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Shahzina Kanwal

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Thesis submitted towards fulfillment of the requirement for
degree of doctorate

Université Paris Descartes

Ecole doctorale Gc2ID (Ed 157)

**Effect of O-GlcNAcylation on tamoxifen sensitivity in
breast cancer derived MCF-7 cells**

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List of abbreviations

AD:	Alzheimer's disease
ATM:	Ataxia telangiectasia mutated
ATP:	Adenosine Triphosphate
AIs:	Aromatase inhibitors
BRCA1:	Breast cancer susceptibility gene 1
BRCA2:	Breast cancer susceptibility gene 2
CTLs:	Cytotoxic T lymphocytes
CDKs:	Cyclin dependent kinases
CBP:	CREB binding protein
DISC:	Death-inducing signaling complex
DCIS:	Ductal carcinoma in situ
EGFR:	Epidermal growth factor receptor
ER:	Estrogen receptor
E2:	Estradiol
ECM:	Extra cellular matrix
FADD:	Fas associated death domain protein
FDA:	Food and drug administration
FSH:	Follicle stimulating hormone
FGF:	Fibroblast growth factor
FBP:	Fructose-1,6-bisphosphate
FDG:	2-deoxy-2-(18F)fluoro-D-glucose
PFK:	Phosphofructokinase
GLUT:	Glucose transporter
GFAT:	Glutamine fructose-6-phosphate amidotransferase

HAT: Histone acetyl transferase
HDAC: Histone deacetylase inhibitors
HIF: Hypoxia-inducible factor
HK: Hexokinase
HBP: Hexosamine biosynthetic pathway
IGF-1: Insulin like growth factor-1
IGFBP: IGF binding protein
IL-6: Interleukin-6
IDC: Invasive ductal carcinoma
LH: Luteinizing hormone
LKB1: Liver kinase B1
MDM2: Mouse double minute-2 homolog
mTOR: Mammalian target of rapamycin
NK: Natural killer
NAD: Nicotinamide adenine dinucleotide
OGT: O-linked N-acetylglucosamine (GlcNAc) transferase
OGA: O-GlcNAcase
PTEN: Phosphatase and tensin homolog
PIP3: Phosphatidylinositol Trisphosphate
PCD: Programmed cell death
pRb: Retinoblastoma protein
PET: Positron emission tomography
PAI-1: Plasminogen activator inhibitor-1
PTMs: Posttranslational modifications
PR: Progesterone receptor
PRLR: Prolactin receptor
SERMS: Selective estrogen-receptor modulators

STK11: Serine/threonine kinase 11
STZ: Streptozotocin
TNFR: Tumor Necrosis Factor Receptor
TEB: Terminal end buds
TNBCs: Triple negative breast cancer
TPRs: Tetratricopeptide repeats
TSP-1: Thrombospondin-1
TCA: Tricarboxylic acid cycle
UDP-GlcNAc: Uridine diphosphate N-acetylglucosamine
VEGF: Vascular endothelial growth factor

INTRODUCTION

Chapter 1

Hallmarks of cancer

Cancer is the second leading cause of death. About 16 million new cancer cases were expected to be diagnosed in 2012 and survival rate is 60-65% in the developed countries. Cancer may affect people at all ages, even fetuses. However, the risk of being diagnosed with cancer increases with age. Most cases occur in adults who are middle aged or older. About 77% of all cancers are diagnosed in persons 55 years of age and older. Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. Different factors are involved in transformation of normal cells into tumor or malignant cells. These include genetic mutations and carcinogens such as tobacco smoke, radiation, chemicals. Moreover it can also be caused by infectious agents. Other cancer-promoting genetic abnormalities may be randomly acquired through errors in DNA replication, or are inherited from parents. DNA methylation and miRNAs are the additional emerging factors causing cancer. Other factors can also play role in increasing cancer risk such as hypercaloric diet, lack of physical activity or smoking.

In a normal adult body, cells grow and divide in a strictly controlled manner through homeostatic regulation between proliferation and death. Complex intracellular machinery is involved to maintain the balance between these mechanisms. This machinery is comprised of several regulatory circuits which allow normal somatic cells to grow to a certain limit. During cell division, cell passes through different phases of the cell cycle, each of which is tightly regulated by different check points. Moreover, after attaining a certain number of divisions, cells either enter into the process of differentiation or in case of damage enter into a regular process of repair or of programmed cell death.

In a normal adult body, old or damaged cells are replaced by new cells and the number of cells remains constant. However, any abnormality in these steps can result in imbalance between cell proliferation and cell death and consequently leads to the development of tumors and can cause cancer.

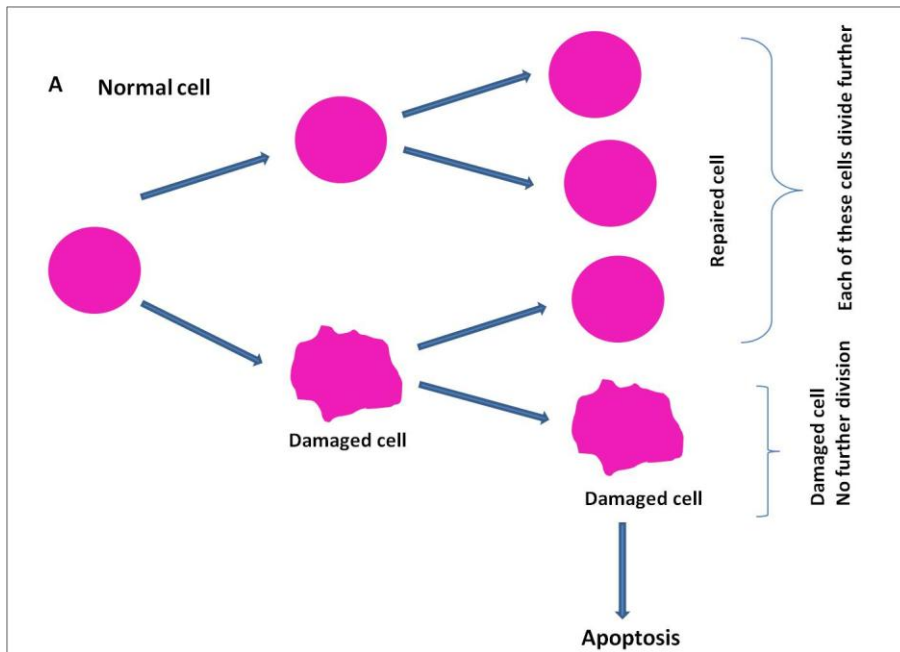
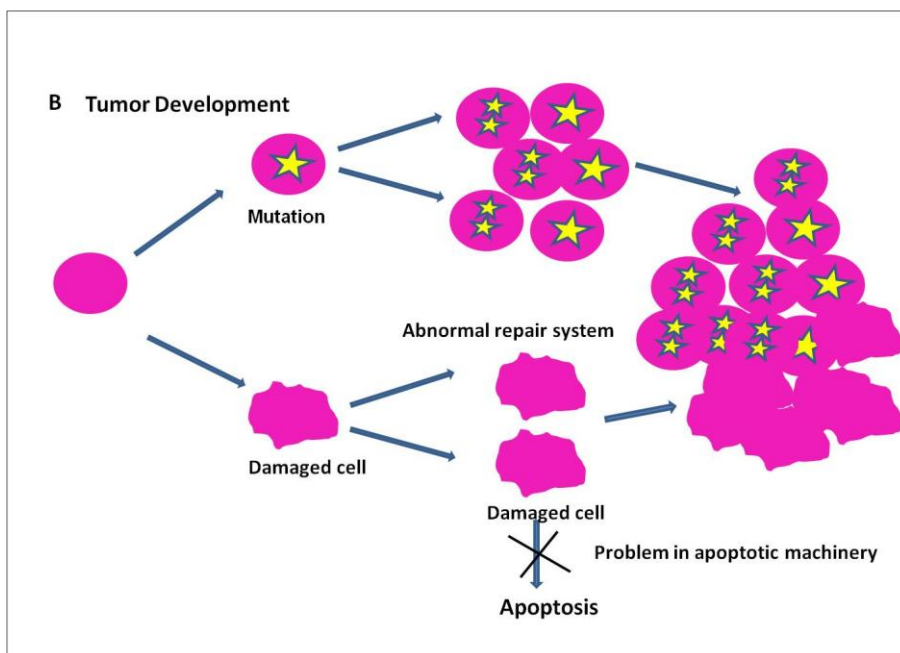


Figure 1: Loss of normal growth control in tumor development

(A) In case of damage, normal cells either enter into the process of apoptosis or cell repair



(B) In case of abnormality in repair system or in apoptotic machinery, damaged cells or cells having mutations exhibit abnormal cell growth and develop tumor.

Overgrowth of normal somatic cells leads to the development of tumors but not all types of tumors are cancerous. Cancer is a heterogeneous group of diseases characterized by uncontrolled growth, tumor formation, and invasion of the surrounding body parts. Some kinds of tumors stay at one site and demonstrate limited growth. They do not invade neighbouring tissues and do not spread throughout the body. This type of tumors is called benign tumors and is non-cancerous as is shown in figure 2.

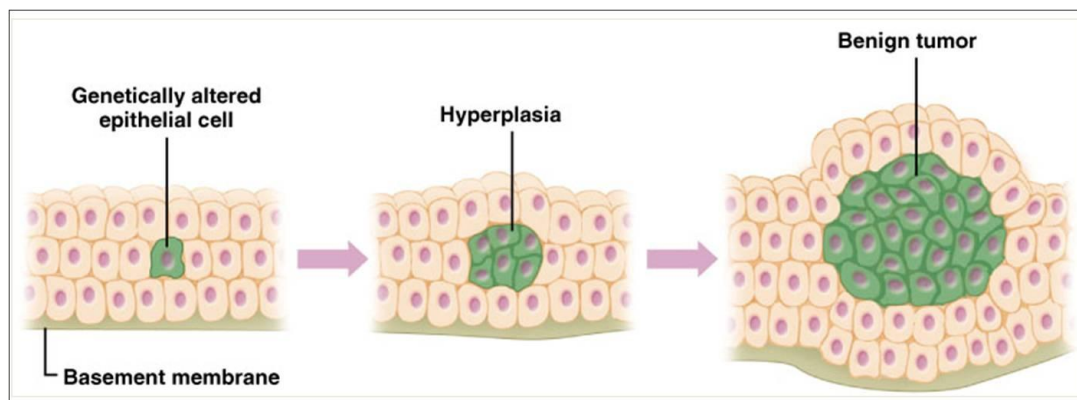


Figure 2: Development of benign tumor

Genetically altered or abnormal cells show uncontrolled and rapid growth resulting in the development of benign tumor.

This figure is adapted from 2003 Pearson Education, Inc. publishing as Benjamin Cummings

A second kind of tumors is known as malignant tumor, it is also called cancerous tumor or neoplasma. This kind of tumors does not stay at one site and spread to the neighbouring tissues or to the other parts of the body as is shown in figure 3. The more devastating form of malignant tumors is known as metastatic tumors, in which tumors move to the distant parts of the body through the lymphatic system or blood stream. Metastasis is the cause of more than 90% of

cancer-related deaths. In order to create a metastatic lesion, primary tumor cells have to invade the local environment and penetrate the walls of lymphatic and/or blood vessels, survive in the circulation, and invade and adapt to the environment of distant organs. The following figures depict the process of invasion and metastasis.

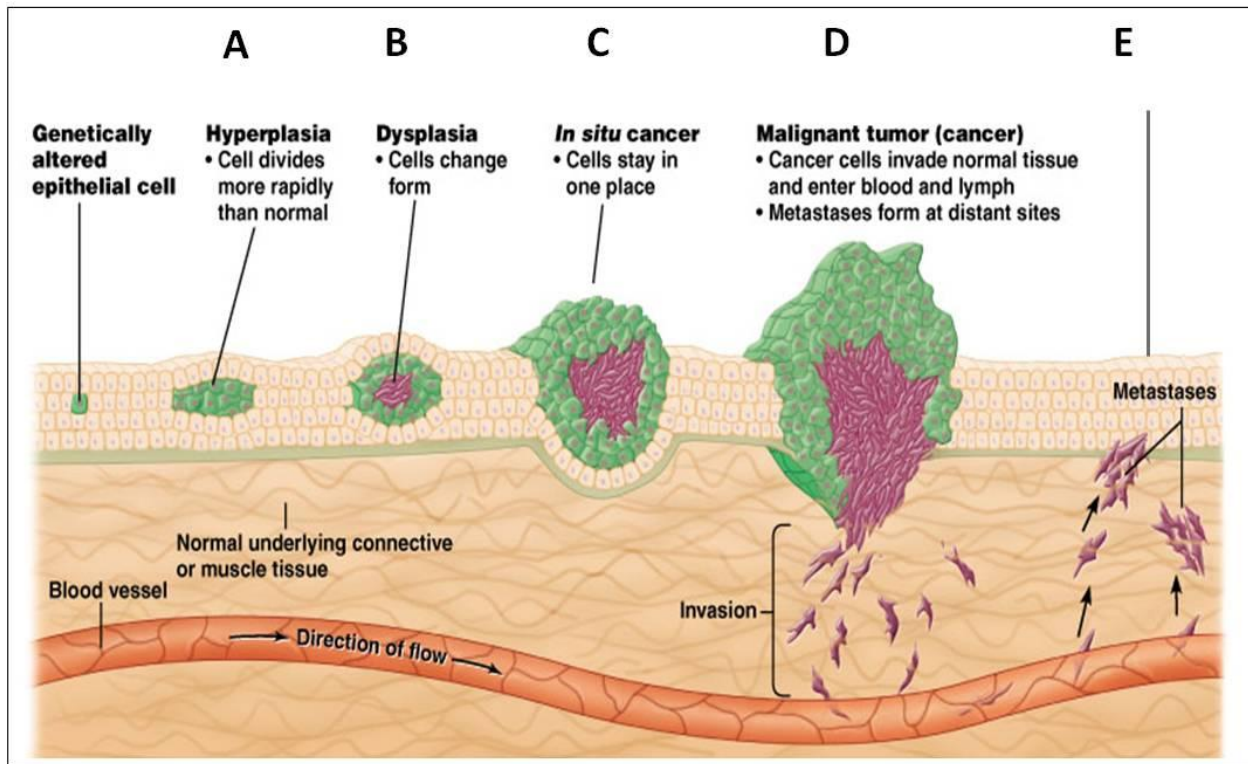


Figure 3: Stages of tumor development and mechanism of metastasis

Tumors development takes place in different steps. (A) Hyperplasia is the stage where genetically altered or abnormal cells show uncontrolled and rapid growth. (B) Dysplasia, stage of tumor development where overgrowing cells change their original form. It consists of more immature cells than mature. (C) In situ cancer represent neoplastic lesion where cells do not go into the process of maturation, have lost their tissue identity and grow without regulation. (D) With malignant tumor, overgrowing cells invade other areas by rupturing basal membrane. (E) Metastases occur when cancer cells reach to the distant parts through lymphatic system and blood circulation. This figure is adapted from 2003 Pearson Education, Inc. publishing as Benjamin Cummings

Cancer is a diverse and versatile type of disease and can occur virtually in all parts of the body. To date more than 200 types of cancer have been documented. Cancer is typically classified by its anatomic origin (breast cancer, colon cancer, prostate cancer, lymphoma, leukemia, lung cancer, etc...). Each of these is further classified into different subtypes. Although cancer is an immensely complex and diverse disease, a set of characteristics are shared among almost all malignancies. These characteristics are unified set of capabilities that are acquired during tumorigenesis. In 2000, Hann and Weinburg tried to simplify the complexity and diversity of malignancies by proposing that most of them have six common characteristics (hallmarks of cancer).

There are six characteristics which were initially proposed as hallmarks of cancer: self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis [1]. This list has been further extended with emerging hallmarks such as deregulating cellular energetics and avoidance of immune response as is described in figure 4. Additional characteristics proposed are, tumor promoting inflammation, and genome instability and mutation [2]. Each of these characteristics is described in detail in the following paragraphs.

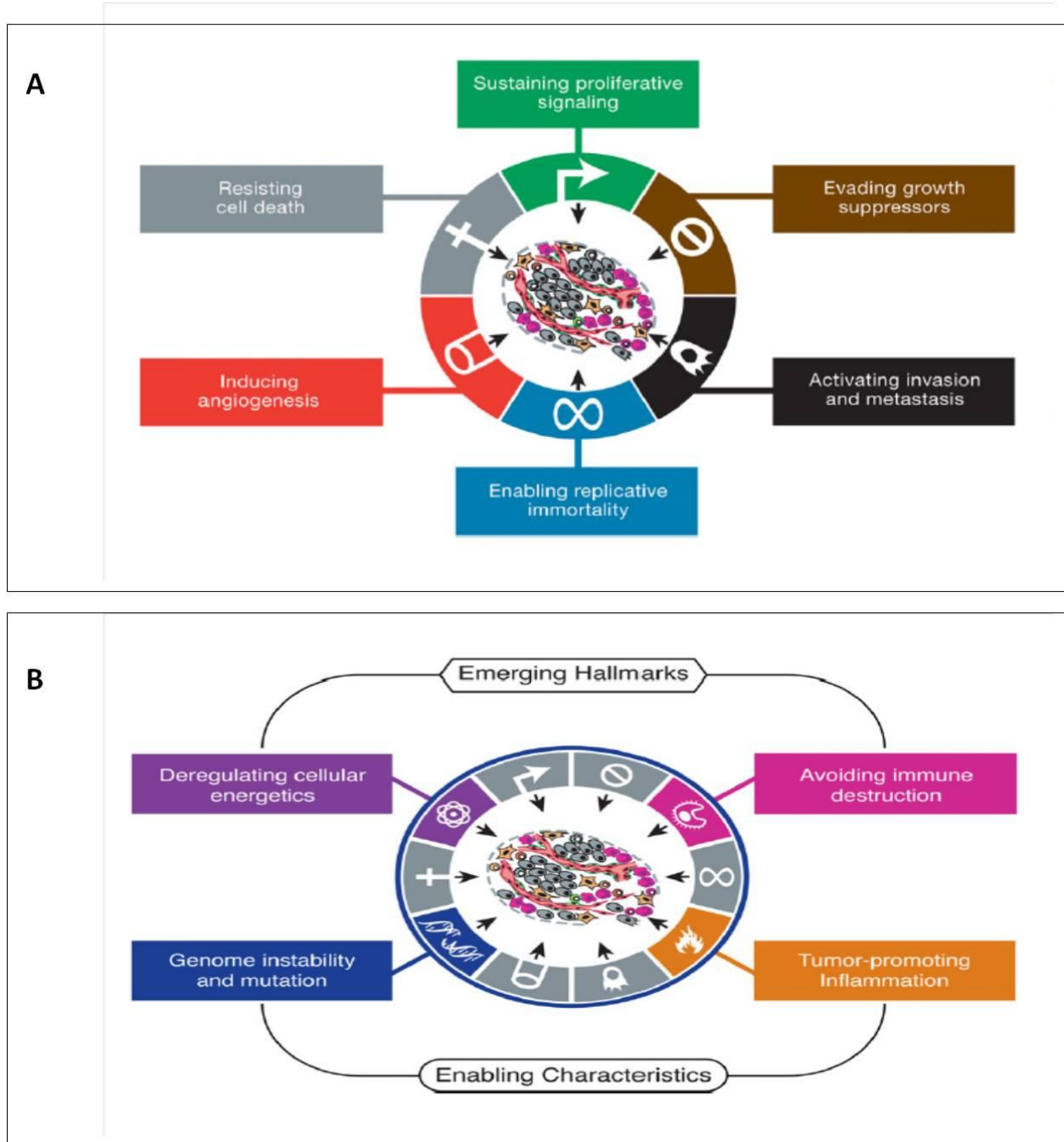


Figure 4: Hallmarks of cancer

(A) The basic hallmarks of cancer presented by Hanhan and Weinberg in 2000. (B) The emerging hallmarks of cancer presented by Hanhan and Weinberg in 2011.

1.1. Self sufficiency in growth signals

A crucial step in transformation of normal cells to malignant cells is the acquisition of self sufficiency in growth signals [1]. Growth of normal cells depends upon the availability of exogenous growth factors produced by their microenvironment including neighbouring cells. In the absence of growth factors, cells enter into quiescent stage and stop growing. However, when cells become hypersensitive to these growth factors or become independent of their availability, they start growing uncontrollably resulting in the development of a tumor. Independence of growth factors is one of the hallmarks of cancer that disrupts homeostasis within a tissue. Cancer cells can attain independence of growth factors through different mechanisms.

Expression of integrins

Within a normal tissue, multiple anti-proliferative signals operate to maintain cellular quiescence and tissue homeostasis. However, cancer cells evade these anti-proliferative signals in order to sustain high proliferation. In normal cells, integrins and other cell adhesion molecules send antigrowth signals from extra cellular matrix to the cells to regulate growth, motility, proliferation and apoptosis. In contrast, cancer cells switch off the expression of integrins and other cell adhesion molecules that send antigrowth signals and favour those molecules which transmit progrowth signals to stimulate proliferation [3]. The Wnt signalling pathway is involved in transmission of progrowth signals and genetic alterations of its components are associated with melanomas and carcinomas of the breast and colon [4]

Deregulation of growth factors signaling

In normal cells, exogenous growth factors signals are transmitted to cells through transmembrane receptors (Tyrosine kinases). Activation of these receptors transmits signals to other intracellular machinery, for example they activate Ras-Raf-MAP kinase pathway. In normal condition, growth factors act on cells either through paracrine or endocrine mechanism. Normal cells exhibit heterotypic signaling where most of the soluble mitogenic growth factors made by one cell are used to stimulate proliferation of another. However in contrast to normal cells, malignant cells show less growth factors (GFs) dependency on their neighbouring cells. They acquire the ability to synthesize GFs to which they are responsive, creating a positive feedback signaling loop named autocrine stimulation. In autocrine stimulation cells start responding to their own growth factors by expressing their cognate receptors. The production of tumor growth factors α (TGF- α) by sarcoma cells is one of the example. Similarly, glioblastomas-produced platelet-derived growth factor (PDGF) stimulates growth through an autocrine mechanism [5]. Alternatively, cancer cells can also modulate their microenvironment to make it favourable to them. For instance, cancer cells can stimulate their neighbouring cells to produce growth factors which can be used to enhance their proliferation. Moreover, malignant cells can acquire ligand-independence by deregulation of signal transducing receptors. Overexpression of growth factor receptors is present in different kind of cancers. For instance, HER2/*neu* receptor is overexpressed in stomach and mammary carcinomas [6, 7]. Thus, by upregulation of signal receptors, cancer cells become over sensitive to the available growth factors. Structural alterations in growth factor receptors can also facilitate growth factors independence. For example, a truncated form of epidermal growth factor receptor is constitutively active [8].

Alteration of downstream factors

Cancer cells can acquire growth factor independence via alteration of downstream factors which transmit signals from growth factor receptors and integrins. It has been suspected that all types of cancers have alterations in one or the other components of growth factor signaling pathways. For example 40% of human melanomas have structurally mutated B-Raf protein (active form). Thus, this alteration results in constitutive signaling through Raf to mitogen activated protein (MAP)-kinase pathway [9]. Similarly, SOS-Ras-Raf-MAP kinase has been shown to be one of the most important cascade pathways that play a role in developing cancer. For example, structurally altered forms of Ras have been found in almost 25 % of human cancer, where it transmits mitogenic signals without stimulation of its normal upstream regulators [10]. PI-3 Kinase pathway is also one of the most deregulated signaling cascades in cancer. The detailed description of each of these pathways is given in the following paragraphs.

RAS-RAF-MAP kinase pathway

In normal cells Ras has transitory signaling whereas in cancer cells it becomes constitutively active. Ras protein acts in “on” and “off” states which are determined through binding of GTP and GDP. In active form it is bound with GTP while in inactive state it is present as Ras-GDP. In normal cells, the hydrolysis of GTP is regulated by guanine nucleotide exchange factors (GEFs) and GAPs (GTPase activating proteins), respectively. Ras has an intrinsic GTPase activity which is stimulated by GAP (GTPase activating proteins) and after transmitting signals to its downstream effectors, it becomes inactive by hydrolyzing GTP. However in cancer cells, mutated Ras becomes insensitive to GAPs resulting in constitutive activation.

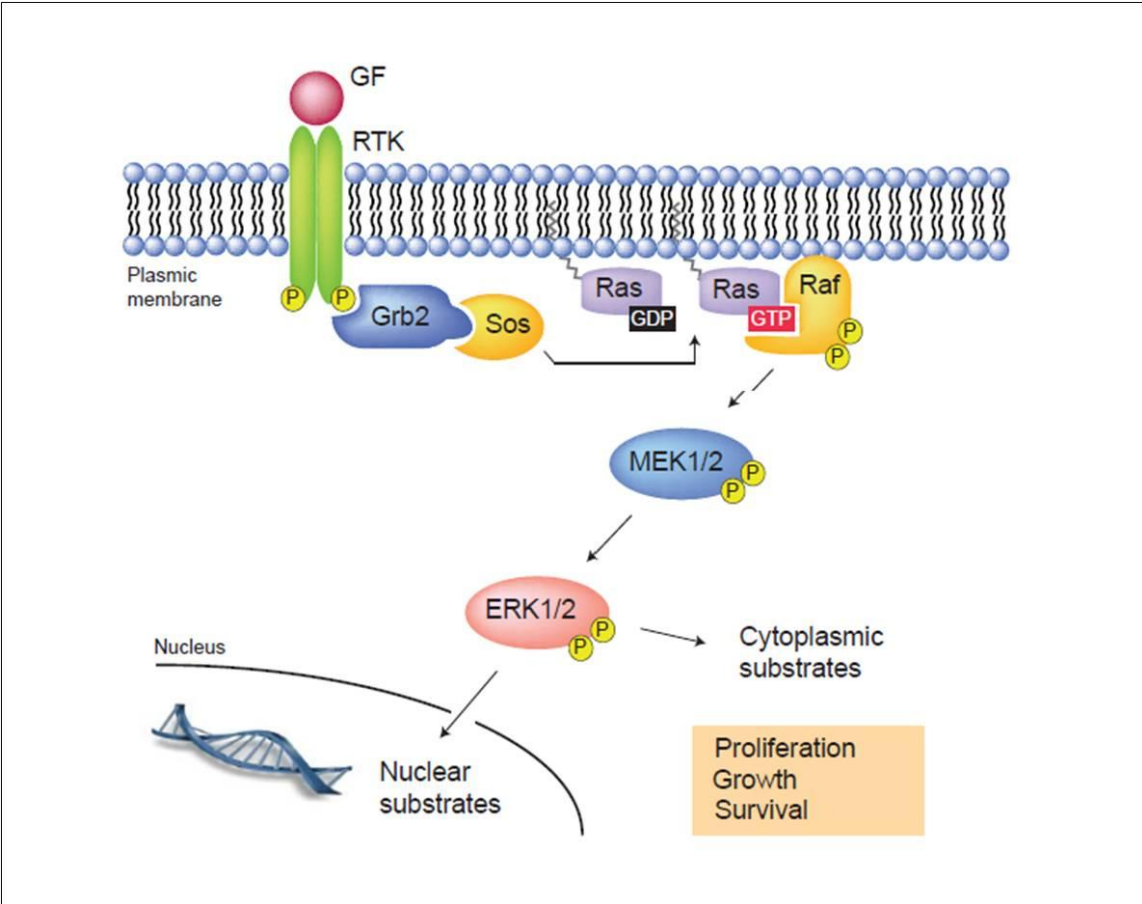


Figure 5: SOS-RAS-RAF-MAP kinase pathway

Tyrosine kinase receptors are activated upon binding of extracellular growth factors. Activated tyrosine receptors (phosphorylated forms) transmit signals to the downstream effectors. Grb2-SOS binds to activated tyrosine receptors and transmit signal to Ras through SOS. GDP-Ras is converted to GTP-Ras. Hydrolysis of Ras-GTP transmits signals to Raf. Phosphorylated Raf transmits the signal to MEK and MAPK. This figure is adapted from Frémin and Meloche Journal of Hematology & Oncology 2010.

PI-3 kinase pathway

PI-3K signaling pathway PIP3 transduces proliferative signals through PIP3 production. The production of PIP3 is regulated by two enzymes PI-3 Kinase and PTEN phosphatase. PI3-kinases are members of phospholipids and are categorized into three classes: I, II and III. Class

IA PI3-K is the most studied class of PI3-Ks in cancer. It is a heterodimer that contain a regulatory subunit (p85) and a catalytic subunit (p110). PI3-K is activated by binding of growth factors or ligands to their cognate receptors (tyrosine kinase and G-coupled receptors). Tyrosine kinase receptors include human epidermal growth factor receptor (HER) family, insulin and insulin like growth factor receptors (IGF-IR). Upon activation of these receptors the regulatory subunit of PI3-K (p85) interacts with their intracellular portion leading to the activation of catalytic subunit of PI3-K (p110). PI3-K is frequently mutated in several cancers including breast cancer. PI3-K activity is upregulated through amplification of catalytic subunit. It can also be enhanced by inhibition of regulatory subunit (p85).

Activated PI3-K phosphorylates phosphatidylinositol biphosphate (PIP₂) to produce a second messenger phosphatidylinositol triphosphate (PIP₃). The negative regulation of PI3-K pathway is conferred by PTEN (Phosphatase and Tensin Homolog deleted on Chromosome 10), which hydrolyzes PIP₃ into PIP₂. PTEN has both lipid and protein phosphatase activity. Mutations in the catalytic subunit of the PI-3 Kinase pathway have been detected in many tumors. This leads to the hyperactivation of the PI-3 Kinase pathway and its downstream effectors such as AKT/PKB signal [13, 14]. Mutations causing functional defects in PTEN results in amplification of PI-3 Kinase signaling and has been found to enhance tumorigenesis. Moreover, PTEN expression is lost by promoter methylation in many human tumors and this can also be responsible for amplification of PI3-K pathway [15-17].

The recruitment of (PH) domain-containing proteins (PDK1, serine/threonine-protein kinase and Akt) to the plasma membrane results in their activation. The membrane colocalization of PDK1 and AKT results in phosphorylation at Thr308 and partial activation of Akt kinase. Complete

activation of Akt takes place upon phosphorylation of Ser473 by PDK2. Akt activates mammalian target of rapamycin (mTOR)–containing complex 1(TORC1) which regulates protein synthesis and cell growth.

Akt, an important component of PI-3 K pathway is a threonine/serine kinase protein which acts as an important regulator of proliferation, apoptosis and cell survival. To date several studies demonstrated a significant role of Akt in cancer pathology and its inhibitors are shown to reduce tumor growth. Moreover, it is involved in the inhibition of apoptosis through regulating important proteins involved in apoptosis. For instance Akt is indirectly involved in regulating proteasome degradation of p53 (tumor suppressor), it inhibits apoptosis through inhibition FOXO mediated transcription of proapoptotic proteins and by directly inactivating proapoptotic proteins such as BAD. Akt can also act as a prosurvival protein by promoting cell cycle progression through inhibition of FOXO mediated transcription of cell cycle inhibitors e.g. p27Kip1(cell cycle inhibitor). It can also regulate stability of Myc and cyclin D1 by inhibiting GSK and through activation of NF-kappa B pathways. Akt activation (pAkt) is associated with tumor size, prognosis and histological grade of breast cancer. PI-3K pathway is described in figure 6.

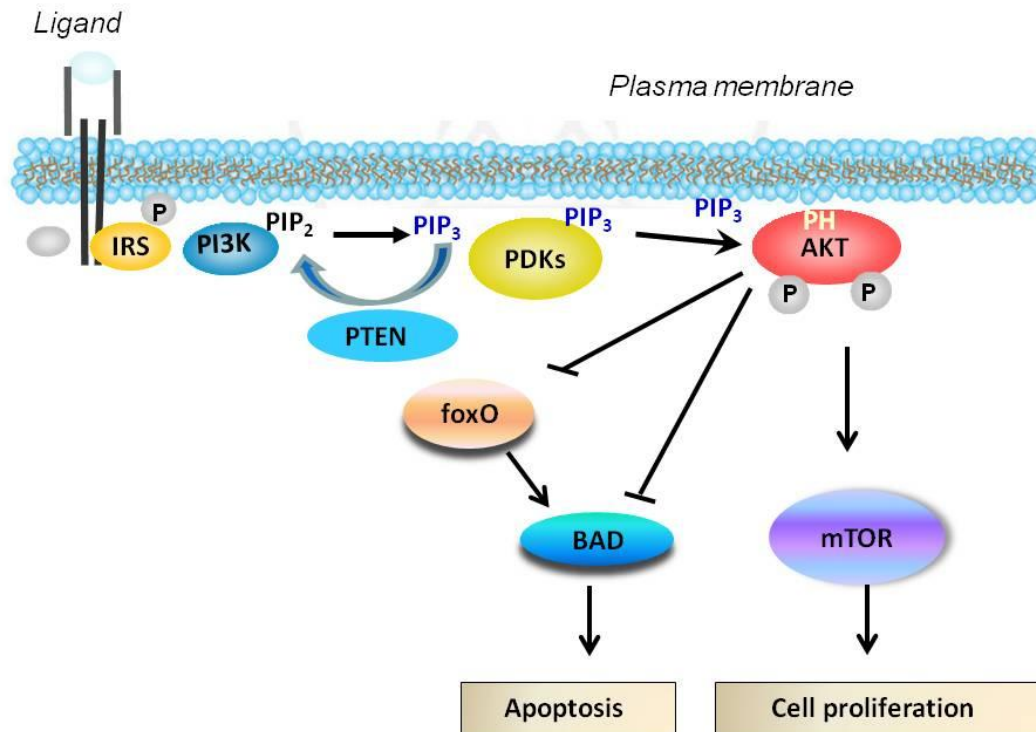


Figure 6: PI3 kinase pathway

PI3 kinase pathway is comprised of PI3-K and PTEN and results in production of PIP₃. PIP₃ production is regulated by two enzymes: i) PI3-K which phosphorylates PIP₂ (phosphatidylinositol 4,5 biphosphate) to give rise PIP₃ Phosphatidylinositol (3,4,5)-triphosphate and leads to the activation of Akt. ii) PTEN is a phosphatase which removes phosphate from PIP₃ to convert it to PIP₂.

Moreover, signaling cascades also exhibit crosstalk interactions with other signaling pathways that enable extracellular growth signaling to elicit multiple intracellular responses. For example Ras can evoke survival pathway as it has direct interaction with cell survival promoting PI-3 Kinase [12] as is shown in figure 7.

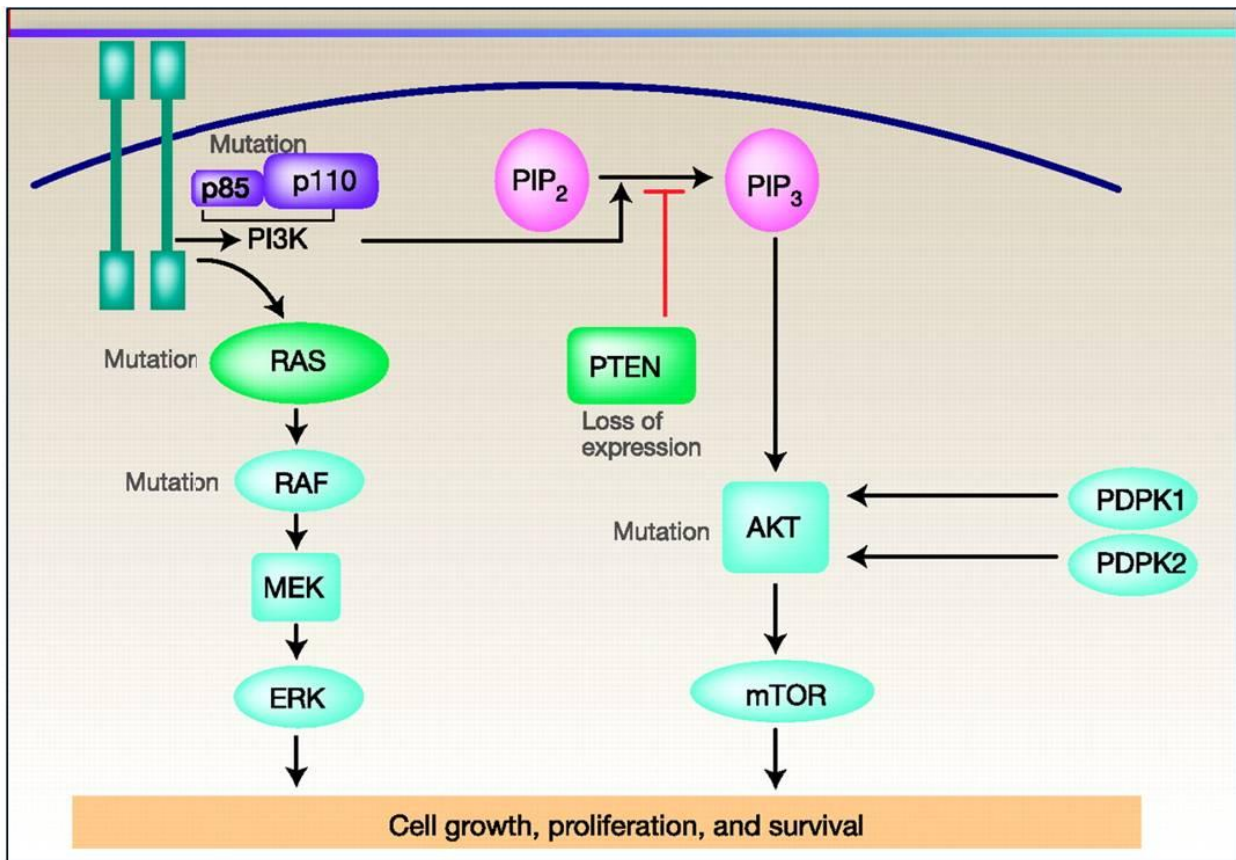


Figure 7: Crosstalk between PI3 kinase and Ras-Raf pathways

Mutations in different components of PI-3 Kinase and in Ras-Ras-MAP kinase pathways are involved in progression of different types of cancers. These pathways can interact to elicit proliferation signal. This figure is adapted from Dasari A, and Messersmith W A Clin Cancer Res 2010;16:3811-3818

mTOR kinase which co-ordinates cell growth and metabolism, presents an example of deregulation of a negative regulatory mechanism. mTOR lies both upstream and downstream of PI3-K signaling pathway. Enhanced mTOR activation results in the inhibition of PI3-K signaling via negative feedback mechanisms. Therefore, when mTOR is inhibited through pharmacological inhibitors (e.g. rapamycin) it results in amplification of activity of PI3-K signaling pathway and its downstream effectors. In this way, the anti-proliferative effect of inhibition of mTOR is overcome by lack of feedback inhibition. It shows that disruption of

negative feedback mechanisms not only results in increase of proliferation but can also contribute in developing resistance toward drugs targeting mitogenic signaling [18, 19].

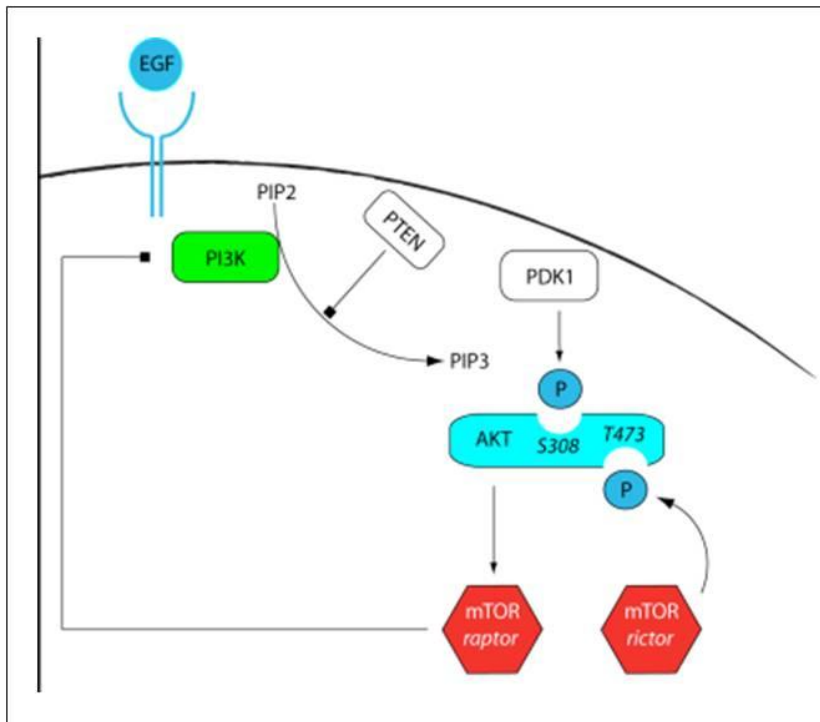


Figure 8: mTOR pathway

PIP3 production leads to the activation of Akt, resulting in activation of downstream effector mTOR. mTOR can also regulate the activity of Akt through negative feedback inhibition.

1.2. Insensitivity to antigrowth signals

In a normal tissue, multiple antiproliferative signals and other antiproliferative components are present to maintain tissue homeostasis. These antiproliferative components are present either as soluble growth inhibitors or immobilized inhibitors which are embedded in the membrane. In normal tissue these antigrowth factors regulate proliferation. According to the physiological conditions, they may promote proliferation or induce cell arrest. On receiving antigrowth signals, cells enter either into quiescent state or into the post-mitotic differentiation

state. However, cancer cells show constitutive proliferation. It suggests that cancer cells may evade the effect of these negative growth signals.

Similar to the proliferative signals, cellular growth inhibitory factors transmit signals through transmembrane cell surface receptors. TGF- β is the best characterized example of soluble antigrowth signals. It can inhibit cell proliferation by different mechanisms. One of the well known mechanisms involves TGF- β signaling through retinoblastoma protein pRb [20, 21]. pRb is a tumor suppressor which regulates cell cycle progression [22]. Deregulation of pRb signaling can cause constitutive proliferation and has been found in different types of cancers. Deregulation of pRb signaling can occur through different mechanism. This protein acts as a tumor suppressor in its hypophosphorylated form. In its active form, pRb inhibits the activity of E2F transcription factor by sequestering it. Thus, E2F transcription factor cannot promote progression of cell from growth phase gap 1(G1) into synthesis phase (S phase). The deregulation of functioning of pRb results in liberation of E2F and it causes progression of cells from G0 phase to S phase [23]. In normal cells, TGF- β prevents pRb phosphorylation and it inhibits its inactivation. For example TGF- β causes the synthesis of inhibitors of CDK complexes such as p21, which are responsible for phosphorylation of pRb. The pRb signaling circuit, governed by TGF- β and by other growth inhibitory factors can be disrupted in a variety of different ways in different types of tumors [24, 25]. TGF- β signaling can be disrupted either through downregulation and dysfunctioning of TGF- β receptors or due to defects in its downstream signaling effectors like Smad4 protein [26, 27].

There are other negative regulatory mechanisms which can influence cell proliferation as well as differentiation. Differentiation can be regulated through different mechanisms. One of the mechanisms involves oncogenes that have been shown to play an important role in inhibition of

differentiation process and thereby favor proliferation. For instance, the Myc oncogene (transcription factor) can favor proliferation by forming a complex with another factor Max protein. In normal cells, this complex has homeostasis with another complex which is made by the interaction of Max with a group of Mad transcription factor. Max-mad complex favors differentiation [28]. However, in most of tumors Myc oncogene is found to be over-expressed. Therefore, it favors Myc-Max complex resulting in increase in proliferation.

1.3. Resisting cell death

Programmed cell death is a natural barrier to the development of the cancer and resistance to apoptosis is one of the highly significant hallmarks of cancer [29-31]. Growth of cells is not only regulated by cell proliferation but is also determined by the programmed cell death. Programmed cell death is a fundamental process in the development of organisms and normal cells die regularly to maintain homeostasis. Three distinct processes are involved in programmed cell death: apoptosis, autophagy and programmed necrosis. Apoptosis and necrosis always lead to cell death while autophagy can result in promoting cell proliferation or cell death depending upon the conditions. Apoptosis is one of the major mechanisms in programmed cell death processes.

