

O-GLCNAC POST TRANSLATIONAL MODIFICATION OF HYPOXIA-INDUCIBLE  
FACTOR (HIF) PROTEINS

by  
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## ABSTRACT

HAILEY ELISABETH GRISHAM: O-GlcNAc Post Translational Modification of Hypoxia-Inducible Factor (HIF) Proteins  
(Under the direction of Dr. Yu-Dong Zhou & Dr. Dale G. Nagle)

O-linked  $\beta$ -N-acetylglucosamine (O-GlcNAc) post-translational modification (O-GlcNAcylation), plays a vital role in many metabolic pathways, including those of numerous diseases. New research methods and basic functional roles of O-GlcNAc post-translational modification on metabolism, human health, and cancer are quickly developing, but a vast amount of research remains on the specificity of this pathway towards specific proteins. In this study, T47D breast cancer cells were used as an in vitro model to investigate the effects of O-GlcNAc modification on hypoxia-inducible factor (HIF) proteins.

## TABLE OF CONTENTS

LIST OF ABBREVIATIONS.....	vi
LIST OF FIGURES.....	viii
CHAPTER 1: INTRODUCTION.....	1
1.1 O-GLCNAC BACKGROUND.....	1
1.2 O-GLCNACYLATION AND RESEARCH METHODS.....	2
1.3 O-GLCNAC IN HUMAN HEALTH.....	4
1.4 O-GLCNAC IN CANCER.....	5
1.5 O-GLCNAC AND HIF.....	8
CHAPTER 2: O-GLCNAC POST TRANSLATIONAL MODIFICATION OF HYPOXIA-INDUCIBLE FACTOR (HIF) PROTEINS.....	10
2.1 BACKGROUND AND INTRODUCTION.....	10
2.2 MATERIALS AND METHODS.....	11
2.3 RESULTS.....	20
2.4 CONCLUSION AND DISCUSSION.....	25
LIST OF REFERENCES.....	27

## LIST OF ABBREVIATIONS

BME	$\beta$ -mercaptoethanol
BSA	bovine serum albumin
CNS	central nervous system
CER	cytoplasmic extraction reagent
DPBS	Dulbecco's phosphate buffered saline
EMT	epithelial-mesenchymal transition
FCS	fetal calf serum
GFAT	glutamine fructose-6-phosphate aminotransferase
HPB	hexosamine biosynthetic pathway
HIF	hypoxia inducible factor
HRP	horseradish peroxidase
IP	immunoprecipitation
M-PER	mammalian protein extraction reagent
MS	mass spectrometry
NER	nuclear extraction reagent
OGA	O-GlcNAcase
OGT	O-GlcNAc transferase
O-GlcNAc	<i>O</i> -linked $\beta$ - <i>N</i> -acetylglucosamine
PBS	phosphate buffered saline
PI	protease inhibitor

PPP	pentose phosphate pathway
PTM	post translational modification
P/S	penicillin and streptomycin
PUGNAc	O-(2-Acetamido-2-deoxy-D-glucopyranosylidnamino)N-phenylcarbamate
SDS	sodium dodecyl sulfate
WR	working reagent

## LIST OF FIGURES

Figure 2.1	Experimental Flow Chart.....	21
Figure 2.2	Standard Curve for Protein Concentration Determination.....	22
Figure 2.3	Western Blot for HIF-1 $\alpha$ and HIF-2 $\alpha$ in T47D cells.....	23
Figure 2.4	Western Blot for O-GlcNAc.....	24
Figure 2.5	Western Blot for HIF-1 $\alpha$ and HIF-2 $\alpha$ .....	24



## **Chapter 1**

### **Introduction**

Hailey Grisham

#### **1.1 O-GlcNAc Background**

Post translational protein modification (PTM) allows cells to quickly respond to internal and external changes by diversifying protein functionality. Some of the most well characterized PTMs include phosphorylation, acetylation, glycosylation, and ubiquitination. While these modification pathways have been extensively studied, a number of critical PTMs and their functions are only beginning to be discovered and more broadly appreciated (Yang and Qian, 2017). Discovered in the 1980s, O-linked  $\beta$ -N-acetylglucosamine (O-GlcNAc) post-translational modification (O-GlcNAcylation) plays a vital role in many crucial biological functions. The extent of these functions is only beginning to be uncovered as researchers are looking for new methods of site-mapping specific O-GlcNAc proteins and discovering new methods to profile these proteins (Ma and Hart, 2014).

## 1.2 O-GlcNAcylation and Research Methods

Comparable to glycosylation, O-GlcNAcylation attaches O-GlcNAc components to serine and threonine residues within proteins of the cytoplasm, mitochondria, and nucleus. The hexosamine biosynthetic pathway (HBP), which metabolizes various biological nutrients, creates uridine diphosphate GlcNAc (UDP-GlcNAc) as a substrate to be used for O-GlcNAcylation to modify sugars onto the serine and threonine hydroxy groups. Thus, O-GlcNAc works as a stress and nutrient sensor capable of using cellular metabolism combined with other PTMs to signal and conduct a vast variety of biological processes (Ma and Hart, 2014; Yang and Qian, 2017).

The process of O-GlcNAcylation is controlled by a pair of enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). These enzymes control the addition and removal of O-GlcNAc to and from the amino acid residues, serine and threonine (Ma and Hart, 2014). Though researchers are lacking in knowledge of all of its sequencing, OGT is known to be responsible for the transfer of Glc-NAc from UDP-GlcNAc to the amino acid residues. The enzyme OGA has been found in all tissues and is responsible for removing Glc-NAc by catalyzing hydrolysis (Vaidyanathan et al., 2014; Yang and Qian, 2017). This enzyme pair is cable of recognizing hundreds of protein substrates, but researchers continue to search for techniques to determine substrate specificity to OGT and OGA (Yang and Qian, 2017).

One major goal in building a foundational knowledge on the effects of O-GlcNAcylation aims to explicitly identify the role of this process on specific proteins. Typical methods to prove the presence of O-GlcNAc include Western blot,

autoradiography, Edman sequencing, and mass spectrometry (MS). Because the effects of O-GlcNAcylation occur at many substrate sites with differing functions, defining the characteristics of each location will help to better understand the role of O-GlcNAc.

