

Identification of KSR1 as a novel target and decoding tyrosine kinase proteome in breast cancer

By
Hua Zhang

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Faculty of Medicine,
Department of Surgery and Cancer, 1st floor ICTEM
Imperial College London
Hammersmith Hospital, Du Cane Road
London, W12 0NN

Supervisor: Prof Justin Stebbing

Co-supervisor: Dr Georgios Giamas

Dedicated

to my family and everyone who loves me

to the 1,278 days

to the stars in the night

and the beautiful eyes in my heart

Abstract

Kinase suppressor of Ras-1 (KSR1), originally identified as a novel protein kinase in the Ras-Raf cascade, plays a role in activation of mitogen-activated protein kinases (MAPKs). Although efforts have been devoted to study the role of KSR1 in certain tumour types, its biological functions in breast cancer have remained largely undefined.

A SILAC-based proteomic analysis was conducted to identify the KSR1-regulated phosphoproteins in breast cancer. Our results revealed that KSR1 overexpression decreases deleted in breast cancer 1 (DBC1) phosphorylation. We then demonstrated that KSR1 decreases transcriptional activity of p53 by reducing phosphorylation of DBC1, which leads to a reduced interaction of DBC1 with sirtuin 1 (SIRT1); this in turn enables SIRT1 to deacetylate p53.

We further examined the correlation between KSR1 expression and clinical outcome in breast cancer. Our results showed that patients with breast cancer with high KSR1 in our cohort (n>1000) had better disease free- and overall survival. Moreover, in KSR1-transfected stable cells, fewer and smaller size colonies were formed in comparison to parental cells, while an *in vivo* study demonstrated that the growth of xenograft tumours overexpressing KSR1 was inhibited. Mechanistically, the tumour suppressive action of KSR1 is BRCA1 dependent, which was shown by *in vitro* 3D matrigel and soft agar assays. KSR1 regulates BRCA1 ubiquitination through elevated BRCA1-associated RING domain 1 (BARD1) expression and increased BRCA1-BARD1 interaction.

Deregulation of tyrosine kinases (TKs) signalling can contribute to tumourigenesis. A combined approach of RNAi and SILAC-based quantitative proteomics was employed to decode the TKs-regulated proteomes upon silencing individually each 65 validated TKs in MCF7 breast cancer cells. Bioinformatics analysis identified 10 new distinctive clusters based on similarity in the TKs-regulated proteomes. The biological relevance of our proteomic study in interpreting the TKs-regulated proteomes supports the essential role of TKs in regulating all aspects of cellular activities.

Statement of originality

All the experimentation presented in this thesis has been conducted by myself, aside from the following:

In result sections, SILAC proteomics analysis was performed by Dundee Cell Products Ltd. Immunohistochemistry staining of tumour tissues from Nottingham Cohort was carried out by Dr Pritesh Trivedi at Histopathology Department, Hammersmith Hospital. Statistical analysis of the significance of KSR1 expression with clinical outcome in breast cancer patients was performed by Dr Andrew Green at Department of Pathology, Nottingham City Hospital and Dr Filipovic, Aleksandra.

In vivo animal studies were conducted by Dr Manikandan Periyasamy, Dr Ylenia Lombardo and myself. Immunohistochemistry and histopathological analysis of tissue samples isolated from mouse mammary primary tumour was performed by Dr Pritesh Trivedi and Dr Aleksandra Filipovic.

SNP analysis of samples from Nottingham cohort was undertaken by Prof Heinz-Josef Lenz's lab at University of Southern California. LOH analysis was performed by Dr Kevin Blighe and Prof Jacqui Shaw at University of Leicester. cDNA samples from 24 different breast cancer cell lines were kindly provided by Prof Simak Ali. Soft agar and 3D-matrigel assays were carried out by myself with the help from Mrs Arnhild Grothey and Dr Ylenia Lombardo. RNAi screen in TKs was performed by Dr Georgios Giamas, Mrs Arnhild Grothey, Mr Yichen Xu and myself.

Bioinformatics analyses of TKs clustering, GO classification and STRING network were conducted by Dr Nicos Angelopoulos.

I am appreciative of the help from the aforementioned.

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In the introduction chapter, some paragraphs of sections 1.2 and 1.3 have been published on two separate articles with myself as the first author. These two papers are entitled “The role of pseudokinases in cancer” on Cellular Signalling, (2012) 24, 1173-1184 and “The dual function of KSR1: a pseudokinase and beyond” on Biochemical Society Transactions, (2013) 41, 1078-1082.

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In the result chapter, some parts of section 3.1 have been published with myself as the first author on a research article entitled “SILAC-based phosphoproteomics reveals an inhibitory role of KSR1 in p53 transcriptional activity via modulation of DBC1” on British Journal of Cancer, (2013) 109, 2675-2684.

Hua Zhang

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CONTENTS

LIST OF FIGURES	13
LIST OF TABLES	16
ABBREVIATIONS	17
CHAPTER 1: INTRODUCTION.....	22
1.1 Cancer	23
1.1.1 Cancer is a major cause of disease and death	23
1.1.1.1 Breast cancer.....	25
1.1.2 The hallmarks of cancer.....	25
1.2 Protein kinases	28
1.2.1 The origins of phosphorylation.....	28
1.2.2 Kinase domain structure	29
1.2.3 Human kinome	31
1.2.3.1 Classification and phylogeny of the human kinome	32
1.2.4 Pseudokinases.....	36
1.2.4.1 Pseudokinases that have kinase activity.....	38
1.2.4.1.1 ERBB3	38
1.2.4.1.2 CASK.....	39
1.2.4.1.3 ILK.....	39
1.2.4.2 Pseudokinases that exert allosteric regulation and scaffolding function	40
1.2.4.2.1 STRAD α	40
1.2.4.2.2 Tribbles family.....	41
1.2.4.2.3 TRRAP	41
1.2.4.3 Future perspectives	42
1.3 Kinase suppressor of Ras 1 (KSR1)	43
1.3.1 KSR1 structure	43
1.3.2 KSR1 as a scaffold protein	45
1.3.3 KSR1 as an active kinase.....	49
1.3.4 Regulation of KSR1	51

1.3.4.1 Phosphorylation and protein-protein interaction.....	51
1.3.4.2 Transcription regulation.....	53
1.3.5 The physiological role of KSR1	53
1.3.6 KSR1 in cancers	54
1.3.6.1 KSR1 in solid tumours.....	54
1.3.6.2 KSR1 in non-solid tumours	56
1.3.7 KSR1 in other biological processes	57
1.3.7.1 KSR1 in immune system	57
1.3.7.2 KSR1/2 in metabolism.....	59
1.3.7.3 KSR1 in central nerve system.....	61
1.3.8 Closing remarks	62
1.4 Tyrosine kinases (TKs)	63
1.4.1 Receptor tyrosine kinases (RTKs)	63
1.4.1.1 Activation of RTK extracellular domain by ligands	63
1.4.1.2 Activation of intracellular kinase domain	66
1.4.1.3 RTKs in cancers.....	68
1.4.1.3.1 EGFR/ERBB family in cancer	70
1.4.2 Cytoplasmic tyrosine kinases (CTKs)	72
1.4.2.1 Activation of CTKs.....	73
1.4.2.1.1 Activation of c-SRC.....	73
1.4.2.1.2 Activation of c-ABL	74
1.4.2.1.3 Activation of Jaks	75
1.4.2.2 CTKs in cancer	75
1.4.2.2.1 Jaks	76
1.4.2.2.2 c-ABL	77
1.4.2.2.3 Other CTKs in cancer	78
1.5 Aims.....	79
CHAPTER 2: MATERIALS AND METHODS	80
2.1 Materials	81
2.1.1 Buffer and solutions.....	81
2.1.2 Mammalian cell lines.....	82

2.1.3 Plasmid constructs	84
2.1.4 Antibodies.....	86
2.1.5 Patient characteristics	88
2.2 Methods.....	90
2.2.1 Mammalian cell culture	90
2.2.1.1 Maintaining and passaging cells	90
2.2.1.2 Cell transfections	90
2.2.1.3 Cell treatments.....	91
2.2.1.4 Generation of stable cell lines.....	91
2.2.2 Protein extraction and western blotting	92
2.2.2.1 Total protein extraction.....	92
2.2.2.2 Nuclear and cytoplasmic protein extraction.....	92
2.2.2.3 Protein quantification and lysate preparation.....	93
2.2.2.4 SDS-polyacrylamide gel electrophoresis and western blotting.....	93
2.2.3 RNA extraction and RT-qPCR	94
2.2.3.1 RNA extraction from cells.....	94
2.2.3.2 cDNA synthesis	94
2.2.3.3 Quantitative real-time PCR.....	94
2.2.4 RNA interference.....	95
2.2.5 Plasmids transformation and DNA extraction	96
2.2.6 Luciferase reporter assay	96
2.2.7 Cell proliferation assay	96
2.2.8 Neddylation assay.....	97
2.2.9 Ubiquitination assay	97
2.2.10 Soft agar assay	97
2.2.11 Three-dimensional overlay culture in Matrigel	98
2.2.12 Single nucleotide polymorphisms and genotyping	98
2.2.13 Protein immunoprecipitation	98
2.2.14 Immunofluorescence staining	99
2.2.15 Genotyping and the determination of LOH	99
2.2.16 Tumour tissue microarray.....	100

2.2.17 Immunohistochemistry	100
2.2.18 <i>in vivo</i> tumourigenicity assay in breast cancer xenografts in nude mice	101
2.2.19 Proteome analysis (SILAC)	101
2.2.19.1 SILAC cell culture	101
2.2.19.2 Protein digestion and peptide fractionation	101
2.2.19.3 Protein digestion and phosphopeptide enrichment	102
2.2.19.4 Mass spectrometry methods	102
2.2.19.5 Proteome quantification	103
2.2.19.6 Bioinformatic analysis	104
2.2.20 Statistical analysis	104
CHAPTER 3: RESULTS.....	105
3.1 KSR1 regulates p53 transcriptional activity through deleted in breast cancer 1 (DBC1) modulation	106
3.1.1 Phospho-proteomic analysis of KSR1-regulated proteins using SILAC	106
3.1.1.1 Strategy of studying KSR1-regulated phospho-proteome in breast cancer	106
3.1.1.2 Profile of KSR1-modulated phospho-proteomics in MCF7	108
3.1.2 Gene ontology analysis of KSR1 regulated phospho-proteins in breast cancer	109
3.1.3 Phospho-proteomics analysis shows a decrease of phospho-DBC1 after KSR1 overexpression	112
3.1.4 KSR1 regulates p53 transcriptional activity	112
3.1.5 KSR1 does not affect p53 mRNA, protein, subcellular localisation and neddylation levels	114
3.1.6 KSR1 decreases p53 acetylation by reducing phosphorylation of DBC1	117
3.1.7 The effects of KSR1 on p53 acetylation is dependent on DBC1 and requires its intact kinase domain	119
3.1.8 KSR1 is important for breast cancer proliferation	121
3.1.9 Discussion	122
3.2 KSR1 regulates BRCA1 degradation and inhibits tumour growth	126
3.2.1 KSR1 is an independent predictor of overall survival in breast cancer	126
3.2.1.1 KSR1 expression in breast cancer patients	126
3.2.1.2 Clinical significance of KSR1 expression in the European cohort	127
3.2.1.4 Correlations between KSR1 expression and tumour biomarkers in European breast cancer patients	131
3.2.1.5 Oncomine analysis of <i>KSR1</i> gene expression between normal breast tissues versus breast carcinoma	133

3.2.1.6 Kaplan Meier Plotter analysis supports the good prognostic outcome of <i>KSR1</i>	135
3.2.2 The <i>KSR1</i> gene interval shows loss of heterozygosity (LOH) in primary breast tumour DNA.....	136
3.2.3 KSR1 is not a positive regulator of the canonical Ras-Raf-MAPKs pathway in breast cancer	137
3.2.3.1 KSR1 is ubiquitously expressed in breast cancer cell lines.....	137
3.2.3.2 KSR1 is not a positive regulator of the canonical Ras-Raf-MAPKs pathway in breast cancer.....	138
3.2.4 KSR1 overexpression inhibits tumour formation and growth <i>in vitro</i> and <i>in vivo</i>	141
3.2.4.1 Generation of KSR1 stably overexpressing breast cancer cell lines	141
3.2.4.2 KSR1 overexpression inhibits tumour formation <i>in vitro</i>	141
3.2.4.3 KSR1 overexpression inhibits tumour growth <i>in vivo</i>	143
3.2.5 KSR1 stabilizes BRCA1 by reducing BRCA1 ubiquitination and inhibits tumour growth through BRCA1	145
3.2.5.1 KSR1 affects the protein abundance of BRCA1 but not the mRNA levels	145
3.2.5.2 KSR1 stabilizes BRCA1 through reducing BRCA1 ubiquitination	147
.....	148
3.2.5.3 The inhibitory effect of KSR1 on tumour growth is through BRCA1	148
3.2.6 KSR1 regulates BRCA1 stability via elevated BARD1 abundance and increased BRCA1-BARD1 interaction	150
3.2.6.1 KSR1 affects the protein abundance of BARD1 not its mRNA level	150
3.2.6.2 The effect of KSR1 on BRCA1 expression is partly through BARD1	151
3.2.6.3 The interaction between BRCA1 and BARD1 is increased upon KSR1 overexpression.....	153
3.2.6.4 BARD1-BRCA1 nuclear abundance is increased upon KSR1 overexpression.....	154
3.2.7 Discussion.....	155
3.3 Global mapping of TKs signalling by combined use of RNAi and quantitative proteomics.....	159
3.3.1 Strategy for identification of TKs signalling by combined use of quantitative proteomics and RNAi	159
3.3.2 Expression profile of TKs family in MCF7 breast cancer cells.....	161
3.3.3 A high-throughput RNAi screen of TKs family in MCF7 breast cancer cells.....	163
3.3.4 Identification of TKs regulated proteomes in MCF7.....	164
3.3.5 Global proteomic portrait of TKs signalling.....	166
3.3.5.1 Proteomic alteration after knockdown of TKs	166
3.3.5.2 Identification of 10 distinctive clusters of TKs	167

3.3.5.3 Characterization of a proteomic signature and functional portrait for each cluster.....	170
3.3.6 Discussion.....	176
CHAPTER 4: DISCUSSION	181
4.1 The role of KSR1 in breast cancer	182
4.1.1 KSR1 in p53 regulation	182
4.1.1.1 Summary of findings	182
4.1.1.2 Future direction.....	183
4.1.1.3 Conclusion	184
4.1.2 KSR1 in BRCA1 regulation	185
4.1.2.1 Summary of findings	185
4.1.2.2 Future direction.....	186
4.1.2.3 Conclusion	187
4.2 Proteomic analysis of TKs signalling in breast cancer	188
4.2.1 Summary of findings	188
4.2.2 Future direction.....	190
4.2.3 Conclusion	190
REFERENCES	191
APPENDICES	216

LIST OF FIGURES

Figure 1 Cancer incidence in the UK.	24
Figure 2 Cancer mortality in the UK.	24
Figure 3 Hallmarks of cancer.	26
Figure 4 The common mechanism and fold of protein kinases.....	31
Figure 5 The phylogenetic tree of the complete superfamily of human protein kinases.....	33
Figure 6 Similarity in sequences of pseudokinases.	37
Figure 7 Schematic depiction of KSR1 structure and identified binding sites.....	45
Figure 8 Schematic model for the function of KSR1.	48
Figure 9 Receptor Tyrosine Kinase Families.	64
Figure 10 Receptor Tyrosine Kinase Dimerization.	66
Figure 11 Cytoplasmic Tyrosine Kinase Families.	73
Figure 12 Strategy of studying KSR1-regulated phospho-proteomics in breast cancer.	107
Figure 13 Scatter plot of KSR1-regulated phospho-proteome in MCF7.	109
Figure 14 Gene Ontology (GO) classification of the KSR1-regulated phospho-proteome in MCF7 cells.	111
Figure 15 Effect of KSR1 on p53 transcriptional activity in the presence or absence of etoposide by luciferase assays.	113
Figure 16 Effect of KSR1 overexpression on p53 mRNA and total protein levels.....	115
Figure 17 Immunofluorescence staining of p53 after KSR1 overexpression.....	115
Figure 18 Effect of KSR1 overexpression on p53 subcellular localisation and neddylation.	116
Figure 19 Effect of KSR1 on DBC1 phosphorylation.....	118
Figure 20 Mechanism of KSR1-regulated p53 acetylation.	120
Figure 21 Effect of KSR1 silencing on breast cancer cell proliferation <i>in vitro</i>	121
Figure 22 Schematic model illustrating the role of KSR1 on p53 transcriptional activity in breast cancer cells.....	125
Figure 23 KSR1 expression in breast cancer tissues by IHC.	126

Figure 24 Clinical significance of KSR1 in breast cancer.....	128
Figure 25 KSR1 expression in the paired adjacent normal breast and invasive breast cancer tissues.	130
Figure 26 Correlations of KSR1 polymorphisms with overall survival and disease free survival in breast cancer patients.....	130
Figure 27 Oncomine data-mining analysis of <i>KSR1</i> expression in different datasets.....	134
Figure 28 Kaplan Meier Plotter analysis of <i>KSR1</i> expression in breast cancer.	135
Figure 29 Loss of heterozygosity (LOH) reported in KSR1 and surrounding genes in primary breast tumour and circulating cfDNA.	136
Figure 30 Expressions of KSR1 mRNA and protein in mammary epithelial cell MCF10A and 24 breast cancer cell lines.....	137
Figure 31 Effect of KSR1 on activation of canonical Ras-Raf-MAPKs pathway in breast cancer.....	139
Figure 32 Effect of KSR1 on activation of ERK upon EGF treatment.	140
Figure 33 Validation of MCF7 stably overexpressing KSR1 cell lines and its effect on tumourigenesis in 3D matrigel assay.....	141
Figure 34 Effect of KSR1 on colony formation in soft agar assay.....	142
Figure 35 Effect of KSR1 on tumourigenesis in MDA231 cells by 3D matrigel assay.....	143
Figure 36 Effect of KSR1 overexpression on tumour growth <i>in vivo</i>	144
Figure 37 Effect of KSR1 on BRCA1 mRNA/protein levels.....	146
Figure 38 Effect of KSR1 overexpression on BRCA1 protein levels in the presence of proteasome inhibitors.	147
Figure 39 Effect of KSR1 on BRCA1 ubiquitination.	148
Figure 40 BRCA1 is required for the tumour suppressive effect of KSR1.....	149
Figure 41 Effect of KSR1 on BARD1 mRNA/protein levels.	151
Figure 42 Effect of KSR1 overexpression on BRCA1 protein levels after BARD1 silencing.	152
Figure 43 Effect of KSR1 on BRCA1 and BARD1 interaction.	153

Figure 44 Effect of KSR1 on subcellular localization of BRCA1 and BARD1.....	154
Figure 45 Proposed model of BRCA1 regulation by KSR1.....	158
Figure 46 Strategy for identification of TKs signalling by combined use of quantitative proteomics and RNAi.....	160
Figure 47 Expression profile of TKs family in MCF7 breast cancer cells.....	162
Figure 48 Expression levels of targeted TKs in this study.....	163
Figure 49 Validation of siRNAs targeting TKs using RT-qPCR.....	163
Figure 50 Validation of siRNAs targeting TKs using western blotting.....	164
Figure 51 Log ₂ ratios distribution of siABL2 vs siControl from SILAC quantitative proteomics.....	165
Figure 52 Heatmap of quantified proteins after silencing TKs.....	167
Figure 53 Correlation heatmap of the distance metric between all TKs in this study.....	168
Figure 54 Hierarchical clustering of the 65 TKs expressed in MCF7.....	169
Figure 55 Number of TKs in each identified cluster.....	169
Figure 56 Number of proteins significantly up or down-regulated in each identified cluster.....	170
Figure 57 Functional portrait of protein-related biological processes in each cluster.....	171
Figure 58 Functional portrait of protein-related molecular functions in each cluster.....	172
Figure 59 Representatives of defined functional networks in each classified TK cluster.....	174

LIST OF TABLES

Table 1 List of buffers and solutions.....	81
Table 2 List of mammalian cell lines.....	82
Table 3 Normal growth media.....	83
Table 4 SILAC Media.....	84
Table 5 Transfection media and reagents.....	84
Table 6 List of plasmids and constructs.....	84
Table 7 Primary antibodies.....	86
Table 8 Secondary antibodies.....	87
Table 9 Patient characteristics data.....	88
Table 10 Cox-proportional hazards analysis for predictors (model including KSR1 expression) of OS and DFS.....	128
Table 11 Patients' characteristics and tumour biomarkers and correlations with KSR1 expression in European breast cancer patients.....	131
Table 12 Key features from the SILAC analyse of siABL2/siControl.....	164

ABBREVIATIONS

ACC	Acetyl-CoA Carboxylase
AGC	Protein Kinases A, G and C families
AKAP	A-Kinase-Anchoring Protein
AKT	Protein Kinase B
ALK	Anaplastic Lymphoma Kinase
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
AMPK	Adenosine Monophosphate Activated Protein Kinase
APRIN	Androgen-Induced Proliferation Inhibitor
AS	Age Standardised
ATF4	Activating Transcriptional Factor 4
ATM	Ataxia-Telangiectasia Mutated
ATP	Adenosine-5'-Triphosphate
ATR	Ataxia-Telangiectasia and Rad3-Related
BARD1	BRCA1 Associated RING Domain 1
BCR	Breakpoint-Cluster Region
BDNF	Brain-Derived Neurotrophic Factor
BP	Biological Process
BRCA1	Breast and Ovarian Cancer Susceptibility Protein 1
Brk	Breast Tumour Kinase
BSA	Bovine Serum Albumin
CaM	Calmodulin
CAMK	Calcium/Calmodulin-dependent Protein Kinases
cAMP	Cyclic Adenosine Monophosphate
C/EBPα	CCAAT/Enhancer-Binding Protein-A
CASK	Ca ²⁺ /Calmodulin-Dependent Serine Protein Kinase
CC	Cellular Component
CCK4	Colon Carcinoma Kinase 4
CDK	Cyclin-dependent Kinases
cfDNA	Cell-Free DNA
CK1	Casein Kinase 1
CMGC	CDKs, MAPKs, GSK3, CLK families
CNK	Connector Enhancer of KSR
C-TAK1	Cdc25C-Associated Kinase 1
CTK	Cytoplasmic Tyrosine Kinase
DBC1	Deleted in Breast Cancer 1
DDR	Discoidin Domain Receptor
DFG	Asp-Phe-Gly
DFS	Disease Free Survival

