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2012

# The Regulation of Mitochondrial DNMT1 During Oxidative Stress

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THE  REGULATION OF MITOCHONDRIAL DNMT1 DURING OXIDATIVE STRESS


A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University


by


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May, 2012

## Acknowledgement

I would like to acknowledge a special group of individuals who have played a significant role throughout this journey in my graduate career. First and foremost, I would like to thank Dr. Shirley Taylor, for being a great mentor, teacher and most importantly a wonderful friend. She not only guided me throughout my experience in the graduate school, but she also helped me overcome obstacles beyond the laboratory. Her "tough love" style of teaching certainly made me become a more independent, strong-willed individuals. She had made a tremendous impact in both my professional and personal life. I am extremely grateful to have had the opportunity to work for such a compassionate, intelligent investigator.

I would like to also express my gratitude to the member of my graduate committee; Dr. Gail Christie and Dr. Joyce Lloyd for their dedication and continuous guidance throughout my master work. Each members of my committee provided me with immense help and constructive challenges that made me become a more conscious, conceptual, well-rounded thinker. I especially want to thank Dr. Gail Christie for also be an encouraging director and to the Molecular Biology and Genetics departments for their overwhelming support.

I would also like to give special thanks the members of the Taylor laboratory, both past and present; Prashant Thakkar, Dr. Timothy Lochmann, Dr. Lisa Shock. These wonderful individuals not only trained me to become a meticulous, efficient student but also provided me unconditional support. I also would like to thank the members of the Moran laboratory, both past and present; Catherine Bell, Stuti Agarwal, Audrey Thacker, William Buchwald, Dr. Chen Yang, Dr, Dr. Courtney Lawrence and Dr. Scott Lawrence. All these amazing people from both the Taylor and Moran laboratory had made my two years experience in the graduate program enjoyable and amusing. They provided the comic relief that I certainly needed, especially during challenging times. I am very appreciative to have work beside a wonderful group of individuals and most importantly very grateful gain such a strong friendship with all of them.

I would like to acknowledge my family and friends who have been my main support throughout this experience. To my friends; Manuel Ramirez, Audrey Sunga, Kimberly Le, Ailene Dumo, Catherine Bell and Meredith Wreck, who have devoted time and effort to make sure my time here in VCU was memorable. To all my family members, who continuously supported me and motivated me to pursue my dreams. Lastly, I want to express my utmost gratitude to my parents, Josephine and Rodel Balinang for being the greatest supporters and fans. They are the main reason for my passion and I am deeply grateful for their contribution and unconditional love.

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**Abbreviations**

| | |
|---|---|
| °C | degrees Celsius |
| $O_2$ | oxygen |
| 5hmC | 5-hydroxymethylcytosine |
| 5mC | 5-methylcytosine |
| 8-ox-dG | 8-Oxo-2'-deoxyguanosine |
| APC | adenomatous polyposis coli |
| AR | androgen receptor |
| ATP | adenosine triphosphate |
| ATPase 6 | ATPase subunit 6 |
| bp | base pair |
| BRCA1 | breast cancer 1 |
| BSA | bovine serum albumin |
| C-5 | carbon 5 |
| $CO_2$ | carbon dioxide |
| cAMP | cyclic adenosine monophosphate |
| ChIP | chromatin immunoprecipitation |
| $CoCl_2$ | cobalt chloride |
| D.C. | dibutyrl cAMP |
| DAP-Kinase | death-associated protein- Kinase |
| DFO | deferoxamine mesylate |
| DNA | deoxyribonucleic acid |
| DNMT1 | DNA methyltransferase 1 |
| DNMT2 | DNA methyltransferase 2 |
| DNMT3a | DNA methyltransferase 3a |
| DNMT3b | DNA methyltransferase 3b |
| DTT | Dithiothreitol |

| | |
|---|---|
| ECL | enhanced chemiluminescent |
| EDTA | ethylenediaminetetraacetic acid |
| ER | estrogen receptor |
| ETC | electron transport chain |
| FBS | fetal bovine serum |
| FMR1 | Fragile X mental retardation 1 |
| gDNA | genomic DNA |
| $H_2O_2$ | hydrogen peroxide |
| H3k4me3 | histone H3 lysine 4 trimethyl |
| HCT116 | human colon carcinoma cell line |
| HDAC | histone deacetylase |
| HIFα | hypoxia-inducible factor 1, alpha subunit |
| HSP | heavy strand promoter |
| IP | immunoprecipitate |
| kb | kilobase |
| kDa | kilodalton |
| LiCl | lithium chloride |
| LSP | light strand promoter |
| MBD | Methyl-CpG-binding domain protein |
| MeCp2 | Methyl-CpG-binding domain protein 2 |
| mM | milimolar |
| mRNA | messenger RNA |
| mtDNA | mitochondrial DNA |
| mtDNMT1 | mitochondrial DNA methyltransferase 1 |
| mTFA | mitochondrial transcription factor A |
| NAD | nicotinamide adenine dinucleotide, |
| NADPH | nicotinamide adenine dinucleotide phosphate |

| | |
|---|---|
| ND1 | NADH dehydrogenase subunit 1 |
| ND6 | NADH dehydrogenase subunit 6 |
| NE | norepinephrine |
| NO | nitric oxide |
| NRF-1 | nuclear respiratory factor 1 |
| NRF-2 | nuclear respiratory factor 1 |
| NT | no treatment |
| OH | hydroxide |
| PCR | polymerase chain reaction |
| PD | Parkinson's disease |
| PGC1α | Peroxisome proliferator-activated receptor gamma coactivator 1-alpha |
| PPARγ | Peroxisome proliferator-activated receptor gamma |
| qPCR | quantitative polymerase chain reaction |
| ROS | reactive oxygen species |
| rRNA | ribosomal RNA |
| SAM | s-adenosylmethionine |
| SDS | sodium dodecyl sulfate |
| SDS-Page | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SH-SY5Y | neuroblastoma cells |
| SOD | superoxide dismutase |
| TAP | tandem affinity purification |
| TBS | tris base saline |
| TET | ten:eleven translocation protein |
| tRNA | transfer RNA |
| UCP-2 | uncoupling protein 2 |
| μg | microgram |

| | |
|---|---|
| **µl** | **microliter** |
| **µM** | **micromolar** |
| **UV** | **ultra violet** |
| **VDAC** | **voltage dependent anion channel** |
| **VHL** | **von Hippel-Lindau** |
| **WC** | **whole cell** |
| **WCL** | **whole cell lysates** |
| **WT** | **wild-type** |

**ABSTRACT**


The Regulation of Mitochondrial DNMT1 During Oxidative Stess

Joyce M. Balinang , B.S.


A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University


Virginia Commonwealth University, 2012


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Epigenetics is the study of heritable gene expression patterns due to alterations in the DNA structure other than the underlying DNA sequence. DNA methylation is one of the three types of epigenetic modifications found in the eukaryotic system. It involves the incorporation of a methyl group at the 5-position of cytosine residues in the DNA. DNA methylation is associated with several disorders and diseases including Fragile X Syndrome, neurodegenerative disease (Parkinson's, Alzhiemer, etc), diabetes and cancer. Cytosine methylation of mitochondrial DNA (mtDNA) was first demonstrated several decades ago but the mechanism of generating cytosine modification and its functional importance remain elusive. Our laboratory recently demonstrated that the enzyme involved in

cytosine modification of mtDNA is a novel mitochondrial isoform of DNA

Methyltransferase 1, mtDNMT1.  This protein is encoded in the nucleus and targeted

to the mitochondria via a N-terminal targeting sequence.  Bioinformatic analysis of

the DNMT1 coding sequence showed a consensus NRF1 binding site that  overlaps a

p53 binding site within the promoter region, previously shown by our laboratory to

repress DNMT1 expression.  Studies in the Taylor laboratory also showed that

mtDNMT protein expression was regulated by the transcription factor NRF1 as well

as its coactivator PGC1α.  PGC1α and NRF1 stimulate a large body of genes that are

involved in mitochondrial biogenesis and cellular respiration in response to

environmental stress.   Considering the previous findings in our laboratory

regarding mtDNMT1 regulation and the importance of PGC1α and NRF1 in oxidative

homeostasis, we asked whether there is a mitochondrial epigenetic component in

the cell's response to cellular stress and whether up-regulation of mtDNMT1 might

be part of the general response to this stress.  To investigate the relationship

between mtDNA methylation and oxidative homeostasis we examined the

regulation of mtDNMT1 by transcription factors that respond to oxidative stress.

Conditions that induced oxidative stress were applied to HCT 116 and SH-SY5Y cell

lines and the protein expression of DNMT1 was observed.  Ethanol and hypoxia-

induced oxidative stress resulted in an increase in protein level of mtDNMT1 while

total DNMT1 level either remained constant or decreased. The protein level of

PGC1α and NRF1 remained low in HCT 116 cells exposed to hypoxic stress, despite

elevated mtDNMT1 protein level.  ChIP analysis of HCT 116 cells exposed to hypoxic

stress demonstrated that NRF1 and PGC1α are not regulating the transcription of

DNMT1[i] in the mitochondria.  However, we observed that p53 dissociated from the

DNMT1 promoter upon hypoxic stress, indicating that the up-regulation of

mtDNMT1 is through the relief of p53 suppression.  The findings of this

investigation indicated that mtDNMT1 is sensitive to oxidative stress through the

regulation by p53 and suggested that mitochondrial epigenetics may be playing an

integral role in the cellular stress response to hypoxia.

**Chapter 1: Introduction and Overview**

**I.    Overview of epigenetics: mechanism, function and significance.**

Epigenetics is the study of heritable changes in gene expression due to DNA modifications other than the underlying DNA sequence.   There are three types of epigenetic modification found in eukaryotic organisms: 1) DNA methylation, 2) histone modification, and 3) nucleosome positioning.   Each modification contributes to the stability and dynamic nature of chromatin structure, creating extra layers of control of gene expression and inheritance (1, 2).  In histone modification, the tails of histone proteins undergo multiple different posttranslational modifications including acetylation, methylation, phosporylation, and ubiquitination.   These posttranslational modifications result in the formation of either highly compact or open chromatin regions that can inhibit or promote binding of essential transcription factors, respectively (1).  Nucleosomes, which consist of approximately 165 bp of DNA wrapped around octamers of core histones (Figure 1-1), can be physically moved by chromatin-remodeling complexes, to create a more accessible region for transcription factors. Changes in nucleosome positioning can lead to both inhibition and activation of gene expression (2).

DNA methylation directly alters cytosine bases after DNA synthesis through incorporation of a methyl group donated from S-adenosylmethionine (SAM) to the C-5 position of cytosine, resulting in the formation of 5-methylcytosine (5mC) (Figure 1-2).  Methylation patterns are established early in embryogenesis and maintained throughout development.  DNA methylation commonly occurs in

1
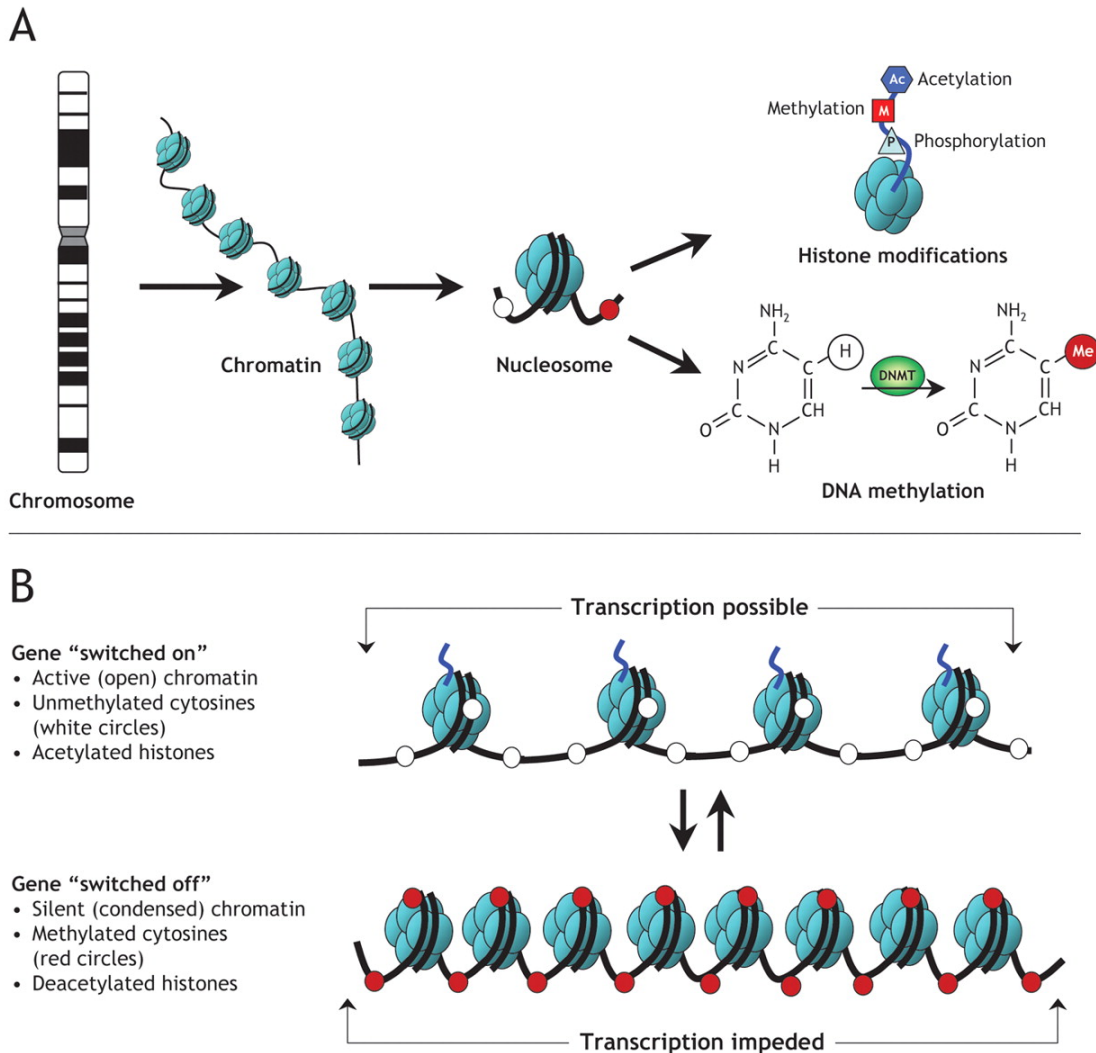
**Figure 1-1: Schematic diagram of epigenetic modifications.** (A) DNA wraps around octamers of core histones to form a nucleosome, which can be altered by three types of modification; nucleosome positioning, histone modification and DNA methylation. (B) All three modifications can result in either stimulation or inhibition of gene transcription, depending on the orientation of the nucleosome relative to the transcription machinery (55).

repetitive DNA sequence and sparse CpG regions of somatic cells (1). The consequence of methylated CpG sites is inhibition of gene transcription mainly due the recruitment of HDACs and other histone modifying enzymes, leading to the establishment of a silenced chromatin configuration. The enzymes responsible for *de novo* and maintenance methylation of mammalian nuclear genome form a family of four active DNA methlytransferases. DNMT3a and DNMT3b are involved in *de novo* methylation during early development while DNMT1 is responsible for maintaining established methylation patterns during DNA replication (4). DNMT2 is a tRNA methyltransferase and does not methylate DNA (5).

Many reports on the role of DNA methylation in the progression of human disease demonstrate its profound influence on both physiological and pathological processes via gene regulation (5,6). When abnormal methylation patterns manifest due to either dysfunction of the DNMT enzymes or other factors involved in DNA methylation such as MBD proteins, inappropriate gene expression can occur. This change in gene expression can dictate the development of many human disorders and diseases by changing transcription of vital genes. For example, Fragile X syndrome is an inherited disorder that presents with the expansion of CGG repeats in the 5'-untranslated region of the fragile X-mental retardation 1 (FMR1) gene, an important regulator of synaptic plasticity in the brain. The full mutation range results in an expansion of CCG repeat that virtually covers the entire FMR1 gene including CpG islands. The formation of new CpGs from such expansion promotes methylation of the promoter region of the gene.
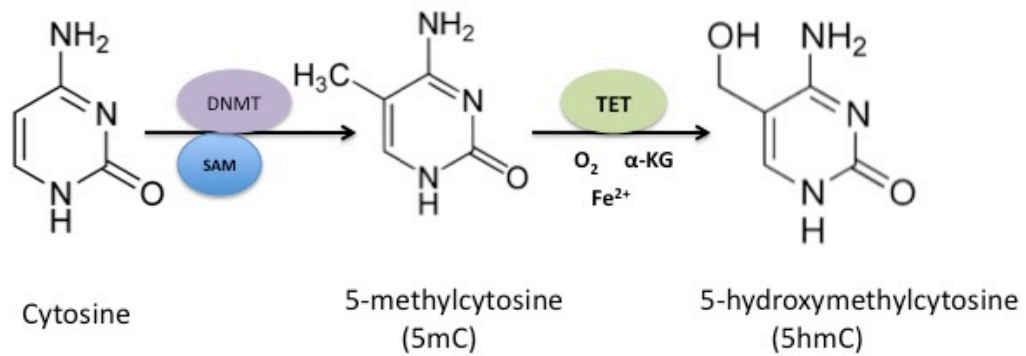
**Figure 1-2: Mechanism of DNA methylation.** The cytosine base in DNA can be methylated by DNA Methyltransferase to form 5-methylcytosine (5mC). The methyl group from SAM is transferred to the 5' carbon of cytosine. 5mC can further be modified by the family of TET enzymes to form 5-hydroxymethylcytosine (5hmC). 5hmC, which appears to be important for DNA demethylation.

The methylation of the CCG repeats as well as CpG islands within the promoter region attracts Methyl-C binding protein (MeCp2) to the methylated DNA region. MeCp2 recruits HDAC and other chromatin remodeling proteins, resulting in a closed chromatin that is inaccessible to the transcription machinery, preventing expression of the FMR1 gene (9). Several neurodegenerative diseases are associated with disruption of the methylation pattern. One of the characteristics of Parkinson's disease (PD) is the accumulation of α-synuclein protein, a key component of Lewy bodies, which are abnormal intracellular inclusions that can be found in neuronal cells. Aggregates of Lewy bodies in the brain are present in the majority of patients with this disease. The α-synuclein gene is hypomethylated at intron-1 in PD, contributing to the increased expression of the gene. The accumulation of α-synuclein along with amyloid-β is associated with pathophysiology of PD (6).

