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Obesity and diabetes are major public health concerns that contribute to cardiovascular disease, hypertension, and stroke. It is now widely accepted that chronic inflammation is an important element of pathogenic mechanisms linking obesity to diabetes. Moreover, clinical and experimental evidence has established the mitogen-activated protein kinase (MAPK) signaling pathway as a pivotal mediator during inflammatory stress in coupling obesity to insulin resistance (IR). While numerous studies have examined the upstream kinase activation of MAPKs, few have examined mechanisms that dephosphorylate, and thus, deactivate these pathways, potentially affording protection against adipose tissue (AT) inflammation and obesity-induced IR. Data presented in this dissertation demonstrate that several MAPK-specific dual-specificity phosphatases (DUSPs) are induced in AT under conditions of genetic and diet-induced obesity that is associated with increased inflammation and IR. While AT is composed of multiple cell types, evidence suggests an essential role for preadipocytes (PAs) and adipocytes (ADs) in the development of AT inflammation and IR. Therefore, we further report phenotypic differences in DUSP expression where four of the ten MAPK-specific DUSPs are more abundant in PAs compared to ADs while two of the ten DUSPs are more abundant in ADs compared to PAs, suggesting a regulatory role for these phosphatases that is cell type specific. Moreover, phenotypic differences were observed regarding MAPK signaling and DUSP expression in PAs and ADs exposed to TNFα-mediated inflammatory stress, where ERK, JNK, and p38 phosphorylation was markedly elevated and transient in PAs while ERK and JNK phosphorylation was prolonged in ADs concomitant with the phenotypic differences in inducible DUSPs. As

induction of DUSPs in PAs kinetically mirrored MAPK dephosphorylation, we further show that de novo mRNA synthesis was essential for MAPK dephosphorylation, suggesting a role for inducible DUSPs in the modulation of MAPK signaling. Based on these data, we present empirical evidence that DUSP knockdown markedly increased the magnitude and duration of ERK, JNK, and p38 phosphorylation in response to inflammatory stress, subsequently elevating MAPK-dependent pro-inflammatory cytokine and chemokine gene expression. Collectively, these findings demonstrate an essential role for DUSPs in the timely modulation of MAPK signaling, highlighting prospective therapeutic targets linking obesity with metabolic inflammatory diseases.

ROLE FOR DUAL-SPECIFICITY PHOSPHATASES ON MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING IN ADIPOCYTES

by

Bradley S. Ferguson

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Approved by

Committee Chair

In dedication to my family, for your continued support and love.

APPROVAL PAGE

This dissertation has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

_____

_____

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Significance of Research

Obesity and diabetes mellitus are major public health concerns that lead to cardiovascular disease, hypertension, and stroke. Globally, obesity rates are expected to increase from 1.6 billion to 2.3 billion, while deaths due to diabetes are expected to jump by over 80% in developed countries between 2006 and 2015. As the prevalence and costs of obesity and diabetes rise, improved therapeutic strategies that target these diseases will be crucial for future generations. Early evidence established a critical role for inflammation in the coupling of obesity to insulin resistance (IR), ultimately contributing to diabetes. It has been further determined that the mitogen-activated protein kinase (MAPK) signaling pathway plays a central role in linking inflammatory stress to obesity-induced IR. Numerous studies have examined the role of upstream kinases that phosphorylate and thus activate MAPK signaling, yet a critical gap exists in the literature, as few studies have examined mechanisms that dephosphorylate (i.e., deactivate) this pathway, potentially providing protection from obesity-induced IR. Collectively, the proposed research is significant, as it will advance our understanding of how phosphatases can modulate or even control MAPK signaling, establishing a platform for the identification of new therapeutic targets for the treatment of obesity-induced IR. The objective of this dissertation is to determine the mechanistic role of MAPK-specific dual-specificity phosphatases (DUSPs) on the timely modulation of MAPK dephosphorylation and its biological consequences in adipocytes under

conditions of differentiation and inflammatory stress. The literature relevant to this research project is reviewed below.

Review of Literature

Obesity and Diabetes. The World Health Organization (WHO) Expert Consultation on Obesity forewarned the global community in 1997 of the emerging overweight and obesity epidemic currently afflicting many nations worldwide. Moreover, the expert panel warned that lack of action would result in epidemic increases of many non-communicable diseases. Since that time, global populations have seen a pandemic rise of overweight and obesity, with over 1 billion adults (age 15+) overweight and 400 million obese or having a body mass index (BMI) of 30 kg/m^2 or greater (1). Concomitant with these statistics, prevalence rates for obese individuals in the United States are continually increasing with one in three people classified as obese. More alarming is the racial division seen in the United States, where half of African American and Hispanic adults are obese (1-6). Correspondingly, the clinical manifestations related to obesity (e.g., diabetes and heart disease) have risen, decreasing an individual's quality of life while pushing the health care system to its limits with current medical costs estimated at well over $140 billion/year (1,3).

Similar to obesity, diabetes is a major cause of morbidity and mortality contributing to rising health care costs and a poor quality of life. While diabetes mellitus consists of a group of chronic metabolic conditions characterized by elevated blood glucose levels via the body's inability to produce or respond to insulin, or both, 90-95% of diabetes burden in the United States is due to type 2 diabetes (7-9). Type 2 diabetes

generally begins as IR, eventually producing a hyperinsulinemic and hyperglycemic state that subsequently leads to pancreatic β-cell failure and/or exhaustion, ultimately decreasing insulin secretion. It is currently estimated that over 20 million people in the United States have diabetes, with 1.5 million new cases of diabetes diagnosed each year (7-10). Moreover, it has been estimated that some 12 million people in the United States are pre-diabetic (i.e., fasting blood glucose (FBG) of 100-125 mg/dL). These individuals have elevated blood glucose levels but do not meet the diagnostic criteria for diabetes (i.e., FBG>126 mg/dL). In addition to the sharp rise in diabetes prevalence over the last two decades, it is expected that global incidence rates of type 2 diabetes will double to 350 million cases by 2030 as the population ages and obesity rates continue to climb for adults and children (7-11).

While the precise link between obesity and its associated type 2 diabetes remain poorly understood, evidence supports a casual role for excess fat mass and IR (12-15). Increased adiposity in humans and animals highly correlates to decreased insulin signaling and the development of diabetes (15-17), whereas fat mass loss reduces type 2 diabetes and premature mortality rates (18-22). Unfortunately, few individuals (~10%) who lose weight keep it off (19,20,23). Therefore, research is clearly needed to delineate effective strategies for the treatment and prevention of obesity and associated type 2 diabetes. However, socioeconomic and molecular complexities in studying obesity and diabetes impede these research efforts. Furthermore, while evidence supports a role for obesity in the development of diabetes, this association appears highly dependent on genetics and/or environment, as many obese subjects do not progress to the diabetic state (14,15,18-20,23-26) Nonetheless, research over the past several years has implicated adipose tissue (AT) as a central mediator of whole-body glucose homeostasis

in normal and diseased states, altering our view of the adipocyte from that of a mere storage depot for lipid (23).

Adipose tissue as an endocrine organ. AT is an innervated connective tissue composed of distinct cell types: preadipocytes (PAs), mature adipocytes (ADs), and macrophages, among others, that contribute to AT function. Classically, it is understood that AT plays an essential role in the storage and release of fatty acids during periods of caloric abundance and restriction. In addition, AT provides insulation and padding to the body. While the role for AT as an energy source has been known for centuries, the idea that it acts in an endocrine manner, to centrally regulate energy expenditure and food intake, was not developed until 1953 (27). Since that time, studies involving genetically obese (ob/ob) and diabetic (db/db) mice demonstrated that a circulating factor, absence in ob/ob and ineffective in db/db mice was involved in obesity, diabetes, hyperphagia, and reduced physical activity (28). However, it was not until 1994 that this lipostatic signal known as leptin, an adipocyte-specific protein essential for whole-body energy homeostasis, was identified and AT recognized as an endocrine organ (29). Not long after, studies demonstrated that injection of ob/ob mice with leptin reduced food intake, body weight, and fat mass while maintaining lean body mass. In addition, these mice had increases in energy expenditure and restored euglycemia, confirming a role for leptin as a mediator of energy intake and storage (30,31).

It is now well established that AT secretes various bioactive peptides or proteins, collectively termed ‘adipokines,’ that play a key role in energy metabolism, vascular homeostasis, and immunity (Fig.1.1). Moreover, studies demonstrate that most adipokines are dysregulated in obesity and type 2 diabetes (17,32). For example, circulating levels of the pro-inflammatory cytokine tumor necrosis factor α (TNFα) are

elevated in AT of obese individuals (33). In contrast, anti-inflammatory or insulin-sensitizing adipokines like adiponectin (Fig.1.1) are markedly attenuated with obesity (32,34). While the didactic view that some adipokines exhibit beneficial versus deleterious effects is an oversimplification of a complex process, it appears that dysregulation of adipocyte-derived proteins may play a significant role in the pathogenesis of diabetes with increased adiposity. Moreover these early studies led to well established understanding that AT is a dynamic endocrine organ, critical for regulating metabolism in health and disease.

Inflammation, obesity, and insulin resistance. The identification that IR occurred in response to infections such as sepsis provided some of the first associations between IR and the immune response (35,36). Since then, it has been demonstrated that many inflammatory diseases such as HIV, hepatitis C, and rheumatoid arthritis promote IR (37-39). Treatment with the anti-inflammatory drug salicylate improved insulin sensitivity in patients, laying an early foundation that inflammation plays a key role in the pathogenesis of diabetes (40,41).

Around the same time as the discovery of leptin, Hotamisligil et al. (42) provided the first molecular link between inflammation and obesity with the observation that the inflammatory cytokine TNFα is overexpressed in AT of rodent models of obesity. Others have since shown that TNFα is overexpressed in AT and muscle of obese humans (43,44), while circulating levels of TNFα fall after weight loss (45). Subsequently, TNFα administration in cell culture or to whole animals inhibits insulin action (46-49), while ablation of this cytokine or its receptors in obese animals improves insulin sensitivity (50-53). These studies clearly illustrate the importance of TNFα as a master 'adipo-cytokine' linking obesity to IR. Since the initial discovery of TNFα, other inflammatory mediators

(e.g., MCP-1 and IL-6) have been identified that increase with obesity and have been shown to play a causal role in the development of IR, establishing the well accepted paradigm that obesity is a chronic condition of low-grade inflammation (44).

Similar to TNFα, monocyte chemoattractant protein-1 (MCP-1)/ chemokine (C-C motif) ligand 2 (CCL2) is elevated in AT of animal and human obesity (54-56). MCP-1 and its cognate receptor CCR2 are required for macrophage infiltration into AT. Macrophage infiltration and inflammatory gene expression are markedly reduced with weight loss in obese subjects (54,55). Mice deficient in MCP-1 or CCR2 have less macrophage accumulation, a lower inflammatory gene profile, and improved insulin sensitivity on a high fat diet compared to wild-type counterparts (57,58). In contrast, transgenic mice expressing MCP-1 have the reverse phenotype (57). Similarly, diet-induced obese (DIO) mice treated with a CCR2 antagonist presented with less adipose macrophage accumulation and improved hyperglycemia (58). Furthermore, in vitro treatment of 3T3-L1 ADs with MCP-1 attenuated insulin-stimulated glucose uptake and expression of key adipogenic genes (59). While MCP-1 appears essential for obesity-induced IR, it has become increasingly clear that other CC chemokines (e.g., CCL3, CCL8, CCL9) are upregulated in genetic and diet-induced obesity models (56). As recent evidence demonstrates that AT inflammation is derived from activated AT macrophages (60), adipocyte-secreted chemokines may play a key role in the early development of adipose inflammation.

Inflammatory cells of AT. The initiation and maintenance of immunity is energy demanding and inefficient during periods of energy deficit or starvation (49,61). For example, it has been reported that fever increases energy demand by 7-13%, per 1°C (e.g., 98.6°F to 100.4°F) increase in temperature. Increased energy consumption

associated with fever approximates energy costs of a 70 kg man walking 27 miles (49,61-64). Thus, evolutionary pressures to survive would have favored the co-development of organ systems and signaling pathways that mediate this process. Evidence for this evolutionary development have been observed in lower organisms, such as Drosophila melanogaster, in which the fat body controls immune and metabolic responses (65), and higher organisms where immune cells reside alongside other cell types including ADs (49). Regarding integration of metabolic and immune responses, cells within AT show evidence of co-evolutionary development and coordination. In particular, PAs, ADs, and macrophages share many functions, as they all secrete cytokines and can be activated by pathogens such as lipopolysaccharide (LPS) and fatty acids via toll-like receptors (44,49,66). Moreover, traits primarily attributed to macrophages, phagocytosis and expression of membrane-bound NADPH oxidases, have also been shown in PAs (67), while transcriptional profiling suggests macrophages and PAs are genetically related (67,68). While studies suggest that PAs are more inflammatory than ADs (66) and that PAs can trans-differentiate into macrophage-like cells (67,68), there is also extensive overlap between insulin-responsive ADs and macrophages regarding inflammation (e.g., TNFα, IL-6, PAI-1) and metabolic regulation (e.g., PPARγ, carnitine acetyltransferase, FABP4) (49). Thus, periods of chronic overnutrition may tip the balance, reactivating the primordial inflammatory potential of AT as observed with increased expression of TNFα and other pro-inflammatory cytokines in obese individuals.

AT cross-talk. As co-evolutionary development and coordinated efforts between ADs and macrophages would have allowed Paleolithic man to store excess nutrients for time of energy deprivation and to mount a potent immune response against infection,

modern man has seen dramatic dietary and lifestyle changes that may convert this beneficial interaction into a pathological relationship (49). Collectively, evidence suggest that cross-talk in AT (Fig.1.2) during times of nutrient overload contributes to adipocyte dysregulation and obesity-induced IR. In particular, evidence demonstrates that both PAs and hypertrophied ADs secrete increased amounts of MCP-1 (59) that results in macrophage infiltration of obese mice and humans (16,69). Consistent with this, macrophage accumulation in AT of lean individuals (5-10%) is substantially less than that of obese patients, where macrophages can make up to 50% of the total number of cells within AT (60). Thus, increased secretion of MCP-1 and/or other chemokines from ADs may initiate the pro-inflammatory state (17). This appears evident as fractionation of AT from DIO mice demonstrates that chemokine expression is primarily secreted from isolated ADs (56). Subsequently, macrophages and to a lesser extent ADs secrete the pro-inflammatory cytokine TNFα (33,70) that has been shown to induce inflammatory gene expression, increase fatty acid release, and inhibit insulin signaling in ADs (17,42). Moreover, increasing evidence demonstrates that AT sits at the nexus of whole-body IR, as increased fatty acid release and decreased triglyceride storage during periods of chronic, low-grade inflammation directly contribute to skeletal muscle IR (17).

Inflammatory signaling. Metabolic effects of inflammatory mediators have been primarily attributed to two major inflammatory signaling pathways, that of nuclear factor-kappa B (NF-κB) and MAPK signaling pathways involving extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (17,71). These signaling pathways are activated in AT in response to inflammatory stimulation and serve as mediators of localized IR through transcriptional mechanisms involving adipocyte gene expression as well as the expression and secretion of other pro-inflammatory molecules

(49,71). In addition to nuclear events involving c-Jun/AP-1 mediated gene expression, MAPK activity has also been linked to cytosolic suppression of proximal insulin signaling through IRS-1 and PI3K/AKT disruption demonstrating that compartmentalized signaling events can impact different properties of AT dysfunction (71-74).

At the molecular level, insulin binds to and activates its tyrosine kinase transmembrane insulin receptor. Once activated, the insulin receptor will phosphorylate select tyrosine residues on downstream targets including the family of insulin receptor substrate (IRS) proteins (i.e., IRS-1, -2, -3, and -4). Tyrosine phosphorylated IRS-1 activates the phosphatidylinositol 3-kinase (PI3K/AKT) pathway that serves to stimulate glucose transporter 4 (GLUT4) translocation to the plasma membrane in order to increase glucose uptake (75-77). Inflammatory signals interfere with these signaling events downstream of the insulin receptor leading to decreased insulin sensitivity. In contrast to the PI3K pathway, MAPK activation inhibits glucose uptake via the phosphorylation of IRS-1 on serine-307 (S307), decreases RNA and protein synthesis of IRS-1, and decreases RNA and protein synthesis of GLUT4. S307 phosphorylation further inhibits tyrosine phosphorylation leading to the dissociation of IRS proteins from the insulin receptor, and thus, impairs GLUT4 translocation and cellular glucose uptake (72-74).

MAPKs, inflammation, and IR. MAPKs are comprised of multiple protein isoforms where activation of specific MAPKs is regulated by a cascade of phosphorylation events mediated by sequential and concerted activation of upstream kinases (Fig.1.3A). For instance, JNK is phosphorylated by upstream MAPK kinases (MAP2Ks), which are phosphorylated by MAPK kinase kinases (MAP3Ks) in response to diverse external stimuli. Phosphorylation of both the threonine and tyrosine residues of

the (T-X-Y) motif within the activation loop by upstream MAP2Ks is essential and sufficient for MAPK activation, and ultimately leads to the phosphorylation of target proteins and transcription factors within the cytosolic and nuclear compartments (78-80). While it is generally accepted that ERK is activated by growth factors and mitogens and is associated with cell survival and that JNK and p38 are stress activated (e.g., pro-inflammatory cytokines, UV irradiation, and reactive oxygen species) and associated with cellular stress and apoptosis (81), biological processes regulated by MAPKs are cell-type dependent.

ERK was the first MAPK identified, as it was shown that partially purified microtubule-associated protein-2 (MAP-2) protein kinase obtained from insulin-stimulated 3T3-L1 ADs could phosphorylate and reactivate phosphatase treated ribosomal protein S6 kinase (RSK) from Xenopus eggs (82-84). Subsequent confirmation of this observation implicated MAP-2 kinase as an S6 kinase kinase (85-87). Since that time, the original name has changed to MAPK and later, when cloned and recognized as an insulin-stimulated kinase similar to yeast kinases involved in cell cycle, designated as ERK (88,89). The ERK family contains a TEY (Thr-Glu-Tyr) activation motif and can be divided into two groups: classic MAPKs consisting of ERK1 and 2, and large MAPKs consisting of ERKs 3, 5, 7, and 8 that contain a kinase domain and c-terminal domain and range in size from 60 to over 100 kDa (90-92). While it is generally concluded that ERK signaling is associated with cell survival and proliferation, depending on the cell line and stimulation this pathway may also regulate non-canonical and even opposing function. Thus, ERK may respond to stress and apoptosis (93,94). Regarding ERK signaling, it has been reported that mice deficient in ERK1 were protected against diet-induced obesity and IR by mechanisms involving decreased

adipogenesis and increased energy expenditure (81). In contrast, mice deficient in signaling adapter p62, an ERK inhibitor, developed mature-onset obesity and IR concomitant with increased ERK activity (95), thus implicating ERK in obesity-induced IR and disease.

JNK was initially identified as a protein kinase activated in the liver of rodents exposed to cycloheximide (96). Since that time, ten isoforms of JNK have been identified that arise from alternative splicing of three genes: JNK1, JNK2, and JNK3, all of which belong to the MAPK family of signaling proteins (97,98). Moreover, studies have identified JNK as a stress-activated protein kinase that phosphorylates c-Jun on two sites in the NH_2-terminal domain (99,100), with subsequent studies demonstrating that JNK is essential for inflammatory signaling and phosphorylation of transcription factors c-Jun/AP-1, required for inflammatory gene expression (101,102). JNK has recently emerged as a major contributor in the inflammatory development of IR in obesity (103,104), where JNK activation is elevated in liver, muscle, and AT during obesity while loss of JNK activity protects animals from IR. For example, deletion of JNK in dietary and genetic (ob/ob) mouse models of obesity led to decreased adiposity, improved insulin sensitivity, and enhanced insulin receptor signaling. Moreover, JNK activity is elevated in ADs during inflammatory stress, triggering IRS-1 inhibition via S307 phosphorylation and decreased glucose uptake (71,74,105,106). While JNK activation can quickly suppress insulin signaling in the cytosol, it can also translocate into the nucleus to regulate gene expression of other pro-inflammatory molecules, further promoting insulin resistance (107,108).

p38α was the first member of the p38 MAPK family identified as a 38 kDa protein (p38) that was rapidly phosphorylated on tyrosine in response to LPS, as a target of

pyridinylimidazole drugs that inhibited pro-inflammatory cytokine production, and as an activator of MAPKAPK2 in cells stimulated with heat shock, arsenite, or interleukin-1 (109-113). Additional p38 family members, which share 60% sequence homology, were subsequently cloned and named p38β, p38δ, p38γ and ERK6. While p38α is ubiquitously expressed in most cell types, other p38 MAPKs are encoded by different genes and have different tissue expression patterns, where p38β is found primarily in the brain, p38γ in skeletal muscle, and p38δ in endocrine glands (114-120). Similar to JNK, p38 has emerged as a key player in IR, where p38 activity is increased in response to hyperglycemia and in diabetes (121). It has also been reported that p38 activity is higher in ADs isolated from type 2 diabetes patients and participates in the downregulation of GLUT4 expression (122). Consistent with this, studies report that p38 inhibition improves glucose uptake in L6 myocytes and 3T3-L1 ADs, suggesting a role for this signaling pathway in obesity-induced IR (123). While p38 can inhibit insulin signaling, it can also translocate to the nucleus and activate pro-inflammatory genes involved in AT inflammation (124).

While MAPK pathways are viewed as relatively simple and linear, it is now apparent that MAPKs have antagonistic effects (e.g., cell growth vs. cell-cycle arrest) that are cell-type dependent. Mounting evidence also indicates that MAPK magnitude, duration, and compartmentalization contribute to the diverse and opposing outcomes observed in response to external stimuli (125-127) (Fig.1.3B). Consistent with the idea of how signal duration elicits different biological outcomes, it has been observed that transient ERK activation results in AD differentiation while sustained ERK activation inhibits AD differentiation (81,128,129). Similarly, transient activation of JNK provides a critical negative feedback for the insulin response while increases in JNK signaling

magnitude and duration leads to IR (76). Kinase localization also plays a major role in specifying cell fate. For example, uncoupling of nuclear JNK activation from its cytosolic effects on IRS-1 suppression led to improved insulin sensitivity in mice (130). Until recently, the differences in MAPK magnitude, duration, and localization were traditionally attributed to protein scaffolding and upstream kinase activation. However, recent evidence demonstrates a critical role for phosphatases in the modulation and even control of MAPK signaling (125-127).

MAPK Phosphatases. While a large proportion of intracellular proteins (30%) are subject to dephosphorylation, all 14 known ERK, JNK, and p38 kinases are completely inactivated by phosphate removal (125-127,131). Evidence now suggests that MAPK deactivation (i.e., dephosphorylation) via phosphatases play a central role in mediating signal transduction and cellular outcome (125-127). This is readily apparent in the complex changes observed in multiple MAPK signaling pathways, where immune function, stress response, and metabolic regulation are altered in animals lacking a specific MAPK phosphatase (132). As phosphorylation of the threonine and tyrosine residues within the activation loop are needed for MAPK activation, dephosphorylation of one or both residues results in inactivation of these molecules (78,108,127).

Protein phosphatases are classified into three structurally distinct gene families: the phosphoprotein phosphatase (PPP) and protein phosphatase M (PPM) families encode protein serine/threonine (S/T) phosphatases, whereas the protein tyrosine (Y) phosphatase (PTP) family includes both tyrosine-specific and dual-specificity (S/T and Y) phosphatases (DUSPs). While protein tyrosine-specific phosphatases (e.g., PTP-SL), and serine/threonine phosphatases (e.g., PP2A) can regulate MAPKs in vivo, the largest group of phosphatases specific for MAPK deactivation are DUSPs (126,131,133,134).

Of the more than 30 DUSPs identified, at least 16 have been shown to dephosphorylate MAPKs (133-136). These DUSPs belong to one of two subfamilies that share a highly conserved C-terminal catalytic DSP domain, homologous to the VH1-like phosphatase isolated from the vaccinia virus (Fig.1.4A). The MAPK-specific DUSPs constitute a structurally distinct group of ten proteins identified by an N-terminal domain characterized by a non-catalytic MAP Kinase Binding (MKB) domain that functions in the docking of MAPKs to the phosphatase (79,126,127) (Fig.1.4B). The MKB domain consists of two CH2 domains flanking a kinase interactive motif (KIM) that confers specificity for select MAPKs. Consequently, this group of DUSPs displays minimal catalytic activity in the absence of substrate. This group of DUSPs can be divided into five classes based on gene structure and localization (Fig.1.4A). Group I DUSPs localize to the nuclear compartment via a nuclear localization sequence (NLS) proximal to the N-terminus. Group II DUSPs encode for a nuclear export sequence (NES) that directs cytoplasmic localization. Group III DUSPs contain a longer C-terminal domain that houses an NLS and NES allowing access to the cytoplasmic and nuclear compartments. Group III DUSPs also contain a PEST sequence, important in the modulation of protein stability. Dusp10 is classified as group IV and is unique as it contains an extended N-terminus with potential regulatory motifs. In contrast to the above DUSPs that specifically target MAPKs, group V consists of the ‘atypical’ DUSPs, a class of poorly characterized enzymes that tend to be smaller and lack the MAPK targeting motifs as they do not contain the MKB domain (79,126,127,134).

DUSPs also display distinct patterns of tissue distribution, expression, compartmentalization, and MAPK specificity. For instance, dusp8 is predominantly expressed in the eye, brain, heart, and lung and is most specific for JNK deactivation,

while dusp4 is abundant in the placenta and highly specific for ERK deactivation. In addition, select DUSPs (e.g., dusp1) are classified as immediate early genes rapidly induced in response to serum, while others respond to different stimuli. While many DUSPs are regulated at the level of gene expression, others are also regulated by protein stability and degradation (137-144). For instance, ERK phosphorylation of dusp1 on S359 and S364 stabilized the phosphatase from ubiquitin-directed degradation in CCL39 fibroblasts (138). As dusp1 localizes to the nuclear compartment (Fig.1.4A), ERK activated protein stability appears to be an important component in dictating localized MAPK activity. Similarly, recent reports demonstrate that p38 phosphorylation of S446 on dusp16 or ERK phosphorylation of dusp4 on S386 and S391 stabilized the proteins from degradation, but did not affect functional phosphatase activity (143,144). Related reports are now emerging demonstrating a complex regulation of DUSPs involving transcription, translation, mRNA stability, and protein stability, mediated in part by post-transcriptional and/or post-translational modifications (144-147). These dynamic complexities suggest that DUSPs play a pivotal role in modulating localized MAPK signaling, potentially affording protection against obesity-induced IR.

DUSPs, Cancer, and Inflammation. While the physiological role for many DUSPs are still largely unknown, emerging reports indicate phosphatase function in cancer and the control of immunity (135,148,149). Regarding cancer outcome, mounting reports reveal dusp1 overexpression in early stages of prostate, colon, and bladder cancer, followed by a loss of expression as tumors become more aggressive or invasive. Moreover, empirical evidence demonstrates that dusp1 overexpression negatively correlates with JNK activity and apoptosis in prostate cancer (150-153). While controversy for JNK and p38 signaling regarding cancer exists in the literature, gene

ablation studies suggest that the stress-activated kinases act as tumor suppressors in vivo (149,154-156). Additionally, others have shown that p38 and JNK activity are essential for cell apoptosis, while activity does not appear to affect tumor metastasis (157). Collectively, temporal regulation of dusp1 appears to play an essential role in cancer cell development and invasion, while indirectly making tumor cells chemo-resistant. Dusp2, dusp4, dusp6, and dusp16 have also been shown to play a role in various cancer types. High dusp2 levels in patients with serous ovarian carcinomas correlated with poor overall survival compared to patients with low expression levels of the phosphatase (158). Concerning dusp4, reports indicate this phosphatase is co-expressed with dusp1 in breast cancer and overexpressed in serous borderline ovarian tumors, and pancreatic and liver cancer (159-162). In pancreatic cancer, it was recognized that MEK-mediated dusp4 expression led to suppressed ERK activity in cells harboring a K-ras mutation (160). Similar to dusp4, dusp6 activity is associated with Ras mutation in pancreatic tumors, although this involves loss of dusp6 with disease progression (163,164). In this case, loss of dusp6 may synergize with K-ras to increase ERK activity, thus contributing to pancreatic tumor development and invasiveness. Finally, overexpression of dusp16 was shown to down-regulate JNK activity and thus suppress BCR-ABL induced Rat-1 fibroblast transformation (165). In addition, down-regulation of dusp16, which has been linked to acute myeloid leukemia, led to increased JNK and p38 activity that stimulated myeloid cell growth and inhibited differentiation (166). Thus, dusp6 and dusp16 may act as potent tumor suppressors via actions on ERK and JNK signaling. Taken together, these reports indicate a critical role for DUSPs in the regulation of cellular growth and differentiation. As hyperplastic obesity involves

PA replication and differentiation, DUSPs may represent key targets that are essential in the development of obesity.

Parallel to studies involving cancer, reports have emerged addressing a role for DUSPs regarding inflammation (135,148). Knockout of dusp1 from macrophages, splenocytes, and dendritic cells (DCs) resulted in increased cytokine production that was largely dependent on amplified p38 and JNK activity, ultimately making mice hyper-responsive to endotoxic shock in vivo (167-170). However, the negative relationship attributed to dusp1 loss on increased cytokine production was not ubiquitous, as IL-12 production was specifically reduced in response to LPS insult (167,168). Adding to this complexity, activation of pro-inflammatory or anti-inflammatory cytokines in dusp1 deficient macrophages occurred in a temporal manner, whereby TNFα induction occurred early (<2hr) and IL-10 occurred late (>2hr) suggesting dual roles for this phosphatase in the regulation of immunity (169). Similar to dusp1, dusp10 deficient mice exhibited increased cytokine production in toll-like receptor (TLR) stimulated macrophages, but decreased T helper cell proliferation and protection from autoimmune encephalomyelitis, revealing a positive and negative role in cellular outcome (171). As with the complexities seen for other DUSPs, ERK and p38 activity actually decreased in dusp2 deficient mice while JNK activity increased in activated macrophages and mast cells. Interestingly, this resulted in reduced inflammation and protected mice from inflammatory arthritis (172). While it has been reported that dusp4 negatively regulates the inflammatory response in sepsis, others demonstrate that dusp4 plays an essential role in protection from infection against *Leishmania Mexicana* (173,174). Dusp4 deficient mice had improved survival in response to LPS and CLP models of sepsis that was attributed to increased ERK activation and decreased p38 and JNK signaling. As dusp4

knockout led to increased dusp1 expression, the authors concluded that activation of ERK in dusp4 deficient mice stimulated dusp1 induction subsequently deactivating p38 and JNK signaling and ultimately contributing to decreased inflammation (173). Conversely, following infection with the parasite *Leishmania mexicana*, dusp4 deficient mice had increased lesion size and parasite burden due in part to increased signaling magnitude and duration of p38 and JNK. In addition, deletion of dusp4 potentiated LPS induction of pro-inflammatory cytokines IL-6, TNFα, and IL-12, collectively contributing to macrophage susceptibility to infection with the parasite (174). Based on the observed complexities for the various DUSPs, it is plausible that modulatory actions of DUSPs on MAPK signaling contribute to feedback inhibition and intracellular signaling cross-talk, ultimately dictating biological outcome. Collectively, these data demonstrate a potential role for DUSPs regarding inflammatory response, and highlight the complexities involving phosphatase modulation of MAPK signaling.

While phosphatases have been classically regarded as switches that regulate 'over-signaling,' evidence now demonstrates a more complex role for these molecules in MAPK regulation and physiological outcome (148,149). It has been estimated that enzymatic activity of phosphatases is markedly greater (>100 x) than that of kinases, as MAPKs require both ATP and MAP2Ks for phosphorylation, while dephosphorylation is direct (175). Thus, signaling magnitude and duration may be more easily regulated by phosphatases than kinases, providing potential targets for obesity and obesity-induced IR.

DUSPs, Obesity, and Insulin Resistance. The function of DUSPs in adipocytes is largely unknown and a role for MAPK phosphatases in obesity and IR is just beginning to emerge. Dusp6 was identified as a gene that antagonized insulin suppression of the

gluconeogenic gene, PEPCK. Additionally, dusp6 was expressed in insulin responsive tissues of liver, AT, skeletal muscle, and heart (176). In vivo studies of diet-induced obesity demonstrate that dusp6 expression is markedly increased in liver, while adenoviral-mediated overexpression of dusp6 in lean mice promote gluconeogenesis and increased fasting blood glucose levels (177). Conversely, knockdown of dusp6 in lean and obese mice decreased fasting blood glucose levels, attributed to down-regulation of gluconeogenic genes, PEPCK and G6Pase (177). Others have shown that down-regulation of dusp1 in diabetic Sprague-Dawley rats may contribute to decreased cardiac function leading to diabetic cardiomyopathy (178) as well as play a role in the pathogenesis of diabetic dementia (179). Furthermore, dusp1 appears to play a critical role in insulin expression from pancreatic β-cells, where dusp1 inhibition decreased insulin expression. Conversely, adenoviral-mediated dusp1 overexpression decreased JNK phosphorylation and increased insulin expression (180).

Our understanding of the DUSPs in ADs is limited to five published works. Dusp9 was identified as a candidate gene involved in insulin signaling via a functional expression screen examining PEPCK promoter activity as a measure of insulin action. Dusp9 was expressed in insulin-responsive tissues with markedly increased dusp9 expression levels observed in obese insulin-resistant rodent models (181). Similarly, dusp9 ectopic expression in ADs reversed insulin-stimulated glucose uptake (182). Others have demonstrated that dexamethasone-induced IR in ADs inhibits GLUT4 activity via dusp1 and dusp9 attenuation of p38 MAPK (183). An inverse relationship was observed between ERK dephosphorylation and dusp1 expression during adipocyte differentiation. Moreover, dusp1 antisense expression led to sustained ERK activation and suppressed adipocyte development (184). In a dusp1 knockout study, JNK activity

was enhanced in white AT, liver, and skeletal muscle of mice on a high fat diet. Furthermore, dusp1 knockout mice were resistant to diet-induced obesity via enhanced energy expenditure, yet still developed glucose intolerance on a high fat diet. However, it was further observed that dusp1 knockout did not inhibit adipocyte differentiation. Interestingly, dusp1 deficient mice exhibit no insulin resistance in the presence of enhanced JNK signaling. As dusp1 is restricted to the nuclear compartment, the authors concluded that elevated JNK activation, present only in the nucleus, is spatially uncoupled from JNK's cytosolic ability to suppress IR (130). Others have shown that dusp1 is down-regulated during adipocyte hypertrophy, concomitant with increased ERK activity and MCP-1 expression and secretion. In addition, dusp1 adenoviral-mediated overexpression markedly decreased MCP-1 expression, suggesting that down-regulation of dusp1 plays a critical role in macrophage recruitment within AT (185).

Review Summary. Obesity and diabetes mellitus are major risk factors that lead to cardiovascular disease, stroke, and hypertension. Studies now demonstrate that dysregulation of adipokines play a pivotal role in the development of obesity-induced IR (1,17), placing AT at the nexus of disease pathology. Moreover, evidence clearly demonstrates that obesity is characterized by a state of chronic, low-grade inflammation that has been attributed to nutrient overload and a hyper-responsive activation of AT immunity (186). Early evidence demonstrated a role for inflammation, where it was discovered that TNFα was overexpressed in AT of obese and insulin resistant mice, in the coupling of obesity with IR (42). Since then, evidence has established the MAPK signaling pathway as critical in linking inflammatory signals to obesity-induced IR (71,81). While numerous studies have examined the upstream pathways that regulate MAPK signaling, few have examined the downstream deactivation of this pathway and

its effects on biological outcome. MAPK-specific DUSPs have emerged as potential therapeutic targets in obesity-related diseases (148,149). While in vitro assessment of DUSPs demonstrates these phosphatases have high specificity for MAPK signaling, limited work in vivo has examined their functional role on MAPKs and biological fate (126). Moreover, observations of DUSPs with respect to obesity and IR are limited and conflicting. Therefore, we have proposed the following study objectives below to elucidate our central hypothesis that DUSPs play a regulatory role on the extent of MAPK signaling in ADs under conditions of differentiation and inflammatory stress, subsequently affecting MAPK-dependent biological processes.

Study Objectives

Chapter II. Little is known about the regulation of DUSPs with regards to obesity in insulin responsive tissues. Therefore, the objective of the study summarized in chapter II is to examine DUSP regulation in insulin responsive tissues of genetic and diet-induced obese mouse models. We will 1) establish relative DUSP expression across tissue types, 2) determine which DUSPs are induced in genetic ob/ob models of obesity in these insulin responsive tissues, and 3) determine regulation of DUSPs during the development of ob/ob and diet-induced obesity as these animals transition to an inflamed, insulin resistant state.

