Abstract

POST-TRANSCRIPTIONAL REGULATION OF mRNA METABOLISM DURING DIFFERENTIATION OF 3T3-L1 CELLS: ROLE OF HuR

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MAY, 2010

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Our studies address early control points in the differentiation process that are necessary for expression as well as the maintenance of the adipocyte phenotype. We have focused on the post-transcriptional regulation of mRNA metabolism by the RNA binding protein HuR. Depending on the particular mRNA, HuR has been suggested to control polyadenylation, translocation to the cytosol, mRNA stability, and/or translational efficiency. Using the 3T3-L1 preadipocyte cell line, we have demonstrated a critical role for HuR in the differentiation process by use of siRNA mediated suppression which resulted in an inhibition of differentiation. We have identified several mRNAs that serve as HuR ligands early in the differentiation process, one of which is C/EBPβ, an important adipocyte transcription factor, whose expression is highly controlled and essential for proper acquisition of the adipocyte phenotype. Within minutes of induction of differentiation, HuR forms an mRNP complex with C/EBPβ mRNA in the nucleus followed by translocation to the cytoplasm. HuR remains associated with C/EBPβ mRNA suggesting a role in mRNA stability and

translation efficiency. Our data suggest that formation of this complex in the nucleus serves as a regulator/attenuator of polyadenylation and that this interaction leads to a controlled metabolism of the C/EBPβ mRNA by determination of the quantity of message translocated to the cytosol and available for translation. Additionally, our data have directed us toward the Zfp206 mRNA as an important HuR ligand whose regulation is predicted to be responsible for maintenance of the differentiation potential of the cells and whose expression is terminated as the cells express PPARγ and establish the adipocyte phenotype. Our mechanistic analysis of these issues will identify novel control points in the initial stages of adipogenesis and thus fundamental to the pathological states of obesity and diabetes.

Post-transcriptional Regulation of mRNA Metabolism during Differentiation of 3T3-L1 Cells: *Role of HuR*

A Dissertation

Presented To

The Faculty of the Department of Biochemistry and Molecular Biology

East Carolina University

In Partial Fulfillment

Of the Requirements of the Degree

Doctor of Philosophy in Biochemistry

By

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May, 2010

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Acknowledgement/Dedication

I wish to thank my parents Howard and Deborah Karschner, and my fiancé William Chappell for their love and support. I wish to thank the members of my committee, Dr. Phillip Pekala, Dr. Joseph Chalovich, Dr. John M. Lehman, and Dr. Maria Ruiz-Echevarria for their scientific guidance.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

Page

LIST OF FIGURES

CHAPTER 1 **Page**

CHAPTER 3

CHAPTER 4

LIST OF FIGURES (continued) **Page**

CHAPTER 5

CHAPTER 6

LIST OF TABLES

Page

CHAPTER 2

ABBREVIATIONS

ARE	**Adenine-uridine rich element**
ATCC	**American type culture collection**
ATP	**Adenosine triphosphate**
BAT	**Brown adipose tissue**
BMP	**Bone morphogenetic protein**
BSA	**Bovine serum albumin**
bZIP	**Basic-leucine zipper transcription factors**
C/EBP	**CCAAT/Enhancer binding protein**
CDK	**Cyclin-dependent kinase**
cDNA	**Complementary deoxyribonucleic acid**
CUGBP	**CUG repeat binding protein**
DEX	**Dexamethasone**
DMEM	**Dulbecco's modified Eagle's medium**
DMSO	**Dimethylsulfoxide**
DNA	**Deoxyribonucleic acid**
DOX	**Doxycycline**
DTT	**Dithiothreitol**
EDTA	**Ethylenediaminetetraacetic acid**
ELAV	**Embryonic lethal abnormal vision**
ESC	**Embryonic stem cell**
FBS	**Fetal bovine serum**

ABBREVIATIONS (continued)

FFA	**Free fatty acids**
GSK3β	**Glycogen synthase kinase 3β**
HEPES	**N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid**
HNS	**HuR Nucleocytoplasmic shutting sequence**
kDa	**Kilo Daltons**
LAP	**Liver-enriched transcriptional activator protein**
LIP	**Liver inhibitory protein**
MAPK	**Mitogen-activated protein kinase**
MC	**Melanocortin**
MCE	**Mitotic clonal expansion**
MCP-1	**Monocyte chemoattractant protein 1**
MDI	**MIX, DEX and Insulin**
MEF	**Mouse embryonic fibroblasts**
MIX	**1-Methyl-3-isobutylxanthine**
mRNA	**Messenger ribonucleic acid**
MSC	**mesenchymal stem cell**
NE-PER	**Nuclear and cytoplasmic extraction reagent**
NIDDM	**Non-insulin dependent diabetes mellitus**
NPY	**Neuropeptide Y**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**

ABBREVIATIONS (continued)

POMC	**Proopiomelanocortin**
PPAR	**Peroxisome proliferator-activated receptor**
qPCR	**Quantitative PCR (Real Time)**
RBP	**RNA binding protein**
RIP	**RNA immuno precipitation**
RNA	**Ribonucleic acid**
RRM	**RNA recognition motif**
RT-PCR	**Reverse transcription- PCR**
SDS	**Sodium dodecyl sulfate**
SREBP	**Sterol regulatory element binding protein**
TAG	**Triacylglycerols**
TNF-α	**Tumor necrosis factor alpha**
uORF	**Upstream open reading frame**
UTR	**Untranslated region**
WAT	**White adipose tissue**
ZFP	**Zinc Finger Protein**
αMSH	**Alpha melanocyte stimulating hormone**

Chapter 1- Introduction and Literature Review

The Epidemic Occurrence of Obesity

Obesity, world wide has reached epidemic proportions. The World Health Organization reported in 2008, that over 400 million adults are obese (96). In the United States alone, obesity affects up to one third of the adult population (about 60 million) and will claim more than 300,000 lives a year (3). The number of obese Americans has continued to increase at an alarming rate and is the second leading cause of unnecessary deaths (3). Obesity is projected to escalate to affect approximately 2.3 billion people by 2015 (96). This alarming rate of obesity heightens concern due to its implication with increased risk of many diseases and health conditions, such as, hypertension, coronary artery disease, dyslipidemias and even cancers (12). Obesity results when energy input exceeds expenditure, giving rise to both hypertrophy and hyperplasia of adipocytes. Hyperplasia is essential to the pathology of obesity through the enlistment and proliferation of preadipocytes followed by differentiation to adipocytes in the vascular stroma of adipose tissue, while hypertrophy simply refers to increased cell size (25, 61).

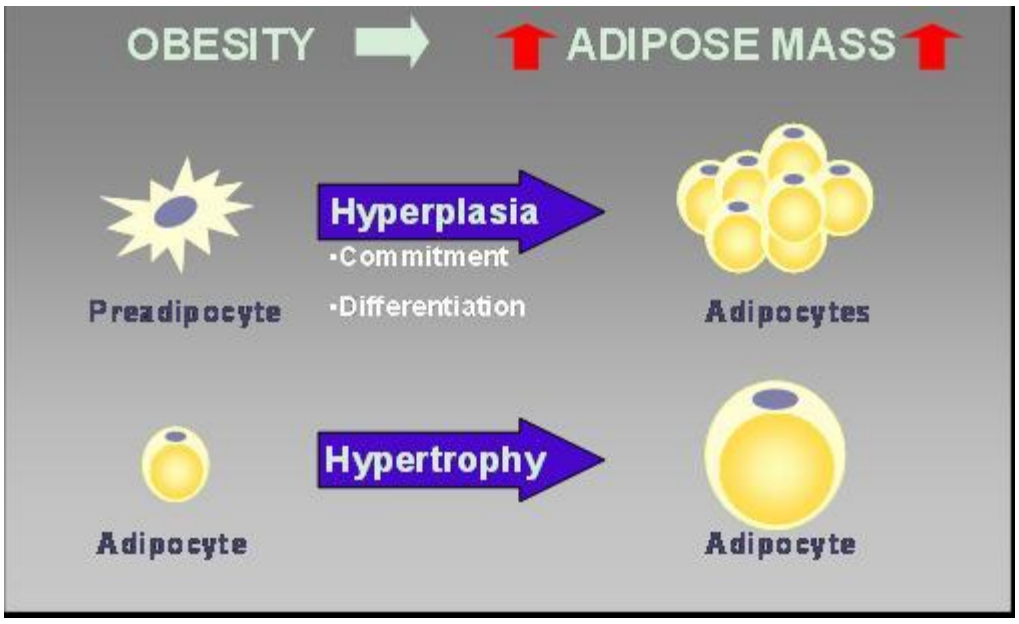


Figure 1 - Hyperplasia and hypertrophy of adipocytes leads to the increase of adipose tissue associated with obesity (Adapted from Otto and Lane (61)).

Diabetes Associated with Obesity.

The prevalence of obesity among adult type II diabetics is approaching 90 percent, which illustrates a strong correlation between obesity and diabetes type II (60). According to the World Health Organization, more than 180 million people worldwide suffer from diabetes (96). The National Diabetes Information Clearinghouse estimates that in the United States $174 billion dollars are spent every year for the treatment of diabetes (58).

Type II diabetes, previously known as adult-onset diabetes, maturity-onset diabetes, or non-insulin dependent diabetes mellitus (NIDDM) is a disorder of carbohydrate metabolism that is characterized by persistent hyperglycemia. Normal blood glucose levels range between 4 to 6 mmol/L, levels of glucose as seen in type II diabetes are in excess of 20 mmol/L. The elevated glucose levels are a result of insulin resistance, when normal or higher amounts of insulin are inadequate to produce an insulin response from fat and muscle resulting in the diminish ability of these tissues to transport glucose across the cellular membrane, ultimately leading to type II diabetes (3).

In a lean healthy individual, small adipocytes store free fatty acids (FFA) as triacylglycerols (20), these triacylglycerols can then be mobilized to generate ATP via mitochondrial β-oxidation in the muscle during caloric deprivation (27). However, obesity alters adipose tissue metabolism resulting in the elevation of inflammatory mediators and the initial insulin resistance in skeletal muscle (27). During times of excess caloric intake an increase in TAG storage results in adipocyte hypertrophy (27).

Yet, in a non-diabetic individual this increase of TAG storage and β-oxidation in the muscle can be maintained and insulin resistance is prevented (27). Conversely, excessive TAG overload in an overweight individual will result in adipocyte hypertrophy and increased secretion of monocyte chemoattractant protein-1 (MCP-1), which augments macrophage infiltration, adding to the pro-inflammatory state (27). Furthermore, the infiltrating macrophages secrete tumor-necrosis factor α (TNF-α), which in turn impairs TAG deposition and increases lipolysis (27). The over abundance of ectopic TAG and FFA accumulation in the muscle disrupts mitochondrial oxidative phosphorylation and insulin-stimulated glucose transport, thus triggering insulin resistance (27).

It is necessary to understand the development and regulation of adipogenesis in order to manage the health implications of adipose tissue in obesity and diabetes (61). This review will address key components involved in the regulation of adipocyte differentiation and therefore provide insight into the development of obesity.

Adipose Tissue, the Adipocyte and Obesity.

Practically all members of the animal kingdom, from C. elegans to Homo sapiens, have evolved means to store energy during excess caloric intake for future need (25). In most species, fat is stored as triacylglycerol in the adipocyte and can be mobilized via lipolysis, resulting in secretion of free fatty acids and glycerol (1). In mammals, two distinct types of adipose tissue have been classified: white adipose tissue(36), the primary site of energy storage, and brown adipose tissue which is

specialized to metabolize fatty acids and generate heat (32, 62). White adipose tissue is distributed throughout the body in multiple depots, both subcutaneously (buttocks, thighs, and abdomen) and intra-abdominally (omentum, intestines, and perirenal area) (25, 87). It is important to note that mature adipocytes are not the only cell type that constitutes the adipose organ; fibroblasts, endothelial cells and macrophages compose up to 50% of the total cellular content (87).

Adipose tissue is important not only because it is the body's largest energy reservoir but it is now recognized as playing a major role in the regulation of metabolism and homeostasis through paracrine and endocrine functions (27, 61). Adipocytes and adipose tissue produce a diverse range of secreted factors termed adipokines or adipocytokines, that effect numerous functions, including glucose metabolism (e.g. adiponectin, resistin), feeding behavior (leptin), lipid metabolism (e.g. cholesteryl ester transfer protein, CETP), inflammation (e.g. TNFα, IL-6), coagulation (PAI-1), blood pressure (e.g. angiotensinogen, angiotensin II) , as well as, influencing reproductive functions, insulin sensitivity and insulin secretion (28, 61).

A key adipocytokine in this system is leptin, a 16 kDa secreted hormone that acts on the hypothalamus of the brain inducing satiety. Leptin informs the brain on the status of body fat abundance that results in an alteration in feeding behavior (99). Leptin was first discovered in 1994, as the product of the *Ob* gene in genetically obese (*ob/ob)* mice (87). Plasma levels of leptin are generally proportional to adipose tissue mass, in a lean individual circulating levels are between 5-15 ng/ml (99). Increased expression of leptin is induced by overfeeding, insulin, glucocorticoids, and cytokines.

Leptin expression is decreased by fasting, testosterone, thyroid hormone and exposure to cold temperatures (99). To induce satiety, leptin binds to leptin receptors (Ob-R) in the basomedial hypothalamus. Neurons in this region are stimulated to synthesize proopiomelanocortin (POMC), which is cleaved to produce α-melanocyte stimulating hormone (αMSH), which then binds downstream melanocortin (MC) receptors. Signaling through MC-3 and MC-4 receptors limits food intake and body fat mass (73). Leptin also signals through another hypothalamic signaling pathway that decreases food intake by inhibiting neuropeptide Y (NPY) production. NPY, the most potent orexigenic substance in our body, stimulates food intake and decreases energy expenditure while inducing lipogenic enzymes in liver and adipose tissue (73).

Adipose tissue also secretes cytokines like TNF-α and peptides such as angiotensinogen. TNF-α is involved in inflammation, immune modulation and type II diabetes (as described previously), while angiotensinogen is implicated in wound healing, vascular remodeling, and regulation of blood pressure (27, 61).

Deregulation of adipose cells can cause obesity, type II diabetes, cardiovascular disease, hypertension, sleep apnea and muscular skeletal problems (1, 61). Obesity develops when energy intake persistently exceeds energy expenditure. Obesity is associated with marked changes in adipocyte gene expression that affects many pathways. Equally important to the development of obesity is the alteration of the secretory functions and the disregulated cellular homeostasis associated with larger adipocytes due to hypertrophy (67). Dysregulation of adipokine expression is now thought to be one of the key events in the pathogenesis of this metabolic disease (89).

There is a great need to understand the molecular mechanisms regulating this dynamic cell type and its involvement in disease states. The adipocyte can no longer be considered a inert cell whose exclusive function is to store lipids due to the emergence of new roles in diverse pathways of physiological and pathological processes (55).

Adipose Development and Regulation

Adipose tissue, bone and muscle originate from a multipotent stem cell population of mesodermal origin (25, 62). Mesodermal formation begins with the migration of cells during gastrulation, these cells form an additional germ layer between the primitive endoderm and ectoderm cell layers of the developing embryo (25). A great deal of our understanding of these events comes from studies using the C3H10T1/2, a multipotent stem cell line, isolated from 14 to 17 day C3H mouse embryos. The current model for stem cell commitment suggests that a common mesoderm/mesenchymal stem cell (MSC) gives rise to adipoblasts, osteoblasts, chondrocytes, myoblasts, and connective tissue in response to the appropriate developmental cues (25, 62). A MSC gives rise to an early precursor of the adipose cell lineage, the adipoblast, which then develops into committed white and brown preadipocytes in the vascular stroma (25, 61). One of these developmental cues necessary for this transition has been suggested to be bone morphogenetic protein-2 (BMP2) and BMP4, members of the transforming growth factor β superfamily, which are cytokines that have been shown to induce commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage (34, 80).

Adipogenesis is the processes by which preadipocytes differentiate into mature fat cells. This progression includes the expression of genes needed for the alteration of cell shape, glucose metabolism, the acquisition of insulin sensitivity, expression and secreting of adipogenic products, and the appearance of cytoplasmic lipid droplets (55). This shift in gene expression is regulated by a cascade of transcription factors that interact in an intricate manner to control expression of hundreds of adipogenic genes. There are two families of transcription factors critical to the process that have been studied at length, they include the CCAAT enhancer-binding proteins (C/EBPs) and the peroxisome proliferator-activated receptor (PPAR) family of nuclear hormone receptors, Fig 2 (29, 55).

The C/EBPs belong to a large family of basic-leucine zipper (bZIP) transcription factors. The general structure of the C/EBPs includes a C-terminal leucine zipper domain that allows dimmer formation, and a basic domain that binds to a consensus sequence on DNA. Six members of this family have been characterized, α, β, δ, γ, ε, and ζ; they are all capable of forming hetero- and homodimers that will bind to promoters/enhancers of different genes to alter expression. C/EBPα, β and δ are three family members that have been extensively studied due to their important involvement in the regulation of adipogenesis (29, 55, 70). Freytag and colleagues demonstrated that ectopic expression of C/EBPα in a variety of fibroblastic cells could induce adipogenesis (20). Furthermore, total ablation of C/EBPα (except liver) in knockout mice demonstrated that C/EBPα is required for white adipose tissue formation, and not brown adipose tissue formation (50). Ectopic expression of C/EBPβ alone induces

adipogenesis in nonadipogenic NIH 3T3 fibroblasts (19). McKnight and colleagues agree that C/EBPδ alone possesses minimal adipogenic activity, but together with C/EBPβ, play important roles in the induction of C/EBPα (11).

The PPARs represent a subset of the nuclear hormone receptor superfamily whose transcriptional activity is altered by ligand-receptor interactions (55). The PPAR family members include PPARα, PPARγ, and PPARδ, which bind to the peroxisome proliferator response elements on DNA. PPARγ has been implicated as the only PPAR family member to play a major role in adipogenesis. Studies have shown that ectopic expression of PPARγ in nonadipogenic mouse fibroblasts can initiate the entire adipogenic program, giving rise to mature adipocytes (86). PPARγ is expressed in two isoforms generated by alternative promoter usage of the same gene, PPARγ1 and PPARγ2 (19). These isoforms gives rise to four distinct mRNAs, pparγ1-4. All four mRNAs encode the PPARγ1 polypeptide, while pparγ2 mRNA encodes the PPARγ2 polypeptide, which is identical to PPARγ1 with the exception of an additional 30 amino acids at the N-terminus (19). PPARγ1 is expressed in many tissues, however PPARγ2 expression is almost exclusively found in adipose tissue (19). Both PPARγ1 and PPARγ2 isoforms form heterodimers with retinoid X receptor (RXR) then bind to promoters/enhancers of target genes (90).

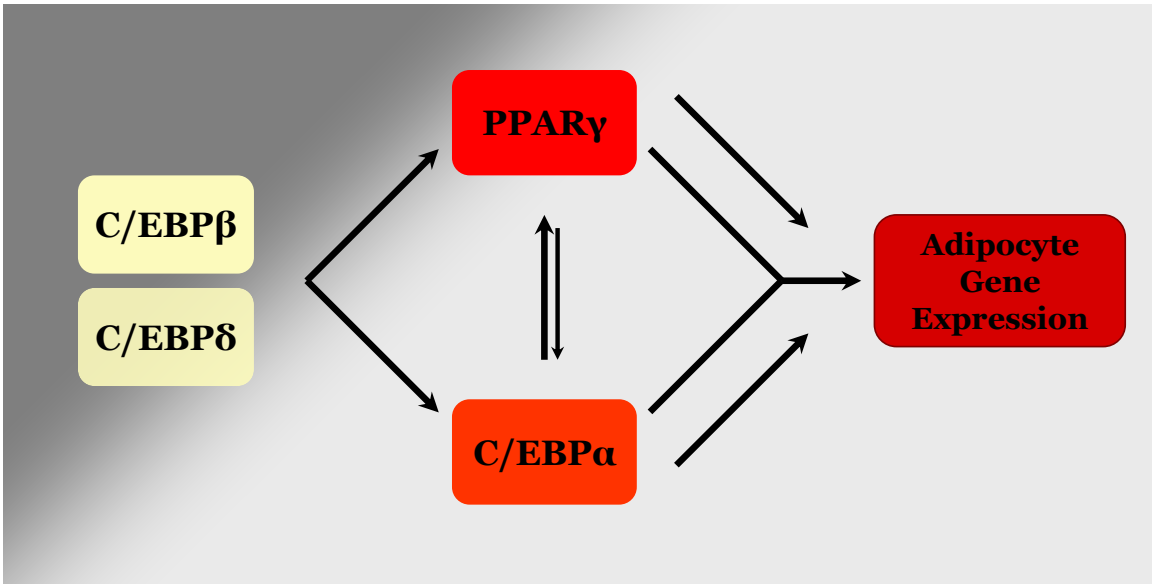


Figure 2 - Transcription factor pathway that mediates adipocyte differentiation (Adapted from Morrison and Farmer (55)). C/EBPβ and C/EBPδ transcription factors transactivate expression of PPARγ and C/EBPα. PPARγ and C/EBPα co-regulate each others expression and together transactivate the expression of the genes responsible for the acquisition of the adipocyte phenotype.

The PPARγ-RXR complex binds ligands, such as, 9-cis-retinoic acid, 15d-PGJ2 or fatty acids in the C-terminal hydrophobic pocket (29, 72, 90). Recent work by Spiegelman and Walkey, suggest that ligand binding is not a requirement for PPARγ function in the adipose differentiation process (90).

The regulation of gene expression is important for the acquisition of the adipocyte phenotype and depends on a cooperative interplay and transactivation between the C/EBPs and PPARγ transcription factors. In knockout mice, Tanaka et al demonstrated that mice lacking both C/EBPβ and -δ were defective in the ability to generate adipose tissue (75). McKnight and associates showed that C/EBPβ and -δ are expressed earlier than C/EBPα during adipogenesis and are responsible for the expression C/EBPα (19). It was hypothesized that C/EBPβ and δ simultaneously controlled both PPARγ and C/EBPα (19). Others hypothesized that C/EBPβ induces C/EBPα and together those two factors regulated PPARγ. Recently, studies with retroviral expression of C/EBPβ in ppar-/- MEFs, showed that C/EBPβ was incapable of stimulating C/EBPα expression in the absence of active PPARγ (107). Furthermore, ectopic expression of C/EBPβ in Swiss fibroblasts was able to induce expression of PPARγ but was unable to induce C/EBPα expression to any significant degree in the absence of a potent PPARγ ligand (19). Therefore, as shown in figure 3, the cascade of transcription factors of adipogenesis involves the induction of C/EBPβ and C/EBPδ, which then mediates the expression of PPARγ (107). PPARγ along with C/EBPβ and δ then activates C/EBPα by binding to C/EBP consensus sequences in the promoter

region. Once PPARγ and C/EBPα are activated, they can then maintain each others gene expression through cross-regulation, even after C/EBPβ and C/EBPδ expression has diminished (55, 66). The co-expression of PPARγ and C/EBPα leads to the expression of adipogenic specific genes.

An additional transcription factor family known as the sterol regulatory element binding proteins (SREBPs) is also recognized to play a role in the modulation of transcription of numerous genes encoding proteins essential in both cholesterol and fatty acid metabolism (55). The SREBP family consists of three members, SREBP-1a, -1c, and -2, which contain two transmembrane domains that anchor the protein to the endoplasmic reticulum. When sterol levels are low, two proteolyic events occur and cause the release of the N-terminal fragments of the SREBP protein into the cytoplasm. The SREBP fragments then translocate to the nucleus where they can bind to promoters of target genes. SREBP-1a and -1c have been primarily implicated in the regulation of fatty acid biosynthesis and SREBP-2 regulates cholesterol metabolism (55). Studies with SREBP1c in regulating adipogenesis demonstrated that its expression significantly augmented the 3T3-L1 adipocytes ability to respond to insulin. Moreover, ectopic expression of a dominate-negative SREBP1c was shown to inhibit differentiation. Others have shown that SREBP1c is linked to the induction of PPARγ1 expression through SREBP binding sites in the promoter regions responsible for pparγ1 and γ3 transcription (19). Many additional transcription factors are components to this complex cascade of factors that are responsible for induction of adipogenesis, the factors outlined above are considered the main players.

3T3-L1 preadipocyte: A model for the mechanism of adipogenesis

Adipogenesis follows a highly ordered temporal sequence of events. The knowledge of these events and the factors involved have been greatly enhanced by the development of cell lines that differentiate from fibroblastic like cells into mature adipocytes (55). The 3T3-L1 preadipocyte cell line derived from Swiss 3T3 cells, has been extensively characterized and is one of the most notable of these cell lines that are committed to the adipocyte lineage (61). In the first steps of adipogenesis *in vitro*, the proliferating 3T3-L1 preadipocytes exhibit contact inhibition of growth. These growth-arrested cells reenter the cell cycle after addition of the differentiation inducers (MDI), a combination of isobutylmethylxanthine (M, a cAMP phosphodiesterase inhibitor), dexamethasone (D, a glucocorticoid), insulin (I, which acts through the insulin-like growth factor-1 receptor), and fetal bovine serum (FBS); the cells then undergo several rounds of cell division referred to as mitotic clonal expansion (29, 65, 72). Mitotic clonal expansion mimics the increase in cell number (hyperplasia) observed in obesity (19). Recent evidence, demonstrating that inhibition of proliferation with drugs that block cell cycle at S phase prevent adipogenesis, is consistent with MCE being required for terminal adipocyte differentiation (19). It is believed that re-organization of chromatin during mitosis is needed to facilitate accessibility of cis-elements to trans-factors essential for transcription of genes that give rise to the acquisition of the adipocyte phenotype (19, 61). In 3T3-L1 preadipocytes, the onset of MCE is characterized by changes in expression of cyclin E/A, cdk2 and turnover of cyclin-depended kinase inhibitors p18, p21 and p27 (61). As the cells traverse the G1/S check point, the

initiation of the transcription factor signaling cascade, as shown in Fig. 2, is started when C/EBPβ acquires DNA-binding activity (81). Figure 3, displays the protein levels of the three members of the C/EBP family involved in the differentiation program. Although it has been shown that C/EBPβ gene is rapidly expressed after induction of differentiation with protein found in the nucleus within 2 hours, DNA-binding activity is not acquired until dual phosphorylation by mitogen-activated protein kinase (MAPK) and glycogen synthase kinase 3β (GSK3β) that occurs within 14 hrs (76, 101). This event triggers the transcription factor cascade as described in the previous section. Terminal differentiation is maintained, at least in part, through the expression of C/EBPα and PPARγ. The expression of C/EBPα has been shown to be antimitotic and is thought to be responsible for termination of MCE as the cells exit the cell cycle (61). This anti-mitotic activity elicited by C/EBPα is through direct interaction with cyclin-dependent inhibitor p21 and cyclin-dependent kinase cdk2 to directly inhibit the cdk enzymatic activity required for cell cycle progression (30). Importantly, it is pertinent to note that C/EBPβ expression is essential for the movement of the cells from a quiescent stage through MCE and terminal differentiation.

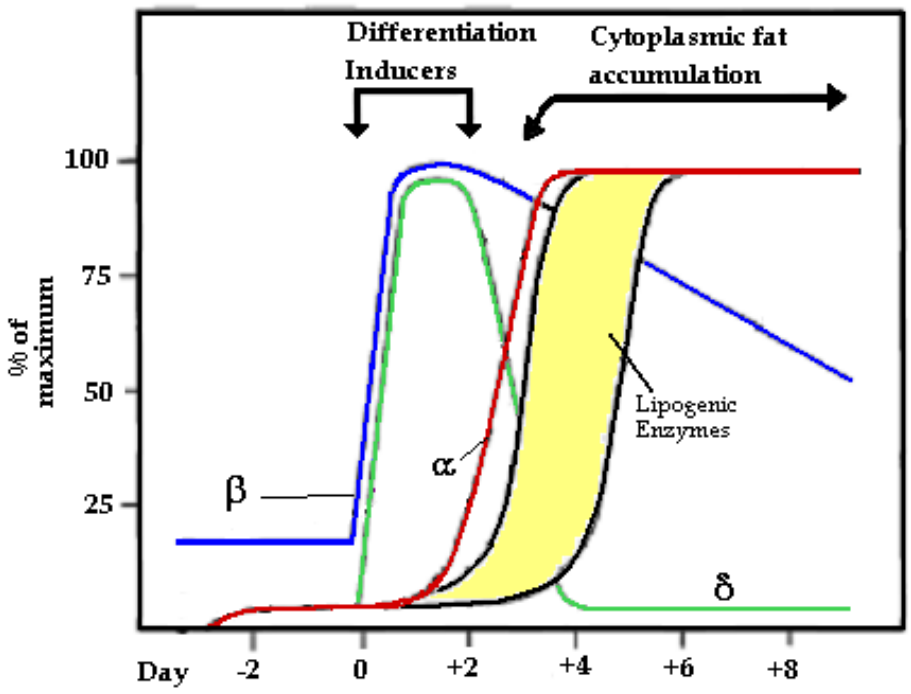


Figure 3 - CCAAT/ enhancer binding protein (C/EBP) family members involved in adipogenesis. Basal level C/EBPβ protein is found -4 days prior to cell confluence. Together C/EBPβ and C/EBPδ protein levels rise after treatment with differentiation inducers. C/EBPα protein levels dramatically rise 2 days post induction of differentiation and remain at 100% activity. As C/EBPα reaches 100% activity C/EBPβ protein levels begin to steadily decline and lipogenic enzyme gene expression increases. C/EBPδ protein levels dramatically decrease after induction of C/EBPα expression.

CCAAT/ Enhancer Binding Protein β (C/EBPβ) mRNA

C/EBPβ is our transcription factor of interest, as there is considerable data to support its role as a key regulator of adipogenesis (79, 81, 102). C/EBPβ is encoded by an intronless gene and its mRNA produces several protein isoforms due to alternative translation initiation sites (4, 68). These in frame AUG start codons produce three N-terminal truncated isoforms corresponding to protein species of, 38, 35, and 20 kDa (4, 68). The p35 protein, the full length transcription factor, is referred to as LAP (liver-enriched transcriptional activator protein), which is a potent transactivator of gene expression. The p38 isoform (LAP*) is also a full length transcription factor but is not produced as frequently as p35. The p20 protein is referred to as LIP (liver inhibitory protein), which lacks the amino terminal transactivation domain but contains the bZIP domain (29, 79). LIP can act as a dominate-negative repressor of LAP activity and can actually block adipogenesis of preadipocytes in culture if the ratio of LAP to LIP is < 1 (29).

The molecular mechanisms that regulate the selection of alternative translational start sties of C/EBPβ are not well understood. Three mechanisms have been proposed. First, selection is controlled by an out of frame upstream open reading frame (uORF) in the 5′ untranslated region (UTR) (29, 79). Xiong *et al* (98) demonstrated that multiple C/EBPβ isoforms are produced by alternative translation initiation at the multiple AUG start sites, and demonstrated a role for the uORF in the regulation of alternative translation initiation by a ribosomal scanning mechanism. Work by Calkhoven *et al* (10) suggests that the LAP to LIP ratio is controlled by the 5′ uORF and

the availability/activity of translation initiation factors eIF2α and eIF4E. They demonstrated using COS-1 cells transfected with C/EBPβ constructs, that overexpression of eIF2α and eIF4E enhanced expression of the truncated isoform (59). Secondly, Welm *et al* have proposed an alternate mechanism involving a proteolytic cleavage of LAP to generate LIP, *in vivo* and *in vitro* (5, 95). Finally, data by Timchenko et al, suggests that translation of LIP and LAP is controlled by CUG repeat binding protein -1 (CUGBP1), which binds to the 5' untranslated region of the C/EBPβ mRNA and regulates a leaky ribosomal scanning mechanism (83, 84). Although the role of CUGBP1 has been studied in myogenesis, nothing is known about the role it plays in preadipocyte differentiation (4).

Post-Transcriptional Gene Regulation and RNA Binding Proteins.

In recent years post-transcriptional processes have gained recognition as key points of precise gene regulation. Post-transcriptional regulation is an adaptive mechanism that allows the cell to adjust gene expression according to environmental changes and physiological processes such as, stress, proliferation, and differentiation (14, 16, 31). Steps in the post-transcriptional mRNA processing, including splicing, polyadenylation, stability, editing, localization, decay and translation, can provide points of regulation to modulate changes in protein levels of a specific gene (31, 63).

Post-transcriptional regulation is dependent on the activity of a variety of *trans*-acting factors, known as RNA-binding proteins (RBPs). It has been estimated that there are more than one thousand RBPs in the human genome. From the beginning of a pre-

mRNA molecule's journey in the nucleus to the ribosome in the cytosol, RBPs are a constant accessory. RBPs bind to cis-elements (protein binding sites) which are frequently present within the 3' untranslated region (UTR) of the messenger RNA but can also be located in the 5'UTR, in introns, and even in coding sequences. The 3'UTR of mRNA is widely known for bearing sequence motifs that determine the rate of mRNA decay. The most commonly studied group of destabilizing motifs are the adenine- and uridine-rich elements (AREs), which are characterized by a highly conserved AUUUA pentamer sequence (16, 63). Many mRNAs subject to rapid response to cell environmental stimuli, such as, growth-response genes, cytokines and proto-oncogenes, contain these AREs (91). While intrinsic instability of these mRNAs may be mediated by RBPs such as AUF-1, a family of proteins known as the Hu-proteins bind to the AREs and this results in stabilization (31, 47).

