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# AEBP1: A Signaling Modulator of MAPK Activation for Adipogenesis

by

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Submitted in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
February, 2000

**DALHOUSIE UNIVERSITY**

**FACULTY OF GRADUATE STUDIES**

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "AEBP1: A Signaling Modulator of MAPK Activation of Adipogenesis"

by Sungwoo Kim

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dated: April 6, 2000

External Examiner

Research Supervisor

Examining Committee

DALHOUSIE UNIVERSITY

Date: August 10, 2000.

AUTHOR Sungwoo Kim

TITLE " AEBP1: A Signaling Modulator of MAPK Activation for Adipogenesis."

DEPARTMENT Biochemistry and Molecular Biology

DEGREE Ph.D. Convocation October Year 2000

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## Abbreviations

| | |
|---|---|
| ACLP | aortic carboxypeptidase-like protein |
| AE-1 | adipocyte enhancer-1 |
| AEBP1 | adipocyte enhancer binding protein 1 |
| aP2 | adipose P2 (422) gene |
| ARF6 | adipocyte regulatory factor 6 |
| BAT | brown adipose tissue |
| cAMP | cyclic adenosine 3',5'-monophosphate |
| CAT | chloramphenicol acetyltransferase |
| C/EBP | CCAAT/enhancer binding protein |
| CHO | Chinese hamster ovary |
| CMV | cytomegalovirus |
| DEX | dexamethasone |
| DLD | discoidin-like domain |
| EGF | epidermal growth factor |
| ERK | extracellular signal-regulating kinase |
| FABP | fatty acid-binding protein |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GPCR | G protein-coupled receptors |
| GLUT | glucose transporter |
| Grb2 | growth factor-bound protein 2 |
| Gγ5 | G protein γ subunit 5 |
| HMG | high mobility group protein |
| HSP27 | heat shock protein 27 |
| IGF-1 | insulin like-growth factor-1 |
| IRS1 | insulin receptor substrate 1 |
| JIP1 | JNK protein 1 |
| JNK/SAPK | c-Jun N-terminal kinase/ Stress-activated protein kinase |
| LTR | Long Terminal Repeat |

| | |
|---|---|
| MAPK | mitogen activated protein kinase |
| MAPKKK | mitogen activated protein kinase kinase kinase |
| MBP | myelin basic protein |
| MEK | MAPK/ERK kinase |
| MKP | MAPK phosphatase |
| MIX | 1-methyl-3-isobutylxanthine |
| MP1 | MEK partner 1 |
| NGF | nerve growth factor |
| PDGF | platelet derived growth factor |
| PGJ2 | prostaglandin J2 |
| PI3K | phosphoinositide 3-kinase |
| PPAR | peroxisome proliferator-activated receptor |
| Rb | retinoblastoma protein |
| RTK | receptor tyrosine kinase |
| SCD | steroyl-CoA desaturase |
| SH2/3 | Src-homology 2/3 |
| SHPTP1 | SH2 domain-containing tyrosine phosphatase |
| TNFα | tumor necrosis factor α |
| WAT | white adipose tissue |

## ABSTRACT

The activation of mitogen-activated protein kinase (MAPK) is a vital step in signaling cascades which regulate cell growth and cell differentiation. MAP kinase activation is a reversible process in which protein phosphatases play a crucial role in controlling cellular activities. Thus, the regulation of prolonged or transient MAP kinase activation may be through phosphatases rather than through the activity of MEK which is itself transiently activated by phosphorylation. In adipogenesis, a complex process in which multiple hormones and factors regulate the conversion of progenitor cells into adipocytes, MAPK activation inhibits the differentiation process. The regulatory mechanisms or the cellular factors that regulate the switch from growth to differentiation in the adipogenic lineage are largely unelucidated. Furthermore, AEBP1, a transcription factor that is down-regulated during adipogenesis, is now known to interact with MAPK.

In this thesis, I show that AEBP1 complexes with MAPK through its N-terminal domain. This interaction protects MAPK activity from dephosphorylation by its specific phosphatase and sustains MAPK activation. AEBP1 levels decrease during adipocyte differentiation in 3T3-L1 cells and as a result of this the associated MAPK decreases. Overexpression of AEBP1 blocks the adipocyte differentiation by enhancing MAP kinase activity whereas the inhibition of protective activity of endogenous AEBP1 stimulates the adipocyte differentiation. These results suggest that enhancement of MAPK activation by the protective effect of AEBP1 may constitute a critical part in the determination of either cell growth or differentiation in the adipogenic lineage. The proposed mode of action by which a transcription factor regulates MAPK activation is novel.

**Acknowledgments**

Thanks to my supervisor Dr. H.S. Ro for all his support, assistance, and encouragement to make this thesis possible. I would also thank his lab members: Dr. Gong Ping He, J.K. Park, Dr. Alexio Muise, Jing Zhang, for their friendship, assistance, and cooperation. Collaborative works (Figure 3.1 and 3.7) are presented in this thesis.

I would like to specially thank my supervisory committee and readers, Drs. C. Lazier, C. Too, N. Ridgeway, and N. Brewste, who have supported, guided, and taken extra time during the research. I would especially like to thank Drs. R. A. Graves, C. B. Lazier, C. R. McMaster, F. B. St. C. Palmer, and M. W. Nachtigal for critical reading of this thesis.

I would like to thank my wife and son for being always with me.

# Chapter 1. Introduction

## *1.I. Obesity and The Adipocyte*

### 1.I.A. Obesity and The Adipocyte

Obesity, the accumulation of an excessive amount of fat storage cells (known as adipocytes), is a major risk factor for heart disease, diabetes, high blood pressure, stroke, and some forms of cancer (Gregoire et al., 1998). Life style and societal pressures can play a significant role in obesity. However, it is now becoming evident that obesity has a physio-pathological component determined by genetic factors.

Adipose tissue is largely composed of adipocytes which store excess calories in the form of triacylglycerol that is synthesized from fatty acids and glycerol. Accumulation of adipose tissue can arise through increases in adipose cell size and/or cell number (Roncari, 1984). While the size of adipocytes is principally determined by the amount of stored triacylglycerol, the number of adipose cells is determined by the differentiation of specialized fibroblasts (preadipocytes) into new mature adipose cells (adipocytes). The size of the adipocyte can be reduced by dietary restriction, but there is little evidence that new adipocytes, once formed, can be lost by dietary intervention. Furthermore, adipocytes may vary in size and number in individuals. For instance, mild obesity involves an increase in the size of the adipocyte (hypertrophic obesity) whereas severe obesity, or obesity in childhood, typically involve an increase in the number of adipose cell (hyperplastic obesity). Therefore, a detailed knowledge of the molecular mechanisms that regulate preadipocyte cell growth and adipocyte differentiation is essential in understanding the physiological nature of obesity.

### 1.I.B. Adipocyte as a Secretory Cell

In addition to its role in fatty acid storage and release, the adipocyte is known to play a role in the control of appetite and glucose homeostasis through the secretion of leptin (the

product of the *obese* gene) and tumor necrosis factor α (TNFα) (Campfield et al., 1996; Hotamisligil and Spiegelman, 1994). Increased leptin, produced in adipose tissue, controls appetite by binding to the brain leptin receptor which is encoded by the *diabetes* or DB gene in the brain (Tartaglia et al., 1995). This protein also decreases the expression of neuropeptide Y that stimulates feeding behavior (Stephens et al., 1995), resulting in the loss of body weight. In addition to leptin, TNFα induces insulin resistance during obesity by interfering tyrosine kinase activity of insulin receptor in insulin signaling, suggesting that the adipocyte cell may also influence glucose homeostasis (Figure 1.1) (Hotamisligil and Spiegelman, 1994).

### 1.I.C. Adipogenesis: Cell Culture Model

Adipogenesis is a complex process regulated by a variety of hormones, growth factors, and cytokines that act via specific receptors to transduce differentiation signals through a cascade of intracellular events. Preadipocytes can be made to differentiate *in vitro* and this differentiation leads to activation of most of the same set of genes characteristic of adipose tissue differentiation *in vivo* (Gregoire et al., 1998).

Clonal cell lines largely divide into three categories; (i) totipotential embryonic stem cells generating all lineages (ES cells), (ii) multipotential stem cells that differentiate into myogenic, chondrogenic and adipogenic lineages, and (iii) cells that have been committed to the adipocyte lineage (preadipocyte) through adipoblasts (unipotential cells) (Cornelius et al., 1994). The multipotential and preadipose cell lines have been developed from mouse embryonic tissue and used extensively for analysis of the adipocyte differentiation program. NIH-3T3 and C3H10T1/2 are well characterized to be maintained as fibroblast-like cells, but to have the ability to differentiate into multiple cell types categorized in (ii). However, the widely studied 3T3-L1, 3T3-F442A, and Ob17 cells are already committed to the adipocyte lineage. These cell lines are defined by their ability to express early differentiation markers but are incapable of storing triacylglycerol or expressing late adipocyte genes (Ailhaud et al., 1992). Differentiation of the preadipocytes, the cell lines categorized in (iii), is mainly discussed in this section.

**Figure 1.1. Major components of the body weight regulatory system**

The adipocyte plays an active role through the regulated secretion of a number of secreted products such as leptin and TNFα. Leptin acts through receptors in the hypothalamus to regulate appetite and insulin secretion via sympathetic nervous system (SNS) output, and neuroendocrine function including reproduction. In addition, leptin decreases the expression of neuropeptide Y. Ingested nutrients stimulate insulin secretion. TNFα induces insulin resistance during obesity by interfering insulin signaling. Together these control adipose differentiation and influence glucose homeostasis.

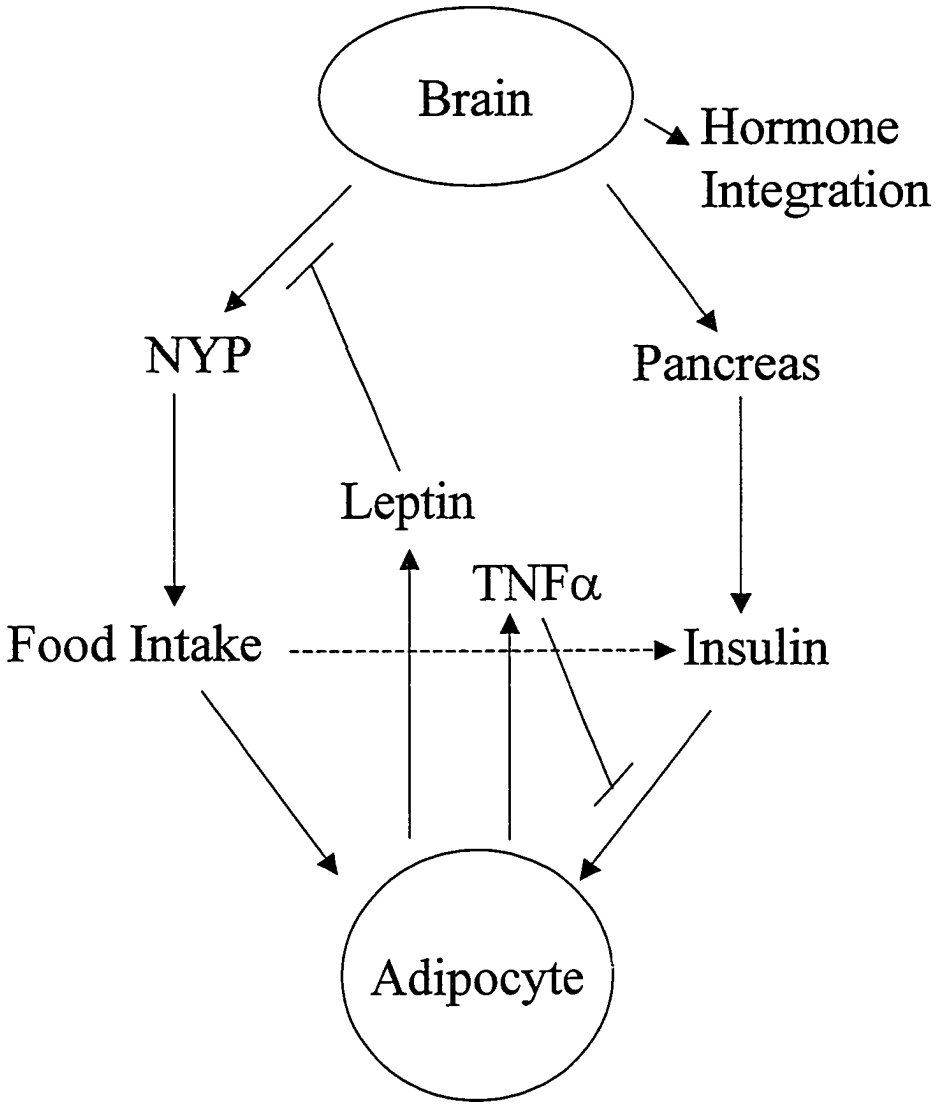


Figure 1.1. Major components of the body weight regulatory system

The first step of adipocyte differentiation involves arrest at confluence (Scott, 1982). At this stage, 3T3-L1 preadipocytes differentiate into mature adipocytes by treatment with appropriate doses of adipogenic mixtures including insulin or insulin-like growth factor 1 (IGF-1). However, the mechanistic actions of insulin and other ligands on differentiation are not clearly defined (reviewed in Ailhaud et al., 1992; Cornelius et al., 1994; MacDougald and Lane, 1995; Gregoire et al., 1998). Preadipocytes possess few insulin receptors but have large numbers of IGF-1 receptors (Smith et al., 1988), a receptor tyrosine kinase (RTK) which can be activated by non-physiologically high concentrations of insulin (Norgues et al., 1993) to influence differentiation of 3T3-L1 preadipocytes.

In addition, dexamethasone (DEX), a synthetic glucocorticoid agonist, and 1-methyl-3-isobutylxanthine (MIX), a cAMP phosphodiesterase inhibitor, are generally considered necessary for the induction of differentiation, along with insulin. DEX and MIX regulate the early induction of two CAAT enhancer binding proteins (C/EBPδ and C/EBPβ) respectively in the differentiation of 3T3-L1 (Yeh et al., 1995). DEX also significantly increases peroxisome proliferator-activated receptor γ (PPARγ) expression that is induced by C/EBPβ and C/EBPδ (Wu et al., 1996). These observations suggest an important regulatory cascade in which hormonal signals first induce C/EBPβ and δ and subsequently activate PPARγ and C/EBPα.

The requirement for adipogenic hormones differs between established clonal lines. For example, NIH-3T3 cells treated with MIX have little or no C/EBPβ expression and are not able to differentiate into adipocytes. However, ectopic expression of C/EBPβ in treatment with DEX and MIX can convert NIH-3T3 cells into adipocytes (Wu et al., 1995). In 3T3-L1 cells, MIX increases C/EBPβ expression, by elevation of cAMP levels, and an induction of PPARγ. This suggests that induction of C/EBPβ may be responsible for commitment to the adipogenic lineage. In contrast, 3T3-F442A preadipocytes do not require MIX or DEX to differentiate into adipocytes, suggesting that these cells are slightly further along in the differentiation process than 3T3-L1 cells. Therefore, the nature of the induction of differentiation is dependent on both the culture conditions and the specific cells investigated.

## 1.I.D. Adipogenesis: Main Features

The committed preadipocyte has to withdraw from the cell cycle before adipose conversion. During adipocyte differentiation, acquisition of the adipocyte phenotype is characterized by chronological changes in the expression of numerous genes. This is reflected by the expression of early, intermediate, and late mRNA/protein markers and triglyceride accumulation. In addition, those genes whose products are inhibitory to adipogenesis or unnecessary for adipose cell function are repressed. The main features of adipogenesis, including gene expression during several distinct steps in preadipocyte cell lines, will be described below.

### *Growth Arrest*

First of all, growth arrest is an essential prerequisite step prior to entry into the adipocyte differentiation pathway but cell-cell contact does not appear to be necessary. This was supported by some studies showing that preadipocytes at a low density in serum-free medium can differentiate in the absence of cell-cell contact (Pairault and Green, 1979; Vanderstrateten-Gregoire, 1989).

### *Clonal Expansion*

After growth arrest at confluence, preadipocytes undergo at least one round of DNA replication and cell doubling. Although it is not clear why cells undergo this step, it seems to be necessary to maintain adipocyte phenotypes (Christy et al., 1991). Retinoblastoma proteins (pRB), p107 and p103, bind to the E2F/DP-1 complex to inactivate growth-promoting transcriptional activities. The binding activity of p107 and p103 to E2F during clonal expansion is different from that during preconfluent cell growth (Richon et al., 1997). E2F predominantly binds to p130 in the confluent cells treated with insulin, DEX, and MIX on day 0. On day1, during mitotic clonal expansion, E2F dissociates with p130 and forms a new complex with p107 (E2F-107) or remains uncomplexed. This switch is associated with transiently increased levels of p107 protein along with decreased p130. Following differentiation, the day 0 pattern was reestablished. Thus, the expression of p107 is specific for clonal expansion.

### *Early Changes in Gene Expression*

Growth arrest and clonal expansion are accompanied by complex changes in the pattern of gene expression that can differ in the cell culture models and with specific differentiation protocols employed. At least two families of transcription factors, C/EBPα and PPARγ, are induced early during adipocyte differentiation. PPARγ is largely adipocyte specific and is expressed at low but detectable levels in preadipocytes. Its expression rapidly increases after hormonal induction of differentiation. It is easily detectable during the second day of 3T3-L1 adipocyte differentiation, and maximal levels of expression are attained in mature adipocytes (Brun et al., 1996). A transient increase in the expression of C/EBPβ and C/EBPδ isoforms precedes the increase in PPARγ expression (Wu et al., 1996). The subsequent decrease of C/EBPβ and C/EBPδ in early to mid stages of differentiation is concomitant with the induction of C/EBPα mRNA. This increase in C/EBPα expression occurs slightly before the expression of adipocyte-specific genes.

### *Terminal Differentiation*

During the terminal phase of differentiation, adipocytes in culture show a marked increase in lipogenesis and acquire sensitivity to insulin. The protein activity and mRNA levels for enzymes involved in triacylglycerol metabolism increase 10- to 100-fold. At this stage, PPARγ and C/EBPα cooperatively activate adipocyte specific genes, such as aP2, GLUT4, SCD1 and leptin (Fajas et al., 1998).

The precise stage beyond which adipocytes can be considered terminally differentiated is not clearly defined. The progressive dedifferentiation followed by cell division has also been reported (Torti et al., 1989). Tumor necrosis factor-α treatment of mature 3T3-L1 adipocytes results in decreased expression of adipocyte markers and loss of lipid, resulting in the development of morphological changes characterized by long, spindle-shaped cytoplasmic extensions (Torti et al., 1989). Although these cells appear to resemble preadipocytes in terms of similar morphological characteristics and gene expression pattern, recent evidence indicates that they in fact differ considerably. For instance, Preadipocyte factor-1 (Pref-1) levels are not restored by TNFα in mature adipocytes. Pref-1 expression may reflect a fundamental difference between preadipocytes and those that result from TNFα treatment (Xing et al., 1997).

## *1.II. Transcriptional Control of Adipogenesis*

The full complement of proteins involved in the differentiation process remains to be determined. Ultimately the PPAR and C/EBP family of transcription factors must function cooperatively to transactivate adipocyte genes. ADD1/SREBP1 may also be required to initiate adipocyte differentiation.

### 1.II.A. PPAR Family

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily. PPARγ is the most adipocyte specific of the PPAR family, and its expression is induced early in the differentiation program (Tontonoz et al., 1994a). Initially, two PPARγ isoforms, PPARγ1 and PPARγ2, were identified and they are derived from the same gene which produces two alternatively spliced products. PPARγ2 is identical to PPARγ1 except for 28 additional amino acids at its N-terminus (Zhu et al., 1995). PPARγ1 seems ubiquitously expressed at low levels whereas PPARγ2 is markedly increased in adipocytes, suggesting that PPARγ2 is important for adipocyte differentiation. Indeed, ectopic expression and activation of PPARγ2 in fibroblastic cell lines leads to a high percentage of adipose conversion (Tontonoz et al., 1994b).

The PPARγ activity can be modulated by interaction with other nuclear receptors, by the binding of ligands, by post-translational modifications and by various cofactors. PPARγ forms a heterodimer with RXRα and controls several crucial adipocyte genes by binding directly to an adipocyte specific-enhancer element that is located upstream of a range of genes. Genes containing this site include aP2 (Tontonoz, et al., 1994), stearoyl-CoA desaturase (Miller and Ntambi, 1996), and fatty acid synthase (FAS) (Schoonjans et al., 1997). This factor also decreases the expression of leptin (Kellen and Lazar, 1996).

PPARγ has two transcription activation domains, namely, a ligand-dependent transcription activation domain (AF-2) in the C-terminus and a ligand-independent activation domain (A/B domain) in N-terminus. Using these domains, PPARγ can modulate gene transcription. Furthermore, synthetic or natural ligands, such as thiazolidinediones (antidiabetic agents) and prostaglandin J2 derivatives, also activate PPARγ (Lehmann et al.,

1995; Kliewer et al., 1995). Finally, PPARγ2 has more ligand-independent transcriptional activity than PPARγ1 (Werman et al., 1997).

Mitogen activated protein kinase (MAPK), which is activated by EGF or PDGF, phosphorylates PPARγ and inhibits its transcription activity and blocks adipogenesis (Hu et al., 1996; Adams et al., 1997; Camp and Tafuri, 1997). This suggests that post-translational modification of PPARγ is also an important regulating component of adipogenesis.

### 1.II.B. C/EBP Family

Three members of the CAAT enhancer-binding protein (C/EBP) family, such as C/EBPα, β, and δ, are expressed at specific times during adipogenesis. These transcription factors have C-terminal basic region/leucine zipper (bZIP) domains, which confer DNA binding ability (basic region) and provides the ability for homo- or heterodimerization with other family members. In 3T3-L1 preadipocytes, C/EBPβ and δ levels are transiently increased in early adipogenesis by MIX and DEX, respectively (Yeh et al., 1995). C/EBPβ, in synergy with C/EBPδ, then induces PPARγ expression and triggers mature adipocyte differentiation (Wu et al., 1996). Recently, it has been reported that the PPARγ2 promoter has two C/EBP-binding sites (Fajas et al., 1997).

In contrast to the early effects of C/EBPβ and δ on PPARγ expression and adipocyte differentiation, C/EBPα functions in the termination of the mitotic clonal expansion that occurs early in the differentiation program. It has been reported that ectopic expression of C/EBPα stimulates the expression of two growth arrest-associated proteins, GADD45 (Constance et al., 1996) and p21(WAF-1/CIP-1/SDI-1) (Timchenko et al., 1996). In addition, C/EBPα binds and transactivates the promoters of several adipocyte-specific genes, including aP2, SCD1, GLUT-4, leptin, as well as the insulin receptor (reviewed by Gretchen et al., 1998). The blocking of C/EBPα induction in 3T3-L1 cells by antisense RNA, thus, inhibits adipocyte differentiation (Lin and Lane 1992). Although NIH-3T3 fibroblasts cannot differentiate into adipocytes even in the presence of hormonal stimulants that induce the 3T3-L1 differentiation, constitutive ectopic expression of C/EBPα in the fibroblasts results in adipose conversion in the absence of hormonal stimulants (Freytag et al., 1994). In an animal model, C/EBPα-deficient mice have reduced mass of both brown adipose tissue (BAT) and

white adipose tissue (WAT) (Wang et al., 1995). Overall, this evidence demonstrates that C/EBPα is essential for both *in vitro and in vivo* adipocyte differentiation.

Lastly, C/EBP homologous protein-10 (CHOP-10) is another member of the C/EBP family. Since CHOP-10 and other C/EBP heterodimers compete to bind to a common class of C/EBP sites (Ubeda et al., 1996), CHOP-10 seems to function as a negative modulator of the activity of C/EBP transcription factors.

Another important mode of regulation of C/EBPs is through the use of alternative translation start sites. Multiple translation start sites give rise to 42, 40, and 30 kDa proteins for C/EBPα, and to 35, 32, and 20 kDa proteins for C/EBPβ. Although both p42 and p30 C/EBPα transactivate C/EBPα-responsive promoters, p42 C/EBPα appears to be a more potent transcription factor and is more capable of inducing adipogenesis and blocking mitosis (Lin et al., 1993). In contrast, p20 C/EBPβ (liver inhibitory protein) functions as a dominant-negative inhibitor of p32 C/EBPβ (liver activator protein) and other C/EBPs. The ratio of p42 C/EBPα to p30 C/EBPα (and liver activator protein to liver inhibitory protein) changes over the course of adipocyte differentiation. This suggests that alternative translation may be a regulated process and may play a role in the control of adipogenesis (Lin et al., 1993).

C/EBPs are also post-translationally modified. CHOP is phosphorylated by p38 mitogen-activated protein kinase at serine 78 and 81 in the transactivation domain, and this phosphorylation is required for the full inhibitory effect on adipogenesis (Wang and Ron, 1996).

### 1.II.C. ADD1/SREBP1

Adipocyte determination and differentiation factor 1 (ADD1) is a member of the basic helix-loop-helix (bHLH) family of transcription factors and is expressed predominantly in WAT, BAT, and liver. Its expression is induced at a very early stage of adipogenesis (Tontonoz et al., 1993). The human homolog of ADD1 has been identified as the sterol regulatory element binding protein 1 (SREBP1), which binds to the sterol regulatory element (SRE) 1 (Yokoyama et al., 1993).

ADD1/SREBP1 increases fatty acid and fat synthesis, suggesting that it may be required, in part, to induce PPARγ activity through the generation of endogenous ligands for

PPARγ (Brun, et al., 1996a). In addition, the adipogenic effect of ADD1/SREBP1 is enhanced by PPARγ and its activators (Kim et al., 1996). Therefore, the activation of adipocyte genes is regulated by a cascade of transcriptional factors acting in concert with each other to request adipogenesis.

### 1.II.D. Cascade and Cooperation of Transcriptional Factors

Adipogenic inducers, MIX and DEX, increase transient expression of C/EBPβ and C/EBPδ, respectively. These inducers stimulate the expression of PPARγ and C/EBPα. Moreover, ADD1/SREBP1 expression is increased by insulin (Streicher et al., 1996) and these factors may increase the activity of PPARγ, possibly through the production of ligands.

C/EBPα and PPARγ act synergistically by triggering adipocyte differentiation and by reciprocally activating transcription of one another (Figure 1.2). This is supported by the finding that ectopic expression of either C/EBPα or PPARγ in NIH 3T3 fibroblasts promotes only partial differentiation, while co-expression of both factors provokes full differentiation (Brun et al., 1996b). These factors also act cooperatively in the transcriptional activation of several crucial adipocyte genes including aP2 which contain C/EBP- and PPAR-binding sites in their promoter regions (Mandrup and Lane, 1997).

## *1.III. AEBP1: A Multifunctional Protein*

### 1.III.A. Novel Transcriptional Repressor of aP2

AEBP1 was originally cloned from a cDNA which encoded a protein that interacts at the AE-1 (adipocyte enhance 1) site of the aP2 gene in the 3T3-L1 preadipocyte. This protein functions as a novel transcription factor that represses the aP2 gene expression through its intrinsic carboxypeptidase activity (He et al., 1995). The aP2 gene encodes fatty acid binding protein (FABP) which is highly expressed in adipose tissues. The study of aP2-deficient mice showed that the absence of aP2 interferes with the development of insulin resistance or diabetics induced by dietary obesity (Hotamisligil et al., 1996a). These mice also failed to

**Figure 1.2. Cascade and cooperation of transcriptional factors during adipocyte differentiation**

Hormonal signals initiate a transient increase in expression of C/EBPβ and C/EBPδ. These factors stimulate the expression of PPARγ and C/EBPα. In addition, ADD1/SREBP1 increases the activity of PPARγ, possibly through the generation of a ligand. Activation of PPARγ by a ligand leads to differentiation, cooperating with C/EBPα which subsequently is induced by PPARγ.

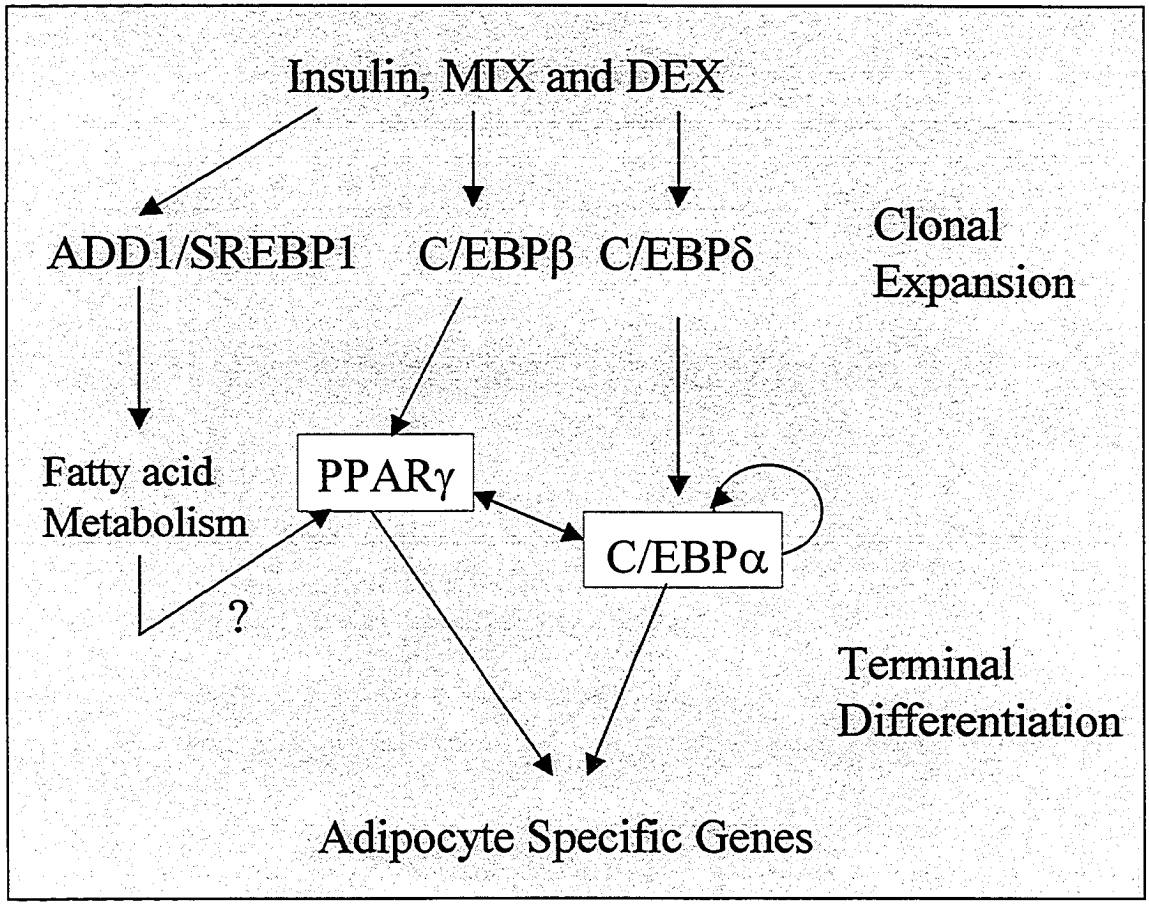


Figure 1.2. Cascade and cooperation of transcriptional factors during adipocyte differentiation of 3T3-L1 cells

express TNFα, which plays a central role in inducing insulin resistance by inhibiting tyrosine kinase activity of insulin receptor substrate 1 (IRS-1) (Hotamisligil et al., 1996b; Uysal et al., 1997). Since free fatty acids (FFA) may induce insulin resistance by increasing the expression of TNFα or other proteins which interfere with insulin action (Hotamisligil et al., 1996a), FABP is proposed to play a critical role in regulating gene expression by binding and shuttling these FFAs to the target cellular compartments. This series of studies suggest that AEBP1 may play a role in the development of insulin resistance by regulating aP2 gene expression. In addition, AEBP1 is expressed in preadipocytes, but its expression is down-regulated during adipocyte differentiation and abolished in the mature adipocyte (He et al., 1995; Kim et al., 2000). These observations further support the view that AEBP1 may have an important role in adipogenesis.

### 1.III.B. AEBP1 Isoforms and Three Distinct Domains

The mouse AEBP1 consists of 748 amino acids (He et al., 1995; Ro et al., 2000) and is almost identical to the human AEBP1 (Ohno et al., 1996) (Figure 1.3). The mouse AEBP1 is expressed in many cell and tissue types including preadipocytes and adipose tissue (a mixture of fibroblast-like cells including preadipocytes and adipocytes), but not in fully differentiated adipocytes. Recently, an isoform of the mouse AEBP1 has been found in mouse adult aorta and identified as the aortic carboxypeptidase-like protein (ACLP) which has an additional 380 amino acids in the N terminus (Figure 1.3). This protein is expressed predominantly in the smooth muscle of aorta but not in skeletal muscle (Layne et al., 1998). While ACLP is shown to be up-regulated during vascular smooth muscle cell differentiation (Layne et al., 1998), AEBP1 is down-regulated during adipocyte differentiation (He et al., 1995).

Recently, Ro and his group reported that the mouse AEBP1 cDNA contains the 3'-end portion of the 9$^{th}$ intron, which is removed from the mature mouse ACLP mRNA and has an in-frame stop codon (TAG) at 43 bases upstream from the alternate start codon (ATG) (Ro et al., 2000). This alternating spliced transcript that retained 9$^{th}$ intron was identified by RT-PCR analysis (Ro et al., 2000). Interestingly, the mouse ACLP, which has a signal peptide, a lysine- and proline-rich 11 amino acid repeating motif within the N-terminal extension, is known as a non-nuclear protein (Layne et al., 1998). In contrast, the mouse AEBP1 is

**Figure 1.3. Comparison of amino acid sequences of the human and mouse AEBP1, and their isoforms**

The arrows indicate the initiating site in mouse and human AEBP1 and their isoforms (mouse ACLP and human ACLP), respectively. The bolded and underlined sequence indicates a signal peptide, the underlined sequence shows the putative phosphorylation site by MAP kinase, and the bolded sequence represents the nuclear localization sequence.

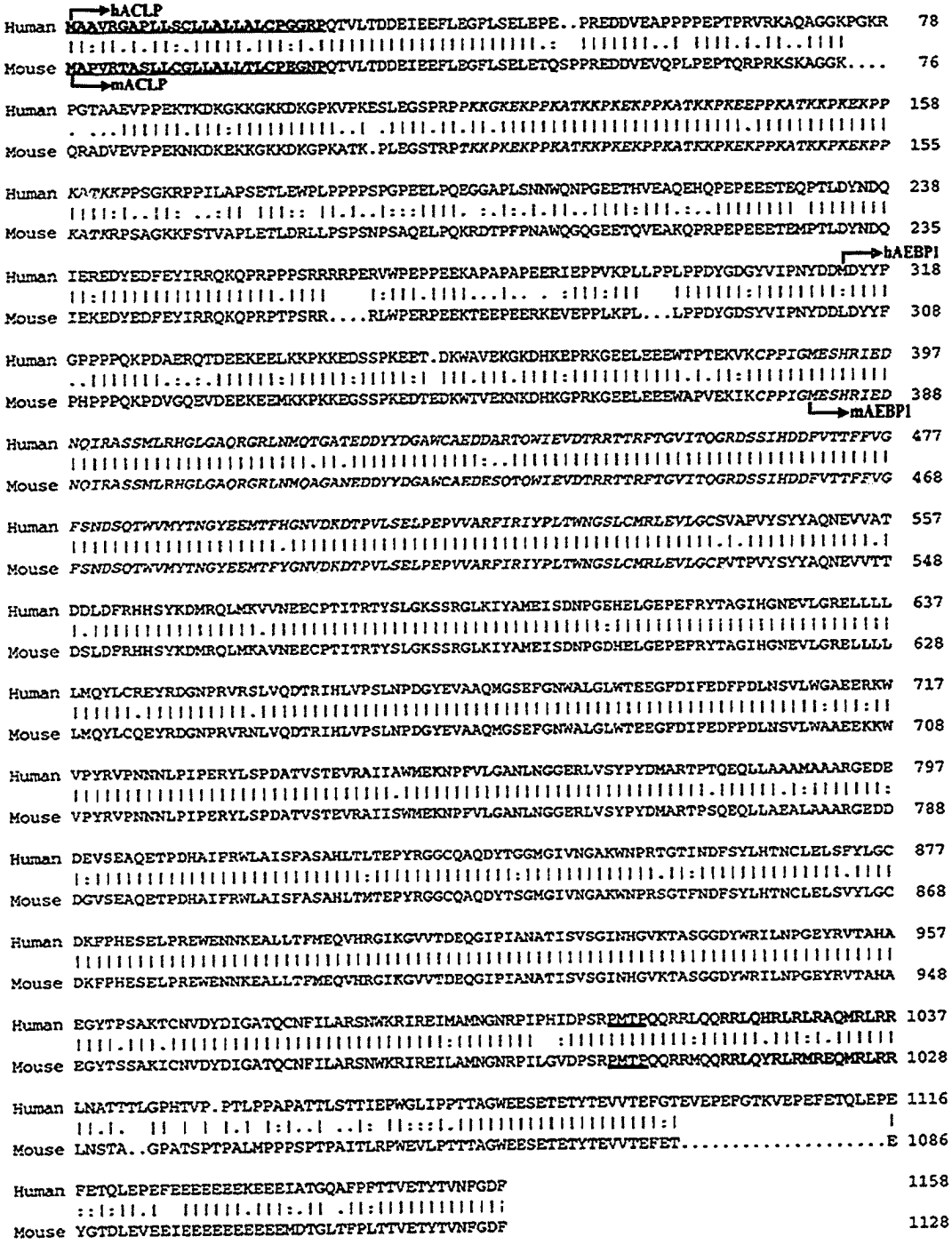


**Figure 1.3. Comparison of amino acids sequences of the human and mouse AEBP1, and their isoforms.**

localized in the nuclear and soluble fractions (Park et al., 1999). This suggests that the N-terminal extension may prevent the nuclear localization of ACLP.