Chapter III. A role for DUSPs in the development of hyperplastic obesity (i.e. PA replication and differentiation) remains unknown. As preadipocyte proliferation and differentiation are highly dependent on the timely modulation of MAPK signaling (81), the objective of the study summarized in chapter III will examine the regulation and role for DUSPs during adipogenesis. While recent evidence suggests a protective role for dusp1

on diet-induced obesity, studies examining the mechanisms of dusp1 actions on adipocyte differentiation are conflicting (130,184,187). Therefore, chapter III will further address a role for dusp1 on MAPK signaling and preadipocyte proliferation and differentiation.

Chapter IV. While a role for select DUSPs on MAPK signaling has been established in classical immune cells, no one has examined a role for all MAPK-specific DUSPs on MAPK signaling in the macrophage-like preadipocyte and insulin responsive adipocyte in response to inflammation. Therefore, the objective of the study summarized in Chapter IV is to: 1) establish which DUSPs are induced, 2) determine which DUSPs regulate MAPK signaling, 3) evaluate their role on pro-inflammatory gene expression, and 4) determine which DUSPs are downstream targets of MAPK signaling in response to TNFα as proposed by our working model (Fig.1.5).

Chapter V. It is well-known that the MAPK-regulated MCP-1 plays a critical role in obesity-induced inflammation that contributes to IR (57,58). As recent evidence suggests that other chemokines also contribute to macrophage infiltration and obesity-induced IR (56), the objective of the study summarized in chapter V is to: 1) establish CC chemokine expression in response to TNFα, 2) elucidate which chemokines are downstream targets of MAPK signaling, and 3) examine the regulatory role of DUSPs on nuclear events involving MAPK-dependent chemokine gene expression as proposed in part of our working model (Fig.1.5).

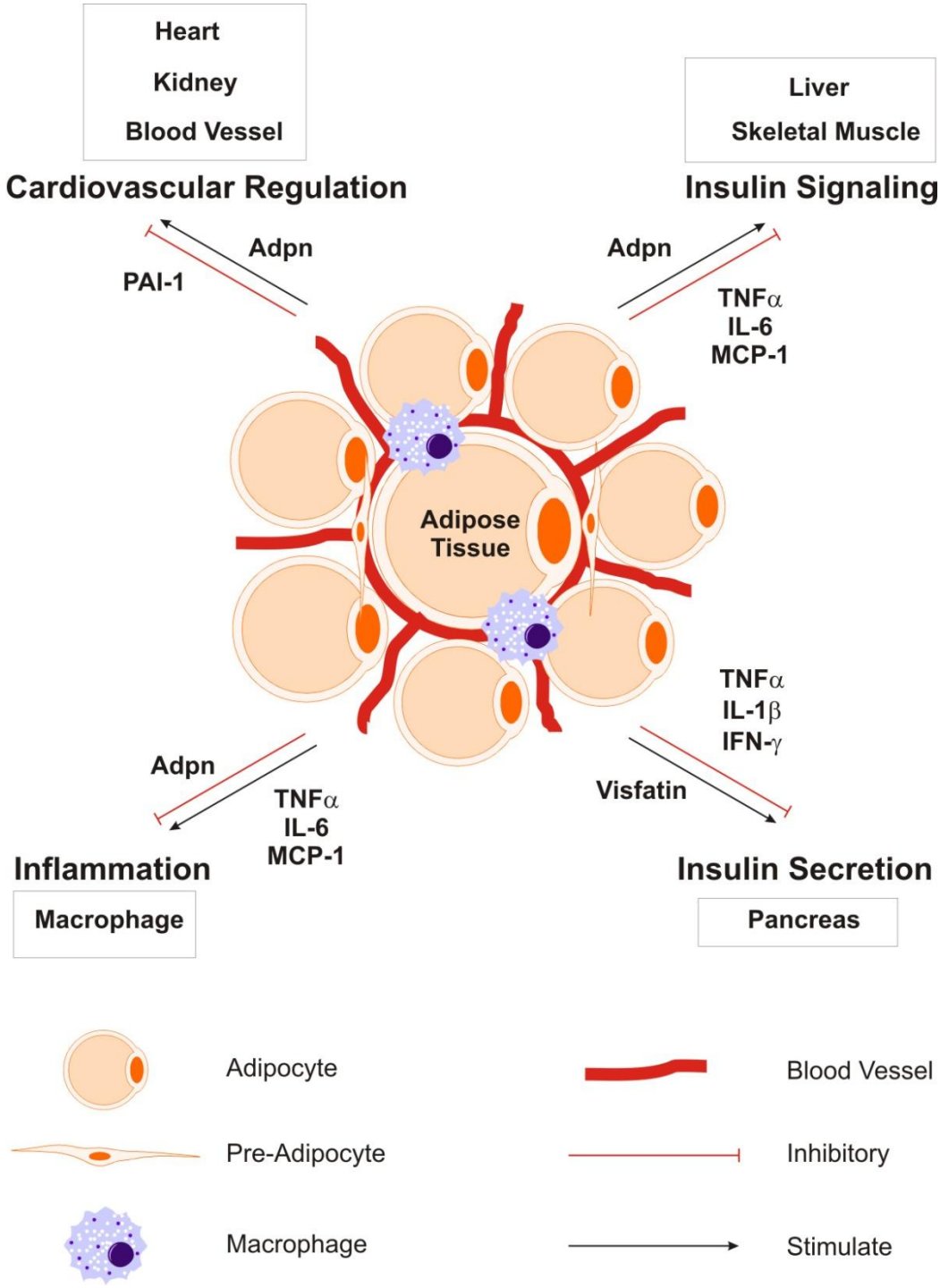

Figure 1.1. Endocrine actions of AT.

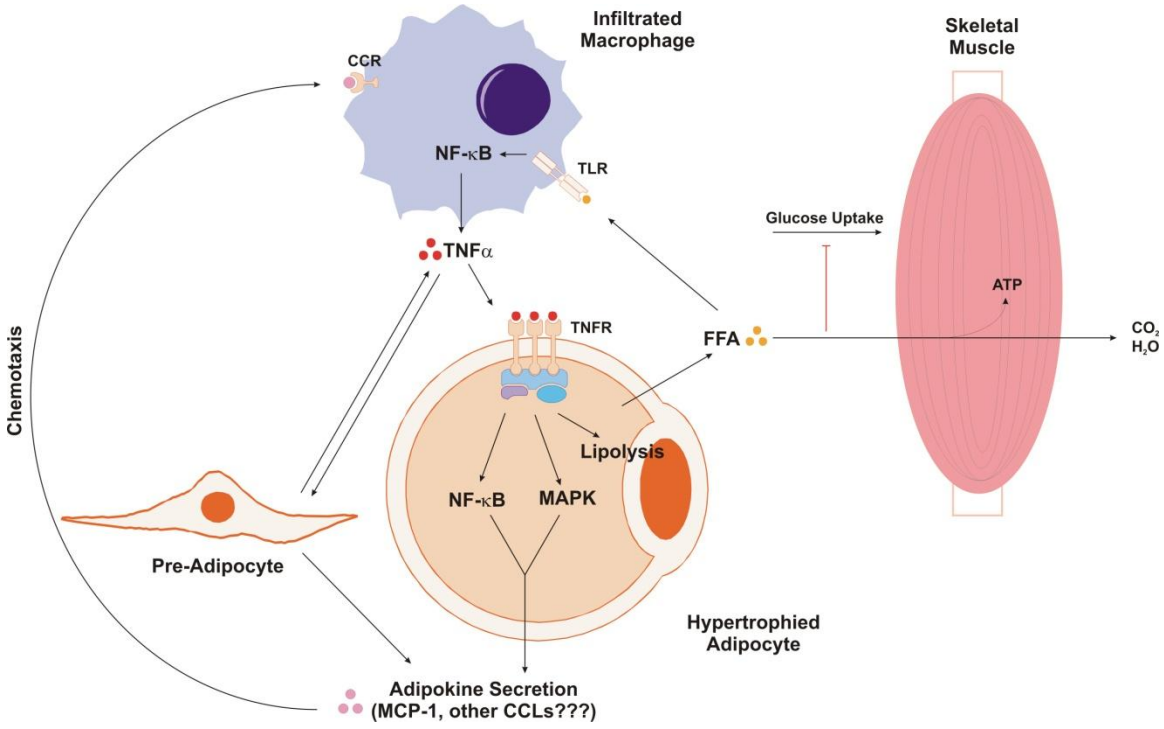

Figure 1.2. Adipocyte-Macrophage cross-talk

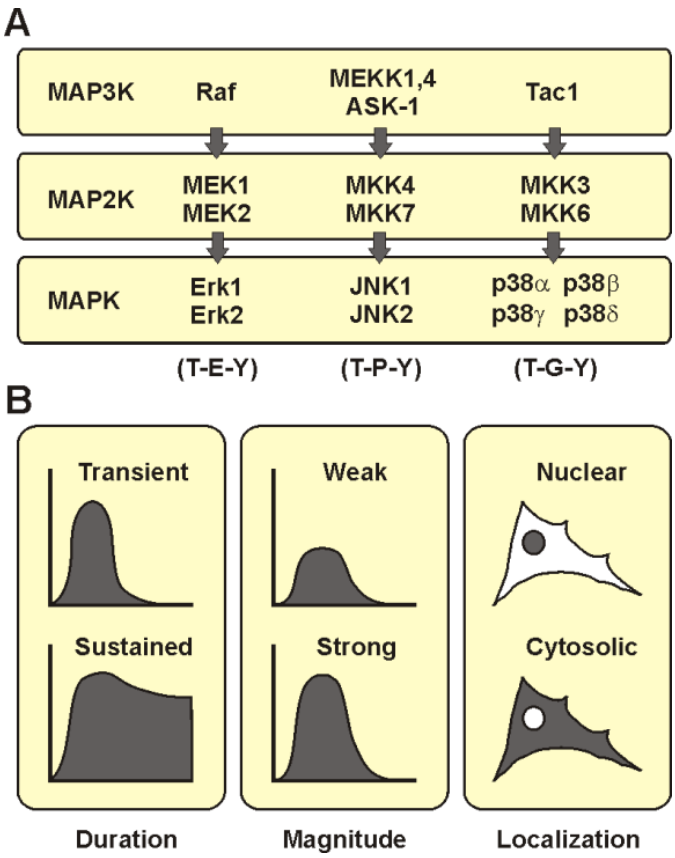

Figure 1.3. MAPK Signaling Cascade

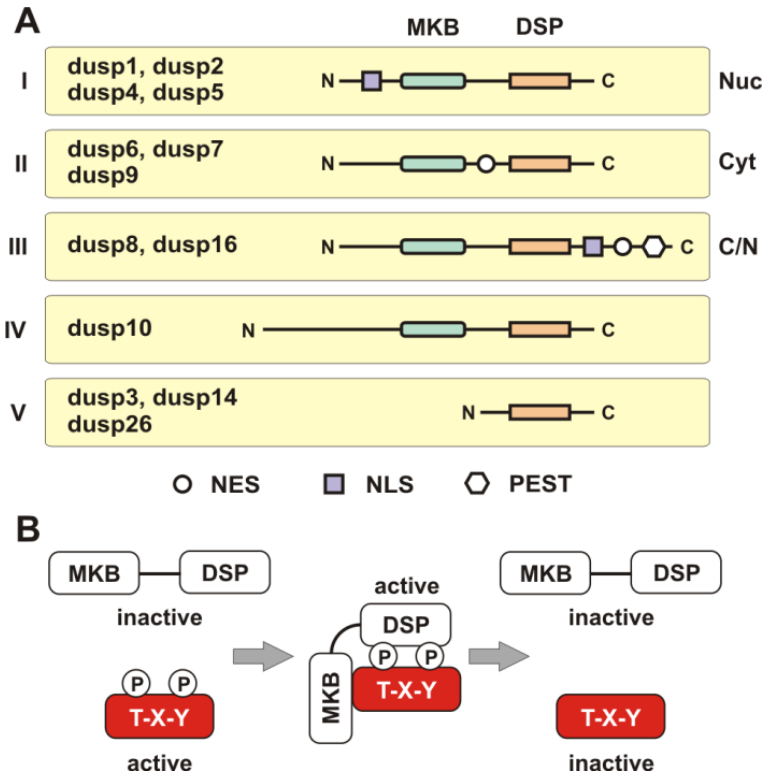

Figure 1.4. DUSP Domain Structure

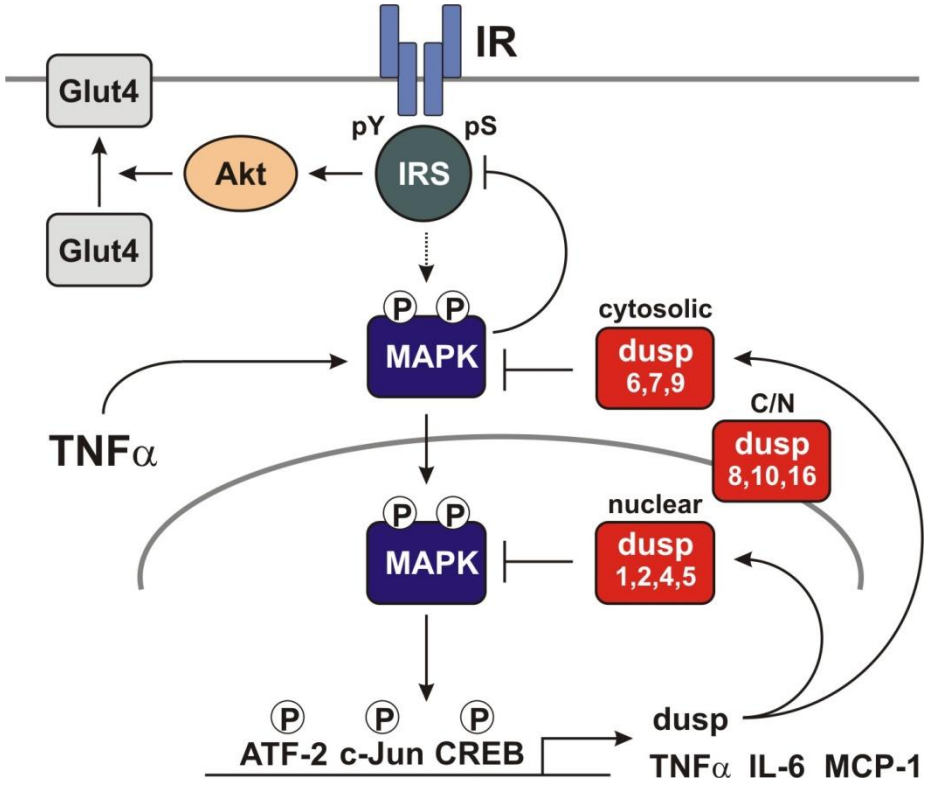

Figure 1.5. Working model of DUSPs on MAPK signaling regulation and function in adipocytes in response to TNFα

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CHAPTER II

REGULATION OF DUAL-SPECIFICITY PHOSPHATASES IN INSULIN-RESPONSIVE TISSUE OF GENETIC AND DIET-INDUCED MODELS OF OBESITY

Abstract

Obesity is characterized by chronic, low-grade inflammation in metabolic tissues that leads to insulin resistance (IR) and type 2 diabetes. Mounting evidence demonstrates that signaling cascades, such as mitogen-activated protein kinases (MAPKs), link inflammation to obesity-induced IR. Moreover, recent reports demonstrate a key role for MAPK-specific dual-specificity phosphatases (DUSPs) in the regulation of MAPK signaling and control of inflammation. This study investigates DUSP mRNA regulation under conditions of genetic and diet-induced obesity (DIO) in insulin-responsive adipose tissue (AT), liver, skeletal muscle, and heart, as dysfunction of these tissues in obesity triggers IR and consequently premature death. Data presented here demonstrate that DUSP expression in lean C57BL/6J mice is tissue specific, where a majority of these phosphatases (dusp2, dusp4, dusp5, dusp9) are most abundantly expressed in AT. These data further demonstrate divergent regulation of DUSPs in insulin-responsive tissues of genetic obesity. Finally, we show that DUSPs were divergently regulated in AT based on the developmental stage (i.e., early vs. mid-stage) and model of obesity (i.e., genetic vs. DIO) as well as the inflammatory environment resulting from obesity. Collectively, these results suggest that tissue-specific regulation of DUSPs contributes to obesity-associated inflammation and IR. Moreover, these data

collectively establish a platform for future studies investigating a role for DUSPs in metabolically critical tissues linking obesity with metabolic inflammatory diseases.

Introduction

Obesity is a modern pandemic that plays a causal role in the development of IR and type 2 diabetes (1). Mounting evidence over the last several years demonstrates that inflammation is a central mediator that links obesity with IR (2-4). Based on the hallmarks of obesity-induced inflammation, obesity is increasingly being associated with the phenomenon termed ‘metaflammation’, which is described as a state of chronic, low-grade inflammation coordinated by metabolic cells such as adipocytes in response to nutrient overload that consequently leads to metabolic dysfunction (5). While AT is the predominant site of cytokine expression during obesity (2), it is now recognized that other metabolically active, insulin-responsive tissues such as liver experience increased inflammation in the obese state (4,6-9). Unlike liver and AT, obesity-derived inflammation from muscle and heart remains a point of debate (7,10,11). However, inflammatory mediators from liver and adipose can alter skeletal muscle and heart metabolism (7,10,11), ultimately impacting systemic glucose homeostasis (4) as well as cardiac contractility and vascular function (9).

Investigations upstream of inflammatory mediators have identified MAPKs, consisting of extracellular signal-regulated kinase (ERK), p38, and c-Jun N terminal kinase (JNK), as major signaling pathways that contribute to metabolic tissue dysfunction (4,12). MAPKs have been shown to be activated in these metabolic tissues in response to obesity-associated inflammation and serve as mediators of IR through transcriptional mechanisms involving adipocyte gene expression as well as the

expression and secretion of other proinflammatory molecules (4,13-16). In addition to nuclear events involving AP-1 mediated gene expression, MAPK activity has also been linked to cytosolic suppression of proximal insulin signaling through JNK phosphorylation of specific serine residues of IRS-1 demonstrating that compartmentalized signaling events can impact different properties of tissue dysfunction (16-18).

While upstream kinases were traditionally viewed as major controllers of MAPK activity, recent evidence demonstrates that downstream phosphatases play a central role in mediating MAPK signaling and biological consequences involving inflammation (19-21). As phosphorylation of threonine and tyrosine residues within the activation loop are needed for MAPK activation, dephosphorylation of one or both residues results in inactivation of these molecules (14,21,22). MAPK-specific dual-specificity phosphatases (DUSPs) have recently emerged as central modulators of MAPK signaling critical for biological processes involving inflammation and IR (23,24), where DUSP loss of function results in increased MAPK signaling that corresponds to increased inflammation along with diabetic cardiomyopathy and diabetic dementia (25-29). This subclass of protein tyrosine phosphatases constitutes a structurally distinct group of ten proteins identified by a non-catalytic MAP Kinase Binding (MKB) domain that functions in the specific docking of DUSPs to MAPKs, subsequently dephosphorylating threonine and tyrosine residues within the activation loop (20,21,30).

In this report, we investigated DUSP regulation in key metabolically active, insulin-responsive tissues of AT, liver, skeletal muscle, and heart under conditions obesity. These tissues were selected as evidence demonstrates that they act as major regulators of blood glucose homeostasis and vascular function (6,9). Moreover, we examined the developmental role of obesity on DUSP regulation in AT using both

genetic and diet-induced models of obesity. This report demonstrates that DUSPs are regulated in a tissue specific manner based on the degree of obesity, the stage of obesity, the model of obesity as well as the inflammatory environment resulting from obesity. Collectively, these data suggest that divergent regulation of DUSPs may contribute to tissue-specific metaflammation and obesity, providing prospective therapeutic targets for obesity-associated metabolic inflammatory diseases.

Materials and Methods

Mice and experimental diets. Animals used for this study include genetically obese male B6.V-Lepob/J (B6-ob/ob) mice and their lean littermates as well as C57BL/6J mice rendered obese by diet and their lean controls. All mice were housed and treated by the supplier (Jackson Laboratories, Bar Harbor, Maine) until shipment 1 wk prior to tissue harvest. B6-ob/ob mice and lean littermates were purchased for experimentation at 6 wks and 10 wks of age and given free access to standard laboratory chow diet. C57BL/6J mice subjected to diet-induced obesity (DIO) were fed a high fat diet (HFD) consisting of 60% kcal from fat (Research Diets Inc. D12492) from 6 wks of age until shipment. Lean C57BL/6J control mice were fed a control diet (CD) consisting of 10% kcal from fat (Research Diet Inc. D12450B) from 6 wks of age until shipment. Both diets contained 10% kcal from protein with the balance in caloric value provided by differences in carbohydrate content. Mice receiving both diets had free access to food and shipped for experimentation at 18 wks and 24 wks of age. All animals were euthanized by CO_2 gas asphyxiation and epididymal AT, liver, skeletal muscle, and heart collected and processed for preparation of total RNA. Animal care and use was in compliance with the Institute of Laboratory Animal Research Guide for the

Care and Use of Laboratory Animals and approved by the institutional Animal Use and Care Committee of the Louisiana State University. Animals were weighed and tissues flash frozen in the laboratory of Jacqueline Stephens (Biological Sciences, Louisiana State University) and shipped to UNCG for further processing.

Real-Time RT-PCR: Total RNA was isolated from epididymal AT, liver, skeletal muscle, and heart by utilizing Trizol reagent according to manufacturer's protocol, and processed as described by Qiagen RNA clean-up protocol. Total RNA quality was assessed via RNA integrity gels and total RNA was quantified with a Nanodrop ND-1000 spectrophotometer. Total RNA was reverse-transcribed to cDNA in a 10 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems). The reverse transcription (RT) master mix containing RT buffer, deoxyribonucleotide triphosphate (dNTP) mix, RT random primers, RNase inhibitor (1.0 U/µl), and MultiScribe RT was added to 1 µg RNA and RNase-free water. Reverse transcription reaction conditions followed the protocol (25°C for 10 min, 37°C for 120 min, 85°C for 5 sec, followed by 4°C indefinitely/ RT complete) and utilized the Gene Amp PCR System 9700 thermal cycler (Applied Biosystems) for cDNA synthesis.

PCR amplification was run utilizing the 7500 fast system (Applied Biosystems) that consisted of enzyme activation at 95°C for 20 sec, followed by 40 cycles of denaturation at 95°C for 3 sec combined with annealing/extension at 60°C for 30 sec. All data were analyzed with the ABI 7500 real time PCR system (Applied Biosystems). TaqMan primer probes used in this study (Table 2.1) were purchased from Applied Biosystems. Data were recorded and analyzed with Sequence Detector Software (Applied Biosystems) and graphs visualized with SigmaPlot software. All data were presented as mean ± standard error of the mean (SEM) and representative of at least

two experiments performed in duplicate. Data were normalized to 18S and measured as relative differences using the $2^{-\Delta\Delta C_T}$ method as previously described (31,32).

Statistical analyses were conducted using SPSS v18. Differences in gene expression between lean and obese animals were determined via student's *t*-test where a p-value of <0.05 was considered significant. mRNA tissue distribution data were analyzed using analysis of variance, with Tukey's post-hoc analysis used when the *p* value for the respective parameter was statistically significant ($p < 0.05$).

Results

DUSP expression in insulin-responsive tissues of C57BL/6J mice. A role for select DUSPs (e.g., dusp1 and dusp6) has recently been demonstrated under conditions of genetic and diet-induced models of obesity (33-35). As activation of MAPKs increase in multiple tissues during the state of metaflammation contributing to obesity-induced IR and premature death (16,18,36,37), we initially compared relative mRNA expression of all known MAPK-specific DUSPs in insulin-responsive tissues from 10 wk old C57BL/6J wildtype mice using qRT-PCR. All ten DUSPs were measurable in lean AT within the detectable limits of 36 threshold cycles (C_T) with dusp5, dusp7, dusp8, and dusp9 least abundantly expressed (27 cycles) and dusp1, dusp6, and dusp10 most abundantly expressed (24 cycles) (Table 2.1). Only dusp2 fell outside this narrow range of expression at 30 cycles. Using DUSP structural differences involving the presence of nuclear localization sequences (NLS) or nuclear export sequences (NES), we and others have grouped DUSPs based on the probability of localization to the nucleus (group I) or cytosol (group II), respectively (Table 2.1). DUSPs containing both NLS and NES were designated as group III phosphatases that may localize to either compartment (20,21).

To assess differences in tissue distribution, DUSPs were normalized to 18S ribosomal RNA and expressed as fold-differences relative to AT. DUSPs were differentially expressed in insulin-responsive tissues with dusp2, dusp4, and dusp5 most abundantly expressed in white AT (WAT) regarding group I phosphatases (Fig.2.1A). Further examination of group I DUSPs, demonstrated that dusp4 expression was markedly more abundant (~14-fold) in WAT than in all other tissue types. Similarly, dusp5 was 20-fold greater in WAT than in liver or skeletal muscle, but not different in heart (Fig.2.1A). While dusp2 was significantly more abundant in WAT, its expression relative to the other tissue types was less marked with 4-fold, 1.5-fold, and 2-fold greater than liver, skeletal muscle, and heart, respectively. In contrast to other group I phosphatases, expression of dusp1 was more abundant in heart and skeletal muscle than in WAT or liver.

Regarding group II phosphatases, expression of dusp6 and dusp9 were also markedly more abundant in WAT, with expression of dusp9 ~50-fold greater in WAT compared to liver, skeletal muscle, and heart (Fig.2.1B). While expression of dusp6 was significantly greater in WAT (~2.5 fold) relative to skeletal muscle and heart, it was expressed equivalent to that of liver. Conversely, dusp7 was significantly more elevated in heart tissue relative to liver (~20 fold), AT (~3 fold), and skeletal muscle (~3 fold). In contrast to group I and group II DUSPs, expression of group III phosphatases was most abundant in skeletal muscle or heart where dusp8 was approximately 4-fold greater than WAT and 86-fold higher than liver (Fig.2.1C). Skeletal muscle had the highest expression of dusp10 compared to WAT (~4-fold), liver (~30-fold), and heart (~6-fold). Interestingly, dusp16 was the only phosphatase examined of any group that displayed similar levels of mRNA abundance across all four tissue types. Collectively, these data

demonstrated divergent tissue distribution of MAPK-specific DUSPs suggesting the probability of tissue-specific regulation and function.

DUSP regulation in insulin-responsive tissues under conditions of genetic obesity. The impact of genetic obesity on the expression of DUSPs in insulin-responsive tissues was further examined in 10 wk old B6-ob/ob mice, where leptin-deficient mice were given free access to standard chow for 10 wks presented with 83% greater body weight compared to wildtype littermates (Table 2.2). Using this genetic model of obesity, we compared the expression of each DUSP between lean and obese animals in all four insulin responsive tissues. Regarding the effect of genetic obesity on group I phosphatases, dusp1 was induced in WAT and liver, while dusp5 was elevated in liver and skeletal muscle. Obesity also increased dusp2 and dusp4, but only in WAT and skeletal muscle, respectively. Conversely, obesity suppressed dusp1, dusp2, and dusp5 in heart tissue as well as dusp4 in the liver. Of group II phosphatases, dusp6 was elevated only in liver tissue and suppressed in the heart. Dusp7 was the only phosphatase examined whose expression was completely unaffected by obesity in all four tissues. In contrast, dusp9 was markedly elevated ~5-fold in WAT, ~4-fold in liver and more than 30-fold in skeletal muscle, while remaining constant in heart tissue. As for group III phosphatases, dusp8 and dusp10 were only elevated in the liver while being suppressed by obesity in skeletal muscle. Dusp8 and dusp16 were also suppressed in skeletal muscle and WAT, respectively.

Collectively, these data demonstrated that inducible MAPK-specific DUSPs were divergently regulated by genetic obesity at the level of gene expression in a tissue-specific manner. Several generalizations were noted. As a group, group I phosphatases were the most affected by obesity with all four members being induced modestly, but

significantly, by obesity in one or more insulin responsive tissues. In contrast, dusp9 (group II) was markedly elevated by obesity in three of the four tissues examined, where gene expression exceeded a 30-fold induction in skeletal muscle. Dusp8 (group III) was also increased markedly with obesity, but only in the liver. While some phosphatases (i.e., dusp7 and dusp16) were mostly unaffected by obesity, select DUSPs increased in one tissue while decreasing in another. Of the four insulin responsive tissues tested, the heart was the only tissue, in which obesity did not lead to an increase in any DUSP, rather it actually led to a suppression of 5 of the 10 phosphatases examined.

Developmental regulation of DUSPs in AT of genetic and diet-induced obesity. Other reports have suggested that AT is the predominant site for the development of obesity-associated inflammation leading to systemic metabolic disruption. As data presented here demonstrated that DUSPs were most abundantly expressed in AT relative to other insulin responsive tissues, we next examined the developmental role of obesity on AT gene expression using both genetic and diet-induced models of obesity. For genetic obesity development, B6-ob/ob mice and wildtype littermates were purchased (Jackson Laboratory) for studies conducted at 6 wks and 10 wks of age. According to the supplier, mice homozygous for the obese mutation exhibit hyperplasia with ensuing obesity notable at 1 month of age and transient glucose intolerance that begins at ~6 wks of age and subsides between 12-16 wks of age. Thus, ages of mice chosen for this study represented models of early stage (6 wks) and mid-stage (10 wks) obesity with developing obesity-related metabolic disorders. As illustrated in Table 2.2, 6 wk and 10 wk old B6-ob/ob mice used in this study presented with 67% and 83% increases in body weight, respectively, relative to lean littermates. For development of diet-induced obesity (DIO), C57BL/6J males were

fed by the supplier a high fat diet (HFD; 60% kcal) starting at six weeks of age. Control B6 males were fed a control diet (CD) containing 10% kcal from fat and the same protein content as the HFD. The source of increased fat in the HFD was from lard, which contains abundant proinflammatory saturated fatty acids. Studies were conducted at 18 wks and 24 wks of age, representing 12 wks and 18 wks of specialized diet, respectively. As shown in Table 2.2, 18 wk and 24 wk old mice fed a HFD in this study presented with 13% and 26% increases in body weight, respectively, relative to lean mice fed the control diet.

RNA was isolated from AT harvested at two stages of obesity development from epididymal fat pads from male B6-ob/ob and DIO mice and assessed by qRT-PCR, normalized to 18S rRNA, and presented relative to lean controls. Initially, relative differences in adipsin were assessed to confirm that both stages of development in each animal model represented cellular changes indicative of the onset of obesity. It is well established that this adipocyte-specific gene is markedly suppressed in adipose tissue isolated from obese animals relative to lean controls (38). As illustrated in Fig.2.3, both models of obesity presented with marked suppression of adipsin at both stages of development. Moreover, the suppression was progressively more pronounced with time, with 40-fold and 80-fold suppression determined for early and late stage obesity in B6-ob/ob animals and 7-fold and 25-fold suppression for early and late stage obesity in DIO animals. To further characterize the stage of development, relative mRNA abundance was determined for MCP-1, IL-6 and TNFα as inflammatory markers critical to the development of chronic AT inflammation during obesity (6,39,40). The chemokine MCP-1, which has been shown to play an early role in macrophage recruitment to AT during the onset of obesity (39,40), increased greater than 8-fold at both stages of development

and in both models of obesity (Fig.2.3). Conversely, IL-6 and TNFα were elevated only during late stages in B6-ob/ob mice fed standard chow. Both cytokines were elevated in DIO mice fed HFD at both stages of development with progressive increases in TNFα correlating to either time on HFD or the stage of obesity development. Along with changes in body weight, relative changes in mRNA abundance of these four genes characterized both animal models as early to mid-stage development of obesity.

Relative DUSP gene expression was further examined in AT during the development of obesity as assessed above. Several generalizations could be made. First, dusp9 (group II) was the only phosphatase examined where mRNA abundance increased in both models of obesity and at both stages of development, including early stage B6-ob/ob where the onset of obesity preceded the increase in inflammatory cytokines. Moreover, dusp9 gene expression progressively increased with the stage of development in both animal models. These data were consistent with other observations from our laboratory where dusp9 was shown to increase during adipocyte differentiation, but displayed no changes in gene expression in undifferentiated preadipocytes or mature adipocytes over time following TNFα stimulation (data not shown; Ch.3&4). Second, dusp4 (group I) increased only in early stage B6-ob/ob mice that were shown here to have no changes in TNFα expression and was suppressed in late stage DIO mice that presented with the greatest increase in TNFα expression. These data were also consistent with other observations from our laboratory where dusp4 was observed to increase with adipocyte differentiation, but was markedly suppressed with TNFα stimulation (data not shown; Ch.3&4). Third, dusp1 and dusp2 (group I) were elevated in obese relative to lean AT only during conditions that also displayed an increase in TNFα; an observation that was again consistent with unpublished data demonstrating that

dusp1 increased at the level of gene expression with TNFα stimulation (Ch.4). Fourth, dusp5 and dusp6 increased during both stages of development in DIO mice, but not B6-ob/ob mice. Neither phosphatase was markedly affected by TNFα stimulation in adipocytes (data not shown, Ch.4), suggesting a role for other inflammatory mediators associated with HFD unrelated to obesity development. Fifth, dusp7, dusp8 and dusp16 displayed no increases in gene expression in either model at either stage of development. Finally, except for a modest decrease in dusp16 only in late stage B6-ob/ob, the development of obesity did not result in suppression of DUSP gene expression in AT. Collectively, these data demonstrated DUSP gene expression was divergently regulated based on the degree of obesity, the stage of obesity, the model of obesity as well as the inflammatory environment resulting from obesity.

Discussion

This investigation presents the first empirical evidence that MAPK-specific DUSPs are divergently regulated in metabolically active, insulin responsive tissues under conditions of genetic obesity, and differentially regulated in AT based on the degree, stage, and model of obesity as well as the inflammatory environment resulting from obesity. First, we show that DUSPs are divergently regulated in all four tissue types analyzed and that DUSPs were most abundantly expressed in AT. Second, we present evidence that DUSPs were differentially regulated in genetic obesity in a tissue-specific manner. Finally, these data show that DUSP regulation in AT was dependent on the developmental stage of obesity (i.e., early vs. mid), the model system that characterized obesity (i.e., genetic or diet-induced), and the development of obesity-associated inflammation. Collectively, these data provide novel insight into DUSP regulation in

insulin responsive tissues involved in blood glucose homeostasis and vascular function, potentially establishing therapeutic targets for obesity-associated metabolic inflammatory diseases.

Mounting evidence demonstrates that DUSPs show different patterns of tissue expression, allowing individual DUSPs to regulate tissue-specific MAPK-dependent biological processes (23). This investigation presents the first empirical evidence demonstrating that all ten MAPK-specific DUSPs were differentially regulated in metabolically active, insulin-responsive tissues, supporting a tissue-specific functional role for individual DUSPs on the modulation of MAPK signaling. Tissue-specific regulation of DUSPs, therefore, may also contribute to MAPK-dependent processes that link obesity with metabolic inflammatory diseases. Data presented here demonstrate that DUSPs were divergently regulated by genetic obesity at the level of gene expression in a tissue-specific manner. Recent studies have identified dusp6 as a candidate gene involved in insulin signaling in the liver, where loss-of-function in lean and obese mice decreased fasting blood glucose levels that was attributed to down-regulation of gluconeogenic genes, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (33,35). While we report that dusp6 was expressed most abundantly in AT and liver compared to skeletal muscle and heart, we further show that dusp6 was only induced in the liver under conditions of genetic obesity, suggesting that tissue-specific regulation of dusp6 is essential for liver gluconeogenesis. Of the four tissues examined, the heart was the only tissue in which obesity did not lead to an increase in any DUSP, but actually led to a suppression of 5 of the 10 phosphatases examined. While loss-of-functions studies demonstrate that induction of individual DUSPs protects against vascular endothelial inflammation and atherosclerosis (41-43), these data suggest that suppression of

DUSPs with genetic obesity potentially contributes to obesity-mediated heart disease. Collectively, these data highlight the therapeutic potential for individual DUSPs in the tissue-specific regulation of MAPK-dependent processes that link obesity to metabolic inflammatory diseases.

While metaflammation leads to metabolic instability, AT remains the predominant site of proinflammatory cytokine expression during obesity. Moreover, evidence demonstrates that AT inflammation happens gradually over time, where cytokine and chemokine expression as well as macrophage infiltration increase with the development of obesity (2,12,12,39,40). Recent reports, for instance, demonstrate that macrophage-related genes MIP-1α and MCP-1 increased within 3 weeks of a high fat diet (HFD) and continued to rise at 16 and 26 weeks on a HFD concomitant with macrophage infiltration and the development of systemic IR (39). Data presented above demonstrate that proinflammatory chemokine and cytokine mRNA was increased in AT of C57BL/6J mice at 10 wks, while TNFα continued to rise at 18 wks on a HFD. We also show that only MCP-1 was increased in 6 wk old B6-ob/ob mice, while MCP-1, IL-6, and TNFα were increased in 10 wk old B6-ob/ob mice. Similar to the gradual development of AT inflammation, we observed a progressive decline in the adipocyte-specific gene adipsin, which has previously been reported to decrease with the degree of obesity (38). Collectively these data support the hypothesis that AT inflammation progressively occurs with the development of obesity.

