Nitric Oxide: An Epigenetic Regulator

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B.S. University of Washington, Seattle, 2012

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Masters of Science in Medicinal Chemistry
in the Graduate College of the
University of Illinois at Chicago, 2014

Chicago, Illinois

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This thesis is dedicated to my parents, family and friends.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Douglas D. Thomas, for his guidance and enthusiasm to help me complete my thesis. I would also like to thank my thesis committee members: Dr. Pavel Petukhov for his encouragement and Dr. Joanna Burdette for her support during my project.

I'd like to acknowledge the past and current members from the Thomas Lab: Jason, Divya, Kate, and Rhea for all their assistance during my experiments. Without them, long hours at lab wouldn't have been so enjoyable. Special thanks to all my colleagues and friends for being my source of inspirations and delight.

Lastly, I warmly thank my parents for their motivations, words of wisdom and unconditional love throughout my graduate studies.

-VTP

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LIST OF ABBREVIATIONS

•NO	Nitric oxide
2-OG	2-oxoglutarate
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
AG	Amminoguanidine
BRCA1	Breast cancer 1, early onset
CaM	Calmodulin
cGMP	Cyclic-guanylyl monophosphate
CH_3	Methyl group
DETA/NO	Diethylene triamine nonoate
DNA	Deoxy Ribonucleic Acid
DMEM	Dulbecco's Modified Eagle's Medium
DNIC	Dinitrosyl iron complex
DNMT	DNA methyl transferase
DUSP1	Dual specificity phosphatase 1
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
EPR	Electro Paramagnetic Resonance
FAD	Flavin Adenine Dinucleotide
FBS	Fetal Bovine Serum
$Fe(NH_4)_2(SO_4)_2$	Ammonium iron(II) sulfate
H_2SO_4	Sulfuric acid

LIST OF ABBREVIATIONS (continued)

H3K9me1/2/3	Histone 3 Lysine 9 mono/di/tri-methylatiation
H3K9ac	Histone 3 Lysine 9 acetylation
H3K4me1/2/3	Histone 3 Lysine 4 mono/di/tri-methylation
H3K4ac	Histone 3 Lysine 4 acetylation
HCl	Hydrochloric acid
HTM	Histone methyltransferase
IFN-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
JMJC	Jumonji C
KDM	Histone lysine demethylase
L-NAME	NG-nitro-κ -arginine methyl ester
LPS	Lipopolysaccharide
LSD	Lysine-specific demethylases
MGMT	O-6-methylguanidine-DNA methyltransferase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
nNOS	Neuronal Nitric Oxide Synthase
NOA	Nitric Oxide Analyzer
NO_3^-	Nitrate
NO_2^-	Nitrite
NOS	Nitric Oxide Synthase
OD	Optical density
PBS	Phosphate buffer saline
PIC	Protease inhibitor cocktail

LIST OF ABBREVIATIONS (continued)

PMSF	Phenylmethanesulfonyl fluoride
PS	Penicillin/Streptomycin
PVDF	Polyvinylidene fluoride
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcription polymerase chain reaction
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SDS	Sodium dodecyl sulphate
sGC	Soluble guanylyl cyclase
SPER/NO	Spermine nonoate
TET	Ten-eleven translocation

SUMMARY

Previously, our lab had discovered three distinct mechanisms in which •NO could affect methylation patterns of histone 3 lysine 9 (H3K9): direct inhibition of JMJC lysine demethylases (KDM) by forming dinitrosyl iron complexes (DNIC) with Fe(II) that are required for KDM catalytic activities, reduction in iron cofactor availability, and regulation of KDM and histone lysine methyltransferase (HMT) gene expression. Here, we will determine the reversibility of these mechanisms on KDM and HMT by the removal of •NO donor. We will also look further into another lysine of interest, H3K4, and confirm the ability of •NO to decrease overall acetylation level via the overall increase in methylation of a specific lysine. And lastly, the role of inducible nitric oxide synthase (iNOS) in mediating histone modifications will be determined by inducing iNOS with cytokines and/or inhibiting this enzyme with aminoguanidine to alter •NO production. This study provides important insights on how this endogenously-produced molecule can possibly regulate transcription of genes such as oncogenes and tumor-suppressor genes by changing global methylation and acetylation levels of histones.

TETs are dioxygenase enzymes that convert 5-methylcytosine (5mC) containing DNA to 5-hydroxymethylcytosine (5hmC) containing DNA. 5mC has been associated with transcription repression whereas, elevated 5hmC levels are associated with increased gene expression. TET-mediated conversion of 5mC to 5hmC is considered as an important epigenetic component of transcriptional regulation. Similar to JMJC domain-containing demethylases, TETs are oxygen-dependent and require α-ketoglutarate and Fe (II) for their catalytic activities. In addition to determining the modifications of histones by •NO, we will demonstrate the ability of •NO to

inhibit TETs functions and decrease the overall conversion of 5mC to 5hmC. Furthermore, the degree of methylations on specific breast cancer related genes will be accurately quantified using pyrosequencing to demonstrate •NO-mediated changes in DNA methylation. Overall, our study reveals novel roles of •NO in regulating the epigenetics landscape via altering DNA modifications and histone modifications.

CHAPTER 1

INTRODUCTION

1.1 Nitric Oxide

Nitric oxide (•NO, nitrogen monoxide), an important short-lived ubiquitous endogenous-produced agent, under standard temperature and pressure is a gas[1]. It is an uncharged, free radical molecule with eleven valence electrons and its one paramagnetic unpaired electron is the reason for the molecule's unique property. Therefore, •NO has only two primary targets, transition metals and other free radicals, in order to stabilize its unpaired electron[2]. Due to its ability to be soluble in nonpolar conditions[3,4], •NO can diffuse selectively in the membrane and lipid phases of cells and acts as a signaling molecule that mediates many processes such as angiogenesis, smooth muscle tone, immune response, apoptosis, and synaptic communication[5]. For these reasons, this molecule of versatility and significance was named Molecule of the Year in 1992[6].

1.1.1 Nitric oxide and oxygen species

The reaction of •NO with O_2 has been of interests because of its important implications in atmospheric pollution. Two molecules of •NO react with one molecule of O_2 to produce two molecules of another paramagnetic radical, nitrogen dioxide, that is a brown gas much more reactive than •NO. This reaction is called autooxidation.

$$2 \bullet NO + O_2 \rightarrow 2 \bullet NO_2$$

•NO_2 will react further with another •NO_2 to form dinitrogen tetroxide (N_2O_4) or with another •NO to form dinitrogen trioxide (N_2O_3).

The formation of N_2O_3 can lead to nitrosative stress since N_2O_3 is considered as a nitrosating agent.

$$\bullet NO_2 + \bullet NO_2 \rightarrow N_2O_4$$

$$\bullet NO_2 + \bullet NO \rightarrow N_2O_3$$

In aqueous solution, transfer to hydroxyl yields nitrous acid. Dissolution of N_2O_3 yields nitrite (NO_2^-) and dissolution of N_2O_4 yields nitrite (NO_2^-) and nitrate (NO_3^-)[2,7].

$$[NO^-] + OH^- \rightarrow HNO_2 \rightarrow H^+ + NO_2^-$$

$$[NO^-][NO_2^-] + H_2O \rightarrow 2NO_2^- + 2H^-$$

$$[NO^+][NO_3^-] + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$$

1.1.2 Nitric oxide and other radicals

If an electron is gained, nitroxyl anion is formed.

$$\bullet NO + e^- \rightarrow NO^-$$

Even though •NO reacts slowly with most biological molecules[11], •NO can react extremely rapidly with superoxide to produce the reactive species peroxynitrite that can lead to oxidative stress[2, 11].

$$\bullet NO + O\bullet_2^- \rightarrow ONOO^-$$

Peroxynitrite is a strong nitrating and oxidizing compound which can react with membrane lipids to cause more complex radicals by initiating lipid peroxidation[12]. The results of nitric oxide reactions with other free radicals in the cell lead to various phenotypic effect. These biological reactions can be classified by the indirect effect of •NO[13]. Another method to classify the biological reactions of •NO is called the direct effect which will be mentioned in the next section.

1.1.3 Nitric oxide and metals

Direct reactions of •NO involve the binding of •NO to the biological target molecule[13]. More examples of direct and indirect reactions of •NO can be seen in Fig.1[15]. Concentration of •NO affect its reactions. Direct effects generally occur at low concentration while indirect effects occur at a higher concentration. •NO can react with many iron-containing enzymes due to its affinity to react with iron[19]. One of the most important reactions in this category is the binding of •NO to the ferro-heme enzyme, soluble guanylyl cyclase (sGC), via a heme-nitrosyl adduct[14]. Another example includes the binding of •NO to deoxyhemoglobin:

$$HbFe^{2+} + \bullet NO \rightarrow HbFe^{2+}NO$$

Nitric oxide can also react with oxygenated hemoglobin to form nitrate and oxidized hemoglobin (methemoglobin)[2].

$$Hb(Fe^{2+})O_2 + \bullet NO \rightarrow Hb(Fe^{3+}) + NO_3^-$$

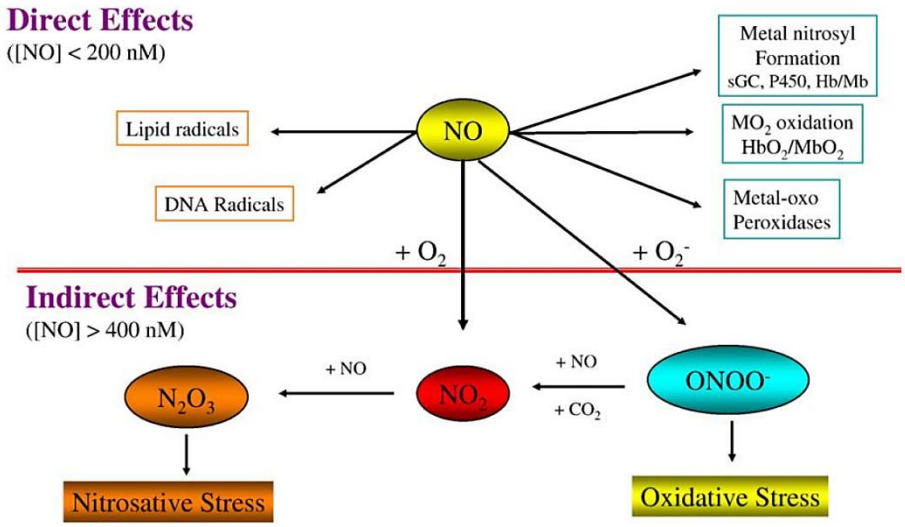


Figure 1: Direct and indirect reactions of •NO[15].

The formation of dinitrosyl iron complexes (DNIC) is another important direct reaction of •NO. It was demonstrated that DNIC are produced under physiologic low concentrations of •NO and

they are the largest intracellular •NO-derived adduct with a long half-life[16]. In biological systems, DNIC are predominantly represented by protein bound mononuclear forms with a characteristic EPR signals at 2.03 (Fig. 2)[17].

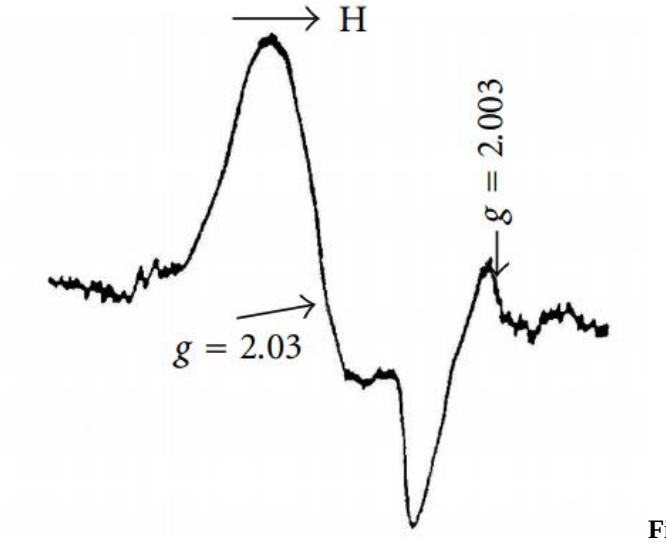


Figure 2: EPR signal of DNIC.

DNIC formation includes the binding of two •NO molecules to Fe^{2+} ions; their d-orbitals favor electron migration between •NO molecules and formation of paramagnetic DNIC. The overall reaction can thus be presented as follows:

$$H^+ + 2L^- + 3NO + Fe^{2+} \rightarrow \{(L^-)_2 Fe^{2+}(NO^+)_2\}^{+1} + \frac{1}{2}(N_2O + H_2O)$$

where L^- are anionic ligands (including thiols) (Fig. 3)[20].

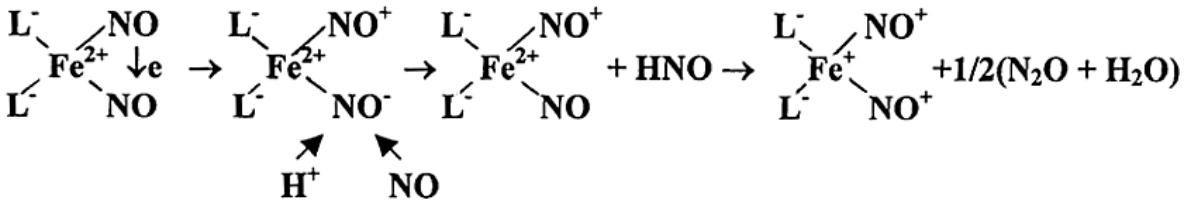


Figure 3: Overall reaction formation of DNIC

1.2 Nitric Oxide Synthase (NOS)

•NO is synthesized from L-arginine by three major isoforms of nitric oxide synthase (NOS) which are neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). nNOS and eNOS are constitutively expressed and activated by Ca^{2+}/CaM-dependent signaling in mammalian cells while iNOS expression is induced by immunological or inflammatory stimulation, such as inflammatory cytokines and bacterial endotoxins, independent of intracellular Ca^{2+} levels.[8,9]

All three NOS isoforms utilize NADPH and O_2 as co-substrates for •NO synthesis. Hydroxylation of L-arginine generates N-hydroxy-L-arginine as an intermediate. The second step converts N-hydroxy-L-arginine to produce •NO and citrulline (Fig. 4).[10,18]

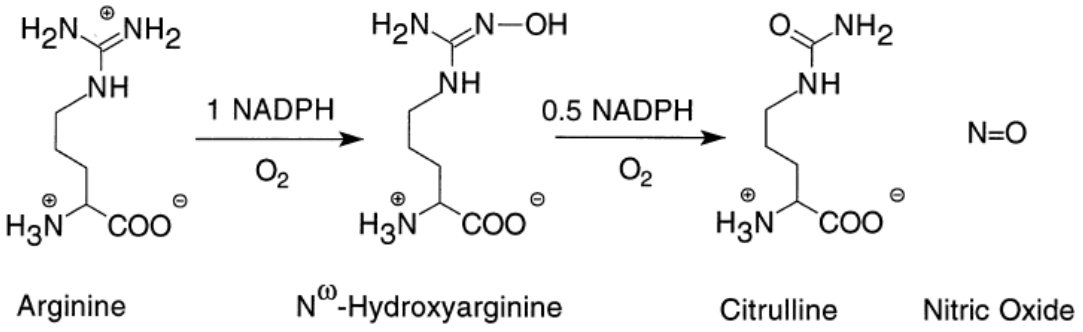


Figure 4: NOS catalyzes •NO synthesis from L-arginine[10].

1.3 •NO and Histone Modifications

The most studied histone modification has been acetylation, but it is now recognized that methylation, phosphorylation, ubiquitination, and SUMOylation all have regulatory functions[21,22]. Histone methylation is catalyzes by histone lysine methyltransferases (HMTs) that

can methylate either lysine or arginine residues. All HMTs utilize a similar reaction mechanism in which S-adenosyl methionine (SAM) donates a methyl group to form N-methyl protein adducts and the byproduct S-adenosyl homocysteine (SAH) (Fig.5)[28].

It was previously published that •NO can affect histone methylation as followed: direct inhibition of Jumonji C (JMJC) demethylase activity by binding to the non-heme iron atom, reduction in iron cofactor availability, and regulation of expression of methyl-modifying enzymes[23]. The first family of demethylases is composed of the lysine-specific demethylases LSD1 and LSD2, which are flavin adenine dinucleotide (FAD)-dependent amine oxidases[29,32]. The other type belongs to a family of the JMJC domain-containing demethylases (KDMs) that are 2-oxoglutarate (2-OG)-(also known as α-ketoglutarate) and Fe(II)-dependent dioxygenases (Fig. 6)[30,31,32]. The majority of the demethylases in this group target specific lysine residues on the N-terminal tail of histone 3 (H3)[25,26,27].

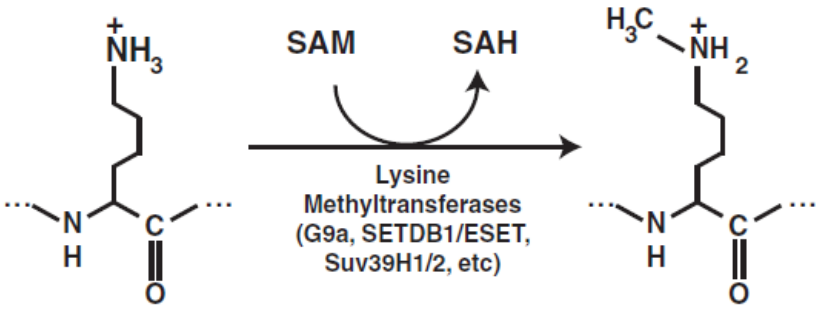


Figure 5: Histone lysine methyltransferase mechanism[32].

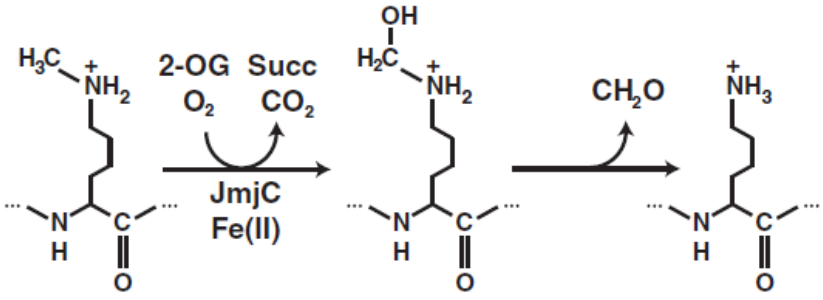


Figure 6: JMJC demethylase mechanism[32].

The alteration of histone methylations by •NO modifies the epigenetic landscape significantly and affects gene expression through activation or repression. •NO also has an effect on the expression level of a number of histone-modifying enzymes including methyltransferase G9a and other histone modifications including acetylation. This novel mechanism of •NO in changing the degree and the location of post-translational histone modifications has the potential to alter gene transcription leading to tumor phenotype regulation (Fig. 6)[33].