Combinations of classic research techniques along with more recent research methods has continued to expand the number of recognized O-GlcNAcylation sites. Western blot, a technique useful for identifying specific proteins, utilizes antibodies capable of distinguishing O-GlcNAc properties on proteins. This technique appears more precise than autoradiography, but in order to increase the range of results, multiple immunoassays using many antibodies are needed to distinguish O-GlcNAc patterns within any given protein sample. Immunoblotting is often combined with immunoprecipitation (IP) to isolate the protein of interest from the rest of the sample. While Western blotting and autoradiography may be useful in identifying the presence of the O-GlcNAc of proteins, Edman sequencing and MS techniques are strategies useful in determining the exact function of specific modification sites. Edman sequencing has been found to be most helpful in differentiating isobaric masses of peptides with O-GlcNAcylation. New MS techniques allow researchers to gain a range of information about the proteins and peptides of interest. As the use of this more advanced method develops, specified details of O-GlcNAc should become more accessible.

Overall, O-GlcNAcylation process is utilized in a vast number of biological processes, such as transcription, translation, epigenetics, signaling, and other vital system responses. The relationship of O-GlcNAc with so many fundamental mechanisms creates a link between the causes of various health issues and aberrant protein O-GlcNAcylation.

It is believed that as researchers discover more about the specificity of the PTM and its direct roles, they will begin to unveil more insights into the causes of many disorders and diseases (Ma and Hart, 2014).

### **1.3 O-GlcNAc in Human Health**

As research continues to uncover the roles of O-GlcNAcylation on certain sites in specific proteins, a notable amount of attention has shifted towards looking at the role of this PTM on various diseases caused by metabolic dysfunctions. Numerous epigenetic studies have led to the discovery that O-GlcNAc PTM functions in many important biological processes. These functions include the role of O-GlcNAc in transcription (Gambetta et al., 2009; Kelly & Hart, 1989), gene silencing (Yang et al., 2002), gene deletion in embryonic stem cells (Shafi et al., 2000), and cell cycle control. Disruption of any of these functions may lead to aberrant proteins that could greatly affect human health (Vaidyanathan et al., 2014).

As described by Fehm and coworkers, the central nervous system (CNS) expends around half of all metabolized glucose (Fehm et al., 2006), suggesting that O-GlcNAc may occur in the brain. Further studies indicated the ubiquitous neuronal presence of this PTM by discovering two presynaptic zone proteins to be some of the most substantially O-GlcNAc modified proteins ever observed (Trinidad et al., 2012). The significant decrease of glucose metabolism in the brain that occurs with Alzheimer's disease has pointed attention toward the potential role of O-GlcNAcylation in that disease. While changes in HBP flux and the enzymes of O-GlcNAc have been shown to impact the

disease (Liu et al., 2012), more research is needed to establish exactly how this PTM works to protect the brain and how a decline in this modification may be involved in the etiology of Alzheimer's disease. Additionally, other critical CNS processes involving synaptic communication and memory development have established the importance of O-GlcNAcylation on these processes (Vaidyanathan et al., 2014).

Another major biological process involving O-GlcNAc focuses attention on the effect of O-GlcNAc PTM in cardiac functioning. Because O-GlcNAc functions as a stress response sensor, studies have found its importance in protection of the heart after a cardiac injury (Lima et al., 2009; Laczy et al., 2009). The O-GlcNAc PTM further enhances cardiac protection through its role in cell signaling to defend against programmed cell death (apoptosis) and other cellular damage (Vaidyanathan et al., 2014).

While O-GlcNAcylation clearly plays a number of crucial roles in maintaining and protecting necessary bodily functions, the over-expression or under-expression of this modification and its enzymes may lead to serious issues. As research continues, more is beginning to be understood about the role of O-GlcNAc PTM on health and disorders.

#### **1.4 O-GlcNAc in Cancer**

Cancer research is a quickly evolving field in which new discoveries are consistently being made. One major aspect of this research is a renewed focus on the energy metabolism of the cancer cells that allows them to survive, grow, and, in some cases, even spread to other places in the body. The basis of unique cancer metabolism was first discovered by Otto Warburg, who found that cancer cells utilize glycolysis to

create energy despite being in a normal oxygenic environment. Because glycolysis is a much less efficient energy-creating pathway, many researchers began looking into why cancer cells are able to thrive through this mechanism. Although only relatively low levels of ATP are generated from glycolysis, large amounts of carbon and nitrogen can be absorbed from the byproducts of this pathway (e.g., lactate and ammonia). Nearby cancer cells can use the absorption of such products as energy sources to increase their organic mass and facilitate the growth and spread of tumor cells (Ferrer et al., 2016).

Because the glycolytic pathway of cancer cells has been characterized, emerging studies examine the mechanisms through which the cells receive excess glucose to generate and maintain this metabolic pathway. Because of the increased levels of glycolysis, increased flux must occur through pathways such as the hexosamine biosynthetic pathway and pentose phosphate pathway (PPP) to deliver the glucose needed for the tumor cells to survive. The substrate UDP-GlcNAc is a product of the hexosamine biosynthetic pathway. Therefore, increased levels of this product lead to increased O-GlcNAcylation of proteins, also known as hyper-O-GlcNAcylation. This increased O-GlcNAc PTM has been found to occur ubiquitously in human cancers.

Because O-GlcNAc modification plays such a vital role in cancer metabolism, potential treatments may be able to target components of this PTM pathway. Hyper-O-GlcNAcylation of cancer cells suggests that OGT levels would also be increased in the cellular biosynthetic pathways. Targeting the OGT enzyme through knockdown lowered the levels of O-GlcNAcylation which established contribution of this pathway to tumor growth (Ma and Voessler 2014). More specific studies were performed by Ferrer and

coworkers, where this group demonstrated that pharmacological inhibition of O-GlcNAc PTM reduced OGT levels and induced breast cancer cell apoptosis without affecting the localized non-cancerous epithelia (Ferrer et al., 2014). This relationship between OGT levels and cancer cell viability has led to further study of its metabolic pathways and its interactions with O-GlcNAc in cancer. Because O-GlcNAc plays such a wide variety of roles in biological processes, its effects on cancer cells can be readily tied to several hallmarks of cancer such as cell proliferation, survival, and metastasis (Ferrer et al., 2016; Ma and Vosseller, 2014).