Increased reports demonstrate a functional role for DUSPs in the regulation of inflammation, where DUSP loss-of-function studies demonstrate that MAPK-dependent inflammation of immuno-modulating cells is markedly activated (25-27,44). Data presented here demonstrate that DUSPs were divergently regulated in AT based on the

degree, stage, and model of obesity as well as the inflammatory environment resulting from obesity. We show that dusp1 and dusp2 were only induced under conditions of obesity where inflammatory genes IL-6 and TNFα were elevated. Furthermore, we report that dusp4 was only induced under conditions of obesity with no inducible inflammatory gene expression of TNFα, while dusp4 was suppressed with DIO where TNFα was markedly elevated. Consistent with these observations, reports have shown that dusp1 and dusp4 regulate MAPK-dependent inflammation in response to endotoxic shock and parasitic infection (25-27,44), where loss-of-function promoted inflammation and worsened animal response to shock or infection. We further report that dusp5 and dusp6 were upregulated under conditions of diet-induced obesity and not genetic obesity. As others have reported a role for fatty acids in the regulation of individual DUSPs (45,46), these data suggest that high fat feeding, and not obesity per se, promotes inducible dusp5 and dusp6 expression. Moreover, reports show that saturated fatty acids bind to toll-like receptors leading to increased AT inflammation (47,48). As obesity occurs in the presence of chronic, positive energy intake, these data suggest that select DUSP regulation in vivo reflects diet-induced models of obesity. Collectively these data suggest that inflammatory progression in AT resulting from obesity and/or high fat feeding divergently regulate DUSPs potentially linking obesity with metabolic dysfunction.

Of the phosphatases examined, dusp9 was the only DUSP induced under all conditions of genetic and diet-induced obesity in AT. Moreover, dusp9 was upregulated in AT, liver, and muscle with genetic obesity. Others have also shown that dusp9 is upregulated in AT under conditions of obesity (24,49), while gain-of-function studies demonstrate that dusp9 blocks adipogenesis (24,34,49). While no study has examined dusp9 loss of function on adipogenesis, these data collectively suggest that obesity

regulates dusp9 mRNA expression. Furthermore, reports have identified Dusp9 as a candidate gene involved in insulin signaling, while others show that dusp9 overexpression improved stress-induced IR (33,35,49). Collectively, these data suggest a functional role for dusp9 in the development of obesity and metabolic regulation.

In summary, previous reports (20,23) demonstrate the importance of phosphorylation and dephosphorylation by upstream kinases and downstream phosphatases in the regulation of MAPK signaling and metabolic function. Data are presented demonstrating that DUSPs are divergently regulated in a tissue-specific manner, and that obesity-associated induction of DUSPs is highly dependent on the tissue examined. Furthermore, we show that progression of obesity and inflammation resulting from obesity as well as diet divergently regulate DUSPs in AT. Collectively, these data suggest that differential regulation of DUSPs potentially contribute to MAPK-dependent tissue-specific biological processes linking obesity with metabolic inflammatory diseases.

Table 2.1. DUSP, adipocyte, and inflammatory genes analyzed in this study.

symbol	name/alias					accession	ABI number	C_T
dual specificity phosphatases (DUSPs)		GP	MKB	NLS	NES			
Dusp1	MKP-1, hVH1	I	●	●		NM_013642	Mm00457274_g1	24
Dusp2	PAC1	I	●	●		NM_010090	Mm00839675_g1	30
Dusp4	MKP-2, hVH2	I	●	●		NM_176933	Mm00723761_m1	25
Dusp5	hVH3	I	●	●		NM_001085390	Mm01266104_m1	27
Dusp6	MKP-3, rVH6	II	●		●	NM_026268	Mm00650255_g1	24
Dusp7	MKP-X	II	●		●	NM_153459	Mm00463228_m1	27
Dusp9	MKP-4	II	●		●	NM_029352	Mm00512646_m1	27
Dusp8	M3/6, hVH5	III	●	●	●	NM_008748	Mm00456230_m1	27
Dusp10	MKP-5	III	●	●	●	NM_022019	Mm00517678_m1	24
Dusp16	MKP-7, MKP-M	III	●	●	●	NM_130447	Mm00459935_m1	25
adipocyte and inflammatory genes								
Cfd	complement factor D, adipsin					NM_013456	Mm00442664_m1	17
Ccl2	monocyte chemoattractant protein-1 (MCP-1)					NM_011333	Mm00441242_m1	26
IL-6	interleukin-6					NM_031168	Mm99999064_m1	32
TNFα	tumor necrosis factor-alpha					NM_013693	Mm99999068_m1	28
reference gene								
18S	18 ribosomal RNA					X03205	4342930E	9

Table 2.1. DUSP, adipocyte, and inflammatory genes analyzed in this study. DUSPs that contained the MAPK binding domain (MKB) were assigned to groups (GP) based on structural differences involving the presence or absence of a nuclear localization sequence (NLS), nuclear export sequence (NES), or both that may dictate subcellular localization to the nucleus (group I), cytosol (group II), or either compartment (group III).

Table 2.2. Final body weight of C57BL/6J and B6.V-Lep^{Ob}/J mice used in this study.

	Final body weight (g)		Difference in body weight (g)	% Change in final body weight
Age	**Lean**	**Ob/ob**	**Ob/ob-Lean**	**Ob/ob**
6 wk	21.8	36.4	14.6	67%
10 wk	28.6	52.4	23.8	83%
Age	**CD**	**HFD**	**HFD-CD**	**HFD**
18 wk	30.8	34.7	3.9	13%
24 wk	32.2	40.6	8.4	26%

CD=Control Diet

HFD=High Fat Diet

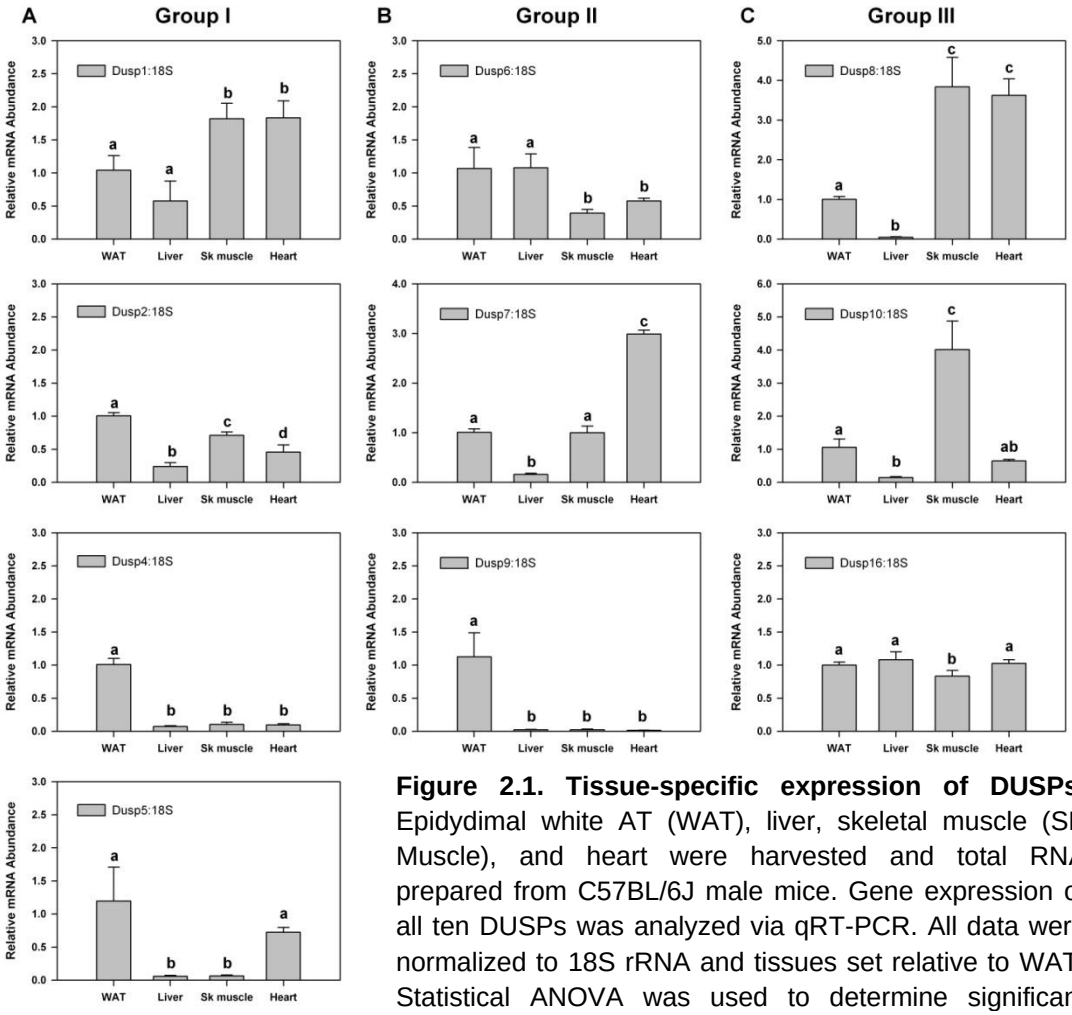

Figure 2.1. Tissue-specific expression of DUSPs. Epididymal white AT (WAT), liver, skeletal muscle (SK Muscle), and heart were harvested and total RNA prepared from C57BL/6J male mice. Gene expression of all ten DUSPs was analyzed via qRT-PCR. All data were normalized to 18S rRNA and tissues set relative to WAT. Statistical ANOVA was used to determine significant differences, $p<0.05$. Tukey's post-hoc analysis was performed when the *p* value for the respective parameter was statistically significant ($p < 0.05$). Additionally, DUSPs were grouped based on localization sequences: group I (NLS), group II (NES), and group III (NLS/NES).

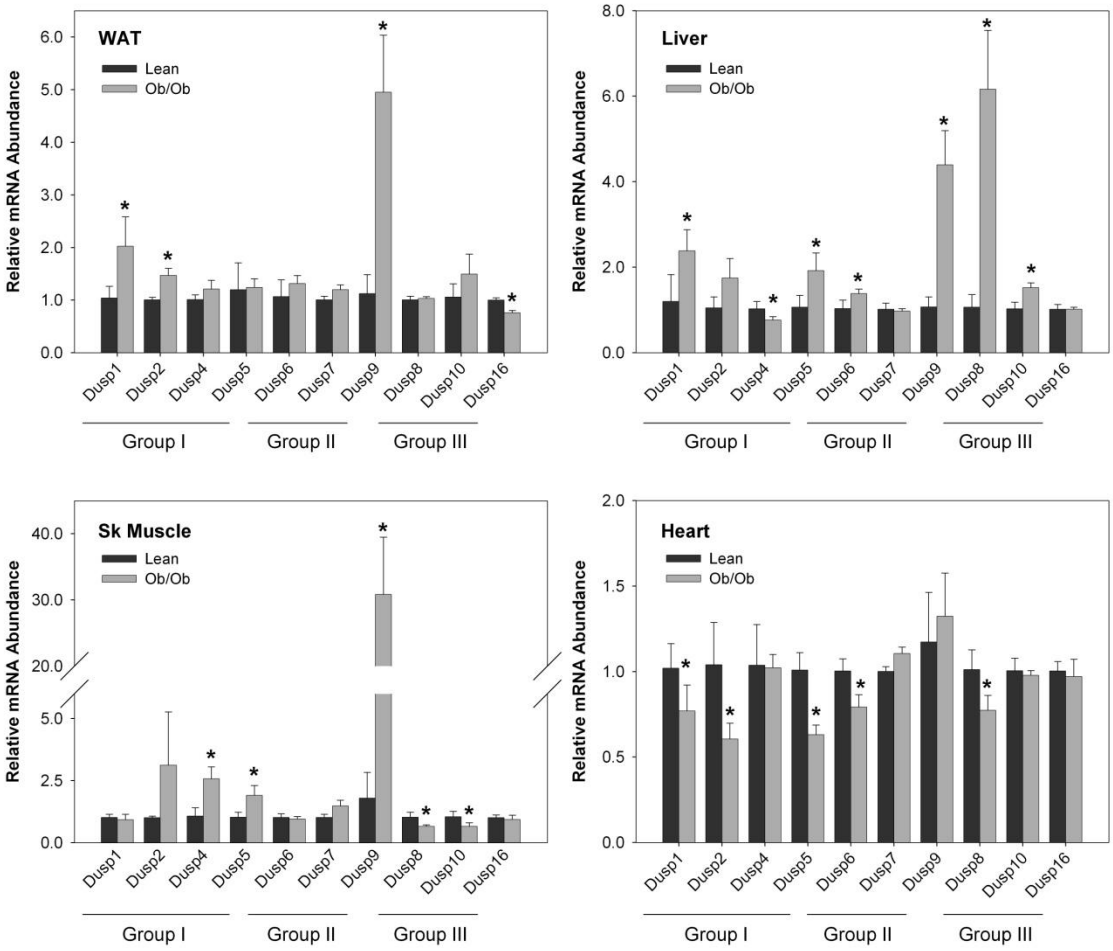

Figure 2.2. Tissue-specific expression of DUSPs under conditions of leptin-deficient obesity. White AT (WAT), liver, skeletal muscle (Sk Muscle), and heart were harvested and prepared for total RNA from 10-wk-old male B6.V-Lep^{Ob}/J (ob/ob) mice and their lean litter mates. Relative DUSP mRNA abundance was measured via qRT-PCR and statistical significance determined at $p<0.05$ via student's *t*-test. All data were normalized to 18S rRNA and expressed relative to lean littermates. Graphical grouping of DUSPs is based on localization sequence, where group I (nuclear) contains and NLS, group II (cytosolic) contains and NES, and group III (cytosolic/nuclear) contains NLS and NES sequences.

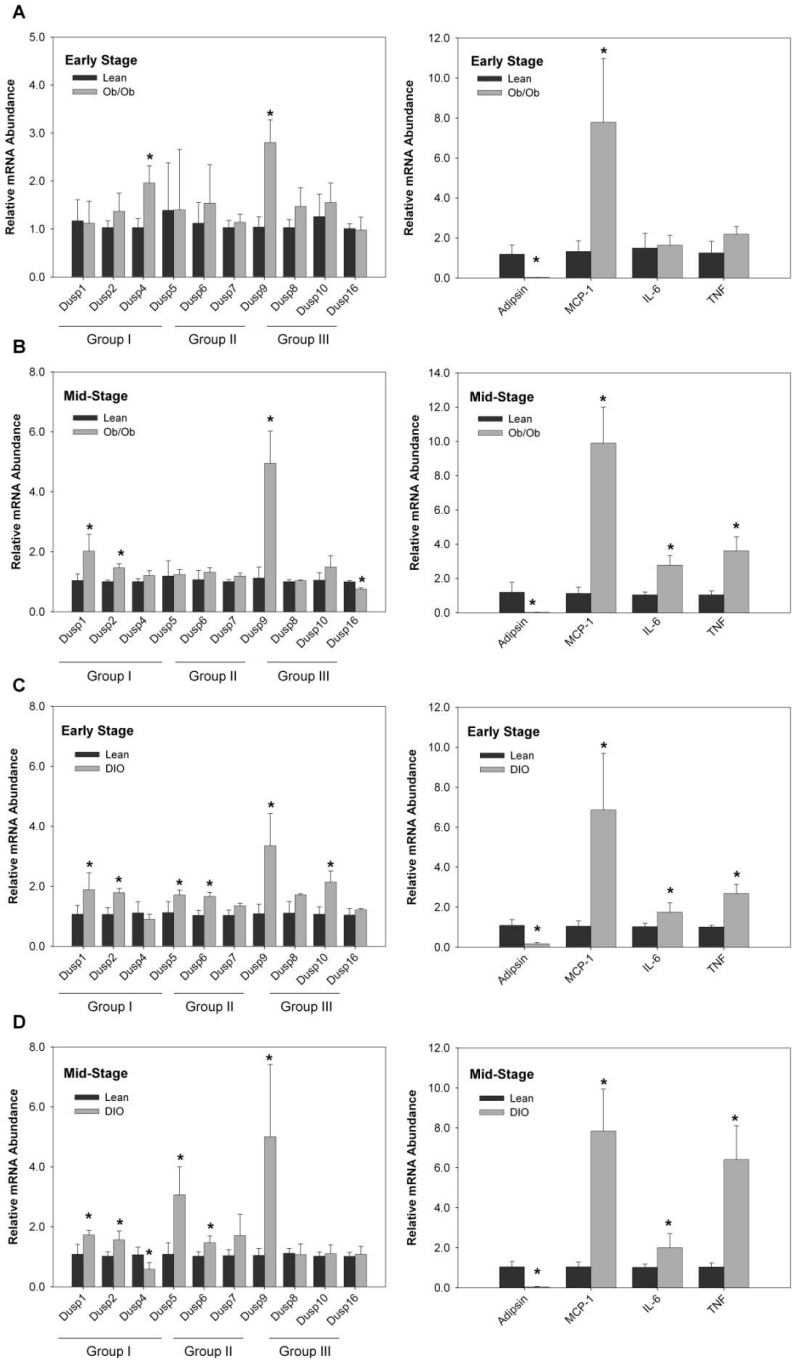

Figure 2.3. AT specific regulation of DUSPs and inflammatory mediators during the development of obesity under conditions of genetic and diet-induced obesity. AT was harvested and RNA prepared from A) 6-wk-old and B) 10-wk-old male B6.V-Lep^{Ob}/J (ob/ob) mice and their lean litter mates, along with male C57B116J C) 18-wk-old and D) 24-wk-old DIO mice and their lean controls. Relative DUSP mRNA abundance was measured via qRT-PCR and statistical significance determined student's *t*-test ($p<0.05$). All data were normalized to 18S rRNA and expressed relative to lean littermates.

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CHAPTER III

ROLE FOR DUAL-SPECIFICITY PHOSPHATASES ON MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING DURING ADIPOCYTE DIFFERENTIATION

Abstract

Knowledge concerning mechanisms that control proliferation and differentiation of preadipocytes is essential to our understanding of adipocyte hyperplasia and the development of obesity. Timely regulation of mitogen-activated protein kinase (MAPK) phosphorylation and dephosphorylation by upstream kinases and downstream phosphatases is critical for coupling extracellular stimuli to cellular growth and differentiation. Using differentiating 3T3-L1 preadipocytes as a model of adipocyte hyperplasia, we examined a role of dual-specificity phosphatases (DUSPs) on the timely modulation of MAPK signaling during states of growth arrest, proliferation, and differentiation. Using real-time reverse transcription PCR (qRT-PCR), we report that several DUSPs were induced during early (i.e., dusp1, dusp2, dusp5, and dusp6), mid (i.e., dusp4), and late (i.e., dusp9 and dusp16) stage adipocyte differentiation concomitant with ERK and p38 dephosphorylation. As timely modulation of ERK and p38 activation during early differentiation is essential for adipogenesis, we further demonstrate that de novo mRNA synthesis was required for ERK and p38 dephosphorylation, suggesting a modulatory role for 'inducible' phosphatases in MAPK signaling. Moreover, we show that pharmacological inhibition of early DUSPs markedly increased ERK and p38 signaling magnitude and duration during early adipogenesis. As conflicting reports have emerged for dusp1 regarding adipocyte differentiation, we

further demonstrate that dusp1-specific knockdown plays a modulatory role in MAPK signaling during early adipocyte differentiation, but did not block adipocyte differentiation. Collectively, these data suggest that cooperative actions of DUSPs is essential for modulating timely dephosphorylation of MAPK signaling during adipocyte differentiation, providing prospective therapeutic targets for the treatment of obesity and its co-morbidities.

Introduction

Obesity is a global pandemic that contributes to the development of stroke, heart disease, and type 2 diabetes (1). Obesity develops from an increase in adipose tissue (AT) mass due to enlargement of adipocyte size (hypertrophy) and cell number (hyperplasia) (2,3). As mature adipocytes (ADs) are resistant to cell division, hyperplasia generally refers to the recruitment of new ADs through proliferation and subsequent differentiation of adipocyte precursor cells referred to as ‘preadipocytes’ (PAs). While PA replication slows during adulthood to provide a constant balance of PAs to ADs when weight remains constant, adipocyte hyperplasia plays a critical role in the onset of obesity throughout all stages of life, particularly during childhood obesity and in adults under morbid conditions (4-7). Unlike hypertrophic obesity, exercise and caloric restriction are less effective in treating obesity derived from adipocyte hyperplasia (4,8,9). Despite evidence suggesting that hyperplastic obesity is associated with the poorest prognosis of treatment, mechanisms regulating adipocyte hyperplasia remain largely unknown.

Intracellular signaling molecules play a critical role in linking environmental cues to increases in AT mass involving proliferation and differentiation of PAs. Major signaling

transduction cascades essential for adipocyte hyperplasia include phosphatidylinositol-3 kinases (PI3Ks) and mitogen-activated protein kinases (MAPKs) (10,11). MAPK activity is regulated by a cascade of phosphorylation events mediated by sequential activation of upstream kinases, where MAPK is phosphorylated by upstream MAPK kinases (MAP2Ks), which are phosphorylated by MAPK kinase kinases (MAP3Ks) in response to diverse external stimuli. Phosphorylation of both threonine and tyrosine residues of the (T-X-Y) motif within the activation loop is essential and sufficient for MAPK activity, leading to the phosphorylation of target proteins and transcription factors within the cytosolic and nuclear compartments (12-14) linking environmental cues to PA proliferation and differentiation.

Of the major MAPK signaling pathways, only extracellular signal-regulated kinase (ERK) and p38 MAPK have been shown to regulate AD differentiation (10). Evidence suggests that timely activation (i.e., phosphorylation) as well as deactivation (i.e., dephosphorylation) of ERK and p38 is necessary for adipogenesis (10,15-19). Loss-of-function studies have demonstrated a critical role for rapid and transient ERK activity regarding the induction of the early adipogenic transcription factor CCAAT/enhancer-binding protein β (C/EBPβ). Activation of C/EBPβ is necessary for PA replication as well as the induction of C/EBPα and peroxisome proliferator-activated receptor (PPAR) γ; two master regulators of adipocyte-specific gene expression (18,20,21). Conversely, prolonged ERK activity has been shown to phosphorylate and inactivate PPARγ blocking AD differentiation (22). Similar evidence suggests that transient p38 activity is also critical for AD differentiation, whereas prolonged p38 activity results in cell death (16,23,24). Collectively, these observations suggest that timely activation of MAPKs,

involving a precise balance in phosphorylation/dephosphorylation mechanisms, is essential for PA proliferation and differentiation.

While differences in MAPK signaling have traditionally been attributed to upstream kinases, recent evidence suggests that MAPK deactivation via phosphatases plays a central role in mediating signal transduction and cellular outcome (25-27). As phosphorylation of threonine and tyrosine residues within the activation loop is needed for MAPK activation, dephosphorylation of one or both residues results in inactivation of these molecules (12,27,28). MAPK-specific DUSPs constitute a structurally distinct group of ten proteins identified by a non-catalytic MAP Kinase Binding (MKB) domain that functions in the docking of MAPKs to the phosphatase (13,26,27). Moreover, the MKB domain consists of two CH2 domains flanking a kinase interactive motif (KIM) that confers specificity for select MAPKs. Recent attention has been given to these MAPK-specific DUSPs regarding both proliferation and differentiation (29). While some reports demonstrate that overexpression of select DUSPs correlate to JNK and p38 deactivation and resistance to cell death (30-32), others show that loss of DUSP expression contributes to increased MAPK signaling and cellular growth (33-36). Reports also demonstrate an essential role for DUSPs in myocyte, neuronal, and osteoblast differentiation (37-39), where the precise balance of MAPK phosphorylation and dephosphorylation was necessary for differentiation of each cell type. Taken together, these data suggest that DUSP-MAPK interaction is essential for cellular growth and differentiation.

Dusp1 has recently emerged in reports examining mouse models of diet-induced obesity where dusp1 knockout mice had enhanced nuclear JNK, p38, and ERK activity in white adipose tissue, liver, and skeletal muscle (40). Furthermore, these mice were

resistant to diet-induced obesity on a high fat diet and presented with increased energy expenditure. However, mouse embryonic fibroblasts from wildtype and dusp1 knockout mice differentiated in a similar manner demonstrating that dusp1 is not essential for adipocyte differentiation (40). Conversely, others have shown that dusp1 antisense expression in 3T3-L1 PAs led to sustained ERK activation and inhibition of adipogenesis (41). Considering the role DUSPs play in proliferation and differentiation, we explored the regulation and role of DUSPs during adipocyte differentiation using the murine 3T3-L1 cell line. When grown to a state of density-arrest, quiescent PAs stimulated with a cocktail containing MDI synchronously re-enter the cell cycle for 1-2 rounds of proliferation prior to terminal growth arrest and AD differentiation (18,42). In this report, we demonstrate that MAPK-specific DUSPs are differentially expressed in PAs and ADs. Moreover, we show that several DUSPs are induced during early (i.e., dusp1, dusp2, dusp5, and dusp6), mid (i.e., dusp4), and late (i.e., dusp9 and dusp16) stages of AD differentiation concomitant with MAPK dephosphorylation. Additionally, pharmacological inhibition of early DUSPs increased MAPK signaling duration suggesting a cooperative role for DUSPs on MAPK dephosphorylation. Collectively, these data represent the first study in any cell type that examined all ten MAPK-specific DUSPs during differentiation and highlight an essential role for DUSPs in the timely modulation of MAPK dephosphorylation that is critical for adipogenesis.

Materials and Methods

Materials: Dulbecco's Modified Eagle's Medium (DMEM), calf bovine serum (CS), and trypsin-EDTA were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from HyClone. Antibodies used for immunoblotting include phospho-ERK,

phosho-p38, total p38, aP2, GAPDH, and α-tubulin purchased from Cell Signaling and dusp1, PPARγ, and C/EBPα purchased from Santa Cruz Biotechnology. Enhanced chemiluminescence (ECL) reagents were obtained from Perkin-Elmer Life Sciences. Actinomycin D was purchased from Sigma Aldrich triptolide was obtained from Calbiochem. All Taqman primer probes (Table 3.1) used in this study were purchased from Applied Biosystems. The murine 3T3-L1 cell line was purchased from Howard Green, Harvard Medical School (43).

Cell Culture: 3T3-L1 preadipocytes were propagated in DMEM supplemented with 10% CS until density-induced growth arrest, as previously described (42). At 2 days post-confluence, growth medium was replaced with DMEM supplemented with 10% FBS, 0.5mM 1-methyl-3-isobutylxanthine, 1 µM dexamethasone, and 1.7 µM insulin (MDI). Throughout the study, 'time 0' refers to density arrested cells immediately before the addition of MDI to the culture medium. Experiments described herein were conducted within the period of differentiation spanning from density arrest (d0) through 8 days (d8) post-MDI. All experiments were repeated at least 3 times to validate results and ensure reliability.

Immunoblotting: Cell monolayers were washed with phosphate-buffered saline (PBS) and scraped into ice-cold lysis buffer containing 1.0 M Tris, pH 7.4, 150 mM NaCl, 1% Triton X, 0.5% Nonidet P-40 (NP40), 1 mM EDTA, 1 mM EGTA, and 10 mM *N*-ethylmaleimide. Phosphatase (20 mM ß-glycerophosphate, 10 mM NaFl, and 2 µM sodium vanadate), as well as protease (0.3 µM aprotonin, 21 µM leupeptin, 1 µM pepstatin, 50 µM phenanthroline, and 0.5 µM phenylmethylsulfonyl fluoride) inhibitors were added to lysis buffer prior to cell harvest. Cell lysates were sonicated, centrifuged (13,000 x *g*, 10 min, 4°C), and the supernatant transferred to a fresh tube. Bicinchoninic

acid assay (Pierce, Rockford, IL) was used to determine protein concentration. Cell lysates were resuspended in loading buffer containing 0.25 M Tris, pH 6.8, 4% sodium dodecyl sulfate (SDS), 10% glycerol, 10% dithiothreitol, and 0.01% bromophenol blue and heated for 5 min at 80°C. Proteins were resolved on SDS-polyacrylamide gel electrophoresis gels (PAGE) and transferred to polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA). After transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4°C. Membranes were subsequently probed with horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Immunoblots were immersed in ECL and visualized by autoradiography using CL-XPosure film (Pierce).

Real-Time qRT-PCR: Total RNA was extracted and genomic DNA contamination was removed using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's protocol. Total RNA was quantified with a Nanodrop ND-1000 spectrophotometer and reverse-transcribed to cDNA in a 10 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems). The reverse transcription (RT) master mix containing RT buffer, deoxyribonucleotide triphosphate (dNTP) mix, RT random primers, RNase inhibitor (1.0 U/µl), and MultiScribe RT was added to 1 µg RNA and RNase-free water. Reverse transcription reaction conditions followed the protocol (25°C for 10 min, 37°C for 120 min, 85°C for 5 sec, followed by 4°C indefinitely/RT complete) and utilized the Gene Amp PCR System 9700 thermal cycler (Applied Biosystems) for cDNA synthesis.

PCR amplification was run utilizing the 7500 fast system (Applied Biosystems) that consisted of enzyme activation at 95°C for 20 sec, followed by 40 cycles of denaturation at 95°C for 3 sec combined with annealing/extension at 60°C for 30 sec. All

data were analyzed with the ABI 7500 real time PCR system (Applied Biosystems). Data were recorded and analyzed with Sequence Detector Software (Applied Biosystems) and graphs visualized with SigmaPlot software. All data were presented as mean ± standard error of the mean (SEM) and representative of at least two experiments performed in duplicate. Data were normalized to 18S previously validated by this lab as a suitable reference gene under these experimental conditions (44). Relative differences between treated and untreated control samples were analyzed by the $2^{-\Delta\Delta C_T}$ method as previously described (44,45).

Statistical analyses were conducted using SPSS v18. Phenotypic differences were determined via student's *t*-test where a p-value of <0.05 was considered significant. Knockdown and Inhibitor data were analyzed using analysis of variance, with Tukey's post-hoc analysis used when the *p* value for the respective parameter was statistically significant ($p < 0.05$).

Flow Cytometry: Cell monolayers were washed with PBS and trypsinized. Detached cells were diluted in ice-cold PBS, and gently pelleted by centrifugation (300 x *g*, 5 minutes, 4°C). PBS was decanted, and cells were fixed and permeabilized by drop-wise addition of 70% ethanol at –20°C while vortexing. Cells were washed in PBS and incubated in the dark for 30 min with a propidium iodide staining solution (50 µg/mL propidium iodide and 100 µg/mL RNase A in PBS). DNA fluorescence was measured with using the Guava easyCyte HT flow cytometer (Millipore) equipped with a 488-nm argon laser. Width (FL2W) and area (FL2A) of propidium iodide fluorescence were recorded for at least 5,000 counts, and DNA histograms were extracted from FL2W-FL2A dot plots. Histograms were analyzed with Modfit software.

RNA Interference. SMARTpools containing four different short interfering RNAs (siRNAs) for dusp1 specific sequences as well as non-targeting sequences were transfected using DharmaFect 3 transfection reagent according to manufacturer's (Dharmacon) protocol. Briefly, 3T3-L1 preadipocytes were propagated in 6-well culture dishes until reaching density-induced growth arrest. Growth medium was then replaced with DMEM supplemented with 10% CS, 3 µl DharmaFect 3 reagent and either 100 nM dusp1 specific siRNA or non-targeting siRNA for 72 hr. Growth medium was subsequently switched to differentiation medium containing MDI as described above.

RESULTS

Regulation of MAPK dephosphorylation by mechanisms involving de novo mRNA synthesis during 3T3-L1 adipocyte differentiation. While it is well established that ERK and p38 play functional roles during AD differentiation, the extent (i.e., magnitude and duration) of MAPK activity during distinct stages of differentiation remains uncertain (10,18,24). As others have reported that MAPK activity is required for early stages of differentiation and inhibits late stages of differentiation, the 'timing' of activity is critical for proper differentiation. To elucidate a role for phosphorylation and dephosphorylation mechanisms regarding MAPK activity during adipocyte hyperplasia, we utilized the well-established 3T3-L1 murine cell line for this study. This cell line synchronously progresses through distinct stages of the cell cycle before entering a state of terminal growth arrest concomitant with activation of a cascade of transcription factors that mediate late stage adipocyte gene expression and acquisition of the functional AD phenotype. To determine the extent of MAPK activity during these distinct stages of adipocyte differentiation, density-arrested PAs were stimulated with MDI and

cell lysates harvested over early (0hr-24hr) and late (d0-d8) stages of differentiation and immunoblotted with phosphospecific antibodies that recognize MAPKs only when phosphorylated on both amino acid residues that are sufficient and essential for MAPK activity. As illustrated in Fig.3.1A, ERK and p38 were rapidly phosphorylated (15 min) and then completely dephosphorylated (<4hr) during early adipocyte differentiation. Also shown is the phosphorylation state of MEK, the upstream MAPK kinase responsible for ERK phosphorylation, demonstrating that ERK was completely dephosphorylated 6-10 hrs before MEK phosphorylation (i.e., activity) returned to baseline. The disconnect between the phosphorylation state of ERK and its upstream kinase is further illustrated in Fig.3.1.B, where phospho-ERK dropped below baseline following the initial activation throughout later stages of differentiation even though MEK retained some degree of activity at or exceeding baseline throughout 8 days of differentiation. It should also be noted (Fig.3.1.B) that the decrease in ERK activity below baseline levels occurred kinetically with the induction of PPARγ which is a known target of ERK whose transcriptional activity is suppressed when phosphorylated by ERK (22). Lastly, while the duration of p38 phosphorylation was nearly identical to ERK during the first 4 hrs of differentiation (Fig.3.1.A), the phosphorylation state of p38 displayed a biphasic pattern that was not similar to ERK during later stages of differentiation (Fig.3.1.B). Immunoblot analysis of total p38 remained unchanged during all stages of adipocyte differentiation which served as a loading control (Fig.3.1A-B).

Data presented above suggest a role for phosphatases in the deactivation of MAPK signaling. As others have shown several DUSPs act as immediate early genes to regulate MAPKs (26), we developed the working hypothesis that ‘inducible’ DUSPs mediate the dephosphorylation of ERK and p38 during adipocyte differentiation. To

initially test our working hypothesis, we examined if ERK and p38 signaling involved de novo mRNA synthesis. Preadipocytes were pretreated with and without 1 ng/ml actinomycin D for 30 mins to inhibit mRNA synthesis prior to stimulation with MDI. Cell lysates were harvested over time during early adipocyte differentiation (<24h) and immunoblotted for bisphosphorylated ERK and p38 as discussed above. As illustrated in Fig.3.1C, blocking RNA synthesis resulted in a marked increase in the duration of ERK extending from less than 4 h in the absence of actinomycin D to more than 24 h when RNA synthesis was inhibited. No discernable effects were noted regarding the magnitude of peak ERK phosphorylation. Conversely, inhibition of RNA synthesis had a marked effect of p38 magnitude and minimal impact of p38 duration. The increase in p38 phosphorylation at 24 hrs post-MDI was likely due to prolonged exposure of actinomycin D. While MAPK dephosphorylation (i.e., ERK duration and p38 magnitude) was dependent on de novo mRNA synthesis, initial MAPK phosphorylation was completely unaffected by actinomycin D. Collectively, these data suggest that the extent of MAPK signaling is regulated by a balance of upstream kinases and downstream inducible phosphatases.

Inducible DUSP gene expression during early and late adipocyte differentiation. As the magnitude and duration of MAPK signaling duration were dependent on de novo mRNA synthesis, we tested the hypothesis that inducible DUSPs modulate the extent of MAPK phosphorylation during adipocyte differentiation. For this, we initially compared relative mRNA expression of all known MAPK-specific DUSPs in PAs (d0) versus ADs (d8) using qRT-PCR. As illustrated in Table 3.1, all ten DUSPs were measurable in PAs within the detectable limits of 36 threshold cycles (C_T), with dusp2, dusp8, and dusp9 least abundantly expressed at 32 cycles and dusp1, dusp6

and dusp7 most abundantly expressed at 24 cycles. This 8 cycle difference was equivalent to a 256-fold difference (i.e., 2^8) in base-line mRNA abundance in PAs.

DUSPs were grouped based on structural differences involving the presence of a nuclear localization sequence (NLS) or nuclear export sequence (NES) that may facilitate localization to the nucleus (group I) or cytosol (group II), respectively. DUSPs containing both sequences were grouped as phosphatases that may be localized to either compartment (group III) (26,27). Fig.3.2-3.4 illustrates comparative data from DUSPs for each group where DUSP mRNA was normalized to 18S ribosomal RNA and expressed as fold-differences relative to untreated controls. Panel A for Fig.3.2-3.4 illustrates relative difference between PAs (d0) and ADs (d8). Panels B and C depict relative changes in DUSP mRNA abundance during early and late stages of differentiation, respectively. Relative mRNA levels of dusp4 and dusp9 were significantly elevated in ADs compared to PAs, while dusp1, dusp2, dusp6, and dusp7 were significantly elevated in PAs compared to ADs. Dusp5, dusp8, dusp10, and dusp16 showed no marked differences between cell phenotypes (Fig.3.2A-3.4A). To determine which DUSPs were induced at the level of gene expression during differentiation, PAs were stimulated with MDI and relative mRNA abundance assessed by qRT-PCR over time representing early (Panel B) and late stage (Panel C) differentiation. Prior to these studies, we set an arbitrary threshold of 2.5-fold change as a conservative measure of biological differences versus technical variation as measured by qRT-PCR (46). Thus, DUSP gene expression was only considered ‘inducible’ when relative mRNA levels exceeded this threshold. Determinations were examined over time to establish when select DUSPs were induced as well as whether the induction was transient versus sustained as insight to functional roles. For characterization purposes, DUSPs were also

considered ‘suppressed’ when relative values fell below 0.5-fold differences in mRNA abundance.

