

THE ROLE OF DARPP-32 IN BREAST CANCER PROGRESSION

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Abstract

Breast cancer accounts for one in every eight females with cancer, and it is the fifth most common cause of cancer death. Previously, our group demonstrated that low expression of DARPP-32 is associated with adverse breast cancer specific survival. The principle aim of this study is to explore the role of DARPP-32 in breast cancer progression.

In this study, in addition to the assessment of mRNA expression within the METABRIC breast cancer cohort, Immunohistochemistry was used in order to assess the expression levels of PKA, PP1, Cdk5, *Sp7, GRB7, DKK1* and the phosphorylation of DARPP-32 at Threonine-34 in a large cohort of early-stage primary breast cancer patients.

To investigate DARPP-32 signalling in breast cancer, purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin were utilised. The effect of compounds on the activity of Cdk5, PP1 and PKA was determined using ELISA, as was the effect on DARPP-32 Threonine-34 and Threonine-75.

RNA sequencing was utilised to analyse transcriptomic changes caused by DARPP-32 knockdown in combination with estradiol17β (E2) stimulation or treatment with PKA inhibitor fragment (6-22) amide.

The effects of combined expression of PKA, PP1, DARPP-32 and Cdk5 on breast cancer specific survival were assessed. Although PP1 protein expression alone was not associated with patient survival, combined expression of cytoplasmic and nuclear low PP1 and low DARPP-32, and low PP1 and low Cdk5 were significantly associated with adverse breast cancer specific survival. Low expression levels of PKA and Cdk5, as well the combination of low PKA and low Cdk5 expression were significantly associated with adverse breast cancer specific survival. Low expression levels of *DKK1*, *Sp7* and phosphorylated DARPP-32 at Threonine-34 were significantly associated with adverse breast cancer specific survival

whereas low protein expression of *GRB7* was significantly associated with improved breast cancer specific survival. In addition, low mRNA expression of Cdk5, *PRKAR1A, PRKAR2A, PRKACB, PPP1CB*, and *PPP1CC* were significantly associated with adverse breast cancer specific survival.

MDA-MB-231 cells were significantly sensitive to all drugs (purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin) at 72 hours. In T47D cells, all compounds were cytotoxic at 24 and 48 hours. In contrast, MCF7 were significantly sensitive to all drugs at 24, 48 and 72 hour time points. Significant reductions in PKA activity was observed following treatment with PKA inhibitor fragment (6-22) amide (P=0.05). Significant reductions in Cdk5 activity were observed following treatment with Cdk5 inhibitor, purvalanol B (P=0.05). Significant reductions in DARPP-32 Threonine-34 phosphorylation were observed following treatment with PP1/PP2A inhibitor, Tautomycetin (P=0.05). Significant reductions in DARPP-32 Threonine-75 phosphorylation were observed following treatment with PKA activator, Forskolin (P=0.05).

DARPP-32 knockdown led to identification of 202 differentially expressed transcripts using RNA sequencing when compared against the control cells; *ENST00000443236.6 (CPAMD8), ENST00000521309.5 (YWHAZ*), and *ENST00000651564.1 (CPAMD8)* transcripts were identified as the most significantly altered transcripts.

In summary, all biomarkers were considered to be involved in the development of breast cancer and our data highlights the importance of DARPP-32 as a regulator of cancer associated signalling pathways which may hold a significant therapeutic potential in breast cancer.

Publications

- **Saidy B,** Kotecha S, Butler A, Rakha EA, Ellis IO, Green AR, Martin SG, Storr SJ. PP1, PKA and DARPP-32 in breast cancer: A retrospective assessment of protein and mRNA expression. *J Cell Mol Med.* 2021;25(11):5015-5024

- **Saidy B**, Rakha EA, Green AR, Ellis IO, Martin SG, Storr SJ. Retrospective assessment of cyclin-dependent kinase 5 mRNA and protein expression and its association with patient survival in breast cancer. *J Cell Mol Med.* 2020;24(11):6263-6271

- Martin SG, Zhang S, Yang S, **Saidy B**, Deen S, Storr SJ. Dopamine and cAMP-regulated phosphoprotein 32kDa (DARPP-32), protein phosphatase-1 and cyclin-dependent kinase 5 expression in ovarian cancer*. J Cell Mol Med.* 2020;24(16):9165-9175

Conference Presentations

- Poster Presentation "The role of Cdk5 in breast cancer progression". Cancer Research Nottingham (CRN) September 16, 2019, Nottingham, UK
- Poster Presentation "The role of Cdk5 in breast cancer progression". BACR British Association for Cancer Research (BACR) Tumour Microenvironment conference, July1-3, 2019, Nottingham, UK
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Abbreviations

ALK	anaplastic lymphoma kinase
BDNF	Brain-derived neurotrophic factor
CCND1	cyclin-D1
DKK1	Dickkopf WNT signaling pathway inhibitor 1
HER2-E	HER2-enriched
METABRIC	molecular taxonomy of breast cancer international consortium
MMP-9	matrix metalloproteinase-9
REC	Research Ethics Committee
RIPA	Radioimmunoprecipitation assay buffer
RT-PCR	Real Time- Polymerase Chain Reaction
siRNA	small interfering RNA
Sp1-6	six other proteins
AH	aqueous humour
AJCC	American Joint Committee on Cancer
Ape1	apurinic/apyrimidinic endonuclease 1
AR	androgen receptor
ATCC	American type culture collection
ATF1	transcriptional activator 1
ATP	Adenosine triphosphate
BCL2	B-cell lymphoma 2
BER	base excision repair
BLBC	Basal-like breast cancer
BMI	Body mass index
BMP2	bone morphogenic protein-2
BRCA1	Breast Cancer gene 1
BRCA2	Breast Cancer gene 2
BZIP	basic leucine zipper
Cdk-5	Cyclin dependent kinase 5

Cdna	complementary DNA
CHF	Congestive heart failure
ChIP	Chromatin immunoprecipitation
CI	Confidence interval
CK2	Casein kinase 2
CMIP	C-Maf Inducing Protein
CREB	cAMP Response Element Binding Protein
CREM	cAMP responsive element regulatory protein
CREs	cAMP response elements
CXCR4	CXC chemokine receptor type 4
DALYs	Disability Adjusted Life Years
DARPP-32	Dopamine and cAMP regulated phosphoprotein 32 kDa
DCIS	Ductal carcinoma in situ
DDR	DNA damage response
DDR1	Discoidin Domain Receptor-1
DLX5	distal-less homeobox 5
DNA	deoxyribonucleic acid
E2	estradiol17β
E-cadherin	Epithelial cadherin
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
EGR-1	Growth response 1
ELISA	Enzyme linked immunosorbent assay
EMT	epithelial mesenchymal transition
EPIC	European Prospective Investigation into Cancer and Nutrition
ER	Estrogen receptor
ErbB	Erythroblastic leukaemia viral oncogene homolog
ERBB2	Erb-B2 Receptor tyrosine kinase 2

ERK1	p44/MAPK
ERK2	p42/MAPK
ESR1	Estrogen Receptor 1
EU	European Union
F-actin	filamentous actin
FAK	focal adhesion kinase
FFPE	Formalin fixed paraffin embedded
FSH	Follicle stimulating hormone
GAPDH	anti-Glyceraldehyde 3-Phosphate Dehydrogenase
GnRH	Gonadotrophin-releasing hormone
GRB7	Growth Factor Receptor Bound Protein 7
HCC	hepatocellular carcinoma
HER2	Human epidermal growth factor receptor-2
HER2-E	HER2-enriched
HPRT	Human qPCR primer pair control
HR	Hazard ratio
IC50	half maximal inhibitory concentration
ICC	Intraclass correlation coefficients
IDC	Invasive ductal carcinoma
IGFs	Insulin-like growth factors
IHC	Immunohistochemistry
IKKα	inhibitory kappa B kinase-α
IL-6	interleukin-6
IMS	Immersion in industrial methylated spirits
kDa	kilodalton
KID	kinase inducible domain
LCIS	Lobular carcinoma in situ
LH	Luteinising hormone
LRP5/6	Low-density lipoprotein receptor-related protein 5 and 6

LVEF	Left ventricular ejection fraction
MAPK	mitogen-activated protein kinases
mCRPC	metastatic castration resistant prostate cancers
MDM2	Mouse double minute 2 homolog
METABRIC	molecular taxonomy of breast cancer international consortium
MMP-9	matrix metalloproteinase 9
MNAR	Modulator of Non-genomic Action of estrogen Receptor
MRI	Magnetic resonance imaging
MSX2	Msh homeobox 2
MT1-MMP/MMP-2	membrane type 1-matrix metalloproteinase
MYOD	myogenic differentiation
NFAT	nuclear factor of activated T cells
NF-Kb2	nuclear factor kappa-light-chain enhancer of activated B cells 2
NGS	Nottingham Grading System
NMADR	N-methyl-D-aspartate receptor
NPI	Nottingham prognostic index
NTCs	No template controls
ORR	Objective response rate
OSA	Ovarian surgical ablation
Pakt	phosphorylation of AKT
PH	leckstrin homology
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKAi	PKA inhibitor fragment (6-22) amide
PP1	Protein phosphatase-1
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase-2B

PPP1R1B	Phosphoprotein phosphatase-1 regulatory subunit 1B
PR	progesterone receptor
PRRX1	factor paired related homeobox 1
PTH	Parathyroid hormone
RCPath	The Royal College of Pathologists
REC	Research Ethics Committee
RIPA	Radioimmunoprecipitation assay buffer
Ror2	Receptor tyrosine kinase like orphan receptor 2
RPE	RNA Pre-Elutio
RRBS	reduced representation bisulfite sequence
RT-PCR	Real Time- Polymerase Chain Reaction
RW1	RNA Wash
S100A4	S100 calcium binding protein A4
SD	standard deviation
SH2	src homology 2
SHBG	sex hormone binding globulin
STAT3	signal transducer and activator of transcription 3
TNT	Treating to new targets
TGF-β1	transforming growth factor beta 1
TNBCs	Triple negative breast cancers
TNFα	Tumour necrosis factor alpha
TP53	tumour protein 53
VEGF	Vascular endothelial growth factor
WHO	World helath Organisation
XKLF	Sp subgroup of Krüppel-like family of transcription factors
β-catenin	Beta catenin

Table of Contents

Abstract i
Publications iii
Acknowledgements iv
Abbreviations v
List of Tables xiv
List of Figurers xvi
Chapter 1 General Introduction 1
1.1 Introduction of Breast Cancer 2
1.1.1 Epidemiology 2
1.1.2 Risk factors 3
1.1.3 Breast cancer classification 9
1.1.4 Prognostic and predictive factors 15
1.1.5 Treatment 19
1.2 DARPP-32 26
1.2.1 Discovery of DARPP-32 26
1.2.2 The function of DARPP-32 27
1.2.3 PKA 29
1.2.4 PP1 30
1.2.5 Cdk5 32
1.2.3 DRAPP-32 /t-DARPP in cancer 33
1.2.4 DARPP-32, Wnt-5a signalling pathway and drug resistance 36
1.2.5 Interaction between DARPP-32, DDR1 and Wnt-5a 38
1.3 Hypotheses, aims and objectives 40
Chapter 2 Materials and Methods 43
2.1 Methods and Materials 44
2.1.1 Patient Cohorts 44
2.1.2 Immunohistochemistry 45
2.1.3 Scoring 46
2.2 Cell culture 47
2.2.1 Cryopreservation and cell recovery 47
2.2.3 Compound preparation 48
2.2.4 Proliferation assay 49

2.3 Kinase activity ELISA **50**
2.4 Cell lysis and protein extraction **51**
2.4.1 Western blot **52**
2.5 Chromogenic *in situ* RNA hybridisation **53**
2.6 Transfection **55**
2.6.1 Measurement of protein expression **56**
2.6.2 Measurement of mRNA expression **57**
2.7 Sample preparation for RNA-Seq **60**
2.7.1 RNA-Sequencing and Quality control **61**
2.7.2 Differential expression analysis **62**
2.7.3 Analysis of gene overrepresentation **62**
2.8 Statistical analysis **62**
Chapter 3 Canonical DARPP-32 Signalling in Breast Cancer **64**
3.1 Introduction **65**
3.1.1 PKA in cancer **65**
3.1.2 PP1 in cancer **67**
3.1.3 Cdk5 in cancer **71**
2.2.7 Hypothesis, aims, and objectives **73**
3.2 Results **74**
3.2.1 Protein staining, frequency and correlation of expression **74**
3.2.2 Association between protein expression and clinicopathological variables **77**
3.2.3 Protein expression and breast cancer specific survival **81**
3.2.4 Cdk5 expression and ER status **85**
3.2.5 Combination expression and clinicopathological criteria **87**
3.2.6 Combination expression and patient survival **94**
3.2.7 mRNA expression level in the METABRIC cohort **97**
3.3.8 Cytotoxic effect of purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin on proliferation **116**
..... **119**
3.4.8 Cdk5, PKA enzyme ELISA and DARPP-32 Threonine-34 and Threonine-75 phosphorylation ELISA **120**
3.4.10 The effect of Purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide, and forskolin on protein expression **123**
3.5 Discussion **124**
3.6 Summary **133**

Chapter 4 DARPP-32 and Identified Signalling partner Expression in breast Cancer 134

4.1 Introduction 135

4.1.1 *Osterix/Sp7* **137**

4.1.2 *Sp7* in cancer **139**

4.1.3 Growth factor receptor bound protein 7 ..... **141**

4.1.4 *GRB7* in Cancer **142**

4.1.5 *DKK1* **145**

4.1.6 *DKK1* in Cancer **147**

4.1.7 Hypothesis, aims and objectives **148**

4.3 Results 149

4.3.1 Chromogenic *in situ* RNA hybridisation **149**

4.3.2 Protein staining, frequency and correlation of expression **151**

4.3.3 Protein expression and associations with clinicopathological variables **153**

4.3.4 Protein expression on breast cancer specific survival **161**

4.3.5 mRNA expression in the METABRIC Cohort **165**

4.3.6 Association between mRNA expression and breast cancer specific survival **177**

4.5 Discussion 180

4.6 Summary 187

Chapter 5 Transcriptomic Effects of DARPP-32 knockdown in Breast Cancer 188

5.1 Introduction 189

5.1.2 DARPP-32 Knockdown **189**

5.1.3 RNA-Seq and DARPP-32 ..... **191**

5.2.1 Hypothesis and Aims **193**

5.3 Results 194

5.3.1 DARPP-32 knockdown Western blot and, Real-Time PCR **194**

5.3.2 RNA Sequencing Data Analysis **196**

5.3.3 Heat maps **201**

5.3.5 Overlapping of differential gene expression **208**

5.3.6 Gene Enrichment Pathway analysis **211**

5.5 Discussion 219

5.6 Summary 227

Chapter 6 General Discussion, Limitations, and Future Work . 228
6.1 Discussion 229
6.2 Limitations and future work 236
6.3 Conclusions 238
References..... 240
Appendix A: Supplementary information for Chapter 3..... 270
Appendix B: Supplementary information for Chapter 4..... 282
Appendix C: Supplementary information for Chapter 5..... 284

List of Tables

Table 1.1: The pattern of gene expression, tumour grade and the survival rate associated with the five subtypes of breast cancer. 11
Table 2.1: List of Antibodies used in Immunohistochemistry…… 46

Table 2.2: List of drugs used in this study. 49
Table 2.3 : List of ELISA used in kinase activity.....51
Table 2.4: Specifications of the antibodies used in Western blotting. .53
Table 2.5 : The primers list we used in this experiments.....60

Table 3.1: Association between various clinicopathological parameters with cytoplasmic and nuclear PP1 and cytoplasmic PKA 78
Table 3.2: Association between various clinicopathological parameters with cytoplasmic and nuclear Cdk5 80
Table 3.3: Association between various clinicopathological parameters with combined cytoplasmic PKA and Cdk5 87
Table 3.4: Association between various clinicopathological parameters with combined nuclear PKA and Cdk5 90
Table 3.5: Probes corresponding to genes encoding various subunits of PKA and PP1.....97
Table 3.6: The correlation between the level of mRNA expression of *PRKAR1A, PRKAR1B, PRKAR2A* or *PRKAR2B*, and various clinicopathological parameters.....99
Table 3.7: Association between various clinicopathological parameters of *PRKACA, PRKACB, and PRKACG* mRNA expression in the METABRIC cohort. 104
Table 3.8: Association between various clinicopathological variables and *PPP1CA, PPP1CB* and *PPP1CC* mRNA expression in the METABRIC Cohort.....109
Table 3.9: Association various clinicopathological parameters of Cdk5 mRNA expression in the METABRIC cohort.....113
Table 3. 10: Proliferation IC_{50} values of purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin in MDA-MB-231, T47D, and MCF7 breast cancer cells. 119

Table 4.1: Association between various clinicopathological parameters and cytoplasmic and nuclear *Sp7* 154
Table 4.2: Association between various clinicopathological parameters and cytoplasmic and nuclear *GRB7*.....156
Table 4.3: Association between various clinicopathological parameters and cytoplasmic and nuclear *DKK1*.....158
Table 4.4: Association between various clinicopathological parameters and cytoplasmic and nuclear level of phosphorylated DARPP-32 at Threonine-34 160

Table 4.5: Associations between various clinicopathological variables and Sp7 mRNA expression in the METABRIC cohort. 165
Table 4.6: Associations between various clinicopathological variables and *GRB7* (probe 1, 2 and 3) mRNA expression in the METABRIC cohort. 168
Table 4.7: Associations between various clinicopathological variables and *DKK1* (probe 1 and 2) mRNA expression in the METABRIC cohort. 172
Table 4.8: Associations between various clinicopathological variables and *PPP1R1B* (probe 1,2 and 3) mRNA expression in the METABRIC cohort. 175

Table 5.1: The lists of top 10 transcripts with the most significant alterations that were either down or up regulated in the DARPP-32 knockdown cells compared with control cells. 203
Table 5.2: The lists of top 10 transcripts with the most significant alterations that either were down or up regulated in the DARPP-32 knockdown cells compared with control cells..... 206

List of Figurers

Figure 1.1: Schematic representation of DARPP-32 and phosphorylation sites.....28
Figure 1.2: Schematic representation of the common structural features within the CREB protein family. 30
Figure 1.3: The catalytic subunit of PP1 has 6 protein variants that have the ability to interact with over 200 other proteins.....31
Figure 1.4: Demonstration of various sections of DARPP-32 35
Figure 1.5: Suppression of breast cancer cell migration through modulation of DARPP-32 mediated by Wnt-5a.....40

Figure 2.1: Demonstration of the hydrophobic line surrounding a section.. 54

Figure 3.1: Various combinations of proteins that interact with PP1γ .70
Figure 3.2: Representative photomicrographs of PKA, PP1, and Cdk5 staining using immunohistochemistry.. 76
Figure 3.3: Kaplan-Meier analysis of PKA expression (A) and PKA in oestrogen receptor positive breast cancer; PP1 expression in the cytoplasmic and nuclear, Cdk5 expression in the cytoplasmic and nuclear 84
Figure 3.4: Kaplan-Meier analysis of cytoplasmic expression in ER positive and ER negative breast cancers and their association with survival. The impact of nuclear expression of Cdk5 in ER positive and ER negative breast cancer on survival 86
Figure 3.5: Kaplan-Meier analysis and the influence of different combinations of protein expression on survival. Expression of PKA combined with cytoplasmic expression of DARPP-32 s.....95
Figure 3.6: Kaplan-Meier analysis and the influence of different combinations of protein expression on survival. Expression of PKA combined with nuclear expression of DARPP-32.....96
Figure 3.7: Low expression of *PRKAR1A* and PRKAR2A were significantly associated with adverse survival. Low expression of PRKAR1B was significantly associated with improved breast cancer specific survival There was no significant association between breast cancer survival and PRKAR2B expression..... 102
Figure 3.8: Low expression of *PRKACA* and *PRKACG* were significantly associated with improved breast cancer survival and low expression of *PRKACB* was significantly associated with adverse breast cancer survival 107

Figure 3.9: Low expression of *PPP1CA* was significantly associated with improved breast cancer specific survival, low expressions of *PPP1CB, PPP1CC* 112
Figure 3.10: Kaplan-Meier analysis of the association between patient survival and Cdk5 mRNA expression level, patient survival and Cdk5 mRNA expression in ER positive disease and ER negative disease..... 115
Figure 3.11: The effect of Purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin on cell proliferation in three breast cancer cell lines MDA-MB-231, T47D and MCF7. 118
Figure 3. 12: The activity of PKA following treatment with PKA inhibitor fragment (6-22) amide, Cdk5 activity following treatment purvalanol B, DARPP-32 Threonine-34 following treatment PKA inhibitor fragment (6-22) amide or tautomycetin, DARPP-32 Threonine-75 following treatment purvalanol B or forskolin..... 122
Figure 3.13: The expression of DARPP-32 and beta actin in the presence and in the absence of purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin in T47D and MDA-MB-231 cells were monitored in a single experiment using Western blotting. 123

Figure 4.1: Schematic representation of interactions that link various proteins within cells. These proteins include Wnt5A, PKA, Cdk5, *DKK1*, *Sp7*, DARPP32, t-DARPP, and DARPP delta3/4.....137
Figure 4.2: A demonstration of the four members of the *DKK* family. 146
Figure 4.3: Staining for negative control, staining of positive control,staining of DARPP-32 in and t-DARPP 150
Figure 4.4: Representation of expression patterns of *Sp7, GRB7, DKK1* and DARPP-32 at Threonine-34 using Immunohistochemistry 152
Figure 4.5: Kaplan-Meier analysis of *Sp7, GRB7*, *DKK1*, and phosphorylation of DARPP-32 at Threonine-34. 164
Figure 4.6: Kaplan-Meier analysis of breast cancer patients demonstrating the effect of *Sp7* expression, *PPP1R1B* probe 1, 2 and 3, *GRB7* probe 1, 2 and 3, and *DKK1* probe 1,2. 179

Figure 5.1: A comparative demonstration between DARPP-32 expression level in DARPP-32 knockdown cells, and beta actin expression (control) in the T47D cell line following treatment with DARPP-32 siRNA oligo duplex at 24, 48 and 72 hour time points. 195
Figure 5.2: Data from DESeq2 analysis where transcript expression is compared between: DARPP-32 knockdown cells and control cells, E2 treated and untreated DARPP-32 knockdown cells, E2 treated and untreated control cells, E2 treated DARPP-32 knockdown cells and E2 treated control cells, PKAi treated and untreated DARPP-32 knockdown

cells, PKAi treated and untreated Control cells, and PKAi treated DARPP-32 knockdown cells and PKAi treated control cells 198
Figure5.3: Principal component analysis demonstrating the contrast in DESeq2 dispersion estimates between: DARPP-32 knockdown and control cells, E2 treated and untreated DARPP-32 knockdown cells, E2 treated and untreated control cells, E2 treated DARPP-32 knockdown cells and E2 treated control cells, PKAi treated and untreated DARPP-32 knockdown cells, PKAi treated and untreated Control cells, and PKAi treated DARPP-32 knockdown cells and PKAi treated control cells..... 200
Figure5.4: Identification and heat map analysis of the 50 most common transcripts with significant (*P*-adjusted) expression alterations in DARPP-32 knockdown cells compared to that of control 202
Figure 5.5: Identification and heat map analysis of the 50 most common transcripts with significant (*P*-adjusted) expression alterations (in DARPP-32 knockdown cells compared to that of DARPP-32 knockdown cells treated with E2. 205
Figure5.6: Identification and heat map analysis of the 50 most common transcripts with significant (P-adjusted) expression alterations in DARPP-32 knockdown cells compared to that of DARPP-32 knockdown cells treated with PKAi. 208
Figure5.7: Venn diagram was adopted in order to demonstrate the overlapping of transcripts that were expressed in the presence and in the absence of E2 treatment. 210
Figure5.8: Venn diagram to demonstrate the overlapping transcripts that expressed in the presence and absence of PKAi treatment. 211
Figure5.9: The 10 most significantly (threshold $P<0.05$) altered pathways are shown above. 215
Figure5.10: Addition of *PPP1R1B* (DARPP-32) to the Tight junction canonical pathway for DARPP-32 knockdown cell versus control cell, DARPP-32 knockdown cell versus DARPP-32 knockdown cell treated with E2, and DARPP-32 knockdown cell treated with E2 versus control cell treated with E2 218

Figure 6.1: Summary diagram of DARPP32 pathway demonstrating regulation of DARPP-32 phosphorylation state by protein kinases and phosphatases. 230

Chapter 1 General Introduction

1.1 Introduction of Breast Cancer

1.1.1 Epidemiology

Breast cancer is the most common type of cancer in women across the world (Sharma et al., 2010, Balekouzou et al., 2016). In the UK alone, over 55,000 new cases of breast cancer are reported annually, with an 18% increase in breast cancer incidence since the early 1990s; this is predicted to rise by a further 2% by 2035. Metastasis accounts for the majority of breast cancer deaths, which stand at >11,500 per year in the UK (Cancer Research UK, 2021).

On a much larger scale, and based on statistical data, the World Health Organisation (WHO) has estimated a collective Disability Adjusted Life Years (DALYs) of patients with various conditions across the world. For all malignant neoplasms, the WHO has estimated 107.8 million DALYs, of which 19.6 million DALYs are attributed to breast cancer (Łukasiewicz et al., 2021). In the five years leading up to the end of 2020, around 7.8 million new cases of breast cancer were recorded globally, including 2.3 million cases in 2020 which led to 685,000 deaths (Sung et al., 2021, WHO, 2021).

Breast cancer is the most prevalent type of cancer in most countries (159 of 185 countries), and it is the leading cause of cancer related deaths in 110 countries (Sung et al., 2021). In Central Africa, breast cancer related deaths accounted for 24.5% of all deaths in females in 2012 (Balekouzou et al., 2016). According to Global Cancer Statistics 2020, breast cancer accounts for 25% of all cancer cases, and it is responsible for one in six cancer related deaths (Sung et al., 2021).

The populations of the Asia pacific region account for almost a quarter of all breast cancer incidence in the world, with hotspots in China, Japan and Indonesia. In 2012 alone, some 277,054 new cases of breast cancer were recorded in East Asia. Data shows that between 1988 and 2006, the Korean population has had the highest prevalence of breast cancer in the

region (Momenimovahed and Salehiniya, 2019, Youlden et al., 2014, Ghoncheh et al., 2016).

Worldwide, five-year survival rates are worse for patients living in less/moderately developed countries; five-year survival rates following diagnosis are much lower in Brazil (58.4%), India (52%), Algeria (38.8%), and Gambia (12%), when compared to the United States (83.9%), Sweden (82.0%), Japan (81.6%), and Australia (80.7%) (Rivera-Franco and Leon-Rodriguez, 2018).

Development of modern diagnostic and treatment methods in the recent years has been the result of research and improved understanding of breast cancer biology. This has paved the way for the discovery of more target-orientated methods of treatment. Promotion of public awareness, combined with improved diagnostic methods, has led to increasingly earlier detection and more accurate diagnosis; this has improved prognosis and the survival rate in patients (Sharma et al., 2010). Despite the recent monitoring and therapeutic advancements, the high incidence of breast cancer and the mortality rate associated with it continue to have serious impact on the quality and longevity of life in patients (Patel et al., 2016).

1.1.2 Risk factors

There is a variety of risk factors that are known contributors to the development of breast cancer; some of these risk factors are avoidable (Łukasiewicz et al., 2021).

1.1.2.1 Genetic implications and family history

A study led by the Institute of Cancer Research in London UK has identified 110 genes associated with increased risk of developing breast cancer (The Institute of Cancer Research, 2021). Almost one in every four

patients diagnosed with breast cancer have a family history of the condition, and around 13-19% of patients have a first-degree family member affected (Sun et al., 2017, Łukasiewicz et al., 2021). One study investigated the relation between breast cancer and family history in over 113,000 patients and it estimated a 1.75-fold higher chance of developing breast cancer in women who have at least one first-degree family member with the disease compared to those that do not. This risk factor increases significantly to 2.5-fold in females with more than one first-degree family member affected (Sun et al., 2017, Brewer et al., 2017).

Some individuals are prone to developing breast cancer through inheritance of certain predisposing genetic mutations, especially those that code for tumour suppressors breast cancer gene 1 (BRCA1) and breast cancer gene 2 (BRCA2) (Sun et al., 2017). Mutations of BRCA1 and BRCA2 could have a direct impact on the DNA repair mechanism through impairing interactions with tumour protein 53 (TP53), and RAD51 amongst other proteins associated with the cell cycle and DNA damage response. This results in irregularities in DNA synthesis (Godet and Gilkes, 2017, Davis and Lin, 2011). Annual mammography and breast magnetic resonance imaging (MRI) inspections are strongly advisable to families with a history of BRCA gene abnormalities (Metcalfe et al., 2009). BRCA mutations can act in an autosomal dominant manner and cause abnormalities even in the presence of a second healthy allele. The importance of BRCA genes is further highlighted by the fact that up to 25% of hereditary breast cancers and up to 10% of all breast cancers carry BRCA1/2 mutations. It has been estimated that there is up to 72% chance of breast cancer development in women with either or both BRCA1/2 mutations (Sun et al., 2017, Mehrgou and Akouchekian, 2016, Breast cancer org, 2021).

Human epidermal growth factor receptor-2 (HER2), a member of the membrane tyrosine Kinase family encoded by the proto-oncogene *Erb-B2* receptor tyrosine kinase 2 (*ERBB2*) (located on chromosome 17q21), is an important receptor implicated in the development and progression of

some breast cancers (Iqbal, 2014, Xie et al., 2000, Ukita et al., 2002). Certain mutations can activate the oncogene, resulting in increased cell proliferation. *ERBB2* is also known as the HER2/neu gene with "neu" referring to the rat/mouse homolog of HER2. Existence of this gene in other species has provided scientists the opportunity to carry out several *in vivo* studies focused on analysing and targeting tumour cells (Iqbal, 2014, Moasse, 2007, Tai et al., 2010).

1.1.2.2 Gender

Breast cancer can develop in both men and women; however, only <1% of the cases are reported to affect men. Male breast cancer often occurs in men with hormonal imbalance, or in those with family history of breast cancer characterised by BRCA2 gene mutation (Momenimovahed and Salehiniya, 2019).

1.1.2.3 Age

Similar to most other malignancies, the prevalence of breast cancer is directly proportional to the age of individuals (Sun et al., 2017). Macmillan Cancer Support has identified age as the strongest risk factor for the development of invasive breast cancer (Macmillan Cancer Support, 2021). The incidence of breast cancer increases with age until it reaches its peak at menopause, and gradually reducing thereafter (Momenimovahed and Salehiniya, 2019). Around 80% of all women diagnosed with breast cancer are over the age of 50. Breast cancer is rare in women under the age of 30 (Macmillan Cancer Support, 2021). Data also suggests that in 2016, around 99.3% of breast cancer related mortalities in the US were reported in women who were over the age of 40, and 71.2% in women over 60 (Sun et al., 2017, Siegel et al., 2017).

1.1.2.4 Reproductive factors

Hormone imbalance caused by events such as early menarche, late menopause and not having children/late age first pregnancy are major risk factors associated with increased chances of developing breast cancer (Sun et al., 2017, Colditz and Bohlke, 2014). The risk of developing breast cancer in women with delayed menopause increases by 3% for each year of delay. Delayed onset of menstrual cycle reduces the risk of breast cancer by 5% per year, and giving birth reduces the risk by 10% per birth. A hazard ratio (HR) of 1.54 is associated with late first labour (greater than 35 years) and early first labour (less than 20 years) (Sun et al., 2017, Rosato et al., 2014).

These reproductive factors have a significant impact on the regulation of endogenous oestrogen, and in some cases they facilitate prolonged exposure to oestrogen. Furthermore, excessive endogenous oestrogen exposure is strongly associated with development of breast cancer, highlighting the importance of factors influencing oestrogen regulation.

Oestrogen induces proliferation through estrogen receptor (ER)α, ERβ and G protein-coupled membrane receptor, GPR30. ERα and ERβ dimerise, bind ligands and activate downstream transcription factors that regulate gene transcription through binding to target genes, resulting in altered breast cancer phenotype and disease progression. Signal transducer and activator of transcription 5 (STAT5) is activated within cell cytoplasm in response to oestrogen and it translocated to the nucleus where it can bind to the promoter of several proliferative genes. In addition, interaction of growth factors with their tyrosine kinase transmembrane receptors induces a signalling cascade across multiple cells, leading to the activation of proteins such as c-Src, MAPK (ERK1/2) and Akt which accelerate cellular growth. Other signaling molecules that are stimulated by the activity of oestrogen include Src-homology and collagen homology (Shc), Phosphoinositide 3-kinase (PI3K), Modulator of Non-genomic Action of estrogen Receptor (MNAR), and p130 Cas (Fox et al., 2019, Nilsson et al., 2001). Therefore, high oestrogen levels can

result in enhanced rate of cellular proliferation through increased activity within the cellular transcription sector, as well as stimulation of carcinogenesis through DNA binding and up-regulation of cellular metabolic activity (Carroll, 2016, Tarannum et al., 2019).

1.1.2.5 Lifestyle

Life-style factors mainly refer to avoidable risk factors associated with the development of breast cancer and its progression. For instance, alcohol consumption has been associated with an increased risk of breast cancer as it induces elevated oestrogen related hormones in blood, activating oestrogen pathways (McDonald et al., 2013, Liu et al., 2015).

Examination of data from 53 independent epidemiological studies revealed that daily alcohol consumption of around 35-44 grams of alcohol is associated with a 32% increase in the risk of developing breast cancer, with a further 7.1% per additional 10 grams of alcohol per day (McDonald et al., 2013, Sun et al., 2017). Previously, the European Prospective Investigation into Cancer and Nutrition (EPIC) found a link between alcohol intake and development of hormone receptor-positive and hormone receptor-negative breast cancers, identifying those with higher alcohol intake as higher risk individuals, especially women who consume alcohol before their first full-term pregnancy (Momenimovahed and Salehiniya, 2019, Romieu et al., 2015).

Obesity is another lifestyle led risk factor which can contribute to the development of breast cancer through elevated levels of oestrogen. Adipose tissue expresses high levels of aromatase enzyme as a part of its metabolic activity which converts androgenic precursors to oestrogen leading to production of high levels of oestrogen. High levels of obesity-induced oestrogen adversely correlates with favourable clinical outcomes in breast cancer patients (lee et al., 2019, Ewertz et al., 2011). In addition, high levels of insulin and insulin like factors related to diet in obese individuals support the abnormal growth of malignant cells

(Momenimovahed and Salehiniya, 2019). Other obesity-induced cellular activities include adipose tissue inflammation and expression of pro-inflammatory mediators, tumour necrosis factor alpha (TNFα) and interleukin-6 (IL-6), through activation of macrophages (lee et al., 2019).

Obesity has also been associated with increased risk of developing triple-negative breast cancer. This suggests other underlying mechanisms that are independent of endogenous oestrogen (lee et al., 2019). The close relation between obesity and large tumour mass, high lymph node metastasis and high-grade tumours indicate a strong connection between obesity and development of more aggressive tumours with higher risk of disease recurrence (Jiralerspong and Goodwin, 2016).

A recent observational study on weight and risk of breast cancer reported that a weight gain of >5 kg past the age of 18 is associated with 17% of oestrogen Receptor positive / progesterone receptor positive (ER+/PR+) postmenopausal breast cancers as well as with 14% of postmenopausal breast cancers in a cohort consisting of 74,177 women with breast cancer. Meanwhile another study identified pre-pregnancy obesity as an independent risk factor promoting development of tumours of ovaries and breast (adjusted hazard ratio (HR), 1.4; 95% confidence interval (CI), 1.1–1.9). Similarly, body mass index (BMI) is linked with the outcome and prognosis of breast cancer patients (Momenimovahed and Salehiniya, 2019, Rosner et al., 2017, Kessous et al., 2017, Berclaz et al., 2004).

Smoking is an additional life-style risk factor, increasing the risk of breast cancer through prolonged exposure to certain chemical components of tobacco that have been shown to promote uncontrolled cell proliferation (Kispert and McHowat, 2017, Medical News Today, 2021). There are both existing and emerging data that highlight the relation between smoking and breast cancer as mammary tissue is known to be capable of uptaking carcinogenic chemical components of tobacco including polycyclic aromatic hydrocarbons, aromatic amines and N-nitrosamines (Goldvaser et al., 2017, Terry and Goodman, 2006, Reynolds, 2013). Upon transportation to mammary tissue, these components may modulate

oncogenes and suppressor genes vital for the healthy state of cells such as the tumour suppressor gene p53. This makes even transient exposure to smoking potentially harmful to mammary cells as this facilitates exposure to pro-carcinogenic stimuli (Łukasiewicz et al., 2021). Several *in vitro* and *in vivo* studies have also demonstrated development of aggressive breast cancers following exposure to tobacco carcinogens (Goldvaser et al., 2017).

1.1.3 Breast cancer classification

1.1.3.1 Histological classification

The histological classification of breast cancer accounts for the appearance and morphological features of cells. The current guides set by the WHO in 2003 include classification of 20 major tumour types and 18 minor subtypes. Adoption of these classification guides unifies the approach in identification of the types of breast cancer across the globe (Viale, 2012, Malhotra et al., 2010).

More than 95% of all breast cancers are adenocarcinomas, which most commonly originate from milk ducts and their associated lobules (Makki, 2015). In general, breast carcinoma is mainly subcategorised into invasive ductal carcinoma (IDC) and ductal carcinoma *in situ* (DCIS), where DCIS comprises localised proliferation of epithelial cells within ducts. Histopathological features of DCIS include pleomorphism, sporadic regions of necrosis, and development of prominent multi-nucleoli cells (Pinder, 2010, Makki, 2015).

Invasive ductal carcinomas are characterised by the proliferation of neoplastic cells that penetrate through the duct wall and invade stroma. Histopathological features of IDC have a wide scope of variation and 75% of cases do not exhibit a distinct set of morphological features. Lobular carcinoma *in situ* (LCIS) are tumours that originate in the lobules rather than ducts and they have a much lower prevalence in comparison to DCIS and IDC (Makki, 2015, Salvatorelli et al., 2020).

Invasive ductal carcinoma is the most prevalent type of invasive breast cancer accounting for 55% of all diagnosed breast cancers (Makki, 2015). The most common sub-types of invasive breast cancer include medullary carcinoma, meta-plastic carcinoma, apocrine carcinoma, mucinous carcinoma, cribriform carcinoma, tubular carcinoma, neuroendocrine carcinoma, classic lobular carcinoma, and pleomorphic lobular carcinoma (Weigelt et al., 2010, Nascimento and Otoni, 2020). Accurate diagnosis of each type requires detailed examination of cellular morphology, as well as analysis of the clinical, epidemiological, and molecular features of the cancer (Sinn and Kreipe, 2013).

In one study, the risk of tumour metastasis was investigated in a cohort of patients with invasive lobular carcinoma. Out of the 530 patients that took part, 57% of patients represented the classic sub-type of invasive lobular carcinoma, 19% with the alveolar type, 11% with the solid type, and the remaining 13% were characterized by pleomorphic, signet ring cell, histiocytoid or apocrine features. It was demonstrated that those with the classic lobular carcinoma were at a lower risk of axillary lymph node metastases, and they were more likely to have a lower tumour grade. Patients with other variants of lobular carcinoma had a reduced disease-free survival and overall survival (Orvieto et al., 2008, Viale, 2012). Histopathological classification of breast cancer along with tumour grading and stage determination remains a vital piece of analysis essential for accurate diagnosis and prognosis (Viale, 2012).

1.1.3.2 Molecular subtypes

The five main intrinsic molecular subtypes of breast cancer are Luminal A, Luminal B, HER2-enriched (HER2-E), Basal like breast cancer (BLBC) and Normal-like breast epithelium. Each subtype is comprised of a number of genetic mutations (Dai et al., 2015, Al-thoubaity, 2019).

Table 1.1: The pattern of gene expression, tumour grade and the survival rate associated with the five subtypes of breast cancer are summarised in Table1. It is important to note that the prevalence covers all cases up to premenopausal period and it does not include all cases of breast cancers (Adapted and Modified from Dai et al., 2015).

Intrinsic subtype	IHC status	Grade	Outcome	Prevalence
Luminal A	[ER+|PR+] HER2-KI67-	1|2	Good	23.7%
Luminal B	[ER+|PR+] HER2-KI67+ [ER+|PR+] HER2+KI67+	2|3	Intermediate Poor	38.8% 14%
HER2 over-expression	[ER-PR-] HER2+	2|3	Poor	11.2%
Basal	[ER-PR-] HER2-, basal marker+	3	Poor	12.3%
Normal-like	[ER+|PR+] HER2-KI67-	1|2|3	Intermediate	7.8%

Note. ER-estrogen receptor, PR-progesterone receptor, HER2-human epidermal growth factor receptor2.

1.1.3.3 Luminal A and B

Luminal A is the single most common subtype of breast cancer which accounts for more than half of the cases, while luminal subtype B is the most common subtype detected in premenopausal females (Yersal and Barutca, 2014, Nascimento and Otoni, 2020). Ki-67 index was introduced as a proliferation marker differentiating the two luminal subtypes based on growth fraction observed in the breast tissue; no standardised cut-off point has been set to clearly distinguish between luminal A and B subtypes (Nishimura et al., 2010, Yersal and Barutca, 2014).

According to the St. Gallen consensus in 2013, luminal A subtype is characterised by the expression of ER (≥1%), PR (≥20%), HER2 (≤10%), and low levels of Ki-67 (<14%). Also, similar to luminal epithelial cells found in breast tissue, luminal A subtype exhibits high expression levels of cytokeratins (Nascimento and Otoni, 2020, Yin et al., 2020). Despite this, luminal A subtype has the most favourable prognosis as it is associated with lower tumour grade and better patient outcome with an 80% five-year survival rate. In part, the desirable patient outcome seen in the luminal A subtype is thought to be due to the expression of steroid

hormone receptors making cancer cells highly responsive to hormone therapy (Trop et al., 2014).

Luminal B Breast cancers are usually associated with a higher tumour grade, faster tumour progression, and poor prognosis. This subtype is characterised by low ER and PR expression levels, high tumour grade, and high recurrence rate (Li et al., 2016, Tran and Bedard, 2011). Luminal B subtype accounts for 15-20% of all cases of breast cancer. Shortly after the characterisation of this subtype in 2001, five-year survival rates for this subtype were estimated to be around 40% (Trop et al., 2014, Yersal and Barutca, 2014).

Studies have identified several genes with potential roles in the development and progression of luminal A and B breast cancer, among which *CCNB1*, *MKI67* and *MYBL2* were found to be highly expressed in luminal B compared with luminal A subtype (Tran and Bedard, 2011, Cheang et al., 2009). Although overexpression of HER2/neu is not a characteristic that is often associated with luminal B breast cancers, around 30% of patients exhibit up-regulation of HER2 cell receptors (Tran and Bedard, 2011).

There is histological variance within the Luminal B subtype as 6% of the cases are negative for ER and HER2 while the rest of the cases are either ER positive, HER2 negative or positive for both ER and HER2 with a high Ki-67 (Nishimura et al., 2010, Yersal and Barutca, 2014).

In an attempt to design a universal criterion that can be applied to clinical practice, several studies have bid on finding ways to differentiate luminal A and luminal B subtypes. In one study by Sorlie *et.al* (2003), different subtypes of breast cancer were distinguished using their distinct patterns of gene expression; in this study, a total of 115 malignant breast tumours were classified using hierarchical clustering based on patterns of expression of 534 intrinsic genes that were divided into gene cluster associated with each breast cancer subtypes, luminal subtype A and B, HER2-enriched, basal-like breast cancer and normal-like breast epithelium. Selection of the genes used for classification was based on

their similar level of expression in different samples taken from the same tumour after a 15-week neoadjuvant treatment interval (Sorlie et al., 2003).

1.1.3.4 HER2-enriched

While the Immunohistochemistry (IHC) profile of HER2-E breast cancer in many cases is associated with ER and PR negative status, around 30% of cases are hormone receptor positive. Interestingly, HER2-E encompasses 40-50% of all breast cancer incidence that are ER or PR positive, whereas the rest are mainly linked with luminal A and B subtypes. HER2-E represents 80-90% of all breast cancer that are ER and PR negative, and the remaining 10-20% of cases are linked with the basal like subtype (Prat et al., 2017, Godoy-Ortiz et al., 2019).

Tumours that overexpress HER2 are susceptible to HER2 targeted treatments. HER2-E patients show a much higher sensitivity to HER2 therapy compared to those who are diagnosed with other subtypes (Brasó-Maristany et al., 2020).

Molecular features of HER2-E tumours include overexpression of genes such as *ERBB2*, growth factor receptor bound protein 7 (*GRB7*), oestrogen receptor 1 (ESR1) and PR (Prat et al., 2017). The *GRB7* gene is located at the 17q12-21 amplicon, directly adjacent to the HER2 gene (Bivin et al., 2017). Aside from detection of *GRB7* and HER2 co-expression in some breast cancers, studies have also reported formation of *GRB7*/HER2 complexs in some breast cancer cell lines (Nadler et al., 2010).

1.1.3.5 Basal-like

Morphological features associated with Basal-like breast cancers are often characterised by the lack of ER, PR, and HER2 expression, also known as the triple negative immune phenotype (Hubalek et al., 2017). Up-

regulation of at least one of the basal cytokeratins (CK5/6, CK14, and CK17) is another known characteristic of Basal-like breast cancer which is usually accompanied by the overexpression of epidermal growth factor receptor (EGFR). Core basal phenotype is one of the simplest IHC criteria proposed so far and it is defined as negative ER, PR, and HER2, and positive CK5 and/or positive EGFR status (Badve et al., 2010, Cheang et al., 2008, Badowska-Kozakiewicz and Budzik, 2016). Absence of ER, PR, and HER2 receptors reduces treatment options for patients, which in turn leads to poor prognosis. Several clinical trials have been designed to find other targets in triple negative breast cancers (TNBCs) to improve efficacy of therapy in patients (Masood, 2016). While TNBC is not confined to basal-like subtype, they represent the majority of cases within this subtype.

Inherited abnormalities of BRCA1 have been associated with Basal-like breast cancers. BRCA1 dysfunction phenotype in the absence of BRCA1 specific abnormalities in patients may suggest involvement of other genetic and epigenetic impairments of BRCA associated proteins that may affect the function of BRCA1 further down the line (Botti et al., 2019, Testa et al., 2020).

Mutations of TP53 gene are observed in 44-82% of all basal like breast cancers. Given that TP53 protein is a critical tumour suppressor whose main function is DNA repair and initiation of apoptosis when DNA damage is beyond repair, mutation of TP53 could result in impairment of those functions. Thus, breast cancer cells sustain uncontrolled growth and they pass on altered DNA to daughter cells in the absence of a functional DNA repair mechanism. This result in a catastrophic genetic instability. Studies have demonstrated that the loss of a single TP53 allele in in BRCA1-null animal models is enough for the promotion of breast cancer, indicating that impairment of both of these genes favours evolution of basal like breast cancers (Botti et al., 2019, Liu et al., 2007, Xu et al., 1999, Hu et al., 2019).

1.1.3.6 Normal-like

Similar to luminal A, normal-like breast cancers are characterised by hormone receptor ER and/or PR positive status, HER2 negative status and low levels of Ki-67. Normal-like breast cancers make-up 5-10% of all breast carcinomas and they are associated with positive patient outcome (Breast cancer Org, 2021, Abubakar et al., 2019, Yersal and Barutca, 2014). In comparison to other breast cancer subtypes, pragmatic identification of normal-like breast cancer has proven to be more challenging as they mostly tend to express genes normally active in healthy adipose tissues. There is limited research data available on the mechanisms involved in development of normal like breast cancer as the clinical importance of this subtype remains disputed (Yersal and Barutca, 2014).

1.1.4 Prognostic and predictive factors

One of the most well-known and validated conventional predictive factors for breast cancer is the staging system which is assessed through monitoring tumour size, invasiveness of tumour, metastasis and nodal status (Allred et al., 1998). Other key prognostic indicators include histological grading, the status of HER2, EGFR, Vascular endothelial growth factor (VEGF), ER and PR status, Epithelial cadherin (E-cadherin), and p53 as well as the age of patients, and their Ki-67 index (Shen et al., 2018).

Predictive factors can aid healthcare professionals in giving patients advice when making crucial decisions including selection of appropriate therapeutic strategies. Healthcare professionals are also able to make predictions with regards to patient response to different therapies and their chances of survival based on data previously obtained from a large group of patients with similar conditions (Bundred, 2001, Halabi and Owzar, 2010). Like many other tumours, early detection of breast cancer

is associated with improved patient outcome through effective early treatment. Early detection also helps preventing further cancer development and metastasis to axillary nodes which directly affect prognosis (Synnestvedt et al., 2012).

1.1.4.1 Histological tumour grade

Determination of histological tumour grading depends on a number of factors including the rate of cancer cell proliferation, nuclear grade and the level of differentiation observed in malignant cells. Studies have highlighted the association of high tumour grading and cancer recurrence in breast cancer patients (Bundred, 2001, Silvestrini et al., 1995, Todd et al., 1987).

The level of tumour cell differentiation has a crucial impact on the grading score in breast cancer patients. Evaluation of tumour cells' morphological features involves examination of hematoxylin-eosin-stained tumour tissue sections in a semi-quantitative manner using a well-defined standard protocol (Rakha et al., 2010). The Nottingham Grading System (NGS), is a well-defined and popular grading system approved by a number of internationally recognised institutions such as the WHO, American Joint Committee on Cancer (AJCC), European Union (EU), and the Royal College of Pathologists (RCPath). NGS was introduced as a refined version of Bloom-Richardson system following addition of a three-grade numerical scoring system for the assessment of three morphological parameters (Cao and Lu, 2016, Elston and Ellis, 1991). The three morphological features that this grading system was set up to evaluate in malignant cells include, the extent of growth in tubule or gland, nuclear pleomorphism, and the rate of proliferation in the form of mitotic count. This is where each variable is given a score between 1 and 3 before the scores are combined and a final grade is assigned (Rakha et al., 2010, Cao and Lu, 2016). One of the main reasons that NGS has become the grading system of choice is its reliability in providing relevant

prognostic information for invasive ductal carcinomas and lobular carcinomas which account for over 80% of all breast cancers. Meanwhile, other complicated and expensive systems including the 70 gene prognostic signature MammaPrint™ have shown inconsistencies in some patients suffering from certain types of breast cancer (Rakha et al., 2010, Mook et al., 2009, Brandão et al., 2019).

1.1.4.2 Tumour size and staging

In 1959, the TNM staging system was introduced by the AJCC staging end results reporting. In this system a score of 1 to 4 is given in relation to the status of the primary tumour, lymph node invasion and the spread of tumour to secondary sites which are represented by letters T, N and M, respectively (Giuliano et al., 2017, Amin et al., 2017). The scores reflect the stage of tumour progression where stage 0 represents localised non-invasive breast cancers such as ductal carcinoma in situ and lobular carcinoma *in situ*, stages 1 to 3 refer to localised malignancies with an enhanced aggressive behaviour, and stage 4 encompasses highly complex and developed tumours that exhibit migration of malignant cells to secondary sites (Cao and Lu, 2016, Kiely et al., 2011).

Patients with stage 4 breast cancers have a life expectancy of around 21.7 months, demonstrated by a meta-analysis of 36 clinical trials that took place between 1999 and 2009 (Cao and Lu, 2016, Kiely et al., 2011). In 2019, another meta-analysis calculated the median life expectancy of breast cancer patients with distant metastases as 26.0 months from diagnosis. This study was comprised of a total of 18,322 patients whose median survival ranged from 8.0 months (95% CI 5.65–10.35 months) in those with brain metastasis to 36.0 months (95% CI 34.74–37.27 months) in those with bone metastasis (Wang et al., 2019).

The probability of nodal metastasis by a tumour with a diameter of 1.0 cm is around 10-20% and those patients that evade nodal metastasis from such tumours have a 90% chance of living a disease-free life for at

least 10 years following treatment. Accurate examination of tumour mass is vital for tumour stage determination and prognostic purposes (Fitzgibbons et al., 2000). Recent improvements in public awareness have resulted in enhanced participation in mammography screening which has led to increased detection of smaller cancers, commonly referred to as the pT1 cancers with sizes between 0.1 and 2 cm (Fitzgibbons et al., 2000, Seely and Alhassan, 2018).

1.1.4.3 Lymphovascular invasion

Penetration of breast cancer cells through blood vessels and lymph vessels, commonly known as blood vascular invasion lymphatic vessel invasion, or cumulatively lymphovascular or vascular invasion, is a strong indication of potential tumour migration and it has direct impact on tumour grading, recurrence, and patient outcome (Mohammed et al., 2007, Rakha and Ellis, 2006). The sensitivity of detection of vascular invasion can sometimes be hindered in individuals who are exposed to neoadjuvant chemotherapy. As the use of neoadjuvant chemotherapy is becoming more common, interference with clinical techniques remains a potential risk (Shen et al., 2018, Ryu et al., 2018).

A number of studies, including one by Agarwal *et.al* (2018), have investigated the practicality of IHC in detection of lymphovascular invasion in order to assess the limitations of this technique, and they have ultimately suggested its use for prognostic purposes. (Agarwal et al., 2018, Song et al., 2011). Storr *et.al* (2011) studied the importance of blood and lymphatic vascular invasion as prognostic markers in 202 cutaneous melanoma patients. Use of IHC improved detection of vascular invasion from 8-30% in a direct comparison to haematoxylin and eosin staining. IHC was used to reveal occurrence of a higher rate of lymphatic vessel invasion in comparison to blood vessel invasion in patients that were examined (27% and 4% of patients, respectively) (Storr et al., 2011).

1.1.4.4 Oestrogen and progesterone receptors

Hormone receptor status is a crucial prognostic biomarker that aids determination of treatment options in breast cancer (Cao and Lu, 2016). ER positive status is detected in approximately 70% of all primary breast cancers. The ER positive status can be detected through techniques such as radio-ligand assays and IHC (Cao and Lu, 2016, Bundred et al., 2001).

A positive PR status is observed in 50% of breast tumours, principally in tumours that are also ER positive. (Łukasiewicz et al., 2021). PR and ER status are linked through the influence of the ER pathway on the regulation of PR. While a positive correlation between the overexpression of ER/PR and the inherited susceptibility to breast cancer has been observed, detection of these biomarkers allows for a target-oriented hormone therapy aimed at regulation of such receptors (Cianfrocca and Goldstein, 2004, Carroll et al., 2016, Łukasiewicz et al., 2021).

1.1.5 Treatment

Treatment options for breast cancer patients depends predominantly on the type of cancer as highlighted in the breast cancer classification section 1.1.3, a treatment regimen for breast cancer usually involves surgery, chemotherapy, radiotherapy, hormone therapy and biological therapy (Nounou et al., 2015).

1.1.5.1 Surgery

One of the most effective strategies in eradicating breast cancer is surgical removal of the affected section (lumpectomy) or, in some cases, removal of the entire breast tissue (mastectomy). Breast conserving surgery procedures are also known as partial/segmental mastectomy, wide local excision, or quadrantectomy. In cases where excision of a

cancer-free margin is not achieved following surgery, further surgery may be necessary to reduce the risk of relapse (Morrow et al., 2009, Łukasiewicz et al., 2021).

1.1.5.2 Chemotherapy

Chemotherapy is a conventional therapeutic method that involves administration of agents that target proliferating cells. The suitability of chemotherapy for administration is assessed based on factors such as type and stage of the cancer, individual drug tolerance, age, status of menopause, overall state of health, medical history, existence of cardiovascular disease, and other potential health complications that can increase the risk of further health deterioration (Sharma et al., 2010).

According to the Cancer Research UK official website, combination of 2 or 3 chemotherapy drugs is a common practice for the treatment of breast cancer in the UK; some patients may only receive one type of chemotherapy based on the strategy of treatment. Examples of such drugs include Docetaxel, Eribulin or Paclitaxel as a single chemotherapy treatment method, or a combination of Docetaxel and Cyclophosphamide, Epiribicin and Cyclophosphamide, Doxorubicin and Cyclophosphamide, or Cyclophosphamide, Methotrexate and Fluorouracil (5FU). The examples of chemotherapy administered for secondary breast cancer include Capecitabine, Epirubicin, Cyclophosphamide or Vinorelbine as a single chemotherapy treatment method, or a combination of Cyclophosphamide and Methotrexate, Gemcitabine and Paclitaxel, Paclitaxel and Carboplatin, or Nab, paclitaxel and Carboplatin (Cancer Research UK, 2022).

In a randomised phase III trial known as the treating to new targets (TNT) trial, which was conducted from 2008 to 2014, 376 patients were subjected to treatment with either carboplatin (n=188) or docetaxel (n=188) in order to assess sensitivity of different breast cancer subtypes to those drugs. Patients with advanced TNBC who exhibited BRCA1 or

BRCA2 germline mutations showed a greater sensitivity to carboplatin than docetaxel (objective response rate (ORR): 17/25 (68·0%) vs. 6/18 (33·3%), respectively, absolute difference 34·7%, *P*=0·03), hence the growing enthusiasm for the use of carboplatin as a first line of treatment in patients with BRCA1 or BRCA2 germline mutations. Patients with non-basal-like tumours showed a significantly better response to docetaxel when compared against carboplatin (ORR: 13/18 (72·2%) vs. 3/18 (16·7%), respectively, absolute difference -55·5%, *P*=0·002). Therefore, determination of breast cancer subtype prior to administration of chemotherapy could aid selection of suitable treatment options (Tutt et al., 2018).

1.1.5.3 Hormonal therapy

Hormone therapy is an effective neoadjuvant and adjuvant treatment used to treat/prevent tumour metastasis and tumour recurrence in hormone receptor positive cancers through blockage of signalling pathways promoting cancer progression downstream of oestrogen and progesterone (Łukasiewicz et al., 2021).

Positive ER status, in the presence of other related markers such as PR, serves as a robust prognostic factor and is an indication of positive patient response to hormone therapy (Louie and Sevigny, 2017, Łukasiewicz et al., 2021). Positive response to hormone-therapy has been reported in 60-70% of patients with ER and PR positive status. In contrast, positive response to hormone therapy is achieved in less than 10% of patients with ER and PR negative status, and in 40% of patients with ER positive/PR negative status (Bundred, 2001).

In some cases, continuation of hormone therapy may be necessary for a period of up to 10 years or more in order to minimise the risk of tumour recurrence. Combination of adjuvant tamoxifen and aromatase inhibitor during hormone therapy reduces the risk of relapse by almost 50% for

the initial four years, and by more than 30% for up to 9 years following treatment (Moo et al., 2018).

Data suggests that despite initial positive response, approximately half of hormone receptor positive patients may eventually develop resistance and instigate relapse if hormone therapy is not combined with chemotherapy (Łukasiewicz et al., 2021). Until recently, administration of tamoxifen was the preferred option for all hormone receptor positive premenopausal patients. However, this strategy was revised following introduction of a more effective regimen which involves combination of exemestane, ovarian suppression and chemotherapy; the superiority of this combination over the use of tamoxifen in premenopausal patients was demonstrated following a randomized 3-arm SOFT trial (McDonald et al., 2016, Pagani et al., 2014).

Other Hormone receptor modulators include aromatase inhibitors and gonadotrophin-releasing hormone (GnRH) agonists (Lumachi et al., 2011). Aromatase inhibitors are known to be effective and are generally well tolerated in postmenopausal patients (Chumsri et al., 2011). GnRH agents such as goserelin ('Zoladex') are mainly used for the treatment of ovarian surgical ablation (OSA) breast cancer in women; goserelin modulates the release of ER in ovaries through downregulation of pituitary GnRH receptors and suppression of luteinising hormone (LH) and follicle stimulating hormone (FSH) (Robertson and Blamey, 2003).

1.1.5.4 Biological therapy

Biological therapy refers to selective therapy targeting specific cancer cell biomarkers. In HER2-positive breast cancer, trastuzumab, pertuzumab, trastuzumab deruxtecan, lapatinib, and neratinib are HER2 targeted agents administered throughout the course of the treatment as neoadjuvant therapy and as adjuvant therapy (Łukasiewicz et al., 2021, Thompson and Moulder-Thompson, 2012). Other biological therapeutics

include angiogenesis inhibitors such as humanized monoclonal anti-VEGF antibody (rhuMAb VEGF) which is also known as bevacizumab (Łukasiewicz et al., 2021, Sun et al., 2017). Trastuzumab emtansine, also known by its brand name Kadcyla, is another type of targeted cancer drug used for the treatment of early breast cancer, post-surgery, metastasised breast cancer and cancer relapse.

Trastuzumab is a monoclonal antibody that acts through blockage of HER2 receptors on the surface of cancer cells, impairing the downstream pathways that would otherwise promote cancer progression and metastasis. Use of trastuzumab is common for the treatment of early stage and metastatic breast cancer (Waks and Winer, 2019, Lavaud and Andre, 2014). Co-administration of trastuzumab with pertuzumab, as well as with other agents such as neratinib during adjuvant therapy, or lapatinib in metastatic cancers, has improved overall response in HER2 positive patients in comparison to the use of trastuzumab alone (Brandão et al., 2018). Administration of dual HER2 agents before surgery is a common practice in the treatment of stage II-III HER2 positive breast cancers (Moo et al., 2018).

Sacituzumab govitecan (Trodelvy), an antibody-drug conjugate approved by the Food and Drug Administration (FDA) in April 2020 for the treatment of metastatic TNBC, selectively targets Trophoblast cell-surface antigen-2 (Trop-2) expressed in epithelial cancers (Kalinsky et al., 2020). Despite demonstration of its therapeutic effects during clinical trials and its consequent approval by the FDA, use of Sacituzumab govitecan for the treatment of patients with TNBC is not being recommended by the National Institute for Health and Care Excellence (NICE) in England; this is predominantly due to financial limitations as this drug is not considered a cost-effective treatment for National Health Service (NHS) England (Cancer Research Uk, 2022).

1.1.5.5 Radiotherapy

Since the discovery of X-rays in 1895 by Wilhelm Conrad Röntgen and the consequent realisation of its clinical application in cancer treatment, over time, radio therapy has emerged as a cost effective technique used to destroy cancer cells. Recent advancements in innovation of modern radiation treatment machines with improved X-ray production and treatment delivery has boosted the effectiveness of this technique in cancer therapy. Today, radiation therapy is utilised as a part of treatment regimen for more than 50% of all cancer patients and it accounts for 5% of the total cost of cancer care (Baskar et al., 2012, Ringborg et al., 2003).

Exposure to high-energy radiation induces formation of electrically charged particles causing DNA damage and cell death. Although ionising energy is focused on delivering maximum dose of radiation to cancer cells, it can also cause genetic damage to the normal cells surrounding the area or those in the path of radiation. However, in comparison to healthy cells, cancer cells are believed to be less efficient in recovering from such damage (Baskar et al., 2012, Begg et al., 2011).

Combination of radiotherapy with surgery, chemotherapy or immunotherapy is a common practice for the treatment of breast cancer in both neoadjuvant therapy and adjuvant therapy. For ductal carcinoma *in situ,* systemic irradiation is required following lumpectomy instead of whole breast irradiation. Chest wall irradiation is recommended following mastectomy in patients with primary tumour stage T3-T4, with additional irradiation directed at affected lymph nodes as required. Internal mammary irradiation is assessed separately in individuals on a case by case basis to assess the benefits and risks (cardiac toxicity) associated with this treatment (Hennequin et al., 2022 Baskar et al., 2012).

1.1.5.6 Side effects

All currently available cancer treatment options are associated with a range of side effects. Despite the significant therapeutic benefit of surgical removal of tumours, surgery can instigate significant discomfort in patients, both physically and mentally, and can be associated with chronic lymphoedema and seroma as a result of fluid build-up in the surrounding tissues (Sampathraju and Rodrigues, 2011, Collins et al., 2011, Toyserkani et al., 2017).

Some of the general side effects commonly observed in patients due to exposure to chemotherapy include hair loss, disruption of nail growth, mouth sores, memory loss, weight loss, diarrhoea, nausea and vomiting (Andreopoulou and Sparano, 2013).

Administration of chemotherapy can also lead to much more serious side effects such as increased risk of developing leukaemia, heart failure, neuropathy, and immunosuppression due to the lack of white blood cell production which, in turn, induces extreme vulnerability to infections (Shakir and Rasul, 2009, American Cancer Society, 2022). Radiotherapy is associated with numbness around the breast and under arms, as well as skin darkening and irritations in patients following exposure to high energy radiation. Other common side effects induced by radiotherapy include fatigue and lymphoedema (Łukasiewicz et al., 2021).

Hormone therapy can cause episodes of hot flashes, tiredness, stomach sensitivity and vomiting, joint and muscle pain, vaginal discharge, dryness or irritation in females, whereas sexual impotence in males has been one of the major undesirable side effects (Sert et al., 2014). In contrast to other types of therapy, side effects associated with trastuzumab treatment are often mild and manageable; however, in rare cases some patients can experience congestive heart failure (CHF) as a result of reduction in left ventricular ejection fraction (LVEF) (Ewer et al., 2005).

Considering the side effects associated with each of the treatment methods currently available, and taking into account the prevalence of breast cancer, there is a desperate need for novel, selective and effective treatment strategies for the treatment of this condition. This could be achieved through a better understanding of the underlying mechanisms associated with breast cancer.

1.2 DARPP-32

1.2.1 Discovery of DARPP-32

Recent improvements in the understanding of biological pathways involved in the development and progression of breast cancer has led to the discovery of many promising therapeutic targets. There is growing evidence suggesting that dopamine and cAMP regulated phosphoprotein 32 kDa (DARPP-32), encoded by phosphoprotein phosphatase-1 regulatory subunit 1B (*PPP1R1B)*, may play an important role in the development a number of tumours including breast cancer (Belkhiri et al., 2016). Emerging evidence suggests that dysregulations of protein kinase A (PKA) and protein phosphatase-1 (PP1), regulated by DARPP-32, have direct impacts on pathways relevant to breast cancer, including the cAMP response element binding protein (CREB) pathway, and processes such as metabolism, gene expression and apoptosis (Michalides et al., 2004, Saidy et al., 2021). The diverse interaction of PKA and PP1 with a large number of cellular proteins potentiates the catastrophe of their dysregulation which is thought to influence several cellular processes associated with tumorigenesis (Ruvolo, 2019, Sapio et al., 2014, Sakamoto and Frank, 2009).

DARPP-32 was originally discovered as a result of investigations on characterisation of cAMP dependant cascade and neurotransmitters in the nervous system. Activated PKA was identified as a crucial contributor to the cascade downstream of dopamine receptor and adenylyl cyclase (Walaas et al., 1983) and subsequent investigations led to the discovery

of various proteins, including a 32 kilo Dalton protein which was found in striatal medium size spiny neurons and was later named DARPP-32 (Fernandez et al., 2006, Walaas and Greengard, 1984). DARPP-32 was first described as a substrate of PKA in the neostriatum region of the brain (Belkhiri et al., 2016); Paul Greengard was awarded the Nobel Prize (2000) for his work which demonstrated that DARPP-32 can function as a central signalling switch within the brain (Ravindran and Greengard, 2011).

1.2.2 The function of DARPP-32

The regulation and functionality of DARPP-32 is influenced by its interactions with various cellular proteins as demonstrated in Figure 1.1. PKA induced phosphorylation at its Threonine-34 residue allows DARPP-32 to act as a potent inhibitor of PP1 (Yger and Girault, 2011, Aggen et al., 2000). Cyclin dependent kinase 5 (Cdk5) phosphorylates DARPP-32 at Threonine-75 to allow DARPP-32 to inhibit PKA, thereby acting as a crucial balancing figure of biochemical, electrophysiological, and behavioural effects (Belkhiri et al., 2016, Fernandez et al., 2006). Ca2+ dependant Protein phosphatase 2B (PP2B) dephosphorylates DARPP-32 at Threonine-34, whereas protein phosphatase 2A (PP2A) dephosphorylates Threonine-75 (Ahn et al., 2007, Fernandez et al., 2006).

Casein kinase 2 (CK2) induced phosphorylation of Serine-97 enables cyto-nuclear shuttling of DARPP-32 which promotes enhanced interactions between DARPP-32 and PKA (Fernandez et al., 2006, Yger and Girault, 2011). Serine-102 and Serine-137 are also susceptible to phosphorylation through the activity of CK2 and CK1, respectively. Addition of phosphate groups to these residues has crucial impacts on the conformational state of DARPP-32, where phosphorylation of Serine-102 enhances the availability of Threonine-34 for phosphorylation, and phosphorylation of Serine-137 results in reduced PP2B induced

Threonine-34 dephosphorylation (Fernandez et al., 2006, Belkhiri et al., 2016).

Considering the influence of each phosphorylation site on the function of DARPP-32 and the complex protein interactions that are important in maintaining a healthy biochemical cellular environment, DARPP-32 can act as either kinase or a phosphatase inhibitor. Any aberrations affecting the expression or the activity of DARPP-32 could result in cellular imbalance and impairment of complex mechanisms involved in cAMP/PKA and Ca2+/PP2A/2B pathways (Wang et al., 2017, Yger and Girault, 2011).

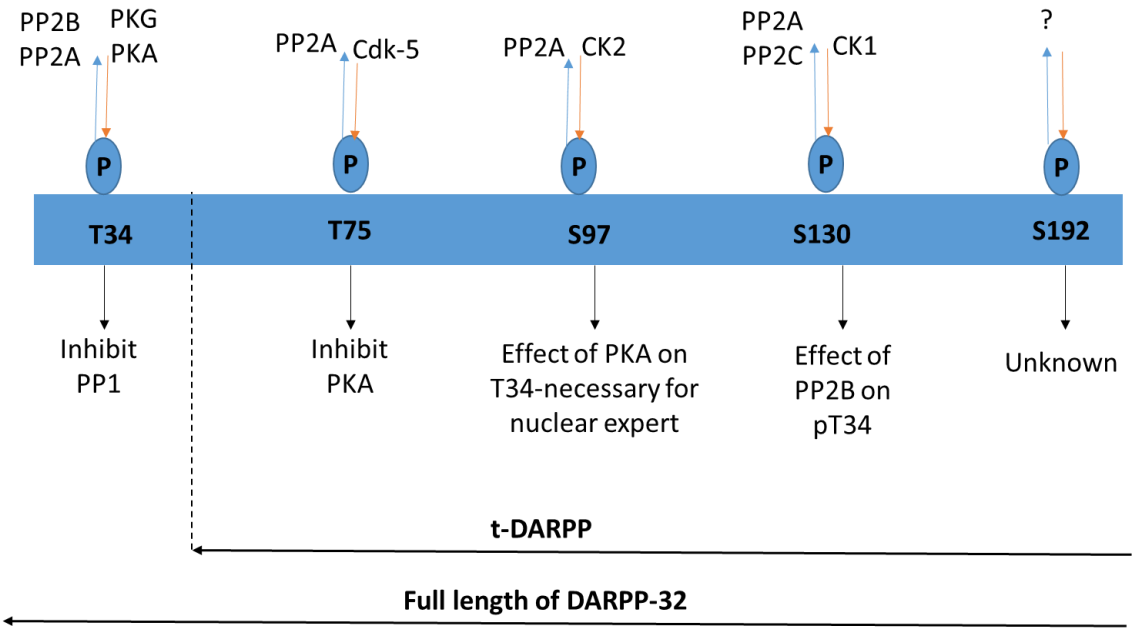


Figure 1.1: Schematic representation of DARPP-32 and phosphorylation sites. Absence of Threonine-34 residue leaves t-DARPP unable to inhibit PP1. Interaction of some of protein kinases and phosphatases are demonstrated using red arrows and blue arrows (respectively). Outcome of each of these interactions is displayed beneath each of the residues. The dotted line represents deletion of Threonine-34 residue in t-DARPP (Adapted and Modified from Yger and Girault, 2011).