As O-GlcNAc has been found to play a role in cell cycle control, cancer cells are known to push cell cycle progression and skip vital checkpoints. FoxM1, a regulator of cell cycle progression, is suggested to be sustained by O-GlcNAc PTM, but in oncogenes, this regulator is often over-expressed. The cell cycle regulator, Cyclin D1, displays a similar pattern of expression. Therefore, hyper-O-GlcNAcylation is correlated to the over-expression of both FoxM1 and Cyclin D1 which can cause the cell cycle to bypass necessary checkpoints, leading to excessive proliferation and tumor cell growth.

Another approach that oncogenic cells take to ensure their survival is the promotion of anti-apoptosis mechanisms. Studies have indicated that O-GlcNAc plays a role in evading death. Because O-GlcNAc PTM is a stress sensor, it is able to respond to many of the stresses that cancer cells must endure, such as a lack of nutrients, endoplasmic reticulum stress, and hypoxia. While normal cells are typically unable to survive under these stressful environments, glycolytic mechanisms supported by O-

GlcNAcylation are able to help tumor cells endure the stresses and avoid apoptosis (Ma and Vosseller, 2014).

More recent studies have begun to look at the potential role of O-GlcNAcylation in tumor cell metastasis (Ferrer et al., 2016; Ma and Vosseller, 2014). Metastasis, the development of a secondary site of cancer cell growth, occurs because of the culmination of multiple processes. One specific process is epithelial-mesenchymal transition (EMT), which is associated with cellular hypoxia and the loss of certain tumor suppressors. The inhibition of OGT can actually reduce EMT and the metastasis of both breast and prostate cancer cells. This outcome established a relationship between roles of both EMT and O-GlcNAc in the tumor metastasis (Ferrer et al., 2016). Research into the mechanistic details of how these processes work together and how O-GlcNAc functions with other processes is just beginning to yield exciting new findings (Ma & Vosseller, 2014).

The process of O-GlcNAcylation is a common PTM among cancers. Therefore, strategies have emerged that target tumor cell hyper-O-GlcNAcylation with the aim to inhibit OGT to prevent excessive O-GlcNAc PTM. This specific approach may become beneficial in developing therapies to treat malignant tumors. Overall, O-GlcNAcylation research appears to have great promise in potential development of novel methods for treatment of cancer (Ferrer et al., 2016; Ma & Vosseller, 2014).

### **1.5 O-GlcNAc and HIF**

The process of O-GlcNAcylation has been found in numerous studies to reprogram metabolic pathways of cancer cells. Hypoxia-inducible factor-1 (HIF-1) is a



transcriptional factor that mainly controls glycolytic enzymes, and thus may play some part in promoting the Warburg effect of cancer cells when increased O-GlcNAcylation occurs (Ma and Vosseller, 2014). Specifically, one study found that reduction of O-GlcNAcylation in cancer cells caused apoptosis of these cells and stress of their endoplasmic reticulum, which further caused degradation of HIF-1 $\alpha$  protein. This finding emphasized the regulation of glycolysis in cancerous cells through HIF-1 $\alpha$  and the transcriptional target GLUT1. Ferrer and his coworkers noted that breast cancer patients with high HIF-1 $\alpha$  levels also have increased OGT and decreased OGA levels. These findings have been correlated with poorer outcomes for patients (Ferrer et al., 2014; Ferrer et al., 2016).

Due to oncogenes, glutamine fructose-6-phosphate aminotransferase (GFAT), a rate-limiting enzyme in the HBP, has been found to have up-regulated levels in cancer cells. These levels are related to an excessive uptake of glutamine that serve to drive the increased HBP flux so that the cancer cells can continue to receive the nutrients necessary to rapidly reproduce. The GFAT expression increases the output of the hexosamine biosynthetic pathway, UDP-GlcNAc. The transcription factor, HIF-1 $\alpha$ , induces GFAT expression, which further shows its relation to O-GlcNAcylation (Ma and Vosseller, 2014).

## **Chapter 2**

### **Post-translational O-GlcNAc Modification of Hypoxia-Inducible Factor (HIF) Proteins**

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#### **2.1 Background and Introduction**

Post translational O-GlcNAc modification (O-GlcNAcylation) plays a vital role in many metabolic pathways, including those implicated in a number of diseases.

Abnormalities in this pathway have been connected with cancer pathogenesis. In this study, T47D human breast cancer cells were tested through various experiments to investigate the effects of O-GlcNAc modification on hypoxia-inducible factor (HIF) proteins. Cultured T47D cells were treated with 1,10-phenanthroline (an Fe<sup>++</sup> chelator that activates HIF-1 in normoxic cells to induce a state of "chemical hypoxia") and PUGNAc (an inhibitor of O-GlcNAcase). Nuclear and cytoplasmic extraction was performed and followed by protein concentration quantification. Western blot analysis was first performed to determine the sensitivity of the extracted proteins to anti-HIF-1 $\alpha$  and anti-HIF-2 $\alpha$  antibodies, respectively. Immunoprecipitation was subsequently performed with anti-HIF-1 $\alpha$  and anti-HIF-2 $\alpha$  antibodies as specified, followed by Western Blot to test the sensitivity of the precipitated proteins to an O-GlcNAc-specific

antibody. A final re-blot was performed using anti-HIF-1 $\alpha$  and anti-HIF-2 $\alpha$  antibodies to confirm the presence of HIF $\alpha$  proteins in the immunoprecipitated samples.

## **2.2 Materials and Methods**

### *2.2.1 Cell Culture and Compound Treatment*

Human breast cancer T47D cells (ATCC) were derived from a metastatic site of human mammary glands. Two million T47D cells were seeded into each well of a six-well polystyrene tissue culture dish (CytoOne) in a volume of 2 mL of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Corning), supplemented with 10% fetal calf serum (FCS, Hyclone) and antibiotics (50 units/mL of penicillin and 50  $\mu$ g/mL of streptomycin, GIBCO). The cells were cultured at 37°C overnight in a humidified environment under 5% CO<sub>2</sub>/95% air, treated with the following compound combinations: 1,10-phenanthroline (10  $\mu$ M, Sigma) + *O*-(2-Acetamido-2-deoxy-D-glucopyranosylideneamino) *N*-phenylcarbamate (PUGNAc, 30  $\mu$ M, Sigma); 1,10-phenanthroline (10  $\mu$ M); PUGNAc (10  $\mu$ M); or PUGNAc (30  $\mu$ M) for 4 hours at 37°C. All compounds were purchased from Sigma, dissolved in DMSA, and the stock solutions stored at -20°C.