DNA-PKcs	DNA-Dependent Protein Kinase Catalytic Subunit
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	EGF Receptor
EphR	Ephrin receptor
ERα	Oestrogen Receptor alpha
ERK	Extracellular-signal Regulated Kinase
ERRα	Oestrogen-Related Receptor alpha
FAK	Focal Adhesion Kinase
FAO	Fatty Acid Oxidation
FCS	Fetal Calf Serum
FDR	False Discovery Rate
FGFR	Fibroblast Growth Factor Receptor
FOXO3a	Forkhead Box O3a
g	Gram
GBM	Glioblastoma Multiforme
GIST	Gastrointestinal Stromal Tumour
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GO	Gene Ontology
GSK3	Glycogen Synthase Kinase 3
GTP	Guanosine (guanine) Triphosphate
h	Hour
HAT	Histone Acetyltransferase
HB-EGF	Heparin-Binding EGF
HDAC1	Histone Deacetylase 1
HRD	His-Arg-Asp
HSP	Heat Shock Protein
IFN-γ	Interferon Gamma
IHC	Immunohistochemistry
IL-9	Interleukin-9
ILK	Integrin-Linked Kinase
IMP	Impedes Mitogenic Signal Propagation
iNOS	inducible Nitric Oxide Synthase
InsR	Insulin Receptor
JAK	Janus Tyrosine Kinase
JMML	Juvenile Myelomonocytic Leukaemia
JNK1	C-Jun Amino Terminal Kinase-1
KSR1	Kinase Suppressor Of Ras1
LCK	Lymphocyte-specific Protein Tyrosine Kinase

LC-MS	Liquid Chromatography-Mass Spectrometry
LIM	Lin11, Isl-1 And Mec-3
LIMK	Lim domain-containing Kinase
LKB1	Liver Kinase B1
LOH	Loss Of Heterozygosity
LPS	Lipopolysaccharide
LTK	Leukocyte Tyrosine Kinase
LTP	Long-Term Potentiation
MAGUK	Membrane Associated Guanylate Kinase
MAPK	Mitogen-Activated Protein Kinase
MAP2K	Mitogen-Activated Protein Kinase Kinase
MARK	Microtubule Affinity-Regulating Kinase
MBP	myelin basic protein
MCM	Mini-Chromosome Maintenance
MEF	Mouse Embryo Fibroblast
MF	Molecular Function
mg	Milligram
ml	Milliliter
mM	Millimolar
MMC	Mitomycin C
MO25α	Mouse Protein 25 A
MPD	Myelo-Proliferative Disorder
MS	Mass Spectrometry
MT1-MMP	Membrane Type-1 Matrix Metalloproteinase
mTOR	mammalian Target Of Rapamycin
mTORC1	mTOR complex 1
MuSK	Muscle-Specific Kinase
NDCT	Non-Determinant Chromosomal Translocation
NF- κB	Nuclear Factor κ B
NGF	Nerve Growth Factor
NK	Natural Killer
NMR	Nuclear Magnetic Resonance
NRG	Neuregulin
ODNs	Oligonucleotides
OS	Overall Survival
PAGE	Polyacrilamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
PDGF	Platelet-Derived Growth Factor
PFA	Paraformaldehyde
PGC1α	Peroxisome Proliferative activated receptor gamma coactivator 1 alpha
PgR	Progesterone Receptor

PI3K	Phosphatidylinositol-3-OH Kinase
PIKK	Phosphatidylinositol 3-Kinase-Related Kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PJS	Peutz-Jeghers Syndrome
PKA	Protein Kinase A
PLCγ	Phospholipase C γ
PP2A	Protein Phosphatase 2A
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
PRD	PIKK-Regulatory Domain
qPCR	Quantitative Polymerase Chain Reaction
Ret	Rearranged during Transfection
RGS19	Regulator of G-Protein Signalling 19
RIPK	Receptor-Interacting Protein Kinase
RNAi	RNA interference
RSK	Ribosomal Protein S6 Kinase
RTK	Receptor Tyrosine Kinase
RTK7	Receptor Tyrosine Kinase 7
RYK	Receptor Related to Tyrosine Kinases
SCC	Squamous Cell Carcinoma
Ser	Serine
SFKs	SRC Family Kinases
SH	SRC Homology
SILAC	Stable Isotope Labelling Amino Acids in Cell Culture
SIRT1	Sirtuin 1
SRB	Sulforhodamine B
STATs	Signal Transducers And Activators Of Transcription
STE	homologs of the yeast Sterile 7, Sterile 11 and Sterile 20 kinases
STKR	Serine/Threonine Kinase Receptors
STRADα	STE-20-Related Adaptors A
STRING	Search Tool for the Retrieval of Interacting Genes
STYK1	Serine/Threonine/Tyrosine Kinase 1
TBS	Tris-Buffered Saline
TCGA	The Cancer Genome Atlas
TESK	Testis-Specific Kinase
TF	Tissue Factor
TFF1	Trefoil Factor 1
TGF-β	Transforming Growth Factor-B
Thr	Threonine
TIE	Tyrosine Kinase Receptor in Endothelial Cells
TKD	Tyrosine Kinase Domain
TKI	Tyrosine Kinase Inhibitor

TKL	Tyrosine Kinase-Like
TMA	Tumour Tissue Microarray
TNFα	Tumour Necrosis Factor A
TP53	Tumor protein p53
Trb	Tribbles Homolog
Tris	Tris(hydroxymethyl)aminomethane
TRRAP	Transformation/Transcription Domain-Associated Protein
TTBK	Tau-Tubulin Kinase
Tyk2	Tyrosine Kinase 2
Tyr	Tyrosine
v	Volume
VAIK	Val-Ala-Ile-Lys
VDR	Vitamin D Receptor
VEGFR	Vascular Endothelial Growth Factor Receptor
VRK-3	Vaccinia-Related Kinase 3
w	Weight
WHO	World Health Organization
°C	Celsius Degrees

CHAPTER 1: INTRODUCTION

1.1 Cancer

1.1.1 Cancer is a major cause of disease and death

Cancer is a major health problem and remains a leading cause of death around the globe. In 2012, 14.1 million people were newly diagnosed with cancer (excluding non-melanoma skin cancer), and 8.2 million died from cancer worldwide [1]. It is predicted that the number of annual cancer cases will increase from 14 million to 22 in the next two decades [1]. Lung, liver, stomach, colorectal and breast cancers are responsible for causing the most cancer-related deaths each year [1, 2]. Data from the World Health Organization (WHO) shows that five leading behavioural and dietary risks including high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco and alcohol use are responsible for about 30% of cancer deaths [1, 2].

More specifically, in 2011 in the UK, more than 331,000 people were diagnosed with cancer [3-7]. The European Age-Standardised (AS) incidence rates for all cancers (excluding non-melanoma skin cancer) in the UK increased by 23% in males and by 43% in females during the period 1975-1977 to 2009-2011. However, there has been a slowdown in the rate of increase over the past ten years, with the AS incidence growing by 3% in males and 7% in females (**Figure 1**) [3-7]. Approximately 159,000 people died from cancer in the UK in 2011 [8]. Despite an increase in cancer incidence, cancer mortality is decreasing in the UK. Between 1990-1992 and 2009-2011, the European AS mortality rates decreased by 26% in males and 20% in females. In the last ten years, the rate of reduction has started to decelerate, with the AS mortality rates declining by 12% and 9% in males and females, respectively (**Figure 2**). A further 17% decrease of cancer deaths is expected between 2011 and 2030 in the UK [9, 10].

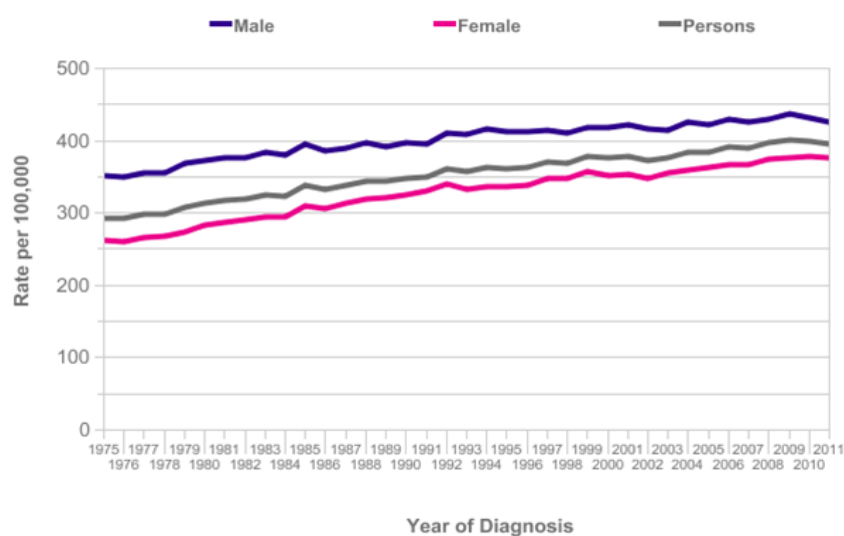


Figure 1 Cancer incidence in the UK.

All cancers excluding non-melanoma skin cancer, European Age-Standardised Incidence Rates, UK, 1975-2011. Modified from Cancer Research UK website, 2014.

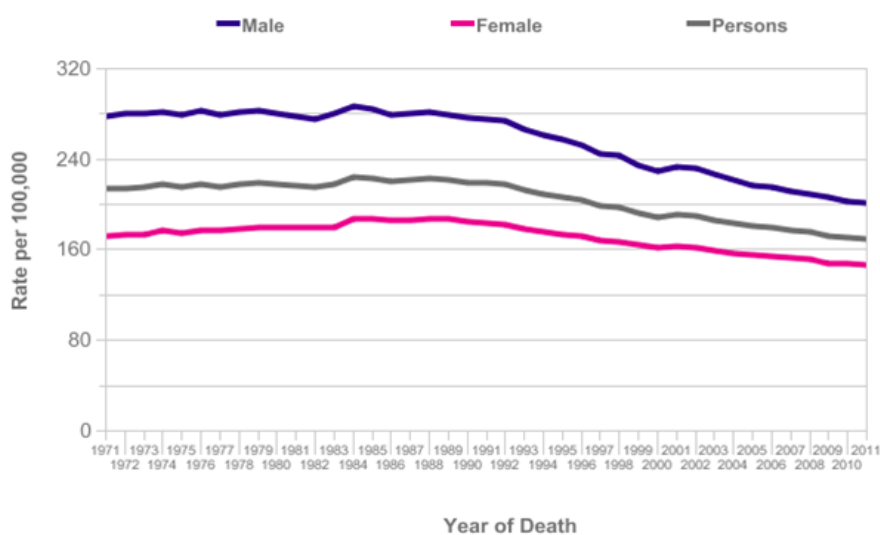


Figure 2 Cancer mortality in the UK.

All cancers, European Age-Standardised Mortality Rates, UK, 1971-2011. Modified from Cancer Research UK website, 2014.

1.1.1.1 Breast cancer

Breast cancer is the second most common cancer after lung cancer worldwide. In females, it is the most frequent malignancy both in more and less developed regions with an estimated 1.67 million people newly diagnosed globally in 2012, accounting for 25% of all cancers [1]. Breast cancer is the fifth cause of death from all cancers (522,000 deaths). However, in females, it is the most common cause of cancer death in less developed regions (324,000 deaths, 14.3% of all) and the second cause in more developed regions (198,000 deaths, 15.4%) after lung cancer [1]. In the UK, breast cancer is the most common cancer in women with a lifetime risk of 1 in 8 females being diagnosed with breast cancer [3-7]. More than 49,500 women and 400 men in the UK were diagnosed with breast cancer in 2010. Since 1970s, breast cancer incidence rates in women have increased by almost 70% in the UK, partly owing to the awareness of early signs and symptoms and mammography screening, which lead to early detection. Around 11,700 females and 80 males died from breast cancer in the UK in 2011 [8]. Female breast cancer death rates have fallen by 40% in the UK since the mid-1980s, despite increases in incidence rates. Numerous factors have contributed to better survival rates of breast cancer patients, including early diagnosis, improvements in surgery practice and radiation-therapy delivery, development of systemic chemotherapy, endocrine therapy, and targeted therapies. Despite that, breast cancer still remains the second most common cause of death from cancer in women. A better understanding of the mechanisms underlying carcinogenesis, tumour progression and metastasis, resistance to therapies will help us develop more effective treatments and continue to improve patient prognosis.

1.1.2 The hallmarks of cancer

Cancer is believed to stem from one single cell. Oncogenic transformation from a normal cell into a malignant cell in humans is a multistage process. Typically, these steps are manifested in genetic changes that contribute to the progressive conversion of normal human cells into malignant derivatives [11, 12].

Due to the remarkable progress in cancer research over the past decades, our understanding of the biology of cancer has greatly improved. Scientists have now realized that tumours are more than a mass of sustaining proliferative cancer cells. Importantly, they are multifaceted entities comprising a collection of distinct cell types, including normal cells forming tumour-related stroma, which function actively to contribute to the initiation, development and metastasis of tumour cells. The conceptual progress has allowed Hanahan and Weinberg to

revisit their original elaboration of hallmarks of cancer and to recognise the emerging hallmark traits. Together with the initial six, they have recently summarised in total ten biological capabilities and enabling characteristics attained during the multistep development from normal cells to human tumours [11, 12]. These hallmarks are:

- Self-sufficiency in growth signals leading to sustaining proliferation
- Resistance to cell death signals
- Avoidance of anti-growth signals
- Uncontrolled replicative immortality
- Sustained angiogenesis replicative immortality
- Activation of invasion and metastasis
- Instability and mutation of genome
- Deregulation of cellular energy metabolism
- Tumour-promoted inflammation
- Escaping from immune destruction

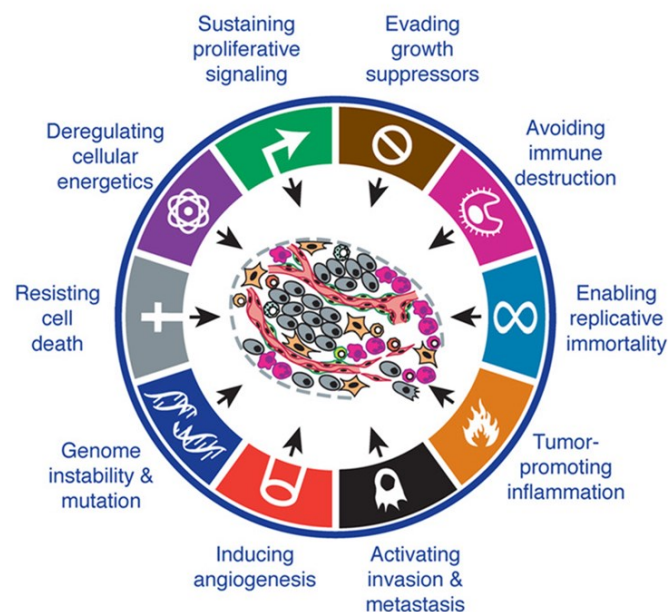


Figure 3 Hallmarks of cancer.

Biological capabilities that were proposed by Hanahan and Weinberg as hallmarks of cancer are shared in common by all human cancers. Modified from Hanahan and Weinberg, 2011

Notably, protein kinases play a fundamental role in all aspects of cell organization and behaviour, whereas deregulation of protein kinases can contribute to the development of almost all hallmarks of cancer [13]. A detailed description of protein kinases, particularly tyrosine kinases (TKs) is presented in sections 1.2 and 1.4, respectively.

Focusing on the main scope of this thesis, the origins of protein phosphorylation and the classifications of protein kinases in the human kinome is discussed. Particularly, I will describe the biological function of an interesting group in the human kinome, termed “pseudokinases”, as well as summarize the current understanding of one member from this group, kinase suppressor of Ras 1 (KSR1). In the last section, TKs are reviewed, highlighting with a few examples regarding their structural and functional importance, and their involvement in carcinogenesis.

1.2 Protein kinases

Ever since the discovery of reversible phosphorylation regulating the activity of glycogen phosphorylase, mounting evidence has shown that the reversible phosphorylation of proteins is essential to the modulation of almost all aspects of cell function [14]. Protein kinases are conserved enzymes that are capable of transferring a phosphate group from a phosphate contributor onto a receiver amino acid in a target protein. In general, the contributor is the γ phosphate of adenosine-5'-triphosphate (ATP), or another nucleoside triphosphate, but different enzymes may have other phosphate donors. Due to the fact that most protein kinases have more than one substrate, classification of protein kinases is thus based on the receiver amino acid specificity rather than protein substrate specificity and two main families are hence defined as serine/threonine (Ser/Thr) and tyrosine (Tyr) kinases [15, 16]. Phosphorylation can lead to activation or inhibition of biological activity, enhancement or decrease of molecule movement and facilitation or disruption of protein-protein interaction, depending on the target substrates and associated networks.

1.2.1 The origins of phosphorylation

The first evidence of protein kinase activity was reported in 1954, which described that an enzyme present in liver mitochondrial can phosphorylate casein [17]. Shortly after, two different groups identified that a phosphorylation/dephosphorylation mechanism was involved in the conversion of phosphorylase b to phosphorylase a [18-20]. One of the groups led by Fischer and Krebs showed that the b form could be converted to the a form in cell-free muscle extracts dependent of Mg^{2+} and ATP and an enzyme named as phosphorylase kinase by the authors [18, 19]. Subsequently, this kinase was revealed to facilitate the transfer of the γ -phosphoryl group of ATP to a specific serine residue on phosphorylase b [14, 21].

It was not easy to purify protein kinases in the early days. In 1959, phosphorylase kinase was the first protein kinase purified from rabbit muscle [22]. After nine years, another protein-serine kinase, the cyclic adenosine monophosphate (cAMP)-dependent protein kinase (also known as protein kinase A (PKA)), was purified from rabbit skeletal muscle [23]. In 1979, the first protein-tyrosine kinase, pp60^{v-src}, was purified using ion exchange or immunoaffinity chromatography [24], followed by purification of the epidermal growth factor (EGF) receptor kinase from shed plasma membrane vesicles [25]. With the development of molecular cloning and gene sequencing in the late twentieth century, a great body of evidence supported that a large family of eukaryotic protein kinases exists, and the burgeoning numbers of protein

kinases identified or characterized further suggested that the mammalian genome may encode as many as a thousand different protein kinases [26, 27]. The achievement of human genome sequence finally allows scientists to be able to identify all human protein kinases, catalogued as human kinome by Manning and colleagues. A total of 518 putative protein kinases constituting about 1.7% of all human genes have been characterized [27]. The human kinome will be discussed in details in the next part of this introduction (Section 1.2.3).

1.2.2 Kinase domain structure

The exponentially increasing rate of identified protein kinases resulting from molecular cloning and sequence has uncovered the fact that all protein kinases share prominent sequence similarity in their catalytic domains [26]. The first evidence of this similarity came from a surprising discovery that a region of approximately 300 amino acids in the catalytic subunit of the cAMP-dependent protein kinase and pp60^{v-src} was related [28]. This finding indicates that the cAMP-dependent protein kinase and pp60^{v-src} may originate from the same ancestral protein kinase. It is thus reasonable to conclude that some if not all eukaryotic protein-serine/threonine and protein-tyrosine kinase genes arose from a single archetypal gene given the identified sequence similarities [26]. Soon catalytic domains consisting of 250-300 amino acid residues within protein kinases were characterized and subsequently the first systematic classifications of protein kinases on account of their homologous kinase domains (catalytic domains) were presented by Hanks and Hunter [16]. Their monumental work that analysed and aligned the amino acid sequences of reported protein kinases allowed the identification of conserved structural features responsible for their enzymatic activity. These revolutionarily conserved domains in turn revealed the common mechanism underlying the regulation of transferring the γ phosphate of a purine nucleotide triphosphate to the hydroxyl groups of their associated substrates, even if diversities of structures, regulation modes, and substrate specificities among the protein kinases exist [16].

In 1991, Knighton and colleagues solved the first crystal structure of the cAMP-dependent protein kinase (PKA), the hallmark for all successive structural studies of kinases [29]. A great body of elegant work subsequently dissected the crystal structures of other protein kinases including cyclin-dependent kinase 2 (CDK2), the mitogen-activated protein kinase (MAPK) or extracellular-signal regulated kinase 2 (ERK2) and the tyrosine kinase c-SRC [29-34]. These comprehensive studies uncovered that classical protein kinases have a canonical catalytic domain of ~250 amino acids in length, consisting of a small N-terminal

lobe of β -sheets and a larger C-terminal lobe of α -helices [35]. From the crystal structures of identified protein kinases, Hanks and Hunter summarized that the conserved kinase domains contain 12 canonical subdomains that fold into a common catalytic core structure and two main subdivisions within the superfamily: the protein-serine/threonine kinases and the protein-tyrosine kinases [16]. The three main roles endorsed to the kinase domains of protein kinases to exert catalytic activity are: a) allowing interaction and orientation of the ATP phosphate donor as a complex with Mg^{2+} ; b) allowing interaction and orientation of the protein (or peptide) substrate; c) allowing the transfer of the γ -phosphate from ATP to the receiver hydroxyl residue (serine, threonine, or tyrosine) of the protein substrate [16].

The common mechanism and fold shared by protein kinases are described here as an example of the basic catalytic cycle for substrate phosphorylation by a kinase (**Figure 4**). Firstly, ATP binds in a cleft between the N-terminal and C-terminal lobes to enable the adenosine moiety to place in a hydrophobic pocket with the phosphate backbone positioned towards the outside of the solution. Then, the protein substrate integrates along the cleft and this consequently allows the conserved residues within the kinase catalytic domain to catalyse the transfer of γ -phosphate of ATP to the hydroxyl oxygen of the Ser, Thr or Tyr residue of the substrate. Finally, after phosphorylation, the substrate and ADP are released from the active site [35].

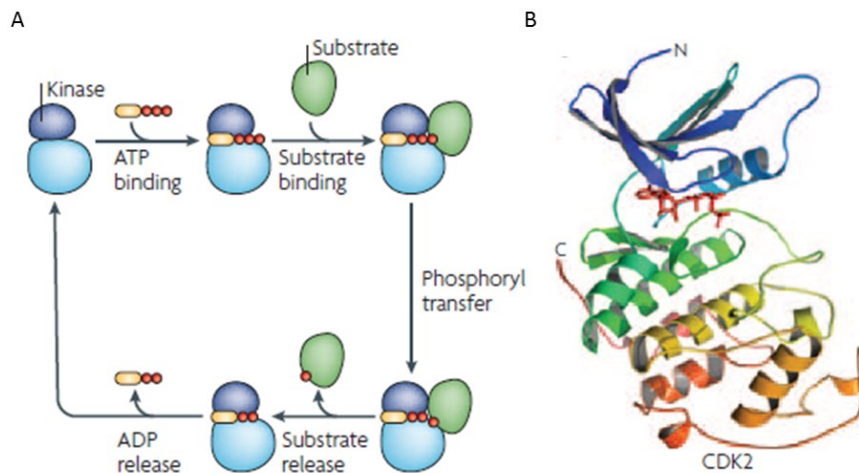


Figure 4 The common mechanism and fold of protein kinases.

A. A representative catalytic cycle of substrate phosphorylation by a protein kinase. Starting clockwise from top left, ATP binds to a cleft between the N-terminal and C-terminal lobes that is the active site of the kinase. Subsequently, the complex is joined by the substrate binding to the active site. After it is bound, the active domain within the kinase catalyses the transfer of the γ -phosphate of ATP (red) to a Ser, Thr or Tyr residue of the substrate. Once phosphorylation is completed, the substrate and ADP are then detached from the kinase. The order of the steps may vary depending on different kinases. For instance, some kinases bind to their protein substrates first and followed by ATP and others release ADP before releasing the protein substrate. **B.** A similar protein fold shared by protein kinase are presented here. It comprises two lobes: one lobe consists of mainly β -sheet structure (blue) and the other lobe consists of α -helices (green, orange and yellow). This lobe structure produces an ATP-binding cleft that creates the active site. The representative fold is shown by the crystal structure of CDK2 (Protein Data Bank (PDB) ID: 1QMZ). ATP is modelled bound in the cleft (red ball and stick model). This figure is adapted from published work from Ubersax et al, 2007.

