ESTROGEN RECEPTOR ALPHA SIGNALLING REGULATES THE EXPRESSION OF THE TAXANE-RESPONSE BIOMARKER PRP4K

by

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To my parents,

without whom none of my success would be possible

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ABSTRACT

The hormone estrogen, when bound to its receptor (ER) triggers a cascade of signalling events that result in the proliferation of normal mammary glands. This hormone can also regulate gene expression at the transcriptional level by activating ER-dependent genes and post-transcriptionally by modulating the expression of microRNAs (miRNAs) that alter mRNA stability and translation. The pre-mRNA splicing factor 4 kinase PRP4K (PRPF4B), is an essential kinase that is a component of the U5 snRNP and functions in spliceosome assembly. In this study I demonstrated that PRP4K is expressed in the normal mammary duct epithelial cells of the mouse, and that estrogen induces PRP4K gene and protein expression in ER^+ human MCF7 breast cancer cells. Furthermore, I found that this hormonal regulation is via the estrogen receptor alpha, encoded by the *ESR1* gene. Thus by modulating levels of PRP4K, estrogen may affect pre-mRNA splicing in tissues expressing ESR1. As a first step towards the characterization of this novel mode of pre-mRNA splicing regulation, I sought to determine the mechanisms behind the hormonal regulation of PRP4K. Although my promoter studies indicated that estrogen does not regulate PRP4K directly at the transcriptional level, I identified several putative binding sites for miRNAs in the 3′-UTR of PRP4K. Several of these miRNAs, including miR-21, are estrogen regulated and/or deregulated in breast cancer. Using ER+ MCF7 breast cancer cells and a dual luciferase reporter system, I have demonstrated that the 3′-UTR of PRP4K can indeed regulate luciferase gene expression and that miR-21 over expression modulates this regulation. PRP4K has been recently shown as a novel biological marker for taxane response in ovarian cancer patients and reduced levels of PRP4K correlate with intrinsic and acquired taxane resistance in both breast and ovarian cancer. Here, I have demonstrated that treatment with tamoxifen, an inhibitor of estrogen signalling, can decrease PRP4K levels in MCF7 breast cancer cells reducing cell sensitivity to the taxane paclitaxel. Thus, this study suggests that PRP4K is novel estrogen regulated kinase, whose expression can be inhibited by tamoxifen in ER+ breast cancer cells which in turn impact cell response to taxanes. These data raise the possibility that by treating ER+ breast cancer patients with anti-estrogen therapies such as tamoxifen, we may inadvertently alter the response of cancer cells to taxane therapy.

LIST OF ABBREVITIONS USED

4OHT	4-hydroxytamoxifen
AF-1/2	Activation Function ½
APC/C	Anaphase Promoting Complex/Cyclosome
BCS	Breast-conserving surgery
BRCA1 and BRCA2	Breast Cancer early onset 1 and 2
CDK1	Cyclin-dependent kinase 1
ChIP	Chromatin Immuno-Precipitation
DBD	DNA binding Domain
DCIS	Ductal Carcinoma In Situ
DGCR8	DiGeorge critical region 8
DMSO	Dimethyl sulfoxide
DNR	Daunorubicin
DOX	Doxorubicin
DSB	Double-Strand Break
E2	17-β-estradiol
ECD	External Cellular Domain
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor

ER	Estrogen Receptor
ErbB2	erb-B2 receptor tyrosine kinase 2
ERE	Estrogen Receptor Elements
H3K27ac	Histone H3 acetylation on lysine 27
Her2/neu	Human epidermal growth factor receptor 2
IGF-I	Insulin-Like Growth Factor-I
IR	Ionizing Radiation
IRES	Initial Ribosome Entry Site
JNK	c-Jun N-terminal Kinase
LBD	Ligand Binding Domain
LCIS	Lobular Carcinoma In Situ
LDL	Low Density Lipoprotein
LDL-R	Low Density Lipoprotein-Receptor
MAD	Mitotic Arrest Deficient
MAPK	Mitogen-Activated Protein Kinase
MAPT	Microtubule-Associated Protein-Tau
MET	Mesenchymal-Epithelial Transition
miRNA	MicroRNA
mRNA	Messenger RNA

ncRNA	Non-coding RNA
NSCLC	Non-small cell lung carcinoma
NST	No-special-type
OGs	Oncogenes
ORF	Open Reading Frame
PARP	Poly ADP Ribose Polymerase
PI	Phosphoinositol
PI3K	Phosphotidylinositide 3-Kinase
PKB/Akt	Protein Kinase B
PKC	Protein Kinase C
PR	Progesterone Receptor
PRP4K	Pre-mRNA Splicing Factor 4
PTH	Parathyroid Hormone
qPCR	Quantitative Polymerase Chain Reaction
RBP	RNA Binding Protein
RNP	Ribonucleoprotein
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction

SAC	Spindle Assembly Checkpoint
SERMS	Selective Estrogen Receptor Modulator
SH2	Src homology 2
shRNA	Short hairpin RNA
SHRs	Steroid Hormone Receptors
SSB	Single-Strand Break
TAM	Tamoxifen
TGFα/β	Transforming Growth Factor α/β
TGGs	Tumour Suppressors
TKIs	Tyrosine kinase inhibitors
TNBC	Triple Negative Breast Cancer
TNC	Triple Negative Cancer
TNM	Tumour-Lymph Node-Metastasis
TOPO II	Topoisomerase II
TRE	TPA Responsive Element
UTR	Untranslated Region
UVA	Ultraviolet A

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CHAPTER 1: INTRODUCTION

1.1 Breast cancer

Breast cancer is the most frequently occurring cancer among women and the second cause of cancer-related death, after lung cancer, in the female population (1). Even with early diagnosis and treatment, 15-20% of patients develop recurrent or metastatic disease (2). Multiple biological and epidemiological risk factors have been found to be associated with breast cancer occurrence. Epidemiological variables include age, marital status, geographical location and racial and ethnic group. For example, the frequency of the disease is higher in North America, Europe and Australia compared to Africa and Eastern Asia (3). Among biological causes are hormonal influences that play a role in etiology of breast cancer as long exposure to human sex hormones can enhance the risk by stimulating proliferation of epithelial cells that have undergone partial malignant transformation (2-3).

Most cancers do not result from inherited gene mutations but rather arise sporadically from damage to the DNA during a person's life. Genetic damage may result from internal factors, such as hormones or the metabolism of nutrients within cells, or from external factors, such as tobacco, or excessive exposure to chemicals, sunlight, or ionizing radiation. However, there is a small proportion of cancers that do occur as a result of inherited genetic alterations that confer a very high risk in developing cancer (4). For example, mutations in the breast cancer 1 or 2 early onset genes, otherwise known as *BRCA1* and *BRCA2*, confer an increased risk of breast cancer. The most common

mutations in *BRCA1* and *BRCA2* include insertions or deletions that lead to protein truncation. However, larger deletions or loss of function mutations are among less common mutations in the genomes of carriers of familial breast cancer (5).

Breast cancer may be detected before symptoms have developed, for example during screening by self-examination or mammography or after physical signs have appeared. Typical symptoms of breast cancer may be the appearance of a small tumour in the breast or sometimes in underarm areas, where a lump or swelling can be felt (7). In the past two decades, the mortality from breast cancer has significantly decreased primarily due to early detection methods (6). For example, routine screening by mammography for women, beginning in the 4^{th} and 5^{th} decades of their lives, has been demonstrated to improve survival but does carry the additional risks of false positive diagnoses and radiation exposure (7). Early stage breast cancers found through routine screening can often be cured surgically with breast conserving methods. However, if left untreated, the disease can progress to a higher stage making the breast cancer difficult or sometimes impossible to treat (10). With regard to its etiology and pathological characteristics, breast cancer is highly heterogeneous disease (11). In some cases tumours grow slowly with good prognosis, whereas in other cases patients might need highly aggressive clinical courses. The degree of cellular differentiation and nuclear pleomorphism help in defining the histological grade and further sub-classification of breast tumours. In the following sections, I will discuss in detail the classification and treatment of breast cancer.

1.2 Morphological Classification of Breast Tumours

Breast cancer is categorised as a neoplasm with benign and invasive forms. More than 95% of breast malignancies are adenocarcinomas. Rregardless of being neoplasia or carcinoma breast cancer can be divided into two types: carcinomas *in situ* and invasive carcinomas. Carcinomas *in situ* refers to a neoplastic proliferation which is limited to ducts (i.e. ductal *in situ* carcinomas or DCIS) and lobules (Lobular *in situ* carcinomas or LCIS) (12,13). DCIS is a broad range of cancer abnormalities starting in the cells lining the breast ducts. DCIS is a non-invasive form of cancer and cells do not grow beyond the layer they originated from. DCIS may or may not progress to invasive carcinoma. LCIS is in fact a neoplasia and not a true form of cancer but an indicator of high risk for developing cancer. More aggressive tumours are invasive carcinomas, which refer to infiltrating carcinomas in which cancerous cells penetrate and broken through basement membrane (the ductal or glandular wall where they originated from) and go into surrounding tissues. Invasive carcinomas are categorised into ductal or no-special-type (NST) carcinoma, lobular, tubular/cribriform, mucinous (colloid), and medullary, papillary and metaplastic carcinomas.

Breast tumours are also classified by stage as the extent of spread of infiltration at the time of diagnosis defines the stage of the disease and has a strong influence on prognosis. There are two main staging systems for cancer. The Tumor-Lymph node-metastasis (TNM) classification and the 0-IV stage levels (14). The TNM classification of tumours which is commonly used in clinical diagnosis is based on tumour size and how far it has spread within the breast (T), the extent of spread to the surrounding lymph

nodes (N), and the presence or absence of distant metastases (spread out into other organs) (M). Once the T, N, and M are determined, a stage of 0, I, II, III, or IV is assigned, with stage 0 being *in situ*, stage I being early stage invasive cancer, and stage IV being the most advanced disease. The staging information helps to choose the best treatment plan option and to predict patient's prognosis. Although each individual is different, cancers with the same stages tend to have similar prognosis and are treated with similar approaches. For example patients in earlier stage of cancer will normally be treated with surgery or radiotherapy while higher stages will need chemotherapy.

In the past decades, breast cancer treatment has experienced a new phase due to the discovery of specific prognostic and predictive biomarkers that enable the application of more individualized therapies to different molecular subgroups.

1.3 Molecular Classification of Breast Tumours

In addition to morphological and histopathological differences, gene expression profiling provided techniques to identify specific subtypes of breast cancer based on different molecular biomarkers such as receptor tyrosine kinases (RTKs) and hormone receptors. Hormone receptors such as estrogen receptor and progesterone receptor have played a significant role in the selection of patients benefiting from endocrine therapy for many years. More recently, a receptor tyrosine kinase, the human epidermal growth factor receptor 2 (HER2), has been validated to be not only a prognostic factor, but also a predictor of response to HER2 targeting therapy.

These subgroups show specific differences regarding biological clinical behavior which in addition to classical clinical prognostic factors influence clinical outcome and the treatment strategy. These well characterized subtypes will be described in more details according to the study by Perou and colleagues (15).

1.3.1 Luminal A

This is the largest group of cancer which comprises 40%-50% of NST carcinomas and mainly occurs in postmenopausal women. These tumours are ER-positive (ER+) and HER2/neu negative (HER2-), normally well differentiated, slow growing and respond well to hormonal therapy.

1.3.2 Luminal B

This type of cancer is also ER+ but is a higher grade cancer with higher proliferative rate and is often associated with overexpression of Her2/neu. Endocrine resistance is a barrier for these patients. To overcome this problem combination of endocrine and receptor targeted drugs are recommended. Luminal A and luminal B cancer patients are both ER+. They generally have a better prognosis and are treated with adjuvant endocrine therapy such as tamoxifen and aromatase inhibitors which target ER (16).

1.3.3 Normal breast-like

This type is mainly well differentiated form of ER+/HER2- cancers with similar gene expression pattern for normal tissue.

ER-negative subtypes, including basal-like and Her2+ (ERBB2+), are characterized by overexpression of HER2-related cluster genes and have the poorest prognosis. HER2+ patients usually respond to monoclonal antibodies, trastuzumab, in combination with conventional chemotherapy drugs.

1.3.4 Triple negative breast cancer (TNBCs)

Triple Negative Breast Cancers (TNBCs), also called as basal-like tumours refers to those that normally do not express the genes for estrogen receptor (ER), progesterone receptor (PR) and HER2. These tumour cells which are associated with poor prognostic are the most challenging type of breast cancer to treat and in need of therapeutic advances. This subtype constitutes approximately 15-20% of all breast cancers and primarily occurs in younger women. Triple negative tumours are classified as aggressive carcinomas with poor outcome. TNBCs tend to be high grade and are initially responsive to chemotherapy. However, this type of cancer is normally aggressive with high recurrence, metastasis and low survival (17). The absence of ER, PR and HER2/neu makes these tumours very difficult to treat with non-surgical therapies as they do not respond to any anti-hormonal treatments. (18,19). So treatment options are limited to traditional systemic cytotoxic chemotherapy. Current approach is a combination of therapies such as surgery, radiation therapy, and chemotherapy. Meanwhile molecular

targeted therapies have been developing and strategies such as targeting poly adenosine diphosphate ribose polymerase (PARP) and EGFR inhibitors and antiangiogenic agents are among potential future therapies.

1.3.5 Claudin-low (CL)

This subtype of TNC is not very well describe yet compared to other types of breast cancer. This subtype consist of tumours with low expression of tight junction proteins most dominant of which are claudin 3, 4 and 7, E-cadherin and a calcium-dependent cell-cell adhesion glycoprotein. In term of phenotype, these tumours are among the least differentiated tumours and express more mesenchymal characterization than luminal (20,21). Due to the lack of epithelial characteristics and being enriched in epithelial-to-mesenchymal transition (EMT), CL tumours are believed to have tumour initiating features and being more frequent in the post-treatment residual mammary tumour tissue after adjuvant chemotherapy and hormone therapy (22).

1.3.6 HER2-enriched

This group comprises ER- and HER2/neu+. Over expression of HER2 is due to amplification of DNA loci that carries ErbB2/neu gene. HER2 over activation has been seen in about 15-20% of breast cancers (23). Several therapeutic agents targeting either HER2 or EGFR are approved for the treatment of HER2 positive breast cancer. Identifying receptor tyrosine kinases and hormone receptor status have become more and more essential in cancer therapy in order to predict suitable treatment regimen for each

individual. Here, I summarize the most well-known receptor tyrosine kinase, HER2, as well as the most important hormones and hormone receptors involved in breast cancer.

1.3.6.1 HER2

HER2 (or ErbB-2) is a member of the epidermal growth factor receptor (EGFR) family. EGFR family comprises of four related but distinct subfamily of receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her3 (ErbB-3) and Her4 (ErbB-4). These proteins belong to trans-membrane growth factor receptors with intrinsic tyrosine kinase activity that in response to extracellular signals activate intracellular signalling pathways (24). There is structural homology between all members of the ErbB family. All members of the family contain an extracellular domain with two cysteine-rich sequences and a cytoplasmic domain with tyrosine kinase activity which is flanked by hydrophobic tails carrying tyrosine autophosphorylation sites. The latter provides autophosphorylation sites on several tyrosine residues in the C-terminal domain of EGFR. The phosphorylated residues serve as a docking site for cytoplasmic proteins with Src homology 2 (SH2) domains (25). EGFR is located on the cell surface and is activated by binding of its ligands including epidermal growth factor (EGF) and transforming growth factor α (TGFα). Following the activation by its specific ligands, EGFR undergoes homo- or heterodimerisation which is a transition from inactive to an active form of the receptor (26). Heterodimerization of EGFR may occur with another member of the ErbB receptor family, such as ErbB2/Her2/neu, to create an activated heterodimer EGFR dimerization stimulates its intrinsic intracellular protein-tyrosine

kinase activity (27). In addition to forming dimers after ligand binding, there is also evidence to suggest the formation of EGFR dimers before ligand binding or in clusters, although it is yet about to reveal that whether this clustering is important for activation itself or occurs subsequent to activation of individual dimmers (28). Unlike other members of this family, the extracellular domain of HER2 lacks ligand binding activity and does not need a ligand to switch to active conformation (29). Instead this receptor is either constitutively active or undergoes heterodimerization with other members of the receptor family such as EGFR to become active (56). Alternatively, Her-2/neu activation has been demonstrated to occur as a consequence of spontaneous cleavage of the receptor extracellular domain (ECD) which results in the production of a truncated membrane-bound fragment (p95) with kinase activity (30,31).

1.3.6.2 HER2 in cancer

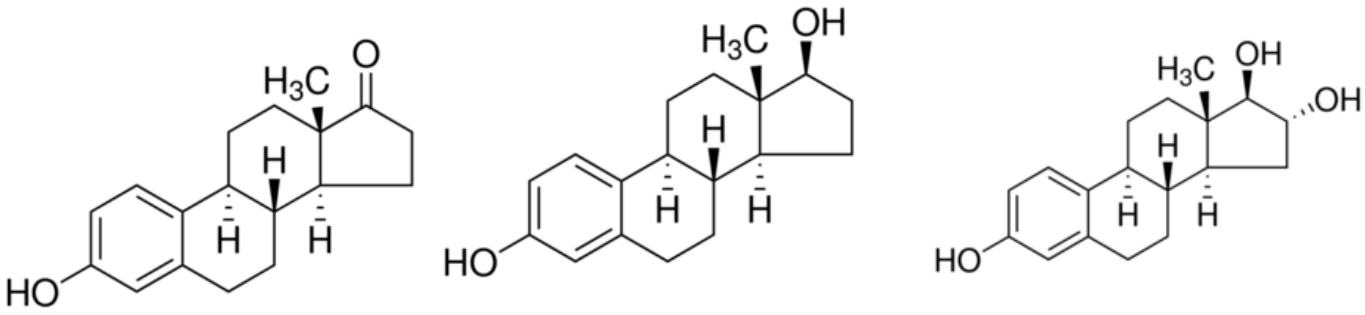
HER2 is the best studied oncogene in the HER family whose overexpression is predictive for breast cancer patients with more aggressive outcomes and poor survival (32). HER2 has been introduced as an important biomarker for breast cancer diagnosis as patients with elevated levels of HER2 are normally the patients with the poor outcome and the most aggressive disease. Mutations that lead to amplifications or dysregulations of EGFR cause constitutively active state of the receptor and produce uncontrolled cell division (33). Over expression of HER2 leads to hyperdimerization of the receptor within the cell membrane. HER2 can become either homo- or heterodimerized with other receptors in the membrane which ultimately activate different mitogenic pathways in

particular phosphatidylinositide 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) (34). The constant activation of EGFR is implicated in about 30% of all epithelial cancers including head and neck cancer, gliomas (35), non-small cell lung cancers (NSCLC)(36), breast and ovarian cancer (33). Similarly, amplification and overexpression of HER2 has been observed in breast (23) and lung cancer (37).

1.4 Important Hormones in Breast Cancer

1.4.1 Estrogens

Estrogens are steroid hormones that play roles in cell growth, differentiation, reproduction and function of their target tissues. The most dominant steroid in human body is estrogen. The main source of the hormone estrogen in pre-menopausal women is ovaries and placenta during pregnancy. In post-menopausal women body's fat tissue carries smaller amounts of hormone which is made by steroid producing cells in the cortex layer of adrenal gland. Estrogens exist in three physiologically active forms: estrone (E1), estradiol (E2), and estriol (E3) with 17β –estradiol (known as E2) being the most predominant form (**Figure 1.1**) (38). The normal function of estrogen is to regulate cell proliferation and differentiation of mammary glands, uterine endometrium and ovarian cells. However, estrogen-mediated signalling provides a mechanism by which the growth of hormone-related cancers may be regulated (39). Estrogen exerts its effect by binding to one of the two estrogen receptors (ERs), alpha and beta, which are major drug targets in breast cancer therapy. Binding to receptor molecules is essential for induction of ERs as the main player of the estrogen receptor pathway. The role of estrogen in stimulating receptor molecules and triggering the downstream regulatory pathway will be discussed in detail later in the chapter.



Estrone (E1) **Estradiol (E2)** **Estriol (E3)**

Figure 1.1 Structure of the three main active forms of estrogen

1.4.2 Progesterone

Progesterone is a steroid hormone that primarily is produced in ovaries and to lesser extends in adrenal gland or during pregnancy in placenta. Progesterone and progesterone receptors (PRs) are essential for development of reproductive system. This hormone is also one of the critical regulators of breast cancer. In breast tissue progesterone has a proliferative role and acts jointly with estrogen and estrogen receptor towards the expansion of mammary gland structure during breast development. Similar to ER signalling pathway, progesterone is necessary to induce progesterone receptor in PR-positive cells. Progesterone receptor is a member of the steroid-receptor superfamily of nuclear receptors and exists in two isoforms: PR-A and PR-B. The two isoforms share many similarities in structural domains. However they are in fact two functionally distinct transcription factors associated with regulation of their own target genes and physiological effects (40). It is believed that ER expression is required, if not necessary, for PR action in PR responsive tissues and PR is a downstream target of ER signalling pathway. In breast, ER and PR crosstalk with each other towards similar functions, for example inducing the growth and proliferation, whereas in ovary and endometrium progesterone shows anti-proliferative effects and induce tissue regression (40). Like ER, PR is also a prognostic marker in breast and ovarian cancer as differential expression of this receptor in breast and ovarian tumours provide valuable information to detect tumour behavior that could be used for endocrine therapy. The structure of progesterone molecule is shown in **Figure 1.2.**

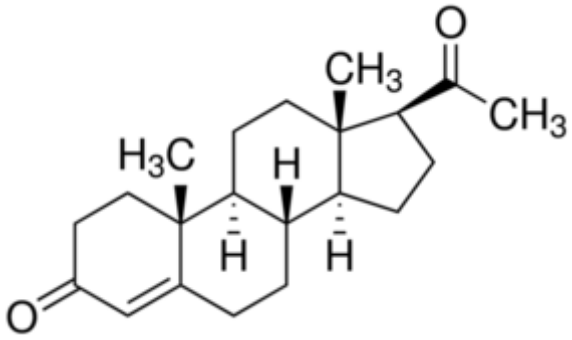


Figure 1.2 Molecular structure of progesterone

1.5 Estrogen Receptors

Estrogen and its receptor (ER) are involved in development of mammary gland. About 60% of breast tumour cells express ERs (1). There are two types or ERs: alpha (ERα) and beta (ERβ). The gene for ERα, *ESR1*, is located on chromosome 6, and the gene for ERβ, ESR2, is found on chromosome 14. These receptors belong to the superfamily of nuclear receptors and in respond to estrogen act as ligand activated transcription factors and regulate a broad range of genes expression (41). The members of nuclear receptor family share common structures comprising of five domains (A-F): The N terminal or A/B domain, the DNA binding domain (domain C), the hinge domain (domain D) and the C-terminal E/F domain. The A/B domain encodes an activation function (AF-1) which is essential for protein-protein interaction with co-activators and ligand-independent transcriptional activation. The DNA binding domain (DBD) contains two zinc finger motifs which plays role in receptor dimerization and sequence-specific binding of ER to DNA. The C-terminal E/F domain is the ligand binding domain (LBD) which mediates transcription via ligand binding, ER dimerization and interaction of ERs with co-activator proteins. This domain also harbors activation function2 (AF-2) and may affect the agonist/antagonist activity of selective ER modulators (SERM) (42). ER and its ligands are essential actors of ER signalling pathway and because breast cancer is a hormone dependant disease (43), it is important to point out the mechanisms through which estrogen and its receptor mediate cell signalling pathways in the cell.

1.5.1 Estrogen receptor signalling pathway

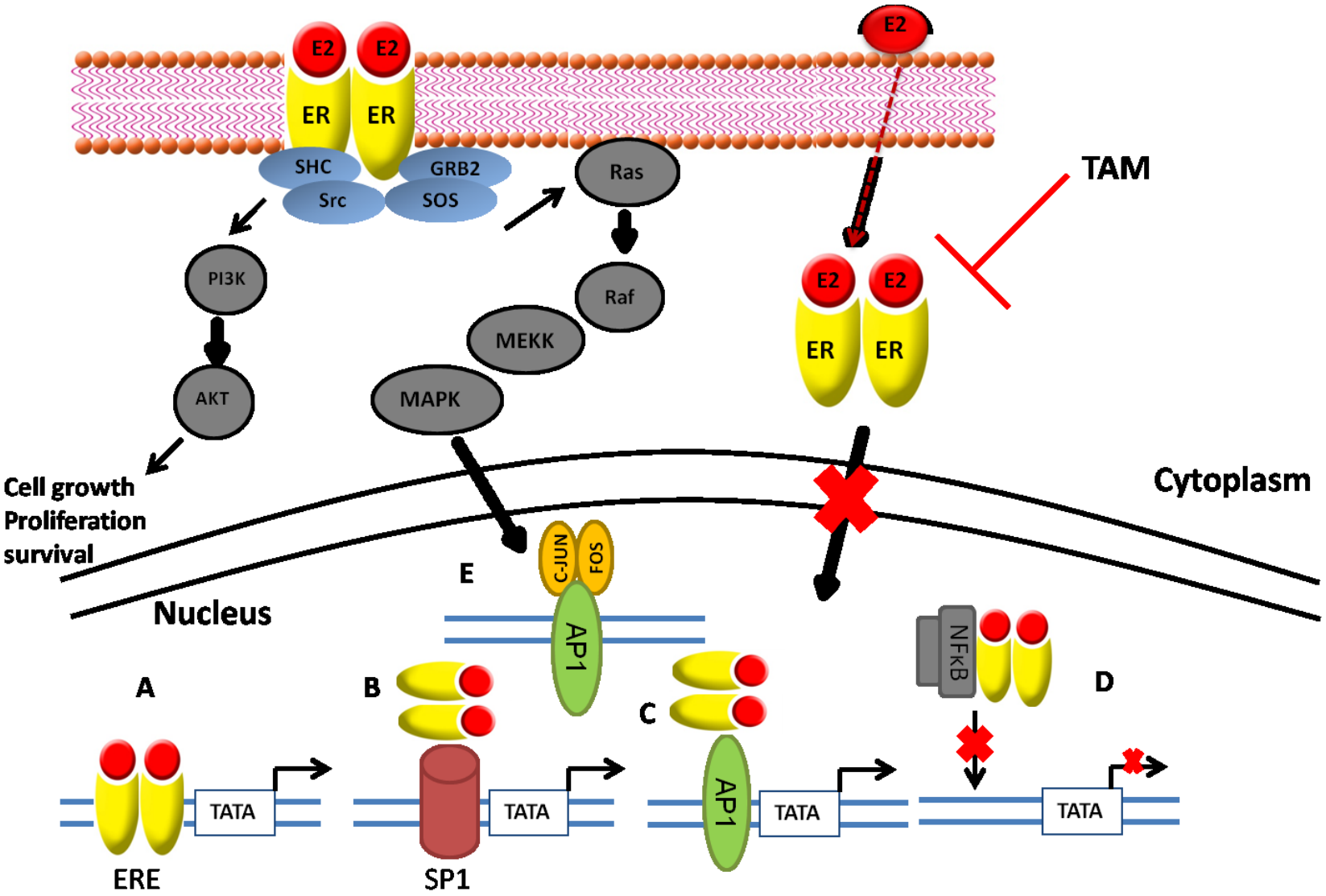
There are multiple mechanisms by which ER exerts its effect on gene expression which mainly fall into four main categories: Classic or genomic pathway, ERE-independent genomic pathway, ligand-independent pathway and non-genomic pathway. Below each of these pathways will be summarized.

1.5.2 Genomic vs. non-genomic pathway

1.5.2.1 The Classical or Genomic pathway

In the classical (genomic) pathway, binding to the ligand (e.g. 17β –estradiol or E2) triggers conformational changes in ligand binding domain and homo- or hetero-dimerization of the receptor. Receptor dimers then bind to the specific DNA response elements known as Estrogen Response Elements (EREs) within an estrogen-regulated gene's promoter. The ERE motif sequence is a conserved regulatory sequence following the sequence of 5'-GGTCAnnnTGACC, and usually found next to the TATA box and transcription start site to which ERα binds within the promoter of an estrogen-regulated gene. This direct binding recruits chromatin remodeling factors and other transcription factors (co-activators or co-repressor) to the promoter and triggers the formation of transcription initiation complex or hinder the transcription process (**Figure 1.3**). The regulatory regions of some estrogen responsive genes have ERE half sites (EREs that are positioned next to SP1 binding sites) (44,45) or nested in AP-1 sites (46). As such, ERα and AP-1 cross talk is also an important regulatory mechanism underlying estrogen-

regulated transcription. During breast cancer progression, ER expression has been shown to be upregulated in ER+ breast cancers and this over expression is associated with more aggressiveness and resistance to anti-estrogen drug tamoxifen (47).


