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AMPK Targets Involved in Energy Homeostasis and Epigenetic Regulation of Mitochondrial Function-related Genes

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ABSTRACT OF THE DISSERTATION

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Mitochondria serve to regulate energy and redox homeostasis, ultimately determining cell fate. While mitochondrial dysfunction is characteristic of many metabolic, cardiovascular and neurodegenerative diseases, AMP-activated protein kinase (AMPK), an energy and stress sensor, is known to regulate mitochondrial biogenesis and function. This work is to study a novel mechanism by which AMPK regulates both energy and redox homeostasis through of NAD^+ synthetase 1 (NADSYN) and two epigenetic modulators, DNA methyltransferase 1 (DNMT1) and retinoblastoma binding protein 7 (RBBP7). This study combines bioinformatics screening and experimental validation to discover novel substrates of AMPK that are involved in energy and mitochondrial homeostasis in the cell.

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Abbreviations

[^{32}P] γ: radio labeled gamma phosphate

ACE2: angiotensin converting enzyme 2

AICAR : 5-aminoimidazole-4-carboxymide-1-β-d-ribofuranoside

AMP : adenosine monophosphate

AMPK : ATP-activated protein kinase

ARRB1: beta-arrestin 1

ATP: adenosine triphosphate

ATM: ataxia telangiectasia mutated

Bcl-6: B-Cell CCL/Lymphoma 6

CALNX: calnexin

CaMKKβ: calcium/calmodulin dependent protein kinase kinase beta

Cav-1: caveolin 1

CBP: creb binding protein

CDK: cyclin-dependent kinase

CHMP1b: charged multivesicular body protein 1B

ChIP: chromatin immunoprecipitation

CPSF2: cleavage and polyadenylation specific factor 3

CSA: E3 ubiquitin-protein ligase complex

DDX56: DEAD (Asp-Glu-Ala-Asp) box helicase 56

DHSR9: dehydrogenase/reductase SDR family member 9

DNA: deoxyribonucleic acid

DNMT1: DNA methyltransferase 1

EDTA: ethylenediaminetetraacetic acid

EGTA: ethylene glycol tetraacetic acid

ER: endoplasmic reticulum

ERCC: excision repair cross-complementing rodent repair deficiency, complementation group 8

ERK: extracellular signal-regulated kinase

FK: FK866 hydrochloride

GLUT4: glucose transporter 4

GPCR: G-protein coupled receptor

FAD: flavin adenine dinucleotide

FoxO3a: forkhead binding protein O3a

eNOS : endothelial nitric oxide synthase

H_2O_2: hydrogen peroxide

HAT: histone acetyltransferase

HDAC5: histone deacetylase 5

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

H2B: histone 2B

HMGA1: high-mobility group protein HMG-I/HMG-Y

HSP: heath shock protein

HUVEC: human umbilical vein endothelial cell

IkBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

INSR: insulin receptor

KLF2: krüppel like factor 2

LPA: lysophosphatidic acid

LKB1: liver kinase B 1

MAPK: mitogen activated kinase

MEF: mouse embryonic fibroblast

mitoKATP: mitochondrial ATP-dependent potassium channel

MnSOD : manganese superoxide dismutase

mRNA : messenger ribonucleic acid

mtDNA: mitochondrial DNA

NAD^+ : nicotinamide adenine dinucleotide

NaAD: nicotinamide

NADPH : nicotinamide adenine dinucleotide phosphate

NADSYN1: NAD^+ synthetase 1

NAMPT: nicotinamide phosphoribosyltransferase

NFκB : nuclear factor kappa B

NO : nitric oxide

Nox: NADPH oxidase

NRF: nuclear respiratory factor

NUCKS: nuclear ubiquitous casein and cyclin-dependent kinase substrate 1

p21CIP: cyclin-dependent kinase inhibitor 1

PAR: poly [ADP-ribose]

PARP-1: poly [ADP-ribose] polymerase 1

PCG: polycomb-group

PGC-1α : peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PKC: protein kinase C

PMSF: phenylmethanesulfonylfluoride

PP2A: protein phosphatase 2A

qPCR: quantitative polymerase chain reaction

RA: retinoic acid

RAR: retinoic acid receptor

RBBP7: retinoblastoma binding protein 7

ROS : reactive oxygen species

SERCA: sarco endoplasmic reticulum calcium ATPase

siRNA : silencing RNA

SEM: standard error of the mean

SIRT1: sirtuin 1

SMC: smooth muscle cell

SOD: superoxide dismutase

TCOF: Treacher Collins-Franceschettie syndrome 1(Treacher)

tfam: transcription factor A

TOP: 5'-terminal oligopyrimidine

UBF: upstream binding factor

UCP: uncoupler protein

ULK1: UNC-51-like kinase 1

UPR: unfolded protein response

UV: ultraviolet

XAB2: pre-mRNA-splicing factor SYF1

ZRF1: DnaJ homolog subfamily C member 2

Chapter 1

Introduction

1.1 Mitochondrial Function and Metabolic Disease

Metabolic syndrome has become an epidemic in the US. According to the National Health Statistics Report 2009, approximately 34% (47 million) adult and 9.2% pediatric Americans meet the criteria for metabolic syndrome. [1, 2] Metabolic syndrome is a systemic response to alteration in metabolic function that presents with a combination of three or more symptoms such as hypertension, hyperglycemia/glucose intolerance, hyperinsulinemia, insulin insensitivity, hyperlipidemia, non-alcoholic liver disease, and typically increased hypertrophied visceral adipose tissue. [3, 4] Although metabolic syndrome is in part due to over nutrition and lack of physical activity, the underlying causes are complex and include a spectrum of physiological alterations that involves interplay between skeletal muscle, adipose tissue, and their coordinated regulation. Foundational to the systemic pathology of metabolic syndrome is the dysregulation of mitochondrial functions at the molecular and cellular levels. [5]

Mitochondria mediate a myriad of functions such as proliferation, apoptosis, synthesis of heme and steroids, calcium signaling, and redox homeostasis. [6-11] Mitochondria are the major source of ATP production and fatty acid oxidation. [6] In the context of metabolic syndrome, however, mitochondrial dysfunction manifests as an increase in oxidative stress, or excessive production of reactive oxygen species (ROS),

and reduced ATP synthesis. [12] Mitochondrial oxidative phosphorylation is a major source of ROS within the cell, which generates unpaired electrons in the form of reduced molecular oxygen ($O_2^{\bullet -}$, superoxide). [11] Although ROS act as important signaling molecules in many biochemical pathways, supraphysiological levels of ROS result in DNA damage and inflammation. [5, 13] Therefore, it is essential that the level of mitochondrial ROS production be stringently regulated by a concerted effort between several intermediates and enzymes including magnesium superoxide dismutase (MnSOD), glutathione, glutathione peroxidase, glutathione reductase, catalase, and thioredoxin; all of which are present in the mitochondrial matrix and convert superoxide to hydrogen peroxide (H_2O_2). [14-19] Other major sources of ROS in the cell are superoxide and H_2O_2 generated by NADPH oxidases (Nox) in the cytosol, which catalyze the electron transfer from NADPH to molecular oxygen. Interestingly, there are reports that Nox4 plays a role in increasing mitochondrial ROS. [19-21] This may be due to an increase in mitochondrial sensitivity to redox balance through regulation of thioredoxin 2, protein kinase C epsilon isoform (PKCε), and ATP-dependent potassium channel (mitoKATP). [22]

At the organ level, disruption of these mitochondrial redox signaling networks, due to imbalanced energy storage and expenditure characteristic of metabolic syndrome, leads to increased ROS with concomitant reduced ATP demand. [23-26] Skeletal muscle biopsies taken from patients with insulin insensitivity, a key feature of metabolic syndrome, have altered expression of mitochondrial genes, particularly those involved in oxidative phosphorylation. [27] However, this alteration is reversed with adequate

physical activity and restriction of refined, high glycemic caloric intake. [28-29] Exercise increases mitochondrial enzyme activity and expression, ultimately improving oxidative capacity, regeneration of ATP aerobic metabolism, and sensitizing cells to insulin-regulated glucose utilization. [29] Importantly, adenosine monophosphate (AMP)-activated protein kinase (AMPK), a key mediator in mitochondrial health, is activated during exercise and caloric restriction. [30-31] It may be that the improvement of the metabolic syndrome phenotype by exercise and caloric restriction is in part due to AMPK's regulation of mitochondrial biogenesis membrane potential.

1.2 AMPK and Mitochondrial Biogenesis and Function

AMPK regulates energy homeostasis by phosphorylating key enzymes involved in metabolic pathways. AMPK is a heterotrimeric protein that consists of α-, β-, and γ-subunit. The heterotrimeric composition of AMPK is complicated by two α, two β, and three γ isoforms that create 12 possible combinations of AMPK heterotrimers. [32] Each AMPK heterotrimer maintains unique but overlapping functions in the cell. Further, there are distinct cellular locations of the α_1- α_2-subunits upon activation. [32] The α_1-subunit is currently believed to function primarily in the cytosol; while the α_2-subunit translocates to the nucleus. It has also been reported that different isoforms of the β-subunit play differential roles in nuclear translocation, AMPK stability, and AMPK activity. [32] AMPK is activated by elevated level of intracellular AMP, which binds to the γ-subunit of AMPK initiating its allosteric activation. [32-34] This conformational change leads to the phosphorylation of Thr172 in the α-subunit by an AMPKK, namely liver kinase B1 (LKB1) or Ca^{2+}/calmodulin-activated protein kinase kinase (CaMKKβ),

while protecting it from dephosphorylation by the phosphatase PP2A. [35-37] AMPK can also be pharmacologically activated by (5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), metformin, and statins. [38-39]

The general characterization of AMPK activation is that it downregulates anabolic pathways while upregulating catabolic pathways to replenish cellular energy. [33-34] This function results in increased NADH and $FADH_2$ production, which increases the demand of mitochondrial oxidative phosphorylation resulting in ATP production. However, there is emerging evidence that the role of AMPK may be more accurately described as a stress responder that balances particular anabolic and catabolic pathways to mitigate cellular and organismal stress for cell survival. For example, AMPK induces anti-inflammatory pathways, facilitates angiogenesis and cell growth, and regulates redox status of the cell, all of which require protein synthesis and degradation as well as energy production and utilization. [40-45] Additionally, AMPK increases mitochondrial biogenesis and function, which not only meets the increased demand from the augmentation of catabolic pathway but also results in decreased ROS production. [46]

AICAR increases exercise endurance in mice in part by increasing the expression of genes necessary for mitochondrial biogenesis and function. [47] Currently, the main accepted mechanism by which AMPK increases mitochondrial biogenesis is through direct phosphorylation of peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α). [47-48] AMPK phosphorylation of PGC-1α also primes it for deacetylation and further regulation by the NAD^+-dependent deacytelase, sirtuin 1 (SIRT1). [49] AMPK also increases NAD^+ concentration to increase SIRT1 activity. However, the underlying

mechanism is unclear. [49] Moreover, AMPK plays a direct role in maintaining a healthy mitochondrial population through mitophagy, a form of autophagy in which dysfunctional mitochondria are degraded, by phosphorylating and activating UNC-51-like kinase 1 (ULK1). [50] This process not only reduces aberrant ROS production by dysfunctional mitochondrial but also ensures effective ATP production.

PGC-1α is considered a 'master regulator" of mitochondrial biogenesis because it functions as a co-activator to increase the action of many transcription factors for nuclear-encoded mitochondrial genes, including PGC-1α. [51-52] All regulatory factors of mitochondrial protein expression are nuclear genes, and only about 10% of genes coding for mitochondrial proteins are encoded in the mitochondrial genome. [53-56] Further, nuclear located mitochondria genes can influence the expression of proteins located in the mitochondria. For example, PGC-1α associates with nuclear respiratory factor 1 and 2 (NRF-1, NRF-2) to increase their transcriptional activity on *cytochrome C oxidase* and other mitochondrial proteins in the nucleus. [54] The NRFs also increase the expression of mitochondrial transcription factor A (Tfam) in the nucleus. Tfam then translocates to mitochondria where it binds to mtDNA at both the heavy- and light-strand promoters to initiate the transcription of genes located in the mitochondria. [54] Significant to this project is the study of AMPK's role in mitochondrial biogenesis and membrane potential.

1.3 Epigenetics and Metabolic Disease

Epigenetics is defined as an inheritable or acquired pattern of chromatin structure that causes the temporal and spatial modulation of chromatin structure plasticity and

recruitment or inhibition of transcriptional regulators upon intra and extra cellular stimuli. [57-58] The constituents of the epigenetic code include a host of chromatin modifications such as DNA methylation and histone posttranslational modifications (methylation, ubiquitination, acetylation, ADP-ribosolyation, phosphorylation, etc.) that produce an "epigenetic" chromatin mark that may be replicated during mitosis and meiosis. [59-63]

Given that gene expression is modulated by circadian-, nutritional-, and environmental- cues, epigenetic alterations are, for the most part, dynamic and reversible. Thus, there is a strong link between nutritional status and metabolic disorders, from intrauterine life through adulthood. [64-67] For instance, metabolism of amino acids (glycine, histidine, and methionine) and vitamins (B6, B12, and folate) provides methyl donors for DNA and protein methylation. [68] The bioavailability of necessary factors and environmental cues results in a fine tuning of epigenetic networks, leading to altered gene expression and the corresponding phenotypic changes. These alterations can be permanent due to irreversible processes of epigenetic imprinting during differentiation and organogenesis. [69] Often, this occurs with untargeted stochastic DNA methylation errors, associated with hyper- or hypophysiological levels of S-adenosylmethionine, the main methyl group donor. [64] An example of nutritional imbalance resulting in metabolic imprinting can be demonstrated in pregnant and neonatal rats fed a high-carbohydrate diet. [70] Pregnant rats fed a high-carbohydrate diet not only developed hyperinsulinemia, but they pass on the hyperinsulinemia phenotype to their offspring. A similar inheritable example is observed in humans, particularly

with type 2 diabetes and metabolic syndrome. [71] These phenotypes can be attributed to aberrant methylation patterns in promoters of genes important for insulin signaling. [71] Epigenetic shifts comprehensively alter metabolic gene expression profiles that result in metabolic syndrome phenotypes. Epidemiological studies have demonstrated that environmental factors and increased nutrient uptake may produce the inheritable dysregulation of gene expression. [65-71] Since mitochondrial dysfunction is a major contributor in metabolic syndrome, epigenetic regulation of genes involved in mitochondrial biogenesis and function is an important topic of study. It is well established that skeletal muscle is sensitive to external perturbations such as glycemic load and the level of muscle use, which ultimately determines mitochondrial health. [27, 73] It is also well known that endurance exercise and caloric restriction improve mitochondrial biogenesis and function. [28-29] Calorie restriction, defined as restriction of daily food intake by 30-–40%, increases the mean lifespan in a variety of species, but the mechanisms remain elusive. During calorie restriction, there is an acute increase in ROS production, which serves to decrease oxidative stress by activating redox-sensitive genes, resulting in upregulation of the antioxidant system. [74] On the other hand, chronic endurance training increases the activity of antioxidant enzymes (i.e. SODs) and heat shock proteins (i.e. Hsp72), reducing basal H_2O_2 production, and decreasing overall oxidative damage upon systemic perturbations or stress. [29,75]

1.4 AMPK and Epigenetic Modulation

AMPK is activated during exercise and caloric restriction. [30, 31] Thus, AMPK downregulation may be a causal factor in metabolic syndrome consequence of low physical activity and high caloric load. Several studies have alluded to AMPK's potential as a therapeutic target for the treatment of metabolic syndrome. [76] AMPK's role in transcription has been primarily studied at the level of posttranscriptional modification of transcription factors. For example, AMPK phosphorylates the transcription factor p53 to induce cell-growth arrest and/or apoptosis through the transcriptional regulation of p53 response genes such as cyclin-dependent kinase inhibitor 1 (p21CIP). [77-78] However, recent reports have indicated that AMPK functions as an epigenetic regulator through direct chromatin association and modification. Bungard et al., demonstrated that AMPK can phosphorylate histone H2B near the promoter region of the *p21* gene facilitating transcription. [79] Histone deacetylase 5 (HDAC5) reduces the acetylation status of histone to inhibit transcription. AMPK activation has been shown to phosphorylate and inhibit HDAC5 activity at the promoter region of the *GLUT*4 gene in vitro, increasing mRNA expression. [80] This epigenetic mechanism may also occur in humans during exercise. [81] In addition, our previous work demonstrated that AMPK can phosphorylate and activate poly [ADP-ribose] polymerase 1 (PARP-1). [41] Unpublished supporting data shows that AMPK's activation of PARP-1 resulted in histone Poly-ADP-ribosylation (PARylation) to facilitate gene transcription. This knowledge led to the hypothesis that AMPK is important, not only for transcription factor regulation, but also for posttranslational modification of

epigenetic factors involved in chromatin relaxation at the promoters of genes AMPK regulates, which is the focused topic in Chapter 3.