### *2.2.2 Nuclear and Cytoplasmic Extract Preparation*

Following compound treatment, the cells were washed once with 1x Phosphate Buffered Saline (PBS), scraped to be detached, and the cell suspension transferred to a 1.5 mL centrifuge, and the cell pellet collected by centrifugation at 500 x g for 5 minutes.

The pellet was washed twice with 1x PBS (two rounds of re-suspension and centrifugation). The supernatant was removed, leaving a dry cell pellet. Based on the packed cell volume of 10  $\mu$ L, 100  $\mu$ L of ice-cold cytoplasmic extraction reagent (CER I, Thermo Scientific) was added to the pellet. To ensure the integrity of the proteins, a protease inhibitor cocktail (Sigma) was added to CER I and NER solutions at the manufacturer-recommended concentration.

The CER I and cell pellet mixture was vortexed at the highest setting of a bench-top vortexer for 15 seconds to completely resuspend the pellet. This mixture was incubated on ice for ten minutes before 5.5  $\mu$ L of CERII (Thermo Scientific) was added. After this addition, the tube was vortexed for five seconds and placed on ice for one minute. After being vortexed one final time on the highest setting for five seconds, the mixture was centrifuged for five minutes in a refrigerated (4°C) in a micro-centrifuge at 16,000x g. The supernatant was immediately removed and transferred to a pre-chilled tube that was placed on ice. The pellet, which is the insoluble portion that contains the nuclei, was suspended in 50  $\mu$ L ice-cold nuclear extraction reagent (NER, Thermo Scientific). The tube was vortexed on the highest setting for 15 seconds and placed on ice with an additional 15 seconds of vortex period every ten minutes for the entirety of 40 minutes. A final centrifugation of the tube at maximum speed for ten minutes was performed to obtain the supernatant (nuclear extract) portion to be transferred into a new pre-chilled tube and placed on ice to store in the freezer at  $-80^{\circ}\text{C}$  until later use.

### 2.2.3 Protein Concentration Quantification

The Micro BCA Protein Assay Kit (Thermo Scientific) provided three micro BCA reagents (MA, MB, MC) and bovine serum albumin (BSA) standard ampules which contain 10 x 1 mL BSA at 2.0 mg/mL in a mixture of 0.9% saline and 0.05% sodium azide. To prepare the BSA standards, different volumes of BSA standard and distilled water were divided into separate 5 mL tubes with micropipettes. Table 2.1 below shows the exact volumes utilized in the experiment.

Table 2.1 - BSA Standard Preparation

Vial	Volume of Diluent ( $\mu\text{L}$ )	Volume & Source of BSA ( $\mu\text{L}$ )	Final BSA Concentration ( $\mu\text{g}/\text{mL}$ )
A	450	50	200
B	400	100 of vial A	40
C	510	90 of vial A	30
D	250	250 of vial B	20
E	250	250 of vial C	15
F	250	250 of vial D	10
G	250	250 of vial E	7.5
H	250	250 of vial F	5
I	250	250 of vial H	2.5
J	250	250 of vial I	1.25
K	250	0	0

The micro BCA working reagents (WR) were then prepared by mixing 25 parts Reagent MA (1750  $\mu\text{L}$ ) and 24 parts Reagent MB (1610  $\mu\text{L}$ ) with 1 part Reagent MC (140  $\mu\text{L}$ ). Micropipettes were used to add 100  $\mu\text{L}$  of each of the prepared standards into separate wells of a polystyrene 96-well microplate with an equal amount of the working

reagent added. A plate shaker was used for 30 seconds to thoroughly mix the standards. The microplate was sealed and incubated at 37°C for one hour and then cooled to room temperature. At this point, the absorbance was measured at 492/620 nm on a spectrophotometer using Magellan software. The average absorption reading of the blank standard was subtracted from the absorbance reading of each of the individual standards. Microsoft Excel was used to prepare a standard curve by plotting the blank reading for each BSA standard versus its concentration to determine the protein concentration of each individual sample.

#### *2.2.4 Western Blot*

To prepare the samples for SDS-PAGE gel electrophoresis, a sample buffer solution was prepared using 95  $\mu\text{L}$  of 2x Laemmli sample buffer (Bio-Rad) with 5  $\mu\text{L}$  of  $\beta$ -mercaptoethanol (BME, Sigma). Protein quantification was performed to determine how much volume of the four NE and the four CE samples were necessary to get 15  $\mu\text{g}$  of each sample to be mixed with the buffer solution. The sample amounts utilized are displayed in Table 2.2 and were added to the USA Scientific's 1.5 mL tubes. Following mixing within the pipette, 10.2  $\mu\text{L}$  of the prepared buffer was added to each sample tube. The tubes were then placed in a hot water bath to boil for 5 minutes in order to denature the protein samples. After boiling, the tubes were placed on ice.

Table 2.2 - Protein Quantification

<b>Sample</b>	<b>Volume necessary for 15<math>\mu</math>g (<math>\mu</math>L)</b>	<b>NER (<math>\mu</math>L)</b>	<b>CER (<math>\mu</math>L)</b>
<b>NE1</b>	8.52	1.68	----
<b>NE2</b>	8.93	1.27	----
<b>NE3</b>	10.2	0	----
<b>NE4</b>	8.2	2.00	----
<b>CE1</b>	3.55	----	6.65
<b>CE2</b>	3.88	----	6.32
<b>CE3</b>	4.14	----	6.06
<b>CE4</b>	3.65	----	6.55

To prepare the running buffer for SDS-PAGE gel electrophoresis, 20 mL of premade 10x Tris/Tricine/SDS buffer (Bio-Rad) were mixed and added to 180 mL of distilled deionized water (ddH<sub>2</sub>O, equivalent to a 1/10 dilution). The denatured NER and CER samples were thawed, centrifuged, and placed on ice. A Bio-Rad precast 4-20% gradient SDS-PAGE minigel was loaded with samples in the following order: molecular weight marker, NE1, NE2, NE3, NE4, CE1, CE2, CE3, CE4. The gel was run at 100 volts for an hour.

A 5x transfer buffer was prepared by dissolving 15.15 g of Tris Base and 72.1 g of glycine in 1 L ddH<sub>2</sub>O. To obtain 1x transfer buffer, the following solutions were mixed in a beaker: 200 mL of 5x transfer buffer, 100 mL of methanol (Sigma), and 700 mL of ddH<sub>2</sub>O. The gel was soaked in the transfer buffer and a transfer 'sandwich' that contains a black base, filter, filter paper, the gel, 45  $\mu$ m nitrocellulose membrane, another filter paper, and a white base, was assembled under the surface of the transfer buffer. The

electrotransfer was run at 140 milliamps for one hour on a stir plate. Ponceau S solution (Sigma) was added to the membrane to ensure the transfer efficiency by staining the proteins.