E2
E2
ER
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SHC
GRB2
Src
SOS
Ras
PI3K
Raf
MEKK
AKT
MAPK
E2
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TAM
Cell growth
Proliferation
survival
Cytoplasm
Nucleus
E
C-JUN
FOS
AP1
A
B
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NFkB
TATA
TATA
TATA
TATA
ERE
SP1
AP1

Figure 1.3 Estrogen receptor signalling pathways. Depicted are the different signalling pathways through which estrogen and ER can mediate gene expression. **A)** In classical genomic pathway E2 diffuses through cell membrane and binds to ER which is located in cytoplasm resulting in receptor dimerization and association with ERE on the promoter of E2-regulated genes. **B,C,D)** in ERE-independent pathway, E2-ER complex binds to non-ERE promoter elements such as AP-1 and SP1to activate target genes or to repressors such as NFkB to suppress the expression of target genes. **E)** The non-genomic pathway includes binding of E2 to membrane-associated ERs and activation of protein kinase cascade that leads to activation of ER-mediated genes through other transcription factors such as c-FOS and JUN.* ERE: Estrogen Receptor Elements

1.5.2.2 ERE-independent genomic pathway

Although many estrogen-responsive genes contain EREs in their promoter, a number of estrogen-regulated genes do not. In this case, hormonal regulation of such genes occurs indirectly via protein-protein interaction between ERα or ERβ and other transcription factors that facilitate promoter binding. An example of such indirect interaction is the association of ERα with the FOS and JUN proteins which form the activator protein-1 (AP-1) complex. In this ERE-independent pathway liganded ERα is tethered to DNA by interacting with other transcription factors such as AP-1or specificity protein-1 (SP-1) (48,49). AP-1 is a dimeric transcription factor complex comprising of members of the JUN and FOS protein families. Similar to ERα, components of AP-1, such as FOS, need to form heterodimers to become transcriptionally active complexes. Following heterodimerization, the AP-1 complex binds to DNA at response elements such as the TPA responsive element (TRE) and cAMP response elements (CREs) (48,50,51). Examples of AP-1-regulated estrogen-responsive genes are ovalbumin , IGF-I , collagenase and cyclin D1(52–55). Genes with CG-rich promoter sequence are regulated in a similar way via SP-1 binding sites (48). In this case, interaction of ERα and SP-1 is capable of regulating the low-density lipoprotein (LDL) receptor (LDL-R) (53), c-*fos* (56), cyclin D1(57) and vitellogenin A1(58) in response to estrogen. Finally, the E2-ER pathway can also indirectly regulates gene repression. One example is repression of NFkB gene. In response to estrogen, ERα interacts with the c-*rel* subunit of nuclear factor *k*B (NFkB) complex and with CCAAT/enhancer binding protein beta (C/EBPβ). In this way, E2 inhibits expression of the cytokine IL-6 (59–61). Another example is repression

of erythropoiesis by estrogen signalling pathway. The ligand-dependent interaction of ERs with erythroid transcription factor GATA-1 transcription factor can repress the process of erythropoesis and reduce the number of erythoid progenitor cells in human bone-marrow (62).

1.5.2.3 Estrogen-independent

ER signalling pathway can become activated in the absence of estrogen. Estrogen-independent signalling can be modulated by the intracellular phosphorylation signalling pathways or by extracellular cell cycle regulators such as growth factors and cytokines. (42). Phosphorylation of ERα can be conducted by protein kinase A (PKA) or protein kinase C (PKC). This ligand-independent pathway provides pipelines between ER signalling pathway and other growth factor signalling pathways in the cells. An important growth factor that can mimic and replace estrogen is Epidermal Growth Factor (EGF) while insulin, insulin-like growth factor I (IGF-I) and transforming growth factor (TGF)-β are among other growth factors with similar effect that can activate ER signalling and regulate cell growth (42). The crosstalk between ER and EGF signalling can go through different pathways. One of the main pathways includes the guanine nucleotide binding protein $p21^{ras}$ and the MAPK as the mediator. P21 acts as intermediate between tyrosine kinases and MAPK in the membrane. These kinases can bind to AF-1 domain on ERα. Alternative downstream cascades including other growth stimulating factors such as insulin have been reported to engage AF-2 region of ERα.

1.5.2.4 Non-genomic pathway

Estrogen receptors are predominantly located in cytoplasm and in the nucleus, however a small portion of ER molecules are associated with the cell membrane. Regardless of the sub-cellular localization of ER, the ligand binding domain of ERα will provide binding site for estrogen while other domains become involved in protein-protein interactions that mediate the non-genomic function of ER. Unlike many other membrane receptors, ER proteins do not contain plasma membrane domain. Yet, these receptors are capable of interacting with plasma membrane via palmitoylation (63). Both plasma membrane associated ER and the ERs in the cytoplasm exist in dimer formation upon estrogen activation. Membrane-bound ERα is in the proximity of a variety of other signalling proteins such as Src kinase, RAS and G proteins and can directly interact with IGF-1 and HER2 which in turns leads to activation of MAPK signalling pathway. Hence, membrane-associated ERs, when induced by hormonal ligand, can play role in non-genomic pathway. Non-genomic properties of hormones can explain some of the rapid responses to the hormone. Cellular response in non-genomic pathway is sometimes too rapid to be involved in any RNA transcription regulation or protein synthesis, rather this response typically involves activation of protein kinase cascades, mobilization of intracellular calcium and the stimulation of adenylate cyclase activity and cAMP production (42). Activation of MAPK and phosphoinositol (PI) 3-kinase signalling pathway has been well studied in several cell types including breast cancer cells. However molecular mechanisms behind these pathways are specific to each cell type and depend on the corresponding set of signal transduction network and downstream target

cascades. A possible mechanism underlying non-genomic pathways is that a sub-population of ERα and ERβ, more likely the spliced isoforms might be responsible for non-genomic properties of steroids.

With any of the above mechanism and in a common pattern estrogen binds to its receptor (ER) and triggers a cascade of signalling events that result in the proliferation and differentiation of normal mammary glands. Therefore, any disruption in physiological balance of hormones could have very critical impact in breast cancer initiation and progression. This makes ERα on of the important prognostic factor in breast cancer and is over expressed in about 60-80% of human primary breast tumours (64). As discussed before, estrogen receptor status is also predictive of response to hormone therapies and drugs designed to block estrogen signalling or the production of estrogen are key agents in treating ER positive breast cancer.

1.7 Breast cancer treatment

There are different types of treatment for patients with breast cancer. Today, treatment plans are designed to meet individual needs although traditional treatment approaches are still common. Common forms of treatment are surgery, radiotherapy, chemotherapy, and hormonal therapy. Traditionally, treatment for early stage breast cancers (stage I and II) may be surgery followed by adjuvant therapy and patients at higher stages (stage III and IV) or with higher grade tumours would undergo radiotherapy and chemotherapy. However, due to the heterogeneous nature of the disease, breast cancer sub-types respond differently to therapy. Even tumours with similar morphology may respond differently to a given therapy due to molecular or genetic differences between tumours. Although this heterogeneity makes treatment difficult, the detection of more specific molecular targets (i.e. biological markers) underlying the therapeutic response of each tumour population could bring personalized treatment to individual cancer patients. Currently, tumour heterogeneity is addressed by using combination therapies consisting of two or more treatments that may target different subpopulations of cancer cells in the patient. For example, patients are often given adjuvant chemotherapy prior to surgery or radiation. This is known as neoadjuvant therapy, and it is given to many breast cancer patients to shrink tumours size and allow clearer margins during surgery, or given to patients with signs of metastasis such as lymph node involvement. In this section we will discuss in detail the major modalities of cancer therapy in summary.

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1.7.1 Surgery

Most women with stage I or II breast go through surgical therapy to remove the breast tumour. The different options for surgery depend on where the tumour is formed and how far it has spread. Surgical approaches include partial of full mastectomy.

Lumpectomy or partial mastectomy- This method is a breast-conserving surgery (BCS) that removes only the part of breast that contains the tumour. Regardless of how small or large the tumour is some cancer cells might be left behind after surgery in which case re-excision is needed to remove the remaining cancerous cells.

Full Mastectomy – Mastectomy is removing the entire breast containing tumour and sometimes nearby lymph nodes. In a more extensive mastectomy (modified radical mastectomy) other surrounding tissues such as muscle under the breast might be removed as well (65).

1.7.2 Radiotherapy

Radiation therapy is treatment with high-energy beams or particles that destroy cancer cells through ionizing radiation (IR). Gamma rays, X-rays and the UVA component of solar light are among ionizing radiations. One of the mechanism through which ionizing radiation works is damaging the DNA of exposed tumours resulting in cell death. These beams carry high energy to electrons from atoms and molecules thereby generating free radicals known reactive oxygen species (ROS) (66). Reactive oxygen species are chemically reactive molecules containing free radicals of oxygen. Examples

of ROS include the unreactive superoxide radical ($O_2^{\bullet-}$), which is the precursor of non-radical hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($^{\bullet}OH$). ROS can be generated from a variety of endogenous and exogenous sources. In living systems, ROS can be formed during the normal metabolism of oxygen via diverse enzymatic pathways. Free radicals in ROS react with biomolecules such as lipids, proteins and DNA molecules causing chemical modifications. These modifications may modulate survival signaling cascades. Modified lipids and proteins can be removed by normal cellular turnover while DNA damage requires specific repair mechanisms. For example, hydroxyl radical reacts with DNA and causes damage to the heterocyclic DNA bases and the sugar moiety by a number of different mechanisms that if remained unrepaired, will cause genomic instability and thus disease process such as carcinogenesis (67). In fact, nuclear DNA and other molecules in cells are continuously exposed to oxygen species that are endogenously generated in living systems by aerobic metabolism. However, exogenous sources such as ionizing radiation can also cause oxidative damage to the bases and the sugar moieties in the backbone of DNA molecules. In normal cells, most of the damages caused by ROS are repaired by a variety of repair mechanisms while in tumour cells; DNA damage to exposed tumour tissue will cause genomic instability and cell death which is a beneficial consequence of radiotherapy. One of the main types of lesions formed by IR is clustered DNA damage. Clustered lesions are distinct from double-strand breaks (DSBs) and can be tandem on the same strand or on opposite strands of DNA within one or two helical turns of DNA (68). Other types of lesions include adjacent, interstrand and intrastrand base-base tandom lesions (for review see (68)). Radiation therapy can be given externally (external beam radiation)

or internally (brachytherapy). Systematic radiation therapy is also used to treat cancer that has spread to other areas, for example to the bones or brain. Radiation to the breast is almost always given as a post-surgical treatment to help lower the chance that the cancer recurrence in the breast or nearby lymph nodes. Most women with primary tumours (stage I or II) who need BCS will receive radiotherapy on entire breast with an extra boost to the area where the tumour cells were removed. This post-surgical radiotherapy lowers the risk or even prevents cancer from coming back in the area. For women who need post-surgical chemotherapy, radiotherapy is often delayed until after the chemotherapy treatment is completed (69).

1.7.3 Chemotherapy

Chemotherapy is a pharmacological approach in which cancer-killing drugs are given either intravenously or orally. One anti-cancer drug can be used by itself or in combination with other drugs to improve effectiveness. Chemotherapy drugs can be given either intravenously or orally and have different mechanisms of action depends on which pathway or molecules they target in cancer cells. Chemotherapy can be given either before surgery (neoadjuvant chemotherapy) or after (adjuvant chemotherapy) depends on the tumour size and stage and the area in the body which is affected. This approach is normally useful for patients with both lower and higher grade tumours cancer however the risk or recurrence is high. The most common chemotherapy drugs used for early breast cancer are anthracyclines such as doxorubicin (Adriamycin®) and epirubicin (Ellence®) (70,71) and the taxanes such as paclitaxel (Taxol®) and docetaxel (Taxotere®)

(72). These may be used alone or in combination with other drugs, like fluorouracil (5-FU), cyclophosphamide (Cytoxan®) and carboplatin (73).

1.7.3.1 Anthracyclines and mechanism of action

The anthracyclines are anticancer compounds that were originally derived from *Streptomyces peucetius.* The first two anthracyclines compounds discovered in early 1960s and were named doxorubicin (DOX) (Adriamycin®) and daunorubicin (DNR) (or Cerubidine) (70,71). A number of different mechanisms have been proposed for the anticancer actions of these agents. These include intercalation with DNA which results in the inhibition of macromolecular biosynthesis, interference with DNA unwinding or DNA strand separation and helicase activity, DNA binding and alkylation, DNA cross-linking and inhibiting the repair process in DNA damage. Damage to DNA could be a consequence of elevated level of free radicals as a response to drug exposure or due to the inhibition of topoisomerase II (TOPO II) and thus preventing TOPO II-dependant re-ligation after single-strand (SSB) or double-strand breaks, a mechanism that ultimately induces apoptotic cell death (74). These drugs are among the most effective anti-cancer drugs for a broad range of cancers including breast cancer. However, the clinical use of anthracyclines is associated to some main adverse effects including cardiac toxicity and and congestive heart failure (75). Anthracyclines side effects are mostly related to formation of ROS which limits the effectiveness of these drugs. The oxidative stress resulting from increased free radical formation in cardiomyocytes may result in multiple side effects such as activation of stress-related signaling pathways or accumulation of

P53 which eventually lead to apoptosis (75). Epirubicin, Idarubicin and Valrubicin are examples of other anthracyclines with proven therapeutic effects (71,76).

1.7.3.2 Taxanes and their mechanisms of action

Taxanes are one class of chemotherapeutic agents used to treatment of breast cancer. Taxane agents include paclitaxel (trade name: Taxol) and docetaxel (trade name:Taxotere). Taxanes are commonly used alone or in combination with other anti-cancer drugs such as anthracyclines. Taxanes (docetaxel, paclitaxel and cabazitaxel) function by disrupting normal microtubules dynamics. Upon active diffusion through cell memebrane, taxane molecules bind the beta subunit of tubulin within polymerized microtubules, and stabilize them by inhibiting their depolymerisation. (77,78). As a cell progresses through mitosis, taxanes inhibit the ability of sister chromatids to properly segregate which triggers activation of the SAC and arrests the cell in prometaphase (79).Thus, by stabilizing microtubules, taxanes disrupt centrosome organization and interfere with spindle formation which in turn leads to prolonged activation of the Spindle Assembly Checkpoint (SAC), increased cyclin-dependent kinase activity and cell cycle arrest in mitosis which eventually results in cell death by apoptosis Taxanes specifically disrupt the metaphase to anaphase transition in mitosis by triggering the SAC. Prolonged mitotic arrest in response to paclitaxel results in apoptosis. Paclitaxel triggers the phosphorylation of Bcl2 by a number of kinases including CDK1, PKA, Raf-1, mTor and JNK. Phosphorylation of Bcl2 prevents it from binding to and inhibiting pro-apoptotic factors such as Bax which in turn promotes apoptosis by destabilizing the

mitochondrial outer membrane triggering cytochrome C release and activation of the caspase cascade. Thus taxanes by targeting microtubules and triggering both SAC activation and Bcl2 phosphorylation, can induce cell cycle arrest and apoptosis in cancer cells (respectively) (77).

1.7.3.3 Mechanism of taxane resistance in cancer

Chemotherapeutics agents are the most effective drugs for metastatic tumours. However, the ability of cancer cells to become resistance to chemotherapy drugs has remained a significant barrier in successful chemotherapy. Resistance to chemotherapy drugs can be an intrinsic characteristic of cancer cells (intrinsic resistance) or it can be acquired (acquired resistance) which mean cancer cells become resistant to a certain drug or combination of drugs after exposure to cytotoxic agents. Taxanes are among the most important components of chemotherapy regimens to treat recurrent breast cancer. Regardless of being intrinsic or acquired, there are two general classes of resistance to anticancer drugs: those that disrupt delivery of anticancer drugs to tumour cells, and those that affect drug sensitivity due to genetic or epigenetic alterations.

Multidrug resistance (MDR) is a trait in cancer cells which results in the loss of activity of multiple chemotherapy drugs. A family of proteins known as the ATP-binding cassette (ABC) transporters has been reported to confer a multidrug-resistant phenotype. Three members of this family P-glycoprotein (P-gp), MRP1, and ABCG2 have been reported to impact chemotherapy resistance (80,81) . The most common form of *in vitro* derived taxane resistance involves the overexpression of ATP-binding drug efflux P-

glycoprotein (P-gp), which actively pumps taxanes out of the cell (82). PGP is the product of the *ABCB1* (or *MDR1*) gene (83) with 12 transmembrane regions and two ATP-binding sites (81). Drugs bound to this protein will be removed from the cell when the protein is exported (84). Impaired drug delivery can be the result of several mechanisms which broadly fall into 4 class; 1) those that affect entry/efflux of the drug into/from cells; 2) those that affect the mode of action of the drug (i.e. alter interaction between taxanes and microtubules); 3) those that affect the ability of taxanes to trigger the SAC; and 4) those that prevent apoptosis or promote cell survival after taxane treatment (77). Other mechanisms of resistance observed *in vitro* include alterations in beta-tubulin that affects microtubule stability or taxane-binding. Once taxane has entered the cell it binds microtubules to disrupt cell division. Therefore, any alteration in microtubules themselves including mutations, isotype switching and post-translation modifications of tubulin can impact taxane efficiency as the affinity of microtubules to bind taxane molecules might change. (85). In addition, microtubule-binding proteins such as Microtubule-Associated Protein-Tau (MAPT) (86) and stathmin (87), when over-expressed, can alter microtubule stability and binding to taxanes, and is correlated with paclitaxel resistance. MAPT binds to both inner and outer surface of microtubules leading to reversible microtubule stabilization. MAPT binding to microtubules competes with taxane binding and low expression of tau is correlated with taxane sensitivity and pathological complete response to paclitaxel-containing regimes (86). Another mechanism through which taxane resistance can impact cancer cells is alteration in SAC function. The status of the mitotic spindle checkpoint exhibits a significant relationship with susceptibility to anti-microtubule agent-induced apoptosis. SAC malfunction is one

of the leading paths to suppression of paclitaxel- induced apoptosis and increased resistance to taxanes. The mechanisms that lead to impaired SAC function include but not limited to mutation in SAC components such as Mad2 and BubR1 or other proteins that directly or indirectly monitor mitotic spindle assembly (88). One example is Aurora A, a serine/theonine kinase with elevated level of expression in cancer cells. Aurora A gene amplification is associated with an alternative route to SAC dysfunction and inducing chomosomal instability. This overexpression disrupt kinetochore-microtubule dynamics and hence cells enter anaphase despite remaining defects in spindle formation (89). The association between Aurora A overexpression and chromosome instability has been specifically reported in breast cancer (90). Moreover, the relationship between SAC impairment and resistance to anti-microtubule agent–induced apoptosis has been studied in different cancers including lung and breast cancer (91).

1.7.3.4 Taxanes and chemotherapy resistance in breast cancer

Paclitaxel and docetaxel, alone or in combination with anthracyclines like doxorubicin, are the most common taxanes used to treat breast cancer. Although taxanes are highly effective as a first line chemotherapy, breast cancer cells often show intrinsic or acquired resistance to these drugs. (77,78). High expression of MAPT in ER+ breast cancers may account for the general resistance of these cancers to taxane treatment. However, Rouzier and colleagues found that not all ER+ breast cancer cell lines express MAPT suggesting that a subset of ER+ breast cancers have no or low MAPT expression and therefore may be comparatively more sensitive to taxanes (86). Stathmin, on the other

hand, destabilizes microtubules and/or sequesters tubulin, and when overexpressed, it can inhibit the cellular uptake of taxanes as well as mitotic entry leading to increased taxane resistance (92). Therefore normal expression of MAPT and stathmin is required for proper microtubules formation and cell cycle arrest inhibition. Furthermore, spindle check proteins such as MAD1, MAD2 and BubR1 are also involved in taxane resistance. For instance in absence of MAD2 and Burb1, MCF7 cells show greater resistance to paclitaxel (88). Finally dysregulation of apoptotic pathways including alteration in genes involved in apoptosis such as Bcl-2 and P53 are linked to chemoresistance. For example increased levels of Bcl2 phosphorylation in tumour cells correlates with sensitivity to taxanes (93). So, drug resistance pathways are tightly regulated in different levels in tumour cells and dysregulation in any of those steps could affect the response of those cells to taxanes. MAPT is also transcriptionally regulated by estrogen signalling in ER+ breast cancer cells. MAPT protein level is increased by estrogen in ER-positive cells and increased level of MAPT by certain doses of chemotherapy drugs (tamoxifen and fulvestrant) is associated with taxane sensitivity (94). While there is substantial evidence supporting the role of mentioned biomarkers and underlying these mechanisms of resistance *in vitro*. The prediction of response to taxane in human breast cancer has yet to be improved clinically as each approach has its own limitation. To resolve the existing conflict in clinical reports and to overcome multidrug resistance the development of more reliable diagnostic methods or combination of methods and biomarkers is required to increase the accuracy of clinical application (95).

1.7.4 Anti-EGFR/Anti-HER2 therapy

Anti-EGFR therapies including tyrosine kinase inhibitors (TKIs) and monoclonal antibodies have been used in treatment of a variety of tumour types which have developed resistance to conventional therapies. These two categories of therapeutic agents are good candidates for ER-negative breast tumours where anti-ER or anti-hormonal drugs are not effective due to lack of target receptors. While both categories of agents inhibit the EGFR pathway, they act via different mechanisms. Monoclonal antibodies bind to the extracellular domain of EGFR, prevent ligand binding and interrupt the downstream signalling cascade. Tyrosine kinase inhibitors bind to the intracellular domain of EGFR and inhibit the downstream effects of EGFR ligand binding. Expression of the epidermal growth factor receptor (EGFR) system has been deregulated in a wide range of epithelial cancers. Therefore, anti-EGFR therapies represent important treatment options in patients ineligible for chemotherapy due to drug resistance. Ongoing trials and investigations have led to discovery of several kinds of FDA-approved anti-EGFR drugs against different types of cancer including trastuzumab and Lapatinib as a very effective drug to treat breast cancer). Trastuzumab is a monoclonal antibody that interferes with the HER2/neu receptor by binding the extracellular domain of HER2 (96);whereas, the most well characterized kinase inhibitor treatment has been lapatinib, a dual tyrosine kinase inhibitor which interrupts both HER2/neu and EGFR signalling pathways. This drug is used alone or in combination therapy in advanced metastatic HER2-positive or triple negative breast cancer patients.

1.7.5 Anti-Hormonal therapy

Hormone therapy is a form of systemic therapy which is most often used as an adjuvant therapy after surgery to help reduce the risk of the cancer returningor as neoadjuvant treatment before surgery. This type of therapy has a low risk of recurrence and is used for early stage breast cancer or cancers that have come back or have spread after treatment. Most types of anti-hormonal therapy for breast cancer include method to enforce lower levels of estrogen or stop estrogen from acting on breast cancer cells. This kind of treatment is helpful for hormone receptor-positive breast cancers, but it does not help patients with hormone receptor negative tumours (ER- or PR-negative). Anti-estrogenic activity of SERMS haven been used widely in hormonal therapy to block ER from binding to its proper ligand. It is well established that in response to hormonal therapy ER+ patients benefit the most. However, there is a subgroup of breast cancer patients that express ER but fail to respond to hormonal therapy. Failure in response to anti-hormonal drugs can be either due to non-functional receptors or the presence of spliced variants with lack of functional response to estrogen and consequently no respond to hormonal therapy (97). The gold standard in anti-estrogen therapy is Tamoxifen which is an antagonist of estrogen receptor in breast tissue. It is well established that both early and advanced stage breast cancer respond to Tamoxifen. Tamoxifen and it active metabolites like 4-hydroxytamoxifen (4OHT) belong to a group of compounds named Selective Estrogen Receptor Modulators (SERMs).

1.7.5.1 Selective estrogen receptor modulators (SERMs)

Selective estrogen receptor modulators (SERMs) are a class of compounds that act as ER ligands. These compounds are able to exert differential effects in different tissues, a characteristic that distinguishes between SERMs and other ER agonist or antagonist drugs. In other words, SERMs are able to selectively stimulate or inhibit ER in various organs. For example, SERMs are ER antagonist in breast tissue where they block the effect of estrogen and act as an agonist to estrogen in some other tissues where estrogen action is desirable (e.g. bone). The importance of SERM drugs was identified after discovery of Tamoxifen (TAM) and its selective effect on different organs. Tamoxifen has carcinogenic effects in some organs and anti-carcinogenic in some others (98).

1.7.5.1.1 Tamoxifen

Tamoxifen, ICI 46,474, is the first clinically approved SERM that has been used for the treatemtn of breast cancer for over ten decades. The anti-tumour activity of tamoxifen relies on its anti-estrogen properties, making it ideally suited as an endocrine therapy to treat breast cancer. The discovery and development of this drug as a gold standard in treating breast cancer came about through two separated streams of research. The first research was in the early 1900's when investigators sought to determine the reasons for differential response of women with breast cancer to non-steroidal anti-estrogen drugs (99,100). Due to toxicity, the further drug development of these early anti-estrogens was discontinued in early 1960's. The second stream of research on anti-

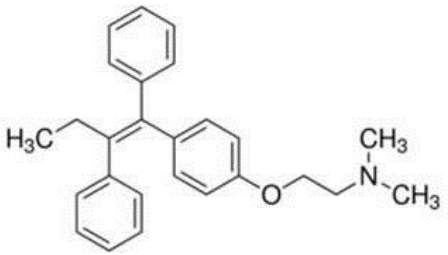
estrogens began in the 1960s with studies of tamoxifenfor use in treating metastatic breast cancer in postmenpausal women. Although tamoxifen was first known as an anti-estrogen drug to treat cancers such as breast cancer in which estrogen was indicated to have a growth impact, later in 1980's it started to be used specifically in ER-positive patients as adjuvant or primary chemotherapy. The clinical concept of chemoprevention was developed in 1987. Following extensive clinical trials to improve the drug efficiency and lower the risk incidence, tamoxifen (trade name Nolvadex) was successfully approved by FDA in 1990 as an anticancer drug (Nolvadexw (tamoxifen citrate) Prescribing Information, AstraZeneca). Once approved by FDA, tamoxifen was extensively studied to examine the drug's chemoprevention potential. Studies in mouse and rat models of carcinogenesis showed long-term exposure to tamoxifen suppress tumourigenesis in both virgin and pregnant animals (100). In particular, tamoxifen usage in early stages after carcinogen induction was highly effective in reducing the number of breast tumours and delaying the onset of the disease (101,102). Since then Tamoxifen has been used to treat metastatic breast cancer, used as adjuvant therapy with chemotherapy, adjuvant therapy alone, used in the treatment of ductal carcinoma *in situ*, and used in risk reduction in higher risk pre- and postmenopausal women as well as breast cancer treatment in men (103). The use of tamoxifen in risk reduction arose from the premise that tamoxifen has a broad range of functions yet has a relatively low risk of side effects. However, like any other cancer treating agent, tamoxifen does have side effects and toxicity, mostly due to its partial agonistic properties. Although it has been effective on treatment of breast cancer through its antagonistic ability to inhibit breast epithelial cells proliferation, tamoxifen has estrogenic agonist effects in tissue other than breast. Similar

to animal models, breast cancer patients and healthy high risk individuals treated with tamoxifen as chemoprevention developed higher incidence of endometrial hyperplasia and uterine cancer. However premenopausal women have shown no increased risk of uterine sarcomas (104). In addition, standard doses of tamoxifen have been linked to higher risk of liver cancer in rats (105). These side effects and uncertainty about tamoxifen led to discovery and development of alternative drugs with enhanced efficacy and reduced toxicity such as like fixed ring compounds and selective estrogen receptor down-regulators (SERDs). Despite all positive and negative effects, tamoxifen has remained the gold standard in endocrine therapy for all stages of breast cancer and in chemoprevention in patients with high risk of developing breast cancer (103). Tamoxifen is also beneficial for bone mineral density and serum lipids in post- menopausal women.

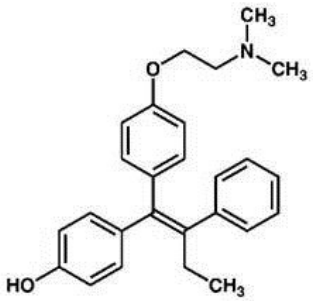
Despite advances in breast cancer therapy, the efficacy of therapy for each individual is still variable. Many factors could affect a patients response, as well as drug toxicity, including the dosage of the drug, the susceptibility of the patient tumour to the drug (e.g. requiring high dose of drug), and the time course of treatment. Moreover, it is still not clear as to the best combination of anti-cancer drugs for a given patient. Finally, when combining drugs, it is also not clear if sequential or concomitant usage will demonstrate more or less efficacy for the treatment of a certain sub-population of cancer patients.

1.7.5.1.2 Pharmacokinetics of tamoxifen and raloxifene

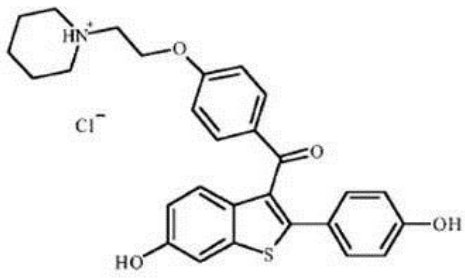
Tamoxifen is the pure trans-isomer of triphenylethylene and is the main member of a family of the drugs named nonsteroidal antiestrogens. Despite hormone-like activity, tamoxifen compete for estrogen binding site on ER and inhibit estrogen-induced tissue proliferation. Tamoxifen exist in more than one metabolically active isoforms. Active metabolites of tamoxifen are including 4-hydroxy-N-desmethyl-tamoxifen (endoxifen), N-desmethyltamoxifen (NDMT), alpha-hydroxytamoxifen (α-OHTAM) and 4-hydroxytamoxifen (4-OHT). Tamoxifen's main metabolites bind to the estrogen receptor preventing estrogen from binding to these receptor. So the mechanism of breast cancer cell growth is interrupted. Tamoxifen is a long lasting drug whereas 4OHT has a shorter half-life but with much higher biding affinity to ER (106).