One indicator of early tumorigenesis is aberrant *de novo* methylation of CpG islands surrounding gene promoters, as well as a general loss of CpG dinucleotide methylation resulting in global hypomethylation (Figure 1-3). The majority of genes that are associated with aberrant hypermethylation in cancer cells are tumor suppressor genes, such as p16 and VHL (7). In addition, a large body of genes involved in the fundamental pathways leading to cancer showed aberrant hypermethylated CpG islands in their promoter regions. Pathways including cell cycle control (Rb, p16), DNA damage repair (BRCA1), inhibition of apoptosis (DAP-kinase), growth factor response (ER, RARβ) and tumor invasion (E-caherin, APC), are all affected by hypermethylation in many different types cancer (7,8).

**A**

Normal / Cancer diagram

nonmethylated Cytosine
methylated Cytosine

**B**

Normal / Cancer diagram

nucleosome
repressive complex (e.g. PcG)
methylated DNA binding protein
H3K4 methyl mark
H3K9/K27 methyl mark
acetylation mark
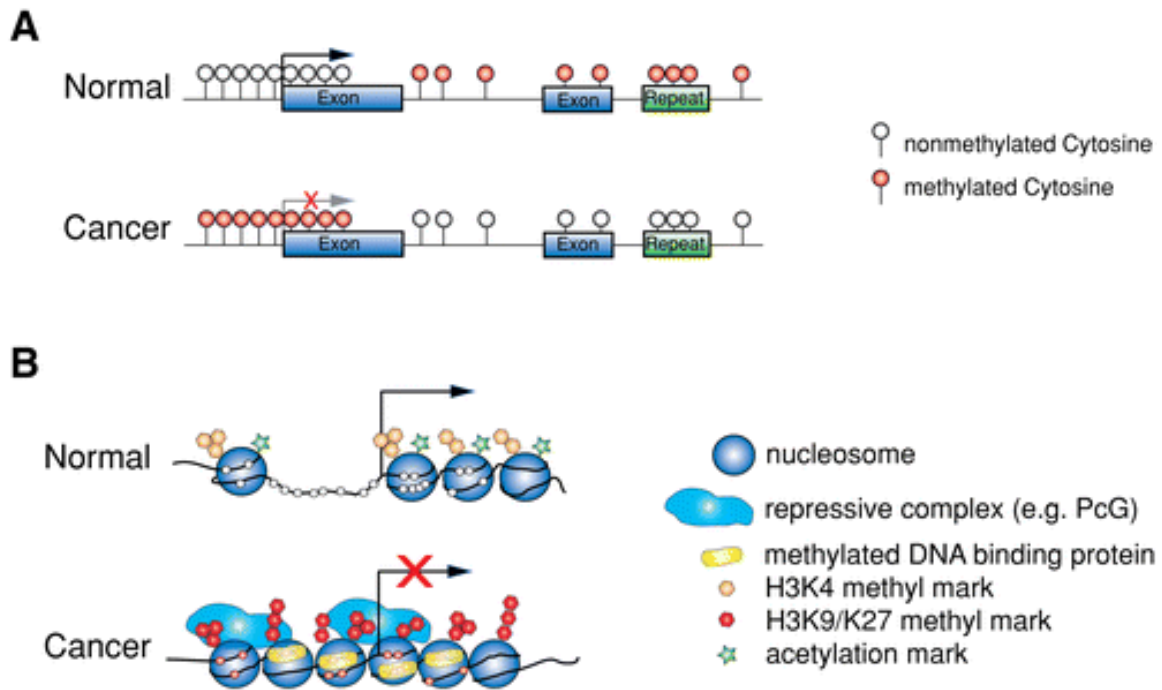
Gal-Yam EN, et al. 2008.
Annu. Rev. Med. 59:267–80

**Figure 1-3: DNA methylation pattern in normal and cancer cells.** (A) Compared to normal somatic cells, the pattern of methylation in the majority of cancer cells is characterized by hypermethylation of CpG islands within the promoter region as well as global hypomethylation. (B)Methylation of CpG islands promotes inhibition of transcription by recruiting repressive complex to the chromatin region (56).

It is evident that DNA methylation plays a crucial role in the development

and progression of human disease through transcription regulation.   Thus,

understanding the underlying mechanism of DNA methylation, especially the

regulation of the enzyme responsible for the modification, is important in terms of

finding potential biomarkers for diagnosis or drug targets for therapeutic

treatments.

**II.      Mitochondrial structure, function and epigenetics.**

Although DNA methylation has been extensively studied at the nuclear level,

our understanding of this modification in the mitochondrial DNA (mtDNA) remains

elusive.   Mitochondria are intracellular, membrane-enclosed organelles responsible

for producing ATP for the energy needs of the cells through electron transfer and

oxidative phosphorylation.  These organelles contain DNA stored in a double

membrane compartment, independent of the nuclear genome.   With a size of

approximately 16.5 kb, mtDNA encodes only 13 proteins, 2 rRNAs and 22 tRNAS.

All of the protein products of mtDNA are involved in the respiratory chain, such as

cytochrome b and NADH dehydrogenase subunit 6 (ND6). Mitochondrial encoded

RNA are critical for the mitochondrial translational system (Figure 1-4).  The

remaining >1500 proteins found in the mitochondria and required for

mitochondrial biogenesis, maintenance, and function, are encoded in the nucleus

and transported to the mitochondria, often via a mitochondrial targeting signal (10).

It has been known for some time that mtDNA contains 5mC at CpG dinucleotides,

although at very low levels, but the mechanism for generating this modification was
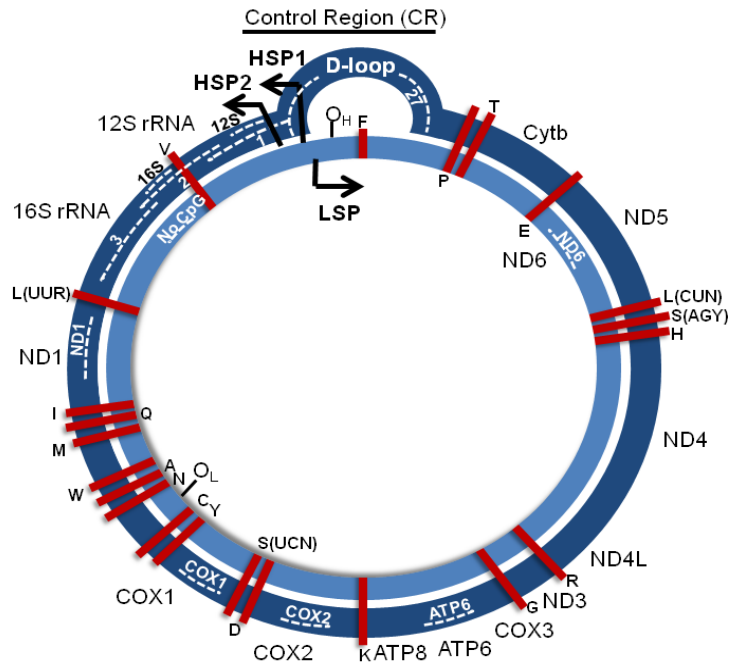
**Figure 1-4: Diagram of mitochondrial DNA (mtDNA), highlighting its general features and encoded genes.** MtDNA is double stranded containing a light strand and heavy strand. Its size is approximately 16.5 kb, encoding 13 proteins involved in cellular respiration and metabolism. Each mitochondrion can contain many individual genomes and there are hundreds to thousands of mitochondria per cell (3).

unknown (3,4).  Our lab recently demonstrated that the protein responsible for cytosine methylation in mtDNA is a novel isoform of DNA Methyltransferase 1, mtDNMT1.   This protein is encoded in the nucleus and targeted to the mitochondria via a N-terminal targeting sequence.  Through the use of a TAP-tagged DNMT1 cell line developed by Lisa Shock in our lab, it was discovered that DNMT1 is efficiently translocated to the mitochondrial matrix and interacts with mtDNA in a CpG dependent manner.  In addition to cytosine modification in the mtDNA, we also demonstrated that mtDNMT1 plays a role in the regulation of mtDNA transcription. Our data demonstrated that, when mtDNMT1 is over expressed, the transcription of the only protein-coding gene in the light strand, NADH-dehydrogenase chain 6 (ND6) is reduced, while the transcription of the heavy strand remained unchanged, except for the expression of the NADH dehydrogenase subunit 1 gene (ND1), which is also enhanced (3).  Considering the functional significance of nuclear DNA methylation in dictating gene transcription and its role in control of development, these findings raise the possibility that a mitochondrial epigenetic system plays a role in mtDNA transcription, biogenesis and overall function.

**III.     Nuclear regulation of mitochondrial function and biogenesis.**

Mitochondrial function and biogenesis are regulated by several nuclear factors in response to external stimuli such as temperature, oxygen level, chemicals and diet. Two proteins in particular are considered to be key players in nuclear-mitochondrial regulation, PGC1$\alpha$ and NRF1 (11).  PPAR$\gamma$ coactivator-1, or PGC1$\alpha$ is a member of a family of co-activators that are responsible for inducing signaling

pathways in response to environmental stimuli. PGC1$\alpha$ is induced by external

stimuli such as cold temperature (12), low glucose level (14,15), endurance exercise

(13) and cellular stress (11). Wu and colleagues demonstrated the importance of

PGC1$\alpha$ in mitochondrial biogenesis and cellular respiration. Through ectopic

expression of PGC1$\alpha$ in pre-adipocytes and myoblasts, Wu and colleagues showed

that both nuclear and mitochondrial gene expression increased which led to an

increase in mtDNA. The ectopic expression of PGC1$\alpha$ also correlated with the

increase in both total respiratory capacity and mitochondrial uncoupling, proving

the functional significance for PGC1$\alpha$ in the mitochondria (14). In addition to its

role in mitochondrial biogenesis and cellular respiration, PGC1$\alpha$ is a vital player in

the antioxidant defense system. In the presence of reactive oxygen species (ROS),

which are byproducts of oxidative stress, PGC1$\alpha$ is immediately induced and

stimulates the expression of a large body of genes involved in the removal of ROS

and repair of damaged cellular components due to oxidative stress (16, 17)

PGC1$\alpha$ links external stimuli directly to mitochondrial biogenesis and

respiration through activation of downstream targets. PGC1$\alpha$ activates the

transcription of various nuclear encoded genes including PPAR$\gamma$, UCP-2, NRF-1 and

NRF-2. The transcriptional effect of PGC1$\alpha$ on NRF-1 (Nuclear Respiratory Factor -

1) is mediated by a direct binding of NRF1 protein on a region near the amino-

terminal activation domain of PGC1$\alpha$. This interaction correlates with stimulation

of cellular growth and respiration through activation of target genes including

NADH
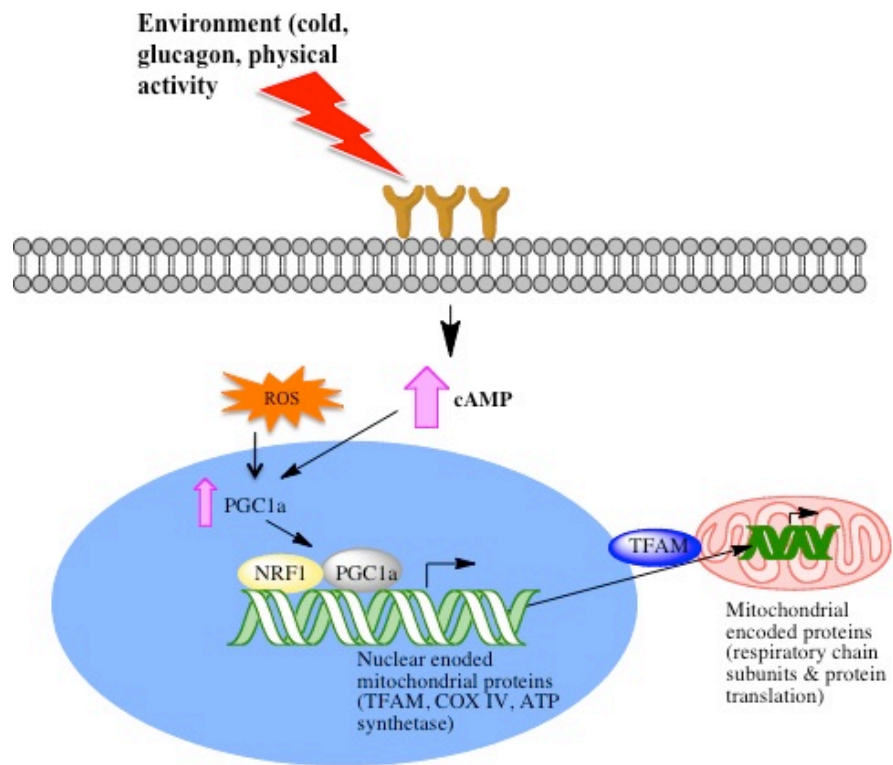
**Figure 1-5: Schematic diagram of the pathway of mitochondrial biogenesis through PGC1a and NRF1.**

dehydrogenase, cytochrome oxidase subunit and ATPase 6, as well as genes involved in mtDNA transcription and replication (Figure 1-5) (12,14). In vivo studies demonstrated that NRF-1 controls respiratory chain expression, early embryonic development and mitochondrial replication, indicating its significance in the nucleo-mitochondrial interaction (15,18,19). Induction of NRF-1 by serum growth factor treatment corresponded to rapid induction of cytochrome *c* mRNA, increased mitochondrial oxidative capacity and mitochondrial density (15). Homozygous gene disruption of NRF-1 is lethal in mice at embryonic day 3.5 -6.5. The blastocysts of these homozygous null nice also showed defects in mitochondrial membrane potential maintenance, severely reduced mtDNA and increased apoptosis (18). In terms of mtDNA transcription, NRF-1 directly activates the TFAM gene, which encodes a potent mitochondrial transcription factor that regulates mtDNA replication and transcription. In vitro studies showed that TFAM binds to sequence elements in both the heavy strand (HSP) and light strand (LSP) promoters within the D-loop region and stimulates transcription of mtDNA (15).

We recently demonstrated that the coding sequence of human DNMT1 contains a consensus NRF-1 binding site within the promoter region. This binding site overlaps with a putative p53 binding which was previously shown to repress DNMT1 mRNA and protein levels (26). Since DNMT1 contained this NRF-1 binding site, we asked whether DNMT1 was regulated by this transcription factor. Through a transient transfection experiment in HCT 116, the lab demonstrated that NRF-1 and PGC1α overexpression increased both mRNA and protein levels of mtDNMT1; the most substantial increase was evident when the two were transfected together

(3).  We therefore sought to determine whether mitochondrial DNA methylation plays an integral role in the cell's response to conditions in which PGC1α and NRF-1 are induced.   Overall, the investigation aimed at finding the link between epigenetic modification and cellular response to environmental stimuli such as oxidative stress.

**IV.     Overview of oxidative stress and the mitochondrial response mechanism.**

In a state of oxidative stress, where the concentration of reactive oxygen species or ROS in the cell is excessive due to an imbalance of ROS production and removal, PGC1α is induced and acts on its target genes including NRF-1.  Together they regulate expression of several nuclear-encoded mitochondrial proteins to maintain oxidative homeostasis (21).  In vivo, oxygen is reduced to superoxide anion ($O_2\bullet$) by NADPH oxidase and NO synthase, in a tightly regulated process. Superoxide anion gets reduced further to the different species of ROS including hydrogen peroxide ($H_2O_2$), and a powerful oxidant hydroxyl radical ($OH\bullet$), by various enzymes (Figure 1-6).  Oxidative insult, such as UV irradiation, can lead to gain/loss of function and/or dysregulation of NADPH oxidase and NO synthase, which ultimately elevates the production of ROS in the cell (17).   However, the bulk of ROS production is in the respiratory chain, largely due to the fact that it is less regulated compared to NADPH oxidase/NO synthase ROS production (Figure 1-6).
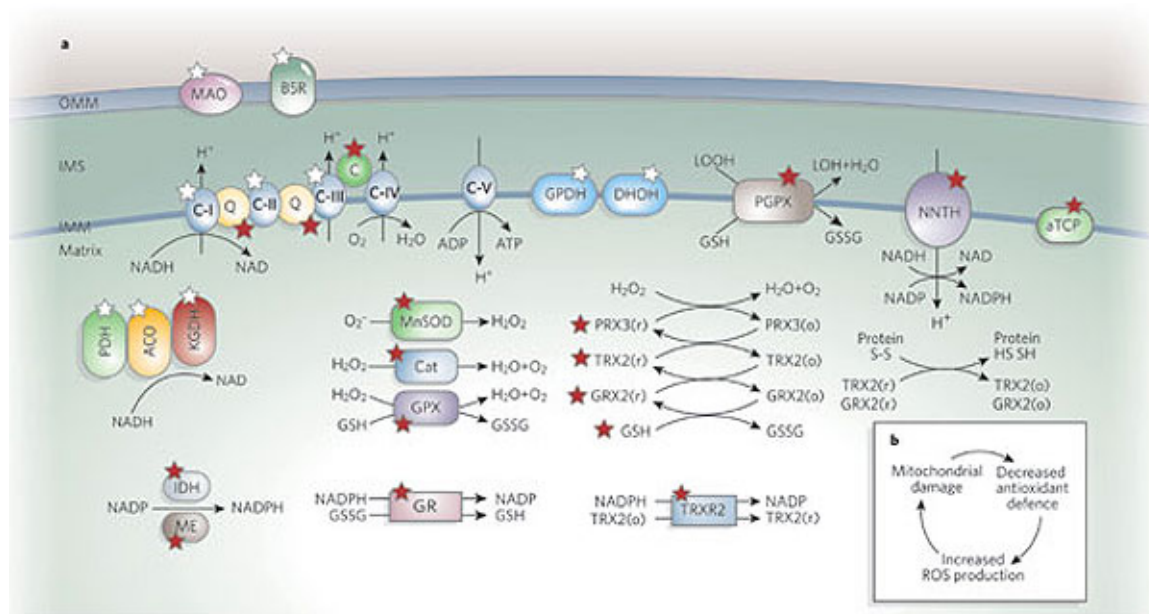
**Figure 1-6: Schematic diagram of the various mechanism for ROS formation in the mitochondria.** The mitochondria are the main source and target of ROS. ROS are produced at different sites of the respiratory chain as well as by separate enzymatic reactions within the inner mitochondrial membrane mitochondrial matrix. (A) The red stars mark the various site of ROS production. (B) Feedback pathway of the effect of an increase level of ROS in the mitochondria. (54)

ROS are produced when there is an elevated influx of electrons in the electron transport chain (ETC) causing some to escape from different complexes and react with molecular oxygen in the mitochondria matrix, forming superoxide radicals (19,22).   In addition, many of the complexes, especially cytochrome c oxidase (complex IV), can produce increased levels of ROS without the presence of oxygen by reducing nitrate to nitric oxide.  This is significant when cells are exposed to low oxygen availability or hypoxia and the oxidative stress is further enhanced (27).

