Roles of EEF1A2 & PTK6 in Breast Cancer

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To my aunt

Fatima J. Fakhro

who passed away from breast cancer in Jan 2008

Declaration

I here declare that this thesis has been composed by me and that all the work presented within is my own unless clearly stated. This work has not been submitted for any other degree or professional qualification.

Mariam Fida, MD

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Abbreviations & Symbols

BMI	Body Mass Index
18S rRNA	18S ribosomal RNA
aCGH	array Comparative Genomic Hybridization
Akt	Serine/threonine protein kinase
ALDH3A1	Aldehyde dehydrogenase 3 family, member A1 (Human gene)
Aldh3a1	Aldehyde dehydrogenase 3 family, member A1(Mouse gene)
APL	Acute promyelocytic leukemia
AR	Androgen receptor gene (Human gene)
ATM	Ataxia Telangiectasia gene (Human gene)
AURKA	Aurora A kinase (Human gene)
B2m	Beta-2-microglobulin (Mouse gene)
BIRC5	Baculoviral inhibitor of apoptosis repeat-containing 5
bp	Base pairs
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BSA	Bovine Serum Albumin
CCND1	Cyclin D1 (Human gene)
CDH1	E-Cadherin
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CLL	Chronic lymphocytic leukemia
CpG	Cytosine and guanine with a phosphodiester bond between them
CREB	cAMP response element-binding
DARPP32	Dopamine- and cyclic AMP-regulated phosphoprotein, Mr = 32000 (Human gene)
DCIS	Ductal Carcinoma *In Situ*
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferases
DRFS	Distant relapse-free survival
Dusp6	Dual specificity phosphatase 6
eEF1A	Eukaryotic elongation factor 1A
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1 (Human gene)
EEF1A2	Eukaryotic translation elongation factor 1 alpha 2 (Human gene)
Eef1a2	Eukaryotic translation elongation factor 1 alpha 2 (Mouse gene)
eEF1B	Eukaryotic translation elongation factor 1B
eEF2	Eukaryotic translation elongation factor 2
Egr1	Early growth response factor 1
EIF2B	Eukaryotic Translation Initiation Factor 2B
ER	Estrogen receptor gene
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (Human gene)
FACS	Fluorescence-activated cell sorting
FAK	Focal Adhesion Kinase

Fas	TNF receptor superfamily, member 6
FBS	Fetal bovine serum
FC	Fold Change
FGF	Fibroblast growth factors
FISH	Fibroblastic growth factor
Gadd45	Growth arrest and DNA-damage-inducible protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GFP	Green Fluorescent Protein
GTP	Guanosine triphosphate
H60a	Histocompatibility 60a
HAT	Histone acetyltransferases
HER2/neu	Human Epidermal growth factor Receptor 2 (Human gene)
hMLH1	Human mutL homolog 1
HRT	Hormone replacement therapy
IBC	Irritable bowel syndrome
IFN	Interferon
IFs	Initiation factors
Ig	Immunoglobulin
IHC	Immuohistochemistry
Itga11	Integrin alpha-11
kb	Kilobase
KCNQ2	Potassium voltage-gated channel, KQT-like subfamily, member 2
kD	kiloDalton
LCIS	Lobular cancer in situ
LOH	Loss of heterozygosity
Mb	Megabase
mdm2	Murine double minute
MeCP	Methyl-CpG–binding proteins
miRNA	Micro ribonucleic acid
mRNA	Messenger Ribonucleic acid
MYBL2	v-myb myeloblastosis viral oncogene homolog (avian)-like 2 (Human gene)
NBCS	Newborn calf serum
NFkB	Nuclear Factor-KappaB
ORF	Open reading frame
PAP	Phosphatidic acid phosphatase
PCR	Polymerase chain reaction
PCT	Mouse plasmacytomas
PET	Paraffin Embedded Tissue
PFDN4	Prefoldin 4
PI3K	Phosphatidylinositol-3 kinase
PML	Promyelocytic leukemia
Ppap2c	Phosphatidic acid phosphatase type 2C
PPM1D	Protein phosphatase 1D (Human gene)
PR	Progesterone receptor gene

PTEN	Phosphatase and tensin homolog (Human gene)
PTK6	Tyrosine-protein kinase 6 (Human gene)
PUM1	Pumilio homolog 1
RARα	Retinoic acid receptor alpha
RB1	Retinoblastoma gene
Renbp	Renin Binding Protein
Rhox5	Reproductive homeobox-5 gene
RNAi	RNA interference
ROMA	Representational Oligonucleotide Microarray Analysis
rpm	Revolutions per minute
RPPA	Reverse Phase Protein Array
rRNA	Ribosomal RNAs
RT	Reverse transcriptase
SH3	Src homology
SILAC	Stable Isotope Labeling by Amino acids in cell Culture
siRNA	small interfering RNA
SRMS	Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal
STAT	Signal Transducer and Activator of Transcription
TBP	TATA binding protein
TMAs	Tissue MicroArrays
TOP2A	Topoisomerase 2-alpha (Human gene)
TP53	Tumor protein 53 (Human gene)
TPD52	Tumor protein D52
tRNAs	Transfer RNAs
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WB	Western Blots
wst	wasted
ZNF217	Zinc finger protein 217 (Human gene)

Abstract

Eukaryotic Translation Elongation Factor 1 Alpha (EEF1A) exists as two forms with different tissue specificities and encoded by separate loci: eEF1A1 on 6q13 and eEF1A2 on 20q13.3. eEF1A1 is ubiquitously expressed whereas eEF1A2 expression is normally limited to the heart, brain and skeletal muscles. eEF1A proteins are GTP-binding proteins that recruit an amino-acylated tRNA to the ribosome during the elongation phase of protein translation.

eEF1A2 mRNA and protein are highly expressed in 50–60% of primary human breast tumors and metastases but not in normal breast epithelium. Since it is also overexpressed in 30% of primary human ovarian tumors, transforms rodent fibroblasts and increases their tumorigenicity in nude mice, eEF1A2 is considered to be a potential human oncogene. The mechanism of eEF1A2 expression is yet to be determined. Studies showed no gene mutation and no correlation between locus amplification or methylation and gene expression.

Phosphate Tyrosine Kinase-6 (*PTK6*) is also located on 20q13.3. It is 48kb upstream of *EEF1A2*. PTK6 is a non-receptor tyrosine-kinase that is normally expressed in epithelial linings, prostate, skin and oral epithelium but it is not detected in the normal human mammary epithelium.

PTK6 has been found to be expressed in many breast cancer cell lines and in approximately 60% of primary human breast tumors but it has not been detected in normal human breast tissue nor in fibroadenomas. Like other tyrosine kinases, PTK6 phosphorylates and activates downstream substrates that would be predicted to lead to increased transcriptional activity and therefore mediates proliferation of breast cancer cells. PTK6 is considered a prognostic marker of metastasis-free survival in breast cancer independent of the classical markers of tumor size, lymph node involvement and HER2 status.

The aim of this project was to characterize for the first time the genomic region containing the two mentioned breast cancer oncogenes and understand their various roles- whether they act in tandem or independently in breast tumorigenesis.

Immunohistochemistry was performed on tissue microarrays from 300 breast cancer patients to detect the expression levels of eEF1A2 and PTK6. Tumors that showed a high co-expression were analyzed for the genes' copy number. An increased copy number of PTK6 was detected but not of eEF1A2 nor of adjacent genes on the 20q13.3 amplicon.

To understand the effect of EEF1A2 expression on other genes, microarray analysis was performed on NIH-3T3 cells stably transfected with EEF1A2. Many upregulated genes were associated with different types of cancers. This was further confirmed by real-time PCR. However, when the NIH-3T3 cells were transiently transfected with EEF1A2, the genes that were upregulated in the microarray study showed no change in expression.

In conclusion, EEF1A2 and PTK6 act independently and each acts through a different mechanism in breast tumorigenesis.

Chapter 1: Introduction

1.1 Breast Cancer

Breast cancer is a complex and heterogeneous disease. It is the most common form of cancer in women and the third leading cause of cancer death in Europe (Ferlay et al., 2007). According to a study published in 2007 by Ferlay, *et al*, in Europe in 2006, it was estimated that 3,191,600 patients were diagnosed with cancer and 1,703,000 died from it. The most common type of cancer was breast cancer (429,900 cases, 13.5% of all cancer cases), followed by colorectal cancers (412,900 cases, 12.9%) and lung cancer (386,300 cases, 12.1%). Lung cancer was the most common cause of cancer death with an estimated 334,800 deaths (19.7% of total), followed by colorectal (207,400 deaths), breast (131,900 deaths) and stomach (118,200 deaths) cancers (Ferlay et al., 2007).

According to the Cancer Research UK statistics, it is estimated that more than 44,000 women and 300 men are diagnosed with breast cancer each year in the UK. While different modalities of treatment (i.e. chemotherapy, hormonal therapy, radiotherapy and surgery) have improved significantly in the past decade decreasing the mortality and morbidity rates, each year in the UK more than 12,000 women and around 100 men die from breast cancer. Of these women, around 1,400 are under the age of 50.

1.1.1 Risk factors for Breast Cancer

Many risk factors play an important role in developing breast cancer. Age is one of the best documented risk factors in breast cancer. The incidence is extremely low before the age of 30 and increases until the age of 80. Unlike the direct link of cigarette smoking to lung cancer, no factors of this kind have yet been identified that have a major effect on the risk of breast cancer. There are, however, several factors that have a more limited effect. Alcohol consumption might increase the risk of breast

cancer. Numerous studies have shown that consumption of one drink per day or less (approximately 12 g alcohol) does not significantly affect the risk of breast cancer (Harvey et al., 1987; Longnecker, 1999; Mannisto et al., 2000). However, Royo-Bordonado *et al.* reported that the relative risk associated with alcohol consumption was higher for those with body mass (BMI) index above the median (Royo-Bordonada et al., 1997). In premenopausal women, BMI was not a risk factor for breast cancer incidence whereas in post-menopausal women, the risk is increased (Tretli, 1989). This is attributed to adipose tissue being a source for extragonadal estrogens. It is also shown that that women who began menstruating before the age of 12 had a relative risk for invasive breast cancer of 1.3 compared to those who began after the age of 15 (Kyriazis et al., 1988). Hormone replacement therapy (HRT) and oral contraceptive use also increase the risk of breast cancer whereas prolonged lactation and physical exercise are protective against the disease. Other factors that increase breast cancer risk include: exposure to radiation, benign breast disease and mammographic density, a positive family history, previous history of neoplastic disease or hyperplasia in the breast and a genetic predisposition.

1.1.2 Breast cancer classification

Breast tumors are classified as non-invasive and invasive (Figure 1.1). Most breast cancers occur in the ducts of the breast which supply milk to the nipple. Breast cancer in this region is called ductal carcinoma. The less common form of breast cancer occurs in the lobules - where breast milk is made and is called lobular carcinoma.

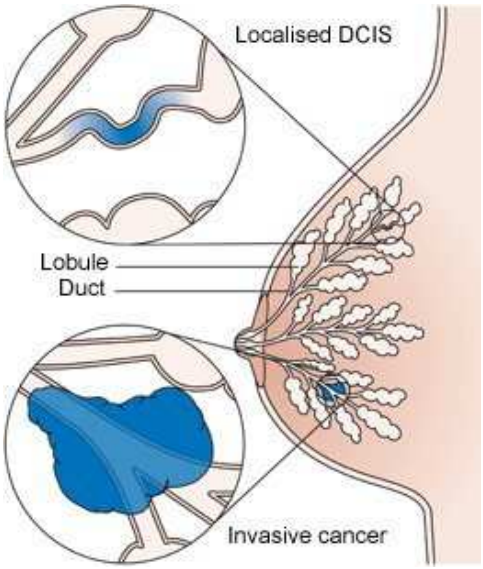

Figure 1.1 Breast cancer classification: Non-invasive (DCIS) and invasive (CancerHelp UK).

1.1.2.1 Non-invasive breast cancer

Non-invasive breast cancer is an abnormal growth of cells within the area in which it started. These cancer cells have not invaded the surrounding breast tissue. The two types of non-invasive tumors are ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). DCIS is a non-invasive breast cancer and referred to as stage 0. In the UK national health screening program for which figures are available annually, diagnosis of DCIS now form some 20% of all breast cancers diagnosed by screening. LCIS on the other hand is not considered a true cancer, but rather one whose presence indicates an increased risk of developing cancer in the future.

1.1.2.2 Invasive breast cancer

When breast cancer cells invade the surrounding breast tissue from the ducts or lobules, the cancer is called invasive. This increases the chance for cancer cells to metastasize to the lymph nodes. Inflammatory breast cancer (IBC) and Paget's disease of the nipple are two rare types of invasive breast cancer. Other less common forms of invasive breast cancer are medullary, mucinous, papillary and tubular carcinoma.

1.1.3 Male breast cancer

Male breast cancer accounts for 0.7% of all breast cancers (Jemal et al., 2004) and 0.17% of all cancers in men (Appelbaum et al., 1999). It is found to be on the rise with the increasing incidence of breast cancer in women (Jemal et al., 2004). The incidence of breast cancer in men has increased by approximately 26% over the past 25 years, affecting approximately one per 100,000 men per year in the United States (Giordano, 2005). The average age for male breast cancer is 67 years at diagnosis which is 5 years older than the average age for women diagnosed with the same disease (Giordano et al., 2004).

The etiology of breast cancer in men is unclear; it has been suggested that hormonal levels play a role in the development of this disease. Several medical conditions have been associated with the increased risk of breast cancer in men. This includes testicular abnormalities such as undescended testes, congenital inguinal hernia, orchiectomy, orchitis and infertility (Fentiman et al., 2006; Sasco et al., 1993; Thomas et al., 1992), and conditions that result in increased estrogen levels, such as advanced age, poor liver function and Klinefelter's syndrome (Giordano, 2005; Yang et al., 2001a). Klinefelter's syndrome, an XXY sex chromosome anomaly may predispose up to 7.5% male breast cancers. This rate is higher than the 3% rate previously reported with a 50-fold increased risk of developing breast cancer relative to normal men (Hultborn et al., 1997).

Gynecomastia is not believed to be a risk factor for breast cancer (Fentiman et al., 2006), whereas radiation therapy to the chest in men –as in women- increases the risk for developing the disease (Jellici et al., 2005). Men with a family history of breast cancer in a female relative have 2.5 times higher odds of having breast cancer than men without a family history (Fentiman et al., 2006).

1.1.3.1 Male breast cancer genetics

Male breast cancer is more commonly associated with germline mutations of *BRCA2* than *BRCA1* (Fentiman et al., 2006). In population-based series, it was shown that between 5% and 15% of men with breast cancer unselected for family history have *BRCA2* mutations, while 0–4% have *BRCA1* mutations (Basham et al., 2002; Frank et al., 2002). Men who inherit the germline *BRCA2* mutation have around 6% higher risk of developing breast cancer. This type of mutation tends to present at a younger age and may be associated with poorer survival (Kwiatkowska et al., 2003).

Other genes have also been linked to male breast cancer. Mutations in the androgen receptor gene (*AR*), *PTEN* tumor suppressor gene, and mismatch repair genes (i.e., hMLH1) have been reported in males with breast cancers (Boyd et al., 1999; Fackenthal et al., 2001). Pich *et. al* have shown that in men with breast cancer, the overexpression of c-myc, c-erbB-2, and p53 proteins is associated with poor prognosis (Pich et al., 2000).

1.1.4 Genes associated with breast cancer

Although environmental factors, hormonal and reproductive factors such as pregnancy history, age at menarche and age at menopause, play an important role in increasing breast cancer risk (Colditz et al., 2006), there is also strong evidence for a genetic component. First-degree relatives of breast cancer patients have approximately a 2-fold increase in risk for developing breast cancer and most of the excess risk is likely to be caused by genetic factors rather than shared environment (Lichtenstein et al., 2000; Peto and Mack, 2000).

1.1.4.1 Oncogenes

MYC is a gene that encodes a transcription factor involved in cell growth, differentiation and cell apoptosis. *MYC* is amplified and overexpressed in 15-20% of breast cancers (Aulmann et al., 2006). Even though gene amplification is always associated with protein expression, Blancato *et al.* have shown that in human breast

tumor tissues, myc overexpression is not always associated with gene amplification (Blancato et al., 2004). Even though no explanation in the literature suggests the reason for the overexpression, many mechanisms could be attributed to protein overexpression with normal gene copy number. These include gene mutation, signalling pathways being activated by other mechanisms such as gene fusion, ligand stimulation cross-activation by other substrates, and epigenetic factors. (General mechanisms of overexpression without amplification discussed in section 3.3.2)

ERBB2 or *HER2/neu* is a gene encoding a tyrosine-kinase transmembrane receptor. The products of this gene are either amplified and/or overexpressed. ERBB2 is overexpressed in 20-40% of breast cancer patients (Paik et al., 1990) and amplified in 20% of invasive breast cancers (Clark and McGuire, 1991). In most cases, HER-2 overexpression is caused by amplification of the HER-2 gene (Pauletti et al., 1996). However, Koo *et al*. reported that HER-2 protein overexpressed breast cancer was detected without HER-2 gene amplification (Koo et al., 2009). The overexpression of this oncogene in breast cancer cell lines has shown an increase in the invasion properties and metastatic activity of the transfected cells (Tan et al., 1997).

CCND1 is another oncogene that encodes cyclin D1. It is overexpressed in 40-50% of breast cancer patients and amplified in only 20% of cases (Buckley et al., 1993). Reis-Filho *et al.* found a good correlation between *CCND1* amplification and cyclin D1 overexpression. However, few breast cancers that lacked *CCND1* amplification still showed a high expression of cyclin D1 (Reis-Filho et al., 2006).

The fibroblastic growth factors (FGFs) and their receptors are involved in cell transformation by deregulating tyrosine-kinase receptor activity. *FGF1* and *FGF2* are frequently co-amplified with *CCND1* in breast cancer but are much more rarely expressed than cyclinD1. *FGFR1* is amplified in 10% of breast cancers (Adnane et al., 1991) and overexpressed in 20% whereas FGFR4 is overexpressed in 30% of breast tumors (Penault-Llorca et al., 1995).

1.1.4.2 Tumor suppressor genes

TP53 or p53 is a transcription factor that was first recognized when a germline mutation was found to be responsible for Li-Fraumeni Syndrome (Malkin et al., 1990). Patients with this syndrome have an increased risk of several cancers with the most frequent being breast cancer (Hollstein et al., 1991). *TP53* mutations are found in 15-35% of sporadic breast cancer cases and are often accompanied by loss of the wild-type allele (LOH) (Greenblatt et al., 1994). Most *TP53* alterations found in breast carcinomas are point mutations leading to the synthesis of a mal-functional protein.

RB1 is a retinoblastoma gene that regulates the cell cycle. In the absence of the gene, cells may undergo uncontrolled proliferation leading to tumour formation. RB1 showed a loss of expression in 20% of breast cancer cases (Varley et al., 1989).

BRCA1 and *BRCA2* are the most studied tumor suppressor genes associated with breast cancer. Mutations of these genes in sporadic breast cancers are rare but deletions of one or more exons have been found in the inherited form. A decreased expression of BRCA1 is due to hypermethylation (Dobrovic and Simpfendorfer, 1997) whereas no hypermethylation of BRCA2 was reported (Collins et al., 1997). BRCA2 is overexpressed in 20% of breast cancers. This overexpression is correlated with poor prognosis (Bieche et al., 1999).

PTEN is a tumor suppressor gene encoding a lipid phosphatase that negatively regulates the cell-survival signaling pathway. It dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P_3), the product of phosphatidyl inositol 3-kinase (PIK). Data suggests that the phosphatase activity of PTEN is essential for its function as a tumor suppressor. The activation of Akt/PKB is regulated by the phosphorylation of Akt on Thr^{308} and Ser^{473} by phosphoinositide-dependent kinase (PDK) and integrin-linked kinase (ILK), respectively (Simpson and Parsons, 2001). Inactivation of PTEN allows constitutive and unregulated activation of the Akt/PKB signaling pathway. In addition to regulating the Akt/PKB signaling pathway, PTEN also inhibits growth factor (GF)-induced Shc phosphorylation and suppresses the MAP kinase signaling pathway. PTEN interacts directly with FAK and is able to dephosphorylate activated FAK. PTEN-induced down-regulation of

$p130^{CAS}$ through FAK results in inhibition of cell migration and spreading (Besson et al., 1999). Somatic mutations are rare in sporadic breast cancer (Rhei et al., 1997). However, a germ-line mutation in this gene is responsible for Cowden disease which is associated with a high risk of breast cancer (Lynch et al., 1997).

ATM is a kinase that is activated by DNA damage which initiates the DNA damage checkpoint by phosphorylating a number of key proteins. Once activated, the checkpoint leads to cell cycle arrest and either DNA repair or apoptosis. Mutations of the *ATM* gene are responsible for Ataxia Telangiectasia, an autosomal recessive disorder characterized by cerebellar ataxia, telangiectasia, immunodeficiency and a predisposition to malignancies including breast cancers. Women with *ATM* mutations causing Ataxia Telangiectasia were found to have a 2-fold increase risk of developing breast cancer (Renwick et al., 2006).

E-Cadherin (CDH1) is a cell adhesion molecule. It is mutated in 20-40% of cases of lobular breast carcinoma (Berx et al., 1995). In invasive breast cancer, no mutation has been reported but a decreased expression of CDH1 is observed (Rimm et al., 1995). The low expression could be due to the hypermethylation of the promoter region (Graff et al., 1995).

The cyclin-dependant kinases inhibitors (Cdki) are cell cycle regulators that inhibit the cyclin/cdk complex. Some of these genes act as tumor suppressor genes such as *p16* and *p27*. Mutations in *p27* have been reported in breast cancer (Spirin et al., 1996). p27 is expressed at high levels in normal breast tissue but shows low expression in breast cancers and is associated with poor prognosis (Catzavelos et al., 1997).

1.1.5 Genomic regions altered in breast cancer

Many regions have been frequently reported to be altered in breast cancer. These regions are either amplified or deleted. Many cancers have been analyzed in genome-wide array Comparative Genomic Hybridization (aCGH), these include both common cancer types, such as breast (Naylor et al., 2005) and colorectal cancers (Kim et al., 2006), as well as more rare cancer types such as gastrointestinal stromal

tumors (Assamaki et al., 2007). These studies analyzed copy number changes in various cancer types to understand the patterns of copy number alterations occurring during cancer development. A number of regions that are frequently involved in gains and losses have been identified (section 1.1.5.1 & 1.1.5.2). Naylor *et al.* compared the location, frequency and size of copy number changes in 47 primary breast cancers versus 18 breast cancer cell lines using aCGH (Figure 1.2). They identified gains on chromosomes 1q, 8q, 11q, 17q, 20q, and losses on 6q, 8p, 9p, 13q, 16q (Naylor et al., 2005).

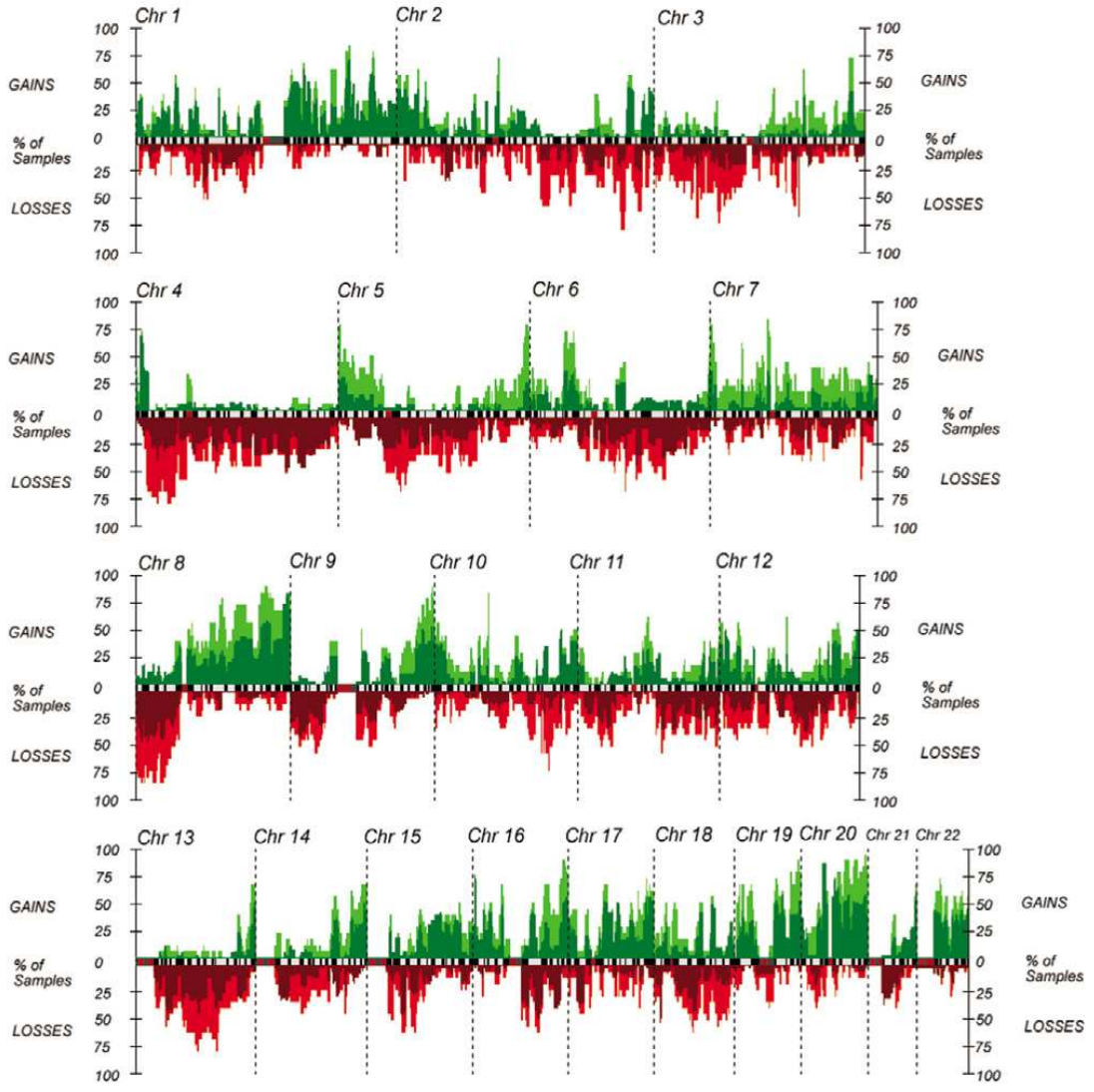

Figure 1.2 Array-based comparative genomic hybridization (aCGH) frequency plots of breast cancers and cell lines. Plots of primary breast cancers (light) overlaid onto breast cell lines (dark) with gains in green and losses in red (Naylor et al., 2005).

1.1.5.1 Amplification - Gain of genetic material

Amplification can be due to gain of part of a chromosome or a whole chromosomal arm. The gain of an entire chromosome arm could be the result of mitotic non-disjunction whereas gene amplification could be the result of the dominant action of such genes.

Hicks *et al*. have used Representational Oligonucleotide Microarray Analysis (ROMA) to detect genomic amplifications in breast tumors. They identified a pattern characterized by multiple closely spaced amplicons they called "firestorms" where tumors were profiled for copy number at a resolution of <50kb (Hicks et al., 2006). This term was given to amplified regions in the genome that were close to one another on the same region of the chromosome. The multiple amplifications on a single chromosome arm were highly correlated with aggressive disease and poor survival in patients.

Comparative genomic hybridization (CGH) has shown frequent gain of chromosome arms of 1q and 8q (Tirkkonen et al., 1998) in approximately 40% of cases. The most frequently amplified regions in breast cancer are 16p11, 17q11.2, 17q24 and 20q13. These amplifications are seen in over 15-20% of breast cancers (Kallioniemi et al., 1994).

1.1.5.1.1 Amplification of 20q13 in cancer

Amplification is an important mechanism for oncogene overexpression in breast cancers. Several amplified regions may participate to breast cancer initiation and/or progression. Of these regions, 20q13 - which is amplified in 8.5-10% of tumors- plays an important role in breast cancer (Letessier et al., 2006). High level of 20q13 amplification was associated with short disease-free survival in lymph node-negative patients (Tanner et al., 1995). In breast cancer and other types of malignancies, several potential oncogenes have been reported including *EEF1A2* (Lee and Surh, 2009), *BRK/PTK6* (Zhang et al., 2005)*, MYBL2* (Forozan et al., 2000), *STK6/AURKA/Aurora A* (Sen et al., 1997) and *ZNF217* (Nonet et al., 2001). All these genes are within a 20Mb region (Figure 1.3).

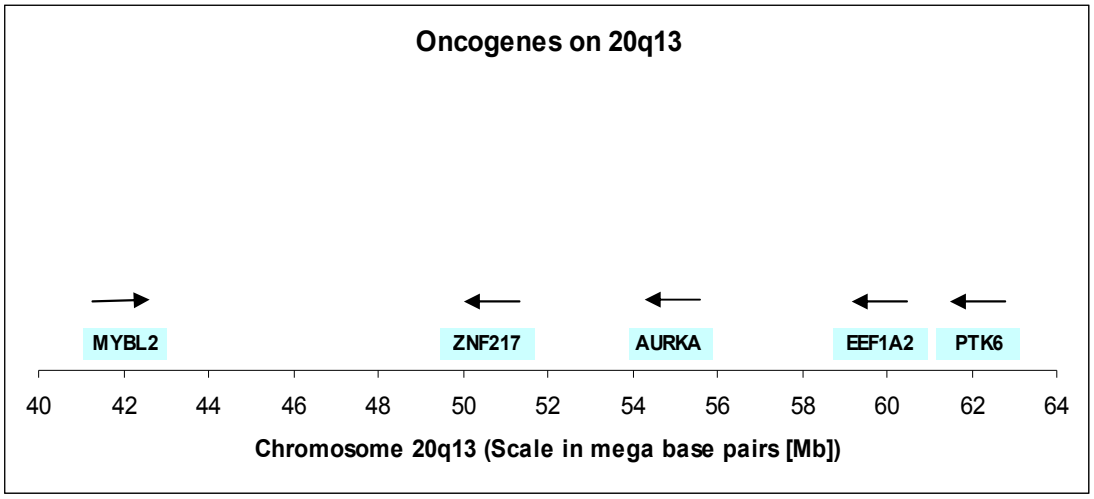

Figure 1.3 20q13 region showing potential oncogenes in breast cancer. *PTK6* (8.8kb), *EEF1A2* (11kb). *AURKA* (23kb), *ZNF217* (16kb) and *MYBL2* (49kb). All genes are within a 20Mb region. Arrows indicate the orientation of gene expression.

In breast cancer, correlations between levels of amplification and gene expression have been reported. Xiang *et al.* have reported the associated co-amplification and correlated overexpression of *BRK* and *ERBB2* in breast cancer. They found that ERBB2 protein interacts with BRK protein, and increases its intrinsic kinase activity. Expression of Brk enhanced the ErbB2-induced activation of Ras/MAPK signalling and cyclin E/cdk2 activity resulting in proliferation of cultured breast cells (Xiang et al., 2008). These studies demonstrated the potential for genes located on distinct amplicons to co-operate in the induction of transformation.

High level of 20q13 amplification was associated with faster cancer progression and worse patient survival in colon and ovarian cancer (Aust et al., 2004; Tanner et al., 2000). Amplification of the *EEF1A2* gene at this locus have been reported in human lung adenocarcinoma (Li et al., 2006). siRNA knockdown inhibited proliferation and promoted apoptosis and expression of EEF1A2 protein was associated with shorter survival time.

1.1.5.1.2 Mechanisms of gene amplification

Gene amplification is likely to occur due to a DNA double-strand break, genome rearrangements and in cells lacking checkpoints. The initiation of amplification has been attributed to defects in DNA replication, telomere dysfunction and chromosomal fragile sites.

1.1.5.1.2.1 DNA replication

Eukaryotic cells normally replicate their DNA only once every cell cycle. This regulation is important because re-replication would threaten genome stability and therefore eventually lead to increased cell division and cancer development. This would explain the case of the ERBB2 gene for example. Due to the importance of DNA replication in cancer, many anticancer drugs target various aspects of DNA replication. Role of DNA replication in cancer proliferation has been reported in many malignancies such as the hereditary cancers of the colon, breast, ovary and skin. These can be caused by mutations in DNA replication genes, such as translation synthesis, intra- S-phase signaling or mismatch repair genes (Sancar, 1994; Marra and Boland, 1995; Bertwistle and Ashworth, 1998; Masutani et al., 1999). In somatic cancers, such early mutations would probably have a lesser effect as the disease progresses, therefore making the relationship less obvious.

1.1.5.1.2.2 Telomere dysfunction

Telomeres prevent the loss of DNA sequences that occurs due to incomplete replication of linear DNA at a chromosomal end. At the end of each replication, the DNA is shortened by 40-50bp. Telomeres are replicated by the enzyme telomerase which is inactive in somatic cells. When telomeres get shortened to a critical length, cells undergo senescence and therefore stop proliferating. In tumors, telomerase is re-activated and cells maintain telomere length. However, before these tumor cells adapt to telomere dysfunction, they could have proliferated with such short telomeres risking the susceptibility of fusion and therefore abnormal cell division (Londono-Vallejo, 2004).

Telomere dysfunction is one of the mechanisms leading to amplification through the breakage-fusion-bridge (BFB) where a chromosomal break or a dysfunctional telomere leads to chromosomal ends fusion resulting in a dicentric chromosome formation. During anaphase, a chromosomal break occurs which leads to the generation of an inverted duplication of terminal sequences. This process will be repeated in following cell divisions until the chromosomal ends are stabilized by addition of telomeric sequences probably by translocation to another chromosome. This BFB event will eventually result in generating an amplicon at the site of the gene where the units are arranged as inverted repeats.

1.1.5.1.2.3 Fragile sites

Fragile sites are chromosomal regions prone to breakage under conditions of replication stress such as exposing cells to agents interfering with DNA replication. More than 120 different fragile sites have been identified in the human genome (Lukusa and Fryns, 2008). These regions are likely to be flexible and therefore associated with high frequency of recombination leading to chromosomal aberrations associated with different types of cancer (Glover et al., 2005). In effect, they are regions prone to breakage that could initiate the amplification process. Miller *et al*. have reported that fragile sites participate in amplification in human cancers where they showed that the boundaries of *MET* amplicon map within the fragile site FRA7G in esophageal cancer (Miller et al., 2006).

1.1.5.1.3 Overexpression due to amplification

Overexpression is usually associated with gene amplification. An example of a gene overexpressed due to amplification –as mentioned in section 1.1.4.1- is HER-2. *HER-2* is a gene located on chromosome 17q12 that encodes a transmembrane protein with tyrosine kinase activity. Overexpression of HER-2 in breast cancer and is associated with poor prognosis, aggressive disease and poor response to hormonal therapy (Carr et al., 2000).

1.1.5.1.4 Overexpression without amplification

Varis *et al.* have evaluated the amplification and expression levels of three target genes, *HER-2*, *TOP2A* and *DARPP32*, located on the 17q12 amplicon in gastric cancer. They showed that of the three genes, *DARPP32* was the most highly overexpressed gene (Varis et al., 2004). Several cancers with gene overexpression were found but lacked *DARPP32* amplification. This suggests that mechanisms other than gene amplification are responsible for *DARPP32* upregulation.

Another gene reported to be overexpressed in breast cancer but its overexpression was not correlated with amplification is *PPM1D*. *PPM1D* is one of the drivers of the 17q23.2 amplicon which is associated with several types of cancers. Lambros *et al*. reported that it is amplified in only 6% of cases and that *PPM1D* amplification was strongly associated with the mRNA expression levels (Lambros et al., 2010). However, they show that PPM1D overexpression is more prevalent than gene amplification.

HDM2 gene, the human homologue of murine *mdm2* is an oncogene that maps to chromosome 12q13. HDM2 blocks the function of p53 by binding to its transcriptional activation domain. Melanoma cell lines were reported to express HDM2 protein but there was no evidence of gene amplification (Polsky et al., 2001). The mechanisms driving HDM2 overexpression are yet to be investigated.

1.1.5.2 Deletion - Loss of genetic material

Many deleted chromosomal regions have been associated with cancer. Deletion of the same loci in different tumors suggests the existence of tumor suppressor genes in this region. The loss of function of tumor suppressor genes requires the loss or inactivation of both copies of the gene. This usually occurs by mutation of one allele and LOH of the wild type one. The most frequently lost chromosomal arms that have been reported using CGH are 1p, 3p, 6q, 8p, 9p, 11p, 13q, 16q, 17p. These deletions are observed in more than 20-25% of breast cancers. On the other hand, deletions at 1q, 7q, 11p, 17q, 18q and 22q have been reported in 15–20% of breast cancers by LOH studies (Devilee et al., 1991).

1.2 Mechanisms of gene expression

Gene expression is modulated by many epigenetic markers that are involved in transcriptional and translational regulation. The nucleosome is the basic unit of the chromatin. It is composed of the four core histones H2A, H2B, H3 and H4 as well as 146 basepairs of DNA wrapped around the histones (Luger et al., 1997). Each core histone is composed of a structured domain and a tail of unstructured amino-terminal of 25-40 residues. Histone tails provide sites for a variety of posttranslational modifications, including acetylation, phosphorylation and methylation (Figure 1.4). Such modifications of histone tails determine the interactions of histones with other proteins, which may in turn also regulate chromatin structure. MicroRNAs (miRNAs) are another class of mediators that are important in gene expression and cancer development.

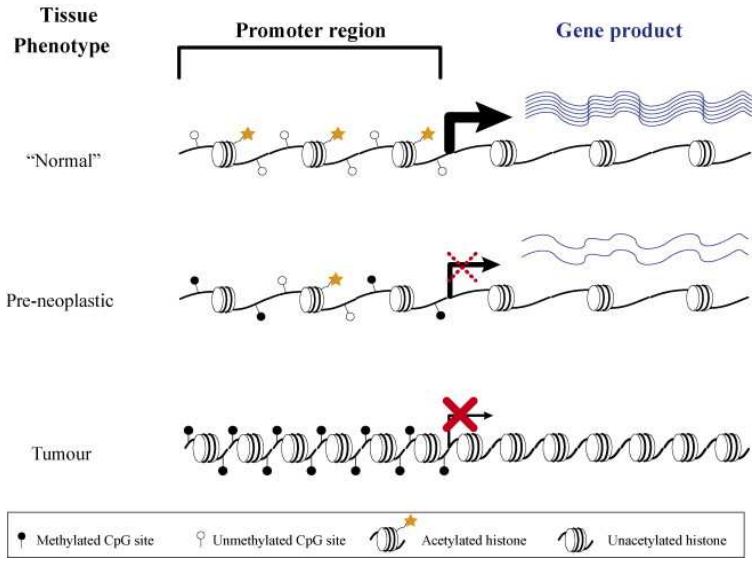

Figure 1.4 Epigenetic gene silencing during tumourigenesis proceeds gradually. In normal tissue the CpG island is typically unmethylated and histones are acetylated. As tissue progresses from normal to pre-neoplastic and tumour state, gradual deacetylation and methylation at CpG island is accompanied by a progressive loss of gene expression (Vaissiere et al., 2008).

1.2.1 Phosphorylation

Histone phosphorylation has been shown to play an important role in processes such as transcription, DNA repair, apoptosis and chromosome condensation (Cheung et al., 2000). The mechanism by which phosphorylation contributes to transcriptional activation is not well understood. The addition of negatively charged phosphate groups to histone tails neutralizes their basic charge and therefore reduces their affinity for DNA. Phosphorylation of serine 10 (Ser-10) in histone H3 has been associated with gene activation in mammalian cells (Thomson et al., 1999). It has been reported that many acetyltransferases have increased HAT activity on serine 10-phosphorylated substrates. Also, mutation of serine 10 decreases activation of Gcn5-regulated genes (Lo et al., 2000). Therefore, phosphorylation may contribute to transcription through HAT activation on the same histone tail. Clayton *et al*. have reported that upon gene activation, histone H3 on the oncogenes c-*fos*- and c-*jun*-associated nucleosomes becomes doubly-modified where the same H3 tail becomes both phosphorylated and acetylated (Clayton et al., 2000). Histone H3 phosphorylation also occurs after activation of DNA-damage signaling pathways

whereas phosphorylation of H2A is associated with mitotic chromosome condensation (Cheung et al., 2000).

1.2.2 Methylation

1.2.2.1 DNA methylation and gene expression

DNA methylation is one of the most commonly occurring epigenetic events that take place in the mammalian genome. It plays a critical role in the control of gene activity. DNA methylation occurs in CpG-rich regions known as CpG islands, which span the 5′ end of the regulatory region of many genes. These islands usually range from 0.5 to 5 kb, occurring on average every 100 kb and are unmethylated in normal cells (Weber et al., 2007). CpG islands are enriched in promoters or the first exon of the gene. They are found in the promoter regions of around 60% of protein-coding mammalian genes. They are normally unmethylated in transcriptionally active genes like housekeeping genes but developmental and tissue-specific genes appear to be methylated and silenced in differentiated tissues (Bird, 2002). Hypermethylation of repetitive genomic sequences prevents gross chromosomal changes, such as translocations or amplifications, which lead to chromosomal instability. In cancer, many unmethylated genes in the normal tissue become methylated in the tumor.

DNA methyltransferases (DNMT) are a group of enzymes important in maintaining DNA methylation. Cells that lack the stabilizing effect of DNA methylation due to spontaneous defects in DNMTs have prominent nuclear abnormalities (Xu et al., 1999). It was reported that mice deficient in the methyltransferase gene die early in development or immediately after birth (Robertson, 2002).

Several mechanisms have been shown to cause transcriptional repression by DNA methylation. The first mechanism involves direct binding of specific transcription factors to their recognition sites in their promoter regions. Many transcription factors such as AP-2, c-Myc/Myn, CREB, E2F, and NFkB, recognize and bind to sequences that contain CpG residues. The binding of each has been shown to be inhibited by methylation (Tate and Bird, 1993). The second mechanism

of transcriptional repression involves the binding of specific transcriptional repressors to methylated DNA. The DNA methylation signals are "read" by the methyl-CpG–binding proteins (MeCP), that bind to the methylated CpG sequence (Prokhortchouk and Hendrich, 2002). Several proteins have been reported to bind to the methylated CpG region. These include MeCP1, MeCP2, MBD1, MBD2, MBD4, and Kaiso (Prokhortchouk and Hendrich, 2002).

1.2.2.2 Hypermethylation in breast cancer

Hypermethylation results in loss of expression of a variety of genes that are important in the development of breast cancer. These include steroid receptor genes, cell adhesion genes, and inhibitors of matrix metalloproteinases (Yang et al., 2001b). Many genes have been reported to be hypermethylated in breast cancer. These include *p16*, estrogen receptor (*ER*) alpha, progesterone receptor (*PR*), *BRCA1*, *GSTP1*, *TIMP-3*, and E-cadherin. Two of these genes, *ER* and *PR* are steroid receptor genes that have long been associated with breast cancer. Methylation studies have shown that the *ER* gene has a CpG island in its promoter region and first exon (Yang et al., 2001b). Even though the *ER* gene is unmethylated in normal cells, it is reported to be hypermethylated in more than half of primary breast cancers. The *BRCA1* gene, which is a gene commonly associated with breast cancer was reported to have a reduced or absent protein product. This has been attributed to DNA methylation as a cause of its inactivation (Catteau and Morris, 2002).

1.2.2.3 Hypomethylation in breast cancer

Hypomethylation in breast cancers mostly occurs in repetitive DNA sequences and pericentromeric satellite DNA, which are normally heavily methylated in non-malignant cells (Bernardino et al., 1997). Unlike in ovarian cancers where increased satellite DNA hypomethylation is associated with tumor progression (Widschwendter et al., 2004), in breast cancer, satellite DNA hypomethylation is associated with early tumor development (Costa et al., 2006). Sat2 and Satα repeats

are frequently hypomethylated in ovarian and breast cancer (Costa et al., 2006; Widschwendter et al., 2004). Hypomethylation of Sat2 affects 50% of breast tumors whereas SatR-1 hypomethylation affects 86% (Costa et al., 2006; Narayan et al., 1998). The melanoma associated cancer/testis antigens MAGE are methylated and silenced in adult tissues, but hypomethylated and expressed in several tumors and breast cancer cells (Weber et al., 1994). Other hypomethylated genes associated with breast cancer are the plasminogen activator uPA (*PLAU*) gene, the breast cancer specific protein 1/synuclein-γ gene (*SNCG*), and the multidrug resistance 1 gene (*MDR1*) (Guo et al., 2002; Gupta et al., 2003; Sharma et al., 2010). Even though it has been shown that in cancer cells there is no reduction of DNA methyltransferase activity (Girault et al., 2003), knockdown of the gene in animal models or deficiencies of DNMTs activity would lead to genome wide DNA hypomethylation and chromosomal instability (Gaudet et al., 2003).

1.2.3 MicroRNAs (miRNAs)

MicroRNAs are small non-coding double-stranded RNAs that play a major role in the expression regulation of many genes (Figure 1.5). It is estimated that miRNAs may target about 60% of mammalian genes (Friedman et al., 2009). As of May 2011, more than 1100 human miRNAs have been identified (http://www.microrna.org/). Mature miRNAs usually down-regulate gene expression by recognizing the target mRNAs complementary sequence. They bind to the 3' untranslated region (UTR) or the open reading frame (ORF) of the target mRNA, or to 5'UTRs (Lee et al., 2009) resulting in inhibition of protein translation and/or degradation of the mRNA. Besides being involved in development and normal function, miRNAs have also been associated with many human diseases. Since miRNAs are important in regulating cellular differentiation and proliferation, any misregulation may be linked to tumorigenesis.

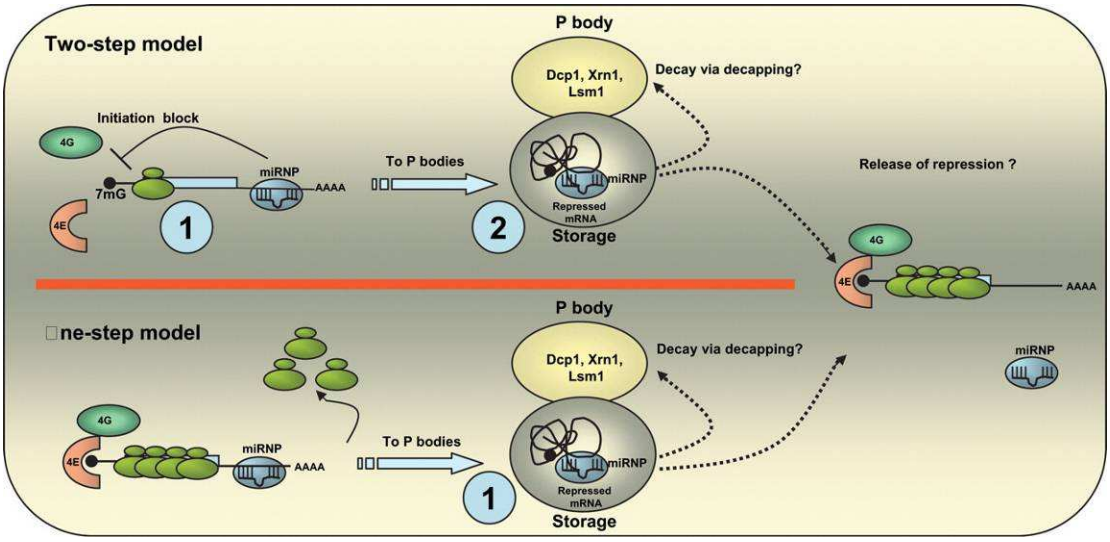

Figure 1.5 Models of miRNA function. The two-step model: Upon binding to the 3′-UTR of a target mRNA, the miRNP complex inhibits translation initiation, presumably by interfering with the 7mG cap function in recruiting eIF4E. Next, the repressed mRNA:miRNP complex is moved to the P-bodies (PBs) for storage. One-step model: Binding of the miRNP to the target mRNA directs the mRNA to the PBs. Disassembly of ribosomes on the message occurs prior to entry into the PBs. The mRNA can then be either degraded or stored for later reuse. 7mG, 7-methyl guanine; AAAA, polyadenosine tail; 4E, eIF4E; 4G, eIF4G (Pillai, 2005).

1.2.3.1 MicroRNAs and cancer

miR15 and *miR16* are the first two miRNAs that were linked to cancer in patients with chronic lymphocytic leukemia (CLL) (Calin et al., 2002). Both the miRNA genes are located at 13q14 and were found to be deleted or down-regulated in the majority of CLL cases (Calin et al., 2002). miRNAs function as regulatory molecules where they can act as oncogenes or tumor suppressors (Liang et al., 2007).

1.2.3.1.1 MicroRNAs as oncogenes

miRNAs can act as oncogenes when amplified or overexpressed. They can downregulate a tumor suppressor gene or other genes involved in cell differentiation and tumor development by stimulating proliferation, angiogenesis and invasion (Miska, 2005). Several miRNAs have been shown to act as oncogenes in pancreatic cancer. Increased expression of miR-103 and miR-107 were detected in pancreatic tumors (Lee et al., 2005). MiRNA-21 is more highly expressed in pancreatic cancer

when compared to benign tumors (Dillhoff et al., 2008). The miRNA miR-155 also acts on the activity of Tumor Protein 53-Induced Nuclear Protein 1 (TP53INP1) by inhibiting its antitumor actions (Gironella et al., 2007).

