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The growing prevalence of obesity has resulted in a huge impact on global health. Obesity contributes to the development of cardiovascular diseases, insulin resistance (IR) and other comorbidities. Research over the past few decades has revealed important roles for adipose tissue (AT) in regulating whole-body metabolism under normal physiological and pathological states. It is well accepted that obesity-mediated chronic, low-grade inflammation contributes to the development of metabolic dysfunctions via the mitogen-activated protein kinase (MAPK) signaling pathways. While the upstream activation of MAPKs has been extensively investigated, how MAPK deactivation modulates cellular responses remains largely unsolved. In recent years, increasing attention has been given to the MAPK-specific, dual-specificity phosphatases (DUSPs) as key regulators that negatively control MAPK activity, potentially provide treatment against obesity-induced AT inflammation and IR.

Early evidence has suggested a role for DUSP4 towards MAPKs signaling in other cell types. However, no study has addressed the role of DUSP4 in 3T3-L1 adipocytes under any condition. Data presented in this dissertation demonstrate that DUSP4 was induced via the MEK/ERK signaling pathway while JNK or p38 did not affect DUSP4 expression. Subsequently, we show that DUSP4 protein was also stabilized by ERK activation and proteasomal activity was partially involved in its protein degradation. Moreover, the accumulation of DUSP4 correlated with the suppression of nuclear ERK

signal and protein-protein interactions were observed between DUSP4 and ERK, suggesting a role for this phosphatase in spatiotemporal regulation of ERK activity in adipocytes.

We further demonstrate that DUSP4 is suppressed in AT under conditions of genetic and diet-induced obesity that is associated with increased inflammation and IR. We report biphasic inductions of DUSP4 during adipocyte differentiation, with the early phase induction being ERK-dependent and the later phase induction being ERK-independent and differentiation-specific. Moreover, DUSP4 was suppressed in preadipocytes and adipocytes exposed to tumor necrosis factor-α. Additionally, DUSP4 knockdown did not inhibit adipocyte differentiation, suggesting a role for this phosphatase that is independent from adipogenesis. Collectively, data presented in this dissertation provide new insights into regulation and potential function of DUSP4 in adipocytes, highlighting new therapeutic targets for the treatment of obesity-mediated inflammation and metabolic disorders.

NOVEL ROLE FOR DUAL-SPECIFICITY PHOSPHATASE 4 IN ADIPOCYTES

by

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Approved by

_____
Committee Chair

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

APPROVAL PAGE

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

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CHAPTER I

INTRODUCTION

Significance of Research

Obesity is the most prevalent nutrition-related health concern that leads to the development of many chronic diseases, such as cardiovascular disease, hypertension, cancers, and insulin resistance (IR). Globally, over 600 million people are obese. Obesity also contributes to the development of diabetes, and the rates are expected to increase from 415 million to 642 million between 2015 and 2040. As obesity and diabetes greatly threaten health care spending and economy growth, advanced therapeutic strategies against these diseases will be very important for our future generations. Early evidence has demonstrated that obesity-induced inflammation leads to IR, a key etiological factor for type 2 diabetes mellitus (T2D). Since then, numerous studies have established a pivotal role for the mitogen-activated protein kinases (MAPKs) in modulating obesity-induced IR due to inflammatory stress response mediated through MAPK signaling pathways. While studies have largely examined upstream signaling for MAPK activation, few have addressed how MAPK deactivation alters biological outcomes, which could potentially provide novel targets against obesity-associated metabolic diseases. The MAPK-specific Dual-specificity phosphatases (DUSPs) are known to negatively regulate MAPK signaling. Although early studies have investigated the role of DUSPs in various

types, information regarding the regulation and function of these DUSPs, especially DUSP4 in adipocytes remains limited. Collectively, the proposed research is significant, as it establishes novel mechanisms that characterize functional significance of DUSP4 in adipocytes, providing a platform for the development of potential therapeutic strategies against obesity-associated IR. The objective of this dissertation is to determine the regulation and mechanistic role of DUSP4 in 3T3-L1 adipocytes under conditions of agonist-specific MAPK activation, obesity-mediated inflammation and differentiation. The literature relevant to this research project is reviewed below.

Review of Literature

Obesity and Diabetes. Obesity is defined as excessive fat accumulation occurs to an extent that affect an individual's health conditions. The prevalence of obesity and overweight has been increasing at an alarming rate worldwide, with the number rising from 857 million in 1980 to 1.9 billion in 2014 [1-3]. It is a major risk factor for the development of many chronic diseases, such as cardiovascular disease, hypertension, stroke, cancers, arthritis, IR and diabetes [1-6]. In addition to these serious health consequences, obese individuals have been reported to have an impaired quality of life and shortened life expectancy by up to 8 years [2, 6]. The World Health Organization (WHO) lists obesity as one of the five leading causes of mortality in the world [7]. While the onset and development of obesity have been attributed to genetics, ethnicity, culture and socioeconomic status, it is generally accepted that excess energy intake and

inadequate physical activity together contribute to the expansion of adipose tissue (AT) [8, 9]. According to the Centers for Disease Control and Prevention (CDC), in the United States, more than 70% of adults aged 20 or older are overweight and almost 37% are clinically obese or having a body mass index (BMI) of 30 kg/m^2 or higher [10, 11]. Meanwhile, the estimated annual health care costs of obesity-related illness are a staggering $190.2 billion, which accounts for more than 20% of annual medical spending in the United States [12]. Researchers have further projected that by 2030, if obesity rates continue to increase, obesity-associated medical costs will rise by $48 to $66 billion a year in the U.S [13].

Similar to obesity, diabetes is another major health problem that affects an individual's quality of life, decreases life expectancy and increases health care spending. Diabetes refers to a group of chronic metabolic diseases characterized by high levels of blood glucose resulting from defects in insulin production, insulin response or both [14, 15]. The global epidemic of obesity has been shown to largely contribute to the rapid increase in the prevalence of diabetes [14, 16]. The number of adults with diabetes worldwide has quadrupled from 108 million in 1980 to 422 million in 2014, and this number is still expected to reach 642 million by 2040 [17-19]. The growing prevalence of diabetes is also associated with increased incidence of cardiovascular diseases, stroke, kidney failure, neuropathy and amputation. In the U.S., prevalence estimate from CDC concluded that approximately 29.1 million Americans are affected by diabetes (i.e., fasting blood glucose (FBG) > 126 mg/dL), while another 86 million have prediabetes

(i.e., FBG > 126 mg/dL) [20, 21]. Among those diabetic patients, T2D accounts for 90-95% of all diagnosed cases [22, 23]. The estimated annual diabetes-related medical costs have risen to $332 billion and is projected to reach $490 billion by 2030 [24].

These statistics clearly indicate that obesity and diabetes are major causes of morbidity and mortality and place a tremendous burden on the health care system. Whereas these diseases were once considered as health problems of adults, becoming more prevalent with aging, they have now become health problems among children, due to the increased incidence in childhood obesity [25, 26]. Therefore, reversing the epidemic of obesity and diabetes is of utmost important. Increased knowledge concerning mechanisms involved in the modulation of obesity-induced IR will become crucial for disease prevention and treatment for our future generations.

Adipose Tissue as an Endocrine Organ. Obesity is the consequence of unhealthy and excessive AT expansion. AT is a highly innervated and complex organ that is mainly composed of mature adipocytes (ADs), preadipocytes (PAs) and various types of other cells, including fibroblasts, macrophages, endothelial cells, nerve cells, smooth muscle cells and lymphocytes [27, 28]. Historically, AT is mostly recognized as a depot in human body to store extra energy in the form of triglycerides during food abundance, and to release free fatty acids as energy supply in times of starvation for survival. Another function of AT is to act as an insulating layer that helps the body to maintain its core temperature. In addition, AT also provides protective padding and support around

major inner organs [27, 29, 30]. For centuries, obesity was even regarded as a sign of well-being or a symbol of higher social status by many societies [27].

Since the beginning of twentieth century, the biology and functionality of AT started to draw growing attention, with accumulating evidence demonstrating that AT has a much more complex function beyond a repository for storing excess energy. A German doctor first suggested a role for AT in glycogen storage in 1905 [27]. Research conducted in the late 1980s identified AT as a major site of metabolism for sex hormones and secretion of adipsin, an endocrine factor found to be significantly suppressed with the development of obesity [31, 32]. A few years later, the identification and characterization of leptin, a hormone predominantly produced in AT that regulates whole-body energy homeostasis, led to the confirmation of AT as an active endocrine organ [33]. It is now well-recognized that AT expresses and secretes a variety of peptides, hormones, growth factors, cytokines and other biologically active molecules [34]. The production of each molecule is precisely controlled and this balance allows AT to regulate energy metabolism, immunity and vascular homeostasis in tissues such as liver, muscle, kidney and heart [27-30, 35]. While a number of factors have been found to affect AT function and trigger IR, abnormal expression of pro-inflammatory genes has been clearly identified as a crucial event coupling obesity to IR.

Inflammation, Obesity and Insulin Resistance. Numerous studies over the past two decades have linked inflammation within AT to IR and subsequently T2D. Unlike acute inflammation that occurs quickly in response to a body injury and typically induces

pain and swelling, inflammation associated with obesity is a chronic, low-grade metabolic condition that can lead to insidious results. Evidence of the association between inflammation and IR can be dated back to over a century ago, however, the idea of IR and its role in the development of T2D was not well recognized due to inconclusive findings during that time [36]. A few decades later, several investigators reported that increased levels of inflammatory markers and mediators such as C-reactive protein (CRP), interleukin-6 (IL-6), fibrinogen and plasminogen activator inhibitor-1 correlate with occurrence of T2D [37-41]. Treatment with a high-dose of the anti-inflammatory drug, sodium salicylate, ameliorated IR and improved glucose tolerance in type 2 diabetic patients [36, 42, 43]. These observations suggested that inflammation is highly involved in the pathogenesis of T2D.

Prior to the identification of leptin, Hotamisligil and colleagues reported the first molecular evidence linking inflammation to obesity with the discovery that adipose-tissue derived tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, was overexpressed in AT of rodent model of obesity and had been shown to contribute to impaired insulin signaling [44]. These observations were revolutionary because it demonstrated for the first time that a molecule produced by expanding AT had systemic effect on metabolism. Since then, other studies reported elevated level of TNF-α in muscle and AT of obese patients, while weight loss intervention ameliorated circulating levels of TNF-α in the blood [45-47]. Administration of TNF-α has also been reported to suppress insulin sensitivity in models of animal and cell culture studies [48-50], whereas

deletion of TNF-α or its receptors improved insulin sensitivity in obese animals [51-53]. These observations clearly suggested an important role for TNF-α in linking obesity to IR. Interestingly, however, several other studies have reported that chronic TNF-α neutralization or administration of TNF-α antagonist does not improve insulin sensitivity in overweight subjects with metabolic syndrome and IR [54, 55], despite an observed improvement of inflammatory status [55]. The absent effect of TNF-α on insulin sensitivity may be due to a compensatory role of other inflammatory molecules produced by AT. In fact, since the initial identification of TNF-α, several other inflammatory mediators, such as MCP-1 and IL-6 have been shown to increase with obesity and contribute to the disruption of insulin signaling.

Monocyte chemoattractant protein-1(MCP-1)/ Chemokine (C-C motif) ligand 2 (CCL2) is a member of the C-C chemokine family. Since its discovery in 1989, MCP-1 has become one of the most extensively studied C-C chemokine, with interest in its role in the etiologies of obesity-related metabolic problems has grown exponentially over the past two decades [56-59]. MCP-1 acts as a potent chemotactic factor for migration and infiltration of monocytes/macrophages into AT [57]. Macrophage infiltration is a major determinant of AT inflammation, as this process leads to the production of other inflammatory molecules by macrophages [36, 57] (Fig.1.1). Similar to TNF-α, MCP-1 was also overexpressed in AT of animal and human obesity [59-61]. Weight loss significant reduced macrophage accumulation and inflammatory gene expression [60, 61]. When challenged with high fat diet, mice lacking MCP-1 or its cognate receptor CCR2 are

partially protected from IR due to decreased macrophage recruitment into AT, lower pro-inflammatory gene profile, improved insulin sensitivity and reduced susceptibility to obesity compared to their wild-type littermates [58, 62]. Similarly, systemic administration of MCP-1 in mice was shown to induce IR, and this adverse effect was attenuated by a CCR2 antagonist [62, 63]. Consistent with these observations, an in vitro study on 3T3-L1 ADs revealed that MCP-1 can disrupt insulin-stimulated glucose uptake and expression of key adipogenic genes [64]. Important findings from a more recent study revealed that other members of the C-C chemokine family, such as MCP-2, MCP-3, are also upregulated in animal models of diet and genetic-induced obesity [59]. Collectively, these results suggest that chemokines produced by AT may play a crucial role in the initiation of adipose inflammatory reactions that lead to IR.

Inflammatory Signaling Pathways and IR. As obesity-mediated inflammation induces the production and secretion of inflammatory mediators in AT that contribute to the development of IR and T2D, identifying key inflammatory signaling pathways is important to our understanding of the root mechanisms involved in obesity-associated metabolic dysfunctions. Since the early 2000s, several studies have reported that the activity of two kinases, IκB-kinase β (IKKβ) and c-Jun N-terminal kinase (JNK), is elevated in metabolic tissues during obesity [65-68]. While IKKβ is a component of the nuclear factor kappa B (NF-κB) pathway and participate in the production of inflammatory cytokines via transcription factor NF-κB, JNK is a member of mitogen-activated protein kinase (MAPK) signaling pathway and capable of activating transcription factor AP-1 for

inflammatory cytokine production [36]. These two signaling pathways are activated in AT in response to inflammatory stress and lead to local IR (Fig.1.1). The produced inflammatory cytokines can continuously activate IKKβ and JNK, leading to a feed-forward amplification mechanism [36, 68].

Under normal physiological conditions, insulin first binds to its tyrosine kinase transmembrane receptor, inducing a conformational change of the receptor that leads to auto-phosphorylation on a number of tyrosine residues. The activated insulin receptor then mediates phosphorylation of selected tyrosine residues on members of the IRS family, such as IRS-1, IRS-2 and IRS-3. Tyrosine phosphorylated IRS-1, for example, then activates the phosphoinositide 3-kinase (PI3K/Akt) pathway, which stimulates translocation of glucose transporter 4 (GLUT 4) to the plasma membrane which subsequently increases glucose import into the cell [68, 69]. Under the pathological condition of IR, aberrant activation of MAPK signaling impacts cytosolic insulin signaling by phosphorylation of IRS-1 on its inhibitory serine site 307 (S307), leading to an inhibition of IRS-1 tyrosine phosphorylation, which further contributes to disrupted GLUT4 translocation and cellular glucose uptake [68, 70-72]. In addition, inflammatory signals mediated by MAPKs also directly influence RNA and protein synthesis of IRS-1 and GLUT4.

MAPK Signaling Pathways, Obesity and IR. MAPKs are important mediators of signal transduction pathways and play a pivotal role in the regulation of many cellular processes including cell growth and proliferation, differentiation, migration,

inflammation and apoptosis, as well as other short-term hormonal changes required for homeostasis [73-76]. In mammalian cells, activation of MAPK signaling pathways is initiated by a variety of different stimuli, including growth factors, hormones, vasoactive peptides, cytokines and environmental stress factors such as ionizing radiation and osmotic shock [75, 77, 78]. As of today, most of the studies conducted to elucidate the function of MAPKs are focused on three major groups: extracellular signal-regulated kinases (ERKs), JNKs and p38 kinases [73-75, 77, 78] (Fig.1.2). It is generally accepted that ERK is activated by mitogens and growth factors and serves as an important regulator for cell cycle and proliferation. In contrast, JNK and p38 are activated by stress stimuli such as inflammatory cytokines, UV radiation and reactive oxygen species, and are associated with cellular stress and apoptosis [75, 77-80]. Therefore, JNK and p38 are also referred to as stress activated protein kinases [81]. All three MAPK signaling pathways are comprised of a three-tier kinase module (Fig.1.2A) in which a MAPK is activated upon phosphorylation by an upstream MAPK kinase (MAPKK), which is in turn activated when phosphorylated by a MAPKK kinase (MAPKKK) in response to extracellular stimuli [73-75, 77, 78]. MAPKs are dually phosphorylated by MAPKKs on tyrosine and threonine residues of a conserved Thr-X-Tyr motif within the kinase activation loop. Activation of MAPKs leads to the phosphorylation of numerous downstream target proteins and transcription factors within the cytosolic and nuclear compartments that further promote differential cellular outcomes.

The best characterized member of the MAPK family is ERK, which was first discovered over three decades ago for its ability to phosphorylate microtubule-associated protein-2 (MAP-2) in 3T3-L1 ADs under treatment with insulin [80]. The ERK family is comprised the classical ERK1 and ERK2 (referred to as ERK1/2), and several other isoforms [77, 80]. While proteins encoded by ERK1/2 genes have molecular weights of 44 and 42 kDa, respectively, the other isoforms, such as ERK3, ERK5, ERK 7 and ERK8, have relatively larger molecular weights ranging from 60kDa to 110 kDa [82, 83]. All ERK family members contain a conserved TEY (Thr-Glu-Tyr) motif within the activation loop. ERK1/2 proteins are ubiquitously expressed, although their relative abundance varies in different tissues [73]. They are nearly 85% identical overall and share most of the signaling activities. However, they are not fully functionally redundant as demonstrated by gene knockout studies [84, 85]. Inactive ERK1/2 are reported to be sequestered by its upstream MAPKK, MAPK/ERK kinase 1 and 2 (MEK1/2), in the cytosol [86]. Phosphorylation of ERK leads to dissociation of the MEK-ERK complex and this activated ERK is then able to phosphorylate cytosolic and nuclear target proteins [77, 86, 87]. As mentioned previously, ERK signaling is well known to be associated with cell survival and proliferation. Nevertheless, studies have also reported that, depending on stimulation and cell type, ERK may act in an opposite manner that leads to inflammation and apoptosis [88, 89].

A decade after the discovery of ERK, JNK was identified as a second member of the MAPKs for its ability to phosphorylate MAP-2 in rodent liver in response to injection

of protein synthesis inhibitor cycloheximide [90]. It was further reported that, following exposure to UV radiation, JNK was able to phosphorylate the transcription factor c-Jun on two serine residues within the N-terminal transactivation domain, thus increasing transcriptional activity of c-Jun [91, 92]. Since then, ten separate JNK isoforms have been identified as the products of three genes: JNK1/SAPKγ, JNK2/SAPKα, and JNK3/SAPKβ, with JNK1 and JNK2 each having two alternative splice sites and JNK3 having one splice site [73, 77, 93]. All JNK isoforms contain a conserved TPY (Thr-Pro-Tyr) motif within the activation loop and are more than 80% identical. While JNK1 and JNK2 are ubiquitously expressed, JNK3 is reported to be predominately found in tissues such as brain, heart and testis [94, 95]. Although the JNK isoforms may share functions in some circumstances, evidence from in vivo gene knock experiments has revealed specific roles for each isoform [96, 97]. In contrast to ERK activation, JNK is known to be directly and robustly phosphorylated by upstream MAPKK: MKK4 and MKK7, in response to inflammatory cytokines and cellular stress such as hyperosmolarity, heat shock, UV radiation, oxidative stress, and DNA damage [93, 98]. There is also evidence demonstrating JNK can be activated to a lesser extent by serum, growth factor, transforming agents, and other non-canonical signaling pathways [73, 77, 99].