Under normal conditions, the level of ROS is closely regulated in the cell. Antioxidant enzymes such as superoxide dismutase (SOD) and catalase are constantly scavenging for the presence of ROS and transforming them into less reactive species (19).  However, uncontrolled increase of ROS either by a defective antioxidant defense system, a change in the condition of the mitochondrial matrix that encourages electron leakage, or exogenous sources such as UV irradiation or heavy metals, can lead to oxidative stress.   In this case, excess ROS can escape the mitochondrial matrix and interact with DNA, protein and lipids causing detrimental effects on protein activity, lipid composition and membrane integrity.   ROS can also create DNA lesions by strand breakage, base deletion and chromosomal rearrangement (22,24).  In response to damage induced by ROS, the cell activates enzymatic and nonenzymatic defense mechanisms to eliminate excess ROS and repair damage.  Being the main intracellular source and target for ROS, mitochondria immediately suffer from ROS mediated damage.  All mitochondrial constituents including the machinery for energy production and cellular respiration

can potentially be affected by ROS.  Prolonged harmful exposure to ROS can lead to mitochondrial dysfunction and ultimately programmed cell death (24,25)

In response to stressors such as temperature, low glucose level and elevated ROS concentration, PGC1$\alpha$ stabilization represents an early and immediate response.  In order to alleviate elevated ROS levels in the mitochondria, PGC1$\alpha$ activates a large body of nuclear genes, including NRF-1, that are involved in enzymatic and non-enzymatic antioxidant defense mechanisms.  The genes that encode superoxide dismutase and catalase are also downstream targets of PGC1$\alpha$. These enzymes are considered ROS scavengers, converting  ROS into less harmful forms (19,24).  Most importantly, PGC1$\alpha$, in response to oxidative stress, stimulates mitochondrial biogenesis and respiration as a compensatory action against the damage done by oxidative stress. (Figure 1-6)

**V. Summary and Objectives**

Our lab recently discovered that the coding sequence of DNMT1 contains a NRF-1 binding site immediately upstream of the published transcriptional start site. Bioinformatic analysis demonstrated that this NRF-1 binding site coincidently overlaps with a p53 binding site (Figure 1-7).  This binding site is conserved among mammalian species, suggesting its functional importance (Table 1-2).  Transient transfection of HCT 116 cells with NRF-1 and PGC1$\alpha$ demonstrated that these two transcription factors up regulate mtDNMT1 protein level, particularly when NRF-1 and PGC1$\alpha$ are present together (Figure 1-8) (3).  In an earlier study, the Taylor lab showed that p53 was able to bind to this particular region of the DNMT1 promoter,

resulting in suppression of DNMT1 transcription. The association of p53 at this

binding site decreased after DNA damage, contributing to an increase in DNMT1

levels in the cell (26). With these findings, we hypothesize that oxidative stress, a

condition that induces PGC1$\alpha$, alters mtDNMT1 protein expression and mtDNA

methylation, largely through the regulation of transcription factors such as NRF-1

and p53, via the NRF-1 binding site within the DNMT1 promoter. This study aimed

to understand the functional importance of this consensus NRF-1 binding site and

whether it plays a role in the cellular response to oxidative stress. Taking into

account the significance of PGC1$\alpha$ and NRF-1 in oxidative homeostasis through the

activation of several downstream nuclear and mitochondrial genes, we

hypothesized that DNMT1 was one of the critical targets during oxidative stress is

likely. The studies described in this thesis aimed to: 1) determine whether oxidative

stress in the cell alters the level of expression of mtDNMT1 and 2) investigate

whether NRF-1 and PGC1$\alpha$ play a role in the transcriptional regulation of mtDNMT1

during oxidative stress.

| NRF1 consensus | YGCGCAYGCGCR | Base Pair Position |
| --- | --- | --- |
| Mouse | CGCGCATGCGCA | -164 to -153 |
| Human | TGGGCATGGCCG | -191 to -180 |
| Chimp | TGGGCATGGCCG | -191 to -180 |
| Rat | TGCGCATGCGCA | -164 to -153 |
| Cow | CGCGCATGCGCG | -205 to -194 |

**Table 1-2: Consensus sequence of putative NRF-1 binding sites within the mitochondrial leader sequence for DNMT1 in different species** (3).

**HUMAN DNMT1**

```
            1
  X    M    N    E    C    L    G    H    R    T    H    L
  tga  ATG  aat  gaa  tgc  ctc  ggg  cac  cgt  gcc  cac  ctc

  P    A    N    R    G    A    W    T    S    P    L    L    R    V    G
  cca  gca  aac  cgt  gga  gct  tgg  acg  agc  cca  ctg  ctc  cgc  gtg  ggg
                                                                2
  G    V    C    A    R    L    A    H    A    C    S    L    G    M    A
  ggg  gtg  tgt  gcc  cgc  ctt  gcg  cat  gcg  tgt  tcc  ctg  ggc  ATG  gcc

  tss 2
  G    S    V    P    S    F    C    T    G    Y    R    L    S    P    F
  GGC  TCC  GTT  CCA  TCC  TTC  TGC  ACA  GGG  TAT  CGC  CTC  TCT  CCG  TTT

  G    T    S    P    P    P    P    R    P    D    W    G    G    R    R
  GGT  ACA  TCC  CCT  CCT  CCC  CCA  CGC  CCG  GAC  TGG  GGT  GGT  AGA  CGC

  R    L    R    S    S    P    L    P    I    G    F    R    A    K    S
  CGC  CTC  CGC  TCA  TCG  CCC  CTC  CCC  ATC  GGT  TTC  CGC  GCG  AAA  AGC

  R    G    A    C    A    A    A    A    A    S    A    E    A    S    E
  CGG  GGC  GCC  TGC  GCT  GCC  GCC  GCC  GCG  TCT  GCT  GAA  GCC  TCC  GAG
  3
  M    P    A    R    T    A    P    A    R    V    P    T    L    A    V
  ATG  CCG  GCG  CGT  ACC  GCC  CCA  GCC  CGG  GTG  CCC  ACA  CTG  GCC  GTC
```

**Figure 1-7: Genomic sequence showing the upstream mitochondrial leader sequence of DNMT1.** Bionformatic analysis of human DNMT1 demonstrated that upstream of the mitochondrial leader sequence (red font) is a NRF-1 binding site (highlighted pink) that overlaps a half site of the p53 binding site (underlined font). Green font indicates the coding sequence of nuclear DNMT1 including the published start codon, ATG3. Upstream and in frame of the published start codon are two potential ATG codons, ATG2 and ATG1. Use of either of these translation start sites results in a protein with a mitochondrial leader peptide. TSS2 represent the published transitional start site for nuclear DNMT1, while the start site for mitochondrial DNMT1 has not been indentified (3).
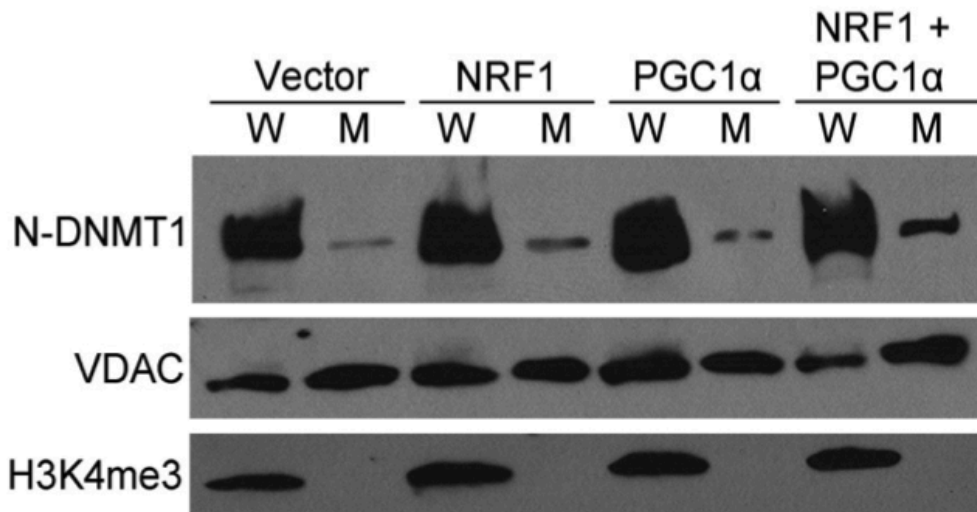
**Figure 1-8: Regulation of DNMT1 protein expression by PGC1α and NRF-1.**
Immunoblot of sub-cellular fractions of HCT 116 cells transfected transiently with
vectors containing NRF-1, PGC1α and NRF-1+PGC1α.  Total DNMT1 expression
stayed relatively constant, while mtDNM1 protein expression substantially
increased, especially when NRF-1 and PGC1α are present together.  VDAC was used
as a loading control and H3K4me3, a nuclear marker was used to indicate purity of
mitochondrial sample (3).

## Chapter 2: Materials and Methods

### Materials

SH-SY5Y cells were obtained from Dr. Michael Miles, Department of
Pharmacology and Toxicology, VCU.  HCT 116 cells were obtained from American
Type Culture Collection ( ATCC, Manassas, VA). Antibodies were purchased from
Abcam (Cambridge, MA),  Calbiochem ( Rockland, MA), Cell Signaling ( Danvers,
MA), Upstate Biotechnology ( Billerica, MA), Santa Cruz ( Santa Cruz, CA), Abnova (
Walnut, CA) , Thermo Scientific (Rockford, IL). A Bioruptor water bath sonicator
(Diagenode, Denville, NJ) was utilized to sonicate genomic DNA for MeDIP assay.
BioRAD DNA Engine Peltier thermal cycler connected to Chromo 4 Real-Time
Fluorescence Detector was used for qPCR studies.

### Cell Culture

SH-SY5Y cells were grown in DMEM medium (Gibco, cat #11995-065)
supplemented with 10% FBS, 5% PenStrep (Gibco, cat # 15140) and 5% L-
glutamine (Gibco, cat # 25030), at 37°C in 10% $CO_2$. HCT 116 cells were grown in
RPMI 1640 medium (Gibco, cat #11875093 ) supplemented with 10% FBS, at 37°C
in 5% $CO_2$.  Four T150 flasks (CytoOne, cat #CC7682-4815) with 80-90% confluent
cells were replated into 15 plug-cap T75 flasks (CytoOne, cat # CC762-4175) each
containing approximately 4 million cells.   Plug-cap flasks were used for ethanol
treatment to minimize evaporation.  Treatment was initiated 24 hours after
replating and cells were harvested 24 hours after treatment initiation.  Five
treatment conditions were used; no treatment control, 1mM Dibutyryl cAMP (Sigma,

D0627), 50 mM, 100 mM and 150 mM ethanol (Pharmco-AAPER, cat # 11ACS200).

Three plug-cap T75 flasks were used for each treatment, one flask for MeDIP

analysis and the remaining two for immunoblotting.

### Subcellular Fractionation

After treatment, the cells were placed on ice, washed twice with 5 ml of cold

PBS (Gibco, cat # 10010-023) then scraped using a sterile scraper.   For MeDIP

analysis, the cells (one flask for each condition) were collected in a pre-weighed 1.5

ml microcentrifuge tube. These tubes were stored at -80°C until MeDIP analysis.

The cells in the remaining two flasks were collected into a pre-weighed 15 ml Falcon

tube (VWR, cat # 21009-216) for immunoblotting.  The cells were pelleted at 2200

rpm for 5 minutes at 4°C.  The supernatant was removed and the pellet was

resuspended in 5 ml cold PBS.  Ten percent (500 µl) of this suspension was

transferred into a pre-weighed 1.5 ml centrifuge tube to generate whole cells lysates

(WCL). The aliquots were spun at 4500 rpm at 4°C  for 5 minutes, pellet weights

were recorded and samples were resuspended in SDS Lysis buffer (62.5 mM Tris pH

6.8, 5% glycerol , 2% SDS, 5% β-mercaptoethanol and 1x EDTA-free complete

protease inhibitor cocktail (Roche, cat # 11873580001)) at a volume 7.5x  the pellet

weight.   The lysates were thoroughly homogenized using a 1 ml syringe equipped

with a 21 G needle to shear DNA and reduce viscosity.

The remaining 90% of each sample was resuspended in 3 ml mitochondrial

homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl ph 7.0, 1mM EDTA ph 6.8)

with 1 tablet EDTA-free complete protease inhibitor cocktail per 25 ml.  The

samples were incubated on ice for 5 minutes to allow the cells to swell in the

hypotonic buffer, then transferred into type B Dounce homogenizer.  The samples

were processed with ten strokes to break open the cells then transferred back to the

original 15 ml falcon tubes.  Samples were centrifuged at 2500 rpm for 5 minutes

and the supernatant containing post-nuclear components  (mitochondrial and

cytosolic fractions) were collected in new 15 ml Falcon tubes.   The pellets were

subjected to two additional homogenization steps with collection of the post nuclear

supernatant.  The pooled post-nuclear supernatant fraction from each sample was

spun at 2200 rpm at 4°C for 5 minutes to remove any remaining nuclear

components.  The supernatant was transferred into a 14 ml round-bottom tube (BD

Falcon, cat # 352059), leaving approximately 2 ml behind to avoid nuclear

contamination.  The mitochondrial fraction from post nuclear supernatant samples

was pelleted by spinning the tubes at 9000xg at 4°C for 15 minutes.   The

mitochondrial pellet was washed with 700 µl mitochondrial homogenization buffer

and transferred into a preweighed 1.5 ml microcentrifuge tube.  The mitochondrial

fractions were pelleted and weighed.   SDS Lysis buffer at a volume of 5x of the

pellet weight was added and the samples were thoroughly homogenized by passage

through a 23G needle.

**SDS Page and Immunoblotting**

The protein concentration in both mitochondrial and whole cell lysates was

determined using a Bradford Protein assay.  To obtain an accurate equal cell

equivalent in both mitochondrial and whole cell lysates, 75 µg of whole cell and 18

μg of mitochondrial lysates were used for immunoblotting. These quantities reproducibly yielded equivalent signals for the mitochondrial marker VDAC, and allowed an estimate of the relative abundance of DNMT1 in each compartment. The samples were mixed with an equal volume of Laemmli sample buffer (BioRad, cat#161-0737) with 5% β-mercaptoethanol and boiled for 5 minutes. 10 μl of protein marker (BioRad Precision Dual Color, cat #161-0374 ) and the lysates were loaded on a 4-15% SDS-Page Mini Protean TGX gel (BioRad, cat# 456-1084). The gel was assembled into a Mini-Protean electrophoretic box unit and the gel box was filled with approximately 1L of 1x running buffer (25 mM Tris Base, 250 mM glycine, 0.1% SDS). Power was supplied at 150 V and the gel was run for approximately 1 hour.

After SDS-PAGE, the gel was removed from the gel cast and equilibrated in cold 1X transfer buffer (25mM Tris, 192 mM glycine, 10% methanol, 0.1% SDS, pH 8.3) for 15 minutes at 4°C with gentle shaking. PVDF membrane (Millipore, cat# IPVH00010) was prepared by soaking in 100% methanol for 1 minute, then with deionized water for 2 minutes. The membrane was then equilibrated in cold 1X transfer buffer at 4°C for 15 minutes with gentle shaking. The wet transfer sandwich was assembled a using transfer plate, 6 pre–cut 3mm Whatman papers and 2 sponges all soaked in cold 1x transfer buffer. One sponge and 3 pre-cut Whatman papers were placed on top of the black face of the transfer plate. The SDS-PAGE gel was placed on top of the 3 3mm Whatman papers followed by the PVDF membrane and the remaining 3 3mm Whatman papers and sponge. Each layer of the sandwich was rolled smooth with a roller to remove any bubbles that can impair

the transfer process.  The transfer sandwich was inserted in the electrode holder and placed into BioRad Mini Protean transfer box (BioRad, 525BR).  The transfer process was carried out at 100 V for 1 hour with approximately 800 ml transfer buffer.

The membrane was carefully removed from the transfer sandwich and rinsed with 100% methanol, then soaked in deionized water for 2 minutes.  To check the efficiency of the transfer the membrane was stained with 20 ml Ponceau S stain (0.1% Ponceau S in 5% acetic acid) (Sigma, cat # P7170) for 5 minutes.  When the bands were clearly visible, the Ponceau stain was removed and the membrane was washed with deionized water until the background was white.  The membrane was blocked overnight in T20 StartingBlock blocking buffer (Fisher, ca # 37543) at 4°C with gentle shaking.

The primary and secondary antibodies were diluted in Starting Block blocking buffer at the optimized dilution (Table 2-1).  The membrane was removed from blocking buffer and cut, using a sterile razor, into strips to separate the expected regions for visualization of DNMT1, VDAC and H3K4me3 bands.  The blots were incubated in the appropriate primary antibody for 1hour at room temperature with gentle shaking.   After 1 hour, the primary antibody was collected for storage at -20 °C and the strips were washed three times with approximately 20 ml of 1x TBS-

| Antibody | Company | Species | Blocking Buffer | Primary Conc | Secondary Conc | Secondary Ab | Size (kDa) |
|----------|---------|---------|-----------------|--------------|----------------|--------------|------------|
| N-DNMT1 | Abcam | Rabbit | StartingBlock | 1:1000 | 1:10,000 | Goat anti-rabbit | ~185 |
| VDAC | Cell Signaling | Rabbit | StartingBlock | 1:2500 | 1:3000 | Goat anti-rabbit | ~32 |
| H3K4me | Upstate | Rabbit | StartingBlock | 1:2000 | 1:15,000 | Goat anti-rabbit | ~17 |
| PGC1a | Santa Cruz | Rabbit | StartingBlock | 1:500 | 1:2,500 | Goat anti-rabbit | ~90 |
| NRF-1a | Abnova | Mouse | StartingBlock | 1:200 | 1:2,500 | Goat anti-mouse | ~55 |
| HIF1a | Thermo Scientific | Rabbit | StartingBlock | 1:500 | 1:5000 | Goat anti-rabbit | ~120 |

**Table 2-1: Optimal conditions for all the antibodies used in immunoblotting**

T for 10 minutes at room temperature with vigorous shaking.   The membrane strips were then placed into the appropriate secondary antibody solution (Table 2-1) and incubated for 1 hour at room temperature with gentle shaking.   The strips were again washed three times with 20 ml 1x TBS-T for 10 minutes at room temperature with vigorous shaking.   The ECL working solution was prepared by mixing reagent 1 and reagent 2 of the SuperSignal West Pico Chemiluminescent Substrate (Pierce, cat# 34080) at a 1:1 ratio.  The membrane was assembled back into original shape, and it was incubated in the ECL working solution for 5 minutes under a foil cover. Blots were developed using autoradiography film (ISC Bioexpress, cat#F-9024-8X10) and a Konica SRX-101A detector.