AEBP1 is made up of three distinct domains, which are the discoidin-like domain (DLD) located in N-terminus, the carboxypeptidase-homology domain (CP), and the carboxyl terminal domain (Figure 1.4). Discoidin is a lectin produced by the slime mold *Dictyostellium discoideum* (Poole et al., 1981) and is thought to facilitate cellular aggregation and migration (Springer, et al., 1984). This domain has been found in coagulation factors V and VIII (Kane et al., 1986), milk fat globule membrane proteins (Stubbs et al., 1990), the discoidin domain tyrosine kinase receptor (Johnson et al., 1993), the endothelial cell protein del-1 (Hidai et al., 1998), and the neuronal surface protein, A5 (Kolodkin et al., 1997). These proteins are believed to be involved in protein-protein, protein-phospholipid, and protein-carbohydrate interactions.

The main body of AEBP1 is about 40% identical to the regulatory carboxypeptidase that cleaves C-terminal amino acids. Kinetic studies have characterized AEBP1 as a member of the regulatory B-like carboxypeptidase (CP) family (Muise and Ro, 1999). This type of carboxypeptidase plays important role in the processing of active peptides, alteration of receptor specificity for substrates, and the termination of polypeptide activity (Skidgel, 1988), while the digestive carboxypeptidase cleaves basic amino acids and plays a role in general protein degradation (Fricker, 1988). A mutational analysis of the CP domain of AEBP1 demonstrates that the loss of CP activity always abolishes the ability of AEBP1 to repress transcription activity. Likewise, if an AEBP1 mutant had CP activity, it is also able to repress transcription function. This indicates that the CP activity of AEBP1 is essential for its transcription regulation function (He et al., 1995). The mechanism by which AEBP1 mediates transcriptional repression is currently unknown. However, this suggests that transcriptional repression may occur through cleavage of an unidentified general transcription factor, although studies to determine potential substrates for the CP domain has not, as yet, provided any candidates.

The C-terminal domain of AEBP1 may be responsible for DNA binding because the AEBP1 mutant deleted in the C-terminus failed to bind DNA. This domain also has STP motifs with several possible MAP kinase proline-directed phosphorylation sites in the basic and STP rich regions (Pro-Xaa-Ser/Thr-Pro, discussed in 1.IV.A). Indeed, an *in vitro* kinase assay showed that AEBP1 is phosphorylated by MAP kinase whereas no phosphorylation was detected with the AEBP1 mutant that lacks the C-terminal domain (Muise, 1997).

**Figure 1.4. Three distinct domains of AEBP1**

AEBP1 consists of three distinct domains including the discoidin-like domain (DLD), the carboxypeptidase-homology domain (CP), and the carboxyl terminal domain. The carboxyl terminal domain consists of three subdomains including the basic amino acid-rich region (Basic) and the S/TP-rich region (STP), and the acidic amino acid-rich region.


Figure 1.4. Three distinct domains of AEBP1

## 1.III.C. Proteins Interacting with AEBP1

The yeast two-hybrid studies have revealed that AEBP1 interacts with several proteins including a high mobility group (HMG)-like protein, heat shock protein 27 (HSP27), and the Gγ5 subunit of a heterotrimeric G protein (Muise, 1997). Further study using co-immunoprecipitation shows that AEBP1 associates with Gγ5 but not with Gγ7 in mammalian cells (Park et al., 1999). This study also demonstrates that Gβ is present in the AEBP1-Gγ5 complex. This result is not unexpected, since G protein β and γ subunits are thought to be tightly associated as a dimer *in vivo*. This study further describes an important functional role for these interactions by showing that the binding of Gγ5 to AEBP1 attenuates its transcriptional repression activity. Interestingly, the Gγ5 level is selectively reduced by adipogenic stimulation. Decreased Gγ5 enhances the transcriptional repression activity of AEBP1 during mitotic clonal expansion at the onset of adipogenesis. Therefore, the actions of Gγ5 and AEBP1 support a model that a signaling molecule directly regulates transcription during differentiation (Park et al., 1999).

HMG proteins are non-histone chromatin proteins which bind to both single- and double-stranded DNA. Although they do not have transcriptional activity by themselves, they change the conformation of DNA and may hence influence transcription (Baxevanis and Landsman, 1995). A defect in the HMGI-C gene has been reported to reduce body fat content (Zhou et al., 1995). It suggests that there is a possible role for HMG proteins in adipogenesis.

TNF-α stimulates HSP27 phosphorylation and oligomerization (Mehlen et al., 1995). Thus, it is possible that HSP27 may also be involved in the regulation of adipocyte differentiation. HSP27 is a small actin-binding protein, and is a member of the heat shock protein family which functions to protect proteins against stress such as heat, oxidative stress, and apoptosis-inducing agents. Under stress, HSP27 is phosphorylated by MAPKAPK-2 (MAP kinase activating protein kinase-2), and oligomerizes to molecular masses of up to 1,000 kDa.

In addition, AEBP1 is phosphorylated by MAP kinase. Since many MAP kinase substrates, including Elk-1 and KSR, also interact with MAP kinase, it is possible that AEBP1 may interact with MAP kinase. For example, Elk-1, a transcription factor regulating

c-fos expression, is phosphorylated by ERK1/2, and also interacts with these kinases. Furthermore, Elk-1 appears to regulate the activity of MAP kinase by interacting with this kinase (Rao & Reddy, 1994), indicating that non kinase proteins can activate MAP kinase. Indeed, when anti-AEBP1 immunoprecipitates were immunoblotted with anti phosphotyrosine antibody, two phosphoproteins with molecular weights approximating the MAPKs (ERK1 and ERK2) were detected (Muise, 1997), suggesting a possible interaction between AEBP1 and MAP kinase. Thus, it would be interesting to determine whether AEBP1 interacts directly with MAP kinase and whether the interaction has a functional role in adipocyte differentiation.

### *1.IV. Receptor Activation of the MAP Kinase Pathway and Adipogenesis*

MAP kinase is an important signaling molecule for cell proliferation, differentiation, and apoptosis (reviewed in Widmann et al., 1999). Many different receptor types including receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) are capable of activating MAP kinase.

### 1.IV.A. RTKs and Adipogenesis

Stimulation of RTK by growth factors, including IGF-1 and insulin, activates different intracellular signaling pathways involving phosphoinositide 3-kinase (PI3K), 70S6K, PLCγ, JAK/STAT, and MAP kinase pathways. Stimulation of RTK also leads to autophosphorylation (Seger and Krebs, 1995). Among the signaling pathways, the MAP kinase pathway has been most extensively studied. Tyrosine phosphorylation of RTK leads to recruitment of an adaptor protein, Shc, which possesses a phosphotyrosine-binding (PTB) domain, a Src homolgy 2 (SH2) domain, and a Src homology 3 (SH3) domain. After Shc binds to the receptor at a specific phosphotyrosine via the PTB domain, another adaptor protein, growth-factor-binding-protein 2 (Grb2), which consists of a SH2 domain and two SH3 domains, binds to Shc (Downward, 1996). The association between Shc and Grb2 is mediated by SH2 domain in Grb2 (Pawson and Schlessinger, 1993). Grb2 also recruits Sos (son of sevenless), a guanine nucleotide exchange factor. The localization of Sos to RTK

leads to the exchange of Ras-GDP for GTP at the plasma membrane (Downward, 1996). The GTP-bound Ras interacts with Raf1 or other MEKK which activates downstream molecules such as MEK and MAP kinase (Figure 1.5).

Insulin receptor substrate 1 (IRS1) serves as a docking site for important signaling molecules, including PI3K, SH2 domain-containing tyrosine phosphatase 2 (SHPTP2) and Grb2 (reviewed in White, 1999). IRS1 phosphorylation is also capable of activating the Ras/MAP kinase pathway. However, its phosphorylation may not be sufficient to activate the Ras/MAP kinase pathway since both Shc and IRS1 can compete for Grb2 (Yamauchi and Pessin, 1994). Furthermore, point mutations in the insulin receptor (tyrosine 1162 and 1163 to phenylalanine), which prevent phosphorylation of IRS1 and IRS1-Grb2 formation, have no effect on the phosphorylation of tyrosine in the 52 kDa Shc, and show little attenuation of Ras activation (Ouwens et al., 1994). These data indicate that the predominant pathway activating Ras occurs through an insulin receptor-Shc interaction rather than an IRS1-Grb2 interaction.

In the mitogenic signaling pathway, the Shc module is shown to be predominant, however both Shc- and IRS1-Grb2-mediated modules have been shown to play an important roles. For example, introduction of IRS1 antisense RNA or antibodies to IRS1 into cells or a point mutation in the Grb2-binding site of IRS1 attenuates the effect of insulin on DNA synthesis (Myers et al., 1994). Microinjection of specific antibodies against Shc also inhibits DNA synthesis induced by insulin (Sasaoka et al., 1996).

Nonphysiologically high concentrations of insulin can convert 3T3-L1 preadipocytes, which have few insulin receptors but have large numbers of IGF-1 receptors (Smith et al., 1988), into mature adipocytes (Norgues et al., 1993), suggesting that insulin (or IGF-1) is involved in adipocyte differentiation. These molecules activate the Ras/MAP kinase pathway through two modules, RTK-Shc-Grb2-Sos module, as described above, and RTK-IRS1-Grb2-Sos (Myers and White, 1996).

IGF-1 stimulation of MAP kinase is rapid and maximal by 5 minutes in 3T3-L1 preadipocytes, but decreases greatly in differentiating cells whereas EGF still maintains MAP kinase activity in differentiating cells. There are apparent differences between the effect of EGF and IGF-1 on the Shc-Grb2 complex. The Shc-Grb2 complex is formed in IGF-1-treated proliferating, but not differentiating, cells. In contrast, this complex is present in EGF-treated proliferating and differentiating cells (Boney et al., 1998). Another mechanism that describes the attenuation of MAP kinase activity during the differentiation process by IGF-1,

**Figure 1.5. Receptor activation of MAP kinase pathway**

MAP kinase cascades are an important signaling module for cell proliferation, differentiation or apoptosis. They typically are organized in a three-kinase architecture consisting of a MAP kinase, a MAP kinase activator (MEK or MAPKK), and a MEK activator (MEKK or MAPKKK) (see text for detail).

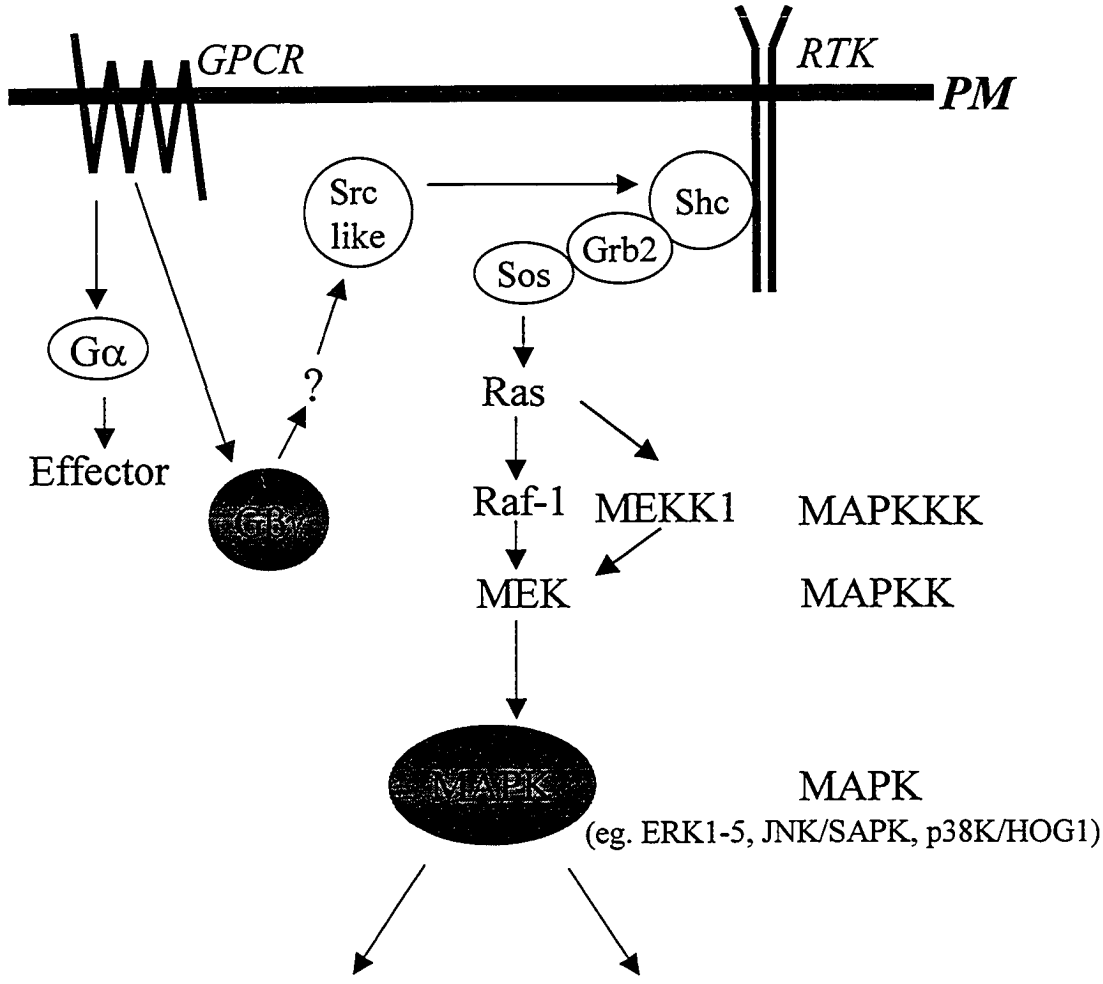


Figure 1.5. Receptor activation of MAPK pathway

but not by EGF, was presented by Kusari et al., (1997). They showed that insulin increases the expression of MAP kinase phosphatase-1 (MKP-1) which acts as a negative regulator of insulin signaling. These data suggest that insulin acts in 3T3-L1 adipocytes, in part, through MAP kinase activation.

### 1.IV.B. Heterotrimeric G Proteins and Adipogenesis

GPCRs (G protein-coupled receptors), which are also known as seven transmembrane receptors, are also able to activate the MAP kinase pathway. In the inactive state, GPCRs associate with heterotrimeric G proteins which consist of the GDP-bound α subunit and the βγ heterodimer. Upon stimulation by variety of hormones and neurotransmitters, the exchange of GDP for GTP bound to the G protein α subunit causes the dissociation of the trimer into the α monomer and βγ dimer. The Gα subunits are divided into four classes: Gsα which activates adenylyl cyclase, Giα which inhibits adenylyl cyclase, Gqα which activates phospholipase C, and G12α (G12α and G13α) (reviewed by Hamm, 1998).

There are numerous reports showing that receptors that signal via pertussis toxin (PTX)-sensitive Giα proteins and PTX-insensitive Gqα proteins could activate the MAP kinase cascade (Koch et al.,1994; Crespo et al., 1994). These studies further demonstrated that Giα- and Gqα-coupled receptors stimulate MAP kinase, and are mediated by Gβγ subunits which allow tyrosine protein phosphorylation and the activation of Ras. In this process, one of the candidate tyrosine kinases was shown to be Src. Src links βγ to MAP kinase activation through the phosphorylation of Shc and the subsequent recruitment of Grb2 and Sos (Luttrell et al., 1996). Other non-receptor tyrosine kinases linking GPCRs to MAP kinase have been reported. These include Src-like kinases such as Fyn, Lyn, Yes and the more distantly related Syk, as well as a novel $Ca^{2+}$-and PKC-dependent protein tyrosine kinase called Pyk2. Additional links in the Gβγ and MAP kinase pathway have also been recently identified and these links include interaction with SH-PTP and PI3Kγ (reviewed by Gutkind, 1998). The latter is a novel PI3K isotype which was shown to act downstream of Gβγ and upstream of the Src-like kinases. Therefore, the combination of various Gα and Gβγ subunits and diverse downstream effectors can elicit various cellular effects on G protein signaling.

## *1.V. MAP Kinase*

### 1.V.A. MAP Kinase and Its Substrate

The MAP kinase family is a critical component of a central switchboard that coordinates incoming signals generated by a variety of extracellular and intracellular mediators. MAP kinase is activated by upstream kinases consisting of a MAP kinase activator (MEK or MAPKK), and a MEK activator (MEKK or MAPKKK) (see Figure 1.5). Several members of the MAP kinase family have been identified, including the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), ERK3, ERK4, ERK5, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38/HOG1 (Widmann et al., 1999). Each member of the MAP kinase family is activated by MEK, a distinct upstream kinase which phosphorylates specific tyrosine and threonine residues on MAP kinase molecule (reviewed in Pelech and Sanghera, 1992; Hill and Treisman, 1995; Marshall, 1995). Classical MAPKs (ERK1 and ERK2), which are activated by various mitogens, contain a Thr-Glu-Tyr (TEY) sequence in a dual phosphorylation motif. JNK and p38, which respond to a variety of cellular stresses and pro-inflammatory cytokines, have Thr-Pro-Tyr (TPY) and Thr-Gly-Tyr (TGY) sequences, respectively (Schaeffer and Weber, 1999; Widmann et al., 1999).

Among the MAP kinase family, ERK1 and ERK2 have been extensively characterized for their substrate specificity. Upon activation by dual phosphorylation on Thr183 and Tyr 185, the activation lip of ERK2 undergoes a conformational change, thereby creating a surface pocket that is specific for proline residue in the MAP kinase phosphorylation site, PXS/TP, of the substrates. This proline-specific pocket is occupied by the side chain of Arg 192 when ERK2 is in an inactive state. Therefore, accessibility to this proline at the catalytic site is blocked when the enzyme is not activated, preventing enzyme-substrate interaction (Canagarajah et al., 1997).

So far, more than 50 different substrate proteins are known to be phosphorylated by these kinases. Many of these substrates are signaling proteins, which may involve a MAP kinase module such as MEK, and pp90 $^{rsk}$, or transcription factors such as c-Fos, GATA-2, c-Myc, and Elk-1, or proteins modulating a wide variety of other processes (Treisman, 1996).

These substrates contain the consensus sequence, Pro-Xaa-Ser/Thr-Pro (Davis, 1993). However, phosphorylation is unfavorable when Xaa of this consensus sequence is proline. In addition to these substrates, AEBP1 also contains several PXS/TP consensus sequences in the C-terminus (He et al., 1995), and it is phosphorylated by MAP kinase (Muise et al., 1997). This wide range of substrates indicates a complex role for MAP kinase in cellular signal transduction. Very recently, some of these substrates have been reported to contain a conserved docking site for MAPKs. The FXFP motif is a specific site for ERK and is termed a DEF (Docking Site for ERKs) whereas the L/I-X-L/I motif is a docking site for both ERK and JNK and is termed a DEJL (Docking Site for ERKs and JNK) (Jacobs et al., 1999). Elk-1 has both docking sites, GATA-2 and c-Fos each contains only a DEF, while MEKK and c-Jun each possesses only a DEJL. In contrast, c-Myc, Sos, and $pp90^{rsk}$ have neither a DEF nor a DEJL. The existence of this class of reported substrates raises the possibility that MAP kinase interacts with one or more yet-to-be defined docking sites.

### 1.V.B. MAP Kinase: A Key Element for Cell Proliferation & Differentiation

In a cell culture system, there is a good correlation between ERK activation and cellular proliferation. Growth factors such as EGF and PDGF which stimulate cell proliferation also activate ERK1/2. Interference of the ERK pathway using dominant negative mutant proteins or antisense constructs for Raf1 or ERK1 hinder cell proliferation (Minshull et al., 1994; Pages et al., 1993), while constitutively activated MEK1 persistently stimulates ERK1 activity and enhances cell proliferation (Seger et al., 1995) (see Figure 1.5). However, ERK1/2 activation may not always be required for cell proliferation. In some cells such as PC12 neuronal cell and K562 erythroleukemia cells, highly active MEK1 or ERK2 induce differentiation (Traverse et al., 1992; Racke et al., 1997). Treatment of PC12 cells with either EGF or NGF activates the MAP kinase signaling pathway. In these cells, EGF stimulates proliferation while NGF induces growth arrest and differentiation. This difference in the outcome between EGF and NGF treatment has been postulated to be related to differences in the duration and magnitude of the MAP kinase activation. The EGF-stimulated MAP kinase activity is transient, returning to basal or near-basal levels within 1-2 h. In contrast, the NGF stimulated activity is more sustained. Overexpression of the EGF receptor in these cells changes the response to EGF from proliferation to differentiation (Qui and

Green, 1992). Taken together, these results indicate that the strength and duration of the signals transmitted through MAP kinase will help to determine whether a cell proliferates or differentiates in response to a specific stimulus.

Since MAPKs activate a variety of substrates, the kinases should have diverse functions in cells. Indeed, many studies have shown that ERK1/2 induces cell proliferation or differentiation whereas JNK or p38K leads to apoptosis and an immune response (Widmann et al., 1999). However, this is not always the case. In certain cell types including B cells, interleukin-4 does not significantly activate ERKs but still induces proliferation (Smith et al., 1997). Activation of ERKs may also provide protection against apoptosis in some cell types. In PC12 cells, the withdrawal of NGF led to the inhibition of ERK1/2 activity and cell death while constitutive activation of the ERK pathway in these cells inhibited apoptosis (Xia et al., 1995). These studies suggest that the relationship between a certain MAP kinase and the induced biological response in mammalian cells may be cell-type specific.

### 1.V.C. MAP Kinase and Adipogenesis

Constitutive expression of Ras or Raf-1 can induce adipocyte differentiation in the absence of insulin or IGF1 (Benito et al., 1991; Porras et al., 1994), suggesting that differentiation may be mediated through MAP kinase activation since Ras and Raf-1 are upstream kinases of MAP kinase. Direct involvement of MAP kinase in adipocyte differentiation was shown by an experiment in which 3T3-L1 preadipocyte was treated with oligonucleotide antisense mRNA to deplete ERK1 and ERK2. These cells did not differentiate into adipocyte (Sale et al., 1995).

In sharp contrast, Santos and his group demonstrated that the activation of MAP kinase was not required for the differentiation process, but rather it antagonized the differentiation process (Font de Mora et al., 1997). These results seem to be reasonable because cytokines (eg., TNFα and TGFα) and many growth factors, including EGF and PDGF which stimulate MAP kinase activity, inhibit adipocyte differentiation (Adams et al., 1997). The antagonistic relation between MAP kinase activation and 3T3-L1 adipocyte differentiation also contrasts with observations for PC12 cells, where MAP kinase is required for neuronal differentiation, as described in the previous section (1.V.B). A possible mechanism to explain the negative modulation of adipocyte differentiation by MAP kinase is

the convergence of the MAP kinase pathway on transcriptional machinery. MAP kinase phosphorylates and alters the functions of transcriptional factors, including Jun, Fos, Myc, ATF2, Elk-1, PPARγ and C/EBPs. For example, insulin represses C/EBPβ mRNA and protein levels through the activation of the MAP kinase signaling pathway (Hemati et al., 1997). CHOP is phosphorylated by MAP kinase and this phosphorylation inhibits adipogenesis (Wang and Ron, 1996). Recently, several researchers have also demonstrated that the inhibition of adipocyte differentiation of 3T3-L1 and NIH-3T3 cells results, in part, from MAP kinase-mediated phosphorylation of the dominant adipogenic transcription factor PPARγ and reduction of its transcriptional activation function (Adams et al., 1997; Camp and Tafuri, 1997; Hu et al., 1996). Lazar and his group (Reginato et al., 1998) further showed that PGF2α, a prostaglandin derivative, blocked adipocyte differentiation through activation of MAP kinase which phosphorylated PPARγ and inhibit its transcriptional activity. In contrast to these results, PPARγ's phosphorylation in Chinese hamster ovary cells (CHO), expressing insulin receptors enhances its transcriptional activity and, furthermore, this phosphorylation is mediated by MAP kinase (Zhang et al., 1996). This discrepancy may be due to the use of different growth factors and different cell lines.

There is an evidence that Raf-1, which acts downstream of Ras, is involved in 3T3-L1 preadipocyte differentiation. This was supported by the fact that transfected Raf-1 induces adipocyte differentiation and a dominant-negative Raf-1 blocks this process. However, this transfection of Raf-1 induces only partial differentiation, indicating that a Raf-1-independent pathway, downstream of Ras, may be involved in adipocyte differentiation. In this case, the signals generated by Raf-1 do not activate the MAP kinase or RS kinase (Porras and Santos, 1996), suggesting a functional dissociation between Raf-1 and MAP kinase/RSK activation in Ras signaling pathways that lead to 3T3-L1 differentiation. This implies that MEK is activated by another MEKK in 3T3-L1 preadipocytes. Haystead et al. (1994) have identified a novel insulin responsive MEKK (I-MEKK) which is distinct from Raf in adipocyte and so I-MEKK may be involved in MEK activation during adipocyte differentiation.

### 1.V.D. Mammalian Scaffolding Proteins

Several reports show that upstream kinase activation may not be sufficient for successful MAP kinase activation. For example, a constitutively active MEKK1 can efficiently phosphorylate and activate MEK, but the activated MEK does not activate ERK fully (Xu et al., 1995). In addition, mutationally active MEK has ample kinase activity *in vitro* but fails to activate ERKs *in vivo* and to transform cells (Catling et al., 1995). These reports imply that other, as yet unknown, mechanisms affecting MAP kinase activation may exist. Recent studies have provided a clue that putative accessory molecules such as adaptor or scaffolding proteins may play a part in enhancing MAP kinase activity (Schaeffer and Weber, 1999). There are three well-known scaffolding proteins; one in yeast and two in mammalian cells (Figure 1.6).

In yeast *S. cerevisiae*, Ste5 was first identified to function as a scaffolding protein that mediates pheromone-induced mating (Choi et al., 1994). This protein interacts with many signaling molecules associated with RTK and GPCR. These molecules include Ste20 (MEKKK), Ste11 (MEKK), Ste7 (MEK), Fus3 and Kss1 (MAPK). Ste5 also associates with G protein βγ subunits (Whiteway et al., 1995). Although genetic studies showed that the binding of Gβ to Ste5 was not a prerequisite for oligomerization of Ste5, Gβ appears to be essential for the activation of Ste11 (Pryciak and Huntress, 1998). Ste5 also binds to several other proteins including Bem1, Cdc24 and Cdc42. These proteins regulate the actin cytoskeleton (Leeuw et al., 1995). This oligomeric protein complex of Ste5 was proposed to insulate a given MAP kinase module from related pathway, thereby increasing the specificity of signaling (Yashar et al, 1995). Ste5 is absolutely required for signaling through this module since deletion of this gene, or overexpression of a Ste5 mutant that can not bind MAP kinase, blocks the pheromone response (Herskowitz, 1995).

In mammalian cells, two scaffold-like molecules, MEK partner 1 (MP1) and JNK interacting protein 1 (JIP1) have been identified and appear to enhance MAP kinase activity (Schaeffer et al., 1998; Whitmarsh et al., 1998). MP1, a very small protein of 14 kDa, selectively associates with ERK1 and MEK1 but not with ERK2 or MEK2 (Schaeffer et al., 1998). *In vitro*, MP1 enhances the activation of MEK by Raf (see also Figure 1.5). When overexpressed in cells, MP1 can selectively enhance the activation of ERK1 but not ERK2, suggesting that MP1 helps to discriminate between these two MAP kinase isoforms.

JIP1 selectively interacts with multiple components of the JNK signaling pathway including JNK, JNK activator MKK7 (MEK isoform, MAPKK), and MKK7 activator mixed-

**Figure 1.6. Scaffolding proteins and AEBP1**

Scaffolding proteins are thought to promote the formation of oligomeric protein complexes with components that function in a specific MAP kinase module. AEBP1 interacts with two important signaling molecules, ERK1/2 and Gβγ.

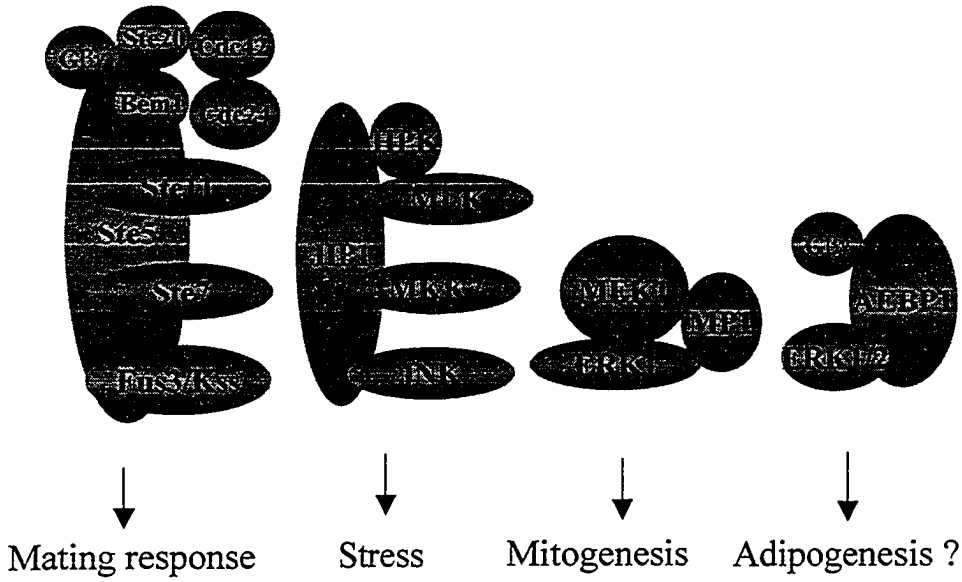


Figure 1.6. Scaffolding proteins and AEBP1

lineage protein kinase (MLK, MAPKKK) (Whitmarsh et al., 1998). The Ste20-like protein kinase, HPK1, also associates with JIP1, but it is not clear whether HPK1 binds directly to JIP1 or whether this binding is mediated by MLK. In addition, JIP1, which is a cytoplasmic protein, is known to cause cytoplasmic retention of JNK and to inhibit gene expression mediated by the JNK signaling pathway (Dickens et al., 1997). Further characterization has revealed that JIP increases JNK activation by the MLK-MKK7 signaling pathway (Whitmarsh et al., 1998). Like Ste5, JIP1 is also phosphorylated by JNK. However, the significance of JIP1 phosphorylation as a requirement for the assembly of the complex is not clear. Recently, the JIP1 isoform, IB1, has been identified to have an additional 47 amino acids in the C-terminal region and to function as a transcription factor that binds a regulatory element in the GLUT2 gene promoter (Bonny et al., 1998). Therefore, it would be interesting to determine whether JIP1 is a protein with multiple physiological functions or whether JIP1 acts as a MAP kinase scaffold to contribute to GLUT2 gene expression.

Since both MP1 and JIP1 bind selectively to a subset of components of the MAP kinase module and specifically enhance signaling that is routed through these components, they exhibit properties of scaffolding proteins. Interestingly, the known MAP kinase scaffold proteins do not share any obvious sequence homology. This raises the possibility that these molecules did not originate from a common ancestor but have evolved independently.

### 1.V.E. Regulation of MAP Kinase Activation

As a result of the pleiotropic nature of MAPKs, their activities are tightly controlled by both positive and negative mechanisms. A variety of factors are known to modulate MAP kinase activity including substrate specificity, its subcellular localization, its dephosphorylation by protein phosphatases, as well as protein-protein interactions. The mechanism regulating the extent and duration of MAP kinase activation will provide an important control on cell function.

In general, MEK1 and MEK2 are the only known activators of ERK1 and ERK2, while other MEKs are highly selective in phosphorylating JNK/SAPK or p38K (Cobb and Goldsmith., 1995; Raingeaud, et al., 1995). The TXY motif in the activation loop, as well as the presence of a different docking site, can regulate the affinity for the MAP kinase counterpart (Jacobs et al., 1999) as mentioned in section 1.V.A..

Subcellular localization undoubtedly plays an important part in directing ERK signaling and in limiting cross-activation between related modules. In unstimulated cells, ERK1/2 are found in the cytoplasm and are associated with microtubules (Chen et al., 1992; Alessi et al., 1995). Simulation of cells with mitogens results in activation of ERKs within the cytoplasm. Subsequently, the activated ERKs are translocated to the nucleus (Lenormand et al., 1993). A similar situation is unfolding for JNK/SAPK and p38. The kinetics of ERK activation influences the efficiency of nuclear translocation and, therefore, access to nuclear substrates. As mentioned in section 1.V.B., if MAP kinase is transiently activated by EGF stimulation of PC12 cells, it may not enter into the nucleus. In contrast, a more prolonged period of activation of MAP kinase by NGF in the cells results in nuclear retention. Dimerization is also an important factor for nuclear accumulation of the kinase. Cobb and her co-workers (Khokhlatchev et al., 1998) showed that phosphorylated MAP kinase forms dimers with phosphorylated and unphosphorylated MAP kinase partners. Disruption of dimerization by mutagenesis of ERK2 reduces its ability to accumulate in the nucleus, suggesting that dimerization is essential for its normal ligand-dependent relocalization. In contrast, MEK1 and MEK2, which are not only upstream activators but also contain nuclear export signals (NES), serve as cytoplasmic anchors for ERKs and, therefore, may regulate the subcellular distribution of ERKs (Fukuda et al., 1997; Gotoh et al., 1999). These series of experiments provide a clue that AEBP1, which contains a putative nuclear localization signal (NLS) in its C-terminus, may regulate the distribution of MAP kinase.

In contrast to MAP kinase activation and translocation into the nucleus, MEK is transiently phosphorylated and remained permanently in the cytoplasm (Alessi et al., 1995). MAP kinase phosphorylation (activation) is a reversible process, in which protein phosphatases play a crucial role in controlling cellular activities. The fact that MEK activation is quickly down-regulated and not translocated with MAP kinase to the nucleus suggests that the regulation of prolonged or transient MAP kinase activation may be through both cytoplasmic and nuclear protein phosphatases, and not through continuous re-activation by MEK (Alessi et al., 1995; Grameer and Blenis, 1997).

Since phosphorylation of both tyrosine and threonine is required for MAP kinase activation, individual Ser/Thr or Tyr protein phosphatases can regulate MAP kinase activity by dephosphorylating only one of the two phosphorylated residues and thereby inactivating the enzyme (Alessi et al., 1995). However, certain dual-specificity protein phosphatases appear better suited to regulate the MAP kinase family members by dephosphorylating one or

both sites (Alessi et al., 1995). These phosphatases include CHR, PAC-1, B23 (also termed hVH-3), MKP-1 (also known as CL100, 3CH134, Erp, and hVH-1), MKP-2 (also known as hVH-2 and TYP-1), and MKP-3 (also known as PYST1 and rVH6). Among these dual-specificity phosphatases, MPK-3 is found predominantly in the cytosol and is highly specific for EKR1 and ERK2 (Groom et al., 1996; Muda et al., 1996).