1.2.3 PKA

PKA, a well-studied multi-unit protein, is a member of the serine threonine protein kinase superfamily. PKA, whose activity largely depends on the cellular levels of cAMP, has an important role in transduction of cellular signals downstream of G-protein coupled receptors (Sapio et al., 2014). Examples of regular cellular processes influenced by PKA include metabolism, gene expression and apoptosis (Michalides et al., 2004, Saidy et al., 2021). The heterotetrameric structure of PKA consists of two catalytic subunits encoded by *PRKACA, PRKACB* and *PRKACG*, and two regulatory subunits encoded by *PRKAR1A, PRKAR1B, PRKAR2A* and *PRKAR2B* (Saidy et al., 2021). Cα and Cβ encoded by *PRKACA* and *PRKACB* genes, respectively, represent the two predominant types of catalytic subunits with multiple splice variants. This diversity is integral for the function of PKA in interacting with various downstream proteins such as CREB, Raf, Bad and glycogen synthase kinase (GSK3), thereby exerting influence over patterns of gene expression and cell survival (Wu et al., 2007, Zhang et al., 2020, Häfner et al., 1994). Another distinctive feature which diversifies the function of PKA is the variation of its regulatory subunit, giving rise to two closely related types (type I and II) that comprise four isoforms in total: RIα, RIβ, RIIα, and RIIβ (Cho et al., 2000, McKnight et al., 1988, Levy et al., 1988).

PKA predominantly targets the CREB family of transcription factors within the nucleus (Sapio et al., 2014). The CREB protein as well as other similar proteins such as cAMP responsive element regulatory protein (CREM) and transcriptional activator 1 (ATF1) are members of the basic leucine zipper (bZIP) superfamily of transcription factors. CREB and ATF1 are present in various tissues have the ability to form homodimers or heterodimers, whereas CREM is predominantly present in the neuroendocrine tissues (Foulkes et al., 1991, Zhang et al., 2020). The common structural features across CREB family proteins are: bZIP, two glutamate domains (Q1 and Q2), and a 60 amino acid long kinase inducible domain (KID)

situated near the centre of the protein which consists of a PKA phosphorylation site (Figure 1.2) (Zhang et al., 2020, Sandoval et al., 2009).

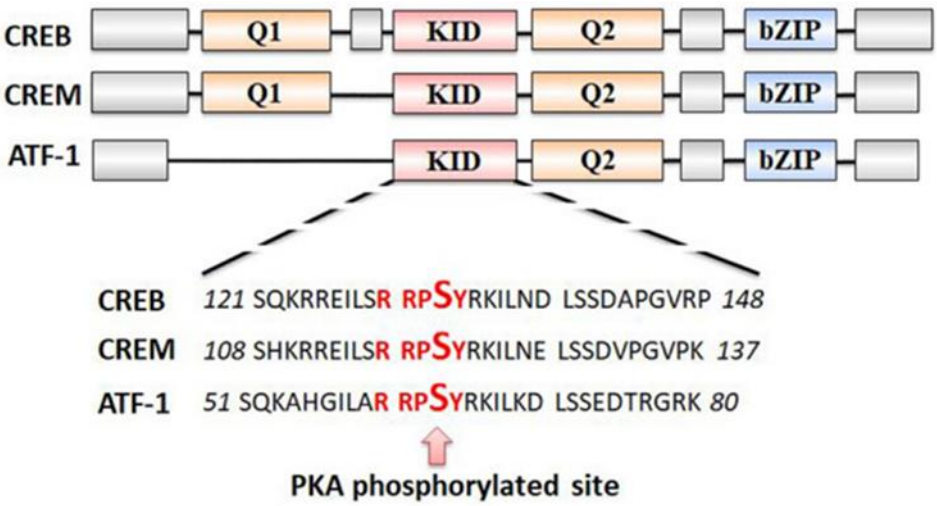


Figure 1.2: Schematic representation of the common structural features within the CREB protein family. (Image taken from Zhang et al., 2020), permission by Creative Commons Attribution 4.0 International license (CC-BY-4.0).

Upon phosphorylation by PKA, CREB proteins interact with the coactivator CREB binding protein and induce gene expression by binding to a specific DNA sequence (TGACGTCA) known as the cAMP response elements (CREs) located within the promoter region of genes (Sapio et al., 2014, Schmidt et al., 2013, Sands and Palmer, 2008). There have been up to 4000 genes identified to date as targets of CREB and each of these targets can influence several cellular processes. Over the years, studies have indicated a potential role of CREB in tumorigenesis (Sapio et al., 2014, Sakamoto and Frank, 2009).

1.2.4 PP1

PP1 is an important regulator of cell division, protein synthesis, muscle function, carbohydrate metabolism, RNA splicing, transcription and neuronal signalling (Peti et al., 2013, Aggen et al., 2000). Balanced

regulation of PP1 is crucial to a notable proportion of cellular processes. The functional diversity of this protein primarily stems from its structure which comprises a catalytic subunit encoded by *PPP1CA*, *PPP1CB* or *PPP1CC* (Moura and Conde, 2019, Saidy et al., 2021). The catalytic subunit of PP1 has the ability to interact with over 200 proteins that serve a regulatory role and determine its substrate specificity (Figure 1.3) (Ruvolo, 2019).

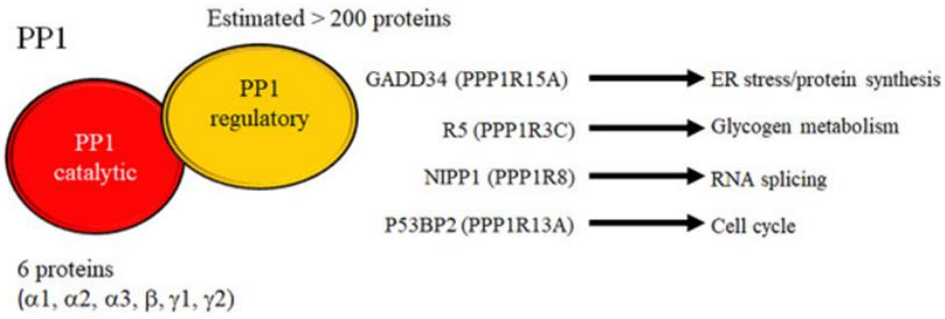


Figure 1.3: The catalytic subunit of PP1 has 6 protein variants that have the ability to interact with over 200 other proteins. Some of these secondary proteins and their functional roles are summarised above (Image taken from Ruvolo, 2019). Copyright© 2019 by Elsevier Publishing Group. Permission was obtained by Copyright Clearance Centre's RightLink ®service.

Three isoforms of PP1 (α, β and γ) are conserved across various species, further highlighting the vitality of its role in key cellular processes. These three isoforms possess similarities in their catalytic domain and they show structural differences at the amino and carboxy-terminals (Winter et al., 2007). The carboxy-terminus of PP1 is deemed to be the point of interaction with downstream proteins. Suppression of sperm production in PP1γ knock out mice is an example of abnormalities that arise in the absence of a functional PP1 isoform. This also suggests that the function of each subtype is unique and their absence cannot be compensated for even in the presence of other isoforms. Furthermore, detection of polyploid spermatids in mice indicates a correlation between the absence

of PP1γ and development of meiosis abnormalities (Winter et al., 2007, Varmuza et al., 1999).

1.2.5 Cdk5

Cdk5 is a serine/threonine kinase and a member of Cyclin-dependent kinase family. Initially, abundance of its activator, p35, in post-mitotic neurons had led to the conclusion that Cdk5 was a neuron-specific kinase (Liu et al., 2017); however, findings have indicated non-neuronal participation of Cdk5 in a variety of biological processes such as gene expression, cell migration, apoptosis and myogenesis, as well as in pathological processes such as cancer, senescence, diabetes, immune dysfunction and inflammation (Arif, 2012, Contreras-Vallejos et al., 2012).

Activation of Cdk5 depends on the activity of p35, a short-lived protein with a life span of 20-30 minutes (Asada et al., 2012). Activation of Cdk5 has negative feedback on the activity of p35 in order to prevent hyper-activation of Cdk5 as this is required for the maintenance of cellular homeostasis. This process involves Cdk5-mediated phosphorylation of p35 which promotes p35 ubiquitination and proteasome-dependant degradation of this protein (Takasugi et al., 2015). N-methyl-D-aspartate receptor (NMDAR) found on cellular membranes is one of many cellular components affected by internal/externals cellular signals. For instance, in the presence of apoptotic signals NMDAR is activated and membrane permeability to calcium is increased. This leads to the activation of Calpain protein, a proteolytic enzyme which cleaves p35 into C-terminal p25 fragment. Formation of Cdk5/p25 complex results in translocation to the nucleus. Cdk5/p25 complex is involved in the regulation of gene expression through activation and stabilisation of transcription factor p53 following translocation to nucleus. Cdk5/p25 induces phosphorylation of p53 which increases the transcriptional activity and expression of p53 as well as the expression of p53-responsive gene p21 and pro-apoptotic proteins Bax and Fas. This, along with other apoptotic events, induces

pathological signalling pathways of cell death (Lee et al., 2000). Considering the importance of p53 in regulating cell cycle progression and apoptosis in response to genetic aberrations observed in breast cancer, upregulation of this protein downstream of Cdk5 could have a therapeutic potential in treatment of breast cancer (Lee et al., 2000, Thie et al., 2011, Zhang et al., 2002)

Unlike most of the conventional Cyclin-dependent kinases, Cdk5 is not directly involved with the regulation of cell cycle. Instead, the function of Cdk5 is deemed essential for differentiation in post mitotic cells. An indirect involvement of Cdk5 with cell cycle regulation and DNA repair mechanism includes regulation of DNA damage response (DDR), a series of events that aids maintaining genomic stability through regulation of DNA replication and DNA repair (Liu et al., 2017). Cdk5 regulates DDR through phosphorylation of various key regulators of DDR such as apurinic/apyrimidinic endonuclease 1 (Ape1). Ape1 is an important enzyme essential for DNA repairing mechanism through the base excision repair (BER) pathway (Goldstein and Kastan, 2015). Hyperactivity of Cdk5 can lead to phosphorylation of Ape1 at Threonine-232 which results in the impairment of DNA repair (Huang et al., 2010). Another way through which the DDR process is influenced by Cdk5 is via signal transducer and activator of transcription 3 (STAT3) regulation which mediates constitutive activation of a series of genes require for rapid proliferation such as cyclin D1 gene and myc gene (Ouédraogo et al., 2016).

1.2.3 DRAPP-32 /t-DARPP in cancer

To date, levels of DARPP-32 expression have been detected in a number of tumours including breast and gastric cancers (Hansen et al., 2006, Kopljar et al., 2015). Truncated DARPP, t-DARPP, was first described in gastric and oesophageal adenocarcinomas, where the truncated isoform

was unable to inhibit PP1, but retained the ability to inhibit PKA (El-Rifai et al., 2002, Chen et al., 2016); this was found to be due to alternate splicing, meaning t-DARPP lacks the Threonine-34 phosphorylation site due to the loss of its 36 N-terminal amino acids (Vangamudi et al., 2011). Following the discovery of t-DARPP, co-expression of t-DARPP and DARPP-32 was reported in a number of patients suffering from adenocarcinomas of breast, prostate, colon, and stomach (Beckler et al., 2003, Christenson et al., 2015). Previously, breast cancer cells that express t-DARPP have been associated with a more aggressive growth and poor patient outcome. t-DARPP has been linked with cancer cell survival, invasion, angiogenesis and drug resistance in gastric and breast cancers (Belkhiri et al., 2016, Avanes et al., 2019).

Our research group has a long-standing interest in the Calpain system in cancer. A small number of publications linked DARPP-32 and Calpain, and our research group originally sought to investigate this link further in our breast cancer cohort. Although no link was found in our studies, DARPP-32 proved to be an important protein in breast cancer independently; we demonstrated that DARPP-32 protein expression was significantly associated with survival of breast cancer patients in over 3000 patients, an observation that was repeated in an addition 1980 patients when looking at mRNA expression. Importantly, DARPP-32 expression was more important in ER positive breast cancer; an observation we wanted to investigate further.

Most of the previous studies on *PPP1R1B* gene transcriptional regulation have exclusively focused on the transcript responsible for the production of full-length DARPP-32, and there seems to be a gap in literature regarding the mechanisms involved in the generation of transcripts that lead to the production of t-DARPP (Avanes et al., 2019).

Analysis of Catalogue of Somatic Mutations in Cancer (COSMIC database) which describes coding mutations across various cancers (over 4 million coding mutations as of September 2016) is indicative of high expression of DARPP-32 and t-DARPP in 9% of breast cancers compared to normal

breast tissues, whereas the frequency of point-mutations associated with *PPP1R1B* stands at 0.08%. This strongly suggests that in the majority of cases high levels of DARPP-32 and t-DARPP expression are due to high level of transcription rather than point-mutation (Forbes et al., 2017).

Although amplification of the 17q12 locus which accommodates the *PPP1R1B* gene is frequently associated with high levels of DARPP-32 in breast and gastric cancers, high DARPP-32 expression have been reported in the absence of PPP1R1B gene amplification in gastric cancer. While *PPP1R1B* gene-associated transcript encompassing exon 1 is associated with production of DARPP-32, two transcripts with a start site within intron 1 of *PPP1R1B* gene which includes exon 1b or exon 1c are responsible for the generation of the truncated protein version of DARPP-32 that lacks the first 36 residues (Figure 1.4) (Avanes et al., 2019).

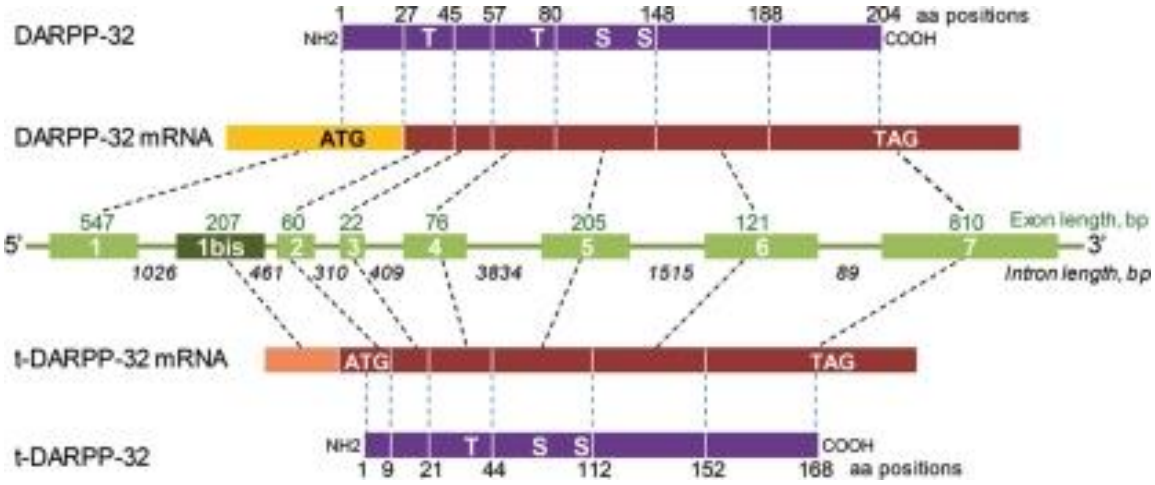


Figure 1.4: Demonstration of various sections of DARPP-32 (PPP1R1B) gene (in green) where introns are presented as green lines and exons as green boxes, and numbers above or below representing their length in base pairs. DARPP-32 and t-DARPP proteins are shown in purple and mRNA in brown. This figure is based on El-Rifai *et al.* (2002) and NCBI sequences (Image taken from Yger and Girault, 2011). Copyright© 2011 Yger and Girault.

Brain-derived neurotrophic factor (BDNF) has been previously studied for its involvement in the upregulation of DARPP-32, and existing evidence confirm that in BDNF null mice the expression levels of DARPP-32 are much lower compared to wild-type models. In addition to BDNF, analysis of *PPP1R1B* gene sequence has unveiled intron 4 of this gene as the corresponding growth response 1 (EGR-1) site both *in vitro* and *in vivo*. High levels of EGR-1 induce a similar increase in the level of DARPP-32 as demonstrated in primary striatal neurons isolated from mice. BDNF has also been shown to promote expression of EGR-1, and EGR-1 binding to *PPP1R1B* intron 4, thereby increasing the expression of DARPP-32. Studies have also indicated that BDNF can induce expression of DARPP-32 through PI3K/AKT pathway (Avanes et al., 2019, Ivkovic et al., 1997, Keilani et al., 2012).

Another important factor with a significant influence over the expression of full-length DARPP-32 transcript is TNF-α. TNF-α treatment has been shown to increase expression of DARPP-32. Transcriptional activation reporter assays and chromatin immunoprecipitation (ChIP) assay have identified an element located −1008 to −996 upstream of the PPP1R1B transcriptional start site as the corresponding genomic location responsible for NF-κB interaction with this gene (Avanes et al., 2019, Keilani et al., 2012).

1.2.4 DARPP-32, Wnt-5a signalling pathway and drug resistance

Based on the findings of previous studies, there is a strong indication that expression level of DARPP-32 protein may serve as potential biomarker of drug resistance in gastric cancer and other malignancies. While existing set of data suggests reversal of the multidrug resistance in Adriamycin resistant gastric adenocarcinoma cells (SGC7901) as a result of DARPP-

32 upregulation, some studies have reported links between high DARPP-32 expression and development of drug resistance in cancers (Hong et al., 2008, Belkhiri et al., 2016). Thus, the involvement of DARPP-32 in the process of drug resistance may be cell type specific. Moreover, inhibition of apoptosis in cancer cells by DARPP-32 through a p53-independent mechanism has been reported in gastric cancer cells against camptothecin, sodium butyrate, and ceramide treatment; this is deemed to be due to the release of anti-apoptotic B-cell lymphoma-2 (Bcl2) protein as a consequence of mitochondrial transmembrane potential perseverance induced by the expression of DARPP-32 and t-DARPP. Another study highlighted the importance of Threonine-75 phosphorylation in the anti-apoptotic function of these proteins through activation of AKT kinase and high levels of phospho-AKT (Serine-473) and phospho-GSK3β (Serine-9) proteins in gastric cancer cells. Additionally, the physical interaction of DARPP-32, Bcl2 and calcineurin in a complex with inositol 1,4,5-triphosphate receptor has been found to promote reduction of Ca+2 release and apoptosis by the reduction in InsP3R phosphorylation in chronic lymphocytic leukaemia cells (Belkhiri et al., 2016, Belkhiri et al., 2005, Belkhiri et al., 2008).

In one study, the role of Wnt-5a signalling pathway in regulating DARPP-32 dependent activity of cAMP-response element binding (CREB), a known PP1 substrate, was investigated; it was shown that Wnt-5a-induced expression of cAMP leads to a higher activity of PKA and phosphorylation of DARPP-32 Threonine-34 (Belkhiri et al., 2016). Considering that PP1 is a major phosphatase of CREB at serine-133, the consequent inhibition of PP1 by Phospho-Threonine-34 DARPP-32 increases the availability of phospho-serine-133 CREB which promotes expression of CREB proteins (Yang et al., 2015). Other studies have found associations between high levels of CREB and drug resistance through activation of the human multidrug resistance 1 (mdr1), P-glycoprotein *and* Bcl2 *(*Stati et al., 2021, Belkhiri et al., 2008).

In addition to cancer cell survival, DARPP-32 and t-DARPP have been implicated in resistance to certain anti-cancer therapies. In one study, it was demonstrated that introduction of t-DARPP to trastuzumab sensitive cells results in acquisition of resistance, whereas co-expression of both t-DARPP and DARPP-32 restores the response to trastuzumab in those cells. This behaviour further illustrates the importance of a balanced regulation of DARPP-32 on the outcome of therapy. In an attempt to investigate the involvement of t-DARPP in acquisition of resistance to lapatinib, Christenson and colleagues (2015) monitored the role of the truncated protein isoform in HER2 positive SKBR3 cells. They noticed that, unlike the t-DARPP null cells, t-DARPP positive cells were able to produce colonies following a four-week incubatory period in the presence of lapatinib. Development of drug resistance was demonstrated to be partially due to hampering lapatinib-dependant accumulation of pro-apoptotic BIM (Bcl2 homology domain 3 (BH3) only protein), which leads to repression of apoptosis (Christenson et al., 2015, Belkhiri et al., 2016).

1.2.5 Interaction between DARPP-32, DDR1 and Wnt-5a

While the exact role of DARPP-32 in maintaining mammary cells in a healthy state remains unclear, its interaction with Discoidin domain receptor-1 (DDR1), a tyrosine kinase receptor involved in promotion of cell migration, is implicated in the suppression of metastasis. Furthermore, upregulation of DARPP-32 in MCF7 cells has been shown to result in a reduced rate of cell migration (Hansen et al., 2006). DARPP-32 transfection of MDA-MB-231 cells that lack expression of DDR1 does not impair cell migration; re-introduction of DDR1 to DARPP-32 transfected MDA-MB-231 cells leads to suppression of cell migration. In addition, inhibition of PKA and modification of Threonine-34 or Threonine-75 residue abolishes the therapeutic effects associated with co-expression of DARPP-32 and DDR1 (Hansen et al., 2006, Hansen et al., 2009).

Since the therapeutic effects of DARPP-32 in suppressing cell migration is facilitated through interactions with DDR1, the availability of DDR1 is integral to initiation of this process. Availability of DDR1 is noticeably influenced by the activity of Wnt-5a which has the ability to induce activation of Frizzled-3/Gαs/cAMP signalling pathway upstream of DARPP-32 (Hansen et al., 2009, Dejmek et al., 2003). Down-regulation of Wnt-5a has been associated with reduced activity of DDR1, whereas up-regulation of this protein has been found to promote activation of DDR1 in breast epithelial cells (Säfholm et al., 2006). Other studies have shown that Wnt-5a influences the development of cancers through interactions with various receptor proteins including Frizzled G-protein-coupled receptor, Receptor tyrosine kinase like orphan receptor 2 (Ror2) and Low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6) (Li et al., 2002, Roarty and Serra, 2007, Liang et al., 2003). Furthermore, Wnt-5a-induced activation of cAMP is responsible for the activation of PKA which results in enhanced interaction with DARPP-32 and contributes to repression of cancer cell migration (Figure1.2). The consequent DARPP-32-induced Inhibition of PP1 results in the activation of PP1 substrate CREB, enhancing the therapeutic effects of DARPP-32 (Hansen et al., 2009, Cho et al., 2015).

These findings demonstrate existence of complex molecular interactions that control key signalling pathways within cells. Better understanding of these molecular interactions could provide further insight into the involvement of DARPP-32 and t-DARPP in tumour invasion and cell mobility.

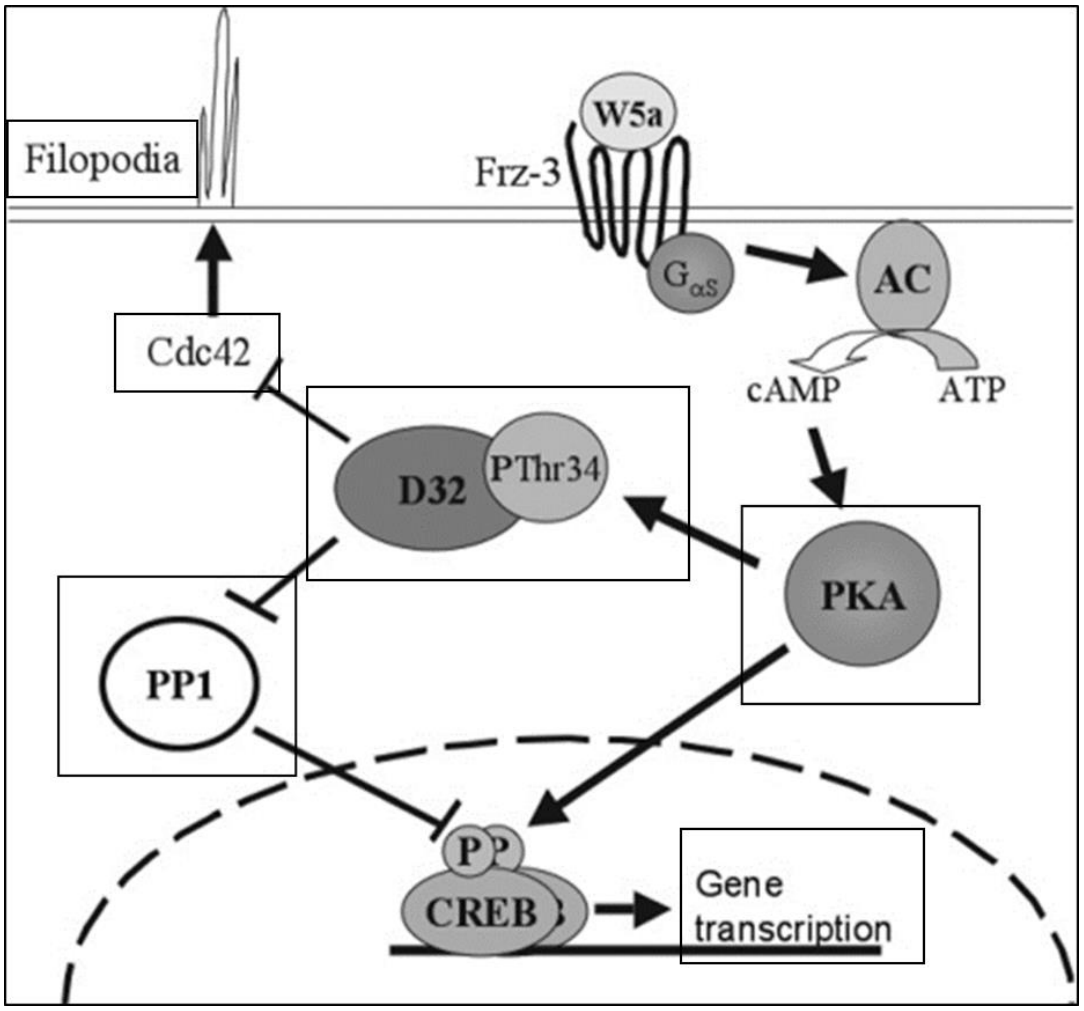


Figure 1.5: Suppression of breast cancer cell migration through modulation of DARPP-32 mediated by Wnt-5a (Image taken from Hansen et al., 2009), permission by Creative Commons Attribution 4.0 International license (CC-BY-4.0).

1.3 Hypotheses, aims and objectives

Hypothesis

We hypothesise that the imbalance of DARPP-32 and t-DARPP is associated with poor breast cancer patient outcome and tumour metastasis by altering cancer cell behaviour. Reduction in DARPP-32 signalling, either through loss of DARPP-32 or a switch to t-DARPP

expression, results in poor patient prognosis through the loss of PP1 inhibition, allowing for development of more aggressive tumours. We have used artificial neural networks to identify *GRB7* and *DKK1* as genes whose expression is associated with DARPP-32 expression in the METABRIC dataset and we hypothesise that their protein expression are associated with patient clinical outcome. In addition, we hypothesise that the reduction of DARPP-32 expression in ER positive breast cancer results in alteration of transcriptome and activation of pathways that mediate phenotype changes, aggravating tumour growth and metastasis.

Aims

The principle aim of this study is to demonstrate how low DARPP-32 expression alters cancer cell behaviour to affect breast cancer patient survival through a deeper understanding of DARPP-32 and t-DARPP-induced changes in cellular signalling. DARPP-32 and its signalling partners are important in breast cancer, and to facilitate understanding of how this affects patient outcome we aim to assess protein expression of Cdk5, PKA, PP1, *Sp7*, *DKK1, GRB7* and DARPP-32 Threonine 34 phosphorylation in a large group of breast cancer patients via Immunohistochemistry. We are using RNA-Seq to identify transcriptomic changes that occur in the absence and in the presence of DARPP-32, with further investigation of changes that occur following estradiol17β stimulation and treatments with a PKA inhibitor fragment (6-22) amide.

Objectives

1. Evaluate the expression of Cdk5, PKA, PP1 and the phosphorylation of DARP-32 at Threonine-34 in a large cohort of breast cancer patients to understand DARPP-32 signalling. In addition, we will

determine the expression of related and newly identified associated genes, *DKK1*, *SP7*, and *GRB7* in a large cohort of early-stage breast cancer patients.

2. To determine the relationship between t-DARPP and DARPP-32 expression in a large cohort of early-stage breast cancer patients using chromogenic *in situ* RNA hybridisation.

3. To use Cdk5, PKA and PP1 inhibitors to determine the effect on DARPP-32 phosphorylation in ER positive breast cancer cell line, T47D, and determine the cytotoxic effect of these compounds in triple negative MDA-MB-231 and luminal phenotype T47D and MCF7 cells.

4. To reduce expression of DARPP-32 in T47D breast cancer cells using siRNA, and validate these call lines as a model of reduced DARPP-32 expression using Western blotting and real-time PCR.

5. Identification of transcriptomic alterations following the loss of DARPP-32 expression in an ER positive breast cancer cells using RNA-Seq.

6. Use of RNA-Seq to assess the effects of PKA inhibition and oestrogen signalling on the cellular transcriptome in DARPP-32 knockdown cell.

Chapter 2 Materials and Methods

2.1 Methods and Materials

2.1.1 Patient Cohorts

Data analysed in this study corresponds to breast cancer patients who were treated at Nottingham University Hospitals between 1998 and 2006. All participants had either undergone breast conserving surgery or a complete removal of the breast tissue based on the requirements of each therapy and based on patient preferences. Patients received radiotherapy where required. Parameters such as the Nottingham prognostic index (NPI), ER and menopausal status were assessed prior to administration of adjuvant therapy. Whilst ER negative and pre-menopausal patients with a NPI score of at least 3.4 were qualified for the administration of combination chemotherapy comprising cyclophosphamide, methotrexate and 5-fluorouracil, individuals with NPI score of below 3.4 did not receive adjuvant therapy. ER positive patients received additional hormonal therapy. The median follow up time was defined as the median time between primary surgery and occurrence of death as a result of breast cancer and was determined to be 148 months using the reverse Kaplan-Meier method. Ethical approval was issued by Nottingham Research Ethics Committee (REC) 2, under the title of 'development of a molecular genetic classification of breast cancer'.

The molecular taxonomy of breast cancer international consortium (METABRIC) database was utilised to assess gene expression levels in patients (n=1980) who where treated between 1977 and 2005 at five centres in the UK and Canada (Curtis et al., 2012). In this study, the association of PKA, PP1, Cdk5 *SP7*, *GRB7*, *DKK1* and *PPP1R1B* mRNA expression with various clinicopathological parameters such as tumour size and tumour grade were assessed. A median patient follow-up of 141 months was determined using the reverse Kaplan-Meier method. Almost all patients exhibiting ER negative and/or lymph node positive status were

given adjuvant chemotherapy and trastuzumab was not available to patients with HER2 overexpression. In brief, RNA and DNA were isolated from samples and were hybridized to the Affymetrix SNP 6.0 and Illumina HT-12 v3 platforms for genomic and transcriptional profiling. Detailed information regarding this approach is described by Curtis *et.al* (Curtis et al., 2012).

2.1.2 Immunohistochemistry

Novolink Polymer Detection kit (Leica) was performed according to the manufacturers' instructions. In brief, slides were heated on a 60°C hotplate (10 minutes) then allowed to cool before deparaffinisation in xylene and ethanol (2 x xylene for 5 minutes; 3 x ethanol for 5 minutes) followed by a 5-minute wash in water. Tissue was microwaved in a 0.01molL-1 sodium citrate buffer for 10 minutes at 750W followed by 10 minutes at 450W for antigen retrieval. Novolink Peroxidase Block was applied to tissue for five minutes, followed by washing with Tris-buffered saline (TBS), and followed by the application of Novolink Protein Block solution for five minutes.

Cdk5, PKA C-α, PP1-α, DARPP32 (phospho Threonine-34), anti-*Sp7*, *DKK1*, and anti-*GRB7* antibodies were incubated on tissue for one hour at room temperature. Tissue was then washed with TBS, and Novolink Polymer solution was applied for 30 minutes. Tissue was stained with 3,3'diaminobenzidine and haematoxylin, then dehydrated in sequential ethanol and xylene baths (3x ethanol for 5 minutes, 2x xylene for 5 minutes). Positive DARPP-32 and negative control samples were included in each staining run. A list of antibodies used in Immunohistochemistry are shown in Table 2.1.

Table 2.1: List of Antibodies used in Immunohistochemistry.

Name	Dilution	Catalogue Number	Company
DARPP-32	1:500	ab40801	Abcam
Cdk5	1:500	12134	Cell Signalling Technology
PKA C-α	1:50	4782	Cell Signalling Technology
PP1- alpha	1:100	clone 10C6-3	Life technologies
DARPP32 (phospho Threonine-34)	1:500	b254063	Abcam
Anti-*Sp7 / Osterix*	1:500	ab22552	Abcam
Anti-*GRB7*	1:500	ab183737	Abcam
DKK1	1:1000	MA5-32229	Thermo Fisher Scientific

2.1.3 Scoring

Slides were scanned using a Nanozoomer Digital Pathology Scanner (Hamamatsu Photonics) and then were analysed at 200x magnification. Cytoplasmic staining was examined using a semi-quantitative immunohistochemical H-score system which classified tumours by the level of staining intensity. In this method, the intensity of staining is given a score from a scale of 0 to 3 where scores represent none, weak, medium and strong, intensity staining. Nuclear staining was assessed based on the percentage of cells exhibiting any degree of staining intensity. An independent second analyst was appointed to assess more than 30% of tissues without the knowledge of the initial assessment score and the eventual clinical outcome of the corresponding patients. Single measure intraclass correlation coefficients (ICC) were determined to highlight the concordance between scorers, where values above 0.7 were considered reliable.

2.2 Cell culture

T47D, MCF7 and MDA-MB-231 breast cancer cell lines were purchased from the American type culture collection (ATCC). The cells were maintained in either Dulbecco's Modified Eagle's Medium-high glucose (Sigma) (T47D), RPMI-1640 (MCF7), or in Medium and in Minimal Essential Medium Eagle (Sigma, UK) supplemented with 1% non-essential amino acids (Sigma) and 2mM L-Glutamine (Sigma) (MAD-MB-231), and 1% Penicillin/Streptomycin (Sigma) and 10% iron supplemented Donor Calf Serum (Gibco).

All tissue culture experiments were performed in a class II biological safety hood, with 70% ethanol solution spray used for hood disinfection before, during and after each use. All cells were incubated at 37°C under humidified conditions, at 5% CO_2. Cells were allowed to achieve 70-80% confluency prior to passage. In brief, cells were passaged by aspirating spent media from the adherent cell layer then gently washed with PBS (Sigma). 0.5 mg/ml trypsin EDTA (Sigma) solution was added to cover the cell monolayer and the flask was incubated at 37°C for 1-5 minutes, depending on the cell line. Detachment of cells was monitored using a microscope. Cells were harvested after deactivation of trypsin-EDTA using complete medium. The harvested cells were split at a ratio of 1:5 to 1:10 for MDA-MB-231, 1:3 to 1:5 for T47D, and 1:5 for MCF7, and were transferred into a fresh tissue culture flask and fresh medium was added (5, 15 or 25mL of media in a T25, T75 or T175 cm^2 flask, respectively). Cells were incubated at the conditions specified previously.

2.2.1 Cryopreservation and cell recovery

Working cell stocks were prepared using a master cell stock and were stored frozen in liquid nitrogen, limiting the chances of cross contamination, genetic drift and ensuring low passage number for

subsequent experiments. Cells were harvested using trypsin EDTA after achieving 70-80% confluency, and the percentage of viable cells was determined using a haemocytometer. Cells were centrifuged (170 Relative Centrifugal Force (RCF) for 5 minutes) and the supernatant was discarded. The cell pellet was resuspended in freezing medium 10% dimethyl sulfoxide DMSO and 90% complete medium to achieve a final approximate concentration of $1X10^6$ cells per mL. Cells were stored frozen overnight (-80°C) in a freezing container (Mr Frosty) designed to achieve the optimal cooling rate for cell preservation purposes prior to long term storage in liquid nitrogen (-180°C) as needed. Cells were thawed before culture in a 37°C water bath immediately after retrieval from liquid nitrogen. Cells were then transferred into a tube containing pre-warmed complete cell culture medium prior to centrifugation at 170 RCF for 5 minutes and disposal of supernatant. Cell pellet was resuspended in fresh growth medium and transferred into a new tissue culture T25 cm^2 tissue culture flask which was incubated at the conditions specified previously in section 2.2. The media was replaced the next day before recounting cell culture and cells were passaged at least three times (with a maximum of 15 passages) for experimental use. The status of mycoplasma was monitored monthly in all cell lines by senior members of the laboratory and using Mycoplasma Detection Kits. The authenticity of each cell line was verified at 6-8 months intervals using Short Tandem Repeat (STR) DNA profiling (Promega Powerplex®).

2.2.3 Compound preparation

Separate solutions of purvalanol B (50mM in DMSO) (Tocris) (stored at 4°C), forskolin (12.5mM in DMSO) (Tocris) (stored at -20°C), and PKA inhibitor fragment (6-22) amide (1 mg/ml in water) (Tocris) (stored at -20°C) were prepared for use in proliferation and Enzyme linked immunosorbent assay (ELISA). Tautomycetin (Tocris) was pre-dissolved

in DMSO at 10mg/ml (stored at -20°C). A list of the all compounds used in this study are shown in Table 2.2.

Table 2.2: List of drugs used in this study.

Name	Catalogue number	Company
Purvalanol B	1581	Tocris
Forskolin	1099	Tocris
PKA inhibitor fragment (6-22) amide	1904	Tocris
Tautomycetin	2305	Tocris

2.2.4 Proliferation assay

Proliferation assays were performed to assess the growth of cancer cell lines and to determine the toxicity of forskolin, purvalanol B, PKA inhibitor fragment (6-22) amide and tautomycetin. $1x10^5$ cells were plated per well in 6 well plates before incubation for a period of 24 hours in order to achieve 70-80% confluency. Cells were then treated with forskolin, purvalanol B, PKA inhibitor fragment amide and tautomycetin at a range of concentrations (0, 1.5, 3, 4.5 μM) and cell proliferation was assessed in triplicate at 24, 48, and 72 hour intervals following treatment. At each time point, cells were trypsinised and counted using a haemocytometer in order to assess the effect of each compound on cellular proliferation. Inclusion of untreated control cells at each time point allowed for direct comparison of proliferation rate in the presence and in the absence of each compound, and was later used to normalise data. The half maximal inhibitory concentration (IC_{50}) for each drug was determined where possible. This analysis was carried out using GraphPad Prism 9 software,

and IC_{50} was calculated using the simple linear regression equation in this software. To enhance the reliability of the data, each experiment was repeated three times, and the results were expressed as the average cell number ± standard deviation (SD) of three separate experiments.

2.3 Kinase activity ELISA

Kinase activity ELISAs were performed on breast cancer cell lines by transferring 200 µL of $2x10^4$ adherent breast cancer cells in culture medium to each well in 96 well-plates. Poly-L-lysine coating was applied to each well by adding 10 µg/mL poly-l-lysine and incubating for 30 minutes at 37 ^{0}C prior to the addition of cells in order to facilitate optimal cell adhesion for otherwise loosely bound cells. Cells were incubated overnight in order to achieve full confluency prior to treatment with forskolin, purvalanol B, PKA inhibitor fragment amide or tautomycetin at 0, 1.5, 3, 4.5 µM concentrations for 24 hours. Cells were fixed to the plates and quench buffer and blocking buffer were used to stop the kinase reaction following treatment with drugs.

Primary antibodies (Anti-DARPP-32 Threonine-34, Anti-DARPP-32 Threonine-75, Anti-Cdk5, and Anti-PKA) or anti-Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) antibody were added to each well containing compound treatment and control samples, respectively, prior to 2 hour of incubation at room temperature and gentle shaking. Cells were incubated for 1.5 hours following the addition of secondary antibodies (HRP conjugated anti-Rabbit IgG antibody for wells with anti-DARPP-32 Threonine-34, Anti-DARPP-32 Threonine-75, Anti-Cdk5 and Anti-PKA antibodies, or HRP conjugated Anti-Mouse IgG antibody for wells with anti-GAPDH) to the samples under conditions specified in the previous step. The substrate included in the ELISA test Kit was then added

before a final incubation of 30 minutes in the dark due to the sensitivity of the substrate to light. The experiment was terminated using the stop solution which was included in the ELISA test kit, and the plate was immediately analysed using a VersaMax microplate reader at 450 nm. This experiment was repeated three times, in five replicates, and the results were expressed as the mean ± standard deviation of three separate experiments. A list of ELISA kits used to determine kinase activity are shown in Table 2.3.

Table 2.3 : List of ELISA used in kinase activity.

Name	Catalogue number	Company
Cdk5	CB5131	Assay bio Tech
PKA	EIAPKA	Thermo Fisher Scientific
DARPP-32 (Threonine 34)	CBCAB00394	Assay Genie
DARPP-32 (Threonine-75)	CBCAB0010	Assay Genie

2.4 Cell lysis and protein extraction

Breast cancer cells were harvested and transferred into a suitable container for centrifugation at 170 RCF for 5 minutes. The pellet was re-suspended in radioimmunoprecipitation assay buffer (RIPA) buffer (Sigma) containing 10% protease and phosphatase inhibitor cocktail and ethylenediamine tetraacetic acid (EDTA) solution (Thermo Fisher Scientific). Cell lysates were placed on ice for 10 minutes before another centrifugation step (170 RCF for 10 minutes). The supernatant was carefully collected and the supernatant was stored at either -20°C or -80°C for short term or long term storage, respectively. Lysates were

thawed gently on ice prior to each use, while keeping the frequency of freeze thaw cycles to a minimum.

2.4.1 Western blot

Western blotting was utilised to confirm the specificity of primary antibodies in the cell lysates derived from various breast cancer cell lines. Western blotting was also used for assessing the impact of administration of purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin on the level of DARPP-32 protein expression across MDA-MB-231 and T47D cell lines using cell lysate which was added to Bolt™ LDS Sample Buffer (Thermo Fisher Scientific), and Bolt™ sample reducing agent (Thermo Fisher Scientific). Proteins within the mixture were denatured by incubation at 100°C for 5-10 minutes followed by a centrifugation step (10000 RCF for 1-2 minutes). Samples were kept on ice until protein loading onto precast polyacrylamide Bolt™ 4-12% Bis-Tris plus gel (Novex Life Technology).

A total of 20 µL of each sample and SeeBlue® plus2 prestained standard (Invitrogen) were loaded onto each well after placing the gel into a Bolt™ mini gel tank (Life technologies) containing 1X Bolt™ MES SDS running buffer (a mixture of Blot™ MES Running Buffer (20X) and distilled water at a volume ratio of 50:950). Protein separation was performed at 200V for 25 minutes, and proteins were transferred onto a 0.2µm nitrocellulose membrane (Whatman GE Healthcare) via the electroblotting using Bolt™ transfer buffer (blot™ transfer buffer (20x) and methanol in distilled water) at 10V for 90 minutes.

The nitrocellulose membrane was incubated in blocking buffer (0.05% phosphate buffered saline (PBS)/Tween® 20 containing 3% non-fat milk) for 1 hour at room temperature to prevent non-specific protein binding. The blocking buffer was then removed, before samples and negative

controls were incubated overnight at 4°C in the presence of primary antibodies and β-actin (Abcam), respectively. Samples were washed in 0.1% PBS /Tween ® 20 three times (5 minutes each time) and they were incubated for 1 hour at room temperature in the presence of secondary antibodies Donkey anti-rabbit and Donkey anti-mouse (LI-COR Biosciences)). List of antibodies used in Western blotting in Table 2.4. Samples were washed once more as previously described. Samples were then analysed on an Odyssey FC Imager (LI-COR Biosciences) operated using Image Studio software (V4.1).

Table 2.4: Specifications of the antibodies used in Western blotting.

Name	Dilution	Catalogue Number	Company
Anti-beta Actin	1:1000	ab8226	Abcam
Cdk5	1:500	12134	Cell Signalling Technology
PKA C-α	1:50	4782	Cell Signalling Technology
PP1- alpha	1:100	clone 10C6-3	Life technologies
DARPP-32	1:500	ab40801	Abcam
DARPP32 (Phospho Threonine-34)	1:500	b254063	Abcam
Anti-*Sp7 / Osterix*	1:500	ab22552	Abcam
DKK1	1:1000	MA5-32229	Thermo Fisher Scientific
Anti-*GRB7*	1:500	ab183737	Abcam
Donkey anti-Rabbit	1:10000	926-32213 LI-COR	LI-COR
Donkey anti-Mouse	1:10000	926-68072	LI-COR

2.5 Chromogenic *in situ* RNA hybridisation

Chromogenic in situ RNA hybridisation (RNAscope, ACD bio-techne) was utilised to detect RNA of interest within intact cells using Formalin fixed

paraffin embedded (FFPE) tissue. Slides were incubated on a 60°C hotplate for 60 minutes and then immersed in xylene for 5 minutes at room temperature before immersion in industrial methylated spirits (IMS) for 2 minutes at room temperature. Slides were air dried for 5 minutes at 60°C. RNAscope Hydrogen Peroxide (RNAscope, 322381) was added to the tissue and incubated for 10 minutes and slides were then washed with distilled water. RNAscope 1X Target Retrieval Reagent (RNAscope, 322381) was prepared according to manufacturor's instructions and slides microwaved in the solution at 750W for 10 minutes followed by 450W for 10 minutes. Slides were rinsed using distilled water and placing in a dish containing IMS for 3 minutes. Slides were dried in the incubator and a barrier was created using a hydrophobic pen as demonstrated in Figure 2.1 which was allowed to dry overnight at room temperature.

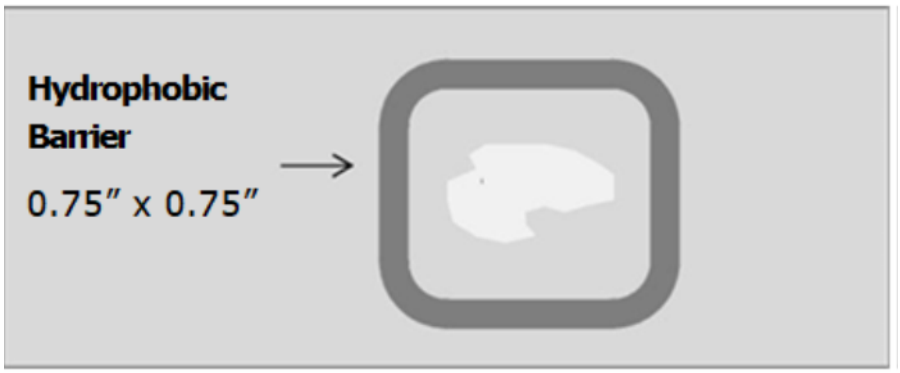


Figure 2.1: Demonstration of the hydrophobic line surrounding a section. Image modified from company protocol (ACD bio-techne).

RNAscope Protease III (322381) was added to slides and inserted into a HybEZ oven rack cover. The rack was then placed in a humidity control tray for 30 minutes and incubated at 40°C.

Negative control, positive control, DARPP-32 and t-DARPP probes were added to individual slides and incubated for 2 hours at 40°C before placing in Tissue-Tek Staining Dish filled with 1X RNAscope Wash Buffer that was prepared from RNAscope 50X Wash Buffer (310091) previously. In order to hybridise BaseScope, AMP 1 was incubated with each slide for 30 minutes at 40°C. AMP 2, AMP 3, AMP 4, AMP 5 and AMP 6 were all added and washed off on separate incubations, exactly as described for AMP 1. Addition of AMP 7 and AMP 8 was followed by a 60-minute and 15-minute incubation periods (respectively) at room temperature. BaseScope™ Fast RED-B was mixed with BaseScope™ Fast RED-A in a ratio of 1:60 (B-A). Slides were placed in the HybEZ Slide Rack in the HybEZ humidity control tray, and were treated with 120µL of the solution and incubated for 10 minutes at room temperature. Slides were washed twice with tap water, then stained by submerging into Haematoxylin for 1 minute at room temperature. Slides were washed 3-5 times with tap and then dried in a 60°C oven for 15 minutes. Slides were mounted using VectaMount before being visualised under a microscope.

2.6 Transfection

T47D cells, purchased from the American Type Tissue Collection (ATTC), were cultured in Dulbecco's Modified Eagle Medium with high glucose content (Sigma) and 10% iron supplemented Donor Calf Serum (Gibco) without antibiotics. Cells were allowed to achieve 70-80% confluency over approximately 24 hours. For more information on cell culture refer to section 2.2. Approximately, $3x10^6$ T47D cells were seeded onto a T75cm^2 tissue culture flask and were transfected using three pre-designed siRNA (OriGene), SR313371A, SR313371B and SR313371C, for DARPP-32 knockdown. SR313371C siRNA was selected for further experiments as it induced the highest level of DARPP-32 knockdown. Negative control

samples were also prepared using (SR30004) siRNA (OriGene) for comparison purposes. Cell transfection was conducted over a period of 24, 48 or 72 hours by introducing 8nM human DARPP-32 (SR313371C, OriGene) siRNA oligo duplex or 8nM negative control (SR30004,OriGene) siRNA to cells, using Lipofectamine RNAiMax (13778030, Thermo Fisher Scientific) in Opti-MEM reduced serum medium (31985062, Thermo Fisher Scientific). After 30 minutes, cells were harvested by trypsinisation, cells were counted, and $3x10^6$ cells were added to fresh media without antibiotics; this suspension was transferred onto a flask containing RNAiMax transfecting complex, and the mixture was incubated at 37^0C at 5% CO_2 for 24, 48 or 72 hours. Following transfection, cells were washed, counted and stored frozen at -80°C until they were required for performing Western blot, real-time PCR and RNA-Seq.

2.6.1 Measurement of protein expression

DARPP-32 knockdown in T47D breast cancer cells following transfection was verified using Western blot. This analysis was carried out on the lysates that were isolated from transfected cells and stored as described in section 2.4. DARPP-32 protein level was determined at 24, 48 and 72 hours post transfection using Western blotting described in section 2.4.1. Protein expression following Western blotting was determined using an Odyssey FC Imager (LI-COR Biosciences) using the Image Studio software (V4.1). The signal was corrected through normalisation against β-actin expression. This experiment was done three times using lysates derived from cells with independent passage numbers. Results were expressed as the average ± standard deviation (SD) of three independent experiments.

2.6.2 Measurement of mRNA expression

2.6.2.1 Total RNA

The workstation and equipment such as micropipettes and microcentrifuge were all cleaned using RNaseZap™ cleaning agent (Sigma) to ensure absence of RNase. All other equipment and consumables including pipette tips and collection tubes were purchased RNase-free. Collection of samples from subconfluent cell culture was carried out on three separate occasions, and DARPP-32 mRNA knockdown was carried out with the aid of RNAprotect Cell Reagent (76526, Qiagen) and RNeasy Plus Mini Kit (74134, Qiagen) for the stabilisation and purification of total RNA.

Cells were seeded into T25cm^2 flasks and incubated under humidified conditions at 37°C for 24 hours, media was then removed, and cells were washed three times using PBS, before addition of RNA protect cell reagent in order to detach cells. The mixture was then transferred into a suitable sterile container and stored at -80°C. Isolation and purification of total RNA included gentle thawing of cell/RNAprotect reagent mixture and dilution using DNase/Rnase free distilled water at a ratio of 1:10, followed by a centrifugation step at 1700 RCF for 5 minutes. The supernatant was carefully removed, and RNA lysis buffer (RLT) Plus was added to the pellet to initiate cell lysis. RLT was used for the lysis of up to 5x10^6 cells, or 600µL RLT for 5x10^6 to 1x10^7 cells as required. The pellet was fully dissolved with the aid of vortexing for approximately 1 minute before transferring into a QIAshredder spin column in a collection tube and centrifugation at 8000 RCF for 2 minutes for homogenisation. The homogenised samples were centrifuged again using a gDNA eliminator spin column in a collection tube at 8000 RCF for another 30 seconds.

The nucleic acids present within the mixture were precipitated by adding an aliquot of 70% ethanol and the mixture was transferred into RNeasy spin column in a collection tube for centrifugation at 8000 RCF for 15 seconds. RNA Wash (RW1) Buffer and RNA wash buffer with ethanol (RPE) Buffer were used to wash the retained RNA within the RNeasy spin column, and the associated collection tube was replaced with a new one before centrifuging 8000 RCF for 1 minute. The collection tube was replaced again with a new collection tube, RNase free water was added to the column membrane and it was centrifuged again exactly as the previous step. The eluted RNA was assessed for quantity and purity using a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific), and stored at -80°C.

2.6.2.2 cDNA synthesis

RNA was isolated from the transfected cells using the RT2 First Strand Kit (330404, Qiagen,) for the synthesis of complementary DNA (cDNA). To avoid genomic DNA contamination, gDNA elimination mix was incubated with each sample at 42°C for 5 minutes before placing on ice for at least 1 minute.

Reverse transcription was initiated by addition of the reverse transcription mix to gDNA elimination mix, and gently mixed using a pipette. The mixture was incubated at 42°C for 15 minutes exactly to allow for annealing before the reaction was terminated by incubation at 95°C for 5 minutes. Samples were stored at -20°C until required for real-time PCR analysis.

2.6.3 Real-time quantitative polymerase chain reaction

The real-time PCR reaction mixture was prepared by the addition of cDNA to Master mix composed of SYBR Green ROX qPCR Mastermix (2x) (330253, Qiagen), RNase-free water (AM9935, Invitrogen), human qPCR primer pair control (HPRT) (Eurofins), t-DARPP (Eurofins), 171 amino acids (Eurofins), and DARPP-32 primers (249900, QIAGEN).

In order to detect presence of DARPP-32, primers corresponding to DARPP-32 and HPRT (Table 2.5) were used. All real-time PCR experiments on DARPP-32 including those on standards, samples and no template controls (NTCs) were conducted in triplicates in an optical 96-well PCR plate (Thermo Fisher Scientific) and using Viia7TM Real-Time PCR system (Applied Biosystems) operated under Quant StudioTM Real-Time PCR software. This reaction included an initialisation and denaturation step with a set temperature of 95°C for 15 seconds in order to activate the DNA polymerase and to denature the double strands of DNA within the mixture. This was followed by annealing and strand elongation at 60°C for 1 minute. The cycle from denaturation to elongation was set to repeat 40 times. The level of mRNA expression in each sample was determined relative to the level of HPRT expression in those samples, expressing the data obtained as an average of DARPP-32 gene expression relative to the respective control ± SD of three independent experiments. A single real-time PCR experiment was also employed in order to assess presence of DARPP-32 splice variant t-DARPP, and the 171 amino acid variant identified by our research group. The list of primers shown in Table 2.5.

Quant StudioTM Real-Time PCR software was used to produce the Ct values on Viia7TM Real-Time PCR system (Applied Biosystems). The normalisation of Ct values against the housekeeping genes (HRPT) enabled calculation of the relative gene expression levels. The fold changes of gene expression levels was calculated using the comparative

Ct (2-ΔΔCt) method, and GraphPad Prism 9 software was used for the analysis and to produce data which was presented as fold induction/repression over the control. Comprehensive information on this qPCR data analysis strategy has been previously presented by Livak and Schmittgen, 2001.

Table 2.5 : The primers list we used in this experiments.

Genes	Primers sequences	Company
HPRT	Forward primer 5'-AAATTCTTTGCTGACCTGCTG-3' Reverse primer 5'-TCCCCTGTTGACTGGTCATT-3'	Eurofins
DARPP-32	Forward and reverse Primers were pre-designed by QIAGEN in single tube (249900, QIAGEN) and no details regarding the sequences were provided.	QIAGEN
t-DARPP	Forward primer 5'-TGAAGCCCTTGAGCTGATCT-3' Reverse primer 5'-TCCAATGTGGGCTGTCTCAC-3'	Eurofins
171 Amino acid variants	Forward primer 5'-ACTCCTCACCAGCTGTGCAG -3' Reverse primer 5'-TCCAATGTGGGCTGTCTCAC -3'	Eurofins

2.7 Sample preparation for RNA-Seq

Following transfection of T47D cells for 48 hours, described in section 2.6, DARPP-32 knockdown cells and control cells were separately prepared in phenol red free media containing 10% charcoal stripped FBS (Gibco) to eradicate potential interference of endogenous serum steroids as well as oestrogen activity of phenol red in the experiments that followed in preparation for RNA sequencing. A solution containing 10nM estradiol17β (E2) (50-28-2, Sigma) dissolved in absolute ethanol was used to treat cells for 24 hours. The same procedure was followed for a 24 hour long

treatment of DARPP-32 knockdown cells and control cells with 3μM PKA inhibitor (PKA inhibitor fragment (6-22) amide, 1904, Tocris) dissolved in distilled water. ELISAs were performed to determine PKA activity as previously described in Chapter 2 Section 2.7. Following transfection and treatment, $3x10^6$ cells were frozen as cell pellets in 500μl Trizol for RNA-Seq. 18 samples were packed and sent to Novogene (UK) Ltd, Cambridge for RNA sequencing on dry ice.

2.7.1 RNA-Sequencing and Quality control

All activities concerning RNA extraction, sample quality control, mRNA library preparation and RNA sequencing were performed by Novogene (UK) Ltd in Cambridge. RNA-Seq bioinformatics were conducted in collaboration with Dr Storr. FastQC, a quality control analysis tool which was developed by Simon Andrews of Babraham Bioinformatics, was used for a basic initial assessment of quality control on raw sequence data. FastQC provided a modular set of analysis enabling a basic insight into potential analytical flaws within the data set before any further comprehensive analysis was undertaken.

https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Trim Galore, a comprehensive quality control analysis tool developed by Felix Krueger of Babraham bioinformatics, was used for a more in-depth quality control analysis. Trim Galore also provided automation of quality and adapter trimming, as well as removing biased methylation positions for reduced representation bisulfite (RRBS) sequence files (for directional, non-directional or paired end sequencing).

https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

2.7.2 Differential expression analysis

Assessment of differential expressed transcripts is an integral part of RNA-Seq data analysis.The assessment of differential expression of transcripts was carried out using DESeq2 (1.36.0) in statistical environment RStudio (version 4.2, and Bioconductor version 3.15) coupled with Kallisto using Tximport. DESeq2 enables comparative assessment of RNA-Seq data using shrinkage estimators for distribution and fold change important for hypothesis tests (Glaus et al., 2012).

2.7.3 Analysis of gene overrepresentation

QIAGEN IPA software was used to assess the influence of altered gene expression on biological processes and signalling pathways by utilising extensive records of literature as knowledge based tool to find associations between certain changes attributed to the expression of two or more genes and development of various health conditions. The significance of such association is expressed as *P* value. This software applies a pre-determined gene cut-off point in the data set via right-tailed Fisher's exact test $P < 0.05$ and a ratio change of more than 2-fold. The likelihood of the suppression or activation of a pathway as a result of changes in the expression pattern of two or more genes is expressed as the z-score, where negative and positive scores signify pathway suppression and activation, respectively (Kramer et al., 2014).

2.8 Statistical analysis

IBM SPSS Statistics (version 28) software was utilised for statistical analysis. X-tile software was utilised in order to determine cut-points (Camp et al., 2004). The Pearson χ^2 test of association was utilised to examine the association between protein expression and

clinicopathological criteria. The Kaplan-Meier method was used to plot survival curves, and statistical significance was determined using the log-rank test where $P \leq 0.05$ was considered statistically significant. Broad Institute Morpheus software was utilised to visualise correlation data in heatmaps (https://software.broadinstitute.org/morpheus). The reporting format of this study is set out in accordance with REMARK criteria recommendations (McShane et al., 2005). The results obtained from all *in vitro* experiments were calculated as the average ± standard deviation of at least three independent experiments. The variation between the drug-treated samples and control samples were assessed via the one-way ANOVA test using GraphPad Prism 9 software (GraphPad Software Inc.) Data were obtained from at least three independent experiments.

Chapter 3 Canonical DARPP-32 Signalling in Breast Cancer

3.1 Introduction

DARPP-32 can function as an inhibitor of PP1 or PKA depending on its state of phosphorylation at Threonine-34 and Threonine-75 residues; PKA induced phosphorylation of Threonine-34 transforms DARPP-32 into an inhibitor of PP1, whereas Cdk5 induced phosphorylation of Threonine-75 residue transforms DARPP-32 into an inhibitor of PKA. DARPP-32, PKA, PP1 and Cdk5 are multifunctional proteins with vital roles in cell signalling. Dysregulation of these proteins may have implications in various cancers (Fernández et al., 2006).

3.1.1 PKA in cancer

The diversity in the function of PKA in different cell types and its involvement in various signalling pathways regulating vital physiological and pathological functions highlight the importance of this protein in various cellular functions. It is therefore not surprising that cellular and genetic aberrations that affect the normal functioning of PKA have been implicated in cancer development and progression (Sapio et al., 2014, Saidy et al., 2021). Evidence suggesting the involvement of the cAMP/PKA signalling pathway in various tumours has been verified across multiple independent studies which may indicate its significance in tumour therapy and diagnosis (Sapio et al., 2014, Naviglio et al., 2009).

The expression and activity of PKA has been associated with poor prognosis in various tumours including colorectal, breast and lung cancers (Khor et al., 2008, Bradbury et al., 1994, Shi et al., 2004). High levels of extracellular PKA found in the serum of some cancer patients are usually the result of PKA overexpression and secretion by transformed cells. Detection of high extracellular levels of PKA could serve as a biomarker in these tumours (Caretta and Mucignat-Caretta, 2011, Nesterova et al.,

2006). *In vitro* PKA gene silencing using antisense oligonucleotides has been found to slow the growth and progression of colorectal, breast and gastric carcinomas, as well as promoting sensitivity to radiation therapy and chemotherapy in colorectal and ovarian cancer cell lines *in vivo* (Khor et al., 2008, Yokozaki et al., 1993, Bianco et al., 2000, Vassileva, 2009).

High cellular levels of PKA in prostate cancer are linked with patient response to radiotherapy and androgen deprivation therapy. This was demonstrated as a part of an investigation comprising a total of 456 clinical prostate cancer specimens where the abundance of PKA RIα was adversely proportional to the efficacy of radiotherapy and androgen deprivation therapy; in this study, overexpression of PKA RIα was detected in 17.5% of the specimens. Distant metastasis and abnormal biochemical index were other important factors found to be associated with high levels of PKA RIα (Pollack et al., 2009, Zhang et al., 2020). In another study, downregulation of the PKA type Iα regulatory subunit combined with radiation and androgen deprivation therapy was shown to cause an extensive impairment of growth in prostate cancer cells both *in vitro* and *in vivo* (Vassileva, 2009).

Abundance of the RI subunit of PKA has been found to favour tumour growth and poor prognosis in breast cancer, as well as promoting transformation of healthy breast epithelial cells. One study involving 66 patients illustrated the link between abundance of nuclear PKA and the spread of breast cancer to secondary sites (Paul et al., 2020, Zhang et al., 2020). Another study has drawn links between the function of PKA and mechanotransduction in cells, and described PKA as the actomyosin contractility regulated effector facilitating mechanically guided cell migration (McKenzieet al., 2020, Zhang et al., 2020). Other investigations have indicated existence of a relationship between the activation of PKA and development of trastuzumab resistance in HER2 breast cancers (n=5) (Moody et al., 2015).

PKA induced phosphorylation of Serine-305 of ERα has been associated with tamoxifen resistance as well as altering cellular transcriptome dynamics via redirection of ERα to alternative transcriptional start sites (Michalides et al., 2004, de Leeuw et al., 2013, Saidy et al., 2021).

Low cellular levels of *PRKAR1A* and high levels of SRC are reportedly associated with poor survival in breast cancer which was demonstrated using the NKI-295 data set (n=295) (Beristain et al., 2015). Whilst there is a lack of evidence suggesting the involvement of *PRKACG* and *PRKAR1B* in breast cancer, activities of the pair have been associated with the progression of acute myeloid leukaemia and poor patient outcome in advanced renal cell cancer patients, respectively (Zhu et al., 2018, Saidy et al., 2021, Garrigos et al., 2017).

3.1.2 PP1 in cancer

Given the functional diversity of PP1 which predominantly stems from its ability to interact with over 200 proteins within cells as previously described, it is not surprising that cellular and genetic aberrations that either reduce or increase the expression/activity of PP1 have been implicated in cancer development and progression. Another factor to note is that implications associated with the development of cancer could be cancer-type and/or species specific, and this has given rise to existence of contradictory evidence regarding the effects of PP1 expression in various cancers. (Moura and Conde, 2019, Saidy et al., 2021, Ruvolo, 2019).

Suppression of PP1 activity to less than 25% using non-specific phosphoprotein phosphatase inhibitors such as okadaic acid, calyculin A, and microcystin has been associated with the development and progression of cancer in mouse skin tissue where n>80% was considered

a high percentage of tumour-bearing mice following treatment compared to 0% in untreated control groups, rat glandular stomach and liver where n>75% was considered a high percentage of tumour-bearing rats following treatment in the presence of N-methyl-N'-nitro-N-nitrosoguanidine compared to 0% in untreated control groups (Fujiki and Suganuma, 2009, Fujiki et al., 2018).

PP1 interacts with BRCA1 through its regulatory proteins. PP1/BRCA1 interactions can influence the activity of PP1 which also serves as a scaffold protein aiding dephosphorylation of BRCA1 associated proteins. In one study, the pattern of expression of BRCA1 and the PP1 isoforms were monitored in primary human breast cancers. Low levels of BRCA1 mRNA were deemed to be the main reason for the reduced BRCA1 protein expression rather than existence of mutations that would alter the function of BRCA1. Also, a significantly lower level of expression for PP1β and γ was observed in transformed tissues in comparison to that of healthy breast tissue (BRCA1 P=0.01, PP1β: P=0.03, PP1γ, P=1.9 × 10-6). In this experiment, changes in gene expression were compared against the housekeeping gene HPRT-1, where the mRNA/HPRT-1 ratios of less than 0.5× the mean level of expression were considered to be under expressed, and those that were greater than 2× the mean level of expression were considered to be overexpressed (Winter et al., 2007).

In another study, amplification of *PPP1CA* gene was estimated to be present in 7% of non-invasive prostate cancer (LPC) in a data set of 59, and 17% of metastatic castration resistant prostate cancers (mCRPC) in a data set of 35. The same study also suggested the existence of a link between the amplification of *PPP1CA* gene and that of G1/S specific cyclin-D1 (CCND1), which raises the suspicions over the potential pro-metastatic and proto-oncogenic role of *PPP1CA* in prostate cancer (Chen et al., 2018, Felgueiras et al., 2014).

PPP1CA amplification in oral squamous cell carcinoma cells has been reported to correlate with increased expression of *PPP1CA* and Protein phosphatase PP1α (Hu et al., 2021). Allelic loss of *PPP1CA* has been reported in thyroid, larynx, kidney and colorectal tumours highlighting the diverse implications linked with the aberrations of *PPP1CA* gene (Castro et al., 2008). In one study, Barker *et.al (*1990), successfully mapped the *PPP1CA* gene to 11q13. Interestingly, translocation of the *PPP1CA* gene involving breakpoints at this location has been previously observed in lymphoma, chronic lymphocytic leukaemia of the B- cell type and multiple myeloma (Barker et al., 1990, Castro et al., 2008). Cloning of chromosomal breakpoints in chronic lymphocytic leukaemia cells by Tsujimoto *et.al* in 1984 led to the identification of a breakpoint on 14q which was also mapped to chromosome 11 using a probe specific for 11q13, suggesting t (11;14) translocation (Tsujimoto et al., 1984).

While a comprehensive evaluation of the exact role of *PPP1CB* in tumorigenesis and tumour progression is still under way, some studies have pointed to the potential influence of *PPP1CB* over uncontrolled growth, migration and invasion of prostate cancer, gastric cancer, and melanoma (Hu et al., 2021). The expression of *PPP1CB* has been associated with tumour mobility and metastasis through facilitating mechanisms such as actomyosin contraction (Zhu et al., 2020, Hu et al., 2021). The importance of PP1c in the regulation of oncogenic pathways is further highlighted by the evidence of PP1 complex dysregulation in carcinogenesis and cancer progression (Figueiredo et al., 2014, Matos et al., 2021). Some examples of different sets of PP1 catalytic and regulatory proteins combinations suspected to have a role in the promotion/inhibition of cancers are presented in Figure 3.1 (Matos et al., 2021).

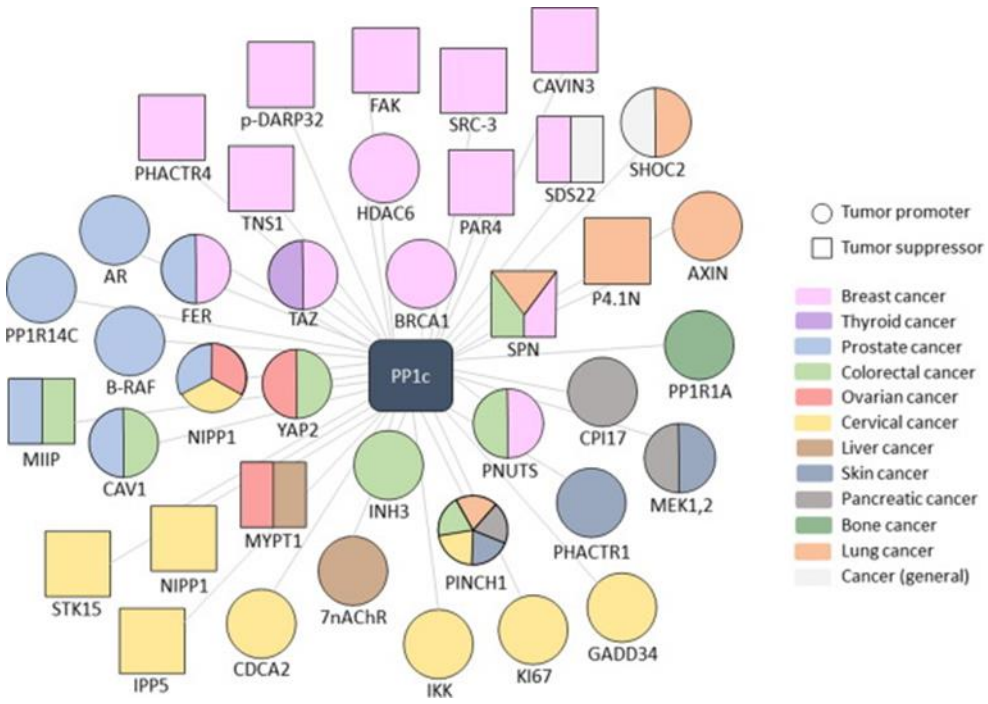


Figure 3.1: Various combinations of proteins that interact with PP1γ are shown above. Proteins demonstrated as circles represent those that could potentially promote cancer development, and proteins demonstrated as squares represent tumour-suppressors. These interactions are colour coded to the relevant cancer types (Matos et al., 2021). Copyright© 2021 by Elsevier Publishing Group. Permission was obtained by Copyright Clearance Centre's RightLink ®service.