The Hu-proteins are a group of classical RBPs that share homology with the Dropsphila ELAV proteins. The Hu family consists of four highly homologous members, HuR (HuA), HuB (Hel-N1), HuC (PLE21), and HuD. Hu proteins B, C and D are expressed specifically in neurons and serve as very early markers for the commitment stage of developing neuronal cells (63). HuB is also expressed in the nervous tissue, testis, and ovaries. HuR is the most widely expressed of the Hu antigens (63). Hu proteins were first identified as target antigens of paraneoplastic neurological syndrome. The presence of certain types of tumors, like small cell lung carcinoma, ectopic expression of antigenic neuronal Hu proteins on the cell surface elicits an autoimmune response, resulting in the production of antibodies. These

antibodies cross the blood-brain barrier and lead to sub-acute sensory neuropathy, dementia and/or encephalomyelopathy (63).

Structurally, the four vertebrate Hu proteins contain three ~90 amino acid–long RNA recognition motifs (RRMs 1-3). The first two RRMs are positioned at the N-terminus and are separated from the third RRM at the C-terminus by a flexible hinged region (63, 85). RRMs are the most common RNA-binding domain and are characterized by two α-helices packed against four anti-parallel β-strands. Structural studies have shown that RRM1 and 2 interact with RNA through amino acids located on the β-strands, in a manner similar to other RBPs (63, 85). RRM3 has been shown to aid in maintaining the stability of the RNA-protein complex, implicated in protein-protein interactions, and might also bind to poly (A) tails (31). There is a substantial body of evidence that demonstrates that the Hu proteins are involved in the regulation of the essential steps of mRNA processing. Some of these studies include mRNA export, stability, translation efficiency (46, 64), alternative splicing (105), and polyadenylation (106).

HuR, the focus of our studies, is a 36 kDa protein that is ubiquitously expressed and found predominantly in the nucleus. HuR contains a shuttling sequence in the hinge region between RRM2 and RRM3, deemed the HNS (HuR nucleocytoplasmic shuttling sequence). The HNS contains both a nuclear localization signal and a nuclear export signal, that allows for shuttling between the nucleus and cytoplasm (31). HuR was initially suggested to function as a chaperon for mRNA ligands, accompanying them into the cytoplasm and providing protection from degradation (7). Strengthening

this argument, HuR has been found to associate with other RBPs, such as, SETα, SETβ, pp32 and APRIL proteins, which have nucleocytoplasmic shuttling capabilities (63). Fan and Steitz revealed that overexpression of HuR resulted in an increase in the stability of ARE-containing mRNAs (18) Several others have described the ability of HuR to stabilize specific mRNA targets including, GLUT1 (37), p27 (92), TNFα (49), cyclin D1, p21 (47) and cyclins A and B1 (91). In addition, previous studies from our lab demonstrated that HuR binding to the GLUT1 mRNA results in an increased efficiency of translation initiation (24). Additionally, Lou and Zhu established that HuR is able to block polyadenylation of the SV40 late (SVL) mRNA in transfected cells (106). Conversely, in a recent study Meisner *et al* demonstrated that RRM3 of HuR contains a functionally conserved Me^{2+}-binding motif in which ATP is a natural ligand (54). They further show that this HuR-ATP interaction is associated with terminal adenosyl transferase activity and RNA-modification (54). As the investigation of HuR function continues our understanding of the multiple mechanisms by which HuR acts to modify gene expression are coming to light.

C/EBPβ mRNA and the RNA Binding Protein HuR.

Previous studies have shown that HuR is constitutively expressed in 3T3-L1 cells and localized predominantly to the nucleus. Within 30 minutes of induction of differentiation, there was a 30% increase in the cytosolic content of HuR protein (23). This rapid translocation of HuR into the cytosol on induction of differentiation suggests

that HuR plays an important function early in adipogenesis. Gallouzi and Steitz (22) proposed that HuR may function as a mRNA chaperon protein during nuclear export. We then began to examine for potential mRNA ligands expressed very early in the differentiation program to determine if HuR plays a role in regulation of adipogenesis through binding to mRNA ligands. Cole *et al.* (13) established that within 2 hours of induction of differentiation, C/EBPβ protein could be found in the nucleus, suggesting that C/EBPβ mRNA must be present in the nucleus shortly after induction of differentiation. Examination of the C/EBPβ mRNA sequence in the 3′ UTR revealed a substantial ARE which we predicted could serve as a HuR binding site (Fig 4A). Binding of HuR to the ARE was confirmed *in vitro* (23). Further analysis of the entire message supports the existence of a single HuR binding site to ARE in the 3′-UTR, as shown in figure 4B (38).

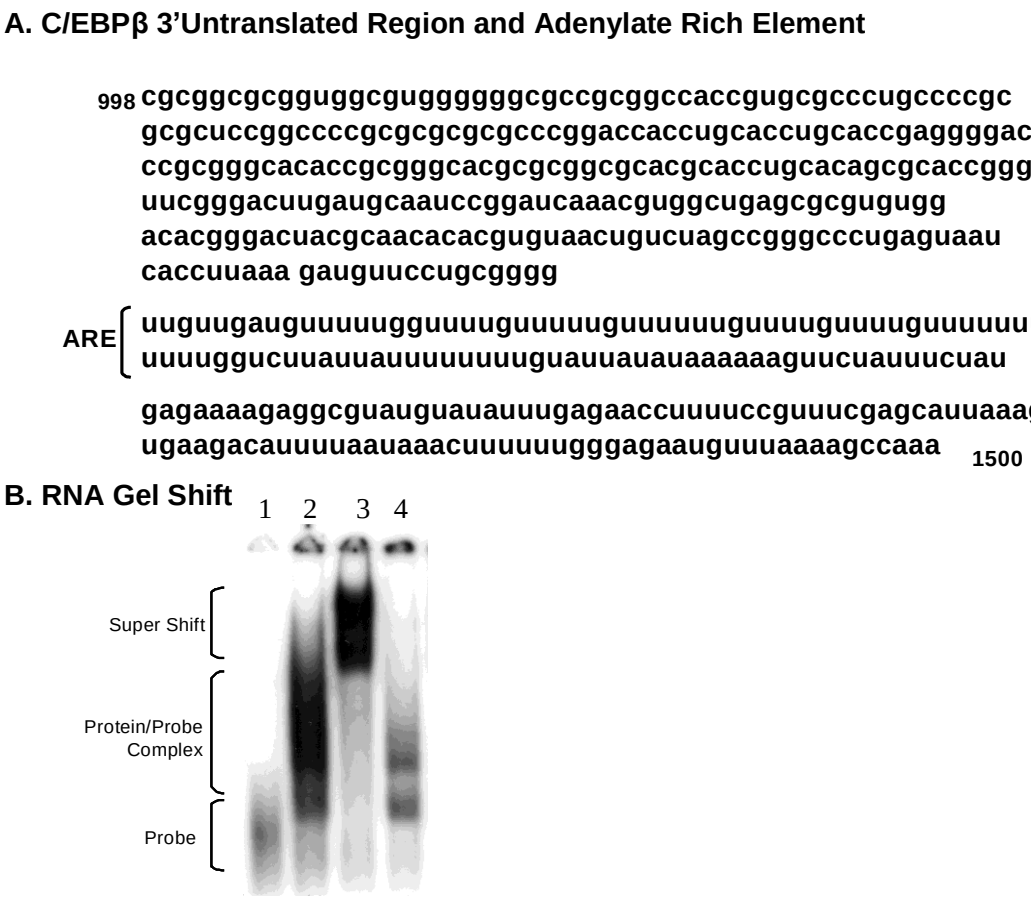


Figure 4 - C/EBPβ mRNA is a Ligand for HuR *in vitro*. (A) 3'UTR and ARE of C/EBPβ mRNA (B) RNA gel shift analysis of HuR binding to C/EBPβ 3'UTR ARE (23). Lane 1 is radioactive probe for C/EBPβ 3'UTR only, Lane 2 is radioactive probe and 3T3-L1 protein lysate, Lane 3 is radioactive probe, 3T3-L1 protein lysate, and HuR 3A2 antibody, Lane 4 is the addition of non-radioactive probe (competition control) .

Statement of Hypothesis

Based on the accumulated data as well as substantial work in the field, we proposed that HuR binding to the C/EBPβ mRNA represents a control mechanism in the metabolism of this message and that HuR may play a role in the onset and maintenance of the adipocyte phenotype. The work described in the body of this dissertation addresses this hypothesis.

Chapter 2 – Experimental Procedures

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco/Invitrogen (Grand Island, New York). Bovine calf serum and fetal calf serum were purchased from Hyclone Laboratories (Logan, Utah). The 3T3-L1 cells used in this work were obtained from Howard Green (Harvard University, Boston, MA). Antibodies directed against: Cyclin A were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclin D1 and p53 were from Cell Signaling (Danvers, MA). Cyclin E antibodies were from Upstate (Charlottesville, VA). β-actin antibodies were from Sigma (St. Louis, MO). p21 antibodies were from Calbiochem (LaJolla, CA). The β-tubulin antibody was a generous gift of Ted Bertrand, Department of Medicine, Brody School of Medicine (Greenville, NC). The antibody used as a control, anti-Armenian–Syrian hamster monoclonal antibody was obtained from BD Pharmingen (San Diego, CA). The rabbit Zfp206 antibodies Ab-1, Ab-2 and Ab-3 were the generous gift of Lawrence Stanton, Department of Stem Cell and Developmental Biology, Genomic Institute of Singapore, Singapore. The 3A2 monoclonal antibody directed against HuR as well as the VSV-G, C/EBPβ, and PPARγ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The BCA protein assay kit, NE-PER™ cell fractionation kit, and HALT™ protease inhibitor mix were from Pierce. The siGENOME SMARTpool™ reagent and siCONTROL™ non-targeting siRNA were obtained from Dharmacon (Lafayette, CO) Opti-MEM I reduced serum medium, RNase inhibitor, Lipofectamine 2000 were purchased from Invitrogen. The BD Retro-X

Universal packaging system, RevTet-Off™ system, MEF/3T3 Tet-Off cell line, vesicular stomatitis virus G protein (VSV-G) and enhanced green fluorescent protein expression vectors were obtained from BD Biosciences/Clontech. pENTR223.1-Zscan10 vector obtained from Open Biosystems. pcDNA-DEST53 Gateway™ Vector, OneShot® ccdB Survival™ T1 Phage-Resistant cells, and Gatway® Clonase™ II Enzyme Mix were obtained from Invitrogen. The QuikChange-XL site-directed mutagenesis kit and XL-10 Gold Ultracompetent cells were purchased from Stratagene (Cedar Creek, TX). The plasmid midi kit was obtained from Qiagen (Valencia, CA). The MAXIScript T7 kit and KinaseMax™ 5'end labeling kit was from Ambion (Austin, TX). SDS-PAGE Electrophoresis Gel reagents (Protogel™) were purchased from National Diagnostics (Atlanta, GA). All other chemicals were of reagent grade and purchased from Sigma–Aldrich Biochemical (St. Louis, MO). Reagents for molecular biology were purchased from Invitrogen.

Table 1. Primers and oligonucleotides used for experimental procedures.

Gene	Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Size
Mutagenesis-poly(A)				
C/EBPβ	TOPO T34A G38C and anti-	5'-GGTGACACTATAGCGGGGTTGT**A** GAT**C**TTTTTGGTTTTGTTTTTGTTTT-3'	5'- AAAACAAAAACAAAACCAAAAAGA TCTACAACCCCGCTATAGTGTCACC-3'	N/A
C/EBPβ	Beta T1389A and anti	5'-GTCTTATTATTTTTTTTGTATTATATAAAAAA GA**T**CTATTTCTATGAGAAAAGAGGCGTATG-3'	5'-CATACGCCTCTTTTCTCATACAAATAGATC TTTTTTATATAATACAAAAAAAATAATAAGAC-3'	N/A
Polyadenylation Determination				
C/EBPβ	Oligo dT Adapter	GCGAGCTCCGCGGCCGGCGTTTTTTTTTTT		N/A
C/EBPβ	275NT5PA	AAACGTGGCTGAGCGCGTGT		N/A
Real Time PCR				
C/EBPβ	CEBPBETA For 1173 and CEBPBETER Rev 1256	CGGGTTTCGGGACTTGATGCAAT	GACAGTTACACGTGTGTTGCGTAG	88
β-Laminin	Laminin For and rev	TCGCTTGTTCTCATGCCCTACTGT	AGGTTTCCCAGGCACTATTGGTGA	
Zfp206 exon 6	Zfp qPCR Fwd and Rev	AGTTGCACCACACGGTCTTACG	CAGGAGTACCTCCCCAACCA	71
Zfp206 exon 2	Zfp qPCR2 Fwd and Rev	GTGGTACAGCTATTGGAGGG	CATTCTTGCCAGCACTGAAG	72
PCR				
C/EBPβ cDNA	Human Beta UTR For and Rev	TGTTCCTACGGGCTTGTT	GGCTTTGTAACCATTCTCAAAA	~300
C/EBPβ gDNA	Human Beta SP6 and Rev	GGATCCATTTAGGTGACACTATAGTGTTCCTACGGGCTTGTT	GGCTTTGTAACCATTCTCAAAA	~300
C/EBPβ cDNA	Human Beta gene	GGATCCATTTAGGTGACACTATAGCCAAACCAACCGCACATG	CACCAAAACCTCCAAAAATAACAGCAGCCCCC	~442
C/EBPβ	Beta H site F and Rev	GTTTCGGGACTTGATGCAAT	GGCTTTTAAACATTCTCCCAAA	322
Zfp206	ZFP Iso Fwd and Rev	GACGGAGAGGAGGTGGTACA	AGCTGCGTCCAAAAGTCTTC	FL= 844
Oct4	Oct 4 Fwd and Rev	GGCGTTCTCTTTGGAAAGGTGTTC	CTCGAACCACATCCTTCTCT	313
Sox2	Sox 2 Fwd and Rev	ATGGCCCAGGAGAACCCCAA	TCGTAGCGGTGCATCGGTTG	428

Methods

Cell Culture

3T3-L1 cell culture - 3T3-L1 preadipocytes were cultured, maintained, and differentiated as described previously (37). Differentiation was induced 2 days post confluence by exposure of the cells to 115 µg/ml isobutyl methyl xanthine (MIX), 40µg/ml dexamethasone(50), and 10µg/ml insulin in the presence of 10% fetal bovine serum, referred throughout the text as "MDI" or "differentiation inducers". Two days later, the culture medium was changed to medium lacking MIX and DEX, yet supplemented with Insulin. Two additional days later, the culture medium was supplemented with a decreased concentration of insulin (2.5 µg/ml); 48hrs later, insulin was omitted.

Transfections - GP2-293 packaging cells were maintained in growth medium consisting of DMEM with 10% fetal bovine serum (FBS) on collagen I-coated plates. Upon reaching 90% confluency, the medium was changed to Opti-MEM I reduced serum medium with 10% FBS (no antibiotics). Co-transfections of the particular β construct along with an expression construct for VSV-G were performed utilizing Lipofectamine 2000™ according to the manufacturer's recommendations. All transfections were accompanied by an enhanced green fluorescent protein transfection efficiency control. At 6 hours post-transfection, the medium was changed to fresh DMEM with 10% FBS. At 48 hours after the medium change, the virus particle-containing supernatants were harvested, filtered through a 0.45-µm filter, and either used immediately or stored at -80 °C. A second harvest was carried out at 120 hrs.

Production of virus particles was confirmed by Western blot analysis for VSV-G in the culture supernatants.

Transductions - The multipotential MEFs (MEF/3T3 Tet-Off cell line) express the tetracycline-controlled transactivator and were cultured in growth medium consisting of DMEM containing 10% calf serum and 100 ng/ml doxycycline on 6-well plates. Cells were plated and transduced at 15–20% confluency by first removing one ml of spent media followed by the addition of 2 ml of virus particle-containing medium plus 6μg/ml polybrene. Cells were then spinoculated via centrifugation at room temperature for 90 min at 2000 rpm. Following an overnight incubation, the medium was changed to DMEM with 10% FBS and 100ng/ml doxycycline. For cells that would follow the differentiation protocol, doxycycline was removed at 50% confluency. Cells were allowed to reach confluency and then subject to the differentiation protocol as previously described with the addition of 10μM troglitizone. Notably, because of the high transduction efficiency (always in excess of 90%, similar to reports by Neal and Clipstone (59), no drug selection was required. In every experiment, control transductions were performed in duplicate using a construct in which expression of enhanced green fluorescent protein was quantified by flow cytometry.

SiRNA treatment of the 3T3-L1 preadipocytes - Transfection of the cells was performed using Dharmacon siGENOME SMARTpool™ reagent (catalog number M-053812-00-0020) as we have previously described (23), a four-siRNA oligo system designed specifically for HuR (mouse ELAV1; GenBank™ accession number

NM_010485 [GenBank]) by Dharmacon. Cells were transfected with siRNA using Lipofectamine 2000™ as a carrier according to manufacturer's instructions (Invitrogen). Control transfections were carried out with Lipofectamine 2000™ alone. Briefly, preadipocytes were transfected in 12-well plates at 60% confluency and again 24h later while the cells remain pre-confluent. The cells were exposed to the transfection mixture for 6 h, at this time the transfection medium was replaced with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. At 24 h after the second transfection, two monolayers were combined to generate immediate confluency, 48 h after the second transfection differentiation was initiated as we have previously described. Cells were then analyzed for acquisition of the adipocyte phenotype and harvested at 0 time, 8, 12, 16, 20 and 24h post MDI.

Similar procedure was followed for Zfp206 suppression, transfection of the cells was performed using Dharmacon ON-TARGETplus SMARTpool™ reagent (catalog number J-054446-09), a four-siRNA oligo system designed specifically for Zfp206 (mouse Zfp206; GenBank™ accession number NM_001033425 [GenBank]) by Dharmacon.

DNA Procedures

Preparation of C/EBPβ 3'UTR Polyadenylation Analysis constructs - Amplification of the 324bp 3'UTR/3'end of C/EBPβ (βwt) was cloned into pCR4 TOPO TA vector (Invitrogen). Primers used for procedure and site directed mutagenesis are described in Table 1. Briefly, the mutant C/EBPβ construct with point mutations

flanking the HuR binding site (βpm) was created by site directed mutagenesis using QuikChange-XL™ site-directed mutagenesis kit (Stratagene). This resulted in the creation of BglII restriction sites flanking the HuR binding site. Removal of the 101-base BglII fragment and subsequent replacement with a 113-base BglII fragment derived from the GLUT1 3'-UTR (bases 1758–1871) resulted in the creation of the mutant C/EBPβ construct with the HuR binding site deleted and substituted (βd/s). Confirmation of mutations and appropriate orientation was obtained by sequencing at the Genomics Core Facility East Carolina University, Greenville, NC. Constructs were sent to Hua Lou, Case Western Reserve, Columbus, OH for further analysis.

Preparation of ZFP206-GFP fusion Construct – Gateway™ Technology system was utilized to create a Zfp206-GFP fusion construct, a universal cloning method that uses site-specific recombination properties to move gene of interest into multiple vector systems. Briefly, the open reading frame of murine Zfp206 was inserted into pENTR223.1 vector by Open Biosystems. pENTR-zfp206 was used in a LR Clonase II reaction with pcDNA-DEST53, a Gateway™ destination N-terminus GFP fusion vector. Example reaction: attL1-gene-attL2 (entry clone) x attR1-ccdB-attR2(destination clone) = attB1-gene-attB2 (expression clone) x attP1-ccdB-attP2 (pDONR). This reaction resulted in a pcDNA-DEST53-ZFP206 where GFP is fused to the N-terminus of the ZFP206 ORF.

RNA Procedures

Immunoprecipitation of mRNP Complexes—The mRNP complexes from either nuclear or cytosolic fractions were immunoprecipitated and mRNA isolated using RNase- and DNase-free conditions by a modification of the protocol described by Tennenbaum et al (82). Protein A-Sepharose beads were resuspended overnight at 4 °C in 10 ml of HNTM buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM MgCl2, and 1% Triton X-100). For immunoprecipitation, 200 μg of cytosolic lysate or 100 μg of nuclear lysate was utilized. Seven samples were prepared for each immunoprecipitation, six for the isolation of RNA and one as a Western blot analysis control. Starting with the protein extract in HNTM buffer, the following were added to an RNase/DNase-free microfuge tube: Halt protease inhibitor (10 μl), vanadyl ribonucleoside complex (10 μl), 40 units/μl RNase inhibitor (10 μl), 0.1 M dithiothreitol (1 μl), and 0.5 M EDTA (33 μl). The antibody was then added; for the isotype control, anti-Armenian/anti-Syrian hamster IgG1 monoclonal antibody was used, and for HuR, monoclonal 3A2 was used. Finally, the appropriate volume of lysate was added and the mixture rotated for 3 h at room temperature followed by the addition of protein A-Sepharose beads with continued mixing for an additional 2 h at room temperature. This was followed by two washes with HNTM buffer alone. The mixture was then centrifuged (13,200 rpm at 4 °C) and washed three times with cold 1 M urea in HNTM buffer. One of the samples was taken at this time for the Western analysis control, and RNA was extracted from

the remaining samples using the TriZol RNA isolation protocol as per the manufacturer's instructions. Extracted RNA was stored at -80 °C.

Real Time PCR - Real-time PCR analysis was performed as described previously (24) with minor modifications. Briefly, total RNA (0.5 μg, integrity demonstrated by ethidium-stained agarose gels) was subjected to reverse transcription with random primers, oligo d(T) and reverse transcriptase reagents from the iScript™ cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed with specific primers designed for each gene with the Beacon Designer tool (Bio-Rad). All primers were subjected to Blast search to ensure specificity and fold analysis to eliminate any primers with potential to form secondary structure. Amplification and detection were done with the iCycler IQ real-time PCR detection system with IQ SYBR Green Supermix (Bio-Rad). Standard curves were prepared for each target gene, and PCR efficiency was determined to be in excess of 90% for all primer sets. Threshold temperatures were selected automatically, and all amplifications were followed by melt-curve analysis, *i.e.* plot of the negative first derivative of the fluorescence *versus* temperature with the software assigning the melt temperature. Single-melt temperatures were recorded in all cases. To calculate relative C/EBPβ mRNA, the threshold cycle (C_T) determined for the cells transduced with the empty vector (EV) (endogenous C/EBPβ) was subtracted from the average C_T for βwt and βd/s (ΔC_T), thus correcting for the minor levels of endogenous expression. The standard curve was generated for each real-time PCR determination using a dilution series (50 ng, 33.3 ng, 11.1 ng, 3.7 ng, 367 pg, 120 pg) of

total RNA from 3T3-L1 adipocytes (day 2). A plot of log starting quantity (ng) on the *x* axis and the C_T on the *y* axis was utilized to determine the arbitrary C/EBPβ mRNA levels of the unknown samples. Real-time PCR analyses with 90% or higher efficiency were utilized for quantification. For multiple independent runs after normalization to β-laminin expression, results were corrected for endogenous C/EBPβ expression using the C_T values obtained from the cells harboring the EV.

Polyadenylation Length Assay - Polyadenylation of the C/EBPβ mRNA was determined using the primer/adapter reverse transcription-PCR method as described by Huarte *et al.* (35). The primer/adapter and primers utilized for this procedure are described above in Table 1. Briefly, RNA was isolated from βwt, β d/s and pRT (empty vector control) transduced MEF cells using TriZol™ RNA isolation protocol as per the manufacturer's instructions. Reverse transcription was preformed on 3ug of RNA using Superscript II reverse transcriptase (Invitrogen) with oligo d(T) adapter/primer. An aliquot of oligo d(T) adapter primer was then 5'end labeled with [γ^{32}P] ATP using KinaseMax™ kit (Ambion) as per manufacturer's instructions. The labeled oligo d(T) adapter/primer was then purified by MicroSpin Columns (GE Healthcare) as per manufacturer's instructions. Reverse transcription products (cDNA) from above were then used in a PCR reaction with 7.5E+05 CPM of labeled adapter/primer and 25pmol 275nt5PA primer using illustra™ puReTaq Ready-To-Go PCR beads (GE Healthcare). PCR products were then extracted using phenol:chloroform:isoamyl alcohol (25:24:1) then precipitated for 1h with 3M sodium acetate and ethanol. Purified PCR products

were then separated on a 6% Acrylamide 8M Urea SequaGel™ (National Diagnostics) as per manufacturer's instructions. Gels were dried under vacuum and exposed to x-ray film.

C/EBPβ mRNA Half Life Determination - The MEF-3T3 cells were transduced with the series of constructs and induced to differentiate as described above. At 24 h after induction of differentiation, doxycycline was added at a final concentration of 0.2 μM to the cultures to stop transcription of Tet-off constructs. Total RNA was then isolated with respect to time, and analysis of C/EBPβ mRNA content was carried out using real-time PCR as described above. The data were plotted as log RNA content *versus* time. The equation $y = ae^{-bx}$ was fitted to the data, and half-lives were calculated.

RIP-CHIP (RNP Immunoprecipitation) - The mRNP complexes from total 3T3-L1 lysate and cytosolic fractions were immunoprecipitated and mRNA isolated using RNase- and DNase-free conditions by a modification of the protocol described by Keene et al (43). Briefly, protein A-Sepharose beads were resuspended overnight at 4 °C in 10 ml of NT2 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl2, and 0.5% IGEPAL). Beads were incubated with antibody at 4°C overnight; for the isotype control, anti-Armenian/anti-Syrian hamster IgG1 monoclonal antibody was used, and for HuR, monoclonal 3A2 was used. Four samples were prepared for each fraction and time point. A typical RIP contains the following: NT2 buffer, Halt protease inhibitor, vanadyl ribonucleoside complex, 40 units/μl RNase OUT™, 0.1 M dithiothreitol (DTT), and 0.5 M EDTA. Finally, 3mg of protein lysate was added. The protein sepharose beads incubated with antibody was then combined with the IP mixture and rotated for 2 h at

room temperature. The beads were then washed twice with NT2 buffer alone. The mixture was then centrifuged (13,200 rpm at 4 °C) and washed three times with cold 1 M urea in NT2 buffer. RNA was extracted from the IP using the TriZol™ RNA isolation protocol as per the manufacturer's instructions. Extracted RNA was stored at -80 °C. RNA was sent to Duke University Array Facility (Durham, NC) for microarray analysis.

Protein Procedures

Isolation of Cytosolic and Nuclear Fractions - The NE-PER™ cell fractionation kit was used to isolate cytosolic and nuclear fractions from the 3T3-L1 cells as per the manufacturer's (Pierce) instructions with minor modifications. Briefly, (5) 10-cm culture dishes were used as a source of material for the separation. In addition to the standard protease inhibitors, an RNase inhibitor (Invitrogen) was added to the lysate. Incubation time for the initial extract was increased from 10 to 15 min. Once the cytosolic fraction was isolated, the nuclear fraction was subjected to a brief centrifugation, and the interface was removed to reduce cytoplasmic contamination. The isolated fractions were stored at -80C° until use. Because of high salt concentrations, nuclear fractions were dialyzed prior to use.

Western blot analysis - Protein extracts were prepared by scraping cells off culture dishes in 1x PBS followed by centrifugation (3000 rpm, 3min) to isolate cell pellets. Cell pellets were resuspended in cell lysis buffer (50mM Tis-HCl, 100mM NaCl, 1% deoxycholic acid (Na-deoxycholate), 4% IGEPAL, 0.4% sodium dodecyl sulfate (SDS) and 1% Halt™ proteinase inhibitor) and frozen -80°C for at least 1h. Lysates were

then subjected to BCA assay and stored at -80°C until needed. Proteins were separated by sodium dodecyl sulfate (SDS)-PAGE, Protogel™ (National Diagnostics) (10% gel) and electro-transferred to a Polyvinylidene Difluoride (PVDF) membrane (Invitrogen). The membranes were processed as instructed by the manufacturer, and the proteins were detected by enhanced chemiluminescence (LumiGlo™).

Bioinformatics

Microarrays - RNA samples from 0 time and 30min cytosolic RIPs, prepared as previously described above, were utilized for spotted array analysis. Oligonucleotide arrays were printed at the Duke Microarray Facility (Durham, NC) using Operon's Mouse Genome Oligo sets, which consists of 35,852 oligonucleotide probes representing 25,000 genes and approximately 38,000 transcripts. All arrays were subject to Loess normalization within each array and across all arrays using the Array Magic package in R (9). Replicate probes were collapsed to one probe corresponding to the median value of all the replicates. Probes were filtered for a raw signal 2x greater than background. Log (10) ratios were then converted to log (2) ratios and IgG control IP values were subtracted from each matched HuR IP value. Data was gated to greater than 2 fold enrichment across all IPs. The resulting data, 495 genes, were then used for further analysis.

Chapter 3

An Early Event in Adipogenesis, the Nuclear Selection of the CCAAT/ Enhancer-binding Protein β (C/EBPβ) mRNA by HuR and its Translocation to the Cytosol*

*Gantt, K., Cherry, J., Tenney, R., Karschner, V., and Pekala, P. H. (2005) *J Biol Chem* 280(26), 24768-24774

Introduction

When confluent 3T3-L1 preadipocytes are treated with differentiation inducers, they synchronously reenter the cell cycle and undergo approximately two rounds of cell division as they enter a process that has become known as mitotic clonal expansion (29, 65, 72). This increase in cell number and commitment to differentiation mimics the increase in adipose mass in obesity (19, 81). In the 3T3-L1 preadipocytes, mitotic clonal expansion has been demonstrated to be required for terminal adipocyte differentiation (19, 61). A transcription factor expressed prior to the start of the process, C/EBPβ plays an essential role in the mitotic clonal expansion as well as in subsequent events in the differentiation program (78, 79). Transcription of the C/EBPβ gene is increased shortly after exposure of the cells to the differentiation inducers, and the C/EBPβ protein can be detected in the nucleus within 2 h (76, 101). When termination of the mitotic clonal expansion phase begins, C/EBPβ has been proposed to be responsible for the transactivation of C/EBPα and PPARγ genes, two transcription factors responsible for establishing and maintaining the adipocyte phenotype (70, 78). As C/EBPα expression is increased, C/EBPβ expression is attenuated (77). Thus, C/EBPβ plays a critical early regulatory role in the differentiation process.

HuR is an RNA-binding protein belonging to the Hu/ELAV family of mRNA-binding proteins and is expressed in adipose (23), intestine, spleen, thymus, and testis with minor expression in liver and uterus (51, 52). HuR contains a nucleocytoplasmic shuttling sequence and functions as an adapter protein in the nuclear export of mRNAs that contain adenylate-uridylate-rich elements (AREs) in their 3'-untranslated regions

(31). Depending on the specific message, the nuclear HuR-containing messenger ribonucleoprotein (mRNP) is bound by APRIL and/or pp32 and then CRM1, which is recognized by a specific binding domain on the nuclear pore complex, facilitating nucleocytoplasmic transport. An alternative to the CRM1 route is mediated by transportin-2 binding to the HuR mRNA complex for exit through the nuclear pore (8, 22). Once in the cytosol, HuR functions to stabilize mRNA ligands (18, 37, 49, 91, 92). Based on results obtained with the ectopic expression of the neuronal Hu family homolog HuB in both the 3T3-L1 adipocytes and human teratocarcinoma cells, HuR may also participate in control of translation initiation (2, 37). The selection of ligands and the translocation of HuR to the cytosol have been proposed to be under tight control (49).

HuR has been suggested to be involved in control of the cell cycle through stabilization of the mRNAs for cyclins A and B1 during S and G2, leading to increased expression of these proteins (91). HuR involvement in differentiation was supported by RNA interference knockdowns of the protein in C2C12 cells, which resulted in an inhibition of myogenesis coincident with decreased expression of MyoD and myogenin mRNAs, which are known ligands for HuR (88). Thus, it might be argued that HuR, by selecting specific mRNA ligands and controlling their expression, is capable of controlling critical events in the cell cycle as well as the differentiation process. In the current study we demonstrate that the C/EBPβ message is a ligand for HuR and that the time frame for cytosolic translocation of the complex is consistent with an early regulatory event in the differentiation process.

Results

HuR Expression in 3T3-L1 - In the 3T3-L1 cells HuR is constitutively expressed and localized predominantly to the nucleus in the preadipocytes with HuR expression increasing ~3-fold as the cells differentiate. Notable is the 30% increase of HuR content in the cytosol with respect to differentiation. This data is consistent with HuR regulating the availability of relevant mRNAs for translation. Examination of the C/EBPβ mRNA sequence demonstrates the presence of an ARE in the 3' UTR that might serve as a potential binding site for HuR. Using *in vitro* RNA gel shifts, we have demonstrated that the C/EBPβ message is a ligand for HuR (data not shown).