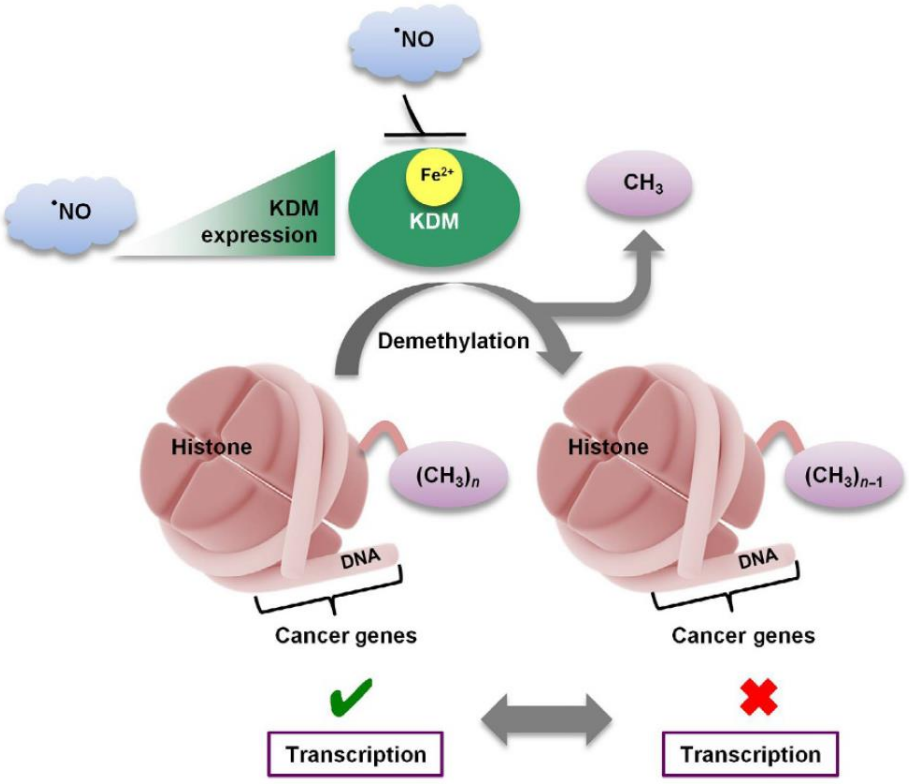


Figure 7: Mechanisms of epigenetic regulation by •NO[33].

1.4 •NO and DNA Modifications

5-methylcytosine (5mC), formed at CpG islands by DNA methyl transferases (DNMT), has been associated with gene repression[34] since dense methylation of gene promoters is associated with gene silencing[38]. Alterations in DNA methylation patterns have been implicated in autoimmune diseases[39], neurological and psychiatric conditions[40] and cancer[41,42]. However, several gene-regulatory mechanisms depend entirely on DNA methylation, including those responsible for X-chromosome inactivation in females, allele-specific silencing of imprinted genes and transcriptional repression of transposons[37]. All DNMT enzymes have the same mechanism as HMT enzymes. This involves methyl transfer from the donor SAM to the 5-carbon of the cytosine ring, yielding SAH. This occurs through enzyme linked thiolate attack of the 6-carbon, which primes the cytosine ring for enamine attack on the methyl group of SAM. The resulting enzyme-linked product then undergoes β-elimination to release 5mC and regenerate active DNMT[32].

5mC had long been thought as a crucial and stable epigenetic modification until 2009, when 5-hydroxymethylcytosine (5hmC) was found to be abundant in the genomic DNA of mouse embryonic stem cells[46] and in mouse Purkinje neurons and brain[47]. The enzymes ten eleven translocation (TET1-3), which are α-ketoglutarate- and Fe(II)-dependent dioxygenases, can bind to CpG island and catalyze the enzymatic conversion of 5mC to 5hmC (Fig. 7)[35,36,37]. TET1 is of interests because it was found that TET1 is required to maintain high levels of 5hmC in mouse genomic regions close to CpG proximal promoters[48]. TET1 and TET3 share a similarity in which they both contain a CXXC domain, unlike TET2. CXXC domains in TET enzymes specifically target and bind to 5mC and 5hmC[53].

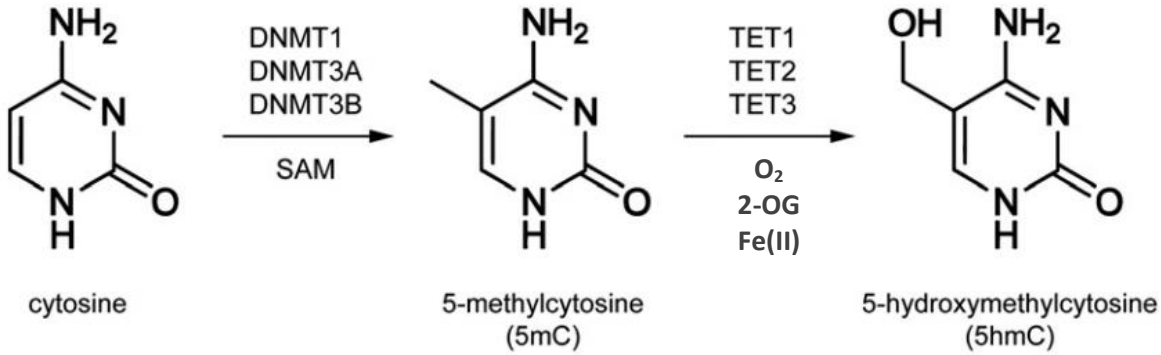


Figure 8: Cytosine in mammalian DNA modifications[37].

5hmC is considered as an intermediate in DNA demethylation pathways[45] and has been defined as the 6th nucleobase[51]. In addition to 5hmC, evidence was found for the enzymatic formations of 5-formylcytosine (5fC) and also 5-carboxylcytosine (5caC) as TET oxidation products [49]. The identifications of 5fC and 5caC emphasized significances of demethylation pathway[52]. Further studies are being conducted to examine biological functions of 5hmC and TET enzymes as key epigenetic modifiers and possible guardians of CpG islands[50].

1.4.1 Pyrosequencing

DNA sequencing to study DNA methylation is one of the most useful tool to study biological systems. Pyrosequencing is a novel sequencing-by-synthesis method that measures the incorporation of nucleotides through the enzymatic conversion of released pyrophosphate into a proportional light signal during DNA synthesis[55,56]. The utilization of pyrosequencing can accurately measures the degree of methylation at several CpGs in close proximity. Cycles of four deoxynucleotide triphosphates (dNTPs) are separately added to the reaction mixture and the reactions require four enzymes to catalyze: Klenow fragment of the DNA polymerase I, ATP sulfurylase, luciferase and apyrase.

The standard for 5-methylcytosine measurements had been bisulfite sequencing in which cytosine is converted to uracil using sodium bisulfite. Using pyrosequencing, converted cytosines are read as thymines and methylated cytosines are read as cytosine since they were not converted to uracil. The downfall of bisulfite sequencing is the inability of the method to quantify 5-hydroxymethylcytosine since it is read as cytosine. Therefore, a new approach had been found in which a specific oxidation of 5-hydroxymethylcytosine to 5-formylcytosine step had been added and the next conversion of 5-formylcytosine to uracil using bisulfite conversion would result in the discrimination of 5hmC (Fig. 9)[57].

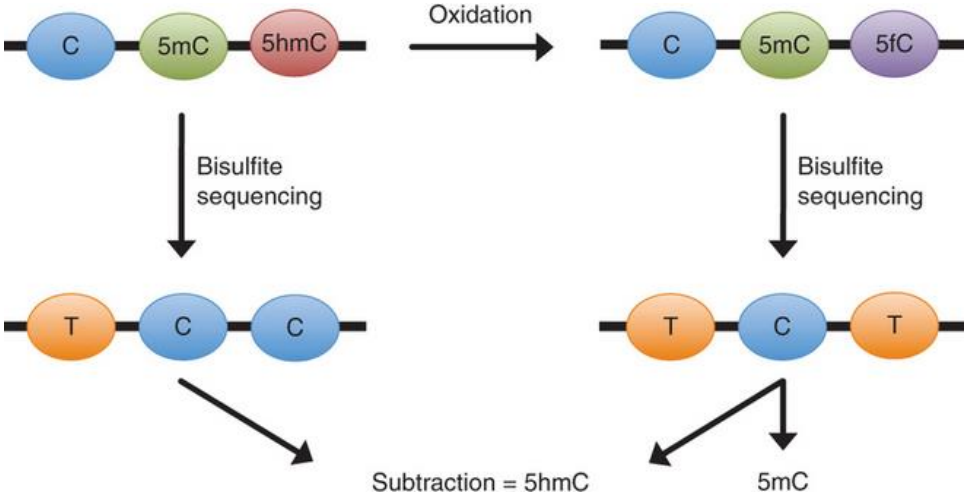


Figure 9: Workflow of oxidative bisulfite sequencing.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Reagents:

Phosphate Buffer Saline (PBS), Phenylmethanesulfonyl fluoride (PMSF), Tris, H_2SO_4, urea, ethylenediaminetetraacetic acid (EDTA), Tween 20, potassium iodide (KI), vanadium (III) chloride, acetic acid, hydrochloric acid (HCl), lipopolysacchride (LPS), aminoguanidine (AG), L-NAME, $Fe(NH_4)_2(SO_4)_2$, ascorbic acid, alpha-ketoglutarate and mineral oil were purchased from Sigma-Aldrich Corporation. Dry non-fat milk was purchased from Nestle. All cell culture reagents were purchased from Invitrogen with the exceptions of methionine-free DMEM and arginine-free DMEM (AthenaES). HEPES was purchased from Invitrogen. Protease inhibitor cocktail set III was purchased from Calbiochem. TFN-γ was from R&D Systems. RIPA Lysis Buffer System was from SantaCruz.

2.2 Cell Culture:

Cells were grown at 37°C and 5% CO_2 in a tissue culture incubator. MDA-MB-231 were incubated with DMEM supplemented with 10% FBS and 1% PS unless otherwise indicated as methionine-free or arginine-free. SK-N-BE (2) were cultured in RPMI media supplemented with 10% FBS and 1% PS.

2.3 Histone Extraction:

Approximately 6 million cells were lysed in cold RIPA Lysis Buffer System (Santa Cruz, 24948) supplemented with PIC, PMSF and Sodium Orthovanadate. The sample was then vortexed every 2 minutes for 15 minutes to ensure complete lysis followed by centrifuging at 10,000g for 10

minutes. A white pellet is expected to be seen at the bottom of the tube. This pellet was then washed with Buffer W (10mM Tris-Cl, pH 7.4 and 13mM EDTA), centrifuged down and resuspended in 450µL of 0.4N H_2SO_4 overnight at 4^0C with shaking. After a centrifuge step the next day at 14,000g for 15 minutes, the white shrunken pellet was discarded. Histone containing supernatant was mixed with 2.25mL of acetone overnight at 20^0C and collected the next day by centrifugation at 14,000g for 15 minutes. Purified histones were then washed once with ice-cold acetone, air dried and resuspended in 4M urea[43]. BCA Bio-Rad DC protein assay was used and the reaction plate was read using the microplate reader to measure protein concentration.

2.4 Protein Extraction:

Whole cell lysates were collected using CelLytic™ M cell lysis reagent (Sigma) supplemented with PIC (Calbiochem) and PMSF (Sigma).

2.5 DNA Extraction:

DNA was collected using DNeasy Blood and Tissue Kit (Qiagen 69504) and quantified using NanoDrop Spectrophotometer (Thermo Scientific).

2.6 Western Blotting:

Proteins were denatured by heating for 10 min in boiling water and then quenching on ice before loading onto 4-15% Mini-PROTEAN TGX gel (BioRad). After gel electrophoresis (BioRad Power Pac Basic), the protein was transferred onto PVDF membranes using the iBlot™ transfer system (Invitrogen). The membranes were blocked with 5% non-fat dry milk in PBS solution containing 0.1% Tween and incubated overnight with primary antibodies: H3K9me2 (Cell

Signaling), H3K9ac (Cell Signaling), H3K4me1, 2 or 3 (Cell Signaling), H3K4ac (Millipore), KDM3A (Abcam), G9a (Cell Signaling), or β-actin (Cell Signaling). The blots were analyzed in a FluorChem HD2 imager (Alpha Innotech) or FluorChemE Fe0443 imager (Cell Biosciences) using SuperSignal West Femto or Pico maximal sensitivity substrate (Thermo Scientific). After imaging, the membranes were stained with Coomassie Blue (BioRad) to confirm accurate protein loading.

2.7 RT-PCR

RNA was extracted using RNAqueous 4PCR kit (Ambion) and reverse-transcribed using the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science). PCR was performed using the Fast SYBR® Green master mix (Applied Biosystems). Standard manufacturer's protocol was followed during each stage. All PCRs were performed on the Step One Plus real time PCR system (ABI). The oligonucleotide primer sequences are: *KDM1A* forward, 5'-ggccattctcaaagggattt-3' and reverse, 5'-ccaacgagacaccacagtttc-3'; *KDM1B* forward, 5'-ccctacccatccctagtcct-3' and reverse, 5'-ggcaatcacagacatcagca-3'; *KDM5A* forward, 5'-ccggcatctgcaaaattc-3' and reverse, 5'-acgaaagctttttacttcacagg-3'; *KDM5B* forward, 5'-actgaccgaagctcaccagt-3' and reverse, 5'-cttccctcggcaacagtc-3'; *KDM5C* forward, 5'-gcggatcttggacctctaca-3' and reverse, 5'-cgacggtccttgcagatag-3'; *KDM2B* forward, 5'-cgacggtccttgcagatag-3' and reverse, 5'- caccttcgccttcaaaag-3'.

2.8 Nitric Oxide Analyzer

Nitric oxide quantification was performed by chemiluminescence using the Sievers Nitric Oxide Analyzer 280i (NOA). 25µl of media were injected into a reaction chamber containing vanadium

(III) chloride in HCl to determine total NO_3^-/NO_2^- concentrations. To determine only nitrite concentrations, 25µl of media were injected into a reaction chamber containing potassium iodide or sodium iodide in acetic acid.

2.9 Enzymatic TET1 Activity Assay:

Mouse recombinant TET1 was purchased from Active Motif (31363). 10uL reactions of enzymes and 5mC containing DNA (Active Motif, 55008) were conducted at 37^0C for 3 hours in 50mM HEPES, pH 6.8, 50uM $Fe(NH_4)_2(SO_4)_2$, 2mM ascorbate and 1mM alpha-ketoglutarate using a digital dry bath (SouthWest Science SH-1002) with a thin layer of mineral oil on top to prevent evaporations. Reaction products were used for dot blotting.

2.10 Dot Blotting:

DNAs were loaded onto nitrocellulose membrane (Bio-Rad 162-0091) using Bio-Dot SF Microfiltration Apparatus (Bio-Rad, 170-6542). After drying, the membrane was then blocked with 5% non-fat dry milk in TBS solution containing 0.05% Tween, incubated with suitable primary antibodies overnight at 4^0C, 5mC (CalbioChem, 16233D3) or 5hmC (Active Motif, 40000). Blot images were then taken using SuperSignal West Femto or SuperSignal West Pico maximal sensitivity substrate (Thermo Scientific) using FluorChemE Fe0443 imager (Cell BioSciences).

2.11 ELISA-based TET1 Activity Assay:

Active human recombinant TET1 (Epigentek E12002) activity was measured by colorimetric-based ELISA kit (Epigentek P3086) according to the manufacturer instructions. To study TET1

activity *in vitro*, MDA-MB-231 or SK-N-BE(2) cells were nuclear extracted using a nuclear extraction kit (Epigentek, OP-0002-1) and TET1 activity was measured using the same *Epigenase 5mC Hydroxylase TET Activity/Inhibition Assay* kit as instructed. TET1 enzyme inhibition activity by Sper/NO was also calculated.

2.12 Pyrosequencing:

DNA was collected using DNeasy Blood and Tissue Kit (Qiagen 69504). Extracted DNA was then bisulfate converted and cleaned-up using EpiTect Fast DNA Bisulfite Kit (59824). PCR was performed using BioRad C1000 Touch Thermal Cycler to amplify converted DNA using the sequences as follow: *BRCA1* forward, 5'- GGAAAGAGTGGGGGATTGG-3' and reverse, 5'-ACCCTCTACCCTCTACTCTTCCAATTA-3'; *DUSP1* forward, 5'-AGGGGTATAAGAGTATGTAAAAGTATAG-3' and reverse, 5'-AAAACTCTCTACCAACTAAAACTAACCT-3'. Pyrosequencing protocol is as instructed by Pyro Mark MD System using the following sequencing primers: *BRCA1* sequence, 5'-AGAGTGGGGGATTGGG-3' and *DUSP1* sequence, 5'- GGAAGGAGAGAGGGAGGA-3'. *MGMT* was purchased as PyroMark Q96 CpG MGMT (Qiagen 972032). All data was collected and analyzed using PSQ^{TM}96MA Software.

2.13 Statistical Analysis:

The data were reported as the Mean ± Standard Deviation. One way ANOVA analysis with Fisher's LSD test was done using OriginPro9.1 software.

CHAPTER 3

RESULTS

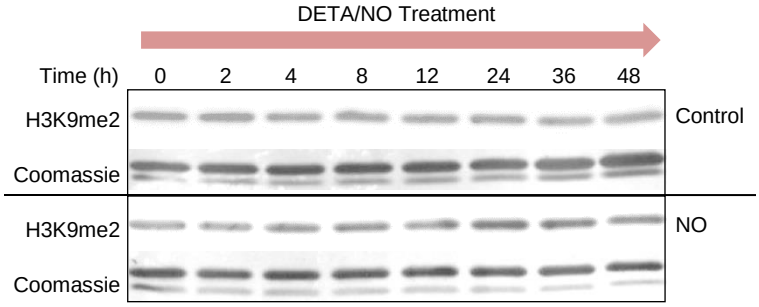
3.1 Effect of Nitric Oxide on Histone Modification is Reversible.

Previously, our lab had confirmed that nitric oxide can inhibit the JMJC class of lysine-specific histone demethylases and affects global histone methylation leading to altered gene expression by forming DNIC. The aim of this study is to observe if continuous exposure of DETA/NO is necessary for this phenomenon to occur. We will be focusing on the epigenetic marks H3K9ac and H3K9me2 and also, the enzymes KDM3A and G9a, demethylase and methyltransferase of H3K9me2, respectively. We hypothesized that an increase in H3K9me2 would lead to a decrease in H3K9ac since increasing methylation at a specific lysine would prevent the acetylation of the same residue.

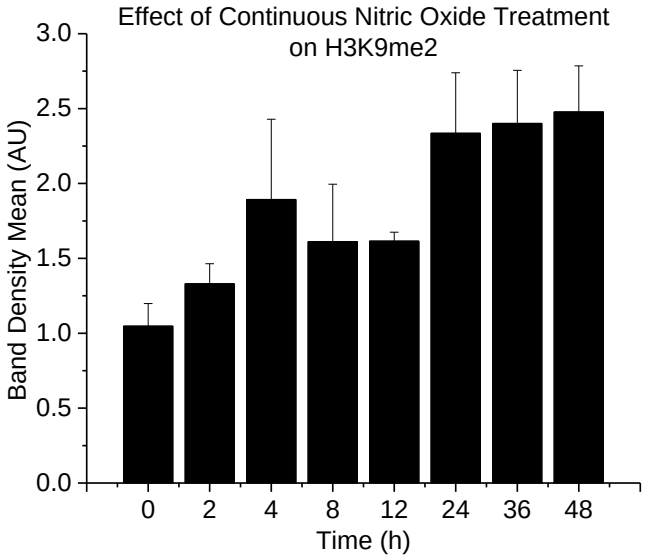
3.1.1 Continuous Exposure of •NO is required for Epigenetic Effect

MDA-MB-231 was grown to 80% confluent then serum starved for 15 hours before treatment with 500µM of DETA/NO. The cells were treated with •NO for 24 hours before a wash step with PBS and let recovered for 24 hours. Histones were collected at 0, 2, 4, 8, 12, 24, 26, 28, 32, 36 and 48 hours for Western Blotting. The changes in H3K9me2 and H3K9ac are shown in Figure 10 and 11, respectively, along with densitometry data for quantitative measurements. Respective coomassie images are attached to show consistencies in protein loading.

