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In vivo and In vitro Expression and Interaction of the Mediator Kinase Module Subunits During Adipogenesis

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IN VIVO AND *IN VITRO* EXPRESSION AND INTERACTION OF THE MEDIATOR KINASE MODULE SUBUNITS DURING ADIPOGENESIS

by

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ABSTRACT

Selective gene expression is crucial in maintaining the self-renewing and differential potential of stem cells. Especially differentiation process requires a constellation of proteins that regulate expression of genes necessary for differentiation towards a specific lineage. Adipogenesis is a process of formation of mature fat cells from precursor cells that is tightly regulated by a number of transcription factors and their cofactors. Dysregulation in the process often leads to metabolic disorders, which is common due to prevalence of obesity. Mediator is a large, evolutionarily conserved, multi-subunit protein complex that functions as a transcriptional coactivator that modulates gene expression by relaying signals from cell type-specific transcription factors to RNA polymerase II. In humans, this complex consists of 30 subunits arranged in four modules. One critical module of the Mediator complex is the kinase module consisting of four subunits: MED12, MED13, CDK8, and CCNC. The kinase module exists in variable association with the 26-subunit Mediator core and affects transcription through phosphorylation of transcription factors and by controlling Mediator structure and function. Many studies have shown the kinase module to be a key player in the maintenance of stem cells that is distinct from a general role in transcription. Genetic studies have revealed that dysregulation of this kinase subunit contributes to the development of many human diseases. This research aims to demonstrate the importance of the Mediator kinase module by examining their expression and interaction with key

adipogenic regulators during adipogenesis. It is hypothesized that the kinase module subunits have a role in adipogenesis of hASCs. As we look to use stem cells to understand human development and treat human disease through both cell-based therapies and tissue engineering, this research will address critical gaps in knowledge related to the molecular mechanisms that control cell fate.

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Author: Sree Lakshmi Venigalla

Date: 06/02/2021

DEDICATION

This dissertation is dedicated to my son, Pranav Swaroop Kanuri, the light of my life and my brother, Jagadeesh Chandra Venigalla, my biggest encouragement and support.

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CHAPTER 1

INTRODUCTION

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Gene expression in eukaryotes is a complex process involving coordination among numerous factors to regulate the expression of a specific gene. The intricate task of regulating gene expression is primarily controlled during initiation of transcription through binding of transcription factors and their cofactors to DNA elements located in promoter-enhancer regions that then regulate RNA polymerase.[6,7] This binding of specific transcriptional regulatory proteins is responsible for development and differentiation of cell types in response to hormones or growth factors. The interplay between transcription factors and their cofactors with their target sites on the genome serves as building blocks for the transcriptional network that governs cell proliferation, cell fate, and cell state.[8,9] Especially in stem cells, regulatory mechanisms support the transcriptional framework and serve as governing agents of self-renewal and pluripotency.[10,11] A detailed understanding of the mechanisms controlling pluripotency/multipotency and lineage commitment is essential in order to be able to use stem cells for clinical applications.

1.1 Stem Cells

Stem cells are undifferentiated cells which can give rise to specialized cells and divide to produce more stem cells. Stem cells are found in multicellular organisms and are characterized by their ability to self-renew and differentiate into different cells (potency). The property of self-renewal in stem cells maintains the undifferentiated state through many cycles of cell division.[12] Potency is described as the potential to differentiate into several types of cells. Stem cells can be totipotent, pluripotent, multipotent, oligopotent, and unipotent (**Figure 1-1**). Totipotent stem cells can differentiate into embryonic layers, which can make a complete living organism. Pluripotent stem cells are derived from totipotent cells and can differentiate into nearly all cells excluding extra embryonic tissue. Multipotent stem cells can differentiate into only cell types of a specific lineage. Oligopotent stem cells can differentiate into only a few types of cells. Unipotent stem cells can give rise to only cells of their own kind, which have self-renewal capacity that distinguishes them from normal cells.[13]

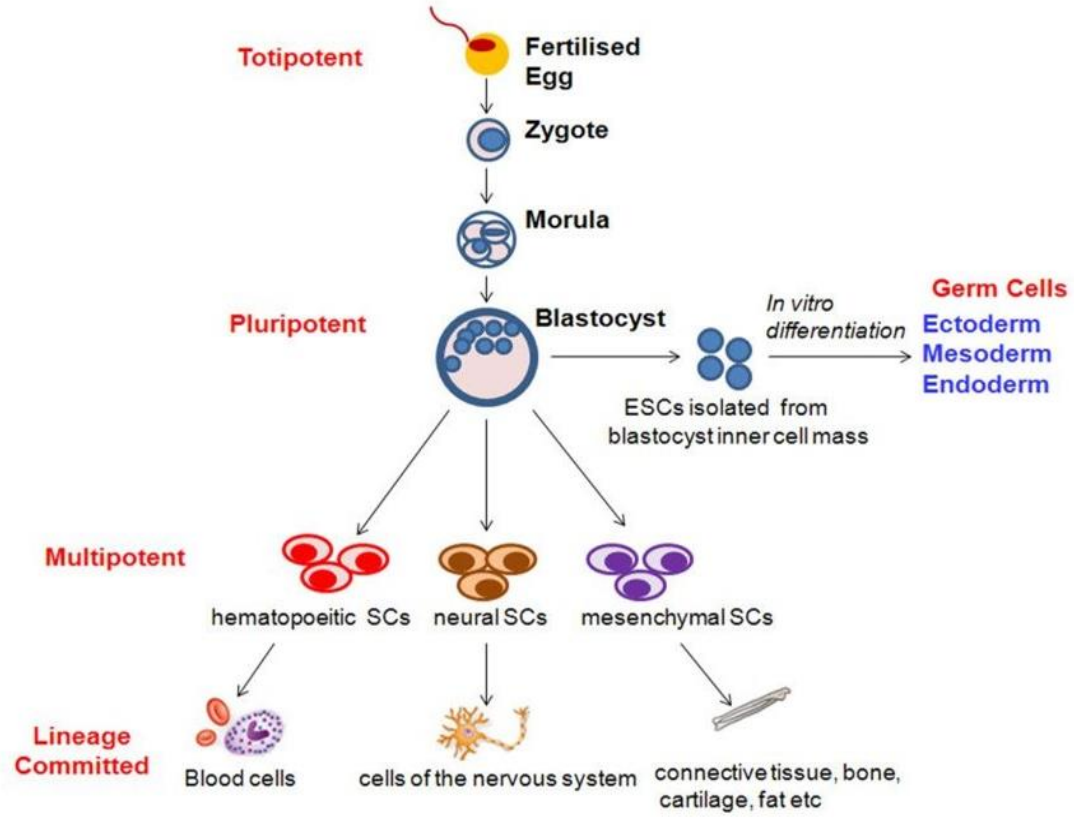

Figure 1-1: A hierarchal structure showing the differentiation potential of stem cells based on their potency. Totipotent stem cells have the most differentiation potential while unipotent cells have limited differentiation potential into tissues they are found in, and are fully committed to becoming that tissue.[1]

Stem cells act as a repair system to aid in regeneration following tissue damage in adults and are responsible for formation of embryonic layers and development of organs in embryos. Today, stem cells are also used for treatment and prevention of disease in the form of stem cell therapy; for example, bone marrow transplantation, which is widely used in the treatment of patients with blood or bone marrow cancers, replaces a patient's diseased hematopoietic stem cells with a healthy donor's cells.[14]

1.1.1 Types of Stem Cells

Based upon the unique properties i.e., self-renewal and potency, stem cells are categorized into two broad types: embryonic and adult stem cells. Embryonic stem (ES) cells are found in the inner layer of the blastocyst of embryos and are pluripotent which means they can give rise to the three germ layers: ectoderm, mesoderm, and endoderm, that develop into organs during development.[15] Adult stem cells, also called somatic stem cells, are multipotent and are named after the origin of tissue: mesenchymal stem cells, adipose-derived stem cells, endothelial stem cells, etc. Both embryonic and adult stem cells can be used in regenerative medicine. Adult stem cells are also used in medical therapies such as bone marrow transplantation. Unlike adult stem cells, ES cells remain a questionable choice of source in regenerative medicine because of their ability for unlimited expansion and pluripotency that are implicated in malignancy.[16]

Adult stem cells can give rise to all the cell types in which they originate from and have the potential to regenerate the whole organ from a few cells. Depending upon the types of cells they arise from, there are distinct types of adult stem cells with the most well characterized and clinically useful being hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and adipose-derived stem cells (ASCs). HSCs are found in bone marrow and umbilical cord blood, and they can give rise to all blood cell types. MSCs are multipotent somatic stem cells which can differentiate into bone, muscle, cartilage, and adipose cells.[17] ASCs, the focus of this research are multipotent and can differentiate into cell types of multiple lineages, making them of great interest to the field of regenerative medicine.[18]

1.1.2 Adipose-derived Stem Cells (ASCs)

ASCs differentiate into cells of the adipogenic, chondrogenic, and osteogenic lineages through a complex cascade of transcriptional and non-transcriptional pathways (**Figure 1-2**). ASCs can be isolated, expanded and differentiated *in vitro*, through the addition of specific chemicals and growth factors.[19] It has been shown that adipose tissue has properties suitable for tissue engineering and regenerative medicine as they are an easily accessible, non-controversial stem cell source. When transplanted into wounded or damaged sites, ASCs are able to interact with their adjacent cells leading to new committed progenitor cells. They secrete cytokines, chemokines, exosomes, and miRNA to restore tissue defects through stimulation of moelcular mechanisms involved in angiogenesis, immunomodulation, cell proliferation, and cell differentiation.[18,20] Altogether ASCs have raised significant interest for therapeutic applications in regenerative medicine.

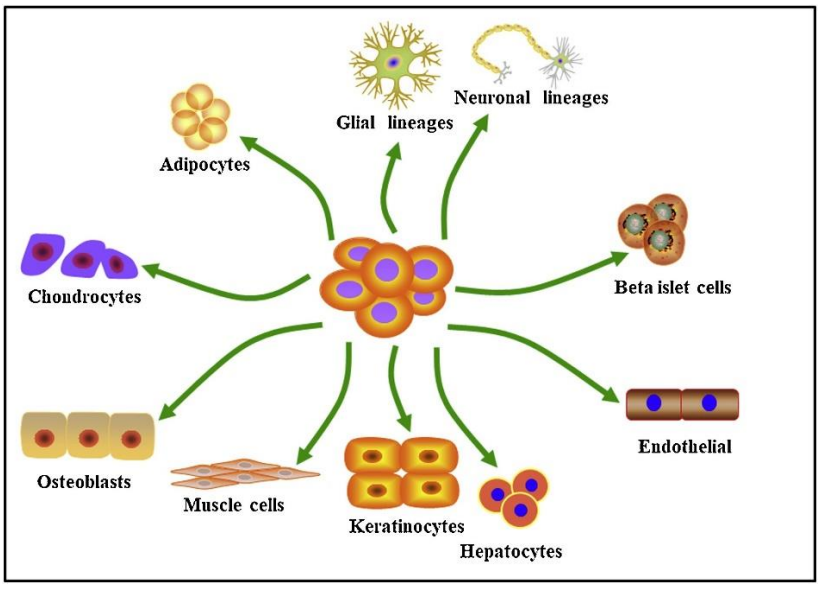

Figure 1-2: A graphical representation showing the differentiation potential of Adipose-derived stem cells. Adipose-derived stem cells have the ability to differentiate into cells of different lineages including adipogenic, chondrogenic, myogenic, and osteogenic.[2]

To assure their self-renewal capacity, stem cells undergo symmetric and asymmetric cell divisions. Symmetric division produces two identical cells with full self-renewal ability, while asymmetric division gives rise to one stem cell and a progenitor cell, which has limited self-renewal capacity.[21] Progenitor cells ultimately can differentiate into distinct cell types. The potency of stem cells can be identified by the presence of surface markers and expression of specific transcription factors. Stem cells depend on transcription factors to determine gene expression and cell fate. Self-renewal and potency of the stem cells are maintained by a complex regulatory network, with transcription factors, Oct4, Sox2, and Nanog, as major contributors.[22] These transcription factors, in association with some coactivators such as Mediator, ensure that transcription is performed without any errors and that the right set of genes are activated or repressed as needed.

1.2 The Mediator Complex

Eukaryotes, being far more complex than prokaryotes, owe their incredible complexity to the regulation of gene expression provided, in large part, by the Mediator complex.[23] Mediator is a large, 1 million Dalton[23] protein complex composed of between 20-30 subunits depending on species[24], and is arranged in four distinct modules: head, middle, tail, and kinase.[25] Bioinformatic and biochemical analyses have identified most of the *H. sapiens* Mediator subunits as similar in sequence, structure, and function to those found in *S. cerevisiae* and *D. melanogaster*, indicating a high degree of sequence conservation across eukaryotic species(**Figure 1-3**).[26]

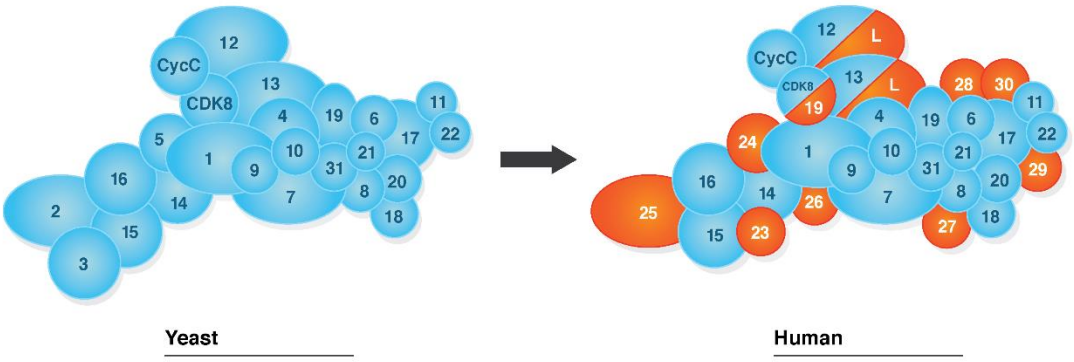

Figure 1-3: A graphical representation of structural differences between *Saccharomyces cerevisiae* (yeast) and *Homo sapiens* (human) Mediator complex. Shared and highly conserved subunits are shown in blue, whereas additional subunits that exist in humans but not yeast are highlighted in orange. This includes subunits that can substitute for each other in the kinase domain, including CDK8/CDK19, MED12/MED12L, and MED13/MED13L.[3]

Mediator functions as a bridge-like adaptor to facilitate the interaction between activators and RNAP II during activator-dependent transcription.[27] Each module—and in some cases individual subunits—of the *H. sapiens* Mediator complex has a unique role in the regulation of transcription and interactions with the transcriptional machinery.[24,28] Recent studies have indicated that Mediator is a key regulator and functional unit of super-enhancers that are responsible for properly regulating cell type-specific gene expression profiles.[29,30] The roles of the head, middle and tail modules of the Mediator complex have been largely defined within the context of directly regulating RNAP II activity.[24,27] The head module, together with the middle module, plays an essential role in stabilizing the interaction of transcription factors and RNAP II during pre-initiation complex (PIC) assembly on a cell type-specific promoter. The head module changes conformation to accommodate the subunit organization that controls the Mediator-RNAP II and Mediator-promoter interactions.[31,32] While the head and middle modules bind RNAP II and the PIC, the tail module interacts with transcription factors bound to

peripheral gene regulatory elements.[24,27] In addition, the tail module along with the kinase module regulates the head and middle modules, whereas the core complex (head, middle, and tail modules) represents the essential function of the Mediator complex.[33] The kinase module of the Mediator complex, composed of subunits MED12/L, MED13/L, CDK8/19, and CCNC, is reversibly associated with the core complex[34–36] and is perhaps the most versatile and mysterious of all of Mediator's modules.

1.2.1 Kinase Module

The Mediator complex exists in two forms: The Mediator complex with the kinase module (30 subunits) and the core Mediator (26 subunits), which lacks the four-subunit kinase module. The 600 K Da kinase module is stable and able to exist and function independently of the core Mediator complex and can transiently associate with the core complex through MED13.[8,37] The structural and functional aspects of the kinase module, including information on paralogs of the kinase module subunits and the importance of kinase activity in transcriptional regulation, is described below.