As illustrated in Fig.3.2, all four DUSPs representing Group I phosphatases were rapidly and transiently induced following induction of differentiation with dusp1, dusp2, and dusp5 reaching greater than 10-fold changes in mRNA by 2 hrs post-MDI. Dusp2 and dusp5 mRNA rapidly declined with values remaining at or below baseline from 12 hrs through d10 of differentiation. While dusp1 also declined following a 45-fold peak in mRNA abundance at 2 hrs, the decline was more gradual with values remaining above baseline through d6 of differentiation. Dusp4 was unique among these phosphatases with biphasic expression with a transient, small peak (3-fold) occurring at 4 hrs and a more sustained, larger peak (7-fold) occurring at 4d and remaining above baseline through d10. While the kinetic profile of all four Group I DUSPs was consistent with the timing of MAPK dephosphorylation during early stages of differentiation, the mid-stage biphasic induction of dusp4 occurred concomitantly with the time period when ERK phosphorylation falls below baseline levels and PPARγ is expressed.

Dusp6 was the only group II phosphatase determined to be rapidly and transiently induced during early adipocyte differentiation where a 3.5-fold induction peaked at 1 hr and returned to baseline within 12 hrs post-MDI (Fig.3.3). Interestingly, dusp6 and dusp7 mRNA abundance fell markedly below baseline values from d1 through d10 of differentiation. In contrast, dusp9 displayed a reciprocal pattern of expression where mRNA fell below baseline during early stages of differentiation followed by a gradual induction during late stages remaining markedly elevated (8-fold) through d10 of differentiation. While all three Group III phosphatases were suppressed during early differentiation (Fig.3.4), dusp8 and dusp10 returned to baseline by d4 of

differentiation. Dusp16 was the only group III phosphatase transiently induced during late stages with peak mRNA abundance observed at d4 of differentiation.

Inhibition of DUSPs during early adipocyte differentiation correlates with prolonged MAPK signaling. Data presented above demonstrate that select DUSPs were induced in a manner consistent with functional roles in MAPK dephosphorylation during early stages and maintained suppression during late stages of adipocyte differentiation. To determine a role for early 'inducible' phosphatases on the extent (i.e., magnitude and duration) of MAPK phosphorylation during early stages of differentiation, PAs were pretreated with or without 1 μM triptolide (TRP) for 30 min prior to MDI stimulation. TRP is a bioactive compound purified from the Chinese herb *Tripterygium wilfordii* and shown to be effective as an immunosuppressant and anti-inflammatory agent and a potent inhibitor of dusp1 gene expression (47). RNA was isolated at 0 hr and 1 hr post-MDI in the absence or presence of TRP and expression of all DUSPs induced within 2 hrs post-MDI examined by qRT-PCR. MDI-induced gene expression of dusp1, dusp2, dusp5, and dusp6 was completely ablated in the presence of TRP (Fig.3.5A). To determine if TRP influenced the extent of MAPK phosphorylation, cell lysates were harvested over time and immunoblotted for phosphorylated ERK and p38. MDI stimulation markedly increased ERK and p38 phosphorylation. In the absence of TRP, MDI stimulation also resulted in the accumulation of dusp1 protein in a manner kinetically consistent with the dephosphorylation phase of both MAPKs, which returned to baseline by 6 hrs post-MDI (Fig.3.5B). In contrast, TRP pretreatment abolished the accumulation of dusp1 and prolonged the duration of phosphorylated ERK and p38 through 24 hrs post-MDI stimulation. While TRP also resulted in a modest increase in phospho-ERK magnitude, pretreatment of this inhibitor did not block the phosphorylation

of either MAPK by upstream MAPK kinases following MDI stimulation. Collectively, these data suggest that one or more inducible DUSP plays a functional role in modulating the extent of MAPK phosphorylation during adipocyte differentiation.

Dusp1 is induced during early adipocyte differentiation. While data presented above suggest a cooperative role for DUSPs on MAPK deactivation, discrepancies on dusp1 expression reported here and elsewhere (23,41,48) led us to further examine the regulation of this phosphatase during adipocyte differentiation. While some, including this report, show an immediate induction of dusp1 that decreases over the course of differentiation (23,48,49), others report a progressive increase in dusp1 mRNA and protein expression over the course of adipogenesis (41). Therefore, we examined dusp1 mRNA and protein expression over the complete course of adipocyte differentiation. Total RNA and whole cell lysates were collected over time and dusp1 mRNA and protein expression as well as MAPK phosphorylation examined via qRT-PCR and immunoblot analysis, respectively. Dusp1 gene expression was rapidly (<1 hr) and robustly induced post-MDI stimulation that gradually decreased over the course of differentiation (Fig.3.6). Correspondingly, dusp1 protein expression mirrored mRNA expression, with a rapid increase at 2 hr followed by a gradual decrease in protein by 8h post-MDI. Moreover, protein and mRNA abundance returned to or fell below baseline expression by d6 of differentiation. Collectively, these data clearly demonstrate an expression profile for dusp1 that was consistent with a functional role in limiting the extent of MAPK phosphorylation previously shown to be essential for adipocyte differentiation.

Dusp1 knockdown amplifies MAPK signaling during 3T3-L1 preadipocyte differentiation. To establish a mechanistic role for dusp1 on MAPK signaling, 3T3-L1

PAs were transfected with siRNA targeted to dusp1 or non-targeting control sequences for 72 h prior to stimulation with MDI. Total RNA and whole cell lysates were harvested over time and analyzed for relative dusp1 mRNA abundance and MAPK phosphorylation, respectively. Dusp1 gene expression was markedly induced during early differentiation in cells transfected with control siRNA (Fig.3.7A). In contrast, both basal (>70%) and MDI-stimulated (>60%) induction of dusp1 mRNA was significantly suppressed in cells treated with dusp1 specific siRNA. Specific knockdown of dusp1 mRNA nearly ablated the accumulation of dusp1 protein resulting from MDI stimulation (Fig.3.7B). Moreover, specific knockdown of dusp1 mRNA and protein markedly increased magnitude and duration of ERK phosphorylation as well as the magnitude of p38 phosphorylation following MDI stimulation.

DUSP1-mediated regulation of MAPK signaling is not sufficient for inhibition of adipocyte hyperplasia. Data presented above demonstrate that dusp1 regulates MAPK dephosphorylation during early adipocyte differentiation. As recent reports differ on the role of dusp1 during adipogenesis (40,41), we further examined the biological role of dusp1 on PA proliferation and differentiation as essential components of adipocyte hyperplasia. A continuum of sequential events culminates in the activation of PPARγ and C/EBPα in order to regulate adipocyte gene expression and acquisition of the mature adipocyte phenotype during adipogenesis (50-52). Among the early events leading to PPARγ and C/EBPα, is an ‘obligatory’ prerequisite period of mitotic clonal expansion, in which density-arrested PAs synchronously re-enter the cell cycle prior to activation of adipocyte-specific genes and terminal growth arrest (18). To determine if dusp1 plays a regulatory role during clonal expansion, density-arrested PAs were transfected with dusp1 specific or non-targeting siRNA for 72 hrs prior to stimulation with

MDI. Cells were harvested at 0 hr and 20 hr post-MDI, fixed, and stained with propidium iodide for flow cytometric analysis of cell cycle progression. As shown in Fig.3.8A, two DNA peaks in the 2n and 4n range, representing G0/G1 and G2/M cell populations, were present in all histograms. Stimulation with MDI resulted in similar shifts from G0/G1 to S and G2/M phase populations demonstrating synchronous cell cycle progression in cells transfected with dusp1 or control siRNA. To confirm these results, cell lysates were harvested under identical conditions and analyzed for cyclin A protein expression, which we have previously shown to accumulate during S phase in this cell model (53). Cyclin A protein accumulated with both dusp1-specific and control siRNA, with modestly elevated cyclin A protein accumulation with dusp1 knockdown (Fig.3.8B). Collectively, these data demonstrate that dusp1 does not block cell cycle progression, which is a known prerequisite for adipocyte differentiation. To the contrary, the modest increase in cyclin A following dusp1 knockdown would be indicative of more efficient S-phase entry, possibly resulting from elevated MAPK phosphorylation.

To elucidate a role for dusp1 on adipocyte differentiation, density-arrested PAs were transfected with control siRNA or dusp1 siRNA 72 hrs prior to stimulation with MDI. Phase contrast microscopy was used to assess changes in cell morphology and lipid accumulation between undifferentiated PAs (d0) and fully mature ADs (d8). As shown in Fig.3.8C, PAs (d0) transfected with siRNA for dusp1 or non-targeting sequences displayed nearly identical fibroblast-like morphology. Similarly, transfection with dusp1-specific or non-targeting sequences prior to differentiation resulted in no discernable differences in the lipid-filled morphology of fully mature ADs (d8). To determine if dusp1 expression was essential for accumulation of proteins that mediate adipocyte differentiation, cell lysates were harvested from cells transfected with dusp1 and control

sequences and immunoblotted for PPARγ, C/EBPα, and aP2 as illustrated in Fig.3.8D. PPARγ and C/EBPα represent major transcription factors that mediate the expression of functional proteins such as the fatty acid binding protein aP2 during adipogenesis. As shown in Fig.3.8D, PPARγ, C/EBPα, and aP2 increased similarly with differentiation in cells transfected with dusp1-specific or non-targeting control siRNA. Similarly, PPARγ, C/EBPα, and aP2 increased with differentiation (d8) in cells transfected with dusp1 siRNA. As dusp1 protein accumulation was markedly attenuated at 2 hr post-MDI with dusp1 siRNA, these data clearly demonstrate that knockdown of dusp1 alone was not sufficient to block adipogenesis suggesting the possibility of redundant or collective roles between multiple phosphatases in limiting the extent of MAPK phosphorylation that is essential for adipocyte differentiation.

Discussion

This report presents evidence demonstrating that the MAPK-specific DUSPs are regulated in a manner consistent with MAPK dephosphorylation during adipocyte differentiation. First, we demonstrate that the extent of MAPK signaling was dependent on de novo mRNA synthesis suggesting a role for 'inducible' phosphatases. Second, we show that several DUSPs were induced during adipocyte differentiation concomitant with dephosphorylation of ERK and p38 signaling. Third, we demonstrate that pharmacological inhibition of early inducible DUSPs markedly prolonged ERK and p38 signaling during early adipocyte differentiation. Finally, we show that while dusp1 played a regulatory role in ERK and p38 signaling magnitude and duration, dusp1 specific knockdown was not sufficient to inhibit adipocyte hyperplasia. Collectively, these data

demonstrate that cooperative actions of several DUSPs play modulatory roles in timely MAPK dephosphorylation during adipocyte hyperplasia.

Mounting evidence demonstrates that the timely phosphorylation as well as dephosphorylation of ERK and p38 is critical for adipogenesis (10,15-19), where the rapid and transient activation (i.e., phosphorylation) of ERK and p38 is essential for preadipocyte proliferation as well as the induction and activation of C/EBPβ, both of which are required for adipogenesis (18-21). Conversely, prolonged ERK activity has been shown to phosphorylate and inactivate PPARγ blocking AD differentiation (22), while prolonged p38 activity results in cell death (16,23,24). However, the extent of MAPK phosphorylation during distinct stages of adipocyte differentiation remains uncertain. Data presented here demonstrate a rapid and transient activation of ERK signaling during early adipocyte differentiation. Consistent with previous reports (10,17), these data further demonstrate that ERK phosphorylation is suppressed during mid and late stages of adipocyte differentiation falling below basal expression, concomitant with the induction of PPARγ. As prolonged phosphorylation of ERK inhibits PPARγ activity and subsequently adipogenesis (54), these data suggest that transient activation during early differentiation and suppression of ERK phosphorylation during late differentiation are necessary for the mature adipocyte phenotype. While phosphorylation of p38 during early adipocyte differentiation was similar to ERK, it displayed a biphasic pattern of induction during late stage differentiation. While some have reported suppression of p38 phosphorylation during late adipocyte differentiation (15,16), others have noted similar biphasic activity (55). As reports demonstrate that ectopic expression of p38 drives adipocyte differentiation in 3T3-L1 preadipocytes and NIH 3T3 fibroblasts (16,23,24), it remains uncertain if biphasic p38 activity or loss of activity during late differentiation is

required for adipocyte differentiation. Collectively, these observations suggest that timely phosphorylation and dephosphorylation of MAPKs is essential for adipogenesis.

Until recently, upstream kinases rather than phosphatases were viewed as major regulators of MAPK signaling. However, many phosphatases are now recognized as key modulators and even controllers of cellular signaling and biological processes involving proliferation and differentiation (56,57). This is evident in reports that demonstrate that kinases can be dephosphorylated in the continued presence of stimulation, where protein tyrosine phosphatases have been shown to control protein tyrosine kinase signaling in response to oxidative stress (56). Data presented in our report demonstrate disconnect between the phosphorylation state of ERK and its upstream kinase MEK, where ERK was completely dephosphorylated in the presence of MEK phosphorylation (i.e., activity) during early differentiation. Moreover, phospho-ERK dropped below baseline following the initial activation throughout later stages of differentiation even though MEK retained some degree of activity, suggesting a role of phosphatases in the timely modulation of MAPK signaling. Recently, DUSPs have been shown to play a critical role in MAPK dephosphorylation of myocyte, osteocyte, and neuronal cells (37-39), where the timely balance of MAPK phosphorylation and dephosphorylation dictates cellular growth and differentiation within these cell types (37,39,58). For instance, timely modulation of ERK activation is essential for myogenesis, where decreased ERK activity, concomitant with increased dusp1, is necessary for muscle-specific gene expression. While ectopic expression of dusp1 was sufficient to block inhibitory actions of ERK on muscle-specific genes, prolonged induction also blocked myogenesis, suggesting that timely modulation of phosphorylation and dephosphorylation is critical for myocyte growth and differentiation (37). As we further report that inhibition of *de novo*

mRNA synthesis markedly prolonged ERK and p38 phosphorylation, these data collectively suggest that ‘inducible’ DUSPs may account for the timely balance of MAPK phosphorylation and dephosphorylation that is critical for adipocyte differentiation.

Data presented here demonstrate that several DUSPs were induced during early, mid, and late stages of adipocyte differentiation, consistent with the dephosphorylation of ERK and p38 during early differentiation and continued suppression of ERK phosphorylation during mid and late stages of differentiation. As the kinetic patterns of the ‘inducible’ DUSPs were markedly different, it is conceivable that phosphatases play a cooperative role in the modulation of MAPK dephosphorylation. Reports now demonstrate that increased expression of immediate early DUSPs plays a key role in the dephosphorylation of MAPKs in other cell types (26,59,60). Consistent with this, we demonstrate that inhibition of the immediate early ($\leq$2hr) DUSPs led to marked increases in ERK and p38 signaling duration. Unpublished observations from our lab further demonstrate that DUSP inhibition with the bioactive compound TRP also blocked preadipocyte proliferation and differentiation in the presence of sustained MAPK phosphorylation. While TRP has been shown to be effective as an immunosuppressant and anti-inflammatory agent, these data provide convincing evidence that DUSPs cooperatively work to modulate timely MAPK signaling that is critical for adipocyte differentiation (18,47,54). As DUSPs may contribute to the transient phosphorylation of MAPKs during early differentiation, induction of DUSPs during late stage adipocyte differentiation may also play a crucial role in the continued suppression of ERK phosphorylation that is pivotal for PPARγ activity and adipogenesis (22). Evidence for this can be seen in nude mice treated with parathyroid hormone and PTH-related protein (PTHrP), in which dusp1 is upregulated followed by increases in bone formation. Loss of

dusp1 markedly increased ERK phosphorylation during late osteoblast differentiation, in which ERK dephosphorylation is required for terminal growth arrest and bone formation (58). Data presented here demonstrate that dusp4, dusp9, and dusp16 were induced during mid- and late-stage adipocyte differentiation, where ERK phosphorylation fell below basal expression of untreated PAs (d0). While some evidence suggests that ectopic expression of dusp9 blocks adipocyte differentiation (61), a role for these late stage phosphatases regarding MAPK signaling and adipogenesis remains unknown.

Dusp1 is an immediate early gene whose expression is induced rapidly and transiently by various extracellular stimuli including agents used to differentiate 3T3-L1 PAs such as insulin, glucocorticoids, and cAMP analogs (62-64). As conflicting reports regarding dusp1 during adipocyte differentiation have recently emerged (23,40,41,48,49), we report that dusp1 was induced rapidly and transiently during early stages of adipocyte differentiation where mRNA decayed slowly over the course of differentiation eventually returning to baseline by d6. While this expression profile of dusp1 is consistent with some reports in 3T3-L1 PAs (23,48,49), it is inconsistent with others (41) where it was reported to increase during late stage adipocyte differentiation. We further show that loss of dusp1 via siRNA knockdown markedly increased the magnitude and duration of ERK and p38 phosphorylation during early adipocyte differentiation, but did not block preadipocyte proliferation or differentiation. Moreover, examination of cyclin A suggests that S-phase transition was more efficient in the presence of increased MAPK signaling, consistent with the role of MAPKs on cellular proliferation (18). Consistently, ERK, JNK, and p38 phosphorylation was increased in AT of dusp1 deficient mice compared to wild-type counterparts (40), while MEFs from wild-type and dusp1 knockout mice differentiated in a similar manner demonstrating that

dusp1 is not essential for adipocyte differentiation (40). While our data demonstrate a role for dusp1 on MAPK dephosphorylation during early adipocyte differentiation, others have shown that dusp1 antisense expression in 3T3-L1 PAs led to sustained ERK activation during late differentiation and inhibition of adipogenesis (41). Collectively, these data demonstrate an essential role for dusp1 regarding MAPK dephosphorylation, although its role with differentiation remains unresolved.

In summary, data outlined here and elsewhere (10,26,57) demonstrate that the timely modulation of MAPK phosphorylation and dephosphorylation, essential for proliferation and differentiation, is regulated in part by downstream DUSPs. Data are presented demonstrating that several 'inducible' DUSPs are associated with the dephosphorylation of ERK and p38 during adipocyte differentiation. While loss of dusp1 via siRNA, alone, increased the duration and magnitude of ERK and p38 phosphorylation, it did not block adipogenesis, suggesting that DUSPs cooperatively modulate MAPK dephosphorylation and adipocyte differentiation. While further studies are needed regarding the cooperative actions of DUSPs during adipocyte differentiation, this report establishes a platform for future investigations regarding timely MAPK signaling during adipogenesis. As work on DUSP regulation and function continues, elucidation into the molecular actions of these phosphatases may provide therapeutic targets for the treatment and prevention of obesity and obesity-induced insulin resistance.

Table 3.1. DUSP genes analyzed in this study.

symbol	name/alias					accession	ABI number	C_T
dual specificity phosphatases		GP	MKB	NLS	NES			
Dusp1	MKP-1, hVH1	I	●	●		NM_013642	Mm00457274_g1	24
Dusp2	PAC1	I	●	●		NM_010090	Mm00839675_g1	32
Dusp4	MKP-2, hVH2	I	●	●		NM_176933	Mm00723761_m1	27
Dusp5	hVH3	I	●	●		NM_001085390	Mm01266104_m1	27
Dusp6	MKP-3, rVH6	II	●		●	NM_026268	Mm00650255_g1	24
Dusp7	MKP-X	II	●		●	NM_153459	Mm00463228_m1	24
Dusp9	MKP-4	II	●		●	NM_029352	Mm00512646_m1	31
Dusp8	M3/6, hVH5	III	●	●	●	NM_008748	Mm00456230_m1	32
Dusp10	MKP-5	III	●	●	●	NM_022019	Mm00517678_m1	26
Dusp16	MKP-7, MKP-M	III	●	●	●	NM_130447	Mm00459935_m1	26
reference gene								
18S	18 ribosomal RNA					X03205	4342930E	9

Table 3.1. DUSP genes analyzed in this study. DUSPs that contained the MAPK binding domain (MKB) were assigned to groups (GP) based on structural differences involving the presence or absence of a nuclear localization sequence (NLS), nuclear export sequence (NES), or both that facilitate subcellular localization to the nucleus (group I), cytosol (group II), or both compartments (group III).

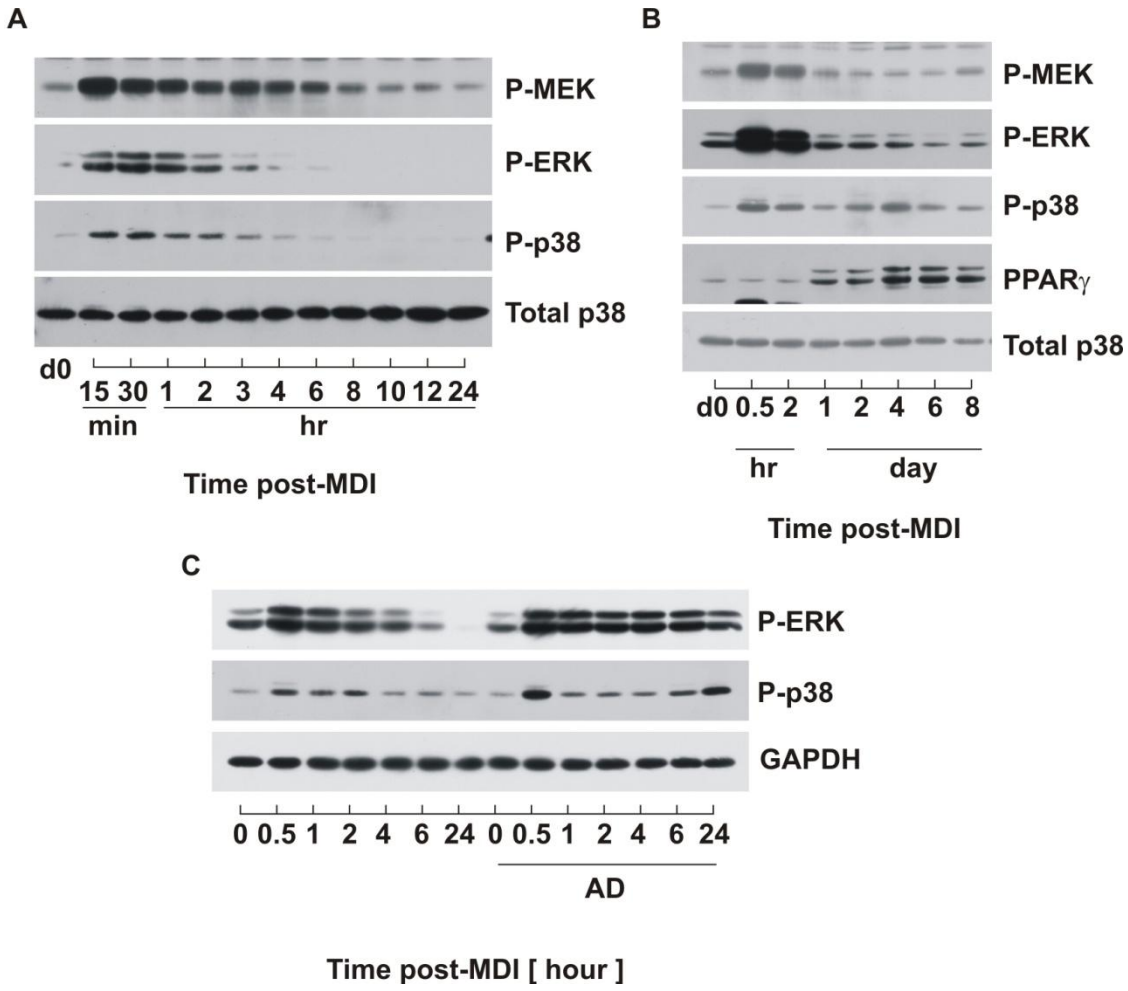

Figure 3.1. MAPK dephosphorylation is regulated by de novo mRNA synthesis. Preadipocytes were stimulated to differentiate with the hormonal cocktail MDI as outlined above and cell lysates harvested during A) early (0h-24h) and B) late differentiation (d0-d8) prior to immunoblot analysis of bisphosphorylated MEK, ERK, and p38 as well as total p38 and PPARγ. C) Preadipocytes were stimulated with MDI in the absence or presence of 1 ng/ml Actinomycin D (AD). Cell lysates were collected at indicated times and protein expression of bisphosphorylated ERK and p38 along with GAPDH analyzed via immunoblotting.

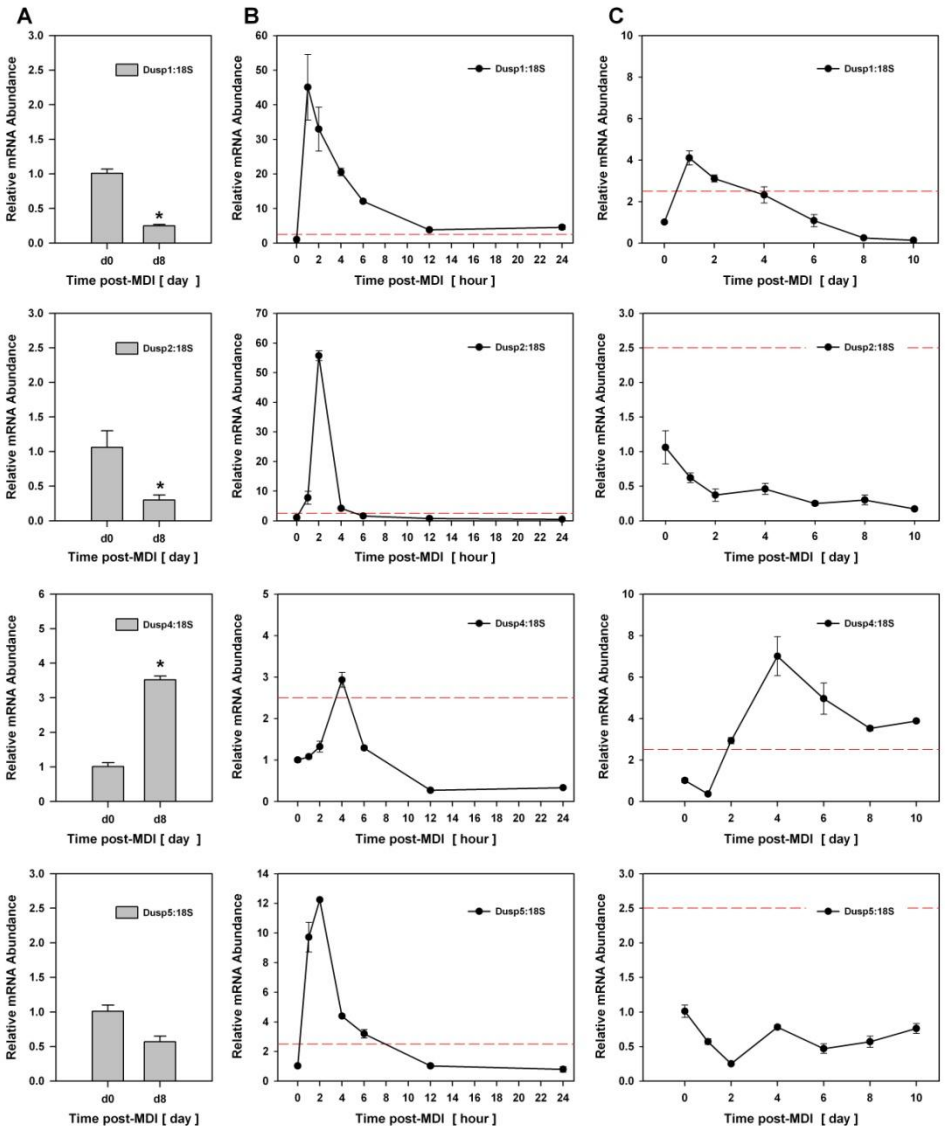

Figure 3.2. Induction of group I DUSP mRNA during adipocyte differentiation. A) Total RNA was harvested from preadipocytes (d0) and adipocytes (d8) and mRNA expression of group I DUSPs measured using qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated preadipocytes (d0). Statistical significance was determined by Student's *t*-test (***** $p<0.05$). Preadipocytes were stimulated to differentiate and total RNA was harvested during B) early and C) late differentiation prior to mRNA analysis of nuclear DUSPs with qRT-PCR. All data were normalized to 18S rRNA and expressed relative to untreated cells (d0/0h). Genes selected as 'inducible' were upregulated above a 2.5-fold criterion indicated by a dashed line on the graph.

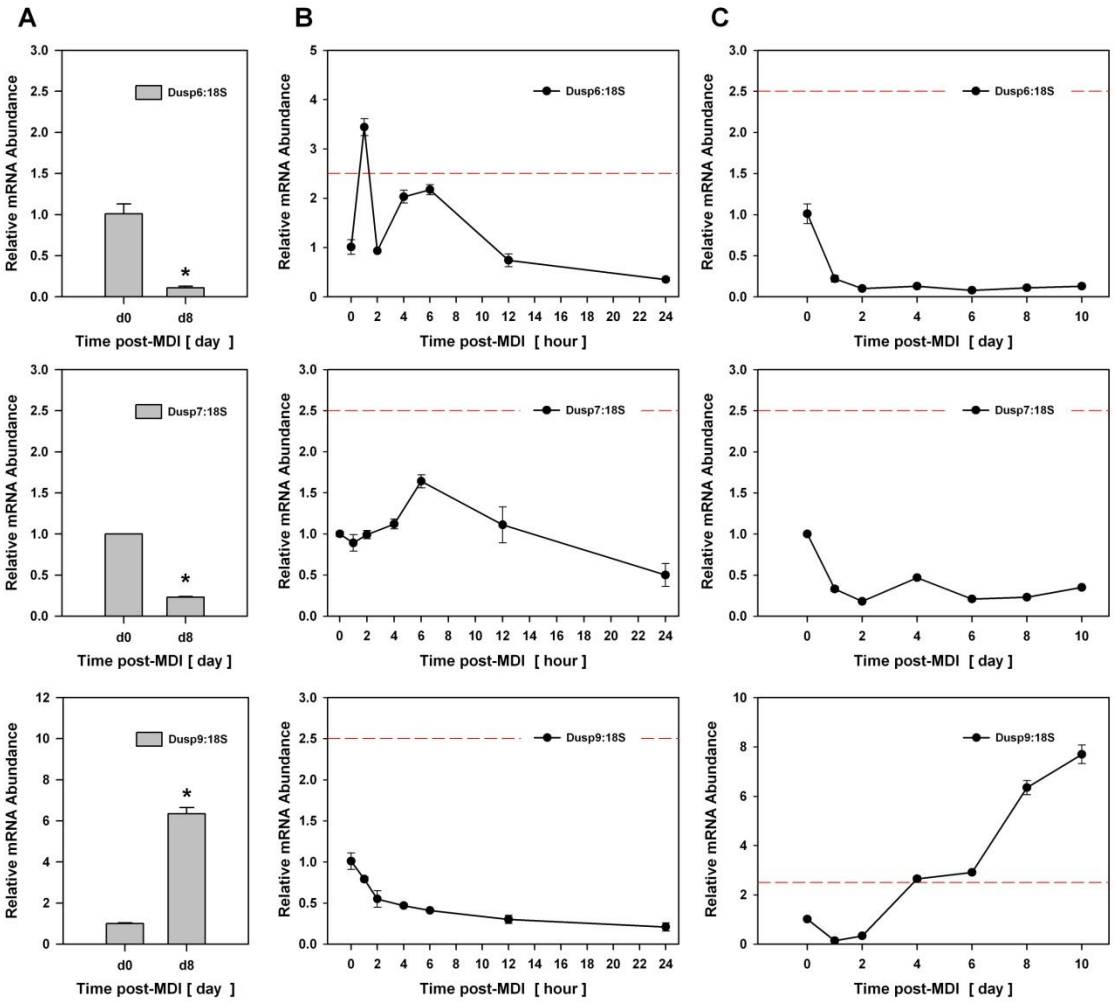

Figure 3.3. Induction of group II DUSP mRNA during adipocyte differentiation. A) mRNA expression group II DUSPs was measured in untreated preadipocytes (d0) and mature adipocytes (d8) using qRT-PCR. Data were normalized to 18S rRNA and expression measured as relative differences compared to untreated preadipocytes (d0). Statistical significance was determined by Student's *t*-test (***** $p<0.05$). Preadipocytes were stimulated to differentiate and total RNA was harvested during B) early and C) late differentiation prior to mRNA analysis of cytosolic DUSPs with qRT-PCR. All data were normalized to 18S rRNA and expressed relative to untreated cells (d0/0h). Genes selected as 'inducible' were upregulated above a 2.5-fold criterion indicated by a dashed line on the graph.

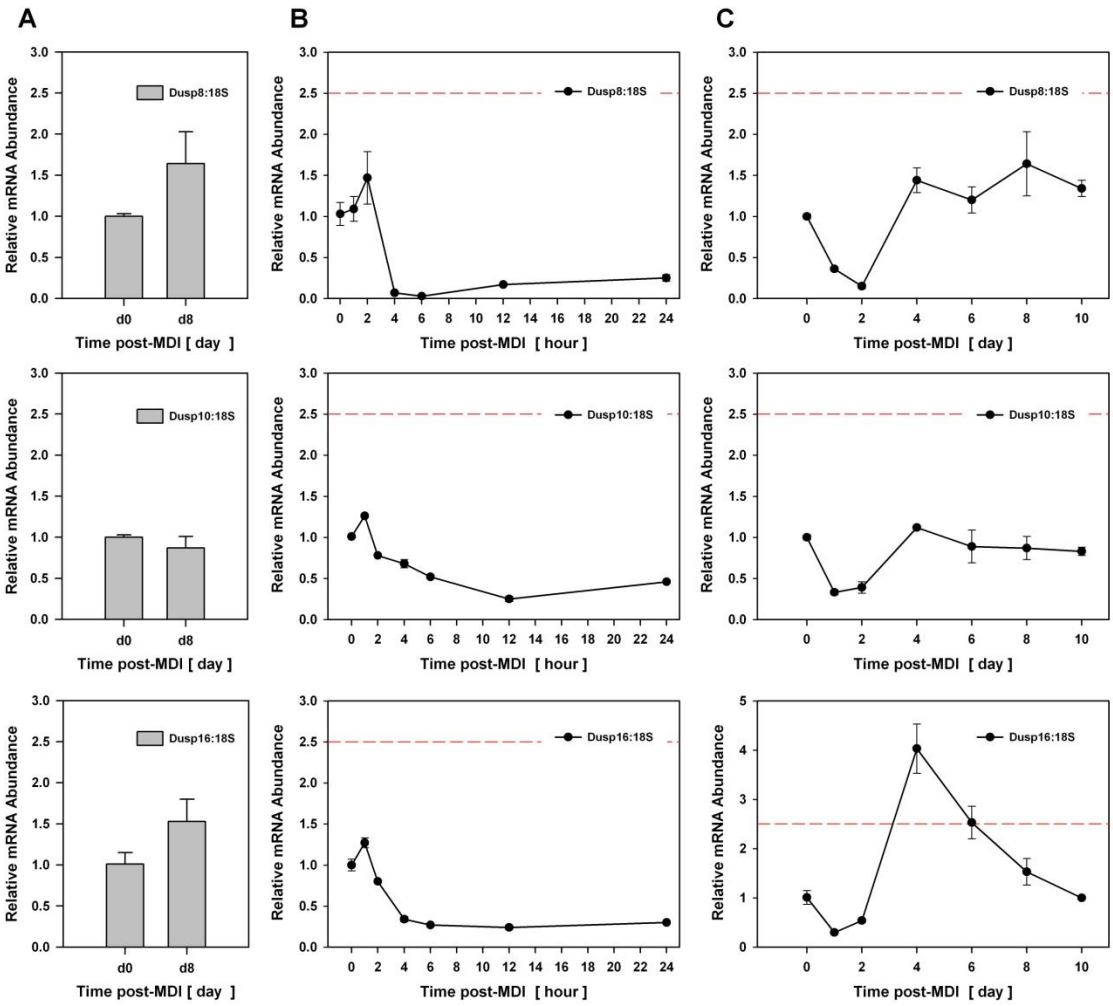

Figure 3.4. Regulation of group III DUSP mRNA during adipocyte differentiation. A) Total RNA was harvested from preadipocytes (d0) and mature adipocytes (d8) and mRNA expression of group III DUSPs measured using qRT-PCR. Data were normalized to 18S rRNA and expression measured as relative differences compared to untreated preadipocytes (d0). Significance was determined by Student's *t*-test (***** $p<0.05$). In addition, preadipocytes were stimulated to differentiate and total RNA harvested during B) early and C) late differentiation prior to mRNA analysis of cytosolic/nuclear DUSPs with qRT-PCR. All data were normalized to 18S rRNA and expressed relative to untreated cells (d0/0h). Genes selected as 'inducible' were upregulated above a 2.5-fold criterion indicated by a dashed line on the graph.