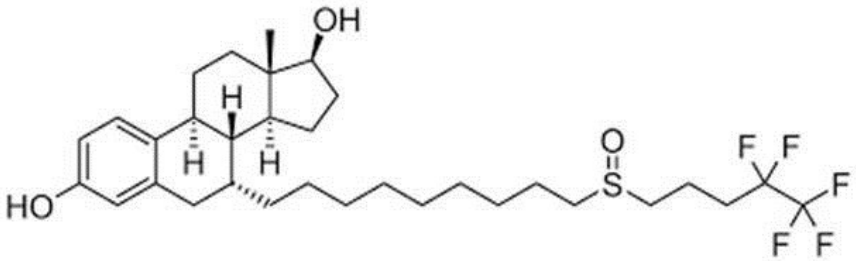
Tamoxifen



4-hydroxytamoxifen



Raloxifen



Fulvestrant

Figure 1.4 Molecular structure of SERMs

1.6.5.1.3 Raloxifene

Raloxifen (RAL), is a fixed ring compound and is used in both prevention and treatment of osteoporosis and breast cancer in postmenopausal women, especially in women with high risk of invasive tumours. Raloxifene was initially developed as an anti-cancer drug on 1980s at that time had shown poor performance compared to tamoxifen but after endometrial proliferative effect of tamoxifen was revealed raloxifene became more applicable again specially because of bone-preserving properties (107). Raloxifene (RAL) is as efficient as tamoxifen in lowering the risk of invasive breast cancer and like tamoxifen acts as estrogen antagonist in breast tissue. Like tamoxifen, raloxifene also binds to and interacts with estrogen receptors, acting as an estrogen agonist in some tissue such as bone and liver while acting as estrogen antagonist in other tissues like breast and uterus. Therefore, raloxifene represents a potentially important alternative to hormone replacement therapy in postmenopausal women for both the prevention and treatment of osteoporosis and cardiovascular disease.

1.7.5.1.4 Fulvestrant

Fulvestrant (trade name Faslodex) is an antagonist of ER with no agonist effects. It is used in treatment of hormone-receptor positive breast cancer especially in postmenopausal women with metastatic cancer.

1.7.6 Mechanisms of SERM action and resistance

Unlike estrogen that is uniformly an agonist, SERMs can selectively choose between agonistic or antagonistic effect depending on the target tissue. The selective effect of SERMs can be explained, in part, by elaborating on their mechanism of action. Both estrogens and SERMs are ligands for estrogen receptors (ERs). Therefore, they have high affinity for ER and can specifically interact with the ligand binding domain in the receptor (108). The ligand-receptor complex binds to EREs on DNA and recruits other co-regulators to the promoter of the target gene. Upon binding to the receptor, each ligand can change the receptor conformation in a unique way that either promote or block the recruitment of transcription co-regulators. Once bound to the ER, each ligand changes the conformation of ER ligand-binding domain mainly by repositioning of helix 12. This structural modification hinders the interaction between transcriptional factors with the AF-2 domain in ER and thus inhibit the subsequent transcription of target genes (109) Depending on the type of the ligand, either co-activators or co-repressors are recruited to the promoter and selectively inhibit or stimulate estrogen-like action in various tissues. The relative concentration of co-activators such as SRC family and co-repressors such as SMRT will dictate the downstream regulatory path through which hormone-regulated genes are expressed. ER antagonists mediate their inhibitory effect by calling co-repressors and inhibit the expression of estrogen-regulated genes many of which are involved in cell growth and proliferation pathways whereas ER agonists promote overexpression of growth genes which might result in development of tumour cells. Each

SERM drug had a unique way of regulation and this differential regulation will contribute to the different cell and tissue-specific activities of SERMs (98).

1.7.6.1 Mechanism of resistance to SERM

Despite the relative safety and significant efficiency of SERMs, These drugs can only be effective in ER+ tumours as there is no ER in ER- cells that can provide the binding site for SERMs. Furthermore, resistance to any therapeutic agents is a natural characteristic of cancer cells meaning that even ER+ tumours can become resistance to SERMs. Resistance can be intrinsic or acquired. In intrinsic resistance ER+ cells initially do not respond to antiestrogen treatment while in acquired resistance, ER+ cell initially respond but gradually lose their ability to respond to a certain anti-hormonal drug. This could be because ER or ER expression maintained but would not respond to a certain drug. Most often patients with relapse on tamoxifen will still respond to fulvestrant (as a pure ER antagonist) or aromatase inhibitors. The effective treatment of tamoxifen-resistant breast cancer requires a better understanding of the mechanisms underlying this resistance.

1.7.6.2 ER signalling and SERM resistance

Since tamoxifen functions primarily target ER, lower expression or loss of function of ER are predictors for tamoxifen resistance. Breast cancer cell with no

estrogen and progesterone receptor (ER-/PR-) are intrinsically resistance to antiestrogen therapy (110). Moreover, ER+ patients who were pre-treated with tamoxifen seem to become ER- on relapse while acquired resistance during treatment. This group of patients sometimes responds to aromatase inhibitors or pure ER antagonists such as fulvestrant which means ER is still in charge of growth regulation in those cells (111). Mutations in ER genes that lead to negative conformational changes in the ligand binding domain can also exert resistance in the host cells. However, these kinds of mutations are mostly generated *in vitro* and are rare in clinical samples from breast cancer patients (112,113). In addition to genetic changes, epigenetic modifications can affect post-transcription regulation of ER and cause ER inactivation (114).

1.7.6.3 HER2 signalling and SERM resistance

Acquired resistance to SERMs can be a result of alteration in ER signalling pathway that converts the ER-SERMs inhibitory function to growth stimulating signals. One of the signalling pathways with an important role in creating resistance to SERMs is the HER2 signalling pathway. Since there is a reverse correlation between ER and HER2 expression, SERM agents such as tamoxifen are less effective on ER+ patients with elevated level of HER2. Activate HER2 triggers a cascade of phosphorylation events including those of Akt and MAPK (115). Phosphorylated MAPK then activates SRC-3 (AIB1), an ER co-activator that can interact with both steroid responsive and nonresponsive nuclear receptors. AIB1 overexpression combined with higher levels of HER2 increases the agonist activity of tamoxifen in ER+ cells (116). Interestingly, in

ER+ patients with higher levels of HER2 tamoxifen acts more like an agonist and causes resistance to adjuvant therapy (111).

1.7.6.4 AKT signalling and SERM resistance

Another signalling pathway that influences ER signalling is phosohatidyl-inositol-3-OH kinase (PI3K) pathway. The crosstalk between ER and PI3K pathway can potentially affect resistance. Similar to ER signalling, upon activation by growth factor stimulation, PI3K triggers the activation of a downstream cascade which has a critical role in many cellular functions including cell growth, proliferation, transcription and protein synthesis. One of the downstream targets of PI3K is PKB/Akt whose increased expression in breast cancer promotes the process of oncogenic transformation including cell proliferation and anti-apoptotic responses (117). In breast cancer cells, PKB/Akt constitutes activation prohibits the tamoxifen-induced apoptosis and facilitates hormone-independent growth. This ligand-independent activation of ER pathway begins with a reciprocal interaction between PI3K and ER, in that PI3K activates AKT. Activated AKT then phosphorylates ER resulting in a hormone-independent activation (118). Therefore, alteration of PKB/Akt can suppress tamoxifen-induced apoptosis. Alternatively, both estrogen and tamoxifen can activate AKT, MAPK, HER2 and EGFR, which triggers a cell survival signal even in the presence of tamoxifen bound to ER. (119). Overall, any alteration in the activation of growth factor signalling components could lead to resistance to SERMs.

1.7.7 Overcoming SERM resistance

1.7.7.1 Aromatase inhibitors

The majority of breast tumours are ER+ with estrogen being the main stimulant for tumour growth. Although blocking the estrogen signalling pathway by SERMS has been the main approach, it has deficiencies and side effects. For example not all ER+ patients respond to tamoxifen and even those who respond will almost always relapse due to acquired resistance following longer exposure to the drug. It has been suggested that tamoxifen deficiencies at least in part could be because of its partial antagonistic properties as the agonistic properties of tamoxifen might be responsible for post-treatment relapse. Therefore, development of other drugs that target estrogen synthesis pathway without having any estrogenic activity would be beneficial to overcome resistance to SERMs. The main agents of this type are the aromatase inhibitors. Aromatase is the enzyme that converts androgens (such as testosterone and androstenedione) to estrogens (such as estradiol and estrone, respectively) and which is the ultimate source of all estrogens after menopausal. The rationale behind using aromatase inhibitors (AIs) is to remove the source of estrogen which has been proven to be superior to tamoxifen with prolonged recurrence time. However, the combination of tamoxifen with AIs, in a sequential manner, seems to be the most effective therapeutic regimen to reduce the risk of returning cancer especially when AIs were used after tamoxifen treatment (120).

Although routine clinical approaches for cancer were based on pathological variables including tumour size, presence or absence of lymph node metastasis and histological grade, today, most treatment decision is made based on molecular marker status namely hormone receptors.

Besides hormone receptor and receptor tyrosine kinase-regulated pathways that predominantly regulate the initiation of gene transcription, post transcriptional regulatory elements are part of gene regulation machinery that define the fate of mRNA and control the amount of gene product that is made. In this chapter post-transcriptional events involving non-coding RNA-mediated and RNA binding protein-mediated controls will be discussed with relation to cancer.

1.8 mRNA post-transcriptional regulation in Cancer

Regulation of gene expression is a multi-step process includes a wide range of mechanisms used by cells to increase to decrease the production of specific genes. In principle, every step required for the process of gene expression could be controlled. Two general types of genes involved in progression of cancers are tumour suppressors and oncogenes. Gene expression is mostly controlled at the level of transcription on promoter regions of protein-coding genes. However, transcriptional regulation is only one part of the extensive interplay of regulatory mechanisms. Post-transcriptional regulation will add another level of complexity to the gene expression at the RNA level. Regulation at the post-transcriptional level normally controls the stability of RNA molecules and the distribution of variant transcripts.

. In this section, I will review how non-coding RNAs and mRNA binding proteins play an important role in the post-transcriptional regulation of gene expression in cancer through interactions with mRNA, particularly through the 3'untranslated region (3'-UTR).

1.8.1 Regulatory regions found within mRNA

Messenger RNA (mRNA) in eukaryotes is initially synthesis as pre-mRNA in nucleus by RNA polymerase II (PolII). This pre-mRNA then undergoes several post transcriptional modifications before becoming mature mRNA and export from nucleus to the cytoplasm. The maturation process of mRNA is a very well regulated mechanism which determines differential gene expression. The 5'- and 3'- untranslated regions (UTRs) are the two main platforms where mRNA regulatory elements interacts with regulatory protein complexes and play important roles in post transcriptional regulation of gene expression mostly by conferring mRNA stability and translation regulation. While the 5'UTR is primarily involved in controlling mRNA translation (121), the 3'-untranslated region (3'UTR) regulates multiple aspects of mRNA biology, including nuclear export, cytoplasmic localization, translational efficiency and mRNA stability (122). The addition of (m^7G[5']ppp[5']N) cap to the 5'- and the poly (A) tail to the 3'-end are among mRNA post transcriptional modifications that confer stability. These elements are also associated with translation initiation factors such as eIF4F and poly-(A)-binding protein (PABP). Cleavage and polyadenylation of pre-mRNA molecules at the 3'end is also mediated by the interaction between a number of RNA-binding proteins (RBPs) and cis-regulatory elements. The 3'UTR of the transcripts located downstream of the open reading frame and controls gene expression via interaction with specific trans-acting factors, including RNA binding proteins and non-coding RNAs, on the regulatory cis-acting RNA sequence elements (**Figure 1.6**). The combination of these

factors can regulate translation either in a positive or negative way and also affect RNA stability and mRNA localization and degradation.

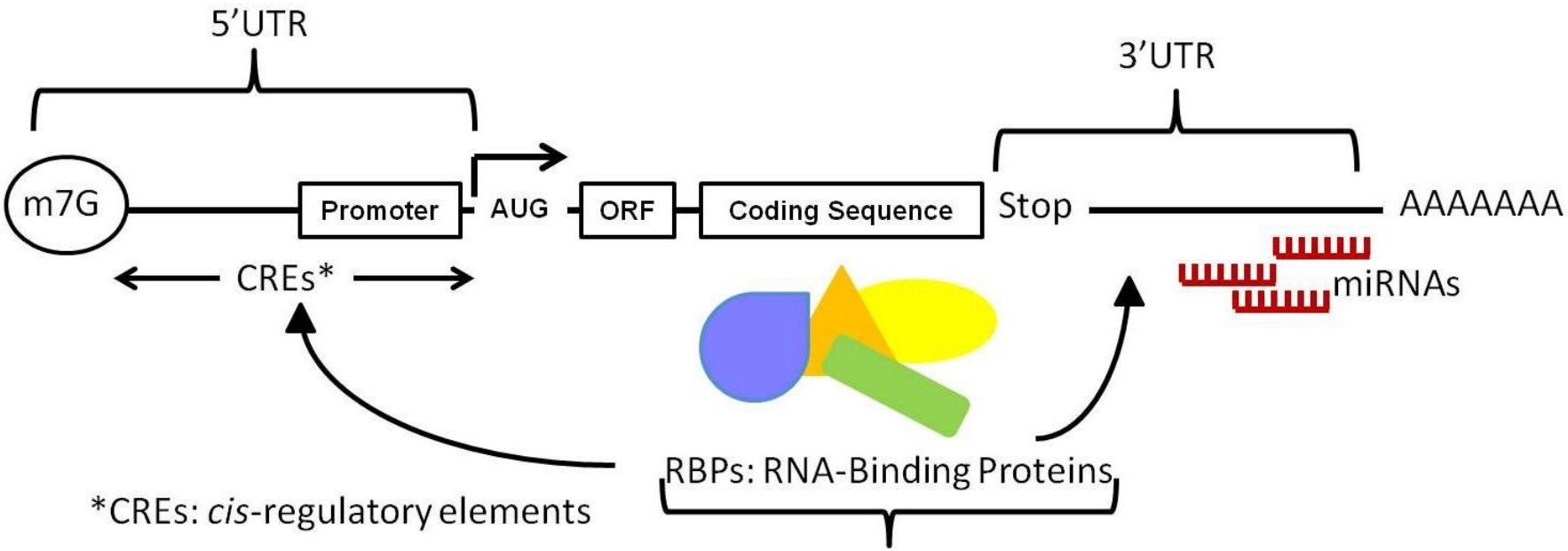

5'UTR
3'UTR
m7G
Promoter
AUG
ORF
Coding Sequence
Stop
AAAAAAA
CREs*
miRNAs
RBPs: RNA-Binding Proteins
*CREs: *cis*-regulatory elements

Figure 1.5 Overview of the regulatory sequences within mRNA

1.8.2 RNA binding proteins

RNA-binding proteins (RBPs) are a large group of proteins (800- 1,000 in humans) that often interact with the 5' or 3′ UTRs of mRNAs on specific regulatory sequences. These regulatory factors function in a broad spectrum of RNA biology from RNA processing (including transcription, pre-mRNA splicing and polyadenylation) to RNA modification, localization, storage, degradation, and translation efficiency and turnover. (123–125). From very early steps in their lifetimes to then end, mRNAs are largely escorted by a group of associated factors which either permanently or transiently bound to mRNA. These accompanying factors constitute of proteins and non-coding RNAs which together form messenger ribonucleoprotein particles (mRNPs). In this way, RBPs act as mRNA chaperones to export mature mRNAs to their target cytoplasmic locations where they undergo more mRNA processing and translation. Once an mRNAs arrived at cytoplasm its half-life determines the level of protein synthesis. For each subset of mRNA with similar or relatively close cellular function there is a specific set of accompanying particles whose relative position on mRNA dictates almost everything that will happen to that mRNA. RNA binding proteins in particular, are one of the main regulators of functionally related mRNA transcripts. In the other word, the fate of a given mRNA is defined by a series of coordinated events from splicing to nuclear export, stabilization, localization and eventually translation (122). This post transcriptional regulation is a mechanism through which RBPs create another level of complexity to cellular phenotype.

1.8.2.1 mRNA binding domains

Many RBPs interact with mRNAs via a limited set of RNA-binding domains. Some well-characterized RNA-binding domains include:

RNA-binding domain (RBD, also known as RNP domain and RNA recognition motif, RRM); zinc finger domain (ZnF, mostly C-x8-X-x5-X-x3-H); K-homology (KH) domain (type I and type II); DEAD/DEAH box;double stranded RNA-binding domain (dsRBD); RGG box (Arg-Gly-Gly); Sm domain; cold-shock domain; Pumilio/ FBF (PUF or Pum-HD) domain; and the Piwi/Argonaute/Zwille (PAZ) domain(126). Although the number of known binding motifs is limited, a number of events can cause higher level of variability to the RNA-protein binding pattern. For examples, often times, several RNA-binding domains are found within one RBP. Also multiple binding proteins can bind one cis-element. In addition, alternative splicing remove some sequence on mRNA and post translation modification of RNA binding proteins might change their affinity or ability to bind RNA. Some of the RNA binding proteins that are related to this study will be reviewed in this section.

1.8.2.2 CSDE1

The human unr (upstream of N-ras) is a transcription unit immediately upstream N-ras gene. This gene which is evolutionary conserved between several mammalian species was identified as an essential gene in embryonic development as its deficiency is lethal in mice embryos (127). The protein product of this gene is an 85kD RNA-binding

protein named Csde1 (cold shock domain containing E1) which is ubiquitously expressed and is implicated in the post-transcriptional regulation of a subset of cellular mRNA (128,129). CSDE1 contains five cold shock domains as RNA recognition motifs and is capable of binding to single-stranded RNA. CSD is one of the most conserved nucleic acid binding domain from prokaryotic to eukaryotic proteins (130). CSD-containing proteins are capable of binding single-stranded DNA and RNA (128).

In eukaryotes, proteins containing CSD function as chaperones, also implicated in various cellular processes such as temperature adaptation, cellular growth and nutrition stress. Some of these proteins are involved in transcription regulation and some others have cytoplasmic roles which affect the mRNA splicing, RNA turnover and translation. CSDE (also known as Unr) has been shown to regulate internal ribosome entry site (IRES)-mediated translation or (IRES)-dependant translation is a cap-independent regulated translation during which CSDE1 interacts with cis-elements on the RNA sequences on the 5'UTR called internal ribosome entry sites (IRESs). Eukaryotic genes containing IRES sequences can recruit the translation machinery independent of the mRNA 5'-cap (121). One example is translation activation of Apaf-1 (Apoptotic protease activating factor 1) by Unr. Upon mRNA binding, Unr acts as a RNA chaperon and changes the conformation of the Apaf-1 IRES to permit the ribosomal formation and translation initiation. Other auxiliary co-factors named IRES-transacting factors (ITAFs) are present at the site of assembly to ensure efficient three-dimensional structure for ribosomal assembly and translation initiation (131). CSDE1 also is capable of binding the 3'UTR and convey mRNA stability. There are very few genes whose 3'UTR has been studies for CSDE1 cis-elements. One example is parathyroid hormone (PTH) gene.

The *cis*-acting element in the PTH mRNA 3'UTR are binding platform for CSDE1 as a component of mRNA stabilizing complex which regulates PHT mRNA stability.

1.8.2.3 ELAVL1 (HuR)

The ELAV/Hu family of RBPs is a highly conserved family consists of four members. Three of these members, HuB/Hel-N1, HuC and HuD, are predominantly localized in cytoplasm and HuA/HuR (also known as ELAVL1) is expressed primarily in the nucleus of all human cells (132). HuR is a ubiquitously expressed protein that exhibits specific affinities for ARE-containing RNA sequences *in vitro*. Although AREs are among destabilizing elements on the 3'UTR of mRNA, under most conditions HuR binding to A/G-UUU rich sequences stabilizes mRNA and promotes translation of targeted transcript (133). This function of HuR has been shown to be in combination with miRNA regulatory machinery. MicroRNA seed elements on the 3'UTR are in proximity or even overlapping with HuR binding sites and thus HuR competes with RISC for binding to AU-rich elements. Binding of one HuR molecule recruits cooperative binding of multiple HuR which eventually hindering miRNA assembly by displacing RISC. This combination regulation by HUR and miRNA affects mRNA stability by removing the hindrance degradative function of miRNA (133). Another mechanism through which HuR helps with mRNA stability is its ability to shuttle between nucleus and cytoplasm. HuR is normally localized within the cell nucleus and hence is associated with the mature mRNA as a very first step in the life of an mRNA. HuR-bound mRNA then export from the nucleus to the cytoplasm where the accompanied mRNA is transferred to the

translation apparatus. In this way HuR transiently shuttle between nucleus and cytoplasm protect its target mRNA from degradation (134).

1.8.2.4 ZRANB2

Zinc finger Ran-binding domain-containing protein 2 (ZRANB2) was originally identified in rat (135). The gene encoding this protein, *ZNF265,* is localized on chromosome 1p31 (136). ZRANB2 is one of the regulator for alternative splicing and interacts with several splicing factors (137). There are two zing finger domains in the N-terminus of ZRANB2 which are categorized as Ran-binding domain. Ran is a small GTP-binding protein that binds proteins with a role in shuttling and export process between nucleus and cytoplasm. Therefore, ZRANB2 is found in nucleus of human cells as well as in splicing speckles. ZRANB2 is expressed ubiquitously and has conserved amino acid sequence among species from human to Xenopus. Besides its role in splicing, ZRAB2 is one of the mRNA binding proteins (103-105). Moreover, this protein was shown to have some relevance to human diseases including cancer. High stage ovarian cancer patients have shown over-expression of ZRANB2 (137).

A possible explanation for mechanism involved in ZRANB2-mediated tumourigenesis are changes in concentration, localization or activity of splicing factors which in turn affect the process of splicing and give rise to protein isoforms with tumourigenic properties (139).

1.9 Regulatory non-coding RNA and Cancer

Non-coding RNA (ncRNA) genes produce RNA transcripts that do not have any Open Reading Frame (ORF) and hence do not encode proteins. Instead these RNAs are functional molecules involved in a variety of cellular regulatory processes on their own or as a part of ribonucleoprotein particles (RNPs). In addition to splicing, the presence of non-coding RNAs in eukaryotic transcriptome explains the high level of complexity of the genome. Non-coding RNAs can be classified into three main sub-group according to their sizes. microRNA with 19-25 nucleotides, small RNAs with 100-200 nucleotides and long non-coding RNAs with over 200 up to 10,000 nucleotides in lenght (for details review (140). Non-coding RNAs are important components in post-transcriptional modification in eukaryotic cells and play roles in a wide range of epigenetic events such as in X-chromosome inactivation, chromatin structure, DNA imprinting, DNA demethylation, and transcription (141). In addition to molecular function, there are growing evidence of ncRNA being involved in cellular and developmental events in eukaryotes. A topic which will be not discussed here.

The discovery of long non-coding RNAs is dated back to 1990s when inactivation of chromosome X in mammals was shown to be mediated by a non-coding RNA. XIST gene was cloned as a large ncRNA expressed exclusively by the inactive chromosome X (142). Another example of epigenetic regulation of ncRNA is loss of imprinting is H19 gene. H19 is the first identified large imprinted ncRNA which mediates the imprinting of insulin-like growth factor 2 (*IGF2*) gene and its deletion mediates imprinting of *IGF* (143). RNA-dependant DNA demethylation is another epigenetic modification mediated

by ncRNAs. KHPS1a is an RNA transcript whose overexpression results in demethylation of CgG-rich island on the sphingosine kinase-1, *sphk1,* gene. The product of this gene, SPHK1 is involved in calcium mobilization process (144). Another example of ncRNA roles in gene regulation is Steroid Receptors co-Activator (SRA). SRA is a selective activator of steroid receptors and is involved in post-translational regulation of nuclear receptor activity and hence in the regulation of ukaryotic gene exprexxion (145). Over times, more functional roles for non-coding RNAs have been found including the ones in maintenance of chromatin structure. For example, several non-coding RNAs (ncRNAs) have been isolated and are associated with the main components of eukaryotic heterochromatin and euchromatin (141). All the above examples have provided the principles for the RNA interference (RNAi) and RNA silencing techniques that has been used broadly in gene knock down methods to study molecular pathways.

1.9.1 Long non-coding RNAs in cancer

The importance of the non-coding part of the genome in gene expression regulation and post-transcription modifications has been studies extensively in the past two decades. LnRNAs, among others, have been shown to function directly as structural and catalytic regulators rather than protein encoding mRNAs. Therefore, changes in lnRNA expression levels have been reported in different human disease including cancer. The role of lnRNAs in cancer has mostly been investigated in the context of ncRNA overexpression but also deletion and down regulation. Examples of ncRNAs overexpressed in cancer are HIS-1 and BIC (Leukemia and lymphoma) (146), PCGEM1

and DD3 (prostate) (147) MALAT-1 (lung) (148), and loss of imprinting and overexpression of H19 and overexpression of BC200 (breast) (149). Therefore, lnRNAs can be used as a powerful diagnostic marker in different types of cancer as well as a therapeutic target in cancer treatment. However, extensive clinical trials are required to improve the application of these ncRNAs in cancer therapy.

1.9.2 MicroRNAs

MicroRNAs (miRNAs) are a class of small single-stranded RNA molecules of 19-22 nucleotides. These non-coding RNA molecules interfere with gene expression regulation by affecting the stability of messenger RNA (mRNA) or translational efficiency of target mRNAs (46,150,151)**.** The discovery of miRNAs is dated back to 1993 when Victor Amber and his colleagues found that *lin-4* which is an essential gene in *C.elegans* postembryonic developmental events did not encode a protein but two small *lin-4* transcripts of approximately 22 and 61 nt (152). These transcripts contain complementary sequences to 3' untranslated region (3'-UTR) of lin-14 mRNA by which and via an antisense RNA-RNA interaction regulates the lin-14 translation post-transcriptionally (152,153). Although this finding did not attract much attention at the time, later on when more non-coding RNA molecules were discovered by other laboratories in plants, animals and human, the importance of these tiny regulatory RNAs came into focus. Over time a large class of small RNAs were found which gave rise to introducing "microRNAs" as a family of small RNA molecules of 17-27 nucleotides.(31,144-145). These single-stranded RNA molecules interfere with gene expression by either directly

degrades mRNA molecules or by inhibiting translation through base-pairing with so called “seed elements” on the 3’-UTR of targeted mRNA(152–154). This antisense complementary feature causes repression in the amount of protein without changing the transcript level (153). Human genome encodes over 1000 miRNA genes (155). miRNA coding sequence which is evolutionary conserved among species such as human, mouse, worm and flies, are found within or in proximity with known genes which are referred to “host genes” (156). Although not fully characterized yet, it is likely for those miRNA coding sequences which are located in introns to share regulatory elements, including promoter, with their host genes. It is also possible that miRNAs have their own promoters and are transcribed independently from their own promoters (156,157). Another aspect of miRNA genes is that their coding genes exist as either single units or as gene clusters. Cluster genes are prone to transcribe together as polycistronic transcripts which usually control miRNAs with related functions (158). Genomic studies revealed that the expression pattern of microRNAs (miRNAs) varies in different cell types and also in different developmental stages in one specific cell type. In addition, the differential expression profile of miRNAs might be due to the abundance of certain miRNAs in the cells with a range from very low to very highly expressed miRNAs (156).

1.9.2.1 MicroRNA biogenesis

MicroRNAs are initially transcribed from the genome as primary miRNAs (pri-miRNAs) which are long molecules with typically several hundred to a thousand (1kb) nucleotides (159). Most of miRNAs are transcribed by RNA polymerase II, however there are evidence that RNA polymerase III might also be a candidate (156). Whether as an independent unit or overlapped with an annotated gene, miRNA coding sequences encode pri-miRNA molecules which undergo folding to form stem-loop structures(46,150,151). Under further maturation processing in the nucleus this long hairpin structure is cleaved by Drosha complex. Drosha cofactor, DGCR8 (DiGeorge critical region 8) activity is required for this cleavage which produce a 60-70 nt stem-loop intermediate molecule named miRNA precursor (pre-miRNA). All pre-miRNA molecules are similar in their hairpin-shaped secondary structure (150,156). Drosha activity also leaves a 5'- phosphate at one end and a 2-nt 3'-overhang at the other end of pre-miRNAs (158,160,161). The 2-nt overhang end of pre-miRNA is recognized by Exportin-5 which is responsible for transferring pre-miRNAs from nucleus to cytoplasm during an active GTP-coupled transfer mediated by Ran cofactor (162). . When transported to cytoplasm, the loop structure of pre-miRNA molecule is cleaved with a cytoplasmic RNase III endonuclease enzyme named Dicer (161). Dicer in association with TRBP/PACT, a double strand RNA binding domain which helps with the enzyme stability, cleaves pre-miRNAs into intermediate miRNA:miRNA* (guide:passenger) duplex of ~22nt which is then unwound into mature single-stranded miRNAs by helicase. This subsequent cleavage by Drosha and Dicer then expose mature miRNAs to

Argonaute proteins (Ago1-4). Ago proteins are components of RNA-induced silencing complex (RISC) while one strand (guide strand, also called sense strand) forms the Ago-RNA complex, the other strand (passenger strand or anti-sense strand) will often be degraded (163,164). Mature single-stranded miRNAs, incorporated into RISC, are able to target mRNA via 3'-UTR region and regulate gene expression by either mRNA degradation or translation inhibition. **Figure 1.7** shows a schematic view of general steps in miRNAs biogenesis. Although the 3'-UTR of mRNA contains the most common target sequences for miRNAs bindings, there are miRNAs that interact with the coding regions (CDS) on mRNA, such as members of miR-103/107 family which preferably bind to their target coding regions, miR-181a which binds to 8-mer-matched site in the CDS of zinc finger genes (ZNF83, ZNF37A,ZNF 180 and ZNF 265) and miR-24 which binds to CDS of FAS-associated factor 1 (FAF1) (165,166).