1.2.2 Structure and Function

A tightly organized network of both physical and functional subunit interactions maintains the structural integrity of the kinase module. Detailed studies of this complex structure have revealed the subunit organization within the kinase module, adding critical insight into the mechanism of CDK8's enzymatic activity. Specifically, early electromagnetic analysis in *S. cerevisiae* showed the kinase module structure having two bent protruding ends, identified as CDK8-CCNC, MED13, and a central globular protein, MED12. This model correlated with the previously observed human kinase module

structure, with both models confirming the connection of the kinase module to the Mediator core through MED13.[37]

The association of the kinase module through MED13/MED13L allows for the adoption of different conformations and reversible interactions not just with the core Mediator but with other factors involved in transcription and cell state regulation.[38] In addition, physical interactions between kinase module subunits and the core Mediator leads to the adoption of different complex conformations, which also plays a role in activating CDK8 kinase activity. Compiling data from different studies, it has been shown that CDK8 kinase activity is activated through a series of sequential steps: the first is the binding of CDK8 to its cyclin partner, Cyclin C (CCNC), resulting in partial activation of CDK8. This is then followed by the binding of MED12 which results in the final necessary conformational change.[39,40] Biochemical studies revealed that CCNC possesses a surface groove that acts as a MED12 docking site. Mutations in either CCNC or the MED12 interface located at the N-terminal of MED12 leads to the dissociation of CCNC-CDK8 from the core Mediator complex followed by impaired kinase activity of CDK8.[41] These findings suggest that the MED12-CCNC binding interface serves as an anchor that also activates CCNC-CDK8.

1.2.3 Kinase Module Paralogs

Although highly conserved as a complete complex, the kinase module in vertebrates is unique and more complex when compared to the core complex as three of the four subunits have paralogs. CDK8 can be replaced by CDK19, MED12 can be replaced by MED12L, and MED13 can be replaced by MED13L.[42,43] The replacement of these subunits by their paralogs is mutually exclusive, meaning that the kinase module

can possess either MED12 or MED12L but not both subunits simultaneously, leading to eight different forms of the kinase module subunits (**Figure 1-4**).[44]

Figure 1-4: A graphical representation of mutually exclusive paralogs of subunits from the Mediator kinase domain. These paralogs, CDK19, MED12L, and MED13L, are replaced by CDK8, MED12, and MED13, respectively, under specific conditions, although the full details of how and why remain unknown.[3]

CDK19, the homolog of CDK8, shares a 91% sequence homology with CDK8. There is an especially high degree of sequence conservation in the kinase activity domain and cyclin binding domain, with more divergence in the C-terminal sequence. The high degree of similarity suggests that CDK8 and CDK19 have overlapping functions and that one may be able to compensate for loss or absence of the other as it relates to kinase activity. For example, isolation and purification of CDK8/CDK19 interacting proteins demonstrates that both CDK19 and CDK8 interact with PRMT5 during repression of

transcription in HeLa cells. However, given that the tail portion of CDK8/CDK19 interacts with transcription factors and cofactors, the difference in the C-terminal sequence suggests that CDK8 and CDK19 more likely regulate different transcriptional programs.[42,45] Supporting this is the fact that CDK8 and CDK19 are differentially expressed across tissues, with CDK19 expression restricted to prostate, testis, thymus, and salivary glands, while CDK8 is expressed more ubiquitously across tissues.[46]

Homology studies reveal that MED12 and MED12L share a 67% gene sequence identity, sharing two of four protein domains: PQL (proline/glutamine/leucine-rich) and OPA (C-terminal opposite paired domain). Interestingly, according to the human protein atlas, after analyzing approximately 37 human tissue samples, MED12L is found to be most highly expressed in the brain while MED12 is expressed ubiquitously in all tissues.[47,48] Though they only share similarity in two of the domains, there does appear to be functional redundancy between MED12 and MED12L. For example, a study by Vogl et al. suggests MED12 and MED12L have the same binding sites for SOX10, which is an essential component of the transcriptional network that regulates the development and terminal differentiation of myelinating glia. From a series of pull-down experiments of MED12/MED12L and SOX10 proteins, it is evident that the C-terminal region of both MED12 and MED12L interact with SOX10. This discovery led to the assumption that both proteins have the same function in the regulation of transcription during the differentiation of myelinating glia.[49] However, there is currently little research in this area, making it difficult to evaluate the complete functional redundancy between MED12 and MED12L.

Finally, MED13L shares a 51% sequence similarity to MED13[50] and they both serve to link the kinase module to core Mediator by associating with middle module subunits, MED14 and MED19.[51] Notably, this interaction of MED13/MED13L with core Mediator is regulated by SCF-FBW7 ubiquitin ligase through the proteasomal degradation of MED13/MED13L. Immunoprecipitation and *in vitro* ubiquitylation assays reveal that MED13/MED13L are directly ubiquitylated by SCF-FBW7 *in vitro.*[34] This degradation of MED13/MED13L prevents the kinase module from associating with the core complex suggesting that MED13/MED13L serves as an anchor for the kinase module. Interestingly, MED13L, but not MED13, associates with MED26, indicating there may be unique functions for each subunit. When co-purified, MED13L is accompanied by a high abundance of MED26; however, MED13L is not present in MED26 isolations. This association is found to be specific to MED13L, as the same set of pull-down assays does not show a robust association between MED13 and MED26.[42] According to the human protein atlas, MED13L is primarily expressed in heart and brain while MED13 is expressed in all human tissues. More recent evidence supports previous studies indicating that mutations in *med13l* are associated with neurodevelopmental defects and heart diseases.[52,53] Knockdown and conditional knockout studies of MED13 in murine zygotes suggest that MED13L can partially substitute for MED13 and function during the development of embryo.[54] This study demonstrates that MED13 is required for both transcriptional activation and repression during zygote genome activation, as demonstrated through the up- and downregulated transcripts observed in MED13-knockdown embryos. Furthermore, *med13* knockout embryos showed arrested development post-implantation. MED13L compensated for MED13 function during the

OET (oocyte to embryo transition) enough to support embryo development to the blastocyst stage during preimplantation in *med13* knockout murine zygotes. Clearly, the functional compensation of MED13 by MED13L is limited in context, as evident by its lack of compensation during post-implantation development and other studies showing mutations of MED13/MED13L that lead to disruptions of cellular functions resulting in respective disorders.[55,56]

Together, studies surrounding the kinase module and each of the seven subunits indicate that the kinase module of the Mediator complex can present itself in many forms by changing its subunit configuration to diversify its function. Although it appears that there is some amount of functional overlap between paralogs, there is no complete functional redundancy for any of these kinase module subunits. Some of these paralogs may in fact play different roles in development and cell-type specific transcription programs. In addition, individual genetic mutations of kinase subunits leads to embryonic lethality in mice and many human disorders with clear developmental disruptions.[54,57–59] In addition, research to date excludes the possibility of complete functional redundancy among the paralogs as they failed to substitute for their paralogous subunits (except for the partial functional compensation of MED13 by MED13L in murine zygote development as described in MED13 paralog section) when the other is genetically disrupted. The mutually exclusive nature, the unique expression profiles, and the relative functional contribution of each kinase module paralog still requires more thorough investigation to understand the structural interactions and functional relationships within the kinase module and what role each subunit plays in human development and disease.

1.2.4 Kinase Module and Human Diseases

The Mediator kinase module is a critical component of the transcriptional machinery required for proper regulation of gene expression and lineage commitment of cells during development and tissue differentiation.[23,27,60] Kinase module subunits, as part of the Mediator complex, have been linked to many key developmental and oncogenic signaling pathways including Wnt, mTORC1, EGF, SHH, and Notch.[26,30,58,61] Alterations of individual subunits of the kinase module have been associated with developmental defects and diseases including Lujan syndrome, schizophrenia, breast and uterine cancers, and cardiovascular diseases.[62] Studies have found that the kinase module subunits are direct targets of genetic alteration in human tumors and specifically, MED12, the largest subunit of the kinase module, has been consistently implicated in many female cancers and cognitive developmental conditions. Research indicates that it is the role of MED12 in regulating the kinase activity of the module that leads to a number of these outcomes.[63] Specific roles for each of the kinase module subunits in development and disease can be found in **Table 1-1**.

Table 1-1: Currently known roles for the Mediator kinase subunits in development or disease. Most cancers noted in this table were previously compiled and reviewed by Clark et al. in 2015.[5]

Subunit	**Role and diseases**
Cyclin C	Multiple deletions associated with T-cell acute lymphoblastic leukemia; function as a tumor suppressor[64] Deletion associated with osteosarcoma; function as an inhibitor of cell growth[65] Regulation of adipogenesis[66] G_0 to G_1 transition in $CD34^+$ cord blood cells[67]
CDK8	Phosphorylation of TFIIH to activate transcription[68]

	Phosphorylation of E2F to activate transcription[69] Phosphorylation of RNA Pol II C-terminal domain to repress transcription[70] Maintenance of ESC pluripotency mediated by MYC protein[71] Colorectal cancer oncogenesis[72,73] Melanoma oncogenesis[73] Breast tumorigenesis[38] Endometrial cancer tumor suppression[74]
CDK19	Upregulated in prostate cancer[75] Intellectual disability[76]
MED12	Designated a cancer driver gene[77] Uterine leiomyoma oncogenesis[41,63,78–80] Breast fibroadenoma oncogenesis and phyllodes tumorigenesis[63,81,82] Prostate cancer oncogenesis[83,84] Interaction with NANOG and SOX2 C-terminals to regulate ESC pluripotency[85] Interaction with Wnt/β-catenin to recruit core Mediator to target genes[58] Interaction with PRC1 to repress differentiation genes during pluripotency[86] Super-enhancer-associated co-activator[57] Maintenance of HSC viability[57] FG syndrome[87,88] Lujan syndrome[89] Ohdo syndrome[90]

MED12L	Designated a cancer driver gene[91]
MED13	Neurodevelopmental disease[53] Breast cancer oncogenesis[92] Regulation of early embryogenesis[93] Interaction with Smad7 to regulate myogenesis[94]
MED13L	Congenital heart defects[50,56,95]

1.2.5 Kinase Module in Transcriptional Control

The kinase module functions as both an activator and repressor of gene transcription, making the study of the module that much more complicated. Initial functional studies revealed a repressive function for the kinase module in *S. cerevisiae* where the Mediator core, together with the kinase module, repressed transcription and the Mediator core alone enhanced activator-dependent transcription.[96] Early electromagnetic structural, and subsequent functional studies in human HeLa cells showed that the kinase module repressed transcription by preventing the binding of RNAP II to Mediator thereby blocking the formation of the PIC-scaffold complex. This inhibition was achieved via multiple mechanisms, including kinase-independent regulation of Mediator-RNAP II interaction[39], kinase-dependent inactivation of TFIIH by phosphorylation[96], and gene silencing through the recruitment of histone methyl-transferases.[38] Later, biochemical and molecular studies supported the view that the kinase module has a context dependent role in both gene repression and activation.[29,97] It now appears that when the Mediator core is absent, the kinase module acts to inhibit core Mediator function within the pre-initiation complex (PIC).[38,97] Furthermore, the kinase module appears to mediate transcriptional activation through ncRNA-a (noncoding RNA-a, a class of ncRNAs which activate

neighboring genes) by interacting with MED12 and chromatin.[98] Finally, a new role for the kinase module in the regulation of transcription elongation has been reported where the kinase module appears to coordinate with positive transcription elongation factor b (P-TEFb) by regulating its kinase activity. ChIP analysis and genome occupancy profiles of elongation factors in human cells indicate that CDK8 orchestrates key events in the formation of a functional elongation complex. CDK8 is required for RNAP II dependent elongation by phosphorylating the C-terminal domain of RNAP II as they demonstrate that RNAP II elongation is impaired upon knockdown of CDK8.[99] This study also suggests that the kinase module may facilitate the interaction of P-TEFb with core Mediator to regulate RNAP II phosphorylation and transcription elongation. These studies represent a marked advance in our understanding of how the kinase module acts to both repress and promote gene expression, while also revealing additional questions about the role the kinase module has as part of the core Mediator complex in regulating transcription.

Although the precise mechanism that regulates the reversible association of the kinase module with core Mediator is not clearly understood, a few studies offer some insight into why the regulatory association of the kinase module with core Mediator occurs. Initial clues were provided by studies in yeast suggesting that the reversible association between the kinase module and core Mediator existed as way of regulating the output of signaling-dependent transcription. Similarly, later studies in mammals suggest that specific cellular signals and signaling pathways regulate Mediator-kinase module association. For example, an *in vivo* study by Mo et al. in human HeLa cells showed that in response to Ras signaling, the repressed promoter of the C/EBPβ is kinase

module-bound whereas, upon activation, the kinase module is lost.[35] Another study by Pavri et al. demonstrated the switch from inactive to active Mediator executed by PARP-1 during retinoic acid (RA)-induced gene expression *in vivo*. ChIP analysis of promoter occupancy in PARP-1 present and absent cells shows that in the absence of PARP-1, Mediator did not attain its active conformation (accompanied by loss of kinase module) upon RA induction as evidenced by the retention of CDK8 after RA treatment.[36] Although these studies do not prove the exact mechanism for what triggers the kinase module to dissociate from the core, they do suggest that the kinase-containing Mediator complex requires an interaction with other factors in order to detach from the kinase module and adopt the conformation required to facilitate transcriptional activation. Indeed, Davis et al. demonstrated mechanistic evidence of kinase module dissociation involving SCF-FBW7 ubiquitin ligase mediated proteasomal degradation of MED13/MED13L, which anchors the kinase module to core Mediator.[34] A recent study by Youn et al. also demonstrated that the Mediator complex in mouse liver undergoes dynamic physiologic regulation through nutrient signaling-dependent downregulation of the kinase module to core Mediator. This dissociation and degradation of the kinase module is induced by SCF-FBW7 E3 ligase.[100]

Finally, the Mediator kinase module has been implicated as a gene regulator in physiological processes from development and differentiation to the maintenance of cell fate and function. Several genetic studies have revealed critical roles for kinase subunits in regulating signal-dependent gene expression during development.[58,81,101] In mice, kinase module subunits are found to be critical in early development, as evident by embryonic lethality resulting from mutations in kinase subunits. For example, embryo

implantation failure is observed when CDK8 is inactivated through gene trap insertions, suggesting that CDK8 is necessary for preimplantation of mouse embryos.[59] CCNC knockout murine embryos failed to survive past implantation due to severe developmental retardation and an underdeveloped placental layer.[64] MED12 mutant and knockout embryos failed to survive later embryonic stages as they suffered from acute defects in developmental processes including neural tube closure and heart formation.[55] Several studies show that these developmental disruptions arise from defects in key developmental signaling pathways, including Wnt, Notch, mTORC1, and TGFβ. The kinase module subunits are found to be involved in expression of signaling pathway target genes and when mutated, lose their ability to activate or repress expression of required target genes for respective signaling pathways, leading to impaired responses of signaling pathways as illustrated by a growing number of developmental disorders. For example, loss of CDK8 in murine embryos is shown to disrupt Wnt target gene expression.[101] Mutant MED12 murine embryos showed aberrant Wnt/β-catenin target gene expression, indicating that MED12 is essential for Wnt signaling during embryogenesis where MED12 mutant embryos recapitulated phenotypes similar to those observed in the absence of β-catenin.[55] Recently, CDK8/CDK19 has been shown to have a role in negatively regulating Notch 1 signaling, a developmental pathway which regulates self-renewal and differentiation in several cell types including stem cells.[64] Kinase subunits have also been linked to TGFβ, a developmental signaling pathway which regulates cell proliferation, differentiation, cell fate, and apoptosis. CDK8-CCNC plays a critical role in regulation of SMADS in TGFβ driven transcriptional responses by limiting the SMAD2/3-dependent induction of mesodermal cell fate in response to TGFβ

signaling.[102] Recently, a study by Youn et al. demonstrated that when mice were fasted and refed, the kinase module dissociated and degraded upon nutrient activation of mTORC1 in mouse livers. This dissociation and degradation of the kinase module is necessary for the induction of lipogenic gene expression because genetic/pharmacological inhibition of mTORC1 in the fed state restores the kinase module suggesting that the kinase module plays a role in repressing lipogenic gene expression. In addition, genetically insulin resistant and obese mice in the fasted state showed elevated levels of lipogenic gene expression and loss of the kinase module was reversed following mTORC1 inhibition.[61] In agreement with these studies, the kinase module has been involved as the terminal factor of cell signaling pathways due to its representation as a final and functional target for transcription factors. Together, these studies imply a highly targeted role of individual Mediator subunits in the regulation of cell state and lineage commitment through the regulation of developmental signaling pathways.