C/EBPβ mRNA and HuR Complex Formation in the Nucleus and Translocation into the Cytosol - To determine whether the C/EBPβ message was present in an mRNP complex with HuR in the intact 3T3-L1 cells, immunoprecipitations were performed on both nuclear and cytosolic extracts prepared at 0 and 30 min after the addition of the differentiation inducers, MDI. As shown in Fig.5A. western blot analysis indicates that the HuR protein was detected in the nuclear fractions at both time points. Additionally, a decrease nuclear presence of the HuR protein 30 min after induction of differentiation was readily apparent. Analysis of the mRNA present in the immunoprecipitated mRNP complex shown in Fig. 5B, indicates that prior to the addition of the differentiation inducers, HuR-C/EBPβ complexes could not be detected.

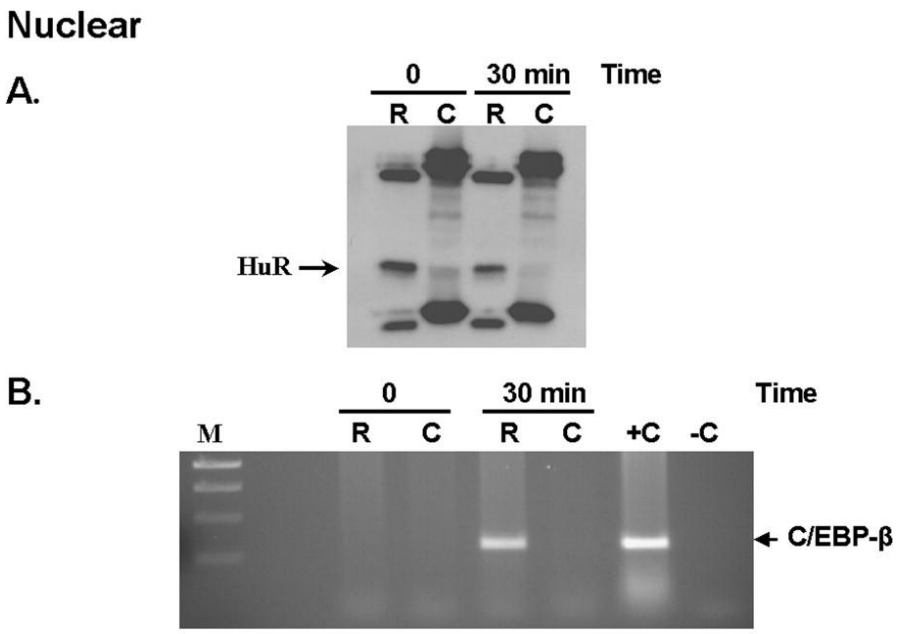


Figure 5 - Formation of nuclear HuR-C/EBPβ complexes. *Panel A*, time course Western analysis confirms the presence of HuR in the immunoprecipitation. Shown is the preadipocyte at time 0 and 30 min after exposure to the differentiation inducers. *R*, immunoprecipitation performed with the HuR monoclonal antibody; *C*, immunoprecipitation performed with control antibody (*arrow* indicates HuR. The other bands are the heavy and light immunoglobulin chains from the immunoprecipitations). *Panel B*, using six identical immunoprecipitations, RNA was extracted and subjected to reverse transcription and PCR analysis for the presence of C/EBPβ (*arrow* indicates C/EBPβ). Designations and times are as stated in *panel A*. *M*, marker of DNA ladder. +*C,* positive control total day 2 cDNA. –*C*, no cDNA template negative control.

However, within 30 min of addition of the inducers C/EBPβ mRNA was found to be present in the immunoprecipitated complex. Examination of the cytosol for the presence of these complexes over the same time period demonstrates the presence of HuR protein in the cytosol (Fig. 6A) with a detectable increase 30 min after the induction of differentiation, consistent with translocation. In Fig. 6B the presence of the C/EBPβ mRNA in the immunoprecipitated cytosolic complex at the 30 min time point is demonstrated. The data presented in Fig. 5 and 6 support a model in which treatment of the preadipocytes with the differentiation inducers results in formation of a HuR-C/EBPβ message complex in the nucleus, which is then followed by translocation to the cytosol. This incident represents a very early event in the differentiation program.

Effect of HuR Depletion on Adipocyte Differentiation - The proximity of the mRNP complex formation event, described above, to the introduction of adipogenic stimuli and the importance of C/EBPβ to the differentiation process has led us to hypothesize a role for HuR in the regulation of the onset of adipogenesis. To support this hypothesis, small interfering RNA suppression of HuR protein content was preformed. When HuR expression was reduced the cells retained their preadipocyte morphology, failed to express normal levels of C/EBPβ, and did not accumulate lipid droplets through a 5-day time course (data not shown).

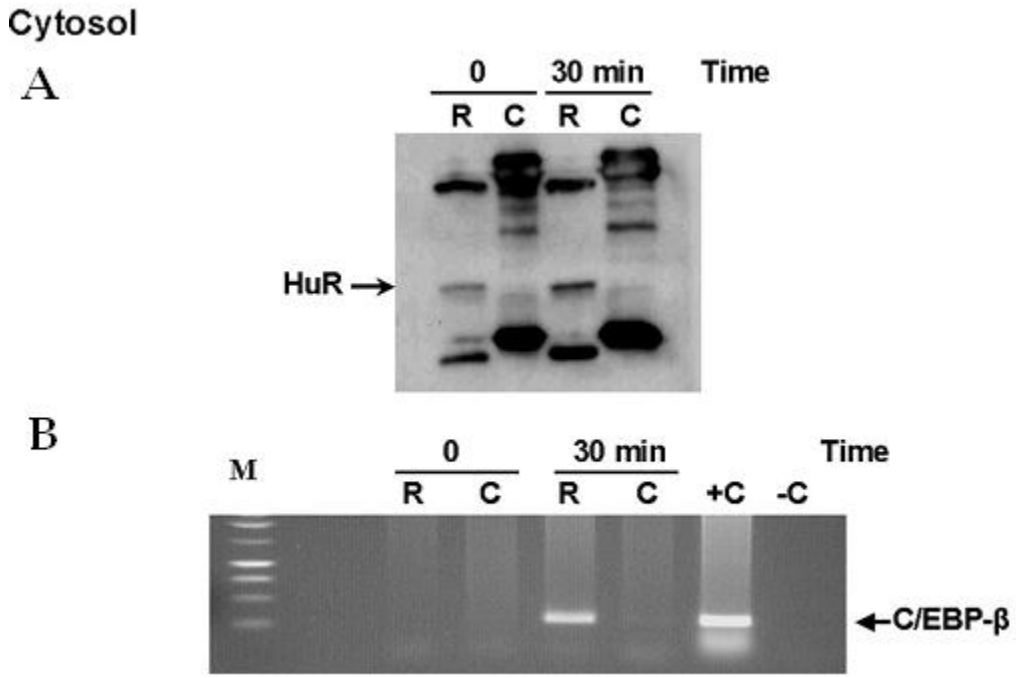


Figure 6 - The translocation of HuR-C/EBPβ complex to the cytosol. *Panel A,* time course Western analysis demonstrates the presence of HuR protein in the cytosol with an increase detectable at 30 min after the induction of differentiation, consistent with translocation. *R*, immunoprecipitation performed with the HuR monoclonal antibody; *C*, immunoprecipitation performed with control antibody *Panel B* shows the presence of the C/EBPβ mRNA in the immunoprecipitated complex at the 30-min time point. Designations and times are as stated in *panel A*. *M*, marker of DNA ladder. +*C,* positive control total day 2 cDNA. –*C*, no cDNA template negative control.

Discussion

Our results describe the constitutive expression of the RNA-binding protein HuR in the 3T3-L1 preadipocytes with a modest 3-fold increase in protein content with respect to differentiation. At confluence, the majority of the HuR protein is retained in the nucleus, in a preadipocyte. However, upon exposure of the cells to the differentiation inducers there is a rapid formation of a nuclear HuR-C/EBPβ complex followed by a translocation of the complex to the cytosol. When HuR protein expression was reduced by siRNA, the cells exhibited an inhibition of C/EBPβ protein expression and an attenuation of the differentiation process.

These observations are consistent with maintenance of HuR protein content and the ability of the cells to express C/EBPβ to progress through the differentiation program. Our data are consistent with a step involving the HuR-mediated movement of the C/EBPβ message to the cytosol. Looking deeper into this process reveals that the proposed major pathway of cytosolic translocation from the nucleus involves recognition of the exon-junction complex by adapter proteins that are recognized by specific nucleoporins (7, 18, 22, 48). However, the *C/EBP*β gene has no introns and must utilize an alternative export mechanism, which our data would suggest is likely mediated by HuR recognition of the 3'-ARE (7). This selection of the C/EBPβ mRNA by HuR and mediation of its translocation to the cytosol becomes a potentially critical control step in the onset of adipogenesis. In addition, these data are consistent with other models where cytosolic translocation of Hu proteins and their associated mRNAs correlated with the onset of myogenesis or neural development (82, 88).

Although decreasing HuR expression using siRNA resulted in a significant decrease in C/EBPβ expression, we anticipate numerous ligands in which HuR binds to at this early event that may influence the differentiation program. We would suggest that the tight temporal control of specific mRNA selection and translocation to the cytosol during the differentiation process would be an absolute necessity and that these data support the existence of a new control point in the differentiation process. In the next chapter we will explore the following major questions:

1. What is the importance of the interaction between HuR and C/EBPβ?
2. What is HuR regulating post transcriptionally?
3. What is its function?
4. Is it important to the adipocyte differentiation process?

Chapter 4

Post-transcriptional Control of CCAAT/Enhancer-binding Protein β (C/EBPβ) Expression: Formation of a Nuclear HuR-C/EBPβ mRNA Complex Determines the Amount of Message Reaching the Cytosol*

***Karschner, V.A., Cherry, J., Jones, H., and Pekala, P. H. (2008) *J Biol Chem* 283(45), 30812-30820**

Introduction

Adipocyte differentiation is a complex process regulated in large part by the temporally controlled expression and activation of numerous transcription factors (61). Among these proteins, the CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor (PPAR) families of transcriptional activators have been identified as critical to initiation of the differentiation process as well as to maintenance of the adipocyte phenotype (61). Functional roles for these factors have been established at least in part through use of the 3T3-L1 preadipocyte model system (61). When 3T3-L1 preadipocytes are induced to differentiate, the cells reenter the cell cycle and undergo mitotic clonal expansion followed by growth arrest and expression of the adipocyte phenotype (61), as previously described in the literature review. Briefly, the transcription factor C/EBPβ is expressed coincidentally with induction of differentiation and is essential for both mitotic clonal expansion and the transcriptional activation of PPARγ and C/EBPα genes (29, 81, 101, 102, 107). The indispensable nature of suitable C/EBPβ expression was demonstrated in studies with C/EBPβ$^{-/-}$ murine embryonic fibroblasts (MEFs), which when treated with the differentiation inducers could neither reenter the cell cycle and undergo mitotic clonal expansion nor express the adipocyte phenotype (81). Similar results were observed in 3T3-L1 cells expressing a dominant-negative C/EBP (102).

Messenger RNA export from the nucleus, mRNA turnover, and translation initiation are important control points in the post-transcriptional regulation of gene

expression. Control of these processes, at least in part, is exerted through recognition of *cis* elements in the mRNA by specific binding proteins. One of these proteins is HuR, a 36 kDa protein that belongs to the Hu/ELAV (embryonic lethal abnormal vision) family of RNA-binding proteins (69). HuR is ubiquitously expressed, localized predominantly to the nucleus, and has been demonstrated to shuttle between the nucleus and cytoplasm. The shuttling activity suggests but has not yet proved that HuR functions by binding to nascent mRNAs in the nucleus and protecting them from degradation by actively participating in their nucleocytoplasmic transport (2, 7, 8, 18, 21, 22, 37, 42, 52, 57). Once in the cytosol, there is compelling evidence to suggest that HuR functions to control the stability and translational efficiency of its ligand mRNAs (2, 7, 8, 18, 21, 22, 37, 42, 52, 57). Recent data have also supported a role for HuR in the regulation of polyadenylation by competitively inhibiting the binding of the cleavage and polyadenylation specificity factor, thereby attenuating polyadenylation and nuclear export (106). It is not clear that any one mRNA is subjected to all four HuR-mediated regulations, *i.e.* 1) control of polyadenylation, 2) translocation to the cytosol, 3) stability, and 4) translational initiation/efficiency. It is important to realize that HuR is a regulatory protein involved in the post-transcriptional processing of certain mRNAs and that the particular function(s) may depend on the particular message.

In 3T3-L1 preadipocytes, HuR is constitutively expressed and localized predominantly to the nucleus (23). Within 30 min of exposure to the differentiation stimulus, the HuR content in the cytosol increases, consistent with HuR regulating the

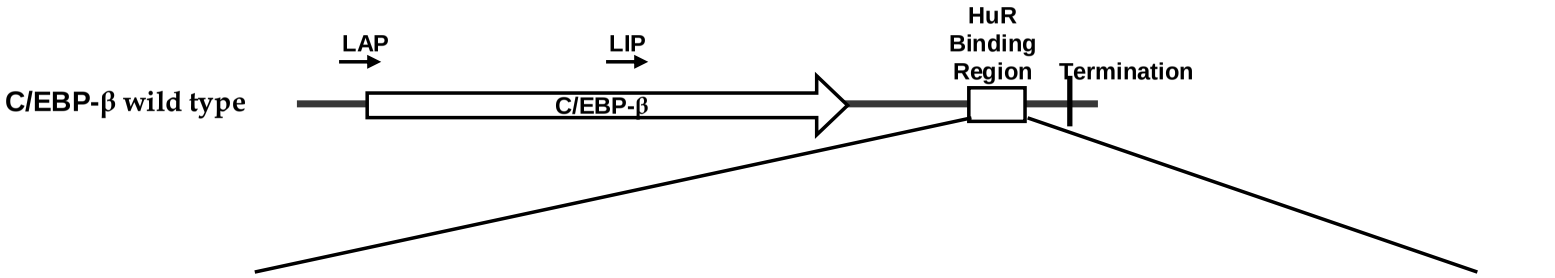
availability of relevant mRNAs for translation. We have demonstrated in the previous chapter (23) that one of the relevant mRNAs forming a nuclear complex with HuR upon induction of the differentiation program and translocating to the cytosol as a messenger ribonucleoprotein (mRNP) complex with HuR is the C/EBPβ message.

In this study, we address the functional significance of the interactions between HuR and the C/EBPβ 3'-untranslated region (UTR). Using non-adipogenic MEF-3T3 cells, we examine the effects of conditional ectopic expression of C/EBPβ and mutants unable to bind HuR on the metabolism of the C/EBPβ message.

Results

Ectopic Expression of Both Wild Type and Mutant C/EBPβ mRNAs in the Multipotential Precursor MEF-3T3 Cells - Our previous work (23) demonstrated that upon exposure of the cells to the differentiation inducers there is a rapid formation of a nuclear HuR-C/EBPβ complex followed by a translocation of the complex to the cytosol. Our recent detailed analysis demonstrated the presence of a single binding site for HuR in the C/EBPβ mRNA. That site is in the AU-rich element (ARE) in the 3'-UTR of the message (38). Therefore, to examine the function of HuR in the translocation and expression of C/EBPβ mRNA, we created constructs that expressed wild type C/EBPβ as well as mutants that could not bind HuR, Fig 7, 1) full-length wild type C/EBPβ cDNA (βwt), 2) C/EBPβ cDNA with point mutations flanking the ARE (βpm), 3) deletion of the ARE (βdel), and 4) deletion of the ARE and substitution with a sequence that does not contain a HuR binding site (βd/s). Expression of these constructs in murine embryonic fibroblasts resulted in significant adipose conversion relative to those cells expressing wild type C/EBPβ. C/EBPβ protein content was increased markedly in both βdel and βd/s, which correlated with the acquisition of the adipocyte phenotype (Data not shown). Analysis of the βd/s cell line demonstrated a robust expression of C/EBPα coincident with peroxisome proliferator-activated receptor-γ expression (Data not shown).

Figure 7 - A Schematic of C/EBPβ Mutant Constructs- Representative diagram of C/EBPβ mRNA indicating the approximate translation initiation sites for liver activating protein 1 and 2 (*LAP*) and liver inhibitory protein forms of C/EBPβ, the HuR binding domain, and termination of transcription is shown. The graphic below describes the sequence alterations in the mutants. In βwt, the *uppercase* bases indicate the sequence altered to form βpm, in which the *uppercase* bases indicate the mutations used to create BglII restriction sites in βwt. βdel is a re-ligation of the construct after removal of the BglII fragment. βd/s consists of removal of the BglII fragment and insertion of a 101-base fragment that does not bind HuR.



C/EBP-β PM 1281 taaagat**C**ttcctgcggggttgttgatgtttttggttttgtttttgtttttgtttgtt ttgtttttttttttggtcttattatttttttgtattatataaaaaag**A**tctatttctat 1400

C/EBP-β Delete HuR Binding Region Deleted

C/EBP-β Del/Sub 1281 ggagccatcaaagtcctgaatatcctcctggacttcactgctggctgaggaaaggcgcccaggcccggcttgcccgagatctatttctatgagaaaa 1400

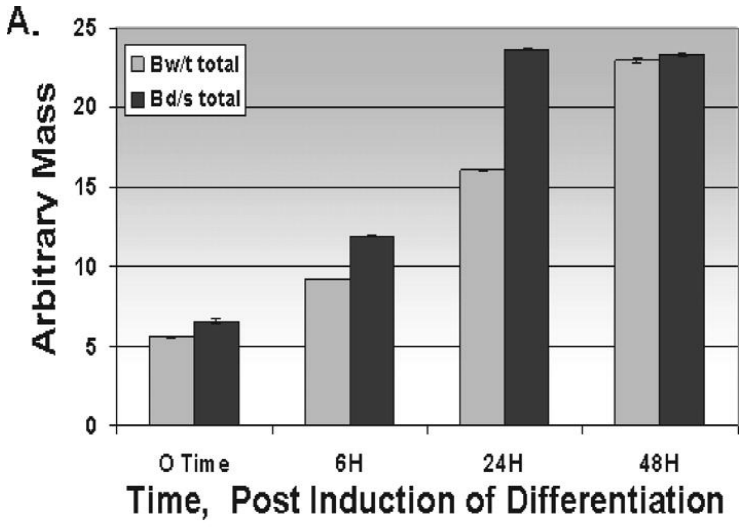
Accumulation of C/EBPβ mRNA in βwt and βd/s Cell Lines- We examined the accumulation of total cellular C/EBPβ mRNA after removal of doxycycline and exposure of the cells to the differentiation mixture. The real time PCR results shown in Fig 8A indicate that total RNA in both βwt and βd/s cells accumulated in a similar manner. The loss of the HuR-binding ARE in βd/s had no effect on the accumulation of total cellular message. This might be expected as the constructs were driven by the same tetracycline/doxycycline-regulated promoter. We next examined the appearance and accumulation of C/EBPβ mRNA in the cytosolic compartment. Using the Pierce NE-PER™ kit, we isolated cytosolic and nuclear compartments prior to and after removal of doxycycline and induction of differentiation, as we have described previously (23). The real-time PCR data shown in Fig. 8B indicate that at all time points βd/s mRNA accumulated in the cytosol to a greater degree than the βwt message. Thus, the loss of the ability to bind HuR at the canonical ARE (present in βwt, but absent in βd/s) did not hinder the movement of the C/EBPβ mRNA into the cytosol. The previous data (Fig. 8*A*) indicated that total cellular C/EBPβ mRNA accumulated to a similar degree in both βwt and βd/s. These data (Fig. 8B) would suggest that a greater percentage of the total βd/s mRNA is in the cytosol, available for translation and driving the accumulation of C/EBPβ protein seen earlier. To confirm this hypothesis, we selected the 48 hr time point and performed five independent isolations and subsequent determinations of the cytosolic *versus* nuclear distribution of βwt and βd/s. Those data are displayed in Fig. 8C and demonstrate that there is approximately twice the βd/s mRNA in the cytosol relative to the βwt mRNA. We note that in separate

experiments a minimum 2-fold differential has been demonstrated to be maintained at least through day 8 of the differentiation program. Thus, in the absence of HuR binding, more C/EBPβ mRNA accumulates in the cytosol.

C/EBPβ mRNA Half-life—The accumulation of the βd/s mRNA in the cytosol could be a result of stabilization of the message upon removal of the ARE. To examine for this possibility, half-life determinations were made 24 h after induction of differentiation (Fig. 9). The βwt and βd/s mRNAs exhibited half-lives of 120 and 60 min, respectively, calculated using exponential decay regression. With the consideration that the βd/s mRNA has a more rapid half-life, the increased cytosolic accumulation is all the more significant.

C/EBPβ mRNA Polyadenylation- The data presented to this point are consistent with involvement of HuR in nuclear processing of the C/EBPβ message. It would appear that once this mRNP complex forms, its translocation to the cytosol is attenuated. To investigate the influence of HuR on nuclear processing of the C/EBPβ mRNA, we examined nuclear polyadenylation of the C/EBPβ transcripts from the βwt and βd/s cell lines. To accumulate the data presented in Fig 9, RNA was isolated from the nuclear fraction and subjected to reverse transcription using an oligo (dT) primer/adapter followed by PCR using a forward primer located 298 nucleotides upstream of the site of poly(A) addition of the C/EBPβ mRNA in conjunction with the ^{32}P-labeled oligo(dT) primer/adapter.

Figure 8 - C/EBPβ mRNA from βwt and βd/s cell lines. *A*, total RNA was isolated with respect to time after removal of doxycycline from the culture medium and exposure of the cells to the differentiation protocol. Accumulation of total C/EBPβ mRNA was determined by real-time PCR analysis as described under Experimental Procedures. The experiment was performed three times with identical results. *B*, cytosolic accumulation of C/EBPβ mRNA in βwt (*Bw/t*) and βd/s (*Bd/s*) cell lines is shown. The cytosolic fraction (*cyto*) of the cells was isolated using the Pierce NE-PER™ kit as we have described under Experimental Procedures. Cytosolic RNA was isolated using the TRIZol™ reagent. Accumulation of C/EBPβ mRNA was determined by real-time PCR analysis as described under Experimental Procedures. The experiment was performed three times with similar results. Data are plotted as the mean ± S.D. *C,* the levels of βwt and βd/s mRNAs in the cytosol at the 48-h time point (five independent determinations) are shown. Normalization was to β-laminin mRNA, the expression of which does not change over the time course of the experiment, followed by correction for endogenous expression as discussed under Experimental Procedures. βwt content was defined as 100%, and βd/s is expressed relative to that value.


A.
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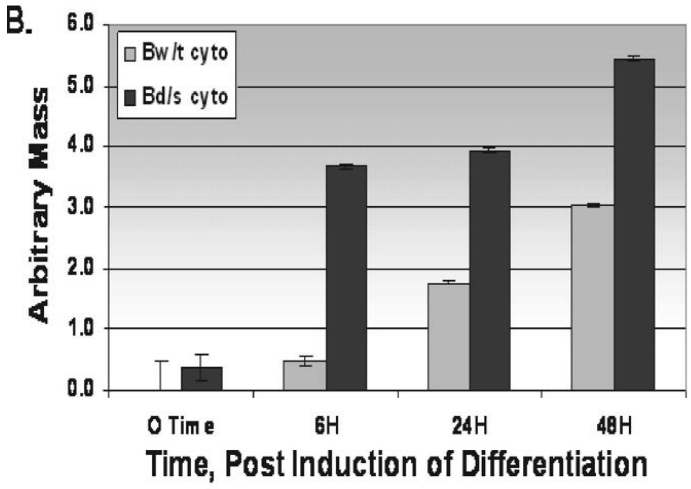
Time, Post Induction of Differentiation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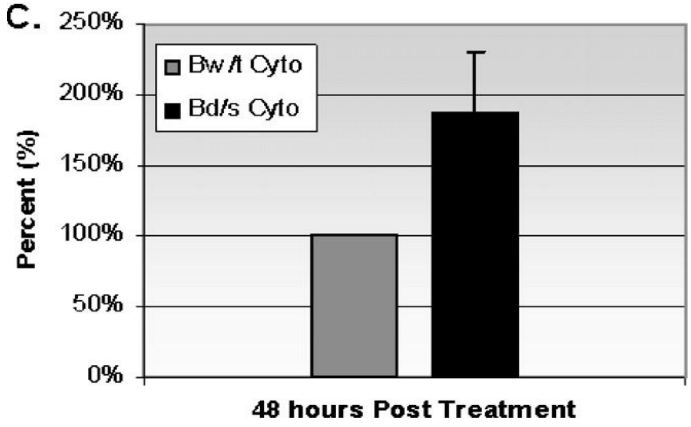
C.
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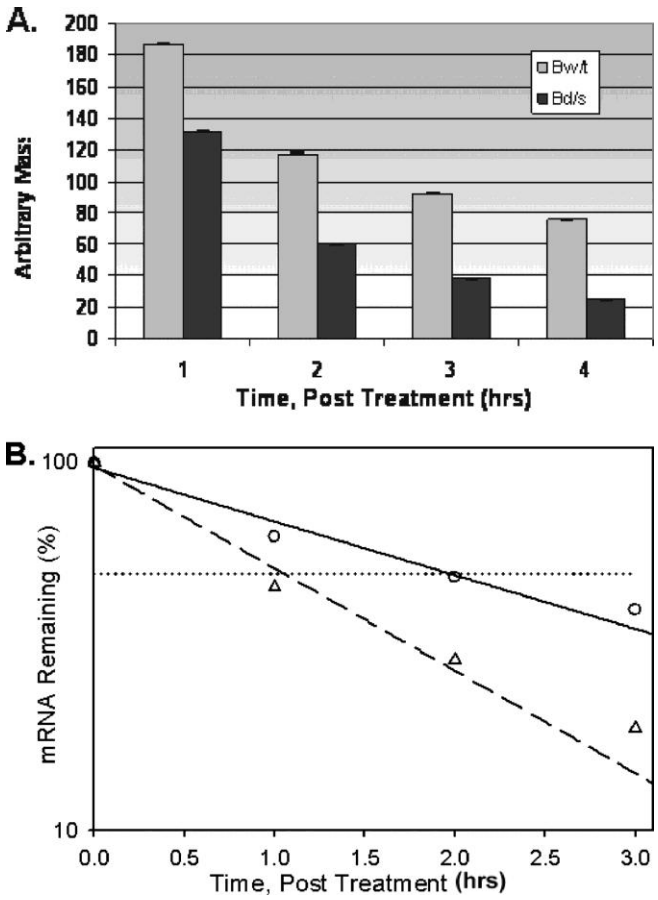


Figure 9 - C/EBPβ mRNA half-life of βwt and βd/s cell lines. At 24 h after induction of differentiation, doxycycline was added to the culture medium, and total RNA was isolated with respect to time. Real-time PCR analysis was utilized to quantify the C/EBP βwt (*Bw/t*) and βd/s (*Bd/s*) mRNAs remaining after addition of the doxycycline. The half-lives of the C/EBPβ mRNAs were determined graphically using a plot of log[mRNA] *versus* time as described under Experimental Procedures. *A*, Real time results are plotted as the mean ± S.D. of two independent experiments analyzed in triplicate. *B*, half-life plot of real-time results, βwt (O) and βd/s (Δ) mRNAs remaining after addition of doxycycline with respect to time are shown. The equation $y = ae^{-bx}$ was fitted to the data, and half-lives were calculated. The experiment was performed twice with similar results.

Reverse transcription-PCR with these primers of the mRNA from the βd/s cells (Fig. 10, *lane 4*) produced a smear of products ranging in size from ~300 to almost 400 nucleotides. The minimal size predicted was 329 nucleotides (298 bp of C/EBPβ plus 31 nucleotides of the primer/adapter). Whereas there is evidence of polyadenylation with βwt (Fig. 10, *lane 2*), densitometric analysis indicated that it is ~35% less than that found in the βd/s. Notably, there is no evidence of polyadenylation occurring when mRNA was isolated from cells containing the empty vector (Fig. 10, *lane 6*). The data suggest that in the absence of HuR binding, the C/EBPβ mRNA is more extensively polyadenylated, leading to translocation to the cytosol. However, in βwt, which binds HuR, polyadenylation appears to occur to a lesser extent, with approximately one-third of the RNA (relative to βd/s) reaching the cytosol. Similar results were obtained using an RNase H-based approach for determination of poly (A) tail size for total cellular RNA.

The process of polyadenylation is a closely coupled set of reactions by which the 3′ end of mRNA is formed. The first step of polyadenylation factors bind to the pre-mRNA to be processed. These factors include: cleavage polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factors I & II (CFI & II) and polyadenylation polymerase (98). Next, the pre-mRNA is endonucleolytically cleaved at a specific site about 10-30 nucleotides downstream of the poly (A) signal. Then during the slow step approximately 10 adenosine residues are polymerized to the cleaved end.

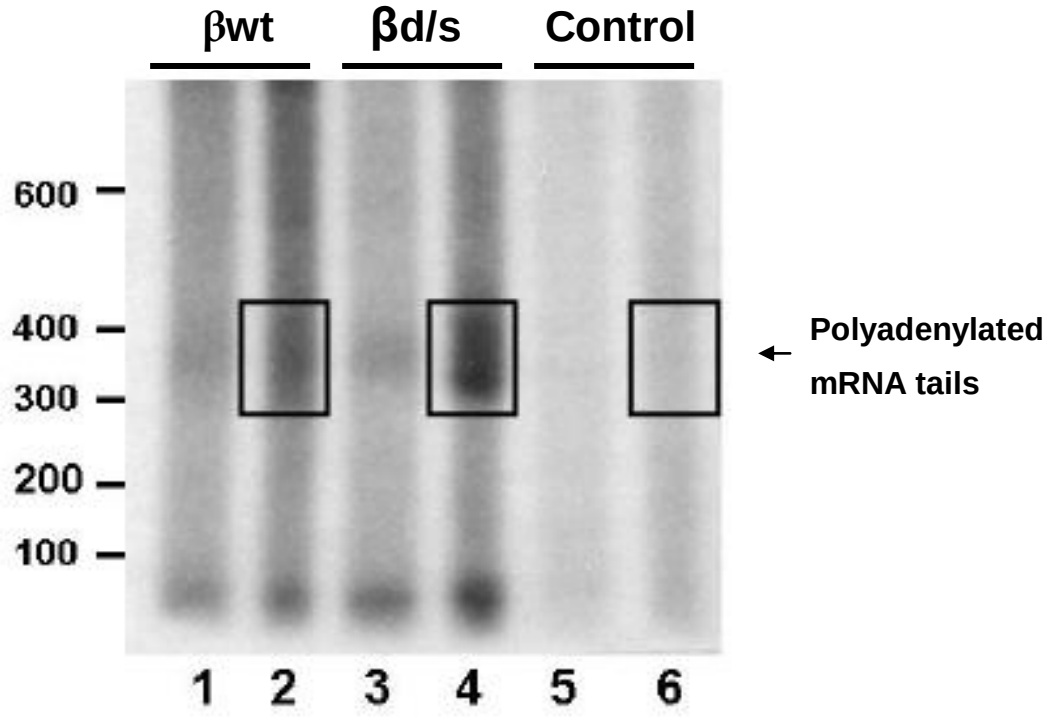


Figure 10 - Identification of a differential nuclear polyadenylation of C/EBPβ mRNA from βwt and βd/s cell lines. Poly (A) tail lengths on βwt and βd/s were determined by reverse transcription-PCR poly (A) analysis of a preparation of total nuclear RNA from βwt and βd/s cell lines. Reverse transcription was primed with an oligo (dT) primer/adapter as described, and poly (A) tail lengths on the βwt and βd/s mRNAs were determined by PCR using a 5'-^{32}P-labeled primer/adapter for the reverse primer and a gene-specific primer for the forward primer. The products of the PCRs were visualized by autoradiography after electrophoresis on 6% polyacrylamide/urea gels. Size markers are in nucleotides. The regions corresponding to polyadenylation of the C/EBPβ mRNA are *boxed* for comparison. *Lanes 2*, *4*, and *6* contain the full PCRs, whereas *lanes 1*, *3*, and *5* contain 20% of the reactions. The autoradiogram presented is representative of an experiment performed 3 times with similar results.