A)



B)



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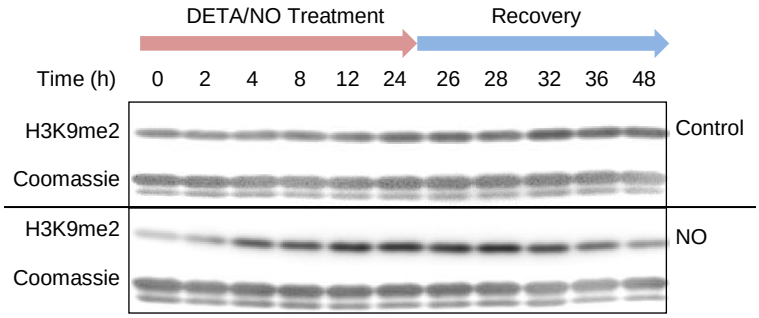


D)

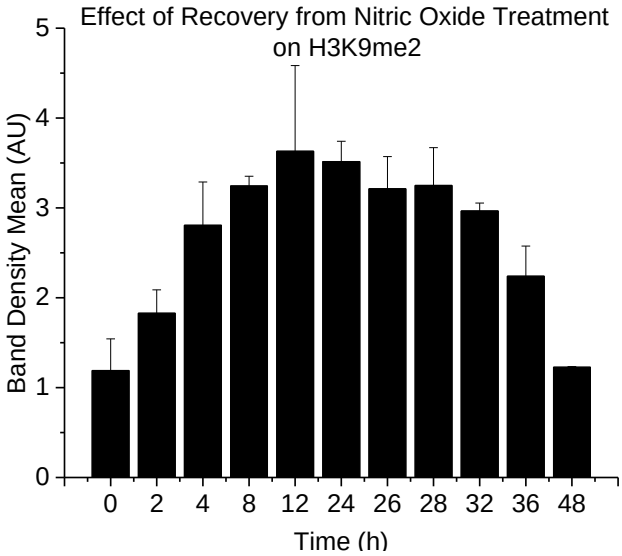
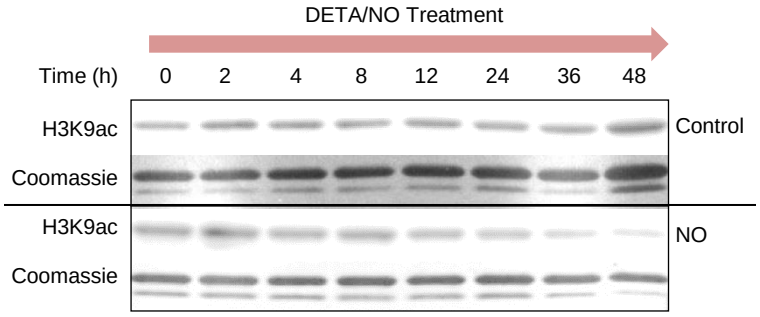
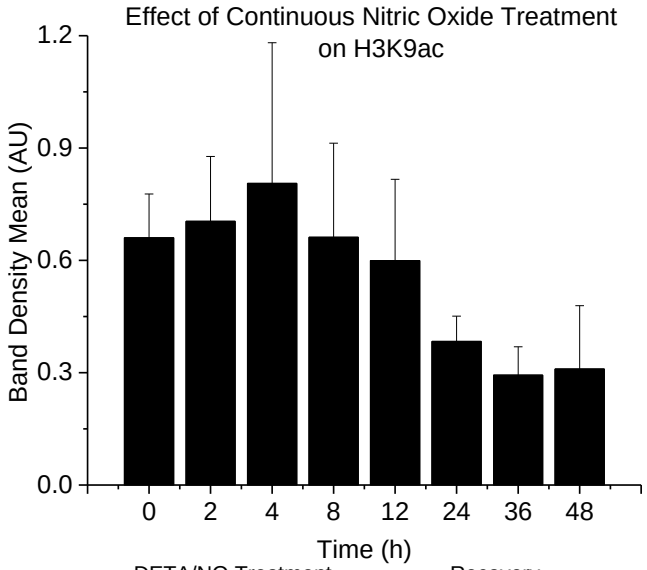


Figure 10: Long term Effect of Nitric Oxide on H3K9me2 Time Course. Western Blot (A) of 48h treatment of 500uM DETA/NO to MDA-MB-231 and (B) its densitometry data. (C) 24h treatment of 500uM DETA/NO to MDA-MB-231 followed by a wash step and recovery for another 24h (24-48h) and (D) its densitometry data. n=3.

A)



B)



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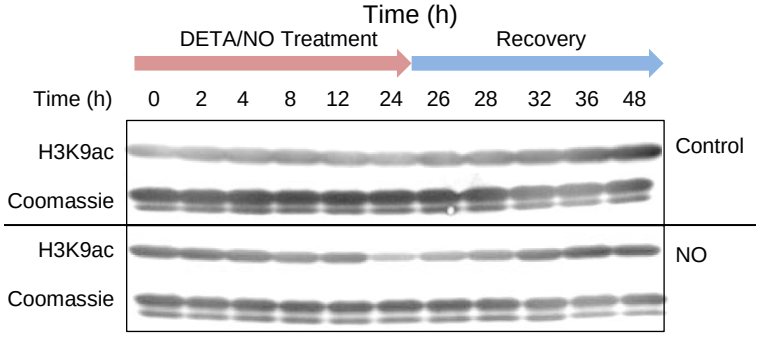

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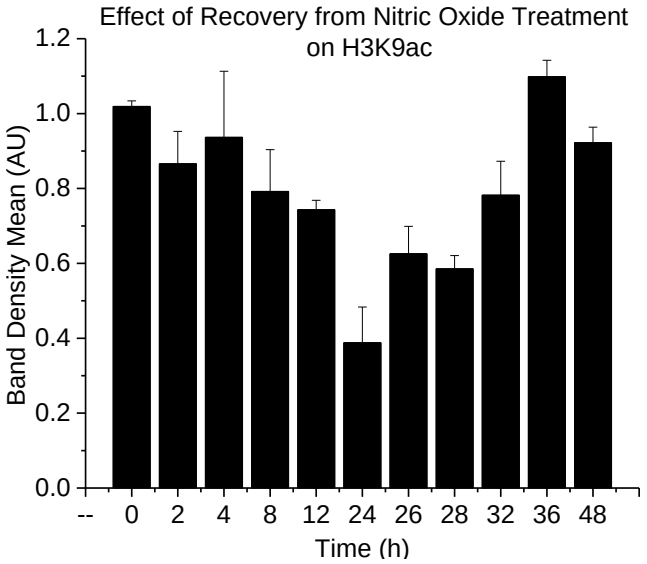
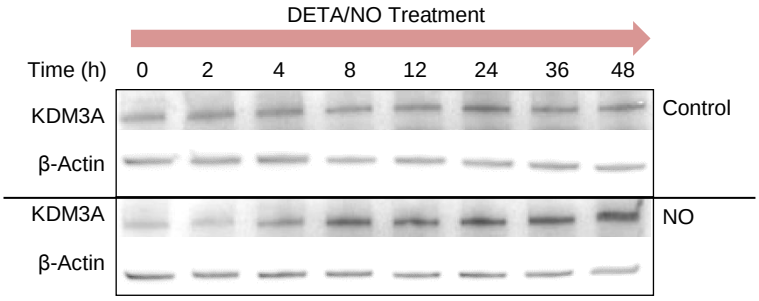


Figure 11: Long term Effect of Nitric Oxide on H3K9ac Time Course. Western Blot (A) of 48h treatment of 500uM DETA/NO to MDA-MB-231 and (B) its densitometry data. (C) 24h treatment of 500uM DETA/NO to MDA-MB-231 followed by a wash step and recovery for another 24h (24-48h) and (D) its densitometry data. n=3.

3.1.2 Long term Effect of Nitric Oxide on Histone Methylase and Demethylase

MDA-MB-231 was grown to 80% confluent then serum starved for 15 hours before treatment with 500µM of DETA/NO. The cells were treated with •NO for 24 hours before a wash step with PBS and let recovered for 24 hours. Whole cell lysates were collected at 0, 2, 4, 8, 12, 24, 26, 28, 32, 36 and 48 hours for Western Blotting against KDM3A and G9a. The changes in KDM3A and G9a are shown in Figure 12 and 13, respectively, along with densitometry data for quantitative measurements.

A)



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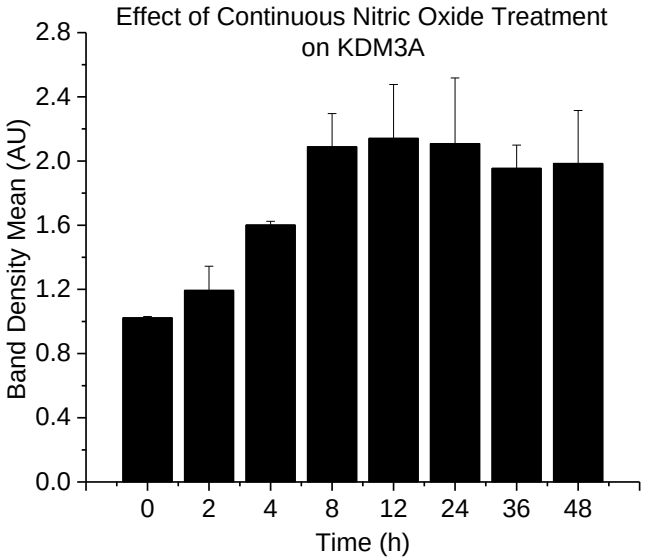

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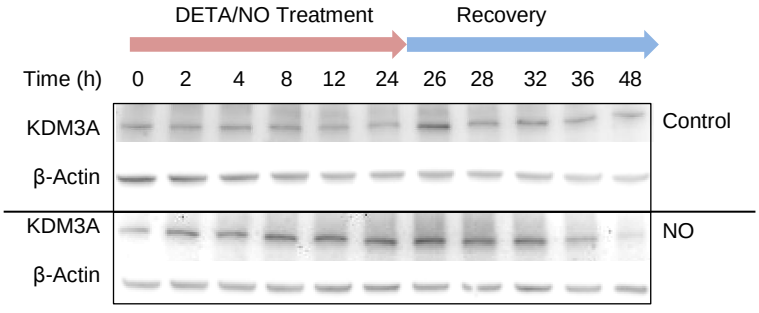

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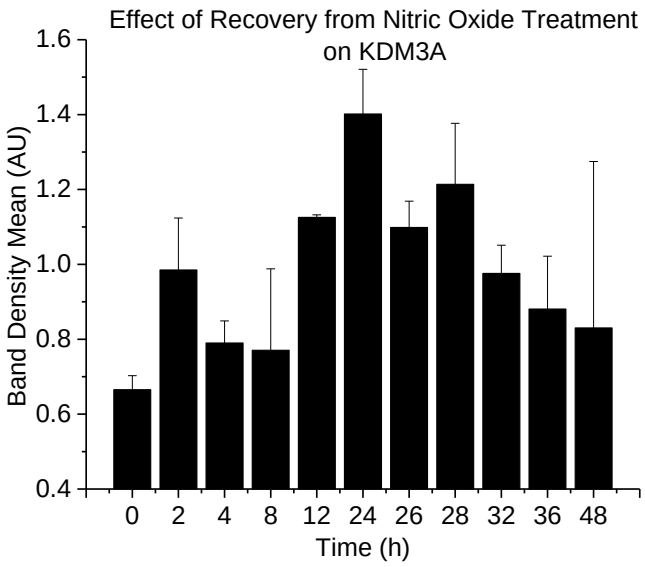
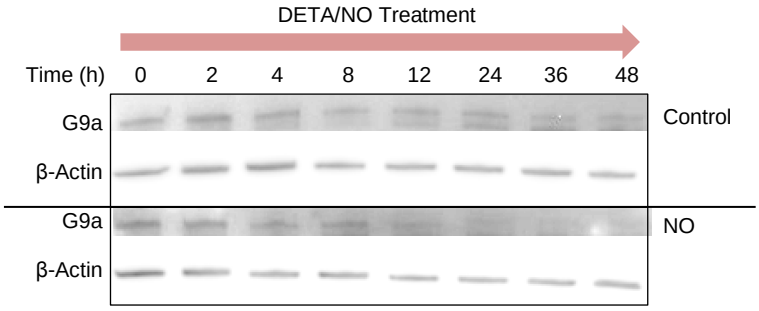
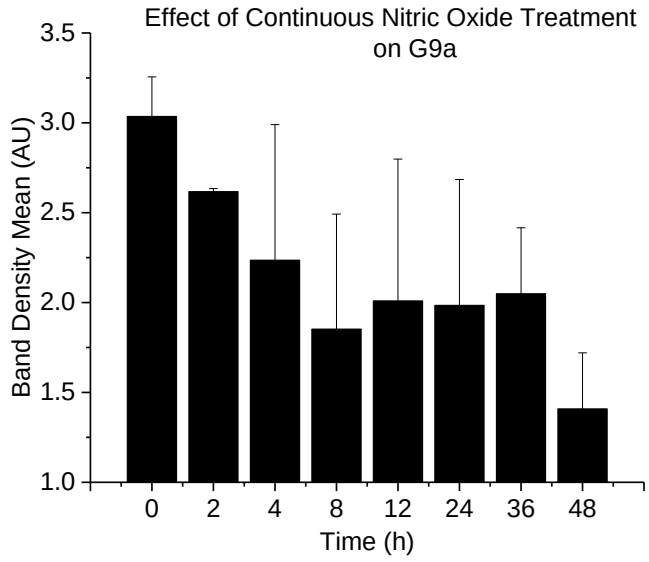


Figure 12: Long term Effect of Nitric Oxide on KDM3A Time Course. Western Blot (A) of 48h treatment of 500uM DETA/NO to MDA-MB-231 and (B) its densitometry data. (C) 24h treatment of 500uM DETA/NO to MDA-MB-231 followed by a wash step and recovery for another 24h (24-48h) and (D) its densitometry data. n=3.

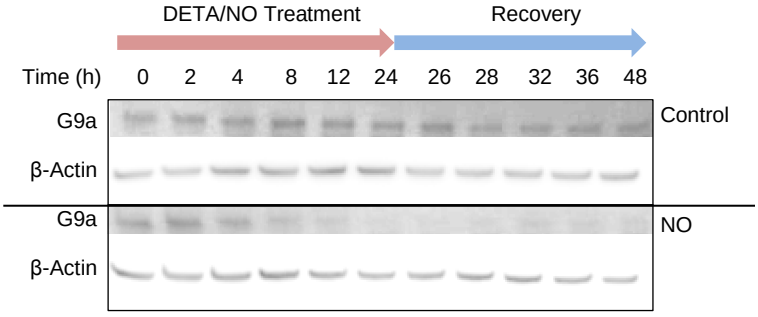
A)



B)



C)



D)

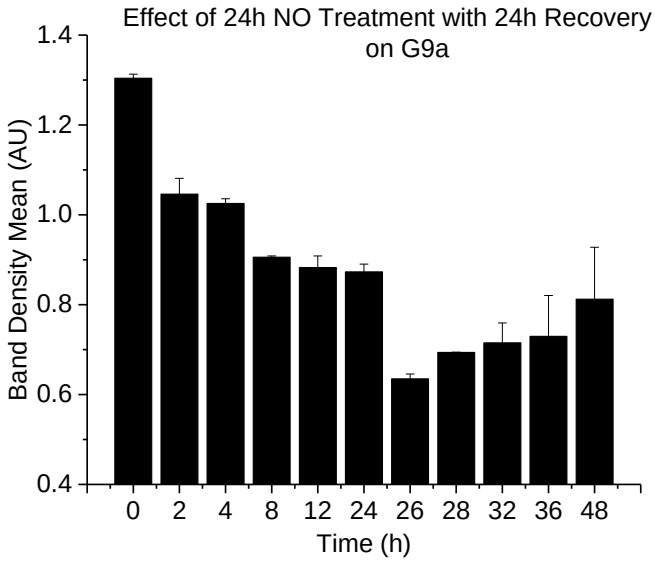


Figure 13: Long term Effect of Nitric Oxide on G9a Time Course. Western Blot (A) of 48h treatment of 500uM DETA/NO to MDA-MB-231 and (B) its densitometry data. (C) 24h treatment of 500uM DETA/NO to MDA-MB-231 followed by a wash step and recovery for another 24h (24-48h) and (D) its densitometry data. n=3.

3.2 Nitric Oxide affects Histone Modifications on H3K4

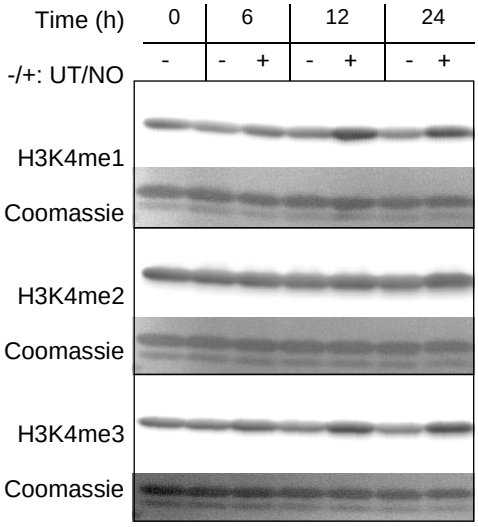
The aim of this study to determine if •NO affects other epigenetic marks through the same mechanism of inhibiting JMJC-class of lysine-specific histone demethylases. Our focus was on H3K4 and the modifications the lysine residue can have, which is acetylation and mono, di or tri-methylation. We hypothesized that there will be identical effects of •NO on the acetylation and methylation of H3K4, similar to H3K9 modifications, which demonstrates that •NO can inhibit the JMJC-containing demethylases of H3K4, increases methylation and leads to the

corresponding effect of decreasing acetylation. We also seek to identify specific demethylases responsible for H3K4me1/2/3 that are affected by DETA/NO treatment.

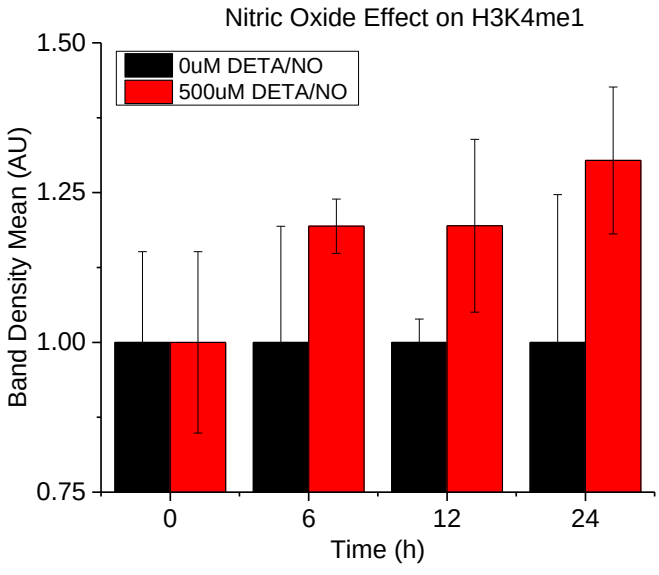
3.2.1 •NO-Mediate Changes in H3K4 Modifications in MDA-MB-231 Cells

MDA-MB-231 was grown to 80% confluent then serum starved for 15 hours before treatment with 500µM of DETA/NO for 24 hours. Histones were collected at 0, 6, 12 and 24 hours for Western Blotting. The changes in H3K4me1, H3K4me2 and H3K4me3 are shown in Figure 14 along with densitometry data for quantitative measurements. Respective coomassie images are attached to show consistencies in protein loading.

