectively referred to as MID. As a robust model of preadipocyte differentiation, 3T3-L1 cells are the subject of several reviews (Cornelius 1994, Rosen 2006) and it is important to note that their differentiation is well characterized and recapitulates the differentiation of adipocytes *in vivo* .

In the 3T3 model, precursor cells are grown in tissue culture plates and allowed to reach confluence and growth arrest through contact-inhibition. Two days post-confluence, the arrested cells are induced with MID for 48 hours, triggering mitotic clonal expansion (MCE) and the

upregulation and activation of the early adipogenic transcription factors C/EBP $\beta$  and C/EBP $\delta$ . C/EBP $\beta$  is critical for MCE, which is an important step during preadipocyte differentiation . MCE is believed to allow reorganization of chromatin during cell division, which facilitates adipogenic gene induction . Consistent with this, use of drugs which inhibit the synthesis phase of mitosis and are administered during this critical 48 hour treatment, prevent adipogenesis .

IBMX is a cyclic nucleotide phosphodiesterase inhibitor, whose important role in 3T3-L1 adipogenic induction was demonstrated as early as 1985 . During the first 48 hours of differentiation, IBMX inhibits the breakdown of cyclic adenosine monophosphate (cAMP) , which in turn relieves the cytosolic inhibition of cAMP dependent Protein Kinase A (PKA) . The activation of PKA promotes the phosphorylation of cAMP Response Element Binding Protein (CREB), which is a transcriptional regulator of C/EBP $\beta$  such that activation of CREB, leads to increased expression of C/EBP $\beta$  . In culture, ectopic expression of C/EBP $\beta$  is capable of inducing adipogenesis in 3T3-L1 preadipocytes in the absence of the hormonal induction cocktail, whereas ectopic expression of C/EBP $\delta$  cannot , thereby making C/EBP $\beta$  important for committing cells to adipogenesis . However, even though cAMP phosphodiesterase inhibition is important in the upregulation of this commitment factor, 3T3-L1 cells do not differentiate as efficiently when treated solely in the presence of IBMX. Studies have demonstrated the necessity of glucocorticoids (dexamethasone) for differentiation and insulin sensitization of 3T3-L1 preadipocytes . In the absence of glucocorticoid treatment, C/EBP $\beta$  is associated with a corepressor complex that includes histone deacetylase 1 (HDAC1), leading to an inhibition of its transcriptional activity. Glucocorticoids induce both the proteasomal degradation of HDAC1 and the acetylation of C/EBP $\beta$  by histone acetyl-transferases PCAF and GCN5 resulting in the potentiation of target gene transcription, notably C/EBP $\alpha$  . Since C/EBP $\beta$  has been shown to not

activate transcription of PPAR $\gamma$  directly, its actions on early adipogenesis are likely mediated via initiation of C/EBP $\alpha$  expression . It is important to note that, in addition to the stimulation of C/EBP $\beta$  transcriptional activity, dexamethasone also upregulates C/EBP $\delta$  expression which can regulate both C/EBP $\alpha$  and PPAR $\gamma$  expression . The actions of C/EBP $\beta$  and C/EBP $\delta$  during the first four days of differentiation, allows the upregulation of the downstream master regulators of adipogenesis C/EBP $\alpha$  and PPAR $\gamma$ .

C/EBP $\beta$  is implicated in the transcriptional regulation of C/EBP $\alpha$  at a response element within its proximal promoter (-197 to -178) . As C/EBP $\alpha$  levels begin to increase, the anti-mitotic activity of C/EBP $\alpha$  inhibits mitosis, allowing for further cellular commitment to adipogenesis . However, it appears that after C/EBP $\alpha$  levels increase (day 4 of differentiation) it is capable of auto-regulating its own expression .

After day 4, when the expression of C/EBP $\beta$  and C/EBP $\delta$  is downregulated, C/EBP $\alpha$  and PPAR $\gamma$ , act to drive terminal differentiation (Cao 1991, Yeh, Cao et al. 1995, Wu, Rosen et al. 1999, Farmer 2006). C/EBP $\alpha$  and PPAR $\gamma$  appear to cross-regulate each other's expression, thereby maintaining the differentiated state . To study the regulation and transcriptional importance of C/EBP $\alpha$  and PPAR $\gamma$ , Wu et al. used mouse embryonic fibroblast models that were null for either C/EBP $\alpha$  or PPAR $\gamma$  . They found that in a C/EBP $\alpha$  knockout cell line, PPAR $\gamma$  over-expression was sufficient to drive adipogenesis, suggesting that it was the final factor in promoting adipogenesis; with the exception of a lack of insulin sensitivity in the final adipocytes . In contrast, in a PPAR $\gamma$  knockout model, C/EBP $\alpha$  overexpression was insufficient to promote adipogenesis, suggesting that C/EBP $\alpha$  acts at least in part to upregulate PPAR $\gamma$  expression . Interestingly, it has been demonstrated that preadipocyte cells lacking PPAR $\gamma$

expression also have reduced levels of C/EBP $\alpha$ , suggesting that PPAR $\gamma$  may also be able to regulate the expression of C/EBP $\alpha$ .

In the adipogenesis program, IBMX and DEX have immediate and transient functions for inducing the adipogenic transcription factors C/EBP $\beta$  and C/EBP $\delta$ . After their removal from the cell culture medium, the necessity and the effects of their target proteins gradually begin to decline as other transcription factors, notably C/EBP $\alpha$  and PPAR $\gamma$ , take their place. During the latter, it is insulin's role to continue the rest of the program. Insulin signalling is very complex and involves several pathways including, but not exclusively: PI3-Kinase, Protein Kinase B and MAP Kinase, which are all capable of phosphorylating a plethora of proteins. From the moment insulin binds its receptor tyrosine kinase (RTK) it triggers a signalling cascade, culminating in increased cellular glucose uptake through the Glut4 transporter, stimulation of glycogen synthesis, regulation of transcription and translation etc., eventually leading to a fully differentiated adipocyte.

Taken together the overall adipogenic cascade involves early induction of C/EBP $\beta$  and C/EBP $\delta$  with MID treatment, resulting in maximal expression after two days post induction. C/EBP $\beta$  and  $\delta$  act to upregulate C/EBP $\alpha$  expression, resulting in maximal expression after four days post induction. Once induced C/EBP $\alpha$  relieves C/EBP $\beta$  of its function and begins to autoregulate its own expression and that of PPAR $\gamma$  (Legraverend, Elmer et al. 1993, Yeh, Cao et al. 1995). C/EBP $\alpha$  and PPAR $\gamma$  persist into the differentiated state, upregulating the expression of adipocyte markers such as ap2, adipsin and PEPCK. Together, these three critical cell culture components are utilized to mimic differentiation signals *in vivo*. Within 7-10 days in the continuous presence of insulin, the cells accumulate neutral lipid droplets that can be labelled using Oil Red O.

## **Retinoic Acid Effect On Adipogenesis**

### **Retinoic Acid (RA) signalling**

Retinoic acid receptors (RARs), like PPARs, are members of the nuclear hormone receptor superfamily. Binding as heterodimers with RXRs, they direct transcription of genes bearing response elements in their promoters in response to all- trans retinoic acid (ATRA). RARs and RXRs have several different isoforms: RAR $\alpha/\beta/\gamma$  and RXR $\alpha/\beta/\gamma$ , which mediate the ATRA signal. In the absence of ligand RARs and RXR heterodimers interact with retinoic acid response elements (RAREs) of target promoters (direct repeat of the sequence GGTTCA separated by anywhere from 1-5 nucleotides) . These heterodimers are transcriptionally inactive, bound to corepressor complexes containing N-CoR and SMRT mSin3A, mSin3B, HDAC1 and 2, which maintain the chromatin in a repressed state and therefore inaccessible to transcriptional activator and/or basal transcriptional machinery . Interestingly, even though RXRs are capable of binding 9-cis RA, it is a scarce product of retinoic acid processing and as a result has no physiological role as a ligand . Furthermore, *in vivo* studies using Raldh2-deficient mice incapable of synthesizing ATRA demonstrated that ligand binding to the RAR receptor is sufficient to induce wild-type level transcription, as RXR-specific ligand binding could not rescue the phenotype . Upon ligand binding to RARs, a conformational change is induced within the C-terminal activation domain which triggers dissociation of the corepressor proteins and the association with coactivator protein complexes containing SWI/SNF, p160 (SRC1-like), p300, MN1, CREB-Binding Proteins and Additional Sex-like 1 (ASXL1) . Together with RARs they induce the transcription of RA target genes.

### **RA inhibits adipogenesis**

RA possesses potent anti-adipogenic ability that has been demonstrated in several instances both *in vitro* and *in vivo*. *In vitro* experiments have demonstrated that treatment with RA results in inhibited expression of adipocyte genes such as C/EBP $\alpha$ , even under inductive conditions; while *in vivo* experiments in the WNIN/Ob obese rat model showed that rats fed diets containing retinoic acid had significant decreases in white adipose tissue depots while maintaining similar food intake to controls (Jeyakumar, Vajreswari et al. 2006). Consistent with this is the common side-effect of weight loss when retinoids are used to treat skin conditions such as psoriasis and acne. From *in vitro* experiments it appears that RA has a critical window of effectiveness, and only when added within the first 48hrs of hormonal induction can it have anti-adipogenic ability. In order for RA to be effective after this time point, the cells must express ectopic retinoic acid receptor (RAR); however, even with ectopically expressed RAR, if RA is added 72hrs after the cells have been induced to differentiate, it is not capable of inhibiting adipogenesis. This data suggests that *in vivo*, RA exerts its inhibition on the formation of new fat cells, before high fat diets can induce their differentiation.

### **C/EBP $\beta$ DNA binding is affected by RA**

The ability of C/EBP $\beta$  to bind and activate the transcription of C/EBP $\alpha$  is a critical process in early adipogenesis: without C/EBP $\alpha$  expression, PPAR $\gamma$  expression is reduced, resulting in an inhibition of differentiation. Previous studies have demonstrated the potential of RA to interfere with C/EBP $\beta$  transcriptional activation of C/EBP response elements and of the C/EBP $\alpha$  promoter. RA's inhibition of 3T3-L1 preadipocytes that have been induced to differentiate occurs during the first 48 hours of the differentiation process, when MID induces

C/EBP $\beta$  to upregulate C/EBP $\alpha$  expression . More recent experiments have shown that a prolonged RA treatment of 48hrs results in abrogated C/EBP $\beta$  DNA binding to its response element within the C/EBP $\alpha$  promoter and that this process is cycloheximide (CHX) sensitive, showing unaltered C/EBP $\beta$  DNA binding with CHX cotreatment . A similar inhibition of C/EBP $\beta$  DNA binding has also been demonstrated in the early regulation of the osteogenesis master regulator Runx2 where C/EBP $\beta$  is capable of binding a response element within the runx2 promoter to repressing runx2 expression . In this model, treatment with RA results in a release of transcriptional inhibition and the potentiation of osteogenic differentiation. This effect occurred both in the absence of interaction between RAR, and C/EBP $\beta$  proteins or between RAR and the runx2 promoter . These findings, in association with the requirement for a prolonged RA treatment and its cycloheximide sensitivity, led our lab to believe that an RA target gene was responsible for inhibiting C/EBP $\beta$  DNA binding. This protein was later identified to be Smad3 .

## **The Smad Proteins**

### **TGF $\beta$ signalling**

The canonical transforming growth factor beta (TGF $\beta$ ) pathway involves the binding of dimerized TGF $\beta$  family members to a TGF $\beta$  type II receptor, followed by association with a type I receptor, which is phosphorylated and activated by the type II receptor . The activated type I receptor then goes on to phosphorylate receptor Smads (R-Smads 1, 2, 3, 5 and 8) which dimerize with the Co-Smad Smad4 and translocate to the nucleus to effect transcription of genes, thus effectively transmitting a signal from the extracellular environment to the nucleus . TGF $\beta$  signalling works through Smad proteins 2 and 3, whereas Bone Morphogenetic Protein signalling works through Smads 1, 5 and 8 .

## **Smad proteins**

Receptor-associated Smads and co-Smad Smad4 share two highly conserved Mad homology domains: the MH1 domain (N-terminal) and the MH2 domain (C-terminal) . The MH1 domain confers upon Smads the ability to bind DNA and also houses their nuclear localization signal, while the MH2 domain allows for receptor-Smad and Smad-Smad interactions . Furthermore the MH1 domain of Smad3 appears to be the only Smad protein in the TGF $\beta$  pathway with a functional NLS-like basic motif in its N-terminus, which confers upon the isolated MH1 domain the ability to be localized to the nucleus . In GST-pull down experiments performed in our lab, Smad3 was capable of interacting with C/EBP $\beta$  through its MH1 domain . However, in similar assays Smad2 was unable to support this same interaction, possibly due to an extra exon encoded within its MH1 domain .

## **Smad3 nucleocytoplasmic shuttling**

Smad protein shuttling during active TGF $\beta$  signalling is highly dynamic, with R-Smad proteins being dephosphorylated and relocated from the nucleus to the cytoplasm . Dephosphorylation results in R-Smad and Co-Smad complex dissociation within the nucleus, followed by export of individual Smad proteins to the cytoplasm . However, if signalling is still active at the cell membrane, these Smads are rephosphorylated, re-heterodimerize with Smad4 and reaccumulate in the nucleus; this process continues until the absence of signal, but allows the ability for intracellular proteins to continuously monitor their activation signal .

All R-Smads contain a highly conserved lysine-rich NLS-like motif (KKLKK) within their N-terminus, . Karyopherins are proteins which can interact with FG dipeptide motifs commonly found in nuclear porins, allowing them to transport cargo through the nuclear pore and into the

nucleus. Smad3 has been demonstrated to have a strong interaction with importin  $\beta$ 1, through the NLS located in its MH1 domain, which is dependent upon the phosphorylation of two C-terminal serine residues . Though Smad2 and Smad3 share high sequence homology, Smad2 has an extra exon (Ex3) encoded in its MH1 domain which could interfere with its NLS-like basic motif.

### **Transcriptional regulation of Smad3**

Although there is a vast amount of literature discussing the regulation of Smad3 activation, there is very little literature characterizing the transcriptional regulation of Smad3. Initially it was demonstrated that Smad3 regulation was under the control of the prenylation pathway, which mediates the addition of hydrophobic prenyl groups (3-methyl-2-buten-1-yl) to proteins. When A549 cells were treated with isoprenyl transferase inhibitors (farnesyl transferase inhibitor FTI-277 and geranylgeranyl transferase inhibitor GGTI-286), there was a significant increase in Smad3 protein expression . After creating deletion mutants of a Smad3 promoter luciferase construct, it was determined that prenylation activity was confined to a critical region upstream of the proximal Smad3 promoter (-849 to -408). This region contained six specificity protein 1 (Sp1) binding sites, and was demonstrated through an electrophoretic mobility shift assay (EMSA) to interact with purified recombinant human Sp1 proteins. Furthermore, treatment with mithramycin, an inhibitor of Sp1 binding, was capable of reducing Smad3 expression from a Smad3 promoter reporter construct .

Mitogen-activated protein kinase A (MAPK) has also been implicated in the expression of Smad3. When A549 cells were treated with PD98059 or UO126, specific mitogen-activated protein kinase kinase 1 (MEK1) inhibitors, there was a dose dependent inhibition of Smad3

expression . Using a Smad3 promoter luciferase construct, treatment with a MEK1 inhibitor significantly reduced basal luciferase expression . Similar to the prenyl transferase pathway, this activity was mapped to the Sp1 binding region between -849 to -408 of the Smad3 promoter and Sp1 binding was inhibited alongside MEK1 inhibition .

Overall, it appears that a core region of three Sp1 binding sites located in the -849 to -408 region of the Smad3 promoter and conserved among the mouse, rat and human, is important for Smad3 regulation . These Sp1 sites have been implicated in the regulation of Smad3 by two separate pathways, one inhibiting (prenyl pathway) and one stimulating (MAPK pathway) the expression of Smad3. Although inhibition of either pathway perturbs the expression of Smad3, how, or if, they directly influence the Sp1 proteins binding to the promoter and the activation of transcription remains to be elucidated.

### **Smad3 as a novel effector of RA**

In T-cell development, RA is known to play a synergistic role with TGF $\beta$  thereby enhancing TGF $\beta$  signalling and T-cell differentiation . In a study performed in 2008, Xiao et al. serendipitously discovered that treatment of T-cells with RA alone upregulated Smad3 mRNA levels . Interestingly, RA appeared to only regulate Smad3 and not its TGF $\beta$ -responsive partner Smad2 . At the time that RA was demonstrated to effect Smad3 transcription, it had already been demonstrated by Choy et al. that TGF $\beta$  signalling was capable of inhibiting adipogenesis and acted as an endogenous throttle by regulating the rate of adipogenesis . In 2003, Choy et al. specifically demonstrated that TGF $\beta$ -inhibition of adipocyte differentiation was dependent upon Smad3 interaction with C/EBP transcription factors, most importantly C/EBP $\beta$  and C/EBP $\delta$ , the early adipogenic regulators . In light of this information, our lab believed that RA was

upregulating the expression of Smad3 over the 48 hour timepoint, which in turn was disrupting C/EBP $\beta$  DNA binding and crippling adipogenesis.

When 3T3-L1 cells were treated with RA there was a specific upregulation of Smad3 mRNA, similar to T-cells . Consistent with this was the upregulation of Smad3 protein levels . Furthermore, *in vitro* translated Smad3 was capable of interfering with C/EBP $\beta$  binding to a synthetic C/EBP response element . When Smad3 expression was disrupted in 3T3-L1 preadipocytes by a shRNA, RA no longer inhibited adipogenesis . This inhibition was attributed to the inability of RA to interfere with C/EBP $\beta$  DNA binding. However, when 3T3-L1 cells were transduced to express ectopic Smad3, there was only a mild reduction in C/EBP $\beta$  DNA binding of the C/EBP $\alpha$  promoter indicating that ectopic Smad3 was not sufficient to recapitulate the crippling anti-adipogenic effect of RA .