1.2.3 Human kinome

Protein phosphorylation is the most common type of post-translational modification in the signal transduction of eukaryotic cells. Through phosphorylation of substrates, protein kinases modulate almost every aspect of cellular processes, including development, metabolism, transcription, differentiation, cell cycle progression, proliferation and apoptosis [27, 35]. Given their essential role in signal transduction, protein kinases are one of the largest families of genes in eukaryotes and have been extensively studied [36-40]. Moreover, scientists have estimated that about 30% of all cellular proteins are phosphorylated on at least one residue and assumedly there are approximately 700,000 potential phosphorylation sites (P-sites) for any given kinase [35, 41, 42]. With the advent of comprehensive information from human genome sequence, a total of 518 human protein kinases, constituting about 1.7% of all human genes, have been identified, which are catalogued as “human kinome” by Manning and

colleagues [27]. It has now been widely recognised that mutations and dysregulation of protein kinases result in impaired cellular function and play a critical role in virtually every human disease, particularly in malignancies [43, 44]. The complete mapping of human protein kinases (kinome) has greatly improved our understanding of the origins of human diseases and benefited us in identifying therapeutics targets against them.

1.2.3.1 Classification and phylogeny of the human kinome

The classification of protein kinases has greatly increased over the past twenty years. In 1995, Hanks and Hunter classified protein kinases into five broad groups, 44 families, and 51 subfamilies, based on the comparison of the sequences in the catalytic fragment [16]. To further advance our understanding of kinase function and evolution, the milestone work by Manning and colleagues has catalogued and classified the complete complement of protein kinases in the human genome and added four new groups, 90 families, and 145 subfamilies [27]. These new classifications are clustered primarily by comparing the sequences of their catalytic domains, assisted by information of similarities in sequence and domain structure outside of the catalytic domains, defined biological functions, and a conserved classification of the yeast, worm, and fly kinomes [38].

The human kinome is catalogued into seven main groups, including PKA, PKG and PKC families (AGC); calcium (Ca^{2+})/calmodulin (CaM)-dependent protein kinases (CAMK); casein kinase 1 (CK1); containing CDKs, MAPKs, glycogen synthase kinase 3 (GSK3), Cdc2-like kinase (CLK) families (CMGC); homologs of the yeast Sterile 7, Sterile 11 and Sterile 20 kinases (STE); tyrosine kinases (TK); tyrosine kinase-like (TKL) (**Figure 5**). Other small groups such as atypical protein kinases are not discussed here due to limited studies available. Moreover, most family members within each main kinase group share evolutionarily conserved sequences, structure, as well as functions.

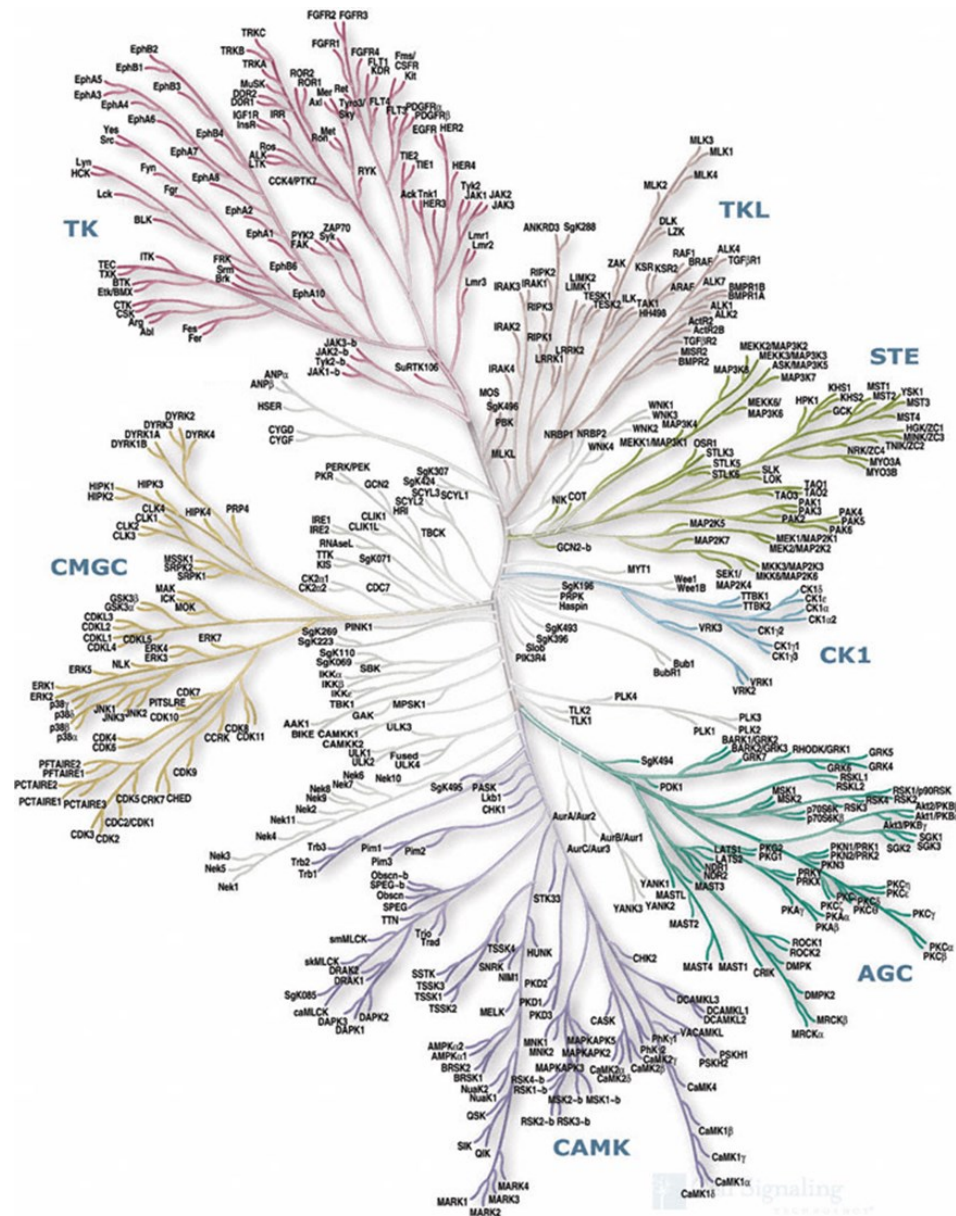


Figure 5 The phylogenetic tree of the complete superfamily of human protein kinases.

A total of 518 human protein kinases are classified based on similarity between the protein sequences of these catalytic domains. Each kinase is at the tip of a branch, and the similarity between various kinases is inversely related to the distance between their positions on the tree diagram. Most kinases fall into small families of highly related sequences, and most families are part of larger groups. The seven major groups are labelled and coloured distinctly. Other kinases are shown in the centre of the tree, coloured gray. This figure is adapted from published work from Manning et al, 2002.

The AGC kinase group of protein kinases comprises 60 members, including PKA, PKG and PKC families [16, 27]. From the information of the solved kinase domain structures so far, it is now recognised that the structures of active AGC kinases are highly similar [45]. In the bilobal kinase fold, ATP, which serves as the phosphate donor during phosphorylation, is forked between a small N-lobe and a large C-lobe. The activation segment of the kinase containing catalytic elements such as the Asp-Phe-Gly (DFG) motif for phosphoryl transfer is located in the C-lobe, adjacent to the ATP-binding site, whereas through α C helix the activation segment connects to the N-lobe [46]. Upon phosphorylation, conformational changes within the active and regulatory elements coordinate the formation of a set of interactions required for the catalytic activity of the kinase [47, 48]. As one of the most studied kinase groups, the AGC family are implicated in multiple important cellular functions and their dysregulation contributes to the pathogenesis of many human diseases, including cancer [45].

The CAMK group of protein kinases comprises a number of important and well-studied subgroups including CaMKs (CaMKI, CaMKII, CaMKIV and CaMKK), check point kinases (CHKs), AMP-activated protein kinases (AMPKs) and microtubule affinity-regulating kinases (MARKs) [27]. Although the CAMK group is named after Ca^{2+} /CaM-dependent protein kinase, only some of the CAMK group protein kinases are known to be activated by Ca^{2+} /CaM binding to a domain located near the C-terminus to the catalytic domain, such as CaMKI, CaMKII, CaMKIV, CaMKK and MLCK [16, 49]. Studies of structure and regulation of CaMKs have provided insights in understanding this interesting group of kinases. CaM is a ubiquitous 16-17 kD protein enclosing four helix-loop-helix motifs that can bind Ca^{2+} with high specificity and affinity. CaM has two globular domains with two Ca^{2+} binding sites individually and coupled by a flexible α -helix. Upon Ca^{2+} binding to CaM, a conformational change is induced, thus exposing hydrophobic residues to allow interaction of the Ca^{2+} /CaM complex with other proteins such as Ser/Thr protein kinases [50]. Moreover, when there is no Ca^{2+} /CaM in the system, the CaMKs are auto-inhibited. Apart from activation by Ca^{2+} /CaM, phosphorylations including auto-phosphorylation or phosphorylation by some other kinase can also modulate their kinase activity [50].

The CMGC group includes key kinases such as CDKs, ERKs, GSKs, CLKs and CK2s, which have a diversity of functions in cell cycle control, stress-response, cell survival and metabolism regulation. Some members of this group tend to phosphorylate substrates at sites lying in proline (Pro)-rich environments and are known as proline-directed kinases [16]. For

example, studies from the CDK family such as Cdc2 and CDK2 indicate a requirement of phosphate acceptors lying directly N-terminal to a Pro residue, which is similar in the case of MAPK family [16]. However, some other kinases may not obey the proline-directed specificity, such as the CK2 family that shows a strong preference for Ser residues positioned N-terminal to a cluster of acidic residues [16]. Further structural analysis will improve our understanding regarding the regulation of this well-conserved group and shed light on their biological function as well.

The CK1 group is a small family originally known as casein kinase 1 but now renamed as cell kinase 1, comprising CK1s, vaccinia related kinases (VRKs), and tau-tubulin kinases (TTBKs). Casein kinase 1 subgroup is a family of monomeric serine/threonine (Ser/Thr)-selective protein kinases, which is the most extensively studied within this group [27]. To date, casein kinase 1 family has been shown to be important in regulating various cellular processes including DNA replication, DNA damage response, circadian rhythms, Wnt signalling, membrane trafficking, cytoskeleton maintenance and RNA metabolism [51-53]. Both *in vitro* and *in vivo* studies have shown that the CK1 family prefers pre-phosphorylated substrates. Indeed, their most preferable motif contains a phospho-residue in the N-3 position, such as pSer/pThr-X-X-Ser/Thr, where pS/pT is a phospho-serine or phospho-threonine, X can be any amino acid, and the underlined residues refer to the target site. It is indicated that the priming phosphoryl group may cooperate with a basic pocket on the surface of the kinase, thus placing the substrate for phosphorylation [54].

The STE group consists of MAPK cascade families (Ste7/mitogen-activated protein kinase kinase (MAP2K), Ste11/MAP3K and Ste20/MAP4K), which could serially activate each other and then consequently phosphorylate the MAPK family [27]. Specifically, the Ste7 family also known as MEK kinases can directly phosphorylate MAPKs and are typically dual-specificity kinase; Ste11 members also known as MEKK kinases can phosphorylate Ste7 kinases; a number of Ste20 members can activate Ste11 kinases and then transduce the signal down the cascade [55]. These families are named after members of the canonical pheromone-responsive MAPK cascade in yeast. Distinctive members of Ste7 and Ste11 kinases are associated with different MAPKs as substrates, such as ERK, c-Jun amino terminal kinase (JNK). This highly interactive cascade plays an important role in multiple cellular processes and its deregulation contributes to human diseases, particularly cancers [55].

The TK group is extensively studied and diverse, and has about 90 members, the largest number of distinct families of any group [27]. There are two main subgroups: one is classified

as receptor tyrosine kinases (RTKs), which localize in the plasma membrane and are activated by binding of an extracellular molecule; the other is known as cytoplasmic tyrosine kinases (CTKs), containing SRC homology 2 (SH2) and/or SH3 domains essential for activation by activated receptor tyrosine kinases motility [56]. A detailed description about the structure and function of TKs is presented in a separate chapter below (Section 1.4). TKL group has approximately 43 members, and is also a very diverse group of families that resemble both tyrosine and serine/threonine kinases. This group comprises the mixed-lineage kinase (MLK), Lim domain-containing kinase (LIMK), testis-specific kinase (TESK), Raf, receptor-interacting protein kinase (RIPK) and serine/threonine kinase receptors (STKR) families, which are mainly involved in regulating cellular proliferation, survival, differentiation, function, and motility [56].

Interestingly, there are about 50 human kinases predicted to be inactive kinases and classified as pseudokinases owing to their lack of one or more conserved catalytic residues required for enzymatic activity [27]. They have been postulated to function as scaffolding proteins or allosteric regulators in signal transduction. A more detailed discussion about this group in human kinome is found in the below section 1.2.4.

1.2.4 Pseudokinases

The term “pseudokinase” originated from the concept that these protein kinases lack one or more conserved residues which are crucial for enzymatic activities compared with classic protein kinases such as PKA, therefore they are not predicted to be able to phosphorylate substrates. Based on sequence analysis, the excellent work conducted by Manning and colleagues demonstrated that approximately 50 protein kinases in the human kinome are classified as pseudokinases, which require the intact three motifs (the Val-Ala-Ile-Lys (VAIK), the His-Arg-Asp (HRD), the DFG) for ATP and peptide binding, and phosphotransfer to exert the catalytic activity (**Figure 6**) (reviewed by Boudeau and Alessi, Zeqiraj and Aalten [57-59]). Interestingly, kinase suppressor of Ras 1 (KSR1), which is the main focus of my PhD project, has been classified as a pseudokinase. Its structure and associated biological functions is extensively described in section 1.3.

It appears that these pseudokinases are widely situated in every branch in phylogenetic tree, which hints that from the evolution perspective, they might function similarly as active protein kinases. Increasing evidence from structural investigation suggests that this might be the case. For example, with-no-K(Lys) kinase (WNK) in which the key lysine residue in

VAIK motif in subdomain II of the catalytic domain was missing was shown that it can function actively by using another neighbouring lysine in the β strand 2 [60, 61]. Ca^{2+} /CaM-dependent serine protein kinase (CASK) is another good example where despite the absence of key enzymatic residues in DFG motif indispensable for Mg^{2+} binding, CASK can still implement itself in a constitutively active conformation, which can exhibit the capability of autophosphorylation and phosphorylating specific substrate, the synaptic protein neurexin-1. Unlike other kinases which require Mg^{2+} for activity, CASK works independently of Mg^{2+} which surprisingly inhibits its catalytic activity [62, 63]. The elucidation of receptor tyrosine protein kinase ERBB3 crystal structure challenged the concept that it was an inactive pseudokinase, since ERBB3 lacks the intact conserved motif HRD required for phosphorylation. In fact, in order to promote phosphoryl transfer, ERBB3 can adopt itself in an active conformation through a unique mechanism by its kinase-defective domain [64, 65].

CASK	38 F A V K 41	139 H R D 141	162 G F G 164
CCK4	827 V L V K 830	928 H K D 930	947 A L G 950
EphB6	684 V A I Q 687	778 H R S 780	798 R L G 800
EphA10	674 V A V H 677	768 H R G 770	788 G F G 790
HER3	739 V C I K 742	832 H R N 834	852 D F G 854
ILK	217 I V V K 220	317 R H A 319	- - -
KSR1	634 V A I R 637	729 H K D 731	751 L F G 753
STRADα	97 V T V R 100	193 H R S 195	213 G L R 215
Trb1	117 L R C K 120	203 L G D 205	225 S L E 227
Trb2	87 L V C K 90	173 L R D 175	195 S L E 197
Trb3	94 Y T C K 97	180 L R D 182	202 N L E 204
TRRAP	3499 Y L V M 3502	3659 L N R L N 3663	3860 Y F R 3862
VRK3	- - - -	304 H G N 306	326 G Y G 328
Conserved Motifs	VAIK	HRD	DFG

Figure 6 Similarity in sequences of pseudokinases.

Multiple sequence alignment of pseudokinases comparing three conserved motifs (VAIK, HRD and DFG) in the catalytic domain essential for enzymatic activity. Highlighted in black are the indicated missing motifs for each pseudokinase. This figure is modified from published work from Zhang et al, 2012.

No matter what functions these pseudokinases perform either as scaffold proteins, allosteric regulators or active kinases, emerging evidence has shown that they are greatly involved in cell proliferation, differentiation, apoptosis and metastasis in malignancies [43]. Here we are discussing some examples of well-established pseudokinases.

1.2.4.1 Pseudokinases that have kinase activity

1.2.4.1.1 ERBB3

ERBB3 belongs to the human epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, which includes EGFR, ERBB2, ERBB3 and ERBB4. A group of growth factors as extracellular ligands bind to these receptors to form dimmers with a preference for heterodimers leading to the activation of downstream signalling pathways in cell proliferation and differentiation, whereas, abnormal activation contributes to pathogenesis of many cancers [66, 67]. Among these diverse homodimer and heterodimer complexes, ERBB2-ERBB3 is shown to be the most active form [68]. The uniqueness of ERBB3 is that it has long been shown to be kinase-inactive because of amino acids substitutions within the conserved kinase domain compared with the other members in this family. Thus, ERBB3 functions allosterically as a catalytic partner to activate the other family members [69, 70].

A recent study showed that the kinase domain of ERBB3 was able to bind ATP and to trans-autophosphorylate its intracellular region by forming a heterodimer between the intact intracellular kinase domain and the kinase domain [64]. However, compared to other EGFR family members, kinase activity of ERBB3 was significantly decreased and no phosphorylation ability on exogenous peptides was shown. Moreover, Shi and colleagues demonstrated that although ERBB3 lacked several key conserved residues, it might employ a distinctive mechanism to transfer the phosphoryl group by using the existing kinase-like conformation instead of the catalytic base aspartate [64, 65]. A further study suggested that although the kinase activity of ERBB3 was far lower than the other members such as EGFR, it may be adequate to activate phosphatidylinositol-3-OH kinase (PI3K)/Akt pathway and contribute to the resistance of tyrosine kinase inhibitors (TKIs) [71]. Considering that ERBB3 may play a critical role in overcoming the resistance to EGFR or ERBB2 inhibitors in cancers, more detailed functional and mechanistic studies are still needed to focus on the interaction between family members and identification of unique substrates.

1.2.4.1.2 CASK

CASK is a pseudokinase belonging to the membrane-associated guanylate kinase protein family (MAGUK) which function as multiple domain adaptor (scaffolding) proteins [72]. CASK was originally described in *C. elegans* and in *Drosophila* (Caki) [73, 74]. CASK is one of 10 subfamilies consisting of a PDZ domain, a catalytically inactive guanylate kinase (GUK) domain and a SH3 domain [75]. Resulting from its numerous domains, CASK acts as a scaffold protein and plays a role in protein organization. In mammals, it is located at cell junctions and synapses and is thought to play a critical role in brain development and central nervous system function [76, 77]. It can bind to more than 20 cellular proteins in different subcellular regions of neurons and form protein complexes for the interaction with the trans-membrane protein neurexin at pre-synaptic sites leading to downstream events [78]. Mutations lead to brain malformation, mental retardation and neonatal lethality [79]. In a single study, it was demonstrated that CASK functions as an active protein kinase that phosphorylates neurexins providing further evidence to suggest that at least some “pseudokinases” retain kinase activity in addition to scaffold properties [62].

1.2.4.1.3 ILK

Integrin-linked kinase (ILK) is a serine/threonine kinase which was originally discovered using a yeast two-hybrid screen system and found to interact with the cytoplasmic domain of $\beta 1$ integrin [80]. It has an N-terminal ankyrin repeat domain, a pleckstrin-homology (PH)-like domain and a C-terminal catalytic domain [81]. The ankyrin repeat domain binds to LIM found in scaffold protein domains of the particularly interesting new cysteine-histidine rich protein (PINCH) family. The C-terminal domain reacts with parvin forming the ILK-PINCH-Parvin (IPP) complex, which forms a scaffold with other factors [82]. ILK has a role to play in adhesion of cells by linking integrin (transmembrane receptor) to the actin cytoskeleton [82, 83].

The argument as to whether ILK is a pseudokinase or possesses kinase activity has recently been discussed in an excellent review by Hannigan *et al*, as there has been some debate in recent years relating to its true function [84]. In some studies, no substrates were found for ILK and it was considered to be a pseudokinase as it lacks the HRD and DFG motifs in its kinase domain which are required for phosphate group transfer [82]. Furthermore, the crystal structure of the kinase domain, particularly its molecular folds differ from those of active kinases [83]. However, ILK has been shown to phosphorylate a variety of different substrates,

including GSK3 and Akt [85]. Taking everything into consideration, it is most plausible that ILK is a true kinase and therefore requires re-classification, while it is likely to have a novel mode of activity resulting from changes to its catalytic domain.

ILK has many important functions in signal transduction pathways associated with cell survival, migration, cell adhesion and extracellular matrix, differentiation, cell survival and angiogenesis and is essential for embryonic development and tissue homeostasis, as deregulation leads to abnormal tissue development [81].

1.2.4.2 Pseudokinases that exert allosteric regulation and scaffolding function

1.2.4.2.1 STRAD α

STE-20-related adaptors α and β (STRAD α and STRAD β) isoforms (also known as STLK5 and STKL6), which belong to the STE20 protein kinase family, also lack kinase activity [57]. These pseudokinases are upstream components in signal-transduction pathways controlling a wide range of biological processes. The function of STRAD α is to allosterically activate the tumour suppressor Liver Kinase B1 (LKB1) in a complex with mouse protein 25 α (MO25 α) scaffolding protein, resulting in the activation of AMPK family members. Similarly, the STRAD β and MO25 β isoforms also stabilise LKB1 [86, 87]. Activation of AMPK leads to the regulation of cellular energy status, fatty acid synthesis, cell polarity and proliferation. The kinase domain structure of STRAD α consists of N and C lobes with a cleft between them making up the active site. The activation loop is found in the C terminal lobe and it is phosphorylated at specific sites when the kinase is in an active state, allowing substrate binding. In turn, LKB1 is activated by binding to STRAD α thus not relying on activation by phosphorylation. Direct interaction of their kinase domains, strongly suggests an allosteric mechanism of activation [88]. ATP and MO25 α binding to STRAD α changes its kinase domain to an active-like kinase conformation that is characterized by extending its A-loop, allowing STRAD α to bind LKB1. This results in the kinase domain of LKB1 to adopt an active kinase conformation, which is further stabilized by the binding of MO25 α to the A-loop of LKB1 [89]. Moreover, its cellular distribution is determined by STRAD α and MO25 α . Re-localization of LKB1 is induced by STRAD α from the nucleus to the cytoplasm [90]. Mutant forms of STRAD α that are unable to bind MO25 α and ATP fail to activate LKB1 [91, 92].