## Methylated DNA Immunoprecipitation

Cells from one T75 flask were pelleted at 4600 rpm at 4°C for 5 minutes.  The pellet was resuspended in 400 µl cold PBS.  Genomic DNA (gDNA) was isolated and purified from these samples using a QIAPrep Spin Miniprep Kit (Qiagen, cat# 27104).  The purified genomic DNA samples were quanitated using NanoDrop ND-1000 V3.1.2 software.   Three antibodies were used for the pull down; IgG (Millipore, cat#12-371), anti 5-methylcytidine (Active Motif, cat#39649) and anti 5-hydroxymethylcytidine(Active Motif, cat#39769).  Four micrograms of DNA were used per immunoprecipitation, a total of 12 µg per condition.   Aliquots of 12 µg gDNA from each condition were sonicated using the Diagenode Bioruptor water bath sonicator at high pulse for 15 minutes with 30 second off/on cycles.  The samples were sonicated for a total of 2 rounds to achieve the optimal fragment size

of 200-600 bp.  The size of the sonicated gDNA was validated using gel electrophoresis and a 1% TAE-EtBr agarose gel.

Once sheared to the appropriate size, the samples were diluted in 1X TE Buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and boiled for 10 minutes.  51 µl of 10X IP Buffer (100 mM Na-Phosphate, pH 7.0, 1.4 M NaCl, 0.5% Triton X-100) and 2 µl of the appropriate antibody were added to the boiled gDNA samples.  The tubes were incubated overnight at 4°C with rotation.  260 µl of IgG sepharose beads (GE Healthcare, cat # 17-0969-01) were pipetted from stock using a 2-200 µl tip with a cut off end.  The beads were pelleted at 4600 rpm for 5 minutes at 4°C and ethanol supernatant removed.  The IgG beads were washed with 400 µl fresh 0.1% BSA-PBS buffer, rotated for 5 minutes at 4°C and repelleted.  The beads were washed for a total of three rounds.  The IgG beads were resuspended in an equal volume of 1X IP Buffer.  To avoid non-specific binding in the immunoprecipitation, 5 µg of BSA and 5 µg of sonicated lambda DNA was added per 30 µl of bead/buffer mixture.  The beads were incubated overnight at 4°C with rotation.

The blocked beads were washed with 400 µl of fresh 0.1% BSA-PBS buffer three times.  The beads were resuspended in equal volume of 1X IP Buffer.  To each DNA/antibody sample, 20 µl of the bead/buffer mixture was added.  The samples were incubated for 2 hours at 4°C .  The beads were collected after incubation and washed three times with 500 µl 1X IP Buffer.  The pelleted beads were resuspended in 250 µl Proteinase K Digestion buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 0.5% SDS) with 3.5 µl of 20mg/ml Proteinase K (Bioline, cat# Bio-37037) and incubated in 50 °C water bath for 3 hours.  The processed samples were subjected to

phenol/chloroform extraction followed by ethanol precipitation with 1 μl of glycogen, 20 μl of 3M sodium acetate (1/10 of total volume), 500 μl of 100% ethanol (2.5X of total volume).   Precipitated DNA samples were washed with 70% ethanol then resuspended in 60 μl TE Buffer for PCR analysis.

### Hypoxia Induction Using Chemical Inducers.

HCT 116 cells in 2 T175 flasks at 90% confluency were replated into six 150 mm culture dishes corresponding to the three treatment; no treatment, cobalt chloride treatment and deferoxamine mesylate treatment.   Two 150 mm culture dishes were used for each condition.  Cells were fed with 20 ml media and allowed to grow for 24 hours under the appropriate treatment.   The media was replaced after 24 hours and 100 μM cobalt chloride (Sigma, cat# C8661) or 100 μM deferoxamine mesylate (Sigma, cat #D9533) was added drop-wise to the appropriate samples.   The cells were harvested 24 hours after initiation of treatment.

Samples were placed on ice and whole cell and mitochondrial lysates were isolated as described above.  Samples were subjected to SDS-PAGE and immunoblotting as described in section 4.

### Time course of induction of hypoxia using cobalt chloride and DFO.

For each of the three treatment (control, cobalt chloride and DFO), two 150 mm culture dishes were assigned to one time point.  A total of four time points were included in the experiment; 12, 24, 36 and 48 hours.  HCT 116 cells were replated

from 4 x T174 flask containing approximately 90 million cells to 24 x 150mm culture dishes.   The number of cells plated for each sample corresponded to a total of 32 million cells at the end of the time point. The cells were fed with 20 ml RPMI media supplemented with 10% FBS and allowed to grow for 24 hours in the appropriate condition.  Media was replaced after 24 hours and the drugs (100 μM cobalt chloride, 100 μM DFO) were carefully reapplied drop wise.   The cells were allowed to grow at the appropriate condition until the end of the time point. The samples were fed every 24 hours, replacing the media and drugs until the end of the time point.

At each time point, two 150 mm culture dishes were obtained and whole cell and mitochondrial lysates were obtained as described in section 3.   The purified lysates were subjected to SDS-PAGE and immunoblotting as described in section 4.

**Chromatin Immunoprecipitation**

HCT 116 cells from one T175 flask at 90% confluency were replated into 3 150 mm culture dishes corresponding to the three conditions: no treatment, cobalt chloride treatment, and DFO treatment.  Each culture dish contained 7.5 million cells, 1.5 million per immunoprecipitation.  The cells were fed with 20 ml media and allowed to grow for 24 hours at the appropriate condition.  The media was replaced after 24 hours and 100 μM cobalt chloride or 100 μm DFO was added drop-wise to the appropriate samples.   The cells were prepared for ChIP 24 hours after treatment initiation.

Samples were placed on ice and media was removed. The cells were cross-linked with 20 ml of 1% formaldehye in RPMI without FBS (Fisher, cat # F79-500) at room temperature for 10 minutes with gentle shaking. The cross-linking process was quenched with 3 ml of 1 M glycine for 5 minutes at room temperature with gentle shaking. The solution was removed and the cells were washed with 5 ml ice cold PBS supplemented with 1 mM PMSF (Sigma, cat# P7626). The cells were scraped using a sterile scaper into individual 15 ml Falcon tubes and repelleted at 2000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet was washed with 1 ml Wash Buffer I (0.25% Triton-X, 10 mM EDTA pH 8, 0.5 mM EGTA pH 7.5, 10 mM Hepe pH 7.5). The cells were repelleted, supernatant was removed and the pellet was washed with 1 ml Wash Buffer II (0.25 mM NaCl, 1 mM EDTA pH 8, 0.5 mM EGTA pH 7.5, 10 mM HEPES pH 7.5). The cells were repelleted and resuspended in 1 ml lysis buffer (150 mM NaCl, 25 mM Tris pH 7.5, 5mM EDTA pH 8, 1% Triton X-100, 0.1% SDS, 0.5% NaDoC). From each condition, the cells were divided into 5 x 1.5 microcentrifuge tube (200 µl) each containing 5 million cells, corresponding to the 5 immunoprecipitations. The samples were sonicated using the Diagenode Bioruptor water bath sonicator at high pulse for 20 minutes with 30 second off/on cycles. The samples were sonicated for one round to achieve the optimal fragment size of 200-600 bp. An aliquot from each sample was obtained to check the size of the chromatin samples. Prior to checking, the samples were RNase A and proteinase K treated then subjected to phenol/chloroform extraction followed by ethanol precipitation with 0.5 µl of glycogen, 2 µl of 3M sodium acetate and 200

μl of 100% ethanol.   The size of the sonicated DNA was validated using gel electrophoresis in a 1% TAE-EtBr agarose gel.

To reduce non-specific binding, the chromatin samples were pre-cleared prior to immunoprecipitation.  Two 300μl aliquots of IgG sepharose beads were obtained; one set for preclearing the chromatin sample and one for immunoprecipitation.  The beads were pelleted at 4500 rpm for 5 minutes at 4°C to remove the residual ethanol.  The beads were washed twice with 400 μl lysis buffer, then repelleted.  The lysis buffer wash was removed and the beads were resuspended in equal volume of lysis buffer.  To prevent nonspecific binding, 8 μg of BSA and 8 μg of sonicated salmon DNA was added per 30 μl of bead/buffer mixture. The beads for pre-clearing were incubated at 4°C for 3 hours while the beads for immunoprecipitation were incubated under the same conditions overnight.   The aliquot of beads for pre-clearing was spun to repellet the beads.  The buffer was removed and the beads were washed three times with 400 μl lysis buffer.  The beads were then resuspend in an equal volume of lysis buffer.  To each sonicated chromatin sample, 30 μl of the bead/buffer mixture was added.  The samples were incubated at 4° for 1 hour with rotation.  The chromatin/bead samples were centrifuged and the supernatant, which contained the pre-cleared chromatin, was transferred to a new 1.5 ml microcentrifuge tubes.  5 μg of the appropriate antibody (IgG, NRF-1, PGC1α and p53) was added to each chromatin sample.   The tubes were incubated overnight at 4°C with rotation.

The pre-blocked beads for immunoprecipitation were washed three times with 500 ul lysis buffer and resuspended in an equal volume lysis buffer. 30 µl of the bead/buffer mixture was added to each chromatin sample and incubated for 1 hour at 4°C with rotation. The tubes were centrifuged to pellet the beads. The supernatant of the no antibody immunoprecipitation from the control condition was saved. This was used as the input for the standard curve in qPCR analysis. The supernatant from the rest of the tubes was discarded and the pellet was washed twice with 500 µl RIPA buffer (150 mM NaCl, 50 mM Tris pH 8, 0.1% SDS, 0.5% NaDoC, 1.0% NP-40). The beads were repelleted and washed with 400 µl high salt buffer (500 mM NaCl, 50 mM Tris pH 8, 0.1% SDS, 1.0% NP-40). The beads were repelleted and washed with 400 µl LiCl buffer (250 mM LiCl, 50 mM Tris pH 8.0, 0.5%NaDoc, 1.0% NP-40). The beads were repelleted and washed twice with 400µl TE buffer (1 M Tris pH 8, 0.5 M EDTA pH 8). The beads were repelleted and the chromatin was eluted with 200 µl Elution buffer (2% SDS, 10 mM DTT, 0.1M NaHCO3) for 15 minutes at room temperature with rotation. The supernatant, which contained the eluted chromatin, was transferred into a new 1.5 ml microcentrifuge tube. An additional elution step was performed and the supernatant were pooled. The eluted chromatin samples were subjected to reversal of cross-linking by adding 20 µl 4M NaCl to each sample and incubating overnight at 65°C.

The tubes were removed from incubation and 1 ml of 100% ethanol was added to each sample. The tubes were vortexed briefly, then incubated at -80°C for an hour. The precipitated DNA was pelleted at 16,000 x g for 10 minutes at 4°C. The

pellet was washed with 1 ml 70% ethanol.  The DNA was repelleted and the ethanol wash was removed completely. The samples were let to dry at room temperature for 5 minutes.  The dried pellet was resuspended in 100 μl TE buffer and 1 μl RNAse (10mg/ml) (Qiagen, cat # 19101) and incubated at 37 °C for 30 minutes.   The samples were then treated with 20 μl of 5x Proteinase K digestion buffer (50 mM Tris pH 7.5, 25 mM EDTA pH 8, 1.25% SDS) and 2 μl Proteinase K (20mg/ml) (Bioline, cat# BIO-37037).  The samples were incubated for 1 hour at 42°C.  The processed samples were subjected to phenol/chloroform extraction followed by ethanol precipitation with 1 μl of glycogen, 20 μl 3M sodium acetate (1/10 of total volume) and 500 μl of 100 % ethanol (2.5 X of total volume).  Precipitated DNA samples were washed with 70% ethanol, and resuspended in 100 μl TE buffer for PCR analysis.

## End Point and Quantitative PCR of ChIP and MeDIP/ hMEDIP products.

The immunoprecipitated DNA from the ChIP or MeDIP assay was subjected to end point PCR.  For each sample, 25 μl PCR reactions were prepared using 1 μl of template DNA, 10.5 μl of HPLC water, 12.5 μl of HotStarTaq Master Mix (Qiagen, cat # 203446) and 0.5 μl of each sense and antisense primers (15 μM).  The primers used for ChIP assay were designed to target the region around the NRF-1 binding site in the promoter region of DNMT1, while the primers used for MeDIP assays was a region of the ND6 gene (Table 2-2).  The samples were amplified using annealing temperature of 57°C for a total of 37 cycles.   The PCR products were separated on a

1% TAE agarose gel stained with ethidium bromide and visualized by UV transillumination.

The immunoprecipitated DNA samples were also analyzed by quantative PCR using Quantitect SYBR Green mastermix (Qiagen, cat #204163). Input DNA samples were quantitated on the NanoDrop spectrophotometer and diluted to make 100 µl working stocks of 10 ng/ul, 1 ng/ul, 0.1 ng/ul and 0.01 ng/ul each, to be used as a standard curve. 25 µl qPCR reactions were prepared using 1 ul of template DNA, 10.5 µl HPLC water, 12.5 µl SYBR Green master mix and 0.5 µl of each sense and antisense primer (15 µM). qPCR was performed using BioRad Peltier thermal cyclers, equipped with Chromo 4 detectors, at an annealing temperature of 57°C for a total of 45 cycles.

| Primer Set | Assay | Forward | Reverse |
|---|---|---|---|
| Hu DNMT1 Promoter | ChIP | 5'-ATC CCC ATC ACA CCT GAA AG-3' | 5'-CCT GTG CAG AAG GAT GGA AC-3' |
| Hu ND6 | Mito MeDIP | 5'-AAA CAC TCA CCA AGA CCT CAA CCC-3' | 5'-ATT GAT TGT TAG CGG TGT GGT CGG-3' |

Table 2-1: Sequence of the primer sets utilized in the study.  Primers for the ChIP assay were specific for the DNMT1 promoter region and were used in end point and qPCR analysis.  Primers used for MeDIP assay were specific for the ND6 coding region of mtDNA and were used for end point and qPCR analysis.

## Chapter 3: Results

### 1. Ethanol-induced oxidative stress increased DNMT1 expression in HCT 116 cells but not in SH-SY5Y cells

Previous studies suggested that a high concentration of ethanol alters the NADH/NAD+ ratio during ethanol metabolism by alcohol dehydrogenase. This change leads to an influx of electrons to the respiratory chain, facilitating the binding of electrons to molecular oxygen and ultimately forming superoxide anions (19,20, 25). We aimed to investigate the effect of ethanol-induced oxidative stress on the expression of total and mitochondrial DNMT1 in ethanol treated SH-SY5Y and HCT 116 cells. HCT 116 cells were used in this study for consistency with previous studies, since all previous data on DNMT1 regulation were carried out with these cells. The SH-SY5Y neuroblastoma cell line was utilized in this investigation due to a study done by the laboratory of Dr. Michael Miles (Department of Pharmacology and Toxicology, VCU) on ethanol-responsive genes in SH-SY5Y cells. They demonstrated that treating these cells with various concentrations of ethanol (50, 100, 150mM) produced a specific pattern of gene expression, including genes involved in apoptosis and oxidative stress response (glutathione metabolism) (26). Using the same conditions, we investigated whether DNMT1 was expression altered in both SH-SY5Y and HCT116 cells.

Immunoblot analysis of both cell lines showed the presence of DNMT1 (185 kDa) in both purified whole cell and mitochondrial fractions, using an antibody against N-terminal DNMT1 (Figure 2-1, 2-2). Total DNMT1 expression in SH-SY5Y cells treated with ethanol moderately decreased compared to control, while DNMT1
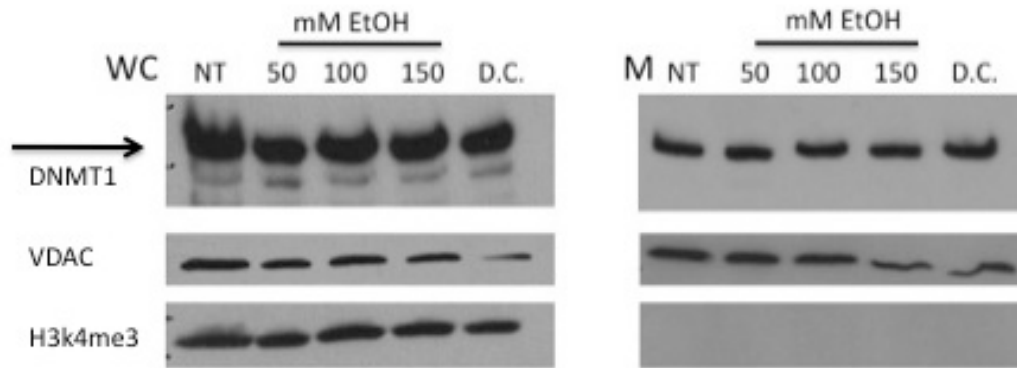
**Figure 3-1:  DNMT1 expression in SH-SY5Y alters in response to ethanol exposure.** Immunoblots of whole cell (WC) and mitochondrial (M) fractions from SH-SY5Y cells treated with ethanol for 24 hours.  The blot showed a moderate decrease of DNMT1 expression in the WC fraction but remained relatively equal in the M fraction compared to control.   The immunoblots are representative of 2 independent biological repeats.  Dibutyryl cAMP (D.C.) served as the positive control. VDAC was used as loading control and H3K4me3 was used to ensure that mitochondrial fractions were free from nuclear contamination. Exposure at 5 seconds using ½ Dura ECL substrate.

levels in all mitochondrial samples were similar to that of control (Figure 3-1). This observation suggested that upon ethanol exposure, DNMT levels in SH-SY5Y cells did not significantly change. Dibutryl cAMP (D.C.) is an analog of cAMP that can stimulate the cAMP-dependent PKA transduction pathway that regulates expression of genes required for cell growth (52). Studies have shown the D.C. can also activate stress response pathways through the regulation of heat shock proteins (HSP) (52). Various studies, including those of Dr. Miles (27) have used D.C. to mimic cellular stress response in culture cells. We expected therefore, that D.C. treatment would elicit a stress response in both SH-SY5Y and HCT 116 cells and subsequently increase DNMT1 protein expression. The result showed that level of total DNMT1 in D.C. treated cell decreased slightly while mtDNMT1 protein level remained equal to no treatment control. It appears that the effect of dibutyryl cAMP moderately affected the expression of total DNMT1 but not mtDNMT1. Overall the data gathered from the immunoblots of SH-SY5Y samples demonstrated that ethanol exposure did not significantly alter the expression of either total or mitochondrial DNMT1 in SH-SY5Y cells and that the effect of ethanol-induced oxidative stress is independent of the regulation of DNMT1 in these cells.