Finally, the elongation step or the fast step, adds approximately 250 adenosine residues forming the poly (A) tail. Recent findings indicate that U-rich sequences upstream of the highly conserved polyadenylation signal AAUAAA can have significant effects on regulation of polyadenylation (41). The Hu proteins are known to bind to AU-rich regions within the same proximity as these U-rich elements (106). Recent *in vitro* assays by Zhu *et al*, show that mammalian Hu proteins regulate polyadenylation by blocking U-rich sequences (106). These U-rich sequences were found to be essential for full activity of the downstream poly(A) signal sequence (41). In Figure 10, our data show that when HuR cannot bind to C/EBPβ mRNA, polyadenylation is more extensive, resulting in more message exiting the nucleus. These data are consistent with the hypothesis that competitive binding of HuR for U-rich regions in the 3'UTR of C/EBPβ may cause attenuation of polyadenylation. Therefore, to examine the function of HuR in the regulated polyadenylation of C/EBPβ mRNA, we created genomic constructs that contain the 3'UTR and the downstream signal sequences of the wild type C/EBPβ (βwt) as well as a mutant where the ARE was deleted and substituted with a sequence that does not contain a HuR binding site (βd/s). Expression of both constructs was under control of an SP6 promoter as detailed in Experimental Procedures. These genomic constructs were then used to create riboprobes for a polyadenylation and cleavage assay to be preformed in collaboration with Hua Lou, Ph.D. Department of Molecular Genetics, Case Western Reserve University. The addition of 2ug of rHuR during the incubation time course resulted in no significant difference in cleavage or polyadenylation between the βwt and the βd/s

riboprobes (Fig. 11). The band visible in figure 11 is the precursor; no visible cleaved product band was present. This negative result would suggest that HuR does not regulate the polyadenylation event of C/EBPβ mRNA in this *in vitro* assay system. It is important to note that the polyadenylation and cleavage assay was preformed under controlled conditions that may have lacked in vital components specific to the 3T3-L1 cells or this particular message required for proper regulation of polyadenylation. With the consideration of the complexity of polyadenylation and plethora of components required for adequate measurement of HuR involvement this set of reactions, we made the decision to focus our efforts on the involvement of HuR in other areas of the differentiation program and not pursue the polyadenylation of C/EBPβ mRNA at this time.

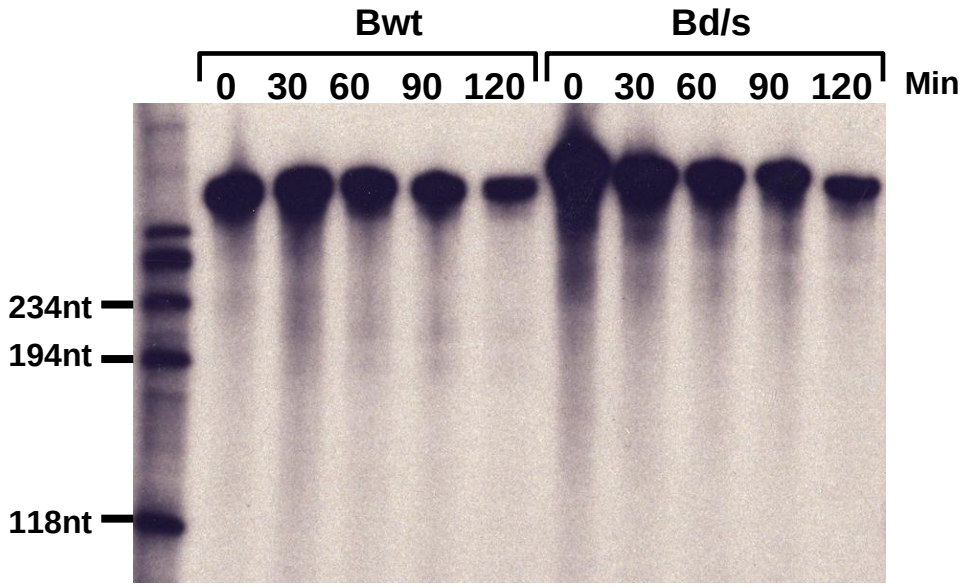


Figure 11 - Polyadenylation and cleavage assay. Riboprobes of the 3'UTR and polyadenylation signal sequences were created from C/EBPβ wild type (βwt) and C/EBPβ delete substitute (βd/s) genomic constructs. Each probe was incubated with 2ug of recombinant GST-HuR for 0, 30, 60, 90, or 120 minutes at 30°C. Precursor riboprobe band visible at ~334 nt, size markers in nucleotides.

Discussion

As preadipocytes differentiate, controlled expression of C/EBPβ is essential to acquisition of the adipose phenotype. Transcriptional activation of the C/EBPβ gene in 3T3-L1 cells occurs within minutes of exposure to the differentiation inducers and is controlled, at least in part, by the cAMP-response element-binding protein (26, 45). In the differentiation program of 3T3-L1 cells, C/EBPβ first controls the entry of the cells into mitotic clonal expansion, and then the expression of C/EBPα and PPARγ (29, 56, 81, 97, 101, 102, 107). The timing of expression during these processes is critical because C/EBPβ is promitotic and C/EBPα is antimitotic, and thus C/EBPβ expression must attenuate as C/EBPα expression initiates. This study describes a critical early post-transcriptional regulation initiated in the nucleus involving formation of a HuR-C/EBPβ mRNA complex. Formation of this mRNP appears to control the rate of C/EBPβ mRNA translocation to the cytosol but is not essential for the translocation process itself. In our previous work, we identified the HuR binding site in the C/EBPβ 3'-UTR and demonstrated that it is the only site within the entire message (38). As evidenced in the data presented in Fig. 8, deletion of this HuR binding site did not disrupt nuclear to cytoplasmic translocation of the message. Indeed, in the absence of HuR binding, 2–7-fold (dependent on the time point) more C/EBPβ mRNA was localized to the cytosol. Conceivably, accumulation of βd/s in the cytosol could be a consequence of deletion of the ARE instability element, resulting in a stabilized message. However, as displayed in Fig. 9, the βd/s mRNA actually has a shorter half-

life than the βwt message, making its accumulation more difficult. Overall, our data are consistent with C/EBPβ mRNA translocation to the cytosol occurring more readily when HuR is not bound, consistent with HuR functioning as an attenuator of movement of the complex to the cytosol. When HuR binds to the C/EBPβ message in the nucleus, movement to the cytosol is not prohibited, simply diminished. The C/EBPβ mRNA reaches the cytosol in lower quantities and is translated into protein. The differentiation program is initiated and maintained. When the binding site is altered such that HuR cannot bind, more C/EBPβ mRNA per unit time is found in the cytosol. The presence of more message drives the overexpression of C/EBPβ protein, resulting in overexpression of C/EBPα protein and a more robust differentiation program.

In the previous chapter, siRNA reduction of HuR resulted in a significant decrease in C/EBPβ expression and an attenuated differentiation program. In this chapter we demonstrated that the absence of a HuR binding site on C/EBPβ resulted in an increase amount of message in the cytosol, increased C/EBPβ protein, as well as, increased C/EBPα and the acquisition of the adipocyte phenotype. Taken together these data are consistent with HuR functioning at an earlier time during adipogenesis, prior to formation of the HuR-C/EBPβ complex. As we have stated previously, we predict that HuR associated with numerous mRNA ligands, during the early phase of the differentiation program any of which may influence the process. We suggest that HuR may be influencing mitotic clonal expansion, which is required for proper

acquisition of the adipocyte phenotype. To begin to understand the exact role of HuR in the differentiation process, we will address the following questions in the next chapter:

1. Does HuR influence mitotic clonal expansion?
2. Will suppression of HuR affect cell cycle?

Chapter 5

HuR Involvement in Mitotic Clonal Expansion during Acquisition of the Adipocyte Phenotype*

Vesna A. Karschner, Phillip H. Pekala.** ***Biochemical and Biophysical Research Communications **383 (2009) 203-205**

Introduction

Fundamental to obesity is adipocyte hyperplasia that occurs through recruitment and proliferation of pre-adipose cells present in the vascular stroma of adipose tissue (25). This hyperplasia is mimicked in culture by 3T3-L1 preadipocytes which when induced to differentiate, synchronously reenter the cell cycle and undergo several rounds of mitotic clonal expansion prior to growth arrest and expression of the adipocyte phenotype (61). Critical to the differentiation process is the RNA-binding protein, HuR (23). HuR is a 32 kDa protein containing three RNA recognition motifs (RRMs) and belongs to the Hu/ELAV family (embryonic lethal, abnormal vision) of RNA-binding proteins (31). Unlike the other three family members, HuB, HuC and HuD, which are exclusively neuronal, HuR is ubiquitously expressed, localized predominantly to the nucleus and demonstrated to shuttle between the nucleus and cytoplasm (17). In the nucleus, recent data have supported roles for HuR in the splicing as well as in the regulation of polyadenylation by competitively inhibiting the binding of the cleavage and polyadenylation specificity factor, thereby attenuating polyadenylation and nuclear export (31, 106). In the cytosol there is compelling evidence to suggest that HuR functions to control the stability and translational efficiency of its ligand mRNAs (31).

Our previous work demonstrated that siRNA mediated suppression of HuR resulted in an inhibition of 3T3-L1 preadipocyte differentiation (23). The inhibition was transient, as the effect of the siRNA was lost after several days and the cells began to

express the adipocyte phenotype (23). Even with that consideration, those data suggest that during the differentiation process, there is formation of a HuR–mRNA complex that is essential for the differentiation program to progress. Whether the function of the complex is export from the nucleus or stabilization of the message, disruption of the interaction leads to alteration of expression and attenuation of the differentiation program. In terms of timing, formation of the HuR–mRNA complex has to be early in the process, because as we have previously demonstrated disruption of this interaction must precede expression of C/EBPβ (39). In addition, more than one ligand mRNA may be involved in the process. Our interest is in identifying these HuR ligands and characterizing their interaction with HuR in the context of mitotic clonal expansion and the differentiation program.

Results and Discussion

Suppression of HuR expression leads to attenuation of β-actin expression- Results from previous studies indicated that the β-actin mRNA was a major ligand for HuR on induction of differentiation (Karschner and Pekala, unpublished results). These data led us to examine the effect of decreased HuR expression on β-actin levels during the early phase of the differentiation process. Cells were transfected with siRNA directed against HuR as described in Experimental Procedures and induced to differentiate. As shown in Fig. 12, an 86% suppression of HuR expression was observed 48 h after induction of differentiation (Lane 1) relative to mock transfected control (Lane 2). β-actin expression decreased by 47% (Lane 1) relative to control (Lane 2). At 72 h (Lane 3) HuR suppression maintained at 78% of control (Lane 4) while β-actin levels were down by 38%. β-tubulin content was used for normalization as its levels do not change during the course of the experiment, moreover, it does not bind HuR. While β-actin expression is known to decrease as the cells attain the adipocyte phenotype, this early decrease prior to mitotic clonal expansion might be expected to slow cell division. Indeed recent observations in HeLa cells by Dormoy-Raclet et al. (15) found that siRNA suppression of HuR led to decreased actin expression resulting in an increased proliferation time. In a parallel experiment, we inhibited actin polymerization by

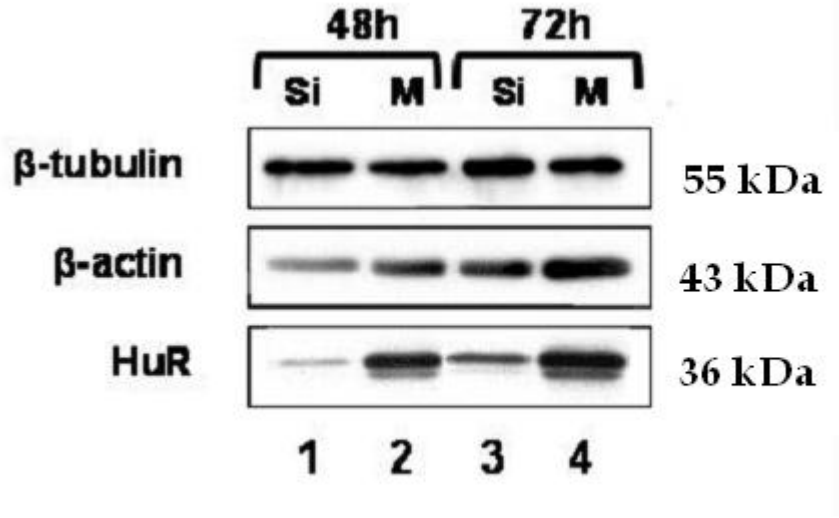


Figure 12 - Suppression of HuR expression leads to attenuation of β-actin expression. Cells were transfected with siRNA directed against HuR and induced to differentiate as described in Materials and Methods. Extracts were prepared at 48 hr and 72 hrs post-induction of differentiation and Western blot analysis of β-tubulin, β-actin, and HuR performed. *Si,* siRNA transfected cell lysates. *M*, mock transfected cell lysates. Quantification of protein levels was performed using scanning densitometry.

exposure of the cells to cytochalasin D and examined the effect on differentiation and used C/EBPα as marker for terminal differentiation. The cytochalasin D treated cells maintained an altered morphology and never accumulated lipid, in addition, on day 8 post-induction of differentiation, C/EBPα protein levels were only 30% of that found in the untreated cells (Karschner and Pekala, unpublished results). These data were similar to what we observed for C/EBPα expression in cells treated with siRNA directed against HuR, providing further confirmation of the importance of actin expression (polymerization) for appropriate induction of differentiation. In their study, Dormoy-Raclet et al. (15) observed that key β-actin mediated functions including cell adhesion, migration and invasion, were markedly decreased and these defects correlated with the loss of the actin stress fiber network. In addition there is significant evidence to link down-regulation of β-actin expression to signaling processes that control transcription (33, 71).

Effect of decreased HuR expression on the expression of Cyclins A, E, and D1 - To examine the influence of HuR on mitotic clonal expansion, we used siRNA to suppress HuR expression, induced differentiation and examined three cell cycle components. As shown in Fig. 13A, suppression of HuR was in excess of 85% at 0, 8, and 12 h after induction of differentiation and 70% for the 16, 20, and 24 h time points. With this degree of HuR depletion, Cyclins A, E, and D1 were expressed at apparent lower levels and on a slightly delayed (4–8 h) time course (Fig. 13C) than that observed for the mock transfected cells (Fig. 13B).

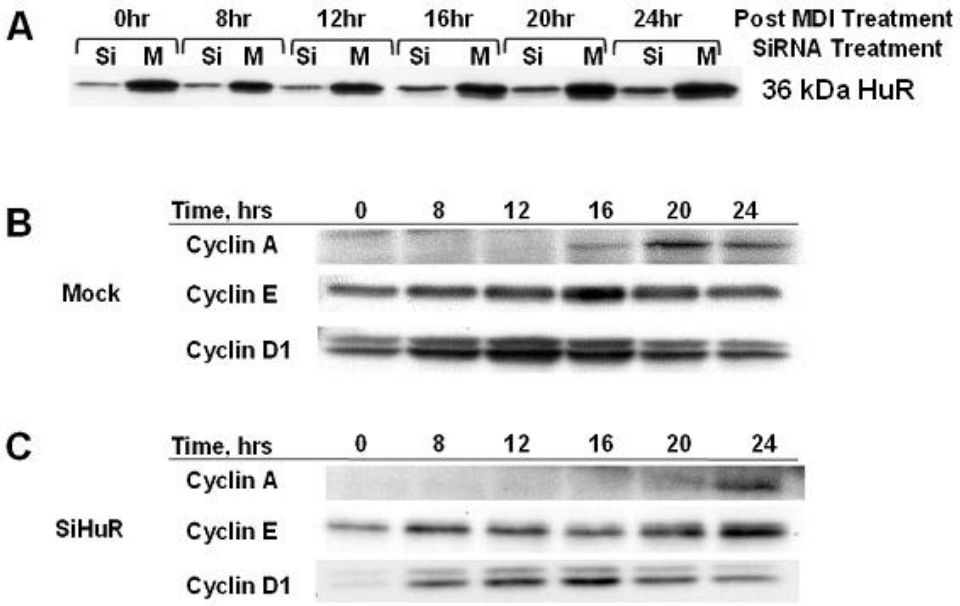


Figure 13 - Effect of decreased HuR expression on the expression of Cyclins A, E, and D1. siRNA mediated suppression of HuR expression was performed as detailed in Materials and Methods. Cells were induced to differentiate and extracts prepared over a 24 h period. Western blot analysis was performed to determine HuR content (A) as well as that of Cyclins A, E, and D1 in mock (B) and siRNA (C) treated cells. *Si*-siRNA transfected cell lysates. *M*- Mock transfected cell lysates. Quantification was by scanning densitometry.

The data are similar to those reported by Dormoy-Raclet et al. (15), a study in which depletion of HuR lead to an attenuation rather than an inhibition of movement through the cell cycle. However, one must consider that Dormoy-Raclet et al. (15) were working with cells that had lost contact inhibition of growth and were constantly moving through the cell cycle, while in our study, the 3T3-L1 cells had attained contact inhibition of growth and on induction of differentiation synchronously reenter the cell cycle, also known as, the mitotic clonal expansion phase of the differentiation program.

siRNA mediated suppression of HuR leads to a transient suppression of p53 and p21 - Using the same extracts we examined for alterations in the expression of p53, reported to be activated as a consequence of down-regulation of actin expression and/or loss of cytosolic actin filaments (71). As shown in Fig. 14, we observed a transient, near 60% decrease in p53 expression at the time of induction of differentiation. The p53 mRNA has a HuR binding site in the 5'-UTR and the interaction is thought to control translational efficiency, thus, loss of HuR led to decreased p53. We also examined for expression of the adipocyte phenotype. We should note that both p53 and p21 are expressed at significant levels throughout the differentiation program (36). While the level of expression does not address activation there is ample evidence consistent with a requirement for both proteins for a productive differentiation program. Interestingly, in the next chapter we examined for potential mRNA ligands of HuR using an RNA Immunoprecipitation–CHIP assay (43).

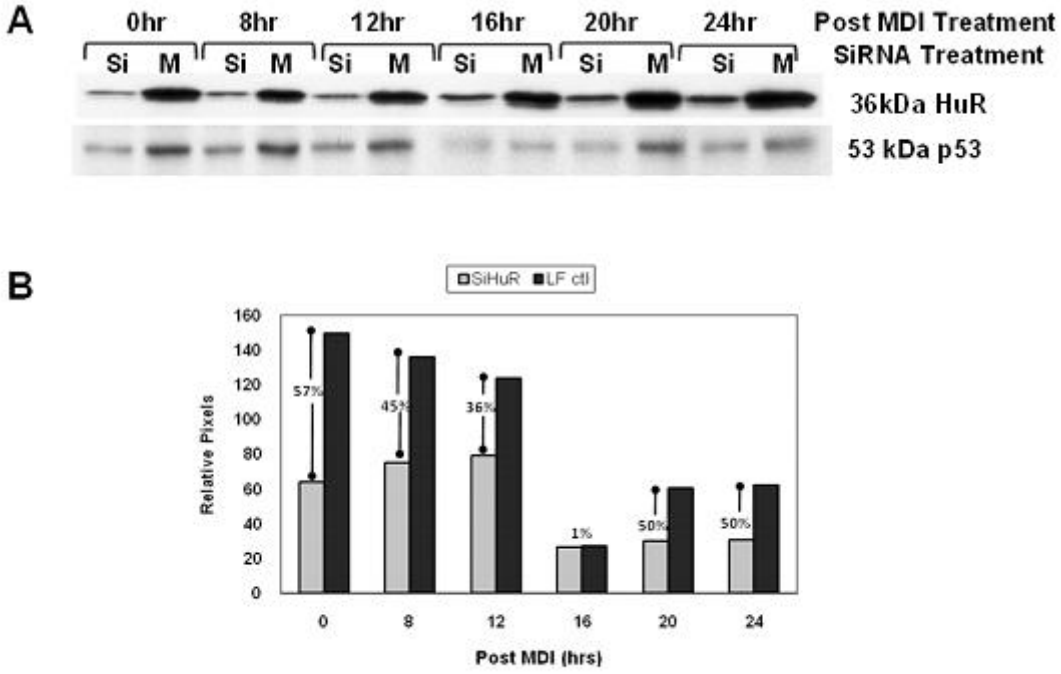


Figure 14 - siRNA mediated suppression of HuR leads to a transient suppression of p53. Cells were prepared for experimentation as detailed in the legend to Fig. 2. Extracts were prepared from siRNA and mock transfected cells and Western blot analysis performed for p53 (A). *Si*-siRNA transfected cell lysates. *M*- Mock transfected cell lysates. Quantification was by scanning densitometry (B).

In this analysis both β-actin and p53 were demonstrated to be ligands of HuR, while p21 was not (see Appendix p119). This would suggest that when HuR protein content was decreased, the resulting mRNAs coding for β-actin and p53 were also decreased leading to decreased β-actin and p53 protein. It appears likely that the decrease in p21 protein may be mediated by loss of p53 and thus decreased transactivation of the p21 gene. Taken together down-regulation of β-actin, p53 and p21 expression most certainly leads to a decreased ability of the cells to undergo mitotic clonal expansion efficiently. Thus, our data are consistent with HuR controlling the expression of a number of gene products that individually could influence/attenuate the progress of the differentiation program. To discover additional mRNA ligands of HuR that are critical to the differentiation process, we will address the following in the next chapter:

1. Microarrays, can we dissect out HuR mRNA ligands (gene sets) that are different between 0 time and 30 min post induction of differentiation?
2. Identify new gene products that are crucial to adipogenesis.

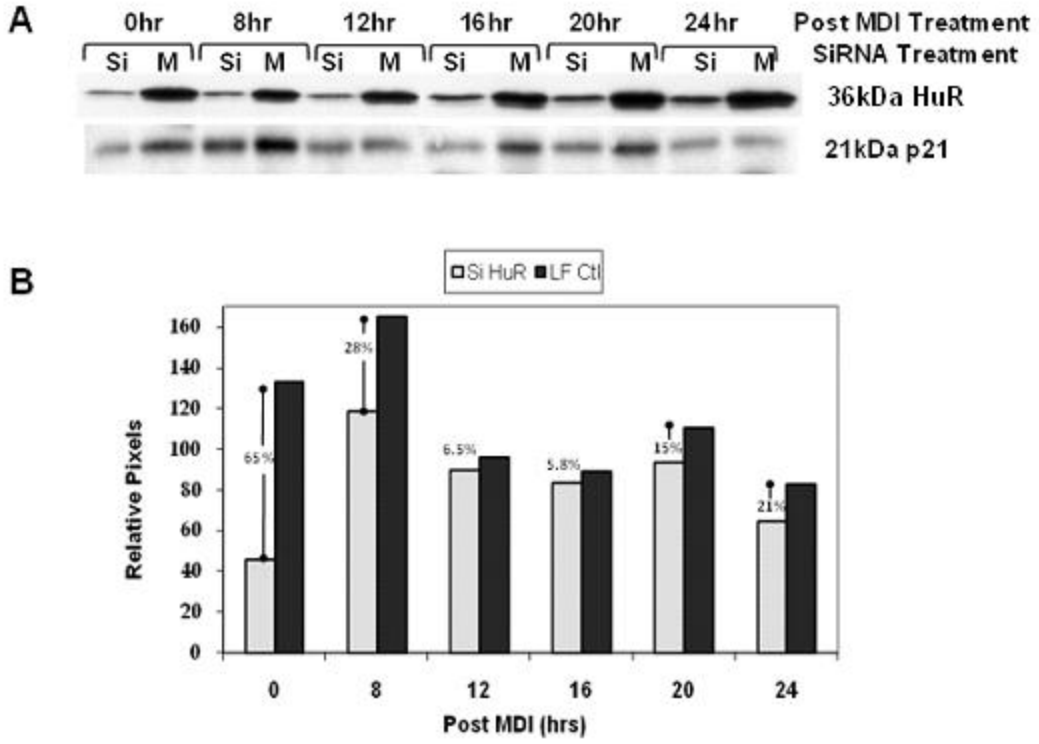


Figure 15 - siRNA mediated suppression of HuR leads to a transient suppression of p21. Cells were prepared for experimentation as detailed in the legend to Fig. 13. Extracts were prepared from siRNA and mock transfected cells and Western blot analysis performed for p21 (A). *Si*-siRNA transfected cell lysates. *M*- Mock transfected cell lysates. Quantification was by scanning densitometry (B).

Chapter 6

RIP-CHIP Analysis of HuR Ligands in 3T3-L1 preadipocyte cells reveals the expression of *Zfp206*, a transcription factor involved in ES cells pluripotency.*

***Vesna A. Karschner and Phillip H. Pekala**

Introduction

Fundamental to obesity is adipocyte hyperplasia that occurs through recruitment and proliferation of preadipose cells present in the vascular stroma of adipose tissue (25, 74). This hyperplasia is mimicked *ex vivo* by post-confluent 3T3-L1 preadipocytes which when induced to differentiate, synchronously reenter the cell cycle and undergo several rounds of mitotic clonal expansion prior to growth arrest and expression of the adipocyte phenotype (81, 101).

The 3T3-L1 adipocyte differentiation program is controlled by a cascade of transcription factors (fig 2 & 3), that includes the CCAAT/enhancer binding proteins (C/EBPs) and a nuclear hormone receptor, peroxisome proliferator activated receptor γ (PPARγ) (19). Superimposed on this signal cascade of C/EBPs and PPARγ expression is the embryonic stem cell transcription factor, *Zfp206*, which we have recently identified through a RIP-CHIP microarray analysis.

Zfp206 (NM_00103345), belongs to the family of SCAN domain-containing poly C_2H_2 zinc finger proteins, which is associated with a large family of transcription factors found exclusively in vertebrates (93, 104). *Zfp206* is located in a gene cluster on chromosome 17 and encodes a 782 amino acid protein (88.4 kDa) that contains 14 zinc fingers and a SCAN-domain. Generally, transcription factors that contain many C_2H_2 zinc fingers, one of the most versatile DNA-recognition motifs, are thought to have multiple ligand specificities and in some cases bind to single or double stranded RNA (93, 104). The SCAN domain is a highly conserved motif (84 residues) that functions as a protein interaction domain and mediates self-association or selective association with

other proteins by mediating homo- and hetero-oligomerization (93, 104). Proteins containing SCAN domains with multiple zinc-fingers represent a subgroup of eukaryotic transcription factors that have been implicated in cell survival and differentiation.

Functional gene expression surveys of the mouse genome suggest that *Zfp206* is expressed almost exclusively in embryonic stem cells (ESC) (103). Detailed analysis indicated that upon differentiation of human and mouse ESC, *Zfp206* (ZNF206 in human) is highly expressed in pluripotent ESC and throughout certain areas of the developing embryo, but was absent in differentiated or adult tissues (93). Zhang et al. confirmed this observation by demonstrating that *Zfp206* expression decreased rapidly upon differentiation of cultured mouse ESC and during development of mouse embryos (104). Furthermore, they demonstrated that *Zfp206* strongly regulates the levels of a handful of transcription factors whose expression is also specific to ESC and early stages of development. These genes encode apparent regulators, such as, eIF-1A, Tcstv1 and Zscan4 (104).

The current data has demonstrated that the previously uncharacterized *Zfp206* gene functions as a positive regulator of pluripotency in ESC and is down-regulated upon differentiated states (6, 104). The following study describes the expression of the transcription factor *Zfp206* in preadipocytes and the role it plays in the regulation of adipogenesis.

Results

RIP-CHIP Analysis Reveals New HuR Ligands during Adipogenesis - In the previous chapters we described the involvement of the RNA binding protein HuR in the regulation of adipocyte differentiation. Our work demonstrated siRNA suppression of HuR expression led to an inhibition of the 3T3-L1 differentiation program; an observation consistent with a critical role for HuR in adipogenesis (23). Previous to this, our studies have focused on one HuR ligand, the C/EBPβ mRNA. In order to identify other HuR ligand mRNAs early in the differentiation program, we used antibodies directed against HuR to perform an RNP immunoprecipitation−microarray (RIP-Chip) analysis, as described in Experimental Procedures. This permitted the identification of a discrete subset of mRNAs associated with HuR, as the population of mRNPs changed markedly during the initial 30 minutes of the adipocyte differentiation program. The RIP-Chip analysis identified 495 individual mRNAs that served as ligands for HuR in this time frame, see appendix A. Figure 16A, shows the distribution of HuR ligand mRNAs at 0 time and 30 minutes post differentiation. The data describe a unique subset of 193 mRNA ligands found only at 0 time, and 30 min post induction of differentiation 45 mRNA ligands unique to this time point were identified. We focused our efforts on the subpopulation of 45 mRNAs that were not ligands at 0 time but become markedly enriched 30 minutes after induction of differentiation. One such ligand identified was *Zfp206* (Fig. 16B).

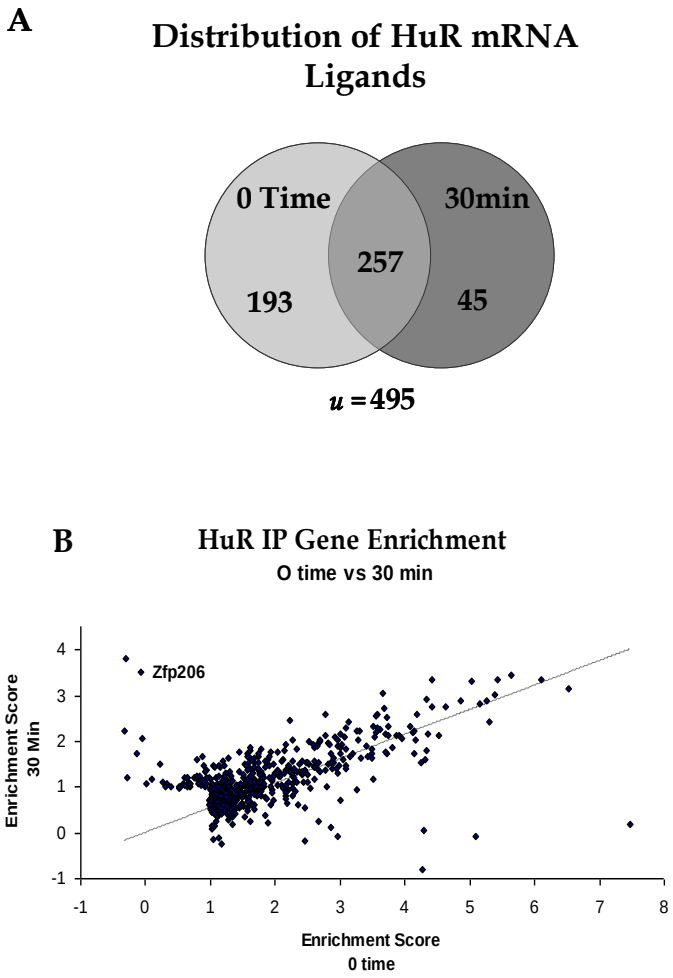


Figure 16 - Charts of HuR RIP-CHIP Gene Enrichment Data. A. Venn diagram showing distribution of genes (mRNA ligands) at 0 time and 30min post MDI treatment. 450 mRNA ligands identified for 0 time, 302 mRNA ligands identified at 30min, 257 mRNA ligands are common between 0 and 30 min, 193 mRNA ligands are unique to 0 time, and 45 mRNA ligands are unique to 30 min. B. Scatter plot of gene enrichment scores of HuR mRNA ligands at 0 time vs. 30 min post MDI treatment. The position of *Zfp206* is shown on plot, enrichment score (-0.082, 3.49).

Characterization of *Zfp206* in 3T3-L1 Preadipocyte and Adipocyte - Detection of Zfp206 mRNA from the RIP-Chip assay was confirmed by RT-PCR using RNA isolated from the 3T3-L1 cells during a differentiation time course. As shown in Fig. 17A, Zfp206 mRNA was detectable at day 0 and expressed at a consistent level through day 7 of the differentiation program, after which decreased to undetectable levels by day 9. Expression of PPARγ mRNA during the same differentiation time course is displayed in Figure 17B for comparison.

Western blot analysis of *Zfp206* protein levels during the differentiation time course detected two protein species. As shown in Fig. 18, an 88 kDa protein corresponding to the full length gene product was detected at day 0 which was maintained through day 3. At which (day 3), a transient ~ 32 kDa isoform was detected at low levels reaching maximal expression by day 7, and then decreasing to undetectable levels by day 11.

Examination of the gene annotation using the Ensembl Gene Browser (http://www.ensembl.org) indicated that *Zfp206* is also known as Zscan10 and genomic evidence suggests the existence of up to 8 potential splice variants (Fig. 19). One of these, Zscan10.201, is approximately 33.5 kDa making it a potential candidate for the ~ 32 kDa protein species identified in figure 18. Recent work in embryonic stem cells has identified at least nine isoforms of *Zfp206*, including seven splice variants (93, 104).

However, the description of the variant Zscan10.201 has not been identified. As shown in Figure 19, the alternative splicing event leading to the formation of Zscan10.201 results in the preservation SCAN domain and the loss of the zinc finger domain of the protein.