MicroRNAs play an important role in colorectal cancer. Increased expression level of let-7f, miR-181b and miR-200c was detected in patients with colorectal cancer (Nakajima et al., 2006). It was also shown that miR-21 downregulates the tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in the human colon cancinoma cells Colo206f, indicating its oncogenic function (Asangani et al., 2008).

Hayashita *et al.* have shown that the *miR-17-92* cluster, which is composed of seven miRNAs and resides in intron 3 of the *C13orf25* gene, is frequently overexpressed in lung cancers (Hayashita et al., 2005). The oncogenic effect of the *miR-17-92* cluster results in inhibition of apoptosis in lung cancer cells (Matsubara et al., 2007).

1.2.3.1.2 MicroRNAs as tumor suppressors

MicroRNAs also function as tumor suppressors in different types of cancers. In pancreatic cancer cells, miR-34a, which is induced by the P53 tumor suppressor protein is frequently absent (Chang et al., 2007). Its expression is also silenced in several types of cancers due to the methylation of the CpG island of the promoter (Lodygin et al., 2008). The miR-34a responsive genes play a role in cell-cycle progression, DNA repair, apoptosis and angiogenesis.

In colon cancer, as well as in colon cancer cells, miR-145 and miR-143 expression levels were reported to be reduced (Akao et al., 2006; Michael et al., 2003). The expression of the let-7 miRNAs have not only been shown to be reduced in both colon tumors and cancer cell lines but also in lung cancer. Members of the let-7 family, which includes let-7b, let-7c, let-7d, let-7f, and let-7g are all down-regulated in lung cancer and linked with shortened survival (Takamizawa et al.,

2004). This has been attributed to their ability to inhibit the RAS oncogene (Johnson et al., 2005).

Many other miRNAs have been reported to be reduced in expression in other types of cancers. These include miR-15a and miR-16-1 in chronic lymphocytic leukemia (Cimmino et al., 2005), miRNA-143 in human bladder tumor tissues (Lin et al., 2009) and miR-101 in progressive prostate and breast cancers (Varambally et al., 2008).

1.2.3.2 MicroRNAs and breast cancer

Many miRNAs have been predicted to regulate important breast cancer genes like *BRCA1/2*, *ATM*, *PTEN* and *CHEK2*. These miRNAs can be divided into miRNAs that function as oncogenes and those that act as tumor suppressors. The miRNAs that act as oncogenes are: miR-27a which targets the transcriptional cofactor ZBTB10/RINZF, miR-10b overexpression was shown to promote cell migration and invasion and miR-21 knockdown in the breast cancer cell line MCF-7 and mouse xenograft tumors decreased cell growth and inhibited tumor proliferation (Zhu et al., 2007b). miR-21 was also reported to down-regulate the tumor suppressor tropomyosin 1 (TPM1) therefore supporting the notion that these miRNAs function as oncogenes (Zhu et al., 2007b).

Several other miRNAs act as tumor suppressors in breast cancer. mir-125a and miR-125b were reported to be significantly reduced in ERBB2-amplified breast cancers (Mattie et al., 2006) leading to cell migration and invasion through the AKT pathway (Scott et al., 2007). miR-206 is another miRNA that is decreased in expression in ER-α positive human breast cancer tissues (Adams et al., 2007).

miRNAs play important roles in breast cancer metastasis. Ma *et al*. have shown that miR-10b is highly expressed in breast cancer metastatic cell lines when compared to nonmetastatic cells. Its overexpression was associated with invasion and metastasis but not proliferation (Ma et al., 2007). miR-373 and miR-520c have been

reported as metastasis-promoting miRNAs in MCF7 cells as they are also involved in tumour migration and invasion (Huang et al., 2008).

1.2.4 Transcription

The process of transcription is initiated when the RNA polymerase (RNA pol) enzyme binds to the template DNA strand and begins the production of complementary RNA. Three different types of RNA polymerase are reported: RNA pol I which transcribes genes encoding most of the ribosomal RNAs (rRNAs) and RNA pol III which transcribes the genes for one small rRNA and transfer RNAs (tRNAs) that play an important role in the translation process, as well as other small regulatory RNA molecules. RNA pol II is the key enzyme that transcribes the mRNAs that serve as templates for protein production. Transcription also requires transcription factors and proteins which specifically recognize short DNA sequences (*cis*-acting elements) in the non-coding region of the gene (Ptashne, 1986). These sequences could be upstream of the transcription start site, where they bind to the promoter for accurate and efficient initiation of transcription, or they could be several thousand bases upstream or even within introns or downstream of the gene, forming enhancer elements, which modulate promoter function (Maniatis et al., 1987).

1.2.5 Translation

1.2.5.1 Initiation

The mechanism of translation initiation can be divided into several steps (Figure 1.6). The pathway starts with the recycling of post-termination complexes where the separated 40S and 60S ribosomal subunits bind to form the 80S ribosomal initiation complex. The large subunit of the ribosome has three sites at which tRNA molecules can bind. The A (amino acid) site is where the aminoacyl-tRNA anticodon binds to the mRNA codon, ensuring that correct amino acid is added to the growing

polypeptide chain. The P (polypeptide) site is where the amino acid is transferred from its tRNA to the growing polypeptide chain. The E (exit) site is the location at which the tRNA -that is not carrying any amino acids- binds before being released back into the cytoplasm to bind to another amino acid and repeat the process. The initiator methionine tRNA is the only aminoacyl-tRNA that can bind in the P site of the ribosome, and the A site is aligned with the second mRNA codon. The ribosome is thus ready to bind the second aminoacyl-tRNA at the A site, which will be joined to the initiator methionine by the first peptide bond.

The initiating Methionine (Met-tRNA $^{Met}_{i}$) is base paired with the initiation codon in the ribosomal P-site which is competent to start the translation elongation step. This is followed by the eukaryotic initiation factor 2 (eIF2)–GTP–Met-tRNA$^{Met}_{i}$ complex formation. Next, formation of a 43S preinitiation complex comprising a 40S subunit, eIF1, eIF1A, eIF3, eIF2–GTP–Met-tRNA$^{Met}_{i}$ and probably eIF5 takes place followed by mRNA activation, during which the mRNA cap-proximal region is unwound in an ATP-dependent manner by eIF4F with eIF4B. The 43S complex will be attached to this mRNA region. Scanning of the 5′ UTR in a 5′ to 3′ direction by 43S complexes will take place. This is followed by recognition of the initiation codon and 48S initiation complex formation, which switches the scanning complex to a ‘closed’ conformation and leads to displacement of eIF1 to allow eIF5-mediated hydrolysis of eIF2-bound GTP and Pi release. 60S subunits will bind to 48S complexes leading to displacement of eIF2–GDP and other factors (eIF1, eIF3, eIF4B, eIF4F and eIF5) mediated by eIF5B and GTP hydrolysis by eIF5B and release of eIF1A and GDP-bound eIF5B from assembled elongation competent 80S ribosomes. Termination follows elongation and leads to recycling, which generates separated ribosomal subunits (Jackson et al., 2010).

1.2.5.2 Elongation

The elongation phase is the second phase in translation. It is controlled by three main elongation factors: eEF1A (Discussed in section 1.3), eEF1B, and eEF2. The ribosome moves along the mRNA in the 5' to 3' direction with the help of the

elongation factor eEF2. The tRNA that corresponds to the second codon binds to the A site. This step requires the elongation factors eEF1A and eEF1B, as well as guanosine triphosphate (GTP) as an energy source for the process. Upon binding of the tRNA-amino acid complex to the A site, GTP is cleaved to form guanosine diphosphate (GDP), then released along with eEF1A to be recycled by eEF1B for the next round.

Peptide bonds are now formed between the first and second amino acids. After the bond formation, the ribosome translocates again, therefore causing the tRNA to occupy the E site. The tRNA is then released to the cytoplasm to bind to another amino acid whereas the A site is now empty and ready to receive another tRNA for the next codon.

This process is repeated until all the codons in the mRNA have been read by tRNA molecules, and the amino acids attached to the tRNAs are linked together in the growing polypeptide chain in the appropriate order. At this point, translation must be terminated, and the protein must be released from the mRNA and ribosome complex.

1.2.5.3 Termination

There are three termination codons that are employed at the end of a protein-coding sequence in mRNA: UAA, UAG, and UGA. tRNAs do not recognize these codons. Therefore, in the place of these tRNAs, one of several release factors binds and facilitates release of the mRNA from the ribosome and subsequent dissociation of the ribosome back into the cytoplasm.

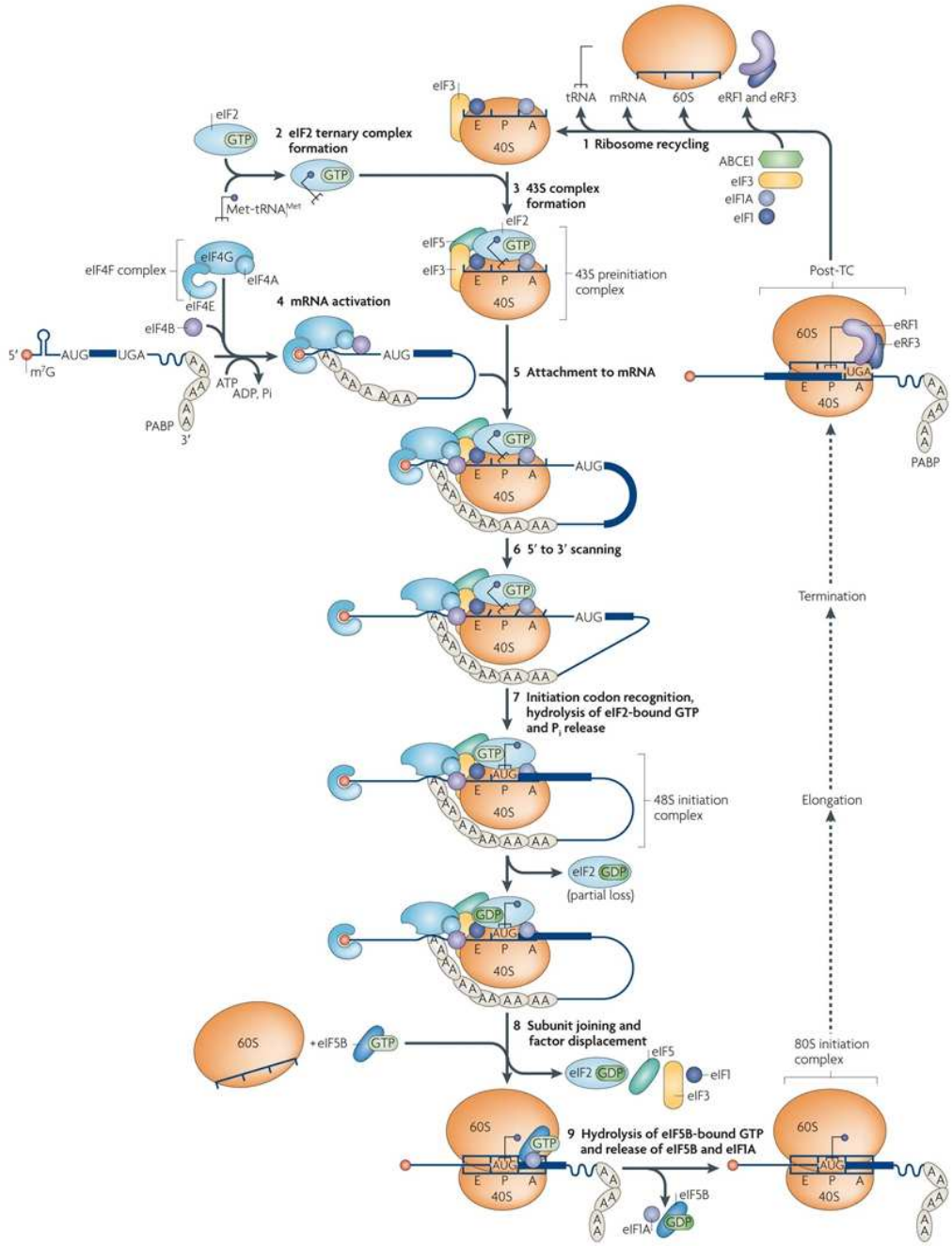

Figure 1.6 Pathway of eukaryotic translation regulation. 1) Recycling of post-termination complexes (post-TCs; 1) to yield separated 40S and 60S ribosomal subunits, and result in the formation of an 80S ribosomal initiation complex, 2) eIF2 ternary complex formation, 3) 43S complex formation, 4) mRNA activation, 5) Attachment of the 43S complex to the mRNA, 6) scanning of the 5′ UTR in a 5′ to 3′ direction by 43S complexes, 7) Initiation codon recognition, hydrolysis of eIF2-bound GTP and P_i release, 8) Joining of 60S subunits to 48S complexes and concomitant displacement of eIF2–GDP and other factors (eIF1, eIF3, eIF4B, eIF4F and eIF5) mediated by eIF5B, 9) GTP hydrolysis by eIF5B and release of eIF1A and eIF5B (Jackson et al., 2010).

1.3 Breast cancer genes studied in this project

1.3.1 EEF1A2

The elongation phase of protein synthesis can be divided into two stages: a) amino-acylated tRNA recruitment to a ribosome where eEF1A2 plays a major role (Figure 1.7) and b) ribosome translocation along the RNA as amino acids are added to the polypeptide chain (Thornton et al., 2003).

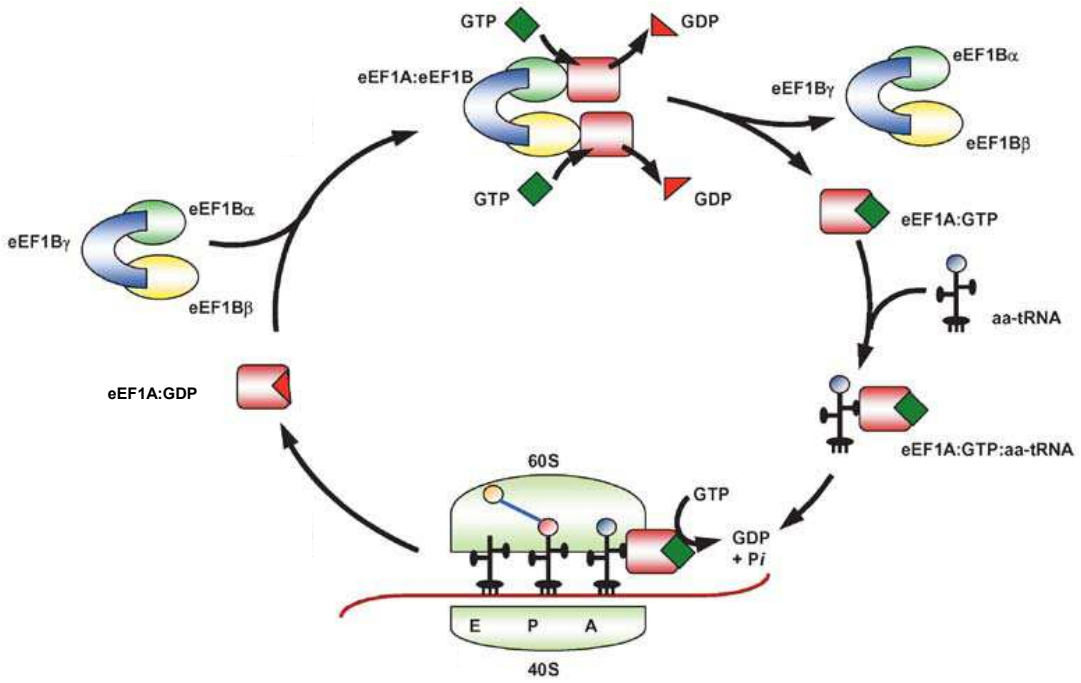

Figure 1.7 The role of eEF1A in the elongation phase of translation. eEF1A recruits the aa-tRNA to the A site of the ribosome where peptide elongation takes place. This requires the release of GDP. The eEF1A:GDP complex binds to eEF1B to allow for the exchange of GDP back to GTP. eEF1B is released again for eEF1A:GTP complex to bind to another aa-tRNA (Thornton *et al.* 2003).

Eukaryotic translation elongation factor 1 alpha 2 (*EEF1A2*) is a 11kb gene consisting of 8 exons and encoding a 463 amino acid protein with a molecular weight of 50.5kD. eEF1A2 is one of the two members of the eEF1A family of proteins,

eEF1A1 and eEF1A2. eEF1A1 and eEF1A2 share 98% amino acid homology but are encoded by different chromosomes (*EEF1A1* in 6q14 and *EEF1A2* in 20q13) (Lund et al., 1996).

eEF1A proteins -as shown in figure 1.7- are GTP-binding proteins that recruit an amino-acylated tRNA to the ribosome during the elongation phase of protein translation (Thornton et al., 2003). While eEF1A1 is ubiquitously expressed, eEF1A2 expression is limited to the normal brain, heart and skeletal muscles (Knudsen et al., 1993). Their translational elongation activities are similar but there is a difference in their binding affinity for GDP and GTP where eEF1A2 binds GDP more strongly than GTP and the opposite is true for eEF1A1 (Kahns et al., 1998).

1.3.1.1 eEF1A2 in wasted mice

In 1972, the autosomal recessive mutation wasted (*wst*) arose spontaneously in the Jackson Laboratory in an inbred mouse colony (Shultz et al., 1982). Wasted mice have a 15.8 kb deletion that abolishes the expression of eEF1A2 and is therefore responsible for the mutant mouse phenotype (Chambers et al., 1998). Mice homozygous for this mutation are born completely normal but show loss of muscle bulk, tremors and gait abnormalities at 20 days of age and die by 4 weeks. Such a phenotype resembles many of the signs seen in motor neuron disease in humans, but with early onset and a quick disease progression (Chambers et al., 1998).

1.3.1.2 eEF1A2 and cancer

eEF1A2 plays an important role in carcinogenesis, possibly by acting as a tumor oncogene. eEF1A2 is overexpressed in several types of cancers. Anand *et al.* have reported that eEF1A2 is overexpressed in 30% of ovarian tumors (Anand et al., 2002). They have shown that the overexpression of Eef1a2 in the mouse fibroblasts cell line NIH-3T3 results in oncogenic activity and also increases the growth rate of ovarian cancer cells ES-2 when overexpressed in xenograft nude mice. Tomlinson *et al.* reported a similar figure where at the RNA level, 33% of ovarian tumors showed overexpression of eEF1A2 (Tomlinson et al., 2007). The authors reported that *EEF1A2* copy number does not correlate with the expression level of the gene, the

EEF1A2 gene is unmethylated in both normal and tumor DNA and that no mutations were detected. This indicates that overexpression is not dependent on genetic or epigenetic modifications at the EEF1A2 locus. The expression of eEF1A2 has also been measured using tissue microarray from 500 ovarian tumors. This showed eEF1A2 overexpression in about 30% of all primary ovarian tumors (Pinke et al., 2008).

Tissue microarray analysis showed that eEF1A2 was overexpressed in lung cancer patients. Elevated protein expression correlated with the increased level of both DNA and mRNA transcripts (Li et al., 2006; Zhu et al., 2007a). High expression of eEF1A2 correlates with increased expression of Ki-67 and is associated with poor prognosis (Li et al., 2006).

Even though little or no expression of eEF1A2 was detected in normal human pancreatic tissue, overexpression was detected in 83% of pancreatic tumors (Cao et al., 2009). Cao *et al.* have shown that overexpression of eEF1A2 promoted cell growth, survival, and invasion in pancreatic cancer.

eEF1A2 was also reported to be amplified and overexpressed in about 50% of hepatocellular carcinomas (Schlaeger et al., 2008). The same group also inhibited EEF1A2 expression using small interfering RNAs (siRNA) in two hepatocellular carcinoma cell lines; HepG2 and Hep3B. Gene silencing led to reduced cell viability and proliferation and increased apoptosis rates in those cells.

Most studies have shown that miRNAs downregulate gene expression by binding to the 3'UTR and either degrading the mRNA or binding to the mRNA and therefore inhibiting translation. However, in 2008, Dahiya *et al*. have reported the overexpression of eEF1A2 in the ovarian cancer cell line BG-1 when transfected with the miRNA let-7f (Dahiya et al., 2008).

1.3.1.3 eEF1A2 and breast cancer

It was shown that eEF1A2 is expressed at low levels in the normal breast epithelium but was overexpressed in 60% of primary human breast tumors (Tomlinson et al., 2005). This overexpression tends to be associated with ER-positive

tumors. Kulkarni *et al.* have reported a similar figure where eEF1A2 mRNA and protein were highly expressed in 50–60% of primary human breast tumors and metastases but not in normal breast epithelium (Kulkarni et al., 2007). Since it is also overexpressed in 30% of primary human ovarian tumors, transforms rodent fibroblasts and increases their tumorigenicity in nude mice, eEF1A2 is considered to be a potential human oncogene (Anand et al., 2002).

Furthermore, eEF1A2 was shown to be overexpressed in rat metastatic mammary adenocarcinoma cell lines when compared to non-metastatic cell lines (Edmonds et al., 1996). High expression level of eEF1A2 in primary breast tumors is associated with a significantly increased 20-year survival in patients when compared to those whose tumor does not express substantial eEF1A2. This is thought to be due to the effect of the high expression of eEF1A2 in reducing the aggressiveness of breast tumors (Kulkarni et al., 2007). They also reported that eEF1A2 protein overexpression predicts increased survival probability in those breast cancer patients independent of HER-2 protein expression, ER status, tumor size and lymph node involvement. Their study suggests that high eEF1A2 expression could be a significant prognostic marker of breast cancer.

In 2001, Ruest *et al.* also showed that continuous expression of eEF1A2 protects differentiated myotubes from apoptosis by delaying cell death, therefore suggesting that eEF1A2 has a prosurvival function in skeletal muscle (Ruest et al., 2002).

1.3.1.4 Effect of eEF1A2 overexpression/knockdown

eEF1A2 is a translation elongation factor that seems to be involved in different pathways and affects the expression of a number of proteins. Amiri *et al.* reported that eEF1A2 is an activator of the protein kinase Akt in a phosphatidylinositol-3 kinase (PI3K)-dependant manner (Amiri et al., 2007). The same group showed that the expression of eEF1A2 in BT549 cells stimulated the formation of filopodia in these human breast cancer cell lines and in non-transformed Rat2 cells. Filopodia production enhances cell invasion and migration and therefore

indicates the importance of eEF1A2 in promoting tumor development through phosphatidylinositol signalling, actin remodeling and cell motility (Amiri et al., 2007). However, even though Amiri *et al.* suggested the involvement of eEF1A2 in tumorigenesis, other groups reported that high eEF1A2 protein expression was associated with increased survival (Pinke et al., 2008; Ruest et al., 2002). EEF1A has been shown to be a component of the acting cytoskeleton (Edmonds et al., 1996) therefore these results correlate with the results published by Edmond *et al.* who reported that the eEF1A protein is overexpressed in rat metastatic cells compared to the nonmetastatic cells and whole tumors (discussed on page 30). In both types of tumors, eEF1A binds to F-actin but eEF1A from metastatic cells has a reduced affinity for actin. The authors reported that a weak association between eEF1A and actin could be related to the metastatic process through a different organization of the actin cytoskeleton and the differential translation of mRNAs associated with the cytoskeleton (Edmonds et al., 1996).

Even though normal mouse and human plasma cells do not express eEF1A2, it has been reported to be overexpressed in plasma cell neoplasms of both mice and humans (Li et al., 2010). Mouse plasmacytomas (PCT) expressing eEF1A2 contributes to cell transformation by cell cycle progression and inhibiting apoptosis. A previous study showed that non-PCT cell lines overexpressing eEF1A2 enhanced cell growth and resisted apoptosis (Ruest et al., 2002). Li et al. showed that knockdown of *Eef1a2* expression in PCT cell lines affected the JAK/STAT pathway and the PI3K-AKT-mTOR pathway. They reported that phosphorylation of STAT3 and AKT was delayed and that PI3K expression was reduced (Li et al., 2010). These data indicate that eEF1A2 may play an important role in the induction or progression of plasma cell neoplasms in mice and humans.

Many genes showed altered expression with the knockdown of eEF1A2. Of these genes were ones involved in tumor invasion (*Tgfbr2*, *Mmp13*, *Itgal*), proliferation (*Pik3cg*, *Fosb*, *Fos*, *Mapk1*, *Flt3*, *Wnt1*, *Cdk6*, *Ccnd2*), survival (*Ifi202b*, *Tnfsf13b*, *Bcl2*, *Bcl2l1*), and cytokine and interferon signaling (*Jak2*, *Stat1*, *Stat2*, *Stat3*, *Irf2*, *Irf8*, *Socs3*) (Li et al., 2010). Some of these genes (*JAK2*, *STAT1*, *STAT2*,

STAT3, *SOCS3*, *FYN*, *LYN*, *PIK3CG*) contain both the SH2 and SH3 domains which are the protein domains to which eEF1A2 is reported to bind (Kim et al., 1999; Panasyuk et al., 2008).

1.3.2 PTK6

Phosphate Tyrosine Kinase-6 (PTK6), also known as Brk/Sik or Brinker, is encoded by a gene consisting of 8 exons giving rise to a protein of 451 amino acids with a predicted molecular weight of 52kD. It encodes a non-receptor tyrosine kinase that contains SH2, SH3 and catalytic domains. The Src homology (SH3) domain bind proline-rich sequences and is involved in significant pathways that regulate kinase activity, protein-protein interactions and cellular localization. The SH2 domain on the other hand is essential in controlling interactions. It recognizes and binds to phosphorylated tyrosine residues. The intracellular localization of PTK6 is flexible and can be found to be present in the nucleus as well as the cytoplasm or at the membrane (Haegebarth et al., 2004). PTK6 is normally expressed in epithelial linings, prostate, skin and oral epithelium but it is not detected in the human normal mammary epithelium (Mitchell et al., 1994).

1.3.2.1 PTK6 and cancer

PTK6 expression has been detected in many different types of cancers. It is not expressed in normal ovaries but has been detected in 70% of human ovarian tumors (Schmandt et al., 2006). It has also been detected in primary and metastasized colon tumors (Llor et al., 1999), prostate tumors (Derry et al., 2003), head and neck squamous cell carcinoma (Lin et al., 2004), B- and T-cell lymphomas (Kasprzycka et al., 2006), and the HeLa cervical cancer cell line (Shen et al., 2008). *PTK6* gene expression has been detected in the cancers of the lung (Rikova et al., 2007), bladder (Ruhe et al., 2007), pancreas (Kubo et al., 2009a), and gastric cancer (Kubo et al., 2009b).

PTK6 mutations have also been detected in human melanomas (Prickett et al., 2009), human bladder carcinoma cell lines and lung cancer cell lines (Ruhe et al., 2007).

1.3.2.2 PTK6 and breast cancer

PTK6 has been found to be expressed in many breast cancer cell lines and in approximately 60% of primary human breast tumors but it has not been detected in normal human breast tissue nor in fibroadenomas (Barker et al., 1997). Like other tyrosine kinases, PTK6 phosphorylates and activates downstream substrates (i.e. Sam68, SLM-1, SLM-2, STAP-2, paxillin, STAT3, STAT5a and STAT5b) that would lead to the increased transcriptional activity and therefore mediates proliferation of breast cancer cells (Weaver and Silva, 2007).

Amplification of the *PTK6* gene has been correlated with the amplification of *ERBB2* in human breast cancers (Xiang et al., 2008). The co-amplification induces prolonged activation of the Ras/MAPK pathway and promotes cell proliferation.

Aubele *et al.* have also demonstrated that PTK6 is a prognostic marker of metastasis-free survival in breast cancer and that it is independent of the classical markers of tumor size, lymph node involvement and HER2 status (Aubele et al., 2007). This has also been reported regarding eEF1A2 where authors showed that high eEF1A2 protein expression was associated with increased probability of 20-year survival in ovarian tumors (Pinke et al., 2008).

1.3.2.3 PTK6 signaling

PTK6 plays an important role as a mediator for many signaling pathways (Figure 1.8). It can lead to activation of substrates, the EGF pathway and the PIK3/Akt pathway.

Multiple PTK6 substrates have been identified. These can be divided into the RNA-binding proteins like Sam68 (Lukong et al., 2005), SLM-1 and SLM-2 (Haegebarth et al., 2004), transcription factors such as STAT3 (Liu et al., 2006) and STAT5a/b (Weaver and Silva, 2007) and different signalling molecules that include paxillin (Chen et al., 2004), Akt (Zhang et al., 2005) and KAP3A (Lukong and Richard, 2008). PTK6 phosphorylation of reported substrates has been shown to promote cell division and migration.

PTK6 was found to phosphorylate and negatively regulate the RNA-binding protein Sam68 (Derry et al., 2000) and the Sam68-like mammalian proteins SLM-1 and SLM-2 (Haegebarth et al., 2004).

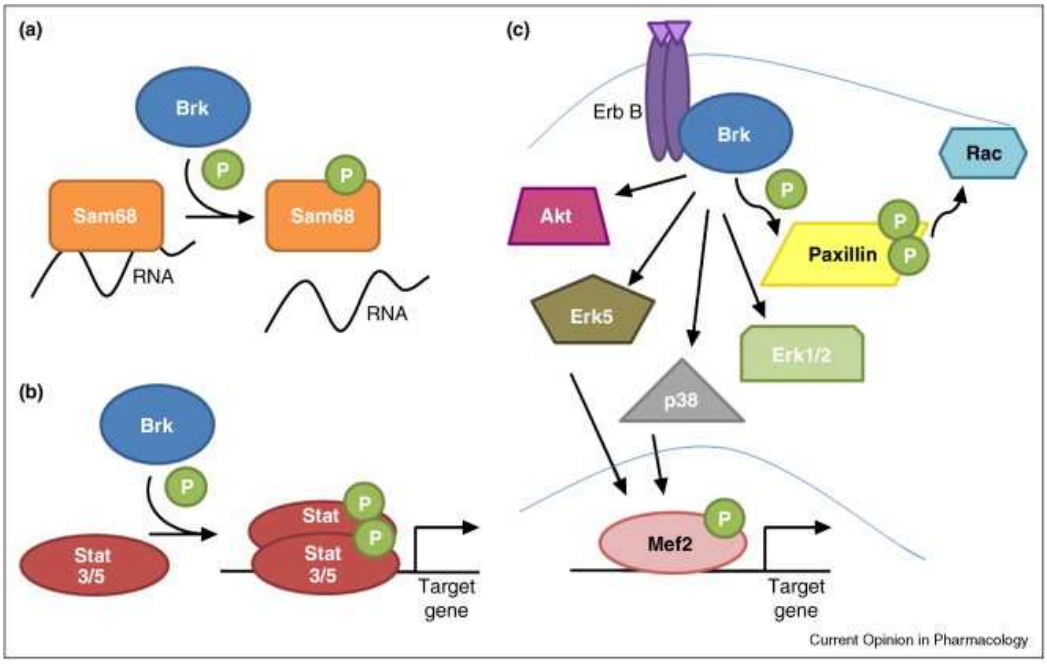

Figure 1.8 PTK6 acts as a mediator for many signaling pathways. a) Tyrosine phosphorylation by PTK6 decreases Samm68 RNA-binding activity. b) PTK6 phosphorylation enhances Stat3 and Stat5 transcriptional activity. c) PTK6 signaling downstream of ErbB receptors activates multiple signalling pathways which can lead to changes in the gene programs expressed by cells (Li et al., 2010).

Many reports have shown that the overexpression of PTK6 in normal mammary epithelial cells increase EGR-induced proliferation (Kamalati et al., 1996). It was further shown that PTK6 interacts and binds to the ErbB family members (Figure 1.8), ErbB2, ErbB3 and ErbB4 (Aubele et al., 2008). This in turn would enhance EGR-induced PTK6 autophosphorylation which then phosphorylates the downstream substrate paxillin leading to Rac1 activation and cell migration (Chen et al., 2004).

PTK6 overexpression also activates the PI3K pathway (Kamalati et al., 2000). It limits the Akt activity in normal cells but interacts with it in breast cancer cell lines leading to the activation of the PI3K/Akt pathway. As described in section 1.3.1.4, Eef1a2 knockdown in PCT cells lines also affected the PI3K/AKT pathway where phosphorylation of AKT was delayed and the expression of PI3K was reduced (Li et al., 2010).

PTK6 has also been shown to play a role in the activation of the STAT signaling pathways. The expression of PTK6 phosphorylates STAT3 and STAT5 leading to increasing transcriptional activity (Weaver and Silva, 2007).

1.3.3 EEF1A2 & PTK6 similarities

EEF1A2 and *PTK6* are two genes located on 20q13.3. Several studies reported the amplification of this region in a various number of tumors including breast cancer. *EEF1A2* and *PTK6* are less than 50kb apart and share many common properties. Both are reported to be potential oncogenes (Lee and Surh, 2009; Zhang et al., 2005), overexpressed in 60% of breast cancers (Barker et al., 1997; Kulkarni et al., 2007), they promote cell growth, migration and inhibit apoptosis by activating the PI3K/AKT pathway (Kamalati et al., 2000; Li et al., 2010) and both were reported to be associated with increased survival (Aubele et al., 2007; Pinke et al., 2008). Being on the same amplicon and sharing all the characteristics mentioned, it is hypothesized that EEF1A2 and PTK6 act in tandem through the same mechanism in breast cancer and that one of these genes could be the driver in this amplicon.

1.4 Main project aims

Many studies have shown that EEF1A2 and PTK6 are overexpressed in 60% of breast cancer patients. The aim of this project was to understand the various roles of the two genes mentioned- whether they act in tandem or independently, and how they contribute to breast cancer development and disease progression. The study focused on characterising for the first time the genomic region those two breast cancer oncogenes are located on and study their role in tumorigenesis. The ultimate aim is to identify new biomarkers and/or therapeutic targets for the treatment of breast cancer.

The main specific aims of this project are:

- To relate the copy number changes in the genes encoding EEF1A2 and PTK6 to their expression levels in human breast tumors to determine whether one of the genes is the driver in this amplicon.
- To assess the extent of the region on 20q13.3 commonly amplified in breast tumors.
- To assess the effect of eEF1A2 overexpression on PTK6 and other breast cancer associated genes.

Chapter 2: Materials & Methods

2.1 Materials

2.1.1 Buffers and solutions

All buffers and solutions used are listed in table 2.1 below.

Table 2.1 All buffers and solutions used in the methods described in this chapter

RIPA Buffer	1.752g NaCl, 2ml NP-40, 1g deoxycholic acid, 1ml 20% SDS, 6.67ml 1.5M tris pH 8.0, up to 200ml dH_2O
Phosphate Buffer Saline (PBS)	1 PBS tablet dissolved in 100ml of dH_2O and autoclaved
PBS-Tween-20 (PBS-T)	1 PBS tablet dissolved in 100ml of dH_2O and 0.1% (v/v) Tween-20 added after autoclaving
0.5M Tris HCL	3g Tris HCl, 50ml dH_2O, pH 6.8
1.5M Tris HCL	9g Tris HCl, 50ml dH_2O, pH 8.8
25% AMPS	25g Ammonium Persulfate (AMPS), 100ml dH_2O
2X Laemmli loading buffer	60nM Tris HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol
10X Laemmli running buffer	250nM Tris HCl (pH 8.3), 1.9M glycine, 10% SDS
Transfer Buffer (10x)	30.3g Tris base, 144.2g glycine, up to 1L dH_2O, pH 8.3
TBE (Tris Borate-EDTA)	108g Tris, 55g Boric acid, 9.3g Na_4EDTA in 1L dH_2O, pH 8.3
Luria-Bertani (LB) medium	1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0, medium autoclaved and allowed to cool down before adding the antibiotic
LB Agar Plates	As above with adding additional 15g/L agar before autoclaving
Peroxidase Blocking Solution	2ml 30% dH_2O_2, 10% sodium azide, up to 400ml dH_2O
10x DNA sample loading buffer	20g sucrose, 100mg of Orange G, up to 50ml dH_2O

2.1.2 Antibodies

Antibodies used in this project are mentioned in table 2.2 below along with their different concentrations for different methods. All primary antibodies are monoclonal whereas the secondary antibodies (HRP labeled) are polyclonal.

Table 2.2 List of antibodies used and conditions under which they were used for Western Blots (WB), Immuohistochemistry (IHC) and Reverse Phase Protein Array (RPPA).

Name	Specificity	Species	Source	Dilution
Western Blots (WB)				
eEF1A2-3	eEF1A2	Sheep	Helen Newbery[1]	1:2000
PTK6 (C-17)	PTK6	Rabbit	Santa Cruz Biotechnology	1:200
Serpine2 (Y-20)	Serpine2	Rabbit	Santa Cruz Biotechnology	1:200
ALDH3A1 (M-19)	Aldh3a1	Rabbit	Santa Cruz Biotechnology	1:200
TPD52 (K-17)	Tpd52	Rabbit	Santa Cruz Biotechnology	1:200
MKP-3 (C-20)	Dusp6	Rabbit	Santa Cruz Biotechnology	1:200
Egr1	Egr1	Goat	R&D Systems[2]	1:2000
GAPDH	GAPDH	Mouse	Chemicon	1:30,000
HRP Rabbit-anti-mouse	Mouse Ig	Rabbit	Dako cytomation	1:1000
HRP Goat-anti-rabbit	Rabbit Ig	Goat	Dako cytomation	1:1000
HRP Rabbit-anti-goat	Goat/Sheep Ig	Rabbit	Dako cytomation	1:1000
Immunohistochemistry (IHC)				
eEF1A2-3	eEF1A2	Rabbit	Helen Newbery[1]	1:15
PTK6 (C-17)	PTK6	Rabbit	Santa Cruz Biotechnology	1:150
Reverse Phase Protein Array (RPPA)				
eEF1A2-3	eEF1A2	Sheep	Helen Newbery[1]	1:150
eEF1A2-3 (14.1.05)	eEF1A2	Rabbit	Helen Newbery[1]	1:50

[1] Medical Genetics, Molecular Medicine Centre, University of Edinburgh
[2] Given by Gráinne Gernon, MRC Human Genetics Unit, University of Edinburgh

2.1.3 Cell lines

Experiments were done on many cell lines. Table 2.3 shows a list of cell lines used in this project.

Table 2.3 List off cell lines, species, cell type, source and media in which cells were grown.

Cell line	Species	Cell Type	Source	Medium
MCF7	Human	Breast adenocarcinoma	John Bartlett[1]	DMEM + 10% FBS
T47D	Human	Breast ductal carcinoma	John Bartlett[1]	DMEM + 10% FBS
MDA-MB-231	Human	Breast adenocarcinoma	John Bartlett[1]	DMEM + 10% FBS
MDA-MB-453	Human	Metastatic breast carcinoma	John Bartlett[1]	DMEM + 10% FBS
BT549	Human	Breast ductal carcinoma	Elad Katz[2]	DMEM + 10% FBS
HeLa	Human	Cervix adenocarcinoma	Justyna Janikiewicz[3]	Given as pellet
NIH-3T3	Mouse	Embryonic fibroblast	Yuan Cao[3]	DMEM + 10% NBCS
NIH-3T3 stable cell lines	Mouse	Embryonic fibroblast expressing eEF1A2	Justyna Janikiewicz[3]	DMEM + 10% NBCS + Zeocin

[1] Edinburgh Cancer Research UK Centre, University of Edinburgh
[2] Breakthrough Research Unit, Cancer Research Centre, University of Edinburgh
[3] Medical Genetics, Molecular Medicine Centre, University of Edinburgh

2.1.4 Primers

Oligonucleotide primers used for this project are listed in table 2.4. DNA primers were used for gene copy number detection while cDNA primers were used for gene expression analysis. Primers were designed using the primer design tool Primer3 unless clearly stated and purchased from Sigma-Aldrich. All primers were stored in TE buffer in -20^{o}C at a concentration of 100µM and were diluted to 0.5µM in dH_2O as a working dilution unless otherwise specified.

Table 2.4 List of primers' names and sequences used in RT-qPCR

Target	Primer	Sequence 5' to 3'	Product	Source
DNA Primers				
EEF1A2	EEF1A2-5F	GGCATCTCTGTGTGCACTGT	134bp	Designed
	EEF1A2-5R	TACTGGGTCTCCACCCTCAC		
PTK6	PTK6F2-7	GTCCCCACTGTCCCTGACT	144bp	Designed
	PTK6-R2-7	CACACCTGCACCATTCTCTG		
KCNQ2	KCNQ2-2F	GCTGTAACTCAGCCCTCACC	129bp	Designed
	KCNQ2-2R	TCTCCGGCTCCAGAGATAAA		
SRMS	SRMS-3F	AGTGGGGAAAGGAGACCTGT	149bp	Designed
	SRMS-3R	CAGGTGCAGCATCTTGTGAT		
D5S478	D5S478-F	CCGTTTGCACATTTGAGGCT	121bp	Ginzinger et al., 2000
	D5S478-R	CTGAAAAAGAAGGCAACGTC		
D11S913	D11S913-F	CTAGATTTCTGAAACTCAAATGCA	164bp	Ginzinger et al., 2000
	D11S913-R	TAGGTGTCTTATTTTTTGTTGCTT		
cDNA Primers (Human)				
EEF1A2	EEF1A2	AGGACGTGTACAAGATTGGC	155bp	Tomlinson,(Tomlinson 2007
	EEF1A2	CACCTCAGTGGTGATGTTCA		
PTK6	PTK6 qPCR	GGACTTCGGGTTAGCCAGG	118bp	Zhao et al., 2003
	PTK6 qPCR	GGATTTGGTGGAGTAATGGCC		
PUM1	PUM1qPCR	TGAGGTGTGCACCATGAAC	187bp	Szabo et al., 2004
	PUM1	CAGAATGTGCTTGCCATAGG		
TBP	TBP qPCR	TGCACAGGAGCCAAGAGTGAA	132bp	Yamagata et al., 2009
	TBP qPCR	CACATCACAGCTCCCCACCA		
cDNA Primers (Mouse)				
Eef1a2	Q m1A2 F	GCTCCAGGACACCGAGACTT	141bp	Justyna Janikiewicz[1]
	Q m1A2 R	GAGTGCGTGTTCCCGGGTT		
Ptk6	mPTK6 F	CTGGCTTCTTCCTCCCTTCT	118bp	Designed
	mPTK6 R	TGTGGACAGTCAAGCTCCAA		
Serpine2	Serpine2 F	CATCAAGTCACGGCCTCATG	112bp	Designed
	Serpine2 R	CGTGGAGAGCTGCTTCTTTGT		
Egr1	Egr1 F	AACACTTTGTGGCCTGAACC	118bp	Designed
	Egr1 R	AGGCAGAGGAAGACGATGAA		
Aldh3a1	Aldh3a1 F	CCCCTGGCACTCTATGTGTT	116bp	Designed
	Aldh3a1 R	GTGGGCACAGTGATGTGAAC		
Tpd52	Tpd52 F	CCTCGCTTCAGGAGTTCAAG	138bp	Designed
	Tpd52 R	CTGAGCCAACCGATGAAAAT		

Dusp6	Dusp6 F	GGCAAAAACTGTGGTGTCCT	106bp	Designed
	Dusp6 R	CATCGTTCATGGACAGGTTG		
Tbp	mTBP F	CCCCACAACTCTTCCATTCT	103bp	Justyna Janikiewicz[1]
	mTBP R	GCAGGAGTGATAGGGGTCAT		
B2M	mB2MG F	CATGGCTCGCTCGGTGACC	166bp	Justyna Janikiewicz[1]
	mB2MG R	AATGTGAGGCGGGTGGAACTG		
18s rRNA	m18S F	CGGACAGGATTGACAGATTG	89bp	Justyna Janikiewicz[1]
	m18S R	CAAATCGCTCCACCAACTAA		
miRNA Primers				
Let-7f	Let-7f FP	GCCCCTGAGGTAGTAGATTGTATAGTT	-	Invitrogen
U6	U6 FP	CGCTTCGGCAGCACATATAC	48bp	Perkins et al., 2007
	U6 RP	TTCACGAATTTGCGTGTCAT		2007

[1] Medical Genetics, Molecular Medicine Centre, University of Edinburgh

2.2 Methods

2.2.1 Western Blots

2.2.1.1 Preparation of protein lysates

Protein lysates from cells were prepared from cells plated in 10cm^2 culture plates. Media was removed from the cell culture plate and the cells washed with 10ml of pre-chilled PBS twice. The plate was put on ice and 1ml of pre-chilled RIPA buffer was added for five minutes. Cells were harvested with a cell scraper and disrupted using vortex. After 10 minutes on ice, cells were centrifuged at 12,000rpm at 4°C for 20 minutes. Supernatant was transferred into a new tube and stored at -20^{o}C. The sample was heated at 100^{o}C for 10 minutes before loading to denature the proteins and disrupt protein-protein interactions.

2.2.1.2 Protein concentration measurement

The concentration of total protein lysates was measured using the Lowri method using the Protein Assay Kit from BioRad following the manufacturer's protocol. Before measuring the lysate concentration, a standard curve of a serial dilution of BSA from 0 to 4.0mg/ml in RIPA buffer was carried out. Reagent A' was created by adding 20µl of reagent S to 1ml of reagent A. In triplicate, 10µl of each dilution was added to a 1.5ml tube along with 50µl of reagent A' and mixed. Next, 200µl of reagent B was added to each tube and kept in room temperature for 15 minutes. Absorbance readings were measured at 750nm and plotted against the protein concentration to create a standard curve. This curve was then used to determine the protein concentrations of all protein lysates.

2.2.1.3 SDS-PAGE gel electrophoresis

The BioRad Mini TransBlot Cell apparatus was used according to the manufacturer's protocol. First, a 10% acrylamide separating gel (enough for two gels) was made as follows:

30% Acrylamide	5.2ml
1.5M Tris pH 8.8	4ml
dH_2O	6.68ml
20% SDS	80µl
TEMED	10µl
25% AMPS	40µl

The gel components were mixed and poured in between two BioRad glass plates. The separating gel was overlaid with dH_2O to disperse bubbles and prevent the upper part of the gel from drying out. It was kept for 30 minutes to set. The water was removed and replaced by the 4.3% stacking gel as follows:

30% Acrylamide	1.45ml
0.5M Tris pH 6.8	2.5ml
dH_2O	5.95ml
20% SDS	50µl
TEMED	5µl
25% AMPS	50µl

The stacking gel was then poured on top of the separating gel, 10 or 15 wells combs (BioRad) were inserted and the gel was allowed to set for 15 minutes. The plates were placed in the gel tank apparatus which was filled with 1x Tris-Glycine-SDS buffer (BioRad). The combs were removed from the gels and the wells were washed out with a pipette. 20µg of protein was mixed with 2x Laemmeli loading buffer and loaded into the wells along with a protein size marker (Fullrange Rainbow - BioRad). The gels were then run at 120V for about 45 minutes.

The gel was removed from the plates and soaked in transfer buffer along with two filter papers (Whatman) and two sponges. A Hybond-P PVDF membrane (Amersham) was soaked in methanol and then soaked in transfer buffer. A sponge, one piece of filter paper, the membrane, the gel, another piece of the filter paper and the second sponge were placed on the transfer cell and assembled into the apparatus. The tank was filled with the transfer buffer and run at 400A at 4^{o}C for one hour in order for the proteins and protein marker to transfer from the gel to the membrane. After an hour, the blots were removed and the non-specific sites on the membrane were blocked with 5% powdered milk in PBS-T overnight at 4^{o}C.

2.2.1.4 Western Blotting

After blocking, the membranes were incubated for one hour with the primary monoclonal antibody and 5% milk at room temperature. The membranes were then washed 3 times, each for 10 minutes with PBS-T. This was followed by incubating the membranes with the HRP-labeled secondary antibody (dilution shown in table 2.2) in 5% milk for one hour at room temperature. The membranes were again washed 3 times for 10 minutes with PBS-T. Protein detection was performed using the enhanced chemiluminescence (ECL) detection kit (Amersham).

2.2.2 Immunuhistochemistry (IHC)

2.2.2.1 Tissue MicroArrays (TMAs)

To visualize the expression pattern of both EEF1A2 and PTK6 in breast tumors, commercial tissue arrays of both normal human breast tissues and cancers (AccuMax Array, Stretton Scientific) were stained with different monoclonal antibody dilutions before deciding on the best dilution shown in table 2.2. This was followed by performing IHC on breast tumor tissue arrays in triplicate from 300 patients obtained from the tissue bank provided by John Bartlett at the Edinburgh Cancer Research UK Centre, University of Edinburgh.