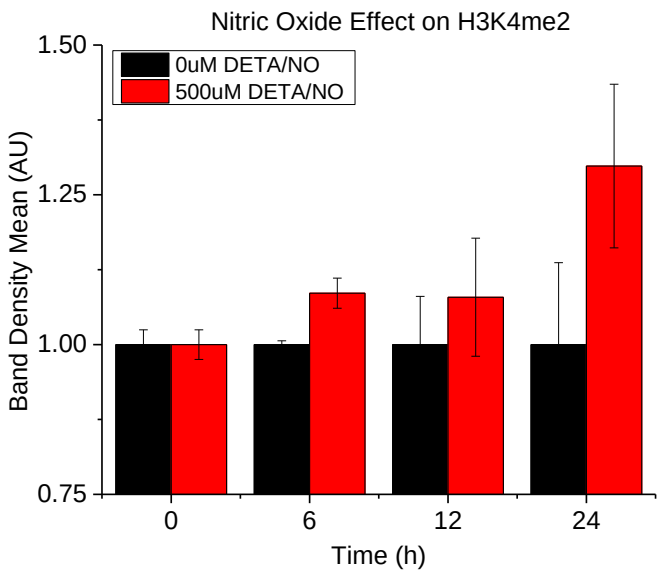
A)



B)



C)



D)

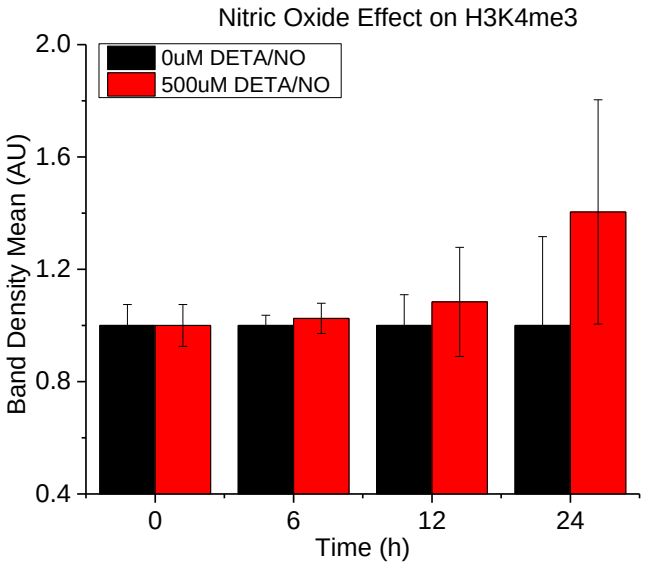


Figure 14: DETA/NO Effect on H3K4me1, H3K4me2 and H3K4me3 Time Course. (A) Western blot data and respective coomassie images for H3K4me1/2/3. Desitometry data for H3K4me1 (B), H3K4me2 (C) and H3K4me3 (D). n=2.

3.2.2 Nitric Oxide Increases H3K4 Demethylases Gene Expression

The aim of this experiment is to confirm the ability of •NO to inhibit JMJC-class of lysine-specific histone demethylases in 6 different demethylases responsible for H3K4me1/2/3 demethylation. MDA-MB-231 were treated with 500µM DETA/NO for 24 hours and quantitative RT-PCR analysis was conducted for known H3K4 demethylase genes: *KDM1A, KDM1B, KDM5A, KDM5B, KDM5C* and *KDM2B*.

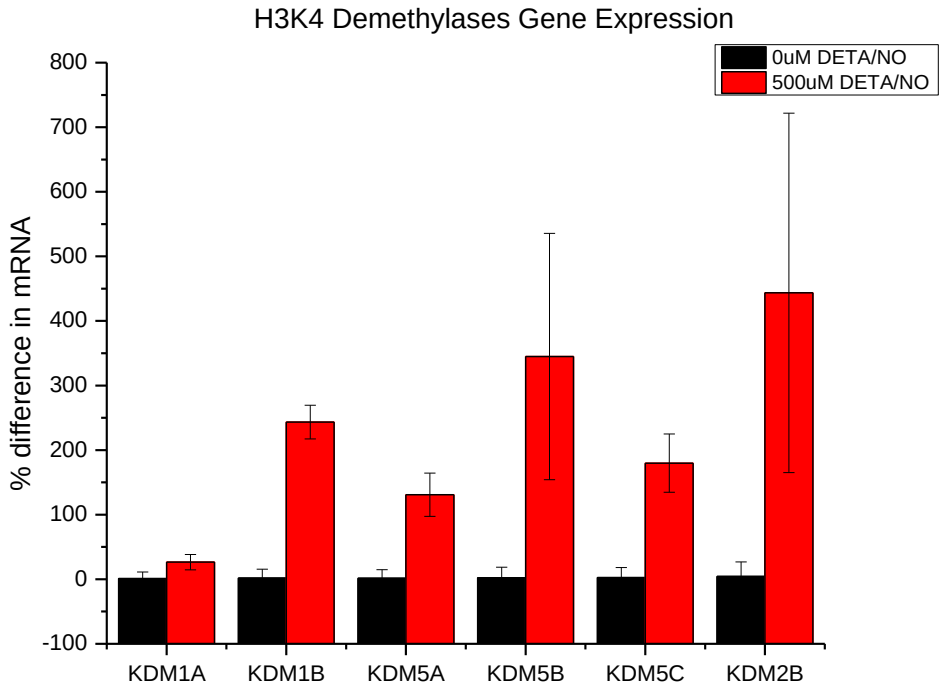


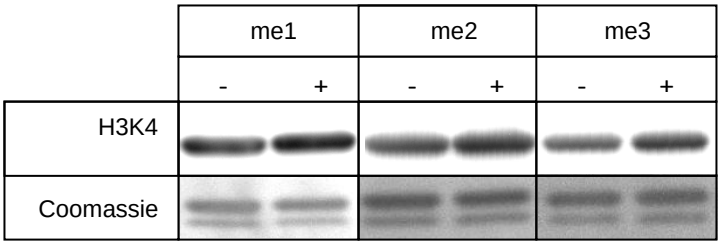
Figure 15: RT-PCR of known H3K4 demethylases. MDA-MB-231 were collected after 500uM treatment of DETA/NO for 24h. n=3.

3.2.3 Methylation by •NO is not a result from increase in methylation

To determine that the increases in •NO-mediated methylation is not a result from increase in overall methylation in the cell by methyltransferases, we incubated MDA-MB-231 in methionine-free media prior to DETA/NO treatment of 500µM for 24 hours then proceeded to

histone extraction and immunoblotted agaisnt H3K4me1, H3K4me2 and H3K4me3. Coomassie images showed no discrepancies in proteins loading.

A)



B)

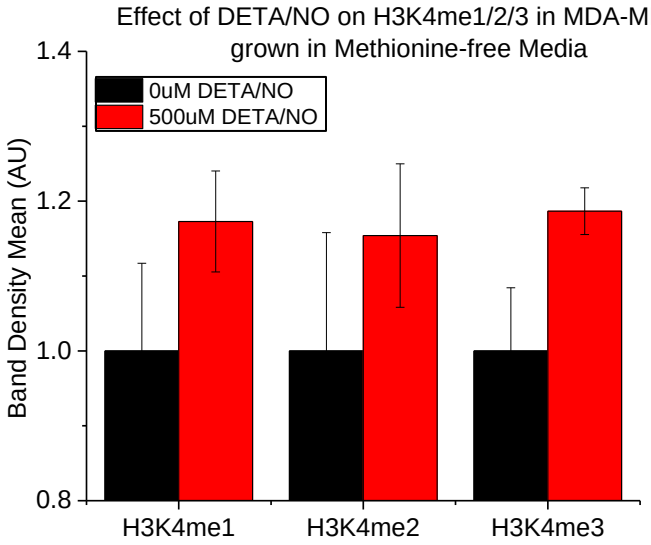
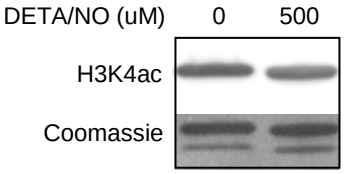


Figure 16: Methylation pattern by NO is not a result of increase in overall methylation by methyltransferases. (A) Western blots of H3K4me1/2/3 with and without 500uM DETA/NO for 24h and their respective coomassie blots. (B) Densitometry data of H3K4me1/2/3 for comparison purposes. n=3.

3.2.4 Nitric Oxide Decreases H3K4ac

Along with measuring changes in H3K4me1/2/3, a parallel experiment to observe H3K4ac was conducted to show the decrease in H3K4ac with DETA/NO treatment, opposite to the increase in H3K4 methylation. Serum starved MDA-MB-231 were treated with 500µM of DETA/NO and histone extracted after 24 hours of treatment. Coomassie image is shown to clarify protein loading for gel electrophoresis and Western blot transferring. Densitometry data shows a decrease in H3K4ac in DETA/NO treated cell samples.

A)



B)

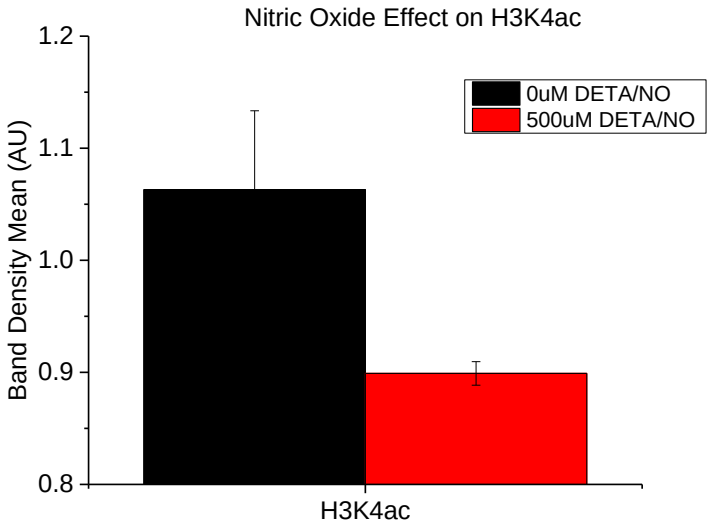


Figure 17: Effect of NO on H3K4ac (A) Western Blot of H3K4ac after 24h DETA/NO Treatment at 500uM. (B) Respective densitometry data. n=3.

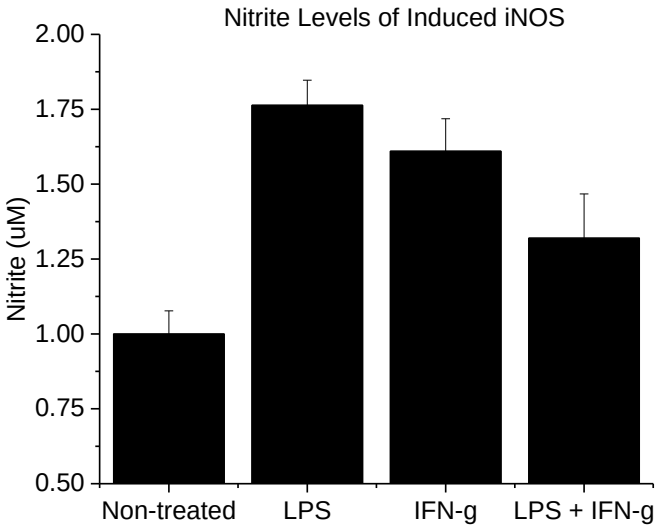
3.3 The Role of iNOS in Mediating Histone Modification

The aim of this experiment is to determine how endogenous production •NO in MDA-MB-231 modifies methylation or acetylation of H3K9. We were interested in stimulating iNOS by cytokines, especially LPS, IFN-γ or a combination of both, to increase •NO production and determine if this treatment can modify our epigenetic marks of interest. We also seek to learn if this endogenous production of •NO can be inhibited by aminoguanidine, L-NAME or the use of arginine-free media as compared to the untreated MDA-MB-231. Nitrite/nitrate analysis was conducted using Nitric Oxide Analyzer to quantify the total amount of •NO produced by the cell from the media solutions.

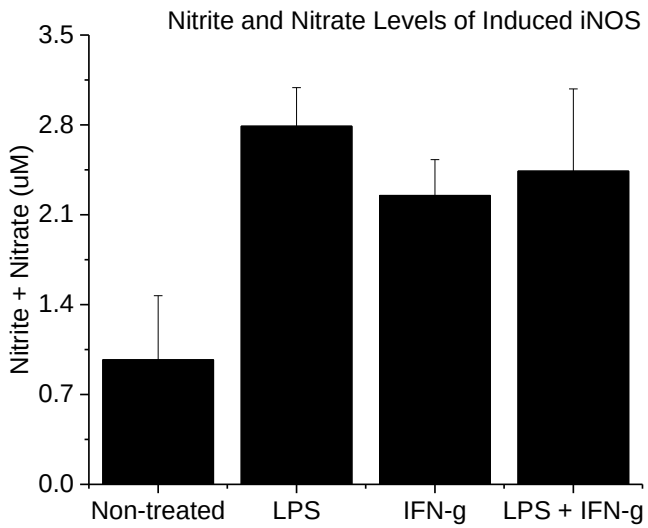
3.3.1 Cytokine Treatments Increases Nitric Oxide Production

Here, we tested the effect of iNOS stimulation with cytokines to increase the production of nitric oxide. MDA-MB-231 grown in DMEM media with 10% FBS and 1% PS were treated with: 1ug/mL LPS, 2ng/µL IFN-γ or a combination of both LPS and IFN-γ for 24 hours before media collection for nitrate and nitrite measurements using the NOA. Using nitrite and nitrate level, the amount of •NO (nmol) was calculated. There is about 2 fold increase in •NO with LPS treatment. MDA-MB-231 cells grown without FBS did not have an increase in •NO level after LPS, IFN-γ or LPS+IFN-γ treatment (data not shown). Whole cell lysate was extracted from treated MDA-MB-231 in serum for Western blotting agaisnt iNOS to observe if there are any changes in iNOS level.

A)



B)



Non-treated	4.85 nmol
LPS	13.95 nmol
IFN-γ	11.25 nmol
LPS + IFN-γ	12.2 nmol

Table I: NITRIC OXIDE PRODUCTION BY INDUCING MDA-MB-231.

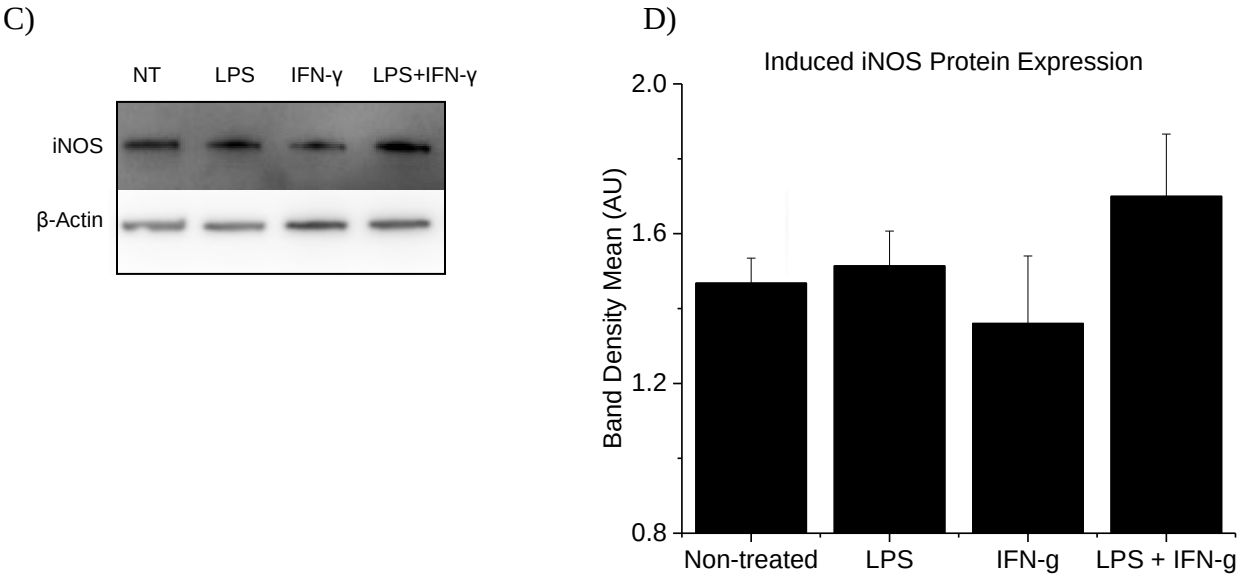


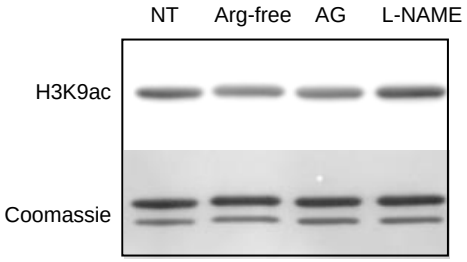
Figure 18: Cytokine stimulations in MDA-MB-231. (A) Nitrite and (B) Nitrate Data of LPS, IFN-γ and LPS+IFN-γ treated cells. (C) Western blot measuring iNOS expression and its densitometry data (D). n=2.

3.3.2 Regulation of iNOS in MDA-MB-231 by Aminoguanidine Leads to Changes in H3K9ac and H3K9me2 Levels

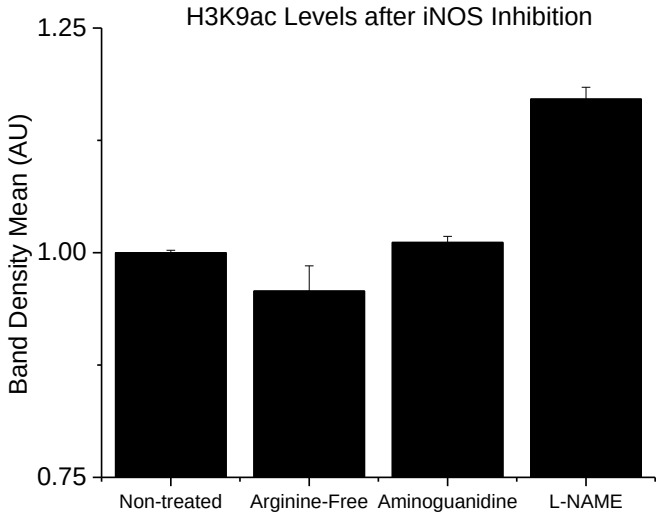
Increased expression of inducible nitric oxide synthase (iNOS) has been associated with disease aggressiveness and poor survival in breast cancer patients. A recent study had found that mice treated with aminoguanidine, an iNOS inhibitor, had a significantly reduced tumor size, 59% as compared to the control, in a xenograph model of green fluorescent protein-tagged MDA-MB-231 breast cancer cells[44]. Here, we want to correlate the effect of inhibiting iNOS with changes

in H3K9ac and H3K9me2 levels in MDA-MB-231 since inhibiting iNOS leads to a decrease in cellular level of nitric oxide. Inhibitors used for this experiment include aminoguanidine (1mM), L-NAME (2mM) and arginine-free media. Since arginine is the substrate NOS enzymes act on to produce citrulline and •NO, the use of arginine-free media results in a decrease of •NO-production. MDA-MB-231 cells were treated with inhibitors for 24 hours before histone extraction and Western blotting. Nitrite was measured from aminoguanidine-treated cells media to confirm the decrease in nitric oxide level.