Around the same time that JNK was identified, another group of the MAPK family with a molecular weight of 38 kDa was discovered [73, 77]. p38α was the archetypal member of the p38 MAPK family reported simultaneously by three research groups In 1994 as 1) a tyrosine phosphorylated protein present in macrophages treated

with inflammatory cytokine LPS, 2) a molecule that binds to pyridinylimidazole drugs that block the production of pro-inflammatory cytokines, and 3) a reactivating kinase for MAP kinase-activated protein kinase -2 (MAPKAPK2) [100-103]. Since then, three other p38 isoforms have been identified, including p38β, p38γ, and p38δ [104-106]. All p38 family members contain a conserved TGY (Thr-Gly-Tyr) motif within the activation loop and share over 60% structural similarity. While p38α and p38β are reported ubiquitously expressed, the other two isoforms have different tissue expression patterns, where p38γ is expressed primarily in skeletal muscle and p38δ is found in organs such as lung, kidney, pancreas, and small intestine [107]. MKK3 and MKK6 are the upstream MAPKK thought to be mainly responsible for p38 MAPKs activation. Similar to JNK, p38 can be activated in response to a number of stress stimuli including pro-inflammatory cytokines, heat and osmotic shock, UV radiation and oxidative stress [107].

Over the past 15 years, a number of in vivo studies have been conducted to elucidate the mechanism of MAPK signaling in the development of obesity and IR. Evidence has revealed that, compare to wild type littermates, ERK 1 knockout mice were protected against diet-induced obesity and IR by mechanisms involving decreased adipogenesis and increased energy expenditure [79]. In contrast, mice lacking adapter p62, an ERK inhibitor, had increased ERK activity and developed mature-onset obesity and IR [108]. Increased activity of JNK is observed in liver, muscle, and AT during obesity [109]. Deletion of JNK in diet and genetic-induced rodent obesity led to decreased fat accumulation, less ADs formation, enhanced insulin receptor signaling, and improved

insulin sensitivity [110, 111]. Activity of p38 is also elevated in ADs isolated from T2D patients and involves in the suppression of GLUT4 expression [112]. Inhibition of p38 improves glucose uptake [113]. Consistent with these findings, studies further report that both JNK and p38 can also translocate into the nucleus and activate other pro-inflammatory genes, further promoting AT inflammation and IR [114, 115].

MAPK Signaling in Adipogenesis. Hyperplasia (cell number increase) and hypertrophy (cell size increase) are crucial mechanisms for the development of obesity, both of which are dependent on adipocyte differentiation [116]. Therefore, many research labs have extensively investigated the role of MAPKs in normal and pathological adipogenesis [117-123].

The first molecular evidence linking ERK pathway to adipogenesis was published in 1991 when a group of scientists found that ectopic expression of an active Ras mutant led to growth arrest and terminal differentiation of 3T3-L1 PAs in the absence of insulin, while overexpression of an inactive Ras mutant suppressed differentiation [124]. Since the active form of Ras protein is a potent EKR pathway activator, these observations suggested a positive role for ERK in adipogenesis. A subsequent study using antisense technology against ERK also confirmed that ERK was required for 3T3-L1 adipocyte differentiation [121]. Nevertheless, other research groups had demonstrated that ERK could inhibit adipogenesis through suppressing the activity of peroxisome proliferator-activated receptor γ (PPARγ), an transcription factor highly expressed in AT and considered a master regulator of adipocyte differentiation, by direct phosphorylation

[119, 125]. The hypothetical contradictory findings that ERK could both induce and suppress adipogenesis were built into a working model for temporal regulation of ERK. Supporting evidence later demonstrated that, upon adipogenic stimulation, ERK pathway was necessary for the early proliferative phase of differentiation, termed mitotic clonal expansion (MCE) [126]. Blockade of EKR activity with a specific and potent MEK-inhibitor, U0126, during MCE was shown to inhibit differentiation [126]. PPARγ was not detected during this proliferative phase, and was significantly induced during terminal differentiation of ADs. On the other hand, ERK activity returned to basal level during later stages of differentiation [120], which would be necessary to avoid negative impact towards PPARγ [79]. In addition, other studies have further demonstrated that ERK activity during MCE is necessary for inducing expression of key adipogenic transcription factors: C/EBPβ, C/EBPδ and C/EBPα [122, 127-129]. Collectively, these results indicate the importance of ERK signaling in adipogenesis.

Similar to ERK, p38 activation is also required for adipocyte differentiation. The first positive role for p38 in adipogenesis was elucidated in 3T3-L1 cells in the late 1990s [120, 123]. Inhibition of p38 was found to suppress differentiation of PAs into mature ADs, which was attributed to a decrease in C/EBPβ phosphorylation and transactivation [123]. Furthermore, it was observed that overexpression of an active MKK6 mutant, an upstream activator of p38, was sufficient to induce adipocyte differentiation of 3T3-L1 cells without any hormonal inducer [120]. However, persistent activation of p38 leads to

massive cell death [120], indicating p38 activation has differential effects depending on the stage of differentiation.

To a lesser extent, JNK has also been reported to play a role in adipocyte differentiation Evidence indicates that PPARγ is phosphorylated by JNK and that this phosphorylation results in PPARγ protein degradation and a reduction in PPARγ-dependent transcriptional activity [130]. Moreover, JNK activity is elevated in ADs during inflammatory stress, which triggers IRS-1 phosphorylation on S307 and decreases glucose uptake [70, 131, 132].

MAPK Phosphatases. An interesting feature of MAPK signaling is that the activation of a single MAPK signaling pathway leads to diverse cellular outcomes. With mounting evidence demonstrating that differences in the magnitude, duration, and subcellular localization of MAPK activities modulate cell signaling specificity (Fig.1.2B), the precise regulation of MAPK signaling is critical in determining various cellular physiological and pathological processes. As dual phosphorylation within the MAPK activation loop is required for MAPK activation, desphosphorylation of one or both residues leads to termination of MAPK activity.

In mammalian cells, inactivation of all known MAPKs can be achieved by a group of phosphatases that remove phosphate groups from threonine and/or tyrosine residues within the MAPK activation loop [133, 134]. These phosphatases include protein tyrosine phosphatases (PTPs) (e.g. PTP-SL, and STEP), serine/threonine phosphatases (PPs) (e.g., PP2A, and PP2C) and dual-specificity phosphatases (DUSPs)

[133, 135-138]. While the first two subgroups of phosphatases dephosphorylate only single tyrosine or threonine residues on MAPKs, DUSPs dephosphorylate both tyrosine and threonine residues in the activation loop of MAPKs [133, 135, 138]. Because abnormal MAPK activities lead to adipocyte dysfunction and IR, numerous studies are underway to identify phosphatases involved in the regulation of MAPKs activity that contribute to the prevention and treatment of obesity-induced inflammation and metabolic dysfunction. Of these phosphatases, the DUSP family has recently emerged.

DUSPs. Since DUSPs were first identified in the early 1990s, knowledge regarding molecular mechanisms and substrates of DUSPs has been emerging rapidly [139, 140]. More than 40 DUSPs have been identified so far, and at least 16 have displayed dephosphorylating activity towards MAPKs [135, 141, 142]. Ten of those 16 DUSPs have been documented to be MAPK-specific in mammalian cells. They are comprised of two domains, the C-terminal dual-specificity phosphatase domain (DSP) and the N-terminal CH2 MAPK-binding (MKB) domain (Fig.1.3A). Evidence shows that the MKB domain plays a critical role in regulating enzymatic specificity through docking interaction that helps individual phosphatases to determine binding specificity towards activated MAPK proteins [143, 144] (Fig.1.3B). The MKB domain also contains sequences that determine the subcellular localization of DUSPs. A number of studies have provided molecular evidence that several members of the DUSP family can be catalytically activated by substrate binding to its MKB domain, resulting in a conformational change of the phosphatase, thereby largely enhancing its catalytic activity.

The 10 MAPK-specific DUSPs can be further divided into three subfamilies based on their sequence homology, substrate specificity and localization (Fig.1.3A). Members identified in this first group includes DUSP1, DUSP2, DUSP4 and DUSP5. They localize exclusively to the nuclear compartment with a nuclear localization signal (NLS) sequence in the N-terminus and are induced by growth factors or stress signals that activate all three MAPKs. The second group is comprised of DUSP6, DUSP7 and DUSP9. These DUSPs localize predominantly in the cytoplasm due to a nuclear export signal (NES) sequence and preferentially target ERK1 and ERK2. The third group of DUSPs consists an extended region either in the N-terminus (e.g., DUSP10) or in the C-terminus (e.g., DUSP8, and DUSP16) in addition to the DSP and MKB domain. The extended C-terminus contains both the NLS and NES, allowing these members to be localized within the cytoplasmic and nuclear compartments. They are known to preferentially regulate the activity of JNK and p38 [134, 143-145]. In addition, group III phosphatases also contains PEST sequences that is known to be associated with protein turn over [146, 147]. Most members of these DUSPs display wide tissue distribution, however, some exhibit a more restricted expression pattern. For example, DUSP2 is found to be predominantly expressed in hematopoietic tissue [148], while DUSP8 is mainly expressed in tissues such as brain, eye, heart and lung [149, 150]. Expression of DUSP9 is detected in placenta, kidney and fetal liver [151], while DUSP10 reported to be restricted to skeletal muscle and liver [152].

DUSP4. One member of the MAPK-specific DUSPs that has gained increasing interest in the last few years is DUSP4, a phosphatase found to be expressed in a wide variety of tissues [153, 154]. Evidence from *in vitro* studies has revealed that DUSP4 can be induced by growth factors, oxidative stress, oncogenes, phorbol 12-myristate 13-acetate (PMA), UV irradiation and LPS [155-159]. While DUSP1 is identified as an immediate-early nuclear inducible gene in response to extracellular stimulation, DUSP4 exhibits a relatively late phase induction [160, 161]. Several studies have demonstrated that DUSP4 is localized to the nuclear compartment due to two distinct NLS's and dephosphorylates either ERK or JNK, depending on cell type [158, 162, 163]. Unlike DUSP1 that only dephosphorylates and release ERK back to the cytosol, DUSP4 displays a higher binding affinity for ERK and retain this kinase in the nucleus, suggesting a more potential role in spatiotemporal regulation of ERK that could subsequently influence cellular outcomes [164]. Nevertheless, a couple of studies have provided contradictory findings on regulatory function of DUSP4 towards p38. One group reported that DUSP4 did not dephosphorylate p38, but exhibited strong binding affinity towards this kinase [165], while the other observed that DUSP4 knockdown led to over-activated p38 under oxidative stress [166].

Previous studies have demonstrated a role for DUSP4 in cancer, apoptosis and senescence. Evidence has linked DUSP4 to the development of oesophagogastric rib metastasis, ovarian cancers, liver carcinoma, and acute myeloid leukemia [167-170]. In contrast, others have identified DUSP4 as a tumor-suppressor gene with the observation

that the expression of DUSP4 is suppressed in primary breast cancer, prostate cancer, and lung cancer [171-173]. Overexpression of DUSP4 has been shown to negatively regulate JKN activity, and as a result, causes JNK-dependent apoptosis [163, 173]. In other cell types, expression of DUSP4, regulated by transcription factors p53 or E2F-1, has been shown to modulate H_2O_2-induced apoptosis by inactivating ERK [174, 175]. Furthermore, it suggested that ERK phosphorylation of DUSP4 on T361/T363 and S390/S395 residues highly stabilized this phosphatase from ubiquitin-associated degradation [156, 157]. Once being activated by ERK, DUSP4 could regulate cell senescence by inactivating nuclear ERK [176, 177].

Interestingly, G_2/M-phase growth arrest and enhanced cyclin B1 expression have been observed in embryonic fibroblasts (MEF) of DUSP4 knockout mice [159]. Similar results have also been obtained from mice mammary tumor cells using DUSP4 siRNA [168]. This newly discovered feature of DUSP4 function is distinct from DUSP1, which is shown to be associated with the entry into cell cycle. It has been reported that DUSP4 appears to potentiate inflammation in response to LPS-mediated septic shock and acute lung injury [178, 179]. In contrast, another investigation has demonstrated a role for DUSP4 in protecting against intracellular infection by parasites in macrophages derived from DUSP4 knockout mice [180]. These findings support potential roles for DUSP4 in the regulation of cell cycle and in the response to inflammation.

Although the MAPKs have been extensively studied in the regulation of insulin action and adipocyte functions, the role of DUSPs, especially DUSP4, on chronic, low-

grade inflammation in the development of obesity-induced IR and subsequent T2D remains largely unknown. It was not until recently that a growing number of investigations have been conducted to better understand roles of DUSPs in adipocyte regarding MAPK signaling and insulin sensitivity [165, 181, 182]. For instance, DUSP1 is immediately induced during the early phase of adipocyte differentiation in response to hormonal inducers and its induction controls the magnitude and duration of ERK and p38 activity [161]. Genetic ablation of DUSP1 leads to prolonged ERK activation and inhibited adipocyte differentiation [182]. Expression of DUSP9 is induced during adipocyte differentiation. It has been reported that overexpression of DUSP9 in 3T3-L1 cells suppressed ERK and JNK activation, leading to a recovery of insulin-mediated PI3K/Akt pathway and improved glucose uptake [181]. Interestingly, evidence suggests that DUSP4 suppresses gluconeogenesis. Under low energy states, AMP-activated kinase (AMPK) activates transcription factor Egr-1, which binds and induces DUSP4 expression, leading to dephosphorylation of p38 and repression of phosphoenolpyruvate carboxykinase (PEPCK) [165]. A recent macrophage-adipocyte co-culture study has provided evidence for the first time that DUSP4 ectopic expression in macrophages inhibits the production of inflammatory cytokines mediated by free fatty acid (FFA) and suppresses inflammatory activation of macrophages by reducing JNK and p38 activity [183]. In addition, overexpression of DUSP4 in macrophages also suppressed inflammation during macrophage-adipocyte interaction [183]. However, there is still a lack of understanding the molecular basis of DUSP4 in the development of obesity and

IR in ADs. As evidence above demonstrated potential the role of DUSP4 in regulating cell-cycle progress, apoptosis, gluconeogenesis, and inflammation in various cell types, it will be of great interest to elucidate the molecular role and physiological function of DUSP4 in regulation of obesity-mediated inflammation and metabolic disorder in ADs.

Study Objectives

Chapter II. Little is known regarding the regulatory mechanism and function of DUSP4 in adipocytes. Therefore, the objective of the study summarized in chapter II is to examine the regulation and function of DUSP4 in 3T3-L1 cultured adipocytes. We will 1) determine how DUSP4 is regulated in response to agonist-specific MAPK activation, 2) determine the mechanisms involved in DUSP4 protein stabilization and degradation, and 3) elucidate the potential role for DUSP4 in regulating ERK activity within specific cellular compartment.

Chapter III. While others have studied the regulation of function of several DUSPs, including DUSP1 and DUSP9 with regards to obesity, inflammation and adipogenesis, a role for DUSP4 under these conditions remains unknown. Therefore, the objective of the study summarized in chapter III is to: 1) establish relative DUSP4 expression in major insulin responsive tissues of lean mice, 2) examine how DUSP4 is regulated during the development of genetic and diet-induced mouse models of obesity as these animals transition to a more inflammatory state, 3) determine the regulation and role for DUSP4 in adipocyte differentiation.

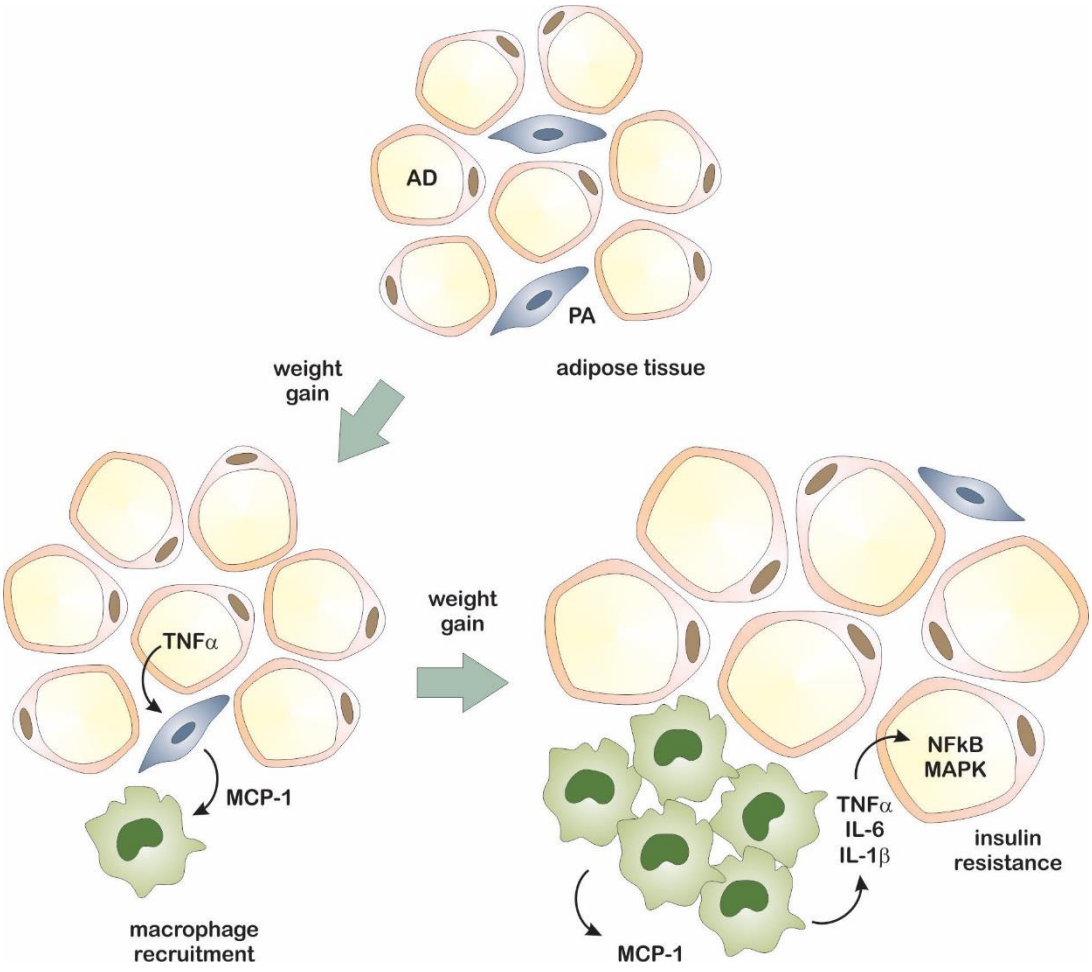

Figure 1.1. Obesity-induced Adipose Tissue Inflammation. Obesity is characterized by expansion of adipose tissue (AT). As AT mass continues to expand, adipocytes can secrete chemoattractant proteins (i.e., MCP-1) to recruit macrophages into AT. Infiltrated macrophages and enlarged adipocytes secrete a large amount of inflammatory cytokines, including TNF-α and IL-6 that stimulates PAs and ADs to activate inflammatory signaling pathways (i.e., MAPK) and gene expression, leading adipocyte dysfunction and IR.