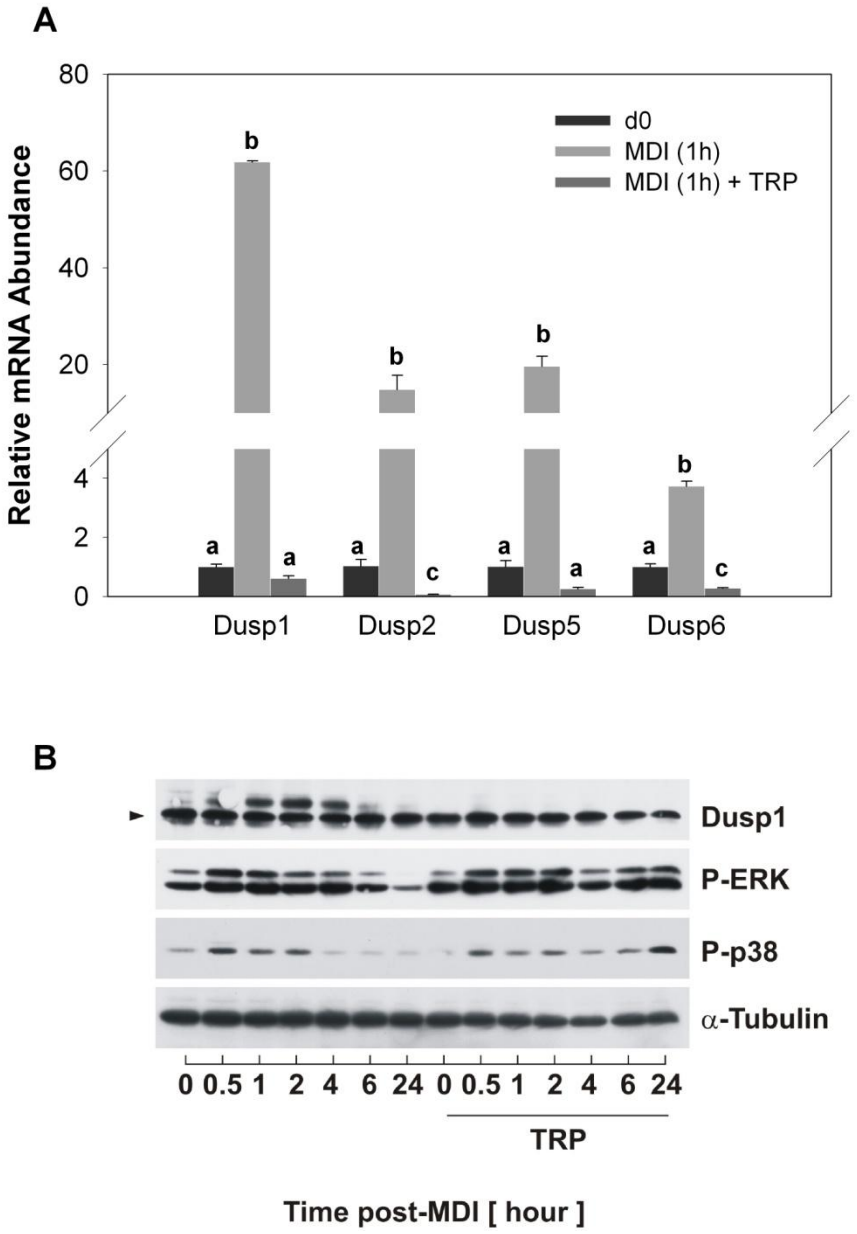

Figure 3.5. Inhibition of DUSPs enhances ERK and p38 phosphorylation during early adipocyte differentiation. Preadipocytes were stimulated to differentiate with MDI in the presence or absence of 1 μM triptolide (TRP) pretreatment (30 min). A), RNA was isolated at 0h and 1h post-MDI stimulation and mRNA expression of dusp1, dusp2, dusp5, and dusp6 analyzed via qRT-PCR. Data were normalized to 18S rRNA and changes in gene expression measured as fold differences relative to untreated preadipocytes (0h). Statistical differences were determined by ANOVA. Tukey's post-hoc analysis was performed when the *p* value for the respective parameter was statistically significant ($p < 0.05$). B) Cell lysates were collected over time post-MDI stimulation and protein expression of bisphosphorylated ERK and p38 along with dusp1 and α-Tubulin examined via immunoblotting.

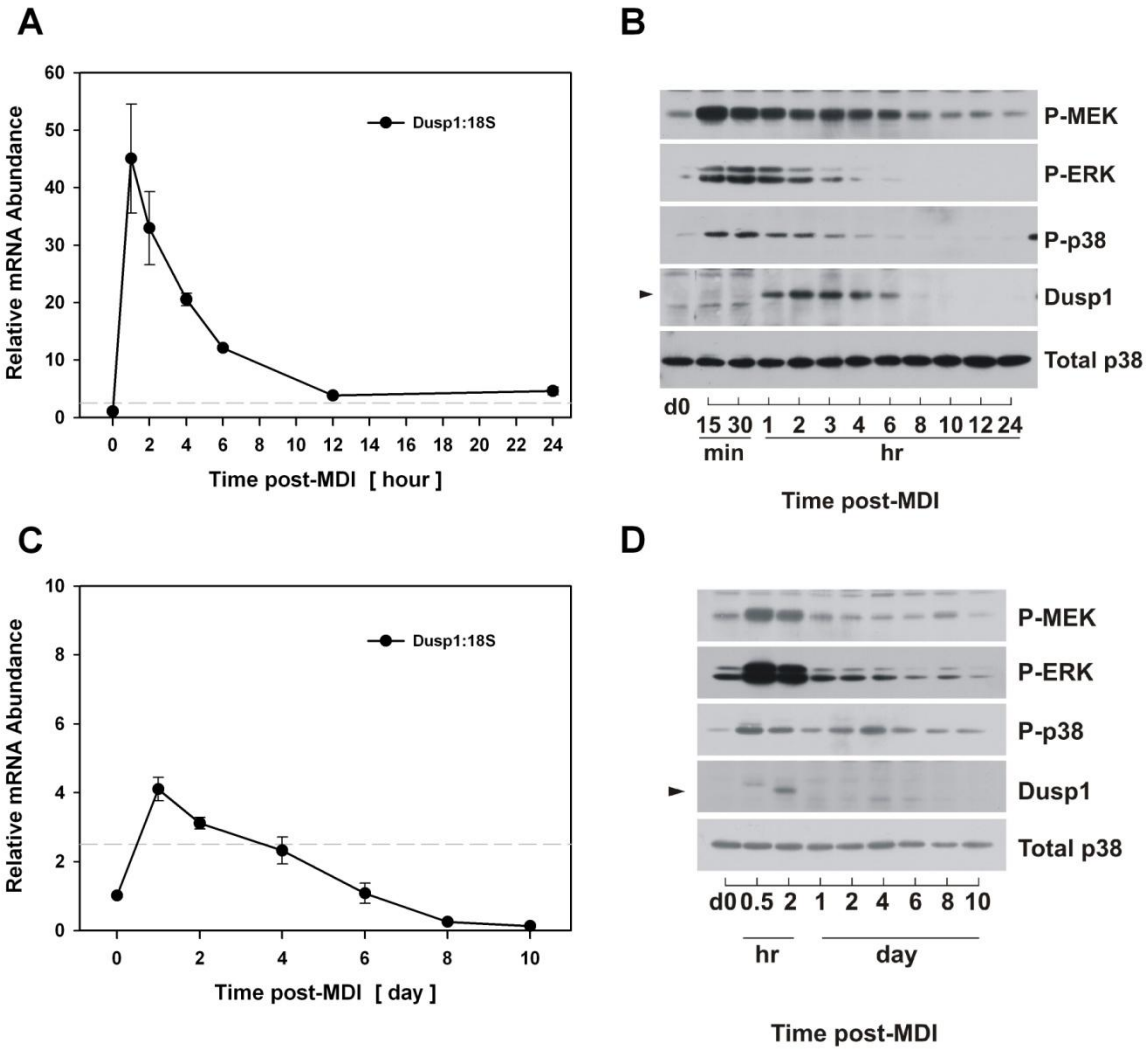

Figure 3.6. Dusp1 kinetically follows MAPK dephosphorylation during early adipocyte differentiation. Preadipocytes were stimulated to differentiate with MDI and RNA isolated during A) early and C) late adipocyte differentiation prior to mRNA analysis of dusp1 via qRT-PCR. Data were normalized to 18S rRNA and expressed as relative differences compared to untreated preadipocytes (d0). Dusp1 gene expression was considered inducible when upregulated above a 2.5 fold criterion indicated by a dashed line. Cell lysates were harvested during B) early and D) late adipocyte differentiation prior to immunoblot analysis of bisphosphorylated MEK, ERK, and p38 as well as dusp1, and total p38 expression.

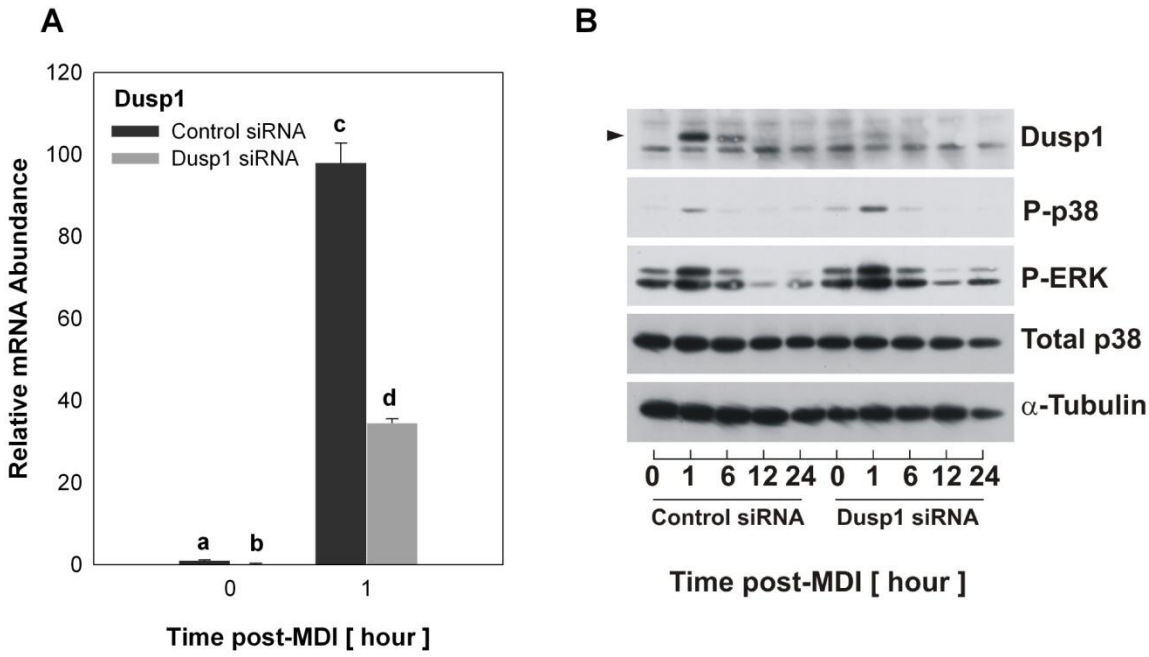

Figure 3.7. Dusp1 knockdown markedly increases ERK and p38 signaling magnitude and duration during early adipocyte differentiation. Preadipocytes were transfected with DharmaFect 3 transfection reagent in the presence of non-targeting control siRNA or siRNA specific for dusp1 for 72 hours prior to stimulation with MDI. A) Cell lysates were harvested for RNA at 0h and 1h post-MDI stimulation and mRNA expression of dusp1 analyzed via qRT-PCR. Data were normalized to 18S rRNA and changes in gene expression measured as fold differences relative to untreated control siRNA (0h). Statistical differences were determined by ANOVA. Tukey's post-hoc analysis was performed when the *p* value for the respective parameter was statistically significant ($p < 0.05$). B) Cell lysates were collected over time post-MDI stimulation for control siRNA and dusp1 siRNA and protein expression of bisphosphorylated ERK and p38 along with dusp1, total p38, and α-Tubulin examined via immunoblotting.

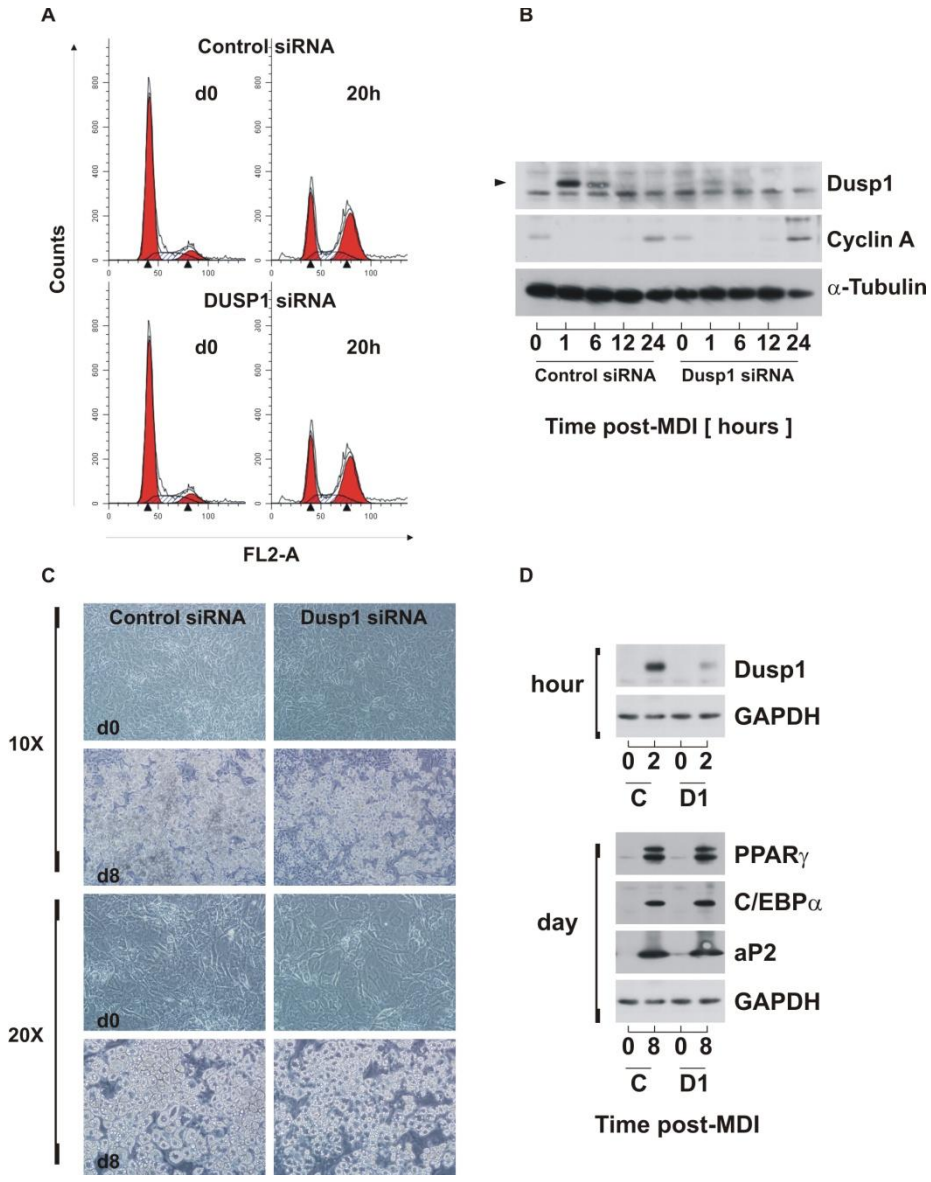

Figure 3.8. Dusp1 knockdown does not inhibit 3T3-L1 adipogenesis. Preadipocytes were transfected with non-targeting control siRNA or siRNA specific for dusp1 for 72 hrs prior to stimulation with MDI. A) Cells were fixed at d0 and 20h post-MDI and DNA stained with propidium iodide. DNA histograms were assessed via flow cytometry. B) Cell lysates were collected over time and protein expression of dusp1, cyclin A, cyclin D1, and α-Tubulin examined via immunoblotting. C) Examination of cell morphology of untreated preadipocytes and mature adipocytes treated with control siRNA or dusp1 siRNA was assessed for lipid accumulation using phase contrast microscopy at 10X and 20X magnitude. D) Cell lysates were collected from preadipocytes (d0) and mature adipocytes (d8) treated with control siRNA (C) or dusp1 siRNA (D1) and protein expression of adipocyte genes PPARγ, C/EBPα, and aP2 examined via immunoblot analysis.

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CHAPTER IV

ROLE FOR DUAL-SPECIFICITY PHOSPHATASES ON MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING IN ADIPOCYTES IN RESPONSE TO INFLAMMATORY STRESS

Abstract

Obesity is a major risk factor for heart disease, stroke, insulin resistance, and type 2 diabetes. Early evidence demonstrated an essential role for the proinflammatory cytokine tumor necrosis factor α (TNFα) during obesity and obesity-induced insulin resistance (IR). Since then, reports have established mitogen-activated protein kinases (MAPKs) as major signaling pathways linking inflammation to obesity-induced IR. While upstream kinases were traditionally viewed as major regulators of MAPK signaling, many phosphatases are now recognized as key modulators and even controllers of cellular signaling and biological processes involving inflammation. Using 3T3-L1 adipocytes, we examined a role for dual-specificity phosphatases (DUSPs) on TNFα-induced activation of three major MAPKs, ERK, JNK, and p38. We report that all three MAPKs examined were phosphorylated in preadipocytes (PAs) and adipocytes (ADs) in response to TNFα, where signaling magnitude and duration were phenotype-specific. We observed that MAPK dephosphorylation included mechanisms involving de novo mRNA synthesis, suggesting a role for 'inducible' phosphatases. We further report that mRNA abundance of several DUSPs was induced in PAs and ADs in response to TNFα, concomitant with dephosphorylation of all three MAPKs. Moreover, we demonstrate that RNA interference (RNAi)-mediated DUSP knockdown of dusp1, dusp8,

and dusp16 led to a marked increase in signaling magnitude and duration of ERK, JNK, and p38 that subsequently resulted in significant increases in MAPK-dependent mRNA expression of MCP-1, IL-6, and Cox-2 in response to TNFα. Collectively, this study demonstrates cooperative actions of DUSPs on MAPK dephosphorylation and regulation of MAPK-dependent inflammation, highlighting the therapeutic potential of DUSPs on MAPK-dependent processes involved in adipose tissue (AT) inflammation and IR.

Introduction

Obesity is an irrefutable risk factor for the development of type 2 diabetes mellitus which develops following prolonged IR predominantly in liver, muscle, and AT (1). While the mechanistic link between obesity and systemic IR is a matter of ongoing investigation, there is mounting evidence demonstrating that AT dysfunction plays a central role in the sequelae of whole-body metabolic changes that result in the loss of insulin sensitivity (1,2). Central to this argument is the development of obesity-induced chronic, low grade inflammation within AT that leads to localized IR with ensuing elevated plasma levels of proinflammatory cytokines and free fatty acids that impart peripheral IR and altered glucose homeostasis in liver and muscle (1-3). Molecular mechanisms that underlie the initiation of AT inflammation during the onset of obesity include the expression and release of various chemokines, such as monocyte chemoattractant protein-1 (MCP-1), that promote infiltration of macrophages that are considered to be the primary source of inflammatory mediators of IR (1,4).

Tumor necrosis factor α (TNFα) is well established in a causal role responsible for adipocyte IR within targeted AT (3,5). This proinflammatory cytokine is expressed in macrophages as well as in cells of adipocyte lineage as a 26 kDa transmembrane

monomer that is enzymatically cleaved to a 17 kDa soluble form of TNFα. Both forms of TNFα have been shown to exert biological activity suggesting that this cytokine may impinge upon insulin sensitivity through autocrine, paracrine, or endocrine modalities (5,6). TNFα has been shown to suppress insulin signaling, alter glucose homeostasis, promote lipolysis, and inhibit lipogenesis in cultured adipocytes (1,3,6,7). Regarding a role in metabolic disease, others have reported that TNFα is elevated in AT of obese subjects and that genetic ablation of TNFα action can restore insulin sensitivity in vitro and in vivo (1,6,8). While the contribution of AT to circulating levels of TNFα has been debated, there is consensus that AT-derived TNFα can impact whole-body insulin sensitivity by impairing AT function through localized IR as well as through TNFα-mediated effects on proinflammatory gene expression (1,2).

Metabolic effects of TNFα are predominantly mediated through nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways involving extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. All four pathways have been shown to be activated in AT in response to TNFα stimulation and serve as mediators of localized IR through transcriptional mechanisms involving adipocyte gene expression as well as the expression and secretion of other proinflammatory molecules (2,9-12). In addition to nuclear events involving AP-1 mediated gene expression, MAPK activity has also been linked to cytosolic suppression of proximal insulin signaling through JNK phosphorylation of specific serine residues of IRS-1 demonstrating that compartmentalized signaling events can impact different properties of AT dysfunction (12-14).

It is well recognized that all MAPK family members are activated when both threonine and tyrosine residues of the (T-X-Y) motif within the activation loop are

phosphorylated (10,15). While upstream MAPK kinases have historically been considered the principal regulators of MAPK activity, recent literature has pointed to phosphatases as powerful and even dominant controllers of many MAPK-dependent biological processes (16,17). Dual specificity phosphatases (DUSPs) are a subclass of protein tyrosine phosphatases that specifically inhibit MAPK activity through dephosphorylation of both threonine and tyrosine residues. Within this subclass, there are ten known DUSPs that are MAPK-specific as they contain a MAP kinase-binding (MKB) domain that selectively imparts catalytic activity toward ERK, JNK, or p38 (17,18). These MAPK-specific DUSPs are further classified based on nuclear localization sequences (NLS) as well as nuclear export sequences (NES) that may direct DUSP localization and activity to the nucleus, cytosol, or both. DUSPs are highly inducible at the level of gene expression in a cell type and stimulus-dependent manner (17,18). For immune cells, these stimuli commonly include cytokines, reactive oxygen species, and pathogen-associated molecules that engage toll-like receptors (17).

In this report, we examined the impact of DUSPs on experimental outcome during TNFα-mediated inflammatory stress using 3T3-L1 murine adipocytes. This cell line provided the opportunity to examine nearly pure populations of undifferentiated preadipocytes as well as mature adipocytes representing cellular effectors of AT dysfunction without macrophage contamination. This study identifies individual DUSPs as key regulators of inflammatory stress in adipocytes and highlights the potential for therapeutic control of MAPK-dependent biological processes associated with AT inflammation.

Materials and Methods

Materials: Dulbecco's Modified Eagle's Medium (DMEM), calf bovine serum, and trypsin-EDTA were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from HyClone. The following antibodies were used for western blot analysis: phospho-ERK, phosho-p38, phosphor-JNK, total JNK, total p38, α-Tubulin, and GAPDH (Cell Signaling). Pharmacological inhibitors of ERK (U0126), JNK (SP600125), p38 (SB203580), and JNK-VIII were purchased from CalBiochem. Enhanced chemiluminescence (ECL) reagents were obtained from Perkin-Elmer Life Sciences. All Taqman primer probes (Table 4.1) used in this study were purchased from Applied Biosystems. The murine 3T3-L1 cell line was purchased from Howard Green, Harvard Medical School (19).

Cell Culture: 3T3-L1 preadipocytes were propagated in DMEM supplemented with 10% CS until density-induced growth arrest, as previously described (20). At 2 days post-confluence, growth medium was replaced with DMEM supplemented with 10% FBS, 0.5mM 1-methyl-3-isobutylxanthine, 1 µM dexamethasone, and 1.7 µM insulin (MDI). Throughout the study, 'time 0' refers to density arrested cells immediately before the addition of MDI to the culture medium. Experiments described herein were conducted in density-arrested preadipocytes (d0) and mature adipocytes (d8) stimulated in parallel with 100 pM TNFα. All experiments were repeated at least 3 times to validate results and ensure reliability.

Immunoblotting: Cell monolayers were washed with phosphate-buffered saline (PBS) and scraped into ice-cold lysis buffer containing 1.0 M Tris, pH 7.4, 150 mM NaCl, 1% Triton X, 0.5% Nonidet P-40 (NP40), 1 mM EDTA, 1 mM EGTA, and 10 mM *N*-

ethylmaleimide. Phosphatase (20 mM ß-glycerophosphate, 10 mM NaFl, and 2 µM sodium vanadate), as well as protease (0.3 µM aprotonin, 21 µM leupeptin, 1 µM pepstatin, 50 µM phenanthroline, and 0.5 µM phenylmethylsulfonyl fluoride) inhibitors were added to lysis buffer prior to cell harvest. Cell lysates were sonicated, centrifuged (13,000 x *g*, 10 min, 4°C), and the supernatant transferred to a fresh tube. Bicinchoninic acid assay (Pierce, Rockford, IL) was used to determine protein concentration. Cell lysates were resuspended in loading buffer containing 0.25 M Tris, pH 6.8, 4% sodium dodecyl sulfate (SDS), 10% glycerol, 10% dithiothreitol, and 0.01% bromophenol blue and heated for 5 min at 80°C. Proteins were resolved on SDS-polyacrylamide gel electrophoresis gels (PAGE) and transferred to polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA). After transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4°C. Membranes were subsequently probed with horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Immunoblots were immersed in ECL and visualized by autoradiography using CL-XPosure film (Pierce).

Real-Time qRT-PCR: Total RNA was extracted and genomic DNA contamination was removed using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's protocol. Total RNA was quantified with a Nanodrop ND-1000 spectrophotometer and reverse-transcribed to cDNA in a 10 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems). The reverse transcription (RT) master mix containing RT buffer, deoxyribonucleotide triphosphate (dNTP) mix, RT random primers, RNase inhibitor (1.0 U/µl), and MultiScribe RT was added to 1 µg RNA and RNase-free water. Reverse transcription reaction conditions followed the protocol (25°C for 10 min, 37°C for 120 min, 85°C for 5 sec, followed by 4°C indefinitely/RT complete) and utilized

the Gene Amp PCR System 9700 thermal cycler (Applied Biosystems) for cDNA synthesis.

PCR amplification was run utilizing the 7500 fast system (Applied Biosystems) that consisted of enzyme activation at 95°C for 20 sec, followed by 40 cycles of denaturation at 95°C for 3 sec combined with annealing/extension at 60°C for 30 sec. All data were analyzed with the ABI 7500 real time PCR system (Applied Biosystems). Data were recorded and analyzed with Sequence Detector Software (Applied Biosystems) and graphs visualized with SigmaPlot software. All data were presented as mean ± standard error of the mean (SEM) and representative of at least two experiments performed in duplicate. Data were normalized to 18S previously validated by this lab as a suitable reference gene under these experimental conditions (21). Relative differences between treated and untreated control samples were analyzed by the $2^{-\Delta\Delta C_T}$ method as previously described (21,22).

Statistical analyses were conducted using SPSS v18. Phenotypic differences were determined via student's *t*-test where a p-value of <0.05 was considered significant. Knockdown data were analyzed using ANOVA, with Tukey's post-hoc analysis used when the *p* value for the respective parameter was statistically significant ($p < 0.05$). Inhibitor data were analyzed using analysis of variance, with Dunnett's post-hoc analysis conducted to assess differences from controls (TNFα) when $p<0.05$.

RNA Interference. SMARTpools containing four different short interfering RNAs (siRNAs) for dusp1, dusp8, and dusp16 specific sequences as well as non-targeting sequences were transfected using DharmaFect 3 transfection reagent according to manufacturer's (Dharmacon) protocol. Briefly, 3T3-L1 preadipocytes were propagated in

6-well culture dishes until reaching density-induced growth arrest. Growth medium was then replaced with DMEM supplemented with 10% CS, 3 µl DharmaFect 3 reagent and either 100 nM dusp1, dusp8, and/or dusp16 specific siRNA or non-targeting siRNA for 72 hr. Growth medium was subsequently switched to differentiation medium containing MDI as described above.

Results

Phenotypic differences between preadipocytes and adipocytes regarding TNFα-mediated changes in magnitude and duration of MAPK activity. AT is comprised of lipid-laden, insulin-sensitive adipocytes (ADs) and a stromovascular fraction containing numerous metabolically relevant cell types including undifferentiated adipocyte precursor cells known as preadipocytes (PAs), as well as smooth muscle cells, endothelial cells, fibroblasts, leukocytes, and macrophages (1). The onset of obesity is associated with marked AT recruitment of macrophages that likely represent the major source of elevated TNFα that imparts AT dysfunction by increasing proinflammatory cytokine and chemokine gene expression in PAs and insulin resistance in ADs (1). As PAs and ADs are both responsive to TNFα action, we examined the effect of TNFα stimulation on MAPK activity in these functionally diverse cell types of adipocyte lineage. This phenotypic comparison of MAPK activity was accomplished using the 3T3-L1 murine adipocyte cell line that yielded undifferentiated, quiescent PAs prior to differentiation and greater than 90% conversion to mature ADs following differentiation. Analyses were cell type-specific as this well-established cell line was devoid of other cell types (e.g., macrophages) that comprise AT. Cells were differentiated by established protocol for 8 days to yield functionally mature ADs and compared to undifferentiated,

density-arrested PAs of similar passage. PAs and ADs were stimulated concurrently with increasing concentrations of murine TNFα. Total cell lysates were harvested at 15 mins post-TNFα stimulation and assayed for MAPK activity by immunoblotting with phosphospecific antibodies that recognize ERK, JNK, and p38 only when bisphosphorylated on both residues that are known to be both essential and sufficient for catalytic activity (10). As illustrated in Fig.4.1A, the magnitude of phosphospecific protein accumulation representing all three MAPKs increased in a concentration-dependent fashion in both PAs and ADs, with an estimated ED_{50} of approximately 100 pM. This concentration was selected for further analyses as it closely approximates the concentration of TNFα in human and animal models of obesity and has been shown to suppress insulin signaling in culture (3,6). By comparison, the 'magnitude' of MAPK accumulation was markedly more robust in PAs relative to ADs for any given concentration. This was especially noted for p38 where ADs were nearly refractory to TNFα stimulation. To compare phenotypic difference in the 'duration' of MAPK signaling, PAs and ADs were stimulated with 100 pM TNFα in parallel and lysates harvested over time and immunoblotted for ERK, JNK, and p38 phosphorylation. As shown in Fig.4.1B, TNFα stimulation resulted in a rapid, but transient activation of all three MAPKs in PAs where robust phosphorylation was observed at 15 mins with complete dephosphorylation by 60 mins post-TNFα. Conversely, the same concentration of TNFα produced markedly lower magnitude, but sustained duration of JNK and ERK with no phosphorylation observed for p38 in mature ADs. As the abundance of total p38 protein was not affected by TNFα, rapid changes in magnitude and duration regarding MAPK phosphorylation likely reflected a balance of kinase and phosphatase mechanisms that were regulated in a phenotype-specific manner.

Regulation of MAPK de-phosphorylation by mechanisms involving de novo mRNA synthesis. Data presented above demonstrated that TNFα stimulation resulted in robust and transient phosphorylation of all three MAPKs in a cell type-specific manner. As each kinase was rapidly dephosphorylated in the presence of TNFα, these data supported the premise that MAPK phosphatases modulate magnitude and/or duration of MAPK activity. As many MAPK phosphatases have been classified as immediate early genes capable of rapid changes in gene expression (17), we developed the working hypothesis that 'inducible' expression of DUSPs mediates MAPK dephosphorylation in response to inflammation. To test this hypothesis, we initially determined if magnitude and/or duration of MAPK signaling were dependent on de novo mRNA synthesis. These determinations were performed in PAs as all three MAPKs were robustly and transiently phosphorylated and dephosphorylated in this cell type. With this reasoning, PAs were pretreated with and without actinomycin D for 30 mins prior to TNFα stimulation at a concentration (1 ng/ml) previously shown to effectively block RNA synthesis (23,24). Cell lysates were harvested over time following 100 pM TNFα stimulation and immunoblotted for ERK, JNK, and p38 phosphorylation. As illustrated in Fig.4.2, preventing de novo RNA synthesis resulted in marked increases in both magnitude and duration of ERK and JNK phosphorylation extending from less than 60 mins in the absence of actinomycin D to more than 2 hrs when RNA synthesis was inhibited. In contrast, while the magnitude of p38 phosphorylation was increased in the presence of actinomycin D, no effect on duration was observed. These effects were not attributed to generalized stress as actinomycin D treatment for more than 4 hrs in the absence of TNFα produced no increase in MAPK phosphorylation (data not shown). It is important to note that while rapid MAPK dephosphorylation was dependent on de novo mRNA synthesis, initial

MAPK phosphorylation was completely unaffected by actinomycin D. Collectively, these data suggested that the magnitude and duration of MAPK phosphorylation was modulated by a balance of upstream kinases and downstream 'inducible' phosphatases.

Cell type-specific induction of select DUSP gene expression following TNFα stimulation. As the extent of MAPK phosphorylation was dependent on de novo mRNA synthesis, we tested our hypothesis that inducible DUSPs modulate MAPK activity following TNFα stimulation in a phenotype-specific manner. For this, we initially screened unstimulated PAs for mRNA abundance of all known DUSPs that contain MKB domains responsible for selective catalytic activity only toward MAPKs. As illustrated in Table 4.1, all ten DUSPs were easily measured by qRT-PCR within the detectable limits of 36 threshold cycles (C_T) with dusp1, dusp6, and dusp7 most abundantly and dusp8 and dusp9 least abundantly expressed. This screen was extended to compare differences in basal DUSP expression between unstimulated PAs and ADs. DUSPs were grouped based on structural differences involving the presence or absence of NLS and NES sequences that may dictate subcellular localization to the nucleus (group I) and cytosol (group II), respectively (Table 4.1). DUSPs that contain both NLS and NES sequences were grouped as phosphatases that could localized to either compartment (group III) (17). DUSP mRNA abundance was normalized to 18S ribosomal RNA and expressed relative to the value observed in PAs. As illustrated in Fig.4.3, relative mRNA abundance of dusp4 and dusp9 was significantly elevated in ADs compared to PAs. Dusp1, dusp2, dusp6, and dusp7 were significantly elevated in PAs compared to ADs. Dusp5, dusp8, dusp10, and dusp16 showed no marked differences in basal expression between these cell types.

To determine which DUSPs were induced at the level of gene expression during inflammatory stress, PAs and ADs were stimulated in parallel with 100 pM TNFα and relative mRNA abundance assessed by qRT-PCR over time. Prior to these studies, we set an arbitrary threshold of 2.5-fold change (dashed line) as a conservative measure of biological differences versus technical variation as measure by qRT-PCR (25). Thus, DUSP gene expression was only considered 'inducible' when relative mRNA levels exceeded this threshold. Determinations were examined over time to establish when select DUSPs were induced as well as whether the induction was transient versus sustained as insight to functional roles. As illustrated in Fig.4.4, Dusp1 was the only group I phosphatase induced in PAs with a 20-fold transient increase in relative gene expression peaking at 1 hr post-TNFα kinetically consistent with a functional role in the rapid dephosphorylation of MAPK as shown in Fig.4.1B. In addition, dusp8 and dusp16 (group III) were rapidly induced in PAs, but in contrast to dusp1, mRNA levels remained elevated ≥4h following TNFα stimulation suggesting the possibility of divergent roles in mediating MAPK magnitude and duration. Parallel determinations in ADs revealed that dusp1, dusp5, and dusp8 were also induced within 2 hrs post-TNFα stimulation. It should be noted, however, that while dusp8 met our criterion for induction in both cell types, the magnitude of induction was dramatically greater in PAs versus ADs. No member of group II phosphatases was induced following TNFα stimulation in either cell type. It is also important to note that while there were similarities between cell types, there were marked difference such as the induction of dusp5 in ADs, but not PAs and the induction of dusp16 in PAs, but not ADs. While this study was focused on understanding the role of inducible DUSPs during inflammatory stress, these data further demonstrated that select DUSPs were suppressed following TNF stimulation. Using a

criterion of 0.5 fold changes in mRNA abundance as an arbitrary threshold for gene suppression (dotted line), mRNA levels for dusp2 and dusp4 of group I phosphatases were markedly suppressed in both cell types where the degree of suppression was sustained for more than 24 hrs. Dusp9 (group II) was also suppressed, but transiently and only in ADs, not PAs.