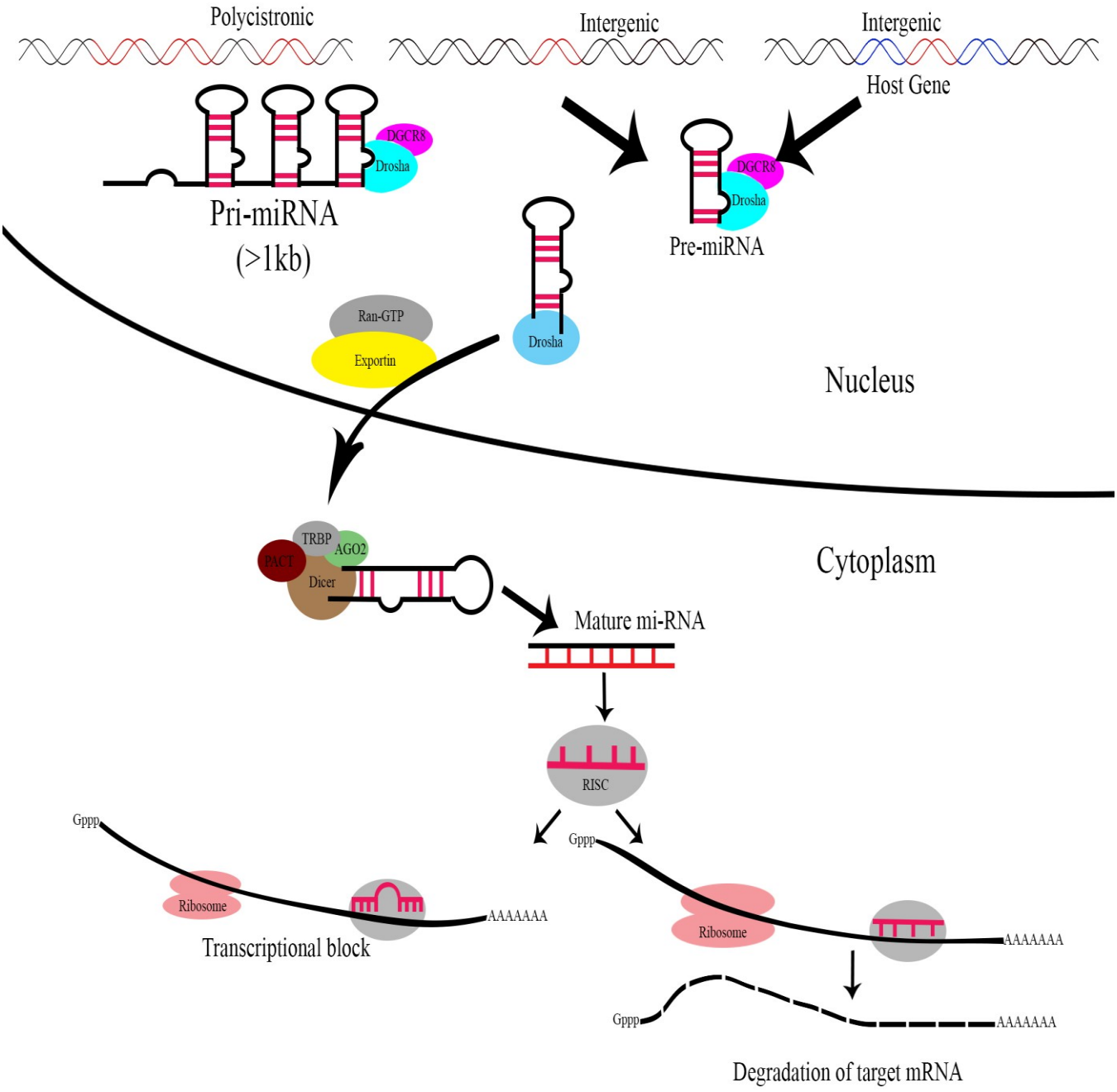


Figure 1.6 MicroRNA biogenesis and function

1.8.2.2 MicroRNAs in carcinogenesis

In past decade many efforts have been put in studying the critical role of miRNAs in important biological processes and human diseases. Cancer has been the most extensively investigated disease with miRNAs connected to its initiation, progression and therapeutic applications. In addition, there are evidences that show miRNAs could function as tumour suppressors or oncogenes. Many studies have revealed that different types of cancer have distinct miRNA profiles due to miRNA abundance and/or variation in miRNA gene expression. This diversity in miRNA expression allows distinction from adjacent normal tissue (167). Moreover, expression studies of various tumour types, and specific cell populations, has also revealed alterations in miRNA expression profiles within one specific tumour (168,169). This miRNA signature has become the main focus of miRNA-related cancer studies. First cancer-related miRNA genes, *miR-15a* and *miR-16-1*, were identified in 2002 in B-cell chronic lymphocytic leukemia (B-CLL). These two miRNA genes, located on chromosome 13q14, are either deleted or downregulated in B-CLL patients (170,171). Other types of cancers that are shown to be associated with miRNA abundance are colorectal neoplasia and Burkitt's Lymphoma with decreased level of *miR-143* and *miR-145,* in colorectal cancer (172) and increased level of *miR-155* and its non-coding RNA host gene, *BIC*, in Burkitt's lymphoma patients (173).

In 2005, three independent studies reported miRNA contribution in carcinogenesis: Two studies introduced *mir-17-92*, a polycistronic cluster located on chromosome 13q31, as an oncogenic miRNAs with increased level of expression in

small-cell lung cancer and human B-cell lymphomas (174–176) and one study reported *Let-7* miRNA gene, located on as a tumour-suppressor-like miRNA whose reduced expression was observed in lung cancer in human (177). Over time, more miRNAs deregulation or mutation was reported to be associated with other human diseases including other types of cancer. There are several aspects to miRNA-associated cancers: First discovered miRNAs in *C.elegans* and *Drosophila* were involved in cell proliferation and apoptosis suggesting that any over-expression or under-expression of these miRNAs could cause proliferation diseases. Second, despite mammalian genome contains only 1% miRNA genes, around 50% of them are mapped to the fragile loci in which are susceptible to breakage and translocation or chromosomal regions that are usually deleted, amplified or genetically altered in human cancers (152,178). In other words there is correlation between the genetic location of cancer-associated genes and the loci carrying miRNAs coding genes in the genome. Third, miRNA expression is aberrant in tumour cells lines and malignant tumours. And fourth, there are evidences that miRNA expression is not only tissue-specific but also tumour progression stage-specific (171-173) . This property of miRNAs can be applied to classify cell populations within a tumour based on the developmental stage of a cell population in one tissue. The list of miRNA alterations in human cancers has been growing over time and miRNAs were proposed to contribute to oncogenesis functioning as tumour suppressor genes (TSGs); for example, as is the case of miR-15a-16-1 cluster, miR-143-145 cluster or the let-7 family. These tumour suppressor-like miRNAs (tsmiRNAs or tsmiRs) target those genes whose products are involved in cell cycle, cell proliferation, differentiation, invasion and angiogenesis. In contrast, oncogenic miRNAs inhibit or down regulate tumour suppressor

genes, for example, as is the case of miR-155, miR-21 or miR17-92 cluster) (168,181). Knowing the above and using different miRNA-specific expression- and analytical techniques, researchers studied miRNA expression in different types of cancer cells. These unique patterns of altered miRNA expression provide complex fingerprints that may serve as molecular biomarkers for tumour diagnosis, prognosis of disease-specific outcomes, and prediction of therapeutic responses.

1.8.2.3 MicroRNAs and estrogen signalling

Estrogen can regulate gene expression at the transcriptional level by activating ER-dependent genes and post-transcriptionally by modulating the expression of microRNAs (miRNAs) that alter mRNA stability and translation. In general, the total level of most miRNAs is lower in tumour tissue versus normal tissue and in ERα-negative versus ERα-positive tumour cells. This global decrease in miRNAs expression level could be, at least in part, an outcome of estrogen regulation in of miRNA biogenesis. Since both steroid hormone receptors (SHRs) and miRNA have regulatory effect on genes, it is not surprising that there is a tight interaction between steroid hormone receptor pathways and miRNA regulation. The direction of this regulation i.e. weather miRNAs regulate steroid hormone receptors or vice versa, has been under debate but there are evidences for both. In some cases there is one or a set of miRNAs that regulates SHs expression. This regulation could be directly via inhibitory effect of miRNAs on gene expression or indirectly through other genes that affect SHs expression. Conversely, in some other cases SHRs can either repress or induce miRNA expression.

For example, the 3'-UTR of ERα gene has been shown to have seed elements for miR-206 (182) in MCF7 and for miR-221/222 in MCF7 and T47D cells (183). As a result, these miRNAs directly suppress the expression of ERα protein without significant changes in mRNA level. In addition, these miRNAs can indirectly regulate the expression of ERα in MCF7 via targeting the downstream targets in ER signalling pathway such as $p27^{Kip1}$ (184). As previously stated, regulation of miRNA expression might be induced by SHs. ERα-regulated miRNAs are the ones that are regulated by E2 and are mostly identified in breast cancer patient samples or human breast cancer cell lines. One of the well-known E2-regulated miRNAs is miR-21 which is over-expressed in breast cancer (185). MiR-21 expression has been shown to be down-regulated by estradiol and this suppression appears to be ERα-mediated (247). However, it is not very clear yet how estrogen receptor signalling is linked to miR-21 and hypothesis on relationship between miR-21 expression level and estrogen signalling pathway is contradictory. miR-17-92 cluster is another E2-induced family of miRNAs although some uncertainty remains between studies (186). Another miRNA with connection to ER is miR-135b. The 3'UTR of ERα contains binding sites for miR-135b and overexpression of this miRNA has been demonstrated to be associated with decreased level of ERα mRNA and protein in breast cancer cells (187).

1.8.2.4 MicroRNAs in breast cancer

Phenotype diversity in breast tumour subtypes is due to several groups of co-expressed genes which are involved in activities of similar signalling pathways and

regulatory system. This classification which is based on mRNA expression level defines breast cancer more as multiple neoplastic disorders instead of one single disease. Similarly, miRNA expression profile can be used to classify breast cancer subtypes (185). In the past decade, miRNA microarray technology identified miRNA expression signature which is correlated to clinical and pathological features of human diseases including breast cancer. A global decrease of miRNAs has been observed in human cancers. Recognition of those differentially expressed miRNAs between normal and tumour tissue could help to unraveled pathogenic role of miRNAs in different types of cancer and to discriminate a specific miRNA signature for breast cancer. Like other cancers, miRNA expression profile in breast cancer tissue is distinct and significantly different from normal breast tissue. For example in a study of 10 normal and 76 neoplastic breast tissue, 29 miRNAs were identified whose expression was deregulated in breast cancer tissue vs. normal tissue including Let-7, miR-10b, miR-125b, miR145 (down-regulated) and miR-21 and miR 155, miR-191 and miR-206 (up-regulated) (185).

Furthermore, expression of different miRNAs can be categorized based on association with ER status (miR-26, miR30, miR-185, miR-191, miR-206, miR-212), PR status (let-7c, miR26a, miR29b,miR-30), tumour stage (miR-21, miR-181, miR-30a, miR-213) and lymph node metastasis (let-7 and miR-9-3) (185,188). Therefore, similar miRNA signature across cancerous tissue or normal tissue could be used to classify normal and tumour tissues. For example, miR-17-5p, miR-20a, miR-21, miR-92, miR-106a and miR-155 overexpression is shown to be associated with tumour development in different types of cancers including breast cancer (189). In addition, each step in cancer

development such as initiation, progression, epithelial– mesenchymal transition (EMT) and metastasis are associated with certain sub-populations of miRNAs (190). Role of miRNAs in cancer initiation and/or progression is more related to the essential characteristics of these molecules in selecting target transcripts. In general, most of the miRNAs inhibits the expression of their target genes. However, depends on the functional importance and molecular pathways that the target genes are involved in, the overall effect of a miRNA of a miRNA family could be either inducing or inhibitory (191). Unique patterns of altered miRNA expression provide complex fingerprints that may serve as molecular biomarkers for tumour diagnosis, prognosis of disease-specific outcomes, and prediction of therapeutic responses. Using genome-wide miRNA microarray, Iorio et al. found that the target genes for those down-regulated miRNAs are the ones with oncogenic function and target genes for up-regulated miRNAs are potentially tumour suppressors. In the other word, those tumour suppressor-like miRNAs are expressed endogenously in lower level in tumour cells compared to normal cell. In contrast, oncogenic miRNAs are expressed in higher level in tumours versus normal tissue (192).

1.9 PRP4K: Pre-mRNA Processing Kinase

1.9.1 PRP4K is an evolutionary conserved dual specificity kinase

Pre-mRNA processing 4 kinase (PRP4K) was originally isolated from *Schizosaccharomyces pombe.* The *prp4* gene of *S. pombe* was identified in a bank of temperature-sensitive (ts) pre-mRNA processing (*prp*) mutant strains. These conditional mutants were identified by accumulation of pre-mRNA at the restrictive temperature (193). Prp4 belongs to the Cdc-like kinase (Clk) sub-family in the eukaryotic protein kinase superfamily (194). This sub-family includes the mammalian SRPK1 and Clk/Sty protein kinases (195,196). The homolog of Prp4k gene in human is *PRPF4B* which encodes *a 150KDa* phosphoprotein that belongs to the dual-specificity tyrosine-regulated kinase *(DYRK)* family (194,197).

1.9.2 Gene and Protein structure of PRP4K

In humans, the *PRPF4B* gene that encodes PRP4K is located on chromosome 6 (ch6p25.2) (194). The most abundant transcript of human PRP4K has 15 exons which are translated into a protein of 1007 amino acid residues. The predicted molecular mass of PRP4K is 117 kDa but Western blot analysis with anti-PRP4K antibodies detects bands of about 152 kDa in nuclear extracts from human and mouse cells (194). PRP4K is a

phosphoprotein in mammalian cells and phosphorylation of PRP4K can be a result from either autophosphorylation or phosphorylation by kinases like CLK and SRPK1 that target the arginine-serine (RS) dipeptides in the N-terminus of PRP4K (198). Phosphatase treatment of nuclear extracts reduces most PRP4K to the 147-kDa fast-migrating hypo-phosphorylated form. Mammalian PRP4K has an extended N-terminal domain characterized by four evolutionarily conserved sub-domains or motifs. These include: the lysine-histidine-rich KKHK box, the arginine/serine (RS)-like domain, a domain containing the motifs MI and MII, and the dual-specificity kinase domain (**Figure 1.8**). The KH-rich KKHK box is also present in Protein ACTivator of the interferon-induced protein kinase (PACT), an snRNP associated protein that interacts with p53, Rb and POP101(199). POP 101 is a component of the splicing-associated inter-chromatin granules. The ORF of POP contains many RS/SR dipeptide repeats. Human homolog of POP, srm160, is a human splicing co-activator localized to speckles (200,201) The second conserved domain in PRP4K protein structure is an RS-like domain which is rich in arginine and serine. RS domains are found in many splicing factors and as discussed above are target sites for phosphorylation by SR protein kinases such as CLK and SRPK1. The RS domain is also believed to mediate protein-protein interactions between splicing factors in spliceosomal complex as well as the transcriptional machinery (198). These KKHK box and RS-like domain are very similar to those found in several splicing factors and also conserved among other specious including metazoan homologues of PRP4K (*Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana*) but is not present in *S. pombe* Prp4p. The third conserved domain that consist of two evolutionary conserved sequence motifs, MI (DDMFA) and MII (DNWTDAEGYYRV)

adjacent to the kinase domain is found in all Prp4 kinases from *S. pombe* to humans (**Figure 1.8).** The fourth conserved domain in PRP4K is a dual specificity kinase domain, similar to what is found in the CLK/STY protein kinase family. The kinase domain of PRP4K like other eukaryotic kinase domains has three main roles in phosphorylation: 1) binding to the phosphate group donor in ATP (or GTP) with divalent cation (usually Mg2+ and Mn2+), 2) binding and orientation of the protein or peptide substrate, and 3) transferring the phosphate group from ATP (or GTP) to the protein substrate. This reaction involves the addition of a covalently bound gamma- phosphate to the acceptor hydroxyl (-OH) group on Ser, Thr or Tyr side chains, of the protein substrate (202).

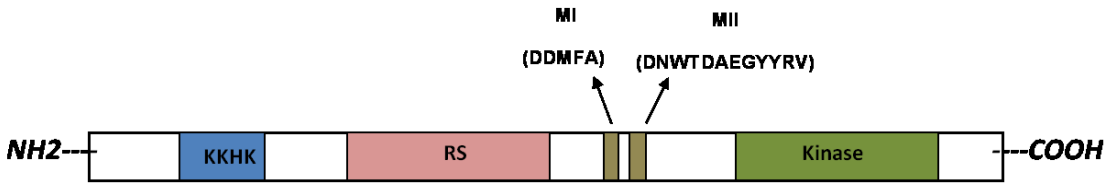


Figure 1.7–The conserved features of primary protein structure in human PRP4K

1.9.3 PRP4K cellular and tissue localization

In human and mouse cells indirect immunofluorescence (IF) has shown PRP4K is mainly located in the nucleus, excluding the nucleolus, and is enriched in multiple speckles. Sequences within the RS-like domain of PRP4K are responsible and sufficient for colocalization of PRP4K within SC35-containing splicing speckles. In interphase, PRP4K is predominately nuclear and localizes in these splicing speckle domains (194). However, during mitosis it is associated with kinetochores, where it plays its role in regulating the accumulation of Spindle Assembly Checkpoint (SAC) proteins such as MAD1 and MAD2 at the kinetochore in response to microtubule targeting drugs (203). Northern blot and immunohistochemistry analysis indicates that PRP4K is ubiquitously expressed in all tissues of the body but is particularly enriched in the brain and in glandular tissue (204,205).

1.9.4 Function of PRP4K

PRP4K is implicated in a number of cellular functions from pre-mRNA splicing to cell cycle checkpoint control. In this section I summarize the evidence supporting an essential role for PRP4K in these processes.

1.9.4.1 The role of PRP4K in pre-mRNA splicing

The most well studied cellular role of PRP4K is in pre-mRNA splicing, a process that occurs co-transcriptionally during eukaryotic transcription. During transcription, the initial RNA (pre-mRNA) that is transcribed from a gene must be processed to produce the mature messenger RNA (mRNA), which is transported from the nucleus to the cytoplasm for translation (206). One of the steps in this processing is joining together of coding exons by the removal of intervening sequences called introns. This process results in the "splicing out" of the intronic sequences producing a final mRNA that is ready for transport to the cytoplasm where it will be translated into protein. Besides this form of “constitutive splicing” which uses defined splicing junctions, alternative splicing also occurs allowing several protein isoforms to be encoded by one gene. Alternative splicing produces multiple transcripts encoding different proteins from the same RNA transcript by including or excluding introns and/or alternative exons at various positions along the transcript. Alternative splicing occurs in an estimated 95% of human genetic transcripts greatly enhancing transcriptome complexity and proteome diversity in higher eukaryotes (207).

The molecular machine that regulates this process is called the spliceosome, a macromolecular machinery consist of five small nuclear ribonucleic proteins (snRNPs), named U1, U2, U4, U5, U6 and several associated cofactors (207). The mRNA contains specific conserved sequence elements that are recognized and utilized for spliceosome assembly. These include the 5' end splice, the branch point sequence, the polypyrimidine tract, and the 3' end splice site. Spliceosome assembly is a complex, step-wise process

that takes place at sites of transcription. The first step involves recognition of the 5' (5'ss) and 3'(3'ss) splice sites located on adjacent exons by U1 and U2 snRNP respectively; a process which is mediated by the carboxy-terminal domain of polymerase II and the 2'OH group of the branch adenosine of the intron. This early recognition of the splice sites which begins with binding of U1asnRNP and weakly associated U2 snRNP to the 5' splice site forms complex E. This binding is mediated by the Ser/Arg (SR)-rich proteins in mammalian cells. In this process, the RS domain of SR proteins interacts with the RS motif of U1-70K. SRF1 is one of the SR-rich splicing factors that ensure the accuracy of splicing and regulating alternative splicing. The SR-rich domain in SRF1interacts with other spliceosome components to form a bridge between the 5' splice site and the U1 snRNP. This interaction is regulated by CLK1 and SRPK1(208,209). Once U1 and U2 snRNP have bound their target splice site, they interact with each other to form the pre-spliceosome (complex A). The next step in assembly involves the recruitment of pre-assembled U4/U6-U5 tri-snRNP to the pre-spliceosome to form complex B; a reaction catalyzed by several splicing factors including pre-mRNA processing factors PRP28, PRP6 and PRP31. PRP28 association with the tri-snRNP is dependent on its phosphorylation by SRPK2 (210). Complex B next undergoes a series of substantial remodeling events resulting in the release of U1 and U4 snRNPs, creating a catalyticially active complex B. Once this catalycally active complex formes, complex B carries out the first catalytic step (the first transesterification reaction) in splicing to form complex C. After a series of rearrangements, Complex C carries out the second step of splicing reaction resulting in a post-spliceosomal complex containing the two exons spliced together from the 5' splice site and the intron-exon lariat intron intermediate. Finally, the

remaining U2, U5 and U6 snRNPs are released from the transcript to be re-used in additional rounds of splicing.

PRP4K's role in splicing was first discovered in 1991 when a temperature sensitive strain of *Schizosaccharomyces pombe* that exhibited a splicing defect was found to harbor a mutation in the yeast gene *prp4* (193). Further analysis of the gene that suppressed Prp4 revealed that this gene encodes a serine/theonine kinase. This finding made *prp4* the first kinase to play a role in splicing (211). Later the mammalian homologue of *prp4* (PRP4K) was shown to interact with pre-mRNA splicing factors including the U5 snRNPassociated protein PRP6 and the Suppressor-of-White-Apricot (SWAP, also known as SFSWAP) which regulates the alternative splicing of the *CD45* and fibronectin genes (194). Finally, PRP4K has been shown to be a key regulator of U4/U6-U5 tri-snRNP assembly and activation of the spliceosome through the phosphorylation of PRP6 and PRP31(212).

1.9.4.2 PRP4K's role in chromatin remodeling

In addition to its splicing role, PRP4K has been shown to play roles in chromatin remodeling by interacting with chromatin remodeling complex. PRP4K is a component of the N-CoR-2 complex and has been purified with proteins involved in gene expression through the modulation of chromatin structure (194)**.** The N-terminal of PRP4K interacts with a mammalian homologue of the *Drosophila* protein Brahma, BRG1, which is a chromatin remodeling protein. BRG1 is the catalytic component of a mammalian Swi/Snf complex and has been implicated in the transcriptional activation of several hormone-

regulated genes. This protein regulates gene expression in the fly and shares homology with its yeast homologue Swi2/Snf2 which is also a chromatin remodeling factor (213). By interacting and phosphorylating BRG1, PRP4K may bridge and coordinate the regulation of chromatin organization during transcription with pre-mRNA splicing. The potential role of PRP4K in chromatin remodeling is also supported by evidence that the affinity purified PRP4K/N-CoR complex exhibits robust histone deacetylase (HDAC) activity (194).

1.9.4.3 PRP4K's role in the spindle assembly checkpoint

PRP4K has been implicated in the regulation of mitosis. The overexpression a truncated form of the kinase Prp4k in *S. pombe* was shown to induce mitotic aberrations (214). Depletion of PRP4K causes defects in chromosome segregation and prevents the normal accumulation of the SAC proteins MAD1 and MAD2 at the kinetochore, which in turn inhibits SAC activation in response to microtubule targeting drugs. Although localized in nucleus during interphase (194), PRP4K is also component of the phosphoproteome in mitotic spindle which contains kinetochore-associated proteins in human cells. PRP4K is enriched in kinetochores during mitosis, where it is believed to play a role in regulating the SAC. In this way, mammalian PRP4K acts as a regulator of the mitotic spindle assembly checkpoint (SAC) through its ability to recruit checkpoint proteins MPS1, MAD1 and MAD2 to the kinetochore (203). In *S.Pombe*, Prp4 kinase was also found to be required for proper segregation of chromosomes during meiosis

(215) as inhibition of Prp4k protein causes massive defects in chromosome segregation during meiosis.

1.9.4.4 PRP4K role in taxane resistance

The Dellaire laboratory recently demonstrated that low levels of PRP4K correlate with increased paclitaxel resistance in ovarian and breast cancer cells (216). This finding has important implications for therapeutic response to taxanes, a family of anti-cancer agents that depend on SAC activity for cell killing. This research has also shown that PRP4K functions downstream of the receptor tyrosine kinase HER2 to regulate paclitaxel response in breast and ovarian cancer, and that its expression is decreased in ovarian cancer patients that have relapsed from taxane treatment. Importantly, Corkery et al., also demonstrated that, amongst ovarian cancer patients with low HER2 expressing tumours, PRP4K expression can be used as a predictive marker to identify patients likely to benefit from taxane therapy (216)

1.10 Hypothesis

Based on previous data indicating a role of PRP4K in taxane drug resistance and the regulation of PRP4K expression by the HER2 growth receptor, I hypothesize that PRP4K may also be regulated by hormone receptor signaling in response to estrogen. As a corollary, anti-estrogen treatments may alter the sensitivity of breast cancer cells to taxanes.

CHAPTER 2: MATERIALS AND METHODS

2.1. Cell culture, drug and hormone treatment

MCF7, T47D and MDA-MB-231 cells were purchased from American Type culture Collection (ATCC) and tested by STR profiling periodically during the study. Cells were seeded in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, Canada) and 1% penicillin/streptomycin at 37°C with 5% CO2. For estradiol experiments cells were grown in phenol free Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 5% carbon- striped FBS and 1% penicillin/streptomycin at 37°C with 5% CO2. Cells were grown for 48 hours before 10nM or 100nM of 17-β-estradiol (E2) (Sigma) and vehicle (Ethanol) was added to cells. Cells were harvested 24 hours after treatment for either protein lysate extraction or total RNA extraction. For tamoxifen cells seeded in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin were treated with two different doses (1μM or 5μM) of 4-hydroxytamoxifen (4-OHT)(Sigma) or vehicle (DMSO) for 24h before protein lysaae or total RNA were extracted for Western blotting and qPCR respectively. For cycloheximide treatment, cells seeded in DMEM supplemented with 5% cs-FBS and 1% penicillin/streptomycin were treated with or 75 μg/ml cycloheximide (Sigma) or vehicle (DMSO) for 2h. Then media was switched to fresh media with or without 10nM E2 depends on the experiment.

2.2. Western Blot Analysis and Densitometry

Cells were harvested and lysed in lysis buffer (300nM KCl, 20mM Tris-HCl pH8, 10% Glycerol, 0.25% Nonidet P-40, 0.5mM EGTA, 0.5mM EDTA, 1X protease inhibitors). Total protein concentration was quantified using Bradford assay with Bradford protein reagent (Bio-Rad) and 1% BSA (Bio-Rad) to generate serial dilutions and create standard curve. Lysates were mixed with 2X sample buffer (2.5 ml 0.5M Tris,PH 6.8, 2.0 ml 20% SDS, 2.0 ml glycerol (100%), 0.25 ml 0.4% Bromophenol Blue, 2.25 ml ddH2O, 100µl, Betamercaptoethanol per 1 ml solution) and boiled at 90°C for 5min. Protein quantification was conducted by Bradford assay (BioRad) using a spectrophotometer (Eppendorf). Total protein concentration of each sample was measured using Bovine Serum Albumin (BSA) as standard control. An equal amount of protein was loaded onto 7% (or 10% for loading control) SDS denaturing polyacrylamide gel and was separated by electrophoresis (SDS-PAGE). Separated proteins were transferred to a nitrocellulose membrane and blocked with 5% skim milk powder in TBST (0.01M Tris HCl, 0.15M NaCl, 0.05% Tween-20) for 1h at room temperature prior to overnight incubation with sheep anti-PRP4K primary antibody (H143) (1:1000) (194,216) or rabbit anti-actin (1:10,000)(Sigma) diluted in TBST supplemented with 5% bovine serum albumin (BSA) overnight at 4ºC. The membranes were then washed with TBST and incubated with goat anti-sheep secondary antibody conjugated to horseradish peroxidase (HP) (1:5000) (Sigma) or mouse anti-actin (Sigma) or rabbit anti-tubulin (Sigma) for 1h at room temperature (RT) before being briefly washed in TBST prior to protein detection by enhanced chemiluminescence (ECL) (Bio Rad). GAPDH antibody

(1:10,0000) (abcam) was used as the loading control with incubation time of 1h at RT. Chemiluminescence was then visualized either by exposing radiographic film (Kodak) or using a VersaDoc imaging system (MP4000; Bio Rad). Densitometry was performed using the QuantityOne softwared (version 4.6.8), where the chemiluminescence image intensity of the protein of interest was normalized to actin, tubulin or GAPDH and at least three separate experiments were used to determine mean fold changes in protein expression. For estimation of significance the Student's T-test in Microsoft Excel was used.