To prepare the TTBS solution, 5 mL of 1M pH 6.8 Tris, 2.5 mL of 5M NaCl, and 0.13 mL of Tween 20, were mixed with ddH<sub>2</sub>O to obtain a final volume of 125 mL. The 5% milk/TTBS solution was prepared by dissolving 5 grams of Blotting-Grade Blocker (nonfat dry milk, Bio-Rad) in 100 mL TTBS solution. For blocking, the membrane was placed in the 5% milk/TTBS solution and incubated at 4°C overnight with gentle shaking.

The primary anti-HIF-2 $\alpha$  polyclonal antibody (Novus) was added to the membrane in a 1:1000 dilution (10  $\mu$ L of the antibody with 10 mL of the 5% milk/TTBS) for an hour at room temperature while rocking on a Boekel Scientific Rocker II. The membrane was washed with 5% milk/TTBS for ten minutes while shaking and rocking, and then washed again with the same solution for five minutes while rocking. The same steps utilized with the first antibody were followed with a secondary antibody of goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP, Jackson Immunolab), that was prepared in a 1:3000 dilution (3.5  $\mu$ L of the antibody in 10 mL). The membrane was rocked for an hour and then washed once for ten minutes in TTBS and subsequently twice more for five minutes extra. The extra liquid was drained and 1.5 mL of West Femto Max sensitivity substrate (Thermofisher) was added to the membrane to soak for five minutes before draining the excess liquid once again. The membrane was placed in a



plastic membrane protector and placed in a Chemi Gel Doc (Bio-Rad) to perform chemiluminescence to acquire the Western Blot.

### *2.2.5 Immunoprecipitation*

In order to separate the protein of interest in T47D cells, Abcam's Immunoprecipitation protocol was followed. One point five million T47D cells were cultured overnight in a 6 cm plate with 3 mL of 10% FCS, DMEM/F12, and P/S (penicillin and streptomycin). The treatment conditions were: 1) media control, 2) 10  $\mu$ M 1,10-phenanthroline, 3) 30  $\mu$ M PUGNAc, 4) 10  $\mu$ M 1,10-phenanthroline plus 30  $\mu$ M PUGNAc. The NE and CE samples were prepared as described in the previous section, using the NE-CE isolation kit from ThermoFisher. The protein concentrations were determined as described using the Micro BCA kit from ThermoFisher.

Over ice, the NE samples were divided into two sets of tubes for immunoprecipitation with anti-HIF-1 $\alpha$  and anti-HIF-2 $\alpha$  antibodies, respectively. The solutions were separated into these tubes in the amounts listed in Table 2.3 below. Then 250  $\mu$ g/ $\mu$ L of anti-HIF-1 $\alpha$ , purified monoclonal antibody, and 1 mg/ $\mu$ L of anti-HIF-2 $\alpha$ , purified polyclonal antibody, were added to the appropriate tubes. The samples with the antibodies were incubated for four hours at 4°C under gentle rotation.

Table 2.3 - Isolation of Nuclear Extract

NE Wells	Original Protein Concentration	
1	1.76	14.2 $\mu$ L ex / 2.8
2	1.68	14.9 $\mu$ L ex / 2.1
3	1.47	17 $\mu$ L ex
4	1.83	13.7 $\mu$ L ex / 3.3

For the monoclonal anti-HIF-1 $\alpha$  antibody, protein G-coupled Sepharose beads were used, while a protein A-coupled Sepharose beads were used for anti-HIF-2 $\alpha$  polyclonal antibody. Then 400  $\mu$ L of beads were used and spun quickly to separate and remove the supernatant. An equal volume of M-PER (Mammalian Protein Extraction Reagent, PIERCE) with 1x protease inhibitor cocktail (PI, Sigma) were added and mixed for an hour to create a slurry. The slurry was then mixed and 55  $\mu$ L of beads was added to each tube and kept on ice. This lysate bead mixture was incubated at 4°C for five minutes and centrifuged at 3000 rpm (800 x g). The supernatant was removed after the incubation and the beads were washed three times with 150  $\mu$ L/tube of lysis buffer made up of a 9:1 mixture of M-PER and 10x PI. Each washing was followed with centrifugation for five minutes (first spin at 3000 rpm; second spin at 4000 rpm, third spin at 5000 rpm) at 4°C and removal of the supernatant. Once the last supernatant was removed, 35  $\mu$ L/tube of 2x Laemmli buffer (loading buffer) was added. The mixture was boiled at 94°C for 5 minutes to denature the protein and separate it from the beads. The mixture was spun at 10000 rpm at 4°C for 5 minutes to retrieve the supernatant containing the protein. This was placed in a new tube and stored at -20°C overnight.

### *2.2.6 O-GlcNAc Western Blot*

To prepare the running buffer for gel electrophoresis, 20 mL of premade 10x Tris/Tricine/SDS buffer (Bio-Rad) were mixed with 180 mL of ddH<sub>2</sub>O. The already prepared tubes of immunoprecipitated NER samples were centrifuged and placed on ice. The 4-20% Mini-Protean TGX Precast Protein Gel (Bio-Rad) was loaded with the following: molecular weight marker, HIF-1 $\alpha$ /NE1, HIF-1 $\alpha$ /NE2, HIF-1 $\alpha$ /NE3, HIF-1 $\alpha$ /NE4, molecular weight marker, HIF-2 $\alpha$ /NE1, HIF-2 $\alpha$ /NE2, HIF-2 $\alpha$ /NE3, HIF-2 $\alpha$ /NE4. The gel was run at 105 volts for an hour.