Dusp1, dusp8, and dusp16 modulate MAPK phosphorylation following TNFα stimulation. Data presented above demonstrated that select DUSPs were induced in a manner that would be consistent with divergent regulatory roles in modulating the extent of MAPK phosphorylation in a cell type-specific manner. As dusp1, dusp8, and dusp16 were induced in PAs following transient phosphorylation of all three MAPKs, we focused additional effort in this study toward understanding the mechanistic role and regulation of these DUSPs in PAs only. To determine a mechanistic role for these DUSPs on MAPK signaling, PAs were transfected with short interfering RNA (siRNA) targeted to either dusp1, dusp8, or dusp16 as well as non-targeting control siRNA for 72 hrs prior to stimulation with 100 pM TNFα. Total RNA and whole cell lysates were harvested over time and analyzed for relative mRNA abundance and phosphorylated MAPK protein, respectively. Targeted siRNA dramatically suppressed TNFα-mediated induction of each DUSP to 70-85% of that observed for non-targeting control siRNA (Fig.4.5A, C, E). Moreover, 72 hr exposure to targeted siRNA decreased basal dusp1 and dusp16, but not dusp8 mRNA. Fig.4.5(B,D,F) depicts the effect of mRNA knockdown for each individual DUSP on the phosphorylation state of MAPK from protein harvested from the same experiments. As illustrated, suppression of dusp1 mRNA increased basal levels of phosphorylated ERK and JNK as well as magnitude of TNFα-mediated JNK phosphorylation through 1 hr post-stimulation. While

dusp8 siRNA had no discernable effect on basal phoshorylation, targeted knockdown markedly increased the duration of ERK and JNK through 4 hrs post-TNFα stimulation. Dusp16 siRNA yielded only a modest increase in magnitude of ERK and JNK (Fig.4.5F). No discernable effects were noted regarding p38 phosphorylation for dusp1, dusp8, or dusp16. As individual DUSP knockdown demonstrated a functional role regarding MAPK signaling, we further investigated the cooperative action of inducible DUSPs on MAPK dephosphorylation. For this, PAs were transfected with pools of oligonucleotides containing siRNAs for each DUSP 72 hrs prior to TNFα stimulation. As illustrated in Fig.4.6A, pooled siRNA for dusp1, dusp8 and dusp16 attenuated TNFα-mediated mRNA induction for each DUSP similar to that shown above for individual DUSP siRNAs. Combined targeted knockdown of all three phosphatases resulted in marked increases in the magnitude and duration of ERK, JNK, and p38 phosphorylation when compared to control (Fig.4.6B). Collectively, these data demonstrated synergistic actions of phosphatases in the regulation of MAPK signaling. The rapid and transient induction of dusp1 kinetically matched the functional effect on 'magnitude' of ERK and JNK phosphorylation while the more delayed and prolonged induction of dusp8 was consistent the functional role on 'duration' of ERK and JNK phosphorylation. Moreover, pooled siRNA had an additive effect on both magnitude and duration of all three MAPKs including p38 relative to that observed when each DUSP was targeted individually.

DUSP-modulation of MAPK phosphorylation alters TNFα-mediated proinflammatory gene expression. Data presented above demonstrated that induction of select DUSPs modulated both magnitude and duration of MAPK phosphorylation. To determine if these effects on MAPK activity resulted in MAPK-dependent changes in biological function, we examined changes in mRNA abundance over time following

TNFα stimulation of four proinflammatory genes known to be expressed in adipocytes under conditions of inflammatory stress. As illustrated in Fig.4.7A, TNFα stimulation resulted in rapid and transient changes in MCP-1, IL-6, Cox-2, and TNFα gene expression in density-arrested PAs. To determine a role for MAPK activity regarding these changes in gene expression, PAs were pretreated with U0126, SP600125, and SB203850, representing potent and selective inhibitors of ERK, JNK, and p38, respectively, for 1 hr prior to stimulation with TNFα. Total RNA was harvested after 2 hrs post-TNFα stimulation corresponding to the time of peak induction for all four proinflammatory genes. As shown in Fig.4.7B, TNFα induction of MCP-1, IL-6, and Cox-2 was significantly suppressed by individual MAPK inhibitors; an effect that was magnified when all inhibitors were used in combination. To determine a role for DUSPs on these MAPK-dependent changes in gene expression, PAs were transfected with a combination of dusp1, dusp8, and dusp16 siRNA 72 hrs prior to stimulation with TNFα. As illustrated in Fig.4.8, knockdown of these inducible DUSPs significantly elevated TNFα-mediated induction of each proinflammatory gene that was shown above to be downstream of MAPK activity. In direct contrast, TNFα-mediated induction in TNFα gene expression was significantly suppressed following DUSP knockdown. While TNFα gene expression was notably suppressed by MAPK inhibitors, the effects were not significant, suggesting that other non-MAPK signaling pathways played more dominant roles in regulating the expression of this proinflammatory gene.

JNK activity is required for Dusp8 mRNA synthesis in response to TNFα. Other studies have proposed a canonical role for DUSPs whereby MAPK-mediated changes in DUSP gene expression result in the expression of targeted phosphatases that specifically modulate MAPK activity (26,27). To determine if MAPKs play a role in

TNFα-mediated changes in DUSP gene expression, PAs were pretreated with pharmacological inhibitors as discussed above and total RNA harvested at 1 hr and 2 hrs post-TNFα stimulation corresponding to peak induction of dusp1 and dusp8/16, respectively. Surprisingly, JNK signaling was the only MAPK pathway involved and only in regards to dusp8 gene expression (Fig.4.9A). Pharmacological blockade of each MAPK pathway, or a combination thereof, did not significantly suppress TNFα induction of dusp1 or dusp16. As all pharmacological inhibitors have off-target effects, we confirmed these data regarding dusp8 by comparing the effect of two structurally independent inhibitors of JNK activity (SP600125 and JNK Inhibitor VII). As illustrated in Fig.4.9B, both inhibitors suppressed TNFα-mediated changes in dusp8 gene expression in a similar, concentration-dependent manner, collectively demonstrating a regulatory feedback loop modulating the extent of MAPK phosphorylation and activity. While TNFα stimulation clearly induced dusp1 and dusp16 gene expression, the induction appeared to be independent of MAPK signaling.

Discussion

Our report presents the first empirical evidence demonstrating that regulating the extent of MAPK phosphorylation by inducible MAPK-specific phosphatases has a profound influence on biological outcome downstream of MAPK signaling under conditions of inflammatory stress in cells of adipocyte lineage. First, we show that TNFα stimulation leads to cell type-specific phosphorylation of ERK, JNK, and p38 with robust, but transient effects on magnitude in PAs and subtle, but prolonged effects on duration in ADs. Second, we illustrate that in contrast to MAPK phosphorylation, mechanisms leading to rapid MAPK dephosphorylation are dependent on de novo RNA synthesis demonstrating a role for inducible gene expression in regulating the extent of MAPK

activity. Third, we show that DUSP gene expression is regulated following TNFα stimulation in a cell type and phosphatase-specific manner where kinetics of dusp1, dusp8, and dusp16 mRNA accumulation are consistent with determined roles on magnitude and duration of MAPK phosphorylation as well as biological function stemming from MAPK activity. Fourth, we illustrate MAPK-dependent and -independent regulation of these inducible DUSPs following TNFα stimulation establishing a feedback modulation of MAPK activity and functional outcome. Collectively, these data establish mechanistic roles for select, inducible, MAPK-specific phosphatases in mediating the extent of MAPK-dependent changes in gene expression highlighting potential therapeutic targets linking obesity with metabolic inflammatory diseases.

It is now well established that obesity is associated with AT inflammation, where cells within AT including PAs, ADs, and macrophages produce and secrete various pro-inflammatory cytokines in response to pathogens and cellular stress (28-30). Evidence further demonstrates that both PAs and ADs play a central role in AT inflammation and IR. While PAs are generally considered more inflammatory than ADs (28,31), others have shown that PAs share traits with macrophages such as phagocytosis and expression of membrane-bound NADPH oxidases (32). Consist with this notion, transcriptional profiling suggests that macrophages and PAs are genetically related (33), while further reports demonstrate that PAs can trans-differentiate into macrophage-like cells (32) and that PAs may, in part, originate from AT macrophages (34). Data presented in this investigation demonstrate that the magnitude of MAPK phosphorylation was markedly greater in PAs compared to ADs, while ERK and JNK signaling duration was prolonged in ADs compared to PAs in response to inflammatory stress. While p38 phosphorylation was observed in PAs, it was not seen in ADs stimulated under the same

conditions, suggesting that PAs are more responsive to external inflammatory cues. As reports demonstrate that activation of MAPKs results in increased inflammation, others have shown that the extent (i.e., duration) of MAPK phosphorylation is also important in controlling biological processes, where prolonged phosphorylation of JNK inhibits insulin signaling (12,13). Therefore, timely modulation of JNK and ERK phosphorylation in ADs suggests that diverse inflammatory regulation (i.e., magnitude and duration) between different cell types may be equally important in AT inflammation and obesity-induced IR.

Traditionally, upstream kinases rather than phosphatases were viewed as major regulators of MAPK signaling. However, phosphatases are now recognized as key modulators and even controllers involved in timely modulation of MAPK signaling and inflammation (16,17). As recent reports demonstrate that 'inducible' DUSPs modulate MAPKs dephosphorylation in various cell types (26,35,36), we show that all MAPK-specific DUSPs examined were expressed in both PAs and ADs. Moreover, we demonstrate clear phenotypic differences in basal DUSP expression, as four of the ten DUSPs were markedly elevated in PAs compared to ADs while two of the ten DUSPs were markedly elevated in ADs compared to PAs. Data presented here further report phenotypic differences in TNFα-induced DUSP gene expression, where dusp1, dusp8, and dusp16 were induced in PAs and dusp1, dusp5, and dusp8 induced in ADs. As TNFα-mediated induction of dusp1 and dusp8 was greater in PAs compared to ADs, these data suggest that phenotypic differences in the duration (i.e., transient vs. prolonged) of ERK and JNK phosphorylation may reflect DUSP expression. Collectively, these data suggest that both upstream kinases and downstream phosphatases may influence the observed phenotypic differences in PAs and ADs regarding inflammatory signaling and inflammatory status.

Molecular mechanisms that underlie AT inflammation during the onset of obesity include the expression and release of various chemokines (i.e., MCP-1) and cytokines (i.e., TNFα and IL-6) that promote infiltration of macrophages and IR (1,3,4). While it is well established that MAPKs serve as central mediators linking inflammation to adipocyte IR (2,12,14), they also contribute to the activation of downstream transcriptional programs (e.g. AP-1) involved in proinflammatory cytokine production, further exacerbating AT inflammation (2,30). Recent reports not only demonstrate a role for DUSPs in the modulation of MAPK signaling, but also the downstream regulation of MAPK-dependent processes including inflammatory gene expression, where mice deficient in select DUSPs have increased JNK and p38 signaling that corresponds to increased expression of TNFα and IL-6 (36-39). Upon further examination of inflammatory PAs, where MAPK activation was transient, we demonstrate that DUSP induction kinetically favored MAPK dephosphorylation in the presence of continued TNFα, suggesting a functional role for 'inducible' phosphatases on MAPK signaling. Moreover, we show that mechanisms leading to MAPK dephosphorylation were dependent on de novo mRNA synthesis, further highlighting a role for 'inducible' DUSPs in the regulation of MAPKs. As RNAi-mediated knockdown of DUSPs in PAs resulted in increased magnitude and duration of ERK, JNK, and p38 phosphorylation, these data confirm that DUSPs play a pivotal role in the modulatory timing of MAPK signaling under conditions of inflammatory stress. While no MAPK-dependent cytosolic change was examined in this study, data presented here demonstrate that the modulatory actions of DUSPs on MAPK signaling significantly increased nuclear events involving pro-inflammatory gene expression of MAPK-dependent MCP-1, IL-6, and Cox-2 in PAs challenged with TNFα. Collectively, these data demonstrate that DUSPs play a central

role in the modulation of MAPK dephosphorylation, ultimately affecting downstream regulation of MAPK-dependent nuclear processes involved in AT inflammation.

Regulation of DUSPs was originally thought to occur in a 'feedback' manner (26,27,35), in which MAPKs induce DUSP expression that, in turn, dephosphorylates MAPKs. We report that only dusp8 was downstream of JNK phosphorylation, while dusp1 and dusp16 were not regulated by MAPK signaling. Recent evidence suggests that MAPK-independent pathways also regulate DUSPs expression under conditions of inflammatory stress, where protein kinase A (PKA) and PKC have been shown to regulate dusp1 mRNA expression (40-42). Thus, MAPK-independent regulation of DUSPs may play a critical role in cellular signaling cross-talk. This is evident in studies that demonstrate MAPK cross-talk between the janus kinase–signal transducer and activator of transcription (JAK-STAT) and phosphatidylinositol 3-kinase (PI3K) pathways (10,43). Moreover, data presented here suggest that MAPK-dependent regulation of dusp8 is essential for feedback and cross-talk modulation of MAPKs, as it was shown that dusp8 modulates JNK and ERK dephosphorylation. Consistently, others demonstrate that sustained JNK phosphorylation in response to mitogenic stimuli blocks ERK activation in COS-7 cells via transcriptional mediated processes (44), with recent reports suggesting a positive regulation of ERK from the JNK phosphatases dusp10 and dusp16 (45). Interestingly, DUSP knockdown significantly attenuated TNFα-induced TNFα gene expression in PAs. While these data demonstrate that TNFα regulation is MAPK-independent, it is possible that modulatory actions of DUSPs on MAPK signaling altered other signaling pathway(s) involved in TNFα regulation. Taken together, these findings suggest that cross-talk within and between signaling modules occur in part

through the regulation of inducible DUSPs, potentially contributing to MAPK-dependent and -independent regulation of AT inflammation and IR.

In summary, data outlined here and elsewhere (17,35) highlight the importance of timely MAPK phosphorylation and dephosphorylation resulting from upstream kinases and downstream phosphatases in the regulation of inflammatory signaling and inflammatory status. We demonstrate that DUSPs regulate MAPK dephosphorylation and MAPK-dependent inflammatory gene expression in 3T3-L1 adipocytes in response to inflammatory stress. Moreover, we report that MAPK-dependent and MAPK-independent regulation of DUSPs is critical in signaling feedback inhibition and cross-talk that potentially contributes to AT inflammation. As work on DUSP regulation and function continues, elucidation into the molecular actions of these phosphatases may provide therapeutic targets for the treatment and prevention of AT inflammation and obesity-induced IR.

Table 4.1. DUSPs and inflammatory genes analyzed in this study.

symbol	name/alias					accession	ABI number	C_T
dual specificity phosphatases		GP	MKB	NLS	NES			
Dusp1	MKP-1, hVH1	I	●	●		NM_013642	Mm00457274_g1	24
Dusp2	PAC1	I	●	●		NM_010090	Mm00839675_g1	32
Dusp4	MKP-2, hVH2	I	●	●		NM_176933	Mm00723761_m1	27
Dusp5	hVH3	I	●	●		NM_001085390	Mm01266104_m1	27
Dusp6	MKP-3, rVH6	II	●		●	NM_026268	Mm00650255_g1	24
Dusp7	MKP-X	II	●		●	NM_153459	Mm00463228_m1	24
Dusp9	MKP-4	II	●		●	NM_029352	Mm00512646_m1	31
Dusp8	M3/6, hVH5	III	●	●	●	NM_008748	Mm00456230_m1	32
Dusp10	MKP-5	III	●	●	●	NM_022019	Mm00517678_m1	26
Dusp16	MKP-7, MKP-M	III	●	●	●	NM_130447	Mm00459935_m1	26
inflammatory genes								
Ccl2	monocyte chemoattractant protein-1 (MCP-1)					NM_011333	Mm00441242_m1	21
IL-6	interleukin-6					NM_031168	Mm99999064_m1	29
Ptgs2	cyclooxygenase-2 (COX-2)					NM_011198	Mm00478372_m1	29
TNFα	tumor necrosis factor-alpha					NM_013693	Mm99999068_m1	35
reference gene								
18S	18 ribosomal RNA					X03205	4342930E	9

Table 4.1. DUSP and inflammatory genes analyzed in this study. DUSPs that contained the MAPK binding domain (MKB) were assigned to groups (GP) based on structural differences involving the presence or absence of a nuclear localization sequence (NLS), nuclear export sequence (NES), or both that may dictate subcellular localization to the nucleus (group I), cytosol (group II), or either compartment (group III).

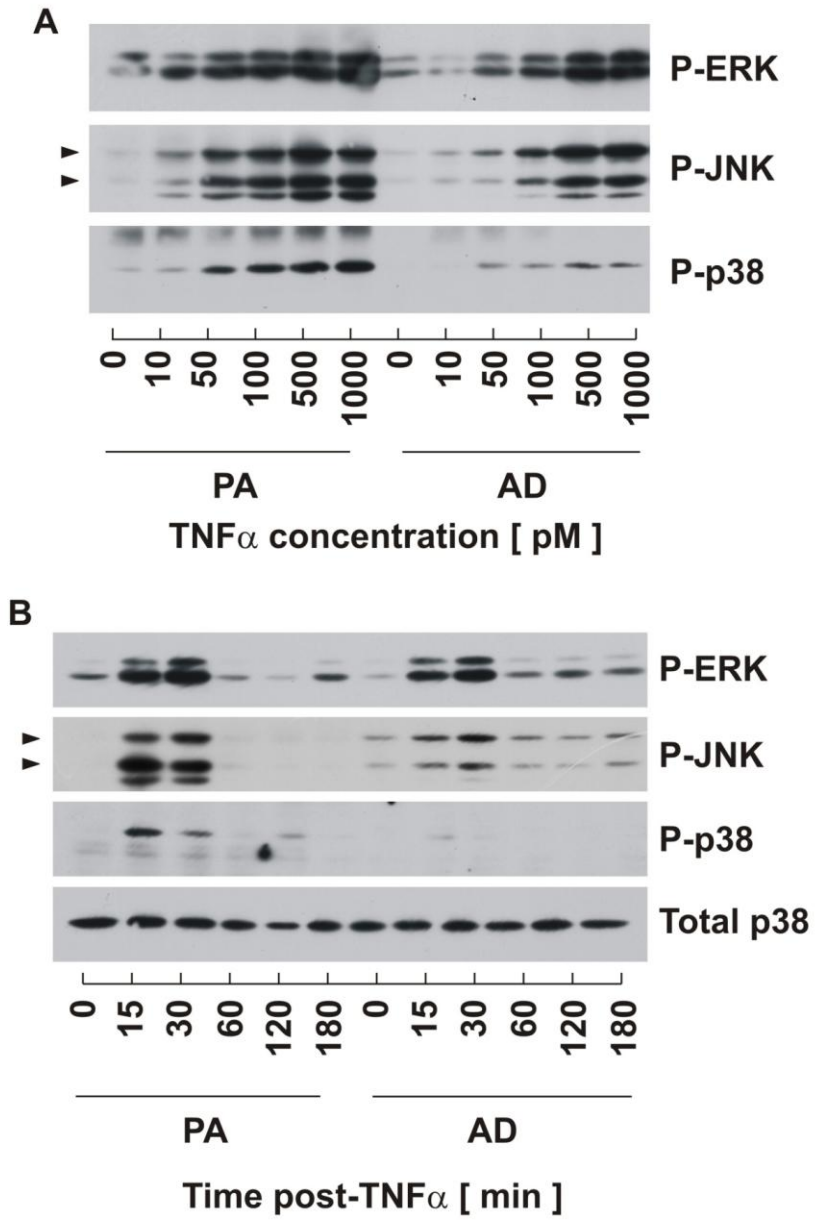

Figure 4.1. Phenotypic differences in TNFα-stimulated MAPK signaling between PAs and ADs. A) PAs and ADs were stimulated in parallel with increasing doses of TNFα and cell lysates harvested at 15 min post-TNFα stimulation prior to immunoblot analysis of bisphosphorylated ERK, JNK, and p38. B) 100 pM TNFα was added to PAs and ADs in parallel. Cell lysates were collected over time post-TNFα and immunoblotted for phospho-ERK, JNK, and p38 as well as total p38.

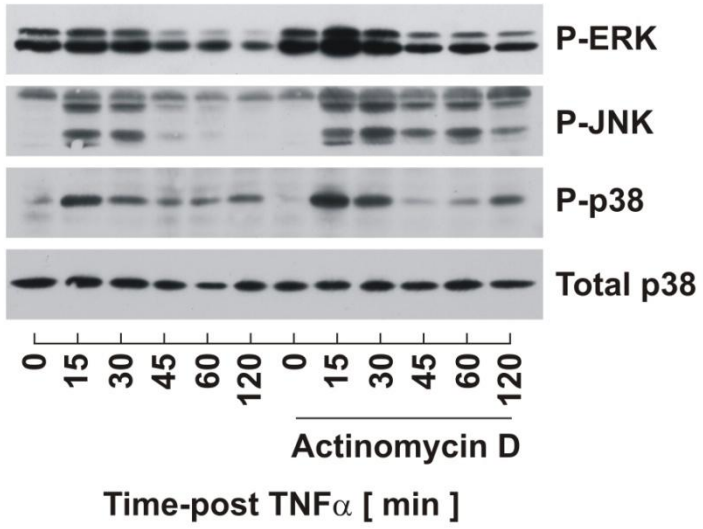

Figure 4.2. MAPK dephosphorylation is regulated by mechanisms involving de novo mRNA synthesis. PAs were stimulated with 100 pM TNFα over time pretreated in the absence or presence of 1 ng/ml Actinomcyin D (AD). Cell lysates were collected over time post-TNFα and protein expression of bisphosphorylated ERK, JNK, and p38 along with total p38 analyzed via immunoblotting.

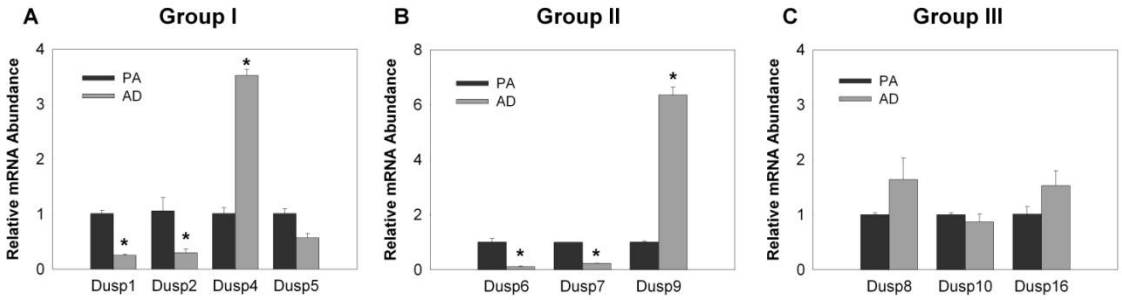

Figure 4.3. DUSPs are phenotypically regulated in PAs versus ADs. Total RNA was harvested from untreated PAs (d0) and mature ADs (d8) prior to mRNA analysis of A) group I, B) group II, and C) group III DUSPs via qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated PAs. Statistical significance was determined by Student's *t*-test (***** $p<0.05$).

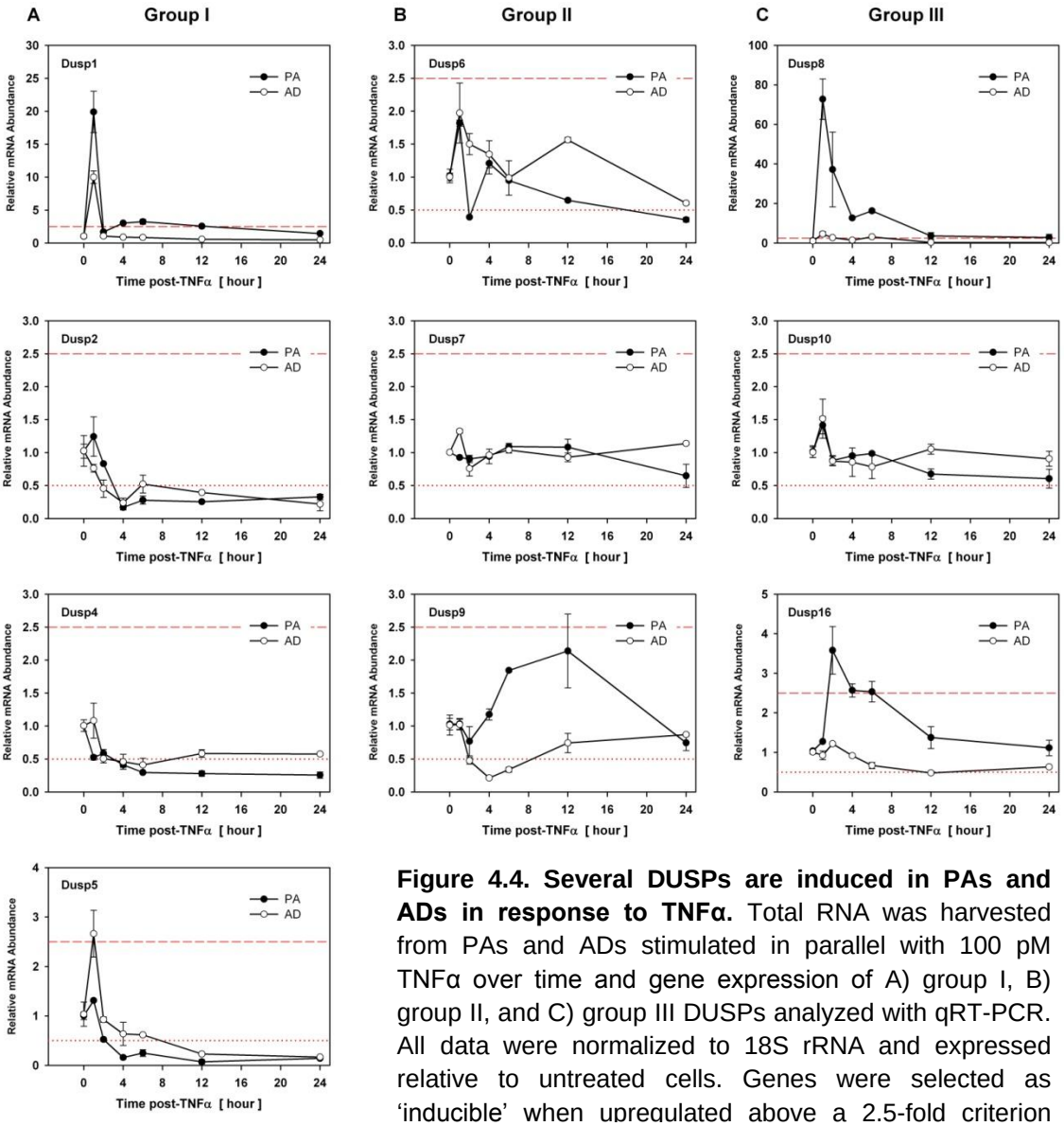

Figure 4.4. Several DUSPs are induced in PAs and ADs in response to TNFα. Total RNA was harvested from PAs and ADs stimulated in parallel with 100 pM TNFα over time and gene expression of A) group I, B) group II, and C) group III DUSPs analyzed with qRT-PCR. All data were normalized to 18S rRNA and expressed relative to untreated cells. Genes were selected as 'inducible' when upregulated above a 2.5-fold criterion indicated by the dashed line, while genes were considered suppressed that fell below a 0.5 criterion indicated by the dotted line on the graph.

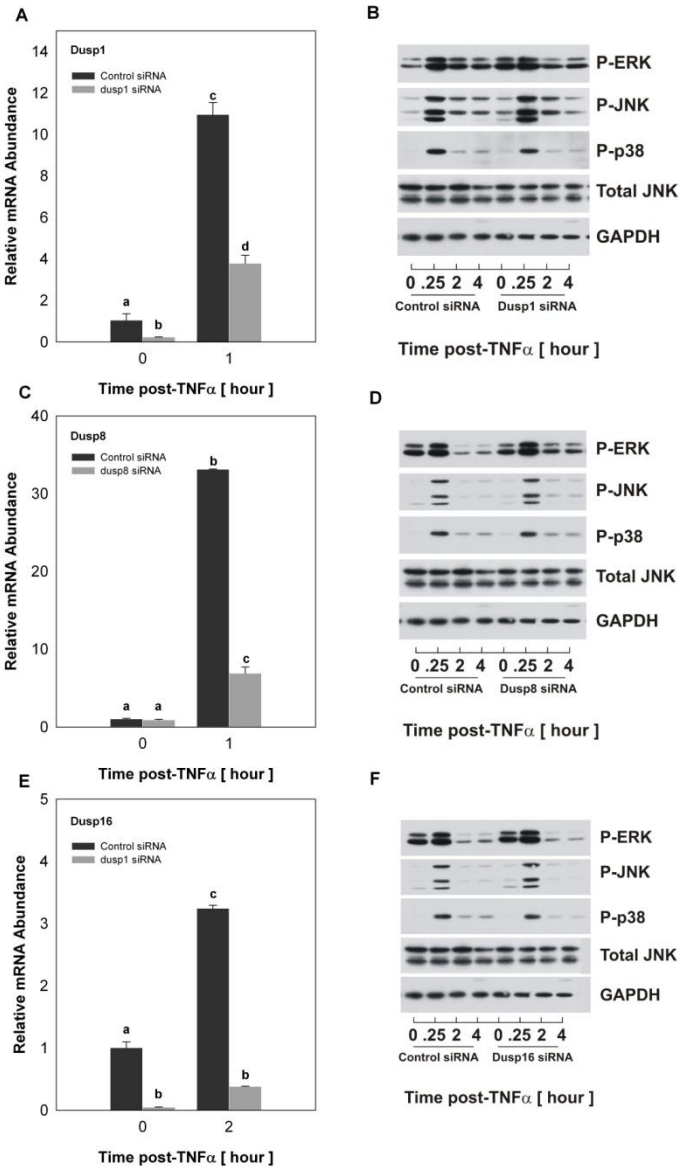

Figure 4.5. Individual DUSPs modulate MAPK signaling in 3T3-L1 PAs in response to TNFα. PAs were transfected with DharmaFect 3 transfection reagent in the presence of non-targeting control siRNA or siRNA specific for dusp1, dusp8, or dusp16 for 72 hrs prior to stimulation with 100 pM TNFα. Cell lysates were then harvested for RNA at 0h, 1h, or 2h post-TNFα stimulation and mRNA expression of A) dusp1, C) dusp8, and E) dusp16 analyzed via qRT-PCR. Data were normalized to 18S rRNA and changes in gene expression measured as fold differences relative to untreated control siRNA (0h). Statistical differences were determined by ANOVA. Tukey's post-hoc analysis was performed when the *p* value for the respective parameter was statistically significant ($p < 0.05$). In addition, cell lysates were collected over time post-TNFα stimulation for control siRNA and B) dusp1, D) dusp8, or F) dusp16 specific siRNA and protein expression of bisphosphorylated ERK, JNK, and p38 along with total JNK and GAPDH examined via immunoblotting.

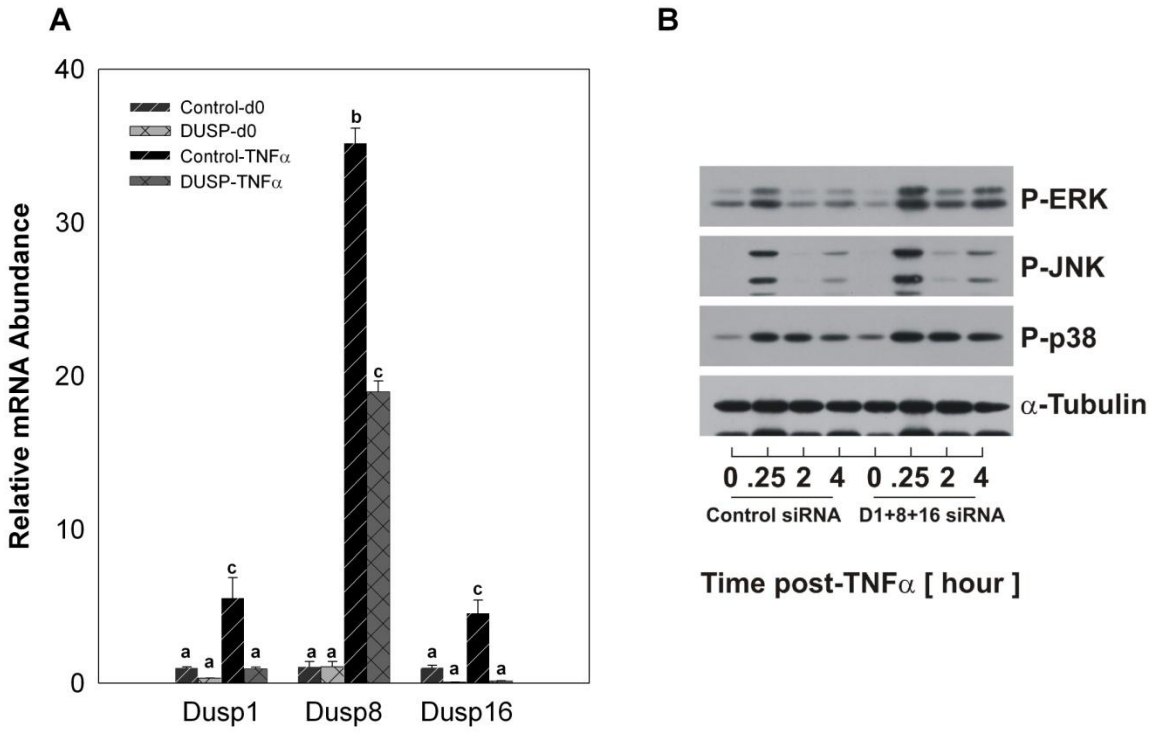

Figure 4.6. Combination DUSP knockdown modulates MAPK signaling magnitude and duration in PAs in response to TNFα. PAs were transfected with DharmaFect 3 transfection reagent in the presence of non-targeting control siRNA or siRNA specific for dusp1, dusp8, and dusp16 for 72 hours prior to stimulation with 100 pM TNFα. A) Total RNA was harvested at 0hr, 1hr, or 2hr post-TNFα stimulation and mRNA expression of dusp1 (1h), dusp8 (1h), and dusp16 (2h) analyzed via qRT-PCR. Data were normalized to 18S rRNA and changes in gene expression measured as fold differences relative to untreated control siRNA (0h). Significance differences were determined by ANOVA. Tukey's post-hoc analysis was performed when the *p* value for the respective parameter was statistically significant ($p < 0.05$). B) Cell lysates were collected over time post-TNFα stimulation for control siRNA or combination DUSP siRNA and protein expression of bisphosphorylated ERK, JNK, and p38 along with α-Tubulin examined via immunoblotting.

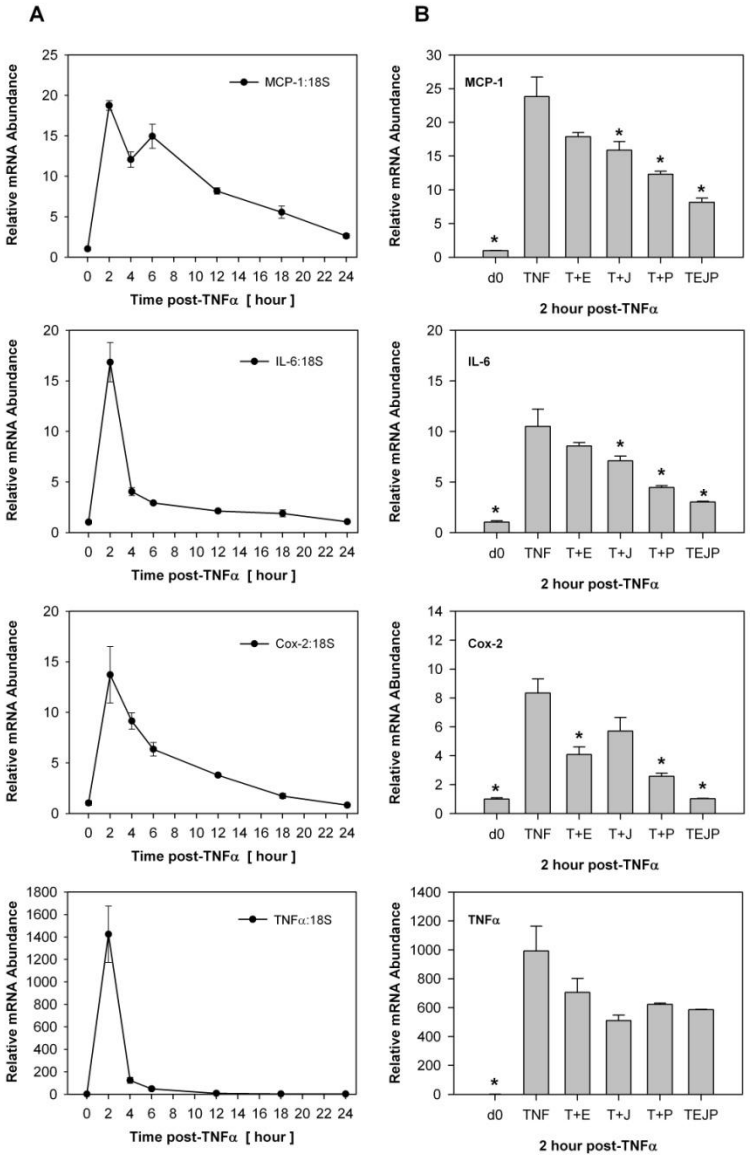

Figure 4.7. MAPK-dependent regulation of proinflammatory genes in response to TNFα. A) Total RNA was harvested over time from PAs stimulated with 100 pM TNFα and gene expression of MCP-1, IL-6, Cox-2, and TNFα mRNA expression examined via qRT-PCR. B) PAs were pretreated for 1hr in the presence of inhibitors for ERK (U0126, 10 μM), JNK (SP600125, 20 μM), or p38 (SB203850, 10 μM) prior to TNFα stimulation. Total RNA was harvested at 2hr post-TNFα and mRNA expression analyzed for the above mentioned genes. Nomenclature related to the graph is assigned: T (TNFα), T+E (TNFα + ERK), T+J (TNFα + JNK), T+P (TNFα + p38), or TEJP (TNFα +ERK+JNK+p38). Data were expressed relative to untreated cells and normalized to the 18S rRNA. Statistical significance was determined by ANOVA with Dunnett's post-hoc analysis conducted to assess differences from control (TNFα) when $p<0.05$.