### 1.V.F. MAP Kinase and G protein Pathways and AEBP1

As mentioned in 1.III.B, AEBP1 binds to two signaling molecules, ERK and Gγ5. Many studies concerning scaffolding proteins indirectly imply that AEBP1 may affect the crosstalk between the major signaling pathways. For example, two mammalian scaffolding proteins such as JIP1 and MP1 have been shown to enhance MAP kinase activation by binding with the signaling molecules involving in MAP kinase modules (Schaeffer et al., 1998; Whitmarsh et al., 1998). In yeast, a scaffolding protein, STE5, also interacts with a Gβ subunit, MAP kinase, and upstream kinases of MAP kinase and functions to facilitate signals from Gβγ to the MAP kinase cascade (Choi *et al*, 1994). Moreover, an isoform of JIP1 was recently shown to be a transcription factor (further described in section 1.V.G)(Bonny et al., 1998). These reports suggest that AEBP1, which was originally described as a transcription factor, may have functional analogy to the scaffolding proteins.

In addition, Ro and his co-workers (Park et al., 1999) showed that transcriptional activity of AEBP1 on aP2 gene expression was regulated by Gγ5. In their studies, recombinant Gγ5 attenuated the DNA binding activity of AEBP1 to the AE-1 site located in the proximal promoter region of the aP2 gene, and inhibited transcriptional repression activity of AEBP1 on aP2 gene expression *in vitro*. Furthermore, Gγ5, which temporarily increases at the initiation of adipocyte differentiation, was shown to be capable of activating transcription of adipogenic genes by inhibiting AEBP1's repression function *in vivo*. Earlier work in Ro's lab (Muise, 1997) also showed that ERK could stimulate the binding of AEBP1 to the AE-1 site in DNA. Overall, these data support the possibility that AEBP1 may be involved in the regulation of the adipogenesis signaling pathway and may act as a nuclear effector regulating the transcription of adipogenic genes (Figure 1.7).

**Figure 1.7. Crosstalk of the MAP kinase pathway and AEBP1 for adipogenesis**

AEBP1 interacts with two signaling molecules, ERK and Gγ5. Gγ5 regulates transcriptional activity of AEBP1 on adipocyte specific gene at the initiation of adipogenesis (see Text). MAP kinase activation is an important anti-adipogenic factor, in part functioning through PPARγ's phosphorylation that results in inhibition of its transcriptional activity.

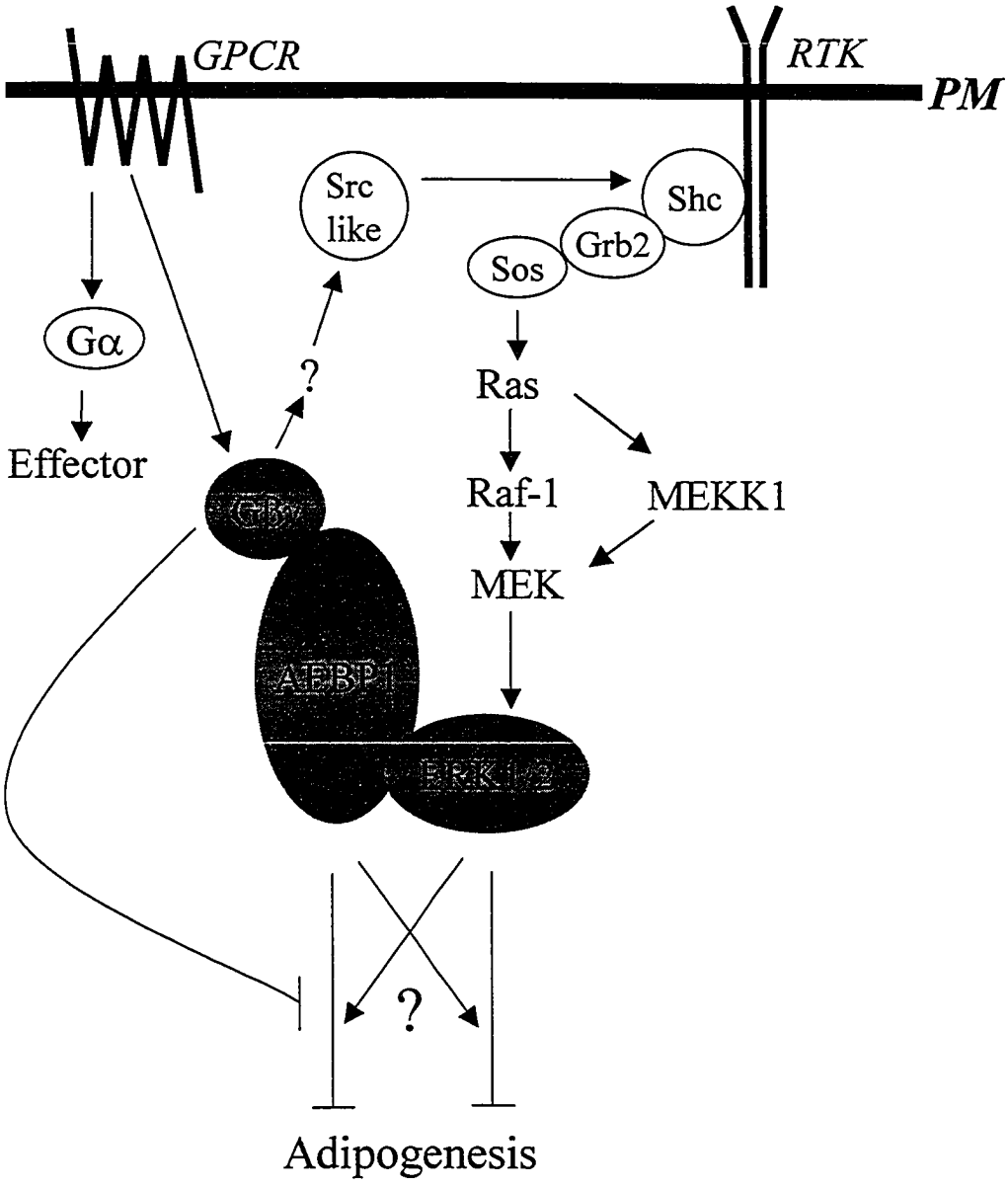


Figure 1.7. Crosstalk of the MAPK pathway and AEBP1 in adipogenesis

### *1.VI. Purpose of Thesis*

The activation of MAP kinase has generally been implicated as a vital step in signaling pathways which determine whether a cell differentiates or proliferates. MAP kinase activation is a reversible process in which protein phosphatases play a crucial role in controlling cellular activities. Thus, the regulation of prolonged or transient MAP kinase activation may be through phosphatases rather than through the activity of MEK which is itself transiently activated by phosphorylation (discussed in section 1.V.F). The role of MAP kinase activation in adipocyte differentiation is still controversial (discussed in section 1.V.D) and the precise mechanism for differentiation is not clear. As a result, a number of questions have been raised concerning the maintenance of the preadipocyte phenotype and the regulation of sustained MAP kinase activity at the onset of adipogenesis. Furthermore, AEBP1, which was originally identified as a transcription factor, is now known to interact with two important signaling molecules, the ERKs and Gγ5.

Therefore, the purpose of this thesis was to investigate whether AEBP1 acts as a mediator in the MAP kinase pathway and whether AEBP1 is an essential factor in the regulation of adipocyte differentiation through MAP kinase modules. In order to test protein-protein interaction, a co-immunoprecipitation assay was performed. Using this method, the present study examined the possibility of a direct interaction between AEBP1 and ERKs as well as a possible association between AEBP1 and other upstream molecules of the MAP kinase pathway. Studies were also carried out to determine whether AEBP1 enhances MAP kinase activation in a manner similar to the known mammalian scaffolding proteins such as JIP1 and MP1. These possibilities were tested using a kinase assay. Finally, using stable transfection techniques, the involvement of AEBP1 in adipocyte differentiation was assessed.

# Chapter 2. Materials and Methods

## *2.I. Materials*

### 2.I.A. Reagents

Restriction enzymes were purchased from New England Biolabs (NEB, Mississauga, ONT) or Life Technologies, Inc (GIBCO/BRL, Burlington, ONT). [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol) and [$\gamma$-$^{32}$P ]ATP were purchased from Amersham Inc (Oakville, ONT). All proteinase inhibitors including aprotinin, leupeptin, phenylmethylsulfonyl fluoride were obtained from American Bioanalytical (Natick, MA). All materials used for preparation of bacteria culture medium were purchased from Difco Laboratories. Cell culture media, including fetal bovine serum (FBS), bovine calf serum, and cosmic calf serum, were purchased from Hyclone, Logan, Utah except for Dulbecco's modified Eagle's medium (DMEM, Life Technologies). BRL49653 was a kind gift from Dr. John Johnson (Parke-Davis/Warner Lambert, Ann Arbor, MI). All other materials were purchased from Sigma Chemical Co., unless otherwise indicated.

### 2.I.B. Antibodies

Protein-A agarose, all secondary antibody and monoclonal antibodies directed against Myc epitope and PPAR$\gamma$ were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA), and monoclonal anti phospho-p44/42 MAP kinase (Thr 202/Tyr204) were obtained from NEB, respectively. All commercial polyclonal antibodies against HA epitope, ERK1, MEK1, and IGFR were from Santa Cruz, and the others including p44/42 MAP kinase, and phospho-MEK1 were from NEB. Polyclonal antibodies against AEBP1 were generated against the entire amino acid sequence of mouse AEBP1 (He et al., 1995).

## *2.II. Construction and Characterization of Recombinant Plasmids*

### 2.II.A. Plasmid Construction

Plasmids constructed and used in this study are listed in Table 2.1. The parental HA tagged vector, pJ3H, was obtained from Dr. J. Chernoff (Fox Chase Cancer Center, PA). To construct the plasmids that encode the wild-type and mutant derivatives of HA-tagged AEBP1 (pJ3H-AEBP1), EcoRI restriction fragments of pG4AEBP1 or its mutant derivatives (He et al., 1995) containing either wild type or mutant derivatives of AEBP1-coding sequence (Figure 3.1) were cloned into the EcoRI site of pJ3H.

The parental retroviral vectors, pWZLneo and pBabe-puro, were obtained from Dr. S.O. Freytag and Dr. B.M. Spiegelman, respectively. In order to generate stable cell lines expressing either the wild-type AEBP1 or its mutant derivatives, pAEBP1/Neo was constructed by insertion of full length of AEBP1 into the *Bam*HI-*Xho*I site of pWZLNeo. To construct the mutant derivatives of pAEBP1/Neo, respective *Eco*RI restriction fragments from the mutant derivatives of pJ3H-AEBP1 were cloned into the *Eco*RI site of pWZLNeo. pAEBP1DLD/Puro or pAEBP1DLD(-)/Puro was constructed by cloning of *Bam*HI restriction fragment of pJ3H-AEBP1 into the *Bam*HI site of pBabe-puro in both orientations. pAS was constructed by cloning the *Bam*HI restriction fragment (opposite orientation of AEBP1 fragment) of pBAEBP1into the BamHI site of pREP4. For construction of the antisense AEBP1 plasmid, pAS/Neo, the *Eco*RI and *Bam*HI restriction fragment of pBlueAEBP1 was cloned in the opposite orientation into the BamHI and EcoRI sites of pWZLNeo. The antisense constructs, pAS and pAS/Neo, were used to attenuate the expression level of AEBP1 in AEBP1/Neo-7 and 3T3-L1 cells.

For the MAP kinase protection assay, several plasmids were used. The AEBP1 expression vector, pSVAEBP1, and the control vector, pSVAEBP1(-), were previously described (He et al., 1995). pSG5/PYST-Myc was obtained from Dr. S. Keyse. This plasmid is an expression vector encoding a Myc-tagged version of a dual specific MAP kinase phosphatase, and pECE/HA-ERK1 encodes an HA-tagged version of ERK1. The reporter construct, paP2(3AE-1/-120)CAT, contains three copies of the AE-1 sequence at 5' to the promoter (-120 to +21) of aP2 gene (He et al., 1999). This construct was used in transcriptional analysis (CAT assay) of wild-type and mutant derivatives of HA-AEBP1 (pJ3H-AEBP1).

| *Plasmid* | *Parental vector* | *Usage* | *Reference* |
|---|---|---|---|
| pJ3H-AEBP1<br>pJ3H-AEBP1(-)<br>pJ3H-AEBP1ΔDLD<br>pJ3H-AEBP1DLD<br>pJ3H-AEBP1ΔHic<br>pJ3H-AEBP1ΔSty | pJ3H | Immuno complex study *in vivo*, CAT assay, or Kinase assay | Sells and Chernoff, 1995 |
| pAEBP1/Neo<br>pAEBP1ΔHic/Neo<br>pAEBP1ΔSty/Neo<br>pAS/Neo | pWZLNeo | Stable cell lines | Freytag et al., 1994 |
| pAEBP1DLD/Puro<br>pAEBP1DLD(-)/Puro | pBabe-puro | Stable cell lines | Pear et al., 1993 |
| pREP-AS | pREP4 | Stable cell lines | Invitrogen |
| pSVAEBP1<br>pSVAEBP1(-) | pSV | Kinase assay | He et al., 1995 |
| pSG5/PYST-Myc | pSG5 | Kinase assay | Groom et al., 1996 |
| pECE/HA-ERK | pECE | Kinase assay | Brunet & Pouyssegur, 1996 |
| paP2(3AE-1/-120)CAT | paP2 | Control reporter for CAT assay | He et al., 1999 |
| pETAEBP1<br>pETAEBP1ΔDLD<br>pETAEBP1DLD<br>pETAEBP1ΔHic<br>pETAEBP1ΔSty | pET-16b | *In vitro* interacting assay and kinase assay | Muise, 1997 |

Table 2.1. The plasmids used for various experiments

### 2.II.B. Transformation

Competent bacterial cells were transformed using the calcium chloride method as described by Ausubel et al., (1994) with minor modifications. pET constructs containing either AEBP1 or its mutant derivatives were transformed into DE3 *E.coli* cells to induce recombinant proteins, whereas DH5α *E.coli* cells were used to propagate all other plasmids. Briefly, competent cells (100 µl) were mixed with the appropriate amount of DNA (20-100 ng), incubated for 30 minutes on ice, heat shocked for 30 seconds at a 37 °C, and placed for 2 minutes on ice. After supplementing with 900 µl of Luria-Bertani medium (LB: 0.5 % [w/v] yeast extract, 1% [w/v] peptone, 1 % [w/v] Nacl), the cells were incubated in a shaker (225 rpm) at 37 °C for 1 hour. Aliquots (250 µl) of the cells were spread onto LB/ampicillin plates (LB containing 2 % [w/v] agar and ampicillin [25 µg/ml]) and grown overnight at 37 °C.

### 2.II.C. Preparation and Characterization of Plasmid DNA

Bacterial colonies were inoculated and grown overnight at 37°C with shaking (225 rpm) in 5 ml of LB medium supplemented with 50 µg/ml ampicillin. Plasmid DNA was then prepared with the QIAprep Spin Miniprep Kit (QIAGEN). To screen for the right plasmid, DNA was digested with the appropriate restriction enzymes (NEB or Life Technologies) in 10 µl of distilled $H_2O$ containing 1X reaction buffer (NEB or Life Technologies). After digestion, samples were mixed with 10X loading buffer (20% Ficoll 400, 0.1 M EDTA, 1% SDS, 0.25% bromphenol blue, 0.25% xylene cyanol; pH 8.0) and analyzed on 1% agarose gel containing ethidium bromide (2 µl of 10mg/ml stock per 40 ml of gel) in 1X TAE buffer (4 mM Tris-acetate, 1 mM EDTA). Lambda-HindIII fragments (250 ng, Life Technologies) were used as DNA size markers. The gel was scanned using the Gel-Doc system (BioRad). For large preparations of plasmid DNA, QLAfilter plasmid Maxi-Kits (Qiagen) were used.

### *2.III. Cell Culture and Differentiation*

### 2.III.A. Cell Lines

The COS-7 cell line (ATCC CRL-1651) was generated from the CV-1 cell line (ATCC CCL-70) by transformation with an origin-defective mutant of SV40 which codes for the wild-type T antigen. 3T3-L1 is a sub-strain from a Swiss-3T3 fibroblast and the NIH-3T3 cell line was established from a Swiss mouse embryo culture (ATCC Catalogue of Cell Lines and Hybridomas, 7$^{th}$ edition, 1992). Several cell lines (see Table 2.2) were generated from 3T3-L1 preadipocytes by stable transfection (described in 2.V.B). AEBP/Neo-P, AEBP1ΔHic/Neo-P, AEBP1ΔSty/Neo-P, and AS/Neo are resistant to G418 (Life Technologies), while AEBP1DLD/Puro and AEBP1DLD(-)/Puro are resistant to puromycin (Sigma). REP-AS is resistant to both hygromycin (Boehringer Mannherim, Laval, Que) and G418 (see Table 2.2).

### 2.III.B. Cell Culture

Frozen cell stocks were quickly thawed at 37 °C and immediately plated in 100-mm dishes (Sarstedt) containing growth medium [DMEM containing 10% calf serum (Hyclone) or 10% cosmic calf serum (Sigma) with 1% penicillin-streptomycin solution (Sigma)] and incubated at 37 °C with 5% $CO_2$ atmosphere. One day after plating, the growth medium was replaced with fresh medium and then every two days thereafter.

At 80% confluence, cells were washed twice with 5 ml of PBS (0.137 M NaCl, 4.2 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, pH 7.3) and were treated with 1 ml 1x trypsin (Life Technologies) for 1 minute. After removal of the trypsin solution, cells were lifted from the plates by gentle tapping. The cells were divided to an appropriate number of plates as required for specific experiments.

To make frozen cell stocks, the detached cells were resuspended in the freezing medium [the growth medium containing 10% DMSO (Sigma)] and frozen at -70 °C for 24 hours. Cells were then put into a liquid nitrogen tank the following day.

### 2.III.C. Adipocyte Differentiation and Lipid Staining

| *Stable Cell Line* | *Parental Cell Line* | *Drug Resistance* | *Cell Line* |
|---|---|---|---|
| AEBP1/Neo-3<br>AEBP1/Neo-7<br>AEBP1/Neo-11<br>Neo-1<br>Neo-9 | 3T3-L1 | G418 | Clone |
| AS-4<br>AS-6 | AEBP1/Neo-7 | Puromycin<br>G418 | Clone |
| AS/Neo-7<br>AS/Neo-11<br>Neo-12 | 3T3-L1 | G418 | Clone |
| AEBP/Neo-P<br>AEBP1ΔHic/Neo-P<br>AEBP1ΔSty/Neo-P<br>Neo-P-P | 3T3-L1 | G418 | Pool |
| AEBP1DLD/Puro<br>AEBP1DLD(-)/Puro | AEBP1/Neo-P | Puromycin | Pool |
| 3T3-L1/DLD(+)<br>3T3-L1/DLD(-) | 3T3-L1 | G418 | Pool |

Table 2.2. Stable Cell lines and Drug Resistance

Stable cell lines transfected with pWZLneo, pBabe-puro, or pREP vectors were maintained in the growth medium with 200 ng/ml G418 (Life Technologies), 1 μg/ml puromycin (Sigma), and 200 ng/ml hygromycin (Boehringer Mannherim), respectively. At confluence, designated as day zero, adipocyte differentiation was induced by treating confluent cells with differentiation-inducing medium [DMEM containing 10% fetal bovine serum (FBS, Life Technologies) or cosmic calf serum with 5 μg/ml insulin (Sigma), 1μM DEX (Sigma), and 0.5 mM MIX (ICN)] for 2 days. The cells were then maintained in DMEM containing 10% FBS and 5 μg/ml insulin every 2 days for 5 additional days. Stable cells expressing antisense-AEBP1 were made to differentiate by DMEM containing 10% cosmic calf serum, 5 μg/ml insulin, and various amount (0.05, 0.5, or 5 μM) of BRL49653 every 2 days for 7 days.

For staining of lipid droplets, the cells were washed three times with PBS and fixed by incubating in 4% paraformaldehyde (ICN) for 10 min. The fixed cells were then permeabilized by incubating in 50% methanol for 5 min and stained with a saturated oil Red O (ICN Biomedicals, Inc., Costa Mesa, CA) in 60% triethyl phosphate solution (BDH) for 30 min.

### *2.IV. Transfection*

### 2.IV.A. Transient Transfection

When NIH-3T3 or COS-7 cells reached 60 – 80% confluence in 60-mm dishes (Sarstedt) they were used for transfection. The cells were incubated with 2 ml DMEM, containing 30 μg/ml of polybrene (Aldrich) and the appropriate plasmids, for 6 hours at 37 °C.

Ten micrograms of plasmid encoding either HA-AEBP1 or HA-AEBP1 mutant derivatives were transfected into the cells in order to carry out co-immunoprecipitation studies. For kinase protection assay, 5 μg of pSG5/PYST-Myc and 1 μg of pECE/HA-ERK1 plasmids were co-transfected into the cells, along with various amount (1 – 8 μg) of pJ3H-AEBP1 (or its mutant derivatives). For studies of transcriptional activity of AEBP1, 5 μg of

the reporter plasmid paP2(3AE-1-120)CAT and 10 μg of pJ3H-AEBP1 (or its mutant derivatives) were used. For an internal control, pHermes-lacZ (1 μg), a plasmid that expresses the *lacZ* gene under the control of the CMV promoter was used.
Following the 6 hour incubation with plasmids, cells were shocked with 2 ml of 30% DMSO in DMEM for 1 - 2 minutes at room temperature, replaced with 4 ml DMEM supplemented with 10% Cosmic calf serum, and cultured for another 48 hours.

### 2.IV.B. Stable Transfection Using Retroviral Infection

The viral packaging cells, Ψ2, were cultured in 100-mm plates containing fresh DMEM with 10% cosmic calf serum until 80% confluence. Then, 20 μg of retroviral vectors (pWZLneo, pREP-hygro, or pBabe-puro) encoding AEBP1 or its mutants were transfected into the cells.

Two days after transfection, the culture media (viral supernatants) were collected and filtered through a 0.45 μm (pore size) filter (Millipore Corp.). 3T3-L1 cells were infected by incubating a 10-mm plate containing ~5 x $10^5$ cells with 2 ml of viral supernatants in the presence of polybrene (8 μg/ml) for 2.5 hours. Fresh medium (8 ml) was added, and the cells were then grown for 2 days to reach confluence. The infected cells were reseeded at a ratio of 1:12 in selective medium containing G418 (400 μg/ml), puromycin (300 μg/ml) or puromycin (1 mg/ml). The selection medium was changed every 4 days for 10 – 14 days to select for resistant colonies. The virus titers obtained using this method were $10^3$-$10^4$ colonies/ml of viral supernatant. The colonies were pooled and used for further analysis. The stable cell lines representing either individual clones or pool population were generated as described in Table 2.2. The selection markers (or drug resistant marker) for stable cell lines were also presented (see Table 2.2).

For the generation of individual stable clones, 3T3-L1 cells were transfected with 20 μg of pWZLneo vector expressing AEBP1 at 70% confluence. The cells were selected with G418 (400 μg/ml) as described above. In order to attenuate AEBP1 expression in the stable clone overexpressing AEBP1 (clone pAEBP1/Neo-7), cells was transfected with 20 μg of pREP-hygro vector expressing antisense AEBP1 at 70% confluence. The cells were selected with puromycin (300 μg/ml).

### 2.IV.C. β-Galactosidase Assay

β-Galactosidase activity was assayed 48 h after transfection to normalize transfection efficiency. Cells were washed twice with cold PBS, collected in 1 ml PBS, and centrifuged at 6,500 rpm for 5 minutes at 4°C. The pellet was then resuspended in 100 µl CAT/TE buffer (250 mM Tris pH 8.0, 5 mM EDTA). Cells were lysed by freezing in liquid nitrogen and thawing at 37°C for 3 minutes three times. The supernatant was collected after centrifugation at 14,000 g at 4°C for 10 minutes and stored at -70°C until the assay.

For β-Galactosidase activity, the supernatant (30 µl) were incubated in reaction buffer [3 µl 100x Mg buffer (100 µl 1M $MgCl_2$, 350 µl β-mercaptoethanol, 550 µl $H_2O$), 66 µl 1x ONPG (4 mg/ml in 0.1 M $NaHPO_4/Na_2PO_4$), and 201 µl 0.1 M $NaHPO_4/Na_2PO_4$] for approximately 6 hours and measured OD at $A_{410}$.

### 2.IV.D. Chloramphenicol Acetyltransferase (CAT) Assay

To assay for AEBP1 transcriptional activity, the CAT assay was used as described (He et al., 1995). The remaining supernatant (70 µl) was heated at 60°C for 10 minutes, and incubated with 10 µl of reaction buffer [2 mg acetyl-CoA (Pharmacia), 100 µl CAT/TE, 5 µl $^{14}C$-chloramphenicol (0.5 µCi; ICN); for 10 reactions]. The reaction was stopped by adding 500 µl ethyl acetate after incubation for 4 hours at 37°C. The samples were vortexed for 30 seconds and centrifuged for 1 minute. The top organic layer was collected and dried in a Speed Vac (Savant). Dried samples were dissolved in 10 µl ethyl acetate, spotted on a thin layer chromatography (TLC) silica plate (Sigma), and run in a saturated TLC tank (5 ml methanol, 95 ml chloroform) for 30 minutes. The plate was air-dried and exposed in the phosphorimager (BioRad), and the % conversion of chloramphenicol to acetyl-chloramphenicol was measured by computerized analysis with the phosphorimager software.

## *2.V. Isolation and Analysis of RNA*

### 2.V.A. Isolation of RNA

Cells were washed twice with PBS and were treated with 1 ml of TRIZOL Reagent (Life Technologies) for 5 minutes. The cell lysates were incubated with chloroform (0.2 ml) for 2 minutes with vigorous shaking and centrifuged at 12,000 x g for 15 minutes at 4°C. To precipitate RNA, the aqueous phase were transferred to a fresh tube and mixed with isopropyl alcohol (0.5 ml) for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA pellets were washed twice with 75% ethanol (1 ml). Following air drying, the pellets were dissolved in 1% DEPC(diethyl pyrocarbonate, Sigma)-treated $H_2O$ and incubated for 10 minutes at 60°C and stored at -70°C for later use. RNA concentration was measured at $A_{260/280}$ using a UV/VIS spectrophotometer.

### 2.V.B. Agarose Gel Electrophoresis

Agarose/formaldehyde gels with ethidium bromide (1 μg/ml) were prepared as described (Ausubel et al., 1994). RNA samples (20 μg) were mixed with 7 μl of sample buffer [2 μl of 10X MOPS buffer (0.2 M MOPS, 0.5 M sodium acetate, 0.01 M EDTA, pH 7.0), 3 μl of 12.3 M formaldehyde, and 2 μl of loading buffer [(1 mM EDTA, 0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, 50% (v/v) glycerol]. After adjusting to 20 μl total volume with DEPC-treated $H_2O$, samples were denatured for 15 minutes at 65°C and placed on ice for 30 seconds. Then samples were loaded onto the gel in 1X MOPS buffer. Following electrophoresis, the gel was washed twice with $H_2O$ for 5 minutes and soaked in 10X SSC (1.5 M NaCl, 0.15 M $Na_3Citrate.2H_2O$, pH 7.0). RNA in the gel was then transferred onto duralon-UV membranes (Stratagen) overnight as described (Ausubel et al., 1994) or for 30 – 60 minutes using PosiBlot™ Pressure Blotter (Stratagen) at the pressure of 75 mm Hg. The transferred RNA was UV-crosslinked for 30 seconds at 245-wavelength using a UV stratalinker (Stratagen).

### 2.IV.C. Hybridization Analysis

RNA blots were incubated with the hybridization buffer [50 mM PIPES (pH 6.5), 100 mM NaCl, 50 mM sodium phosphate (pH 7.0), 1 mM EDTA (pH 7.0) and 5% SDS] for 15 minutes at the appropriate hybridization temperature (62 – 65°C) as described (Virca et al., 1990). The hybridization buffer was then discarded and replaced with fresh hybridization buffer containing the specific probes ($10^6$ cpm/ml) that has been denatured by incubating with 0.1 M NaOH at 37°C for 30 seconds. Hybridization was carried out overnight in a hybridization oven (Bio/Scan Hybridization). Following hybridization, blots were rinsed twice for 15 minutes in 50 ml of 2x SSC and 0.1% SDS at room temperature. Blots were then washed twice for 20 minutes in 0.2x SSC and 0.1% SDS at the hybridization temperature and then exposed X-ray film at –70 °C or to phosphorimager (BioRad).

### 2.IV.D. Radiolabeling Probes

DNA (25 ng) was denatured by heating for 5 minutes and immediately cooling on ice. DNA was radiolabeled ([$\alpha$-$^{32}$P] dCTP at >3000 Ci/mmol) using the Random Primers DNA Labeling System (Life Technologies) at least for 3 hours at room temperature. The radiolabeling reaction was stopped by adding 5 µl of stop buffer (Life Technologies). The labeled probe was purified from unincorporated nucleotides through a MicroSpinTM G-50 column (Pharmacia) by centrifugation for 1 minutes at 3000 rpm. The probe's specific activity was measured by liquid scintillation counter.

The major probes used in this thesis were as follows: For the AEBP1 probe, pG4AEBP1 was digested by BamHI, released 700 bp fragments and used as a probe. PPAR$\gamma$, a full length cDNA (1.2 kb) was cut by EcoRI from pSV.SPORT-PPAR$\gamma$. The C/EBP$\alpha$ probe (2.0 kb) was the full length cDNA obtained by digesting pSV.SPORT-C/EBP$\alpha$ with EcoRI and HindIII. The Neo probe was a 700 bp fragment cut from pWZLneo with HindIII and SphI.

## *2.VI. Analysis of Proteins*

### 2.VI.A. Preparation of Total Cell Lysate

Cells were washed twice with PBS and incubated in 1 ml cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 50 mM $Na_2PO_7 \bullet 7H_2O$, 0.23%[w/v] sodium deoxycholate, 0.1% Nonidet p-40, 1 mM $Na_3VO_4$, 1 mM NaF, 5 mM EDTA, and 5 mM EGTA, pH 7.4) containing proteinase inhibitors (1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin) on ice for 30 minutes. Cells were transferred to an Eppendorf tube and disrupted by repeated pipetting through a 21 gauge needle. PMSF (10 μl of 10 mg/ml stock) was added to cells. The cell lysates were then centrifuged at 12,000 x g for 20 minutes at 4°C and the supernatant was collected.

### 2.VI.B. Determination of Protein Concentration

Sample (10 μl) were mixed with 790 μl $H_2O$ and 200 μl Coomassie brilliant blue G-250 dye (BioRad) and incubated at room temperature for 10 minutes. The mixture was measured at $A_{595}$ using a UV/VIS spectrophotometer. Protein concentration was calculated from a standard curve which was obtained by measuring the absorbance of various concentrations of bovine serum albumin (BSA) in $H_2O$.

### 2.VI.C. Polyacrylamide Gel Electrophoresis (PAGE) and Western Blot Analysis

Proteins (20μg) were mixed with 3X sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromphenol blue] to final volume of 30 μl $H_2O$ and denatured by heating at 95 °C for 5 minutes. After pelleting for 2 minutes at 13,000 rpm, the samples were loaded into 8.5, 10, or 15% SDS-PAGE mini-gels (Bio-Rad). Pre-stained protein markers (NEB) were used to determine molecular weights. The nitrocellulose membranes (Micron Separations, Westborough, MA) were soaked in the transfer buffer [48 mM Tris, 39 mM glycine, and 20% methanol; pH 9.2] for 15 minutes. The

gels were electro-transferred onto the membranes at 7 V for 30 – 60 minutes using Trans-Blot SD cell (BioRad). Membrane blocking was performed with blocking buffer [5% milk powder in TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Tween-20)] for 1 hour at room temperature with constant shaking. Membranes were washed three times with TBST for 15 minutes and incubated with 100 ng/ml of commercial antibodies for 1 hour at RT or with 200 ng/ml of anti-AEBP1 antibody for overnight at 4 °C in the blocking buffer. The membranes were then washed three times with TBST, and then incubated with horseradish peroxidase (HRP) conjugated anti-rabbit or anti-mouse secondary antibodies (1:5,000 dilution) for 1 hour in the blocking buffer. For immuno-detection, membranes were incubated with either Amersham ECL reagents for 1 minute or ECL plus reagents for 15 minutes. The membranes were wrapped in SaranWrap and exposed to X-ray film for 10 seconds to 4 hours.

## *2.VII.* Interacting Assay

### 2.VII.A. Immunoprecipitation and Kinase Analysis

Total proteins were collected as above and incubated with protein A-agarose for 1 hour at 4°C and centrifuged at 5,000 rpm for 5 minutes. The beads were then discarded and the supernatant was incubated with specific antibodies (2 μg/ml anti-HA, anti-ERK1, anti-MEK1, or anti-IGFR, or 2.5 μg/ml of either affinity-purified anti-AEBP1 antibodies or IgG from preimmune serum) for 1 hour and then overnight with protein A-agarose for 1 hour. The agarose beads were pelleted by centrifugation at 5,000 x g for 5 minutes and washed 4 times with RIPA buffer. The precipitated proteins were released with 1 x loading buffer (NEB) by incubating in boiling water for 5 minutes. Samples were then resolved by SDS-PAGE (8.5, 10, or 15%) and analyzed by Amersham's ECL or ECL plus Western blotting system with specific antibodies (200 ng/ml) as described in section 2.VI.C.

For the immuno-complex kinase assay, MAP kinase or MEK was immunoprecipitated from cell lysates with specific antibodies overnight. The pelleted agarose beads were washed three times with RIPA buffer and equilibrated three times in kinase buffer [30 mM Tris-HCl (pH7.4), 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1mM sodium orthovanadate, 1

mM dithiothreitol], then 15 μCi [$\gamma^{32}$P]ATP and specific substrate were added and incubated for 30 min at 37 °C. Reactions were stopped by adding SDS-PAGE loading buffer. Samples were resolved by either 8.5% (AEBP1), 12% (p42), or 15% (MBP) SDS-PAGE and transferred to nitrocellulose membranes. Bands were detected and quantitated by the Molecular Imager system (Bio-Rad Laboratories). Total ERK1 and MEK1 were measured by the ECL Western blotting system with anti-ERK1 and anti-MEK1 antibodies (200 ng/ml), respectively. Pre-stained protein markers (NEB) were used to determine molecular weights.

### 2.VII.B. *In vitro* Protein-Protein Interaction Assay

The His-tagged recombinant AEBP1 protein or the mutant derivatives (300 ng) was mixed with or without 300 ng of the recombinant ERK2 protein for 1 hour at 30 °C. The reaction mixture was immunoprecipitated with the anti-p42/44 MAP kinase antibody (1 μg/ml) for 1 hour, then incubated with protein-A agarose for 30 minutes at 4 °C. The pellet was collected, washed, and resolved by 8.5% SDS-PAGE as described in 2.VII.A. AEBP1 and its mutant derivatives were detected by immunoblotting with anti-AEBP1 antibody (200 ng/ml).

## *2.VIII. MAP Kinase Activity Assay*

### 2.VIII.A. Stimulation of MAP Kinase Activation

COS-7 or 3T3-L1 cells were starved in DMEM containing 0.1% calf serum for 2 to 24 hours. The cells were then stimulated with DMEM containing 15% FBS for a various lengths of time or containing 10 nM of EGF (Sigma) for 15 minutes as described previously (Muda et al., 1996). Five microgram of total protein was resolved by SDS-polyacrylamide gel (8.5 %) electrophoresis and transferred to a nitrocellulose membrane as described in section 2.VI.C. Both unphosphorylated and phosphorylated forms of ERK1 and 2 were detected by immunoblotting with anti-ERK1 antibody (200 ng/ml).

### 2.VIII.B. Phosphatase Protection Assay *in vivo*

COS-7 cells were transfected with plasmids expressing the MAP kinase specific phosphatase, PYST1 (pSG5/PYST-Myc), and an HA-tagged version of ERK1 (pECE/HA-ERK1) along with various plasmids encoding AEBP1 or its mutant derivatives as described in Table 1. Two days after transfection, cells were starved in DMEM containing 0.1% calf serum, then stimulated for 15 min with 10 nM of EGF as described in Section 2.VIII.A. The cell lysates in cold RIPA buffer were immunoprecipitated with anti-HA antibody (2 μg/ml) for 1 h and incubated with protein-A agarose for 30 minutes. The assay for MAP kinase activity was performed with of Myelin Basic Protein substrate (MBP, Sigma) as described in 2.VII.A. Alternatively, phosphorylated ERK was detected by immunoblotting with Phospho-p44/42 MAP kinase monoclonal antibody (1:10,000 dilution, NEB).