Overall, experiments in our lab recently identified Smad3 as a RA-target gene that interferes with C/EBP $\beta$ 's interaction with DNA. In the presence of RA, both mRNA and protein levels of Smad3 are up-regulated. *In vitro* experiments using a C/EBP $\beta$  response element have shown diminished occupancy in the presence of Smad3. Furthermore, the up-regulation of Smad3 is transient and concurrent with the optimal disruption of C/EBP $\beta$  from endogenous target gene promoters (~48hours post RA treatment), suggesting that during the first 48 hours of treatment with RA, Smad3 was the effector of inhibition.

## **EXPERIMENTAL RATIONALE**

Experiments in our lab have recently identified Smad3 as a novel RA-target gene that interferes with C/EBP $\beta$ 's interaction with DNA. This inhibition is dependent upon Smad3 expression, as Smad3 deficient cells differentiate efficiently in the presence of RA, and is

believed to be a result of a direct interaction between Smad3 and C/EBP $\beta$ . As demonstrated previously, Smad3's interaction with C/EBP $\beta$  is the principal reason why TGF $\beta$  signalling is capable of inhibiting adipogenesis . *In vitro* GST- pulldown assays in our lab have demonstrated that this interaction is mediated by the MH1 domain of Smad3, because in the absence of the MH1 domain Smad3 is no longer able to interact with C/EBP $\beta$  . It is our belief that the MH1 domain itself could enter the nucleus by virtue of its NLS and inhibit C/EBP $\beta$  mediated transcription, thereby recapitulating the effects of RA.

## **HYPOTHESIS AND OBJECTIVES**

### **Hypotheses**

We hypothesize that expression of the Smad3 MH1 domain alone could recapitulate the effects of retinoic acid treatment on adipogenesis by specifically interfering with C/EBP $\beta$  DNA binding of target promoters.

### **Objectives**

The objectives of this thesis were:

1. To assess the impact of the Smad3 MH1 domains expression on adipogenesis and C/EBP $\beta$  DNA-binding.

We predicted that the MH1 domain of Smad3 would interfere with C/EBP $\beta$  DNA binding, resulting in reduced C/EBP $\alpha$  expression and inhibition of adipogenesis.

2. To further characterize the regulation of Smad3 transcription by retinoic acid
3. To further characterize the mechanism of Smad3 nuclear accumulation in the presence of retinoic acid

## MATERIALS AND METHODS

### Expression Constructs

Select plasmids were constructed using directional cloning molecular techniques. Inserts were cut or PCR amplified from existing plasmids, ensuring that the appropriate restriction enzyme sites were present or introduced for insertion according to the Table 1. After amplification or excision, the inserts were restriction digested and directionally ligated into the desired vector.

Table 1. Created constructs: either through per amplification or site-directed mutagenesis.

Vector Name	Functional Use	Cloning Strategy	Vector and Insert Digestion	Insert
pLXSN-MH1-GFP	Retroviral Expression/Stable expression of the MH1-GFP fusion protein	PCR amplification of insert and insertion into plasmid. MH1 was cloned from pLPCX-Flag-Smad3, with the desired restriction sites on each primer.	EcoRI XhoI	5'- GGAGAATTCATGGGTCGA CAATCG TC-3' 3'- ATTCTCGAGAGGAGGTAG AACTGGT -5'
pLPCX-Flag-MH1	Retroviral Expression/Stable expression of the Flag-MH1 fusion protein	Site-directed Mutagenesis of pLPCX-Flag-Smad3 plasmid. Inserting a stop codon and EcoRI restriction site after the MH1 domain.	EcoRI (used for digest confirmation)	5'- GAGTAGAGACACCAGTTC TACCTCCTTAGGAATT CCCACGCCACACAGAGAT CCCGGCC- 3' 3'- CTCATCTCTGTGGTCAAG ATGGAGGAATCCTTAAGG GT GCGGTGTGTCTCTAGGGC CGG- 5'
pEGFP-N3-MH1	Mammalian Expression of MH1-GFP fusion protein	PCR amplification of insert and insertion into plasmid. MH1 was cloned from pLPCX-Flag-Smad3, with the desired restriction sites on each primer	KpnI SacI	5'- GGAGAGTTCATGGGTCGA CAA TCGTCC-3' 3'- ACACCAGTTCTACCTCCT GGTACCAAT-5'

Site-Directed Mutagenesis of constructs was achieved using the Agilent Technologies Quick Change® Site-Directed Mutagenesis Kit (Mississauga, Ontario, Canada). Mutagenesis primer pairs were constructed using the ‘Agilent Genomics Primer Design Tool.’ PCR amplification of the plasmid was performed using PfuTurbo™ (Agilent Technologies, Mississauga, Ontario, Canada). Following amplification the products were digested for 1hr with DpnI (Agilent Technologies, Mississauga, Ontario, Canada) in order to remove parental plasmid DNA. After digestion the PCR products were transformed into XL-1Blue supercompetent bacteria via heat-shock. To see plasmids constructed using this method, consult Appendix A.

Construct integrity was assessed and confirmed using restriction digest patterns, followed by DNA sequencing (StemCore Laboratories, Sprott Centre).

### **Bacterial Transformation, Culture and Plasmid Preparation**

Heat-shock competent *Escherichia coli* of the DH5 $\alpha$  and XL-1 Blue strains were transformed by incubating 20-40  $\mu$ l of competent cells on ice for 30 minutes with plasmid DNA. Following incubation on ice the cells were heat shocked at 42°C for 25-45 seconds. Immediately following heat shock, warmed LB broth was added to cells and these were allowed to recover for 30 minutes at 37°C with shaking. Following recovery the cells were plated onto on agar plates containing 100mg/ml of ampicillin or 50mg/ml of kanamycin, depending upon the resistance gene encoded by the plasmid. After growth on antibiotic plates, individual colonies were picked from the plate and grown in LB broth-antibiotic (100mg/ml) overnight.

The next day, plasmid DNA was purified from the bacteria by alkaline lysis using the following solutions sequentially: Solution I (50mM glucose, 25mM Tris-HCl (pH8.0), 10mM EDTA (pH 8.0)), Solution II (0.2N NaOH, 1% SDS), Solution III (5M potassium acetate, glacial

acetic acid, ddH<sub>2</sub>O). Following lysis, ethanol precipitation was performed to remove salt and the dried DNA pellet was resuspended in a TE-RNase buffer.

### **Cell Culture**

3T3L1 cells ( American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) which contained 4.5g/L glucose, 110 mg/L sodium pyruvate and 584mg/L L-glutamine (Wisent Bioproducts, St-Bruno, QC, Canada), and was supplemented with 10% heat inactivated calf serum (HI-CS) (Invitrogen Canada Inc., Burlington, ON, Canada) and 81.4g/L MEM Non-essential Amino Acid solution (1X NEAA) (Wisent Bioproducts, St-Bruno, Qc, Canada). Cells were passaged every two days (1:4 split) to maintain them at subconfluent levels.

Phoenix™ Ampho packaging cells (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM which contained 4.5g/L glucose, 584mg/L L-glutamine, 110mg/L sodium pyruvate, 10% heat inactivated fetal bovine serum (HI-FBS) (Invitrogen Canada Inc., Burlington, ON, Canada) and 1X NEAA. Cells were passaged every two days (1:4 split) to maintain them at subconfluent levels.

10T1/2 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM which contained 4.5g/L glucose, 584mg/L L-glutamine, 110 mg/L sodium pyruvate, 10% HI-FBS and 1X NEAA. Cells were passaged every two days (1:4 split) to maintain them at subconfluent levels.

Human Mesenchymal Stem cells (Lonza, Walkersville, MD, USA) were maintained in Mesenchymal Stem Cell Growth Medium (MSCGM) (Lonza, Walkersville, MD, USA), supplemented with Mesenchymal Cell Growth Supplement (MCGS) (Lonza, Walkersville, MD,

USA), L-glutamine (Lonza, Walkersville, MD, USA) and gentamicin sulphate and amphotericin B (Lonza, Walkersville, MD, USA). Cells were passaged to maintain them at subconfluent levels.

## **Retroviral Infection**

### **Phoenix<sup>TM</sup> Ampho packaging cell Transfection**

Calcium phosphate transfection was used to create replication incompetent pLPCX, pLPCX-Smad3, pLPCX-MH1, pLXSN-GFP and pLXSN-MH1-GFP retroviruses (Clontech Laboratories, Inc., Mountain View, CA, USA) from Phoenix<sup>TM</sup> Ampho packaging cells. Prior to DNA transfection, medium containing 25  $\mu$ M chloroquine diphosphate (Sigma-Aldrich, Oakville, ON, Canada) was added to  $1.5 \times 10^6$  Phoenix cells in a 60mm dish, to inhibit lysosomal fusion with plasmid-containing endosomes. 10 $\mu$ g of Plasmid DNA was diluted in 428  $\mu$ l of ddH<sub>2</sub>O and 62  $\mu$ l of CaCl<sub>2</sub> was added to the diluted DNA. Subsequently, 500  $\mu$ l of 2X HBS (10mM KCl, 50mM HEPES pH 7.05, 12mM dextrose, 1.5mM Na<sub>2</sub>HPO<sub>4</sub> and 280mM NaCl) was added. The DNA solution was mixed quickly by inversion and added dropwise to the Phoenix cells, within two minutes of HBS addition. While adding dropwise, the Phoenix dish was swirled gently to evenly distribute the DNA mixture. The Phoenix cells were incubated for 48hours following transfection and medium containing virus was removed from the Phoenix cells and filtered using a 0.45  $\mu$ m syringe filter (Millipore, Billerica, MA, USA). After filtering, the fresh virus was used for transduction or stored at -80°C.

### **Infection of 3T3-L1 and 10T1/2 cells**

Early in the day, approximately 50% confluent 10cm dishes of 3T3-L1 and 10T1/2 cells were infected with 1mL of fresh virus in 5mL DMEM which contained 6 ug/ $\mu$ L of polybrene (Sigma-Aldrich, Oakville, ON, Canada). At the end of the day, 4mL of DMEM was added to each dish to dilute polybrene and stop infection. Selection began 48 hours after transduction in DMEM containing either 400  $\mu$ g/mL final of G418 or 2  $\mu$ g/mL final of Puromycin (Sigma-Aldrich, Oakville, ON, Canada) depending on the plasmids resistance (G418 for pLXSN and Puromycin for pLPCX). Selection was continued for 7 days in order to eliminate uninfected cells.

### **Differentiation of 3T3-L1 cells**

$1 \times 10^5$  3T3-L1 cells were plated in each well of a 6-well plate and allowed to reach confluence in maintenance medium. Two days post-confluence, the cells were treated with DMEM supplemented with a hormonal cocktail (MID) containing 100nM insulin (Roche, Mannheim, Germany),  $1 \times 10^{-6}$ M dexamethasone (Sigma-Aldrich, Oakville, ON, Canada) and 500  $\mu$ M isobutylmethylxanthine (Sigma-Aldrich, Oakville, ON, Canada) in the presence or absence of  $1 \times 10^{-6}$ M retinoic acid (RA) (Sigma-Aldrich, Oakville, ON, Canada), using equivalent volume of 100% ethanol vehicle in its absence. Two days post MID treatment, the medium was changed to DMEM containing 100nM insulin in the presence or absence of RA. This medium was used to replace the cell medium every two days as required by differentiation, for a total of 8-10 days. After 8-10 days, differentiation was assessed through Oil Red O staining and Western Analysis.

### **Oil Red O staining**

Once 3T3-L1 adipogenic differentiation was achieved, the cells were rinsed once with 1x phosphate buffered saline (PBS) and fixed in 10% phosphate buffered formalin for 30 minutes. Following fixing, the cells were rinsed once with tap water, then again with distilled water, draining excess water after rinsing. Next, the cells were washed twice with 1,2-propandiol for 5 minutes after which the cells were incubated with oil red o (ORO) (Sigma-Aldrich, Oakville, ON, Canada) for 30 minutes, or until neutral lipid staining could be verified under the microscope. Following ORO incubation, the cells were washed once with 85% 1,2-propandiol for 3 minutes, to remove excess ORO stain, and rinsed with distilled water. Cells were then stained for 1 minute with hematoxylin, which was subsequently rinsed off with tap water and distilled water. At this point the cells were ready to be visualized under the microscope.

### **Western Analysis and Immunodetection**

#### **Preparation of cell protein extracts**

Cells were initially washed twice with 1x PBS and scraped from the dish using a rubber policeman in either IPH buffer (50mM Tris pH 7.5, 150mM NaCl, 0.5% NP-40, 5mM EDTA and freshly added 1X Protease Inhibitor and 1mM DTT) or 8M Urea (Fisher, Ottawa, ON, Canada). Following removal from the dish, the cell suspensions were incubated on ice for 30 minutes after which the samples were sonicated for 10 seconds at 10% power output, using a Branson Sonifier-450 (Branson, Danbury, CT, USA). Following sonication, the samples were centrifuged for 10 minutes at 13 000 rpm (4°C), to insoluble cellular components.

#### **SDS-PAGE and PVDF membrane transfer**

The concentration of protein in each sample was measured using a Beckman spectramax M2 spectrophotometer. Following protein concentration measurements the desired amount of protein from each sample (25-5 $\mu$ g) was mixed in a 3:1 ratio with 3X Sodium Dodecyl Sulfate (SDS) loading dye, followed by heating to 95°C to induce protein denaturation and allow the SDS to bind the proteins. After the SDS-PAGE was complete (voltage and time varied), the proteins were transferred onto a PVDF membrane (Kankakee, Illinois, USA) at 110V for 1hr. After transfer, the membranes were incubated with blocking solution for one hour, followed by incubation in primary antibody or primary antibody in blocking solution, overnight. The next day, the blots were removed from primary antibody and washed with 1X PBS +0.25% Tween-20 (PBS-T) five times, for 5 minutes each time. Following washing, the membrane was incubated with secondary antibody for one hour at room temperature and subsequently washed five times, for 5 minutes each, with PBS-T. Subsequently, the western blot was analyzed using the ECL chemiluminescent detection system (GE Healthcare, Waukesha, WI, USA). Images were taken using a Fujifilm LAS-4000 chemiluminescent detection system (Fujifilm, Fremont, CA, USA).

### **Antibodies**

The following primary antibodies were used from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, at a 1:400 concentration, to immunoblot membranes: C/EBP $\alpha$  (14AA), C/EBP $\beta$  (C-19), Smad2/3 (FL-425), p-Smad2/3 (Ser 423/425), HDAC1 (C-19),  $\alpha$ -Tubulin (B-7, 1:10 000), PPAR $\gamma$  (H-100). The following secondaries were used to visualize protein migration through chemiluminescence: anti-rabbit and anti-mouse (each used at a concentration of 1:50 000 from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); and anti-goat (used at a concentration of 1:50 000 from GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK).

The following primary antibodies were used from Sigma Aldrich, Inc., St. Louis, MO, USA, for either immunoblotting membranes or indirect immunofluorescence (IIF):  $\beta$ -Actin (AC-74, 1:10 000), FLAG (SIG1-25, 1:100). For  $\beta$ -Actin, used during immunoblotting, the secondary antibody used was anti-mouse(used at a 1:50 000 dilution from GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK). For the FLAG antibody, which was used during IIF, the secondary anti-rabbit antibody DyLight™ 488 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was used.

The GFP antibody (clones 7.1 and 13.1; 1:1 000 dilution; Roche, Mannheim, Germany) was used for immunoblots.

The  $\beta$ -Tubulin antibody (1:100 dilution), was produced in the lab using the E7 Hybridoma cell line (Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences, Iowa City, IA).

### **Image Analysis**

After taking the images, band quantification was performed using ImageJ v1.44-Image analysis software or, Multiguage 2.0 Image analysis software. Protein bands were quantified into arbitrary units and normalized using either an Actin or  $\beta$ -Tubulin loading control.

### **RNA Isolation and cDNA synthesis**

All RNA harvests were performed using a Qiagen RNeasy minikit (supplier). Cells were grown, ensuring that they did not reach confluence. At the time of RNA harvest, the medium was

aspirated and the cells were briefly washed with 1X PBS. After washing, 350  $\mu$ l of freshly prepared RLT Buffer (with  $\beta$ -mercaptoethanol) was added to the cells. Cell lysate was collected using a rubber policeman and pipetted to a sterile microtube. Following harvest, the cells were homogenized for 30 seconds using a tissue homogenizer. Following homogenization, 350  $\mu$ l of 70% ethanol was added and the suspension mixed immediately by pipetting. The homogenate was then spun down in an RNeasy spin column for 15 seconds at 12 000RPM. The column was then washed with 700  $\mu$ l of buffer RW1 and centrifuged again for 15 seconds at 12 000RPM. 500  $\mu$ l of buffer RPE was then added to the RNeasy spin column and centrifuged for 15 seconds at 12 000RPM, this last step was repeated, spinning for 2 minutes. Following this spin, a dry spin was performed for 1 minute at 12 000RPM, to remove residual RPE buffer in the column. The RNeasy spin column was then placed in a 1.5 ml tube and RNA eluted using 50  $\mu$ l of RNase-free water, spinning for 1 minute at 12 000RPM. Concentration of RNA was further increased by repeating this same elution with the eluate of the first spin.

Complementary DNA (cDNA) was synthesized using the BioRAD iScript cDNA synthesis kit (BioRAD, Hercules, CA, USA) according to manufacturer's instructions, which contains random hexamers, capable of amplifying non-poly-A tail mRNA's (18S rRNA-loading control).