1.2.4.2.2 Tribbles family

Tribbles was first shown as a regulator in coordinating mitosis and morphogenesis during *Drosophila* development. Through promoting the degradation of Cdc25/String, tribbles blocked mitosis at a critical point to control cell cycle progression [93-95]. There are three mammalian trb isoforms (trb1, trb2 and trb3) homologues to the *Drosophila* tribbles and they all share the highly conserved kinase domain, which lacks catalytic residues.

Despite the conserved structure of the tribble family members, their actions differ: trb1 and trb2 are mainly involved in the development of leukemia, while trb3 is upregulated in non-haematopoietic malignancies. Dedhia *et al* generated mice using hematopoietic stem cells expressing all three tribbles isoforms separately. Only those overexpressing trb1 or trb2 developed acute myeloid leukaemia (AML). In addition, this data also showed that trb1 and trb2, but not trb3, have the ability of promoting the degradation of CCAAT/enhancer-binding protein- α (C/EBP α) by resulting in their differential functions in leukaemogenesis [96].

1.2.4.2.3 TRRAP

Transformation/transcription domain-associated protein (TRRAP), a large 434 kDa protein, is a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family that shares a high homology to the PI3Ks [97, 98]. TRRAP was originally discovered in 1998 where it was found to interact with c-Myc and E2F oncoproteins [99]. In humans, it is one of six family members, which also include mammalian target of rapamycin (mTOR), ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3-related (ATR), DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and suppressor of morphogenesis in genitalia (SMG-1) [100].

TRRAP has been reported to act as a scaffold protein for numerous regulatory factors including GCN5, p53, BRCA1, E2F and c-Myc [101, 102]. It acts as a mediator, being part of several different histone acetyltransferase (HAT) complexes [102-106]. In turn, these complexes act as a platform for different regulatory factors. Transcriptional profiling revealed that TRRAP knockdown affected numerous signalling pathways and cell cycle components. In one study TRRAP was associated with repair of DNA double-strand breaks [102]. Collectively, TRRAP plays a role in forming part of the complex necessary for *c-Myc* oncogene activation and is involved in the regulation of tumour suppressor gene *p53*.

1.2.4.3 Future perspectives

Our knowledge of this enigmatic family of protein kinases has increased in recent years where approximately 10% of these proteins have been classified as pseudokinases. Their initial classification was largely based on sequence analysis and the discovery that pseudokinases lacked at least one highly conserved amino acid from their kinase domain. It appears that pseudokinases can have different functions, having regulatory roles. Some provide a molecular platform, steering enzymes and their substrates into critical positions allowing cellular reactions to proceed, and others are able to activate kinases by allosteric changes of their “substrates”. In addition, some pseudokinases, although they have evolved “inactive” kinase domains, still exhibit catalytic activity by various unique mechanisms, indicating they are evolutionary counterparts of protein kinases. New experimental facts will in future allow a more precise classification of pseudokinases.

New insights gained from structure analysis of WNK, CASK and ERBB3 suggest that it would be interesting to re-evaluate the remaining pseudokinases to delineate possible novel mechanisms in regulating phosphorylation. Thus, uncovering the three-dimensional structure of pseudokinases will improve our understanding of the intramolecular interactions in signalling transduction and possibly classify some pseudokinases to active protein kinases. Re-instating known active domains within pseudokinases may shed more light on their functions. In addition, further evidence on elucidating the pathways they participate in and their protein partners they cooperate with may reshape our perspectives on their biological functions.

1.3 Kinase suppressor of Ras 1 (KSR1)

KSR1 was originally identified nearly 20 years ago as a novel protein kinase evolutionarily conserved in the Ras signalling pathway functioning between Ras and Raf in *Drosophila* and *Caenorhabditis elegans* [107-109]. Interestingly, in *C. elegans*, deletion of *KSR1* alleles repressed activated Ras showing by a blockade of inducing multiple vulvas, but did not affect wild-type counterparts. Similarly, in *Drosophila*, defects in KSR1 inhibited the effect of activated Ras, but not activated Raf, indicating that KSR1 functions either downstream of Ras and upstream of Raf or in parallel pathways [110]. By comparing the sequences between mammalian and *Drosophila* KSR1, similarity through the kinase domain has been observed [107]. However, instead of a lysine at its subdomain II, which is required for kinase activity, an arginine residue is invariantly present in the mouse and human sequences. Owing to this fact, mammalian KSR1 are extensively referred to as a pseudokinase. Subsequently, the role of KSR1 as a scaffolding protein was revealed. Murine KSR1 (mKSR1) was first reported to cooperate with activated Ras to facilitate MEK and MAPK activation thereby promoting *Xenopus* oocyte maturation and cellular transformation [111]. In this report, the authors concluded that the activity of KSR1 is Ras-dependent and it can also interact with Raf. However, KSR1 is not a Raf kinase, but a crucial part of this signalling complex, even though it can bind to Raf. In addition, KSR1 was observed to translocate from the cytoplasm to the plasma membrane in the presence of activated Ras, during which it forms a complex involving Raf-1, MEK1 and 14-3-3. This in turn leads to the activation of Raf-1, which is independent of its enzymatic activity, highlighting its role as a scaffold in the MAPKs pathway [112, 113]. Meanwhile, the notion KSR1 as an active kinase was described from the finding that tumour necrosis factor alpha (TNF α) and ceramide were shown to significantly increase KSR1 autophosphorylation and its capacity to phosphorylate and activate Raf-1 [114]. Emerging evidence thus supports dual activity of KSR1 as an active kinase as well as a scaffold protein in the Ras-Raf-MAPK pathway. Here the recent advances in unravelling its structure and function, especially in cancers are discussed.

1.3.1 KSR1 structure

The KSR family proteins are conserved from invertebrates to mammals, and two mammalian members namely KSR1 and KSR2 have been identified [107, 115]. From sequence comparison, the similarity of amino acids between KSR1 and KSR2 is approximately 61% [115]. As the aim of this project is about the function of KSR1, we will focus mainly on

KSR1. It is closely related to the Raf kinase family containing five conserved areas (CA) named CA1-CA5 [107].

CA1 domain is located in the N-terminus of 40 amino acids exclusive to KSR1 but lacking in KSR2 [115]; CA2 is a proline-rich domain with unrevealed function; CA3 is a cysteine-rich atypical C1 motif mediating its membrane recruitment with phospholipids and this domain is essential for its activity to enhance Ras-dependent signalling and localization to the plasma membrane [113, 116]. The function of KSR1 C1 domain was further studied through elucidating its three-dimensional solution structure using nuclear magnetic resonance (NMR) [116]. This domain is similar to the C1 domains of Raf-1 and PKC γ , although not functionally interchangeable between them. Moreover, it does not directly bind to Ras indicating its distinctive role comparing with Raf-1 and PKC γ [116]. CA4 is a serine/threonine-rich region with an FXFP motif that interacts with ERK [111, 117, 118]; CA5 missing the conserved lysine residue at subdomain II required for phosphorylation is kinase-like domain [107, 117]. Notably, in *Drosophila* and *C. elegans*, the lysine residue is conserved and exists within the kinase domain for phosphotransfer reaction [107-109]. One recent study identified another domain composed of a coiled coil (CC) and a sterile α motif (SAM) at the N-terminus of KSR1. In fact, by binding directly to micelles and bicelles, the CC-SAM domain can guide KSR1 to certain sites at plasma membrane upon growth factor stimulus. Furthermore, nuclear magnetic resonance spectroscopy and *in vitro* assays demonstrated that the helix α of the CC motif is essential for modulation of membrane binding, indicating that combined with the atypical C1 domain, CC-SAM domain is indispensable for KSR1 cellular translocation [119]. A schematic depiction of KSR1 structure is shown in **Figure 7**.

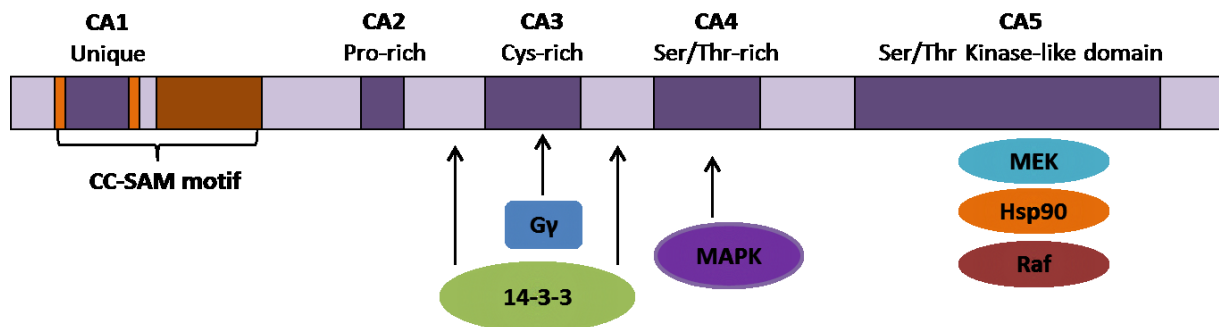


Figure 7 Schematic depiction of KSR1 structure and identified binding sites.

Five conserved domains are identified in the KSR family members: CA1, a domain unique to the KSR1 protein; CA2, a proline-rich region; CA3, a cysteine-rich domain; CA4, a serine/threonine-rich region, and CA5, a putative kinase domain (Therrien et al, 1995 and Morrison et al, 2001). 14-3-3 proteins bind to two phosphoserine residues (Ser297 and Ser392) located on either side of the CA3 domain (Cacace et al, 1999). G protein γ subunits (G γ) bind to the CA3 domain (Bell et al, 1999). ERK1/2 (MAPK) interacts with an FxFP motif in the CA4 domain (Cacace et al, 1999; Muller et al, 2001). MEK1/2 (MEK), Raf, Hsp90 and p50cdc37 (Hsp90/p50) interact with the CA5 domain (Denouel-Galy et al, 1998; Yu et al, 1998; Stewart et al, 1999 and reviewed by Morrison, 2001). A new identified domain comprises of a coiled coil (CC) and a sterile α motif (SAM) required for membrane binding (Koveal et al, 2012). This figure is modified from published work from Morrison, 2001 and Koveal et al, 2012.

1.3.2 KSR1 as a scaffold protein

The function of KSR1 as a scaffold protein has been well described in numerous cellular contexts upon various stimulations, although in some cases contradictory downstream effects have been reported comparing to the initial notion of a positive regulator in the Ras-Raf-MAPKs pathway. Murine KSR1 was first described to cooperate with activated Ras to stimulate *Xenopus* oocyte maturation and cellular transformation through elevated MEK and MAPK activity [111]. In the same study, authors also observed an interaction between mKSR1 with Raf at the plasma membrane in a Ras-dependent manner [111]. This finding was also verified by another study showing that KSR1 increased Raf activity via its cysteine-rich CA3 domain binding to Raf [113]. Cell fractionation assays revealed that KSR1 can form a complex with 14-3-3 in both the membrane and cytoplasmic fractions of cell lysates and with Raf in the membrane fraction, through which it can positively modulate the Ras pathway in vertebrate organisms [112].

Conversely, an inhibitory effect of KSR1 on Ras-induced transformation was reported. KSR1 was shown to cooperate with MEK1/2, but not Ras or Raf-1, and this interaction resulted in an inhibition of proliferation of embryonic neuroretina cells induced by Ras [120]. Yu and

colleagues described that KSR1 can directly interact with MEK1 and ERK, and expression of KSR1 in COS-7 cells inhibited serum-stimulated MAPK activation [121]. Moreover, ectopic expression of KSR1 decreased MAPK activation induced by growth factors, activated Ras and Ras effectors in fibroblasts [122]. KSR1 was also able to suppress EGF and Ras-induced phosphorylation of ternary complex factors (TCF), which are substrates of MAPK. However, no effects of KSR1 on activation of MAPK itself were observed [123]. Bell and colleagues demonstrated that KSR1 can cooperate with G-protein $\beta\gamma$ subunits, which mediates the translocation of KSR1 to the plasma membrane. In COS-7 cells, expression of wild-type KSR1 repressed G-protein $\beta\gamma$ -induced MAPK activation [124]. Furthermore, KSR1 was shown to form a multimolecular signalling complex comprised of heat shock protein 90 (HSP90), HSP70, HSP68, p50CDC37, MEK1/2 and 14-3-3 in human embryonic kidney 293T cells. This study also reported that KSR1 did not affect the MEK activity or activation but the membrane fraction of MEK, indicating the scaffolding role of KSR1. KSR1 was also shown to function as a scaffold protein in the Ras-Raf-MAPK pathway *in vivo* [125]. In fact, the MAPK activation was decreased in KSR1-null mice and high-molecular-weight complexes comprising KSR, MEK, and ERK were not detected in the depletion of KSR1 [125].

Interestingly, it appears that the expression levels of KSR1 determine its biological effects on the downstream signalling pathways, which further supports its role as a scaffold protein in modulating various pathways. Indeed, at a higher expression, KSR1 acted to decrease Ras-induced activity, but increased the Ras signalling pathway when lower levels were expressed in *Xenopus* oocytes [126]. A following study in *KSR1*^{-/-} mouse embryo fibroblasts (MEFs) confirmed this observation when re-introducing a titration of KSR1 abundance. With low KSR1 expression, ERK activity was enhanced as well as cell growth and RasV12 oncogenic transformation. Conversely, all these effects were markedly reduced with higher KSR1 expression highlighting the importance of expression levels of a molecular scaffold in the modulation of cellular signalling [127]. Moreover, the similar phenomenon of its scaffolding role was also implicated in regulating adipogenesis in MEFs [128]. While removal of KSR1 inhibited adipogenesis *in vitro*, re-introduction of low levels of KSR1 restored adipogenesis. Optimal levels of KSR1 were shown to be necessary for organizing activation of ERK and ribosomal protein S6 kinase (RSK) with C/EBP β synthesis essential for the adipogenic program. However, increased levels of KSR1 were able to suppress adipogenesis through indirectly regulating peroxisome proliferator-activated receptor gamma (PPAR γ) [128].

More studies have revealed a dynamic interplay that KSR1 is involved in as an allosteric regulator in Ras-Raf-MAPKs signalling cascade. In non-stimulated cells, Cdc25C-associated kinase 1 (C-TAK1) phosphorylates KSR1 at S392, which creates the binding site for 14-3-3, resulting in sequestration of KSR1 in the cytosol, where it constitutively interacts with MEK and ERK. Upon growth factor stimulation, activated Ras triggers the dephosphorylation of KSR1 at S392 by protein phosphatase 2A (PP2A), leading to the release of 14-3-3 from its binding sites. This in turn allows KSR1 to translocate to the cell membrane, where KSR1 forms a complex with Raf, MEK and ERK. KSR1 thus potentially enhances the phosphorylation of Raf, MEK, and ERK, facilitates the upstream signalling transduction as well as multiple substrates phosphorylation in the cytoplasm and nucleus, and through this manner regulates proliferation, cell cycle and apoptosis [129-131] (**Figure 8**). A series of follow-up studies added complexity to the molecular scaffold KSR1-involved signalling regulation. Douziech and co-workers reported that connector enhancer of KSR (CNK) together with Hyphen (HYP), a novel SAM domain-containing protein, can recruit KSR1 to Raf before signal activation. Subsequently, the interaction of this ternary can enhance KSR1's capability to facilitate the Ras-dependent Raf-activation indicating a potential role of KSR1 as an allosteric modulator of Raf catalytic function [132]. CK2 was identified as an element of the KSR1 scaffold complex contributing to Raf kinase activity [133]. The basic surface region of the KSR1 atypical C1 domain was shown to be required for CK2 binding to KSR1 and interruption of KSR1/CK2 complex caused a significant inhibition of growth factor induced Raf activation [133]. The Ras effector protein impedes mitogenic signal propagation (IMP) was capable of deactivate KSR1 or disturbing KSR1 homooligomerization and B-Raf/c-Raf hetero-oligomerization in order to regulate assembly of mitogenic signalling complexes and MAPK activation [134, 135].

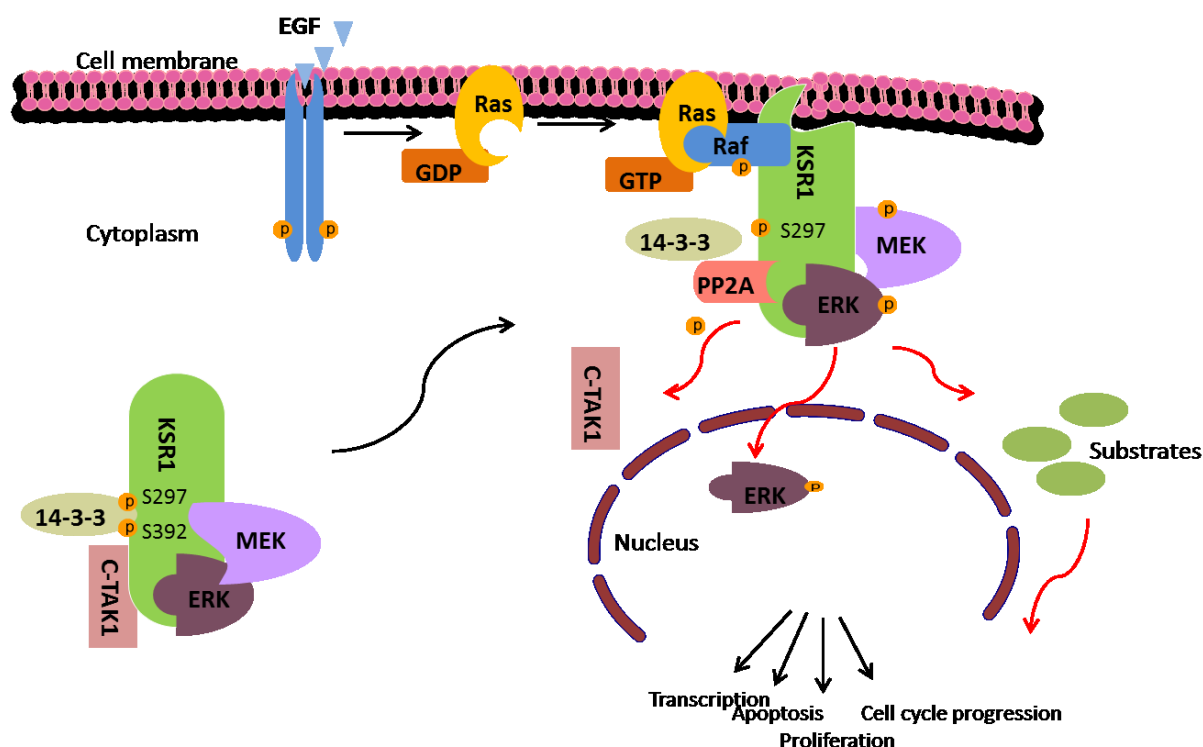


Figure 8 Schematic model for the function of KSR1.

In quiescent cells, KSR1 remains in the cytoplasm with 14-3-3 binding at S297 and S392 phosphorylated by C-TAK1. KSR1 also constitutively interacts with MEK and ERK. Upon stimulation by growth factors such as EGF, KSR1 is dephosphorylated by PP2A at S392 resulting in the release of 14-3-3 and exposure of the CA3 domain on KSR1. This leads to cell membrane translocation where KSR1 forms an active complex with phosphorylated Raf, MEK and ERK. The activated ERK can in turn phosphorylate various substrates in the cytoplasm and nucleus regulating proliferation, cell cycle and apoptosis. This figure is modified from published work from Zhang et al, 2012

Recent advances in KSR1 structure further demonstrated its involvement in multiple scaffold complexes. Upon growth factor stimulation, a functional CA1 region of KSR1 was reported to be required for the assembly of a ternary complex with B-Raf and MEK, resulting in activation of MEK and ERK. This in turn allows ERK to phosphorylate KSR1 and B-Raf on several feedback Ser/Thr P-sites, which leads to the dissociation of KSR1 from the plasma membrane [136]. This finding again highlights the scaffolding role of KSR1 in the signalling dynamics, where KSR1 behaves as both a stimulator and an inhibitor in ERK cascade activation regulating the intensity and duration of the signalling. Using structural biology analysis, Rajakulendran and colleagues generated the KSR1-Raf side-to-side heterodimer. In forming this complex, KSR1 can directly regulate Raf activation, despite missing catalytic

function. Moreover, mutations in the dimer interface of KSR1 suppressed the activity of Raf [137]. A-kinase-anchoring protein (AKAP)-Lbc was lately identified to coordinate with KSR1 in forming the central parts of the MAPKs signalling. Together with KSR1, the complex enables signal transmission from Raf, through MEK, and on to ERK1/2 efficiently [138]. The scaffolding role of KSR1 is further supported by the observation that Raf inhibitors can trigger KSR1/B-Raf complex formation, which is dependent on conserved dimer interface residues in each partner but not relying on interaction of B-Raf to activated Ras [139]. In addition, this study demonstrated that KSR1 was able to compete with C-Raf for inhibitor-induced dimerisation to B-Raf, thereby modulating downstream ERK signalling [139].

1.3.3 KSR1 as an active kinase

A body of evidence supports the notion of KSR1 as an active kinase, despite suggestions of an incomplete catalytic domain. Zhang and colleagues first showed mouse KSR1 is competent to autophosphorylate as a 100 kDa membrane-bound molecule [114]. Moreover, TNF α and ceramide can induce the kinase activity of KSR1 to autophosphorylate and transactivate Raf-1 at Thr269 *in vivo* and *in vitro* [114]. Shortly, EGF was shown to be able to stimulate the kinase activity of KSR1 in a two-stage *in vitro* kinase assay when overexpressing Flag-tagged KSR1 in COS-7 cells. Its enzymatic activation of c-Raf-1-dependent activity was only observed in full-length KSR1, but not kinase inactive and C- and N-terminal deletion mutants. In addition, endogenous KSR1 isolated from A431 cells expressing high levels of activated EGF receptor was also shown a constitutive enrichment of catalytic activity [140]. Direct phosphorylation of Raf-1 by KSR1 was demonstrated to be essential for TNF α -induced ERK1/2 activation in intestinal epithelial cells, revealing a protective role of KSR1 in the inflammatory process, which required its regulatory kinase activity [141, 142]. Conversely, by overexpressing a dominant-negative kinase-inactive KSR1 in young adult mouse colon cells, decreased cell survival and increased apoptosis were observed upon TNF α treatment [143]. Moreover, kinase-dead KSR1 also suppressed nuclear factor κ B (NF- κ B) activation and ERK/MAPK activation in the same context further supporting its regulatory role as a kinase [143].