2.2.2.2 IHC on breast tissue arrays

At room temperature, parrafin embedded sections of human breast tumors were deparaffinised with xylene, rehydrated in 75% and 100% ethanol and microwaved in citric acid pH 6. Slides were washed in dH_2O, treated with 3% hydrogen peroxidase, washed with PBS and loaded into a sequenzer. Three drops of Protein Block Serum-Free (Dako) were added and kept for 10 minutes. 100µl of the primary antibody was added overnight at the dilution mentioned in table 2.2. Slides were washed with PBS for five minutes, incubated in three drops of Biotinylated Link Universal (Dako) for 30 minutes, washed with PBS and incubated in Streptavidin-HRP (Dako) for another 30 minutes. After washing with PBS for five minutes, the slides were removed from the sequenzer and treated with 3, 3-Diaminobenzidine (DAB) for two minutes. Finally, the slides were washed in dH_2O, counterstained in haematoxylin, stained with lithium carbonate, dehydrated in 100% and 75% ethanol, cleared in xylene and mounted in pertex. The slides were left to dry overnight and sections were viewed by light microscopy using the DP software (Olympus) on Olympus BX51.

2.2.2.3 Immunohistological scoring

Cores on the breast tumors TMAs were scored based on the protein expression as shown below:

Expression	Histoscore	Intensity
Negative	0	0
Weak	1-100	1
Moderate	101-200	2
Strong	201-300	3

Sections were histoscored using a score of 1 to 3 for staining intensity multiplied by the percentage of tumor tissue staining, therefore giving a maximum score of 300 per core. Blind scoring was carried out by two researchers.

2.2.2.4 Statistical Analysis

After scoring all the breast TMAs, histoscore analysis was carried out to check for the correlation between EEF1A2 and PTK6 using the SPSS analytical program (IBM). A two sample paired t-test was carried out as it has greater power when compared to unpaired tests. P values of 0.05 or less were considered to be statistically significant. A pearson correlation of 0.3 – 0.5 would signify a positive moderate association whereas a correlation of 0.6 – 1.0 would signify a positive strong association.

2.2.3 RT-PCR

2.2.3.1 mRNA extraction from cell lines

Cells were washed with PBS, trypsinised and centrifuged. The supernatant was removed and cells were stored in -70^{o}C until used. RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol.

2.2.3.2 miRNA extraction from cell lines

Total RNA (miRNA and mRNA) was extracted from peletted cells using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's protocol.

2.2.3.3 RNA concentration measurement

Concentration of RNA was measured using the NanoDrop™ 1000 spectrophotometer (Thermo Scientific). A nanodrop is a spectrometer that passes a beam of narrow-band light through a droplet of water and measures the intensity of light transmitted through the sample. It measures the absorbance reading at 260nm giving an RNA concentration in ng/µl. The ratio of absorbance at 260nm and 280nm is used to assess the purity of RNA. A ratio of ~2.0 is generally accepted as "pure" for RNA. RNA concentration of 10ng/µl was used as a working dilution.

2.2.3.4 cDNA synthesis

cDNA was produced from RNA using two different kits depending on the experiment it will be used in. To produce cDNA for mRNA reactions, the First Strand cDNA Kit (Roche) was used as follows:

Reaction	+RT	-RT
RNA	10ng	10ng
10X reaction buffer	2µl	2µl
25mM $MgCl_2$	4µl	4µl
dNTP mix	2µl	2µl
Oligo (dT)	2µl	2µl
RNase Inhibitor	1µl	1µl
AMT Reverse Transcriptase	0.8µl	-
RNase free dH_2O	To 20µl	To 20µl

The reaction is kept at room temperature for 10 minutes, followed by 42^{o}C for 1 hour and then 5 minutes at 99^{o}C. The reaction was kept at -20^{o}C until used.

As for cDNA synthesis from miRNA, the NCode VILO miRNA cDNA Synthesis Kit (Invitrogen) was used as per manufacture's protocol. The following reaction was carried out:

5X Reaction Mix	4µl
10X SuperScript Enzyme Mix	2µl
Total RNA (10ng)	Xµl
DEPC-treated water	To 20µl

The reaction mix was gently mixed by vortexing and centrifuged briefly to collect the contents. It was incubated at 37°C for 60 minutes and terminated at 95°C for 5 minutes. The cDNA was then stored at -20°C until used.

2.2.4 Patient Samples

2.2.4.1 DNA extraction from Paraffin Embedded Tissue (PET)

Breast tumors were obtained from the tissue bank provided by John Bartlett, PET sections was cut by Ria Kishen and the DNA extraction protocol was provided by Vicky Sabine, all from the Edinburgh Cancer Research UK Centre, University of Edinburgh. PET samples were cut and put in 1.5ml tubes. 200µl of 5% Tween 20 was added to the 1.5ml tube containing the sample and spun briefly to collect the material at the bottom of the tubes. To remove the paraffin wax, the tubes were agitated by inverting them and heating them to 90°C for 10 minutes in a heating block. Tubes were cooled to 55°C by transferring them to a separate heating block for five minutes. 4µl (10mg/ml) of Proteinase K was added to the mixture and incubated at 55°C for one hour. This step was repeated four times. Next, 4µl (10mg/ml) of Proteinase K was added again and incubated at 55°C overnight. 300µl of 1X PBS was added and mixed by vortexing for ten seconds. The mixture was heated to 99°C for ten minutes in a heating block. The hot tubes were transferred briefly to ice to cool, placed into a microfuge at 4°C and spun at 10,500rpm for 15 minutes. After spinning, the sample was placed on ice again for five minutes to further harden the paraffin wax. The liquid was aspirated from below the now hard wax layer and transferred to a clean pre-labelled 1.5ml centrifuge tube. Next, the DNA extraction was carried out using the QIAamp DNA Mini Kit (QIAgen) as per the manufacturer's protocol and stored at -20°C.

2.2.4.2 DNA concentration measurement

Measuring DNA concentration was carried out the same way as the RNA (section 2.2.3.3). A 260/280 ratio of ~1.8 is generally accepted as "pure" for DNA. DNA concentration of 10ng/µl was used as a working dilution.

2.2.5 Real-Time PCR

The double delta Ct (ΔΔCt) method (Livak and Schmittgen, 2001) was used to detect the gene copy number for EEF1A2, PTK6, SRMS and KCNQ2 and the expression level of several genes using the Bio-Rad MyiQ iCycler (Discussed in section 3.2.3). The amplification efficiency was first determined on the breast cancer cell lines MCF7, T47D, MDA-MB-231 and MDA-MB-453 (Standard curves shown in Appendix A). Primers with efficiencies between 90% and 110% were used for the copy number analysis as well as detecting the expression level. As shown in table 2.4, primers were designed to amplify a product of the size between 100-200bp with no secondary structure and no primer-dimer formation. All genes were normalized against two reference genes (copy number analysis) or three reference genes (expression level analysis). Normal human blood (provided by Pippa Thomson at the Molecular Medicine Centre, Edinburgh) was used as a control for the copy number analysis as it should contain normal two copies of the genes studied. Samples were done in triplicate and the mean Ct value was taken for the final analysis. For the qPCR reaction, the DyNAmo Flash SYBR Green qPCR Kit (New England Biolabs) was used as follows:

Master Mix	10µl
Forward Primer	0.5nM
Reverse Primer	0.5nM
DNA or cDNA	10ng
dH_2O	To 20µl

Normal control DNA was extracted and provided by Pippa Thomsons. It was extracted from 10ml whole blood using a Nucleon™ BACC Genomic DNA Extraction kit (GE Healthcare Life Sciences).

The cycling program used is shown below:

Step	Cycles	Temperature	Time
Step 1	1x	95°C	5 minutes
Step 2	40x	95°C	45 seconds
		60°C	30 seconds
		72°C	30 seconds
Step 3	1x	95°C	1 minute
Step 4	1x	60°C	1 minute
Step 5	80x	60°C	10 seconds

2.2.6 Cell Transfection

2.2.6.1 Cell culture and cell count

Cells were grown in T175cm^2 flasks at 37°C in a 5% CO_2 incubator to a confluency of around 80%. The media was aspirated, cells washed with PBS and 5ml trypsin was added. After 2-3 minutes, the medium was added and the cell suspension was transferred into a 50ml Falcon™ tube. Cell suspension was centrifuged at 2000rpm for 5 minutes. Supernatant was aspirated, 5ml media was placed in a 50ml tube and it was added and mixed with the cells. For cell counting, 200µl of the mixture was placed into the counting chamber of the cell counter Cellometer Auto T4 (Nexcelom).

2.2.6.2 EEF1A2 construct preparation

To produce bacteria containing the EEF1A2 construct, 80mls LB agar was heated in the microwave for 6 minutes and left to cool for 15 minutes, 2µl of Zeocin (100mg/µl) was then added. 10mls of the LB agar with Zeocin was poured into 10cm^2 plates and left to cool. The bacteria containing the pcDNA3.1/GS-EEF1A2 construct from Invitrogen (Figure 2.1) was placed on ice. A loop was dipped in the frozen bacteria and spread on the LB agar with Zeocin plate and the plate was sealed with a paraffin tape. Plates were incubated at 37^{o}C overnight. 4mls of LB with 1µl of Zeocin (100mg/µl) was placed in a 10ml tube. One colony from the plate was picked and inoculated the 4ml LB starting culture. This was then incubated at 37^{o}C with shaking at 180rpm for two hours in an Innova 4300 incubator/shaker (New Brunswick Scientific). 150mls of LB with 37.5µl Zeocin (100mg/µl) was prepared for the next step. To dilute the starting culture 1:1000, 4µl of it was added to the 150ml LB/Zeocin and left in the shaker overnight. A maxi prep was then performed using the HiSpeed Plasmid Maxi Prep Kit (QIAgen) to produce high copy plasmid DNA as per the manufacturer's protocol. The presence of the EEF1A2 DNA construct was confirmed by loading 5µl of DNA with 5µl of loading buffer on a 0.8% agarose gel. The correct band size was detected (Figure 2.2). The DNA was stored at -20^{o}C. Both BT549 and NIH-3T3 cells were transfected with the EEF1A2 DNA construct.

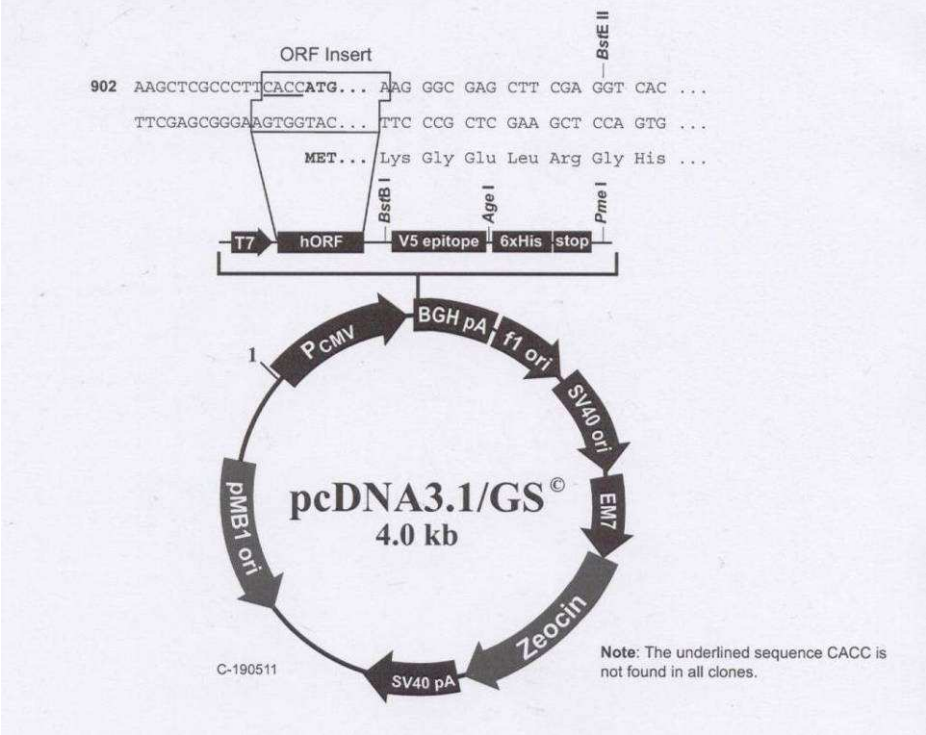

Figure 2.1 EEF1A2 expression construct (Invitrogen). Vector pcDNA3.1/GS for expression in mammalian cells includes the CMV promoter and enhancer sequences for stable expression; and the Zeocin antibiotic for selection of bacterial transformants and of stably transfected mammalian cells. Vector also includes the V5 epitope tag after the insert to allow detection of proteins for which antibodies are not available, a carboxy-terminal polyhistidine tag $(His)_6$ to allow metal-affinity protein purification, and the T7 promoter to enable in vitro transcription and translation of the target gene.

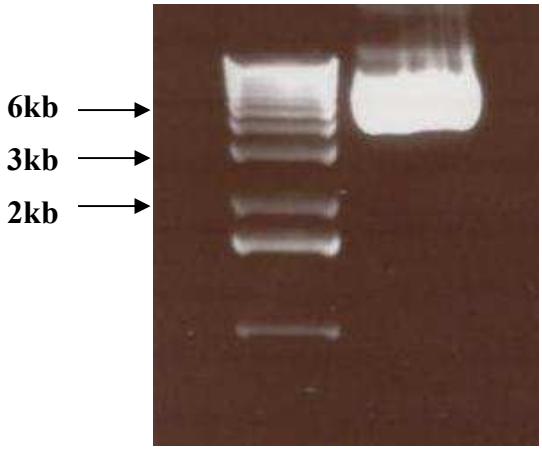

Figure 2.2 Agarose gel showing the 6kb EEF1A2 expression construct band. 3µl of the EEF1A2 construct was loaded to determine the correct band size after extraction.

2.2.6.3 Let-7f miRNA

BT-549 cells were transfected with the microRNA Let-7f to check if the EEF1A2 expression level is affected by the presence of Let-7f. The mature hsa-let-7f was purchased from Invitrogen. 6µl (30nM) of let-7f was used for each reaction.

2.2.6.4 Transfection using a nucleofector

A nucleofector (Amaxa) was used for the transfections into BT549 and NIH-3T3 cells. BT549 cells were transfected with the EEF1A2 DNA construct to check for the change in PTK6 expression. BT549 cells were also transfected with let-7f miRNA to check for its effect on EEF1A2. NIH-3T3 cells were transfected with the EEF1A2 DNA construct to check for its effect on the expression level of PTK6 and the expression level of the top genes in the microarray study (chapter four): Serpine2, Egr1, Aldh3a1, Tpd53 and Dusp6. After cell counting (section 2.2.6.1), 1 x 10^6 cells were centrifuged at 900rpm for 5 minutes and the pellet resuspended in 300µl of the appropriate nucleofector solution (Amaxa) to perform the experiment in triplicate. BT549 cells were suspended in solution T whereas NIH-3T3 cells were suspended in solution R as recommended and provided in the Cell Line Nucleofector® Kit (Amaxa). 10µl/reaction (3µg) of DNA vector was added to 100µl of the solution which was transferred to a cuvette. Cells were electroporated in the nucleofector using the appropriate program. Program A-023 was used for BT549 whereas program U-030 was used for NIH-3T3 cells. These are programs that have been pre-configured by Amaxa. The cuvette was removed immediately after electroporation from the nucleofector and 500µl of medium was added. The cells were then transferred with a pipette into a 10cm^2 plate containing 10ml of medium. The same was done with the remaining 200µl of solution as every plate required 3 reactions. Controls included cells with no electroporation and cells with the nucleofector solution but lacking DNA.

2.2.7 Microarrays

2.2.7.1 RNA preparation

Total RNA was extracted using the AllPrep DNA/RNA/Protein Mini Kit (QIAgen) according to the manufacturer's instructions. An aliquot of 150ng of total RNA was converted to single-stranded cDNA using an oligo (dT) tagged with a phage T7 promoter to enable in vitro transcription (Ambion). Single-stranded cDNA was converted to produce double-stranded cDNA. This was followed by transcription *in vitro* where the now cRNA is amplified and labelled by an *in vitro* transcription reaction that incorporates biotin UTP. This was done using the TotalPrep RNA Amplification kit (Ambion) recommended by Illumina. All twelve RNA samples were quantified and monitored with the Agilent 2100 Bioanalyzer (Agilent) to check the high-quality RNA.

2.2.7.2 Sample processing

Samples were processed and data acquired by Louise Evenden at the Wellcome Trust Clinical Research Facility/Genetics Core of Edinburgh University using two mouse WG-6 v2.0 Expression BeadChips for the analysis of more than 47,000 probes. From each of the twelve samples, 1.5µl of labelled cRNA was aliquoted into 0.2µl tubes prior to the Agilent procedure. Once the concentrations are measured, decision is made whether to dilute or concentrate down the sample to get the 1500ng (10µl of 150ng/µl) needed for the Mouse WG-6 arrays. The sample was diluted or concentrated down and resuspended with RNase free water (SIGMA). The sample needed is 10µl of 150ng/µl and this is what was used for the Gene Expression protocol. The twelve cRNA targets were hybridised to the BeadChips, which were scanned on an Illumina BeadStation. Image processing and raw data extraction were performed using the Illumina Beadstudio software. A number of pre-processing steps including background adjustment, probe filtering, normalization and quality control checks were performed using the R package, lumi (www.bioconductor.org). Boxplots

of the microarray data before and after quantile normalization demonstrate how the distributions of the intensities are scaled to have equal means and variances across samples (Chapter 4, figure 4.6). To determine pathways affected by the overexpression of EEF1A2, the "DAVID pathway viewer" tool was used (Chapter 4, section 4.3.1).

2.2.8 Reverse Phase Protein Array (RPPA)

The protocol for the RPPA was created by Peter Mullen from the Breakthrough Research Unit, Edinburgh.

2.2.8.1 EEF1A2 antibody optimization

2.2.8.1.1 EEF1A2 antibody incubation

A FAST-slide (Whatman) was placed in a suitable container in x1 Whatman wash buffer for five minutes on a rocking platform (All buffers and reagents were provided by Peter Mullen). Wash buffer was replaced with LiCor Blocking Buffer diluted 50:50 in PBS and incubated for 1 hour at room temperature on a rocking platform. Two monoclonal EEF1A2 antibodies were tested: one raised in sheep and the other in rabbit. 125µl of each of the EEF1A2 primary antibodies was prepared in LiCor Blocking Buffer diluted 50:50 in PBS at the serial dilutions: 1:50, 1:100, 1:250, 1:500 and 1:1000 and kept on ice. Slides were mounted in the FAST Frame slide holder (Whatman) so that a tight seal is formed between the slide and the incubation chamber. Residual buffer was removed from wells and 80µl of the EEF1A2 antibodies was added to the respective wells. Slide and chamber were placed into a sealed wet box and incubated on a rocking platform overnight at 4^{o}C.

2.2.8.1.2 Secondary antibody incubation

At room temperature, the EEF1A2 antibodies were carefully removed from each well. 80µl of PBS-T was added to the wells and slides were washed on a rocking platform at room temperature for five minutes. The wash was repeated three times. The fluorescently-labelled polyclonal secondary antibodies were prepared by diluting them in Odyssey Blocking Buffer (diluted 50:50 in PBS) with 0.01% SDS at 1:2000 dilution (1µl/2ml). The sheep-derived EEF1A2 antibody is detected using an anti-goat fluorescently-labelled secondary antibody (either 680nm or 800nm wavelength) whilst the rabbit-derived EEF1A2 antibody is detected using an anti-rabbit fluorescently-labelled secondary antibody (either 680nm or 800nm wavelength). By combining a sheep primary and a rabbit primary along with their respective secondary antibodies (one of 680nm and the other of 800nm), dual-labelled RPPA Fast-Slides can be obtained. After preparing the secondary antibodies, the buffer was removed from the wells, 80µl of the fluorescently-labelled secondary antibodies were added to the respective wells and incubated at room temperature in the dark for 45 minutes with gentle shaking. Secondary antibodies were removed from the wells and briefly washed three times in 80µl PBS-T at room temperature. Slides were further washed in excess PBS-T for 15 minutes in the dark. PBS-T was removed and the membrane was washed with PBS at room temperature in the dark for 15 minutes to remove residual Tween 20. FastSlides were dried in a 50^{o}C oven for ten minutes and then scanned on the Li-Cor Odyssey scanner.

2.2.8.1.3 Determining efficient antibody dilutions for RPPA

Based on the results of the antibody optimization, it was decided that the anti-sheep EEF1A2 should be used at a dilution of 1:150 whereas the anti-rabbit EEF1A2 should be used at a dilution of 1:50.

2.2.8.2 RPPA protocol

2.2.8.2.1 EEF1A2 antibody incubation

A FAST-slide (Whatman) was placed in a suitable container in LiCor Blocking Buffer diluted 50:50 in PBS and incubated for 1 hour at room temperature on a rocking platform. EEF1A2 antibodies were prepared in LiCor Blocking Buffer diluted 50:50 in PBS at the desired concentrations and kept on ice. Slides were mounted in the Fast Frame slide holder (Whatman). Residual buffer was removed from wells and 700µl primary antibody was added to the respective wells. Slides and chamber were placed into a sealed wet box and incubated on a rocking platform.

2.2.8.2.2 Secondary antibody incubation

At room temperature, the EEF1A2 antibodies were carefully removed from each well. 700µl of PBS-T was added to the wells and slides were washed on a rocking platform at room temperature for five minutes. The wash was repeated three times. The fluorescently-labelled secondary antibodies were prepared by diluting them in Odyssey Blocking Buffer (diluted 50:50 in PBS) with 0.01% SDS at 1:2000 dilution (1µl/2ml). The buffer was removed from the wells, 700µl of the fluorescently-labelled secondary antibodies were added to the respective wells and incubated at room temperature in the dark for 45 minutes with gentle shaking. Secondary antibodies were removed from the wells and briefly washed three times in 700µl PBS-T at room temperature. Slides were further washed in excess PBS-T for 15 minutes in the dark. PBS-T was removed and the membrane was washed with PBS at room temperature in the dark for 15 minutes to remove residual Tween 20. FastSlides were dried in a 50^{o}C oven for ten minutes and then scanned on the Li-Cor Odyssey scanner.

2.2.8.3 RPPA analysis

All data obtained from the scanned membrane were analyzed on Microsoft Excel.

Chapter 3: EEF1A2 & PTK6 expression in breast cancer cell lines/tumors

3.1 Introduction

Clinical parameters are mostly assessed and analyzed for the diagnosis or prediction of breast cancer outcome. This is usually done by either FISH or IHC on tumor TMAs. Protein expression is correlated with clinical markers such as grade and size of tumors, ER status, PR status, HER-2 status lymph node involvement, treatment modality and disease free survival which are typically assessed. Retrospective studies can be performed on archival breast cancer samples from patients with long follow-up and then compared to the protein expression. Overexpression is usually the result of gene amplification. However, several studies have reported protein overexpression without amplification. This might be due to the role miRNAs play in the translation process as intermediates in different types of cancers, but many other causes are possible.

In this part of the project, I focused firstly on the clinical correlations in breast cancer in regards to EEF1A2 and PTK6. Clinical correlations were assessed for each protein separately on breast tumor TMAs. Secondly, I analyzed the copy number of both genes to further understand the correlation between their protein overexpression and gene amplification. Finally, the role of miRNAs in regards to EEF1A2 was assessed in a breast cancer cell line.

3.2 Results

3.2.1 Significant correlation between eEF1A2 and PTK6 in breast cancer TMAs

Concentrations of the EEF1A2 and PTK6 antibodies were first optimized for IHC using different dilutions on breast cancer cell lines, normal human breast tissues and tumor sections. EEF1A2 antibody concentration was decided at 1:15 whereas the

concentration for PTK6 was 1:150. In order to assay the level of expression of both eEF1A2 and PTK6 in breast cancer, around 300 different breast tumors on two slides were stained in triplicate for eEF1A2 and another set of the same identical TMA slides were stained for PTK6 (i.e core A1 for example was scored 6 times on identical TMA slides; 3 times for eEF1A2 and 3 times for PTK6). As shown in figure 3.1, slide A has 146 primary breast tumors from patients treated with chemotherapy after the biopsy was taken whereas slide B has 152 primary breast tumors from patients treated later with both chemotherapy and hormonal treatment.

Figures 3.2 and 3.3 Show the breast conservation series array maps for both slides A (breast tumors from patients treated with chemotherapy only after the biopsy was taken) and slides B (breast tumors from patients treated with both chemotherapy and hormonal therapy after the biopsy was taken).

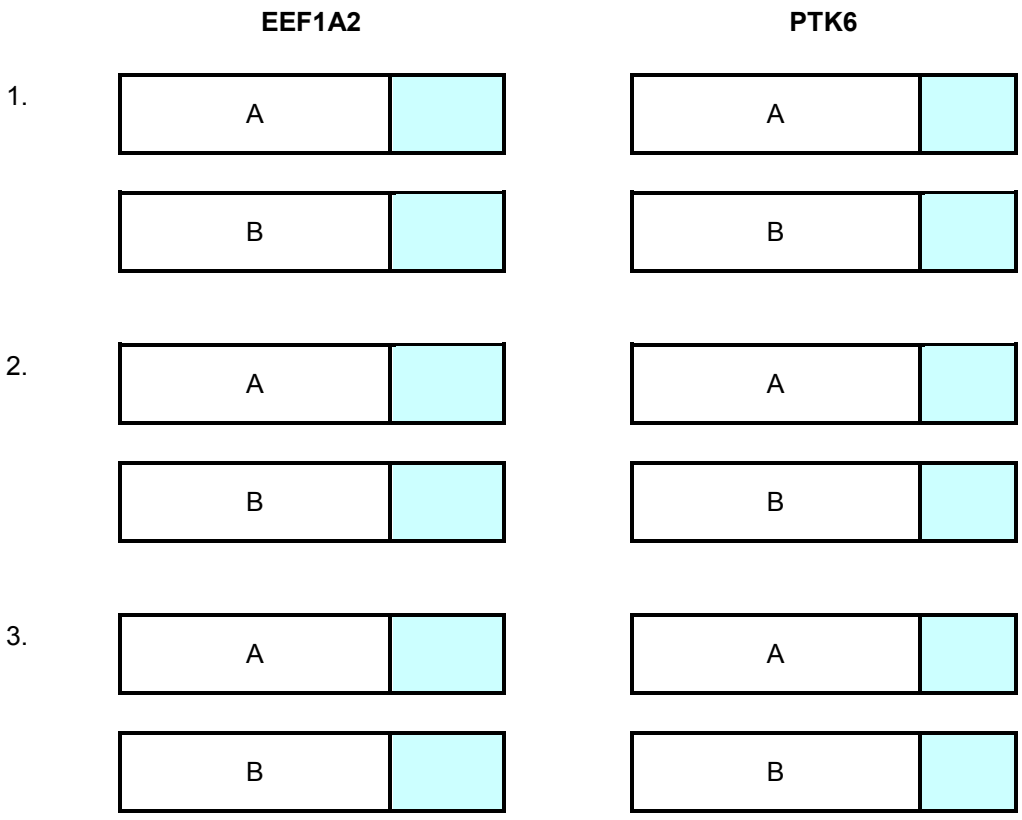

Figure 3.1 Breast conservation series array maps of both EEF1A2 & PTK6. Slide A has 146 breast tumors from patients treated with chemotherapy only whereas slide B contain 152 breast tumors from patients treated with both chemotherapy and hormonal therapy after biopsy was taken.

I	275	1641															
H	554	161	1493	986	476	1646	276	1637	569	1652	225	228	351	501	182	148	1380	1520
G	543	535	385	567	521	537	529	598	99	215	212	190	77	323	337	443	448	619
F	469	284	457	194	192	174	245	255	390	386	586	570	589	311	326	257	282	627
E	1112	1586	901	1271	115	1333	230	238	236	207	314	317	218	418	445	98	499	471
D	1478	1501	1491	1387	1455	1413	1628	735	670	725	1602	1521	1572	1119	1792	708	1664	1687
C	1562	989	1054	1540	1590	1599	1561	1708	1789	1622	665	1781	1773	1760	1604	1519	1511	1477
B	1381	1247	1284	1293	820	783	1750	1751	1722	1679	1692	1701	1702	1705	1712	1373	969	970
A	1075	1077	1088	1104	1118	1126	1146	1151	1127	1213	1215	1216	1224	1238	1314	1312	1315	1259
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**

Figure 3.2 Breast conservation series array map. A map of slide A which shows 146 breast tumors from patients treated with chemotherapy alone after the biopsy was taken.

I	1759	1611	1272	1607	1037	966	1153	1286										
H	1345	1102	1209	1115	682	1438	1796	1101	1459	1617	1166	1465	1623	1419	1518	1063	1047	424
G	1564	1566	1557	1565	1507	1499	1495	1334	1388	1443	1417	1406	1417	1391	1429	1452	1440	1445
F	1574	1783	1806	1807	1763	1658	1661	1719	1665	1768	1771	1655	1331	1592	1589	1582	1581	1546
E	1433	867	1747	1753	1756	1757	1685	1726	1728	1730	1731	1737	1735	1468	1500	1472	1617	1482
D	1250	1246	869	890	897	881	809	1688	1669	1672	1674	1711	1691	1693	1698	1485	1476	1618
C	1244	1302	1317	1319	1323	1377	1359	1368	1372	1370	1254	882	1711	1699	1729	900	1752	1643
B	1103	1109	1125	1130	1156	1158	1169	1180	1185	1187	1201	1203	1204	1219	1225	1228	1239	1241
A	953	956	993	999	1021	1023	1029	962	967	974	1035	1137	1086	1067	1026	1139	1096	1098
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**

Figure 3.3 Breast conservation series array map. A map of slide B which shows 152 breast tumors from patients treated with both chemotherapy and hormonal therapy after the biopsy was taken.

IHC was performed as described in section 2.2.2. Each core on the TMA slide was then scored for the intensity of the expression level in the cytoplasm. Cores were scored by two researchers using a histoscore method which involves scoring the intensity at a level of 0 to 3 and then multiplying it by the percentage of tumor cells stained at that level; stromal tissue was discounted. This should give a maximum score of 300. Cores were then assigned to a category based on their histoscores. Tumors with a histoscore of 0 were considered "negative", 1-100 were defined as "weak", 101-200 were defined as "moderate" and histoscores of 201-300 were labeled as tumor with a "strong" expression (Figure 3.4). Since scoring was done in triplicate, the average of the 3 histoscores was calculated to get the final score for the expression level of each tumor on the TMA slide.

Differences in the histoscores between eEF1A2 and PTK6 were analysed by Student's t-test and showed a significant correlation (*p*-value 0.006) between the two proteins in these 300 breast tumor TMAs.

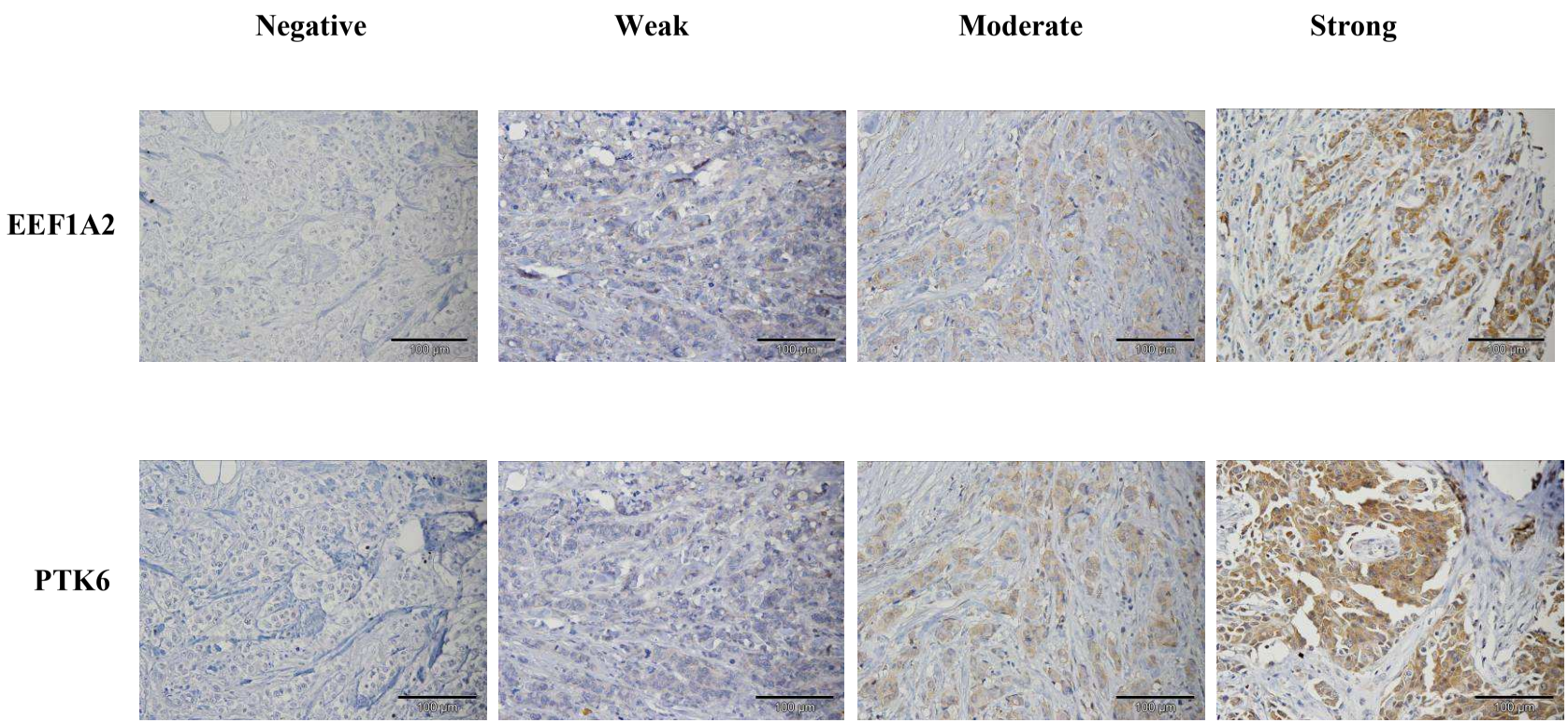

Figure 3.4 Example of breast tumors scored showing weak, moderate, strong and no expression of eEF1A2 and PTK6. Magnification: 20x.

3.2.2 Expression of eEF1A2 and PTK6 in breast tumors is not associated with any clinicohistopathological parameters

To determine whether the expression of eEF1A2 and/or PTK6 was correlated with any histopathological parameters, tumors were divided into the ones with low expression (scores of 0 and 1) and tumors with high expression (scores of 2 and 3). All scoring classifications were determined based on the Allred scoring method (which is based on staining intensity and proportion of positive cells) (Allred et al., 1998). The parameters that were looked at were: grade and size of tumors, ER status, PR status, lymph node involvement, treatment modality (chemotherapy alone or chemotherapy and hormonal therapy) and disease free survival.

In the case of this database that contained all the above mentioned parameters, all markers were scored by John Bartlett's lab (Edinburgh Cancer Research UK Centre, University of Edinburgh) using the AllRed method of scoring.

As mentioned in section 1.3, both EEF1A2 and PTK6 are proteins expressed in the cell cytoplasm. Different monoclonal antibody dilutions were carried out before deciding on the best concentrations (Table 2.2).

3.2.2.1 Correlation of eEF1A2 and PTK6 expression with tumor size

Tumor size was divided into tumors <20mm and tumors ≥20mm. No association was found between tumor size and EEF1A2 (*p*-value 0.221) nor with PTK6 (*p*-value 0.155).

EEF1A2

Tumor size		Weak expression	High expression	Total
0 (<20mm)	Count	46	106	152
	%	51.7%	59.6%	56.9%
1 (≥20mm)	Count	43	72	115
	%	48.3%	40.4%	43.1%
Total	Count	89	178	267
	%	100%	100%	100%

PTK6

Tumor size		Weak expression	High expression	Total
0 (<20mm)	Count	42	112	152
	%	50.6%	59.9%	57%
1 (≥20mm)	Count	41	75	116
	%	49.4%	40.1%	43%
Total	Count	83	187	270
	%	100%	100%	100%

3.2.2.2 Correlation of eEF1A2 and PTK6 expression with tumor grade

Tumor grade was classified as grade 1 (well differentiated), grade 2 (moderately differentiated) and grade 3 (poorly differentiated). EEF1A2 showed no significant correlation with tumor grade (*p*-value 0.55) whereas increased expression of PTK6 was associated with low grade tumors (*p*-value 0.003).

EEF1A2

Tumor grade		Weak expression	High expression	Total
1	Count	8	20	28
	%	8.9%	11%	10.3%
2	Count	33	75	108
	%	36.7%	41.4%	39.9%
3	Count	49	86	135
	%	54.4%	47.5%	49.8%
Total	Count	90	181	271
	%	100%	100%	100%

PTK6

Tumor grade		Weak expression	High expression	Total
1	Count	6	23	29
	%	7.2%	12%	10.6%
2	Count	23	86	109
	%	27.7%	45%	39.8%
3	Count	54	82	136
	%	65.1%	42.9%	49.6%
Total	Count	83	191	274
	%	100%	100%	100%

3.2.2.3 Correlation of eEF1A2 and PTK6 expression with the estrogen receptor status

Estrogen receptor was another marker examined. Tumors were divided into estrogen receptor positive (code = 1) and estrogen receptor negative (code = 0). There was no association between estrogen receptor status and EEF1A2 (*p*-value 0.64). However, there was a statistically significant association between PTK6 overexpression and the estrogen receptor positive tumors (*p*-value 0.037).

EEF1A2

Estrogen receptor status		Weak expression	High expression	Total
0 (ER-)	Count	44	93	137
	%	47.8%	50.8%	49.8%
1 (ER+)	Count	48	90	138
	%	52.2%	49.2%	50.2%
Total	Count	92	183	275
	%	100%	100%	100%

PTK6

Estrogen receptor status		Weak expression	High expression	Total
0 (ER-)	Count	50	89	139
	%	59.5%	45.9%	50%
1 (ER+)	Count	34	105	139
	%	40.5%	54.1%	50%
Total	Count	84	194	278
	%	100%	100%	100%

3.2.2.4 Correlation of eEF1A2 and PTK6 expression with the progesterone receptor status

Another marker was the progesterone receptor. Tumors were either progesterone receptor positive (code = 1) or negative (code = 0). There was no association between EEF1A2 and the progesterone receptor (*p*-value 0.08) nor was there an association with PTK6 (*p*-value 0.74).

EEF1A2

Progesterone receptor status		Weak expression	High expression	Total
0 (PR-)	Count	26	71	97
	%	28.3%	39%	35.4%
1 (PR+)	Count	66	111	177
	%	71.7%	61%	64.6%
Total	Count	92	182	274
	%	100%	100%	100%

PTK6

Progesterone receptor status		Weak expression	High expression	Total
0 (PR-)	Count	31	66	97
	%	36.5%	34.4%	35%
1 (PR+)	Count	54	126	180
	%	63.5%	65.6%	65%
Total	Count	85	192	277
	%	100%	100%	100%

3.2.2.5 Correlation of eEF1A2 and PTK6 expression with the lymph node status

To look at the association of EEF1A2 and PTK6 with lymph node status, breast tumors were categorized as 0, 1, 2 and 3 based on the number of lymph nodes involved where 0 is node negative, 1 = 1 to 3 nodes involved, 2 = 4 to 9 nodes involved and 3 = 10+ lymph node involvement. There was no association between EEF1A2 and lymph node involvement (*p*-value 0.15) nor was there an association with PTK6 (*p*-value 0.08).

EEF1A2

Lymph node status		Weak expression	High expression	Total
0	Count	28	57	85
	%	30.4%	31%	30.8%
1	Count	41	100	141
	%	44.6%	54.3%	51.1%
2	Count	15	15	30
	%	16.3%	8.2%	10.9%
3	Count	8	12	20
	%	8.7%	6.5%	7.2%
Total	Count	92	184	276
	%	100%	100%	100%

PTK6

Lymph node status		Weak expression	High expression	Total
0	Count	34	51	85
	%	39.5%	26.4%	30.5%
1	Count	35	108	143
	%	40.7%	56%	51.3%
2	Count	9	22	31
	%	10.5%	11.4%	11.1%
3	Count	8	12	20
	%	9.3%	6.2%	7.2%
Total	Count	86	193	279
	%	100%	100%	100%

3.2.2.6 Correlation of eEF1A2 and PTK6 expression with distant relapse-free survival (DRFS)

Distant relapse-free survival (DRFS) was also examined. There was no association between overexpression of EEF1A2 and DRFS (*p*-value 0.6) nor was there an association between PTK6 overexpression and DRFS (*p*-value 0.55) (Figure 3.5). There was also no association between the overexpression of both EEF1A2 and PTK6 combined with DRFS (*p*-value 0.9) (Figure 3.8 top graph).

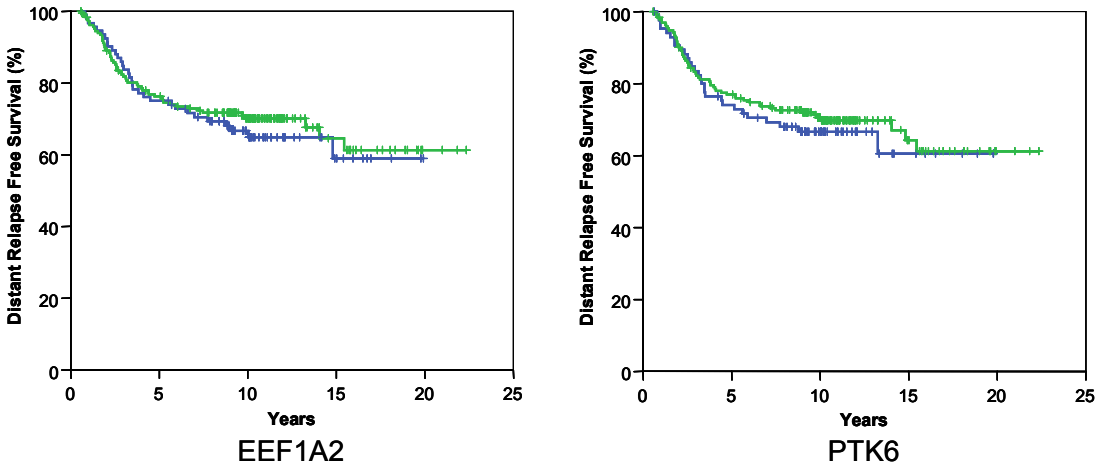

Figure 3.5 Kaplan-Meier curve showing no association between distant relapse-free survival and EEF1A2/PTK6 overexpression over 20 years of follow-up. Green line indicates tumors with high expression whereas the blue line indicates tumors with low expression.

To check if patient survival is affected based on the treatment received patients were split into two groups: those treated with chemotherapy only and those treated with chemotherapy and hormonal therapy. There was no association between survival in patients treated with chemotherapy alone and EEF1A2 overexpression (*p*-value 0.2) nor was there an association with chemotherapy/hormonal treatment (*p*-value 0.7) (Figure 3.6).

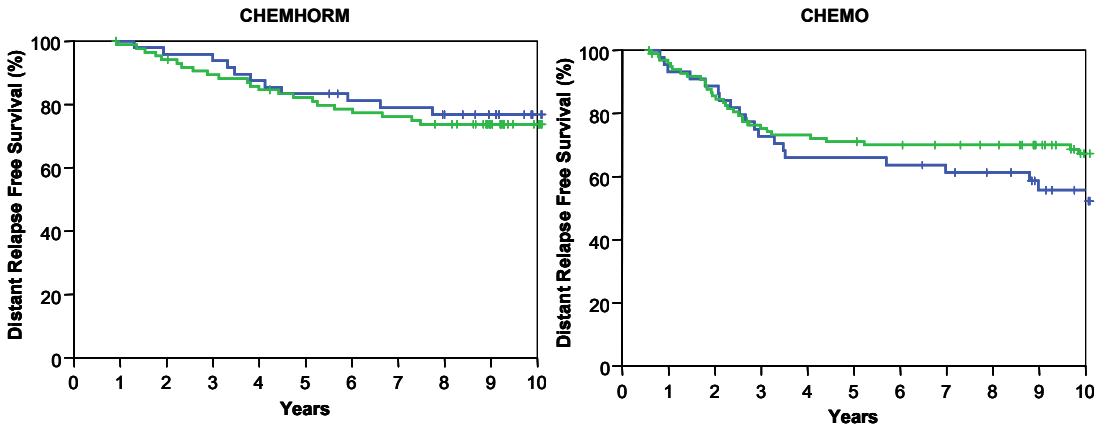

Figure 3.6 Kaplan-Meier curve showing patients treated with either chemotherapy and hormonal or chemotherapy alone with overexpression of EEF1A2. There is a slight trend towards increased survival in patients treated with chemotherapy and high EEF1A2. Green line indicates tumors with high expression whereas the blue line indicates tumors with low expression.

As for PTK6, there was also no association between survival in patients treated with chemotherapy alone and PTK6 overexpression (*p*-value 0.3) nor was there an association with chemotherapy/hormonal treatment (*p*-value 0.9) (Figure 3.7).

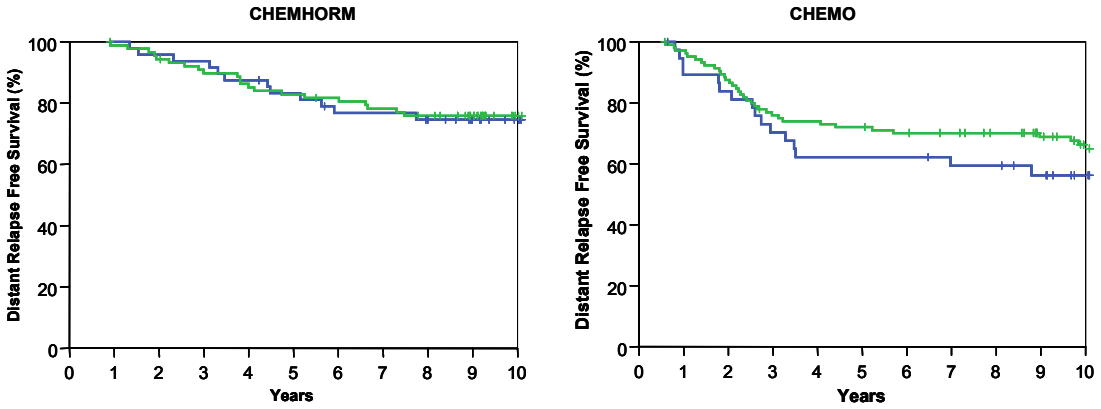

Figure 3.7 Kaplan-Meier curve showing patients treated with either chemotherapy and hormonal or chemotherapy alone with overexpression of PTK6. There is a slight trend towards increased survival in patients treated with chemotherapy and PTK6 overexpression. Green line indicates tumors with high expression whereas the blue line indicates tumors with low expression.

Next, the correlations of both EEF1A2 and PTK6 combined were assessed in all patients and then in patients treated with chemotherapy alone and the chemotherapy/hormonal group. Overall, there was no association between the overexpression of both EEF1A2 and PTK6 combined and DRFS in both groups of patients (*p*-value 0.9) nor was there an association between the overexpression of those two proteins and the treatment modality: chemotherapy/hormonal group (*p*-value 0.9) and the chemotherapy only group (*p*-value 0.6) (Figure 3.8).

In figure 3.9, the axis was truncated at 10 years to get a better indication of the trend. The chemotherapy only graph shows that patients with overexpression of both EEF1A2 and PTK6 have an increased survival. This is decreased slightly by having either protein, whereas the worst prognosis is in patients with neither at 10 years. However, in the previous figure (Figure 3.8), the graph shows that as patients approach 25 years after diagnosis, survival is increased with both proteins overexpressed and a similar prognosis is evident in patients with neither or either.

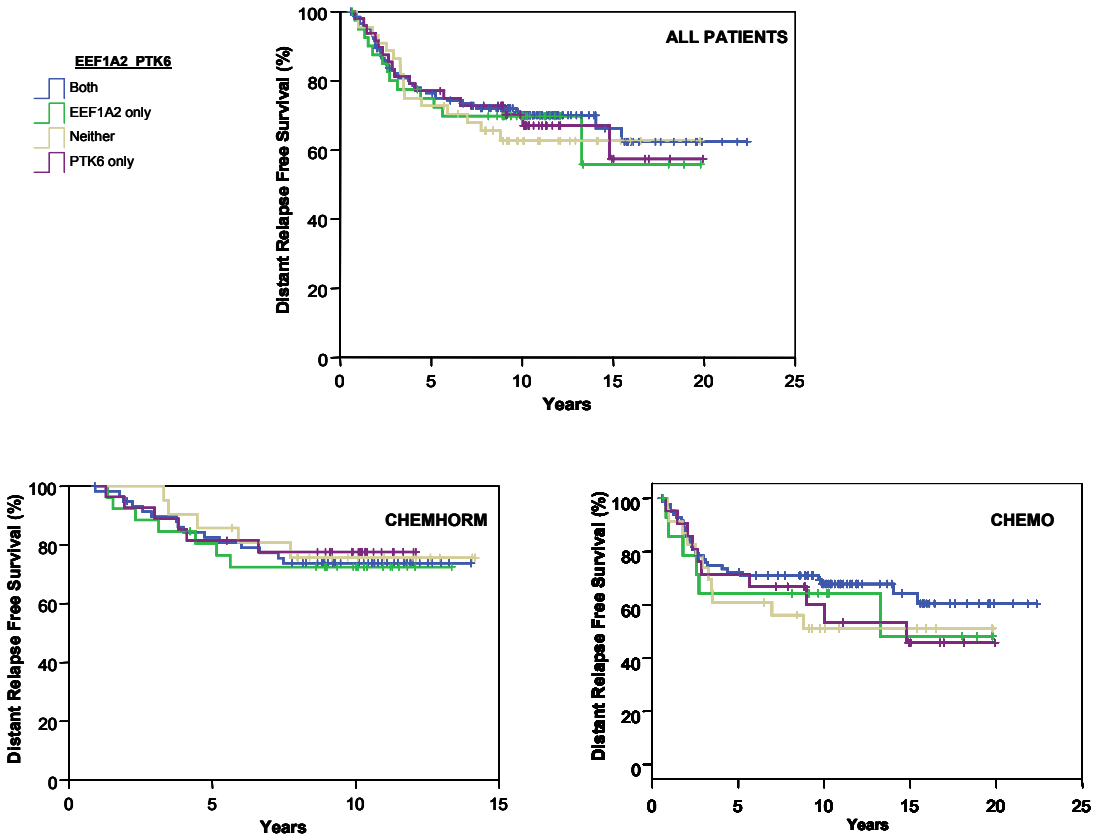

Figure 3.8 Kaplan-Meier curve showing the association of EEF1A2 and PTK6 combined in all patients, in patient treated with chemotherapy/hormonal and in patients treated with chemotherapy alone in relation to disease free survival.