## **Cell Treatments**

### **Actinomycin-D**

Human mesenchymal stem cells were grown to 70% confluence in 6cm plates and subsequently treated in the presence or absence of Actinomycin-D (10 µg/ml) (Sigma-Aldrich, Oakville, ON, Canada). Cells were harvested at 1, 2, 4 and 8 hours after treatment and RNA was isolated using the Qiagen RNeasy mini kit.

## **MG132**

Pooled 3T3-L1 stables expressing MH1-GFP, were grown to 70% confluence and treated with MG132 (25µM) (Sigma-Aldrich, Oakville, ON, Canada), and protein harvested at 0, 8 and 16 hours. Protein analysis was performed using SDS-PAGE as previously described.

## **5-Azacytidine**

3T3-L1 cells were incubated with growth medium containing 3µM 5-Azacytidine (Sigma-Aldrich, Oakville, ON, Canada) for 24 hours. The next day, the medium was replaced and cells were treated with Retinoic Acid or vehicle. 24hours post RA treatment total RNA was harvested from the cells.

## **Nucleocytoplasmic Fractionation**

10T ½ cells were grown to 100% confluence and treated with RA for 48 hours post confluence. After 48 hours the cells were washed with 1X PBS and scraped off the dish, using a rubber policeman, into a microtube. The microtube was centrifuged at 4°C, 2000 rpm for five minutes, to pellet the cells. The pellet was then washed using 1ml of ice cold 1X PBS and spun at 4°C, 10 000 rpm for one minute. Following centrifugation the pellet was dissolved in 60 µl of hypotonic buffer (10mM Tris-base, 10mM KCl, 1.5mM MgCl<sub>2</sub>, ddH<sub>2</sub>O and β-mercaptoethanol). Next, the cells were spun at 4°C, 10 000 rpm for 30 seconds and the pellet was dissolved in 80 ul

of lysis buffer (Hypotonic Buffer and 0.4% NP-40). The suspension was incubated on ice for 10 minutes. Following incubation, the lysate was spun at 4°C, 10 000 rpm for one minute. At this point the supernatant containing the cytosolic extract was stored. The pellet was further washed with 0.02M KCl buffer (20mM Tris-base (pH 7.3), 21.75% glycerol, 1.5mM, 1.5mM MgCl<sub>2</sub>, 0.2mM EDTA, 0.02M KCl, ddH<sub>2</sub>O and β-mercaptoethanol) and spun at 4°C, 10 000 rpm for one minute. Following this centrifugation, the pellet was dissolved in 15 µl of 0.02M KCl buffer, followed by dropwise addition of 60 µl of 0.6M KCl buffer (20mM Tris-base (pH 7.3), 21.75% glycerol, 1.5mM, 1.5mM MgCl<sub>2</sub>, 0.2mM EDTA, 0.6M KCl, ddH<sub>2</sub>O and β-mercaptoethanol), gently shaking at 4°C for 30 minutes. After shaking, the suspension was centrifuged at 4°C, 10 000rpm for 15 minutes. At this point the supernatant containing the nuclear extract was stored. Both the nuclear and cytosolic extracts were then resolved by SDS-PAGE.

## **Immunofluorescence**

### **Preparation of Poly-L-lysine Coverslips**

Briefly, coverslips were dipped in ethanol, flame sterilized, then dipped in poly-l-lysine and left to dry. After drying, the coverslips were rinsed with sterilized ddH<sub>2</sub>O and left to dry before storage.

### **Staining**

Cells were grown to desired confluency on poly-l-lysine (Sigma-Aldrich, Oakville, ON, Canada) coated coverslips, in 6-well tissue culture plates. Cells were rinsed once with 1X PBS

and fixed in ice-cold methanol for 15 minutes at room temperature with gentle shaking. Following fixation, the cell coated coverslips were washed with ice-cold PBS for 3 minutes with gentle shaking, twice. The fixed cells are then incubated for 10 minutes with PBS containing 0.25% Triton X-100 to permeabilize them. Following permeabilization, the coverslips were washed three times with ice-cold PBS for 3 minutes. This step is optional, but the cells were incubated with 2% Donkey Serum in 1X PBS-Tween for 30 minutes to block non-specific binding. Following this blocking step, the cells were treated overnight with primary antibody at a desired concentration.

After overnight incubation with Rabbit anti-FLAG antibody (1:100 dilution, Sigma Aldrich, Inc., St. Louis, MO, USA), the coverslips were washed with 1X PBS three times, 3 minutes each and then incubated with a desired dilution of fluorescent secondary antibody, anti-Rabbit DyLight™ 488 (1:200 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for one hour (in the dark). Following this incubation, the cells were washed three times with 1X PBS for 3 minute each and mounted on microscope slides using vectashield (Vector Laboratories Inc., Burlingame, CA, USA), which contains the nuclear stain 4',6-diamidino-2-phenylindole (DAPI). Coverslips were sealed onto the microscope slides with nail polish.

## **Studies of Protein-Protein Interaction**

### **Whole cell harvest**

PLXSN-GFP and PLXSN-MH1-GFP pooled stable clones were grown to approximately 70-90% confluence in a 15cm dish. The cells, still attached to the 15cm dish, were washed once with 2ml of 1X PBS. Following washing, 2ml of 1X PBS was added again to the cells and there were scraped from the dish with a rubber policeman. After scraping, the cells were collected into a 15ml tube and spun down at 2000rpm (4°C) for 5 minutes. Following centrifugation, the cell pellet was resuspended in 100ul of TEDG buffer (10mM Tris-base (pH 7.4), 1mM EDTA, 10% glycerol, 20mM Molybdate; and 1mM DTT and 1X protease inhibitor, added fresh before use).

### **Non-denaturing protein extraction or Whole cell protein extraction**

Following resuspension in 100 ul TEDG buffer the cells were allowed to swell for 10 minutes at 4°C. This mixture was then subjected to four freeze/thaw cycles as follows: 2 minutes in dry ice methanol bath, 2 minutes in 37°C waterbath, vortex 10 seconds, repeat. After four freeze/thaw cycles, the samples were spun at 13 000rpm for 2 minutes at 4°C to pellet cell debris.

### **GST and GST-fusion protein preparation**

Briefly, bacteria containing the pGEX2T-C/EBP $\beta$  plasmid (Appendix table 1) were grown to an OD of 0.6-0.8 at 37°C and 220 rpm shaking. Once the bacteria reached this optical density, they were induced using 300mM Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) and further incubated for two hours at 37°C and 220 rpm shaking. After shaking the bacteria were lysed using GST-lysis buffer (25mM Hepes (pH7.9), 100mM KCl, 2mM EDTA, 20% Glycerol, ddH<sub>2</sub>O, 2mM DTT, 2X Protease Inhibitor (Roche, Mannheim, Germany), Nonidet P-40 (NP-40)) and the lysates were incubated with glutathione-agarose beads (BD Biosciences Pharmingen, Mississauga, ON, Canada), to capture GST-tagged proteins for 60 minutes at 4°C. Following

capture, the beads were washed with GST-lysis buffer to remove non-specific interacting proteins. The GST and GST-fusion proteins were quantified on an SDS-PAGE gel stained with Coomassie blue, alongside several BSA dilutions: 0.25 µg, 0.5 µg, 1 µg, 2 µg, 5 µg, 7.5 µg. GST fusion protein yield was determined by densitometric analysis.

### ***In vitro* pulldown**

0.5 ug of GST, or GST-CEBPbeta protein, were used. To correct for differing bead amounts between the highly expressed GST and the lower expressed GST-C/EBPβ, an equalizing volume of empty beads was used in each pull-down. Equivalent amounts of both proteins were incubated with 50 of whole cell protein extract in 120 µl of Lysis buffer and ddH<sub>2</sub>O. This mixture was incubated at 4°C, rotating for 90 minutes. Following incubation the beads are washed four times with 500 µl of binding buffer (0.6X GST lysis buffer), centrifuging at 4000 rpm for 2 minutes, and resolved by SDS-PAGE.

### **Statistical Analysis**

Statistical analysis was performed using a student's two tail T-TEST with a confidence interval of 95%. Therefore only data with a p-value of <0.05 was considered significant.

## RESULTS

### Chapter 1

#### Expression of a Flag-tagged MH1 domain inhibits C/EBP $\beta$ DNA binding

Given that Smad3 had been demonstrated by our lab to be an important effector of RA action during adipogenesis and that the MH1-domain is important for the interaction between Smad3 and C/EBP $\beta$ , our lab sought to determine whether the MH1 domain alone could recapitulate the effects of RA on adipogenesis .

To investigate the effect of Smad3 MH1 domain expression on adipogenesis, we created a retroviral construct driving the expression of an N-terminally FLAG-tagged MH1 fusion protein. Primers were designed with the Agilent Genomics Primer Design Tool to introduce a stop codon in the pLPCX-FLAG-SMAD3 retroviral construct cloned by Choy et al. . An EcoRI restriction site was introduced after the stop codon ensuring efficient screening of mutagenized clones. Following confirmation of mutagenesis by DNA sequencing, the mutagenized construct was packaged into retroviruses using the Phoenix<sup>TM</sup> Amphi packaging cell line and replication incompetent retrovirus was used to transduce 3T3-L1 mouse preadipocytes. As a negative control, retrovirus produced with the parent vector was created (pLPCX).

Following transduction, expression of the MH1 domain was assessed by Western blotting using an antibody specific for the FLAG epitope tag. Despite extensive troubleshooting to ensure that the FLAG antibody was functioning and to optimize the Western conditions, detection of the MH1 construct was unsuccessful in preadipocytes. However, human mesenchymal stem cells (hMSCs) infected with the FLAG-MH1 virus had detectable levels of the fusion protein, confirming that the construct was functional (Figure 1). In silico analysis of

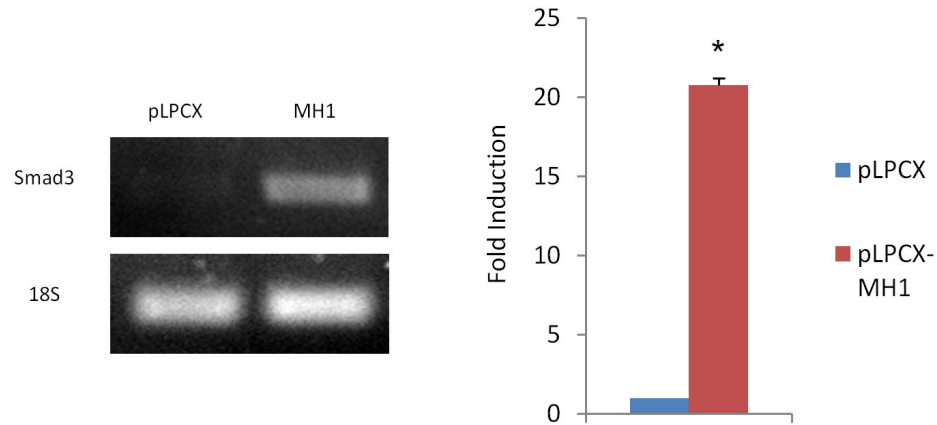
the predicted FLAG-MH1 protein stability in mammalian cells using the ExPASy ProtParam tool, determined an instability index value of 41.86 (unstable proteins > 40, stable proteins < 40), leading to the prediction that rapid protein turnover was responsible for the inability to visualize the protein product by immunoblotting. We analyzed the level of expression of FLAG-MH1 mRNA as a means of demonstrating that the MH1 construct was indeed expressed in preadipocytes. After total RNA harvest of 3T3-L1 stable cell lines, the expression of the FLAG-MH1 mRNA was determined by RT-qPCR (Figure 2). The FLAG-MH1 mRNA was expressed approximately 20-fold over background empty vector pLPCX, suggesting that the retroviral construct was able to drive MH1 expression in preadipocytes.

### **Expression of the FLAG-MH1 construct inhibits C/EBP $\beta$ DNA binding and adipogenesis**

Before we could assess the ability of the MH1 domain to inhibit C/EBP $\beta$  DNA-binding and adipogenesis, we wished to ensure that it was capable of entering the nucleus. Due to the inability to visualize the FLAG-MH1 construct through Indirect Immunofluorescence, we decided to add a GFP tag to the MH1 domain to both increase its stability and facilitate its visualization through indirect immunofluorescence within the cells. To determine intracellular localisation of the MH1 protein, we created a new MH1 construct, fused to the N-terminus of eGFP, in the parent mammalian expression plasmid peGFP-N3. Following cloning, we



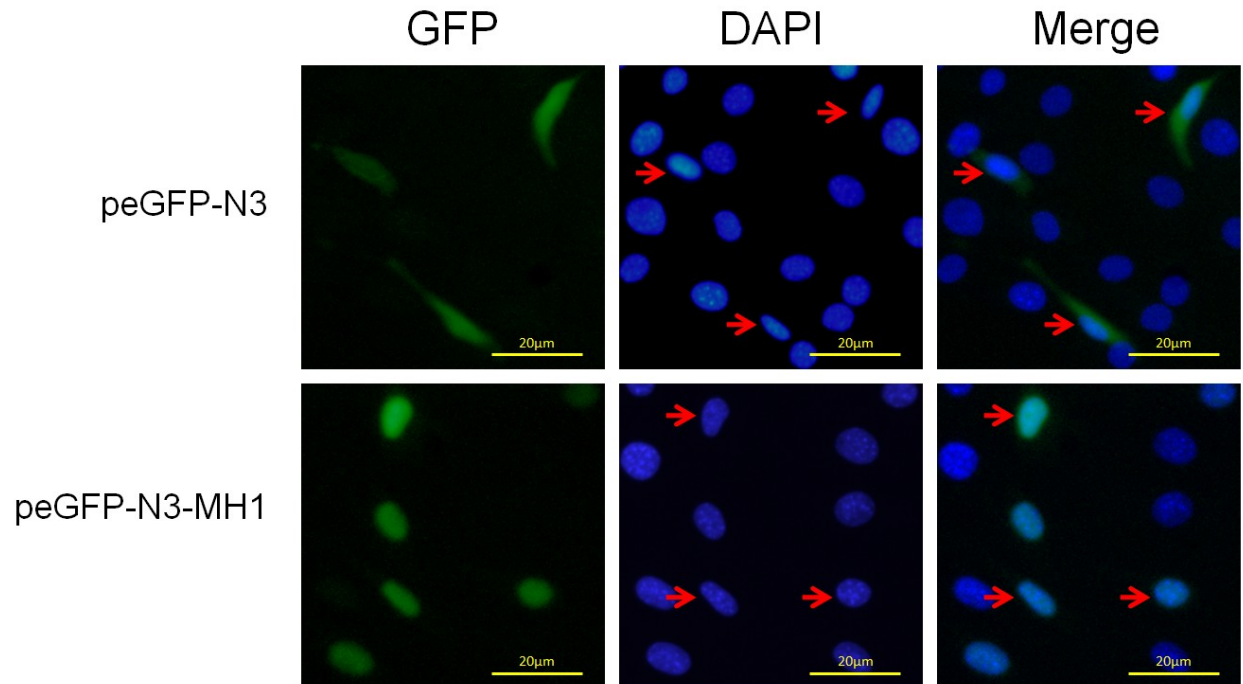
**Figure 1. FLAG-MH1 construct is capable of expression in human mesenchymal stem cells (hMSCs).** hMSCs were retrovirally transduced with empty vector pLPCX or pLPCX-FLAG-MH1. The expression of FLAG-MH1 was detectable in hMSCs using the mouse monoclonal anti-FLAG M2 antibody. Actin is used as a loading control.



**Figure 2. FLAG-MH1 mRNA is expressed.** RT-PCR of total RNA from 3T3-L1 stables infected with empty vector pLPCX or pLPCX-FLAG-MH1. Primers specific for the FLAG-MH1 mRNA were used for RT-PCR, visualized by agarose gel electrophoresis (left) and quantified by RT-qPCR (right) to determine the fold induction over empty vector.

transfected the empty vector peGFP-N3 and the cloned peGFP-N3-MH1 construct into 3T3-L1 cells. Twenty-four hours post-transfection, the cells were fixed and fluorescence assessed (Figure 3). In the empty vector peGFP-N3 control transfected cells, eGFP signal was dispersed evenly throughout the cell. This observation was consistent with other published reports indicating that GFP is equally distributed in the nucleus and cytoplasm. However, in the MH1-eGFP expressing cells, the eGFP signal was located solely in the nucleus and not dispersed throughout the cytoplasm like wild-type eGFP. This result is consistent with previous reports as the MH1 domain of Smad3 contains a nuclear localisation sequence (NLS) which is responsible for the nuclear localization of the MH1-eGFP fusion construct.

In early adipogenesis, C/EBP $\beta$  and C/EBP $\delta$  are regulators of commitment and promote the transcription of downstream target genes such as C/EBP $\alpha$ , an important target gene necessary for sensitization of adipocytes to insulin signalling and the induction of PPAR $\gamma$  expression. There is one C/EBP response element within the C/EBP $\alpha$  promoter to which C/EBP $\beta$  and C/EBP $\delta$  are able to bind and activate transcription of C/EBP $\alpha$ . Previous studies in our lab demonstrated that RA inhibited C/EBP $\beta$  DNA binding to this element after 48 hours of hormonal induction treatment, which depended upon the presence of Smad3. Furthermore, our lab determined that the MH1 domain was required for the interaction between C/EBP $\beta$  and Smad3. Given that the MH1 domain is localized to the nucleus in the absence of RA or TGF $\beta$  signalling, we wanted to determine whether the MH1 domain could interfere with C/EBP $\beta$  DNA binding at the C/EBP $\alpha$  promoter in the absence of RA.