These data align with the findings of enhanced cellular *PPP1CA* and *PPP1CB* mRNA availability in pancreatic adenocarcinoma compared to normal tissues. In one investigation, Kaplan Meier analysis of patients in a pancreatic cancer cohort (n=55) was used to illustrate the correlation of high mRNA levels of *PPP1CA, PPP1CB,* and *PPP1CC* and undesirable patient outcome based on prognosis (P<0.01) (Hang et al., 2021).

PP1 has been found to regulate actin cytoskeleton through interactions with intermediate proteins, thereby influencing cellular adhesion and mobility (Martin-Granados et al., 2012, Hendrickx et al., 2009). Another

recent study unveiled the involvement of PP1 in modulating enteric nerve cell migration (Martin-Granados et al., 2012, Zhang et al., 2012).

Further research is underway in order to discover the exact role of PP1 in regulation of cell growth and migration. Overall, the regulation of PP1 activity and/or its partnership with a vast variety of proteins may prove to be an effective method for targeted cancer therapy. Further research could potentially unveil a novel approach which utilises specific PP1 complexes in order to initiate interactions that have crucial therapeutic benefits (Matos et al., 2021).

3.1.3 Cdk5 in cancer

Given the involvement of Cdk5 in critical cellular process in cells, dysregulation of this protein can have adverse effects on the normal functioning of those cells. Studies have demonstrated that dysregulation of Cdk5 plays an important role in the development and progression of tumours, whereas inhibition of Cdk5 in some cancers is believed to play an anti-cancer role and improves response to chemotherapy. This makes Cdk5 a novel biomarker and a promising target for anti-cancer therapy (Jeggo et al., 2016, Liu et al., 2017).

Overexpression of Cdk5 in lung cancer was demonstrated to be associated with poor prognosis in a study comprising a total of 395 samples of lung tissue, including samples of non-small cell lung cancer (n=339), small cell lung cancer (n=26) and normal lung tissue (n=30) (Wei et al., 2016). Overexpression of Cdk5 in head and neck squamous cell carcinoma (n=60) has also been associated with tumour cell motility and metastasis (Sun et al., 2015). In pancreatic cancer cells, inhibition of Cdk5 has proven to be effective in impairing oncogenic Ras-induced neoplasia, tumour progression and metastasis (Feldmann et al., 2010). In prostate

cancer, Cdk5 induces cancer progression through STAT3 and androgen receptor (AR) signalling (Hsu et al., 2013).

Previous studies have demonstrated that in breast cancer both p35 and Cdk5 are up-regulated and hyperactivated in the presence of transforming growth factor beta 1 (TGF-β1), a factor known to induce epithelial mesenchymal transition (EMT) and cancer cell motility through downstream phosphorylation of focal adhesion kinase (FAK) at Serine-732 (Liang et al., 2013). In support of these findings, a study by Shupp *et.al* (2017) describes the aberrant overexpression of Cdk5 and p35 in breast cancer as the incentive for the emergence of inhibitors (Shupp et al., 2017). Other studies including one by Liu *et.al* (2017) (n=108) have also reported the common trend of increased Cdk5 expression across a number of cancers including breast cancer (Liu et al., 2017, Liang et al., 2013).

Cellular acquisition of motility and metastasis can be achieved through regulation of actin and microtubule cytoskeleton as well as focal adhesions. In breast cancer, Cdk5 has been found to modulate the formation of F-actin (filamentous) bundles (Liang et al., 2013), and effect actin dynamics regulators such as caldesmon, an actin- and calmodulin-binding protein, in order to facilitate cell migration and metastasis (Bisht et al., 2015). Furthermore, Cdk5 is also capable of interacting with other microtubule associated proteins including Tau (Takashima et al., 2001, Rouzier et al., 2005).

EGF is another factor that facilitates cancer cell motility through activation of Cdk5. When activated, Cdk5 can phosphorylate the Gα-interacting vesicle associated protein, Girdin, thereby activating downstream G-coupled receptor-dependent signalling mechanisms that promote cell migration (Pozo and Bibb, 2016).

Evidence indicates that Cdk5 participates in tumorigenesis, progression and metastasis of different cancers and through manipulating various signalling pathways. In contrast, in majority of cases downregulation of Cdk5 has been associated with normalisation of cellular processes vital for the progression of various cancers (Liu et al., 2017).

2.2.7 Hypothesis, aims, and objectives

Hypothesis

We hypothesise that reduction in DARPP-32 signalling, either through loss of DARPP-32 or switch to t-DARPP expression results in poor patient prognosis through the loss of PP1 inhibition, allowing the development of more aggressive tumours.

Aims

The aim of the current study is to demonstrate that DARPP-32 and its signalling partners are important in breast cancer, and to facilitate understanding of how this affects patient outcome.

Objectives

1. Evaluate expression of Cdk5, PKA, and PP1 to understand DARPP-32 signalling in a large cohort of breast cancer patients.

2. To use Cdk5, PKA and PP1 inhibitors to determine the effect on DARPP-32 phosphorylation in T47D breast cancer cell line, and determine the cytotoxic effect of these compounds in triple

negative MDA-MB-231 and luminal phenotype T47D and MCF7 cells.

3. To determine the effects of purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin on DARPP-32 protein expression in T47D and MDA-MB-231 breast cancer cells by Western blotting.

3.2 Results

3.2.1 Protein staining, frequency and correlation of expression

The expressions of PKA, PP1 and Cdk5 were determined in both the cytoplasm and the nucleus and were compared with the previously determined DARRP-32 protein expression (Kotecha et al., 2019). The expression of PKA, PP1 and Cdk5 were determined via a semi-quantitative scoring method using an Immunohistochemical score (H-score). Scores which ranged between 0 and 300 were evaluated by multiplying staining intensity by the percentage of positive cells in tissues. The percentage of positive cells was determined subjectively per intensity. The score index ranking was expressed as 0, 1, 2, and 3 representing negative, weak, moderate, and strong staining intensity, respectively.

Representative examples of tissue staining are demonstrated in Figure 3.2. Expression of PKA was determined in a total of 1159 patient specimens. PKA expression was most abundant in cytoplasm and exhibited a weak to strong staining intensity in tumour cells (Figure 3.2 B and C). The median H-score for cytoplasmic expression of PKA was 160

(ranging between 0 and 290). PP1 expression was assessed in both the cytoplasm and the nucleus in 1488 patient specimens (Figure 3.2 D and E). The median H-score for cytoplasmic expression of PP1 was 0 (ranging between 0 and 160), the median H-score for nuclear PP1 expression was 0 (ranging between 0 and 100). Cdk5 expression was determined in 1110 tumour specimens with the median H-score for cytoplasmic expression of Cdk5 being 105 (ranging between 0 and 240) and the median H-score for nuclear Cdk5 expression being 5 (ranging between 0 and 85); representative tissue staining is shown in Figure 3.2 F and G. Western blot analysis were used to confirm specificity of the antibodies used for Immunohistochemistry (Appendix A-Figure A1).

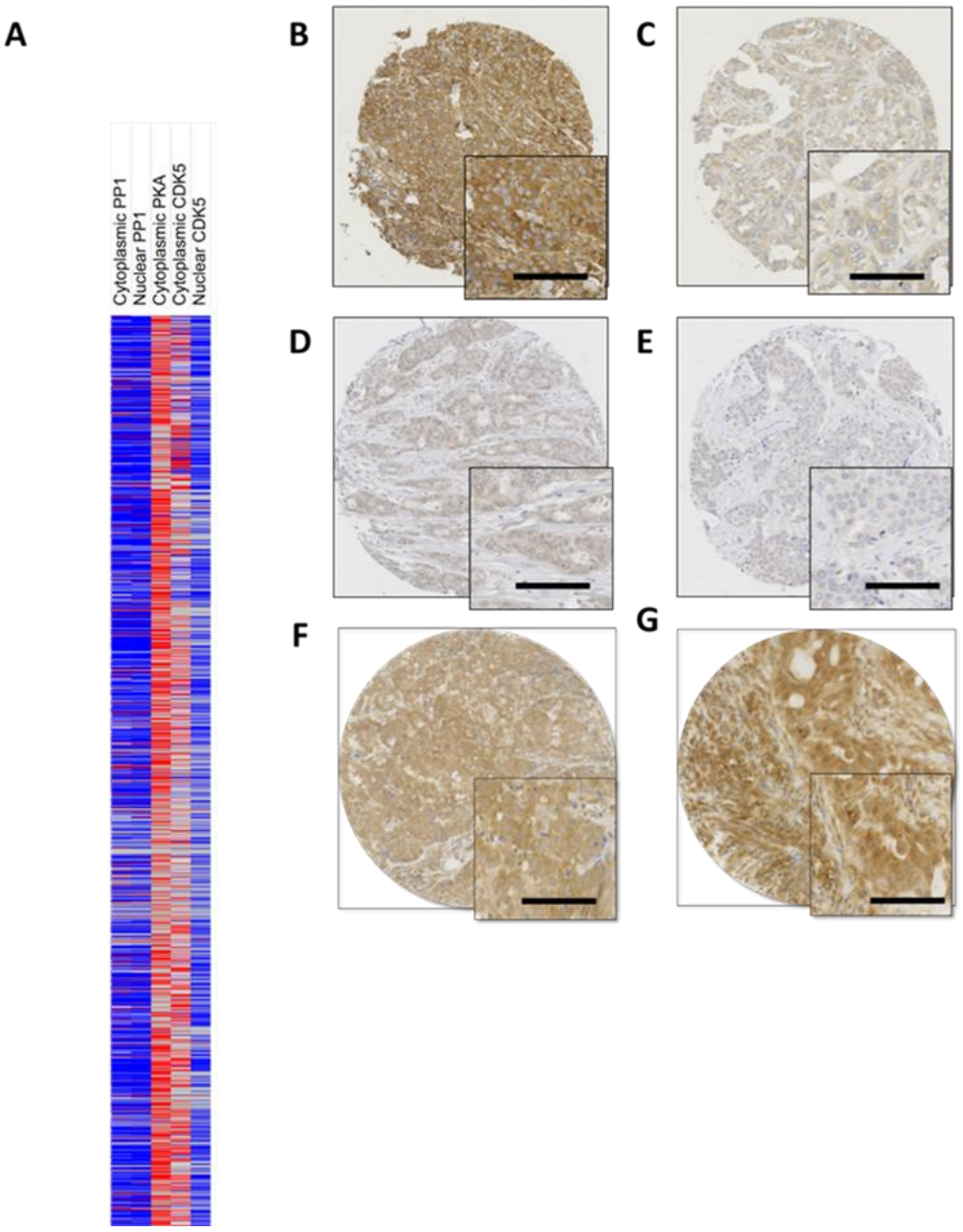


Figure 3.2: Representative photomicrographs of PKA (B, C), PP1 (D, E), and Cdk5 (F, G) staining using immunohistochemistry. Photomicrographs are taken at 10×magnification with a 20× magnification inset box; scale bar represents 100 μm. (A) shown scores for each patient for each immunohistochemistry marker shown in a heatmap.

3.2.2 Association between protein expression and clinicopathological variables

Low expression of PKA was significantly associated with vascular invasion (χ^2=6.353, d.f.=1, *P*=0.012). There was no significant association between the expression of PKA and other clinicopathological variables (Table 3.1). Low cytoplasmic PP1 expression was significantly associated with pleomorphism (χ^2=6.918, d.f.=2, *P*=0.031) and mitosis (χ^2=8.849, d.f.=2, *P*=0.012). Low nuclear PP1 expression was significantly associated with patients age (χ^2=4.112, d.f.=1, *P*=0.043). There was no significant association between nuclear and cytoplasmic expression of PP1 and other clinicopathological variables (Table 3.1).

Low cytoplasmic Cdk5 expression was significantly associated with NPI (X^2=7.334, d.f.=2, *P*=0.026). Low nuclear Cdk5 expression was significantly associated with the development of tumour size (X^2=7.858, d.f.=1, *P*=0.005), higher tumour grade (X^2=7.630, d.f.=2, *P*=0.022), mitosis (X^2=7.702, d.f.=2, *P*=0.021), NPI (X2=8.386, d.f.=2, *P*=0.015), ER positive tumours (X^2=6.754, d.f.=1, *P*=0.009), and triple receptor negative tumours (X^2=5.927, d.f.=1, *P*=0.015). (Table 3.2).

Table 3.1: Association between various clinicopathological parameters with cytoplasmic and nuclear PP1 and cytoplasmic PKA level of expression are demonstrated below. The *P* values highlighted in bold are <0.05.

		PKA Cytoplasmic			PP1 Cytoplasmic			PP1 Nuclear		
		Low	**High**	***P* value**	**Low**	**High**	***P* value**	**Low**	**High**	***P* value**
Patient age										
	<50 years	41 (3.6%)	328 (28.6%)	0.919	428 (25.9%)	71 (4.3%)	0.140	428 (25.9%)	71 (4.3%)	**0.043**
	≥ 50 years	84 (7.3%)	695 (60.4%)		1019(61.7%)	134 (8.1%)		1032(62.4%)	124 (7.5%)	
Tumour size										
	< 2cm	71 (6.2%)	617 (53.7%)	0.499	891 (54.0%)	135 (8.2%)	0.242	902 (54.5%)	127 (7.7%)	0.371
	≥ 2cm	54 (4.7%)	406 (35.4%)		555 (33.6%)	70 (4.2%)		557 (33.7%)	68 (4.1%)	
Tumour grade										
	1	21 (1.8%)	144 (12.5%)		231 (14.0%)	30 (1.8%)		229 (13.8%)	32 (1.9%)	
	2	50 (4.4%)	384 (33.4%)	0.501	599 (36.3%)	75 (4.5%)	0.245	601 (36.3%)	76 (4.6%)	0.838
	3	54 (4.7%)	495 (43.1%)		616 (37.3%)	100 (6.1%)		629 (38.0%)	87 (5.3%)	
Tubule formation										
	1	12 (1.1%)	69 (6.1%)		104 (6.3%)	15 (0.9%)		103 (6.3%)	16 (14.1%)	
	2	29 (2.6%)	297 (26.1%)	0.257	418 (25.5%)	67 (4.1%)	0.473	429 (26.1%)	58 (3.5%)	0.855
	3	82 (7.2%)	648 (57.0%)		914 (55.8%)	120 (87.7%)		914 (55.7%)	121 (7.4%)	
Pleomorphism										
	1	2 (0.2%)	10 (0.9%)		21 (1.3%)	0 (0.0%)		20 (1.2%)	1 (0.1%)	
	2	43 (3.8%)	265 (23.3%)	0.084	447(435.7%)	50 (3.1%)	**0.031**	439 (26.8%)	59 (3.6%)	0.594
	3	78 (6.9%)	739 (65.0%)		968 (59.1%)	152 (9.3%)		987 (60.1%)	135 (8.2%)	
Mitosis										
	1	63 (5.5%)	454 (40.0%)		720 (44.0%)	99 (6.1%)		719 (43.9%)	103 (6.3%)	
	2	18 (1.6%)	208 (18.3%)	0.233	280 (17.1%)	25 (1.5%)	**0.012**	281 (17.1%)	24 (1.5%)	0.050
	3	42 (3.7%)	351 (30.9%)		434 (26.5%)	78 (4.8%)		444 (27.1%)	68 (4.1%)	

Table Continued

		PKA Cytoplasmic			PP1 Cytoplasmic			PP1 Nuclear		
		Low	**High**	***P* value**	**Low**	**High**	***P* value**	**Low**	**High**	***P* value**
Vascular invasion										
	Definite	50 (4.4%)	297 (25.9%)	**0.012**	415 (25.2%)	64 (3.9%)	0.461	426 (25.8%)	53 (3.2%)	0.614
	No/probable	75 (6.5%)	726 (63.2%)		1030 (62.4)	141 (8.5%)		1033(62.5%)	141 (8.5%)	
Stage										
	1	74 (6.5%)	649 (56.7%)		926 (56.2%)	118 (7.2%)		931 (56.4%)	113 (6.8%)	
	2	32 (2.8%)	271 (23.7%)	0.278	376 (22.8%)	70 (4.2%)	0.050	383 (23.3%)	66 (4.0%)	0.082
	3	18 (1.6%)	101 (8.8%)		141 (8.6%)	17 (1.0%)		142 (8.6%)	16 (1.0%)	
NPI										
	Good	37 (3.2%)	328 (28.7%)		515 (31.3%)	63 (3.8%)		516 (31.3%)	62 (3.8%)	
	Medium	60 (5.2%)	526 (46.0%)	0.303	704 (42.7%)	111 (6.7%)	0.315	716 (43.4%)	102 (6.2%)	0.597
	Poor	27 (2.4%)	166 (14.5%)		223 (13.5%)	31 (1.9%)		223 (13.5%)	31 (1.9%)	
ER status										
	Negative	21 (1.8%)	215 (18.7%)	0.273	274 (16.6%)	43 (2.6%)	0.507	281 (17.0%)	36 (2.2%)	0.797
	Positive	104 (9.1%)	809 (70.4%)		1174(71.0%)	162 (9.8%)		118(71.3%)	159 (9.6%)	
PR status										
	Negative	52 (4.8%)	414 (38.3%)	0.831	579 (37.1%)	82 (5.3%)	0.865	586 (37.5%)	75 (4.8%)	0.597
	Positive	66 (6.1%)	548 (50.7%)		784 (50.3%)	114 (7.3%)		790 (50.6%)	110 (7.0%)	
HER2 status										
	Negative	105 (9.8%)	867 (80.8%)	0.978	1249(80.5%)	176 (11.3%)	0.529	1262(81.2%)	166 (10.7%)	0.251
	Positive	11 (1.0%)	90 (8.4%)		108 (7.0%)	15.8 (1.2%)		107 (6.9%)	19 (1.3%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.0

Table 3.2: Association between various clinicopathological parameters with cytoplasmic and nuclear Cdk5 level of expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Cdk5 Cytoplasmic			Cdk5 Nuclear		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**
Age						
<50 years	289 (26.3%)	70 (6.4%)	0.748	234 (21.4%)	124 (11.3%)	0.500
≥50 years	588 (53.6%)	150 (13.7%)		467 (42.6%)	271 (24.7%)	
Tumour Size						
<2.0 cm	507 (46.3%)	143 (13.0%)	0.055	393 (35.9%)	256 (23.4%)	**0.005**
≥2.0 cm	369 (33.7%)	77 (7.0%)		307 (28.0%)	139 (12.7%)	
Grade						
1	131 (12.0%)	41 (3.7%)		102 (9.3%)	70 (6.4%)	
2	331 (30.2%)	91 (8.3%)	0.127	256 (23.4%)	166 (15.2%)	**0.022**
3	414 (37.8%)	88 (8.0%)		342 (31.2%)	159 (14.5%)	
Pleomorphism						
1	12 (1.1%)	3 (0.3%)		8 (0.7%)	7 (0.6%)	
2	247 (22.8%)	65 (6.0%)	0.909	191 (17.6%)	121 (11.2%)	0.316
3	609 (56.1%)	149 (13.7%)		494 (45.6%)	263 (24.3%)	
Mitosis						
1	398 (36.7%)	113 (10.4%)		306 (28.3%)	205 (18.9%)	
2	170 (15.7%)	41 (3.8%)	0.224	137 (12.7%)	74 (6.8%)	**0.021**
3	299 (27.6%)	63 (5.8%)		249 (23.0%)	112(10.3%)	
Vascular invasion						
Definite	265 (24.2%)	53 (4.8%)	0.072	210 (19.2%)	107 (9.8%)	0.308
No/probable	611 (55.7%)	167 (15.2%)		490 (44.7%)	288 (26.3%)	
Stage						
1	549 (50.1%)	142 (13.0%)		440 (40.2%)	251 (22.9%)	
2	234 (21.4%)	64 (5.8%)	0.167	188 (17.2%)	109 (10.0%)	0.780
3	92 (8.4%)	14 (1.3%)		71 (6.5%)	35 (3.2%)	
NPI						
Good	274 (25.0%)	85 (7.8%)		208 (19.0%)	151 (13.8%)	
Medium	444 (40.6%)	110 (10.1%)	**0.026**	370 (33.9%)	183 (16.7%)	**0.015**
Poor	156 (14.3%)	25 (2.3%)		121 (11.1%)	60 (5.5%)	
ER status						
Negative	186 (16.9%)	38 (3.5%)	0.186	160 (14.6%)	64 (5.8%)	**0.009**
Positive	691 (62.9%)	183 (16.7%)		542 (49.4%)	331 (30.2%)	
PR status						
Negative	373 (36.2%)	83 (8.1%)	0.103	303 (29.5%)	153 (14.9%)	0.243
Positive	445 (43.2%)	128 (12.4%)		360 (35.0%)	212 (20.6%)	

Table Continued

	Cdk5 Cytoplasmic			Cdk5 Nuclear		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**
HER2 status						
Negative	748 (72.3%)	192 (18.6%)	0.769	603 (58.4%)	336 (32.5%)	0.628
Positive	76 (7.4%)	18 (1.7%)		58 (5.6%)	36 (3.5%)	
Triple negative status						
Negative	728 (67.6%)	189 (17.5%)	0.365	572 (53.2%)	344 (32.0%)	**0.015**
Positive	132 (12.3%)	28 (2.6%)		116 (10.8%)	44 (4.1%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.05.

3.2.3 Protein expression and breast cancer specific survival

Kaplan-Meier survival curves were used to assess the impact of PKA, PP1 and Cdk5 protein expression on breast cancer specific survival. Low protein expression of PKA was significantly associated with adverse breast cancer specific survival with a stronger impact in ER positive breast cancer patients (P=0.040 and P=0.003 respectively, (Figure 3.3 A and B)). Cytoplasmic and nuclear expression of PP1 was not associated with breast cancer specific survival (P=0.217 and P=0.428 respectively, (Figure 3.3 C and D)). Low nuclear and cytoplasmic expression of Cdk5 was significantly associated with adverse breast cancer specific survival (P=0.004 and P=0.001 respectively, (Figure 3.3 E and F)).

Multivariate analysis was performed via Cox's proportional hazard method, and included tumour size, tumour grade, tumour stage, NPI, ER status, PR status, HER2 status and vascular invasion (each individually significantly associated with patient survival). Ten-year survival rates for breast cancer patients with high and low levels of PKA expression were 82.3% (95% CI=0.768-0.848) and 77.6% (95% CI=0.675-0.835), respectively. However, PKA expression level was not found to be significant to the overall survival of patients when other potentially crucial

factors such as tumour size, grade, nodal stage, ER status, PR status, HER2 status, and LVI status were included in multivariate Cox regression analysis (*P*=0.201, HR=0.768; 95% CI=0.513-1.151). A Hazard ratio of 0.612 and 95% confidence interval of 0.418-0.896 (*P*=0.011) for nuclear Cdk5 expression, and a hazard ratio of 0.507 and 95% confidence interval of 0.318-0.809 (*P*=0.004) for cytoplasmic Cdk5 expression were observed.

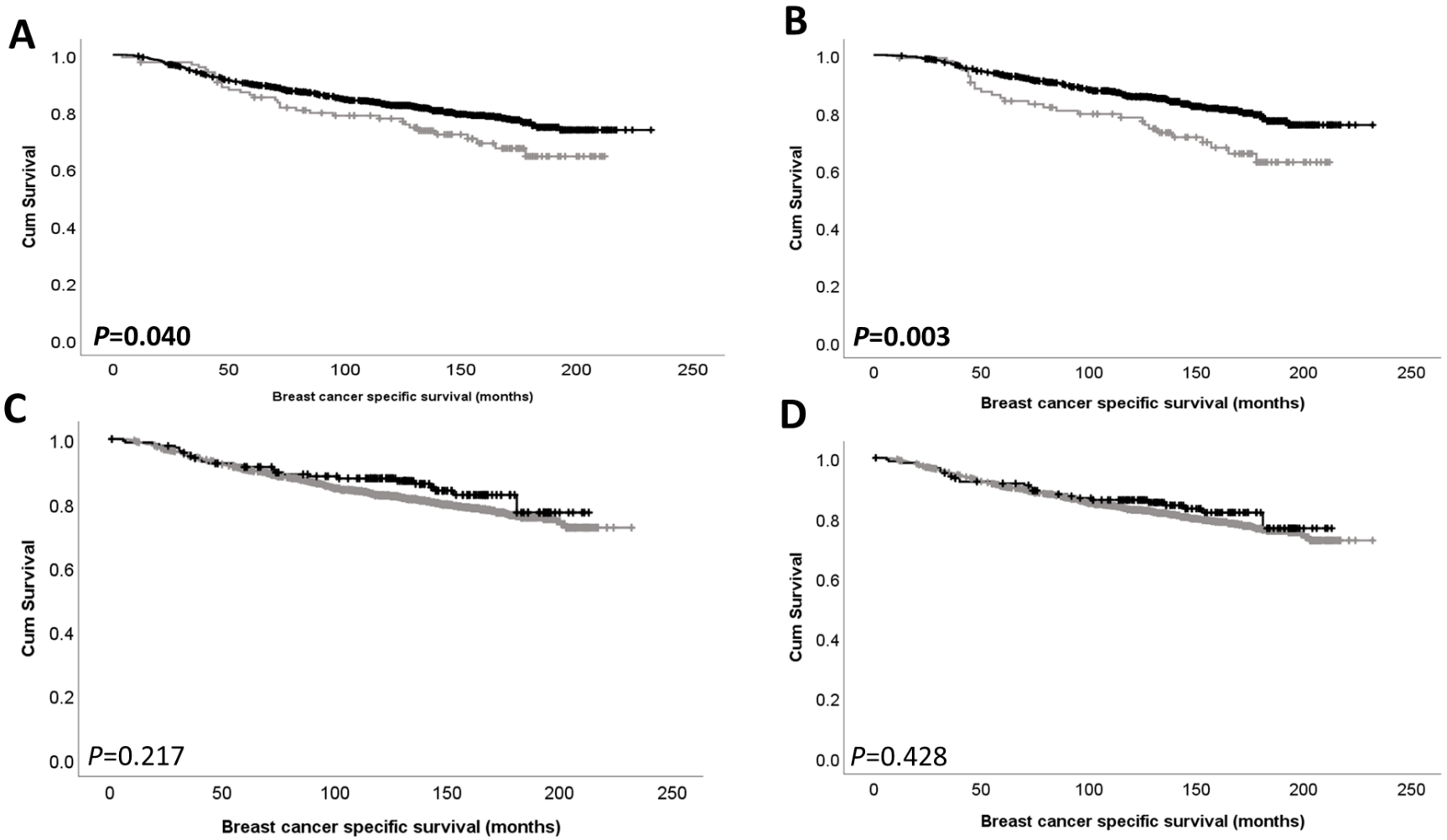


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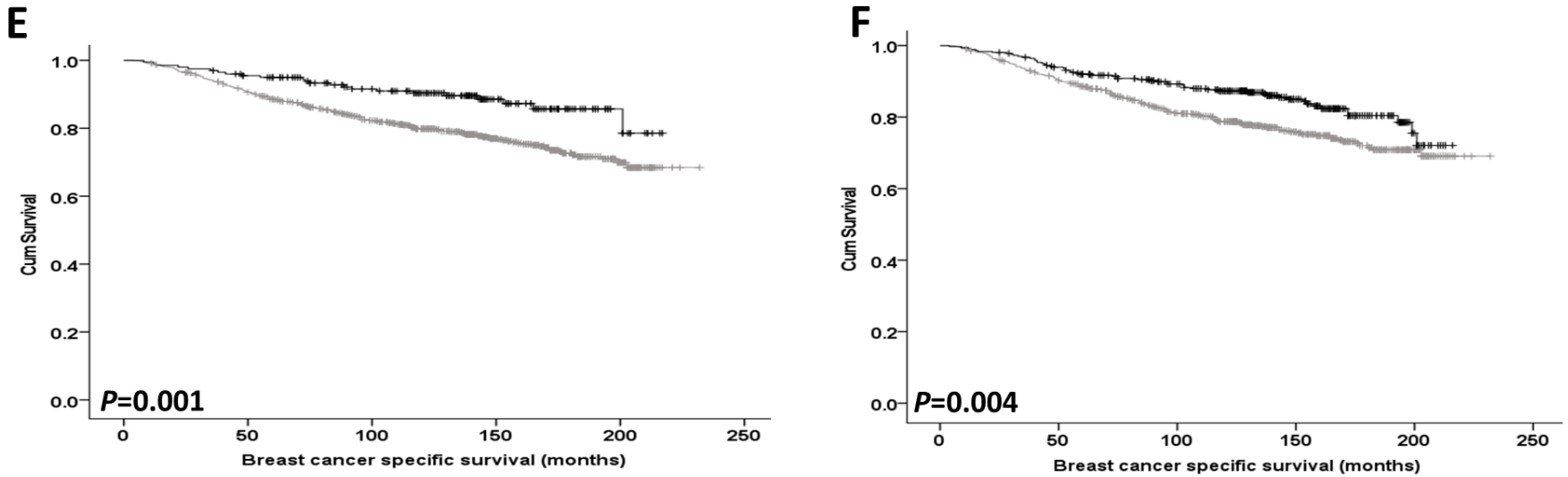


Figure 3.3: Kaplan-Meier analysis of PKA expression (A) and PKA in oestrogen receptor positive breast cancer (B); PP1 expression in the cytoplasmic and nuclear (C and D), Cdk5 expression in the cytoplasmic and nuclear (E and F). The grey line shows the effects of low expression and the black line shows the effect high protein expression.

3.2.4 Cdk5 expression and ER status

Patients were divided into ER positive and negative subgroups in order to investigate the impact of Cdk5 expression against survival in ER positive patients; this was principally due to the previous observations on DARPP-32 in this subgroup of patients (Kotecha et al., 2019). Cytoplasmic expression of Cdk5 was found to be significantly associated with the survival of ER positive patients (P=0.002), a pattern that was absent in ER negative patients (P=0.245) (Figure 3.4 A and B, respectively). Nuclear expression of Cdk5 did not have a significant impact on the survival of ER positive or negative subgroups (P=0.079 and P=0.065, respectively) (Figure 3.4 C and D, respectively).

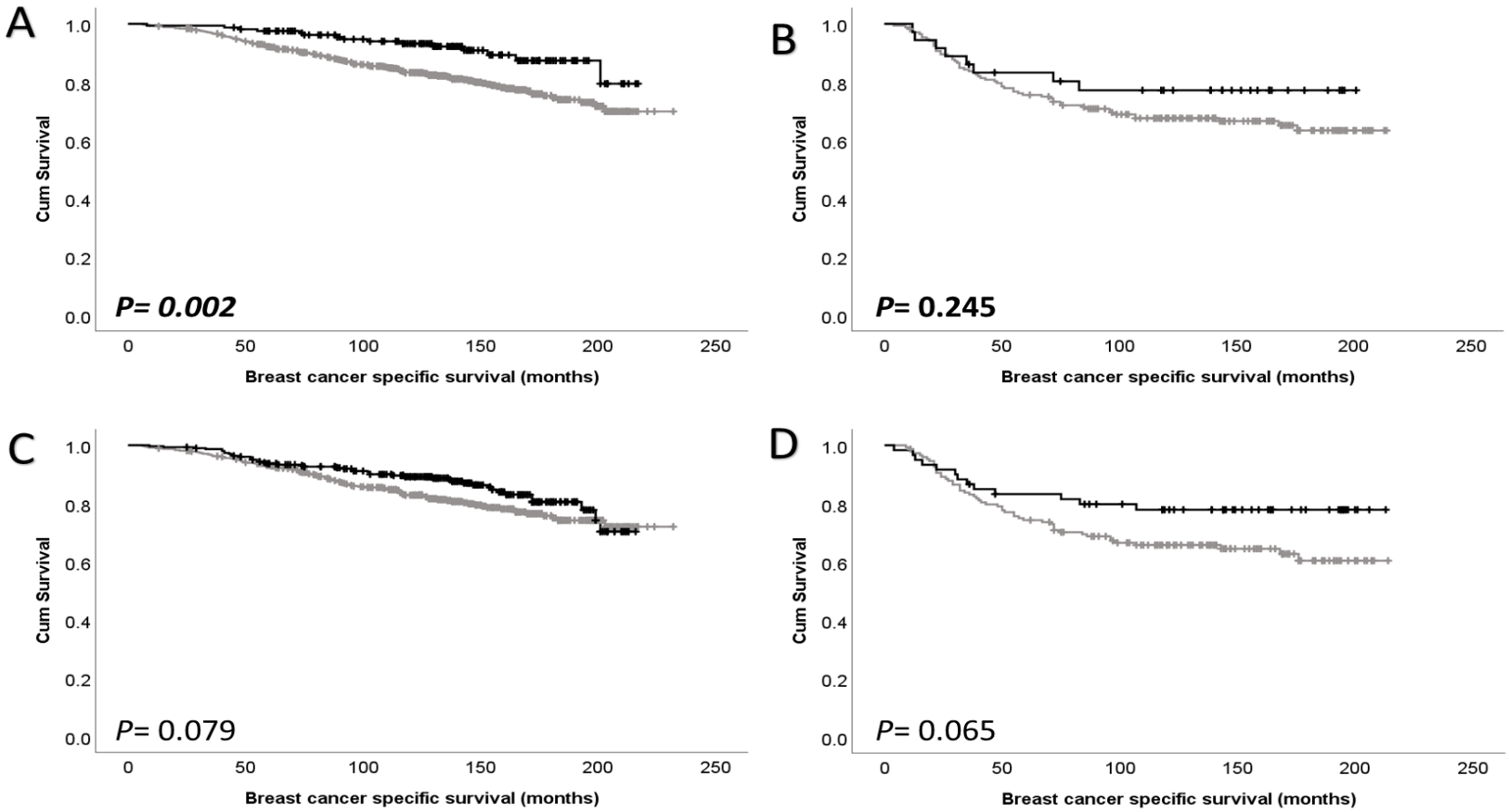


Figure 3.4: Kaplan-Meier analysis of cytoplasmic expression in ER positive (A) and ER negative breast cancers (B) and their association with survival. The impact of nuclear expression of Cdk5 in ER positive and ER negative breast cancer on survival are demonstrated in C and D, respectively.

3.2.5 Combination expression and clinicopathological criteria

PKA, PP1, cdk5 and DARPP-32 expression combinations were assessed for association with clinicopathological variables. The combination of low PKA and cytoplasmic low cdk5 expression were significantly associated with the development of tubule formation (χ^2=13.049, d.f.=6, *P*=0.042), mitosis (χ^2=17.509, d.f.=6, *P*=0.008), and tumour type (χ^2=36.051, d.f.=18, *P*=0.007) (Table 3.3).

Table 3.3: Association between various clinicopathological parameters with combined cytoplasmic PKA and Cdk5 level of expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Cytoplasmic				
	Low PKA and low Cdk5	**High PKA and low Cdk5**	**Low PKA and high Cdk5**	**High PKA and high Cdk5**	***P* value**
Age					
<50	23(8.5%)	198(72.8%)	1(0.4%)	50(18.4%)	0.623
≥50	50(9.2%)	386(71.0%)	7(1.3%)	101(18.6%)	
Tumour Size					
<2 cm	44(9.2%)	332(69.5%)	5(1.0%)	97(20.3%)	0.415
≥2 cm	29(8.6%)	252(74.6%)	3(0.9%)	54(16.0%)	
Grade					
1	12(9.9%)	81(66.9%)	3(2.5%)	25(20.7%)	0.141
2	29(9.8%)	203(68.4%)	4(1.3%)	61(20.5%)	
3	32(8.0%)	300(75.4%)	1(0.3%)	65(16.3%)	
Tubule formation					
1	7(11.7%)	39(65.0%)	1(1.7%)	13(21.7%)	**0.042**
2	13(5.5%)	166(70.6%)	5(2.1%)	51(21.7%)	
3	52(10.1%)	375(73.1%)	2(0.4%)	84(16.4%)	
Pleomorphism					
1	1(12.5%)	6(75.0%)	0(0.0%)	1(12.5%)	0.248
2	27(13.0%)	137(65.9%)	3(1.4%)	41(19.7%)	
3	44(7.4%)	437(73.8%)	5(0.8%)	106(17.9%)	

Table Continued

	Cytoplasmic				
	Low PKA and low Cdk5	**High PKA and low Cdk5**	**Low PKA and high Cdk5**	**High PKA and high Cdk5**	***P* value**
Mitosis					
1	39(11.0%)	237(66.6%)	6(1.7%)	74(20.8%)	**0.008**
2	5(3.1%)	127(78.4%)	2(1.2%)	28(17.3%)	
3	28(9.7%)	215(74.4%)	0(0.0%)	46(15.9%)	
Tumour type					
Ductal	52(7.3%)	518(73.1%)	8(1.1%)	131(18.5%)	
Lobular	14(23.3%)	33(55.0%)	0(0.0%)	13(21.7%)	
Medullary-like	0(0.0%)	8(100.0%)	0(0.0%)	0(0.0%)	
Miscellaneous	0(0.0%)	5(83.3%)	0(0.0%)	1(16.7%)	**0.007**
Mixed &lobular	0(0.0%)	1(100.0%)	0(0.0%)	0(0.0%)	
Special type	7(23.3%)	18(60.0%)	0(0.0%)	5(16.7%)	
Tubular	0(0.0%)	0(0.0%)	0(0.0%)	1(100.0%)	
Vascular invasion					
Definite	25(10.4%)	181(75.4%)	3(1.3%)	31(12.9%)	0.057
No/Probable	48(8.3%)	403(70.0%)	5(0.9%)	120(20.8%)	
Stage					
1	49(9.4%)	370(70.7%)	4(0.8%)	100(19.1%)	0.239
2	13(6.3%)	151(72.6%)	4(1.9%)	40(19.2%)	
3	11(13.1%)	62(73.8%)	0(0.0%)	11(13.1%)	
NPI					
GPG	22(8.7%)	172(68.0%)	4(1.6%)	55(21.7%)	0.312
MPG	37(8.8%)	302(71.7%)	3(0.7%)	79(18.8%)	
PPG	14(10.0%)	108(77.1%)	1(0.7%)	17(12.1%)	
ER Status					
Negative	14(8.0%)	137(77.8%)	0(0.0%)	25(14.2%)	
Positive	59(9.2%)	447(69.7%)	8(1.2%)	127(19.8%)	0.110

Table Continued

	Cytoplasmic				
	Low PKA and low Cdk5	**High PKA and low Cdk5**	**Low PKA and high Cdk5**	**High PKA and high Cdk5**	***P* value**
PR Status					
Negative	36(10.6%)	248(72.7%)	1(0.3%)	56(16.4%)	0.104
Positive	34(8.1%)	293(69.6%)	6(1.4%)	88(20.9%)	
HER2 Status					
Negative	60(8.8%)	489(71.7%)	7(1.0%)	126(18.5%)	0.881
Positive	5(6.3%)	57(72.2%)	1(1.3%)	16(20.3%)	
					0.145
	61(9.0%)	476(70.2%)	8(1.2%)	133(19.6%)	
Triple Negative	10(8.3%)	95(79.2%)	0(0.0%)	15(12.5%)	
Negative					
Positive					

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.05.

The combination low levels of PKA and nuclear Cdk5 expression is significantly associated with the development of smaller tumour size (χ^2=8.121, d.f.=3, *P*=0.044), mitosis (χ^2=15.779, d.f.=6, *P*=0.015), moderate NPI (χ^2=13.085, d.f.=6, *P*=0.042), ER positive tumours (χ^2=9.784, d.f.=3, *P*=0.020), and triple receptor negative tumours (χ^2=8.994, d.f.=3, *P*=0.029) (Table 3.4).

Table 3.4: Association between various clinicopathological parameters with combined nuclear PKA and Cdk5 level of expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Nuclear				
	Low PKA and low Cdk5	**High PKA and low Cdk5**	**Low PKA and high Cdk5**	**High PKA and high Cdk5**	***P* value**
Age					
<50	17(6.3%)	163(60.1%)	7(2.6%)	84(31.0%)	0.817
≥50	41(7.5%)	310(57.0%)	16(2.9%)	177(32.5%)	
Tumour Size					
<2 cm	36(7.5%)	258(54.1%)	13(2.7%)	170(35.6%)	**0.044**
≥2 cm	22(6.5%)	215(63.6%)	10(3.0%)	91(26.9%)	
Grade					
1	9(7.4%)	66(54.5%)	6(5.0%)	40(33.1%)	0.212
2	24(8.1%)	159(53.5%)	9(3.0%)	105(35.4%)	
3	25(6.3%)	248(62.5%)	8(2.0%)	116(29.2%)	
Tubule formation					
1	6(10.0%)	28(46.7%)	2(3.3%)	24(40.0%)	0.076
2	9(3.8%)	135(57.4%)	9(3.8%)	82(34.9%)	
3	43(8.4%)	306(59.8%)	11(2.1%)	152(29.7%)	
Pleomorphism					
1	1(12.5%)	3(37.5%)	0(0.0%)	4(50.0%)	0.088
2	20(9.6%)	108(51.9%)	10(4.8%)	70(33.7%)	
3	37(6.3%)	358(60.0%)	12(2.0%)	184(31.1%)	
Mitosis					
1	31(8.7%)	185(52.0%)	14(3.9%)	126(35.4%)	**0.015**
2	6(3.7%)	102(63.0%)	1(0.6%)	53(32.7%)	
3	21(7.3%)	181(62.8%)	7(2.4%)	79(27.4%)	

Table Continued

	Nuclear				
	Low PKA and low Cdk5	**High PKA and low Cdk5**	**Low PKA and high Cdk5**	**High PKA and high Cdk5**	***P* value**
Tumour type					
Ductal	42(5.9%)	422(59.6%)	18(2.5%)	226(31.9%)	
Lobular	10(16.7%)	26(43.3%)	4(6.7%)	20(33.3%)	
Medullary-like	0(0.0%)	5(62.5%)	0(0.0%)	3(37.5%)	0.054
Miscellaneous	0(0.0%)	5(83.3%)	0(0.0%)	1(16.7%)	
Mixed &lobular	0(0.0%)	1(100.0%)	0(0.0%)	0(0.0%)	
Special type	6(20.0%)	13(43.3%)	1(3.3%)	10(33.3%)	
Tubular	0(0.0%)	0(0.0%)	0(0.0%)	1(100.0%)	
Vascular invasion					
Definite	20(8.4%)	144(60.3%)	8(3.3%)	67(28.0%)	0.385
No/Probable	38(6.6%)	329(57.1%)	15(2.6%)	194(33.7%)	
Stage					
1	41(7.8%)	297(56.8%)	12(2.3%)	173(33.1%)	0.434
2	11(5.3%)	125(60.4%)	6(2.9%)	65(31.4%)	
3	6(7.1%)	50(59.5%)	5(6.0%)	23(27.4%)	
NPI					
Good	17(6.7%)	136(53.8%)	9(3.6%)	91(36.0%)	**0.042**
Medium	34(8.1%)	248(59.0%)	6(1.4%)	132(31.4%)	
Poor	7(5.0%)	88(62.9%)	8(5.7%)	37(26.4%)	
ER Status					
Negative	11(6.3%)	120(68.2%)	3(1.7%)	42(23.9%)	**0.020**
Positive	47(7.3%)	354(55.3%)	20(3.1%)	219(34.2%)	
PR Status					
Negative	28(8.2%)	200(58.7%)	9(2.6%)	104(30.5%)	0.859
Positive	28(6.7%)	246(58.6%)	12(2.9%)	134(31.9%)	
HER2 Status					
Negative	48(7.0%)	402(59.0%)	19(2.8%)	212(31.1%)	0.513
Positive	4(5.1%)	42(53.2%)	2(2.5%)	31(39.2%)	
Triple Negative					
Negative	49(7.2%)	378(55.8%)	20(3.0%)	230(34.0%)	**0.029**
Positive	8(6.7%)	84(70.0%)	2(1.7%)	26(21.7%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.05.

The combination of low expression of PP1 and low cytoplasmic expression of DARPP-32 was significantly associated with tumour grade (χ^2=19.392, d.f.=6, *P*=0.004), tubule formation (χ^2= 33.405, d.f.=6, *P*<0.001), pleomorphism (χ^2=17.733, d.f.=6, *P*= 0.007), mitosis (χ^2=16.641, d.f.= 6, *P*=0.011), tumour type (χ^2= 32.409, d.f.=18, *P*=0.020), vascular invasion (χ^2=10.065, d.f.=3, *P*=0.018), tumour stage (χ^2=18.126, d.f.=6, *P*=0.006), poor NPI (χ^2=17.230, d.f.=6, *P*=0.008), ER positive tumours (χ^2=39.663, d.f.=3, *P*<0.001), PR positive tumours status (χ^2=16.063, d.f.=3, *P*=0.001), and triple receptor negative tumours (χ^2=29.259, d.f.=3, *P*<0.001). Association between various clinicopathological parameters with combined cytoplasmic PP1 and DARPP-32 level of expression are demonstrated in Appendix A-Table A1.

The combination of low expression of PP1 and low nuclear DARPP-32 expression was significantly associated with the tumour grade (χ^2=16.924, d.f.=6, *P*=0.010), tubule formation (χ^2=24.736, d.f.=6, *P*<0.001), mitosis (χ^2=17.020, d.f.=6, *P*=0.009), tumour size (χ^2=32.612, d.f.=18, *P*=0.019), vascular invasion (χ^2=12.191, d.f. =3, *P*=0.007), stage (χ^2=19.315, d.f.=6, *P*=0.004), poor NPI (χ^2=19.069, d.f.=6, *P*=0.004), ER positive tumours (χ^2=41.473, d.f.=3, *P*<0.001), PR positive tumours (χ^2=18.135, d.f.=3, *P*<0.001), and triple receptor negative tumours (χ^2=31.342, d.f.=3, *P*<0.001). Association between various clinicopathological parameters with combined nuclear PP1 and DARPP-32 level of expression are demonstrated in Appendix A-Table A2.

The combination of low expression of PP1 and low cytoplamic Cdk5 expression was significantly associated with the presence of mitosis (χ^2=15.423, d.f.=6*,* *P*=0.017), poor NPI (χ^2=13.877, d.f.=6, *P*=0.031), and PR negative tumours (χ^2=10.670, d.f.=3, *P*=0.014). Association between various clinicopathological parameters with combined cytoplasmic PP1 and Cdk5 level of expression are demonstrated in Appendix A-Table A3.

The combination of low expression of PP1 and low nuclear Cdk5 expression was significantly associated with the development of larger tumour size (χ^2=8.751 d.f.=3, *P*=0.033), mitosis (χ^2= 20.599, d.f.=6, *P*=0.002), moderate NP1 (χ^2=13.581, d.f.=6, *P*=0.035), ER negative tumours (χ^2=10.869, d.f.=3, *P*=0.012),PR negative tumours (χ^2=10.283, d.f.=3, *P*=0.016), and triple receptor positive tumours (χ^2=11.331, d.f.=3, *P*=0.010). Association between various clinicopathological parameters with combined nuclear PP1 and Cdk5 level of expression are demonstrated in Appendix A-Table A4.

The combination low levels of PKA and cytoplamic low DARPP-32 expression was significantly associated with the development of tubule formation (χ^2=13.362, d.f.=6, *P*=0.038), tumour type (χ^2=37.912, d.f.=18, *P*=0.004), vascular invasion (χ^2=10.064, d.f.=3, *P*=0.018), ER positive tumours (χ^2=43.521, d.f.=3, *P*<0.001), PR positive tumours (χ^2=23.762, d.f.=3, *P*<0.001), and triple receptor negative tumours (χ^2=35.135 d.f.=3, *P*<0.001). Association between various clinicopathological parameters with combined cytoplasmic PKA and DARPP-32 level of expression are demonstrated in Appendix A-Table A5.

The combination low levels of PKA and nuclear low DARPP-32 expression was significantly associated with the development of tubule formation (χ^2=16.375, d.f.=6, *P*=0.012), tumour type (χ^2=38.928, d.f.=18, *P*=0.003), vascular invasion (χ^2=12.059, d.f.=3, *P*=0.007), stage (χ^2=14.231, d.f.=6, *P*=0.027), poor NP1 (χ^2=13.901, d.f.=6, *P*=0.031), ER positive tumours (χ^2=42.946, d.f.=3, *P*<0.001), PR positive tumours (χ^2=16.586, d.f.=3, *P*<0.001), and triple receptor negative tumours (χ^2=31.261, d.f.=3, *P*<0.001). Association between various clinicopathological parameters with combined nuclear PKA and DARPP-32 level of expression are demonstrated in Appendix A-Table A6.

3.2.6 Combination expression and patient survival

The association between combinations of cytoplasmic and nuclear expression of PKA, PP1, Cdk5 and DARPP-32 and breast cancer specific survival were investigated. Combined expression patterns of PKA and cytoplasmic DARPP-32 were not significantly associated with breast cancer specific survival (*P*=0.139) (Figure 3.5 A). In contrast, the combination of low PKA and low Cdk5 expression (Figure 3.5 B), low PP1 and low DARPP-32 (Figure 3.5 C), and low PP1 and low Cdk5 (Figure 3.5 D) were significantly associated with adverse breast cancer specific survival (*P*=0.006, *P*=0.001 and *P*=0.010, respectively).

The combination of low PKA and low Cdk5 expression (*P* =0.049; Figure 3.6 B), low PP1 and low DARPP-32 (*P*=0.007; Figure 3.6 C) and combination of low PP1 and low Cdk5 (*P*=0.010; Figure 3.6 D) were associated with adverse breast cancer specific survival. The combined expression of PKA and nuclear DARPP-32 was not associated with breast cancer specific survival (*P*=0.072) (Figure 3.6 A).

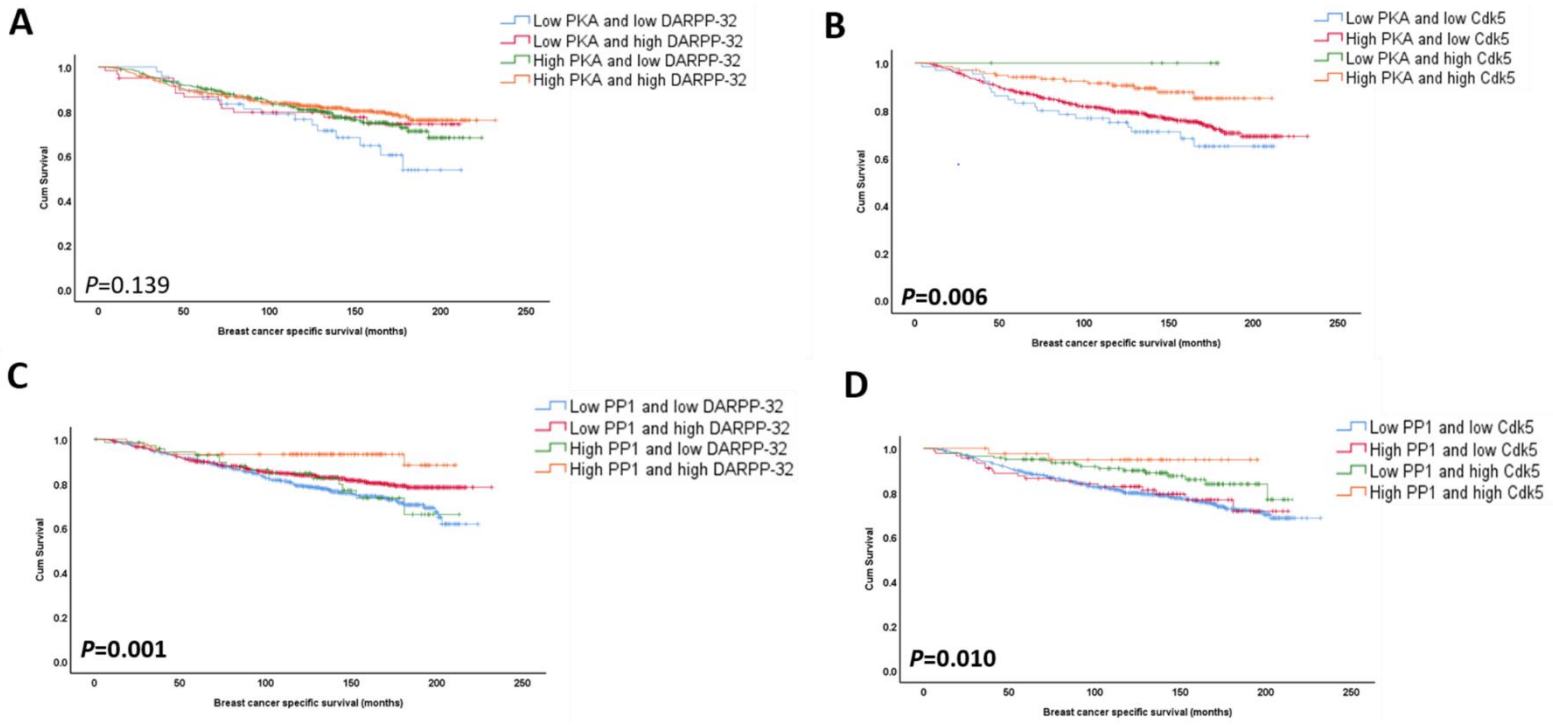


Figure 3.5: Kaplan-Meier analysis and the influence of different combinations of protein expression on survival. High expression of PKA combined with high and low cytoplasmic expression of DARPP-32 shown in orange and in green, respectively, and low levels of PKA combined with high and low cytoplasmic expression of DARPP-32 shown in pink and in blue, respectively, are illustrated in Figure 3.5 A. High expression of PKA combined with high and low cytoplasmic expression of Cdk5 shown in orange and in pink, respectively, and low levels of PKA combined with high and low cytoplasmic expression of Cdk5 shown in green and in blue, respectively, are illustrated in Figure 3.5 B. High expression of PP1 combined with high and low cytoplasmic expression of DARPP-32 shown in orange and in green, respectively, and low Levels of PP1 combined with high and low cytoplasmic expression of DARPP-32 shown in pink and in blue, respectively, are illustrated in Figure 3.5 C. High expression of PP1 combined with high and low cytoplasmic expression of Cdk5 shown in orange and in Pink, respectively, and low levels of PP1 combined with high and low cytoplasmic expression of Cdk5 shown in green and in blue, respectively, are illustrated in Figure 3.5 D.

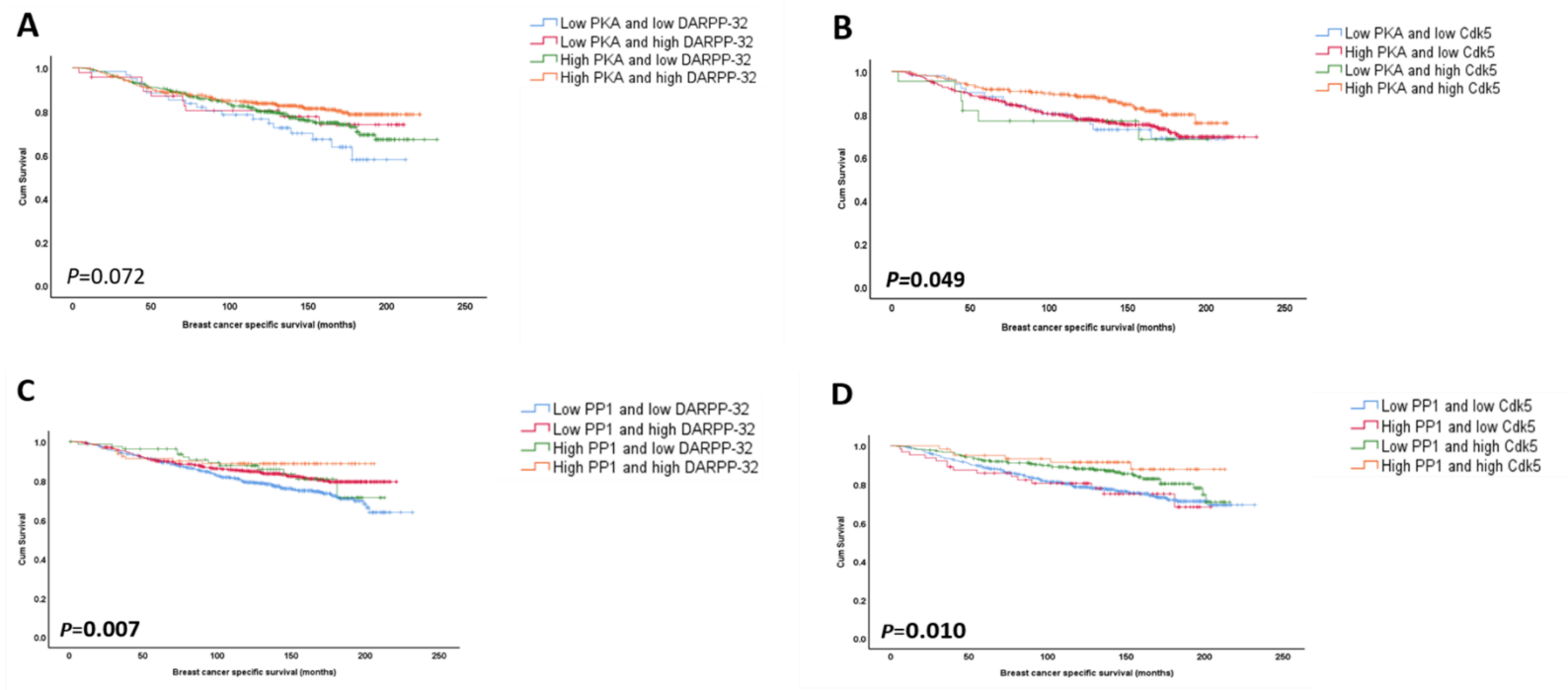


Figure 3.6: Kaplan-Meier analysis and the influence of different combinations of protein expression on survival. High expression of PKA combined with high and low nuclear expression of DARPP-32 shown in orange and in green, respectively, and low Levels of PKA combined with high and low nuclear expression of DARPP-32 shown in pink and in blue, respectively, are illustrated in Figure 3.6 A. High expression of PKA combined with high and low nuclear expression of Cdk5 shown in orange and in pink, respectively, and low Levels of PKA combined with high and low nuclear expression of Cdk5 shown in green and in blue, respectively, are illustrated in Figure 3.6 B. High expression of PP1 combined with high and low nuclear expression of DARPP-32 shown in orange and in green, respectively, and low Levels of PP1 combined with high and low nuclear expression of DARPP-32 shown in pink and in blue, respectively, are illustrated in Figure 3.6 C. High expression of PP1 combined with high and low nuclear expression of Cdk5 shown in orange and in Pink, respectively, and low levels of PP1 combined with high and low nuclear expression of Cdk5 shown in green and in blue, respectively, are illustrated in Figure 3.6 D.

3.2.7 mRNA expression level in the METABRIC cohort

Association of PKA/PP1 subunit and Cdk5 mRNA expression levels and patient survival in the METABRIC cohort were assessed. The probe level information and the association between the expression of genes encoding various subunits of PKA/PP1 and patient survival within the METABRIC cohort are illustrated in Table 3.5 The probes that were associated with patient survival included all of those corresponding to *PRKACG (ILMN-1808368), PRKAR1B (ILMN-1674390)* and *PPP1CA (ILMN-1695827 and ILMN-2377980*) genes, one out of the five *PRKACB (ILMN-1690314)* probes, one out of the three *PRKAR1A (ILMN-2277077)* probes, and one of two probes for *PPP1CB (ILMN-2405018).*

Table 3.5: Probes corresponding to genes encoding various subunits of PKA and PP1 are presented below, and *P* value, hazard ratio and the 95% confidence interval associated with Cox regression analysis (proportional hazards) against survival for each gene are tabulated. *P* values that were considered 'significant' are highlighted in bold.

Gene	Probe	*P* value	Hazard ratio	95% Confidence interval
PRKACA	ILMN-1786195	0.685	0.892	0.513-1.552
	ILMN-2395314	0.171	1.411	0.862-2.311
PRKACB	ILMN-1690314	**0.021**	**0.451**	**0.230-0.885**
	ILMN-1759922	0.189	1.576	0.800-3.103
	ILMN-1771523	0.434	0.874	0.624-1.224
	ILMN-2278112	0.546	0.871	0.556-1.364
	ILMN-2374234	0.161	0.924	0.827-1.032
PRKACG	ILMN-1808368	**0.045**	**1.616**	**1.011-2.582**
PRKAR1A	ILMN-1738632	0.065	0.898	0.801-1.007
	ILMN-2277077	**0.003**	**0.395**	**0.215-0.724**
	ILMN-2389590	0.146	0.953	0.893-1.017
PRKAR1B	ILMN-1674390	**0.001**	**1.612**	**1.205-2.156**
PRKAR2A	ILMN-1681888	0.347	0.863	0.635-1.173
PRKAR2B	ILMN-1684850	0.465	0.797	0.433-1.467
PPP1CA	ILMN-1695827	**0.017**	**1.215**	**1.035-1.426**
	ILMN-2377980	**>0.001**	**1.732**	**1.416-2.118**
PPP1CB	ILMN-2405018	**0.003**	**0.772**	**0.651-0.916**

Table continued

Gene	Probe	*P* value	Hazard ratio	95% Confidence interval
	ILMN-2405023	0.208	0.779	0.527-1.150
PPP1CC	ILMN-1701855	0.605	0.977	0.758-1.175

Note. *P* values highlighted in bold are <0.05.

3.2.7.1 *PRKAR1A, PRKAR1B, PRKAR2A* and *PRKAR2B* mRNA expression and clinicopathological variables

Expression of *PRKAR1A, PRKAR1B, PRKAR2A and PRKAR2B* mRNA were assessed for associations with available clinicopathological variables. Low expression of *PRKAR1A* was significantly associated with Genefu (an R/Bioconductor software for computation analysis of gene expression in breast cancer) ER+/HER2 low prolif (χ^2=10.495, d.f.=4, *P*=0.033), ER positive tumours (χ^2=19.100, d.f.=1, *P*<0.001), PR negative tumours (χ^2=19.558, d.f.=1, *P*<0.001), and luminal A Prediction Analysis of Microarray 50 (PAM50), an assay used for the analysis of 50 genes in tumour samples, subtype (χ^2=29.193, d.f.=5, *P*<0.001). Low expression of *PRKAR1B* was significantly associated with tumour grade (χ^2=81.822, d.f.=2, *P*<0.001), Genfu ER+/HER2 low prolif (χ^2=53.181, d.f.=4, *P*<0.001), ER positive tumour (χ^2=92.900, d.f.=1, *P*<0.001), PR positive tumours (χ^2=66.119, d.f.=1, *P*<0.001), and luminal A PAM50 subtype, (χ^2=134.885, d.f=5, *P*<0.001). Low expression of *PRKAR2A* was significantly associated with grade (χ^2=13.068, d.f.=2, *P*=0.001), ER positive tumours (χ^2=4.265, d.f.=1, *P*=0.039), PR negative tumours (χ^2=10.732 d.f=1, *P*=0.001), and luminal A PAM50 subtype (χ^2=40.552 d.f.=5, *P*<0.001). Low expression of *PRKAR2B* was significantly associated with tumour grade (χ^2=16.978, d.f.=2, *P*<0.001), Genefu ER+/HER2 low prolif (χ2=29.372 d.f.=4, *P*<0.001), ER positive tumours (χ^2=24.634 d.f.=1, *P*<0.001), HER2 negative tumours (χ^2=7.108 d.f.=1, *P*=0.008), PR negative tumours (χ^2=13.876 d.f.=1, *P*<0.001), and luminal A PAM50 subtype (χ^2=38.414, d.f.=5, *P*<0.001) (Table 3.6).

Table 3.6: The correlation between the level of mRNA expression of PRKAR1A, PRKAR1B, PRKAR2A or PRKAR2B, and various clinicopathological parameters (acquired from the METABRIC Cohort). P values <0.05 are highlighted in bold.

	PRKAR1A **mRNA expression**			***PRKAR1B*** **mRNA expression**			***PRKAR2A*** **mRNA expression**			***PRKAR2B*** **mRNA expression**		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**	**Low**	**High**	***P* value**	**Low**	**High**	***P* value**
Grade												
1	36 (1.9%)	134 (7.1%)	0.094	145 (7.7%)	25 (1.3%)	**<0.001**	61 (3.2%)	109 (5.8%)	**0.001**	93 (4.9%)	77 (4.1%)	**<0.001**
2	170(9.0%)	600 (31.7%)		567 (30.0%)	203(10.7%)		285(15.1%)	485 (25.6%)		378(20.0%)	392(20.7%)	
3	249(13.2%)	703 (37.2%)		544 (28.8%)	408(21.6%)		428(22.6%)	524 (27.7%)		562(29.7%)	390(20.6%)	
Genfu												
ER-/HER2-	45(2.3%)	106 (5.4%)	**0.033**	76 (3.8%)	75 (3.8%)	**<0.001**	68 (61.1%)	83(4.2%)	0.072	102(5.2%)	49(2.5%)	**<0.001**
ER+/HER2-High Prolif	69 (3.5%)	299 (15.1%)		228 (11.5%)	140(7.1%)		129(6.5%)	239 (12.1%)		164(8.3%)	204(10.3%)	
ER+/HER2-Low Prolif	88 (4.4%)	280 (14.1%)		295(14.9%)	73 (3.7%)		140 (7.1%)	228(11.5%)		196(9.9%)	172(8.7%)	
HER2+	32 (1.6%)	78 (3.9%)		68 (3.4%)	42 (2.1%)		45(2.3%)	65 (3.3%)		70(3.5%)	40 (2.0%)	
ER status												
Negative	151 (7.6%)	323 (16.3%)	**<0.001**	230(11.6%)	244(12.3%)	**<0.001**	211(10.7%)	263 (13.3%)	**0.039**	305(15.4%)	169 (8.5%)	**<0.001**
Positive	331(16.7%)	1175(59.3%)		1091(55.1%)	415(21.0%)		590(29.8%)	916 (46.3%)		773(39.0%)	733(37.0%)	
HER2status												
Negative	416(21.0%)	1317(66.5%)	0.352	1165(58.8%)	568(28.7%)	0.204	694(35.1%)	1039(52.5%)	0.327	924(46.7%)	809(40.9%)	**0.008**
Positive	66 (3.3%)	181(9.1%)		156 (7.9%)	91(4.6%)		107 (5.4%)	140(7.1%)		154 (7.8%)	93(4.7%)	

Table Continued

	PRKAR1A **mRNA expression**			***PRKAR1B*** **mRNA expression**			***PRKAR2A*** **mRNA expression**			***PRKAR2B*** **mRNA expression**		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**	**Low**	**High**	***P* value**	**Low**	**High**	***P* value**
PR status												
Negative	271(13.7%)	669 (33.8%)	**<0.001**	542 (27.4%)	398(20.1%)	**<0.001**	416(21.0%)	524(26.5%)	**0.001**	553(27.9%)	387(19.5%)	**<0.001**
Positive	211(10.7%)	829 (41.9%)		779 (39.3%)	261(13.2%)		385(19.4%)	655 (33.1%)		525(26.5%)	515(26.0%)	
PAM50subtype												
Basal	111(5.6%)	218 (11.0%)	**<0.001**	140(7.1%)	189 (9.5%)	**<0.001**	164(8.3%)	165(8.3%)	**<0.001**	207(10.5%)	122(6.2%)	**<0.001**
HER2	67(3.4%)	173 (8.7%)		152 (7.7%)	88 (4.4%)		112(5.7%)	128(6.5%)		154(7.8%)	86(4.3%)	
Luminal A	158(8.0%)	560 (28.3%)		565 (28.5%)	153(7.7%)		243(12.3%)	475 (24.0%)		365(18.4%)	353(17.8%)	
Luminal B	91 (4.6%)	397(20.1%)		327 (16.5%)	161 (8.1%)		181(9.1%)	307 (15.5%)		227(11.5%)	261(13.2%)	
Normal	54 (2.7%)	145 (7.3%)		132 (6.7%)	67 (3.4%)		100(5.1%)	99(5.0%)		121(6.1%)	78(3.9%)	
NC	1(0.1%)	5(0.3%)		5(0.3%)	1(0.1%)		1(0.1%)	5(0.3%)		4(0.2%)	2(0.1%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.05

3.2.7.2 Associations between *PRKAR1A, PRKAR1B, PRKAR2A* and *PRKAR2B* mRNA expression and breast cancer survival in the METABRIC cohort

Kaplan-Meier analysis was used to assess the impact of *PRKAR1A, PRKAR1B, PRKAR2A* and *PRKAR2B* mRNA expression on breast cancer specific survival. Low expression of both *PRKAR1A* and *PRKAR2A* were significantly associated with adverse breast cancer specific survival (P=0.014 and P=0.017, respectively) (Figure 3.7A and C, respectively), low expression of *PRKAR1B* was significantly associated with improved breast cancer specific survival (P<0.001, Figure 3.7B), and expression of *PRKAR2B* was not significantly associated with breast cancer survival (P=0.076, Figure 3.7D).