### 2.VIII.C. *In vitro* Phosphatase Protection Assay

The MAP kinase protection activity of AEBP1 was tested by utilizing the active recombinant murine ERK2 (NEB) protein as described (Alessi et al., 1995). The recombinant phospho-ERK2 (2.5 ng) protein was mixed with 100 ng of recombinant AEBP1 (or its mutant derivatives) protein for 1 hour in RIPA buffer on ice. The mixture was then treated with 100 units of λ-PPase (NEB) for 30 minutes at 30 °C in phosphatase reaction buffer supplemented with 2 mM $MnCl_2$. The reaction was stopped by adding SDS-PAGE loading buffer (NEB). Samples were resolved in 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The treated kinases were analyzed by immunoblotting with Phospho-p44/42 MAP kinase monoclonal and p44/42 MAP kinase antibodies, respectively.

# Chapter 3. Results and Discussion

This chapter comprises four main sections. Since previous studies by Dr. Muise provided a clue for an interaction between AEBP1 and MAP kinase, the first section characterizes the interaction. The specificity of AEBP1 binding to MAP kinase was investigated by *in vitro* and *in vivo* co-immunoprecipitation assays. Furthermore, the domain of AEBP1 responsible for interaction with MAP kinase was also examined.

The second section deals with the effect of AEBP1 on MAP kinase activation. Using individual clones expressing different levels of AEBP1, I determined whether or not the AEBP1 expression can modulate the activation of MAP kinase or its upstream kinase (MEK). Furthermore, since AEBP1 is abolished in mature adipocytes, I determined whether AEBP1 could modulate MAP kinase activity under physiological conditions.

In the third section, a possible mechanism for AEBP1 modulation of MAP kinase activation is explored. Since AEBP1 was originally known as a transcriptional repressor, I considered whether the modulation of MAP kinase activation is dependent on the physical interaction of AEBP1 with MAP kinase or on the transcriptional activity of AEBP1.

The last section deals with the critical role of MAP kinase activation by AEBP1 in adipogenesis. Using stable cell lines overexpressing AEBP1 or antisense AEBP1 mRNA, I investigated whether the modulated MAP kinase activation by AEBP1 stimulates or inhibits adipogenesis. The above questions are discussed at the end of each section.

## *3.I. AEBP1 Association with MAP Kinase*

### 3.1.A. AEBP1 interacts with MAP Kinase *in vitro* and *in vivo*

MAP kinase is a prominent component of signaling cascades that regulate intracellular activities in response to physiological changes. The MAP kinase family is divided into subfamilies based on conserved residues, particularly a TXY (Thr-Xaa-Tyr) motif in the activation loop which can be phosphorylated by MEK (or MAP kinase kinase)

(Hill and Treisman, 1995; Marshall, 1995). Among the MAP kinase family members, ERK1 and ERK2 are well known kinases that are activated in a mechanism involving dual phosphorylation at both tyrosine and threonine, and are involved in many differentiation processes (discussed in section 1.V). AEBP1, which is a novel transcription factor (He et al., 1995) and an interacting partner of the γ5 subunit of a heterotrimeric G protein (Park et al., 1999), has a number of structurally and functionally distinctive features. In the N-terminus, there is a discoidin-like domain (DLD) that is believed to be involved in protein-protein, protein-phospholipid, or protein-carbohydrate interactions (discussed in section 1.III). In the C-terminus, there are serine, threonine, proline (STP)-rich regions with a few potential MAP kinase proline-directed phosphorylation sites which are flanked each side by a putative nuclear localization signal (RRLQYRLRMREQMRLRR) and a glutamic acid-rich region (see Figure 1.3). Another potential MAP kinase site (PMTP), which is identical to the consensus sequence PXS/TP (for review, see Davis 1993; Songyang et al., 1996), is located N-terminally from the putative nuclear localization signal.

An earlier study showed that AEBP1 is phosphorylated by MAP kinase *in vitro* and suggested that the site for phosphorylation of AEBP1 by MAP kinase is possibly located at the C-terminus, since no phosphorylation was detected with a AEBP1-truncated mutant that lacks the C-terminus (Muise, 1997). Furthermore, using immunoprecipitation and immunoblot experiments with antibodies toward AEBP1 and phospho tyrosine, this study also showed that AEBP1 was not phosphorylated by a tyrosine kinase. Interestingly, during these procedures, two phosphoproteins with the approximate molecular weights of the MAPKs, ERK1 (44 kDa) and ERK2 (42 kDa), were detected when AEBP1 immunoprecipitates were probed with anti-phosphotyrosine antibody. These observations prompted us to examine the possibility that MAP kinase may have co-immunoprecipitated with AEBP1.

To ascertain whether AEBP1 interacts with MAP kinase directly or via intermediates, the bacterially produced recombinant proteins, AEBP1 and ERK2, were made. The recombinant proteins were purified by binding to a metal affinity column (Clontech), eluted, and renatured. The purification process allowed the isolation of nearly homogeneous recombinant protein of an estimated molecular weight of 83 kDa for AEBP1 and 42kDa for ERK2 (Figure 3.1).

**Figure 3.1. Expression and purification of recombinant His-tagged AEBP1 and ERK2**

The recombinant His-tagged AEBP1 and ERK2 proteins were measured by immunoblotting with either anti-AEBP1 or ERK1 antibody that can cross-react with ERK2. Lanes 1, 2 , and 3 shows different amounts (20, 10, 5 μg) of AEBP1 loaded. Lane 4 shows ERK2. kDa, molecular weight in thousands.

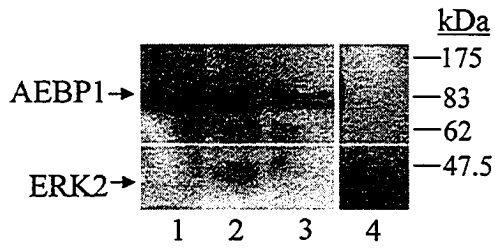


Figure 3.1. Expression and purification of recombinant His-tagged AEBP1 and ERK2

Since co-immunoprecipitation systems have been widely used to show a protein-protein interaction, the recombinant proteins, AEBP1 and ERK2, were co-immunoprecipitated. The His-tagged recombinant AEBP1 proteins were incubated with the recombinant ERK2 protein, then the mixture was immunoprecipitated with anti-p44/42 MAPK antibody (NEB). As shown in Figure 3.2, AEBP1 (lane 3) was detected in the immunoblot. When the mixture containing the recombinant AEBP1, but without the recombinant ERK2, was immunoprecipitated with anti-p44/42 MAPK antibody (lane 2) or pre-immune serum (lane 1), AEBP1 was not detected in the immunoblot experiment. These results suggest the direct association of AEBP1 with ERK2 *in vitro*.

To test the interaction between AEBP1 and MAP kinase in mammalian cells, I subcloned the AEBP1 cDNA into a mammalian expression vector (pJ3H) and created an expression plasmid (pJ3H-AEBP1) encoding an HA-tagged version of AEBP1. The plasmid was transfected into COS-7 cells, followed by the preparation of cell lysates and immunoprecipitation with anti-HA antibody. The immunoprecipitates were then subjected to western blotting and was probed with anti-ERK1 antibody. Figure 3.3 (bottom panel) shows that ERK1 was detected in the immunoprecipitates from cells transfected with pJ3H-AEBP1 (lane 1), but not from cells transfected with the control plasmid pJ3H-AEBP1 (-) (lane 4). MAP kinase was not detected in the negative control immunoprecipitates (anti-IGFR) using cells transfected with either pJ3H-AEBP1 (lane 3) or pJ3H-AEBP1 (-) (lane 6). Reciprocal immunoprecipitation experiments with anti-ERK1 antibody and immunoblotting with anti-HA antibody (Figure 3.3, top panel) demonstrated that HA-AEBP1 was detected in pJ3H-AEBP1 transfected cells (lane 2), but not in cells transfected with the control plasmid (lane 5). Again, HA-AEBP1 was not detected in the control immunoprecipitates from cells transfected with pJ3H-AEBP1 (lane 3) or pJ3H-AEBP1 (-) (lane 6). These results indicate that AEBP1 also interacts with MAP kinase in mammalian cells.

I also tested whether AEBP1 interacts with an upstream component (MEK1) of the MAP kinase pathway. The same plasmid (pJ3H-AEBP1) as described above was

**Figure 3.2. Direct binding of AEBP1 to ERK2 *in vitro***

To test direct binding of AEBP1 to ERK2, *in vitro* co-immunoprecipitation analysis was carried out. Three hundreds nanograms of the His-tagged recombinant AEBP1 (lanes 1-3) were individually mixed with (lane 3) or without (lanes 1 and 2) 300 ng of the recombinant ERK2 protein. The mixture was immunoprecipitated either with pre-immune IgG (lane 1) or p44/42 MAPK antibody (lanes 2 and 3; NEB). The precipitates were immunoblotted with anti-AEBP1 antibody.

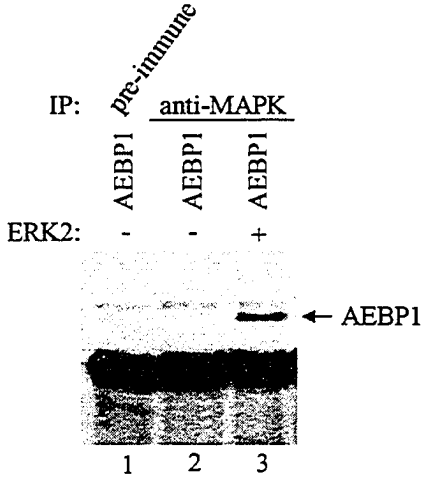


Figure 3.2. Direct binding of AEBP1 to ERK2 *in vitro*

**Figure 3.3. Interaction of AEBP1 with ERK1 *in vivo***

Equal amounts of COS-7 cell extracts, transfected with 10 μg of pJ3H-AEBP1 (lanes 1-3) or the control plasmid pJ3H-AEBP1 (-) (lanes 4-6), were used in immunoprecipitation studies with anti-HA antibody (lanes 1 and 4), anti-ERK1 antibody (lanes 2 and 5), or anti-IGFR antibody (lanes 3 and 6). The immunoprecipitated samples were immunoblotted with either anti-HA antibody (top panel) or anti-ERK1 antibody (bottom panel). IP: immunoprecipitation.

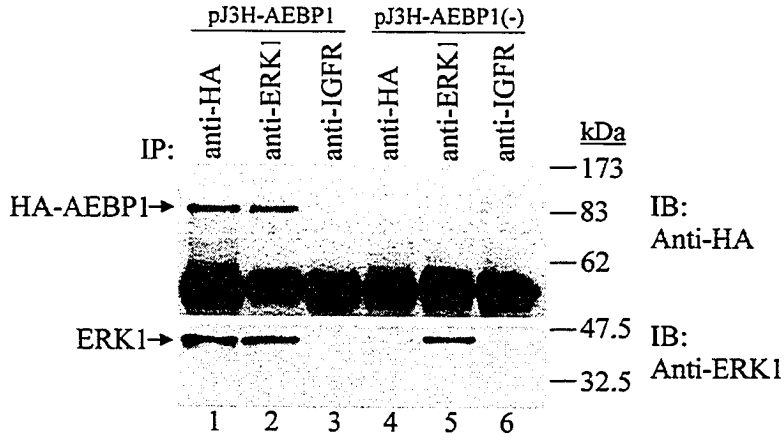


Figure 3.3. Interaction of AEBP1 with ERK1 *in vivo*

transfected into COS-7 cells. Cell extracts were collected at preconfluent stage, then immunoprecipitated with anti-HA, anti-ERK1, anti-MEK1, or anti-IGFR antibody, and then immunoblotted with anti-HA. As shown in Figure 3.4, HA-AEBP1 was not detected in MEK1 immunoprecipitates (lane 3) whereas it was detected in both the HA and ERK1 immunoprecipitates (lanes 1 and 2). HA-AEBP1 was not detected in the immunoprecipitates from anti-IGFR (lane 4).

If AEBP1 interacts with only a phosphorylated MEK1, then there may be little chance of identifying the interaction in the unstimulated cells (Alessi et al., 1995). Cells were stimulated with serum for 5 minutes to phosphorylate MEK1. Then the cell extracts were analyzed by the co-immunoprecipitation assay described above. In this experiment, AEBP1 also did not interact with phosphorylated MEK1 (data not shown). However, I can not rule out the possibility that AEBP1 interacts with other upstream components of the MAP kinase pathway.

In the series of experiments described above, the amount of AEBP1 or MAP kinase that remains unbound in the transfected cells is not indicated. Therefore, it was necessary to show how much of the total AEBP1 interacts with MAP kinase, and how much of the total MAP kinase interacts with AEBP1. To further assess the interaction between AEBP1 and MAP kinase, I carried out reciprocal immunoprecipitation and immunoblotting experiments. 3T3-L1 cells were grown until the preconfluent stage. To assess how much of the total AEBP1 and MAP kinase interact with each other, cell extracts were immunoprecipitated with either anti-AEBP1 or anti-ERK antibodies, and then post-precipitated cell extracts were further immunoprecipitated with the opposite antibodies. Figure 3.5 shows that the amount of AEBP1 that remains unbound (top, lane2) is approximately similar to the amount of AEBP1 associated with ERK1 and ERK2 (top, lane 1). Conversely, the amount of MAP kinase that remains unbound (bottom, lane 4) is approximately similar to the amount of MAP kinase associated with AEBP1 (bottom, lane 3). These results further confirm that AEBP1 and MAP kinase interact in mammalian cells.

Because AEBP1 associates with MAP kinase, MAP kinase may be involved in the regulation of AEBP1, or AEBP1 may participate in the activation of the MAP kinase signaling pathway. AEBP1 may interact with the unphosphorylated (inactive) form of MAP kinase and either inhibit or enhance MAP kinase activation, which occurs by phosphorylation at specific threonine and tyrosine residues on MAP kinase by the upstream kinase, MEK (Hill and Treisman, 1995; Plelech and Sanghera, 1992). It therefore would be interesting to

**Figure 3.4. AEBP1 does not interact with MEK**

The co-immunoprecipitation assay (see legend of Figure 3.3) was carried out to test the interaction of AEBP1 with MEK1. Cell extracts, transfected with pJ3H-AEBP1 (lanes 1-4) or pJ3H-AEBP1 (-) (lanes 5-8), were immunoprecipitated with anti-HA (lanes 1 and 5), anti-ERK (lanes 2 and 6), anti-MEK1 (lanes 3 and 7), and anti-IGFR (lanes 4 and 8) antibodies. The immunoprecipitated samples were probed with anti-HA antibody after western blotting.

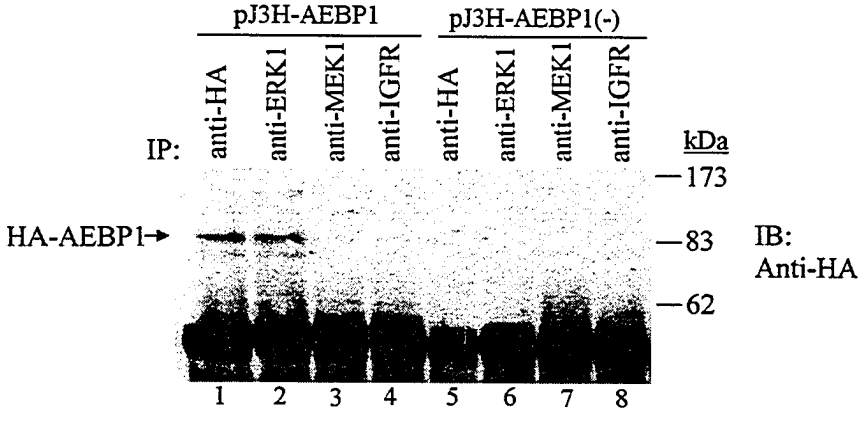


Figure 3.4. AEBP1 does not interact with MEK

**Figure 3.5. Reciprocal co-immunoprecipitation in 3T3-L1 cells**

Total protein (1 mg) from 3T3-L1 cell extracts was subjected to immunoprecipitation with either anti-AEBP1 or anti-ERK1 antibodies ($1^{st}$), and the post-immunoprecipitation supernatants were further immunoprecipitated with the opposite antibodies ($2^{nd}$). Both sets of precipitated samples were analyzed by immunoblotting (IB) with anti-AEBP1 (top panel) and anti-ERK1 (bottom) antibodies, respectively. As a control, the same amount of total protein was immunoprecipitated with either anti-IGFR antibody (lane 5) or pre-immune IgG (lane 6).

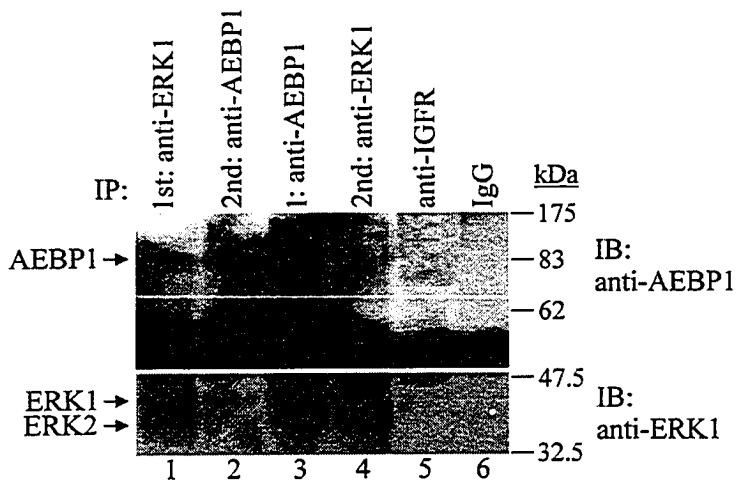


Figure 3.5. Reciprocal co-immunoprecipitation in 3T3-L1 cells

determine whether AEBP1 interacts with the phosphorylated (active), unphosphorylated (inactive), or both forms of MAP kinase. Though many techniques have been developed to show MAP kinase activation, a gel-shift mobility assay was chosen (Muda et al., 1996). The phosphorylation of MAP kinase can be detected by slower migration of the phosphorylated form in SDS-polyacrylamide gels as reported previously. I used cell extracts from quiescent and serum-stimulated 3T3-L1 cells. As expected, the phosphorylated forms of ERK1 (44kDa) and ERK2 (42kDa) were slightly shifted and observed after serum stimulation (Figure 3.6. lanes 2 to 5), compared with the unphosphorylated form found in quiescent cells (lanes 1 and 6). These data show the difference in the electrophoretic mobilites of the phosphorylated and unphosphorylated forms of ERK1 and ERK2.

To determine the phosphorylation state of MAP kinase that complexes with AEBP1, an immunoprecipitation experiment was done with quiescent or serum-stimulated 3T3-L1 cells at preconfluent stage by Dr. A. Muise. AEBP1 was isolated from cell extracts by immunoprecipitation with the anti-AEBP1 antibody, and MAP kinase was then detected by immunoblot analysis. As Figure 3.7 illustrates, the MAP kinases, ERK1 and ERK2, were detected in the AEBP1-immunoprecipitates prepared from both quiescent (lane 3) and the serum-stimulated cells (lane 4). Thus, AEBP1 associates with both forms of MAP kinase.

Since AEBP1 stably associates with the phosphorylated form of MAP kinase, AEBP1 may be involved in the regulation of MAP kinase activation (discussed in section 3.II).

### 3.I.B. Domain of AEBP1 Responsible for MAP Kinase Interaction *in vivo* and *in vitro*

To locate a domain(s) of AEBP1 responsible for MAP kinase interaction, AEBP1 cDNA or its mutant derivatives were subcloned into a pJ3H vector for transcription experiments and into a pET-16b vector to generate recombinant proteins for *in vitro* co-immunoprecipitation assays. The constructs encoding mutant derivatives of AEBP1 are illustrated in the Figure 3.8. AEBP1DLD contains the discoidin-like domain (DLD) and a part of carboxypeptidase domain, while AEBP1ΔDLD deletes the N-terminal portion. AEBP1 ΔHic does not have a part of carboxypeptidase domain which is known to be vital for transcriptional repression function(He et al., 1995). AEBP1 ΔSty does not contain the

**Figure 3.6. MAP kinase activation (phosphorylation) in 3T3-L1 cells by serum stimulation**

Quiescent cells (lanes 1 and 6) were serum-stimulated for a various lengths of time as indicated (lanes 2-5). Equal amounts of cell extracts were resolved in a 12% SDS-PAGE gel and then analyzed by immunoblotting with anti-ERK antibody. The phosphorylated form of MAP kinase migrates slower than the unphosphorylated form.

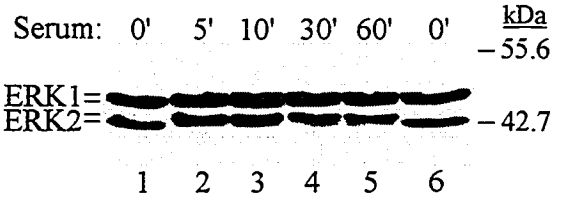


Figure 3.6. MAPK activation (phosphorylation) in 3T3-L1 cells by serum stimulation

**Figure 3.7. AEBP1 interacts with the phosphorylated and unphosphorylated forms of MAP kinase**

Cell extracts from quiescent (lanes 1 and 3) or serum-stimulated (lanes 2 and 4) cells were used in immunoprecipitation studies with either anti-ERK1 (lanes 1 and 2) or anti-AEBP1 (lanes 3 and 4) antibodies, and immunoprecipitates were analyzed by immunoblotting with anti-ERK antibody. Protein samples were resolved in 12% SDS-PAGE gels and then transferred to nitrocellulose for immunoblotting. IP indicates the antibody used to immunoprecipitate the samples, either α-ERK1 (protein A-conjugated anti-ERK1 antibody, Santa Cruz Biotechnology) or α-AEBP1 (anti-AEBP1 antibody).

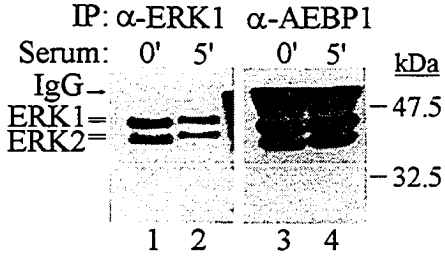


Figure 3.7. AEBP1 interacts with the phosphorylated and unphosphorylated forms of MAPK

**Figure 3.8. Schematic representations of the AEBP1 coding region in the chimaeric mutant proteins**

The small black boxes represent sequences created by frameshift mutations. The numbers indicate amino-acid residues of AEBP1. Due to a sequencing correction at the 5' untranslated region of AEBP1 cDNA, an extra 29 amino-acid residues are added at the N terminus of the published sequence (He et al., 1995). The presence (+) and absence (-) of AEBP1 interaction with MAP kinase is shown.

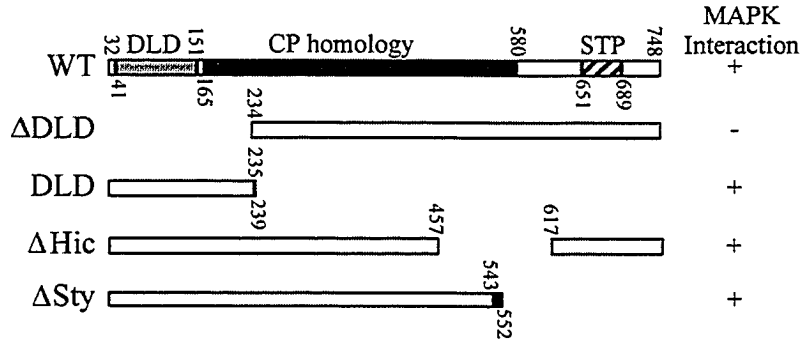


Figure 3.8. Schematic representations of the AEBP1 coding region in the chimaeric mutant proteins

carboxyl terminus and may not have transcriptional activity since this mutant failed to bind DNA (Muise, 1997).

The pJ3H constructs were transfected individually into COS-7 cells and analyzed for the ability of the encoded AEBP1 polypeptides to interact with MAP kinase by co-immunoprecipitation experiments. Figure 3.9 shows that the wild-type and mutant derivatives of HA-AEBP1 were detected in cells transfected with the respective plasmid, but not in the control cell transfected with the empty vector pJ3H (top panel). Next, lysates prepared from cells transfected with these plasmids were immunoprecipitated with anti-HA antibody, and the immunoprecipitates were immunoblotted with anti-ERK1 antibody. Figure 3.9 (bottom panel) shows that ERK1 was co-immunoprecipitated from cell lysates containing the wild-type or most mutant derivatives (lanes 1, 3, 4, and 5). However, ERK1 was not co-immunoprecipitated from cell lysates prepared from cells producing the mutant derivative HA-AEBP1 ΔDLD (lane 2) that lacks the N-terminal 233 amino acids (see Figure 3.8), or cells transfected with the empty vector pJ3H (lane 6). Interaction with MAP kinase was not abolished either by deletion of the carboxypeptidase domain (pJ3H-AEBP1 ΔHic, lane 4) or by the truncation at the C terminus (pJ3H-AEBP1 ΔSty, lane 5). Significantly, ERK1 was co-immunoprecipitated from cell lysates containing the mutant derivative HA-AEBP1DLD (lane 3) which expresses only the N-terminal 204 amino acids (residues 32 to 235) of AEBP1 (see Figure 3.8). Therefore, these results indicate that a domain of AEBP1 responsible for MAP kinase interaction is located at the N terminus, which contains sequences that were previously termed discoidin-like domain (DLD; He et al., 1995), and suggest that phosphorylation is not required for AEBP1 to interact with MAP kinase.

Next, I assessed a direct interaction between the AEBP1 mutant derivatives and MAP kinase in an *in vitro* co-immunoprecipitation experiment, utilizing the bacterially produced recombinant proteins as described earlier. The wild-type and mutant derivatives of the His-tagged recombinant AEBP1 proteins (He et al., 1995) were mixed individually with the recombinant ERK2 protein, then the mixture were immunoprecipitated with anti-ERK antibody. The precipitates were then analyzed by immunoblotting with anti-AEBP1 antibody. As shown in Figure 3.10, the wild-type (lane 3) and all the mutant derivatives of His-AEBP1 (lanes 5-7), except the mutant ΔDLD with amino-terminal deletion (lane 4), were detected in the immunoblot. Specifically, a mutant derivative which consists of only the amino-terminal 204 amino-acids (DLD) was able to co-immunoprecipitate with the recombinant ERK2

**Figure 3.9. AEBP1 interacts with MAP kinase through its N-terminal domain *in vivo***

COS-7 cells were individually transfected with 10 μg of pJ3H-AEBP1 (lane 1) and its mutant derivatives as indicated (lanes 2-5). Lane 6 contains material from cells transfected with the empty vector pJ3H. Cell extracts from each of the transfected cells were analyzed by immunoblotting with anti-HA antibody (top panel). * indicates HA-AEBP1 and its mutant derivatives. Cell extracts were also immunoprecipitated with anti-HA antibody and then analyzed by immunoblotting with anti-ERK1 antibody (bottom panel).

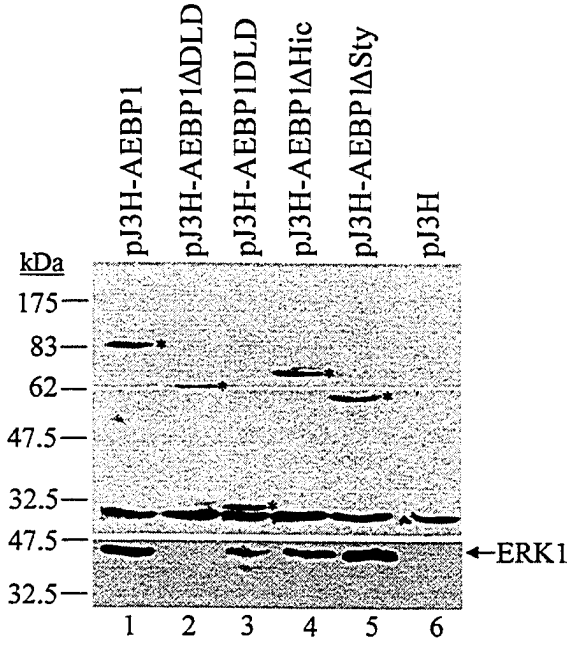


Figure 3.9. AEBP1 interacts with MAPK through its N-terminal domain *in vivo*

**Figure 3.10. Direct interaction of the N-terminus of AEBP1 with ERK2 *in vitro***

As previously described, the *in vitro* co-immunoprecipitation assay (see legend of Figure 3.2) was carried out. Three hundred nanograms of the His-tagged recombinant AEBP1 (lanes 1-3) and its mutant derivative (lanes 4-7) proteins were individually mixed with (lanes 3-7) or without (lanes 1 and 2) 300 ng of recombinant ERK2 protein. The mixtures were immunoprecipitated either with pre-immune IgG (lane 1) or p44/44 MAPK antibody (lanes 2-7: NEB). The precipitates were immunoblotted with anti-AEBP1 antibody. * indicates His-AEBP1 and its mutant derivatives.

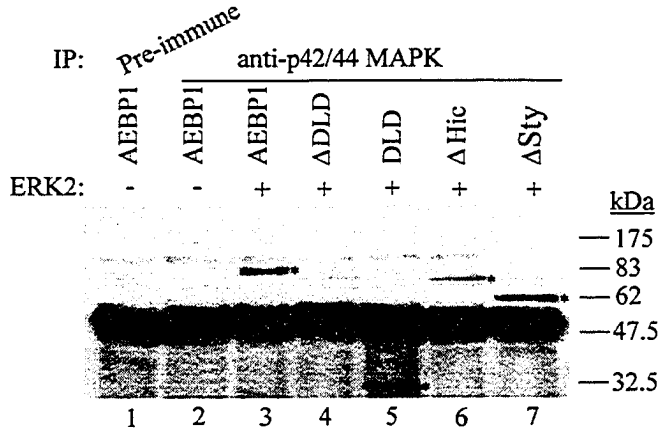


Figure 3.10. Direct interaction of the N-terminus of AEBP1 with ERK2 *in vitro*

protein (lane 5). Furthermore, when a protein mixture containing the wild-type AEBP1, but not the recombinant ERK2 protein, was immunoprecipitated with anti-ERK antibody (lane 2) or pre-immune serum (lane 1) AEBP1 was not detected in the immunoblot experiment. Lack of interaction of the N-terminal deletion mutant with MAP kinase was not due to neither by its inability to be recognized by the anti-AEBP1 antisera nor by preventing immunoprecipitation by anti-ERK antisera. Taken together these results suggest that MAP kinase directly interacts with AEBP1 at the N terminus. Examination of the sequences in the N-terminal domain of AEBP1 did not reveal any MAP kinase docking sites described recently (Jacobs et al., 1999; Yang et al., 1998a, b). Other MAP kinase substrates, including c-Myc, Sos, and pp90rsk, also do not have such defined docking sites. The existence of this class of reported substrates raises the possibility that MAP kinase may interact with other yet-to-be-defined docking sites.

The interaction between MAP kinases and the transcription factors that they phosphorylate is not unprecedented. Several transcription factors, such as c-Fos, GATA-2, c-Myc, and Elk-1, bind to, and are phosphorylated by, MAP kinase (Jacobs et al., 1999). One of the examples is the Elk-1 protein which is a ternary complex factor. In association with serum response factor (SRF), this factor forms a ternary complex on the serum response element of the c-fos promoter (Hipskind et al., 1991) and regulates c-fos transcription. Both *in vitro* and *in vivo*, this protein is a strong substrate for MAP kinase (Rao and Reddy, 1994) and, more interestingly, acts as an activator of MAP kinase (Rao and Reddy, 1993). This suggests that a target of a signaling molecule could also function as its activator and, furthermore, MAP kinase activators, apart from being MEK, can also be non-kinase proteins that might associate with MAP kinase.

Another example is the scaffold protein, JIP-1, which interacts with multiple components of the JNK signaling pathway (discussed in section 1.V.D). JIP-1 binds to and is phosphorylated by JNK, although the significance of this phosphorylation for the assembly of the complex is not clear yet. Initially, JIP-1 was identified in the cytoplasm and characterized as an inhibitor of JNK signaling, by virtue of its ability to prevent the translocation of JNK to the cell nucleus. Overexpression of JIP-1 in PC12 cells thereby prevented JNK mediated gene expression (Dickens et al., 1997). Further characterization of JIP-1 using transient transfection assays has revealed that JIP-1 increases JNK activation by the JNK signaling pathway, (Whitmarsh et al., 1998), although the mechanism of regulation and the precise cellular location of JIP-1 are not understood. Interestingly, the JIP1 isoform, IB1, is a

putative transcription factor that binds to a regulatory element in the GLUT2 gene promoter (Bonny et al., 1998). Further studies are required to determine whether JIP-1 is a protein that has multiple physiological functions or whether the role of JIP-1 as a MAP kinase scaffold contributes to GLUT2 gene expression. Despite a number of unanswered questions, this evidence suggests that transcription factors maybe involved in the MAP kinase signaling pathway to regulate MAP kinase activation. In light of these studies, since AEBP1 stably associates specifically with MAP kinase, it would be interesting to examine whether AEBP1 may participate in the MAP kinase signaling pathway.

## *3.II. Modulation of MAP Kinase Activity by Alteration of AEBP1 Expression*

### 3.II.A. Isolation of 3T3-L1 Clones Stably Overexpressing AEBP1

To determine whether MAP kinase activity can be modulated by altering the abundance of AEBP1, I ectopically overexpressed AEBP1 and examined the activation state of MAP kinase. A single clone stable transfection may not be suitable, since individual clones overexpressing a given protein show cellular variation among individual cellular clones. Routinely, investigators use either retroviral or adenoviral technology or any inducible expression system. However, the major purpose of this study was to determine whether a different level of AEBP1 expression can affect MAP kinase activation and whether there is any correlation between AEBP1 expression and MAP kinase activation. Therefore, I first adopted a single clone stable transfection approach to overexpress AEBP1, by generating individual clones with differentially expressed AEBP1. This was further tested with pooled stable cells generated by retroviral vector (see section 3.IV).

An expression plasmid, pAEBP1/Neo, that constitutively expresses AEBP1 was stably transfected into 3T3-L1 cells and stable cell lines expressing high levels of AEBP1 were isolated through drug selection with G418. Since the plasmid construct encodes the fusion neo-AEBP1 transcript, it can be detected by probing with the Neo gene sequence. This transcript contains an internal ribosome entry site (IRES) which allows for translation of the neomycin and AEBP1 gene products from the same transcript (Boris-Lawire and Temin, 1993). Three pAEBP1/Neo-transfected clones (AEBP1/Neo-3, -7, and –11), along with the

parental plasmid pWZLNeo-transfected clones (Neo-1 and 9), were selected for further analysis. As shown in Figure 3.11, the clone pAEBP1/Neo-7 expresses the highest level of the AEBP1/Neo fusion transcript in the Northern blot analysis (top panel, lane 2) and also AEBP1 protein in the Western blot analysis (bottom panel, lane 2). Two clones (pAEBP1/Neo-3 and –11) express much lower levels of the AEBP1/Neo transcript (lanes 1 and 3) than in pAEBP1/Neo-7 cells (lane 2), which reflects the lower protein level that is, however, still higher than the level in the control cells (lanes 4 and 5).

I next asked whether MAP kinase activity was modulated by the alteration of AEBP1 expression with these cell lines.