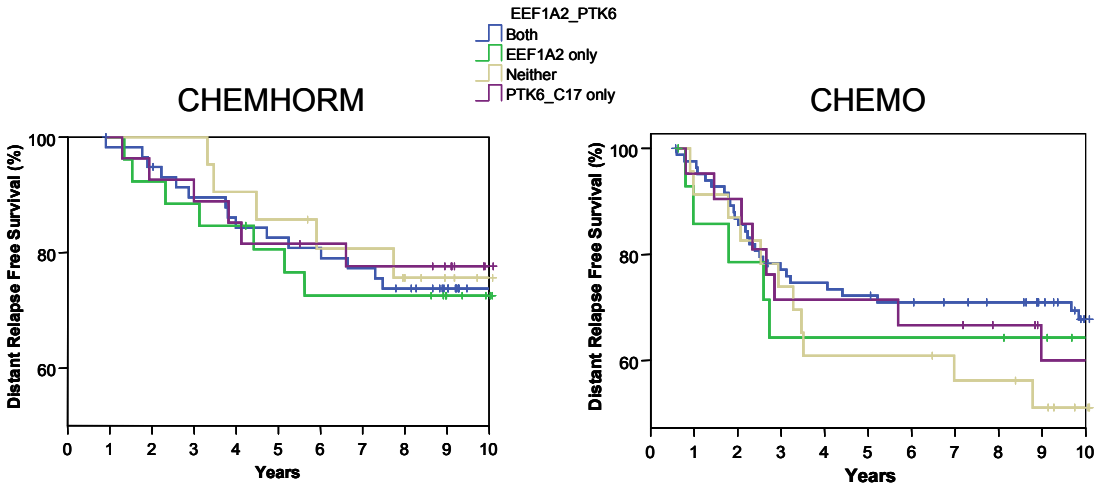

Figure 3.9 Same Kaplan-Meier curve as above but with axis truncated to give a better indication of the trend. The chemotherapy only graph shows patients with overexpression of both EEF1A2 and PTK6 have an increased survival. This is decreased slightly by having either protein, whereas the worst prognosis is in patients with neither at 10 years.

3.2.3 Gene copy number analysis in cell lines

To assay the gene copy number of EEF1A2 and PTK6, primer optimization was first carried out on the breast cancer ER positive cells MCF7 and T47D and the ER negative cells MDA-MB-231 and MDA-MB-453 along with the cervical cancer cell line HeLa (Figure 3.10 & Table 3.1). Optimal primer concentration was determined to be 0.5µM and the gene copy number analysis was carried out on those cell lines. As discussed in section 2.2.5, normal human blood was used as a calibrator/control as it normally has two copies of each gene. The microsatellite markers D5S478 (5p15.2) and D11S913 (11q13.1) were used as reference genes (Ginzinger et al., 2000) as these loci are located in regions of the genome that usually did not show alterations in human breast tumors. To detect the copy number changes, the double delta Ct method ($2^{-\Delta\Delta Ct}$) was used where first the difference between the mean Ct value of the genes in the control sample and ΔCt of the reference gene in the control sample was determined to get ΔCt. To get ΔΔCt, ΔCt would be subtracted from the same value and therefore it equals zero and 2^0 equals one which is equivalent to the number of copies in a haploid cell. This number would be multiplied by 2 to get the gene copy number in a diploid cell (Livak and Schmittgen, 2001). For the tumor samples, evaluation of $2^{-\Delta\Delta Ct}$ indicates the gene copy number difference relative to the control sample where the same calculations were used. No

copy number changes were detected for both the genes in all the breast cancer cell lines as they all showed normal two copies but HeLa cells showed an increased copy number of both *EEF1A2* and *PTK6* (Table 3.1 and Figure 3.10).

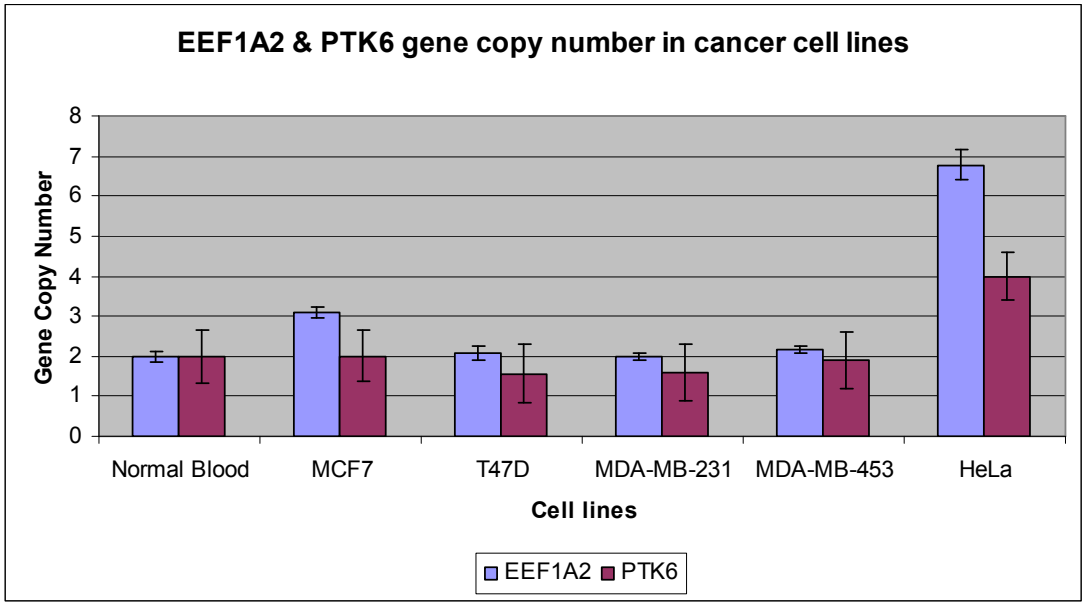

Figure 3.10 EEF1A2 and PTK6 gene copy number in cancer cell lines. EEF1A2 and PTK6 were normalized to the reference genes (PUM1 and TBP). Normal human blood was used as a calibrator.

Table 3.1 Gene copy number analysis in cancer cell lines using the $2^{-\Delta\Delta Ct}$ method.

	EEF1A2		
	ΔCT	**ΔΔCT**	**$2^{-\Delta\Delta Ct}$**
Normal Blood	0.15	0.00	2
MCF7	-0.05	-0.20	3
T47D	0.09	-0.06	2
MDA-MB-231	0.16	0.01	2
MDA-MB-453	0.04	-0.11	2
HeLa	-1.61	-1.76	7
	PTK6		
	ΔCT	**ΔΔCT**	**$2^{-\Delta\Delta Ct}$**
Normal Blood	3.97	0.00	2
MCF7	3.77	-0.20	2
T47D	4.33	0.36	2
MDA-MB-231	4.31	0.34	2
MDA-MB-453	4.04	0.07	2
HeLa	3.15	-0.82	4

3.2.4 PTK6, but not EEF1A2, is amplified in breast cancer

After scoring the breast tumor TMAs and dividing them based on the expression level of eEF1A2 and PTK6 into tumors with weak, moderate and strong expressions (section 3.2.1), tumors that showed a weak, moderate and strong co-expression of both genes were selected (Table 3.2), DNA was extracted from the original breast tumors' paraffin embedded tissue (PET) from which the cores were obtained, DNA concentration was measured and copy number analysis was carried out using real-time PCR. Normal blood was also used as a calibrator along with the same reference genes used for the copy number detection in cell lines as described in section 3.2.3.

In total, 55 breast tumors were analyzed for copy number changes in the *EEF1A2* and *PTK6* genes. More tumors were initially selected which were later excluded as not enough tumor material was found in the paraffin blocks. Analysis using the $2^{-\Delta\Delta Ct}$ method showed an increased copy number of *PTK6* in all tumors when compared to *EEF1A2* (Table 3.3). This was observed for all the three tumor categories: weak, moderate and strong, and all showed a significant correlation between EEF1A2 and PTK6 gene copy number especially in the tumors with the strong co-expression (all p-values were <0.05). In those tumors that showed a strong co-expression, copy numbers of *PTK6* ranged between 8 and 92 copies whereas the copy numbers for *EEF1A2* were surprisingly between 2 and 14. In the tumors with moderate co-expression, *PTK6* copy number was lower than that seen in the tumors with strong expression (between 2 and 27), yet still higher that *EEF1A2* copy number (between 2 and 19 copies). In the tumors with weak expression, *PTK6* copies were between 2 and 20 whereas *EEF1A2* copies were the same as in the tumors that showed strong and moderate expression tumors (Figure 3.11).

Table 3.2 Breast tumors co-expressing EEF1A2 and PTK6.

Weak		Moderate		Strong	
Core ID	Tumor ID	Core ID	Tumor ID	Core ID	Tumor ID
B9C	1722	A4	1104	A5	1118
B14W	1705	A6C	1126	A7	1146
B18	970	A9	1127	D1	1478
C17	1511	B15	1712	E3	901
E8C	238	D10	725	D14	1119
F8	255	E1	1112	F5	192
G6	537	E6	1333	G1	543
G11	212	E10	207	G2	535
G13	77	F4	194	H1S	554
G16	443	G4	567	H5	476
G17	448	H4	986	B1	1103
A12	1137	A1	953	F2	1783
A17	1096	A6H	1023	H2	1102
B6	1158	B14M	1219	I2S	1611
B9H	1185	C10	1370		
C9	1372	D18	1618		
C12	882	E2	867		
D11	1674	F9	1665		
E8H	1726	G6	1499		
E17	1617	H1	1345		
I5	1037				

Table 3.3 EEF1A2 and PTK6 gene copy number in breast tumors. Copy number analysis was carried out on breast tumors that showed weak, moderate and strong co-expression of EEF1A and PTK6.

Weak		
Tumor	EEF1A2	PTK6
B9C	7	10
B14W	9	8
B18	9	9
C17	7	7
E8C	2	5
F8	4	6
G6	3	9
G11	5	11
G13	5	15
G16	4	11
G17	6	10
A12	3	8
A17	3	7
B6	7	20
B9H	3	9
C9	3	10
C12	4	11
D11	4	8
E8H	2	5
E17	3	6
I5	3	10
Average	4	9

Moderate		
Tumor	EEF1A2	PTK6
A4	5	13
A6C	7	7
A9	3	15
B15	3	6
D10	4	12
E1	9	27
E6	3	8
E10	2	8
F4	5	11
G4	3	8
H4	2	8
A1	4	8
A6H	2	15
B14M	8	18
C10	6	13
D18	5	7
E2	5	8
F9	4	10
G6	3	4
H1	4	9
Average	4	11

Strong		
Tumor	EEF1A2	PTK6
A5	4	16
A7	5	13
D1	2	8
E3	1	8
D14	4	10
F5	3	8
G1	14	92
G2	6	53
H1S	4	35
H5	3	23
B1	4	37
F2	3	8
H2	7	15
I2S	4	12
Average	5	24

As shown in Table 3.3, the average number of *EEF1A2* gene copies is almost the same in all tumors with weak, moderate and strong expression at the protein level (4, 4, and 5 gene copies respectively). However, the average copy number for *PTK6* was the highest in the tumors with strong expression. *PTK6* amplification could explain its overexpression at the protein level. This leads to phosphorylation of its substrates which in turn increases cell division and breast tumorigenesis. As for *EEF1A2*, having an average of 4-5 gene copies does not explain the high expression at the protein level detected from the breast tumors TMAs. Therefore it is suggested that other modulators –such as miRNAs, a strong promoter, an enhancer within the amplified region- might play a role in eEF1A2 overexpression (discussed in section 3.3.2).

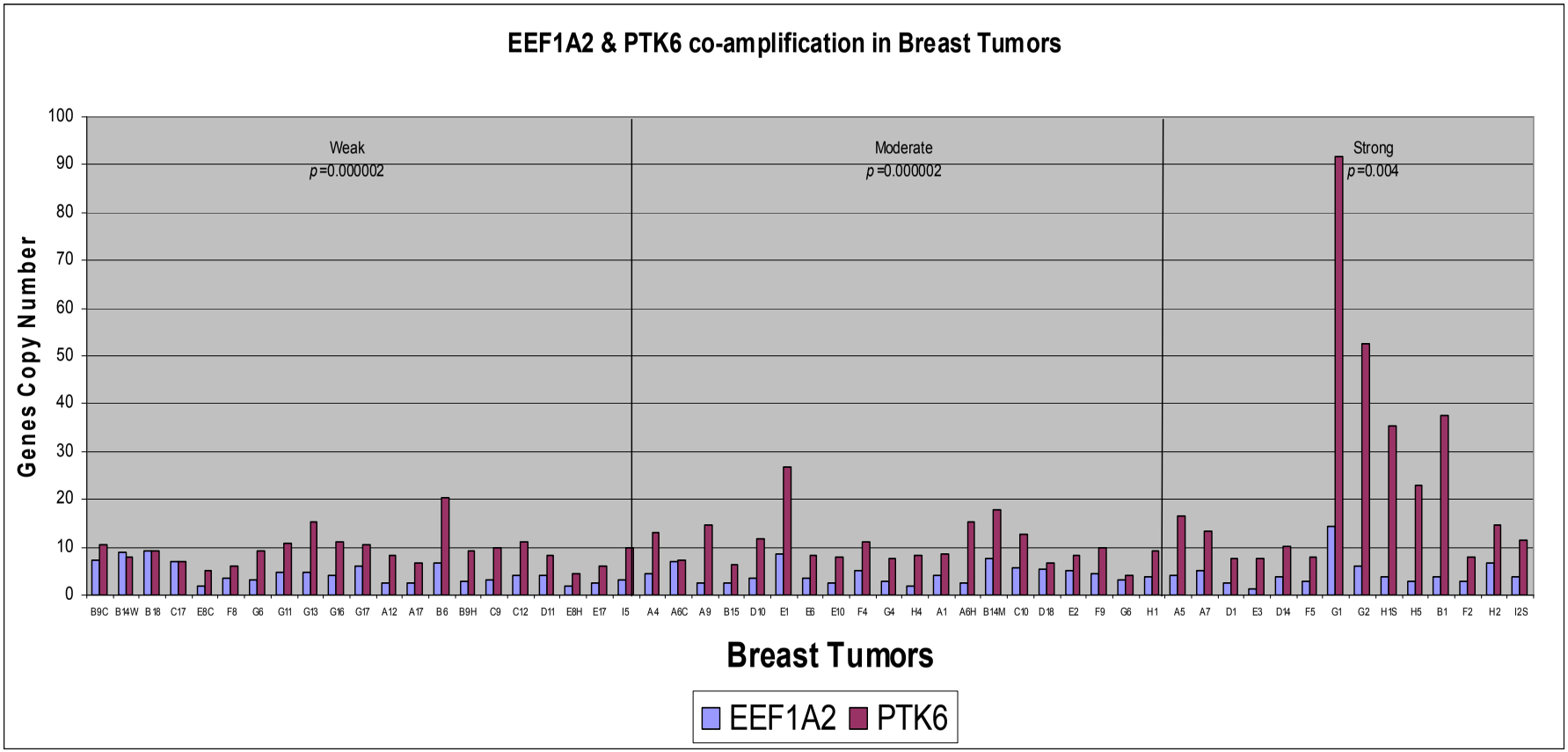

Figure 3.11 Co-amplification of EEF1A2 and PTK6 in breast tumors. PTK6 shows an increased copy number when compared to EEF1A2 especially in the tumors with strong expression. The genes were normalized to the reference genes (PUM1 and TBP). Normal human blood was used as a calibrator.

3.2.5 SRMS and KCNQ2 have normal copy numbers

It was shown in the previous section that *PTK6* showed a copy number increase higher than *EEF1A2*. Since 20q13.3 is a region known to be amplified in breast cancer, the hypothesis was that many genes in this region will also show an increased copy number. Therefore both genes that flank *EEF1A2* and *PTK6* were analyzed for copy number changes: *SRMS* and *KCNQ2* (Figure 3.12). Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites (SRMS) is a non-receptor tyrosine kinase that is involved in proliferation or differentiation of keratinocytes in the skin. *KCNQ2* (Potassium voltage-gated channel subfamily KQT member 2) is a gene important in the regulation of neuronal excitability. Mutations in the *KCNQ2* gene was shown to be associated with epilepsy (Singh et al., 1998).

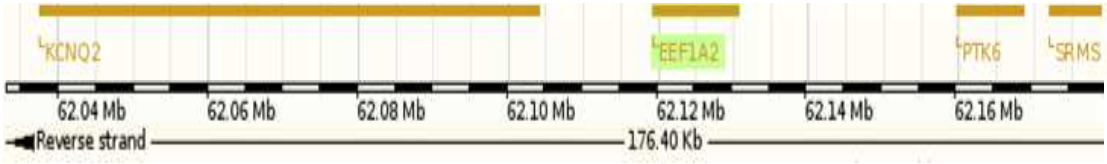

Figure 3.12 20q13.3 amplicon showing the two genes flanking EEF1A2 and PTK6: SRMS and KCNQ2 (Ensembl).

To detect the copy number changes in *SRMS* and *KCNQ2*, real-time PCR was carried out on seven of the tumors that showed an increased copy number of *PTK6* in the strong co-expression category. Since this is a region known to be amplified in breast cancer, *SRMS* and *KCNQ2* might be expected to have an increased gene copy number, especially if *PTK6* and *EEF1A2* were part of a single amplicon. However, when copy number analysis was carried out, both genes showed normal two copies with no sign of amplification (Table, 3.4, Figure 3.13, 3.14 and 3.15).

Tumor ID	KCNQ2	EEF1A2	PTK6	SRMS
Calibrator	2	2	2	2
A5	2	4	16	1
A7	1	5	13	1
G2	2	6	53	3
H1S	2	4	35	3
H5	1	3	23	2
B1	2	4	37	2
I2S	1	4	12	2

Table 3.4 qPCR analysis showing copy number changes in KCNQ2, EEF1A2, PTK6 & SRMS.

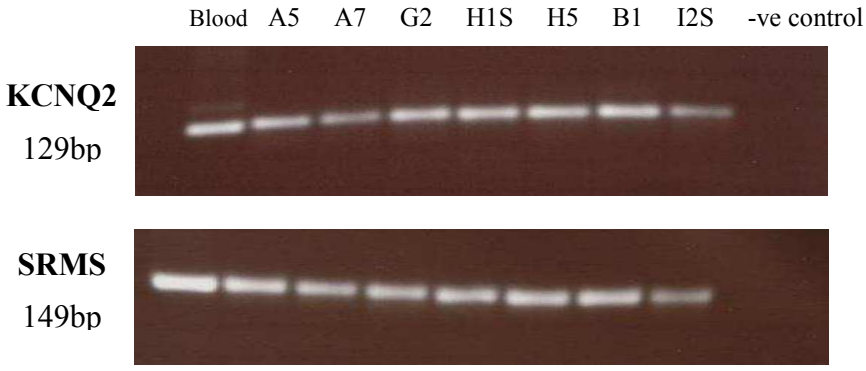

Figure 3.13 Real-time PCR product run on an agarose gel after analysis. KCNQ2 and SRMS show a single band of the right size.

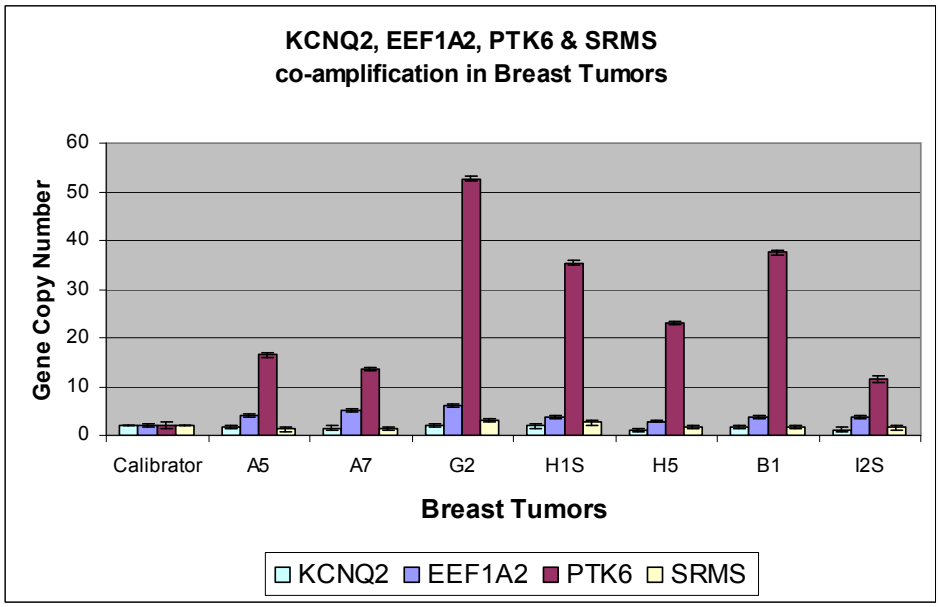

Figure 3.14 Copy number analysis of 4 genes in the 20q13.3 region: KCNQ2, EEF1A2, PTK6 and SRMS in 7 different breast tumors. All four genes were normalized to the reference genes (PUM1 and TBP). Normal human blood was used as a calibrator.

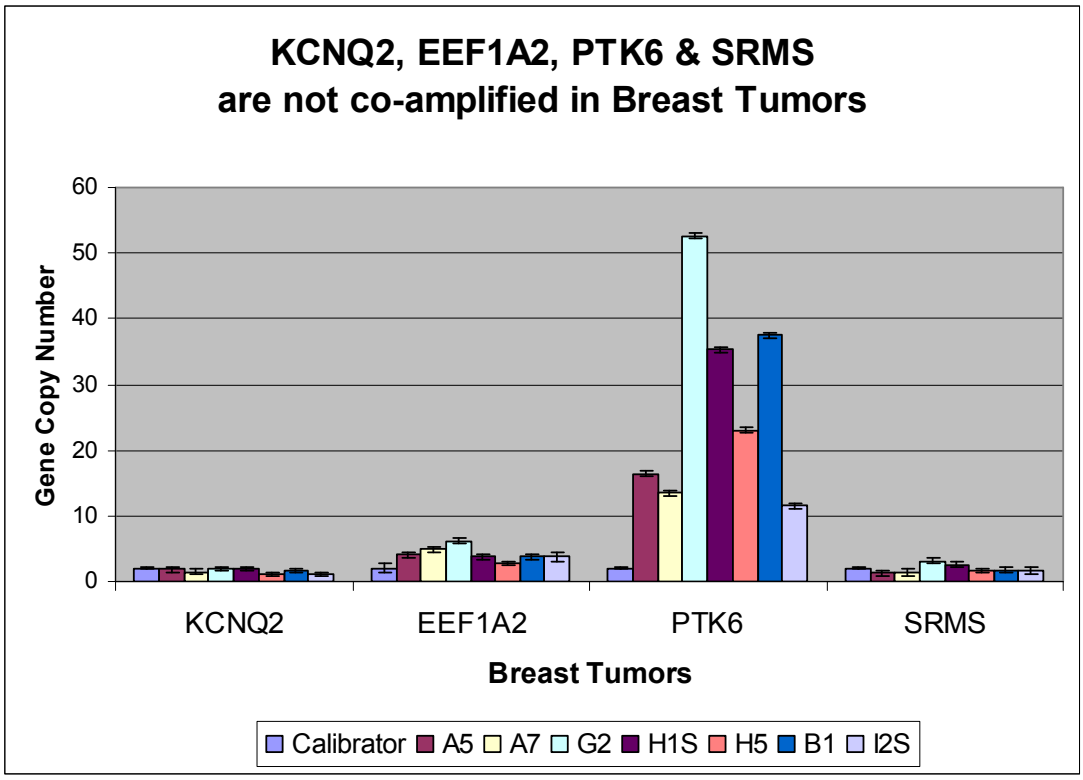

Figure 3.15 Copy number analysis of KCNQ2, EEF1A2, PTK6 and SRMS based on their location on the 20q13.3 amplicon. Only PTK6 appears to be amplified amongst those genes. The four genes were normalized to the reference genes. Normal human blood was used as a calibrator.

Since *EEF1A2* gene copy number did not correlate with the protein overexpression detected in the breast tumors TMAs (section 3.2.4), one hypothesis was that miRNAs might play a role in its overexpression. Most studies have shown that miRNAs downregulate gene expression by binding to the 3'UTR and either degrading the mRNA or inhibiting translation. However, in 2008, Dahiya *et al*. reported the overexpression of eEF1A2 in the ovarian cancer cell line BG-1 when transfected with the miRNA let-7f (Dahiya et al., 2008).

Alexey Larionov (Breakthrough Breast Cancer Research Unit, Western General Hospital, Edinburgh) has kindly provided the figure below (Figure 3.16) where he looked at the association between eEF1A2 mRNA and Let-7f miRNA in 60 breast tumors. His analysis was based on data obtained with Illumina HT-12 mRNA microarrays and Illumina miRNA arrays. There was no association between eEF1A2 mRNA and Let-7f miRNA expression in those breast tumors.

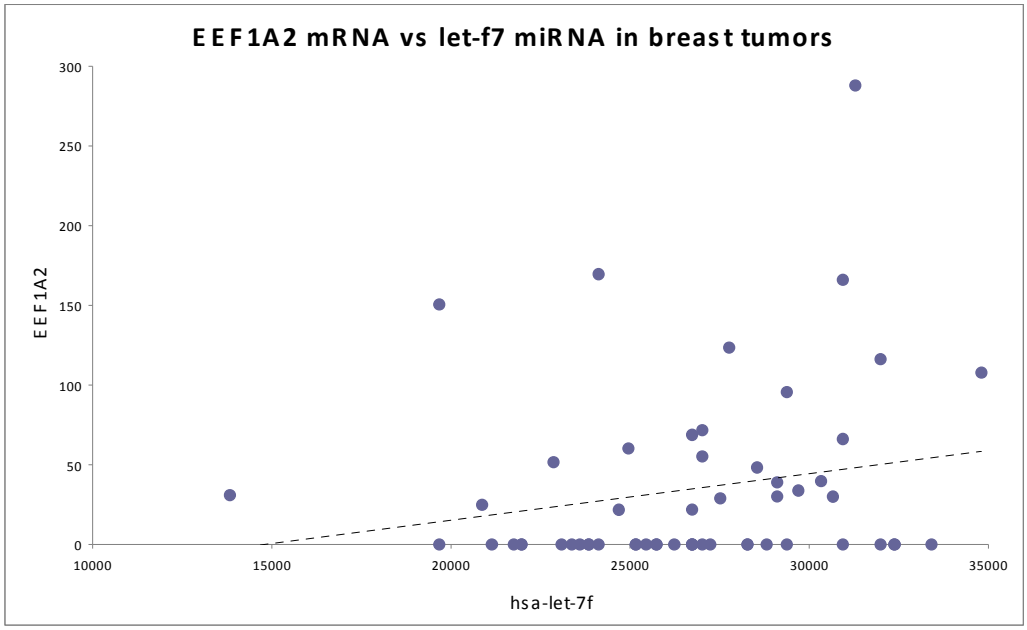

Figure 3.16 Microarray study analyzing the association between eEF1A2 mRNA and Let-7f miRNA in 60 breast tumors. No significant correlation between eEF1A2 mRNA and let-7f miRNA in breast tumors (Alexey Larionov, Breakthrough Breast Cancer Research Unit, Western General Hospital, Edinburgh).

The graph shows many breast tumors expressing let-7f but not eEF1A2 indicating that let-7f alone is not sufficient to increase the expression level of eEF1A2, at least at the RNA level. On the other hand, tumors expressing eEF1A2 appear to have relatively an increased level of let-7f.

3.2.6 EEF1A2 expression in breast tumors and breast cancer cell lines using RPPA

Reverse Phase Protein Array (RPPA) is a technique used to detect the presence or absence of a certain protein in a large set of tumors and/or cells lines in a single slide. This was carried out on 107 breast tumors along with 26 breast cancer cell lines to detect the presence of EEF1A2 at the protein level. Protein extraction and slides' generation was carried out by Peter Mullen from the Breakthrough Research Unit, Edinburgh. Before carrying out the RPPA, antibody optimization was carried out for two EEF1A2 antibodies to determine the best concentration to be used (described in section 2.2.8). eEF1A2 anti-sheep antibody was used at a concentration of 1:150 while eEF1A2 anti-rabbit antibody was used at a concentration of 1:50.

EEF1A2 is not usually expressed in normal breast tissue. However, it was shown to be increased in 60% of breast tumors. As shown in figure 3.17, eEF1A2 is expressed in all breast tumors with exceptionally increased expression in two breast tumors detected by the anti-sheep antibody. The anti-rabbit antibody showed similar results of increased eEF1A2 expression in all breast tumors, it also showed a significant increase in the same tumors as the ones detected by the anti-sheep antibody along with 2 other tumors (Figure 3.18). EEF1A2 showed increased expression in all 26 breast cancer cell lines as shown in figure 3.19 and 3.20. This was detected using both the anti-sheep and anti-rabbit antibodies.

STAT3 is one of the intermediates affected by the downregulation of EEF1A2. Even though Li *et al.* reported that knockdown of *Eef1a2* expression resulted in delayed phosphorylation of STAT3 in PCT cell lines, the actual level of STAT3 was not affected (Li et al., 2010). In this RPPA study, Peter Mullen has been working on STAT3 and has kindly provided figures 3.21, 3.22, 3.23 and 3.24 where he used two STAT3 antibodies: STAT3 and pSTAT3 (Tyr705) (Cell Signaling Technology®). As shown in figures 3.21 and 3.22, using both antibodies, many breast tumors show an increased expression of STAT3. Interestingly, both tumors that showed an exceptionally increased expression of EEF1A2 also show a high expression of STAT3. However, when pSTAT3 (Tyr705) antibody was used to detect the phosphorylated form of STAT3, it was noticed to be expressed in all 107

breast tumors. As for the 26 breast cancer cell lines, all showed an increased STAT3 and pSTAT3 (Tyr705) level but lower than the expression detected in breast tumors (Figures 3.23 and 3.24).

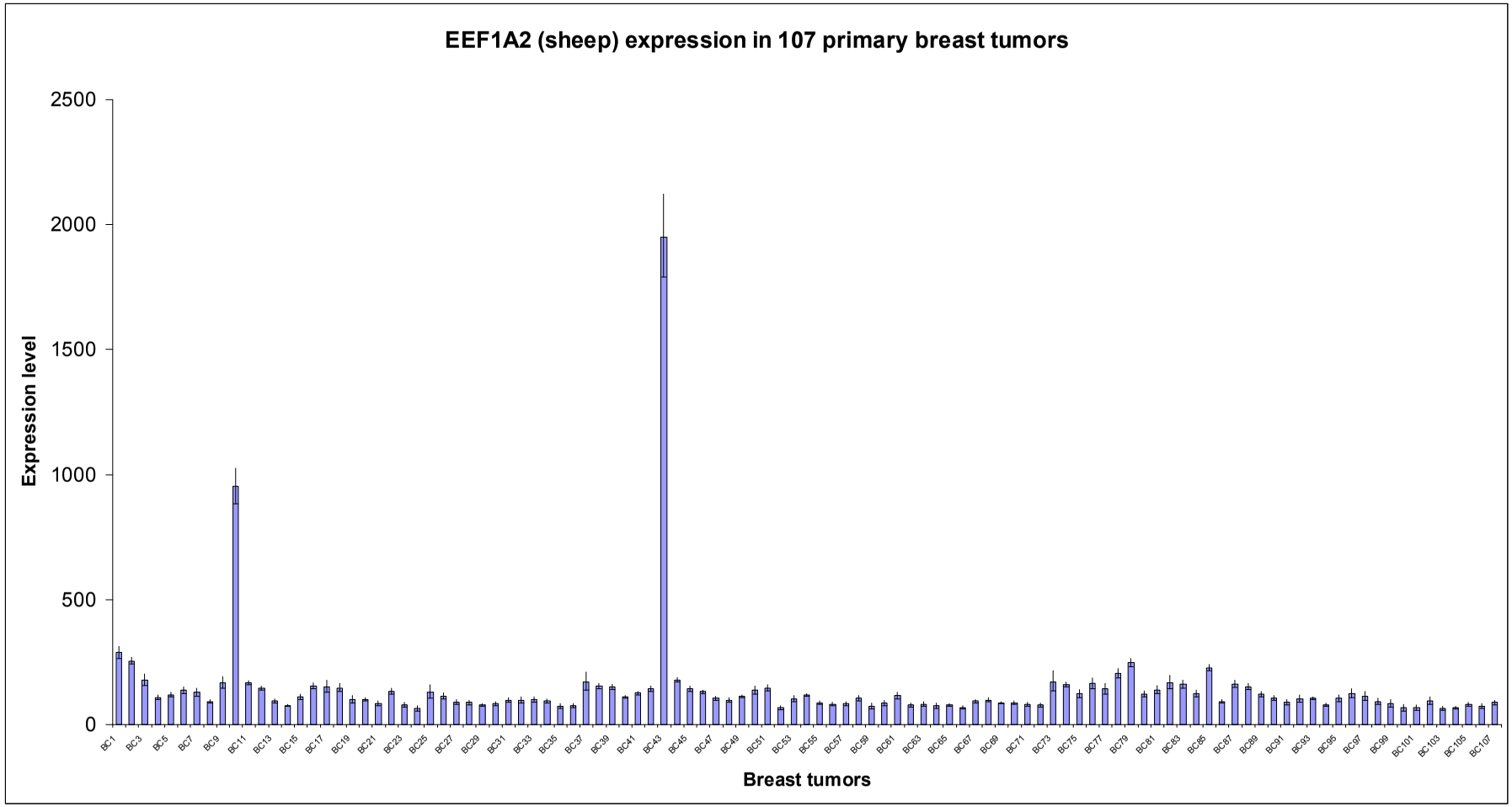

Figure 3.17 RPPA analysis using EEF1A2 anti-sheep antibody. Results show varying eEF1A2 expression in 107 breast tumors. Two tumors show exceptionally high levels of eEF1A2.

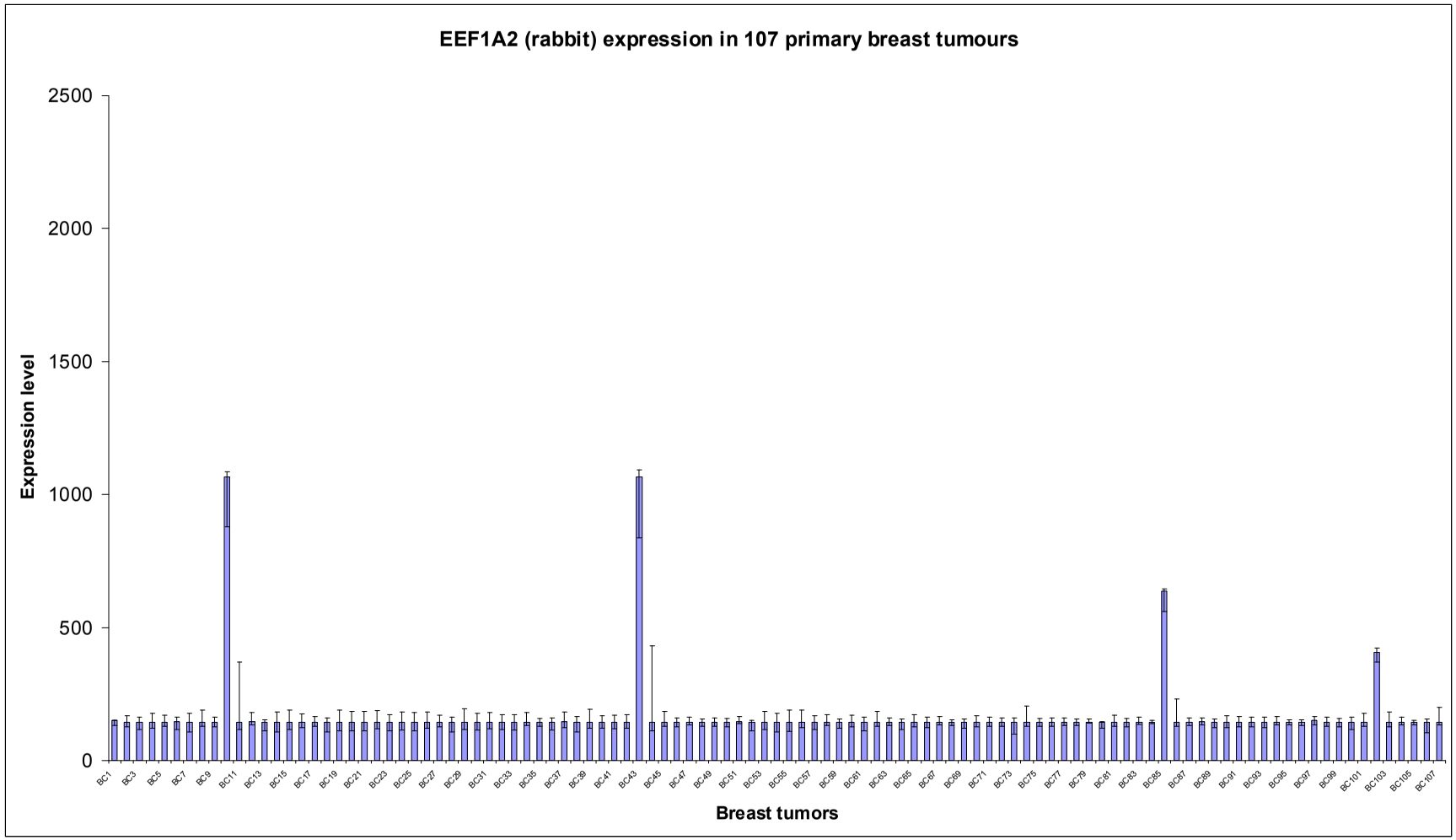

Figure 3.18 RPPA analysis using EEF1A2 anti-rabbit antibody. Results show varying eEF1A2 expression in 107 breast tumors. Four tumors show exceptionally high levels of eEF1A2.

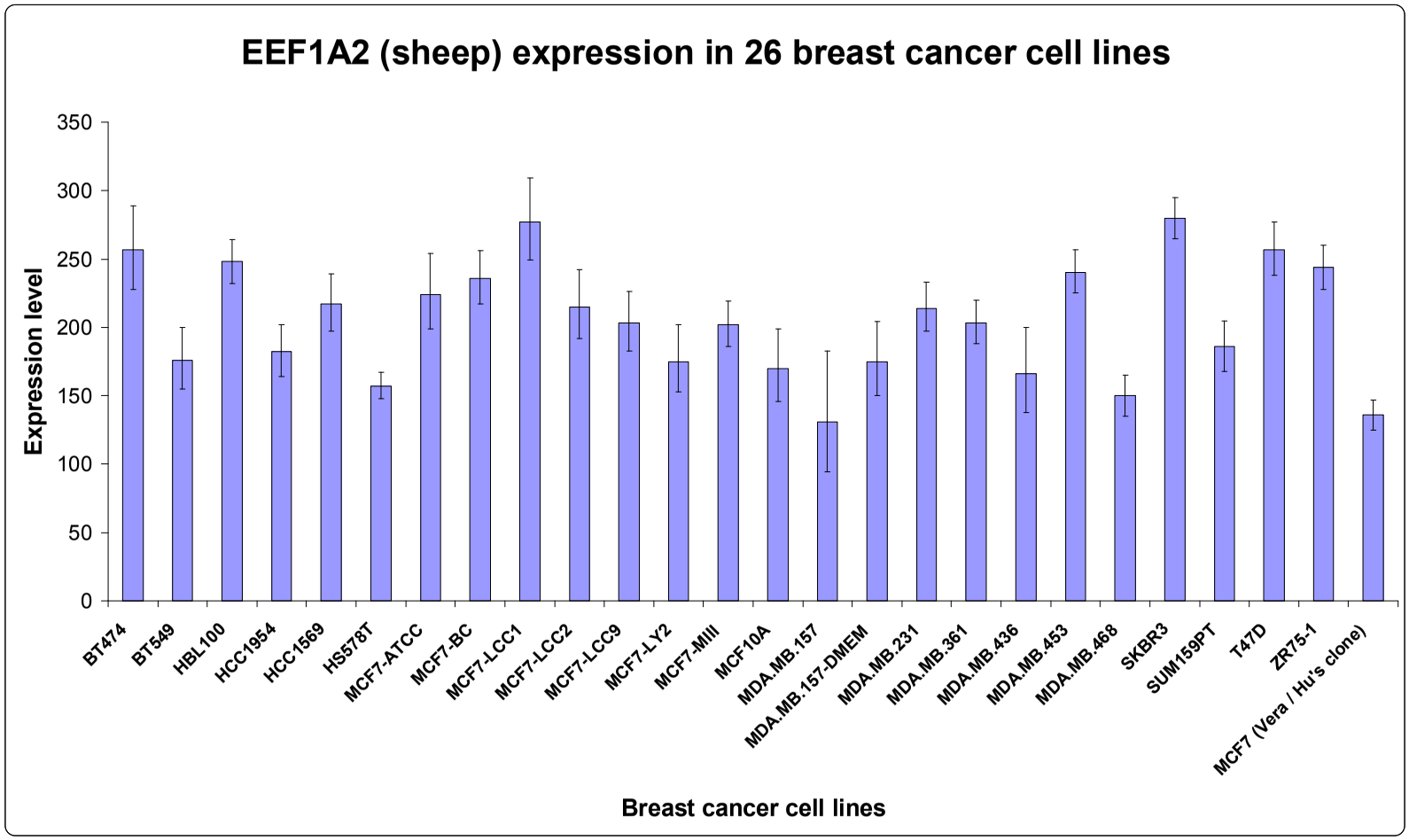

Figure 3.19 RPPA analysis using EEF1A2 anti-sheep antibody. Results show varying eEF1A2 expression in 26 breast cancer cell lines.

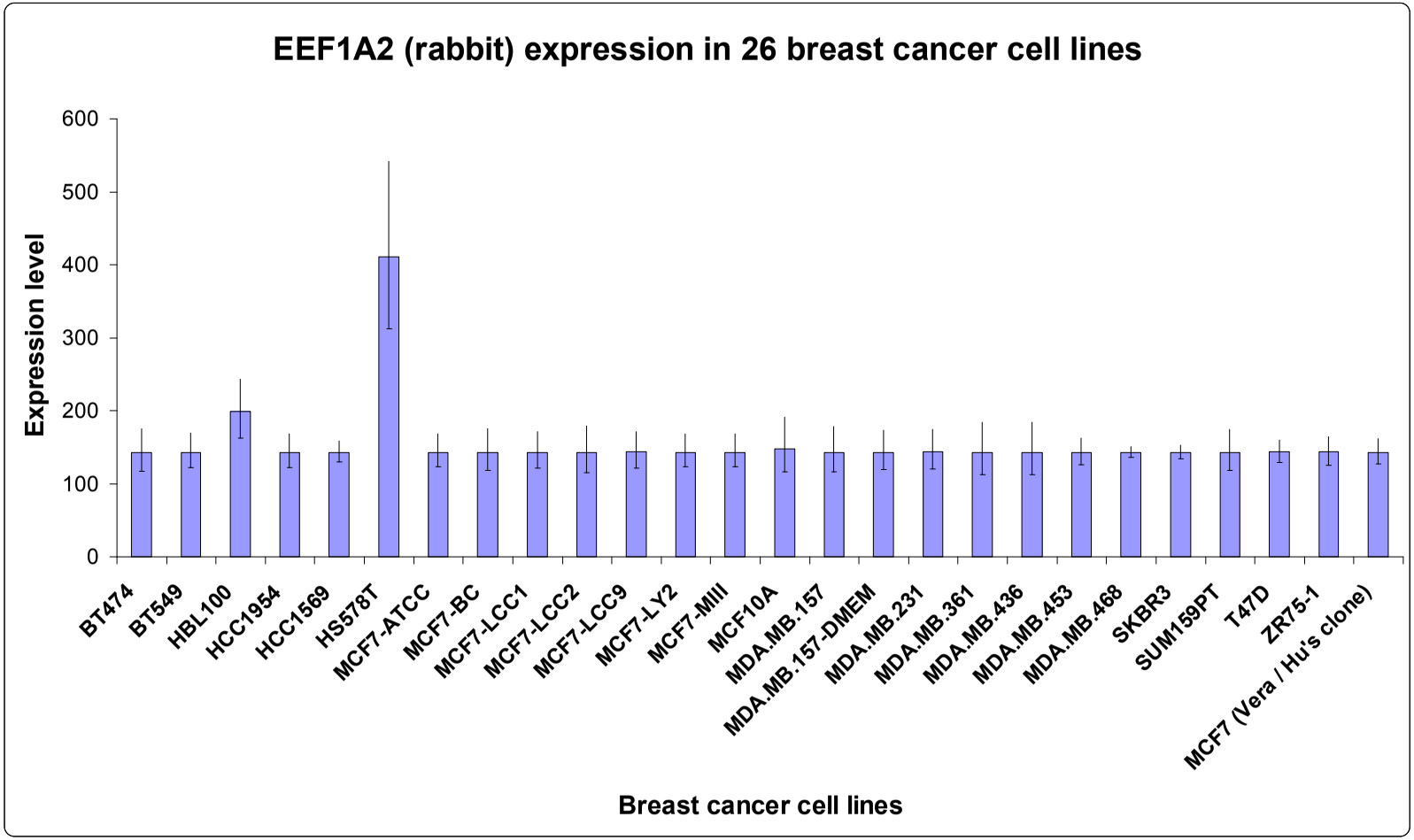

Figure 3.20 RPPA analysis using EEF1A2 anti-rabbit antibody. Results show varying eEF1A2 expression in 26 breast cancer cell lines.

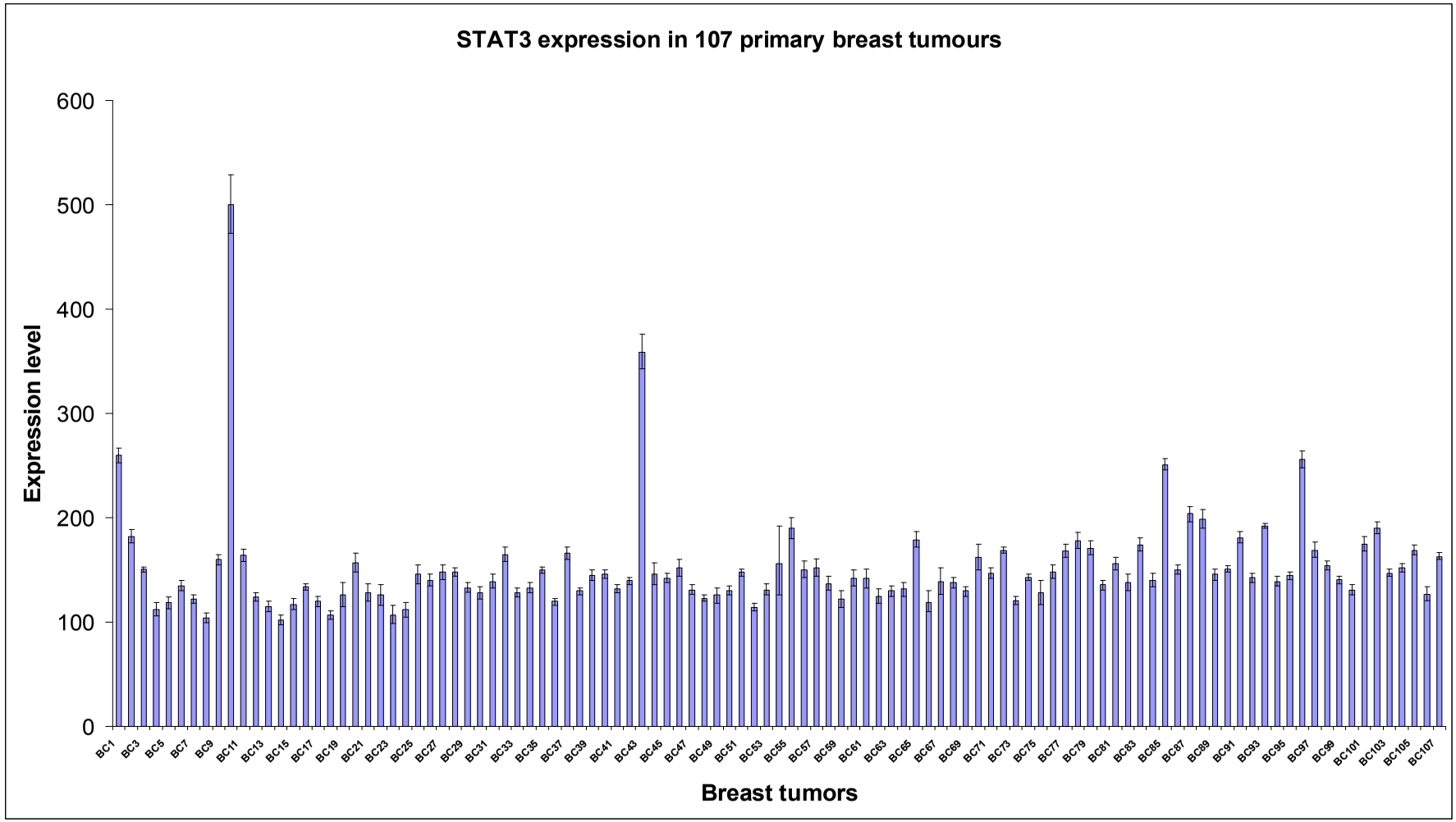

Figure 3.21 RPPA analysis using STAT3 antibody. Results show varying STAT3 expression in 107 breast tumors (Figure provided by Peter Mullen).