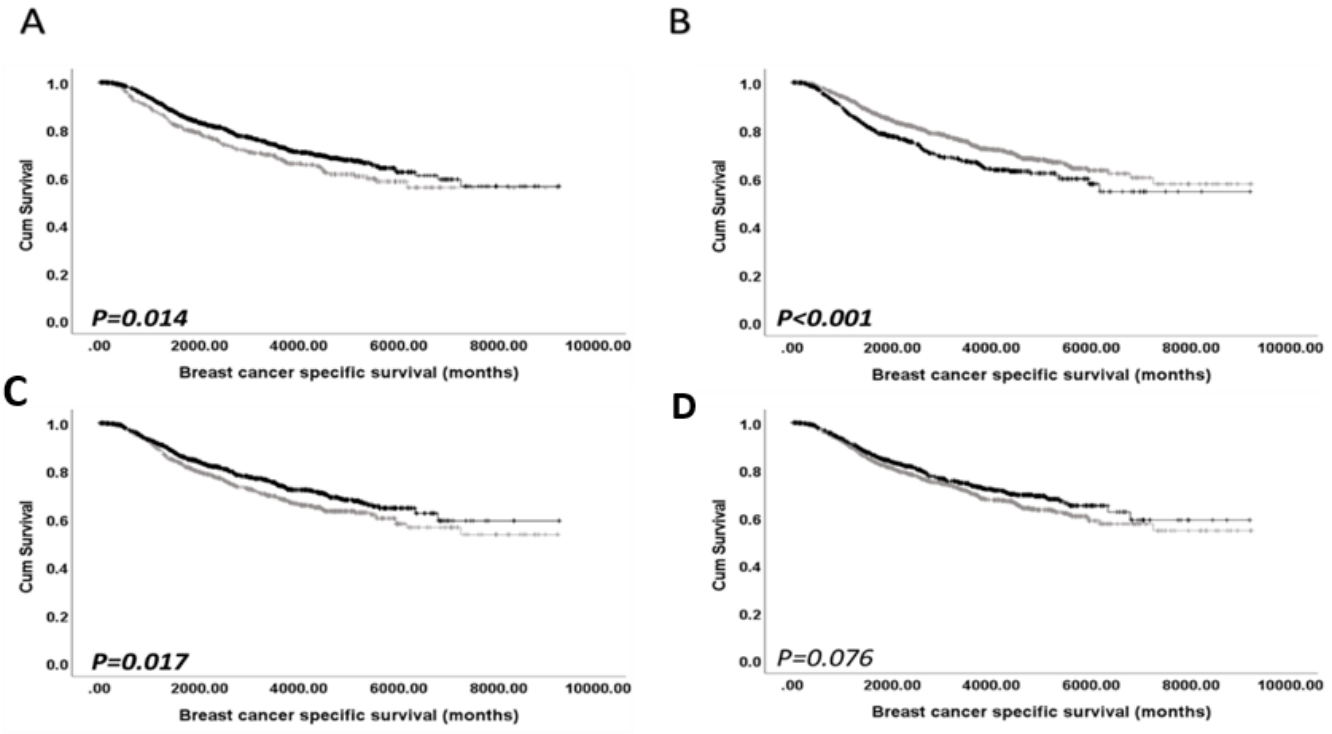


Figure 3.7: Kaplan-Meier analysis where the grey lines denote low expression and the black lines denote high expression. Low expression of PRKAR1A (Figure 3.7A) and PRKAR2A (Figure 3.7C) were significantly associated with adverse survival (P=0.014 and P=0.017, respectively). Low expression of PRKAR1B was significantly associated with improved breast cancer specific survival (P<0.001, Figure 3.7B). There was no significant association between breast cancer survival and PRKAR2B expression (P=0.076, Figure 3.7 D).

3.2.7.3 Associations between *PRKACA, PRKACB,* and *PRKACG* mRNA expression and clinicopathological variables in the METABRIC Cohort

Expression of *PRKACA, PRKACB,* and *PRKACG* were assessed for associations with available clinicopathological data (Table 3.7). Low expression of *PRKACA* was significantly associated with high tumour grade (χ^2=7.098, d.f.=2, *P*=0.029), ER positive tumours (χ^2=9.726, d.f.=1, *P*=0.002), PR positive tumours (χ^2=5.971, d.f.=1, *P*=0.015), and luminal A PAM50 subtype (χ^2=22.485, d.f.=5, *P*<0.001). Low expression of *PRKACB* was significantly associated with Genfu ER+/HER2-high prolif (χ^2=15464, d.f.=4, *P*=0.004), and luminal A PAM50 subtype (χ^2=14.402, d.f.=5, *P*=0.013). Low expression of *PRKACG* was significantly associated with HER2 negative tumours (χ^2=5.652, d.f.=1, *P*=0.017), and luminal A PAM50 subtype (χ^2=19.724, d.f.=5, *P*=0.001) no association with other clinicopathological parameters such as ER and PR status were observed.

Table 3.7: *Association between various clinicopathological parameters of PRKACA, PRKACB, and PRKACG mRNA expression in the METABRIC cohort. The P values highlighted in bold are <0.05.*

	***PRKACA* mRNA expression**			***PRKACB* mRNA expression**			***PRKACG* mRNA expression**		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**	**Low**	**High**	***P* value**
Grade									
1	159(8.4%)	11(0.6%)	**0.029**	10 (0.5%)	160 (8.5%)	0.117	119(6.3%)	51 (2.7%)	0.088
2	696(36.7%)	76(4.0%)		76 (4.0%)	694(36.7%)		471(24.9%)	299(15.8%)	
3	832(44.0%)	120(6.3%)		105(5.5%)	847(44.8%)		587(31.0%)	365(19.3%)	
Genfu									
ER-/HER2-	135(6.8%)	16 (0.8%)	0.336	9 (0.5%)	142 (7.2%)	**0.004**	93 (4.7%)	58 (2.9%)	0.579
ER+/HER2-High Prolif	328 (16.6%)	40 (2.0%)		43(2.2%)	325(16.4%)		234(11.8%)	134 (6.8%)	
ER+/HER2-Low Prolif	337(17.0%)	31 (1.6%)		25 (1.3%)	343(17.3%)		235(11.9%)	133(6.7%)	
HER2+	94 (4.7%)	16 (0.8%)		6 (0.3%)	104(5.3%)		61(3.1%)	49 (2.5%)	
ER status									
Negative	403(20.4%)	71 (3.6%)	**0.002**	47(2.4%)	427 (21.6%)	0.782	296 (14.9%)	178 (9.0%)	0.949
Positive	1358(68.6%)	148(7.5%)		156(7.9%)	1350(68.2%)		938(47.4%)	568(28.7%)	
HER2 status									
Negative	1549(78.2%)	184(9.3%)	0.096	180(9.1%)	1553(78.4%)	0.602	1097(55.4%)	636(32.1%)	**0.017**
Positive	212 (10.7%)	35(1.8%)		23 (1.2%)	224(11.3%)		137 (6.9%)	110(5.6%)	
PR status									
Negative	819 (41.4%)	121(6.1%)	**0.015**	90 (4.5%)	850 (42.9%)	0.344	579 (29.2%)	361(18.2%)	0.525
Positive	942 (47.6%)	98 (4.9%)		113(5.7%)	927 (46.8%)		655 (33.1%)	385(19.4%)	
PAM50 subtype									
Basal	288 (14.5%)	41(2.1%)	**<0.001**	37(1.9%)	292 (14.7%)	**0.013**	207 (10.5%)	122(6.2%)	**0.001**
HER2	194 (9.8%)	46(2.3%)		37(1.9%)	203 (10.3%)		127 (6.4%)	113(5.7%)	

Table Continued

	PRKACA **mRNA expression**			***PRKACB*** **mRNA expression**			***PRKACG*** **mRNA expression**		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**	**Low**	**High**	***P* value**
Luminal A	647 (32.7%)	71 (3.6%)		68 (3.4%)	650 (32.8%)		462 (23.3%)	256(12.9%)	
Luminal B	442 (22.3%)	46 (2.3%)		51(2.6%)	437 (22.1%)		292 (14.7%)	196(9.9%)	
Normal	185 (9.3%)	14 (0.7%)		10 (0.5%)	189 (9.5%)		143 (7.2%)	56(2.8%)	
NC	5(0.3%)	1(0.1%)		0(0.0%)	6(0.3%)		3(0.2%)	3(0.2%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.05

3.2.7.4 Associations between *PRKACA, PRKACB*, and *PRKACG* mRNA expression and breast cancer survival in the METABRIC cohort

Kaplan Meier analysis was used to assess the impact of *PRKACA, PRKACB*, and *PRKACG* mRNA expression on breast cancer specific survival. Low expression of *PRKACA* and *PRKACG* were significantly associated with improved breast cancer specific survival (P=0.016 and P=0.005, respectively, Figure 3.8 A and C), and low expression of *PRKACB* was significantly associated with adverse breast cancer specific survival (P=0.039, Figure 3.8 B).

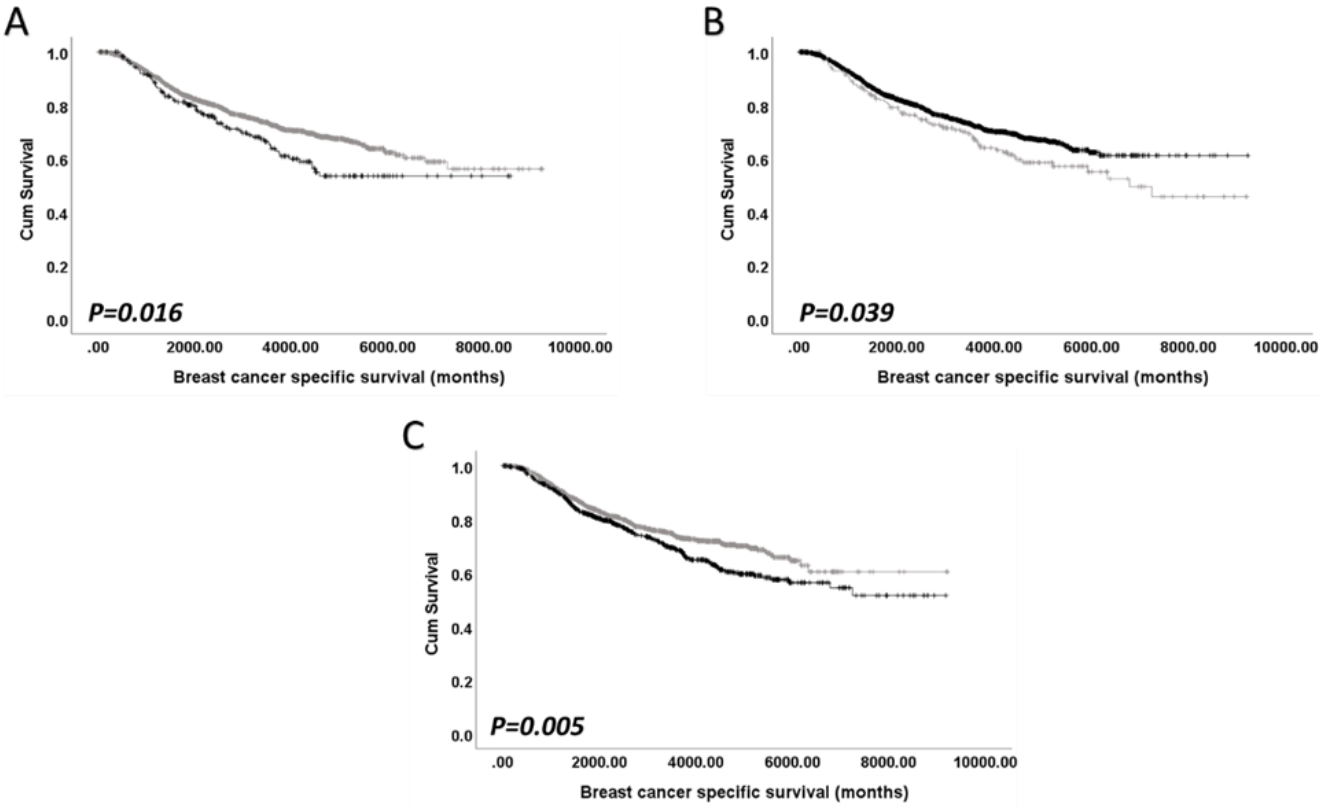


Figure 3.8: Kaplan-Meier analysis where the grey line denotes low expression and the black line denotes high expression. Low expression of *PRKACA* and *PRKACG* were significantly associated with improved breast cancer survival (*P*=0.016 and *P*=0.005 respectively, Figure 3.8A and C) and low expression of *PRKACB* was significantly associated with adverse breast cancer survival (*P*=0.039, Figure 3.8B).

3.2.7.5 Associations between *PPP1CA, PPP1CB* and *PPP1CC* mRNA expression and patient survival in the METABRIC cohort

Expression of mRNA that corresponds to PP1 catalytic subunit (*PPP1CA, PPP1CB* or *PPP1CC*) was assessed for associations with available clinicopathological data to understand links with components of the PP1 enzyme (Table 3.8). Low expression of *PPP1CA* was significantly associated with higher tumour grade (χ^2=32.075, d.f.=2, *P*<0.001), Genfu ER+/HER2 low prolif (χ^2=66.211, d.f.=4, *P*<0.001), PR positive tumours (χ2=5.509, d.f.=1, *P*=0.019), and luminal A PAM50 subtype (χ^2=94.025, d.f.=5, *P*<0.001).

Low expression of *PPP1CB* was significantly associated with higher tumour grade (χ^2=16.099, d.f.=2, *P*<0.001), Genfu ER+/HER2 high prolif (χ^2=25.574, d.f.=4, *P*<0.001), ER positive tumours (χ^2=40.318, d.f.=1, *P*<0.001), PR positive tumours (χ^2=22.394, d.f.=1, *P*<0.001), and luminal A PAM50 subtype (χ^2=67.918, d.f.=5, *P*<0.001).

Low expression of *PPP1CC* was significantly associated with higher tumour grade (χ^2=9.269, d.f.=2, *P*=0.01), Genfu ER+/HER2 low prolif (χ^2=18.817, d.f.=4, *P*<0.001), and luminal A PAM50 subtype HER2 (χ^2=25.508, d.f.5, *P*<0.001); no other significant association with other clinicopathological parameters such as ER and PR status were observed.

Table 3.8*:* Association between various clinicopathological variables and *PPP1CA*, *PPP1CB* and *PPP1CC* mRNA expression in the METABRIC Cohort. The *P* values highlighted in bold are <0.05.

	***PPP1CA* mRNA expression**			***PPP1CB* mRNA expression**			***PPP1CC* mRNA expression**		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**	**Low**	**High**	***P* value**
Grade									
1	148(7.8%)	22 (1.2%)	**<0.001**	60 (3.2%)	110 (5.8%)	**<0.001**	68 (3.6%)	102 (5.4%)	**0.01**
2	625 (33.0%)	145 (7.7%)		391 (20.7%)	379 (20.0%)		375 (19.8%)	395 (20.9%)	
3	683 (36.1%)	269 (14.2%)		493 (26.1%)	459 (24.3%)		400 (21.1%)	552 (29.2%)	
Genfu									
ER-/HER2-	122 (6.2%)	29 (1.5%)	**<0.001**	53 (2.7%)	98 (4.9%)	**<0.001**	55 (2.8%)	96 (4.8%)	**<0.001**
ER+/HER2-High Prolif	230 (11.6%)	138 (7.0%)		215 (10.9%)	153 (7.7%)		134 (6.8%)	234 (11.8%)	
ER+/HER2-Low Prolif	318 (16.1%)	50 (2.5%)		172 (8.7%)	196 (9.9%)		173 (8.7%)	195(9.8%)	
HER2+	84 (4.2%)	26 (1.3%)		53 (2.7%)	57 (2.9%)		50(2.5%)	60 (3.0%)	
ER status									
Negative	367 (18.5%)	107 (5.4%)	0.951	176(8.9%)	298 (15.1%)	**<0.001**	199 (10.1%)	275 (13.9%)	0.207
Positive	1164 (58.8%)	342 (17.3%)		811 (41.0%)	695 (35.1%)		682 (34.4%)	824 (41.6%)	
HER2 status									
Negative	1348 (68.1%)	385 (19.4%)	0.194	854 (43.1%)	879 (44.4%)	0.179	772 (39.0%)	961 (48.5%)	0.902
Positive	183 (9.2%)	64(3.2%)		133 (6.7%)	114(5.8%)		109 (5.5%)	138(7.0%)	
PR status									
Negative	705 (35.6%)	235 (11.9%)	**0.019**	416 (21.0%)	524 (26.5%)	**<0.001**	424 (21.4%)	516 (26.1%)	0.603
Positive	826 (41.7%)	214 (10.8%)		571 (28.8%)	469 (23.7%)		457 (23.1%)	583 (29.4%)	
PAM50 subtype									

Table Continued

	***PPP1CA* mRNA expression**			***PPP1CB* mRNA expression**			***PPP1CC* mRNA expression**		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**	**Low**	**High**	***P* value**
Basal	245 (12.4%)	84 (4.2%)	**<0.001**	125 (6.3%)	204 (10.3%)	**<0.001**	128 (6.5%)	201 (10.2%)	**<0.001**
HER2	185 (9.3%)	55 (2.8%)		139 (7.0%)	101 (5.1%)		125 (6.3%)	115 (5.8%)	
Luminal A	616 (31.1%)	102 (5.2%)		366 (18.5%)	352 (17.8%)		352 (17.8%)	366 (18.5%)	
Luminal B	309 (15.6%)	179 (9.0%)		290 (14.6%)	198 (10.0%)		183 (9.2%)	305 (15.4%)	
Normal	171 (8.6%)	28 (1.4%)		64 (3.2%)	135 (6.8%)		90 (4.5%)	109(5.5%)	
NC	5(0.3%)	1(0.1%)		3(0.2%)	3(0.2%)		3(0.2%)	3(0.2%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.05

3.2.7.6 Associations between *PPP1CA, PPP1CB* and *PPP1CC* mRNA expression and patient survival in the METABRIC cohort

Kaplan Meier analysis was used to assess the impact of *PPP1CA*, *PPP1CB* and *PPP1CC* mRNA expression on breast cancer specific survival to understand the impact of mRNA expression of components of the PP1 enzyme. Low expression of *PPP1CA* was significantly associated with improved prognosis breast cancer specific survival ($P<0.001$, Figure 3.9A), and low expressions of *PPP1CB* and *PPP1CC* were significantly associated with adverse breast cancer specific survival ($P<0.001$ and $P=0.138$, respectively, Figure 3.9 B and C).

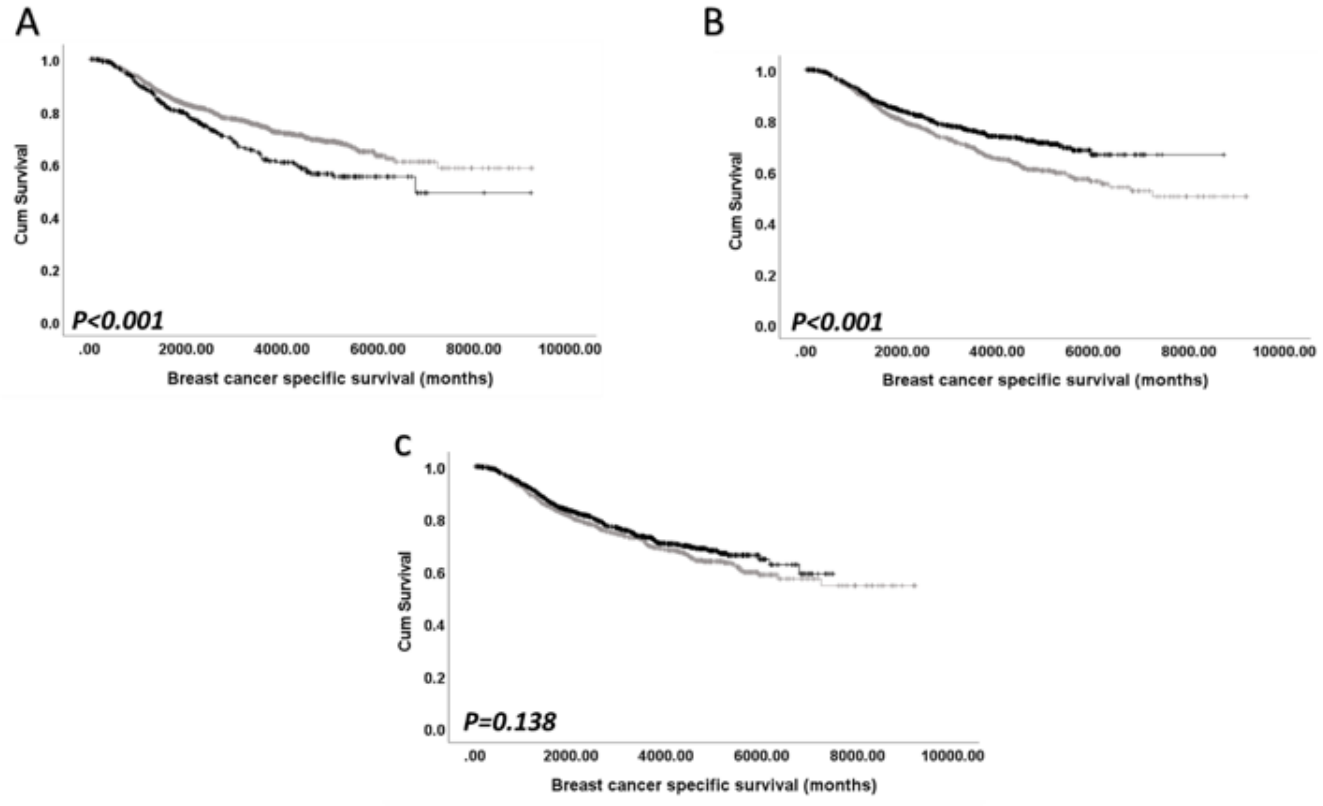


Figure 3.9: Kaplan-Meier analysis where the grey line denotes low expression and the black line denotes high expression. Low expression of PPP1CA was significantly associated with improved breast cancer specific survival (*P*<0.001 Figure 3.9A), low expressions of PPP1CB, PPP1CC were significantly associated with adverse breast cancer specific survival (*P*<0.001 and *P*=0.138, respectively, Figure 3.9B and C).

3.2.7.7 Cdk5 mRNA expression and clinicopathological variables in the METABRIC cohort

In order to investigate the relation between the expression of Cdk5 and clinicopathological implications in breast cancer, data from the METABRIC patient cohort was analysed (Table 3.9). high Cdk5 mRNA expression was significantly associated with higher tumour grade (χ^2=12.898, d.f.=2, *P*=0.002), luminal B PAM50 subtype (χ^2=88.278, d.f.=5, *P*<0.001), ER positive tumours (χ2=8.588, d.f.=1, *P*=0.003), and HER2 negative tumours (χ^2=9.922, d.f.=1, *P*=0.002).

Table 3.9: Association various clinicopathological parameters of Cdk5 mRNA expression in the METABRIC cohort. The *P* values highlighted in bold are <0.05.

	Cdk5 mRNA expression		
	Low	**High**	***P* value**
Grade			
1	141 (7.5%)	29 (1.5%)	**0.002**
2	548 (29.0%)	222 (11.7%)	
3	661 (34.9%)	291 (15.4%)	
Stage			
1	374 (25.7%)	127 (8.7%)	0.544
2	589 (40.5%)	236 (16.2%)	
3	89 (6.1%)	29 (2.0%)	
4	7 (0.5%)	3 (0.2%)	
ER status			
Negative	365 (18.4%)	109 (5.5%)	**0.003**
Positive	1055 (53.3%)	451 (22.8%)	
HER2 status			
Negative	1222 (61.7%)	511 (25.8%)	**0.002**
Positive	198 (10.0%)	49 (2.5%)	
PR status			
Negative	671 (33.9%)	269 (13.6%)	0.754
Positive	749 (37.8%)	291 (14.7%)	

Table Continued

	Cdk5 mRNA expression		
	Low	**High**	***P* value**
PAM50 subtype			
Basal	240 (12.1%)	89 (4.5%)	**<0.001**
HER2	185 (9.3%)	55 (2.8%)	
Luminal A	535 (27.0%)	183 (9.2%)	
Luminal B	278 (14.0%)	210 (10.6%)	
NC	5 (0.3%)	1 (0.1%)	
Normal	177 (8.9%)	22 (1.1%)	

Note. *P* values highlighted in bold are <0.05.

3.2.7.8 Cdk5 mRNA expression and patient survival breast cancer survival

Low Cdk5 mRNA expression was significantly associated with adverse breast cancer specific survival (*P*=0.005, Figure 3.10A), and high expression of Cdk5 mRNA was associated with adverse breast cancer specific survival in ER positive patients (*P*=0.027), a pattern that was absent in ER negative patients (*P*=0.231) (Figure 3.10B and C).

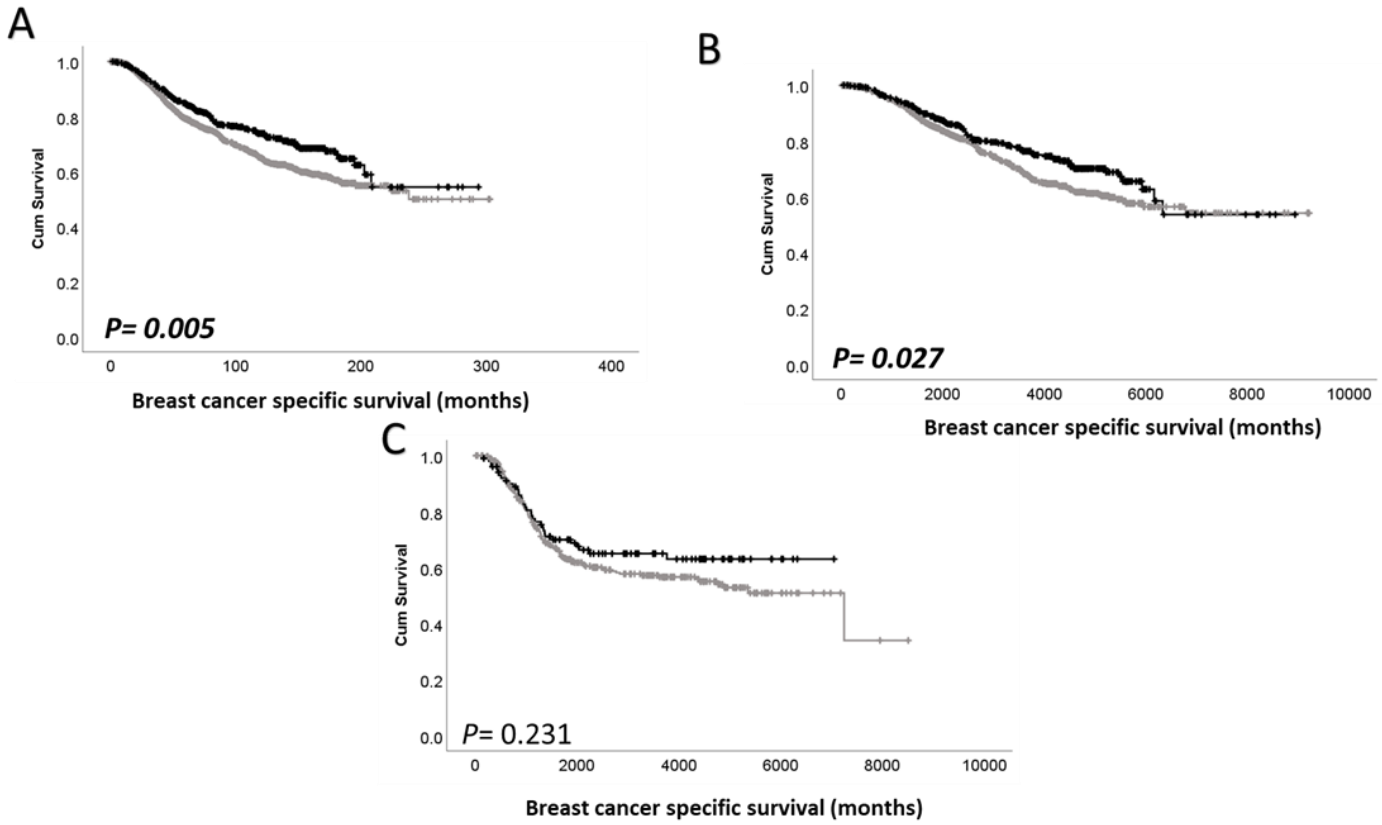


Figure 3.10: Kaplan-Meier analysis of the association between patient survival and Cdk5 mRNA expression level (A), patient survival and Cdk5 mRNA expression in ER positive disease (B) and ER negative disease (C) Low expression is denoted using grey lines, and high expression is denoted with black lines.

3.3.8 Cytotoxic effect of purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin on proliferation

Proliferation of MDA-MB-231, MCF7 and T47D breast cancer cells were assessed following treatment with purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin in a time-dependant (24, 48, and 72 hours) and dose-dependent (0, 1.5, 3 and 4.5 μM) manner. Cells were counted and dose response curves determined the IC_{50} for each interval period following exposure (Figure 3.11).

IC_{50} values associated with purvalanol B induced inhibition of proliferation were similar across all three breast cancer cell lines. The individual IC_{50} values in MDA-MB-231 vs T47D vs MCF7 cells were 3.07 ± 0.06 μM vs 3.02 ± 0.13 μM vs 3.05 ± 0.07 μM at 24 hours, 2.47± 0.09 μM vs 2.45 ± 0.06 μM vs 2.71 ± 0.08 μM at 48 hours, and 1.70± 0.02 μM vs 1.81 ± 0.07 μM vs 1.90 ± 0.05 μM at 72 hours, respectively (Figure 3.11A).

IC_{50} values of tautomycetin in MDA-MB-231 vs T47D vs MCF7 cell lines were 2.97 ± 0.27 μM vs 3.03 ± 0.10 μM vs 3.33 ± 0.42 μM at 24 hours, 2.54 ± 0.24 μM vs 2.48 ± 0.12 μM vs 2.8 ± 0.23 μM at 48 hours, and 1.84 ± 0.09 μM vs 1.99 ±. 0.32 μM vs 2.2 ± 0.17 μM at 72 hours, respectively (Figure 3.11B).

IC_{50} values associated with PKA inhibitor fragment (6-22) amide induced inhibition of proliferation were similar across all three breast cancer cell lines where the individual IC_{50} values in MDA-MB-231 vs T47D vs MCF7 cells were 3.45 ± 0.27 μM vs 3.67 ± 0.16 μM vs 3.63 ± 0.41 μM at 24 hours, 2.71 ± 0.39 μM vs 2.77 ± 0.17 μM vs 2.73 ± 0.09 μM at 48 hours, and 2.01 ± 0.13 μM vs 2.07 ± 0.13 μM vs 2.21 ± 0.03 μM at 72 hours respectively (Figure 3.11C).

forskolin induced 50% inhibition at similar concentration levels in in MDA-MB-231 vs T47D vs MCF7 cells with IC_{50} values of 3.15 ± 0.08 μM vs 3.95

± 0.37 μM vs 4.05 ± 0.20 μM at 24 hours, 2.48 ± 0.10 μM vs 3.22 ± 0.26 μM vs 3.26 ± 0.14 μM 48 hours, and 1.92 ± 0.03 μM vs 2.05 ± 0.18 μM vs 2.08 ± 0.15 μM at 72 hours respectively (Figure 3.11D). Results confirmed that MDA-MB-231 cells were significantly more sensitive to all drugs at 72 hours (P=0.008), than 24 or 48 hours, indicating that cytotoxicity in this cell line is time dependant. T47D cells were significantly sensitive to all four drugs at 24 and 48 hours (P=0.001 and P=0.002 respectively), but not after 72 hours of exposure. In contrast, MCF7 cells displayed a significant sensitivity to all drugs at all time points (P=0.002, P=0.005, P=0.045 respectively). The proliferation IC_{50} values of purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin in MDA-MB-231 T47D, and MCF7 breast cancer cells are shown in Table 3.10.

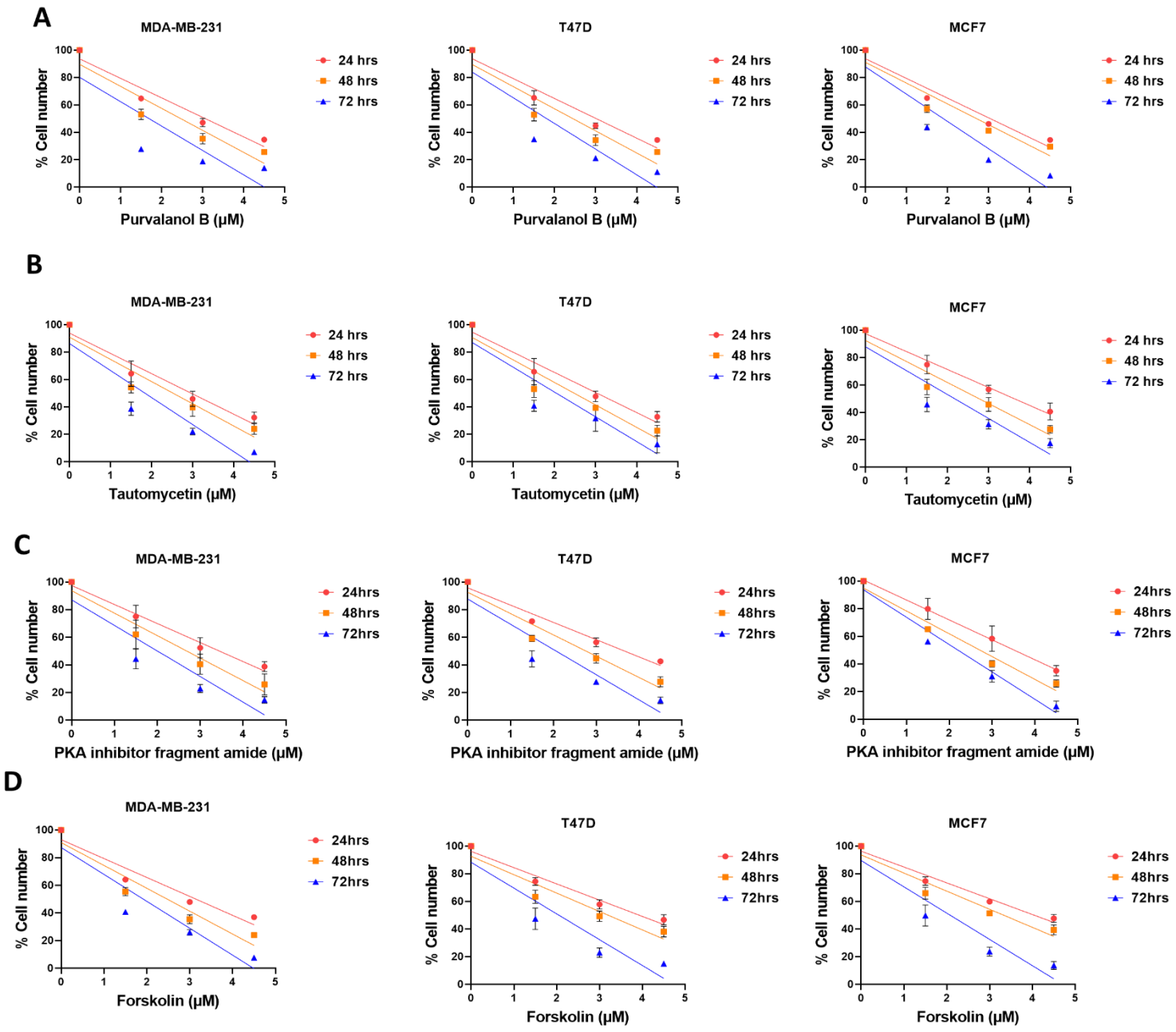


Figure 3.11: The effect of Purvalanol B (A), tautomycetin (B), PKA inhibitor fragment (6-22) amide (C) and forskolin (D) on cell proliferation in three breast cancer cell lines MDA-MB-231, T47D and MCF7. Each cell line was separately exposed to various concentrations of drugs and proliferation was assessed after 24, 48 and 72 hours. Data represent the proliferation assays ± SD of three independent experiments and were analysed by one-way ANOVA test.

Table 3. 10: Proliferation IC_{50} values of purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin in MDA-MB-231, T47D, and MCF7 breast cancer cells.

Drugs	Proliferation Assay IC_{50} MDA-MB-231 (µM)		
	24h	48h	72h
Purvalanol B	3.07 ± 0.06	2.47± 0.09	1.70± 0.02
Tautomycetin	2.97 ± 0.27	2.54 ± 0.24	1.84 ± 0.09
PKA inhibitor fragment (6-22)amide	3.45 ± 0.27	2.71 ± 0.39	2.01 ± 0.13
Forskolin	3.15 ± 0.08	2.48 ± 0.10	1.92 ± 0.03

Drugs	Proliferation Assay IC_{50} T47D(µM)		
	24h	48h	72h
Purvalanol B	3.02 ± 0.13	2.45 ± 0.06	1.81 ± 0.07
Tautomycetin	3.03 ± 0.10	2.48 ± 0.12	1.99 ±. 0.32
PKA inhibitor fragment (6-22)amide	3.67 ± 0.16	2.77 ± 0.17	2.07 ± 0.13
Forskolin	3.95 ± 0.37	3.22 ± 0.26	2.05 ± 0.18

Drugs	Proliferation Assay IC_{50} MCF7 (µM)		
	24h	48h	72h
Purvalanol B	3.05 ± 0.07	2.71 ± 0.08	1.90 ± 0.05
Tautomycetin	3.33 ± 0.42	2.8 ± 0.23	2.2 ± 0.17
PKA inhibitor fragment (6-22)amide	3.63 ± 0.41	2.73 ± 0.09	2.21 ± 0.03
Forskolin	4.05 ± 0.20	3.26 ± 0.14	2.08 ± 0.15

3.4.8 Cdk5, PKA enzyme ELISA and DARPP-32 Threonine-34 and Threonine-75 phosphorylation ELISA

The effects of purvalanol B, forskolin, PKA inhibitor fragment (6-22) amide and tautomycetin on the activities of Cdk5 and PKA, and the levels DARPP-32 Threonine-34 and Threonine-75 were determined by ELISA in T47D breast cancer cells following a 24 hour period exposure to a range of drug concentrations. T47D cells were selected in this experiment in order to further investigate the importance of DARPP-32 expression in an ER positive breast cancer cell line. This decision was based on a combination of results obtained from immunohistochemistry highlighting the importance of DARPP-32 expression in ER positive cells and observations regarding expression of DARPP-32 in T47D cells.

Approximate percentage reduction in expression for each protein is estimated relative to the untreated control. These results were in line with expectations from each drug. We observed 50% reduction in PKA activity following treatment with 1.5 μM PKA inhibitor fragment (6-22) amide; when the concentration of PKA inhibitor fragment (6-22) amide was increased to 3 μM and 4.5 μM, significant (P=0.05) reductions (60% and 80%, respectively) in PKA activity were observed (Figure 3.12A). Significant reductions in Cdk5 activity were observed following treatment with 1.5 μM, 3 μM, and 4.5 μM Cdk5 inhibitor, purvalanol B (60%, 70%, and 80%, respectively, P=0.05), relative to untreated control (Figure 3.12B). Approximately, 30% reduction in DARPP-32 Threonine-34 phosphorylation was observed following treatment with 1.5 μM PKA inhibitor fragment (6-22) amide, versus 60% reduction following treatment with the same drug at 3 μM concentration; increasing the concentration of PKA inhibitor fragment (6-22) amide to 4.5 μM led to reduction of DARPP-32 Threonine-34 phosphorylation by approximately 70%, relative to untreated control (Figure 3.12C). Approximately, 50% reduction in DARPP-32 Threonine-34 phosphorylation was observed

following treatment with 1.5 μM PP1/PP2A inhibitor, Tautomycetin; significant reduction in DARPP-32 Threonine-34 phosphorylation (P=0.05) were observed at higher concentrations of this drug where treatment with 3 μM and 4.5 μM concentrations led to 70% and 80% reduction, respectively (Figure 3.12D). Approximately, 20% reduction in DARPP-32 Threonine-75 phosphorylation was observed following treatment with 1.5 μM Purvalanol B; increasing the concentration of Purvalanol B to 3 μM and 4.5 μM led to reduction in DARPP-32 Threonine-75 phosphorylation by 40% and 60%, respectively (Figure 3.12E). Approximately, 60% reduction in DARPP-32 Threonine-75 phosphorylation was observed following treatment with 1.5 μM PKA activator, Forskolin; significant reductions in DARPP-32 Threonine-75 phosphorylation (P=0.05) were observed following treatment with 3 μM and 4.5 μM Forskolin (80% and 90% reduction, respectively) (Figure 3.12F).

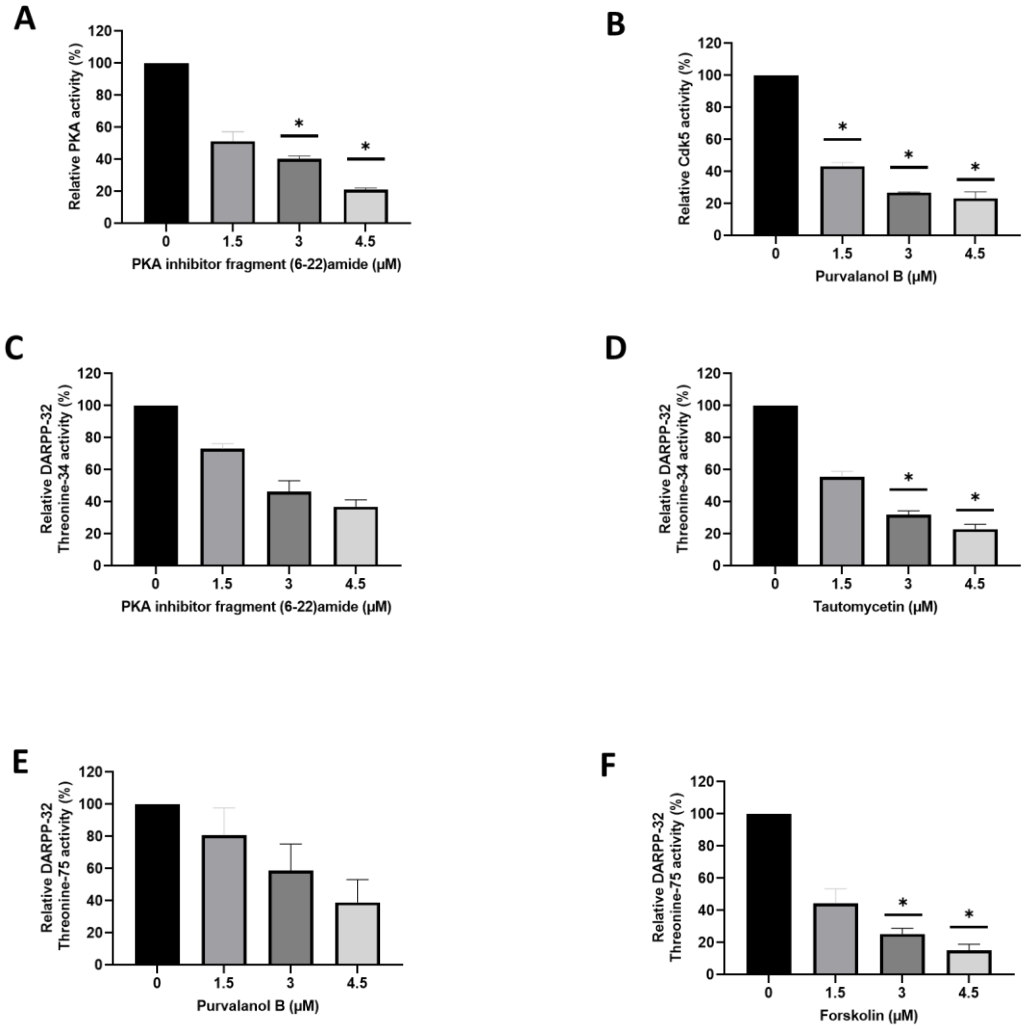


Figure 3. 12: The activity of PKA following treatment with PKA inhibitor fragment (6-22) amide (A), Cdk5 activity following treatment purvalanol B (B), DARPP-32 Threonine-34 following treatment PKA inhibitor fragment (6-22) amide or tautomycetin (C and D, respectively), DARPP-32 Threonine-75 following treatment purvalanol B or forskolin (E and F, respectively) in the presence various concentrations. Data represent the relative kinase activity (%) ± SD of three independent experiments and were analysed by one-way ANOVA test (*P<0.05).

3.4.10 The effect of Purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide, and forskolin on protein expression

The effects of purvalanol B (cdk5 inhibitor), tautomycetin (PP1 inhibitor), PKA inhibitor fragment (6-22) amide (PKA inhibitor) and forskolin (PKA activator) on DARPP-32 protein expression were assessed in MDA-MB-231 and T47D breast cell lines by Western blotting following 24 hours of exposure. DARPP-32 was expressed in T47D and MDA-MB-231 control cells, and its expression was not affected following exposure to any of the drugs (1.5μM concentration). This provided assurance that potential alterations in cellular activity of DARPP-32 induced by each drug are due to the phosphorylation state of DARPP-32, and not as a result of expression inhibition (Figure 3.13).

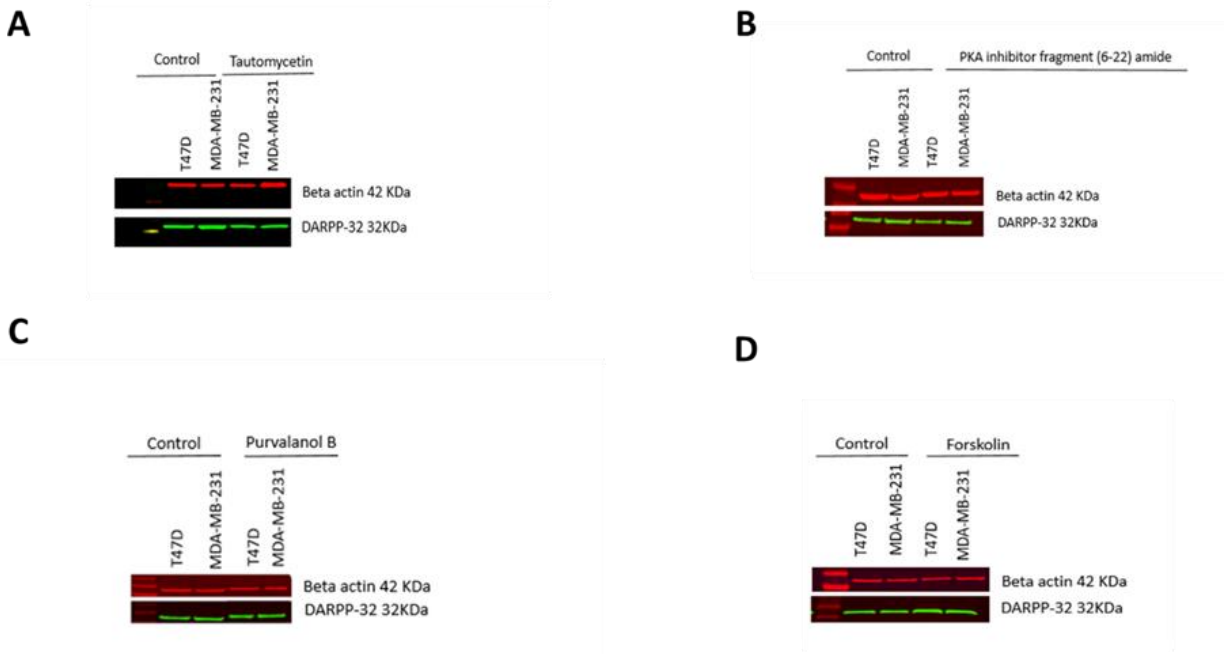


Figure 3.13: The expression of DARPP-32 and beta actin (loading control) in the presence and in the absence of purvalanol B (cdk5 inhibitor), tautomycetin (PP1 inhibitor), PKA inhibitor fragment (6-22) amide (PKA inhibitor) and forskolin (PKA activator) in T47D and MDA-MB-231 cells were monitored in a single experiment using Western blotting.

3.5 Discussion

In this study, the expression and the clinical significance of key DARPP-32 signalling pathways were investigated in a large cohort of breast cancer patients. We provide evidence that low protein expression of PKA is significantly associated with adverse breast cancer specific survival with a more intense adverse survival effect in ER positive breast cancer patients. While the expression of PKA protein in breast cancer provides important information regarding cancer cell behaviour and it has a potential clinical importance as a therapeutic target, our data suggests that it cannot be considered as an independent prognostic marker, but instead as a prognostic marker which is possibly dependent on different clinopathological parameter of tumours such as tumour grade and tumour size.

In this study, low mRNA expression of PKA subunits, *PRKAR1A, PRKAR2A,* and *PRKACB*, were significantly associated with adverse breast cancer specific survival. Low expression of *PRKAR1B, PRKACA*, and *PRKACG* were significantly associated with improved prognosis breast cancer specific survival, and *PRKAR2B* was not significantly associated with breast cancer survival.

Previously, low expression of *PRKAR1A*, and overexpression of SRC have been reported as be common traits amongst basal like and HER2 positive breast cancer patients with reduced survival period (Beristain et al., 2015). Despite a lack of sufficient evidence regarding the role of *PRKACG* and *PRKAR1B* in breast cancer, abnormalities associated with these genes have been implicated in other malignancies. The activity of *PRKACG* has been shown to contribute to the characterisation of acute myeloid leukaemia by acting as a hub gene for the mutation of transcription factor RUNX1, and single nucleotide polymorphisms of *PRKAR1B* have been associated with poor survival in advanced renal cell cancer patients (Zhu et al., 2018, Garrigos et al., 2017).

In the current study, expression of PP1 protein was not significantly associated with breast cancer specific survival. While the expression of PP1 protein in breast cancer provides important information regarding cancer cell behaviour.

Low mRNA expression of *PPP1CB* and *PPP1CC* were significantly associated with adverse breast cancer specific survival. Low expression of *PPP1CA* was significantly associated with improved prognosis of breast cancer specific survival.

Previous studies have reported formation of *PPP1CB* anaplastic lymphoma kinase (ALK) fusion gene in a number of malignancies including spindle cell tumour, paediatric glioma and a leiomyosarcoma (Lopez-Nunez et al., 2020, Ng et al., 2019, Ptakova et al., 2018). In addition, reduced expression of *PPP1CB* in MDA-MB-231 breast cancer cells has been shown to be involved in cytoskeleton remodelling preceding metastasis (Madsen et al., 2015). One study reported *PPP1CA* amplification in both localised and metastatic castration-resistant prostate cancers (mCRPC) comprising 7% and 17% of the cases in data sets of 59 and 35, respectively. In this investigation, amplification of *PPP1CA* was found to frequently coincide with the amplification of G1/S-specific cyclin-D1 gene (CCND1), indicating the possibility of a proto-oncogenic behaviour associated with *PPP1CA* (Chen et al., 2018, Felgueiras et al., 2014). In oral squamous cell carcinoma cells, amplification of *PPP1CA* correlates with the amplification and overexpression of PP1A. In contrast, *PPP1CA* allelic loss has been reported in thyroid, larynx, kidney and colorectal tumors (Hsu et al., 2006, Castro et al., 2008).

Our study demonstrated a significant association between low nuclear and cytoplasmic Cdk5 expression and adverse disease specific survival indicating that the expression of Cdk5 could be considered as an independent prognostic marker and it may have clinical applications in treatment of breast cancer. These findings were further supported

following the investigation of the relation between Cdk5 mRNA expression and survival of ER positive patients within the METABRIC cohort. There are limited studies that have investigated Cdk5 expression and its impact on patient survival; often limited in patient numbers. Previous studies have demonstrated that low nuclear and cytoplasmic Cdk5 expression is associated with poor prognosis and poor patient outcome in various cancers (Sun et al., 2016, Zeng et al., 2018). In one investigation, expression of Cdk5 was monitored in a cohort comprising 244 patients (180 men and 64 women) with gastric adenocarcinoma. Low levels of Cdk5 were shown to be prevalent amongst females as well as patients exhibiting lymph node metastasis (Zeng et al., 2018). Another study detected low expression of Cdk5 in liver and brain cancer cell lines (Zeng et al., 2018).

In contrast to the studies that have identified low expression of Cdk5 as an indication of cancer progression, one study involving 456 patients demonstrated that high levels of Cdk5 mRNA are associated with shorter patient survival and that reduced levels of nuclear and cytoplasmic Cdk5 promoted longer survival period in breast cancer patients following treatment with PARP inhibitor and ionizing radiation (Chiker et al., 2015). It is important to note that patients in the study by Chiker *et.al* (2015) were subjected to a different treatment method which may explain the difference in the observations in that study in comparison to our study. One study by Liang and colleagues (2013) on breast cancer (n=108) indicated association between high Cdk5 protein expression and ER negative status (Liang et al., 2013). In comparison, our investigation showed that low Cdk5 protein expression is associated with ER positive status, signifying the need for further investigation in larger patient cohorts. However, these findings indicate that Cdk5 expression may be of clinical relevance in breast cancer. Both low and high Cdk5 expression levels could favour development of cancer depending on the type of cancer and the involvement of crucial cellular pathways. Therefore, both

extremes may constitute a threat to the longevity of patient survival through promotion of cancer (Liang et al., 2013).

In addition to the assessment of individual protein expression, the impact of the co-expression of various pairs of proteins on breast cancer specific survival was investigated. Investigation of the potential clinicopathological implications associated with combined expression of PKA, PP1, DARPP-32 and Cdk5 requires consideration of the complex interplay between each of these proteins. Phosphorylation of DARPP-32 at the Threonine-75 residue by Cdk5 can lead to the inhibition of PKA, antagonising dopamine signalling and influencing the activity of calcium/calmodulin kinase II and PP2A. This forces a decrease in Threonine-75 phosphorylation in response as a part of normal homeostasis in healthy cells (Fukunaga et al., 2000, Vangamudi et al., 2011). Reduced levels of both Cdk5 and DARPP-32 may be the consequence of an abnormal cellular switch to t-DARPP expression (Lenz et al., 2017). Phosphorylation of Threonine-34 residue by PKA can lead to inhibition of PP1. The imbalance of each one of these proteins may result in dysregulation of the others (Scheggi et al., 2018).

In this study, associations between combined PKA, PP1, Cdk5 and DARPP-32 cytoplasmic/nuclear expression and breast cancer specific survival were assessed. Combined expression of PKA and DARPP-32 were not significantly associated with breast cancer specific survival. In contrast, the combination of low PKA and low Cdk5 expression, low PP1 and low DARPP-32, and low PP1 and low Cdk5 were significantly associated with adverse breast cancer specific survival.

Proliferation assays were utilised to determine the cytotoxicity of exposure to purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin in in triple negative MDA-MB-231 and luminal T47D and MCF7 cells over 72 hours. MDA-MB-231 cells were significantly sensitive to all drugs at 72 hours, but not at 24 or 48 hours. In T47D cells

sensitivity to all drugs was significant at 24 and 48 hours, but not after 72 hours of exposure. MCF7 cells were significantly sensitive to all drugs at all time-points up to 72 hours.

The extent of cytotoxicity associated with the exposure to purvalanol B was similar across all three breast cancer cell lines. Studies have described purvalanol B's ability to induce cell cycle arrest at the G2/M checkpoint. Purvalanol B has also been reported to induce activation of apoptosis through inhibition of the ATP binding site of Cdk1 and Cdk2. In gastric cancer, this process has been shown to cause activation of caspases and cytochrome-c release in cancer cells (Knockaert et al., 2002, Coker-Gürkan et al., 2015). Purvalanol B was shown to reduce cancer cell proliferation and induce apoptosis through MMP loss in colon cancer, adding to the existing optimism regarding the efficacy of purvalanol B (Coker-Gürkan et al., 2015). In a separate investigation, with the exception of minor unwanted inhibitory effect on p42/MAPK (ERK2) and p44/MAPK (ERK1) at high concentrations, purvalanol B was able to selectively interact with Cdk1, 2 and 5 at nanomolar concentrations (Knockaert et al., 2002, Chang et al., 1999). Given that there are a significant number of cellular components whose activities depend on interactions with ATP, selectivity of such therapeutic drugs must be comprehensively studied prior to administration in humans (Knockaert et al., 2002).

Tautomycetin induces apoptosis through inhibition of tyrosine kinase and PP1 in cells. Some studies have described this event as an activated T cell-specific apoptosis where the interaction of PP1 and microtubule-associated protein τ leads to the phosphorylation and deactivation of actin-binding protein cofilin in human T lymphocytes, inhibiting T cell activation; this gives tautomycetin an immunosuppressive characteristic. Therefore, administration of tautomycetin as a therapeutic agent could be associated with unintended side effects such as immunodeficiency (Shim et al., 2002, Liao et al., 1998, Ambach et al., 2000).

In an effort to further investigate the relationship between the induced cytotoxicity of tautomycetin and its PP1 inhibitory activity *in vivo*, phosphatase activity of PP1 in MCF7 cell extracts following exposure to the drug was determined in a previous study. It was noted that exposure to tautomycetin at 1 and 2 μM concentrations reduced the activity of PP1 by 60% and 81%, respectively (Niu et al., 2012). Although the activity of PP1 was not monitored in the current study, these findings support the findings of previous studies that have described tautomycetin as a potent inhibitor of PP1 (Niu et al., 2012).

PKA inhibitor fragment (6-22) amide-induced inhibition of PKA stems from its ability to bind to the substrate site of the protein kinase. Moreover, previous findings have indicated that PKA inhibitor fragment (6-22) amide exerts its anti-proliferative effects through impairment of the cAMP-PKA pathway and the reduced ability of cells to nullify the effects of oxytocin in MDA-MB-231 cells. cAMP plays an important role in the promotion or the suppression of cell growth depending on the type of cells (Cassoni et al., 1997). These observations provide further insight into the mechanism of action of PKA inhibitor fragment (6-22) amide, and therefore direct comparisons are not possible since in the current study the cellular toxicity in MDA-MB-231, T47D and MCF7 cell lines were determined instead.

Forskolin (PKA activator) is known for its ability to supress the growth of the gastric cancer cells through inhibition of c-myc and cyclin D1 oncogenes expression, and has been shown to inhibit cell growth and induce apoptosis in rhabdomyosarcoma, neuroblastoma, and hepatoblastoma cells via impairment of the Hedgehog signalling pathway and reduced c-myc expression (Yamanaka et al., 2010, Wang et al., 2019). Emerging evidence suggests possibility of forskolin's involvement in promotion of sensitivity to conventional chemotherapy in myeloma and in colorectal cancer cells (Illiano et al., 2017, Follin-Arbelet et al., 2015). Forskolin is generally well tolerated by patients and it is approved for the

treatment of various conditions, including heart disease, diabetes, hypertension, and asthma (Huerta et al., 2010, Ríos-Silva et al., 2014 Wang et al., 2019).

Overall, there is a limited amount of comparable data available on cellular toxicity associated with the use of purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin in breast cancer cell lines in literature. However, some studies have estimated the IC_{50} of purvalanol B and tautomycetin for the inhibition of their respective individual target proteins (Cdk5 and PP1, respectively) to be 6 nM and 1.6 nM, respectively. (Mitsuhashi et al., 2001, Gicenas et al., 2014). Forskolin was demonstrated to have an estimated IC_{50} of 41 nM and EC50 (Half maximal effective concentration) of 0.5 μM for its interactions with type I adenylyl cyclase in membranes from stably transfected Sf9 cell (Robbins et al., 1996). These results indicate the potency of each drug with regards to their respective cellular targets, but they do not represent inhibitory concentration required for inducing cellular toxicity.

In the current study, the kinase activity of Cdk5 and PKA, and the levels of DARPP-32 Threonine-34 and Threonine-75 were determined in T47D breast cancer cells following exposure to purvalanol B, forskolin, PKA inhibitor fragment amide and tautomycetin. Threonine-34 and Threonine-75 are the two main phosphorylation sites of DARPP-32 that promote inhibition of PP1 and PKA, respectively. These events depend on the interactions of PKA and Cdk5 with Threonine-34 and Threonine-75. The activity of PP2A leads to dephosphorylation of Threonine-75 and Serine-97 to reduce PKA, whereas suppression of PP2A reverses this process. Importantly, Threonine-34 residue is only present in full length DARPP-32, whereas Threonine-75 residue is present in full length DARPP-32 and in t-DARPP. PKA inhibitor fragment (6-22) amide caused a reduction in Threonine-34 phosphorylation (approximately 60% to 70%) as expected through significant (P=0.05) inhibition of PKA at 3 μM and 4.5 μM

concentrations (approximately 60% and 80% PKA activity reductions, respectively), relative to untreated control cells.

Given that purvalanol B is a potent inhibitor of Cdk5, administration of this drug was expected to result in a reduced level of DARPP-32 Threonine-75 phosphorylation. Our results confirmed that exposure to purvalanol B leads to a notable (approximately, 20% to 60%) reduction in Threonine-75 phosphorylation through significant (P=0.05) reduction of Cdk5 activity at 1.5 μM, 3 μM, and 4.5 μM concentrations (approximately 60%, 75%, and 80% Cdk5 activity reductions, respectively), relative to untreated control cells.

Treatment with 3 μM and 4.5 μM PP1/PP2A inhibitor, tautomycetin, led to significant reduction in Threonine-34 phosphorylation (70% and 80%, respectively; P=0.05), relative to untreated control. Given that phosphorylated Threonine-75 inhibits PKA, and that PP2A dephosphorylates Threonine-75, tautomycin-induced inhibition of PP2A results in an indirect inhibition of PKA. This results in reduced phosphorylation of Threonine-34 by PKA. PKA activator, forskolin, caused a significant reduction in Threonine-75 phosphorylation (P=0.05) following treatment at 3 μM and 4.5 μM concentrations (80% and 90% reduction of Threonine-75 phosphorylation, respectively), potentially through inhibition of Cdk5 increased availability of PKA. Such drug-induced alterations indicate the possibility of regulation of DARPP-32 phosphorylation in breast cancer in a dose dependent manner through inhibition or activation of up-stream proteins.

Western Blotting was used to determine the expression of DARPP-32 in MDA-MB-231 and T47D breast cell lines following exposure to purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin. DARPP-32 was expressed in MDA-MB-231 and T47D control cells and its expression levels were not affected following exposure to any of the drugs. This indicated that the changes observed in ELISA kinase activity

were due to modulation of DARPP-32 phosphorylation state, and not as a result of gene expression alterations.

Conclusion

Overall, the data obtained in this study demonstrate the association of cytoplasmic/nuclear expression levels of Cdk5, PP1 and PKA with breast cancer specific survival.

IC_{50} values associated with purvalanol B, tautomycetin, PKA inhibitor fragment (6-22) amide and forskolin at 24, 48 and 72 hour time points in MDA-MB-231, T47D and MCF7 indicated that MDA-MB-231 cells were only significantly sensitive to all drugs at 72 hours, suggesting that cytotoxicity in this cell line is time dependant. In contrast, the sensitivity of T47D cells to all four drugs was found to be significant only at 24 and 48 hours, suggesting a potential initialisation of resistance. The sensitivity of MCF7 cells to all drugs was significant at all time-points.

We successfully demonstrated a large reduction in Threonine-34 phosphorylation induced by both tautomycetin (PP1/PP2A inhibitor) and PKA inhibitor fragment (6-22). Observations regarding the effects of Cdk5 inhibitor purvalanol B and PKA activator forskolin on the state of DARPP-32 phosphorylation were indicative of large reductions in Threonine-75 phosphorylation. Our data suggests that the expression of PKA, PP1 and Cdk5 are vital to breast cancer specific survival and that modulation of these proteins is possible through regulation of the phosphorylation state of DARPP-32.

3.6 Summary

- Expression of PP1 protein was not significantly associated with breast cancer specific survival.
- Low expressions of Cdk5 and PKA are significantly associated with adverse Breast cancer specific survival.
- The combination of low PKA and low Cdk5 expression, low PP1 and low DARPP-32, and low PP1 and low Cdk5 were significantly associated with adverse breast cancer specific survival.
- MDA-MB-231 cells were significantly sensitive to all drugs (purvalanol B (Cdk5 inhibitor), tautomycetin (PP1 inhibitor), PKA inhibitor fragment (6-22) amide (PKA inhibitor) and forskolin (PKA activator)) at 72 hours, but not at 24 or 48 hours. T47D cells were sensitive to all drugs at 24 and 48 hours, but not after 72 hours of exposure. MCF7 cells were significantly sensitive to all drugs at all time-points up to 72 hours.
- PKA inhibitor fragment (6-22) amide caused a reduction in Threonine-34 phosphorylation through significant inhibition of PKA.
- PP1/PP2A inhibitor, Tautomycetin caused a significant reduction in Threonine-34 phosphorylation
- PKA activator, forskolin, caused a significant reduction in Threonine-75 phosphorylation potentially through inhibition of Cdk5 increased availability of PKA.
- Western Blotting demonstrated that the expression of DARPP-32 in MDA-MB-231 and T47D control cells were not affected following exposure to any of the drugs. Therefore, the changes observed in ELISA kinase activity were not as a result of gene expression alterations, but due to modulation of DARPP-32 phosphorylation state.

Chapter 4 DARPP-32 and Identified Signalling partner Expression in breast Cancer

4.1 Introduction

Recent discoveries in breast cancer research have led to the discovery of a number of promising targets with potentially important roles in carcinogenesis and cancer-specific survival. There is an increasing evidence suggesting that dopamine and cAMP regulated phosphoprotein 32 kDa (DARPP-32), encoded by phosphoprotein phosphatase-1 regulatory subunit 1B *(PPP1R1B),* may play an important role in tumours, including breast cancer (Belkhiri et al., 2016). The activity of DARPP-32 mainly depends on its state of phosphorylation at Threonine-34 and Threonine-75 residues. (Vangamudi et al., 2011).

Since the study of DARPP-32 and its signalling partners provide a profound understanding of some of the important biological mechanisms involved in breast cancer, we aim to assess protein expression of *Sp7*, *DKK1, GRB7* and DARPP-32 Threonine-34 phosphorylation in a large group of breast cancer patients. This will provide further insight into the potential role of DARPP-32 and its signalling partners in influencing patient outcome.

Previously, our group identified a number of genes associated with *PPP1R1B* expression through Artificial neural network analysis and using three *PPP1R1B* probes and commonalities within the top 200 genes identified for each probe where *CDC42 (ILMN_1696041), DKK1 (ILMN_1773337), GRB7 (ILMN_1740762), PNMT (ILMN_1710027)* and *GPER (ILMN_1795298)* were amongst the most common transcripts (Kotecha et al., 2019). These data led us to pursue further investigations with regards to *GRB7* and *DKK1* in particular in the current study in order to determine the pattern of protein and mRNA expression and to assess whether their levels of protein expression are associated with patient clinical outcome. However, associations between *PPP1R1B* and *GRB7* have been previously described in upper gastrointestinal adenocarcinomas where they, often along with other genes such as

TARD3, *TCAP, PNMT, PERLD1, ERBB2, C17orf37*, have been amplified at locus 17q (Maqani et al., 2006, Kotecha et al., 2019). DKK1, an important regulator of the wnt-5A pathway, is a protein that functions downstream of DARPP-32. Dysregulation of *DKK1* has been implicated in various types of cancer including liver cancer, lung cancer, oesophageal cancer, breast cancer, glioma, and cervical cancer (Zhu et al., 2021, Huang et al., 2020) (Figure 4.1).

In the current study, we have investigated the complex and diverse molecular interactions of DARPP-32 with a range of proteins, namely *Sp7, GRB7,* and *DKK1,* which often results in mediation of key cellular pathways. To fulfil the objectives of this study, the potential role of *Sp7, GRB7,* and *DKK1* in the stimulation of important signalling nexus in cancer that promotes metastasis is being investigated. One of the aims of the current study is to investigate the interrelation between these proteins in order to provide detailed insight into their roles and their involvement in complex biological mechanisms in cancer.

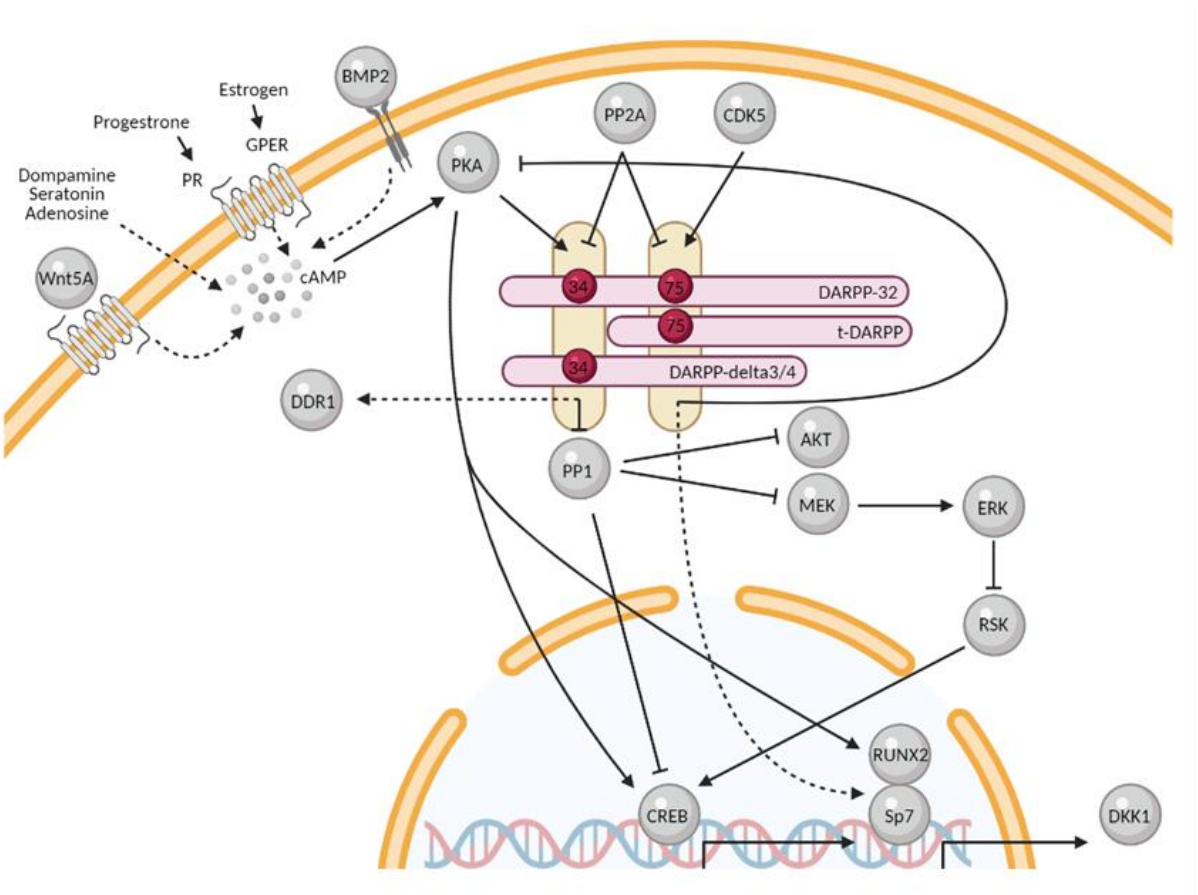


Figure 4.1: Schematic representation of interactions that link various proteins within cells. These proteins include Wnt5A, PKA, Cdk5, *DKK1*, *Sp7,* DARPP32, t-DARPP, and DARPP delta3/4. This figure also highlights the impact of DARPP-32 splicing on the downstream effectors and the consequent gene expression. Image was created on biorender.com.