1.2.6 Kinase Activity

One of the most important and highly conserved functions of the kinase module is its kinase activity. The best characterized of these activities is the kinase module's phosphorylation of the C-terminal domain of RNAP II during transcription initiation, elongation, and RNA processing.[24] Human CDK8 appears to negatively regulate transcription by phosphorylating TFIIH[96], while inhibition of CDK8 kinase activity suppresses the RNAP II CTD phosphorylation thereby preventing elongation of transcription.[103] Despite the requirement for CDK8 and CCNC to interact and bring the kinase module together, the CDK8-CCNC interaction is not sufficient for CDK8 kinase

activity. In the past two years, a series of studies by two different labs have provided evidence that MED12 is required for CDK8 kinase activity.[94,104] With a combination of Hi-C and cryo-EM studies along with knockdown studies in murine embryonic stem cells, MED12 knockdown cells showed acute depletion of Mediator and RNAP II, indicating that MED12 is required for proper phosphorylation of RNAP II. Another recent study by Klatt et al. described the binding location of MED12 and CDK8-CCNC dimer providing insight into activation of CDK8 by MED12. *In vitro* biochemical and *in vivo* studies together with cross-linking coupled with mass spectroscopy, demonstrated that the N-terminal of MED12 wraps around CDK8 at its T-loop to form an activation helix, which activates the enzymatic activity of CDK8.[104] With MED12 now known to activate CDK8 as a kinase, the association of MED12 with the CDK8-CCNC dimer provides a critical element of regulation and prevents uncontrolled and inappropriate substrate targeting.

Overall, the genetic and biochemical analysis of the kinase module is consistent with the functional studies indicating a role for this unique module in both gene activation and repression. The kinase activity of CDK8 is required for activator-dependent transcription and has been shown to direct multiple steps in transcription including initiation, elongation, and re-initiation. Although several studies have begun to shed light on the signaling and mechanistic role for the kinase module and Mediator core, there is still much to be explored. Involvement of kinase subunits in many physiological processes and pathways indicates the complexity and the functional implications of the kinase module subunits either individually or as a complete structure. The significant functional involvement of the Mediator kinase module in regulating aspects of

transcription has a significant impact on human development as illustrated by an increasing number of diseases and developmental disorders that have been associated with Mediator subunit mutations.

1.3 Kinase Module and Stem cells

Gene regulation is tightly controlled in order to ensure proper transcription for healthy organismal development. Aberrant gene expression is implicated in a multitude of developmental defects and diseases and would be more rampant if not for the highly specialized, complex method of cell type-specific transcriptional control.[105,106] The unique properties of stem cells to both self-renew and differentiate under controlled laboratory conditions allows for the effects and functional characterization of deliberate perturbations in the regulation of gene expression.[13] This includes altering the function and expression of the general transcription factors (GTFs) that assemble the transcription pre-initiation complex on gene promoters, activators and repressors that bind to gene regulatory elements located upstream or downstream of promoters, and the essential co-activator of cell type-specific genes like the Mediator complex. The fate of any stem cell is ultimately determined by regulating the transcription of specific genes, a feature largely facilitated by the Mediator complex. Stem cell self-renewal and the role of the Mediator complex kinase module in this process, followed by the kinase module's regulation of differentiation down the various possible lineages is described below.

1.3.1 Kinase Module in Self-renewal and Differentiation of Stem Cells

Self-renewal is the process by which stem cells replicate themselves to maintain a stable population of undifferentiated clones. For individual stem cells, self-renewal and differentiation are mutually exclusive in that lineage commitment down a differentiation

pathway which alters a stem cell's gene expression profile away from self-renewal permanently. If a stem cell is not self-renewing, it is differentiating, and vice versa[107]—until the stem cell reaches quiescence, temporarily halting further self-renewal.[108] Work by Miyata et al. revealed that CCNC plays a role in maintaining expression of CD34 levels in human cord blood CD34+ cells by keeping the cells in G0 phase.[76] CDK8 has also been shown to play a role in stem cell self-renewal where knockdown of CDK8 in murine ESCs resulted in a loss of ESC pluripotency.[80] Furthermore, knockdown of MED12 in murine ESCs also resulted in loss of pluripotency.[114] Med12 is also shown to be critical for maintaining HSC viability in murine ESCs where MED12 knockout revealed the inability of murine HSCs to form colonies highlighting a failure in self-renewal.[33] Finally, out of all Mediator subunits, MED13 was the most highly translated Mediator subunit during oocyte maturation with MED13L and MED12L ranking at second and third, respectively. RNA-seq analysis revealed that the knockdown of MED13 results in 1201 upregulated and 2203 downregulated genes. RNA processing, cell cycle, transcription, protein catabolism, and chromatin modification were among the categories of the downregulated genes discovered during GO analysis.[54] Given that these sets of genes are important for determining cell state, these results point toward the Mediator kinase module as an integral cell state regulator.

Differentiation is the process by which a stem cell alters its transcription program in response to the external environment and internal regulation to commit to one of its many potential lineages. In normal, healthy stem cells, differentiation is a permanent epigenetic transformation that, once started, causes a stem cell to lose its potency and self-renewal capacity while gaining specialized forms and functions important to the

operation of tissues and organs. This research investigates the lineage commitment especially adipogenic lineage and the role of kinase module in supporting the critical role of Mediator in directing cell fate and need for continued research in this area.

1.3.2 Kinase Module in Adipogenesis

Work by Song et al. involved screening adipocytes with shRNA libraries to identify genes whose expression was altered during adipogenesis. CCNC and CDK19 were downregulated while CDK8 was upregulated during adipogenesis. CCNC and CDK19 expression in brown adipose tissue of 2-year-old mice was down to 25% of the expression levels present in 3-month-old mice. Conversely, CDK8 expression was twice as high in brown adipose tissue of older mice when compared to younger mice. Since brown adipose tissue is responsible for producing heat, groups of mice were exposed to either 4°C or 22°C conditions. The transcription levels of *ccnc*, *cdk8*, and *cdk19* mRNA were determined to be unaffected by these differences in temperature; however, CCNC and CDK19 protein expression fell slightly while CDK8 rose in the 4°C group compared to 22°C. After performing siRNA knockdown of *ccnc*, the expression of adipogenesis markers including PPARΓ, FABP4, and CEBPA fell during the first 2 days of adipogenesis, but expression of these genes increased after 5 days to the levels in the control group. After switching to an inducible knockout system for CCNC, cells undergoing CCNC knockout did not undergo adipogenesis and did not express the previously tested adipogenic markers as well as a host of other genes associated with brown adipose tissue, mitochondria, and lipogenesis. Retroviruses expressing CCNC only partly rescued adipogenesis in CCNC knockout cells but did not improve adipogenesis in cells unaffected by CCNC knockout. The most down regulated pathway in the absence of

CCNC was the PPAR pathway, a master regulator of adipogenesis. PPARΓ-2 overexpression rescued both lipid vesicle formation and adipogenic marker expression in CCNC knockout cells, showing that PPARΓ's activity does not depend on CCNC. C/EBPα, a co-regulator of adipogenesis along with PPARΓ, could not rescue adipogenesis upon overexpression in CCNC knockout cells. Due to C/EBPα being an important regulator of white adipose tissue, siRNA knockdown of CCNC was also performed in 3T3-L1 cells, revealing a similar decrease in lipid accumulation and adipogenic marker expression as was seen in brown adipose tissue.[66] In summary, CCNC regulates adipogenesis by interacting with C/EBPα.

1.4 Conclusion

It's been three decades since the discovery of the Mediator complex, expanding our understanding of cell complexity which led to a multitude of further questions. Because of that, we now know how Mediator functions to regulate cell type-specific gene transcription. The kinase module has expanded the complexity of this regulatory relationship given its transient nature, and its certain because of the kinase module's subunit CDK8/19 possessing kinase activity. It is understood that each kinase module subunit plays an integral role in the modules over all stability, thereby enhancing its regulatory and kinase activity. Unfortunately, mutations in these subunits have been implicated in developmental disorders and diseases, including many cancers, wherein lies the importance of proper kinase module functioning to maintain healthy development and cell fate determination.

Despite what we have learned, we still do not have a complete understanding of how the kinase module interacts with nuclear machinery and signaling molecules to drive

cell state into a particular direction of cell proliferation or to differentiate into a particular lineage while avoiding oncogenic state. A large focus has been on MED12, warranted due to its importance in oncogenesis and development. The web of interactions between Mediator kinase module, the various transcription factors, and the magnitude of signaling molecules is falling into place piece-by-piece but work is far from finished. The benefits will likely extend beyond molecular biology, stem cell biology, and biochemistry and into clinical application. Given a more complete understanding of cell state regulation, we may one day see the efforts of this research pay off in the form of regenerative medicine involving stem cell therapies that are tailorable to individual patients, therefore maximizing patient benefit.

CHAPTER 2

REGULATION OF KINASE MODULE SUBUNIT EXPRESSION AND INTERACTION IN MOUSE ADIPOSE TISSUE

2.1 Introduction

According to the WHO more than 650 million people in the world are obese and the prevalence of obesity is approximately 42.4% in the United States by 2018.[109] In 2000, there were an estimated 300 million obese adults worldwide and the number of obese adults had doubled by 2018. Obesity is a global epidemic in all age groups and in both developed and developing countries, placing a large burden on the health care system and economy. Obesity carries serious health implications due to its associated disorders including insulin resistance, type 2 diabetes mellitus (T2D), cardiovascular diseases, metabolic disorders, and some types of cancer (**Figure 2-1**).[110,111] Obesity is characterized by excess body fat accumulation, to the extent that health is impaired. The amount and distribution of excess fat accumulation is associated with a greater risk of disability or premature death. The additional energy that results from caloric excess is stored as lipids in adipose tissue and may accumulate in other metabolic organs such as liver and skeletal muscle.[112] That increased amount of lipid significantly alters normal metabolism and creates an environment that chronically transmits a signal of nutrient excess to the cell. As a result, signaling cascades that drive glucose uptake, cell growth, cell proliferation, and angiogenesis are activated. An increase in adipose tissue associated

with obesity correlates with an increase in levels of leptin and with reduction in levels of adiponectin. Leptin and adiponectin are adipokines, a set of cytokines secreted by adipose tissue that have pleiotropic effects on metabolism, cellular signaling, and inflammatory pathways.[113] Leptin regulates appetite and energy balance through activation of multiple signaling cascades including the PI3K, MAPK, mTOR, and JAK/STAT pathways. Adiponectin balances many of the effects of leptin by activating AMP, which has a variety of cellular effects including induction of cell cycle arrest and inhibition of mTOR activity. This opposing effect of leptin and adiponectin and the change in ratio of the two adipokines is a critical change linked to obesity.[114,115]

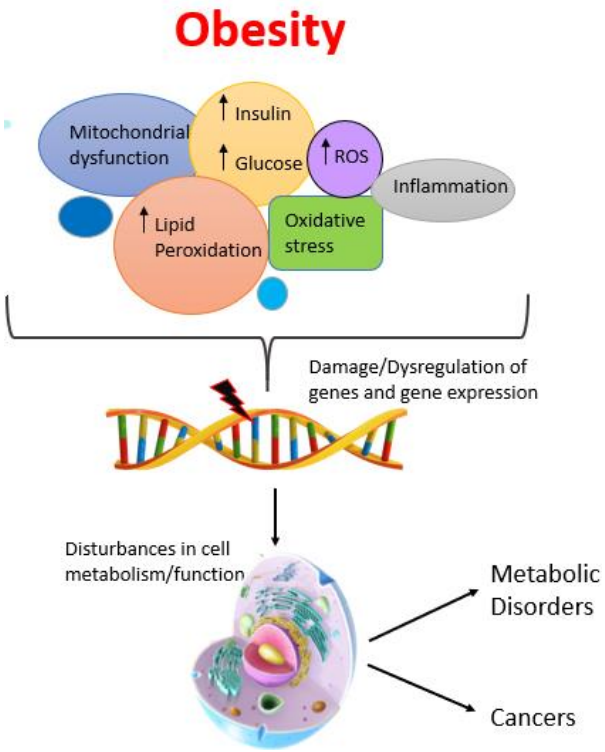

Figure 2-1: A pictorial representation of molecular disturbances resulting due to obesity. Obesity causes dysregulation of metabolic gene expression leading to disorders. Increase in insulin, blood glucose, lipid peroxidation causes oxidative stress and inflammation which leads to dysregulation of genes and gene expression causing metabolic disorders.

In mammals, adipose cells exist as three types: brown, white, and beige. Brown adipose tissue (BAT) specializes in heat production by exerting its thermogenic function mainly in newborn babies. BAT is present in the interscapular region and posteriorly, and can transform into white adipose tissue (WAT).[116] Beige adipose tissue is believed to originate from white adipocytes trans-differentiation within the same cellular lineage and is still under investigation to uncover additional properties. Genetically, beige adipocytes are found to have an intermediate behavior between brown and white adipose tissues, where they can store the excess energy in the form of lipids and when stimulated dissipate energy to produce heat.[116–118] WAT is the most abundant adipose tissue and is responsible for lipid storage in the form of triglycerides. Most WAT is distributed throughout the body in subcutaneous and visceral depots and their characteristics are important for differential susceptibility to metabolic diseases. Subcutaneous WAT is located beneath the skin and has been associated with protective metabolic diseases; whereas visceral tissue surrounds the vital organs and is contained within the gonadal, perirenal, retroperitoneal, and pericardial depots (**Figure 2-2**), linked to metabolic disorders including T2DM and cardiovascular disease.[114,119,120]

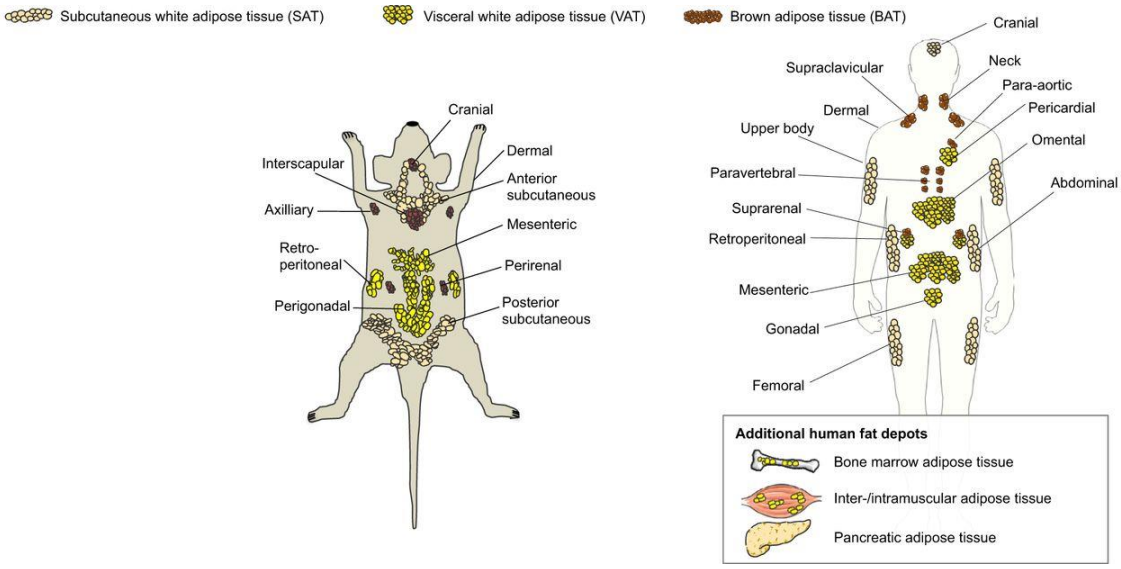

Figure 2-2: A graphical representation showing different adipose tissue depots in mice (left) and humans (right). Showing are white and brown adipose tissue deposited in different places of mice and human bodies. White adipose tissue is situated either subcutaneous or visceral regions of the body of an organism.[4]

The expansion of adipose tissues through hypertrophy of mature adipocytes and differentiation of preadipocytes through a process called adipogenesis to store excess triacylglycerols often leads to obesity. Impairment in adipogenesis leads to ectopic deposition of fat in several organs including skeletal muscles, liver, and kidneys, characterized by impaired secretion of various adipokines, and resulting in insulin resistance and an increased risk for type2 diabetes.[4,115] The process of transforming preadipocytes into adipocytes is regulated by an elaborate network of transcription factors that coordinate the expression of hundreds of proteins responsible for establishing the mature fat cell phenotype. The two main factors of this network are PPARγ and C/EBPα, which oversee the entire terminal differentiation process. PPARγ in particular is considered the master regulator of adipogenesis.[121–123] When an adipogenic program is activated, the stimulated cells reenter the cell cycle and progress through clonal

expansion, where the cells express specific adipogenic transcription factors and cell-cycle regulators that facilitate the expression of PPARγ and C/EBPα . These adipogenic-committed cells now undergo terminal differentiation manifested by production of lipid droplets and expression of various metabolic programs characteristic of mature fat cells. Emphasis on research to understand the transcriptional processes controlling the conversion of precursor cells into fully functional adipocytes has shown that various coactivators and corepressors control the activity of the adipogenic transcription factors that induce a well-defined component of the adipogenic process.[122] A number of factors enhance adipogenesis and serve as molecular switches in controlling the fate of the progenitors. One such crucial coactivator that facilitates the communication of adipogenic transcription factors with the transcriptional machinery is the Mediator complex.[124]

Mediator is a multi-subunit protein complex that functions as a bridge between RNA Polymerase II and cell-type specific transcription factors. The Mediator complex is conserved in all eukaryotes from yeast to humans, consisting of 25-31 subunits and comprised of four modules: head, middle, tail and kinase domain.[125–127] Mediator controls and coordinates multiple steps in transcription including initiation and elongation and has roles in gene activation and repression. It plays a key role in both activator dependent and activator independent transcription. In activator independent transcription it controls the formation of the initiation complex.[128–131] Based on previous findings, mutations or alterations to the Mediator complex also has a role in many types of cancer, as well as developmental, neurological, and metabolic disorders.[132] Different Mediator subunits seem to control distinct types and stages of cancer through specific signaling pathways.