1.5 Bioinformatics and AMPK

Although a few reports have shown the regulation of epigenetic players by AMPK, the scope that AMPK plays at regulating chromatin remodeling is not well defined. To explore how AMPK mediates cellular responses following stress cues, we conducted a bioinformatic search using the accepted consensus motif recognized by AMPK as a phosphorylation site, **βφβ**XXX**S/T**XXX**φ**, where φ refers to a hydrophobic residue (M, L, I, F, or V) and β to a basic residue (R, K, or H).[82, 83] Using an R script, this consensus motif was mapped to human and mouse proteomes imported from ENSEMBL proteome database. The R Project uses a specific language to create an environment for a wide variety of statistical and graphical techniques. For example, it can be used for sequence alignment, clustering and mapping. It is free software (http://www.r-project.org/) that runs on a variety of systems such as UNIX, Windows, or MacOS. [84] The ENSEMBL project is a joint effort between the European Bioinformatics Institute and the Wellcome Trust Sanger Institute. The Ensemble data base is created by pattern-matching of protein to DNA. This is accomplished by sequencing data into a software pipeline that generates a set of predicted locations, which are saved in a database for future analysis (www.ensembl.org).

A Scansite search was also performed to compare with the ENSEMBL search. However, the consensus sequence used for this query is less stringent as it did not specify which amino acids would be in the hydrophobic or basic positions. Scansite

detects short protein motifs, represented as a position–specific scoring matrix, which may be recognized by serine/threonine or tyrosine kinases. Scansite incorporates identification of putatively interacting enzymes and links to their functions. It also predicts a measure of surface accessibility, and produces a ranking of candidate motifs that reflects the strength of their prediction. However, Scansite can also be used to match a particular sequence to a proteome databases such as SWISS-PROT, ENSEMBL, TrEMBL, and Genpept. [85] SWISS-PROT was used for comparison. The SWISS-PROT is a manually curetted protein knowledgebase that connects amino acid sequences with the current knowledge in the Biological (Life) Sciences (http://www.expasy.org/sprot/ and http://www.ebi.ac.uk/swissprot/). The data for each protein entry consists of an interdisciplinary effort to provide relevant information regarding protein function, domain structure, post-translational modification, and much more. [86]

The generated data bases were integrated into the Java open-source software system Gaggle to predict and display pathways involving AMPK. Gaggle enables the integration of many different data sources and bioinformatic programs. Standard programs and data bases integrated into Gaggle include KEGG, BioCyc, String Cytoscape, DataMAtrixViewer, R statistical environment, and TIGR Microarray Expression Viewer. However, integration into Gaggle can be expanded by utilizing "plug-ins", which can be any compatible software or data base (http://gaggle.systemsbiology.net). [87] Ultimately, our screening approach identified AMPK as a key mediator of a comprehensive group of networks important for total

cellular function and survival including a spectrum of epigenetic regulators, which is described in Chapter 2.

From the bioinformatics screen, it can be illustrated that AMPK is not only a regulator of energy homeostasis but a key modulator in all cellular processes. This dissertation presents a new mechanism by which AMPK's regulates energy homeostasis via increasing NAD^+ synthetase1 (NADSYN1) expression and activity to increase [NAD^+] as validation of the screening methods; and exposes AMPK as an important epigenetic regulator of mitochondrial biogenesis and function through direct phosphorylation of DNA methytransferase 1 (DNMT1) and retinoblastoma binding protein 7 (RBBP7) followed by subsequent gene transcription, which is the content in Chapter 3.

Chapter 2

AMPK is a Key Regulator in Cellular Responses to Stress: A Bioinformatic Approach

2.0 Introduction

AMPK regulation is multi-faceted and dependent upon various stimuli that trigger cellular stress. For instance, AMPK activation is facilitated by elevated intracellular AMP, which binds to the γ-subunit of AMPK allosterically promoting phosphorylation in its activation loop and protecting it from dephosphorylation. [33-34] Thus, AMPK is often characterized as an energy sensor. Although increased AMP/ATP ratio indicates decreased energy supply, it also suggests an increase in metabolic demand and deviation from homeostasis during multiple forms of cellular stresses. Calcium influx, which increases under physiologic stress, can also activate AMPK but in an AMP-independent manner by activating CaMKKβ, as occurs under pulsatile shear stress in endothelium. [37]

Once activated, AMPK propagates signaling cascades that are protective against pathological phenotypes highlighting AMPK as a target for therapeutic intervention. However, understanding how AMPK exerts its protective effects is currently speculative because its spectrum of targets and their functions are unknown. The accepted consensus phosphorylation motif recognized by AMPK is βφβXXXS/TXXXφ (hydrophobic, φ = M, L, I, F, or V; basic, β = R, K, or H). [82, 83] However, there are known AMPK substrates that offer slight variability to this consensus sequence. For example, there may be only one basic residue present or none. Also, the hydrophobic residues may be positioned ± one

amino acid. From an enzymology point of view, these deviations still allow optimal *Kcat* and *Km* for phosphate transfer catalyzed by AMPK. To explore the putative AMPK substrates at the genome-wide scale, we mapped this consensus motif to both human and mouse proteomes. Delineation of networks involving AMPK included integration of these data into the Java open-source software system, Gaggle, to predict pathways comprising the putative targets. Through this bioinformatic approach, we showed that AMPK is a central node for cellular stress response, requiring its modulation of a variety of pathways that both consume and generate energy. Assessing a cohort of these predicted novel targets to validate our screen led us to further explore downstream effects of AMPK activation on NADSYN1.

2.1 Methods

Bioinformatics Approach

Using an R script, the AMPK phosphorylation consensus motif, βφβXXXS/TXXXφ (where φ = M, L, I, F, or V and β = R, K, or H), was mapped to human and mouse proteomes imported from ENSEMBL proteome database. To find the proteins containing an AMPK consensus motif, each peptide sequence obtained from the R script mapping was individually pasted into the BLAST algorithm provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast). Using the less stringent consensus motif, in which φ and β were not specified to be a specific amino acid, we also performed a Scansite search to compare results from the ENSEMBL search to the SWISS-PROT protein data base. The generated data bases were integrated into the Java open-source software system Gaggle to predict and display pathways involving AMPK (Fig. 2.1).

Kinase Assay

AMPK kinase assays were performed in 50 mM HEPES, pH 7.4, 1 mM AMP, 1 mM (γ-^{32}P)ATP, 5 mM $MgCl_2$, 1 pM recombinant AMPK, and 1 nM recombinant substrate protein in a 50 µl reaction volume at 37 ^{o}C for 1 hr. Proteins were then resolved using SDS-PAGE, stained with Coomassie blue, and submitted to autoradiographic analysis. Peptide assays were performed under the same conditions but with 3 nM peptide, (γ-^{32}P)ATP, and 1 pM AMPK. SAMS (3 nM) peptide was used as positive control and no peptide as negative control. Peptide reactions were terminated by blotting samples onto Whatman P81 ion exchange chromatography paper and rinsed in 1% phosphoric acid. Individual filter papers were then washed in acetone and allowed to air dry. Filter papers were placed into separate scintillation vials with 1 ml scintillation fluid and measured with a Beckman LS 6500 scintillation counter. Peptides used for kinase assays: NADSY1 S641, KVKRFFSKYSMNRRR; NADSY1 S641A, KVKRFFAKYSMNRRR.

ChIP assay

Chromatin immunoprecipitation (ChIP) assays were performed using protocol by ABcam. Protein was cross linked to DNA using formaldehyde, washed and harvested in lysis buffer (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and halt protease inhibitors). The resulting cell lysates were sonicated. Protein was immunoprecipitated overnight at 4 ^{o}C with protein A conjugated sepharose beads and respective antibody. The beads were washed with wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 150 mM Nail, 20 mM Tris-

HCl, pH 8.0, and eluted in 100 µl elution buffer (1% SDS, 100 mM $NaHCO_3$). DNA was purified using Qiagen PCR purification kit prior to qPCR analysis. The following primers were used for qPCR analysis of immunoprecipitated DNA: mouse promoter NADSYN1 forward 1740F, ATTCCTTGGCTTCCTACTGC; mouse promoter NADSYN1 reverse 2054R, GTGTCTTGATAGATGGGCTACAG.

Genomic DNA isolation

Cells were lysed in 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM PMSF, and protease inhibitors. Cell lysates were extracted with phenol:chloroform (1:1) and the RNA was removed with the addition of RNase A suspended in 20 mM Tris, pH 7.4. After 1 h incubation at 37 ^oC, reactions were extracted again with phenol:chloroform and precipitated with an equal volume of isopropanol and a 1:10 volume of potassium acetate, washed three times with 75% EtOH, and re-suspended in 50 µl nuclease free H_2O.

mRNA Quantification

RNA was purified using TRIzol reagent from Life technologies. An amount of 2 ng of RNA was converted to cDNA using Promega reverse transcriptase according to the manufacturer's instructions. cDNA was then quantified via qPCR using cyber green qPCR master mix purchased from Bio-Rad. Results were calculated using the delta-delta ct method. The following primers were used for qPCR analysis: mouse NADSYN1

forward 1740F AGAGCCTTTGTCCAGTTTTG; mouse NADSYN1 reverse 2054R GTTGTCATCTTGTGCCTGTTC.

Immunobloting

Following treatment, cells were lysed in 10 mM Tris (pH 7.4), 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 1mM NaF, 20 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate (Na_3VO_4), 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X-100, 10% Glycerol, 1 mM PMSF and protease inhibitors. Cell lysates were resolved via 10% SDS-PAGE, blocked with 5% milk, rinsed with TBST, probed with respective primary and secondary antibodies. Signal was generated by Emerson chemiluminescence substrate (ECL) and image captured on HyBlot CL autoradiography film.

NADSYN1 Assay

NAD^+ concentration was measured in C2C12 cells using EnzyChromTM NAD^+/NADH assay kit (E2ND-100) and protocol from BioAssay Systems. Cells were washed with cold PBS and lysed with 100 uL NAD extraction buffer. Extracts were heated for 5 minutes at 60°C. An amount of 100 uL of the opposite extraction buffer and 20 uL of assay buffer were added to neutralize the reaction. Extracts were then vortexed and spun at 14,000 rpm for 5 minutes. Supernatants of each sample were placed in a 96-well plate along with 8 standard dilutions. Following addition of the working reagent (Assay buffer, lactate, enzyme A, enzyme B, MTT), optical density was read at 565 nm at 0 min and 15 min.

Statistical Analysis

Data are expressed as means ± SEM of at least three independent experiments. Comparisons of mean values between two groups were evaluated using a two-tailed Student's t test, Wilcoxon signed-rank test or Mann-Whitney U test. Unless otherwise indicated, * $p < 0.05$ was considered statistically significant.

2.2 Results

AMPK is a Central Node in Cellular Survival

Bioinformatics and Systems Biology

The results of mapping the most stringent consensus sequence to the ENSEMBL mouse proteome yielded 8121 protein matches, from over 7000 peptide sequences. Mapping to the ENSEMBL human proteome yielded 866 proteins, from over 9000 peptide sequences (Fig. 2.1.1A). Matching the less stringent consensus motif to the SWISS-PROT protein data base, in which φ and β were not specified to be a specific amino acid, yielded 3158 proteins from the mouse proteome and 4505 from the human proteome (Fig. 2.1.1B). These predicted AMPK targets were then integrated into Gaggle to generate predictive and known pathways or functions (Fig. 2.1.2) and represented in a Cytoscape display (Fig. 2.1.3). Each predicted target is accompanied by proteins that have high probability to associate with the respective target. Putative protein targets were then categorized by their known function (Fig. 2.2).

Novel Targets and Predicted Module Regulation

Predicted targets of AMPK representing several categorical functions were chosen for validation by kinase assays using (γ- ^{32}P)ATP and the respective, full-length recombinant proteins. The categories selected included both well-explored functions of AMPK for screen validation and unknown functions to prompt novel hypotheses. Out of 18 proteins tested for phosphorylation, the 15 proteins yielding a positive autoradiograph from phosphate transfer (Fig. 2.3, Table 1). Utilizing information gathered from gaggle and PubMed (http://www.ncbi.nlm.nih.gov/pubmed), we generated testable hypotheses of the functional outcomes produced by AMPK phosphorylation (Fig. 4).

Arrestin, Beta 1 (ARRB1)

ARRB1, commonly referred to as β-arrestin 1, regulates many signaling cascades by steric hinderance of G protein coupling of seven-transmembrane receptors (GPCRs) and acts as a scaffold for several signaling molecules, such as mitogen-activated protein kinases (MAPKs) and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα)-nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) complexes.[88, 89] ARRB1 is present in both the cytosol and nucleus implying its multicellular function.[89] Although extensive study of its nuclear function is lacking, ARRB1 has been shown to bind directly to c-Fos and p27 promoters, recruiting histone acetyltransferases (HATs), such as p300, increasing transcription.[90] AMPK regulation of ARRB1 could indeed facilitate all of these processes. AMPK has also been shown to decrease ERK1/2 and IκBα-NFκB signaling. Further, AMPK does regulate p27 function

and might therefore facilitate its transcriptional activation through modulation of its promoter chromatin acetylation state (Fig. 2.4A). [91]

Cleavage and Polyadenylation Specific Factor 2 (CPSF2)

CPSF2 is the 100 kDa subunit of CPSF complex that recognizes the AAUAAA signal sequence and interacts with factors such as the poly(A) polymerase to prompt cleavage of the template mRNA and initiation of poly(A) addition in pre-mRNA 3'-end formation. [92] This process inhibits mature mRNA degradation and activates translation. [92] The regulation of CPSF2 by AMPK is likely dependent upon the stimuli. In the presence of stress, AMPK phosphorylation may increase CPSF2 association with the CPSF complex, directing CPSF's recognition of the AAUAAA sequence increasing translation of genes necessary for homeostatic balance. One the other hand, AMPK could of course inhibit this process in genes that encourage excessive energy expenditure causing their degradation (Fig. 2.4B).

DEAD (Asp-Glu-Ala-Asp) Box Helicase 56 (DDX56)

DDX56, also known as nucleolar helicase of 61kDa (NOH61), is a putative RNA helicase implicated in a number of cellular processes involving alteration of RNA secondary structure. DDX56 is a constituent of free nucleoplasmic 65S preribosomal particles and is thought to be necessary for ribosome synthesis at the level of large (60S) ribosomal subunit assembly. [93] Transcriptional inhibitors, such as actinomycin D, cause complete dissociation of DDX56 from nucleolar components. [93] Exercise-induced AMPK activation is accompanied by decreased translation of 5'-terminal oligopyrimidine (TOP)

genes. [94] Importantly, all vertebrate genes encoding for the ribosomal proteins, as well as some other genes important for translation, are TOP genes. [95] The mechanism through which exercise-induced AMPK activation acts to represses translation of mRNAs encoding ribosomal proteins is unclear but is independent of ribosomal protein S6 kinase beta-1 (S6K1) or ribosomal protein S6 (rpS6) phosphorylation, both of which are known to regulate translation. [94] It may be that AMPK decreases the translation of TOP genes through phosphorylation and inhibition of DDX56 function in the nucleolus (Fig. 2.4C).

Excision Repair Cross-complementing Rodent Repair Deficiency, Complementation Group 8 (ERCC8)

ERCC 8 is the substrate-recognition component of the E3 ubiquitin-protein ligase complex (CSA complex), involved in transcription-coupled nucleotide excision repair. [96] Upon UV stimulation, it promotes the ubiquitination and proteasomal degradation of ERCC6, which is necessary for continued RNA synthesis post transcription-coupled repair of damaged DNA. The CSA complex also associates with a variety of proteins, such as pre-mRNA-splicing factor SYF1 (XAB2), involved in removing RNA polymerase II-blocking lesions from the transcribed strand of active genes. [97] AMPK has been strongly implicated in DNA repair and cell proliferation upon UV radiation. [98] For example, AMPK regulates insulin receptor ionizing radiation-induced G2-M checkpoint through the ataxia telangiectasia mutated (ATM)-AMPK-p53/p21cip1 signaling pathway. [99] We have shown that AMPK positively regulates the DNA repair enzyme PARP-1. [41] ERCC 8

regulation by AMPK may prove to be the next novel mechanism by which AMPK facilitates DNA repair through assembly and activation of the CSA complex (Fig. 2.4D).

Treacle (TCOF1)

Treacle is a nucleolar protein involved in ribosomal gene transcription through its interaction with upstream binding factor (UBF) and promoter selectivity factor SL1, which together promote RNA polymerase I activity. [100, 101] Treacle deficiency is accompanied by poor neural crest cell formation and proliferation due to neuroepithealial apoptosis consequence of decreased 28S ribosomal subunit of rRNA expression. [102] This ultimately results in decreased expression of genes important for structural development. AMPK's regulation of protein synthesis remains to be fully elucidated, but it is generally accepted as an inhibitor of protein synthesis. AMPKα1- and α2-null embryos are lethel~10.5 days post-conception due to poor expression of genes important for growth and survival. [103] If AMPK does indeed regulate the treacle protein, it may do so to prevent aberrant protein synthesis allowing normal embryonic development (Fig. 2.4E).