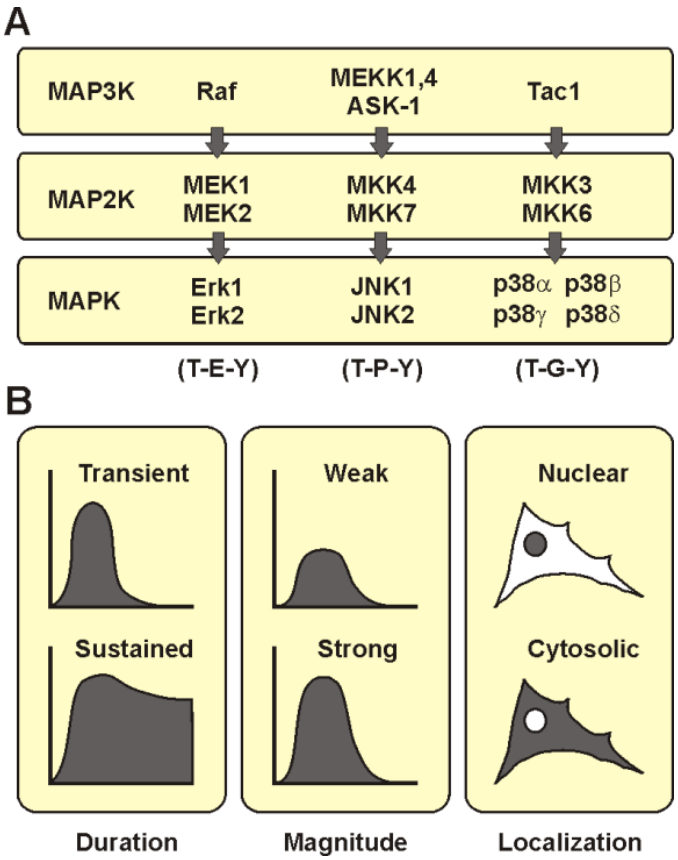

Figure 1.2. Overview of MAPK Signaling Pathways.

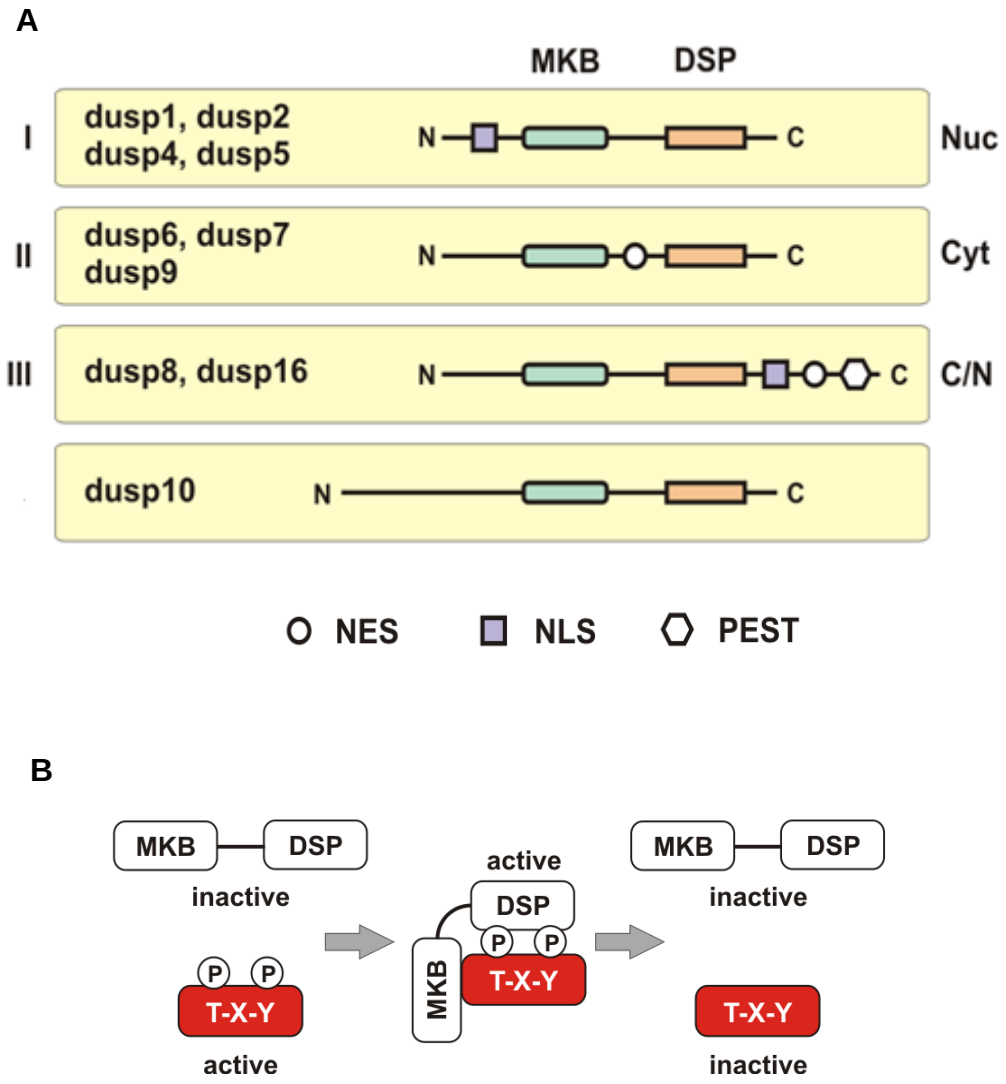

Figure 1.3. DUSPs Classification and Domain Structure.

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CHAPTER II

REGULATION OF ENDOGENOUS DUAL-SPECIFICITY PHOSPHATASE 4 BY AGONIST-SPECIFIC MAPK ACTIVATION IN ADIPOCYTES

Abstract

Activation of the ERK, JNK and p38 mitogen activated protein kinases (MAPKs) by different extracellular stimuli leads to a variety of cellular outcomes, such as proliferation, inflammation, differentiation and apoptosis. The signal specificity is shown to be dependent upon duration, magnitude and localization of MAPK activity. While numerous studies have examined upstream signaling for MAPK activation, few have addressed how MAPK deactivation alters biological responses. Evidence now supports potential roles for MAPK-specific, dual-specificity phosphatases (DUSPs) in the deactivation of MAPK signaling and modulation of obesity-associated metabolic dysfunction. However, the precise mechanisms underlying the regulation of DUSPs remain poorly understood. In this study, we have investigated regulatory mechanisms of endogenous DUSP4 by several MAPK agonists: tumor necrosis factor-α (TNF-α), hydrogen peroxide (H_2O_2), ultraviolet (UV) irradiation and phorbol 12-myristate 13-acetate (PMA). We found that PMA stimulation resulted in robust and sustained ERK phosphorylation up to 8 hours, leading to a stronger induction of endogenous DUSP4. Treatment with the ERK inhibitor U0126 suppressed ERK phosphorylation and abolished

DUSP4 expression. In contrast, TNF-α, H_2O_2, and UV irradiation that led to transient ERK phosphorylation were not able to induce DUSP4 protein accumulation. In addition, PMA-mediated sustained ERK phosphorylation highly stabilized DUSP4 protein, while blocking ERK activity resulted in a much shortened DUSP4 half-life. Furthermore, we demonstrated that the induction of DUSP4 correlates with the loss of ERK activation in the nucleus while cytosolic ERK remains activated. Finally, protein-protein interactions were observed between DUSP4 and ERK. Collectively, these results demonstrate 1) induction of endogenous DUSP4 is tightly regulated by the duration and magnitude of ERK activity; 2) phospho-ERK is involved in DUSP4 protein stability; 3) DUSP4 only modulates ERK activity in a compartment-dependent manner as a nuclear phosphatase; and 4) DUSP4 serve as an anchor protein that retains ERK in the nucleus, preventing reactivation of this kinase by upstream kinases.

Introduction

Obesity is the result of excessive fat accumulation in the body due to an imbalance between caloric intake and energy expenditure[1]. It is the most prevalent nutrition-related health concern worldwide as it contributes to the increased risk of numerous chronic diseases, such as cardiovascular disease, hypertension, cancer, insulin resistance and type 2 diabetes [2-4]. Studies over the past three decades have demonstrated that obesity is associated with a progressive infiltration of macrophages that results in increased production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which leads to chronic, low-grade

inflammation[3, 5, 6]. These inflammatory cytokines further contribute to the initiation and development of many metabolic disorders by aberrantly activating intracellular signaling molecules, such as the mitogen-activated protein kinases (MAPKs) [7, 8].

MAPKs are important mediators of signal transduction pathways and play a pivotal role in the regulation of numerous cellular processes including cell growth and proliferation, differentiation, inflammation and apoptosis. Most of the functional studies of MAPKs are focused on three major groups: extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs) and p38 kinases [9]. The MAPK signaling cascade is directed by sequential kinases activity, for instance, MAPK is phosphorylated by upstream MAPK kinases (MAPKKs), which are phosphorylated by MAPK kinase kinases (MAPKKKs) in response to diverse extracellular signals. Dual-phosphorylation of tyrosine and threonine residues within the T-X-Y motif of the activation loop is required for MAPK activation, leading to the phosphorylation of cytosolic and nuclear targets [9, 10]. While MAPK activation by upstream signaling has been extensively investigated, mechanisms regarding MAPK deactivation are still poorly understood. Growing evidence now suggests that MAPK inactivation by phosphatases is also a critical event that modulates MAPK-associated biological consequences in response to extracellular signals [11-13]. A subgroup of the protein tyrosine phosphatases superfamily, known as the dual-specificity phosphatases (DUSPs), dephosphorylate both tyrosine and threonine residues of MAPKs [11, 14]. Within this group, 10 DUSPs share a common MAPK binding domain (MKB), allowing them to carry

out catalytic activity through docking interaction specifically towards MAPKs [11, 15, 16]. These MAPK-specific DUSPs can be further divided into three subgroups based on their sequence homology, substrate specificity and subcellular localization. The first group includes DUSP1, DUSP2, DUSP4 and DUSP5. They localize exclusively to the nuclear compartment with a nuclear localization signal (NLS) sequence and are induced by growth factors or stress signals that activate all three MAPKs. The second group, comprised of DUSP6, DUSP7 and DUSP9, are ERK-specific and localize predominantly in the cytoplasm with a nuclear export signal (NES) sequence. The third group contains DUSP8, DUSP10 and DUSP16, which are JNK/p38-specific and localized in both the cytoplasm and nucleus [11, 15-19].

In recent years, there is growing interest in the role for DUSPs in adipocytes metabolism and obesity-mediated inflammation. For instance, genetic ablation of DUSP1 leads to prolonged ERK and p38 activation without any interference on adipocyte differentiation [20]. Overexpression of DUSP9 in 3T3-L1 cells suppressed ERK and JNK activation leading to improved glucose uptake under inflammatory stress [21]. DUSP4, one of the earliest DUSPs identified, is another candidate that has drawn attention for its role in regulation of MAPK signaling in numerous cell types. Evidence from *in vitro* studies show that DUSP4 can be induced by growth factors, oxidative stress, oncogenes, lipopolysaccharide (LPS) and UV radiation [22-25]. Others have shown that DUSP4 is localized in the nucleus due to two NLS and dephosphorylates either ERK or JNK, depending on cell type [22, 25, 26]. Studies also demonstrate potential roles for DUSP4

in regulating cell proliferation, inflammation, and gluconeogenesis in other cell types [23, 27, 28]. However, relatively little attention was given to its role in adipocytes. A recent study using macrophage-adipocyte co-culture revealed that ectopic DUSP4 expression in macrophages inhibits production of inflammatory cytokines mediated under free fatty acid stimulation, and subsequently reduces JNK and p38 activity [29]. In addition, overexpression of DUSP4 in macrophages also suppressed inflammatory stress mediated by macrophages during macrophage-adipocyte interaction [29].

Despite the potential biological function demonstrated by others, the regulatory mechanism of DUSP4 in adipocytes remains poorly understood. Since DUSP4 ectopic expression has been reported to artificially shift the balance of its subcellular localization [30], herein, we sought to elucidate the regulatory mechanisms of endogenous DUSP4 by MAPK activity and subsequent spatiotemporal regulation of MAPK activity by DUSP4 in adipocytes. We found that induction of endogenous DUSP4 requires strong and sustained ERK activation. We also demonstrated that ERK activation stabilizes DUSP4 protein while blockade of ERK activity leads to rapid DUSP4 degradation mediated partially by proteasome activity. Moreover, we observed that DUSP4 was exclusively localized in the nucleus, and the accumulation of DUSP4 correlated with the suppression of nuclear ERK phosphorylation. Additionally, we revealed that DUSP4 co-immunoprecipitated with ERK, suggesting an important role for DUSP4 in spatiotemporal regulation of nuclear ERK activation via negative feedback mechanism.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DMEM), calf bovine serum (CS), Trypsin-EDTA, and recombinant murine TNF-α were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from HyClone. The following antibodies were used for immunoblot analysis: phospho-MEK, phospho-ERK (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), total ERK and α-tubulin, were purchased from Cell Signaling; DUSP4 (SC-1200) were purchased from Santa Cruz Biotechnology. Pharmacological inhibitor of ERK (U0126) was purchased from CalBiochem. Epoxomicin was purchased from Boston Biochem. Cycloheximide was purchased from Sigma. Enhanced chemiluminescence (ECL) reagents were obtained from Perkin-Elmer Life Sciences. All TaqMan primer probes used in this study were purchased from Applied Biosystems.

Cell Culture. The murine 3T3-L1 cell line was purchased from Howard Green, Harvard Medical School. Cells were propagated in DMEM supplemented with 10% CS until reaching density-induced arrest, as previously described. Throughout the study, '0h' refers to density arrested cells immediately prior to stimulation as described in figure legend. Experiments described herein were conducted in density-arrested preadipocytes. All experiments were repeated 2- 3 times to validate results and ensure reliability.

Immunoblotting. Cell monolayers were washed with phosphate-buffer saline (PBS) and scraped into ice-cold lysis buffer containing 0.1 M Tris (pH 7.4), 150 mM NaCl,

10% sodium dodecyl sulfate (SDS), 1% Triton X, 0.5% Nonidet P-40 (NP40), 1 mM EDTA, 1 mM EGTA. Phosphatase inhibitors (20 mM β-glycerophosphate, 10 mM sodium fluoride and 2 μM sodium orthovanadate) and protease inhibitors (0.3 μM aprotinin, 21 μM leupetin, 1 μM pepstatin, 50 μM phenanthroline, 0.5 μM phenylmethylsulfonyl fluoride) were added to lysis buffer immediately prior to cell harvest. Cell lysates were sonicated and centrifuged (15,000*g,* 10 min, 4°C), and the supernatant transferred to a fresh tube. Protein content was determined by bicinchoninic acid (BCA) procedures according to manufacturer's (Pierce, Rockford, IL) instructions. Equal amounts of whole cell lysate protein were separated by SDS-PAGE electrophoresis. Cell lysates were mixed with loading buffer containing 0.25M Tris (pH6.8), 4% SDS, 10% glycerol, 0.01% bromophenol blue, and 10% dithiothreitol, then heated at 80°C for 5 min prior to electrophoresis. Proteins were resolved on SDS-polyacylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) 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 2 hrs at room temperature. Membranes were immersed in ECL and data 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, Valencia, CA), according to manufacturer protocol. 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 in definitely/ 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. All TaqMan primer probes used in this study were also purchased from ABI. Data were recorded and analyzed with ABI Sequence Detector Software and graphs visualized with Excel software. All data were presented as mean ± standard error of the mean (SEM) and representative of duplicate determinations. Data were normalized to 18S and measured as relative differences using the $2^{-\Delta\Delta CT}$ method as previously described [31, 32].

Immunofluorescence. Cells, cultured in 4-well glass chamber slides (Nunc Lab-Tek II System; Thermal Scientific Co.) at 37ºC overnight, were washed with PBS, fixed with methanol free 4% Formaldehyde (Polysciences) for 15 min at room temperature, permeabilized with 0.2% Triton X for 5 min, blocked with 10% BSA for 20 min, and

incubated overnight with primary antibody at 4°C. Cells were subsequently washed and incubated with fluorescently-conjugated secondary antibodies, Alexa Fluor® 488 anti-mouse and Alexa Fluor® 594 anti-rabbit (Molecular Probes), in dark for 30 min at room temperature. Slides were then washed with PBS, mounted using Antifade mounting solution (Molecular Probes) and visualized by confocal microscopy.

Immunoprecipitation/Co-immunoprecipitation. Co-immunoprecipitation (co-IP) was performed using the Thermo Fisher Scientific Pierce co-IP kit (26149) following the manufacturer's protocol. Briefly, total ERK and DUSP4 antibodies were first immobilized for 2 hrs using AminoLink Plus coupling resin. Cells were washed with PBS and incubated with IP Lysis/Wash buffer containing 25 mM Tris, pH 7.4, 150 mM NaCl, 1% NP40, 1 mM EDTA and 5% glycerol on ice. Cell lysates were collected by brief centrifugation (13,000*g*, 10 mins, 4°C). The antibody-coupled resin was then washed and incubated with cell lysate at 4°C overnight. After incubation, the resin was again washed and protein complex eluted using elution buffer. Protein complexes were then resuspended in loading buffer, resolved on SDS-polyacrylamide electrophoresis gel, and immunoblotted as discussed above.

Results

Sustained Activation of the MEK/ERK Signaling Pathway Induces Endogenous DUSP4 mRNA and Protein in 3T3-L1 Adipocytes. Early evidence has demonstrated that DUSP4 can be upregulated in response to growth factors, inflammatory stress, oncogenes and UV radiation [24, 33-36]. Many of these stimuli are also known to

activate MAPK signaling pathways. Therefore, we investigated the effects of several MAPK agonists, TNF-α, H_2O_2, UV irradiation and PMA on DUSP4 expression. Density-arrested 3T3-L1 adipocytes were first stimulated and harvested over time and then immunoblotted with phospho-specific antibodies to identify MAPKs exclusively when being dually phosphorylated on the T-x-Y motif of the activation loop. As illustrated in Figure 2.1A, compared to 0h, treatment with either TNF-α or H_2O_2 caused a rapid and transient induction of p-ERK, p-p38 and p-JNK that was gone by 1 hr. However, DUSP4 expression was not detectable under either conditions as compared to the positive control sample for DUSP4. As shown in Figure 2.1B, UV exposure led to strong induction of p-p38 and p-JNK with sustained activity over 4 hrs while only minimal p-ERK induction was observed when compared to 0 hr, no treatment sample. Expression of DUSP4 was not detectable under this condition. PMA stimulation resulted in robust and sustained MEK/ERK activation up to 8 hrs while no induction of p38 or JNK was observed (Figure 2.1C). The accumulation of DUSP4 protein was observed at 2 hrs post-PMA and remained upregulated up to 8 hrs. Interestingly, the level of DUSP4 mRNA peaked at 2 hrs post PMA treatment, but decreased significantly by 4 hrs and remained low throughout the remainder of the experimental period (Figure 2.1D). To confirm that the induction of DUSP4 is dependent on the MEK/ERK signaling pathway, we pre-treated cells with MEK inhibitor U0126 for 15 min before addition of PMA (Figure 2.2A). Although a sustained MEK-independent p-ERK induction was observed, the magnitude was weaker as compared to control cells. DUSP4 protein expression was greatly

suppressed under this condition. To explore p-ERK magnitude versus duration, we treated cells with PMA for 1 hr before blocking ERK activation with U0126 (Figure 2.2B). We found that even though we initially induced the magnitude of p-ERK level to the same extent as control cells, shortened duration of ERK activity abolished DUSP4 induction. Collectively, these data suggest that endogenous DUSP4 induction requires strong and sustained ERK activation in adipocytes.