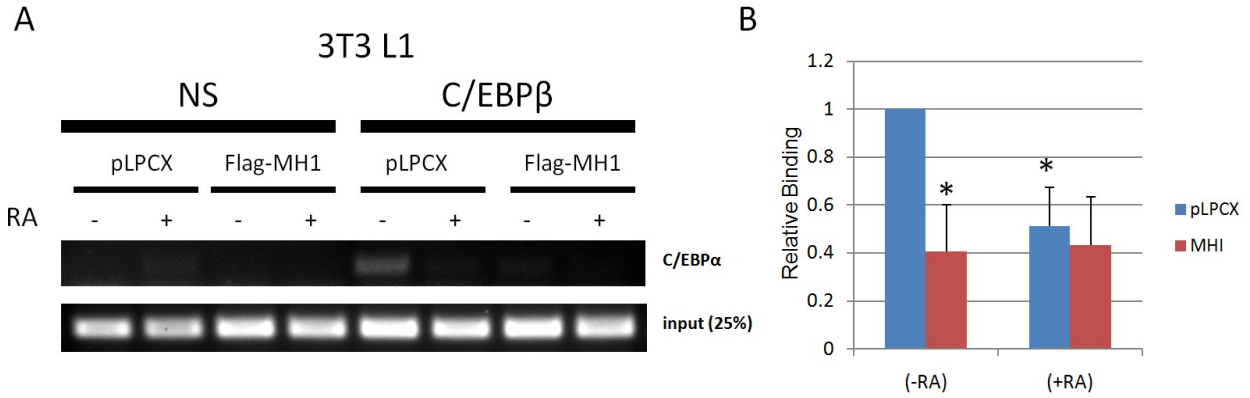


**Figure 3. MH1-eGFP is predominantly nuclear.** 3T3-L1 cells were transiently transfected with parent vector (GFP) or MH1-eGFP fusion construct. Twenty-four hours following transfection GFP expression was analyzed by direct fluorescence. Nuclei were visualized with DAPI staining. Scale bar is 20µm.

To evaluate the ability of C/EBP $\beta$  to interact with the C/EBP $\alpha$  promoter in the presence of ectopic MH1 domain, a chromatin immunoprecipitation (ChIP) assay of C/EBP $\beta$  at the C/EBP $\alpha$  promoter was performed on MH1-expressing and empty vector control stable cell lines (Figure 4). In the pLPCX control cells in the absence of RA C/EBP $\beta$  interacted with the C/EBP $\alpha$  promoter, whereas RA reduced C/EBP $\beta$  occupancy (Figure 4).

Both findings are consistent with our lab's previous results . In the MH1-expressing stables, there was reduced binding in both the presence and absence of RA, to levels comparable to RA treatment alone. This data demonstrates that the MH1 domain is capable of recapitulating the inhibition on C/EBP $\beta$  DNA binding in the absence of RA.

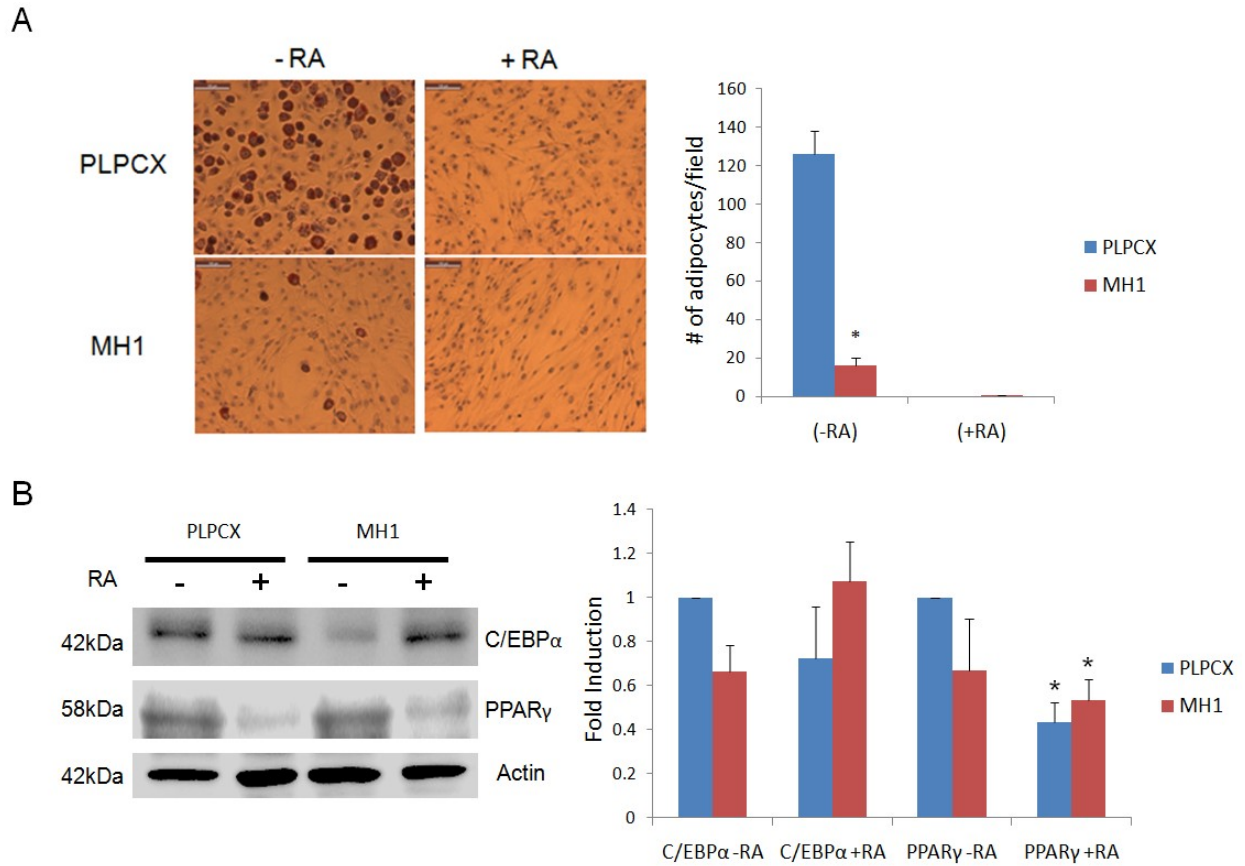
The 3T3-L1 stable cell lines were next induced to differentiate into adipocytes to determine whether expression of the MH1 domain could inhibit adipogenesis (Figure 5). Briefly, empty vector (pLPCX) and MH1-expressing cultures were differentiated according to standard procedures and stained with the lysochrome dye Oil Red O to reveal neutral lipid vesicles within mature adipocytes (Figure 5A). Adipocyte counts for four randomly selected fields revealed a significant six-fold decrease in differentiated adipocytes in the MH1-expressing cells as compared to empty vector pLPCX controls. As previously shown, mature adipocytes were absent in the RA treated wells. The cells were harvested at day 10 after induction to differentiate and Western analysis was performed to measure the expression of the master regulators of adipogenesis: C/EBP $\alpha$  and PPAR $\gamma$  (Figure 5B). In the vehicle-treated condition, the MH1 stable cells had reduced C/EBP $\alpha$  protein expression as compared with empty vector cells but this effect was not statistically significant. In the +RA condition, there was high



**Figure 4. The Smad3 MH1 domain reduces C/EBPβ occupancy of the C/EBPα promoter *in vivo*.**

**A)** Chromatin immunoprecipitation (ChIP) analysis of C/EBPβ occupancy of the C/EBPα promoter in stable 3T3-L1 cells, expressing empty vector (pLPCX) or Flag-MH1 fusion protein, following treatment with ethanol vehicle or RA for 48hrs. Isolated chromatin was immunoprecipitated using anti-C/EBPβ antibody (C/EBPβ) or with a non-specific type matched Rabbit IgG antibody (NS).

**B)** Quantitative PCR analysis of the C/EBPα promoter following ChIP. pLPCX (-RA) corrected Ct value set at 1 and all other corrected Ct values were measured relative to pLPCX (-RA). Data represents three independent experiments and error bars are the standard error of the mean (\* p<0.05).



**Figure 5. MH1 mediated inhibition of adipogenesis is detectable through Oil Red O staining**

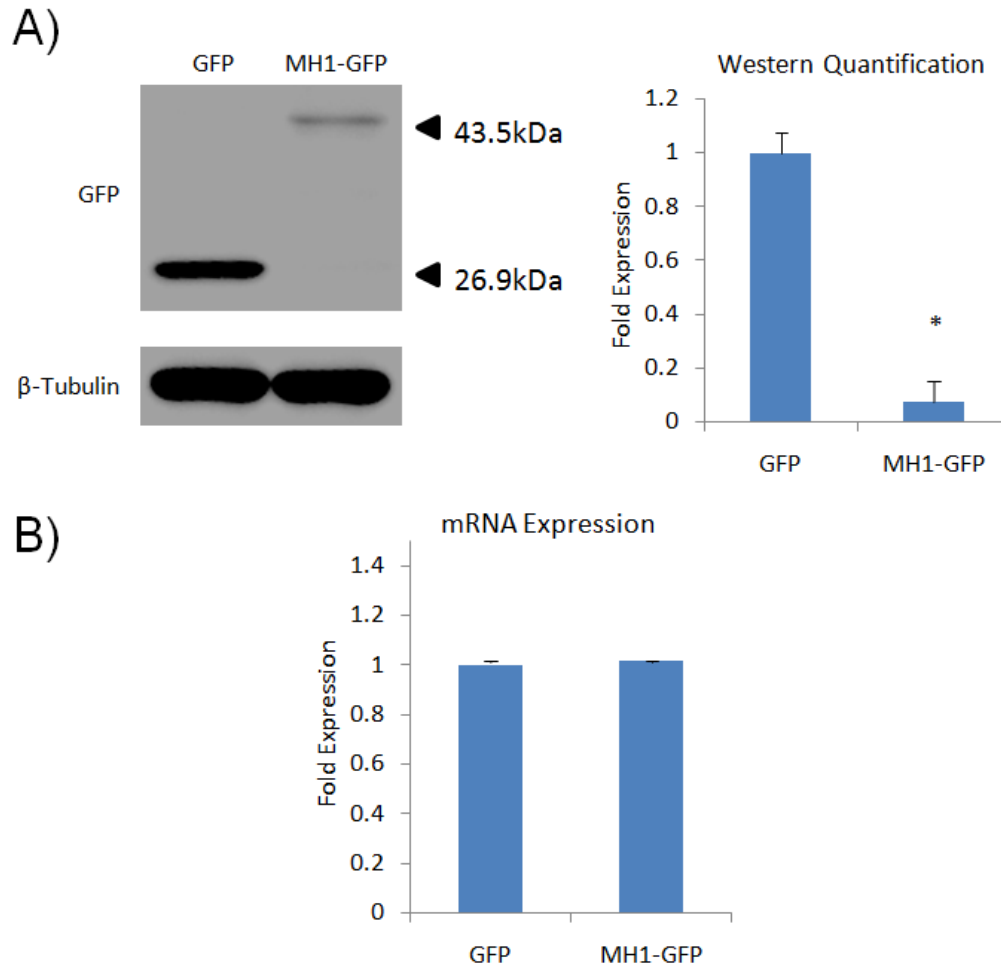
A) Oil Red O staining of empty vector and MH1-expressing 3T3-L1 pooled stable cell lines. Stable cells were subjected to differentiation in the presence of MID; after ten days of differentiation, the cells were fixed and stained with Oil Red O. Pictures were taken of four random fields in each dish and the number of adipocytes was enumerated. Numbers represent the average of four fields and error bars represent the standard error of the mean (\*  $p < 0.05$ ). ( $n=4$ ). Scale bar represents 100  $\mu\text{m}$  and field area represents 401, 111.11  $\mu\text{m}^2$ .

B) Western blot and quantification of four independent adipogenesis trials. Cells were grown according to the adipogenesis protocol and the expression of master regulators of adipogenesis (C/EBP $\alpha$  and PPAR $\gamma$ ) was quantified using the multigaugue analysis program. Protein levels were normalized to an actin loading control and compared to the PLPCX -RA condition for each protein. Error bars represent the standard error of the mean (\*  $p < 0.05$ ).

variability in C/EBP $\alpha$  levels in both the pLPCX and MH1 stables and no decrease in C/EBP $\alpha$  expression due to RA treatment was observed. The highly variable levels of C/EBP $\alpha$  at day 10 of differentiation could potentially be the result of alternative pathways which are capable of inducing C/EBP $\alpha$  expression . In accordance, high levels of C/EBP $\alpha$  have been demonstrated in mice null for both C/EBP $\beta$  and C/EBP $\delta$  . These mice demonstrate wild-type levels of both C/EBP $\alpha$  and PPAR $\gamma$  in undifferentiated cells of fat pads, suggesting that other pathways are capable of promoting the expression of C/EBP $\alpha$  .

### **MH1-GFP protein is expressed but actively degraded**

Due to the lability of the MH1 protein and inability to detect it through Western analysis, we decided to tag the MH1 construct with GFP in an attempt to improve its stability. The MH1-GFP fusion protein, predicted instability index value of 33.73, was created in a retroviral vector (pLXSN) and virus was created using the Phoenix<sup>TM</sup> Amphi packaging cell line. 3T3-L1 preadipocytes were transduced with virus prepared using the pLXSN-GFP or pLXSN-MH1-GFP constructs. Western analysis was performed using a GFP antibody to determine the expression level of the pLXSN-MH1-GFP construct in 3T3-L1 stable cells. The MH1-GFP fusion protein was detected (Figure 6A), however a notable discrepancy between the expression of GFP alone from the same promoter and the MH1-GFP construct was noted (significant 10-fold reduction). Analysis of the mRNA expression of these two constructs demonstrated that there was no significant difference in the transcription of these genes and that both genes were transcribed equally (Figure 6B). This suggested that the MH1 domain was destabilizing the GFP protein.



**Figure 6. GFP and MH1-GFP expression in pooled stable cell lines.**

**A)** Pooled stable cell lines were created using the vectors PLXSN-GFP and PLXSN-MH1-GFP, and protein expression analyzed via western analysis, immunoblotting for GFP and  $\beta$ -tubulin. Bands were subsequently quantified using ImageJ gel analysis software. Fold Expression, represents the  $\beta$ -tubulin loading control corrected intensity values. Data represents three independent experiments and error bars are the standard error of the mean (\*  $p < 0.05$ ).

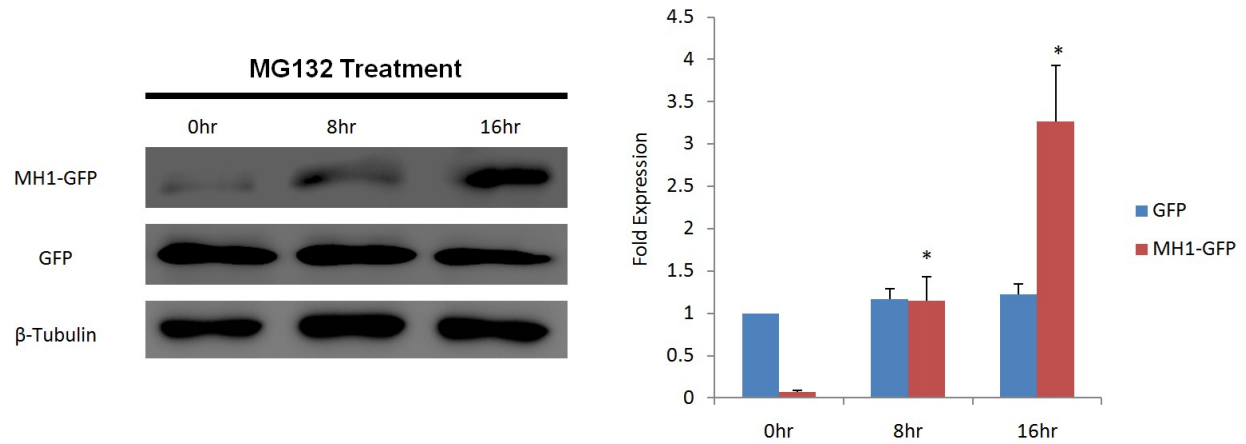
**B)** RT-qPCR analysis of GFP and MH1-GFP mRNA expression. Primers amplifying a region within the GFP cDNA were used. mRNA Ct values were corrected using the 18S loading control Ct value and data is shown as fold expression over the GFP control. Data represents three independent experiments and error bars are the standard error of the mean.

To further investigate whether the low levels of MH1-GFP expression was a result of active degradation of the MH1 domain, we treated these pooled stable cell lines with MG132, a potent 26S proteasome inhibitor (Figure 7). At time 0, the 10-fold disparity between MH1-GFP and GFP expression was observed. At 8 hours post-MG132 treatment, MH1-GFP expression was increased 10-fold as compared to the 0hr timepoint, while GFP levels remained unchanged. After 16 hours of MG132 treatment, MH1-GFP levels continued to increase reaching a 30-fold increase as compared to time 0. This result demonstrates that fusion of MH1 to GFP contributes to the increased turnover of the MH1-GFP fusion protein, resulting in a baseline expression that is 10-fold lower than the GFP control. Therefore, the MH1 domain is actively degraded.