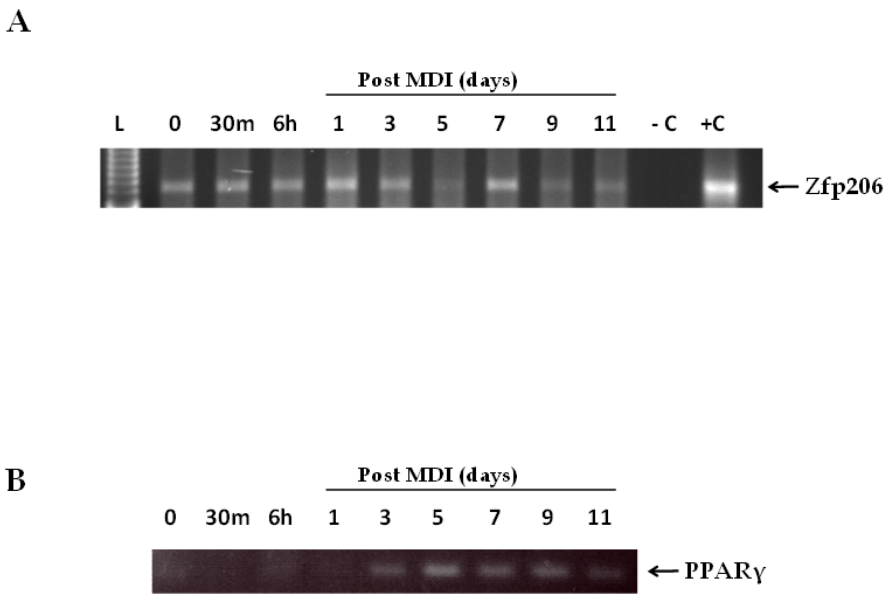


Figure 17 - Zfp206 and PPARγ mRNA expression in 3T3-L1 A, RT-PCR analysis of Zfp206 mRNA expression over a 3T3-L1 differentiation time course. – Ctr lane is primers only. + Ctr lane is ESC RNA. B, RT-PCR analysis of PPARγ mRNA expression over the same 3T3-L1 differentiation time course in A.

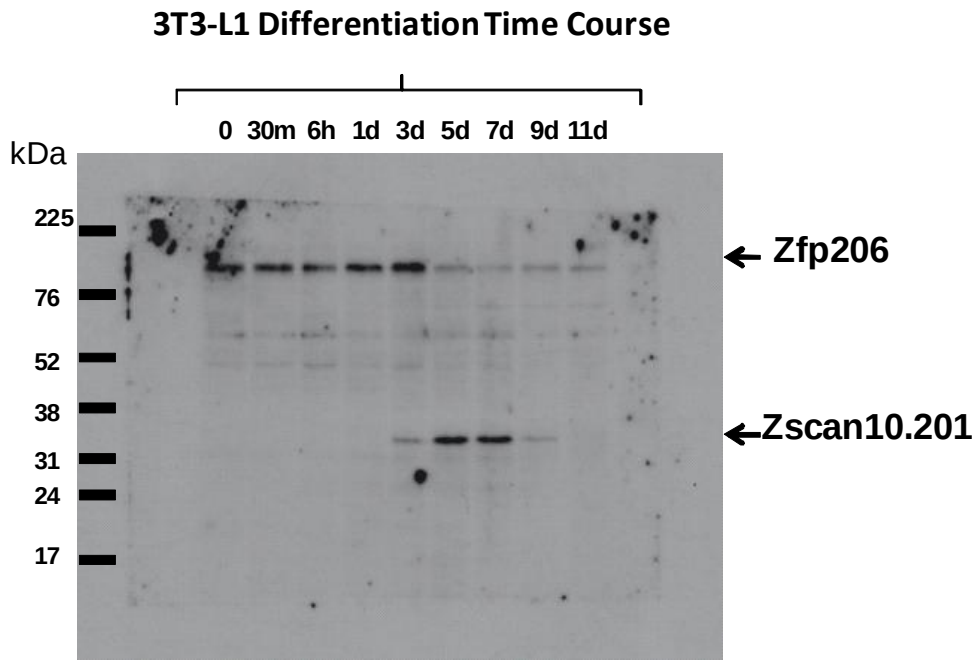
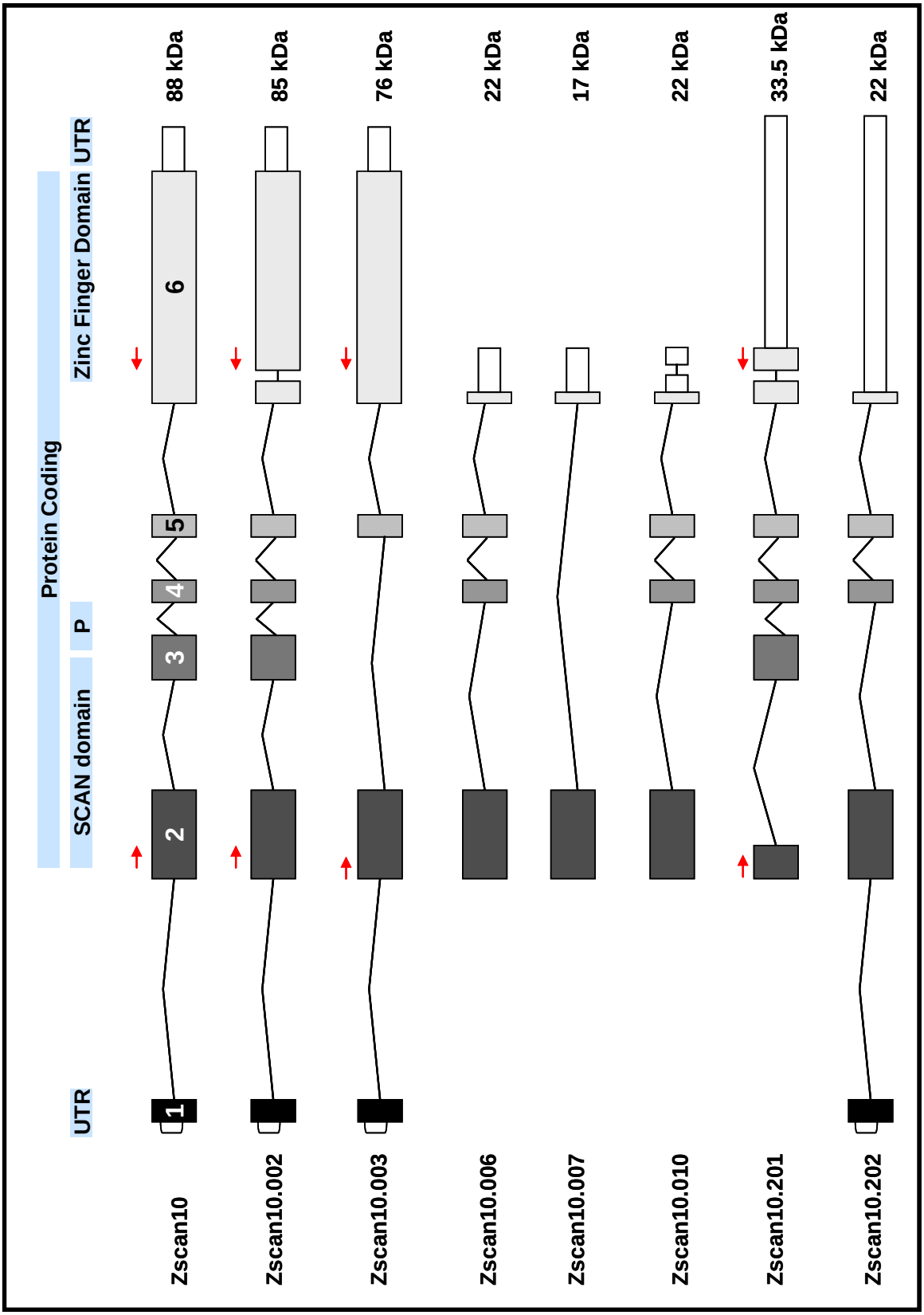


Figure 18 - *Zfp206* protein expression in 3T3-L1 cell line. Western Blot analysis of *Zfp206* protein expression over a 3T3-L1 differentiation time course. *Zfp206* full length (88 kDa) and Zscan10.201 isoform (33 kDa) shown by arrows.

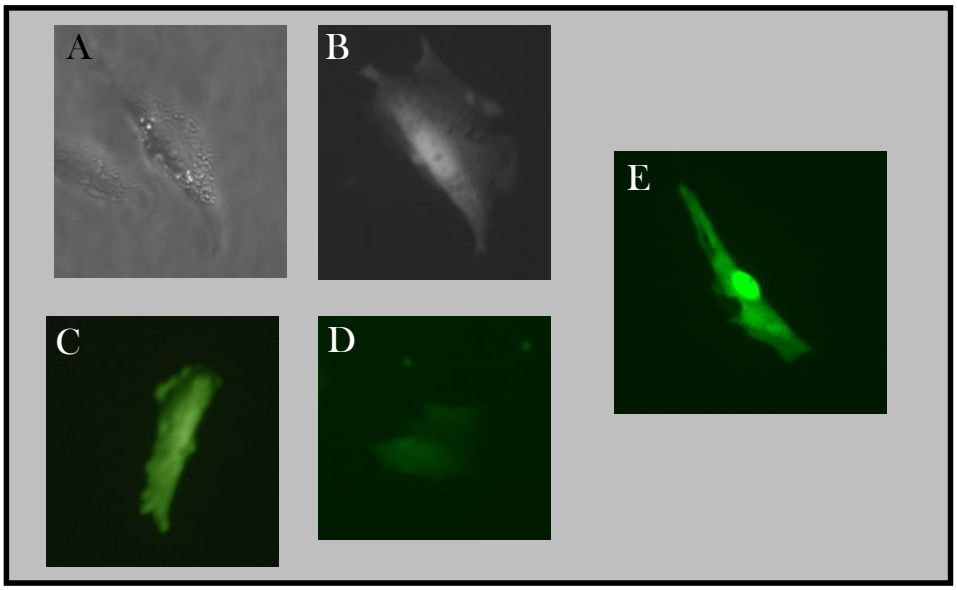
Figure 19. Gene Annotation of Zscan10. Adaptation of Ensembl genomic annotation for Zscan10 Mus musculus. Black to gray scale boxes represents exons 1-6. Protein coding, SCAN and Zinc finger domains illustrated in blue. Red arrows depict primers used in RT-PCR analysis.


Protein Coding
UTR
SCAN domain
P
Zinc Finger Domain
UTR
1
2
3
4
5
6
Zscan10
88 kDa
Zscan10.002
85 kDa
Zscan10.003
76 kDa
Zscan10.006
22 kDa
Zscan10.007
17 kDa
Zscan10.010
22 kDa
Zscan10.201
33.5 kDa
Zscan10.202
22 kDa

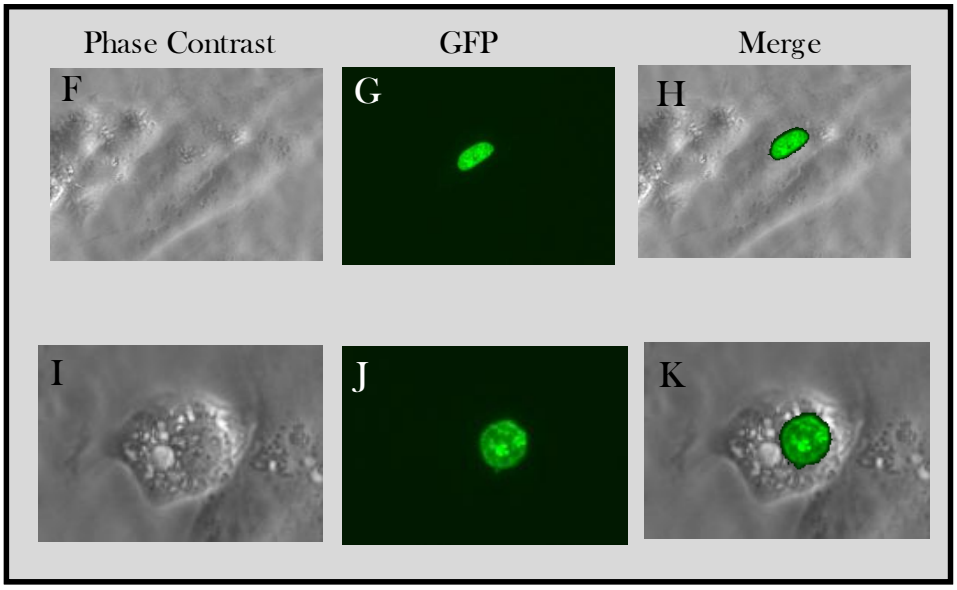
Zfp206-GFP fusion protein localizes to the nucleus of 3T3-L1 - *Zfp206* has been characterized as a transcription factor that is highly expressed in embryonic stem cells and is promptly repressed upon differentiation into to various tissue lineages (93). Based on our recent data, *Zfp206* follows a similar expression pattern (Fig. 18). We hypothesized that overexpression of *Zfp206* in the 3T3-L1 cell line would prevent or lessen the degree of differentiation into adipocytes. To begin to test this, we have created a Zfp206-GFP fusion construct, by inserting the open reading frame of *Zfp206* into an N-terminal GFP fusion vector. The Zfp206-GFP plasmid was then transfected into 3T3-L1 cells and monitored by microscopy for GFP at 48hrs post transfection. As shown in Fig. 20J&G, cells transfected with the Zfp206-GFP construct demonstrated GFP expression localized to the nucleus. Conversely, the control IRES-EGFP vector (Fig. 20A-E) demonstrates GFP expression throughout the entire cell. This data suggests that *Zfp206* is localized to the nucleus of the 3T3-L1 cell and supports *Zfp206* functioning as a transcription factor. Further examination of how *Zfp206* overexpression affects differentiation is currently being tested.

Figure 20. Zfp206-GFP fusion protein localizes to Nucleus. Phase and fluorescent images of 3T3-L1 cells, 48hrs post transfection with either IRES-EGFP or ZFP-GFP plasmids. A. Phase contrast, IRES-EGFP. B. Fluorescent clear filter, cell expressing IRES-EGFP (same cell as A). C. Fluorescent GFP, cell expressing IRES-EGFP. D. Fluorescent GFP, cell expressing IRES-GFP. E. Fluorescent GFP, cell expressing IRES-EGFP. F. Phase contrast, ZFP-GFP. G. Fluorescent GFP filter, ZFP-GFP (same cell as F). H. Merge of F and G images. I. Phase contrast, ZFP-GFP. J Fluorescent GFP, ZFP-GFP (same cell as I). K. Merge of I and J images.

IRES-EGFP



ZFP –GFP Fusion



Zfp206 gene expression in 3T3-L1 is not regulated by Oct4/Sox2 - In embryonic stem cells, *Zfp206* gene expression is regulated by Oct4 and Sox2, which are embryonic transcription factors known to play important roles in the maintenance of embryonic stem cell pluripotency (94). The presence of two composite sox-oct binding elements within the first intron of *Zfp206* has been shown to activate transcription synergistically (94). To determine if Oct4 and Sox2 maybe relevant to the control of *Zfp206* expression in the 3T3-L1 cells we examined for their expression, RNA was isolated from a differentiation time course and examined for the presence of both Sox2 and Oct4 mRNA via RT-PCR. As shown in figure 21, neither Sox2 mRNA nor Oct4 mRNA were present in 3T3-L1 cells relative to that found in the D3 murine ESC line. These data suggest that *Zfp206* gene regulation is cell type specific and Oct4/Sox2 is not required for transcriptional activation of *Zfp206* in the 3T3-L1 preadipocyte cell line.

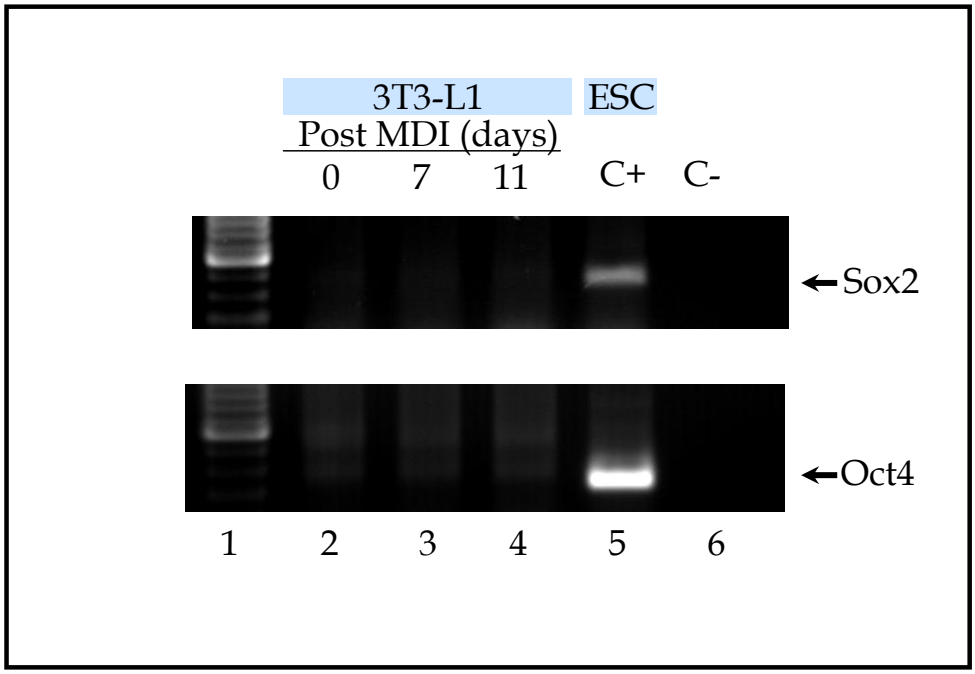


Figure 21 - Oct4 and Sox2 mRNA Expression in the 3T3-L1 Differentiation Time Course. 3T3-L1 RNA was harvested at 0 time, day7, and day 11 post MDI treatment and analyzed by RT-PCR with primers for Sox2 and Oct4. Lane 1, DNA 100bp ladder. Lane 2-4, 3T3-L1 0 time, day 7, and day 11, respectively. Lane 5, positive control embryonic stem cell line D3. Lane 6, negative control H_2O.

***Zfp206* is not down-regulated in C/EBPβ null MEF cell line -** To further investigate the regulation of *Zfp206* we utilized C/EBPβ null mouse embryonic fibroblasts (C/EBPβ$^{-/-}$). These cells were harvested from 13.5 day embryos that had been derived by mating C/EBPβ $^{+/-}$ mice. The C/EBPβ$^{-/-}$ MEFs will not differentiate when stimulated with induction cocktail. Cells were grown to confluency and induced to differentiation as described in Experimental Procedures. Cells were maintained for 11 days post induction of differentiation and the preadipocyte morphology was conserved. As shown in Fig. 22, western blot analysis of C/EBPβ$^{-/-}$ MEFs at days 0, 7 and 11 post induction of differentiation demonstrated that *Zfp206* expression was maintained throughout the differentiation time course. This expression pattern differs from that of the 3T3-L1 cells where *Zfp206* is down regulated after day 7 of induction of differentiation (Fig.18). Interestingly, expression of Zscan10.201 splice variant was not observed.

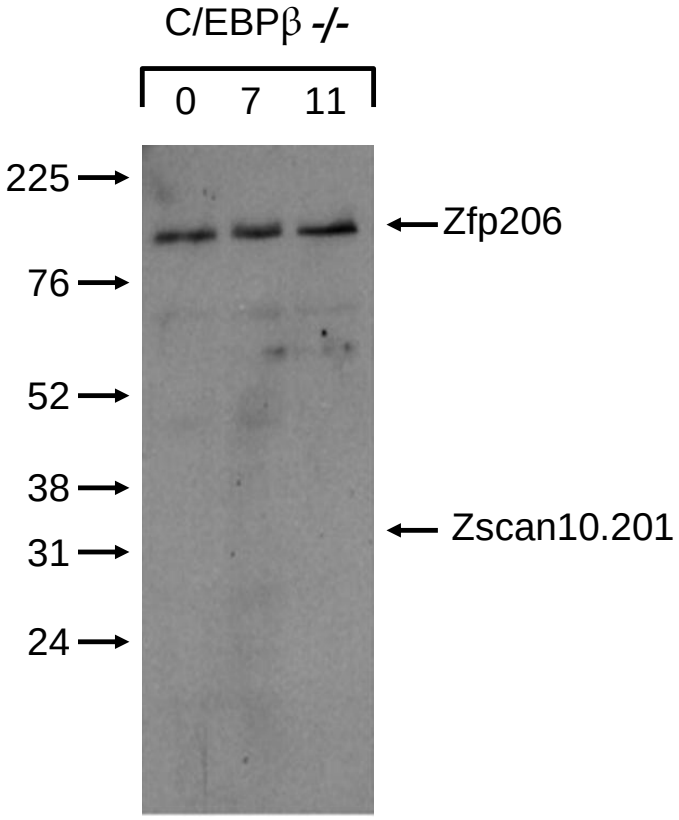


Figure 22 . *Zfp206* expression in C/EBPβ$^{-/-}$ MEF cell line. C/EBPβ-/- MEF cells were induced to differentiate and harvested at day 0, 7, and 11. Protein samples were analyzed by western blot for *Zfp206* (88 kDa). Absence of Zscan10.201 observed (32 kDa).

***Zfp206* Expressed in Human Preadipocytes but not in Adipocytes -** Cultures of human subcutaneous preadipocytes and adipocytes (2 wks post differentiation) were obtained from Zen-Bio, Inc. and maintained as described in Experimental Procedures. Analysis of *Zfp206* protein expression in the human cell was carried out by use of the murine *Zfp206* antibody which shares 60% homology with the human protein. As shown in Fig. 23A, the 88 kDa full length species was detected in the preadipocyte and similar to that observed in the 3T3-L1 cells (Fig.18), down regulation was observed in the mature human adipocytes. Further confirmation was obtained by examination of Zfp206 mRNA levels by RT-PCR. As shown in Figure 23B, Zfp206 mRNA was detected in the human preadipocyte and was absent in the adipocyte. Importantly, these data demonstrate that *Zfp206* expression is not an artifact of the 3T3-L1 cells or the culture system; and its expression and down regulation in primary human cells is consistent with the involvement of this embryonic stem cell transcription factor in the differentiation program of human preadipocytes.

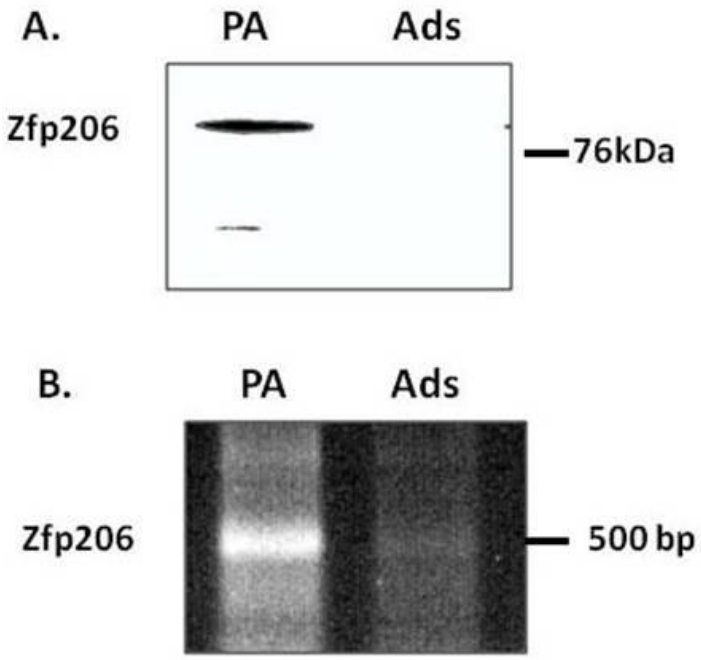


Figure 23 - *Zfp206* Expression in Human Subcutaneous Preadipocyte and Adipocytes. *A*- Western blot analysis of preadipocyte (PA) and adipocyte (Ads) lysates. *B*- Coomassie Brilliant Blue R Staining of the blot shown in Panel A to demonstrate protein loading. *C*- RT-PCR of RNA isolated from 2 week post differentiated adipocytes.

Analysis of Suppression of Zfp206 mRNA by Quantitative PCR - Our hypothesis, based on the current but limited literature on *Zfp206*, is that expression of *Zfp206* is part of a mechanism for maintenance of the preadipocyte differentiation potential, and as the adipocyte phenotype is established, expression of *Zfp206* is diminished. It may be that as PPARγ expression achieves homeostatic levels, locking in the adipocyte phenotype, and *Zfp206* expression is down regulated. To begin to address our hypothesis, we have attempted to suppress *Zfp206* via several methods as described below, with minimal to no effect of *Zfp206* expression.

Attempts at suppression of *Zfp206* by use of siRNA-*Zfp206* transiently transfected into 3T3-L1 cells resulted in no significant suppression of protein when examined by western blot analysis (data not shown). Six independent troubleshooting trials were preformed, the following are the conditions tested: Single dose transfections of siRNA *Zfp206* at 60% and 90% confluency, Double dose (consecutive transfections 24hrs apart) of siRNA *Zfp206* at 60% and 90% confluence, varying harvest times at 48hr, 72hr and 96 hrs post transfection, and with or without combination of transfected monolayers for immediate confluence. The reason for these failures is unknown. We have used this same siRNA system (Dharmacon SMARTpool) for suppression of HuR in previous studies with higher then 80% suppression of the protein.

Next we utilized two *Zfp206* shRNA constructs (gifts from Larry Stanton) for transiently transfections into 3T3-L1 cells. Two independent troubleshooting trials were preformed testing the following conditions: 1. Cells were harvested at 48hrs and 72hrs post transfection, with combined monolayers for immediate confluence 2. Cells were

harvested at 92hr, 144hr, and 192hrs, without combining monolayers. Treatments included sh1 & sh2 independently and collectively. These trials resulted in no significant suppression of the protein when examined by western blot (data not shown). The failures of these attempts are assumed to be caused by low transfection efficiencies.

Cell lines were created by stable transfections of shRNA constructs into the 3T3-L1 cell line to select for a pure shRNA expressing population. However, no effect was observed. Both 3T3-L1-sh1Zfp and 3T3-L1-sh2Zfp expressed *Zfp206* protein equal to that of the control 3T3-L1 cells (data not shown).

Larry Stanton was able to show that Zfp206 mRNA levels decrease during differentiation of ES cells by quantitative PCR (qPCR) (104) Using the same qPCR primers for *Zfp206* we attempted to show suppression of Zfp206 mRNA by siRNA and shRNA independently. Zfp206 mRNA content was first determined by qPCR on days 1, 7 and 11 of a differentiation time course. Zfp206 mRNA content was observed to be maximal on day 1 after induction of differentiation; by day 7 mRNA levels were significantly diminished and remained reduced through day 11 (Fig. 24A). These data are consistent with the *Zfp206* protein expression profile, (Fig. 18).

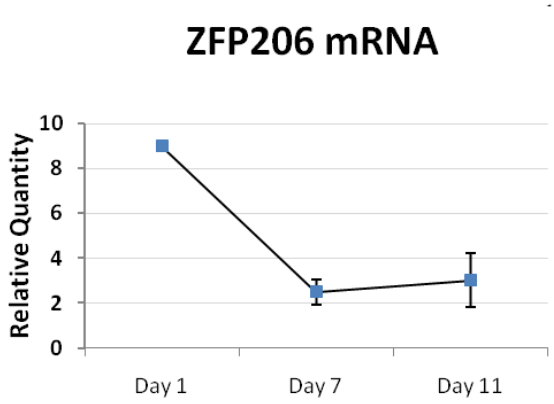
In our next qPCR trial we attempted to suppress *Zfp206* expression by siRNA and shRNA, and then analyze Zfp206 mRNA content at 24 hour and 48 hours post transfection, as displayed in Fig. 24B&C. Approximately 60% and 80% suppression of Zfp206 mRNA was observed at 24hrs and 48hrs, respectively. The results demonstrate a possible trend towards mRNA suppression. However, data from these trials had extremely variable threshold values and therefore are not reliable. To better understand

the variability seen in the qPCR results we sought help from the qPCR specialist at BioRad, Inc. It was their opinion that the primer melt curves for β-laminin (control) primer and Zfp206 were valid. They suggested that we should alter the annealing temperature from 64°C to 60°C; this had no overall effect on results.

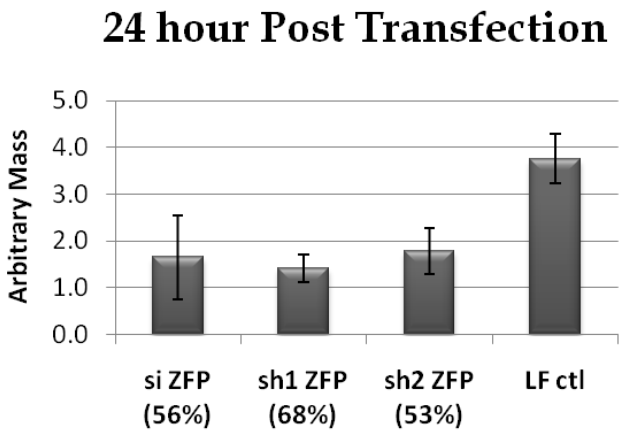
Analysis of standard curve data for the two sets of primers utilized a dilution series ranging from 500ng of 5pg of Day 1 3T3-L1 cDNA. The standard curve generated from the β-laminin primers is depicted in figure 25A, which shows a valid standard curve and equation.

Figure 24. Quantitative PCR analysis of Zfp206 mRNA suppression. A. At day 1, 7 and 11 post induction of differentiation, total RNA was isolated from 3T3-L1 cells and subjected to RT-qPCR with Zfp206 specific primers. B. Total RNA isolated from siRNA, shRNA or Lipofectamine 2000 control treated 3T3-L1 cells 24hrs post transfection was subjected to RT-qPCR with Zfp206 specific primers. C. Forty-eight hour time point, same conditions as panel B. B&C are representative of the best data from 5 independent qPCR trials. Percentages in parentheses are percent reduction in expression.

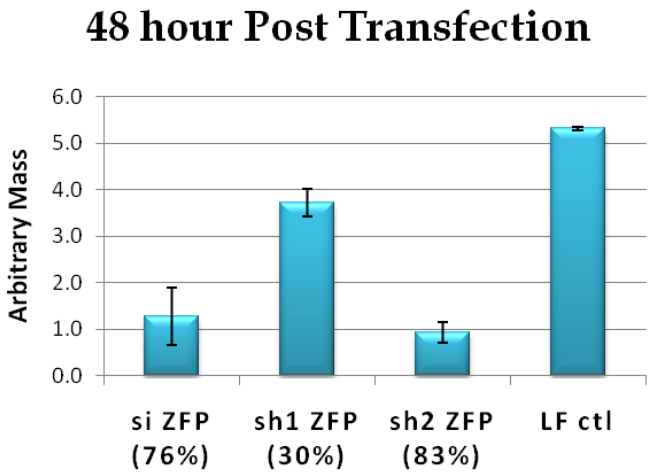
A


ZFP206 mRNA
Relative Quantity
10
8
6
4
2
0
Day 1
Day 7
Day 11

B


24 hour Post Transfection
Arbitrary Mass
5.0
4.0
3.0
2.0
1.0
0.0
si ZFP (56%)
sh1 ZFP (68%)
sh2 ZFP (53%)
LF ctl

C


48 hour Post Transfection
Arbitrary Mass
6.0
5.0
4.0
3.0
2.0
1.0
0.0
si ZFP (76%)
sh1 ZFP (30%)
sh2 ZFP (83%)
LF ctl

However, the standard curve data for the Zfp206 primers with the same serial dilution of cDNA shows an erratic display of threshold cycle values (Ct) independent on concentration of starting material (Fig 25B). The data produced do not constitute an acceptable standard curve and the equation of y=-0.229x + 29.35 is almost a straight horizontal line. It was concluded that the Zfp206 primers used fail to amplify a true amplicon in 3T3-L1 cells and therefore all data produced with Zfp206 primers are null and void. It is possible that the targeted region of mRNA of these primers is highly structured and difficult to access. Further analysis of these primers using RNA fold prediction software showed a hairpin structure formed between the forward and reverse primers. Future attempts at qPCR for Zfp206 mRNA will require new primer design.

Further analysis is needed to determine whether suppression of *Zfp206* can be established and whether its suppression will affect the 3T3-L1 differentiation protocol. Studies using Lentiviral shRNA constructs are underway.