2.3. Indirect immunofluorescence detection of PRP4K in murine mammary tissue

Mammary glands were dissected from mice and mammary tissue was fixed in 4% paraformaldehyde (PFA) at 4°C, paraffin-embedded, sectioned (at 5 μm), and stained with hematoxylin and eosin. For the immunofluorescence detection of PRP4K, the primary sheep anti-PRP4K antibody H143 (1:200) (194,216) was incubated with (control) or without a blocking recombinant peptide (1 ug/ml)(504 to 688 aa of human PRP4K) before being used for the immunodetection of PRP4K on tissue sections, followed by donkey anti-goat Cy3 secondary antibody (1:500)(Jackson Immunoresearch). Tissue slides where then imaged on a Nikon Eclipse E600 equipped with a Nikon DXM1200F CCD camera using the software ACT-1.

2.4. RNA isolation and quantitative real-time PCR (q-RTPCR)

TRIzol Reagent (Invitrogen) was used to lyse cultured cells. Total RNA was extracted according to the manufacturer's manual. RNA was quantified using a spectrophotometer (BioRad). One microgram of RNA was converted to cDNA using the QuantiTect Reverse Transcription kit (Qiagen) by employing universal hexamer primers and Reverse Transcriptase. Quantitative PCR (qPCR) was then performed on the cDNA using primers specific to PRPF4B and RPLP0 (endogenous control gene) (Qiagen) and the QuantiFast SYBR green PCR kit (Qiagen). Q-PCR reactions were perforemd using a Maxpro3000 real-time PCR detection system (Stratagene). The relative expression levels were then calculated between experimental treatments by normalizing PRPF4B to RPLP0 expression using ΔΔCt method (217). All experiments were done in three independent replicates. For estimation of significance the Student's T-test in Microsoft Excel was used.

2.5. MicroRNA extraction and quantitative real-time PCR (q-RTPCR)

Large and small RNAs from MCF7 cell line were isolated with the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's protocol. For miRNA quantitation, 2 μg of small RNA was used as the template. cDNA was synthesized from 5 ng total RNAusing TaqMan microRNA transcription kit and miRNA-specific primers for miR-21 and snRNA U6 as an endogenous control (Invitrogen) according to the TaqMan miRNA Assay protocol (Applied Biosystem). The relative expressions of miR-21 were measured using Real-Time PCR according to the TaqMan protocol, using triplicate reactions for each biological replicate including reverse transcription product, miRNA-specific primer and probe assay mix for miR-21 (Invitrogen), and 1X RotorGene Master Mix (QIAGEN). Thermal cycling was performed using a Rotor-Gene thermocycler

(QIAGEN). Results were normalized relative to the endogenous U6 as a control (assay ID). Relative expression levels were calculated from Ct values.

2.6. MicroRNA transfection

To study the effect of miR-21 on PRP4K gene expression, MCF7 cells were transfected with 25nM miR21-5p mirVana mimics or miR-1 mimic (Ambion) using using Dharmafect 1 (Dharmacon). PRP4K and PTK1 gene expression was monitored 24 hours post-transfection by qPCR using specific primers for "Hs_*PRPF4B"* and "Hs_*WTF1"* (Qiagen) as the positive control. To study the effect of miR-21 on PRP4K protein translation, MCF7 cells were transfected with miR-21or scrambled miRNAs as negative control and PRP4K protein level was measure with Western blot analysis and normalized to GAPDH level as housekeeping marker. For luciferase assay, MCF7 cells were co-transfected with HSV-TK-Renilla, pMIR-4kbUTR or pMIR-4kbUTR-mut, in combination with miR-21 mimics using Dhamfect1 (Dharmacon). Alternately, cells were co-transfected with mir-21 expression vector pCMV-miR21 with the pMIR-4kbUTR reporters or pCMV-luc-miR21(p), which contains a containing a miR-21 site, as a positive control (Addgene #20381 and #20382,respectively) (218) (data not shown).

2.7. Plasmid constructs and luciferase assays

2.7.1. Cloning of promoter constructs

The putative full length human PRPF4B promoter genomic region encoding ~5.0 kb of sequence 5' to the start-codon and flanked by a 5' AflII and 3' SacII restriction site was cloned from BAC RP11-208-G3 into the promoter-less vector PGL4.14 encoding Firefly luciferase (Promega) between restriction sites KpnI and EcoRV. The construct was named "*full promoter*" A truncated 2.3 kb promoter, flanked by a 5' *ClaI* and a 3' *SacII* restriction site, was also cloned and named "*ClaI* promoter"

2.7.2. Promoter assay

For promoter assays, empty PGL4.14 vector or that containing either the 2.3 kb truncated *ClaI* promoter or the full length 5.0 kb promoter were transfected into MCF7 breast cancer cells in media supplemented with 5% carbon stripped serum, as described for estrogen experiments, using Lipofectamine 2000 (Invitrogen). As a normalization control, cells were concurrently transfected with a plasmid encoding Renilla luciferase (HSV-TK-Renilla). Relative Firefly luciferase expression level was measured before and after estrogen treatment over time and normalized to Renilla as an internal control. After 24 hours cells were lysed in 1X passive lysis buffer (Promega) and Firefly and Renilla expression were determined using the Dual-Reporter Luciferase Reporter System (Promega) and a Promega GloMax lumminometer.

2.7.3. Cloning of 3'UTR constructs

For 3'UTR experiments, a human brain cDNA (AB011108) encoding PRP4K or cDNA isolated from MCF7 cells was used to clone the full length 3' UTR of the PRP4K transcript. The human brain-derived UTR was subcloned as three fragments into pTRE-PGL4.14 (Promega), UTR1 (position 3092-4277 bp relative to AB011108), UTR2 (4278-5498 bp relative to AB011108) and UTR3 (5499-6680 bp relative to AB011108) within the 3' UTR region of the vector at the FseI restriction site. These three brain derived UTR fragments were initially used to optimize the 3'UTR assay and the corresponding luciferase activity. MCF7 TET-OFF cells (gift of Dr. Craig McCormick, Dalhousie University) were co-transfected with either pTRE-PGL4.14- UTR1, -UTR2, or -UTR3 and HSV-TK-Renilla, using Lipofectamine 2000 (Invitrogen). Firefly luciferase expression was monitored 24-48 hours post-transfection and normalized to Renilla expression as described above (data not shown). Meanwhile, The full length PRP4K 3' UTR was amplified from cDNA derived from MCF7 using DNA polymerase "Phusion Hot start" (Invitrogen) and CG-rich amplification PCR program: 98°C for 1min; [98°C for 30", 58°C for 30", 72° for 1.3 min] (X5); [98°C for 30", 60°C for 30", 72°C for 1min](X25) and 72°C for 10min. To make cDNA, total RNA extracted from MCF7 cells was converted to cDNA using oligo dT primers and superscript III first strand synthesis system for RT-PCR (Invitrogen) according to the manufacture manual. Two sets of primers were designed according to *PRF4B* transcript (NM-003913.4) to amplify cDNA for further cloning purposes. (primer set 1- F:5-GGCATATTTTATTCAGTTTCC-3, R: 5- GCTAAAGGACTTGTTGGACCA-3; primer set 2- F: ATAAAGTCTTTGTGAAC

AAGGCATT-3 and R: TGATAATAAGAGGCATATTTATTC-3. Purified 3'UTR fragments were then sub-cloned into the pCR-4-TOPO (Life Technologies) vector using TA-cloning kit (Invitrogen), verified with sequencing and then cloned into pMIR-REPORT-Luc vector (Life Technologies) between restriction sites *SacI* and *SpeI* to create pMIR-4kbUTR for 3'UTR assays. A truncated 3'UTR was made using following forward and reverse primers and the pMIR-REPORT-Luc-4kbUTR construct as a template to generate a shorter 3'UTR construct names pMIR-LUC-UTR1 which still contains intact miR21 seed elements (5-TAAGCTA-3). For miRNA studies, using overlap PCR reactions and using the following primers, the miR-21 binding site within pMIR-UTR1 was altered from "TAAGCTA" to "GAACGAT" to create pMIR-UTR1-mut. All sequences were verified by sequencing and analyzed using Clone Manager Software (Version 9.0).

2.7.4. 3'UTR assay

For pMIR-REPORT-LUC-4kbUTR, pMIR-REPORT-LUC-UTR1 and pMIR-REPORT-LUC-UTR1-mut experiments, MCF7 cells were co-transfected with pMIR-REPORT-LUC-4kbUTR, -REPORT-LUC-UTR1 or pMIR-REPORT-LUC-UTR1-mut and HSV-TKE-Renilla as the transfection control using Lipofectamine 2000 (Invitrogen) the day before cells were lysed and harvested for luciferase assay.

For experiments involving treatment with 17-β-estradiol (E2), MCF7 cells were seeded (for 24h) and transfected (8-12h) in phenol free DMEM supplemented with 5% charcoal stripped FBS and 1% Peniciline/Steptomycin before treated with (10nM) or

without (vehicle) 17-β-estradiol (E2) for another 24h. Then cells were lysed in 1X passive lysis buffer (Promega) and Firefly and Renilla luciferase activity was measured using the Dual-Reporter Luciferase Reporter System (Promega) and a Promega GloMax lumminometer.

2.8 Cell transfection and shNA Lentiviral Transduction

For ESR1 transfections, MDA-MB-231 cells were transfected the ESR1 expression vector pcDNA-HA-ER WT (Addgene plasmid #49498) using Neon® transfection system (Invitrogen) according to the manufactures protocol. After 12-18h cells were harvested for protein lysate preparation and Western blot analysis as described above. To knockdown ESR1 in the T47D cell line, ESR1 targeting GIPZ Lentiviral shNAs (shESR1-1 = clone: V2LH5_239590, shESR1-2 = clone: V2LH5_239351) were purchased from Thermos Scientific. Lentivirus was obtained by co-transefection of theTRIPZ shNA, pMD2.G, pCMV-8.92, and pCMV-8.93 vectors (described previously (219)) into human HEK-293T cells via calcium-phosphate transfection (Promega), according to the manufacturer's protocol. After 48 h, media from the transfected cells was filter sterilized using a 0.45μ filter, and the viral media added to T47D cells for 48 h, after which the media was exchanged for media supplemented with 2 μg/ml of puromycin and positively transduced cells were selected in the presence of this antibiotic over 72 h prior to further experimentation.

2.9 Tamoxifen and taxane treatment and cell viability assay

Paclitaxel (Sigma, T7402) and 4-OHT (Sigma, H7904) and tamoxifen were reconstituted in dimethyl sulfoxide (DMSO) and diluted in growth media so that the DMSO concentration was 0.05% or less. To evaluate MCF7 cell response to combined doses of tamoxifen and taxane *in vitro*, 5,000 cells were plated in individual wells of a 96 well plate, and allowed to adhere for 24 h prior to incubation with the indicated concentration of tamoxifen. After 24h cells were treated with 1μM 4-OHT or DMSO as vehicle for another 24h. The drug was removed after 24h and media was replaced by DMEM containing indicated doses of paclitaxel for 90min. Following acute paclitaxel treatment, the drug was removed and cells were allowed to recover in fresh medium for 72 h, at which point cell viability was measured using the alamarBlue cell viability assay (Life Technologies, DAL1100) according to the manufactures protocol. To determine the effect of tamoxifen treatment on paclitaxel response, fluorescence was measured using SpectraMax M2 plate reader (Molecular Devices) 4 h after the addition of alamarBlue reagent. Values were normalized to DMSO-treated cells in three replicates.

2.10 RNAseq and Splicing primers

RNA sequencing was done in collaboration with Dr. Stephen Montgomery in University of Stanford, CA. Specific primers were designed according to the exon-exon junctions and alternative spliced variants were quantified using qPCR.

Specificity of each primer was determined using NCBI BLAST module. Real time PCR was performed with Maxpro3000 real-time PCR detection system (Stratagene). Using SYBR Green Mastermix (QIAGEN) and the data was analyzed with MaxPro 3000 software using Delta Ct method.

CHAPTER 3: RESULTS

3.1. PRP4K is expressed in mammary epithelial cells and is induced by 17-β-estradiol in breast cancer cells

The pre-mRNA splicing kinase PRP4K is an essential kinase that is ubiquitously expressed, with the highest expression in the brain and hematopoetic cells (194,204). Unpublished observations of the immunohistochemical detection of PRP4K available from the Protein Atlas web resource (**http://www.proteinatlas.org**) indicated that PRP4K may also be highly expressed in the epithelial cells lining the ducts of the ovary and breast. Using indirect immunofluorescence detection of PRP4K in murine mammary tissue, we confirmed that PRP4K is predominately expressed in the epithelial layer of lobular ducts (**Figure 2.1A**). These epithelial cells of the milk duct express the estrogen ERα (ESR1), suggesting that perhaps PRP4K protein expression may be regulated by estrogen signalling via this receptor. Both estrogen and progesterone (PR) receptors are used as prognostic biomarkers in prediction of proper therapeutic response to anti-hormone therapy in breast cancer(3). Considering that gene-chip analysis showed that *PRP4K* gene expression can be induced 2-fold by estrogen in osteosarcoma cells expressing the ERα (220), I sought to confirm if ERα+ (ER+) breast cancer cells treated with 17beta-estradiol (E2) would exhibit increased PRP4K protein levels (**Figure 2.1B**). Indeed, PRP4K protein levels increased by ~1.9-fold in ER+ positive MCF7 breast cancer cells (**Figure 3.1B**) and both 10 and 100 nM E2 resulted in a similar increase in protein expression of 2-2.5 fold (1.8 ± 0.2 and 2.2 ± 0.4, respectively) (Figure 3.1C). Since PRP4K protein levels responded similarly to 10 and 100 nM E2, all other

experiments were conducted using 10 nM of E2. In agreement with the Western blot analysis of PRP4K protein levels, treatment of the ERα-positive breast cancer cell line MCF7 with E2 at a concentration of 10 nM for 24 h resulted in a 2-3 fold increase in PRP4K gene expression (2.7 ± 0.6) as measured by quantitative PCR (qPCR) **(Figure 3.1D)**.

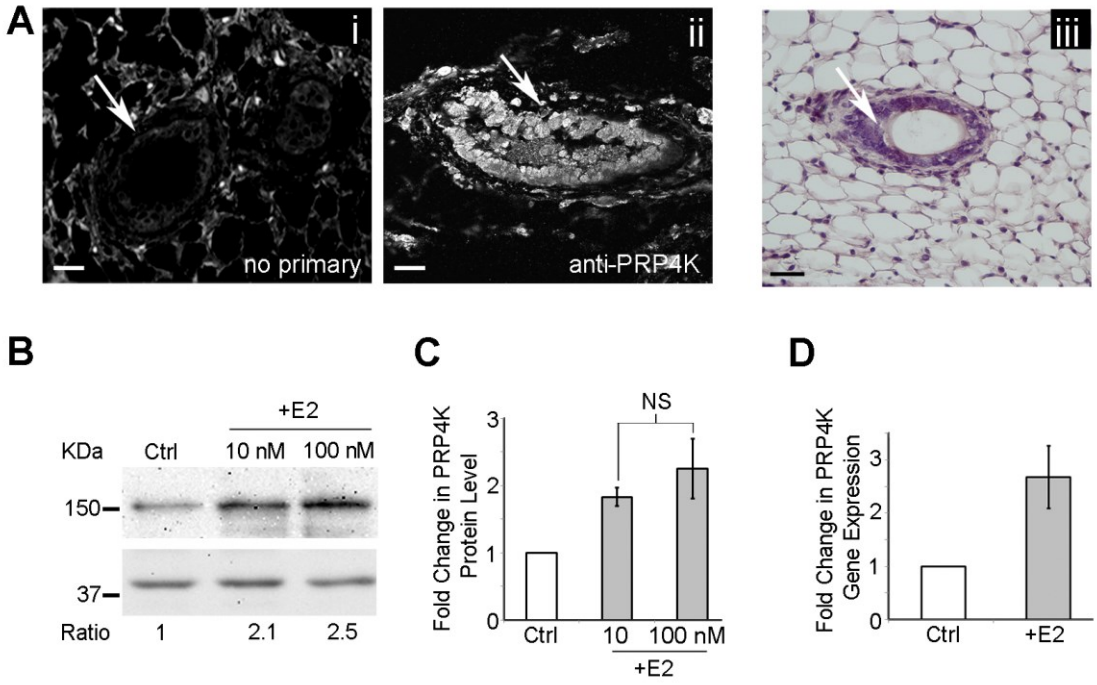
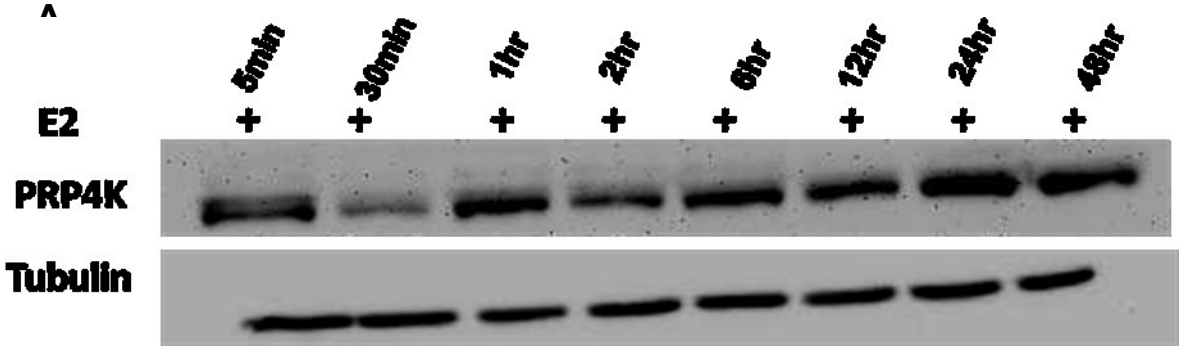


Figure 3.1- PRP4K is expressed in mammary duct epithelial cells and induced by E2 in breast cancer cells. A) PRP4K is expressed in mammary duct epithelial cells**.** Murine mammary tissue was harvested, fixed, embedded in paraffin and processed for immunohistological analysis of PRP4K localization by indirect immunofluorescence **i)** without primary antibody and **ii)** with sheep anti-PRP4K antibody, and **iii)** general tissue morphology of the mouse mammary tissue by hematoxylin and eosin (H&E) staining is shown at the far right. Milk ducts lined with mammary epithelial cells are indicated by white arrows**. B-D**) MCF7 cells were treated for 24 h with either vehicle (0.01% EtOH) (Ctrl) or 17-β-estradiol (E2) at the indicated concentration before being processed for Western blot analysis and densitometry analysis. A representative Western blot is shown in (**B**) and a bar graph of the relative fold change in PRP4K protein level after 24h treatment with E2 in shown in (**C**). Error bars = SE (where n = 4), *$p<0.05$, NS = not significant. **D**) Q-PCR was also performed on MCF7 cells treated as in A with vehicle (Ctrl) or E2 (10nM) to determine the relative levels of PRP4K gene expression in response to estrogen as indicated in the bar graph. Error bars = SEM (where n = 3), *$p<0.05$.

3.2. Estradiol regulation of PRP4K over a 48 h time course

To further investigate the effect of estradiol on PRP4K protein and transcript regulation and to determine the long and short-term effects of estradiol stimulation on PRP4K, PRP4K mRNA and protein expression were observed over a 48 h time period. Serum starved MCF7 cells were treated with E2 (10nM) or vehicle (0.01 % EtOH) and protein and transcript level were measured between 5min and 48h. As shown in Figure 3.2 the maximum response for transcript and protein appeared at 12h and 24h, respectively. In addition, estradiol induced a rapid increase in PRP4K protein levels within 5 min after hormone induction (**Figure 3.2 A-B**). The stimulated effect of E2 on PRP4K gene expression and protein levels was maintained after 6h and appeared to reach the peak by 24h although the changes were not statistically significant for all time points. The estradiol-induced increase in PRP4K transcript seems to appear at earlier time points compared to protein up-regulation (i.e. a peak in transcript at 12h proceeded the peak increase in protein level at 24h). The effect of estradiol seems to decrease after 24h for both protein and transcript but still higher compared to the control level in this particular experiment **(Figure 3.2B-C)**.

A


5min
30min
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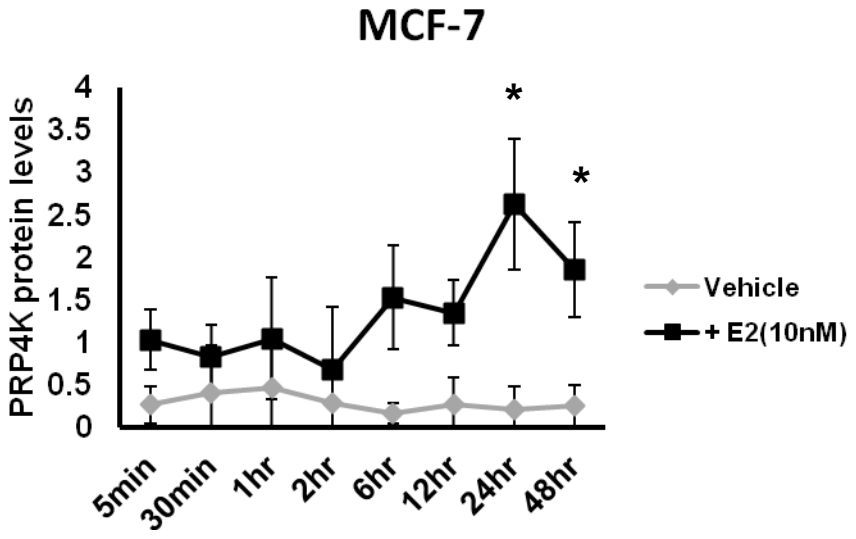
12hr
24hr
48hr
E2
+ + + + + + + +
PRP4K
Tubulin

B


MCF-7
PRP4K protein levels
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
5min
30min
1hr
2hr
6hr
12hr
24hr
48hr
Vehicle
+ E2(10nM)

C

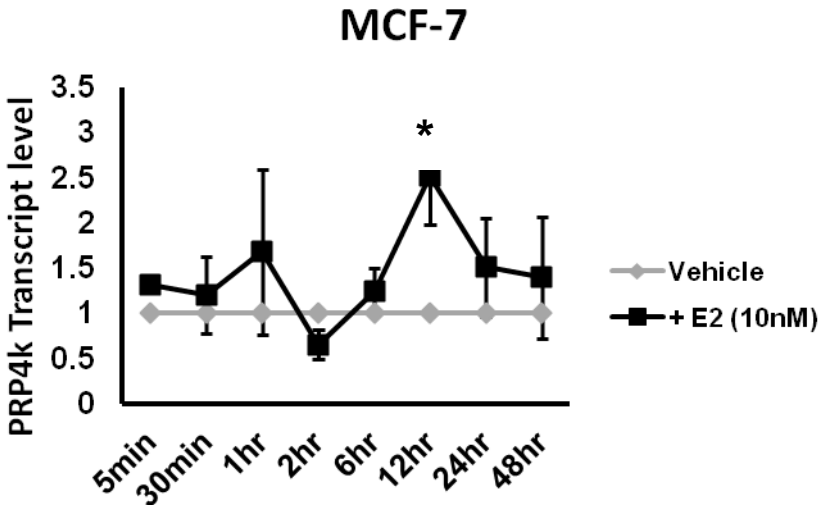

MCF-7
PRP4k Transcript level
3.5
3
2.5
2
1.5
1
0.5
0
*
5min
30min
1hr
2hr
6hr
12hr
24hr
48hr
Vehicle
+ E2 (10nM)

Figure 3.2- Time course of induction of PRP4K mRNA and protein levels by E2. MCF7 cells were seeded and grown in charcoal-stripped media for 48h before they were treated with 10nM of E2 or vehicle (0.01% EtOH). Protein lysates and total RNA were extracted in different time points between 5 min and 48h. For 5 min time point vehicle treated value was the same as non-treated values so only 5 min time point is shown here. All data is normalized to 5min (-E2). **A)** Western blot analysis of changes in PRP4K protein levels over time in the presence of E2. **B)** Quantification of protein level changes in response to E2. Error bars = SE (where n = 3), *p≤0.05. **C)** Quantification of transcript levels by RT-qPCR in response to E2. Error bars = SEM (where n=3). *P ≤0.02.

3.3. Regulation of PRP4K expression in breast cancer is ERα-dependent

3.3.1.ERα knockdown is associated with PRP4k downregulation

In response to estrogen signalling, ERα normally promotes the downstream signalling pathway which is responsible for estrogen-stimulated cell proliferation and development of ER-positive breast cancer (45). The overexpression of ERα is frequently observed in the early stage of breast cancer. However, many rapid E2-stimulated effects are shown to be ERα- independent so it is important to investigate the correlation between ERα and PRP4K expression in breast cancer cells. To investigate whether or not the estrogen response in ER+ cells is via ERα, ERα was knocked down in T47D cell line using a retrovirus carrying shNA directed against the ESR1 mRNA **(Figure 3.3).** shESR1 transduced T47D cells exhibit ~30-40% reduction in the amount of PRP4K suggesting that ERα is likely the key receptor in regulating PRP4K protein expression.

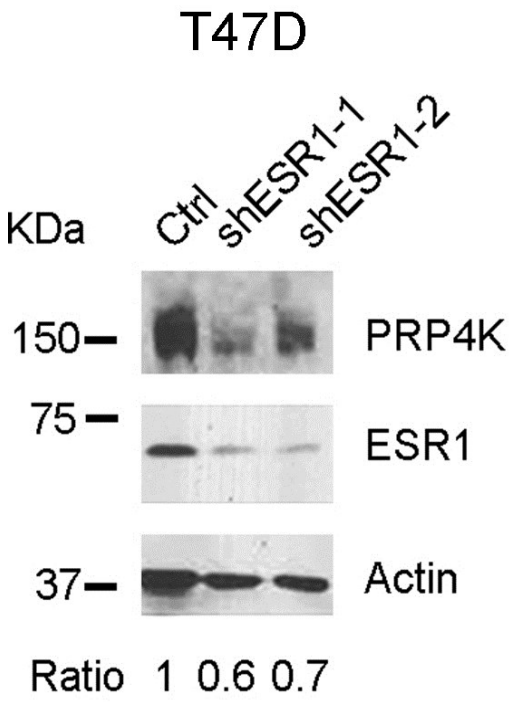


Figure 3.3- PRP4K is down-regulated by knock-down of ERα in T47D breast cancer cells. Western blot analysis of PRP4K and ESR1 levels in ER+ breast cancer cell line T47D following transduction with retrovirus carrying a control short-hairpin RNA (shNA)(Ctrl) or one of two different shRNAs directed against the ERα mRNA (shNA-ESR1-1 and -2, respectively). Actin was used as a loading control and the ratio of PRP4K in control cells normalized to actin is shown.

3.3.2.ERα overexpression is associated with PRP4K upregulation

Estrogen receptor signalling is the major pathway by which breast cancer cells respond to estrogen in ER-positive cell lines. ER-negative breast cancer cells like the MDA-MBA-231 cell line do not express ERα and therefore are ideal for testing the dependence of PRP4K protein expression on the ERα. Thus, I ectopically expressed ERα in the MDA-MB-231 cell line by transient transfection with *ESR1* expression vector and then monitored changes in PRP4K level by western blot analysis. I observed a positive correlation between overexpression of ERα and upregulation of PRP4K, suggesting that ERα could be the estrogen receptor regulating PRP4K expression.

In a separate experiment, wild type HepG2 liver cells and a HepG2 variant cell line stably expressing ERα developed by Barkhem and colleagues called HepG2-ER3(221), were treated with estradiol for 24h. PRP4K protein level and transcript levels increased only in the HepG2-ER3 cell line expressing ERα after treatment with E2 (2.2 +/- 0.2 and 2.5 +/- 0.7 respectively), which again implicates ERα in the regulation of PRP4K **(Figure 3.4)**. Thus, taken together these data indicate that the ability of E2 to stimulate increased PRP4K gene expression is likely ERα- dependent.