Calnexin (CALNX)

The type I integral endoplasmic reticulum (ER) transmembrane chaperone, CALNX, transiently binds to newly synthesized glycoproteins to assist in protein folding and transport. [104] CALNX is protective against ER stress, hallmarked by high concentration of unfolded proteins in the ER. [105] It is a key component of the unfolded protein response (UPR) or ER stress response, which increases protein folding/transport chaperones and decreases protein synthesis. [106] Upon phosphorylation of its cytoplasmic

domain, CALNX also regulates sarco endoplasmic reticulum calcium ATPase (SERCA) 2b, controlling Ca^{2+} signaling and Ca^{2+}-sensitive chaperone functions in the ER. [107] Inhibition of SERCA and subsequent Ca^{2+} efflux is known to causes ER stress. [108] Importantly, AMPK functions to decrease SERCA oxidation, maintaining its activity and intracellular Ca^{2+} homeostasis. [109] However, the direct mechanism is unknown. AMPK's regulation of Ca^{2+} and SERCA activity may be through phosphorylation of CALNX facilitating a protective interaction that inhibits oxidation and maintains Ca^{2+} efflux. (Fig. 2.4F).

High-mobility Group Protein HMG-I/HMG-Y (HMGA1)

HMGA1 is a transcriptional regulator that preferentially binds to the minor groove of A/T-rich regions in double-stranded DNA to form a transcriptionally active multiprotein-DNA complex that includes HMGA1, transcription factor Sp1, and the CCAAT enhancer binding protein beta (C/EBPb). [110] The insulin receptor (INSR) promoter is activated by this mechanism. HMGA may also potentiate recruitment of transcriptional complexes at the solute carrier family 2, facilitated glucose transporter member 3 (SLC2A3/Glut3) promoter but through calveolin 1 (Cav-1) signaling. Expression of Cav-1 increases glucose uptake and ATP production by stimulating transcription of SLC2A3/GLUT3 via an HMGA1-binding site within the promoter. [111] Depletion of Cav-1 decreases glucose uptake, intracellular ATP level and lactate accumulation, and triggers autophagy through activation of AMPK-TP53/p53 signaling. [111] Importantly, Cav-1 expression is necessary for metformin induction of AMP binding to AMPK and activation. [112] This knowledge encourages the investigation of Cav-

1/AMPK/HMGA1 signaling pathway in the transcriptional regulation of genes important for insulin signaling and glucose transport (Fig. 2.4G).

Dehydrogenase/Reductase SDR Family Mmember 9 (DHRS9)

DHRS9 is among a class of enzymes that oxidize retinaldehyde to retinoic acid. [113] Retinoic acid binds to the retinoic acid receptor initiating the induction of ATP-binding cassette transporter A1 and G1 (ABCA1, ABCG1) both of which are cholesterol efflux transporters. [114-116] AMPK has emerged as a key regulator of endothelial homeostasis by preventing cholesterol deposition in the vascular wall. [117] Therefore, it is likely that the phosphorylation of DHRS9 by AMPK facilitates it activation, increasing production of retinoic acid, triggering cholesterol transport from the vascular wall (Fig. 2.4H).

Charged Multivesicular Body Protein 1B (CHMP1b)

CHMP1b has been implicated in the formation of vesicle-filled endosomes (multivesicular bodies) and trafficking that targets proteins to the interior of lysosomes. [118] CHMP1b also plays a role in gene regulation through chromatin structure maintenance. It associates with nuclease-resistant, condensed chromatin and the polycomb-group (PcG) proteins, which are required for maintenance of gene silencing during development. CHMP1b induction causes cell-cycle arrest and increases S-phase cell number. [119] However, CHMP1b also forms a shell around chromatin that frequently has histone H3 phosphorylation and acetylation, which is associated with transcriptional activity. [120] The regulation of CHMP by AMPK may play a role in forming a demarcation between active

and inactive chromatin domains and therefore determining the heritable epigenetic marks (Fig. 2.4I).

Nuclear Ubiquitous Casein and Cyclin-Dependent Kinase Substrate 1 (NUCKS1)

NUCKS has been shown to play a functional role in cell cycle as a substrate for casein kinase and cyclin-dependent kinase (CDK). [121] Upon mitosis, NUCKS translocates from the nucleus to the cytoplasm. [121] Aberrant overexpression of NUCKS is correlated with breast carcinomas. [122] Importantly, cancer is highly correlated to disrupted cellular circadian rhythm, which serves to maintain mechanisms involved in normal positive and negative transcriptional feedback loops. Both casein kinase and CDK are key regulators of cellular circadian rhythm as is AMPK. [123, 124] Although poorly characterized, NUCKS is subjected to extensive phosphorylation. Therefore, AMPK may indeed phosphorylate NUCKS to regulate circadian rhythm (Fig. 2.4J).

DnaJ Homolog Subfamily C Member 2 (ZRF1)

ZRF1 serves both cytosolic and nuclear functions. In the nucleus, it facilitates H2AK119 ubiquination resulting in chromatin remodeling and transcription activation and binds the DNA sequence: 5'-GTCAAGC-3'. [125] In the cytoplasm, it acts as a molecular chaperone facilitating protein folding of the nascent polypeptide chain as it exits the ribosome by stimulating the ATPase activity of heat shock 70 kDa protein 14 (HSPA14) chaperones. [126, 127] Because AMPK represses translation on a large scale, the phosphorylation of ZRF1 by AMPK is not only likely to result in dissociation of ZRF

from the ribosome but also lead to its nuclear accumulation to participate in epigenetic remodeling of promoters required for establishing cellular homeostasis (Fig. 2.4K).

Angiotensin Cconverting Eenzyme 2 (ACE2)

ACE 2 is an exopeptidase that catalyzes the conversion of angiotensin I to the nonapeptide angiotensin or the conversion of angiotensin II (Ang II) to angiotensin 1-7 (Ang-(1-7). [128, 129] A major consequence of Ang-(1-7) binding to its G protein-coupled receptor Mas as well as through decreased angiotensin II bioavailability, is the production of the vasodilator nitric oxide (NO), identifying the Ang-(1-7)/ACE2/Mas axis as a therapeutic target for hypertension. [130-132] Further, activation of the Ang-(1-7) Mas receptor produces anti-inflammatory effects in both asthma and arthritis. [133, 134] Although not well defined; there is a positive correlation to Ang-(1-7) and AMPK activation. [135] It is possible that AMPK phosphorylates ACE 2 to increase its cleavage of Ang II to increase NO production and inhibit inflammatory pathways (Fig. 2.4L).

Kruppel-like factor 2 (KLF2)

KLF2 is a transcription factor that regulates diverse biological processes by direct binding to DNA or association with transcription coregulators such as acetyltranferases cAMP response element binding protein (CBP) and p300. [136-138] KLF2 is highly expressed in vascular endothelial cells during embryonic development. [139] *Klf2* genetic knockout is lethal in utero due to impaired vasculogenesis secondary to defective recruitment of pericytes and vascular smooth muscle cells (SMCs). [140] However, the

mechanism by which KLF2 recruits SMC is not completely understood. Lysophosphatidic acid (LPA) is a potent lipid mediator with a wide variety of biological actions, particularly SMC cell recruitment in vasculogenesis, mediated through G protein-coupled receptors (LPA(1-6)). [141] The G(13) protein-coupled receptor, lysophosphatidic acid receptor 4 (LPA4), has proven to be essential for blood vessel formation and recruitment of SMCs and contains a KLF2 binding sequence in its promoter. [141] Although the transcriptional expression of KLF2 by AMPK activation has been largely explored, it might be that AMPK modulates KLF2 transcriptional activity to increase LPA4 expression. [142] However, AMPK phosphorylation of KLF2 may simply lead to its stability (Fig. 2.4M).

NAD^+ Synthetase 1 (NADSYN1)

NADSYN is a glutamine-dependent NAD^+-synthetase that catalyzes the final step in the biosynthesis of nicotinamide adenine dinucleotide (NAD) from nicotinic acid adenine dinucleotide (NaAD). NAD is a cofactor and precursor for a spectrum of signaling molecules in a variety of cellular processes, particularly redox reactions. [143] AMPK's regulation of NADSYN1 was chosen for further validation because NAD^+ is a necessary cofactor for many proteins regulated by or serve a parallel function to AMPK, such as PARP-1 and SIRT1 in the regulation of NF-κB and p53 (Fig. 2.4N). [144-150]

AMPK has the reputation of being an energy sensor serving as a key mediator in energy homeostasis. Cantó C *et al.* reported that AMPK activation increases cellular [NAD^+] levels independent of nicotinamide phosphoribosyltransferase (NAMPT) activity, a key enzyme in NAD^+ synthesis. [49] Our bioinformatics approach suggests a novel link

between AMPK and NADSYN1 that is also upregulated by AMPK-dependent FoxO3a nuclear translocation and perpetuated by AMPK autophosphorylation (Fig. 2.5A). Autoradiography of activated AMPK alone with (γ- ^{32}P)ATP reveals AMPK autophosphorylation on both the α and β subunits. The predicted peptides autophosphorylated in the respective subunits are also shown in Fig. 2.4B. Further, we confirmed NADSYN1 is an AMPK substrate, through peptide kinase assays using peptides containing the AMPK consensus sequence and potential phosphorylation site Ser641, as well as the full length NADSYN1(Fig. 2.3). Peptides with the wild-type sequence had comparable signal to the SAMS peptide positive control, while those containing a S641A substitution had no significant phosphorylation, indicating Ser641 is likely phosphorylated by AMPK (Fig. 2.5C).

AMPK Promotes FoxO3a Binding to the NADSYN1 Promoter and NADSYN1 Expression

AMPK is known to increase FoxO3a transcriptional activity through phosphorylation. [151] Since the NADSYN1 promoter contains a FoxO3a binding site, we investigated the possibility of increased NADSYN1 transcription and translation by AMPK and FoxO3a. [152] ChIP of FoxO3a at the NADSYN1 promoter in C2C12 cells treated with AICAR showed increased binding of FoxO3a at the NADSYN1 promoter. This interaction was significantly decreased in cells transfected with AMPK siRNA (Fig. 2.6A). Furthermore, NADSYN1 mRNA expression increased following treatment with AICAR, which was blocked with AMPK and FoxO3a siRNA as analyzed by RT-qPCR (Fig. 2.6B). Similarly, phosphorylated FoxO3a and NADSYN1 protein levels were

increased in AMPK$^{+/+}$ mouse embryonic fibroblasts (MEFs) and C2C12 cells treated with AICAR; while cells transfected with AMPK or FoxO3a siRNA or AMPK$^{-/-}$ MEFs showed no such increase (Fig. 2.6C, 2.6D).

AMPK Activation Increases NAD$^+$ through NADSYN1

To investigate AMPK activation of NADSYN1, an [NAD$^+$]/[NADH] concentration assay was performed in AMPK$^{+/+}$ or AMPK$^{-/-}$ MEFs (Fig. 2.7A) and C2C12 cells. With AICAR treatment, [NAD$^+$] bioavailability was increased in AMPK$^{+/+}$ MEFs and C2C12 cells, but not in AMPK$^{-/-}$ MEFs or C2C12 cells transfected with NADSYN1 siRNA. In addition to NADSYN1, NAMPT is an additional source of cellular NAD$^+$. [49] Treating C2C12 cells with FK688, a NAMPT inhibitor had little effect on the AICAR-induced NAD$^+$ bioavailability. Taken together, these results indicate that AMPK can activate NADSYN1 resulting in increased [NAD$^+$] independent of NAMPT (Fig. 2.7B). Further, the basis for AMPK activation of NADSYN1 is bimodal: increased expression through FoxO3a -dependent transactivation and direct phosphorylation and activation.

2.3 Discussion

To date, AMPK continues to be primarily classified as an energy sensor. However, this study presents a new paradigm for AMPK involving all aspects of cellular functions and reveals its obligatory role in total cellular stress response. The fundamental characteristic of any cellular response is a cascade of events that initiates gene expression, processing, translation, stabilization and or recycling. Our results indicate that AMPK is involved in the fine-tuning of these processes to facilitate cell cycle control, apoptosis or

cell survival, intra and extra cellular signaling, cell motility, energy and redox homeostasis and many more (Fig. 2.2, Fig. 2.3, Table 1). In particular, we identified new AMPK targets involved in various cellular functions as discussed in the following.

Epigenetic and Transcriptional Regulation

Knowledge of AMPK in epigenetic regulation is limited to its phosphorylation of H2B and HDAC5 to increase expression of genes necessary for stress response. [79, 89] We postulate that AMPK is a key mediator in epigenetic modulation through the recruitment of HATS by way of HMGA1 and KLF2, ubiquitin transferases via ZRF1, and poly (ADP) ribosylases such as PARP-1. [41, 90, 136-138, 125] Regulation of HMGA2, KLF2, ZRF1, CHMP1b, and NUCKS may, however, simply result in direct DNA interaction to increase expression of AMPK-related genes. Most interestingly, AMPK is likely to maintain the heritable epigenetic mark through regulating CHMP1b's protective replication effects at domains of both condensed and transcriptionally active chromatin. [120] Further, AMPK regulation of transcriptional complex formation, such as treacle association with UBF and SL1 is a possible mechanism by which AMPK regulates transcription to prevent aberrant embryonic development. [100-103]

Translation, Protein Synthesis, and Cytokinesis

Although AMPK does decrease protein synthesis on a large scale, augmented AMPK activity must not inhibit translation of genes necessary for function during stress response. Its regulation of CPSF2 is likely to facilitate specific mRNA processing and translation necessary for stress response. [92] On the other hand, the opposite may perhaps

be true for genes not needed for stress response. For example, AMPK's regulation of DDX56 may decrease ribosomal biogenesis through repression of TOP gene transcription as well as modulation of treacle to decrease expression of rRNA, yet promote expression of mRNA necessary for growth and survival. [102]

In addition to transcription and translation of proteins important for stress response, AMPK likely plays a role in their folding and transport. The chaperone ZRF1 increases HSPA14 ATPase chaperone activity necessary for nascent peptide folding as a whole. [126, 127] Thus, AMPK probably serves a inhibitory function in this regard. However, this mechanism many be dependent upon the peptide requiring folding. Serving as an ER transmembrane chaperone, CALNX assists not only in protein transport but also a responder in ER stress by increasing calcium efflux through SERCA2b. Both of these mechanisms wane ER stress. AMPK may in fact regulate CALNX protein transport function but possibly aids in reducing ER stress through ER calcium efflux. In addition, AMPK conceivably plays a cellular recycling role through the regulation of CHMP1b's transport of proteins to the lysosome. Moreover, AMPK initiates cytokinesis necessary for embryonic development, particularly in angiogenesis. [100] Given that both LPA signaling and KLF2 are also necessary for SMC recruitment necessary for normal angiogenesis, the AMPK-KLF2-LPA4 pathway is a novel mechanism for future study. [140, 141]

Inflammation and Redox Regulation

AMPK is known to decrease the inflammation and oxidative stress through Bcl-6 transcription and inhibition of NF-κB. [41, 146] Furthermore, AMPK may also control the inflammatory response in part through ARRB1 and Ang-(1-7) inhibition of the IκB-NF-κB complex. [90-91] Complimentarily, AMPK potentially increases ACE2 cleavage of Ang II to Ang-(1-7) and KLF2 stability, decreasing oxidative stress though eNOS activity or NO production. [131-132, 140] In addition to these anti-oxidative and anti-inflammatory effects, AMPK may engage in cholesterol transport. Cholesterol oxidation is a key instigator of the inflammatory response. Interestingly, retinoic acid induces cholesterol transport through the ABCA1 and ABCG1 transporters in macrophages and endothelium. AMPK possibly activates DHRS9 to increase retinoic acid availability for cholesterol transport mechanisms. [113, 115-116] To potentially augment this pathway, our bioinformatics screen revealed ABCA1 and ABCG1 as putative AMPK substrates, which could increase cholesterol transport. [113, 115-116]

AMPK and Homeostasis through NADSYN1

AMPK functioning as a stress responding kinase can be demonstrated by its ability to regulate NF-κB and p53 to control inflammation and oxidative stress. This stress response occurs in conjunction with SIRT1 and PARP-1, both of which increase AMPK activation. [71, 144-150] PARP-1 prompts AMP binding to AMPK priming it for phosphorylation and activation. In the meantime, SIRT1 deacetylates and activates LKB1 to subsequently phosphorylate and activate AMPK. [149, 150] NAD^+ is a common substrate of SIRT1 and PARP-1. Intuitively, AMPK must acutely increase NAD^+ to sustain this

stress response triangle (Fig. 4N). Here we provide a mechanism by which AMPK perpetuates an anti-inflammatory and anti-oxidative stress through the AMPK-PARP1-SIRT1 regulation of p53 and NF-κB (Fig. 5A). This occurs via 3 mechanisms: 1) autophosphoryation to self-modulate its response to stress as shown in Fig. 5B; 2) phosphorylation of NADSYN at Ser641 increasing its catalytic activity, as shown in Fig. 5C; and 3) modulation of FoxO3a binding to the NADSYN1 promoter increasing its expression (Fig 6A). All of these processes regulate SIRT1 and PARP-1 by mediating the substrate NAD^+ availability. While AMPK activation has been shown to increase cellular [NAD^+] levels, the underlying mechanism remains unclear. Previous studies have shown that AMPK has no profound effect on NAMPT, the other main enzyme important for NAD^+ production. [49] Here we show that AMPK's ability to increase [NAD^+] is at least in part due to its up regulation of NADSYN1 expression and activity. To correlate this pathway to the role of AMPK as an energy sensor, through phosphorylation of NADSYN, AMPK increases NAD^+ so it can continue its oxidizing role in glycolysis, the TCA cycle, and amino acid catabolism to maintain cellular energy homeostasis.