4.1.1 *Osterix/Sp7*

Transcription factor *Sp7,* also known as Osterix, is a protein with an important role in the regulation of osteoblast differentiation and bone formation (Wang et al., 2021, Qu et al., 2019). This protein was cloned by Nakashima and his colleagues in 2002 (Nakashima et al., 2002). *Sp7* was isolated from osteoblasts following differentiation of C2C12 skeletal muscle progenitor cells in the presence of bone morphogenetic protein 2

(BMP-2) (Sinha and Zhou, 2013, Nakashima et al., 2002). *Sp7* falls into the Sp subgroup of Krüppel-like family of transcription factors (XKLF) that feature a three zinc-finger structure located near the carboxy-terminus facilitating DNA interactions. There are six other proteins (Sp1-6) that have been identified in this subgroup; aside from the homological features in the zinc-finger DNA-binding domain, there is a high degree of structural variation among these proteins (Milona et al., 2003).

Abnormalities associated with *Sp7* can lead to the development of serious conditions such as recessive types of brittle bone disease osteogenesis imperfecta. Other variants of *Sp7* have been associated with bone mineral density variations in humans (Wang et al., 2021 Lapunzina et al., 2010, Fiscaletti et al., 2018). Many skeletal abnormalities are linked with the genetic network associated with *Sp7,* and a number of osteocytes related genes associated with *Sp7* are also expressed in neurons. The activity of *Sp7* in different cellular environments points to the possible similarities in the underlying mechanisms regulating the developments in multiple tissue networks (Wang et al., 2021).

Although the exact role of *Sp7* in promoting osteocyte differentiation remains to be uncovered, the interaction of this protein with the nuclear factor of activated T cells (NFAT) is known to trigger activation of collagen type Iα1 which induces bone formation (Wang et al., 2021, Dai et al., 2015). Until recently, the expression and function of *Sp7* was thought to be confined to bone tissues; this perception was drastically changed as the overexpression of *Sp7* was first detected in tumours including osteosarcoma, prostate cancer and breast cancer (Qu et al., 2019).

Since then, there have been a limited number of studies focused on investigating the role of *Sp7* in cancer. The extent of *Sp7* involvement in the process of osteoblast differentiation depends on the level of its expression and availability (Barbuto and Mitchell, 2013). The expression of *Sp7* is modulated by the influence of proteins such as bone

morphogenic protein-2 (BMP2), Msh homeobox 2 (MSX2), myogenic differentiation (MYOD), and distal-less homeobox 5 (DLX5). BMP2 in particular, is a crucial player in the differentiation of C2C12 cells into osteoblasts through expression of a series of osteoblast genes (Sinha and Zhou, 2013). So far, a number of *in vivo* studies have investigated the role of Sp7, where severe skeletal deformities and prenatal death was reported in *Sp7* knockout mice in particular. Such deformities were thought to be a result of failure in maturation of osteoblasts in the absence of *Sp7* (Chen et al., 2019).

4.1.2 *Sp7* in cancer

While *Sp7* is mainly expressed in bone tissues where it plays a crucial role in modulating differentiation of osteoblasts, several studies have pointed to the potential involvement of this protein in tumour development and progression (Dai et al.,2019).

Activity of *Sp7* is suspected to influence regulation of various proteins involved in the promotion of tumour invasion, especially in breast cancer. These proteins include VEGF, matrix metalloproteinase-9 (MMP-9), Beta-catenin (β-catenin), and Epithelial-cadherin (E-cadherin). The exact mechanism that modulates the expression and activity of *Sp7* in tumour invasion is not clear and further investigations are required (Qu et al., 2019, Dai et al., 2019).

Microarray analysis of a set of genes that code for MMP enzymes in mice animal models revealed that osteoblasts isolated from mice expressing *Sp7* exhibited a very high expression level of MMP9 compared to those isolated from *Sp7* knockout mice. This discovery indicated the potential interaction between *Sp7* and MMP proteins which have been associated with cancer invasion, and osteoclast differentiation and activation. Collaboration of *Sp7,* MMP9, MMP13 and VEGF in promotion of breast

cancer metastasis to bone tissue remains a possibility. This could be facilitated through a complex mechanism characterised by regulation of proteins involved in the metastatic cascade and angiogenesis (Yao et al., 2019, Pivetta et al., 2011).

In 2019, Qu *et.al* reported findings from their investigation into the role of *Sp7* in potentiating metastasis and angiogenesis in breast cancer both *in vitro* and *in vivo*. They detected a high level of *Sp7* expression in metastatic breast cancer cells. *Sp7* was also found to contribute to cell migration and angiogenesis through up-regulation of S100 calcium binding protein A4 (S100A4). Therefore, designing therapeutic methods that target *Sp7* may prove to be an effective strategy in prevention of tumour metastasis in the future (Qu et al., 2019).

Other studies have referred to *Sp7* as a tumour suppressor gene with a role in prevention of cancers that originate in the bone-forming cells. In contrast, some studies have reported high level of *Sp7* expression in kidney, prostate and lung cancers that have developed into metastatic tumours, thereby identifying it as a target for the treatment of such conditions (Dai et al., 2015, Cao et al., 2005, Logothetis and Lin, 2005, Steeg, 2006).

Whilst the activity of certain factors have been found to supress the expression of *Sp7,* the activity of proteins such as Insulin-like growth factors (IGFs) has been reported to stimulate expression of *Sp7* in human mesenchymal cells (Barbuto and Mitchell, 2013, Celil and Campbell, 2005). The TNF-α is amongst inhibitors of *Sp7* and its activity has a negative impact on the maturation of osteoblast. TNF-α exerts its inhibitory effect on *Sp7* through regulation of EGFR via the activity of transcription factor paired related homeobox 1 (PRRX1). Parathyroid hormone (PTH), a peptide hormone secreted by the parathyroid glands that regulates the serum calcium concentration, is another important player with a crucial role in the maturation of both osteoblasts and

osteocyte. Further studies are required to investigate the possible influence of PTH and its downstream effectors on *Sp7* (Barbuto and Mitchell, 2013, Lu et al., 2006).

In 2019, Yao *et.al* reported their findings from an investigation focused on the role of *Sp7* in breast cancer invasion and bone metastasis. During their investigation they demonstrated that the expression of *Sp7* in patients with breast cancer (n=154) was associated with lymph node metastasis and poor prognosis, and that down-regulation of *Sp7* together with suppression of MMP9, MMP13, VEGF, IL-8, and PTHrP impeded the process of invasion and metastasis. Their investigation also verified the influence of *Sp7* in the regulation of MMP9's activity, thereby providing evidence for a role for *Sp7* in promoting invasion in breast cancer cells (Yao et al., 2019). Overall, *in vitro* and *in vivo* studies have demonstrated that downregulation of *Sp7* could hinder abnormal growth in malignant cells, which makes it a potentially valuable target for the treatment of patients suffering from breast cancer (Dai et al., 2015, Cao et al., 2005).

4.1.3 Growth factor receptor bound protein 7

Growth factor receptor bound protein 7 (*GRB7*) is a 532 amino acid adaptor molecule with a crucial role in the activation of multiple intracellular pathways through transmission of signals from cell membrane receptors. The structure of *GRB7* comprises an amino-terminal proline rich domain, a RA domain, a central pleckstrin homology (PH) domain important in membrane interactions, a BPS motif and a carboxyl-terminal SH2 (src homology 2) domain crucial for interaction with the downstream intracellular proteins (Shen et al., 2004, Han et al., 2001, Porter et al., 2007). These regions are conserved across different species highlighting their importance for the function of the protein. Co-

existence of these regions within *GRB7* allows this protein to be involved in the transmission of signals across multiple cellular pathways (Vermehren-Schmaedick et al., 2020, Zheng et al., 2020).

GRB7 interacts with several cell receptors and other key players involved in the regulation of cell proliferation including the Erythroblastic leukaemia viral oncogene homolog (ErbB) receptor family, platelet-derived growth factor receptor, insulin receptor and RAS GTPase (Zheng et al., 2020, Paudyal et al., 2013). The ability of *GRB7* to interact with multiple cellular pathways makes it an important player with a key role in the regulation of cell growth, development of cancer, cancer invasion and the spread of malignant cells (Vermehren-Schmaedick et al., 2020, Luoh et al., 2019).

The gene that codes for *GRB7* is located on the 17q11–21, a locus positioned in the immediate proximity of the HER2 gene where it forms a section of HER2 gene amplicon. In healthy individuals, detectable levels of *GRB7* are expressed in various organs including pancreas, kidney, placenta, prostate, intestine, colon, liver, lung and testis (Frantz et al., 1997, Nadler et al., 2010). Although the exact role of *GRB7* is not fully understood, various studies have linked its activity with the promotion of cell growth and carcinogenesis. This is further complicated by the fact that *GRB7* adaptor molecule is known to interact with over 20 downstream effectors from various cellular pathways including non-receptor tyrosine kinases, and receptor tyrosine kinases such as EGFR and HER2, most of which interact with *GRB7* through its SH2 domain (Nadler et al., 2010, Pawson et al., 2001).

4.1.4 *GRB7* in Cancer

Evidence presented by a number of recent studies indicates existence of a connection between irregularities in *GRB7* expression and development

of various cancers including breast cancer, oesophageal cancer, and gastric cancer (Gotovac et al., 2020, Vermehren-Schmaedick et al., 2020). Other studies have identified an abnormal variant of the protein, *GRB7v*, isolated from oesophageal cancer cells (Vermehren-Schmaedick et al., 2020, Ekman et al., 2007). While different types of cancers may involve distinctive tumorigenic pathways under the influence of various different proteins, there is emerging evidence suggesting possible involvement of *GRB7* in cancers originating from the ovaries and bladder (Wang et al., 2010).

GRB7 induced promotion of cancer cell migration has been shown to involve focal adhesion kinase (Wang et al., 2010). Expression of *GRB7* has been associated with tumour cell migration to lymph nodes in oesophageal and pancreatic carcinomas. Up-regulation of *GRB7*, which is detected especially in older patients, has also been associated with negative patient outcome, development of large and advanced tumours (high stage), invasion and lymph node metastasis in hepatocellular carcinoma (n=64) and cervical cancer (n=120) (Vermehren-Schmaedick et al., 2020, Zhao et al., 2017). In breast cancer, overexpression of *GRB7* is associated with negative patient outcome and cancer recurrence (Vermehren-Schmaedick et al., 2020, Ramsey et al., 2011, Nadler et al., 2010).

The importance of a balanced regulation of *GRB7* is further highlighted by the impact of its suppression in tumours. Studies have found that suppression of *GRB7* in gastric cancer and oral squamous cell carcinoma leads to reduced cancer growth, cell invasion and overall tumour aggressiveness. While the interactions between *GRB7* protein and certain biomarkers in invasive breast tumours including HER2 have been noticed previously, the level of *GRB7* expression has been found to be proportional to mortality rate and development of high-grade tumours in breast cancer and ovarian cancer, respectively (Vermehren-Schmaedick et al., 2020, Gotovac et al., 2020). In bladder cancer, the significance of

GRB7 overexpression is not very well understood, whereas in cervical cancer abundance of *GRB7* has been found to contribute to apoptosis suppression and distant metastasis (Zheng et al., 2020). For other tumours such as oesophageal cancer, a shortfall in preclinical and clinical experimental data seems to be the limiting factor hindering the understanding of the role of *GRB7,* and potential discovery of targeted therapy (Gotovac et al., 2020).

Involvement of GRB7 in breast cancer progression through expression of HER2 has been reported in a number of studies. In most cases, high expression level of HER2 correlated with the expression of *GRB7.* This was demonstrated as a part of several small cohort studies (<80 patients) that investigated the relation of *GRB7* and HER2 in primary breast tumours using Real Time- Polymerase Chain Reaction (RT-PCR) and Western blot techniques. Further investigations not only led to the discovery of *GRB7/* HER2 complex in breast cancer, but they also illustrated formation of complexes with HER2 conjugates HER3 and HER4 in some breast cancer cell lines (Nadler et al., 2010, Kauraniemi et al., 2001, Bièche et al., 2003, Fiddes et al., 1998).

Considering existence of limited evidence for *GRB7* involvement in HER2 positive breast cancers, further investigations may pave the way for the discovery of novel treatment methods focused on regulation of *GRB7* which may prove to be highly effective in eradicating tumour resistance to therapy and prevention of metastasis in patients (Luoh et al., 2019).

In TNBC, high levels of *GRB7* expression was demonstrated to be strongly associated with poor survival rates during a clinical study involving 246 patients. In this study, overexpression of *GRB7* was shown to serve as a predictive sign for development of drug-resistance, with a 5-year recurrence rate of 20.4% (95% CI 16.5%, 25.0%) compared to 10.5% (95% CI 7.8%, 14.1%) in the low *GRB7* group (Sparano et al., 2011).

GRB7 has been found to be activated through epidermal EGFR signalling pathway in inflammatory breast cancer. Given the importance of the SH2 domain in *GRB7* in enabling interactions with EGFR signalling pathway, development of therapeutic agents that target this region may induce an extensive anti-tumour effect (Morlacchi et al., 2014, Zhang et al., 2013).

4.1.5 *DKK1*

Dickkopf WNT Signaling Pathway Inhibitor 1 (*DKK1*), a glycoprotein encoded by the *DKK1* gene which is located on chromosome 10q11.2.26 in humans, was first discovered in the African clawed frog (Xenopus). This protein was found to have an important role in the development of head and limb during early development (Zhu et al., 2021, Niu et al., 2019). The 1815kb structure of *DKK1* consists of two highly conserved cysteine-rich domains and a 50–55 amino acids long region that connects the domains together (Zhu et al., 2021). While most members of the DKK family (*DKK1*, *DKK2* and *DKK4*) are highly similar to one another, *DKK3* is more distinguishable as it contains a shorter amino acid linker region (Figure 4.2) (Igbinigie et al., 2019, Krupnik et al., 1999).

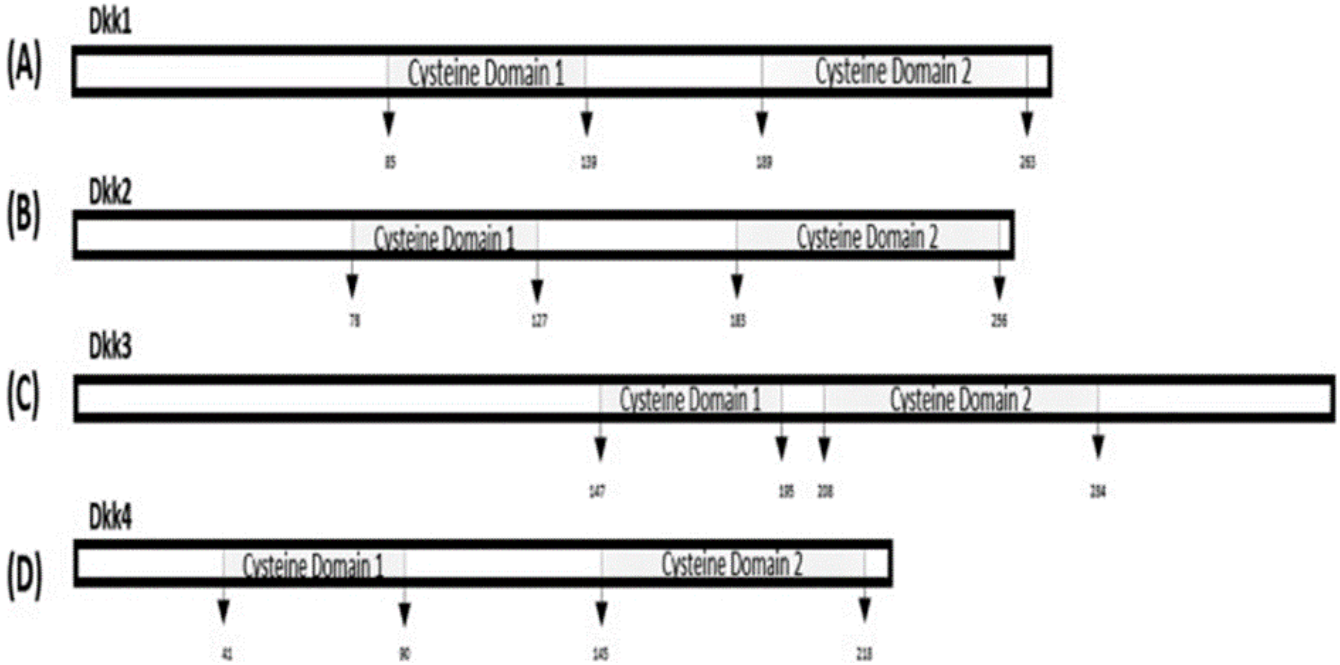


Figure 4.2: *A demonstration of the four members of the* DKK *family: (A)* DKK1 *is made up of 266 amino acids in total with its first and second domain located between the 85th and 139th, and 189th and 263rd amino acid sections, respectively (B)* DKK2 *contains 259 amino acids in total with its first and second domain located between the 147th and 195th, and 183rd 256th amino acid sections, respectively. (C)* Dkk3 *contains 350 amino acids in total with its first and second domain located between the 78th and 127th, and 208th and 284th amino acid sections, respectively. (D)* Dkk4 *contains 224 amino acids in total with its first and second domain located between the 41st and 90th, and 145th and 218th amino acid sections, respectively (Igbinigie et al., 2019). Copyright© 2019 by ELSEVIER Publishing Group. Permission was obtained by Copyright Clearance Centre's RightLink ®service.*

Shortly following its discovery in humans, *DKK1* was found to act as an inhibitor of Wnt signalling pathway which is an important regulator of cell growth and cell survival (Zhu et al., 2021). A number of studies have indicated that dysregulation of Wnt signalling pathway induced via the activity of *DKK1* is crucial to cancer cell migration and bone metastasis in breast cancer and prostate cancer (Pang et al., 2018, Bu et al., 2008,

Thudi et al., 2011). Other studies have reported the up-regulation of *DKK1* in HCC and myeloma, where it was found to contribute to tumour progression. In renal cell carcinoma and colorectal cancers the activity and expression of *DKK1* have been detected at considerably lower levels indicating potential variation in the underlying mechanisms involved (Niu et al.,2019, Feng et al., 2019, Chen et al., 2018, Rui et al., 2019).

4.1.6 *DKK1* in Cancer

In contrast to the role of *DKK1* in the promotion of metastasis and its high level of expression in various types of cancer including liver cancer, lung cancer, oesophageal cancer, breast cancer, glioma, and cervical cancer, this protein can also act as a tumour suppressor by inhibiting proliferation and inducing apoptosis under different circumstances and environment (Zhu et al., 2021, Huang et al., 2020). Furthermore, low levels of *DKK1* have been reported in colon cancer, renal cell cancer and leukaemia, where re-introduction of this protein is believed to initiate anti-tumour effects (Huang et al., 2020). A group of researchers led by Qi *et.al* (2012), illustrated that the therapeutic effects of *DKK1* in colon cancer is associated with the inhibition of metastasis through suppression of epithelial mesenchymal transition (EMT) where expression of this protein is inversely proportional to tumour stage and rate of metastasis (Qi et al., 2012, Fezza et al., 2019). In support of these findings, other studies have also reported reduced levels of *DKK1* in patients suffering from colon cancer; re-introduction of *DKK1* in those patients triggered some therapeutic effects through suppression of β-catenin, which would otherwise activate expression of various genes including the proto-oncogene c-Myc (Fezza et al., 2019, González-Sancho et al., 2005).

High levels of *DKK1* in prostate tissue and in non-small cell lung carcinoma have been found to promote tumour progression where its activity is modulated by proteins such as p38 mitogen activated protein kinases (MAPK) and p53, respectively (Igbinigie et al., 2019). In one

study, the level of *DKK1* expression present in the serum of healthy individuals was compared to that of patients with gastric cancer. Out of a total of 153 patients with gastric cancer, 144 were found to exhibit high levels of *DKK1,* whereas in the 173 healthy volunteers that participated the expression of this protein was found to be much lower (Zhu et al., 2021, Lee et al., 2012). In a similar type of study, it became apparent that *DKK1* serum level was far greater in patients with breast cancer compared to that of healthy individuals. In the same study, it was also shown that the level of *DKK1* expression in patient with bone metastasis was superior compared to those without (Voorzanger-Rousselot et al., 2009, Zhu et al., 2021). *DKK1* could serve as a diagnostic biomarker and a predictive factor for tumour behaviour under various circumstances (Yu et al., 2009, Tung et al., 2011, Zhu et al., 2021).

4.1.7 Hypothesis, aims and objectives

Hypothesis

Our hypothesis is that reduced DARPP-32 Threonine-34 phosphorylation favours promotion of metastasis, and that the regulation of DARPP-32 signalling pathway may have therapeutic benefits. We have used artificial neural networks to identify *DKK1* and *GRB7* as genes whose expressions are associated with DARPP-32 expression in the METABRIC. We therefore hypothesise that their protein expression will correlate with DARPP-32 expression, and associate with patient clinical outcome.

Aims

The aim of the current study is to further demonstrate the significance of low DARPP-32 expression in breast cancer, and to facilitate a better

understanding of how this affects patient outcome through understanding DARPP-32 and t-DARPP-induced changes in cellular signalling.

Objectives

1. To investigate the expression of DARPP-32 and t-DARPP in a large group of breast cancer patients using chromogenic *in situ* RNA hybridisation.

2. To assess the pattern of *Sp7,* DARPP-32 Threonine 34, *DKK1,* and *GRB7* protein expression in a large group of breast cancer patients. In addition, the expression of *Sp7*, *PPP1R1B, DKK1* and *GRB7* will be determined at mRNA level.

4.3 Results

4.3.1 Chromogenic *in situ* RNA hybridisation

In order to determine DARPP-32 and t-DARPP expression in large groups of patients with primary breast cancer Chromogenic *in situ* RNA hybridisation was utilised. We were unable to stain DARPP-32 and t-DARPP at sufficient levels suitable for scoring. Detection of DARPP-32 and t-DARPP was unsuccessful despite employing different incubation times and repeated attempts in probe staining using the advice provided by the kit supplier and in the presence of a representative of the supplying company ACD. In addition, attempts in re-designing and utilisation of longer probes (2ZZ or 3ZZ) by the supplying company were unsuccessful. However, the accuracy of the technique and the sensitivity of RNAscope

in situ hybridization assay were demonstrated through successful identification of positive and negative controls. Figure 3.4 demonstrates examples for the identification of negative control (A), positive control (B), staining of DARPP-32 (C) and staining of t-DARPP (D). This also provided reassurance on the successful preservation of cell integrity with an adequate strong positive control signal.

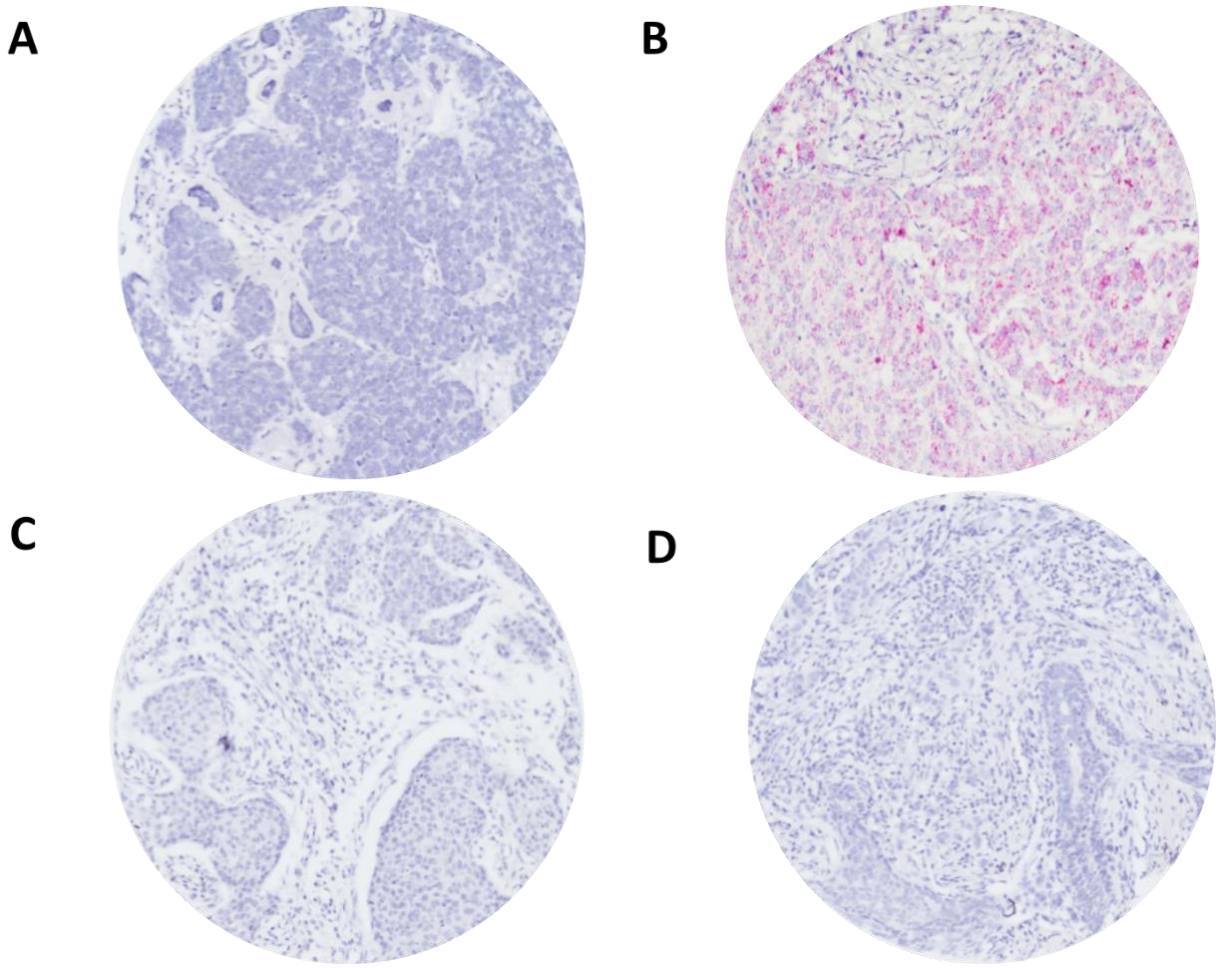


Figure 4.3: Staining for negative control in (A), staining of positive control in (B) staining of DARPP-32 in (C) and t-DARPP in (D) represent the sensitivity of chromogenic in situ RNA hybridisation. Three independent experiments were conducted to ensure experimental validity. Photomicrographs are taken at 10×magnification.

4.3.2 Protein staining, frequency and correlation of expression

Cytoplasmic and nuclear expression patterns of proteins which were assessed using immunohistochemistry are presented in Figure 4.4. To determine *Sp7* expression, 1340 patient tissue specimens were available for assessment. The median H-score for cytoplasmic expression of *Sp7* was 140 (ranging between 50 and 240), and the median H-score for nuclear *Sp7* expression was 30 (ranging between 0 and 90); representative tissue staining is shown in Figure 4.4A. To determine *GRB7* expression, 1408 patient were assessed where the median H-score for cytoplasmic expression of *GRB7* was 0 (ranging from 0 to 290), and the median H-score for nuclear *GRB7* expression was 0 (ranging between 0 and 90); representative tissue staining is shown in Figure 4.4B. To determine *DKK1* expression, 1036 tissue specimens were available for assessment. The median H-score for cytoplasmic expression of *DKK1* was 110 (ranging between 10 and 260), and the median H-score for nuclear *DKK1* expression was 25 (ranging between 0 and 100); representative tissue staining is shown in Figure 4.4C. To determine levels of phosphorylated DARPP-32 at Threonine-34, 1274 patient tissue specimens were available for assessment. The median H-score for cytoplasmic expression of phosphorylated DARPP-32 at Threonine-34 was 100 (ranging between 0 and 290), and the median H-score for nuclear phosphorylated DARPP-32 at Threonine-34 expression was 25 (ranging between 0 and 110); representative tissue staining is shown in Figure 4.4D. Western blot analysis confirmed the specificity of *Sp7, GRB7*, *DKK1*, and DARPP-32 Threonine-34 proteins as demonstrated in Appendix B-Figure B1.

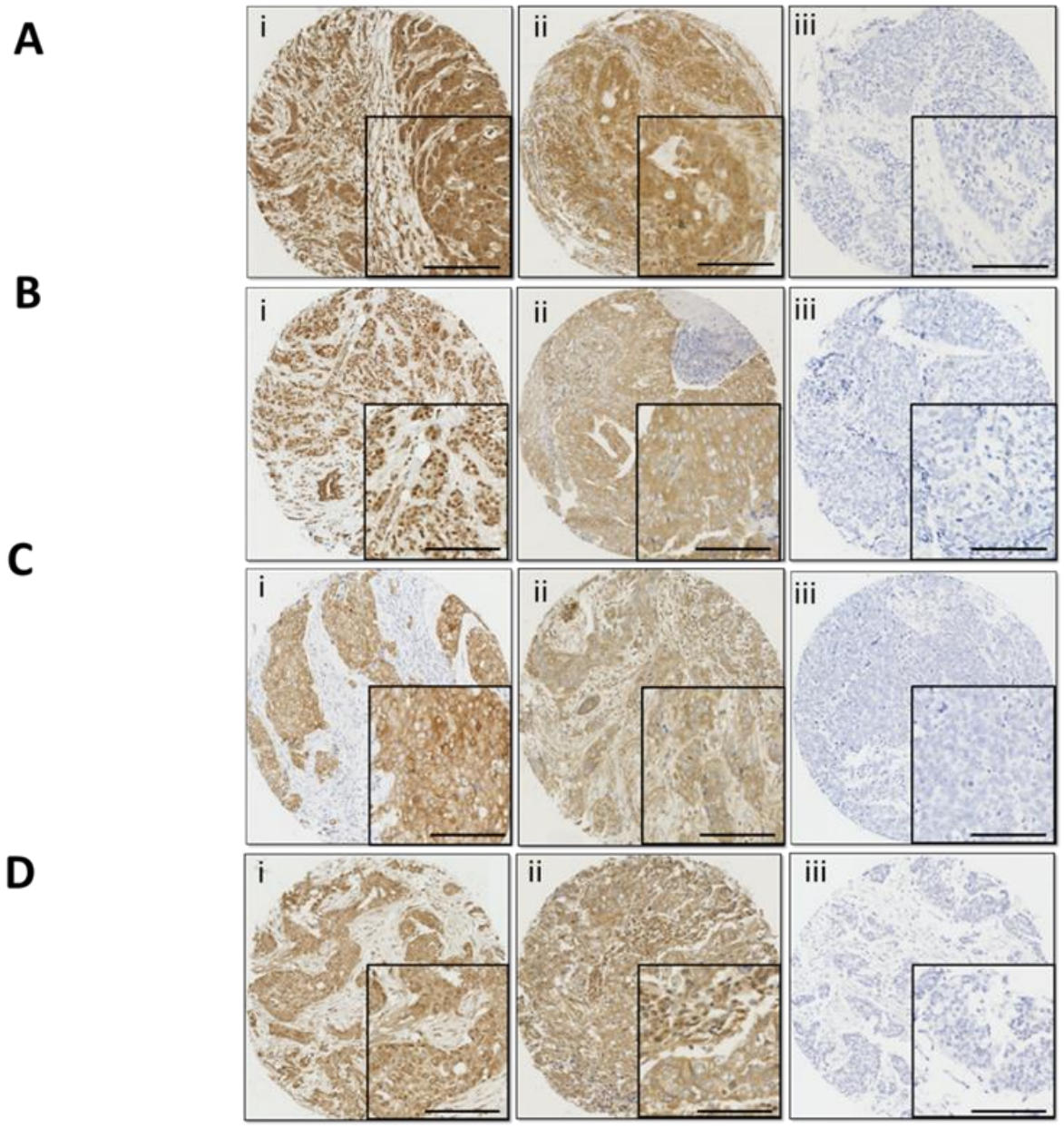


Figure 4.4: Representation of expression patterns of *Sp7, GRB7, DKK1* and DARPP-32 at Threonine-34 using Immunohistochemistry where Figure A, B, C and D correspond to each biomarker, respectively. Figure A, B, C and D are subdivided further into ii, iii and iii that represent: staining of the relevant biomarker, positive and negative control, respectively. The representative graphs are taken at 100× magnification with a 200× magnification inset box in the right bottom corner; scale bar represents 50 μm.

4.3.3 Protein expression and associations with clinicopathological variables

Associations between cytoplasmic and nuclear expression of *Sp7,* phosphorylated DARPP-32 at Threonine-34, *GRB7* and *DKK1* protein and clinicopathological variables were assessed. Low levels of cytoplasmic *Sp7* expression was significantly associated with the development of larger tumour size (X^2=6.220, d.f.=1, *P*=0.013), higher tumour grade (X^2=25.975, d.f.=2, *P*<0.001), pleomorphism (X^2=6.284, d.f.=2, *P*=0.043), mitosis (X^2=9.234, d.f.=2, *P*=0.010), and poor NPI (X^2=6.592, d.f.=2, *P*=0.037). Low nuclear expression of *Sp7* was found to be significantly associated with a larger tumour size (X^2=4.479, d.f.=1, *P*=0.034), higher tumour grade (X^2=14.153, d.f.=2, *P*<0.001), pleomorphism (X^2=9.490, d.f.=2, *P*=0.009), mitosis (X^2=6.732, d.f.=2, *P*= 0.035), positive vascular invasion (X^2=6.506, d.f.=2, *P*=0.011), poor NPI (X^2=11.687, d.f.=2, *P*=0.003), ER negative tumours (X^2=8.165, d.f.=1, *P*=0.004), PR negative tumours (X^2=7.876, d.f.=1, *P*= 0.005), and triple receptor positive tumours (X^2=5.177, d.f.=1, *P*=0.023) (Table 4.1).

Table 4.1: Association between various clinicopathological parameters and cytoplasmic and nuclear *Sp7* expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Cytoplasmic *SP7* expression			Nuclear *SP7* expression		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**
Age						
<50 years	322(78.3%)	90 (21.8%)	0.241	199(48.3%)	213(51.7%)	0.891
≥50 years	751 (80.9%)	177(19.0%)		444(47.9%)	483(52.1%)	
Tumour size						
<2.0 cm	634(77.9%)	180 (22.1)	**0.013**	372(45.7%)	442(54.3%)	**0.034**
≥2.0 cm	439(83.5%)	87(16.5%)		271(51.6%)	254(48.4%)	
Grade						
1	132 (68.0%)	62(32.0%)	**< 0.001**	71 (38.6%)	123(63.4%)	**< 0.001**
2	422 (79.2%)	111(20.8%)		253(47.6%)	279(52.4%)	
3	519 (84.7%)	94(15.3%)		319(52.0%)	294(48.0%)	
Pleomorphism						
1	12(60.0%)	8 (40.0%)	**0.043**	4(20.0%)	16(8.0%)	**0.009**
2	288(78.5%)	79 (21.5%)		163(44.5%)	203(55.5%)	
3	773(81.1%)	180(18.9)		476(49.9%)	477(50.1%)	
Mitosis						
1	485(76.6%)	148(23.4%)	**0.01**	281 (44.5%)	351(55.5%)	**0.035**
2	228 (84.1%)	43 (15.9%)		144(53.1%)	127(46.9%)	
3	360(82.6%)	76 (17.4%)		218(50.0%)	218(50.0%)	
Vascular invasion						
Negative	760 (79.3%)	198(20.7%)	0.281	439(45.8%)	519(54.2%)	**0.011**
Positive	313(81.9%)	69 (18.1%)		204(53.5%)	177(46.5%)	
Stage						
1	657 (79.8%)	166(20.2%)	0.144	383(46.5%)	440(53.5%)	0.297
2	297 (78.4%)	82(21.6%)		188(49.0%)	191(54.4%)	
3	118(86.1%)	19 (13.9%)		72(52.9%)	64(47.1%)	
NPI						
Good	342(77.0%)	102(23.0%)	**0.037**	185(41.7%)	259(58.3%)	**0.003**
Medium	542 (80.3%)	133(19.7%)		340(50.4%)	335(49.6%)	
Poor	188 (85.5%)	32 (14.5%)		118(53.9%)	101(46.1%)	
ER status						
Negative	209(77.7%)	60(22.3%)	0.278	150 (55.8%)	119 (44.2%)	**0.004**
Positive	863(80.7%)	207(19.3%)		492(46.0%)	577(54.0%)	
PR status						
Negative	461 (82.5%)	98 (17.5%)	0.06	293(52.4%)	266(47.6%)	**0.005**

Table Continued

	Cytoplasmic *SP7* expression			Nuclear *SP7* expression		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**
Positive	863 (78.3%)	207 21.7%)		345(44.6%)	428(55.4%)	
HER2 status						
Negative	930(80.0)	232(20.0%)	0.953	547(47.1%)	614(52.9%)	0.104
Positive	142(80.2%)	35 (19.8%)		95 (53.7%)	82 (46.3%)	
Triple negative						
Negative	909(80.0%)	227(20.0%)	0.978	529(46.6%)	606(53.4%)	**0.023**
Positive	153(80.1%)	38(19.9%)		106(55.5%)	85(44.5%)	
Lymph node status						
Negative	657(79.8%)	166(20.2%)	0.79	383(46.5%)	440(53.5%)	0.16
	415(80.4%)	101(19.0%)		260(50.5%)	255(49.5%)	
positive						
Ki67 index Groups						
Low	406(77.6%)	117(22.4%)	0.231	233(44.6%)	290(55.4%)	0.419
High	389(80.7%)	93(19.3%)		227(47.1%)	255(52.9%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.05.

Low levels of *GRB7* cytoplasmic expression was significantly associated with older patient age (X^2=12.308, d.f.=1, *P*<0.001), low tumour grade (X^2=103.669, d.f.=2, *P*<0.001), pleomorphism (X^2=51.487, d.f.=2, *P*<0.001), mitosis (X^2=96.619, d.f.=2, *P*<0.001), absence of vascular invasion (X^2=5.833, d.f.=1, *P*=0.016), low tumour stage (X^2=32263, d.f.=2, *P*<0.001), good NPI value (X^2=63.142, d.f.=2, *P*<0.001), ER positive tumours (X^2=58.281, d.f.=1, *P*<0.001), PR positive tumours (X^2=65.761, d.f.=1, *P*<0.001), HER2 negative tumours (X^2=655.473, d.f.=1, *P*<0.001), triple receptor positive tumours (X^2=4.586, d.f.=1 , *P*=0.032), negative lymph node status (X^2=6.784, d.f.=1, *P*=0.009) , and low ki67 index (X^2=34.862, d.f.=1, *P*<0.001). Low nuclear expression of *GRB7* was found to be significantly associated with older patient age (X^2=7.007, d.f.= 1, *P*=0.008), low tumour grade (X^2=94.413, d.f.=2, *P*<0.001), pleomorphism(X^2=41964, d.f.=2, *P*<0.001), mitosis (X^2 =88.882, d.f.=2, *P*<0.001), negative vascular invasion (X^2=6.817, d.f.=1, *P*=0.009), low tumour stage (X^2=22.317, d.f.=2, *P*<0.001), good

NP1 (X^2=48.610, d.f.=1, *P*<0.001), ER positive tumours (X^2=58.682, d.f.=1, *P*<0.001), PR positive tumours (X^2=64.840, d.f.=1, *P*<0.001), HER2 negative tumours (X^2=521347, d.f.=1, *P*<0.001), negative lymph node status (X^2 =6.81, d.f.=1, *P*=0.009), and low Ki67 index (X^2 =18.637, d.f.=1, *P*< 0.001)(Table 4.2).

Table 4.2: Association between various clinicopathological parameters and cytoplasmic and nuclear *GRB7* expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Cytoplasmic *GRB7* expression			**Nuclear *GRB7* expression**		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**
Age						
<50 years	371(84.3%)	69 (15.7%)	**<0.001**	334 (76.8%)	101 (23.2%)	**0.008**
≥50 years	878 (90.7%)	90 (9.3%)		794 (82.8%)	165(17.2%)	
Tumour size						
<2.0 cm	773(89.4%)	92 (10.6)	0.326	698 (81.4%)	160 (18.6%)	0.602
≥2.0 cm	476(87.7%)	67(12.3%)		430 (80.2%)	106(19.8%)	
Grade						
1	213 (98.2%)	4(1.8%)	**<0.001**	198 (91.7%)	18 (8.3%)	**<0.001**
2	537 (95.7%)	24 (4.3%)		445 (89.5%)	58 (10.5%)	
3	499 (79.2%)	131(20.83%)		435 (69.6%)	190 (30.4%)	
Pleomorphism						
1	22(100.0%)	0 (0.0%)	**<0.001**	21(95.5%)	1(4.5%)	**<0.001**
2	395(97.8%)	9 (2.2%)		364(91.0%)	36 (9.0%)	
3	832(84.7%)	150(15.3)		743(76.4%)	229 (23.6%)	
Mitosis						
1	656(96.9%)	21(3.1%)	**<0.001**	605 (90.6%)	63 (9.4%)	**<0.001**
2	239 (85.7%)	40 (14.3%)		217(78.3%)	60 (21.7%)	
3	354(78.3%)	98 (21.7%)		306 (68.2%)	143(31.8%)	
Vascular invasion						
Negative	915 (90.0%)	102 (10.0%)	**0.016**	832(82.6%)	175 (17.4%)	**0.009**
Positive	334(85.4%)	57 (14.6%)		296(76.5%)	91(23.5%)	
Stage						
1	792 (90.4%)	84 (9.6%)	**<0.001**	724 (83.3%)	145 (16.7%)	**<0.001**
2	352 (90.0%)	39(10.0%)		311 (81.0%)	73 (19.0%)	
3	104(74.3%)	36 (25.7%)		93(66.4%)	47(33.6%)	
NPI						
Good	471(97.1%)	14 (2.9%)	**<0.001**	433 (90.2%)	47 (9.8%)	**<0.001**

Table Continued

	Cytoplasmic *GRB7* expression			Nuclear *GRB7* expression		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**
Medium	601 (86.2%)	96 (13.8%)		532(78.2%)	150 (21.8%)	
Poor	176(78.2%)	49 (21.8%)		156 (69.6%)	68(30.4%)	
ER status						
Negative	213(75.8%)	68(24.2%)	**<0.001**	180 (64.7%)	98 (35.3%)	**<0.001**
Positive	1035(91.4%)	91(8.1%)		947 (84.9%)	168(15.1%)	
PR status						
Negative	462 (80.5%)	112(19.5%)	**<0.001**	404 (70.9%)	166 (29.1%)	**<0.001**
Positive	780 (94.4%)	46 (5.6%)		719 (88.1%)	97(11.9%)	
HER2 status						
Negative	1186(97.1)	35 (2.9%)	**<0.001**	1091(90.3%)	117 (9.7%)	**<0.001**
Positive	62(33.3%)	124 (66.7%)		36 (19.5%)	149 (80.5%)	
Triple negative						
Negative	1048(88.1)	142 (11.9%)	**0.032**	953(80.9%)	225 (19.1%)	0.523
Positive	191(93.2%)	14(16.8%)		168 (82.8%)	35(17.2%)	
Lymph node status						
Negative	792(90.4%)	84(9.6%)	**0.009**	724(83.3%)	145(16.7%)	**0.004**
Positive	456(85.9%)	75(14.1%)		404(77.1%)	120(22.9%)	
Ki67 index Groups						
Low	543(97.8%)	12(2.2%)	**< 0.001**	498(91.0%)	49(9.0%)	**< 0.001**
High	442(88.9%)	55(11.1%)		404(81.9%)	89(18.1%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.05

Low levels of *DKK1* cytoplasmic expression was significantly associated with the development of larger tumours (X^2=5.177, d.f.=1, *P*=0.023), higher tumour grade (X^2=49.148, d.f.=1, *P*<0.001), pleomorphism (X^2=17.603, d.f.=2, *P*<0.001), mitosis (X^2 =49610, d.f.=2, *P*<0.001), intermediate NPI (X^2=6.592, d.f.=2, *P*=0.037), ER negative tumours (X^2=66.761, d.f.=1, *P*=0.001), negative PR status (X^2=45.770, d.f.=1, *P*<0.001), HER2 positive tumours (X^2=6.632, d.f.=1, *P*=0.010), triple receptor positive tumours (X^2=56.570, d.f.=1, *P*<0.001), and high Ki67 index (X^2=21.096, d.f.=1, *P*<0.001). Low nuclear expression of *DKK1* was found to be significantly associated with younger patient age (X^2=7.924, d.f.=1, *P*=0.005), larger tumour size (X^2=6.621, d.f.=1,

P=0.010), high tumour grade (X^2=14.153, d.f.=2, *P*<0.001), pleomorphism (X^2=27.793, d.f.=2, *P*<0.001), mitosis (X^2=48.622, d.f.=2, *P*<0.001), poor NPI value (X^2=41.645, d.f.=2, *P*<0.001), ER negative tumours (X^2=82.423, d.f.=1, *P*=0.001), PR negative tumours (X^2 =33.601, d.f.=1, *P*<0.001), triple receptor positive tumours (X^2=68.76, d.f.=1, *P*<0.001), and high Ki67 index (X^2=16.339, d.f.=1, *P*<0.001) (Table 4.3).

Table 4.3: Association between various clinicopathological parameters and cytoplasmic and nuclear *DKK1* expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Cytoplasmic *DKK1* expression			**Nuclear *DKK1* expression**		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**
Age						
<50 years	247(73.1%)	91 (26.9%)	0.055	137 (40.5%)	201 (59.5%)	**0.005**
≥50 years	469 (67.2%)	229 (32.8%)		221 (31.7%)	477(68.3%)	
Tumour size						
<2.0 cm	414 (66.5%)	209 (33.5)	**0.023**	196 (31.5%)	427 (68.5%)	**0.01**
≥2.0 cm	302 (73.1%)	111 (26.9%)		162 (39.2%)	25 (60.8%)	
Grade						
1	87 (54.4%)	73 (45.6%)	**<0.001**	34 (21.3%)	126(78.8%)	**<0.001**
2	245(62.3%)	148 (37.7%)		105 (26.7%)	288 (73.3%)	
3	384 (79.5%)	99 (20.5%)		219 (45.3%)	264 (54.7%)	
Pleomorphism						
1	6(40.0%)	9 (60.0%)	**<0.001**	2(13.3%)	13(86.7%)	**<0.001**
2	175(61.6%)	109 (38.4%)		68 (23.9%)	216 (76.1%)	
3	535(72.6%)	202 (27.4)		288(39.1%)	449(60.9%)	
Mitosis						
1	280 (58.8%)	196(41.2%)	**<0.001**	120 (25.2%)	356 (74.8%)	**<0.001**
2	146 (71.6%)	58 (28.4%)		66 (32.4%)	138 (67.6%)	
3	240(81.5%)	66 (18.5%)		172 (48.3%)	184(51.7%)	
Vascular invasion						
Negative	500 67.6%)	240 (32.4%)	0.089	250(33.8%)	490 (66.2%)	0.409
Positive	216(73.0%)	80 (27.0%)		108 (36.5%)	188(63.5%)	

Table Continued

Table continued

	Cytoplasmic *DKK1* expression			Nuclear *DKK1* expression		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**
Stage						
1	425 (67.5%)	205 (32.5%)	0.17	207 (32.9%)	423 (67.1%)	0.168
2	215 (73.4%)	78 (26.6%)		103 (35.2%)	190 (64.8%)	
3	75(67.0%)	78 (26.6%)		47(42.0%)	65(58.0%)	
NPI						
Good	187(55.5%)	150 (44.5%)	**0.037**	70 (20.8%)	267(79.2%)	**<0.001**
Medium	399 (76.1%)	125(23.9%)		215(41.0%)	309 (59.0%)	
Poor	129(74.1%)	45 (25.9%)		72 (41.4%)	102(58.6%)	
ER status						
Negative	204(91.5%)	191(8.5%)	**0.001**	134 (60.1%)	89 (39.9%)	**0.001**
Positive	511(62.9%)	301 (37.1%)		223 (27.5%)	589 (72.5%)	
PR status						
Negative	346 (80.7%)	831 (19.3%)	**<0.001**	191 (44.5%)	238 (55.5%)	**<0.001**
Positive	366 (60.9%)	235 (39.1%)		163 (27.1%)	438 (72.9%)	
HER2 status						
Negative	603(67.6)	289 (32.4%)	**0.01**	299(32.5%)	593 (66.5%)	0.1
Positive	112(78.3%)	31(21.7%)		58 (40.6%)	85 (59.9%)	
Triple negative						
Negative	556(64.4)	307(35.6%)	**<0.001**	250(29.0%)	613 (71.0%)	**<0.001**
Positive	155(93.9%)	10 (6.1%)		103 (62.4%)	62(37.6%)	
Lymph node status						
Negative	425(67.5%)	205(32.5%)	0.159	207(32.9%)	423(67.1%)	0.167
Positive	290(71.6%)	115(28.4%)		150(37.0%)	255(63.0%)	
Ki67 index Groups						
Low	236(60.7%)	153(39.3%)	**<0.001**	100(25.7%)	289(74.3%)	**<0.001**
High	292(76.0%)	92(24.0%)		151(39.9%)	233(60.7%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.05

Low levels of phosphorylated DARPP-32 at Threonine-34 cytoplasmic expression were significantly associated with the development of larger tumour size (X^2=7.057, d.f.=1, *P*=0.008), pleomorphism (X^2=8.778, d.f.=2, *P*=0.012), positive vascular invasion (X^2=5.843, d.f.=1,

P=0.016), higher tumour stage (X^2=17.966,d.f.=2, *P*<0.001), poor NPI (X^2 =12.216, d.f.=2, *P*=0.002), positive lymph node status(X^2 =11.980, d.f.=1, *P*<0.001), and low Ki67 index (X^2=6.671, d.f. =1, *P*=0.010). Low nuclear expression of phosphorylated DARPP-32 at Threonine-34 expression was found to be significantly associated with a moderate tumour stage (X^2=8.644, d.f.=2, *P*=0.013), and positive lymph node status (X^2=8.541, d.f.=1, *P*=0.003) (Table 4.4).

***Tab*le 4.4:** Association between various clinicopathological parameters and cytoplasmic and nuclear level of phosphorylated DARPP-32 at Threonine-34 expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Cytoplasmic DARPP-32 Threonine-34 expression			**Nuclear DARPP-32Threonine-34 expression**		
	Low	High	*P* value	Low	High	*P* value
Age						
<50 years	87 (20.7%)	334 (79.3%)	0.35	340 (80.8%)	81 (19.2%)	0.477
≥50 years	196 (23.0%)	657 (77.0%)		702 (82.4%)	150 (17.6%)	
Tumour size						
<2.0 cm	152 (19.7%)	619 (80.3)	**0.008**	625 (81.2%)	145 (18.8%)	0.433
≥2.0 cm	131 (26%)	372 (74%)		417 (82.9%)	86 (17.1%)	
Grade						
1	30 (15.7%)	161 (84.3%)	0.064	150 (78.5%)	41 (21.5%)	0.399
2	115 (23.4%)	377 (76.6%)		403 (81.9%)	89 (18.1%)	
3	138 (23.4%)	453 (76.6%)		484 (82.9%)	101 (17.1%)	
Pleomorphism						
1	2 (11.1%)	16 (88.9%)	**0.012**	15(83.3%)	3 (16.7%)	0.836
2	59(17.2%)	285 (82.8%)		278 (80.8%)	66 (19.2%)	
3	222(24.3%)	690 (75.5)		799(82.2%)	162 (17.8%)	
Mitosis						
1	125 (21.3%)	462 (78.8%)	0.36	476 (81.1%)	111 (18.9%)	0.480
2	67 (25.5%)	196 (74.5%)		222 (84.4%)	41 (15.6%)	
3	91(21.5%)	333 (78.5%)		344 (81.3%)	79(18.7%)	
Vascular invasion						
Negative	185 (20.4%)	721 (79.6%)	**0.016**	731 (80.7%)	175 (19.3%)	0.089
Positive	48 (26.6%)	270 (73.4%)		311 (84.7%)	56(15.3%)	
Stage						
1	147 (19.0%)	627 (81.0%)	**<0.001**	613 (79.3%)	160 (20.7%)	**0.013**

Table Continued

	Cytoplasmic DARPP-32 Threonine-34 expression			Nuclear DARPP-32Threonine-34 expression		
	Low	High	*P* value	Low	High	*P* value
2	90 (24.5%)	277 (75.5%)		316 (86.1%)	51 (13.9%)	
3	46(34.8%)	86 (65.2%)		112(84.8%)	20 (15.2%)	
NPI						
Good	77 (18.6%)	337 (81.4%)	**0.002**	325 (78.5%)	89 (21.5%)	0.072
Medium	141 (21.8%)	507 (78.2%)		536(82.8%)	111 (17.2%)	
Poor	65 (30.8%)	146 (69.2%)		180 (85.3%)	31 (14.7%)	
ER status						
Negative	67 (25.0%)	201(75.0%)	0.22	221 (82.5%)	47 (17.5%)	0.766
Positive	216 (21.5%)	789 (78.5%)		820 (81.7%)	184 (18.3%)	
PR status						
Negative	373 (36.2%)	83 (8.1%)	0.276	303 (29.5%)	153 (14.9%)	0.287
Positive	445 (43.2%)	128 (12.4%)		360 (35.0%)	212 (20.6%)	
HER2 status						
Negative	125 (23.9%)	399 (76.1%)	0.446	436 (83.2%)	88 (16.8%)	0.331
Positive	158(21.3%)	585 (78.7%)		600 (80.9%)	142 (19.1%)	
Triple negative						
Negative	233 (21.9%)	833 (78.1%)	0.416	868(81.5%)	197 (18.5%)	0.469
Positive	48 (24.5%)	148 (75.5%)		164 (83.7%)	32 (16.3%)	
Lymph node status						
Negative	147(19.0%)	627(81.0%)	**<0.001**	613(79.3%)	160(20.7%)	**0.003**
Positive	136(27.3%)	363(72.7%)		428(85.8%)	71(14.2%)	
Ki67 index Groups						
Low	116(23.3%)	382(76.7%)	**0.01**	412(82.7%)	86(17.3%)	0.102
High	76(16.6%)	382(83.4%)		359(78.6%)	98(21.4%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, P values highlighted in bold are <0.05

4.3.4 Protein expression on breast cancer specific survival

Kaplan-Meier analysis was used to assess the impact of *Sp7, GRB7*, *DKK1* and phosphorylated DARPP-32 at Threonine-34 protein expression on breast cancer specific survival. Low *Sp7* nuclear expression was significantly associated with adverse breast cancer specific survival (*P*=0.016) (Figure 4.5B). Low nuclear and cytoplasmic expression of *GRB7* was significantly associated with improved prognosis of breast cancer patients (Figure 3.6C and 3.6D) (*P*=0.012, *P*=0.003 respectively). Low *DKK1* nuclear/cytoplasmic expression was significantly associated

with adverse breast cancer specific survival (Figure 4.5E and 4.5F) (*P*=0.002, *P*=0.031 respectively). Low nuclear and cytoplasmic expression of phosphorylated DARPP-32 at Threonine-34 was significantly associated with adverse breast cancer specific survival (*P*=0.002, *P*<0.001 respectively) (Figure 4.5G and 4.5H).

Multivariate analysis was performed via Cox's proportional hazard method, and included tumour size, tumour grade, tumour stage, NPI, ER status, PR status, HER2 status and vascular invasion. Hazard ratio of 0.826 with 95% confidence interval of 0.644-1.058 (*P*=0.130) was observed for nuclear *Sp7* expression, and hazard ratio of 1.076 with 95% confidence interval of 0.774-1.495 (*P*=0.664) was observed for cytoplasmic *Sp7* expression. Hazard ratio of 0.953 with 95% confidence interval of 0.617-1.472 (*P*=0.828) was observed for nuclear GRB7 expression, and hazard ratio of 1.090 with 95% confidence interval of 0.656-1.809 (*P*=0.740) was observed for cytoplasmic *GRB7* expression. Hazard ratio of 0.834 with 95% confidence interval of 0.625-1.113 (*P*=0.217) was observed for nuclear *DKK1* expression, and hazard ratio of 0.934 with 95% confidence interval of 0.668-1.304 (*P*=0.687) was observed for cytoplasmic *DKK1* expression. Hazard ratio of 0.658 with 95% confidence interval of 0.443-0.978 (*P*=0.038) was observed for nuclear phosphorylation of DARPP-32 at Threonine-34 expression, and a hazard ratio of 0.763 with 95% confidence interval of 0.579-1.008 (*P*=0.057) was observed for cytoplasmic phosphorylation of DARPP-32 at Threonine-34 expression.

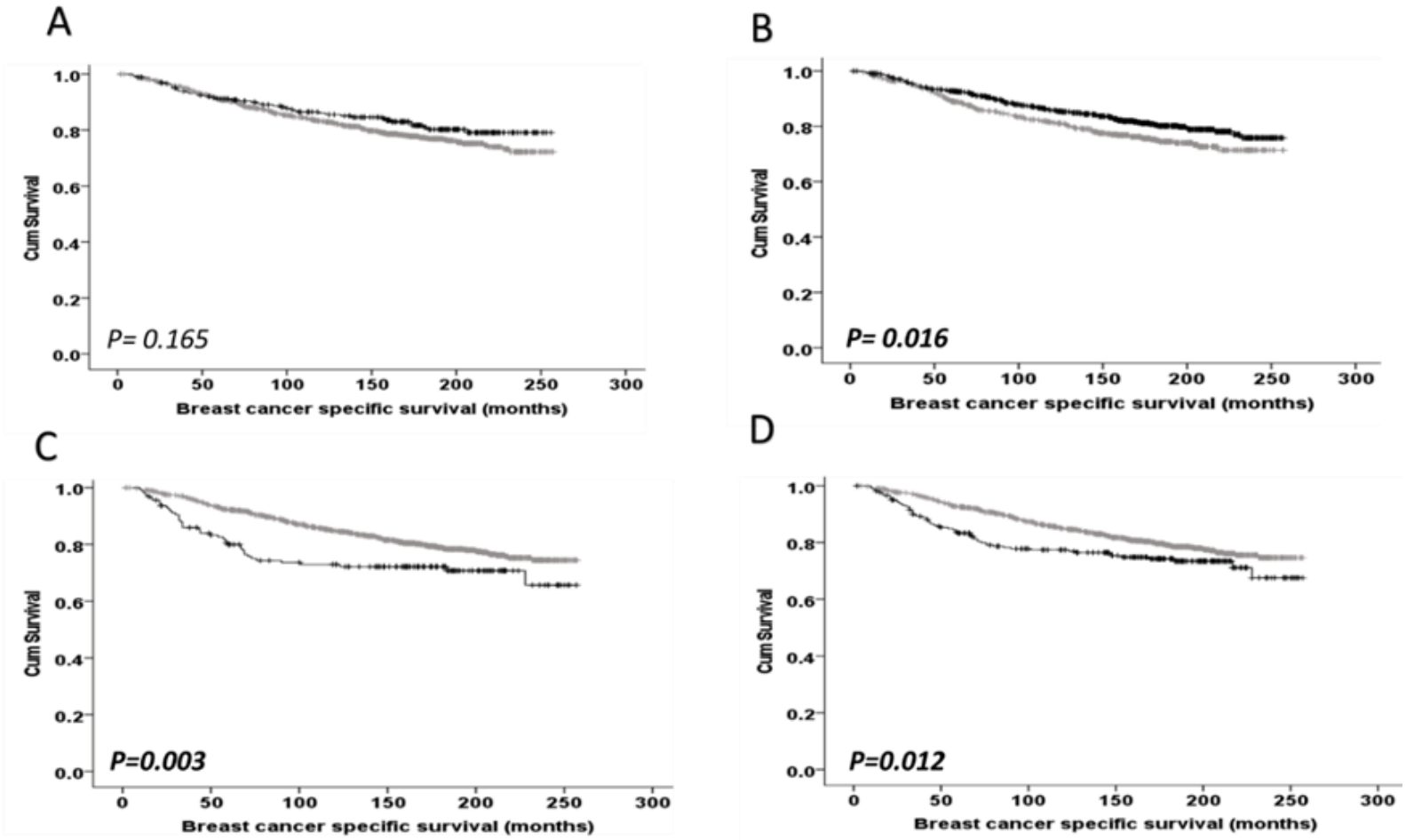


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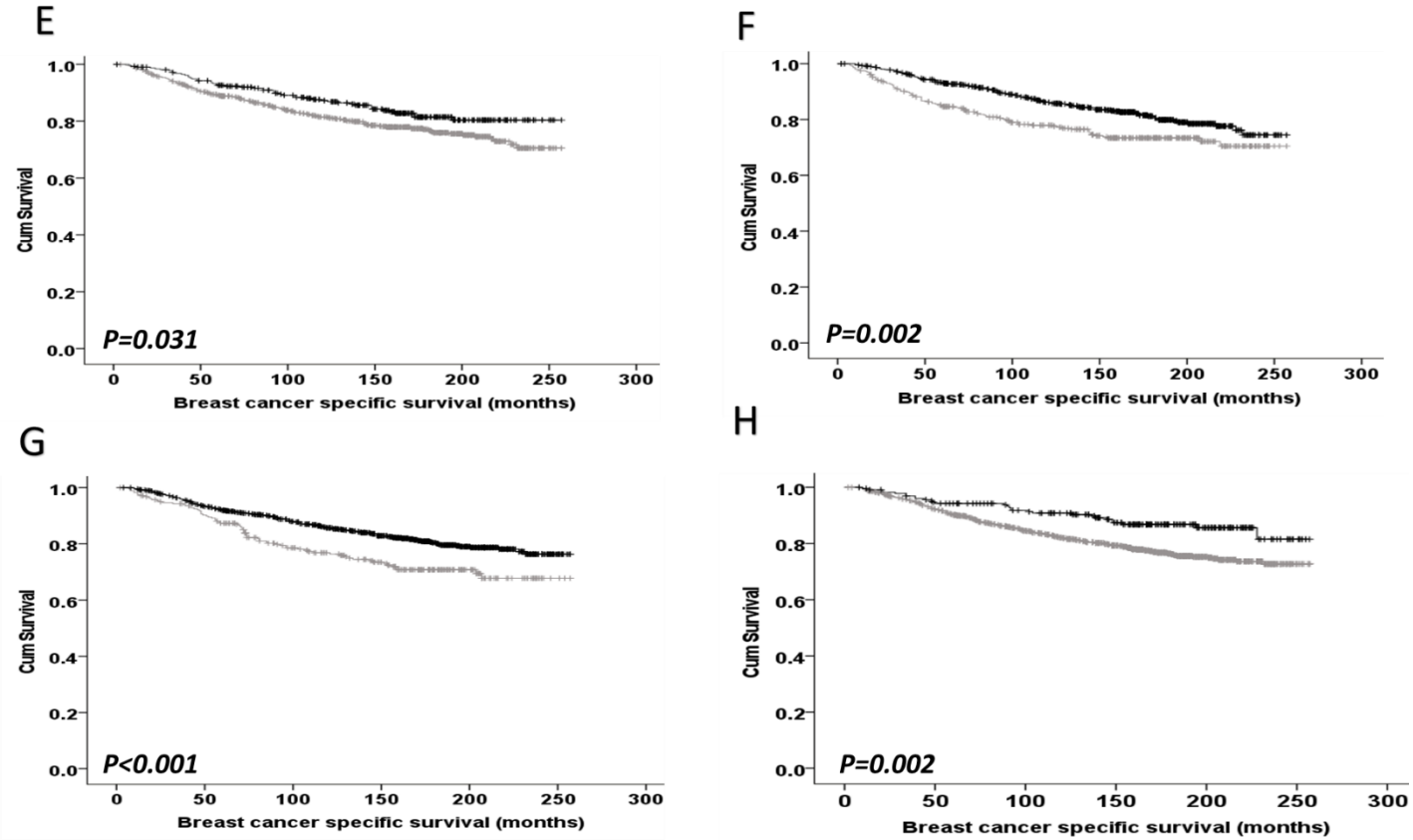


Figure 4.5: Kaplan-Meier analysis where the grey lines denote low expression and the black lines denote high expression within cytoplasm and the nucleus: *Sp7* (Figure 4.5A and B), *GRB7* (Figure 4.5C and D), *DKK1* (Figure 4.5 E and F), and phosphorylation of DARPP-32 at Threonine-34 (Figure 4.5 G and H).

4.3.5 mRNA expression in the METABRIC Cohort

The association between *Sp7, GRB7, DKK1* and *PPP1R1B* mRNA expression levels and patient survival in the METABRIC cohort were assessed. One probe was available for *SP7* assessment within the METABRIC cohort; *Sp7* probe (ILMN_1689461) was not associated with clinicopathological parameters (Table 4.5).

Table 4.5: Associations between various clinicopathological variables and *Sp7* mRNA expression in the METABRIC cohort. The *P* values highlighted in bold are <0.05.

	***SP7* mRNA expression**		
	Low	**High**	***P* value**
Age			
<40 years	59(3.0%)	61 (3.1%)	0.851
≥40 years	931 (47.0%)	929 (46.9%)	
Cellularity			
High	493(25.7%)	474(24.7%)	
Moderate	368(19.2%)	366(19.1)	0.712
Low	103(5.4%)	112(5.8%)	
Grade			
1	89 (4.7%)	80(40.2%)	0.794
2	390 (20.6%)	380 (20.1%)	
3	475 (25.1%)	477(25.2%)	
Genefu			
ER-/HER-	76(7.6%)	74(7.4%)	
ER+/HER-High	183(18.4%)	184 (18.5%)	0.965
ER+/HER-Low	181(18.2%)	187 (18.8%)	
HE2+	57(5.7%)	53(5.3%)	
Pam50 subtype			
Basal	161(8.2%)	170(8.6%)	
HE2	113(5.7%)	126(6.4%)	
Luminal A	358(18.1%)	357(18.1%)	0.455
Luminal B	261 (13.2%)	299 (11.6%)	
Normal	94(4.8%)	105 (5.3%)	

Table Continued

	***SP7* mRNA expression**		
	Low	**High**	***P* value**
ER status			
Negative	768 (38.8%)	740 (37.4%)	0.14
Positive	222(11.2%)	250 (12.6%)	
Stage			
0	241(15.7%)	249(16.3%)	
1	179(11.7%)	192(12.5%)	
2	272(17.8%)	299 (19.5%)	0.758
3	46 (3.0%)	44(2.9%)	
4	3(0.2%)	7 (10.5%)	
NPI			
Good	347(17.5%)	344 (16.9%)	0.715
Medium	548 (27.7%)	552 (27.9%)	
Poor	95(4.8%)	104 (5.3%)	
HER2 status			
Negative	123(6.2%)	123(6.2%)	1
Positive	867(43.8%)	867 (43.8%)	
Tumour size			
<2 cm	455(22.7%)	414 (21.1%)	0.17
≥2 cm	536(27.3%)	565(28.8%)	
Lymph node status			
Negative	532(27.0%)	503(25.5%)	0.216
Positive	456(23.1%)	482(24.4%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.05

Three probes were available for *GRB7* assessment including probe 1 *(ILMN-2405254),* probe 2 *(ILMN-1740762)* and probe 3 *(ILMN-1798582).*

Probe 1 maps to *GRB7* transcript IDs that are identified as protein coding: *ENST00000309156.9*, *ENST00000394211.7*, *ENST00000445327* .6, *ENST00000394209.6,* and *ENST00000394204.1* (ILMN-2405254). Probe 2 maps to *ENST00000309156.9, ENST00000394211.7,* ENST00000445327.6, and *ENST00000394204.1,* but

not *ENST00000394209.6* (ILMN_1740762). Probe 3 maps to *GRB7* transcript ID *ENST00000309156.9,* although it also maps to a number of GRB7 transcripts that do not result in protein *(ILMN-1798582).* There is little evidence on the function of functional changes resulting from different *GRB7* transcripts. According to genome browser Ensembl (http://www.ensembl.org/) all transcript IDs mentioned previously belong to members of the fourteen splice variant products of *GRB7* gene *ENSG00000141738.*

Expression of *GRB7* in probe 1, 2 and 3 mRNA were assessed for associations with various clinicopathological data (Table 4.6). Low *GRB7* probe1 expression was associated with high tumour grade (X^2=24.778, d.f.=2, *P*<0.001), ER+/HER-low proliferation genefu (X^2=146.453, d.f.=4, *P*<0.001), luminal A PAM50 subtype (X2 =120.683, d.f.=5, *P*<0.001), stage (X^2=11.544, d.f.=5, *P*=0.042), NPI (X^2=11.649, d.f.=2, *P*=0.003), and HER2 negative tumours (X^2=271.839, d.f.=1, *P*<0.001). Low *GRB7* probe 2 expression was associated with high tumour grade (X^2=17.637, d. f.=2, *P*<0.001), ER+/HER-low proliferation genefu (X^2=144.088, d.f.=4, *P*<0.001), luminal A PAM50 subtype (X^2=113.789, d.f.=5, *P*<0.001), ER positive tumours (X^2=10.693, d.f.=1, *P*<0.001), stage (X^2=16.130, d.f. =5, *P*=0.006), intermediate NPI (X^2=7.432, d.f.=2, *P*=0.024), and HER2 negative tumours (X^2=267.364, d.f.=1, *P*<0.001). Low *GRB7* probe 3 expression was associated with high tumour grade (X^2=16.721, d.f.=2, *P*<0.001), ER+/HER-low proliferation genefu (X^2=113.833, d.f.= 4, *P*<0.001), luminal A PAM50 subtype (X^2 =68.089, d.f.=5, *P*<0.001), ER positive tumours (X^2=5.886, d.f.=1, *P*=0.015), intermediate NPI (X^2=26.925, d.f.=2, *P*<0.001), HER2 negative tumours (X^2=212.573, d.f.=1, *P*<0.001), and lymph node status (X^2=11.272, d.f.=1, *P*<0.001).