### 3.II.B. Enhanced MAP Kinase Activity by Overexpression of AEBP1

To examine MAP kinase activity in these overexpressing AEBP1 cells, I carried out the kinase assay. The cell extracts from each of these cell lines were treated with anti-ERK1 antibody, then the immunoprecipitates were incubated with the MAP kinase-substrate, MBP, and [$\gamma$-$^{32}$P ]ATP. The kinase activity was detected by visualizing a radiolabeled band which was quantitated by a Molecular Imager (Bio-Rad). As shown in the top panel of Figure 3.12, the kinase activity in AEBP1/Neo-7 cells was highest among the stable clones (lane 1). This activity was at least 8 times higher than the control cells (lanes 4 and 5). Two other clones (AEBP1/Neo-3, and –11) exhibited significantly lower activities than the activity in AEBP1/Neo-7 (lanes 2 and 3), but about 4 times higher than the activity in control cells (lanes 4 and 5). These results closely reflect the relative level of AEBP1 proteins in the stable clones (Figure 3.11). To normalize the total amounts of ERK1 and ERK2 precipitated from each of the cell lines, the above immunoprecipitates were immunoblotted with anti-ERK1 antibody that also cross-reacts with ERK2. As shown in the bottom panel of Figure 3.12, there was no difference in the total amount of MAP kinase protein in these cell lines, and thus the difference in MAP kinase activity is not due to the amount of MAP kinase. These results suggest that AEBP1 interacts with MAP kinase and results in increased MAP kinase activity.

The potential problem of the above studies is that AEBP1 overexpression could be eliciting expression of a growth factor that is activating the MAP kinase pathway (Seger and Krebs, 1995; Charlotte et al., 1998). To address this possibility it is critical to analyze the phosphorylation and/or activity of upstream components of the MAP kinase pathway,

**Figure 3.11. Ectopic Overexpression of AEBP1 mRNA and Protein in 3T3-L1 cells**

The AEBP1 expression vector pAEBP1/Neo or the parental vector (pWZLneo, Freytag et al., 1994) were stably transfected into 3T3-L1 cells. Levels of AEBP1/Neo fusion transcript and Neo mRNA were determined by Northern blot analysis using the Neo probe (top panel). A GAPDH probe was used to normalize the amount of RNA loaded. The AEBP1 protein level in each stable cell line was determined by Western blot analysis (bottom panel). Lanes 1-3, AEBP1/Neo-transfected clone 3, 7, and 11, respectively, lanes 4, 5, the parental vector-transfected clones 1 and 9.

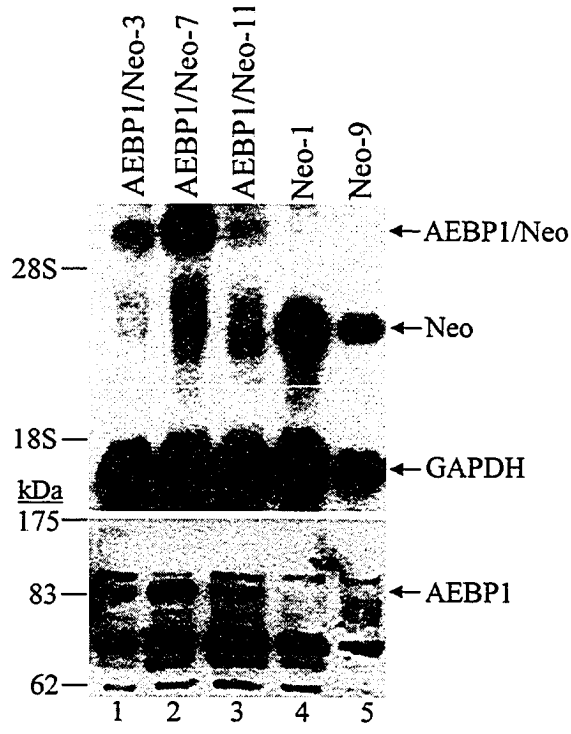


Figure 3.11. Ectopic Overexpression of AEBP1 mRNA and protein in 3T3-L1 cells

**Figure 3.12. Overexpression of AEBP1 increases MAP kinase activity**

Cell extracts collected from above clones (see legend of Figure 3.11) were immunoprecipitated with anti-ERK1, and the MAP kinase activity (top panel) were determined by the immuno-complex assay using the myelin basic protein (MBP; Sigma). The amount of precipitated MAP kinase from each cell line was determined by immunoblotting with anti-ERK1 antibody (bottom panel). The radiolabeled MBP bands were quantitated by a Molecular Imager (Bio-Rad), and the percent activity was estimated for each sample.

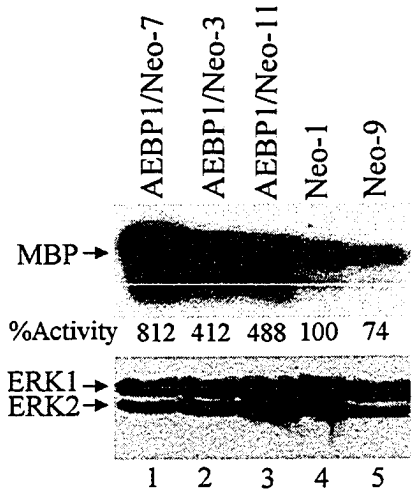


Figure 3.12. Overexpression of AEBP1 increases MAPK activity

especially MEK, in AEBP1-expressing and control cells. If AEBP1 is directly regulating MAP kinase activation, MEK activity would be identical in the overexpressing and control cell lines. Therefore, the activity profile of MEK was examined. The samples from each of the stable cell lines were immunoprecipitated with anti-MEK1 antibody (New England Biolab), then the kinase activity was measured using the recombinant MAP kinase p42 (Santa Cruz biotechnology) as a MEK substrate. As shown in Figure 3.13 (top panel), no difference in the MEK activity was observed among these clones. Therefore, these results indicate that MAP kinase activation is regulated by AEBP1, mostly likely mediated by direct interaction between AEBP1 and MAP kinase.

Next, I examined whether the increased activity of MAP kinase in the overexpressing cell lines is due to a sustaining effect of AEBP1 on MAP kinase activation. The quiescent cells were stimulated with serum for various lengths of time, then MAP kinase was detected by the gel-shift mobility assay as previously described. The top panel of Figure 3.14 shows that the MAP kinase activation in the control cells is transient, since it lasted about 2 hours. After 4 hours of serum-stimulation, about 50% of MAP kinase was unphosphorylated, and after 8 hours all of MAP kinase was unphosphorylated. In contrast, the activation of MAP kinases in AEBP1/Neo-7 cells was sustained for a much longer time, for instance 50% of MAPKs still remained phosphorylated 8 hours after the serum induction (bottom panel). The sustained MAP kinase activation in AEBP1/Neo-7 cells may not be due to MEK activation since MEK activation is not affected by AEBP1 expression (see Figure 3.13). Furthermore, the differences in the duration of MAP kinase activation appear not to be the result of MEK activation but rather a MEK-independent pathway involving phosphatases which may inactivate MAP kinase both in the cytoplasm and nucleus (Alessi et al., 1995; Brondello et al., 1997; Grammer and Blenis, 1997). This will further discussed further in section 3.III.

Sustained activation of MAP kinase would allow phosphorylation of different subsets of proteins in comparison to the situation of the transient activation. The activation of different subsets of transcription factors may thus determine which genes are turned on, thereby determining cell fate. It has been demonstrated that in fibroblasts, sustained activation of MAP kinase is associated with proliferation, not differentiation (Meloche et al., 1992; Mansour et al., 1994; Cowley et al., 1994). In other cell types, the converse may be true. For example, in PC12 cells, the sustained activation of MAP kinase by fibroblast growth factor or nerve growth factor induces differentiation whereas the transient activation by epidermal growth factor induces proliferation (Marshall, 1995). Our results further add to this

**Figure 3.13. Overexpression of AEBP1 does not affect MEK activity**

A kinase assay (as in the legend of Figure 3.12) was carried out. Immunoprecipitates with anti-MEK1 antibody (NEB) were analyzed for the MAP kinase activity (top panel) by the immunocomplex assay using the MEK substrate, the recombinant MAPK p42 (Santa Cruz Biotechnology). The total amounts of precipitated MEK from each cell line were determined by immunoblotting with anti-MEK1 antibody (bottom panel). The radiolabeled p42 bands were quantitated by a Molecular Imager (Bio-Rad), and the percent activity was estimated for each sample.

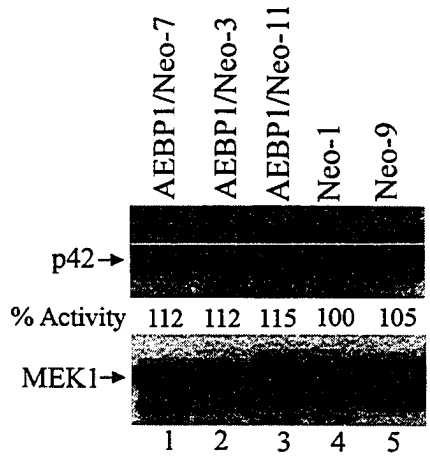


Figure 3.13. Overexpression of AEBP1 does not affect MEK activity

**Figure 3.14. Overexpression of AEBP1 results in sustaining of MAP kinase activation**

The quiescent cells (lanes 1 and 9) from the control cell line Neo-1 (top panel) and AEBP1/Neo-7 (bottom panel) were serum-stimulated for various lengths of time as indicated (lanes 2 –8). Cell extracts were analyzed by immunoblotting with anti-ERK1 antibody. This experiment was repeated three times with similar results.

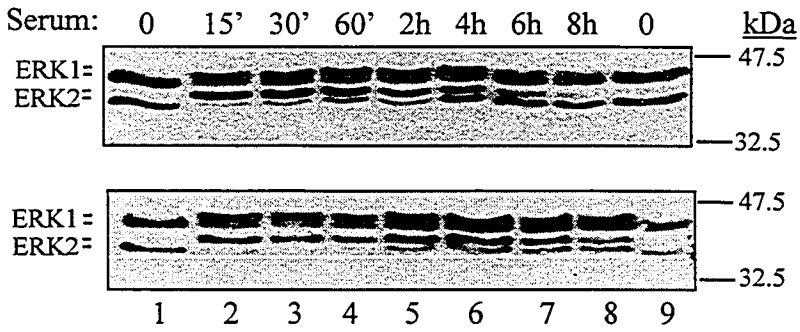


Figure 3.14. Overexpression of AEBP1 results in sustaining of MAPK activation

regulation in which AEBP1 may regulate the duration of MAP kinase activation through an unknown mechanism. Therefore it would be interesting to determine how AEBP1 regulates MAP kinase activity. These questions were explored by *in vitro* and *in vivo* kinase assays (section 3.III).

I asked whether MAP kinase activity could be decreased by reducing the level of expression of AEBP1 in the AEBP1 overexpressing cells (pAEBP1/Neo-7) by overexpressing antisense AEBP1 RNA. This experiment can also rule out the possibility that the increased MAP kinase activity in AEBP1/Neo-7 cells may be due to an intrinsic property of the stable clone. The AEBP1/Neo-7 cells were stably transfected with two expression plasmids, pREP-AS and pAS/Neo, that constitutively express an antisense RNA corresponding to the 5' 700-bp region of the AEBP1 transcript. After drug (Puromycin) selection, several stable clones were expanded and screened for AEBP1 expression by Northern and Western blot analyses. I selected two clones (AEBP1/Neo-7/AS-4 and-6) which showed decreased level of AEBP1 in comparison to the level in AEBP1/Neo-7 cells (Figure 3.15). Of the two clones, AS-6 exhibited a lower level of AEBP1 expression (lane 4), similar to the level in the control cell line Neo-1 (lane 1). The clone AS-4 exhibited a much higher level of AEBP1 (lane 3), only slightly lower than the level in AEBP1/Neo-7 (lane 2).

MAP kinase was immunoprecipitated from each of these cell lines and kinase activity was measured by checking the ability to phosphorylate MBP as described earlier. As shown in Figure 3.16 (top panel), MAP kinase activity in AS-4 cells (lane 3) was similar to the level of activity in AEBP1/Neo-7 cells (lane 2). However, clone AS-6 (lane 4) exhibited much lower MAP kinase activity than the activity in AEBP1/Neo-7; in fact, the activity in AS-6 cells was similar to the activity in Neo-1 cells (lane 1). These results further suggest that AEBP1 can modulate MAP kinase activation.

Unphysiologically high levels of AEBP1 in the AEBP1-overexpressing cell lines may enhance the kinase activity artificially. To circumvent this problem, it would be better to carry out an experiment which modulates the endogenous level of AEBP1. Since AEBP1 is down-regulated during adipocyte differentiation (He et al., 1995), a useful experiment would be to examine MAP kinase activity during adipocyte differentiation, thus determining whether ERK activity is correlated with AEBP1 expression (discussed in section 3.II.D). Another complementary experiment would be to ablate endogenous AEBP1 expression using antisense RNA inhibition and to examine if suppression of endogenous AEBP1 by antisense RNA expression can lead to decreased MAP kinase activity (discussed in section 3.II.C).

**Figure 3.15. Reduced AEBP1 level by antisense AEBP1 transfection in stable cell lines selected from pAEBP1/Neo-7 cells**

The stable cell line highly overexpressing AEBP1, pAEBP1/Neo-7 cells, was stably transfected by the plasmids (pREP-As and pAS/Neo). After puromycin selection, the stable clones that constitutively express an antisense RNA of the AEBP1 transcript were established. The AEBP1 protein level in each stable cell line was determined by Western blot analysis. Lane 1, the parental vector-transfected clones 1, lane 2, AEBP1/Neo-transfected clone 7, and lanes 3 and 4, antisense AEBP1 RNA expressing clone 3 (AS-4) and 4 (AS-6), respectively.

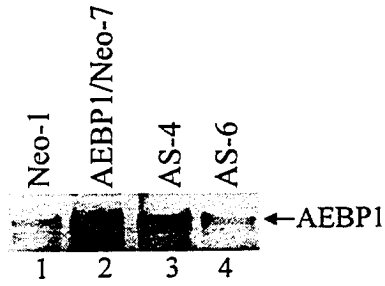


Figure 3.15. Reduced AEBP1 level by antisense AEBP1 Transfection in stable cell lines selected from pAEBP1/Neo-7 cells

**Figure 3.16. Reduction of MAP kinase activity in pAEBP1/Neo-7 cells by reduced AEBP1 expression**

Cell extracts collected from above clones (see legend of Figure 3.15) were analyzed by the immunocomplex assay. The MAP kinase activity (top panel) and immunoblotting (bottom panel) analyses were same as described in the legend of Figure 3.12.

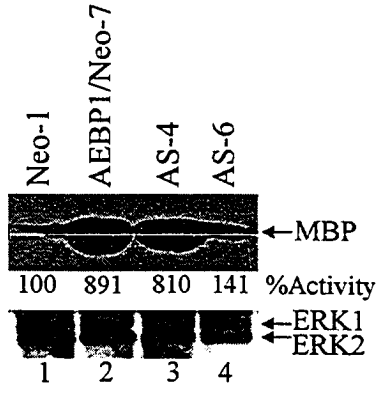


Figure 3.16. Reduction of MAPK activity in pAEBP1/Neo-7 cells by reduced AEBP1 expression

This series of experiments was needed to establish the role of AEBP1 in modulating MAP kinase activity in physiological conditions.

### 3.II.C. Attenuated MAP Kinase Activity by Reducing the Endogenous AEBP1 Level

To decide whether AEBP1 operates similarly at physiological concentrations as in ectopically overexpressing cells, the endogenous AEBP1 expression was suppressed by antisense RNA procedure. The antisense AEBP1 RNA expression vector, pAS/Neo, was stably transfected into 3T3-L1 cells and several stable clones were expanded and screened for the antisense AEBP1/Neo fusion transcript and AEBP1 protein expression (Figure 3.17). Two clones (AS/Neo-7 and -11) showed high expression of the antisense AEBP1/Neo fusion transcript (top panel, lanes 1 and 2) which results in decreased level of AEBP1 protein (bottom panel) in comparison to the level in the control cell line that was transfected with the empty vector (Neo-12). Of the two clones, AS/Neo-11 exhibited a higher level of the fusion transcript (Figure 3.17, top panel, lane 3) and a lower level of AEBP1 expression (bottom panel).

MAP kinase activity in each of these cell lines was measured as described earlier. As predicted, clone AS/Neo-11 exhibited the lowest MAP kinase activity (24% of the control level) among these clones (Figure 3.18, top panel). Similar amounts of ERK1 and ERK2 were precipitated from each of these cell lines (upper-middle panel). The MAP kinase activity thus is directly related to the endogenous AEBP1 level. However, the MEK activity was not affected by changes in AEBP1 (lower-middle panel). These results suggest that AEBP1 specifically modulates MAP kinase activation, and that the modulation by AEBP1 may be physiologically relevant.

### 3.II.D. Modulation of MAP Kinase Activity Mediated by AEBP1 During Adipocyte Differentiation

The extracts from mouse tissues were subjected to Western blot analysis. As shown in Figure 3.19, AEBP1 was expressed in a wide variety of tissues. As expected, the highest abundance of AEBP1 protein was detected in 3T3-L1 preadipocytes (lane 2), but not in the

**Figure 3.17. Attenuation of endogenous AEBP1 expression by the expression of antisense AEBP1 mRNA**

The antisense AEBP1 RNA expression vector, pAS/Neo was stably transfected into 3T3-L1 cells. After G418 selection, stables cell lines were generated. Levels of AS/Neo fusion transcript and Neo mRNA were determined by Northern blot analysis using a Neo probe (top panel). A GAPDH probe was used to normalize the amount of RNA loaded. The AEBP1 protein level in each stable cell line was determined by Western blot analysis (bottom panel). Lane 1, the parental vector-transfected clones 12, lanes 2 and 3, AS/Neo-transfected clone 7 and 11, respectively.

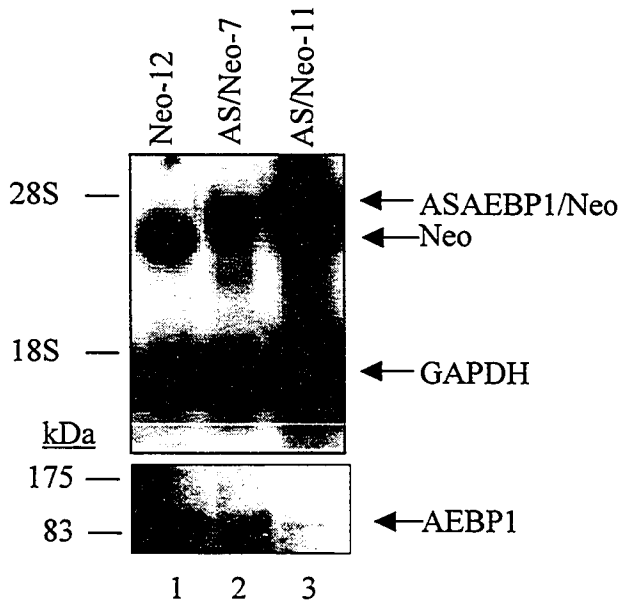


Figure 3.17. Attenuation of endogenous AEBP1 expression by the expression of antisense AEBP1 mRNA

**Figure 3.18. Modulation of MAP kinase activity by attenuation of endogenous AEBP1 expression**

Cell extracts (see the legend of Figure 3.17) were immunoprecipitated with either anti-ERK1 or anti-MEK1 antibodies (NEB). The MAP kinase activity (top panel) and the MEK1 activity (lower-middle panel) were analyzed by the ability to phosphorylate either MBP or the recombinant MAPK p42 (Santa Cruz Biotechnology). Total amounts of ERK1 and ERK2, or MEK1 were determined by immunoblotting with either anti-ERK1 (upper-middle panel) or anti-MEK1 antibodies (bottom panel).

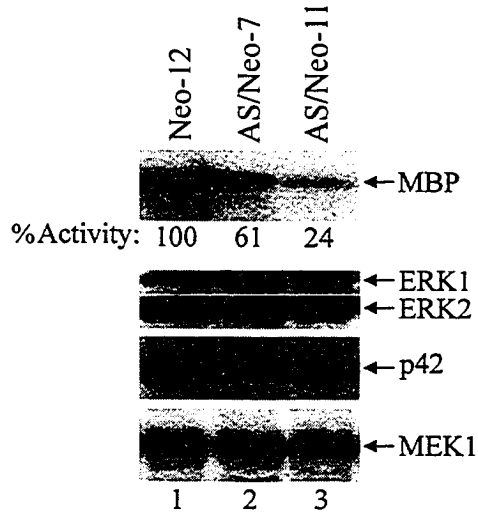


Figure 3.18. Modulation of MAPK activity by attenuation of endogenous AEBP1 expression

**Figure 3.19. AEBP1 protein expression in a variety of mouse tissues**

Total protein extracts were prepared from 10 different mouse tissues isolated from a 7 week-old male mouse (Lanes 3 to 12). AEBP1 was immunoprecipitated from extracts containing an equal amount (1 mg) of protein for each and immunoblotted with anti-AEBP1 antibody. Lanes 1 and 2, AEBP1 immunoprecipitated from 3T3-L1 preadipocytes and adipocytes, respectively.

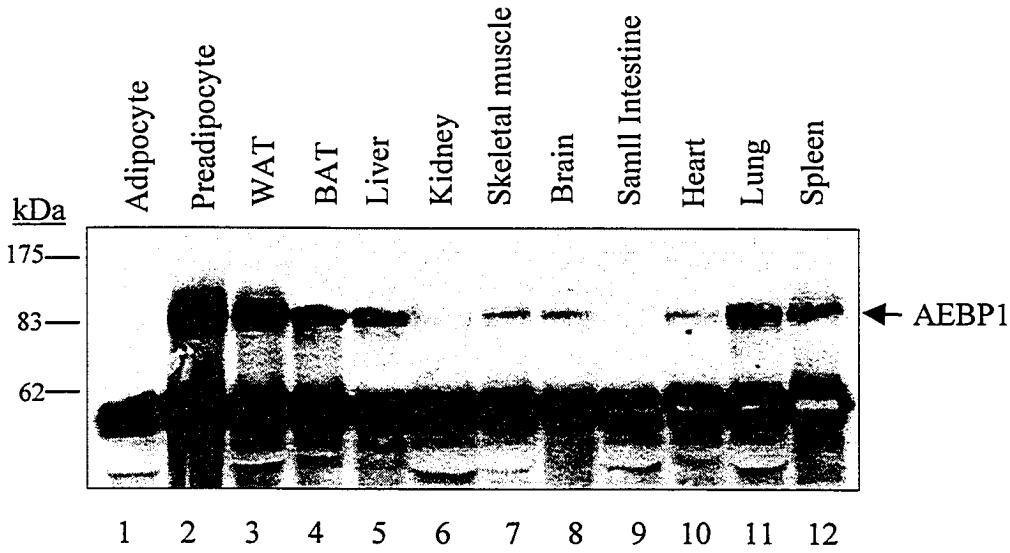


Figure 3.19. AEBP1 protein expression in a variety of mouse tissues

terminally differentiated 3T3-L1 adipocytes (lane 1). This is consistent with the previous results (He et al., 1995). The AEBP1 protein level in the liver (lane 5) was similar to the level in BAT (lane 4). The amount of AEBP1 protein detected in the liver, lung, and spleen (lanes 5, 11, 12) was substantially higher than the amount detected in the other three tissues, skeletal muscle, brain and heart (lanes 7, 8, 10). AEBP1 protein was barely detectable in the kidney (lane 6) or not detectable in small intestine (lane 9). AEBP1 expression was detected in both white and brown adipose tissues (lanes 3 and 4), whereas its expression was abolished in terminally differentiated adipocytes (lane 1). The majority of adipose tissue is composed of adipocytes, with intermingled blood cells, endothelial cells, pericytes, fibroblasts, and precursor adipocytes which are cells with varying degrees of differentiation (Geloen et al., 1989). The discrepancy between adipose tissue and the cultured cells is, therefore, explained by the possibility that the AEBP1 expression in the adipose tissues may be from non-adipocyte cells resident within adipose tissue (stroma-vascular fraction).

Though AEBP1 expression is abolished in the mature adipocyte, the precise expression pattern of AEBP1 during adipogenesis has not been defined. I therefore examined AEBP1 expression during adipocyte differentiation and tested whether MAP kinase activity is really modulated by the changes in the endogenous level of AEBP1 during adipocyte differentiation. To test when AEBP1 expression is down-regulated during adipocyte differentiation, 3T3-L1 cells at the confluent stage (Day 0) were allowed to differentiate for 7 days (as described in section 2.III.C). Samples were collected daily and were analyzed by Western blotting. Figure 3.20 shows the Western blot analysis of AEBP1 abundance during 7 days after exposure to the differentiation conditions. Expression of AEBP1 decreased on Day 1 and remained unchanged until 4 days after exposure to the differentiation medium, then further decreased on Day 5 and was subsequently abolished at the terminal stage of adipocyte differentiation on Day 6 and 7 as observed previously (Figure 3.19; He et al., 1995). Since AEBP1 is undetectable on Days 6 and 7, I would not expect to observe any co-precipitation of MAP kinase on these days. Indeed, MAP kinase was not detected in the AEBP1-immunoprecipitates from Day 6 and 7 (Figure 3.21, top panel). These results further confirm the specific interaction between AEBP1 and MAP kinases. Interestingly, the relative amounts of ERK1 and ERK2 coprecipitated with AEBP1 are different in comparison to the situation in preconfluent cells (compare Figure 3.7, and Figure 3.21, top panel). These different levels of ERK proteins may reflect different expression of MAP kinases in the different cell conditions (preconfluent vs confluent cells). However, Western blotting of cell lysates

**Figure 3.20. Expression of AEBP1 is abolished at the terminal stage of adipogenesis**

Confluent 3T3-L1 cells (Day 0) were subjected to the differentiation protocol described in the materials and methods section (2.III.C). Total cell extracts were then prepared at the times indicated during differentiation. The extracts were immunoprecipitated with anti-AEBP1 antibody and immunoblotted with anti-AEBP1 antibody to identify the AEBP1 expression.

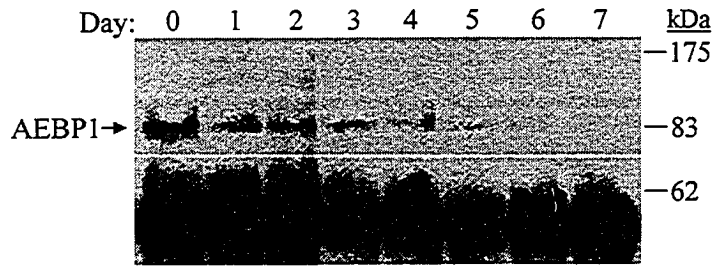


Figure 3.20. Expression of AEBP1 is abolished at the terminal stage of adipogenesis

**Figure 3.21. MAP kinase co-immunoprecipitates with AEBP1 during adipocyte differentiation**

Samples from each cell extract as described in Figure 3.20 were immunoprecipitated with anti-AEBP1 antibody, and then immunoblotted with anti-ERK1 antibody (top panel). The band above the ERK1 band (top panel) represents a partial image of the IgG band. The bottom panel shows Western blot analysis of the cell extracts with anti-ERK1 antibody (Santa Cruz Biotechnology) that also cross-reacts with ERK2.

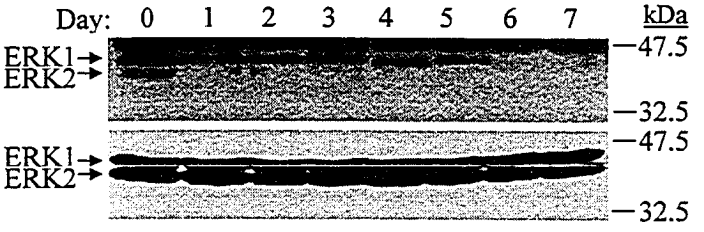


Figure 3.21. MAPK co-immunoprecipitates with AEBP1 during adipocyte differentiation

showed no significant change in the MAP kinase content of either form in confluent or preconfluent conditions.

Next, I determined whether MAP kinase activity is also modulated by the change in abundance of AEBP1 during adipocyte differentiation. Confluent 3T3-L1 cells (Day 0) were treated with the differentiation inducers (insulin, DEX, and MIX) for two days, and total cell extracts were prepared everyday from Day 3 to Day 7 of the differentiation periods (see section 2.III.C). Just prior to the preparation of cell extracts, cells were stimulated with EGF after serum-starvation for 2 hours. MAP kinase was immunoprecipitated from each of these cells undergoing differentiation, then the kinase activity was measured by its ability to phosphorylate the myelin basic protein (MBP). Figure 3.22 shows that the MAP kinase activity was relatively constant until Day 5 when it fell significantly to about 50% of the activity in Day 0, then further decreased to 6% and 2% on Days 6 and 7, respectively. The extent of reduction in MAP kinase activity was precisely correlated with the expression level of AEBP1 during this period. The MAP kinase activity dropped markedly on the same day when AEBP1 expression was abolished, when MAP kinases would be free from AEBP1. The total MAP kinase content in the samples prepared during differentiation showed no significant difference (bottom panel). These results strongly suggest that the endogenous AEBP1 expression modulates MAP kinase activity in physiological conditions.

### *3.III. AEBP1: Significant Enhancer of MAP Kinase Activity*

So far I have tested the hypothesis that AEBP1 may be involved in the regulation of MAP kinase activation, since AEBP1 stably associates with MAP kinase. I found that MAP kinase activity is enhanced by the AEBP1 expression through the complex of AEBP1 with MAP kinase.

Next, I explored how MAP kinase activation is modulated by AEBP1. The possible mechanisms for the enhanced MAP kinase activation by AEBP1 in these situations are as follow 1) AEBP1 may stimulate growth factor expression which induces a MAP kinase module 2) AEBP1 may influence the expression of MAP kinase, or other proteins which modulate MAP kinase activation (e.g. MAPK-specific phosphatases). Finally AEBP1 may protect MAP kinase from inactivation by its specific phosphatase. As I considered in section 3.II.B, enhanced MAP kinase activation appeared not to be the result of either persistent

**Figure 3.22. Decrease in MAP kinase activity correlates with loss of AEBP1 expression during adipocyte differentiation**

Confluent 3T3-L1 cells (Day 0) were subjected to the differentiation protocol and cell extracts were prepared at the times indicated. Just prior to cell extract preparation the cells were serum-starved for 2 hours, then stimulated with 10 nM EGF for 15 minutes. Cell extracts were immunoprecipitated with anti-ERK1 antibody, then the MAP kinase activity was determined by the immuno complex assay using MBP (top panel). The radiolabeled MBP was quantitated by the Molecular Imager system (Bio-Rad), and the percent MAP kinase activity was estimated in each sample as indicated. Cell extracts were also analyzed by immunoblotting with anti-ERK1 antibody for the total content of ERKs during this period (bottom panel).

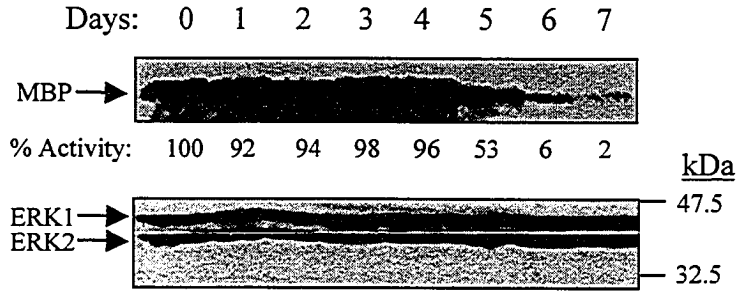


Figure 3.22. Decrease in MAPK activity correlates with loss of AEBP1 expression during adipocyte differentiation

MEK activation or increased MAP kinase expression. Within five minutes of growth factors binding to RTKs, MAP kinase and MEK are activated in the cytoplasm. If the activation of MAP kinase is prolonged for approximately 15-30 minutes, MAP kinase is translocated to the nucleus. However, MEK is quickly down-regulated upon activation of MAP kinase, and does not accompany MAP kinase as it translocates to the nucleus (Alessi et al., 1995; Chen et al., 1992). The fact that MEK is down-regulated and not translocated with MAP kinase to the nucleus suggests that the regulation of enhanced MAP kinase activation occurs through both cytoplasmic and nuclear protein phosphatases, and not through continuous re-activation by MEK. Therefore AEBP1 may modulate MAP kinase activation by protection from dephosphorylation by MAP kinase-specific phosphatases. I therefore tested this hypothesis using *in vivo* and *in vitro* phosphatase protection assays.

### 3.III.A. AEBP1 Protects MAP Kinase Inactivation from MAP Kinase-Specific Phosphatase *in vivo*

MAP kinase phosphorylation (or activation) is a reversible process, in which protein phosphatases play a crucial role in controlling cellular activities. I therefore determined if the interaction of AEBP1 with MAP kinase enhanced MAP kinase activity in cells through its protective role against dephosphorylation by phosphatases. An emerging class of dual-specificity phosphatases, that directly and specifically regulate the MAP kinase family members, has been characterized. Among them, the dual-specificity phosphatase PYST1 (MKP-3) that is selective for inactivation of ERK family MAP kinases have been identified (Groom et al., 1996; Muda et al., 1996).

To test this hypothesis I used ectopically expressed AEBP1, along with ERK1 and a MAPK-specific phosphatase (PYST1), to demonstrate that AEBP1 attenuates ERK1 inactivation. To establish a system to allow assessment of MAP kinase regulation by AEBP1, I first co-transfected PYST1 with a range of pSG/PYST1-Myc plasmid levels (0.5-5.0 μg) (Groom et al., 1996) and 1 μg of pECE/HA/ERK1 plasmid (Muda et al., 1996) into COS-7 cells. Cells were then stimulated with EGF after serum-starvation and extracts were immunoprecipitated with anti-HA antibody. The samples from these cells were used to measure mitogen-stimulated ERK1 activity by the immunocomplex assay using MBP. The activity was quantitated with the use of a Molecular Imager (Bio-Rad), and the percent

activity was estimated. Figure 3.23 shows that PYST1 expressed in COS-7 cells blocked heterologously expressed ERK1 (pECE/HA-ERK1) activation after stimulation with EGF, and this inhibition increased when cells were transfected with an increased amount of PYST1 plasmid (top panel). The total HA-ERK1 amounts immunoprecipitated were not different in these samples (bottom panel). These results show that PYST1 is active in dephosphorylating ERK1 in these systems.

I next tested whether expressing AEBP1 restored the EGF-stimulated ERK1 activation under conditions where the PYST1-mediated inhibition would be maximal. I co-transfected either pSVAEBP1 or pSVAEBP1 (-) along with the aforementioned plasmids tested in the above experiment (see Figure 3.23, top panel, lane 5). The EGF-stimulated ERK1 activation was measured as described before. The top panel of Figure 3.24 shows that ERK1 activation was recovered in a dose dependent manner when COS-7 cells were transfected with the plasmid expressing AEBP1 (lanes 4-6). However, the ERK1 activation was not recovered when the cells were transfected with the control plasmid pSVAEBP1 (-) (lane 1-3). The PYST1 inhibition is unlikely mediated by AEBP1 binding to the phosphatase, since co-immunoprecipitation experiments did not reveal such interaction (data not shown). These results suggest that the interaction of AEBP1 with MAP kinase stabilizes the phosphorylation status of MAP kinase, making it resistant to dephosphorylation by the MAP kinase-specific phosphatase.

Since there was no sustained MEK activity by AEBP1 (see Figures 3.13 and 3.18), the above results were not affected by the possibility that AEBP1 may enhance the growth factor or upstream kinase activity as I discussed earlier. However AEBP1 was initially found as a transcription factor. It is still possible that the transcriptional repression activity of AEBP1 could maintain ERK1 activation in a manner independent of PYST1 in this system. AEBP1 may down-regulate endogenous PYST levels or other known or unknown MAPK-specific phosphatases and may lead to the same results as above. To rule out this possibility, I attempted to identify mutant derivatives of AEBP1 that are defective in transcriptional activity, but still able to interact with MAP kinase. Then the protective function of these mutant derivatives of AEBP1 could be examined (discussed in section 3.III.C). First I carried out transient transfection experiments using a CAT reporter system to test transcriptional activity as described previously (He et al., 1995). Plasmids encoding the wild-type (pJ3HAEBP1) or the mutant derivatives (pJ3H-AEBP1ΔDLD, pJ3H-AEBP1DLD, pJ3H-

**Figure 3.23. Inhibition of MAP kinase activation by PYST1**

COS-7 cells were transfected with pECE/HA-ERK1 (1 μg) plasmid together with 0.5, 1.0, 2.5, or 5.0 μg of pSG5/PYST1-Myc plasmids as indicated. Following 48 hours of growth and 2 hours of serum starvation, cells were stimulated with 10nM EGF for 15 minutes. ERK1 was immunoprecipitated with anti-HA antibody (bottom) were carried out. The percent MAP kinase activity was estimated as in Figure 3.12.