Future Work

Cellular response to stress is very dynamic and complex involving coordinated, parallel, and temporal network interplay, adjustment and adaptation. Therefore, investigation of AMPK's temporal regulation during a variety of stress responses is a topic for future study. Such investigations are required to delineate the transient, chronic, and combinatorial modulation of key networks AMPK influences. Another aspect of future work is the validation of the deduced AMPK substrates in vitro and in vivo. Their

functional consequences in terms of translational relevance in health and diseases deserve further investigations.

2.4 Figures

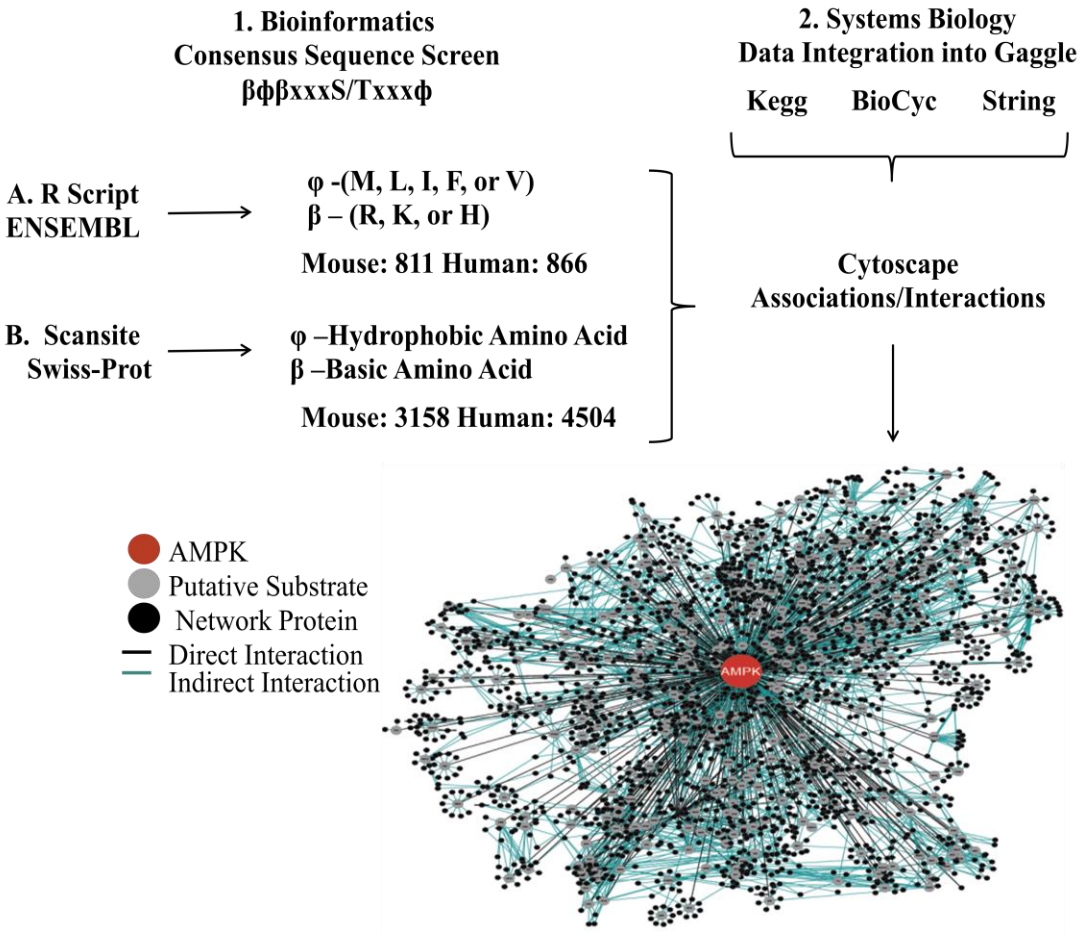

Figure 2.1. Prediction of AMPK Involvement in Total Cellular Function and Response to Stress by Bioinformatics Screening of Mouse and Human Proteomes. AMPK consensus sequence was mapped to Ensembl and Swiss-Prot proteomes (1.1). These data were integrated into Gaggle to determine categorical function of each putative substrate of AMPK (1.2). Derived network of AMPK regulation was uploaded and displayed in Cytoscape.

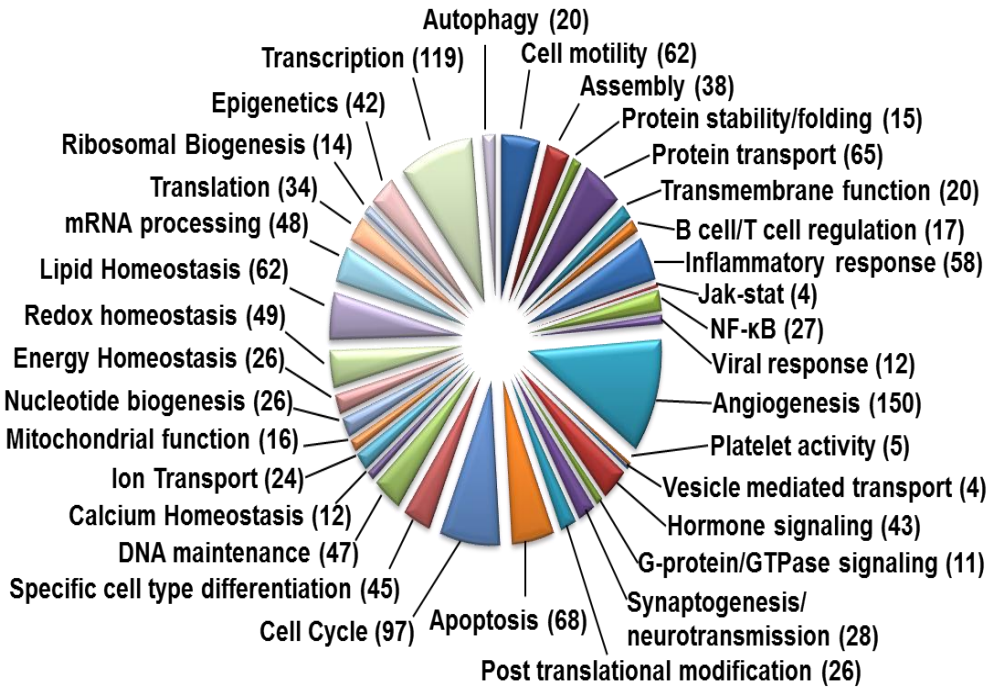

Figure 2.2 AMPK Regulation of Cellular Function. Diagram representing categorization of a large portion of proteins containing AMPK consensus sequence.

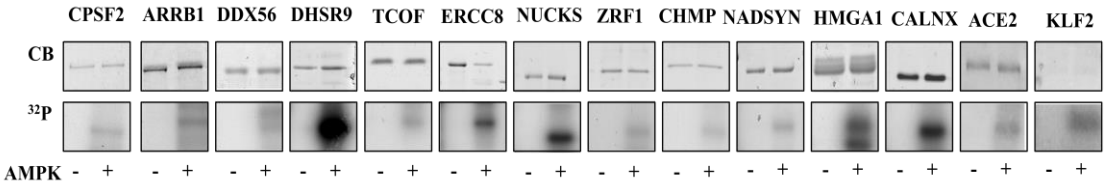

Figure 2.3. **Kinase Assay of AMPK Putative Substrates.** Autoradiography of kinase assays. Top: Coomassie blue stained SDS-PAGE gel showing protein levels of the various putative AMPK substrates. Bottom: autoradiography representing γ-^{32}P phosphate transfer. Reactions containing active AMPK and (γ-^{32}P)ATP are on the right of each substrate depiction. Reactions containing only the putative full-length substrate and (γ-^{32}P)ATP are located on the left as a negative control.

Table 2.1. Putative AMPK Targets and Predicted Phosphorylation Sequence(s) and Function

Symbol	Protein Name	Putative Phosphorylated Sequence	Functional Category
ARRB1	Arrestin, Beta 1	LGIIVSYKVKVKLVVS$_{330}$RGGLLGDLASSDVAVE	Transcription, Epigenetics, MAPK, Inflammation
CPSF2	Cleavage and polyadenylation specific factor 2	VDFNHKREIHLN-GCS$_{188}$LEMLSRPSLLITDSFN	mRNA processing , Splicing, Translation
DDX56	DEAD (Asp-Glu-Ala-Asp) box helicase 56	RKKLSSSCRKAKRAKS$_{526}$QNPLRSFKHKGKKFRP	rRNA Processing, Helicase
ERCC8	Excision repair cross-complementing rodent repair deficiency, complementation group 8	RQTGLEDPLRLRRAES$_{23}$ TRRVLGLELNKDRDVE	DNA repair, Oxidative Stress
TCOF	Treacle/TCOF1 T reacher Collins-	QEKSLGNILQAKPTSS$_{583}$PAKGPPQKAGPVAVQV	Ribosomal Biogenesis

	Franceschetti syndrome 1		
CALNX	Calnexin	DKKTHLYTLILNPDNS$_{247}$FEILVDQSVVNSGNLL VFLVILFCCSGKKQTS$_{510}$GMEYKKTDAPQPDVKE	Protein Folding and Exocytosis
HMGA1	High-mobility group protein HMG-I/HMG-Y	SEVPTPKRPRGRPKGS$_{64}$ KNKGAAKTRKTTTTPG	Transcription, Viral Replication, Chromatin Structure
NADSYN1	NAD synthetase 1	ICTPRQVADKVKRFFS$_{641}$KYSMNRHKMTTLTPAY	NAD+ synthesis
DHSR9	Dehydrogenase/reductase SDR family member 9	EGYIEKSLDKLKGNKS$_{255}$YVNMDLSPVVECMDHA	Biosynthesis of Retinoic Acid
CHMP1b	Charged multivesicular body protein 1B	AARVQTAVTMGKVTKS$_{91}$ MAGVVKSMDATLKTMN	Protein Transport
NUCKS1	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	EKKMPKPRLKATVTPS$_{181}$PVKGKGKVGRPTASKA	Mitosis, Cell Cycle
ZRF1	DnaJ homolog subfamily C member 2	MEEVEKLCDRLE-LAS$_{391}$LQCLNETLTSCTKEVG NSRWEVIANYMN-IHS$_{488}$SSGVKRTAKDVIGKAK	Chromatin Structure, Protein

			Folding, ATPase Activity
ACE2	Angiotensin converting enzyme 2	HLHAYVRAKLMNAYPS$_{254}$YISPIGCLPAHLLGDM GEIMSLSAATPKHLKS$_{420}$IGLLSPDFQEDNETEI TDWSPYADQSIKVRIS$_{623}$LKSALGDKAYEWNDNE FGEEDVRVANLKPRIS$_{680}$FNFFVTAPKNVSDIIP	Vasodilation, Converts Angiotensin I to Angiotensin 1-7
AKT2	Protein Kinase B beta	SPSDSSTTEEMEVAVS$_{141}$KARAKVTMNDFDYLKL FVMEYANGGELFFHLS$_{242}$RERVFTEERARFYGAE RFYGAEIVSALEYLHS$_{268}$RDVVYRDIKLENLMLD FTAQSITITPPDRYDS$_{458}$LGLLELDQRTHFPQFS	Metabolism, Proliferation, Cell survival, Growth, Angiogenesis
KLF2	Krüppel-like factor 2	PPAFYYPEPGAPPPYS$_{85}$ APAGGLVSELLRPELD GPRGLKREGAPGPAAS$_{158}$CMRGPGGRPPPPPDTP SPDGPARLPAPGPRAS$_{192}$FPPPFGGPGFGAPGPG GLAPPAARGLLTPPAS$_{248}$PLELLEAKPKRGRRSW	Transcription, Anti-inflammation, Redox Regulation, Vasodilation

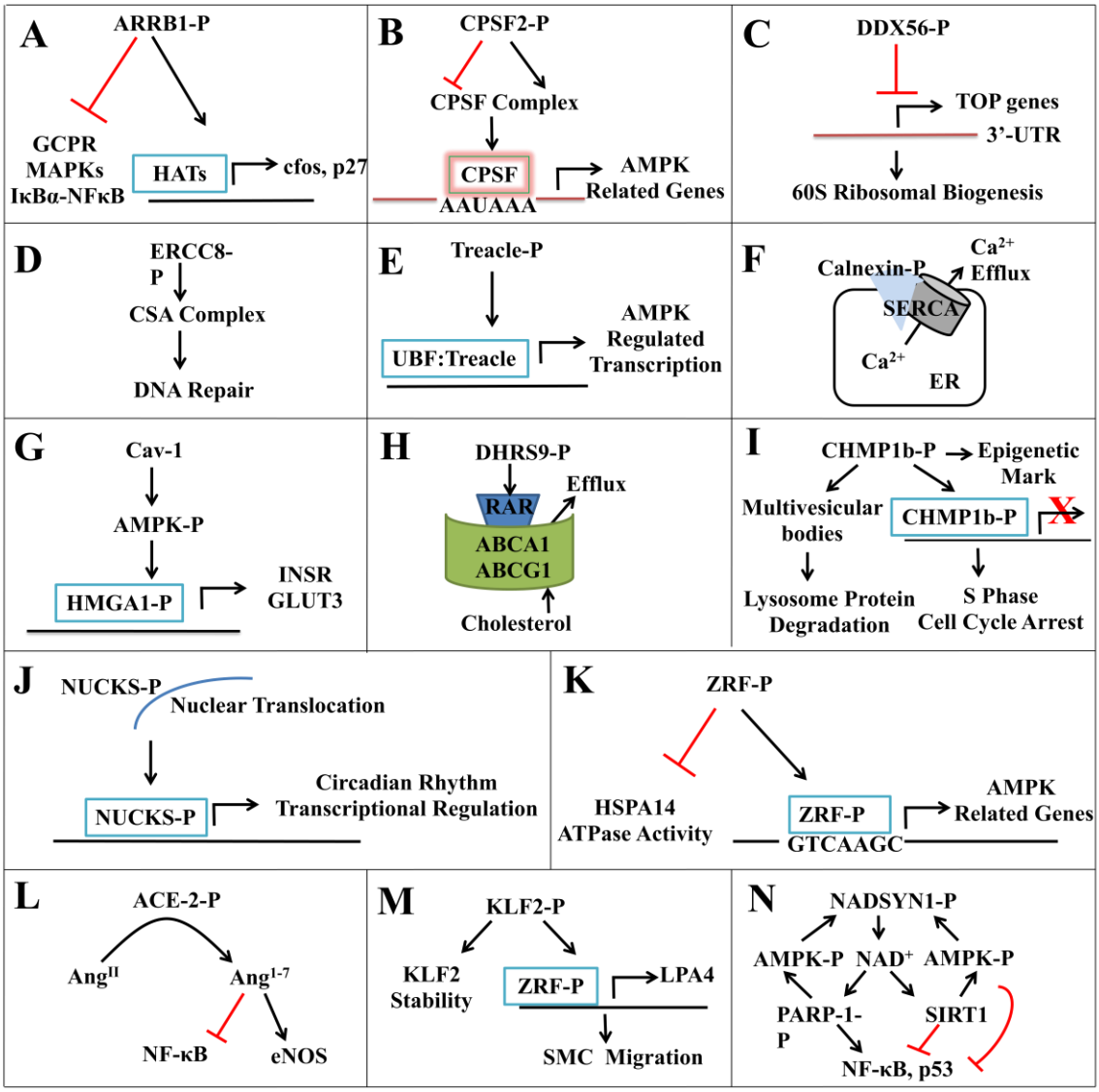

Figure 2.4. Predicted Modules of AMPK Phosphorylated Substrates and The Related Functions. (A) AMPK phosphorylation of ARRB1 inhibits GPCR signaling and increases histone acetylation activity at c-Fos and p27 promoters. (B) AMPK phosphorylation of CPSF2 increases translation of AMPK related genes. (C) AMPK phosphorylation of DDX56 TOP gene translation and subsequent 60S ribosomal

biogenesis. (D) AMPK phosphorylation of ERRC8 participates in CSA complex formation and subsequent DNA repair. (E) AMPK phosphorylation of Treacle associates with UBF to increase AMPK-related gene transcription. (F) AMPK phosphorylation of Calnexin acts a chaperone to positively regulate ER Ca^{2+} efflux through SERCA2b. (G) AMPK phosphorylation of HMGA1 increases INSR and Glut3 transcription through the Cav-1/AMPK pathway. (H) AMPK phosphorylation of DHRS9 increases cholesterol efflux through increased retinoic acid production and ABCA1- and ABCG1-mediated transporter mechanisms. (I) AMPK phosphorylation of CHMP1b facilitates lysosomal recycling of proteins and increases S1 cell cycle arrest. (J) AMPK phosphorylation of NUCKS transcriptionally regulates the circadian rhythm. (K) AMPK phosphorylation of ZRF1 inhibits ATPase activity but increases transcription of AMPK-related genes. (L) AMPK phosphorylation of ACE 2 increases its cleavage of Ang II to Ang-(1-7), resulting in decreased NF-κB activity and increased NO production. (M) AMPK phosphorylation of KLF2 increases LP4R transcription and SMC recruitment. (N) AMPK phosphorylation of NADSYN1 increases the level of NAD^+, hence promoting PARP-1, SIRT1 and AMPK regulation of NF-κB and p53.