The MEK/ERK Signaling Pathway Regulates Endogenous DUSP4 Protein Stabilization. Data presented above demonstrate that endogenous DUSP4 is induced via the MEK/ERK signaling pathway. While DUSP4 mRNA only peaked at 2 hrs post-PMA, its protein remained abundant up to 8 hrs suggesting post-translational stabilization of DUSP4 protein may be involved. It has been reported that ERK activation affects protein stability of several DUSPs, including DUSP4, in other cell types [22, 24, 37]. Therefore, we speculated that endogenous DUSP4 protein can be stabilized by ERK activation in adipocytes. To test this hypothesis, DUSP4 was maximally induced with 2 hrs PMA pretreatment before stimulation with the protein synthesis inhibitor cycloheximide (CHX) to prevent additional protein synthesis. As illustrated in Figure 2.3A, although DUSP4 protein levels decreased upon CHX treatment, the remaining DUSP4 proteins were very stable as long as the levels of phospho-ERK remained elevated (18 hrs post-CHX), indicating a long protein half-life of this phosphatase under the condition of sustained ERK activation. However, when cells were concomitantly treated with CHX and U0126 in combination, ERK activation was significantly suppressed, and DUSP4

protein stability decreased dramatically, yielding an estimated half-life of 1.5 hrs (Figure 2.3B). These results suggest that DUSP4 protein stability depends on MEK/ERK activation.

Proteasomal Activity is Involved in the Degradation of Endogenous DUSP4 Protein. Previous studies have shown that proteasomal activity is involved in protein degradation of DUSPs, such as DUSP1, DUSP5 and DUSP6 [38-40]. To investigate the involvement of proteasomal activity towards DUSP4 protein degradation in adipocytes, DUSP4 was induced with PMA for 2 hrs before treating with U0126, CHX and proteasome inhibitor epoxomicin (EPOX). As illustrated in Figure 2.3C, when ERK activation was suppressed by U0126, addition of EPOX partially attenuated DUSP4 protein degradation, extending its protein half-life to 4 hrs, as compared to cells in Figure 2.3D. Our results indicate that DUSP4 protein degradation is mediated by mechanisms involving proteasomal activity.

Induction of DUSP4 Correlates with the Loss of ERK Phosphorylation in the Nucleus. It has been well established that phospho-ERK is a target of DUSP4 [33, 41, 42]. However, as shown in Figure 2.1C, the level of PMA-stimulated ERK phosphorylation did not decrease following DUSP4 induction, suggesting that persistent ERK phosphorylation may be exclusively localized to the cytoplasmic compartment. To test this hypothesis, subcellular localization of phospho-ERK and DUSP4 following PMA stimulation was analyzed by immunofluorescence microscopy with DAPI counterstaining used for defining nuclear compartment. As illustrated in Figure 2.4, phospho-ERK was detected in

the cytosol, but not the nucleus in quiescent, non-stimulated 3T3-L1 cells. DUSP4 was not detectable in either compartment under quiescent conditions. PMA stimulation for 10 min induced rapid phospho-ERK accumulation in the nucleus prior to induction of DUSP4. With PMA stimulation for 2 hrs, nuclear phospho-ERK was significantly suppressed concomitant with nuclear accumulation of DUSP4 while cytosolic phospho-ERK remained elevated. Additionally, phospho-ERK signal remained suppressed in the nucleus at 4 hrs post-PMA while DUSP4 continued to be expressed. Collectively, these data suggest that DUSP4 modulate ERK activity in a compartment-dependent manner that the phosphatase only controls ERK activation in the nucleus while not interfering with cytosolic ERK activation.

Protein-protein Interactions Between DUSP4 and ERK. Data presented above demonstrate the loss of nuclear ERK signal is associated with the induction of DUSP4 in adipocytes. A number of studies have reported that while some DUSPs only dephosphorylate and release ERK, other DUSPs can anchor ERK protein within specific cellular compartments [42-44]. To further examine if DUSP4 can anchor and retain ERK protein in the nucleus, density-arrested 3T3-L1 cells were first treated with PMA and harvested at indicated times. Lysates were immunoprecipitated and immunoblotted for both ERK and DUSP4. As illustrated in Figure 2.5, under basal conditions where there was no DUSP4, ERK did not co-immunoprecipitate with this phosphatase (lane 3). PMA stimulation for 4 hrs led to DUSP4 induction and DUSP4-ERK co-immunoprecipitation (lane 4). Non-specific binding of lysate proteins to agarose coupling resin was ruled out

(lane 5). These data strongly suggest that DUSP4 can serve as an anchor protein for ERK, retaining the kinase in the nucleus.

Discussion

In this study, we demonstrated the regulation of endogenous DUSP4 by MAPKs activation in 3T3-L1 adipocytes. First, we report that DUSP4 induction required strong and sustained ERK activation. Blockade of ERK activity completely abolished DUSP4 expression while JNK or p38 activation did not play a role in DUSP4 upregulation. Second, we show that sustained ERK activation also promoted DUSP4 protein stability. Third, we demonstrate proteasome activity was involved in DUSP4 protein degradation. Fourth, we present nuclear phospho-ERK was absent following induction of DUSP4 in the nucleus. Finally, we reveal protein-protein interaction between DUSP4 and ERK. Taken together, data presented in this report demonstrate that endogenous DUSP4 is regulated via the MEK/ERK signaling pathway and DUSP4 regulates ERK activation in a compartment-dependent manner in 3T3-L1 adipocytes.

It has been well established that obesity-mediated chronic inflammation in adipose tissue leads to the production of pro-inflammatory cytokines such as TNF-α and IL-6. These inflammatory molecules can interfere with insulin signaling pathway and cause other obesity-associated metabolic disorders by activating intracellular signaling pathways including the MAPKs [5-7]. Traditionally, upon receiving appropriate extracellular signals, the MAPK signaling pathways mediate normal biological responses [9, 10]. However, aberrant MAPK activation by obesity-mediated inflammatory stress

has been shown to cause antagonist effects [3, 7, 45]. As MAPK signaling cascades are often depicted as simple and linear signaling transduction networks, a growing number of studies have demonstrated that differences in the duration, magnitude and subcellular localization of MAPK activities all contribute to specific and diverse cellular outcomes [46, 47]. Investigations in the past extensively focused on upstream activation of MAPK signaling. Accumulating evidence now suggests that MAPK deactivation by phosphatases also play a pivotal role in regulating signal specificity [15, 18]. The MAPK-specific DUSPs are plausible candidates that have been shown to modulate MAPK dephosphorylation and subsequent cellular outcomes in various cell types [14-16, 48, 49]. Studies have further demonstrated that, besides dephosphorylating MAPKs, several DUSPs were able to anchor their substrates in subcellular compartments adding to their versatility as MAPK regulators [19, 42, 50].

Although DUSP4 was one of the first DUSPs to be identified, information delineating its regulation and function in adipocytes has not been described. Previous studies have shown that DUSP4 can be induced by several extracellular signals that are also known to activate MAPKs, but the precise mechanism and involvement of MAPK activity in DUSP4 induction remains unclear. Data presented in this investigation demonstrate that PMA-mediated strong and sustained ERK activation led to significant induction of endogenous DUSP4 mRNA and protein. Blockade of ERK activation with U0126 completely abolished DUSP4 induction. In contrast, TNF-α and H_2O_2-mediated transient ERK activation could not induce DUSP4. In addition, regardless of the signal

duration and magnitude, JNK or p38 activation did not contribute to DUSP4 upregulation. Consistent with our observation, Cagnol and colleague also reported sustained oncogenic ERK activation promotes DUSP4 expression [22]. Taken together, our data demonstrate that the upregulation of DUSP4 is solely dependent on MEK/ERK signaling.

Evidence from early studies indicates that ERK activation plays an important role in post-translational modification of DUSPs protein. For instance, DUSP1, DUSP5 and DUSP16 were reported to be stabilized by ERK phosphorylation [24, 39, 51], while DUSP6, on the other hand, is destabilized when phosphorylated by ERK [38]. These studies also suggest that DUSPs protein degradation is associated with proteasomal activity. Our findings demonstrated that without ERK activation, the protein half-life for endogenous DUSP4 was approximately 1.5 hrs. In contrast, DUSP4 protein half-life was significantly increased beyond 18 hrs with sustained ERK activation. In addition, under the condition of ERK suppression, inhibition of proteasomal activity by EPOX could extend DUSP4 protein half-life from 1.5 hrs to 3 hrs. Similarly, others have also shown that DUSP4 protein only yielded a short half-life of 7.5 min when ERK activity was suppressed, while its protein half-life was markedly extended to 1.5 hrs with sustained ERK activation in other cells [22]. Peng et al. also reported blockade of ERK phosphorylation promotes proteasome degradation of DUSP4 protein [37]. Collectively, our findings suggest that DUSP4 protein stability requires ERK phosphorylation in adipocytes and its protein degradation is partially mediated by proteasomal activity.

Growing evidence now clearly suggests that the magnitude, duration and localization of ERK signaling modulate diverse cellular outcomes [13, 47, 52-54]. Thus, ERK activity needs to be precisely controlled both spatially and temporally to prevent adverse effects due to aberrant MEK/ERK pathway activation. Data presented above demonstrate DUSP4 is induced in a MEK/ERK-dependent manner by sustained ERK activation and DUSP4, canonically functions in a negative feedback manner to dephosphorylate and inactivate ERK signaling [22, 25, 42]. However, we observed that PMA-induced ERK phosphorylation in whole cell lysates did not decrease following the induction of DUSP4 as visualized by immunoblotting. This led us to hypothesize that DUSP4 regulates ERK activity in the nucleus and that sustained ERK phosphorylation following DUSP4 induction is exclusively localized to the cytosolic compartment. Our observations supported the hypothesis by showing that nuclear ERK inactivation occurred concomitantly with nuclear induction of DUSP4. Furthermore, we have shown protein-protein interactions between ERK and DUSP4 by Co-IP. However, this DUSP4-ERK interaction was only observed when we first immunoprecipitated DUSP4 protein and then immunoblotted for ERK. We were unable to detect protein-protein interactions when immunoprecipitated ERK and immunoblotted for DUSP4. One explanation would be that DUSP4 is exclusively localized to the nuclear compartment, as we immunoprecipitated sufficient amount of this phosphatase, a relatively strong DUSP4-ERK interaction signal could be detected by western blot. In contrast, ERK proteins are localized in both compartments of a cell and the ERK antibody recognizes

both activated and inactivated forms of ERK. Therefore, when we immunoprecipitated ERK proteins that comes from both cytosolic and nuclear compartments, those ERK proteins that attached to DUSP4 might be limited. As a result, the DUSP4-ERK association could be too weak to be detected. Collectively, these data support the hypothesis that DUSP4 regulate ERK phosphorylation in a compartment-dependent manner and it also serves as an anchor protein to retain ERK in the nucleus. It further suggests that sustained nuclear ERK activation could mediate adverse effects in quiescent cells. Therefore, specific nuclear regulator is expressed to modulate ERK signal at the appropriate time. Based on these preliminary findings, we developed the following working model (Fig.2.6), where sustained activation of ERK leads to its translocation from the cytosol to the nucleus that further induces nuclear DUSP4 expression, and DUSP4 subsequently dephosphorylates and retains ERK in the nucleus from reactivation (Fig.2.6A). Meanwhile, ERK phosphorylation stabilizes DUSP4 from proteasome-mediated protein degradation (Fig.2.6B).

In summary, this study provides the first evidence regarding regulatory mechanisms of endogenous DUSP4 by MAPK activation in adipocytes. Data presented here demonstrate that induction of endogenous DUSP4 requires strong and sustained ERK activation. We also report that ERK activity controls DUSP4 protein stability with the observation that, while loss of ERK signal led to rapid DUSP4 protein degradation, sustained ERK activation could maintain protein levels of DUSP4 during our experimental period. In addition, inhibition of proteasomal activity partially rescued the

loss of DUSP4 protein under the condition of ERK inactivation. Our data further show that nuclear ERK phosphorylation was suppressed following induction of DUSP4 while cytosolic ERK remained activated, and DUSP4 co-immunoprecipitated with ERK, underlining the importance of negative feedback mechanisms by DUSP4 in spatiotemporal regulation of ERK activity in the nucleus. These data collectively suggest that ERK plays an important role in regulation of DUSP4 and DUSP4, in turn, provides a negative feedback control of nuclear ERK activation in 3T3-L1 cells.

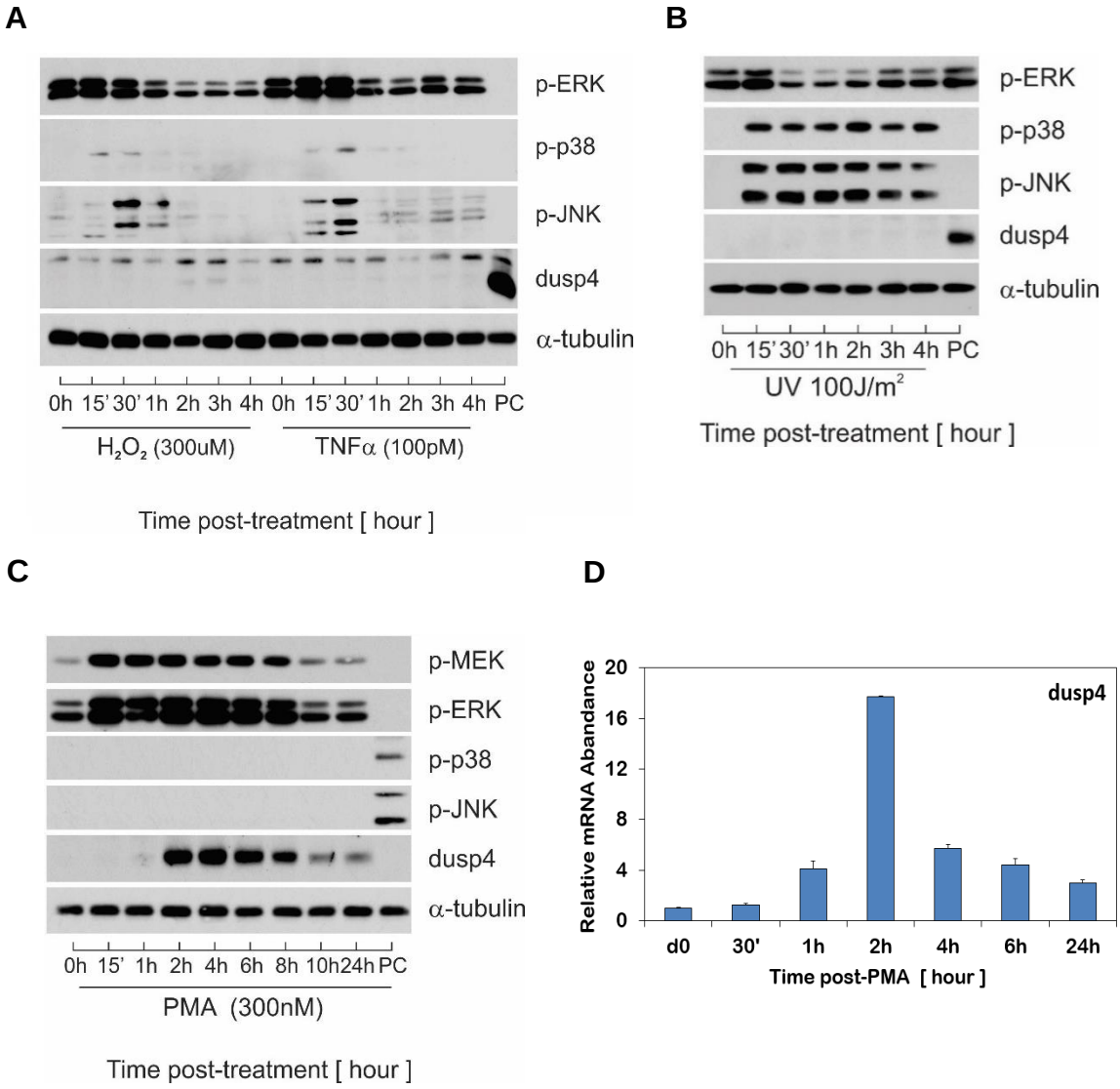

Figure 2.1. DUSP4 Induction in Response to Agonist-specific MAPKs Activation. Preadipocytes were stimulated with A) 300 μM H_2O_2 or 100 pM TNF-α, B) 100J/m^2 UV radiation and C) 300 nM PMA. Cell lysates were collected at indicated times and protein expression of bisphosphorylated MEK, ERK, p38 and JNK as well as DUSP4 and α-tubulin was analyzed via immunoblotting. PC: positive control for DUSP4. D) Total RNA was harvested from PMA-stimulated preadipocytes and mRNA expression of DUSP4 measured using qRT-PCR. Data were normalized to 18S rRNA and changes in gene expression measured as fold differences relative to untreated preadipocytes (0h).