In HCT 116 cells, the pattern of DNMT1 expression was different from that observed in SH-SY5Y cells. The level of total DNMT1 remained unchanged relative to the no treatment control (50 and 150 mM ethanol) except at 100 mM, where total DNMT1 expression increased by approximately 2 fold. The mitochondrial fraction also showed an approximately 2-fold increase in DNMT1
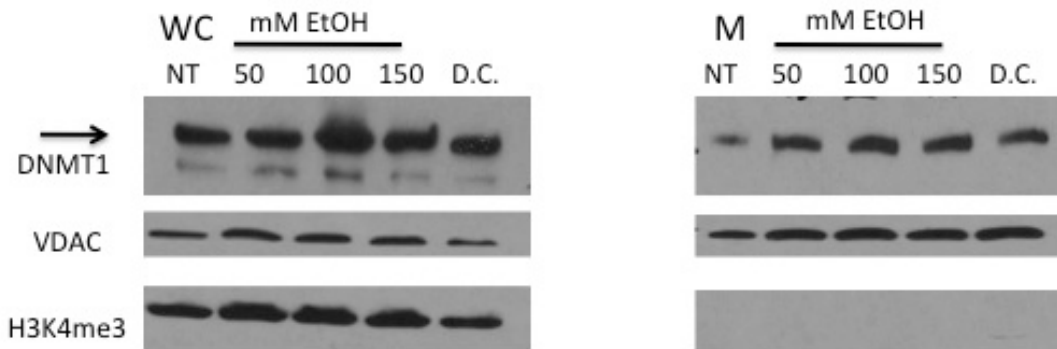
**Figure 3-2: DNMT1 expression increased in whole cell and mitochondrial fractions of alcohol treated HCT116 cells.** Immunoblot of whole cell (WC) and mitochondrial (M) lysates of HCT 116 cells with and without ethanol treatment (24 hours). The blot showed a dramatic increase in whole cell lysates that were treated with 100 mM ethanol and moderate increase of mtDNMT1 expression in all concentrations of ethanol. The immunoblots are representative of 2 independent biological repeats. VDAC was used for the mitochondrial loading control and H3K4me3 served as control for nuclear contamination. Exposure at 5 seconds using ½ Dura ECL substrate.

expression in all treated samples compared to control, including D.C. treatment (Figure 3-2).   Although the VDAC signals from the ethanol treated cells appeared to be bigger than no treatment control, the difference in VDAC band intensity between treated and non-treated control appeared to be smaller than DNMT1 signal between treated and non-treated samples.  Thus, if we double the intensity of no treatment to achieve equal VDAC signal, the DNMT1 signal will still be less than treated samples. We can determine the precise difference in band intensity by performing a densitometry to quantitate DNMT1 protein level of each sample.  Based on the data in figure 3-2, mtDNMT1 protein expression appeared to be selectively sensitive to ethanol exposure compared to total DNMT1.  The increase in mtDNMT1 expression was observed in conditions as low as 50 mM ethanol, while total DNMT1 expression did not increase until 100 mM ethanol.   The level of expression of total DNMT1 in whole cell lysates treated with D.C. was comparable to untreated cells.  This observation was similar to what was gathered from the SH-SY5Y cell lines, indicating that the effect of D.C. in the cell is not targeting nuclear DNMT1 expression.  Overall, the results indicated that in the HCT 116 cell line, ethanol induced oxidative stress results in the up-regulation of mtDNMT1.  The immediate increase of mtDNMT1 expression may be due to the induction PGC1α and NRF1 during oxidative stress.

Antibodies against specific nuclear and mitochondrial markers were used to demonstrate the purity and loading of the sub cellular fractions.  Voltage-dependent anion channel (VDAC), is a protein found on the outer membrane of the
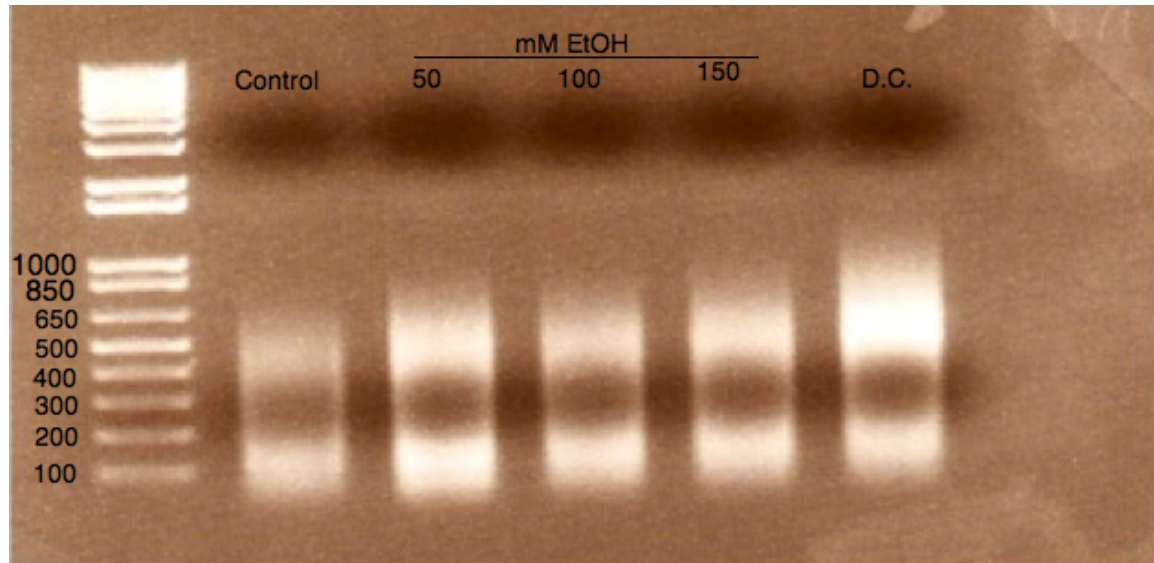
**Figure 3-3: Sonication of HCT 116 genomic DNA showed the appropriate fragment size for MeDIP.** 12 µg of genomic DNA isolated from ethanol treated cells was sonicated for 15 minutes with 30 seconds off/on to fragment the DNA to 200-600bp. Lane 1 represents the size ladder (1 kb+ ladder). Lane 2 represents the the no treatment control. Lane 2-4 correspond to the three ethanol conditions and Lane 5 represents D.C. samples. 5 µL of sonicated genomic DNA was loaded in each lane.

mitochondria and serves as a reliable mitochondrial marker(~32 kDa). Histone H3

Lysine 4 trimethyl (H3K4me3), a histone protein found exclusively in the nucleus

(17 kDA), was used as a nuclear marker. No signal for H3K4me3 antibody in our

mitochondrial fraction indicated the purity of the sample (Figure 3-2).

**MeDIP analysis of ethanol treated HCT116 showed elevated 5mC and 5hmC of mtDNA.**

We next asked whether the alcohol-induced increase of mtDNMT1 protein

level was paralleled by altered cytosine modifications in mtDNA. We measured the

level of cytosine modifications in mtDNA using DNA immunoprecipitation with

antibodies specific for 5mC and 5hmC. Total genomic DNA (gDNA) from ethanol

treated HCT 116 cells (50, 100, 150 mM) and D.C. treated cells was purified and

sonicated to fragment the DNA to an average size of 300 bp (Figure 3-3). DNA was

immunoprecipitated and the presence of cytosine modifications was quantitated by

end point and qPCR. Primers that target a region within the ND6 coding sequence in

mtDNA were used to amplify the DNA IP product (Table 2-2). End point PCR

indicated only a single amplification product in each sample and suggest a slightly

higher level of 5mC and 5hmC in the IP material from ethanol treated samples

compared to control (Figure 3-4). We quantitated 5mC enrichment through qPCR

with the same primer set and the results indicated an approximately 6-fold increase

of 5mC in mtDNA from cells treated with low ethanol concentration (50 mM). At

high ethanol concentrations, we did not observe a strong increase of 5mC content in
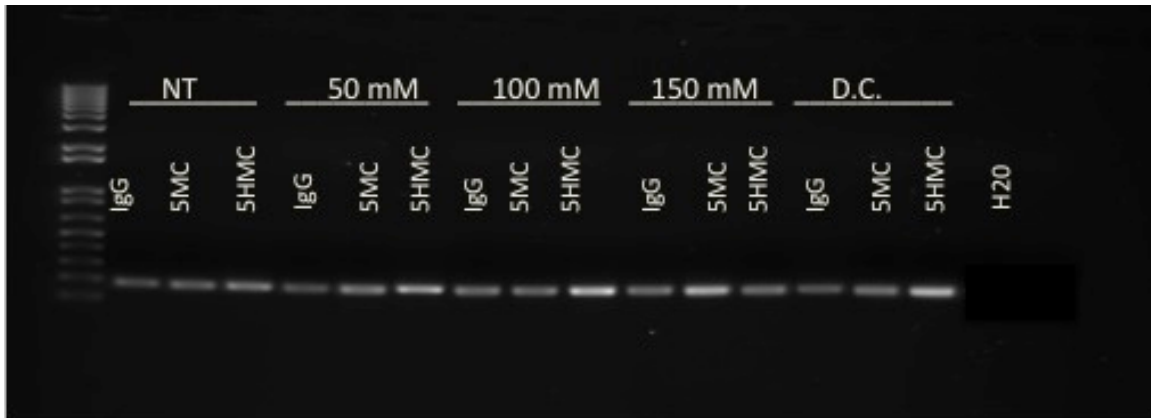
**Figure 3-4: DNA immunoprecipitation using 5mC and 5hmC antibodies shows increased mtDNA modification in ethanol treated cells.** Immunoprecipitated material from ethanol treated HCT 116 cells amplified for a total of 37 cycles using primers specific for a region in mtDNA (ND6 coding region). PCR products (5 µl) were resolved on a 1% nusieve agarose gel containing ethidium bromide (05.µg/ul). These data are representative of 2 independent biological repeats.

mtDNA. As a matter of fact, at 100 mM ethanol concentration, the level of 5mC moderately decreased, while at 150 mM, the level of 5mC slightly increased by approximately 2-fold compared to no treatment control. The level of 5mC content in mtDNA did not change in D.C. treated cells compared to control (Figure 3-5a).

The qPCR analysis also showed altered level of 5hmC content in mtDNA of ethanol treated cells. At low ethanol concentration (50 mM), we detected a small increase of 5hmC level compared to no treatment control. But at high ethanol concentrations (100 mM and 150 mM) the level of 5hmC in mtDNA dramatically increased as much as 25-fold. Dibutyryl cAMP treatment also increased the level of 5hmC in mtDNA by approximately 5-fold. Looking closely at the qPCR analysis of both 5mC and 5hmC, we observed a reciprocal pattern of both cytosine modifications in mtDNA. In low ethanol concentration, where we detected the highest level of 5mC, the level of 5hmC was relative equal to no treatment control. At high ethanol concentrations (100 mM and 150 mM) where we measured low level of 5mC, the level of 5hmC in both concentrations was significantly high (Figure 3-5b). Overall, the results indicated that ethanol exposure alters overall cytosine modification in mtDNA in a concentration dependent manner. It is possible that at high ethanol concentrations, the enzymes responsible for the conversion of 5mC to 5hmC are selectively stimulated, yielding more 5hmC in mtDNA and a decrease of 5mC level.
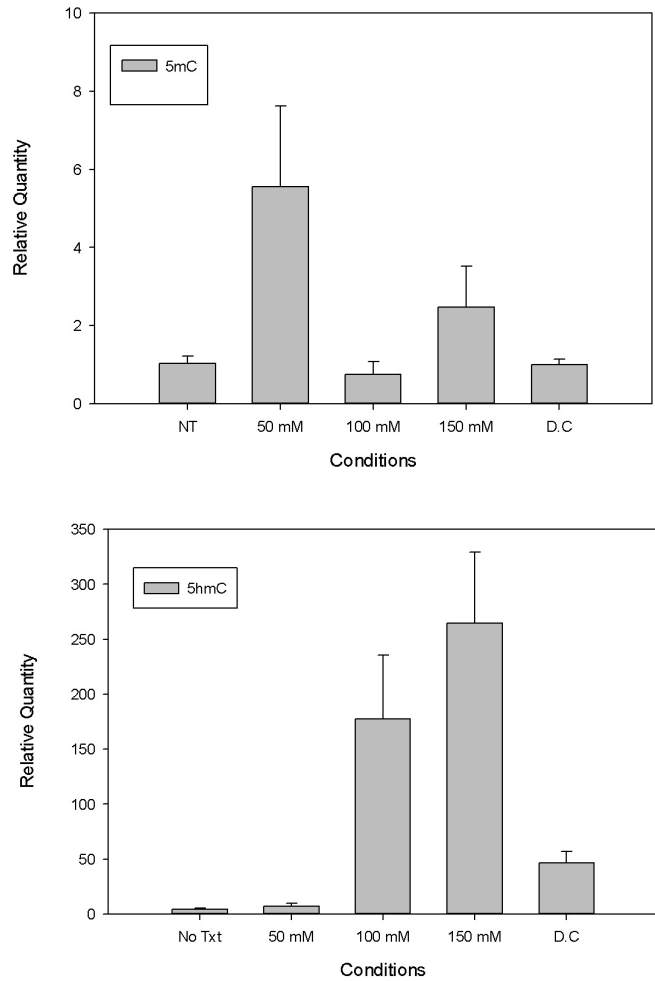
**Figure 3-5: Quantitation of 5-methylcytosine and 5-hydroxymethylcytosine demonstrated increased modification of cytosine in alcohol treated cells.**
qPCR analysis of immunoprecipitated DNA using primers specific for the ND6 coding region in mtDNA. Values of 5mc (A) and 5hmc (B) in nanograms (ng) are normalized to IgG values, with no treatment control set to 1. qPCR analysis demonstrated a high level of 5mC in samples treated with low ethanol concentration (50mM) compared to no treatment control (NT), with an increase in 5hmC evident only at higher ethanol concentration, suggesting a precursor (5mC)-product (5hmC) relationship. Data from 2 independent repeat experiments was combined.

**Chemical inducers of hypoxia up-regulate mtDNMT1 protein expression in HCT116 cells.**

In addition to ethanol-induced oxidative stress, we utilized hypoxia or low oxygen availability as a model of oxidative stress. Studies have demonstrated that low oxygen condition stimulates formation of ROS, such as nitric oxide (NO) from the respiratory chain, leading to oxidative stress (38,39). In this part of the project we induced hypoxia in HCT 116 cell culture using chemical inducers of hypoxia. Several sources suggested that cobalt chloride, a transition metal, and deferoxamine mesylate (DFO), an iron chelator, stimulate a hypoxia-like response in cultured cells (47). Cobalt chloride stabilizes HIF1α through two mechanisms. First, cobalt chloride prevents iron from binding to HIF-specifc proline hydroxylase by binding to the iron center of the enzyme. This interaction blocks the activation of HIF-specifc proline hydroxylase, preventing the hydroxylation and degradation of HIF1α (48). The second action of cobalt chloride is binding to hydroxylated HIF1α at a site overlapping the pVHL-binding site, where it prevents the interaction of HIF1a and pVHL, a tumor suppressor gene that targets HIF1α for degradation (53). DFO works to stabilize HIF1α by chelating iron required for the activity of HIF-specific proline hydroxylase (48). The removal of iron by DFO also enhances the activity of cobalt chloride by making the iron-binding site of HIF-specific proline hydroxylase free for cobalt chloride to bind (30). The stabilization of HIF1α activates downstream targets that are involved in the cell's response to hypoxia, such as erythropoietin, vascular endothelial growth factors, and several glycolytic enzymes (53).

HCT116 cells were treated with cobalt chloride or DFO for 24 hours at the concentration used in other studies (100 μM) (30), and subcellular fractions were obtained from each condition.   The immunoblots of whole cell and mitochondrial fractions showed distinct pattern of DNMT1 expression.  Total DNMT1 decreased upon treatment with the chemical inducers, especially with DFO, by approximately 3 fold.  The decrease in total DNMT1 expression may be due to the cell's response to stimulate hypoxic response genes that are regulated by DNA methylation.  On the other hand, DNMT1 in the mitochondria showed an approximately 4-5 fold increase after treatment with cobalt chloride and DFO (Figure 3-6).   The data demonstrated that chemically induced hypoxia altered the levels of both total and mitochondrial DNMT1. These results supported our hypothesis that oxidative stress alters the protein expression of DNMT1.   The VDAC signal in both whole cell and mitochondrial fractions indicated equal loading and the band corresponding to H3K4me3 in whole cells but not in the mitochondria indicates the lack of nuclear contamination in the mitochondrial fraction.