Table 4.6: Associations between various clinicopathological variables and *GRB7* (probe 1, 2 and 3) mRNA expression in the METABRIC cohort. The *P* values highlighted in bold are <0.05

	***GRB7* mRNA**								
	***GRB7* Probe 1**			***GRB7* Probe 2**			***GRB7* Probe 3**		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**	**low**	**High**	***P* value**
Age									
<40 years	60(3.0%)	60 (3.0%)	1	53(2.7%)	67 (3.4%)	0.187	55(2.8%)	65(3.3%)	0.346
≥40 years	930 (47.0%)	930(47.0)		937 (47.3%)	923(46.6%)		935(47.2%)	925(46.7%)	
Cellularity									
Low	102(5.3%)	113(5.9%)		107(5.6%)	108(5.6%)		124(6.5%)	91(4.7%)	
Moderate	376(19.6%)	358(18.7%)	0.581	384(20.0%)	350(18.3%)	0.258	369(19.3%)	365(19.1%)	0.048
High	479(25.0%)	488(25.5%)		467 (24.4%)	500(26.1%)		468(24.4%)	499(26.0%)	
Grade									
1	102 (5.4%)	67(3.5%)	**<0.001**	101 (5.3%)	68 (3.6%)	**<0.001**	90(4.8%)	79(4.2%)	
2	417 (22.1%)	353 (18.7%)		408 (21.6%)	362(19.1%)		423(22.4%)	347(18.4%)	**<0.001**
3	422 (22.3%)	530(28.0)		431 (22.8%)	521(27.6%)		431(22.8%)	521(27.6%)	
Genefu									
ER-/HER-	95(4.8%)	55(2.8%)		94(4.7%)	56(2.8%)		98(4.9%)	52(2.6%)	
ER+/HER-High	184(9.3%)	183 (9.2%)	**<0.001**	183(9.2%)	184(9.3%)	**<0.001**	176(8.9%)	191(9.6%)	
ER+/HER-Low	232(11.7%)	136 (6.9%)		231(11.7%)	137 (6.9%)		201(10.2%)	167(8.4%)	**<0.001**
HE2+	0(0.0%)	110(5.6%)		0(0.0%)	110 (5.6%)		4(0.2%)	106(5.45%)	

Table Continued

	GRB7 mRNA								
	GRB7 Probe 1			*GRB7* Probe 2			*GRB7* Probe 3		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**	**low**	**High**	***P* value**
Pam50 subtype									
Luminal A	400(20.3%)	315(16.0%)		397(20.1%)	318(16.1%)		386(19.6%)	329(16.7%)	
Luminal B	250(12.7%)	240(12.2%)		243(12.3%)	247(12.5%)		236(12.0%)	254(12.9%)	
HER2	42(2.1%)	197(10.0%)	**<0.001**	45 (2.3%)	194 (9.8%)	**<0.001**	64(3.2%)	175(8.9%)	**<0.001**
Basal	193(9.8%)	138 (7.0%)		190(9.6%)	141 (7.1%)		194(9.8%)	137(6.9%)	
Normal	101(5.1%)	98 (5.1%)		110 (5.6%)	89(4.5%)		106(5.4%)	93(4.7%)	
ER status									
Negative	206 (10.4%)	266 (13.4%)	0.002	205(10.4%)	785(39.6%)	**0.001**	231(10.8%)	259(13.1%)	**0.015**
Positive	784(39.6%)	724 (36.6%)		267 (13.5%)	23(36.5%)		777(39.2%)	731(36.9%)	
Stage									
0	221(14.4%)	269(17.6%)		221(14.4%)	269(17.6%)		264(17.2%)	226(14.8%)	
1	186(12.1%)	185(12.1%)		178(11.6%)	193(12.6%)		189(12.3%)	182(11.9%)	
2	314 (20.5%)	257 (16.8%)	**0.042**	324 (21.1%)	247 (16.1%)	**0.006**	286(18.7%)	285(18.6%)	
3	47 (3.1%)	43(2.8%)		45 (2.9%)	45 (2.9%)		40(2.6%)	50(3.3%)	0.275
4	6(0.4%)	4 (0.3%)		5(0.3%)	5(0.3%)		3(0.2%)	7(0.5%)	
NPI									
Good	83(4.2%)	116 (5.9%)	**0.003**	85 (4.3%)	114 (5.8%)	**0.024**	73(3.7%)	126(6.4%)	
Medium	536 (27.1%)	564 (28.5%)		542(27.4%)	558 (28.2%)		532(26.9%)	568(28.7%)	**<0.001**
Poor	371 (18.7%)	310 (15.7%)		363 (18.3%)	318(16.1%)		385(19.4%)	296(14.9%)	

Table Continued

	***GRB7* mRNA**								
	***GRB7* Probe 1**			***GRB7* Probe 2**			***GRB7* Probe 3**		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**	**low**	**High**	***P* value**
HER2 status									
Negative	988(49.9%)	764 (37.7%)	**<0.001**	987(49.8%)	747 (37.7%)	**<0.001**	947(49.2%)	750(38.4%)	**<0.001**
Positive	2(0.1%)	244 (12.3%)		3 (0.2%)	243 (12.3%)		16(0.8%)	230(11.6%)	
Lymph node status									
Negative	518(26.3%)	517(20.2%)	0.87	510(25.8%)	525(26.6%)	0.577	555(28.1%)	480(24.3%)	**<0.001**
Positive	466(23.6%)	472(23.9%)		474(24.0%)	464(23.5%)		432(21.9%)	506(25.6%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.0

The two probes that were available for *DKK1* were: probe *1 (ILMN-1773337)* and probe 2 *(ILMN-2174189).* Probe 1 maps to DKK1 transcript IDs that are identified as protein coding; *ENST00000373970.4*, although it also maps to a number of *DKK1* transcripts that do not result in protein such as *ENST00000467359.5*, and *ENST00000476752.1 (ILMN-1773337)*. Probe 2 maps to similar transcript IDs to probe 1 *(ILMN-2174189)*. There is little evidence on the function of functional changes resulting from different *DKK1* transcripts. Transcript *ENST00000373970.4* is one of the four splice variant products of the *DKK1* gene *ENSG00000107984.*

Expression of *DKK1* probe 1, and probe 2 were assessed for associations with various clinicopathological data (Table 4.7). Low *DKK1* probe 1 expression was associated with older patient age (X^2=20.439, d.f.=1, *P*<0.001), high cellularity (X^2=26.800, d.f.=4, *P*<0.001), grade (X^2=51.050, d.f.=2, *P*<0.001), ER+/HER-high proliferation Genefu (X^2=108.325, d.f.=4, *P*<0.001), luminal A PAM50 subtype (X^2=267.899, d.f.=5, *P*<0.001), positive ER status (X^2=188.048, d.f.=1, *P*<0.001), stage (X^2=13.043, d.f.=5, *P*=0.023), intermediate NPI value (X^2=24.766, d.f.=2, *P*<0.001), HER2 negative tumours (X^2=76.050, d.f.=1, *P*<0.001), and negative lymph node status (X^2=3.833, d.f.=1, *P*=0.050). Low *DKK1* probe 2 expression was associated with luminal A PAM50 subtype (X^2=18.936, d.f.=5, *P*=0.002), ER positive tumours (X^2=12.117, d.f.=1, *P*<0.001), and HER2 negative tumours (X2=4.178, d.f.=1, *P*=0.041) (Table 4.7).

Table 4.7: Associations between various clinicopathological variables and *DKK1* (probe 1 and 2) mRNA expression in the METABRIC cohort. The *P* values highlighted in bold are <0.05

	***DKK1* mRNA expression**					
	***DKK1* Probe 1**			***DKK1* Probe 2**		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**
Age						
<40 years	36(1.8%)	84 (4.2%)	**<0.001**	64(3.2%)	56 (2.8%)	0.451
≥40 years	954 (48.2%)	906(45.8%)		926(46.8%)	934(47.2%)	
Cellularity						
Low	79(4.0%)	136(7.1%)		109(5.7%)	106(5.5%)	
Moderate	365(19.1%)	369(19.3%)	**<0.001**	384(20.0%)	350(18.3%)	0.314
High	523(27.3%)	444(23.2%)		470(24.5%)	497(25.9%)	
Grade						
1	92 (4.9%)	77(4.1%)	**<0.001**	92 (4.9%)	77 (4.1%)	0.461
2	455 (24.1%)	315(16.7%)		391 (20.7%)	379(20.0%)	
3	400 (21.2%)	952(50.3%)		470(24.9%)	482(25.5%)	
Genefu						
ER-/HER-	37(1.9%)	113(5.7%)		71(3.6%)	79(4.0%)	
ER+/HER-High	235(11.9%)	132 (6.7%)	**<0.001**	199(10.1%)	168(8.5%)	0.128
ER+/HER-Low	220(11.1%)	148 (7.5%)		196(9.9%)	172 (8.7%)	
HE2+	29(1.5%)	81(4.1%)		49(2.5%)	61(3.1%)	
Pam50 subtype						
Luminal A	423(21.4%)	292(14.8%)		377(19.1%)	38(17.1%)	
Luminal B	350(17.7%)	140(7.1%)		267(13.5%)	223(11.3%)	
HE2+	60(3.0%)	179(9.1%)	**<0.001**	96 (4.9%)	143 (7.2%)	**< 0.001**
Basal	89 (4.5%)	242(12.3%)		150(7.6%)	181 (9.2%)	
Normal	65(3.3%)	134 (6.8%)		98 (5.0%)	101(5.1%)	
ER status						
Negative	106 (5.4%)	366(18.5%)	**<0.001**	203(10.3%)	269 (13.6%)	**<0.001**
Positive	844(44.6%)	624(31.5%)		787(39.7%)	721(36.4%)	
Stage						
0	274(13.8%)	216(10.9%)		256(12.9%)	234(11.8%)	
1	179(9.0%)	192(9.7%)		185(9.3%)	186(9.4%)	
2	267 (13.5%)	304(15.4%)	**0.023**	278(14.0%)	293(14.8%)	0.834
3	37(1.9%)	59(2.7%)		41 (2.1%)	49 (2.5%)	
4	6(0.3%)	4 (0.2%)		5(0.3%)	5(0.3%)	

Table Continued

	DKK1 mRNA expression					
	DKK1 Probe 1			*DKK1* Probe 2		
NPI						
Good	390(19.7%)	291(14.7%)	**<0.001**	341(17.2%)	340(17.2%)	0.519
Medium	519 (26.2%)	581(29.3%)		557(28.1%)	534(27.4%)	
Poor	81 (4.1%)	118 (6.0%)		92 (4.6%)	107(5.4%)	
HER2 status						
Negative	931(47.0%)	803(40.6%)	**<0.001**	882(44.5%)	852(43.0%)	**0.041**
Positive	59(3.0%)	187 (9.4%)		108 (5.5%)	138 (7.0%)	
Lymph node status						
Negative	540(27.4%)	166(20.2%)	**0.05**	506(25.6%)	529(26.8%)	0.289
Positive	448(22.7%)	490(24.8%)		481(24.4%)	457(23.2%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.05

The three probes were available for *PPP1R1B* were: Probe 1 (*ILMN-1690096)* which represents the coding region that corresponds to the N-terminal of DARPP-32, and probe 2 *(ILMN-1759012)* and probe 3 *(ILMN-2304495)* which represent untranslated regions (5′ and 3′, respectively). The mRNA corresponding to DARPP-32 would align with probe 1 and probe 3, whereas mRNA corresponding to t-DARPP would align with probe 2 and probe 3. Expression of *PPP1R1B* probe 1, 2 and 3 were assessed for associations with various clinicopathological data (Table 4.8). Low *PPP1R1B* probe 1 was associated with high cellularity (X^2= 16.48, d.f.=4, *P*=0.002), high tumour grade (X^2=6.966, d.f.=2, *P*=0.031), luminal A PAM50 subtype (X^2=36.782, d.f.=5, *P*<0.001), positive ER status (X^2=31.256, d.f.=1, *P*<0.001), ER+/HER-high proliferation Genefu (X^2=42.275 d.f.=4, *P*<0.001), and negative HER2 status (X^2=39.288, d.f.=1, *P*<0.001). Low *PPP1R1B* probe 2 expression was associated with high cellularity (X^2=18.162, d.f.=4, *P*<0.001), ER+/HER-high proliferation Genefu (X^2=84.518, d.f.= 4,*P*<0.001), luminal A PAM50 subtype (X^2=144.682, d.f.=5, *P*<0.001), ER negative tumours (X^2=48.470, d.f.=1, *P*<0.001), and HER2 negative tumours (X^2=60.324, d.f.=1, *P*<0.001). Low *PPP1R1B* probe 3 expression was

associated with high tumour grade (X^2=10.537, d.f.=2, *P*=0.005), ER+/HER-high proliferation Genefu (X^2=32.758 ,d.f.=4, *P*<0.001), luminal A PAM50 subtype (X2=34.403, d.f.=5, *P*<0.001), ER positive tumours (X^2=16.924, d.f.=1, *P*<0.001), and HER2 negative tumours (X^2=130.992, d.f.=1, *P*<0.001).

Table 4.8: Associations between various clinicopathological variables and *PPP1R1B* (probe 1,2 and 3) mRNA expression in the METABRIC cohort. The *P* values highlighted in bold are <0.05

	***PPP1R1B* mRNA expression**								
	***PPP1R1B* Probe 1**			***PPP1R1B* Probe 2**			***PPP1R1B* Probe3**		
	Low	**High**	***P* value**	**Low**	**High**	***P* value**	**low**	**High**	***P* value**
Age									
<40 years	54(2.7%)	66 (3.3%)	0.258	54 (2.7%)	66(3.3%)	0.258	61(3.1%)	59(3.0%)	0.851
≥40 years	936(47.3%)	924(46.7%)		936 (47.3%)	924(46.7%)		929(46.9%)	931(47.0%)	
Cellularity									
High	523(27.3%)	444(23.2%)		517(27.0%)	450(23.5%)		500(26.1%)	476(24.4%)	
Moderate	351(18.3%)	383(20.0%)	**0.002**	363 (18.9%)	371 (19.4%)	**0.001**	366(19.1%)	368(19.2%)	0.105
Low	88(4.6%)	127(6.6%)		83 (4.3%)	132(6.9%)		94(4.9%)	121(6.3%)	
Grade									
1	83 (4.4%)	86(4.5%)	**0.031**	78 (4.1%)	91 (4.8%)	0.064	92(4.9%)	77(4.1%)	
2	413 (21.8%)	357(18.9%)		409 (21.6%)	361 (19.1%)		417(22.1%)	353(18.7%)	**0.005**
3	450 (23.8%)	502(26.5%)		457 (24.2%)	495 (26.2%)		445(23.5%)	507(26.8%)	
Genefu									
ER-/HER-	72(7.2%)	78(7.8%)		68(6.8%)	82(8.2%)		73((7.3%)	77(7.7%)	
ER+/HER-High	223(22.4%)	144(14.5%)	**<0.001**	251(25.2%)	116(11.7%)	**<0.001**	207(20.8%)	160(16.1%)	
ER+/HER-Low	184(18.5%)	184(18.5%)		177(17.8%)	191 (19.2%)		187(18.8%)	181(18.2%)	**<0.001**
HE2+	29(2.9%)	81(8.1)		26(2.6%)	84 (8.4%)		28(2.8%)	82(8.2%)	
Pam50 subtype									
Basal	138 (6.7%)	193(9.8%)		133(6.7%)	198(10.0%)		152(7.7%)	179(9.1%)	
HE2	100(5.1%)	139(7.0%)		76 (3.9%)	163(8.3%)		84(4.3%)	155(7.9%)	
Luminal A	386(19.6%)	329(16.7%)	**<0.001**	368(18.6%)	347 (17.6%)	**<0.001**	378(19.1%)	337(17.1%)	**<0.001**

Table Continued

	***PPP1R1B* mRNA expression**								
	***PPP1R1B* Probe 1**			***PPP1R1B* Probe 2**			***PPP1R1B* Probe3**		
Luminal B	281 (14.2%)	209 (10.6%)		343(17.4%)	147 (7.4%)		276(14.0%)	214(10.8%)	
Normal	82(4.2%)	117 (5.9%)		67 (3.4%)	132(6.75%)		96(4.9%)	103(5.2%)	
ER status									
Positive	807 (40.8%)	701(35.4%)	**<0.001**	820(41.4%)	688 (34.7%)	**<0.001**	793(40.1%)	715(36.1%)	**<0.001**
Negative	183(9.2%)	289(14.6%)		170 (8.6%)	302(15.3%)		197(9.9%)	275(13.9%)	
Stage									
0	254(16.6%)	236(15.4%)		236(15.4%)	254(16.6%)		221(14.4%)	269(17.6%)	
1	177(11.6%)	194(12.7%)		182(11.9%)	189(12.3%)		188(12.3%)	183(11.9%)	
2	294 (19.2%)	277(18.1%)	0.236	301 (19.6%)	270 (17.6%)	0.579	303(19.8%)	268(17.5%)	0.106
3	36 (2.3%)	54(3.5%)		42 (2.7%)	48 (3.1%)		49(3.2%)	41(2.7%)	
4	5(0.3%)	5(0.3%)		5(0.3%)	5(0.3%)		5(0.3%)	5(0.3%)	
NPI									
Good	351(17.0%)	330(16.7%)	0.605	337 (17.0%)	344 (17.4%)	0.78	347(17.5%)	334(16.9%)	
Medium	542 (27.4%)	558(28.2%)		557(28.1%)	543 (27.4%)		546(27.6%)	554(28.0%)	0.806
Poor	97 (4.9%)	102 (5.2%)		96 (4.8%)	103(5.2%)		97(4.9%)	102(5.2%)	
HER2 status									
Positive	77(3.9%)	169 (8.5%)	**<0.001**	66(3.3%)	180 (9.1%)	**<0.001**	68(3.4%)	178(9.0%)	**<0.001**
Negative	913(46.1%)	821(41.5%)		924 (46.7%)	810 (40.9%)		922(46.6%)	812(41.0%)	
Lymph node status									
Negative	524(26.6%)	511(25.9%)	0.606	507(25.7%)	528(26.8%)	0.287	504(25.2%)	531(26.9%)	0.252
Positive	464(23.5%)	474(24.0%)		482(24.4%)	456(23.1%)		481(24.4%0	457(23.2%)	

Note. NPI, Nottingham Prognostic Index, ER, oestrogen receptor, and PR, progesterone receptor, *P* values highlighted in bold are <0.05

4.3.6 Association between mRNA expression and breast cancer specific survival

The Kaplan-Meier method was used to assess the impact of *Sp7, PPP1R1B, GRB7* and *DKK1* mRNA expression on breast cancer specific survival. *Sp7* and *PPP1R1B* (probes 1-3) mRNA expression were not significantly associated with breast cancer survival (Figure 4.6A, B, C and D). Low *GRB7* probe 1 and 2 expression were associated with improved breast cancer survival ($P<0.001$ and $P<0.001$ respectively) (Figure 4.6E and F), and low *DKK1* probe 1 expression was associated with improved breast cancer specific survival ($P=0.022$) (Figure 4.6H).

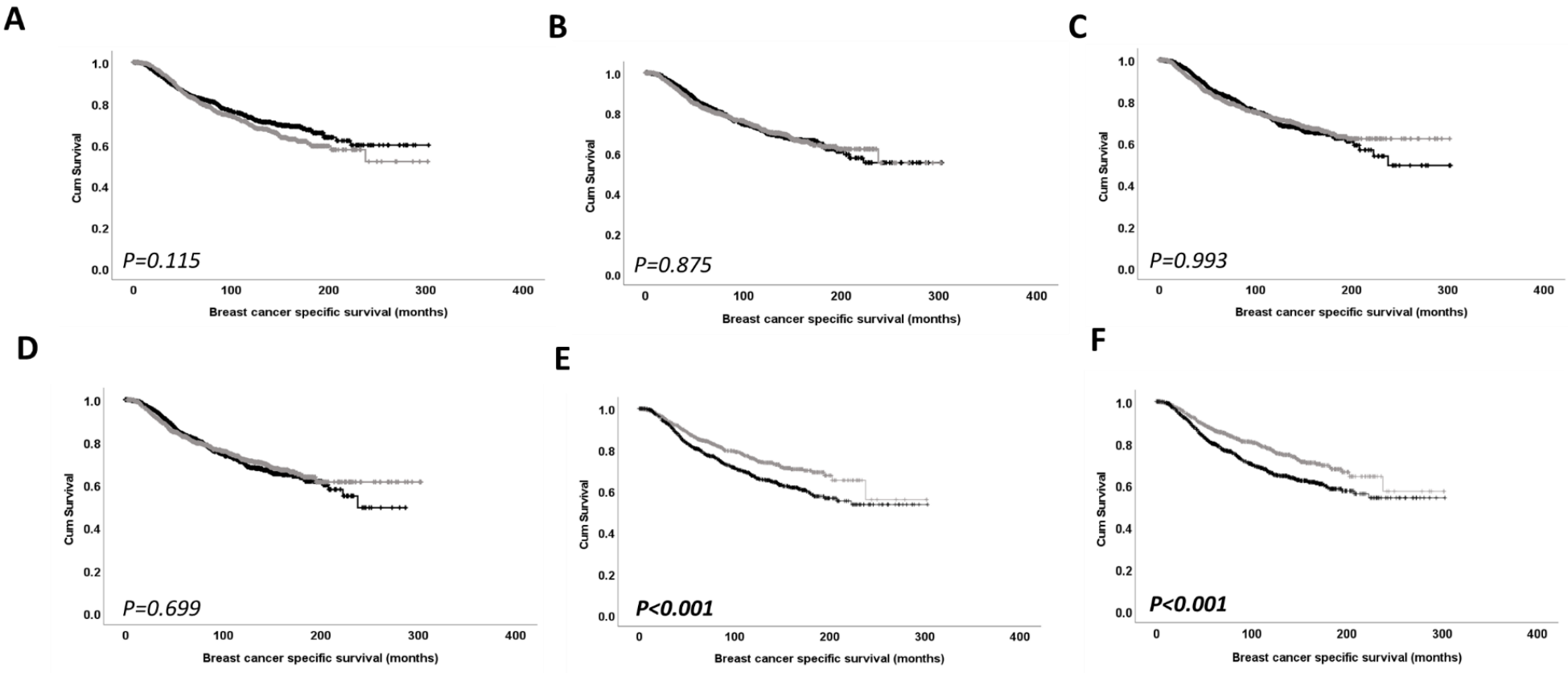


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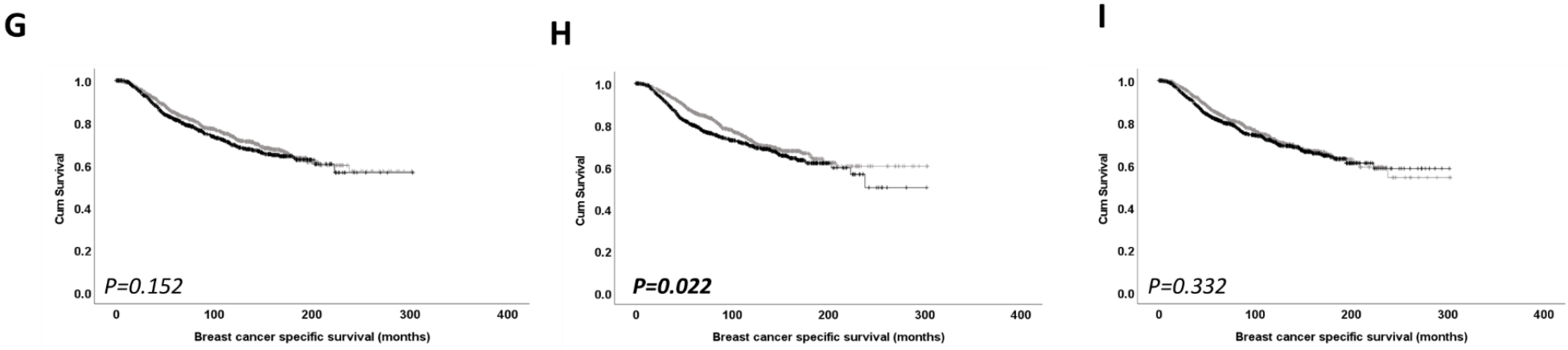


Figure 4.6: Kaplan-Meier analysis of breast cancer patients demonstrating the effect of *Sp7* expression figure 4.6-A (*P*=0.115), *PPP1R1B* probe 1, 2 and 3 figure 4.6 B, C and D (P=0.875, P=0.993 and P=0.699, respectively), *GRB7* probe 1, 2 and 3 figure 4.6 E, F and G (P<0.001, *P*<0.001, and *P*=0.152, respectively), and *DKK1* probe 1, 2 figure 4.6-H and I (*P*=0.022 , *P*=0.332 respectively). For each plot, low expression and high expression are illustrated by the grey and the black lines, respectively. The *P* values highlighted in bold are <0.05.

4.5 Discussion

As described in previous chapters, the function of DARPP-32 depends largely on its state of phosphorylation through interaction with other proteins. Threonine-34 and Threonine-75 residues, in particular, are vital to the activity of various downstream proteins (Bateup et al., 2008, Greengard et al., 1999). We initially sought to determine t-DARPP expression directly using chromogenic *in situ* RNA hybridisation. Unfortunately, this technique produced unsatisfactory result; therefore, expression of phosphorylated DARPP-32 at Threonine-34 was determined instead. DARPP-32 Threonine-34 phosphorylation is only present in full length DARPP-32, as t-DARPP is missing this phosphorylation site. DARPP-32 Threonine-75 phosphorylation is present in both DARPP-32 and t-DARPP. DARPP-32 Threonine-75 phosphorylation level was not determined as the specificity of the relevant antibody was not deemed sufficient. Therefore, protein expression of DARPP-32 Threonine-75 phosphorylation was not determined.

In this study, protein expression of *Sp7, GRB7*, *DKK1* and DARPP-32 at Threonine-34 were determined using Immunohistochemistry in a large cohort of early-stage invasive breast cancer patients. Furthermore, expression of *Sp7*, *GRB7*, *DKK1* and *PPP1R1B* mRNA were evaluated in the METABRIC data set. Low nuclear expression of *Sp7* was significantly associated with adverse breast cancer specific survival. Interestingly, cytoplasmic expression of *Sp7* was not significantly associated with breast cancer specific survival suggesting a different function of *SP7* in the nucleus, perhaps as a transcription factor. Analysis of data from the METABRIC data set showed that *Sp7* mRNA was not associated with breast cancer specific survival and clinicopathological variables.

One study by Yao *et.al* (2019) demonstrated that high expression of *Sp7* is associated with breast cancer specific survival. In this study, immunohistochemistry was used to assess *Sp7* expression in 154 breast

cancer patients; *Sp7* was expressed in 57.1% of the tissues. *Sp7* was significantly associated with positive lymph node, but was not associated with other clinicopathological variables. (Yao et al., 2019).

Other studies have also suggested that *Sp7* may be involved in unregulated activity of several pro-metastatic genes including those encoding VEGF, MMP-9, β -catenin, and E-cadherin in human breast cancer cells (Qu et al., 2019, Dai et al., 2015). Since *Sp7* is also regarded as a contributor to inducing chemo-sensitivity in breast cancer cells, further investigatory work focused on discovering the exact mechanism of its action could be crucial to the treatment of breast cancer (Wu et al., 2017).

In a study by Dai *et.al* (2017), high expression of *Sp7* was detected in MDA-MB-231 and MDA-MB-361 cell lines which are known to have metastatic capability. In contrast, non-metastatic breast cancer cell line MCF7 did not exhibit detectable levels of *Sp7* (Dai et al., 2015).

Low nuclear and cytoplasmic levels of *GRB7* were significantly associated with improved prognosis of breast cancer specific survival. These data are aligned with the findings of previous studies which have reported prevalence of high *GRB7* levels along with high expression of HER2 in breast cancer. Amplification of 17q12-21 amplicon in particular was found to be associated with the overexpression of this protein, which is adjacent to the HER2 gene (Vermehren-Schmaedick et al., 2020, Giricz et al., 2012, Ramsey et al., 2011).

A significant association between high expression levels of *GRB7* and mortality has previously been described in a study comprising 638 breast cancer patients (P=0.0034); furthermore, a significant association between high expression levels of *GRB7* and mortality in patients with tumour invasion to lymph node was demonstrated (P=0.0019), a trend that was absent in node-negative subset alone (Nadler et al., 2010).

Since high levels of *GRB7* have also been reported in various tumours including ovarian cancer, *GRB7* may have a role as a prognostic biomarker (Vermehren-Schmaedick et al., 2020). Currently, *GRB7* is included in the 21 gene set as a part of Oncotype DX assay which is used for the prognosis of early-stage breast cancer (Nadler et al., 2010, Paik et al., 2004).

In one study, high cytoplasmic level of *GRB7* was detected in 19.15% of samples from a total of 30 samples isolated from normal epithelial tissues; 15.14% of the samples had medium expression, and 25.17% had weak expression. *GRB7* was undetected in 40.53% of the samples. In contrast, samples obtained from various breast cancer tissues (n=419) exhibited high, medium and low cytoplasmic expression in 52.33%, 17.65% and 2.65% of the cases, respectively (Vermehren-Schmaedick et al., 2020). Data obtained from the analysis of METABRIC data in *GRB7* demonstrated that low expression levels of *GRB7* probe 1 and 2 were associated good prognosis of breast cancer specific survival, whereas probe 3 was not significantly associated with breast cancer specific survival. Probe 3 maps to *GRB7* transcript *ENST00000309156.9* and a number of other non-protein coding transcripts. In contrast, Probe 1 maps to a number of protein coding *GRB7* transcripts identified as *ENST00000309156.9*, *ENST00000394211.7*, *ENST00000445327.6, E NST00000394209.6,* and *ENST00000394204.1* (ILMN-2405254), as well as probe 2 which maps to *ENST00000309156.9*, *ENST00000394211.7, ENST00000445327.6*, *ENST00000394204.1*. There is little evidence on the implications of functional changes resulting from different *GRB7* transcripts. Furthermore, low *GRB7* probe 1, 2 and mRNA expression were associated with improved survival of patients in the METABRIC cohort.

Two independent investigations by Cobleigh *et.al* and Vinatzer *et. al* demonstrated that presence of high levels of *GRB7* mRNA were strongly associated with worse survival in two separate groups composed of 78

and 85 breast cancer patients, respectively. Furthermore, the effect of *GRB7* expression in the five year survival of triple negative breast cancer patients following a standard adjuvant anthracycline and taxane therapy showed that low and high *GRB7* mRNA expression groups had a survival rate of 10.5% and 20.4%, respectively (Vinatzer et al., 2005, Cobleigh et al., 2005). Abundance of *GRB7* was also associated with resistance to neoadjuvant doxorubicin and taxane therapy (Sparano et al., 2013). Co-expression of *GRB7* and *ERBB* family of proteins has also been demonstrated in studies investigating breast cancers, cervical cancers, invasive Barrett's carcinoma, advanced oesophageal carcinoma, and gastric cancers (Chu et al., 2019).

Interestingly, associations between *PPP1R1B* and *GRB7* genes have been previously demonstrated in upper gastrointestinal adenocarcinomas where the pair, often along with other genes such as *TARD3*, *TCAP*, and *PNMT,* have been amplified at locus 17q (Maqani et al., 2006, Kotecha et al., 2019). Thus, based on the findings of the current study, and the supporting evidence obtained in various other studies, up-regulation of *GRB7* is indicative of the development of more aggressive tumours and it defines a subset of breast cancer patients with reduced survival rate.

As previous studies have demonstrated, *DKK1* could have a potential role in the promotion of tumour metastasis in various types of malignancies with a direct impact on cancer-specific survival. Therefore, expression of this protein may serve as a biomarker for prognostic purposes (Kasoha et al., 2018, Zhu et al., 2021, Han et al., 2015).

In the current study, low protein expression level of *DKK1* was significantly associated with adverse breast cancer specific survival *DKK1* expression was also associated with a number of clinicopathological criteria including larger tumour size, high tumour grade, pleomorphism, mitosis, intermediate NPI value, negative ER status, negative PR status, positive HER2 status, triple negative tumours and tumours with a high

Ki67 index. This may indicate that reintroduction and regulation of healthy *DKK1* levels may contribute to a better patient outcome, probably through inhibition of Wnt signalling pathway which is an important regulator of cell growth and cell survival in breast cancer (Kasoha et al., 2018).

Analysis of data from the METABRIC data set showed that low expression level of *DKK1* probe 1 was significantly associated with good prognosis of breast cancer specific survival, whereas probe 2 was not significantly associated with breast cancer specific survival. Probe 1 maps to protein coding *ENST00000373970.4 DKK1* transcript ID which is one of the four splice variant products of the *DKK1* gene *ENSG00000107984.* High expression of *DKK1* gene *ENSG00000107984* has been associated with promotion of proliferation, invasion and growth in cancer (Shang et al., 2017).

Previously, upregulation of *DKK1* has been shown to reduce the rate of proliferation, migration and the overall aggressiveness in human colon cancer (González-Sancho et al., 2005, Chen et al., 2013). High levels of *DKK1* have also been reported in a number of malignancies including breast cancer, liver cancer, lung cancer, glioma, and cervical cancer where this protein may be involved in inducing apoptosis through suppression of cell growth and transformation (Zhu et al., 2021). In one study, upregulation of *DKK1* was detected in 26 out of 180 human non-small cell lung cancers, 59 of 85 small cell lung cancers, and 51 of 81 oesophageal squamous cell carcinoma patients (Chen et al., 2013, Yamabuki et al., 2007). In contrast to our findings with regards to the association of low *DKK1* expression with a number of clinicopathological criteria, a previous study found high levels of *DKK1* in breast cancer patient's serum in comparison to healthy volunteers, with even higher concentrations detected in breast cancer patients with bone metastasis. It was also shown that high expression of *DKK1* was also common in triple

negative breast cancer patients with poor prognosis (Zhu et al., 2021, Voorzanger-Rousselot et al., 2009, Xu et al., 2012).

Another investigation found strong association between high levels of *DKK1* and lymph metastasis in a group of patients suffering from Intrahepatic cholangiocarcinoma (ICC). Down-regulation of *DKK1* using small interfering RNA was found to reduce the rate of tumour migration and invasion (Chen et al., 2013, Shi et al., 2013). This indicates that dysregulation of *DKK1,* whether too high or too low, could result in devastating cellular events with a broad set of implications in various tumours.

The expressions of *SP7, GRB7* and *DKK1* in breast cancer provides important information regarding cancer cell behaviour and it has a potential clinical importance as a therapeutic target, our data suggests that it cannot be considered as an independent prognostic marker, but instead as a prognostic marker which is possibly dependent on different clinopathological parameter of tumours such as tumour grade and tumour size.

Protein expression of phosphorylated DARPP-32 at Threonine-34 was assessed in a large group of breast cancer patients (n=1274). The expression of Threonine-34 could be regarded as an independent prognostic marker with potential clinical applications in the treatment of breast cancer. This may also suggest that low nuclear/cytoplasmic expression levels of phosphorylated DARPP-32 at Threonine-34 and the consequent inability of DARPP-32 in effective inhibition of PP1 protein could be crucial in promoting adverse breast cancer specific survival.

In continuation of the work previously undertaken by our research group which led to the identification of a number of genes including *DKK1 and GRB7* as gene candidates that may be linked to *PPP1R1B* (Kotecha et al., 2019), the current study demonstrates the corresponding association of low expressions of *Sp7* (nuclear), *DKK1* (nuclear/cytoplasmic), and

phosphorylated DARPP-32 at Threonine-34 (nuclear/cytoplasmic) with adverse breast cancer specific survival. We have also demonstrated that unlike *Sp7* (nuclear), *DKK1* (nuclear/cytoplasmic), and DARPP-32 Threonine-34, lower expression level of *GRB7* is associated with improved breast cancer specific survival. In addition, the association of low expression of phosphorylated DARPP-32 at Threonine-34 and adverse breast cancer specific survival which was demonstrated in the current study supports our group's previous findings that low expression of DARPP-32 is significantly associated with poor breast cancer specific survival (P=0.041); this indicates that insufficient inhibition of PP1 downstream of phosphorylated DARPP-32 at Threonine-34 may have a crucial role in patient survival.

Expression of *PPP1R1B* probe 1, 2 and 3 within the METABRIC dataset (n=1980) were not significantly associated with breast cancer specific survival; however, there were a number of significant associations with clinicopathological variables including cellularity, high tumour grade, and ER positive status, HER2 negative status and luminal A subtype.

In conclusion, *Sp7*, *DKK1*, DARPP-32 Threonine-34 and *GRB7* were associated with a number of clinicopathological criteria indicative of poor prognosis. Analysis of the METABRIC cohort showed that *GRB7* (probe 1 and 2) and *DKK1* (probe 2) mRNA expression were associated with improved breast cancer specific survival. Our data indicates correspondence of low expressions of *Sp7*, *DKK1,* and phosphorylated DARPP-32 at Threonine-34 in association with adverse breast cancer specific survival. In contrast, low expressions of cytoplasmic and nuclear *GRB7* were associated with improved prognosis and a better overall patient outcome. These observations could serve as a platform for future investigations aiming to discover potential links and correlations that may exist between these proteins. *Sp7, DKK1*, *GRB7* are involved in a variety of cellular processes and our data indicate that their level of expression may play an important role in patient outcome.

4.6 Summary

- *Sp7* mRNA is not associated with breast cancer specific survival and clinicopathological variables.
- Low nuclear and cytoplasmic levels of *GRB7* were significantly associated with improved prognosis of breast cancer specific survival
- Low mRNA expression level of *DKK1* was significantly associated with good prognosis of breast cancer specific survival
- Low protein expression level of *DKK1* was significantly associated with adverse breast cancer specific survival
- Low nuclear and cytoplasmic expression of phosphorylated DARPP-32 at Threonine-34 was significantly associated with adverse breast cancer specific survival

Chapter 5 Transcriptomic Effects of DARPP-32 knockdown in Breast Cancer

5.1 Introduction

El-Rifai and colleagues described the expression of a truncated variant of DARRP-32, t-DARPP in gastric carcinoma (El-Rifai et al., 2002, Christenson and Kane, 2014). Subsequent studies led to the validation of such observation in numerous other types of cancer including breast tumours (Beckler et al., 2003, Christenson and Kane, 2014). DARPP-32 and t-DARPP proteins have been detected in abundance in adenocarcinomas of the breast, prostate, colon, and stomach. Dysregulation of DARPP-32 and t-DARPP has been demonstrated to aggravate tumour growth, promote resistance to chemotherapy through activation of pro-oncogenic signal transduction pathways, and reduce tumour-specific patient survival (Belkhiri et al., 2016). Previously our group identified that low levels of DARPP-32 protein expression was significantly associated with adverse breast cancer specific survival, which was particularly apparent in breast cancer patients with ER positive tumours (Kotecha et al., 2019). Therefore, in this study we aim to monitor the transcriptomic changes that occur in the absence and in the presence of DARPP-32 following E2 stimulation in order to enable a better understanding of the link between DARPP-32 and oestrogen signalling and to see how the combination of ER signalling and DARPP-32 can alter cancer cell behaviour and patient survival. Utilisation of tissue specimens which have not previously been treated with oestrogen inhibiting agents such as tamoxifen allows for the study of the relevant transcriptomic changes without the interference of external factors, mimicking a clinical representation of samples.

5.1.2 DARPP-32 Knockdown

Utilisation of small interfering RNA (siRNA) for the purpose of targeted protein knockdown is a widely used technique which has provided crucial

insight into the role of various proteins in different pathways. This has contributed to a better understanding of the biology of diseases over the years. In cancer, reducing the expression of various oncogenes aids further understanding of the complex biological mechanisms involved paving the way for the discovery of novel therapeutic agents (Pileczki et al., 2016, Fakhr et al., 2016, Al-Lazikani et al., 2012).

One study exploited xenotransplantation of DARPP-32 knockdown SNU-16 gastric tumour cells to mice in order to investigate the role of DARPP-32 in gastric tumour resistance to gefitinib. Results revealed that reduced expression of DARPP-32 in SNU-16 cells significantly increased the sensitivity to gefitinib ($P<0.01$). Furthermore, it was demonstrated that DARPP-32 promotes resistance to gefitinib by promoting *EGFR* and *ERBB3* interactions and through activation of PI3K-AKT signalling (Zhu et al., 2011)

In gastric cancer, downregulation of *CXC chemokine receptor type 4 (CXCR4*) using small molecule inhibitors and siRNA has been shown to impair DARPP-32 dependent signalling and cancer invasion. This is due to *CXCR4's* role in the activation of the *membrane type 1-matrix metalloproteinase* (*MT1-MMP/MMP-2*) pathway which is crucial for enabling DARPP-32-mediated tumour invasion (Zhu et al., 2013). Formation of DARPP-32/*CXCR4* complex has shown to reduce availability of *CXCR4* for ubiquitination, prolonging its half-life (Zhu et al., 2013, Belkhiri et al., 2016).

In pancreatic ductal carcinomas, where elevated levels of DARPP-32 have been associated with poor patient survival, knockdown of DARPP-32 protein significantly reduces metastasis to lung tissues *in vivo*. Previously, the role of DARPP-32 in the promotion of p53 breakdown has been described as a result of *Mouse double minute 2 homolog* (*MDM2*) Ser166 residue phosphorylation which stabilise the *p53* specific E3 ubiquitin ligase. This highlights the link between DARPP-32 overexpression and

deterioration of health accompanied by poor survival prospects in pancreatic ductal carcinomas (Tiwari et al., 2020, Moll and Petrenko, 2003).

A previous study by Belkhiri *et.al* (2008) demonstrated the correlation between enhanced expression of t-DARPP and increased phosphorylation of *AKT (pAKT)* along with increased *BCL2* protein levels in BT-474 and SKBR-3 cell lines. In this study, knockdown of endogenous t-DARPP was shown to not only reduce *ERBB2*, *pAKT* and *BCL2* protein levels, but also increase production of cleaved caspase-3, leading to a notable increase of apoptosis in BT-474 cells (Belkhiri et al., 2008).

It has also been shown that high expression of t-DARPP is associated with trastuzumab resistant in *ERBB2* positive breast cancer cells. Interestingly, knockdown of t-DARPP has been shown to cause a reversal in the sensitivity of those cells to trastuzumab, leading to the activation of caspase3 and apoptosis (Belkhiri et al., 2008, Belkhiri et al., 2016).

5.1.3 RNA-Seq and DARPP-32

RNA sequencing has been firmly integrated into cancer research as it provides useful information about cancer microenvironment, cancer development and its variation amongst patients, causes of drug resistance, pattern of gene expression, and identification of cancer biomarkers and neoantigens (Hong et al., 2020, Moon and Nakai, 2016). This technique also facilitates additional monitoring steps and provides the capability for early detection of high-risk mutations. Therefore, utilisation of this technique aids identification of new biomarkers as well as guiding targeted therapy during early treatment (Hong et al., 2020, Wang et al., 2020, Jiang et al., 2019).

A recent investigation by Alam *et.al* (2018), reported stimulation of cell migration as a result of the interaction between DARPP-32 and *inhibitory*

kappa B kinase-α (*IKKα*) which activates *nuclear factor kappa-light-chain enhancer of activated B cells 2* (*NF-κB2*) signalling in non-small cell lung carcinoma. The analysis of Kaplan–Meier survival curves, with the aid of RNA sequencing in a total of 201 human lung adenocarcinoma tissue samples, illustrated association of high NF-*κB2* and *IKKα* transcripts expression with shorter patient survival, a pattern that was absent in patients with lower levels of these transcripts (Alam et al., 2018). Another recent study by Murad *et.al (2021*) identified the *PPP1R1B* gene as one of the most commonly overexpressed genes in trastuzumab resistant breast cancer cell lines using RNA-Sequencing (Murad et al., 2021).

High expression levels of t-DARPP have been described in association with enhanced Notch signalling in small-cell lung carcinoma. The abundance of transcripts corresponding to DARPP-32 and t-DARPP were determined in a RNA-Seq dataset comprising data from a cohort of 29 patients with small cell lung cancer. Unlike in normal lung tissue where t-DARPP was not detectable, t-DARPP transcripts were highly expressed in transformed tissues (Rudin et al., 2012, Alam et al., 2018).

DARPP-32 can influence the function of various key cellular pathways giving this protein a role in the maintenance of normal cell homeostasis, and abnormalities associated with the expression of DARPP-32 and t-DARPP have implications in the promotion of various malignancies. Current evidence strongly suggests the suitability of DARPP-32 both as a biomarker for the prediction of drug resistance, and as a therapeutic target in various cancers including gastric and lung cancers (Alam et al., 2018, Belkhiri et al., 2016).

In breast cancer, we have demonstrated that DARP-32 may be involved in the underlying mechanisms associated with breast cancer (Kotecha et al., 2019). However, the details of how various cellular pathways are affected due to DARPP-32 imbalance in breast cancer are yet to be fully understood. Current data suggests that the state of DARPP-32

phosphorylation at various phosphorylation sites could lead to contrasting cellular events; PKA induced phosphorylation of Threonine-34 residue transforms DARPP-32 into a potent inhibitor of PP1, whereas Cdk5-induced phosphorylation of DARPP-32 Threonine-75 residue promotes PKA suppression (Kotecha et al., 2019). Therefore, cellular pathways that influence the activity of PKA and Cdk5, such as the Wnt-5a pathway, could also influence the function of DARPP-32 in breast cancer (Hansen et al., 2019)

A better understanding of the cellular interactions that influence the expression and activity of DARPP-32 and t-DARPP would further expose the role of DARPP-32 as a regulator and/or activator of various important cancer associated signalling pathways in breast cancer, leading the way to the discovery of novel approaches for the treatment of this condition (Belkhiri et al., 2016).

5.2.1 Hypothesis and Aims

Hypothesis

We hypothesise that the reduction of DARPP-32 expression in ER positive breast cancer results in alteration of transcriptome and activation of pathways that aggravate tumour growth and metastasis.

Aims

The aim of this study is to identify transcriptomic changes that occur in the absence and in the presence of DARPP-32, with further investigation of changes that occur following E2 stimulation and treatments with a PKAi.

Objectives

- Use siRNA to reduce expression of DARPP-32 in T47D breast cancer cells and validation of DARPP-32 knockdown in those cells compared with that of control cells using Western blot and real-time PCR.

- Identification of transcriptome alterations following loss of DARPP-32 expression in an ER positive breast cancer cell line using RNA-Seq.

- Use of RNA-Seq to assess the effects of PKA inhibition and oestrogen signalling on the cellular transcriptome in DARPP-32 knockdown cells.

5.3 Results

5.3.1 DARPP-32 knockdown Western blot and, Real-Time PCR

The Protein and mRNA levels of DARPP-32 were assessed via western blotting and Real-time PCR. Western blotting demonstrated that, relative to the negative control group, the expression of DARPP-32 was reduced by 95% after 24 hours following siRNA treatment (Figures 5.1A and 5.1B). Real-time PCR analysis showed a 60% fall in the level of DARPP-32 mRNA at 24 hours following siRNA treatment (Figure 5.1C). In contrast, no significant difference in DARPP-32 expression was observed in the negative control group or the wild-type T47D cells. In addition, the

mRNA levels of DARPP-32 splice variant t-DARPP and 171 amino acid were assessed. Real-time PCR revealed a 40% reduction in 171 amino acid mRNA and 95% reduction in t-DARPP mRNA following 24 hour siRNA treatment (Appendix-C Figure C1).

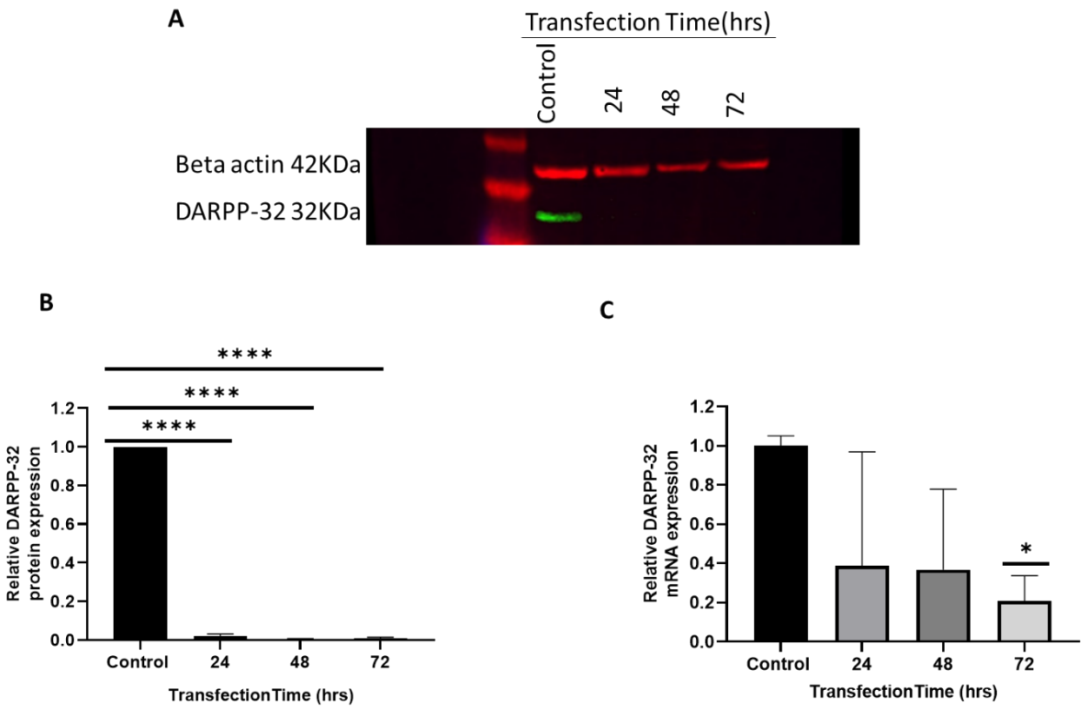


Figure 5.1: A: A comparative demonstration between DARPP-32 expression level in DARPP-32 knockdown cells, and beta actin expression (control) in the T47D cell line following treatment with DARPP-32 siRNA oligo duplex at 24, 48 and 72 hour time points. The results of DARPP-32 knockdown and control determined by Western blot quantified analysis using Li-Cor (B) and Real-time PCR (C) demonstrate expression of DARPP-32 in untreated cells and following DARPP-32 knockdown treatment at various time points. Data presented the average ± SD of three independent experiments analysed using one-way ANOVA. **** $P<0.0001$, * $P<0.05$.

5.3.2 RNA Sequencing Data Analysis

5.3.2.1 DESeq2

Data obtained from DESeq2, visualising count assessment and dispersion estimates are demonstrated in Figure 5.2 and Figure 5.3, respectively. Data illustrates emergence of differentially expressed transcripts (greater than 2-fold change with a Benjamini-Hochberg *P* adjusted value of <0.05) in both DARPP-32 knockdown and control cells within the E2 and PKAi groups. The largest sums of differentially expressed genes were attributed to DARPP-32 knockdown and control cells that were treated with E2.

There were a total of 202 transcripts differentially expressed in DARPP-32 knockdown cells compared with DARPP-32 control cells. Interestingly, the number of differentially expressed transcripts between the DARPP-32 knockdown group and the DARPP-32 knockdown group treated with E2 was 42641. Similarly, 40065 differentially expressed transcripts were found in the control cells versus control cells treated with E2; this was not unexpected as estrodiol is known to have a significant impact. Differences in 193 transcripts were observed when comparing the E2 treated DARPP-32 knockdown cells and E2 treated control cells. PKA inhibition in DARPP-32 knockdown cells resulted in 1155 differentially expressed transcripts when compared to the untreated DARPP-32 knockdown cells. 1704 differentially expressed transcripts were found in the control cells versus control cells treated with PKA inhibitor. Differences in expression was observed in 181 transcripts when comparing the DARPP-32 knockdown treated with PKA inhibitor and control cells that were treated with PKA inhibitor. Volcano plots are shown in Appendix-C Figure C2.

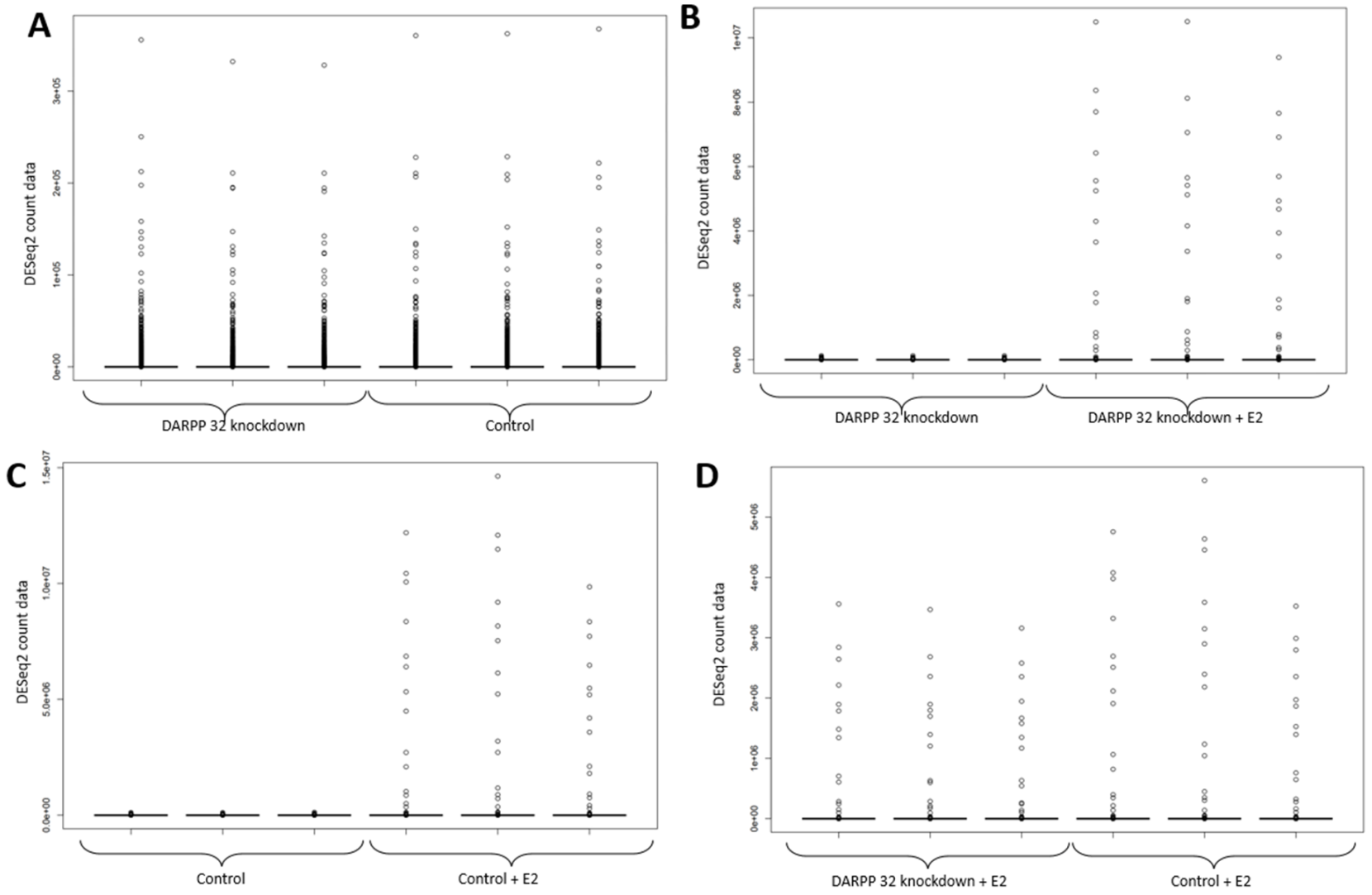


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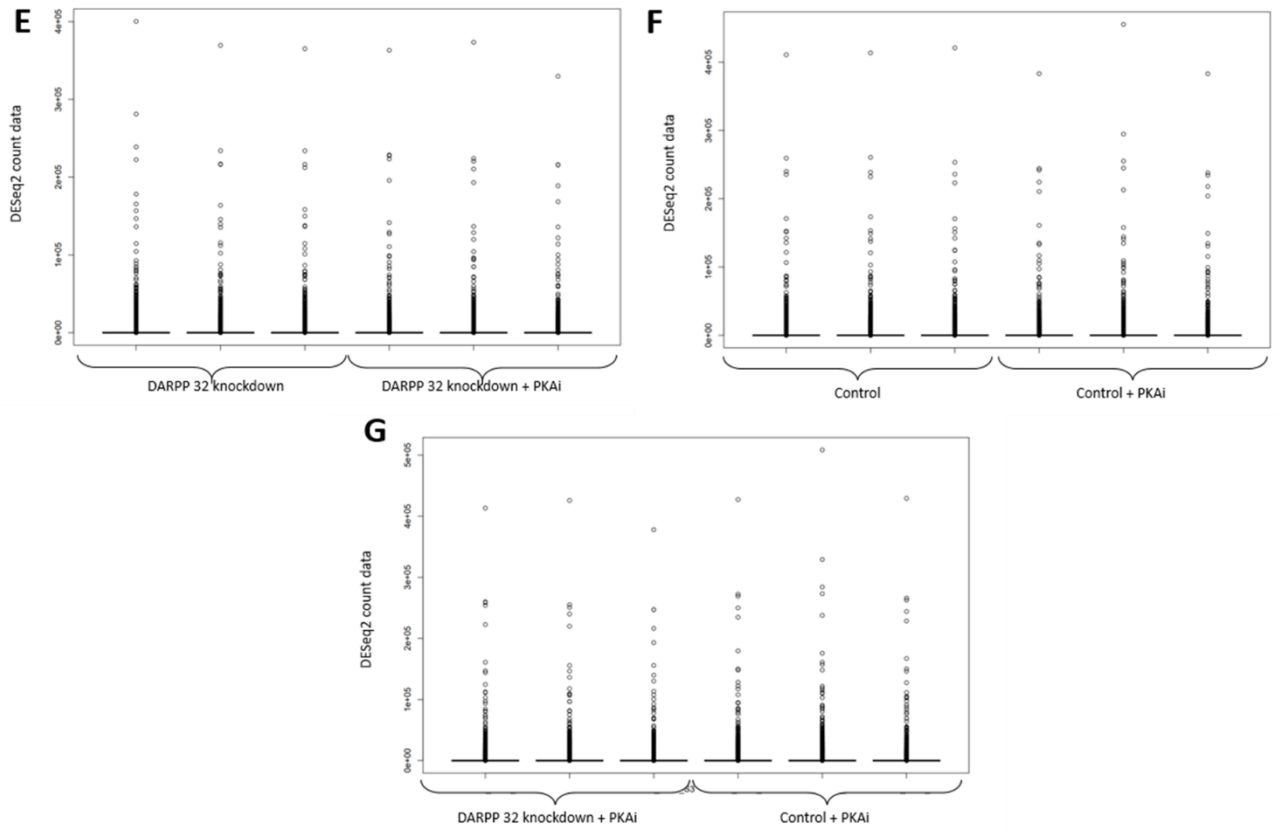


Figure 5.2: Data from DESeq2 analysis where transcript expression is compared between: DARPP-32 knockdown cells and control cells (A), E2 treated and untreated DARPP-32 knockdown cells (B), E2 treated and untreated control cells (C), E2 treated DARPP-32 knockdown cells and E2 treated control cells (D), PKAi treated and untreated DARPP-32 knockdown cells (E), PKAi treated and untreated Control cells (F), and PKAi treated DARPP-32 knockdown cells and PKAi treated control cells (G). Analysis was performed by Dr Storr.

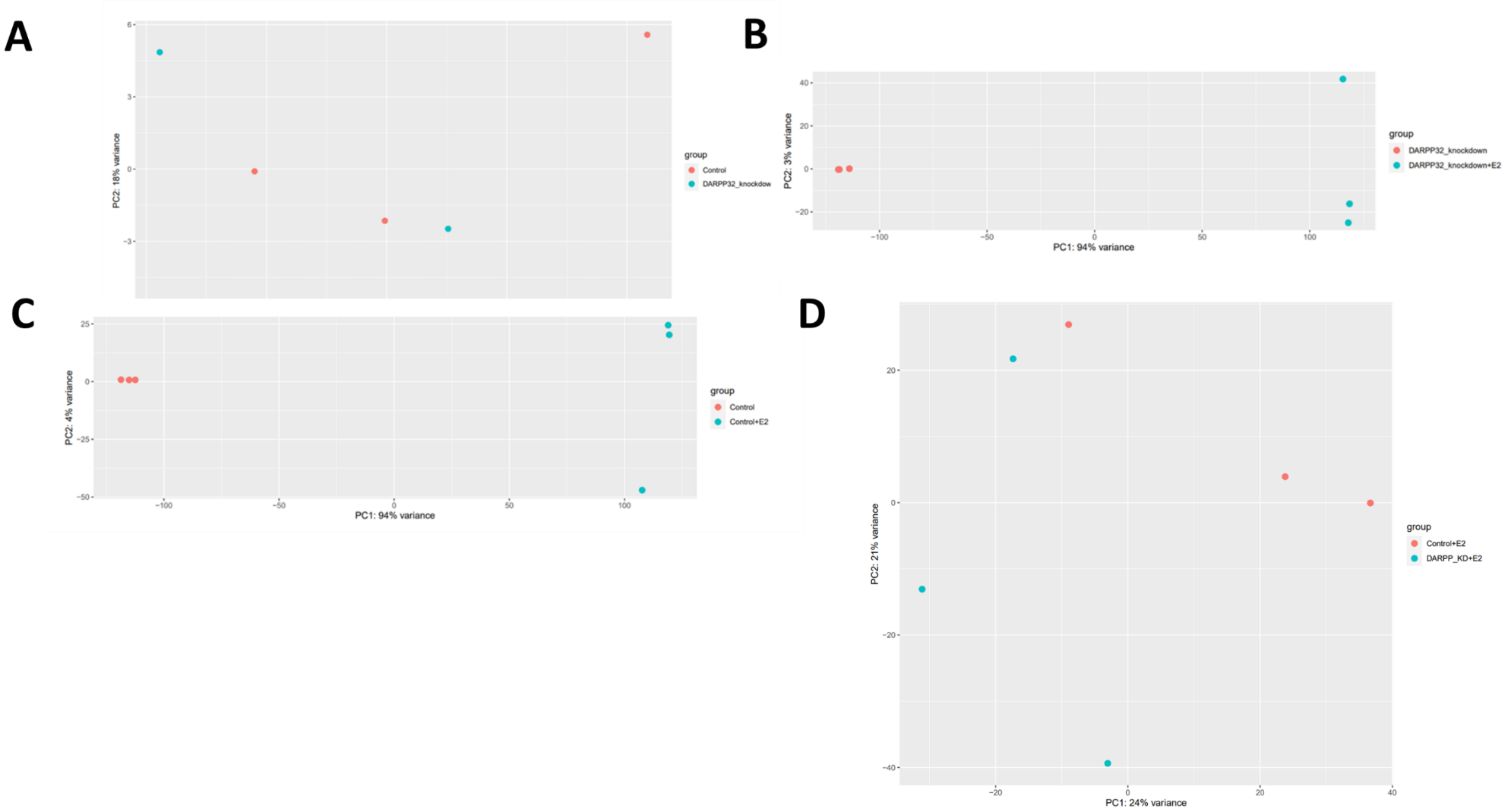


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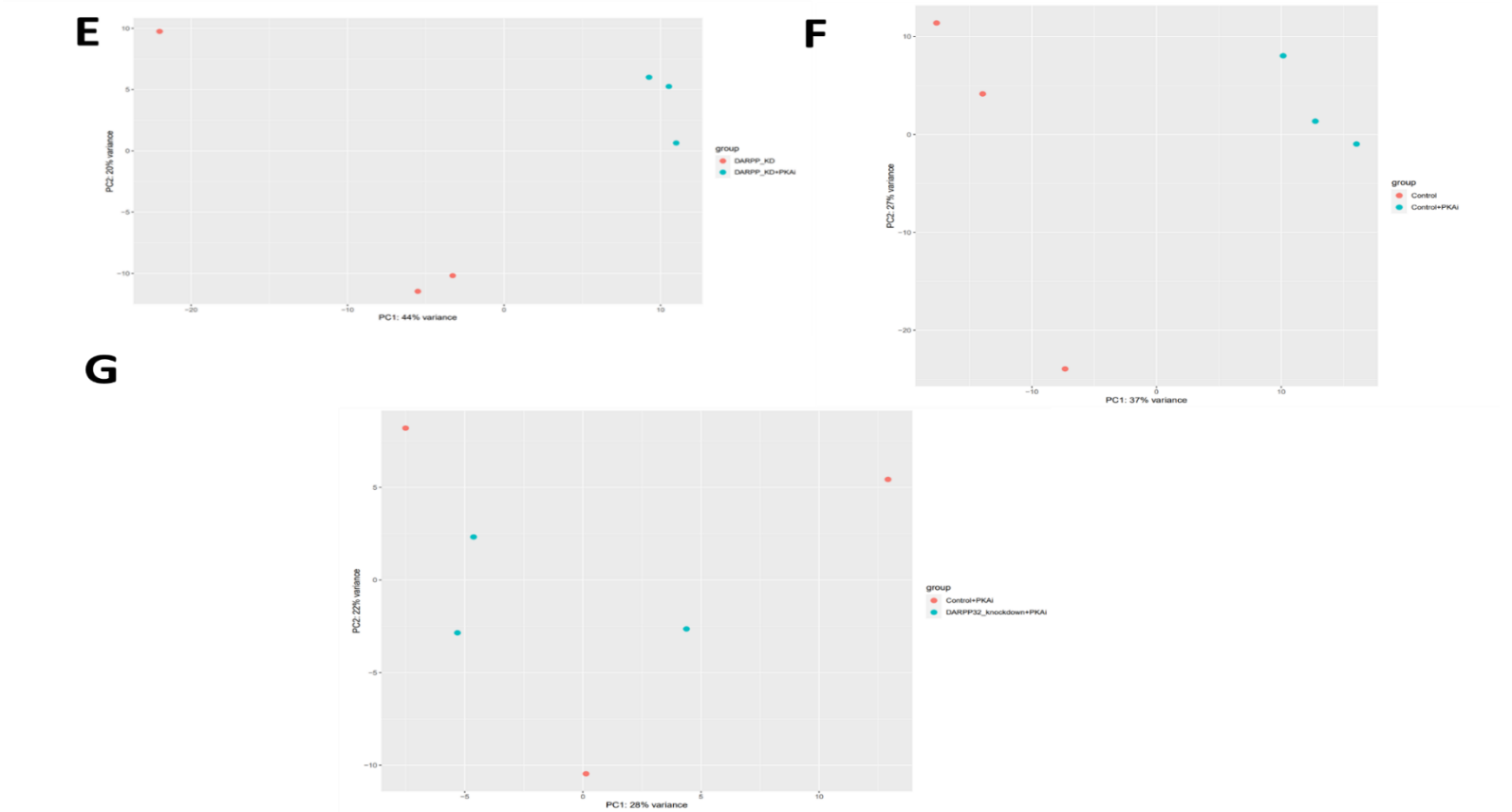


Figure5.3: Principal component analysis demonstrating the contrast in DESeq2 dispersion estimates between: DARPP-32 knockdown and control cells (A), E2 treated and untreated DARPP-32 knockdown cells (B), E2 treated and untreated control cells (C), E2 treated DARPP-32 knockdown cells and E2 treated control cells (D), PKAi treated and untreated DARPP-32 knockdown cells (E), PKAi treated and untreated Control cells (F), and PKAi treated DARPP-32 knockdown cells and PKAi treated control cells (G). Analysis was performed by Dr Storr.

5.3.3 Heat maps

The pattern of gene expression was analysed across the samples using heat maps with the aim of exploring transcript alterations induced by DARPP-32 knockdown. This facilitated identification of the 50 most common transcripts with significant (*P*-adjusted) expression alterations compared to that of control samples (Figure 5.4). Alterations in the three most significant transcripts were highly reproducible, and included reduced *ENST00000443236.6* (*CPAMD8*), reduced *ENST00000521309.5* (*YWHAZ*), and increased *ENST00000651564.1* (*CPAMD8*) (Note that the *CPAMD8* has difference in transcript number as they represent two different isoforms) in the DARPP-32 knockdown cells in comparison to that of control cells (Figure 5.4). *ENST00000443236.6* and *ENST00000651564.1* transcript identifiers correspond to a 1885 amino acid protein and a 1815 amino acid protein, respectively. The details of the top 10 transcripts with the most significant differential alterations that were either down or up regulated in the DARPP-32 knockdown cells compared with control are summarised in Table 5.1.

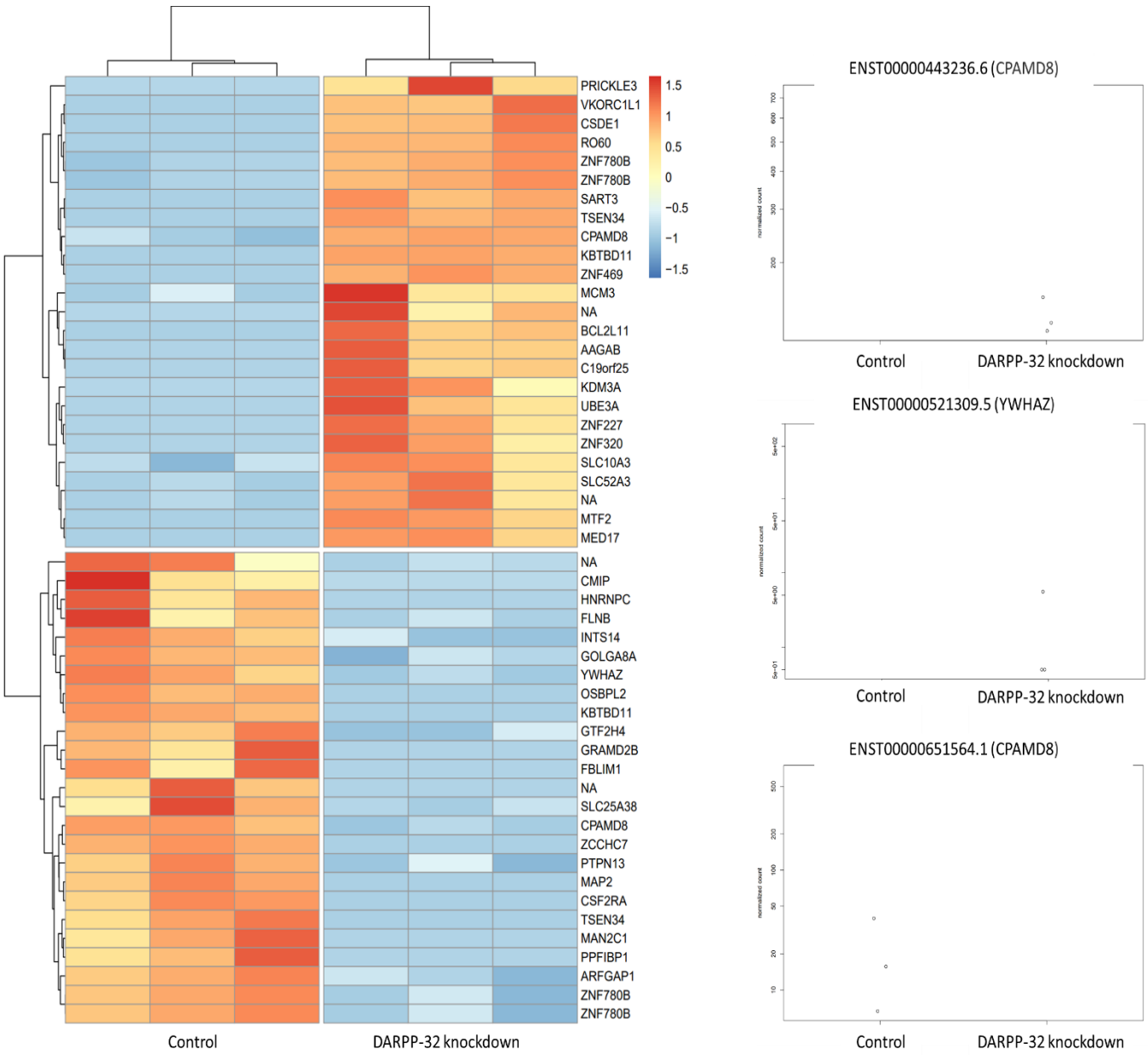


Figure5.4: Identification and heat map analysis of the 50 most common transcripts with significant (P-adjusted) expression alterations (greater than 2-fold) in DARPP-32 knockdown cells compared to that of control samples. The three most significant transcript and their corresponding gene identifiers are displayed on the right. Analysis was performed by Dr Storr.