Apoptosis

Apoptosis is a vital process that occurs during development and aging. It is also involved in maintaining tissue homeostasis. Moreover, it can be used as a defense mechanism in immune reactions or when cells are damaged by disease [32]. Notably, its deregulation can lead to cancer, autoimmune and degenerative diseases [33]. As most of the cancer therapies depend on the

apoptosis, its deregulation not only causes the development of cancer but it can also play a role in developing resistance against these therapies.

Apoptosis was first described by Kerr, Wyllie, and Currie in 1972 as a morphological distinct form of programmed cell death. However, certain components of the apoptosis concept were explicitly described in later years [34, 35]. In normal condition, apoptosis is present in latent form in all types of cells. However, it triggers on receiving different extracellular or intracellular physiological signals such as irreparable DNA damage and normally takes only 24h to complete [36]. Apoptosis involves a series of different morphological and biochemical changes such as cell shrinkage, blebbing, condensation and degradation of chromosomes, formation of apoptotic bodies and finally their engulfment by neighboring cells and phagocytes.

Apoptotic pathways

Apoptosis is a highly complex and sophisticated process that can occur through two well characterized pathways: extrinsic or death receptor pathway and intrinsic or mitochondrial pathway as shown in figure 9. Each of these pathways is comprised of two types of components: upstream regulators and downstream effectors [29]. Upstream regulators are the sensors that receive different extracellular and intracellular stimuli and transmit signals to downstream regulators. Signals are transmitted according to the physiological conditions, resulting in cell survival or cell death.

Extrinsic pathway

The extrinsic pathway involves direct signal transduction by binding of the death receptors with their extracellular death ligands to make death complex (DC). Death receptors belong to the superfamily of TNFR (Tumor Necrosis Factor Receptor), which are characterized by a cysteine

rich extracellular domain and a homologous intracellular domain known as the death domain. The best-characterized death receptors are Fas and TNFR1 (Tumor Necrosis Factor Receptor-1). Fas ligand binding to its cognate transmembrane receptors Fas Receptor and TNF α binding to its cognate receptor TNF-R1 are classical examples of the extrinsic apoptotic pathway [37]. Each of these pathways leads to the activation of a cascade of different proteases known as caspases. These caspases play different roles in disruption of cell integrity and cause programmed cell death.

Binding of Fas ligand to its death receptor causes the recruitment of Fas associated death domain protein (FADD). FADD then associates with procaspase-8 and leads to the formation of death-inducing signaling complex (DISC). DISC containing Fas, FADD and ProCaspase8, results in autocatalytic activation of procaspase-8 [38]. Similarly, the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TRADD (Tumor Necrosis Factor Receptor-1-Associated Death Domain) with recruitment of FADD and RIP (Receptor-Interacting Protein) [39-41]. This results in the activation of procaspase-8. Activated caspase-8 then triggers execution phase of the apoptosis by recruitment and activation of executioner caspases such as caspases-3.

Intrinsic pathway

The second pathway is the intrinsic pathway, a mitochondrial energy dependent apoptotic process. There are different extracellular and intracellular stress signals which can trigger this pathway such as DNA damage, radiations, toxin, viral infections, hypoxia, signaling imbalance provoked by oncogenes or survival factors insufficiency [31]. The release of cytochrome c is a critical event in mitochondrial dependent apoptosis. These stimuli alter permeability of mitochondrial membrane and result in release of internal cytochrome c into the cytosol [42, 43].

The cytochrome c then recruits Apaf-1 (apoptotic peptidase activating factor-1) and pro-caspase-9, resulting in oligomerization of Apaf-1 and formation of apoptosome which activates caspase-9. Activated caspase-9 then cleaves caspase-3 resulting in downstream events involved in cell death [44]. The release of cytochrome c is regulated through counterbalancing between different proapoptotic and antiapoptotic members of Bcl-2 family. The proapoptotic members of the Bcl-2 family are: Bax, Bak, Bid, Bim. The antiapoptotic proteins are BCL2, Bcl-XL, Bcl-W [29, 45, 46]. The proapoptotic members of the Bcl-2 family are embedded in the inner membrane of the mitochondria and are inhibited by the antiapoptotic members of the family. On receiving proapoptotic signals, they are released from the inhibition caused by antiapoptotic members. This results in disruption of mitochondrial membrane and the release of proapoptotic signaling proteins like cytochrome c. The released cytochrome c, in turn, activates initiator caspases such as caspase-9 which further activates executioner caspases such as caspases-3. These caspases induce different cellular apoptotic processes through their proteolytic activities

The intrinsic apoptotic pathway is found to be deregulated in most of the cancers. The cellular conditions that trigger apoptosis are not yet fully revealed. However, several abnormalities in sensors that play key roles in tumor development have been identified [29, 30]. One of the critical sensors is DNA damage, which functions through p53 tumor suppressor [47]. The DNA checkpoints proteins, ATM (Ataxia Telangiectasia Mutated protein) and Chk2 (Checkpoints Factor-2) directly phosphorylates and stabilize p53 and inhibit its MDM2 (Mouse Double Minute-2 Homolog) mediated ubiquitination. p53 promotes apoptotic cell death by regulating expression of different proapoptotic Bcl2 family members like BAX and Bid and by repressing antiapoptotic Bcl2 proteins. It also regulates other proteins like Noxa and PUMA (p53-Upregulated Modulator of Apoptosis). Moreover p53 also transactivates other genes that may

contribute to apoptosis including PTEN (Phosphatase and Tensin Homolog Deleted on Chromosome-10).

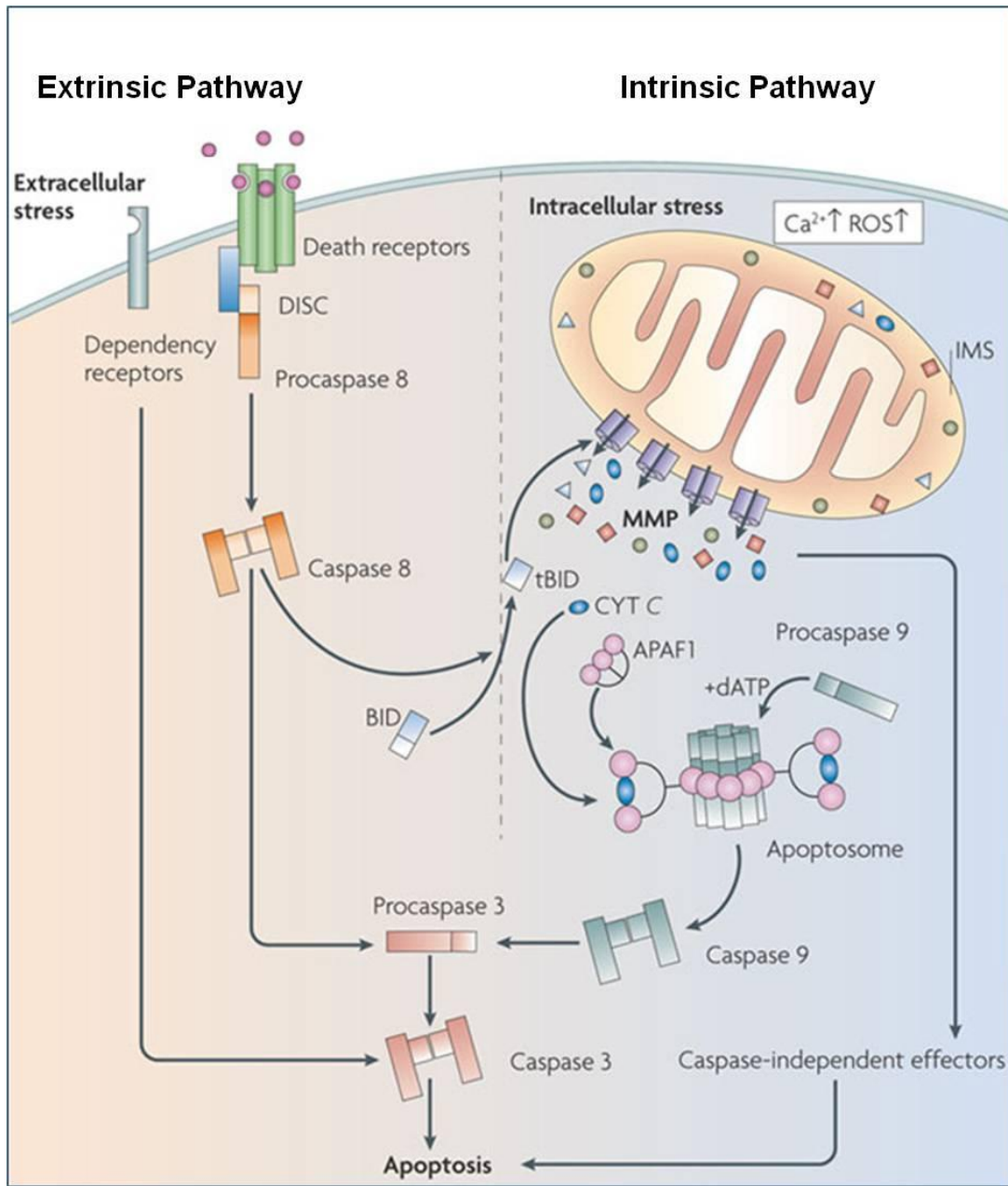


Figure 9: Extrinsic and Intrinsic Pathways of apoptosis

Apoptosis can result from the activation of two biochemical pathways which are known as extrinsic and intrinsic pathways. The extracellular apoptotic pathway is initiated at the plasma membrane by specific transmembrane

receptors, whereas mitochondrial apoptosis is triggered by intracellular stimuli such as Ca^{2+} overload and overgeneration of reactive oxygen species (ROS). Extrinsic pathway/ death receptor pathway leads via caspase 8 and intrinsic pathway is carried out through initiator caspase 9, both resulting in activation of procaspase 3 which leads to apoptosis. In intrinsic pathway, cytochrome c (CYTC) released from mitochondria interacts with the adaptor protein apoptotic peptidase activating factor 1 (APAF1) as well as with procaspase 9 to form the apoptosome. One of the major links between extrinsic and mitochondrial apoptosis is provided by the BCL-2 homology domain 3 (BH3)-only protein BID. BID can promote MMP ((Mitochondrial membrane permeabilization) following caspase-8-mediated cleavage. dATP, (deoxyadenosine triphosphate) ; DISC (death-inducing signaling complex); tBID (truncated BID); MMP (Mitochondrial membrane permeabilization); IMS mitochondrial intermembrane space. This figure is adapted from Lorenzo Galluzzi, Klas Blomgren & Guido Kroemer Nature Reviews Neuroscience 10.

Despite the presence of sophisticated mechanisms for apoptosis, cancer cells can acquire resistance to apoptotic cell death through different strategies. One of the commonly found mechanisms is the mutation in *TP53* gene that is found in 50% the human cancers [48]. Alternatively, cancer cells may also achieve resistance to cell death by others strategies like increased expression of anti-apoptotic regulators such as (Bcl-2, Bcl-xL) or by down regulation of proapoptotic factors like Bax, Bim and Puma [49, 50].

Increase in survival signals can also play an important role in developing resistance against apoptosis through regulation of different genes by FOXO transcription factors. FOXO family is an important downstream target of protein kinase PKB/Akt that is one of the most deregulated pathways found in different cancers [51, 52]. This pathway is found to be upregulated in different human cancers with disrupted apoptosis. It is activated either by extracellular growth factors such as IGF-1/IGF-2 and by interleukin (IL-3) [31], or by intracellular signals originating from Ras signaling pathway [12]. It can also be activated by loss of the tumor suppressor phospholipid phosphatase (PTEN), which normally attenuates Akt signaling pathway through dephosphorylation of PIP3 [53].

Autophagy

The best known form of programmed cell death is apoptosis. However, recent research indicated other forms of programmed cell death. Autophagy is among recently accepted programmed cell death mechanism. It refers to the cell's self-eating process. Autophagy is a highly regulated catabolic mechanism that involves the lysosomal degradation pathway. Autophagy is essential for different cellular processes like cell survival, differentiation, development, and homeostasis. Like apoptosis, autophagy is present in a less active state in normal conditions while it can be strongly induced by different intracellular or extracellular signals. Growth factor deficiency is one of the important stimuli to trigger autophagy [54, 55].

When nutrients are scarce, cell undergoes through a process of autophagy as a defense mechanism. This process involves degradation of cellular components and cellular organelles such as ribosomes and mitochondria. Autophagy provides the energy required for minimal cell functioning in stressed, nutrient-limited condition. During the process of autophagy, cellular components are enclosed by small intracellular vesicles called autophagosomes and are isolated from the rest of cell. Autophagosomes then fuse with lysosomes where degradation process takes place. It generates low molecular weight metabolites and provides energy to support cell survival in nutrient limited conditions.

Autophagy is mainly a cell survival mechanism that responds to extracellular or intracellular stress signals. However, over-activation of autophagy can also result in autophagic cell death [56]. Autophagy can induce cell death either directly or via interaction with apoptosis. Recent research has demonstrated the relationship between autophagy, cancer and metabolism in different ways. For example, stimulation of Akt/mTOR pathway (a survival pathway) inhibits

not only apoptosis but also autophagy. Similarly, downregulation of Akt/mTOR pathway as a response to insufficient growth factors results in induction of both apoptosis and autophagy [54, 57, 58]. Moreover, these two processes also share regulatory factors, for example stress transducing BH3 proteins (Bid, Bad, Bim etc) can induce both processes or triggers any one of these depending upon the physiological state of the cell. Similarly, defects in autophagy regulatory proteins such as beclin-1 protein not only affect autophagy but also increase cell's susceptibility to tumorigenesis [54, 59]. However, autophagy can also exhibit beneficial effect for the survival and progression of the cancer. For example, autophagy can provide cytoprotection to cancer cells against nutrient starvation, radiotherapy, and certain cytotoxic drugs. It can also help cancer cells to develop reversible dormancy in severe stressed conditions induced by anti-cancer treatments [60-62]. In this way, autophagy helps in developing resistance against cancer treatment. Briefly, these studies showed that autophagy has conflicting effects on progression of cancer as, on one hand it acts as a barrier against tumorigenesis and on the other hand it plays role in cancer cell survival under stressed conditions.

Necrosis

Necrosis is another cell death mechanism in which cell bloated and explodes as a response to different kind of stresses, e.g. infection, trauma and toxin. The necrotic cell death causes release of cell content and results in local inflammation. Previously, it was considered a random, uncontrolled and accidental death process. However, recent research has shown that necrosis can also be a programmed cell death mechanism [63, 64]. Moreover, there are multiple lines of evidences showing that necrotic cell death is beneficial in promoting cancer cell proliferation. For example, necrotic cell death releases pro-inflammatory signals like IL-1 α into their microenvironment which can stimulate neighboring viable cells to proliferate [65].

1.4. Limitless Replicative Potential

In order to develop cancer, cells have to overcome the barrier to limitless replication abilities. Normal cells can grow to a certain limit and after certain number of doublings they enter into a senescence state in which they remain viable but stay in a non-proliferative state. However, those cells which can circumvent the senescence phase, enter into another state where massive cell death occurs as a result of karyotypic disarray. This is called the crisis phase [66]. This phenomenon is also observed in cultured cells, where after a certain number of doublings cells enter into the senescence phase. If they can overcome the first barrier (senescence phase) then they enter into the crisis phase which leads to cell death. However, some cells emerge from this population of crisis phase, with limitless replicative potential and acquire immortalization trait.

Most of tumor cells which propagate in culture are immortalized [67]. This suggests that they may have acquired this trait during their *in vivo* progression which was probably required for the development of their malignant growth state [68]. This immortalization state has been associated with maintenance of telomeres length. In contrast to the normal somatic cells, all types of malignant cells have maintained their telomeres. Telomeres consist of several thousand hexanucleotide repeats, present at the ends of chromosomes which shorten progressively in non-immortalized cells propagated in culture. The progressive shortening of telomeres with successive division results in unprotected chromosomes, leading to the chromosomal fusions and ending with cell death via crisis. Measurement of telomeres length is used as to count the number of generations that each cell undergoes before entering into senescence state.

As cancer cells require limitless replication, they need a mechanism to maintain their telomeric DNA. Different factors are involved in maintaining telomeres in malignant cells. One

of the most abundantly found is the higher expression of telomerase enzyme. This enzyme is responsible for adding hexanucleotides at the end of the chromosomes. In normal cells telomerase is either completely absent or it is present at low expression level while it is present at detectable expression levels in 80-90 % of the tumor cells [69]. Those tumor cells which lack telomerase enzymes, maintained their telomeres by another less known mechanism called alternative lengthening of telomeres (ALT). ALT is a recombination-based telomere maintenance mechanism in which a telomere sequence is used as a template to elongate a short telomeric extremity [70]. In this way, it allows tumor cells to maintain telomeres to the extent which can protect them from senescence and crisis.

1.5. Sustained Angiogenesis

Sustained angiogenesis is in another characteristic exhibited by most cancers. Angiogenesis refers to the growth of blood vessels from the existing vasculature. In normal conditions, angiogenesis and vasculogenesis take place during embryogenesis. These are two distinct mechanisms: vasculogenesis which involves birth of the endothelial cells from progenitor cells and angiogenesis that involves sprouting of new capillaries from the existing vessels. However, in normal adult body vasculature becomes largely quiescent except during certain physiological processes where angiogenesis is turned on transiently, such as wound healing and female reproductive cycle. In contrast, angiogenesis remains activated during progression of tumors and cause continuous sprouting of new vessels which helps to sustain tumor expansion. Similar to normal tissues, tumors require sustenance in the form of nutrients and oxygen, as well as an ability to evacuate metabolic wastes and carbon dioxide. Owing to high proliferation rate, cancer cells require stimulation of angiogenesis to provide ample nutrients and oxygen for their cell survival and growth and for elimination of cell wastes [71, 72].

Angiogenesis is regulated by a balance between positive and negative signals which can stimulate or block it [73, 74]. These signals can be conveyed through soluble factors and their receptors displayed on the surface of endothelial cells. Vascular endothelial growth factor (VEGF), acidic and basic fibroblast growth factors (FGF1/2) are examples of angiogenic stimuli. Each of these factors binds to its tyrosine kinase receptor and conveys signals for the development of angiogenesis [75]. Thrombospondin-1 (TSP-1) is a prototypical angiogenesis inhibitor that binds to transmembrane receptors displayed by endothelial cells (CD36) and stimulates suppressive signals that can counteract proangiogenic stimuli [76, 77]. Most tumors appear to induce angiogenesis by changing the balance between positive and negative angiogenic signals. The balance can be shifted either by upregulating stimulatory signals expression (VEGF and/or FGFs) or by downregulating endogenous inhibitors of angiogenesis (TSP-1). Moreover, both changes can also occur in the same tumor [78, 79]. VEGF gene expression can be upregulated by hypoxia, oncogene signaling and by modulation of proteolysis. For example, activation of the *ras* oncogene or loss of the von Hippel-Lindau (VHL) tumor suppressor gene in certain cell types causes up regulation of VEGF expression [80, 81]. Inhibition of VEGF impairs neovascularization and growth of subcutaneous tumors in mice [82, 83]. Similarly, the inhibitory signals are also regulated by the p53 tumor suppressor protein in some cell types. For instance loss of *P53* gene can cause downregulation of TSP-1 gene expression and liberate endothelial cells from its inhibitory effect [84]. Collectively, these observations suggest that the inhibition of angiogenesis could be one of the therapeutic strategies against cancer.

1.6. Genome Instability

Development of cancer is a highly complex and multistep process in which cells acquire features that enable them to divide uncontrollably and to metastasize. Many factors play crucial roles in

the transformation of normal cells into tumor cells. One of the major factors is the genetic mutation; however epigenetic mechanism (DNA methylation and histone modifications) and alteration in gene expression can also cause cancer. Genetic mutation is quite frequent in normal somatic cells as DNA is vulnerable to different kinds of intrinsic and extrinsic damages, e.g. abnormality in chromosomes replication, damage induced by different types of carcinogens or by exposure to ultra violet rays. In normal cells these damages are overcome by strict regulation of different DNA repair systems and checkpoint proteins. Owing to these strict regulations, the rate of genetic mutations is very rare in normal human cells. Thus, a normal human cell may need several decades or whole span of human life to acquire the full array of mutant alleles that are required to develop neoplastic state [85]. These facts suggest that cancer may occur rarely. However, cancer occurrence at substantially high rate and is mostly characterized by genomic instability [86, 87]. The genetic instability has various forms, chromosomal instability is the major form although other forms have also been described, like microsatellite instability and increased frequencies of base pair mutations [88-90]. Collectively, these facts suggest that preneoplastic cells may have high rate of genetic mutability and studies have revealed that indeed cancer cells have increased rate of mutations [91, 92]. Different mechanisms may lead to the increased rate of genetic mutability such as increased sensitivity to mutagenic agents, breakdown of one or several components of genomic maintenance machinery or by compromising surveillance system that normally monitor genomic instability [93-95]. Genomic instability occurs by inactivation of checkpoint proteins and DNA repair system. DNA repair system is comprised of different components encoded by three classes of genes: caretakers, gatekeepers and landscapers [96, 97].

Caretaker genes

Caretaker genes encode proteins involved in: (i) detection of DNA damage and in activation of DNA repair machinery, (ii) in reparation of damaged DNA, (iii) inactivation or interception of mutagenic molecules before they have damaged the DNA [91, 98, 99].

As these proteins are essential to maintain genome stability of the normal cell, mutation in their genes can give rise to genomic instability. Genomic instability can result in abnormal cell growth and finally can lead to tumor formation. One of the most frequently mutated caretaker genes is Cockayne syndrome type B (also known as *ERCC6* and *RAD26*). This gene encodes a chromatin remodeling factor that functions in transcription coupled nucleotide excision repair. Moreover, BRCA1 and BRCA2 are also known as caretaker genes. However, high throughput genomic studies revealed that mutations in caretaker genes are infrequent in sporadic cancer. These can cause hereditary cancer such as breast cancer BRCA1 but their role in non-hereditary cancer is less evident.

Gatekeepers genes

The second type of genes is called gatekeepers genes. These include genes encoding proteins that are involved in monitoring check and balance between cell proliferation and cell death. They are comprised of both tumor suppressors and oncogenes. The detailed description of these tumor drivers is given in the following paragraph.

Oncogene

An oncogene is an altered form of a normal cellular gene, with dominant transforming properties that can transform a normal cell to a malignant cell. These genes are critical for normal cell growth, signal transduction, proliferation and differentiation. In their normal state, they are known as proto-oncogenes. Mutations in proto-oncogenes render them oncogenes that can transform normal cells into malignant cells. Oncogenes can participate in tumorigenesis if one allele is mutated or overexpressed [97]. Oncogenes encode different types of cellular proteins that control cell proliferation, apoptosis or both. These include transcriptional factors, chromatin remodelers, growth factors, signal transducers and apoptosis regulators [100]. There are several oncogenes discovered so far, e.g. Ras oncogene, Raf, myc, Src-family, Her2/*neu* etc.

Ras is one of the earliest discovered oncogene, it belongs to small GTPase family. In normal cells, Ras delivers signals from cell surface receptors, such as growth factor receptors or G-protein coupled receptors, to different proteins in different pathways which ultimately affect different processes such as lipid metabolism, DNA synthesis, cytoskeleton organization, cell survival and apoptosis. In normal condition Ras uses guanine nucleotide to toggle between “on” (Ras-GTP) and “off” (Ras-GDP) state. Activated Ras has been found in several cancers. Oncogenic mutation makes Ras insensitive to the state of the nucleotide and makes it permanently active, leading to deregulation of different mechanisms and finally can cause cancer [101, 102].

Myc proto-oncogene is another strong activator of carcinogenesis which is involved in the regulation of cell check points and cell proliferation. In normal cells, the level of *Myc* remains low while it increases to a moderate level during cell division. In non-transformed condition, the

expression of Myc is tightly regulated at transcriptional and translational level and is exquisitely responsive to the extracellular growth signals. But deregulation of these mechanisms results in overexpression of Myc, which can cause abnormal cell proliferation, consequently leads to the development of cancer [103, 104].

Tumor suppressors

Tumor suppressor is another class of tumor susceptibility genes which regulate cell growth either by inhibiting cell proliferation or by inducing apoptosis. In contrast to proto-oncogenes which induce cancer in their hyperactive state, tumor suppressors trigger cancer as null or inactive alleles. The best studied tumor suppressor genes include Retinoblastoma (Rb) and *TP53* genes. *TP53* is one of the most frequent targets for genetic alterations in cancer and mutation of *TP53* tumor suppressor gene occurs in about 50% of all human tumors. In normal condition, p53 remains latent while it get activated in response to different kinds of stresses such as DNA damage, hypoxia or heat shock and oncogenes such as Myc and Ras can also regulate p53. But genetic mutation of *TP53* leads to aberrant functioning of the tumor suppression, resulting to a net increase in cell growth [105]. However, mutated tumor suppressor genes are less likely to play a major role in the development of sporadic cancer, because they are present as recessive genes and mutation on both alleles is required to confer a phenotypic change of the cell.

Landscapers genes

Third types of cancer susceptibility genes are called landscapers. This class includes proteins that control the microenvironment in which cells grow e.g. SMAD4. Normal functioning of extra-cellular matrix (ECM) proteins is essential for controlled proliferation of cells because cells communicate with each other either directly, or through different proteins

in ECM or through different signals i.e. growth factors. Deregulation of these proteins cause abnormal signalling between neighbouring cells and with their ECM.

1.7. Evading immune destruction

Another highly emerging hallmark of cancer is evasion from immune destruction. According to the immune surveillance theory, cells and tissues are constantly monitored by the immune system. This immune system always remains active and is responsible for recognition and eradication of incipient cancer cells and nascent tumors. This suggests that cancer cells need to avoid this surveillance system. This hypothesis is also supported by a study which demonstrated that some cancers are significantly increased in immunocompromised individuals. For instance, immunosuppression increases melanoma risk development [106]. The majority of such cancers are viral-induced cancers. However, evidences from genetically engineered mice showed that it can also exist in non-viral cancer. For example, carcinogen-induced tumor grows more rapidly in immunodeficient mice compared to immunocompetent controls. Studies demonstrated that both innate and adaptive immune systems are important for providing surveillance against cancer development. For example, deficiencies in the development or function of CD8⁺ cytotoxic T lymphocytes (CTLs), CD4⁺ Th1 helper T cells or natural killer (NK) cells, each led to significant increases in the development of cancer [107, 108]. In addition to mice studies, epidemiological studies also supported the existence of antitumoral immune responses in some cancers. For example, colon or ovarian cancer patients with higher expression of CTLs and NK exhibit better prognosis compared to those who lack such abundant natural killer lymphocytes [109, 110]. The molecular mechanism involved in evading immune surveillance is still in its infancy. However

studies showed that cancer cells may inactivate CTLs and NK cells, by secreting TGF- β or other immunosuppressive factors such as CCL21 [111, 112]. Collectively, these studies suggest that antitumor immunity may serve as a significant barrier to tumor formation and progression in humans.

1.8. Alteration in cellular Bioenergetics

An emerging hallmark of cancer is the alteration in cellular bioenergetics. Cancer cells are characterized by high proliferation rate compared to normal cells. This increase in proliferation is maintained by increase in metabolic rate. Studies have shown that altered cellular metabolism is a defining feature of cancer. The first tumor-specific alteration, deregulated metabolism, was discovered by the Nobel Prize winner Otto Warburg in 1920s. Since then numerous studies have demonstrated different roles of metabolism in the development of cancer. It is a huge area of research and is discussed in the next chapter under topic of metabolism and cancer.

Chapter 2

Metabolism and cancer

All cells need energy for maintenance of basic cellular processes such as cytoskeletal dynamics, DNA repair, basal transcription and translation, protein turnover and vesicle trafficking. They obtain this energy either from sunlight or from different nutrients. A major source of cellular energy and new cell mass is glucose. Glucose is metabolized through a process of glycolysis to produce pyruvate. Pyruvate is further metabolized to yield energy, either through oxidative phosphorylation or via fermentation. Oxidative phosphorylation is a high energy yielding process in which pyruvate is oxidatively metabolized to CO₂ in the tricarboxylic acid (TCA) cycle. However, pyruvate can also be reductively metabolized through fermentation to produce organic acids or alcohols (e.g. lactate, acetate, or ethanol) as depicted in figure 10 and 11. The amount of ATP produced by fermentation is 18 fold less than that produced through oxidative phosphorylation. In aerobic conditions, normal cells metabolize glucose through oxidative phosphorylation whereas fermentation process is favored in anaerobic condition.

Highly proliferating cells need additional energetic processes for cell growth and division in addition to the above mentioned basic cellular processes. More energy is needed to build new biosynthetic blocks and to construct macromolecules which are used for the formation of new cells. As cancer cells are highly proliferating cells, they need to find a solution to achieve high energy demand by modulating different metabolic pathways. Studies have shown that cancer cells adjust their metabolism to fuel their high proliferation rate [113]. Previous decades brought huge information on association of cancer with glucose metabolism and nutrition. The deregulation of cellular energetics or reprogramming of energy metabolism has reshaped cancer

research and has become one of the emerging hallmarks of cancer. However, the first study regarding modulation of metabolic pathways in cancer cells was done in 1924, by Otto Warburg and his colleagues. They observed that cancer cells consume much larger quantity of glucose compared to their normal partners. Moreover, they observed that cancerous cells reprogrammed their energy metabolism to aerobic glycolysis to produce high yield of lactate [114]. This phenomenon is called “Warburg effect”, and its detailed description is given in the following paragraph.

2.1. Warburg Effect

Warburg and his colleagues observed that tumor cells metabolized 10-fold more glucose than normal cells. Moreover, they observed that in presence of oxygen, rat liver carcinoma produced 2-fold more lactate compared to their normal partners in which inhibition of lactate production occurs in the presence of normal oxygen tension [114, 115], shown in figure 10.

In later years research, Warburg effect was also observed in several human carcinoma and in other highly proliferating cells. However, the advantage provided by this paradoxal metabolic switch of cancer cells from a high energy producing process to a relatively low energy producing process is not obvious. Glycolysis is inefficient in terms of ATP production as it generates only two ATP molecules per molecule of glucose, whereas one glucose molecule produces 36 ATP molecules by complete oxidation through the process of oxidative phosphorylation. One of the hypotheses proposed stated that the deregulation of mitochondrial respiration is the reason of this paradoxal metabolic switch in cancer. However, studies showed that several cancers have intact

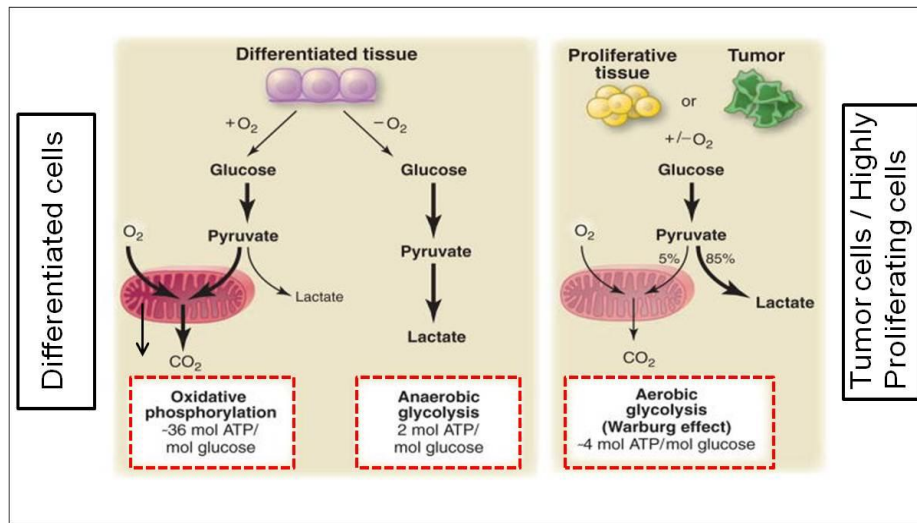


Figure 10: Aerobic Glycolysis (Warburg effect)

This figure is presenting the differences between anaerobic and aerobic glycolysis (Warburg effect). In the presence of

oxygen, normal (differentiated) non proliferating cells metabolize glucose first into pyruvate and then completely oxidize most of the pyruvate to produce CO_2 in mitochondria. In limited oxygen availability, glucose is metabolized through anaerobic glycolysis resulting in lactate generation. Tumor cells and highly proliferating cells switch to lactate formation regardless of the presence of oxygen (aerobic glycolysis). This figure is adapted from Vander Heiden et al, Science 2009.

Cancer cells enhance glucose uptake and lactate production through different mechanisms. One of these mechanisms is the upregulation of upstream processes of glycolysis, for instance overexpression of glucose transporters. Glucose enters into mammalian cells through glucose transporters (GLUT1-GLUT5). GLUT1 and GLUT3 are expressed in nearly all mammalian cells, whereas the expression of GLUT2, GLUT4 and GLUT5 is tissue specific. Tumor cells and other highly proliferating cells frequently modulate the expression of glucose transporter GLUT1 to increase the glucose uptake [116-119]. Additionally, it has been demonstrated that suppression of GLUT1 expression in a human gastric cancer cell line can decrease the number of cells in S phase and inhibit tumor growth [120]. In the cell, glucose is converted to glucose-6-phosphate by the hexokinase (HK), preventing glucose transport out of the cell. Four mammalian HK isoforms (HKI-IV) are known, and HK I is expressed in most of the normal cells and at particularly high

levels in brain tissue [121]. HK II expression is more limited and is normally found mainly in skeletal muscle and adipose tissue. However, cancer cells frequently overexpress HK II [122] and some glioblastoma cells are specifically dependent on HKII over other isoforms of the enzyme [123]. The advantage provided by selection of HK II over HK I is still not clear. Glucose-6-phosphate is further metabolized in subsequent reactions regulated by different enzymes. For example, phosphofructokinase (PFK) irreversibly converts fructose-6-phosphate to fructose-1,6-bisphosphate (FBP). PFK has been found to be overexpressed in various human cancer cell lines [124].

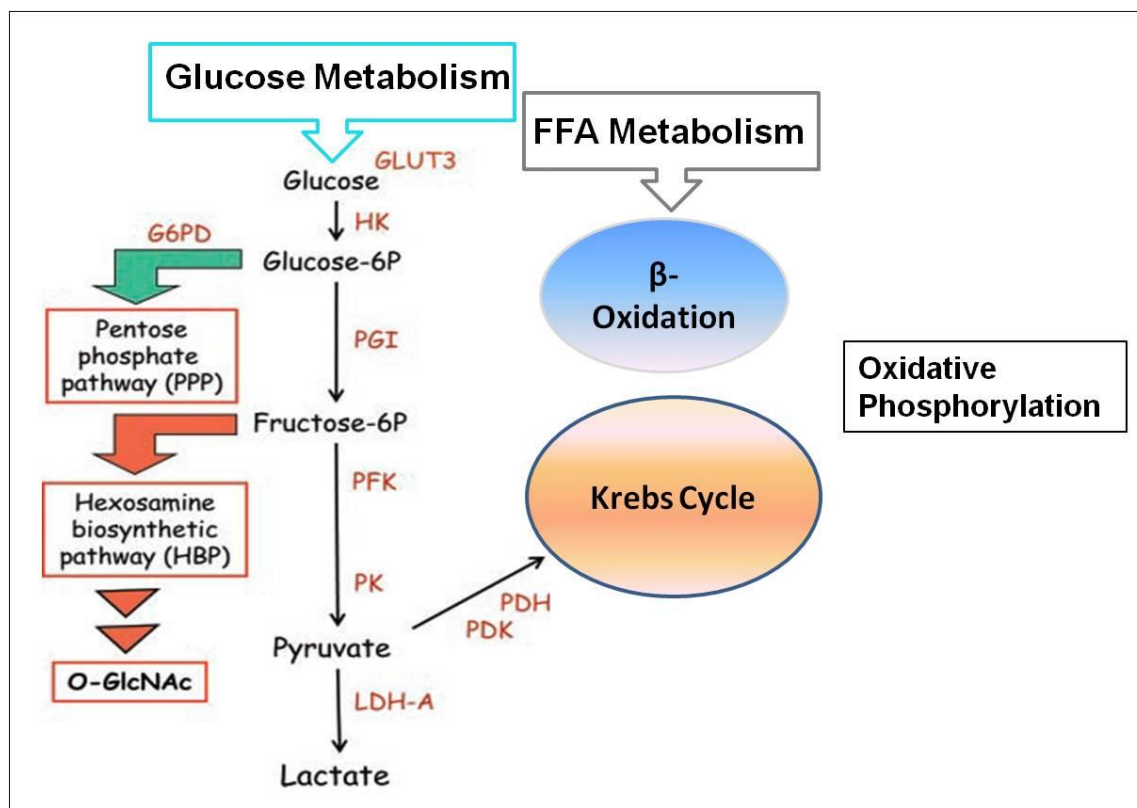


Figure 11: Glucose metabolism

Glucose entering into the cell is metabolized through different enzymes in different mechanisms. Two major pathways of glucose metabolism are glycolysis and oxidative phosphorylation. Pentose phosphate pathway and

hexosamine biosynthetic (HBP) pathway are quantitatively minor pathways. Glucose entering into the cells leads to glucose-6-phosphate by hexokinase. Glucose-6-phosphate can enter into the pentose phosphate pathway. The major part of glucose-6-phosphate is metabolized into fructose-6-phosphate. Glucose metabolism can bifurcate here and leads to the production of pyruvate or enters into HBP. Pyruvate can be further hydrolyzed into lactate or can enter into Krebs cycle leading to oxidative phosphorylation. (This figure is adapted from Salminen et al., 2010. J Cell Physiol: 1-6).

FDG-PET

Increase uptake of glucose has been translated into clinical utilization by using FDG-PET (2-deoxy-2-[18F] fluoro-d-glucose positron emission tomography) for cancer imaging in patients. FDG revealed that glucose uptake is immensely increased in different cancers. FDG imaging proved to be useful in several cancers such as breast cancer, colorectal cancer melanoma, while other types of cancer like primary prostate cancer, hepatocarcinoma and pancreatic cancer are difficult to visualize by this technique. PET scan is being used since 1980 and now has become one of the routinely used tools in oncology: for diagnosis, to determine different stages of cancer and to monitor therapeutic treatments of several cancers. FDG molecule enters into the cell via GLUT1 and GLUT3 glucose transporters. It is phosphorylated into FDG-6-Phosphate by the same enzyme which processes glucose into glucose-6-Phosphate (hexokinase). However, unlike glucose-6-phosphate which is further processed to fructose-6-phosphate via the glycolytic pathway or through oxidation to 6-phosphogluconolactone in the pentose phosphate, FDG is not metabolized beyond phosphorylation and FDG-6-Phosphate accumulates in the cell. This accumulation depends on both glucose transporter and hexokinase activity allowing assessment of glucose uptake by the cell.

As mentioned before that the Warburg effect could not fully explain advantages of aerobic glycolysis (as glycolysis produces less molecules of ATP per molecule of glucose). Another hypothesis was proposed to explain the advantage gained by cancer cells through aerobic glycolysis. According to this hypothesis, aerobic glycolysis provides advantage by higher production rate of ATP molecules. This hypothesis stated that although aerobic glycolysis produce low yield of ATP but it is comparatively faster than oxidative phosphorylation. In this way, cancerous cells have advantage over their neighbouring cells in a tissue [125].

However, this hypothesis was also challenged as other studies showed that glycolysis is not the major contributor of ATP in most cells [126]. This fact does not support the hypothesis that cancer cells exhibit aerobic glycolysis to generate ATP at faster rate. Collectively, there are different studies with controversial hypotheses and results to explain the advantage that aerobic glycolysis confers on cancer cells. These studies demonstrated that aerobic glycolysis provides advantage to the cancer cells not merely for the production of ATP molecules but for several other benefits, some of them are described in the following paragraphs.

2.2. Role of glycolysis in the production of biomolecules

Different studies showed that aerobic glycolysis goes beyond ATP production and the major function of enhanced glycolysis in proliferating cells is to maintain constant levels of glycolytic intermediates as macromolecular precursors. It provides carbon source for the production of different nucleotides, lipids and amino acids which form building blocks of RNA, DNA and proteins. The cellular machinery involved in the production of these biomolecules is altered in transformed cells to enhance their biosynthesis. Studies have shown that the incorporation of nucleotides into DNA increases with increasing glucose concentration in

mitogen-stimulated rat thymocytes [127]. Furthermore, study of cell proliferation in the absence of glucose has demonstrated that HeLa cancer cells can grow at nearly maximum growth rate if they are provided with glutamine and uracil [128]. However, this study did not discuss about other nutrients that HeLa cells may have used in addition to glutamine to compensate carbon source. In the same context, a recent study demonstrated that cancer cells can grow in size by using glutamine as the only major carbon source. However, cancer cells could not maintain TCA cycle metabolite pools in absence glucose uptake and could not proliferate [129]. In addition to support nucleotide biosynthesis, glycolysis also provides carbon source for lipid precursors. Lipogenesis has been shown to increase in cancer cells. Moreover, altered expression of lipid biosynthetic enzymes is implicated in tumorigenesis. For example, fatty acid synthase is overexpressed in cancer cells [130], and inhibition of fatty acid synthase activity has been shown to kill cancer cells and hinder the growth of tumors in xenograft models [131]. Glycerol-3-phosphate (a product of glucose metabolism) is crucial for biosynthesis of phospholipids and the elevated levels of glycerol and glycerol-3-phosphate have been reported in human peripheral lymphocytes after mitogen stimulation [132].

A large fraction of mammalian cell biomass is composed of proteins. Accordingly, highly proliferating cells have higher requirement of amino acid synthesis to provide higher requirement of proteins. Glycolysis can play a role in the synthesis of different amino acids as glycolytic intermediates are direct precursors for the biosynthesis of some amino acids. For instance, the carbons of four non-essential amino acids are derived from glycolytic intermediates: 3-phosphoglycerate provides carbons for cysteine, glycine, and serine, and pyruvate provides the carbons for alanine. In addition to provide building blocks for protein synthesis, amino acids availability provides the dominant input for the cell growth signal transduction machinery. For

example, the target of rapamycin (TOR) protein (mTOR in mammalian cells) is a serine/threonine kinase that coordinates activation of the cell growth machinery with amino acid availability [133].

2.3. Functions of lactate beyond ATP production

As described in previous paragraphs aerobic glycolysis leads to lactate production. However, lactate can perform other functions than providing energy in the form of ATP molecules. Cancer cells exhibit increase in glucose uptake but the major part of it is used for lactate formation and only a minor part is used for the biosynthesis of macromolecules [134]. This excreted lactate is not wasted and is used by cancer cells to gain other advantages. Biosynthesis of macromolecules is highly sensitive to the decrease in the glycolytic pathway [135] and increased production of lactate plays an important role to maintain glycolytic flux. For instance the NAD⁺/NADH redox balance must be maintained to continue glycolysis. The production of lactate maintains this balance by regenerating NAD⁺. Moreover lactate production can provide cancer cells with secondary benefits by altering their microenvironment [136]. It provides cancer cells with acidic extracellular environment. Excreting high levels of lactate may support survival, growth and invasion of cancer cells. As mentioned before, lactate generates NAD⁺ and that is essential for tumor proliferation [137]. It can also suppress anticancer T cell immune responses [138] and can promote tumor invasion [139, 140].

2.4. Metabolic reprogramming of proliferating cells

Cancer cells modulate several cellular mechanisms to increase their glycolytic flux and to enhance the production of biosynthetic molecules, as described in previous paragraphs. However, deregulation of cancer cells machinery can also reprogram metabolic pathways. For

instance, mutation in PI-3K signaling pathway (one of the major survival pathways) increases transport of glucose into the cells through Akt. Akt enhances the uptake of glucose by increasing expression of glucose transporters GLUT1, or by preventing internalization of glucose transporters [141-144]. Recent research in cancer biology revealed that glycolytic fueling is associated with activated oncogenes (RAS, MYC) and with tumor suppressors like p53 [118, 145]. Metabolic reprogramming provides cancer with other benefits like high proliferation, decrease of apoptosis and escape from cytostatic controls.