New biochemical techniques such as protein purification and molecular modelling have enabled further aspects of KSR1 function to be probed, generating new perspectives on its catalytic function. In order to demonstrate that KSR1 is an active kinase for Raf-1, Zafrullah

and colleagues generated a specific monoclonal antibody against phospho-Raf-1 (Thr269). Using this antibody, the immuno-purified KSR1 was shown to be capable of phosphorylating FLAG-Raf-1 and kinase-dead FLAG-Raf-1(K375M) but not Raf-1(K375M/T269V), altered with a Thr269 to valine substitution [144]. The authors further purified KSR1 to homogeneity and confirmed that KSR1 can phosphorylate BSA-conjugated Raf-1 peptide at Thr269 [144]. Consistently, recombinant wild-type KSR1, but not kinase-inactive KSR1, can autophosphorylate on its serine residues, phosphorylate myelin basic protein (MBP) as a generic substrate, and directly activate MEK1 through phosphorylation, regulating cell survival in response to TNF α [145]. In addition, the authors overexpressed FLAG-tagged wild-type KSR1 (+KSR1) and KSR1 (+D683A/D700A) in KSR1^{-/-} colon epithelial cells and observed that only FLAG immunoprecipitates from cells encoding wild-type KSR1 were able to phosphorylate MEK1, suggesting MEK1 is an *in vitro* substrate of KSR1 [145].

Furthermore, in order to distinguish between the scaffold and kinase function of KSR1, Hu and co-workers generated a mutant (A587F) by adding a bulky phenylalanine at the ATP binding pocket of KSR1 to impair ATP binding. However, this mutant can still maintain a closed, active conformation, but is unable to interact with ATP. Indeed, the KSR1 mutant was not able to phosphorylate MEK, although it can constitutively bind to its partner as a scaffold. On the other hand, the wild-type KSR1 was shown to phosphorylate MEK induced by c-Raf, indicating a requirement of an active kinase activity in this process [146]. At the same time, structural and biochemical studies were utilized to understand the interaction between KSR2 and MEK1. Crystal structure analysis of the kinase domain of KSR2 illustrated that the side-to-side interaction between KSR2 with MEK1 is through their respective activation segments and C-lobe α G helices. Upon ATP bound to KSR2 catalytic site, KSR2 was shown to phosphorylate MEK1 by *in vitro* assays and chemical genetics [147]. Moreover, BRAf can form a side-to-side heterodimer with KSR2 (KSR2-BRAf), in which BRAf can allosterically increase the kinase activity of KSR2. The KSR2-BRAf complex thereby facilitates MEK phosphorylation by BRAf achieved via the KSR2-involved transmission of a signal from BRAf to allow conformational adjustment of MEK for phosphorylation [147]. The notion highlighting that KSR coordinates with a regulatory Raf molecule in *cis* to trigger a conformational alteration of MEK, assisting phosphorylation of MEK by another enzymatic Raf molecule in *trans* sheds new light on our understanding the function of KSR family members [147].

1.3.4 Regulation of KSR1

Given the fact that KSR1 is an important player in the Ras-Raf-MAPKs pathway, extensive efforts have been devoted to study the mechanisms regulating KSR1 protein as well as associated biological effects. Amongst these are post-translation modifications of KSR1 and the protein-protein interactions with other regulatory molecules.

1.3.4.1 Phosphorylation and protein-protein interaction

Phosphorylation was first shown to affect KSR1 function and its interaction with binding partners. Cacace and colleagues identified five phosphorylation sites, two of which (Ser297 and Ser392) are constitutively phosphorylated, whereas phosphorylations of Thr260, Thr274, and Ser443 are induced by activated Ras. Indeed, in serum-starved medium, Ser297 and Ser392 of KSR1 stayed constitutively phosphorylated, regulating its binding capacity to the 14-3-3 family proteins. Upon Ras activation, three other sites Thr260, Thr274, and Ser443 were shown phosphorylated by MAPK and subsequently were blocked by MEK inhibitor PD98059 [126]. Similarly, Volle and co-workers demonstrated that murine KSR1 remained phosphorylated in cells and in an immune complex on at least 10 distinct serines and threonines [148]. In this study, all the phosphorylated sites identified are restricted to the N-terminus of KSR1 excluding the kinase domain. Interestingly, wild-type mouse KSR and the kinase-inactive KSR (R598M) proteins were shown to have identical phosphorylated sites in intact cells and in the immune complex, indicating that the kinase domain of KSR1 does not contribute to the phosphorylation of KSR1 itself [148].

As shown here, phosphorylation plays an important role in subcellular localization of KSR1 resulting in regulation of associated signalling pathways. In quiescent cells, C-TAK1 was shown be able to constitutively interact with KSR1 and phosphorylate KSR1 on Ser392 leading to 14-3-3 binding and cytoplasmic sequestration of KSR1 [129]. Upon growth factor activation, de-phosphorylation of KSR1 by serine/threonine protein phosphatase PP2A was shown to trigger a disassociation of KSR1 and 14-3-3 allowing KSR1 membrane translocation and regulation of Ras-Raf-MAPKs signalling [131]. On the other hand, Brennan and colleagues first observed nuclear fluorescence of KSR1 in cells expressing GFP-KSR1. Subsequently, the authors showed that of the phosphorylated sites identified, mutations of Thr274 and Ser392 caused the re-shuttle of nuclear localisation of KSR1 indicating a role of phosphorylation in modulating KSR1 nucleocytoplasmic distribution [149]. Moreover, when fluorescent forms of KSR1 and MEK were co-transfected in cells, it was shown that each

protein facilitated the cytoplasmic localization of the other highlighting the notion that MEK interaction is also crucial in regulation of KSR1 subcellular distribution [149]. Intriguingly, NM23-H1, a tumour metastasis suppressor, was described to associate with KSR1 in 293T cells and MDA-MB-435 breast carcinoma cells. *In vitro* kinase assays demonstrated that autophosphorylated recombinant Nm23-H1 phosphorylated KSR1 on Ser392 and Ser434, whereas Ser392 mutation of KSR1, either alone or in combination with Ser297 inhibited the phosphorylation ability of Nm23-H1 [150]. Moreover, NM23-H1 overexpression prompted an increase in Hsp90 binding to cytoplasmic KSR1 and enhanced degradation of KSR1 protein in MDA-MB-435 breast carcinoma cells [151]. Tso and colleagues reported that an elevated level of Nm23-H1 and -H2 and an increase in phosphorylated KSR in the nuclear fractions were observed in HEK293 cells stable expressing regulator of G-protein signalling 19 (RGS19), indicating RGS19 as a new regulator of Nm23H1/2-mediated KSR1 phosphorylation [152]. Recently, one report showed that VRK2A can interact with KSR1 and MEK1 by forming a stable high molecular size (600-1,000 kDa) complex on the surface of the endoplasmic reticulum (ER). VRK2A silencing led to a dissociation of these high molecular size complexes, whereas VRK2A overexpression increased KSR1 abundance in the particulate fraction and inhibited ERK1/2 incorporation into the complex upon EGF stimulation [153]. The finding that KSR1 complex regulated by VRK2A in the ER can affect MAPK signalling output provides a new mechanism of KSR1 subcellular modulation and its associated downstream effects.

In addition, Yoder and co-workers reported that high Zn^{2+} levels can enhance KSR1 phosphorylation in mammalian tissue culture cells, which was not affected by EGF stimulus and a MEK inhibitor (PD98059) [154]. The observed effect on KSR1 phosphorylation was also shown to be Zn^{2+} specific as adding high concentrations of Co^{2+} , Cu^{2+} , Mg^{2+} or Ca^{2+} did not achieve the same result [154]. To better understand the biological effects of KSR1 phosphorylation in regulating the Ras-Raf-MAPKs pathway, mutations of Ser392 and Thr274 of KSR1 (KSR1.TVSA) were generated and used for multiple functional analyses [155]. KSR1.TVSA resulted in constant ERK activation and cell cycle progression with either platelet-derived growth factor (PDGF) or EGF treatment. It was also shown that KSR1.TVSA cells grew faster and a higher density than wild-type KSR1 cells, and KSR1.TVSA was more stable than wild-type KSR1 [155].

Very recently, Sibilski and colleagues reported Tyr728 as a novel regulatory phosphorylation site of KSR1, which is phosphorylated by lymphocyte-specific protein tyrosine kinase (LCK,

a member of SRC family) suggesting a potential link between SRC kinases and MAPK signalling via KSR1 [156]. Moreover, the authors revealed that Tyr728 is essential in retaining the conformation of the KSR1 kinase domain obligatory for its interaction with MEK. Phosphorylation of Tyr728 initiated rearrangements of conformation in the MEK interface and reorganized structural elements required for catalytic activity of KSR1. Phosphorylation of Tyr728 may negatively affect Raf-mediated MAPK activation resulting in an inhibition in cell proliferation [156].

1.3.4.2 Transcription regulation

1, α 25-dihydroxyvitamin D₃ (1,25D), was reported to be able to selectively increase *KSR1* gene expression in human leukaemia HL60 cells, whereas other inducing agents including retinoic acid or dimethyl sulfoxide did not [157]. Importantly, the authors revealed that a vitamin D responsive element (VDRE) exists in the promoter region of the human *KSR1* gene, allowing vitamin D receptor (VDR) to bind, in a 1,25D-dependent manner [157]. This finding suggests a new mechanism of regulating *KSR1* in the transcriptional level as well as an involvement of KSR1 in 1,25D-induced monocytic differentiation of human myeloid leukaemia cells [157]. The same group showed that oncoprotein Cot1 inhibited KSR1 and 1, 25-Dihydroxyvitamin D₃-induced differentiation of human AML cells [158]. In fact, silencing Cot1 resulted in a marked increase in KSR1 mRNA in HL60 and U937 cells and overexpression of Cot1 caused a decrease in KSR1 abundance induced by 1,25D [158].

1.3.5 The physiological role of KSR1

Expression profiles of KSR1 protein and gene were determined by western blotting and RT-qPCR in the normal adult mouse. Western blotting showed that KSR1 protein is higher expressed in the adult brain and lower expressed in bladder, ovary, testis, and lung, whereas it is not detected in other adult tissues. However, *KSR1* transcripts are identified in all adult tissues except liver shown by RT-PCR [159]. Another study later also reported that *KSR1* mRNA is expressed in neutrophils, macrophages, thymus, and spleen in mice [160].

In addition, a splice variant of murine KSR1 (B-KSR1) highly expressed in brain-derived tissues was characterized and it is evident in mouse brain throughout embryogenesis [161]. Introduction of B-KSR1 in PC12 cells caused an increase in nerve growth factor (NGF)-induced neuronal differentiation and EGF-induced neurite outgrowth, which required MEK binding and activation [161]. This finding demonstrates that B-KSR1 may be important in

Ras-dependent neuronal differentiation and involved in regulating the mature central nervous system [161].

To study the *in vivo* function of KSR1, mouse models of homozygous deletion for KSR1 were generated [125, 162]. The data revealed *KSR1*^{-/-} mice were viable and phenotypically normal, and did not develop any major defects, although a distinctive hair follicle phenotype was observed in one study, similar to *EGFR* deficient mice [125, 162]. Consistently, both groups reported that there were no main histological abnormalities of the major organs in young mice or in adults up to 1 year of age, and the weight, behaviour, and brood size were also unaffected in the mice [125, 162]. However, in comparison to MEFs from wild-type *KSR1* mice, MEK and ERK activation was attenuated in MEFs derived from *KSR1*^{-/-} mice, as well as in KSR1 depleted T cells [125].

KSR1 was also shown to be essential for maximal ERK activation induced by UV light, ionizing radiation, or the DNA cross-linking agent mitomycin C (MMC) [163]. Of note, in the absence of KSR1, cells underwent but did not recuperate from MMC-induced G2/M arrest, whereas restoration of KSR1 enabled *KSR1*^{-/-} cells to return to cell cycle upon MMC treatment [163]. Cells expressing a mutated form of KSR1 unable to bind ERK showed no recovery from MMC-induced cell cycle arrest, indicating a requirement of the KSR1-ERK interaction. Moreover, only cells expressing KSR1 were capable of re-programming from MMC-induced cell cycle arrest, as in *KSR1*^{-/-} cells constant activation of ERK was not adequate to promote cell cycle re-initiation upon MMC-treatment [163].

1.3.6 KSR1 in cancers

1.3.6.1 KSR1 in solid tumours

As KSR1 plays an essential role in the Ras-Raf-MAPKs module, which is one of the well-known oncogenic pathways, studies are beginning to explicate its biological characteristics in different cancers. Polyomavirus middle T-antigen (MT) can trigger oncogenic potentials in a Ras-dependent manner through the involvement of the adaptor protein, Shc, and the lipid kinase, phosphatidylinositol 3-kinase [164, 165]. An initial study reported that in MT⁺KSR1^{+/-} mice, the onset of tumour formation was 35±8 days whereas the tumour was not detected until 65±9 in MT⁺KSR1^{-/-} mice [125]. This indicates that the absence of KSR expression can slow tumour formation although it might not be indispensable in this mouse model. Interestingly, Lozano and colleagues showed that in a v-Ha-Ras-mediated skin cancer mouse model, KSR1 contributed to tumourigenesis as 70% of Tg.AC transgenic mice in a KSR1^{+/+} background

developed papillomas, whereas only 10% in a KSR1^{-/-} background demonstrated papillomas [162]. It has been noticed that the discrepancies from the two models may be due to different genomic background and distinctive tumourigenesis signals. As in Tg.AC transgenic mice, c-Raf-1/MAPK cascade is predominant requiring KSR1, MT-driven mammary tumourigenesis relying on SRC and PI3K is KSR1 independent. This finding also supports the notion that KSR1 is selectively required for some forms of oncogenic Ras-transduced MAPK mediated tumourigenesis [162].

Subsequent work from the same group reported that targeting KSR1 by continuous infusion of phosphorothioate antisense oligonucleotides (AS-ODNs) reduced tumour growth of K-Ras-dependent human PANC-1 pancreatic and A549 NSCLC xenografts in nude mice, suggesting inhibition on KSR1 as a potential therapeutic target in Ras-dependent malignancies [166]. To further assess the correlation between ODNs uptake and therapeutic effects, an ultrasensitive noncompetitive hybridization-ligation enzyme-linked immunosorbent assay (NCHL-ELISA) was developed to quantify KSR1 ODNs uptake in plasma and tumour tissues [167]. The ELISA assay was performed to measure plasma and tumour KSR1 ODN levels in PANC-1 derived pancreatic cancer mice and showed that tumour suppression associated with KSR1 ODN uptake in tumour tissues not in plasma [167]. Introduction of KSR1 into KSR1^{-/-} MEFs induced cell proliferative and oncogenic potential and removal of KSR1 inhibited Ras(V12)-dependent transformation [127]. Furthermore, the increase in transformation by ectopic expression of KSR1 required an optimal concentration range of KSR1, as it started to inhibit the proliferative action and activity of Ras-Raf-MAPKs cascade at higher levels [127]. KSR1 is also involved in cell sensitivity to cisplatin-induced apoptosis. Specifically, in comparison to wild type MEFs, KSR1 depletion in MEFs correlated with reduced ERK activation by cisplatin and elevated resistance to cisplatin-stimulated apoptosis. Expression of KSR1 into KSR1^{-/-} MEFs and MCF7 cells increased ERK activation and sensitivity to cisplatin [168].

A screen to characterize KSR1 expressions and the effects on drug sensitivity using a collection of cancer cell lines and the NCI60 anticancer drugs supported an important role of KSR1 in defining cellular sensitivity [169]. The results showed that KSR1 expression levels varied nearly 30-fold difference between the highest and lowest expressing cell lines in the NCI60 [169]. In comparison to KSR1^{-/-} MEFs, reintroduction of KSR1 expression in MEFs enhanced resistance of cells to tunicamycin and cytochalasin H [169]. In EGFR-driven A431 squamous cell carcinoma (SCC) cells, targeting KSR1 was shown to down-regulate elevated tissue factor (TF), which is involved in tumour cell survival, growth, and angiogenesis [170].

Conversely, overexpression of KSR1 in A431 cells resulted in an increase in TF expression and tumourigenicity in mice and this effect was suppressed by pan-EGFR inhibitor, CI-1033, suggesting the fact that the oncogenic potentials of KSR1 and TF are EGFR dependent [170]. In addition, KSR1 was also observed to play an important role in targeting Ras-mediated radioresistance. Indeed, silencing KSR1 or overexpressing kinase-inactive KSR1 abolished ionizing radiation-induced Raf/MEK/ERK2 cascade activation, improved the cytotoxic effect of radiation, gained radiosensitization and facilitated clonogenic death in A431 cancer cells [171]. In mouse models, pharmacologic deletion of KSR1 by infusing KSR1 AS-ODN resulted in radiosensitization in EGFR-dependent A431 SCC and in oncogenic K-Ras-driven A549 human NSCLC [171].

Additionally, through modulation of peroxisome proliferative activated receptor gamma coactivator 1 alpha (PGC1 α) and oestrogen-related receptor alpha (ERR α), KSR1 was demonstrated to induce oncogenic Ras-dependent anchorage-independent growth [172]. Interestingly, the authors showed that KSR1 required the presence of H-RasV12 to regulate the expression of metabolic regulators PGC1 α and ERR α [172]. In comparing *KSR1* cDNA expression between normal endometrium and endometrial carcinoma (ECC), Llobet and co-workers performed a cDNA array and reported that *KSR1* was up-regulated in ECC, validated using RT-qPCR. Moreover, tumour tissue microarray (TMA) confirmed that KSR1 protein was significantly elevated in endometrial carcinoma in comparison to normal endometrium [173]. Deletion of KSR1 by small hairpin RNA led to decrease in both proliferation and anchorage-independent cell growth of endometrial cancer cells. Importantly, KSR1 silencing re-sensitized TRAIL- and Fas-induced apoptosis in resistant endometrial cell lines, which might partly be regulated through down-regulation of FLICE-inhibitory protein FLIP [173]. Recently, our kinome screen using RNA interference to identify novel regulators of ER α revealed a potential involvement of KSR1 in breast cancer, which led to a main part of my PhD study on its function and associated signaling in breast cancer [174].

1.3.6.2 KSR1 in non-solid tumours

The work to study the rationale for deltanoids in therapy for myeloid leukaemia revealed a KSR1-involved signalling associating with MAPK and C/EBP. Upon deltanoid activation of VDR, KSR1 expression and the activity of MAPK pathway were up-regulated, partially contributing to monocytic differentiation of human myeloblastic HL60 cells [175]. As discussed in the previous part, oncoprotein Cot1 was shown to down-regulate KSR1

expression and 1,25-Dihydroxyvitamin D³-induced differentiation of human AML cells, thus providing extended information of KSR1's involvement in non-solid tumours [158].

KSR1 was recently indicated to contribute to activating protein tyrosine phosphatase, non-receptor type 11 (PTPN11)-induced granulocyte-macrophage colony-stimulating factor (GM-CSF) hypersensitivity, the characteristic phenotype of juvenile myelomonocytic leukaemia (JMML). To address this, bone marrow progenitors from WT and KSR1^{-/-} mice expressing WT Shp2, and *PTPN11* gain-of-function mutants (Shp2E76K, or Shp2D61Y) were assessed. Increased KSR1 activation and enhanced interaction between KSR1 and active ERK were observed in Shp2D61Y- and ShpE76K-expressing macrophage progenitors. While KSR1 loss partially suppressed Shp2E76K-induced GM-CSF hypersensitivity, the similar effect was not observed in Shp2D61Y-expressing cells suggesting a distinctive role of KSR1 in GM-CSF hypersensitivity in different mutants [176]. In bone marrow-derived mast cells from KSR1-deficient mice, KSR1 depletion caused a decrease in kit-ligand-mediated proliferation and degranulation, as well as a decline in migration [177]. It was shown that effect of KSR1 loss on migration was through activation of p21-activated kinase, a modulator of F-actin polymerization key to mast cell migration [177].

Furthermore, KSR1 depletion in B cells resulted in a reduction in proliferation and an increase in cytokine deprivation-induced apoptosis. Although overexpression of Myc restored the proliferation capability of KSR1-null B cells, the inactivation of KSR1 in Myc overexpressing mice led to an enhancement in B cell apoptosis as well as an impediment in the onset of B cell tumourigenesis, further suggesting that KSR1 modulates the cooperation of Ras/MAPK signalling pathway and Myc to drive oncogenic transformation and lymphoma development [178]. Together, these results indicate a significant role of KSR1 in various cancers and it might be a potential therapeutic target in Ras-dependent cancers. However, in breast cancer, which seems to have few Ras mutations, the role of KSR1 remains to be thoroughly investigated.

1.3.7 KSR1 in other biological processes

1.3.7.1 KSR1 in immune system

Several lines of evidence demonstrate that KSR1 is an important regulator of immune functions. In particular, KSR1 null mice displayed impairment in MEK and ERK activities, thus, resulting in a marked reduction in T cell proliferation [125]. Moreover, KSR1 overexpression suppressed the DP to SP transition during positive selection in reaggregate

fetal thymic organ culture (rFTOCs) and structural analysis of KSR1 displayed that an interaction between KSR and MEK was required for the action of KSR overexpression in this system, supporting that KSR coordinates Ras signalling to ERK activation during positive selection of T cells [179]. To address the role of KSR1 in ERK activation upon proinflammatory stimuli and its function in physiological inflammatory process *in vivo*, Fusello and colleagues first showed that a defect in ERK activation by osmotic shock or after stimulation with proinflammatory cytokines or LPS was observed in KSR1-deficient [160]. The results from arthritis development after arthritogenic antiserum induction revealed that in comparison to KSR1 wild-type mice, KSR1-null counterparts displayed a significant decrease in the ankle and forepaw swellings [160].

Several studies have documented the protective role of KSR1 against cytokine-induced apoptosis in intestinal epithelium inflammatory conditions [142, 143]. Initial *in vitro* studies showed that inactivation of KSR1 resulted in a decrease in survival and an increase in apoptosis of TNF-treated cells through modulating NF- κ B activation, cIAP2 expression and MAPK kinase activity, supporting KSR1 as an essential regulator for TNF-stimulated survival pathways in intestinal epithelial cell lines [143]. To examine the function of KSR1 in regulating intestinal cell fate during cytokine-mediated inflammation, KSR1-deficient mouse model was generated [142]. The authors showed that KSR1 depletion caused an increased susceptibility to chronic colitis and an enhancement of TNF-induced apoptosis in mouse colon epithelial cells *in vivo*, correlated with an inability to initiate antiapoptotic signals including Raf-1/MEK/ERK, NF- κ B, and Akt [142]. The aforementioned effects can be inverted by introducing wild-type KSR1, but not kinase-inactive further confirming a potential requirement of a catalytic activity of KSR1 [142].

Lin and colleagues described that MAPK activity is digital upon T-cell-receptor stimulation and the system output of the MAPK cascade in T cells is highly fluctuated and modulated by upstream signalling of the MAPK module, thus regulating various biological functions [180]. However, it appears that although KSR1 was shown to modulate the threshold required for MAPK activation in T cells, it did not affect the fundamental system outputs [180]. Filbert and co-workers examined thymocyte selection in KSR1-deficient mice and showed although negative selection in the HY model was marginally diminished in KSR1-null mice, positive selection in two different TCR transgenic models, HY and AND, was normal. These results suggest that KSR1 may be essential for full ERK activation in thymocytes but not for thymocyte selection [181]. In addition, Gringhuis and colleagues demonstrated that KSR1

was involved in forming a signalosome complex together with LSP1, CNK and the kinase Raf-1, consequently coordinating the C-type lectin DC-SIGN tailored cytokine production in response to distinct pathogens [182].