To ensure that cobalt chloride and DFO were inducing hypoxia in HCT 116 cells, we analyzed the presence of HIF1-α in whole cell lysates treated with cobalt chloride and DFO.  The immunoblot showed a strong HIF1-α signal at the expected size of 120 kDa in cobalt chloride treated cells, confirming that
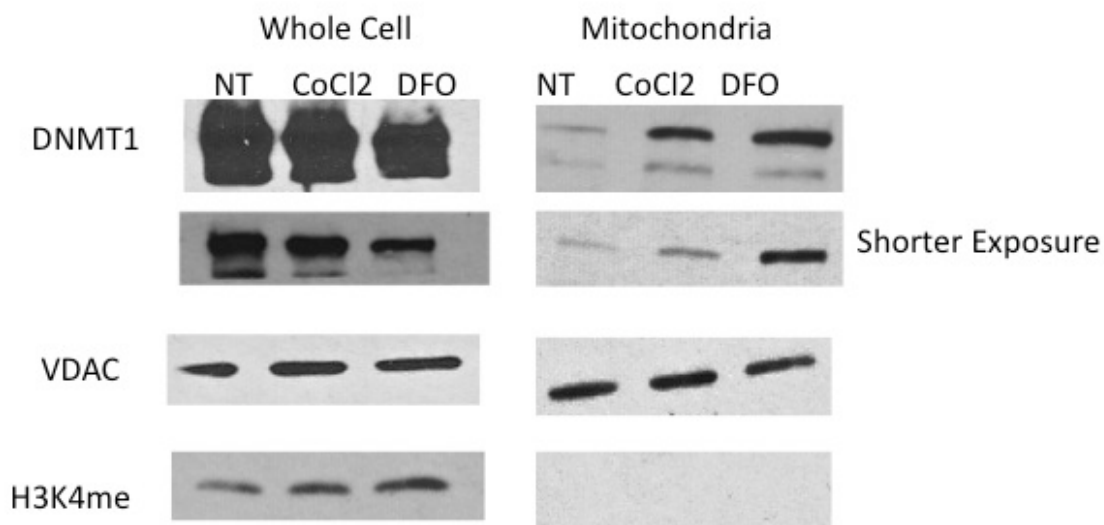
**Figure 3-6:  Response of DNMT1 to chemical inducers of hypoxia in HCT 116 cells.**  Cells were treated for 24 hours with 100 μM cobalt chloride or 100 μM DFO and fractionated for immunoblot analysis using antibodies to DNMT1, VDAC and H3K4me3.  Total DNMT1 protein level decreased in response to cobalt chloride and DFO treatments compared to no treatment (NT) control.  The protein level of mtDNMT1 however, increased after chemical inducer treatments.  The VDAC signal indicated equal loading and the H3K4me3 signal in whole cells but not in the mitochondrial fraction confirmed the purity of the mitochondrial lysates.  These data are representative of 2 independent biological repeats.

hypoxia was induced during this treatment.   However, DFO treatment did not

appear to stabilize HIF1α as much as cobalt chloride (Figure 3-7).  The differences

between cobalt chloride and DFO in terms of stabilization of HIF1α, might be due to

the fact that the two chemicals are stabilizing HIF1α via different mechanisms.

Cobalt chloride directly stabilizes HIF1α by interacting with both HIF1α and the

hydroxylase enzyme, while DFO indirectly stabilizes HIF1α by sequestering iron.

The action of cobalt chloride might be a more immediate response, in comparison to

DFO, thus we detected a stronger signal of HIF1α in cobalt chloride treated samples.

Since DFO treatment showed the highest expression of mtDNMT1, it is also possible

that it induces oxidative stress, independent of HIF1α dependent pathways.  Iron is

certainly an important cofactor for iron-sulfur proteins including ferredoxins and

NADH dehydrogenase, which are major players in oxidative phosphorylation (49).

Many reports have demonstrated that iron deficiency results in oxidative stress

through the formation of NO (50,51).   The ability of DFO to induce oxidative stress

independent of HIF1α stabilization in HCT 116 cells can be tested by examining the

presence of oxidative stress markers such as 8-oxo-2deoxyguanosine (8-oxo-dG).

   With the difference in HIF1α stabilization in cobalt chloride and DFO treated

cells and the overall up-regulation of mtDNMT1 in both treatment, we asked

whether the two chemical inducers are both altering mtDNMT1 protein expression

through PGC1α and NRF1 regulation or an alternative mechanism.   Thus we

proceeded to investigate the regulatory mechanism of DNMT1, following treatment

with these two chemicals.

**PGC1α levels increased while NRF1 levels remained invariable in cells exposed to cobalt chloride and DFO.**

PGC1α expression is activated during different types of oxidative stress, including hypoxia.   This activation promotes the stimulation of a large number of genes that are involved in mitochondrial biogenesis, cellular respiration and antioxidant defense mechanisms.   NRF1 is one of the target genes of PGC1α under conditions of cellular stress.   NRF1 and PGC1α together activate nuclear genes required for mitochondrial biogenesis and mtDNA replication (12,14). With the evidence of a consensus NRF1 binding site found in the leader peptide of the mitochondrial DNMT1, and the up regulation of this protein by hypoxic stress, we aimed to investigate whether NRF1 and PGC1α regulate DNMT1 expression during hypoxia by direct binding to the DNMT1 promoter.   Examining first the level of NRF1 and PGC1α levels in cobalt chloride and DFO treated cells should determine whether the two transcription factors are induced upon treatment and contribute to the up regulation mtDNMT1 expression.   We expected that cobalt chloride and DFO treatment, with their ability to mimic the cellular hypoxic response, would increase the protein levels of PGC1α and NRF1 compared to control.   An immunoblot of HCT 116 cells treated with the two inducers for 24 hours showed an approximately 2-fold increase of PGC1α levels in cobalt chloride treated cells while DFO treatment did not alter PGC1α expression.   It was also observed that PGC1α was present in the mitochondrial fraction at a higher level in treated cells compared to control.   NRF1 levels appeared to be equal in control, cobalt chloride and DFO conditions (Figure 3-7).   Overall, the results suggested that 24 hour cobalt chloride and DFO treatments
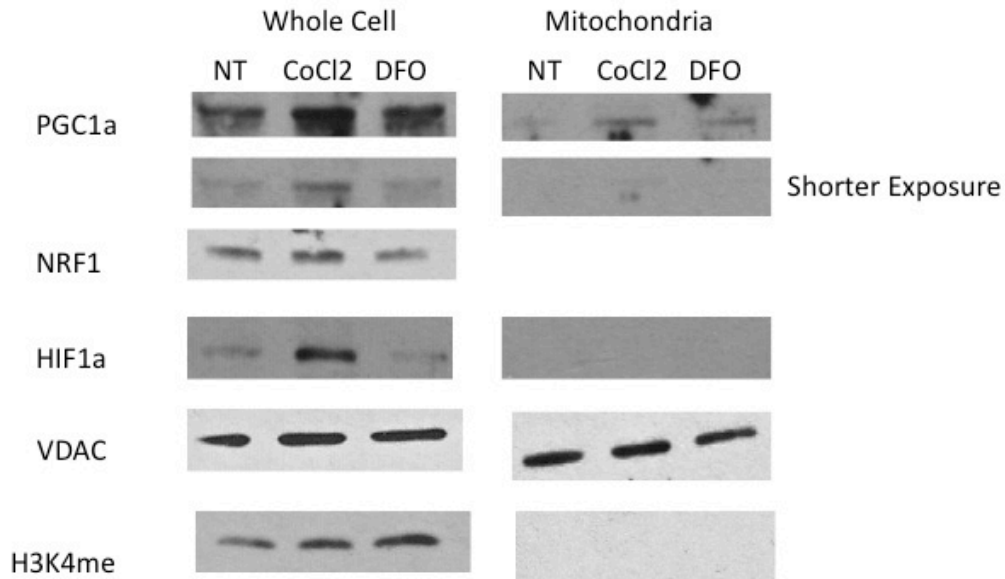
**Figure 3-7: Levels of transcription factors in HCT 116 cells after 24 hour treatment with chemical inducers of hypoxia.**
Immunoblots of subcellular fractions were probed with antibody against PG1α, (90 kDA) NRF1 (55 kDA), HIF1α (120 kdA), VDAC and H3K4me3.  The analysis showed minimal increase of both PGC1α and NRF1 in whole cell fractions following treatment with cobalt chloride and DFO.  Exposure for 2 minutes showed the presence of PGC1α in the mitochondrial sample.  Shorter exposure was for 5 minutes.  HIF1α protein expression increased in cobalt chloride treated cells but not in DFO treated cells. Immunoblot was developed using ½ Dura ECL substrate.  These data are representative of 2 independent biological repeats.

induced PGC1α but not NRF1 in HCT 116 cells.   However, NRF1 was clearly present in all conditions.  Since its ability to activate gene expression appears to depend on co-activation by PGC1α (12), we considered the possibility that an increase in PGC1α protein level would be sufficient to activate DNMT1 expression, as well as other genes of mitochondrial biogenesis.

**PGC1α and NRF1 protein levels remain constant throughout 48 hours of hypoxic stress, while DNMT1 levels were variable.**

The data obtained from the 24 hour treatments mentioned above presented conflicting results that did not support our prediction.  First, although we detected an increase in PGC1α upon treatment, NRF1 levels were found to be similar to control levels.  This observation did not correspond to the notion that NRF1 is a downstream target of PGC1α during chemically induced oxidative stress at this time point.  Secondly, the pattern of DNMT1 levels in DFO treated cells was higher than any of the other conditions, while PGC1α and NRF1 remained equal to untreated cells, suggesting alternative mechanisms of DNMT1 induction.   If PGC1α and NRF1 are indeed regulating mtDNMT1, we expected that conditions that gave the highest expression of mtDNMT1 would also increase the levels of PGC1α and NRF1, which was not the case in DFO treated cells. These observations certainly needed to be explored deeper to have a better understanding of the factors contributing to the increase of mtDNMT1 in hypoxic stress.  We therefore investigated whether there might be time dependent changes in the levels of these transcription factors using a time course experiment.  It is possible that PGC1α and NRF1 are induced at a

different time point during cobalt chloride and DFO treatment, which could explain the increase in mtDNMT1 expression. HCT 116 cells were therefore treated with cobalt chloride and DFO for 12, 24, 36 and 48 hours. The purified subcellular fractions were analyzed by immunoblot using DNMT1, PGC1α and NRF1 antibodies.

The results showed that total DNMT1 protein levels decreased in treated cells compared to control, as early as 12 hours post treatment in accordance with our previous observation at 24 hours. The level of total DNMT1 continued to decrease up to 36 hours. At 48 hours, total DNMT1 signal in both control and cobalt chloride conditions were not detected, most likely due to the fact that the cells reached full confluency, while DFO treated cells showed an approximately 2 fold increase in expression at 36 hours (Figure 3-8). These results indicated that over the course of 48 hours, cobalt chloride and DFO altered the accumulation of total DNMT1. The disappearance of bands in both control and cobalt chloride at 48 hours might be a consequence of two factors. The first factor is the passage number of the cells, which may have contributed to a slower proliferation rate at 48 hours, impairing mtDNMT protein production. Although the passage number of the cells used for the time course was slightly higher than the cells used for the previous 24-hour treatment, there is still the possibility of acquiring additional mutations that can contribute to a slower proliferation rate at 48 hours thus impairing mtDNMT1 protein production. This can be tested by running a repeat of the experiment using fresh low and high passage HCT 116 cells and then comparing the protein levels of DNMT1. Secondly, DFO treatment might have had a protective effect on the cells, enabling them to proliferate and produce mtDNMT1 efficiently. To test whether
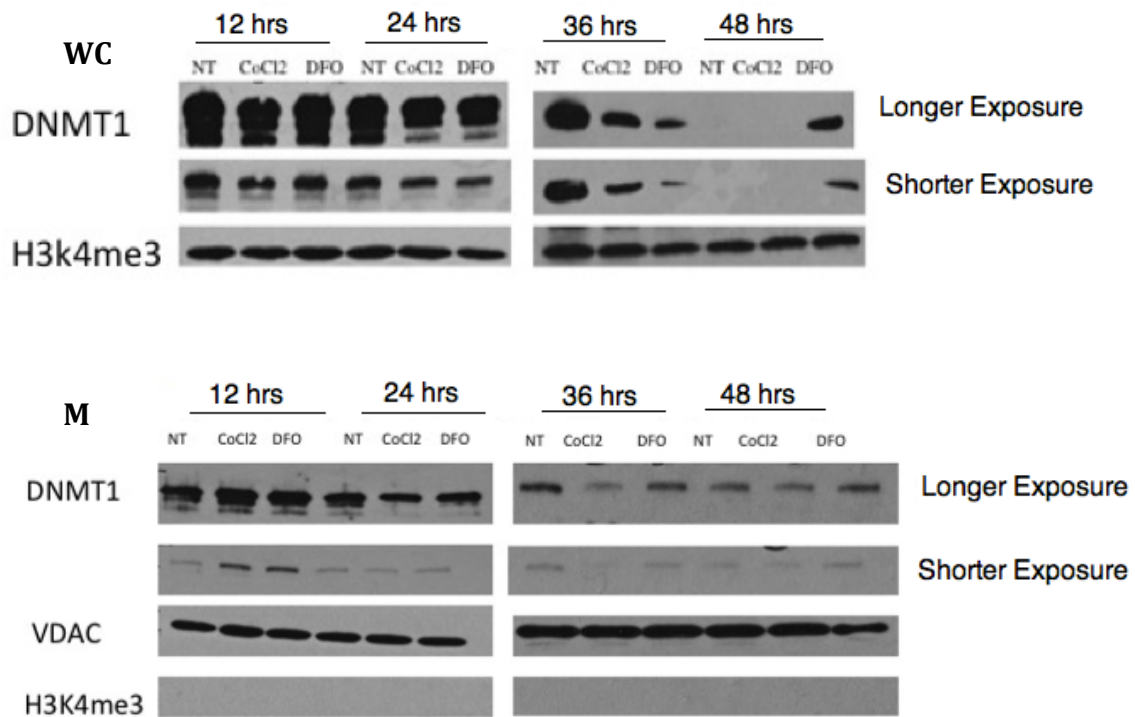
**Figure 3-8: DNMT1 levels in HCT 116 treated with chemical inducers of hypoxia for 12, 24, 36 and 48 hours.** Cells were treated 24 hours after replating with 100 μM cobalt chloride or 100 μM DFO for the indcated times. WC and M lysates were resolved on 4-15% gradient gels and probed with the indicated antibodies. This experiment has been done only once.

DFO protected the cells from the effect of over-passaging, we could repeat the experiment using high passage HCT 116 cells and compare the levels of total DNMT1 in DFO treated and non treated samples. Lastly, a technical error during the transfer process might have contributed to the disappearance of bands around that region. We have observed in the past that an air bubble in the transfer sandwich can affect the ability of proteins to transfer efficiently to the membrane. A repeat from the same aliquot of lysates would indicate technical problems contributing to the expression patterns observed.

The immunoblot showed an increase in mtDNMT1 in cobalt chloride and DFO treated cells after 12 hours, but not at 24 hour. This did not agree with our previous experiment in which a single 24 hour time point was taken. The expression of mtDNMT1 in both treatments appeared to be equal to that of control after 24 hours. The levels of mtDNMT1 subsequently decreased in cobalt chloride treated cells while mtDNMT1 levels in DFO treated cells remained similar to control (Figure 3-9). These results demonstrated that chemically induced hypoxic stress altered mtDNMT1 as early at 12 hours post treatment. The observations gathered from cobalt chloride treated cells after 24 hours might be a consequence of prolonged exposure to this chemical. It is known that cobalt chloride exposure can lead to tissue and cellular toxicity. A study by Vengullar's group, using growth curve analysis and cell toxicity assays on mouse embryonic fibroblasts treated with cobalt chloride for 72 hours, showed that following treatment with the same concentration of cobalt chloride used in this study (100 μM), little to no growth was detected and the toxicity level increased as early as 48 hours (33). The decrease of mtDNMT1 in
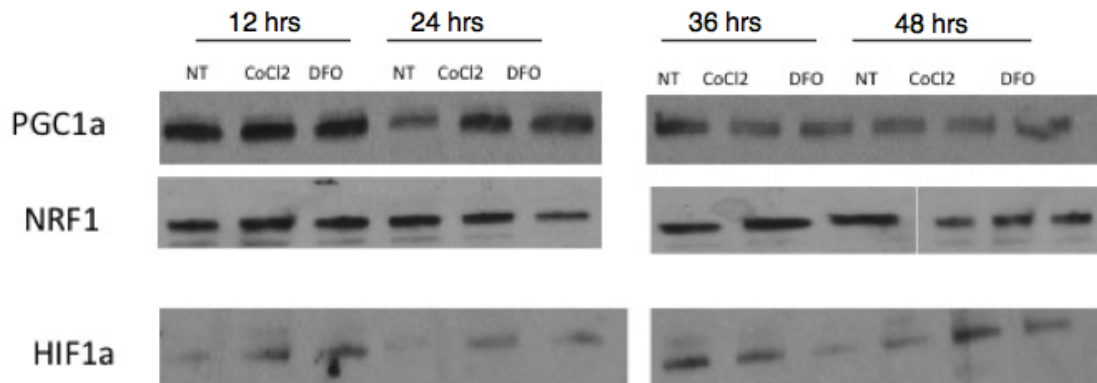
**Figure 3-9: Protein level of PGC1α, NRF1 and HIF1α in cobalt chloride and DFO treated HCT 116 cells over the course of 48 hours.** Immunoblot analysis showed the relatively constant levels of NRF1 and PGC1α throughout 48 hours of hypoxic induction. A slight increase of PGC1α at 24 hours was observed but otherwise expression remained low. HIF1a signals in all the samples confirmed the induction of hypoxia in the samples. ½ Dura ECL subtrate was use to develop the blot. This is a representative of 1 independent biological repeat.

cobalt chloride treated cells after 36 hours might be due to an increase of toxicity in the system, as observed in Vengullar's study and this could potentially affect the proliferation rate of the treated cells.   Running tests similar to those in Vengullar's study, as well a as cell cycle study using flow cytometry on cells that have been treated with cobalt chloride for 48 hours, could test the validity of this conclusion.