In studying the roles of subunits of the Mediator complex it is known that one or more subunits are involved in developmental processes. Knockout studies of the Mediator complex in mice revealed the involvement of its subunits in developmental processes including chondrogenesis, adipogenesis, cardiovascular development, and differentiation of neuron, smooth muscle, luminal cell, and keratinocyte.[30,133] Data from previous studies indicate interaction of subunits with specific transcription factors, which regulate transcription of genes involved in development and cell differentiation.[134,135]

Recently, it has been reported that the kinase module is dissociated and degraded through a nutrient-stimulated mTORC1-dependent regulation in mouse liver.[100] However, it is unclear whether the kinase module expression is regulated in adipose tissue under nutrient-regulated physiological state. In this study, it is demonstrated that the kinase subunits in certain mouse adipose tissue depots undergo physiological downregulation of the entire kinase module. In addition, the interaction of MED12 and CDK8 with different adipogenic regulators reveals the dynamic possible ability of these subunits in regulating the initiation, progression, and maintenance of adipogenic lineage.

2.2 Materials and Methods

2.2.1 Animal Studies

14-week-old female mice (strain:C57BL6/J) were used for fasting and feeding experiments. Mice were provided 5015 mouse chow. For fasting and feeding experiments, the fasting group (n=3) were fasted overnight for 14 hrs and sacrificed the next morning, whereas the fed group (n=3) were provided with food 4 hrs after 14 hrs of overnight fasting and were sacrificed after feeding.

2.2.2 Protein Extraction and Immunoblotting

Fresh mouse fat pad depot tissues were cut and frozen immediately in liquid nitrogen. The tissues were homogenized in IP buffer that contained 10 mM Tris (pH 7.4), 150 mM NaCl, 1mM EGTA, 1 mM EDTA, 1% Triton X-100, and 0.5% IGEPAL CA-630 for 5-10 seconds and immediately placed on ice. A mixture of protease inhibitors (1 mM PMSF, 1 µM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 µM leupeptin, 1 mM 1, 10-phenanthroline) and phosphatase inhibitors (100 µM sodium fluoride and 0.2 mM sodium vanadate) were added to IP buffer. After centrifugation at 20,000 XG for 20 min at 4C, the resulting supernatants were used for immunoblotting. Total fat depots tissue lysates were prepared using the IP lysis buffer with freshly added protease inhibitors and phosphatase inhibitors. Protein was quantified using BCA assay (Sigma-Aldrich cat# BCA1). Protein samples were separated on 5% and 12% SDS gels (acrylamide Cat # EC-890, National Diagnostics) and transferred to nitrocellulose membrane (Cat# 162-0115). Western blots were performed to detect Mediator subunits and adipogenic factors (**Table 2-1**).

Table 2-1. An overview of antibodies used in western blotting analysis.

Antibody	**Dilution**	**Company**	**Catalog number**
MED12	1:1000	Bethyl Laboratories	A301-774A-M
MED13	1:1000	Bethyl Laboratories	A301-278A-M
CDK8	1:1000	Bethyl Laboratories	A302-501A-M
CCNC	1:1000	Bethyl Laboratories	A301-989A-M
Tubulin	1:2000	Cell Signaling	2144S
Adiponectin	1:1000	Thermofisher	PA1-054

PPARγ	1:100	Santa Cruz Biotechnology	SC-7273
CEBPα	1:1000	Abcam	Ab15048
SREBP1c	1:1000	Santa Cruz Biotechnology	SC-365513
STAT5A	1:1000	Abcam	Ab32043
Goat Anti-mouse	1:10,000	Jackson Immunoresearch	115-035-071
Goat Anti-Rabbit	1:10,000	Jackson Immunoresearch	115-035-003

2.2.3 Immunoprecipitation Assay

3T3L1 mouse preadipocytes (originally obtained from Howard Green lab at Harvard Medical School) were cultured using standard cell culture medium (DMEM-Sigma-Aldrich cat# D5030 with 10% bovine calf serum-Thermo Scientific Cat# 16030074). Cells were induced to differentiate using a standard MDI induction cocktail of 0.5 mM 3-isobutyl-1-methylxanthine (MIX), 1 µM dexamethasone, and 1.7 µM insulin in DMEM containing 10% FBS (Thermoscientific Cat# A31605). Cell cultures were collected at stages of preadipocytes and mature adipocytes by adding IP buffer and a mixture of protease and phosphatase inhibitors were added to collected protein samples (same as above). After centrifugation at 13,000 x g for 10 min at 4C, the resulting supernatants were used for immunoprecipitation. A BCA analysis (listed above) was performed to quantify the amount of protein and 200 µg of protein was used. 25 µl of bead slurry (Protein A/G Plus-Agarose Santa Cruz Biotechnology Cat# sc-2003) was added to the sample and rocked for 1 hr in the cold room and were spun at 12,000 rpm for 1 min. 5 µg of the appropriate antibody was added to each sample and incubated on the rocker in the cold room for one hour or overnight. After the incubation, samples were

centrifuged for 1 to 3 mins at 13,000 rpm. Supernatant was carefully aspirated or collected for western blot analysis. Beads were then washed 3 times by adding 1 ml of cold IP buffer and centrifuge for 1-3 mins at 10,000 rpm. A sample of whole cell lysate and an IP buffer with no cell lysate was used as a negative control to ensure that there was no unspecific binding of the beads to protein samples.

2.3 Results

2.3.1 Reduced Expression of the Kinase Module Subunits in Specific Fat Tissue Depots

Previous studies have shown that the kinase module is dissociated from the Mediator complex and degraded upon nutrient signals in mouse liver.[136] To determine whether the kinase module subunits of the Mediator complex are also regulated by nutrient availability in other tissues, the protein levels of different fat tissue depots (iWAT, gWAT, rWAT, mWAT, and BAT) were compared between mice that were fasted overnight and those that were fed for 4 hours following the overnight fast. As observed in the previous study, the mouse liver extracts displayed reduced levels of the kinase module subunits in the fed state. Similarly, there was also a reduction in MED13, MED12, CDK8, and CCNC in tissues iWAT, rWAT, and gWAT (**Figure 2-3A, 2-3B & 2-3C**). Whereas there was no significant change in expression of the kinase subunits in tissues mWAT and BAT (**Figure 2-4A and 2-4B**) suggesting that the transcriptional control is different with nutrient availability in certain fat tissue depots.

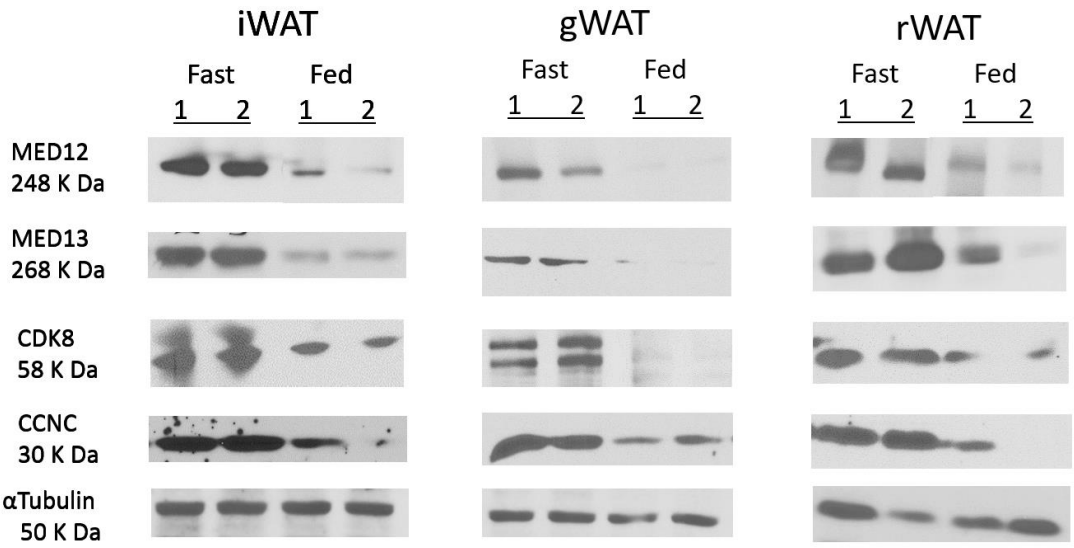

Figure 2-3: Decreased expression of kinase module subunits upon feeding following fasting. 14-week-old female mice were subjected to the fasting and feeding protocol (n=3 per group). Western blot analysis of the Mediator kinase module subunits with fasting and feeding of mouse adipose tissue depots. **A)** representative western blot of iWAT, **B)** gWAT, and **C)** rWAT. 1 & 2 represent the samples collected from two different mice.

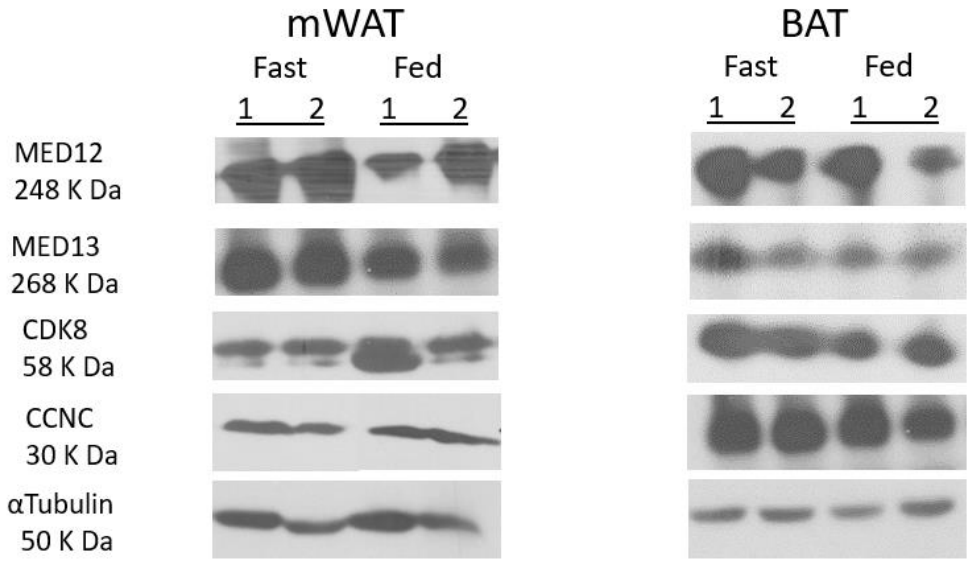

Figure 2-4: Consistent expression of kinase module subunits upon feeding following fasting in mWAT and BAT. 14-week-old female mice were subjected to fasting and feeding protocol (n=3 per group). Western blot analysis of the Mediator kinase module subunits with fasting and feeding of mouse adipose tissue depots. **A)** representative western blot of mWAT and **B)** BAT. 1 & 2 represent the samples collected from two different mice.

2.3.2 Differential Interaction of MED12 and CDK8 with Adipogenic Transcription Factors in Pre- and Mature Adipocytes

To explore the interactions of MED12 and CDK8 with the regulators of adipogenesis, protein-protein interactions were examined using immunoprecipitation analysis. 3T3L1 preadipocytes and mature adipocytes were cultured, and protein was collected. As shown MED12 interacts with PPARγ, C/EBPα, and STAT5A in both pre- and mature adipocytes (**Figure 2-5**). Interestingly, MED12 interacts with PPARγ1 than PPARγ2 in both pre- and mature adipocytes suggesting that MED12 might play a role in driving adipogenesis as it also interacts with C/EBPα, which is known to activate PPARγ for initiation and progression of adipogenesis. MED12 also interacts with STAT5A, known to promote adipogenesis, in both pre- and mature adipocytes. Whereas CDK8 interacts with C/EBPα in both pre- and mature adipocytes, there was no significant interaction with PPARγ and STAT5A (**Figure 2-6**).

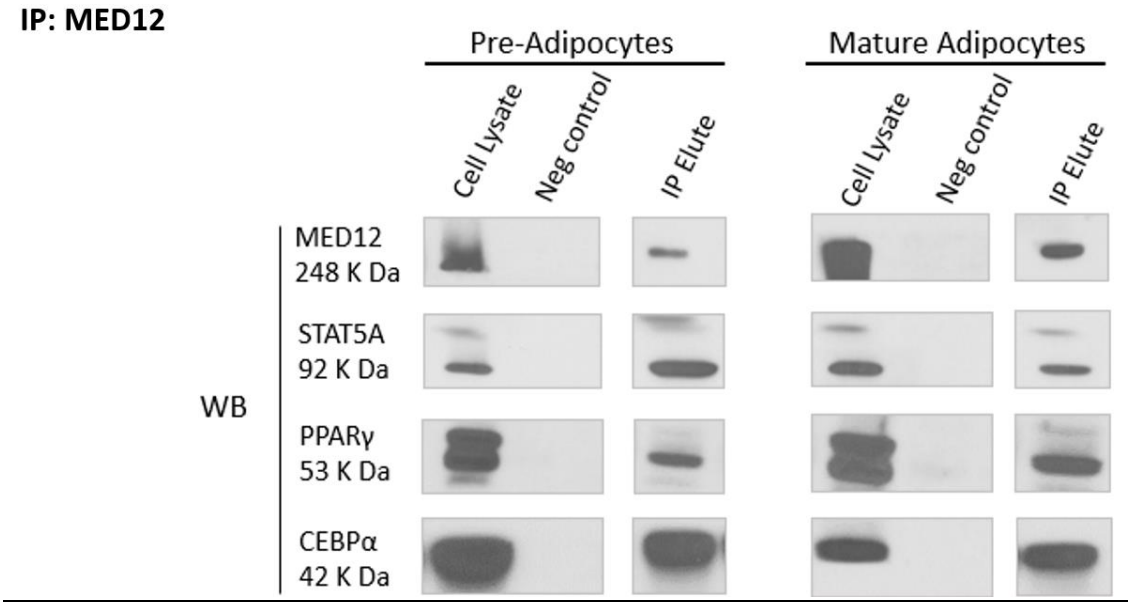

Figure 2-5: Interaction of MED12 with transcription regulators in pre- and mature adipocytes. 200ul of protein lysate collected from cultured 3T3L1 mouse pre- and mature adipocytes were incubated with 5ul of MED12 antibody and the precipitated immunocomplexes were subjected to western blot. Whole cell lysate and IP lysis buffer were used as positive and negative control. IP elute shows presence of respective protein present with the immunoprecipitation.

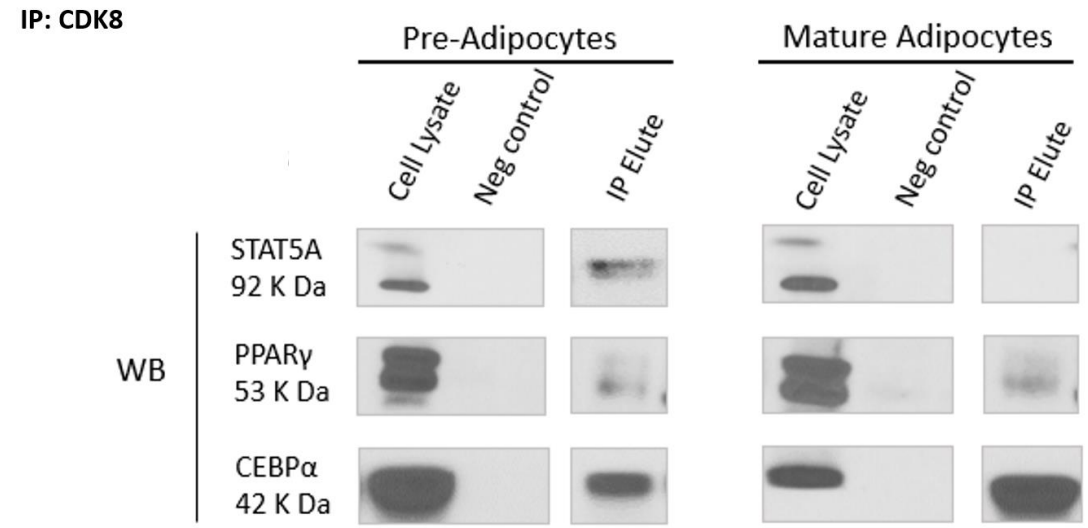

Figure 2-6: Interaction of CDK8 with transcription regulators in pre- and mature adipocytes. 200ul of protein lysate collected from cultured 3T3L1 mouse pre- and mature adipocytes were incubated with 5 ul of CDK8 antibody and the precipitated immunocomplexes were subjected to western blot. Whole cell lysate and IP lysis buffer were used as positive and negative control. IP elute shows presence of respective protein present with the immunoprecipitation.