Most of rapidly growing cells including cancer cells encounter hypoxia condition where cells are deprived of oxygen. Hypoxia increased the expression of glucose transporters and of many glycolytic enzymes [118, 145-147]. Hypoxia can further accelerate dependence of cancer cells on glycolytic fueling by different mechanisms. For instance, hypoxia leads to the activation of hypoxia inducible factor-1 (HIF-1). HIF-1 is a transcription factor that regulates the transcription of several genes involved in cancer metabolism, cell survival/proliferation, angiogenesis, and metastasis [148, 149]. Increased HIF-1 regulates several glycolytic genes resulting in increase of aerobic and anaerobic glycolysis. For instance, HIF-1 activity promotes aerobic glycolysis by upregulating the expression and activity of pyruvate dehydrogenase kinase1. The increased activity of pyruvate dehydrogenase kinase 1 results in inactivation of pyruvate dehydrogenase leading to decrease in oxidative phosphorylation and increase in lactate production [150]. Similarly, hypoxia increase expression of glucose transporters (GLUT1 and GLUT3) via HIF-1. Moreover, HIF-1 increases the expression of vascular endothelial growth factor (VEGF) to facilitate the growth of new blood vessels. It is a heterodimer composed of HIF-1 α and HIF-1 β subunits. HIF-1 α activation has been shown to play an important role in altering the metabolism of cancer cells. Moreover, studies showed that loss of HIF-1 α in cancer

cells dramatically slows their growth as xenograft tumors in nude mice [151-153]. The expression of HIF-1 α can also be regulated by oxygen independent mechanisms. HIF-1 α can be induced by the glycolytic metabolites pyruvate and lactate [154]. RAS oncogene can also increase the expression of HIF-1 α and HIF-1 β by hypoxia-independent mechanism [146, 155].

Metabolic deregulation can cause different diseases including vascular diseases, aging cancer and diabetes. These diseases may have common regulatory components and can influence each other. In previous decades, potential links have been observed between diabetes and cancer. A detailed description of the link between diabetes and cancer is given in the following paragraph.

2.5. Diabetes and Cancer

Diabetes and cancer are worldwide prevalent diseases and incidences for both diseases are increasing every year, particularly in developed countries. Epidemiological studies have shown that patients with diabetes have higher risk of many forms of cancers. Moreover, both diseases share different risk factors and one of the important risk factors is metabolic deregulation. Cancer is the second leading cause of death and according to American organization for cancer about 16 million new cases of cancer were expected to be diagnosed in 2012. The most commonly diagnosed cancers are those of the lung/bronchus, breast, and colorectum, whereas the most common causes of cancer deaths are lung, stomach, and liver cancer. Similarly diabetes is also a commonly occurring disease. According to the annual report of International diabetes federation 2011, the estimated number of adults living with diabetes reached to 366 million i.e. 8.3% of the global adult population. Diabetes is the 12th leading cause of death and the number of deaths caused by diabetes reached 4.6 million by 2011. Diabetes is typically divided into two

major types: type I and type II diabetes. Type II diabetes is more prevalent form that accounts almost 95% of diabetes.

Initially, studies have been conducted for several years to examine the association between diabetes and cancer but no conclusive evidence of a positive association could be established [156]. However, in subsequent years studies have demonstrated a positive association between diabetes and cancer and more recently studies indicated that some cancers developed more frequently in diabetic patients compared to non-diabetic people. For example, diabetic patients have 2-fold or more liver, pancreas, and endometrium cancers compared to the non-diabetic people. Similarly, other types of cancer like colon/rectum, breast, and bladder are more common (approximately 1.2-fold to 1.5- fold) in diabetic patients compared to non-diabetic people. In contrast, lung cancer does not appear to be associated with diabetes whereas prostate cancer occurs less often in male diabetic patients [157]. Moreover, epidemiological studies have shown that mortality in cancer patients is increased with pre-existing diabetes compared to normoglycemic individuals [158, 159]. In another study, 5-year mortality rates have been observed to be significantly higher in patients diagnosed with both breast cancer and diabetes than in comparable breast cancer patients without diabetes [160].

Cancer and diabetes are heterogeneous diseases and both involve several risk factors. It might be possible that association between diabetes and cancer arise because of common factors. There are certain common risk factors between diabetes and cancer such as age, sex, ethnicity/race, diet, obesity, etc... A strong relationship has been demonstrated between obesity and diabetes type II as well as insulin resistance [161]. This relation between obesity and diabetes is supported by the evidence that weight loss lowers diabetes risk. Moreover, several studies have demonstrated that weight loss decreases diabetes incidence and restores euglycemia

in a significant percentage of individuals with type 2 diabetes [162] [163]. Obesity imposes a greater risk of colon cancer for men of all ages and for premenopausal women. Physical activity has been indicated to reduce cancer risk as well as diabetes. Evidences have demonstrated that high physical activity reduce risk factors of colon, endometrial and breast cancer, moreover it may improve survival after diagnosis [164-167]. Similarly, studies suggested that physical activity may reduce risk of developing type II diabetes. The association between weight loss and cancer is less clear. Several studies have demonstrated a relation between weight gain and several forms of cancer, like colon/rectum, endometrium and more particularly breast cancer.

Diabetes and cancer can also have direct association. For instance, hyperglycemia or certain diabetic factors may cause cancer as well such as diabetes such as insulin resistance and hyperinsulinemia. Hyperinsulinemia, a major feature of type II diabetes, is important in the pathogenesis of colon cancer. Since few decades glucose is known as a driving factor for growth of tumors. Moreover, insulin has been shown to promote metastasis [168, 169].

These studies are indicating the role of different factors in development of cancer and diabetes; however more studies are needed to explore the molecular links between these two diseases. The critical question is to find the possible biological links between diabetes and cancer. Despite increasing number of epidemiological studies, there are less experimental studies investigating the link between metabolic alterations and the progression of cancer.

Insulin and insulin like growth factor (IGF) receptors are present on cell surface and play an important role to stimulate insulin and IGF-1 responses. The majority of cancer cells express these receptors and insulin has been shown to play an important role in cancer cells proliferation and metastasis in addition to its metabolic role [170]. Increase in glucose uptake which is considered as hallmark of cancer is independent of insulin binding to its receptor [171]. This

suggests that the effect of insulin receptor activation on tumor development is more likely linked to its mitogenic activity. Activation of insulin and IGF-1 receptors can modulate different pathways involved in tumorigenesis like proliferation, protection from apoptotic stimuli, invasion and metastasis. Moreover, hyperinsulinemia could promote carcinogenesis indirectly through its effects on IGF-I [172]. Insulin reduces the hepatic production of IGF binding protein (IGFBP)-1 [173, 174] and possibly IGFBP-2 [175]. This results in increased levels of circulating free bioactive IGF-I. IGF-I has more potent mitogenic and antiapoptotic activities than insulin [176] and could act as a growth stimulus in preneoplastic and neoplastic cells. Moreover, adipose tissue is an active endocrine organ, producing free fatty acids, interleukin- 6 (IL-6), monocyte chemoattractant protein, plasminogen activator inhibitor-1 (PAI-1), adiponectin, leptin, and tumor necrosis factor-alpha [177]. These factors may play an etiologic role in regulating malignant transformation or cancer progression. For example, the plasminogen system has been linked to cancer, with expression of plasminogen activator inhibitor 1(PAI-1) linked to poor outcome in patients with breast cancer [178]. Similarly, experimental studies indicate that cancers tend to behave more aggressively when animals are fed with hypercaloric diet and less aggressively when animals are calorically restricted [179, 180]. Another study indicated a direct influence of high glucose and triglycerides on the progression of cancer [181].

Diabetic treatments have not been shown to exert significant effect on progression of tumorigenesis except metformin which has been shown to reduce cell proliferation and colony formation of breast cancer cells [182-184].

Collectively, studies have suggested different factors and mechanisms which may link diabetes and cancer, one of the recently proposed associations is through O-GlcNAcylation. O-

GlcNAcylation is the recently characterized posttranslational modification (PTM). The detailed description of O-GlcNAcylation and its role in diabetes and cancer is given in the next chapter.

Chapter 3

O-GlcNAcylation

Several kinds of posttranslational modifications (PTMs) have been described so far including phosphorylation, methylation, acetylation, ubiquitylation, sumoylation and the recently characterized *O*-GlcNAcylation. All of them are known to play important roles in the regulation of diverse biological mechanisms and cellular pathways. *O*-GlcNAcylation involves covalent attachment of β -D-*N*-acetylglucosamine (GlcNAc) moieties to the hydroxyl group of serine or threonine residues on nuclear and cytoplasmic proteins [185, 186]. It was discovered in 1984 by Torres and Hart as GlcNAc addition of single sugar moiety linked through O-Glycosidic linkage. It was found on surface proteins of lymphocytes and was initially believed to modify extracellular proteins but the following year research of the same team showed that O-GlcNAcylated proteins are predominantly nuclear or cytoplasmic and are also present in other organelles [187]. Similarly, John Hanover showed that O-GlcNAc proteins are present in the nuclear pore complex [188]. The vast majority of these proteins are soluble and are modified in response to nutrient fluxes, glucose, signaling molecules and stress. *O*-GlcNAc modification is believed to serve as a nutrient sensor because the level of its substrate uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) does fluctuate depending upon the nutrient flux in the cells. *O*-GlcNAcylation is regulated by two well characterized enzymes, *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA). OGT is the single enzyme that catalyses the addition of *O*-GlcNAc to its target proteins by using a substrate UDP-GlcNAc and OGA antagonizes OGT by catalyzing the removal of the sugar. UDP-GlcNAc is produced in hexosamine biosynthetic pathway which is described in the following paragraph.

3.1. Hexosamine Biosynthetic Pathway (HBP)

The hexosamine biosynthetic pathway integrates the metabolism of carbohydrates (glucose), amino acids (Glutamine), fat (acetyl-CoA) and nucleotides (uridine and ATP) in the synthesis of UDP-GlcNAc (Figure 12). This suggests that HBP may function as a broad-range nutrient-sensing pathway to regulate different mechanisms [189, 190]. HBP leads to the synthesis of UDP-GlcNAc which is used as substrate to transfer O-GlcNAc to several nuclear and cytoplasmic proteins.

For the synthesis of UDP-GlcNAc, glucose serves as one of the major carbon source. Glucose flux potentially varies in different cells. However 3-5% of the glucose entering into the cell leads to hexosamine biosynthetic pathway. On entering into cells, glucose is phosphorylated through hexokinase to produce glucose 6-phosphate. Further, it either participates in glycogen synthesis or enters into glycolytic pathway after its conversion into fructose 6-phosphate by phosphoglucose isomerase. These two steps are shared between HBP and glycolysis but at this point HBP diverges from glycolysis and fructose 6-phosphate is converted into glucosamine-6-phosphate by a rate limiting enzyme GFAT (L-glutamine:D-fructose-6-phosphate amidotransferase). Glutamine is another major metabolite which funneled into the HBP at this point. Furthermore, Glucosamine-6-phosphate is metabolized by acetyltransferase (Emeg32) to N-acetylglucosamine-6-phosphate by using acetyl-CoA [191, 192]. N-acetylglucosamine-6-phosphate is rapidly metabolized to give the end product uridine diphospho-N-acetylglucosamine (UDP-GlcNAc). Monosaccharide glucosamine can also leads to the elevation of UDP-GlcNAc. It enters into HBP downstream of rate limiting step catalyzed by the enzyme GFAT.

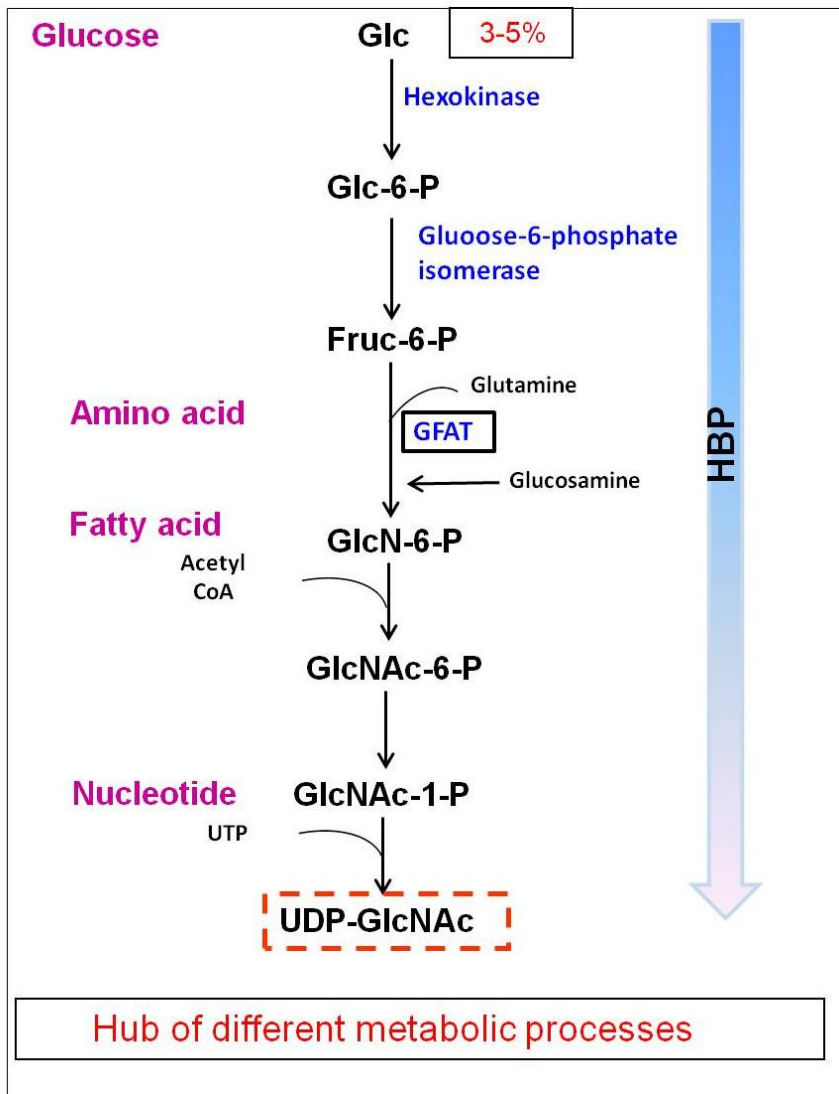


Figure 12: Hexosamine biosynthetic pathway (HBP)

HBP serves as a hub of different metabolic pathways and leads to the production of UDP-GlcNAc. Nutrients or metabolites of different metabolic processes can enter into HBP. Glucose metabolism gives glucose-6-phosphate, which is metabolized to glucose-6-phosphate. GFAT (the rate limiting enzyme) converts glucose-6-phosphate into glucosamine-6-phosphate. Amino acid metabolism provides HBP with glutamine which enters at the rate limiting

step. Glucosamine-6-phosphate is converted into GlcNAc-6-phosphate and fatty acid metabolism provides acetyl CoA. GlcNAc-6-phosphate is converted into GlcNAc-1-phosphate which can be metabolized to give the end product UDP-GlcNAc. In the final step, UTP (from nucleotide metabolism) can enter into HBP. (This figure is adapted from Hart et al., 2011 Annu Rev Biochem: 825-58)

GFAT is regulated through feedback inhibition by the enzymatic product glucosamine 6-phosphate and the final product UDP-GlcNAc [193]. GFAT can also be regulated at transcriptional and posttranslational levels. In addition, the inhibition of glutaminase (the enzyme which first converts glutamine into glutamate and then into α -ketoglutarate in the

mitochondria) also results in the reduction of GFAT activity leading to decrease in O-GlcNAcylation level of different proteins including OGT [194, 195].

Formation of UDP-GlcNAc is an end point of the hexosamine biosynthetic pathway [196]. UDP-GlcNAc is the sugar donor for O-GlcNAcylation. It serves as a substrate for the O-GlcNAc transferase (OGT) and transduces signal to the proteins by addition of O-GlcNAc moiety. Hexosamine biosynthetic pathway (HBP) is a quantitatively minor branch of glucose metabolism, however it has been shown to play a role in insulin resistance and diabetes. Moreover, recent studies suggested the role of HBP in the development of cancer. Glucose metabolism may influence development and growth of cancer through HBP and other interlinked pathways. The potential role of HBP and O-GlcNAcylation in diabetes and cancer is discussed in 3.3.

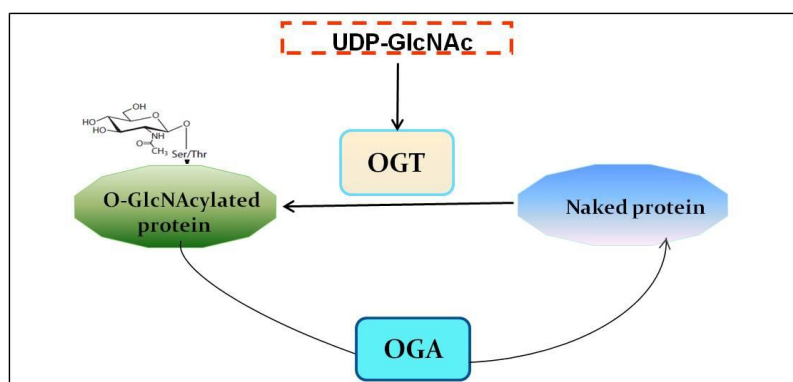


Figure 13: O-GlcNAcylation

UDP-GlcNAc is used as substrate by O-GlcNAc transferase (OGT) to modify proteins with O-GlcNAc. This process is antagonized by another enzyme O-GlcNAcase (OGA). O-GlcNAc modified proteins are involved in regulation of different cellular mechanisms

3.2. General Characterization of O-GlcNAcylation

O-GlcNAcylation is present in all metazoans including *C.elegans*, insects as well as in plants. Moreover, it has been documented in fungi, bacteria and in some eukaryotic infecting viruses (Olszewski NE 2010). But surprisingly O-GlcNAcylation has not yet been detected in yeast.

Most of the O-GlcNAcylated proteins reside in the nucleus including nucleopore proteins, chromatin proteins and transcription factors (Sp1, Ap-1, NFkB, c-myc, ER- β) [187, 188, 197-200]. However, cytosolic, mitochondrial and membrane associated proteins are also modified by O-GlcNAc and these include kinases such as Akt, CaMIV, etc... [201-203], enzymes involved in glycolytic pathways [204], cytoskeleton proteins and their regulatory polypeptides [205, 206]. Two enzymes involved in reversible process of O-GlcNAcylation have different cellular localization. OGT is mainly nuclear and OGlcNAcase (OGA) is predominantly cytosolic but both can also be found in any other intracellular compartment [207, 208]. However, OGT is excluded from nucleolus while OGA resides in it [209]. Mechanisms regulating subcellular localization of these enzymes are not well known. OGT has a nuclear targeting sequence in its catalytic domain [210] and a sequence in the C- terminal domain of OGA may play a role in its sub-cellular trafficking [208, 211].

O-GlcNAcylation is similar to phosphorylation as it is an inducible and reversible process. The finding of O-GlcNAcylation involvement in different cellular processes is demonstrating that O-GlcNAcylation is as abundant as phosphorylation. However, only one couple of enzymes (OGT and OGA) is involved for the addition and removal of O-GlcNAc, unlike phosphorylation which involves 650 different enzymes (kinases and phosphatases) for removal and addition of phosphate on different proteins. The detailed description of these two enzymes is given in the following paragraphs.

3.2.1. OGT

In 1990, G.W. Hart's team discovered an enzyme responsible for the addition of O-linked N-acetyl glucosamine (O-GlcNAc) to proteins, called O-Linked N-Acetylglucosamine

Transferase (OGT) [212]. They used a synthetic peptide YSDSPSPST as a substrate for the enzyme assay. This peptide was made by using sequences of different known O-GlcNAcylated proteins. Apparently OGT does not need a strict consensus sequence but the presence of single proline in proximity of serine/threonine seemed to be necessary. Moreover, characterization of the product showed that GlcNAc is covalently attached to the peptide via O-linkage. This study demonstrated that the enzyme is similar to other previously characterized GlcNAc transferases as it uses UDP-GlcNAc as a sugar donor and is strongly inhibited by free UDP. But in contrast to other GlcNAc transferases, OGT does not need divalent cations for its activity and is mainly localized in the cytosolic compartment of the cell, although it is present throughout intracellular compartments [212, 213].

Two years later the same group purified a uridine diphospho-N-acetyl glucosamine (OGT) from rat cytosol. The apparent molecular weight of this enzyme based on gel filtration was approximately 340,000 and SDS-PAGE revealed that it consists of two subunits, at 110 and 78 kDa. They observed an approximately 2:1 ratio between these two subunits respectively. This suggests that the enzyme is present as a heterotrimer with two 110-kDa subunits and one 78-kDa subunit. The catalytic activity is found only in the larger subunit (110 kDa) [214].

As this ratio between two subunits remained fairly constant during different steps of preparation irrespective of the time consumed in the process, it is unlikely that the small fragment corresponds to the protease fragment of the larger subunit [214]. The catalytic subunit of OGT (p110) was cloned in 1997 by G W Hart team. Its characterization showed that it is a highly conserved gene through evolution. Moreover, it is consistently associated with O-GlcNAc modifications in terms of function. The catalytic activity of OGT is highly sensitive to the

intracellular concentration of UDP-GlcNAc ranging from nano molar to more than 100 milli molar [215].

OGT structure

OGT is present as a single copy gene encoding multiple splice variants in *Caenorhabditis elegans*, *Drosophila melanogaster*, mice and humans. In contrast, in higher plants two OGT-related genes are identifiable. OGT gene is located on the X chromosome in region D in mice, in the region syntenic with human Xq13 [216]. An extracellular form of glycosyltransferase EOGT has been documented recently but it has no sequence homology with OGT [217]. Structurally OGT is comprised of three functional domains.

- (i) An N-terminal tetratricopeptide region (TPR). Splice variants differ in this region. Several studies have shown that the number of TPRs domains in OGT influenced its substrate recognition [218]. This fact suggests that different splice variants may interact with different proteins which can affect their subcellular localization and their functions.
- (ii) A second region is comprised of a linker domain. The linker region of OGT contains a nuclear localization sequence [207].
- (iii) The third part of OGT consists of a catalytic C-terminus. The catalytic domain of OGT has homology with other glycosyl-transferases [219, 220]. Moreover, this region has been indicated to play a role in catalysis as the deletions in C-terminal of OGT lead to dramatic reductions in its enzymatic activity [218].

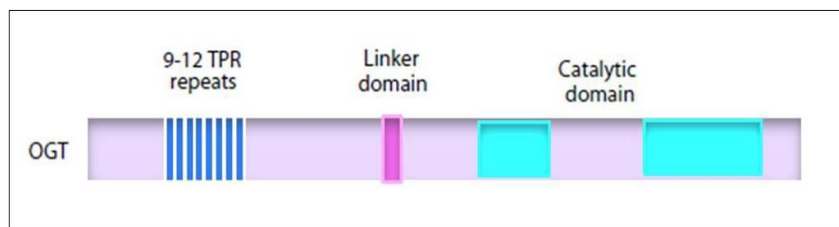


Figure 14: Structure of OGT

OGT consist of three domains: an N-terminal domain consisting of 9-

12 TPR repeats, a linker domain and a catalytic domain. (This figure is adapted from Love et al., 2005, Sci. STKE re13).

As mentioned previously, OGT gene gives rise to different alternatively spliced isoforms. All isoforms contain multiple tandem tetratricopeptide repeats. Presence of these TPRs suggests that OGT may interact with other proteins through these tandem repeats. Isoforms of OGT differ in the length of their amino-terminal tetratricopeptide repeats (TPRs) and in their intracellular localization [210, 215, 218, 221], as shown in figure 15.

The longest isoform, termed nucleocytoplasmic OGT (ncOGT), and the shortest isoform (sOGT) are present in the nucleus and the cytoplasm, whereas the intermediate isoform is targeted to the mitochondria (mitochondrial OGT, mOGT). Mitochondrial isoform has a specific sequence on the N-terminal which targets it to the mitochondria [207, 222]. Mitochondrial OGT has 9 TPRs while nucleocytoplasmic OGT has 12 TPRs and the shortest isoform (sOGT) has only 3 TPRs. Earlier studies showed that O-GlcNAc modification is either absent in mitochondria or it is present at low level, although mOGT was found to be catalytically active [188, 197]. However, a recent study by John A Hanover's team found that overexpression of the mitochondrial isoform has a different impact on cell than the longest nucleocytoplasmic isoform of OGT (ncOGT) [223]. Moreover, studies demonstrated that mitochondria O-GlcNAcylation plays an important role in physiology and pathology of cardiovascular and heart system [224-226].

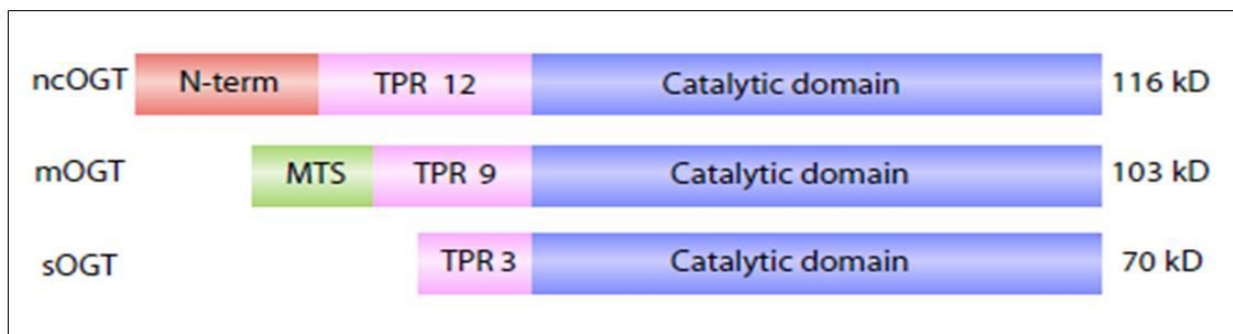


Figure 15: Isoforms of OGT

OGT has three isoforms that differ in their N-terminal: ncOGT (nucleocytoplasmic isoform of OGT), the longest isoform (116 kDa), containing 12 TPRs. mOGT (mitochondrial isoform of OGT), it contains 9 TPRs and sOGT (shortest form of OGT) which has only 3 TPRs. (This figure is adapted from Love et al., 2005, Sci. STKE re13).

OGT gene has been found in several organisms including bacteria, plants and mammals [190, 221, 227]. OGT is highly conserved in evolution, with 99% identity between rat and human OGT, and 61% identity between Rat and *Caenorhabditis elegans* OGT. Levels of OGT mRNA, protein and activity vary in different tissues. However, OGT is expressed in all tissues studied; it appears to be particularly rich in macrophages, T-cells and B-cells, pancreas, thymus and the central nervous system.

OGT shows auto-GlcNAcylation in the catalytic domain and in the ninth TPR. It is also modified through serine and tyrosine phosphorylation. However, the effect of these modifications on OGT activity is not yet clear. OGT can regulate different cellular processes and plays an important role as a nutrient sensor. Moreover, it plays a role in diabetes, insulin resistance and cancer. OGT has been shown to be indispensable for the viability of mice embryonic stem cells [216]. Moreover, OGT has been shown to play an important role in growth responses. The

inhibition of OGT results in deregulation of growth responses, arrest of cell cycle and in decreased expression of different transcriptional factors (factors c-Jun, c-Fos, and c-Myc [213]. In addition, tissue targeted inhibition of OGT has been shown to lead severe motor defects through apoptosis of neuronal cells and it also causes T-cells apoptosis [228].

3.2.2. OGA

β -*N*-acetylglucosaminidase (*O*-GlcNAcase) is a neutral hexosaminidase which catalyses the removal of *O*-GlcNAc from target substrates and is found in both cytoplasm and nucleus. Similar to the OGT, OGA is highly expressed in immune cells and in the central nervous system. It is also expressed in all other tissues including endocrine tissues. Its catalytic site is similar to the family of glycoside hydrolases that specifically catalyzes the removal of β -linked GlcNAc on its substrates. However, it exhibits substantial differences from lysosomal hexosaminidases. OGA has neutral pH optimum whereas lysosomal hexosaminidases have acidic pH optima. Moreover, it selectively cleaves *O*-GlcNAc but not *O*-GalNAc while lysosomal hexosaminidases cleave both *O*-linked *N*-acetylgalactosamine (*O*-GalNAc) and *O*-GlcNAc.

OGA is present in higher metazoan animals, flies, worms and coding sequences related to OGA are also detected in bacteria. However it has not been detected in higher plants. OGA does not share significant homology with any other proteins but has putative acetyl transferase domain at the C-terminus. The C-terminal domain of mouse *O*-GlcNAcase has been suggested to possess acetyltransferase activity *in vitro*, however this could not be confirmed *in vivo* [229-231].

OGA is highly conserved in higher eukaryotic species, especially in mammals. In mammals, it is present as single gene copy (mapped to chromosome 10q24.1-q24.3), and is

annotated as meningioma expressed antigen 5 (*MGEA5*). It is alternatively spliced to give two isoforms which differ in their C-terminals. The longer of these variants (130 kDa) has a histone acetyltransferase (HAT) domain at its C-terminus, while shorter variant (75 kDa) has a much smaller C-terminal domain. The catalytic domain is restricted to the N-terminal domain but *in vitro* studies suggested that the C-terminal domain of OGA is important for full enzymatic activity.

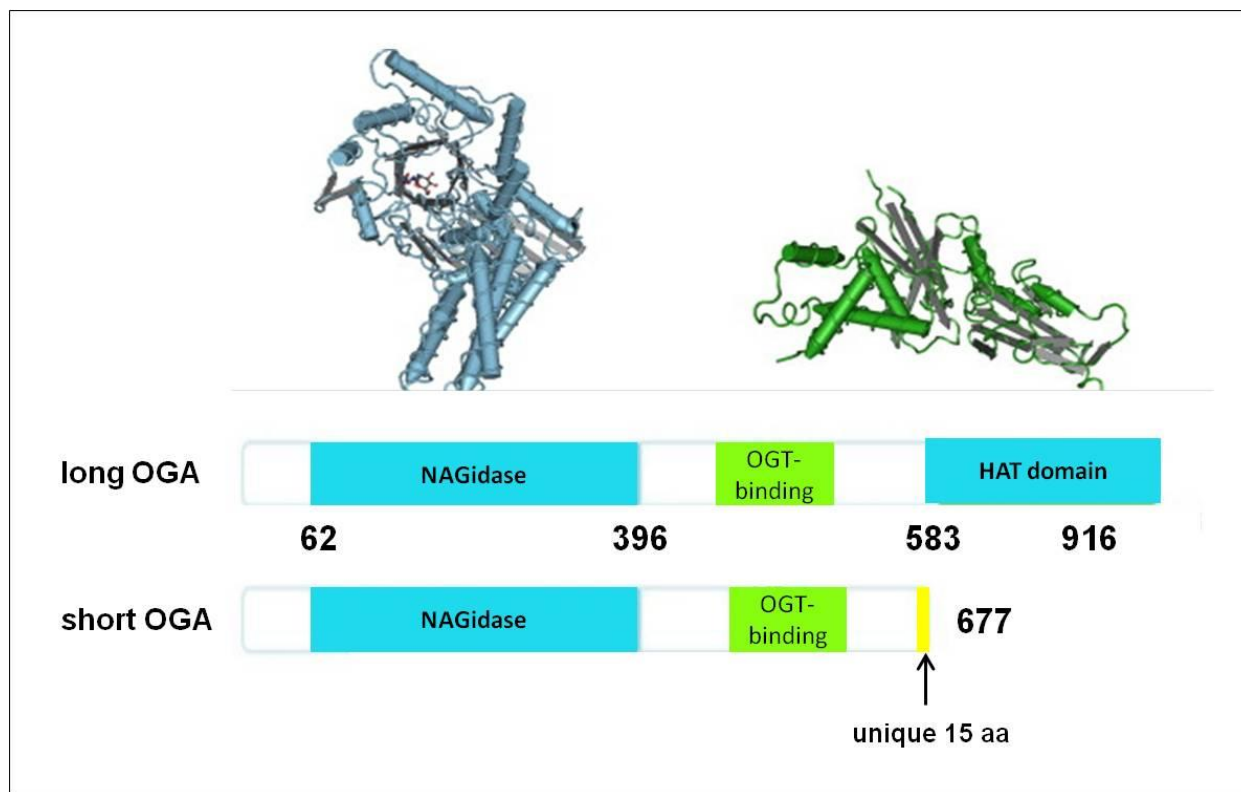


Figure 16: Structure of OGA

OGA is present in two isoforms: the long isoform of OGA is comprised of 916 amino acids and has three major domains i) the N-terminal domain, ii) the middle domain which contains OGT binding domain and C-terminal domain which is also called HAT domain. The short isoform of OGA is comprised of 677 amino acids. It is truncated at C-terminal and lacks HAT domain and has additional 15 amino acids sequence at the end. This figure is adapted from Hanover et al., 2009.

Crystal structure data of two OGA homologues suggest that the human OGA conforms to a TIM-barrel structure with highly conserved catalytic residues. The longer isoforms (130-kDa) is present in both nucleus and cytoplasm but it localizes predominantly in the cytoplasm. The C-terminal domain of the full length isoform exhibits features similar to histone acetyl transferases (HATs). It binds to histone tails and modifies them. The shorter isoform (75-kDa) resides in the nucleus and is associated with lipid droplets. This suggests that it may have a role in lipid droplet assembly and mobilization [232]. MGEA5 gene has been linked to type II diabetes in a Mexican population [233].

OGA is cleaved by caspases-3 both *in vitro* and *in vivo* but this cleavage does not affect its O-GlcNAcase activity [231, 234]. However, it occurs in disordered region that links the N-terminal and C-terminal domains. This region is more susceptible to proteolysis and plays a role in recognition of different substrates [235]. It suggests that O-GlcNAcase may act on different sets of substrates upon its cleavage by caspases-3. O-GlcNAcase has also been shown to be regulated through posttranslational modifications like serine phosphorylation and O-GlcNAcylation [236, 237]. However, the effect of these modifications is still not known. OGT and OGA are present in same complexes and they also interact with each other, suggesting that they may affect each other's activity. A central domain of human OGA has been shown to interact with OGT [238]. One *in vitro* study showed that O-GlcNAcase activity is inhibited when it complexed with OGT [239]. However, the functional role of this interaction still remains to be explored.

Several pharmacological inhibitors of OGA have been shown to inhibit its activity *in vitro* and *in vivo*. They are being preferably used to inhibit O-GlcNAcylation compared to genetic inhibitors. These inhibitors include PUGNAc, GlcNAcstatin, NbutGT, thiamet-G,

GlcNAc-thiazoline, and its derivatives [240-242]. Recent inhibitors (GlcNAc-statin and GlcNAc-thiazoline) are more specific towards O-GlcNAc-ase compared to PUGNAc [243-245]. STZ is a common drug used to induce diabetic states in mice and rats. Streptozotocin (STZ) can also inhibit O-GlcNAc-ase at very high concentrations but it is toxic to cells [246].

3.3. Functional characteristics of O-GlcNAcylation

Glycosylation is one of the most abundant posttranslational modifications (PTMs) in eukaryotic cells, as it is estimated that 1–3% of the human genome is dedicated to encoding enzymes that build and break down complex carbohydrates [247]. O-GlcNAcylation is one of the most abundant eukaryotic glycosyltransferase reactions and occurs in both nucleus and the cytoplasm [248]. To date, more than 2,000 proteins have been identified as targets for *O*-GlcNAc addition. These targets reflect all aspects of cellular function including transcription regulation, nutrient homeostasis, stress response, cell survival, apoptosis, cell cycle and DNA repair. It can regulate localization, ubiquitination and stability of proteins. Disruption of O-GlcNAc in mammalian cells affects cell growth and viability. However, OGT and OGA inhibition has not been shown to affect viability of *C.elegans*. Deregulation of O-GlcNAcylated proteins is involved in several diseases like insulin resistance, other diabetes complications, neurodegeneration, tumorigenesis and cancer. O-GlcNAcylation can also interact with other PTMs to regulate different cellular processes. It exhibits extensive crosstalk with phosphorylation and regulates different cellular processes, such as cell signaling, transcription and cytoskeleton arrangements.

3.3.1. Interplay between phosphorylation and O-GlcNAcylation

O-GlcNAcylation is similar to protein phosphorylation in terms of stoichiometry, localization and cycling. These two PTMs extensively cross interact and influence each other in different

cellular processes either by modifying enzymes of other process or by competing for the occupancy of the same sites on polypeptides. The interplay between phosphorylation and O-GlcNAcylation can occur through different mechanisms which are depicted in the following figure.

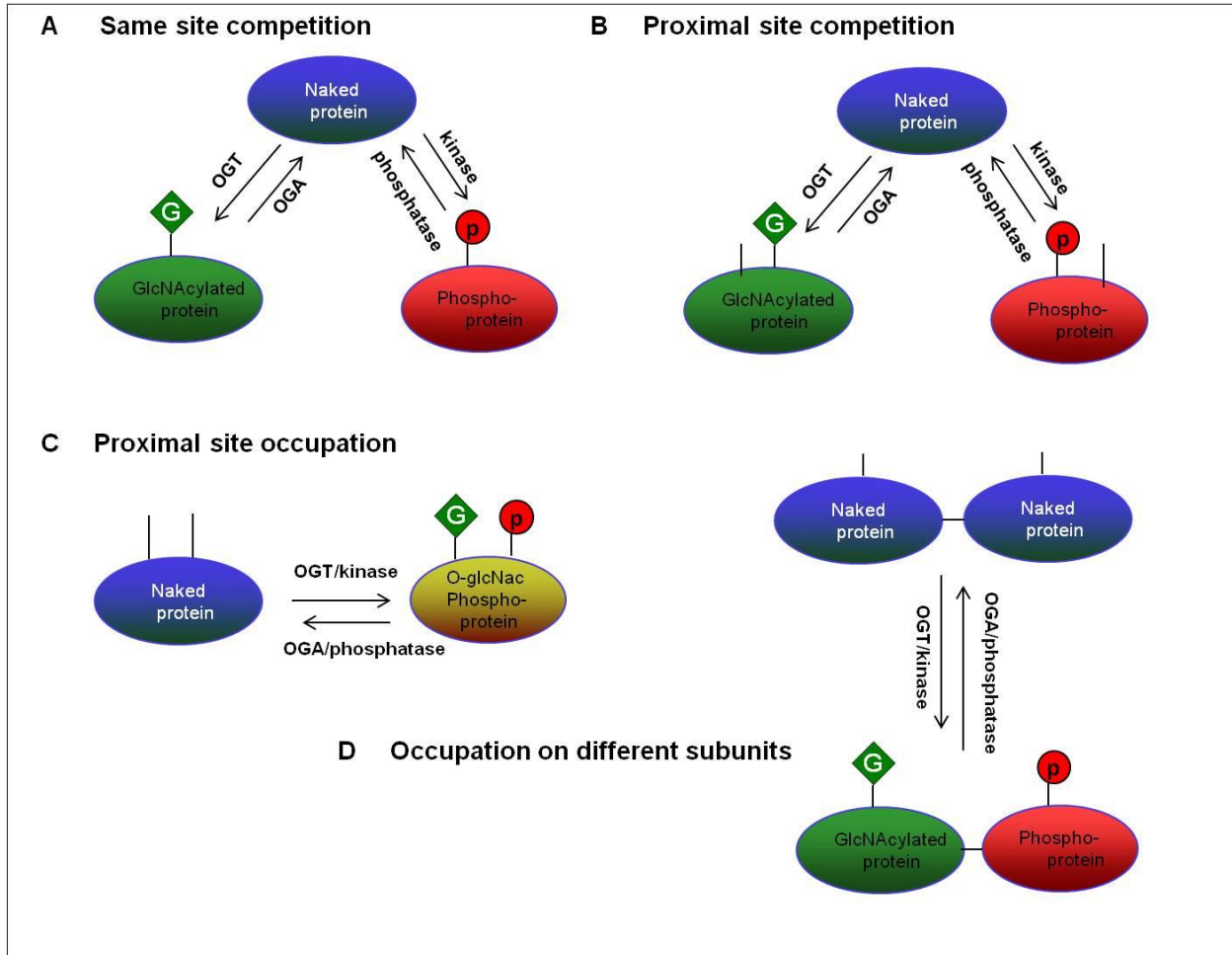


Figure 17: Interplay between O-GlcNAcylation and phosphorylation

O-GlcNAcylation and phosphorylation exhibit different mechanisms of interplay. (A) They can compete for the occupation of same site on protein. (B) They can show competition for proximal sites occupation. (C) They can occupy proximal sites and can co-exist. (D) O-GlcNAc or phosphate can occupy different subunits of protein. This figure is adapted from Butkinaree et al., 2010. *Biochim Biophys Acta*: 96-106.

- 1- In the first type of interplay the same site is reciprocally modified by either O-GlcNAc or phosphate as a response to different physiological conditions. These two modifications are mutually exclusive i.e. only one of these can exist at one time. This competition is known as “Yin-Yang” hypothesis where *O*-GlcNAc moieties could directly oppose *O*-phosphate on serine and threonine residues. Such sites are present on the c-Myc oncogene protein where threonine 58 is reciprocally modified by phosphorylation or by *O*-GlcNAcylation [249, 250]. Serum starvation results in increase of *O*-GlcNAcylation and reciprocal decrease of phosphorylation on Thr-58 while serum stimulation has the opposite effect. Moreover, inhibition of the kinase responsible for Thr-58 phosphorylation (glycogen synthase kinase 3) results in increase of *O*-GlcNAcylation on Thr-58 [251]. Similarly reciprocal occupancy of Ser-16 occurs in the transactivation domain of estrogen receptor β and regulates stability of the protein [252]. The COOH-terminal domain (CTD) of RNA polymerase II is also modified either through phosphorylation or *O*-GlcNAcylation [253].
- 2- In the second type of competitive mechanism, *O*-GlcNAc and phosphate occupy proximal sites instead of the same residue and modulate other modification. This competition may result from the large size of an *O*-GlcNAc residue, by the negative charge of phosphate group or by the induction of conformational changes in the protein. For example, a dynamic interplay exists between phosphorylation and *O*-GlcNAcylation for two sites on p53 and thus regulates its stability. *O*-GlcNAc modified Ser-149 results in decrease phosphorylation of Thr-155 and consequently increases stability of p53 [254].

Similarly, CaMKIV (Calcium/calmodulin-dependent protein kinase type IV) and vimentin also exhibit the same kind of competition between O-GlcNAc and phosphate.

- 3- In the third type of mechanism, both modifications can occur simultaneously at different sites. The insulin receptor substrate (IRS)-1 is phosphorylated at Thr-1045 while O-GlcNAc modification is present on Ser-1036 residue [255]. Similarly, the cardiac myosin light chains are phosphorylated and O-GlcNAcylated at different sites and GlcNAc exposure decreases cardiac myofilament sensitivity to calcium but had no effects on phosphorylation [256].
- 4- In the fourth type of mechanism, proteins are phosphorylated and O-GlcNAcylated at distant sites and even on different subpopulation of molecules. For example phosphorylation and O-GlcNAcylation are segregated on different molecules of cytokeratins and both modifications increase simultaneously [257, 258].

In addition to their crosstalk at the level of site occupancy, O-GlcNAcylation and phosphorylation dynamically modify the enzymes that catalyze these modifications on polypeptides. Several kinases are found to be O-GlcNAcylated. For example, Akt, a serine/threonine kinase that regulates different cellular functions including gene transcription and cell survival is modified by O-GlcNAc. O-GlcNAc modified Akt1 has reduced activity that can be correlated with decrease in its phosphorylation [202, 259, 260]. A cyclic regulation exists between CaMKIV (an important kinase in neurons) and OGT. OGT modifies CaMKIV and O-GlcNAc modified CaMKIV remains inactive. De-O-GlcNAcylation and subsequent phosphorylation of CaMKIV leads to its activation. The activated CaMKIV phosphorylates OGT and activates it [201, 261].