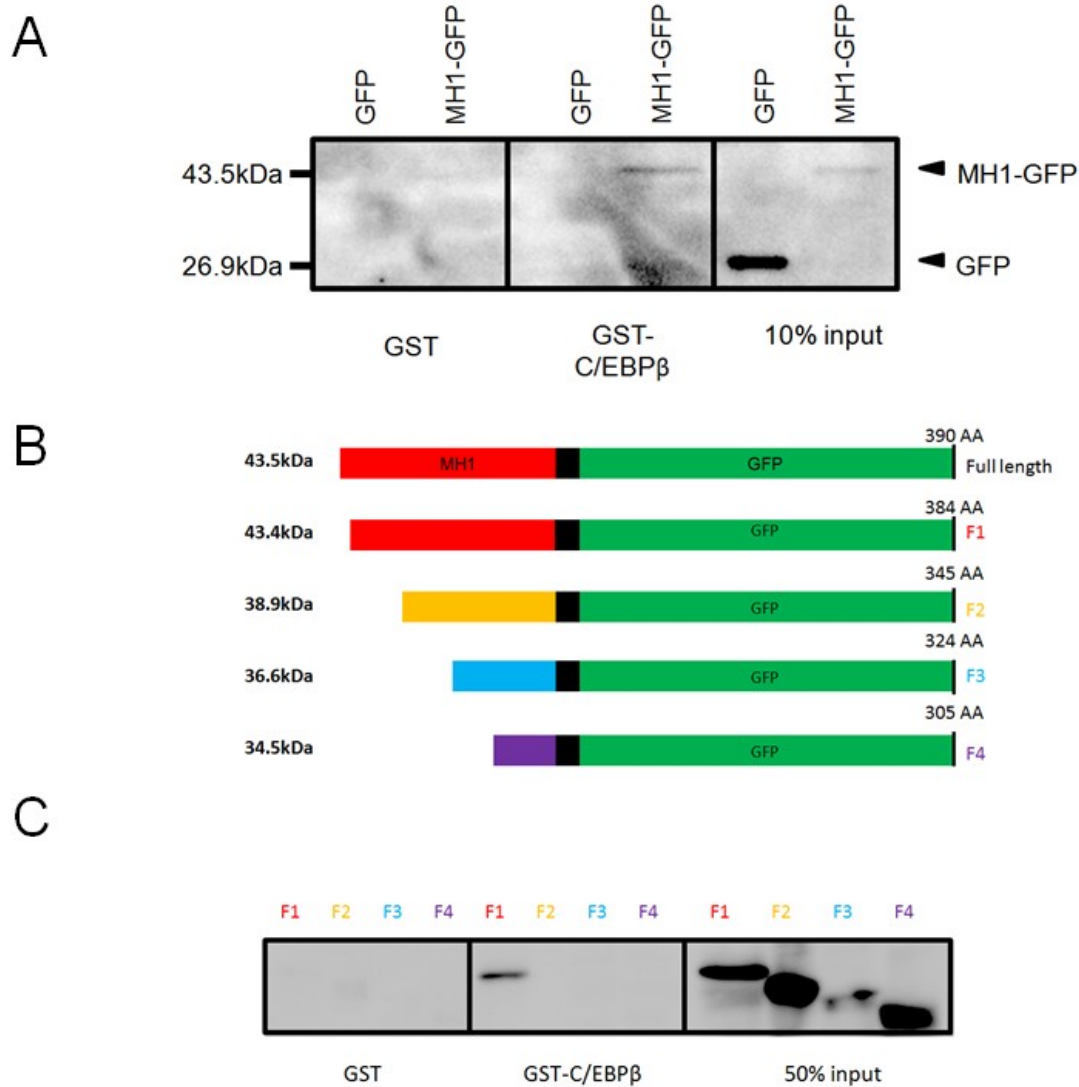
### **MH1-GFP protein is capable of interacting with C/EBP $\beta$**

With the addition of the GFP tag, and the increase in MH1-GFP construct mass, we sought to affirm its ability to interact with C/EBP $\beta$  using a GST-pulldown assay (Figure 8A). Whole cell extracts from GFP and MH1-GFP expressing cells were incubated with GST or GST-C/EBP $\beta$ . GST alone demonstrated no interaction with GFP or MH1-GFP. Incubation with GST-C/EBP $\beta$  revealed no *in vitro* interaction between C/EBP $\beta$  and GFP, but it did show an interaction between MH1-GFP and GST-C/EBP $\beta$  (10% of input) (Fig. 8A). This demonstrated that the MH1 domain was still capable of interacting with C/EBP $\beta$  *in vitro*, despite its GFP tag.

The ability to visualize the MH1-GFP construct via Western analysis allowed us to map the interaction between MH1 and C/EBP $\beta$ . Four MH1-GFP truncated protein fragments were produced by PCR amplification and subsequent *in vitro* translation (Figure 8B). Fragment one is a deletion of the first six amino acids in the MH1 domain, fragment two is a deletion of the first



**Figure 7. MH1-GFP fusion protein is degraded by the proteasome.** Pooled stable cell lines expressing GFP or MH1-GFP were treated with the 26S proteasome inhibitor MG132 for 8 and 16 hours. Following treatment, protein expression was analyzed via Western analysis for GFP and  $\beta$ -tubulin. Quantification of Western analysis for GFP and MH1-GFP expression was performed using Multigauge Image analysis software. Data is shown as fold expression as compared to the 0hr timepoint. All values for GFP and MH1-GFP were corrected using  $\beta$ -tubulin expression to control for loading. Data represents three independent experiments and error bars are the standard error of the mean (\*  $p < 0.05$ ).



**Figure 8. MH1-GFP interacts with C/EBP $\beta$  *in vitro*.**

**A)** Interaction of MH1-GFP fusion protein with GST and GST-C/EBP $\beta$ . 3T3-L1 pooled stables expressing GFP or MH1-GFP were harvested and whole cell lysate was incubated with 0.5  $\mu$ g GST or GST-C/EBP $\beta$ . Following binding and extensive washing, the precipitated MH1-GFP was detected through Western Analysis using an anti-GFP antibody.

**B)** Schematic representation of full length MH1-GFP and the size and mass of the separate fragments generated using *in vitro* translation (IVT).

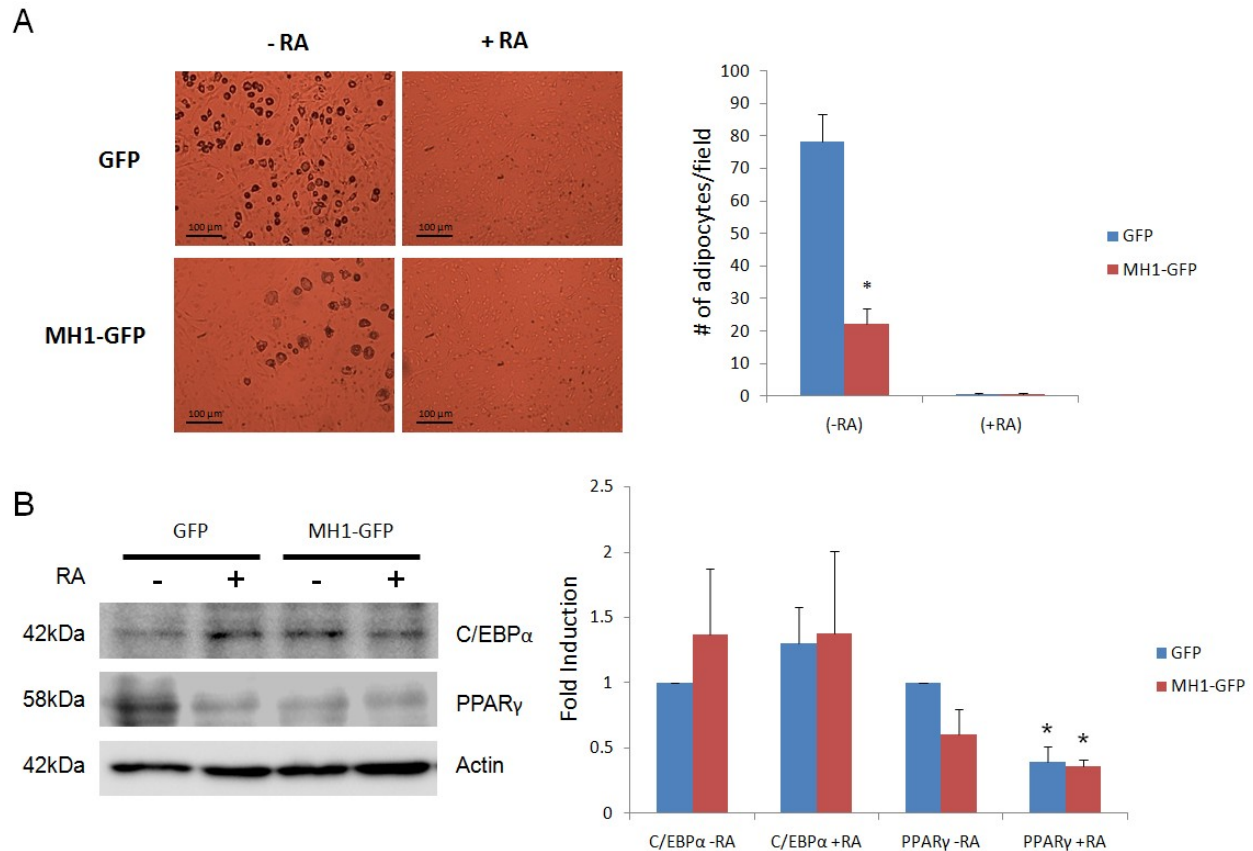
**C)** Interaction of MH1-GFP fusion protein fragments with GST and GST-C/EBP $\beta$ . Fragments were generated using *in vitro* translation and incubated with GST and GST-C/EBP $\beta$ . Following binding, the precipitated MH1-GFP fragments were detected through Western Analysis.

45 amino acids, fragment three a deletion of the first 66 amino acids and fragment four is a deletion of the first 85 amino acids. Following *in vitro* translation, the fragments were incubated with GST or GST-C/EBP $\beta$  and co-precipitated proteins were analyzed by Western blot (Figure 8C). None of the fragments interacted with GST. In contrast, GST-C/EBP $\beta$  interacted with Fragment 1, but none of the other fragments, allowing the mapping of the interaction site to residues between amino acids 6-45. It is interesting to note that this is a region of significant difference between the Smad3 and Smad2 proteins. In the Smad2 MH1 domain there is a 10 nucleotide insertion termed the GAG box, which could account for Smad2's inability to interact with C/EBP $\beta$ .

### **Expression of the MH1-GFP inhibits adipogenesis**

Hypothesizing that the GFP tag would further stabilize the MH1 fragment in cells, we repeated the adipogenesis assay in pooled cell lines expressing either GFP or MH1-GFP (Figure 9A). Oil Red O micrographs taken at day 10 of differentiation demonstrate that in the GFP control, there was significant Oil Red O staining and corresponding cell counts revealed approximately 80 adipocytes per field (Fig. 9A). In the presence of RA there was no Oil Red O staining in GFP-expressing cells, consistent with pLPCX observations.

In the MH1-GFP expressing cells in the absence of RA, there was a four-fold reduction in Oil Red O stained cells, as compared to GFP -RA (Figure 9A). As well, in the presence of RA there was no Oil Red O staining, consistent with the GFP +RA control. Similar to the pLPCX experiment, this demonstrates that there is an important difference in adipocyte differentiation in the presence of the MH1 domain, detectable through Oil Red O staining.



**Figure 9. MH1-GFP mediated inhibition of adipogenesis is detectable through Oil Red O staining**

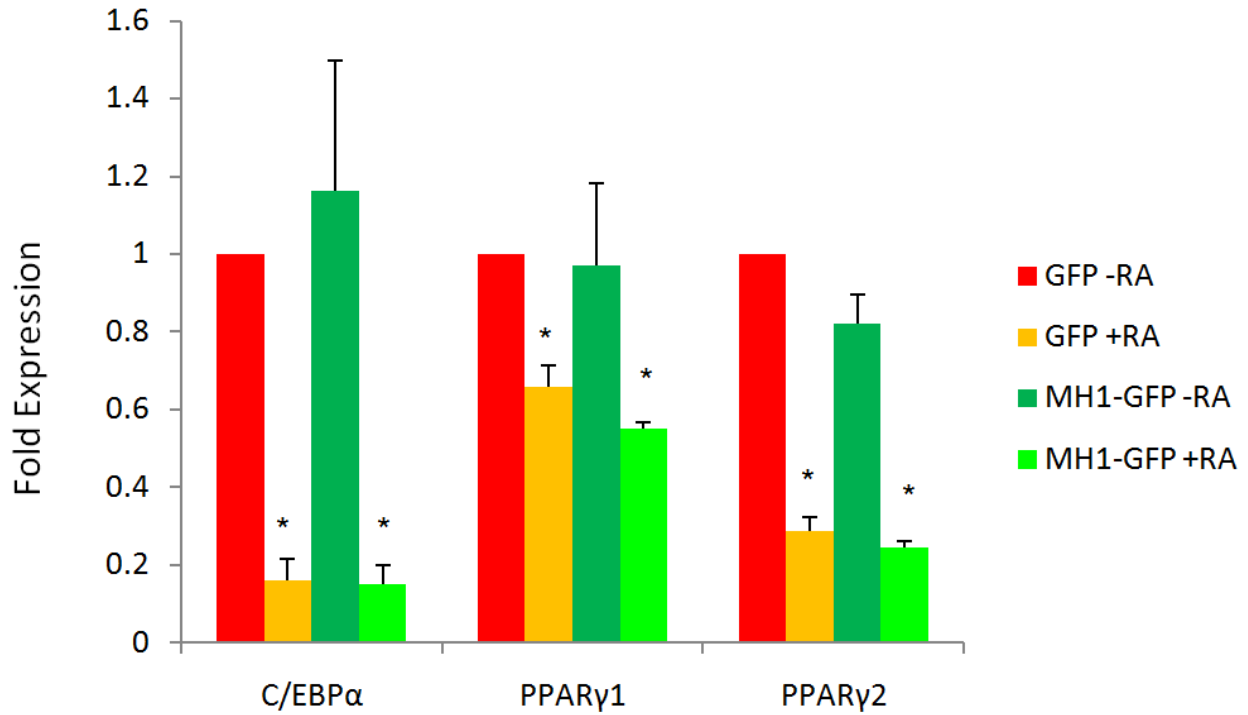
A) Oil Red O micrographs of GFP and MH1-GFP expressing 3T3-L1 pooled stable cell lines. Stable cells were subjected to differentiation in the presence of MID; after ten days of differentiation, the cells were fixed and stained with Oil Red O. Pictures were taken of four random fields in each dish and the number of adipocytes was enumerated. Numbers represent the average of four fields and error bars represent the standard error of the mean (\*  $p < 0.05$ ). ( $n=4$ ). Scale bar represents 100 µm and field area represents 401, 111.11 µm<sup>2</sup>.

B) Western immunoblot and Quantification of Western analysis of four independent adipogenesis trials. Cells were grown according to the aforementioned adipogenesis protocol and the expression of master regulators of adipogenesis (C/EBPα and PPARγ) was quantified using the multiguage analysis program. Protein levels were normalized to an actin loading control and compared to the GFP-expressing vehicle-treated condition for each protein. Error bars represent the standard error of the mean (\*  $p < 0.05$ ).

Following differentiation, Western analysis was performed to analyze the expression of C/EBP $\alpha$  and PPAR $\gamma$  (Figure 9B). Similar to our results with the FLAG-MH1 construct, in the absence of RA the C/EBP $\alpha$  levels in the MH1-GFP stables appear to be highly variable but comparable to the GFP -RA control. Even in the presence of RA, the C/EBP $\alpha$  protein levels appear both highly variable and remain unchanged between the MH1-GFP and GFP expressing cells. However, the PPAR $\gamma$  levels are consistent with the Oil Red O staining. In the absence of RA, the MH1-expressing cells have reduced expression of PPAR $\gamma$ , compared with GFP. This is consistent with the approximate four-fold difference in number of adipocytes between the GFP and MH1-GFP stables. Furthermore, in the presence of RA, both the GFP and MH1-GFP stable cells have significantly reduced expression of PPAR $\gamma$ , consistent with lack of adipocytes in these conditions.

As demonstrated previously through CHIP, the MH1 domain is capable of interfering with C/EBP $\beta$ 's ability to bind the C/EBP $\alpha$  promoter. In the presence of glucocorticoids, C/EBP $\beta$  is a positive regulator of C/EBP $\alpha$  transcription and if its ability to bind DNA is inhibited, we expect a reflection of this in C/EBP $\alpha$  transcript levels. To assess the ability of the MH1-domain to influence C/EBP $\alpha$  transcription, total RNA was harvested from 3T3-L1 stables that had been induced to differentiate using hormonal induction. 48hours after MID treatment the RNA was harvested from these cells and the level of C/EBP $\alpha$ , PPAR $\gamma$ 1 and PPAR $\gamma$ 2 were analyzed (Figure 10).

The GFP control in the absence of RA is considered maximal C/EBP $\alpha$  expression for our system and was assigned a value of 1 and to this the fold expression of C/EBP $\alpha$  in all other



**Figure 10. The expression of C/EBP $\alpha$  remains unaltered by MH1-GFP.** Total RNA was harvested from pooled 3T3-L1 stable clones at day 2. The expression of the master regulators of adipogenesis C/EBP $\alpha$  and PPAR $\gamma$ 2 was assessed. PPAR $\gamma$ 1 expression was also assessed as a control. All expression levels are expressed as a fold induction compared to the GFP -RA condition (\* denotes p<0.05)

treatments was compared and calculated (Figure 10). In the presence of RA, consistent with RA-mediated inhibition of adipogenesis, the levels of C/EBP $\alpha$  were decreased by approximately 10-fold in both the GFP and MH1-GFP expressing cells. However, in the MH1-GFP stables, contrary to Oil Red O and ChIP data in the absence of RA, the level of expression of C/EBP $\alpha$  remained the same and appears equivalent to the GFP -RA condition.