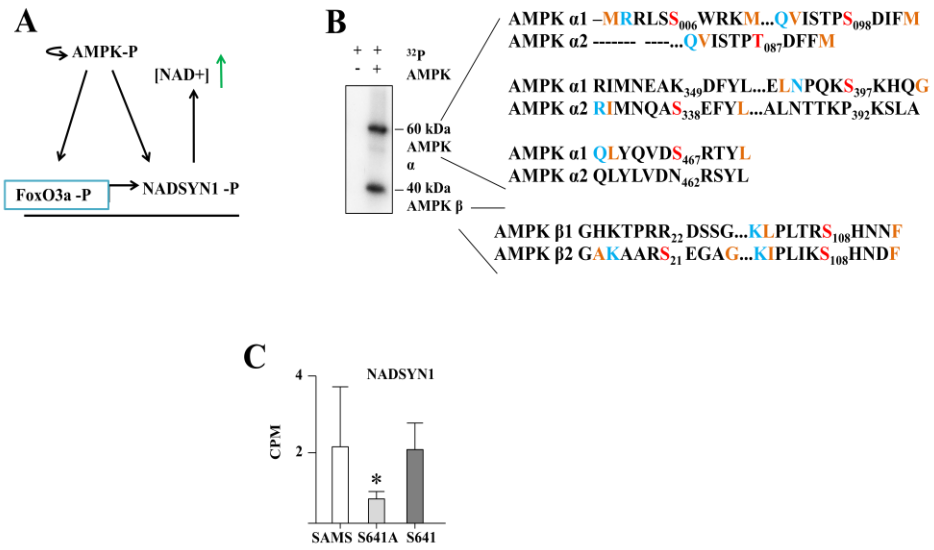

Figure 2.5. AMPK Increases Energy Bioavailability through Autophosphorylation and NADSYN1. **(**A) Graphical abstract of novel AMPK-targeted pathway. (B) Autoradiography demonstrating AMPK autophosphorylation and pile up of predicted phosphorylation sites. (C) Kinase assay using NADSYN Ser641 and Ser641A peptides. *$p < 0.05$.

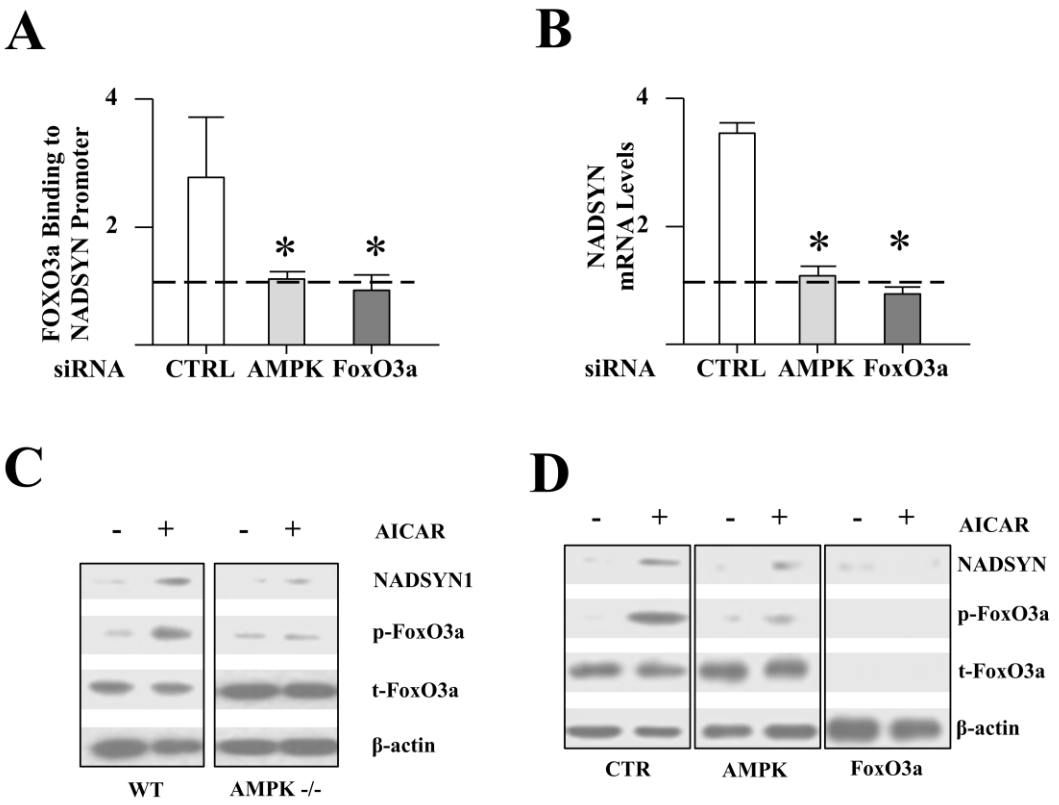

Figure 2.6. AMPK Increases NADSYN1 Expression through Phosphorylation of FoxO3a. (A) Following treatment with AICAR, ChIP assay was performed to assess FoxO3a binding to the NADSYN promoter. (B) Cells were transfected with control, AMPK, or FoxO3a siRNA and then AICAR. NADSYN1 mRNA levels were assessed by qPCR. (C) Immunoblotting of $AMPK^{+/+}$ and $AMPK^{-/-}$ MEFs lysates treated with AICAR or left untreated. (D) Immunoblotting of lysates from cells transfected with control, AMPK, or FoxO3a siRNA then treated with AICAR. *$p < 0.05$.

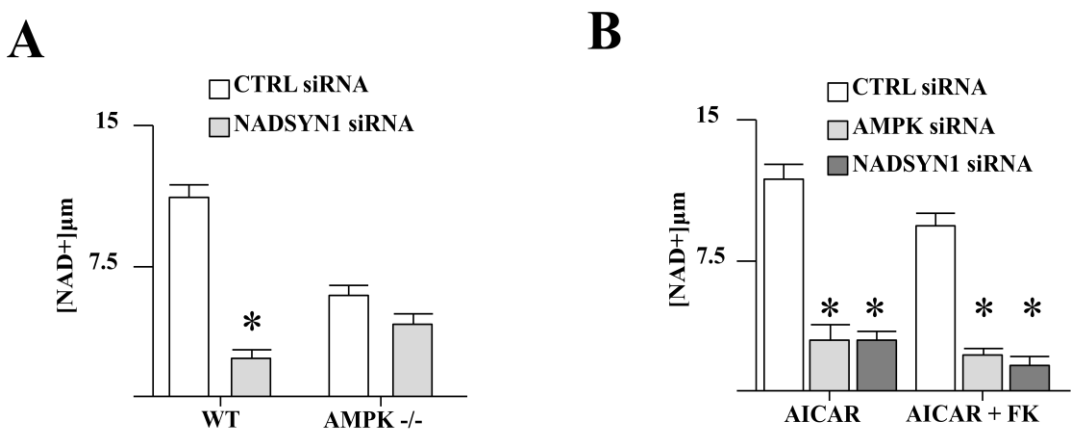

Figure 2.7. AMPK Increases NAD^+ Level through NADSYN1. (A) [NAD^+] in $AMPK^{+/+}$ and $AMPK^{-/-}$ MEFs treated with AICAR. (B) AICAR also increases [NAD^+] in C2C12s transfected with control, AMPK, or NADSYN siRNA then treated with AICAR with or without the NAMPT inhibitor FK. *$p < 0.05$.

Chapter 3

AMPK Regulates The Metabolic Transcriptome Through Epigenetic Mechanism.

3.0 Introduction

Epigenetic Regulation of Metabolic Transcriptome by AMPK

Mitochondria mediate a diverse set of functions including β-oxidation and oxidative phosphorylation that contribute to cellular energy homeostasis [5] Dysregulation of mitochondrial activity and biogenesis causes impaired ATP production, increased ROS, and/or aberrant Ca^{+2} signaling, all of which play important roles in the etiology of cardiovascular, neurological, and metabolic diseases. [7] Mitochondrial biogenesis is regulated by a myriad of mechanisms that include a diverse set of signaling cascades that potentiate the transcription of genes involved in mitochondrial biogenesis and function. [5-12] An increase in oxidative phosphorylation requires enhanced transactivation of mitochondrial function-related genes such as peroxisome proliferator-activated receptor (PPAR), co-activators (PGC-1α), NRF-1/2, uncoupler proteins 1 and 2 (UCP1, UCP2), and Tfam. [47, 51, 52, 155] As a key molecule regulating these genes, AMPK phosphorylates PGC-1α to induce NRF-1 and NRF-2, which coregulate the production of tfam mRNA. [48] To date, there is a lack of detailed understanding of the mechanisms by which AMPK mediates these multifaceted transcriptional events.

As mentioned in previous chapters, AMPK is a heterotrimeric serine/threonine protein kinase whose activity is highly sensitive to cellular energy status. The activation

of AMPK involves an intricate chorography initiated with AMP binding to its γ subunit followed by the phosphorylation of Thr-172 in the activation loop of the α subunit by LKB1 or CaMKKβ. [31-37] Through this regulating cascade, AMPK is both an energy sensor and a switch that modulates energy homeostasis by post-translational modification of targets mentioned above as well as histone H2B.[31] AMPK phosphorylation of H2B leads to increased expression of genes involved in cellular stress. The phosphorylation of H2B suggests that AMPK may also take part in the epigenetic modulations of genes involved in mitochondria functions. Epigenetic regulation involves interactions among multiple molecules including histones, DNA methytransferases, histone acetyltransferases, and polyADP ribosyltransferases (PARP-1) at the genome-wide scale. [59-63] To determine the network by which AMPK could epigenetically modulate mitochondrial functions, we utilized the data generated from bioinformatic analyses aimed at identifying direct AMPK targets on a kinome-wide scale. Based on the identified targets putatively involved in epigenetic regulation, we constructed an AMPK-regulated signaling network that is likely to affect the chromatin structure of genes implicated in mitochondrial function. Functional validation was conducted to determine the relationship between specific nodes of this network and phenotypic outcomes contributed by these AMPK targets in the context of mitochondrial functions.

3.1 Methods

Bioinformatics Approach

As mentioned in Chapter 2, an R script was used to map the AMPK phosphorylation consensus motif to human and mouse proteomes imported from ENSEMBL proteome database. A Scansite search was also performed to compare with the ENSEMBL search. The generated data bases were integrated into the Java open-source software system Gaggle to predict and display pathways involving AMPK.

Kinase Assay

AMPK kinase assays were performed using full-length recombinant proteins in 50 mM HEPES (pH 7.4), 1 mM AMP, 1 mM (γ-^{32}P)ATP, 5 mM $MgCl_2$, 1 pM AMPK and 1 nM recombinant protein in a 50 µl reaction volume at 37 ^{o}C for 1 h. Proteins were then resolved using SDS-PAGE, stained with Coomassie blue, and submitted to autoradiographic analysis. Peptide assays were performed under the same conditions but with 3 nM peptide, (γ-^{32}P)ATP, and 1 pM AMPK. SAMS (3 nM) peptide was used as positive control and no peptide as negative control. Peptide reactions were terminated by blotting samples onto Whatman filter paper and rinsed in 1% phosphoric acid. Individual filter papers were then washed in acetone and allowed to air dry. Filter papers were placed into separate scintillation vials with 1 ml scintillation fluid and measured with a Beckman LS 6500 scintillation counter. Peptides used for kinase assays: RBBP7s314, KLHTFESHKDEIFRR; RBBP7s314a, KLHTFEAHKDEIFRR; DNMT1s730, NIPEMPSPKKMHQRR; DNMT1s730a , NIPEMPAPKKMHQRR

Nuclear Extraction

Nuclear extracts were prepared according to the nuclear extract kit from Active Motif (Cat # 40010). After washing the cells three times with ice-cold PBS, cells were scraped from the surface of the plate in 1 ml ice cold HB buffer and allowed to swell for 15 minutes. The cell membrane was broken open with the addition of Noniodent P-40 to a final concentration of 0.5% followed by vigorous vortexing for 10 seconds. The resulting lysates were centrifuged for 30 seconds at 4^{o}C at 5,000×*g*. The supernatant was removed and the nuclear pellet was resuspended in 50 µl complete lysis buffer. Following 30 minutes of incubation at 4^{o}C with gentile agitation, nuclear extracts were centrifuged at 14,000×*g* at 4^{o}C and the resulting supernatant was saved for further analysis.

DNMT1 Activity Assay

Following appropriate treatments, DNMT activity was quantified from nuclear extracts according to the manufacturer's instructions (Active Motif, Cat# 55006). Five micrograms of nuclear extracts were added to the assay plate and incubated at 37^{o}C for 2 hours. An amount of 100 µl of diluted His-MBD2b was added and the plate was incubated at room temperature for 45 minutes. Following three washes with proprietary buffer AM3, 100 µl of diluted anti-polyHis-HRP antibody was added to the reaction wells and the plates were incubated at room temperature for 45 minutes. After several

washes with wash buffer, a proprietary developing solution was added. After stopping the reactions with acidic stop solution, absorbance was analyzed on a spectrometer.

Genomic DNA isolation

Cells were lysed in 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% Glycerol, 1 mM PMSF, and protease inhibitors. Cell lysates were extracted with phenol:chloroform (1:1) and the RNA was removed with the addition of RNase A suspended in 20 mM Tris, pH 7.4. After 1 hour incubation at 37^{o}C, reactions were precipitated with an equal volume of isopropanol and a 1:10 volume of potassium acetate, and re-suspended in 50 µl nuclease free H_2O.

Promoter Methylation Assay

Following appropriate treatments and DNA isolation, methylated and hemi-methylated DNA was quantified with the EpiMark 5-hmC and 5-mC Analysis kit by New England Biolabs (Cat # E33175). Per the manufacturer guidelines, restriction nucleases, either MspI or HpaII, were incubated with DNA at 37^{o}C for 16 hours followed by incubation with proteinase K for 30 minutes at 40^{o}C. Incubation at 95^{o}C for 10 minutes inactivated proteinase K was inactivated by incubating the reactions at. The level of non-cleaved DNA was quantified using qPCR with the following primer pairs.

Gene	Forward primer sequence	Reverse primer sequence
P38	CTAAAGGGCAAGAGTCGGTC	ACACTGCAAGTAGGTCATTCC

P65 GCACTACCAAACACAACTCAC AGGAAGCTGGGATTTGATCTC

PGC1α GGCAAGGGTGTAGTTACTGTG AACTCCAATCCACTCTGACAC

Tfam GTACCACGGATCTCTAACTTCAG CAATACGGTGCCTATGGACTG

UCP2 AGTATTGCTTTCACGTCCCC GATCTTCCCCTTCTATTCCCG

UCP3 TGTCCTCTACCTTCTCTGTCC CACATCTCTAGGAAGCAGGATC

Formaldehyde assisted isolation of regulatory elements (FAIRE)

After appropriate treatment, Isolation of transcriptionally active euchromatin was achieved using formaldehyde assisted isolation of regulatory elements (FAIRE) analysis as previously described. [157] In short, 1%formaldehyde used to fix protein-DNA interactions prior to cell lysis (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA, containing Halt protease inhibitors (Pierce, Cat. # 87786). Cell lysates were intermittently sonicated with 30 second pulses for 15 minutes using a Bioruptor UCD-200. DNA was extracted And promoters amplified using qPCR.