It was expected with the up-regulation of mtDNMT1 at 12 hours that NRF1 and PGC1α would also increase at the same time point.  However, the immunoblot of the whole cell fraction showed equal signals for PGC1α and NRF1 at 12 hours in all conditions (Figure 3-9).  The level of NRF1 in treated cells remained constant throughout the 48 hour period.  On the other hand, PGC1α levels appeared to increase in cobalt chloride and DFO treated cells after 24 hours, which was similar to what was detected from earlier 24-hour experiments (Figure 3-6).   However, after 36 and 48 hours, the levels of PGC1α reverted to the level of the control sample.  Overall, the data gathered from the time course demonstrated with treatment with cobalt chloride and DFO did not consistently increase levels of NRF1 and PGC1α over the course of 48 hours.  Though we did detect an increase of PGC1α at 24 hours, it did not result in NRF1 up-regulation at the same or later time points. These data indicated that the mtDNMT1 increase upon treatment with hypoxia might be due to factors other than NRF1 and PGC1α.

**Cobalt chloride and DFO treatment do not increase binding of NRF1 and PGC1α to the DNMT1 promoter.**

The data gathered from the time course experiment suggested that NRF1 and PGC1α do not regulate mtDNMT1 protein expression during cobalt chloride and DFO treatments.  However, an earlier transient transfection study with HCT 116 cells expressing NRF1 and PGC1α showed that the two transcription factors together significantly up-regulate levels of mtDNMT1.   The discrepancy between the two studies led us to question the role of PGC1α and NRF1 in the transcriptional regulation of mtDNTM1 during hypoxic stress.   We aimed to investigate whether treatment with cobalt chloride or DFO alters the enrichment of NRF1 and PGC1α at the proximal promoter region of mtDNMT1 using chromatin immunoprecipitation (ChIP).  An increased association of the two transcription factors on the DNMT1 promoter would indicate that NRF1 and PGC1α are playing a role in the transcriptional regulation of mtDNMT1 during hypoxic stress.   Chromatin samples from each condition were immunoprecipitated using antibodies specific for NRF1 or PGC1α.  p53 antibody was also used in the immunoprecipitation as a positive control.  We know from an earlier study that p53 binds to the DNMT1 promoter and represses DNMT1 expression (22).  The level of enrichment was quantitated through qPCR with primers against a region in the DNMT1 promoter that includes the consensus NRF1 binding site (Figure 3-10). The data obtained showed that the association of NRF1 with the promoter region is relatively constant between hypoxic and control samples.   PGC1α was detected at a low level in all samples and its enrichment moderately decreased upon treatment with cobalt chloride or DFO. The level of p53 enrichment also appeared to decrease with treatment of the
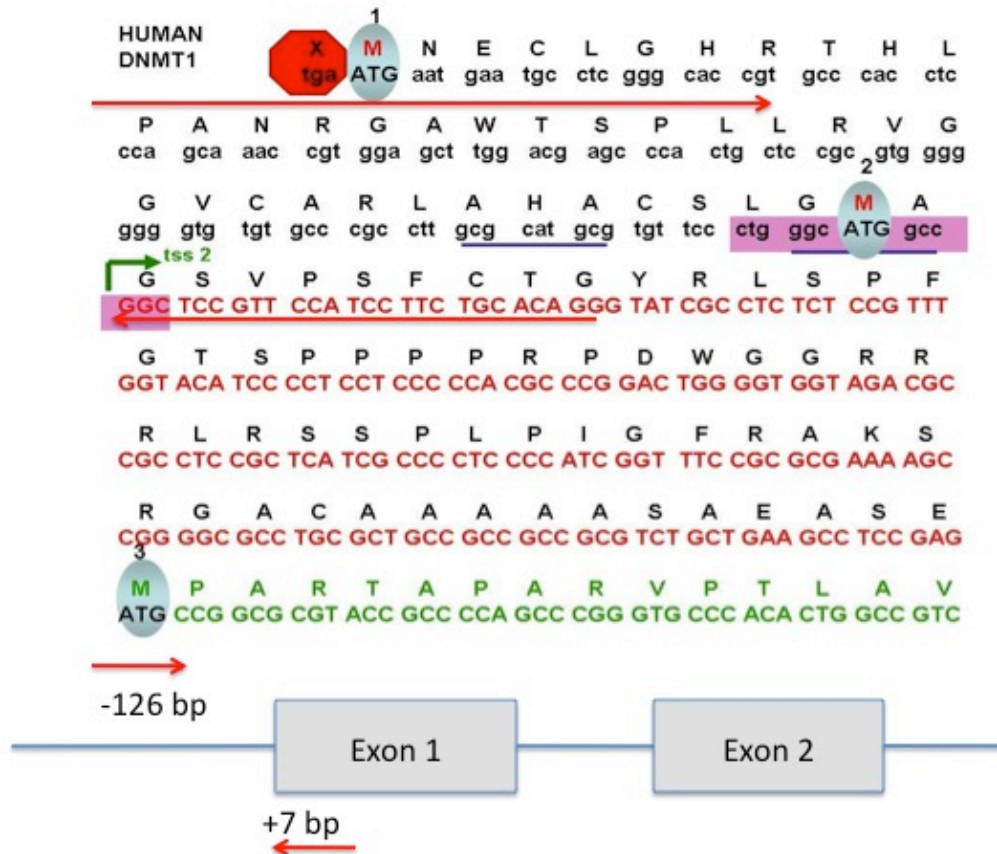
**Figure 3-10: Schematic diagram of primer placement for chromatin IP to analyze the enrichment of binding factors in the DNMT1 promoter** (Adapted from Shock et al)(3).

chemical inducers compared to control (Figure 3-12). The overall result did not demonstrate that cobalt chloride and DFO treatment elevated the enrichment level of NRF1 and PGC1α on the DNMT1 promoter. This suggests that NRF1 and PGC1α are not influencing the up-regulation of mtDNMT1 during oxidative stress. Although we did not achieve the expected result, the data presented compelling evidence that can explain what is contributing to the up regulation of mtDNMT1 during cobalt and DFO treatment. First, the end point and qPCR analysis proved that NRF1 is present on the promoter region of DNMT1 under normal conditions, which has not been previously shown. Most importantly, the qPCR result showed the level of p53 enrichment decreased upon treatment with cobalt chloride or DFO. This observation was evident in the end point PCR result, showing a disappearance of p53 signal in cobalt chloride treated cells and a weak signal in DFO treated cells compared to control (Figure 3-11). We know from an earlier study from our lab that p53 has the ability to suppress DNMT1 mRNA and protein levels, when bound to the same site at the DNMT1 promoter region. Also, our lab discovered that genotoxic stress (UV irridiation and treatment with topoisomerase inhibitors) promotes the dissociation of p53 from this binding site and ultimately increases the level of DNMT1 mRNA and protein (22). Considering this study and the result from both ChIP and immunoblot analysis of cobalt chloride or DFO treated cells, we concluded that the up regulation of mtDNMT1 protein expression was due to the dissociation of p53 from the DNMT1 promoter, as a result of p53 activation following oxidative stress, thus relieving suppression of mtDNMT1.
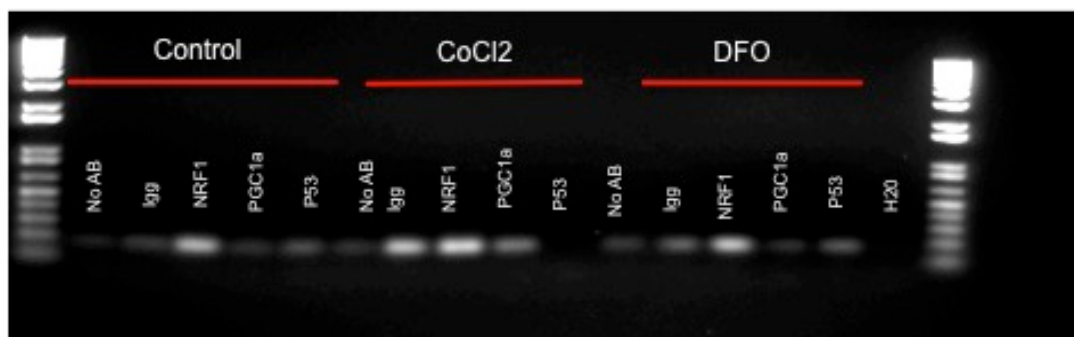
**Figure 3-11: End point PCR of ChIP products from cobalt chloride and DFO treated HCT 116 cells.** Purified DNA from ChIP analysis from all conditions was amplified using a region specific primer for the DNMT1 promoter (Table 1-2). 10 μl of end point PCR products were resolved on a 1% nusieve agarose gel with ethidium bromide. PCR was programmed with annealing temperature at 56°C and a total of 36 cycles. The data are representative of 2 independent biological repeat.
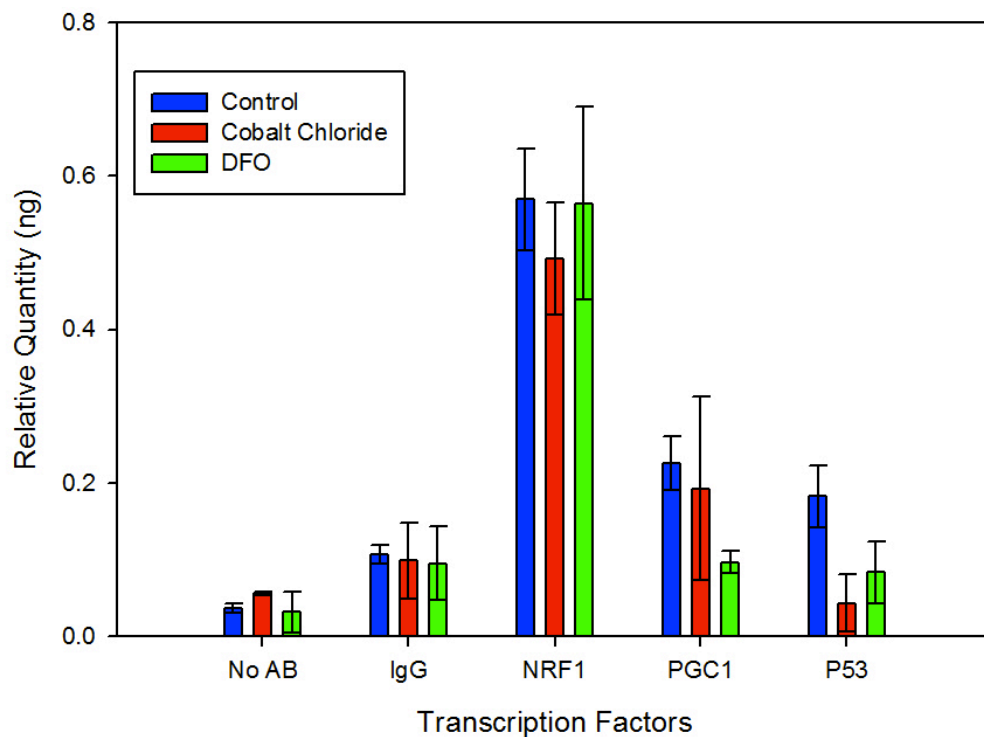
**Figure 3-12: Cobalt chloride and DFO treatment in HCT 116 cells showed no significant changes in the level of NRF1 enrichment on the DNMT1 promoter.** qPCR analysis of chromatin IP product of HCT 116 cells treated with cobalt chloride and DFO for 24 hours. The level of enrichment of NRF1 stayed relatively constant in all conditions. Enrichment of PGC1α on the promoter region decreased upon treatment with chemical inducers. p53 served as the positive control. qPCR was programmed with an annealing temperature of 56°C and 45 total cycles. These data are representative of 2 independent biological repeats.

## Chapter 4: Discussion and Perspectives

### 1. Discussion

The transcriptional regulation of mtDNMT1 is not currently understood, largely because the identification of this isoform as the active DNA methyltransferase in the mitochondria was discovered only recently in our laboratory (3).   The data presented in this thesis provide initial evidence of the regulation of mtDNMT1 during oxidative stress.  Previous bioinformatic analysis of mtDNMT1 suggested that this gene might be regulated by factors that respond to environmental stimuli.  We identified an NRF1 binding site that overlaps with a p53 binding site upstream of the published transcription start site.   This NRF1 binding site was conserved among mammalian species, suggesting its evolutionary significance (3).   Initial studies to test the importance of this binding site in the regulation of mtDNMT1 were done by transiently transfecting HCT 116 cells with expression vectors containing NRF1 and PGC1α.  NRF1 and PGC1α alone moderately increased the levels of DNMT1 protein compared to control.  Most importantly, transfected together, NRF1 and PGC1α upregulated mtDNMT1 substantially, indicating their potent role as coactivators in the regulation mtDNMT1 expression *in vitro*.

With the importance of NRF1 in mitochondrial biogenesis and energy homeostasis, we sought to further investigate the significance of NRF1 and PGC1α in the regulation of mtDNMT1 during cellular stress.   PGC1α, a powerful master regulator of mitochondrial biogenesis and aerobic respiration, is essential for the

activation of NRF1 transcription (17), thus it was also important to study its contribution to mtDNMT1 regulation.  The aims of this study were: (1) to determine whether oxidative stress in the cell alters the level of mtDNMT1 and mtDNA methylation, and (2) to investigate whether NRF1 and PGC1a play a role in the transcriptional regulation of mtDNMT1 during oxidative stress.  The overall hypothesis was that mtDNMT1 and mtDNA modification represent part of the cellular response to oxidative stress.

The results overall demonstrated that oxidative stress induced by ethanol and hypoxia increased the level of mtDNMT1 protein and that this increase was independent of the regulation of NRF1 and PGC1$\alpha$.  ChIP analysis proved that alteration of mtDNMT1 protein expression during ethanol and hypoxia induced oxidative stress was due to the relief of p53 suppression of the DNMT1 promoter.  In addition, increased expression of mtDNMT1 resulted in an increase in 5mC and 5hmC content in mtDNA.

**Cell-specific response to ethanol and hypoxia induced oxidative stress.**

The variable results obtained from HCT 116 and SH-SY5Y cells exposed to ethanol and low oxygen availability indicates that  different cell lines respond differently to oxidative stress.  Immunoblot analysis showed that ethanol treatment did not alter either total or mitochondrial DNMT1 levels in SH-SY5Y cell while DNMT1 levels in HCT 116 cells increased in both whole cell and mitochondrial fractions.  Low oxygen conditions also presented opposing patterns of protein levels between the two cell lines, especially after 48 hours.  Total DNMT1 protein level

after 48 hours of hypoxia appeared to increase in SH-SY5Y but decreased in HCT 116 cells. mtDNMT1 expression was only detected in SH-SY5Y cells, but this observation may be due to technical error during mitochondrial fractionation. The data from both cell lines demonstrated a cell-specific response to the effect of ethanol and hypoxia induced oxidative stress. This finding is supported in various studies investigating the effect of oxidative stress on DNA methylation using different cell lines. Skowronski's group showed that incubating HCT 116 cells in an oxygen chamber under low oxygen condition for 24 and 48 hours showed a decrease in DNMT1 protein level and activity, which corresponds to our findings in hypoxic samples of HCT 116 cell at 48 hour time point (33). In addition, studies done by Shahrzad and colleagues demonstrated that the level of 5mC in HCT 116 nuclear DNA significantly decreased in response to low oxygen conditions (34). These findings on HCT 116 cells corroborated with the results gathered in this study. On the other hand, studies on cell lines other than HCT 116 cells (not SH-SY5Y), showed the opposite results. Studies done by Watson's lab demonstrated that low oxygen conditions increased the level of global DNA methylation in prostate cells. This increase was due to the up regulation of DNMT1 protein levels (29). In various cancer cell lines (lung, breast and renal cancer) DNA methlyation was also elevated under hypoxic conditions, resulting in the hypermethylation and transcriptional inhibition of the erythropoietin gene (35). These findings, compared to those done on HCT 116, presented conflicting results, indicating a cell specific response to low oxygen. Evidently, hypoxia and most likely ethanol exposure present epigenetic alterations that are particular to certain cell lines similar to what

we observed with the variable expression of DNMT1 expression in HCT 116 and SH-SY5Y.

**Possible role of DNMT1 in the cellular response to ethanol-induced oxidative stress.**

For consistency purposes, since previous data and subsequent experiments were done using HCT 116 cells, we concentrated on the results gathered from HCT 116 cells to develop our conclusions on the possible role of DNMT1 in the cell's response to ethanol-induced oxidative stress. Immunoblot analysis showed that with HCT 116 cells, ethanol treatment increased total DNMT1 protein levels, especially at 100 mM ethanol concentration. Most importantly, the levels of mtDNMT1 increased upon ethanol exposure as low as 50 mM concentration. In conjunction with the increase of mtDNMT1 protein, qPCR analysis of MeDIP products from HCT 116 cells treated with ethanol showed elevated enrichment of 5mC in the mtDNA. The results overall suggested that, in response to ethanol-induced oxidative stress, mtDNMT1 methylation increased following the up-regulation of mtDNMT1 protein levels. The changes in DNMT1 levels both in whole cell and mitochondria, might potentially be part of the cell's stress response pathway that is induced by oxidative stress. Though there are no reports demonstrating the role of DNMT1 in the cell's stress response to ethanol exposure, there is evidence proving its functional importance in gene regulation during oxidative stress. A recent study by O'Hagan's group showed that DNMT1 forms a tight complex with SIRT1, DNMT3b and polycomb members on CpG islands of

damaged DNA in response to hydrogen peroxide treatment (36).  Hydrogen

peroxide is one of the potent reactive oxygen species found in the cell system that

can easily induce oxidative stress (28).  Looking closely at the position of the protein

complex, they discovered that it formed close to promoter regions of highly

expressed genes such as Myc and RPL13.  Most importantly, O'Hagan showed that

the assembly of this complex was DNMT1 dependent and that its interaction with

the chromatin resulted in transcription suppression of the targeted genes.  This

study demonstrated the functional role of DNMT1 in silencing genes that cannot

only prolong oxidative stress but can also contribute to cell transformation and

oncogenesis (36).  It is likely that DNMT1 is functioning as a transcription regulator

of nuclear genes that play an important role in promoting ethanol-induced oxidative

stress, such as those involved in ROS formation.

One particular gene that DNMT1 might target during oxidative stress is

alcohol dehydrogenase (ADH).   ADH is a nuclear-encoded gene that plays an

important role in ROS formation through the metabolism of alcohol to acetaldehyde

and acetate.   In conditions of elevated alcohol concentration, ADH continually

oxidizes alcohol leading to the increase of the NADH/NAD+ ratio.  As mentioned

previously, the increase of the electron carrier NADH contributes to the influx of

electrons in the respiratory chain, which is an ideal condition for ROS production.