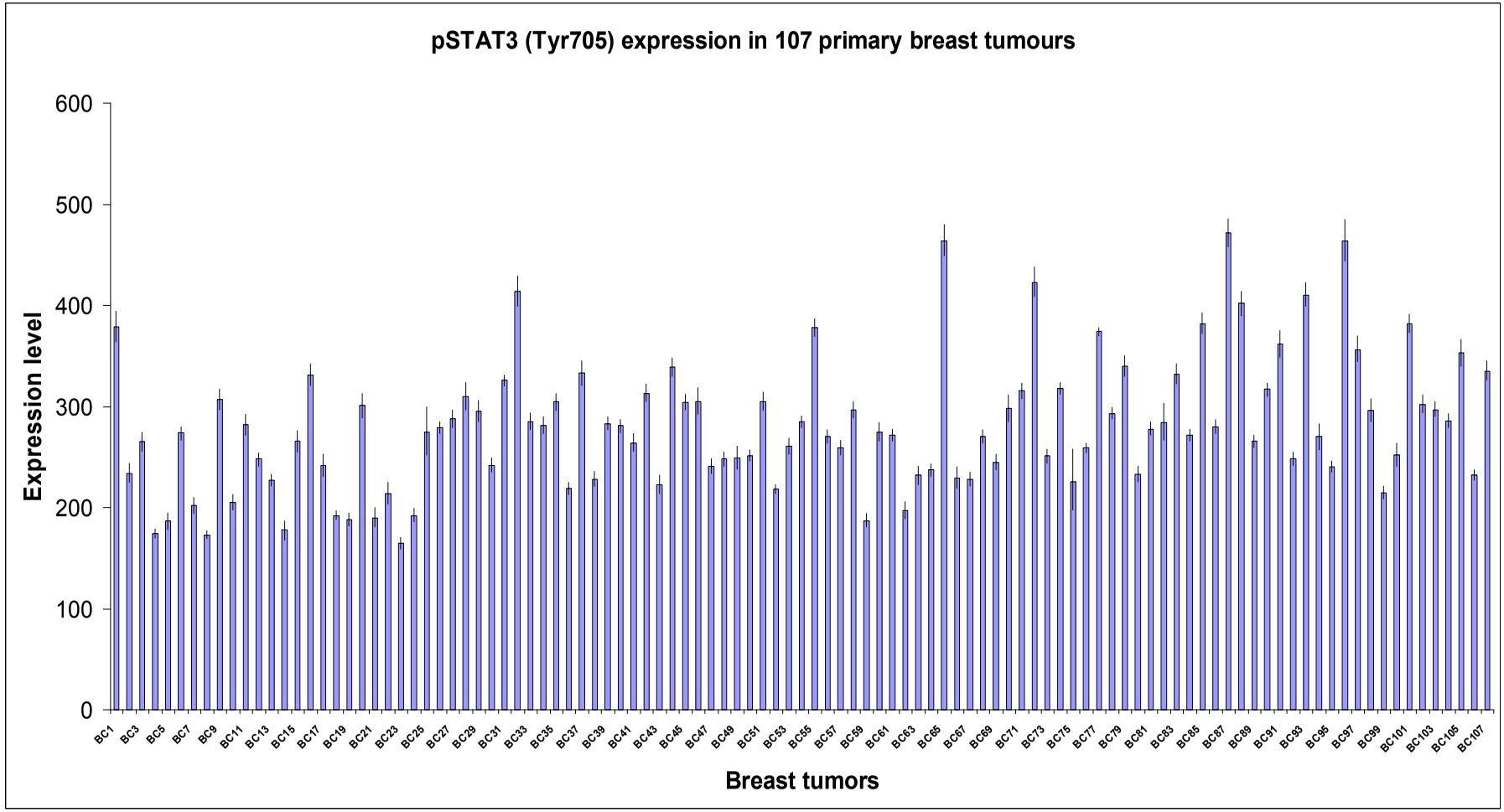

Figure 3.22 RPPA analysis using pSTAT3 (Tyr705) antibody. Results show varying pSTAT3 (Tyr705) expression in 107 breast tumors (Figure provided by Peter Mullen).

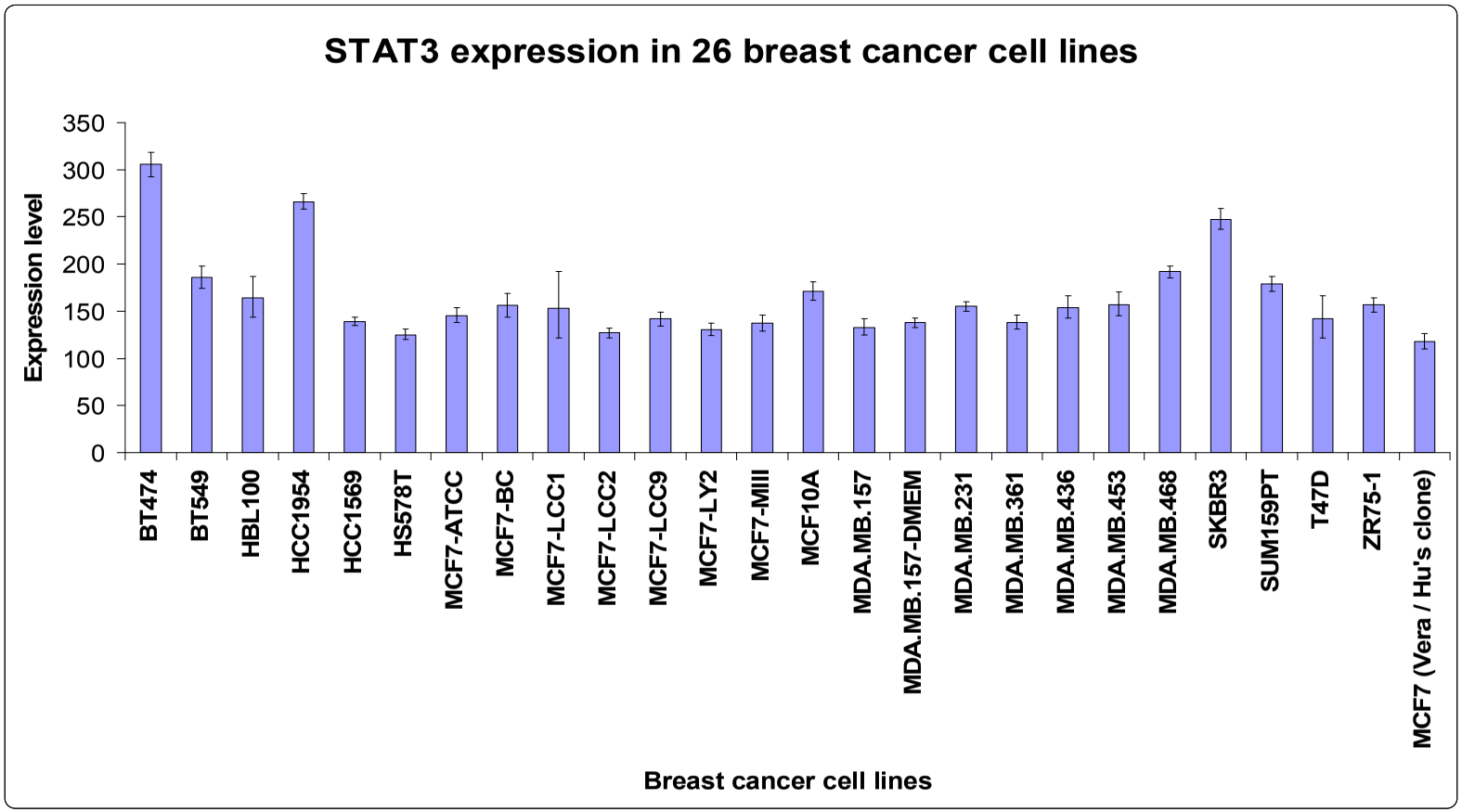

Figure 3.23 RPPA analysis using STAT3 antibody. Results show varying STAT3 expression in 26 breast cancer cell lines (Figure provided by Peter Mullen).

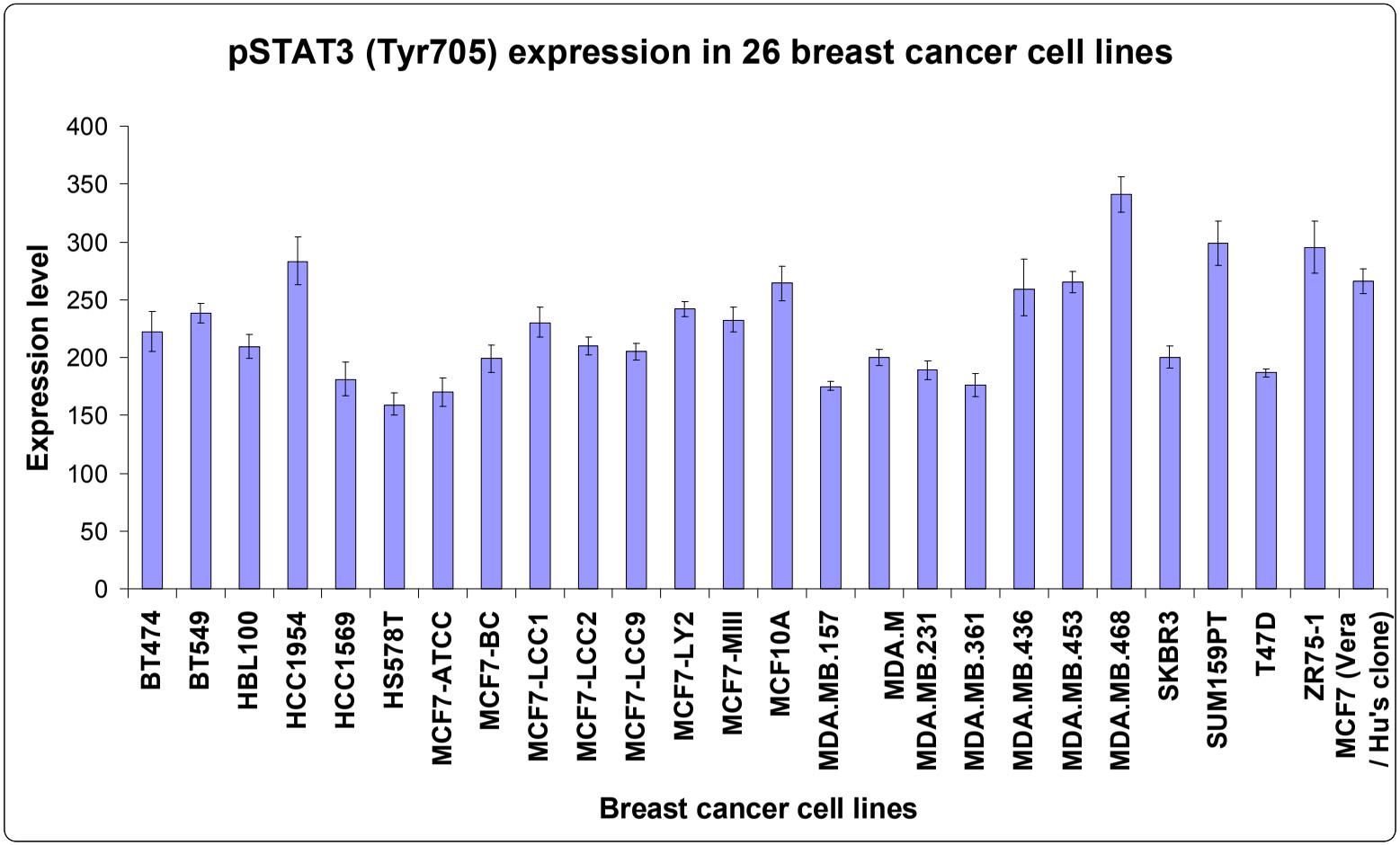

Figure 3.24 RPPA analysis using pSTAT3 (Tyr705) antibody. Results show varying pSTAT3 (Tyr705) expression in 26 breast cancer cell lines (Figure provided by Peter Mullen).

3.3 Discussion

3.3.1 EEF1A2 and PTK6 correlation with histopathological parameters in breast cancer

EEF1A2 and *PTK6* are two oncogenes mapping to the 20q13.3 amplicon. Both eEF1A2 and PTK6 are overexpressed in 60% of breast tumors and since these genes are located on a region known to be amplified in many different types of cancers, it was thought that these genes might have an increased copy number due to co-amplification, resulting in overexpression. Co-expression of PTK6 in conjunction with eEF1A2 has not previously been analyzed in breast cancer tissue. We found a significant correlation between PTK6 and eEF1A2 expression at the protein level (*p*-value 0.006) when IHC was carried out on breast tumor TMAs.

In 2007, Aubele *et al.* demonstrated that PTK6 is a prognostic marker of metastases-free survival in 193 breast tumors, and that it is independent of the classical morphological and molecular markers of lymph node involvement and tumour size (Aubele et al., 2007). In 2008, the same group reported that PTK6 protein expression in 426 breast tumors showed significant statistical correlation with the disease-free survival of patients but no correlation with lymph node status and tumor size (Aubele et al., 2008). However, when I analyzed 300 breast tumors to study the association of the histopathological parameters and PTK6, PTK6 overexpression was associated with only low grade tumors and the estrogen receptor positive tumors. No correlation was found with tumor size, lymph node status or with disease-free survival of patients. This could be attributed to the small number of tumors that I studied or those discrepant findings may be caused by different compositions of the tumor cohorts. This may influence and change the statistical results. Analysis of a larger set of tumors would give a better result of the correlation with those histopathological parameters.

Kulkarni *et al*. have reported that high eEF1A2 expression is a significant predictor of outcome. Individuals with tumors which display eEF1A2 protein overexpression have an increased probability of 20-year survival compared to those women whose tumors do not express substantial eEF1A2. Therefore they concluded that

eEF1A2 protein expression predicts increased survival probability in breast cancer patients (Kulkarni et al., 2007). In the 300 breast tumor that I analyzed, no significant statistical correlation was found between eEF1A2 and any of the histopathological parameters studied. This could be due to the usage of an eEF1A2 antibody different than the one used by their group as our antibody is not commercial (detecting both EEF1A1 and EEF1A2) but generated in our lab and therefore detecting only EEF1A2. However, a study with a larger number of tumors might give a better indication of any trend.

3.3.2 EEF1A2 and PTK6 copy number analysis in breast tumors

Protein overexpression usually correlates with increased gene copy number. However, a few reports showed that overexpression can be detected in the absence of gene amplification as shown with the *PPM1D, HDM2* and *DARPP32* genes (Lambros et al., 2010; Polsky et al., 2001; Varis et al., 2004). Real-time PCR showed that PTK6 has an increased copy number in breast tumors with a high expression in the TMAs. PTK6 amplification would therefore explain the mechanism by which the protein is overexpressed. PTK6 stimulates ErbB receptor tyrosine kinase signaling in cancer cells. Overexpression of PTK6 sensitizes mammary epithelial cells to mitogenic effects of EGF (Kamalati et al., 1996) and therefore its coexpression with ErbB3 enhances EGF signaling through AKT and PI-3 kinase pathways (Kamalati et al., 2000). Different ErbB receptor ligands such as EGF and heregulin, stimulate PTK6 activity (Kamalati et al., 2000; Ostrander et al., 2007).

However, since *EEF1A2* was thought to be located on the same amplicon, the hypothesis was that it would have a similar increase in gene copy number. When these same tumors were analyzed for copy number changes, *EEF1A2* did not show copy numbers as high as *PTK6*. Therefore other factors/modulators might effect EEF1A2 leading to high protein expression. Tomlinson *et al.* reported that at the RNA level, 33% of ovarian tumors showed overexpression of eEF1A2 (Tomlinson et al., 2007). The authors reported that *EEF1A2* copy number does not correlate with the expression level of the gene, the *EEF1A2* gene is unmethylated in both normal and tumor DNA and that no mutations were detected. This indicates that overexpression is not dependent on

genetic or epigenetic modifications at the *EEF1A2* locus at least in ovarian tumors. This has not been tested in breast tumors. However, another hypothesis for the overexpression of EEF1A2 is attributed to the roles miRNAs play in gene expression. Most studies have shown that miRNAs downregulate gene expression by binding to the 3'UTR and either degrading the mRNA or binding to the mRNA and inhibiting translation. However, in 2008, Dahiya *et al*. have reported the overexpression of eEF1A2 in the ovarian cancer cell line BG-1 when transfected with the miRNA let-7f (Dahiya et al., 2008). As mentioned, miRNAs usually repress translation by either binding to the mRNA or degrading it. In the case of EEF1A2, it is thought that let-7f binds to an inhibitor that deactivates EEF1A2 mRNA, leading to a high expression level. Another hypothesis is that EEF1A2 has a strong promoter that affects its expression and therefore would not require many copies of the gene. Bischoff *et al.* analyzed the promoter activity of *EEF1A2* in the human amnion AMA cells and delineated a minimal promoter region that is sufficient to drive transcription of the gene (Bischoff et al., 2000). They have shown that the myocyte enhancer factor-2 (MEF2) site did not affect promoter activity in AMA cells. However MEF2 has been reported to participate in the activation of genes specifically expressed in skeletal muscle, heart, somatic muscle, and neuronal tissues (Black and Olson, 1998). Since eEF1A2 is expressed in equivalent tissues, the authors suggested that MEF2 might act as a factor that activates the expression of eEF1A2.

On the other hand, several other factors including enhancers might affect the expression of eEF1A2. Newbery *et al.* carried out percentage identity plot (PIP) analysis of the human and mouse regions surrounding the gene encoding eEF1A2 (Newbery et al., 2007). This analysis was based on the region contained in a human PAC clone. Figure 3.25 shows a line-up of human and mouse gene sequences covering the whole region between eEF1A2 and PTK6 where the black line shows homology between human and mouse genes between 50 and 100%. No significant region of human/mouse homology was detected other than within known genes, with the exception of a 100 bp region upstream of EEF1A2 that showed 70% homology between human and mouse (circled in red). Homology suggests conservation due to functional elements in the 3kb upstream of eEF1A2 but –as shown in the figure- there is absolutely no homology up until the C20orf149 (Novel gene 2) coding sequence. Also, there is no homology until the 3' end

of *Ptk6*. These data do not conclude that there are no enhancers, but if there are any, they are not conserved and that EEF1A2 amplification is most probably not the mechanism leading to its overexpression.

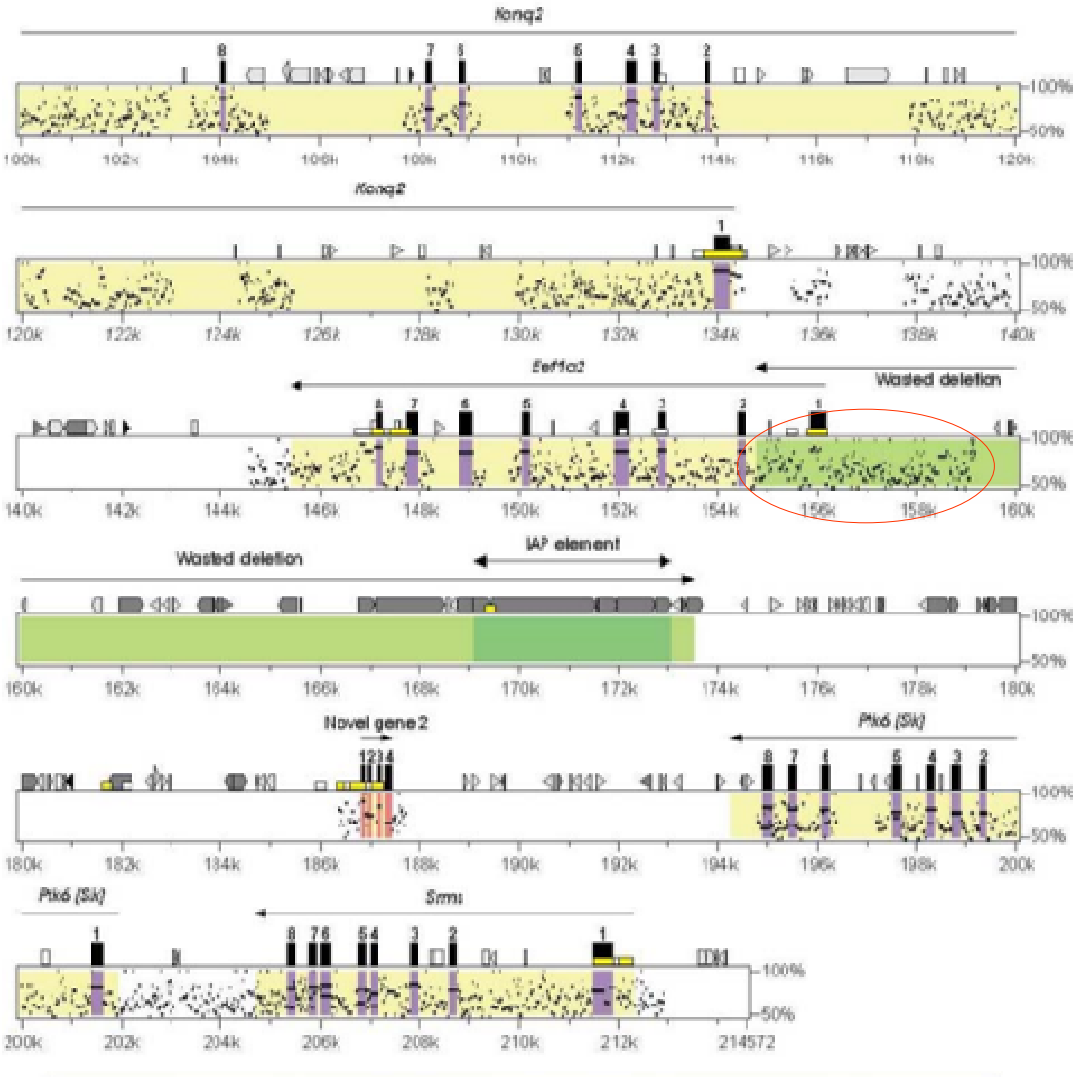

Figure 3.25 Percentage identity plot (PIP) of the region surrounding the wasted locus of *Eef1a2*. The nucleotide positions for the mouse sequence are shown on the x axis, and the percent identity with the corresponding human sequence is shown on the y axis (50-100%). Genes are labeled according to known cDNA sequences. Black lines indicate homology between human and mouse genes suggesting conservation due to functional elements in the 3kb or so upstream of eEF1A2 (circled in red). As shown, there is no homology up until the C20orf149 (Novel gene 2) coding sequence. Also, there is no homology until the 3' end of *Ptk6*. These data do not conclude that there are no enhancers, but if there are any, they are not conserved (Newbery et al., 2007).

SRMS and *KCNQ2* are two genes flanking *EEF1A2* and *PTK6*, therefore located on 20q13.3. Since these genes are within a 200kb distance and located on a region known to be amplified in breast cancer, the hypothesis was that both would show an increased gene copy number as was observed with *PTK6*. Surprisingly, both genes showed normal copies leading to the conclusion that only *PTK6* is amplified in this region.

3.3.3 Effect of Let-7f on EEF1A2 in breast cancer cell lines

EEF1A2 gene copy number did not correlate with high expression of the gene at the protein level detected in the breast tumor TMAs (section 3.2.4) and nor were the copy numbers as high as *PTK6*. Tomlinson *et al.* reported that EEF1A2 copy number does not correlate with the expression level of the gene in ovarian tumors, it is unmethylated in both normal and tumour DNA and that no mutations were detected (Tomlinson et al., 2007). This was not determined in breast cancer, however one of the hypothesis proposed was that miRNA might be involved in the overexpression of eEF1A2. miRNAs usually degrade mRNA or bind to it resulting in inhibition of translation and therefore decreasing protein expression. However, with eEF1A2, the only miRNA that has been directly demonstrated to affect its expression is let-7f, and this increases rather than decreases levels of eEF1A2 mRNA. The hypothesis is thus that the miRNA let-7f probably increases its expression by deactivating an inhibitor that binds to its mRNA. This is a different mechanism by which miRNAs usually repress translation by binding directly to the mRNA.

Dahiya *et al*. have shown that overexpression of the miRNA let-7f in ovarian cancer cell lines was associated with a high expression of eEF1A2 (Dahiya et al., 2008). EEF1A2 expression showed a 600 increase fold-change in the BG-1 let-7f transfected cells. To establish whether this was also true in a breast cancer cell line, I transfected BT549 –a breast cancer cell line that does not express eEF1A2 under normal conditions- with let-7f precursor. Unfortunately, these data were not conclusive (Details and results of the experiment are given in Appendix B).

The microarray results provided by Alexey Larionov (Figure 3.16) also differ from the results Dahiya *et al*. have shown where they reported an overexpression of

EEF1A2 in ovarian cancer cell transfected with let-7f (Dahiya et al., 2008). It is entirely possible that let-7f is not the only miRNA responsible for the overexpression of eEF1A2 in breast cancers. On the other hand, since let-7f is probably deactivating an inhibitor binding to eEF1A2, this intermediate could differ in breast cancer cells and therefore would show a difference in expression in ovarian tumors versus breast tumors.

In conclusion, PTK6 overexpression in breast cancer is the result of gene amplification leading to phosphorylation/activation of downstream substrates (i.e. Sam68, SLM-1, SLM-2, STAP-2, paxillin, STAT3, STAT5a and STAT5b) that would lead to the increased transcriptional activity and therefore mediates proliferation of breast cancer cells (Weaver and Silva, 2007). However, even though *EEF1A2* is mapped to the same 20q13.3 region, its overexpression is not associated with gene amplification. Other mechanisms/intermediates appear to play a role in its overexpression. This could be attributed to the hypothesis of the roles of miRNAs in binding to an inhibitor that deactivates eEF1A2 translation leading to an increased expression of eEF1A2 at the protein level or it could be due to the effect enhancers have on EEF1A2. Another hypothesis is that *EEF1A2* has a strong promoter that does not require several copies of the gene (Discussed in section 3.3.2).

Knockdown of *Eef1a2* expression in PCT cell lines affected the JAK/STAT pathway and the PI3K-AKT-mTOR pathway (Li et al., 2010). The authors reported that phosphorylation of STAT3 and AKT was delayed and that PI3K expression was reduced. This correlates well with previous studies showing that eEF1A2 appears to be upstream of AKT and PI3K (Amiri et al., 2007). These data indicate that eEF1A2 may play an important role in the induction or progression of plasma cell neoplasms in mice and humans. These mechanisms might also be true in breast cancer where induction of those pathways might increase cell division and lead to breast tumor development through activating several modulators, one of which might be the miRNA involvement. In contrast to our initial hypothesis that EEF1A2 and PTK6 act in tandem to increase the risk of developing breast cancer through similar mechanisms, it appears from the work done so far that both genes act independently and that each protein is overexpressed in breast cancer through a different mechanism.

3.3.4 EEF1A2 expression in breast tumors and breast cancer cell lines

As shown in figures 3.17 and 3.18, RPPA was carried out to detect the expression of EEF1A2 in 107 breast cancers using two different eEF1A2 antibodies. One was raised in sheep and the other antibody raised in rabbit. RPPA showed that EEF1A2 was detected in all breast cancers with two cancers having exceptionally high levels of EEF1A2.

Li *et al.* showed that knockdown of *Eef1a2* expression in Plasmacytoma (PCT) cell lines affected the JAK/STAT pathway suggesting that eEF1A2 may play an important role in the induction or progression of plasma cell neoplasms in mice and humans (Li et al., 2010). Peter Mullen has been working on STAT3 and pSTAT3 and has kindly provided the RPPA results shown in figures 3.21 and 3.22. Surprisingly, the same two cancerous tumors that showed an exceptionally high levels of EEF1A2 also showed high levels of STAT3 (Figure 3.17). Li *et al.* reported these finding in PCT cells whereas our results confirm the effect of EEF1A2 on STAT3 in breast cancers.

EEF1A2 was also expressed in all breast cancer cell lines on these RPPA slides but expression levels varied between different cell lines (Figures 3.19 and 3.20). STAT3 and pSTAT (Tyr705) have also been detected in all breast cancer cell lines and as EEF1A2, their expression level varied between cells (Figures 3.23 and 3.24).

In conclusion, as EEF1A2 is not usually known to be expressed in normal breast tissue, based on all the RPPA data mentioned above, it is clear that EEF1A2 protein is expressed in all the breast cancers studied in this experiment. Surprisingly, two of the cancerous tumors which showed an exceptionally high level of EEF1A2 have also detected high levels of STAT3. This supports a previous study published by Li *et al.* indicating that EEF1A2 affects the expression of STAT3 in PCT cells (Li et al., 2010).

Chapter 4: Pathways affected by the overexpression of EEF1A2

4.1 Introduction

The development and progression of cancer are accompanied by complex changes in the pattern of gene expression. Gene expression microarrays provide a powerful tool for studying these complex changes. Illumina BeadChips were used to analyze global gene-expression changes during transformation of NIH-3T3 mouse fibroblast cells by EEF1A2.

Results from the previous chapter showed that EEF1A2 and PTK6 are overexpressed at the protein level. Even though *PTK6* was amplified in those breast tumors, therefore explaining the overexpression pattern, EEF1A2 overexpression did not correlate with the gene copy number.

In this part of the project, I focused on the effect of EEF1A2 expression on cancer-related genes. This was carried out by using microarrays to assess a network of up-regulated and down-regulated cancer genes in NIH-3T3 mouse fibroblast cell lines stably transfected with *EEF1A2*.

In addition, to start to address the question of which gene -*EEF1A2* or *PTK6*- is the cancer driver and which one is the passenger, I carried out *EEF1A2* and *PTK6* transfections on cell lines and measured their expression level using real-time PCR.

4.2 Results

4.2.1 Effect of EEF1A2 expression in NIH-3T3 stable cell lines

4.2.1.1 Generation of NIH-3T3 stable cell lines

NIH-3T3 cells are mouse fibroblasts that do not express eEF1A2 under normal conditions. Justyna Janikiewicz –a previous PhD student in our lab- has generated NIH-3T3 stable cell lines expressing eEF1A2 (Janikiewicz, 2010). The usage of these stable cell lines was critical to my work and therefore has been described below:

To generate a stable cell line that expresses the protein of interest (eEF1A2) from a mammalian expression construct (section 2.2.6.2, figures 2.1 and 2.2), a killing curve was first established. The minimum concentration of selection antibiotic required to kill the NIH-3T3 untransfected host cell line was determined. Cells were plated for 24 hours after which the medium was removed and replaced by DMEM containing varying concentrations of Zeocin^{TM}. The medium was replenished every 3-4 days and the percentage of cells was determined every time. A concentration of 450μg/ml of Zeocin^{TM} was established as the minimal concentration that kills the majority of cells within two weeks of culturing.

Cells were harvested, counted and transfected with the EEF1A2 construct as described in section 2.2.6. Medium (DMEM + 10% NBCS + Zeocin) was replaced 24 hours later. This was repeated every 3-4 days until distinct cell colonies were observed after 2-4 weeks. Once colonies developed, cells from every individual colony were transferred and cultured in a separate plate. The cells were then collected and the presence of the insert was confirmed by PCR as well as by Western blots. Out of many stable cells generated, three that showed different expression level of eEF1A2 were chosen to examine the effect of EEF1A2 on cancer pathways. These three stable cell lines were named after the plate and colony number they were taken from where the first number is the plate number whereas the second number is the colony number: 7.2, 9.6 and 10.2. NIH-3T3 cells that were transfected with the

empty vector pcDNA3.1 GS was used as a negative control whereas EJTF2 cells that stably express the H-Ras^{G12V} oncogene were used as a positive control.

To determine the oncogenic potential of these three cell lines, Justyna Janikiewicz used the focus formation assays where cells that showed multi-layers and crisscrossed growth of spindle shapes were considered transformed (Figure 4.1). NIH-3T3 cells that express EEF1A2 resulted in an increased level of foci formation when compared to the negative control cells. The number of foci in the 7.2, 9.6 and 10.2 cells was 36, 37 and 39 respectively when compared to the NIH-3T3 negative control cells (Janikiewicz, 2010).

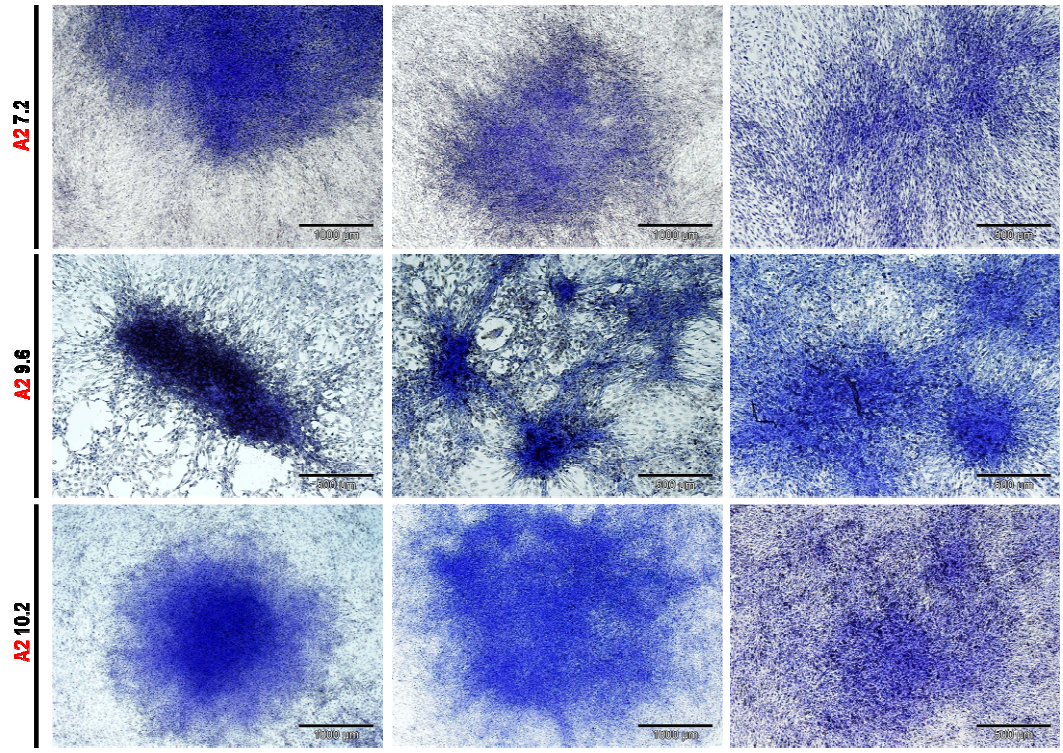

Figure 4.1 Effects of EEF1A2 overexpression on cellular morphology within the stable cell lines 7.2, 9.6 and 10.2. NIH-3T3 cells stably transfected with the EEF1A2 construct were cultured in zeocin-supplemented DMEM for determination of their capacity to produce foci. Three weeks later cells were fixed, stained with crystal violet and their morphology was photographed. Size bars are equal to 500µm and 1000µm (Janikiewicz, 2010).

The other assay conducted by Justyna to examine the oncogenic potential of these cells was the anchorage independent growth assay (Figure 4.2). The same control cells were used as described above. After three weeks in culture, it was noticed that all EEF1A2 overexpressing cells developed colonies in soft agar whereas no colonies were detected in the negative control cells. The number of colonies detected for the 7.2, 9.6 and 10.2 cells was 119, 131 and 207 respectively compared to the zero number of colonies formed by the NIH-3T3 negative control cells (Janikiewicz, 2010).

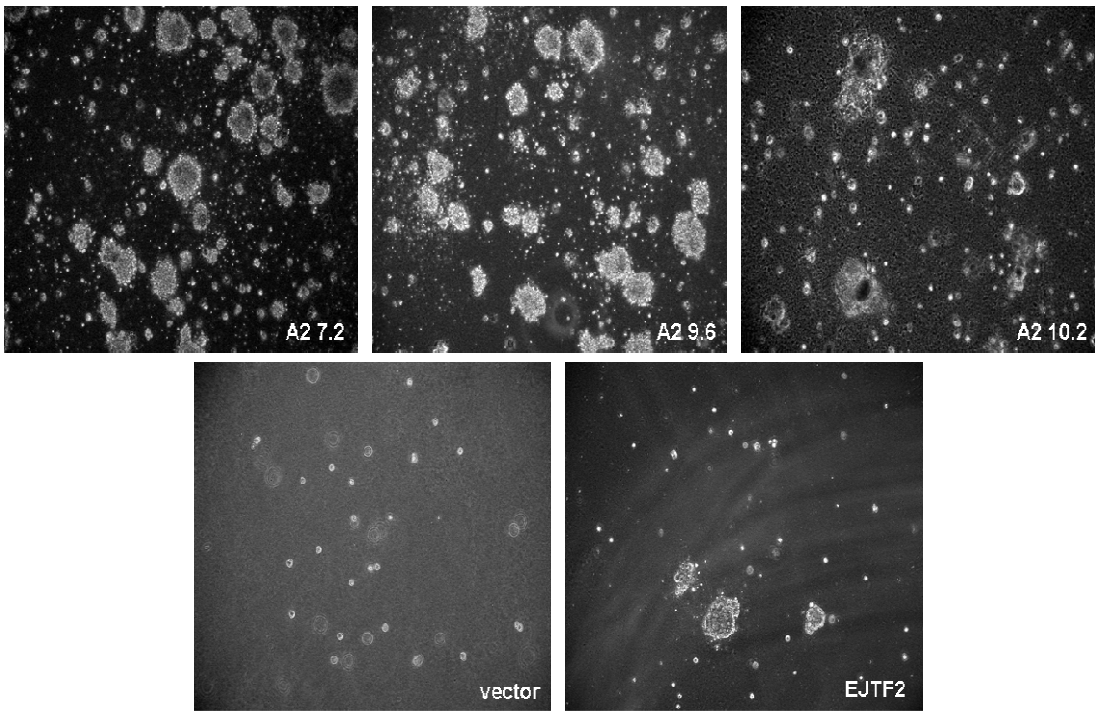

Figure 4.2 Ectopic expression of all EEF1A2 variants in NIH-3T3 cells induces anchorage independent growth. Following 21 days of culture in 0.3% layer of agar, colonies were counted and photographed (magnification 10x). NIH-3T3 cells stably transfected with empty vector were used as a negative control and 3 weeks after plating, single cells were observed within these wells. In contrast, substantial numbers of colonies were detected in dishes of EJTF2 positive control and cell lines stably expressing EEF1A2 (Janikiewicz, 2010)**.**

Cellular proliferation of these cells was also assessed and compared to the control cells (Figure 4.3). Justyna has shown that the proliferation rates of all the three stable cell lines 7.2, 9.6 and 10.2 were considerably higher than that of both control cell lines (Janikiewicz, 2010).

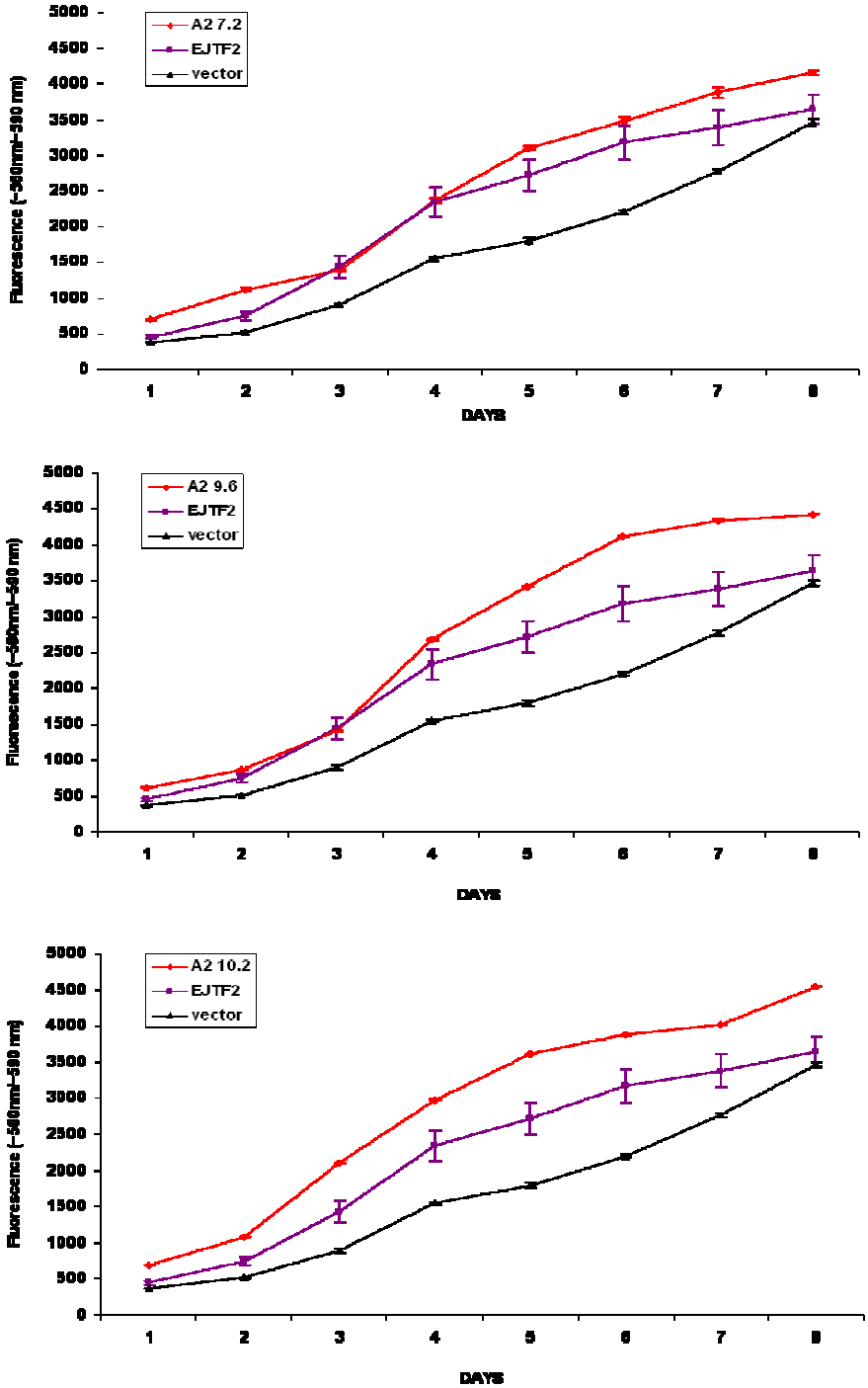

Figure 4.3 Effect of EEF1A2 overexpression on NIH-3T3 cells proliferation. The stable cell lines were subjected to determination of growth rate under standard culture conditions. Proliferation of EEF1A2 clones (7.2, 9.6, 10.2) was compared to the stable lines of empty-vector or EJTF2 cells. AlamarBlue® dye was applied to the culture media of the cells and fluorescence was measured up to 8 days after seeding. The results of growth magnitude are represented as the average fluorescence intensity at indicated time point ±SEM calculated from three separate experiments, each performed in five replicates (Janikiewicz, 2010).

It was critical to assess for continued maintenance and expression of EEF1A2 in these cells and to check the primers before carrying out the experiments mentioned in this chapter, therefore PCR was carried out on the NIH-3T3 control cells, 7.2, 9.6 and 10.2. As shown in figure 4.4, the right EEF1A2 155bp band size was detected in all the stable cells lines but not in the control cells nor in the –RT control reaction.

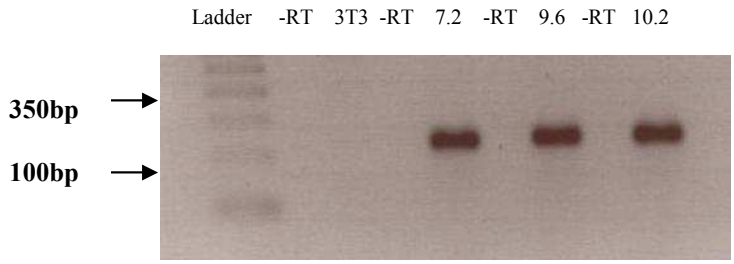

Figure 4.4 PCR on NIH-3T3 stable cell lines detecting EEF1A2 cDNA of the right 155bp band size on an agarose gel. 3T3, 7.2, 9.6 and 10.2 are the stable cell lines tested. No bands detected in the reactions lacking the Reverse Transcriptase (–RT) enzyme in the PCR reactions.

These assays clearly indicate that the three stable cell lines have incorporated EEF1A2 and have developed an oncogenic phenotype. Based on these results, the three stable cell lines were chosen for this project to further understand the role of *EEF1A2* as a potential oncogene.

4.2.1.2 Microarray analysis on the stable cell lines

It was reported that eEF1A2 is involved in tumorigenesis by affecting many cancer-related pathways (Amiri et al., 2007; Li et al., 2010). In this part of the project, this is going to be examined in NIH-3T3 stable cell lines generated in our lab using microarrays to assess a network of up- and down-regulated genes.

To determine the effect of eEF1A2 on other pathways, in particular cancer-related pathways, microarray analysis was performed as described in section 2.2.7. Before labeling the RNA and hybridizing it to the BeadChips, western blots were carried out. V5 tag antibody was used to detect the presence of V5-tagged eEF1A2 protein (Figure 4.5). The EEF1A2 antibody could not be used as the present aliquot was noticed to detect unspecific proteins/bands.

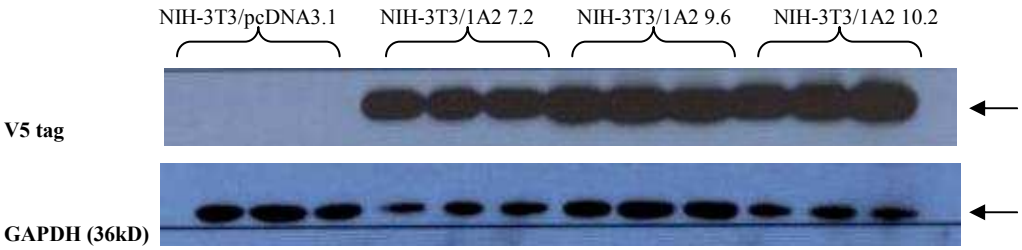

Figure 4.5 Western blots detecting V5 tag that is binding to eEF1A2, indicating the presence of the eEF1A2 protein in NIH-3T3 cells stable cells lines: 3T3, 7.2, 9.6 and 10.2. The experiment was carried out in triplicate hence the three lanes for each stable cell line.

Before performing the analysis on the microarray data, a number of pre-processing steps including background adjustment, probe filtering, normalization and quality control checks were performed using the R package, lumi (www.bioconductor.org). Boxplots of the microarray data before and after quantile normalization demonstrate how the distributions of the intensities are scaled to have equal means and variance across samples (Figure 4.6)

The R software was also used to give an unsupervised overview of gene expression across the three cell lines stably expressing EEF1A2: 7.2, 9.6 and 10.2 (Figure 4.7). Figure 4.7a shows a heatmap generated from the analysis of all probes (more than 47,000 probes) whereas figure 4.7b shows a heatmap of the 500 most variable genes. As shown, the heatmaps produced from the R software allowed us to visualize the differential action of these genes between the three stable cell lines. It is clear that these stable cells can be distinguished from each other in terms of gene expression.