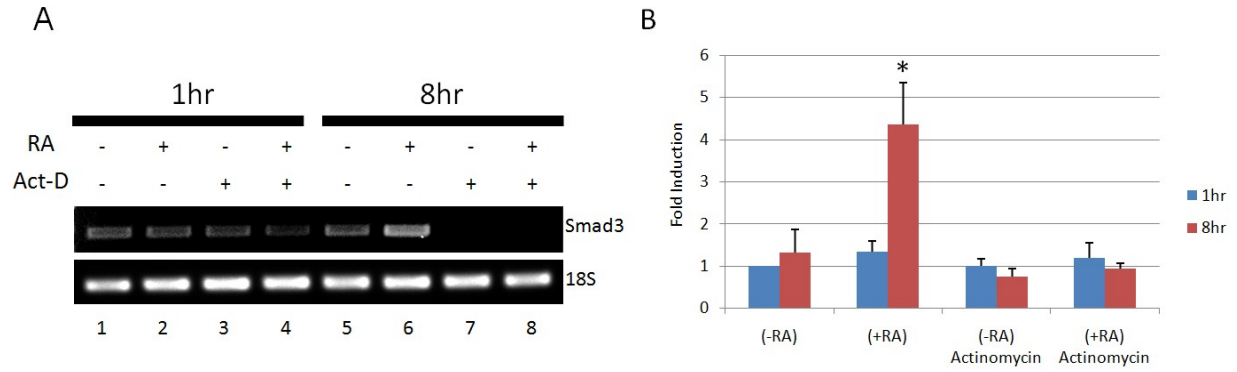
In the adipocyte differentiation model, the expression of PPAR $\gamma$ 2 marks the differentiation of a cell into an adipocyte. When the mRNA from cells at day 2 after hormonal induction was analyzed, it was expected that there would be a marked decrease in PPAR $\gamma$ 2 expression in the presence of RA. Consistent with this, there was a 70-80% reduction in the expression of PPAR $\gamma$ 2 mRNA in both the GFP and MH1-GFP expressing stables (Figure 10). However, in the absence of RA in MH1-GFP expressing cells, there was only a modest reduction in the expression of PPAR $\gamma$ 2 mRNA. As a control, PPAR $\gamma$ 1 levels were also assessed by QPCR. We did not expect RA treatment to regulate the mRNA expression of PPAR $\gamma$ 1, as PPAR $\gamma$ 1 protein expression remained unchanged in previous work done by our lab. Surprisingly, in the presence of RA there was a significant 40% reduction in the expression of PPAR $\gamma$ 1 mRNA in both the GFP and MH1 GFP stables (Figure 10). In contrast, the presence of MH1-GFP in the absence of RA yielded no significant difference in PPAR $\gamma$ 1 expression. Taken together, these results suggest that the MH1 domain of Smad3 acts to inhibit adipogenesis by interfering with the expression of PPAR $\gamma$  and/or its endogenous ligand, rather than C/EBP $\alpha$ .

## Chapter 2

### RA imposes an immediate regulation upon Smad3

As previously described, our lab recently identified Smad3 as a novel retinoic acid target gene after a 48 hour treatment of 3T3-L1 preadipocytes with RA revealed a significant upregulation of Smad3 mRNA levels . However, this time point is quite coarse and allows for the possibility of indirect regulation where RA regulates another protein capable of promoting the transcription of Smad3. Furthermore, in silico analysis of a 5 kb region of the Smad3 promoter and published studies analyzing the immediate 2kb promoter revealed no canonical Retinoic Acid Response Elements (RAREs), leading us to postulate that the Retinoic Acid Receptor (RAR) is incapable of directly binding the Smad3 promoter DNA and thus does not directly influence Smad3 transcript levels.

Wanting to further our understanding of how RA influences Smad3 mRNA levels, we chose to pursue a time-course analysis of Smad3 mRNA expression in RA-treated human mesenchymal stem cells (hMSCs) to determine whether the regulation of Smad3 by RA was conserved. To do so, PCR primers were designed to amplify all four human Smad3 isoforms. A one hour treatment with RA was without effect on Smad3 transcript levels, suggesting that a one hour RA treatment was insufficient to appreciably upregulate Smad3 expression (Figure 11A-lane 2, B). However, eight hours of treatment resulted in a significant 4-fold upregulation of Smad3 transcript (11A-lane 6, B), suggesting that RA's ability to effect Smad3 transcription is conserved at least among humans and mice. To determine if RA mediated upregulation of Smad3 was the result of de novo transcription, the transcriptional inhibitor Actinomycin D (Act-D) was used to perturb transcription. Act-D is a transcriptional inhibitor that intercalates



**Figure 11. RA induces transcription of Smad3.**

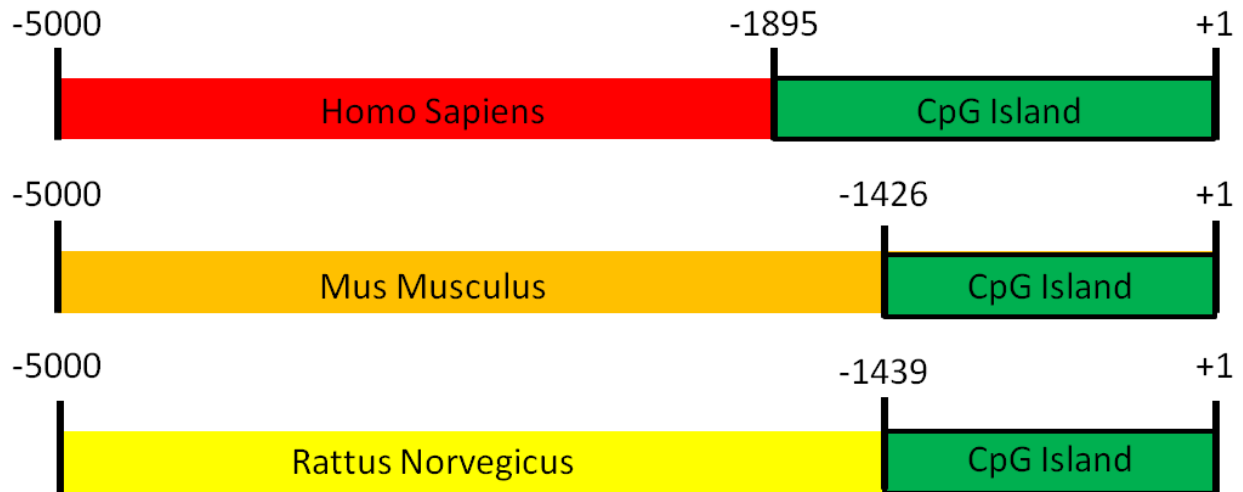
**A)** Reverse Transcriptase PCR followed by agarose gel electrophoresis, after treatment of hMSCs with ethanol vehicle or RA, in the presence or absence of the transcriptional inhibitor Actinomycin-D ( $10\mu\text{g}/\mu\text{l}$ ).

**B)** Quantitative PCR analysis of Smad3 mRNA transcript levels, following treatment with ethanol vehicle or RA, in the presence or absence of Actinomycin-D. Fold Induction represents the 18S loading control corrected Ct value divided by the ethanol vehicle control. Data represents three independent experiments and error bars are the standard error of the mean (\*  $p < 0.05$ ).

within double stranded DNA, protecting it from the melting processes required for RNA elongation by RNA polymerase . This results in the immobilization of the RNA polymerase, thereby interfering with the production of RNA . Therefore, Act-D stalls de novo transcription allowing us to observe changes in Smad3 mRNA in the presence of RA. hMSCs were treated with Act-D for one or eight hours, in the presence or absence of co-treatment with RA (Figure 11). Smad3 transcript levels were unaffected by a short one hour cotreatment with RA and Act-D, which was consistent with an absence of Smad3 upregulation after one hour of treatment (Figure 11- lane 4). Furthermore, after eight hours of RA cotreatment with Act-D, Smad3 transcript disappeared, suggesting that de novo transcription was required to drive Smad3 expression in the presence of RA (Figure 11- lane 8). This experiment demonstrated that RA-mediated upregulation of Smad3 is a result of direct action upon transcription.

### **Demethylation of the Smad3 promoter is not responsible for RA mediated Smad3 upregulation**

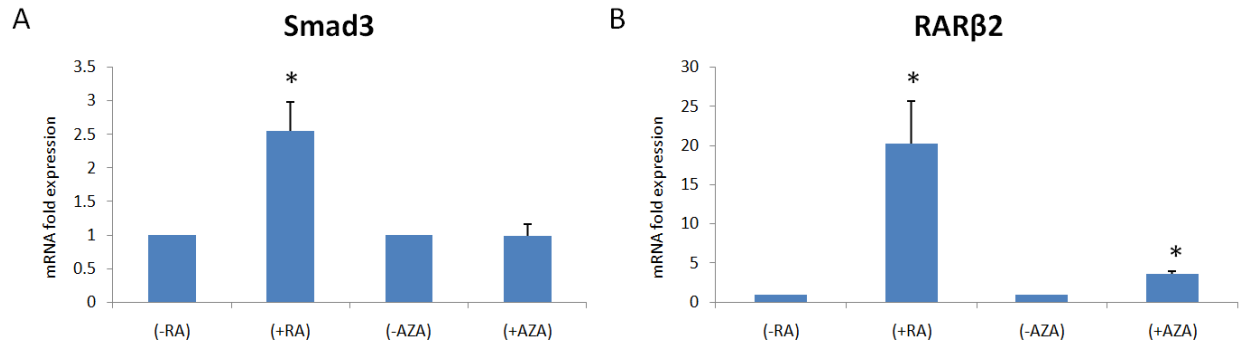
In silico analysis revealed that there were no canonical RAREs in either the rat, human or mouse Smad3 promoters. In fact, all three of these promoters were quite divergent, with the exception of large CpG islands within them. Using the CpG Island Search Algorithm designed by Takai D. et al. and the most stringent search conditions possible, a CpG island of approximately 1.5kb was determined to be conserved among the human, mouse and rat Smad3 proximal promoters (Figure 12). CpG islands are CG rich regions, which can be located proximal, distal or within the promoters of most housekeeping genes . These CG-rich regions are prone to methylation, which is associated with gene silencing.



**Figure 12. Smad3 Promoter CpG Island.** Representation of the CpG island in the 5kb region of the Smad3 promoter of humans, mouse and rat. The program ‘CpG Island Searcher’ was used to identify the CpG islands within the Smad3 promoter using the most stringent settings possible (%GC content >60%, ObsCpG/ExpCpG >0.7, length > 1200bp). The identified CpG island was determined to be highly conserved using the ClutalW2 promoter alignment program.

It has previously been demonstrated that the co-Smad Smad4 is silenced through the methylation of these CG rich regions . Methylation of the Smad3 promoter has also been demonstrated in humans, but the impact remains to be elucidated . Furthermore, in promoter studies of the RAR $\beta$  gene, a retinoic acid target gene that is also regulated through a CpG island, revealed that RA-induced promoter demethylation of CpG islands resulted in increased RAR $\beta$  expression . This same demethylation could be recapitulated by treatment of cells with 5-Azacytidine (AZA), a methylation resistant cytosine analog. Once incorporated into the CpG island AZA replaces cytosine residues thereby reducing CpG island methylation.

To determine if RA treatment was promoting demethylation of the Smad3 CpG island, we treated cycling preadipocytes with AZA or vehicle for 24 hours. An 8 hour RA treatment was used as a positive control for Smad3 induction. Following RA treatment, as previously demonstrated, the level of Smad3 transcripts was rapidly upregulated a significant 2.5-fold over the vehicle-treated control after 8 hours (Figure 13A). However, AZA treatment failed to change Smad3 expression as compared to vehicle controls. To ensure that the AZA treatment was effective in reducing CpG island methylation under these conditions, we examined the expression of the RAR $\beta$  gene, a known RA target gene that is negatively regulated by promoter methylation (Figure 13B). The expression of RAR $\beta$  mRNA increased approximately 20-fold over the vehicle-treated control in the presence of RA. In the presence of AZA, consistent with previous reports, the expression of RAR $\beta$ 2 increased approximately 3.5-fold in the absence of methylation, compared to the vehicle-treated control. This demonstrated that non-specific promoter demethylation was unable to recapitulate the effect of RA upon Smad3 gene transcription, suggesting that RA does not induce CpG island



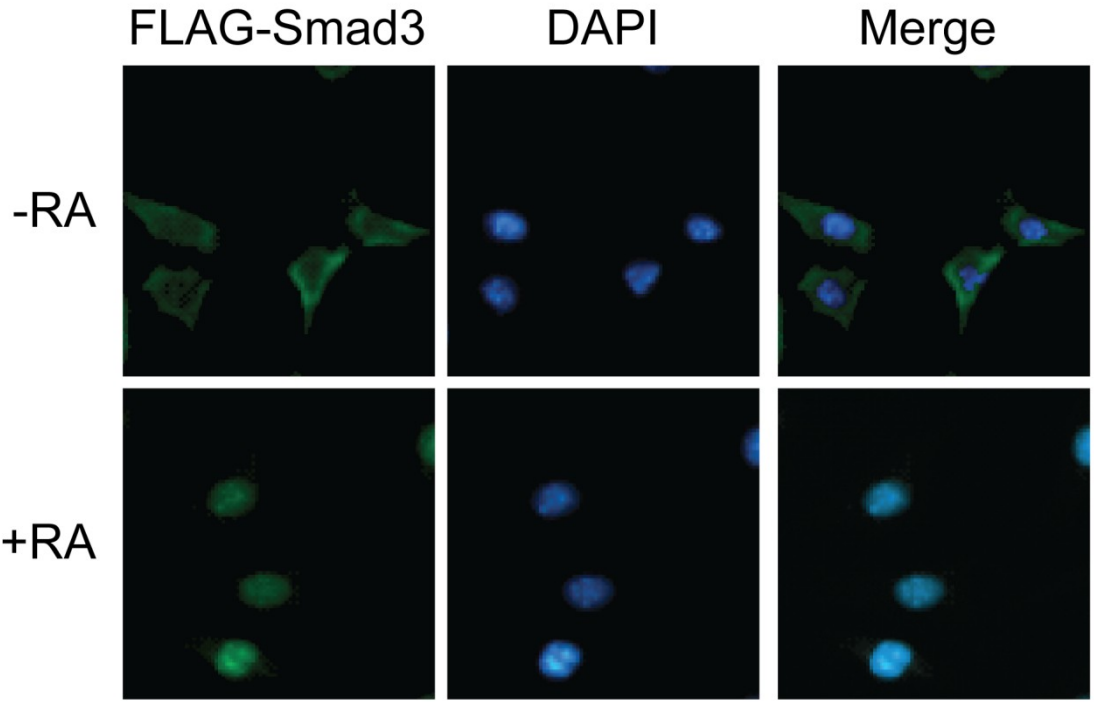
**Figure 13. Changes in DNA methylation status do not contribute to upregulated transcription of Smad3 in the presence of RA.** Quantitative PCR analysis of RARβ2 and Smad3 mRNA following treatment of 3T3 L1 preadipocytes with RA or ethanol vehicle for 8 hours, or azacytidine (AZA) or vehicle (50/50 glacial acetic acid) for 24 hours. RARβ2 is a positive control demonstrating the efficacy of the AZA treatment. Fold expression represents the 18S loading control corrected Ct value, divided by each treatments negative control. Data represents three independent experiments and error bars are the standard error of the mean (\* p<0.05).

demethylation to drive Smad3 expression, but may act directly to influence de novo transcription of the Smad3 gene.

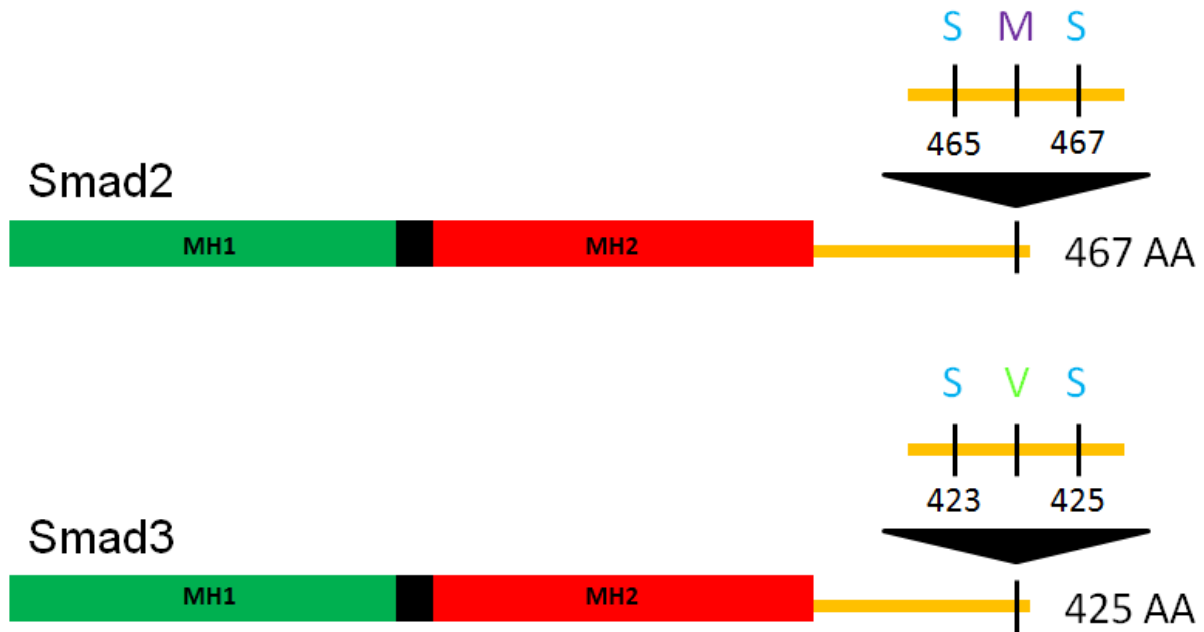
### **RA induces Smad3 shuttling in the absence of phosphorylation**

As previously described, Smad3 is a novel effector of RA action during adipogenesis. However, in 3T3-L1 preadipocytes retrovirally transduced to express ectopic Smad3, there was no detectable inhibition of adipogenesis in the absence of RA treatment. This surprising result led us to speculate that RA, in addition to stimulating the expression of Smad3, also ensured its nuclear localization. In canonical TGF $\beta$  signalling, phosphorylation of Smad3 and its subsequent heterodimerization with Smad4 are required for its nuclear accumulation. In order for Smad3 to perform its duties as the essential effector of RA-mediated abrogation of adipogenesis, it must be localized to the nucleus where it can disrupt C/EBP $\beta$ -mediated transcription. With the 3T3-L1 data in mind, we hypothesized that full length Smad3 was unable to reach the nucleus in the absence of RA. To verify this hypothesis, we created a pooled stable C3H10T1/2 cell line expressing ectopic FLAG-tagged Smad3 and assessed its localisation by indirect immunofluorescence. We determined that in the absence of RA, ectopic Smad3 was predominantly cytoplasmically localized (Figure 14). However, following a 48 hour RA treatment, Smad3 was localized to the nucleus, clearly demonstrating RA's ability to promote increased nuclear presence of Smad3 (Figure 14).