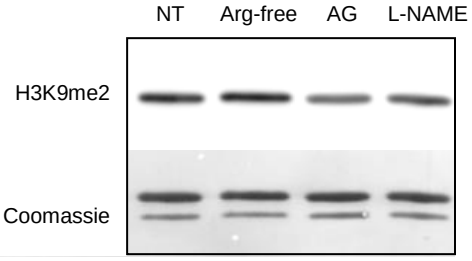
A)



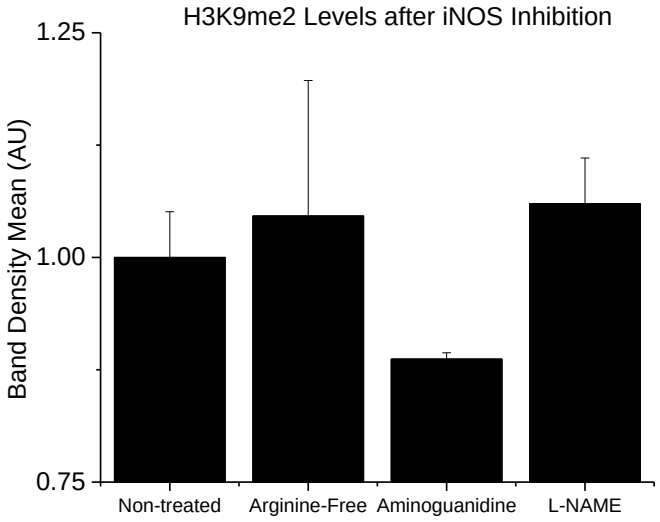
B)



C)



D)



E)

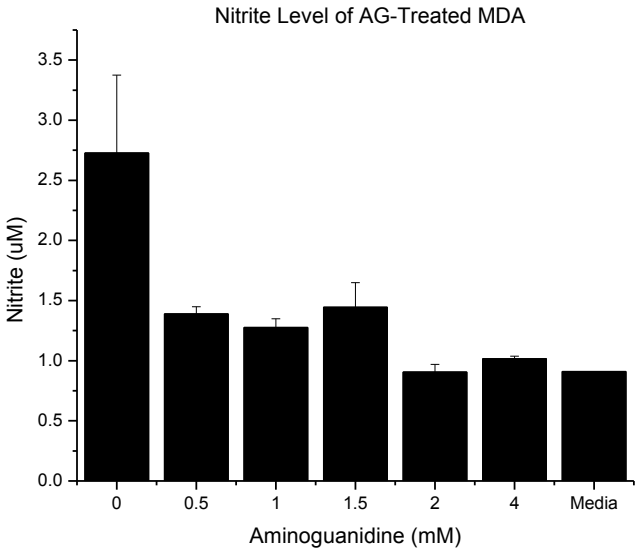


Figure 19: Inhibition of iNOS in MDA-MB-231. (A) H3K9ac and (C) H3K9me2 data of MDAC-MB-231 cells treated with Arginine-free media, Amminoguanidine and L-NAME with respective densitometry data (B) and (D). (E) Nitrite measurement of Aminoguanidine treated MDA-MB-231. n=2.

3.4 Nitric Oxide Inhibits TET Enzymes and Alters 5hmC Level

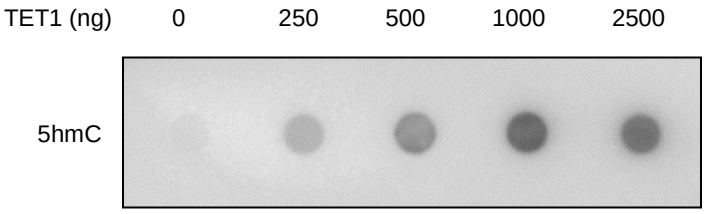
We hypothesized that nitric oxide can inhibit TETs by forming DNIC with iron that is required for the catalytic activity of these enzymes. Since TETs possess enzymatic activity toward the methyl mark of 5mC and convert it into 5hmC, the inhibition of TETs would lead to the decrease of 5hmC. This is a potential novel mechanism in which •NO can mediate DNA modifications.

3.4.1 Dot blot showing TET1 Inhibition by Nitric Oxide

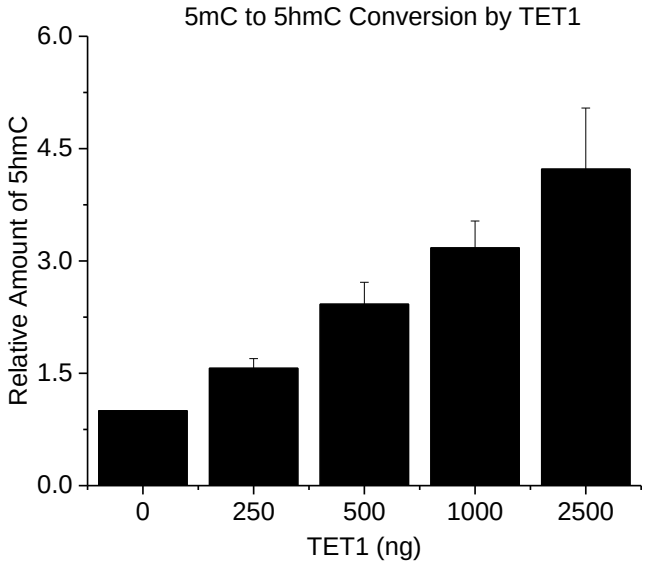
To test enzymatic TET1 Activity, 100ng of DNA containing 5mC (Active Motif) was incubated with various concentration of TET1 protein (Active Motif) (0, 250, 500, 1000, 2000ng) for 3 hours. Reaction products were dot blotted onto nitrocellulose membrane against 5hmC. 5hmC containing DNA was increased with increasing in TET1 concentration which shows that the

protein is active and suitable for the next step. We then tested the inhibition of TET1 by Nitric Oxide using an •NO donor, Sper/NO. Various concentration of Sper/NO (0, 10, 25, 50 and 100μM) was added to the reaction mixture of 100ng 5mC DNA and 1000ng of TET1 for 3 hours. Dot blot results show that there is a decrease in 5hmC which suggests that •NO inhibits the activity of TET1. Further experiments are needed to confirm the mechanisms of TET1 inhibition by •NO through the formation of DNIC and decrease in iron cofactor availability.

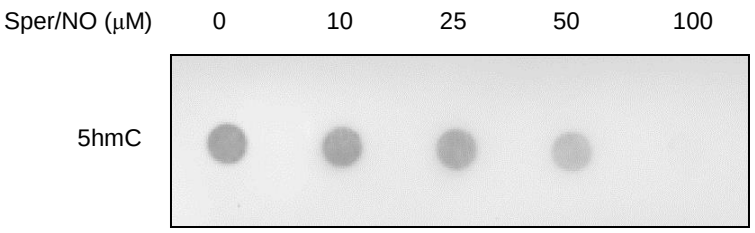
A)



B)



C)



D)

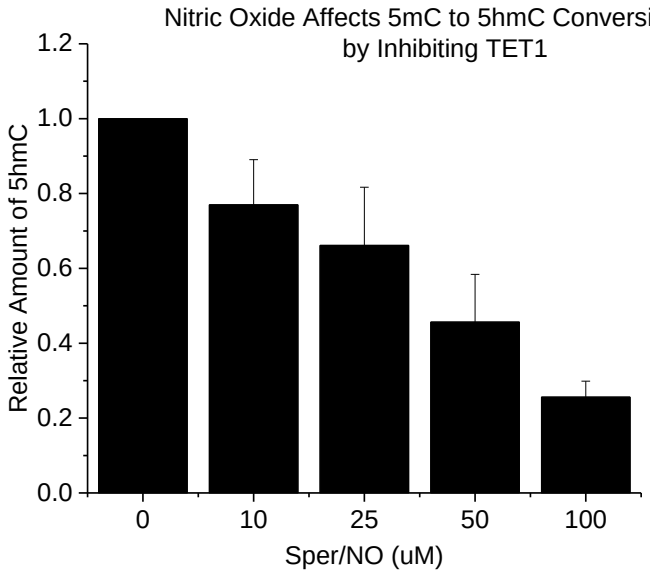
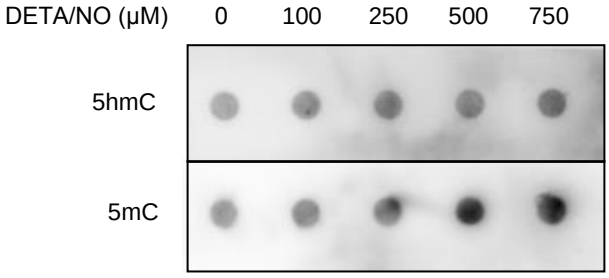


Figure 20: TET1 Inhibition by •NO. (A) Dot blot showing converted 5hmC from 5mC with increase in TET1 active, recombinant enzyme. (B) TET1 dose course densitometry data. (C) Inhibition of TET1 using Sper/NO. (D) Sper/NO dose course densitometry data.

3.4.2 Nitric Oxide Alters 5hmC Level in MDA-MB-231

To observe changes in 5hmC level in genomic DNA, MDA-MB-231 was grown to 80% confluent and treated with DETA/NO for 24 hours at 0, 100, 250, 500 or 750uM. Genomic DNA was then extracted and dot blotted on nitrocellulose membrane against 5hmC or 5mC antibodies. The ratio of 5hmC to 5mC densitometry data was graphed to determine relative changes in these DNA modifications with •NO exposure. Since DNA concentration in the cell could change over the course of the experiment and affect overall amounts of 5mC and 5hmC, 5hmC/5mC data can give a better estimate on how •NO can have an impact on the regulation of these two epigenetic marks.

A)



B)

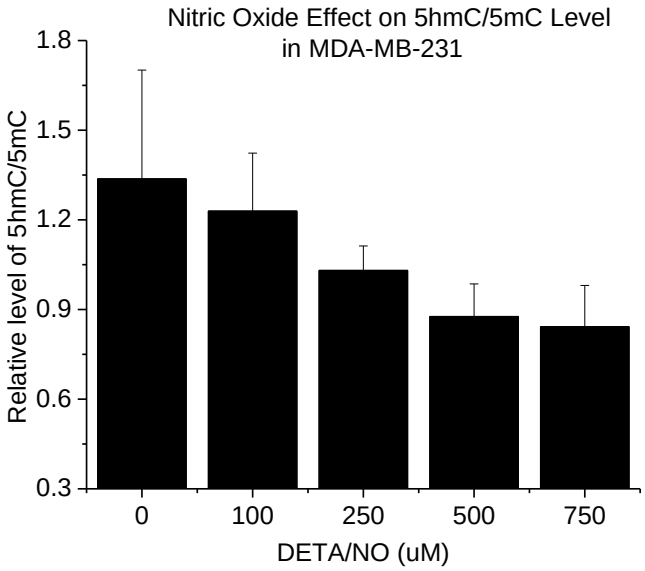


Figure 21: •NO inhibits TET *in vitro*. (A) 5hmC and 5mC data of genomic DNA extracted from DETA/NO treated MDA-MB-231. (B) 5hmC/5mC graph.

3.4.3 ELISA-based Assay to Measure TET1 Enzymatic Activity

To further quantitatively measure TET1 activity (Epigentek), a colorimetric-based ELISA assay was performed (Epigentek) which can be used to calculate the amount of 5hmC formed using 5hmC-specific antibodies. Reaction product, 5hmC, is proportional to the activity of the enzyme and was measured by reading the absorbance at 450nm using a plate reader. Protocol is as instructed by manufacturer. Different amounts of active, human recombinant TET1 enzyme were

used (0, 100, 200 and 300ng) along with 8ng of 5mC starting substrate for each reaction. 5hmC calculation is as followed:

$$5hmC\ (ng) = \frac{(Sample\ OD - Blank\ OD)}{Slope\ of\ standard\ curve}$$

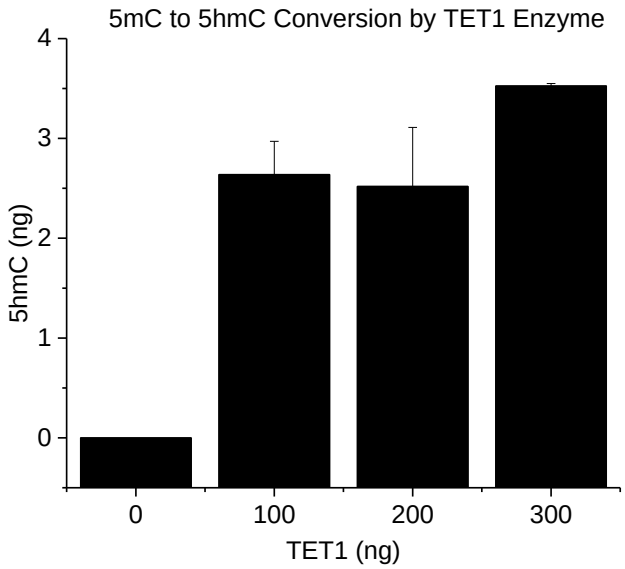


Figure 22: Quantitative analysis of TET1 activity. Amounts of 5hmC formed from active TET1 dose course.

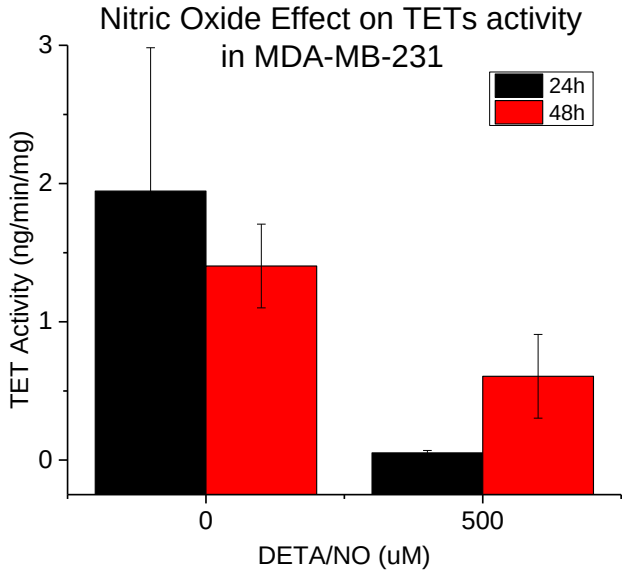
3.4.4 Nitric Oxide Inhibits TETs in MDA-MB-231

Nuclear extract of MDA-MB-231 was collected using EpiQuik Nuclear Extract kit (Epigentek) to measure the effect of nitric oxide on TETs activity in the cell. 5µg of nuclear extract was used to determine 5mC to 5hmC conversion after 24 and 48 hours treatment with 500µM DETA/NO. 5hmC quantification (ng) was calculated using OD450nm data after performing ELISA-based assay (Epigenase 5mC-hydroxylase TET Activity/Inhibition Kit). The % inhibition of •NO to TETs was also calculated as instructed by protocols from manufacturer. The equation for TETs activity and % inhibition are as followed:

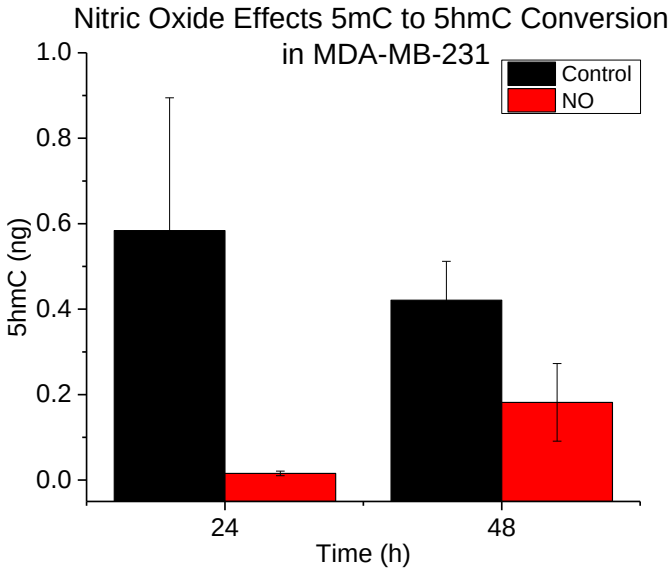
$$TET\ Activity\ (ng/min/mg) = \frac{5hmC\ (ng)}{(protein\ amount\ (ug) \times \min)}$$

$$\%\ inhibition = \left[1 - \frac{Inhibitor\ sample\ OD - Blank\ OD}{No\ inhibitor\ sample\ OD - Blank\ OD}\right] \times 100\%$$

A)



B)



C)

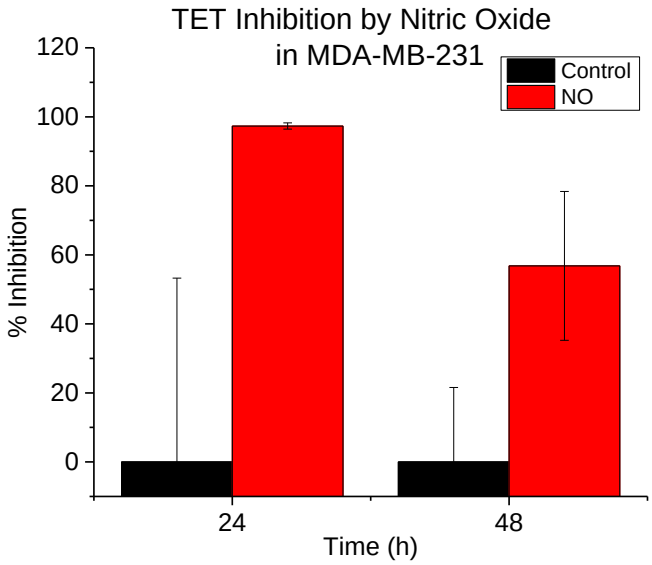
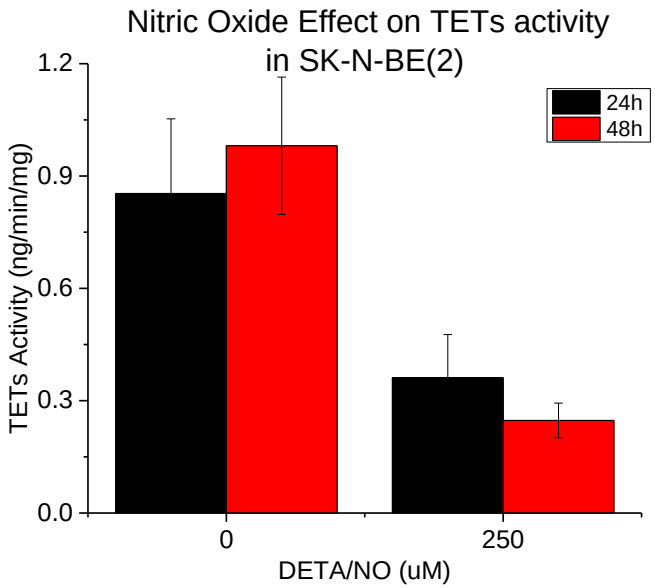


Figure 23: TETs inhibition by •NO in MDA-MB-231. (A) TETs activity of nuclear extract from DETA/NO-treated MDA-MB-231 to show TETs inhibition by •NO. (B) Amounts of 5hmC formed after DETA/NO treatment. (C) % inhibition of Nitric Oxide to TETs.