As for natural killer (NK) cells, it was shown that the loss of KSR1 in NK cells perturbed NK-cell cytolytic capacity. Upon T cell activation, KSR1 was recruited to NK lytic synapse that in turn regulated the localisation of active ERK to the synapse [183]. *KSR1^{-/-} interleukin 10 (IL10)^{-/-}* double knockout mice were shown to be susceptible to an early manifestation of colitis by the age of 4 weeks [184]. Additionally, elevated expression of interferon gamma (IFN- γ) in T cells was detected in the double knockout mice and the inhibition of IFN- γ was able to reduce the extent of severity in colitis [184]. Moreover, KSR1 has been shown to be a protective factor against bacterial infection. In fact, KSR1-deficient mice were highly vulnerable to pulmonary *Pseudomonas aeruginosa* infection, comparing to wild type mice. Mechanistically, upon infection, KSR1 was capable of inducible nitric oxide synthase (iNOS) and Hsp90 recruitment resulting in increased iNOS activity and NO release to eliminate bacteria [185]. In lipopolysaccharide (LPS)-induced acute lung injury model, a very similar response to LPS was observed in both KSR1-deficient and wild-type mice, suggesting a dispensable role of KSR1 in LPS-induced ERK activation in alveolar macrophages [186]. Interestingly, a recent study has established a relationship between MAPK and mTOR pathways through the involvement of KSR1 in T cells. Although KSR1 modulates mTOR complex 1 (mTORC1) activity, KSR1 deficiency has no obvious effects in mTOR -dependent T cell differentiation [187].

1.3.7.2 KSR1/2 in metabolism

Both KSR members are involved in the maintenance of cellular metabolism in which metabolic abnormalities were evident in KSR1 and 2 deficient mouse models. Phenotypically, *KSR1^{-/-}* mice exhibited adipocyte hypertrophy while *KSR2^{-/-}* counterparts were obese and glucose intolerant [128, 188]. As for the role of KSR1 in adipogenesis, the initiation of adipocyte differentiation relied indirectly on suitable amounts of KSR1 that orchestrated the temporal coordination of Raf/MEK/ERK and RSK signalling, and in this manner, controlled the activities of adipogenic transcription factors such as C/EBP β and PPAR γ [128].

KSR1 has been recently implicated in regulating glucose tolerance and insulin sensitivity via the interplay between KSR1 and MARK2. Klutho and colleagues reported an elevated glucose disposal rate in response to exogenous insulin, increased glucose tolerance, and

resistance to diet-induced obesity were observed in mice lacking MARK2 [189]. Interestingly, disruption of KSR1 in MARK2-null mice reversed the increased sensitivity to exogenous insulin resulting from MARK2 removal. As shown, the mode of action in which MARK2 acts as a negatively modulator in insulin sensitivity relies on the direct phosphorylation on S392 of KSR1 [189]. Furthermore, KSR1 and its associated PP2A/KSR1/ERK signalling have been shown to play a role in pravastatin normalized contraction induced by ET-1 in aortic smooth muscle in type 2 diabetic rats [190].

Comparing to a potential involvement of KSR1 in metabolism regulation, KSR2 seems to be an essential modulator in energy expenditure and insulin sensitivity supported by several elegant studies below. Costanzo-Garvey and colleagues have revealed a direct interaction between the two KSR members and AMPK, a prominent energy sensor for metabolic processes [188]. The disruption of KSR2-AMPK interaction prevented AMPK activation and phosphorylation that is imperative to induce fatty acid oxidation and regulate glucose uptake. Mechanistically, in wild-type KSR2^{+/+} mice, KSR2 can cooperate with AMPK to suppress acetyl-CoA carboxylase (ACC) and increase the expression of PGC1 α -dependent OXPHOS genes, and consequently repression of ACC can thus inhibit malonyl CoA synthesis a negative regulator of CPT1. Hence, increased CPT1 activity and OXPHOS gene expression can facilitate the oxidation of fatty acids and decrease their storage as triglycerides (TG) [188]. However, KSR2 loss can result in an impairment of AMPK function, a reduction of OXPHOS gene expression, and deregulation of ACC activity, eventually causing obesity [188]. Of note, a recent study has revealed that KSR2 controls tumour metabolism and cell growth in an AMPK dependent manner. Particularly, the forced expression of KSR2 in KSR1 null MEFs resulted in augmented proliferation and induction of anchorage independent growth. On the other hand, the introduction of AMPK restores the transformed phenotype and tumour metabolic activities upon KSR2 depletion [191].

To investigate the role of KSR2 in humans and identify *KSR2* variants in obese individuals, a comprehensive study was performed by sequencing 2,101 individuals with severe early-onset obesity and 1,536 controls [192]. Pearce and colleagues identified multiple rare variants in *KSR2* in individuals with severe early-onset obesity, which can potentially cause impaired Raf-MEK-ERK signalling or decreased the interaction between KSR2 and AMPK. Furthermore, when transfecting *KSR2* variants in cells, impaired glucose oxidation and palmitate-stimulated fatty acid oxidation (FAO) were observed in comparison to wild-type

KSR2 counterparts [192]. These findings highlight a fundamental role of KSR2 in obesity, insulin resistance, and impaired cellular fuel oxidation.

Collectively, these studies have underlined the significance of KSR members in regulating cellular metabolism and energy homeostasis and KSR1/2 could be potential therapeutic interventions in metabolic disorders, such as obesity.

1.3.7.3 KSR1 in central nerve system

Several studies have investigated the function of KSR1 in regulating associated signalling in the nervous system. Shalin and colleagues first revealed an important role of KSR1 in coordinating hippocampal signal transduction as well as in synaptic plasticity and memory formation [193]. In this study, shortages in contextual and cued associative fear conditioning, passive avoidance, and the Morris water maze were observed in KSR1-depletion mice, as well as decreased theta burst stimulation (TBS)-induced long-term potentiation (LTP) indicating a potential cellular mechanism for the observed behavioral phenotype [193]. Mechanistically, the authors showed that the action of KSR1 is through specifically enhancing activation of a membrane pool of ERK that is triggered by PKC activation but not by cAMP/PKA dependent pathway, revealing a new mechanism of KSR1-regulated ERK activation in specific learning and plasticity paradigms [193].

Szatmari and colleagues studied the contribution of KSR1 associated ERK1/2 activation in neutral survival signalling. In cultured rat cortical neurons, it was shown that brain-derived neurotrophic factor (BDNF) increased KSR1 interaction with activated ERK1/2, whereas KSR1 depletion using a short hairpin RNA decreased ERK1/2 activation induced by BDNF and reduced the BDNF protection against camptothecin (CPT)-mediated apoptosis, but not trophic deprivation-induced apoptosis. On the other hand, KSR1 overexpression enhanced BDNF protection against CPT-induced apoptosis [194]. A similar observation was shown in cortical neurons upon another neuroprotective stimulus, forskolin, which is a cAMP-elevating drug. As reported, Forskolin increased ERK1/2 activation in a PKA dependent manner, accompanied by an increase in association between KSR1 and active ERK1/2, while the inhibition effect of Forskolin on CPT-induced apoptosis was dependent of KSR1 and ERK1/2 [194]. These findings reveal a crucial role of KSR1 in the anti-apoptotic activation of ERK1/2 by BDNF or cAMP/PKA signalling.

Moreover, it has been shown that KSR1 and its phosphorylation by downstream kinase ERK1/2 contribute to maintaining physiological levels of synaptic plasticity in hippocampal

neurons. Canal and co-workers first showed that KSR1 was phosphorylated by ERK1/2 at multiples sites in HEK239T cell and further characterization of the KSR1 phosphorylation sites revealed a feedback regulation of ERK1/2 activity by KSR1. In fact, ERK1/2 activity was considerably decreased after 20 and 60 min and depleted after 4 h of EGF treatment in control or KSR1 wild-type cells, whereas KSR1 mutants on the feedback phosphorylation sites endured a constant activation of ERK1/2, even at 4 hours, indicating a role of feedback phosphorylation of KSR1 in controlling ERK1/2 signalling [195]. Furthermore, electrophysiological recordings in hippocampal neurons expressing wild-type or feedback-deficient KSR1 revealed that the potentiation of excitatory postsynaptic currents was restrained by KSR1 feedback phosphorylation highlighting the function of feedback phosphorylation of KSR1 as a keeper to prevent excessive ERK1/2 signalling in the postsynaptic compartment [195].

1.3.8 Closing remarks

Comprehensive insights regarding the structural and functional studies of KSR1 have started to redefine our previous knowledge, as it is becoming obvious now that the original classification of KSR1 as a pseudokinase may not be representative of its exact biological behaviour. Advances made in the last few years have changed our perspectives in the functions of KSR1, whereas a more complicated conception of KSR1 as a scaffold protein and an active kinase is established. Nevertheless, numerous gaps remain to be filled in order to understand the complexity of KSR1 biological actions. First of all, physiological substrates of KSR1 and its catalytic activity need to be identified and further validated in differential cellular contexts, as well as its subcellular localization where it performs associated functions. Moreover, as a scaffold protein, it is crucial to determine its binding partners in dynamic scenarios since the subsequent interaction will ultimately contribute to various biological outcomes. Finally, the inquiry as to whether KSR1 is an attractive therapeutic target in malignancies that are confined to be Ras-dependent tumours requires further clarification.

1.4 Tyrosine kinases (TKs)

TKs are enzymes that catalyse the transfer of phosphate from ATP to tyrosine residues in substrates. This group lies in the largest branch in the human kinome phylogenetic tree, which is comprised of 90 members [27]. TKs are divided into two main classes: RTKs and CTKs. Receptor TKs are transmembrane proteins with a ligand-binding extracellular domain, a membrane spanning segment and a catalytic intracellular kinase domain, whereas non-receptor TKs lack transmembrane domains and are normally localized in the cytosol, the nucleus, or the inner surface of the plasma membrane [56]. Like all the other canonical protein kinases, the catalytic domains of all TKs have a bilobar structure, with an N-terminal lobe binding ATP and magnesium, a C-terminal lobe enclosing an activation loop, and a cleft between the lobes to which polypeptide substrates bind [196].

1.4.1 Receptor tyrosine kinases (RTKs)

In the human kinome, there are 58 known RTKs, which are categorized into 20 subfamilies (**Figure 9**) [27, 197]. These subclasses are classified based on sequence similarity and characteristic structural features such as two cysteine-rich repeat arrangements in the extracellular domain of monomeric subclass I receptors, disulfide-linked heterotetrameric $\alpha_2\beta_2$ structures with similar cysteine-rich sequences in subclass II receptors, and five or three immunoglobulin-like repeats in the extracellular domains of subclass III and IV receptors, respectively [196]. Due to their structural characteristics, the ligand binding domain and the kinase activity of RTKs are isolated by the plasma membrane. Thus, receptor activation induced by extracellular ligand binding must be transduced through the membrane barrier to activate the intracellular domain functions [196].

1.4.1.1 Activation of RTK extracellular domain by ligands

With recent advances in structural studies of RTKs, our knowledge and understanding have developed from the classic mechanism for ligand-induced dimerization to diverse regulations of their activation by growth factor ligands. The original conceptual idea is that the activation of RTKs and associated signalling potentials is through ligand-induced dimerization [198]. In the absence of ligands, some RTKs are monomeric and disabled with an inactive conformation of their kinase domains. Upon ligands binding to the extracellular domain, a subsequent conformational alteration is induced resulting in receptor oligomerization, which stabilizes a specific contact between adjacent cytoplasmic domains and disrupts the

autoinhibitory interaction. This eventually triggers an activation of catalytic function essential for activating multiple downstream signalling pathways [196, 197].

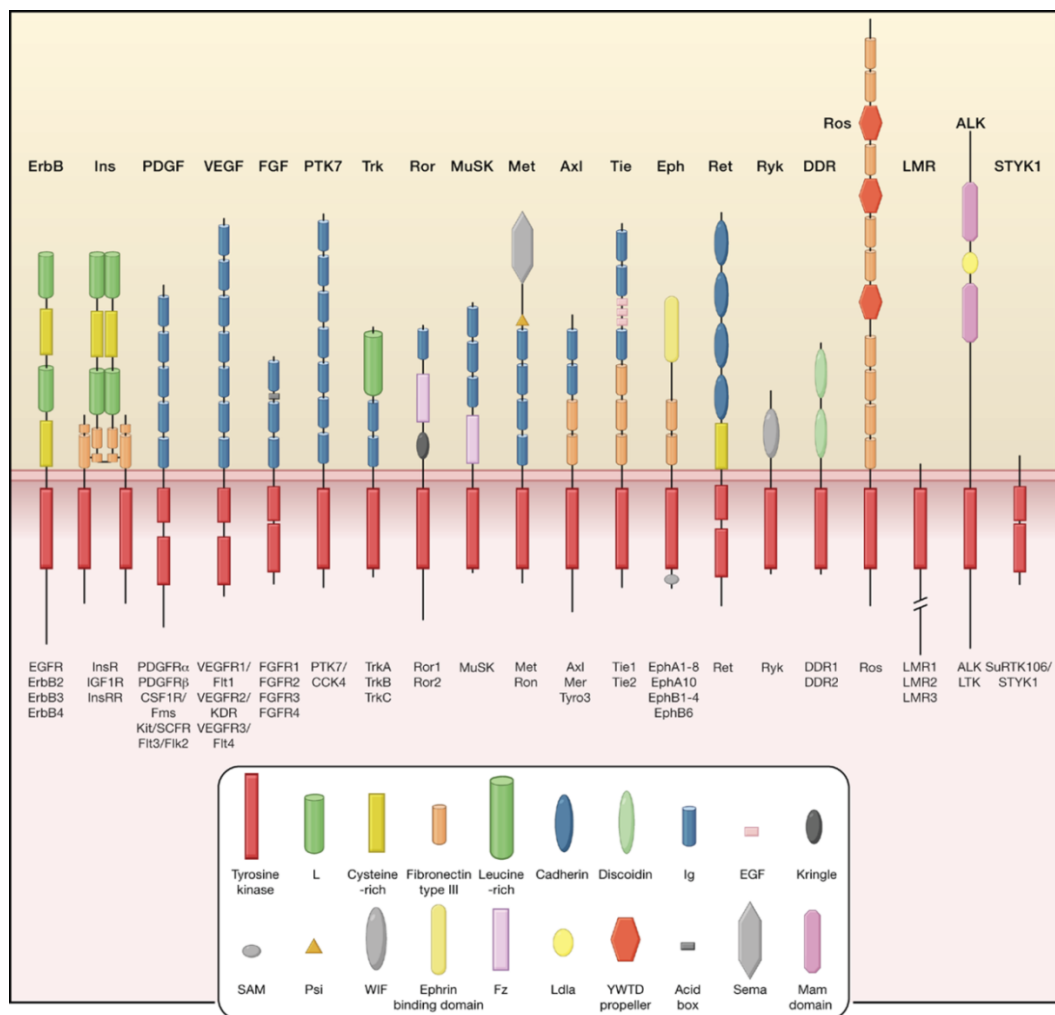


Figure 9 Receptor Tyrosine Kinase Families.

In the human kinome, there are 58 identified RTKs, which contain 20 subfamilies. Illustrated are schematic structures presented with the family members listed beneath each receptor. Structural domains in the extracellular regions, identified by structure determination or sequence analysis, are marked according to the key. The intracellular kinase domains are shown as red rectangles. Abbreviations of the prototypic receptors: EGFR, epidermal growth factor receptor; InsR, insulin receptor; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor; CCK, colon carcinoma kinase; EphR, ephrin receptor; Axl, a Tyro3 PTK; TIE, tyrosine kinase receptor in endothelial cells; RYK, receptor related to tyrosine kinases; DDR, discoidin domain receptor; Ret, rearranged during transfection; ROS, RPTK expressed in some epithelial cell types; LTK, leukocyte tyrosine kinase; ROR, receptor orphan; MuSK, muscle-specific kinase; LMR, Lemur; STYK1, serine/threonine/tyrosine kinase 1. This figure is adapted from published work from Lemmon and Schlessinger, 2010.

Evidence from solved crystal structures of the ligand-binding domains upon RTKs bound to their relevant ligands strongly supports this ligand-modulated dimerization in many cases, such as the Flt1-vascular endothelial growth factor (VEGF) receptor [199, 200], the NGF/neurotrophin receptor TrkA [201], Eph receptors [202], Axl [203], Tie2 [204] and the stem cell factor receptor KIT [205]. All of these studies reveal a consistent mechanism that a bivalent ligand cooperates simultaneously with two receptor molecules and effectively interplays them into a dimeric complex [197].

Furthermore, recent progress in uncovering more complete extracellular regions of RTKs has added into our understanding of the mechanisms for ligand-induced dimerization. Lemmon and Schlessinger have recently categorized and summarized the mechanism into two extremes and two intermediate cases [197]. As shown in **Figure 10A**, TrkA/NGF is an example of one extreme, where receptor dimerization is exclusively “ligand mediated” and the two receptors make no direct contact. As an alternative, the other extreme is shown by the example of ERBB/growth factor, whose dimerization is entirely “receptor mediated” and the ligand has no influence on forming the dimer interface (**Figure 10D**). On the other hand, in some cases such as KIT/stem cell factor and FGFR/growth factor, a combination of ligand-mediated and receptor-mediated components is shown as the main mechanism for dimerization (**Figure 10B and 10C**). Above all, dimerization of most RTKs falls into one of these four modes and more comprehensive studies of other RTK families are needed to provide new insight into this field.

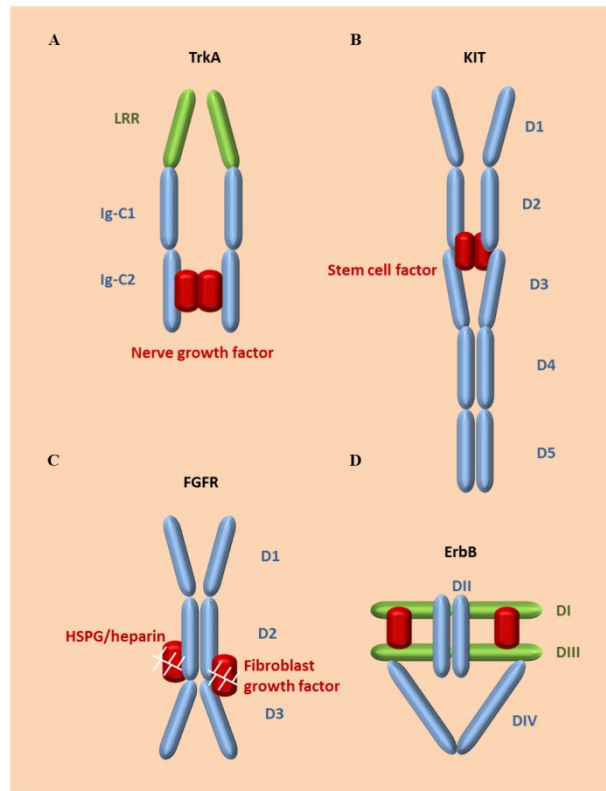


Figure 10 Receptor Tyrosine Kinase Dimerization.

In general, RTKs interact as dimers when ligands (red) bind to their extracellular regions. The bound ligand can form all, a portion, or none of the dimer interface, and it activates the receptors by stabilizing a specific relationship between two individual receptor molecules. **A.** A nerve growth factor dimer (red) interacts with two TrkA molecules without any direct contact between the two receptors (Wehrman et al, 2007). **B.** A stem cell factor dimer (red) interacts with two KIT molecules. Additionally, two Ig-like domains (D4 and D5), re-orienting upon receptor activation, interact across the dimer interface. Hence, a combination of ligand-mediated and receptor-mediated dimerization modes is used by KIT (Yuzawa et al, 2007). **C.** Two fibroblast growth factor receptor (FGFR) molecules interact with one another through the Ig-like domain D2, where the molecule heparin or heparin sulfate proteoglycans (white sticks) also bind (Schlessinger et al., 2000). In addition, each fibroblast growth factor molecule (red) associates with Ig-like domains D2 and D3 of both FGFR molecules. **D.** Dimerization of ErbB receptors is inter-mediated completely by the receptor. Through binding to two sites (DI and DIII) of the same receptor molecule, the ligand initiates conformational changes in EGFR from a previously blocked dimerization site in domain II. This figure is adapted from published work from Lemmon and Schlessinger, 2010.

1.4.1.2 Activation of intracellular kinase domain

The extracellular signalling of RTKs initiated by ligand-induced dimerization subsequently results in the activation of the intracellular tyrosine kinase domain (TKD), thereby transducing the activated signal down the cascade. Multiple excellent examples from KIT, FGFR, the insulin receptor and EGFR have illustrated a comprehensive picture of the activation mechanism that albeit varies in different cases [13, 197]. Although the crystal structures of activated TKDs in most RTKs with N-lobe and a C-lobe are similar, the inactive

forms of TKDs, as well as regulatory mechanisms vary significantly across receptors [206]. Owing to the diversities in regulating the catalytic activities of RTKs, it is therefore likely for them to be activated in a variety of distinctive manners. Generally, in each case, TKD is exclusively *cis*-autoinhibited by numerous intramolecular contacts specific for its receptor, whereas upon receptor dimerization induced by its ligand, *cis*-autoinhibition is unrestricted, thus promoting RTK activation [13, 197]. Here are some brief descriptions of the aforementioned examples.

Juxtamembrane (region between the transmembrane helix and the cytoplasmic kinase domain) autoinhibition is the most known regulatory mechanism for catalytic activity of RTKs. Although juxtamembrane region may vary substantially in length and sequence among RTKs, it frequently comprises tyrosine phosphorylation sites essential for regulating catalytic activity and downstream signalling protein recruitment [207]. For the past decade, biochemical and structural studies of RTKs have provided strong evidence to support this mechanism, represented by Eph family RTKs [208], MuSK [209], Flt3 [210] and KIT [211]. Indeed, in each of these examples, the juxtamembrane region contacts directly with certain parts of the TKD, such as the activation loop, and contribute to maintain an autoinhibited conformation. Subsequent phosphorylation of the tyrosine residues of this region promoted by ligand-induced dimerization releases juxtamembrane autoinhibition and stimulates receptor activation [197, 207]. Moreover, deletions of residues Lys550 to Glu561 or other mutations within the juxtamembrane region of KIT can interrupt the autoinhibitory interactions, prevent the region from maintaining the inactive kinase structure, thus causing a constitutive aberrant kinase activation [212].