Gene	Forward	Reverse
P38	CTAAAGGGCAAGAGTCGGTC	ACACTGCAAGTAGGTCATTCC
P65	GCACTACCAAACACAACTCAC	AGGAAGCTGGGATTTGATCTC
PGC1α	GGCAAGGGTGTAGTTACTGTG	AACTCCAATCCACTCTGACAC
Tfam	GTACCACGGATCTCTAACTTCAG	CAATACGGTGCCTATGGACTG
UCP2	AGTATTGCTTTCACGTCCCC	GATCTTCCCCTTCTATTCCCG
UCP3	TGTCCTCTACCTTCTCTGTCC	CACATCTCTAGGAAGCAGGATC

mRNA Quantification

RNA was purified using TRIzol reagent from Life technologies. Two µg of RNA was converted to cDNA using Promega reverse transcriptase according to the manufacturer's instructions. cDNA was then quantified via qPCR using cyber green qPCR master mix purchased from Bio-Rad. Results were calculated using the delta-delta ct method. The following primers were used for qPCR analysis:

mRNA Primers

Gene	Forward	Reverse
P38	CCAGATGCCGAAGATGAACT	GATAGGTGGACAGACGAACAG
P65	GTTTCCCCTCATCTTTCCCTC	GCATTCAAGTCATAGTCCCCG
PGC1α	CACCAAACCCACAGAAAACAG	GTACAACTCAGATTGCTCGGG
TFAM	CACCCAGATGCAAAACTTTCAG	AGATCACTTCGTCCAACTTCAG
UCP2	TCGTCTCCCAGCCATTTTC	ATTCTGATTTCCTGCTACCTCC
UCP3	ACGGTTGTGAAGTTCCTGG	GGTTCTGTAGGCATCCATAGTC

Immunoblotting

Following treatment, cells were lysed in 10 mM Tris (pH 7.4), 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 1mM NaF, 20 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate (Na_3VO_4), 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM PMSF and protease inhibitors. Cell lysates were resolved via 10% SDS-PAGE, blocked with 5% milk, rinsed with TBST, probed with respective primary and secondary antibodies per manufacturer instructions. Signal was generated by Emerson

chemiluminescence substrate (ECL) and image captured on HyBlot CL autoradiography film.

ChIP assay

Chromatin immunoprecipitation (ChIP) assays were performed using the published ChIP protocol by ABcam. Protein was cross linked to DNA using formaldehyde, washed and harvested in FA lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and Halt protease inhibitors). The resulting cell lysates were sonicated with a Bioruptor 200. ATF-2 OR FoxO3a was immunoprecipitated overnight at $4^{o}C$ with protein A conjugated sepharose beads and respective antibody. The beads were washed with wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 150 mM Nail, 20 mM Tris-HCl, pH 8.0 and eluted in 100 µl elution buffer (1% SDS, 100 mM $NaHCO_3$). DNA was purified using Qiagen PCR purification kit prior to qPCR analysis. The following primers were used for qPCR analysis of immunoprecipitated DNA:

Gene	Forward primer sequence	Reverse primer sequence
P38	CTAAAGGGCAAGAGTCGGTC	ACACTGCAAGTAGGTCATTCC
P65	GCACTACCAAACACAACTCAC	AGGAAGCTGGGATTTGATCTC
PGC1α	GGCAAGGGTGTAGTTACTGTG	AACTCCAATCCACTCTGACAC
Tfam	GTACCACGGATCTCTAACTTCAG	CAATACGGTGCCTATGGACTG
UCP2	AGTATTGCTTTCACGTCCCC	GATCTTCCCCTTCTATTCCCG

UCP3 TGTCCTCTACCTTCTCTGTCC CACATCTCTAGGAAGCAGGATC

Mitochondrial Biogenesis Assay

Cells were grown in chambered cover glass wells. Following treatment, mitotracker green dye was added to the medium according to the manufacturer's instructions. Following staining, cells were imaged using a Leica SP5 inverted confocal microscope. Mitochondrial intensity was quantified using IMARIS imaging software.

Measuring Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured using JC-1 Mitochondrial Membrane Potential Assay kit (Cayman Chemical, cat# 10009172). JC-1 is a lipophilic, cationic dye that enters the mitochondria and reversibly aggregates upon an increase in membrane potential. This causes it the JC-1 to change color from green to red. After appropriate treatments were conducted, JC-1 stain was added to the medium and given 30 minutes in a CO_2 incubator set at 37^oC to infuse into the mitochondria. Images were then taken on a Zeiss 510 confocal microscope with an excitation/emission wave length set to 590/610 respectively.

Animal Experiments

Following administration of AICAR, animals were sacrificed and their tissues were isolated. Following isolation, Tissue was either fixed in 4% paraformaldehyde with gentile agitation for 30 minutes or homogenized in 10mM Tris pH 7.4, 100mM NaCl,

1mM EDTA, 1mM EGTA, 1mM NaF, 20mM $Na_4P_2O_7$, 2mM Na_3VO_4, .1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% Glycerol, 1mM PMSF lysis buffer or Trizol. Fixed tissues were then washed three times with PBS and FAIRE was conducted as previously described. RNA was extracted from tissues homogenized in Trizol according to the manufacturer's instructions. Quantitative PCR was then conducted as previously described. DNA methylation was quantified from samples homogenized in lysis buffer as previously described.

Statistical Analysis

Data are expressed as means ± SEM of at least three independent experiments. Comparisons of mean values between two groups were evaluated using a two-tailed Student's t test, Wilcoxon signed-rank test or Mann-Whitney U test. Unless otherwise indicated, $*p < 0.05$ was considered statistically significant.

3.2 Results

AMPK is a Regulating Node in the Epigenetic Network

A hallmark of transcriptional activation is nucleosome disruption or chromatin remodeling. AMPK's involvement in mitochondrial biogenesis and function, at the least, is through its transcription of PGC-1α and UCP2. [155] However, chromatin remodeling is required to facilitate gene transcription. Therefore, we explored the role of AMPK in epigenetic regulation of chromatin structure of genes involved in mitochondrial biogenesis and function. Using a systems biology approach, we identified that at least 40

proteins involved in epigenetic regulation contain an AMPK phosphorylation motif (Table 3.1). Figure 3.1A depicts a cytoscape network displaying protein targets containing an AMPK phosphorylation consensus sequence involved in epigenetic regulation. A module from this network containing two key proteins functioning in chromatin remodeling and DNA modification was studied thereafter (Fig. 3.1B). While AMPK was predicted to phosphorylate RBBP7 to increases its binding, AMPK might also phosphorylate and inhibit DNMT1 independent of RBBP7. Together, these two mechanisms would result in decreased promoter methlyation, facilitating gene transcription. We thus focus on the role of AMPK in regulating DNA methylation through DNMT1 and RBBP7.

AMPK Phosphorylates RBBP7 and DNMT1

To determine whether the *in silico* predicted sites of DNMT1 and RBBP7 could be phosphorylated by AMPK, peptides containing the AMPK consensus sequence with or without Ser-to-Ala substitution together with (γ-^{32}P)ATP were used for *in vitro* kinase assays. SAM's peptide was used as a positive control (Fig. 1C). RBBP7 Ser-314 and DNMT1 Ser-730 were phosphorylated with a higher level of ^{32}P incorporation, compared to their Ser-to-Ala substituted counterparts. The full length, recombinant proteins were also used in kinase assays with or without active AMPK followed by autoradiograph. Both RBBP7 and DNMT1 with active AMPK kinase reactions yielded positive autoradiographs.

AMPK Associates with DNMT1 and RBBP7

We next examined whether AMPK activation initiates protein-protein interactions of an epigenisome consisting of AMPK, DNMT-1, and RBBP7 in differentiated C2C12 cells, which are capable of myotubule differentiation and a model cell line for mitochondrial studies. [156] CoIP experiments performed on nuclear extracts of C2C12 cells transected with Flag-tagged S730 or S730A DNMT1 or S314 or S314A constructs show that AICAR increases DNMT1, RBBP7, and AMPK (both the α and β subunits) association at 10 min but not in the S730A DNMT1 or S314A RBBP7 transfected cells (Fig. 3.1D,E). These data indicate that AMPK, DNMT1, and RBBP7 likely form a DNA methylation inhibitory complex.

AMPK Phosphorylation of DNTM1 and RBBP7 Inhibits DNMT1

Given the crucial role of DNMT1 in transcriptional regulation and that AMPK is likely to phosphorylate DNMT1, we then examined whether AMPK affects DNMT1 activity. AICAR treatment decreased DNMT1 activity in C2C12 and $AMPK^{+/+}$ MEFs, but not in $AMPK^{-/-}$ MEFs (Fig. 3.2A, B). Similarly, infecting $AMPK^{-/-}$ MEFs with a constitutively activated form of AMPK (Ad-AMPK-CA) inhibited DNAMT. In contrast, infection with a dominant negative AMPK (Ad-AMPK-DN) did not (Fig. 3.2B). Next, we knocked down AMPK, PARP-1, or RBBP7 in C2C12 cells. Because PARP-1, by associating with DNMT1 or ADP-ribosylation of DNMT1, inhibits DNA methylation, its knockdown was used as a control. [156] Additionally, RBBP7 has been reported to be important for DNMT1 activity. [158] As shown in Fig. 3.2A, AICAR treatment decreased DNMT1 activity while AMPK, RBBP7, and PARP-1 knockdown increased DNMT1

activity regardless of AICAR treatment (Fig 3.2C). Further, transfecting DNMT1 S730A, DNMT1 S314A mutant, or RBBP7 plasmid into C2C12 cells resulted in little, if any, inhibition upon AICAR treatment compared to cells transfected with wild-type S730 DNMT and S314 RBBP7 constructs (Fig 3.2D). Because DNMT1 is partly inhibited by binding to DNA via its auto inhibitory domain, ChIP assay was performed to determine the effect of AMPK activation on DNMT1-PGC1-α promoter interaction.

AMPK Activation Decreases Promoter Methylation of Genes Involved in Mitochondrial Biogenesis and Function

To determine the effects of AICAR on methylation through AMPK *in vitro*, $AMPK^{+/+}$ MEFs and $AMPK^{-/-}$ MEFs were treated with AICAR for 4 hours and promoter methylation status was assessed. Mitochondrial precursor genes PGC1-α, tfam, UCP2 and UCP3 had less degree of methylation in their promoters upon AICAR treatment in the $AMPK^{+/+}$ MEFs when compared with those in the $AMPK^{-/-}$ MEFs (Fig. 3.3A). The same effect was seen in C2C12 cells transfected with control RNA but not when transfected with AMPK, PARP-1 or RBBP7 siRNA (Fig. 3.3B). Further, S730 phosphorylation proved to be important for AMPK's ability to decrease promoter methylation. When cells were transfected with the S730A construct, AICAR did not decrease promoter methylation as it did when cells were transfected with S730 or S730D constructs (Fig. 3.3C).

AMPK Activation Decreases Nucleosomal Compaction of Genes Involved in Mitochondrial Biogenesis and Function

To determine if AMPK phosphorylation of DNMT1 and RBBP7 facilitates chromatin relaxation, euchromatin was isolated and quantified using qPCR. Following AICAR treatment, an increase in transcriptionally active chromatin was observed at the PGC1-a, tfam, UCP2 and UCP3 promoters in wild-type MEFS and C2C12 cells transfected with control siRNA. This effect was attenuated in $AMPK^{-/-}$ MEFS (Fig. 3.4A) or in C2C12 transfected with AMPK, PARP-1, or RBBP7 siRNA (Fig. 3.4B). Similarly, the level of euchromatin was increased in DNMT1 siRNA-treated C2C12 cells. Further, transcriptionally active euchromatin was isolated from cells transfected with native DNMT1 or RBBP7, but not in cells transfected with DNMT1 S730A, DNMT1 S314A, or RBBP7, respectively (Fig. 3.4C).

The AMPK Epigenetic Network is Important for Mitochondrial Biogenesis and Mitochondrial Membrane Potential

We treated C2C12 cells with AICAR. AMPK activation by AICAR increased mitochondrial membrane potential at 24 hours but not in C2C12 cells transfected with DNMT1 S730A or RBBP7 S314A (Fig 3.5A). This is likely through the increased transcription of UCP2 and UCP3. DNMT1 S730 is also important for mitochondrial biogenesis upon AICAR treatment as shown by the greater mitotracker staining in the AICAR-treated DNMT1 S730, but not DNMT1 S730A-transfected C2C12 cells (Fig. 3.5B). To show functional consequence of mitochondrial function and biogenesis, both ROS and ATP levels were measured upon AICAR treatment for 24 hours. Both S730

DNMT1 and S314 RBBP7 proved to be important for AICAR's effect on ATP production. AICAR treatment did not increase ATP levels as significantly in cells transected with S730A DNMT1 and S314A constructs compared to those transfected with wild-type constructs. (Fig. 3.5C.) Additionally, AICAR treatment resulted in lower level of ROS in C2C12 cells transfected with DNMT1 S730 or RBBP7S314 but higher level of ROS in cells transfected with DNMT1 A730 or RBBP7A314 (Fig. 3.5D).

AMPK Activation Reduces Methylation Status and Activates Promoters of Genes Important for Mitochondrial Biogenesis and Function *In Vivo*

To determine the effects of AMPK activation by AICAR on methylation *in vivo*, wild-type (AMPK$\alpha 2^{+/+}$) and AMPK$\alpha 2^{-/-}$ mice were treated with saline (control) or AICAR for 24 hours, sacrificed, and gastrocenemius muscle (data shown), aorta, liver, heart, and adipose tissue were isolated. FAIRE analysis revealed active PGC-1α, Tfam, UCP2, and UCP3, but not the control p38 and p65 promoters in the wild-type mice. It is likely that AMPKs epigenetic regulation of promoters is limited to gene regulated by AMPK or important for stress response and energy homeostasis. However, AMPK$\alpha 2^{-/-}$ mice did not reveal a significant difference between saline or AICAR treatment (Fig. 3.6A). AICAR also decreased the methylation status of the respective promoters, but not P38 and p65, in WT mice, but not AMPK$\alpha 2^{-/-}$ mice (Fig. 3.6B). qPCR analysis showed that the PGC-1a, Tfam, UCP2, and UCP3 mRNA levels were higher in AMPK$\alpha 2^{+/+}$ than those in AMPK$\alpha 2^{-/-}$ mice (Fig. 4.6C), which supported results from the promoter activity assay.

3.3 Discussion

AMPK is a therapeutic prospect for metabolic diseases, such as metabolic syndrome, and has potential to alter the epigenetic mark that leads to pathophysiological phenotypes. [57, 58, 155, 160] However, its role as a modulator in adaptive genetic reprogramming of metabolism has predominately been studied in relation to its indirect and direct control of TF-DNA association and bioavailability. [155] To investigate the role of AMPK in gene regulation, this study exposes AMPK as a central node in controlling a myriad of epigenetic factors facilitating chromatin remodeling at promoters of genes important for stress response and energy homeostasis (Table 3.1, Fig. 3.1A). In particular, we demonstrate that AMPK can directly and indirectly affect DNA methylation status of the PGC1-α, Tfam, UCP2, and UCP3 promoters through regulation of both DNMT1 and RBBP7, revealing a novel mechanism by which AMPK alters the epigenetic code.

DNMT1 is the major methyltransferase responsible for regulating cytosine methylation patterns and genome imprinting, providing an inheritable mechanism of genetic regulation irrespective of DNA sequence heterogeneity. [160] Interestingly, DNMT1 function is bimodal in that it has both an active and an autoinhibitory mechanism that ensures the high fidelity of DNMT1-mediated DNA methylation preservation. DNMT1 "self-regulates" to increase methylation at hemi-mCPG regions; while structurally protecting unmethylated CpG sites by association with its CXXC domain and CXXC-BAH1 linker. [161, 162] Located near the auto inhibitory domain,

phosphorylation of Ser-730 by AMPK may impart structural changes that cause constitutive association between unmethylated CpG regions and CXXC domain.

On the other hand, the phosphorylation of Ser-730 may induce conformational changes in DNMT1 to alter its protein-protein interactions. DNMT1 is influenced by several signal transduction pathways that are also regulated by AMPK. For example, Ctcf (CCCTC-binding factor) promotes PARP-1 activity and DNMT1 inhibition by PARylation. [161, 162] Additionally, Ctcf associates with PARP-1 and DNMT1 to preserve the DNA methylation profile, particularly unmethylated regions, of daughter cells. [162]

AMPK may indeed influence PARP-1 and DNMT1 association; however, our study provides a novel AMPK-induced RBBP7-DNMT1 interaction. [41] RBBP7, a known coactivator of the histone acetyltransferase, HAT1, is phosphorylated by AMPK on Ser314. [163] Importantly, we identified HAT1 is a substrate of AMPK in another study. AMPK phosphorylates HAT1, increasing its activity and histone acetylation (data not published). However, RBBP7 is predicted to affect DNMT1's ability to maintain genomic methylation status as well, but the mechanism has not been explored. [159] Our results indicate that DNMT1 and RBBP7 do associate with each other upon AMPK activation, which is dependent upon DNMT1 Ser730 and RBBP7 Ser314 phosphorylation. This association decreases DNMT1's ability to methylate or increases its "protection" of unmethylated CpG regions (Fig. 3.3).