2.4 Discussion

The Mediator complex is a multi-subunit protein complex that directly binds to RNA Polymerase II and multiple DNA sequence-specific transcription factors. The Mediator complex integrates and conveys gene-specific regulation of initiation and elongation of transcription. Biochemical analyses of the Mediator complex have identified at least two major complexes: the large or complete Mediator complex and the small or core Mediator, which lacks the kinase module. Previous studies suggest that the core Mediator primarily functions during initiation, whereas the large Mediator complex participates in elongation of transcription. It has been assumed that the large and small Mediator complexes control different subsets of regulated gene expression.[68,137,138] Although recent studies have shown that the kinase subunit levels are regulated under various nutritional states in the mouse liver, it still remains to be seen if this is true in other tissues. A study by Youn et al. in mouse liver revealed a dynamic oscillation of different forms of the Mediator complexes between fasting and feeding. Their data indicate that the hepatic Mediator complex primarily exists in the form of the large Mediator complex in the fasted state and following feeding, the kinase module undergoes dissociation and degradation, resulting in the formation of the small Mediator complex.[136]

This study of mouse adipose tissue revealed a regulated expression of the kinase subunits of the Mediator complex between fasting and feeding. Based on the western blots, the data demonstrates that the expression of the kinase module subunits during feeding was reduced compared to the expression in the fasted state in certain adipose tissue depots (iWAT, gWAT, and rWAT). However, kinase module expression remains same in mWAT and BAT. This suggests that following feeding, the kinase module

undergoes dissociation and degradation, resulting in the formation of the small Mediator complex. Although the regulation and mechanism of the dissociation and degradation of the kinase module subunits remains unclear, this data suggests that the previous identification of size oscillation of the Mediator complex in mouse livers might be replicated in certain adipose tissues. Although we are yet to identify the specific component regulating expression of the kinase module subunits, it is likely that the feeding induced activation of mTORC1 might be necessary and sufficient for reduced expression of the kinase module subunits in certain adipose tissues correlated with the dissociation and degradation of the kinase module in mouse livers. mTORC1, is an important target of numerous signaling pathways and serves as a critical sensor of nutrient status. Previous research provide evidence that *in vivo* hepatic mTORC1 activation leads to activation of lipogenic gene expression.[61,136] However, whether mTORC1 activation is necessary for activation of gene expression during lipogenesis in adipose tissue is yet to be determined.

Despite this caveat, immunoprecipitation analysis reveals that MED12 interacts with adipogenic transcription factors, PPARγ and C/EBPα in both pre- and mature murine adipocytes, suggesting a possible crucial role for MED12 in initiation and maintenance of adipogenesis. Analysis of interaction between MED12 with PPARγ indicates an interaction with PPARγ1 but not PPARγ2 in both pre- and mature adipocytes. These data demonstrate that of the two isoforms of PPARγ, PPARγ1 appears to have more affinity to bind to MED12 than PPARγ2 and vice versa. Interestingly, MED12 is also found to interact with STAT5A in both pre and mature adipocytes. STAT5A is known to promote adipogenesis in 3T3-L1 murine cells[139] and so this

interaction further supports the possibility of MED12 in promoting and maintaining adipogenesis.

CDK8 controls mechanisms of transcription factor turnover, regulation of CTD phosphorylation and regulation of activator or repressor functions that are associated with both positive and negative effects on transcription[70,97,140]. Furthermore, CDK8 has emerged as a key regulator of multiple transcription programs linked to differentiation control. In agreement with these results, using 3T3-L1 murine pre and mature adipose cells, immunoprecipitation analysis reveals that CDK8 interacts with C/EBPα. However, there is little to no interaction of CDK8 with PPARγ and STAT5A in both pre and mature adipocytes. Although it remains to be determined whether or not CDK8 interacting proteins are phosphorylated, as CDK8 is primarily thought to be negatively regulating the initiation of transcription.[68] This study shows for the first time that the reduced expression of the kinase subunit proteins of the Mediator complex in certain adipose tissue depots under nutrient regulated conditions and that MED12 and CDK8, subunits of same module have differential interaction with adipogenic regulators *in vivo.*

CHAPTER 3

EXPRESSION AND INTERACTION OF THE KINASE MODULE SUBUNITS DURING ADIPOGENESIS OF hASCs

3.1 Introduction

Individuals with obesity are more likely to develop type 2 diabetes and cardiovascular disease. The disproportionate increase of adipose tissue and its resulting inflammation especially in the visceral regions, is linked to development of metabolic disorders. Over the past two decades, researchers have embarked on understanding the function of adipocytes and the transcriptional events regulating adipogenesis *in vitro*. Adipogenesis is a differentiation process of forming adipocytes from preadipocytes or precursor cells and is regulated by an elaborate network of transcription factors that coordinate expression of many proteins responsible for progression and maintenance of fat cell phenotype.[141] Adipose-derived stem cells (ASCs) are a type of adult stem cells known for their high plasticity and their ability to generate cell types of mesodermal and non-mesodermal lineages including adipocytes, osteocytes, and chondrocytes. The ability of stem cells to differentiate into different lineages is the basis for tissue regeneration and repair.[2,18,20] Disruption in the differentiation process often leads to impaired metabolic processes and can result in disease.[142,143] Understanding the complex regulatory

mechanism of adipocyte differentiation can contribute to clinical treatment of human diseases, including those caused by obesity and adipocyte dysfunction.

Several transcription factors have been implicated in this process, including the C/EBP proteins, the nuclear receptor PPARγ, and the helix-loop-helix protein SREBP1c. Several studies in murine adipogenic cell line models have shown that induction of differentiation causes increased expression of C/EBPβ and C/EBPδ.[121,141,144,145] Together these proteins enhance the expression of C/EBPα and PPARγ. C/EBPα and PPARγ are responsible for gene expression changes characteristic of mature adipocytes and are able to induce each other's expression to promote and maintain the adipogenic state. Importantly, precursor cells were able to differentiate into fat cells in the absence of C/EBPα but not in the absence of PPARγ demonstrating that PPARγ is an essential regulator of adipogenesis.[144] Moreover, SREBP1c has been found to promote adipogenesis through PPARγ in addition to activating expression of key genes that regulate fatty acid metabolism.[145] Taken together, the adipocyte differentiation program is regulated by transcription factors (**Figure 3-1)**, along with their cofactors that regulate the expression of genes involved in promoting and maintaining the adipocyte phenotype. Among the transcription coactivators, a multi-subunit protein complex called the Mediator complex has been linked to several of these transcription factors.

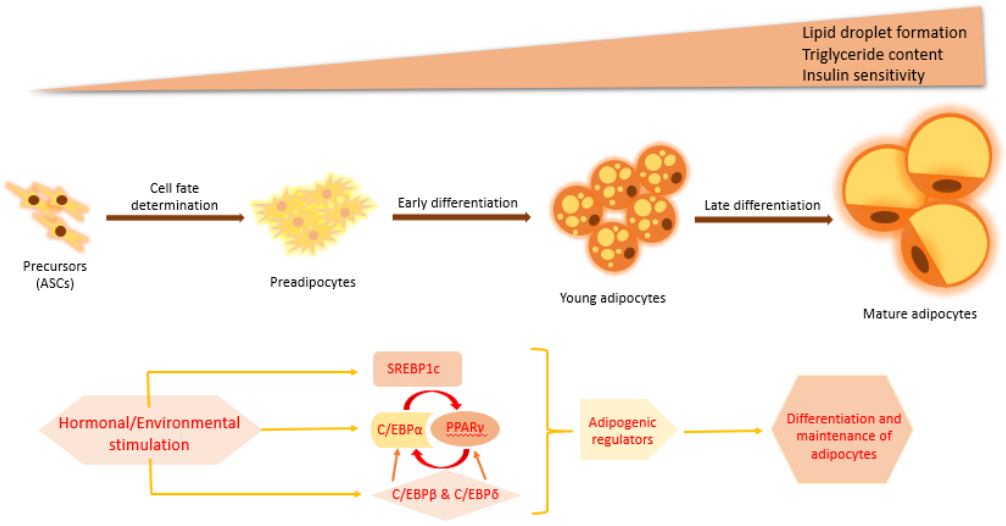

Figure 3-1: Schematic figure of the regulation of adipogenesis. Adipogenesis is a sequential cascade of transcription factors leading to lipid accumulation and the acquisition of insulin sensitivity involving key regulators that help differentiate and maintain adipose state.

The Mediator complex binds to various cell-type specific transcription factors and relays the transcriptional signals to RNA polymerase. The mammalian Mediator is composed of 30 subunits grouped into four modules. The most unique module with reversible binding ability to core Mediator is the kinase module consisting of four conserved subunits MED13, MED12, CDK8, and CCNC. Paralogs have been identified for three of them i.e., MED13L, MED12L, and CDK19 with fewer known functions and are mutually exclusive with MED13, MED12, and CDK8, respectively.[44] The kinase module was originally considered as a transcriptional repressor, but later found to both activate and repress gene expression through kinase dependent and kinase independent mechanisms.[140] Many biochemical, molecular, and physiological studies using cell lines or model organisms have shown diversified functional roles of the Mediator complex. However, the physiological role of the Mediator complex especially the kinase module remains to be explored. Among the four subunits of the kinase module, the CDK8-CCNC

dimer has been studied for its role in lipogenic gene expression, where the dimer negatively regulates de novo lipogenesis through reduction in SREBP1c protein by phosphorylation.[53,61] Furthermore, knockdown of CDK8 in mouse livers showed elevated lipid levels in blood, supporting the CDK8 function in negative regulation of lipogenesis. Recent studies have further shown reduced expression of CDK8 and CCNC in mouse livers through mTORC1 nutrient signaling upon feeding or insulin resistant state or non-alcoholic fatty liver disease state.[61] In addition to CDK8-CCNC studies, MED13 has also been studied for its role in metabolic regulation. MED13 is found to inhibit the expression of genes involved in glucose metabolism in skeletal muscles. Interestingly, MED13 regulates genes involved in energy expenditure and metabolic rate where the deletion of MED13 in cardiac cells resulted in elevated lipid levels independent of food intake levels.[56,146,147] To date, not much information is available on the functions of MED12 individually or as a part of the kinase module in metabolic regulation processes.

This study investigates the gene and protein expression profiles of the kinase module subunits during adipogenesis *in vitro* using cultured hASCs. The mRNA and protein levels of the kinase module subunits were observed to fluctuate through adipogenic differentiation. MED12-knockdown hASCs showed reduced adipogenesis as well as reduced expression of PPARγ, indicating a role for this protein in directing stem cell fate. Finally, we observed that MED12 interacts with PPARγ and C/EBPα at all stages of adipogenesis, but it interacts with SREBP1c only in mature state of

adipogenesis. Altogether, this study suggests a possible indispensable role for MED12 *in vitro* in promoting and maintaining adipogenesis.

3.2 Materials and Methods

3.2.1 Cell culture and Differentiation

Human Adipose-derived Stem Cells (hASCs) were cultured on 10 cm tissue culture treated polystyrene dishes in complete culture medium (CCM) containing αMEM 1x (Life Technologies, Cat# 12561049), 16.5% Fetal Bovine Serum (FBS, Atlanta Biologicals, Cat# S11550), 1% L-glutamine 200 mM (100x) (Gibco Cat #25030-081), and 1% Penicillin Streptomycin (Life Technologies Cat#15140122). Cells were incubated at 37°C at 5% CO_2 and media was changed every 48 hours to ensure the cells were receiving proper nutrients. hASCS cultured till they reached 70-80% confluency and adipogenesis was induced through the addition of AdipoQual medium (LaCell Cat# LaADM-500). Media was changed every 72 hours until collected at different timepoints (day 7, day14, and day 21).

3.2.2 siRNA Mediated Transfection

hASCs were cultured until they were 30-50% confluent and transfected with negative control (Thermo Scientific Cat#4390843) and MED12-specific siRNA (5nmol/ml) (Thermo Fisher Scientific Cat#s19364) using Lipofectamine RNAiMAX reagent (Life Technologies Cat#13778075) following manufacturer's protocol. Media was changed 24 hours after transfection.

3.2.3 Oil red O Staining

Oil Red O stock solution was prepared using 0.5g of solid Oil Red O (VWR Cat#11411-412) and 100ml of 100% isopropanol. Working solution was prepared from

stock solution in a 3:2 ratio of stock solution with distilled water and filtered through a 0.22 µm syringe filter. Cells were fixed with a 10% formalin solution and incubated at room temperature for 15 minutes. Oil Red O working solution was added to cells and incubated for 20 minutes at room temperature. Cells were imaged in a Cytation 5 BioTek Plate Reader.

3.2.4 RNA Extraction and cDNA Synthesis

Cultured cells were washed with PBS twice and RNA was collected using TRIzol reagent (Ambion Cat#15596018). 1 ml of TRIzol reagent is added per 10 cm plate and cells were scraped with a cell scrapper. RNA was extracted following the manufacturer's protocol. RNA was then quantified using a Take3 plate on a Cytation 5 BioTek Plate Reader. qScript cDNA Supermix (VWR Cat#95048-100) was used to synthesize cDNA using 1µg of RNA (used manufacturer's protocol-Quanta Biosciences).

3.2.5 qRT-PCR

qRT-PCR was used to determine transcript levels of the kinase module subunits and adipogenic markers expressed in hASCs at day 0, day 7, day 14, and day 21 after differentiation. qRT-PCR was performed in technical triplicates using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific Cat#A25742), primers, and 1 µl of cDNA. The reaction was run using a StepOnePlus Applied Biosystems machine standard quantitation experiment. The data was plotted in excel using $\Delta\Delta C_T$ curve. The primer sequences used are listed in **Table 3-1**.

Table 3-1: List of primer sequences used for qRT-PCR

Gene	Forward Sequence (5'-3')	Reverse Sequence (3'-5')	Size (bp)
gapdh	CCCCACTTGATTTTGGAGGGA	AGGGCTGCTTTTAACTCTGGT	206
med12	CGAAAAGGGACAGCAGAAAC	CCCATCCTCCCCACCTAAGA	87
med13	TGTCCTGCTCCTTCACCTTTT	GGCATAAGATAACTTGAAATGGGCT	150
ccnc	GCTGATTTGATCGAGGAGCG	ATCCATTGCAAATAGTGGGAGC	148
cdk8	GCCAAGAGGAAAGATGGGAAGG	GCCGACATAGAGATCCCAGTT	77
pparγ	GCTGTTATGGGTGAAACTCTG	ATAAGGTGGAGATGCAGGTTC	151
c/ebpα	TATAGGCTGGGCTTCCCCTT	AGCTTTCTGGTGTGACTCGG	94
tubulin1-α	CCAGGGCTTCTTGGTTTTCC	CGCTCAATGTCGAGGTTTCT	167
srebp1c	CTCTTGAAGCCTTCCTGAG	GCACTGACTCTTCCTTGAT	138

3.2.6 Protein Extraction and Western Blot

Cultured cells were washed twice with ice cold PBS and harvested on ice using 400 uL of RIPA lysis buffer (Thermo Fisher Scientific Cat#89900)) supplemented with Halt Protease and Phosphatase inhibitor Single-Use Cocktail (Thermo Fisher Scientific Cat#78441). Whole cell lysates were then agitated for 30 min at 4 °C and then centrifuged for 20 mins at 12,000 RPM at 4 °C. Protein samples were quantified using BSA standard curve generated using Bradford assay. SDS-PAGE was performed using

standard protocol for Mini-PROTEAN TGX 4-15% (Bio-Rad Cat#456-1084)). Proteins were transferred onto a PVDF membrane (Thermo Fisher Scientific Cat#88518) at 100V for 1 hr. The transferred membrane was blocked in 5% non-fat dry milk in 1x TBS with 0.1% Tween-20. The blocked PVDF membranes were cut according to the size of target protein and were probed overnight at 4 °C with respective antibody. Membranes were washed 4 times with TBST, 5 mins each and probed with secondary antibody for 1 hr at room temperature. Blots were developed using ECL reagents (BioRad Cat#1705060) and images were taken using chemiluminescence Chemdoc machine (BioRad). The list of antibodies used for western blot are listed in **Table 3-2**.