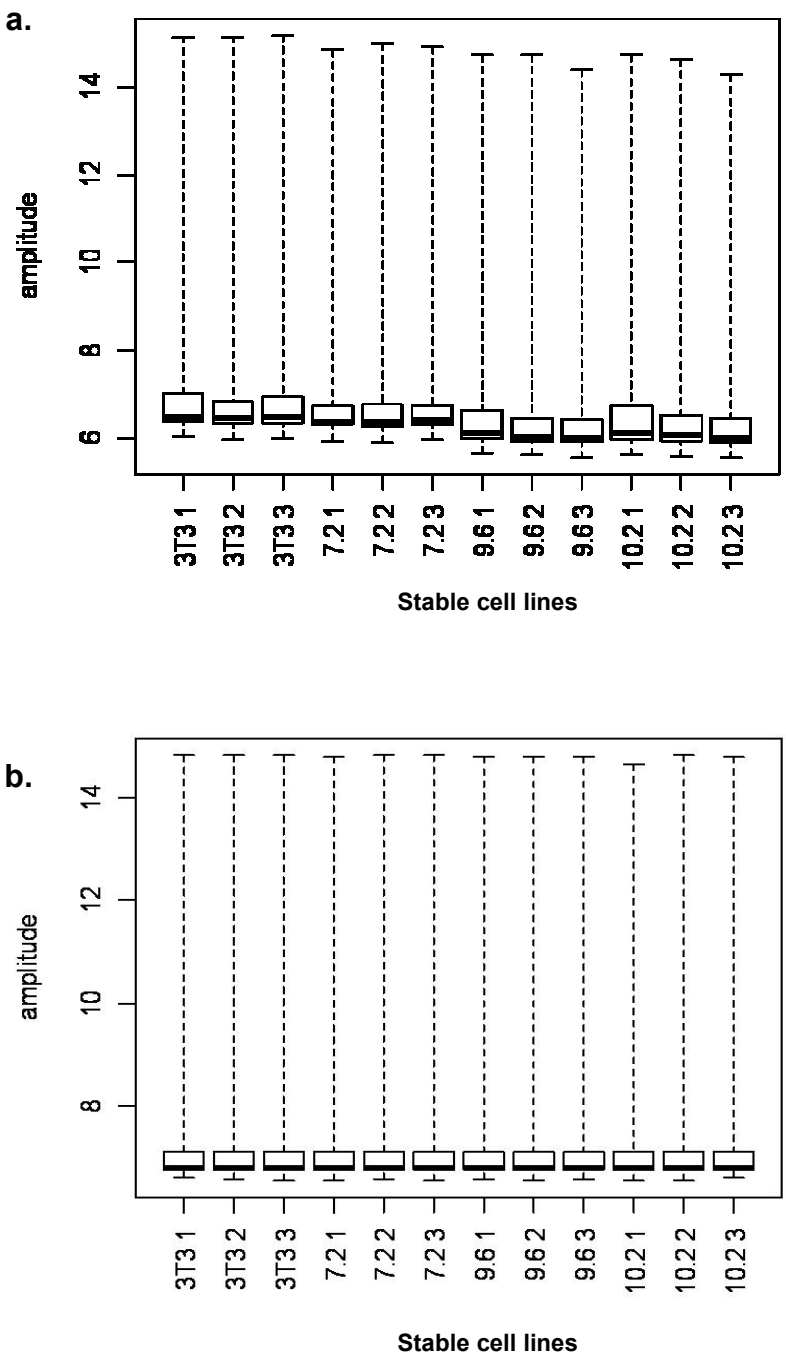

Figure 4.6 Boxplots of microarray intensities a) before normalization, b) after normalization. The experiment was carried out in triplicate on each stable cell lines: 3T3, 7.2, 9.6 and 10.2 hence the numbers 1, 2 and 3 following each stable cell line in the figure.

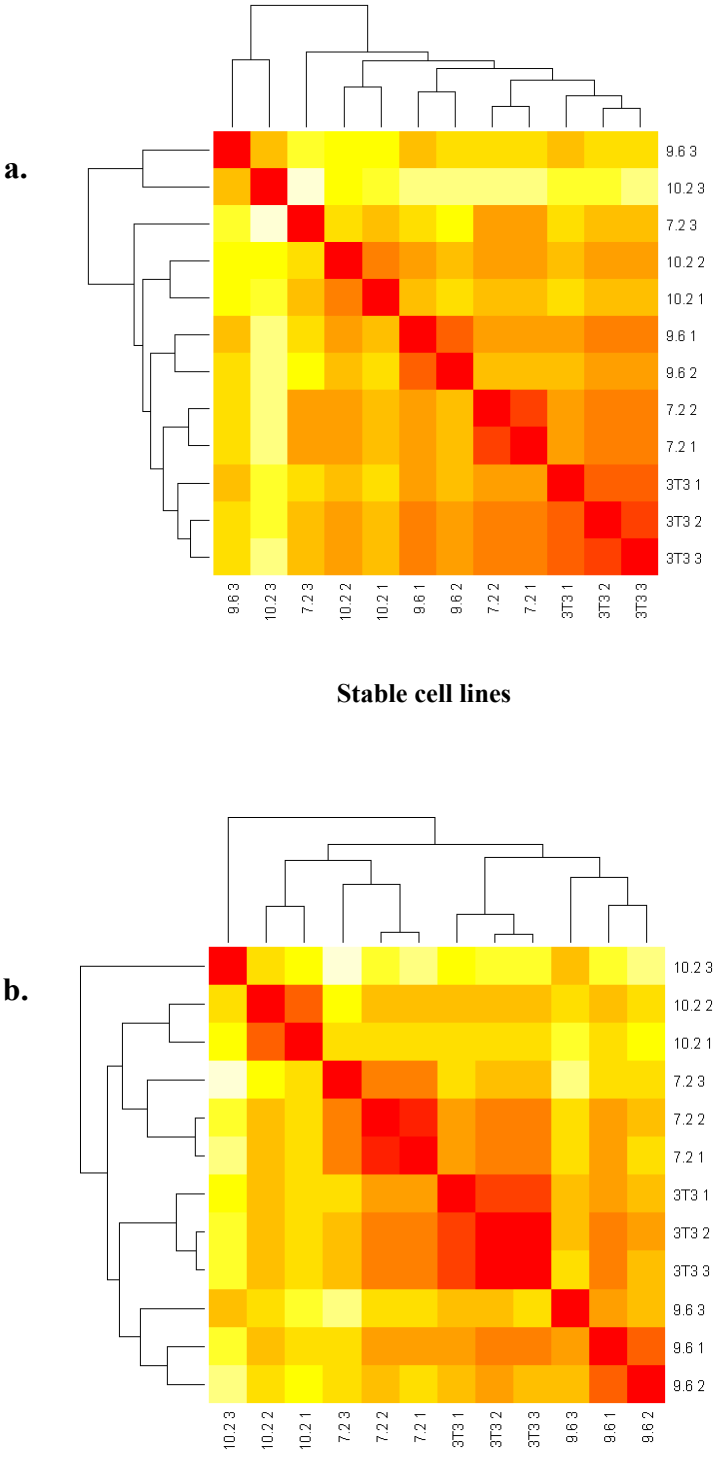

Figure 4.7 Heatmaps produced by the Bioconductor function (R software) from the microarray data of all stable cell lines showing a) a heatmap generated from the analysis of all probes and b) a heatmap of the 500 most variable genes. Red represents the strongest correlation, white the weakest with shades of yellow/orange in between. The experiment was carried out in triplicate on each stable cell lines: 3T3, 7.2, 9.6 and 10.2 hence the numbers 1, 2 and 3 following each stable cell line in the figure.

Microarray analysis comparing NIH-3T3/pcDNA3.1 cells with the stably transfected cells (7.2, 9.6 and 10.2 that express eEF1A2) resulted in a long list of genes that were upregulated (Table 4.1 & 4.2) and downregulated (Table 4.3 & 4.4), potentially due to the expression of EEF1A2. Table 4.1 shows a list of the top 33 upregulated genes, where the cutoff point was an average fold-change increase of 1.9 and higher, whereas table 4.2 shows the same genes along with their individual fold-change increases and the significance in the three stable cell lines compared to the control. As shown in table 4.1, 14 of those 33 upregulated genes are associated with different types of cancers. Since this project is focusing on breast cancer, five of the upregulated genes that are associated with human breast cancer have been chosen for further analysis. These genes are *Serpine2*, *Egr1*, *Aldh3a1*, *Tpd52* and *Dusp6*.

SERPINE2 is located on 2q36.1. It is a serine protease inhibitor gene that has been linked to many cancers especially breast cancer and breast cancer metastasis (Candia et al., 2006). It showed the highest average fold-change increase (FC 19.6) in the microarray list of genes affected by the overexpression of EEF1A2 with a statistically significant *p*-value of 0.0004 and 0.0009 in both 7.2 (FC 24.7) and 10.2 (FC 35.3) stable cell lines respectively (Table 4.2). In the stable cell lines 9.6, there was no significant correlation between Serpine2 and EEF1A2 expression (*p*-value 0.1).

Early growth response factor 1 (*EGR1*) is a nuclear protein mapped to 5q31.1 that functions as a transcriptional regulator. The products of target genes it activates are required for differentiation and mitogenesis. It has also been reported to act as a tumor suppressor gene (Krones-Herzig et al., 2005). It showed an average 2.9-fold change (FC) increase which was statistically significant in the two stable cells lines 7.2 (*p*-value 0.01, FC 1.7) and 9.6 (*p*-value 0.02, FC 3.5) (Table 4.2).

Aldehyde dehydrogenase 3 family, member A1 (*ALDH3A1*) is another gene associated with breast cancer. It is mapped to 17p11.2. It is involved in the detoxification of alcohol-derived acetaldehyde and in the metabolism of corticosteroids, neurotransmitters, and lipid peroxidation. It showed an average fold change increase of 2.4 in the microarray upregulated gene list that was significant in

all three stable cell lines 7.2 (*p*-value 1.21E-06, FC 3.6), 9.6 (*p*-value 0.02, FC 1.5) and 10.2 (*p*-value 0.01, FC 2) (Table 4.2).

Tumor protein D52 (*TPD52*), identified in the 8q21 amplicon, is a gene amplified and overexpressed in many different types of cancers including breast cancer. It is potentially involved in vesicle trafficking. TPD52 showed an average 2.2-fold change increase on the gene list. Its upregulation was statistically significant in all three stable cell lines: 7.2 (*p*-value 0.001, FC 1.1), 9.6 (*p*-value 7.11E-06, FC 1.5) and 10.2 (*p*-value 4.15E-06, FC 4.1) (Table 4.2).

Dual specificity phosphatase 6 (DUSP6) –also known as MKP3- negatively regulates members of the MAP kinase family which are associated with cellular proliferation and differentiation. *DUSP6* is located on 12q21.33 and showed an average fold-change increase of 2 in the stable cell lines. Its overexpression was statistically significant in all stable cell lines: 7.2 (*p*-value 0.001, FC 1.7), 9.6 (*p*-value 7.11E-06, FC 1.5) and 10.2 (*p*-value 4.15E-06, FC 2.9) (Table 4.2).

SYMBOL	DEFINITION	Average FC	Type of cancer
Serpine2		19.6	Beast, Testicular metastasis
Srpx2	Mus musculus sushi-repeat-containing protein, X-linked 2 (Srpx2), transcript variant 2, mRNA.	3.5	
Mgp	Mus musculus matrix Gla protein (Mgp), mRNA.	3.2	
Egr1	Mus musculus early growth response 1 (Egr1), mRNA.	2.9	Suppressor gene, bladder, prostate, breast
Saa3	Mus musculus serum amyloid A 3 (Saa3), mRNA.	2.7	
Pkia	Mus musculus protein kinase inhibitor, alpha (Pkia), mRNA.	2.5	
Actg2	Mus musculus actin, gamma 2, smooth muscle, enteric (Actg2), mRNA.	2.4	
Prelp	Mus musculus proline arginine-rich end leucine-rich repeat (Prelp), mRNA.	2.4	
Slc12a2	Mus musculus solute carrier family 12, member 2 (Slc12a2), mRNA.	2.4	
Aldh3a1	Mus musculus aldehyde dehydrogenase family 3, subfamily A1 (Aldh3a1), mRNA.	2.4	Breast, lung cancers
Ier3	Mus musculus immediate early response 3 (Ier3), mRNA.	2.3	Squamous cell carcinoma
Anxa8	Mus musculus annexin A8 (Anxa8), mRNA.	2.2	Leukemia
Tpd52	Mus musculus tumor protein D52 (Tpd52), transcript variant 5, mRNA.	2.2	Prostate, ovarian, breast, liver, colorectal
Slc12a2	Mus musculus solute carrier family 12, member 2 (Slc12a2), mRNA.	2.2	
Ctgf	Mus musculus connective tissue growth factor (Ctgf), mRNA.	2.2	Breast, rhabdomyosarcoma
Dusp1	Mus musculus dual specificity phosphatase 1 (Dusp1), mRNA.	2.2	Ovarian, lung cancers
Arl6ip1	Mus musculus ADP-ribosylation factor-like 6 interacting protein 1 (Arl6ip1), mRNA.	2.1	
Srpx2	Mus musculus sushi-repeat-containing protein, X-linked 2 (Srpx2), transcript variant 2, mRNA.	2.1	
Hist1h1c	Mus musculus histone cluster 1, H1c (Hist1h1c), mRNA.	2.1	
Cyr61	Mus musculus cysteine rich protein 61 (Cyr61), mRNA.	2.1	Breast cancer
Srpx2	Mus musculus sushi-repeat-containing protein, X-linked 2 (Srpx2), mRNA.	2.1	
Inhba	Mus musculus inhibin beta-A (Inhba), mRNA.	2.0	
Dusp6	Mus musculus dual specificity phosphatase 6 (Dusp6), mRNA.	2.0	Breast, Pancreas cancer
Iap		2.0	
Rgs16	Mus musculus regulator of G-protein signaling 16 (Rgs16), mRNA.	2.0	
Nuak1	Mus musculus NUAK family, SNF1-like kinase, 1 (Nuak1), mRNA.	2.0	Colorectal
Junb	Mus musculus Jun-B oncogene (Junb), mRNA.	2.0	Lymphoma
Hist2h2aa1	Mus musculus histone cluster 2, H2aa1 (Hist2h2aa1), mRNA.	1.9	
Gm773	Mus musculus gene model 773, (NCBI) (Gm773), mRNA.	1.9	
Phlda1	Mus musculus pleckstrin homology-like domain, family A, member 1 (Phlda1), mRNA.	1.9	
Fxyd5	Mus musculus FXYD domain-containing ion transport regulator 5 (Fxyd5), mRNA.	1.9	Gastric, metastasis
Tspan6	Mus musculus tetraspanin 6 (Tspan6), mRNA.	1.9	Breast cancer
Hist2h2aa2	Mus musculus histone cluster 2, H2aa2 (Hist2h2aa2), mRNA.	1.9	

Table 4.1 Microarray analysis. Top 33 upregulated genes with an average fold change increase of 1.9 and higher. Genes highlighted in pink are associated with breast cancer and are further studied in this project (FC = Fold Change).

Stable cells	NIH-3T3/1A2 7.2		NIH-3T3/1A2 9.6		NIH-3T3/1A2 10.2		Average FC
Gene Symbol	FC	*p*-value	FC	*p*-value	FC	*p*-value	
Serpine2	**24.7**	**0.0004**	**-1**	**0.1**	**35.3**	**0.0009**	**19.6**
Srpx2	1.9	0.0004	5	0.00004	3.4	0.0007	3.5
Mgp	3.2	0.0001	2.1	0.02	4.3	0.02	3.2
Egr1	**1.7**	**0.01**	**3.5**	**0.02**	**3.4**	**0.07**	**2.9**
Saa3	1.2	0.05	2.9	0.02	4.2	0.02	2.7
Pkia	1.6	0.0003	2.5	0.002	3.6	0.04	2.5
Actg2	1.5	0.01	1.3	0.03	4.5	0.005	2.4
Prelp	1.1	0.07	1.7	0.01	4.3	0.05	2.4
Slc12a2	1.4	0.02	3.5	0.05	2.3	0.08	2.4
Aldh3a1	**3.6**	**0.000001**	**1.5**	**0.02**	**2**	**0.01**	**2.4**
Anxa8	2.9	0.0003	1.6	0.0003	2.2	0.01	2.2
Tpd52	**1.1**	**0.001**	**1.5**	**0.000007**	**4.1**	**0.000004**	**2.2**
Slc12a2	1.4	0.02	2.8	0.001	2.3	0.000003	2.2
Ctgf	1.8	0.03	1.6	0.01	3.1	0.03	2.2
Dusp1	1.1	0.009	2.2	0.1	3.1	0.05	2.2
Arl6ip1	-1.8	0.1	4.8	0.0002	3.4	0.0006	2.1
Hist1h1c	1.8	0.00001	2.2	0.000005	2.2	0.0008	2.1
Cyr61	1.6	0.0008	1.7	0.003	2.9	0.003	2.1
Srpx2	1.5	0.0001	2.5	0.004	2.2	0.02	2.1
Inhba	2.6	0.003	1.5	0.004	2	0.0009	2.0
Dusp6	**1.7**	**0.03**	**1.5**	**0.0001**	**2.9**	**0.001**	**2.0**
Iap	1	0.003	1.1	0.0009	3.8	0.003	2.0
Rgs16	1.8	0.02	2.2	0.04	1.9	0.01	2.0
Nuak1	1.7	0.6	1.9	0.7	2.4	0.1	2.0
Hist2h2aa1	1.5	0.0001	1.9	0.001	2.3	0.005	1.9
Gm773	3.8	0.0004	1	0.01	1	0.08	1.9
Phlda1	3	0.05	1.1	0.002	1.7	0.002	1.9
Fxyd5	3.1	0.04	1.6	0.05	1.1	0.04	1.9
Tspan6	1.4	0.0001	1.7	0.07	2.6	0.4	1.9
Hist2h2aa2	1.5	0.2	2	0.4	2.2	0.2	1.9
Pdk4	1.2	0.002	2.4	0.06	2	0.6	1.9
Myl9	1.1	0.001	1.5	0.0003	3	0.001	1.9
Tfrc	1.8	0.01	1.9	0.04	1.8	0.05	1.8

Table 4.2 Top 33 genes with the largest fold-changes from the microarray data showing the fold-change increase (in pink) of all the three stable cell lines along with their *p*-value (in blue).
Highlighted genes are breast cancer associated genes studied in this project.

Microarray analysis comparing NIH-3T3/pcDNA3.1 cells with the stably transfected cells (7.2, 9.6 and 10.2 that express eEF1A2) also resulted in a long list of downregulated genes.

Table 4.3 shows a list of the 32 most downregulated genes, where the cutoff point was an average fold-change decrease of 1.7 and lower, whereas table 4.4 shows the same genes along with their individual fold-change decrease and their significance in the three stable cell lines separately. The most downregulated five genes are *Rhox5*, *Renbp*, *Ppap2c*, *H60a* and *Itga11* with only *Ppap2c* being previously associated with cancer in the literature (Tanyi et al., 2003).

The reproductive homeobox-5 (RHOX5) gene encodes a transcription factor which showed the most decreased fold-change in the microarray list with 4.9. Loss of *Rhox5* in mice causes increased male germ-cell apoptosis, reduced sperm count, and reduced sperm motility (Maclean et al., 2005). The authors reported that the reduced sperm count is probably due to apoptosis as cell proliferation was not significantly affected. Rhox5 is expressed in Sertoli cells promoting survival and differentiation of the adjacent male germ cells. In the microarray list, Rhox5 was the most downregulated gene. Since this gene has not been studied in breast cancer previously, its downregulation might play a similar apoptotic role as it does in male germ-cells and therefore act as a good therapeutic target in cancer. Its downregulation was statistically significant in all three stable cell lines: 7.2 (*p*-value 0.0004, FC -2.1), 9.6 (*p*-value 0.005, FC -2.0) and 10.2 (*p*-value 4E-06, FC -11) (Table 4.4).

The second most downregulated gene that showed a 3.1 fold-change decrease is *Renbp* (Renin Binding Protein). Renbp is involved in the regulation of the renin-angiotensin system which plays an important role in cardiovascular and renal physiology and disease. Therefore downregulation of this gene would have a negative impact especially on the renal and cardiovascular systems. *RENBP* is mapped to Xq28 and its downregulation was statistically significant in all three stable cell lines: 7.2 (*p*-value 2.73E-06, FC -3.3), 9.6 (*p*-value 0.002, FC -1.7) and 10.2 (*p*-value 1.72E-06, FC -4) (Table 4.4).

Ppap2c (phosphatidic acid phosphatase type 2C) is the third most downregulated gene in the microarray list with a fold-change decease of 2.3. The protein encoded by this gene is a member of the phosphatidic acid phosphatase (PAP) enzyme family. PPAP2C is significantly overexpressed in ovarian carcinoma (Tanyi et al., 2003), bladder, lung, and prostate cancers compared with normal tissues (Flanagan et al., 2009). Flanagan *et al.* also reported that PPAP2C is particularly highly expressed in breast carcinoma. Therefore its downregulation might be associated with decreased risk of breast tumorigenesis. *Ppap2c* is mapped to 19p13.3 and its downregulation was statistically significant only in 7.2 (*p*-value 5.67E-06, FC -4.4) but not in 9.6 (*p*-value 0.06, FC -1.0) nor in 10.2 (*p*-value 0.2, FC -1.3) stable cell lines (Table 4.4).

Histocompatibility 60a (*H60a*) gene showed a fold-change decrease of 2.2 in the microarray list making it the forth most downregulated gene. H60a is an MHC class I-like glycoprotein and is one of the ligands for the natural killer cell receptor NKG2D. Downregulation of H60a was statistically significant only in 9.6 (*p*-value 0.002, FC -4.1) but not in 7.2 (*p*-value 0.2, FC -1.5) nor in 10.2 (*p*-value 0.6, FC -1.1) stable cell lines (Table 4.4).

ITGA11 (Integrin alpha-11), mapped to 15q23, is a protein that in humans is encoded by the *ITGA11* gene. It is a cell surface adhesion receptor mediating cell-adhesion to extra cellular matrix or to other cells. It showed a 2.2 fold-change decrease in the microarray list of genes from the NIH-3T3 stable cell lines expressing EEF1A2. Since this gene is important in cell-adhesion, its downregulation as detected in this study would probably facilitate cell detachment mediating cell migration. Downregulation of Itga11 was statistically significant in the two stable cell lines 7.2 (*p*-value 2.26E-05, FC -2.7) and 9.6 (*p*-value 2.47E-05, FC -2.7) but not in 10.2 (*p*-value 0.8, FC -1.1) (Table 4.4).

SYMBOL	Definition	Ave FC
Il1rl1	Mus musculus interleukin 1 receptor-like 1 (Il1rl1), transcript variant 2, mRNA.	-1.7
Kcnab1	Mus musculus potassium voltage-gated channel, shaker-related subfamily, beta member 1 (Kcnab1), mRNA.	-1.7
Adi1	Mus musculus acireductone dioxygenase 1 (Adi1), mRNA.	-1.7
2300002D11Rik	Mus musculus RIKEN cDNA 2300002D11 gene (2300002D11Rik), mRNA.	-1.7
Ramp3	Mus musculus receptor (calcitonin) activity modifying protein 3 (Ramp3), mRNA.	-1.7
LOC100044411	PREDICTED: Mus musculus similar to Epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (LOC100044411), mRNA.	-1.7
B230343A10Rik		-1.7
Atp6ap2	Mus musculus ATPase, H+ transporting, lysosomal accessory protein 2 (Atp6ap2), mRNA.	-1.7
Soat1	Mus musculus sterol O-acyltransferase 1 (Soat1), mRNA.	-1.7
Ptn	Mus musculus pleiotrophin (Ptn), mRNA.	-1.7
Rpl31	Mus musculus ribosomal protein L31 (Rpl31), mRNA.	-1.7
Bdh2	Mus musculus 3-hydroxybutyrate dehydrogenase, type 2 (Bdh2), mRNA.	-1.8
Cenpa	Mus musculus centromere protein A (Cenpa), mRNA.	-1.8
Dbp	Mus musculus D site albumin promoter binding protein (Dbp), mRNA.	-1.8
Ctsk	Mus musculus cathepsin K (Ctsk), mRNA.	-1.8
Ramp3	Mus musculus receptor (calcitonin) activity modifying protein 3 (Ramp3), mRNA.	-1.8
Gstm2	Mus musculus glutathione S-transferase, mu 2 (Gstm2), mRNA.	-1.8
Csrp2	Mus musculus cysteine and glycine-rich protein 2 (Csrp2), mRNA.	-1.8
Actr3b	Mus musculus ARP3 actin-related protein 3 homolog B (yeast) (Actr3b), mRNA.	-1.8
2810417K24Rik		-1.9
EG633692	PREDICTED: Mus musculus predicted gene, EG633692 (EG633692), mRNA.	-1.9
Uap1l1	Mus musculus UDP-N-acteylglucosamine pyrophosphorylase 1-like 1 (Uap1l1), mRNA. XM_918982	-1.9
Dhcr24	Mus musculus 24-dehydrocholesterol reductase (Dhcr24), mRNA.	-1.9
Prkg2	Mus musculus protein kinase, cGMP-dependent, type II (Prkg2), mRNA.	-2.0
Spon2	Mus musculus spondin 2, extracellular matrix protein (Spon2), mRNA.	-2.0
Gsta4	Mus musculus glutathione S-transferase, alpha 4 (Gsta4), mRNA.	-2.0
Cyp2c55	Mus musculus cytochrome P450, family 2, subfamily c, polypeptide 55 (Cyp2c55), mRNA.	-2.1
Itga11	Mus musculus integrin, alpha 11 (Itga11), mRNA.	-2.2
H60a	Mus musculus histocompatibility 60a (H60a), mRNA.	-2.2
Ppap2c	Mus musculus phosphatidic acid phosphatase type 2c (Ppap2c), mRNA.	-2.3
Renbp	Mus musculus renin binding protein (Renbp), mRNA.	-3.1
Rhox5	Mus musculus reproductive homeobox 5 (Rhox5), mRNA.	-4.9

Table 4.3 Microarray analysis. Downregulated 32 genes with -1.7 fold-change decrease and lower (FC = Fold Change). Itga11, H60a, Ppap2c, Renbp and Rhox5 are genes studied further in this project.

Stable cells	NIH-3T3/1A2 7.2		NIH-3T3/1A2 9.6		NIH-3T3/1A2 10.2		Average FC
Gene Symbol	FC	*p*-value	FC	*p*-value	FC	*p*-value	
Il1rl1	-1.4	0.006	-2	0.004	-2.0	0.02	-1.7
Kcnab1	-1.4	0.006	-2	0.0002	-1.7	0.002	-1.7
Adi1	-1.3	0.08	-1.5	0.01	-2.2	0.003	-1.7
2300002D11Rik	-1.4	0.0005	-1.9	0.0005	-1.7	0.003	-1.7
Ramp3	-1.1	0.2	-1.8	0.0002	-2.2	0.0003	-1.7
LOC100044411	-2.3	0.003	-1.6	0.04	-1.2	0.2	-1.7
B230343A10Rik	-1.5	0.0006	-1.6	0.001	-2.0	0.0001	-1.7
Atp6ap2	-1.5	0.01	-1.3	0.04	-2.3	0.001	-1.7
Soat1	-1.2	0.1	-1.9	0.0003	-2.1	0.004	-1.7
Ptn	-2.1	0.002	-1.2	0.1	-2	0.0009	-1.7
Rpl31	-1.0	0.7	-1.7	0.01	-2.5	0.001	-1.7
Bdh2	-1.6	0.0002	-1.7	0.02	-2.0	0.008	-1.8
Cenpa	-1.6	0.001	-1.7	0.002	-2.0	0.002	-1.8
Dbp	-1.5	0.002	-1.3	0.02	-2.5	0.0001	-1.8
Ctsk	-2.5	0.00001	-1.7	0.0003	-1.2	0.04	-1.8
Ramp3	-1.2	0.002	-1.9	0.0001	-2.4	0.0003	-1.8
Gstm2	-2.3	0.00003	-1.1	0.5	-2.1	0.003	-1.8
Csrp2	-1.5	0.01	-2.1	0.001	-1.9	0.004	-1.8
Actr3b	-1.0	0.8	-2.1	0.001	-2.4	0.002	-1.8
2810417K24Rik	-1.5	0.2	-2.2	0.02	-2	0.0004	-1.9
EG633692	-1.5	0.03	-1.4	0.05	-2.7	0.002	-1.9
Uap1l1	-1.7	0.001	-1.6	0.0004	-2.4	0.0002	-1.9
Dhcr24	-1.5	0.05	-2.7	0.004	-1.5	0.03	-1.9
Prkg2	-1.2	0.04	-2.0	0.002	-2.7	0.0002	-2.0
Spon2	-1.5	0.04	-3.0	0.001	-1.4	0.09	-2.0
Gsta4	-1.2	0.1	-2	0.003	-3	0.0007	-2.0
Cyp2c55	-2.1	0.0009	-1.6	0.007	-2.5	0.001	-2.1
Itga11	-2.7	0.00002	-2.7	0.00002	-1.1	0.8	-2.2
H60a	-1.5	0.2	-4.1	0.002	-1.1	0.6	-2.2
Ppap2c	-4.4	0.00001	-1	0.06	-1.3	0.2	-2.3
Renbp	-3.2	0.000003	-1.7	0.002	-4	0.000002	-3.1
Rhox5	-2.1	0.0004	-2.0	0.005	-11	0.000004	-4.9

Table 4.4 The 32 most downregulated genes from the microarray data showing the fold-change decrease (in pink) of all the three stable cell lines along with their p-value (in blue). FC= Fold-Change.

4.2.1.3 Expression level of upregulated breast cancer genes

Real-time PCR was carried out on the four stable cell lines to establish the validity of the microarray results (Described in section 3.2.3). Expression level of all five breast cancer-associated genes in the NIH-3T3 stable cell lines (*Serpine2*, *Egr1*, *Aldh3a1*, *Tpd52* and *Dusp6*) were analyzed (Table 4.5, Figures 4.8 & 4.9). Figure 4.8 is shown based on the expression level of each gene in all the stable cell lines, whereas figure 4.9 shows the same results but arranged so that it shows the expression level of all five genes in a single stable cell line instead.

Table 4.5 Analyzing gene expression level of Serpine2, Egr1, Aldh3a1, Tpd52 & Dusp6 in the stable cell lines using the $2^{-\Delta\Delta Ct}$ method. (Ct gene= average Ct values of the gene studied, Ct ref genes= average Ct values of all the reference genes, ΔCT= difference in CT values).

NIH-3T3 cells	Ct gene	Ct ref genes	ΔCT	ΔΔCT	2^-ΔΔCT
Serpine2					
NIH-3T3	37.09	27.93	9.16	0	1
7.2	30.96	28.16	2.80	-6	82
9.6	37.09	27.68	9.41	0	1
10.2	30.91	27.86	3.05	-6	69
Egr1					
NIH-3T3	31.77	27.93	3.84	0	1
7.2	30.16	28.16	2.00	-2	4
9.6	30.18	27.68	2.50	-1	3
10.2	30.19	27.86	2.32	-2	3
Aldh3a1					
NIH-3T3	35.63	27.93	7.70	0	1
7.2	30.70	28.16	2.55	-5	36
9.6	33.92	27.68	6.25	-1	3
10.2	32.37	27.86	4.50	-3	9
Tpd52					
NIH-3T3	35.82	27.93	7.89	0	1
7.2	35.27	28.16	7.11	-1	2
9.6	35.62	27.68	7.94	0	1
10.2	33.14	27.86	5.28	-3	6
Dusp6					
NIH-3T3	31.73	27.93	3.80	0	1
7.2	30.96	28.16	2.80	-1	2
9.6	31.30	27.68	3.62	0	1
10.2	30.22	27.86	2.35	-1	3

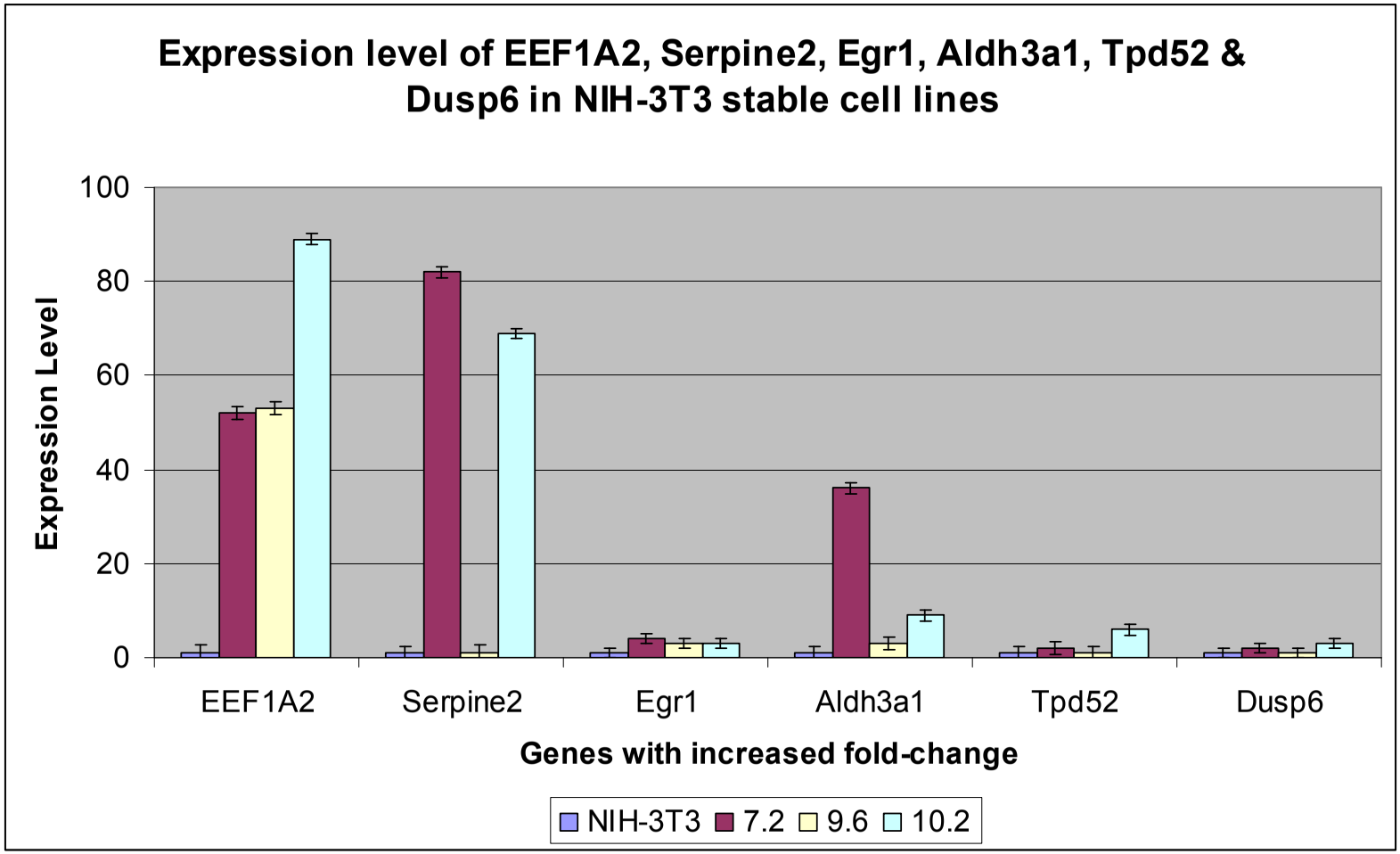

Figure 4.8 Expression level of EEF1A2 and the top 5 upregulated breast cancer-associated genes in the NIH-3T3 stable cell lines. The top five genes were normalized to the reference genes and then expressed relatively to the expression level in NIH-3T3 cells.

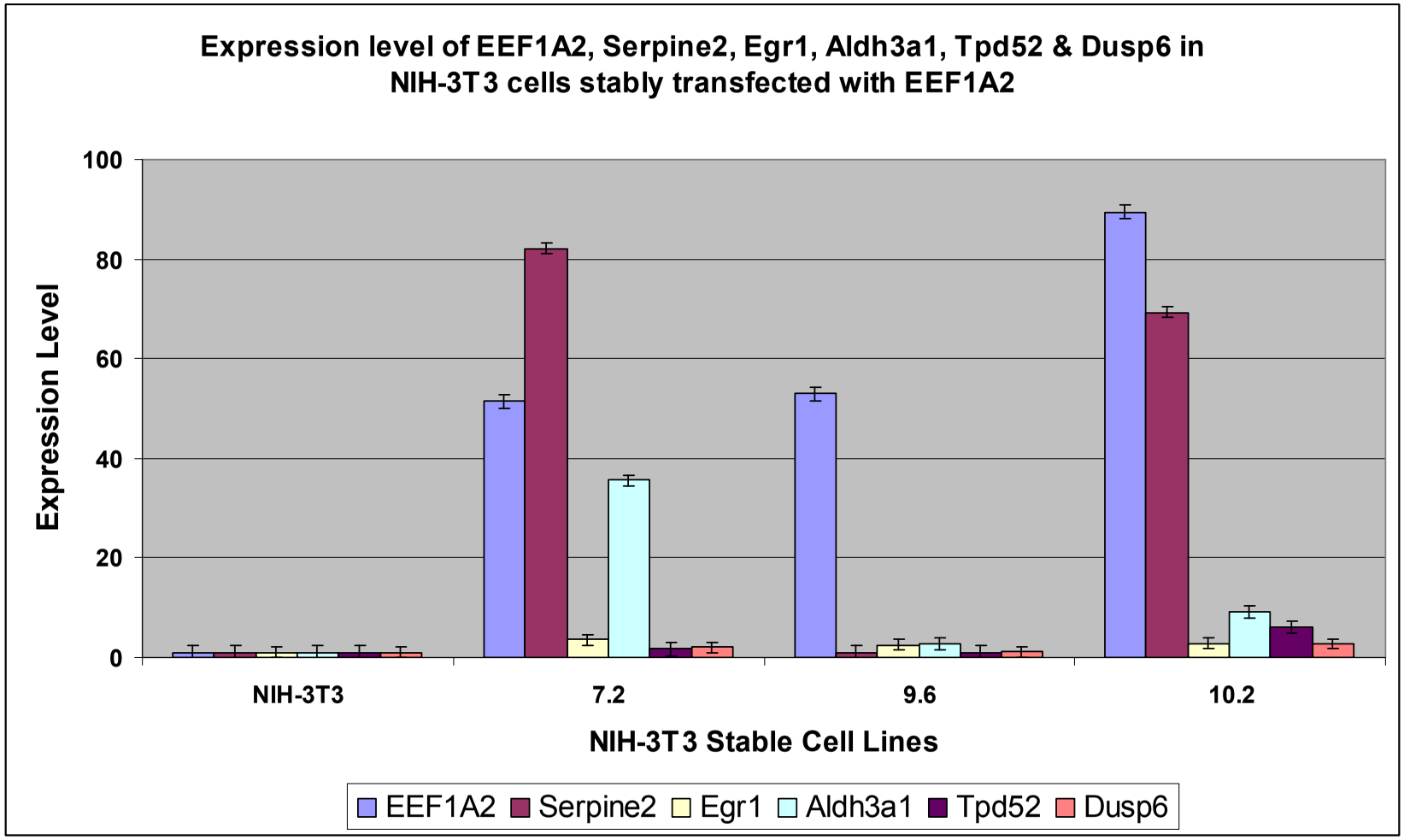

Figure 4.9 Expression level of EEF1A2 along with the top 5 upregulated beast cancer-associated genes varies in the NIH-3T3 stable cell lines. The top five genes were normalized to the reference genes and then expressed relatively to the expression level in NIH-3T3 cells.

As shown in figure 4.8 and table 4.6 below, the expression level of all five genes in the three stable cell lines from the microarray data (7.2, 9.6 and 10.2) corresponded to the expression level detected by real-time PCR. NIH-3T3 cells do not express EEF1A2 under normal conditions. In the stable cell lines (Figure 4.8) EEF1A2 expression level was the lowest in 7.2 cells and the highest in 10.2 cells. Serpine2 –which showed the highest average fold-change increase (FC 19.6) in the microarray list of genes- showed an increased expression in 7.2 and 10.2 cells but it was not detected in 9.6 cells. These results are similar to the fold-change increase detected in the microarray data. Egr1, Tpd52 and Dup6 are not normally expressed in NIH-3T3 cells. In the stable cells lines, all three genes showed a slight increase in the expression level detected in the microarray data and confirmed by the real-time PCR results. As for Aldh3a1 -which showed an average fold-change increase of 2.4- it showed the highest fold-change increase in the 7.2 cells (FC ~ 4) and slightly lower (FC ~ 2) in the 10.2 cells.

Table 4.6 Average fold-change of all 5 breast cancer-associated genes in all three stable cell lines along with their real-time PCR expression level.

Stable cells	7.2 cells		9.6 cells		10.2 cells		Average FC
Gene	FC	qPCR	FC	qPCR	FC	qPCR	
Serpine2	24.7	82	-1.0	1	35.3	69	19.6
Egr1	1.7	4	3.5	3	3.4	3	2.9
Aldh3a1	3.6	36	1.5	3	2.0	9	2.4
Tpd52	1.1	2	1.5	1	4.1	6	2.2
Dusp6	1.7	2	1.5	1	2.9	3	2.0

4.2.1.4 Protein detection of top 5 breast cancer-associated genes from the microarray data in the stable cell lines

Serpine2, *Egr1*, *Aldh3a1*, *Tpd52* and *Dusp6* are five genes that showed an increased fold-change expression in the microarray data. This was confirmed by detecting the same expression at the mRNA level using real-time PCR. To detect whether those 5 genes were expressed at the protein level, Western blots were carried out on the 7.2, 9.6 and 10.2 stable cell lines (Figure 4.10). MCF7 breast cancer cells were used as a control as they are known to express many proteins in cancer.

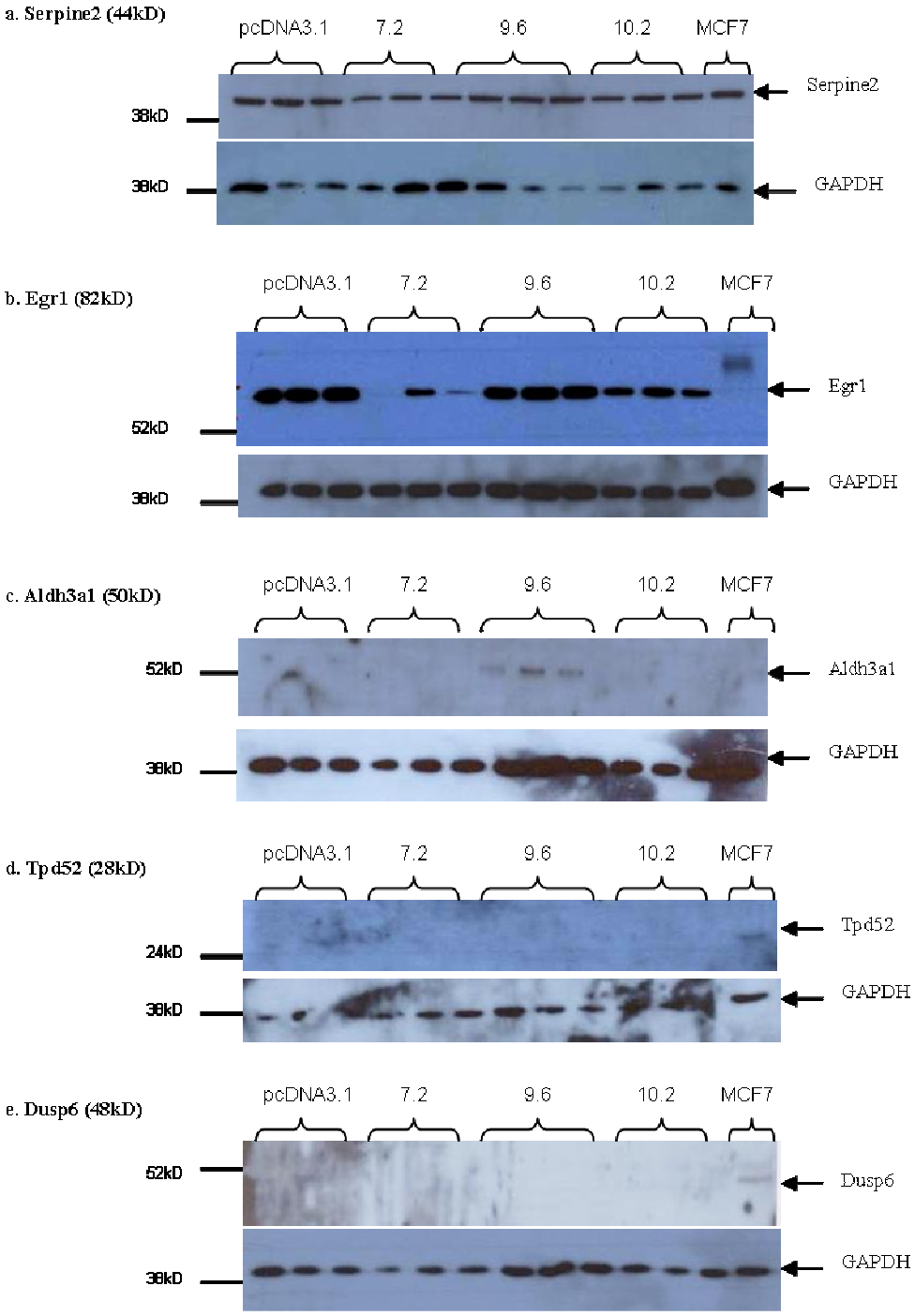

Figure 4.10 Western blots on NIH-3T3/pcDNA3.1, 7.2, 9.6 and 10.2 stable cell lines detecting a) Serpine2, b) Egr1, c) Aldh3a1, d) Tpd52 and e) Dups6. MCF7 breast cancer cells were used as a control as they usually express EEF1A2 and other cancer-related proteins. Each experiment was carried out in triplicate hence the three lanes of proteins for each of the stable cell lines.

SERPINE2 protein was expressed in all stable cells lines including MCF7 cells (Figure 4.10a). Its expression in the 9.6 cells at the mRNA level was similar to the control cells yet as shown from the microarray results, 7.2 and 10.2 cells showed an increased expression when compared to the control cells (Table 4.6 and figure 4.8).

EGR1 which did not show an average fold-change increase as high as Serpine2, was detected at the protein level in all stable cell lines but was slightly decreased in 7.2 cells (Figure 4.10b). The expression level however from the real-time PCR results (Table 4.6) did not show a high increase in the three stable cell lines when compared to the control cells nor was the expression level different between those three cells.

ALDH3A1 showed an increased fold-change at the mRNA level in both 7.2 and 10.2 stable cell lines. The opposite was observed in Western blots. ALDH3A1 was not detected in 7.2 and 10.2 cell lines but a band was seen in the 9.6 cells (Figure 4.10c). This might probably be due to the antibody detecting/cross-reacting with another protein. Aldh3a1 is not usually detected in MFC7 cells as shown.

TPD52 and DUSP5 also showed a fold-change increase at the mRNA level in the microarray data and were slightly increased in the 10.2 cells when real-time PCR was carried out (Table 4.6). These two proteins are not normally expressed in NIH-3T3 cells. As shown in figures 4.10d and 4.10e, neither were detected at the protein level in the NIH-3T3 cells nor in any of the stable cell lines. Both these proteins are expressed in MCF7 cells.

4.2.2 Effect of EEF1A2 expression in NIH-3T3 transiently transfected cells

NIH-3T3 cells were transiently transfected with the same EEF1A2 expression construct used in the stable cell lines. This was done to determine whether EEF1A2 would affect the same genes (*Serpine2*, *Egr1*, *Aldh3a1*, *Tpd52* and *Dusp6*) in the transiently transfected cells when compared to the stable cell lines.

EEF1A2 construct (construct details described in Chapter 2, section 2.2.6.2 and figure 2.1) was transfected into NIH-3T3 cells and cells were collected 24 and 48 hours after transfection. Real-time PCR was carried out as described in section 2.2.5 to detect the expression level of all the five breast cancer-associated genes (Table 4.7).

Table 4.7 Analyzing gene expression level of Serpine2, Egr1, Aldh3a1, Tpd52 & Dusp6 in NIH-3T3 cells transiently transfected with EEF1A2 using the $2^{-\Delta\Delta Ct}$ method.

NIH-3T3 cells	Ct gene	Ct ref genes	ΔCT	ΔΔCT	2^-ΔΔCT
Serpine2					
NIH-3T3	35.42	29.22	6.2	0	1
NIH-3T3/EEF1A2 24h	33.72	28.64	5.09	-1	2
NIH-3T3/EEF1A2 48h	33.96	29.7	4.26	-2	4
Egr1					
NIH-3T3	32.76	29.22	3.54	0	1
NIH-3T3/EEF1A2 24h	32.71	28.64	4.07	1	1
NIH-3T3/EEF1A2 48h	32.93	29.7	3.23	0	1
Aldh3a1					
NIH-3T3	34.51	29.22	5.29	0	1
NIH-3T3/EEF1A2 24h	32.32	28.64	3.68	-2	3
NIH-3T3/EEF1A2 48h	33.70	29.7	4	-1	2
Tpd52					
NIH-3T3	36.88	29.22	7.66	0	1
NIH-3T3/EEF1A2 24h	36.91	28.64	8.27	1	1
NIH-3T3/EEF1A2 48h	36.46	29.7	6.76	-1	2
Dusp6					
NIH-3T3	32.34	29.22	3.12	0	1
NIH-3T3/EEF1A2 24h	31.47	28.64	2.83	0	1
NIH-3T3/EEF1A2 48h	34.30	29.7	4.6	1	0

Tables 4.7 and 4.8, and figure 4.11 show the real-time PCR expression level analysis from the transient transfection of eEF1A2 in NIH-3T3 cells 24 and 48 hours post-transfection. Cell viability was >95% and apoptosis was minimal. As expected, the expression level of EEF1A2 was high 24 hours after transfection and was slightly decreased 48 hours later. Surprisingly, Serpine2 which showed the highest expression level in the stable cell lines in both the microarray data and real-time PCR results, showed no significant expression at the mRNA level in the transiently transfected cells whether 24 or 48 hours after transfection.