DNMT1 localizes to replication forks and copies methylation patterns from parent to daughter cells. Here we demonstrate that AMPK activation decreases DNMT1 activity at 30 minutes and decreases promoter methylation status. Although early TF kinetics at

the promoter of genes are involved, our data indicates that nucleosome remodeling is a structural requirement preceding transactivation of PGC1-α, Tfam, UCP2, and UCP3. This remodeling by AMPK activation is likely a resultant of an entire epigenome regulated to promote gene transcription and protective against the silencing of positive regulators of cell survival and vise versa. In summary, AMPK's epigenetic effects through phosphorylation of DNMT1 and RBBP7, leading to increased PGC1-α, tfam, UCP2, and UCP3 while decreased p65 and p38 expression. As a result of this newly defined pathway, mitochondrial biogenesis as well as mitochondrial membrane potential are increased (Fig. 3.1D, 3.1E). This ultimately improves ATP production with a concomitant control of mitochondrial ROS production (Fig. 3.5).

3.4 Figures and Tables

Table 3.1. Predicted AMPK Targets Involved in Epigenetic Regulation

Gene Symbol	Gene Name	Gene Symbol	Gene Name
ATRX	Alpha thalassemia/mental retardation syndrome X-linked	H3-K9-HMTase 1	Histone-lysine N-methyltransferase, H3 lysine-9 specific 1
ACTL6A	Actin-like 6a	H3-K9-HMTase 2	Histone-lysine N-methyltransferase, H3 lysine-9 specific 2
ARRB1	Arrestin, Beta 1	H3-K9-HMTase 3	Histone-lysine N-methyltransferase, H3 lysine-9 specific 3
BAZ1B	Bromodomain adjacent to zinc finger domain protein 1B	HMGA1	High mobility group AT-hook 1
CBX5	Chromobox protein homolog 5	KAT2A	K(lysine) acetyltransferase 2A
CHD4	Chromodomain helicase DNA binding protein 4	KLF2	Kruppel like factor 2
CHMP1B	Charged multivesicular body protein 1B	MTA2	Metastasis associated 1 family, member 2
DDX56	DEAD (Asp-Glu-Ala-Asp) box helicase 56	NCL	Nucleolin
DNMT1	DNA (cytosine-5-)-methyltransferase 1	NUCKS	Nuclear ubiquitous casein and cyclin-dependent kinases substrate

DNMT3a	DNA (cytosine-5-)-methyltransferase 3a		Nap1l1	Nucleosome assembly protein 1-like 1
DPF3	D4, zinc and double PHD fingers, family 3		Nap1l4	Nucleosome assembly protein 1-like 4
EHMT2	Euchromatic histone-lysine N-methyltransferase 2		PARP1	Poly [ADP-ribose] polymerase-1
GLMN	Glomulin, FKBP associated protein		RBBP2	Retinoblastoma binding protein 2
HAT1	Histone acetyltransferase 1		RBBP4	Retinoblastoma binding protein 4
HDAC1	Histone deacetylase 2		RBBP7	Retinoblastoma binding protein 7
HDAC2	Histone deacetylase 2		RBBP9	Retinoblastoma binding protein 9
HDAC6	Histone deacetylase 6		SMARCE1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1
HDAC7	Histone deacetylase 7		SMYD3	SET and MYND domain containing 3
H1	Histone cluster 1		UBTF	Upstream binding transcription factor, RNA polymerase I
H2	H2A histone family, member X		ZRF1	DnaJ homolog subfamily C member 2

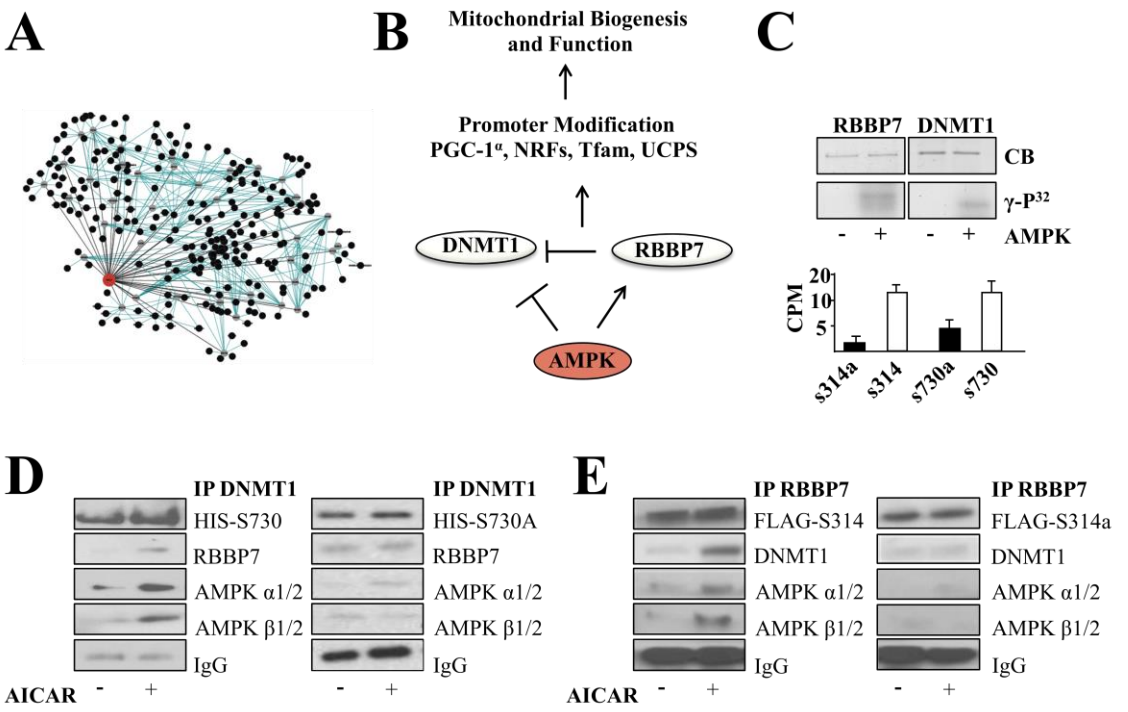

Figure 3.1. AMPK Regulates an Epigenetic Interactome. A) Cytoscape display representing the functional interactome between AMPK and putative targets in epigenetic regulation. B) AMPK epigenetisome involved in chromatin remodeling. C) Kinase assays using recombinant target protein in the presence or absence of activated recombinant AMPK. Top panel represents the Coomassie stained gel for loading and bottom panel represents the autoradiograph. Kinase assays using peptides synthesized corresponding to the AMPK consensus sequence on target proteins. Ser to Ala substituted peptides were used as a negative control and SAMS peptide as a positive control. D, E) AICAR treatment for 10 minutes increases association of DNMT1, AMPK and RBBP7 in C2C12s transfected with S730 DNMT1 or S314 RBBP7 but not when transfected with S730A DNMT1 or S314 RBBP7.

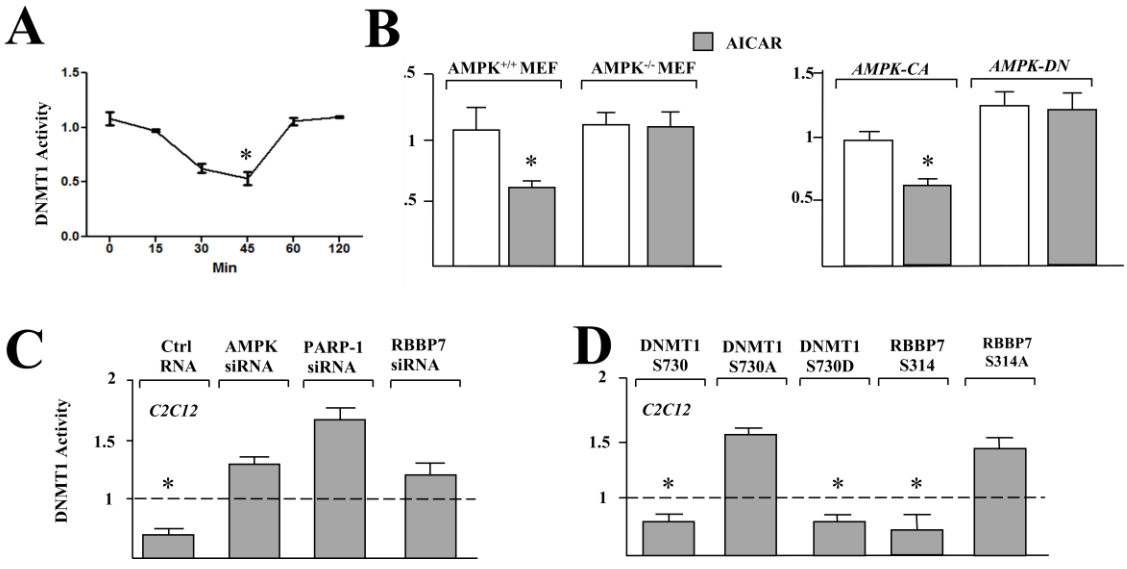

Figure 3.2. AMPK Decreases DNMT1 Activity. A) AICAR (1mM) decreased DNMT1 activity in C2C12 at 30 min. B) AICAR decreased DNMT1 activity in AMPK +/+ cells but not in AMPK -/- MEFs unless transected with constitutively active AMPK (Ad-AMPK CA). AMPK -/- MEFs transfected with dominant negative AMPK (Ad-AMPK-DN) did not change the level of DNMT1 activity. C) C2C12 cells transfected with control, AMPK, PARP-1 or RBBP7 siRNA then treated with AICAR. AICAR decreased DNMT1 in cell transfected with control but not AMPK, PARP-1, or RBBP7 siRNA. D) AICAR decreased DNMT1 activity in C2C12 cells transfected with S730 or S730D DNMT1 and 314 RBBP7 constructs but not with S730A DNMT1 or 314A RBBP7 constructs. *$p < 0.05$.

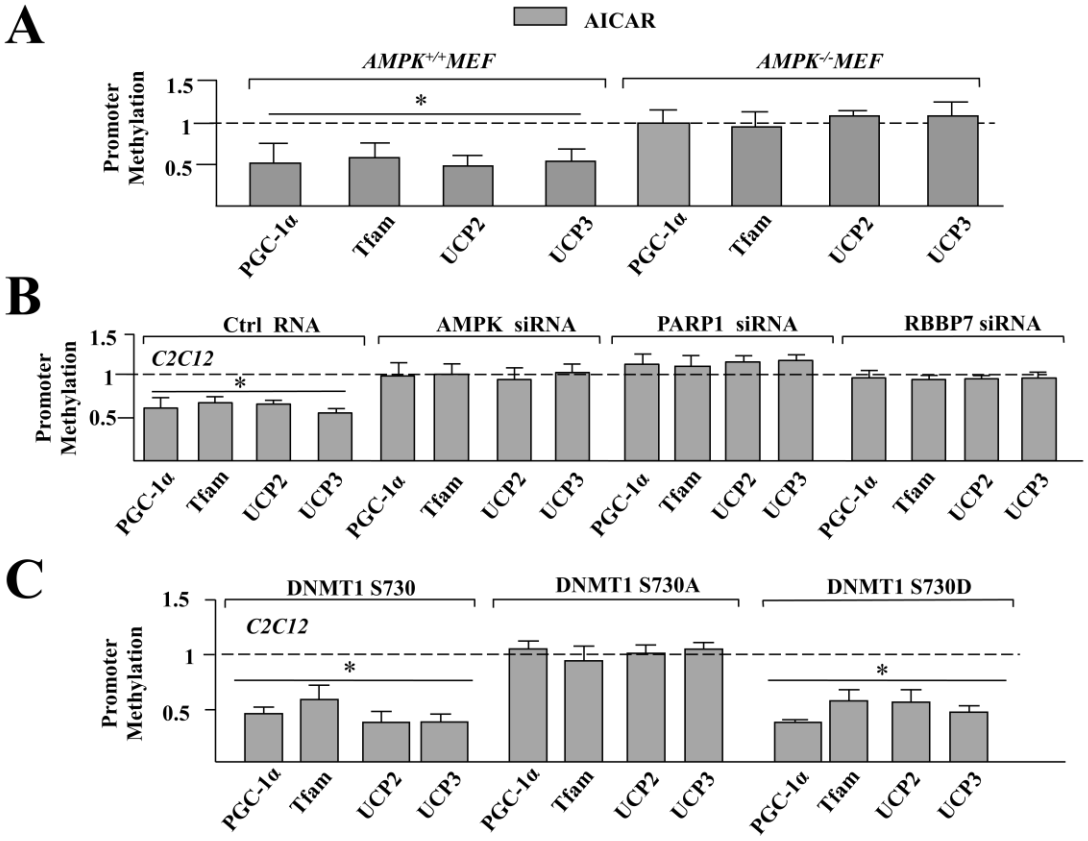

Figure 3.3. AMPK Activation Decreases Promoter Methylation of Genes Important for Mitochondrial Biogenesis and Function. Promoter methylation status was quantified after AICAR treatment in AMPK +/+ or AMPK -/- MEFs (A) and C2C12 (B) transfected with AMPK, PARP-1, or RBBP7 siRNA. C) Promoter methylation status was analyzed in C2C12 cells transfected with S730, S730A, or S730D DNMT1. *$p < 0.05$.

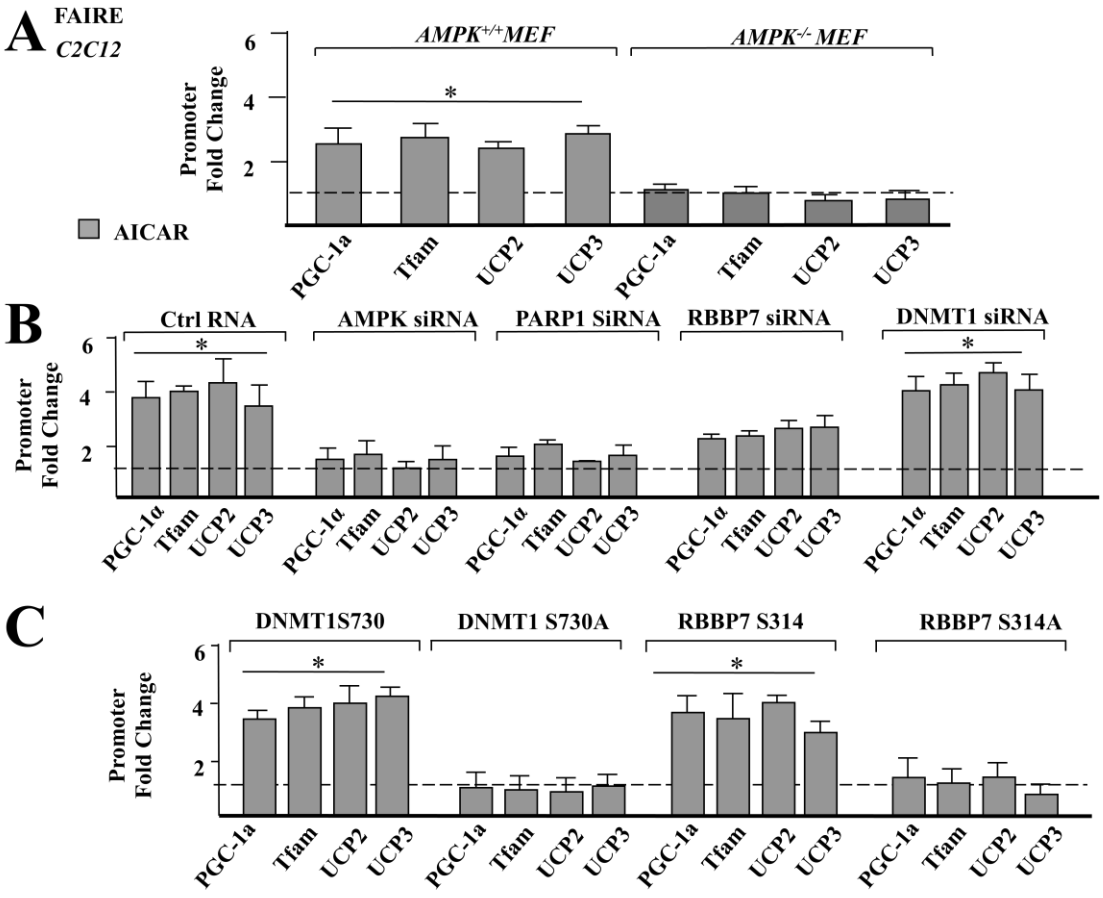

Figure 3.4. AMPK Activation Decreases Nucleosomal Compaction of Genes Involved in Mitochondrial Biogenesis and Function. A) FAIRE followed by qPCR using primers to respective promoters was performed after treatment with AICAR for 60 min in AMPK +/+ or AMPK -/- MEFs and B) C2C12 transfected with AMPK, PARP-1, RBBP7 or DNMT1 siRNA . C) FAIRE was conducted in C2C12 cells transfected with S730 DNMT1 , S730A DNMT, S314 RBBP7 or S314A RBBP7. *$p < 0.05$.