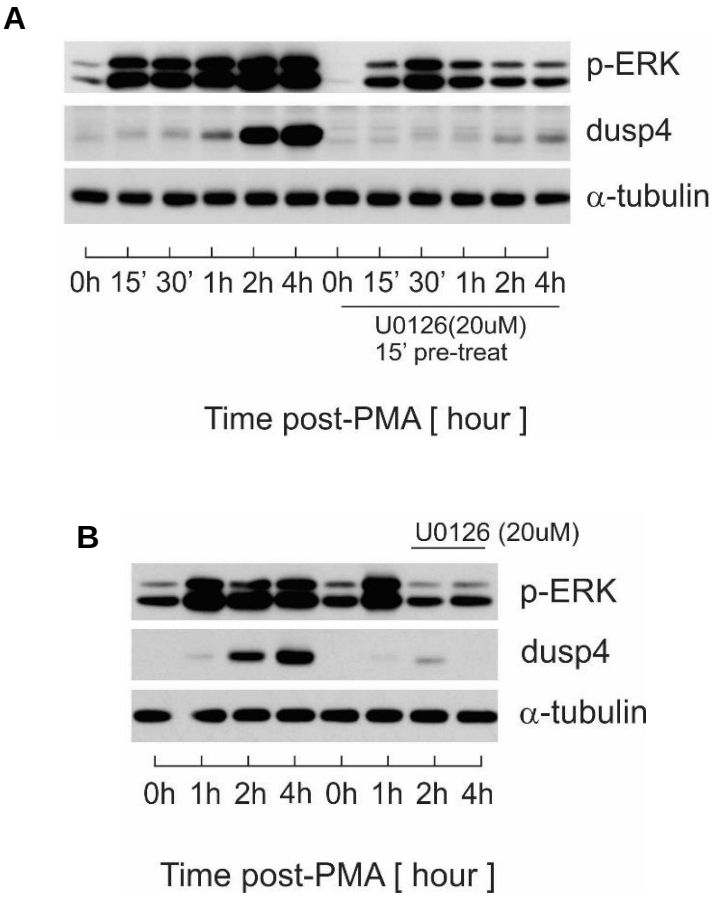

Figure 2.2. Magnitude and Duration of ERK Activity Determines DUSP4 Protein Expression. A) Preadipocytes were stimulated with 300nM PMA in the absence or presence of 20 μM U0126 prior to immunoblot analysis of bisphosphorylated ERK as well as DUSP4 and α-tubulin. B) PAs were stimulated with PMA for 1 hr before addition of U0126 or vehicle. Cell lysates were collected at indicated times and protein expression of bisphosphorylated ERK along with dusp4 and α-tubulin analyzed via immunoblotting.

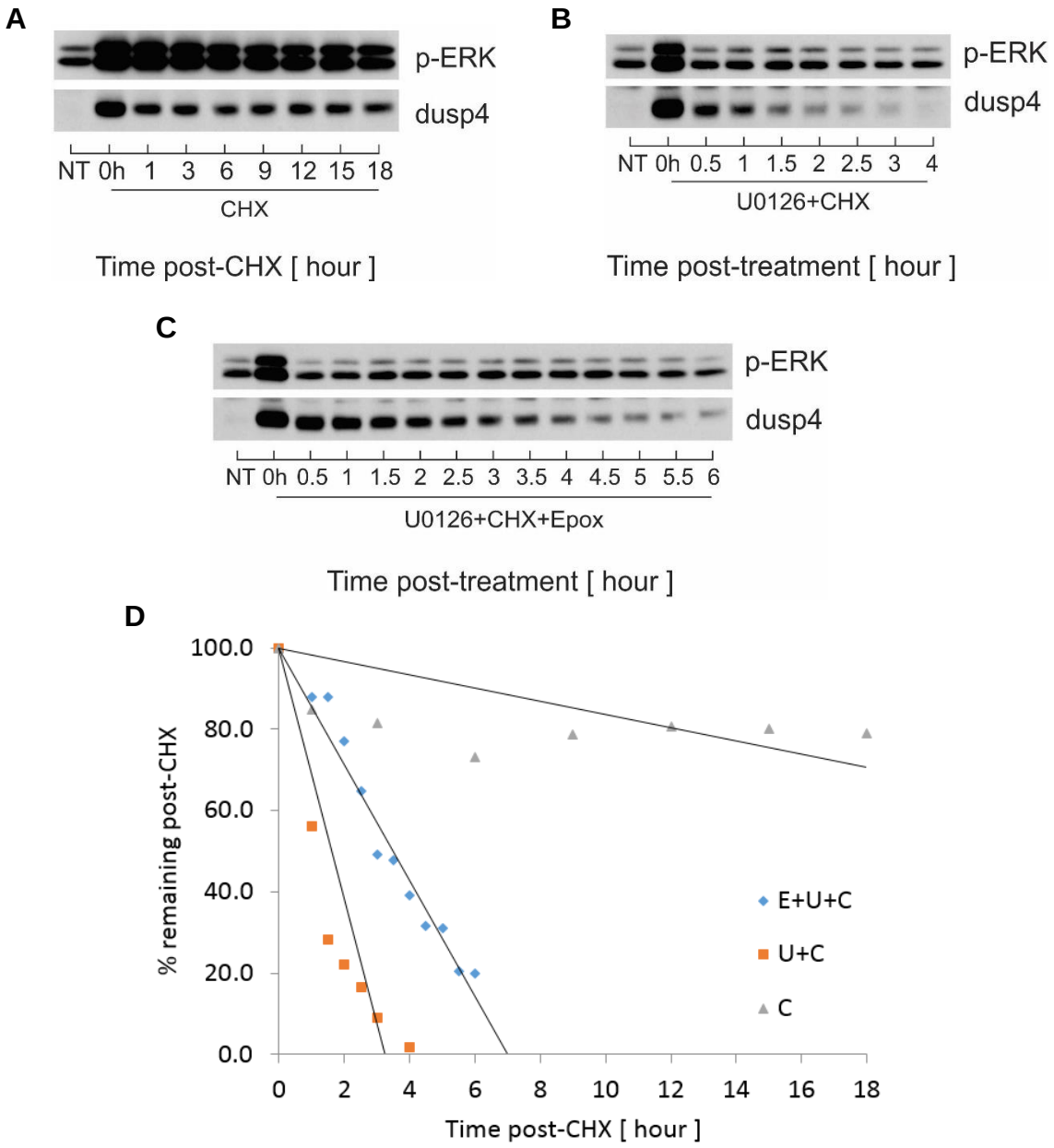

Figure 2.3. ERK Activity Determines DUSP4 Protein Stability and Proteasomal Activity is Involved in DUSP4 Protein Degradation. Preadipocytes were pretreated with PMA for 2h before addition of A) 5μM cycloheximide (CHX), B) U0126+CHX, or C) U0126+CHX+ 5μM Epoxomicin (Epox). Lysates were collected at indicated times and protein expression of bisphosphorylated ERK and DUSP4 analyzed via immunoblotting. NT: no treatment. D) Remaining DUSP4 protein abundance was determined by densitometry and plotted as a percent of control for half-life determination.

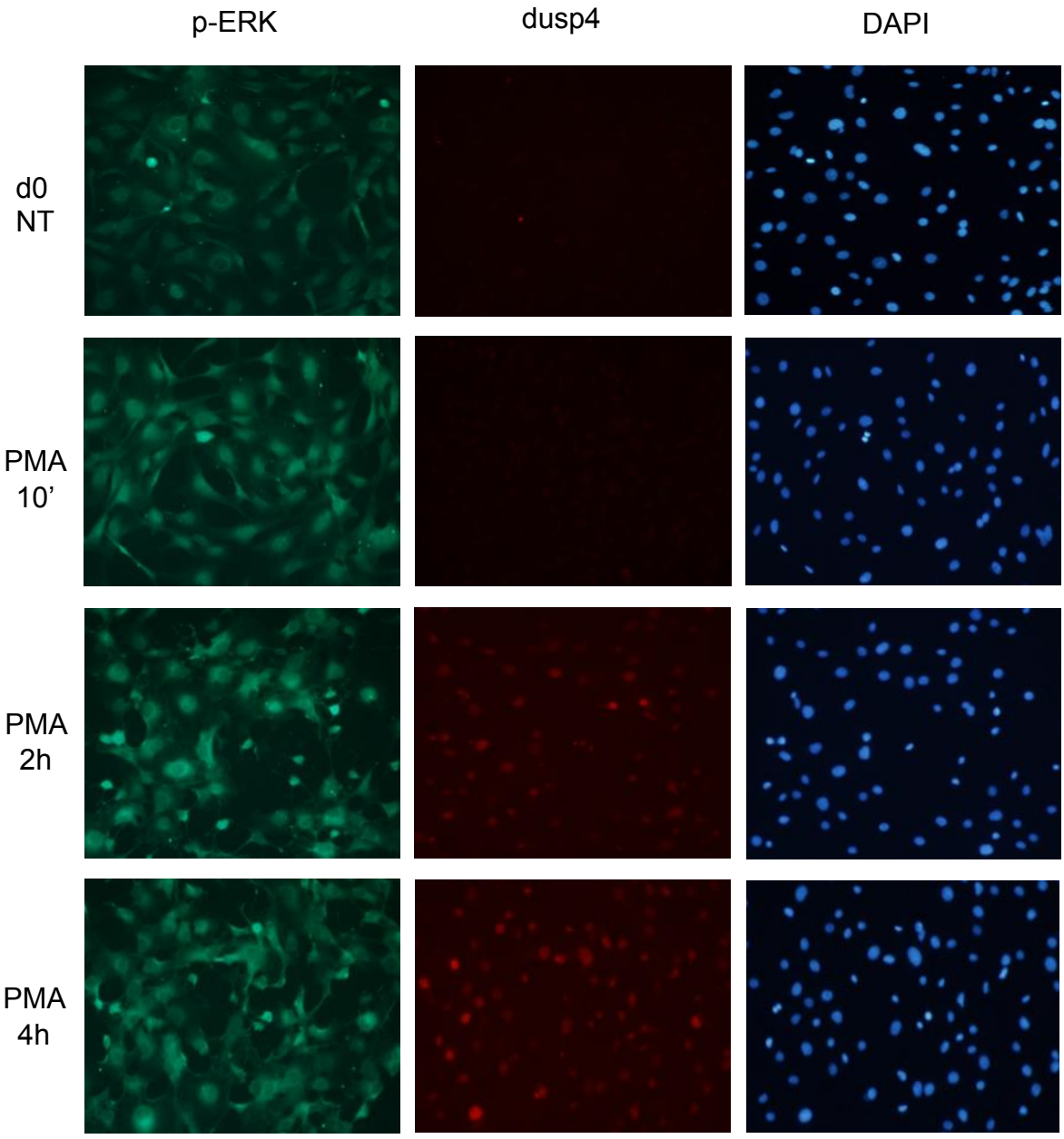

Figure 2.4. Inhibition of ERK Phosphorylation following DUSP4 Expression in the Nucleus. Preadipocytes were grown on a 4-well chamber slide and stimulated with PMA. Subcellular compartmentalization of bisphosphorylated ERK (green) along with DUSP4 (red) was assessed at indicated times by immunofluorescence. DAPI (4',6-diamidino-2-phenylindole) staining (blue) was performed to reveal location of nucleus.

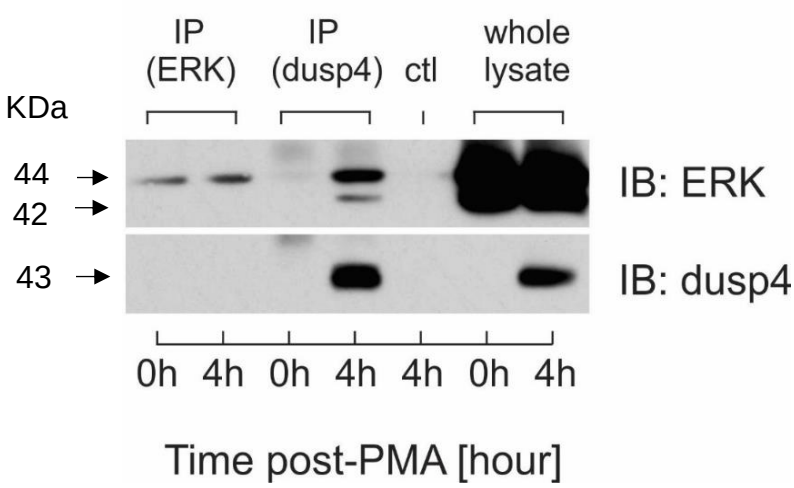

Figure 2.5. Protein-protein Interactions Between DUSP4 and ERK. Cells lysates were harvested from density-arrested 3T3-L1 PA at 0 hr and 4 hrs post-PMA (300nM), immunoprecipitated for ERK (lane 1 and 2) and DUSP4 (lane 3 and 4), immunoblotted for DUSP4 and ERK. Lane 5: control, resin with no antibody attached. Lane 6 and 7: whole cell lysates.

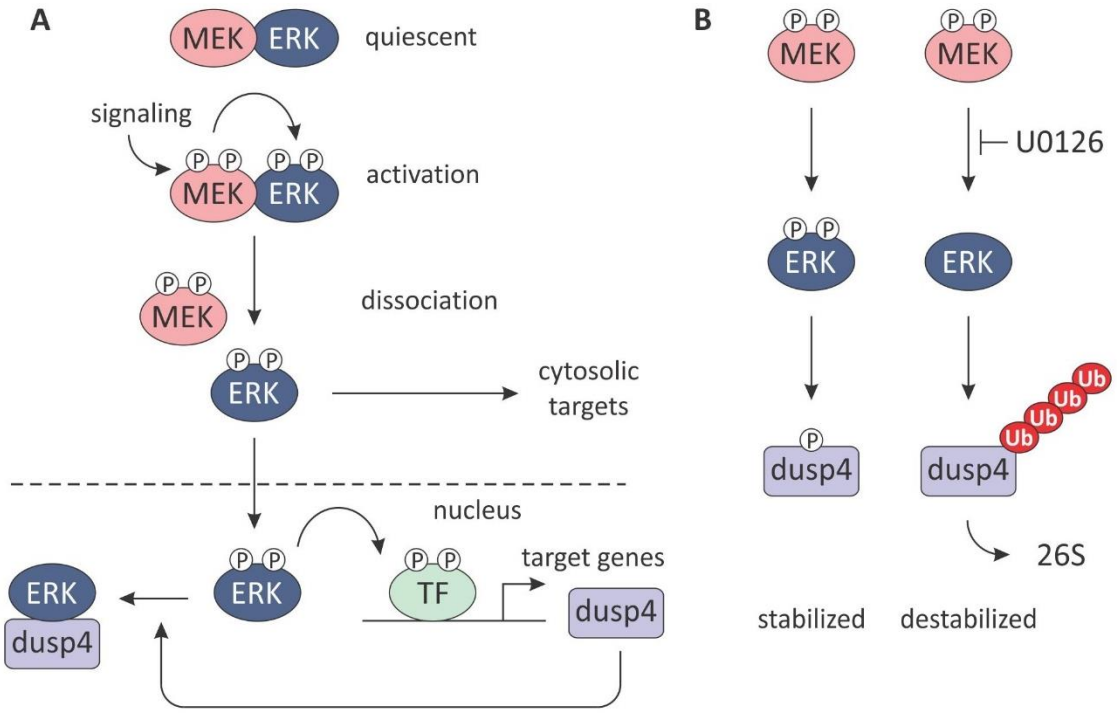

Figure 2.6. Proposed Working Model for Regulation and Function of DUSP4 in Response to Sustained ERK Activation in Adipocytes.

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CHAPTER III

ROLE FOR DUAL-SPECIFICITY PHOSPHATASE 4 IN ADIPOCYTE DIFFERENTIATION

Abstract

Chronic adipose tissue (AT) inflammation is central to pathogenic mechanisms linking obesity and insulin resistance (IR). There is strong evidence demonstrating that mitogen-activated protein kinases (MAPKs) are important signaling mediators linking inflammation to obesity-induced IR. While numerous studies have examined upstream signaling kinases that are critical for MAPK activation, few have examined the biological outcomes of MAPK deactivation by MAPK-specific, dual specificity phosphatases (DUSPs). To address the latter, we determined the expression profile of the 10 known DUSP family members in insulin-responsive tissues of genetic-induced obese (GIO) and diet-induced obese (DIO) mice that are known to have elevated degrees of inflammation. We demonstrated that DUSP4 and DUSP9 mRNAs were significantly and markedly (>10-fold) more abundant in lean AT isolated from C57BL/6J male mice relative to liver, skeletal muscle and heart. Examining sequential stages of GIO and DIO mice, we determined that DUSP9 was the only DUSP with elevated mRNA in obese versus lean AT. In contrast, DUSP4 mRNA inversely correlated with the degree of inflammation associated with both models of obesity. Using 3T3-L1 adipocytes, we further demonstrated biphasic induction of DUSP4 during adipocyte differentiation,

with early phase induction of this phosphatase being ERK-dependent and late stage induction being ERK-independent and differentiation-specific. Moreover, DUSP4 was significantly and markedly suppressed by TNF-α in adipocytes. However, DUSP4 antisense expression did not inhibit 3T3-L1 adipogenesis. Collectively, these results suggest that tissue-specific regulation of DUSP4 may contribute to obesity-mediated inflammation and IR. Furthermore, our findings establish a platform for future investigations towards the function of DUSP4 in obesity-associated inflammation.

Introduction

Obesity is one of the most prevalent nutrition-related physiological disorders that contributes to the development of a variety of chronic health problems such as cardiovascular diseases, dyslipidemia, hypertension, nonalcoholic fatty liver disease, insulin resistance (IR), type II diabetes (T2D) and certain cancers [1-4]. The increasing rate of obesity, and its concomitant health risks, makes it one of the greatest threats to global health [1, 5]. While adipose tissue (AT) has been historically considered as a reservoir for storing excess energy, extensive research over the past few decades has shown that AT is a biologically active endocrine organ which plays an important role in regulating whole-body homeostasis [6, 7]. However, obesity could negatively impact AT function by alteration of major signaling pathways, leading to IR and other molecular dysfunctions [8-10]. A number of studies have shown that pro-inflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6 and tumor necrosis factor-α (TNF-α), are increased with obesity [8, 9, 11-13]. These inflammatory

molecules trigger inflammatory responses by various mechanisms, one of which is through the activation of the mitogen activated protein kinases (MAPKs).

It is well established that *MAPK*s play an important role in various cellular processes. Most of the functional studies of MAPK are focused on three major groups: extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs) and p38 kinases. MAPK activation requires dual phosphorylation of threonine and tyrosine residues of the (T-X-Y) motif within the activation loop by specific upstream MAPK kinases [14, 15]. Activated MAPKs participate in the transcriptional activation of a number of genes that promote differential cellular outcomes such as proliferation, differentiation, inflammation and apoptosis [16]. Adipocyte hyperplasia (cell number increase) and hypertrophy (cell size increase) are crucial mechanisms for the development of obesity, and are dependent on adipocyte differentiation [7, 16-18]. Many research labs have extensively investigated the role of MAPKs in adipocyte development under normal and pathological states. Evidence has revealed that early, transient ERK activation is required for adipocyte differentiation while blocking ERK activity suppresses this process [17, 19, 20]. Similarly, p38 activation is required for adipocyte differentiation, while persistent activation can lead to the death of adipocytes [21-23]. Activation of JNK by free fatty acids has been shown to reduce glucose uptake by alteration of insulin receptor-mediated signal transduction, which leads to suppressed glucose transporter type 4 (GLUT4) translocation [24]. Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear transcription factor that is highly

expressed in AT and considered a master regulator of adipogenesis. Activation of PPARγ initiates transcription of genes that regulate lipid and glucose metabolism as well as suppressing inflammatory genes in adipocytes [24-26]. Studies have shown that phosphorylation of PPARγ by MAPKs suppresses its transcriptional activity, thereby leading to impaired adipogenesis and insulin sensitivity [27-29].