OGT is both tyrosine and serine phosphorylated while OGA is serine phosphorylated [236, 262]. OGT is phosphorylated by insulin receptor, CaMKIV and other kinases [263]. The tyrosine phosphorylation of OGT activates it and may play role in its interaction with other substrates or targeting subunits. In addition to reciprocal modification, OGT, OGA, kinases and phosphatases co-exist in different protein complexes [264]. It suggests that different proteins may have cyclic regulation by both kinds of modifications. Moreover, the balanced O-GlcNAc cycling plays an important role in the regulation of phosphorylation level in the cell. The loss of OGT or OGA results in altered serine/threonine phosphorylation profile and increased in GSK-3 protein and phosphorylation [265]. Abnormal crosstalk between O-GlcNAcylation and phosphorylation is involved in different diseases like diabetes, neurodegenerative disease, cancer and most recently in AIDS. For instance, increased O-GlcNAcylation of IRS1 alters its patterns of phosphorylation, resulting in decrease of insulin signaling. Increased O-GlcNAcylation on IRS1 potentially causes a reduction in its interaction with p85 and attenuation of the activating tyrosine phosphorylation at Y608 of IRS1. O-GlcNAcylation of IRS1 reduces Y608 phosphorylation, and concomitantly promotes IRS1 Ser632 and Ser635 phosphorylation [285]. Other components of insulin signaling pathway are modified with O-GlcNAc including IRS2, PI3-K, PDK1 (*in vitro*). Moreover, Akt is O-GlcNAcylated after insulin stimulation in 3T3-L1 adipocytes. O-GlcNAcylation of AKT lowers phosphorylation on Thr308 and alters downstream signaling [499]. The cross interaction of O-GlcNAcylation with other PTMs is largely unknown.

3.3.2. Regulation of transcription

The regulation of transcription in response to nutrients or stress is a major function of O-GlcNAcylation. Histone proteins are abundantly modified by O-GlcNAc. Moreover, RNA polymerase II and other basal transcriptional machinery proteins are also modified through this

posttranslational modification. Classically O-GlcNAcylation is known as transcriptional silencer as it is mainly present in transcriptionally inactive areas in drosophila and in mammalian cells and OGT is found as a polycomb gene (these genes are master regulators for repressing the expression of subsets of genes during development). However, studies depict that the role of O-GlcNAcylation is more complex than to merely silence transcription. Several O-GlcNAcylated proteins have been identified which are involved in transcription including housekeeping transcription factor Sp1. These include c-Myc, c-Jun, c-Fos, CREB binding protein (CBP) and foxO 1 [237, 266-268].

O-GlcNAcylation regulates gene expression by altering the transcriptional activity of different transcription factors through modulation of their localization, activity or binding partners. For example, localization and transcriptional activity of β -catenin (an intracellular signal transducer in the Wnt signaling pathway that can also act as an oncogene) are regulated through O-GlcNAcylation. Elevation of O-GlcNAcylated β -catenin results in its cytoplasmic localization and consequently leads to decrease of its transcriptional activity [269]. Similarly, localization of the master transcriptional factor Sp1 is also regulated through O-GlcNAcylation. Increase in O-GlcNAcylated Sp1 results in its localization into the nucleus which can lead to an increase or decrease of its transcriptional activity [270, 271]. However, decrease in O-GlcNAcylated Sp1 results its sequestration in the cytoplasm, leading to decrease in its transcriptional activity and increase in its degradation [272].

3.3.3. Role in translation

Several studies have depicted the involvement of O-GlcNAcylation in regulation of translation. Moreover, both OGT and OGA have been found to be associated with ribosomes. Ribosomal proteins are modified through O-GlcNAc, such as ribosomal protein S6. Stress

granules and processing bodies are ribonucleoproteins that play a role in the regulation of translation and mRNA turnover [273]. Studies demonstrated that stress granules and processing bodies are regulated by O-GlcNAc cycling. Most of the stress granules components are directly modified by O-GlcNAc. OGA predominately resides in the nucleolus, the major site of ribosomes synthesis. Moreover, overexpression of OGT leads to the accumulation of 60S subunits and 80S. This suggests that O-GlcNAc plays important roles in the regulation of translation and in ribosome biogenesis. However, more studies are needed to elucidate the functional details of O-GlcNAc modification and its role in the process of translation.

3.3.4. Stability of proteins

O-GlcNAcylation is known to be involved in the regulation of protein degradation and stability. Most of the O-GlcNAc modified proteins have PEST region (Pro-Glu-Ser-Thr) which indicate rapid degradation by ubiquitination. O-GlcNAc modification at this site generally protects proteins against proteasomal degradation. For instance, increase in O-GlcNAcylation in MCF-7 cells results O-GlcNAc modified protein p53 which is more stable [274]. A detailed description of the role of O-GlcNAc in stability of p53 is given in 3.5.3. Similarly, GlcNAcylated forms of murine β -estrogen receptor are more resistant to proteasome degradation than non-GlcNAcylated forms [252]. Moreover, O-GlcNAcylation can also regulate proteasome complex directly. For instance, O-GlcNAcylation of the Rpt2 ATPase, a component of the 19S cap of the proteasome, inhibits its ATPase activity and lowers the proteolytic activity of the proteasome on the transcription factor Sp1 [275]. Moreover, increased O-GlcNAc level results in enhanced ubiquitination and inversely decrease in O-GlcNAc level leads to decreased ubiquitination. This suggests that O-GlcNAcylation and ubiquitination are not strictly antagonistic. Interestingly, E1 family (the enzyme that initiates ubiquitination process), can be

GlcNAcylated and its GlcNAcylation status determines its complex formation with heat shock protein (Hsp70). This suggests that the ratio between these two modifications might play a role in proteolysis through E1 [276].

3.3.5. Proteins localization

O-GlcNAcylation can also regulate proteins activity by regulating their localization. For example, the transcription factor β -catenin which plays an important role in cell adhesion and gene expression is O-GlcNAcylated. Increase in its O-GlcNAcylation state results in decrease of nuclear localization and transcriptional activity. Moreover, O-GlcNAcylation blocks its interaction with E-cadherin (a cell adhesion molecule) [269]. Similarly, PUGNAC treatment of SH-SY5Y cells induces nuclear accumulation of Akt 1 [202]. O-GlcNAcylation appears to regulate the localization of the transcription factor Sp1. Insulin stimulation increased O-GlcNAcylation of Sp1, resulting in its nuclear accumulation and thereby increases Sp1-dependent gene expression [270].

3.3.6. Role in cellular stress response

Several studies have shown a protective role of O-GlcNAcylation against cellular stress and cell death. O-GlcNAc or OGT levels are increased in response to different types of cellular stresses, such as heat shock, exposure to UV light, hypoxia, etc... Moreover, reducing O-GlcNAc level leads to increased sensitivity towards cellular stress [277]. Upon heat shock treatment, O-GlcNAc level is rapidly increased on several proteins. This increase in O-GlcNAcylation appears to be involved in the upregulation of chaperones and heat shock proteins such as heat shock protein 70 (Hsp 70) by increasing their expression and stability. In addition, studies showed that some Hsp70 family proteins have specific lectin activity toward O-GlcNAc, and the activity is

increased after stress [277-279]. Similarly, major components of stress granules are also modified by O-GlcNAc in response to cellular stress. These components include glyceraldehyde-3-phosphate dehydrogenase, receptor for activated C kinase 1 (RACK1) and prohibitin-2. Moreover, several ribosomal proteins are O-GlcNAcylated as a response to cellular stress, suggesting that O-GlcNAc is required for stress granule assembly [273].

The role of O-GlcNAcylation in cellular stress is also supported by different animal models. Increased O-GlcNAc level protects cardiomyocytes after ischemia or reperfusion. Similarly, infarct size of damaged heart can also be reduced by O-GlcNAc elevation [225, 280]. Furthermore, glucosamine treatment can improve cardiac functions in a rat model of trauma-hemorrhage during resuscitation [281] and reduces circulating inflammatory cytokines.

3.4. Role of O-GlcNAcylation in chronic diseases

Since O-GlcNAcylation is a nutrient sensor that exhibits extensive cross talk with phosphorylation, its involvement in diseases is not unexpected. Studies have depicted fundamental roles of O-GlcNAcylation in several chronic diseases and particularly in diabetes, neurodegeneration, cardiovascular disease, and cancer. Moreover, key regulatory proteins of eukaryotic infectious viruses have also been found to be O-GlcNAcylated.

3.4.1. Role of O-GlcNAcylation in diabetes

Several studies have shown a well established role of O-GlcNAcylation in diabetes and glucotoxicity. The link between hexosamine biosynthetic pathway and glucose-induced insulin resistance was first established by a study in adipocytes [196]. This link was further supported by studies revealing the role of glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate limiting enzyme in hexosamine biosynthetic pathway, in the development of insulin resistance.

Studies have shown that glucose and insulin regulate GFAT activity in skeletal muscles. Moreover, overexpression of GFAT leads to defects in glucose transport and to insulin resistance [282, 283]. The end product of the hexosamine biosynthetic pathway (UDP-GlcNAc), serves as a substrate for O-GlcNAcylation and elevated nucleocytoplasmic O-GlcNAcylation results in insulin resistance [284]. However, other studies showed that the inhibition of O-GlcNAcylation does not affect insulin resistance in 3T3 L1 adipocytes. Despite some controversial studies [285, 286], the hexosamine biosynthetic pathway and O-GlcNAcylation have been shown to play an important role in insulin resistance. For instance, hepatic overexpression of OGT impairs the expression of insulin-responsive genes and causes insulin resistance in C57BL/6J mice [287]. In addition, this study has identified the molecular mechanism by which nutritional cues regulate insulin signaling through O-GlcNAc. Insulin activates PI3-K pathway, and PIP3 produced by activated PI3K recruits OGT from the nucleus to the plasma membrane [287]. This leads to O-GlcNAcylation of components of insulin signaling cascade, resulting in modulation of phosphorylation of key components of the pathway and in attenuation in insulin signaling transduction. Another study showed that upon recruitment of OGT to the plasma membrane, insulin receptor phosphorylates OGT through tyrosine phosphorylation resulting in increased activity of OGT [263]. These facts suggest that deleterious effects of hyperglycemia may occur through elevation of O-GlcNAcylation. O-GlcNAcylation can regulate the activity and stability of several transcription factors such as Sp1, foxO1, NFκB, etc... Reduced O-GlcNAcylation of Sp1 increases its activity and susceptibility to proteasomal degradation. Increased Sp1 activity has been shown to play a role in different aspects of hyperglycemia and insulin resistance [200]. Similarly, the activity of NFκB is also associated with its O-GlcNAcylation state under hyperglycemic conditions. Activation of NFκB has been shown to play a role in inflammatory

stress response in diabetic nephropathy. Abnormal elevation of O-GlcNAcylation can cause adverse effects on cell proliferation and metabolism.

3.4.2. Role of O-GlcNAcylation in cancer

A role of O-GlcNAcylation in cancer development is plausible as increased uptake of glucose and enhanced utilization of glutamine are considered as hallmarks of cancer. Indeed 3-5% of the glucose entering into the cell leads to HBP which serves as a substrate for OGT. Similarly, glutamine enters into the HBP at the rate limiting step and is necessary for the production of UDP-GlcNAc. Moreover, O-GlcNAcylation is known to play role in diabetes and diabetes has also been linked to cancer etiology. Therefore, O-GlcNAcylation may also play a role in the development of cancer.

O-GlcNAcylation may also influence cancer development through O-GlcNAc modification of different oncogenes and tumor suppressors such as c-Myc [249, 250, 288], retinoblastoma (Rb) [289], β -catenin [269], estrogen receptor [252, 290, 291] and p53 [274]. Moreover O-GlcNAcylation exhibit cross talk with signaling pathways which plays important roles in cancer etiology. For instance, oncogene c-Myc is reciprocally O-GlcNAcylated and phosphorylated on the same site (Thr-58). This site is mutated in a variety of lymphomas and leads to a more stable Myc. Thr-58 lies within the transactivation domain, and its phosphorylation leads to rapid degradation, resulting in the loss of Myc target gene expression [292]. However, before GSK3 β can phosphorylate Thr58, Ser62 needs to be phosphorylated. Phosphorylation of Ser62 is carried out by a variety of kinases and promotes Myc stability [293]. The Myc oncoprotein promotes both proliferation and apoptosis and mutated Myc plays a role in deregulation of apoptosis. In quiescent cells, Thr-58 is modified through O-GlcNAc. However,

when cells are stimulated to grow, the same site is phosphorylated [251]. Mutations at Thr-58 disrupt the ability of Myc to promote gene expression of the pro-apoptotic protein BIM, by this way it uncouples proliferation from apoptosis [294, 295]. However, it is unclear whether Thr58 *O*-GlcNAcylation or Ser62 phosphorylation alters the status of the other site *in vivo*. Neither has it been clear whether the oncogenic effect of the Thr58 mutation is due to the loss of phosphorylation or *O*-GlcNAcylation or both, as shown in figure 18.

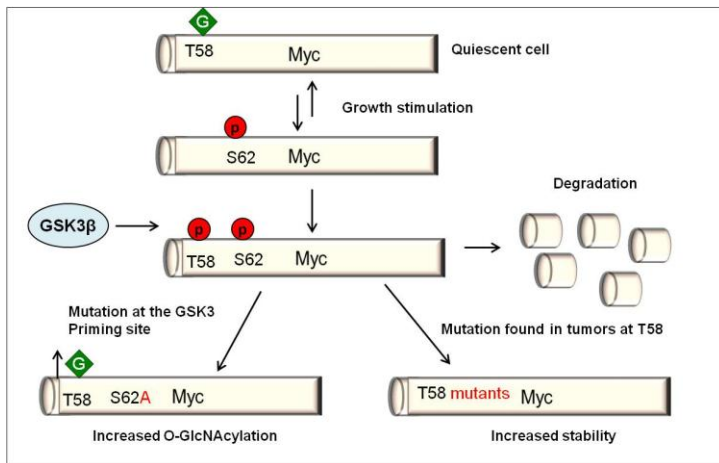


Figure 18: Regulation of activity and stability of Myc through interplay between O-GlcNAcylation and Phosphorylation

Phosphorylation and O-GlcNAcylation exhibit interplay on threonine-58 (T58) residue of Myc and regulate its stability. Phosphorylation

of Myc on serine 62 (S62) makes it stable while Myc phosphorylation on both T58 and S62 leads to its degradation. Occupation of T58 by O-GlcNAc inhibits degradation of Myc. Mutation at T58 results in increase of Myc stability. (This figure is adapted from Slawson et al., *Nature Reviews Cancer* **11**, 678-684)

Similarly, interplay between phosphorylation and O-GlcNAcylation play an important role in stability and activity of tumor suppressor p53. More than 50% of all cancers contain mutations in *TP53* gene locus [296]. In normal conditions, the level of p53 remains low and is tightly regulated by proteolytic degradation [297]. However, in case of environmental stress, DNA damage or cellular stress, p53 expression and stability are increased leading to cell cycle arrest, apoptosis or autophagy [298]. The ubiquitin ligase MDM2 negatively regulates p53 expression by promoting its degradation [299]. Moreover, the stability of p53 is also affected by

phosphorylation on different sites. The amino terminal domain of p53 contains a transactivation domain and has several phosphorylation sites. Phosphorylation at different sites has different impacts on the stability of p53. Phosphorylation at Ser18 and Ser23 promotes p53 stability and tumor suppression, while phosphorylation of p53 at Thr155 promotes p53 degradation [254, 300]. O-GlcNAcylation of p53 at Ser149 is antagonistic to Thr155 phosphorylation, resulting in increase in stability [274]. However, the mechanism by which O-GlcNAcylation blocks phosphorylation is not yet clear.

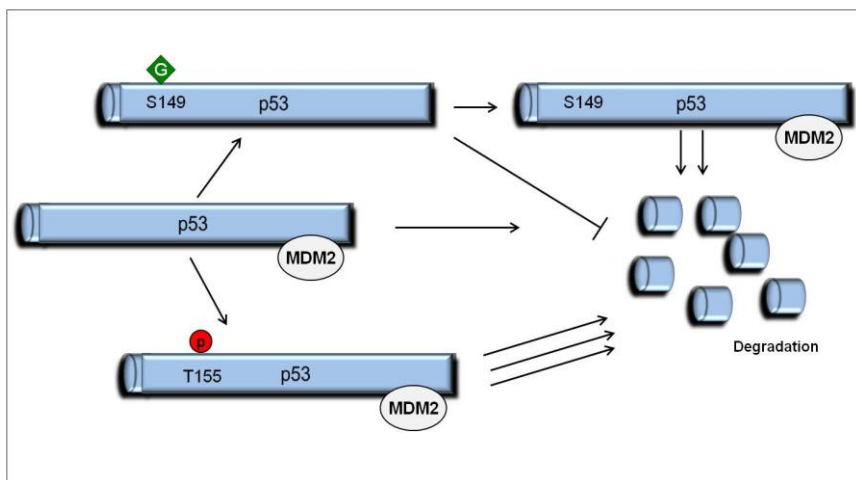


Figure 19: Activity and stability of p53 is regulated through interplay between O-GlcNAcylation and Phosphorylation.

Phosphorylation and O-

GlcNAcylation exhibit interplay on serine-149 (S149) and threonine-155 (T155) residues of p53 and regulate its stability. Naked and phosphorylated forms (T155) of p53 are vulnerable to degradation through MDM2. The occupation of S149 by O-GlcNAc inhibits p53 degradation. (This figure is adapted from Slawson et al., *Nature Reviews Cancer* **11**, 678-684)

O-GlcNAcylation can also affect cancer metastasis by regulating cell adhesion molecules. E-cadherin is the best characterized adherens junction proteins. O-GlcNAc modification leads to the loss of E-cadherin transport to the cell surface of epithelial cells. This results in reduced intracellular adhesion that is a critical mechanisms underlying metastasis of cancer cells [301]. In epithelial cells, the cytoplasmic tail of E-cadherin forms a dynamic complex with catenin and regulates several intracellular signal transduction pathways. O-

GlcNAcylation of β -catenin blocks its association with E-cadherin and affects its signaling. In addition, O-GlcNAcylation can also play a role in regulation of E-cadherin by modifying the transcription repressor SNAIL1. SNAIL1 negatively regulates E-cadherin gene expression. In normal conditions, SNAIL1 is phosphorylated at Ser112 which promotes its degradation and alleviates its repression on E-cadherin. However, SNAIL1 can be O-GlcNAcylated at the same site (Ser112) and elevations in O-GlcNAc levels blocks phosphorylation of SNAIL1, inhibits its degradation and repress E-cadherin [302]. In this way, increased O-GlcNAcylation may results in enhanced metastasis.

O-GlcNAc levels in cancer

Recent studies have shown the presence of increased level of O-GlcNAcylation in breast cancer cells compared to normal epithelial cells. Reduced O-GlcNAcylation has been shown to inhibit tumor growth both *in vitro* and *in vivo*. Higher O-GlcNAcylation is present in metastatic lymph nodes compared to primary tumor tissues and reduced O-GlcNAcylation results in decrease of tumor invasiveness [303]. Another study indicated the presence of enhanced mRNA expression of OGT and lower expression of OGA in breast ductal carcinomas. Moreover, this study demonstrated that the expression of OGT is relatively higher in poorly differentiated tumors (grade II and III) than grade I tumors. In contrast, MGEA5 transcript levels are found to be significantly lower in grade II and III in comparison with grade I tumors [304]. Collectively, these studies suggest that increase in O-GlcNAc level might be implicated in breast cancer progression and invasiveness. OGT is also overexpressed in prostate cancer compared to normal prostate epithelium. Moreover, depletion of OGT in prostate cancer cells leads to decreased invasion and angiogenesis. Moreover, this study showed that the bone metastasis is blocked by reducing expression of OGT in a mouse model of metastasis [305]. These studies suggest that

OGT might represent a therapeutic target for breast cancer and prostate cancer. O-GlcNAcylation is also increased in chronic lymphocytic leukemic lymphocytes [306] and is correlated with aggressiveness of the disease. Patients with the highest levels of O-GlcNAcylation have low grade lymphoma and present a better prognosis, whereas those patients whose lymphocytes have lower levels of O-GlcNAcylation have a more aggressive form of the disease and a poor prognosis. This suggests that O-GlcNAcylation and cancer etiology are intricately linked and O-GlcNAcylation may regulate cancer by complex mechanisms. Similarly, enhanced O-GlcNAc level as well as increased OGT expression has been detected in other cancer cells like lung and colon cancer compared to the adjacent tissues. Moreover, an *in vitro* study showed the involvement of O-GlcNAcylation in anchorage-independent growth of lung and colon cancer [307]. Increased O-GlcNAcylation has also been observed in hepatocarcinoma cells compared to the healthy liver tissue. Additionally, *in vitro* studies demonstrated the involvement of O-GlcNAcylation in migration, invasion and viability of hepatocarcinoma cells [308]. Collectively, these studies strongly suggest the involvement of O-GlcNAcylation in different mechanisms of cancer development and progression. My research work was to study the role of O-GlcNAcylation in breast cancer derived MCF-7 cells. Therefore, a detailed description of breast cancer is necessary to comprehend my thesis work.

Chapter 4

Breast Cancer

Breast cancer is the most common cancer diagnosed in women in Europe and in USA. It is the second leading cause of cancer death. It can also rarely occur in males. More than 1,300,000 cases are being reported each year worldwide. According to estimate of The National Cancer Institute, about 227 thousand women were to be diagnosed with, and about 39 thousand were estimated to die of breast cancer in 2012. Breast cancer is one of the oldest known forms of cancerous tumors in humans. Breast cancer mainly starts in epithelial lining of ducts, called ductal carcinoma. However, it can also start in other parts such as lobular cancers. The lymphatic system is involved in spreading breast cancer to other parts of the body. Mammary ductal carcinoma has two forms, one is the ductal carcinoma *in situ* (DCIS) and the other is called invasive ductal carcinoma (IDC). The ductal carcinoma *in situ* is a non-invasive neoplasm confined to the milk duct where it initially developed. In contrast, invasive ductal carcinoma has a tendency to spread (metastasize) to other tissues of the breast and/or other regions of the body. However, there are some other less common types of breast cancer such as inflammatory breast cancer, characterized by general inflammation of the breast. Other rare types of breast cancer are medullary carcinoma (an invasive breast cancer that forms a distinct boundary between tumor tissue and normal tissue), mucinous carcinoma (formed by the mucus-producing cancer cells) and tubular carcinoma.

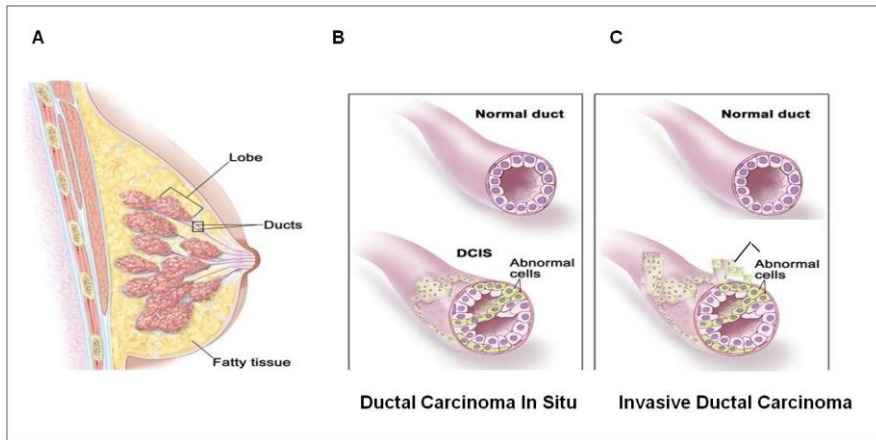


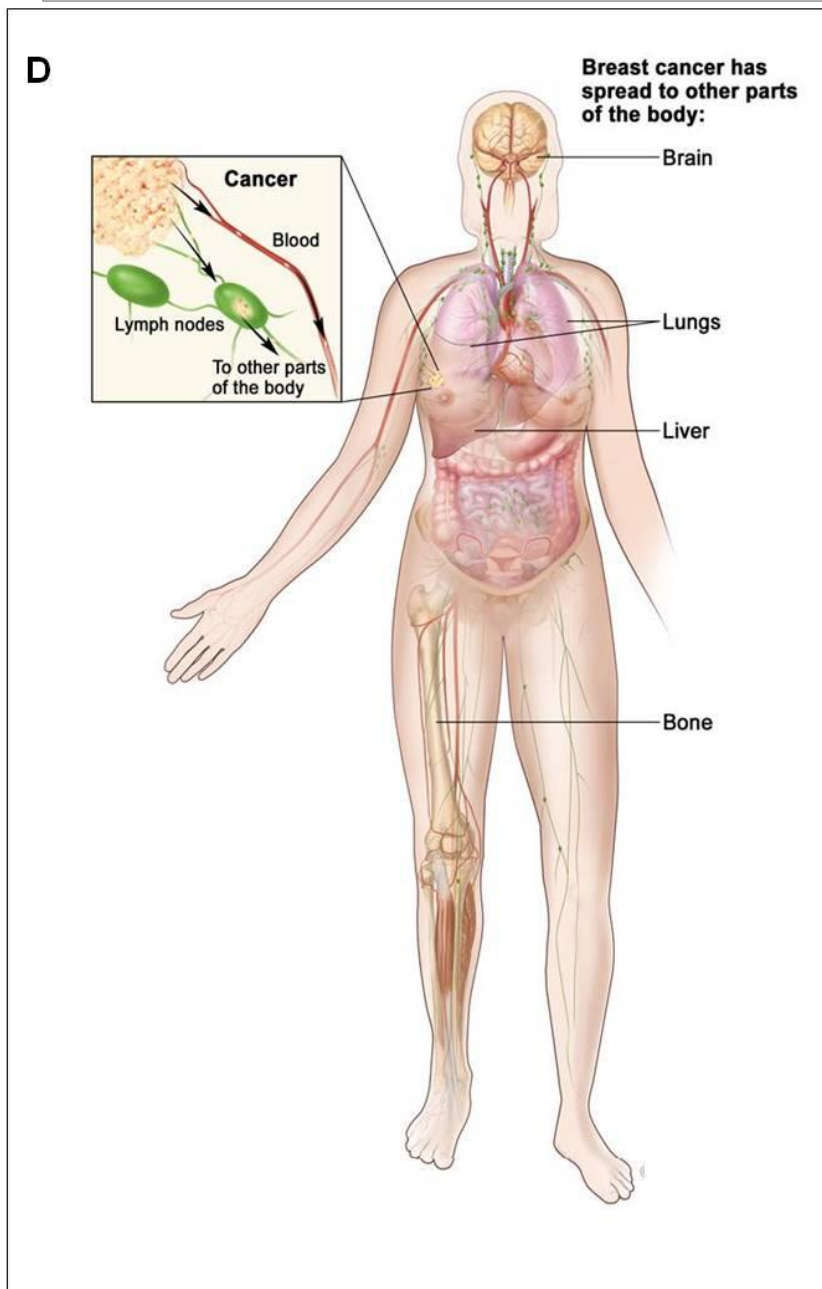
Figure 20: Breast Cancer

Different forms of breast cancer. Breast cancer normally starts in epithelial lining of ducts called ductal carcinoma.

(A) Different parts of human

breast. (B) The non-invasive ductal carcinoma confined to its origin called ductal carcinoma *in situ* (DCIS).

(C) The invasive ductal carcinoma (IDC) which spread to other tissues of the breast. (D) Breast cancer metastasizes to other regions of the body. This figure is adapted from cancer research organization. (www.cancer.gov)



4.1. Structure of the human breast

To better understand breast cancer and its invasiveness, it is important to comprehend different parts of the breast and development of mammary glands. Mammary glands (milk producing glands) are the characteristic of all female mammals. However, they are also present in males but in rudimentary and non-functional form. Mammary glands are regulated by the endocrine system and become functional in response to the hormonal changes associated with puberty. It is a complex structure and is mainly made up of small individual lobes (15-20), called lobules. Each lobule consists of a central epithelial duct (the tiny tube carrying milk from lobules to the nipples) with many tributary ducts branching from it [309]. The stroma is another part of the breast that is made up of fatty and connective tissues surrounding ducts and lobules, blood vessels and lymphatic vessels. The development of mammary glands is a complex and long process. It begins in early embryogenesis and continues throughout life. In contrast to other glands, complete development of mammary glands occurs only during the pregnancy and lactation period and not during fetal development.

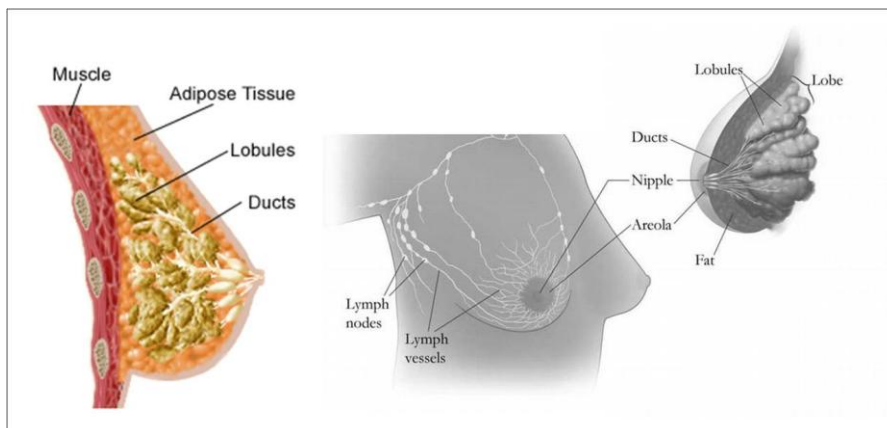


Figure 21: Structure of mammary gland

Human mammary glands are comprised of small individual lobes (lobules) which are surrounded by connective tissues and adipose tissues.

Tubular drains/ ducts called areola originate from each lobule and converge near the nipple. A network of lymph nodes is present around the breast and lymph nodes are inter-connected through tiny tubes called lymph vessels. This figure is adapted from cancer research organization. (www.cancer.gov).

4.2. Stages of development

As mentioned before, the development of human mammary glands begins during embryogenesis. The ductal branches are formed during early embryogenesis forming an epithelial ductal system. However, this system remains rudimentary and does not change throughout the childhood until puberty time. At the time of birth, human breast is indistinguishable in males and females. During puberty, the female breast undergoes different developmental stages whereas the male breast remains quiescent. During this stage, the epithelial ductal branches undergo development and are further divided to form branching ductal bundles that are lined with differentiated luminal and myoepithelial cells. These structures have terminal end buds (TEB) that are made up of multiple layers of luminal body cells and a layer of undifferentiated, multipotent basal cap cells [310, 311]. High proliferation in these ductal end buds results in their elongation. These TEB undergo lengthening, thickening, branching and differentiation which result in the formation of lobules and alveoli (milk producing terminal ducts). These structures undergo several cycles of proliferation and apoptosis. However, no further development takes place before the onset of pregnancy or lactation period. The major portion of mammary growth occurs during pregnancy. The growth process is slow at the beginning of the pregnancy, but it accelerates as the pregnancy advances. Before the onset of pregnancy, mammary glands contain large fatty pads. However, during pregnancy period, they are gradually replaced by ducts, alveoli, blood vessels, and connective tissues. The growth of

mammary gland continues during early lactation and it starts decreasing after the peak of the lactation period.

The development of mammary glands can be divided into three phases: embryonic, adolescent and adult phase, each of which is differentially regulated. Several hormonal changes play important roles in adolescent and adult phases whereas the embryonic phase is hormone independent. The detailed description of different hormones and their role is given below.

4.3. Role of hormones in mammary glands development

Ovarian hormones play major roles in the development of mammary glands, however others types of hormones are also involved, such as pituitary hormone and growth factors [312]. In females, follicle-stimulating hormones (FSH) and lutenizing hormone (LH) are released in a cyclic pattern from the anterior pituitary gland. These hormones stimulate the ovaries to synthesize and release female sex steroid hormones, estrogens (estradiol) and progestins (progesterone). These hormones play important roles in the development of mammary glands as well as in tumorigenesis.

4.3.1. Estrogen

Estrogens are a group of steroid hormones synthesized in ovarian cells that are converted from a testosterone intermediate to a more potent form, 17-beta oestradiol (E2) by aromatase [313]. In women with active menstrual cycles, the ovaries generate E2 in a non-linear periodic rthm, which is then converted to its metabolites: estrone (E1) and estriol (E3), they have weaker estrogenic activity [314, 315]. Estrogen has been shown to play a vital role in the development of normal breast tissue. Women lacking the ability to produce estrogen fail to develop mammary

glands beyond the rudimentary ductal tree [316, 317]. Although estrogens are generated locally, they circulate systemically and are essential for a variety of physiological processes in men and women. Some of these processes include the development and maintenance of secondary sexual organs. The intact functional ovary is indispensable for the breast development and in case of ovaries absence, estradiol-replacement therapy is necessary to induce breast development. Moreover, estrogens exert a variety of biological effects in cardiovascular, musculoskeletal, immune and central nervous systems [313, 314, 318, 319].

Steroid hormones such as estradiol and progesterone are lipophilic and they enter cells and their nuclei primarily by diffusing through plasma and nuclear membranes. Inside the nucleus they interact with their cognate receptors with high affinity and specificity. Estrogens carry out their biological function through estrogen receptors (ERs) [320].

4.3.2. Estrogen Receptors

Estrogen Receptors (ERs) are members of the nuclear receptor superfamily that bind steroids and other ligands, functioning as a ligand-inducible transcription factors [321]. Estrogens mediate their effects in target tissues through two members of the nuclear receptor superfamily, estrogen receptor- α (ER α) and estrogen receptor- β (ER β) [315]. ER α was discovered in late 1950 while ER β was discovered more recently [322]. These two subtypes of ERs are transcribed by two individual genes (ESR1 and ESR2), present on different chromosomes (ESR1 is present on 6q while ESR2 is present on 14q) [323-325]. ER α and ER β vary in their sizes (595 and 530 amino acids, respectively). However, they have high overall sequence homology in the ligand binding domain (53%) and the DNA binding domain (96%) [326].

Structural and Functional Domains of ERs

Similar to the other members of nuclear receptor superfamily, both ER subtypes have a six region structure and contain defined functional domains that have considerable homology [327].

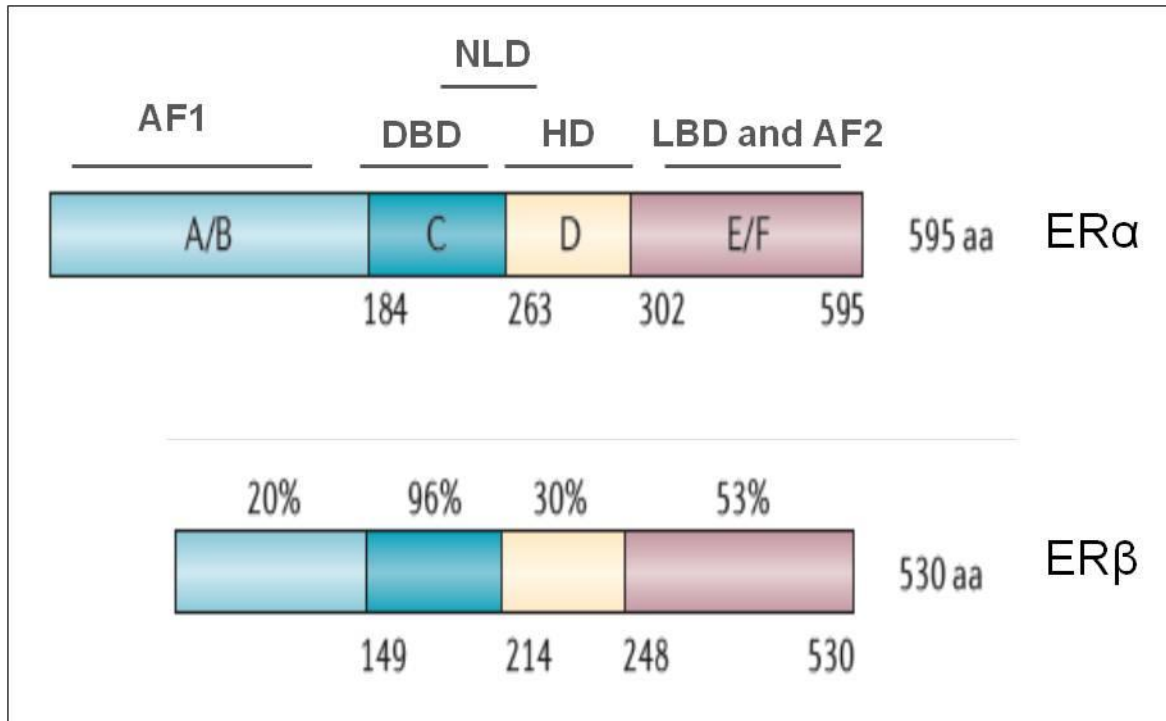


Figure 22: Structural and Functional domains of ERs

ERs contain six structural domains A-F which form different functional domains. Functional domains are: activating functional domain (AF), the DNA binding domain (DBD), the ligand binding domain (LBD), the hinge region and nuclear localization domain (NLD). Different domains of ER α and ER β exhibit homology with 96 % homology in DNA binding domain and 53 % homology in ligand binding domain.

Figure 22 shows that there are six (A-F) structural domains of ER α and ER β . Two domains exhibit high sequence homology in two subtypes of ERs: the DNA binding domain C that has 96% homology and E/F domains which exhibits 53 % homology. However, other domains are not well conserved between ER α and ER β [327-329]. ERs have defined functional domains; the

relative position of the functional domains is presented by solid bars in figure 22. The functional domains are: (i) the activating functional domain (AF-1) domain that is present at amino terminal within region of A and B domains. It contains ligand-independent activation function and a co-regulatory domain which binds various ER coactivators and corepressors that modulate its transcriptional activity [330-333]. (ii) The DNA binding domain (C-domain) that contains two zinc finger motifs. The DNA binding domain is responsible for binding to specific estrogen response elements (EREs) within the promoters of estrogen-responsive genes [330, 334, 335]. (iii) The DNA dimerization domain is splitted between C and E-domains, it plays a role in ER dimerization [330]. iv) The D-domain contains the hinge region, part of nuclear localization signal and the ligand-dependent transactivation domain AF-2a [336, 337]. v) The E-domain, contains the ligand-binding site, a second dimerization domain and ligand-dependent AF-2 transactivation domain [336]. vi) The F-domain, appears to optimize the transcriptional activity of the receptor [321, 338].

Mechanism of action of ERs

ERs are activated upon binding of their ligands and transmit signals to their downstream effectors via classical genomic action, non-classical genomic action or non-genomic action (Figure 23 A, B, C and figure 24). Moreover, ligand independent activation has also been reported. Hyperactive growth factor receptors, such as epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 receptor (IGFIR), can stimulate protein kinase cascades that phosphorylate and activate the ERs in the absence of ligand [339] (Figure 23).

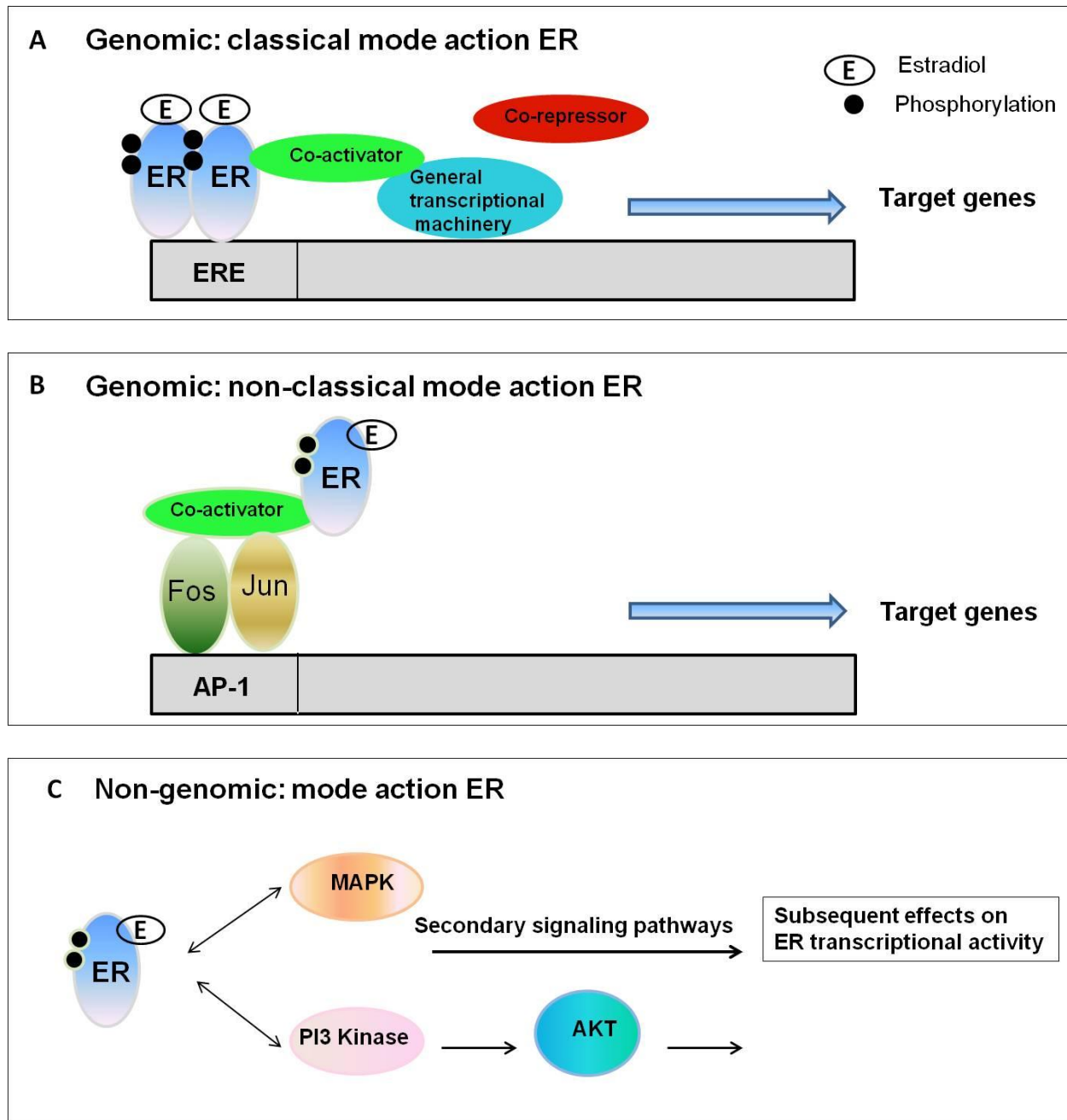


Figure 23: Genomic (classical, non-classical) and non-genomic mode action ER

(A) Classical genomic mode action ER, where it regulates genes expression by directly interacting with ERE elements of target genes. (B) Non-classical genomic mode action ER, where it regulates genes expression indirectly by interacting with other transcription factors. (C) Non-genomic mode action ER, where it interacts with different signaling cascades. This figure is adapted from Ring and Dowsett et al., 2004 *Endocrine-Related Cancer* (2004) 11 643–658.

Genomic action of ER

In the classical genomic action of ER, ligand binds to the ligand binding domain of the receptor leading to ligand specific conformational changes in the receptor. The ligand-bound receptors dimerize and bind to DNA through their zinc finger-containing DNA-binding domains at EREs. The consensus ERE is a 13-base pair inverted repeat sequence (GGTCAnnnTGACC), where “n” represents a random nucleotide, many EREs have variations from the consensus. Once bound to EREs the receptor recruit co-activators or co-repressors.