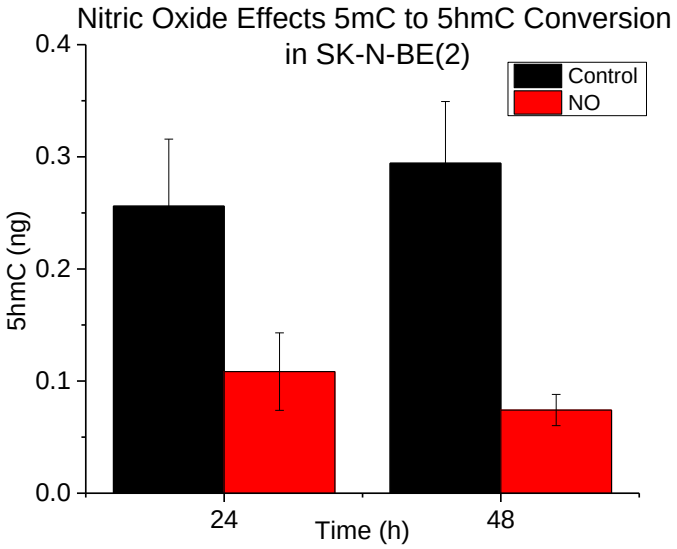
3.4.5 Nitric Oxide Inhibits TETs in SK-N-BE (2)

To further confirm •NO inhibition on TETs activity, a different cell line, SK-N-BE(2), was used. SK-N-BE(2) are neuroblastomas that are derived from neural crest cells and therefore, they have high levels of 5hmC[59]. Recently, it was published that hypoxia increases global 5hmC level by the transcriptional activation of TET1 in SK-N-BE(2)[60]. Genes encoding for the α-ketoglutarate- and Fe(II)-dependent dioxygenases are upregulated in hypoxia[61] similar to •NO response as observed in our RT-PCR for JMJC demethylases. Consequently, it is of interest to determine the expression of TET1 in this cell line in response to •NO since it provides a relevant model to our study. Nuclear extract was collected to measure TETs activity after treatment with 250μM DETA/NO for 24 and 48 hours since nuclear extract contains TET1/2/3. 250uM DETA/NO is corresponded to 80 ± 22nM of $[NO]_{ss}$.

A)



B)



C)

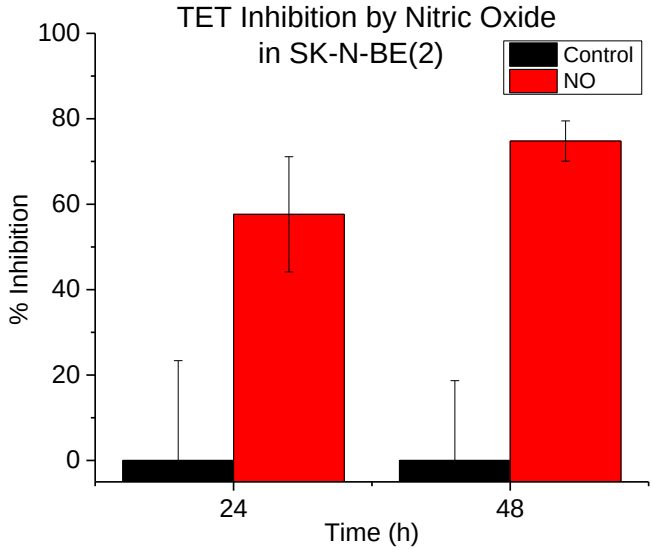


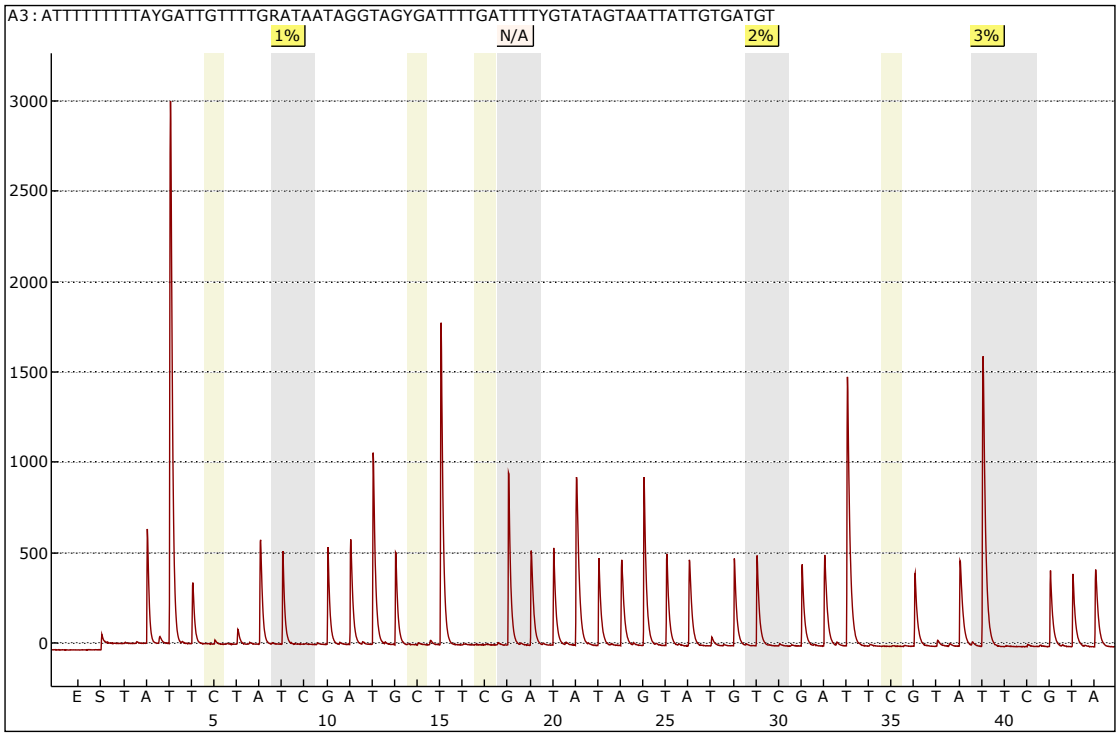
Figure 24: TETs Inhibition by •NO in SK-N-BE (2). (A) OD450nm data of nuclear extract from DETA/NO-treated SK-N-BE (2). (B) Amounts of 5hmC formed after DETA/NO treatment. (C) % inhibition of nitric oxide to TET1.

3.5 •NO Modifies DNA Methylation Pattern

After clarifying the inhibition of TETs by •NO which lead to changes in overall 5hmC DNA levels, we seek to investigate the effect of •NO on DNA methylation of specific genes. Since DNA methylation is associated with gene repression, we want to established a correlation between •NO and DNA methylation to further demonstrate that •NO is an epigenetic regulator in MDA-MB-231. We looked at genes related to breast cancer: *BRCA1*, *DUSP1* and *MGMT*. MDA-MB-231 were treated with 500µM DETA/NO for 24 hours before DNA extracted, bisulfite converted and pyrosequenced. Protocol is as described in the Methods section.

BRCA1:

A)



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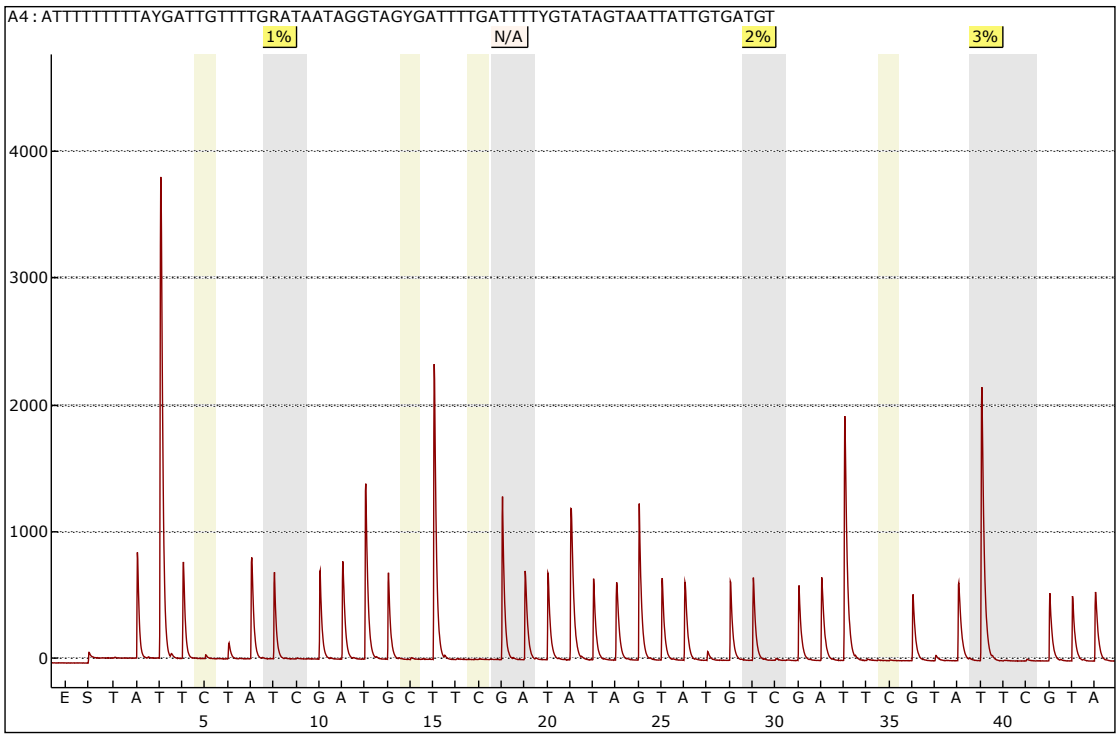

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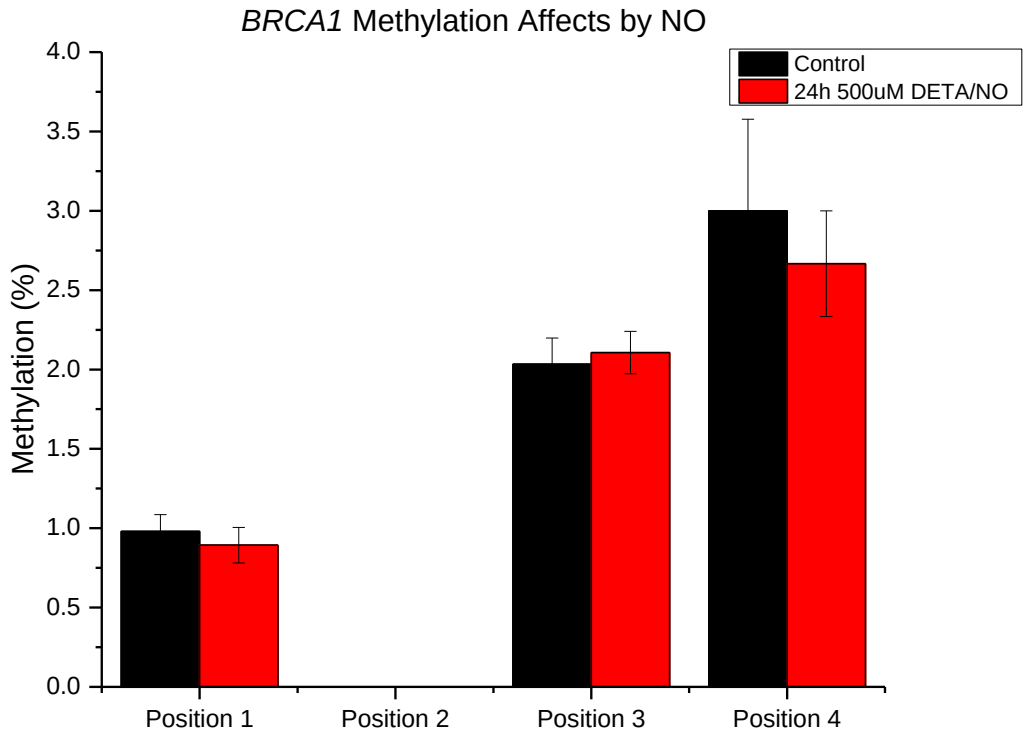
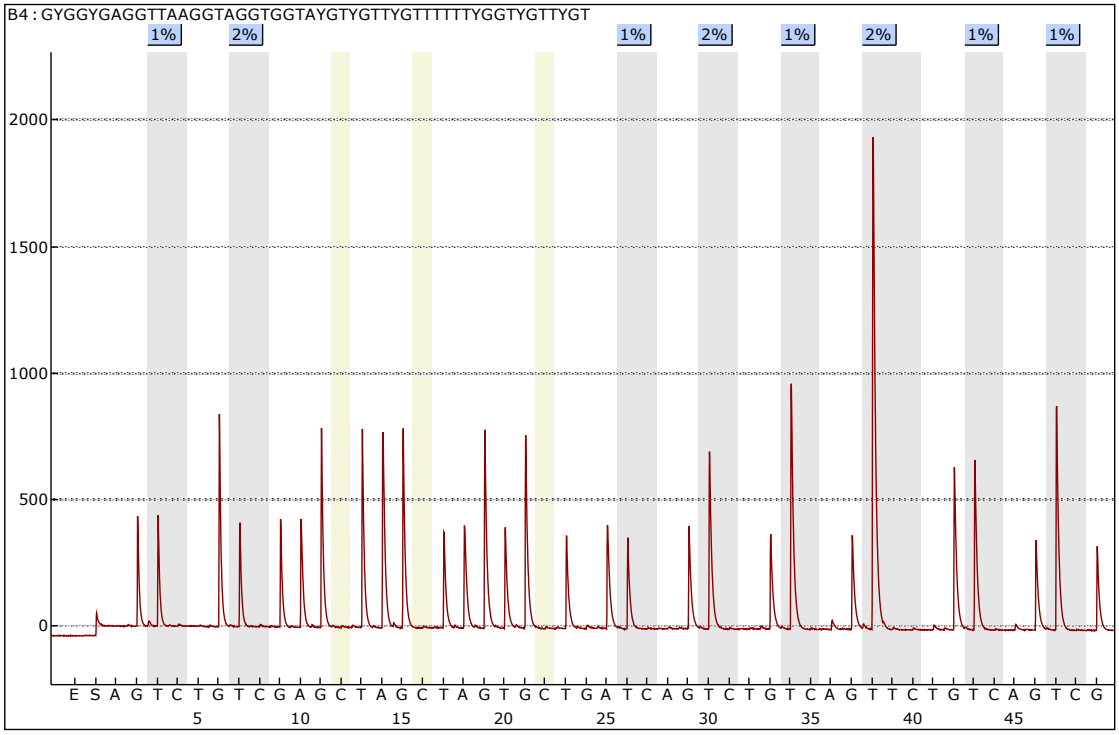


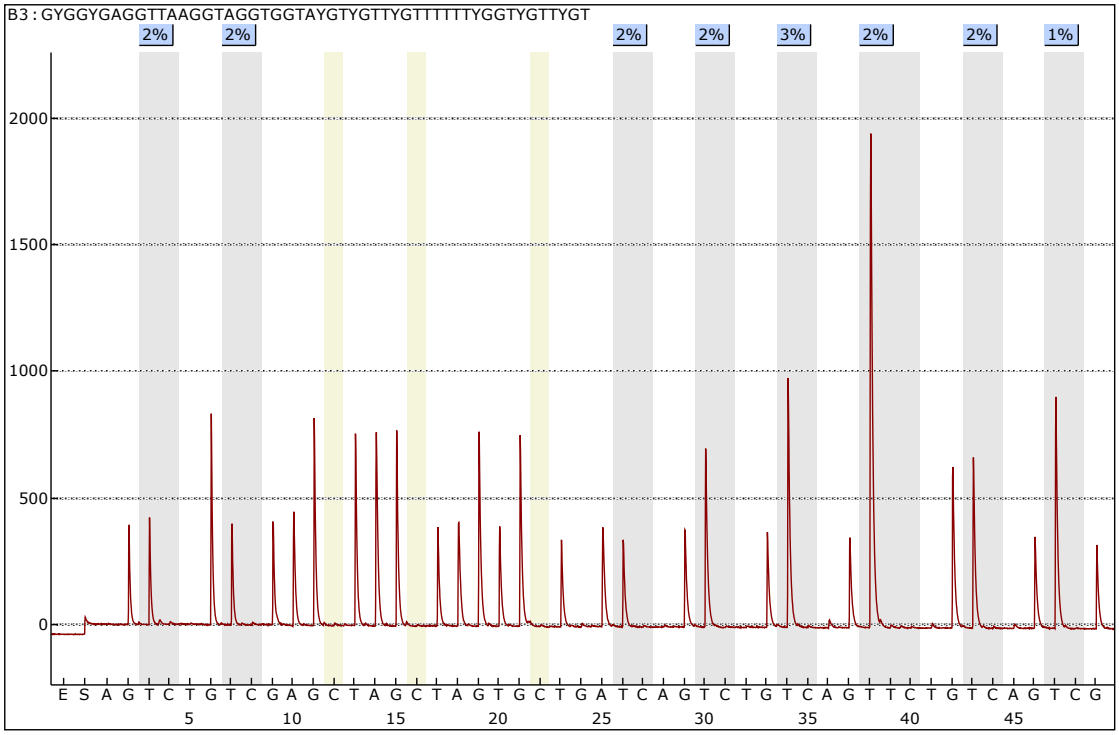
Figure 25: •NO Affects Methylation in *BRCA1*. A) Pyrogram of un-treated MDA-MB-231. B) 500µM DETA/NO-treated MDA-MB-231. C) Total % methylation in *BRCA1*.