ALDH3A1 is not normally expressed in NIH-3T3 cells. In the stable cell lines, ALDH3A1 showed an increased expression, however, in the transiently transfected cells, no expression was observed.

EGR1, TPD52 and DUSP6 did not show a change in expression level whether in the transiently transfected cells or in the stable cell lines expressing EEF1A2.

Proteins were extracted from these transiently transfected NIH-3T3 cell lines and Western blots were carried out. As expected, SERPINE2, EGR1, ALDH3A1, TPD52 and DUSP6 proteins were not detected (data not shown). This was not surprising as no mRNA was detected when real-time PCR was carried out. To further assess and monitor the efficiency of the transient transfection, cells could be co-transfected with the Green Fluorescent Protein (GFP). This would be followed by using fluorescence-activated cell sorting (FACS) or the fluoresence microscope to detect the percentage of the cells expressing the fluorescent protein.

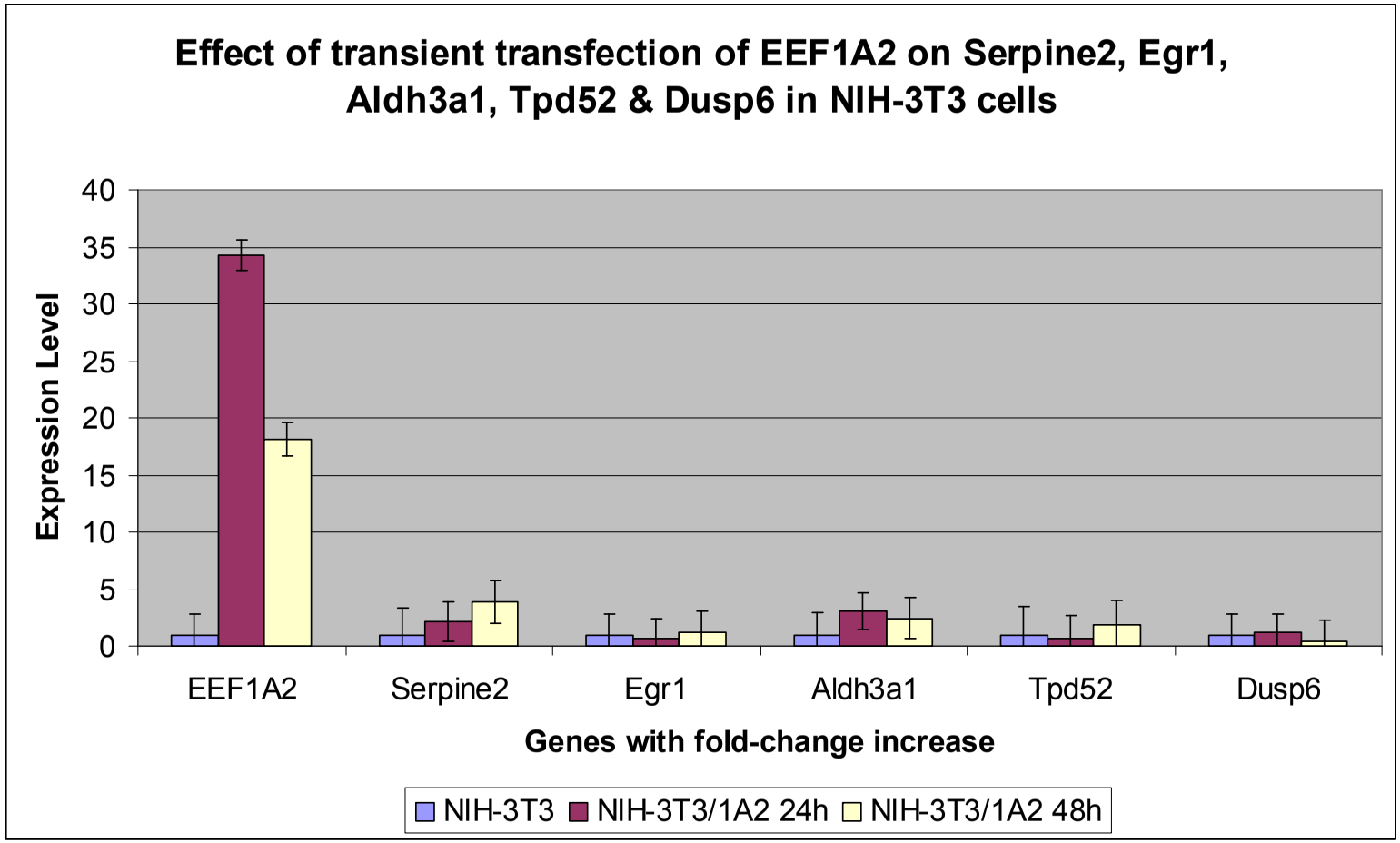

Figure 4.11 Transient transfection of EEF1A2 in NIH-3T3 cells showing the expression level of EEF1A2 along with the top 5 upregulated beast cancer-associated genes detected in the microarray data. The top five genes were normalized to the reference genes and then expressed relatively to the expression level in NIH-3T3 cells.

4.2.3 Effect of EEF1A2 expression on PTK6

EEF1A2 and *PTK6* are both located on 20q13.3 and are less than 50kb apart. Since both are overexpressed in 60% of breast cancer patients and are considered to be oncogenes, it is thought that one gene would be the driver of the other on this amplicon. This hypothesis was examined first in the stable cell lines expressing EEF1A2 and then in two transiently transfected cells that do not express EEF1A2 nor PTK6 under normal conditions: NIH-3T3 and BT549 breast cancer cell line.

4.2.3.1 Effect of EEF1A2 expression on PTK6 in stable cell lines

As shown in section 4.2.1.2, table 4.8 below and figure 4.12, EEF1A2 was expressed in the NIH-3T3 stably transfected cells 7.2, 9.6 and 10.2. The expression level of PTK6 was analyzed in these cells and showed no significant increase at the PTK6 mRNA level using real-time PCR. Since the database indicates the presence of few PTK6 splice variants, it is not certain if these primers would recognize other unidentified isoforms. In the microarray list however, there was only one probe for PTK6 which showed an average fold-change increase of 0.3 which was not statistically significant in any of the stable cell lines.

As shown below in table 4.8, even though eEF1A2 is not known to be detected in NIH-3T3 cells, the $2^{-\Delta\Delta Ct}$ value always gives a value of one. This is because the amount of eEF1A2 was first normalised to the reference genes and then expressed relatively to the expression level in NIH-3T3 cells (=1).

Table 4.8 Analyzing gene expression level of EEF1A2 &PTK6 in NIH-3T3 cells stably transfected with EEF1A2 using the $2^{-\Delta\Delta Ct}$ method.

Sample ID	Ct EEF1A2	Ct ref genes	ΔCt	ΔΔCt	2^-ΔΔCt
NIH-3T3	38.53	27.93	10.60	0	1
NIH-3T3/1A2 7.2	33.07	28.16	4.91	-6	52
NIH-3T3/1A2 9.6	32.55	27.68	4.87	-6	53
NIH-3T3/1A2 10.2	31.98	27.86	4.12	-6	89
Sample ID	**Ct PTK6**	**Ct ref genes**	**ΔCt**	**ΔΔCt**	**2^-ΔΔCt**
NIH-3T3	37.00	27.93	9.07	0	1
NIH-3T3/1A2 7.2	35.36	28.16	7.20	-2	4
NIH-3T3/1A2 9.6	37.00	27.68	9.32	0	1
NIH-3T3/1A2 10.2	35.94	27.86	8.08	-1	2

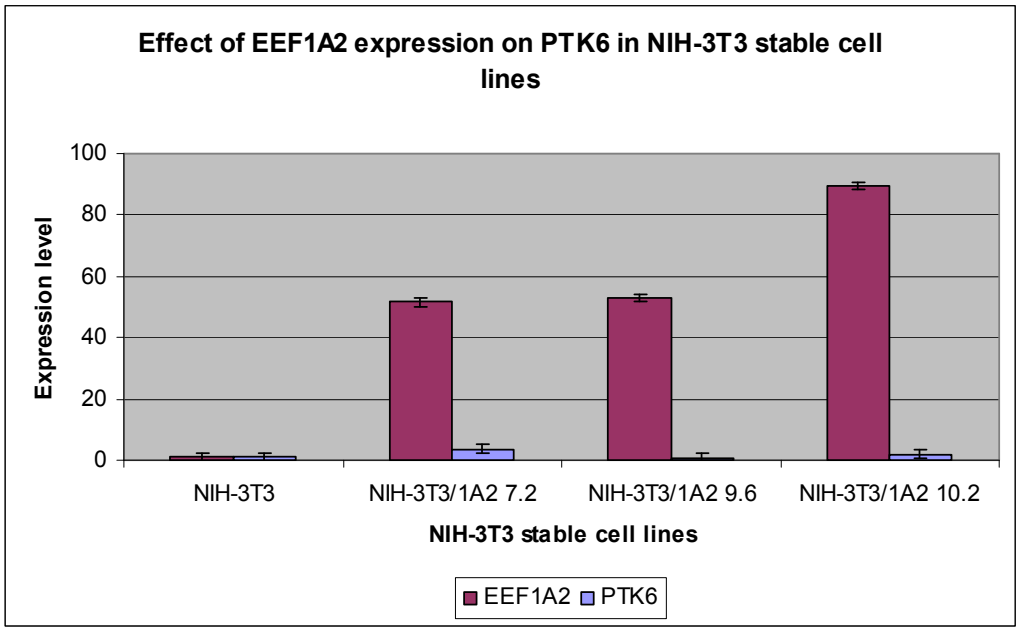

Figure 4.12 Expression of EEF1A2 and PTK6 in NIH-3T3 stable cell lines showing no significant change in PTK6 expression. EEF1A2 and PTK6 transcripts were normalized to the reference genes (Tbp, B2M and 18s rRNA) and then expressed relatively to the expression level in NIH-3T3 cells.

4.2.3.2 Effect of EEF1A2 expression on PTK6 in transiently transfected cells

4.2.3.2.1 Transient transfection of EEF1A2 into NIH-3T3 fibroblast cells

The EEF1A2 construct was transfected into NIH-3T3 cells as described in section 2.2.6 and the expression level of both eEF1A2 and PTK6 was analyzed. The expression level of eEF1A2 was increased after 24 hours and decreased slightly by 48 hours whereas no significant change in PTK6 expression was observed (Table 4.9 and figure 4.13).

Table 4.9 Analyzing gene expression level of EEF1A2 & PTK6 in NIH-3T3 cells transiently transfected with EEF1A2 using the $2^{-\Delta\Delta Ct}$ method.

NIH-3T3 cells	Ct gene	Ct ref genes	ΔCT	ΔΔCT	2^-ΔΔCT
EEF1A2					
NIH-3T3	35.43	29.22	6.21	0	1
NIH-3T3/EEF1A2 24h	29.75	28.64	1.11	-5	34
NIH-3T3/EEF1A2 48h	31.73	29.70	2.03	-4	18
PTK6					
NIH-3T3	38	29.22	8.78	0	1
NIH-3T3/EEF1A2 24h	38	28.64	9.36	1	1
NIH-3T3/EEF1A2 48h	38	29.70	8.30	0	1

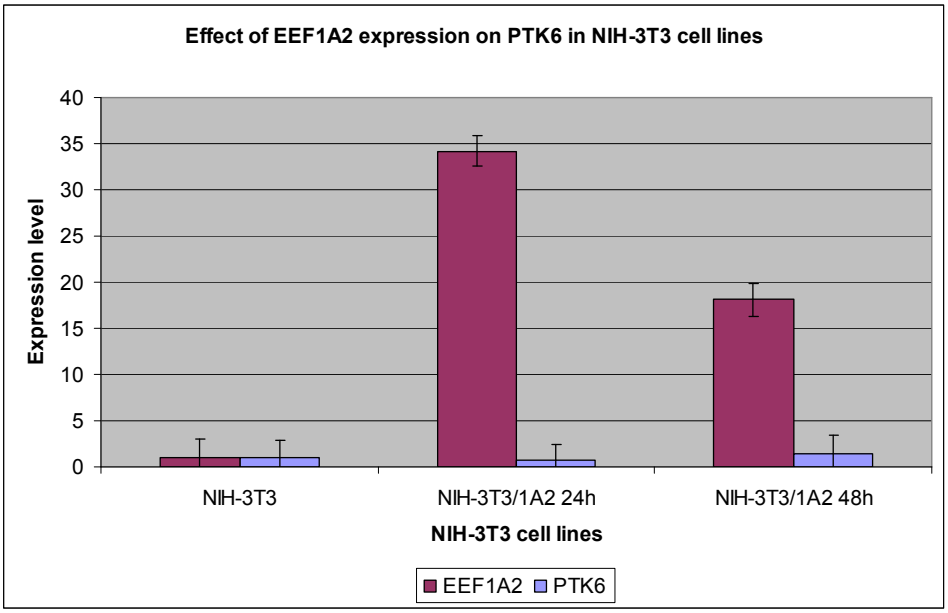

Figure 4.13 Transient transfection of EEF1A2 in NIH-3T3. Expression of EEF1A2 shows no significant change in PTK6 expression 24hrs and 48hrs after transfection. EEF1A2 and PTK6 were normalized to the reference genes (Tbp, B2M and 18s rRNA) and then expressed relatively to the expression level in NIH-3T3 cells.

4.2.3.2.2 Transient transfection of EEF1A2 into BT549 breast cancer cells

The EEF1A2 construct was transfected into the breast cancer cell line BT549. This cell lines also does not express either EEF1A2 or PTK6 under normal conditions. The expression level of both genes was detected using real-time PCR. EEF1A2 showed an increased expression 24 hours after transfection whereas no PTK6 expression was observed (Table 4.10 and figure 4.14).

Table 4.10 Analyzing gene expression level of EEF1A2 & PTK6 in BT549 breast cancer cells transiently transfected with EEF1A2 using the $2^{-\Delta\Delta Ct}$ method.

BT549 cells	Ct gene	Ct ref genes	ΔCt	ΔΔCt	2^-ΔΔCt
EEF1A2					
BT549 cells	36.75	30.21	6.54	0	1
BT549/EEF1A2	26.11	28.57	-2.45	-9	510
PTK6					
BT549 cells	34.51	30.27	4.24	0	1
BT549/EEF1A2	33.59	28.69	4.90	1	1

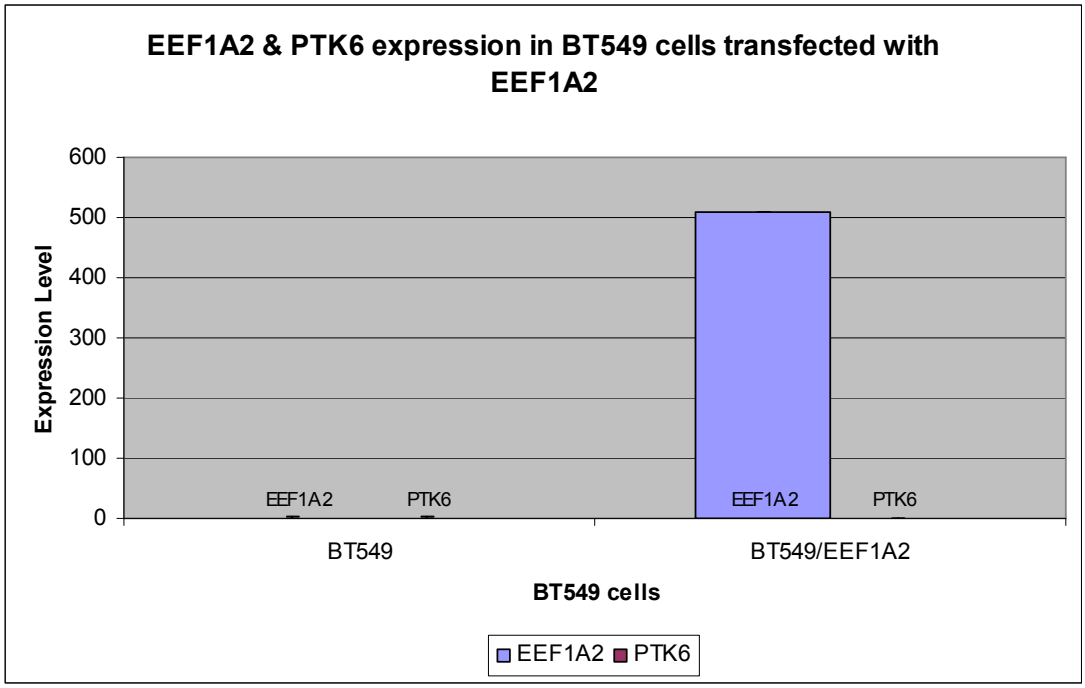

Figure 4.14 Transient transfection of EEF1A2 in BT549 breast cancer cell line. Expression of EEF1A2 shows no significant change in PTK6 expression. EEF1A2 and PTK6 were normalized to the reference genes (PUM1 and TBP) and then expressed relatively to the expression level in BT549 cells.

Western blots were also carried out to check for the protein expression in these cells. Since the V5-tag is part of the EEF1A2 construct, an anti-V5 antibody was used to check for the expression of EEF1A2. As shown below, even though there was an unequal protein loading in the wells, V5 was detected in the BT549 transfected cells but not in the MOCK cells nor in MCF7 cells (Figure 4.15).

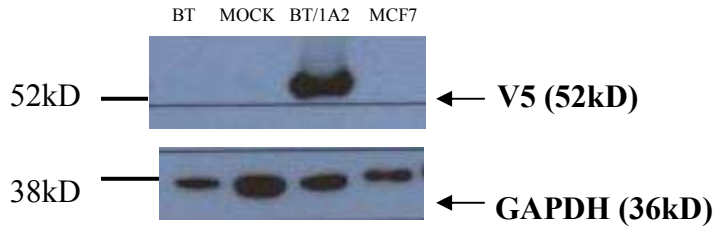

Figure 4.15 Western blot on BT549 breast cancer cells transiently transfected with EEF1A2. Anti-V5 tag antibody was used to detect the presence of EEF1A2. No protein was detected in BT549 cells nor in the MOCK cells whereas a protein band was detected in the BT549 cells transiently transfected with EEF1A2. MCF7 cells were used as a negative control as it was not transfected and therefore the V5 tag would not be detected.

PTK6 was not detected in BT549, MOCK cells nor in the cells transfected with EEF1A2 (Figure 4.16). This confirms the real-time PCR results where EEF1A2 expression did not affect the expression of PTK6. PTK6 was detected in MCF7 cells as two protein bands. This is always the case in this cell line as those two protein bands might indicate the presence of two PTK6 splice variants.

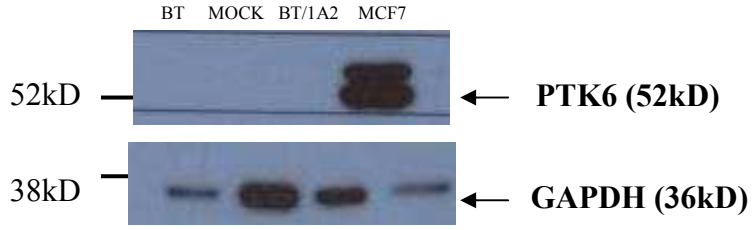

Figure 4.16 Western blot on BT549 breast cancer cells transiently transfected with EEF1A2 to detect a change in PTK6 expression. No PTK6 protein was detected in BT549 cells nor in the MOCK cells or in the BT549 cells transiently transfected with EEF1A2. MCF7 cells were used as a positive control as it always detects two PTK6 bands that might indicate the presence of different splice variant.

4.3 Discussion

4.3.1 Pathways affected by EEF1A2 expression

Microarray data analysis on NIH-3T3 cells stably transfected with EEF1A2 (section 4.2.1) showed a list of up- and down-regulated genes that were affected by the expression of EEF1A2 (Tables 4.1 and 4.3). As shown in section 4.2.1.2, many of these genes are associated with different types of cancers; either as oncogenes or tumor suppressor genes and therefore would be involved in cancer-related pathways.

To determine pathways that are affected by the overexpression of EEF1A2, the "DAVID Pathway Viewer" was used (Huang da et al., 2009). This tool displays the genes on pathway maps to facilitate biological interpretation in a network context. From a list of the 500 most upregulated genes that were loaded, three main pathways from Biocarta were shown to be affected by the overexpression of EEF1A2. These pathways are the p53 signaling pathway (Figure 4.17), PML transcriptional regulation pathway (Figure 4.18) and the VEGF, Hypoxia, and Angiogenesis pathway (Figure 4.19).

In the p53 pathway (Figure 4.17), the four upregulated genes from the microarray data are *Gadd45a* (FC 1.5), *Mdm2* (FC 1.4), *p21* (FC 1.3) and *Rb1* (FC 1.2). As discussed in section 4.3.1, Kato *et al.* have shown that *EEF1A* was one of the genes upregulated by the overexpression of p53 (Kato et al., 1997).

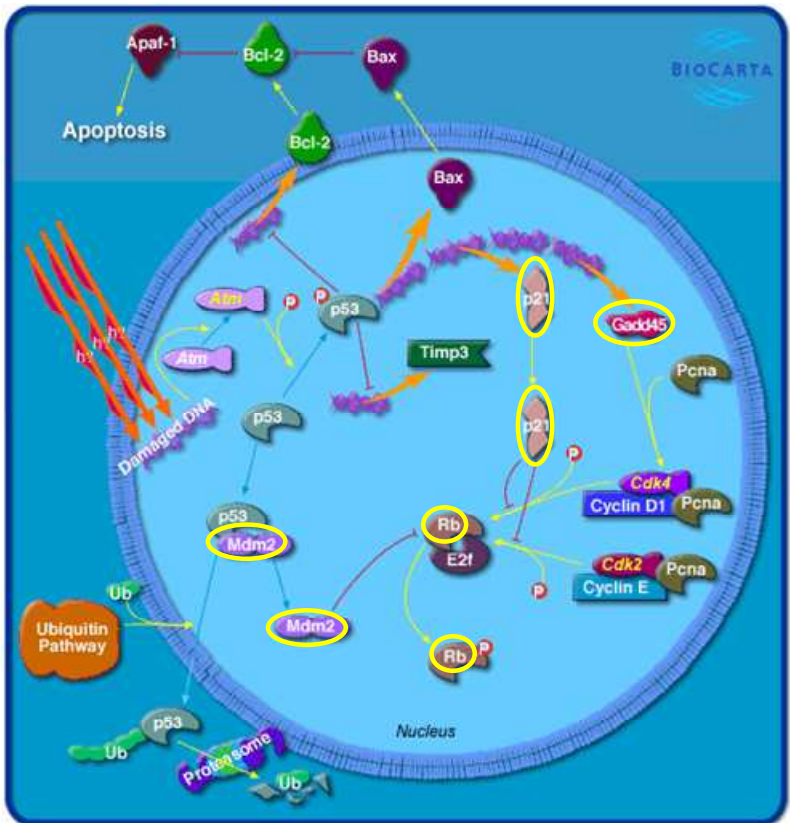

Figure 4.17 p53 pathway showing four upregulated genes from the microarray data where NIH-3T3 cells were stably transfected with EEF1A2. Genes upregulated in the study are circled in yellow: Gadd45, Mdm2, p21 and Rb (Biocarta).

Growth arrest and DNA-damage-inducible protein (*Gadd45*) genes function as stress sensors that modulate the response of mammalian cells to genotoxic and/or physiological stress and modulate tumor formation. These proteins bind to stress modulators such as p21 and p38 kinases.

HDM2 gene, the human homologue of murine double minute (*mdm2*) oncogene, blocks the function of p53 by binding to its transcriptional activation domain (discussed in section 1.1.5.1.4) (Figure 4.17).

P21, also known as cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1 is a protein that in humans is encoded by the *CDKN1A* gene. Its expression is tightly controlled by the tumor suppressor protein p53 as it functions as a regulator of cell cycle progression. The retinoblastoma protein (Rb) is a tumor suppressor protein that is dysfunctional in many types of cancers (discussed in section 1.1.4.2).

Another pathway affected by the overexpression of EEF1A2 is the PML transcriptional regulation pathway (Figure 4.18) which has three of the upregulated microarray genes, one of them is *RB* along with *PML* and *FAS*.

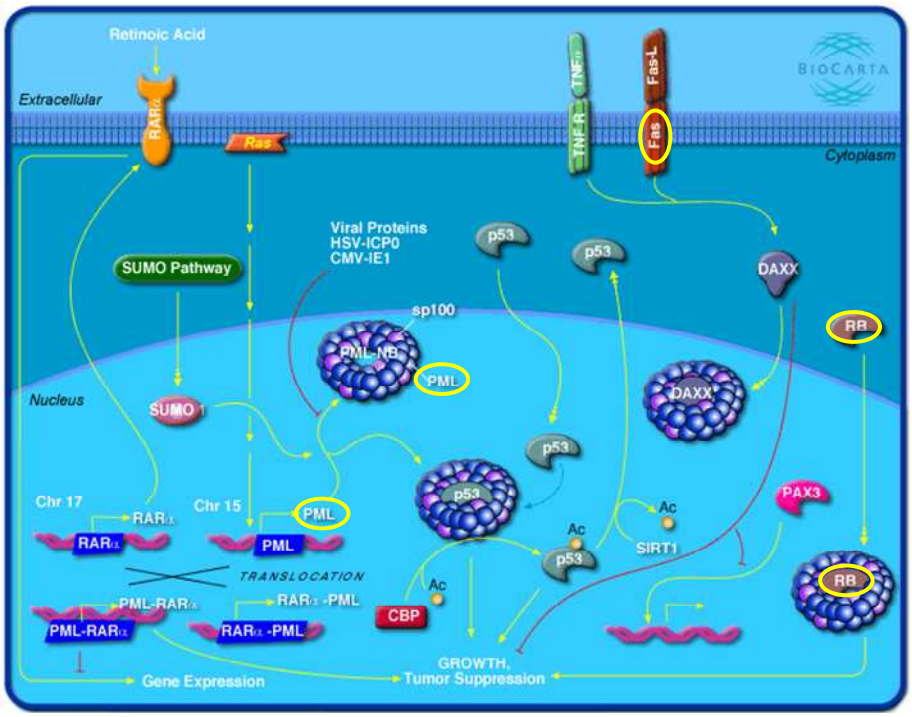

Figure 4.18 Regulation of transcriptional activity by PML. The three upregulated genes from the microarray list where NIH-3T3 cells were stably transfected with EEF1A2 are circled in yellow: RB, PML and Fas (Biocarta).

Both PML and FAS showed a fold-change increase of 1.3 in the microarray list of upregulated genes. Promyelocytic leukemia (PML) protein functions as a transcription factor and tumor suppressor. As shown in figure 4.18, its expression is cell-cycle related and it regulates the p53 response to oncogenic signals. The gene is often involved in the translocation with the retinoic acid receptor alpha (*RARα*) gene associated with acute promyelocytic leukemia (APL).

Fas (TNF receptor superfamily, member 6) encodes a receptor that contains a death domain therefore it plays an important role in the regulation of programmed cell death. It has also been implicated in the pathogenesis of various malignancies and diseases of the immune system. As shown in figure 4.17, Fas activates downstream substrates that inhibit growth.

The third pathway involved in the overexpression of EEF1A2 is the VEGF, hypoxia, and angiogenesis pathway (Figure 4.19). Three upregulated genes from the microarray list are involved in this pathway: VEGF and FAK both showed a 1.3 fold-change increase and EIF2B that had a 1.2 fold-change increase. VEGF (Vascular endothelial growth factor) is a protein involved in angiogenesis. It plays an important role in inflammation and during normal and pathological angiogenesis, a process that is associated with wound healing, embryonic development, and growth and metastasis of solid tumors when overexpressed. Interestingly, as discussed in section 4.3.1, VEGF –like EEF1A2- has been associated with motor neuron degeneration in mice (Sathasivam, 2008).

FAK (Focal Adhesion Kinase), also known as PTK2 is a non-receptor tyrosine kinase involved in cell motility, proliferation and apoptosis (Figure 4.19). Chan *et al.* reported that when FAK was blocked, breast cancer cells became less metastastic due to decreased mobility (Chan et al., 2009).

EIF2B (Eukaryotic Translation Initiation Factor 2B) is another upregulated gene that is involved in this pathway. It is one of the initiation factors (IFs) associated with severe autosomal recessive neurodegenerative disease, described in young children as CACH (childhood ataxia with central nervous system hypomyelination)/VWM (leukoencephalopathy with vanishing white matter) syndrome. In the microarray list of genes, it showed a fold-change increase of 1.2.

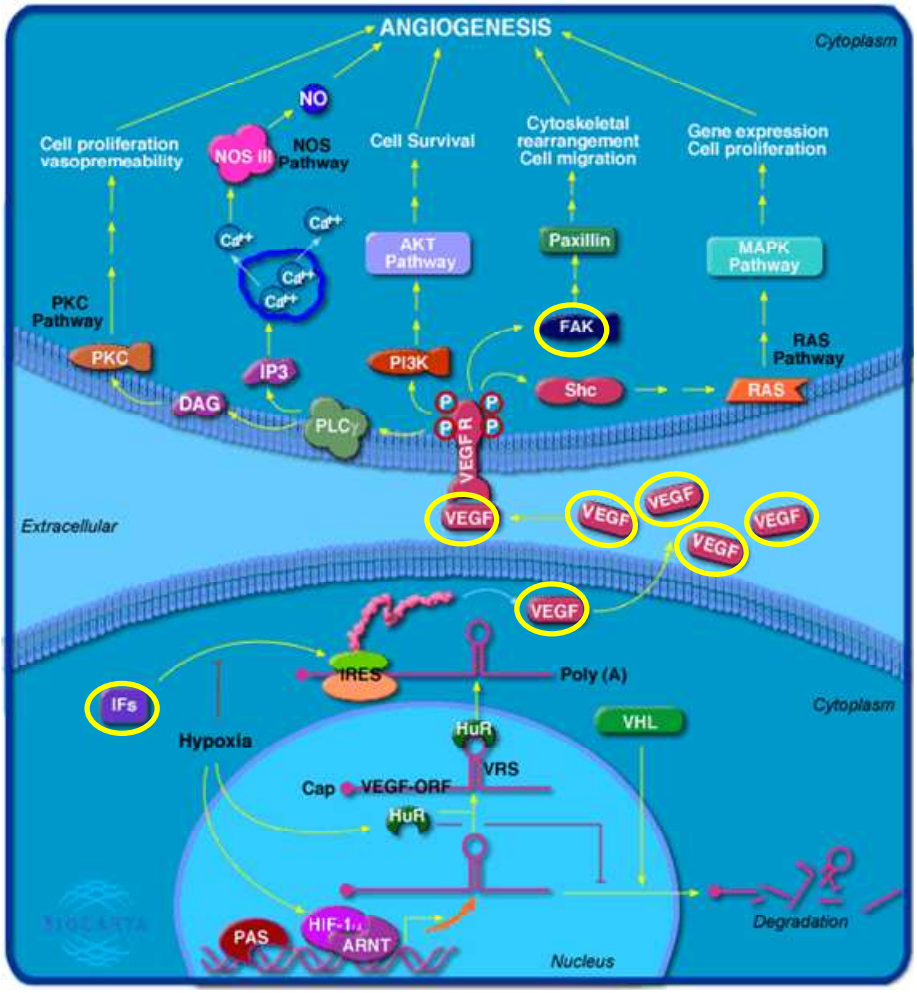

Figure 4.19 VEGF, Hypoxia, and Angiogenesis pathway. The three upregulated genes from the microarray data where NIH-3T3 cells were stably transfected with EEF1A2 are circled in yellow: VEGF, FAK and IFs (Biocarta).

Overexpression of EEF1A2 has also shown to downregulate genes as shown in table 4.3. Using the same "DAVID Pathway Viewer" tool, 200 downregulated genes from the microarray list were analyzed to determine if any were involved in cancer-related pathways. One pathway that was associated with EEF1A2 expression was the B Cell Survival Pathway (Figure 4.20).

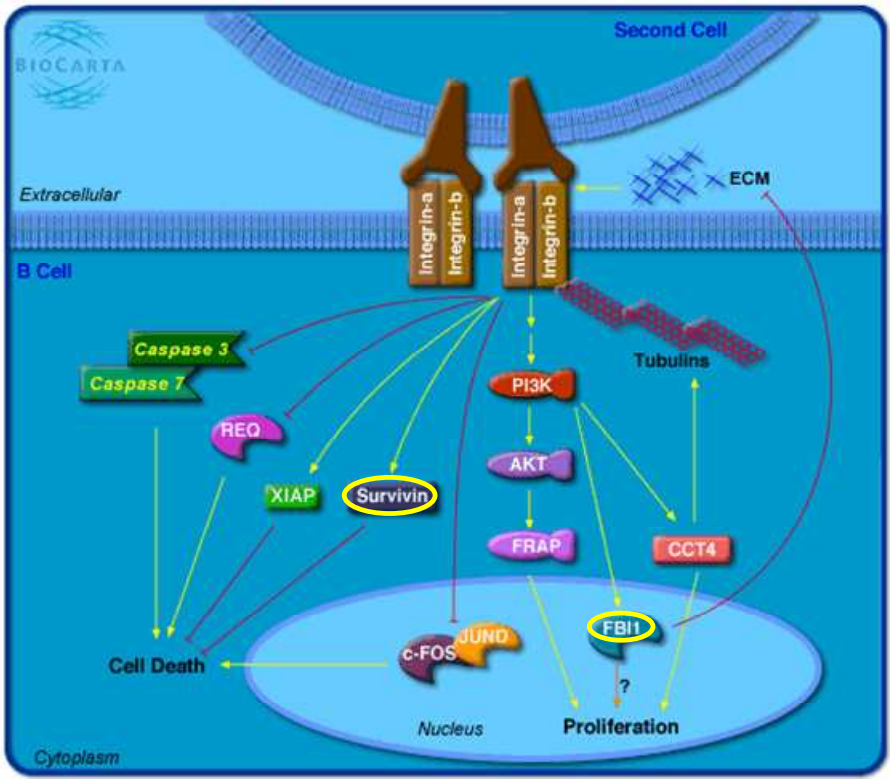

Figure 4.20 The B-cell survival pathway. The two downregulated genes from the microarray data where NIH-3T3 cells were stably transfected with EEF1A2 are circled in yellow: Survivin and FBI1 (Biocarta).

Two of the downregulated genes that are involved in this pathway are *SURVIVIN* and *FBI1*. Survivin, also known as BIRC5 (baculoviral inhibitor of apoptosis repeat-containing 5) showed a 1.6 fold-change decrease in the microarray list. It inhibits caspase activation leading to negative regulation of apoptosis. Since it is downregulated in this study, this would lead to caspase activation and thus promote apoptosis.

FBI1 is a proto-oncogenic protein, which represses tumor suppressor *ARF* gene transcription. FBI1 also represses transcription of the *Rb* gene, a tumor suppressor gene important in cell cycle (Jeon et al., 2008). It showed a 1.4 fold-change decrease in the NIH-3T3 stable cell lines expressing EEF1A2. As shown in figure 4.8, FBI1 is associated with cell proliferation. Therefore as its expression decreases by the effect of EEF1A2 overexpression, FBI1 downregulation could lead to reduced cell proliferation. Together with the downregulation of SURVIVIN, this

could lead to increased survival as reported by other groups (discussed in section 4.3.1).

4.3.2 Effect of EEF1A2 on cancer-related pathways

A number of reports have indicated that eEF1A2 may play an important role in tumorigenesis and therefore act as an oncogene. Amiri *et al.* reported that eEF1A2 is an activator of the protein kinase Akt in a phosphatidylinositol-3 kinase (PI3K)-dependant manner (Amiri et al., 2007) whereas Li *et al.* showed that knockdown of *Eef1a2* expression in PCT cell lines affected the JAK/STAT pathway suggesting that eEF1A2 may play an important role in the induction or progression of plasma cell neoplasms in mice and humans (Li et al., 2010).

To examine which other genes/cancer-related pathways are affected by EEF1A2 expression, microarray analysis was carried out on NIH-3T3 stable cell lines generated in our lab that express eEF1A2. As shown in table 4.1, many upregulated genes were associated with different types of cancer. However, most of the downregulated genes are not known to be associated with tumorigenesis (Table 4.3).

As shown in the p53 pathway (Figure 4.17), the oncogene *Mdm2* is upregulated in the NIH-3T3 stable cells expressing EEF1A2 along with *p21* and *Gadd45*. P21 overexpression blocks the effects of the Rb tumor suppressor gene while Mdm2 and Gadd45 are associated with tumor formation. The overexpression of EEF1A2 would eventually lead to the increased activity of those three genes and therefore make EEF1A2 an important therapeutic target in cancer. Interestingly, Kato *et al.* have shown that EEF1A was one of the genes upregulated by the overexpression of p53 (Kato et al., 1997). The authors reported that since p53 also plays a role in G2/M checkpoint regulation then this suggests that EEF1A might be a key factor in the regulation of cell cycle at G2/M. They have suggested that since p53 upregulates the *EEF1A* gene, this provides new insights into the mechanism of the cell cycle and cell death, and it might also be an important target in cancer therapy.

Figure 4.18 shows the PML transcriptional regulation pathway which involves two upregulated genes from the microarray list PML and FAS. PML protein functions as a transcription factor and tumor suppressor. It is also associated with acute promyelocytic leukemia (APL). Therefore upregulation of this gene might decrease the risk of cancer development. The Fas receptor on the other hand activates downstream substrates that inhibit growth by promoting apoptosis. Therefore Fas could be an important therapeutic target in cancer treatment where its ligands would act as antagonists increasing its apoptotic activity and therefore reducing tumor size.

The third pathway that is affected by the overexpression of EEF1A2 is the VEGF, Hypoxia, and Angiogenesis pathway (Figure 4.19). The overexpression of the initiation factor EIF2B and VEGF would lead to the activation of the PI3K/Akt pathway that in turn leads to cell survival. This has been previously reported by Amiri *et al*. They reported that eEF1A2 is an activator of the protein kinase Akt in a phosphatidylinositol-3 kinase (PI3K)-dependant manner (Amiri et al., 2007). The same group showed that the expression of eEF1A2 in BT549 cells stimulated the formation of filopodia in these human breast cancer cell lines and in non-transformed Rat2 cells. Filopodia production enhances cell invasion and migration and therefore indicates the importance of eEF1A2 in promoting tumor development through phosphatidylinositol signalling, actin remodeling and cell motility (Amiri et al., 2007). However, even though Amiri *et al.* suggested the involvement of eEF1A2 in tumorigenesis, other groups reported that high eEF1A2 protein expression was associated with increased survival (Pinke et al., 2008; Ruest et al., 2002). FAK is another gene in this pathway that is upregulated. It is involved in cell motility, proliferation and apoptosis (Figure 4.19). Chan *et al.* reported that when FAK is blocked, breast cancer cells became less metastatic due to decreased mobility (Chan et al., 2009).

Interestingly, Oosthuyse *et al.* showed for the first time that manipulation of the VEGF gene in mice resulted in a new role for VEGF in the pathogenesis of motor neuron degeneration (Oosthuyse et al., 2001). 60% of mice with the homozygous deletion died before or around birth from vascular abnormalities in the lung whereas

the remaining 40% that survived, begun to show symptoms of motor neuron degeneration at around five months of age. The authors suggested that insufficient VEGF leading to vascular abnormalities may put motor neurons at risk of late-onset neurodegeneration brought on by chronic ischemia (Oosthuyse et al., 2001). Since EEF1A2 is a translation factor also reported to be involved in motor neuron degeneration (section 1.3.1.1). These results further demonstrate the importance of EEF1A2 as a therapeutic target that could affect the downstream substrates and reduce cell proliferation and tumor development.

Figure 4.20 shows the B-cell survival pathway which involves two downregulated genes from the microarray list FBI1 and Survivin. FBI1 is a proto-oncogene which represses two tumor suppressors ARF and Rb. FBI1 is associated with cell proliferation. Therefore as its expression decreases by the effect of EEF1A2 overexpression, FBI1 downregulation could lead to reduced cell proliferation. On the other hand, as shown in figure 4.20, FBI1 is another downstream substrate of the PI3K pathway but not through Akt.

The data reported by Li *et al.* (Discussed on page 136) showed that knockdown of *Eef1a2* expression in PCT cell lines indicate that eEF1A2 may play an important role in the induction or progression of plasma cell neoplasms in mice and humans. In this study, we show that overexpression of EEF1A2 –rather than its knockdown- downregulates FBI1 which is downstream of PI3K but not through the Akt pathway (Figure 4.20). These results are the opposite of what Li *et al.* have reported. This could be due to the fact that FBI1 is not affected by Akt but rather affected directly by PI3K (PI3K-FBI1 pathway).

Survivin, which is another gene downregulated in this pathway (Figure 4.20), inhibits caspase activation leading to negative regulation of apoptosis. Since it is downregulated in this study, this would lead to caspase activation and thus promoting apoptosis.

With both FBI1 and Survivin being downregulated and therefore leading to reduced cell proliferation and apoptosis with the overexpression of EEF1A2, these

results differ from the reports previously published by other groups that showed eEF1A2 having anti-apoptotic properties and that high eEF1A2 protein expression was associated with increased survival (Pinke et al., 2008; Ruest et al., 2002).

Other microarray gene expression studies done by different groups on breast cancers showed different up- and down-regulated genes then the ones shown in our study (Ma et al., 2003; Sotiriou et al., 2006). Sotiriou *et al.* examined whether histologic grade was associated with gene expression profiles of breast cancers and whether such profiles could be used to improve histologic grading. Unlike our study (Tables 4.1 & 4.3), the five most upregulated genes in their study were *UBE2C*, *RACGAP1*, *KPNA2*, *C10orf3* and *PTTG1* whereas the five most down-regulated genes were *TBC1D17*, *FLJ21628*, *SMARCA2*, *DIXDC1* and *KIF13B* (Sotiriou et al., 2006). Another microarray analysis was generated by combining the use of laser capture microdissection and DNA microarrays. They generated *in situ* gene expression profiles of the premalignant, preinvasive, and invasive stages of human breast cancer (Ma et al., 2003). The top five genes with increased expression both in high tumor grade and the ductal carcinoma *in situ*- invasive ductal carcinoma (DCIS-IDC) transition were *IDH2*, *FLJ10540*, *KNSL1*, *ANKT* and *PRO2000* (Ma et al., 2003).

These breast cancer up-/down-regulated genes were not similar to the genes which showed a change in expression from our microarray data (section 4.2.1.2). This is most likely because the change of gene expression in our study is due to the effect of eEF1A2 expression in the NIH-3T3 transfected cells.

4.3.3 Effect of EEF1A2 on cancer-related genes

Table 4.1 shows the top 33 upregulated genes from the microarray list of which 14 genes were associated with different types of cancer. Five of the genes that were associated with breast cancer were studied in this project. These genes are: *Serpine2*, *Egr1*, *Aldh3a1*, *Tpd52* and *Dusp6*. To confirm the microarray data, real-time PCR was carried out on the stable cell lines to assess their expression level. Serpine2 showed the highest average fold-change increase in the microarray data (19.6) and as shown in table 4.5 and figure 4.9, its expression level was high in both

stable cell lines 7.2 and 10.2 but not in 9.6. This might be due to the EEF1A2 construct being incorporated within the *Serpine2* gene and therefore would have altered it expression. This could explain why there was a fold change increase in 7.2 and 10.6 but not in 9.6 cells. Another explanation would be a technical artefact in the microarray chip, however, since real-time PCR results also showed a reduced SERPINE2 expression at the mRNA level in the 9.6 cells, the more valid hypothesis would be that the EEF1A2 construct was integrated within the *Serpine2* gene. All other genes showed a fold-change increase between 2 and 3. Expression level of all these genes which were assessed by real-time PCR confirmed the microarray results. Surprisingly, when the same NIH-3T3 cells were transiently transfected with the same EEF1A2 construct, no change in the expression level of these five genes was detected (Table 4.7 & figure 4.11). This might explain the indirect role EEF1A2 plays in cancer which is the activation of cancer-related pathways which in turn activates downstream intermediates such as SERPINE2. In the transiently transfected cells, no increase of expression in those five genes was detected as cells were collected 24, 48 and 72 hours after transfection which might not be the time scale long enough for these intermediates to be activated and therefore become overexpressed as observed in the stable cell lines.

Western blots were carried out to detect the protein level of the five upregulated gene in the stable cell lines (Figure 4.10). Even though SERPINE2 protein was expressed in all three stable cells along with the control cells, the protein expression was not as high as was detected at the mRNA level when the real-time PCR was carried out. Surprisingly, ALDH3A1 which showed an increased expression at the mRNA level in both the microarray data and in real-time PCR in 7.2 and 10.2 cells, showed no protein expression in these two stable cell lines (Figure 4.10c). This might be the result of miRNAs either degrading ALDH3A1 mRNA or binding to the mRNA and inhibiting protein translation. As for the bands detected in 9.6, the antibody is probably cross-reacting with another protein.

EGR1, TPD2 and DUSP6 only showed 2-3 fold-change increase in the microarray data and a slight increase was detected at the mRNA level when real-time PCR was carried out (Table 4.5 and figure 4.8). The expression level did not show a high increase in the three stable cell lines when compared to the control cells nor did the expression level show much difference between those three cells. Western blots showed no expression at the protein level of TPD52 and DUSP6; this is consistent with the fact that NIH-3T3 do not usually express these proteins but is in contrast to the results of the microarrays. Egr1 however was expressed in all the stable cell lines but not at a higher level as that in the parental cells.

These results provide evidence that EEF1A2 is involved in many cancer-related pathways by indirectly activating many genes. This signifies its role as an oncogene as previously reported (Lee and Surh, 2009) and would therefore be a good therapeutic target for the treatment of breast cancer.

4.3.4 Effect of transformation on cells

NIH-3T3 cells are mouse fibroblast cell lines that do not express EEF1A2 under normal conditions. However, as discussed in section 4.2.1.1, these cells developed an oncogenic phenotype when stably transfected with EEF1A2. Microarray analysis on these stable cell lines generated a list of many upregulated genes which are associated with different types of cancers (Table 4.1). A few were well documented in the literature as being associated with breast cancer as previously mentioned.

Serpine2 is one of the genes associated with breast cancer and showed the largest increase in the microarray list. In another study conducted by Bergeron *et al*., Serpine2 was also the most upregulated gene with more than 28 fold-change increase in cells overexpressing activated MEK1 when compared to cells expressing the wild type MEK in the rat intestinal epithelial cells (Bergeron et al., 2010). MEK1 is one of the kinases that is known to be associated with different types of cancers. The overexpression of SERPINE2 was furthermore confirmed by carrying out real-time PCR. The authors also reported that SERPINE2 protein is secreted; they confirmed its

presence by carrying out Western blots after spinning down the proteins collected from the culture medium they grew in. This was not carried out when our stable cell lines were growing in culture in preparation for the microarrays experiment but cells were harvested and protein was extracted directly from the cells. It is possible that this is the reason for the discrepancy in the results.

In another study where microarrays were conducted on NIH-3T3 cells stably transfected with the *Rho* oncogene, Serpine2 showed a fold-change increase of only 0.15 (Berenjeno et al., 2007). However, they also reported an upregulation of four of the same genes that were upregulated in our microarray study with approximately similar fold-change increases. These genes are *DUSP6*, *GADD45* and *MDM2* (Figure 4.17) and *VEGF* (Figure 4.19).

Another study reported the upregulation of many cancer-associated genes/pathways detected by microarray data when NIH-3T3 cells were transfected with Kaposin A (Chen et al., 2009). A few of the upregulated genes that were involved in the cancer pathways were also the same that were upregulated in our microarray data. Kaposin A is a product of the Kaposi sarcoma-associated herpes virus (KSHV) which when transfected into the NIH-3T3 cells caused these cells to develop oncogenic properties, inducing cell proliferation and tumorigenesis. In the p53 pathway, they reported the upregulation of *p21*, *Mdm2* and *Gadd45*. These are the same upregulated genes detected in our microarray data that showed an overexpression in the p53 pathway (Figure 4.17). Interestingly, Chen *et al.,* have also shown the upregulation of VEGF in the Kaposin A expressing NIH-3T3 cells. Other pathways that have been upregulated in their study are the PI3K/Akt, STAT and MAPK pathways (Chen et al., 2009).