Different classes of ER coactivators have been identified. One class includes members of the steroid receptor complex (SRC)/p160 family: SRC-1, transcriptional intermediate factor 2 (TIF2/SRC-2) and Amplified In Breast cancer 1 (AIB1/SRC3) [314]. The p160 coactivators interact with and recruit other co-regulatory proteins including E1A-binding protein 300/cAMP response element binding protein (EP300 or p300/CBP), p300/CBP-associated factor (PCAF), histone acetyltransferases (HATs) and co-activator-associated arginine methyltransferase-1 (CARM-1), which either bind directly to ER or associate with other coactivators [340-343]. The other class of coactivators is represented by thyroid receptor-associated proteins (TRAP)/vitamin D receptor-interacting proteins (DRIP), a complex initially identified in separate biochemical screens for proteins recruited by thyroid hormone receptor [344] and vitamin D receptor, respectively [345-347].

ER undergoes conformational changes unique to the presence of either agonists or antagonists which in the case of antagonists like tamoxifen and raloxifene, allows ER to associate with corepressors [348, 349]. Some of these include nuclear receptor corepressor 1 (NCoR1) and silencing mediator for retinoid and thyroid hormone receptor (SMRT or NCoR2) and Repressor of ER α Activity (REA) which bind to ER α and inhibit its transcriptional activity [348, 349]. In

other mechanisms of action, corepressors interfere with mRNA processing of ER target genes, inhibit ER dimerization and ER DNA binding.

Increased expression of SRC1 and SRC3, which induce cell proliferation and migration, was found in breast and ovarian cancers [350, 351] and reduced expression of nuclear receptor co-repressor (NCOR) and silencing mediator of retinoid and thyroid receptor (SMRT; also known as NCOR2) has been correlated with tamoxifen resistance in breast cancer [327, 352]. In non-classical genomic action ERs regulate gene expression by interacting and activating other transcription factors such as Fos/ Jun, Ap1, Sp1, NFkB, and CREB, p53, etc...[353-355]

Non-genomic action of ER

In addition to the genomic action, estrogens initiate non-genomic effects via interaction of E2-bound ER with cell signaling molecules that activate the mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3-kinase/Akt (PI3-K-Akt) pathways [314, 356, 357]. The non-genomic action begins outside the nucleus and is independent of gene transcription. Some reports have shown that E2 activates ERs in the plasma membrane and modulates the interaction between ERs and membrane-associated proteins. It activates the MAPK and PI3-K-Akt pathways and modulates cell processes including proliferation and apoptosis [358-360]. Both genomic and non-genomic effects often converge at certain regulatory sites of the adjacent ER-responsive genes [361].

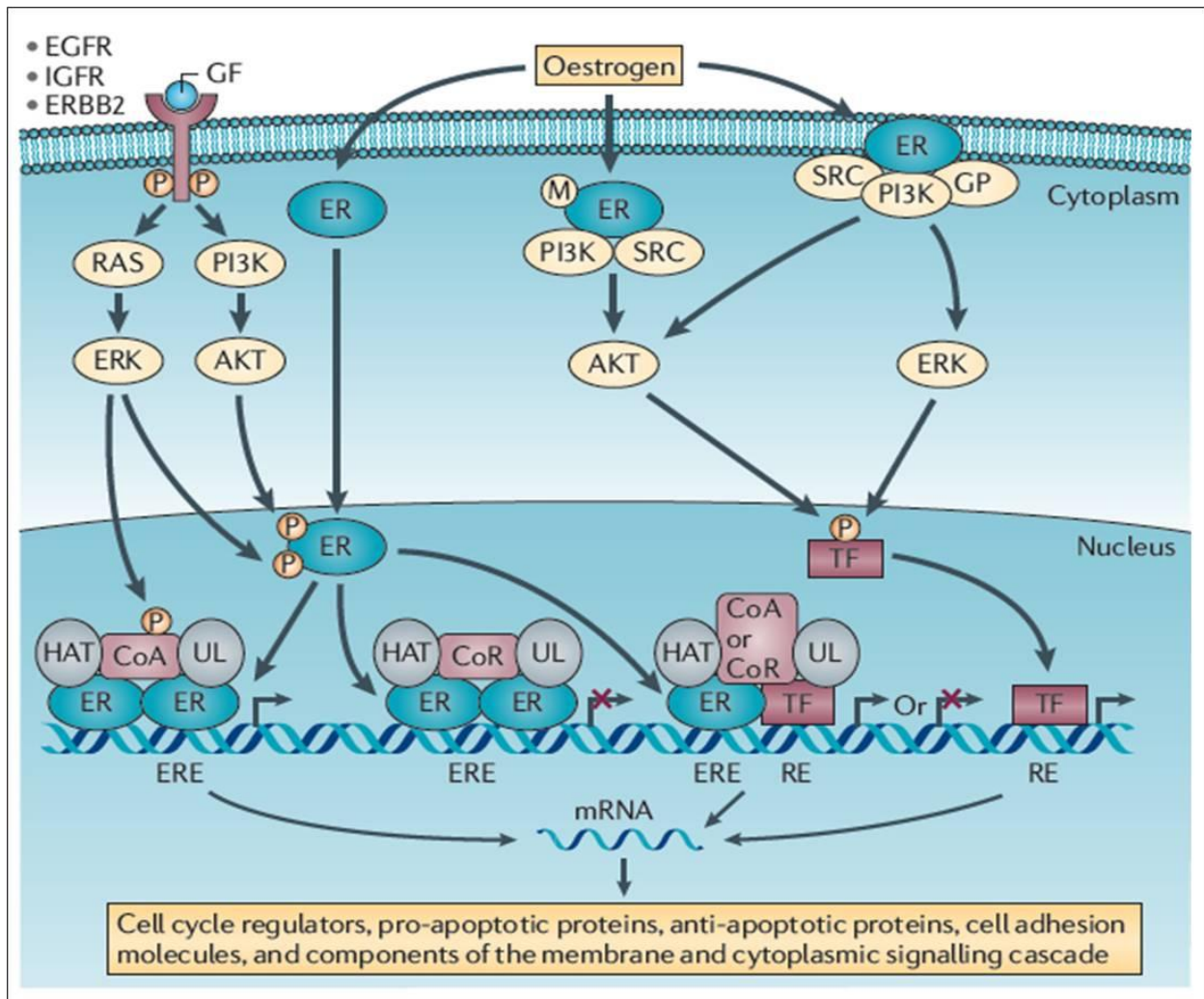


Figure 24: The cellular kinase pathways interacting with ER

This figure is adapted from Thomas et al., 2011. Nature reviews.

Different roles of ERs subtypes

ERs (ER α and ER β) have been shown to exert opposite effects on different cellular processes such as cell proliferation, apoptosis and cell migration [362]. Consequently, they have different influence on the development and progression of cancer. Microarray studies in mouse tissues and in breast cells showed that ER α and ER β exhibit different transcriptional regulation [362]. They can differently regulate the expression of same genes. They show different binding affinity to

different agonists and antagonists. They also exhibit differences at the level of coactivators and corepressors recruitments and in activation of signaling cascade as a response to binding of same ligand. However in predicting response to endocrine therapy, nuclear wild-type ER β has been reported with better overall outcome. However, tamoxifen recruits corepressors when bound to ER α but not to ER β [363, 364]. This might be because of the poor conserved sequence of the activating function 1 (AF 1) domain which recruits co-regulatory proteins. It has only 20% amino acids homology between ER α and ER β . The ratio of ER α /ER β can also vary in cancers of different tissue origins such as breast cancer, prostate cancer and endometrial cancer [365, 366]. Clinical studies have demonstrated that ERs expression also varies depending upon the tumor types and cancer stage [366-369] [370]. ER α is essential for the normal development of mammary glands while the absence of ER β expression does not impairs the normal development of mammary glands in mice model [321, 371, 372].

The expression of ER β has been shown to repress the transcriptional activity of ER α in ER α cells. Moreover, ER β and ER α differently regulate the nuclear transcription factor Myc. Similarly, they oppose each other in the regulation of CCND1 (which encodes cyclin D1). Cyclin D1 is overexpressed in various cancers and can affect the response of these cancers to systemic therapy.

In addition to these two subtypes of ERs, each subtype has several isoforms generated by alternative splicing and promoter usage such as ER α -36, ER β 2 and ER β 5. ER α -36 is the most reported truncated form of ER α which lacks AF 2 domain. It is localized at the plasma membrane or in the cytoplasm. It has been reported to reduce responsiveness to tamoxifen sensitivity in breast cancer [373]. It has also been shown to promote tamoxifen agonist action in endometrial cancer cells through MAPK-ERK and PI3K-Akt mediated upregulation of Myc. The

transcriptional regulation of expression of ER α is regulated by seven promoters [374]. ERs transcriptional regulation involves several transcriptional factors which, in the case of ER α , include GATA-binding factor 3 (GATA3), forkhead box protein O3A (FOXO3A), forkhead box protein M1 (FOXM1) and ER α , which can regulate its own expression [375-378].

4.3.3. Progesterone and progesterone receptors

Although estrogen has been shown to play more important roles in the development of mammary glands, studies suggested that progesterone may also play a role in the development of mammary glands. For instance proliferation rate of breast epithelial cells is higher during the luteal phase of the menstrual cycle when the level of progesterone is at peak compared to the follicular phase when estrogen level is highest [379]. Progesterone signaling is mediated by progesterone receptor (PR). They are members of the steroid/thyroid hormone nuclear receptor superfamily, and they function as ligand-dependent nuclear transcription factors. Upon ligand binding, receptor dimerization occurs and these dimers interact with DNA response elements and regulate gene transcription [380]. There are two isoforms of the progesterone receptors PRA and PRB, encoded by the same gene and they only differ in their N-terminal. PRA is shorter than PRB with truncation of 164 amino acids at N-terminal. Two isoforms are thought to be functionally different and PRB has been shown to be the most transcriptionally active form, although PRA may also regulate transcription [381, 382]. Moreover, it is the dominant repressor of PRB and has also been implicated in repression of ER α [383, 384]. Progesterone receptor is present in 10-15 % of epithelial lining of the breast. The binding of progesterone to progesterone receptor cause a conformational change, leading to the dimerization of two ligand receptor complexes [385-388]. This results in increase phosphorylation of the receptor and leads to the binding of

receptor dimers to the hormone response elements present in the promoter region of target genes [320, 389, 390]. Progesterone has been shown to play a role in the development of mammary glands. The critical significance of progesterone in mammary gland development is demonstrated by progesterone receptor knockout mouse. Progesterone receptor knockout mice exhibited full ductal elongation but showed impaired side branching. Moreover, a significant decrease was observed in the terminal ductal lobular unit (TDLU) development [391-393]. This suggests that progesterone might not be indispensable for ductal development but plays an important role for the functional development of the mammary gland. Progesterone has been suggested to play roles in proliferation and differentiation of mammary glands through regulation of genes involved in cell cycle, such as cyclin D1 and CD59 which are progesterone responsive genes [394]. Progesterone acts through paracrine signaling on PR-negative cells to stimulate their proliferation [395, 396].

4.3.4. Prolactin

Prolactin has been demonstrated to play a significant role in normal development of the mammary gland [397-400]. It interacts with the prolactin receptor (PRLR), mediating paracrine or endocrine signaling. PRLR is a transmembrane receptor present in three isoforms: long, intermediate and short isoform. Isoforms of PRLR differs in their intracellular domains. Long and intermediate isoforms have been shown to play important roles in the induction of differentiation and milk production whereas the short isoform acts as negative regulator [401, 402]. The importance of PRLR in mammary gland development has been demonstrated by PRLR knockout mice. These studies showed that the absence of PRLR leads to the impaired development of TDLU and inhibition of lactation [399]. During organogenesis, PRLR plays a

role in the maturation of mammary gland. In addition, it plays a role in expansion and differentiation of lobuloalveolar system [398].

4.4. Breast cancer risk factors

Breast cancer is a versatile disease with several different causing factors ranging from high risk to moderate and to low risk factors, e.g. hormonal status, age, inheritance and gene expression etc. Each of these is described in more detail in the following paragraphs.

Age

The incidence of breast cancer increases with age, doubling about every 10 years until the menopause. According to the report of cancer research United Kingdom, for women in their forties there is an estimated risk of 2% which rises to over 9 % in their seventies (Cancer Research United Kingdom) [403, 404].

Geographical variation

Breast cancer is more prevalent in certain regions or countries than others. This geographical variation has decreased in last decades. However, a fivefold difference is still observed between Far Eastern and Western countries. This variation can be due to differences in genetic makeup or environmental factors. However, studies of migrants from Japan to Hawaii show that the rates of breast cancer in migrants assume the rate in the host country within one or two generations. It indicates that environmental factors are of greater importance than genetic factors (Cancer Research United Kingdom and global cancer statistics 2002) [405-407].

Hormonal Exposure

Increased hormonal exposure can enhance the risk of breast cancer. It can be either through increase of endogenous or through exposure to exogenous hormones [408]. Each of these is described in the following paragraph.

Endogenous estrogens

Endogenous estrogens are the female sex hormones that are produced by body during menstrual cycle. Increased exposure to the endogenous estrogens is another risk factor of breast cancer. The level of estrogen is highest between puberty and menopause time. Studies showed that obese women also have increased level of estrogens compared to normal women [409]. The endogenous level of estrogen can be increased due early to menarche or because of late menopause. Parity can also affect the level of estrogen.

Hormone Replacement therapy (HRT)

Women are treated with HRT when their ovaries stop producing hormones or in case of ovary removal. HRT is used to provide exogenous hormones: estrogen or combination of estrogen and progesterone. HRT has been shown to increase breast cancer risk. The relative risk is higher when combination of estrogen and progesterone is used relative to using estrogen alone [410, 411].

Family history

Women with one or more affected close first degree relatives have an increased risk of developing breast cancer. Genes associated with inherited breast cancer can be transmitted through either sex and some family members may transmit the abnormal gene without developing cancer themselves. It is not yet known how many genes are involved in family inheritance of breast cancer. However, in 1990's two major breast cancer susceptibility genes

were discovered, BRCA1 and BRCA2. These genes are responsible for 2-5% of all breast cancer cases and women with these mutations have up to an 80% chance of cancer development [412]. The increased risk for breast cancer development, ranges from 59%–87% for BRCA1 and from 38%–80% for BRCA2 mutations [413-416]. BRCA1 and BRCA2 are large genes and mutations can occur at different positions. Moreover, certain mutations are more frequent in particular families or in women of certain region. For instance, some 2% of Ashkenazi Jewish women carry BRCA1 185 del AG (deletion of two base pairs in position 185). Similarly BRCA2 999 del 5 (deletion of five base pairs at position 999), accounts for about half of all familial breast cancer in Iceland. However, the majority of families with multiple breast cancers patients have neither of these mutations. This observation supports the involvement of other genes mutations in breast cancer inheritance. Some other rare gene mutations have also been identified which are associated with the early-onset of breast cancer. These include mutations in genes coding for p53 (Li-Fraumeni syndrome), PTEN (Cowden syndrome) or STK11/LKB1 (Peutz-Jeghers syndrome). According to studies, 5%–10% of all breast cancers in women are associated with hereditary susceptibility due to mutations in autosomal dominant genes. These genes include BRCA1 and BRCA2, p53, PTEN, and STK11/LKB1 [417, 418].

Moreover, some other rare gene mutations have been identified by a number of large scale studies, such as DNA repairing genes: a tumor suppressor gene called check point kinase (CHEK2A), a serine threonine kinase involved in DNA repair called Ataxia telangiectasia mutated (ATM), the tumor suppressor partner and localizer of BRCA2 (PALB2) and BRCA 1 interacting proteins BRIPI [419-422]. These gene mutations are very rare in the population and have incidence levels between 0.7 and 5 %. Similarly, genome wide analysis of single nucleotide polymorphism (SNPs) has identified a number of new genes which confer moderately increased

risk of breast cancer. For instance FGFR2, which encodes fibroblast growth factor receptor 2 that is amplified and overexpressed in 5-10% of breast tumors, and H19 which is involved in the regulation of insulin-like growth factor 2 (IGF2) [412]. Another 15%–20% of female breast cancers occur in women with a family history but without an apparent autosomal dominant inheritance pattern, and are probably due to other genetic factors [423, 424].

Reproductive history

Several epidemiological studies showed that women from developed countries have more risk of breast cancer occurrence than those from developing countries. Many environmental and genetic factors may play roles in this geographic variation, including reproductive history. Different reproductive factors can affect the risk of breast cancer [425]. For instance, age at menarche, age at time of first pregnancy, lack of or less duration of breast feeding, influence of parity and age of menopause.

In developed countries, the average age of menarche has been reduced from 16-17 to 12-13 (from mid 19th to 2000) [426, 427]. It has been shown to be associated with increased breast cancer risk. For every year delay of menarche (after 12 years age), there is 6% reduction of breast cancer risk in pre-menopausal women and 3% reduction in post-menopausal women [428]. Countries with less breast cancer risk (for instance China) have higher average age at menarche (16-17 years). Increased exposure of cyclical hormone could be responsible for increase breast cancer risk associated with early age menarche [429]. Parity has also been proposed to influence breast cancer risk. It was first reported by an Italian physician that the rate of breast cancer is higher in nuns compared to other women. Later, his report was supported by another study done in 1990s which mentioned that nulliparous women have 30% increase risk of

breast cancer compared to parous women [430]. Early aged first pregnancy can further reduce breast cancer risk.

4.4. Types of Breast Cancer

Breast cancer exhibits immense heterogeneity at the level of morphology, histological grades, and in possessing different biomarkers such as estrogen receptor (ER), progesterone receptor (PR) and Epidermal growth factor receptor 2 (HER2). This heterogeneity affects physiopathology, prognosis, outcome and response to treatments. Moreover, it reveals that many interconnecting cellular networks are responsible for this disease. Breast cancer is categorized according to different parameters i.e. tumor size, lymph node involvement, histological grade, age, or by biomarkers like estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2). The expression of biomarkers is being routinely used in the diagnosis and treatment of patients.

According to this classification breast cancer can be categorized into three different classes. One is estrogen receptor positive (ER+) group. This category is comprised of the most diverse group with immense genetic diversity. It shows good prognosis and can be treated by endocrine therapy according to different genetic makeup. The second type of cancer is called HER2-positive cancer which is also known as ERBB2. This group is characterized by the amplification of the HER2 gene. It shows better clinical outcomes as compared to ER+ cancer because of effective therapeutic targeting of HER2. The third group is triple negative breast cancer (TNBCs) which is also known as basal-like breast cancer. It has the worst prognosis as it lacks estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor (HER2).

However, this classification of breast cancer on traditional parameters is not sufficient to develop common therapy for each group. Because breast cancer has far more heterogeneity to be placed in three groups and these basic groups are comprised of many other subgroups. Currently, breast cancer is divided into five major subtypes: normal breast-like, luminal A (ER- α positive, low histological grade), luminal B (low ER- α expression, high histological grade), HER2 (amplification and overexpression of ERBB2 gene) and basal-like or triple-negative breast cancers (negative for ER, PR and HER2) [431].

Luminal A breast cancers are responsive to anti-estrogen therapy. The sensitivity to anti-estrogen decreases in luminal B subtypes due to lower ER α expression levels and possible increased HER2 expression [431, 432]. In the HER2 subtype which accounts for 30% of all breast cancers, HER2, a member of the Epidermal Growth Factor Receptor (EGFR) family, is overexpressed and there is a significant correlation between HER2 overexpression and decreased survival of breast cancer patients [433]. HER2 overexpression also inversely correlates with ER α expression and with a lack of response to endocrine therapy and chemotherapeutic agents. Trastuzumab, a monoclonal antibody against HER2, was developed and approved by the FDA in 1998 for the treatment of HER2 overexpressing cancers in the U.S. [433]. BRCA1 has also been shown to interact with and inhibit ER- α transcription [434]. In basal-like breast cancers, BRCA1 germline mutations frequently occur. Additionally, basal-like cancers express high levels of basal markers including cytokeratin (CK) 5 and 6, EGFR and extracellular matrix (ECM) receptor, $\alpha 6\beta 4$ integrin [431, 435]. Basal-like breast cancers generally have poor prognosis because they are aggressive and do not respond to anti-estrogens or anti-HER2 antibodies [434]. Current therapeutic strategies include use of DNA damaging agents to induce double-stranded

DNA breaks (for example: anthracyclines and etoposide) and kinase inhibitors including imatinib used to downregulate EGFR signalling [435].

4.5. Therapeutic strategies

Owing to extensive heterogeneity in breast cancer, several therapeutic strategies are being used to treat breast cancer such as cytotoxic chemotherapy, radiation therapy and surgical resection. However, here as my thesis work is more concerned with ER and tamoxifen sensitivity, only ER related therapies are described in detail.

4.5.1. Selective Estrogen-Receptor Modulators (SERMs)

Tamoxifen was the first SERM to be developed and used in clinical applications, mostly in the treatment of breast cancer. Tamoxifen competes with E2 for binding to ER α . Tamoxifen and estrogen both bind to ER with different binding affinity, leading to different conformational changes in ER. Tamoxifen has agonist or antagonist effects on ER depending upon the target gene and the cell context. In breast cancer, it acts as an antagonist of estrogen whereas it acts as an agonist of estrogen in endometrial cancer. In endometrial and bone cells, tamoxifen binds ER α and induces the recruitment of coactivators, thereby exhibiting estrogen-like activity. As an antagonist, it binds ER α and brings a distinct conformational change than with binding of estrogens, resulting in the recruitment of corepressors on target gene promoters in mammary cells, instead of co-activators in case of estrogen binding [436]. By this way, tamoxifen deregulate estrogen signaling pathway. Tamoxifen has improved overall survival and has contributed to reduce breast cancer cell death in last decades. However, 20-30% of the breast cancer patients either exhibit *de novo* resistance or acquired resistance. In spite of the beneficial

effects of tamoxifen, resistance is one of the significant clinical problems. Other SERMs include raloxifene, which has bone-protective effects and is inhibitory on breast cancer cell growth, without any growth-promoting activity in the uterus [437]. Coregulators are thought to play a major role in cell-context dependent functions of SERMs [438, 439]. However, approximately 40 to 60% of tumors that are ER positive and PR negative are not responsive to anti-hormonal therapy due intrinsic or acquired resistance [438]. Genetic variations in the cytochrome P450 enzyme 2D6 (CYP2D6) that converts 4-hydroxytamoxifen to the active metabolite, endoxifen, can contribute to metabolic mechanisms of tamoxifen resistance [438, 440]. Intrinsic resistance occurs when ER-positive breast cancers are initially unresponsive to tamoxifen treatment and acquired resistance can occur when changes in ER signal transduction pathways convert inhibitory SERMs into growth stimulatory signals [441].

Mechanisms of Tamoxifen resistance

Owing to complex interaction of tamoxifen and ER signaling, many potential mechanisms can contribute in causing tamoxifen resistance. The effect of tamoxifen is primarily mediated through estrogen receptor (ER), therefore the expression of ER serve as a strong predictor for tamoxifen sensitivity and loss in ER expression can cause tamoxifen resistance. Indeed, studies have shown that the absence of estrogen/progesterone receptor is the dominant mechanism for *de novo* tamoxifen resistance [442-447]. Another study indicated that patients exhibited acquired resistance against tamoxifen, associated with the loss in ER expression [448]. However, other studies demonstrated that tumors can acquire resistance to tamoxifen without loss of ER expression [449]. Moreover, some patients showed resistance against tamoxifen but they respond to Aromatase inhibitors or to the pure downregulators of ER [450, 451]. It suggests that ER continues to regulate growth in some of tamoxifen-resistant patients. *In vitro* studies

demonstrated that mutations in ER gene can also cause abrogation of ER signaling or can modulate the effect of tamoxifen without effecting the expression of ER [452, 453]. However, these mutations are rarely observed in breast cancer patients [454, 455].

Collectively, these studies suggest that in spite of the primary importance of ER expression in determining tamoxifen sensitivity or resistance, other components may also play role in developing tamoxifen resistance. As the transcriptional activity of ER is altered with the recruitment of corepressors or coactivators, they can also play a role in developing tamoxifen resistance. Indeed, some studies have shown that the expression or binding of coactivators and corepressors does affect the response of tumors to tamoxifen. However, the role of corepressors or coactivators in developing tamoxifen resistance remains largely controversial.

Cellular signaling and transduction pathways can also play a role in altering tamoxifen sensitivity. ER interact with different components of several signaling pathways, one of the most documented cross-talk of ER is with growth factor receptor pathways, such as the EGFR/HER2 family and insulin-like growth factor receptor (IGFR) family. HER2 directly interacts with ER. Moreover, ER is phosphorylated by Erk1/Erk 2 (downstream effectors of HER2 pathway). Both *in vitro* and clinical studies indicated the role of increased HER2 expression in alteration of ER signaling as well as in *de novo* and acquired tamoxifen resistance [456-458]. Similarly, PI3K/Akt pathway also exhibit cross-talk with ER pathway and several studies demonstrated its role in tamoxifen sensitivity [459, 460].

4.4.2. Selective estrogen receptor downregulators (SERDs)

The pure antagonists of ER α are also used as therapeutic agents in breast cancer. The pure ER antagonist ICI 182,780 (fulvestrant) is approved by the Food and Drug Administration (FDA) of U.S. It binds to ER α with 100-fold greater affinity than tamoxifen and by binding to ER α , it

inhibits receptor dimerization, resulting in abrogation of estrogen signaling [461]. Studies have shown that ICI 182,780 cause a decrease in ER α protein level [462]. However, by using pure inhibitor of estrogen, there is risk of inhibition of beneficial effects of ER (the anti-invasive effect of ER). Indeed, studies demonstrated that the invasiveness of MCF-7 cells is enhanced by anti-estrogens [463, 464]. Moreover, ER- breast cancers are less differentiated and more aggressive than those containing ER [465].

4.4.3. Aromatase inhibitors (AIs)

Conversion of androgen to estrogens occurs in adipose tissue, muscle, liver and breast tumor in post-menopausal women [466]. AIs act by inhibiting the enzyme aromatase, thereby preventing the formation of estrogens from precursor molecules, androgens, androstenedione and testosterone. Current AIs are approved by the FDA for use as an adjuvant treatment with tamoxifen and other chemotherapeutic agents. These include exemstane, anastrozole and letrozole.

4.4.4. Cyclin D1 and cyclin dependent kinase inhibitors

Cyclin D1 is a member of D-type family of cyclins that associate with a variety of cyclin dependent kinases (CDKs), including Cdk4 and Cdk6. They are involved in the regulation of the G1-S transition phase of cell cycle division in mammals [467]. Mice lacking cyclin D1 are small and have defects in mammary gland and retinal development [468]. Overexpression of cyclin D1 in the mammary gland leads to hyperplasia and carcinogenesis in mice, and cyclin D1 is implicated in both ER- and HER2- mitogenic signaling [433, 468, 469]. The regulation of cyclin D1 transcription by E2 and ER α is known to occur through CRE (cAMP response element) or AP-1 (activator protein-1) sites because a classical ERE (estrogen response elements) is absent in

the cyclin D1 promoter [470]. Cyclin D1 is also overexpressed in approximately 40% of invasive breast cancers, and ER α expression positively correlates with high cyclin D1 expression levels in some breast cancers [471]. It has been widely demonstrated that cyclin D1 is a good prognostic factor associated with better patient outcome especially for ER-positive patients [472]. The growth-promoting activities of cyclin D1 are linked to associated-CDKs. CDKs represent a therapeutic target for breast cancer. Flavopiridol is one of the CDK inhibitors that has been developed and extensively studied. Flavopiridol is a synthetic flavone that has been reported to decrease cyclin D1 expression in breast cancer cells [473]. In acute myelogenous leukemias, flavopiridol has proven to be effective when used in combination with other therapeutic agents [474]. However, in breast cancer, the utility of flavopiridol is yet to be demonstrated and clinical trials with flavopiridol in combination with adjuvant therapies are currently in progress.

4.4.5. Histone deacetylase (HDAC) inhibitors

Histone proteins organize DNA into nucleosomes, which are repeated structures of chromatin [475]. The acetylation status of histones is dictated by activities of histone acetyltransferases (HATs) and HDACs, which alter chromatin structure and influence activation or repression of gene expression, respectively. HDACs keep histones deacetylated and chromatin in a tightly packed state, which leads to the repression of gene expression. Aberrant HDAC activity is associated with the development of cancer [476].

HDAC inhibitors are currently being investigated as a valuable therapeutic option [476, 477] because it is thought that the use of HDAC inhibitors alone or in combination with existing therapies could be an efficient avenue for reversing hormone therapy resistance by re-sensitizing tumors to antiestrogens. In the case of ER, part of the rationale for this comes from evidence that HDAC1 overexpression inhibits ER α transcriptional activity [478] and a retrospective study

showed that HDAC6 expression was associated with increased survival of breast cancer patients who had hormone-responsive tumors and underwent treatment with tamoxifen [479]. Some of the different HDACs currently under investigation in clinical trials include vorinostat, panobinostat and valproic acid [477], though they have only proven to be effective in the case of hematological malignancies and not in solid tumors.

Aim of the study

Endocrine therapies have made considerable progress for treatment of breast cancer in the last few decades. These therapies induce cell cycle arrest or/and apoptosis by regulation of different proteins including tumor suppressors, cell cycle regulatory proteins and proteins involved in pro-survival pathways. About 70 % of breast cancers are ER dependent, and tamoxifen is the most common treatment being used for ER+ breast cancer. However, *de novo* and acquired resistance against tamoxifen is a major obstacle to control the growth and metastasis of breast carcinoma. Different mechanisms are involved in developing tamoxifen resistance, such as lack of estrogen and progesterone receptors expression, alteration in co-regulatory proteins (increased expression of co-activators or decreased expression of co-repressors), deregulation of signal transduction pathways (EGFR, HER2, and IGF-IR signaling pathways), increased activity of different kinases (Akt and ERK), deregulation of cell cycle regulatory proteins (CDKs and CDKs inhibitors e.g. p21 and p27). Several recent studies demonstrated the involvement of O-GlcNAcylation in different mechanisms of tumorigenesis and cancer progression. To date, different studies suggested that the elevated O-GlcNAcylation is linked with increase in tumor growth, invasion and poor differentiation in breast cancer. However, to the best of our knowledge, the effect of O-GlcNAcylation on anticancer chemotherapy had never been investigated. As different signaling pathways and/or proteins involved in tamoxifen resistance have been shown to be regulated by O-GlcNAcylation, it seemed possible that O-GlcNAcylation played role in regulation of tamoxifen effect in breast cancer. Thus, this study was conducted (i) to determine whether O-GlcNAcylation can modulate the effect of tamoxifen in human breast cancer derived MCF-7 cells, and (ii) to explore the potential mechanisms linking O-GlcNAcylation and tamoxifen sensitivity.

RESULTS

1. O-GlcNAcylation protects MCF-7 cells from tamoxifen-induced cell death

In order to determine if O-GlcNAcylation can affect tamoxifen-induced cell death, we treated MCF-7 cells with 4-Hydroxytamoxifen in presence and absence of PUGNAc and glucosamine for 24 and 48 hours. 4-Hydroxytamoxifen (TamOH) is an active metabolite of tamoxifen which is used in breast cancer as SERM. Cells were incubated in DMEM culture media supplemented with 1% fetal bovine serum and with PUGNAc (100µM), glucosamine (5mM) and/or TamOH (10µM). As depicted in the figure 25, glucosamine enters into the hexosamine biosynthetic pathway below the rate limiting step (GFAT) to induce O-GlcNAcylation, and PUGNAc inhibits OGA (the enzyme responsible for removal of O-GlcNAcylation). Each agent resulted in increase of global protein O-GlcNAcylation both in presence and absence of TamOH (Figure 26 A). This increase in O-GlcNAcylation was further enhanced when PUGNAc and glucosamine were added together.

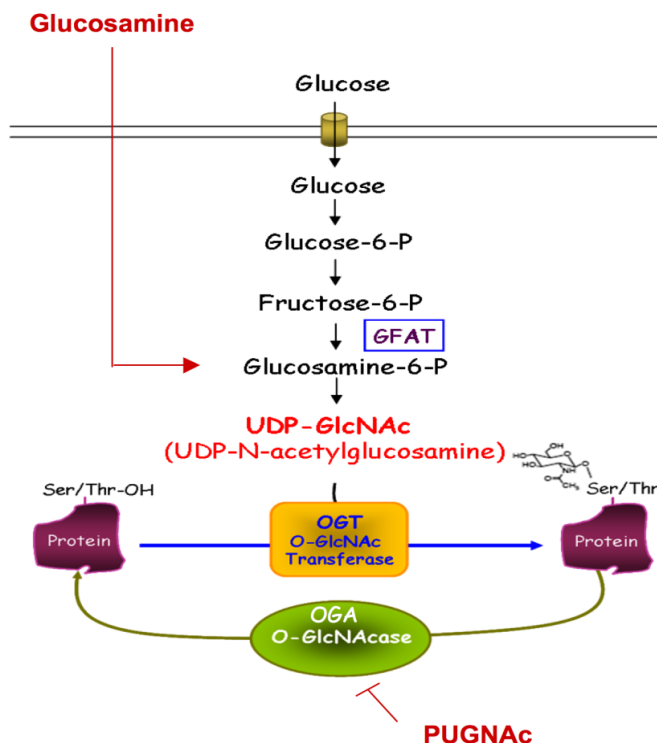


Figure 25: Hexosamine biosynthetic pathway (HBP)

3-5 % of the glucose entering into the cell is directed to the HBP. Glucose entering into the HBP is metabolized into glucosamine-6-phosphate which is then converted into UDP-GlcNAc. UDP-GlcNAc is used as a substrate by OGT to modify proteins with O-GlcNAc. OGA antagonizes OGT and catalyzes the removal of O-GlcNAcylation.

UptiBlue assay allowed us to measure the effect of different treatments on the growth of control versus treated cells. We observed a significant decrease in the number of viable cells with TamOH treatment (figure 26 B, C). The treatment with O-GlcNAcylation-stimulating agents (PUGNAc and glucosamine) resulted in the protection from TamOH-induced cell death.

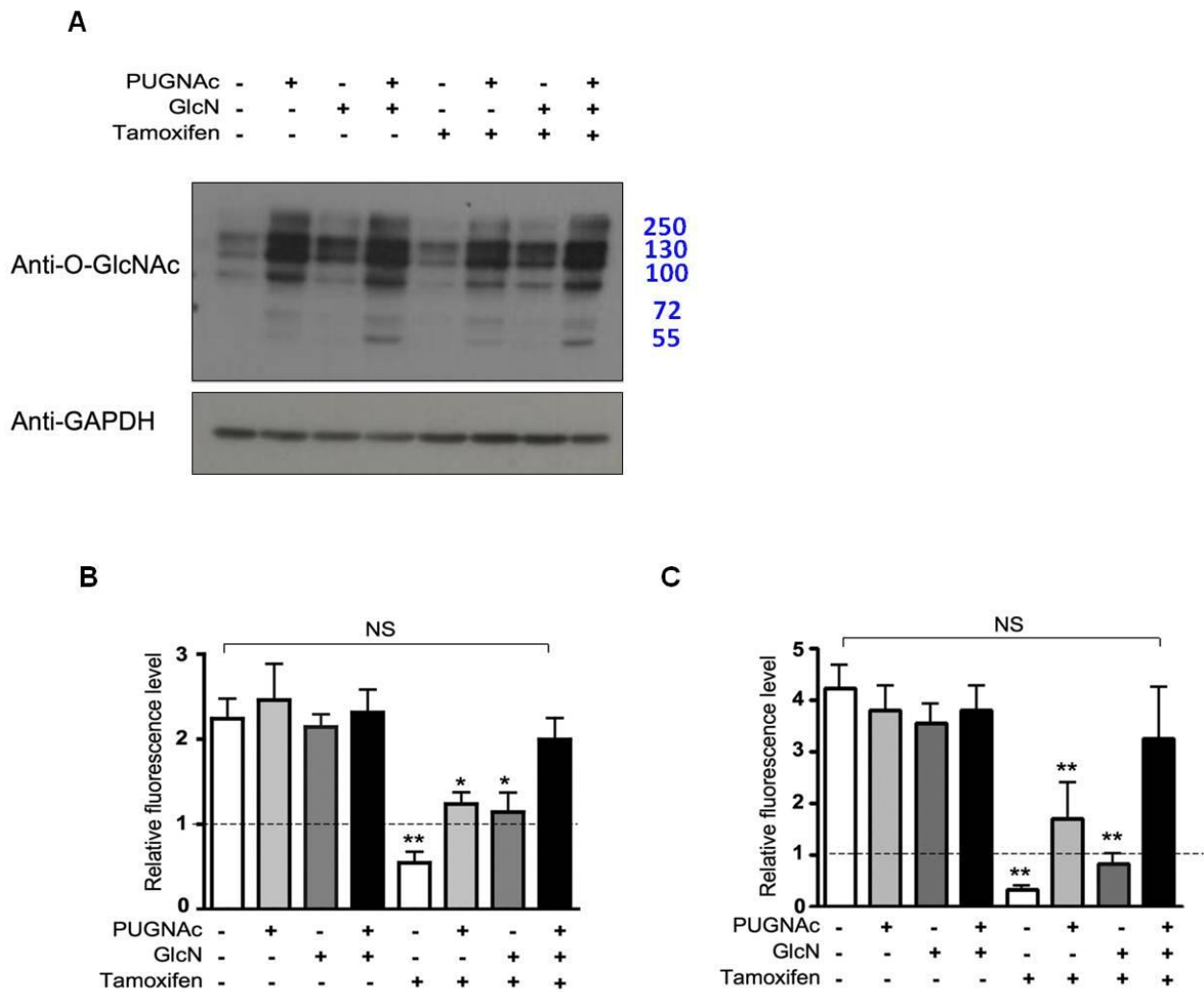


Figure 26: Effect of PUGNAc and glucosamine treatment on tamoxifen-induced cell death

MCF-7 cells were seeded in 96 well plates in DMEM containing 10% fetal bovine serum. After 24h, cells were treated with 10 μ M TamOH, 100 μ M PUGNAc and 5mM glucosamine in DMEM containing 1% fetal bovine serum.

(A) After 24h of treatments, cell lysates were analyzed by western blotting and O-GlcNAc level is detected by using anti-O-GlcNAc antibody (western blot shown is representative of at least three independent experiments). (B), (C), cells were incubated for 24h (B) or 48h (C) in presence of P, G and tamoxifen. The effect of treatments was evaluated by measuring the fluorescence at 580nm. Statistical analysis was performed by ANOVA followed by Dunnet test. Results are mean \pm SEM of eight (B) and 7 (C) independent experiments (*, **; $p < 0,05$, $p < 0,01$, respectively). Control (ctrl), PUGNAc (P), Glucosamine (G), PUGNAc+glucosamine (PG).

Furthermore, we analyzed the effect of these treatments on apoptosis by using flow cytometry assay (FACS) through Annexin V-FITC used in conjugation with propidium iodide (PI). We observed that PUGNAc+glucosamine (PG) per se had no effect on cell apoptosis while tamoxifen significantly induced apoptosis in MCF-7 cells. Tamoxifen-induced apoptosis was abrogated in presence of PUGNAc+glucosamine (tamoxifen +PG).

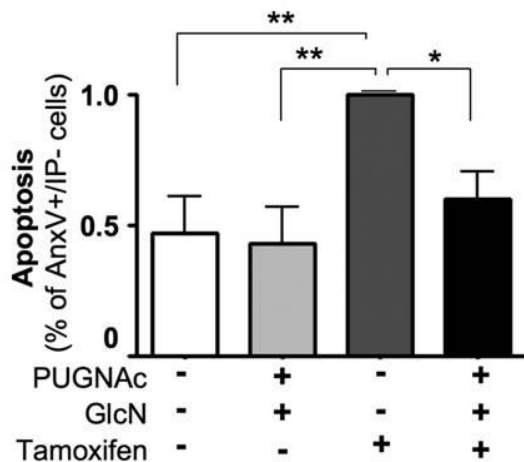


Figure 27: Effect of PUGNAc+glucosamine treatment on tamoxifen-induced apoptosis.

MCF-7 cells were seeded in 6 well plates for 24h in DMEM containing 10% fetal bovine serum. After 24h, MCF-7 cells were treated with 10 μ M TamOH, 100 μ M PUGNAc and 5mM glucosamine in DMEM containing 1% fetal bovine serum. After 24h incubation, apoptosis was measured by using FACS through Annexin V-FITC and propidium iodide (PI). Control (ctrl), PUGNAc+glucosamine (PG). The percentage of apoptotic cells (Annexin V-FITC positive and PI negative) was determined in each condition. Results are mean \pm SEM of four independent experiments. Statistical analysis was performed by ANOVA followed by Tukey multiple comparison test (*, **; $p < 0,05$, $p < 0,01$, respectively).

2. OGT depletion enhanced tamoxifen-induced cell death in MCF-7 cells

To test whether a decrease in O-GlcNAcylation can also modify tamoxifen-induced MCF-7 cell death, we inhibited OGT by using siRNA. MCF-7 cells were transfected with siRNA against OGT and siNEG (non-targeting negative control siRNAs) for 48 hours. The efficiency of inhibition of OGT was confirmed through western blotting which showed a marked decrease in OGT expression (Figure 28 A). We further analyzed whether the inhibition of OGT expression resulted in global decrease of O-GlcNAcylation. Results showed that the depletion of OGT led to a significant decrease in global O-GlcNAcylation compared to control cells transfected with siNEG (Figure 28B). After 48 hours of transfection, cells were treated with TamOH for 24 hours and were analyzed by using flow cytometry assay (FACS) through Annexin V-FITC in conjugation with propidium iodide (PI). These results showed that the depletion of OGT markedly potentiated the effect of tamoxifen on cell death. This suggests that the effect of tamoxifen could be enhanced by decreasing O-GlcNAcylation through OGT inhibition.

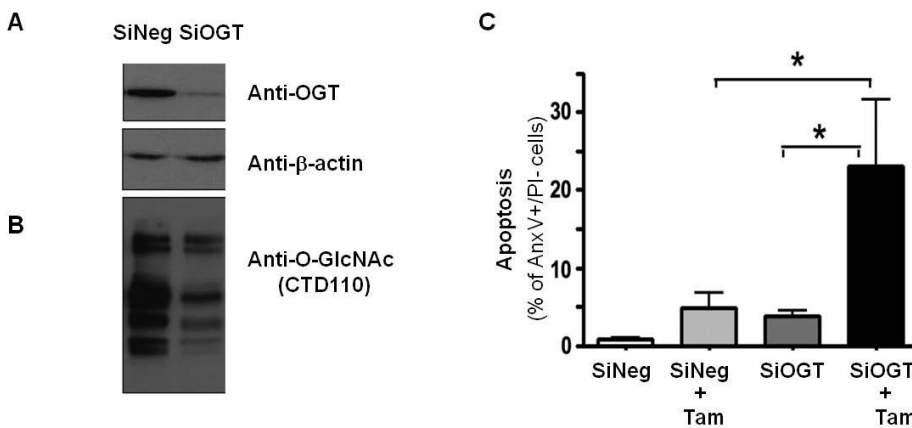


Figure 28: Effect of OGT inhibition on tamoxifen-induced cell death

MCF-7 cells were seeded in 6 well plates in DMEM containing 10% fetal bovine

serum. After 24h, MCF-7 cells were transfected with siNEG or siOGT. Cells were lysed 48h after transfection. (A) The crude extract was analyzed by using western blotting for detection of OGT. β -actin was used as a loading control for western blot. (B) The level of global O-GlcNAcylation was evaluated by using CTD110 antibody. OGT

depletion led to a significant decrease in global O-GlcNAcylation. (C) 48h after siRNAs transfection, cells were treated with 10 μ M TamOH, in DMEM containing 1% fetal bovine serum. After 24h of treatment, apoptosis was measured by using FACS through Annexin V-FITC and propidium iodide (PI). siNEG (siRNA negative control), siOGT (siRNA against OGT), Tam (tamoxifen). Results are the mean \pm SEM of five independent experiments. Statistical analysis was performed by ANOVA followed by Tukey multiple comparison test (*; $p < 0.05$).