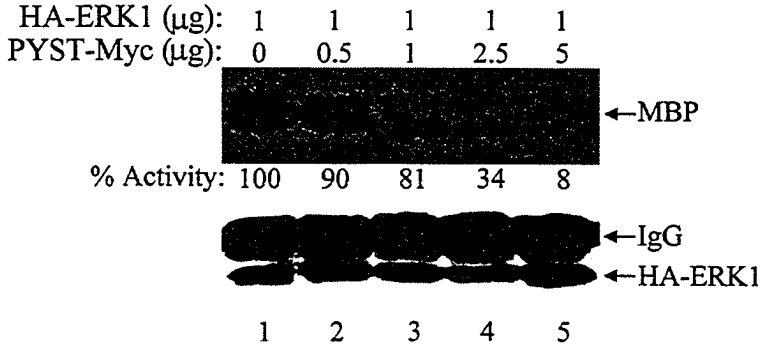


Figure 3.23. Inhibition of MAPK activation by PYST1

**Figure 3.24. AEBP1 attenuates ERK1 inactivation by PYST1**

Cos-7 cells were co-transfected with pECE/HA-ERK1 (1 μg) plasmid and 5 μg of pSG5/PYST1-Myc plasmid along with either pSVAEBP1 (lanes 4 to 6) or pSVAEBP1 (-) (lanes 1-3) plasmids in varying amounts as indicated. The MAP kinase activity (top panel) and immunoblotting analysis (bottom) were as in the legend of Figure 3.23.

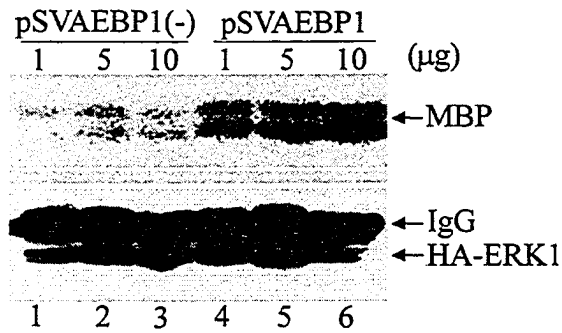


Figure 3.24. AEBP1 attenuates ERK1 inactivation by PYST1

AEBP1ΔHic, or pJ3H-AEBP1ΔSty) were co-transfected with the reporter plasmid, paP2(3AE-1/-120)CAT, into NIH-3T3 cells. Figure 3.25 shows that three mutant derivatives (DLD, ΔHic, and ΔSty) that interact with MAP kinase (see Figure 3.9 and 3.10) did not significantly decrease CAT activity compared to the wild-type control (pJ3H-AEBP1). These may be due to either defective carboxypeptidase activity (ΔHic), which is vital for the transcriptional function, or defective DNA-binding ability (ΔSty) (see section 1.III.B and He et al., 1995).

Since I am mainly interested in AEBP1 mutant derivatives which are defective in transcriptional repression but still able to interact with MAP kinase, the mutant derivatives (ΔHic, and ΔSty) were therefore tested for MAP kinase protection activity.

### 3.III.B. Domain of AEBP1 Responsible for Protection of MAP Kinase Inactivation from MAPK-Specific Phosphatase

The plasmids expressing different AEBP1 mutants (pJ3H-AEBP1ΔSty or ΔDLD) or wild type AEBP1 (pJ3H-AEBP1) were co-transfected with pECE/HA-ERK1 and pSG5/PYST1-Myc plasmids under similar phosphatase protection assay conditions as described previously. After EGF stimulation, cell extracts were immunoblotted with anti-HA antibody to show that the expression of AEBP1 and the mutant derivatives (ΔSty and ΔHic) consistently increased as the amount of corresponding plasmids increased from 1 μg to 10 μg (Figure 3.26, upper-middle panel). After confirming expression of AEBP1 and the mutant derivatives, the extracts were then immunoprecipitated with anti-HA antibody and the immunoprecipitated samples were immunoblotted with anti-phospho-p44/42 MAPK antibody to show the phosphorylated (active) form of ERK1 (Figure 3.26, top panel). Under conditions where inhibition by PYST1 is maximal (see Figure 3.23), EGF-stimulated ERK1 activation was recovered with increasing amount of the wild type plasmid (lanes 7-9) as expected. Moreover, the ERK1 activation was recovered in a dose-dependent manner in cells transfected with the plasmid expressing the mutant derivative ΔHic (lane 4-6), but not with the plasmid expressing the mutant derivative ΔSty (lanes 1-3). The protection by ΔHic was not as strong as that conferred by wild-type AEBP1, which may be due to the large deletion in the ΔHic. I did not observe any protection effect with the ΔSty mutant, which suggests that

**Figure 3.25. Transcriptional analysis (CAT assay) of wild-type and mutant derivatives of HA-AEBP1**

Cells were transiently transfected with the reporter construct psP2(3AE-1/-120)CAT (He et al., 1999) along with an expression vector encoding either wild-type (pJ3H-AEBP1) or mutant (pJ3H-AEBP1ΔDLD, pJ3H-AEBP1DLD, pJ3H-AEBP1ΔHic, pJ3H-AEBP1ΔSty) HA-AEBP1 proteins. The control plasmid pJ3H-AEBP1 (-) contains the AEBP1 cDNA in the opposite orientation. The transcription activity was estimated as levels of CAT activity (% conversion of chloramphenicol to acetylated chloramphenicol) expressed from the aP2 promoter. The values shown represent three separate transfection experiments. The error bars indicate the standard deviations.

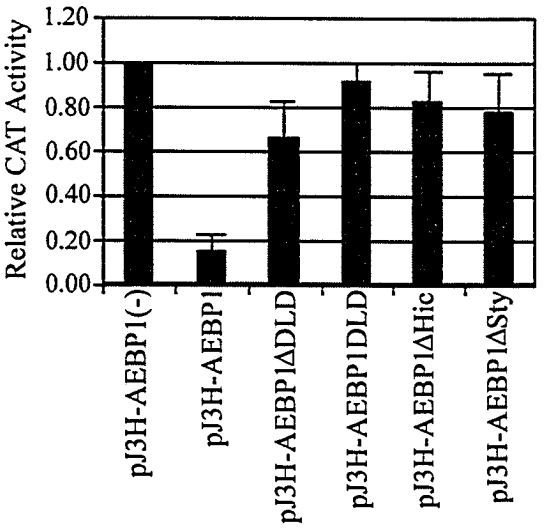


Figure 3.25. Transcriptional analysis (CAT assay) of wild-type and mutant derivatives of HA-AEBP1

**Figure 3.26. AEBP1 attenuates ERK1 dephosphorylation by PYST1 through its C-terminal domain of AEBP1**

To measure MAP kinase phosphorylation in the transfected cells expressing different AEBP1 mutant derivatives in the presence of PYST1, COS-7 cells were co-transfected with pECE/HA-ERK (1 μg) plasmid and 5.0 μg of pSG5/PYST1-Myc plasmid along with either pJ3H-AEBP1ΔSty (lanes 1-3), pJ3H-AEBP1ΔHic (lanes 4-6) or pJ3H-AEBP1 (lanes 7-9) plasmids in varying amounts as indicated. Cells were then stimulated with EGF at 48 hours post-transfection and cell lysates were immunoprecipitated with anti-HA antibody as described in the legend of Figure 3.24. The immunoprecipitated samples were immunoblotted with either anti-phospho-p44/42 MAPK (top panel), anti-phospho-MEK1 (second top panel), anti-HA (middle and second bottom panel), or anti-Myc (bottom) antibodies.

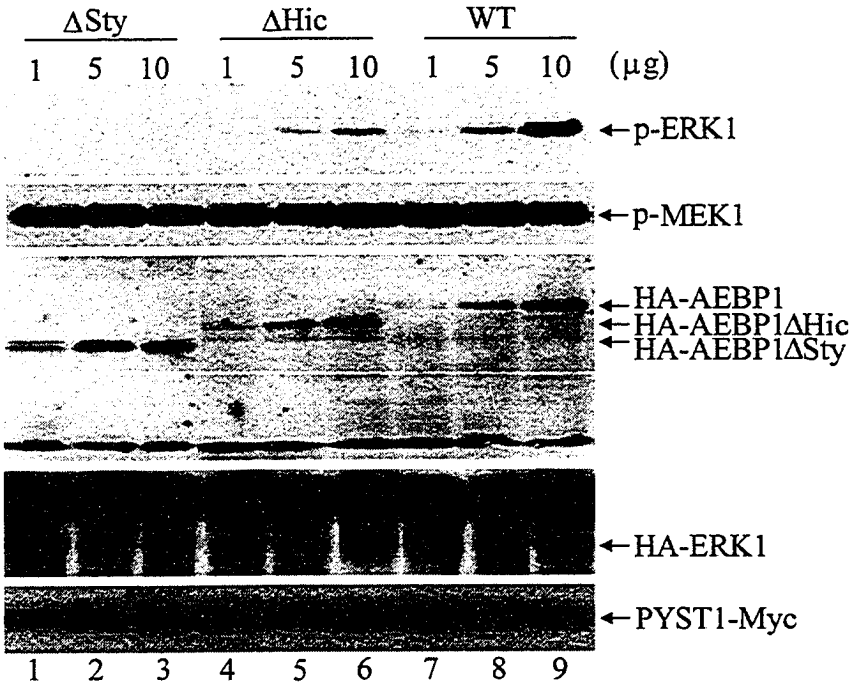


Figure 3.26. AEBP1 attenuates ERK1 dephosphorylation by PYST1 through its C-terminal domaoin of AEBP1

the protection domain is located at the C-terminus that is missing in the ΔSty protein (see Figure 3.8). The decreased protection activity for ΔHic and ΔSty are not due to defects in their expression, since the expression level of each mutant was similar to the wild-type level (upper-middle panel). The total amount of HA-ERK1 and PYST1-Myc were detected by immunoblotting with either anti-HA (lower-middle panel) or anti-Myc (bottom panel) antibodies and showed no difference in these samples. These results indicate that AEBP1 can protect MAP kinase from a MAPK-specific phosphatase, and suggest that the protection is mediated by an interaction of AEBP1 with MAP kinase, not by the transcriptional activity of AEBP1.

### 3.III.C. Attenuation of Protective Effect of MAP Kinase Activity by a Negative Dominant Mutant (DLD)

To further substantiate the conclusion that AEBP1 modulates MAP kinase activation by a protective interaction, I utilized another mutant derivative (DLD), that consists of only the interaction domain (see Figure 3.8), as a dominant-negative mutant. I would predict that this mutant and the wild-type AEBP1 compete for a possible binding site on MAP kinase and that DLD attenuates the protective activity exhibited by the wild-type in a dominant-negative fashion. I co-transfected DLD at a range of pJ3H-AEBP1 DLD plasmid levels (1-8 μg) under the conditions where the protective effects on MAP kinase activity by AEBP1 would be maximal (see Figure 3.24, lane 6). After EGF-stimulation, cell extracts were first immunoblotted with anti-HA antibody to detect the level of HA-DLD and HA-AEBP1 expression. The DLD expression increased with increased dosage of HA-DLD plasmids (Figure 3.27, upper-middle panel). The phosphorylated (active) form of MAP kinase in these samples, immunoprecipitated with anti-HA antibody, was then measured by immunoblotting with anti-phospho-p44/42 MAPK antibody (Figure 3.27, top panel). Importantly, DLD expressed in COS-7 cells attenuated the protective activity of AEBP1 in a dose-dependent manner. Taken together, these results strongly suggest that AEBP1 modulates MAP kinase activity through protective interaction.

**Figure 3.27. The MAP kinase-protective activity of AEBP1 is attenuated by ectopic expression of DLD**

COS-7 cells were co-transfected with pECE/HA-ERK1 (1 μg), pSG5/PYST-Myc (5 μg), and pJ3HAEBP1 (10 μg) along with pJ3H-AEBP1DLD in varying amount as indicated. The band below HA-DLD band (upper-middle panel) represents a non specific band. The cell lysates were analyzed as in Figure 3.26.

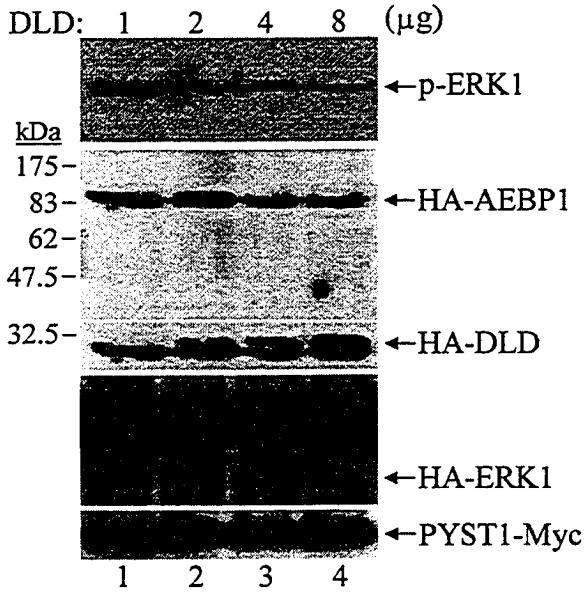


Figure 3.27. The MAPK-protective activity of AEBP1 is attenuated by ectopic expression of DLD

### 3.III.D. MAP Kinase-Protective Activity of AEBP1 *in vitro*

To demonstrate if the interaction of AEBP1 with MAP kinase directly blocks dephosphorylation of activated MAP kinase, an *in vitro* phosphatase assay was designed using recombinant AEBP1 protein. Since the recombinant phospho-ERK2 protein (NEB) is the activated kinase with phosphorylated Thr 183 and Tyr 185, this can be maximally dephosphorylated by Lambda Protein Phosphatase (λ-PPase), which is a protein phosphatase with activity towards phosphorylated serine, threonine and tyrosine residues. Therefore the recombinant phospho-ERK2 protein (NEB) was treated with λ-PPase, after incubation with either wild-type or mutant derivatives of the recombinant AEBP1 protein (see Figure 3.10). As shown in Figure 3.28 (top panel), MAP kinase incubated with either AEBP1 (lane 1) or ΔHic (lane 2) was protected from λ-PPase treatment whereas MAP kinase incubated with ΔSty (lane 3) was not. These results are consistent with those of *in vivo* studies (see Figure 3.26). It is most unlikely that the protection effect is due to non-specific inhibition of λ-PPase activity by AEBP1 or ΔHic, but not by ΔSty. Taken together with *in vivo* data, inhibition of MAP kinase activity by the overexpression of phosphatase is blocked by co-expression of AEBP1. These results strongly provide a mechanism involving protection against dephosphorylation of MAP kinase activity by direct binding of AEBP1.

## 3.IV. AEBP1: A Significant Signaling Modulator for Adipogenesis

MAP Kinase maintained in its active phosphorylated form by protection from phosphatases may cause overstimulation of MAP kinase pathways. Several recent reports show that activation of the MAP kinase pathway phosphorylates PPARγ and inhibits adipogenesis (Hu et al., 1996; Adams et al., 1997; Camp and Tafuri 1997; Reginato et al., 1998). Thus, we tested the hypothesis that the enhancement of MAP kinase activity by AEBP1 is a biologically relevant process in adipogenesis.

**Figure 3.28. AEBP1 directly blocks dephosphorylation of activated MAP kinase**

Recombinant phospho-ERK2 protein (NEB) was incubated with AEBP1 (lane 1), ΔHic (lane 2) or ΔSty (lane 3) and then treated with λ-PPase as described in materials and methods. Protein samples were resolved by SDS-PAGE, and the phosphorylated (top) and total ERK2 protein (bottom) were detected by immunoblotting with anti-Phospho-p44/42 MAPK and anti-p44/42 MAPK antibodies, respectively. Protein sizes were determined by comparison to pre-stained molecular weight markers (NEB).

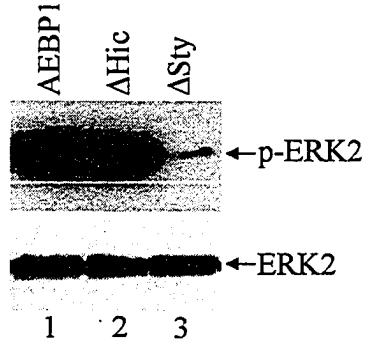


Figure 3.28. AEBP1 directly blocks dephosphorylation of activated MAPK

For this study, several individual stable clones either overexpressing or underexpressing AEBP1 that were already tested in the MAP kinase assay (see section 3.II) were used (Figures 3.29, 3.30, 3.31, 3.36, 3.37, and 3.38). Since these individual clones may have cellular variation in adipogenesis on a clone to clone basis, I further utilized the retroviral technology, which most laboratories use in this field to circumvent these problems, and generated stable clones representing a pooled population of cells. With these clones, I further explored biological effects (adipogenesis) to support the previous evidence (Figures 3.32, 3.33, 3.34, and 3.35). For further confirmation of the direct involvement in enhancement of MAP kinase activation by interaction of AEBP1 with MAP kinase, I also generated the stable cell line expressing the dominant negative mutant, DLD (Figures 3.39, 3.40, and 3.41).

### 3.IV.A. Enhanced MAP Kinase Activity by AEBP1 Overexpression Negatively Modulates Adipocyte Differentiation (with Individual Stable Clones)

First, the overexpressing cell lines described above (see Fig. 3.11) were treated with adipogenic inducers (insulin, DEX, and MIX) at the confluent stage, and allowed to differentiate for 7 days. These cultures were then monitored for the differentiated phenotype, using a lipid staining technique (see section 2.1.C). As shown in Figure 3.29, examination of these cultures indicated striking differences in the ability of the cells to accumulate lipid, a feature of mature adipocytes (top panel). Although the level of lipid accumulation varied, it was clear that the cell line expressing the highest level of AEBP1 (AEBP1/Neo-7) was unable to efficiently convert to the adipocyte phenotype. Two other stable cell lines (AEBP1/Neo-3 and –11), in which AEBP1 expression is much lower than the expression in AEBP1/Neo-7 cells (see Figure 3.11), differentiated more efficiently than AEBP1/Neo-7; however, they differentiated less efficiently than the control and mock-transfected cells, in which more than 90% of the cells accumulated lipid and differentiated (top panel). The decrease in lipid staining in the overexpressing cell lines was due to a decrease in the total number of cells that differentiated to adipocytes, and not in the average amount of lipid per cell (bottom three panels).

**Figure 3.29. Ectopic overexpression of AEBP1 blocks adipogenesis**

The differentiation phenotype was monitored by macroscopic analysis of the cells stained with oil Red O at 7 days after the confluent cells were subjected to the differentiation protocol (top panel). The differentiated phenotype was also monitored by microscopic examination either before (upper-middle) or after (lower-middle and bottom) staining with oil-red O. Magnifications are 300X (upper-middle and lower-middle) and 40X (bottom).

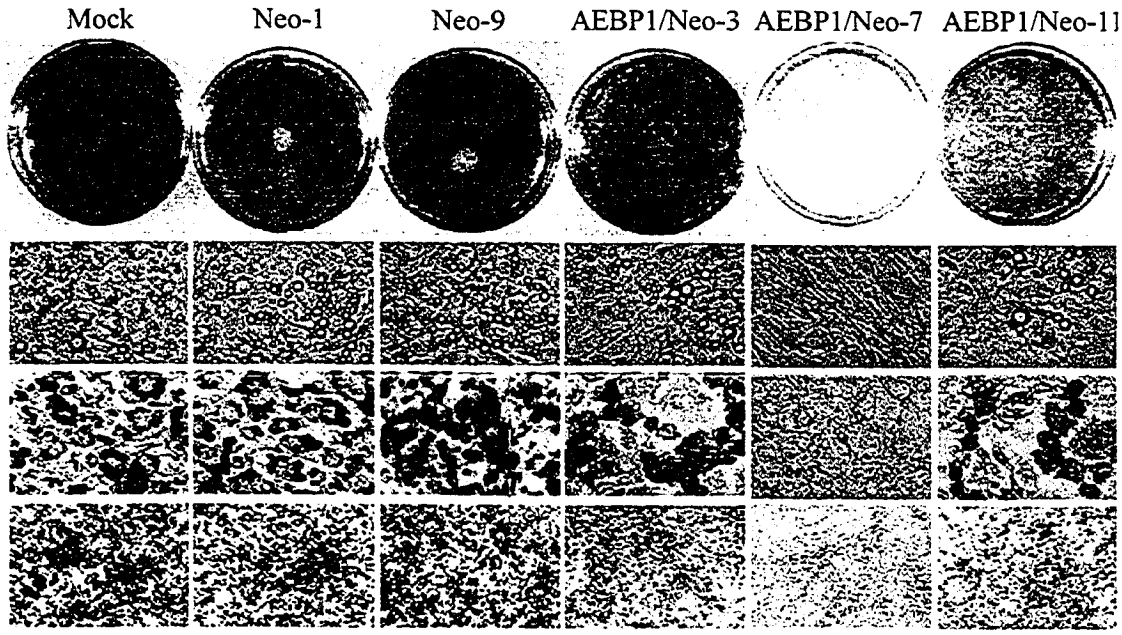


Figure 3.29. Ectopic overexpression of AEBP1 blocks adipogenesis

I then examined the expression of differentiation-specific genes in these cells to conclusively verify that overexpression of AEBP1 blocks the differentiation process. Total RNA in these cells were collected before (preadipocyte stage) and after the differentiation process. The expression of differentiation-specific genes was measured by northern blotting analysis (Figure 3.30). In AEBP1/Neo-7 cells, expression of the differentiation-specific genes PPARγ, C/EBPα, aP2, adipsin, and LPL was very low (lane 7). In agreement with the differentiated phenotype, the expression of these genes was detected in two other stable cell lines (AEBP1/Neo-3, -11), albeit at levels lower than those in the control cell lines (compare lanes 8, 9). Therefore, the extent of differentiation is inversely correlated with the level of AEBP1 expression in these stable cell lines.

The inability of AEBP1/Neo-7 cells to differentiate is unlikely due to an intrinsic property of the stable clone. I tested the two derivative cell lines (AS-4 and AS-6) of AEBP1/Neo-7 described above (see Figure 3.15). As shown in Figure 3.31, only the cell line AS-6, which exhibited AEBP1 abundance and MAP kinase activity at the control level, was able to differentiate. Therefore, the extent of differentiation is inversely correlated with the level of AEBP1 expression and MAP kinase activity in these stable cell lines.

As described earlier, AEBP1 is a transcriptional repressor. It binds to the AE-1 (adipocyte enhancer 1) site in the promoter region of the aP2 gene which encodes the adipose fatty acid binding protein. By doing so, it is capable of repressing aP2 expression (He et al., 1995). Although it is not clear whether down-regulation of aP2 expression in AEBP1/Neo-7 cells (Figure 3.30, lane 7) is affected by the transcription role of AEBP1 or by the sustained MAP kinase activity, the blockage of adipocyte differentiation in these cells is unlikely controlled by the decreased aP2 expression. This is supported by the evidence that the adipose tissue of aP2 knock-out mice was morphologically normal although the gene disruption interferes with the development of dietary obesity-induced insulin resistance (Hotamisligil et al., 1996a).

In the AEBP1 overexpressing cell lines (see Figures 3.29 and 3.30), it is possible that AEBP1 may down-regulate the dominant adipogenic genes, PPARγ and C/EBPα and result in inhibition of adipocyte differentiation. There is accumulated data showing that PPARγ and C/EBPα critically influence adipocyte differentiation. The blocking of C/EBPα induction in 3T3-L1 cells by antisense RNA inhibits adipocyte differentiation (Lin and Lane 1992). Although NIH-3T3 fibroblasts cannot differentiate into adipocytes even in the presence of

**Figure 3.30. Expression of adipocyte specific genes in AEBP1 overexpressing cells**

Expression of two adipogenic "master" genes (PPARγ and C/EBPα) and other differentiation-specific genes (aP2, adipsin, and LPL) was analyzed by Northern blotting before (left) and after (right) the differentiation protocol.

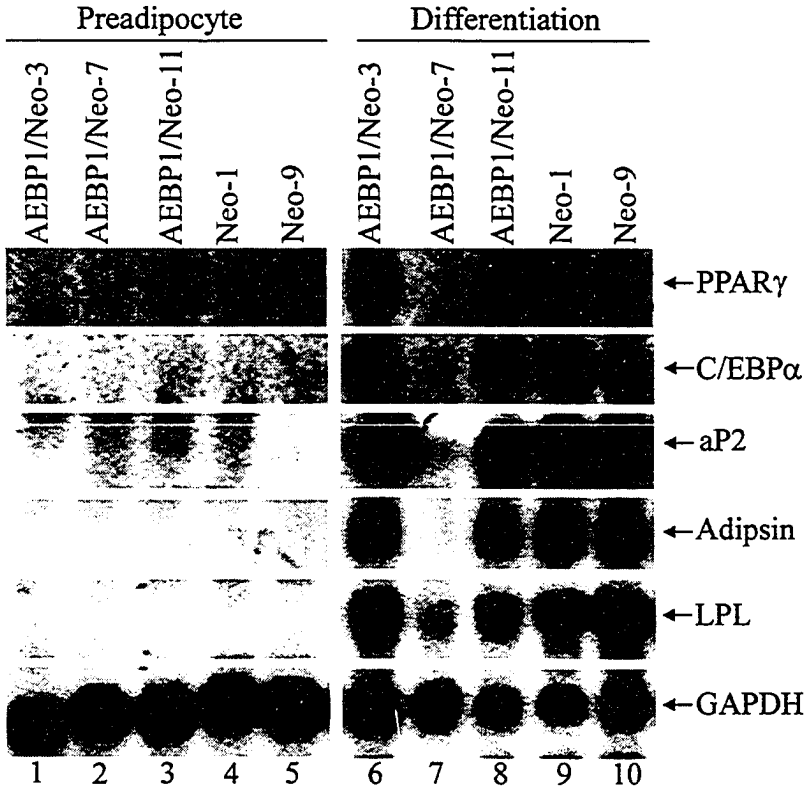


Figure 3.30. Expression of adipocyte specific genes in AEBP1 overexpressing cells

**Figure 3.31. Reacquisition of differentiation ability by decreasing the AEBP1 level in the antisense cell line**

The differentiated phenotype for each stable cell line was monitored by macroscopic analysis of the cells stained with Oil Red O. The cell lines are described in the legend to Figure 3.15.

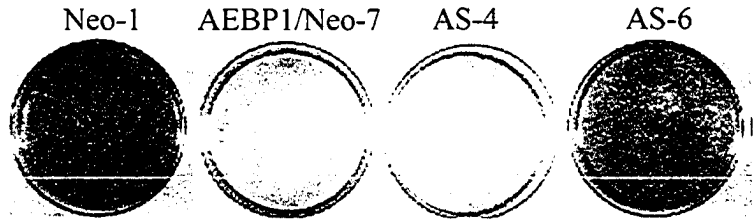


Figure 3.31. Reacquisition of differentiation ability by decreasing the AEBP1 level in the antisense cell line

hormonal stimulants, forced expression of C/EBPα results in adipose conversion in the absence of hormonal stimulants (Freytag et al., 1994). C/EBPα knock-out mice have reduced mass of both brown and white adipose tissue demonstrating that C/EBPα is essential for *in vivo* adipocyte differentiation (Wang et al., 1995). Ectopic expression of PPARγ in the presence of its ligand thiazolidinedione or prostaglandin derivatives stimulates adipocyte differentiation of NIH-3T3 fibroblasts (Yeh et al., 1995). When PPARγ and C/EBPα are expressed together ectopically in NIH-3T3 fibroblasts, they synergistically accelerate the conversion into adipocytes (Tontonoz et al., 1994b).

### 3.IV.B. Experiments with Pooled Stable Cell Lines

Since the possibility exists for clonal variation, I also generated stable cell lines representing a pooled population and tested whether the effects on MAP kinase activity and adipogenesis by AEBP1 in these cells are same as those in the individual clones.
The pooled stable cell lines generated by retroviral vectors that express either the wild-type or mutant derivatives of AEBP1 were also tested for the anti-adipogenic effect 7 days after the adipocyte differentiation process (Figure 3.32). The stable cell line generated by the virus harboring wild-type AEBP1 (AEBP1/Neo-P) was unable to differentiate, as in the case of the clonal cell line AEBP1/Neo-7 (see Figure 3.29). Importantly, the stable cell line (AEBP1ΔSty/Neo-P) generated by the virus harboring the mutant derivative ΔSty, which does not protect MAP kinase (see Figure 3.27), was able to differentiate as efficiently as the control cell line generated by the parental retroviral vector (Neo-P). In contrast the stable cell line generated by the virus harboring the mutant derivative ΔHic (AEBP1ΔHic/Neo-P) was not able to differentiate efficiently (Figure 3.32).

As predicted from the differentiation phenotype, the bottom panel of Figure 3.33 showed that the expression of PPARγ in the AEBP1ΔHic/Neo-P cells (lane 2) was much lower than the level in the control cells following the differentiation protocol (lane 4). The amount of PPARγ in the AEBP1ΔSty/Neo-P cells (lane 3) was similar to the level in the control cells (lane 4), whereas the level in the AEBP1/Neo-P cells (lane 1) was lower than the level in the AEBP1ΔHic/Neo-P cells (lane 2).

**Figure 3.32. Microscopic analysis of the differentiation phenotype of the pooled stable 3T3-L1 cells**

The viral supernatant containing pNeo-P, pAEBP1/Neo-P, pAEBP1ΔHic/Neo-P, or pAEBP1ΔSty/Neo-P was transfected into 3T3-L1 cells using retroviral transfection. After G418 selection, the stable cells were subjected to the differentiation protocol. The differentiated phenotype was monitored by microscopic examination either before (top) or after (mild and bottom panels) staining with oil-red O. Magnification are 300X (top and middle panels) and 40X (bottom panel).

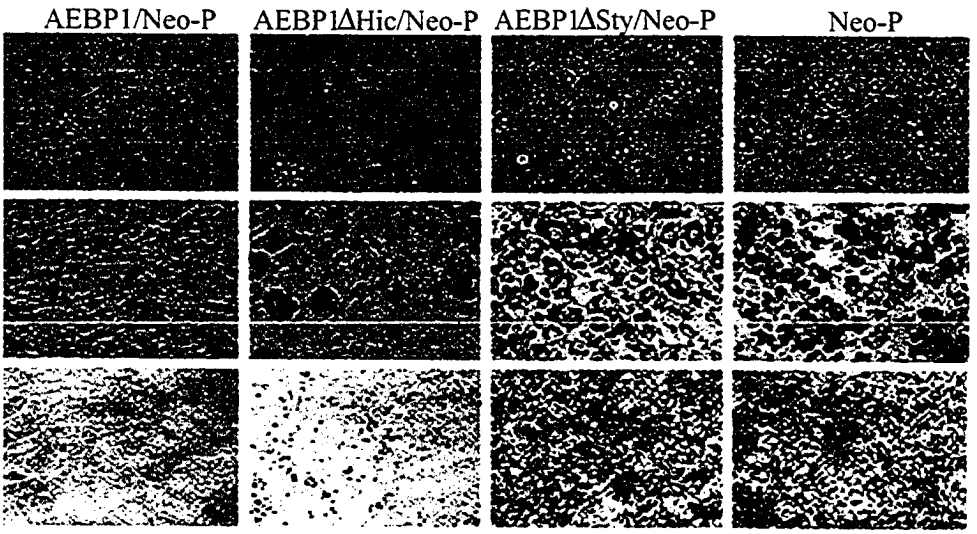


Figure 3.32. Microscopic analysis of the differentiation phenotype of the pooled stable 3T3-L1 cells

**Figure 3.33. Increased phosphorylation of MAP kinase results in inhibition of the adipocyte differentiation process**

Total protein (20 μg) from the stable cell line was analyzed by immunoblotting with anti-AEBP1 (top panel), anti-Phospho-p44/42 MAPK monoclonal (upper-middle panel), and anti-p44/42 MAPK (lower-middle panel) antibodies. Cell extracts were also analyzed by immunoblotting with anti-PPARγ antibody (Santa Cruz Biotechnology) at 7 days after the confluent cells were subjected to the differentiation protocol (bottom panel). The arrows in the top panel indicate AEBP1 or its mutant protein.

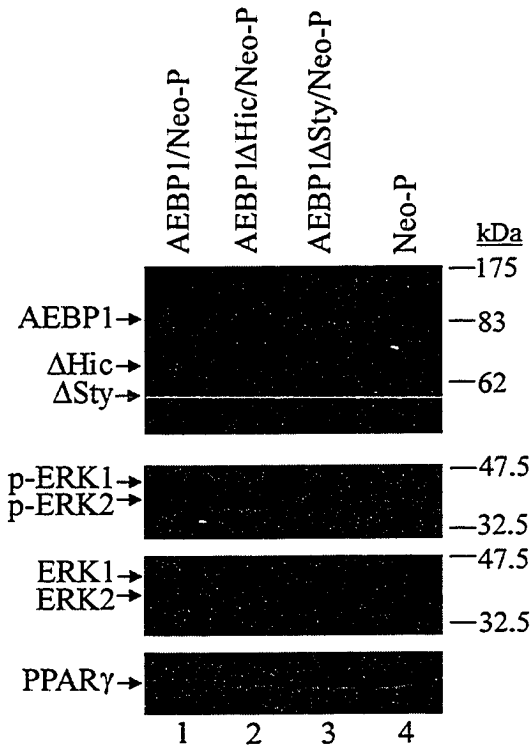


Figure 3.33. Increased phosphorylation of MAPK results in inhibition of the adipocyte differentiation process

Figure 3.33 (upper-middle panel) also shows the amount of phosphorylated ERKs in the stable cells. The AEBP1/Neo-P cells (lane 1) contained the highest level, whereas the AEBP1ΔSty/Neo-P cells (lane 3) contained the lowest level, being similar to the level in the control Neo-P cells (lane 4). Significantly, the amount of phosphorylated ERK1 and ERK2 in the AEBP1ΔHic/Neo-P cells (lane 2) was much higher than the level in the control cells, but lower than the level in the AEBP1/Neo-P cells. These results are consistent with the MAP kinase protection activity exhibited by the AEBP1ΔHic mutant (see Figure 3.26). The expression level of AEBP1 and its mutant derivatives were measured by immunoblotting with anti-AEBP1 antibody (Figure 3.33, top panel). The total ERK1 and ERK2 amounts were also detected by immunoblotting with anti-p44/42 MAPK antibody and found no difference among these cells (Figure 3.33, lower-middle panel). The ΔHic protein that does not have either transcriptional activity (see Figure 3.25) or carboxypeptidase activity (He et al., 1995), showed anti-adipogenic effect. The ΔSty protein, for which interaction with MAP kinase does not protect MAP kinase activity against dephosphorylation, did not show anti-adipogenic effect.

These results are consistent with the previous reports that activation of MAP kinase (ERK1 and ERK2) is known to antagonize adipocyte differentiation (Hu et al., 1996; Adams et al., 1997; Font de Mora et al., 1997). I further showed that this anti-adipogenic effect by activation of MAP kinase is in part due to the protective activity of AEBP1. In general, two adipocyte-specific transcription factors, PPARγ and C/EBPα are known to be essential inducers of adipocyte differentiation by activating other adipocyte-specific genes, such as GLUT4 and aP2. MAP kinase-mediated phosphorylation of PPARγ results in a blockage of its ability to activate the transcriptional events (Hu et al., 1996; Adams et al., 1997) required for progression of the adipocytic differentiation program.

Next I determined whether the protective activity solely affects adipocyte differentiation. Since DLD acts as a dominant negative mutant against wild type AEBP1 by blocking the MAP kinase binding site (see Figure 3.27), this mutant would inhibit only the protective role of AEBP1 on MAP kinase activity, but most likely not the transcriptional activity of AEBP1. If I could demonstrate that the DLD protein could differentiate 3T3-L1 cells overexpressing AEBP1 (AEBP1/Neo-P cells) into adipocytes, this would show that the protective activity of AEBP1 solely influences adipocyte differentiation. To test this hypothesis, I generated two more stable cell lines by retroviral transfection. The pooled stable

cell line AEBP1/DLD(+) was derived from the AEBP1/Neo-P cell line by infection with the virus expressing DLD (AEBP1DLD/Puro). The control pooled cell line AEBP1/DLD(-) was generated with the control virus AEBP1DLD/Puro. First the expression level of DLD in the stable cell lines was checked by immunoblotting with anti-AEBP1 antibody (Figure 3.34, top panel). DLD was detected in AEBP1/DLD(+) cells (lane 3) but not in AEBP1/Neo-P cells (lane 1) or AEBP1/DLD(-) cells (lane 2). MAP kinase activity was measured by detecting the phosphorylated form of MAP kinase using anti-phospho-p44/42 MAPK antibody. Again as predicted, the amount of phosphorylated ERK1 and ERK2 in AEBP1/DLD(+), but not in AEBP1/DLD(-), cells was significantly decreased compared to the level in the parental AEBP1/Neo-P cells (Figure 3.34, upper-middle panel). The total amount of ERK1 and ERK2 was not different in these cells (Figure 3.34, lower middle). These results are consistent with the results of the MAP kinase protection assay shown in Figure 3.27.