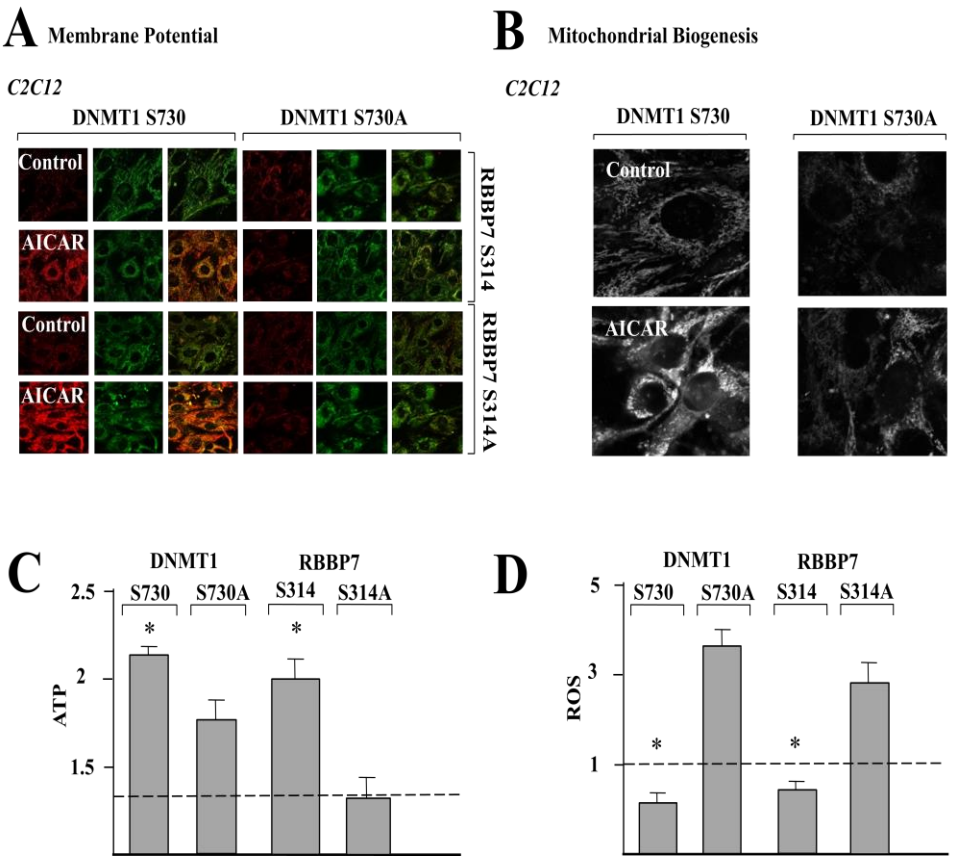

Figure 3.5. AMPK Activation Increased Mitochondrial Biogenesis and Function. Following transfection with native or S730A mutant DNMT1 plasmids, cells were treated With AICAR for 12 hours and mitochondrial membrane potential (A) or abundance (B) was analyzed. C2C12 cells were transfected with native, S730A mutant DNMT1 or native or S314A mutant RBBP7. Resulting cell lysates were analyzed for their ATP content (C) or ROS levels (D). *$p < 0.05$.

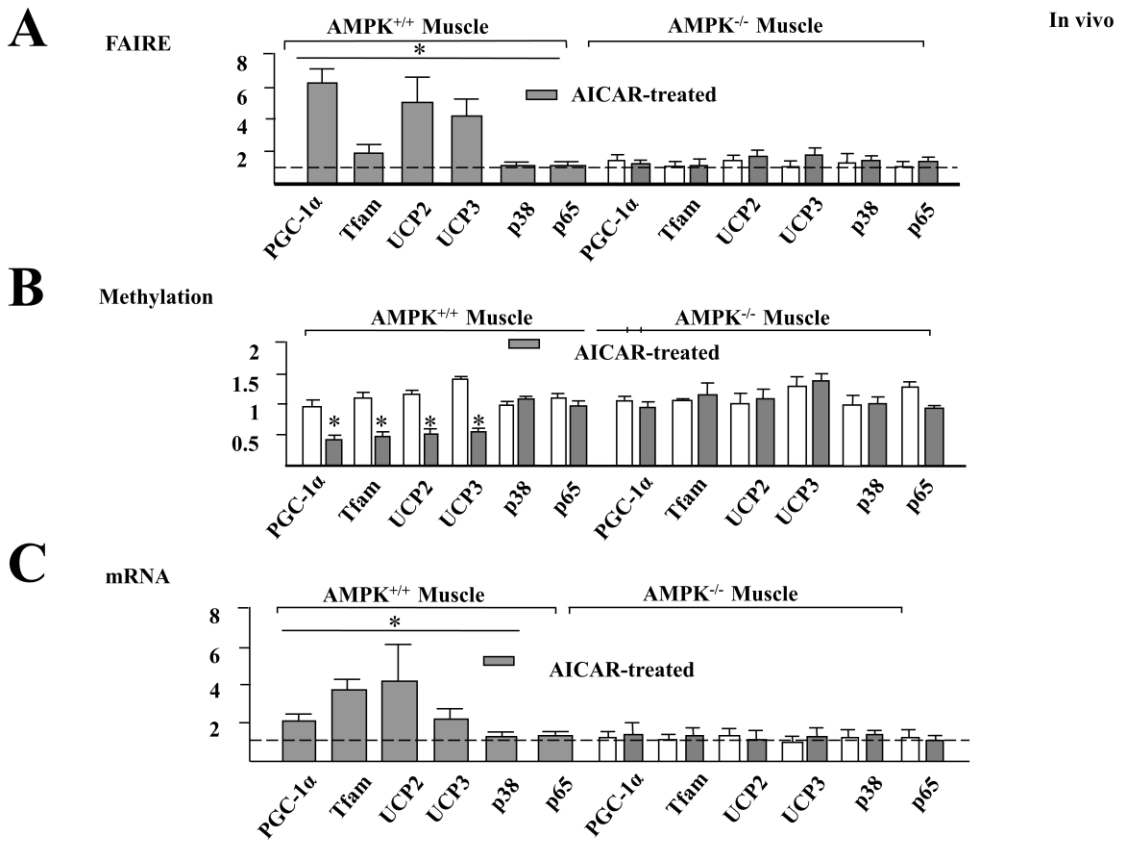

Figure 3.6. AICAR Instigates Epigenetic Regulation through AMPKα2 *In Vivo*

Euchromatin structure (A), methylation status (B), or mRNA abundance (C) were analyzed in muscle isolated from AMPK +/+ and -/- mice following administration of AICAR. *$p < 0.05$.

Chapter 4

Summary and Perspectives

4.1 Introduction

The characterization of AMPK remains as an energy sensor that facilitates catabolic process and inhibits anabolic process to conserve energy. [33, 34] However, its regulation is dependent upon a variety of stimuli aside from high AMP:ATP ratio and hypoglycemia inferring that AMPK's function is much more complex than simply an energy sensor. For example, AMPK promotes mitochondrial biogenesis through transcriptional activation of PGC1-α and mitophagy through ULK1, both of which require energy use but ultimately lead to cellular homeostasis and health. [48, 50] AMPK also positively regulates angiogenesis, by increasing KLF2 expression, which has an even higher energy demand. [142] The studies presented in this dissertation shed light on how AMPK efficiently participates in energy consuming activities to maintain cellular homeostasis and survival through regulation of epigenetic factors while maintaining energy availability.

4.2 AMPK and Target Substrate Specificity

Although screening the consensus sequence for candidates of a kinase is a useful tool, the presence of a consensus sequence does not guarantee its phosphorylation. For phosphate transfer to a target substrate, tertiary elements of protein folding are important for kinase substrate accessibility and specificity along with a sequence flanking the phosphorylation site that mirrors the catalytic pocket of the kinase are required. [164, 165]

Despite the limitations of consensus sequence information, their general success as a guide for experimental validation is a testament of their importance. The AMPK consensus sequence has a large amount of complexity compared to other kinases making it an attractive element for screening the genome. We utilized the consensus sequence to screen potential target candidates for AMPK. Due to variability in the consensus sequence, absence of a protein in our screen does not necessarily omit it as a target of AMPK. Alternatively, a fraction of our candidate list may compose false positive targets. However, our study validates the usefulness of the consensus sequence as a tool for deriving targets of AMPK.

Our screen revealed the potential for AMPK as a central node in total cellular homeostasis (Fig. 2.1, Fig. 2.2.). The 15 novel substrates tested all offer new hypotheses for future study for AMPK function (Table 1, Fig. 2.4). Predicted positive epigenetic or transcriptional regulators include ARRB1, HMGA1, KLF2, CHMP1B, ZRF1, and DDX56. Novel substrates involved in RNA processing and translation include CPSF1, TCOF, and DDX56. CALNX, CHMP1B, and ZRF1 are involved in protein folding and transport. Cell cycle control and growth are partially regulated by CHMP1B, NUCKS and DHRS9. [115, 120-126] ERCC8 is important for DNA repair after oxidative stress damage. [98, 99] KLF2 plays a key role in cell migration. [141-143] Energy homeostasis is in part due to AMPK autophosphorylation and regulation of NADSYN1. Finally, redox and inflammatory regulation might occur through ARRB1, ACE2, DHRS9, and KLF2. [90, 91, 116, 117, 135, 136, 144]

4.3 AMPK regulates NADSYN to Modulate Stress Response.

NADSYN1 was further explored to validate functional outcome from the bioinformatics screening approach. NAD^+ is an important substrate for both PARP-1 and SIRT1. PARP-1 responds to cellular stress, particularly DNA damage and hyperphysiologic oxidative states, to repair DNA and initiate apoptosis if necessary. [149, 151] PARP-1 utilizes NAD^+ as a substrate to PARylate many nuclear enzymes important for total stress response. SIRT1, also a stress responder, directly couples NAD^+ hydrolysis to the deacetylation of many important enzymes in the stress response. [145-146] AMPK, PARP-1 and SIRT1 all regulate NF-κB and p53 to ensure cell stress response and survival. [148-154] Here we show that AMPK maintains the function of these enzymes through autophosphorylation and increasing bioavailability of NAD^+ as a substrate for PARP-1 and SIRT1. AMPK phosphorylates and activates NADSYN1 to increase NAD^+ level in the cell (Fig. 2.5, 2.7) However, AMPK also transcriptionally upregulates NADSYN1 expression through phosphorylating FoxO3a promoting its binding to the NADSYN1 promoter to increases mRNA levels and subsequent protein levels (Fig. 2.6). Although this is just one pathway shown, AMPK likely regulates a spectrum of energy producing pathways to allow it function in synthesis of proteins required for the stress response.

4.4 AMPK and Its Epigenetisome

In chapter 3, elucidation of a mechanism by which AMPK epigenetically regulates mitochondrial function-related genes located in the nucleus was presented. It is well known that chromatin remodeling involves an orchestrated effort between multiple DNA and histone modifying enzymes. For example, histones are subjected to

acetylation by HAT1, phosphorylation by AMPK, and ADP-Ribosylation by PARP-1, while DNA is subjected to methylation by DNMT1. The regulation of these modifications is further complicated by splice variants and functional enzymatic redundancy within the genome. Our bioinformatics approach revealed that AMPK regulates an epigenetisome consisting of at least 40 factors involved in chromatin remodeling/nucleosome disruption (Fig. 3.1A Table. 3.1). The relative contribution from each one of these nodes on the epigenetic regulation of mitochondrial-related genes is currently speculative. Utilizing PGC-1α as a transcriptional eaout, RNA interference studies can be conducted to elucidate the influence of individual noes on the kinetics of nucleosome remodeilin. Double knockdown experiemetns will likely determine pathway epistasis to validate our constructed network.

According to this established epigenetisome, AMPK regulates a variety of genes across the genome epigenetically. Our data indicates that cells treated with AICAR for 24 hours results in PGC-1α, Tfam, UCP2, and UCP3 nucleosome remodeiling and activation, but not p65 and p38, which inhibit mitochondrial biogenesis (Fig. 3.6). [160-162] This demonstrates that the role of AMPK as an epigenetic regulator is gene-specific, most likely regulating genes involved in stress response and energy homeostasis. Assessing chromatin remodeling on a genome wide scale in the future study will uncover novel promoters that are epigenetically regulated by AMPK. Further, the mechanisms that confer specificity of chromatin remodeling remain elusive. Knockdown of individual components to our AMPK- regulated epigenetisome followed

by genome wide screening may reveal the contribution of each component towards chromatin remodeling specificity.

4.5 AMPK Decreases Promoter Methylation through Regulating DNMT1 and RBBP7

AMPK inhibits DNMT1 methyltranferase activity in the active promoters (Fig 3.3). The involved mechanism is that AMPK phosphorylate DNMT1 S730 (Fig. 3.1). However, RBBP7 phosphorylation by AMPK at S314 promotes an inhibitory protein-protein interaction between RBBP7 and DNMT1 (Fig 3.1). As mentioned in Chapter 3, the mechanism by which this is done requires further study given the complexity of DNMT1 regulation. DNMT1 localizes to replication forks suggesting that maintenance of methylation is coupled to cell division and preservation of a hereditable epigenetic mark. [159-163] Complete DNMT1 inactivation results in global demethylation, although additional regulatory elements such as PARP-1, HAT1, etc. are involved in the epigenetic regulatory machinery. [158,160] AMPK inhibition of DNMT1 through phosphorylation and increased RBBP7 association possibly alter the epigenetic code, particularly in the onset of chronic diseases such as diabetes mellitus and metabolic syndrome. The translational relevance in this regard deserves further studies.

4.6 AMPK Increases Mitochondrial Function through Epigenetic Regulation

AMPK is well known for its effects on increasing mitochondrial number and function. However, its ability to transcriptionally regulate these effects has been limited to transcriptional activation of TFs such as PGC1-α through phosphorylation. [46] Here, we

show that AMPK is directly involved in the epigenetic regulation of mitochondrial biogenesis and function by decreasing promoter methylation and decreased nucleosomal compaction of the genes, including PGC1-α, Tfam, UCP2, and UCP3 (Fig. 3.3, Fig. 3.4, Fig. 3.5, Fig. 3.6).

Given that mitochondrial DNA undergoes epigenetic regulation, AMPK may increase mitochondrial function through increasing mitochondrial genes involved in anti-oxidation and ATP production. Similar to nuclear DNA, mtDNA contains 5-methylcytosine (5mC) at CpG dinucleotides. [169] To date, DNMT1 is the only reported methyltranferase that contains a mitochondrial targeting sequence immediately upstream the translational start site. The expression of mitochondrial DNMT1 (mtDNMT1) is up-regulated by NRF1 and PGC1α. [169] This is potentially an unforeseen pathway regulated by AMPK that deserves further investigation.

4.7 Insight to Regulation of PGC-1α

PGC-1α transactivation is heavily regulated by choreographed alterations in chromatin structure coupled to binding and dissociation of co-activators and repressors respectively. The PGC-1α promoter lacks a *TATA box* but contains several regulatory sequences including a GC box, a cyclic AMP responsive element (CRE), several *insulin response sequences* (IRSs), a *serum response element* (SRE), binding sites for GATA, myocyte enhancer factor-2 (MEF2), p53, NF-κB, and E-box elements. [168] The contributions of the GC box, NF-κB, and SRE to PGC-1α transactivation have not been explored. However, in response to protein kinase A (PKA) activation, cyclic AMP response element binding protein (CREBP) is phosphorylated and binds to the PGC-1α

promoter inducing transcription. [169] Insulin stimulation initiates forkhead transcription factor FKHR binding to the IRSs in the PGC-1α promoter, which positively regulates its transcription. [170] MEF2 and p53 have been shown to bind to and activate the PGC-1α promoter. [171-173] Additionally, functional interactions between PKD, HDAC5, and ATF2 result in activation of the PGC-1α promoter. AMPK regulates PGC-1α transactivation by influencing GATA-4 and upstream stimulatory factor 1 (USF-1) binding to GATA/E box elements, and potentially PKA, PKD, HDAC5, p53, and ATF2 all of which contain an AMPK phosphorylation recognition motif. [169, 174] This dissertation work adds a crucial layer to PGC-1α regulation, which involves all TF binding. All together, our data suggest that AMPK regulates the promoters of PGC1-α, Tfam, UCP2, and UCP3 through chromatin remodeling, a necessary requirement for TF binding.

4.8 Summary

This dissertation provides new insight to AMPK's role in total cellular stress response and homeostasis by revealing several novel pathways regulated by AMPK. AMPK functions at every level from transcription, translation, stabilization and transport of key molecules essential for homeostasis. In particular, AMPK was demonstrated as a key epigenetic regulator of genes involved in maintaining energy homeostasis and mitochondrial health. AMPK activation with exercise, caloric restriction, anti-inflammatory foods, metformin and statins, are useful interventional or therapeutic tools to begin reversing the current epidemics of metabolic disease. Although acute regulation of mitochondrial function serves a necessary role in cellular response to stress,

reprogramming of the epigenetic mark may be a mechanism by which AMPK activation will improve mitochondrial function in not only parents, but their offspring as well.

Chapter 5

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