The activation loop of the receptor TKD also contributes to RTK autoinhibition revealed by structural studies of the insulin receptor and FGFR1. In the case of insulin receptor, a crucial tyrosine (Tyr1162) in the activation loop of the insulin receptor TKD positions into the active site, and this direct contact with the active site of the kinase prevents both ATP and protein substrates from accessing to the active site. Therefore, the insulin receptor TKD maintains inactive in *cis* by its own activation loop. However, upon insulin binding to the receptor, phosphorylation of Tyr1162 and other two sites in TKD interrupts the *cis*-autoinhibitory interactions, which allows the receptor to adopt a conformational change from *cis*-autoinhibition to an active state for catalytic function [206, 213]. Similarly, tyrosines in the activation loop of FGFR1 act through an indirectly intramolecular interaction to stabilize the inactive conformation of the kinase, thus blocking the protein-substrate-binding site. Once

FGF binds to the receptor, tyrosines in the activation loop become phosphorylated, which subsequently disrupts the *cis*-autoinhibitory configuration enabling an active transformation for kinase activity [214, 215].

Another form of reversible *cis*-autoinhibition is regulated through a region in the C-terminal of kinases. For instance, tyrosine sites in the C-terminal tail of Tie2 prevent substrate access to the active site [216]. Autophosphorylation of the tyrosines sites in the Tie2 C-terminal tail may destroy these autoinhibitory interactions and hence activate Tie2 [197]. Furthermore, crystallographic and mutational studies of the EGFR/ERBB family have revealed an allosteric mechanism for activation of TKDs [66]. Interestingly, the asymmetric dimer interface formed between the C-lobe of one TKD, (called the “Activator”) and the N-lobe of the second TKD, (called the “Receiver”) is essential for EGFR activation. Thus, conformational changes in the N-lobe of the Receiver kinase induced by this direct contact disrupt the *cis*-autoinhibitory structure observed in the monomer. Eventually, the characteristic active configuration of the Receiver kinase is employed in the absence of phosphorylation of its activation loop. Likewise, another member of the family, ERBB4 kinase, adopts an asymmetric dimer conformation essentially identical to the mechanism observed for activation of the EGFR [217].

Above all, RTKs have developed a variety of *de novo* autoinhibitory mechanisms to inhibit basal-level activity. Upon binding to ligands, a set of characteristic conformational changes occur in multiple regions of RTKs including extracellular and intracellular domains, which subsequently allows RTKs to adopt an active form to perform their enzymatic activity and activate numerous downstream signalling pathways. The prevalence of mutations within the key domains such as juxtamembrane region in RTKs results in constitutively active forms of these receptors independent of ligand binding, contributing to many human diseases particularly malignancies [13, 212].

1.4.1.3 RTKs in cancers

RTKs are central regulators of intracellular signal-transduction pathways mediating almost all aspects of cellular processes, including proliferation, survival, differentiation, apoptosis, metabolism and motility [13]. Deregulation of RTK signalling by amplification (overexpression), mutations or alterations in structure can result in aberrant kinase activity contributing to various human disorders, especially malignant transformation. Considering their vital role in tumourigenesis, RTKs have become one of the most important groups of

drug targets, and plenty of inhibitors or targeted antibodies have been tested and some are already used in clinics [218].

In the human kinome, almost all 20 subfamilies of RTKs have been implicated in regulating various signalling pathways in different cancers and they mainly function as dominant oncogenes [13]. For example, EGFR is amplified/overexpressed or mutated in a number of tumours including breast, oesophageal, glioblastoma, liver, prostate, ovarian and NSCLC, and activated EGFR signalling represents one of the main oncogenic signatures in these cancers [44, 219-221]. In PDGFR family, PDGFR α is amplified or mutated in gastrointestinal stromal tumour (GIST), glioblastoma, ovarian carcinoma, whereas PDGFR β is mutated or overexpressed AML and CMML [13, 212, 222, 223]. Abnormal activity of FGFR family particularly due to genetic alterations has been extensively implicated in the development of a range of cancers. Amplifications in breast, ovarian, bladder, and mutations in melanoma, and translocations of FGFR1 in leukaemia are frequently observed [224-229]. FGFR2 amplifications and mutations are often seen in gastric, breast and endometrial cancer [230-232], whereas FGFR3 is overexpressed or mutated in bladder and cervical cancer [233-235].

Like the ones in the larger groups of RTKs described above, members of the smaller families have also been implicated in oncogenesis. For instance, anaplastic lymphoma kinase (ALK) has been shown as an important oncogenic driver in several solid and hematologic tumours and has thus emerged as a biomarker and potential therapeutic target for certain population of patients. Indeed, numerous alterations of ALK including amplification, mutation, translocation and structural rearrangement have been implicated in contribution to tumour initiation and development in lung, breast, oesophageal, colorectal cancer, neuroblastoma and anaplastic large cell lymphoma [236-239]. Moreover, activation of RET via gain-of-function mutations or through aberrant expression has been greatly implicated in heritable and sporadic tumours. Initial studies revealed that the germline oncogenic RET mutations are associated with multiple endocrine neoplasia type 2, which is an autosomal dominant multi-tumour syndrome characterized by early onset medullary thyroid carcinoma [240-242]. Recently, RET has been shown to be expressed in over 50% of pancreatic ductal carcinoma and more frequently in high grade and metastatic tumours [243, 244]. In breast cancer, increased RET activity can result in an increase in cell proliferation and survival, and elevated levels of RET expression *in vivo* in breast tumours have been linked with resistance to endocrine therapies [245-248].

Mounting evidence has supported the oncogenic potential of RTKs in a wide range of cancers. Here, an example of the most established RTK family-EGFR/ERBB in cancer is presented.

1.4.1.3.1 EGFR/ERBB family in cancer

There are four members in the ERBB family: EGFR, ERBB2, ERBB3 and ERBB4, which all share the conserved RTK's structural features with an extracellular ligand binding region, a single membrane spanning region, and a cytoplasmic tyrosine kinase domain [249]. In general, like other typical RTKs, activation of ERBB receptors is through ligand-induced dimerization. Upon ligand binding, the formation of homo- and heterodimers of ERBB receptors is induced and thus the intrinsic kinase domain is exposed and activated, resulting in phosphorylation of the regulatory tyrosine residues within the cytoplasmic tail. These phosphorylated residues in turn function as binding sites and initiate the recruitment of a variety of molecules essential for the activation of multiple intracellular signalling pathways [250, 251]. Based on their specificity of binding to different ERBB receptor, the EGF family ligands are divided into three groups: the first comprises EGF, TGF- α , amphiregulin and epigen, which attach specifically to EGFR; and the second includes betacellulin, heparin-binding EGF (HB-EGF) and epiregulin, which display dual specificity by binding both EGFR and ERBB4. The third group includes the NRGs, which are comprised of two subgroups based on their capacity to bind ERBB3 and ERBB4 (NRG1 and NRG2) or only ERBB4 (NRG3 and NRG4) [249, 252]. Notably, although none of the EGF ligands bind ERBB2, it can be activated through forming heterodimers with the other ligand-bound receptors in the family and in fact it is the preferred heterodimerization partner for the other activated ERBBs [253].

Of the four EGFR receptors, enormous evidence has demonstrated an influential contribution of EGFR, ERBB2 and ERBB3 to the initiation, development and progression of various types of human cancer, but the role of ERBB4 in oncogenesis remains to be determined. As the involvement of ERBB3 in cancer has been discussed in the previous chapter, this part will focus on EGFR and ERBB2.

Deregulation of EGFR by amplification, overexpression, mutation or structural alterations are frequent in human malignancies and associated with its oncogenic capacity. Elevated expression of *EGFR* gene is detected in numerous cancers including brain, breast, ovary, cervix, bladder, oesophagus, stomach, colon and lung, and is frequently associated with higher grade, higher proliferation and reduced survival. [56, 249, 250]. In addition to overexpression, activating EGFR mutations are crucial drivers of transformation and exist in

certain tumours. In particular, in NSCLC samples, about 10% of cases in North America and Western Europe, and ~30-50% of cases in East Asian descent harbour EGFR mutations [254-257]. Moreover, in both cell culture and transgenic mouse studies, some of the identified mutations such as exon 19 deletions, L858R, G719S and ins 770(NPG)-mutated EGFR proteins have been proven to be capable of inducing oncogenic transformation, resulting from increased EGFR kinase activity and the activation of downstream pro-survival pathways [258-261]. In tumours, constitutively activated EGFR recruit a variety of key regulatory proteins, such as Shc, Grb2 and phospholipase C γ (PLC γ), and stimulate numerous signalling pathways, including PI3K/Akt, mTOR, MAPK, Src kinase, and signal transducers and activators of transcription (STATs) [249]. Given its crucial part in the development and evolution of cancer, targeting EGFR and the associated signalling network has contributed dramatically to the treatment of a range of human cancers [249]. For instance, the first generation of EGFR TKIs, such as erlotinib and gefitinib, have been shown to be effective in a subpopulation of patients with NSCLC that contains mutations in the kinase domain of EGFR, even if acquired resistance occur eventually [262, 263]. Currently, coupled therapy or dual-targeting approach is under development to increase drug efficacy and overcome resistance, leading to better patient outcomes.

In 1987, *ERBB2* amplification was initially detected in a subset of breast tumours and was a significant predictor of both overall survival and time to relapse in patients with breast cancer [264]. To date, *ERBB2* amplification and overexpression have been found in some other cancers, including gastric, ovarian and salivary gland tumours [265-267]. *ERBB* mutations have also been identified in a small proportion of lung cancer and introduction of an *ERBB2* mutant with G776(YVMA) insertion in exon 20 resulted in activating signal transducers, phosphorylating EGFR, and inducing survival, invasiveness, and tumourigenicity [268, 269]. Moreover, the interaction between *ERBB2* and *ERBB3* has been recognised as most active *ERBB* signalling dimer and plays a fundamental role in *ERBB2*-mediated oncogenic signalling [67, 68]. In prostate cancer, the *ERBB2*-*ERBB3* dimer might regulate androgen receptor protein levels as well as its transcriptional activity in hormone-refractory prostate cancer, and might contribute to the proliferation signal in "androgen-independent" tumours [270, 271]. Like EGFR, activated *ERBB2* allows the recruitment of downstream signalling components and the formation of signalling complexes, resulting in aberrant activation of multiple crucial signalling pathways including the MAPK and the PI3K-Akt pathways in tumours [272]. The subsequent activation of the MAPK pathway increases the

transcription of cyclin D1, cyclin D2, Myc and VEGFA, which drive cellular proliferation, migration, differentiation and angiogenesis [250, 251]. On the other side, stimulation of the PI3K-Akt pathway triggers cellular signalling via GSK3 β , NF- κ B, p27 and BAD to facilitate cell survival, as well as inhibition of apoptosis and cell cycle control [273, 274]. Considering its central role in the tumourigenesis of many types of solid tumour, ERBB2 as a target for cancer therapy has been well validated and several agents have been developed against ERBB2 activation [249, 263, 272, 275]. One of the most known and effective is the humanised monoclonal antibody trastuzumab, which has already been approved by the FDA as part of standard treatment of ERBB2 amplified breast cancer patients [276]. Through interaction with the extracellular juxtamembrane region of ERBB2 (domain IV), Trastuzumab inhibits ERBB2 signalling activity, leading to suppression of downstream signalling pathways, which consequently cause anti-tumour effects, such as cell cycle arrest and reduction of angiogenesis [277-279]. Many new therapies including antibodies, inhibitors, and antibody-chemotherapy conjugates are under investigation to target against ERBB2 and its regulated signalling.

The examples of EGFR and ERBB2 highlight the significance of uncovering molecular mechanisms in the discovery of novel drugs, as well as the importance of collaboration between basic research and the oncology clinic. Overall, along with the progress of our understanding of the underlying pathogenesis, more effective, improved and personalized treatments will be available to patients.

1.4.2 Cytoplasmic tyrosine kinases (CTKs)

In the human kinome, 32 CTKs are identified to date and categorised into 10 subfamilies [13, 27]. In comparison to the sequences of RTKs, CTKs lack extracellular and transmembrane spanning regions, thus normally locate in the cytoplasm when inactivated. As shown in **Figure 11**, some members of this family have similar domain organization, including SRC, ABL and TEC, share a common core, which is well recognized by N-terminal SH3/SH1 domain, central SH2 domain and C-terminal kinase domain [280, 281]. SH2 recognizes and binds tyrosine phosphorylated sequences in a sequence-specific context and is therefore important in relaying cascades of signal transduction, whereas SH3 recognizes and binds to sequences typically rich in proline [280, 282]. These regulatory domains are essential for protein-protein interactions and coordination of key components of signalling pathways in response to extracellular signals.

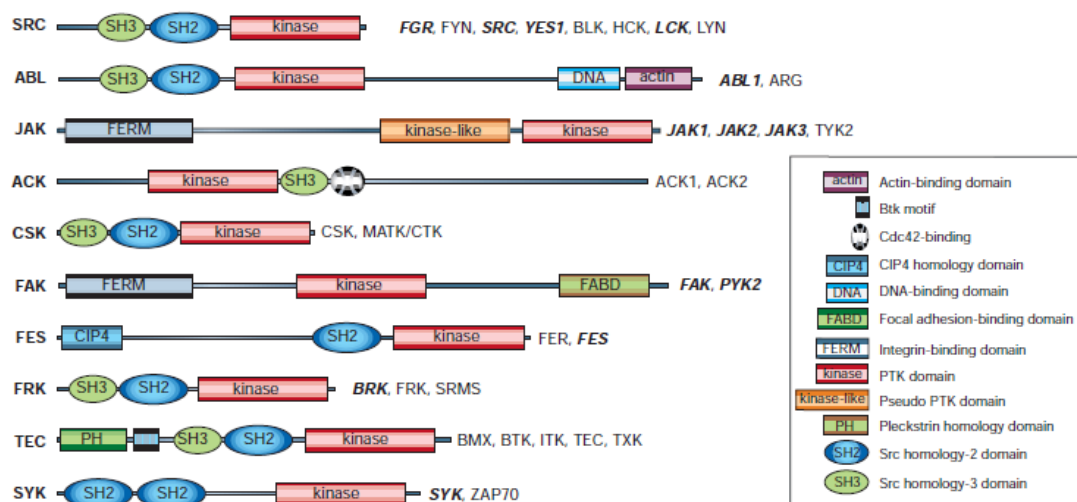


Figure 11 Cytoplasmic Tyrosine Kinase Families.

In the human kinome, there are 32 identified CTKs, which contain 10 subfamilies. Here are schematic structures presented with the family members. Structural domains, identified by structure determination or sequence analysis, are marked according to the key. The kinase domains are shown as pink rectangles. Abbreviations: ACK, activated CDC42 kinase 1; CSK, c-SRC tyrosine kinase; FAK, focal adhesion kinase; FRK, FYN-related kinase; SYK, spleen tyrosine kinase. This figure is adapted from published work from Blume-Jensen and Hunter, 2001.

1.4.2.1 Activation of CTKs

Generally, in unstimulated cells, CTKs such as ABL and SRC stay in an inactive state through tight control including intramolecular autoinhibition by various cellular inhibitory proteins and lipids in the cytoplasm [283, 284]. In response to stimulus, CTKs are activated and subsequent conformational changes occur, which are induced by various intracellular signals through disruption of an autoinhibitory state, including oligomerization and autophosphorylation upon recruitment to transmembrane receptors, and trans-phosphorylation by other kinases [56]. Here are a few examples of the identified mechanisms for activation of some CTKs.

1.4.2.1.1 Activation of c-SRC

c-SRC was the first identified cellular homologue of a viral oncoprotein, which belongs to the SRC family of CTKs [285]. The structural studies revealed that c-SRC consists of several important domains: SH4, unique domain, SH3, SH2-SH3 linker, SH2, SH1 (kinase domain) and C-terminal negative regulatory region [284]. The SH4 domain located on the N-terminal region is a myristoylation sequence crucial for membrane localization [33, 286]. As described

above, the SH3 and SH2 domain binds to proline rich sites and phosphorylated tyrosine sites respectively, both of which are important for protein-protein interactions [286, 287]. The SH1 domain contains the kinase domain and tyrosine residue 419, which can be autophosphorylated by active c-SRC [33].

In its inactive state, tyrosine residue 530 (Tyr530) of the C-terminal of c-SRC is phosphorylated and enables the interaction between the C-terminal with the SH2 domain, whereas the SH3 domain cooperates with the linker region between the SH2 domain and the N-terminal kinase lobe [13, 288]. This allows c-SRC to maintain an isolated conformation thus preventing a direct interaction of substrate proteins with the kinase domain. Numerous factors can contribute to the activation of c-SRC catalytic activity, such as dephosphorylation of phosphorylated Tyr530, SH2 binding and SH3 binding ligands [33]. Dephosphorylation of Tyr530 at the C-terminal region triggers its detachment from the SH2 domain, inducing an active conformation, which enables substrate protein access to the catalytic kinase site [289]. Ligand-activated RTKs allows specific tyrosine autophosphorylation sites to compete for c-SRC SH2 domains resulting in a dissociation of SH2 from phosphorylated Tyr530 [290]. All of these events consequently lead to elimination of inhibitory elements on the kinase domain and activation of c-SRC with an open state.

1.4.2.1.2 Activation of c-ABL

c-ABL belongs to the ABL family of CTKs and its aberrant activity associated with the oncoprotein breakpoint-cluster region (BCR)-ABL contributes to various forms of leukaemia in humans [280]. Similar to SRC family, the c-ABL protein contains a conserved kinase domain preceded by SH3 and SH2 domains, as well as other unique domains including DNA/actin binding domains at the C-terminal and a 'Cap' region located at the N-terminal important for recruiting the protein to membranes through myristoyl and/or palmitoyl groups. These structural configurations potentially determine a tight control of autoinhibition in cells and a brief description is presented here.

In inactive state, c-ABL is engaged in the membrane through a covalent N-terminal myristoyl group and is inhibited through an intramolecular interaction of the SH3 domain with adjacent proline residue, as well as a direct contact of an inhibitory membrane lipid, phosphatidylinositol-4,5-bisphosphate (PIP2) to the catalytic domain. These factors together repress the kinase activity of c-ABL by maintaining the ATP-binding and catalytic lobes of the kinase domain in closed conformation [280, 283]. c-ABL is activated through the

phosphorylation of regulatory tyrosine residues, including one in the activation loop and the other near the SH3 binding site by related kinases. Upon PDGF stimulus, PDGFR β is dimerized and autophosphorylated, subsequently generating binding sites for c-SRC and PLC γ to exert their activity. Activated c-SRC can phosphorylate c-ABL on the regulatory tyrosine 412, while PLC γ can hydrolyze and destroy the lipid inhibitor PIP2 [56, 283]. This in turn induces conformational changes of c-ABL allowing binding of the substrate and ATP to the active site for catalytic reaction.

1.4.2.1.3 Activation of Jaks

The Janus tyrosine kinase (Jaks) family has four members (Jak1, Jak2, Jak3 and tyrosine kinase 2 (Tyk2)). This family is composed of seven Jak homology (JH) domains, named JH1-JH7 from the carboxyl to amino terminus. Interestingly, JH1 is a typical tyrosine kinase domain whereas the neighbouring JH2 region is a pseudokinase domain serving regulatory functions of Jaks activity through binding to JH1 [291-293]. The amino terminus of Jaks contains an SH2 domain (JH3-JH4) and a Band-4.1, ezrin, radixin, moesin (FERM) homology domain (JH6-JH7). The FERM domain plays an important role in modulating interactions with transmembrane proteins such as cytokine receptors and is also implicated in positively regulating catalytic activity via binding the kinase domain [294-296].

Normally, Jaks constitutively interact with cytokine receptors on the cell surface. Upon cytokine binding, receptor dimerization occurs, which leads to an activation of Jaks through a conformational change in the receptor allowing phosphorylation of the two bound Jaks. Activated Jaks can in turn phosphorylate the cytoplasmic domain of the cytokine receptors, resulting in recruitment of SH2-domain containing proteins to the receptor complex, such as STATs whose activation are widely implicated in many cancers [297-300]. When STATs bind the phosphorylated receptor chains, Jaks can subsequently phosphorylate STATs, inducing the formation of stable STATs homodimers and heterodimers through interactions between SH2 domains and phosphorylated tyrosine residues. Activated STATs can then translocate to the nucleus where they activate transcription [297, 301, 302].

1.4.2.2 CTKs in cancer

Given their importance in various cellular processes, such as proliferation, apoptosis, angiogenesis and immune surveillance, deregulation of the kinase activity of certain CTKs has been comprehensively implicated in multiple oncogenic signalling in human malignancies.

Here are some examples of CTKs whose aberrant activation by mutations and other genetic alterations contribute to carcinogenesis.

1.4.2.2.1 Jaks

Jak members are highly associated with hematopoietic malignancies. For example, a gain-of-function mutation (V617F) in the JH2 domain of Jak2 correlates with myelo-proliferative neoplasia [303-306]. Jak1 mutations were also reported in patients with AML and ALL [307, 308]. In one study, approximate 18% of patients with the T cell precursor ALL were shown to have Jak1 mutations, which associates with poor response to therapy and overall prognosis. Three gain-of-function mutations identified were implicated in contributing to IL-9 independent resistance to apoptosis of T cell lymphoma BW5147 cells [307]. In addition, Jak3 pathway genes were shown to be elevated in B-lineage ALL and the increased expression of these genes correlates with steroid resistance and relapse [309].

The Jak family has also been suggested to play a role in solid tumour growth. In breast cancer, Jaks were shown to interact with SRC tyrosine kinase contributing in constitutive activation of STAT3. Inhibition of Jaks by tyrosine kinase selective inhibitors resulted in reduced growth and increased programmed cell death in breast cancer cells [310]. In prostate cancer, Jaks inhibitor, tyrphostin AG490, can suppress STAT3 activation and inhibit the growth of human prostate cancer cells. This indicates that Jaks-STAT3 pathway is crucial in the survival and proliferation of prostate cancer cells [311]. Furthermore, erythropoietin was capable of activating Jak2-STAT5 signalling leading to metastasis in head and neck squamous cell carcinoma [312]. The Jaks-STATs pathway is also involved in lung cancer. Gao *et al* showed that in EGFR constitutively activated human lung adenocarcinoma-derived cells, a pan-Jaks inhibitor (P6) significantly reduced STAT3 phosphorylation. Additionally, P6 suppressed tumourigenesis of human lung adenocarcinoma cells *in vitro* and *in vivo*. Results from this study conclude that STAT3 phosphorylation in lung adenocarcinoma is dependent on the IL-6/gp130/Jaks pathway [313].

Numerous reviews on the role of Jaks in cancer have been published recently [314-319]. Involvement of Jak kinases in the development of malignancies has resulted in a great number of inhibitors currently being evaluated in clinical trials [301, 320, 321].

1.4.2.2.2 c-ABL

The Philadelphia (Ph) chromosome, a t(9;22)(q34;q11) translocation, was first discovered as an abnormal, small chromosome associated with a specific type of leukaemia. This reciprocal translocation generates the oncogenic *BCR-ABL* fusion gene [322]. Subsequently, several lines of evidence support *BCR-ABL* as an oncogene that contributes to CML pathogenesis. In cultured cells, introduction of BCR-ABL has been shown to be capable of driving the transformation of mouse fibroblast cell lines, haematopoietic cell lines and primary bone-marrow cells [323, 324]. Similarly, in human CD34⁺ cells, ectopic expression of BCR-ABL has demonstrated several features of primary CML progenitors, including increased proliferation in primitive progenitor culture, reduced adhesion to fibronectin, and reduced chemotaxis to stroma-derived factor-1alpha [325, 326]. In addition to studies in cultured cells, several animal models of CML also prove its role in leukaemogenesis. A myelo-proliferative disorder (MPD) that resembles CML was shown to be developed when BCR-ABL was introduced in mouse bone-marrow cells using retroviral transduction and bone-marrow transplantation methods [327-329]. This was confirmed by another mouse model in which a murine stem-cell retroviral vector was used to express the *BCR-ABL* oncogene in haematopoietic stem/progenitor cells, and introduction of the transgene in the bone-marrow cells of mice induced a human CML-like disease with 100% efficiency [330, 331]. Moreover, studies in transgenic mice also showed that inducible expression of BCR-ABL in B-cell lymphocytic and megakaryocytic precursors contributed to the development of B-ALL and megakaryocytic myelo-proliferative syndrome [332, 333].