While upstream kinases have been extensively studied regarding MAPK activation in the past, growing evidence now strongly suggests protein phosphatases, including protein tyrosine phosphatases (PTPs), serine/threonine phosphatases (PPs) and dual-specificity phosphatases (DUSPs), as important regulators of MAPK activity [30, 31]. While the first two groups only dephosphorylate single tyrosine or threonine residues, DUSPs dephosphorylate both residues [32]. Of the many identified DUSPs, a group of 10 DUSPs contains a common MAPK-binding (MKB) domain that specifically confers catalytic activity toward MAPKs [32-34]. These DUSPs are further divided into three subfamilies based on their sequence, substrate specificity and localization. Type I DUSPs includes DUSP1, DUSP2, DUSP4 and DUSP5, all of which are localized to the nuclear compartment and can be induced by mitogenic or stress signals. Type II DUSPs comprises DUSP6, DUSP7 and DUSP9, which are ERK-specific cytosolic DUSPs. The last group containing DUSP8, DUSP10 and DUSP16 are JNK/p38-specific phosphatases that are localized within both the cytosolic and nuclear compartment [30, 32].

In recent years, investigators have started to uncover roles of DUSPs in obesity-induced IR and adipocyte dysfunction. For instance, DUSP1 is an immediate early gene

that is induced during adipocyte differentiation [35]. DUSP1 knockdown lead to prolonged activation of ERK and p38 without inhibiting adipocyte differentiation [35]. Mice lacking DUSP1 were protected against diet-induced obesity (DIO) [36]. DUSP9 is also induced during late stages of adipocyte differentiation [35]. Overexpression of DUSP9 in 3T3-L1 cells suppressed ERK and JNK activation, leading to improved insulin sensitivity and glucose tolerance against obesity-induced inflammatory stress [37]. In addition, studies have revealed that DUSP6 is involved in glucose and lipid metabolism and DUSP6-deficient mice are protected against DIO [38, 39].

Considering DUSPs as controllers of MAPK signaling and the potential of DUSPs against obesity-induced chronic inflammation, we explored the role of DUSP4 in adipocyte differentiation. Initial screening of DUSP4 revealed that, among major biologically active insulin-responsive tissues, DUSP4 was expressed more abundantly in white AT relative to liver, skeletal muscle, and heart of lean mice. Expression of DUSP4 was markedly suppressed in animal models of genetic and diet-induced obesity with elevated production of inflammatory markers. Using the murine 3T3-L1 cell line as a model of adipogenesis, we further demonstrated that DUSP4 was induced biphasically during adipocyte differentiation. While DUSP4 induction during early phase of adipocyte differentiation (4 hrs) required ERK signaling, the late phase DUSP4 induction (d4-d5) appeared to be ERK-independent and specific to differentiation, regardless of cell type. Additionally, DUSP4 knockdown was not sufficient to block adipogenesis. Collectively, our findings provide the first molecular evidence demonstrating the regulation of DUSP4

during adipocyte differentiation, highlighting a potential role for DUSP4 as a prospective target for obesity-associated metabolic dysfunctions.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DMEM), calf bovine serum (CS), Trypsin-EDTA, and recombinant murine TNF-α were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from HyClone. The following antibodies were used for immunoblot analysis: phospho-MEK, phospho-ERK (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), and α-tubulin were purchased from Cell Signaling; DUSP4 was purchased from Santa Cruz Biotechnology. Pharmacological inhibitors of ERK (U0126), JNK (SP600125) and p38 (SB203580) were purchased from CalBiochem. Troglitazone was purchased from Cayman Chemical. Enhanced chemiluminescence (ECL) reagents were obtained from Perkin-Elmer Life Sciences. All TaqMan primer probes used in this study were purchased from Applied Biosystems.

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 a 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. Lean C57BL/6J control mice were fed a control low fat diet (LFD) consisting of 10% kcal from fat (Research Diet Inc. D12450B) from 6 wks of age. Both diets contained 10% kcal from protein with the balance in caloric value provided by differences in carbohydrate content. Mice receiving both diets were given 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, calf skeletal muscle, and ventricular heart tissue 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.

Cell Culture. The murine 3T3-L1 cell line was purchased from Howard Green, Harvard Medical School. Cells were propagated in DMEM supplemented with 10% CS until reaching density-induced arrest, as previously described. 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. All experiments were repeated 2-3 times to validate results and ensure reliability.

Immunoblotting. Cell monolayer were washed with phosphate-buffer saline (PBS) and scraped into ice-cold lysis buffer containing 0.1 M Tris (pH 7.4), 150 mM NaCl, 10% sodium dodecyl sulfate (SDS), 1% Triton X, 0.5% Nonidet P-40 (NP40), 1 mM EDTA,

1 mM EGTA. Phosphatase inhibitors (20 mM β-glycerophosphate, 10 mM sodium fluoride and 2 μM sodium orthovanadate) and protease inhibitors (0.3 μM aprotinin, 21 μM leupetin, 1 μM pepstatin, 50 μM phenanthroline, 0.5 μM phenylmethylsulfonyl fluoride) were added to lysis buffer immediately prior to cell harvest. Cell lysates were sonicated and centrifuged (15,000*g,* 10 min, 4°C), and the supernatant transferred to a fresh tube. Protein content was determined by bicinchoninic acid (BCA) procedures according to manufacturer's (Pierce, Rockford, IL) instructions. Equal amounts of whole lysate protein were separated by SDS-PAGE electrophoresis. Cell lysates were mixed with loading buffer containing 0.25M Tris (pH6.8), 4% SDS, 10% glycerol, 0.01% bromophenol blue, and 10% dithiothreitol, then heated at 80°C for 5 min prior to electrophoresis. Proteins were resolved on SDS-polyacylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) 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 2 hrs at room temperature. Membranes were immersed in ECL and data 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, Valencia, CA), according to manufacturer protocol. 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. All TaqMan primer probes used in this study were also purchased from ABI. Data were recorded and analyzed with ABI Sequence Detector Software and graphs visualized with SigmaPlot software. All data were presented as mean ± standard error of the mean (SEM) and representative of duplicate determinations. Data were normalized to 18S and measured as relative differences using the $2^{-\Delta\Delta CT}$ method as previously described [40, 41].

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. Data regarding mRNA tissue distribution were analyzed using analysis of variance (ANOVA), with Tukey's *posthoc* analysis used when the *p*-value for the respective parameter was statistically significant ($p < 0.05$).

RNA Interference. Lentiviruses were generated using the 2nd generation Lentiviral packaging plasmid (psPAX2) and envelope plasmid (pMD2.G) purchased from Addgene. Polyethylenimine (Polysciences) was used to transfect HEK L293 cells either with vectors containing DUSP4-specific shRNA (TRCN0000054351, Signa-Aldrich) or a non-targeted scramble shRNA. Stable knockdown 3T3-L1 cell line was produced by lentiviral transduction with either DUSP4 targeting shRNA or scrambled shRNA in the presence of 10μg/mL polybrene and selected in 10% CS medium containing 5μg/mL puromycin (Invitrogen) for 10 days. The selected clones were subsequently maintained in fresh puromycin-containing medium, analyzed, and used for experiments as described above.

Results

Expression of DUSP4 in Insulin-responsive Tissues of Lean C57BL/6J Male Mice. A number of studies have revealed that obesity-induced chronic inflammation leads to aberrant MAPKs activation which further contributes to the development of IR [28, 42-44]. Correspondingly, accumulating evidence has also shown that select DUSPs play a role in modulating MAPK activity and insulin signaling under inflammatory stress [37, 39, 45, 46]. Thus, we initially sought to examine relative mRNA expression of all 10 MAPK-specific DUSPs in major types of insulin-responsive tissues, such as epididymal white AT (WAT), liver, skeletal muscle and heart, from C57BL/6J wildtype mice using qRT-PCR. DUSPs mRNA abundance were normalized to 18S ribosomal RNA and expressed as fold-

differences relative to WAT. We report that, within the nuclear DUSP group, only DUSP4 mRNA was ~14-fold more abundant in WAT compared to liver, skeletal muscle and heart (Fig.3.1A). Similar tissue distribution was noted for cytosolic DUSP9 where relative mRNA was markedly more abundant (~50 fold) in WAT relative to all the other three tissue types (Fig.3.1B). The remaining DUSPs exhibited divergent tissue distribution (data not shown). Collectively, these data suggest the potential of tissue-specific regulation and function of DUSP4 and DUSP9.

Regulation of DUSP4 and DUSP9 in WAT during the Progressive Dvelopment of Genetic and Diet-induced Obesity. Data presented above demonstrated that both DUSP4 and DUSP9 were significantly more abundant in AT relative to other insulin-responsive tissues. Since accumulating evidence now clearly suggests that AT is the predominant site for the development of obesity-induced metabolic dysfunction, we next examined if expression of DUSP4 and DUSP9 were impacted under pathological progression of obesity using two distinct stages and models of obesity (genetic vs diet-induced obesity). For development of genetic-induced obesity (GIO), B6-ob/ob mice 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, according to the supplier. Thus, to compare the progression of obesity, 6 wk and 10 wk old ob/ob mice and wildtype littermates, representing sequential stages (early vs. mid-stage) of obesity with developing obesity-related metabolic disorders, were chosen for this study. For development of diet-induced obesity (DIO), C57BL/6J male mice were

fed a high fat diet (HFD; 60% kcal from fat) starting at 6 wks of age by the supplier. Control littermates were fed a control diet (CD) containing 10% kcal from fat. Both diets contained 10% kcal from protein with the balance in caloric value provided by differences in carbohydrate content. Studies were conducted at 18 wks and 24 wks of age, representing 12 wks and 18 wks of dietary intervention, respectively. Progression of obesity development in both models were arbitrarily designated as stage I and stage II. Using these two models, we determined relative mRNA abundance for DUSP4 and DUSP9 as well as other well-known inflammatory markers of obesity (i.e., MCP-1, TNF-α, and IL-6) in WAT where fold-differences were determined between obese and lean values at different sequential stages of obesity. Adipsin was also assessed to confirm the onset of obesity at each stage as others have reported a significant suppression of this adipocyte-specific gene in WAT isolated from obese animals compared to their lean littermates [47, 48].

As illustrated in Fig.3.2, adipsin was markedly suppressed as ob/ob mice transitioned from stage I (40-fold reduction) to stage II (80-fold reduction) obesity. Similarly, adipsin expression progressively decreased (7-fold vs. 25-fold) in both stages of DIO mice. MCP-1 has been shown to promote macrophage infiltration into AT during onset of obesity [13, 49-51]. Consistent with others, we observed MCP-1 induction which was greater than 7-fold at both stages of development and in both models of obesity. Relative mRNA abundance of IL-6 and TNF-α was only significantly induced in stage II ob/ob mice fed a standard chow. Conversely, both cytokines were elevated in

DIO mice fed 12-wk HFD (stage I) with progressive increases in TNF-α (3-fold vs. 6-fold) correlating with the progression of obesity (18-wk HFD, stage II). DUSP4 expression increased only in stage I genetic (ob/ob) mice while no significant changes were observed for IL-6 and TNF-α. Induction of DUSP4 was not detected in late stage ob/ob mice with elevated production of IL-6 and TNF-α. Regarding DIO, DUSP4 mRNA expression showed no marked difference after 12 wks of HFD (stage I) and was even suppressed after 18 wks of HFD (stage II) while TNF-α expression progressively increased. Interestingly, in both animal models of obesity, DUSP9 mRNA abundance increased during each stage of development. In addition, DUSP9 gene expression also progressively increased as mice transitioned from stage I to stage II obesity in both models. Collectively, these data demonstrated gene expression of DUSP4 and DUSP9 was divergently regulated in WAT based on the progressive stage of obesity development, the progressive inflammatory environment as well as the model of obesity.

Regulation of DUSP4 during Early and Late Stages of Adipocyte Differentiation. The progression of obesity involves adipocyte hyperplasia and hypertrophy and adipocytes are the most predominant cell type in AT [7, 18]. While others have already studied the role for DUSP9 in adipocytes [37, 52], information regarding regulation and function of DUSP4 in adipocytes is still limited. This led us to investigate the regulation of DUSP4 during the course of adipocyte differentiation. Density-arrested preadipocytes were stimulated with hormonal differentiation cocktail (i.e., MDI). Total RNA and whole

cell lysates were collected over early (0-24hr) and late (d0-d9) stages of differentiation. DUSP4 mRNA and protein expression as well as MAPK phosphorylation and PPARγ expression were examined via qRT-PCR and immunoblot analysis, respectively. As illustrated in Fig.3.3, during the early stage of differentiation, addition of MDI led to immediate, robust ERK phosphorylation that peaked within 10 mins and decayed to basal levels within 6 hrs of stimulation (Fig.3.3A). DUSP4 mRNA and protein levels kinetically and transiently increased (4-fold) at 4 hrs post-MDI in a feedback manner where expression of this phosphatase was inversely proportional to ERK phosphorylation (Fig.3.3A and B). Expression of this phosphatase returned to baseline value by 24 hrs post-MDI. More surprisingly, we observed a more sustained induction of DUSP4 starting at 4d, correlating with the expression of adipogenic marker PPARγ, and remaining above baseline throughout the course of adipocyte differentiation (Fig.3.3C and D).

Using MAPK inhibitors that selectively inhibit ERK, JNK or p38 activation, we demonstrated that induction of DUSP4 during the early phase of differentiation was mediated via MEK/ERK signaling as only ERK inhibitor U0126 suppressed DUSP4 expression (Fig.3.4A). In contrast, induction of DUSP4 during the late stages of adipocyte differentiation was not suppressed by any MAPK inhibitor (Fig.3.4A) as phosphorylation state for all three MAPKs remained unchanged or even decreased throughout this period (Fig.3.4B). To determine the regulation of DUSP4 under inflammatory stress, PAs and ADs were stimulated in parallel with 100 pM TNF-α and relative mRNA abundance

assessed by qRT-PCR over time. As illustrated in Fig.3.5, mRNA levels for DUSP4 were markedly suppressed in both cell types where the degree of suppression was sustained throughout the course of the experiment.

As the ERK-independent DUSP4 induction occurred concomitantly with the expression of adipogenic marker PPARγ, we next stimulated PAs with MDI in the presence or absence of troglitazone (TROG) to determine if DUSP4 is a downstream target of PPARγ. TROG is a potent agonist that increases PPARγ transcriptional activity while also induces its protein degradation [53, 54]. As illustrated in Fig.3.6A, DUSP4 protein expression did not increase with addition of TROG during adipocyte differentiation. We further sought to investigate if the ERK-independent DUSP4 induction was specific to 3T3-L1 cells or to the process of differentiation. First, density-arrested non-adipogenic NIH 3T3 cells [55] were stimulated with MDI and cell lysates harvested over time and immunoblotted for DUSP4 and PPARγ. As illustrated in Fig.3.6B, MDI did not induce NIH 3T3 cell differentiation and expression of PPARγ and DUSP4 was not detected. Second, adipogenic 3T3-F442A cells [56] were induced to differentiation with insulin over time in the presence or absence of TROG and immunoblotted for DUSP4 and PPARγ. Consistent with the MDI experiment above, induction of DUSP4 and PPARγ was observed during F442A cells differentiation and DUSP4 protein expression did not further increase with the addition of TROG (Fig.3.6C). Taken together, these data demonstrate that mid-stage induction of DUSP4 in dependent on differentiation as

opposed to chemical inducers and suggest a potential role for DUSP4 during latter stages of adipogenesis.

DUSP4 Knockdown does not Inhibit Adipocyte Differentiation. Data presented above demonstrate that DUSP4 induction during late stages of adipocyte differentiation is MAPK-independent and specific to differentiation. To examine a role for DUSP4 during differentiation, we first established a stable DUSP4 knockdown 3T3-L1 cell line using short hairpin RNA lentiviral particle and appropriate antibiotic selection before inducing to differentiate. Total RNA was collected over time during differentiation and DUSP4 mRNA expression was examined via qRT-PCR. Phase contrast microscopy was performed to assess changes in cell morphology and lipid accumulation at early (d0), mid (d4) and late (d8) stages of differentiation. As shown in Fig.3.7A, compared to L1 control cells, DUSP4 knockdown cells exhibited 75% less DUSP4 mRNA at the beginning of differentiation. ERK-independent DUSP4 induction during late stages of differentiation was also significantly suppressed in knockdown cells. No discernible differences of morphology or lipid accumulation were observed between these two groups of cells during differentiation (Fig.3.7B). These data suggest a role for DUSP4 in adipocytes independent from adipocyte differentiation.

Discussion

In this study, we present the first empirical evidence regarding the regulation of DUSP4 in major insulin responsive tissues under conditions of obesity, as well as roles for DUSP4 in 3T3-L1 adipocytes. First, we demonstrate that DUSP4 was significantly

more abundant in WAT of lean animals relative to liver, skeletal muscle and heart. Second, we show that DUSP4 in WAT was suppressed with the degree of inflammation in both genetic and diet-induced models of obesity. Third, we reveal biphasic induction of DUSP4 during early and late stages of adipocyte differentiation as well as suppression of DUSP4 by inflammatory cytokine TNF-α in both PAs and ADs. Fourth, we show that while early phase DUSP4 induction was in response to ERK activation, induction of this phosphatase during mid-to-late stages of adipocyte differentiation appeared to be ERK-independent and specific to differentiation regardless of cell type. Finally, we demonstrate that DUSP4 specific knockdown was not sufficient to block adipocyte differentiation. Collectively, these data provide novel insight regarding DUSP4 in WAT under conditions of obesity as well as during adipocyte differentiation, potentially establishing new therapeutic targets against inflammatory stress and metabolic disorders that are associated with obesity.

It is well documented that obesity-associated chronic inflammation is crucial for the development of IR and other systemic metabolic diseases [4, 5, 7]. Accumulating evidence also shows that members of MAPK-specific DUSPs exhibit restricted expression in different tissue types [30, 31], allowing individual phosphatases to modulate MAPK-dependent biological processes involved in insulin signaling [37, 39, 57]. Therefore, we first evaluated expression patterns of these DUSPs in four major metabolically active insulin-responsive tissues under normal physiological condition. Data presented here demonstrate that expression of DUSP4 and DUSP9 was significantly more abundant in

WAT relative to liver, skeletal muscle and heart, highlighting the potential for individual DUSPs in tissue-specific regulation of MAPK events that contribute to obesity-mediated IR and other metabolic inflammatory diseases.

Previous studies have clearly demonstrated that WAT is the predominant site for the production and secretion of inflammatory molecules that cause IR and metabolic dysfunction [7, 10]. Moreover, WAT inflammation develops gradually over the course of obesity, where infiltration of macrophages as well as production of inflammatory cytokine and chemokine increases with the development of obesity [6-9, 13]. Data presented above demonstrate that, in the GIO model, MCP-1 was the only inflammatory marker increased in WAT of 6 wk old B6-ob/ob mice (stage I), while MCP-1, IL-6 and TNF-α were all elevated in WAT of 10 wk old B6-ob/ob mice (stage II). However, in the DIO model, all three inflammatory markers were already elevated in WAT of mice fed a 12wk HFD (stage I). In addition, TNF-α expression continued to increase in mice fed HFD for 18 wks (stage II). In contrast, the expression of adipsin in WAT significantly decreased at these two sequential stages and in both models of obesity, indicating the development of obesity. Collectively, these data demonstrate that the degree of WAT inflammation is positively correlated with the development of obesity.