Table 5.1: The lists of top 10 transcripts with the most significant alterations that were either down or up regulated in the DARPP-32 knockdown cells compared with control cells are shown below. This table also includes details of log fold change and *P*-adjusted value for each transcript.

Downregulated Genes				
Transcript ID	**Gene ID**	**Protein Name**	**Log 2 fold change**	***P*-adj**
ENST00000331222.5	*CLN8*	CLN8 transmembrane ER and ERGIC protein	-11.05803607	2.31E-14
ENST00000542322.5	*GRAMD2B*	GRAM Domain containing 2B	-10.06789274	4.82E-11
ENST00000553753.5	*HNRNPC*	Heterogeneous Nuclear Ribonucleoprotein C	-9.745665809	6.40E-10
ENST00000534928.5	*ZCCHC7*	Zinc finger CCHC type containing 7	-9.69637191	1.94E-11
ENST00000566513.5	*CMIP*	C-Maf Inducing Protein	-9.346226251	1.63E-07
ENST00000643981.1	*OSBPL2*	Oxysterol Binding Protein Like 2	-9.131240716	1.40E-09
ENST00000302937.8	*TSEN34*	TRNA Splicing Endonuclease Subunit 34	-9.119311594	7.32E-09
ENST00000565683.5	*MAN2C1*	Mannosidase Alpha Class 2C Member 1	-8.778196095	1.88E-07
ENST00000457011.5	*NDUFS1*	NADH:Ubiquinone Oxidoreductase Core Subunit S1	-8.72838881	7.28E-04
ENST00000360351.8	*MAP2*	Microtubule Associated Protein 2	-8.687415485	4.24E-08
Upregulated Genes				
Transcript ID	**Gene ID**	**Protein Name**	**Log 2 fold change**	***P*-adj**
ENST00000617214.2	*KBTBD11*	Kelch Repeat And BTB Domain Containing 11	8.636119783	3.25E-08
ENST00000595635.5	*ZNF230*	Zinc Finger Protein 230	8.859972526	3.52E-07
ENST00000615946.4	*BCL2L11*	BCL2 like 11	8.867428652	4.24E-08
ENST00000589005.5	*ZNF227*	Zinc finger protein 227	8.945160201	6.37E-08
ENST00000360768.5	*VKORC1L1*	Vitamin K Epoxide Reductase Complex Subunit 1 Like 1	8.964917161	1.71E-08
ENST00000588871.5	*C19orf25*	Chromosome 19 Open Reading Frame 25	9.129251159	1.63E-08
ENST00000396388.3	*TSEN34*	TRNA Splicing Endonuclease Subunit 34	9.200759381	7.81E-10
ENST00000651280.1	*SART3*	Spliceosome Associated Factor 3, U4/U6 Recycling Protein	9.432837955	2.28E-10
ENST00000538028.1	*AAGAB*	Alpha And Gamma Adaptin Binding Protein	9.439062358	2.61E-09
ENST00000328354.10	*CHEK2*	Checkpoint kinase 2	9.690303267	1.08E-08

The top three most significantly altered transcripts in the DARPP-32 knockdown cells treated with E2 compared with DARPP-32 knockdown cells that were not treated with E2 include *ENST00000361390.2* (*MT-ND1), ENST00000361453.3* (*MT-ND2*), and *ENST00000361624.2* (*MT-CO1*) (Figure 5.5). Interestingly, all three transcripts favour activation of *NADH* dehydrogenase within the mitochondrial respiratory chain. The top three most significantly up regulated transcripts in the control cells treated with E2 compared with those that were not include *ENST00000361681.2 (MT-ND6*), and two transcripts of *MTRNR2L11*, *ENST00000604646.1* and *ENST00000540040.2* (Appendix-C Figure C3).

The top three most significant transcript alterations in the DARPP-32 knockdown cells treated with E2 compared with control knockdown treated with E2 include *ENST00000375258.8* (*METTL8*), *ENST00000593035.5 (TPGS2*), and *ENST00000515059.5 (BMPR1B)* (Appendix-C Figure C4). Interestingly, *METTL8*, otherwise known as methyltransferase 8, was amongst those transcripts that were significantly reduced following application of E2 in DARPP-32 knockdown cells compared with control cells. A function of *METTL8* is to catalyse formation of 3-methylcytidine on mRNA. Similarly, *TGPS2*, otherwise known as tubulin polyglutamylase complex subunit-2, which contains the genetic codes for a section of the neuronal polyglutamase complex required for the addition of glutamate to tubulin tales was reduced. Finally, an enhanced expression of *BMPR1B*, otherwise known as bone morphogenetic protein receptor type 1B, was observed. The details of the top 10 transcripts with the most significant differential alterations that were either down or up regulated in the DARPP-32 knockdown cells compared with control cells (both treated with E2) are summarised in Table 5.2.

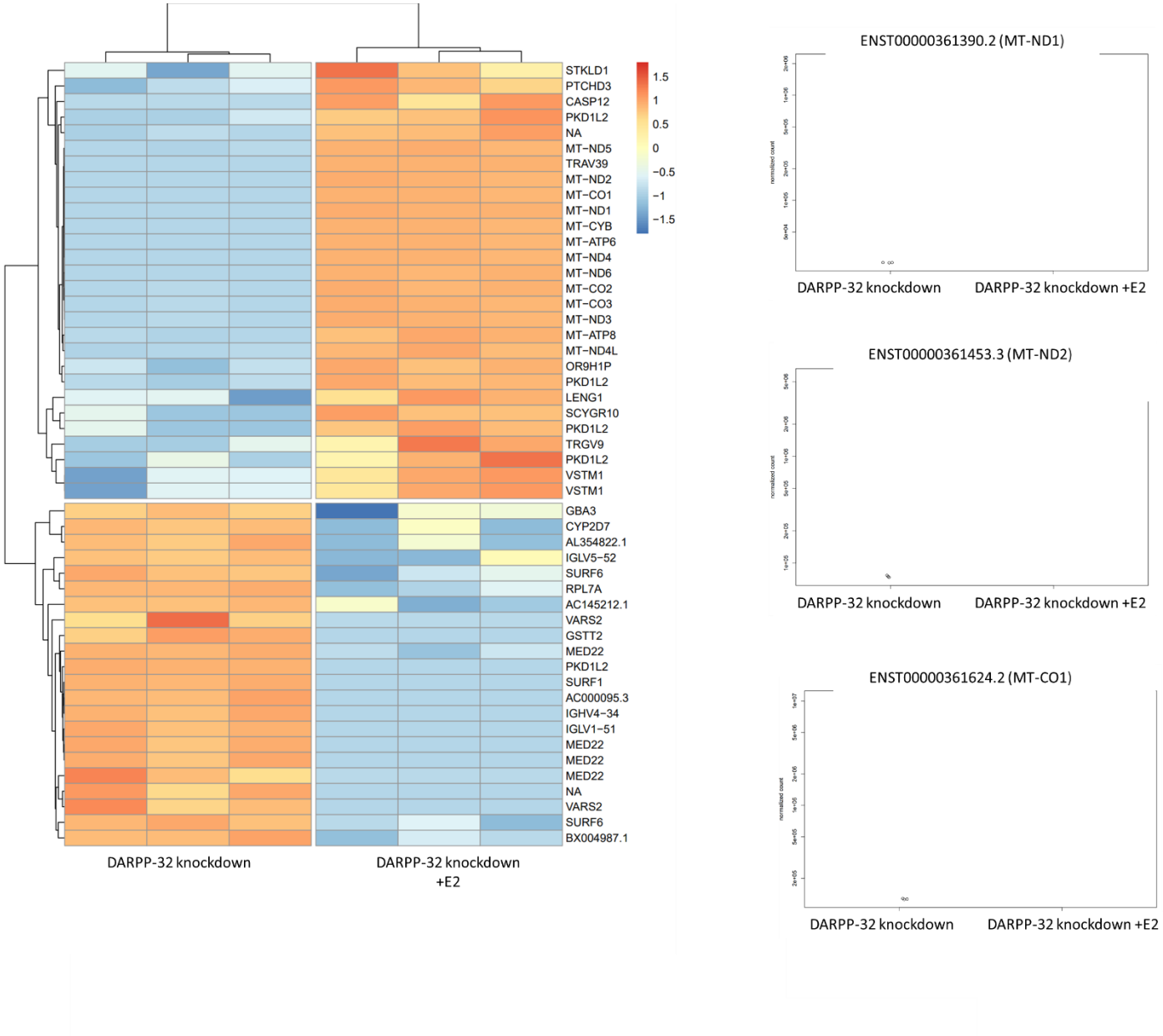


Figure 5.5: Identification and heat map analysis of the 50 most common transcripts with significant (P-adjusted) expression alterations (greater than 2-fold) in DARPP-32 knockdown cells compared to that of DARPP-32 knockdown cells treated with E2. The three most significant transcript and their corresponding gene identifiers are displayed on the right. Analysis was performed by Dr Storr.

Table 5.2: The lists of top 10 transcripts with the most significant alterations that either were down or up regulated in the DARPP-32 knockdown cells compared with control cells (both treated with E2) are shown below. The table includes details of log fold change and *P*-adjusted value for each transcript.

Downregulated Genes

Transcript ID	Gene ID	Protein Name	Log 2 fold change	*P*-adj
ENST00000527613.5	*BCLAF1*	BCL2 associated transcription factor 1	-21.7126001	2.22E-05
ENST00000400345.8	*NEDD4L*	NEDD4 like ubiquitin protein ligase	-21.6917111	2.22E-05
ENST00000598245.2	*MAGEA3*	MAGE family member A3	21.68927794	2.22E-05
ENST00000415942.5	*RBCK1*	RANBP2-tyoe and C3HC4-type zinc finger containing 1	21.67201963	2.22E-05
ENST00000394601.8	*PTBP1*	Polypyrimidine tract binding protein	21.67161156	2.22E-05
ENST00000621279.2	*GON7*	GON7 subunit of KEOPS complex	21.65351941	2.22E-05
ENST00000394201.8		Novel transcript	21.65006664	2.22E-05
ENST00000373489.10	*PBX3*	PBX hormeobox3	21.62863576	2.23E-05
ENST00000373609.1	*TXLNA*	Taxilin Alpha	21.14117586	4.13E-05
ENST00000375258.8	*METTL8*	Methyltransferase8,methylcytidine	9.739816353	8.97E-11

Upregulated Genes

Transcript ID	Gene ID	Protein Name	Log 2 fold change	*P*-adj
ENST00000273179.10	*CTDSPL*	CTD small phosphate like	8.068875732	8.24E-06
ENST00000458464.3	*ARMC1*	Armadillo repeat containing 1	8.089802111	2.11E-06
ENST00000340107.8	*FGFR3*	Fibroblast growth receptor 3	8.095462736	2.11E-06
ENST00000394123.7	*G3BP1*	G38P stress granule assembly factor 1	8.161603622	1.95E-06
ENST00000395799.8	*TNRC6A*	Trinucleotide repeat containing adaptor 6A	8.204367723	2.11E-06
ENST00000477256.5	*EIF3L*	Eukaryotic transition initiation factor 3 subunit L	8.229806348	2.11E-06
ENST00000631604.1	*CDKN1C*	Cyclin dependent kinase inhibitor 1C	8.349326336	1.66E-06
ENST00000453551.6	*PUF60*	Poly(U) binding splicing factor 60	8.714978317	1.41E-07
ENST00000515059.5	*BMPR1B*	Bone morphogenic protein receptor type 1B	9.008851339	2.53E-08
ENST00000547379.6	*SLC16A7*	Solute carrier family 16 member 7	9.555575573	2.49E-06

The effects of treatment with PKAi on transcripts expression in DARPP-32 knockdown cell were assessed by comparing against untreated samples. The top three most significant transcript alterations were *ENST00000260356.5 (THBS1), ENST00000295524.4 (CLDN1),* and *ENST00000224784.10 (ACTA2)* where treatment with PKAi brought on a reduction in the expression of all three transcripts (Figure 5.6). The top three most significant transcript alterations in the control cells compared with control cells treated with PKAi included *ENST00000372764.4 (PLAU), ENST00000451137.7 (CCN1)* and *ENST00000223095.4 (SERPINE1)* where treatment with PKAi brought on a reduction in the expression of all three transcripts (Appendix-C Figure C5). The top three most significant transcript alterations in the DARPP-32 knockdown cells treated with PKAi compared with control cells treated with PKAi included *ENST00000652269.1 (ELMO3), ENST00000643625.1 (TSC1)* and *ENST00000637526.1 (ATP6AP2)* (Appendix-C Figure C6). *ELMO3*, otherwise known as engulfment and cell motility-3 which has a crucial role in cell migration, was down regulated in DARPP-32 knockdown cells treated with PKAi. In contrast, *TSC*1, otherwise known as hamartin, was found more abundantly in PKAi treated DARPP-32 knockdown cells compared to the PKAi treated control cells. *TSC1* is involved in tumour suppression through stabilisation of GTPase activating protein tuberin and inhibition of mammalian target of rapamycin complex 1 *(mTORC1*).

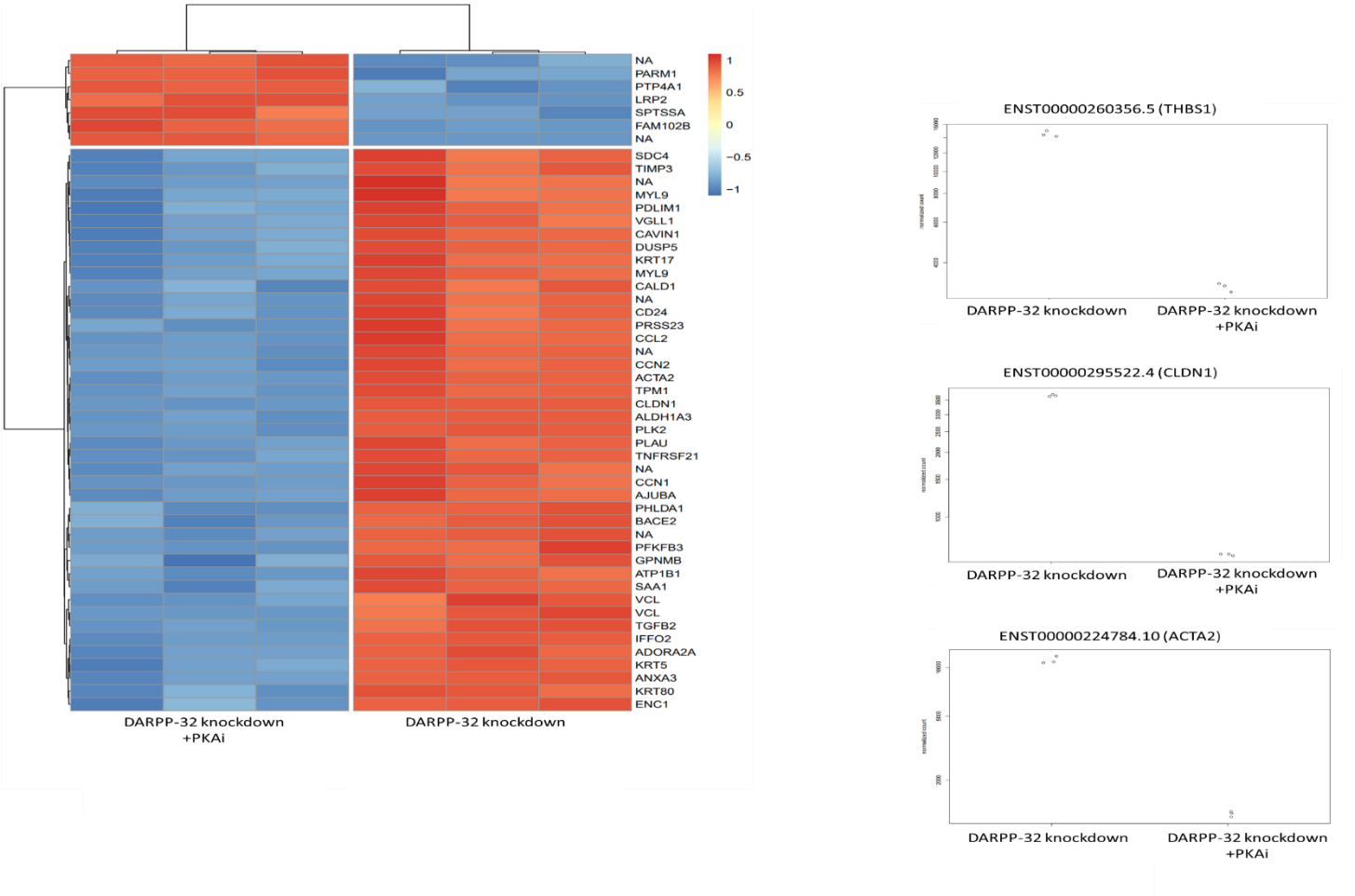


Figure5.6: Identification and heat map analysis of the 50 most common transcripts with significant (*P*-adjusted) expression alterations (greater than 2-fold) in DARPP-32 knockdown cells compared to that of DARPP-32 knockdown cells treated with PKAi. The three most significant transcript and their corresponding gene identifiers are displayed on the right. Analysis was performed by Dr Storr.

5.3.5 Overlapping of differential gene expression

Venn diagram was adopted as a graphical demonstration of the overlapping of transcripts expressed in the presence and in the absence of E2 treatment or PKA inhibition allowing for the identification of differentially expressed transcripts unique to each arrangement. Identification of such transcript could serve as a platform for the initiation

of future investigations focused on the study of transcripts involved in the progression of breast cancer.

The analysis of overlapping transcripts that were differentially expressed in both DARPP-32 knockdown cells versus control cells, and DARPP-32 knockdown cells treated with E2 versus control cells treated with E2 led to identification of transcripts *ENST00000625998.2* and *ENST00000651280.1*, which are associated with gene identifier *PROSER1* (proline and serine rich 1) and *SART3* (spliceosome associated factor 3 U4/U6 recycling protein), respectively. 51 transcripts were found to be differentially expressed in the DARPP-32 knockdown cells/control cells only; whereas, 9 differentially expressed transcripts were unique to the E2 treated DARPP-32 knockdown cells/ E2 treated control cells groups. Stimulation with E2 led to an overlap of 29680 transcripts that were differentially expressed irrespective of DARPP-32 expression status. DARPP-32 knockdown cells compared to DARPP-32 knockdown cells stimulated with E2 exhibited differential expression in 12738 unique transcripts, whereas the control cells with vs without E2 exhibited a differential expression in 10180 unique transcripts as demonstrated in Figure 5.7. In Figure 5.7, each oval corresponds to the number of differentially expressed transcripts specific for separate groups, and areas of overlap point to the overlap of differentially expressed transcripts between the groups.

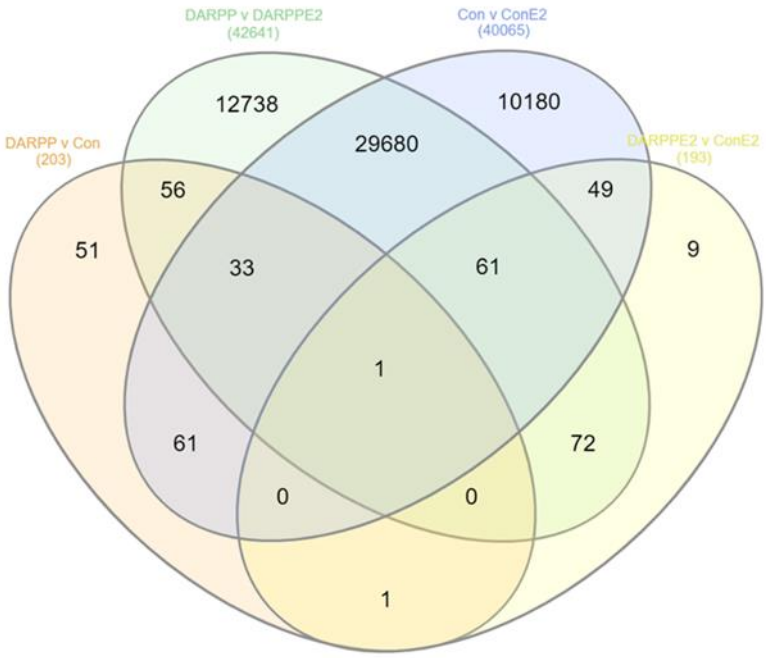


Figure5.7: Venn diagram was adopted in order to demonstrate the overlapping of transcripts that were expressed in the presence and in the absence of E2 treatment. Created using InteractiVenn by Heberle *et.al* (2015). Analysis performed by Dr Storr.

The analysis of transcripts that were differentially expressed in the four groups in the presence and in the absence of PKAi led to the identification of numerous overlapping transcripts in those groups (Figure 5.8). This includes a total of 519 transcripts that were commonly altered in both PKAi treated versus untreated DARPP-32 knockdown cells, and PKAi treated versus untreated control cells. However, the analysis of PKAi treated DARPP-32 knockdown versus PKAi treated control cells led to the identification of 130 transcripts with differential expression which were unique to that group.

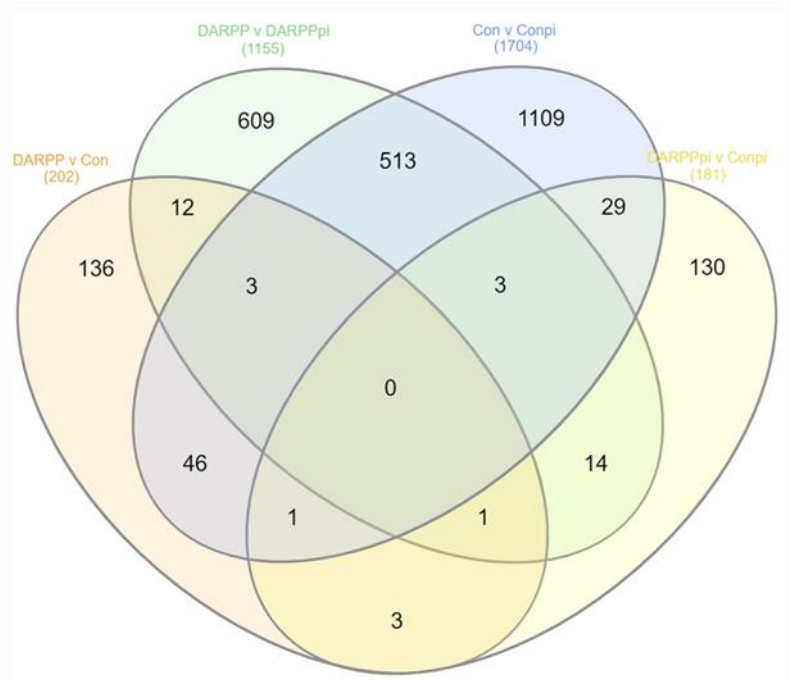


Figure5.8: Venn diagram to demonstrate the overlapping transcripts that expressed in the presence and absence of PKAi treatment. This figure was created using InteractiVenn by Heberle *et.al* (2015). Analysis performed by Dr Storr.

5.3.6 Gene Enrichment Pathway analysis

The relationship between DARPP-32 (*PPP1R1B*) and a wide array of genes that were differentially expressed across a range of previously described experiments was further investigated using Qiagen IPA in order to identify the impact of DARPP-32 knockdown, E2 stimulation and PKAi treatment on various pathways (cut off *P*-adjusted value of $P<1x10^{-22}$). The top 10 pathways induced by each variable are demonstrated in Figure 5.9. Alterations in 14 canonical signalling pathways were detected when DARPP-32 knockdown cells were compared against the control group. Alterations in 105 pathways were detected when DARPP-32 knockdown

cells treated with E2 were compared against DARPP-32 knockdown cells that were not treated with E2. Alterations in 94 pathways were detected when control cells treated with E2 were compared against control cells that were not treated with E2. Alterations in 84 pathways were detected when DARPP-32 knockdown cells treated with E2 were compared against control cells treated with E2.

241 pathways were found to be affected in DARPP-32 knockdown cells that were treated with PKAi compared to DARPP-32 knockdown cells that were not treated with PKAi. 296 pathways were found to be significantly affected when control cells treated with PKAi were compared with those that were not treated with PKAi. The total number of pathways that were significantly affected in PKAi treated DARPP-32 knockdown cells compared to PKAi treated control cells was 38.

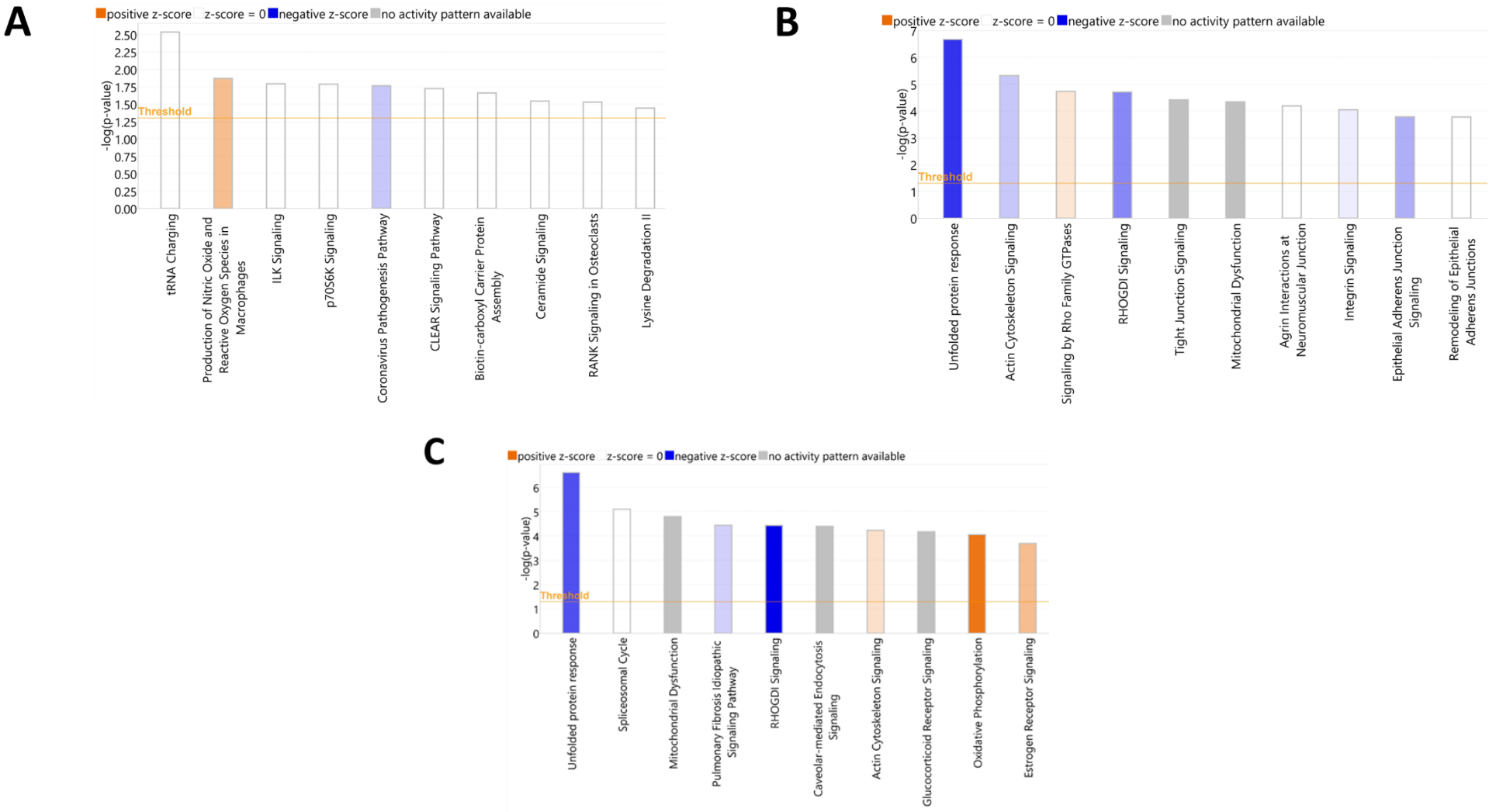
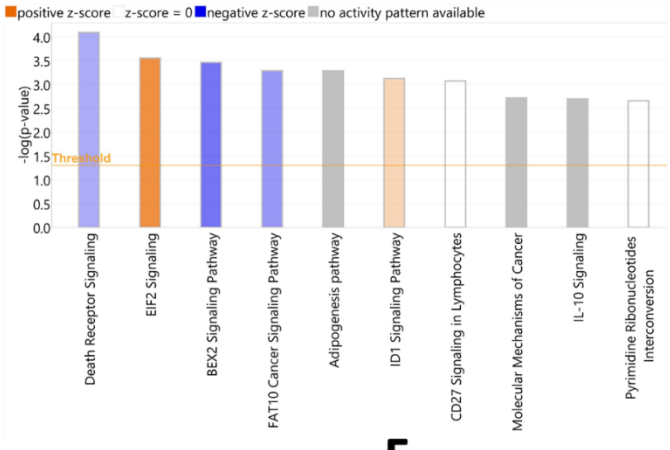
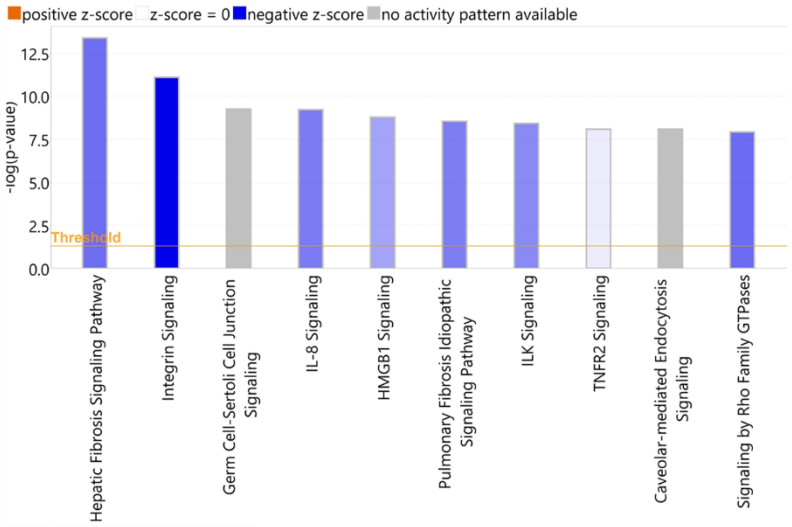


Figure Continued

D



E



F

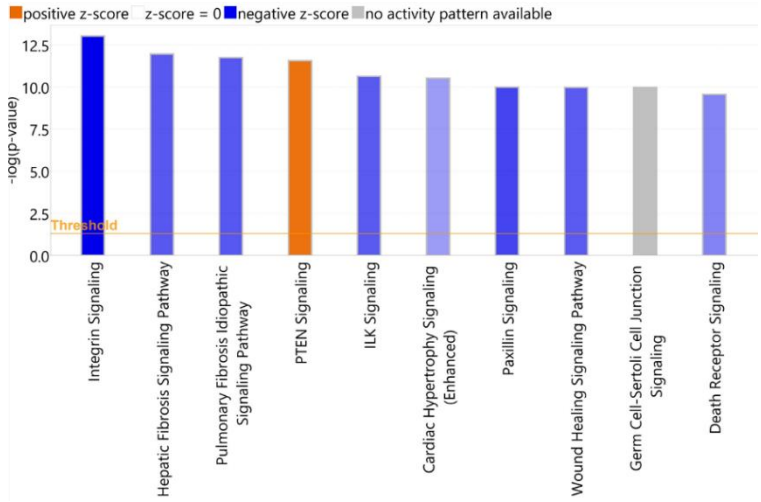


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G

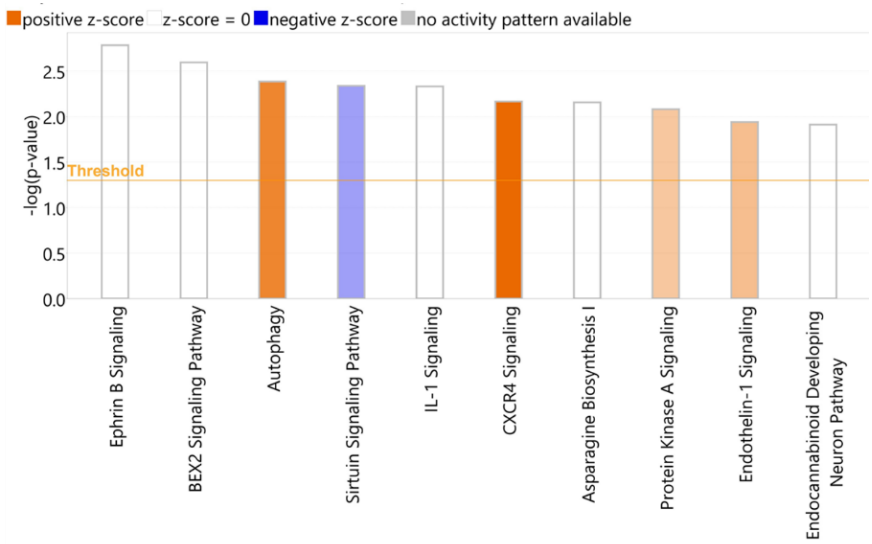
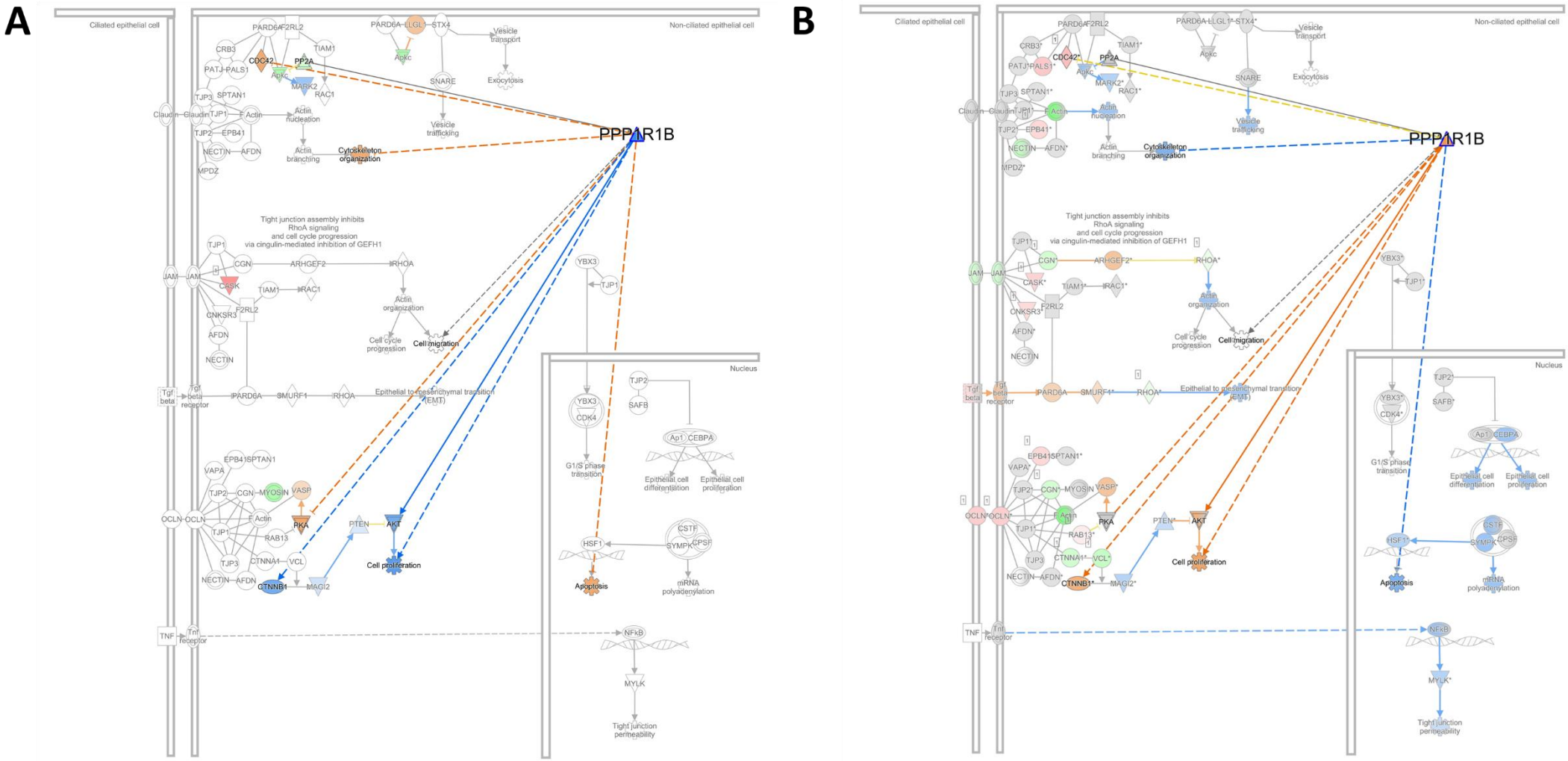


Figure5.9: The 10 most significantly (threshold *P*<0.05) altered pathways are shown above. An increase or a decrease in pathway activity is demonstrated using either positive (orange bar) or negative z-scores (blue bar), respectively. The top 10 pathways with differential activities in DARPP-32 knockdown cells and control cells are shown in A. The top 10 pathways with differential activities in E2 treated and untreated DARPP-32 knockdown cells are shown in B. The top 10 pathways with differential activities in E2 treated and untreated control cells are shown in C. The top 10 pathways with differential activities in E2 treated DARPP-32 knockdown cells and E2 treated control cells are shown in D. The top 10 pathways with differential activities in PKAi treated and untreated DARPP-32 knockdown cells are shown in E. The top 10 pathways with differential activities in PKAi treated and untreated control cells are shown in F. The top 10 pathways with differential activities in PKAi treated DARPP-32 knockdown cells and PKAi treated control cells are shown in G. Analysis was performed by Dr Storr.

While there were a number of unique pathways attributed to each of the four analysis groups, only one pathway (ILK signalling) was found to be common amongst all groups. Tight junction signalling pathway was identified as a common pathway altered in all DARPP-32 knockdown groups regardless of E2 treatment. In tight junction signalling pathway alterations of PP2A expression was observed and the known and putative changes in activation state based on *PPP1R1B* expression were mapped where DARPP-32 was added to the pathway as a node (Figure 5.10). Alteration of PP2A expression that were observed in the DARPP-32 knockdown cell versus control cell analysis was believed to be mediated through alteration of PPP2R5C subunit component (protein phosphatase 2 regulatory subunit B gamma), whereas in DARPP-32 knockdown cell treated with E2 versus control cell treated with E2 this was mediated through alteration of PTPA (protein phosphatase 2 phosphatase activator) expression. Ceramide signalling was identified as the only common pathway altered in DARPP-32 knockdown cells versus control cells, and DARPP-32 knockdown cells treated with E2 versus control cells treated with E2. tRNA charging pathway, coronavirus pathogenesis pathway and IGF-1 signalling pathway were identified as the three common pathways altered in DARPP-32 knockdown cells versus control cells and the DARPP-32 knockdown cells versus DARPP-32 knockdown cells treated with E2, but no common pathways were identified in the control cells versus control cells treated with E2 analysis.


A
Ciliated epithelial cell
Non-ciliated epithelial cell
PPP1R1B
Tight junction assembly inhibits RhoA signaling and cell cycle progression via cingulin-mediated inhibition of GEFH1
Cell migration
Cell proliferation
Epithelial to mesenchymal transition (EMT)
Nucleus
Apoptosis
Tight junction permeability
B
Ciliated epithelial cell
Non-ciliated epithelial cell
PPP1R1B
Tight junction assembly inhibits RhoA signaling and cell cycle progression via cingulin-mediated inhibition of GEFH1
Cell migration
Cell proliferation
Epithelial to mesenchymal transition (EMT)
Nucleus
Apoptosis
Tight junction permeability

C

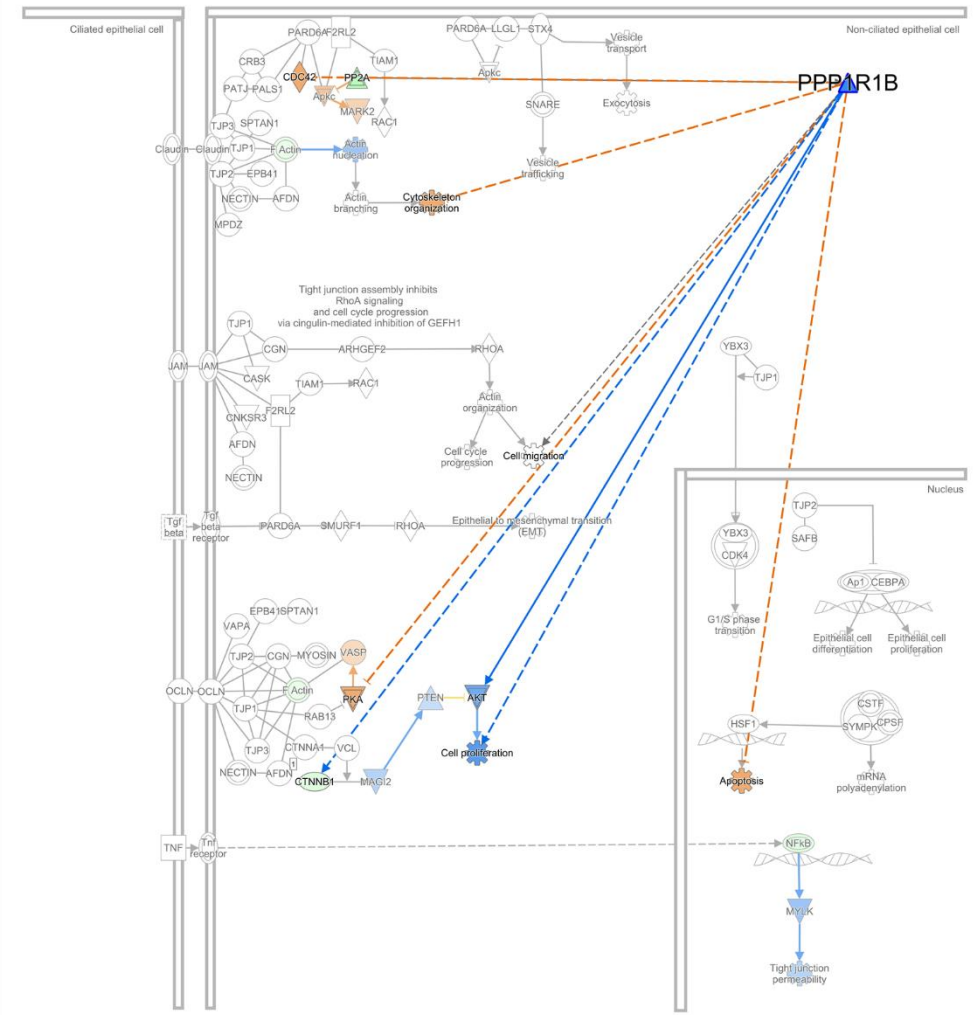


Figure5.10: Addition of *PPP1R1B* (DARPP-32) to the Tight junction canonical pathway for DARPP-32 knockdown cell versus control cell (A), DARPP-32 knockdown cell versus DARPP-32 knockdown cell treated with E2 (B), and DARPP-32 knockdown cell treated with E2 versus control cell treated with E2 (C). In this figure, genes that are highlighted in red represent increased expression, whereas those in green represent decreased expression. The blue dotted lines represent the interactions that are predicted to induce inhibition of the genes (also in blue) that they are connected to. The orange dotted lines represent the interactions that are predicted to induce activation of the genes (also in orange) that they are connected to. Colour yellow represents inconsistent effect on a downstream molecule, and colour grey represent instances where the outcome of an interaction is not predicted. Analysis was performed by Dr Storr.

5.5 Discussion

DARPP-32 knockdown was successfully performed using siRNA, and verified using Western blotting and real-time PCR techniques; protein and mRNA expression of DARPP-32 were shown to drop by more than 95% and 60%, respectively.

The impact of DARPP-32 knockdown on the transcriptome was determined using RNA-seq. The effects of E2 stimulations or PKA inhibition were determined in both DARPP-32 knockdown and control cells. T47D cells were selected in this experiment in order to further investigate the importance of DARPP-32 expression in an ER positive breast cancer cell line. This decision was based on a combination of results obtained from Immunohistochemistry highlighting the importance of DARPP-32 expression in ER positive cells.

The three most significant differentially expressed transcripts identified when comparing DARPP-32 knockdown cells against control cells were *ENST00000443236.6 (CPAMD8), ENST00000521309.5 (YWHAZ*), and *ENST00000651564.1 (CPAMD8).* There is little information available on the implication of *CPAMD8* in development of human disease in literature, except for its involvement in regulating aqueous humour (AH) and development of anterior segment dysgeneses (recessive), a disorder that impairs the development of the anterior segment of the eye (Cheong et al., 2016); for this reason, investigation of CPAMD8 in future work is important.

Increased expression of *YWHAZ* has been associated with cellular proliferation, apoptosis, cell invasion and migration across a variety of tumours including hepatocellular carcinoma, colorectal cancer, lung cancer and breast cancer (Gan et al., 2020). Further evaluation of the implications associated with *YWHAZ* expression would allow for identification of potential links with the DARPP32 pathway in breast

cancer. Previous studies have described the role of *YWHAZ* in the regulation of stress fibres and focal adhesions via the RhoA signalling pathway (Ye et al., 2016, Mei et al., 2021). Other studies have found links between higher levels of *YWHAZ* and poor patient outcome, while down regulation of *YWHAZ* using siRNA in metastatic breast cancer has shown promising results in reducing cancer growth both *in vitro* and *in vivo* (Neal et al., 2009, Mei et al., 2021). There are emerging reports with promising results in reducing tumour progression in gastric cancer cell lines *in vitro* through post transcriptional regulation of *YWHAZ* using microRNA miR-375 (Nishimura et al., 2013, Tsukamoto et al., 2010). Recent discoveries show promising results regarding the effectiveness of protein/gene expression determination as a prognostic tool in malignancies such as breast cancer, lung cancer, and head and neck cancer (Fan et al., 2007, Watanabe et al., 2016).

The top 10 down regulated or top 10 up regulated transcripts identified as a result of DARPP-32 suppression included genes that were previously linked with malignancies as well as genes that were not previously linked with malignancies. High expression of *HNRNPC* (an RNA binding protein) and *CMIP* (C-Maf Inducing Protein) has been reported in a number of tumours with limited information on their exact role in the development of those tumours (Wu et al., 2018). A previous investigation on the role of *HNRNPC* in breast cancer demonstrated a strong anti-proliferative effect associated with the inhibition of *HNRNPC* through interferon cascade activation in MCF7 and T47D cells (Wu et al., 2018). Further evaluation of the implications associated with *HNRNPC* expression could facilitate identification of potential links with the DARPP32 pathway in breast cancer. Similarly, *CMIP* has been identified as a proto-oncogene and its overexpression has been detected in human gastric cancer while its suitability as therapeutic target or a potential biomarker remains under debate (Zhang et al., 2017). The evidence available in literature also supports reduced expression of *KBTBD11* (a negative regulator of

osteoclastogenesis) in cancer; previous studies have found down regulation of *KBTBD11* to favour evasion of apoptosis and enhanced growth in colorectal cancer (Watanabe et al., 2019).

The three most significant differentially expressed transcripts identified when comparing E2 treated and untreated DARPP-32 knockdown cells were *MT-ND1*, *MT-ND2*, and *MT-CO1,* whereas in E2 treated and untreated control cells, *MT-ND6,* and two transcripts corresponding to *MTRNR2L11* were identified as the three most significant differentially expressed transcripts. In support of our findings, previous studies have also reported increased expression of *MT-ND1*, and *MT-ND2* in colorectal cancer, and *MT-ND6* in osteosarcoma cells (Akouchekian et al., 2011, Stoccoro and Coppedè, 2022). Overexpression of both the wild-type and the mutated variants of *MT-CO1* have been associated with promoting cancer phenotype enabled directly through high glucose uptake and lactate release *in vitro* (Singh et al., 2019).

The top 10 down regulated or top 10 up regulated transcripts identified in E2 treated DARPP-32 knockdown cells versus untreated DARPP-32 knockdown cells are shown in Figure 5.5. However, not all of the genes associated with the top 10 down regulated or top 10 up regulated transcripts were previously linked with malignancies. In contrast, *NEDD4L* and *PTBP1,* two genes previously associated with various cancers, were amongst the down regulated transcripts in this group, whereas *ARMC1, FGFR3*, and *PUF60* were amongst the transcripts that had been up regulated. Low levels of *NEDD4L* enzyme have been associated with shortened cancer-specific survival in clear cell renal carcinoma and renal chromophobe cell carcinoma. Additionally, absence of sufficient *NEDD4L* expression favours interstitial and immune cell infiltration in most cancers (Dong et al., 2021). Also, speculations over its role in destabilisation of pro-tumour proteins may add to *NEDD4L's* importance as a tumour suppressor. In contrast, abundance of *NEDD4L* in melanomas has been associated with cancer progression, which adds to the complexity of

biological mechanisms in cancer (Gao et al., 2012, sakashita et al., 2013, Tanksley et al., 2013, Lee et al., 2020). Further investigations focused on evaluating the association of transcripts with currently unknown clinical implications in breast cancer may lead to uncovering new links which would allow us to better understand the mechanisms involved in breast cancer progression. Previous studies have demonstrated that high levels of *PTBP1* are associated with transformation of human mammary epithelial cell, uncontrolled cellular growth, tissue invasion, and enhanced aerobic glycolysis in breast cancer (He et al., 2014). Further evaluation of the implications associated with *PTBP1* expression in breast cancer may provide useful information on potential links with the DARPP32 pathway. Over expression of *ARMC1* has been implicated in invasive breast carcinoma where it can serve as a prognostic and diagnostic biomarker (Gan et al., 2022). Overexpression of *FGFR3* has been implicated in a number of tumours including multiple myeloma, bladder cancer, non-small cell lung cancer, oral cancers, and oropharyngeal squamous cell carcinoma (Li et al., 2019). Overexpression of *PUF60* has been associated with the inhibition of apoptosis, vigorous tumour growth, invasion and metastasis in breast cancer (Sun et al., 2019).

The three most significant differentially expressed transcripts identified when comparing E2 treated DARPP-32 knockdown cells and E2 treated control cells were *METTL8, TPGS2*, and *BMPR1B*. High levels of *METTL8* (Human Methyltransferase 8, methylcytidine) have been previously reported in the majority of isolated breast cancer cell lines where knockdown of its associated transcription factor *Yin Yang 1 (YY1)* has been validated as an effective method of inhibition, leading to a consequent repression of abnormal growth and metastasis (Lee et al., 2021). Mutations of *PTGS2* (Prostaglandin endoperoxide synthase 2) gene have been associated with cancers affecting skin, colon and endometrium; increased expression of this gene inversely correlates with cancer-specific survival and enhances the risk of tumour relapse in

patients (Kunzmann et al., 2013). Aberrant expression of *BMPR1B*, also known as bone morphogenetic proteins receptor type 1B, has been associated with poor prognosis and increased susceptibility to breast cancer, especially the *rs1434536* and *rs1970801* alleles, whereas other alleles such as rs1434536 T and *rs1970801* T promote *BMPR1B* overexpression (Zheng et al., 2022). Further evaluation of the implications associated with *BMPR1B or METTL8* expression in breast cancer may provide useful information on potential links with the DARPP32 pathway.

Previously, high levels of E2 have also been shown to alter expression of genes crucial for cancer progression including *CCND1*, Cathepsin D, EGFR and MYC (Basu and Rowan, 2005). Activation of *MYC* regulator genes has been commonly associated with factors such as the regulation of cell growth, metabolic rate, differentiation, and survival which are crucial determinants for the development and progression of malignant cells (Duffy et al., 2021, Xu et al., 2010). In breast cancer in particular, high levels of MYC seem to be due to sensitivity to the fluctuations in the level of endogenous oestrogen. Stimulation of oestrogen-induced *MYC* gene expression involves signal transduction by the oestrogen receptors and the activation of transcription factor activator protein 1 (AP-1) (Fallah et al., 2017, Shang et al., 2000, Wang et al., 2011).

While there is a limited amount of data currently available in literature that links the function of DARPP-32 and oestrogen, the association between PKA and oestrogen has been well documented; PKA-induced phosphorylation of oestrogen receptor alpha (ERα) at Ser-305 residue has been shown to promote development of tamoxifen resistance in breast cancer through expression of MYC (de Leeuw et al., 2013). Additionally, Dopamine-induced activation of ER in human female brain has been previously described as an indirect association between PKA and oestrogen receptor (Del Rio et al., 2018, Olesen and Auger, 2008)

Previously, our group identified that low DARPP-32 expression in ER positive patients is associated with poor prognosis in breast cancer. Since over 80% of all breast cancers are oestrogen receptor positive where oestrogen contributes to boosting uncontrolled growth, a number of therapeutic agents including tamoxifen and anastrazole have been used to block the effects of oestrogen on these receptors. However, cancer relapse remains a significant issue and employment of new approaches is required. Thus, orchestration of strategies that combine conventional methods with regulation of DARPP-32 may have clinical benefits (Kotecha et al., 2019).

In the current study, Qiagen IPA was utilised to identify the significantly affected pathways following DARPP-32 suppression and/or E2 treatment. Tight junction pathway was identified as a common pathway affected by DARPP-32 knockdown regardless of E2 treatment. This was facilitated through identification of pathways common to DARPP-32 knockdown cell versus control cells, and E2-treared DARPP-32 knockdown cells versus E2-treated control cells. Alteration of PP2A expression in tight junction signalling pathway was independent of E2 treatment. Interaction of tumour suppressor PP2A and DARPP-32 leads to increased availability of PKA by dephosphorylating Threonine-75 residue (Slupe et al., 2011).

In an effort to investigate the importance of PKA on the activity of DARPP-32, PKA inhibitor fragment (6-22) amide which is derived from the active portion of the heat stable PKA inhibitor protein PKI, was used to inhibit PKA (as described in section 2.4.9). As a result, a 60% fall in both the activity of PKA and DARPP-32 Threonine-32 phosphorylation was observed in T47D cells following 24 hours of exposure to the PKA inhibitor (3μM concentration).

The three most significant differentially expressed transcripts identified when comparing PKAi treated and untreated DARPP-32 knockdown cells were THBS1, *CLDN1*, and *ACTA2. THBS1,* also known as Thrombospondin

1, is an angiogenesis suppressor gene that has been found to be up regulated in a number of cancers. *THBS1's* ability to promote tumour invasion and metastasis has been reported in gastric cancer, colorectal cancer and T-cell lymphoma (Zhang et al., 2021). *CLDN1* is a cancer invasion/metastasis suppressor whose expression levels directly correlates with patient outcome in lung adenocarcinoma (Chao et al., 2009). *ACTA2* has a role in enabling metastasis in human lung adenocarcinoma (Lee et al., 2013).

The three most significant differentially expressed transcripts identified when comparing PKAi treated and untreated control cells were *PLAU, CCN1* and *SERPINE1*; the expression levels of all three transcripts were depleted following treatment with PKAi. *PLAU* is known to promote transformation of fibroblasts to inflammatory cancer-associated fibroblasts through uPAR/Akt/NF-κB/IL8 pathway in oesophageal squamous cell carcinoma (Fang et al., 2021). *CCN1* is a pro-angiogenic factor that has been found to be up regulated in 30% of invasive breast carcinomas including triple negative breast carcinoma, where it promotes cancer development, metastasis and chemotherapy resistance (Espinoza et al., 2011). Similarly, up regulation of *SERPINE1* has been associated with tumour progression and poor prognosis in gastric cancer (Chen et al., 2022).

The three most significant differentially expressed transcripts identified when comparing PKAi-treated DARPP-32 knockdown cells with PKAi-treated control cells were *ELMO3, TSC1* and *ATP6AP2*. While the role of *ELMO3* in cancer has already been discussed, expression of *TSC1* has been associated with tumour suppression in liver, lung, bladder, breast, ovarian, and pancreatic cancers (Mallela and Kuma, 2021). Upregulation of *ATP6AP2* in breast cancer has reportedly been associated with tumour progression (Zhao et al., 2020). Further evaluation of the clinical implications of *ELMO3, TSC1* and *ATP6AP2* expression in breast cancer

may provide useful information on potential links with the DARPP32 pathway.

In addition to oestrogen, growth factors are also known to stimulate growth in E2 positive breast cancer cells through interactions with their tyrosine kinase transmembrane receptors which induce a signalling cascade, leading to the activation of proteins such as c-Src, MAPK (ERK1/2) and Akt which accelerate cellular growth as previously described. Convergence of oestrogen and growth factor pathways could result in a more robust and prolonged proliferative response in breast cancer cells. However, an 'alternative pathway' independent of the 'classical pathways' for intracellular steroid receptors has also been described which involves collaboration of sex hormone binding globulin (*SHBG*) and E2 in activation of membrane SHBG-Receptors through cAMP and PKA, leading to inhibition of growth in breast cancer cells (Bowers-Morrow and Ali, 2005).

Previously, the combined effects of PKA inhibitor fragment (6-22) amide and cAMP accumulation induced by *SHBG* was investigated in MCF7 breast cancer cells. Given that cAMP has a role in the inhibition of PKA by promoting dephosphorylation of DARPP-32's Threonine-75 residue through regulation of PP2A, the combination of PKA inhibition methods resulted in a more comprehensive inhibition of the protein (Fortunati et al., 1996).

Conclusion

T47D breast cancer cell line was used as a model for DARPP-32 knockdown in this study, where the success of this procedure was verified using Western blot and real-time PCR. Furthermore, the impact of treatment with E2 and PKA inhibition was assessed by monitoring differentially expressed transcripts and alterations within various pathways that may be implicated in breast cancer. A number of differentially expressed transcripts that were identified in this study have

been previously shown to have a role in deterioration of patient health and reduced survival. Qiagen IPA analysis revealed tight junction pathway as a pathway affected by DARPP-32 knockdown regardless of stimulation with E2. The interactions between tumour suppressor PP2A and DARPP-32 in this pathway leads to increased availability of PKA by dephosphorylating Threonine-75 residue. The findings of this study further highlight the importance of DARPP-32 in coordination of key cellular processes in various cancer associated signalling pathways.

5.6 Summary

- *ENST00000443236.6 (CPAMD8), ENST00000521309.5 (YWHAZ*), and *ENST00000651564.1 (CPAMD8)* were identified as the three most significant differentially expressed transcripts as a result of DARPP-32 knockdown
- *METTL8, TPGS2*, and *BMPR1B* were identified as the three most significant differentially expressed transcripts as a result of E2 treatment of DARPP-32 knockdown cells compared with E2-treated control cells.
- Tight junction pathway was identified as a common pathway affected by DARPP-32 knockdown regardless of E2 treatment.
- *PLAU, CCN1* and *SERPINE1* were identified as the three most significant differentially expressed transcripts as a result of PKAi treatment of control cells
- *ELMO3, TSC1* and *ATP6AP2* were identified as the three most significant differentially expressed transcripts as a result of PKAi treatment of DARPP-32 knockdown compared with PKAi-treated control cells

Chapter 6 General Discussion, Limitations, and Future Work

6.1 Discussion

Breast cancer is the most prevalent type of cancer in the UK which effects one in every seven women at some point in their lifetime (Breast Cancer Now, 2022). 80-90% of breast cancer patients have an expected survival period of more than five years if they are diagnosed and treated early (Yang et al., 2019). The prognosis of breast cancer patients depends predominantly on early diagnosis and effective treatment; therefore, accurate and prompt identification of breast cancer type has a direct impact on patient survival.

The function of DARPP-32 largely depends on the phosphorylation state of Threonine-34 and Threonine-75; PKA induced phosphorylation of Threonine-34 transforms DARPP-32 into a potent inhibitor of PP1, whereas Cdk5 induced phosphorylation of Thronine-75 facilitates suppression of PKA activity (a summary diagram of DARPP-32 pathway is demonstrated in Figure 6.1). The canonical splice variant, truncated-DARPP, or t-DARPP, lacks the Threonine-34 residue, creating a functionally significant variant (Theile et al., 2017, Christenson and Denny, 2015, Vangamudi et al., 2011). Although the exact implications of DARPP-32 splicing in cancer progression and metastasis remain unclear, detection of t-DARPP and DARPP-32 could be perceived as an important biomarker for prognosis of breast cancer and metastasis. While reduced levels of DARPP-32 in breast cancer may be the consequence of an abnormal cellular switch to t-DARPP expression, low expression of Cdk5 could shift the function of DARPP32 towards PP1 inhibition following PKA-induced phosphorylation of Threonine-75 residue; our results illustrate that these events are associated with adverse breast cancer specific survival.

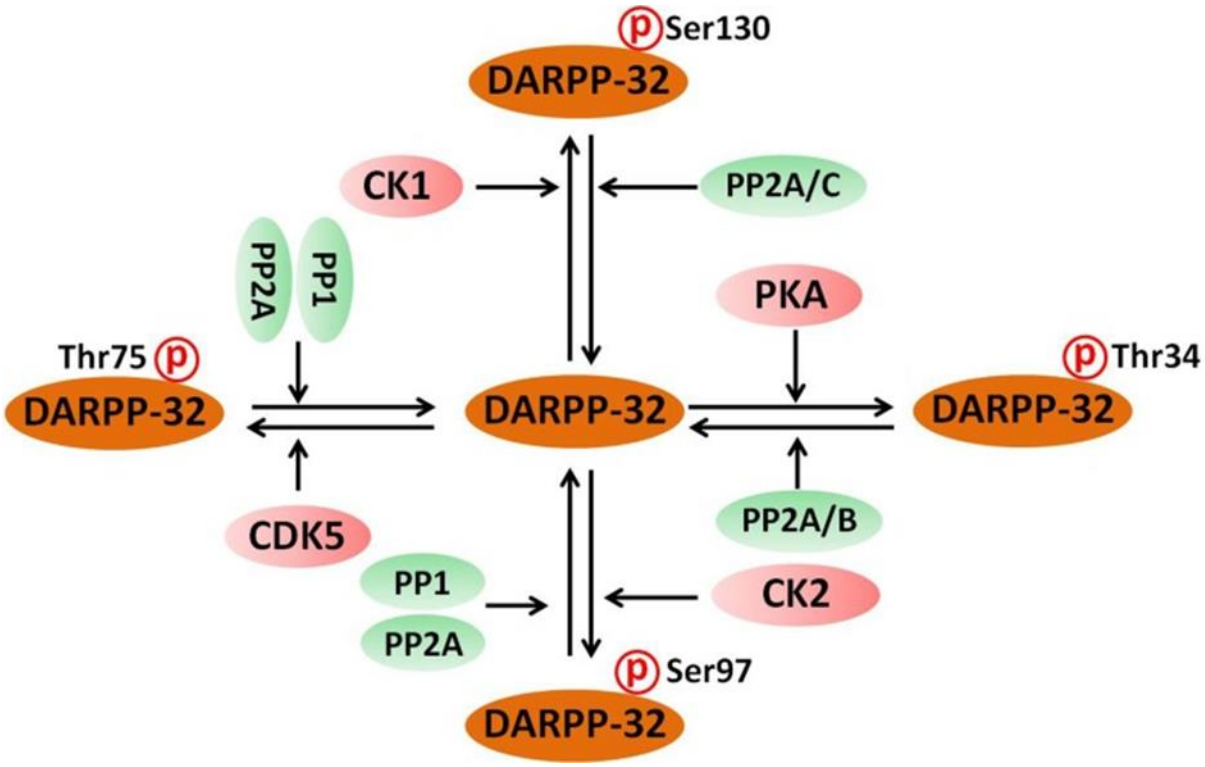


Figure 6.1: Summary diagram of DARPP-32 pathway demonstrating regulation of DARPP-32 phosphorylation state by protein kinases and phosphatases. In this figure, protein phosphatases are shown in green ovals and protein kinases in red ovals (Image taken from Wang et al., 2017), permission by Creative Commons Attribution license (CC-BY-3.0).

Previously, our group has identified DARPP-32 as an important biomarker in breast cancer at both protein and mRNA levels (Kotecha et al., 2019). Also, the analysis of gene expression data using artificial neural network by our research group has led to the identification of a number of genes, including *DKK1 and GRB7*, *as gene candidates* that could be associated *with the expression of PPP1R1B.* We have also demonstrated that DARPP-32 may be involved in facilitating tumour metastasis, primarily to bone tissue, through interactions with various proteins including transcription factor *Sp7.* In order to build on the findings of our previous research, the expression level of important signalling partners of DARPP-32 including

PP1, PKA and Cdk5, as well as potential DARPP-32 signalling partners such as *DKK1, GRB7* and *Sp7* were determined.

The link between DARPP32 and metastasis in colorectal cancer has been previously described by Kopljar *et.al* in 2015. Clinical and histopathological data of 100 colorectal cancer patients analysed as a part of this study indicated that the expression of DARPP-32 was significantly higher in patients exhibiting tumour metastasis *(P*=0.002). Multivariate regression analysis also confirmed that expression of DARPP-32 in the primary cancer tissue serves as a significant predictor of distant metastases, and it negatively impacts both overall and disease free survival (*P*=0.037) with a cut-off point of 76.5% (Kopljar et al., 2015).

Previously, our group demonstrated that low expression of DARPP-32 was significantly associated with poor breast cancer specific survival in large cohorts of early breast cancer patients. In the current study, the level of phosphorylated DARPP-32 at Threonine-34, which is only present in full length DARPP-32, was also significantly associated with survival indicating the importance of DARPP-32 and downstream proteins such as PP1 protein on clinical outcome of breast cancer patients.

While low PP1 protein expression was not associated with patient survival, combined expression of low PP1 and low DARPP-32, and low PP1 and low Cdk5 were significantly associated with adverse breast cancer specific survival.

Low protein expression of Cdk5, was significantly associated with adverse breast cancer specific survival. Low nuclear and cytoplasmic expression of Cdk5 remained associated with survival when other potentially crucial factors such as tumour size, grade, nodal stage, ER status, PR status, HER2 status, and LVI status were included in multivariate Cox regression analysis. There are limited study data available in literature regarding the impact of Cdk5 expression on patient survival and they are often limited in patient numbers. Previously, it has been shown that low expression of

Cdk5 is associated with poor prognosis and adverse cancer specific survival gastric adenocarcinoma, liver and brain cancer cells (Sun et al., 2016, Zeng et al., 2018). In comparison to our study which showed that low Cdk5 protein expression is associated with ER positive status, one study on breast cancer (n=108) demonstrated the association between high Cdk5 protein expression and ER negative status (Liang et al., 2013).

Low protein expression of PKA was significantly associated with adverse breast cancer specific survival. In addition, unlike the combined expression of PKA and cytoplasmic DARPP-32 which was not significantly associated with breast cancer specific survival, the combination of low PKA and low Cdk5 expression were significantly associated with adverse breast cancer specific survival.

Low protein expression of *DKK1* was significantly associated with adverse breast cancer specific survival. Previous studies have indicated that *DKK1* may be involved in promotion of metastasis in various tumours which has a direct impact on cancer-specific survival. Therefore, detection of high levels of this protein may be useful for prognostic purposes (Kasoha et al., 2018, Zhu et al., 2021, Han et al., 2015).

Low protein expression of *Sp7* was significantly associated with adverse breast cancer specific survival. Previously, Yao *et.al* (2019) reported association of high *Sp7* expression with breast cancer specific survival; in this study, *Sp7* was expressed in 57.1% of the samples out of a total of 154 tissues from breast cancer patients (Yao et al., 2019).

Low protein expression of *GRB7* was significantly associated with improved breast cancer specific survival. *GRB7* has been shown to promote cancer cell migration through modulation of focal adhesion kinase (Wang et al., 2010). In breast cancer, high expression of *GRB7* is associated with negative patient outcome and cancer recurrence (Vermehren-Schmaedick et al., 2020, Ramsey et al., 2011, Nadler et al., 2010).

The mRNA expression of *Sp7, DKK1*, *GRB7* and *PPP1R1B* as well as, PKA subunits, PP1 subunits, and Cdk5 were assessed. Low expression levels of PKA subunits, *PRKAR1A, PRKAR2A*, and *PRKACB,* and PP1 subunits, *PPP1CB* and *PPP1CC,* and Cdk5 were significantly associated with adverse breast cancer specific survival. In contrast, low expression of PKA subunits, *PRKAR1B, PRKACA* and *PRKACG, GRB7* (probe 1 and 2) and *DKK1* (probe1) were significantly associated with improved prognosis of breast cancer specific survival. PKA subunit *PRKAR2B, GRB7* (probe1), *Dkk1*(probe 2), PP1 subunit *PPP1R1B* (probe 1, 2, and 3) and *SP7* mRNA expressions were not significantly associated with breast cancer survival. The associations between low mRNA/protein expression levels and breast cancer specific survival indicate existence of a fine balance required for the maintenance of healthy cellular environment and the devastating impact of homeostatic dysregulations on patient outcome.

Interestingly, amplification of *PPP1R1B* and *GRB7* genes at locus 17q has been reported in upper gastrointestinal adenocarcinomas (Maqani et al., 2006, Kotecha et al., 2019). Based on the observations in the current study and the supporting evidence available in literature, high expression of *GRB7* can be an indication of development of tumours characterised by shorter cancer specific patient survival. Some of the probes used in the current study were not associated with survival, indicating that some transcripts may have a greater importance in breast cancer than others, possibly due to their involvement in protein coding. In case of *GRB7,* probe 1 and 2 which were associated improved prognosis of breast cancer specific survival, map to protein coding transcripts such as*ENST00000309156.9*, *ENST00000394211.7*, *ENST00000445327.6, ENST00000394209.6, ENST00000394204.1* (ILMN-2405254), *ENST00000309156.9*, *ENST00000394211.7, ENST00000445327.6*, and *ENST00000394204.1*, whereas probe 1 which was not significantly associated with breast cancer specific survival maps to *GRB7* transcript *ENST00000309156.9* and a series of other non-protein coding

transcripts. There is limited amount of data available on the implications of functional changes as a result of the expression of each of the *GRB7* transcripts. Similarly, low *DKK1* probe 1 expression, which maps to protein coding *ENST00000373970.4* transcript (a splice variant product of the DKK1 gene *ENSG00000107984*), was significantly associated with good prognosis of breast cancer specific survival, and probe 2 was not. Previously, high levels of *DKK1* gene *ENSG00000107984* have been found to favour proliferation, invasion and growth in cancer (Shang et al., 2017).