Finally, I determined whether the anti-adipogenic effect in the AEBP1/Neo-P cell line can be attenuated by ectopic overexpression of the DLD mutant. Figure 3.35 shows that the pooled stable cell line AEBP1/DLD(+) was able to differentiate. The control pooled stable cell line AEBP1/DLD(-) was unable to differentiate as in the case of the parental cell line AEBP1/Neo-P. As predicted, the amount of PPARγ was increased in the AEBP1/DLD(+) cells following the differentiation protocol (Figure 3.34, bottom panel). Since DLD lacks the putative DNA-binding site (Muise, 1997), it can not compete with the endogenous AEBP1 for DNA-binding. Therefore, DLD would not affect the transcriptional activity of AEBP1, and it is unlikely that the induction of PPARγ in AEBP1/DLD(+) cells (bottom panel, lane 3) is due to influence on the transcriptional function of AEBP1. Taken together, these results strongly suggest that the anti-adipogenic effect of overexpressed AEBP1 is mediated by the protective activity through the direct protein-protein interaction of AEBP1 and MAP kinase.

### 3.IV.C. Stimulation of Adipogenesis by Suppression of Endogenous AEBP1 Expression

The results of the above study are obtained from experiments involving ectopic overexpression of AEBP1. This means that the level of AEBP1 is considerably higher than in normal cells. Since AEBP1 is a MAP kinase substrate and therefore binds MAP kinase, there is a concern that the effect of AEBP1 on ERK1/ERK2 activity may be an artifact of substrate overexpression. By binding to MAP kinase, the super-physiological level of AEBP1 may

**Figure 3.34. Phosphorylated MAP kinase level is reduced in AEBP1/Neo-P cells ectopically expressing DLD**

Viral supernatant containing pAEBP1DLD/Puro (lane 3) or pAEBP1DLD(-)/Puro (lane 2) was transfected into AEBP1/Neo-P cells, and the puromycin-resistant stable cells were generated. Neo-P (lane 4) and AEBP1/Neo-P cells were used as controls. Cell extracts were analyzed as in Figure 3.32.

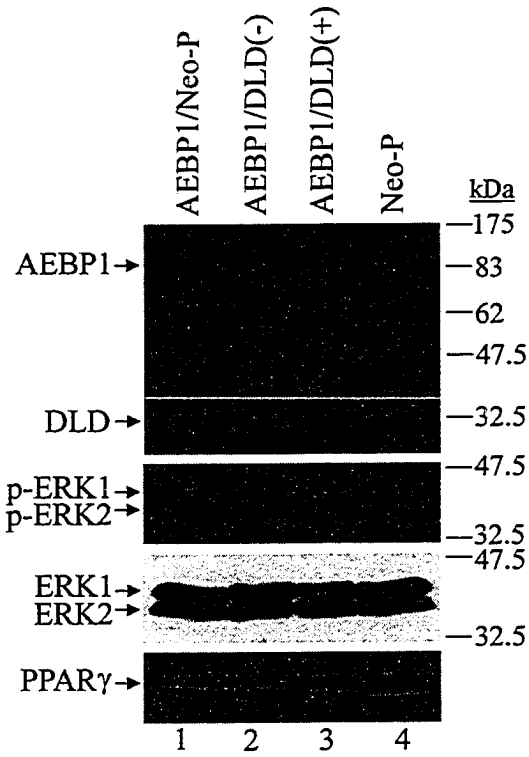


Figure 3.34. Phosphorylated MAPK level is reduced in AEBP1/Neo-P cells ectopically expressing DLD

**Figure 3.35. Reacquisition of differentiation ability by ectopic expression of DLD in the AEBP1/Neo-P cell line**

Microscopic analysis of the differentiation phenotype of the pooled stable cell lines was as previously described in the legend of Figure 3.32.

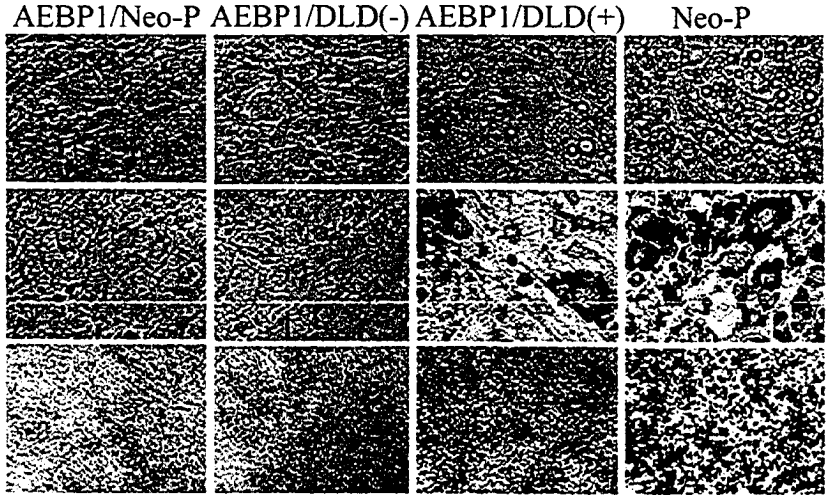


Figure 3.35. Reacquisition of differentiation ability by ectopic expression of DLD in the AEBP1/Neo-P cell line

protect the kinase from dephosphorylation. Since the endogenous level of AEBP1 is down-regulated during adipocyte differentiation (see Figure 3.20), I examined ERK1/ERK2 activity during the differentiation time course (see Figures 3.22 and 3.40). I found that the ERK activity is directly correlated with AEBP1 expression, suggesting that MAP kinase activity modulated by AEBP1 is not an artifact.

Furthermore, a complementary experiment was also carried out to attenuate endogenous AEBP1 expression using antisense RNA inhibition. I found that the MAP kinase activity was attenuated by suppression of endogenous AEBP1 expression and ablation of its protective activity (see Figure 3.18). Next, it would be necessary to establish that the modulation of MAP kinase activity by the attenuation of AEBP1 expression influences adipocyte differentiation. Figure 3.36 (top and middle panels) shows the differentiation morphology profiles in these cells after exposure to differentiation conditions as described earlier. The antisense cells AS/Neo-11, that have significantly reduced endogenous level of AEBP1 and MAP kinase activity in comparison to the control cells (see Figures 3.17 and 3.18), differentiated most efficiently among the stable cell lines. AS/Neo-7 cells had less MAP kinase activity than the control cells but higher activity than As/Neo-11 cells (see Figure 3.18). The extent of differentiation in AS/Neo-7 cells was not significantly different from that in the control cells Neo-12. As predicted, the expression of PPARγ protein in AS/Neo-11 cells was much higher than the control and AS/Neo-7 cells (Figure 3.36, bottom panel). These data support my findings that the extent of differentiation is inversely correlated with the level of AEBP1 expression and MAP kinase activity. Therefore, these results strongly suggest that AEBP1 modulates MAP kinase activity and this modulation is a biologically relevant process in adipogenesis.

However, there is still the possibility that the master transcription factor, PPARγ, may be regulated by the transcriptional function of AEBP1. This may enhance adipocyte differentiation in addition to the effect on MAP kinase activity (see section 3.IV.A and Figure 3.31). As mentioned earlier (see sections 1.II.A, 1.II.B, and 3.IV.A), PPARγ and C/EBPα are master genes for activating other adipogenic genes. PPARγ has two different isoforms. PPARγ1 seems ubiquitously expressed at very low level whereas PPARγ2 is specifically expressed at high levels in adipocytes (Tontonoz et al., 1994b). C/EBPα is expressed in adipocytes but not in preadipocytes (Lin and Lane, 1992). Since AEBP1 is a transcriptional repressor expressed in preadipocytes, AEBP1 may regulate the expression of

**Figure 3.36. Enhanced adipocyte differentiation by attenuation of endogenous AEBP1 expression**

The differentiated phenotype of the AEBP1-antisense cells was monitored by macroscopic (top panel) and microscopic (middle panel, 40X) examination of the cells stained with oil Red O after the confluent cells were subjected to the differentiation protocol for 7 days. The extent of differentiation in the AEBP1-antisense cells was also monitored at the molecular level by immunoblotting with anti-PPARγ antibody (bottom).

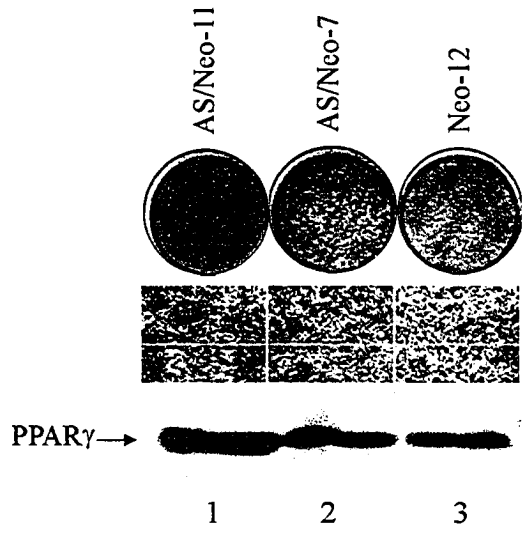


Figure 3.36. Enhanced adipocyte differentiation by attenuation of endogenous AEBP1 expression

PPARγ and C/EBPα in preadipocytes. Using the antisense AEBP1 RNA expressing cell lines, I explored whether the master genes can be induced in preadipocytes by suppression of AEBP1. Total RNA was isolated from these cells, and their expression was analyzed by Northern blotting. As shown in Figure 3.37, C/EBPα (top panel) and PPARγ2 (middle panel) were highly expressed in the differentiated Neo-12 cells (lane 2) and were used as controls. Since PPARγ1 is only expressed in liver (Tontonoz et al., 1994), I used this tissue to distinguish it from PPARγ2 (middle panel, lanes 1 and 6). Interestingly, C/EBPα and PPARγ1 are expressed in AS/Neo-11 cells in the preadipocyte stages (top and middle panel, lane 4). AEBP1 expression in these cells was significantly reduced at preadipocyte stage (see Figure 3.17, lane 3). PPARγ1 expression in AS/Neo-7 was much lower than that in AS/Neo-11 cells, but slightly higher than that in Neo-12 cells (middle panel), showing that PPARγ and C/EBPα expression is inversely correlated with AEBP1 expression. These results suggest that both PPARγ and C/EBPα may be directly or indirectly regulated by AEBP1.

The slightly higher PPARγ expression in AS/Neo-7 cells (lane 3) did somewhat enhance adipocyte differentiation in comparison to the control cells (Figure 3.36, top and middle panels, lanes 2 and 3). The use of BRL49653, a strong PPARγ ligand (Lehmann et al., 1995), made a significant difference to the adipogenic phenotypes in these two antisense cell lines. At confluence, these cells were treated with insulin and BRL49653 instead of the usual adipogenic inducers (insulin, DEX and MIX) as indicated (see the legend of Figure 3.38). As shown in Figure 3.38, the preadipocyte cells 3T3-L1 were unable to differentiate into adipocytes (bottom panel) with any dosage of BRL49653, whereas AS/Neo-7 cells (middle panel) were able to differentiate with 5 μg of the ligand, but not as efficiently as in AS/Neo-11 cells, in which these cells showed robust adipogenesis in the presence of the PPARγ ligand. Taken together with Figures 3.30 and 3.31, these results further support that AEBP1 may synergistically inhibit adipocyte differentiation through its transcriptional activity and protective activity.

### 3.IV.D. Stimulation of Adipogenesis by Ablation of the MAP kinase Protective Activity of the Endogenous AEBP1

**Figure 3.37. Regulation of PPARγ and C/EBPα mRNA expression by AEBP1**

Total RNAs were isolated from antisense AEBP1 expressing clones (AS/Neo-11, lane 1 and AS/Neo-7, lane 2) and the control (Neo-12, lane 3) at confluent stage (preadipocyte). C/EBPα (top panel) and PPARγ (middle panel) mRNA were analyzed as in the materials and methods. As an internal control, 28S and 18S rRNA were used and were detected by ethidium bromide stain.

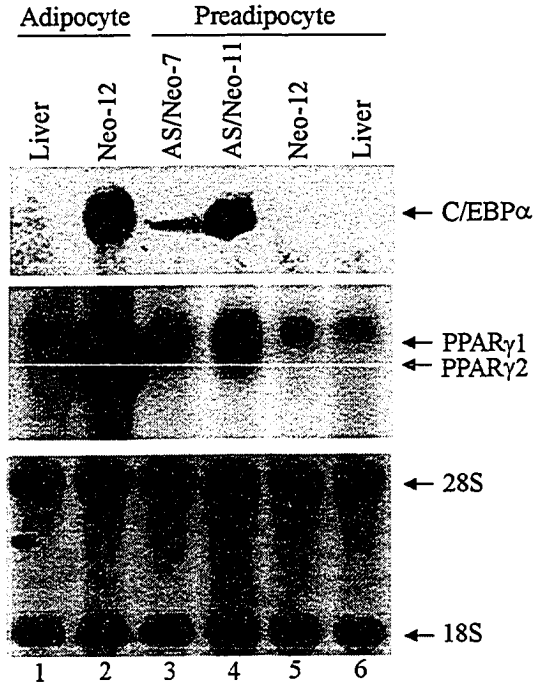


Figure 3.37. Regulation of PPARγ and C/EBPα mRNA expression by AEBP1

**Figure 3.38. Attenuation of AEBP1 stimulates adipocyte differentiation after BRL treatment**

At confluence, the AEBP1-antisense cells and the mock cells (3T3-L1) were treated with different dosages (0.05, 0.5, and 5 μM) of BRL49653 and insulin every 2 days for 7 days, but without DEX and MIX as indicated. As a positive control, these cells were treated with the conventional adipogenic inducers (insulin, DEX, and MIX) as described earlier. The differentiation phenotypes were measured by lipid staining method using oil Red O.

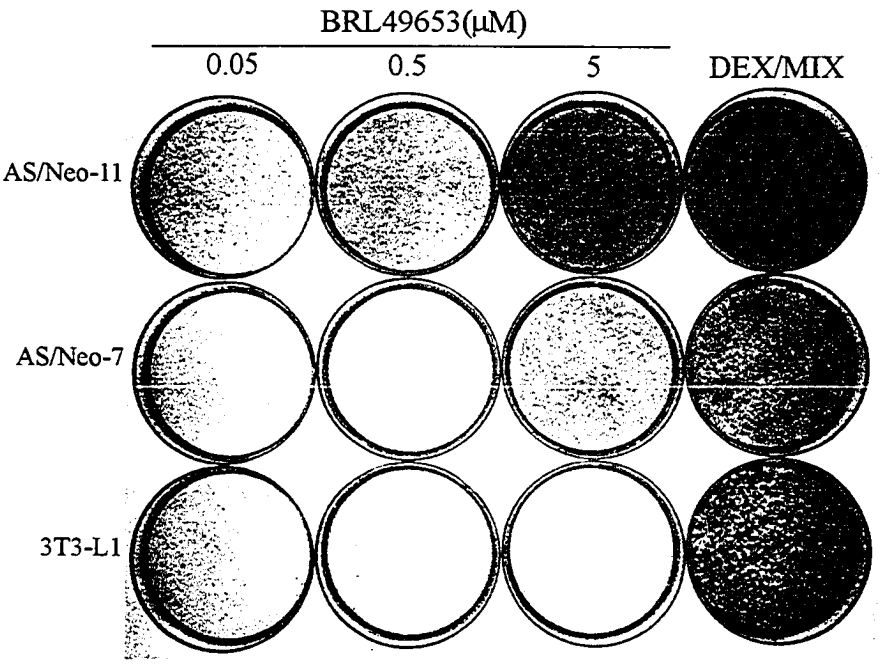


Figure 3.38. Attenuation of AEBP1 stimulates adipocyte differentiation after BRL treatment

To further establish the fact that the protective activity of AEBP1 influences adipocyte differentiation at physiological concentrations, I tested whether ablation of the protective activity of endogenous AEBP1 by ectopic expression of DLD (see Figures 3.27 and 3.34) in 3T3-L1 cells could also lead to accelerated adipogenesis. Stable cell lines were established by retroviral infection using the viruses [AEBP1DLD/puro and AEBP1DLD(-)/puro]. Figure 3.39 shows the DLD expression in 3T3-L1 cells infected with AEBP1DLD/puro [3T3-L1/DLD(+)], but not in the control cells 3T3-L1/DLD(-).

After these cells at the confluence were treated with the adipogenic protocols (see materials and methods), MAP kinase activity and differentiation profiles were examined. As shown in Figure 3.40 (top panel), the amount of phosphorylated MAP kinase in the control cells (-) was markedly reduced on Day 5, then the level further decreased on Days 6 and 7. These results are in agreement with the change in MAP kinase activity during adipocyte differentiation (see Figure 3.22). It is important to note that the differentiation profile correlated inversely with the abundance of AEBP1 and the amount of co-immunoprecipitated MAP kinase during this period (see Figure 3.21). The amount of phosphorylated MAP kinase in the 3T3-L1/DLD(+) cells was lower than the level in 3T3-L1/DLD(-) cells throughout the differentiation period. On Days 6 and 7, the amount of phosphorylated MAP kinase in the 3T3-L1/DLD(+) cells was reduced to the control level when AEBP1 expression is abolished. The lower level of phosphorylated MAP kinase in 3T3-L1/DLD(+) cells was neither due to lower level of precipitated total ERK1 and ERK2 (upper-middle panel), nor due to change in the amount of phosphorylated MEK and total amount of MEK during this period (lower-middle and bottom panels). In agreement with the MAP kinase phosphorylation profile, ectopic expression of DLD in 3T3-L1 cells caused an accelerated differentiation (Figure 3.41). These results establish the role of AEBP1 in enhancing MAP kinase activity by its protective effect, and adipogenic differentiation.

### 3.IV.E. Overall Summary

**Figure 3.39. Mutant DLD Protein Expression**

Cell extracts were analyzed by immunoblotting with anti-AEBP1 antibody to detect the expression of DLD in the 3T3-L1/DLD(+) cells.

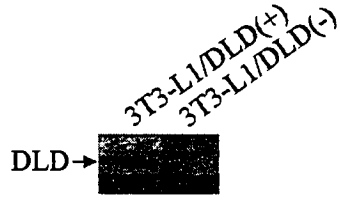


Figure 3.39. DLD Protein Expression

**Figure 3.40. Profile of MAP kinase activity in DLD expressing cells**

Confluent cells were treated with the differentiation inducers for two days, and total cell extracts were prepared every day from Day 3 to Day 7 of the differentiation period (see the materials and methods). Just prior to the preparation of cell extracts, cells were stimulated with 10% FBS and 5 μg/ml insulin for 15 minutes after 2 hours of serum starvation. Phosphorylated MAP kinase (top panel) and MEK (lower middle panel) were detected by immunoblotting analysis of the cell extracts with anti-Phospho-p44/42 MAPK monoclonal and anti-Phospho-MEK1/2 antibodies, respectively.

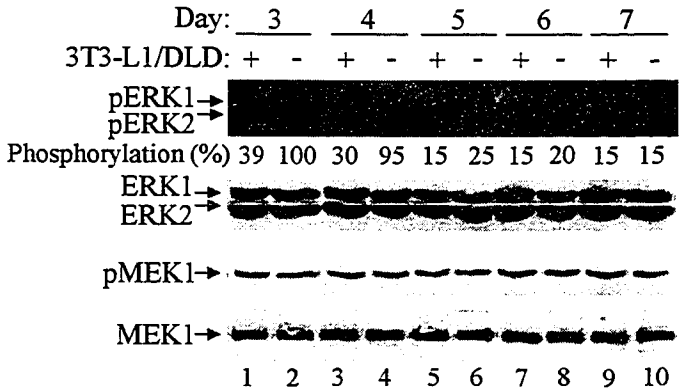


Figure 3.40. Profile of MAPK activity in DLD expressing cells

**Figure 3.41. Inhibition of the protective function of AEBP1 by DLD stimulates adipocyte differentiation and PPARγ expression**

The extent of differentiation in the 3T3-L1/DLD(+) and 3T3-L1/DLD(-) cells was monitored by microscopic (40X) examination of the cells stained with oil Red O and by immunoblotting with anti-PPARγ antibody.

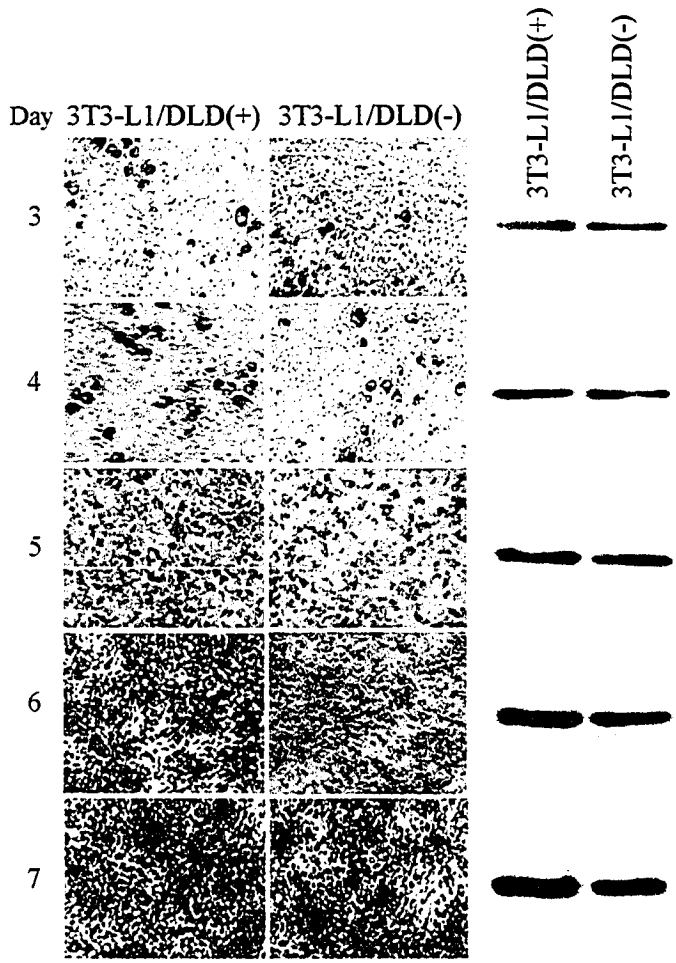


Figure 3.41. Inhibition of the protective function of AEBP1 by DLD stimulates adipocyte differentiation and PPARγ expression

This thesis has explored a transcriptional repressor (AEBP1) that regulates adipocyte differentiation by a novel signaling pathway involving the enhancement of MAP kinase activation. AEBP1 represses transcription of aP2 which encodes a fatty acid binding protein (FABP) (He et al., 1995). AEBP1 levels decrease during adipocyte differentiation in 3T3-L1 cells (see Figures 3.19 and 3.20). AEBP1 associates with MAP kinase in 3T3-L1 cells at the preadipocyte stage, and the level of AEBP1 and the associated MAP kinase decrease as cells undergo differentiation (see Figure 3.21). As indicated in Figure 3.42, AEBP1 interacts with MAP kinase through its N-terminal region, which contains a DLD domain. C-terminal truncated mutants of AEBP1 (DLD and ΔSty) that could still interact with MAP kinase were not able to protect MAP kinase activity from a phosphatase, whereas the ΔHic mutant, which has an internal deletion could still bind and protect MAP kinase activity, albeit less efficiently than wild type AEBP1 (Figure 3.42). Since ΔHic did not have any transcription activity, the effect of this mutant on the protective activity is due to the physical interaction and not due to the transcriptional activity of AEBP1. Moreover, the sustained activation of MAP kinase by ΔHic caused inhibition of adipocyte differentiation, even though the extent of inhibition was not as efficient as wild type AEBP1 (see Figure 3.32). However, ΔSty, which did not have any protective activity, did not inhibit adipocyte differentiation. These results clearly indicate that adipocyte differentiation is attenuated by the MAP kinase activity sustained by AEBP1. Furthermore, the mutant DLD derivative of AEBP1, which lacks transcriptional activity, was shown to interact with MAP kinase and stimulate the rate of adipocyte differentiation. These data strongly suggest a role for AEBP1 in modulating MAP kinase activity by physical association.

To determine whether MAP kinase activity, which is modulated by AEBP1, influences adipocyte differentiation, I used individual clones that stably overexpressed AEBP1. As shown in Table 3.1, using stable individual clones overexpressing AEBP1 (AEBP1/Neo-7) or AEBP1 antisense RNA (AS/Neo-11), I found that MAP kinase activity was modulated by either overexpression or suppression of AEBP1 regulated adipocyte differentiation (see Figures 3.29, 3.31, and 3.36). In these cells, no difference in MEK activity was observed (see Figures 3.13 and 3.18). I then confirmed the effects on adipocyte

**Figure 3.42. Overall summary of modulation of MAP kinase activity and adipocyte differentiation by AEBP1**

Small black boxes represent sequences created by frameshift mutations. The numbers indicate amino-acid residues of AEBP1. + or – indicates presence or absence of interaction of AEBP1 with MAP kinase, its protective activity, or its transcriptional activity. The effect (+) or no effect (-) on regulation of adipocyte differentiation is also shown. N.D. indicates not determined.

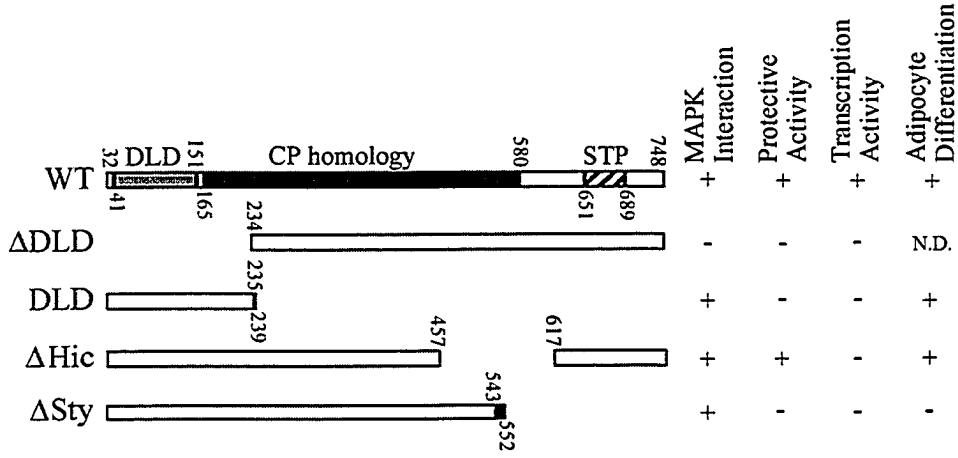


Figure 3.42. Overall summary of modulation of MAPK activity and adipocyte differentiation by AEBP1

differentiation with pooled cells stably overexpressing AEBP1 or its mutant derivatives. The blockage of adipocyte differentiation by overexpression of AEBP1 (AEBP1/Neo-P) (see Figure 3.32) was attenuated by ectopic expression of DLD (AEBP1DLD/Puro) (see Figure 3.35), suggesting that the protective activity of AEBP1 is a critical factor for inhibition of adipogenesis. Furthermore, I showed that adipocyte differentiation was stimulated by attenuation of the protective activity of endogenous AEBP1 by ectopic expression of DLD (see Figure 4.41).

Overall the thesis describes a novel mechanism in the control of adipogenesis. AEBP1 acts as a protective factor that binds to MAP kinase and prevents its dephosphorylation by protein phosphatases. AEBP1 plays a role in adipocyte differentiation by enhancing MAP kinase activity via this protective function. Furthermore, this thesis highlights the possibility that AEBP1 may also regulate adipocyte differentiation through its transcription repression function toward PPARγ and C/EBPα. This thesis provides evidence that both the protective and transcriptional activities of AEBP1 may contribute in the regulation of adipocyte differentiation.

| *Cell Type* | *Stable Cell Line* | *Parental Cell Line* | *MAPK Activity* | *Adipocyte Differentiation* |
|---|---|---|---|---|
| Individual Clone | AEBP1/Neo-7 | 3T3-L1 | Increase | Inhibit |
| | AS-4 | AEBP1/Neo-7 | Decrease | Stimulate |
| | AS/Neo-11 | 3T3-L1 | Decrease | Stimulate |
| Pooled | AEBP1/Neo-P | 3T3-L1 | Increase | Inhibit |
| | AEBP1DLD/Puro | AEBP1/Neo-P | Decrease | Stimulate |
| | 3T3-L1/DLD (+) | 3T3-L1 | Decrease | Stimulate |

Table 3.1. Modulation of MAP kinase activity and adipocyte differentiation in the stable cell lines

# Chapter 4. Conclusions

The first section of this chapter deals with the role of AEBP1 as a protective factor that enhances MAP kinase activity, and the second section explores the role of AEBP1 in adipogenesis through transcriptional and protective activity.

## 4.I. Enhanced MAP Kinase Activity by its Interaction with AEBP1

I show here that AEBP1 complexes with MAP kinase, and as a result of this interaction AEBP1 protects MAP Kinase dephosphorylation by MAPK-specific phosphatase. The persistent activation of MAP kinase, as a result of ectopic overexpression of AEBP1, would allow phosphorylation of different sets of proteins in comparison to the situation for transient activation. The activation of different subsets of transcription factors may therefore determine which genes are turned on to determine cell fate. It has been demonstrated with the use of fibroblasts that the sustained activation of MAP kinase is associated with proliferation, not differentiation (Meloche et al, 1992; Mansour et al., 1994; Cowley et al., 1994). In other cell types, the converse may be true. For example, in PC12 cells the sustained activation of MAP kinase by fibroblast growth factor or nerve growth factor induces differentiation, whereas the transient activation by epidermal growth factor induces proliferation (Marshall, 1995). These differences in the duration of MAP kinase activation appear not to be the result of MEK activation but rather of a MEK-independent pathway in which phosphatases that inactivate MAP kinase both in the cytoplasm and nucleus are regulated (Alessi et al., 1995; Brondello et al., 1997; Grammer and Blenis, 1997). Our results further add to this regulation, in that AEBP1 may regulate the duration of MAP kinase activation through its protective effect against specific phosphatases.

As another possible explanation, the nuclear localization of activated MAP kinase and AEBP1 may lead to increase retention time in this compartment and may sustain MAPK activity. AEBP1, which has a bipartite nuclear localization signal at the carboxyl terminal domain, was phosphorylated by MAP kinase (Muise, 1997) and found in the nucleus and the cytosol fractions (Park, 1998). This phosphorylation may alter the conformation of the

protein or its negative charges may increase the affinity for the proteins in the nuclear pore complex, which then transfers the protein through the nuclear pore. Many proteins have been shown to locate to the nucleus by a mechanism involving phosphorylation near the nuclear localization site (Jans, 1995). MAP kinase may indeed facilitate the nuclear translocation of AEBP1. Alternatively it is also possible that MAP kinase specifically phosphorylates the nuclear pool of AEBP1, as MAP kinase is known to translocate to the nucleus upon activation. The phosphorylated AEBP1 may be specifically retained in the nucleus as a result of this interaction. MAP kinase contains a putative nuclear export sequence (MELDDLPKEKLKEL) (Wen et al., 1995). Interaction of AEBP1 with MAP kinase in the nucleus may block their nuclear export not only because they are too large for diffusion through nuclear pores, but also because interaction masks this putative export signal.

Since a potential MAP kinase site (PMTP) at the C-terminus of AEBP1 is conserved between the human and mouse, it may represent a potential target for modification by MAP kinase that activates AEBP1 in response to extracellular signals. Therefore it is intriguing to speculate that the MAP kinase interaction/protection, nuclear localization of MAP kinase and AEBP1, and transcriptional activity of AEBP1 may be modulated by this phosphorylation.

### 4.II. Novel Regulatory Role of AEBP1 in Adipocyte Differentiation

It has been well documented that adipocyte differentiation is inhibited by a large number of mitogens and growth factors (Navre and Ringold, 1989; Serrero and Mills, 1991). As previously mentioned, inhibition of adipocyte differentiation is, in part, influenced by reduced transcription activity of PPARγ that can be phosphorylated by MAP kinase (Hu et al., 1996; Adams et al., 1997; Camp and Tafuri, 1997). MAP kinase phosphorylation of PPARγ directly impairs the ability of PPARγ to induce adipogenesis in NIH-3T3 cells and in cells ectopically expressing the insulin receptor (Hu et al., 1996).

This phosphorylation of a MAP kinase site in PPARγ inhibits both ligand-independent and ligand-dependent transactivation functions by altering receptor interaction with other unidentified intermediary proteins (Adams et al., 1997). Interestingly, EGF or PDGF induces the phosphorylation of PPARγ through the activation of MAP kinase, and negatively regulates PPARγ activity, thus preventing the progression of adipocyte differentiation (Camp

and Tafuri, 1997). TNFα also stimulates MAP kinase, which in turn phosphorylates and inhibits PPARγ, thus blocking adipogenesis (Adams et al., 1997). These results suggest that negative regulation of adipocyte differentiation by some growth factors might act through the MAP kinase pathway that phosphorylates PPARγ. Insulin, which promotes adipocyte differentiation, also activates MAP kinase like other growth factors. However, insulin stimulation induces the phosphorylation of PPARγ that enhances the transcription activity of the PPARγ (Zhang et al., 1996). Although this discrepancy requires further studies, MAP kinase appears to be an important mediator of cross-talk between insulin signal-transduction pathways and PPARγ activation.

There is some argument concerning the regulation of adipocyte differentiation by MAP kinase activation. MAP kinase activation appears to be required for differentiation of preadipocytes (Sale et al., 1995; Zhang et al., 1996), whereas MAP kinase opposes adipocyte differentiation in 3T3-L1 cells (Font de Mora et al., 1997), possibly through repression of PPARγ function (Hu et al., 1996; Adams et a., 1997). This conflicting role of MAP kinase required for differentiation may be explained by the differences in stimulation of MAP kinase, since MAP kinase translocates into the nucleus upon prolonged activation (Marshall, 1995). Another possible mechanism is that the activation of MAP kinase by different receptor types, although utilizing similar signaling pathways, may cause significant differences in cellular outcomes. For example, adipogenic agents such as insulin causes transient MAP kinase activation while a growth factor, such as EGF, cause prolonged MAP kinase activation (Boney et al., 1998). Although insulin normally induces adipocyte differentiation, overexpression of IRS1, a component of the insulin signaling pathway, causes the activation of MAP kinase and causes NIH3T3 cell transformation (Ito et al., 1996). These results suggest that adipogenesis is, in part, MAP kinase dependent.

In a cell culture model, 3T3-L1 cells generally enter into the terminal stage of adipocyte differentiation around day 4-5 of adipocyte differentiation (see Figure 1.2; Adams et al., 1997; Carel et al., 1996; MacDougald et al., 1995). At this stage, PPARγ and C/EBPα cooperatively activate adipocyte specific genes such as GLUT4 and aP2 (Fajas et al., 1998). MacDougald's group showed that insulin decreases C/EBPα mRNA and protein in fully differentiated adipocytes and the decline of C/EBPα expression is blocked by a MEK inhibitor, suggesting that the process is dependent on MAP kinase activity (Hemati et al., 1997). Therefore a significant down-regulation of AEBP1 that leads to attenuating MAP

kinase activity (see Figures 3.20 and 3.22) may play an important role in establishing of the fully differentiated adipocyte.

Recent studies have led to the finding that AEBP1 interacts with the γ5 subunit of a heterotrimeric G protein (Park et al., 1999). Heterotrimeric G proteins are important mediators of signal transduction in eukaryotic cells, linking ligand-bound seven-transmembrane receptors (also called G protein-coupled receptors, GPCR) with the intracellular machinery (for review, see Clapham and Neer 1997). It is interesting to speculate that AEBP1 may function as a scaffolding protein (for review Schaffer and Weber, 1999; Whitmarsh and Davis 1998) in channeling the GPCR to the MAP kinase signaling module during adipogenesis. Regulation of the AEBP1 activity on MAP kinase activation in conjunction with the Gγ regulation may be important in adipocyte differentiation.