Data presented here also revealed that DUSP4 and DUSP9 were divergently regulated in WAT based on the stage and model of obesity as well as the degree of inflammation associated with obesity. We observed induction of DUSP4 only in WAT of stage I GIO mice where there was no elevated gene expression of TNF-α. DUSP4 was

suppressed at the sequential stage of GIO mice as well as in both stages of DIO mice where TNF-α was markedly induced. In contrast, we demonstrate that DUSP9 was significantly induced in WAT of GIO and DIO mice at both stages, paralleling the elevated inflammatory cytokine gene expression. Consistent with our findings, other studies also reported that DUSP9 was elevated in WAT of mice under conditions of obesity [37, 58]. Collectively, these data suggest that progression of obesity-induced inflammation may be responsible for the divergent regulation of DUSP4 and DUSP9 in WAT.

It is widely accepted that WAT is comprised of many different cell types with ADs being the most predominant cells. While studies in the past extensively investigated the effect of MAPK activation in adipogenesis, recent reports have begun to elucidate roles of DUSPs in adipogenesis with growing evidence pointing to DUSPs as key modulators controlling cell signaling and biological outcomes. For instance, earlier evidence showed that genetic ablation of DUSP1 inhibits adipocyte differentiation [59]. However, contradictory findings from a more recent study demonstrated that genetic loss of DUSP1 leads to sustained ERK and p38 activation during early phase of adipocyte differentiation, but with no effect on adipogenesis[35]. Moreover, DUSP9 gain-of-function studies showed that overexpression of this phosphatase blocks adipogenesis and protects cells against inflammatory stress-induced IR [37, 52]. As the role of DUSP4 in adipocytes remains largely unknown, data presented in our report demonstrate biphasic induction of DUSP4 during adipocyte differentiation. The early phase DUSP4 induction is mediated via the MEK/ERK signaling as blocking ERK activity by U0126

significantly suppressed DUSP4 expression. Interestingly, we find a second peak of DUSP4 induction during mid-to-late stages of adipocyte differentiation occurring concomitantly with the induction of PPARγ. This late phase induction is independent of MAPK activity as phosphorylation of all three MAPKs remained at basal levels comparable to untreated PAs (d0) throughout the entire experimental period. We also observed TNF-α-mediated marked suppression of DUSP4 in PAs and ADs, which is consistent with the finding from our in vivo study that inhibition of DUSP4 is accompanied by the progressive increase of inflammatory cytokine TNF-α during the development of obesity. Upon further examination, we revealed that DUSP4 is not downstream of PPARγ, suggesting a potential role for DUSP4 in protecting PPARγ against aberrant MAPK activation as phosphorylation of PPARγ by MAPKs has been shown to suppress transcriptional activity during adipogenesis [27-29]. In addition, by using NIH-3T3 and NIH-F442A cells, we report that the late phase induction of DUSP4 is not cell-type, but differentiation-specific. By establishing DUSP4 knockout 3T3-L1 cell line, we further demonstrate that suppression of DUSP4 is not sufficient to block adipocyte differentiation. Taken together, these data demonstrate unique regulation of DUSP4 during adipocyte differentiation while its exact role regarding MAPK activity and adipogenesis remain unresolved.

In summary, this study provides the first comprehensive analysis showing that members of the MAPK-specific DUSP family are divergently regulated in a tissue-specific manner under conditions of obesity. Data outlined here and elsewhere [37, 52]

demonstrated that expression of DUSP9 in WAT is upregulated with the developmental stage of obesity. DUSP4, on the other hand, induced during the early-stages of obesity when there are no elevated levels of TNF-α, and significantly suppressed at the sequential stage of obesity characterized by the increased inflammation associated with obesity. Data presented here further demonstrate the biphasic induction of DUSP4 during adipocyte differentiation. While the early phase DUSP4 induction is ERK-dependent, the late phase induction is differentiation-specific, regardless of cell type. Moreover, knockdown of DUSP4 alone did not block adipogenesis, suggesting that DUSP4 may function independently from adipogenesis. Conclusions drawn from our data have led to the following working model (Fig.3.8). Obesity-mediated inflammation leads to PPARγ phosphorylation by aberrant MAPK activation, resulting in degradation of PPARγ and suppression of PPARγ transcriptional activity during adipogenesis (Fig.3.8A). DUSP4 negatively control MAPK activation, which could potentially protect PPARγ from being phosphorylated by MAPK so that PPARγ can maintain normal function during adipocyte differentiation (Fig.3.8B). As investigations on DUSP4 continue, elucidation into the mechanistic role of DUSP4 will shed new lights on the progression and regulation of AT inflammation and provide novel therapeutic targets for treatment of obesity-associated metabolic dysfunction and IR.

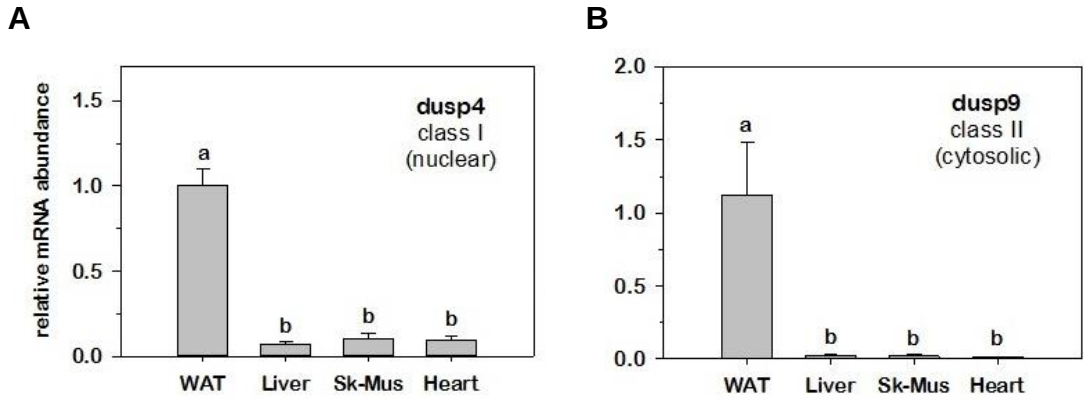

Figure 3.1. Tissue-specific Expression of DUSP4 and DUSP9. Epididymal white AT (WAT), liver, skeletal muscle (SK Muscle), and heart were harvested and total RNA prepared from C57BL/6J male mice. Gene expression of all ten DUSPs was analyzed via qRT-PCR. All data were normalized to 18S rRNA and tissues 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 ($p < 0.05$).

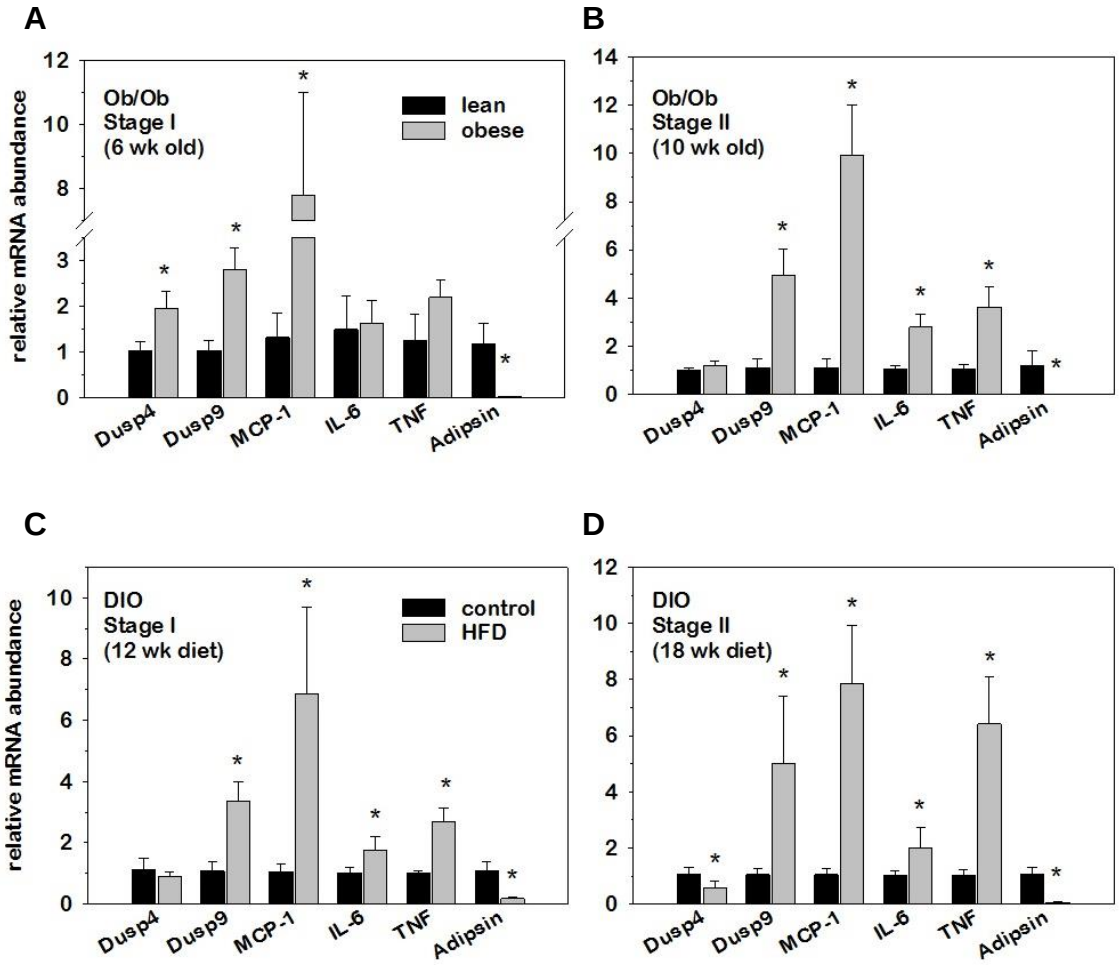

Figure 3.2. AT Specific Regulation of DUSP4 and DUSP9 and Inflammatory Mediators during the Development of Obesity under Conditions of Genetic and Diet-induced Obesity. AT was harvested and RNA prepared from A) 6-wk-old and B) 10-wk-old male B6.V-LepOb/J (ob/ob) mice and their lean littermates, along 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.

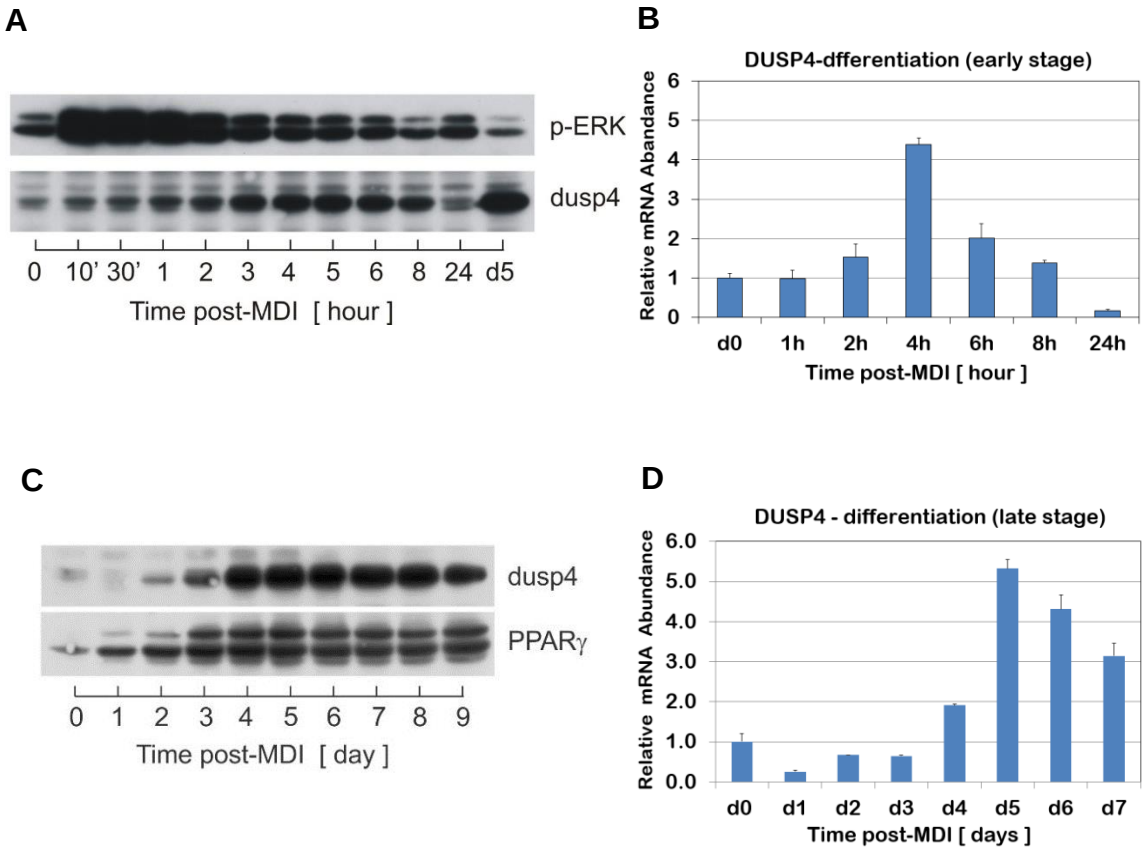

Figure 3.3. Biphasic Induction of DUSP4 during Adipocyte Differentiation. Preadipocytes were stimulated with MDI and cell lysates harvested during A) early and C) late differentiation at indicated times and protein expression of DUSP4 along with bisphosphorylated ERK and PPARγ analyzed via immunoblotting. In addition, total RNA was harvested during B) early and D) late adipocyte differentiation prior to mRNA analysis of DUSP4 using qRT-PCR. Data were normalized to 18S rRNA and expressed as relative differences compared to untreated preadipocytes (d0).

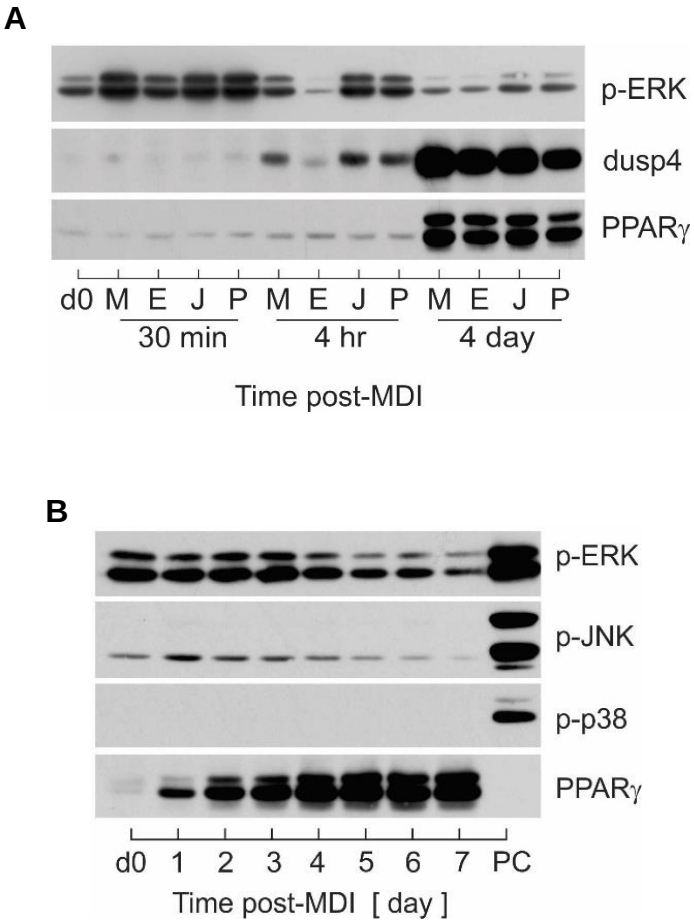

Figure 3.4. DUSP4 Induction in Response to ERK Activation and MAPKs Phosphorylation Status during Adipocyte Differentiation. A) Preadipocytes were stimulated with MDI in the presence or absence of individual MAPKs inhibitors. Cell lysates were harvested at indicated times and protein expression of DUSP4 along with bisphosphorylated ERK and PPARγ analyzed via immunoblotting. M: MDI only; E: U0126+MDI; J: SP600125 +MDI; P: SB203580+MDI. B) Preadipocytes were stimulated with MDI and cell lysates harvested over time prior to immunoblot analysis of bisphosphorylated ERK, JNK, and p38 as well as PPARγ.

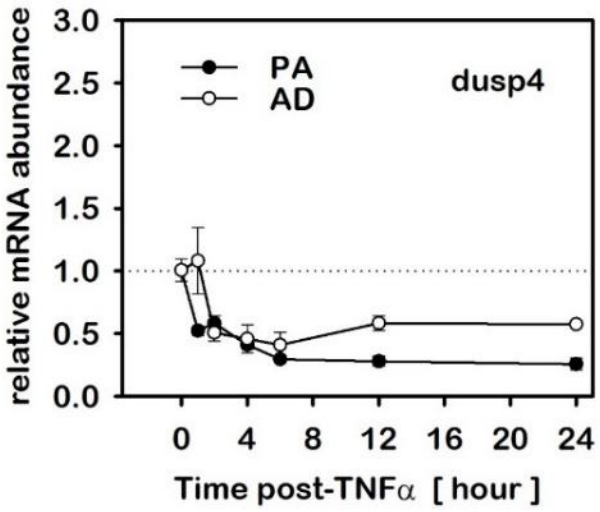

Figure 3.5. DUSP4 is Suppressed in PAs and ADs in Response to TNF-α. Total RNA was collected from PAs and ADs stimulated in parallel with 100pM TNF-α over time and gene expression of DUSP4 measured via qRT-PCR. All data were normalized to 18S rRNA and expressed relative to untreated cells. Genes were considered suppressed when fell below a 0.5 criterion indicated by the dotted line on the graph.