As mentioned previously, canonical TGF $\beta$  signalling requires the direct phosphorylation of Smad3 in its SXS motif, Serine 423/425, which is a necessity prior to nuclear transport (Fig. 15). To begin to elucidate the mechanism by which RA treatment promotes nuclear accumulation of Smad3, we analyzed Smad3 phosphorylation status by Western blotting in RA-treated cells.

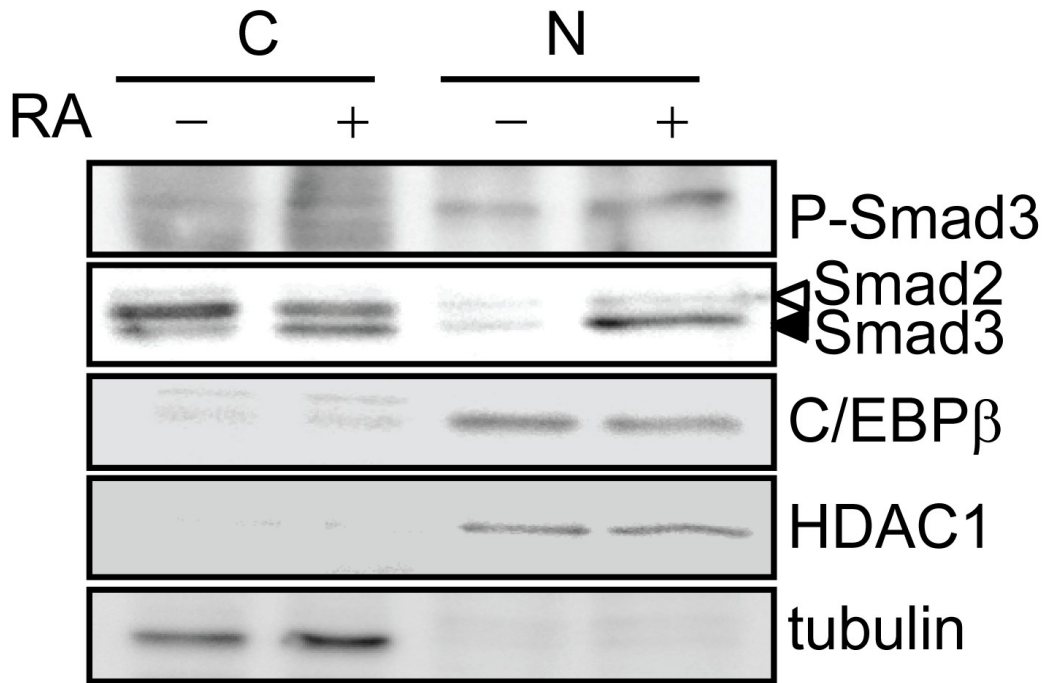


**Figure 14. Nucleo-cytoplasmic Localization of Ectopically expressed Flag-Smad3.** Indirect immunocytochemistry of ectopic Smad3 localization in C3H10T1/2 cells retrovirally transduced to express full length Smad3 bearing a FLAG epitope tag and treated with vehicle (-RA) or with  $10^{-6}$ M RA for 48hrs. DAPI is used as a nuclear stain.



**Figure 15. Receptor Smad2/3 C-terminal Phosphorylation sites.** Schematic representation of the C-terminal serine phosphorylation sites required for canonical receptor-Smad nuclear transport, in relation to the full-length Smad2/3 proteins. Numbers below phosphorylation sites indicate amino acid position. Black region between MH1 and MH2 domains represents the proline rich linker region.

Nucleo-cytoplasmic fractionation was performed in both vehicle and RA-treated C3H10T1/2 mesenchymal stem cells, followed by nucleo-cytoplasmic fractionation of proteins (Fig. 16). While Western analysis revealed an increase in Smad3 nuclear accumulation in the presence of RA, there were no significant differences in Smad3 phosphorylation status between vehicle and RA-treated cells in either the nucleus or cytoplasm. Therefore, RA is capable of inducing Smad3 nuclear accumulation in the absence of its phosphorylation. However, it is interesting to note that this accumulation is slower than the accumulation witnessed in the presence of TGF $\beta$  and results in maximal protein levels in the nucleus after 48 hours, as opposed to 12hours with TGF $\beta$ .



**Figure 16. Nucleo-cytoplasmic fractionation in C3H10T1/2 cells.** Western Blot Analysis, immunoblotting for phospho-Smad3 (P-Smad3), Smad2, Smad3, and C/EBPβ following treatment with ethanol vehicle or RA. HDAC1 and tubulin are used as loading controls and to confirm the integrity of the nuclear and cytoplasmic compartments, respectively.

## Discussion

In this thesis, we provide evidence to suggest that the Smad3 MH1 domain is sufficient to inhibit adipogenesis in the absence of RA. However, while we hypothesized that the MH1 domain would do so by specifically abrogating transcription from the *C/EBP $\alpha$*  promoter, in fact we observe more robust changes in PPAR $\gamma$  protein expression that may account for the inhibition of adipogenesis. Indeed, though there was reduction of *C/EBP $\beta$*  occupancy of the *C/EBP $\alpha$*  promoter in preadipocytes expressing MH1, this did not result in reduced transcription of *C/EBP $\alpha$*  or changes in protein expression. *C/EBP $\delta$*  has also been demonstrated to bind the *C/EBP $\alpha$*  promoter at this same response element and regulate transcription, so it is possible for a compensation mechanism to exist in *C/EBP $\beta$* 's absence. Interestingly, Smad3 has also been demonstrated to interact with *C/EBP $\delta$* , so this could be. Although it is possible for *C/EBP $\delta$*  to bind the *C/EBP* response element within the *C/EBP $\alpha$*  promoter and induce transcription, recent experiments in our lab suggested that RA induced Smad3 expression was capable of inhibiting *C/EBP $\beta$*  DNA binding by virtue of its MH1 domain, which significantly reduced *C/EBP $\alpha$*  protein expression. Taken together, this data suggested that the Smad3 MH1 domain imposed a crippling effect upon adipocyte differentiation as predicted, but not at the level of transcription of *C/EBP $\alpha$* , contrary to what was expected.

There are a few important questions which remain to be answered and should drive future work: (1) How does the MH1 domain inhibit adipogenesis? ; (2) How does RA upregulate Smad3 expression? and; (3) How does RA cause Smad3 nuclear accumulation?

## **(1) How does the MH1 domain inhibit adipogenesis?**

The binding of C/EBP $\beta$  to the C/EBP $\alpha$  promoter and subsequent positive regulation of its activity is important for the early commitment of preadipocytes. In RA-mediated inhibition of adipogenesis, Smad3 is necessary to inhibit this DNA binding process through an interaction with C/EBP $\beta$ . Our lab has demonstrated that the MH1 domain is important for Smad3's ability to bind C/EBP $\beta$  and inhibit transcription, and that full length Smad3 is required for the inhibition of adipogenesis by RA. The isolated MH1 domain localized to the nucleus and could inhibit C/EBP $\beta$  occupancy of the C/EBP $\alpha$  promoter. However, what was most interesting was that, despite this inhibition of DNA binding, corresponding changes in C/EBP $\alpha$  expression were not observed. Taken together, these results suggested that, while the isolated MH1 domain can displace C/EBP $\beta$  from its target promoters, it has further effects which allow C/EBP $\alpha$  expression and mitigate PPAR $\gamma$  expression that are independent of C/EBP $\beta$ . Indeed, other factors are capable of stimulating C/EBP $\alpha$  expression within the first 48 hours of induction, most notably C/EBP $\delta$ . In C/EBP $\beta$  knockout mice, there is a severe reduction in adipose tissue mass, but not an abolishment of fat cells, suggesting that C/EBP $\delta$  is capable of partially compensating for early functions of C/EBP $\beta$ . In accordance, in C/EBP $\beta$ -/- mouse embryonic fibroblasts induced to differentiate into adipocytes, C/EBP $\delta$  is capable of inducing the expression of C/EBP $\alpha$  and PPAR $\gamma$ , but not to the same extent as wild-type cells. This demonstrates that C/EBP $\delta$  activity could compensate for C/EBP $\beta$ 's absence in inducing C/EBP $\alpha$  transcription, but it may not be able to provide full recovery.

In addition, C/EBP $\delta$  is both capable of directly binding to and activating the transcription of the PPAR $\gamma$ 2 promoter. Furthermore, since we have not demonstrated an ability of the MH1 domain to bind C/EBP $\delta$  *in vitro*, our data does not discount the possibility that disruption of

C/EBP $\delta$  activity of the PPAR $\gamma$ 2 promoter is responsible for the inhibition of adipogenesis . Therefore, through the compensatory mechanisms of such proteins as C/EBP $\delta$ , the expression of C/EBP $\alpha$  and PPAR $\gamma$  could be maintained.

Though the expression of PPAR $\gamma$ 2 and C/EBP $\alpha$  are critical for adipogenesis, it appears that in our MH1 expressing cells, the high level of expression of these proteins was not sufficient to promote adipogenesis. Interestingly, this situation is similar to the C/EBP $\beta$  and C/EBP $\delta$  double null mouse. In the dwarfed adipose depots of these mice, even though adipogenesis was inhibited, there were still high levels of expression of both C/EBP $\alpha$  and PPAR $\gamma$ , suggesting that the importance of C/EBP $\beta$  in transcriptional regulation of adipogenesis extends beyond upregulation of C/EBP $\alpha$  . In experiments performed by Hamm and colleagues, inhibition of C/EBP $\beta$  activity through the use of a non-functional bzip transcription factor inhibited differentiation along with C/EBP $\alpha$  and PPAR $\gamma$ 2 expression, without affecting Dexamethasone mediated upregulation of PPAR $\gamma$ 1 . This inhibition of C/EBP $\alpha$  expression and adipogenic differentiation could only be rescued by addition of an exogenous PPAR $\gamma$  ligand, troglitazone . Therefore, antagonizing C/EBP $\beta$  activity not only inhibits the expression of C/EBP $\alpha$  and PPAR $\gamma$ 2, but also renders the cells dependent on an external PPAR $\gamma$  ligand to continue the differentiation process. This suggests that functional C/EBP $\beta$  also regulates the activity of PPAR $\gamma$ 's through the regulation of an endogenous PPAR $\gamma$  ligand which is critical in the differentiating adipocyte. It is possible that the MH1 domains ability to interfere with C/EBP $\beta$  DNA binding extends to other C/EBP $\beta$  regulated genes, for example the ADD1/ SREBP1c gene . The SREBP1c gene is initially initially highly expressed upon hormonal induction and regulates both the expression of PPAR $\gamma$  and its ligand . ChIP experiments performed by Payne et al. have demonstrated the critical early binding of C/EBP $\beta$  to C/EBP elements within the

SREBP1c promoter, which is necessary for SREBP1c upregulation during hormonal induction . This suggests that MH1 inhibits SREBP1c production and the production of the PPAR $\gamma$  endogenous ligand. This is a possible explanation for why we see significantly inhibited differentiation in the presence of PPAR $\gamma$  protein expression.

An important regulatory mechanism of C/EBP function is phosphorylation. Phosphorylation of C/EBP's has been demonstrated to give them greater activity. For example after mitotic clonal expansion, phosphorylation of C/EBP $\beta$  is necessary for its transcriptional activity. Tang et al. demonstrated through both *ex vivo* and *in vitro* experiments that C/EBP $\beta$  phosphorylation by glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) is required for DNA-binding and transcriptional activation of C/EBP $\alpha$  and adiponectin (a late marker of adipogenesis) . Interestingly, C/EBP $\alpha$  is regulated by a similar mechanism involving GSK3 $\beta$ , where phosphorylation by GSK3 $\beta$  allows activation and promotion of adipogenesis. This conclusion was reached after treatment with a GSK3 $\beta$  inhibitor (lithium), which leads to dephosphorylation of C/EBP $\alpha$ , inhibited adipocyte conversion . Therefore, it appears that phosphorylation of C/EBPs is a form of regulation and though their protein levels may be high during adipogenesis, until they are phosphorylated they do not possess enough inherent activity to exert their function. Similarly, Smad3 has also been demonstrated to be phosphorylated by GSK3 $\beta$  as well . Through phosphorylation by GSK3 $\beta$ , Smad3 becomes ubiquitinated and subsequently degraded . Surprisingly, GSK3 $\beta$  recognizes a consensus motif within the Smad3 MH1 domain . Therefore, it is possible that C/EBP $\alpha$  competes with the retrovirally expressed MH1 domain for GSK3 $\beta$ . This competition could result in a significant reduction in GSK3 $\beta$  efficiency in phosphorylation of C/EBP $\alpha$  and thereby reduce its activity. This also aids in explaining why we see high levels of C/EBP $\alpha$ , but an absence of adipogenesis, as C/EBP $\alpha$  is indispensable in sensitizing 3T3-L1

preadipocytes to insulin , in part through the upregulation of cell membrane glucose transport protein Glut4 ; and cooperating with PPAR $\gamma$  in the expression of a subset of adipocyte genes . Besides a competitive inhibition of C/EBP $\alpha$  phosphorylation, GSK3 $\beta$  phosphorylation of Smad3 potentially explains why the MH1 domain destabilizes GFP and we see a 10-fold difference between GFP and MH1-GFP expression.

In RA treatment conditions there was a significant inhibition of both C/EBP $\alpha$  and PPAR $\gamma$  mRNA levels at 48 hours, but at day 10 of differentiation there was no inhibition of C/EBP $\alpha$ , but significantly impaired expression of PPAR $\gamma$ . Though RA has been demonstrated by our lab to reduce C/EBP $\alpha$  expression through Smad3-mediated inhibition of C/EBP $\beta$  DNA binding , it does not discount the possibility of regulation of C/EBP $\alpha$  by other proteins at late time point, most notably PPAR $\gamma$ 1 , which we demonstrated to be only mildly inhibited at the mRNA level by RA. Recently, it had been demonstrated that only Smad3 inhibition of C/EBP $\beta$  DNA binding to the C/EBP $\alpha$  promoter was necessary to inhibit adipogenesis, however, the experiments in this thesis suggest that the full length Smad3 protein must have other functions, capable of inhibiting the aforementioned early redundancy associated with C/EBP $\alpha$  and PPAR $\gamma$  transcription. Consistent with this, in the presence of the MH1 domain alone, we did not see any inhibition of C/EBP $\alpha$  or PPAR $\gamma$  transcription, despite inhibition of C/EBP $\beta$  DNA binding. It is entirely possible that there are other unknown pathways, critical to adipogenesis, that RA inhibits. What is exciting is that there is evidence that these pathways may be common to other nuclear receptors. The ligand of the Vitamin D receptor (VDR), 1,25(OH) $_2$ D $_3$ , is capable of recapitulating the crippling effect of RA upon adipogenesis. In 3T3-L1 cells in the presence of this VDR ligand there was a significant reduction in C/EBP $\alpha$  and PPAR $\gamma$  expression, consistent with the anti-adipogenic abilities of RA . Furthermore, ligands of the estrogen receptor have also

been demonstrated to recapitulate the effects of RA as well, manifested as a reduction in C/EBP $\alpha$  and PPAR $\gamma$  expression . Furthermore, estrogen ligands are also capable of inducing osteogenesis within the treated cells, while they inhibit adipogenesis . Interestingly, our lab has also demonstrated osteogenic conversion after RA treatment of C3H10T1/2 mesenchymal stem cells . A possible means of action could be through the retinoid x receptor (RXR), which is a known communal heterodimerization partner of RARs and VDRs . Therefore, it appears that nuclear receptors share some common pathways with regards to adipogenic regulation which may be mediated, at least in part, through their interaction with RXRs.

Overall, it appears that the MH1 domain inhibits adipogenesis, but behaves completely different from our expectations. Though it inhibits adipogenesis, it does so in a manner consistent with an inhibition of PPAR $\gamma$  ligand production and possibly inhibition of C/EBP $\alpha$  activation through phosphorylation.

## **(2) RA mechanism of Smad3 upregulation**

The regulation of Smad3 expression is poorly understood, with the majority of what is known focusing on regulators of its activity. Previous studies have demonstrated Smad3's regulation by the prenylation pathway , where inhibition of this pathway increased Smad3 transcription, and through a MAPK-dependent pathway , where inhibition of a phosphokinase activator of MAPK, MAPK-Kinase1 resulted in a decrease in Smad3 transcription. Though positive and negative control of the Smad3 promoter have been demonstrated to act through a critical region composed of three well-conserved Sp1 sites, the scientific community has yet to identify factors which can specifically interact with Sp1 proteins and modulate expression of the Smad3 promoter.