3. PUGNAc and glucosamine stimulate PI3-K pathway and increase PIP3 production in MCF-7 cells

As PUGNAc+glucosamine protect MCF-7 cells from tamoxifen-induced cell death, it might be possible that this occurs through upregulation of the pro-survival PI3-K pathway. We studied the PI3-K pathway by using a BRET assay previously developed in the laboratory [480]. This BRET assay is comprised of Akt-PH fused with the Renilla luciferase (Luc) and a membrane-anchoring signal peptide fused with yellow fluorescent protein (YFP). This assay is based on the measurement of Akt-PH domain recruitment to the plasma membrane in response to PIP3 production, which results in energy transfer between Akt-PH-Luc and membrane-YFP. After 48h of the transfection with above mentioned BRET constructs, MCF-7 cells were incubated with PUGNAc and glucosamine for 6h. We observed that PUGNAc or glucosamine treatment individually tend to increase BRET signal compared to untreated cells. This effect was significantly increased when both agents are added together (PUGNAc+glucosamine). This experiment demonstrated that PIP3 production is enhanced by O-GlcNAcylation-inducing agents.

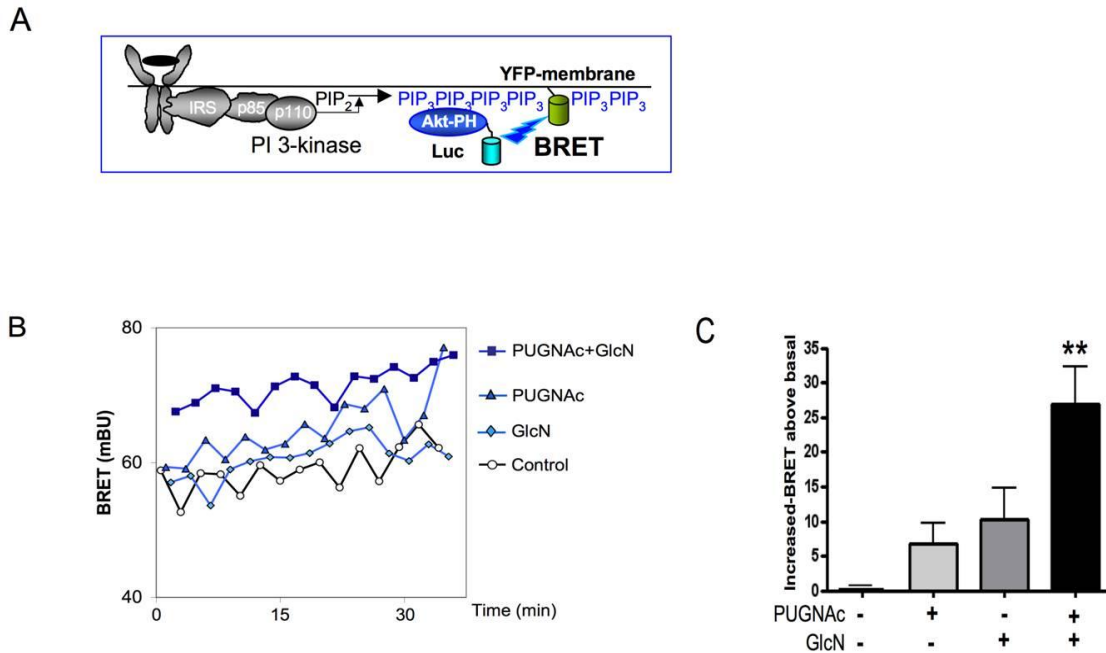


Figure 29: Effect of O-GlcNAc-inducing agents on PIP3 production in MCF-7 cells

MCF-7 cells were seeded in DMEM containing 10% fetal bovine serum. (A) After 24h, MCF-7 cells were transfected with Luc-Akt-ph and membrane-YFP. 24h post-transfection, cells were transferred into a 96 well BRET plate. After 24hrs cells were treated with PUGNac and glucosamine for 6h. (B) Cells were washed with PBS and were incubated with coelentraxine for 10 min. BRET signal was measured by using Fusion luminometer (Perkin Elmer). (C) Results are the mean \pm SEM of at least four independent experiments. Statistical analysis was performed by ANOVA followed by Dunnet test compared to control condition (**; $p < 0.01$). Pug (PUGNac), GLN (Glucosamine), Pug+GLN (PUGNac+ Glucosamine)

Furthermore, the stimulation of PI3-K pathway was confirmed at the level of Akt phosphorylation by western blotting. We observed that PUGNac+glucosamine enhanced the phosphorylation of Akt and this effect was sustained during 24h.

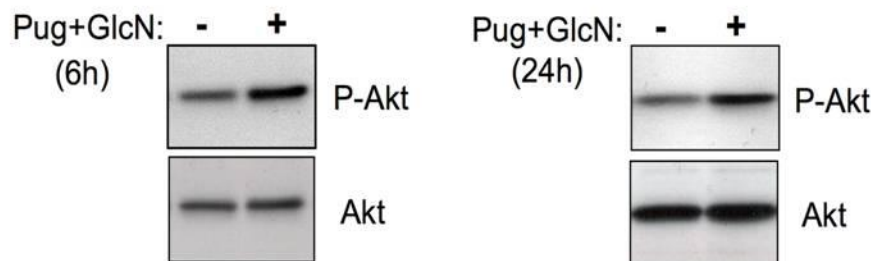


Figure 30: Effect of PUGNAc+glucosamine treatment on Akt phosphorylation

The effect of PUGNAc+glucosamine treatment on PI3-K pathway stimulation was determined by western blotting for phospho-Akt (Serine 473). Cells were treated with PUGNAc+glucosamine for 6h and 24h and the crude extract was analyzed for Akt phosphorylation. Each western blot is representative of three independent experiments.

4. PI3-K inhibition could not impairs the protective effect of PUGNAc+glucosamine against tamoxifen-induced cell death

The result obtained suggested that the effect of PUGNAc and glucosamine against tamoxifen-induced cell death may occur by upregulation of PI3-K pathway. In order to determine the effect of PI3-K pathway in protecting from tamoxifen-induced cell death, we used the PI3-K inhibitor LY294002 and analyzed cell growth by using UptiBlue assay. The result showed that the inhibition of PI3-K pathway did not modify the protection from tamoxifen-induced cell death provided by PUGNAc+glucosamine.

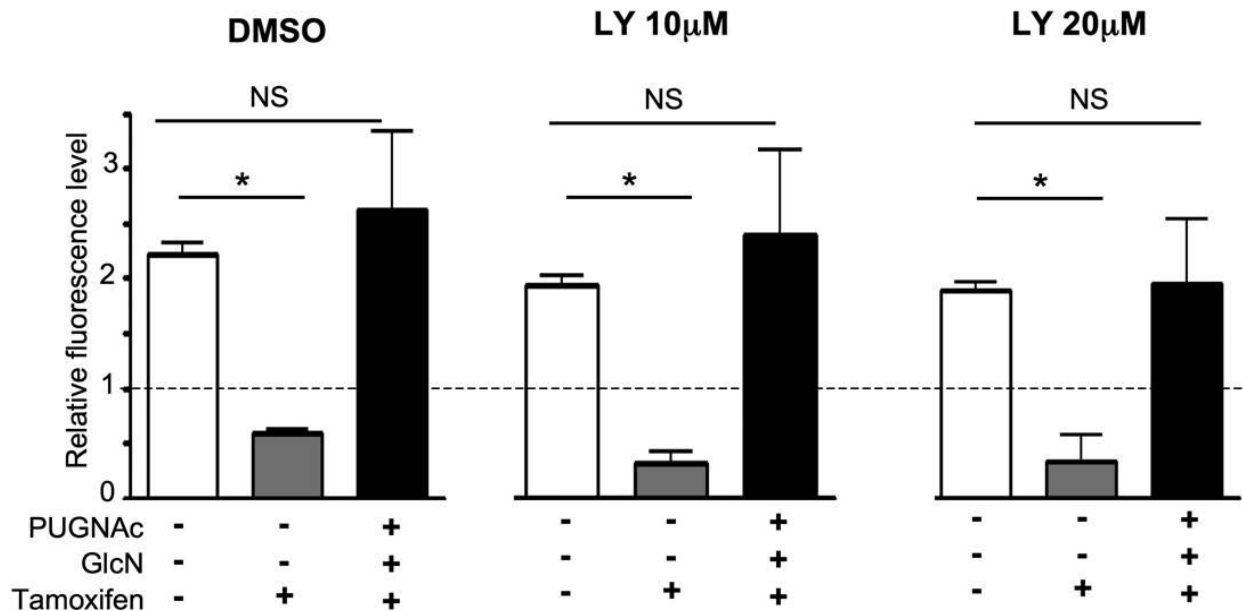


Figure 31: Effect of PI3-K inhibition in protecting MCF-7 cells from tamoxifen-induced cell death by PUGNac+glucosamine

MCF-7 cells were seeded in 96 well plates for 24h in DMEM containing 10% fetal bovine serum. After 24h, MCF-7 cells were treated with 10µM TamOH, 100µM PUGNac and 5mM glucosamine in DMEM containing 1% fetal bovine serum in absence and presence of LY294002 (PI3-K inhibitor). Two different concentrations of LY294002 were used (10 and 20µM). The effect of different treatments on cell growth was analyzed by using UptiBlue assay. Results are the mean ± SEM of three independent experiments. Statistical analysis was performed by ANOVA followed by Dunnet test (*; p<0.05) compared to control condition. Control (ctrl), PUGNac+glucosamine (PG), tamoxifen (tam).

5. Stimulation of PI3-K pathway by IGF-1 does not impair tamoxifen-induced cell death

We also evaluated the effect of insulin-like growth factor 1 (IGF-1) on PIP3 production by using BRET assay. We observed that IGF-1 treatment greatly increased BRET signal and this effect was further enhanced in the presence of PUGNac+glucosamine.

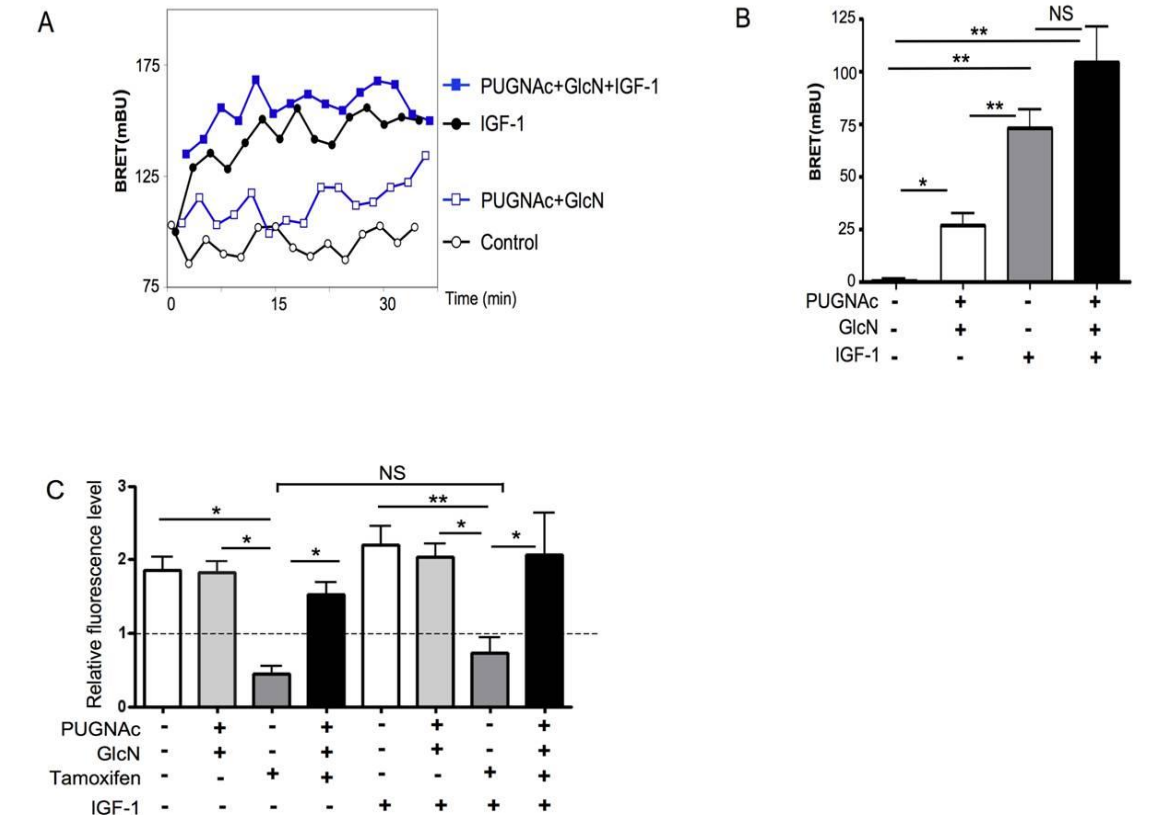


Figure 32: Effect of IGF-1 treatment on tamoxifen-induced cell death

(A, B) MCF-7 cells were seeded for 24h prior to transfection in DMEM containing 10% fetal bovine serum. MCF-7 cells were then transfected with Luc-Akt-ph and membrane-YFP. 24h post transfection, cells were transferred into a 96 well BRET plate. After 24hrs cells were treated with PUGNAc+glucosamine for 6h. Cells were washed with PBS and incubated with coelentrazine for 10 min. 100nM of IGF-1 was added on cells immediately before measuring BRET signal using a Fusion luminometer (Perkin Elmer). (B) Results are the mean \pm SEM of at least 5 independent experiments. Statistical analysis was performed by ANOVA followed by Tukey multiple comparison test (*, **, ***; $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively). (C) MCF-7 cells were treated with 10 μ M TamOH, 100nM IGF-1, 100 μ M PUGNAc and 5mM glucosamine in DMEM containing 1% fetal bovine. The cell growth was analyzed by using UptiBlue assay after 24h treatment. Results are the mean \pm SEM of five independent experiments. Statistical analysis was performed by ANOVA followed by Dunnet test ($p < 0.05 = *$) compared to control condition. Control (ctrl), PUGNAc+glucosamine (PG).

We observed that IGF-1 treatment strongly stimulated PIP3 production in MCF-7 cells compared to cells treated with PUGNAc+glucosamine (Figure 32 A). In order to determine whether the stimulation of PI3-K by IGF-1 could mimic PUGNAc+glucosamine effect on tamoxifen-induced cell death, we performed UptiBlue assay. We observed that IGF-1 treatment could not protect MCF-7 cells from tamoxifen-induced cell death whereas the effect of PUGNAc+glucosamine on tamoxifen-induced cell death was still observed in presence of IGF-1 (Figure 32 C).

Based on the result obtained so far, we concluded:

- i) Elevated O-GlcNAcylation level in MCF-7 cells stimulate PI3-K pathway.
- ii) Elevation in O-GlcNAcylation level leads to the protection from tamoxifen-induced cell death of MCF-7 cells.
- iii) Protection from tamoxifen-induced cell death is independent of PI3-K pathway.

As the result obtained demonstrated that the pro-survival PI3-K/Akt pathway was not involved in inhibition of tamoxifen effect, we hypothesized that the effect of PUGNAc+glucosamine might be directly linked with tamoxifen responsiveness rather than through stimulation of PI3-K pathway. Therefore, we looked at the expression of estrogen receptor-alpha ($ER\alpha$), which is considered as a main biomarker in predicting the response to tamoxifen.

6. Increased level of O-GlcNAcylation modify the expression of estrogen receptor α

To evaluate the effect of O-GlcNAcylation-inducing treatment on the expression of estrogen receptor α , MCF-7 cells were incubated with PUGNAc+glucosamine for 24h. The expression of estrogen receptor α was analyzed at mRNA and protein levels by RT-QPCR and western blotting, respectively. We observed that the increased O-GlcNAc by PUGNAc+glucosamine led to a reduction in estrogen receptor α expression at both mRNA (*ESR1*) and protein ($ER\alpha$) levels. Time course experiments showed that the elevated O-GlcNAc significantly reduced mRNA at 6h and this effect was sustained until 36h with a corresponding decrease in protein. A rapid decrease in mRNA level leading to a subsequent decrease in protein level suggested that the reduction in expression of estrogen receptor α is mainly caused at the transcriptional or posttranscriptional level.

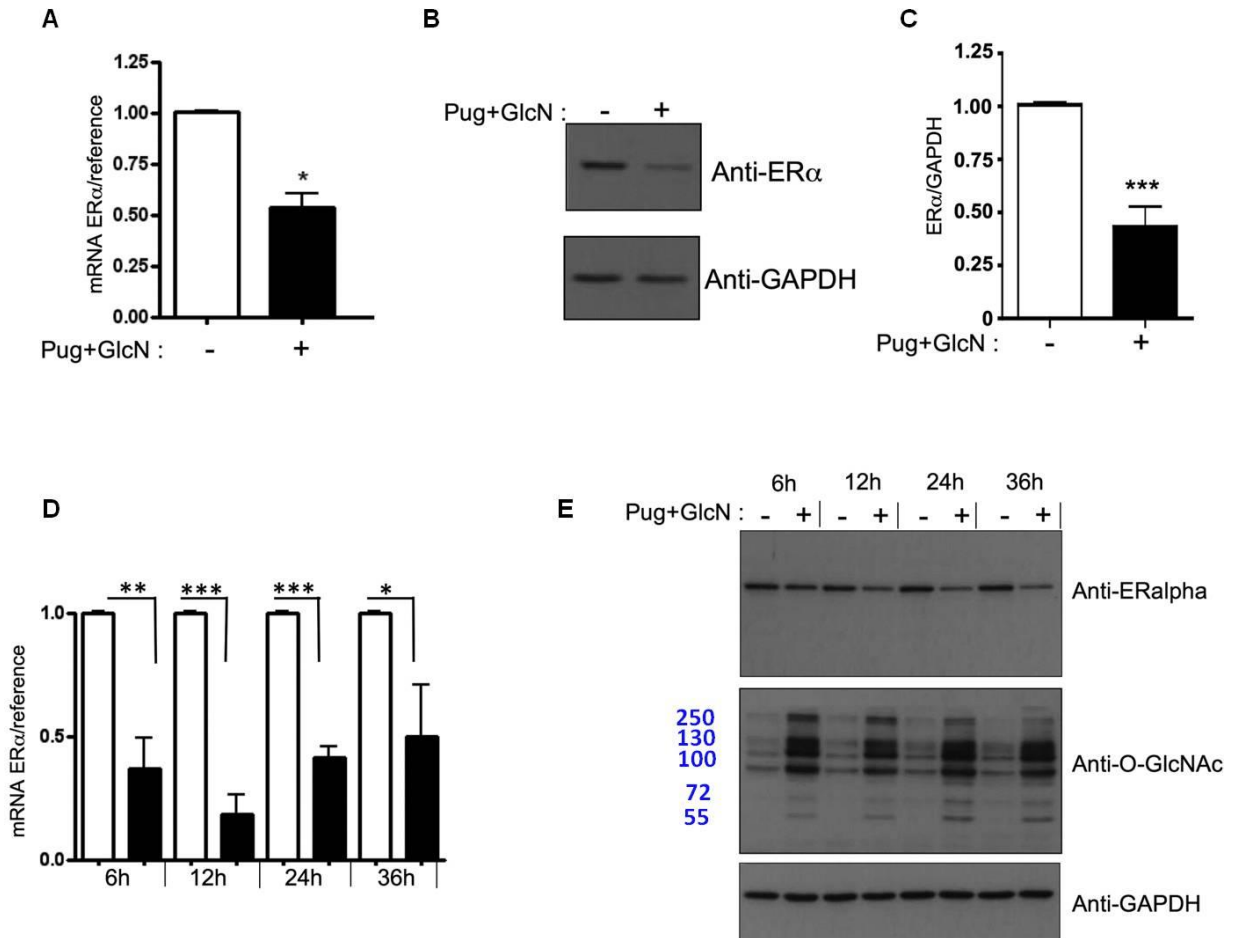


Figure 33: Effect of O-GlcNAcylation-inducing treatments on estrogen receptor alpha expression.

Adherent MCF-7 cells were treated with 100 μ m PUGNAc and 5mM glucosamine. (A) Expression of estrogen receptor was analyzed by RT QPCR for mRNA (*ESR1*). Results are the mean \pm SEM of three independent experiments. Statistical analysis was performed unpaired t-test (*; $p < 0.05$). (B) Whole cell lysates were analyzed by western blotting for ER α expression. (C) Statistical analysis was performed by unpaired t-test. Results are means of 5 independent experiments (D) After 6, 12, 24 and 36h of treatments the expression of estrogen receptor α mRNA (*ESR1*) was analyzed by RT QPCR. Results are the mean \pm SEM of three independent experiments. Statistical analysis was performed by ANOVA followed by Dunnet test (*, **, ***; $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively) compared to control conditions. (E) Crude extract was prepared from time course experiment to analyze the effect of PUGNAc+glucosamine on ER α expression by western blotting. Anti-GAPDH antibody was used as western blot loading control. Results shown in each B and D are representative of at least three independent experiments. Control (ctrl), PUGNAc+glucosamine (PG).

7. The transcriptional activity of *ESR1* promoter is reduced by O-GlcNAc

The next question was to determine whether O-GlcNAc can affect transcriptional activity of *ESR1* promoter. MCF-7 cells were co-transfected with *ESR1*-Luc reporter gene (firefly luciferase coding sequence under the control of the P1 promoter of *ESR1* gene) and Renilla luciferase plasmid (used as a reference gene to normalize for transfection efficiency). 12h after transfection, cells were treated with PUGNAc+glucosamine for 24h to enhance O-GlcNAcylation, cells were lysed and cell lysates were analyzed by using a dual luciferase assay. We observed that PUGNAc+glucosamine induced a significant decrease in transcriptional activity of *ESR1* promoter, as shown in figure 34 (A). A similar result was obtained through elevation of O-GlcNAcylation with OGT transfection as shown in figure 34 (B). OGT reduced the transcriptional activity of *ESR1* promoter compared to cells transfected with pcDNA3. This inhibitory effect was further accentuated by treatment with glucosamine, which provide substrate for OGT. Thus, we concluded that elevation in O-GlcNAcylation level leads to the decrease of *ESR1* promoter activity.

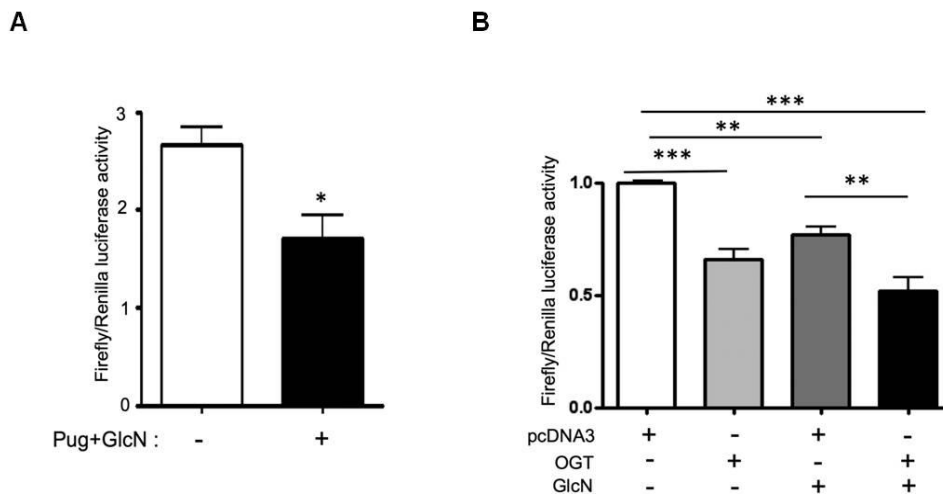


Figure 34: Effect of O-GlcNAcylation on the transcriptional activity of *ESR1* reporter gene

MCF-7 cells were seeded in 12 well plates. (A) 24h later, cells were co-transfected with

ESR1 promoter and Renilla luciferase. 12h after transfection, cells were treated with PUGNAc+glucosamine and

then incubated for 24h. Cells were lysed with passive lysis buffer and the lysate was analyzed by using dual luciferase assay to determine the transcriptional activity *ESR1* promoter. Results are the mean \pm SEM of four independent experiments and statistical analysis was performed by unpaired t-test (*; $p < 0.05$). (B) MCF-cells were transfected with *ESR1* promoter, Renilla luciferase and either pcDNA3 or OGT. pcDNA3 was used as a control plasmid for transfection. 12h after transfection, cells were treated with glucosamine for 24h. The lysates were analyzed by dual luciferase assay. Results are the mean \pm SEM of four independent experiments and statistical analysis was performed by ANOVA followed by Tukey multiple comparison test (**, ***; $p < 0.01$, $p < 0.001$, respectively).

As tamoxifen effect is mainly mediated through estrogen receptor, we were interested to evaluate whether the O-GlcNAc inducing treatments (which reduce the expression of estrogen receptor) could modify the expression of tamoxifen regulated genes.

8. Tamoxifen-mediated increase in p21 and Egr1 expression is abrogated by PUGNAc+glucosamine

First, we investigated p21 expression which is known to be increased by tamoxifen [481, 482]. We observed that tamoxifen enhanced the expression of p21 and this effect was abrogated by the presence of PUGNAc+glucosamine.

We further investigated tamoxifen effect on the expression of Egr1 (another tamoxifen regulated gene). As described in previous studies, tamoxifen treatment significantly enhanced Egr1 expression [483, 484]. We observed that tamoxifen mediated increase in expression of Egr1 was declined by PUGNAc+glucosamine treatment. These results suggest that the reduction by PUGNAc+glucosamine of tamoxifen effect on Egr1 and p21/Cip1 expression might play a role in protection from tamoxifen-induced cell death.

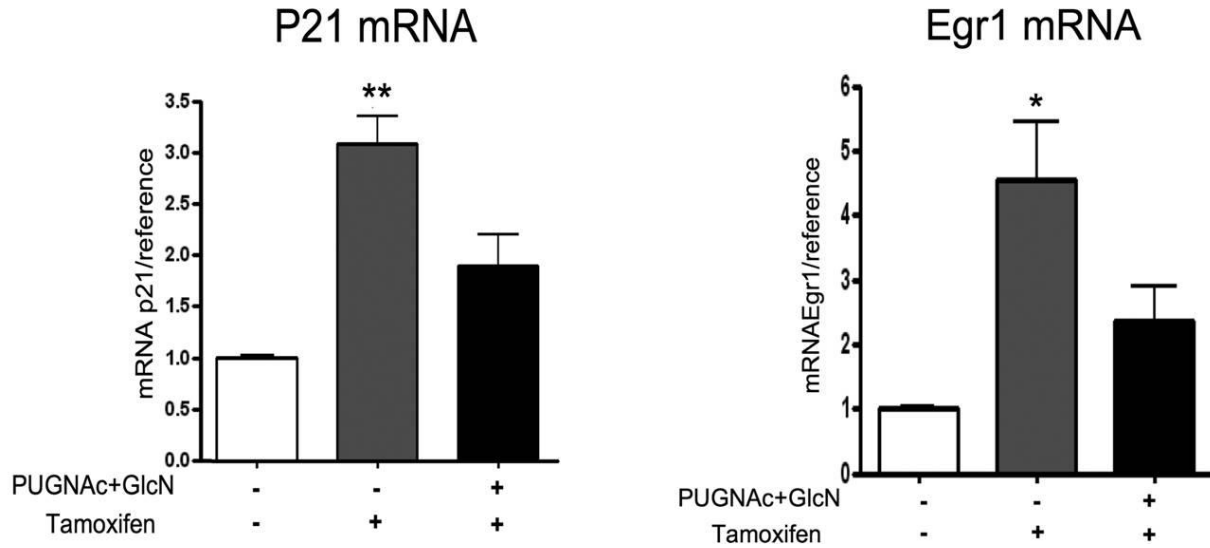


Figure 35: O-GlcNAc elevation abrogated tamoxifen-induced increase in p21 and Egr1 expression.

MCF-7 cells were seeded in 6 well plates for 24h in DMEM containing 10% fetal bovine serum. After 24h, MCF-7 cells were treated with 10 μ M TamOH, 100 μ M PUGNAc and 5mM glucosamine in DMEM containing 1% fetal bovine serum. After 24h of incubation, RNA was extracted by using trizol and was analyzed for the expression of p21 and Egr1 by RT-qPCR. Results are the mean \pm SEM of three independent experiments and statistical analysis was performed by ANOVA followed by Dunnet test (**; $p < 0.01$). Control (ctrl), PUGNAc+glucosamine (PG) and tamoxifen (Tam).

Results obtained in the second part of the study showed that:

- i) The elevation of O-GlcNAcylation by PUGNAc+glucosamine leads to downregulation of expression of ER α at mRNA and at protein level.
- ii) The transcriptional activity of *ESR1* promoter is reduced by enhanced O-GlcNAc level, indicating that the regulation of ER α expression by PUGNAc+glucosamine occurs mainly at transcriptional level.

iii) PUGNac+glucosamine also led to a decrease in tamoxifen-induced expression of Egr1 and p21, suggesting a potential mechanism for the inhibition of tamoxifen effect on cell death.

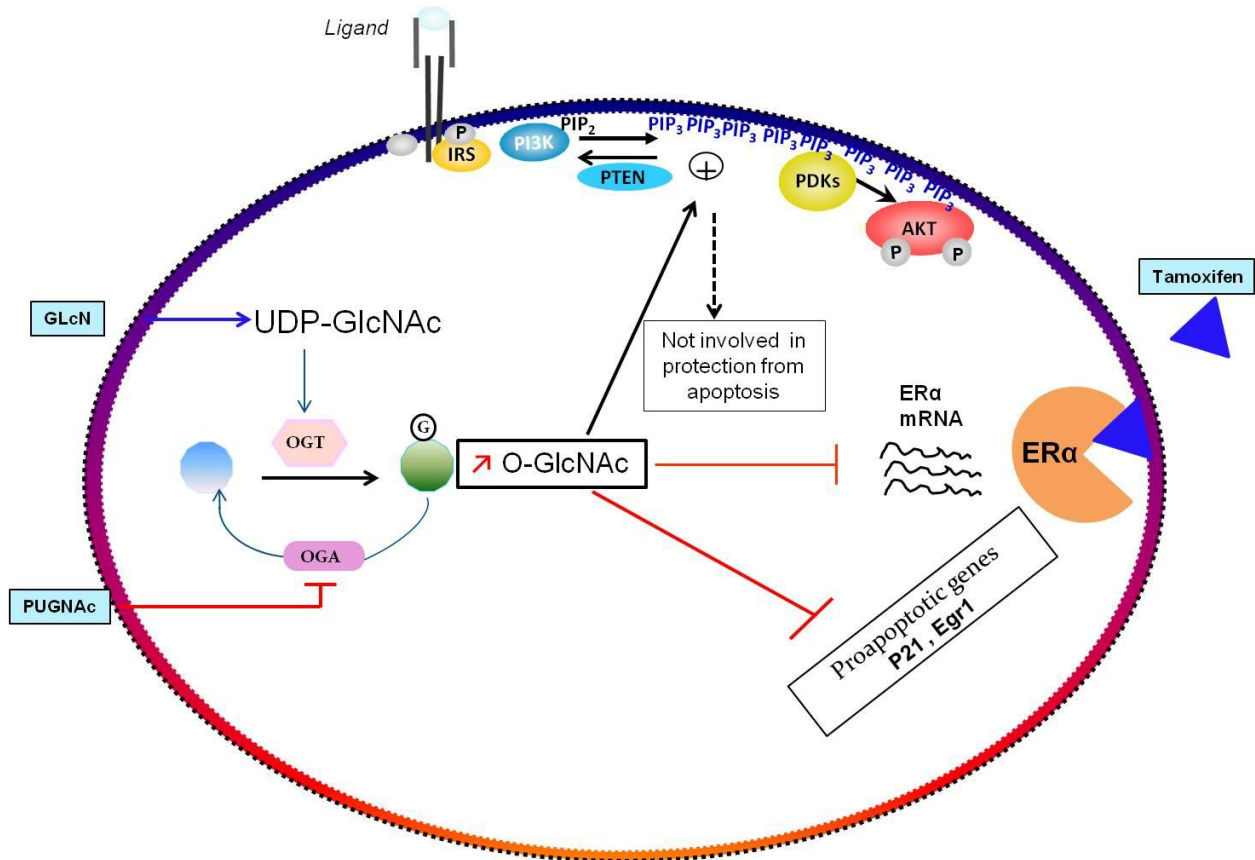


Figure 36: Potential mechanism involved in protection from tamoxifen-induced cell death with enhanced O-GlcNAcylation

DISCUSSION

1. The protective effect of O-GlcNAc against tamoxifen-induced cell death is independent of the PI-3K pathway

The phosphatidylinositol 3-kinase (PI3-k) pathway and its downstream effectors such as Akt and mTOR play essential roles in different cellular processes such as cell survival, proliferation, migration, angiogenesis and apoptosis. Each of these processes is involved in tumor development and cancer. A detailed description of these processes and their role in cancer is given in the first chapter. PI3-K is one of the most frequently mutated pathways in breast cancer. Genetic mutations are found in different components of the pathway. These mutations can result either in the activation of oncogenic components or inactivation of tumor suppressors. The fact that more than 70% of gene mutations lead to constitutive activation of the PI3-K pathway in breast cancer makes it an attractive therapeutic target against breast cancer. Indeed several drugs targeting different components of the PI3-K pathway are in clinical development in breast cancer [485]. In addition, whereas food restriction is known to inhibit tumor growth, a recent study demonstrated that mutations leading to constitutive activation of PI3-K pathway exhibited resistance to the inhibitory effect of food restriction [179].

Akt, an important component of the PI-3 K pathway is a serine/threonine kinase which acts as an important regulator of proliferation, apoptosis and cell survival. To date, several studies demonstrated a significant role of Akt in cancer pathology and inhibitors of this enzyme were shown to reduce tumor growth. Akt is involved in the inhibition of apoptosis through regulation of important proteins involved in apoptosis. For instance Akt is indirectly involved in regulating proteasome degradation of p53 (tumor suppressor) [486, 487]. It can also inhibit apoptosis through inhibition of FOXO mediated transcription of proapoptotic proteins such as BIM, FAS ligand, etc [51]. Akt can also directly inactivate proapoptotic proteins such as BAD [488].

Moreover, Akt can also promote cell cycle progression through inhibition of FOXO mediated transcription of cell cycle inhibitors e.g. p27Kip1 and p21Cip1 [489, 490]. It can also regulate the stability of Myc and cyclin D1 by inhibiting GSK3 [491, 492]. PI3-K and Akt are also involved cell motility and in tumor invasiveness by regulating different proteins e.g. E-cadherin.

In breast cancer the level of pAkt is associated with the size, prognosis and histological grade of tumor [493, 494]. Moreover, Akt activation plays a role in tamoxifen resistance and is associated with poor overall survival in ER α -positive breast cancers [495]. In ER⁺ breast cancer, Akt is involved in both estrogen dependent and estrogen independent transcriptional activity of the ER and may play a role in anti-estrogen resistance. Interestingly, luminal A (ER- α positive, low histological grade), luminal B (low ER- α expression, high histological grade) both are ER⁺ but luminal B benefit less from adjuvant anti-estrogen therapy [496]. It has been shown that the level of p-Akt is enriched in ER⁺ luminal B breast cancer compared to luminal A subtype. This shows that p-Akt plays an important role in modulating the response to anti-hormonal therapy.

Therefore, we investigated that whether treatment with PUGNAc+glucosamine may affect tamoxifen sensitivity through activation of PI3-K pathway. We observed an increase in PIP3 production as well as in the phosphorylation of Akt with PUGNAc and glucosamine treatment. An increase in PIP3 production could be mediated either by upregulation of PI3-kinase expression/activity or because of decrease in expression of PTEN/ loss of its activity.

Most of the key regulatory components of the PI3-K pathway are modified through O-GlcNAc, for instance IRS-1, Akt and PI3-K [497] [255, 498]. However, these studies were conducted in adipocytes and enhanced O-GlcNAc was shown to be negatively associated with activation of PI3-K pathway. Elevation of O-GlcNAc by using PUGNAs has been shown to negatively

regulate phospho-Akt at Thr-308 in 3T3-L1 adipocytes and in rat primary adipocytes [497, 499]. Studies have also demonstrated the link between O-GlcNAcylation and PI3-K pathway. For instance OGT has been shown to directly interact with PIP3 through phosphoinositide-binding domain [287]. However, in this study the recruitment of OGT on the plasma membrane was associated with decrease in insulin signaling through PI3-K pathway. In contrast, in MCF-7 cells we found that phospho-Akt is positively regulated by the elevation of O-GlcNAc level.

Since O-GlcNAcylation of all other components of PI3-K seems to be rather associated with inhibition of PI3-K activation, we tried to evaluate whether increase in PIP3 production with PUGNAc+glucosamine treatment occurred through regulation of PTEN. However, we observed that PUGNAc+glucosamine treatment did not affect the expression of PTEN, as shown in figure 36. We also evaluated the effect of PUGNAc+glucosamine on the lipid phosphatase activity of PTEN by measuring production of free inorganic phosphate in an *in vitro* assay (by using echelon kit available for the measurement of PTEN phosphatase activity). We found in preliminary experiments that PUGNAc+glucosamine did not affect the lipid phosphatase activity of PTEN (data not shown). Therefore, the mechanism by which PUGNAc+glucosamine increases PIP3 production remains unknown at the present time and needs more investigation.

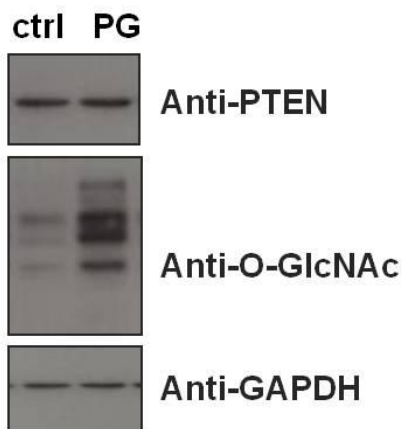


Figure 37: Effect of O-GlcNAc inducing treatments on PTEN expression

MCF-7 cells were seeded in 6 well plates for 24h in DMEM containing 10% fetal bovine serum. After 24h, MCF-7 cells were treated with 100 μ M PUGNAc and 5mM glucosamine in DMEM containing 1% fetal bovine serum. After 24h of incubation, cells were lysed and crude extracts were analyzed by western blotting for PTEN expression. Anti-GAPDH antibody is used as a loading control of western blotting.

The importance of PI3-K pathway in cell survival suggested that the elevation of O-GlcNAcylation may play a role in cell survival of MCF-7 cells through activation of this pathway. Interestingly, a recent study conducted in diverse cancer cell lines showed that the sensitivity/resistance of PI3-K to its inhibitor (GDC-0941) is correlated with the expression of OGT [500]. We hypothesized that PUGNAc+glucosamine might protect MCF-7 cells from tamoxifen-induced cell death by activating the PI3-K pathway. However, by using the pharmacological inhibitor (LY294002) and a potent stimulator (100nM of IGF-1) of the PI3-K pathway, we observed that this pathway is not involved in the protection of tamoxifen-induced cell death.

As the result obtained demonstrated that the pro-survival Akt pathway was not involved in the inhibition of tamoxifen-induced cell death, the next potential target could be some protein which is directly involved in mediating tamoxifen response. The most important biomarker which is used to determine the response of cells to tamoxifen-mediating cell death is the ER α .

2. Elevated O-GlcNAc causes a decrease in expression and promoter activity of estrogen receptor

About 70 % of breast cancers are ER positive and can be treated with anti-estrogen therapies. The presence of ER α is associated with more favorable prognosis, increased differentiation and less invasiveness of the tumor cells. The expression of ER α is also associated with low invasiveness and low motility in human breast cancer cell lines in both nude mice study and in matrigel outgrowth assay [501]. Moreover, *in vitro* studies indicated the anti-invasive effect of tamoxifen is mediated by ER α expression. Both tamoxifen and estrogen bind to ER α but

tamoxifen binding brings a distinct conformational change than that resulting from estrogen binding and leads to the recruitment of corepressors instead of co-activators. By this way, tamoxifen deregulate estrogen signaling pathway. In spite of its beneficial effects, tamoxifen resistance is one of the significant clinical problems encountered in breast cancer therapy.

Our results indicated that the elevation of O-GlcNAc led to the inhibition of ESR1 promoter activity and downregulation of ER α expression at mRNA and protein levels. Owing to previous studies, the expression of ER α is considered as the basic marker for a response to endocrine therapy. This suggests that the protective effect observed with PUGNAc+glucosamine against tamoxifen-induced cell death could be due to downregulation of ER α expression.

3. The decrease in ER α expression with PUGNAc+glucosamine is not inhibited by LY294002.

Our previous results showed that the PI-3K pathway is stimulated by PUGNAc+glucosamine. Moreover, we demonstrated that the PUGNAc+glucosamine led to decreased expression of ER α . Thus, in order to determine whether this decrease in ER α expression was dependent on the PI-3 kinase pathway, we treated MCF-7 cells with PUGNAc+glucosamine in presence and absence of the PI3-K inhibitor LY294002. Interestingly, LY294002 tend to increase basal ER α protein expression. However, the inhibitory effect of PUGNAc+glucosamine on ER α expression was preserved, indicating that this effect was independent of the PI-3K/Akt pathway.

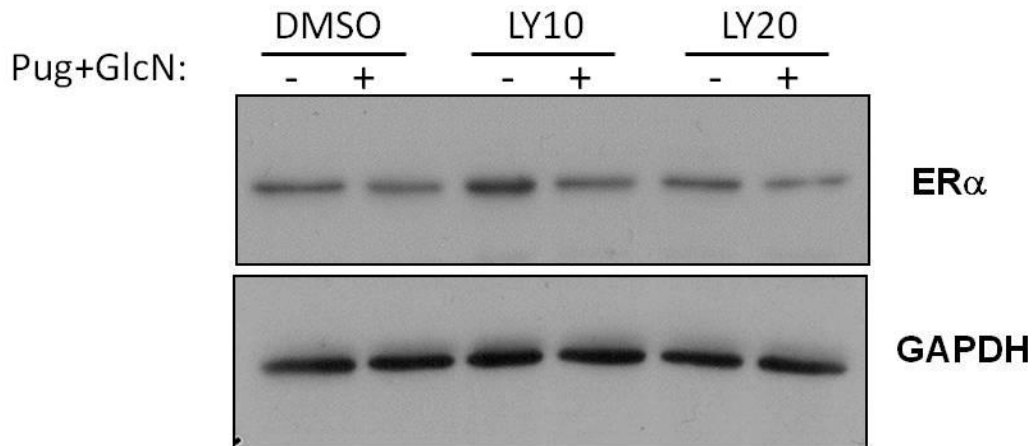


Figure 38: The effect of LY294002 on decrease of ER α expression by PUGNAc+glucosamine.

MCF-7 cells were seeded in 6 well plates for 24 h. They were treated with PUGNAc+glucosamine in absence or presence of 10 or 20 μ M LY294002 (LY) or control (DMSO). After 24h treatment, cells lysate was analyzed for ER α expression by western-blotting. Anti-GAPDH was used as a loading control.