This thesis provides new information suggesting that the enhancement of MAP kinase activation by physical interaction with AEBP1 could constitute a critical part of the molecular mechanism of adipogenesis. For maintaining the preadipocyte phenotype, AEBP1 may block inappropriate signals by balancing the level of activated MAP kinase, in conjunction with the regulation by specific phosphatases. When the cells are stimulated to differentiate, the protective effect of AEBP1 on MAP kinase activation may be attenuated, thus allowing the signal to begin the differentiation process. Furthermore, the decreased MAP kinase activity may enhance the rate of adipogenesis (see Figure 3.41). Overall these results demonstrate a novel function for AEBP1 as an important mediator in the adipogenic signal-transduction pathway.

## References


Adams, M., Reginato, M. J., Shao, D., Lazar, M. A., and Chatterjee, V. K. (1997) Regulation of peroxisome proliferator-activated receptor gamma activity by mitogen-activated protein kinase. J. Biol. Chem. 272:5128-5132

Ailhaud, G., Grimaldi, P. and Négrel, R. (1992) Cellular and molecular aspects of adipose tissue development. Annu. Rev. Nutr. 12:207-233

Alessi, D. R., Gomez, N., Moorhead, G., Lewis, T., Keyse, S. M., and Cohen, P. (1995) Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. Curr. Biol. 5:283-295

Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, L. (1994) Current protocols in molecular biology, John Willy & Sons, Inc.

Baxevanis, A., and Landsman, D. (1995) The HMG-1 box protein family: classification and functional relationships. Nuc. Acid. Res. 23:1604-1613

Benito, M., Porras, A., Nebreds, A. R., and Santos, E. (1991) Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of ras oncogenes. Science 253:565-568

Boney, C. M., B., Smite, R. M., and Gruppuso, P. A. (1998) Modulation of insulin-like growth factor I mitogenic signaling in 3T3-L1 preadipocyte differentiation. Endocrinology 139:1638-1644

Bonny, C., Nicod, P., and Waeber, G. (1998) IB1, a JIP-1-related nuclear protein present in insulin-secreting cells. J. Biol. Chem. 273:1843-1846

Boris-Lawrie, K. A., and Temin, H. M. (1993) Recent advances in retrovirus vector technology. Curr. Opin. Genet. Dev. 3:102-9

Brondello, J.M., et al., (1997) The dual specificity mitogen-activated kinase phosphatase-1 and – are induced by the p42/p44 MAPK cascade. J. Biol. Chem. 272:1368-1378

Brun, R. P., Kim, J. B., Hu, E., Altiok, S., and Spiegelman, B. M. (1996a) Adipocyte differentiation: a transcriptional regulatory cascade. Curr. Opin. Cell Biol. 8:826-832

Brunet, A., and Pouysségur, J. (1996) identification of MAP kinase domains by redirecting stress signals into growth factor responses. Science 272:1652-1655

Burn, R. P., Tontonoz, P., Forman, B. M., Ellis, R., Chen, J., Evans, R. M., and Spiegelman, B. M. (1996b) Differentiation activation of adipogenesis by multiple PPAR isoforms. Genes Dev. 10:974-984

Camp, H. S., and Tafuri, S. R. (1997) Regulation of peroxisome proliferator-activated receptor gamma activity by mitogen-activated protein kinase. J. Biol. Chem. 272:10811-10816

Campfield, L. A., Smith, F. J., and Burn, P. (1996) The OB protein (leptin) pathway – a link between adipose tissue mass and central neural networks. Horm. Metab. Res. 28:619-632

Canagarajah, B. J., Khokhlatchev, A., Coob, M. H., and Goldsmith, E. J. (1997) Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. Cell 90:859-869

Carel, K., Kummer, J.L., Schubert, C., Leitner, W., Heidenreich, K.A., and Draznin, B. (1996) Insulin stimulates mitogen-activated protein kinase by a Ras-independent pathway in 3T3-L1 adipocytes. J Biol. Chem. 271:30625-30

Catling, A. D., Schaeffer, H.-J., Reuter, C. W.M., Reddy, G. R., and Weber, M. J. (1995) A proline-rich sequence unique to MEK1 and MEK2 is required for Raf binding and regulates MEK function. Mol. Cell. Biol. 15:5214-5225

Chen, R. H., Sarnecki, C., Blenis, J. (1992) Nuclear localization and regulation of erk-1 and rsk-encoded protein kinase. Mol. Cell. Biol. 12:915-927

Choi, K. Y., Satterberg, B., Lyons, D. M., and Elion, E. A. (1994) Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in S. cerevisiae. Cell 78:499-512

Christy, R. J., Yang, V. W., Ntambi, J. M., Geiman, D. E., Landschulz, W. H., Friedman, A. D., Nakabeppu, Y., Kelly, T. J., and Lane, M. D. (1991) CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. Proc. Natl. Acad. Sci. USA 88:2593-2597

Clapham, D. E., and Neer, E. J. (1997) G protein beta gamma subunits. Annu. Rev. Pharmacol. Toxicol. 37:167-203

Cobb, M. H., and Goldsmith, E. J. (1995) How MAP kinases are regulated. J. Biol. Chem. 270:14843-14846

Constance, C. M., Morgan, J. I., IV, and Umek, R. M. (1996) C/EBPalpha regulation of the growth-arrest-associated gene gadd45. Mol. Cell. Biol. 16:3878-3883

Cornelius, P., MacDougald, O. A., and Lane, M. D. (1994) Regulation of adipocyte development. Annu. Rev. Nutr. 14:99-129

Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH-3T3 cells. Cell 77:841-852

Crespo, P., Cachero, T. G., Xu, N., and Gutkind, J. S. (1995) Dual effect of beta-adrenergic receptors on mitogen-activated protein kinase. Evidence for a beta gamma-dependent activation and a G alpha s-cAMP-mediated inhibition. J. Biol. Chem. 270:25259-25265

Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997) Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. Nature 390:88-91

Davis, R. J. (1993) The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. 268:14553-14556

Denton, R. M., and Tavaré, J. M. (1995) Does mitogen-activated-protein kinase have a role in insulin action? The cases for and against. Eur. J. Biochem. 227:597-611

Dickens, M., Rogers, J. S., Cavanagh, J., Raitano, A., Xia, Z., Halpern, J. R., Greenberg, M. E., Sawyers, C. L., and Davis. R. J. (1997) A Cytoplasmic Inhibitor of the JNK Signal Transduction Pathway. Science 277: 693-696.

Downward, J. (1996) Control of ras activation. Cancer Surv. 27:87-100

Fajas, L., Fruchart, J. –C., and Auwerx, J. (1998) Transcriptional control of adipogenesis. Current Opinion in Cell Biology 10:165-173

Fajas, L., Auboeuf, D., Raspe, E., Schoonjans, K., Lefebre, A .M., Salsadin, R., Najib, J., Laville, M., Fruchart, J. C., Deeb, S., Vidal-Puig. A., Flier, J., Briggs, M. R., Staels, B., Vidal, H., and Auwerx, J. (1997) Organization, promoter analysis and expression of the human PPARγ gene. J. Biol. Chem. 272:18779-18789

Faure, M., Voyno-Yasenetskaya, T. A., and Bourne, H. R. (1994) cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. J. Biol. Chem. 18:7851-7854

Font de Mora J., Porras, A., Ahn, N., and Santos, E. (1997) Motogen-activated protein kinase activation is not necessary for, but antagonizes, 3T3-L1 adipocyte differentiation. Mol. Cell. Biol. 17:6068-6075

Freytag, S. O., Paielli, D. L., and Gilbert, J. D. (1994) Ectopic expression of the CCAAT/enhancer-binding protein alpha promotes the adipogenic program in a variety of mouse fibroblastic cells. Genes Dev. 8:1654-63

Fricker, L. D. (1988) Carboxypeptidase E. Annu. Rev. Physiol. 50:309-21

Fukuda, M., Gotoh, Y., and Nishida, E. (1997) Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase. EMBO J. 16: 1901-1909

Geloen, A., Collet, A. J., Guay, G., and Bukowiecki, L. J. (1989) Insulin stimulates in vivo cell proliferation in white adipose tissue. 256:C190-196

Gotoh, I., Fukuda, M., Adachi, M., and Nishida, E. (1999) The control of cell morphology and the S phase entry by mitogen-activated protein kinase kinase. J. Biol. Chem. 274:11874-11880

Grammer, T. C., and Blenis, J. (1997) Evidence for MEK-independent pathways regulating the prolonged activation of the ERK-MAP kinases. Oncogene 274:1635-1645

Gregoire, F. M., Smas, C. M., and Sul, H. S. (1998) Understanding adipocyte differentiation. Physiol. Rev. 78:783-809

Groom, L. A., Sneddon, A. A., Alessi, D. R., Dowd, S. and Keyse, S.M. (1996) Differential regulation of the MAP, SAP and RK/p38 kinases by Pystl, a novel cytosolic dual-specificity phosphatase. EMBO J. 15: 3621-3632

Gutkind, J. S. (1998) The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. J. Biol. Chem. 273:1839-1842

Hamm, H. E. (1998) The many faces of G protein signaling. J. Biol. Chem. 273:669-672

Haystead, C. M. M., Gregory, P., Shirazi, A., Fadden, P., Mosse, C., Dent, P., and Haystead. T.A.J. (1994) Insulin activates a novel adipocyte mitogen-activated protein kinase kinase kinase that shows rapid phasic kinetics and is distinct from c-Raf. JBC 269:12804-128

He, G.-P., Musie, A., Li, A. W., and Ro, H.–S. (1995) A eukaryotic transcriptional repressor with carboxypeptidase activity. Nature 378:92-96

He, G. –P., Kim, S. W., and Ro, H. –S. (1999) Cloning and characterization of a novel zinc finger transcriptional repressor. A direct role of the zinc finger motif in repression. J. Biol. Chem. 274:14678-84.

Hemati, N., Ross, S. E., Lerickson, R., Groblewski, G. E., and MacDougald, O. A. (1997). Signaling pathways through which insulin regulates CCAAT/enhancer binding protein α (C/EBPα). J. Biol. Chem. 272:25913-25919

Herskowitz, I. (1995) MAP kinase pathways in yeast: for mating and more. Cell 80:187-197

Hidai, C., Zupancic, T., Penta, K., Mikhail, A., Kawana, M., Quertermous, E. E., Aoka, Y., Fukagawa, M., Matsui, Y., Platika, D., Auerbach, R., Hogan, B. L. M., Snodgrass, R., and Quertermous, T. (1998) Cloning and characterization of developmental endothelial locus-1: an embryonic endothelial cell protein that binds the alphavbeta3 integrin receptor. Genes Dev. 12:21– 33

Hill, C. S., and Treisman, R. (1995) Transcription regulation by extracellular signals: mechanisms and specificity. Cell 80:199-211

Hipskin, R. A., Rao, V. N., Mueller, C. G. F., Reddy, E. S. P., and Nodheim, A. (1991) Ets-related protein Elk-1 is homologous to the c-fos regulatory factor p62TCF. Nature 354:531-534

Hotamisligil, G. S., Johnson, R. S., Distel, R. J., Ellis, R., Papaioannou, V. E., and Spiegelman, B. M. (1996a) Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. Science 271:1377-1379

Hotamisligil, G. S., Peraldi, P., Budavari, A., Ellis, R., White, M. F., and Spiegelman, B. M. (1996b) IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-α and obesity induced insulin resistance. Science 271:665-668

Hotamisligil, G. S., and Spiegelman, B. M. (1994) Tumor necrosis factor alpha: a key component of the obesity-diabetes link. Diabetes 43:1271-1278

Hu, E., Kim, J. B., Sarraf, P., and Spiegelman, B. M. (1996) Inhibition of adipogenesis through MAP-kinase mediated phosphorylation of PPARγ. Science 274:2100-2103

Ito, T., Sasaki, Y., and Wands, J. R. (1996) Overexpression of human insulin receptor substrate 1 induces cellular transformation with activation of mitogen-activated protein kinase. Mol. Cell. Biol. 16:943-951

Jacobs, D., Glossip, D., Xing, H., Muslin, A. J., and Kornfeld, K. (1999) Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. Genes Dev. 13:163-175

Jans, D. A. (1995) The regulation of protein transport to the nucleus by phosphorylation. Biochem. J. 311:705-716

Johnson, J. D., Edman, J. C., and Rutter, W. J. (1993) A receptor tyrosine kinase found in breast carcinoma cells has an extracellular discoidin I-like domain. Proc. Natl. Acad. Sci. USA 90, 5677– 5681

Kane, W. H., and Davie, E. W. (1986) Cloning of a cDNA coding for human factor Vm, a blood coagulation factor homologous to factor VIII ceruloplasm. Proc. Natl. Acad. Sci. USA. 83:6800– 6804

Kellen, C. B., and Lazar, M. A. (1996) Antidiabetic thiazolidinediones inhibit leptin (ob) gene expression in 3T3-L1 adipocyte. Proc. Natl. Acad. Sci. USA 93:5793-5796

Khokhlatchev, A. V., Canagarajah, Wilsbacher, J., Robinson, M., Atkinson, M., Glodsmith, E., and Cobb, M. H. (1998) Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. Cell 93:605-615

Kim, J.B., and Spiegelman, B.M. (1996) ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. Genes Dev. 10: 1096-1107

Kim, S.-W., Muise, A.M., and Ro, H.-S. (2000) Regulation of adipogenesis mediated by a transcriptional repressor that modulates MAPK. J. Cell Biol. (submitted)

Koch, W. J., Hawes, B. E., Allen, L. F., Lefkowitz, R. J. (1994) Direct evidence that G coupled receptor stimulation of mitogen-activated protein kinase is mediated by Gβγ activation of p21ras. Proc. Natl. Acad. Sci. USA 91:12706-12710

Kolodkin, A. L., Levengood, D. V., Rowe, E. G., Tai, Y. T., Giger, R.J ., and Ginty, D. D. (1997) Neuropilin is a semaphorin III receptor. Cell 90:753-62

Kusari, A. B., Byon, J., Bandyopadhyay, D., Kenner, K. A., and Kusari, J. (1997) Insulin-induced mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1) attenuates insulin stimulated MAP kinase activity: a mechanism for the feedback inhibition of insulin signaling. Mol. Endocrinol. 11:1532-1543

Larocca, D., Peterson, J. A., Urrea, R., Kuniyoshi, J., Bistrain, A. M., and Ceriani, R. L. (1991) A Mr 46,000 human milk fat globule protein that is highly expressed in human breast tumors contains factor VIII-like domains. Cancer Res. 51, 4994– 4998

Layne, M. D., Endege, O. W., Jain, M. K., Yet, S. –F., Hsieh, C. –M, Chin, M. –T., Perrella, M. A., Blannar, M. A., Haber, E., and Lee., M. –E. (1998) Arotic carboxypeptidase-like protein, a novel protein with discoidin and carboxypeptidase-like domains, is up-regulated during vascular smooth muscle cell differentiation. J. Biol. Chem. 273:15654-15660

Leeuw, T., Fourest-Lieuvin, A., Wu, C., Chenevert, J., Clark, K., Whiteway, M., Thomas, D. Y., and Leberer, E. (1995) Pheromone Response in Yeast: Association of Bem1p with Proteins of the MAP Kinase Cascade and Actin. Science 270:1210-1213.

Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., and Kliewer, S. A. (1995) An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ) J. Biol. Chem. 270:12953-12956

Lenormand, P., Sardet C., Pages, G., L'Allemain, G., Brunet, A., Pouysségur, J. (1993) Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. J. Cell. Biol. 122:1079-1088

Lin, F. T., and Lane, M. D. (1992) Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. Genes Dev. 6:533-544

Lin, F. –T., MacDougald, O. A., Diehl, A. M., and Lane, M. D. (1993) A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. Proc. Natl. Acad. Sci. USA 90:9606-9610

Luttrell, L. M., Della Rocca, G. J., van Biesen, T., Luttrell, D. K., and Lefkowitz, R. J. (1997) Gβγ subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. J. Biol. Chem. 272:4637-4644

MacDougald, O.A., Cornelius, P., Liu, R., & Lane, M. D. (1995) Insulin regulates transcription of the CCAAT/enhancer binding protein (C/EBP)a, b, and d genes in fully-differentiated 3T3-L1 adipocytes. J. Biol. Chem. 270:647-654

Mandrup, S., and Lane, M. D. (1997) Regulating adipogenesis. J. Biol. Chem. 272:5367-5670

Maniatis, T., Fritsch, E.F., and Sambrook. J. (1982). Electrophoresis of RNA through gels containing formaldehyde. p 202-203. In Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.

Mansour, S. J., Matten, W.T., Hermann, A.S., Candia, J.M., Rong, S., Fukasawa, K., Vande Woude, G. F., Ahn, N. G. (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 265:966-970

Marshall, C. J. (1995) specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179-185

Mehlen, P., Mehlen, A., Guillet, D., Preville, X., and Arrigo, A. (1995) Tumor necrosis factor-α induced changes in the phosphorylation, cellular localization, and oligomerization of human hsp27, a stress protein that confers cellular resistance to this cytokine. J. Cell. Bioc. 58:248-259

Meloche, S., Seuwen, K., Pagès, G., and Pouyssègur, J. (1992) Biphasic and synergistic activation of $p44^{mapk}$ (ERK1) by growth factors: correlation between late phase activation and mitogenicity. Mol. Endocrinol. 658:845-854

Miller, C. W., and Ntambi, J. M. (1996) Peroxisome proliferators induce mouse liver stearoyl-CoA desaturase 1 gene expression. Proc, Natl. Acad. Sci. USA 93:9443-9448

Minshull, J. H., Sun, H., Tonks, N. K., and Murray, A. W. (1994) A MAP kinase-dependent spindle assembly checkpoint in *Xenopus* egg extracts. Cell 79:475-486

Muda, M., Boschert, U., Dickinson, R., Martinou, J. –C., Marinou, I., Camps, M., Schlegel, W., and Arkinstall, S. (1996) MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase. J. Biol. Chem. 271:4319-4326

Muise, A. M. (1997) AEBP1, a multifunctional protein involved in adipogenesis. Ph.D. Thesis, Dept. Biochemistry, Dalhousie University

Muise, A. M., and Ro, H. –S. (1999) Enzymic characterization of a novel member of the regulatory B-like carboxypeptidase with transcriptional repression function: stimulation of enzymic activity by its target DNA. Biochem. J. 15:341-5.

Myers, M. G. Jr., and White, M. F. (1996) Insulin signal transduction and the IRS proteins. Annu. Rev. Pharmacol. Toxicol. 36:615-658

Myers, M. G. Jr., Wang, L. M., Sun, X. J., Zhang, Y. T., Yenush, L., Schlessinger, J., Pierce, J. H., and White, M. F. (1994) The role of IRS-1/GRB2 complexes in insulin signaling. Mol. Cell. Biol. 14:3577-3587

Navre, M and Ringold, G. M. (1989) Differential effects of fibroblast growth factor and tumor promoters on the initiation and maintenance of adipocyte differentiation. J. Cell Biol. 109:1857-63

Norgues, J., Reyne, Y., Barenton, B., Cherry, T., Garamdel, V., and Sariano, J. (1993) Differentiation of Ob17 preadipocytes in a serum-free medium is influenced by glucocorticoids and endogenously produced insulin-like growth factor. Int. J. Obesity 17:159-167

Ohno, I., Hashimoto, J., Shimuzu, K., Takaoka, K., Ochi, T., Matsubara, K., and Okubo, K. (1996) A cDNA cloning of human AEBP1 from primary cultured osteoblasts and its expression a differentiating osteoblast cell line, Biochem, Biophy. Res. Comm. 228:411-414

Ouwens, D. M., van der Zon G. C. M., Pronk, G. J., Bos, J. L., Moller, W., Cheatham, B., Kahn, C. R., and Maassen, J. A. T. (1994) A mutant insulin receptor induces formation of a Shc-growth factor receptor bound protein 2 (Grb2) complex and p21ras-GTP without detectable interaction of insulin receptor substrate 1 (IRS1) with Grb2. J. Biol. Chem. 269:33116-33123

Pages, G., Lenormand, P., L'allemain, G., Chambard, J. C., Meloche, S. and Pouysségur, J. (1993) Mitogen-activated protein kinase p42mapk and p44mapk are required for fibroblast proliferation. Proc. Natl. Acd. Sci. USA 90:8319-8323

Pairault, J., and Green, H. (1979) A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenease as differentiation marker. Proc. Natl. Acad. Sci. USA 76:5138-5142

Park, J. –G. (1998) Gγ5: Nuclear localization and transcriptional regulation during adipocyte differentiation. M.Sc. Thesis, Dept. Biochemistry, Dalhousie University

Park, J. –G., Muise, A., He, G. –P., Kim, S. –W., and Ro, H. –S. (1999) Transcriptional regulation by the γ5 subunit of a heterotrimeric G protein during adipogenesis. EMBO J. 18:4004-4012

Pawson, T., and Schlessinger, J. (1993) SH2 and SH3 domains. Curr. Biol. 3(7):434-159

Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993) Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. U S A. 90:8392-8396

Pelech, S. L., and Sanghera, J. S. (1992) MAP kinases: charting the regulatory pathways. Science 275:1355-1356

Peraldi, P., Xu, M., and Spiegelman, B. M. (1997) Thiazolidinediones block tumor necrosis factor-α-induced inhibition of insulin signaling. J. Clin. Invest. 100:1863-1869

Pierschbacher, M. D., Hayman, E. G., and Ruoslahti, E. (1985) The cell attachment determinant in fibronectin. J. Cell Biochem. 28:115-26

Poole, S., Firtel, R. A., Lammar, E., and Rowekamp, W. J. (1981) Sequence and expression of the discoidin I gene family in Dictyostelium discoideum. J. Mol. Biol. 153:273-289

Porras, A., Muszynski, K., Rapp, U. R., and Santos, E. (1994) Dissociation between activation Raf-1 kinases and the 42-kDa mitogen-activated protein kinase/90-kDa (MAPK/RSK) cascade in the insulin/Ras pathway of adipocyte differentiation of 3T3-L1 cells. J. Biol. Chem. 269:12741-12748

Porras, A., and Santos, E. (1996) The insulin/Ras pathway of adipocyte differentiation of 3T3-L1 cells: dissociation between Raf-1 kinase and the MAPK/RSK cascade. Int. J. Obes. Relat. Metab. Disord. 3:S43-51

Pryciak, P. M., and Huntress, F. A. (1998) Membrane recruitment of the kinase cascade scaffold protein Ste5 by the Gbetagamma complex underlies activation of the yeast pheromone response pathway. Genes Dev. 12:2684-2697

Qui, M. S., and Green, S. H. (1992) PC12 cell neuronal differentiation is associated with prolonged p21ras activity and consequent prolonged ERK activity. Neuron 9:705-717

Racke, F. K., Lewandoswska, K., Goueli, S., and Goldfarb, A. N. (1997) Sustained activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway is required for megakaryocytic differentiation of K562 cells. J. Biol. Chem. 272:23366-23370

Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Hna, R., Ulevitch, J., and Davis, R. J. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J. Biol. Chem. 270:7420-7426

Rao, V. N., and Reddy, E. S. (1993) Elk-1 proteins are phosphoproteins and activators of mitogen-activated protein kinase. Cancer Res. 53:3449-3454

Rao, V. N., and Reddy, E. S. (1994) Elk-1 protein interact with MAP kinases. Oncogene 9:1855-1860

Reginato, M. J., Krakow, S. L., Bailey, S. T., and Lazar, M. A. (1998) Prostaglandins promote and block adipogenesis through opposing effects on peroxisome proliferator-activated receptor $\gamma$. J. Biol. Chem. 273:1855-1858

Richon, V.M., Lyle, R. E., and McGehee, Jr, R. E. (1997) Regulation and expression of retinoblastoma proteins p107 and p130 during 3T3-L1 adipocyte differentiation. J. Biol. Chem. 272:10117-10124

Ro, H. –S., Kim, S. –W., and He, G. –P. (2000) Alternative splicing yields two isoforms of the mouse adipocyte enhancer binding protein (AEBP1) with subcellular localization that function in different cell lineage. Nucleic Acids Res. (submitted)

Roncari D.A.K. (1984) Pre-adipose cell replication and differentiation. TIBS 9:486-489

Sale, E. M., Atkinson, P. G. P., and Sale. G. J. (1995) Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. EMBO J 14:674-684

Sasaoka, T., Ishihara, H., Sawa, T., Ishiki, M., Morioka, H., Imamura, T., Usui, I., Takata, Y., and Kobayashi, M. (1996) Functional importance of amino-terminal domain of Shc for interaction with insulin and epidermal growth factor receptors in phosphorylation-independent manner. J. Biol. Chem. 271:20082-20087

Schaeffer, H. J., Catling, A. D., Eblen, S. T., Collier, L. S., Krauss, A., and Weber, M. J. (1998) MP1: A MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science 281:1668-1671

Schaeffer, H. J., and Weber, M. J. (1999) Mitogen-activated protein kinases; Specific messages from ubiquitous messengers. Mol. Cell. Biol. 19:2435-2444

Schoonjans, K., Martin, G., Staels, B., and Auwerx, J. (1997) Peroxisome proliferator-activated receptors, orphans with ligands and functions. Curr Opin Lipidol. 8:159-166

Scott R. E. (1982) Coupling of preadipocyte growth arrest and differentiation. A cell cycle model for the physiological control of cell proliferation. J. Cell Biol. 94:400-405

Seger, R., and Krebs, E. G. (1995) The MAPK signaling cascade. FASEB J. 9:726-735

Sells, M. A., and Chernoff, J. (1995) Epitope-tag vectors for eukaryotic protein production. Gene 152:187-9

Serrero, G., and Mills, D. (1991) Physiologic role of EGF on adipose tissue development in vivo. Proc. Natl. Acad. Sci. USA. 88:3912-3916

Skidgel, R. A. (1988) Basic carboxypeptidases: regulators of peptide hormone activity. Trends pharmacol. Sci. 9:299-304

Smith, A., Ramos-Morales, F., Ashworth, A., and Collins, M. (1997) A role for JNK/SAPK in proliferation, but not apoptosis, of IL-3-dependent cells. Curr. Biol. &:893-896

Smith, P. J., Wise, L. S., Berkowitz, R., Wan, C., and Rubin, C. S. (1988) Insulin-like growth factor is an essential regulator of the differentiation of 3T3-L1 adipocytes. J. Biol. Chem. 263:9402-9408

Songyang, Z., Lu, L. P., Kwon, Y. T., Tsai, L., Filhol, O., Cochet, C., Brickey, D. A., Soderling, T. R., Bartleson, C., Graves, D. J., Demaggio, A. J., Hoekstra, M. F., Blenis, J., Hunter, and Cantley, L. C. (1996) A structural basis for substrate specificities of protein Ser/Thr kinases: Primary sequence preference of casein kinases I and II, NIMA, phosphorylate kinase, calmodulin-dependent kinase II, CDK5, and Erk. Mol. Cell. Biol. 16:6486-6493

Springer WR, Cooper DN, Barondes SH. (1984) Discoidin I is implicated in cell-substratum attachment and ordered cell migration of Dictyostelium discoideum and resembles fibronectin. Cell 39:557-64

Stephens, T. W., Basinski, M., Briston, P. K., Bue-Valleskey, J. M., Burgett, S. G., Craft, L., Hale, J., Hoffman, J., Hsiung, H., Knausciunas, A., Makellar, W., Roseck, P. R. Jr., Schoner, B., Smith, D., Tinsley, F. C., Zhang, X. Y., and Heiman, M. (1995) The role of neuropeptide Y in the antiobesity action of the *obese* gene product. Nature 377:530-532

Stubbs, J. D., Lekutis, C., Singer, K.L., Bui, A., Yuzuki, D., Srinivasan, U., and Parry, G. (1990) cDNA cloning of a mouse mammary epithelial cell surface protein reveals the existence of epidermal growth factor-like domains linked to factor VIII-like sequences. Proc. Natl. Acad. Sci. U S A 87:8417-821

Takagi, S., Hirata, T., Agata, K., Mochii, M., Eguchi, G., and Fujisawa, H. (1991) The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors. Neuron 7, 295– 307

Tartaglia, L. A., Dembrski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G. J., Campfield, L. A., Clark, F. T., deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K. J., Smutko, J. S., Mays, G. G., Woolf, E. A., Monroe, C. A., and Tepper, R. I. (1995) Identification and expressing cloning of a leptin receptor, OB-R. Cell 83:1263-1271

Timchenko, N.A., Wilde, M., Nakanishi, M., Smith, J. R., and Darlington. G. J. (1996) CCAAT/enhancer-binding protein alpha (C/EBP alpha) inhibits cell proliferation through the p21(WAF-1/CIP-1/SDI-1) protein. Genes Dev. 10:804-815

Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I., and Spiegelman, B. M. (1994a) mPPARγ2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. 8:1224-1234

Tontonoz, P., Hu, E., and Spiegelmam, B. M. (1994b) Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell 79:1147-1156

Tontonoz, P., Kim. J. B., Graves, R. A., and Spiegelman, B. M. (1993) ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. Mol. Cell. Biol. 13:4753-4759

Toole, J. J., Knopf, J. L., Wozney, J. M., Sultzman, L. A., Buecker, J. L., Pittman, D. D., Kaufman, R. J., Brown, E., Shoemaker, C., Orr, E. C., Amphlett, G. W., Foster, W. B., Coe, M. L., Knutson, G. J., Fass, D. N., and Hewick, R. M. (1984) Molecular cloning of a cDNA encoding human antihaemophilic factor. Nature 312: 342– 347

Torti, F. M., Dieckmann, Beutler, B., Cerami, A., and Ringold, G. M. (1989) Modulation of adipocyte differentiation by tumor necrosis factor and transforming growth factor beta. J. Cell. Biol. 108:16:1479-1489

Traverse, S., Gomez, N., Paterson, H., Marshall, C., and Cohen, P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. Biochem. J. 288:351-355

Treisman, R. (1996) Regulation of transcription by MAP kinase cascades. Cur.Opi.Cell.Biol. 8:205-215

Ubeda, M., Wang, X. Z., Zinszner, H., Wu, I., Habener, J. F., and Ron, D. (1996) Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. Mol. Cell. Biol. 16:1479-1489

Uysal., K. T., Wiesbrock, S. M., Marino, M. W., & Hotamisligil, G. S. (1998) Protection from obesity-induced insulin resistance in mice lacking TNFα function. Nature 389:610-614

van Biesen, T., Luttrell, L. M., Hawes, B. E., and Lefkowitz, R. J. (1996) Mitogenic signaling via G protein-coupled receptors. Endocr. Rev. 17:698-714

Vanderstraeten-Gregoire, F. *La differentiation in vitro des precurseurs des cellules adipeuses de Rat* (PhD thesis). Louvain: Unversite Catholique de Louvain, 1989

Virca, G.D., Northemann, W., Shiels, B. R., Widera, G., and Broome, S. (1990) Simplified northern blot hybridization using 5% sodium dodecyl sulfate. BioTechniques 8:370-371

Wadanabe, F., Shirakawa, H., Yoshida, M., Tsukada, K., and Teraoka, H. (1994) Stimulation of DNA-dependent protein kinase activity by high mobility group proteins 1 and 2. Biochem. Biophy. Res. Comm. 202:736-742

Wang, N. D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., and Darlington, G. J. (1995) Impaired energy homeostasis in C/EBP alpha knockout mice. Science 269:1108-1112

Wang, X. Z., and Ron, D. (1996) Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. Science 272:1347-1349

Wen, W., Meinkoth, J. L., Tsien, R. Y., and Tayleor, S. S. (1995) Identification of a signal for rapid export of proteins from the nucleus. Cell 82, 463-473

Werman, A., Hollenberg, A., Solanes, G., Bjorbaek, C., Vidal-Puig, A., and Flier, J. S. (1997) Ligand-independent activation domain in the N terminus of peroxisome proliferator-activated receptor γ (PPARγ). J. Biol. Chem. 272:20230-20235

White, M. F. (1998) The IRS-signaling system: A network of docking proteins that mediate insulin action. Mol. Cell. Biochem. 182:3-11

Whitmarsh, A.J., Cavanagh, J., Tournier, C., Yasuda, J., and Davis, R.J. (1998) A mammalian scaffold complex that selectively mediates MAP kinase activation. Science 281:1671-1674

Whitmarsh, A. J., and Davis, R. J. (1998) Structural organization of MAP kinase signaling molecules by scaffold proteins in yeast and mammals. Trends Biochem. Science 23:481-485

Whiteway, M. S, Wu, C., Leeuw, T., Clark, K., Fourest-Lieuvin, A., Thomas, D. Y., and Leberer, E. (1995) Association of the yeast pheromone response G protein beta gamma subunits with the MAP kinase scaffold Ste5p. Science 269:1572-1575

Widmann, C., Gibson, S., Jarpe, M. B., and Jhonson, G. L. (1999) Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. Physiol. Rev. 79:143-180

Wu, Z., Bucher, N. L., and Farmer, S. R. (1996) Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids. Mol. Cell. Biol. 16:4128-4136

Wu, Z., Xie, Y., Bucher, N. L. R., and Farmer, S. R. (1995) Conditional ectopic expression of C/EBPβ in NIH-3T3 cells induces PPARγ and stimulates adipogenesis. Genes Dev. 9:2350-363

Xia, Z., Dickens, M., RainGeaud, J., Davis, R., and Greenberg, M. E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270: 1326-1331

Xing, H., Northrop, J. P., Grove, J. R., Kilpathrick, K. E., Su, J. L., and Ringold, G. M. (1997). TNF alpha-mediated inhibition and reversal of adipocyte differentiation is accompanied by suppressed expression of PPAR gamma without effects on Pref-1 expression. Endocrinology 138:2776-2783

Xu, S., Robbins, D., Frost, J., Dang, A., Lange-Carter, C., and Cobb, M. H. (1995) MEKK1 phosphorylates MEK1 and MEK2 but does not cause activation of mitogen-activated protein kinase. Proc. Natl. Acad. Sci. USA 92:6808-6812.

Yamauchi, K., and Pessin, J. E. (1994) Insulin receptor substrate (IRS1) and Shc compete for a limited pool of Grb2 in mediating insulin downstream signaling. J. Biol. Chem. 269:31107-31114

Yang, S. –H., Whitmarsh, A. J. Davis, R. J., and Sharrocks, A. D. (1998b) Differential targeting of MAP kinase to the ETS-domain transcription factor Elk-1. EMBO J. 17:1740-1749.

Yang, S. –H., Yates, P. R., Whitmarsh, A. J., Davis, R. J., and Sharrocks, A. D. (1998a) The Elk-1 ETS-domain transcription factor contains a mitogen-activated protein kinase targeting motif. Mol. Cell. Biol. 18:710-720

Yashar, B., Irie, K., Printen. J. A., Stevenson, B. J., Sprague, Jr., G. F., Matsumoto, K., and Errede, B. (1995) Yeast MEK-dependent signal transduction: response thresholds and parameters affecting fidelity. Mol. Cell. Biol. 15:6545-6553

Yeh, W. C., Cao, Z., Classon, M., and McKnight, S. L. (1995) Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev. 9: 168-181

Yokoyama, C., Wang, X., Briggs, M. R., Admon, A., Wu, J., Hua, X., Goldstein, J. L., and Brown, M. S. (1993) SREPB-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. Cell 75:187-197

Yu, W., Fantl, W. J., Harrow, G., and Williams, L. T. (1997) Regulation of the MAP kinase pathway by mammalian Ksr through direct interaction with MEK and ERK. Curr. Biol. 8:56-64

Zhang, B., Berger, J., Zhou, G., Elbracht, A., Biswas, S., White-Carrington, S., Szalkowski, D., Moller D. E. (1996) Insulin and mitogen-activated protein kinase-mediated phosphorylation and activation of peroxisome proliferator-activated receptor gamma. J. Biol. Chem. 271:31771-31774.

Zhou, X., Benson, K. F., Ashar, H. R., and Chada, K. (1995) Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C. Nature 376:771-774

Zhu, Y. Qi, C., Korenberg, J. R., Chen, X. –N., Noya, D., Rao, M. S., and Reddy, J. K. (1995) Structural organization of the mouse peroxisome proliferator-activated receptor gamma (mPPARγ) gene: alternative promoter use and different splicing yield two mPPARγ isoforms. Proc. Natl. Acd. Sci. USA 92:7921-7925