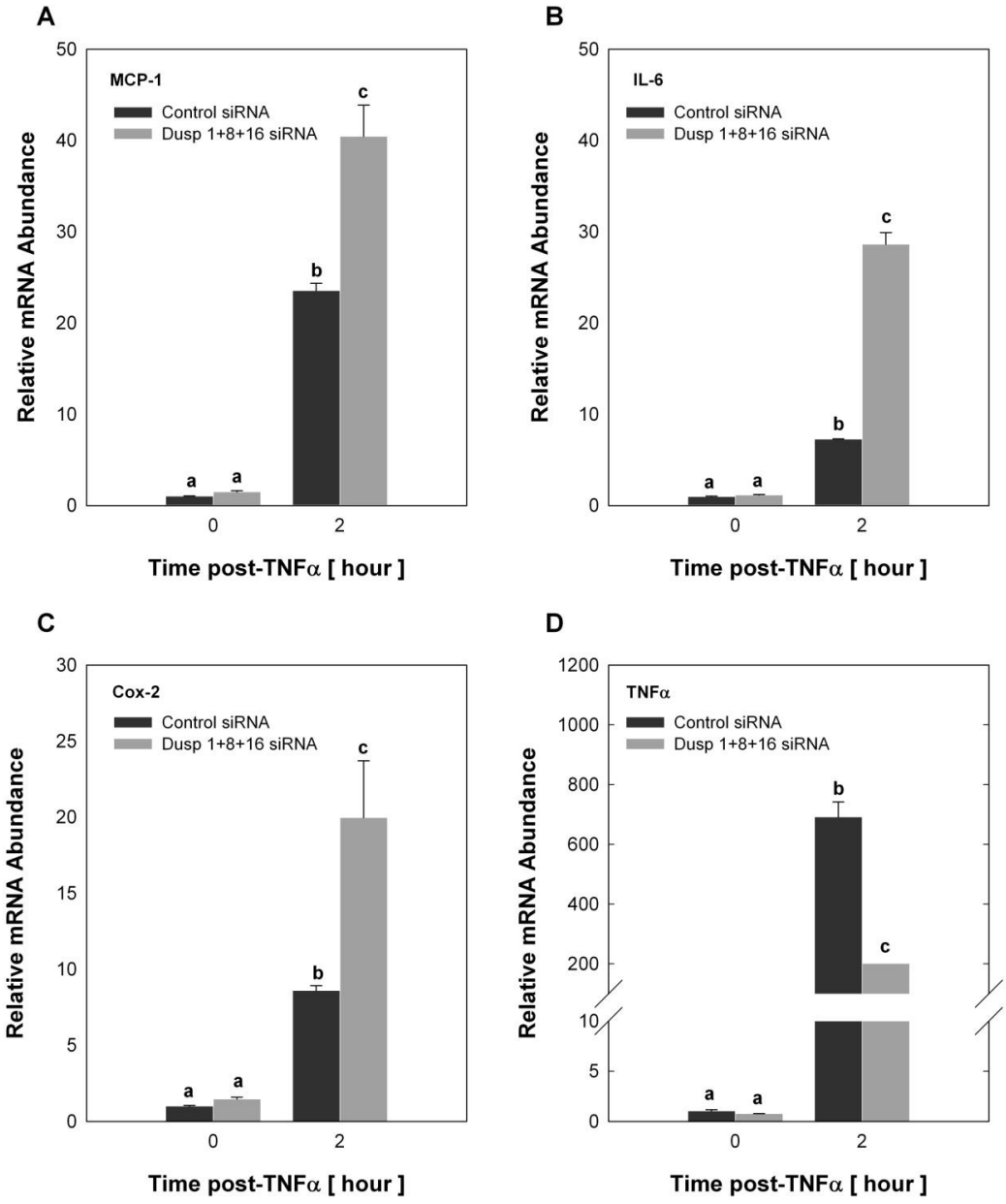

Figure 4.8. DUSPs modulate MAPK-dependent proinflammatory gene expression in response to TNFα. PAs were transfected with 3 µl DharmaFect 3 transfection reagent in the presence of non-targeting control siRNA or siRNA specific for dusp1, dusp8, and dusp16 for 72 hours prior to stimulation with 100 pM TNFα. Total RNA was collected at 0h or 2h post-TNFα stimulation and mRNA expression of A) MCP-1, B) IL-6, C) Cox-2, and D) TNFα analyzed via qRT-PCR. Data were normalized to 18S rRNA and changes in gene expression measured as fold differences relative to untreated control siRNA (0h). Statistical differences were determined by ANOVA. Tukey's post-hoc analysis was performed when the *p* value for the respective parameter was statistically significant ($p < 0.05$).

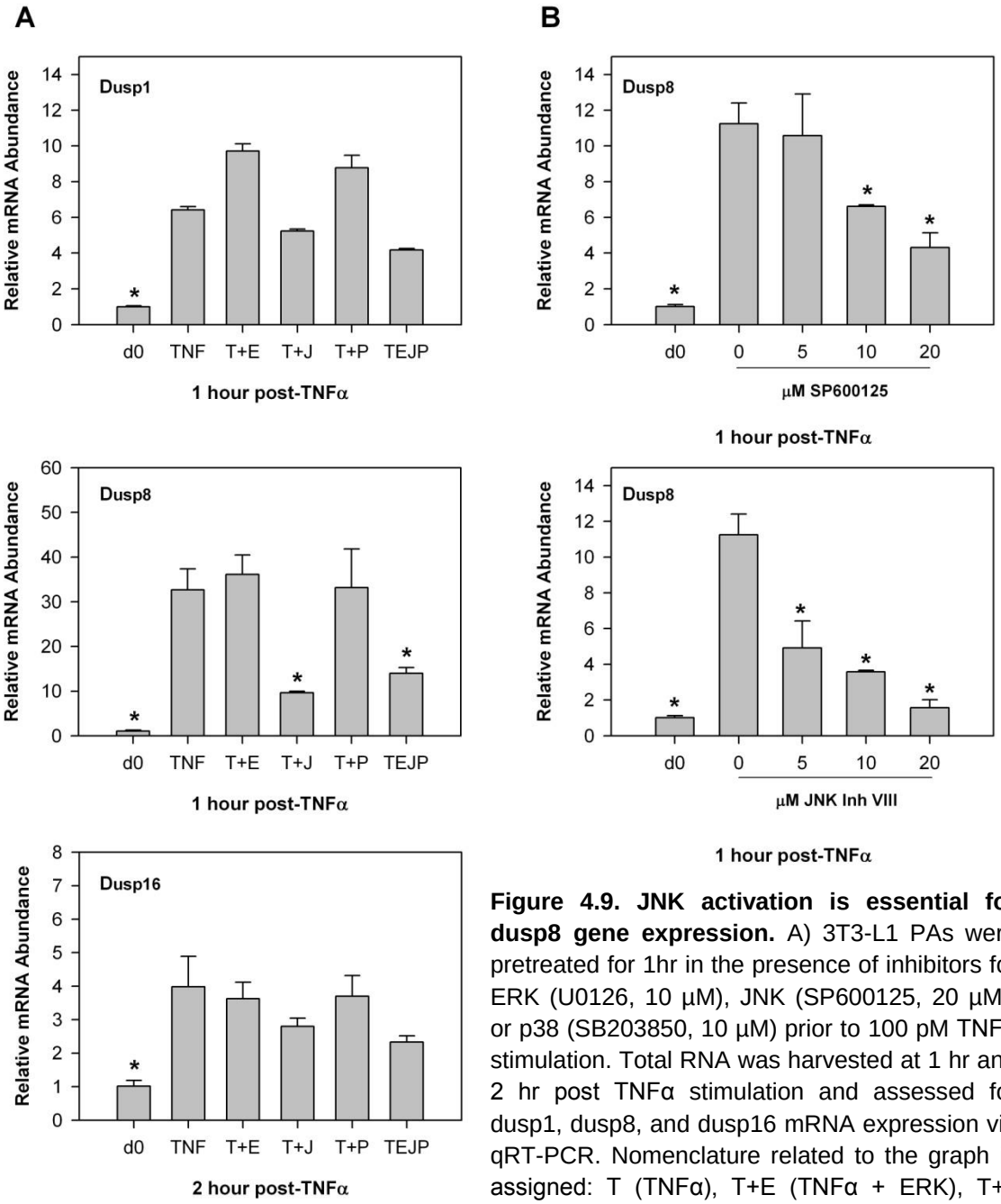

Figure 4.9. JNK activation is essential for dusp8 gene expression. A) 3T3-L1 PAs were pretreated for 1hr in the presence of inhibitors for ERK (U0126, 10 μM), JNK (SP600125, 20 μM), or p38 (SB203850, 10 μM) prior to 100 pM TNFα stimulation. Total RNA was harvested at 1 hr and 2 hr post TNFα stimulation and assessed for dusp1, dusp8, and dusp16 mRNA expression via qRT-PCR. Nomenclature related to the graph is assigned: T (TNFα), T+E (TNFα + ERK), T+J (TNFα + JNK), T+P (TNFα + p38), or TEJP (TNFα +ERK+JNK+p38). B) In addition, PAs were pretreated for 1hr with increasing doses of JNK inhibitors A) SP600125 or B) JNK inhibitor VIII prior to stimulation with 100 pM TNFα. Total RNA was harvested at 1 hr post TNFα stimulation and assessed for dusp8 expression via qRT-PCR. Data were expressed relative to untreated cells and normalized to 18S rRNA. Statistical significance was determined by ANOVA with Dunnett's post-hoc analysis conducted to assess differences from control (TNFα) when $p<0.05$.

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CHAPTER V

ROLE FOR DUAL-SPECIFICITY PHOSPHATASES REGARDING CHEMOKINE EXPRESSION IN ADIPOCYTES IN RESPONSE TO INFLAMMATORY STRESS

Abstract

Obesity is an independent risk factor for heart disease, stroke, insulin resistance (IR), and diabetes. Early evidence demonstrated an essential role for the proinflammatory chemokine monocyte chemoattractant protein-1 (MCP-1) in macrophage recruitment, adipose tissue (AT) inflammation, and obesity-induced IR. However, it is now clear that other proinflammatory chemokines are induced in AT with obesity and are associated with obesity-induced IR. Moreover, reports have elucidated that the modulation of mitogen-activated protein kinase (MAPK) signaling by upstream kinases and downstream phosphatases plays a central role in linking inflammation to obesity-induced IR. This study investigated the role of MAPK-specific dual-specificity phosphatases (DUSPs) on MAPK-regulated CC chemokine expression under conditions of inflammatory stress in 3T3-L1 adipocytes. We report that all CC chemokines (i.e., CCL2, CCL3, CCL4, CCL5, CCL8, CCL9, CCL12, and CCL20) examined were expressed in preadipocytes (PAs) and adipocytes (ADs), where expression for most CC chemokines was greatest in PAs compared to ADs. Additionally, we demonstrated that all CC chemokines examined were induced in response to inflammatory stress (i.e., TNFα). While nuclear factor kappa B (NF-κB) partially regulated the expression of select chemokines, all CC chemokines examined were downstream targets of MAPK signaling. Finally, this report demonstrated that increased MAPK signaling duration and magnitude

via DUSP RNA interference (RNAi) resulted in significant amplification of nuclear MAPK-dependent processes involving CC chemokine expression. Collectively, these data present empirical evidence that timely modulation of MAPK signaling via DUSPs is essential in the regulation of biological processes involving MAPK-dependent CC chemokine expression and AT inflammation.

Introduction

Obesity plays a causal role in IR where increased adiposity in humans and animals highly correlate with decreased insulin sensitivity (1-4). Excessive expansion of AT in obesity triggers inflammatory stress, disrupting adipocyte function that leads to localized and systemic IR (5-10). The emergence of inflammation as a central mediator involved in adipocyte dysfunction and IR developed from observations that tumor necrosis factor α (TNFα) increased during obesity, while genetic attenuation of TNFα or its receptor protects mice fed a high-fat diet from developing IR (11-16). While it is now apparent that obesity is associated with chronic, low-grade inflammation, initiation of the inflammatory response in AT has not been fully elucidated.

Observations of macrophage accumulation and activation in AT of obese humans and animals revealed an important source for TNFα expression in obesity that disrupts adipocyte function and impairs insulin signaling (17). Moreover, reduction of AT macrophage infiltration observed in diet-induced obese (DIO) mice deficient in MCP-1/ chemokine (C-C motif) ligand 2 (CCL2) or its cognate receptor CCR2 demonstrated a decreased expression of inflammatory adipose cytokines (i.e., TNFα) and improved insulin sensitivity (18,19). In contrast, transgenic mice expressing MCP-1/CCL2 have the reverse phenotype (18). MCP-1 is a CC chemokine that belongs to the β-chemokine

family that includes monocyte chemoattractant proteins (MCP), macrophage inflammatory proteins (MIP), and regulated upon activation, normal T cell expressed and secreted (RANTES), among others. These chemokines attract most monocytes, eosinophils, basophils, and lymphocytes under inflammatory conditions (20) including obesity (21). While MCP-1/CCL2 appears essential for obesity-induced IR, it has become increasingly clear that other CC chemokines play an essential role in AT inflammation (21). As reports demonstrate that AT inflammation involves the secretion of TNFα derived from activated AT macrophages (22), adipocyte-secreted CC chemokines may play a key role in the development of chronic AT inflammation.

Two major signaling pathways that have been identified as upstream regulators of TNFα-mediated inflammatory signaling include nuclear factor kappa B (NF-κB) and MAPKs, consisting of extracellular signal-regulated kinase (ERK), p38, and c-Jun N terminal kinase (JNK) (1,23). All four pathways are activated in AT in response to macrophage-mediated TNFα stimulation and serve as mediators of localized IR through transcriptional mechanisms involving adipocyte gene expression as well as the expression and secretion of other proinflammatory molecules (1,24-27). Compared to lean counterparts, AT of obese animals display markedly increased expression of ERK, p38, and JNK (27-30), while genetic loss-of-function studies demonstrate that deletion of these kinases protects against AT inflammation and IR in animals challenged with obesity or high fat diet (HFD) (27,31). For example, deletion of JNK in dietary and genetic (ob/ob) mouse models of obesity led to decreased adiposity, improved insulin sensitivity, and enhanced insulin receptor signaling (27). Moreover, phosphorylated JNK has been shown to regulate nuclear events involving cytokine (e.g., TNFα) and

chemokine (e.g., MCP-1) expression in 3T3-L1 adipocytes that promote macrophage recruitment and AT inflammation (32,33).

Activation of MAPK signaling is regulated by sequential and concerted phosphorylation of upstream kinases where phosphorylation of both the threonine and tyrosine residues of the (T-X-Y) motif within the activation loop by upstream MAPK kinases is essential and sufficient for MAPK activation (25,34,35). While upstream kinases were traditionally viewed as major regulators of MAPK signaling, many phosphatases are now recognized as key modulators and even controllers of MAPK activation and MAPK-dependent processes involving inflammation (25,33,36-38). MAPK-specific DUSPs constitute a group of ten protein tyrosine phosphatases that specifically inhibit MAPK activity through dephosphorylation of both threonine and tyrosine residues. These 10 known DUSPs are MAPK-specific as they contain a MAP kinase-binding (MKB) domain that selectively imparts catalytic activity toward ERK, JNK, or p38 (34,37,38,38,39). DUSPs have recently been identified as potential targets in the regulation of MAPK-mediated inflammation and IR (37,40), where dusp1 was observed to regulate inflammatory protein expression in macrophages in response to lipopolysaccharide (LPS) challenge as well as mitigate IR and MCP-1 expression within ADs (41-44).

Considering the role for CC chemokines on obesity-associated AT inflammation and IR, we examined the impact of DUSPs on MAPK-dependent CC chemokine regulation during TNFα-mediated inflammatory stress in 3T3-L1 adipocytes. This cell line consists of nearly pure populations of undifferentiated PAs as well as mature ADs representing cellular effectors of AT dysfunction without macrophage contamination. This study demonstrated that DUSPs act as key regulators of MAPK-dependent CC

chemokine expression under conditions of TNFα-mediated inflammatory stress in adipocytes, highlighting a potential therapeutic role for DUSPs on macrophage infiltration and chronic AT inflammation.

Materials and Methods

Materials: Dulbecco's Modified Eagle's Medium (DMEM), calf bovine serum (CS), and trypsin-EDTA were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from HyClone. Antibodies used for immunoblotting include phospho-ERK, phosho-p38, phospho-JNK, total p38, and α-tubulin purchased from Cell Signaling. U0126, SP600125, SB203580, and Helenalin were purchased from CalBiochem. Enhanced chemiluminescence (ECL) reagents were obtained from Perkin-Elmer Life Sciences. All Taqman primer probes (Table 5.1) used in this study were purchased from Applied Biosystems. The murine 3T3-L1 cell line was purchased from Howard Green, Harvard Medical School (45).

Cell Culture: 3T3-L1 preadipocytes were propagated in DMEM supplemented with 10% CS until density-induced growth arrest, as previously described (46). At 2 days post-confluence, growth medium was replaced with DMEM supplemented with 10% FBS, 0.5mM 1-methyl-3-isobutylxanthine, 1 µM dexamethasone, and 1.7 µM insulin (MDI). Throughout the study, 'time 0' refers to density arrested cells immediately before the addition of MDI to the culture medium. Experiments described herein were conducted in density-arrested preadipocytes (d0) and mature adipocytes (d8) stimulated in parallel with 100 pM TNFα. All experiments were repeated at least 3 times to validate results and ensure reliability.

Immunoblotting: Cell monolayers were washed with phosphate-buffered saline (PBS) and scraped into ice-cold lysis buffer containing 1.0 M Tris, pH 7.4, 150 mM NaCl, 1% Triton X, 0.5% Nonidet P-40 (NP40), 1 mM EDTA, 1 mM EGTA, and 10 mM *N*-ethylmaleimide. Phosphatase (20 mM ß-glycerophosphate, 10 mM NaFl, and 2 µM sodium vanadate), as well as protease (0.3 µM aprotonin, 21 µM leupeptin, 1 µM pepstatin, 50 µM phenanthroline, and 0.5 µM phenylmethylsulfonyl fluoride) inhibitors were added to lysis buffer prior to cell harvest. Cell lysates were sonicated, centrifuged (13,000 x *g*, 10 min, 4°C), and the supernatant transferred to a fresh tube. Bicinchoninic acid assay (Pierce, Rockford, IL) was used to determine protein concentration. Cell lysates were resuspended in loading buffer containing 0.25 M Tris, pH 6.8, 4% sodium dodecyl sulfate (SDS), 10% glycerol, 10% dithiothreitol, and 0.01% bromophenol blue and heated for 5 min at 80°C. Proteins were resolved on SDS-polyacrylamide gel electrophoresis gels (PAGE) and transferred to polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA). After transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4°C. Membranes were subsequently probed with horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Immunoblots were immersed in ECL and visualized by autoradiography using CL-XPosure film (Pierce).

Real-Time RT-PCR: Total RNA was extracted and genomic DNA contamination was removed using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's protocol. Total RNA was quantified with a Nanodrop ND-1000 spectrophotometer and reverse-transcribed to cDNA in a 10 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems). The reverse transcription (RT) master mix containing RT buffer, deoxyribonucleotide triphosphate (dNTP) mix, RT random primers,

RNase inhibitor (1.0 U/µl), and MultiScribe RT was added to 1 µg RNA and RNase-free water. Reverse transcription reaction conditions followed the protocol (25°C for 10 min, 37°C for 120 min, 85°C for 5 sec, followed by 4°C indefinitely/ RT complete) and utilized the Gene Amp PCR System 9700 thermal cycler (Applied Biosystems) for cDNA synthesis.

PCR amplification was run utilizing the 7500 fast system (Applied Biosystems) that consisted of enzyme activation at 95°C for 20 sec, followed by 40 cycles of denaturation at 95°C for 3 sec combined with annealing/extension at 60°C for 30 sec. All data were analyzed with the ABI 7500 real time PCR system (Applied Biosystems). Data were recorded and analyzed with Sequence Detector Software (Applied Biosystems) and graphs visualized with SigmaPlot software. All data were presented as mean ± standard error of the mean (SEM) and representative of at least two experiments performed in duplicate. Data were normalized to 18S previously validated in this lab as a suitable reference gene under these experimental conditions (47). Relative differences between treated and untreated control samples were analyzed by the $2^{-\Delta\Delta C_T}$ method as previously described (47,48).

Statistical analyses were conducted using SPSS v18. Phenotypic differences in mRNA expression were determined via student's *t*-test where a p-value of <0.05 was considered significant. Knockdown data were analyzed using ANOVA, with Tukey's post-hoc analysis used when the *p* value for the respective parameter was statistically significant ($p < 0.05$). Inhibitor data were analyzed using analysis of variance, with Dunnett's post-hoc analysis conducted to assess differences from controls (TNFα) when $p<0.05$.

RNA Interference. SMARTpools containing four different short interfering RNAs (siRNAs) for dusp1, dusp8, and dusp16 specific sequences as well as non-targeting sequences were transfected using DharmaFect 3 transfection reagent according to manufacturer's (Dharmacon) protocol. Briefly, 3T3-L1 preadipocytes were propagated in 6-well culture dishes until reaching density-induced growth arrest. Growth medium was then replaced with DMEM supplemented with 10% CS, 3 µl DharmaFect 3 reagent and either 100 nM dusp1, dusp8, and/or dusp16 specific siRNA or non-targeting siRNA for 72 hr. Growth medium was subsequently switched to differentiation medium containing MDI as described above.

Results

Phenotypic differences between preadipocytes and adipocytes regarding TNFα-mediated changes in inflammatory signaling. AT is comprised of insulin-sensitive adipocytes (ADs) and a stromal vascular fraction containing numerous metabolically relevant cell types including undifferentiated adipocyte precursor cells known as preadipocytes (PAs). The onset of obesity is associated with marked AT recruitment of macrophages that likely represent the major source of elevated TNFα that imparts AT dysfunction by increasing proinflammatory signaling that leads to cytokine and chemokine gene expression in PAs and insulin resistance in ADs (17). Therefore, we examined the effect of TNFα stimulation on MAPK and NF-κB signaling in these functionally diverse cell types of adipocyte lineage. The 3T3-L1 murine adipocyte cell line was used to assess phenotypic differences, as this cell line yielded undifferentiated, quiescent PAs prior to differentiation and greater than 90% conversion to mature ADs following differentiation. Moreover, phenotypic differences in inflammatory signaling were

cell type specific as this well-established cell line is devoid of other cell types (e.g., macrophages) that comprise AT. Cells were differentiated by established protocol for 8 days to yield functionally mature ADs and compared to undifferentiated, density-arrested PAs of similar passage. To compare phenotypic difference in MAPK and NF-κB signaling, PAs and ADs were stimulated with 100 pM TNFα in parallel and lysates harvested over time and immunoblotted for ERK, JNK, and p38 bisphosphorylation. This concentration of TNFα was chosen, as it was previously determined that inflammatory signaling was increased in a concentration-dependent fashion in both PAs and ADs, with an estimated ED_{50} of approximately 100 pM (data not shown; Ch.4). Furthermore, this dose closely approximates the concentration of TNFα in human and animal models of obesity and has been shown to suppress insulin signaling in culture (11). As shown in Fig.5.1, TNFα stimulation resulted in a rapid, transient activation of all three MAPKs in PAs where robust phosphorylation was observed at 15 min with complete dephosphorylation by 60 min post-TNFα. Conversely, the same concentration of TNFα produced markedly lower magnitude, but sustained duration of JNK and ERK with no phosphorylation observed for p38 in mature ADs. Likewise, degradation of Iκ-Bα, an indicator of increased NF-κB activity, was rapid and transient in PAs where complete degradation was observed at 15 min with return expression by 60 min post-TNFα. In contrast, Iκ-Bα degradation was delayed in ADs where complete degradation was observed at 30 min with a return in expression by 60 min post-TNFα. As the abundance of total p38 protein was not affected by TNFα, phenotypic differences observed regarding magnitude and duration of MAPK phosphorylation and Iκ-Bα degradation suggest that PAs were more responsive to inflammation.

Cell type specific induction of CCL chemokine gene expression following TNFα stimulation. Data presented above demonstrated phenotypic differences in inflammatory signaling. Thus, we examined phenotypic differences between PAs and ADs regarding chemokine expression. We initially screened unstimulated PAs for mRNA abundance of eight chemokines previously shown to be regulated under conditions of genetic and diet-induced obesity, using qRT-PCR. As illustrated in Table 5.1, all chemokines were easily measurable within the detectable limits of 36 threshold cycles (C_T) with CCL2, CCL5, CCL8, and CCL9 most abundantly expressed and CCL3, CCL4, CCL12, and CCL20 least abundantly expressed. Additionally, this screen was extended to examine differences in basal chemokine expression between unstimulated PAs and ADs. Chemokine mRNA abundance was normalized to 18S ribosomal RNA and expressed as relative differences to unstimulated PAs. As illustrated in Fig.5.2, relative mRNA abundance of CCL3 and CCL4 was significantly elevated in ADs compared to PAs. CCL2, CCL5, CCL8, CCL12, and CCL20 were significantly elevated in PAs compared to ADs, while CCL9 showed no marked differences in basal expression between cell types.

Data presented above demonstrated that chemokine expression and inflammatory signaling was more prominent in PAs compared to ADs. Therefore, we next examined which chemokines were induced at the level of gene expression during inflammatory stress in PAs stimulated with 100 pM TNFα using qRT-PCR. Kinetic determinations for chemokine expression were examined over time to establish which chemokines were induced as well as whether the induction was transient versus sustained to gain potential insight into functional roles. As illustrated in Fig.5.3, CCL3, CCL4, and CCL12 were induced rapidly and transiently in PAs with an inducible peak

occurring at 2h post-TNFα, returning to baseline by ≤6h post stimulation. In addition, CCL2 and CCL20 were rapidly induced in PAs, but in contrast to the above chemokines, mRNA levels remained elevated ≥12h following TNFα stimulation suggesting the possibility of divergent roles in inflammation and macrophage recruitment (Fig.5.3). Conversely, induction of CCL5, CCL8, and CCL9 was delayed in PAs stimulated with TNFα where CCL5 peaked at 12h, CCL8 peaked at 24h, and CCL9 peaked at 6h post-TNFα. While the early activation from the above chemokines suggests a direct role for TNFα signaling, late phase induction of CCL5 and CCL8 may be in response to autocrine or paracrine activation by TNFα-induced inflammatory mediators.

Role for MAPK activity regarding chemokine gene expression in response to TNFα. As data presented above suggested that all chemokines were induced under these conditions of inflammatory signaling, we next examined the role of MAPK and NF-κB activity on chemokine regulation in PAs under conditions of inflammatory stress. To address this question, PAs were pretreated with specific, pharmacological inhibitors of ERK (U0126), JNK (SP600125), p38 (SB203580), and NF-κB (Helenalin) for 1h prior to TNFα stimulation. Total RNA was harvested from PAs at 2h, 4h, or 18h post-TNFα stimulation and mRNA abundance assessed by qRT-PCR comparing treated cells to untreated controls. Inhibition of ERK signaling significantly suppressed TNFα-induced CCL5 and CCL9 mRNA accumulation (Fig.5.4). In addition, inhibition of p38 signaling significantly suppressed CCL2, CCL3, CCL4, CCL8, CCL9, CCL12, and CCL20 mRNA accumulation, while JNK inhibition significantly suppressed CCL3, CCL4, CCL5, and CCL9. Suppression of CCL3 and CCL4 by JNK and p38 accounted for the effects seen with the combination of all three MAPK inhibitors. Likewise, suppression of CCL5 by ERK and JNK accounted for the combined effects of all three MAPK inhibitors, while

suppression of CCL8, CCL12, and CCL20 by p38 signaling accounted for the effects of all three MAPK inhibitors combined. The diverse regulation of chemokines by MAPK signaling suggested specificity of pharmacological inhibitors. Interestingly, CCL3, CCL4, CCL5, CCL8, and CCL9 were also significantly suppressed in the presence of NF-κB inhibition by helenalin suggesting that multiple signaling pathways were involved in the regulation of these inflammatory genes. While MAPKs were essential for the upregulation of all the chemokines examined, it played no role in the regulation of interleukin-7 (IL-7).

Role for DUSPs regarding MAPK-dependent chemokine gene expression in response to TNFα. We demonstrated above that all the CC chemokines examined were induced in response to TNFα and downstream of MAPK signaling. Previous observations from our lab (data not shown; Ch.4) demonstrated that dusp1, dusp8, and dusp16 were induced in PAs following transient phosphorylation of all three MAPKs in response to TNFα, while individual knockdown via RNAi led to increased MAPK signaling magnitude and duration. Therefore, we focused additional effort in this study toward understanding a cooperative role of these DUSPs in PAs in response to TNFα. For this, PAs were transfected with pools of oligonucleotides containing siRNAs for dusp1, dusp8, and dusp16 for 72 hrs prior to TNFα stimulation. Pooled siRNAs attenuated TNFα-mediated mRNA induction for each DUSP (Fig.5.5A). Combined targeted knockdown of all three phosphatases resulted in a marked increase in the magnitude and duration of ERK, JNK, and p38 phosphorylation when compared to non-targeting control siRNA (Fig.5.6B).

Data presented above demonstrated that all the proinflammatory CC chemokines examined were MAPK-dependent targets. Therefore, we hypothesized that alterations in

MAPK status observed with DUSP knockdown would exacerbate inflammatory status via upregulation of these proinflammatory genes. To address this question, we examined chemokine expression in the presence of combination DUSP knockdown via qRT-PCR. As DUSP knockdown was essential for early MAPK activity, we chose to focus on those CC chemokines that were rapidly induced ($\leq$2h) in response to TNFα. Thus, CCL5 and CCL8 were not examined in the presence of DUSP knockdown as TNFα-mediated induction of gene expression was markedly delayed (Fig.5.3). While TNFα robustly induced the expression of all inflammatory genes, TNFα-induced CCL2, CCL3, CCL4, CCL9, CCL12, and CCL20 were significantly elevated in the presence of DUSP knockdown (Fig.5.6). However, TNFα-induced IL-7 mRNA expression was not increased with DUSP knockdown (Fig.5.4). As IL-7 was not a downstream target of MAPK signaling, these data suggests that DUSPs modulate MAPK-dependent biological processes involving AT inflammation.

Discussion

This study presents empirical evidence that modulatory actions of DUSPs on MAPK signaling has a profound influence on biological processes involving MAPK-dependent regulation of CC chemokine expression under conditions of inflammatory stress. First, we show phenotypic differences in inflammatory signaling and basal chemokine expression, where MAPK and NF-κB signaling and chemokine mRNA expression were markedly greater in PAs compared to ADs. Second, we demonstrate that stimulation of PAs with TNFα resulted in the induction of all chemokines examined, where most chemokines were inducible in a manner consistent with direct actions of TNFα-mediated signaling. Third, we show that inhibition of NF-κB activity partly

suppressed select chemokines, while all chemokines were downstream targets of MAPK phosphorylation. Finally, we report that increased MAPK signaling duration and magnitude with DUSP knockdown resulted in amplification of TNFα-inducible chemokine gene expression. Collectively, these data demonstrate an important biological role for select DUSPs in the modulation of MAPK signaling and MAPK-dependent biological outcome involving CC chemokine expression, highlighting potential therapeutic targets regarding decreased macrophage infiltration that links obesity with metabolic inflammatory diseases.

It is well established that TNFα plays a critical role in AT inflammation linking obesity with IR (11,12). Cells within AT including PAs, ADs, and macrophages share many functions, including the production and secretion of various cytokines and chemokines in response to TNFα (5,49,50). Moreover, traits primarily attributed to macrophages, such as phagocytosis, have also been shown in PAs (51), while others have shown that PAs can trans-differentiate into macrophage-like cells (51). As transcriptional profiling studies suggest that macrophages and PAs are genetically related (52), other reports demonstrate that the development of new PAs may originate, in part, from AT macrophages (53). Data presented in this investigation demonstrate that MAPK and NF-κB signaling magnitude was markedly greater in PAs compared to ADs, while duration of ERK and JNK signaling was prolonged in ADs in response to TNFα. Moreover, the increase in TNFα-mediated p38 phosphorylation in PAs was not observed in ADs stimulated under the same conditions. While MAPK signaling magnitude highlights the responsiveness of PAs to inflammatory stimulation, the extent of MAPK signaling in ADs may be equally important, as others have shown that both JNK signaling magnitude and duration influence inflammation and insulin signaling (17).

Phenotypic examination of CC chemokine expression further highlights the inflammatory nature of PAs, as five of the eight chemokines examined were significantly higher in PAs compared to ADs. Collectively, these data demonstrate that PAs are more responsive to external inflammatory cues and have a greater inflammatory potential as chemokine expression was markedly greater in this cell type. However, prolonged signaling duration of JNK and ERK as well as high levels of CCL3 and CCL4 mRNA expression in ADs suggests that diverse inflammatory regulation between the different cell types may be equally important in AT inflammation and obesity-induced IR.

Evidence now demonstrates that PAs and hypertrophied ADs secrete increased amounts of the chemokine MCP-1/CCL2 (54) that results in macrophage infiltration, increased AT inflammation, and IR (3,55). Consistently, macrophage accumulation in AT of lean individuals (5-10%) is substantially less than that of obese patients, in which macrophage accumulation can make up to 50% of the total number of cells (22). While chemokine receptor CCR2 deficient mice have severely impaired macrophage accumulation, MCP-1 deficient mice have partial impairment of macrophage accumulation (56), suggesting a role for other CC chemokines in macrophage recruitment. As other CC chemokines can bind to CCR2, it is plausible that increased secretion of MCP-1 and/or other CC chemokines from adipocytes maintain a state of chronic AT inflammation (1). This appears evident as several CC chemokines are upregulated in genetic and dietary models of obesity, while fractionation of adipose tissue from DIO mice demonstrates that CC chemokine expression is primarily secreted from isolated adipocytes (21). In this investigation, we showed that five of the eight CC chemokines examined were expressed more abundantly in PAs compared to ADs. As AT recruitment of macrophages likely represents the major source of elevated TNFα that

imparts AT dysfunction by increasing proinflammatory chemokine gene expression in PAs and insulin resistance in ADs (17), we further show that all eight CC chemokines are induced in response to TNFα-mediated inflammatory stress in PAs. Moreover, we report that TNFα-mediated regulation of CC chemokines resulted in different kinetic profiles of early (i.e., CCL3, CCL4, and CCL12), prolonged (i.e., CCL2 and CCL20), and late (i.e., CCL5, CCL8, and CCL9) induction that may have biological consequences regarding chronic AT inflammation. Collectively, these data suggest a cyclical pattern of macrophage infiltration and adipocyte inflammation resulting in the chronic inflammation associated with obesity.

The rapid mRNA accumulation observed for early, inducible CC chemokines suggests a direct role for TNFα-mediated inflammatory signaling potentially involving MAPKs and NF-κB, while the late induction of CCL5 and CCL8 suggest secondary mechanisms possibly involving autocrine/paracrine feedback from TNFα-induced inflammatory mediators. While cellular signaling cascades play a pivotal role in linking external environmental cues like inflammation to cellular outcome such as IR, MAPKs are recognized for their innate ability to couple inflammation to macrophage recruitment and obesity-induced IR (17). Evidence supports the hypothesis that all three MAPK family members may be activated in response to external stress from proinflammatory cytokines (24-26). Furthermore, reports demonstrate that MAPK signaling is essential for select CC chemokine expression in 3T3-L1 adipocytes stimulated with free fatty acids (21). While we demonstrate that pharmacological inhibition of NF-κB significantly suppressed select CC chemokines gene expression, we further show that all chemokines examined were downstream targets of MAPK phosphorylation. Thus, regulation of select CC chemokines by both MAPK and NF-κB pathways suggest that

multiple signaling pathways can regulate chemokine expression. However, regulation of IL-7 was predominantly downstream of NF-κB and not MAPK signaling, suggesting that pharmacological inhibition was target-specific. As macrophages primarily contribute to TNFα secretion (57,58), these data suggest that MAPK signaling couples TNFα to CC chemokine expression creating a feedback loop involving PA recruitment of macrophages, ultimately contributing to chronic AT inflammation.