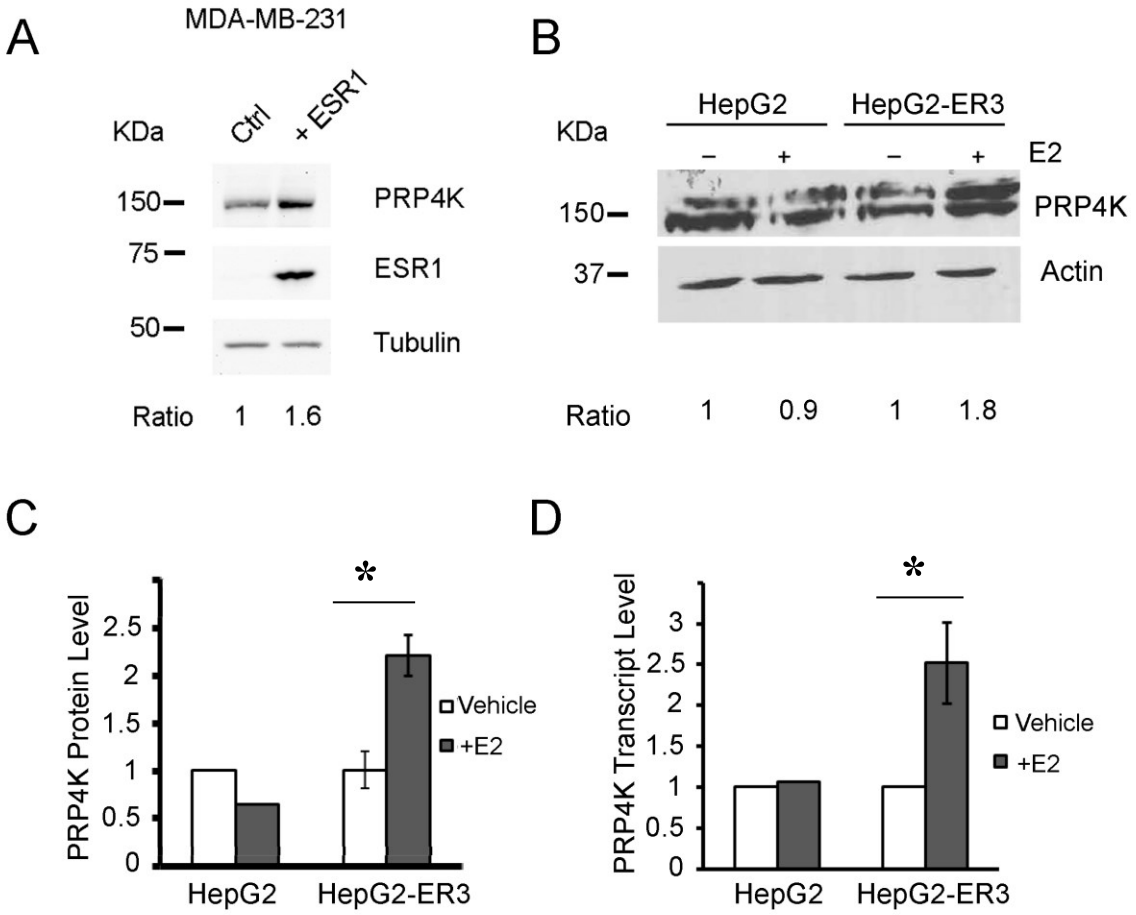


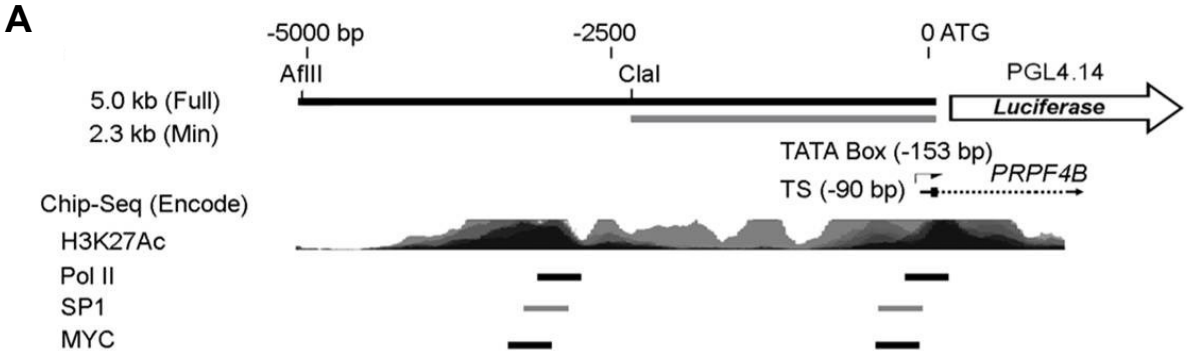
Figure 3.4- ERα overexpression is correlated with increased levels of PRP4K.
A) Expression of ERα (ESR1) in ER-negative MDA-MB-231 breast cancer cells upregulates PRP4K protein expression. MDA-MB-231 cells were transfected with ERα expression vector for 8h before protein lysates were prepared for Western blot analysis. The ratio of PRP4K protein to the loading control tubulin is shown. **B)** HepG2 liver cells (ER-) or those stably expressing ERα (HepG2-ER3) were treated with E2 (10nM) and vehicle (EtOH) for 24h prior to Western blot analysis. The ratio of PRP4K protein to the loading control actin is shown. **(C-D)** To quantify changes in PRP4K protein and mRNA, HepG2 or HepG2-ER3 cells were harvested 24h after E2 or vehicle treatment. The fold change in protein (2.2 +/- 0.2) and transcript level (2.5 +/- 0.7) are presented in the as bar graphs in C and D, respectively. All values are normalized to actin expression as an internal control. Error bars = SE (where n=3). *p value ≤0.05.

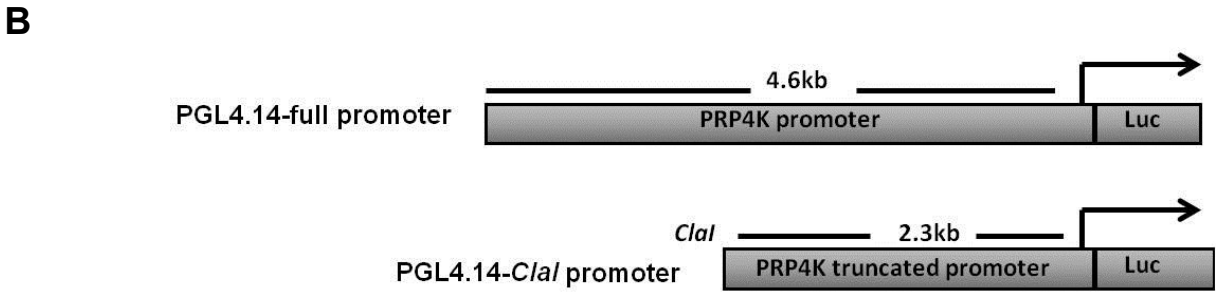
3.4. Cloning and characterization of PRP4K promoter

ERα is a ligand-activated transcription factor and a member of the nuclear receptor superfamily. The classic mechanism of ER alpha action involves estrogen-induced formation of a nuclear ERα homodimer and binding to the 5'-regulatory estrogen response elements (EREs) on the promoter of the target genes. This binding recruits other nuclear transcription factors to activate gene expression.

In ERE-independent pathway, ERα also interacts with Sp1 protein to transactivate genes through binding Sp1-ERE or Sp1-ERE ½ site (ERE half site) where both ERα and Sp1 bind DNA elements (222). Given that PRP4K transcription was upregulated by E2 in an ERα –dependent manner, we sought to determine if the PRP4K promoter sequence might harbour classic DNA elements such as EREs that might explain how estrogen was regulated PRP4K expression. We first did an *in silico* analysis of transcription factor binding sites using the University of California at Santa Cruz (UCSC) Genome Browser and the ENCODE transcription factor chromatin-immunoprecipitation sequencing (ChIP-Seq) datasets available through their web portal **(https://genome.ucsc.edu/) (Figure 3.5A)**. The upstream genomic region containing the putative promoter of PRP4K is notable for having two separate clusters of chromatin enriched in histone H3 acetylated at lysine 27 (H3K27ac) (one from 0 to -2.5kb upstream of the transcription start site and one between -2.5 to -5kb). H3K27ac is a marker of "open chromatin" associated with enhancer regions of promoters (223). We did not find any evidence of ERE binding sites or direct binding of ERα in the ChIP-Seq data. However, we did find evidence of Sp1 and MYC binding sites in the ChIP-seq data,

which are known to either cooperate or to be regulated by ERα themselves (224,225). Despite the lack of clear ERE elements in the promoter region of the PRP4K gene, I sought to better understand how estrogen was regulating PRP4K by cloning fragments of the genomic sequence upstream of the PRP4K gene containing either both of the H3K27ac-enriched regions (which I term the full promoter and is ~4.6kb), or only the first region enriched in H3K27ac, (which I term the "truncated promoter" which is approximately 2.3kb). The full and truncated promoter regions of PRP4K were then cloned into a promoter-less luciferase reporter plasmid (pGL 4.14, Promega) to enable promoter activity to be measured by induction of luciferase activity. These promoter expression vectors, named "PGL4.14-full promoter" and "PGL4.14-*ClaI* promoter", were transfected into MCF7 cells and luciferase expression was monitored following 24h treatment with vehicle (0.01% EtOH) or 10 nM E2. Both the full length and the truncated promoter were able to drive luciferase expression as compared to the promoterless expression vector. I observed significant increase (~30-fold increase in expression for full promoter and almost 70-fold increase for the truncated promoter) in luciferase, which indicates that these genomic sequences were indeed acting as promoters sequence is successfully cloned into the luciferase vector (**Figure 3.5**).


A
-5000 bp
-2500
0 ATG
AflII
ClaI
PGL4.14
5.0 kb (Full)
Luciferase
2.3 kb (Min)
TATA Box (-153 bp)
PRPF4B
TS (-90 bp)
Chip-Seq (Encode)
H3K27Ac
Pol II
SP1
MYC


B
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PGL4.14-full promoter
PRP4K promoter
Luc
ClaI
2.3kb
PGL4.14-ClaI promoter
PRP4K truncated promoter
Luc

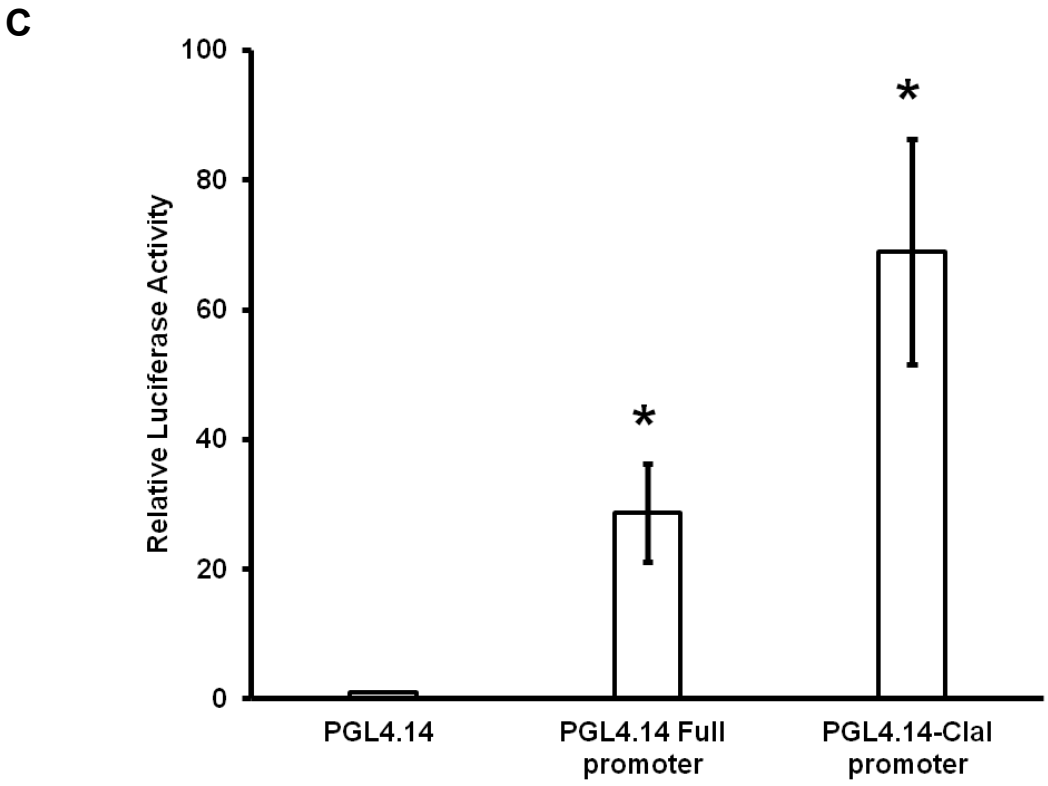

C
100
80
60
40
20
0
Relative Luciferase Activity
*
*
PGL4.14
PGL4.14 Full promoter
PGL4.14-ClaI promoter

Figure 3.5- Cloning and characterization of PRP4K promoter. A) The genomic sequencing of the *PRPF4B* gene encoding PRP4K was screened using the University of California at Santa Cruz Genome Browser (**https://genome.ucsc.edu/**) to scan for and regulatory histone modifications associated with active promoters (i.e. histone H3 acetyl-lysine 27; H3K27ac) and transcription factor binding sites from chromatin immunoprecipitation sequencing data (ChIP-seq) from the ENCODE project (**https://genome.ucsc.edu/ENCODE/).** Although the *PRPF4B* promoter region does not contain Estrogen Receptor Elements (EREs), the promoter region has two prominent H3K27ac-enriched regions, as well as binding sites for the SP1 and MYC transcription factors (positions indicated relative to the start site of the *PRPF4B* gene).
B) The full PRP4K promoter (4.6 kb) and the truncated promoter (2.3 kb) were cloned into a promoter-less *Firefly* luciferase vector (PGL4.14). **C)** MCF7 breast cancer cells were transfected with control promoterless PGL4.14 or the same vector containing the full length (Full Promoter) or truncated PRP4K (*ClaI* Promoter) promoters. As a transfection control, an expression vector encoding *Renilla* luciferase was co-transfected and used to normalize luminescence data. The bar graph depicts relative luciferase activity (FLu/RLu) measured using Dual-Reporter Luciferase Assay for each promoter versus the empty PGL4.14 control. Error bars = SEM (where n=3). *$p < 0.02$

3.5. 17-β-estradiol does not regulate PRP4K expression via its promoter

The presence of transcription factor binding motifs related to estrogen signalling such as Sp1and MYC in the promoter region of the *PRPF4B* gene could indicate that the expression of PRP4K may be regulated by E2 through these factors rather than by direct ERα binding. To begin to explore this possibility it was necessary to evaluate whether the promoter of PRP4K could be activated transcriptionaly by estrogen. To this end, MCF7 cells were transfected with the promotorless *Firefly* luciferase expression vector pGL4.14 or pGL4.14 containing either the full length or truncated PRP4K promoter. After 24 h, these cells were then treated with vehicle (0.01% EtOH) or 10 nM E2 (10nM) for 24 h, followed by measurement of luciferase expression. Although both the full and truncated promoters could drive luciferase expression robustly, there was no significant change in the luciferase expression in the presence of E2 as compared to vehicle control **(figure 3.6).**

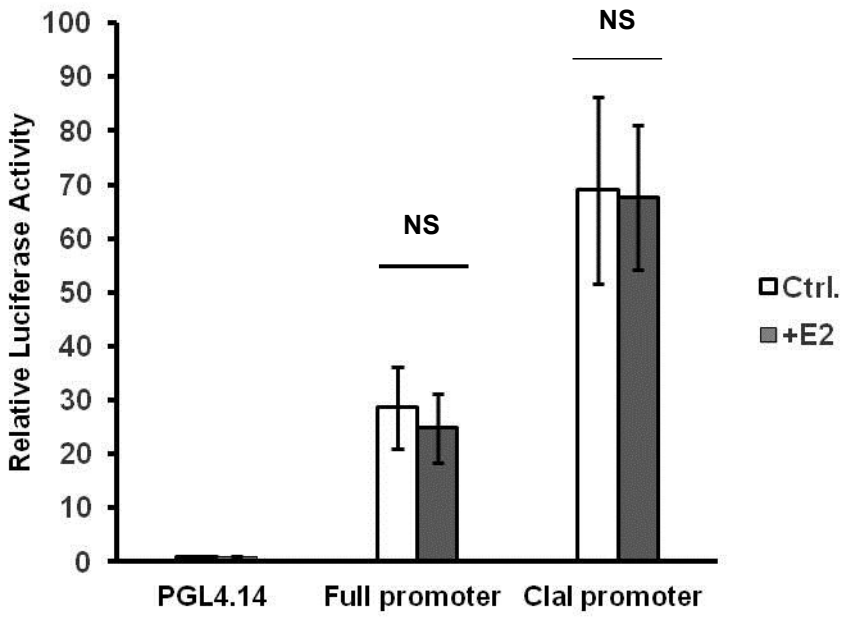


Figure 3.6- Estrogen regulation of PRP4K expression does not occur via the *PRP4FB* gene promoter. MCF7 breast cancer cells were transfected with control promoterless PGL4.14 or the same vector containing the full length (Full Prom) or truncated PRP4K (Min Prom) promoters. 24 h post-transfection, cells were treated for an additional 24 h with either 10 nM E2 (+E2) or 0.01% ETOH vehicle (Ctrl) as a control. Transfection efficiency was accounted for by co-transfection with an expression vector encoding *Renilla* luciferase allowing luminescence data to be normalized. The bar graph depicts relative luciferase (Flu/RLu) activity measured using Dual-Reporter Luciferase Assay for each promoter versus the empty PGL4.14 control treated with vehicle or E2. Error bars = SEM (where n=3). NS: non-significant

3.6. Post-transcriptional regulation of PRP4K

3.6.1. Characterization of PRP4K 3'UTR

Given that our study of the *PRPF4B* gene promoter failed to elucidate a transcriptional mechanism underlying the ability of E2 to stimulate PRP4K expression in ER+ MCF7 cells, we sought to determine if post-transcriptional mechanisms might be regulating the accumulation of PRP4K mRNA after estrogen treatment. Like most mRNA, the PRP4K transcript has both a 5' and a 3' UTR. The 5' UTR of *PRPF4B* gene is very short in sequence (~97 bp) and has no (or very few) RNA motifs or microRNA (miRNA) binding sites. In addition, the 5' UTR of the *PRPF4B* gene was actually included in the promoter expression vectors in section 3.6, and thus was expressed as part of the luciferase transcript in these studies. Given the inability of E2 to stimulate additional luciferase expression in these experiments, it is unlikely the 5' UTR plays a role in the estrogen regulation of PRP4K expression. On the other hand, the 3'UTR of the PRP4K transcript is very large and potentially contains many seed elements for miRNAs. As discussed previously, miRNAs regulate the expression of target genes by binding to the 3'UTR of specific mRNAs and triggering mRNA degradation or translational repression. Using the web service Target Scan 6.2 **(/www.targetscan.org/)** we analyze the miRNA-binding sites within the 3'UTR of PRP4K. The 3'UTR of PRP4K contains seed elements for miRNAs including the ones involved in breast cancer such as miR-21 (**Figure 3.7A**). The seed sequence for miR-21 lies within the first 1 kb of

the 3'UTR of PRP4K following the stop codon, and is highly conserved in diverse vertebrate species **(Figure 3.7B)**.

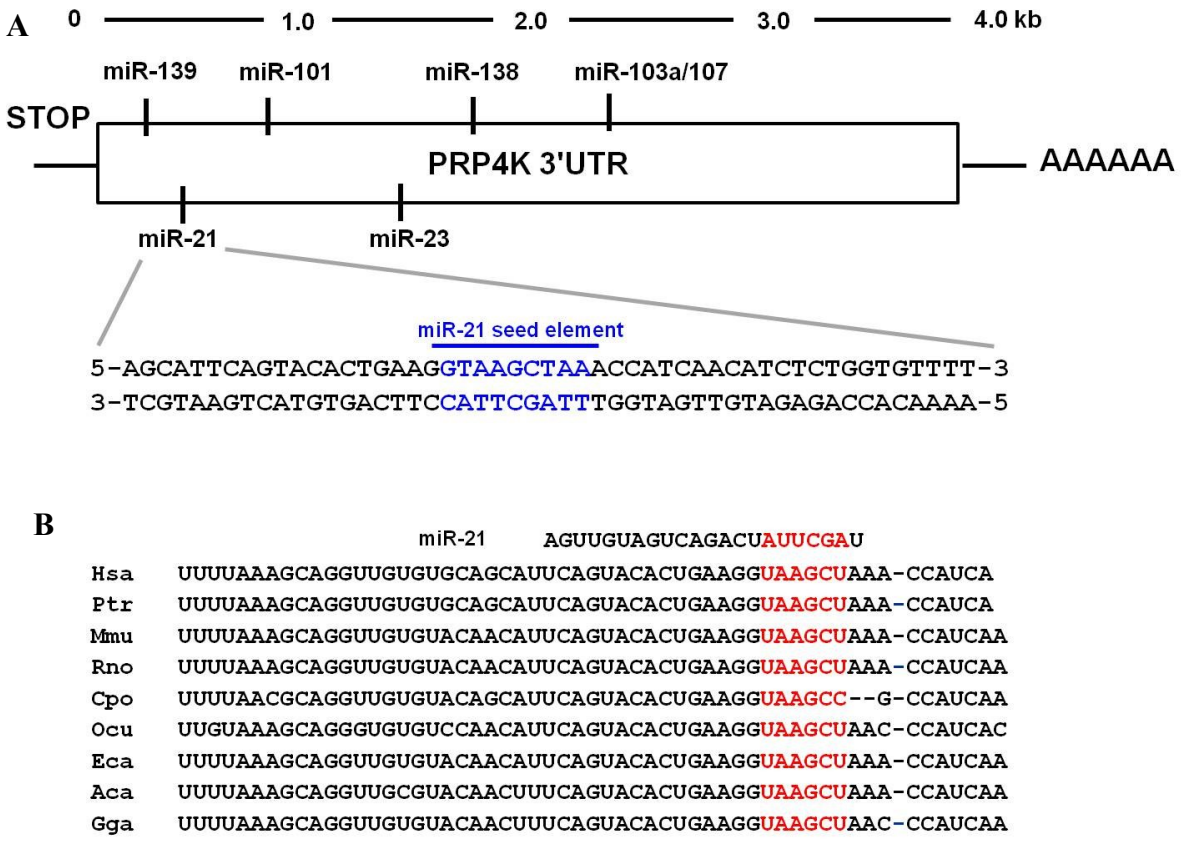


Figure 3.7- Target Scan Analysis of highly conserved miRNA binding sites in the 4.3 kb PRP4K 3′ UTR. A) Analysis of highly conserved miRNA binding sites using TargetScan 6.2 revealed a cluster of miRNA binding sites within the 4.3 kb 3' UTR of PRP4K transcript. The position of these putative miRNA binding sites and the stop codon and poly-A sequences within the UTR are indicated. **B)** Conservation of the miR-21 seed sequence binding site in the 3' UTR of various vetebrates including human (Hsa), chimpanzee (Ptr), mouse (Mmu), rat (Rno), guinea pig (Cpo), rabbit (Ocu), horse (Eca), lizard (Aca), and chicken (Gga).

3.6.2. The effect of miR-21 over expression on PRP4K gene expression

MicroRNA-21 (miR-21) overexpression has been observed in variety types of cancer and is associated with translation inhibition or mRNA decay (226–229). To investigate the effect of miR21 on PRP4K gene expression, MCF7 were transfected with miR-21 RNA mimics or the vector pCMV-miR-21, which expresses miR-21, and PRP4K protein level was analyzed with Western blotting. PRP4K protein decreased in cells transfected with the miR-21 mimic compared to control scrambled miRNA transfected cells (**Figure 3.8A**). Similarly, pCMV-miR21 transfected cells showed reduced PRP4K protein expression compared to control untransfected cells **(Figure 3.8B)**. I also examined PRP4K transcript level in response to miR-21 overexpression. MCF7 cells were transfected with a miR-21 mimic, or with a miR-1 mimics as a control and RNA was extracted for qPCR analysis (**Figure 3.8C**). The *mir*Vana™ miRNA Mimic miR-1 Positive Control is designed to mimic mature miR-1 miRNA in mammalian cells. One of the miR-1 target genes is Protein Tyrosine Kinase 9 (PTK9), also known as twinfilin-1, whose mRNA level is effectively down-regulated by miR-1. As shown in **Figure 3.8C** PRP4K gene expression levels did not change significantly upon miR-21 overexpression; however, this was not a result of failed transfection of the miR mimics as the control miR-1 experiment did show a significant decrease in PTK9 gene expression level upon miR-1 overexpression ($p<0.03$). Thus, these data indicate that miR21 does not decrease PRP4K transcript levels but may be influencing PRP4K translation or PRP4K protein stability.

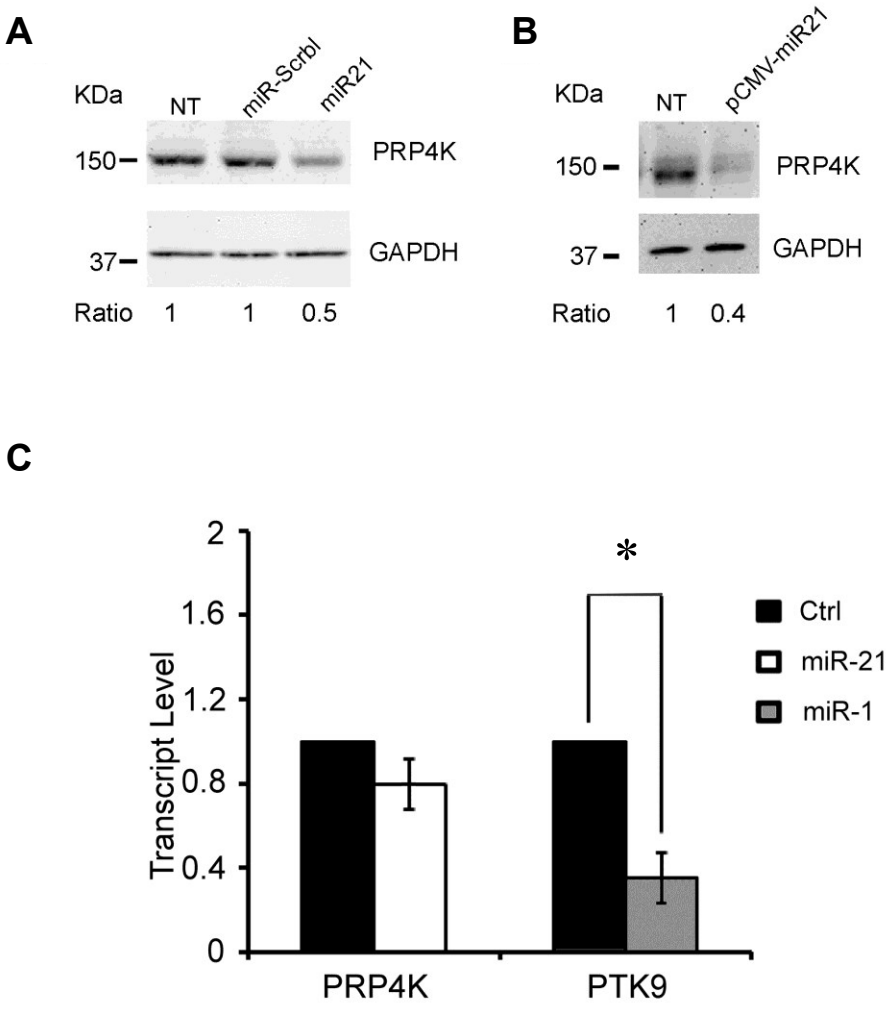


Figure 3.8- miR21 effect on PRP4K gene expression. **A-B**) MCF7 cell were transfected with miR-21 or scrambled miRNA in panel A, or transfected with or without pCMV-miR21 expression vector in panel B. 24h post-transfection PRP4K protein level was measured by Western blotting. The ratio of PRP4K normalized to GAPDH as a loading control is shown below each blot. NT = non-transfected **C)** MCF7 cells were transfected with either a control (Neg. Ctrl) scrambled miRNA mimic, or mimics for miR-21 or miR-1. MiR-1 was used as a positive control for mimic transfection as it directly targets the Protein Tyrosine Kinase 9 (PTK9). After 24 h post-transfection, PRP4K and PTK9 transcript levels were measured by qPCR using specific primers. As expected PTK9 transcript level was reduced by the miR-1 mimic. No significant changes were measured in PRP4K transcript level. Error bars = SE where n=3. *p value =0.03

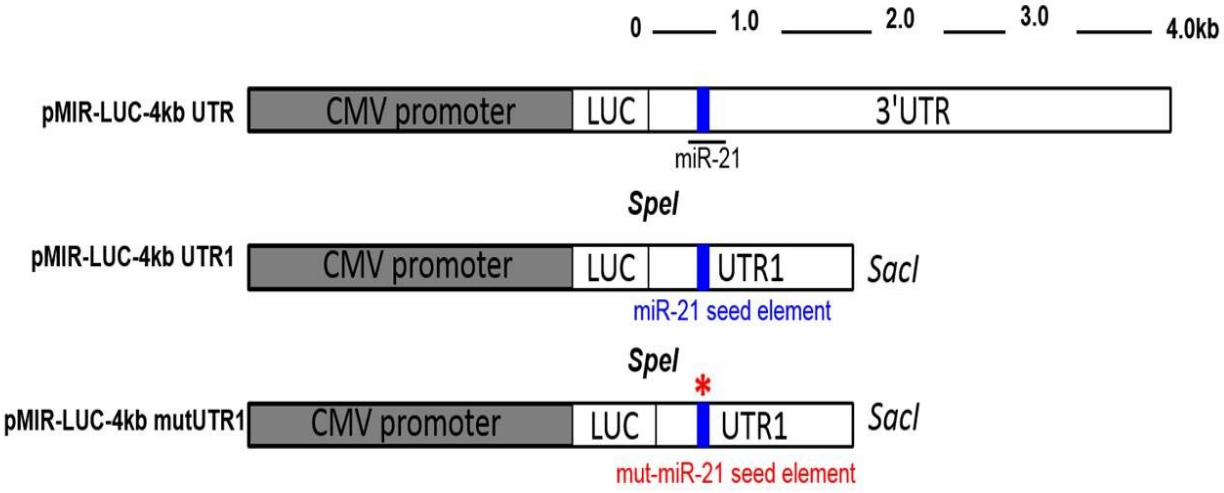
3.6.3. The effect of miR-21 on wild type and mutant 3'UTR of PRP4K

Although miR-21 appeared to affect PRP4K protein levels in cells over-expressing this miRNA, I did not observe changes in PRP4K transcript level under the same conditions **(Figure 3.8).** Nonetheless, it is possible that the 3' UTR of PRP4K could still mediate changes in PRP4K protein expression but at the level of protein translation and/or transcription stability. One method to assay this possibility is to employ luciferase reporters that encode the 3' UTR of PRP4K. These expression reporters require not only the transcription of the luciferase gene but also translation of the protein to produce the enzymatic activity measured in the luminescence assay. Thus, the impact of the 3'UTR of PRP4K on luciferase protein expression can be measured using the standard luminescence assay. To determine whether the PRP4K 3'UTR could still regulate protein expression in response to miR-21, I constructed luciferase expression vectors using the pMIR-REPORT-LUC system (Ambion) containing either the full length 4.2 kb 3'UTR (Full-UTR) of PRP4K or a shorter fragment (UTR1) encoding only the first 1.2 kb of the 3' UTR but still containing the miR-21 binding site (depicted in **Figure 3.9A**). This shorter UTR1 was used to better isolate the impact of miR-21 and to prevent cross-talk from other possible miRNAs binding elsewhere in the full UTR. The UTR1 vector also facilitated the production of a mutant UTR1 (mut-UTR1) that has the miR-21 binding site mutated at 4 nucleotides within the seed sequence. MCF7 cells were transfected with the pMIR-REPORT-LUC vectors encoding the full UTR or UTR1 with and without the miR-21 site mutation and luciferase activity was measured 24h post-transfection. Both the full

and UTR1 wild type 3'UTRs sequences increased luciferase activity in my assay, while the mutated UTR1 actually decreased the luciferase activity compared with control empty pMIR-REPORT-LUC vector (**Figure 3.9B**). .