DUSP1:

A)


B4 : GYGGYGAGGTTAAGGTAGGTGGTAYGTYGTTYGTTTTTTYGGTYGTTYGT
1%
2%
1%
2%
1%
2%
1%
1%
2000
1500
1000
500
0
E S A G T C T G T C G A G C T A G C T A G T G C T G A T C A G T C T G T C A G T T C T G T C A G T C G
5
10
15
20
25
30
35
40
45

B)


B3 : GYGGYGAGGTTAAGGTAGGTGGTAYGTYGTTYGTTTTTTYGGTYGTTYGT
2%
2%
2%
2%
3%
2%
2%
1%
2000
1500
1000
500
0
E S A G T C T G T C G A G C T A G C T A G T G C T G A T C A G T C T G T C A G T T C T G T C A G T C G
5
10
15
20
25
30
35
40
45

C)

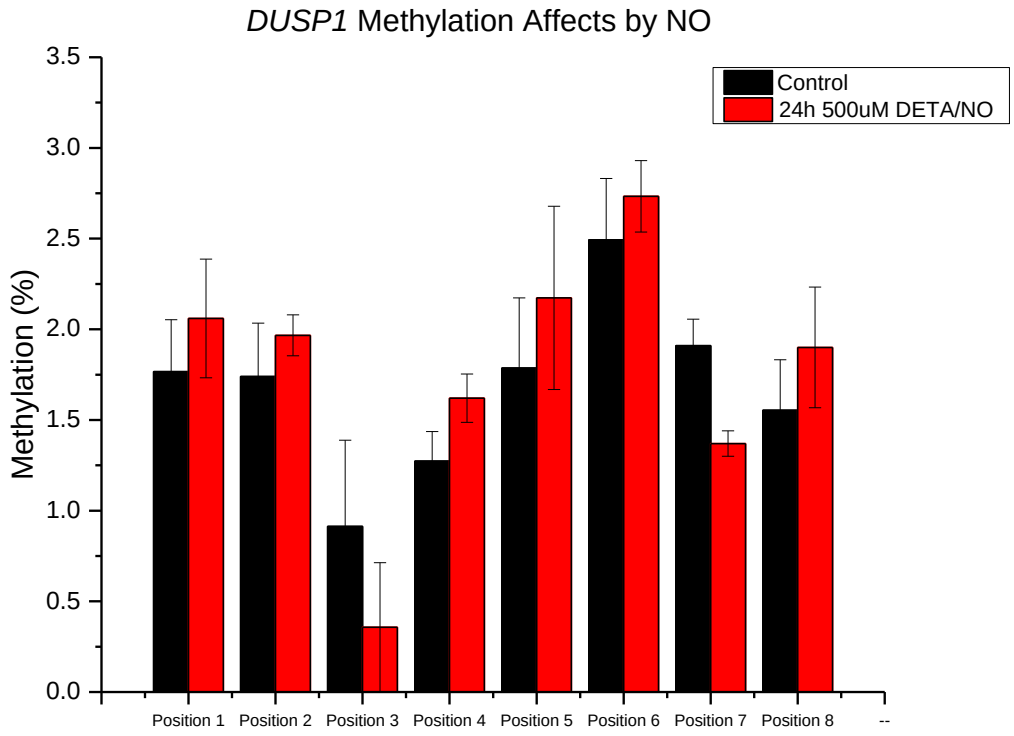
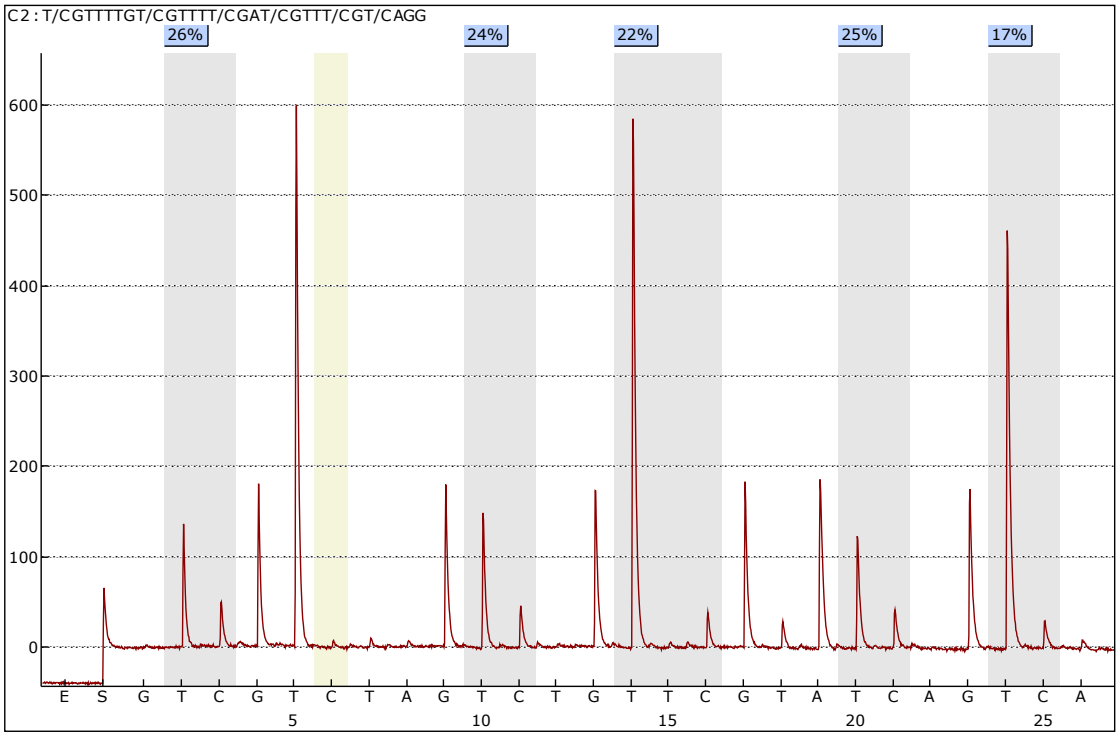


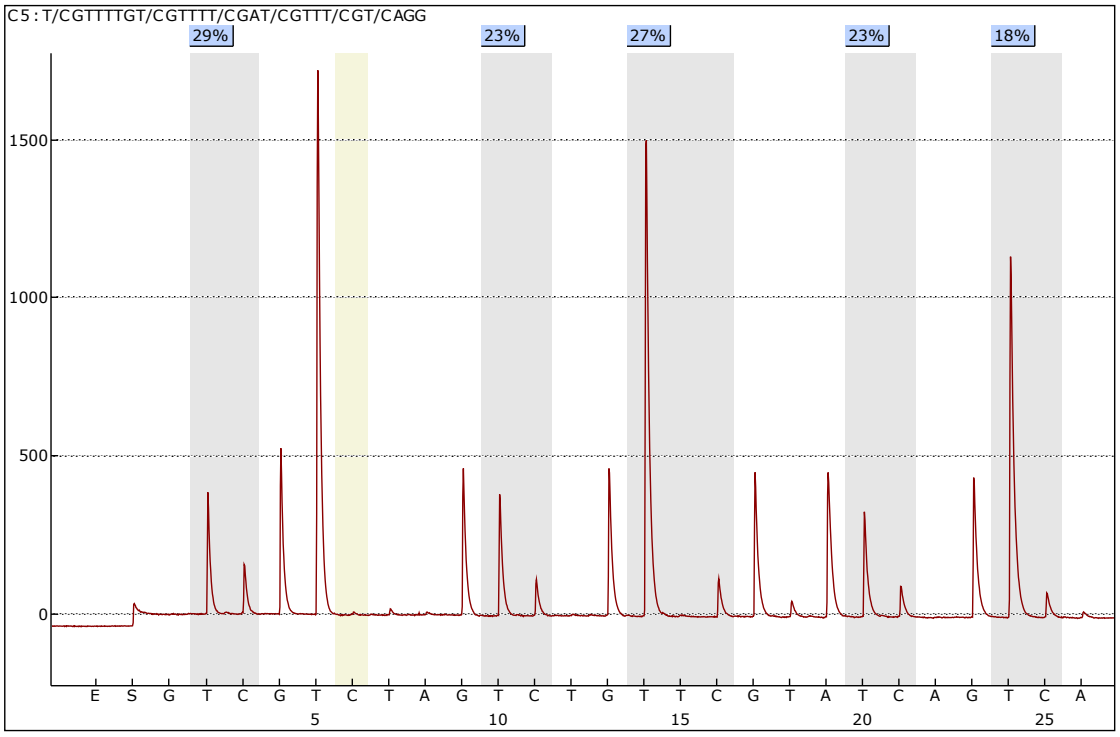
Figure 26: •NO Affects Methylation in *DUSP1*. A) Pyrogram of un-treated MDA-MB-231. B) 500µM DETA/NO-treated MDA-MB-231. C) Total % methylation in *DUSP1*.

MGMT:

A)



B)



C)

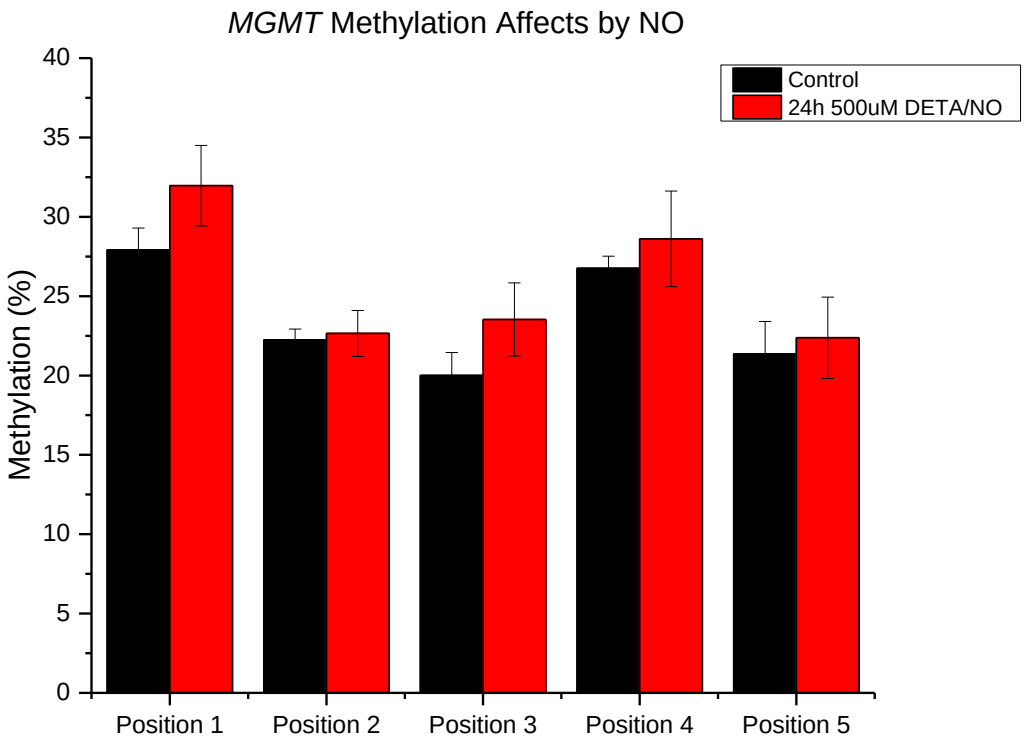


Figure 27: •NO Affects Methylation in *MGMT*. A) Pyrogram of un-treated MDA-MB-231. B) Pyrogram of 500µM DETA/NO-treated MDA-MB-231. C) Total % methylation in *MGMT*.

CHAPTER 4

DISCUSSION

4.1 Reversible Effect of Nitric Oxide on Histone Modification

As a confirmation to our lab's previous publication, nitric oxide can directly inhibit the Jumonji C domain Fe (II) α-ketoglutarate family of demethylases by forming dinitrosyl iron complexes and reduce the iron cofactor availability leading to the global modification of histone methylation within physiologically relevant doses. To continue to the next step of studying this inhibition of demethylases by •NO, a series of time courses were conducted to study the long term effect of •NO treatment on MDA-MB-231 along with recovery periods to observe the stability of •NO inhibition. We also seek to determine if changes in global methylation of H3K9 play a role in modifying the global acetylation of H3K9 since lysine 9 of histone 3 can be methylated along with acetylated.

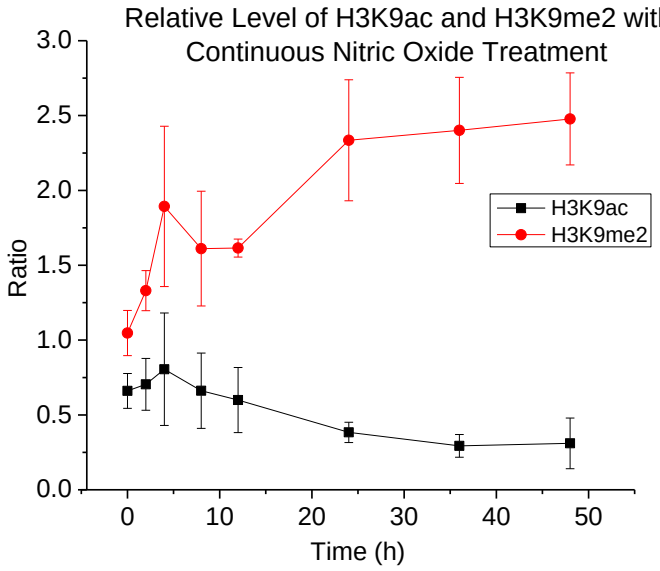
4.1.1 Continuous exposure of •NO is required for inhibition of demethylases

Here, we observed the long-term effect of 500μM DETA/NO, an •NO donor ($[•NO]_{ss}$= 265 ± 21 nM), in MDA-MB-231 in a 48-hour treatment. We found that nitric oxide can continue to inhibit demethylases leading to an increase in global H3K9me2 levels in the cells (Figure 28A). We were also interested in determining the stability of •NO inhibition to demethylases. We hypothesized that without the availability of excess •NO, the inhibition of demethylases will decrease and H3K9me2 mark will return to basal level. As we hypothesized, H3K9me2 increased in a time-dependent manner with •NO treatment and decreased again, also time dependently, during the recovery period without •NO treatment (Figure 28B). This discovery led us to conclude that the inhibition of the Jumonji C domain Fe (II) α-ketoglutarate family of

demethylases by •NO is reversible which is shown by the global increase and decrease of H3K9me2 modification, dependent on the availability of •NO source. We also conclude that there were no critical modifications or destructions to the enzymes from •NO inhibition.

In correlation with changes in H3K9me2 level, an increase in methylation results in a decrease in acetylation level of the same lysine with •NO exposure in a time-dependent manner. After a recovery period from •NO, H3K9ac also returned to basal level similar to post •NO treatments. Since H3K9ac is associated with high levels of gene expression and H3K9me2 is associated with low gene expression, we hypothesized that •NO can act as a molecular switch to turn a gene on and off. Further studies need to be conducted to determine the role of •NO in gene expression.

A)



B)

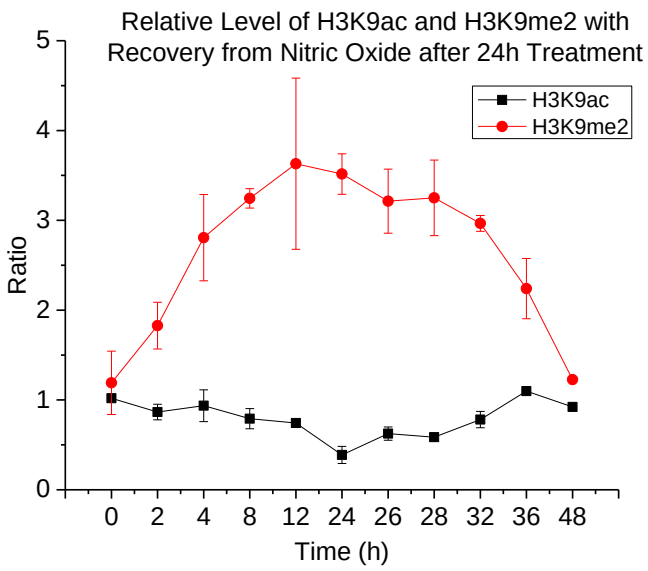


Figure 28: Nitric oxide modifies H3K9ac and H3K9me2 in MDA-MB-231. (A)Effect of 48h •NO exposure on H3K9me2 and H3K9ac. (B) Effect of Recovery from •NO exposure on H3K9me2 and H3K9ac.

4.1.2 The expression of KDM3A is reversible upon •NO removal

We focused on demethylase, KDM3A, for this experiment since KDM3A is the predominant demethylase that acts upon H3K9me2. With 48 hours exposure to DETA/NO, KDM3A was upregulated in a time-dependent manner and the expression of KDM3A from immunoblots show a correlation to H3K9me2. In response to 24 hours DETA/NO treatment and 24 hours of recovery, level of expression decreased to basal level as observed with H3K9me2 mark. The inhibition of KDM3A by •NO does not decrease the expression of the enzyme, but it results in the accumulation and buildup of the enzyme in the cell. The decrease in demethylation by KDM inhibition is the factor that drives up H3K9me2 accumulation. We will look further into why the increase in H3K9me2 is a result of demethylase inhibition by •NO and not due to the increase in methylation by methyltransferase in later experiments that looks closely into the different methylations on H3K4: H3K4me1, H3K4me2 and H3K4me3.

In a parallel experiment with determining the expression of KDM3A in MDA-MB-231 with DETA/NO treatment by immunoblotting, we were also interested in estimating the expression of methyltransferase G9a since this enzyme is the predominant methyltransferase responsible for methylating H3K9. We observed a significant down-regulation of G9a after 24 hours of 500μM DETA/NO treatment in MDA-MB-231. The enzyme was also unable to recover to basal level even after a recovery period of 24 hours without •NO. This further suggests that •NO mediates H3K9me2 modification by inhibiting demethylases KDM3A and the increase in H3K9me2 was not a result from methylation by methyltransferase G9a.

4.2 Modifications of H3K4 by Nitric Oxide

After studying the effect of •NO on H3K9 modification, we were interested in verifying that •NO can form DNIC with JMJC-class of lysine-specific histone demethylases that affect other lysine modifications although our evidence had already suggested the end result. Di-methylation of H3 lysine 9 (H3K9me2) is mainly associated with heterochromatin and gene silencing. The mono-, di- and tri-methylation of histone H3 lysine 4 (H3K4me1, H3K4me2 and H3K4me3, respectively), however, has been associated with euchromatin and ongoing gene expression[54]. Here, we will focus our study on H3K4me1/2/3 and also H3K4ac to determine how •NO can modifies these epigenetic marks. Furthermore, we seek to measure the gene expression of demethylases responsible for H3K4 and add to the clarification that •NO drives the increase in methylation by demethylase inhibition and not through increase in methylation.

4.2.1 •NO Mediates Methylation Modifications in H3K4

We observed a time-dependent increase in H3K4 mono, di and tri-methylation with 500μM DETA/NO treatment in MDA-MB-231. After 24 hours, H3K4me1, me2 and me3 all had increases in expression by 30%, 14% and 40%, respectively. This data further demonstrates our finding that by forming DNIC, NO can inhibit JMJC-containing demethylases and alter global histone modifications. In addition, we detected a decrease in H3K4ac which correlates to our H3K9 data showing a decrease in acetylation level and increase in methylation level.

4.2.2 •NO Increases H3K4 Demethylases Gene Expression

In accordance to the evidence that •NO increases H3K9 JMJC-containing demethylases gene expression, we hope to determine relative changes in H3K4 demethylases with •NO treatment in

MDA-MB-231. The known H3K4 KDMs we were interested in are KDM1A, KDM1B, KDM5A, KDM5B, KDM5C and KDM2B. KDM1A and KDM1B are responsible for the demethylation of H3K4me1 and H3K4me2. KDM5A, KDM5B and KDM5C are responsible for the demethylation H3K4me2 and H3K4me3. KDM2B is responsible for H3K4me3 demethylation. As a result, KDM5B and KDM2A, which are both JMJC-demethylases, showed significant increases in steady-state mRNA levels.

4.2.3 •NO Modifies Methylation Pattern Independently of Methyltransferases

To advance our confirmation that methyltransferases do not play an effect on the increase of methylation with •NO treatment, we cultured MDA-MB-231 cells in methionine-free media then treated with 500μM DETA/NO. Since methionine is required for S-Adenosylmethionine, the methyl donor of methyltransferases, synthesis, the lack of methionine would hinder methyltransferases function and results in a decrease of methylation. Our data showed that even in methionine-free media, methylation pattern of H3K4 still increase by an estimate of 120% by immunoblots. This confirms our theory that methyltransferases do not play a role in the increase methylation of histone in the presence of •NO.