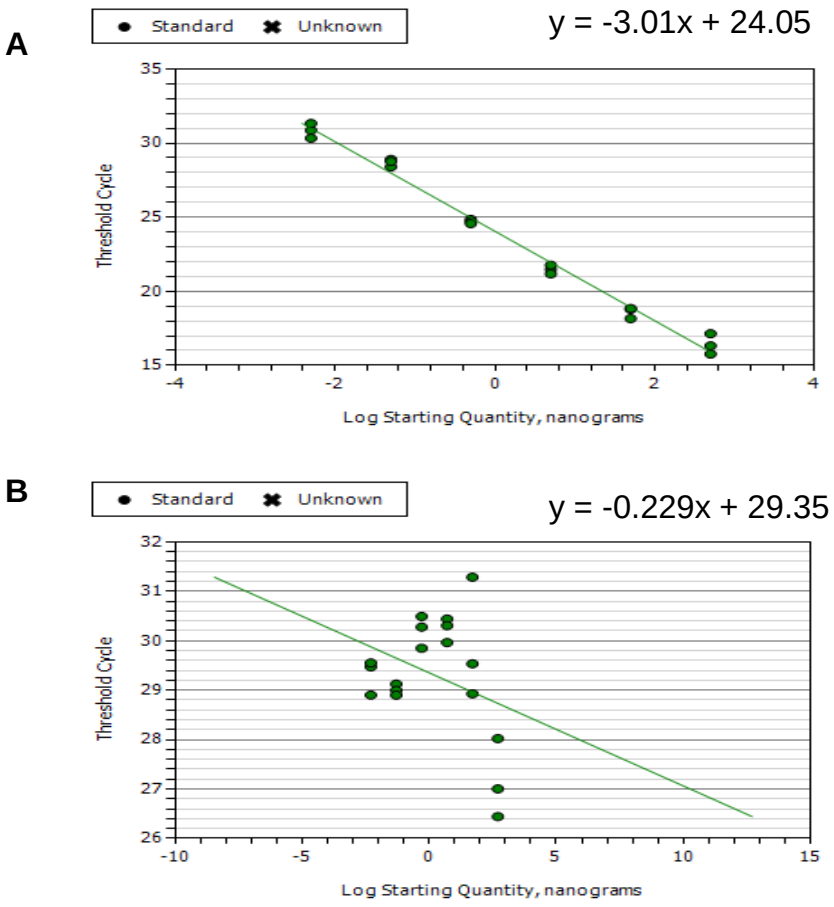


Figure 25. Real Time PCR Standard Curve Data. A. Standard curve of 3T3-L1 day 1 cDNA with β-Laminin primers, standard curve equation for arbitrary RNA mass shown, acceptable standard curve. B. Standard curve of 3T3-L1 day 1 cDNA with *Zfp206* primers, standard curve equation for arbitrary RNA mass shown, not acceptable standard curve, target not amplified.

Discussion

The Zfp206 mRNA was identified among 495 HuR ligands in a RIP-ChIP microarray analysis. Prior to this, *Zfp206* had been described as being exclusively expressed in embryonic stem cells by Wang *et al.* (93) and Zhang *et al.* (103, 104). Our array data indicated that at 0 time, the Zfp206 mRNA was not a ligand for HuR, yet at 30 min after induction of differentiation the complex was detected. Zfp206 mRNA was detected via RT-PCR in the 3T3-L1 preadipocyte at 0 time. The data suggest that the HuR binding site may initially (0 time) be occupied by another protein complex. Indeed, differential occupation of an adenylate-uridylate rich site in other mRNAs has been demonstrated for HuR and AUF-1 (47).

Our findings demonstrate that *Zfp206* protein is expressed in confluent 3T3-L1 preadipocytes and expression is maintained through the first three days post induction of differentiation. As the cells become fully differentiated, *Zfp206* expression is terminated. Additionally, an approximate 32 kDa splice variant (Zscan10.201) was observed on day 3 post induction of differentiation with its expression diminished as the cells reach terminal differentiation. The splice variant Zscan10.201 maintains an intact SCAN domain but has lost the zinc finger domain of the protein, thus it can no longer bind DNA. This combination of domains or lack of domains, leads to the hypothesis that this variant may serve as a dominant-negative form of *Zfp206*. The SCAN domain is preserved and can potentially interact with other protein partners involved in either gene activation or repression. Recent work has demonstrated that *Zfp206* can bind to more than 3,500 sites in the genome of embryonic stem cells (100).

Target genes include those involved in nucleic acid metabolism and several genes that encode pluripotency-regulating transcription factors, such as *Klf2, Klf4, Zfp281, Oct4 and Sox2* (100). Examination of 10 of these genes indicated that *Zfp206* binding could activate, suppress or have no effect on the rate of transcription. Interestingly, Oct4 and Sox2 expression is regulated by *Zfp206* through direct recruitment to their promoter regions. Correspondingly, Oct4 and Sox2 reciprocally regulate the expression of *Zfp206* by directly binding to the *Zfp206* promoter (100).

Zscan10.201 could potentially act as a *Zfp206* binding partner sink, sequestering potential partners inhibiting their binding to the full length *Zfp206* and thus preventing gene activation and/or repression. Our hypothesis would suggest that this inhibition of *Zfp206* function would permit the 3T3-L1 cells to enter a terminally differentiated state. This hypothesis has yet to be proven, but will be the focus of future studies.

In embryonic stem cells, *Zfp206* gene expression is regulated by the transcription factors Oct4 and Sox2 (94). Conversely, our findings show that Oct4 and Sox2 are not expressed during the differentiation of 3T3-L1 cells, suggesting that another mode of regulation must be in effect. Future studies will define the promoter region of the *Zfp206* gene and elucidate the regulatory elements that control expression of *Zfp206* during differentiation.

The *Zfp206* protein expression profile in the C/EBPβ$^{-/-}$ MEFs differs from that of the 3T3-L1 cells, in that, *Zfp206* is not down-regulated as seen in the mature adipocyte. Our C/EBPβ$^{-/-}$ MEF data is consistent with the hypothesis that *Zfp206* plays a role in the differentiation program. The absence of C/EBPβ protein in these cells effectively

prevents activation of the differentiation program and thus the adipocyte phenotype is never established and *Zfp206* is not down-regulated. Interestingly, there are several C/EBPβ and C/EBPα binding sites within the purported enhancer/repressor regions of the *Zfp206* gene (unpublished data). This would be consistent with either direct or indirect C/EBPβ involvement in control of expression of both *Zfp206* and Zscan10.201.

Our data indicate that human preadipocytes, isolated from subcutaneous fat depots of female donors, express *Zfp206*. We also demonstrate that human preadipocytes that were induced to differentiate to adipocytes show a down-regulation of *Zfp206* protein. These findings show a similar pattern of expression as the 3T3-L1 preadipocyte cell line. Further studies of *Zfp206* expression and role in human preadipocyte tissue may lead to clinically significant findings that may be applied to obesity.

Attempts at suppressing Zfp206 mRNA by siRNA/shRNA illustrate the potential to decrease Zfp206 mRNA by eighty percent 48 hours after transfection. Further manipulation of this method is needed such that protein levels are affected and the role of *Zfp206* in the differentiation program is clarified. Based on our hypothesis we predict that loss of *Zfp206* will result in a spontaneous initiation of the differentiation program with a rapid onset of the adipocyte phenotype i.e. early expression of the C/EBPs and PPARγ as well as early deposition of lipid.

Figure 26 summarizes the protein expression pattern of *Zfp206* in context with PPARγ and the C/EBP family of transcription factors during the differentiation process. As PPARγ and C/EBPα protein levels rise and the cells enter into terminal

differentiation, *Zfp206* protein diminishes. Further studies will be required to fully elucidate the mechanism(s) by which *Zfp206* regulates transcription levels of its putative targets and by which it influences adipocyte differentiation. If we are to identify new diagnostic biomarkers and discover new treatment options for obesity, we need to understand the regulation of the differentiation process as well as the maintenance of the differentiated phenotype in this complex tissue.

Chapter 7- DISCUSSION

Gene expression in eukaryotes is extensively controlled at the post-transcriptional level by RNA-binding proteins and ribonucleoprotein complexes modulating the maturation, stability, transport, editing and translation of RNA transcripts. Our studies describe an RNA binding protein, HuR, which interacts with AU-rich elements in the 3' untranslated region of mRNAs. Many of the genes which possess AU-rich elements regulate transient biological responses, including cell growth and differentiation, immune responses, signal transduction, hematopoiesis, apoptosis, and metabolism. Our studies focus on the involvement of HuR in the 3T3-L1 preadipocyte cell and during induction of differentiation into a mature adipocyte.

With the realization that HuR shuttles between the nuclear and cytosolic compartments, our work has demonstrated that upon confluence the bulk of HuR protein is retained in the 3T3-L1 cell nucleus. However, on exposure to the differentiation inducers the distribution is altered with the cytosolic content doubling in the first 30 min. We have further demonstrated that suppression of HuR protein expression resulted in an attenuation of the differentiation program with one of the downstream effects being an inhibition of C/EBPβ protein expression.

As we have stated previously, we predict that HuR associates with numerous mRNA ligands during the early phase of the differentiation program, any of which may influence the process. Adipogenesis requires a sequence of events including growth arrest of proliferating preadipocytes, synchronous reentry into the cell cycle from G_0

followed by limited mitotic clonal expansion, and growth arrest prior to terminal differentiation during which lipid accumulation occurs. Thus, it is reasonable to assume that factors involved in cell cycle regulation may have important roles in the adipocyte differentiation process. We have proposed that HuR may be influencing mitotic clonal expansion. Suppression of HuR lead to lower protein levels of cyclins A, E, and D1 and a lag in the expression pattern compared to the controls. The data are similar to those reported by Dormoy-Raclet et al. (15), a study in which depletion of HuR in HeLa cells led to attenuation of the movement through the cell cycle. In addition to the cyclins, HuR suppression led to decreased protein levels of both p53 and p21. These two factors are expressed at significant levels and have been demonstrated to be required for the differentiation process (40). The p53 mRNA has a HuR binding site in the 3'-UTR and the interaction is thought to control translational efficiency, thus, loss of HuR led to decreased p53. It appears likely that the decrease in p21 protein may be mediated by loss of p53 and thus decreased transactivation of the p21 gene. Taken together down-regulation of cyclins, p53 and p21 expression most certainly leads to a decreased ability of the cells to undergo an effective mitotic clonal expansion efficiently. These observations are consistent with HuR playing a significant role in the onset of adipogenesis.

C/EBPβ is essential for the acquisition of the adipose phenotype and its expression occurs early in the differentiation process, for that reason, we had selected C/EBPβ as a potential target of HuR. We have verified that HuR binds to C/EBPβ

mRNA 3'untranslated region forming an mRNP that translocates to the cytosol upon stimulation with differentiation inducers. Our original hypothesis suggested that HuR binding to the C/EBPβ mRNA was essential for the translocation of the message from the nucleus to the cytosol. However, our data proved quite the opposite as the message was demonstrated to translocate more effectively without the interaction with HuR. This led to increased cytosolic C/EBPβ mRNA and protein resulting in overexpression of the C/EBPβ downstream target, C/EBPα and an enhanced acquisition of the adipocyte phenotype. These observations are consistent with the formation of a HuR-C/EBPβ mRNA complex controlling the rate at which C/EBPβ mRNA is available in the cytosol for protein synthesis. Taken together these data support a function of HuR in the post-transcriptional regulation of C/EBPβ mRNA metabolism during early stages of adipogenesis.

Examination for potential mRNA ligands of HuR using an RNA Immunoprecipitation–Chip assay allowed the identification of a discrete subset of mRNAs associated with HuR. Notably, the population of mRNPs changed markedly during the initial 30 minutes of the adipocyte differentiation program. A unique subset of 193 mRNA targets found only at 0 time and a unique 45 mRNA targets at 30 min post induction of differentiation are genes that encode for transcription factors, as well as other proteins involved in transcriptional regulation, RNA binding proteins, proteins involved in apoptosis, and cell cycle proteins. Similarly, Tenenbaum et al. identified targets of HuB (HuR-related protein) during neuronal differentiation of P19 cells using RIP-ChIP technology (82). This revealed the existence of subsets of mRNAs that were

coordinately regulated at the post-transcriptional level. This functional approach to genomics has been termed “ribonomics" and designed to elucidate functional connections between groups of genes that are expressed concurrently during biological processes (82). The primary hypothesis of this model is that mRNAs are organized into groups sharing common functions and structural features. It is conceivable that a common RNA-binding protein could thus effectively regulate these linked mRNA subpopulations during cellular processes such as proliferation, differentiation, and the stress response. Keene et al. and Tenenbaum et al. (44, 82) recently proposed that mRNA-protein associations share equivalent characteristics to that of operons. The present investigation provides evidence that HuR coordinately regulates a subset of genes with critical functions maintaining preadipocyte potential and during early stages in adipogenesis, thereby lending support to the post-transcriptional operon model.

This analysis also revealed a novel HuR ligand mRNA at the 30min time point, *Zfp206*, a poly-zinc finger transcription factor whose expression has only been described in embryonic stem cells. Our data indicated that *Zfp206* protein is transiently expressed during the differentiation program and its expression is down-regulated as the cells enter a terminal differentiated state. Also documented was a 32 kDa splice variant, Zscan10.201, which lacks the zinc finger domain and is transiently expressed when *Zfp206* is down-regulated at day 3. These observations as well as the proposed regulatory role assigned to *Zfp206* in ES cells have led us to hypothesize that expression of *Zfp206* is essential for maintenance of the adipogenic differentiation potential. *Zfp206* may mediate maintenance directly or by control of specific genes that in turn control

differentiation potential. The suggestion of uncovering a regulatory gene for the maintenance of the preadipocyte state opens up the possibilities to novel therapeutics targeting obesity.

In this study, we provide evidence that expression of C/EBPβ, Cyclins A, E, D, p53, p21, and *Zfp206* during adipocyte differentiation is subject to regulation by direct or indirect interaction with the RNA-binding protein HuR. Our findings support a role for HuR in coordinately regulating the expression of several genes that play pivotal roles in the maintenance or the establishment of the adipocyte phenotype. Previous studies have demonstrated a role for HuR in coordinating the expression of a set of genes involved in the same biological process. For example, HuR influenced the expression of both cyclin A and cyclin B1 during the cell division cycle by proportionally stabilizing their respective mRNAs during the proliferative phases (91). Likewise, cellular response to stresses such as exposure to UV light caused coordinate changes in the stability of several stress-response genes, like the p21 (92).

Based on the accumulated data we have confirmed that HuR plays an important role in the metabolism of several key mRNA ligands during the induction of differentiation of the 3T3-L1 preadipocyte cell line. Moreover, we have described a novel mRNA ligand for HuR that may be responsible for the maintenance of the differentiation potential of the preadipocytes.

Among the metabolic disorders obesity is the most common and significantly increases the risk of the developing type II diabetes. The long-term objectives are to understand the molecular mechanisms that regulate the differentiation of preadipocytes

into fully functional adipocytes. Thus, this brings us one step closer to understanding the relationship between excess adipose tissue and the development of insulin resistance and diabetes. Our recent studies have contributed significantly to the elucidation of the network of control points in the regulation of adipogenesis; control points that could be potential drug targets. Further investigation into the role that HuR plays in regulating genes involved in adipogenesis may provide novel insights into the treatment and diagnosis of obesity and diabetes.

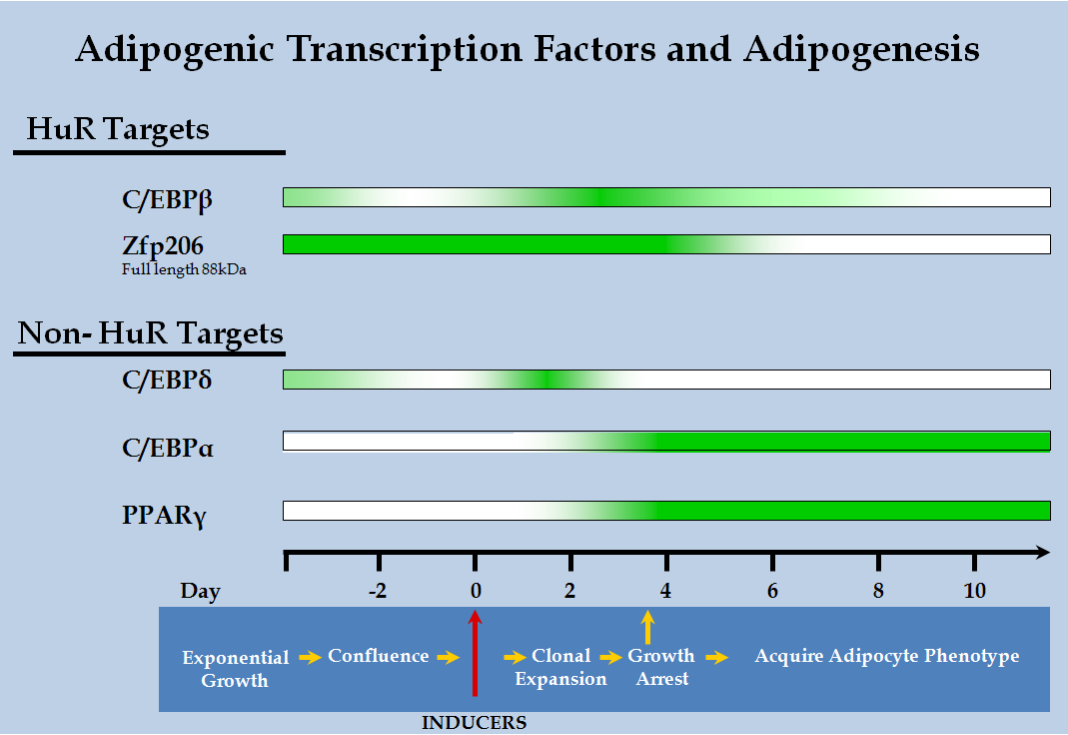


Figure 26 – Expression of adipogenic transcription factors during differentiation of 3T3-L1 preadipocytes. The degree of expression is indicated by the intensity of the green zones on the horizontal band corresponding to each transcription factor. Transcription factors are arranged according to relationship with HuR. The differentiation protocol is as described. (adapted from Mandrup and Lane (53))

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Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M200000084	Cellular tumor antigen p53 (Tumor suppressor p53)	NM_011640	ENSMUST00000005371	1.85347	0.78302
M200000105	Actin, cytoplasmic 1 (Beta-actin)	NM_007393	ENSMUST00000031564	6.11008	3.33452
M200000286	CD81 antigen (26 kDa cell surface protein TAPA-1)	NM_133655	ENSMUST00000037941	5.39336	3.02947
M200000288	Mago nashi protein homolog	NM_010760	ENSMUST00000030348	0.51460	1.00363
M200000316	Ras-related C3 botulinum toxin substrate 1 (p21-Rac1) (Ras-like protein TC25)	NM_009007	ENSMUST00000080537	0.99258	0.94671
M200000424	Transcription factor jun-D	NM_010592	ENSMUST00000061259	2.31902	0.89793
M200000454	Peripheral myelin protein 22 (PMP-22) (Growth-arrest-specific protein 3) (GAS3)	NM_008885	ENSMUST00000018361	2.60984	1.45933
M200000511	MAD protein (MAX dimerizer)	NM_010751	ENSMUST00000001184	0.57298	1.07116
M200000621	Glypican-4 precursor (K-glypican)	NM_008150	ENSMUST00000033450	2.63434	1.51753
M200000730	Cyclin-dependent kinase 6 inhibitor (p18-INK6) (Cyclin-dependent kinase 4 inhibitor C) (p18-INK4c)	NM_007671	ENSMUST00000030278	1.81080	1.28155
M200000746	Calreticulin precursor (CRP55) (Calregulin) (HACBP) (ERp60)		ENSMUST00000003912	1.25435	0.48503
M200000883	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1 (Transducin beta chain 1)	NM_008142	ENSMUST00000030940	3.61338	2.26719
M200000923	Protein disulfide isomerase A4 precursor (ERp72)	NM_009787	ENSMUST00000061626	2.95351	1.89871
M200001010	WD-repeat protein 1 (Actin interacting protein 1) (AIP1)	NM_011715	ENSMUST00000005234	1.29148	0.65939
M200001014	NA	NM_025880	ENSMUST00000071296	0.64402	0.99504
M200001021	Platelet-derived growth factor, A chain precursor (PDGF A-chain) (PDGF-1)		ENSMUST00000076095	1.16643	1.10797
M200001048	Insulin-like growth factor IB precursor (IGF-IB) (Somatomedin)	NM_010512	ENSMUST00000075330	1.09425	0.47959
M200001058	sialyltransferase 4C (beta-galactoside alpha-2,3-sialytransferase)	NM_009178	ENSMUST00000034537	1.62235	1.62291
M200001081	Nucleolar transcription factor 1 (Upstream binding factor 1) (UBF-1)	NM_011551	ENSMUST00000079589	1.01458	0.07037
M200001129	CD9 antigen	NM_007657	ENSMUST00000032492	2.36504	1.50968
M200001143	Transmembrane 9 superfamily protein member 3 precursor	NM_133352	ENSMUST00000025989	1.46884	0.97652
M200001148	ECT2 protein (Epithelial cell transforming sequence 2 oncogene)	NM_007900	ENSMUST00000029248	5.69543	-6.48798
M200001169	Pleiotrophin precursor (PTN) (Heparin-binding growth-associated molecule) (HB-GAM)	NM_008973	ENSMUST00000031864	5.30097	2.43703
M200001209	G1/S-specific cyclin D2	NM_009829	ENSMUST00000000188	2.33036	1.10117
M200001297	14-3-3 protein zeta/delta (Protein kinase C inhibitor protein-1) (KCIP-1)	NM_011740	ENSMUST00000022894	5.25676	2.90007
M200001395	Voltage-dependent anion-selective channel protein 1 (VDAC-1) (mVDAC1) (mVDAC5)	NM_011694	ENSMUST00000020673	2.48076	1.53565
M200001437	Ran-specific GTPase-activating protein (Ran binding protein 1) (RANBP1)	NM_011239	ENSMUST00000052325	1.47221	1.00919
M200001461	Syndecan-4 precursor (Ryudocan core protein)	NM_011521	ENSMUST00000017153	5.03300	3.31248
M200001508	Caspase-2 precursor (EC 3.4.22.-) (CASP-2) (ICH-1 protease) (NEDD2 protein)	NM_007610	ENSMUST00000031895	1.13717	1.02746
M200001516	Eukaryotic translation initiation factor 4E (eIF4E) (eIF-4E) (mRNA cap-binding protein)	NM_007917	ENSMUST00000029803	0.60339	1.03532
M200001637	Ran-binding protein 10	NM_145824	ENSMUST00000041400	1.26937	0.64671
M200001645	protein tyrosine phosphatase, receptor type, S.	NM_011218	ENSMUST00000025037	2.53475	0.98017
M200001805	Sodium/potassium-transporting ATPase beta-1 chain	NM_009721	ENSMUST00000027863	2.86157	1.72316
M200001816	Serine/threonine protein phosphatase PP1-beta catalytic subunit (EC 3.1.3.16) (PP-1B)	NM_172707	ENSMUST00000015100	3.01084	2.14775
M200002024	Protein CBFA2T1 (MTG8 protein)	NM_009822	ENSMUST00000076549	1.50740	1.22025
M200002099	Polyomavirus enhancer activator 3 (PEA3 protein) (ETS translocation variant 4)	NM_008815	ENSMUST00000017868	0.74824	1.19292
M200002183	amyloid beta (A4) precursor protein-binding, family B, member 2	NM_009686	ENSMUST00000068206	1.31327	0.85689
M200002348	protein tyrosine phosphatase 4a2	NM_008974	ENSMUST00000030578	1.01766	0.47170
M200002355	Probable ribosome biogenesis protein NEP1 (C2f protein)	NM_013536	ENSMUST00000004379	1.74695	1.35825

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M200002423	Ubiquitin ligase SIAH1A (EC 6.3.2.-) (Seven in absentia homolog 1a)	NM_009172	ENSMUST00000045296	1.19421	1.58314
M200002499	Long-chain fatty acid transport protein precursor (FATP)	NM_011977	ENSMUST00000034267	2.59422	1.17772
M200002531	Ubiquitin-like protein SMT3C precursor (Ubiquitin-homology domain protein PIC1)	NM_009460	ENSMUST00000027175	1.29623	1.10279
M200002589	Polyadenylate-binding protein 2 (Poly(A)-binding protein 2) (PolyA binding protein II) (PABII)	NM_019402	ENSMUST00000022808	3.67513	2.72940
M200002663	Cyclin-dependent kinase inhibitor 1B (Cyclin-dependent kinase inhibitor p27) (p27Kip1)	NM_009875	ENSMUST00000067327	1.05793	0.57613
M200002684	imprinted and ancient.	NM_008378	ENSMUST00000025290	1.75707	1.30303
M200002764	Heterogeneous nuclear ribonucleoprotein L (hnRNP L)	NM_177301	ENSMUST00000038572	4.63561	2.74070
M200002789	Nuclear factor 1 X-type (Nuclear factor 1/X)(CCAAT-box binding transcription factor)	NM_010906	ENSMUST00000077717	2.15122	0.81486
M200002879	Transcriptional regulator ATRX (X-linked nuclear protein) (Heterochromatin protein 2)	NM_009530	ENSMUST00000033580	1.03404	-0.15934
M200003203	Forkhead box protein C1 (Forkhead-related protein FKHL7) (Forkhead- related transcription factor 3)	NM_008592	ENSMUST00000062292	0.53221	1.00671
M200003218	Ceramide glucosyltransferase (EC 2.4.1.80) (Glucosylceramide synthase)	NM_011673	ENSMUST00000030074	2.96637	1.30622
M200003364	Mothers against decapentaplegic homolog 1 (SMAD 1)	NM_008539	ENSMUST00000066091	1.89192	1.60878
M200003449	BTG1 protein (B-cell translocation gene 1 protein)	NM_007569	ENSMUST00000038377	2.59826	1.87713
M200003641	Butyrate response factor 1 (TIS11B protein)	NM_007564	ENSMUST00000021552	3.19448	1.73667
M200003732	cAMP-dependent protein kinase, alpha-catalytic subunit (EC 2.7.1.37) (PKA C-alpha)	NM_008854	ENSMUST00000005606	2.19728	1.10439
M200003737	fatty acid elongase 1; homolog of yeast long chain polyunsaturated fatty acid elongation enzyme	NM_134255	ENSMUST00000034904	1.24576	1.12824
M200003853	Glutathione reductase, mitochondrial precursor (EC 1.8.1.7) (GR) (GRase)	NM_010344	ENSMUST00000033992	1.70782	0.90444
M200003903	Testis-specific Y-encoded-like protein 1 (TSPY-like 1)	NM_009433	ENSMUST00000061372	1.37632	0.76309
M200004061	LPS-induced TN factor; TBX1 protein; LPS-induced TNF-alpha factoR	NM_019980	ENSMUST00000023143	2.02835	1.38606
M200004252	p66 alpha homolog	NM_145596	ENSMUST00000065169	3.04677	1.61794
M200004300	Yippee homolog (CGI-127)	NM_027166	ENSMUST00000045174	2.15594	1.94080
M200004308	calponin 3, acidic	NM_028044	ENSMUST00000029773	1.31418	0.52944
M200004387	golgi phosphoprotein 3	NM_025673	ENSMUST00000059680	1.44266	1.04267
M200004431	NA	NM_172396	ENSMUST00000058733	1.75439	1.14288
M200004448	Collagen alpha 1(I) chain precursor.	NM_007742	ENSMUST00000001547	1.17843	-0.23394
M200004463	Ras-related protein Rab-18.	NM_011225	ENSMUST00000025128	1.02783	1.08330
M200004500	NA		ENSMUST00000019276	1.62466	1.08120
M200004585	Mitochondrial import receptor subunit TOM34 (Translocase of outer membrane 34 kDa subunit)	NM_025996	ENSMUST00000018466	2.12407	1.59499
M200004686	DNA polymerase delta interacting protein 3; polymerase delta interacting protein 46	NM_178627	ENSMUST00000058793	0.98036	0.72076
M200005043	KDEL endoplasmic reticulum protein retention receptor 1	NM_133950	ENSMUST00000002855	2.01009	1.29203
M200005078	NA	NM_025377	ENSMUST00000020794	2.10721	1.56484
M200005082	NA	NM_145466	ENSMUST00000038075	1.10340	0.50764
M200005113	protein phosphatase 6, catalytic subunit	NM_024209	ENSMUST00000028087	1.61846	1.40487
M200005125	NA	NM_203507	ENSMUST00000033973	0.89309	1.12779
M200005292	Homeobox protein Hox-D9 (Hox-4.4) (Hox-5.2)	NM_013555	ENSMUST00000059272	0.92633	0.99789
M200005650	Protein FAM3C precursor	NM_138587	ENSMUST00000081288	3.57721	2.58613
M200005696	coiled-coil transcriptional coactivator; GRIP1-interacting protein	NM_026192	ENSMUST00000023818	1.09767	0.34526
M200005761	FK506-binding protein 1A (EC 5.2.1.8) (Peptidyl-prolyl cis-trans isomerase)	NM_008019	ENSMUST00000044011	3.54130	2.10659
M200005924	Caveolin-1	NM_007616	ENSMUST00000007799	1.10380	0.38624

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M200005936	Cystinosin	NM_031251	ENSMUST00000006103	0.79519	1.22259
M200006155	translocating chain-associating membrane protein 1	NM_028173	ENSMUST00000027068	4.42153	3.33194
M200006351	T-complex associated-testis-expressed 1-like (Protein 91/23)	NM_025975	ENSMUST00000033519	1.69389	0.95324
M200006371	Adenylate kinase isoenzyme 1 (EC 2.7.4.3) (ATP-AMP transphosphorylase)	NM_021515	ENSMUST00000068271	2.60385	1.57619
M200006615	14-3-3 protein gamma.	NM_018871	ENSMUST00000055808	1.73624	1.11285
M200006649	UMP-CMP kinase (EC 2.7.4.14) (Cytidylate kinase)	NM_025647	ENSMUST00000030491	0.64659	1.21745
M200006674	Thioredoxin-dependent peroxide reductase, mitochondrial precursor (EC 1.11.1.-) (Perioredoxin 3)	NM_007452	ENSMUST00000025961	1.02439	1.02727
M200006807	NA	NM_134054	ENSMUST00000056228	0.67568	1.02304
M200006820	Lysosomal-associated transmembrane protein 4A (Golgi 4-transmembrane spanning transporter)	NM_008640	ENSMUST00000020909	1.77753	0.90963
M200006842	Actin-like protein 2 (Actin-related protein 2)	NM_146243	ENSMUST00000000137	1.66543	1.29132
M200006908	Beta-2-syntrophin (59 kDa dystrophin-associated protein A1, basic component 2)	NM_009229	ENSMUST00000047425	1.04075	0.16658
M200006912	26S proteasome non-ATPase regulatory subunit 9 (26S proteasome regulatory subunit p27).	NM_026000	ENSMUST00000060664	1.00177	0.56220
M200007163	Transmembrane 4 superfamily member 9 (Tetraspanin 5)	NM_019571	ENSMUST00000029800	3.54505	2.20534
M200007317	Zinc finger and BTB domain containing protein 1	NM_178744	ENSMUST00000042779	0.59735	1.20748
M200007523	NA	NM_026170	ENSMUST00000001619	0.85259	1.18324
M200007618	Placenta-specific gene 8 protein (C15 protein) (Onzin).	NM_139198	ENSMUST00000031264	2.82415	1.95319
M200007631	tryptophan rich basic protein.		ENSMUST00000023913	1.15676	1.44870
M200007637	actin related protein 2/3 complex, subunit 1A; actin related protein 2/3 complex, subunit 1A	NM_019767	ENSMUST00000031625	1.22520	0.84250
M200007781	sarcoma amplified sequence	NM_025982	ENSMUST00000081003	1.86918	1.52289
M200007799	Ras-related protein Rab-11B.	NM_008997	ENSMUST00000048874	3.70217	2.14597
M200007806	2-4-dienoyl-Coenzyme A reductase 2, peroxisomal.	NM_011933	ENSMUST00000040907	2.16670	1.37865
M200007942	NA	NM_026166	ENSMUST00000020149	0.94411	1.38519
M200007966	similar to human MGC33424 protein.	NM_212445	ENSMUST00000037853	1.25328	1.12910
M200008112	monocyte to macrophage differentiation-associated.	NM_026178	ENSMUST00000004050	2.08691	1.31765
M200008301	neuritin 1; candidate plasticity-related gene 15	NM_153529	ENSMUST00000037623	1.92544	1.76140
M200008310	transcription elongation factor A (SII)-like 1; cDNA sequence BC011290.	NM_146236	ENSMUST00000055104	0.67952	1.06102
M200008625	Tumor necrosis factor receptor superfamily member 10B precursor (Death receptor 5) (MK)	NM_020275	ENSMUST00000022663	0.80912	1.15014
M200008667	Selenoprotein K	NM_019979	ENSMUST00000044239	1.66939	1.53170
M200008896	RE1-silencing transcription factor	NM_011263	ENSMUST00000080359	1.87849	0.22943
M200008915	ELAV-like protein 1 (Hu-antigen R) (HuR) (Elav-like generic protein) (MelG)	NM_010485	ENSMUST00000042830	3.29415	2.22951
M200009353	CDK5 regulatory subunit associated protein 1-like 1	NM_144536	ENSMUST00000006353	1.90459	1.66100
M200009372	Muscleblind-like protein (Triplet-expansion RNA-binding protein)	NM_020007	ENSMUST00000029327	1.15840	0.87889
M200009421	ARP2/3 complex 20 kDa subunit (p20-ARC) (Actin-related protein 2/3 complex subunit 4)	NM_026552	ENSMUST00000032412	1.67634	0.62123
M200009460	bromodomain containing 4 isoform 1; bromodomain-containing 5; bromodomain-containing 4.	NM_020508	ENSMUST00000003726	1.13593	0.48596
M200009519	signal sequence receptor, gamma; translocon-associated protein gamma.	NM_026155	ENSMUST00000029414	2.19560	1.37449
M200009545	Regulator of nonsense transcripts 1 (Nonsense mRNA reducing factor 1) (NORF1)	NM_030680	ENSMUST00000075666	2.50716	1.27626
M200009548	lobe homolog-like; lobe homolog-like (Drosophila); lobe homolog (Drosophila)	NM_026270	ENSMUST00000054343	1.19831	0.67016
M200009682	Calnexin precursor.	NM_007597	ENSMUST00000020637	2.77339	1.37170
M200009955	Probable ATP-dependent RNA helicase p54 (Oncogene RCK homolog)	NM_007841	ENSMUST00000034608	1.40422	0.73153