Western blot was performed as that described in the earlier section, using standard methods. The primary antibody mouse anti-O-GlcNAc (CTD 110.6) monoclonal antibody (Cell Signaling Technology) was used at 1:1000 dilution

### *2.2.7 Stripping of the Membrane and Reblot*

The membrane was rinsed twice with TTBS and then immersed in Restore Western Blot Stripping Buffer (Thermofischer) at room temperature for 10 minutes with rocking. The membrane was washed with TTBS three more times for five minutes each. It was then blocked in 5% milk/TTBS at room temperature with shaking for an hour. The membrane was cut apart to separate the HIF-1 $\alpha$  and the HIF-2 $\alpha$  portions. A primary antibody, rabbit anti-HIF-2 $\alpha$ , is added to the HIF-2 $\alpha$  portion of the membrane in a 1:1000 dilution (10  $\mu$ L in 10 mL). Similarly, a primary mouse anti-HIF-1 $\alpha$  antibody was added to the HIF-1 $\alpha$  portion of the membrane in an identical dilution. Both of these additions

were left overnight on the membrane at 4°C with rocking. The membrane was washed with secondary antibody and blocked with 5% milk/TTBS. The extra liquid was drained and 1.5 mL of West Femto Max sensitivity substrate was added to the membrane to soak for five minutes at room temperature before draining the excess liquid once again. The membrane was placed in a plastic membrane protector and placed in a Chemi-Gel Doc (Bio-Rad) to perform chemiluminescence to obtain the images.

## 2.3 Results

Figure 2.1: Experimental Flow Chart

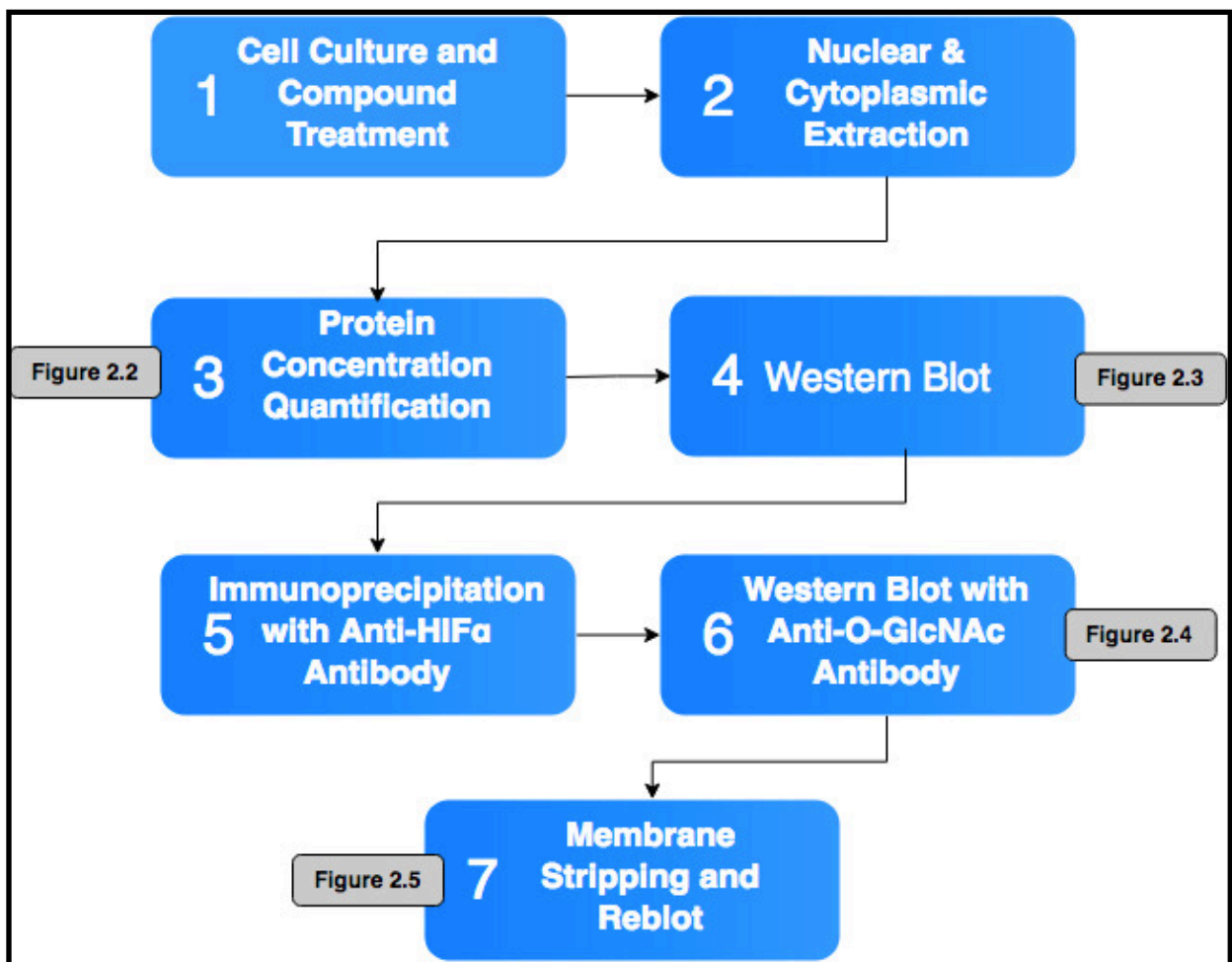
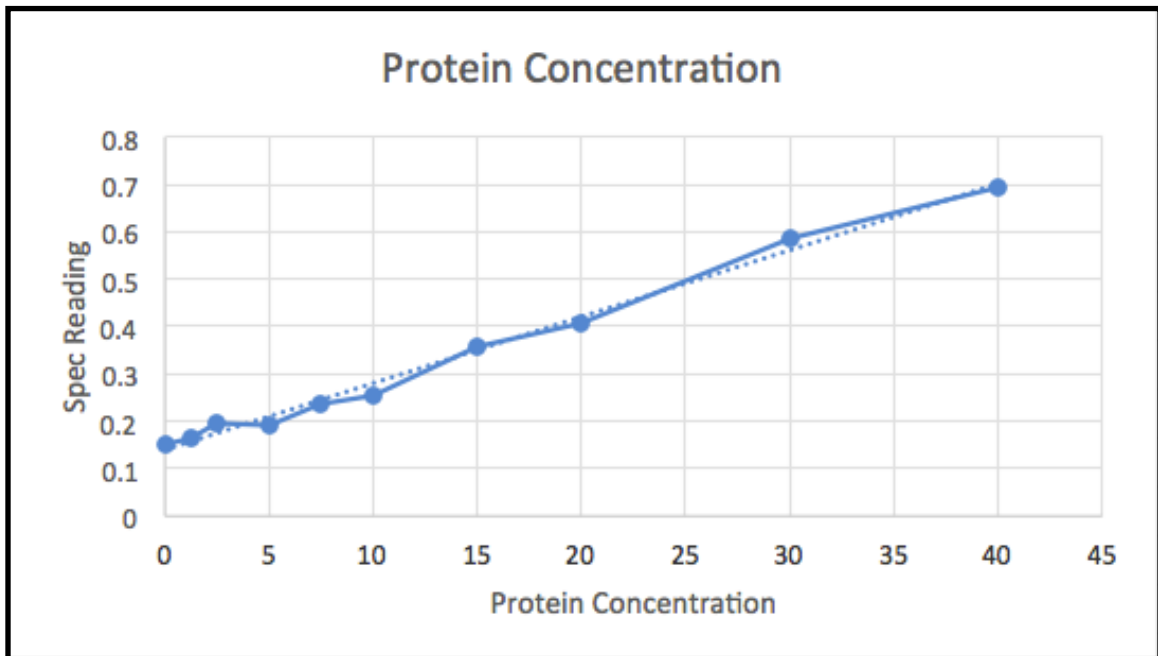