4.3 The Role of iNOS in Mediating Histone Modification in MDA-MB-231

After establishing that •NO can inhibit JMJC-containing demethylases within physiologically relevant doses (~256nM of NO)[23], our next goal was to run a set of experiments to show that the amount of •NO produced endogenously by MDA-MB-231 can modify the epigenetic landscape. Inducible nitric oxide synthase (iNOS) has been associated with breast cancer aggressiveness and

poor survival rate and it is induced by cytokines to produce •NO in the tumor microenvironment. Therefore, it is essential to understand the mechanism of iNOS in the relevant cancer cells we have been using, MDA-MB-231, after treatment with cytokines. We were also interested in how iNOS inhibition can effect histone modification. Aminoguanidine was used to prevent accumulation of nitrate and nitrite in the media; L-NAME was used as an iNOS inhibitor and arginine-free media was used to hinder iMOS activity to produce •NO.

4.3.1 Cytokines treatment increases •NO production

After treatments with LPS, IFN-γ and LPS+IFN-γ, the amount of •NO produced had more than 2-fold increase as compared to the control, untreated, samples. LPS-induced MDA-MB-231 had the highest overall •NO production rate of 288% increase. In a parallel experiment where we incubated the cells in serum-free media then treated them with the cytokines, •NO-production was relatively unchanged as compared to the control which suggests that serum is essential for growth and enzymes functions. iNOS expression by immunoblot shows no relative change in iNOS levels after treatments. Therefore, we determine that LPS and IFN-γ can induce iNOS and elevate •NO production without changing the overall expression levels of these enzymes.

4.3.2 Inhibition of iNOS leads to changes in Histone Modification

Since we have established that iNOS can be induced by cytokines to produce •NO, we want to investigate the effect of iNOS inhibition on the modification of H3K9me2 and H3K9ac. iNOS-derived •NO has been associated with enhanced tumor biomarker expression, tumor growth and metastatic burden; and iNOS had been implicated as a key driver of cancer progression toward metastatic disease[44]. Therefore, inhibiting iNOS is the next approach in determining •NO activity

inside the cell. As an inhibitory effect on iNOS by aminoguanidine, •NO production decreases dramatically which also lead H3K9me2 mark to decrease. The effect suggests that demethylases are not being inhibited by •NO. As a correlation to a decrease in H3K9me2, H3K9ac increases with iNOS inhibition by L-NAME. This result confirms our hypothesis that endogenous level of •NO in MDA-MB-231 can modify global histone methylation and acetylation pattern since no exogenous •NO were treated to the cells. Nitrite data from the NOA serves as a control experiment to show that •NO levels decreases with increases in aminoguanidine treatment.

Even though many studies have been conducted, the actual amount of •NO produced in the tumor is still unknown. Since •NO can both advance and inhibit certain tumors, inhibition of endogenous •NO production or delivery of exogenous •NO have therapeutics potentials and the mechanisms are currently being explored. Future studies focusing on •NO mechanisms will be of interest to yield selective treatments that tailor specific type and stages of cancer[62].

4.4 Nitric Oxide Inhibits TETs and Alters 5hmC Level

Here, we focused on the effect of •NO on TET enzymes (TET1/2/3). TETs are responsible for the conversion of 5mC to 5hmC-containing DNA and they belong to the α-ketoglutarate and iron dependent dioxygenases family similar to JMJC-containing KDMs. Since •NO can function as an epigenetic modulator in histone modifications, we want to make a correlation of •NO to DNA modifications by TETs inhibition. Our hypothesis is that •NO can bind to the iron requires for the catalytic site of TETs and hinders TETs ability to convert 5mC to 5hmC.

4.4.1 TET1 Inhibition by •NO

From our dot blot data, it is shown that the amount of 5hmC produced is correlated to the amount of TET1 enzyme in the reaction. An increase in recombinant TET1 enzyme (0-2500ng) increases 5mC conversion to 5hmC which suggests a dose-dependent conversion rate of this enzyme. We then used a relevant concentration of TET1 (1000ng) with 100ng of 5mC substrate and performed a Sper/NO dose course for this experiment. The result shows that •NO can reduce the amount of 5hmC formed by TET1 in a dose-dependent manner. Since methylation of DNA is associated with gene silencing, the effect of •NO on the alteration of 5mC to 5hmC level predicts •NO's role in gene expression and further confirms our hypothesis that •NO can mediate DNA modification.

We were then focused on the effect of •NO on the global DNA modification of MDA-MB-231. After genomic DNA extraction, the amount of 5mC and 5hmC produced was estimated using immunoblotting. 5hmC to 5mC DNA level decreases with increased treatment of DETA/NO which suggests that •NO acts as an important epigenetic modulator in DNA modifications and possibly the overall gene expressions of MDA-MB-231. To conclude our hypothesis, specific genes related to •NO are of interest for pyrosequencing to accurately measure the location and degree of DNA methylation and hydroxymethylation.

To further quantify changes in DNA modifications by •NO, we applied a colorimetric ELISA-based technique for TETs activity measurements by calculating the amount of 5hmC formed using 5hmC-specific antibody. After performing the colorimetric-based assay, the absorbance was read at 450nm to estimate the amount of 5hmC produced in the reaction wells which is

proportional to the activity of TET1. After a recombinant TET1 dose course, we saw an increase in 5hmC product in a dose-dependent manner. We then tested our hypothesis that •NO can hinder the function of TETs *in vitro*. MDA-MB-231 cells were treated with DETA/NO for 24 or 48 hours before nuclear extraction. TETs activity was measured using the nuclear extract on 5mC substrate using manufacturer protocol since there are TET1/2/3 in the nuclear extract. According to our data, •NO was able to shutdown TETs function in 24 hours by the dramatic decrease of 5hmC product formed. The amount of inhibition was also calculated. 16% inhibition of TETs by •NO was able to decrease overall 5hmC product (ng). We next tested this inhibition on SK-N-BE(2). We found the same inhibitory effect of •NO on the TETs of this cell line. The amount of 5hmC product formed decreased as compared to the control and the % inhibition rate increased as compared to the untreated samples even though we used a lower dose of DETA/NO on this cell line, 250µM vs. 500µM for MDA-MB-231.

Overall, we showed evidence that the presence of •NO hindered the activity of TETs which is calculated by the 5mC to 5hmC conversion. Our *in vitro* study demonstrated the ability of •NO to change the DNA modification landscape in two different cell lines. Further studies are of interest to observe this effect *in vivo,* especially •NO-producing tumor. We are also interested in seeing how this effect can change the overall methylation of specific genes. Pyrosequencing is a method of interest to see this change in genomic DNA modifications.

4.5 Nitric Oxide and DNA Methylation

Since •NO can inhibit TETs and alters 5hmC DNA landscape, we seek to determine the amount of 5mC changes in specific genes. We picked three genes that are associated with breast cancer progression. *BRCA1* is a well-known breast cancer gene associated with being a tumor repressor and is down-regulated by •NO. Therefore, we hypothesized that this gene will have an increase in methylation. *DUSP1* is associated with proliferation and is up-regulated by •NO. Therefore, we hypothesized that *DUSP1* will have a decrease in methylation. *MGMT* is a control gene to confirm our pyrosequencing techniques.

All three genes showed no apparent increase or decrease in DNA methylation with •NO treatment. Further experiments are needed to be conduct to confirm the ability of •NO in changing the DNA methylation landscape since we were only able to observe 45-50 DNA base pairs of a specific gene region. Furthermore, the oxidative bisulfite sequencing step will be added for future experiments to measure both 5mC and 5hmC levels in the DNA sequence of interest. This study shows tremendous potentials for future developments and findings.

CHAPTER 5

CONCLUSION

The roles of •NO in epigenetic proves to be enormously complex. From mediating histone modifications to DNA modifications, future studies are necessary to study how this endogenously-produced epigenetic regulator affects specific genes transcription under physiological and pathophysiological conditions.

Here, we have shown that the inhibition of •NO to JMJC-containing histone demethylases is reversible and this effect is associated to the decrease in expression level of histone methyltransferase with exposure to •NO. Changes in methyl-modifying enzymes expression directly influence the global methylation levels of H3K9 and H3K4 and ultimately, these changes can greatly impact chromatin structure and gene transcription. This mechanism of •NO is capable of mediating the overall methylations of other lysines on histone 3 and histone 4, examples include H3K27, H3K36 and H4K20. In correlation, it results in the decrease of global histone acetylation level. We have also shown that endogenous level of •NO can affect H3K9 methylation and acetylation by inducing and also inhibiting iNOS to alter •NO production. Since elevated level of iNOS expression and •NO production has been associated with aggressive tumor phenotypes and negative prognostic indicators, this study revealed an insight on how •NO has the potential to regulate a variety of cancer-related genes. It would be beneficial in the future to determine if the effect of •NO on histone modification gets passed down from mother to daughter cells. Looking at heritable changes can provide additional understanding of epigenetic markers. It's also of great interest to acquire an overall map of histone modifications affected by

•NO and eventually test our theory on tumor samples to further analyze the effect of this molecule.

DNA methylation has been associated with gene repression and DNA demethylation via oxidation to produce 5-hydroxymethyl cytosine has been linked with gene transcription. DNA methylation patterns have been found to be highly dysregulated in cancer and changes in DNA methylation status have been postulated to inactivate tumor suppressors and activate oncogenes, thus contributing to tumorigenesis[58]. Here, we have demonstrated the ability of •NO to inhibit TET enzymes and decrease the conversion of 5mC to 5hmC. The described novel mechanism of •NO can help in deciphering the many roles of this small signaling molecule in epigenetic regulations. Future direction of this project includes the measurement of TETs expression in different cell lines, both in the protein and mRNA levels. Furthermore, continuation of pyrosequencing using oxidative bisulfite conversion would be advantageous since it will provide accurate quantification of the degree of methylation and hydroxymethylation in accordance with •NO exposure.

Methylation status of lysine as well as changes in the expression of histone modifying enzymes can result in phenotypic consequences. Adding to the complexities is the ability of •NO to mediate changes in DNA methylation. Since •NO increases epigenetic silencing mark, H3K9me2, and also decreases demethylation of 5mC, another mark related to gene silencing, we conclude that •NO has a potential role in global gene repression. Overall, we revealed two novel explanations in which •NO can regulate gene transcriptions. However, •NO-mediated epigenetic effects rely on two properties: steady-state concentration of •NO and duration of •NO exposure[33].

These factors will largely be influenced by the microenvironment of the cells. Understanding the cellular conditions is essential to predict •NO outcomes since its environment depicts the chemistry and molecular targets of this endogenously-produced molecule. Therefore, the design of novel •NO-releasing drugs or the use of iNOS inhibitor to decrease •NO production, depends on the cancer type and stage, represents a promising area of cancer research for personalized medicine and more effective treatment strategy.

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APPENDICES

APPENDIX A

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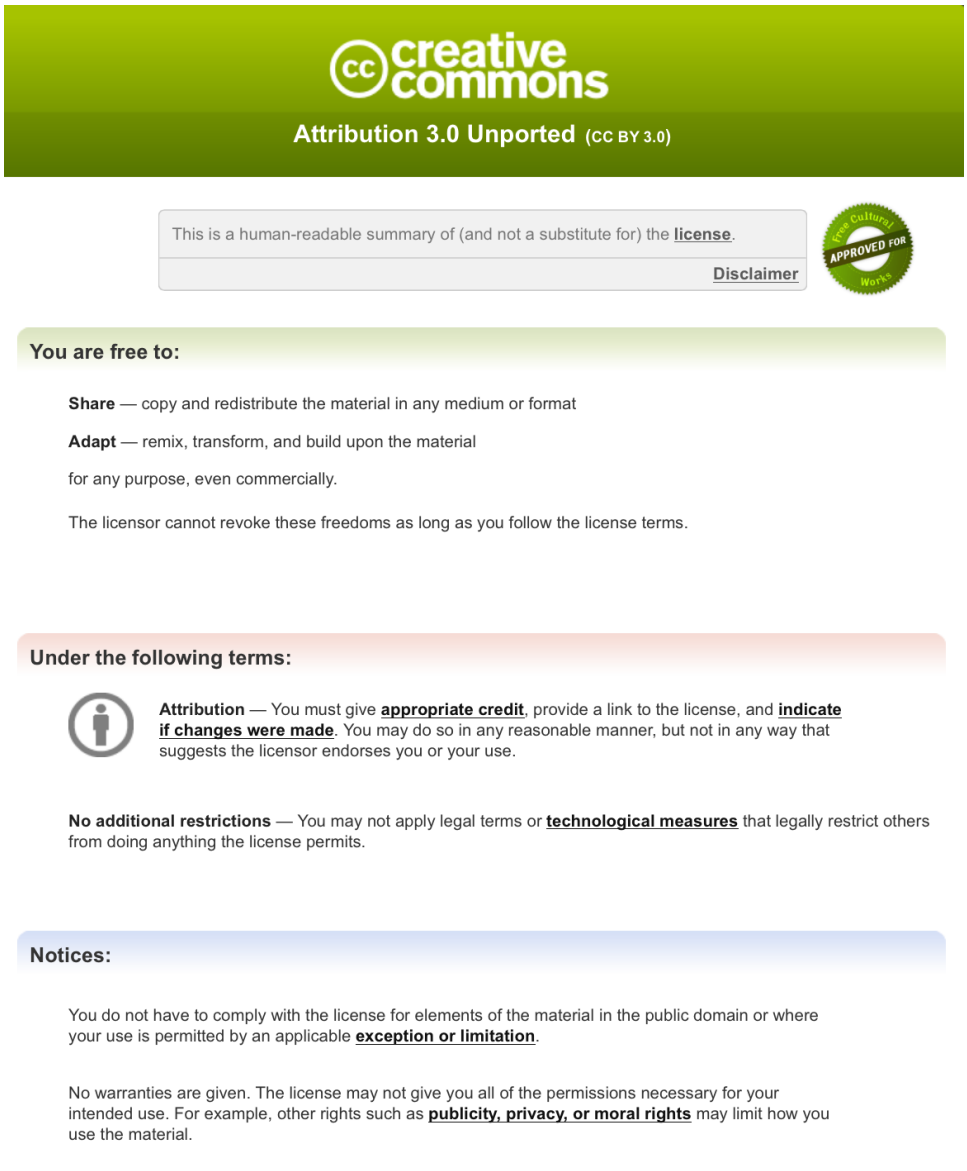

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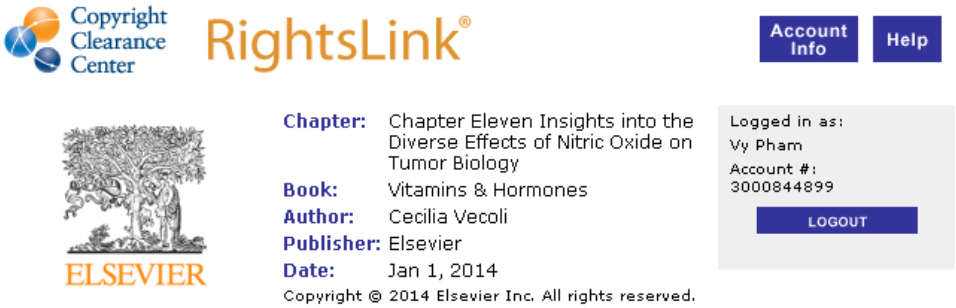

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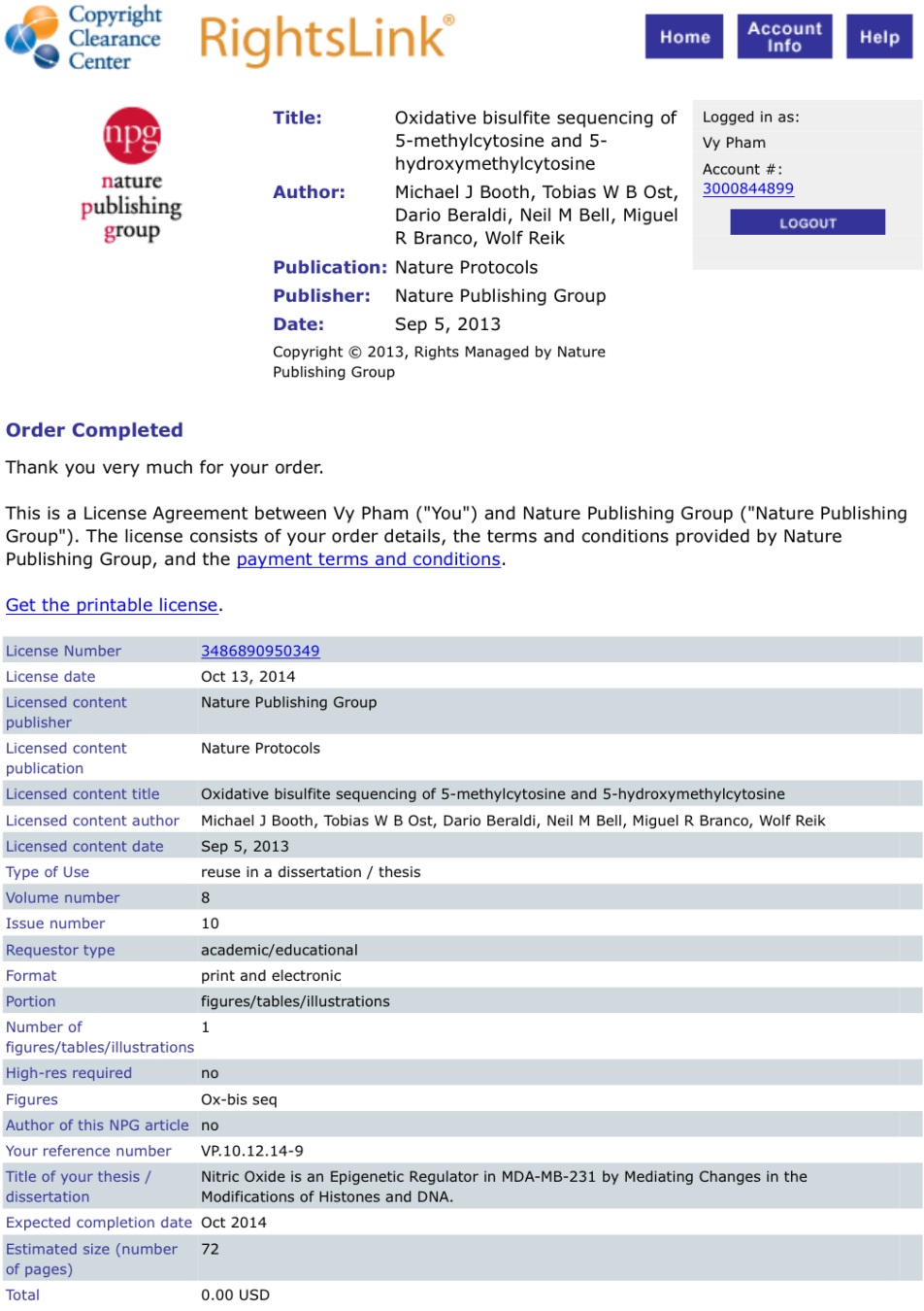

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