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M200011599	NA	NM_153807	ENSMUST00000040228	1.56449	1.36684
M200011784	ADP-ribosylation factor-like 10B	NM_026823	ENSMUST00000027684	1.84366	1.58007
M200011942	Hsc70-interacting protein (Hip) (Putative tumor suppressor ST13)	NM_133726	ENSMUST00000023039	1.08866	0.23830
M200011966	limb-bud and heart	NM_029999	ENSMUST00000024857	2.99638	1.74659
M200012037	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	NM_025841	ENSMUST00000001908	5.43574	3.34827
M200012044	Early growth response protein 1 (EGR-1) (Krox-24 protein) (ZIF268)	NM_007913	ENSMUST00000064795	1.00791	0.62568
M200012509	Serine/threonine protein phosphatase 4 catalytic subunit (EC 3.1.3.16) (PP4C) (Pp4)	NM_019674	ENSMUST00000032936	1.09076	0.38901
M200012559	Nuclear transport factor 2 (NTF-2)	NM_026532	ENSMUST00000008594	1.23218	1.29990
M200012643	Signal peptide peptidase-like 3 (EC 3.4.99.-) (SPP-like 3 protein)	XM_485673	ENSMUST00000031530	1.44959	0.53476
M200012683	acetyl-Coenzyme A acetyltransferase 2; t-complex protein 1, related sequence 1	NM_009338	ENSMUST00000007005	1.77621	1.05978
M200012686	ADP-ribosylation factor 6.	NM_007481	ENSMUST00000050063	3.14799	1.67892
M200012692	myosin regulatory light chain-like.	NM_023402	ENSMUST00000038446	1.21902	0.55468
M200012720	Eukaryotic translation initiation factor 5A (eIF-5A) (eIF-4D) (Rev- binding factor)	NM_181582	ENSMUST00000043419	1.11439	0.54985
M200013007	NA		ENSMUST00000025675	0.97236	1.19035
M200013024	Ubiquitin thiolesterase protein OTUB1 (EC 3.4.-.-) (Otubain 1)	NM_134150	ENSMUST00000025679	1.77983	1.08452
M200013059	Adiponectin receptor protein 1	NM_028320	ENSMUST00000027727	1.24983	0.40086
M200013439	EIB-55kDa associated protein 5.	NM_144922	ENSMUST00000043765	1.26491	0.69241
M200013650	NA	NM_028058	ENSMUST00000026016	1.23020	0.71776
M200013714	NA	NM_029868	ENSMUST00000030460	1.07801	1.20993
M200013777	NA	NM_026417	ENSMUST00000024874	0.91312	1.34793
M200013802	MLN64 N-terminal domain homolog (STARD3 N-terminal like protein)	NM_024270	ENSMUST00000039694	1.43577	0.95019
M200013883	Dihydropyrimidinase related protein-2 (DRP-2) (ULIP 2 protein)	NM_009955	ENSMUST00000022629	3.08982	1.47366
M200013917	Spindlin (30000 Mr metaphase complex) (SSEC P)	NM_011462	ENSMUST00000057478	1.56711	1.73481
M200013976	14-3-3 protein epsilon (14-3-3E)	NM_009536	ENSMUST00000067664	3.42037	1.66914
M200014007	Casein kinase I, alpha isoform (EC 2.7.1.-) (CKI-alpha) (CK1)	NM_146087	ENSMUST00000025469	1.30579	0.75851
M200014111	Sestrin 3	NM_030261	ENSMUST00000034507	1.55570	0.76784
M200014215	Raver1	NM_027911	ENSMUST00000010349	1.19478	0.70562
M200014231	suppressor of Ty 16 homolog; suppressor of Ty 16 homolog (S.cerevisiae).	NM_033618	ENSMUST00000046709	1.05617	0.53670
M200014251	ring finger protein 38.	NM_175201	ENSMUST00000045793	1.31218	0.96457
M200014541	NA	NM_029649	ENSMUST00000021296	1.68532	1.62545
M200014558	Transcription factor SOX-9.		ENSMUST00000000579	1.97465	1.24564
M200014565	NA		ENSMUST00000006851	1.13284	0.63224
M200014632	BCL2-like 12 (proline rich).	NM_029410	ENSMUST00000003290	1.13060	1.23454
M200014840	transcription factor AP-4 (activating enhancer-binding protein 4)	NM_031182	ENSMUST00000005862	1.28675	1.62669
M200015000	kelch repeat and BTB (POZ) domain containing 2; cDNA sequence BC022962	NM_145958	ENSMUST00000081115	1.49188	0.77859
M200015033	Ras-related protein Rab-14	NM_026697	ENSMUST00000028238	1.13055	0.49620
M200015243	repulsive guidance molecule B.	NM_178615	ENSMUST00000057264	2.99877	1.87965
M200015255	potassium channel, subfamily K, member 5	NM_021542	ENSMUST00000024011	1.06787	0.14858
M200015349	Rho-related BTB domain-containing protein 3.	NM_028493	ENSMUST00000022078	2.17893	0.65474

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M200015439	Beta-1,4-galactosyltransferase 5 (EC 2.4.1.-) (Beta-1,4-GalTase 5) (Beta4Gal-T5) (b4Gal-T5)	NM_019835	ENSMUST00000018073	1.08858	0.78092
M200015503	dynactin 4	NM_026302	ENSMUST00000025505	1.16674	0.55562
M200015872	Similar to follicular lymphoma variant translocation 1	XM_203753	ENSMUST00000010049	1.29748	1.25717
M200015881	NA	NM_183186	ENSMUST00000046859	1.78996	0.81287
M200016063	Sprouty homolog 2 (Spry-2)	NM_011897	ENSMUST00000022709	2.26872	1.24843
M200016377	ADP-ribosylation factor-like 10C	NM_026011	ENSMUST00000032196	2.64310	1.93540
M300000003	Insulin-like growth factor II precursor (Multiplication stimulating polypeptide) (IGF-II)		ENSMUST00000053222	1.30419	1.12020
M300000180	Chromatin assembly factor 1 subunit C (CAF-1 subunit C) (Chromatin assembly factor I p48 subunit)	NM_009030	ENSMUST00000001366	1.30841	1.11139
M300000203	Tubulin beta-5 chain	NM_011655	ENSMUST00000001566	1.26189	0.44891
M300000225	ubiquitin-like 3	NM_011908	ENSMUST00000080815	1.67901	1.18529
M300000226	ubiquitin-like 3	NM_011908	ENSMUST00000080815	1.60212	1.08828
M300000295	Ras-related protein Rab-11B	NM_008997	ENSMUST00000002361	4.16867	2.04127
M300000373	MLN64 N-terminal domain homolog (STARD3 N-terminal like protein)		ENSMUST00000039694	0.93882	1.07882
M300000378	Platelet-activating factor acetylhydrolase IB beta subunit		ENSMUST00000003215	1.89841	1.48536
M300000501	polymerase I and transcript release factor		ENSMUST00000044938	1.17853	0.66277
M300000556	Ras-related protein Ral-B.	NM_022327	ENSMUST00000004565	1.54248	1.15542
M300000642	Krueppel-like factor 5 (Intestinal-enriched krueppel-like factor) (Transcription factor BTEB2)	NM_009769	ENSMUST00000005279	0.78850	1.24999
M300000714	breast carcinoma amplified sequence 2	NM_026602	ENSMUST00000005830	0.85147	1.14987
M300000803	Prefoldin subunit 2	NM_011070	ENSMUST00000006579	2.27395	2.02291
M300000851	sarcoma amplified sequence	NM_025982	ENSMUST00000060991	2.69308	2.01020
M300000858	Superoxide dismutase [Mn], mitochondrial precursor (EC 1.15.1.1)	NM_013671	ENSMUST00000007012	1.27851	0.28335
M300000896	ocular development associated gene	NM_026033	ENSMUST00000007559	1.08879	0.78462
M300000921	Transforming protein RhoA	NM_016802	ENSMUST00000007959	2.27692	1.30584
M300000928	NA	XM_354754	ENSMUST00000007980	2.78740	0.99664
M300001011	RNA-binding protein EWS		ENSMUST00000079949	2.28185	1.41150
M300001525	Lysosome-associated membrane glycoprotein 2 precursor (LAMP-2)	NM_010685	ENSMUST00000061755	4.30285	1.61496
M300001526	Lysosome-associated membrane glycoprotein 2 precursor (LAMP-2)	NM_010685	ENSMUST00000061755	1.04727	1.47203
M300001584	proteasome 26S non-ATPase subunit 11	NM_178616	ENSMUST00000017572	3.05949	2.10305
M300001652	Profilin I	NM_011072	ENSMUST00000018437	4.85536	2.87998
M300001678	carcinoma related gene	NM_033562	ENSMUST00000018586	1.57608	1.67583
M300001683	NA	XM_126426	ENSMUST00000018620	1.53518	0.87889
M300001785	NA	NM_007590	ENSMUST00000019514	1.62545	0.86132
M300001921	Serine/threonine-protein kinase Sgk1 (EC 2.7.1.37) (Serum/glucocorticoid-regulated kinase 1)	NM_011361	ENSMUST00000020145	0.83728	1.24295
M300002017	Ras-related protein Rab-21 (Rab-12)	NM_024454	ENSMUST00000020343	1.01616	0.82368
M300002101	Ubiquitin-like protein SMT3A		ENSMUST00000020501	0.99399	0.96668
M300002370	Ubiquitin-like protein SMT3B (Sentrin 2)	NM_133354	ENSMUST00000021084	1.60656	1.24983
M300002374	Growth factor receptor-bound protein 2 (GRB2 adapter protein) (SH2/SH3 adapter GRB2)	NM_008163	ENSMUST00000077165,E	1.07121	0.81438
M300002407	NA	XM_126676	ENSMUST00000047715,E	1.20706	1.19374
M300002473	NA	NM_172947	ENSMUST00000021297	4.33244	2.92994

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M300002564	Poly(A) polymerase alpha (EC 2.7.7.19) (PAP)	NM_011112	ENSMUST00000021535	1.25524	0.80458
M300002647	UPF0143 protein C14orf1 homolog	NM_021446	ENSMUST00000021676	1.49818	1.28742
M300002718	Transcription factor AP-2 alpha (AP2-alpha) (Activating enhancer- binding protein 2 alpha)		ENSMUST00000077859	3.57895	2.28959
M300002876	basic transcription factor 3	NM_145455	ENSMUST00000022163	1.67779	1.12247
M300003298	Twinfilin 1 (A6 protein) (Protein tyrosine kinase 9)	NM_008971	ENSMUST00000023087	2.26892	0.80322
M300003424	NA	XM_156257	ENSMUST00000023313	1.03682	0.67114
M300003426	CD47 antigen; integrin-associated protein; Rh-related antigen	NM_010581	ENSMUST00000023320	2.89137	1.54277
M300003481	NA	NM_171826	ENSMUST00000023426	2.34056	1.82682
M300003525	Follistatin-related protein 1 precursor (Follistatin-like 1) (TGF- beta-inducible protein TSC-36)	NM_008047	ENSMUST00000023511	2.20723	0.93002
M300003535	RAB, member of RAS oncogene family-like 3	NM_026297	ENSMUST00000023524	0.87820	1.66709
M300003601	ATP synthase oligomycin sensitivity conferral protein, mitochondrial precursor (EC 3.6.3.14) (OSCP)	NM_138597	ENSMUST00000023677	2.85222	0.10516
M300003763	Cation-independent mannose-6-phosphate receptor precursor (CI Man-6-P receptor) (CI-MPR)	NM_010515	ENSMUST00000024599	1.34408	0.17799
M300003876	Band 4.1-like protein 3 (4.1B) (Differentially expressed in adenocarcinoma of the lung protein 1)	NM_013813	ENSMUST00000077529	1.70036	0.95864
M300004057	Tapasin precursor (TPSN) (TPN) (TAP-binding protein)		ENSMUST00000025161	2.21485	1.38785
M300004178	NA	NM_028392	ENSMUST00000025377	-0.04203	2.07541
M300004216	NA	NM_172832	ENSMUST00000025472	2.02551	1.76591
M300004390	Splicing factor 1 (Zinc finger protein 162) (Transcription factor ZFM1) (mZFM)		ENSMUST00000076351	1.23418	0.74358
M300004402	programmed cell death 4	NM_011050	ENSMUST00000074371	3.26490	2.22664
M300004412	Transcription factor 7-like 2 (HMG box transcription factor 4) (T- cell-specific transcription factor 4)		ENSMUST00000075651	1.27551	0.86085
M300004437	Tripartite motif protein 8 (RING finger protein 27) (Glioblastoma- expressed RING finger protein)	NM_053100	ENSMUST00000026008	1.44256	0.58771
M300004801	Protein disulfide isomerase A4 precursor (EC 5.3.4.1) (Protein ERp-72) (ERp72).		ENSMUST00000077290	3.95420	2.05315
M300004807	Platelet-derived growth factor, A chain precursor (PDGF A-chain)		ENSMUST00000046901	0.93841	1.19876
M300004854	6130401J04Rik protein (EC 6.3.2.19) (Ubiquitin-conjugating enzyme E2)		ENSMUST00000027075	1.79009	1.83329
M300004860	NA	NM_025964	ENSMUST00000053469	0.67136	1.11708
M300004917	non-catalytic region of tyrosine kinase adaptor protein 2	NM_010879	ENSMUST00000069643	1.27612	1.09694
M300005104	nuclear, casein kinase and cyclin-dependent kinase substrate	NM_175294	ENSMUST00000062264	2.52881	1.50562
M300005193	Sterol O-acyltransferase 1 (EC 2.3.1.26) (Cholesterol acyltransferase 1)	NM_009230	ENSMUST00000051396	1.31556	0.92834
M300005240	Microsomal glutathione S-transferase 3 (EC 2.5.1.18) (Microsomal GST- 3)	NM_025569	ENSMUST00000028005	1.14460	0.72073
M300005817	NA	NM_144901	ENSMUST00000029446	1.82905	0.99332
M300005827	Vesicle trafficking protein SEC22b (SEC22 vesicle trafficking protein- like 1)	NM_011342	ENSMUST00000029476	2.87430	1.38893
M300005877	calcium/calmodulin-dependent protein kinase II, delta; CaMK II	NM_023813	ENSMUST00000066452	1.98476	1.29017
M300005888	secreted frizzled-related sequence protein 2; secreted frizzled-related sequence protein 5		ENSMUST00000029625	3.08973	1.39130
M300006081	Beta-1,4-galactosyltransferase 1 (EC 2.4.1.-) (Beta-1,4-GalTase 1) (Beta4Gal-T1)	NM_022305	ENSMUST00000030121	1.91071	1.22066
M300006113	Golgi-associated plant pathogenesis-related protein 1 (Golgi- associated PR-1 protein)	NM_027450	ENSMUST00000030202	3.13477	2.42911
M300006171	Glycoprotein 38 precursor (GP38) (OTS-8)	NM_010329	ENSMUST00000030317	1.94118	1.12879
M300006194	Protein C1orf8 homolog precursor (Thymic dendritic cell-derived factor 1)	NM_029565	ENSMUST00000030361	1.52917	1.02821
M300006266	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit precursor	NM_007838	ENSMUST00000030538	1.49635	0.63649
M300006729	splicing factor, arginine/serine rich 9; splicing factor, arginine/serine rich 9 (25 kDa)	NM_025573	ENSMUST00000031513	5.15329	2.81727
M300007194	Vasodilator-stimulated phosphoprotein (VASP)		ENSMUST00000032561	2.09810	1.05345

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M300007474	NA	NM_138589	ENSMUST00000033158	1.40282	0.97622
M300007712	Glucocorticoid-induced leucine zipper protein	NM_010286	ENSMUST00000055738	3.71406	2.53035
M300007781	dCMP deaminase.	NM_178788	ENSMUST00000033966	1.10868	0.98253
M300007813	Acid ceramidase precursor (EC 3.5.1.23) (Acylsphingosine deacylase)		ENSMUST00000034000	1.57024	0.98029
M300008017	Amyloid-like protein 2 precursor (CDEI-box binding protein) (CDEBP)	NM_009691	ENSMUST00000072634	1.11761	0.56547
M300008081	Oligosaccharyl transferase STT3 subunit homolog (B5) (Integral membrane protein 1)	NM_008408	ENSMUST00000034627	1.25350	0.72398
M300008124	ring finger 111	NM_033604	ENSMUST00000034739	1.04013	0.79346
M300008358	folliculin.	NM_146018	ENSMUST00000047706	1.27119	0.41287
M300008360	NA	XM_194358	ENSMUST00000036372	1.06123	0.59864
M300008361	Skeletal muscle LIM-protein 2 (SLIM 2) (Four and a half LIM domains protein 3) (FHL-3)	NM_010213	ENSMUST00000038684	2.95157	1.41267
M300008433	Casein kinase II, alpha chain (CK II) (EC 2.7.1.37).	NM_007788	ENSMUST00000041081	1.95315	0.95827
M300008745	Beta-2-microglobulin precursor.	NM_009735	ENSMUST00000080804	1.72294	0.81709
M300008835	RNA binding motif protein 9; fox-1 homolog (C. elegans); Fyn-binding molecule 2.	NM_053104	ENSMUST00000063150	2.69416	1.33635
M300008868	butyrate-induced transcript 1.	NM_021345	ENSMUST00000036615	1.58987	1.54250
M300008920	NA	NM_198625	ENSMUST00000052457	1.28763	0.36366
M300009180	NA	NM_177093	ENSMUST00000078717	1.12398	-0.10295
M300009824	trophoblast glycoprotein	NM_011627	ENSMUST00000006559	2.84270	1.93433
M300010003	leukocyte receptor cluster (LRC) member 5	NM_024168	ENSMUST00000067069	2.80941	1.51714
M300010087	NA		ENSMUST00000045628	2.28099	1.20981
M300010092	Lactoylglutathione lyase (EC 4.4.1.5) (Methylglyoxalase) (Aldoketomutase) (Glyoxalase I)	NM_025374	ENSMUST00000044467	1.02050	0.82976
M300010267	Periphilin 1.		ENSMUST00000049122	3.49408	1.83733
M300010273	Histone H1.2 (H1 VAR.1) (H1C)	NM_015786	ENSMUST00000040914	1.46080	0.90010
M300010327	S-phase kinase-associated protein 1A; transcription elongation factor B (SIII)	NM_011543	ENSMUST00000037324	3.48187	1.74680
M300010609	Ras-related protein Rap-2b	NM_028712	ENSMUST00000049064	1.19554	0.86831
M300011013	spermatogenesis associated, serine-rich 2; serine-rich spermatocytes and round spermatid protein		ENSMUST00000063517	1.56217	0.54485
M300011266	Retinoic acid receptor alpha (RAR-alpha)		ENSMUST00000001334	2.49947	1.11847
M300011580	Reelin precursor (EC 3.4.21.-) (Reeler protein)	NM_011261	ENSMUST00000062372	4.29682	0.04916
M300011699	Lysophosphatidic acid receptor Edg-2 (LPA receptor 1) (LPA-1) (Rec1.3) (VZG-1)	NM_010336	ENSMUST00000055018	1.16851	1.07201
M300011779	NA	NM_145943	ENSMUST00000038091	1.13419	0.84107
M300011994	Ubiquitin-conjugating enzyme E2 H (EC 6.3.2.19) (Ubiquitin-protein ligase H)	NM_009459	ENSMUST00000036826	1.29234	0.70391
M300012013	Recombining binding protein suppressor of hairless (J kappa- recombination signal binding protein)		ENSMUST00000037618	1.21225	0.65301
M300012230	NCAM	NM_010875	ENSMUST00000053131	1.32101	0.82278
M300012256	RNA binding motif, single stranded interacting protein 3; RNA-binding protein RBMS3	NM_178660	ENSMUST00000074547	2.38127	1.34377
M300012485	Large neutral amino acids transporter small subunit 1 (L-type amino acid transporter 1)	NM_011404	ENSMUST00000045557	1.39062	0.40500
M300012510	Telomerase-binding protein p23 (Hsp90 co-chaperone) (Progesterone receptor complex p23)	NM_019766	ENSMUST00000052798	4.13185	2.19908
M300012949	NA		ENSMUST00000038893	1.52782	0.35705
M300013540	NA	NM_172510	ENSMUST00000046658	0.22794	1.51382
M300013796	SHC transforming protein 1 (SH2 domain protein C1)	NM_011368	ENSMUST00000039110	1.96823	0.71048
M300013817	NA	NM_172943	ENSMUST00000044250	2.45291	0.55787

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M300013961	NA	NM_028627	ENSMUST00000041391	1.09814	0.66576
M300016167	NA	NM_007705	ENSMUST00000054666	2.95238	2.02400
M300016892	NA	XM_131400	ENSMUST00000062977	1.03074	0.15604
M300019000	NA	NM_172871	ENSMUST00000079544	1.35469	0.44364
M300019706	NA	NM_198016	ENSMUST00000061490	1.39671	0.94097
M300020021	protein tyrosine phosphatase 4a1	NM_011200	ENSMUST00000027232	4.41993	2.76417
M300020190	Similar to cytochrome P450 monooxygenase	XM_129747	ENSMUST00000060608	1.61470	1.12192
M300020249	NA	XM_148582	ENSMUST00000081933	1.32731	0.98032
M300021114	NA	NM_026242	ENSMUST00000068795	3.87455	2.14024
M300022123	Brain MY039 protein homolog (Fragment)	XM_284454	ENSMUST00000054960	4.32410	1.81402
M300022233	NA	NM_029519	ENSMUST00000062117	1.27060	0.78638
M400000019	NA		ENSMUST00000000672	1.75853	1.00830
M400000066	NA	NM_016957	ENSMUST00000003122	2.65143	1.39155
M400000143	NA		ENSMUST00000065744	2.11388	0.97838
M400000149	NA		ENSMUST00000008286	1.00778	0.51416
M400000220	Stanniocalcin 1 precursor (STC-1)	NM_009285	ENSMUST00000014957	1.64224	1.60615
M400000306	Eukaryotic initiation factor 4A-I (eIF4A-I) (eIF-4A-I)	NM_144958	ENSMUST00000018919	1.95074	1.16959
M400000339	NA	NM_178606	ENSMUST00000020023	1.28594	0.79920
M400000390	NA	NM_007480	ENSMUST00000020717	6.53200	3.15082
M400000425	Presynaptic density protein 95 (PSD-95) (Presynaptic protein SAP90) (Synapse-associated protein 90)		ENSMUST00000018700	1.73896	1.65122
M400000481	Heterogeneous nuclear ribonucleoprotein K		ENSMUST00000058184	1.01961	0.45596
M400000579	CREB-binding protein (EC 2.3.1.48)	XM_358750	ENSMUST00000023165	2.15505	0.93343
M400000637	Cyclic-AMP-dependent transcription factor ATF-1		ENSMUST00000023769	1.16482	0.44359
M400000700	Zinc Finger Protein 206 (zfp206)(Zscan10)		ENSMUST00000048869	-0.08246	3.49907
M400000711	Probable serine/threonine-protein kinase SNF1LK (EC 2.7.1.37) (HRT-20)	NM_010831	ENSMUST00000024839	1.72996	1.71755
M400000721	NA	NM_198937	ENSMUST00000024981	1.17605	0.53454
M400000747	Adapter-related protein complex 3 sigma 1 subunit (Sigma-adaptin 3a)	NM_009681	ENSMUST00000025357	3.03318	1.94585
M400000798	THO complex subunit 4 (Tho4) (RNA and export factor binding protein 1) (REF1-I)	NM_011568	ENSMUST00000026125	3.73619	2.32300
M400000863	NA	NM_133797	ENSMUST00000026989	1.58408	0.94635
M400000910	Septin 2 (NEDD5 protein)	NM_010891	ENSMUST00000027495	4.07524	2.33469
M400000965	Weakly similar to KH type splicing regulatory protein (Fragment)		ENSMUST00000055244	2.61184	1.45227
M400001037	S-adenosylhomocysteine hydrolase-like 1	NM_145542	ENSMUST00000029490	1.17429	0.40709
M400001095	Nuclear factor 1 A-type (Nuclear factor 1/A) (NF1-A) (CCAAT-box binding transcription factor)	NM_010905	ENSMUST00000030295	3.72828	1.73797
M400001197	NA		ENSMUST00000031862	3.48183	1.64415
M400001198	NA		ENSMUST00000072905	3.90824	2.14267
M400001394	Mouse fat 1 cadherin (Fragment)		ENSMUST00000034067	2.15168	0.74589
M400001635	NA	NM_054043	ENSMUST00000070847	1.59151	0.78610
M400001776	guanine nucleotide releasing protein x	NM_023900	ENSMUST00000036805	1.18022	0.99907
M400001825	midnolin	NM_021565	ENSMUST00000042057	1.05208	0.45884

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M400001884	NA	XM_203329	ENSMUST00000036952	1.61201	0.68918
M400002140	Transcription factor Dp-1 (E2F dimerization partner 1)		ENSMUST00000045045	1.30152	0.83627
M400002163	NA		ENSMUST00000079973	1.29421	0.78360
M400002322	high glucose-regulated protein 8	NM_145393	ENSMUST00000040654	2.16995	1.04521
M400002343	heparan sulfate 2-O-sulfotransferase 1		ENSMUST00000043325	1.39638	0.92296
M400002668	NA	NM_0010055(	ENSMUST00000043560	2.14384	1.29199
M400002728	NA		ENSMUST00000058233	1.20591	0.70209
M400002729	NA		ENSMUST00000057643	1.37949	0.68469
M400002865	NA		ENSMUST00000056948	1.76978	1.11846
M400002923	NA		ENSMUST00000053955	1.32044	0.97362
M400003074	NA	NM_023871	ENSMUST00000059660	2.59430	1.43545
M400003152	NA		ENSMUST00000063059	0.84709	1.04499
M400003253	NA	XM_136032	ENSMUST00000056162	1.62286	1.78536
M400003307	NA		ENSMUST00000061554	2.68861	2.03919
M400003390	microfibrillar-associated protein 1	NM_026220	ENSMUST00000056732	1.34015	0.34269
M400003478	NA	XM_485251	ENSMUST00000060134	1.27614	0.69699
M400003764	NA		ENSMUST00000061980	2.67620	1.52573
M400004073	NA	NM_013901	ENSMUST00000015467	3.25854	0.94365
M400004233	NA		ENSMUST00000064770	1.85289	0.39551
M400004236	NA		ENSMUST00000064785	1.32780	0.42652
M400004264	NA		ENSMUST00000063988	1.05040	1.03842
M400004288	Growth-arrest-specific protein 1 precursor (GAS-1)	NM_008086	ENSMUST00000065086	1.61729	0.25654
M400004473	Vitamin K-dependent gamma-carboxylase	NM_019802	ENSMUST00000065906	1.67866	0.85186
M400004482	Tumor necrosis factor-inducible protein TSG-6 precursor (TNF- stimulated gene 6 protein)	NM_009398	ENSMUST00000065927	2.23480	2.44960
M400004498	NA		ENSMUST00000066001	1.10563	0.79356
M400004560	Dual specificity protein phosphatase 7 (EC 3.1.3.48)	NM_153459	ENSMUST00000066312	2.15943	0.86205
M400004798	Microsomal signal peptidase 23 kDa subunit		ENSMUST00000067476	1.39793	1.04005
M400004876	CGG triplet repeat binding protein 1	NM_178647	ENSMUST00000067744	1.49928	0.57228
M400004935	SET protein (Phosphatase 2A inhibitor I2PP2A) (I-2PP2A) (Template activating factor I) (TAF-I)	NM_023871	ENSMUST00000067996	2.45598	1.36013
M400004949	Myeloid-associated differentiation marker (Myeloid up-regulated protein)	NM_016969	ENSMUST00000068052	2.43100	1.12394
M400005175	Transcription factor Maf (Proto-oncogene c-maf)		ENSMUST00000069009	3.00218	0.69942
M400005232	NA	NM_007497	ENSMUST00000054770	1.26309	0.95510
M400005290	NA	NM_177836	ENSMUST00000069502	2.92402	2.09940
M400005455	Ras-related protein Rab-7	NM_009005	ENSMUST00000070298	1.86197	0.73465
M400005528	CCAAT/enhancer binding protein beta (C/EBP beta) (Interleukin-6- dependent binding protein)	NM_009883	ENSMUST00000070642	4.15121	1.74069
M400005534	NADH-ubiquinone oxidoreductase chain 4L		ENSMUST00000070674	1.93061	0.60880
M400005550	NA		ENSMUST00000070771	3.51086	2.32295
M400005551	NA		ENSMUST00000070776	1.66116	0.92832
M400005658	Poly(rC)-binding protein 2 (Alpha-CP2) (Putative heterogeneous nuclear ribonucleoprotein X) (hnRNP X)		ENSMUST00000023811	2.51872	1.99364

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M400005941	Proteasome subunit beta type 3 (EC 3.4.25.1) (Proteasome theta chain)		ENSMUST00000063203	1.16559	0.80315
M400005986	tripartite motif protein 30-like	NM_199146	ENSMUST00000033211	1.02897	0.92704
M400006200	NA		ENSMUST00000072359	1.15445	0.75739
M400006282	Ubiquitin-conjugating enzyme E2 E2 (EC 6.3.2.19) (Ubiquitin-protein ligase E2)	NM_144839	ENSMUST00000076133	1.52433	1.07616
M400006402	NA		ENSMUST00000079808	1.96618	1.23627
M400007498	NA		ENSMUST00000064522	2.96416	-0.08722
M400007551	NA		ENSMUST00000072300	1.60739	0.95799
M400008005	NA	XM_484103	ENSMUST00000081215	0.08702	1.17227
M400008043	NA	NM_010439	ENSMUST00000074307	3.73559	2.13945
M400008071	Cyclin I	NM_017367	ENSMUST00000058550	4.15282	2.22040
M400008081	NA		ENSMUST00000079188	2.21594	1.37214
M400008261	NA	XM_486667	ENSMUST00000071581	2.76273	2.12115
M400008480	NA	NM_175416	ENSMUST00000072496	0.67826	1.07250
M400008505	High mobility group protein 1 (HMG-1) (Amphoterin) (Heparin-binding protein p30)	XM_484795	ENSMUST00000074738	1.97181	1.48666
M400008541	NA		ENSMUST00000077348	2.26208	1.55673
M400008611	Eukaryotic initiation factor 4A-I (eIF4A-I)		ENSMUST00000075434	1.22574	0.88125
M400008643	Chromobox protein homolog 3 (Heterochromatin protein 1 homolog gamma)		ENSMUST00000081455	3.70609	1.76494
M400008677	Putative RNA-binding protein 3 (RNA binding motif protein 3)	XM_485004	ENSMUST00000071901	1.57004	1.17615
M400008984	Poly(rC)-binding protein 2 (Alpha-CP2) (Putative heterogeneous nuclear ribonucleoprotein X) (hnRNP X)	NM_011042	ENSMUST00000078404	5.63786	3.43689
M400009064	Similar to 60S ribosomal protein L30 isolog.	NM_198609	ENSMUST00000066378	2.58609	1.94211
M400009098	Transcription factor 4 (Immunoglobulin transcription factor 2) (ITF-2) (MITF-2) (SL3-3 enhancer factor 2)		ENSMUST00000082063	1.25879	0.56560
M400009143	Transcription factor 7-like 2 (HMG box transcription factor 4) (T- cell-specific transcription factor 4)		ENSMUST00000041717	2.20572	1.47930
M400009294	NA	NM_027453	ENSMUST00000030297	1.15832	0.80732
M400009413	olfactory receptor 458; olfactory receptor MOR257-4	NM_146444	ENSMUST00000082332	5.09486	-0.07563
M400009632	Similar to 60S ribosomal protein L30 isolog		ENSMUST00000034738	3.09211	2.28370
M400009657	RNA binding motif, single stranded interacting protein 3; RNA-binding protein RBMS3		ENSMUST00000044901	0.79939	1.59290
M400009834	guanine nucleotide releasing protein x		ENSMUST00000067576	1.28893	1.34737
M400010118	Tapasin precursor (TPSN) (TPN) (TAP-binding protein)		ENSMUST00000079128	2.06102	1.24506
M400010139	high mobility group nucleosomal binding domain 3 isoform HMGN3b; HMGN3a,HMGN3b,TRIP7		ENSMUST00000082001	2.22077	1.34400
M400010198	Transcription factor 7-like 2 (HMG box transcription factor 4) (T- cell-specific transcription factor 4)		ENSMUST00000073670	2.27665	1.06289
M400010331	Nuclear factor 1 A-type (Nuclear factor 1/A) (NF1-A) (NFI-A) (NF-I/A) (CCAAT-box binding transcription factor)		ENSMUST00000063932	3.31989	1.61921
M400010661	Glucocorticoid-induced leucine zipper protein		ENSMUST00000033807	0.89813	1.45716
M400010745	Chromobox protein homolog 3 (Heterochromatin protein 1 homolog gamma)	NM_007624	ENSMUST00000081455	3.80510	1.86406
M400010838	NA	NM_008538		4.24146	1.53382
M400010859	Platelet-activating factor acetylhydrolase IB beta subunit (EC 3.1.1.47) (PAF acetylhydrolase 30 kDa subu	NM_008775	ENSMUST00000003215	1.56707	0.66642
M400010861	Platelet-derived growth factor, A chain precursor (PDGF A-chain)	NM_008808	ENSMUST00000076095	2.90931	1.64013
M400010872	Prothymosin alpha (Fragment)	NM_008972	ENSMUST00000045897	1.70338	0.99295
M400010880	Recombining binding protein suppressor of hairless (J kappa- recombination signal binding protein)	NM_009035	ENSMUST00000037618	1.40263	0.53525
M400010896	secreted frizzled-related sequence protein 2; stromal cell derived factor 5	NM_009144	ENSMUST00000029625	2.98685	1.46042