As tamoxifen mainly mediate its effect through ER α , we were interested to determine whether treatments affecting expression of ER- α could modify the expression of tamoxifen regulated genes

4. Decrease in ER expression is correlated with impairment of tamoxifen effect on its target genes

We tried to analyze genes known to be regulated by tamoxifen. P21/Cip1 is one of the extensively studied cyclin dependent kinase inhibitors and is known to be upregulated by tamoxifen. Several studies demonstrated the role of p21 in prognosis or in mediating response to anti-estrogen therapy. It has been shown that p21 expression is increased by tamoxifen treatment in MCF-7 cells, resulting in cell cycle inhibition. In contrast, estrogen leads to the

downregulation of p21 expression leading to proliferative effect [502]. Moreover, the abrogation in p21 leads to decrease anti-estrogenic effect of tamoxifen [502]. The absence or decrease expression of p21 is associated with poor prognosis and low survival. In addition, the loss of p21 was shown to be associated with tamoxifen resistance as well as in promoting proliferative effects of tamoxifen [503]. Collectively, these studies suggest that p21 has tumor suppressing capabilities and can also act as a prognostic marker.

Therefore, we were interested in evaluating the effect of PUGNAc+glucosamine treatment on tamoxifen regulation of p21 expression. Our results demonstrated that the increased expression of p21 induced by tamoxifen is reverted back by O-GlcNAc elevation through PUGNAc+glucosamine. This suggests that inhibition of tamoxifen effect on p21 expression through PUGNAc+glucosamine treatment can play a role in abrogation of tamoxifen inhibitory effect on cell growth. We also evaluated the expression of another tamoxifen regulated gene Egr1 (early growth response 1). Breast tumors and cancer cell lines have reduced expression of Egr1 compared to normal tissues and decrease in Egr1 expression is associated with tumorigenesis. Studies demonstrated that the inhibition of Egr1 expression plays a role in chemoresistance [504]. Moreover, it has been shown that the inhibition of tumor growth by tamoxifen treatment is associated with increase in Egr1 expression [483]. In addition, a link between tamoxifen, Egr1 and p21 expression has been documented. It was demonstrated that tamoxifen-induced increase in p21 expression was mediated through Egr1 and was inhibited by Egr1 depletion [484]. These studies suggested Egr1 as an attractive candidate that could be involved in abrogation of tamoxifen effect by PUGNAc+glucosamine. Our results showed that tamoxifen induced the expression of Egr1 in MCF-7 cells and PUGNAc+glucosamine treatment abrogated this effect.

Altogether, results obtained suggested that the abrogation of tamoxifen-induced increase in p21 and Egr1 expression by PUGNAc+glucosamine may play a role in the protection of MCF-7 cells from tamoxifen-induced cell death.

Conclusion and Perspectives

Conclusion and Perspectives

Our results demonstrated that PIP3 production is increased by elevating O-GlcNAcylation in MCF-7 cells, however the mechanism involved still remains unknown. Preliminary results suggested that the lipid phosphates activity of PTEN is not modified through O-GlcNAc elevation. The other potential candidate that could be investigated is PI3-K (catalytic and regulatory subunits). Although studies have demonstrated the O-GlcNAcylation of PI3-K in adipocytes, the effect of O-GlcNAcylation on PI3-K activity is not known in cancer cells. It will be important to determine whether the increased O-GlcNAcylation by PUGNAc+glucosamine can affect expression/activity of PI3-K enzyme in MCF-7 cells. Moreover, we showed that the phosphorylation of Akt is increased by PUGNAc+glucosamine. Further, it will be interesting to determine the biological consequences (cell migration and protein synthesis) of increase in PIP3 production and p-Akt induction by PUGNAc+glucosamine in MCF-7 cells.

We have shown that the elevation of O-GlcNAcylation leads to reduced ER α expression and decreased activity of *ESR1* promoter. It will be important to evaluate the mechanism through which O-GlcNAcylation could affect the expression of ER α . ER- α expression can be regulated by different mechanisms, one of the recently documented mechanisms involves Wilms' tumor 1 WT1 (a transcription factor). In 2008, Han observed that WT1 decreased tamoxifen-induced cell death in MCF-7 cells through downregulation of ER- α . The overexpression of WT1 resulted in decline of ER- α expression and its bona fide downstream targets (progesterone receptor and PS2). WT1 directly binds on ER promoter leading to the decrease in ER- α transcriptional activity [505]. Moreover, several other studies indicated the inverse association between WT1 and of ER- α expression. Owing to these studies, we hypothesized that the downregulation of ER- α expression by PUGNAc+glucosamine may occur by upregulating WT1 expression. However, the

preliminary results demonstrated that these treatments did not modify the expression of WT1 (data not shown). It will be interesting to evaluate whether WT1 is modified through O-GlcNAcylation.

Moreover, we showed that the tamoxifen-mediated p21 and Egr1 expression was abrogated in presence of PUGNAc+glucosamine and this is correlated with decrease in ER α expression. We will investigate whether this abrogation is regulated by ER α . P21 has ERE half elements in its promoter and can be regulated through estrogen receptor signaling. We will further study the effect of PUGNAc+glucosamine treatment on p21 reporter gene by mutating ERE sites on p21 promoter. Another link could be through ER α regulation by p53. Studies showed that ER α is positively regulated by p53 [506]. Thus, in future studies we could work to explore the effect of O-GlcNAcylation on the axis involving ER α , p53 and p21. We could also evaluate whether the inhibition of p21, Egr1 or ESR1 could mimic the protective effect of PUGNAc+glucosamine on tamoxifen-induced cell death. We can target each of these genes with siRNA and can evaluate whether tamoxifen still cause apoptosis in our system. In contrast, we could overexpressed ER α in MCF-7 prior treating with PUGNAc+glucosamine and determine whether it can abrogate the protective effect of PUGNAc+glucosamine on tamoxifen-induced cell death. In future, *in vivo* study can be conducted to evaluate the effect of OGT inhibition in tamoxifen responsiveness. Studies indicated that the level of O-GlcNAcylation is higher in breast cancer tissue compared to normal epithelial tissue, however no study is reported exploring the relation between level of O-GlcNAcylation and tamoxifen resistance. Therefore, we need to compare the level of O-GlcNAcylation in tamoxifen resistant and sensitive breast cancer cells.

Abstract

One of the hallmarks of cancer cells is to exhibit increased uptake and consumption of glucose. 3-5% of the glucose entering into the cell leads to a minor pathway of the glucose metabolism known as the hexosamine biosynthetic pathway (HBP). UDP-N-acetylglucosamine is the end product of HBP and is used as substrate by OGT (O-GlcNAc transferase) to modify diverse range of nuclear and cytoplasmic proteins with a recently characterized post-translational modification called O-GlcNAcylation. It corresponds to the addition of sugar moiety O-linked β -N-acetylglucosamine (O-GlcNAc) on serine or threonine residue of proteins. This process is antagonized by another enzyme called O-GlcNAcase (OGA). Recent studies indicated the presence of increased O-GlcNAcylation level in several cancer cells. Moreover, inhibition of OGT has been shown to reduce *in vivo* and *in vitro* tumor growth of breast cancer cells. However, the relationship between O-GlcNAcylation and the response to anti-cancer therapy has not been studied. Tamoxifen is the oldest and most prescribed selective-estrogen receptor modulator (SERM) for patients with estrogen receptor (ER)-positive breast cancer. Tamoxifen is known to reduce tumor growth and invasion. Despite its beneficial effects *de novo* and acquired resistance are great obstacles in its clinical effectiveness. We found that O-GlcNAc elevation in MCF-7 cells protected them from tamoxifen-induced cell death. Increased O-GlcNAc also increased PI3-K/Akt signaling. However, the protective effect of PUGNAc+glucosamine from tamoxifen-induced cell death is independent of PI3K/Akt pathway. Increased O-GlcNAcylation also led to reduced *ESR1* promoter activity and decreased expression of ER α at mRNA and protein levels. The decrease in ER α expression is correlated with a reduced expression of two tamoxifen regulated genes i.e. early growth response 1 and p21 Waf1/Cip1. In conclusion, this study showed for the first time the involvement of O-GlcNAcylation in reducing tamoxifen-

sensitivity in MCF-7 cells. Thus, OGT can act as a novel therapeutic target for treatment of tamoxifen resistant cells.

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Manuscript submitted

**O-GlcNAcylation-inducing treatments inhibit estrogen receptor α
expression and confer resistance to 4-OH-Tamoxifen.**

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Abstract

O-GlcNAcylation (addition of N-acetyl-glucosamine on serine or threonine residues) is a post-translational modification that regulates stability, activity or localization of cytosolic and nuclear proteins. O-linked N-acetylglucosamine transferase (OGT) uses UDP-GlcNAc, produced in the hexosamine biosynthetic pathway to O-GlcNAcylate proteins. Removal of O-GlcNAc from proteins is catalyzed by the β -N-Acetylglucosaminidase (OGA). Recent evidences suggest that O-GlcNAcylation may affect the growth of cancer cells. However, the consequences of O-GlcNAcylation on anti-cancer therapy have not been evaluated. In this work, we studied the effects of O-GlcNAcylation on tamoxifen-induced cell death in the breast cancer-derived MCF-7 cells. Treatments that increase O-GlcNAcylation (PUGNAc and/or glucosamine) protected MCF-7 cells from death induced by tamoxifen. In contrast, inhibition of OGT expression by siRNA potentiated the effect of tamoxifen on cell death. Since the PI-3 kinase/Akt pathway is a major regulator of cell survival, we used BRET to evaluate the effect of PUGNAc+glucosamine on PIP₃ production. We observed that these treatments stimulated PIP₃ production in MCF-7 cells. This effect was associated with an increase in Akt phosphorylation. However, the PI-3 kinase inhibitor LY294002, which abolished the effect of PUGNAc+Glucosamine on Akt phosphorylation, did not impair the protective effects of PUGNAc+glucosamine against tamoxifen-induced cell death. These results suggest that the protective effects of O-GlcNAcylation are independent of the PI-3 kinase/Akt pathway.

As tamoxifen sensitivity depends on the estrogen receptor (ER α) expression level, we evaluated the effect of PUGNAc+glucosamine on the expression of this receptor. We observed that O-GlcNAcylation-inducing treatment significantly reduced the expression of ER α mRNA and protein, suggesting a potential mechanism for the decreased tamoxifen sensitivity induced by these treatments. Therefore, our results suggest that inhibition of O-GlcNAcylation may constitute an interesting approach to improve the sensitivity of breast cancer to anti-estrogen therapy.

Key words: O-GlcNAcylation, O-GlcNAc glycosylation, Breast cancer, Estrogen Receptor, tamoxifen resistance, Bioluminescence Resonance Energy Transfer, Phosphatidylinositol 3 phosphate, PI-3 kinase, Akt, PTEN.

Abbreviations

BRET, Bioluminescence Resonance Energy Transfer;

DMEM, Dulbecco's Modified Eagle's Medium

EGR1, Early Growth Response protein 1;

ER α , Estrogen Receptor α

ESR1, Estrogen Receptor 1 gene

FBS : Fetal Bovine Serum

FACS, Fluorescence-Activated Cell Sorting

GFAT, Glutamine fructose-6-phosphate amidotransferase,

GlcN, Glucosamine;

GlcNAc, N-Acetyl-glucosamine;

HBP, Hexosamine Biosynthetic Pathway;

IGF1, Insulin-like Growth Factor-1;

Luc, *Renilla* luciferase

mBU, milliBRET Unit;

OGA, O-linked β -N-Acetyl-glucosaminidase;

OGT, O-GlcNAc Transferase;

PI3K, Phosphatidylinositol 3-Kinase

PH, pleckstrin homology;

PTEN, Phosphatase and Tensin homolog

PUGNAc, O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenylcarbamate;

SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis;

YFP, Yellow Fluorescent Protein

Introduction

Growth and proliferation of cancer cells tightly depend on their nutritional environment, particularly on glucose availability, which is necessary for increased biosynthesis of cellular components associated with proliferation (*e.g.* membranes, proteins and nucleic acids) [1]. Nutritional and metabolic conditions are known to influence tumour development. Excess food intake associated with modern lifestyle constitutes an important cancer risk factor [2]. In animals, food restriction has inhibitory effects on the growth of certain tumours [3], whereas in diet-induced obesity models, overfeeding is associated with accelerated development of tumours [4].

Nutritional conditions can modulate tumour development by modifying insulin and IGF-1 concentrations, which affect signalling pathways involved in cell growth, proliferation and apoptosis. However, at the cellular level, glucose can also directly regulate signalling pathways and multiple biological processes through O-GlcNAc glycosylation (O-GlcNAcylation) of cytosolic and nuclear proteins [5]. O-GlcNAcylation is a reversible post-translational modification, analogous to phosphorylation, which controls protein localisation, stability or activity according to the nutritional environment. It corresponds to the addition of N-Acetylglucosamine (GlcNAc) on serine or threonine residues. This reaction is catalysed by O-GlcNAc transferase (OGT), which uses UDP-GlcNAc as a substrate (Fig. 1). UDP-GlcNAc, produced through the hexosamine biosynthetic pathway (HBP), can be considered as a sensor for the nutritional state of the cell, as it integrates glucose, glutamine, fatty acids (acetyl), uridine and ATP metabolism [5-9]. O-GlcNAc is rapidly removed from proteins by O-GlcNAcase (OGA), permitting dynamic regulation of O-GlcNAcylation levels in cells.

A growing amount of studies indicates that O-GlcNAcylation constitutes an important regulator of cancer growth and invasion. A number of transcription factors involved in the control of cell proliferation or cell death can be regulated by O-GlcNAc [10-15]. Moreover, increased protein O-GlcNAcylation has been detected in cells derived from breast, lung, colon, liver and prostate cancers. These modifications, often associated with changes in OGT and/or OGA levels [16-18], favour the growth and/or migration of tumour cells through different mechanisms, including regulation of transcription factors activity [19, 20], E-cadherin [18, 21], and MMP metalloproteases [18, 20].

Breast cancer is the most common cancer in women. Endocrine therapies have permitted important progress for the treatment of hormone-sensitive breast cancers. However, the development of treatment resistance constitutes an important limitation to these therapies.

Thus, tamoxifen, a partial antagonist of the estrogen receptor, has been largely used for the treatment of estrogen receptor positive breast cancers, but resistance to this drug often occurs [22]. The link between nutritional conditions, obesity and breast cancer is well established, particularly in menopausal women [23]. However, few studies have evaluated the consequences of metabolic manipulations on the efficiency of anti-cancer therapy. In this work, we studied the effect of treatments that induce protein O-GlcNAcylation on the sensitivity to tamoxifen, using the human breast cancer derived, estrogen receptor positive MCF-7 cell line.

Methods

Chemicals and antibodies

Glucosamine (GlcN) was from Sigma-Aldrich, O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenylcarbamate (PUGNAc) from Toronto Research Chemicals Inc., and LY294002 from Cell Signaling Technology. Anti-Akt, anti-Phospho-Akt and anti-phospho-Erk1/2 antibodies were from Cell Signaling Technology, anti-ER α (60C) from Millipore, anti-O-GlcNAc (CTD 110.6) from Covance, anti-GAPDH from Life Technology, anti- β -actin from Sigma, anti-Erk2 (sc 154) and HRP-conjugated donkey anti-rabbit (sc 2305) from Santa Cruz, and HRP-conjugated goat anti-mouse from Jackson ImmunoResearch laboratories.

Cell culture and transfection

MCF-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum. Transfection of siRNA and cDNAs were performed with Lipofectamine RNAi Max (Life Technologies) and FuGene 6 (Promega), respectively. The negative control siRNA was from Eurogentec (Seraing, Belgium).

Uptiblu assay

Cells were plated in clear 96-well-plates at a density of 2000 cells/well. 24 h after plating, cell population density was evaluated 2h30 after adding 10% Uptiblu reagent, by measuring fluorescence (initial fluorescence) at 595 nm using Typhoon 9400 imager (GE Healthcare). Cells were then cultured for 24 or 48 h in fresh media containing the different agents, and then the final fluorescence was measured. The cell population growth in each well was expressed as the ratio of the final fluorescence over the initial one in the same culture well [24].

Apoptosis analysis

After treatment in 6-well-plates, cells were harvested by trypsin digestion, washed in PBS and labelled with both Annexin-V-FITC and propidium iodide using the Annexin-V FLUOS kit (Roche Diagnostics) according to manufacturers' instructions. Cells were analyzed by flow cytometry with the FC 500 cytometer (Beckman Coulter).

BRET experiments

Luc-Akt-PH and YFP-Mem cDNAs used for the study of PIP₃ production have been previously described [25]. MCF-7 cells were transfected with 0.7 µg Luc-Akt-PH and 0.3 µg pYFP-Mem cDNAs per 10.3 mm dish and transferred to a 96 well plate 24 h before BRET experiments. BRET experiments were performed as described previously [26, 27]. Results were expressed in milliBRET units as previously described [28, 29].

Luciferase assay

The *ESR1-Luc* reporter gene (firefly luciferase coding sequence under the control of the P1 promoter of *ESR1* gene [30], kindly provided by Dr. M. LE ROMANCER-CHERIFI) was used. Cells were seeded in 12-well-plates and transfected 24 h later with 1 µg of ESR1-Luc plasmid combined with 2 ng of a Renilla luciferase cDNA to normalize for transfection efficiency. After treatment, cells were lysed and luciferase activities were measured with a Centro LB 910 luminometer (Berthold) using the DUAL Luciferase Assay kit (Promega).

Western-blotting

MCF-7 cells were lysed with buffer containing 50 mM Tris-HCl (pH 8), 137 mM NaCl, 10% (v/v) glycerol, 1% (v/v) NP40, 50 mM NaF, 10 mM di-sodium β-glycerophosphate, 1 mM Na₃VO₄, 1 mM streptozotocin and protease inhibitors (1µg/ml pepstatin, antipain, leupeptin, aprotinin and AEBSF). Proteins were then analysed by SDS-PAGE followed by western-blotting [31].

RNA extraction, Reverse Transcription and qPCR

After treatment, cells cultured in 6-well-plate were lysed directly in Trizol reagent (Life Technologies) and RNA were isolated and reverse transcribed as previously described [32].

Levels of the cDNA of interest were measured by qPCR using LightCycler FastStart DNA Master SYBR Green 1 kit. To ensure absence of genomic DNA contamination, RNA samples were treated in parallel without Reverse Transcriptase and controlled for absence of amplification by qPCR. The sequences of the primers used in these experiments are: ESR1: Forward, GCATTCTACAGGCCAAATTCAG, Reverse, GTCGTTATGTCCTTG AATACTTC; p21: Forward, TGTACCCTTGTGCCTCGCTCAG; Reverse, TGTA CCCTTGTGCCTCGCTCAG; EGR1: Forward, GCACCTGACCGCAGAGTCTT, Reverse, AGTGGTTTGGCTGGGGTAACT; Cyclophilin A: Forward, GGTGACTTC ACACGCCATAATG, Reverse, ACAAGATGCCAGGACCCGTAT. Gene expression was normalized using cyclophilin A mRNA level as a reference.

Statistical analysis

Statistical analyses were performed with Prism software (GraphPad) using either a *t* test or ANOVA followed by post-test as indicated in figure legends.

Results

O-GlcNAcylation-inducing treatments protect MCF-7 cells from 4OH-Tamoxifen-induced cell death.

We used the Uptibblue, which monitors the cellular growth within the same well, to evaluate the effect of different treatments on MCF-7 cells [24]. Cells were treated with PUGNAc (inhibitor of OGA) and glucosamine (bypasses the GFAT rate limiting step) in absence or presence of 4-OH-Tamoxifen. Treatment with PUGNAc and glucosamine (GlcN) markedly increase O-GlcNAcylation of proteins in MCF-7 cells, both in the absence and presence of 4-OH-Tamoxifen (Supplementary Figure S1).

Figure 2A shows that treatment with PUGNAc and/or GlcN had no significant effect on cell growth. 4-OH-Tamoxifen markedly reduced the growth of the cell population after 24 h of culture. Interestingly, the effect of 4-OH-Tamoxifen was partially reversed by the presence of either PUGNAc or GlcN, and completely reversed when adding both agents together. Similar results were observed after 48 h of culture with the different agents (Fig. 2B). These results suggest that treatment with PUGNAc and GlcN protects MCF-7 cells from 4-OH-Tamoxifen-induced cell death.

We then studied more specifically the effects of 24h treatments on apoptosis using FACS analysis, after labelling the cells with Annexin V-FITC and propidium iodide. In absence of Tamoxifen, PUGNAc and GlcN had no significant effect on cell apoptosis. Tamoxifen induced a 2-fold increase in apoptosis, which was prevented by the presence of PUGNAc + GlcN (Fig. 2C).

To confirm the involvement of O-GlcNAc modifications in protection from 4OH-tamoxifen-induced cell death, we used siRNA to study the effect of inhibiting OGT expression on apoptosis. As shown in Fig. 3A, the expression of OGT was inhibited in siOGT-transfected cells compared to control (siNeg-transfected) cells, which in turn resulted in a decrease in the level of O-GlcNAcylated proteins. Fig. 3B shows that inhibition of OGT expression increased tamoxifen-induced cell death. Altogether, these results indicate that increased O-GlcNAcylation protects MCF-7 cells from tamoxifen-induced apoptosis, whereas inhibition of OGT sensitizes these cells to the effects of tamoxifen.

O-GlcNAc-inducing treatments stimulate the PI-3 kinase/Akt pathway in MCF-7 cells.

The PI-3 kinase/Akt pathway is known as a major anti-apoptotic pathway in MCF-7 cells. To determine whether O-GlcNAcylation-inducing treatments may affect this pathway, we used our previously developed BRET-based assay to monitor PIP₃ production in intact living cells

[25]. In this assay, cells are co-transfected with cDNA coding for the PH domain of Akt fused to *Renilla* Luciferase and YFP targeted to the plasma membrane (Fig. 4A). Cells were incubated with PUGNAc, GlcN or both for 6h prior to BRET assay. Whereas treatment with PUGNAc or GlcN alone had only modest effects on PIP₃ production, a significant increase was observed when both agents were added together (Fig. 4B, C). In agreement with this result, we observed that PUGNAc+GlcN treatment for 6 or 24h resulted in increased Akt phosphorylation in MCF-7 cells (Fig. 4D).

Protection of 4-OH-Tamoxifen-induced cell death by O-GlcNAc-inducing treatments is independent of PI-3 kinase/Akt pathway.

To determine whether PUGNAc+GlcN protective effects against 4-OH-Tamoxifen-induced cell death were mediated by their stimulatory effect on PI-3 kinase/Akt pathway, we evaluated the effects of these treatments in presence of LY294002, a PI-3 kinase inhibitor. We observed that LY294002, which markedly inhibited Akt phosphorylation in these conditions (Supplementary Figure S2), did not impair PUGNAc+GlcN rescue from 4-OH-Tamoxifen-induced cell death (Fig. 5). This result strongly suggested that the protective effect of O-GlcNAcylation-inducing treatment is not mediated by the PI-3 kinase pathway.

Independence of the PI-3kinase pathway was confirmed by evaluating the effect of IGF-1. As previously demonstrated [25], IGF-1 markedly stimulated PIP₃ production in these cells (Fig. 6A, B). Although IGF-1 had a much higher effect on PIP₃ production than PUGNAc+GlcN (Fig. 6B), no significant rescue from 4-OH-Tamoxifen-induced cell death was observed (Fig 6C). In contrast, the protective effect of PUGNAc+GlcN against tamoxifen induced cell death was still observed, and was similar in absence and presence of IGF1.

Therefore, although these experiments reveal a novel and highly interesting effect of O-GlcNAcylation-inducing treatments on the PI-3 kinase/Akt pathway, protection against 4-OH-Tamoxifen-induced cell death by these treatments appears to be independent of this pathway.

ER α expression is inhibited by increased O-GlcNAcylation in MCF-7 cells

Tamoxifen effects on breast cancer are largely mediated by its antagonistic action against the estrogen receptor. A number of evidences indicate that the breast cancer cell sensitivity to tamoxifen depends on the expression level of the estrogen receptor ER α [33, 34]. Low expression level of *ESR1* gene, which codes for ER α , is an important determinant of tamoxifen resistance in ER-positive breast tumors [35]. We hypothesised that treatment with O-GlcNAcylation-inducing agents may reduce 4-OH-Tamoxifen-induced cell death through inhibition of ER α . Therefore, we evaluated the effect of PUGNAc+GlcN on the expression of

ER α in MCF-7 cells by RT-qPCR and western-blotting. As shown in Fig. 7, 24h treatment of MCF-7 cells with PUGNAc+GlcN markedly reduced ER α expression at both the mRNA and protein levels. Time-course experiments (Fig. 8) indicated that inhibition of ER α expression could already be detected at the mRNA level 6h after of treatment with PUGNAc+GlcN (Fig. 8A), and was readily detected at the protein level after 12 h of treatment (Fig. 8B).

To determine whether O-GlcNAcylation-inducing treatments affect ER α expression through a transcriptional mechanism, we transfected MCF-7 cells with a reporter gene constituting of the firefly luciferase coding sequence under the control of *ESR1* promoter. Twelve hours after transfection, cells were treated with PUGNAc+GlcN for 24h, and then lysed for luciferase activity measurements. As shown in Fig. 9A, PUGNAc+GlcN treatment markedly reduced the expression of *ESR1* promoter reporter gene. To further confirm the role of protein O-GlcNAcylation in the regulation of *ESR1* promoter, a luciferase reporter gene assay was performed after over-expression of OGT. Transfection with OGT cDNA increased OGT expression and protein O-GlcNAcylation in MCF-7 cells (Supplementary Fig. S3). Fig. 9B shows that the *ESR1-Luc* reporter gene expression was significantly reduced when OGT cDNA was co-transfected with the reporter gene. This inhibitory effect was accentuated by glucosamine, which provides UDP-GlcNAc to OGT. We also evaluated the effect of PUGNAc+GlcN on ER α expression in presence of tamoxifen. The inhibitory effect of PUGNAc+GlcN on *ESR1* promoter activity and mRNA expression was still observed when cells were cultured in presence of tamoxifen (Supplementary Figure S4). As previously described [36, 37], tamoxifen treatment increased ER α protein expression level, however, the inhibitory effect of PUGNAc+GlcN on ER α protein level was still observed in these conditions.

To determine whether O-GlcNAc-induced inhibition of ER α expression was associated with an inhibition of tamoxifen effect on gene expression, we studied the mRNA expression of genes coding for p21 and Egr1 by RT-qPCR. p21 and Egr1 negatively regulate cell proliferation and were previously shown to be activated by tamoxifen in MCF-7 cells [38, 39]. We observed PUGNAc+GlcN reduced the stimulatory effect of tamoxifen on the expression of these genes (Fig. 10).

Altogether, these data suggest that O-GlcNAcylation-inducing treatments inhibited ER α expression, and this inhibition was associated with a reduction of tamoxifen effect on key genes involved in regulation of cell proliferation.

Discussion

O-GlcNAcylation has been implicated as an important determinant in cancer cell growth and migration [16-18, 21][19][18]. However, its role in the sensitivity to anti-cancer therapy was not investigated in these studies. In the present work, we demonstrate that increasing O-GlcNAcylation results in important protective effects against tamoxifen-induced cell death in the MCF-7 cell line.

In MCF-7 cells, increased activity of the PI-3 kinase/Akt pathway has been previously shown to participate in resistance to various chemotherapeutic agents, including tamoxifen [40]. To evaluate the potential involvement of this pathway in the protective effects of O-GlcNAc-inducing treatments, we used our previously developed BRET-based assay to assess PIP₃ level in living cells. We observed that PUGNAc+GlcN treatment significantly increased PIP₃ production in MCF-7 cells and was associated with an increase in Akt phosphorylation. Interestingly, recent work also suggested a link between PI-3 kinase activity and OGT expression level in cancer cells [41]. The mechanism by which O-GlcNAc increases PI-3K/Akt pathway activity in MCF-7 cells remains elusive. Whereas O-GlcNAcylation of the p85 subunit [42] of PI-3K has been previously described, its consequences on its activity have not been studied. Moreover, whereas O-GlcNAcylation of Akt has been largely described [5, 9, 43, 44], this modification is rather associated with a decrease in its phosphorylation. PIP₃ level at the plasma membrane depends on the balance between PIP₂ phosphorylation by PI-3 kinase activity and PIP₃ dephosphorylation by the lipid-phosphatase PTEN. Clearly, the effect of O-GlcNAc treatment on PI-3 kinase and PTEN expression and/or activity in MCF-7 cells deserves further investigations.

Whatever the mechanism involved in O-GlcNAc-induced increase in PI-3-kinase/Akt pathway in these cells, it does not appear to play a significant role in the protective effect of O-GlcNAc against tamoxifen-induced cell death. Indeed, pharmacological inhibition of the PI-3 kinase/Akt pathway using LY294002 did not impair the protective effects of PUGNAc+GlcN treatments on 4-OH-tamoxifen induced cell death. Moreover, using IGF1, we demonstrated that activation of PI-3 kinase is not sufficient to rescue MCF-7 cells from tamoxifen effects, whereas PUGNAc+GlcNAc treatment was still efficient under these conditions. This prompted us to investigate whether O-GlcNAcylation, rather than acting through a general anti-apoptotic signalling pathway, may affect a protein more specifically involved in tamoxifen action.

Tamoxifen exerts its effects on estrogen-receptor positive cells by binding to ER α . ER α expression is both necessary and sufficient to predict the responsiveness to anti-estrogen in a

high proportion of breast tumors, and low expression level is generally associated with a poor prognosis [33-35, 45, 46].

We observed that O-GlcNAcylation-inducing treatment results in inhibition of ER α expression, suggesting a potential mechanism for decreased tamoxifen effect. Inhibition of ER α mRNA expression could be detected after only 6 h of treatment, and luciferase reporter gene assay indicated that PUGNAc+GlcN treatments inhibit the activity of the ESR1 promoter reporter gene. This suggests that the inhibition of ER α expression occurred at least in part through a transcriptional repression mechanism. This inhibition appeared to be independent of the effect of O-GlcNAc on PI-3 kinase/Akt pathway, because treatment of MCF-7 cells with LY294002 did not impair inhibition of ER α expression by PUGNAc+GlcN treatment (data not shown).

Positive and negative regulation of gene expression by post-translational modification of transcription factors has been largely documented [8, 47, 48]. Interestingly, Sp1, one the first transcription factors identified to be modified by O-GlcNAcylation [49], is known to bind to and positively regulate the *ESR1* promoter activity [50]. Indeed, Sp1 appears to participate in a transcription complex (comprised of USF1 and ER α itself) that regulates the expression of *ESR1*. O-GlcNAcylation of Sp1 has been largely associated with decreased transcriptional activity on various promoters, generally through dissociation of its interaction with transcription factors or co-activators [51-57]. Therefore, it would be of interest to determine whether the effect of PUGNAc+GlcN in MCF-7 cells is associated with dissociation of this complex.

Expression of the ER α , a good prognostic factor in breast cancer, is associated with higher levels of p21 proteins. Tamoxifen is known to modulate the expression of genes involved in cell proliferation and cell death. Increased expression of the cell cycle regulator p21^{WAF1/CIP1} is believed to play a role in cell cycle arrest upon treatment with tamoxifen and other anti-estrogen drugs [38, 58]. Moreover, down-regulation of p21^{WAF1/CIP1} using antisense RNA abrogates anti-estrogen-mediated cell cycle arrest in MCF-7 cells [38]. Similarly, Egr1 is deleted in ER-negative human breast carcinoma, which is suggested to contribute to the poor prognosis of ER-negative versus ER-positive breast carcinomas [59]. Decreased Egr1 expression in human, mouse and rat mammary cells and tissues correlated with tumor formation, and tamoxifen treatment was shown to restore its expression in rat mammary tumors [39]. Moreover, in the ER-positive breast cancer cells MDA-MB-361, tamoxifen-induced increase in Egr1 expression resulted in activation p21^{WAF1/CIP1} promoter and increased

transcription of $p21^{WAF1/CIP1}$ gene, providing a link between *Egr1* transcriptional activity, *p21* expression and tamoxifen-induced cell cycle arrest [60]. Our results indicated that O-GlcNAcylation-inducing treatment reduced the stimulatory effect of tamoxifen on *Egr1* and *p21* expression, providing a potential mechanism for the protective effects of O-GlcNAc on tamoxifen-induced inhibition of cell growth.

In summary, we have observed that increased O-GlcNAcylation in MCF-7 cells have inhibitory effects on tamoxifen induced cell death. Although O-GlcNAc-inducing treatments stimulate the PI-3 kinase/Akt pathway through an unknown mechanism, protection from tamoxifen-induced cell death appear to be independent of this pathway. Moreover, we observed that increased O-GlcNAcylation reduces the expression of ER α , one of the main determinants of tamoxifen sensitivity. Our results suggest that targeting the O-GlcNAc pathway might be an interesting therapeutic approach for sensitisation of anti-estrogen resistant breast tumors.

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Figure legends

Figure 1: The hexosamine biosynthetic pathway controls O-GlcNAc-modification of proteins. Cytosolic and nuclear O-GlcNAc glycosylation constitutes a dynamic process that regulates the activity, the localisation or the stability of the modified proteins. O-GlcNAc glycosylation of proteins on serine and threonine residues depends on the flux of glucose through the hexosamine biosynthetic pathway (HBP). A fraction (2 to 5%) of the glucose entering the cell is directed into this pathway for the biosynthesis of UDP-GlcNAc. OGT uses UDP-GlcNAc as a substrate to add GlcNAc on serine or threonine residues, and its activity is tightly dependent on the concentration of UDP-GlcNAc in the cell. These modifications can be reversed by O-GlcNAcase (OGA), which removes the O-GlcNAc moiety from O-GlcNAc-modified proteins. Experimentally, the level of O-GlcNAc in proteins can be increased by incubating cells with glucosamine (which bypasses allosteric inhibition of the rate limiting enzyme GFAT (Glutamine Fructose 6-P amidotransferase)), or with PUGNAc (O-[2-acetamido-2-deoxy-D-glucopyranosylidene] amino-N-phenylcarbamate), which inhibits deglycosylation by OGA.

Figure 2: O-GlcNAcylation-inducing treatments protect from tamoxifen-induced cell death. MCF-7 cells were cultured in presence of 1% SVF during 24 h (A) or 48 h (B), in the absence or presence PUGNAc (100 μ M), Glucosamine (GlcN, 5 mM) and 4-OH-tamoxifen (10 μ M). The cell population growth in each well was evaluated using an Uptiblué-based-assay as the ratio of final fluorescence over the initial one (broken line) in the same well. Each determination corresponds to measurements performed in triplicate wells. Results are the mean \pm SEM of at least 7 independent experiments. Statistical analysis was performed using ANOVA followed by Dunnet's post-test. *, P< 0.05; **, P< 0.01 when compared to untreated control; NS, not significant. (C) MCF-7 cells were cultured in presence of 1% SVF during 24 h, in the absence or presence of PUGNAc+GlcN and 4-OH-tamoxifen (10 μ M) and then analysed by FACS after Annexin V-FITC/Propidium Iodide staining. Statistical analysis was performed using ANOVA followed by Tukey's post-test *, P< 0.05; **, P< 0.01. Results correspond to the mean \pm SEM of at least 4 independent experiments.

Figure 3: Inhibition of OGT expression sensitizes MCF-7 cells to tamoxifen-induced apoptosis. MCF-7 cells were transfected with control (siNEG) or anti-OGT siRNA (siOGT, sequence: TGGCATCGACCTCAAAGCA). (A) OGT protein expression and global O-GlcNAc levels were analysed 48h later by western-blot. (B) 48h after transfection with siRNA, cells were cultured for 24 hours in absence or presence of 4-OH-tamoxifen (10 μ M) and then analysed by FACS after Annexin V-FITC/Propidium Iodide staining. Statistical analysis was performed using ANOVA followed by Tukey's post-test *, $P < 0.05$. Results correspond to the mean \pm SEM of 5 independent experiments.

Figure 4: O-GlcNAc-inducing treatments stimulate the PI-3 kinase/Akt pathway in MCF-7 cells. (A) Principle of the BRET assay to measure PIP₃ production in living cells. Activation of PI-3 kinase induces the phosphorylation of phosphatidyl-inositol 2 phosphate (PIP₂) into phosphatidyl-inositol 3 phosphate (PIP₃) and subsequent recruitment of Akt to the plasma membrane through its pleckstrin homology (PH) domain. To monitor the production of PIP₃ induced by receptor activation, cells are co-transfected with cDNAs coding for the PH domain of Akt fused to luciferase (Luc-Akt-PH) and YFP fused to a membrane localization sequence. (B) 48 h after transfection, cells were pre-incubated for 6 h in presence of PUGNAc, GlcN or both. Cells were incubated for 10 min with coelenterazine, then light acquisition at 480 nm and 530 nm started. A typical experiment is shown. (C) PUGNAc and GlcNAc-induced BRET (BRET above basal at the plateau). Results are the means \pm S.E.M. of 4 to 8 independent experiments. Statistical analysis was performed using ANOVA followed by Dunnet's post-test. **, $P < 0.01$ when compared to untreated control. (D) Effect of O-GlcNAcylation-inducing treatment on Akt phosphorylation. MCF-7 cells were incubated in the absence or presence of PUGNAc+GlcN for 6h or 24 h and lysed. The phosphorylation of Akt was evaluated by western-blot using anti-phospho-S473-Akt antibody.

Figure 5: Protection of MCF-7 cells from 4-OH-tamoxifen-induced cell death is not abrogated by inhibition of the PI-3 kinase/Akt pathway. MCF-7 cells were cultured in presence of 1% SVF during 24 hours in the absence or presence PUGNAc (100 μ M), Glucosamine (GlcN, 5 mM), 4-OH-tamoxifen (10 μ M) and either DMSO (vehicle) or the PI-3 kinase inhibitor LY294002 (LY) at a concentration of 10 or 20 μ M. The growth of the cell population in each well was evaluated using Uptiblue-based-assay as the ratio of final

fluorescence over the initial one (broken line) in the same well. Each determination corresponds to measurements performed in triplicate wells. Results are the mean \pm SEM of 3 independent experiments. Statistical analysis was performed using ANOVA followed by Dunnet's post-test. *, $P < 0.05$ when compared to corresponding untreated control; NS, not significant.

Figure 6: IGF-1 treatment does not protect MCF-7 cells from 4OH-tamoxifen-induced cell death. (A) MCF-7 cells were co-transfected with cDNAs coding for the PH domain of Akt fused to luciferase (Luc-Akt-PH) and YFP fused to a membrane localization sequence. 48 h after transfection, cells were pre-incubated for 6 h in the absence or presence of PUGNAc+GlcN. Cells were incubated for 10 min with coelenterazine and then stimulated with 100 nM IGF-1. Light acquisition at 480 nm and 530 nm started immediately after IGF-1 addition. A typical experiment is shown. (B) PUGNAc+GlcNAc and IGF-1-induced BRET (BRET above basal at the plateau). Results are the means \pm S.E.M. of at least 5 independent experiments. Statistical analysis was performed using ANOVA followed by Tukey's post-test. *, $P < 0.05$; **, $P < 0.01$; NS, not significant. (C) MCF-7 cells were cultured in presence of 1% SVF during 24 h in the absence or presence PUGNAc (100 μ M), Glucosamine (GlcN, 5 mM), 4-OH-tamoxifen (10 μ M) and IGF-1 (100 nM). Cell population growth in each well was evaluated using an Uptiblue-based-assay as the ratio of final fluorescence over the initial one (broken line) in the same well. Each determination corresponds to measurements performed in triplicate wells. Results are the mean \pm SEM of 5 independent experiments. Statistical analysis was performed using ANOVA followed by Tukey's post-test. *, $P < 0.05$; **, $P < 0.01$; NS, not significant.

Figure 7: Inhibition of ER α expression level by O-GlcNAcylation-inducing treatments Cells were cultured for 24 h in the absence or presence of PUGNAc+GlcN. (A) RNA were extracted and the expression of ER α mRNA was evaluated by RT-qPCR. (B) Cells were lysed and the expression of ER α protein was analysed by western-blot. GAPDH expression level was used as loading control. (C) ER α /GAPDH signals quantified by densitometric analysis of the autoradiograms of western-blot from 6 independent experiments. Statistical analysis was performed using unpaired t test. *, $P < 0.05$; ***, $P < 0.001$.

Figure 8: Time-course of inhibition of ER α expression by O-GlcNAcylation-inducing treatments. Cells were cultured in absence or presence of PUGNAc+GlcN for 6, 12, 24 and 36 h. (A) RNA was extracted and the expression of ER α mRNA was evaluated by RT-qPCR. Statistical analysis was performed using unpaired *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Results correspond to the mean \pm SEM of at least 4 independent experiments. (B) Cells were lysed at the indicated times and the expression of ER α protein was analysed by western-blot. GAPDH expression level was used as loading control. The effect of PUGNAc+GlcN treatment on O-GlcNAcylation of proteins was controlled using anti-O-GlcNAc antibody. Results are representative of at least 3 independent experiments.

Figure 9: Inhibition of ESR1 promoter activity by O-GlcNAcylation-inducing treatments (A) MCF-7 cells were co-transfected with *ESR1* promoter-Firefly luciferase reporter gene (*ESR1*-luc) and *Renilla* luciferase cDNAs. 12 h after transfection, cells were treated with PUGNAc+GlcN for 24 h and then lysed for determination of Firefly and Renilla luciferase activities. Each determination was performed in triplicate. Results are mean \pm SEM of three independent experiments. Statistical analysis was performed using unpaired *t* test ; *, $P < 0.05$. (B) Cells were transfected as in A, but the *ESR1*-Luc and *Renilla* luciferase plasmids were co-transfected with either pcDNA3.1 control plasmid or OGT expression vector. Cells were then treated with GlcN for 24 h and then lysed for determination of Firefly and Renilla luciferase activities. Each determination was performed in triplicate. Statistical analysis was performed using ANOVA followed by Tukey's post-test **, $P < 0.01$; ***, $P < 0.001$. Results correspond to the mean \pm SEM of 4 independent experiments.

Figure 10: PUGNAc+GlcNAc treatment inhibited the effect of 4-OH-tamoxifen on *p21* and *Egr1* gene expression. Cells were cultured for 24h in absence or presence of 4-OH tamoxifen or in presence of 4-OH-Tamoxifen and PUGNAc+GlcN. (A) RNA were extracted and the level of *p21* and *Egr1* mRNA was evaluated by RT-qPCR and normalised using Cyclophilin A expression levels. Results are the mean \pm SEM of 3 independent experiments. Statistical analysis was performed by ANOVA followed by Dunnet's post-test. *, $P < 0.05$; **, $P < 0.01$ when compared to untreated control.