I next looked at the impact of miR-21 over-expression on luciferase expression and found that although miR-21 did not have significant impact on the expression from the pMIR-REPORT-LUC vector carrying the full PRP4K UTR, the shorter UTR1 and the mut-UTR1 were negatively impacted by miR-21 expression and reduced luciferase expression was seen **(Figure 3.9C)** .

A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1.0
2.0
3.0
4.0kb
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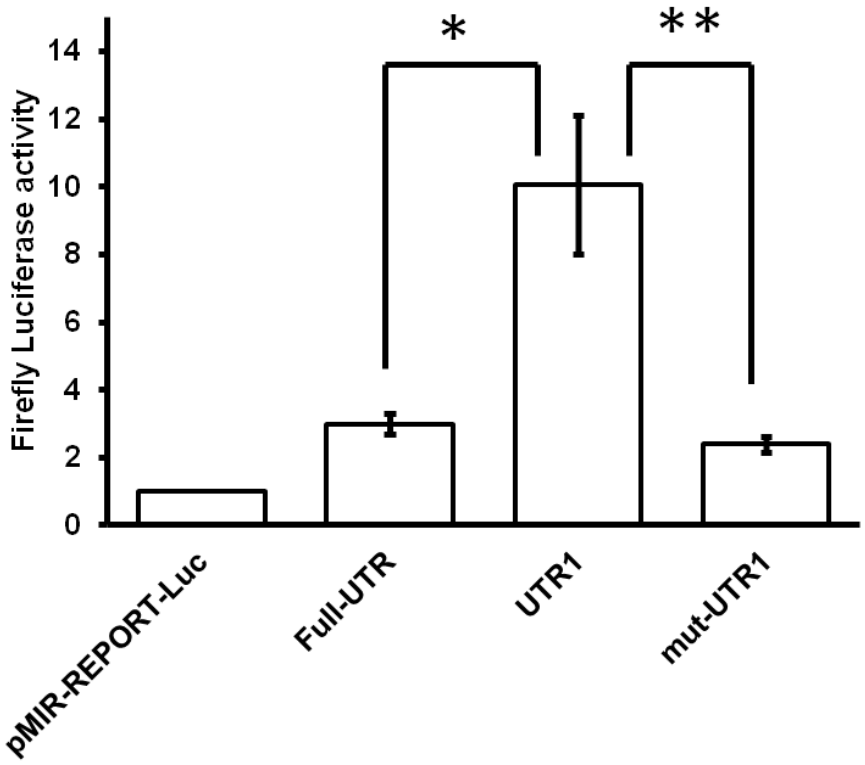
CMV promoter
LUC
3'UTR
miR-21
SpeI
pMIR-LUC-4kb UTR1
CMV promoter
LUC
UTR1
SacI
miR-21 seed element
SpeI
*
pMIR-LUC-4kb mutUTR1
CMV promoter
LUC
UTR1
SacI
mut-miR-21 seed element

B


*
**
14
12
10
8
6
4
2
0
Firefly Luciferase activity
pMIR-REPORT-Luc
Full-UTR
UTR1
mut-UTR1

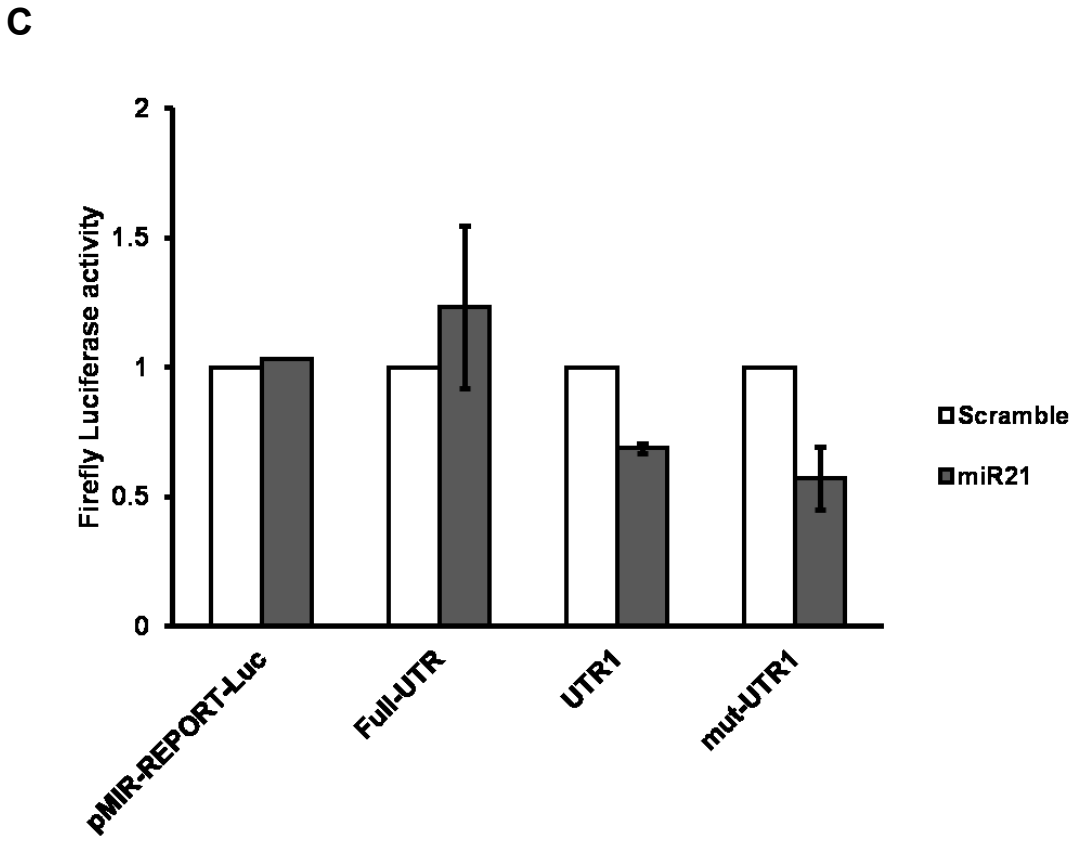


Figure 3.9- The effect of mutant miR-21 seed elements on the 3'UTR of PRP4K.

A) Schematic view of 3'UTR of PRP4K cloned into pMIR-REPORT-LUC vector and with wt and mutant miR21 seed elements. **B)** Luciferase constructs carrying wt (Full-UTR and UTR1) and mutant (mut-UTR) 3'UTR of PRP4K were transfected into MCF7 cells and relative *firefly* luciferase activity (Flu/RLu) was measured and normalized to *Renilla* activity. Error bars: SEM (where n=3). * p value ≤0.04. ** p Value≤0.03. **C)** Putative miR-21 binding sites on 3'-UTR of PRP4K mRNA were predicted by Target Scan. Either the wild-type or mutant PRP4K-3'UTR was co-transfected into MCF7 cells with miR-21 mimic or scrambled for miR-21 mimic as negative control. The normalized luciferase activity in the negative control group was set as 1. Co-transfcetion of luciferase constructs carrying wt- and mut-3'UTR of PRP4K and miR21 mimics decreased luciferase activity of wt-PRP4K-3'-UTR1. However, this effect was not reversed when mut-UTR1was co-transfected with the miR-21 mimic. Error bars= SD.

3.6.4 miR-21 and ZRANB2 binding sites overlaps in the 3'UTR of PRP4K

To better understand the mechanism underlying miR-21 overexpression effect a deeper sequence analysis was done at the binding site of miR-21. Using RNA-binding specificities data base (BPDB) (230), the wild type UTR1 and mut- UTR1 sequences of PRP4K used in our luciferase reporter assay were screened for putative sites of docking RNA-binding protein sites. Sequence analysis revealed that the miR-21 seed element overlapped with a binding site for the RNA binding protein ZRANB2 (**Figure 3.10A**). The human splicing factor zinc finger, Ran-binding domain containing protein 2 (ZRANB2) is a splicing protein that is overexpressed in some cancers (137). Specific endogenous targets for ZRANB2 are currently unknown and this protein is not very well characterized. A recent study showed that ZRANB2 can bind to a sequence containing the consensus AGGUAA via its zing finger motif (231). Therefore, any mutational changes to the miR-21 seed elements AGG-(UAAGCU)-AAA alters the ZRANB2 binding site simultaneously (**Figure 3.10B**). RNA binding proteins can facilitate a number of processes that affect both transcript stability and translation (232). Thus, blocking or mutating the binding site for ZRANB2 could either destabilize the PRP4K mRNA and/or reduce its translation which could explain, at least in part, the unexpected decrease in luciferase expression in the reporter constructs carrying the mutated miR-21 seed sequencing in mut-UTR1 (**Figure 3.10**). If the PRP4K mRNA is indeed regulated by ZRANB2 it would be likely that cells expressing high levels of ZRANB2 may also express higher levels of PRP4K. To test this assumption, I elicited the help of Dr. Daniel Gaston (Molecular Diagnostics, QEII Hospital, Halifax, NS) to carry-out a correlation

analysis for ZRANB2 and PRP4K expression across 877 cell lines in the Broad Cancer Cell Line Encyclopedia (**http://www.broadinstitute.org/ccle/home) (Figure 3.10C**). Spearman correlation analysis across these cancer cell lines from various tissues showed a significant correlation between ZRANB2 and PRP4K mRNA expression (0.48, $p < 1 \times 10^{-51}$). These data strongly suggest that ZRANB2 is most likely a positive regulator of PRP4K protein expression.

A

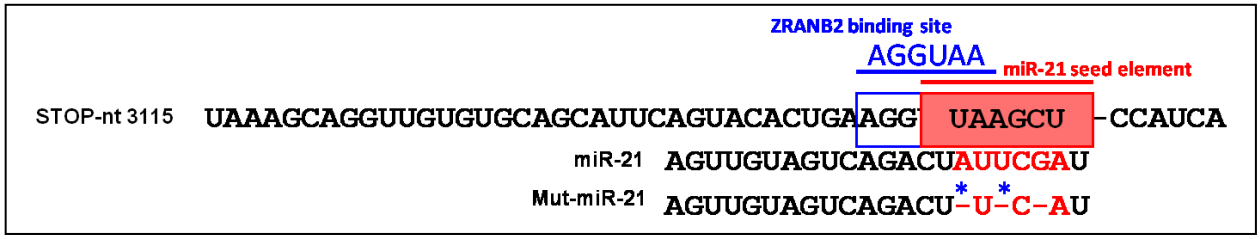
Mutated
1 UGAAGAUUAA CCAGACCAUC AACAUCUCUG GUG

Score	Relative Score	RBP Name		Start	End	Matching sequence
9.4281438	100%	ybx2-a	21	26	AACAUC	114_7499328
9.3741518	100%	ybx2-a	21	26	AACAUC	115_7499328
7.3693752	100%	FUS	30	33	GGUG	637_11098054
5.2682554	100%	RBMX	11	14	CCAG	922_19282290
4.99861593	94%	RBMX	16	19	CCAU	922_19282290
3.787534341	81%	KHDRBS3	6	11	AUUAAC	1174_19561594

WT
1 UGAAGGUAAG CUAAACCAUC AACAUCUCUG GUG

Score	Relative Score	RBP Name		Start	End	Matching sequence
10.3039431	100%	ZRANB2	4	9	AGGUAA	1285_19304800
9.4281438	100%	ybx2-a	21	26	AACAUC	114_7499328
9.3741518	100%	ybx2-a	21	26	AACAUC	115_7499328
7.3693752	100%	FUS	30	33	GGUG	637_11098054
4.99861593	94%	RBMX	16	19	CCAU	922_19282290
3.939327845	84%	KHDRBS3	10	15	GCUAAA	1174_19561594

B



C

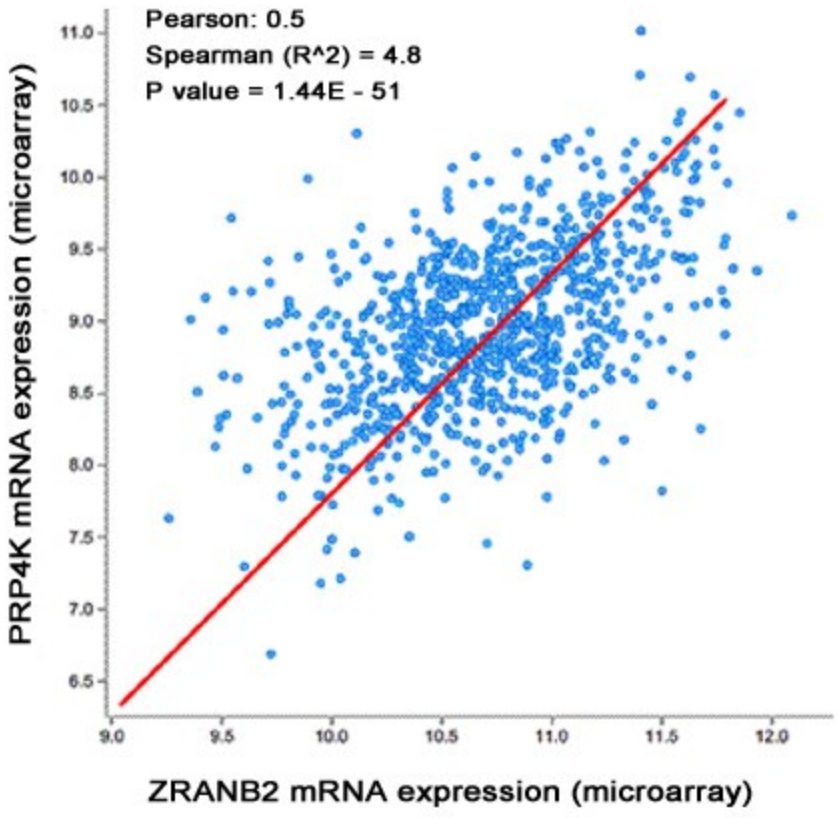
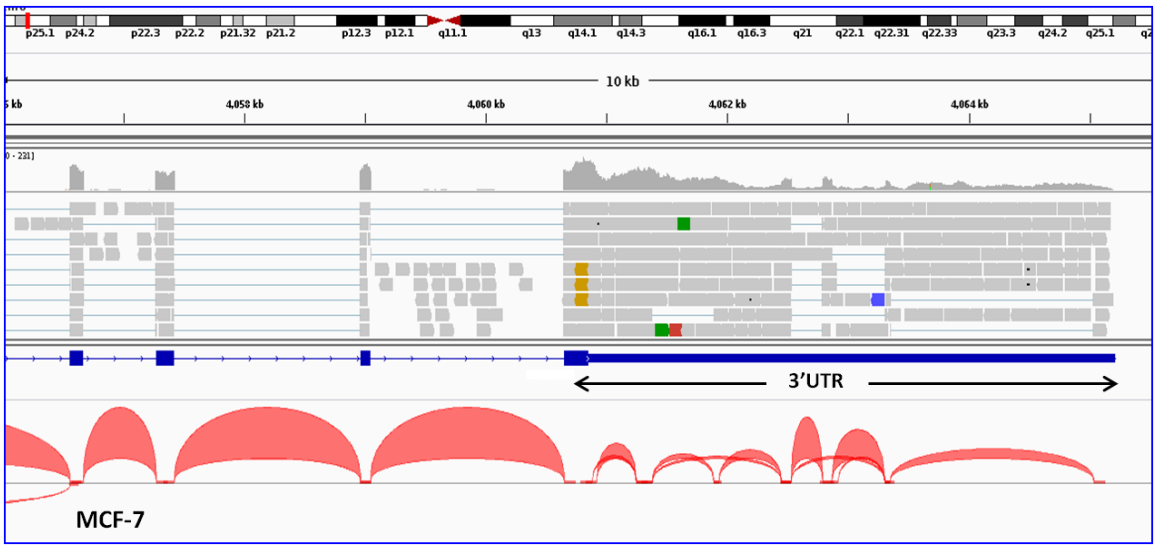


Figure 3.10- MicroR-21 and ZRANB2 binding sites overlap on the 3'UTR of PRP4K. The 3'UTR of PRP4K was screened for RNA-binding protein in proximity of miR-21 seed elements. A and B) miR-21 and ZRANB2 biding sites overlap on the 3'UTR as site directed mutation in the miR-21 seed elements removed the binding site for RANB2 as well. B) miR-21 seed elements and ZRANB2 binding site overlaps on the 3'UTR of PRP4K. C) Spearman correlation analysis for ZRANB2 and PRP4K showed a significant correlation between these two mRNA. R^2:0.48. P value: 1.44E-51. The red line indicates the theoretical distribution expected for the perfect correlation between ZRANB2 and PRP4K gene expression.

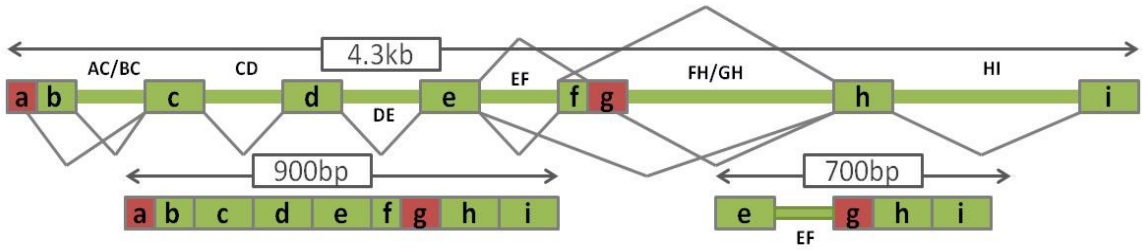
3.6.5. Alternative splicing in the 3'UTR of PRP4K

During the process of 3'UTR cloning, I observed more than one amplicon appeared on the gel after each PCR reaction using the designed primers (set 1 and set 2 in materials and methods). This finding encouraged us to analyze the 3'UTR sequence of PRP4K by RNA sequencing (RNA-seq). Hence, in collaboration with Dr. Stephen Montgomery (Stanford University), and to better understand the post transcriptional regulation of PRP4K, RNA-seq data was analyzed for evidence of alternative pre-mRNA splicing in the 3'UTR of PRP4K. Interestingly, the RNA-seq data showed a very complex splicing pattern in the 3'UTR of the PRP4K transcript (**Figure 3.11**). Therefore, knowing the fact that some of these splicing junctions are at or near miRNA seed elements including miR-21 seed element, alternative splicing of the 3'UTR of PRP4K could represent yet another level of regulation involved in the complex regulation of this kinase.

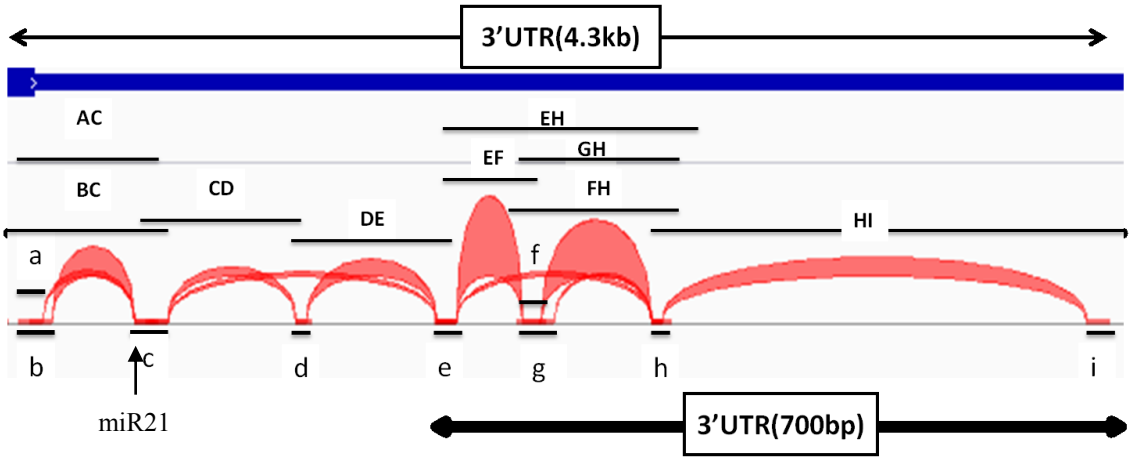
A


10 kb
4,058 kb
4,060 kb
4,062 kb
4,064 kb
3'UTR
MCF-7

B


4.3kb
AC/BC
CD
DE
EF
FH/GH
HI
a b c d e f g h i
900bp
a b c d e f g h i
700bp
e g h i
EF

C


3'UTR(4.3kb)
AC
EH
EF
GH
BC
CD
FH
DE
HI
a
f
b
c
d
e
g
h
i
miR21
3'UTR(700bp)

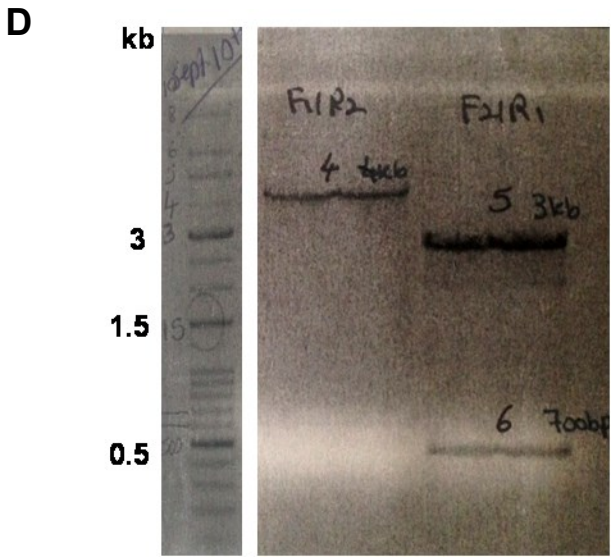


Figure 3.11- Alternative splicing in the 3'UTR of PRP4K. A) RNAseq data on the 3'UTR of PRP4K showed that this UTR is prone to several splicing events. The 3'UTR of PRP4k is subject to several alternative splicing that generates spliced variants of transcript. **B**) Schematic view of PRP4K 3'UTR with all alternative splicing junctions according to the to the RNAseq result. **C)** miR-21 seed element is mapped to 3'UTR of PRP4K. The location is near a splice junction and was confirmed by sequencing. **D)** The Western blog showing more than one fragment size for the 3'UTR of PRP4K. Two sets of primers were designed to amplify full length 3'UTR out of cDNA template. The 4.3kb and the 700bp fragments appears to be the most abundant spliced variants (RNAseq data on fragment abundance are not shown here) .

3.7. The combinatorial effect of hormone drug tamoxifen and taxanes in vitro

Patients with estrogen receptor (ER)-positive breast cancers have a better prognosis than those with ER-negative breast cancers, but often have low sensitivity to chemotherapy and a limited survival benefit. Typically these patients will receive chemotherapy that includes DNA damaging agents (e.g. doxorubicin and cyclophosphamide) and an anti-mitotic drugs such as the taxanes (e.g. paclitaxel). Patients are then treated with anti-hormone therapies such as tamoxifen, an inhibitor of ERα, for 5 to 10 years (233). Unfortunately, a significant number of patients will relapse. Resistance of cancer cells to chemotherapy with taxanes is one of the main causes of treatment failure. Even with long-term anti-hormonal treatment, some tumours develop resistance that are refractory to treatment with tamoxifen and may need to be treated with other hormonal therapies and/or additional systemic chemotherapy, including treatment with taxanes. Because of the significant side-effects such as peripheral neuropathy, cardiotoxicity and neutropenia, taxanes greatly affect the quality of life (234). Thus, there is growing interest in finding biomarkers that predict treatment response to taxanes not only during initial treatment but also post-relapse to prevent unnecessary treatment in potentially non-responsive patients.

To test the effects of tamoxifen on PRP4K expression, I first treated cells with the active metabolite of tamoxifen 4-OHT at varying concentrations for 24 h. I observed a dose-dependent decrease in PRP4K protein levels in response to 4-OHT, with 2 μM drug inhibiting protein expression by 60%. Similarly, 24 h treatment of MCF7 cells with tamoxifen (1 μM) robustly decreased PRP4K protein expression by 60%, and transcript

level by 80% **(Figure 3.12).** Resistance of cancer cells to chemotherapy with taxanes is one of the main causes of treatment failure.

The combination effects of chemotherapeutic agent (paclitaxel) and hormone drug (TAM) was evaluated in ER-positive human breast cancer cell lines (MCF7). A cell viability assay was carried out to assess the anti-proliferative effect of PAX on ER+ cells which are pre-treated with TAM. Fluorescent values were determined for the anti-proliferative activity of paclitaxel before and after treatment with tamoxifen.

Since a concentration of tamoxifen (1 μM) robustly inhibited PRP4K expression, I sought to determine if tamoxifen treatment could alter the taxane response of ER+ breast cancer cells. To this end, ER+ MCF7 breast cancer cells were sequentially treated with tamoxifen (1μM) followed by various concentrations of the taxane paclitaxel. Resistance to a range of doses of paclitaxel (from 0.3 to 1.25 μM; $p < 0.02$) was significantly increased in cells pretreated with tamoxifen as compared to vehicle-treated cells (IC50 of 0.41 versus 0.11 μM paclitaxel, respectively) (**Figure 3.13**).

This data suggests that the reduction of PRP4K expression by tamoxifen can reduce the efficacy of paclitaxel.