Until recently, upstream kinases rather than phosphatases were traditionally viewed as major regulators of MAPK signaling. However, evidence now demonstrates that DUSPs can modulate and even control MAPK signaling in response to inflammation (37). Since the initial identification of dusp1, various DUSPs have been examined as a feedback control mechanism governing the production of proinflammatory chemokines and cytokines by limiting MAPK activity (37,59-61). DUSP knockout animals lend further support to the critical need for these phosphatases in the restraint of the immune response during infection, where animals present with an increased proinflammatory profile and susceptibility to infection (37,42,43,62). In adipocytes, suppression of dusp1 and increased activation of ERK leads to increased secretion of MCP-1, while adenoviral-mediated overexpression of dusp1 markedly decreased MCP-1, suggesting that down-regulation of dusp1 plays a critical role in macrophage recruitment within AT (44). In this report, we demonstrate that knockdown of select DUSPs (i.e., dusp1, dusp8, and dusp16) significantly increased mRNA expression of CCL2, CCL3, CCL4, CCL9, CCL12, and CCL20, concomitant with increased MAPK signaling magnitude and duration under conditions of TNFα-mediated inflammatory stress. Moreover, we report that DUSP knockdown did not affect TNFα-induced IL-7 mRNA expression. As inhibitor data presented above demonstrates that IL-7 was not downstream of MAPKs, these

data suggest that DUSP-mediated effects on chemokine expression were MAPK-specific. Collectively, these data demonstrate that DUSPs act as key regulators of inflammatory stress in adipocytes, potentially influencing AT macrophage accumulation and inflammation.

In summary, data outlined here and elsewhere (37,39) demonstrate the importance of MAPK phosphorylation/dephosphorylation in the regulation of inflammatory signaling and inflammatory status. We show that all CC chemokines were induced in adipocytes in response to TNFα, suggesting a role for PAs in macrophage recruitment contributing to chronic AT inflammation. As all CC chemokines were downstream targets of MAPK signaling, loss of DUSP function resulted in amplification of TNFα-induced MAPK-dependent CC chemokine expression. As work on DUSP regulation and function continues, elucidation into the function of these phosphatases may provide therapeutic targets for the treatment and prevention of obesity-mediated inflammatory diseases.

Table 5.1. DUSP and chemokine genes analyzed in this study.

symbol	name/alias	GP	MKB	NLS	NES	accession	ABI number	C_T
dual-specificity phosphatases								
Dusp1	MKP-1, hVH1	I	●	●		NM_013642.3	Mm00457274_g1	24
Dusp8	M3/6, hVH5	III	●	●	●	NM_008748.2	Mm00456230_m1	32
Dusp16	MKP-7, MKP-M	III	●	●	●	NM_130447	Mm00459935_m1	26
'CC' chemokines								
Ccl2	MCP-1, Scya2					NM_011333	Mm00441242_m1	21
Ccl3	MIP-1α, Scya3					NM_011337	Mm99999057_m1	36
Ccl4	MIP-1β, Scya4					NM_013652	Mm00443111_m1	34
Ccl5	Rantes, Scya5, TCP228					NM_013653	Mm01202428_m1	29
Ccl8	MCP-2, Scya8, HC14					NM_021443	Mm01297184_g1	28
Ccl9	MIP-1γ, MRP-2, Scya9					NM_011338	Mm00441260_m1	24
Ccl12	MCP-5, Scya12					NM_011331	Mm01617100_m1	34
Ccl20	MIP-3A, Scya20, exodus-1					NM_106960	Mm00444228_m1	32
Ccl20						NM_001159738		
reference gene								
18S	18 ribosomal RNA					X03205.1	4342930E	9

Table 5.1. DUSP and chemokine genes analyzed in this study. DUSPs that contained the MAPK binding domain (MKB) were assigned to groups (GP) based on structural differences involving the presence or absence of a nuclear localization sequence (NLS), nuclear export sequence (NES), or both that may dictate subcellular localization to the nucleus (group I), cytosol (group II), or could localize to either compartment (group III).

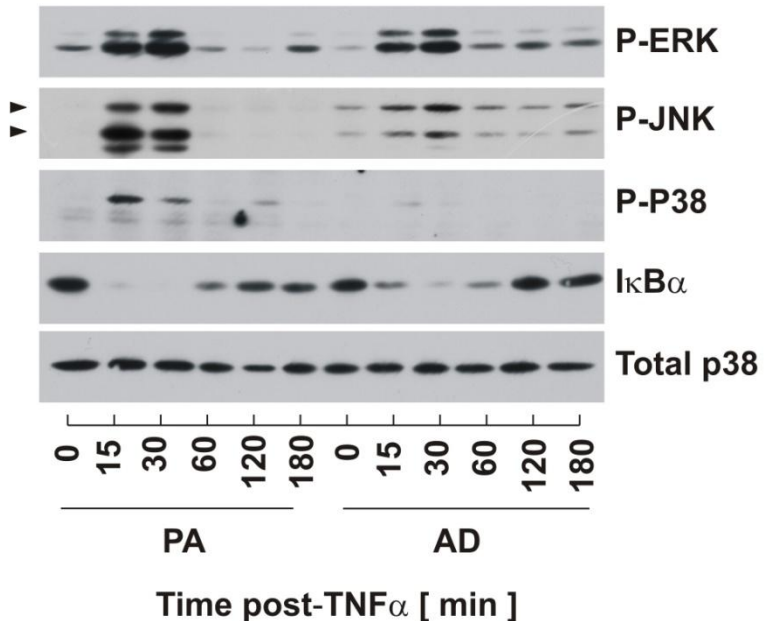

Figure 5.1. Phenotypic differences in MAPK signaling and IkBα degradation in response to TNFα. Cell lysates were harvested over time from 3T3-L1 preadipocytes (PA) and mature (d8) adipocytes (AD) stimulated in parallel with 100 pM TNFα. Phosphorylated state of ERK, JNK, and p38 along with degradation of IkBα was assessed in both cell lineages by immunoblotting.

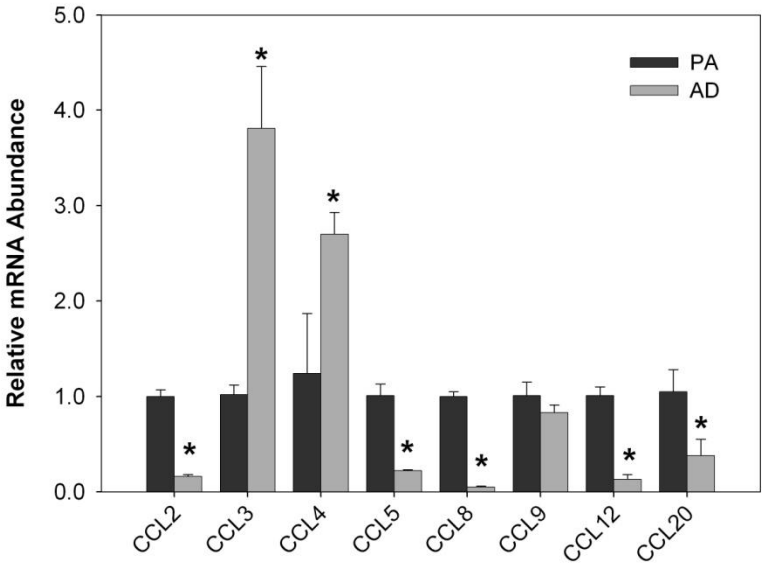

Figure 5.2. Basal chemokine expression in PAs versus ADs. mRNA expression of select CC chemokines was measured in untreated PAs (d0) and mature ADs (d8) using qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated PAs (d0). Statistical significance was determined by Student's *t*-test (***** $p<0.05$).

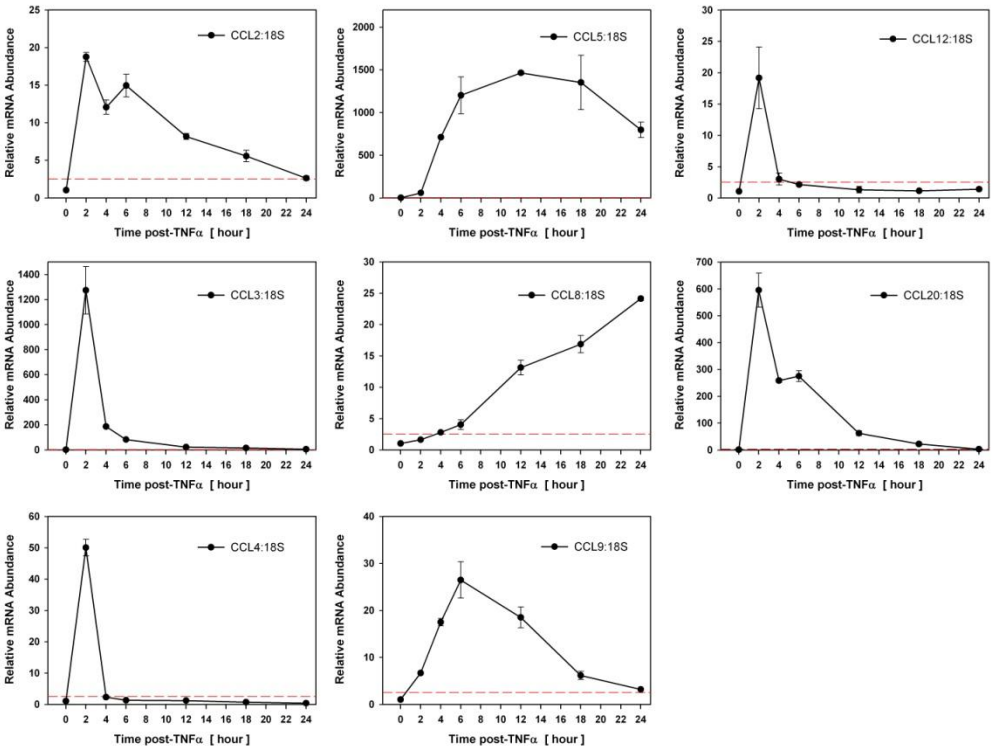

Figure 5.3. TNFα-mediated regulation of chemokine mRNA expression in PAs. Total RNA was harvested from preadipocytes stimulated in parallel with 100 pM TNFα over time and gene expression of select CC chemokines analyzed with qRT-PCR. All data were normalized to 18S rRNA and expressed relative to unstimulated PAs.

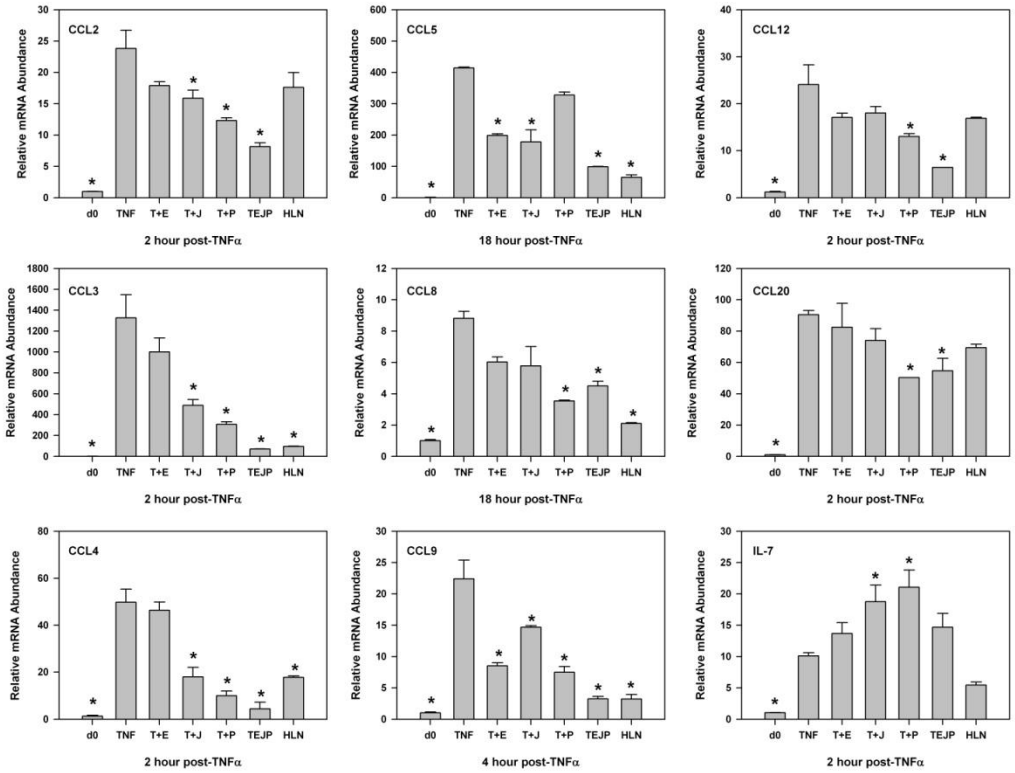

Figure 5.4. MAPK and NF-κB signaling mediate TNFα induced chemokine mRNA expression. Total RNA was harvested at 1h, 2h, 4h, and 18h post TNFα stimulation in the absence or presence of inhibitors for ERK (U0126; 10 μM), JNK (SP600125; 20 μM), p38 (SB203850; 10 μM), or helenalin (HLN; 10 μM). Nomenclature related to the graph is assigned: T (TNFα), T+E (TNFα + U0126), T+J (TNFα + SP600125), T+P (TNFα + SB203850), TEJP (TNFα +U0126+SP600125+SB203850), or T+HLN. mRNA was assessed for select CC chemokine and IL-7 expression using qRT-PCR and values expressed as fold differences relative to untreated PAs. All data were normalized to 18S rRNA and statistical significance determined by ANOVA, with Dunnetts post-hoc analysis performed to assess differences from control (TNFα) when $p < 0.05$.

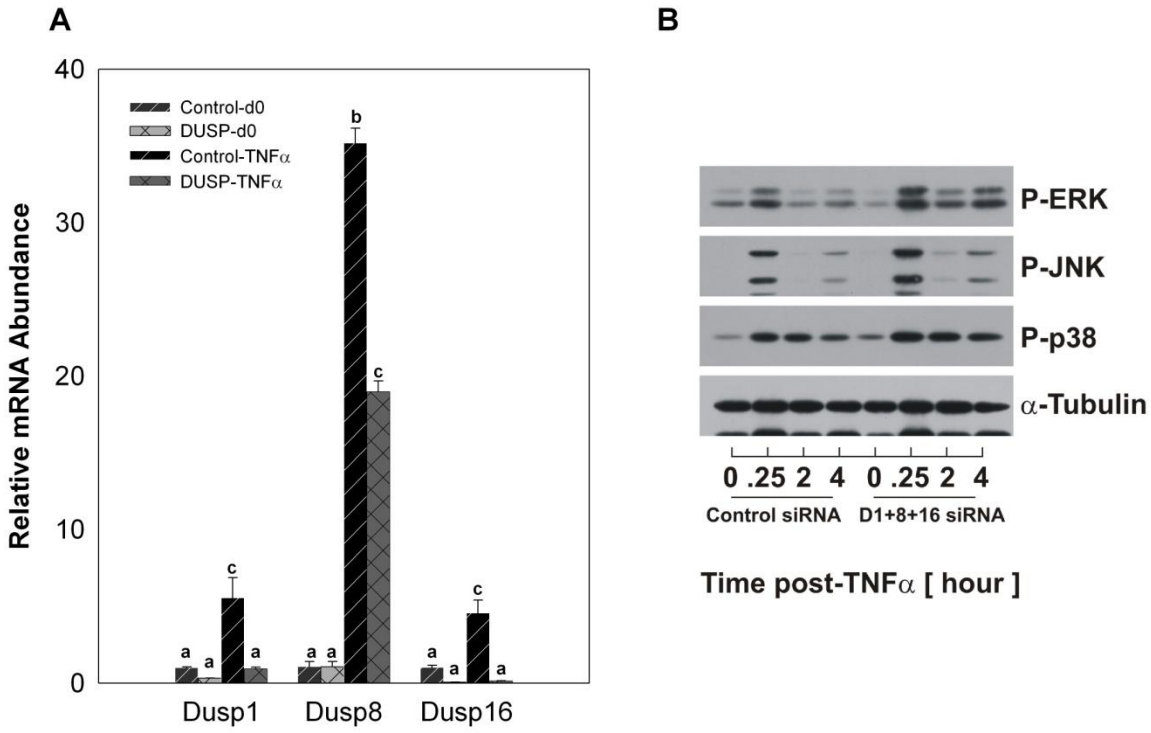

Figure 5.5. Combination DUSP knockdown amplifies MAPK signaling magnitude and duration. Preadipocytes were transfected with DharmaFect 3 transfection reagent in the presence of 100 nM non-targeting control siRNA or 100 nM siRNA specific for dusp1, dusp8, and dusp16 for 72 hours prior to stimulation with 100 pM TNFα. A) Total RNA was harvested at d0, 1h, or 2h post-TNFα stimulation and mRNA expression of dusp1 (1h), dusp8 (1h), and dusp16 (2h) analyzed via qRT-PCR. Data were normalized to 18S rRNA and changes in gene expression measured as fold differences relative to untreated control siRNA (d0). Statistical differences were determined by ANOVA. Tukey's post-hoc analysis was performed when the *p* value for the respective parameter was statistically significant ($p < 0.05$). B) Cell lysates were collected over time post-TNFα stimulation for control siRNA or combination DUSP siRNA and protein expression of bisphosphorylated ERK, JNK, and p38 along with α-Tubulin examined via immunoblotting.

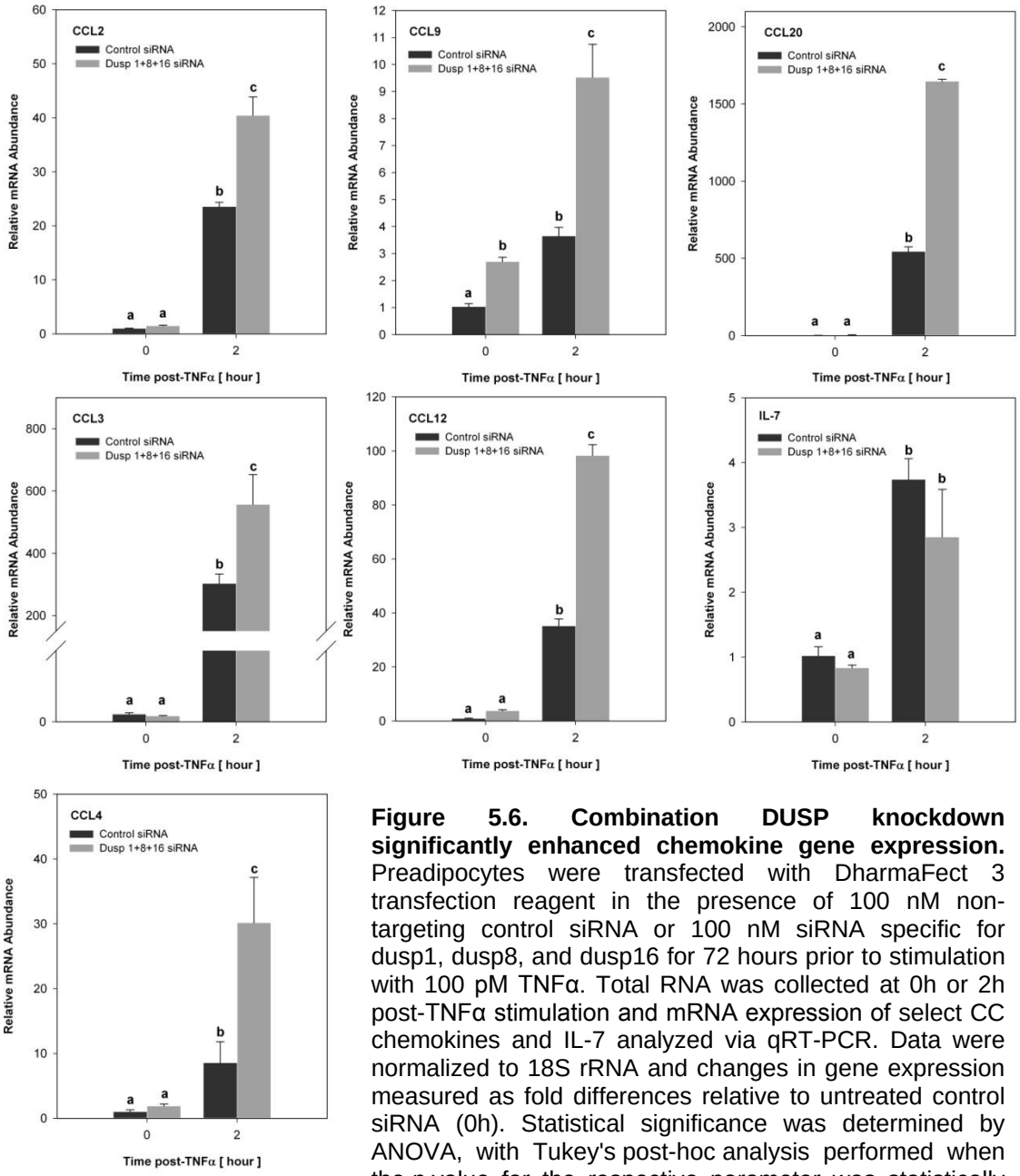

Figure 5.6. Combination DUSP knockdown significantly enhanced chemokine gene expression. Preadipocytes were transfected with DharmaFect 3 transfection reagent in the presence of 100 nM non-targeting control siRNA or 100 nM siRNA specific for dusp1, dusp8, and dusp16 for 72 hours prior to stimulation with 100 pM TNFα. Total RNA was collected at 0h or 2h post-TNFα stimulation and mRNA expression of select CC chemokines and IL-7 analyzed via qRT-PCR. Data were normalized to 18S rRNA and changes in gene expression measured as fold differences relative to untreated control siRNA (0h). Statistical significance was determined by ANOVA, with Tukey's post-hoc analysis performed when the *p* value for the respective parameter was statistically significant ($p < 0.05$).

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CHAPTER VI

EPILOGUE

Data presented in this dissertation represent the first empirical evidence demonstrating that regulation of the extent of MAPK signaling by inducible MAPK-specific DUSPs impacts biological processes downstream of MAPK activation in 3T3-L1 adipocytes. Findings presented in Ch.2 collectively demonstrated that DUSPs were abundantly expressed in adipose tissue (AT) and divergently regulated under conditions of genetic and diet-induced obesity that is associated with AT inflammation and insulin resistance (IR). Furthermore, data from Ch.3 demonstrated kinetic differences in DUSP regulation throughout adipocyte differentiation concomitant with the timely modulation of MAPK signaling and adipogenesis, suggesting a role for DUSPs in the development of obesity. Finally, findings from Ch.4 and Ch.5, demonstrated an essential role for DUSPs on the extent of MAPK signaling and subsequent regulation of MAPK-dependent proinflammatory genes involved in macrophage recruitment, AT inflammation, and IR under conditions of inflammatory stress. Collectively, data presented in this dissertation provide seminal evidence for the regulatory actions of DUSPs on MAPK-dependent biological processes within the adipocyte lineage, potentially linking obesity with metabolic inflammatory diseases.

While this dissertation established a role for DUSPs on MAPK signaling, findings from the previous chapters, along with other observations made by our lab, have generated additional questions for future investigation. These preliminary findings and potential areas of focus will be discussed in detail in this chapter. Data presented in this

dissertation demonstrated that dusp4 was only induced during early genetic obesity, absent of TNFα-mediated inflammation (Ch.2). Consistent with this observation, we further observed that that mRNA expression of dusp4 was markedly upregulated during mid-stage adipocyte differentiation (Ch.3), concomitant with ERK dephosphorylation and peroxisome proliferator-activated receptor γ (PPARγ) induction. Preliminary findings from our lab further demonstrated that regulation of dusp4 required the same activators of adipocyte differentiation as that of PPARγ. PPARγ is a master regulator of adipocyte-specific gene expression (1-3). As ERK dephosphorylation is required for PPARγ activity and adipocyte differentiation (4), it is plausible that dusp4 modulates ERK dephosphorylation critical for adipogenesis. Furthermore, we demonstrated that dusp4 was suppressed in preadipocytes (PAs) and adipocytes (ADs) under conditions of TNFα-mediated inflammation (Ch.4). In addition to its role in adipogenesis, suppression of PPARγ expression and activity in response to inflammatory stress results in adipocyte dysfunction, consequently leading to decreased triglyceride storage, increased fatty acid release, and IR (5). Thus, inflammatory-mediated suppression of dusp4 potentially alters MAPK-dependent regulation of PPARγ activity and adipocyte function. An interesting addition to the story would be to examine a role for dusp4 on MAPK signaling and PPARγ activity in differentiating and fully differentiated adipocytes as well as examine a role for this phosphatase under these conditions in response to inflammatory stress. As loss of dusp4 may negatively affect PPARγ activity and adipocyte function, in vivo investigations of dusp4 knockout animals under conditions of obesity may highlight phenotypic changes regarding inflammation and insulin signaling. Future examination of dusp4 would ultimately expand our knowledge regarding adipogenesis and adipocyte function.

We also presented data that demonstrated a role for early inducible DUSPs on MAPK signaling during adipocyte differentiation (Ch.3). However, time constraints prohibited loss-of-function studies investigating the direct role of these DUSPs individually and cooperatively on timely modulation of MAPK signaling and preadipocyte replication and differentiation. As transient MAPK signaling during early adipocyte differentiation is essential for adipogenesis (6), future examination of the regulatory actions of early DUSPs would add to our understanding of early signaling processes involved in adipocyte differentiation. In addition to the studies addressing a role for early inducible DUSPs during differentiation, examination of the late-stage inducible dusp9 (Ch.3) also poses interesting questions regarding MAPK signaling and adipocyte differentiation. Observations from this dissertation showed that dusp9 was upregulated with genetic and diet-induced obesity (Ch.2) and not affected by TNFα-mediated inflammation in ADs (Ch.4). While MAPK signaling is suppressed in mature ADs (Ch.3), it is conceivable that induction of dusp9 maintains MAPK suppression and therefore maintains the adipocyte phenotype. Loss-of-function studies examining dusp9 under conditions of differentiation, obesity, and inflammation in vitro and in vivo would add to our understanding of the regulatory actions of these phosphatases on MAPK-dependent physiological outcomes (e.g., metabolic dysfunction).

Data highlighted in Ch.3 further showed that dusp1 increased the extent of MAPK signaling during early adipocyte differentiation. Preliminary observations from our lab demonstrated that mRNA induction of dusp1 during early adipocyte differentiation occurred in the presence of methyl-isobutylxanthine (MIX), a component of our differentiation cocktail that primarily regulates cAMP/PKA activity and its downstream transcription factors, cAMP-response element binding protein (CREB) and

CCAAT/enhancer-binding protein β (C/EBPβ) (7,7,8). Subsequently, we observed that treatment with the cAMP/PKA inhibitor H-89 led to a marked and significant decrease in dusp1 during early adipogenesis, suggesting that cAMP/PKA regulates dusp1 mRNA expression. While C/EBPβ is a downstream transcription factor regulated by the cAMP/PKA signaling pathway, others report that C/EBPβ regulates dusp1 in other cell types (9). However, we observed that loss-of-function did not attenuate dusp1 expression, suggesting that C/EBPβ does not regulate dusp1. Similar to C/EBPβ, CREB is a downstream target of cAMP signaling (7) and a regulator of dusp1 in other cell types (10). Preliminary observations from our lab demonstrated that CREB loss-of-function significantly increased dusp1 mRNA and protein expression, suggesting that CREB indirectly regulates MAPK signaling via dusp1 suppression during early adipocyte differentiation. As others report a critical role for CREB in the early regulation of adipogenesis (7), future examination of CREB-mediated dusp1 regulation may highlight a role for transcription factors on feedback regulation of signaling cascades essential for differentiation. Thus, elucidating functional CREB binding sites within the dusp1 promoter, as well as examining CREB and dusp1 loss- and gain-of-function studies would delineate a role for CREB-mediated suppression of dusp1 on MAPK-dependent adipogenesis.

While data presented above generated questions regarding dusp1 mRNA expression, other observations from our lab led to questions regarding dusp1 protein stability. Preliminary findings from our lab demonstrated that MIX was sufficient to induce dusp1 mRNA expression, but protein accumulation only occurred when dexamethasone or insulin was present. As ERK has been shown to phosphorylate dusp1 on S359 and S364 and stabilize the protein from degradation (11), we examined the role of MAPKs on

dusp1 protein stability. Inhibition with all three MAPK inhibitors, SB203850 (p38), UO126 (ERK), or SP600125 (JNK) demonstrated a potential role for p38 in the regulation of dusp1 protein stability (Fig.6.1), where inhibition led to decreased protein independent of gene suppression. Using cyclohexamide to inhibit protein synthesis, we further observed that dusp1 half-life markedly decreased with ERK or p38 inhibition during early adipocyte differentiation, suggesting that kinase-mediated phosphorylation of DUSPs is essential for protein stability. While ERK phosphorylation has been previously shown to stabilize dusp1 protein, investigations of p38 remain unknown. Thus, investigations examining p38-mediated phosphorylation of dusp1 would increase our understanding of dusp1 regulation. For instance, is p38 needed for dusp1 stability, and if so, which phospho-specific site(s) on dusp1 are essential to prevent protein degradation?

Observations presented above highlight future studies involving DUSPs on MAPK signaling during adipogenesis. In this dissertation, we have also identified individual DUSPs that markedly increased MAPK signaling and MAPK-dependent nuclear events involving cytokine and chemokine gene expression in PAs in response to inflammation (Ch.4&5). However, a role for inducible DUSPs on MAPK signaling and biological outcome remains unknown in ADs. Loss-of-function examination of DUSPs on MAPK signaling and MAPK-dependent processes involving inflammation and IR in ADs would prospectively highlight therapeutic targets linking obesity with inflammatory diseases. Furthermore, as MAPK activation can influence cellular fate involving the regulation of cytosolic or nuclear events, examination of compartmentalized DUSP actions in both PAs and ADs would highlight potential therapeutic targets that uncouple MAPK signaling in the cytosol (i.e., insulin signaling) from those events in the nucleus (i.e., inflammatory gene expression).

While we showed that TNFα-mediated inflammation induced individual DUSPs in PAs and ADs, we further showed that several DUSPs were suppressed in response to inflammation (Ch.4). This observation generated additional questions regarding the role of DUSP suppression on MAPK activation and cellular outcome in PAs and ADs in response to inflammation. While this dissertation was limited to investigations of inducible DUSPs, gain-of-function studies involving suppressed DUSPs would contribute to our understanding how phosphatases participate in the balanced regulation of phosphorylation/dephosphorylation that is critical in determining cellular outcome. Moreover, recent research has shown that the anti-inflammatory potential of phytochemicals is mediated in part through the suppression of MAPK signaling in response to inflammatory stress (12), while others have shown that DUSPs contribute to the MAPK-dependent actions of these phytochemicals (13). Therefore, investigation of bioactive food components on the regulation of induced and suppressed DUSPs will potentially link anti-inflammatory actions of phytochemicals with MAPK suppression and improved inflammatory status and insulin signaling in ADs.

While most of our future questions developed from observations related to this dissertation, other questions regarding DUSPs have developed from other studies undertaken in our lab. Recent focus in our lab has begun to address the role of histone deacetylases (HDACs) on the epigenetic regulation of MAPK signaling and inflammation. Anti-inflammatory actions have been ascribed to HDAC inhibitors in vitro and in vivo, mediated in part through select class I and II HDACs (14,15). While many of these anti-inflammatory properties appear to be driven by epigenetic regulation of gene transcription (16), new evidence suggest that lysine acetylation-deacetylation contributes to signaling regulation in part through acetylation-mediated increases or decreases in

phosphorylation (17). Furthermore, reports are emerging involving HDAC regulation of select DUSPs, where HDACs regulate dusp1 and dusp16 expression in response to inflammation (18,19). Preliminary findings from our lab demonstrated that pretreatment of PAs with trichostatin A (TSA), an HDAC class I and IIb inhibitor, for 24 hrs markedly attenuated p38 and ERK signaling, but not JNK. We further observed that TSA pretreatment of <24 hrs did not attenuate MAPK signaling, suggesting a role for new mRNA/protein synthesis in the regulation of phosphorylation. Moreover, pretreatment with TSA was sufficient to ablate TNFα induction of proinflammatory chemokines and cytokines that are downstream targets of MAPK-dependent as well as MAPK-independent pathways, suggesting that anti-inflammatory actions of TSA are mediated in part by MAPK signaling. Based on these preliminary findings, we developed the following working model (Fig.6.1), where HDACs mediate MAPK signaling and MAPK-dependent inflammation through epigenetic regulation of DUSP transcription in response to inflammatory stress.

Data presented in chapters II-V have significantly contributed to the limited body of knowledge regarding DUSPs in adipocytes. Use of the murine 3T3-L1 cell line provided an excellent model to study DUSP regulation and function, as this cell line does not consist of a heterogeneous population of cells (e.g. macrophages, endothelial cells, preadipocytes, adipocytes, etc.) allowing us to delineate signaling events in a homogenous population of PAs or ADs. Moreover, our findings have led to ample questions for future studies related to DUSPs in adipocytes. It is expected that continued examination of DUSPs will provide researchers with a better understanding of signaling networks involved in obesity, inflammation, and IR potentially providing therapeutic targets for the treatment of obesity-related metabolic inflammatory diseases.

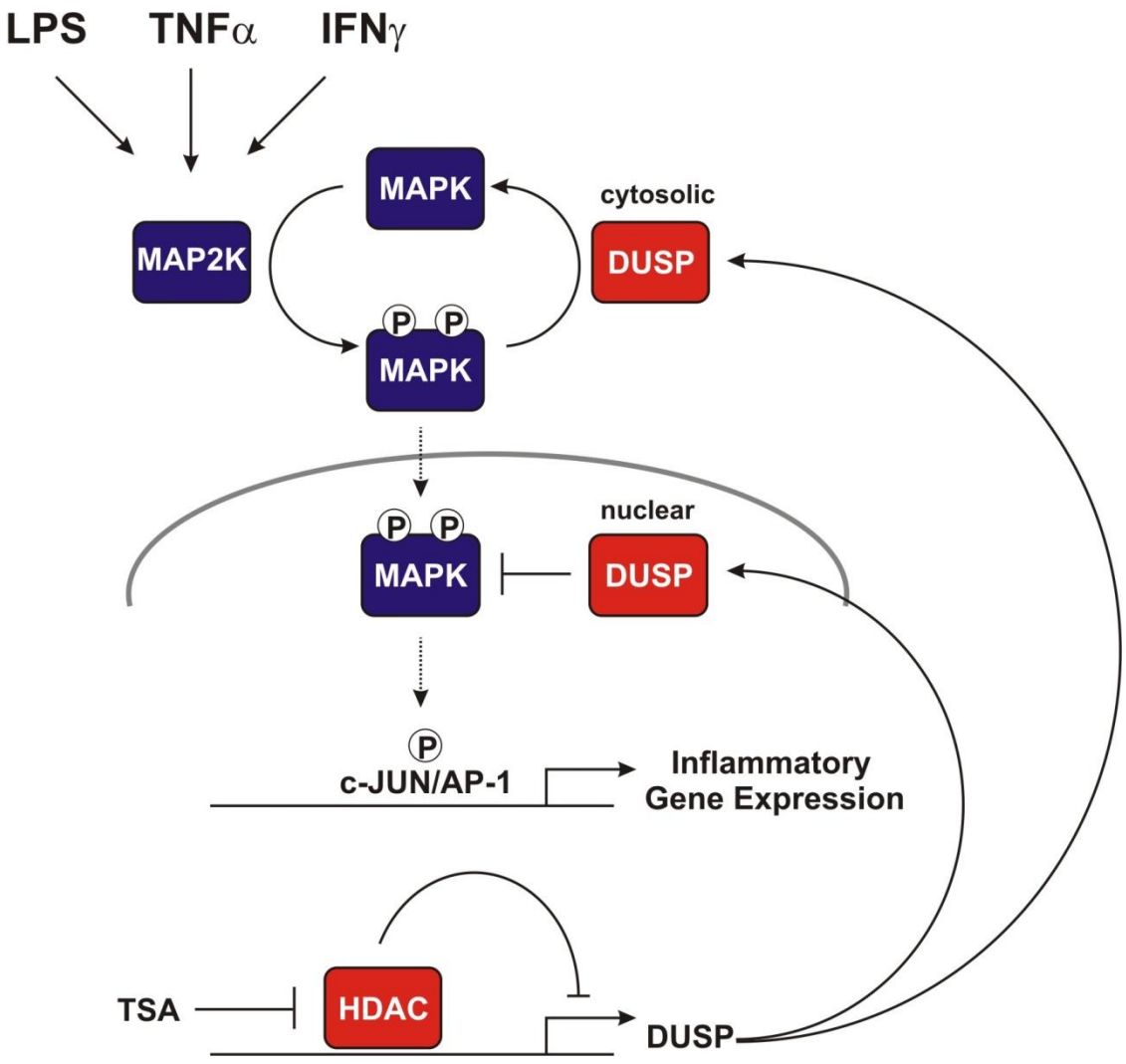

Figure 6.1. Working model examining the role of HDACs on MAPK signaling through epigenetic regulation of DUSP transcription.

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