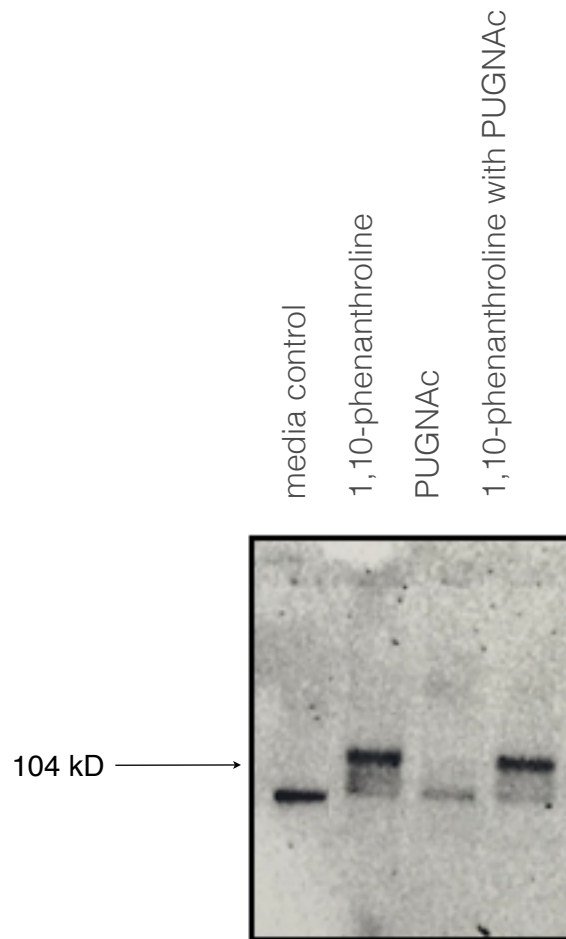
Figure 2.1 displays an overview of the procedural steps taken to complete this experiment. It highlights specific portions of the process that have graphs and data displayed in this section of results.

Figure 2.2: Standard Curve for Protein Concentration Determination



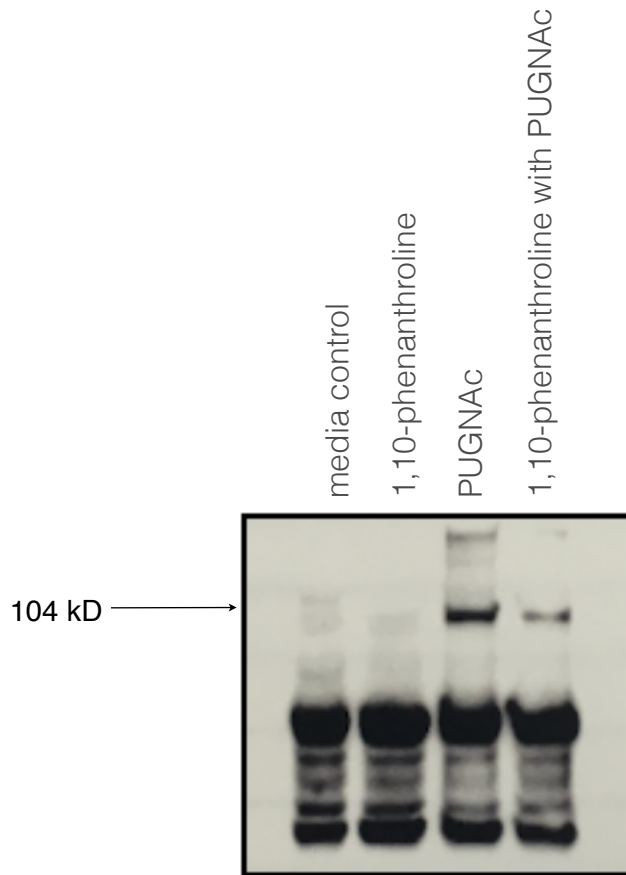
A spectrophotometer using Magellan software was used to measure the concentration of proteins present in the T47D cell culture samples. The average absorption reading of the blank standard was subtracted from the absorbance reading of each of the individual standards. This standard curve was prepared using Microsoft Excel by plotting the blank reading for each BSA standard versus its concentration to determine the protein concentration of each individual sample. The equation  $y = 0.014x + 0.14$  was obtained from the slope of the line, so that the absorbance values can be plugged into the x value to determine the y value that represents protein concentration.