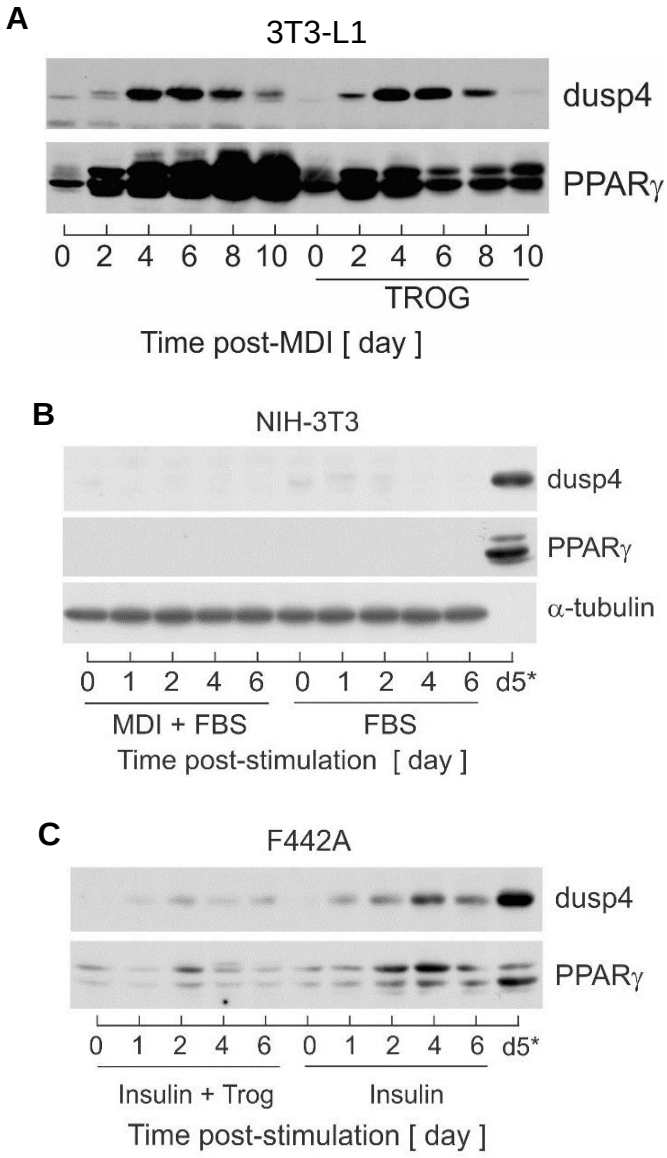

Figure 3.6. ERK-independent DUSP4 Induction during Adipocyte Differentiation is not Mediated by PPARγ and is Specific to Differentiation. A) 3T3-L1 preadipocytes were stimulated with MDI in the presence or absence of Troglitazone (Trog), B) NIH 3T3 cells were treated with MDI, and C) F442A cells were induced to differentiation with insulin in the presence or absence of Trog. Cell lysates were harvested at indicated times during adipocyte differentiation and immunoblotted for dusp4 and PPARγ as well as α-tubulin.

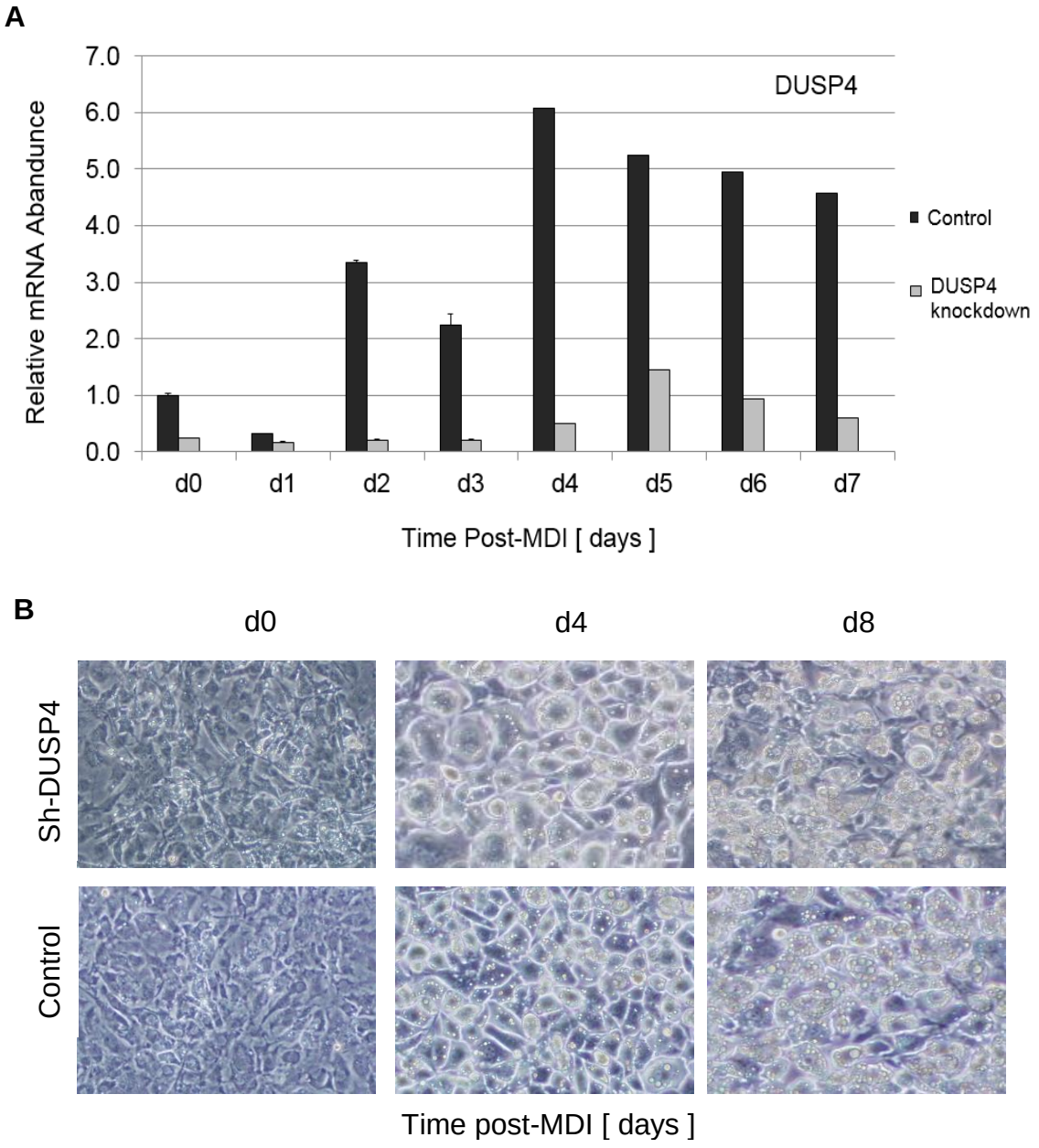

Figure 3.7. DUSP4 Knockdown does not Suppress Adipocyte Differentiation. DUSP4 knockdown or control 3T3-L1 PAs were stimulated to differentiation with MDI. A) Total RNA was collected and gene expression of DUSP4 measured via qRT-PCR. All data were normalized to 18S rRNA and expressed relative to untreated cells (d0). B) Cell morphology and lipid accumulation was assessed with DUSP4 knockdown and control 3T3-L1 cells during adipocyte differentiation using phase contrast microscopy at 10x magnitude.

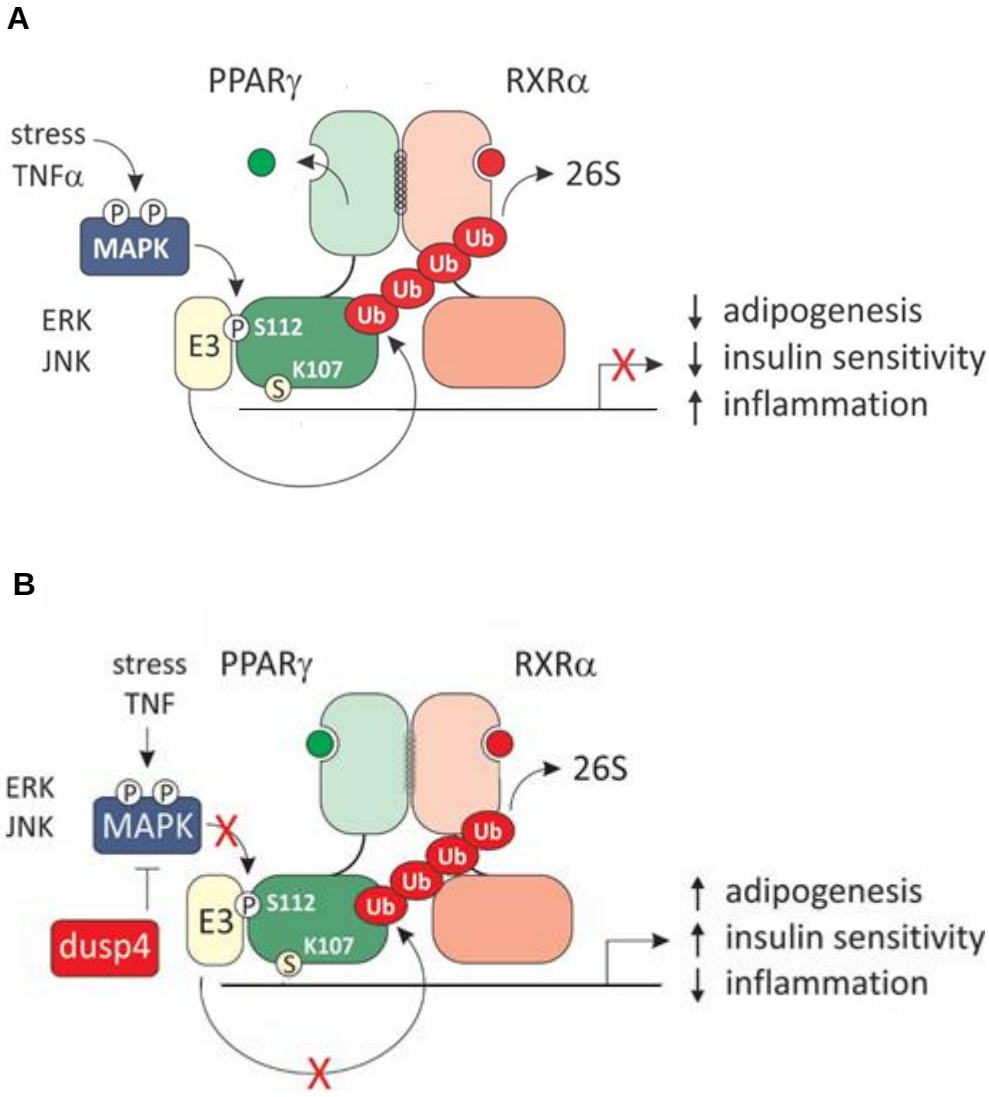

Figure 3.8. Proposed Working Model for Role of DUSP4 during Adipocyte Differentiation. (see text for details)

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CHAPTER IV

EPILOGUE

Data presented in this dissertation represented the first empirical evidence demonstrating the regulatory mechanisms and potential physiological functions of MAPK-specific DUSP4 in 3T3-L1 adipocytes. Moreover, findings from the previous chapters along with other observations made by our lab generated additional questions for future investigation. These preliminary findings and potential areas of focus will be discussed in detail in this chapter.

Our findings presented in Chapter II of this dissertation collectively demonstrated that DUSP4 was only upregulated in response to strong and sustained ERK activation. Besides inducing the expression of DUSP4, ERK activation also directly increased DUSP4 protein stability. In addition, we observed that induction of DUSP4 was highly correlated with the inhibition of ERK phosphorylation in the nucleus and DUSP4 could co-immunoprecipitate with ERK, suggesting a role for DUSP4 in spatiotemporal regulation of ERK activity. As we found unique regulation and function of DUSP4 from Chapter II, we next investigated the regulation and role of DUSP4 in metabolically active insulin responsive tissues under conditions of obesity, as well as during 3T3-L1 adipogenesis. Data presented in Chapter III demonstrated that DUSP4 was abundantly expressed in white adipose tissue (WAT) and suppressed under conditions of genetic

and diet-induced obesity, which is associated with WAT inflammation and IR. Furthermore, we observed an ERK-independent DUSP4 induction during late-stage adipocyte differentiation and this induction was significantly suppressed by TNF-α treatment. However, DUSP4 knockdown did not suppress adipocyte differentiation, suggesting that this phosphatase does not play a direct role in normal adipogenesis. Collectively, data presented in this dissertation provide regulatory mechanisms that have never been studied regarding the novel role of DUSP4 with the adipocyte lineage that potentially links obesity with metabolic inflammatory diseases.

Data presented in this dissertation highlight regulatory mechanisms of DUSP4 in adipocytes in response to specific MAPK agonists (Ch.2). Although studies have reported that DUSP4 can be upregulated in response to growth factors, oncogenes, oxidative stress and UV radiation[1, 2], all of which are also known as MAPK activators, the precise mechanism regarding DUSP4 regulation in adipocytes remains unsolved. Our preliminary findings showed that DUSP4 was markedly up-regulated only in response to strong and sustained ERK activation, while transient ERK activation had no effect on DUSP4 upregulation. On the other hand, activation of JNK or p38, regardless of the signal duration or magnitude, did not induce DUSP4 expression, suggesting that the ERK pathway is the primary signaling cascade responsible for DUSP4 induction. Moreover, early evidence has shown that upon extracellular stimulation, DUSP4 exhibited a relative late phase induction when compared to DUSP1, a prototypical member of the nuclear inducible DUSPs initially identified as an immediate early gene [3]. Consistent with the

report, we further demonstrated that DUSP1 mRNA level peaked 30 min post-PMA while expression of DUSP4 was maximally induced at 2 hrs, suggesting potential coordinated action among nuclear DUSPs in controlling ERK activity. As ERK is only a kinase that phosphorylates numerous transcription factors, future studies identifying potential transcription factor(s) involved in DUSP4 gene expression would enhance our understanding of DUSP4 regulation in adipocytes by MAPK signaling cascades.

Activated ERK has been reported to phosphorylate DUSP4 and stabilize this protein form degradation by proteasome in other cell types [4-6]. We next examined the role of ERK on DUSP4 protein stability in adipocytes (Ch.2). Using cycloheximide to inhibit additional protein synthesis, we observed that DUSP4 protein was very stable throughout the experiment period as long as ERK remained activated. However, we showed that DUSP4 protein half-life markedly decreased when ERK activation was inhibited by U0126, indicating that activation of ERK is essential for DUSP4 protein stability. Moreover, blockade of proteasome activity by epoxomicin partially restored the loss of DUSP4 protein in response to suppressed ERK activation, suggesting DUSP4 protein degradation is partially mediated by proteasome. Therefore, future investigations examining specific phosphorylation site(s) on DUSP4 as well as potential mechanisms involved in DUSP4 protein degradation will increase our understanding of DUSP4 regulation in adipocytes.

Data highlighted in Ch.2 further demonstrated that the accumulation of DUSP4 in the nucleus correlated with the inhibition of nuclear phospho-ERK signal while

cytosolic phospho-ERK remain elevated. In addition, studies have demonstrated that besides dephosphorylating activated ERK, several DUSPs can serve as anchors that retains ERK within certain cellular compartments [7-9]. As the role for DUSP4 in regulation of ERK has not been fully elucidated in adipocytes, we examined the association between DUSP4 and ERK. Using aldehyde-activated agarose beads coupled with anti-DUSP4 antibody, we could co-immunoprecipitate (Co-IP) ERK with DUSP4 only when this phosphatase was expressed. However, when using anti-ERK antibody, DUSP4 could not co-immunoprecipitated with ERK under the same treatment condition. One possible explanation is that because DUSP4 is exclusively localized to the nuclear compartment, pulling down sufficient amount of DUSP4 protein allows us to detect relatively strong ERK signal when protein-protein interactions between these two proteins exist. In contrast, as the anti-ERK antibody detects both phosphorylated and dephosphorylate forms of ERK protein in the whole cell lysate, ERK-DUSP4 complexes are very likely to be mixed with other single ERK molecules. Even if we pull down sufficient amount of ERK protein, those that were coupled with DUSP4 may be very limited, therefore affecting the signal strength of DUSP4 that can be detected. These data suggested a potential role for DUSP4 in retaining ERK and consequently inhibiting ERK reactivation in the nucleus as prolonged activation of ERK in the nucleus may lead to adverse effects in quiescent cells.

Observations presented above highlight future studies involving regulatory mechanisms of DUSP4 in adipocytes. In this dissertation, we demonstrated the

regulation of DUSP4 under conditions of obesity, as well as a role for DUSP4 in adipocyte differentiation (Ch.3). Preliminary findings from our lab demonstrated that DUSP4 was abundantly expressed in WAT of lean mice. Using genetic-induced (GIO) and diet-induced (DIO) mouse models of obesity, we showed that DUSP4 was only induced during early stage GIO with no elevated levels of TNF-α. Expression of DUSP4 was markedly suppressed during the sequential stage of GIO with TNFα-mediated inflammation. During early stage DIO, DUSP4 induction was not observed as TNF-α level was already elevated. DUSP4 expression was further suppressed at the sequential stage of DIO correlating with the progressive increase in TNF-α. Interestingly, observations from our lab further showed that DUSP9 was upregulated in WAT of GIO and DIO mice and TNF-α-mediated inflammation did not affect its expression, suggesting divergent regulation for members of DUSPs under conditions of obesity. As others have already demonstrated a protective role for DUSP9 against inflammation that couples obesity to IR [10], future examination of DUSP4 towards obesity-mediated inflammation would expand our knowledge regarding its regulatory action in WAT under conditions of obesity.

We also presented data demonstrating that mRNA and protein expression of DUSP4 was markedly induced during late stages of adipocyte differentiation, which was consistent with the observation on DUSP4 induction during early stage GIO. This late phase induction is independent of the ERK pathway as ERK phosphorylation status remained at basal condition throughout the course of differentiation. Subsequently, we

demonstrated that expression of DUSP4 was significantly suppressed in preadipocytes (PAs) and adipocytes (ADs) in the presence of TNF-α. Our findings further demonstrated that late phase DUSP4 induction correlated with PPARγ induction during adipocyte differentiation. PPARγ is a nuclear transcription factor highly expressed in AT and considered a master regulator of adipogenesis. Activation of PPARγ initiates transcription of genes that regulate lipid and glucose metabolism in adipocytes [11-13]. Early evidence revealed that phosphorylation of PPARγ by abnormal MAPKs activity affects its transcriptional activity during adipogenesis [13-15], leading to decreased triglyceride storage, increased fatty acids release and IR [16]. Based on these preliminary findings, we hypothesize that under normal physiological conditions, DUSP4 precisely controls MAPK activation critical for adipogenesis. However, as obesity progressively develops, inflammation-mediated suppression of DUSP4 may interrupt the balance between activation and inactivation of MAPKs, leading to impaired PPARγ activity and adipocyte dysfunctions. Finally, we demonstrated that DUSP4 knockdown did not suppress adipocyte differentiation, suggesting that DUSP4 does not directly participate in the process of differentiation, but rather modulates MAPK phosphorylation status so that differentiation can proceed normally. Considering that TNF-α suppresses the expression of DUSP4, gain-of-function study would be a more promising approach to help us delineate the role of DUSP4 towards inflammation-mediated abnormal MAPK activity during adipogenesis.

Data presented in Chapter II and III significantly contribute to the limited body of knowledge regarding regulation and function of DUSP4 in adipocytes. Use of the murine 3T3-L1 cell line provided an excellent model to study and characterize DUSP4, as this cell line does not contain other cell types (e.g. macrophages, endothelial cells, nerve cells, etc.) Such conditions allow us to delineate MAPK signaling events in a homogenous population of cells. Moreover, our findings have led to ample questions for future studies related to DUSP4 in adipocytes. It is expected that continued examination of DUSP4 will provide research scientists with a better understanding of signaling networks involved in obesity, inflammation, and IR potentially, therefore providing new therapeutic targets for the treatment of obesity-associated metabolic dysfunctions.

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