Figure 1

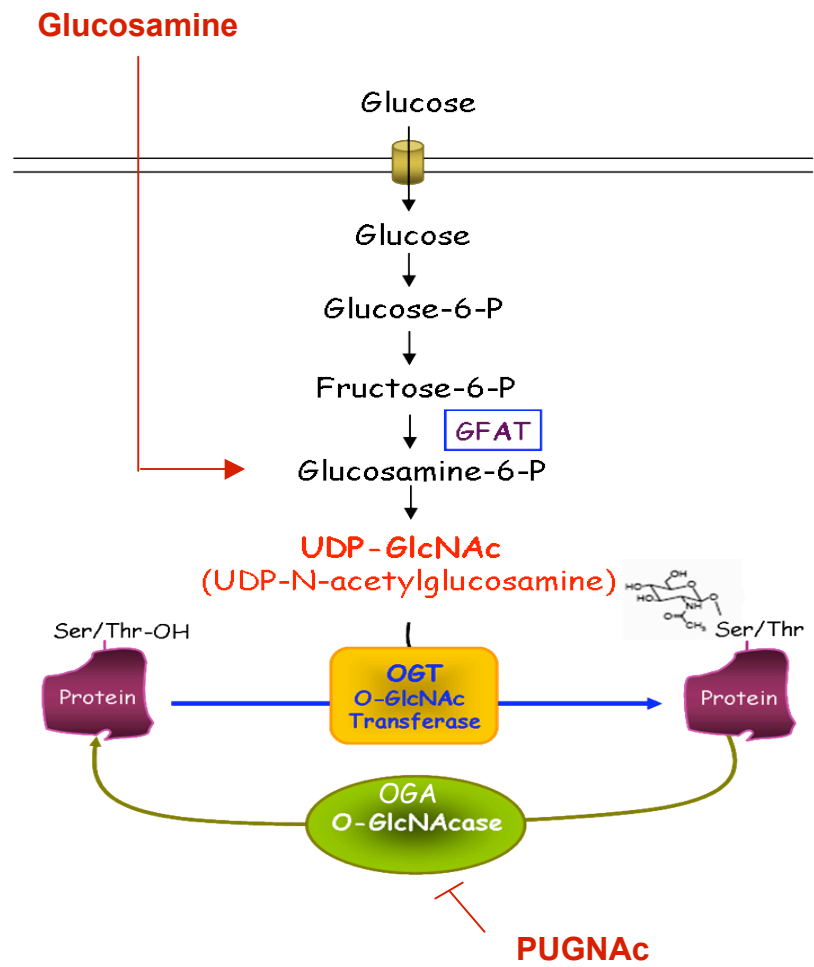
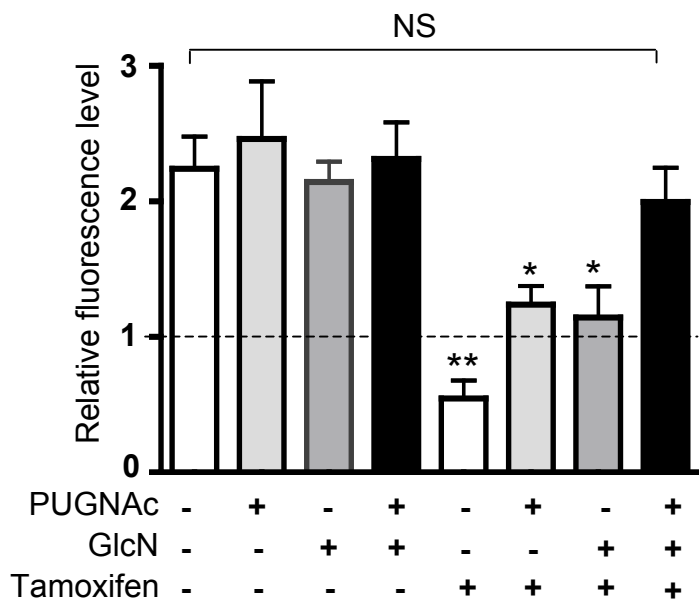
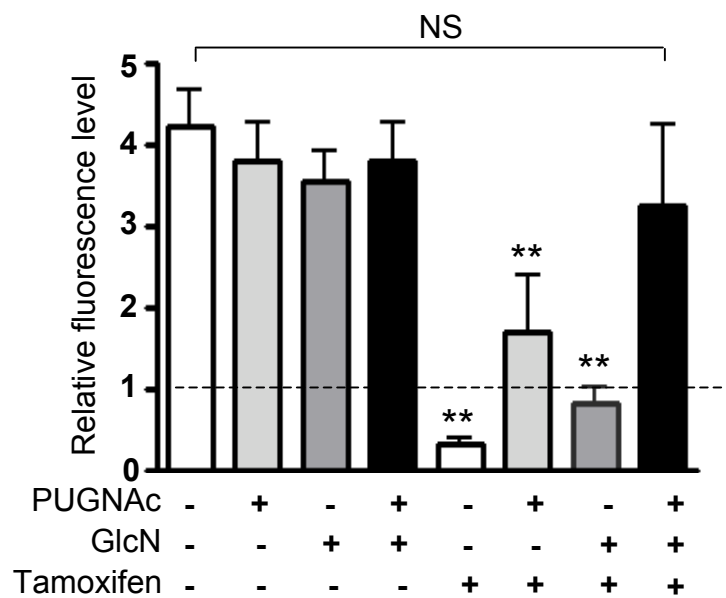


Figure 2

A



B



C

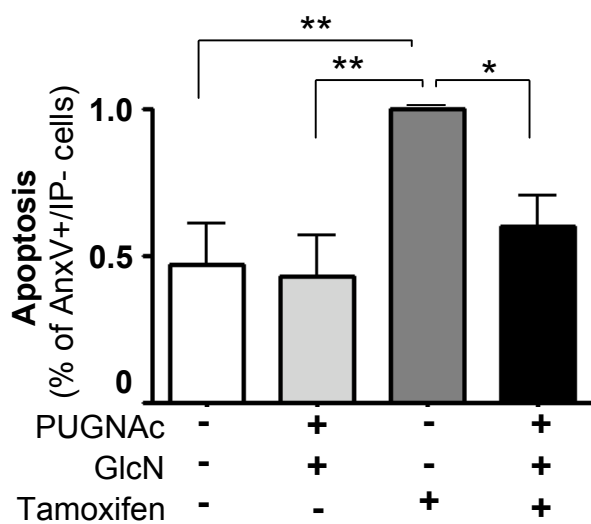
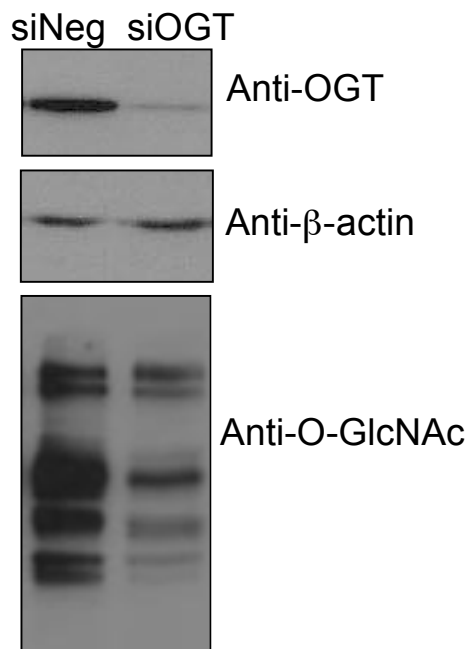


Figure 3

A



B

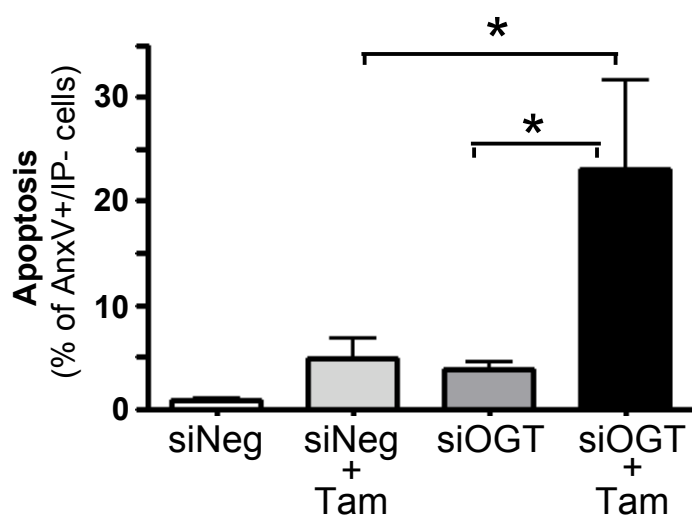


Figure 4

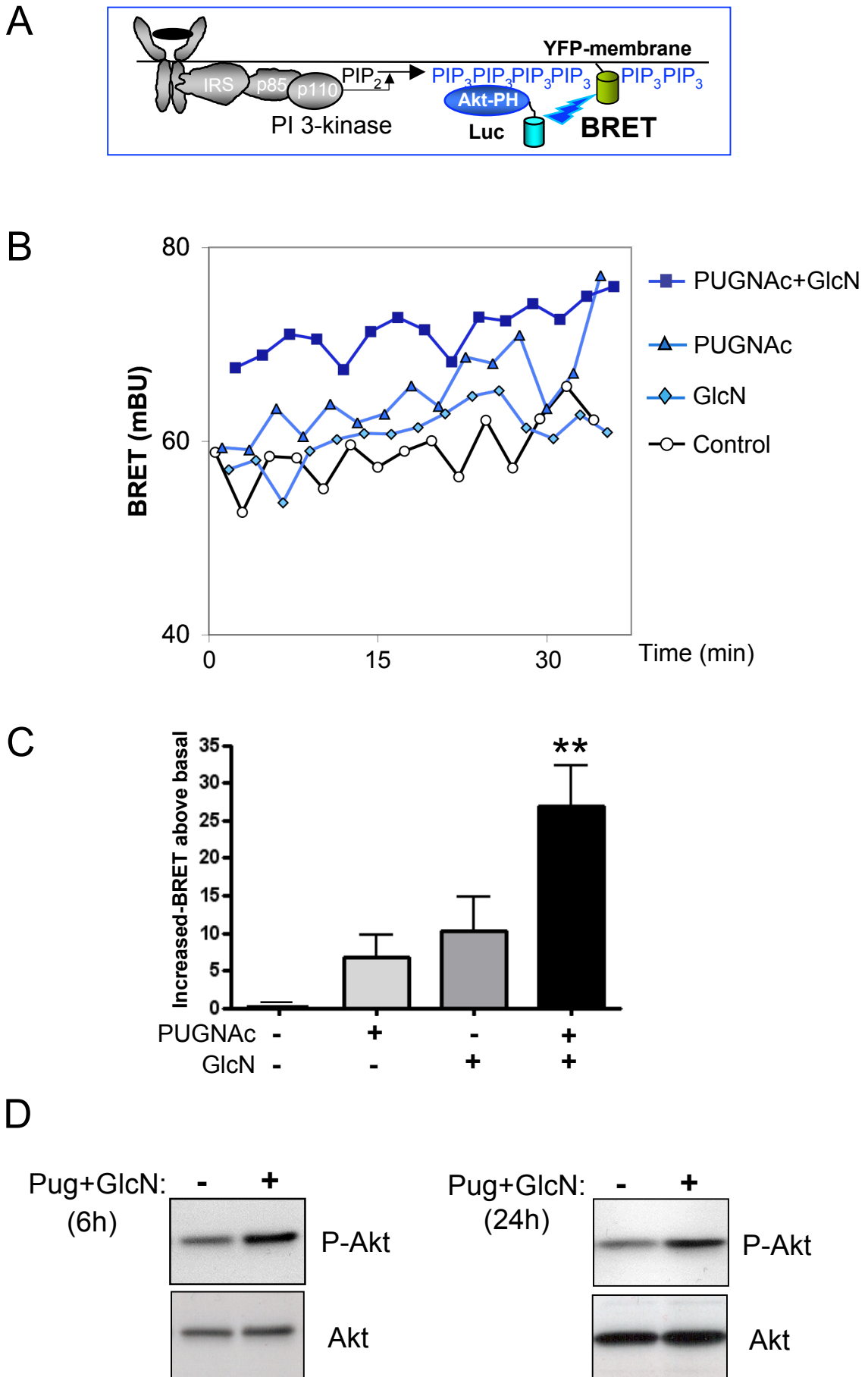


Figure 5

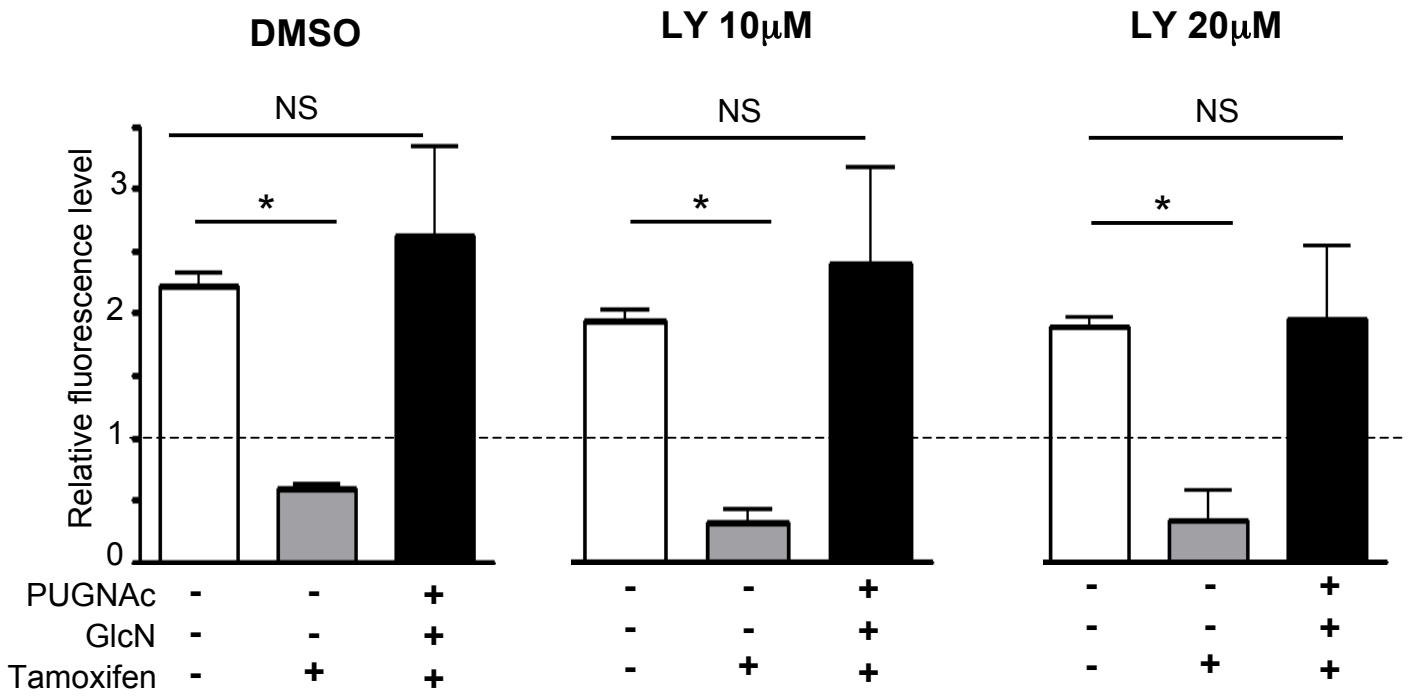


Figure 6

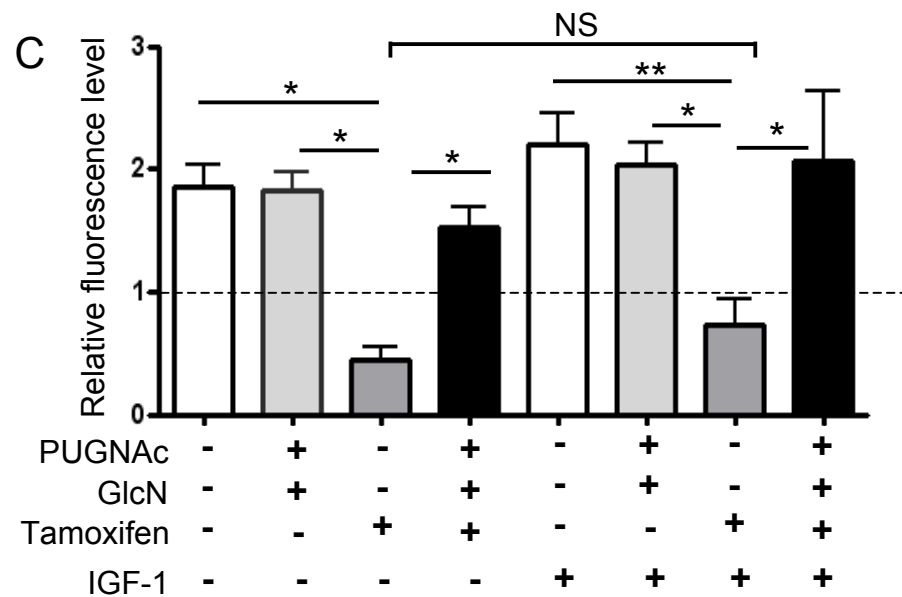
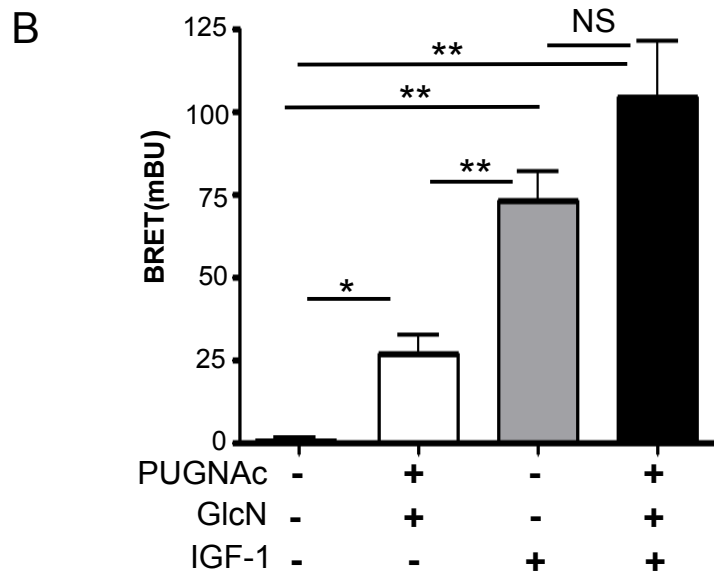
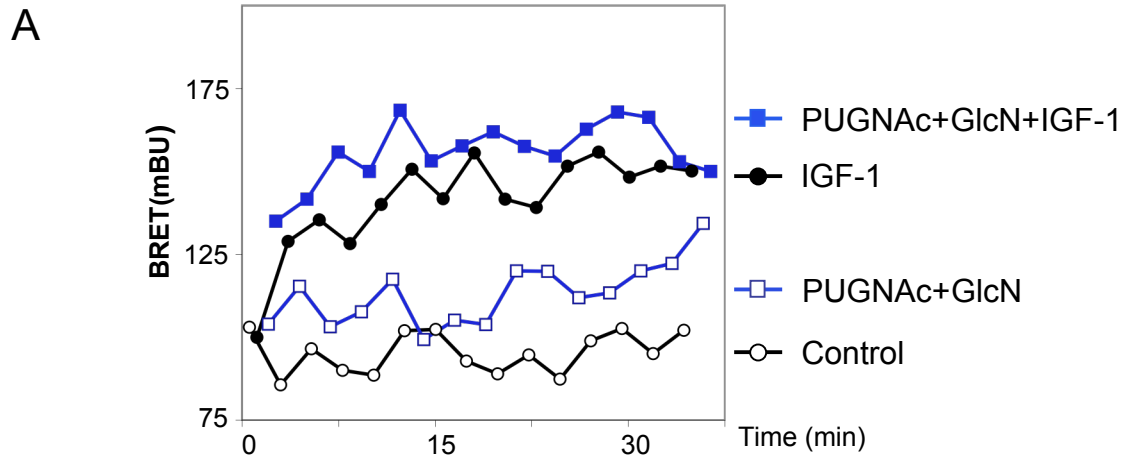
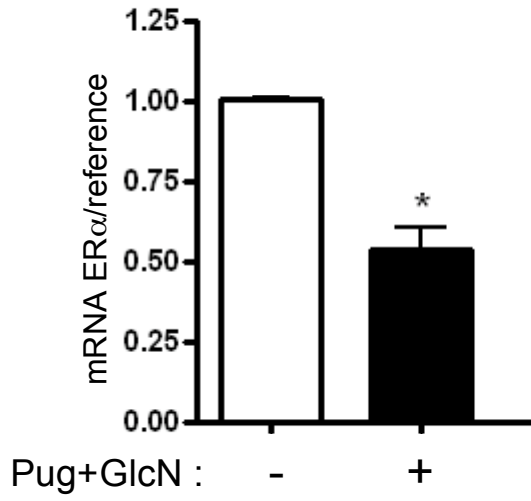
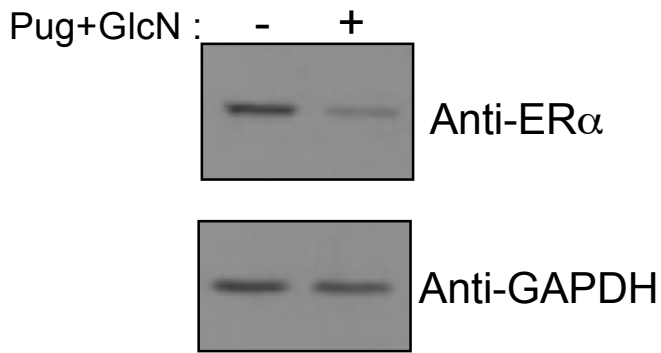


Figure 7

A



B



C

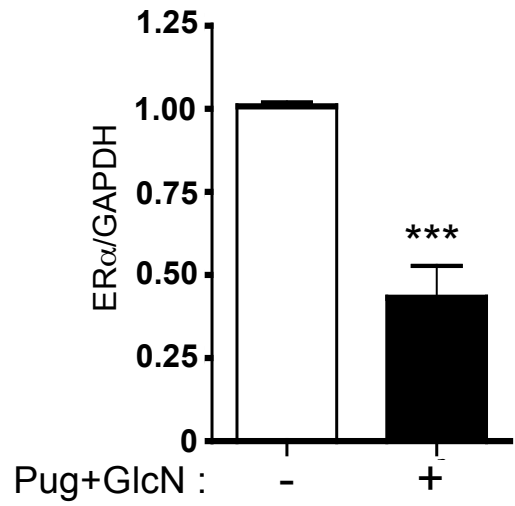
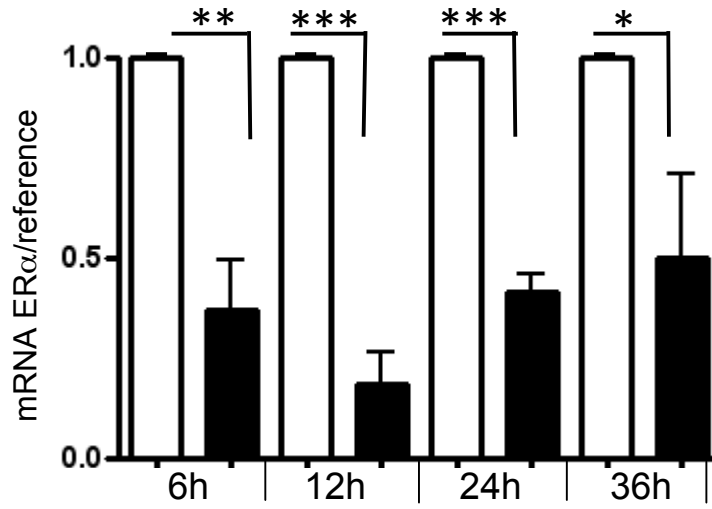


Figure 8

A



B

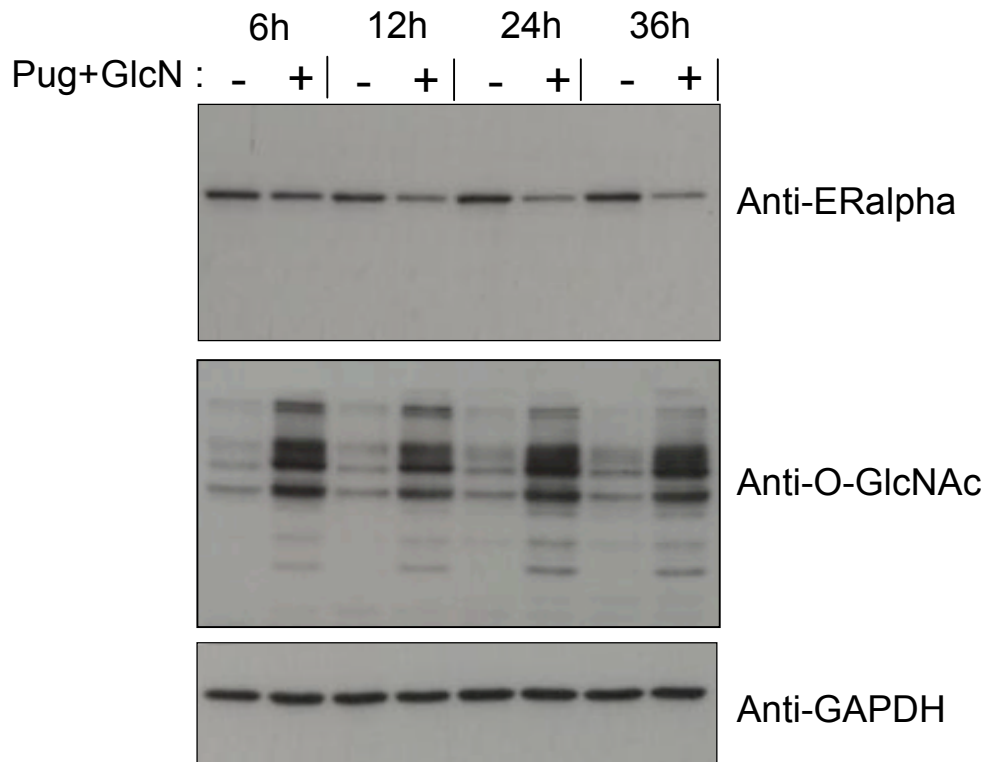
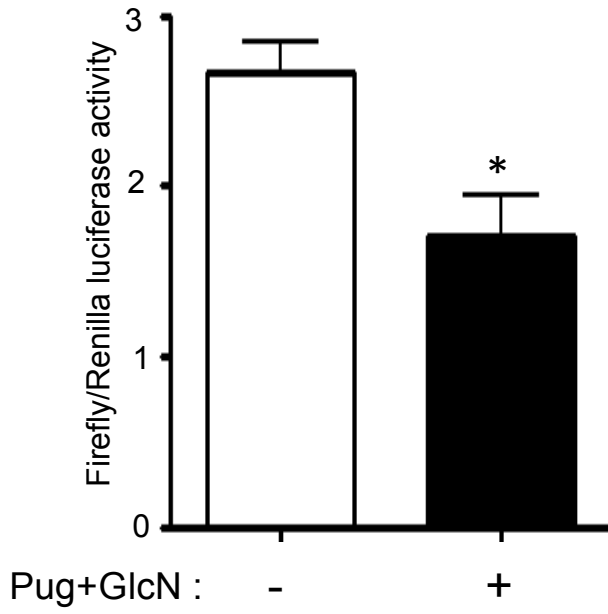


Figure 9

A



B

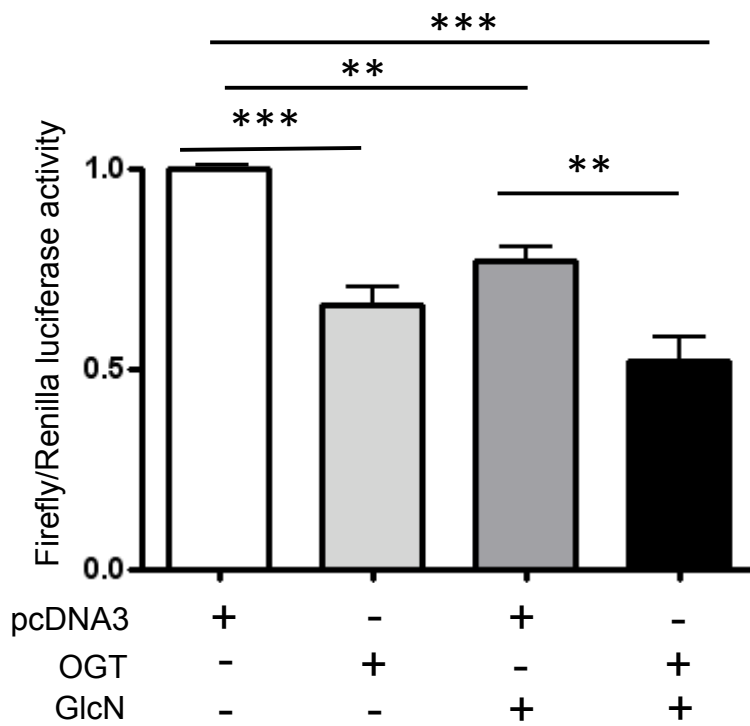
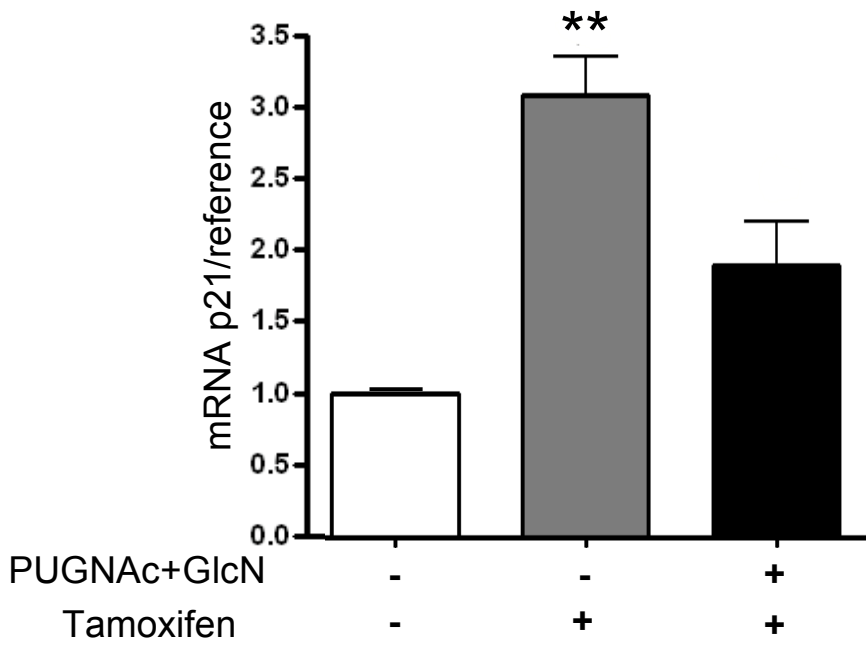
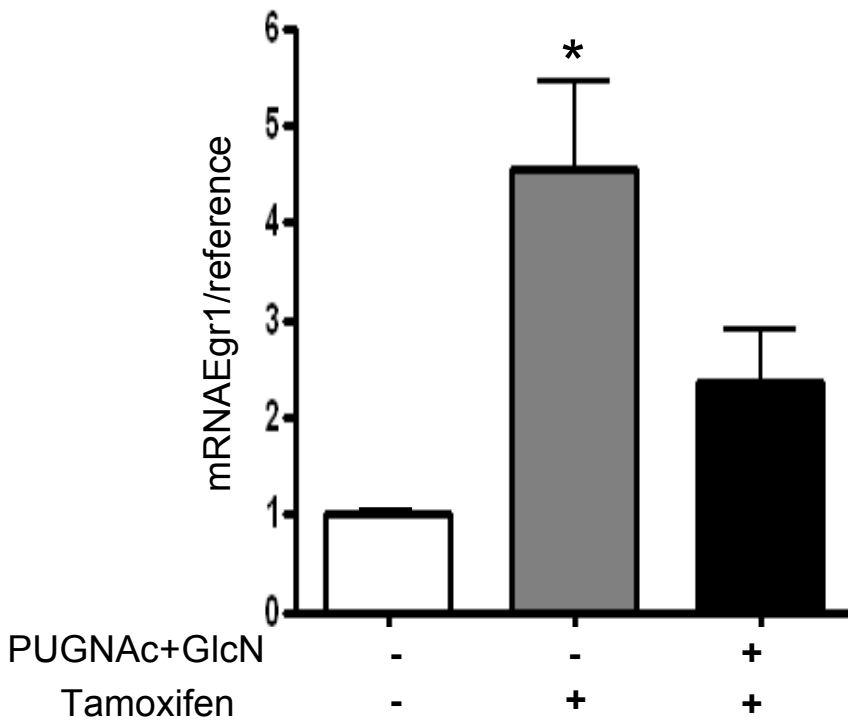


Figure 10

P21 mRNA



Egr1 mRNA



Supplementary Figure legends

Supplementary Figure S1: Effect of PUGNAc and Glucosamine on O-GlcNAc level in MCF-7 cells treated or not with 4-OH-tamoxifen. MCF-7 cells cultured in absence (Ct) or presence of PUGNAc (P), glucosamine (G) or PUGNAc+glucosamine (PG), were treated or not with of 4-OH tamoxifen (10 μ M). After 24 h of treatment, cells were lysed and protein O-GlcNAcylation level was evaluated by western-blotting. GAPDH expression level was used as a loading control.

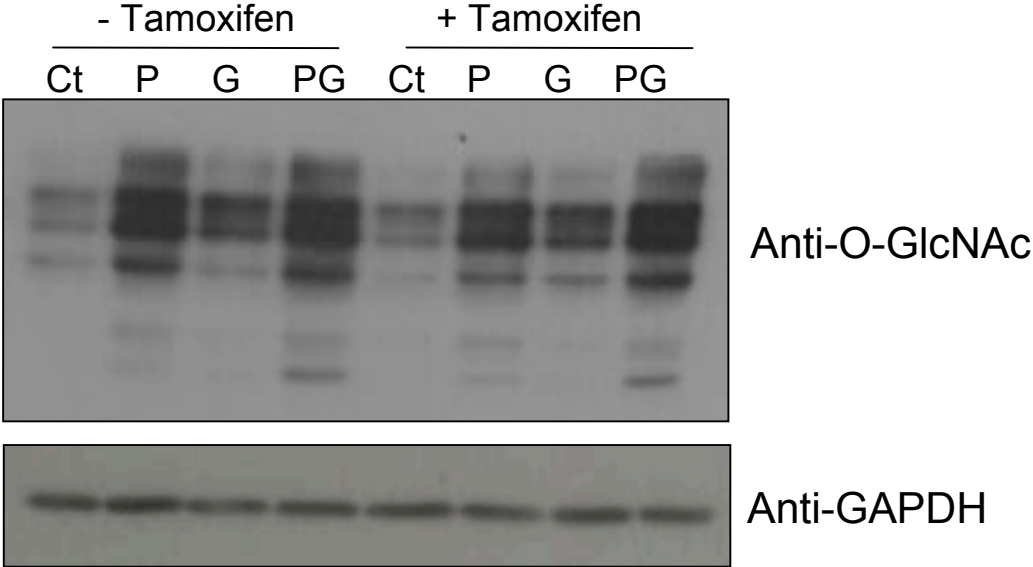
Supplementary Figure S2: Effect of LY294002 on Akt phosphorylation in MCF-7 cells. MCF-7 cells treated with 10 or 20 μ M LY294002 (LY) or vehicle (DMSO) were cultured during 24 h in absence or presence PUGNAc+GlcN and/or tamoxifen. Cells were lysed and Akt phosphorylation level was evaluated by western-blotting. As a control for specificity of PI-3 kinase inhibition, Erk phosphorylation was shown to be unaffected by LY294002 treatment in the same experiments.

Supplementary Figure S3: Effect of OGT transfection on OGT expression and O-GlcNAcylation level in MCF-7 cells. MCF-7 cells were transfected with either pcDNA3 or OGT cDNA. 48 h after transfection, cells were lysed. OGT expression and O-GlcNAcylation level of proteins were evaluated by western-blotting. GAPDH expression level was used as a loading control.

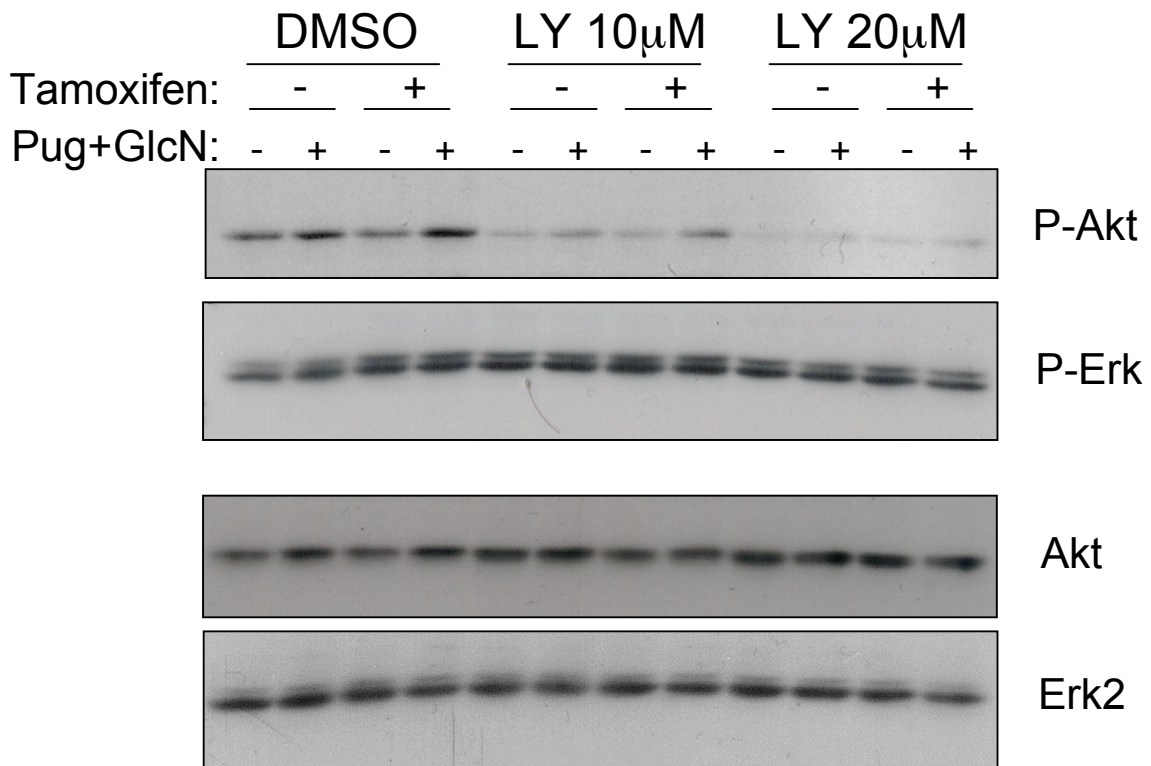
Supplementary Figure S4: Effect of PUGNAc+GlcN on *ESR1* promoter and ER α expression in presence of 4-OH-tamoxifen. (A) MCF-7 cells were co-transfected with *ESR1* promoter-Firefly luciferase reporter gene (*ESR1*-luc) and *Renilla* luciferase cDNAs. 12 hours after transfection, cells were treated with PUGNAc+GlcN in the absence or presence of 4-OH-tamoxifen for 24 h and then lysed for determination of Firefly and *Renilla* luciferase activities. Each determination was performed in triplicate. Results are mean \pm SEM of three independent experiments. Statistical analysis was performed using ANOVA followed by Dunnet's post-test. **, P < 0.01 when compared to untreated control; NS, not significant. (B) Cells were cultured for 24 h in the absence or presence of PUGNAc+GlcN and 4-OH tamoxifen. RNA was then extracted and the expression of ER α mRNA was evaluated by RT-qPCR. Results are the mean \pm SEM of 4 independent experiments.

Statistical analysis was performed using ANOVA followed by Dunnet's post-test. **, $P < 0.01$ when compared to untreated control; NS, not significant. (C) Cells were lysed and the expression of ER α protein was analysed by western-blot. GAPDH expression level was used as loading control. (D) ER α /GAPDH signals quantified by densitometric analysis of the autoradiograms of western-blot from 6 independent experiments. Statistical analysis was performed using ANOVA followed by Tukey's post-test. ***, $P < 0.001$.

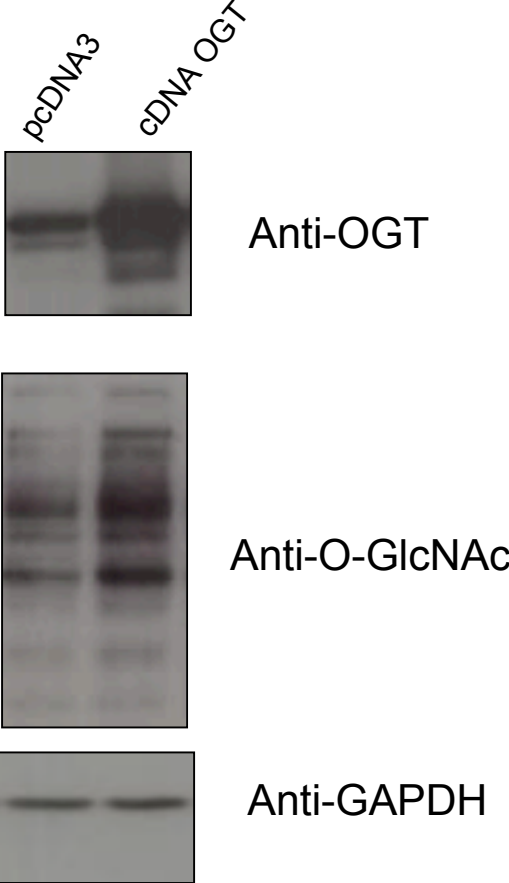
Supplementary Fig. S1



Supplementary Fig. S2

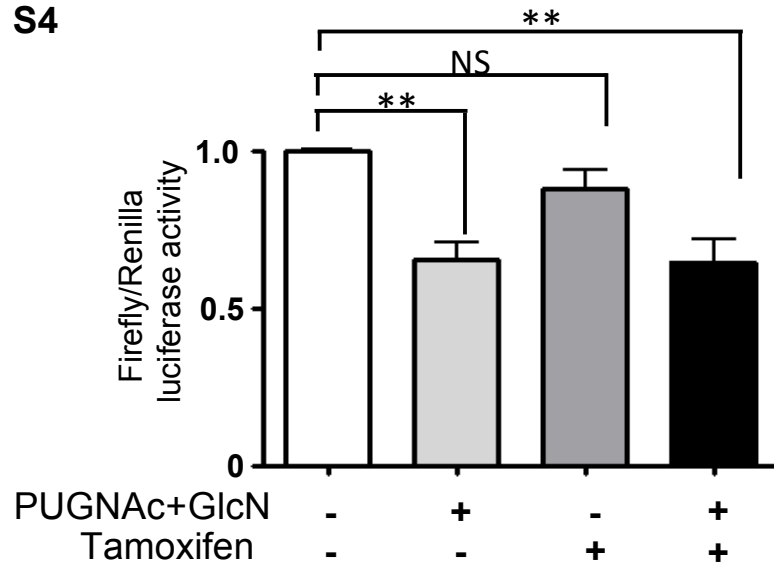


Supplementary Fig. S3

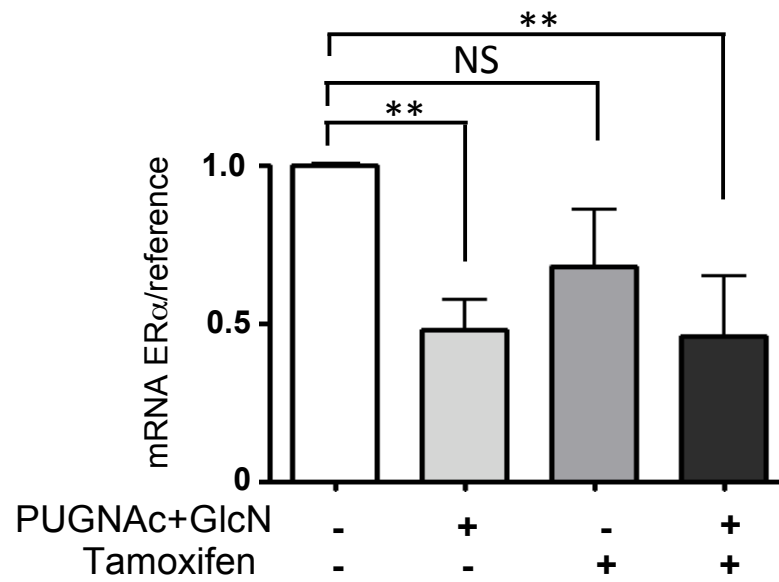


Supplementary Fig. S4

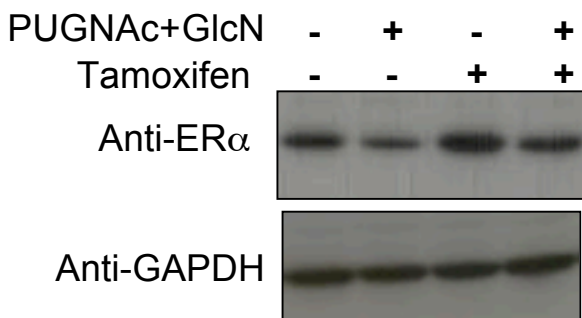
A



B



C



D