Through the use of a transcriptional inhibitor, Actinomycin D, we have demonstrated that RA acts to increase transcription of Smad3. Though our data does not exclude the possibility of the interaction of a RAR-containing protein complex with the Smad3 promoter DNA, due to the absence of canonical RAREs within the promoter, it is possible that RARs cannot physically interact with Smad3 promoter DNA directly.

RA treatment has been demonstrated to stimulate gene expression through demethylation of CG-rich regions and acetylation of histones of promoters of target genes . The regulation of Smad3 by RA is conserved amongst humans and mice but the majority of the Smad3 promoter is highly divergent, the only conservation in the Smad3 promoter is a large proximal CpG island, which contains the Sp1 sites. Given RA's ability to induce promoter demethylation in CG-rich sequences, we evaluated RA's ability to induce demethylation as a cause for this upregulation in Smad3 transcription. Interestingly, even a decrease in methylation of the Smad3 promoter, through the use a methylation resistant cytosine analog, was incapable of recapitulating RA-induced transcription of Smad3. Taken together, this suggested that the actions of RA upon the Smad3 promoter were immediate, but may not have been due to changes in promoter methylation status. This is consistent with a previous report that analyzed Smad3 promoter methylation status in cells from different individuals, where Smad3 transcript expression did not correlate with methylation status of the promoter, suggesting that variations in the activity, localization and/or abundance of trans-acting factors is responsible for differential Smad3 expression . Interestingly, there appears to be contention in the literature as to whether Sp1 binding to DNA is regulated by methylation or not. Some researchers have argued that Sp1 binds DNA and regulates transcription regardless of methylation status, while other have argued that increased methylation hinders Sp1 binding and transcriptional activity; in any case, it

appears that this quality of Sp1 is promoter and/or cell type dependent, as each of the researchers utilized different promoters and cells in their studies . Though we haven't determined that RA actually regulates methylation at the Smad3 promoter, which could be achieved through bisulfite sequencing or restriction enzyme techniques, we postulated that it could, based on documented regulation of RAR $\beta$  gene transcription . From our azacytidine experiment, it appears that methylation-related gene silencing does not affect the Smad3 promoter.

The RARs are nuclear receptors that are thought to be constitutively bound to RARE's, waiting for retinoid signals to activate transcription . In our investigation, we cannot discount the possibility that RARs are interacting with a non-canonical RARE , because we have not performed a Chromatin Immunoprecipitation Assay pulling down with an antibody specific for RARs. Different labs have demonstrated the ability of RARs to interact with Sp1 transcription factors and synergize its transcription at the urokinase and monoamine oxidase promoters . Interestingly, recent experiments suggest that RARs are capable of interacting with Sp1 sites *in vivo* at the monoamine oxidase B promoter, in Sp1 knockout cells . However, this is in contention with experiments performed upon the urokinase and HIV type 1 LTR sequence, which suggest that RARs can only interact with Sp1 sites through their interaction with Sp1 . Therefore, it appears that the ability of RARs to bind Sp1 sequences is context dependent. Perhaps there are bridge factors recruited by surrounding sequences at the MAO B promoter that are not present at the urokinase promoter. In this regard, evidence suggests that RARs can interact with and be recruited by other transcription factors to regulate gene expression, thereby allowing their association with non-RARE's . Even though RARs may be incapable of interacting with Sp1 binding sites, they are capable of direct interaction with Sp1 *in vitro* and this interaction is important in transcription of interleukin-1 $\beta$  and 17 $\beta$ -hydroxysteroid

dehydrogenase type 2 genes . As mentioned previously, studies on the Smad3 promoter identified three highly conserved Sp1 sites in the immediate Smad3 promoter which are part of an important regulatory region . We believe that RA could be acting through this regulatory region via a direct interaction with Sp1. This interaction has been mapped to the zinc finger domains of Sp1 and enhances Sp1's inherent DNA binding ability . Once recruited to the Sp1 site, in the presence of ligand, RARs can recruit other coregulatory proteins, which induce a relaxed chromatin structure and facilitate Sp1 binding and recruitment of RNA Polymerase II to the Smad3 promoter .

Though the Sp1 sites appear to be a viable mechanism for RA-mediated upregulation of Smad3 expression, there are several other unexplored transcription factor binding sites which could also provide a means through which RA could regulate Smad3 gene expression, such as the NF- $\kappa$ B response elements. There are six potential NF- $\kappa$ B binding sites within the immediate 2kb human Smad3 promoter. Recent evidence implicates RA as a potential activator of NF- $\kappa$ B, as RA-mediated activation of an NF- $\kappa$ B reporter gene was inhibited by treatment with an RAR antagonist in a dose dependent manner . Therefore, it is possible that through activation of NF- $\kappa$ B transcription RAR is capable of increasing Smad3 levels, at least in humans. It is interesting to note that several of these NF- $\kappa$ B sites are also in close proximity to Sp1 sites in the human Smad3 promoter .

Overall, we propose that the ability of RARs to have a direct effect upon Smad3 transcription is mediated independently of RARs ability to induce promoter demethylation, but could be the result of another epigenetic modification or RAR interaction with other transcription factors, most notably Sp1.

### **(3) Mechanism of Smad3 nuclear accumulation in the presence of RA**

In canonical TGF $\beta$  signalling, TGF $\beta$  receptor II association with TGF $\beta$  leads to phosphorylation and activation of the type I receptor, which in turn phosphorylates Smad2 and Smad3. Phospho-Smad2 and phospho-Smad3 then interact with Smad4 and enter the nucleus to regulate TGF $\beta$  target genes. Smad3, by virtue of its intact NLS, has been shown to shuttle independent of Smad4 co-association, but overall remains largely cytoplasmic. Similarly, Smad1, another receptor Smad in the BMP pathway, also has a functional NLS within its MH1 domain, which is important for entry into the nucleus. Furthermore, Smad4 contains a classical bipartite NLS, which has been demonstrated to interact with another Importin  $\alpha$ 1, promoting Smad4 nuclear entry, even in the absence of TGF $\beta$  signal. Work from our laboratory has demonstrated that endogenous Smad3 became more nuclear 24-48 hours after RA treatment, reaching maximal accumulation at 48 hours. Our data demonstrates that exogenous Smad3 also localized to the nucleus after a 48 hour treatment with RA but not in vehicle treated cells. Previous experiments performed in developing T-cells suggested that RA was activating TGF $\beta$  signalling through upregulation of Smad3 and promoting its phosphorylation, suggesting that RA induced the phosphorylation of Smad3, which allowed its nuclear accumulation. However, our nucleo-cytoplasmic fractionation data in 3T3-L1 cells demonstrated that RA's effect upon nuclear accumulation of Smad3 was not a result of c-terminal phospho-Smad3 production, as the levels were not significantly different between the +RA and -RA treated subcellular compartments. This suggested the possibility of a non-canonical Smad import mechanism, where RA induces nuclear accumulation of Smad3 in the absence of significant changes in phosphorylation of serines 423/425. As mentioned previously, canonical Smad import involves the phosphorylation of Smads, followed by their association, via their NLS, with Karyopherins

such as Importin  $\beta$ 1, resulting in their association with nuclear pores and entry into the nucleus . It has been demonstrated that this phosphorylation enhances the interaction between Smad3 and Importin  $\beta$ 1 as well as its subsequent nuclear import . In contrast, the NLS-like motif in the Smad2 MH1 domain is non-functional and is incapable of interacting with importin  $\beta$ 1, suggesting that other nuclear import pathways may exist .

Interestingly a Karyopherin-independent mechanism has been proposed for the nuclear import of Smad2, Smad3 and Smad4 . Using *in vitro* assays Xu et al. demonstrated that a series of hydrophobic sites (hydrophobic corridor) within the MH2 domain of both Smad2, Smad3 and Smad4 proteins, were responsible for direct physical interaction between these receptor Smads and the nucleoporins CAN/Nup214 (located on the cytoplasmic side of nuclear membrane) and Nup153 (located on nucleoplasmic side of nuclear membrane) . Furthermore, a similar interaction with this same hydrophobic corridor was demonstrated for a nuclear Smad retention factor (FAST-1) and a cytoplasmic Smad retention factor (SARA) . The authors suggested that a competition was continually occurring between these four proteins and the Smad2/3 –MH2 domains. Upon TGF $\beta$  signalling Smad2/3 phosphorylation liberates them from SARA , allowing them to interact with CAN/Nup214 and be transported into the nucleus. Once in the nucleus, they could be retained by FAST-1 and their phosphorylation would allow it to remain in association with transcriptional regulatory machinery in the nucleus. However, once dephosphorylated, Nup153 could compete for the hydrophobic binding region to allow for nuclear export . It is important to note that the interaction with either of the nucleoporins is not dependent upon phosphorylation. A similar interaction between the Smad4 MH2 domain and these nucleoporins was also demonstrated . Therefore, this suggests a mechanism where RA could induce nuclear accumulation of Smad3 in the absence of its phosphorylation. Due to the

slow accumulation of Smad3 after 24-48 hours , it would be interesting to determine if a RAR-regulated protein was capable of interacting with the Smad cytoplasmic retention factor SARA. If liberated from SARA then the Smad3 MH2 domain would be free to interact with CAN-Nup214, an interaction that is not dependent upon phosphorylation, and be localized to the nucleus .

Though it is possible that the Smad3 protein could be freed from SARA by an RA target protein, it is equally probable that this RA target is capable of shuttling Smad3 itself. If this were the case, the RA target gene would be capable of interacting with free or bound Smad3 in the cytoplasm and involved in its nuclear import. In a review of the literature, it is interesting to note that there is a significant amount of cross-talk between the RA and TGF $\beta$  signalling pathways. For example in U937 and HL-60- leukemia cells and human keratinocytes, RA is believed to upregulate production of TGF $\beta$  and its receptors, which is responsible for growth inhibition . Consistent with this, Smad3 has been demonstrated to interact with two retinoic acid receptor isoforms, RAR $\alpha$  and RAR $\gamma$ , in experiments performed by separate labs . In a co-transfection experiment performed by La et al., RAR $\alpha$  was demonstrated to interact with Smad3, in a manner that appeared to be dependent upon phosphorylation or the presence of a co-mediator . Furthermore, RAR $\gamma$  was demonstrated by Pendaries et al. to also interact with Smad3 and this interaction was mapped to the MH2 domain of Smad3 . Taken together, it appears that Smad3 is capable of interacting with retinoic acid receptors and that the MH2 domain of Smad3 appears to mediate this interaction. In the presence of retinoic acid, an important RA target gene is RAR $\beta$  and data in this thesis as well as other labs have demonstrated its upregulation in the presence of RA, due to an RARE within its promoter . It is also important to note that other specific isoforms of RARs, RAR $\alpha$ 2 and RAR $\gamma$ 1, are also RA-responsive . However, even though RARs

are upregulated, they are believed to be constitutively bound to DNA and their presence in the cytoplasm is quite minimal, so how would they ever come into contact with cytoplasmic Smad3? We propose that RA mediated upregulation of RARs must result in their translation within the cytoplasm. As the receptors move to enter the nucleus, via their nuclear import signals, Smad3 may be able to hitch a ride via interaction between the receptors and its MH2 domain. This could account for the slow accumulation of Smad3 within the nucleus and its nuclear localization in the absence of C-terminal phosphorylation. However, typically it is this same phosphorylation of Smads that liberates them from SARA, but more importantly maintains their nuclear retention. So how can Smad3 accumulate in the nucleus in the absence of significant levels of phosphorylation? In addition to being able to shuttle Smad3 into the nucleus, the RA-target gene would need to sustain its interaction with Smad3 once in the nucleus and perhaps maintain involvement in Smad3 function until Smad3 became targeted for degradation by GSK3 $\beta$ .

Overall, we propose that an RA-dependent shuttling mechanism may exist, which involves the MH2 domain of Smad3 being able to interact with nucleoporins or de novo synthesized RARs.

## **FUTURE DIRECTIONS**

Based on our results, the question of how the MH1 domain inhibits adipogenesis still lingers. From literature analysis, we have discussed several possible avenues which are all capable of explaining the anti-adipogenic capabilities of the MH1 domain or its inability to recapitulate RA inhibited adipogenesis, which have left us with new hypotheses: (1) The MH1 domain inhibits endogenous PPAR $\gamma$  ligand production through interference with SREBP1c transcriptional upregulation by C/EBP $\beta$ , (2) The MH1 domain competitively inhibits C/EBP $\alpha$

phosphorylation activation, rendering cells expressing C/EBP $\alpha$  to be insulin insensitive and (3) RA has additional anti-adipogenic functions, common to all nuclear receptors, during adipogenesis, which are not exerted in MH1-expressing cells.

To further evaluate (1), we could induce the MH1-GFP and GFP expressing cells to differentiate and observe the early transcriptional regulation of SREBP1c by C/EBP $\beta$ . If the MH1 expressing cells are inhibiting C/EBP $\beta$  regulation of SREBP1c, then we would expect diminished binding of C/EBP $\beta$  to the SREBP1c promoter at 8 hours, in addition to decreased transcription over the course of 24 hours.

In the case of hypothesis (2), MH1-GFP and GFP expressing stable cell lines could be induced to differentiate and a timepoint analysis of the level of C/EBP $\alpha$  protein phosphorylation could be assessed. Furthermore, we could analyze the transcription of the GLUT4 glucose transport protein, whose transcription is activated by C/EBP $\alpha$ . An *in vitro* GSK3 $\beta$  phosphorylation assay could also be performed upon C/EBP $\alpha$  in the presence of the MH1 domain.

In order to further evaluate hypothesis (3) a control analyzing the expression of Smad3 in VDR ligand and/or ER ligand treated 3T3-L1 preadipocytes that have been induced to differentiate, would have to be performed. This could give insight into the possibility that these signalling pathways inhibited differentiation through a similar mechanism to RA.

Together these experiments could give clues to determine answers to the questions of how the MH1 domain and nuclear receptors are capable of inhibiting adipogenesis.

In chapter 2 of this thesis we focussed on the regulation of Smad3 and it was important for us to determine how RA both regulates the transcription and induces nuclear accumulation of

Smad3. In the discussion of transcriptional regulation it was suggested that through an RAR interaction with Sp1, RA could both enhance the binding of Sp1 to its response element within the Smad3 promoter and induce the recruitment of coactivators to the Smad3 promoter to promote transcription. To determine if RA acts through an RAR interaction with Sp1, we could employ the use of mithramycin, an inhibitor of Sp1 DNA binding . Through the inhibition of Sp1 DNA binding, it inhibits Sp1 activity, thereby allowing us a quick means of observing changes in Smad3 mRNA in the presence of mithramycin and RA cotreatment. Furthermore, to assess the possibility of a direct interaction with a non-canonical RARE, a Chromatin Immunoprecipitation Assay of RAR at the Smad3 promoter could be performed.

According to our discussion of question 3, we proposed that, due to the slow accumulation of Smad3 in the presence of RA, an RA target gene could potentially explain this slow accumulation. In light of the documented interaction between RARs and Smad3 and that RAR $\beta$  is a known RA target gene , we propose a similar indirect immunofluorescence experiment in cells induced to express siRNA directed to RAR $\beta$ . We could observe the nucleocytoplasmic distribution of ectopic Smad3, in the presence of RA, but the absence of RAR $\beta$ .

## **CONCLUSION**

This thesis provides evidence of the anti-adipogenic abilities of the MH1 domain. However, contrary to our expectations, it did not behave in a manner that recapitulated RA-mediated inhibition of adipogenesis. We propose several mechanisms by which the MH1 domain could be inhibiting adipogenesis, but until further experimentation, it is not possible to comment on how it may be doing so. Furthermore, we demonstrated that RA imposes immediate transcriptional activation upon the Smad3 promoter, which appears to occur in the

absence of RARs ability to induce promoter demethylation. We believe this effect may be through RARs' interaction with the transcription factor Sp1. Lastly, we also demonstrated the potential of RA signalling to induce the nuclear accumulation of Smad3 and how it occurred in the absence of c-terminal serines 423/425 phosphorylation. We propose that an RA target gene, RAR $\beta$ , is mediating this effect.

Therefore, the MH1 domain of Smad3 appears to be a promising anti-adipogenic protein, but its mechanism of action remains to be elucidated. Furthermore, it appears that Smad3 is a novel RA target gene, whose nucleo-cytoplasmic regulation by RA demonstrates a functionally relevant cross-talk between the TGF $\beta$  and RA signalling pathways.

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## APPENDIX

Table 1. Constructs obtained from external sources

Vector Name	Functional Use	Description	Source	Reference
pLXSN-GFP	Retroviral Expression	Plasmid allows expression of GFP in mammalian cells. This was used a control alongside pLXSN-MH1-GFP	Haché lab	-
pLPCX-Flag-Smad3	Retroviral Expression	Plasmid allows the expression of an N-terminal flag tagged Smad3 fusion protein	Add gene	R. Derynck (University of California)
pGEX2T-C/EBP $\beta$	IPTG induced, Bacterial Expression Plasmid	Plasmid allows expression of GST-C/EBP $\beta$ fusion protein, when bacteria are incubated with IPTG.	Haché lab	N. Wiper-Bergeron (University of Ottawa)

Table 2. Base vectors used for cloning

Vector Name	Functional Use	Mammalian Promoter	Source
pLXSN	Retroviral Expression	Moloney murine Leukemia virus 5' LTR	Clontech
pLPCX	Retroviral Expression	Moloney murine Leukemia/sarcoma virus 5' LTR	Clontech
pEGFP-N3	Mammalian Expression Vector	Human cytomegalovirus (CMV) immediate early promoter	Clontech