Dannenberg and his colleagues reported that the human ADH genes, ADH1A, ADH1B

and ADH1C, are all regulated by DNA methylation via CpG sites within their

proximal promoter region.  When methylated, the transcriptional activity of all three

ADH genes is inhibited mainly due to the inability of essential transcription factor

such as USF and C-Myc to bind at an E-box binding site near the methylated CpG sites.  They demonstrated that the inhibition of the ADH genes was directly through the action of the DNMT1 protein (37).  In response to the findings by O'Hagan's group, it is likely that DNMT1 functions as a transcription regulator in response to ethanol-induced oxidative stress and suppresses general transcripts including ADH.  Inhibition of ADH transcription can depress oxidative stress by preventing the formation of ROS through alcohol metabolism.  If this was the case, then the increase of total DNMT1 expression at 100 mM ethanol treatment is part of the cell's stress response to modulate ROS production.

Since that mtDNMT1 protein levels also increased upon ethanol exposure, it is possible that this DNMT1 isoform is functioning in a similar manner to that of nuclear DNMT1 but in the mitochondria.  However, compared to total DNMT1, the regulation of mtDNMT1 might be more sensitive to ethanol-induced oxidative stress.  This is evident from the up regulation of mtDNMT1 levels upon ethanol treatment as low as 50 mM, while total DNMT1 only increased at higher ethanol concentrations.  Because mtDNMT1 is the only active DNA methyltransferase  in the mitochondria, and considering the fact that mitochondria are the main source and immediate target of ROS, it is likely that the immediate increase of mtDNMT1 protein levels is vital for the mitochondria's adaptive response to ethanol exposure.  It is known that the different subunits of the respiratory chain are highly capable of producing ROS, including NADH dehydrogenase (complex I), cytochrome b-cl (complex III) and cytochrome oxidase (complex IV).  If the production of ROS is left uncontrolled, oxidative stress can impair electron transport, proton translocation,

oxidative phosphorylation and ultimately ATP synthesis (19,22).  The most extreme

consequence of oxidative stress in the mitochondria is mitochondrial-mediated cell

apoptosis (19).  Therefore, it would seem important that these subunits be

regulated in conditions that can encourage ROS formation, in order to prevent the

effect of prolonged oxidative stress.   Since the proteins in these complexes are

encoded in the mtDNA, mtDNMT1 can certainly contribute to the inhibition of these

subunits through methylation of CpG islands.  Increased methylation can

correspond to gene specific transcription of these mitochondrial-encoded genes,

especially those specifically involved in the production of ROS.  The selective

transcription of mtDNA due to increased levels of mtDNMT was demonstrated by

the Taylor lab in previous studies (3).  This mechanism can ultimately alleviate the

effect of oxidative stress.  Although this mechanism can impair ATP production, cells

contain hundred to thousands mitochondria, depending on the cell type and are able

to compensate for the depressed ATP level, in order to overcome oxidative stress.

Therefore, the observed increase in mtDNMT1 protein levels in ethanol treated cells

might be part of the stress response mechanism that the cell activates to counteract

the effect of oxidative stress.

**Possible role of mtDNMT1 in hypoxia induced oxidative stress.**

Hypoxia was used in this study as another mode of oxidative stress in the

cell.  Initially, HCT 116 and SH-SY5Y cells were incubated under  low oxygen

conditions to activate hypoxia-induced oxidative stress.  However, with a

malfunctioning oxygen sensor and lack of HIF1α stabilization in protein samples

from cells incubated in the oxygen chamber, the results gathered from this particular experiment were not reliable.  Thus, we explored other methods of inducing hypoxia in the cells.  Several studies demonstrated the effectiveness of cobalt chloride and deferoxamine mesylate  (DFO) in stimulating a hypoxia-like response in cultured cells, largely through the stabilization of HIF1α (30).   The efficiency of the two chemical inducers was tested in our lab using HCT 116 cells, and immunoblot analysis demonstrated that treatment with cobalt chloride or DFO effectively stabilized HIF1α, indicating that a hypoxia-like response was induced in the cells.

The immunoblot analysis of HCT 116 cells treated with either cobalt chloride or DFO for 24 hours presented  evidence supporting our hypothesis.   The immunoblot of the mitochondrial fraction showed an approximately 3-4 fold increase of mtDNMT1 protein levels in both cobalt chloride and DFO treated cells.  While mtDNMT1 increased in response to these treatments, total DNMT1 levels decreased as much as 2 fold.  Clearly hypoxia induced by these two chemicals alters the levels of both total and mitochondrial DNMT1, suggesting a role for the protein during oxidative stress.  The decrease of total DNMT1 in HCT 116 cells treated with cobalt chloride or DFO corresponds to findings of previous studies.  Studies using HCT 116 cells demonstrated that hypoxia decreased the level of global DNA methylation evident from the depression of 5mC levels (34).   Furthermore, the same group studied the levels and activity of all the DNMTs in HCT 116 cells exposed to hypoxia and discovered that both mRNA and protein levels of DNMT1, DNMT3a and DNMT3b is significantly decreased and the overall DNMT activity is

reduced compared to normoxic cells (33).  Taking into account the findings of these studies and the data we obtained, the reduction of total DNMT1 in HCT 116 cells treated with cobalt chloride and DFO may be due to the cell's effort to stimulate the expression of genes, such as antioxidant genes, that are involved in the stress response to oxidative stress.

The change in the level of mtDNMT in chemical induced hypoxic stress may potentially be part of the cell's response similar to ethanol exposure.   It is known that under conditions of low oxygen, components of the respiratory chain produce ROS, similar to ethanol exposure.  Studies with yeast showed that hypoxia transiently increased mitochondrial-generated oxidative stress and elevated the level of protein and DNA oxidation (38).   Yeast and mammalian studies demonstrated that under hypoxic conditions, mitochondrial cytochrome c oxidase contributes heavily to the increased level of nitric oxide (NO), mainly from the oxidation of nitrite (23).   NO at low levels is involved in many biological processes in eukaryotic cells, such as the oxidation of iron-containing proteins, activation of guanylate cyclase, ADP ribosylation of proteins and iron regulatory factor activation (39).  However at elevated concentrations, NO can interact with other proteins, lipids and DNA resulting in oxidative stress (39, 23).  Thus, it is crucial that mitochondria strictly regulate the level and activity of specific mitochondrial encoded proteins, such as cytochrome oxidase, that can add to increased levels of ROS, during hypoxia induced oxidative stress.  In view of the function of DNMT1 in gene silencing during oxidative stress, mtDNMT1 can certainly play a significant role in the transcriptional regulation of mitochondrial genes during hypoxic stress.

**Transcriptional regulation of mtDNMT1 during oxidative stress**

This study indicated that oxidative stress in HCT 116 cells altered the levels of both total and mitochondrial DNMT1. Having seen a robust up-regulation of mtDNMT1 protein levels, we further investigate what factor(s) is(are) contributing to this change during oxidative stress. Using ChIP with antibody specific for NRF1, PGC1α and p53 and qPCR analysis with primers against the region around the consensus NRF1 binding site, we showed that the up regulation of mtDNMT1 was not through the action of NRF1 and PGC1α but largely through the action of p53. It was expected that upon treating the cells with cobalt chloride and DFO, PGC1α gets induced and activates NRF1. Activated NRF1 along with PGC1α was expected to bind to the DNMT1 promoter at the specific consensus-binding site and up regulate mtDNMT1 expression. This response should have been evident in the immunoblot analysis, showing an increase in PGC1α, NRF1 and mtDNMT1 protein level, as well as in the qPCR analysis of the ChIP product, presenting an elevated enrichment of PGC1α and NRF1 on the DNMT1 promoter region. However, the results gathered did not support our prediction. Although we observed an increase in mtDNMT1 and PGC1α protein level upon treatment, NRF1 remained at the normal level. In addition, the ChIP analysis demonstrated that the enrichment of NRF1 at the DNMT1 promoter region in treated cells was similar to control and the level PGCα was reduced. Evidently, NRF1 and PGC1α are not contributing to the up regulation of mtDNMT1 that was observed in cells exposed to hypoxic stress. The conflicting conclusions led us to ask whether an alternative factor might be directing the increase of DNMT1 protein levels in hypoxic conditions. Looking closely at the end

point and qPCR analysis from the ChIP experiment, we discovered that the level of p53 on the DNMT1 promoter decreased upon treatment with cobalt chloride and DFO. The apparent dissociation of p53 from the DNMT1 promoter might be the link to the up regulation of mtDNMT1 during hypoxic stress.

Earlier studies in our lab demonstrated the regulation of DNMT1 expression by p53 during genotoxic stress. It was discovered that p53 directly interacts with DNMT1 at a consensus-binding site within the promoter region and represses DNMT1 mRNA and protein levels. This binding site coincidently overlaps with the NRF1 binding site that was investigated in this study. In response to cellular stress induced by DNA damage, we found that the suppression of DNMT1 is relieved through the activation of p53 and subsequently its dissociation from the DNMT1 promoter (22). It is likely that the hypoxic condition led to the relief of the p53-mediate DNMT1 repression in HCT 116 cells, thus contributing to the increase of mtDNTM1 protein level.

p53 is a tumor suppressor protein with a significant role in regulating the cell cycle. When activated by cellular signals or posttranslational modifications, p53 acts as a transcription factor and stimulates the expression of genes involved in growth arrest and cell apoptosis. Various types of genotoxic stress, including DNA damage, signal the activation of p53 in the cell. Depending on the type of cellular stress, p53 can either arrest cells at the G1 phase through the activation of the p21 gene or promote cell death via apoptosis through the induction of pro-apoptotic proteins such as bax (40). In addition to genotoxic stress, it has been shown that

hypoxia can also activate p53 in the cell.  A study on human lymphoblast (AG1522) and colorectal carcinoma cell lines (RKO) exposed to acute low oxygen conditions showed an increase in p53 protein levels proportional to the duration of exposure and showed that reoxygenating the cells returned p53 to the levels of untreated control.  It was also discovered that the accumulation of p53 protein due to hypoxia was directed to the nucleus rather than to the cytoplasm where it normally resides (41).   These findings correspond to other investigations, including those from Koumenis' group, who also proved that hypoxia induced p53 and the likely mechanism for the accumulation of the protein in the nucleus was the down regulation of Mdm-2 during hypoxia (42).  Mdm2 is a potent negative regulator of p53, functioning as an E3 ubiquitin ligase and targeting p53 for degradation by the proteasome (65).  Perhaps one of the most compelling studies that provided evidence for the activation of p53 in conditions similar to the experiments in this thesis was done by a group at the NCI (43).   They discovered that hypoxia induced by cobalt chloride and DFO increased activated wild type p53 protein levels in both breast cancer (MCF-7) and hepatoma (HepaC1C7) cell lines.  Most importantly, this accumulation of active p53 in these treated cells was found to be HIF1α dependent (43).

All these investigations point to the fact that p53 is induced and activated under low oxygen conditions, similar to what is found in our study.   Thus it is highly possible that p53 is accumulated and activated in HCT 116 cells treated with cobalt chloride and DFO.  These changes in p53, similar to what we observed from a

previous study, result in the dissociation of p53 from the DNMT1 promoter, relieving DNMT1 suppression and up regulating mtDNMT1 expression.

## 2. Perspective

Since its identification as the novel mitochondrial isoform of DNMT1 by our lab, there has been no study on its regulation and potential role in the nucleo-mitochondrial communication in response to environmental changes. The findings in this thesis provided a first step towards understanding the underlying regulatory mechanism of mtDNMT1 by factors that are receptive to oxidative stress. Preliminary data obtained by members in the Taylor lab demonstrated that mtDNMT1 is regulated by transcription factors that stimulate nuclear encoded genes involved in oxidative homeostasis. Bioinformatic analysis of the coding sequence of mtDNMT showed a consensus NRF1 binding site within the promoter region that coincidently overlaps with a p53-binding site. The regulation by NRF1 and PGC1α of mtDNMT1, potentially through this binding site was shown by transiently transfecting HCT 116 cells with vectors expressing NRF1 and PGC1α. This earlier study demonstrated that both NRF1 and PGC1α individually are capable of up regulating mtDNMT1 but when present together, their effect on protein levels is enhanced, suggesting co-activation. PGC1α is considered a master regulator of mitochondrial biogenesis and cellular respiration. This transcription factor also plays an integral role in antioxidant defense mechanisms during oxidative stress. PGC1α's ability to control many mitochondrial functions is through the induction of various downstream nuclear encoded genes including NRF1. NRF1 is also a key

player in mitochondrial biogenesis, mtDNA replication and oxidative homeostasis and its function is mediated by direct interaction with PGC1α. Considering the functional importance of PGC1α and NRF1, as well as the preliminary findings mentioned above, we sought to further investigate the relationship between mitochondrial epigenetics and oxidative homeostasis. More specifically we aimed to understand the regulatory mechanism of mtDNMT1 during oxidative stress and whether mtDNMT1 is important in the cellular response to environmental changes.

The results from this study demonstrated that mtDNMT1 is responsive to oxidative stress, evident from the up regulation of its protein expression in HCT 116 exposed to both ethanol and hypoxia-induced oxidative stress. Under the conditions used to induce hypoxic stress (chemical inducers, cobalt chloride and DFO) the protein expression of NRF1 and PGC1a did not change substantially, indicating that the up regulation of mtDNMT1 was not due to the stimulation of these two transcription factors. A time course experiment of HCT 116 cells treated with cobalt chloride and DFO further demonstrated that the effect of the chemical inducers of hypoxia is independent of the expression of NRF1 and PGC1α. Thus, the increase of mtDNTM1 expression was through other factor(s) induced by oxidative stress. qPCR analysis of chromatin immunoprecipitate from HCT 116 cells exposed to hypoxic stress indicated that the up regulation of mtDNMT1 was due to the relief of p53 suppression from the promoter region.

**Future Studies**

Although the results provided in this thesis did not present evidence on the regulation of NRF1 and PGC1α on mtDNMT during oxidative stress, future studies can certainly provide answers to the contribution of these two transcription factors to the regulation of mtDNMT1 expression. An experiment using a condition or chemical that directly induces PGC1α and/or NRF1 will allow us to understand whether PGC1α and NFR1 directly affect mtDNMT1 expression. Several studies suggested various inducers of PGC1α, such as hydrogen peroxide (12), low temperature (44) and serum activation (57), as well as pharmaceutical stimulators including AICAR and Pioglitazone (45). The main limitation of these inducers is their effect on p53 activation. Since we are trying to establish a system where we bypass the regulation of mtDNMT1 by p53, it is vital that we check the status of p53 in the cells treated with these activators. Once we achieve a condition that effectively induces PGC1α but not p53, we can observe the protein levels of NRF1 and DNMT1, similar to the prior experiment using cobalt chloride and DFO. The expected result is that the induction of PGC1α will increase the level of NRF1 and subsequently up regulate mtDNMT1. We would study both transcript and protein levels of all three factors during the induction and use ChIP assays to validate direct interactions of these factors with the promoter of mtDNMT1.

Testing the functional importance of the consensus NRF1 binding site is also another future study that can broaden our understanding of the regulation of mtDNMT1. This can be done by a reporter gene assays such as the luciferase assays. If this binding site is indeed required for the regulation of mtDNMT1, then disrupting it by site directed-mutagenesis should decrease gene transcription.

There is, however the possibility that an additional NRF1 binding site within the promoter region that can participate in the regulation of mtDNMT1.  We recently analyzed the promoter region of human mtDNMT1 using MatInspector 8.0.5, and discovered another NRF1 binding site approximately 300 bp upstream of the  site studied in this thesis.   The functional significance of this binding site, however, has not been validated by determining its conservation across other mammalian species, thus a separate bioinformatics study is necessary in the future.   If prior ChIP analysis showed that the proximal NRF1 binding site is not contributing to the regulation of mtDNMT1 upon induction of PGC1$\alpha$, then it is possible that the additional upstream NRF1 binding site might be the site of regulation.   Additional ChIP and functional experiments can determine its significance to mtDNMT1 expression.

Ultimately, we want to study the contribution of epigenetic modification in the overall function of the mitochondria.  The main goal of the future studies is to address whether mitochondrial DNA methylation plays an important role in the regulation of mitochondrial processes such as the ETC, ATP synthesis, aerobic respiration and glycolysis.   We propose to address this by measuring the rate of mitochondrial oxidative phosphorylation in cells containing a disruption of mtDNMT1.   This approach can be done by transiently knocking down mtDNMT1 in cells, and then analyzing the rate of ATP production and glycolysis using a power instrument called SeaHorse bioanalyzer.  The data from this experiment will demonstrate the potential role of mtDNMT1 in cellular respiration and energy metabolism.

**mtDNMT1 as potential therapeutic target and biomarker of diseases**

The results gathered from this thesis present valuable information about the role of mitochondrial epigenetics in the cell's response to oxidative stress. The findings that we obtained are certainly valuable in that they allow us to asse the role of mitochondrial epigenetics in diseases that are associated with mitochondrial dysfunction. We know that oxidative stress increases the level of mtDNMT1 and mtDNA methylation. It is also known from previous studies that over expression of mtDNMT1 results in changes in mtDNA transcription. Therefore, it is likely that under oxidative stress, which can ultimately lead to mitochondrial dysfunction, mtDNMT1 is regulating transcription to alter mitochondrial functions. Knowing that mitochondrial dysfunction plays a critical role in the progression of these diseases, it is vital that we begin to understand the underlying mechanism involved in the regulation of mitochondrial function, for potential therapeutic targets.

The alteration of mitochondrial processes that can result in mitochondrial dysfunction requires the input of the nuclear genome, which encodes for most of the enzymes involved in oxidative phosphorylation and all of those involved in mitochondrial biogenesis and maintenance. PGC1α and NRF1 as mentioned before, are vital players in the nucleo-mitochondrial communication that are important in regulating genes vital for the overall health of the mitochondria, especially during cellular stress. Having demonstrated that mtDNMT1 is regulated by these two factors and that it is receptive to oxidative stress, a condition implicated in mitochondrial dysfunction, it is likely that there is an epigenetic component in the

development and progression of diseases including cancer, neurodegenerative diseases and cardiovascular diseases.  Taking all these in consideration, mtDNMT1 can potentially be a therapeutic target specifically against mitochondrial-dysfunction associated diseases.   In addition, by understanding the different factors involved in the disruption of mitochondrial DNA methylation, which can potentially lead to the mitochondrial dysfunction, we can find promising biomarkers that can be helpful in the development of diagnostic testing and intervention treatment.

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