Since the same pathways have been shown to be upregulated by different genes stably transfected into NIH-3T3, this shows that *EEF1A2* is not the only gene affecting these cancer-related pathways and that there are other substrates that are involved. Being a translation elongation factor, EEF1A2 will probably affect the translation of other proteins that in turn activates/upregulates these pathways. The other explanation for the upregulation of the same genes in NIH-3T3 cells is that these genes could simply reflect the general state of transformation of cells. To

further understand whether EEF1A2 targets specific transcripts, SILAC (Stable Isotope Labeling by Amino acids in cell Culture) would be a useful tool to detect the proteins upregulated in our stable cell lines (Chapter 5, Future Directions).

4.3.5 Effect of EEF1A2 expression on PTK6 and vice versa

EEF1A2 and *PTK6* are less than 50kb apart on 20.q13.3 and share many common properties. Both are overexpressed in 60% of breast cancers (Barker et al., 1997; Kulkarni et al., 2007), are reported to be potential oncogenes (Lee and Surh, 2009; Zhang et al., 2005), they promote cell growth, migration and inhibit apoptosis by activating the PI3K/AKT pathway (Kamalati et al., 2000; Li et al., 2010) and both were reported to be associated with increased survival (Aubele et al., 2007; Pinke et al., 2008). Showing all those similar properties, it was hypothesized that EEF1A2 and PTK6 act in tandem to increase the risk of developing breast cancer and that one of them acts as the driver while the other is the passenger.

This was first examined in the NIH-3T3 stable cell lines that express EEF1A2. As shown in figure 4.12, no significant increase was detected in PTK6 expression. Similar results were found in the NIH-3T3 cells which were transiently transfected with EEF1A2. EEF1A2 expression was detected 24 hours after transfection and slightly decreased 48 hours later but PTK6 showed no change in expression (Figure 4.13). The same experiment was repeated where EEF1A2 was transfected into the breast cancer cell lines BT549. As shown in the other cell line, no significant change in PTK6 expression was detected as confirmed by Western blots (Section 4.2.3.2.2, figure 4.15).

Since EEF1A2 expression seems to have no effect on the expression level of PTK6, it was hypothesized that the opposite might be true where PTK6 might affect the expression level of EEF1A2. This hypothesis was considered after we established that the overexpression of PTK6 was due to its increased gene copy number in the primary breast tumors while *EEF1A2* did not show a copy number as high as *PTK6* (Chapter 3). Therefore, to determine whether PTK6 affects EEF1A2 expression, the

same cell lines which were transfected with the EEF1A2 construct were now transfected with the PTK6 construct: NIH-3T3 and BT549. NIH-3T3 cell line showed a slight increase in the expression of PTK6 while BT549 showed a high PTK6 expression with no significant change in EEF1A2 expression. When Western blots were carried out on these cells, PTK6 was not detected even though it showed an increased expression at the mRNA level detected by real-time PCR (Tables and figures are given in Appendix C). We cannot therefore be confident that PTK6 expression has no effect on the expression level of EEF1A2. The construct used was a LacZ-PTK6 construct which should express the LacZ/PTK6 fusion protein. Excluding construct errors (no mutations, correct orientation), one of the reasons why cells that showed an increased PTK6 expression did not show PTK6 at the protein level might be that the fusion protein has a different conformation that the PTK6 antibody could not recognize. Also, PTK6 is not normally expressed in these two cell lines and therefore when they are transfected and thus do express PTK6 at the mRNA level, miRNAs might degrade the mRNA or bind to it inhibiting translation and protein expression.

These results clearly show that our initial hypothesis that EEF1A2 and PTK6 act in tandem is not correct. Based on the studies done so far, we suggest that EEF1A2 and PTK6 act independently and that the expression of one does not affect the expression of the other and therefore each act through a different mechanism in breast tumorigenesis.

These results of EEF1A2 expression in several breast tumors and breast cancer cell lines further confirm the role of EEF1A2 in breast tumorigenesis. As shown in this chapter, EEF1A2 contributes to breast cancer development by indirectly activating several downstream intermediates which in turn promotes cell division and invasion. This shows that EEF1A2 could be an important therapeutic target for the treatment of breast cancer. On the other hand, another set of downregulated genes reduce cell proliferation and promote apoptosis, thus supporting reports published by other groups reporting that high eEF1A2 protein expression was associated with increased survival (Pinke et al., 2008; Ruest et al., 2002).

Chapter 5: Discussion

5.1 Summary of Results

EEF1A2 and *PTK6* are two genes located on 20q13.3. Several studies reported the amplification of this region in a number of tumor types including breast cancer. *EEF1A2* and *PTK6* being less than 50kb apart and sharing many common properties, it was hypothesized that EEF1A2 and PTK6 act in tandem through the same mechanism in breast cancer and that one of these genes could be the driver in this amplicon.

To test this hypothesis - whether EEF1A2 and PTK6 act in tandem or independently (chapter three), the copy number changes in the genes encoding EEF1A2 and PTK6 was related to their expression level using IHC on primary breast tumors TMAs from around 300 patients, 55 of which were analyzed for copy number changes. Being in the same small genomic region, it was hypothesized that both genes would have an increased copy number in the tumors that strongly co-expressed both proteins. Surprisingly, as shown in table 3.3 and figure 3.11, *PTK6* showed an increased copy number in those tumors but not *EEF1A2*. Two other genes –*KCNQ2* and *SRMS*- flanking both *EEF1A2* and *PTK6* were also tested for gene copy number in a smaller group of tumors but showed a normal gene copy number (Table 3.4 and figure 3.14). These results need to be expanded by using a larger set of tumors but from these data, it is clear that *PTK6* is the only gene amplified to any significant degree.

Also, in those 300 breast tumors that were analyzed, no significant statistical correlation was found between eEF1A2 and any of the histopathological parameters studied (grade and size of tumors, ER status, PR status, lymph node involvement, treatment modality (chemotherapy alone or chemotherapy and hormonal therapy) and disease free survival). On the other hand, PTK6 overexpression was associated with only low grade tumors and the estrogen receptor positive tumors. No correlation was found with tumor size, lymph node status or with disease-free survival of patients.

To expand on this work, EEF1A2 and PTK6 were analyzed to understand whether either affects the expression of the other. As shown in the results' chapters where the expression of both genes was analyzed when either is expressed in cell lines, PTK6 expression is clearly not affected by the expression of EEF1A2. Gene copy number analyses on breast tumors have shown that *PTK6* is amplified in this region but not *EEF1A2*. This led us to the conclusion that each has a different mechanism contributing to breast cancer development. This shows that our initial hypothesis that one of those genes is the driver while the other is the passenger is clearly not correct.

EEF1A2 has also been analyzed for its oncogenicity effect to establish its role as a therapeutic target. As shown in chapter four, the overexpression of EEF1A2 led to the upregulation of many cancer-associated genes. We also demonstrated that EEF1A2 could contribute to breast cancer development by indirectly activating several downstream intermediates which in turn promotes cell division and invasion. This shows that EEF1A2 could be an important therapeutic target for the treatment of breast cancer. On the other hand, another set of downregulated genes reduce cell proliferation and promote apoptosis, thus supporting reports published by other groups reporting that high eEF1A2 protein expression was associated with increased survival (Pinke et al., 2008; Ruest et al., 2002).

5.2 Future Directions

5.2.1 Effect of EEF1A2 expression on protein synthesis

Microarray data analysis on NIH-3T3 stable cell lines expressing EEF1A2 have shown that EEF1A2 is potentially responsible for the indirect upregulation of many cancer-related genes/pathways. Being a translation elongation factor, EEF1A2 probably affects many proteins that in turn would lead to the differential regulation of downstream substrates.

Justyna Janikiewicz –a previous PhD student in our lab- measured the rate of global protein synthesis in all these stable cell lines expressing eEF1A2 along with control cells. This involved metabolic [^{35}S] methionine/cysteine labelling of the cell lines stably expressing eEF1A2. The radiolabeled amino acids will incorporate into the newly synthesized polypeptides which are then measured for radioactivity levels. Justyna showed that the rate of protein synthesis in the stable cells is marginally lower than the control cells despite the increased growth rate and the colony and focus formation (Janikiewicz, 2010). This suggests that the oncogenic properties of eEF1A2 might derive from other non-canonical functions of this protein or from affecting translation of specific messages rather than global translation.

To further test this and to understand whether EEF1A2 targets specific transcripts, it would be interesting to perform SILAC (Stable Isotope Labeling by Amino acids in cell Culture) on one of the stable cell lines expressing EEF1A2 in comparison to the control cells. SILAC is a powerful and accurate technique for proteome-wide quantitation by mass spectrometry.

The basic principle of SILAC is exposing a cell line to a given amino acid that will take it up from the growth medium and metabolically incorporate it into its proteome. This concept is exploited by passaging cell cultures in media containing either a normal (light) or a heavy form of a selected amino acid, most frequently Lysine and Arginine, where the light and heavy amino acids differ only in mass as a result of different isotopic content. Once fully labeled (after 5 to 7 doublings), cells can be collected and pooled together before performing any preparative procedures, thus eliminating quantitation inaccuracies introduced during sample handling. Finally, the proteome of the two differently labeled cell cultures (the control cells and one of the stable cell lines) will be analyzed in the same liquid chromatography-mass spectrometry (LC-MS/MS) run and the origin of the proteins or peptides can readily be discriminated based on the mass shift introduced by the different isotopic content conveyed by the labeled amino acid on these stable cell lines. We can then compare the protein expression generated from SILAC with the mRNA gene expression generated from the microarray data analysis.

5.2.2 EEF1A2 knockdown in breast cancer cell lines

Even though normal mouse and human plasma cells do not express eEF1A2, it has been reported to be overexpressed in plasma cell neoplasms of both mice and humans (Li et al., 2010). In mouse plasmacytomas (PCT), eEF1A2 appears to contribute to cell transformation by affecting cell cycle progression and inhibiting apoptosis. A previous study showed that cultured myotube cells overexpressing eEF1A2 had enhanced cell growth and were resistant to apoptosis (Ruest et al., 2002) whereas Li *et al.* showed that phosphorylation of STAT3 in the JAK/STAT pathway was delayed and PI3K expression (part of the PI3K-AKT pathway) was reduced when *Eef1a2* was knocked down in PCT cell lines (Li et al., 2010). The authors suggested that eEF1A2 may play an important role in the induction or progression of plasma cell neoplasms in mice and humans.

It would be interesting to test the same concept but using breast cancer cell lines instead of mouse cell lines to check whether the knockdown of EEF1A2 would affect the same pathways in human cells. EEF1A2 would be knocked down in breast cancer cells that normally express EEF1A2. Since breast tumors respond differently to treatment based on the Estrogen Receptor (ER) status, the RNAi technique would be used on an ER+ and ER- breast cancer cell lines. Once the knockdown was achieved and confirmed by various methods (Western blots, real-time PCR), a microarray gene expression analysis would be performed. This would generate a list of upregulated and downregulated genes that might be involved in many cancer-related pathways. This will further shed light on the role of EEF1A2 as an oncogene and therefore will establish its role as a therapeutic target for breast cancer treatment.

To further expand on this, it would be interesting to know whether the same concept applies on breast tumors. Perou *et al.* used microarrays and clustering to identify patterns of gene expression in human mammary epithelial cells in primary human breast tumors (Perou et al., 1999). This was followed by IHC on proteins encoded by a particular gene in a cluster. They showed that one cluster was found to correlate with variation in cell proliferation rates, another with Interferon (IFN) response. These reports supported the usefulness of studying variation in gene expression patterns in human cancers to classify breast cancer.

5.2.3 Role of MicroRNAs in EEF1A2 expression

Most studies have shown that miRNAs downregulate gene expression by binding to the 3'UTR and either degrading the mRNA or binding to the mRNA and therefore inhibiting translation. However, in 2008, Dahiya *et al*. have reported the overexpression of eEF1A2 in the ovarian cancer cell line BG-1 when transfected with the miRNA let-7f (Dahiya et al., 2008).

We established from this project (Chapter 3) that EEF1A2 gene copy number – unlike PTK6- does not correlate with its expression level in human breast tumors, therefore other factors might contribute to the overexpression of eEF1A2. It would be of interest to determine if miRNAs play a role in EEF1A2 expression. This could be done by transfecting different miRNAs into breast cancer cell lines that express eEF1A2 endogenously. miRNAs could be identified using different databases such as "www.microrna.org" .This will then be followed by measuring the expression of eEF1A2 and that particular miRNA by real-time PCR to determine whether the miRNA has an effect on EEF1A2 expression. This will shed light on whether miRNAs affect the expression of EEF1A2.

5.3 Conclusion

This study shed light on the function of two breast cancer-associated genes – *EEF1A2* and *PTK6*- on the 20q13.3 region. We clearly show that even though both genes are less than 50kb apart and share many oncogenic characteristics, both are associated with breast cancer through different mechanisms. As many other amplified regions in cancer, a specific gene is usually the driver of neighboring genes. Collins *et al.* have shown that analysis of a 1-Mb region on 20q13.2 produced evidence for five genes of which *ZNF217* is probably the driver (Collins et al., 1998). On the other hand, GC analysis of a 1.2-Mb region of amplification at 20q13.2 have identified 6 genes of which prefoldin 4 (*PFDN4*) was identified as the driver gene (Collins et al., 2001). Since both *ZNF217* and *PFDN4* are amplified in the same region, the authors reported for the first time the presence of a duplicon on the 20q13.2 breast cancer amplicon (Collins et al., 2001). We show however that this is not the case with *EEF1A2* and *PTK6* and that neither is the driver nor the passenger in this region as only *PTK6* was amplified. EEF1A2 expression on the other hand led to the upregulation of many cancer associated genes/pathways making it a possible therapeutic target for cancer. Because EEF1A2 can activate multiple signaling pathways, further studies are needed to understand which signal is a key signal to control accelerated tumorigenesis by EEF1A2.

Appendices

Appendix A: Real-time PCR efficiency curves

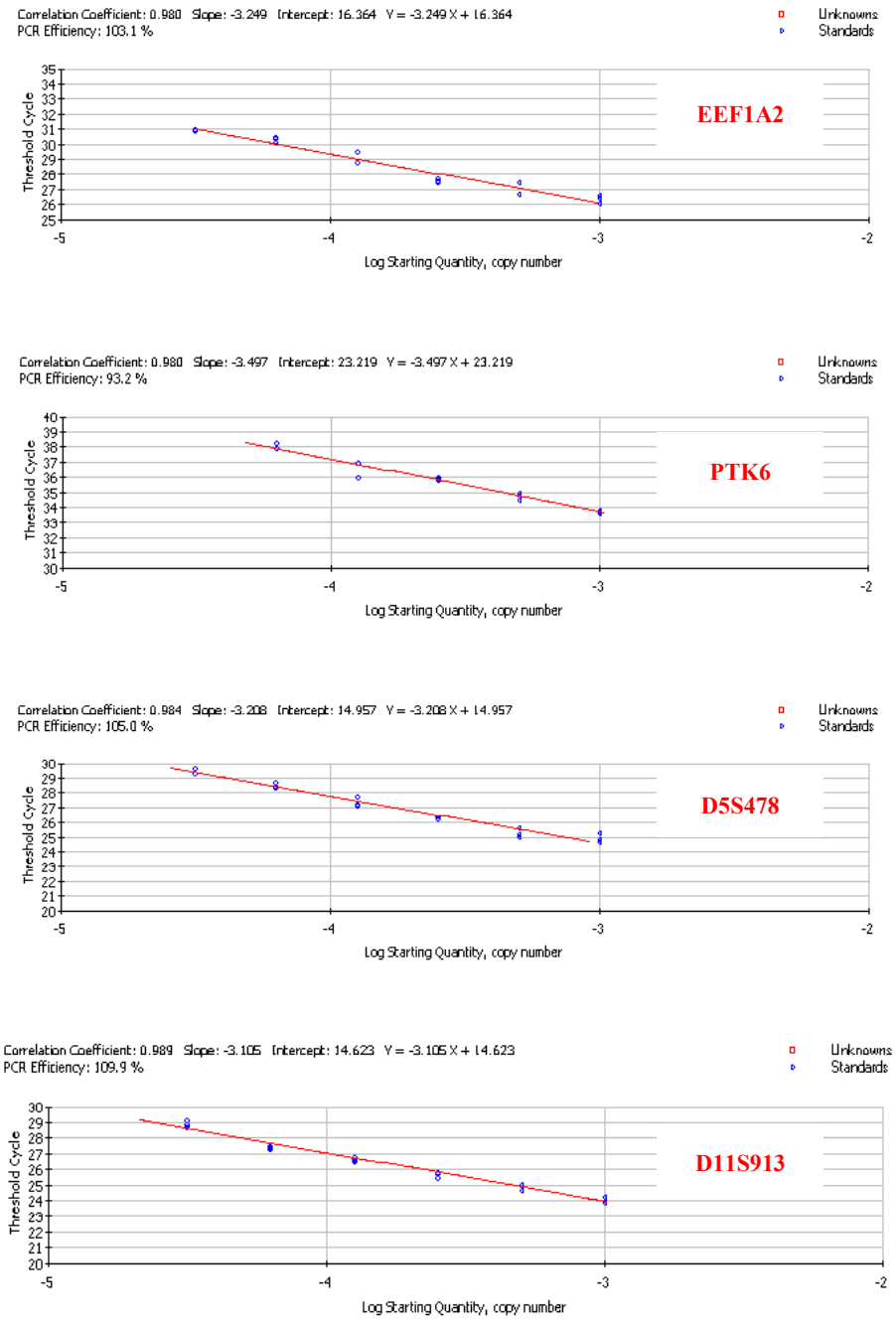

Figure A.1 Standard curve calibration performed for quantitative real-time PCR. Graphs represent standard curves generated for primer sets amplifying EEF1A2, PTK6 and the two reference genes D5S478 and D11S913. Log starting quantity on the x-axis of each curve represents a log10 dilution series of the particular DNA (in triplicate) used for calibration. Amplification of PCR products with efficiency between 90-110 % and correlation coefficient R2 > 0.98 for standard curves were considered acceptable for further analysis.

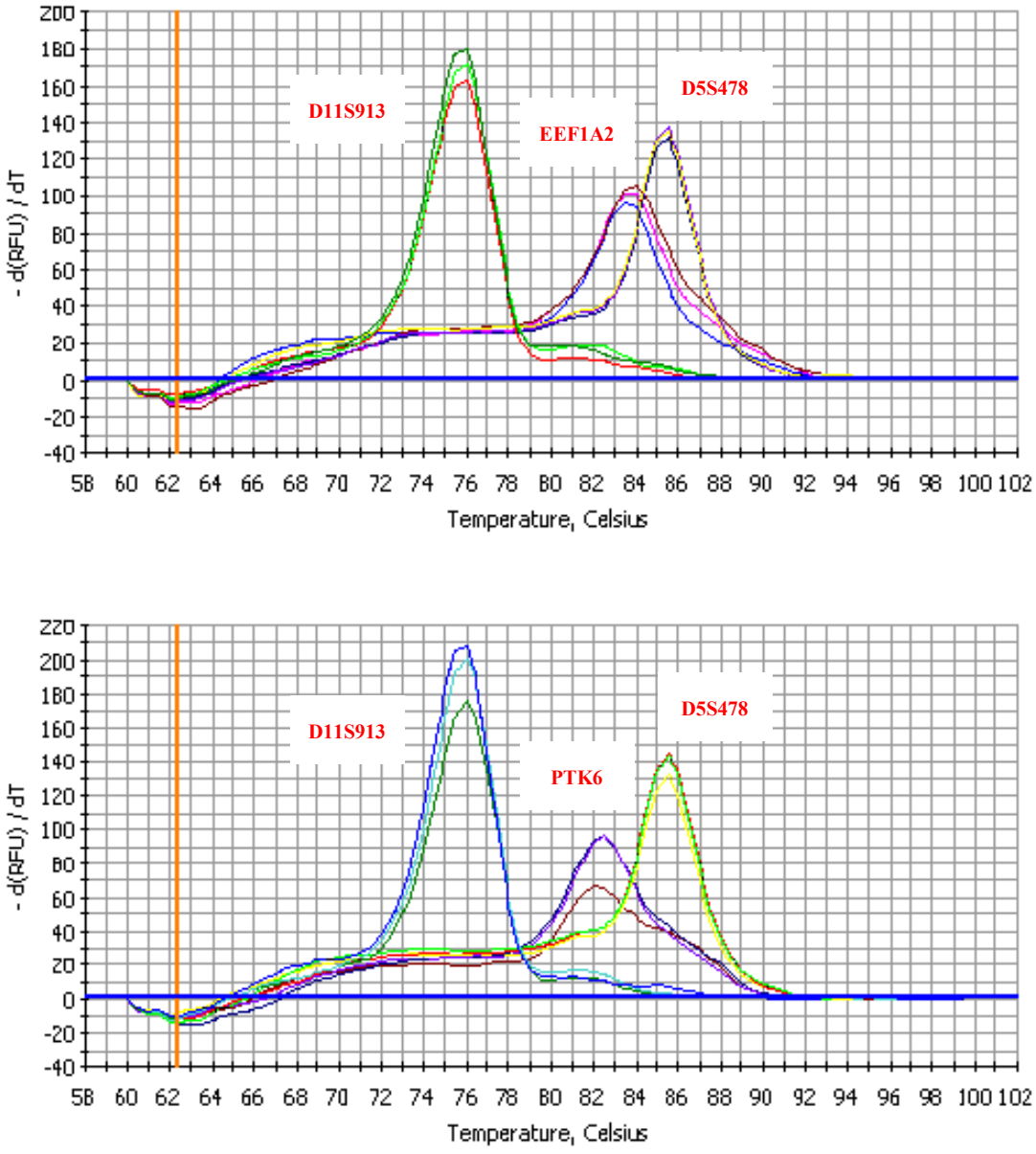

Figure A.2 Illustration of melting curves analysis. Melting curves for EEF1A2, PTK6 and the reference genes D5S478 and D11S913 primer sets were performed in order to confirm their specificity. The presence of a single prominent peak corresponds to single amplicon detection exclusively.

Appendix B: Let-7f miRNA effect on eEF1A2 expression

To examine whether let-7f miRNA might play a role in eEF1A2 overexpression in breast cancer, let-7f miRNA was transfected into the breast cancer cell line BT549 which is a cell line that does not express eEF1A2 under normal conditions. This was done to determine whether let-7f alone could trigger the overexpression of eEF1A2. Cells were collected 24, 48 and 72 hours after transfection. mRNA was isolated to detect the expression level of eEF1A2, and miRNA was purified to detect the expression level of the miRNA let-7f. Finally, protein was extracted to check for the expression of eEF1A2 by Western blotting, as miRNAs can exert their effect at the level of mRNA degradation and/or translational repression. Real-time PCR was performed as described above. The reference genes used for the mRNA were PUM1 (Pumilio homolog 1) and TBP (TATA-box binding protein) whereas the reference gene used for the miRNA was U6 (Perkins et al., 2007; Szabo et al., 2004; Yamagata et al., 2009). The reference genes were selected based on previous reports on RT-qPCR studies and were used as endogenous controls to estimate the amount and integrity of the total RNA in each reaction. Reference genes in general always show a Ct value lower than that of the studied transcript. This correspnds to higher mRNA/miRNA abundance (ie the lower the Ct value, the higher the gene/transcript expression). This experiment was repeated three times to take into account experiment variability and ultimately to confirm results. The first and second transfections showed a slight increase in the expression level of both EEF1A2 and let-7f with no significant correlation (*p*-value 0.2) between the two (Figures B.1 and B.2 & tables B.1 and B.2). In the third transfection, let-7f showed an increased expression 48 hours post-transfection with a slight increase in EEF1A2. The expression of both EEF1A2 and let-7f decreased after 72 hours (Figure B.3 and table B.3). No significant association was found between the two (*p*-value 0.1). Surprisingly, even though EEF1A2 was expressed in the transfected cells at the mRNA level, Western blots showed no eEF1A2 expression at the protein level 24, 48 and 72 hours post-transfection (Figure B.4). Unfortunately, due to the variability in the eEF1A2 and let-7f expression in each of the three repeated experiments, these results cannot be considered conclusive and would need to be followed up by more experiments either in a different cell line or by using another miRNA that might affect the expression level of EEF1A2 and therefore might help in the explanation of the role EEF1A2 plays in breast cancer.

Table B.1 Analyzing gene expression level of EEF1A2 & let-7f miRNA in BT549 breast cancer cell line transiently transfected with let-7f using the $2^{-\Delta\Delta Ct}$ method (Experiment #1).

Cells	Ct gene	Ct Ref G	ΔCT	ΔΔCT	2^-ΔΔCT
BT549 (EEF1A2)	32.52	34.60	-2.09	0	1
BT549/Let-7f 24h	32.03	34.92	-2.89	-1	2
BT549/Let-7f 48h	31.42	34.84	-3.42	-1	3
BT549/Let-7f 72h	31.75	34.41	-2.65	-1	1
BT549 (Let-7f)	35.77	28.07	7.71	0	1
BT549/Let-7f 24h	33.82	27.42	6.40	-1	2
BT549/Let-7f 48h	34.08	27.47	6.60	-1	2
BT549/Let-7f 72h	34.46	27.81	6.65	-1	2

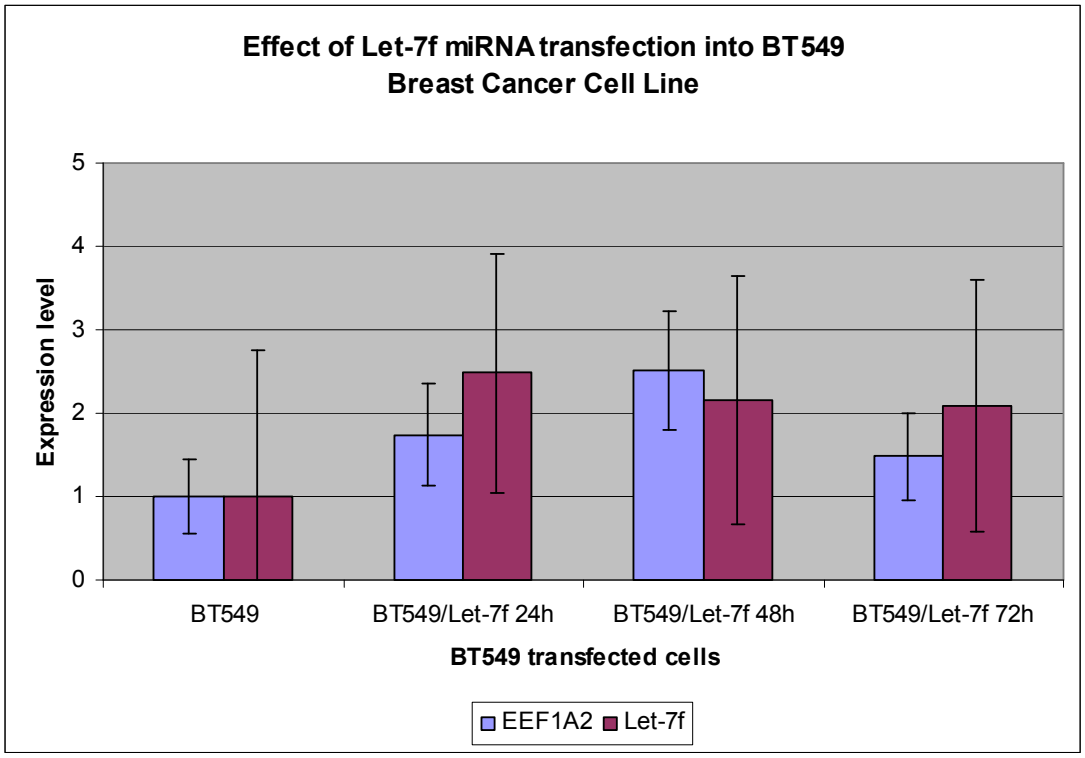

Figure B.1 First transfection: Effect of let-7f miRNA on eEF1A2 expression in BT549 breast cancer cell line. EEF1A2 and let-7f were normalized to the reference genes (PUM1 and TBP for EEF1A2, U6 for let-7f miRNA) and then expressed relatively to the expression level in BT549 cells.

Table B.2 Analyzing gene expression level of EEF1A2 & let-7f miRNA in BT549 breast cancer cell line transiently transfected with let-7f using the $2^{-\Delta\Delta Ct}$ method (Experiment #2).

Cells	Ct gene	Ct Ref G	ΔCT	ΔΔCT	2^-ΔΔCT
BT549 (EEF1A2)	35.58	35.64	-0.06	0	1
BT549/Let-7f 24h	33.29	35.27	-1.98	-2	4
BT549/Let-7f 48h	33.65	34.97	-1.32	-1	2
BT549/Let-7f 72h	33.53	34.70	-1.17	-1	2
BT549 (Let-7f)	36.05	27.66	8.39	0	1
BT549/Let-7f 24h	33.71	26.99	6.72	-2	3
BT549/Let-7f 48h	33.58	26.76	6.82	-2	3
BT549/Let-7f 72h	33.51	27.10	6.41	-2	4

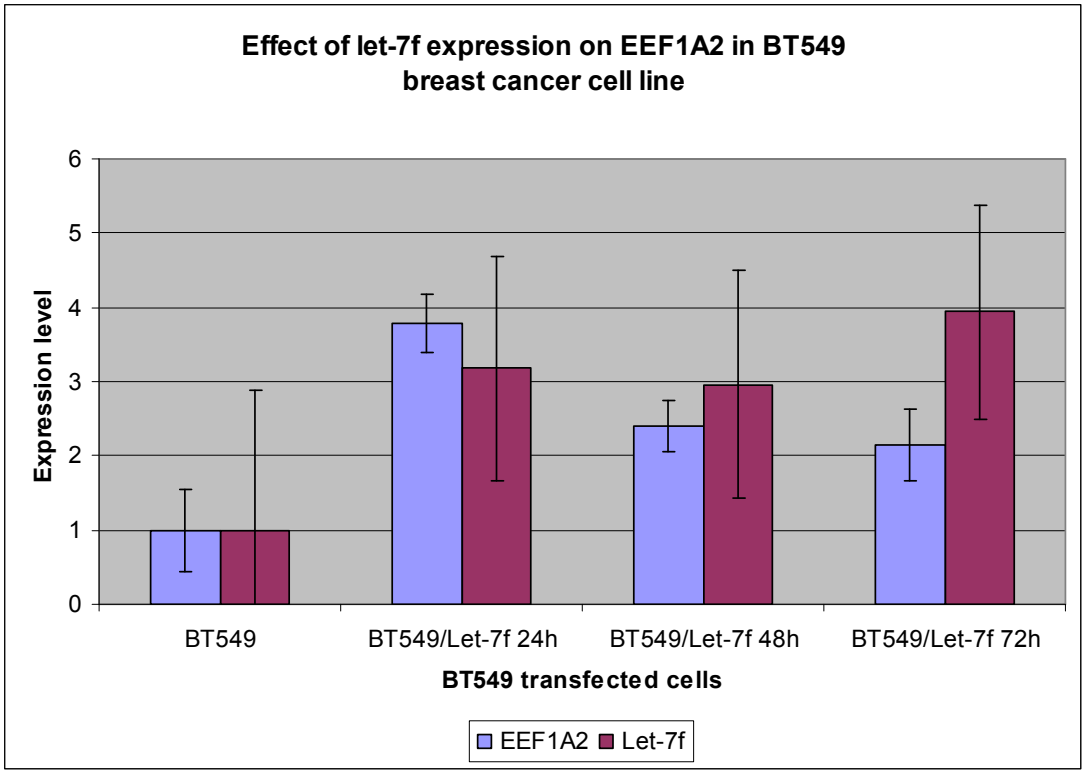

Figure B.2 Second transfection: Effect of let-7f miRNA on eEF1A2 expression in BT549 breast cancer cell line. EEF1A2 and let-7f were normalized to the reference genes (PUM1 and TBP for EEF1A2, U6 for let-7f miRNA) and then expressed relatively to the expression level in BT549 cells.

Table B.3 Analyzing gene expression level of EEF1A2 & let-7f miRNA in BT549 breast cancer cell line transiently transfected with let-7f using the $2^{-\Delta\Delta Ct}$ method (Experiment #3).

Cells	Ct gene	Ct Ref G	ΔCT	ΔΔCT	2^-ΔΔCT
BT549 (EEF1A2)	38.41	36.01	2.40	0	1
BT549/Let-7f 24h	38.07	35.54	2.53	0	1
BT549/Let-7f 48h	35.82	35.71	0.11	-2	5
BT549/Let-7f 72h	37.51	35.75	1.76	-1	2
BT549 (Let-7f)	36.05	27.66	8.39	0	1
BT549/Let-7f 24h	33.71	26.99	6.72	-2	1
BT549/Let-7f 48h	33.58	26.76	6.82	-2	7
BT549/Let-7f 72h	33.51	27.10	6.41	-2	6

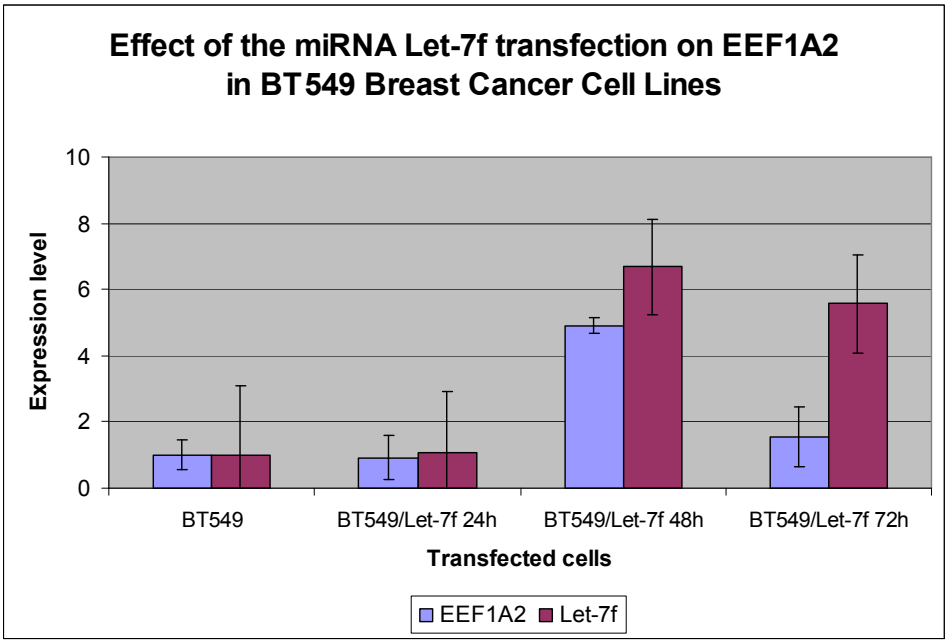

Figure B.3 Third transfection: Effect of let-7f miRNA on eEF1A2 expression in BT549 breast cancer cell line. EEF1A2 and let-7f were normalized to the reference genes (PUM1 and TBP for EEF1A2, U6 for let-7f miRNA) and then expressed relatively to the expression level in BT549 cells.

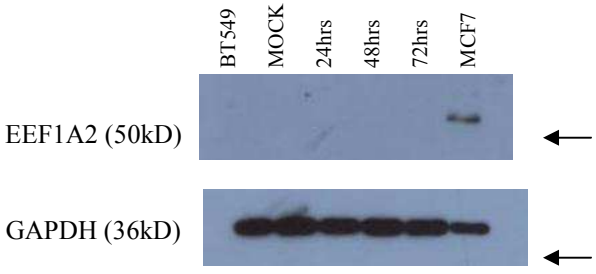

Figure B.4 Western blot on BT549 cells transfected with let7-f to detect a change in EEF1A2 expression. No EEF1A2 was detected in BT549 cells or in the MOCK cells or in the BT549 cells transiently transfected with let-7f. MCF7 cells were used as a positive control. GAPDH was used as a loading control.

Appendix C: Effect of PTK6 expression in transiently transfected cells

Transient transfection of EEF1A2 into NIH-3T3 cells and BT549 cells showed no effect of EEF1A2 expression on PTK6 expression (section 4.2.3). In this section, the opposite was examined with PTK6 being transfected into NIH-3T3 cells and BT549 to assess any changes in the EEF1A2 expression level. This was done to determine if the amplification and resulting overexpression of PTK6 shown in chapter three is the driver of overexpression of EEF1A2 in breast cancer. This would explain the co-overexpression of the two genes in so many individual tumors.

C.1 Transient transfection of PTK6 into NIH-3T3 fibroblast cells

The PTK6 construct was kindly provided by Micaela Aubele and Natalie Ludyga from the German Research Center for Environmental Health in Neuherberg, Germany (Figure C.1). The construct (which has the Lac-Z sequence and would therefore express PTK6-lacZ fusion protein) was transfected into NIH-3T3 cells and the expression level of EEF1A2 was analyzed. As shown in table C.1 and figure C.2, PTK6 level was slightly increased 24 hours after transfection and gradually decreased 48 and 72 hours later. EEF1A2 however did not show an increase in expression at the mRNA level as did PTK6.

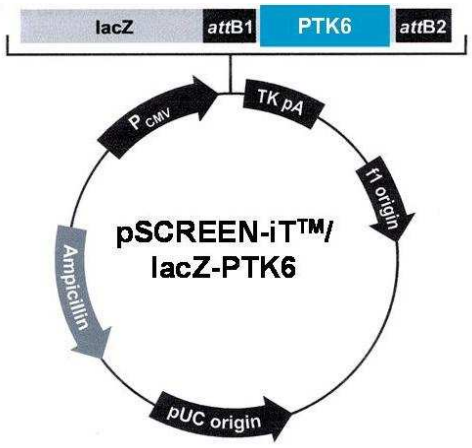

Figure C.1 LacZ-PTK6 expression construct. Construct and figure provided by Micaela Aubele and Natalie Ludyga from the German Research Center for Environmental Health in Neuherberg, Germany. The lacZ-PTK6 fusion vector was constructed by recombination of the pENTR221 hORF vector and the pSCREEN-iT/lacZ-DEST vector (Invitrogen) using the LR clonase II enzyme. *att*B sites immediately following the lacZ ORF for fast and efficient recombination to generate the lacZ/PTK6 gene fusion. The CMV promoter is used for strong expression of the lacZ-PTK6 gene fusion.

Table C.1 Analyzing gene expression level of EEF1A2 & PTK6 in NIH-3T3 cell line transiently transfected with PTK6 using the $2^{-\Delta\Delta Ct}$ method.

NIH-3T3 cells	Ct gene	Ct ref genes	ΔCT	ΔΔCT	2^-ΔΔCT
EEF1A2					
NIH-3T3	35.73	27.62	8.11	0	1
NIH-3T3/PTK6 24h	35.87	27.65	8.22	0	1
NIH-3T3/PTK6 48h	35.3	26.95	8.35	0	1
NIH-3T3/PTK6 72h	35.54	25.63	9.9	2	0
PTK6					
NIH-3T3	33.65	27.62	6.03	0	1
NIH-3T3/PTK6 24h	31.23	27.65	3.58	-2	5
NIH-3T3/PTK6 48h	32.18	26.95	5.23	-1	2
NIH-3T3/PTK6 72h	31.58	25.63	5.95	0	1

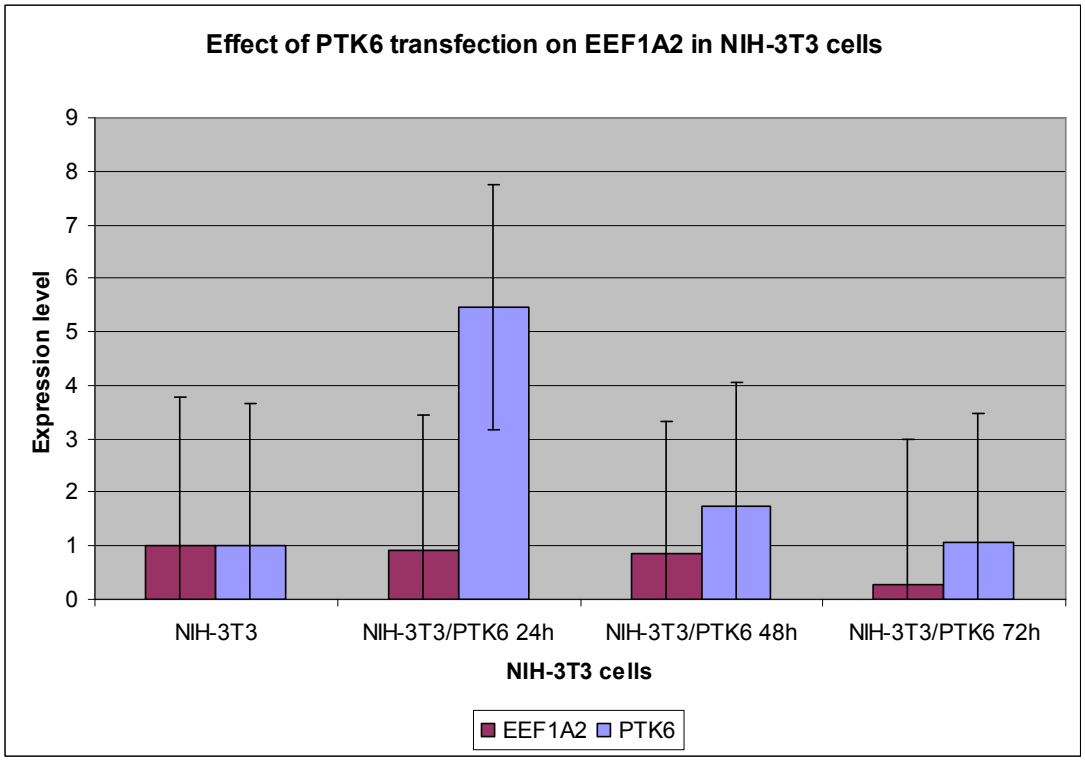

Figure C.2 Transient transfection of PTK6 in NIH-3T3. Expression of PTK6 shows no change in EEF1A2 expression 24, 48 and 72 hrs after transfection of the PTK6 construct. EEF1A2 and PTK6 were normalized to the reference genes and then expressed relatively to the expression level in NIH-3T3 cells

A western blot was carried out to check for the expression of PTK6 in these NIH-3T3 transfected cells (Figure C.3). PTK6 was not detected in the cells showing an increase in expression in the real-time PCR results as NIH-3T3 cells do not normally express this protein (Table C.1 & figure C.2). It was, however, detected as two protein bands in MCF7 cells when Western blots were carried out.

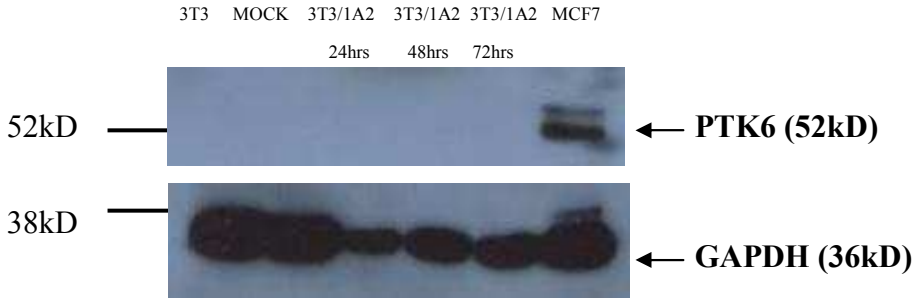

Figure C.3 Western blot on NIH-3T3 cells transiently transfected with EEF1A2 to detect a change in PTK6 expression. No PTK6 protein was detected in BT549 cells nor in the MOCK cells or in the BT549 cells transiently transfected with EEF1A2 24, 48 and 72 hours post transfection. MCF7 cells were used as a positive control as it always detects two PTK6 protein bands that might indicate the presence of different splice variants. GAPDH was used as a loading control.

C.2 Transient transfection of PTK6 into BT549 cells

The same PTK6 construct was transfected into BT549 breast cancer cells to assess the change in EEF1A2 expression as was shown in the NIH-3T3 cells (Table 4.9 and figure 4.1). Real-time PCR analysis showed no increase in EEF1A2 expression and only slight increase in PTK6 expressing cells 24, 48 and 72 hours post-transfection (Table C.2 and figure C.4).

Table C.2 Analyzing gene expression level of EEF1A2 & PTK6 in BT549 breast cancer cells transiently transfected with PTK6 using the $2^{-\Delta\Delta Ct}$ method.

BT549 cells	Ct gene	Ct ref genes	ΔCT	ΔΔCT	2^-ΔΔCT
EEF1A2					
BT549	35.22	30.21	5.01	0	1
BT549/PTK6 24h	37.32	36.16	1.16	-4	14
BT549/PTK6 48h	37.72	35.73	2.00	-3	8
BT549/PTK6 72h	37.96	35.56	2.40	-3	6
PTK6					
BT549	33.16	30.21	2.95	0	1
BT549/PTK6 24h	30.61	36.16	-5.55	-9	362
BT549/PTK6 48h	30.70	35.73	-5.02	-8	251
BT549/PTK6 72h	31.30	35.56	-4.26	-7	148

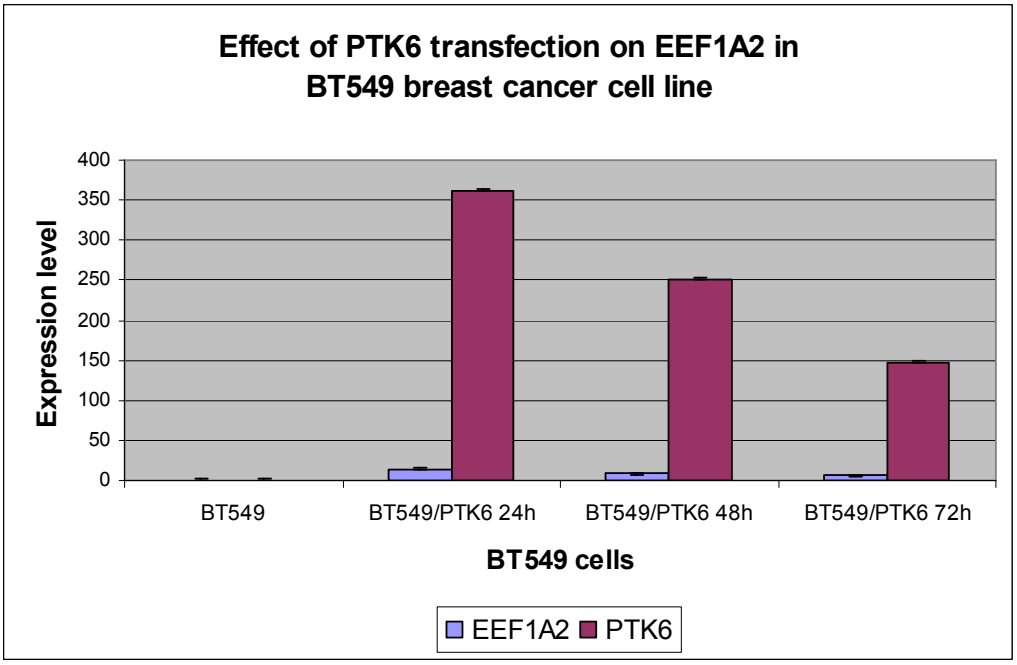

Figure C.4 Transient transfection of PTK6 in BT549 breast cancer cells. Expression of PTK6 shows no change in EEF1A2 expression using real time PCR. EEF1A2 and PTK6 were normalized to the reference genes (PUM1 and TBP) and then expressed relatively to the expression level in BT549 cells.

Western blots were carried out to check for the expression of PTK6 in BT549 transfected cells (Figure C.5). PTK6 expression was not detected in the transfected cells even though it was shown to be increased at the mRNA level when real-time PCR was carried out. Since this is a LacZ-PTK6 construct, it should express the LacZ/PTK6 fusion protein which might have a conformation different from PTK6 and therefore would hinder the PTK6 antibody from recognizing it. This might be one of the reasons why the PTK6 antibody did not detect PTK6 protein in the Western blot. Another reason would be that there is a fault in the construct itself especially if the sequence was not in the correct orientation or if there were mutations in the sequence.

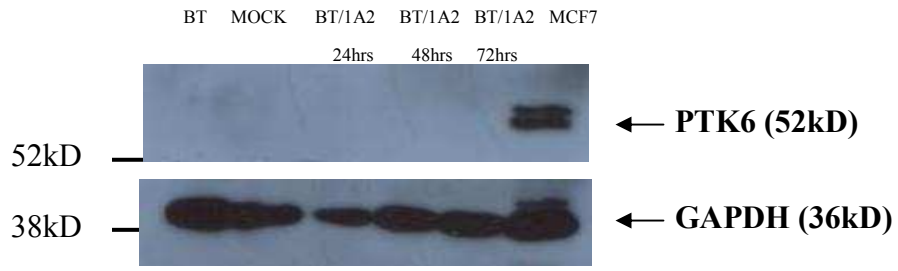

Figure C.5 Western blot on BT549 breast cancer cells transiently transfected with EEF1A2 to detect a change in PTK6 expression. No PTK6 protein was detected in BT549 cells nor in the MOCK cells or in the BT549 cells transiently transfected with EEF1A2 24, 48 and 72 hours post transfection. MCF7 cells were used as a positive control as it always detects two PTK6 protein bands that might indicate the presence of different splice variants. GAPDH was used as a loading control.

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