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M400010899	NA	NM_009155		1.48122	0.61920
M400010912	NA	NM_009238		3.51475	1.18607
M400010924	Tapasin precursor (TPSN) (TPN) (TAP-binding protein) (TAP-associated protein)	NM_009318	ENSMUST00000079128	2.42158	1.25650
M400010926	Transcription factor 7-like 2 (HMG box transcription factor 4) (T- cell-specific transcription factor 4)	NM_009333	ENSMUST00000041717	2.40444	1.34156
M400010928	Transcription factor Dp-1 (E2F dimerization partner 1) (DRTF1- polypeptide-1)	NM_009361	ENSMUST00000045045	1.19665	0.93502
M400010929	NA	NM_009378		0.38658	1.01996
M400011046	Insulin-like growth factor II precursor (Multiplication stimulating polypeptide) (IGF-II)	NM_010514	ENSMUST00000000033	1.31936	1.23164
M400011065	NA	NM_010743		1.01134	1.16396
M400011104	Paired mesoderm homeobox protein 1 (PRX-1) (Homeobox protein MhoX)	NM_011127	ENSMUST00000027878	2.26763	0.97519
M400011129	Splicing factor, arginine/serine-rich 2 (Splicing factor SC35)	NM_011358	ENSMUST00000072341	1.22675	1.07098
M400011134	NA	NM_011406		1.66733	1.44222
M400011137	NA	NM_011435		1.01916	0.85559
M400011139	Transcription factor SOX-9	NM_011448	ENSMUST00000000579	3.41271	1.88818
M400011147	Transcription factor AP-2 alpha (AP2-alpha) (Activating enhancer- binding protein 2 alpha)	NM_011547	ENSMUST00000080429	3.27804	2.03441
M400011157	NA	NM_011657		1.00729	0.74507
M400011182	NA	NM_011971		0.80313	1.06856
M400011214	NA	NM_013685		1.63088	0.60217
M400011239	Zinc finger homeobox protein 1b (Smad interacting protein 1)	NM_015753	ENSMUST00000028229	1.44577	0.85325
M400011333	NA	NM_019734		1.09750	1.07400
M400011334	Cop-coated vesicle membrane protein p24 precursor (p24A) (Sid 394)	NM_019770	ENSMUST00000060226	1.82799	1.07930
M400011345	NA	NM_019929		2.20301	1.47210
M400011390	NA	NM_021389		1.05198	0.46740
M400011409	NA	NM_022314	ENSMUST00000072359	1.36167	0.58041
M400011463	NA	NM_024431		1.48658	0.86118
M400011467	NA	NM_024473	ENSMUST00000019276	1.15753	0.99246
M400011488	NA	NM_025480	ENSMUST00000078838	0.32299	1.08538
M400011681	NA	NM_029595		0.26494	1.09583
M400011722	NA	NM_031249		1.96110	0.88033
M400011724	general transcription factor II A, 1 isoform 2; general transcription factor Iia	NM_031391	ENSMUST00000021345	1.77122	0.83164
M400011821	transmembrane 9 superfamily protein member 4	NM_133847	ENSMUST00000077063	1.05533	0.83813
M400011828	F-box/LRR-repeat protein 14 (F-box and leucine-rich repeat protein 14)	NM_133940	ENSMUST00000032094	1.45181	1.32815
M400011924	Periphilin 1	NM_146062	ENSMUST00000049122	1.74354	1.39191
M400012207	Copine I	NM_170588	ENSMUST00000079312	1.08344	0.83935
M400012212	NA	NM_172116		2.14635	1.80546
M400012309	NA	NM_175395		1.33740	1.14608
M400012319	NA	NM_175553		1.26631	0.44591
M400012431	NA	NM_177730	ENSMUST00000080019	1.92428	1.00032
M400012447	NA	NM_177994	ENSMUST00000045628	2.32524	1.32924
M400012549	NA	NM_183258		1.07398	1.44444

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M400012644	tryptophan rich basic protein	NM_207301	ENSMUST00000023913	1.20680	1.47977
M400012692	F-box/LRR-repeat protein 14 (F-box and leucine-rich repeat protein 14)	NM_025356		1.83567	1.43992
M400012693	NA			1.57359	0.46974
M400012790	NA			4.51718	2.14299
M400012854	NA			4.34394	2.14978
M400012946	NA			3.55885	2.55151
M400012965	NA			1.02565	0.65547
M400012986	NA			2.02838	1.02861
M400013004	NA			1.78164	1.28937
M400013013	NA			1.78614	1.35070
M400013025	NA			1.41672	0.83176
M400013099	NA			2.06788	1.23607
M400013178	NA			1.23485	1.02531
M400013217	NA			1.22048	0.49685
M400013253	NA			1.26814	1.01440
M400013265	NA			1.73656	1.52532
M400013287	NA			1.46995	0.84259
M400013314	NA	NM_021565		1.40456	0.72312
M400013327	NA			1.45973	1.07997
M400013372	NA			1.38096	0.78202
M400013580	NA			1.20101	1.14561
M400013581	NA			1.39027	0.84958
M400013582	NA	NM_008408		1.52965	1.49303
M400013620	NA			1.12455	1.46421
M400013621	NA			1.05968	0.42062
M400013626	NA			0.52542	0.99135
M400013705	NA			1.09067	0.95263
M400013772	NA			1.75274	1.26450
M400013796	NA			3.08087	1.82670
M400013845	NA			1.61034	1.86381
M400013951	NA			2.77269	2.60272
M400013970	NA			1.31110	0.93604
M400014072	NA			1.67382	0.51714
M400014359	NA			3.65108	3.06173
M400014412	NA			0.63713	1.19275
M400014437	NA			0.00632	1.06191
M400014568	NA			1.63471	0.88641
M400014570	NA	XM_132579		2.42434	1.56573
M400014572	NA			1.20412	0.72128

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time	Enrichment Score 30Min
M400014715	NA			1.55727	1.47593
M400015007	NA			2.86100	1.53742
M400015034	NA			0.99951	0.66854
M400015052	NA			1.18723	0.60649
M400015103	NA			1.00261	0.93636
M400015473	NA	NM_025872		2.64561	1.90037
M400015684	NA			2.50894	1.54505
M400015754	NA			1.23379	0.76574
M400015846	NA			1.45756	0.56762
M400015998	NA			2.45256	-0.16677
M400016015	NA			1.21293	1.22468
M400016041	NA			1.40166	1.04426
M400016046	NA			0.31309	0.99891
M400016150	NA			-0.30146	3.81860
M400016182	NA			4.18094	2.57467
M400016183	NA			2.34246	0.62913
M400016386	NA			0.98851	0.62281
M400016461	NA			2.65946	0.25798
M400017308	NA			1.05263	0.97965
M400017682	NA			1.10769	0.99786
M400017954	NA			7.47321	0.19759
M400018177	NA			-0.32441	2.21608
M400018934	NA			-0.13150	1.72177
M400018959	NA			4.26100	-0.81059
M400019378	NA			1.43945	1.76727
M400019404	NA			-0.27565	1.19038
M400019412	NA			1.82881	1.21239

Appendix B - HuR Ligands Unique to 0 Time Post Induction of Differentiation

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time
M200006912	26S proteasome non-ATPase regulatory subunit 9 (26S proteasome regulatory subunit p27).	NM_026000	ENSMUST00000060664	1.00
M300007813	Acid ceramidase precursor (EC 3.5.1.23) (Acylsphingosine deacylase)		ENSMUST00000034000	1.57
M200007637	actin related protein 2/3 complex, subunit 1A; actin related protein 2/3 complex, subunit 1A	NM_019767	ENSMUST00000031625	1.23
M200013059	Adiponectin receptor protein 1	NM_028320	ENSMUST00000027727	1.25
M200002183	amyloid beta (A4) precursor protein-binding, family B, member 2	NM_009686	ENSMUST00000068206	1.31
M300008017	Amyloid-like protein 2 precursor (CDEI-box binding protein) (CDEBP)	NM_009691	ENSMUST00000072634	1.12
M200009421	ARP2/3 complex 20 kDa subunit (p20-ARC) (Actin-related protein 2/3 complex subunit 4)	NM_026552	ENSMUST00000032412	1.68
M300003601	ATP synthase oligomycin sensitivity conferral protein, mitochondrial precursor (EC 3.6.3.14) (OSCP)	NM_138597	ENSMUST00000023677	2.85
M300003876	Band 4.1-like protein 3 (4.1B) (Differentially expressed in adenocarcinoma of the lung protein 1)	NM_013813	ENSMUST00000077529	1.70
M200015439	Beta-1,4-galactosyltransferase 5 (EC 2.4.1.-) (Beta-1,4-GalTase 5) (Beta4Gal-T5) (b4Gal-T5)	NM_019835	ENSMUST00000018073	1.09
M300008745	Beta-2-microglobulin precursor.	NM_009735	ENSMUST00000080804	1.72
M200006908	Beta-2-syntrophin (59 kDa dystrophin-associated protein A1, basic component 2)	NM_009229	ENSMUST00000047425	1.04
M200009460	bromodomain containing 4 isoform 1; bromodomain-containing 5; bromodomain-containing 4.	NM_020508	ENSMUST00000003726	1.14
M200004308	calponin 3, acidic	NM_028044	ENSMUST00000029773	1.31
M200000746	Calreticulin precursor (CRP55) (Calregulin) (HACBP) (ERp60)		ENSMUST00000003912	1.25
M200014007	Casein kinase I, alpha isoform (EC 2.7.1.-) (CKI-alpha) (CK1)	NM_146087	ENSMUST00000025469	1.31
M300008433	Casein kinase II, alpha chain (CK II) (EC 2.7.1.37).	NM_007788	ENSMUST00000041081	1.95
M300003763	Cation-independent mannose-6-phosphate receptor precursor (CI Man-6-P receptor) (CI-MPR)	NM_010515	ENSMUST00000024599	1.34
M200005924	Caveolin-1	NM_007616	ENSMUST00000007799	1.10
M200000084	Cellular tumor antigen p53 (Tumor suppressor p53)	NM_011640	ENSMUST00000005371	1.85
M400004876	CGG triplet repeat binding protein 1	NM_178647	ENSMUST00000067744	1.50
M200005696	coiled-coil transcriptional coactivator; GRIP1-interacting protein	NM_026192	ENSMUST00000023818	1.10
M200004448	Collagen alpha 1(I) chain precursor.	NM_007742	ENSMUST00000001547	1.18
M400012207	Copine I	NM_170588	ENSMUST00000079312	1.08
M400000579	CREB-binding protein (EC 2.3.1.48)	XM_358750	ENSMUST00000023165	2.16
M400000637	Cyclic-AMP-dependent transcription factor ATF-1		ENSMUST00000023769	1.16
M200002663	Cyclin-dependent kinase inhibitor 1B (Cyclin-dependent kinase inhibitor p27) (p27Kip1)	NM_009875	ENSMUST00000067327	1.06
M300007781	dCMP deaminase.	NM_178788	ENSMUST00000033966	1.11
M300006266	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit precursor	NM_007838	ENSMUST00000030538	1.50
M400004560	Dual specificity protein phosphatase 7 (EC 3.1.3.48)	NM_153459	ENSMUST00000066312	2.16
M200015503	dynactin 4	NM_026302	ENSMUST00000025505	1.17
M200012044	Early growth response protein 1 (EGR-1) (Krox-24 protein) (ZIF268)	NM_007913	ENSMUST00000064795	1.01
M200001148	ECT2 protein (Epithelial cell transforming sequence 2 oncogene)	NM_007900	ENSMUST00000029248	5.70
M200013439	EIB-55kDa associated protein 5.	NM_144922	ENSMUST00000043765	1.26
M400008611	Eukaryotic initiation factor 4A-I (eIF4A-I)		ENSMUST00000075434	1.23
M200012720	Eukaryotic translation initiation factor 5A (eIF-5A) (eIF-4D) (Rev- binding factor)	NM_181582	ENSMUST00000043419	1.11
M300008358	folliculin.	NM_146018	ENSMUST00000047706	1.27

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time
M300003525	Follistatin-related protein 1 precursor (Follistatin-like 1) (TGF- beta-inducible protein TSC-36)	NM_008047	ENSMUST00000023511	2.21
M400011724	general transcription factor II A, 1 isoform 2; general transcription factor Iia	NM_031391	ENSMUST00000021345	1.77
M200003853	Glutathione reductase, mitochondrial precursor (EC 1.8.1.7) (GR) (GRase)	NM_010344	ENSMUST00000033992	1.71
M300002374	Growth factor receptor-bound protein 2 (GRB2 adapter protein) (SH2/SH3 adapter GRB2)	NM_008163	ENSMUST00000077165	1.07
M400004288	Growth-arrest-specific protein 1 precursor (GAS-1)	NM_008086	ENSMUST00000065086	1.62
M400001776	guanine nucleotide releasing protein x	NM_023900	ENSMUST00000036805	1.18
M400002343	heparan sulfate 2-O-sulfotransferase 1		ENSMUST00000043325	1.40
M400000481	Heterogeneous nuclear ribonucleoprotein K		ENSMUST00000058184	1.02
M300010273	Histone H1.2 (H1 VAR.1) (H1C)	NM_015786	ENSMUST00000040914	1.46
M200011942	Hsc70-interacting protein (Hip) (Putative tumor suppressor ST13)	NM_133726	ENSMUST00000023039	1.09
M200001048	Insulin-like growth factor IB precursor (IGF-IB) (Somatomedin)	NM_010512	ENSMUST00000075330	1.09
M200015000	kelch repeat and BTB (POZ) domain containing 2; cDNA sequence BC022962	NM_145958	ENSMUST00000081115	1.49
M300010092	Lactoylglutathione lyase (EC 4.4.1.5) (Methylglyoxalase) (Aldoketomutase) (Glyoxalase I)	NM_025374	ENSMUST00000044467	1.02
M300012485	Large neutral amino acids transporter small subunit 1 (L-type amino acid transporter 1)	NM_011404	ENSMUST00000045557	1.39
M200009548	lobe homolog-like; lobe homolog-like (Drosophila); lobe homolog (Drosophila)	NM_026270	ENSMUST00000054343	1.20
M200006820	Lysosomal-associated transmembrane protein 4A (Golgi 4-transmembrane spanning transporter)	NM_008640	ENSMUST00000020909	1.78
M400003390	microfibrillar-associated protein 1	NM_026220	ENSMUST00000056732	1.34
M300005240	Microsomal glutathione S-transferase 3 (EC 2.5.1.18) (Microsomal GST- 3)	NM_025569	ENSMUST00000028005	1.14
M400001825	midnolin	NM_021565	ENSMUST00000042057	1.05
M200013802	MLN64 N-terminal domain homolog (STARD3 N-terminal like protein)	NM_024270	ENSMUST00000039694	1.44
M400001394	Mouse fat 1 cadherin (Fragment)		ENSMUST00000034067	2.15
M200009372	Muscleblind-like protein (Triplet-expansion RNA-binding protein)	NM_020007	ENSMUST00000029327	1.16
M200012692	myosin regulatory light chain-like.	NM_023402	ENSMUST00000038446	1.22
M400005534	NADH-ubiquinone oxidoreductase chain 4L		ENSMUST00000070674	1.93
M300012230	NCAM	NM_010875	ENSMUST00000053131	1.32
M200002789	Nuclear factor 1 X-type (Nuclear factor 1/X)(CCAAT-box binding transcription factor)	NM_010906	ENSMUST00000077717	2.15
M200001081	Nucleolar transcription factor 1 (Upstream binding factor 1) (UBF-1)	NM_011551	ENSMUST00000079589	1.01
M300000896	ocular development associated gene	NM_026033	ENSMUST00000007559	1.09
M400009413	olfactory receptor 458; olfactory receptor MOR257-4	NM_146444	ENSMUST00000082332	5.09
M300008081	Oligosaccharyl transferase STT3 subunit homolog (B5) (Integral membrane protein 1)	NM_008408	ENSMUST00000034627	1.25
M400011104	Paired mesoderm homeobox protein 1 (PRX-1) (Homeobox protein MhoX)	NM_011127	ENSMUST00000027878	2.27
M400010859	Platelet-activating factor acetylhydrolase IB beta subunit (EC 3.1.1.47) (PAF acetylhydrolase 30 kDa subu	NM_008775	ENSMUST00000003215	1.57
M300002564	Poly(A) polymerase alpha (EC 2.7.7.19) (PAP)	NM_011112	ENSMUST00000021535	1.26
M300000501	polymerase I and transcript release factor		ENSMUST00000044938	1.18
M200015255	potassium channel, subfamily K, member 5	NM_021542	ENSMUST00000024011	1.07
M200009955	Probable ATP-dependent RNA helicase p54 (Oncogene RCK homolog)	NM_007841	ENSMUST00000034608	1.40
M400005941	Proteasome subunit beta type 3 (EC 3.4.25.1) (Proteasome theta chain)		ENSMUST00000063203	1.17
M200002348	protein tyrosine phosphatase 4a2	NM_008974	ENSMUST00000030578	1.02
M200001637	Ran-binding protein 10	NM_145824	ENSMUST00000041400	1.27

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time
M200015033	Ras-related protein Rab-14	NM_026697	ENSMUST00000028238	1.13
M300002017	Ras-related protein Rab-21 (Rab-12)	NM_024454	ENSMUST00000020343	1.02
M400005455	Ras-related protein Rab-7	NM_009005	ENSMUST00000070298	1.86
M300010609	Ras-related protein Rap-2b	NM_028712	ENSMUST00000049064	1.20
M200014215	Raver1	NM_027911	ENSMUST00000010349	1.19
M200008896	RE1-silencing transcription factor	NM_011263	ENSMUST00000080359	1.88
M400010880	Recombining binding protein suppressor of hairless (J kappa- recombination signal binding protein)	NM_009035	ENSMUST00000037618	1.40
M300012013	Recombining binding protein suppressor of hairless (J kappa- recombination signal binding protein)		ENSMUST00000037618	1.21
M300011580	Reelin precursor (EC 3.4.21.-) (Reeler protein)	NM_011261	ENSMUST00000062372	4.30
M200015349	Rho-related BTB domain-containing protein 3.	NM_028493	ENSMUST00000022078	2.18
M300008124	ring finger 111	NM_033604	ENSMUST00000034739	1.04
M200014251	ring finger protein 38.	NM_175201	ENSMUST00000045793	1.31
M200012509	Serine/threonine protein phosphatase 4 catalytic subunit (EC 3.1.3.16) (PP4C) (Pp4)	NM_019674	ENSMUST00000032936	1.09
M200014111	Sestrin 3	NM_030261	ENSMUST00000034507	1.56
M300013796	SHC transforming protein 1 (SH2 domain protein C1)	NM_011368	ENSMUST00000039110	1.97
M200012643	Signal peptide peptidase-like 3 (EC 3.4.99.-) (SPP-like 3 protein)	XM_485673	ENSMUST00000031530	1.45
M300011013	spermatogenesis associated, serine-rich 2; serine-rich spermatocytes and round spermatid protein		ENSMUST00000063517	1.56
M300004390	Splicing factor 1 (Zinc finger protein 162) (Transcription factor ZFM1) (mZFM)		ENSMUST00000076351	1.23
M300005193	Sterol O-acyltransferase 1 (EC 2.3.1.26) (Cholesterol acyltransferase 1)	NM_009230	ENSMUST00000051396	1.32
M300000858	Superoxide dismutase [Mn], mitochondrial precursor (EC 1.15.1.1)	NM_013671	ENSMUST00000007012	1.28
M200014231	suppressor of Ty 16 homolog; suppressor of Ty 16 homolog (S.cerevisiae).	NM_033618	ENSMUST00000046709	1.06
M200006351	T-complex associated-testis-expressed 1-like (Protein 91/23)	NM_025975	ENSMUST00000033519	1.69
M200003903	Testis-specific Y-encoded-like protein 1 (TSPY-like 1)	NM_009433	ENSMUST00000061372	1.38
M400009098	Transcription factor 4 (Immunoglobulin transcription factor 2) (ITF-2) (MITF-2) (SL3-3 enhancer factor 2)		ENSMUST00000082063	1.26
M300004412	Transcription factor 7-like 2 (HMG box transcription factor 4) (T- cell-specific transcription factor 4)		ENSMUST00000075651	1.28
M400002140	Transcription factor Dp-1 (E2F dimerization partner 1)		ENSMUST00000045045	1.30
M400010928	Transcription factor Dp-1 (E2F dimerization partner 1) (DRTF1- polypeptide-1)	NM_009361	ENSMUST00000045045	1.20
M200000424	Transcription factor jun-D	NM_010592	ENSMUST00000061259	2.32
M400005175	Transcription factor Maf (Proto-oncogene c-maf)		ENSMUST00000069009	3.00
M200002879	Transcriptional regulator ATRX (X-linked nuclear protein) (Heterochromatin protein 2)	NM_009530	ENSMUST00000033580	1.03
M200001143	Transmembrane 9 superfamily protein member 3 precursor	NM_133352	ENSMUST00000025989	1.47
M400011821	transmembrane 9 superfamily protein member 4	NM_133847	ENSMUST00000077063	1.06
M400005986	tripartite motif protein 30-like	NM_199146	ENSMUST00000033211	1.03
M300004437	Tripartite motif protein 8 (RING finger protein 27) (Glioblastoma- expressed RING finger protein)	NM_053100	ENSMUST00000026008	1.44
M300000203	Tubulin beta-5 chain	NM_011655	ENSMUST00000001566	1.26
M300003298	Twinfilin 1 (A6 protein) (Protein tyrosine kinase 9)	NM_008971	ENSMUST00000023087	2.27
M300011994	Ubiquitin-conjugating enzyme E2 H (EC 6.3.2.19) (Ubiquitin-protein ligase H)	NM_009459	ENSMUST00000036826	1.29
M400004473	Vitamin K-dependent gamma-carboxylase	NM_019802	ENSMUST00000065906	1.68
M200001010	WD-repeat protein 1 (Actin interacting protein 1) (AIP1)	NM_011715	ENSMUST00000005234	1.29

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time
M400011239	Zinc finger homeobox protein 1b (Smad interacting protein 1)	NM_015753	ENSMUST00000028229	1.45
M400018959	NA			4.26
M400015998	NA			2.45
M300009180	NA	NM_177093	ENSMUST00000078717	1.12
M400007498	NA		ENSMUST00000064522	2.96
M300016892	NA	XM_131400	ENSMUST00000062977	1.03
M400017954	NA			7.47
M400016461	NA			2.66
M300012949	NA		ENSMUST00000038893	1.53
M300008920	NA	NM_198625	ENSMUST00000052457	1.29
M400004233	NA		ENSMUST00000064770	1.85
M400013621	NA			1.06
M400004236	NA		ENSMUST00000064785	1.33
M300019000	NA	NM_172871	ENSMUST00000079544	1.35
M400012319	NA	NM_175553		1.27
M400011390	NA	NM_021389		1.05
M400012693	NA			1.57
M400013217	NA			1.22
M200005082	NA	NM_145466	ENSMUST00000038075	1.10
M400000149	NA		ENSMUST00000008286	1.01
M400014072	NA			1.67
M400000721	NA	NM_198937	ENSMUST00000024981	1.18
M300013817	NA	NM_172943	ENSMUST00000044250	2.45
M400015846	NA			1.46
M400011409	NA	NM_022314	ENSMUST00000072359	1.36
M300008360	NA	XM_194358	ENSMUST00000036372	1.06
M400011214	NA	NM_013685		1.63
M400015052	NA			1.19
M400010899	NA	NM_009155		1.48
M400016183	NA			2.34
M200014565	NA		ENSMUST00000006851	1.13
M400012965	NA			1.03
M300013961	NA	NM_028627	ENSMUST00000041391	1.10
M300003424	NA	XM_156257	ENSMUST00000023313	1.04
M400002729	NA		ENSMUST00000057643	1.38
M400001884	NA	XM_203329	ENSMUST00000036952	1.61
M400003478	NA	XM_485251	ENSMUST00000060134	1.28
M400002728	NA		ENSMUST00000058233	1.21
M200013650	NA	NM_028058	ENSMUST00000026016	1.23

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 0 Time
M400014572	NA			1.20
M400013314	NA	NM_021565		1.40
M400011157	NA	NM_011657		1.01
M400006200	NA		ENSMUST00000072359	1.15
M400015754	NA			1.23
M400013372	NA			1.38
M400002163	NA		ENSMUST00000079973	1.29
M400001635	NA	NM_054043	ENSMUST00000070847	1.59
M300022233	NA	NM_029519	ENSMUST00000062117	1.27
M400004498	NA		ENSMUST00000066001	1.11
M400000339	NA	NM_178606	ENSMUST00000020023	1.29
M400009294	NA	NM_027453	ENSMUST00000030297	1.16
M200015881	NA	NM_183186	ENSMUST00000046859	1.79
M400013025	NA			1.42
M300011779	NA	NM_145943	ENSMUST00000038091	1.13
M400013287	NA			1.47
M400013581	NA			1.39
M400011137	NA	NM_011435		1.02
M400011463	NA	NM_024431		1.49
M300001785	NA	NM_007590	ENSMUST00000019514	1.63
M300001683	NA	XM_126426	ENSMUST00000018620	1.54
M400011722	NA	NM_031249		1.96
M400014568	NA			1.63
M400005551	NA		ENSMUST00000070776	1.66
M400013970	NA			1.31
M400015103	NA			1.00
M300019706	NA	NM_198016	ENSMUST00000061490	1.40
M400004073	NA	NM_013901	ENSMUST00000015467	3.26
M400000863	NA	NM_133797	ENSMUST00000026989	1.58
M400013705	NA			1.09
M400005232	NA	NM_007497	ENSMUST00000054770	1.26
M400007551	NA		ENSMUST00000072300	1.61
M400002923	NA		ENSMUST00000053955	1.32
M300007474	NA	NM_138589	ENSMUST00000033158	1.40
M400000143	NA		ENSMUST00000065744	2.11
M400017308	NA			1.05
M300020249	NA	XM_148582	ENSMUST00000081933	1.33

Appendix C- HuR Ligands Unique to 30min Post Induction of Differentiation

Probe ID	Gene	Ref Seq NCBI	Ensembl Transcript ID	Enrichment Score 30 Min	Biological Process
M300000714	breast carcinoma amplified sequence 2	NM_026602	ENSMUST00000005830	1.15	
M200005936	Cystinosin	NM_031251	ENSMUST00000006103	1.22	Transport, glycoprotein
M200001516	Eukaryotic translation initiation factor 4E (eIF4E)	NM_007917	ENSMUST00000029803	1.04	Protein Biosynthesis, RNA binding
M200003203	Forkhead box protein C1 (Forkhead-related protein FKHL7)	NM_008592	ENSMUST00000062292	1.01	Transcription regulation, DNA
M400010661	Glucocorticoid-induced leucine zipper protein	NP_034416.3	ENSMUST00000033807	1.46	Transcription Factor, Anti-apoptotic
M200005292	Homeobox protein Hox-D9 (Hox-4.4) (Hox-5.2)	NM_013555	ENSMUST00000059272	1.00	Transcription regulation, DNA
M300000642	Krueppel-like factor 5 (Intestinal-enriched krueppel-like factor) (BTEB2)	NM_009769	ENSMUST00000005279	1.25	Transcription regulation, DNA
M200000511	MAD protein (MAX dimerizer)	NM_010751	ENSMUST00000001184	1.07	Transcription regulation, DNA
M200000288	Mago nashi protein homolog	NM_010760	ENSMUST00000030348	1.00	RNA binding protein
M300000373	MLN64 N-terminal domain homolog (STARD3 N-terminal like protein)	NP_077232.2.	ENSMUST00000039694,ENS	1.08	Transmembrane protein
M200013007	NA - AC140307.3-201	XM_912399,	ENSMUST00000025675	1.19	na
M400003152	NA - AC158928.6-201	-	ENSMUST00000063059	1.04	retrotransposed,no protein prdt
M400008005	NA - Adipocyte P27 protein LOC432621	XM_484103	ENSMUST00000081215	1.17	-
M400014412	NA - calmodulin binding transcription activator 1 (camta1)	NM_001081557.1	ENSMUSG00000014592	1.19	Transcription regulation, activator
M200007523	NA - endoplasmic reticulum-golgi intermediate compartment (ERGIC)	NM_026170	ENSMUST00000001619	1.18	Vesicle-mediated transport, ER-
M200001014	NA - hypothetical protein LOC668661 Putative uncharacterized protein	NM_025880	ENSMUST00000071296	1.00	na
M300004860	NA - hypothetical protein LOC67099 (Fam119a)	NM_025964	ENSMUST00000053469	1.12	Transmembrane protein
M200007942	NA - IKK interacting protein isoform 1	NM_026166	ENSMUST00000020149	1.39	Induction of Apoptosis, protein
M300013540	NA - major facilitator superfamily domain containing 4	NM_172510	ENSMUST00000046658	1.51	Transmembrane protein, transport
M400011681	NA - phosphatidylethanolamine binding protein 2 (Pbp2)	NM_029595	ENSMUSG00000047104	1.10	
M400011182	NA - proteasome (prosome, macropain) subunit, beta type 3 (Psmb3)	NM_011971	ENSMUSG00000069744	1.07	hydrolase, threonine protease
M300004178	NA - protein phosphatase 2, regulatory subunit B (PR 52), beta isoform (Ppp2r2b)	NM_028392	ENSMUST00000025377	2.08	Apoptosis, signal transduction
M200005125	NA - RWD domain containing 4A	NM_203507	ENSMUST00000033973	1.13	na
M200006807	NA - small subunit of serine palmitoyltransferase A	NM_134054	ENSMUST00000056228	1.02	Transmembrane protein
M400010929	NA - thrombomodulin (Thbd)	NM_009378	ENSMUSG00000074743	1.02	Calcium ion Binding, Blood
M400011488	NA - transmembrane protein 128 (Tmem128)	NM_025480	ENSMUST00000078838,ENS	1.09	Transmembrane protein
M200013777	NA - Yip1 domain family, member 4 (Yipf4)	NM_026417	ENSMUST00000024874,ENS	1.35	Transmembrane protein
M300004807	Platelet-derived growth factor, A chain precursor (PDGF A-chain) (PDGF-1)	NP_032834.1	ENSMUST00000046901	1.20	Growth factor, mitogen,
M200002099	Polyomavirus enhancer activator 3 (PEA3 protein) (ETS translocation variant 4)	NM_008815	ENSMUST00000017868	1.19	Transcription regulation, DNA
M300003535	RAB, member of RAS oncogene family-like 3	NM_026297	ENSMUST00000023524	1.67	GTP-binding
M400009657	RNA binding motif, single stranded interacting protein 3; RNA-binding protein RBMS3	NM_178660	ENSMUST00000044901	1.59	RNA binding protein
M300001921	Serine/threonine-protein kinase Sgk1	NM_011361	ENSMUST00000020145	1.24	Apoptosis, Protein Binding, Protein
M200008310	Transcription elongation factor A (SII)-like 1	NM_146236	ENSMUST00000055104	1.06	RNA Elongation, DNA binding,
M200008625	Tumor necrosis factor receptor superfamily member 10B precursor (Death receptor 5)	NM_020275	ENSMUST00000022663	1.15	Apoptosis, signal transduction
M200006649	UMP-CMP kinase (EC 2.7.4.14) (Cytidylate kinase) (Deoxycytidylate kinase)	NM_025647	ENSMUST00000030491	1.22	ATP binding, Prymidine biosynthesis
M200007317	Zinc finger and BTB domain containing protein 1	NM_178744	ENSMUST00000042779	1.21	Transcription regulation
M400000700	Zinc finger SCAN domain containing 10 (Zfp206)	NM_001033425	ENSMUSG00000023902	3.50	Transcription Factor
M400008480	NA	NM_175416	ENSMUST00000072496	1.07	
M400014437	NA			1.06	
M400016046	NA			1.00	
M400016150	NA			3.82	
M400018177	NA			2.22	
M400018934	NA			1.72	
M400019404	NA			1.19	

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