Table 3-2: List of antibodies used for western blot analysis

Antibody	**Size (k Da)**	**Host species**	**Company**	**Catalog No.**	**Dilution**
MED12	250	Rabbit	Bethyl Laboratories	A300-774A	1:1000
MED13	268	Rabbit	Bethyl Laboratories	A301-278A	1:1000
CDK8	58	Rabbit	Bethyl Laboratories	A302-501A-M	1:1000
CCNC	28	Rabbit	Bethyl Laboratories	A301-989A	1:1000
PPARγ	53, 57	Rabbit	ThermoFisher	PA3-821A	1:500
C/EBPα	30 & 42	Rabbit	ThermoFisher	PA1-337	1:1000
GAPDH	37	Rabbit	Abcam	Ab9485	1:1000

3.2.7 Co-Immunoprecipitation Assay (Co-IP)

Protein for Co-IP assays was collected as described above but used IP lysis buffer instead of RIPA buffer. The collected protein was agitated for 5 mins at 4°C and centrifuged at 13,000 XG for 10 mins. The supernatant was collected and quantified using Bradford assay. Co-IPs were performed using the Pierce Crosslink Magnetic IP/Co-IP kit following the manufacturer's protocol (Thermo Fisher Scientific Cat#88805). 5 µl of polyclonal antibody was used per 200 µg of lysate. Collected elution and unbound lysates were used for western blot analysis.

3.2.8 Statistical Analysis

Data were presented as the mean +/- standard deviation (SD). Graphs were generated using Student's t test with $P<0.05$ considered statistically significant.

3.3 Results

3.3.1 The Expression of Kinase Module Subunits of The Mediator Complex Fluctuates During Adipogenesis of hASCs

To investigate the potential role of the Mediator kinase module subunits in adipogenesis, the gene and protein expression profiles for each of the four subunits of the kinase module were examined in hASCs induced to undergo adipogenesis. Cells were visually examined at days 0, 7, 14, and 21 through phase-contrast imaging and Oil Red O staining (**Figure 3-2**). Differentiation was confirmed by the increased presence of lipid vesicles and changing expression profiles of adipogenic regulators C/EBPα, PPARγ, and SREBP1c (**Figure 3-3**). The transcript levels of the kinase module subunits, i.e., MED12, MED13, CDK8, and CCNC at different timepoints during adipogenesis had different expression patterns with each timepoint (Normalized to *gapdh*) (**Figure 3-4**). The

expression of *med12* and *ccnc* followed the same trend where their expression increased from day 0 to 14 into differentiation and dropped at day 21 upon reaching a mature state. The expression of *med13* increased consistently from day 0 to 21, whereas the expression of *cdk8* dropped at day 7 then increased over day 14 and 21. When examined together, *med12* appears to be transcribed at the highest levels with at least a fold change of 0.5 at each time point. This suggests and supports a kinase-independent activity of MED12 in transcription regulation in adult stem cells.[57]

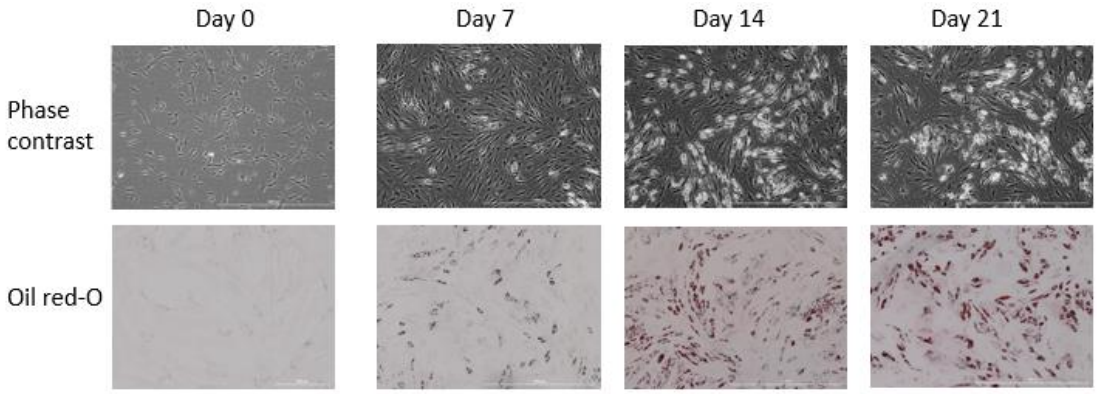

Figure 3-2: Morphological characteristics of hASCs at different stages of adipogenic differentiation. Pictures represent the morphology of differentiating cells in phase-contrast imaging (top row) and staining pattern of Oil Red O (bottom row) induced at day 0, 7, 14, and 21, respectively.

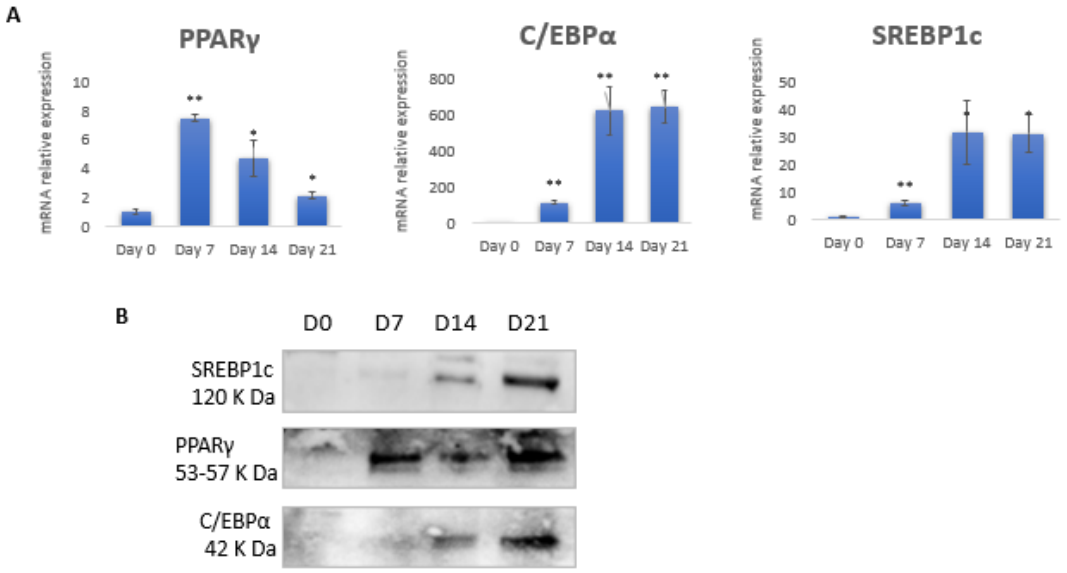

Figure 3-3: Expression of adipogenic markers during adipogenesis of hASCs. A) mRNA expression and B) Protein expression pattern of PPARγ, C/EBPα, and SREBP1c at different timepoints during differentiation of hASCs. The mRNA expression level at day 0 was assigned as the control (Normalized to *gapdh*). Data represented as mean +/- SD (*) $P < 0.05$ and (**) $P < 0.01$.

In contrast, the protein levels of MED12, CDK8, and CCNC demonstrated a distinctive pattern throughout the progression of adipogenesis (normalized for the GAPDH protein level), where the expression decreased during the middle stage and increased again during the late stage of adipogenesis (**Figure 3-5**). However, MED13 remains consistent with transcript levels which steadily increased through day 21. The reduction in protein levels of MED12, CDK8, and CCNC was observed and contrasted with their respective mRNA levels suggesting post-translational modification.

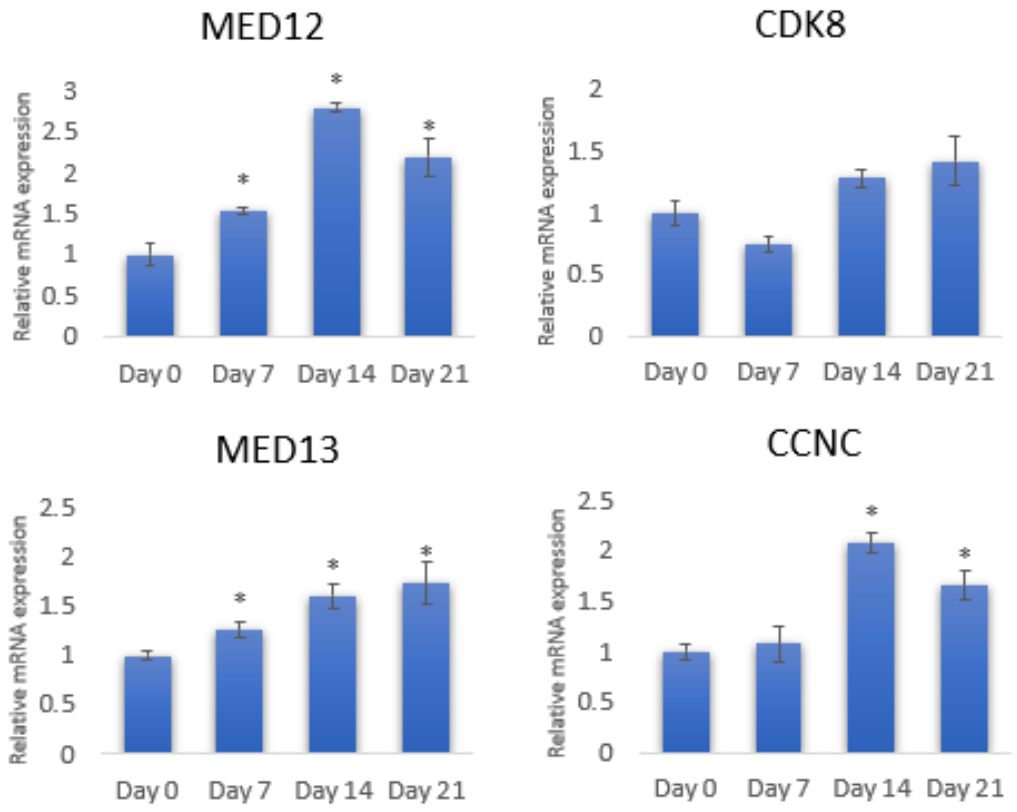

Figure 3-4: The relative mRNA expression of genes of the kinase module subunits during adipogenesis of hASCs. Expression pattern of MED12, MED13, CDK8 and CCNC at different timepoints during differentiation of hASCs. The mRNA expression level at day 0 was assigned as the control. Data represented as mean +/- SD (*) $P < 0.05$.

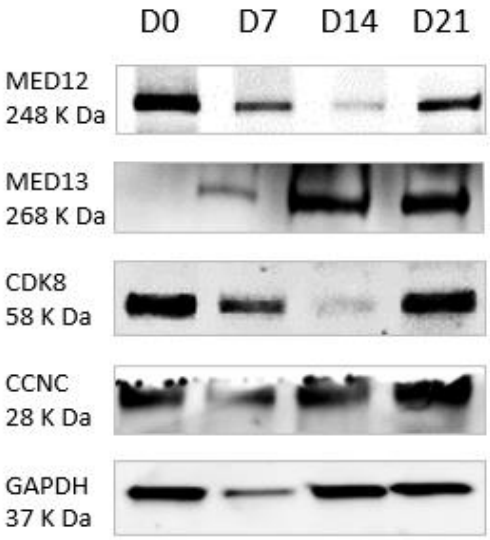

Figure 3-5: The protein expression of subunits of the kinase module during adipogenesis of hASCs. Expression pattern of MED12, MED13, CDK8 and CCNC over 21 days of adipogenic differentiation of hASCs.

3.3.2 MED12 Knockdown Leads to Decreased Adipogenesis in hASCs

As MED12 is a central part of the kinase module of the Mediator complex, we tested whether the decreased expression of MED12 affects adipogenesis. To test this, MED12 was knocked down in cultured hASCs just before inducing adipogenic differentiation. Cells were collected at day 3, 7, and 14 of differentiation, and adipogenesis was assessed based on morphology, Oil Red O staining, and transcription factor profiles. Oil Red O staining indicated less lipid vesicle formation following the decreased expression of MED12 (**Figure 3-6A**). MED12 knockdown was confirmed using qRT-PCR (**Figure 3-6B**) and western blot (**Figure 3-6C**) showing less MED12 at both transcript and protein levels, respectively.

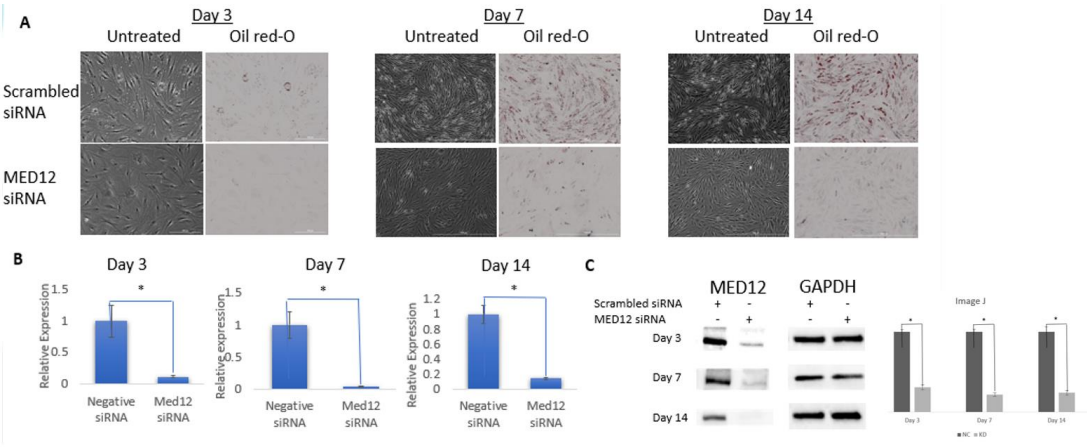

Figure 3-6: MED12 knockdown during adipogenesis of hASCs. A) Morphological characteristics with knockdown of MED12. Morphology of differentiating cells with negative control (top row) and MED12 siRNA (bottom row) with phase-contrast (left column) and Oil Red O staining (right column) during different stages of adipogenesis of hASCs. Validation of MED12 knockdown via B) qRT-PCR analysis showing MED12 mRNA expression and C) Western blot showing MED12 protein expression during adipogenesis of hASCs with MED12 knockdown. Also shows % of MED12 protein remaining with ImageJ analysis. Data represented as (*) $P < 0.05$. Data Credit: Onyekachi Idigo and Joseph Straub.

To uncover additional molecular signatures related to adipogenesis, we examined the expression of PPARγ, a master regulator of adipogenesis. We observed that both mRNA and protein levels of PPARγ decreased with the diminished expression of MED12 (**Figure 3-7A and 3-7B**), suggesting that MED12 plays a role in adipogenic differentiation.

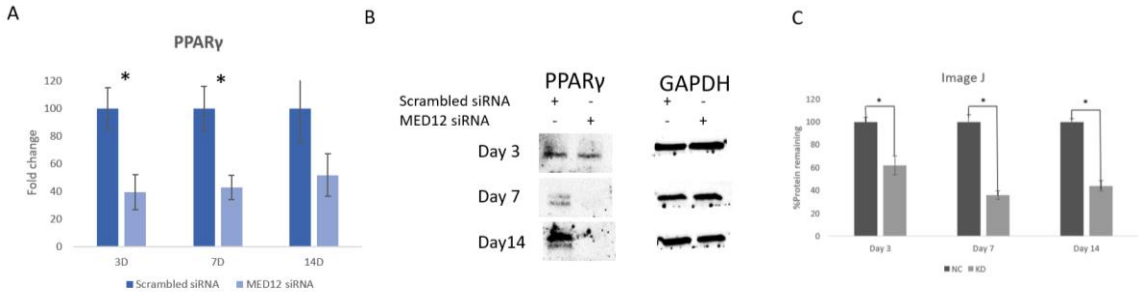

Figure 3-7: Decreased PPARγ expression with MED12 knockdown A) qRT-PCR analysis showing mRNA expression of PPARγ and B) Western blot analysis showing protein expression of PPARγ during different stages of adipogenesis of hASCs with MED12 knockdown. C) Image J analysis of protein levels. Data represented as (*) $P < 0.05$. mRNA Data credit: Onyekachi Idigo.