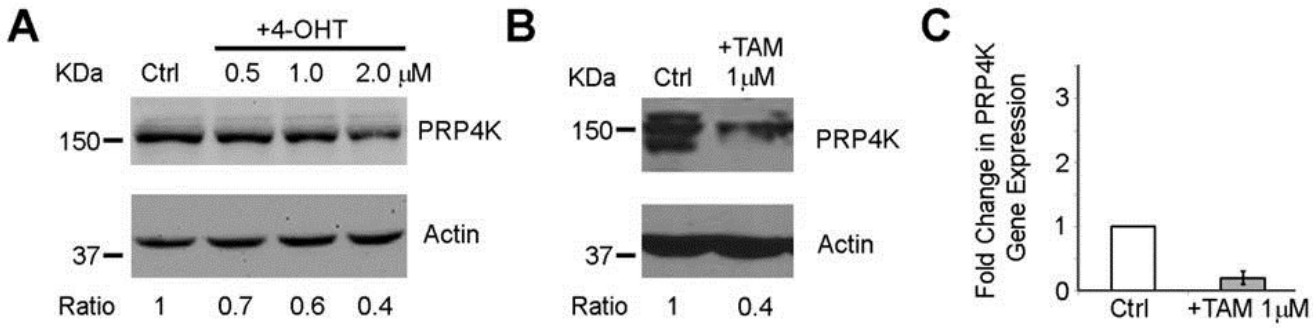


Figure 3.12- Tamoxifen down-regulates PRP4K expression and decreases the sensitivity of MCF7 breast cancer cells to taxane treatment. A,B) MCF7 cells were treated with TAM for 24h and protein lysate was prepared to measure PRP4K protein level in cells treated with or without TAM. **C)** MCF7 cells were treated with 1μM of TAM for 24h followed by indicated doses of PAX for 90min. Error bar = SEM (where n=3) *$p<0.05$

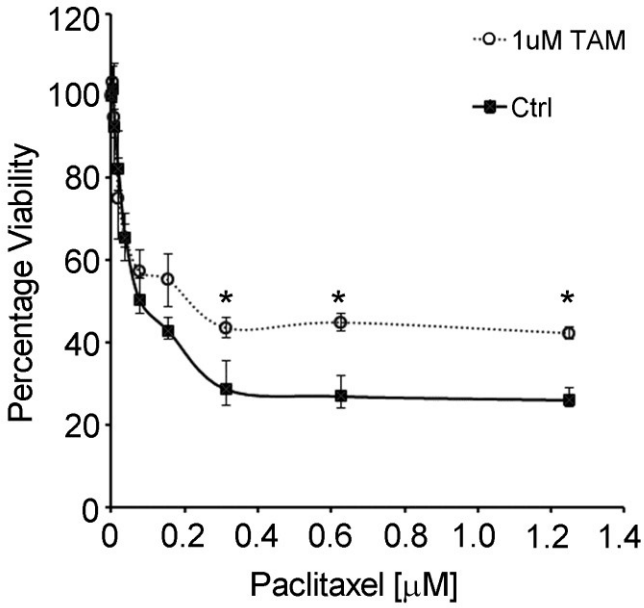


Figure 3.13- Treatment with tamoxifen reduces the sensitivity of ER+ MCF7 cells to paclitaxel. ER+ MCF7 breast cancer cells were treated for 24 h with either vehicle (Ctrl; 0.05% DMSO) or 1μM tamoxifen (TAM) prior to acute treatment with paclitaxel at increasing doses as indicated for 90 min. Cells were allowed to recover after drug treatment for an additional 24 h prior to measurement of cell viability. IC50 of paclitaxel plus vehicle is 0.11 μM (95% CI: 0.07 to 0.17 μM), and in the presence of 1 μM tamoxifen (TAM) was 0.41 μM (95% CI: 0.20 to 0.83 μM). Error bars = SE (N=3) *P<0.05. Percentage viability is shown versus concentration of paclitaxel for vehicle versus tamoxifen treated cells. Error bars = SE (where n=3); * = p <0.02

3.8. The effect of tamoxifen on MAPT

As I demonstrated above, pre-treatment with the anti-hormone drug tamoxifen increases the resistance of MCF7 cells to paclitaxel. These data are in agreement with a study by Ikeda and colleagues that found that tamoxifen was antagonistic and reduced the sensitivity of MCF7 to the taxanes paclitaxel and docetaxel (234).Increased levels of MAPT/tau, BCL2 and p-glycoprotein (MRP1) are associated, *in vitro*, with taxane sensitivity (95). Although the mechanism of tamoxifen antagonistic action with taxanes is not very well understood, the Ikeda study showed a slight increase in BCL2 and MAPT expression consistent with enhanced resistance. However, they also observed a decrease in MRP1, which is inconsistent with enhanced resistance and thus unlikely to play a role in the antagonistic effects of tamoxifen on the taxane response (234). Since taxane resistance is multi-factorial, evaluation of PRP4K protein expression in addition to other modifiers of drug response such as BCL2 and MAPT may prove useful in stratifying patients based on potential benefit from taxane therapy. I also evaluated MAPT expression under our assay conditions; however, I did not see a significant change in MAPT protein levels following tamoxifen treatment (**Figure 3.14**).

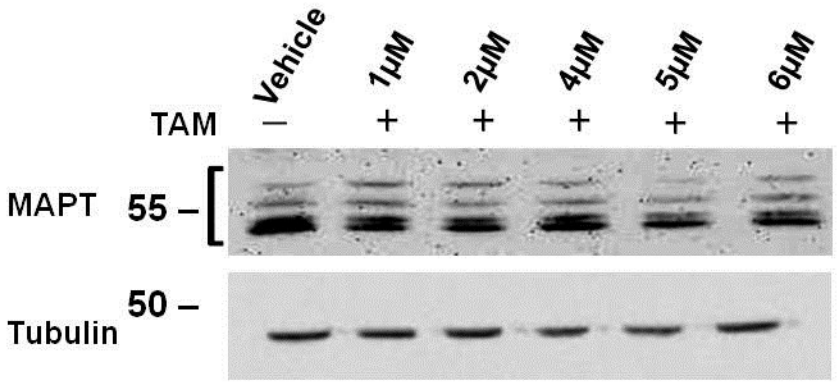


Figure 3.14- The effect of tamoxifen on MAPT. MCF7 cells treated with increasing doses of TAM were harvested after 24h and PRP4K protein level was measured with Western blotting using antibodies against MAPT (tau antibody, Thermo Scientific) and tubulin.

Chapter 4: DISCUSSION

4.1 Regulation of PRP4K gene expression by estrogen signalling

The estrogen signalling pathway is one of the major pathways in normal breast tissue development. Estrogens play important roles in different aspects of human physiology including in reproductive system tissue such as breast, uterine and ovary and non-reproductive tissues such as liver, bone and nervous system (39). Estrogen exerts its effect by interacting with two members of nuclear receptor family: ERα and ERβ. In the canonical (genomic) ER signalling pathway, ligand bound ERs bind to ERE elements on DNA and recruit co-regulatory factors to regulate transcription (42). In non-canonical (non-genomic) pathway, estrogen bind to the membrane associated ERs or ERs in the cytoplasm. Thus, without binding to DNA sequence, the E2-ER complex is still able to regulate transcription of a sub-set of estrogen regulated genes indirectly via other transcription factors (235). Beside the normal development of breast and reproductive tract, exposure to estrogens in particular 17-β-estradiol (E2) is associated with increased risk of breast cancer and proliferation of cancer cells (236,237). Normal mammary epithelial cells and the vast majority of breast tumours express the ERα receptor gene, *ESR1*, and the growth in these cells is mediated by estrogen (117,238). The expression status of ERα and dysregulation of estrogen regulated genes has a strong impact on tumourigenesis from different aspects such as tumour growth, cancer progression and can be used as a prognostic marker for breast cancer survival. Therefore, ER pathway and E2 synthesis have been targeted in various types of treatments and an increasing number of

estrogen regulated genes have been reported as biomarker in breast cancer diagnosis, treatment prediction and overall survival (239). Consequently, ERα expression is a good predictive marker of responses to endocrine therapy. However, the complexity of ER signalling pathway conveys various cellular responses to estrogens and also drug resistance to antiestrogen therapy (240,241). Given the importance of ER status in breast cancer treatment decisions, my research focused on the regulation of a known biological marker of taxane sensitivity, pre-mRNA splicing factor 4 kinase (PRP4K), by estrogen and its impact on the taxane sensitivity of ER+ breast cancer.

4.1.1 PRP4K gene and protein expression is rapidly induced by 17-β-estradiol (E2)

I first became interested in the possible regulation of PRP4K by hormonal signalling in breast cancer after making the observation that this kinase was expressed in mouse mammary gland tissue (**Figure 3.1A**). This result was in agreement with publically available tissue microarray data from the Human Protein Atlas (242) and later research from the Dellaire laboratory indicating high expression of PRP4K in some breast cancer tissues (216). Since mammary epithelial cells express ERα, I sought to determine if PRP4K could be regulated by estrogen-signalling via this receptor. My qPCR and Western blot analysis confirmed that upon treatment of ER+ MCF7 breast cancer cells with 10 nM 17-β-estradiol (E2), PRP4K protein and transcript levels increased ~2-fold after 24 h of E2 treatment (**Figure 3.1 B-C**).

Time course experiments indicated that rather than a gradual increase in PRP4K transcript and protein expression overtime, both transcript and protein levels oscillated over 48 h post-E2 treatment with an initial rise in transcript level at1 h followed by reduced mRNA levels at 6 h and again a peak at 12 h. The "spikes" in PRP4K transcript preceded increases in PRP4K protein levels at 6 h and 24 h post-treatment with E2 (**Figure 3.2B-C**). These results could indicate new transcription of PRP4K following E2 treatment specifically after 2hr time point. However I also observed a decrease or oscillation of transcript and protein levels between the early time points up until 24 h. This oscillatory behavior in PRP4K levels suggests feedback mechanisms that either alters transcript levels or stability, and/or protein stability over the first 24 h after E2 treatment. I will discuss in more detail the possible post transcriptional regulation of PRP4K later in this chapter. The rapid response within 5 min post treatment in protein level could be explained in two ways: first, E2 stimulation opens the Ca2+ channels in the cell membrane which leads to rapid activation of cAMP-dependent protein kinase (PKA). Second, E2 can directly activate PKC which in turn contributes to PKA activation (243). This way E2 exert its effects via non-genomic pathway either by binding membrane-associated ER or by activation the downstream PI3K /Akt pathway components and acts in non-genomic pathway (**Figure 3.2A-B**). This response to E2 stimulation on PRP4K levels indicates a potentially complex regulation of PRP4K, which may include "feed-back" mechanisms to limit PRP4K under- or over-expression. For under expression, it is clear from previous studies that an acute reduction in PRP4K expression greater than 50% from initial levels in MCF7 cells is lethal and studies of

PRP4K loss required tightly controlled regulation of kinase knock-down using inducible shRNA (216). Our research group has also observed that over-expression of the PRP4K protein is also acutely lethal to MCF7 cells (Dale Corkery, unpublished observation). Thus, it appears that there may be an ideal level of PRP4K for a given cell line that the cell must maintain for survival.

How PRP4K is rapidly induced following E2 treatment in MCF7 cells is not clear but previous research indicates that estrogen actions can be mediated by rapid signalling that occurs within seconds to minutes of cell stimulation, in which the rapid actions of estrogen are mediated through membrane associated estrogen receptors i.e non-genomic ER signalling pathway (244). Therefore, following estrogen stimulation both transcriptional and non-transcriptional events might regulate PRP4K transcript and/or protein levels. For example, estrogen stimulation can induce rapid cell membrane-mediated Src-MAPK pathways without inducing transcriptional activity (245). Indeed, a subpopulation of ERα which is localized to the cytoplasm or the plasma membrane is able to rapidly activate intracellular signalling pathways, including MAPK and PI3K, pathways that are typically activated by receptor kinases such as the epidermal growth factor receptor (EGFR/ERBB1), and human epidermal growth factor receptor 2 (HER2/ERBB2) (35). Given the recent finding in our laboratory indicating that HER2 signalling can impact PRP4K expression (216) it may be that growth factor stimulation of PRP4K protein levels, either by epidermal growth factor (EGF) or estrogen may share a common intermediate signalling pathways such as via PI3K or Src-MAPK signalling. In addition to non-genomic regulation of gene expression by estrogen, it has been shown that 10nM of E2 induces rapid ERα translocation from the cytoplasm to nucleus as early

as 5 min and peaking at 30 min with a 3.2-fold increase in the nuclear accumulation of ERα (137). So, it is possible that immediately after E2 induction, both genomic and non-genomic pathways are triggered that impact PRP4K transcript and/or protein levels. However as I will discuss later, my data indicates that the induction of PRP4K expression does not involve direct transcriptional regulation of the PRP4K gene, *PRPF4B*, by ERα. Other pathways that I will consider below are the regulation of the PRP4K mRNA by microRNAs and/or RNA binding proteins that may be themselves regulated by estrogen signalling.

4.1.2 Estrogen regulates PRP4K expression via ERα signalling

We next investigated if the regulation of PRP4K expression by E2 was occurring directly via ERα signalling by knock-down and overexpression of ERα in ER+ and ER- breast cancer cell lines, respectively. We found that knock-down of ERα/ESR1 in ER+ T47D breast cancer cells **(Figure 3.3)**, or over-expression of ERα in triple negative MDA-MB-231 cells **(Figure 3.4A),** correlated with a decrease or increase of PRP4K protein expression (respectively). To determine if the regulation of PRP4K by ERα was a general mechanism that could occur in non-breast cancer cell lines we also employed the liver cell lines HepG2 and HEPG-ER3 (REF), a derivative of HepG2 expressing ERα, to examine the regulation of PRP4K by E2 treatment. In the HepG2-ER3 cell line expressing ERα, E2 treatment resulted in an increase in both PRP4K protein and transcript level **(Figure 3.4B-D)** but E2 stimulation failed to alter PRP4K levels in the parental HepG2 cell line. Thus, the ability of ERα to regulate PRP4K expression is not

tissue specific and together these data strongly indicate that PRP4K is regulated by estrogen-signalling via ERα.

4.2 Characterization of the *PRPF4B* promoter region

In the course of determining how estrogen was regulating PRP4K expression, I cloned and characterized the promoter region of the PRPF4B gene that encodes PRP4K (**Figure 3.5**). *In silico* analysis of the promoter region of PRPF4B via the UCSC genome browser portal (**https://genome.ucsc.edu/)** did not reveal any putative estrogen response element (ERE) sites that could indicate direct binding of ERα Similarly, available chromatin immunoprecipitation sequencing data from the ENCODE project accessed through this portal did not reveal ERα binding; however, it did reveal both Sp1 and c-MYC binding sites, two factors implicated in estrogen signalling (224,225). Despite the possibility of estrogen regulating PRP4K transcriptionally via Sp1 and/or c-MYC, luciferase reporter experiments using reporters containing either the putative full length or truncated *PRPF4B* promoter did not respond to treatment with E2 in ER+ MCF7 cells (**Figure 3.6**). Yet, PRP4K transcript levels did change over time upon E2 treatment in our previous experiments (**Figure 3.1 and 3.2**). One possibility is that long-range genomic interactions, which cannot be reflected in our reporter assay, could be influencing PRP4K expression in response to E2, and/or additional elements in the cloned promoters when taken out of their genomic context do not function the same in our assay as they would as *in situ* within their chromatin context. In partial support of this hypothesis is the fact that the truncated promoter was much more active in inducing luciferase expression than the

full length promoter (**Figure 3.5**), which could indicate DNA elements in the full promoter that inhibit gene expression in our assay.

Another possibility is that the regulation of PRP4K expression by estrogen does not occur directly at the transcription level. Thus, other ER pathways not dependent on the transcriptional activation of the *PRPF4B* promoter are involved in the accumulation of PRP4K mRNA and protein following E2 stimulation of ER+ breast cancer cells. Finally, the failure to induce luciferase expression after E2 treatment in these promoter reporter assays may reflect the fact that other potential regulatory elements found within the *PRPF4B* gene body or 3' UTR may be regulating PRP4K transcript splicing, turn-over or translation in response to estrogen.

4.3 PRP4K post-transcriptional regulation via its 3'UTR

After the *PRPF4B* promoter experiments failed to indicate transcriptional regulation of PRP4K in response to estrogen, I sought to determine what post-transcriptional mechanisms might be regulating PRP4K expression in response to estrogen stimulation of ER+ breast cancer cells. The 3'UTR of PRP4K was analyzed using the web browser Target Scan 6.2 **(/www.targetscan.org/).** The results indicated that although a number of miRNAs may target the 3'UTR of PRP4K, only a handful are highly conserved between vertebrate species including miR-139-5p, miR-21, miR-101, miR-23, miR-138, and miR-103a/107 **(Figure 3.7)** of these I decided to focus on miR-21. miRNA has been studied widely in different types of cancer including breast cancer (246) and its expression level is regulated the by hormone estrogen (247). In this study, I have

shown that miR-21 over-expression decreased PRP4K protein synthesis with no change in the transcript level (**Figure 3.8**). This data indicates that this miR-21 most likely blocks PRP4K translation rather that inducing mRNA degradation. To better understand the mechanism behind miR-21--mediated regulation, I mutated the seed elements for miR-21 within the 3'UTR of PRP4K and generated luciferase construct containing full length wild type (wt) or a truncated UTR containing the first 1.2 kb (termed UTR1) of the 3' UTR with and without mutation of the putative miR-21 seed sequence binding site **(Figure 3.9A)**. This assay integrates both changes in transcript stability and translation, as both will affect the level of luciferase activity.

Surprisingly, both the full and UTR1 wild type 3'UTRs sequences increased luciferase activity in my assay, while the mutated UTR1 actually decreased the luciferase activity compared with control empty pMIR-REPORT-LUC vector (**Figure 3.9B**). There was also a similar inhibition of luciferase activity by miR-21 in the mut-UTR1 reporter, despite the seed site being mutated **(Figure 3.9C)**. These data indicate that other regulatory factors, for example RNA binding proteins or other miRNAs, that bind the 3' UTR of PRPK may be affected by miR-21 and that together these regulatory pathways are controlling luciferase expression in my assay. This data was puzzling as typically mutation of the binding site of a miRNA should prevent inhibition of the luciferase in this assay if the miRNA is negatively regulating the transcript, which I initially assumed from our miR-21 over-expression experiments (**Figure 3.8**). To reconcile this result, a more detailed sequence analysis was carried out on the 3'UTR sequence encoded by the wt and mutated UTR1, which revealed that the putative miR-21 binding site overlaps on the 5' end with a putative binding site for the RNA binding protein ZRANB2. Therefore, it is

likely that when I mutated the miR-21 seed element I inadvertently destroyed the putative binding site for ZRANB2 as well (**Figure 3.10B**). Knowing the fact that ZRANB2 like other mRNA bonding proteins may play role in mRNA stabilization or translation it is very important to investigate the effect of mutated miR-21 seed element and mutated ZRANB2 binding site separately. To our knowledge this is the first study reporting this overlap in miR-21 and ZRANB2 binding sites.

In collaboration with Dr. Stephen Montgomery, Stanford University, we also analyzed the alternative splicing events in the *PRPF4B* gene. We focused on the 3' UTR and found that the miR-21 binding site within the PRP4K 3' UTR is formed at the junction of two non-coding exons by alternatively splicing. Thus, potentially alternative splicing of the PRP4K 3'UTR may represents another layer of regulation of this kinase. With un-spliced 3'UTR (4.3kb) being the most abundant variant, these spliced UTRs provide differential miRNA binding sites including target sequences for some cancer related miRNAs such as such as miR-21 (246), miR-590, miR-103 and miR-107 (248) and several mRNA binding proteins such as HuR, ZRANB2, FUS and RBMX (**Figure 3.10**). Alternative pre-mRNA splicing produces several variants of PRP4K mRNA that differ in the composition of their 3'-untranslated regions (3'-UTRs). This heterogeneity in 3'-UTR sequence could regulate PRP4K expression by two potential mechanisms. First, PRP4K may regulate its own expression by binding either directly regulating alternatively splicing through its known role in the spliceosome (194,212) and/or by interacting with RNA binding proteins that are involved in regulating PRP4K transcript stability or translation. Analysis of possible alternative mRNA isoforms for PRP4K listed in the ENSEMBL database (**http://www.ensembl.org/index.html**) indicates that several

putative splice isoforms may be subject to nonsense-mediated decay (NMD). Thus, the NMD pathway may also provide another potential mechanism to regulate PRP4K protein expression. Second, each splice variant might serve as a unique landing site for miRNAs and mRNA binding proteins. These binding sites might be missing in other splice variants. For example, ZRANB2 targets splice junctions in the 3'UTR. Therefore, any changes due to alternative splicing could affect ZRANB2 binding affinity to the 3'UTR and therefore modifies the post-transcriptional regulation of its target genes (249). Finally, differential expression of these splice variants may also occur under different growth conditions and may themselves respond to estrogen signalling, as has been recently shown for a number of genes regulated by estrogen including EGFR2 and AXIN1(250). Very little is known regarding the impact of 3'UTR alternative splicing on post-transcriptional regulation. Therefore, through our initial findings we have begun to shed light on how splicing events in the 3'UTR could affect post-transcriptional regulation of PRP4K through multiple mechanisms including altering the binding sites for RNA binding proteins like ZRANB2 and miRNAs such as miR-21.

4.4 PRP4K and taxane resistance

Breast cancer is generally classified into two main sub-group: Estrogen-receptor (ER)–positive (ER+) and –negative (ER-). Estrogen receptor and/or progesterone receptor positive patients are the most responsive to antiestrogen drugs (i.e tamoxifen) and normally have a better prognosis than those with ER- breast cancer. However, these patients often suffer from lower sensitivity to chemotherapy and therefore relapse during

or after endocrine therapy (233,251,252). Therefore, patients with ER+ breast cancer usually receive chemotherapy that includes DNA damaging agents (e.g. doxorubicin and cyclophosphamide) and anti-mitotic drugs such as the taxanes (e.g. paclitaxel) (77,253)

Receiving taxanes greatly affect the quality of life due to significant side-effects including peripheral neuropathy, cardiotoxicity and neutropenia (57). Thus, there is growing interest in finding biomarkers that predict treatment response to taxanes not only for initial treatment but also during post-relapse to prevent unnecessary treatment in potentially non-responsive patients.

Our laboratory recently characterized PRP4K as a novel HER2-regulated modifier of taxane response in breast and ovarian cancer whose higher level is associated with taxane sensitivity (216). However, the mechanism through which this kinase controls taxane response is unknown and one of the objectives of this project.

As mentioned above I have shown that PRP4K is regulated by estrogen in breast cancer cell lines. Regulation by ERα-dependent signalling was further confirmed by treatment of cells with tamoxifen, at a certain dose, which reduced PRP4K protein expression (**Figure 3.12**). Since PRP4K is one of the proteins involved in spindle-assembly checkpoint (203), any changes in PRP4K may alter SAC activity and allow cells to escape cell death by "mitotic-slippage"(216). Therefore, I investigated whether or not that pre-treatment with the anti-hormone drug tamoxifen changes the resistance of MCF7 cells to paclitaxel. Previous clinical studies have shown that sequential use of tamoxifen and anthracycline/cyclophosphamide chemotherapy is more efficient than concurrent use of these drugs (254). Moreover, previous *in vitro* studies have reported that anti-hormone drugs can have an antagonistic effect on some chemotherapy regimens

(255). In this study, I have demonstrated that the reduction in PRP4K protein levels by treatment of MCF7 breast cancer cells with tamoxifen correlates with reduced sensitivity of these cells to taxanes (**Figure 3.13**). These data are in agreement with previous study that showed that tamoxifen was antagonistic to taxane and reduced the sensitivity of MCF7 paclitaxel and docetaxel (120).

Although the mechanism of antagonistic action of tamoxifen with taxanes is not very well understood, Ikeda and colleagues showed a slight increase in BCL2 and MAPT expression consistent with enhanced resistance. However, they also observed a decrease in p-glycoprotein (MRP1), which is inconsistent with enhanced resistance and thus unlikely to play a role in the antagonistic effects of tamoxifen on the taxane response. Other in vitro studies also showed that increased levels of MAPT/tau, BCL2 and (MRP1) are associated with taxane sensitivity (95). However, when I evaluated MAPT expression there was no significant change in MAPT protein levels following tamoxifen treatment (**Figure 3.14**), indicating that changes in MAPT are unlikely to explain the differences in taxane sensitivity.

CHAPTER 5: CONCLUSION

In summary, in this study I have demonstrated for the first time that PRP4K is an estrogen regulated gene that is expressed in mammary gland epithelial cells. The data demonstrated that the hormonal regulation of PRP4K is ERα-dependent; however, PRP4K promoter studies did not indicate direct transcriptional regulation by ERα in response to E2 stimulation. Yet PRP4K protein level was still positively correlated with ERα expression in both ER+ and ER- cell lines when the receptor was knocked-down or over-expressed (respectively). It is not clear yet how ERα and estrogen receptor signalling regulates PRP4K gene expression but my work has demonstrated that estrogen regulation of PRP4K is complex and may involve positive and negative feedback regulation of PRP4K transcript and protein levels following E2 stimulation in breast cancer cells. In particular, analysis of the 3'UTR of PRP4K identified regulatory elements including miRNA and RNA binding protein sites, such as miR-21 and ZRANB2 (respectively) which could potentially explain how PRP4K is regulated by estrogen. MiR-21 is an estrogen regulated miRNA whose downregulation has been shown in breast tumours (247) and thereby is categorized as a tumour suppressor miRNA. Here, it has been demonstrated that miR-21 regulates PRP4K as over expression of miR-21 decreased PRP4K protein level. However, the fact that the miR-21 binding sites overlaps with that of ZRANB2 in the 3'UTR of PRP4K, coupled with the existence of alternative splicing events the produce different 3'UTR sequences, some of which lack the miR-21 and other miRNA seed sequences, bring more complexity to post-transcriptional regulation of PRP4K. Finally, I have uncovered a new potential

mechanisms through which tamoxifen might alter the response of ER+ breast cancers to taxanes. When treated with tamoxifen (1 μM), MCF7 cells showed a 60% reduction in the protein level of PRP4K **(Figure 3.12)**; a modifier of taxane sensitivity as discussed above (216). These data indicate that pre-treatment with tamoxifen may modulate the effect of taxanes on ER+ cells in part by altering PRP4K protein levels. Thus, this study not only provides new insight into the possible antagonistic activity of tamoxifen with respect to taxane treatment but also identifies PRP4K as a potentially useful biological marker for taxane treatment response in breast cancer patients receiving or previously treated with tamoxifen therapy.

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APPENDIX I

To test the effect of media on the promoter activity, luciferase expression was done using PGL4.14-full promoter in media supplemented with 10% FBS versus phenol-free medial supplemented with 5% charcoal stripped FBS. I observed ~180-fold increase in luciferase expression for full promoter in complete media and almost 40-fold increase in stripped media (figure 3.5). This observation indicated that this genomic sequence was successfully cloned into the luciferase vector and acting as promoter sequence not only in the complete media and in the presence of supplemented growth factors but also in the stripped media that has been used for all E2 treatment experiments in this study.

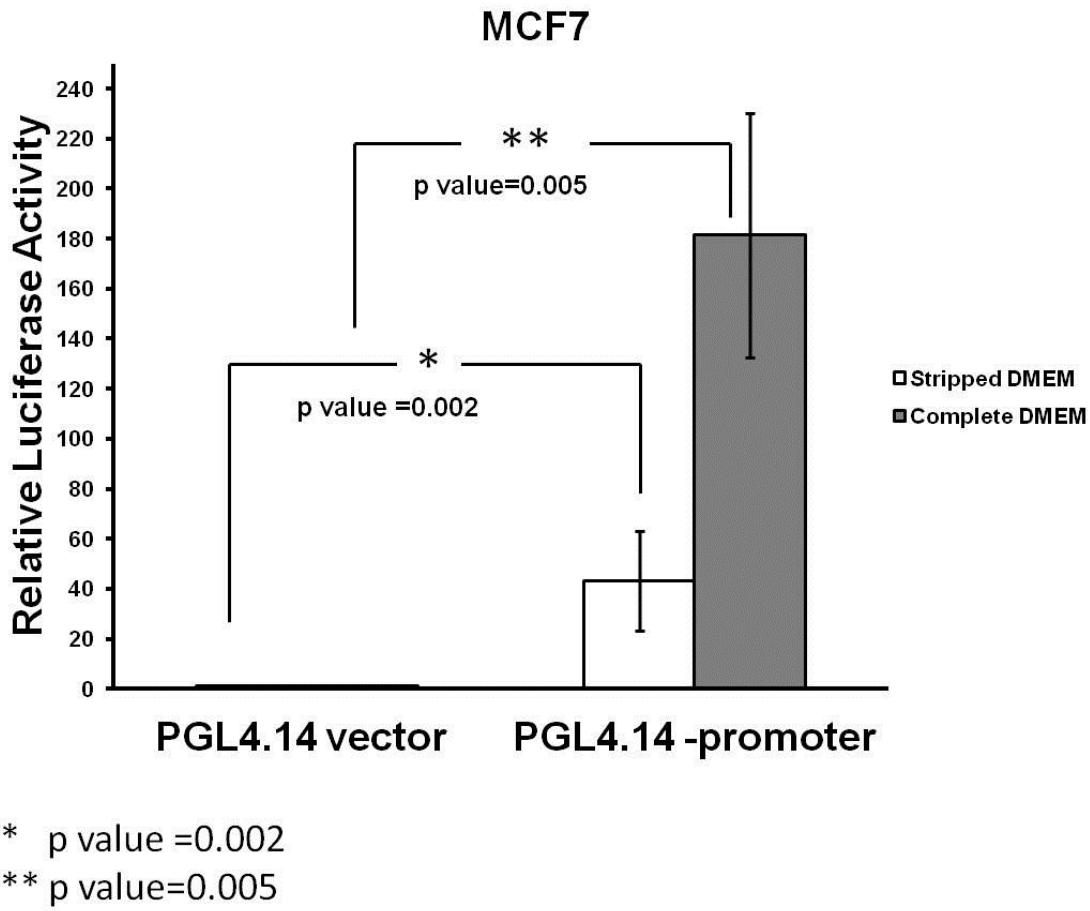

Figure I- PRP4K promoter activity in complete versus stripped media. PRP4K full length promoter luciferase construct was able to drive luciferase expression in both serum-containing (complete) and serum-stripped media. Luciferase activity was measured using Dual-Reporter Luciferase Assay. Error bars = SE, where n=3 p-values are indicated.