The results obtained from the *in vitro* proliferation assay were used to determine the extent of toxicity associated with exposure to purvalanol B (Cdk5 inhibitor), tautomycetin (PP1 inhibitor), PKA inhibitor fragment (6-22) amide (PKA inhibitor) and forskolin (PKA activator) in triple negative MDA-MB-231 and luminal T47D and MCF7 cells over a period of 72 hours. Inhibition of PKA, PP1 and Cdk-5 was cytotoxic to MDA-MB-231 cells at 72 hours, but not at 24 or 48 hours. All compounds were cytotoxic to T47D cells at 24 and 48 hours, whereas all compounds were cytotoxic to MCF7 cells at all time points. The mechanism through which purvalanol B induces apoptosis involves inhibition of the ATP binding site of Cdk1 and Cdk2. This mechanism has been shown to be responsible for the activation of caspases and cytochrome-c release in gastric cancer (nockaert et al., 2002, Coker-Gürkan et al., 2015). In colon cancer, purvalanol B slows cell growth and induces apoptosis through cellular inhibition of MMP (Coker-Gürkan et al., 2015).

The mechanism through which tautomycetin induces apoptosis has been described previously. According to previous studies, this drug interacts with tyrosine kinase and PP1 as a part of a process described as activated T cell-specific apoptosis. In this process, interaction of PP1 and microtubule-associated protein τ blocks T cell activation through deactivation of actin-binding protein cofilin in human T lymphocytes; therefore, administration of tautomycetin is associated with

immunosuppression in patients (Shim et al., 2002, Liao et al., 1998, Ambach et al., 2000).

The pro-apoptotic events that unfold as a result of PKA inhibitor fragment (6-22) amide treatment of MDA-MB-231 cells involve inhibition of the cAMP-PKA pathway and the inability to neutralise the effects of oxytocin (Cassoni et al., 1997). Forskolin (PKA activator) has been shown to reduce cellular growth in gastric cancer cells via inhibition of c-myc and cyclin D1, and promote apoptosis in rhabdomyosarcoma, neuroblastoma, and hepatoblastoma cells through inhibition of c-myc and dysregulation of the Hedgehog signalling pathway (Yamanaka et al., 2010, Wang et al., 2019).

A large reduction in DARPP-32 Threonine-34 phosphorylation was induced by both tautomycin and PKA inhibitor fragment (6-22) amide. This was expected as PKA phosphorylates Threonine-34 directly, and inhibition of PKA-by-PKA inhibitor fragment (6-22) amide would result in reduced Threonine-34 phosphorylation. PP2A would dephosphorylate Threonine-75 by blocking the inhibition of PKA; tautomycin-induced inhibition of PP2A would therefore result in an indirect inhibition of PKA.

Cdk5 inhibitor, purvalanol B, and the PKA activator, Forskolin, caused a large reduction in Threonine-75 phosphorylation. This was expected as inhibition of Cdk5 results in a reduced Threonine-75 phosphorylation, and PKA activity is restored through PP2A mediated dephosphorylation of Threonine-75. These data indicate our ability to regulate DARPP-32 phosphorylation in breast cancer through DARPP-32 up-stream signalling partners in a dose dependent manner.

We sought to identify transcriptomic changes that occur following the loss of DARPP-32 expression in ER positive breast cancer to identify cancer associated signalling pathways that DARPP-32 may be able to modulate. To provide further insights we chose to stimulate DARPP-32 knockdown and control cells with estradiol17β (E2) or inhibit PKA activity with PKA

inhibitor fragment (6-22) amide. The findings in the current study point to an enhanced cellular growth as a result of DARPP-32 knockdown and through alteration of gene expression. These genes may potentially have crucial roles in cancer progression, and include but not limited to *MT-ND1, MT-ND2*, and *MT-CO1, NEDD4L, PTBP1, ARMC1, FGFR3,* and *PUF60, METTL8, TPGS2,* and *BMPR1B.* RNA-sequencing and gene enrichment analysis revealed that a number of pathways were significantly affected by the loss of DARPP-32 and/or in the presence of E2 or PKAi. These findings indicate the involvement of DARPP-32 in changes that are important in the promotion of pro-cancer signalling pathways and poor prognosis.

Tight junction pathway, which regulates epithelial-mesenchymal plasticity in breast cancer cells, was identified as a common pathway significantly altered by DARPP-32 knockdown along with PP2A expression regardless of E2 treatment (Nilchian et al., 2019). Interaction of tumour suppressor PP2A and DARPP-32 leads to availability of PKA through dephosphorylation of Threonine-75 residue (Slupe et al., 2011). These data demonstrate the importance of DARPP-32 in the regulation of vital pathways in breast cancer

Introduction of E2 initiated alteration of a number of pathways previously linked with cancer cell behaviour with a notable effect on patient survival and/or oestrogen signalling. In addition to the observation of previous studies that have found links between DARPP-32 and HER2-positive breast cancers, the current study reports relevant evidence demonstrating the association of DARPP-32 signalling nexus in ER-positive breast cancer.

6.2 Limitations and future work

Although the level of mRNA and protein expression of phosphorylated DARPP-32 at Threonine-34, *Sp7*, *GRB7* and *DKK1* were monitored in the

current study, further investigations would allow for a more comprehensive understanding of the role of DARPP-32 in breast cancer by determination of correlation of phosphorylated DARPP-32 at Threonine-34, Sp7, *GRB7* and *DKK1* expression in breast cancer.

In this study, most of the data was obtained as a part of *in vitro* investigations using a limited variation of breast cancer cell lines which do not represent the scale of diversity in breast cancer. Utilisation of alternative cell lines, including other ER positive and ER negative breast cancer cell lines, in future work would unveil the significance of DARPP-32, Cdk5, PP1, PKA, *Sp7*, *GRB7* and *DKK1* in a broader setting that is inclusive of other molecular subtypes of breast cancer in order to understand their exact functions in various breast cancers. Future work could also include assessment of the effects of combined drug exposure on apoptosis and migration assay through knockdown of genes suspected to have a role in those events.

Other future work could include utilisation of clustered regularly interspaced palindromic repeats (CRISPR), a relatively simple gene editing technique which allows for switching genes on or off within the genome in cells/organisms at a low cost (Redman et al., 2016). In contrast to DARPP-32 knockdown, target-specific CRISPR (clustered regularly interspaced short palindromic repeats) plasmids are available for both DARPP-32 knockout and activation. Although there is limited data currently available in literature regarding the use of CRISPR for the analysis of DARPP-32 in cancer, such techniques enable genome editing with applications in scientific research (Han et al., 2017). This technique could be used for identification of transcriptome alterations due to expression/loss of t-DARPP as well as for verification of RNA-Seq data reported in the current study regarding the transcriptome alterations following the loss of DARPP-32 expression. In order to improve our

understanding of the relation between oestrogen and t-DARPP, the effects of E2 treatment on pathway alteration in ER positive cells transfected with t-DARPP could be assessed. Additionally, the time that it takes for the alterations to occur could be monitored to determine whether or not E2 treatment speeds up this process.

Future work involving further evaluation of the implications associated with transcriptomic alterations which result from the loss of DARPP-32 and E2 stimulations or PKA inhibition in breast cancer (demonstrated by RNA-seq analysis) may provide useful information about potential links with the DARPP-32 pathway. Transcripts such as *YWHAZ, HNRNPC, PTBP1, BMPR1B, METTL8*, *ELMO3, TSC1* and *ATP6AP2* could be of interest in the future studies in particular due to their involvement in breast cancer progression as described by previous studies. The study of transcripts identified in this study with currently unknown clinical implications in breast cancer may lead to uncovering new links allowing us to better understand the mechanisms involved in breast cancer progression.

The findings of the current study contributes to uncovering a wider role of DARPP-32 in breast cancer by providing leads on the importance of DARPP-32 and its partners/potential partners as regulators of cancer associated signalling pathways. Analysis of the impact of DARPP-32 loss in breast cancer, combined with E2 stimulation or PKA inhibition provided an insight into transcriptomic changes that may contribute to clinical implications of breast cancer which may hold a significant therapeutic potential.

6.3 Conclusions

Overall, the findings of the current study could serve as a starting point for future investigations that aim to improve the quality of life and

survival of breast cancer patients, which may lead to identification of previously unknown protein interactions and discovery of novel breast cancer biomarkers with prognostic/therapeutic significance. The study of DARPP-32 and the nuclear events that affect the expression of this protein may facilitate a comprehensive understanding of the underlying cellular mechanisms that influence important signalling pathways in breast cancer; the observations and knowledge that would be acquired from such investigations could provide us with the technical expertise required for the design of novel selective therapeutic agents in breast cancer

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Appendix A: Supplementary information for Chapter 3

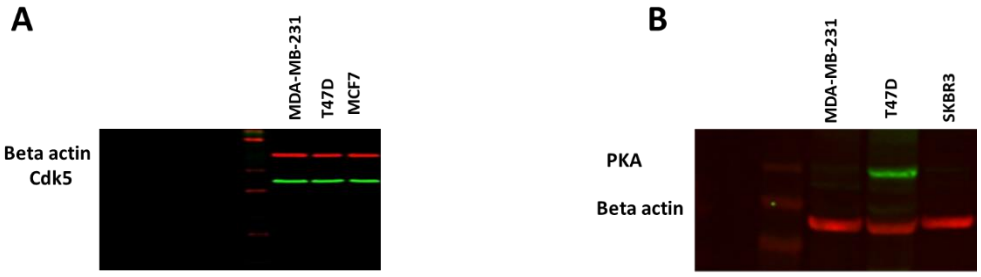


Figure A1: The expressions of Cdk5 (A) and PKA (B) were determined across different breast cancer cell lines in a single experiment by Western blotting. Beta actin was used as the loading control in this experiment. This figure demonstrates the specificity of the antibodies used in Immunohistochemistry.

Table A1: Association between various clinicopathological parameters with combined cytoplasmic PP1 and DARPP-32 level of expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Cytoplasmic				
	Low PP1 and low DARPP-32	**Low PP1 and high DARPP-32**	**High PP1 and low DARPP-32**	**High PP1 and high DARPP-32**	***P* value**
Age					
<50	178(37.3%)	229(48.0%)	28(5.9%)	42(8.8%)	0.289
≥50	422(39.4%)	530(49.4%)	48(4.5%)	72(6.7%)	
Tumour Size					
<2 cm	347(36.2%)	488(50.9%)	50(5.2%)	74(7.7%)	0.079
≥2 cm	252(42.8%)	271(46.0%)	26(4.4%)	40(6.8%)	
Grade					
1	70(28.8%)	148(60.4%)	9(3.7%)	18(7.3%)	**0.004**
2	254(40.7%)	302(48.4%)	28(4.5%)	40(6.4%)	
3	275(40.5%)	309(45.5%)	39(5.7%)	56(8.2%)	
Tubule formation					
1	24(21.2%)	75(66.4%)	3(2.7%)	11(9.7%)	
2	150(33.0%)	242(53.3%)	23(5.1%)	39(8.6%)	**<0.001**
3	420(43.4%)	437(45.1%)	47(4.9%)	64(6.6%)	

Table Continued

	Cytoplasmic				
	Low PP1 and low DARPP-32	**Low PP1 and high DARPP-32**	**High PP1 and low DARPP-32**	**High PP1 and high DARPP-32**	***P* value**
Pleomorphism	5(23.8%)	16(76.2%)	0(0.0%)	0(0.0%)	**0.007**
1	166(35.8%)	253(54.5%)	20(4.3%)	25(5.4%)	
2	423(40.3%)	485(46.2%)	53(5.0%)	89(8.5%)	
3					
Mitosis					
1	281(37.0%)	389(51.3%)	25(4.6%)	54(7.1%)	**0.011**
2	131(45.6%)	134(46.7%)	11(3.8%)	11(3.8%)	
3	182(37.4%)	229(47.0%)	27(5.5%)	49(10.1%)	
Tumour type					
Ductal	540(40.6%)	620(46.6%)	69(5.2%)	102(7.7%)	
Lobular	40(31.5%)	80(63.0%)	3(2.4%)	4(3.1%)	**0.020**
Medullary-like	3(21.4%)	8(57.1%)	1(7.1%)	2(14.3%)	
Miscellaneous	3(25.0%)	8(66.7%)	0(0.0%)	1(8.3%)	
Mixed &lobular	1(100.0%)	0(0.0%)	0(0.0%)	0(0.0%)	
Special type	12(20.3%)	39(66.1%)	3(5.1%)	5(8.5%)	
Tubular	0(0.0%)	3(100%)	0(0.0%)	0(0.0%)	
Vascular Invasion					
Definite	197(43.0%)	199(43.3%)	29(6.3%)	33(7.2%)	**0.018**
No/Probable	401(36.8%)	560(51.4%)	47(4.3%)	81(7.4%)	
Stage					
1	354(36.2%)	513(52.5%)	48(4.9%)	63(6.4%)	**0.006**
2	174(41.2%)	183(43.4%)	22(5.2%)	43(10.2%)	
3	69(47.6%)	62(42.8%)	6(4.1%)	8(5.5%)	
NPI					**0.008**
Good	195(36.1%)	287(53.1%)	23(4.3%)	35(6.5%)	
Medium	284(37.1%)	378(49.9%)	43(5.6%)	60(7.8%)	
Poor	117(49.0%)	93(38.9%)	10(4.2%)	19(7.9%)	
ER Status					
Negative	75(24.9%)	187(62.1%)	8(2.7%)	31(10.3%)	**<0.001**
Positive	525(42.0%)	573(45.9%)	68(5.4%)	83(6.6%)	
PR Status					
Negative	213(34.5%)	330(53.5%)	20(3.2%)	54(8.8%)	**0.001**
Positive	348(41.0%)	394(46.4%)	52(6.1%)	55(6.5%)	

Table Continued

	Cytoplasmic				
	Low PP1 and low DARPP-32	**Low PP1 and high DARPP-32**	**High PP1 and low DARPP-32**	**High PP1 and high DARPP-32**	***P* value**
HER2 Status					
Negative	530(39.6%)	647(48.3%)	69(5.2%)	93(6.9%)	0.153
Positive	37(31.1%)	64(53.8%)	5(4.2%)	13(10.9%)	
Triple Negative					
Negative	535(41.2%)	607(46.7%)	71(5.5%)	86(6.6%)	**<0.001**
Positive	57(25.4%)	138(61.6%)	5(2.2%)	24(10.7%)	

Table A2: Association between various clinicopathological parameters with combined nuclear PP1 and DARPP-32 level of expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Nuclear				
	Low PP1 and low DARPP-32	**Low PP1 and high DARPP-32**	**High PP1 and low DARPP-32**	**High PP1 and high DARPP-32**	***P* value**
Age					
<50	218(45.7%)	190(39.8%)	37(7.8%)	32(6.7%)	0.062
≥50	495(46.2%)	469(43.8%)	52(4.9%)	56(5.2%)	
Tumour Size					
<2 cm	427(44.5%)	417(43.5%)	57(5.9%)	58(6.0%)	0.489
≥2 cm	285(48.4%)	242(41.1%)	32(5.4%)	30(5.1%)	
Grade					
1	87(35.5%)	129(52.7%)	12(4.9%)	17(6.9%)	
2	293(47.0%)	265(42.5%)	35(5.6%)	31(5.0%)	**0.010**
3	332(48.9%)	265(39.0%)	42(6.2%)	40(5.9%)	
Tubule formation					
1	36(31.9%)	62(54.9%)	6(5.3%)	9(8.0%)	**<0.001**
2	185(40.7%)	217(47.8%)	21(4.6%)	31(6.8%)	
3	480(49.6%)	378(39.0%)	62(6.4%)	48(5.0%)	
Pleomorphism					
1	8(38.1%)	12(57.1%)	1(4.8%)	0(0.0%)	0.076
2	191(41.2%)	221(47.6%)	30(6.5%)	22(4.7%)	
3	502(47.8%)	424(40.4%)	58(5.5%)	66(6.3%)	

Table Continued

	Nuclear				
	Low PP1 and low DARPP-32	**Low PP1 and high DARPP-32**	**High PP1 and low DARPP-32**	**High PP1 and high DARPP-32**	***P* value**
Mitosis					
1	324(42.7%)	345(45.5%)	45(5.9%)	45(5.9%)	**0.009**
2	155(54.0%)	111(38.7%)	14(4.9%)	7(2.4%)	
3	222(45.6%)	199(40.9%)	30(6.2%)	36(7.4%)	
Tumour type					
Ductal	642(48.2%)	537(40.3%)	75(5.6%)	77(5.8%)	
Lobular	43(33.9%)	71(55.9%)	9(7.1%)	4(3.1%)	**0.019**
Medullary-like	3(21.4%)	8(57.1%)	2(14.3%)	1(7.1%)	
Miscellaneous	5(41.7%)	7(58.3%)	0(0.0%)	0(0.0%)	
Mixed & lobular	1(100.0%)	0(0.0%)	0(0.0%)	0(0.0%)	
Special type	16(27.1%)	34(57.6%)	3(5.1%)	6(10.2%)	
Tubular	1(33.3%)	2(66.7%)	0(0.0%)	0(0.0%)	
Vascular invasion					
Definite	238(52.0%)	169(36.9%)	30(6.6%)	21(4.6%)	**0.007**
No/Probable	474(43.5%)	490(45.0%)	58(5.3%)	67(6.2%)	
Stage					
1	424(43.4%)	450(46.0%)	53(5.4%)	51(5.2%)	**0.004**
2	202(47.9%)	160(37.9%)	28(6.6%)	32(7.6%)	
3	84(57.9%)	48(33.1%)	8(5.5%)	5(3.4%)	
NPI					
Good	232(43.0%)	251(46.5%)	28(5.2%)	29(5.4%)	**0.004**
Medium	340(44.4%)	333(43.5%)	45(5.9%)	47(6.1%)	
Poor	137(57.3%)	74(31.0%)	16(6.7%)	12(5.0%)	
ER Status					
Negative	97(32.2%)	172(57.1%)	9(3.0%)	23(7.6%)	**<0.001**
Positive	616(49.3%)	488(39.1%)	80(6.4%)	65(5.2%)	
PR Status					
Negative	259(42.0%)	292(47.3%)	24(3.9%)	42(6.8%)	**<0.001**
Positive	413(48.6%)	335(39.5%)	61(7.2%)	40(4.7%)	
HER2 Status					
Negative	631(47.1%)	559(41.7%)	76(5.7%)	73(5.5%)	0.233
Positive	46(38.7%)	54(45.4%)	10(8.4%)	9(7.6%)	
Triple Negative					
Negative	630(48.5%)	518(39.9%)	84(6.5%)	67(5.2%)	**<0.001**
Positive	74(33.0%)	127(56.7%)	5(2.2%)	18(8.0%)	

Table A3. Association between various clinicopathological parameters with combined cytoplasmic PP1 and Cdk5 level of expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Cytoplasmic				
	Low PP1 and low Cdk5	**High PP1 and low Cdk5**	**Low PP1 and high Cdk5**	**High PP1 and high Cdk5**	***P* value**
Age					
<50	234(70.1%)	36(10.8%)	45(13.5%)	19(5.7%)	0.271
≥50	480(70.8%)	62(9.1%)	111(16.4%)	25(3.7%)	
Size					
<2 cm	416(68.5%)	58(9.6%)	102(16.8%)	31(5.1%)	0.193
≥2 cm	297(73.5%)	40(9.9%)	54(13.4%)	13(3.2%)	
Grade					
1	104(67.5%)	13(8.4%)	32(20.8%)	5(3.2%)	0.147
2	280(71.1%)	31(7.9%)	65(16.5%)	18(4.6%)	
3	329(71.1%)	54(11.7%)	59(12.7%)	21(4.5%)	
Tubule formation					
1	51(67.1%)	7(9.2%)	14(18.4%)	4(5.3%)	0.634
2	189(67.0%)	29(10.3%)	49(17.4%)	15(5.3%)	
3	468(72.8%)	60(9.3%)	90(14.0%)	25(3.9%)	
Pleomorphism					
1	11(78.6%)	0(0.0%)	3(21.4%)	0(0.0%)	0.091
2	206(72.5%)	19(6.7%)	51(18.0%)	8(2.8%)	
3	491(69.8%)	77(11.0%)	99(14.1%)	36(5.1%)	
Mitosis					
1	325(69.0%)	43(9.1%)	83(17.6%)	20(4.2%)	**0.017**
2	147(75.4%)	10(5.1%)	32(16.4%)	6(3.1%)	
3	235(70.4%)	43(12.9%)	38(11.4%)	18(5.4%)	
Tumour type					
Ductal	608(69.6%)	90(10.3%)	136(15.6%)	39(4.5%)	
Lobular	65(80.2%)	2(2.5%)	13(16.0%)	1(1.2%)	0.261
Medullary-like	5(62.5%)	2(25.0%)	0(0.0%)	1(12.5%)	
Miscellaneous	5(62.5%)	0(0.0%)	2(25.0%)	1(12.5%)	
Mixed &lobular	1(100%)	0(0.0%)	0(0.0%)	0(0.0%)	
Special type	28(73.7%)	4(10.5%)	4(10.5%)	2(5.3%)	
Tubular	0(0.0%)	0(0.0%)	1(100.0%)	0(0.0%)	
Vascular invasion					
Definite	215(73.6%)	29(9.9%)	34(11.6%)	14(4.8%)	0.205
No/Probable	498(69.3%)	69(9.6%)	122(17.0%)	30(4.2%)	
Stage					
1	459(71.1%)	56(8.7%)	106(16.4%)	25(3.9%)	0.185
2	183(67.0%)	32(11.7%)	41(15.0%)	17(6.2%)	
3	70(76.9%)	10(11.0%)	9(9.9%)	2(2.2%)	
NPI					
Good	229(68.6%)	25(7.5%)	66(19.8%)	14(4.2%)	**0.031**

Table Continued

	Cytoplasmic				
	Low PP1 and low Cdk5	**High PP1 and low Cdk5**	**Low PP1 and high Cdk5**	**High PP1 and high Cdk5**	***P* value**
Medium	360(70.0%)	54(10.5%)	73(14.2%)	27(5.3%)	
Poor	122(75.8%)	19(11.8%)	17(10.6%)	3(1.9%)	
ER status					
Negative	150(72.5%)	23(11.1%)	22(10.6%)	12(5.8%)	0.114
Positive	564(70.0%)	75(9.3%)	135(16.7%)	32(4.0%)	
PR status					
Negative	310(73.3%)	40(9.5%)	49(11.6%)	24(5.7%)	**0.014**
Positive	357(67.6%)	52(9.8%)	99(18.8%)	20(3.8%)	
HER2 status					
Negative	606(70.1%)	83(9.6%)	138(16.0%)	38(4.4%)	0.728
Positive	61(70.1%)	11(12.6%)	11(12.6%)	4(4.6%)	
Triple negative					
Negative	595(70.2%)	80(9.4%)	140(16.5%)	32(3.8%)	0.088
Positive	103(71.0%)	17(11.7%)	15(10.3%)	10(6.9%)	

Table A4: Association between various clinicopathological parameters with combined nuclear PP1 and Cdk5 level of expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Nuclear				
	Low PP1and low Cdk5	**High PP1 and low Cdk5**	**Low PP1 and High Cdk5**	**high PP1 and high Cdk5**	***P* value**
Age					
<50	192(57.7%)	27(8.1%)	86(25.8%)	28(8.4%)	0.067
≥50	390(57.4%)	41(6.0%)	213(31.3%)	36(5.3%)	
Size					
<2 cm	328(53.9%)	40(6.6%)	198(32.6%)	42(6.9%)	**0.033**
≥2 cm	253(62.6%)	28(6.9%)	101(25.0%)	22(5.4%)	
Grade					
1	81(52.6%)	9(5.8%)	52(33.8%)	12(7.8%)	0.089
2	214(54.0%)	27(6.8%)	133(33.6%)	22(5.6%)	
3	286(61.9%)	32(6.9%)	114(24.7%)	30(6.5%)	
Tubule formation					
1	38(50.0%)	5(6.6%)	25(32.9%)	8(10.5%)	0.063
2	145(51.2%)	20(7.1%)	100(35.3%)	18(6.4%)	
3	392(61.0%)	43(6.7%)	170(26.4%)	38(5.9%)	
Pleomorphism					
1	6(42.9%)	1(7.1%)	7(50.0%)	0(0.0%)	0.347
2	157(55.1%)	16(5.6%)	94(33.0%)	18(6.3%)	
3	412(58.6%)	51(7.3%)	194(27.6%)	46(6.5%)	
Mitosis					
1	249(52.6%)	33(7.0%)	158(33.4%)	33(7.0%)	**0.002**
2	117(60.0%)	11(5.6%)	63(32.3%)	4(2.1%)	
3	208(62.5%)	24(7.2%)	74(22.2%)	27(8.1%)	
Tumour type					
Ductal	499(57.2%)	60(6.9%)	258(29.6%)	56(6.4%)	
Lobular	48(58.5%)	5(6.1%)	27(32.9%)	2(2.4%)	0.210
Medullary-like	5(62.5%)	0(0.0%)	0(0.0%)	3(37.5%)	
Miscellaneous	6(75.0%)	0(0.0%)	2(25.0%)	0(0.0%)	
Mixed & lobular	1(100.0%)	0(0.0%)	0(0.0%)	0(0.0%)	
Special type	21(55.3%)	3(7.9%)	11(28.9%)	3(7.9%)	
Tubular	0(0.0%)	0(0.0%)	1(100.0%)	0(0.0%)	
Vascular invasion					
Definite	179(61.5%)	16(5.5%)	78(26.8%)	18(6.2%)	0.368
No/Probable	402(55.8%)	52(7.2%)	221(30.7%)	46(6.4%)	
Stage					
1	371(57.4%)	41(6.3%)	196(30.3%)	38(5.9%)	0.731
2	152(55.5%)	21(7.7%)	79(28.8%)	22(8.0%)	
3	57(62.6%)	6(6.6%)	24(26.4%)	4(4.4%)	
NPI					

Table Continued

	Nuclear				
	Low PP1and low Cdk5	**High PP1 and low Cdk5**	**Low PP1 and High Cdk5**	**high PP1 and high Cdk5**	***P* value**
Good	173(51.8%)	18(5.4%)	121(36.2%)	22(6.6%)	**0.035**
Medium	311(60.4%)	36(7.0%)	133(25.8%)	35(6.8%)	
Poor	96(59.6%)	14(8.7%)	44(27.3%)	7(4.3%)	
ER status					
Negative	136(65.7%)	14(6.8%)	42(20.3%)	15(7.2%)	**0.012**
Positive	447(55.4%)	54(6.7%)	257(31.8%)	49(6.1%)	
PR status					
Negative	262(61.9%)	22(5.2%)	106(25.1%)	33(7.8%)	**0.016**
Positive	290(54.8%)	42(7.9%)	168(31.8%)	29(5.5%)	
HER2 status					
Negative	449(57.6%)	57(6.6%)	254(29.3%)	56(6.5%)	0.887
Positive	48(55.2%)	7(8.0%)	25(28.7%)	7(8.0%)	
Triple negative					
Negative	474(55.9%)	56(6.6%)	267(31.5%)	51(6.0%)	**0.010**
Positive	97(66.9%)	10(6.9%)	26(17.9%)	12(8.3%)	

Table A5: Association between various clinicopathological parameters with combined cytoplasmic PKA and DARPP-32 level of expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Cytoplasmic				
	Low PKA and low DARPP-32	**Low PKA and high DARPP-32**	**High PKA and low DARPP-32**	**High PKA and high DARPP-32**	***P* value**
Age					
<50	16(4.6%)	22(6.3%)	131(37.6%)	179(51.4%)	1.000
≥50	33(4.6%)	45(6.3%)	270(37.6%)	371(51.6%)	
Tumour size					
<2 cm	29(4.5%)	38(5.9%)	234(36.6%)	342(53.2%)	0.608
≥2 cm	20(4.7%)	29(6.8%)	167(39.4%)	208(49.1%)	
Grade					
1	7(4.6%)	14(9.2%)	46(30.3%)	85(55.9%)	0.124
2	25(6.2%)	23(5.7%)	155(38.6%)	199(49.5%)	
3	17(3.3%)	30(5.8%)	200(39.0%)	266(51.9%)	
Tubule formation					
1					
2	4(5.5%)	8(11.0%)	17(23.3%)	44(60.3%)	**0.038**
3	10(3.3%)	18(5.9%)	106(34.8%)	171(56.1%)	

Table Continued

	Cytoplasmic				
	Low PKA and low DARPP-32	**Low PKA and high DARPP-32**	**High PKA and low DARPP-32**	**High PKA and high DARPP-32**	***P* value**
	35(5.2%)	39(5.8%)	271(40.0%)	333(49.1%)	
Pleomorphism					
1	0(0.0%)	2(18.2%)	1(9.1%)	8(72.7%)	0.068
2	18(6.3%)	23(8.0%)	102(35.5%)	144(50.2%)	
3	31(4.1%)	40(5.3%)	291(38.4%)	396(52.2%)	
Mitosis					
1	30(6.3%)	32(6.7%)	171(35.8%)	244(51.2%)	0.166
2	6(2.8%)	9(4.3%)	90(42.7%)	106(50.2%)	
3	13(3.5%)	24(6.5%)	133(36.2%)	197(53.7%)	
Tumour type					
Ductal	39(4.2%)	50(5.4%)	370(39.9%)	468(50.5%)	
Lobular	6(7.9%)	10(13.2%)	16(21.1%)	44(57.9%)	**0.004**
Medullary-like	0(0.0%)	0(0.0%)	3(27.3%)	8(72.7%)	
Miscellaneous	0(0.0%)	0(0.0%)	3(33.3%)	6(66.7%)	
Mixed &lobular	0(0.0%)	0(0.0%)	1(100%)	0(0.0%)	
Special type	4(10.0%)	7(17.5%)	8(20.0%)	21(52.5%)	
Tubular	0(0.0%)	0(0.0%)	0(0.0%)	2(100.0%)	
Vascular invasion					
Definite	22(6.6%)	26(7.8%)	133(39.9%)	152(45.6%)	**0.018**
No/Probable	27(3.7%)	41(5.6%)	268(36.5%)	398(54.2%)	
Stage					
1	28(4.2%)	41(6.1%)	239(35.7%)	362(54.0%)	0.246
2	13(4.6%)	16(5.6%)	114(40.0%)	142(49.8%)	
3	7(6.4%)	10(9.2%)	47(43.1%)	45(41.3%)	
NPI					0.349
GPG	17(5.0%)	20(5.9%)	125(37.1%)	175(51.9%)	
MPG	21(3.8%)	33(6.0%)	197(36.0%)	295(54.1%)	
PPG	10(5.6%)	14(7.8%)	77(43.0%)	78(43.6%)	
ER Status					
Negative	5(2.3%)	15(6.8%)	46(20.7%)	156(70.3%)	**<0.001**
Positive	44(5.2%)	52(6.1%)	355(42.0%)	396(46.7%)	
PR Status					
Negative	13(3.0%))	35(8.0%)	134(30.7%)	254(58.3%)	**<0.001**
Positive	34(5.9%)	27(4.7%)	242(42.2%)	270(47.1%)	
HER2 Status					
Negative	46(5.1%)	51(5.6%)	349(38.5%)	460(50.8%)	0.012
Positive	0(0.0%)	10(10.9%)	28(30.4%)	54(58.7%)	
Triple Negative					
Negative	44(4.9%)	56(6.3%)	365(41.1%)	424(47.7%)	**<0.001**
Positive	5(3.2%)	9(5.7%)	29(18.5%)	114(72.6%)	

Table A6: Association between various clinicopathological parameters with combined nuclear PKA and DARPP-32 level of expression are demonstrated below. The *P* values highlighted in bold are <0.05.

	Nuclear				
	Low PKA and low DARPP-32	**Low PKA and high DARPP-32**	**High PKA and low DARPP-32**	**High PKA and high DARPP-32**	***P* value**
Age					
<50	21(6.0%)	22(6.3%)	131(37.6%)	179(51.4%)	1.000
≥50	33(4.6%)	45(6.3%)	270(37.6%)	371(51.6%)	
Tumour size					
<2 cm	29(4.5%)	38(5.9%)	234(36.6%)	342(53.2%)	0.608
≥2 cm	20(4.7%)	29(6.8%)	167(39.4%)	208(49.1%)	
Grade					
1	7(4.6%)	14(9.2%)	46(30.3%)	85(55.9%)	0.124
2	25(6.2%)	23(5.7%)	155(38.6%)	199(49.5%)	
3	17(3.3%)	30(5.8%)	200(39.0%)	266(51.9%)	
Tubule formation					
1					
2	4(5.5%)	8(11.0%)	17(23.3%)	44(60.3%)	**0.038**
3	10(3.3%)	18(5.9%)	106(34.8%)	171(56.1%)	
	35(5.2%)	39(5.8%)	271(40.0%)	333(49.1%)	
Pleomorphism					
1	0(0.0%)	2(18.2%)	1(9.1%)	8(72.7%)	0.068
2	18(6.3%)	23(8.0%)	102(35.5%)	144(50.2%)	
3	31(4.1%)	40(5.3%)	291(38.4%)	396(52.2%)	
Mitosis					
1	30(6.3%)	32(6.7%)	171(35.8%)	244(51.2%)	0.166
2	6(2.8%)	9(4.3%)	90(42.7%)	106(50.2%)	
3	13(3.5%)	24(6.5%)	133(36.2%)	197(53.7%)	
Tumour type					
Ductal	39(4.2%)	50(5.4%)	370(39.9%)	468(50.5%)	
Lobular	6(7.9%)	10(13.2%)	16(21.1%)	44(57.9%)	**0.004**
Medullary-like	0(0.0%)	0(0.0%)	3(27.3%)	8(72.7%)	
Miscellaneous	0(0.0%)	0(0.0%)	3(33.3%)	6(66.7%)	
Mixed &lobular	0(0.0%)	0(0.0%)	1(100%)	0(0.0%)	
Special type	4(10.0%)	7(17.5%)	8(20.0%)	21(52.5%)	
Tubular	0(0.0%)	0(0.0%)	0(0.0%)	2(100.0%)	
Vascular invasion					
Definite	22(6.6%)	26(7.8%)	133(39.9%)	152(45.6%)	**0.018**
No/Probable	27(3.7%)	41(5.6%)	268(36.5%)	398(54.2%)	
Stage					
1	28(4.2%)	41(6.1%)	239(35.7%)	362(54.0%)	0.246

Table Continued

	Nuclear				
	Low PKA and low DARPP-32	**Low PKA and high DARPP-32**	**High PKA and low DARPP-32**	**High PKA and high DARPP-32**	***P* value**
2	13(4.6%)	16(5.6%)	114(40.0%)	142(49.8%)	
3	7(6.4%)	10(9.2%)	47(43.1%)	45(41.3%)	
NPI					0.349
Good	17(5.0%)	20(5.9%)	125(37.1%)	175(51.9%)	
Medium	21(3.8%)	33(6.0%)	197(36.0%)	295(54.1%)	
Poor	10(5.6%)	14(7.8%)	77(43.0%)	78(43.6%)	
ER Status					
Negative	5(2.3%)	15(6.8%)	46(20.7%)	156(70.3%)	**<0.001**
Positive	44(5.2%)	52(6.1%)	355(42.0%)	396(46.7%)	
PR Status					
Negative	13(3.0%))	35(8.0%)	134(30.7%)	254(58.3%)	**<0.001**
Positive	34(5.9%)	27(4.7%)	242(42.2%)	270(47.1%)	
HER2 Status					
Negative	46(5.1%)	51(5.6%)	349(38.5%)	460(50.8%)	0.012
Positive	0(0.0%)	10(10.9%)	28(30.4%)	54(58.7%)	
Triple Negative					
Negative	44(4.9%)	56(6.3%)	365(41.1%)	424(47.7%)	**<0.001**
Positive	5(3.2%)	9(5.7%)	29(18.5%)	114(72.6%)	

Appendix B: Supplementary information for Chapter 4

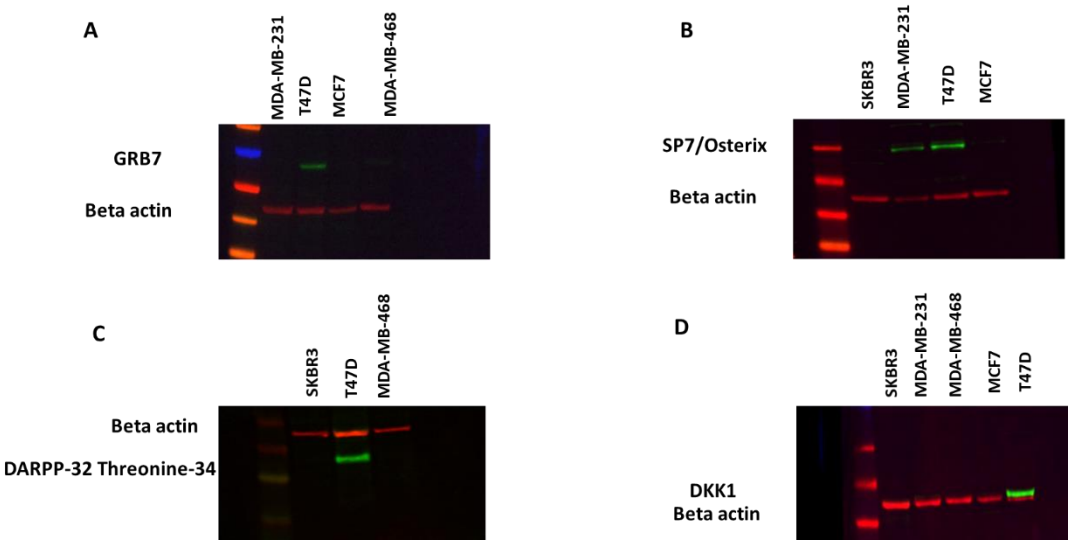


Figure B1: The expressions of *GRB7* (A), *Sp7* (B), DARPP-32 Threonine-34 (C), and *DKK1* (D) were determined across different cell lines in a single experiment by western blotting. Beta actin was used as the loading control in this experiment. This figure demonstrates the specificity of the antibodies used in Immunohistochemistry.

Appendix C: Supplementary information for Chapter 5

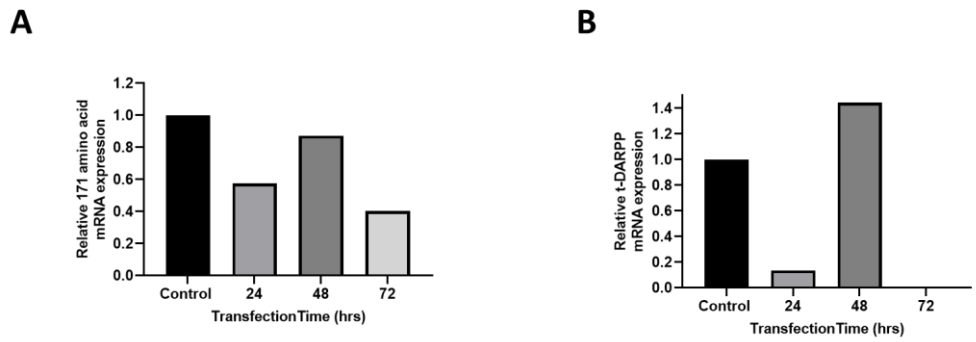


Figure C1:The mRNA expression levels of DARPP-32 splice variants t-DARPP (B) and 171 amino acid (A) following 24, 48 and 72 hours incubation with siRNA oligo duplex.

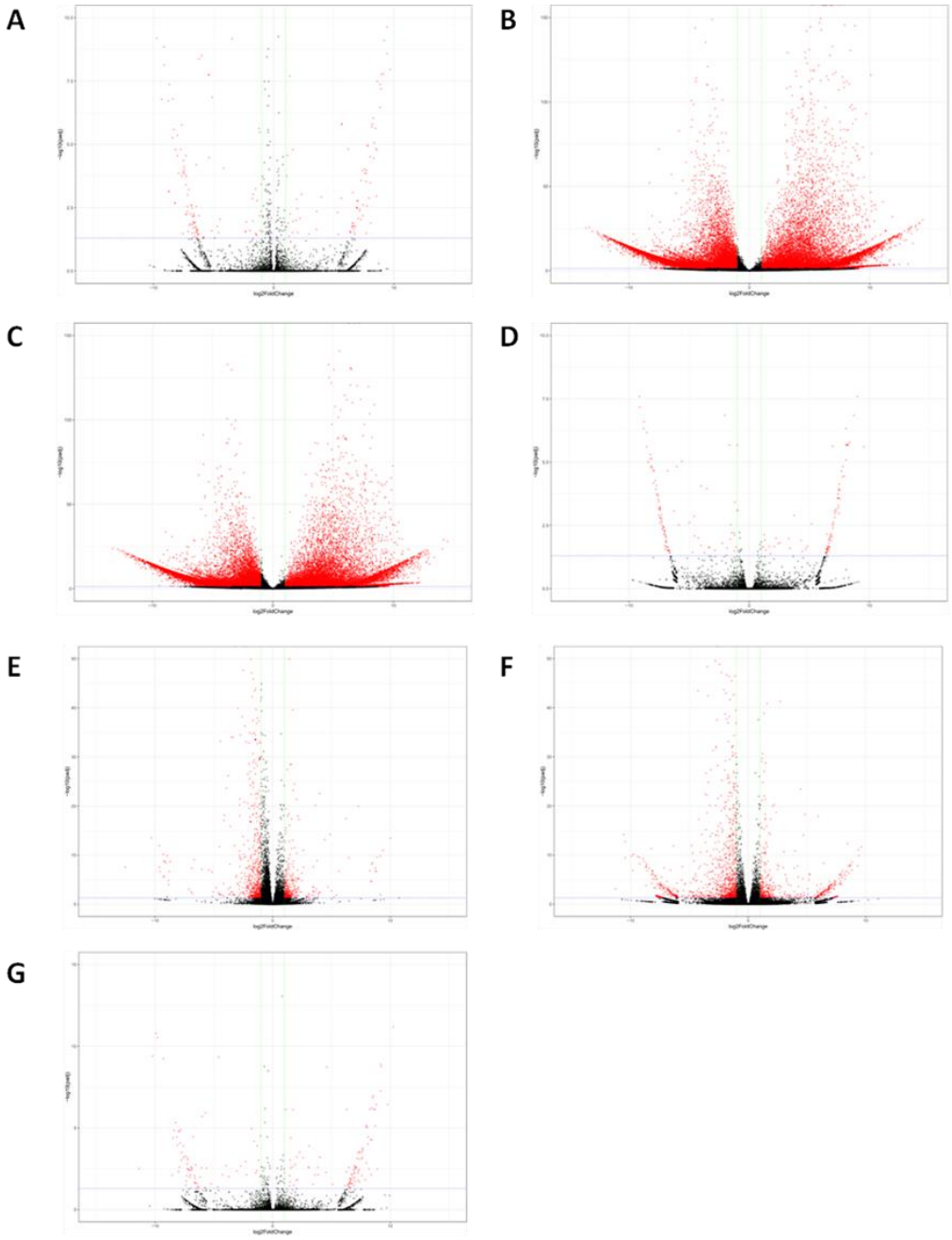


Figure C2: DESeq2 analysis and *P*-adjusted Volcano plots of log10 against log2 fold change of transcripts in A: DARPP-32 knockdown cells and control cells, B: E2-treated and untreated DARPP-32 knockdown cells, C: E2-treated and untreated control cells, D: E2-treated DARPP-32 knockdown cells and E2-treated control cells, E: PKA inhibitor-treated and untreated DARPP-32 knockdown cells, F: PKA inhibitor treated and untreated control cells, G: PKA inhibitor treated DARPP-32 knockdown cells and PKA inhibitor-treated control cells.

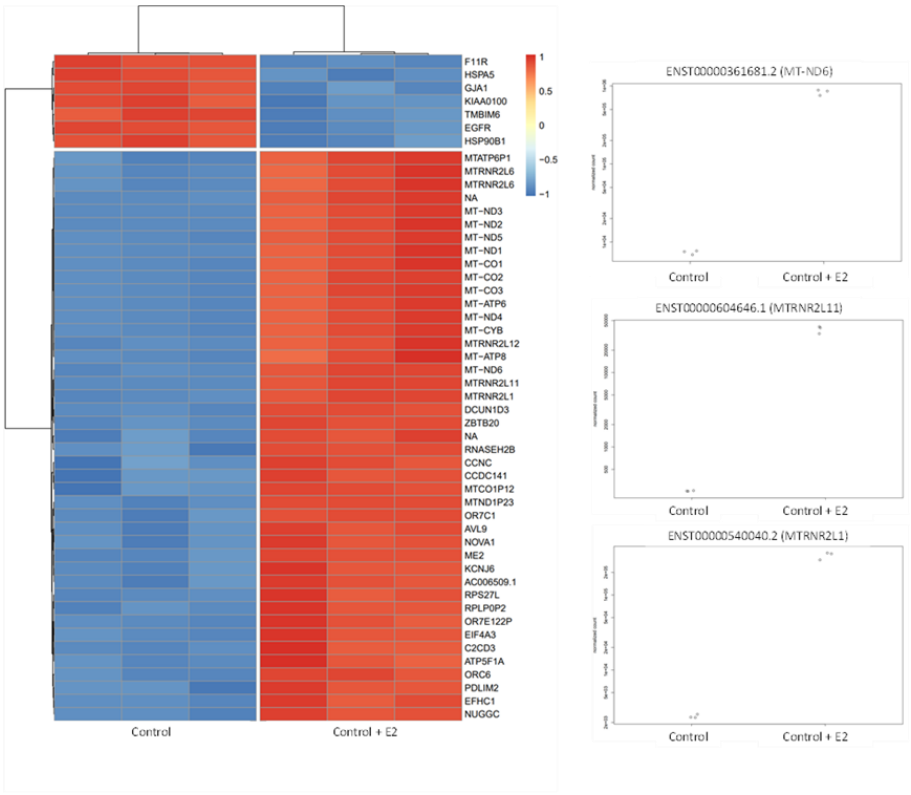


Figure C3: Identification and heat map analysis of the 50 most common transcripts with significant (*P*-adjusted) expression alterations (greater than 2-fold) in control cells compared to that of control cells treated with E2. The three most significant transcript and their corresponding gene identifiers are displayed on the right.

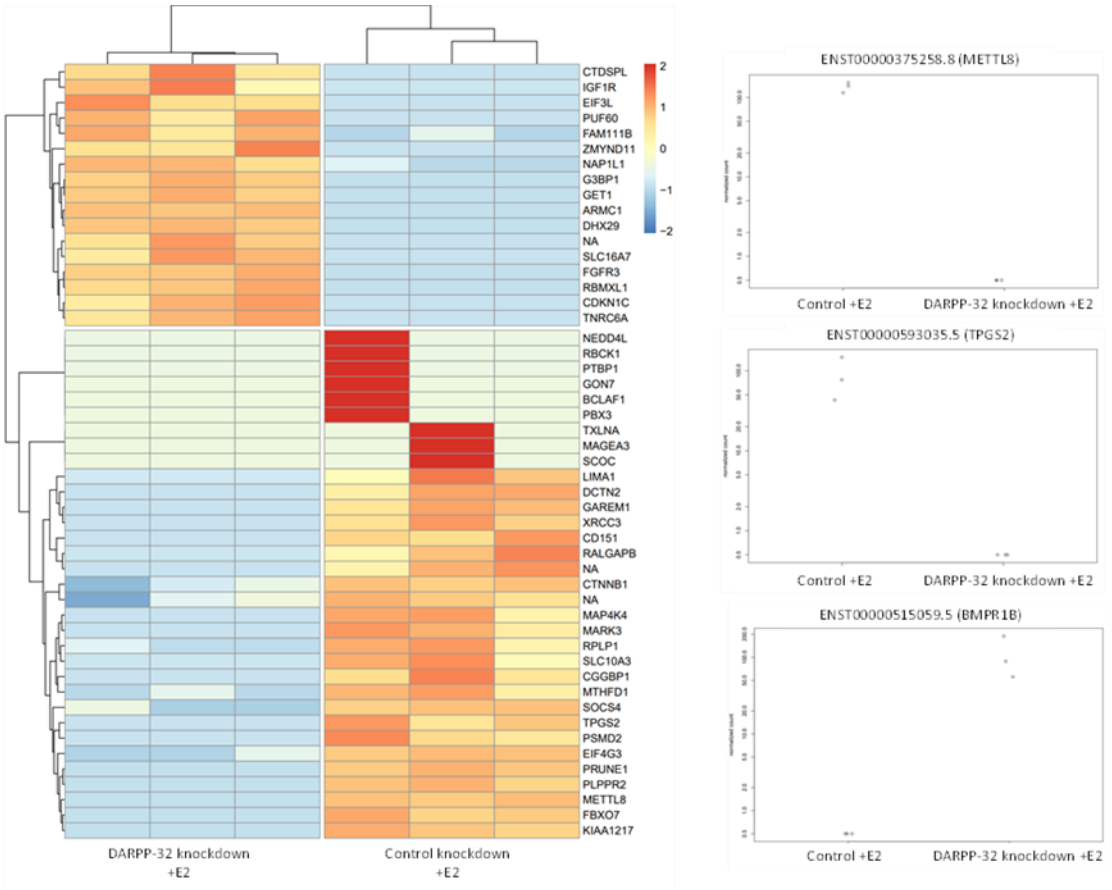


Figure C4: Identification and heat map analysis of the 50 most common transcripts with significant (*P*-adjusted) expression alterations (greater than 2-fold) in DARPP-32 knockdown cells treated with E2 compared to that of control cells treated with E2. The three most significant transcript and their corresponding gene identifiers are displayed on the right.

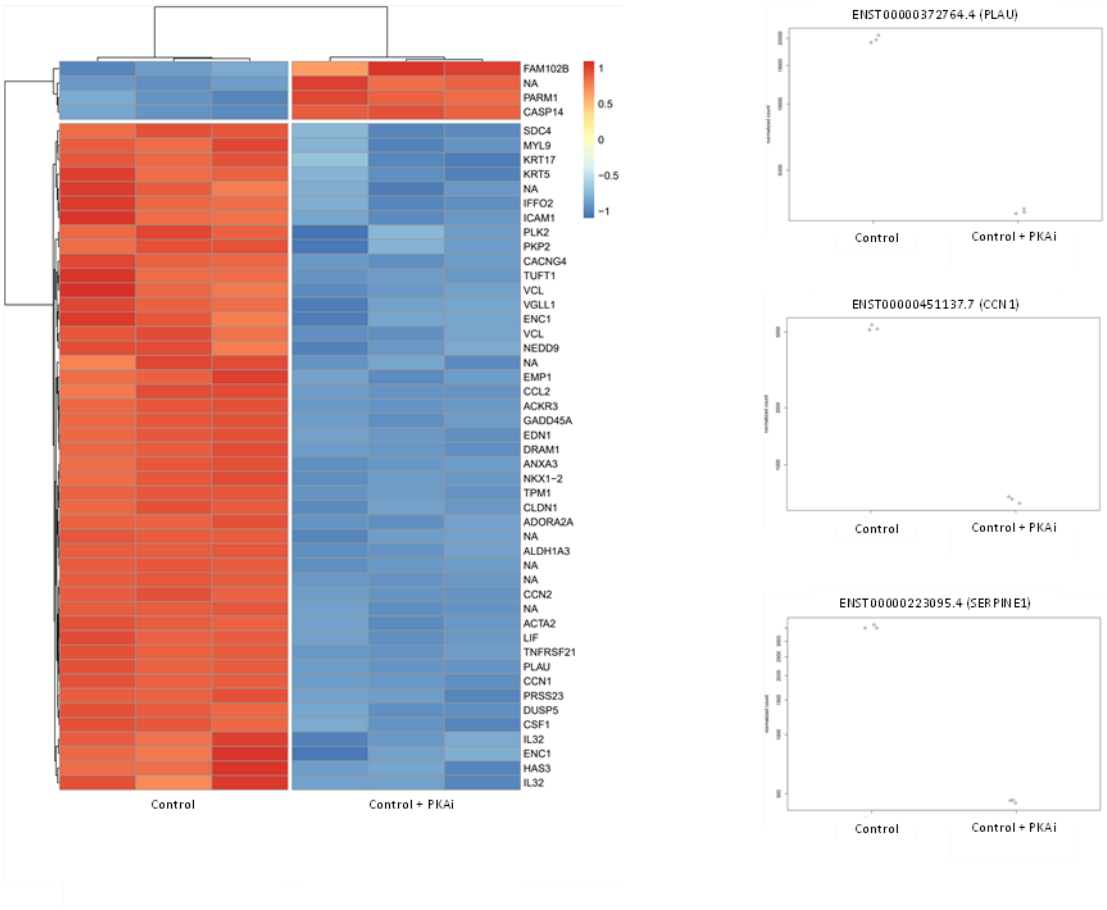


Figure C5: Identification and heat map analysis of the 50 most common transcripts with significant (*P*-adjusted) expression alterations (greater than 2-fold) in control cells compared to that of control cells treated with PKAi. The three most significant transcript and their corresponding gene identifiers are displayed on the right.

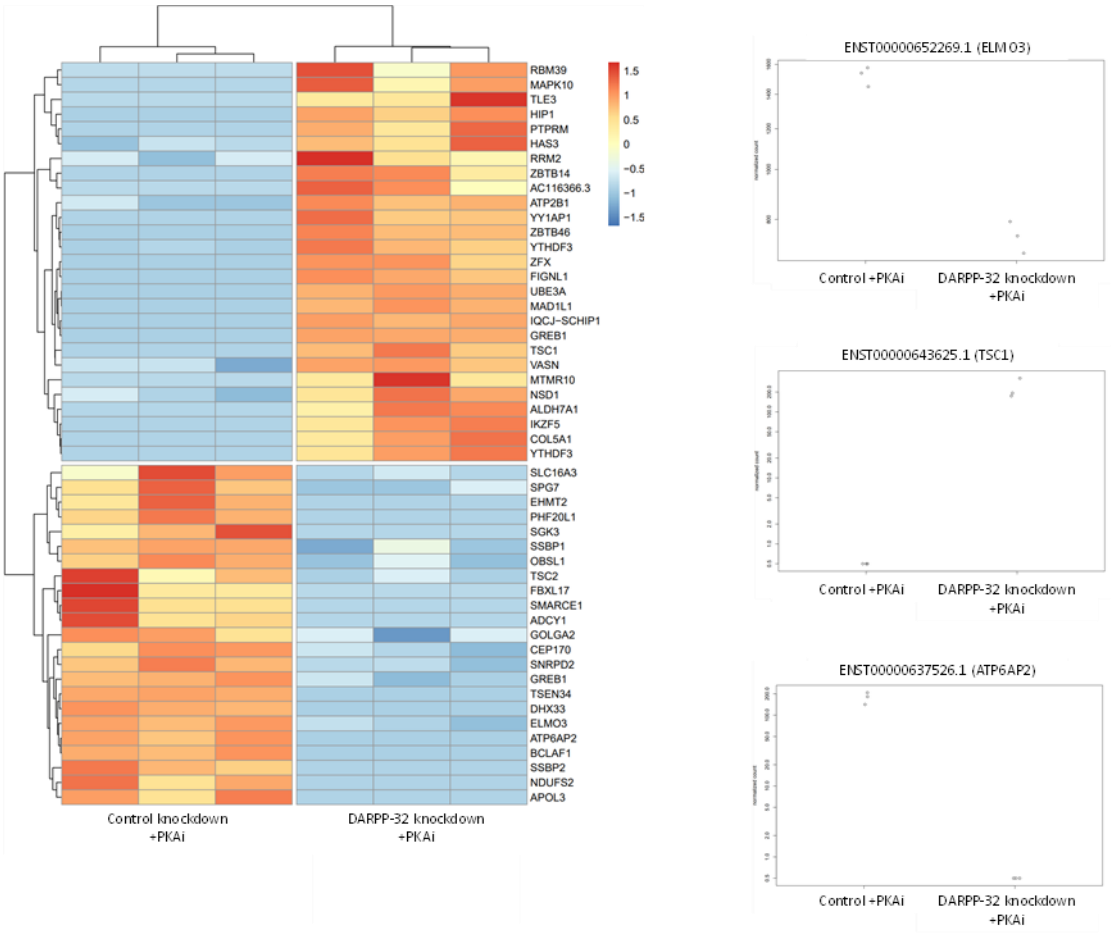


Figure C6: Identification and heat map analysis of the 50 most common transcripts with significant (*P*-adjusted) expression alterations (greater than 2-fold) in knockdown cell treated with PKA inhibitor compared to that of control cells treated with PKA inhibitor. The three most significant transcript and their corresponding gene identifiers are displayed on the right.

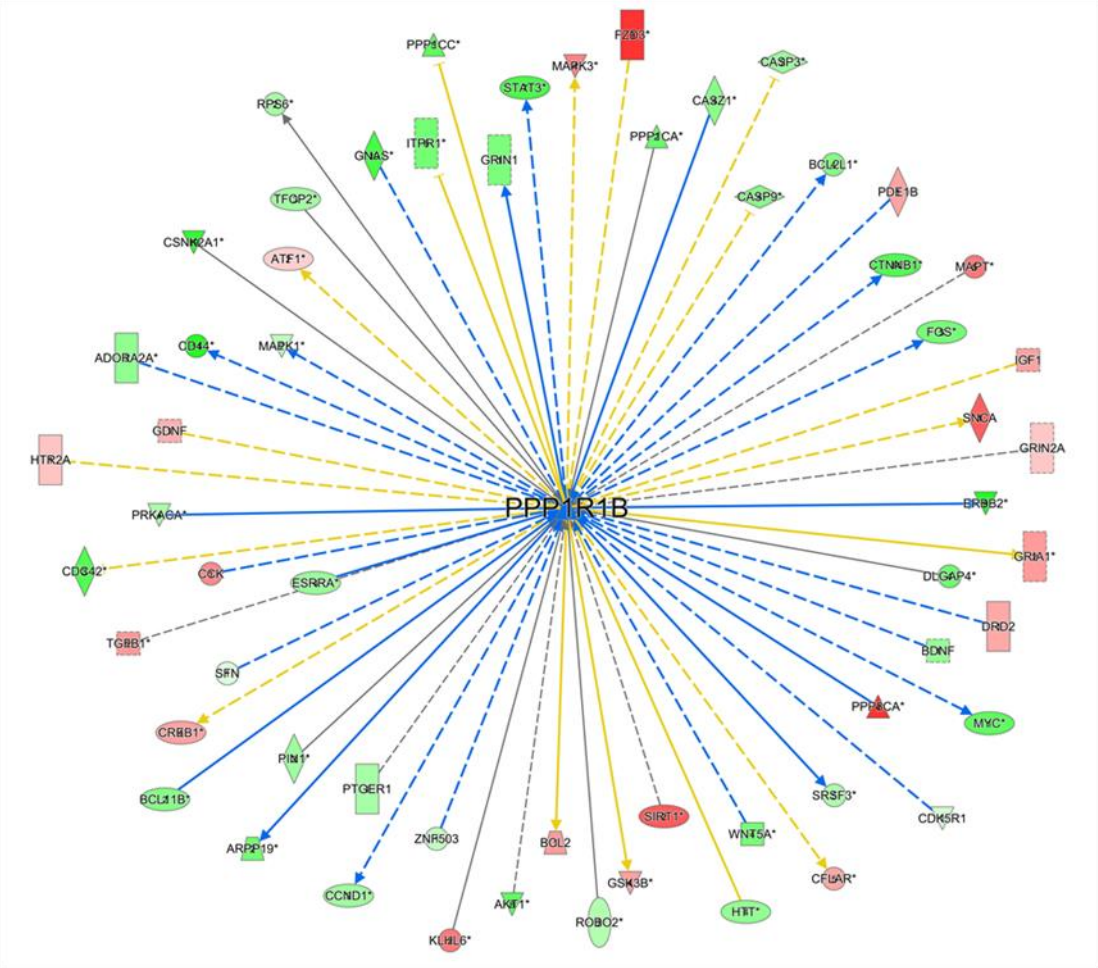


Figure C7: Identification of genes with alterations in expression induced by E2 treatment in DARPP-32 knockdown cells (compared to untreated DARPP-32 knockdown cells). The relationship of these genes with DARPP-32 was reviewed in various other cancers to learn about the previously identified interactions. The orange, blue, yellow and grey colours of the lines that connect *PPP1R1B* to each gene represent a relationship that leads to activation, suppression, inconsistent activity, and unpredicted influence on those genes, respectively.

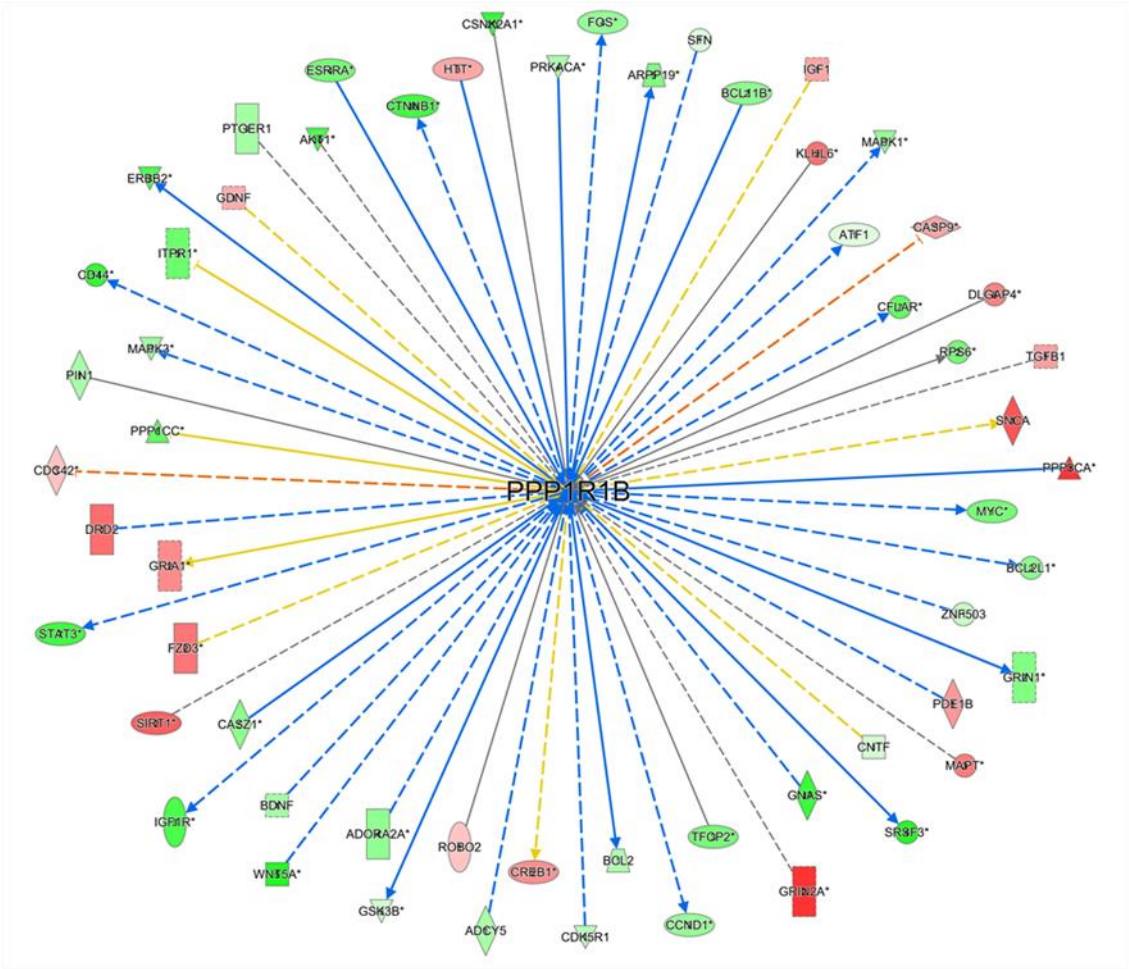


Figure C8: Identification of genes with alterations in expression induced by E2 treatment in control cells (compared to untreated control cells). The relationship of these genes with DARPP-32 was reviewed in various other cancers to learn about the previously identified interactions. The orange, blue, yellow and grey colours of the lines that connect *PPP1R1B* to each gene represent a relationship that leads to activation, suppression, inconsistent activity, and unpredicted influence on those genes, respectively.

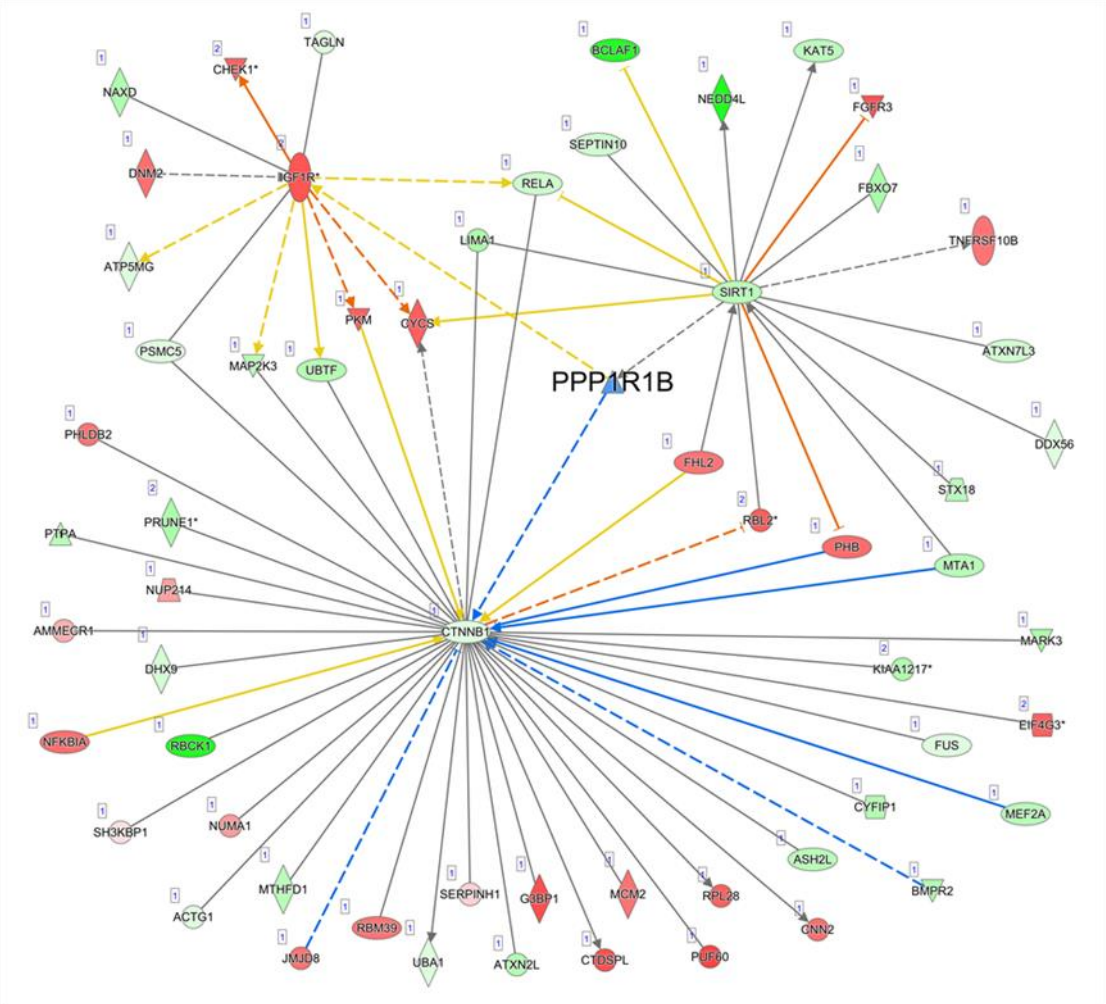


Figure C9: Identification of genes with alterations in expression induced by DARPP-32 suppression in E2-treated DARPP-32 knockdown cells (compared to E2-treated control cells). The relationship of these genes with DARPP-32 was reviewed in various other cancers to learn about the previously identified interactions. The orange, blue, yellow and grey colours of the lines that connect *PPP1R1B* to each gene represent a relationship that leads to activation, suppression, inconsistent activity, and unpredicted influence on those genes, respectively.

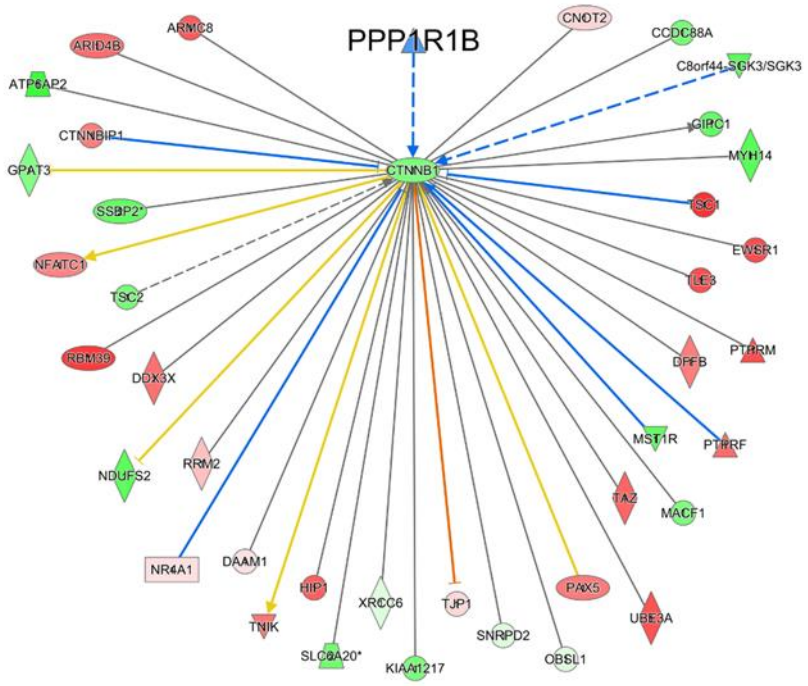


Figure C10 Identification of genes with alterations in expression induced by DARPP-32 suppression in PKA inhibitor treated DARPP-32 knockdown cells (compared to PKA inhibitor treated control cells). The relationship of these genes with DARPP-32 was reviewed in various other cancers to learn about the previously identified interactions. The orange, blue, yellow and grey colours of the lines that connect *PPP1R1B* to each gene represent a relationship that leads to activation, suppression, inconsistent activity, and unpredicted influence on those genes, respectively.