3.3.3 Loss of MED12 Disrupts Kinase Module

To test if the decreased expression of MED12 impacts the other kinase module subunits, we examined both transcript and protein levels of MED13, CDK8, and CCNC. Given the central location of MED12 between MED13 and CDK8/CCNC in the kinase module, the decreased expression of CDK8/CCNC was expected with the knockdown of MED12 as the CDK8/CCNC dimer is attached to MED12 through CCNC and this trio is linked to core Mediator through MED13. Interestingly, qRT-PCR analysis of MED12 knockdown demonstrated significant change in *med13*, *cdk8*, and *ccnc* mRNA levels at day3 and 7 of adipogenesis (**Figure 3-8A**). The protein expression of CDK8 remained

unchanged, where as CCNC and MED13 demonstrated lower expression in MED12 knockdown cells compared to negative control cells throughout adipogenic differentiation (**Figure 3-8B**). This data suggests a Mediator kinase independent role for CDK8 even though its attachment to the Mediator core is disrupted through knockdown of MED12.

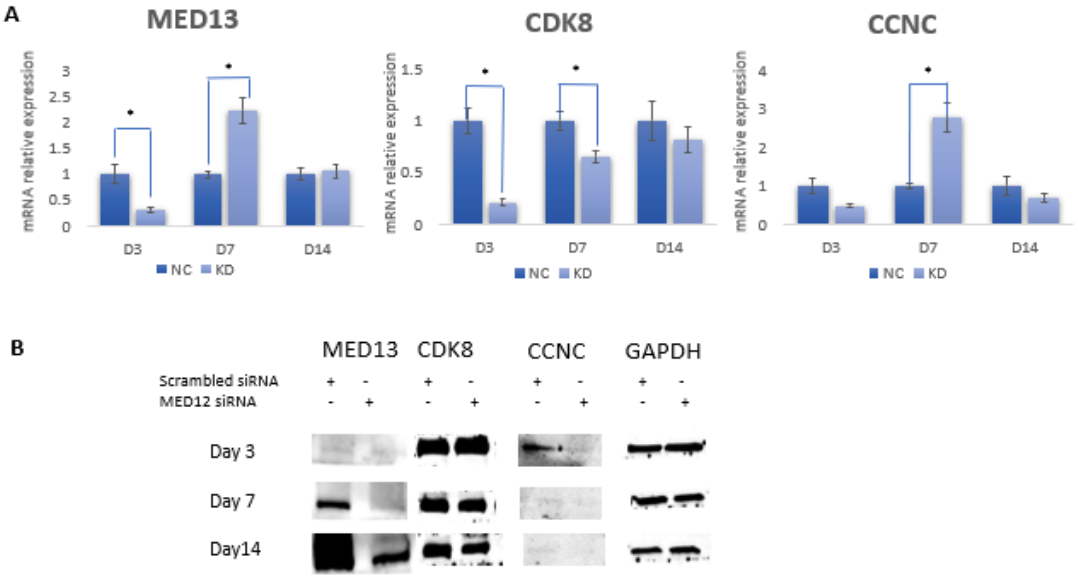

Figure 3-8: Decreased kinase subunit expression with MED12 knockdown A) qRT-PCR analysis showing mRNA expression and B) Western blot analysis showing protein expression of MED13, CDK8, and CCNC with MED12 knockdown. Data represented as (*) $P < 0.05$. mRNA Data Credit: Onyekachi Idigo

3.3.4 MED12 Interacts with Adipogenic Regulators During Adipogenesis of hASCs

To further examine the interaction between MED12 and adipogenic regulators including PPARγ, C/EBPα, and SREBP1c, co-immunoprecipitation (Co-IP) experiments were performed during early, middle, and late stages of adipogenesis. As expected, the MED12 pulldown assay revealed the presence of PPARγ and C/EBPα at all stages of adipogenesis (**Figure 3-9**). Interestingly, MED12 interacts with SREBP1c only in the late

stage i.e., in mature adipocytes (**Figure 3-10**). These results demonstrating the interaction of MED12 with transcription regulators of adipogenesis support the notion that MED12 plays a possible role in initiating adipogenesis as well as maintaining the adipogenic state. Other interaction partners included CDK8, CCNC, and MED13, where the interaction between the kinase module subunits was evaluated. Interaction of MED12 with other subunits was found to be consistent throughout adipogenesis.

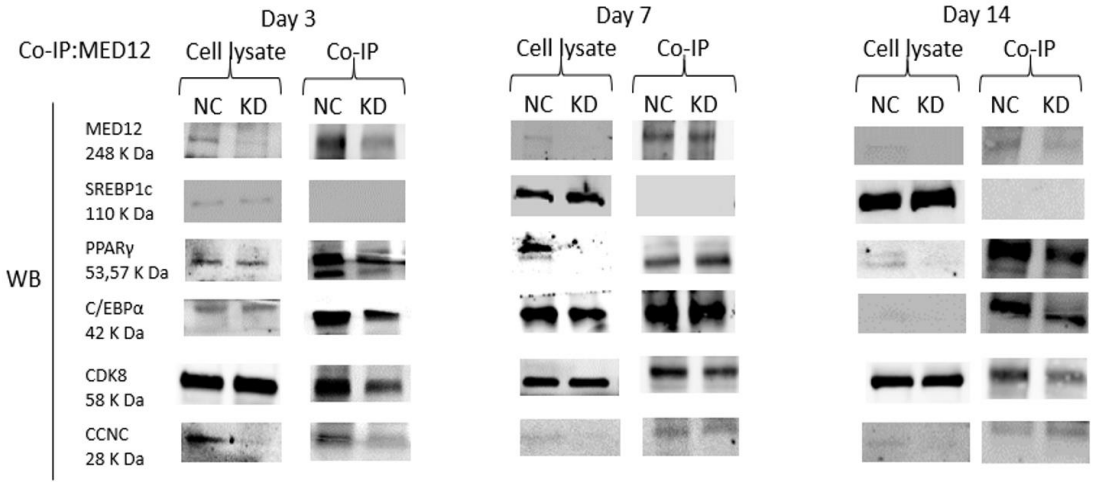

Figure 3-9: Interaction of MED12 with adipogenic regulators. Co-IP of MED12 during different stages of adipogenesis, day 3, 7, and 14 (early, middle, and late). Cell lysates are whole cell extracts of respective timepoints with negative control and MED12 knockdown. Co-IP represents the elution sample obtained after MED12 co-immunoprecipitation.

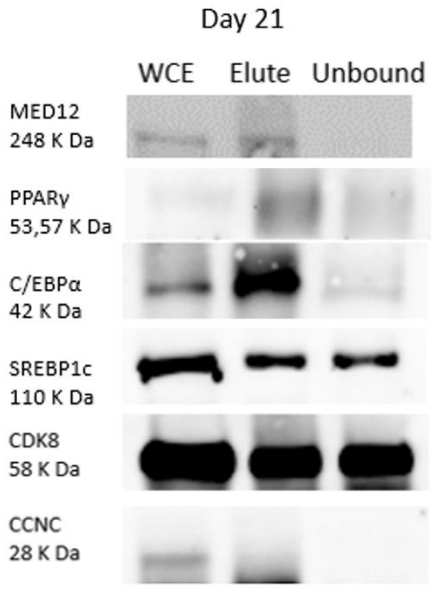

Figure 3-10: Interaction of MED12 with adipogenic regulators and other kinase module subunits at day 21 of adipogenesis. Co-IP of MED12 during day21 i.e., mature stage of adipogenesis. WCE represents whole cell extract at day 21. Elute represents elution sample obtained after MED12 co-immunoprecipitation. Unbound represents the remaining sample content that the target protein doesn't interact with.

Co-IP experiments following MED12 knockdown showed a reduction of PPARγ presence in eluted samples, supporting the reduction in PPARγ and adipogenesis following the decreased expression of MED12. Additionally, no significant reduction in expression of C/EBPα was observed with MED12 knockdown even though the interaction between these proteins is constantly present during all stages of adipogenesis. This suggests that MED12 might be regulating adipogenesis through PPARγ. Overall, these results provide a mechanistic insight for the maintenance of lineage-specific transcription regulation by key adipogenic regulators.

3.4 Discussion

Differentiation is a well-orchestrated process that relies on the convergence of environmental factors, cell-signaling, transcription factors, and gene expression

regulation to produce a nearly irreversible alteration of a stem cell's gene expression profile, leading to an overall change in the stem cell's cellular identity. Adipogenesis is the process of a multipotent stem cell differentiating into a mature adipocyte capable of accumulating and storing fatty acids within lipid vesicles. Mediator is found to play a critical role in cell type-specific gene expression, and its role includes regulating activity on the part of the kinase module. The kinase module plays a prominent role in gene expression, and the results from this study point to possible MED12 involvement beyond cell-fate determination and into maintenance of differentiated adipocytes. The data demonstrating the link between MED12 and PPARγ via protein-protein interactions, has illuminated the possibility that these two critical regulators act together during adipogenesis. This possibility of working in coherence continues further into the later stages of differentiation as shown by the interaction of MED12 with SREBP1c in mature adipocytes.

Studies deciphering Mediator complex regulation are still in the early stages. Altered phosphorylation has been observed as a mechanism for the regulation of Mediator subunits.[148] MED13/MED13L are shown to be regulated by ubiquitination, which targets these subunits for degradation by SCF-Fbw7.[34] It is likely that other post-translational modifications regulate Mediator kinase subunits, and of particular interest are those generated by metabolic pathways. Since Mediator kinase module subunits are implicated as terminal signal transducers for many pathways, it is likely that the expression of subunits is regulated in a context-specific manner. The post-translational modifications resulting in different levels of kinase module subunits during adipogenesis *in vitro* remains to be identified. We speculate that these contexts or metabolically

induced posttranslational modifications are intimately tied to the metabolic demand of specific tissues and rapidly modulate the transcriptional gene network in response to whole cell/tissue demands.

A number of studies have identified MED12 mutations in cancers.[41,78,81] Since mutations drive cancers and other developmental disorders, it is tempting to speculate that MED12 mutations could give rise to metabolic disorders, as MED12 loss led to diminished adipogenesis and PPARγ expression according to this study. Interestingly, several of the adipogenic regulators interacting with MED12 during adipogenesis are also frequently mutated in several metabolic disorders, including PPARγ, C/EBPα , and SREBP1c.[66,149] Future work will test whether these genetic events indeed induce molecular disability by modulating transcription factor activity.

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

Gene expression regulation in mammals is accomplished by coordinated action of several transcription factors and their associated cofactors, which integrate signals from different pathways. In particular, stem cells have numerous factors that can influence stem cell fate including environmental stimulus, chromatin modifiers, signal transduction molecules, and transcription machinery. Modification to even one of these factors will change the genes expressed and the decision to continue self-renewal or differentiate into a specific lineage. In recent years, interest has increased in studying cofactors that bridge transcription factors and RNA polymerase to activate or repress the transcription machinery in response to external signals. One of the recently identified cofactors is the highly conserved Mediator complex, which has 30 subunits grouped into four modules and is involved in various biological processes through interaction with specific transcription factors. The four-subunit kinase module of the Mediator complex consisting of MED12, MED13, CDK8, and CCNC can reversibly associate with the 26-subunit Mediator core. The kinase module is able to alter the structure and function of Mediator and has been established as an essential transcriptional cofactor of numerous

developmental, biochemical, and metabolic processes. Mutations of any of these subunits have been reported in mental diseases, metabolic disorders, and cancers.

Due to the prevalence of obesity, metabolic conditions related to obesity such as type 2 diabetes, cardiovascular diseases, and cancer are becoming more common. The common feature of these obesity-related disorders is dysregulation in lipid metabolism. Adipogenesis, the developmental process of forming adipocytes from precursor cells, is considered an important aspect of lipid metabolism and is tightly regulated by a transcriptional cascade including transcription factors PPARγ, C/EBPα, and SREBP1c, and cofactors including the Mediator complex. Recent studies have shown that Mediator subunits play a crucial role as a cofactor for these transcription regulators, indicating a possible role in adipogenesis/lipogenesis. Despite the growing research in this field, the role of the kinase module subunits in adipogenesis has not yet been investigated. My research focused specifically focused on investigating the role of kinase module subunits during adipogenesis and their interaction with adipogenic transcription regulators. Through molecular biology techniques, I have been able to demonstrate the fluctuations in expression and interactions of the kinase module subunits in murine adipose tissue depots during fasting and refeeding. This research helps bridge the knowledge gap that exists in understanding the regulation of adipogenic differentiation of hASCs through interaction with adipogenic transcription factors.

In Chapter 2 the refed mice demonstrated decreased expression of the kinase module subunits compared to fasted mice in iWAT, gWAT, and rWAT. However, there was no significant change in expression of the kinase module subunits in BAT and mWAT. This could be an indication of activation of specific nutrient sensitive pathways

upon refeeding following fasting that are responsible for the decrease in expression of kinase module subunits. But the nutrient signaling, and the requirement of transcriptional regulation is different for different tissue locations as evident by expression in BAT and mWAT. This study also investigated the interaction of MED12 and CDK8 with transcription regulators including PPARγ, C/EBPα, and STAT5A, where MED12 was found to interact with all three in both murine pre and mature adipocytes. However, CDK8 was observed to only interact with C/EBPα in both pre and mature adipocytes. These interactions indicate that the subunits of the kinase module regulate different metabolic processes through their differential interaction with transcription factors.

Chapter 3 focused on the expression of kinase module subunits during adipogenesis of hASCs and the interaction of MED12 with key adipogenic regulators. By culturing hASCs and differentiating into adipocytes, progression of adipogenesis was evaluated through phase contrast imaging and Oil Red O staining. The mRNA and protein expression of all the kinase module subunits during adipogenesis is inconsistent throughout the differentiation, that is likely due to necessity of the subunits at each stage of the differentiation process. Also, there was no correlation between the transcript and protein levels, which could be because of varied post translational modifications. MED12 knockdown in hASCs resulted in a reduction of adipogenic differentiation in hASCs, as indicated by reduced lipid vesicle formation and Oil red-O staining. Most importantly, MED12 knockdown studies suggest that MED12 is indispensable for the progression of adipogenesis. Co-immunoprecipitation analysis show that MED12 co-precipitates with key adipogenic regulators, such as PPARγ, C/EBPα, or SREBP1c, which may be recruiting MED12 to interact and cooperate with other transcriptional activators and co-

activators to regulate adipogenesis. Supporting this notion, MED12 interacts with PPARγ and C/EBPα at all stages of adipogenesis, an interaction that was reduced following the knockdown of MED12. Thus, this study suggests that MED12 plays a critical role in initiating adipogenesis *in vitro*. Interestingly, MED12 interacts with SREBP1c only during late stage i.e., in mature adipocytes, supporting the idea that MED12 also might play a role in maintenance of adipogenic state *in vitro*. Overall, this is the first study that identified MED12 as a key regulator of adipogenesis *in vitro* and suggest the involvement of MED12 beyond the point of cell fate determination and into the maintenance of differentiated adipocytes.

4.2 Future Directions

The ultimate goal of this research is to gain a better understanding of adipogenesis and its regulation by the Mediator kinase module subunits. From this work a critical role for MED12 during *in vitro* differentiation was revealed that introduces an opportunity for more in-depth mechanistic studies. By using different cell and molecular biology techniques including cell culture, staining, qRT-PCR, western blot, Co-IP, and siRNA mediated knockdown, I was able to demonstrate the importance of MED12 during adipogenesis and its interaction with different adipogenic regulators.[66,124] Having shown that specific mice adipose tissue depots have reduced expression of the kinase module subunits with refeeding following fasting, a possible future study as to why the same adipose tissue at different locations have differential transcriptional control can be explored. In addition, the possible reason behind the regulation of expression can be explored in depth and if any of the kinase module subunits interact with lipogenic or glucogenic genes that were affected by nutrient availability. Furthermore, the differential

interaction of MED12 and CDK8 with transcription regulators can be further explored as to find if they were enhancing or repressing gene expression through their interaction.

The difference in transcript and protein levels of the kinase module subunits during adipogenesis of hASCs offers an additional way to study how post-translational modifications regulate protein expression during adipogenesis. Since adipogenesis in adult stem cells is highly characterized, this *in vitro* study using hASCs opens possible routes of differentiation potential to be explored for clinical purposes. MED12 interaction with master adipogenic regulators opens a whole area of research that can be explored in terms of the Mediator subunits being the downstream target for a number of metabolic pathways and how the mechanism of regulation influences cellular differentiation.

APPENDIX A

OPTIMIZATION OF SARS-COV2 DETECTION IN WASTEWATER

A.1 Introduction

The newly emerged infectious Coronavirus disease 2019 (COVID 19) is caused by a novel coronavirus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This epidemic disease, which started in December 2019, has spread worldwide and turned into a pandemic causing global health emergencies and economic crises.[150] To date, there have been more than 166 million confirmed infections and more than 3.4 million deaths reported globally.[151] Many studies have reported that many of the sickest patients have been obese individuals who are 113% more likely to land in hospital and 48% more likely to die when compared to individuals of healthy weights.[152,153] A number of physiological factors drives the biology of obesity including impaired immunity, chronic inflammation, and blood that's prone to clot, all of which can worsen the symptoms of COVID-19.