Figure 2.3: Western Blot for HIF-2 $\alpha$  Proteins in T47D cells



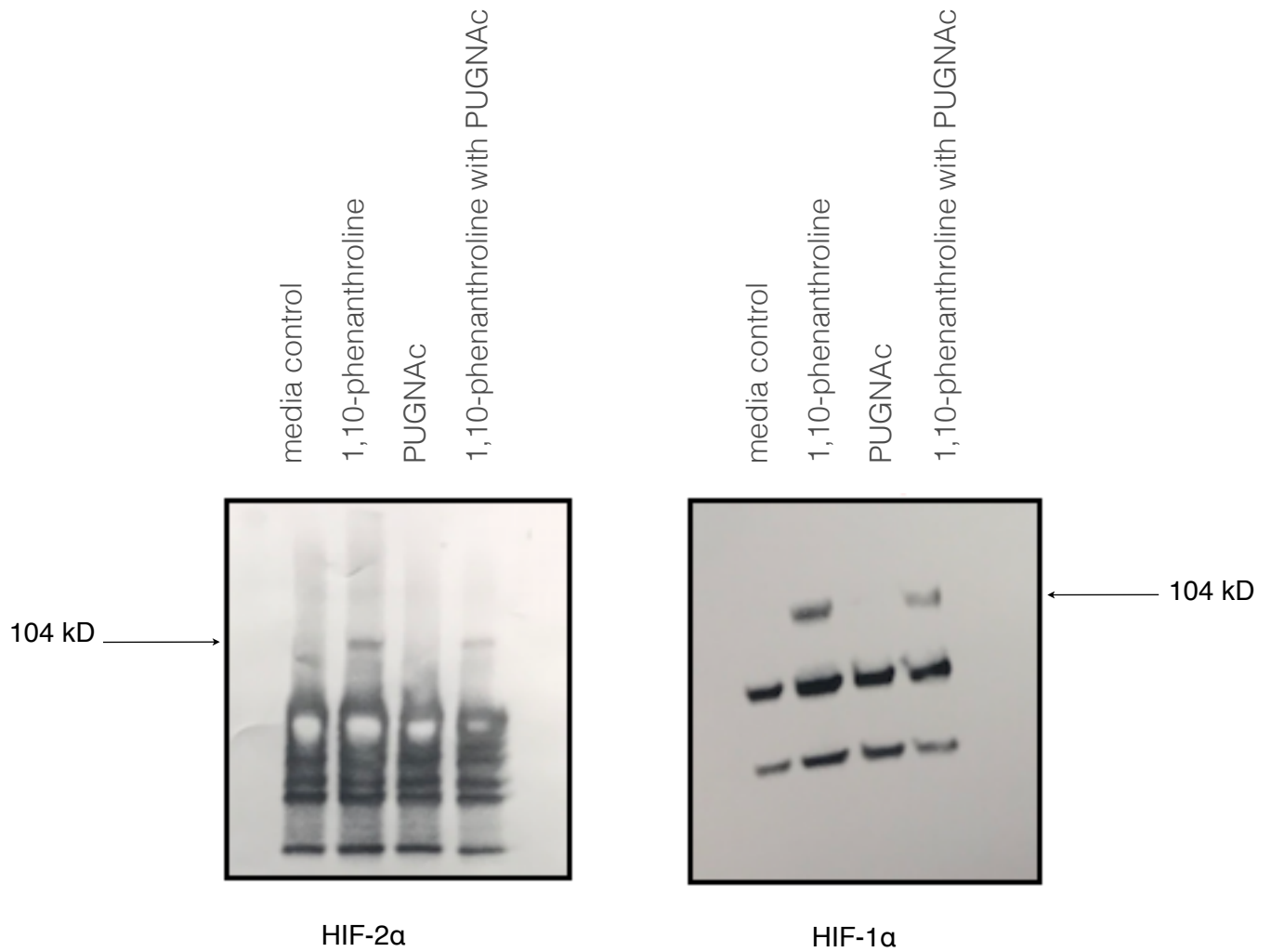
This Western Blot displays varying treatments on T47D cells. The marks on the blot in the order from left to right display: media control, 1,10-phenanthroline, PUGNAC, and 1,10-phenanthroline with PUGNAC. The bands on the blot confirm that proteins are present and that the antibody (HIF-2 $\alpha$  in this figure) was able to detect to the proteins of interest. The signals were stronger for the cells treated with 1,10-phenanthroline and those treated with 1,10-phenanthroline + PUGNAC.

Figure 4: Western Blot for O-GlcNAc



This Western Blot displays the effects of the O-GlcNAc antibody on the varying nuclear extract treatments (the samples were loaded in the same order as that in Figure 2.3). This result shows that the antibody did, in fact, detect the presence of O-GlcNAc modification in the proteins of interest (HIF-1 $\alpha$  protein), in the presence of OGA inhibitor PUGNAC. Similar results were obtained for HIF-2 $\alpha$  protein.

Figure 5: Western Blot for HIF-1 $\alpha$  and HIF-2 $\alpha$



This set of Western Blots confirms that O-GlcNAc post translational modification occurs through hypoxia-inducible factor (HIF-1 $\alpha$  and HIF-2 $\alpha$ ) proteins of T47D breast cancer cells. The subsection of this pathway onto these proteins is displayed by the bands shown on the blot.



## 2.4 Conclusion and Discussion

The extent of O-GlcNAc post translational modification functioning continues to be uncovered as researchers are discovering new methods of site-mapping specific O-GlcNAc proteins and new methods to profile these proteins. Research has shown its etiological influence in a vast range of diseases including: cardiac injuries, diabetes, Alzheimer's, and cancer. One common feature of the role of O-GlcNAc in these disease processes is its control at either or both the cell cycle and transcriptional level. Although these findings display great progress, further research is needed to define the role O-GlcNAc modification plays in specific proteins (Vaidyanathan et al, 2014).

Because hyper-O-GlcNAcylation has been correlated to the over-expression of several cell cycle regulators that cause the cell cycle to bypass necessary checkpoints, researchers believe that it leads to excessive proliferation and tumor cell growth. Further pursuit of this research is necessary to determine which proteins are subjected to O-GlcNAc PTM so that they can be specifically targeted therapeutically. Additionally, the process of O-GlcNAcylation has been found in numerous studies to reprogram the metabolic cancer cell pathways. Hypoxia-inducible factor (HIF) is a transcriptional factor protein that mainly controls glycolytic enzymes, and plays an important role in promoting the Warburg effect of cancer cells when increased O-GlcNAcylation occurs (Ma and Vosseller, 2014). The connection between the O-GlcNAc PTM and HIF proteins highlights an area of research to be further studied.

This particular study focused on the potential of O-GlcNAcylation on HIF proteins in T47D breast cancer cells. After the series of experiments, it was concluded

that the antibodies did, in fact, detect the presence of O-GlcNAc modification in the proteins of interest (HIF-1 $\alpha$  and HIF- $\alpha$  proteins), in the presence of OGA inhibitor PUGNAc. Further research is needed to decipher the direct role of O-GlcNAc in these HIF proteins and to determine how the PTM effects the metastasis of the tumor cells. The study of O-GlcNAcylation and its roles in biological process is a quickly evolving process. As more efficient tools and methods of site-mapping are developed, a deeper clarification of the O-GlcNAc pathway and its specific roles will be possible (Ma and Hart, 2014).

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