SARS-CoV2 is a type of human beta-coronavirus containing single stranded RNA associated with a nucleoprotein within a capsid. Among all known RNA viruses, coronaviruses possess the largest genomes with several conserved genes including spike (S), envelope (E), membrane (M), and nucleocapsid (N), encoding for structural proteins which are responsible for host infection (Figure A-1).[150,154,155] The N protein is responsible for packaging the viral genome into a helical ribo-nucleocapsid and is relatively abundant during virus replication. Because of this targeting, the N gene (N1 and N2) of COVID-19 yielded diagnostic sensitivity and specificity for detecting infections. Many studies showed that the N-gene based qRT-PCR assay was more sensitive than any other genes for detection of COVID-19 soon after emergence of the disease when limited genetic information was available.[155–158] While the timely diagnosis

of the progressing infection is essential to prevent transmission, rapid assays and early testing can have a risk of false-negative results. Keeping track of the full extent of the ongoing pandemic is a constant challenge, as there is insufficient testing, delayed results, and the absence of data from those who are asymptomatic. Researchers around the world began to monitor sewage for traces of pathogen which provides an effective surveillance of an entire community regardless of positivity rate.

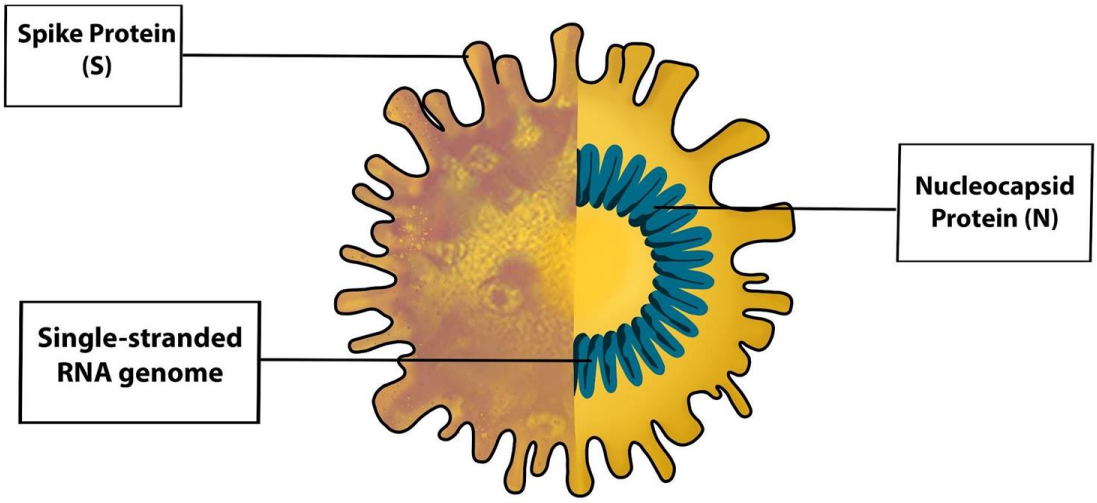

Figure A-1: Graphical representation of SARS-CoV-2 structure. This picture shows the spike protein, nucleocapsid protein and the viral RNA present in the virus. Image credit: Kristin Jackson, VISTA.

Wastewater testing is an effective and widely known application which began in the 1990s with efforts to eradicate poliovirus, and where it was shown to prevent outbreaks.[159] COVID-19, which is spread through respiratory droplets, is detected in patient stool samples even before the onset of symptoms. Wastewater surveillance is expected to lead diagnostic tests by a week given that shedding may occur soon after infection, whereas an infected person may develop symptoms after 5-7 days of infection.[160,161] This study developed a method of tracking infection rates in the City of

Ruston, LA. The samples were collected from the wastewater treatment plant in the city of Ruston and processed to collect viral RNA. qRT-PCR analysis was performed for genes N1 and N2 to assess the amount of SARS-CoV2 genome present in the wastewater. The results demonstrate that a higher viral load is followed by rise in positive cases. It is also evident that the infection rates were higher following events with large gatherings or after breaks in the University's schedule where students travel to see friends and family before returning to campus. These data suggest that wastewater testing may be a preliminary indicator of community transmission.

A.2 Materials and Methods

A.2.1 Sample Collection and Handling

Treated wastewater samples were obtained from the City of Ruston wastewater twice a week. Samples were stored at 4^{o}C or processed immediately after receiving. Samples were centrifuged at 5000 RPM for 30 minutes to pellet the debris. Clear supernatant was collected into another 50 ml tube and 0.9g NaCl and 4g PEG 8000 were added per 40 ml by gentle rocking. Once completely dissolved, the samples were left at least 1 hr or overnight at 4^{o}C and centrifuged at 12,000G for 2 hours at 4^{o}C. The supernatant was poured out carefully and RNA was collected.

A.2.2 RNA extraction and cDNA synthesis

RNA was collected using TRIzol reagent (Ambion Cat#15596018) 1 ml per tube. RNA was extracted using manufacturer's protocol. Resulting RNA concentrations were quantified by a plate reader with a Take3 plate (BioTek). cDNA was synthesized

immediately after quantifying RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Cat#4374966).

A.2.3 qRT-PCR

Determination of the number of viral genome copies per ml of wastewater was analyzed by qRT-PCR. Primers and probes used included the N1 and N2 primers and probe mix (2019-nCoV EUA, Integrated DNA Technologies Cat#10006770). Each 10 µl of qRT-PCR reaction included 1x mastermix (Taqpath Applied Biosystems Cat#A15297), 0.75 µl primer probe mix, 1µg of RNA sample, and PCR grade nuclease free water. Each qRT-PCR run included a six-fold serial dilution of positive control consisting of 2019-nCoV_N_Positive control (Integrated DNA Technologies Cat#10006770) for genes N1 and N2 and was run in triplicates. The qRT-PCR was run in a thermocycler (Applied Biosystems) with the conditions given by Centers for Disease Control (CDC). Virus concentrations were determined by comparing Ct values of samples against the standard curve from dilutions of the positive control. Dilution factor,

volume of RNA used, and the total volume of sample in each well resulted in the calculated genome units/ml of wastewater.

A.2.4 Statistical methods

The viral genome concentration per ml were represented with graphs over time periods and the correlated data with positive cases were represented through Pearson's correlation using SPSS software.

A.3 Results

A.3.1 Optimization of Methodology for Quantitative Detection SARS-CoV-2 in Wastewater

Multiple testing methods and laboratory workflows were used to quantify SARS-CoV-2 in wastewater across the United States.[161,162] All testing methods use sample processing steps, use of laboratory controls, and implementation of biosafety measures to ensure that the resulting data can be used for public health interpretation. Sample processing is the most important part of wastewater testing as it involves the key steps including sample preparation, sample concentration, RNA collection, extraction, and quantification. Methods selected for each step were tailored for use with wastewater depending on the appropriate laboratory conditions. It was initially challenging to produce reliable outcomes as testing was relatively new to our laboratory and at least initially, that time there was no standard operating procedure for SARS-CoV-2 wastewater detection.

Considerations for proper handling of samples include proper storage and transfer of samples, sample concentration, RNA extraction, purification, and quantification, and minimizing RNA degradation. Proper storage and preparing the samples ensures that

SARS-CoV-2 RNA wastewater measurements are accurate. Concentration method selection also depends on the processing time and availability of laboratory personnel. RNA extraction and purification are an essential and crucial step in isolating SARS-CoV-2 RNA from wastewater. Selecting an extraction protocol designed to produce highly purified nucleic acid extracts was a challenging task. Upon using phenol/glycogen standard method and an extraction kit designed specifically to purify viral RNA, we chose standard method as optimal RNA extraction method for our laboratory setting. Although using the extraction kit resulted in high purity of viral RNA, it had low yield/concentration due to volume restriction through column (**Table A-1**). The biggest challenge faced was avoiding degradation of RNA, when the sample was being handled or processed, with of no way of knowing whether degradation occurred because of sample mishandling or from lower concentrations of viral RNA in wastewater. Overcoming these challenges and establishing a routine protocol to follow allowed us to produce complementary COVID-19 surveillance indicator data.

Table A-1: An overview of difference between using different RNA extraction methods for wastewater analysis.

Method/Kit used	RNA Conc ng/ul	Purity 260/280	Steps/Time
TRIzol/Chloroform/Glycogen	~180	~1.8-2.0	5/60mins
Viral RNA Extraction kit-Qiagen	~92	2.0	9/90mins

A.3.2 SARS-CoV2 RNA detection frequency and correlation with COVID-19 cases in Lincoln Parish

During the five-month study from August 2020 to January 2021, SARS-CoV-2 RNA was detected in wastewater from wastewater treatment facility in the City of Ruston

(Figure A-2). In an effort to determine the relationship, if any, between SARS-CoV-2 viral concentrations in wastewater and COVID-19 disease burden, the new COVID-19 case counts reported to the City's department of health were summed by week and case rates were then plotted against the weekly SARS-CoV-2 genome concentration/ml in wastewater.

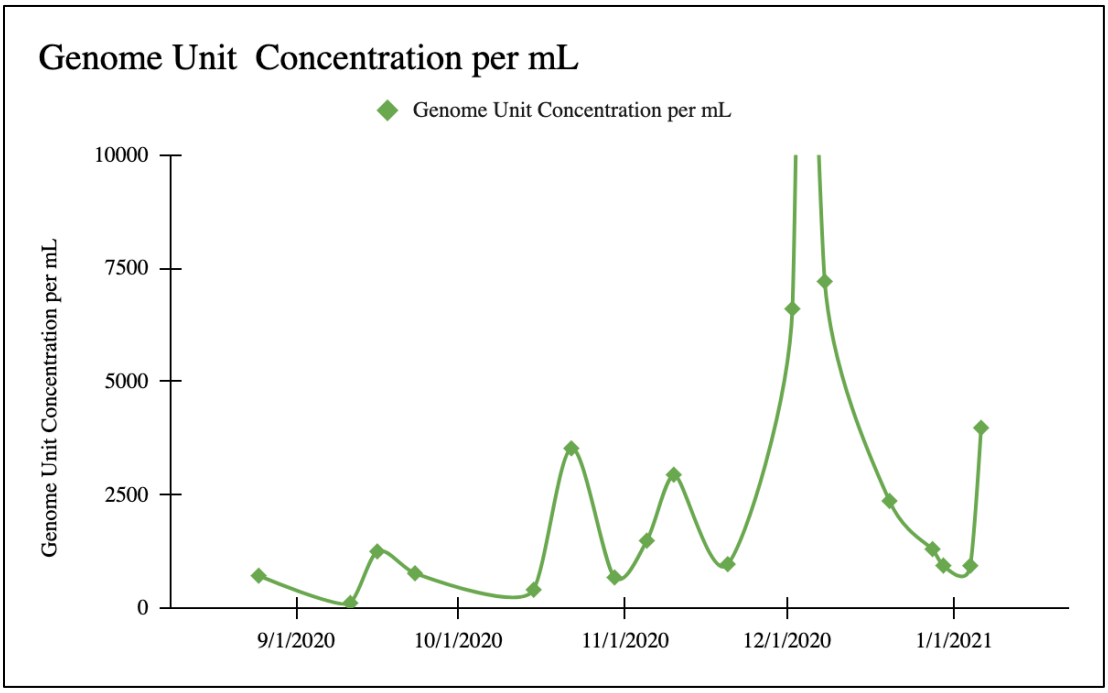

Figure A-2: Average of SARS-CoV-2 viral genome units/ml of wastewater collected from the city of Ruston versus the sampling period. SARS-CoV-2 viral genome units per ml is calculated and plotted against the sampling period for N1 gene.

Abundance of SARS-CoV-2 RNA in wastewater from the city of Ruston correlated with higher COVID-19 caseloads (Figure A-3). Time periods with higher COVID-19 caseloads tended to have higher SARS-CoV-2 in wastewater a week prior to the rise in number of positive cases. Specifically, the reporting period of early December

had over 60 confirmed cases per day and correlated with higher viral concentration as students returned for Winter quarter after Thanksgiving break.

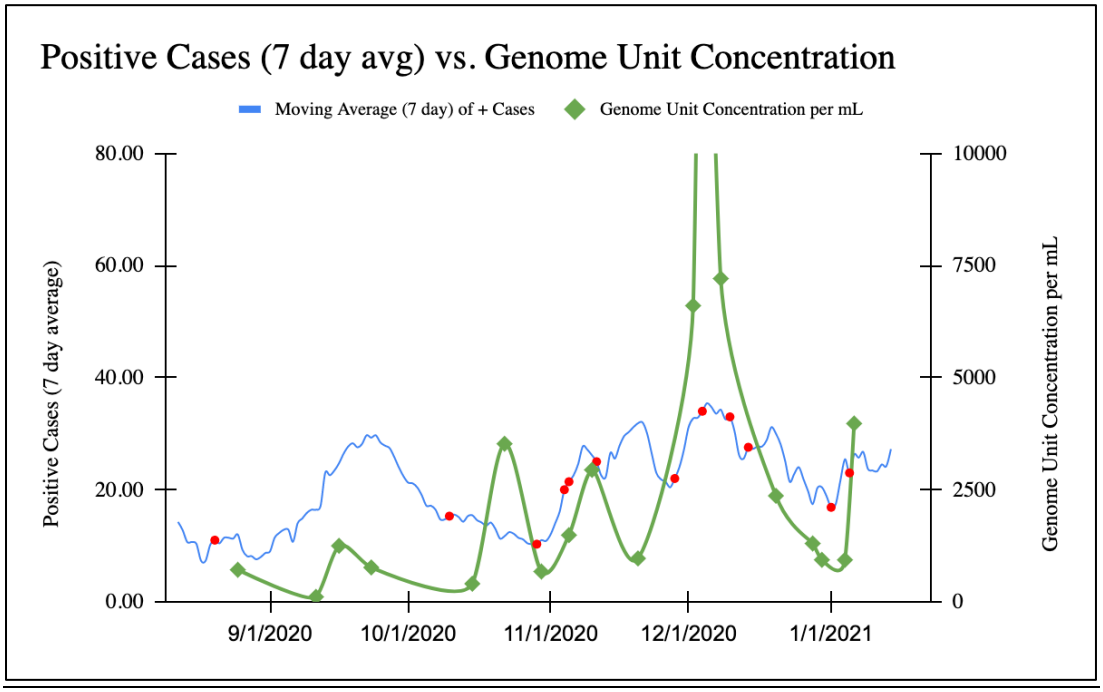

Figure A-3: Correlation between SARS-CoV-2 genome concentration/ml of wastewater and weekly average of positive COVID-19 cases. Red dots indicate any major events at the University (LATECH) such as start date of fall classes, Halloween, football games, quarter breaks etc.

Distinct trends in viral RNA abundance versus case counts were observed throughout the testing period. First, SARS-CoV-2 wastewater loads in August and September were lower compared to actual cases reported. Second, the spike in wastewater viral loads during last week of October (Halloween) is followed by increase in weekly COVID-19 cases from less than 10 to more than 25 per day. These results suggest that the increase in case counts may occur concurrently with or even precede the increase in SARS-CoV-2 RNA in wastewater. This variation in RNA in wastewater versus case counts has been suggested by many other studies. Further, this long-term shedding of the SARS-CoV-2 after negative test result may account for the detection of

RNA in wastewater after the decline in cases. However, additional research would be needed to confirm these observations during outbreaks.

A.4 Discussion

The newly emerged SARS-CoV-2 coronavirus causing COVID-19 developed into a pandemic and is still threatening many countries with its rising infections. With an unusually high number of cases and a limited number of testing facilities available, researchers sought wastewater testing as a reliable tool for monitoring the need to activate further clinical testing or other interventions in a particular area. This may be especially useful in university areas where there is varied influx and outflux of students that make up most of the community. However, there are several concerns with using this analysis with false negative/positive signals from a few infected signals among potentially large number of individual populations. Therefore, caution must be used in designing samples for smaller systems to avoid providing false results that are applicable in a critical public health setting.

In Ruston, LA, weekly sampling of wastewater provided community level surveillance and identification of emerging outbreaks to help maximize the use of targeted clinical testing. This research allowed us to identify certain time periods where the rise in viral genome concentration is followed or correlated with the number of positive cases. The city also plans to sample target smaller areas like specific buildings in the University which increases the specificity of disease prevalence status. Further, this information could support responsible vaccination and reopening local events when appropriate and provide early warnings of breakouts.

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