The kinase domain of ABL as well as other regulatory domains has been shown to be important for its capability of tumourigenesis. *In vivo* studies showed that mice that encode a mutant BCR-ABL (a point mutation in the ATP-binding site of ABL), did not develop leukaemia, indicating that the kinase activity is required for its function in leukaemogenesis [331]. Expression of another mutant ABL with deletion of the SH3 domain, which has increased tyrosine kinase activity, can transform both fibroblast and haematopoietic cell lines *in vitro* and induce a CML-like disease in mice [334, 335]. Furthermore, BCR-ABL has been implicated to interact with many important proteins, including STAT5, PI3K, PLC γ , Ras GTPase activating protein, FAK (also known as PTK2) and FES, which in turn activate multiple signalling pathways such as Ras/Raf/MAPK, PI3K/Akt and Jak/STATs and potentially contribute to oncogenesis [324].

1.4.2.2.3 Other CTKs in cancer

The SRC family kinases (SFKs) have been shown to be important for cell proliferation, survival, adhesion, invasion and angiogenesis during tumour development [284]. SFKs cooperate with many key mediators in multiple signalling pathways to regulate tumourigenesis. For example, SFKs interact with RTKs, including EGFR and VEGFR to influence cell proliferation and angiogenesis. Moreover, SFKs regulate gene expression through transcription factors such as STATs, as well as modulate cell adhesion and migration through interaction with integrins, actins, paxillin, and FAK [284]. In breast cancer, SRC interacts with ERBB2 to enhance cell growth and SRC activity is necessary for the metastatic potential of ERBB2-overexpressing cancer [336]. In lung cancer, it has been reported that about 50-80% of patients have elevated expression of SRC protein and/or increased kinase activity [337]. In prostate cancer, SRC signalling can stimulate cell growth upon androgen treatment, as well as contribute to androgen independent prostate cancer. The addition of SRC inhibitor into anti-androgen deprivation therapy for prostate cancer patients may improve therapeutic results [338, 339]. In head and neck cancer, expression of dominant active SRC in squamous cell carcinoma has shown an increase in growth and invasion in comparison to the vector controls [340].

FAK plays a critical role in tumour initiation, progression and metastasis. It is a main mediator of signal transduction by integrins and is involved in a range of other pathways associated with growth factors, G-protein-coupled receptor agonists, cytokines, and other inflammatory mediators [341, 342]. In breast cancer, it is well documented that FAK is overexpressed in breast cancer patients as well as in cell lines. Moreover, elevated FAK expression and activity are frequently associated with poor prognosis and progression to metastasis in breast cancer specimens [343-345]. In polyoma middle T oncoprotein (PyMT) induced breast cancer model, FAK-deficient PyMT-transformed cells exhibited an arrest of growth and a diminished activity of invasion and metastasis, highlighting its involvement in Ras- and PI3K-dependent mammary tumour initiation [345]. In addition to its high levels in breast cancer, FAK has also been shown to be overexpressed in some other cancer types, including brain, cervical, colorectal, gastric, lung, and head and neck, indicating that FAK is a common oncogene and a potential therapeutic target in a broad range of cancers [341, 342].

1.5 Aims

Although great efforts have been devoted to study the involvement of KSR1 in certain tumour types, its biological functions and associated signalling pathways implicated in breast cancer have remained largely undefined. Therefore, in this work, we sought to provide a comprehensive picture illustrating its role in breast cancer and our aims were to:

- identify a KSR1-regulated phospho-proteomic profile in breast cancer cells using a stable isotope labelling of amino acids in cell culture (SILAC-based) approach;
- characterize the functional portrait of the KSR1-regulated proteins identified from SILAC using Gene Ontology analysis and reveal new KSR1-associated signalling networks;
- examine the expression of KSR1 in a large breast cancer patient cohort (n>1000) and evaluate its clinical association and prognostic significance in breast cancer patients;
- study the functional effects of KSR1 on breast cancer growth using *in vivo* and *in vitro* models;
- gain an insight into the potential mechanisms that contribute to its phenotypic effects and propose new intracellular pathways that KSR1 is involved in.

Although our understanding of TK signalling has developed dramatically over the past decades, only approximately half of them are well understood and much more remains to be investigated in order to achieve a complete picture of their regulated proteomes. Thus, further work in this thesis was addressed at globally decoding tyrosine kinase signalling by combined use of RNAi and SILAC quantitative proteomics in breast cancer. The aims were to:

- determine the expression profile of all 90 TKs in MCF7 cell line using RT-qPCR;
- silence individually each member of TKs expressed in MCF7 cells using a validated RNAi library;
- identify the TKs-regulated proteomes after siRNA treatment by SILAC-based quantitative proteomics;
- propose a new classification of the TKs family based on the similarity in their regulated proteomes;
- characterize a proteomic signature and a functional portrait for each identified cluster by performing various bioinformatic analyses.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Materials

2.1.1 Buffer and solutions

Table 1. List of buffers and solutions

Reagent	Recipe	Storage
1M Tris-HCl	60.5 g Tris in total of 500ml ddH ₂ O and adjusted to desired pH with pure HCl	RT
NP-40	50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 10%(v/v) glycerol; 1% NP40; 5 mM dithiothreitol (DTT); 1 mM EDTA; 1 mM EGTA; 50 µM leupeptin; 30 µg/ml aprotinin	4°C
RIPA	Tris-HCl: 50 mM, pH 7.4; NP-40: 1%; Na-deoxycholate: 0.25%; NaCl: 150 mM; EDTA: 1 mM; PMSF: 1 mM; Aprotinin, leupeptin, pepstatin: 1 µg/ml each; Na ₃ VO ₄ : 1 mM; NaF: 1 mM	4°C
Laemmli buffer	50mM Tris-HCl pH 6.8; 15% glycerol; 0.1% (w/v) bromophenol blue; 4% SDS	-20°C
5x Loading buffer	0.25M Tris-HCl, pH 6.8; 15% SDS; 50% glycerol; 25% beta-mercaptoethanol; 0.01% bromophenol blue	4°C
10x SDS-PAGE Running buffer	10g SDS; 30.3g Tris, 144.1g glycine dissolved in 1 l of ddH ₂ O	RT
10x Semi Dry Transfer buffer	Tris Base 5.8g; Glycine 2.9g; Methanol 200ml and make up to 1 l with ddH ₂ O	RT
10x TBS	24.23g Trizma HCl; 80.06 g NaCl dissolved in 1l of ddH ₂ O and adjusted pH to 7.6 with pure HCl	RT
TBS-Tween	100ml of TBS 10x; 900ml ddH ₂ O; 1ml Tween® 20 (BDH)	RT
Blocking buffer	TBS containing 0.1% (v/v) Tween20 and 5% (w/v) non-fat milk	4°C
0.4% Sulforhodamine B (SRB)	0.4% w/v SRB powder dissolved in 1% acetic acid	RT
40% Trichloroacetic Acid (TCA)	40 w/v TCA dissolved in ddH ₂ O	4°C
IF fixation buffer	4% w/v paraformaldehyde	4°C

IF blocking buffer	10% AB-serum in PBS	-20°C
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2.1.2 Mammalian cell lines

Table 2. List of mammalian cell lines

Cell Type	Tissue	Morphology	Tumourigenicity
MCF7	Breast	Epithelial	Human cell line derived from a pleural effusion of adenocarcinoma
MCF7-vector	Breast	Epithelial	MCF7 cell line stably overexpressing pCMV6-vector plasmid
MCF7-KSR1	Breast	Epithelial	MCF7 cell line stably overexpressing pCMV6-KSR1 plasmid
MDA-MB-231	Breast	Mesenchymal	Human Metastatic and high invasive cell line derived from pleural effusions of ductal carcinoma
MDA-MB-231-vector	Breast	Mesenchymal	MDA231 cell line stably overexpressing pCMV6-vector plasmid
MDA-MB-231-KSR1	Breast	Mesenchymal	MDA231 cell line stably overexpressing pCMV6-KSR1 plasmid
T47D	Breast	Epithelial	Human cell line derived from a pleural effusion of an infiltrating ductal carcinoma
ZR75-1	Breast	Epithelial	Human cell line derived from metastatic site of ductal carcinoma
SKBR3	Breast	Epithelial	Human cell line derived from metastatic site of adenocarcinoma
BT474	Breast	Epithelial	Human cell line derived from a solid, invasive ductal carcinoma
MDA-MB-453	Breast	Epithelial	Human cell line derived from metastatic carcinoma

Table 3. Normal growth media

Cell Type	Media	Additives	Storage
MCF7 MDA-MB-231 T47D ZR75-1 SKBR3 BT474	DMEM (Dulbecco's Modified Eagle's Medium) (Sigma-Aldrich, Dorset, UK)	2mM Glutamine 50 units/ml Penicillin 50µg/ml Streptomycin 10% FCS	4°C, used within one month
MCF7-vector MCF7-KSR1 MDA-MB-231-vector MDA-MB-231-KSR1	DMEM (Dulbecco's Modified Eagle's Medium) (Sigma-Aldrich, Dorset, UK)	2mM Glutamine 50 units/ml Penicillin 50µg/ml Streptomycin 10% FCS 1000-500µg/ml G-418 (Roche Diagnostic Ltd, West Sussex, UK)	4°C, used within one month (G418 added fresh each time)

Table 4. SILAC Media

Name	Media	Additives	Storage
DMEM-14 (R0K0)	Control SILAC DMEM media containing unlabelled arginine and lysine amino acids	10% SILAC dialysed foetal bovine serum	4°C, used within one month
DMEM-15 (R10K8)	SILAC DMEM media containing 13C and 15N labelled arginine, and 13C and 15N labelled lysine	10% SILAC dialysed foetal bovine serum	4°C, used within one month
DMEM-16 (R6K4)	SILAC DMEM media containing 13C labelled arginine and 2D labelled lysine amino acids	10% SILAC dialysed foetal bovine serum	4°C, used within one month

Table 5. Transfection media and reagents

Transfection media	Additives	Transfection reagents	Storage
Normal growth media (NEAT)	NO	HiPerFect Transfection Reagent (Qiagen, West Sussex, UK)	4°C
Opti-MEM [®] I Reduced Serum Medium(Gibco [®])	NO	Lipofectamine [®] 2000 Reagent (Invitrogen, Life Technologies Ltd, Paisley, UK)	4°C

2.1.3 Plasmid constructs

Table 6. List of plasmids and constructs

Vector	Insert (Gene)	Amino Acids	ID	Source
pCMV6 Myc/FLAG	Empty vector	Empty vector	PS10000	Origene
pCMV6 Myc/FLAG	KSR1	full length	RC211770	Origene
pCMV6 Myc/FLAG	KSR1	full length (R502M)	N/A	Site-directed Mutagenesis
PGL3	p53-R2	(luciferase. reporter)	N/A	Vikhanskaya et al., 2007
PGL3	p53-GADD45	(luciferase. reporter)	N/A	Vikhanskaya et al., 2007
PGL3	p53-P21	(luciferase. reporter)	N/A	Vikhanskaya et al., 2007
PGL3	p53-AIP1	(luciferase. reporter)	N/A	Vikhanskaya et al., 2007
PGL3	p53-CYCLIN G1	(luciferase. reporter)	N/A	Vikhanskaya et al., 2007

PGL3	p53-IGFBP3	(luciferase. reporter)	N/A	Vikhanskaya et al., 2007
pCDNA3.1	Ubiquitin	Myc-tag	N/A	Faronato et al., 2013
pCDNA3.1	BARD1	FLAG-BARD1 (aa 1-320)	N/A	Nishikawa et al., 2009
pCDNA3.1	BARD1	FLAG-BARD1 (full length)	N/A	Nishikawa et al., 2009
pCDNA3.1	BARD1	Myc3-BARD1 (full length)	N/A	Nishikawa et al., 2009
pCDNA3.1	BRCA1	Myc-BRCA1 (aa 1-772)	N/A	Nishikawa et al., 2009
pCDNA3.1	BRCA1	FLAG-BRCA1 (aa 1-772)	N/A	Nishikawa et al., 2009
pCDNA3.1	BRCA1	Myc3-BRCA1 (full length)	N/A	Nishikawa et al., 2009
pCDNA3.1	HA-NEDD8	Full length	18711	Addgene

2.1.4 Antibodies

Table 7. Primary antibodies

Name	Dilution	Producer
KSR1 rabbit polyclonal	1:1000	Santa Cruz
KSR1 rabbit polyclonal	1:1000	Cell Signalling
Anti-Flag mouse monoclonal	1:1000	Sigma Aldrich
p53 mouse monoclonal DO-1	1:1000	Santa Cruz
Acety-p53 rabbit polyclonal	1:500	Cell Signalling
p-p53 Ser15 rabbit polyclonal	1:500	Cell Signalling
SIRT1 rabbit polyclonal	1:1000	Cell Signalling
DBC1 rabbit polyclonal	1:1000	Cell Signalling
p-DBC1 Thr454 rabbit polyclonal	1:500	Cell Signalling
β-actin mouse monoclonal	1:20000	Abcam
BRCA1 rabbit polyclonal	1:1000	Santa Cruz
BARD1 rabbit polyclonal	1:1000	Bethyl Laboratories
Ubiquitin mouse monoclonal	1:1000	Cell Signalling
Anti-Myc mouse monoclonal	1:1000	Sigma Aldrich
NEDD8 rabbit polyclonal	1:1000	Cell Signalling
p-MEK1/2 rabbit polyclonal	1:1000	Cell Signalling
p-ERK1/2 rabbit polyclonal	1:1000	Cell Signalling
MEK1/2 rabbit polyclonal	1:1000	Cell Signalling
ERK1/2 rabbit polyclonal	1:1000	Cell Signalling
p-C-Raf rabbit monoclonal	1:1000	Cell Signalling
C-Raf rabbit polyclonal	1:1000	Cell Signalling
p-B-Raf rabbit polyclonal	1:1000	Cell Signalling

B-Raf rabbit monoclonal	1:1000	Cell Signalling
HDAC1 rabbit polyclonal	1:1000	Santa Cruz

Table 8. Secondary antibodies

Antibody	Dilution	Producer	Dilution buffer
Polyclonal goat anti-rabbit IgG/HRP	1:3000	Dako, Cambridge, UK	5% milk in TBST
Polyclonal goat anti-mouse IgG/HRP	1:3000	Dako, Cambridge, UK	5% milk in TBST
Alexa Fluor [®] 555 goat anti-mouse IgG	1:1000	Invitrogen, Paisley, UK	10% AB-serum in PBS
IRDye [®] Donkey anti-mouse IgG	1:10000	LI-COR [®] , USA	5% milk in TBST
IRDye [®] Donkey anti-rabbit IgG	1:10000	LI-COR [®] , USA	5% milk in TBST

2.1.5 Patient characteristics

The study cohort comprised > 1000 primary operable breast cancer cases from the previously validated Nottingham Tenovus Primary Breast Carcinoma Series [174, 346-348]. This well-characterized resource contains information on patients' clinical and pathological data including histological tumour type, primary tumour size, lymph node status, histological grade and data on other breast cancer relevant biomarkers. All patients participated in this study cohort were consented. This study was approved by Nottingham Research Ethics Committee 2.

Table 9. Patient characteristics data

	Patient characteristics	Number (%)
Tumour Stage	1	561 (59.1%)
	2	297 (31.3%)
	3	90 (9.5%)
Grade	1	137 (14.4%)
	2	303 (31.8%)
	3	510 (53.7%)
Age	<50	317 (33.2%)
	=>50	635 (66.7%)
Vascular Invasion	Definitive	332 (35.1%)
	Negative	494 (52.3%)
	Probable	119 (12.6%)
Tumour type	Invasive Ductal	591 (63.3%)
	Classical Lobular	44 (4.7%)
	Tubular Mixed	149 (16.0%)
	Atypical Medullary	23 (2.5%)
ER Status	Negative	270 (29.1%)
	Positive	657 (70.9%)
PgR Status		

	Negative	402 (44%)
	Positive	511 (56%)
cerbB2 Status		
	Negative	794 (85%)
	Positive	140 (15%)
Triple Negative Status		
	No	742 (80.4%)
	Yes	180 (19.5%)

2.2 Methods

2.2.1 Mammalian cell culture

2.2.1.1 Maintaining and passaging cells

Cells were usually maintained in 150 cm² flasks with normal growth medium at 37 °C in a humidified 5% CO₂ incubator. Cells were routinely passaged at a confluence of ~90%. To passage cells, medium was first removed with aspirators and washed 2-3 times with room temperature PBS. 3 ml of 10% trypsin in EDTA was added into each flask and cells were allowed to detach at 37 °C in incubator for 3 min. Medium with FCS was added into flasks to neutralize the trypsin and cell clumps were resuspended gently through pipetting. Suspension of cells was taken out of flask, and transferred in 15 mL sterile centrifuge tube and centrifuged at 1000 rpm for 5 min. Once centrifugation was finished, the supernatant was aspirated and cell pellet was resuspended in a certain amount of medium depending on requirement. The proper dilution of cells was then reseeded into new flasks and fresh media was added. Cell passages used in all experiments were normally between passages 4 and 20 and mycoplasma was tested routinely.

2.2.1.2 Cell transfections

For siRNA transfection, cancer cell lines were seeded into different sizes of dishes (6 cm or 10 cm) or plates (6-well or 24-well) with appropriate amount of medium depending on experiment design. Cells were allowed to attach at 37°C with 5% CO₂ for overnight before transfection. On next day, the named siRNA (20 nM) was firstly added into neat medium or Opti-MEM[®] without additives and this mix was left at RT for 10 min before proceeding to next step. Hiperfect Transfection Reagent (Qiagen) were then put into the previous solution and mixed gently. The master mix was incubated at RT for 15 minutes to enable the formation of transfection complexes. Subsequently, the complexes were added drop-wise onto the cells, and the plates were gently swirled to allow distribution of the transfection complexes evenly. The cells with the different transfection complexes were then incubated under normal growth conditions for indicated time. Each transfection was performed at least three times and the following assays including protein and RNA extractions were implemented.

For plasmid DNA transfection, cells were plated into the indicated dishes or plates for each experiment as aforementioned. On the transfection day, the indicated amount of plasmid DNA was added into Opti-MEM[®] without additives. At the same time, certain volume of

Lipofectamine[®] 2000 (ratio 1 μ l: 2 μ g) was added into same amount of Opti-MEM[®] without additives. Each mix was incubated for 15 min at RM temperature to allow complex formation to occur. After incubation, the two complexes were mixed gently and kept at RM temperature for another 15 min according to manufacturers' protocols. This master mix was then added drop-wise onto the cells, and the plates were swirled to allow distribution of the transfection complexes evenly. The cells with the different transfection complexes were then incubated under normal growth conditions for indicated time. Each transfection was performed at least three times for the experiments described in this work.

2.2.1.3 Cell treatments

For EGF treatment, MCF7 cells were seeded in 60 mm dishes at a 50% confluence and incubated under stripped medium without growth factors. Cells were then transfected with indicated plasmid DNA for 24 hours. EGF (Invitrogen, Life Technologies Ltd, Paisley, UK) or its vehicle DMSO (for the time point 0) was then added at a final concentration of 10 ng/ml for the indicated time points. After each time point, dishes were placed on ice and medium was aspirated. Cells were washed three times with cold PBS, scraped and centrifuged for 5 minutes at 1300 rpm. The supernatant was removed and the cell pellet was processed for western blotting assay.

2.2.1.4 Generation of stable cell lines

MCF7 and MDA231 cells were used to generate cell lines stably expressing human wild type KSR1 (pCMV6-KSR1) and empty vector (pCMV6-vector). These plasmid constructs were purchased from Origene (Table 6) and sequences were confirmed before experiments. To start, a working concentration of G-418 to guarantee selection and maintenance of the neomycin-resistant cells by eliminating non-transfected cells was determined as following. A titration experiment was designed by treating non-transfected MCF7/MDA231 cells seeded at different densities to different doses of G-418 (0, 0.1, 0.3, 0.5, 0.8, 1 mg/ml) for 10 d, and 1 mg/ml showed to be the minimum amount of G-418 that caused 99% cell death at all densities. Next, 1×10^6 cells were seeded in 100 mm dishes in medium in the absence of antibiotics and attached overnight. On the following day, plasmids pCMV6-KSR1 or pCMV6-vector were transfected into cells using protocol described above. After 48 hours, stably transfected cells were treated with normal growth media in the presence of 1 mg/ml of G-418. In 10 days from the addition of the antibiotic, non-transfected cells died and removed. Stably transfected cells were plated at a lower density to allow each single cell form a monoclonal colony with G-418.

When clones were visualized by eyes, 20 clones for each transfection condition were reseeded into 24-well plates: cloning discs were dipped in trypsin-EDTA for 2-3 min, then put on top of a single cell colony and incubated for 5 min at 37 °C to allow cells to detach. Each cloning disc carrying monoclonal cells was then transferred to each well of a 24-well plate in presence of G-418. Cells derived from monoclonal colonies were cultured by changing medium twice a week, and expanded to high confluence before to be transferred to a large scale plate. Finally, selected stable cells were acquired and success of transfection was confirmed by RT-qPCR and western blotting to check the mRNA and protein expression levels.

2.2.2 Protein extraction and western blotting

2.2.2.1 Total protein extraction

Cells were NP40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10%(v/v) glycerol, 1% NP40, 5 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, 50 µM leupeptin and 30 µg/ml aprotinin) was used to extract whole cell lysates. Cell pallets were mixed thoroughly with NP40 lysis buffer, and then incubated in ice for 15 min before centrifuging at 15000 rpm for 15 min at 4 °C.

2.2.2.2 Nuclear and cytoplasmic protein extraction

Nuclear and cytoplasmic extracts were prepared from whole-cell lysis using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Briefly, cells were harvested with trypsin-EDTA and centrifuged at 1000 rpm for 5 minutes to get the pellet. Next, cell pellet was washed twice in cold PBS and supernatant was removed. Certain amount of ice-cold CER I buffer I depending on the cell pellet volume was added to the cell pellet. Cell lysate in the tube was mixed vigorously on the highest setting of vortex for 15 seconds to fully suspend the cell pellet and incubated on ice for 10 minutes. Subsequently, ice-cold CER II was added to the tube and mixed vigorously on the highest setting of vortex for 10 seconds and incubated on ice for 1 minute. Directly, the tube was vortexed for 5 seconds on the highest setting and then centrifuged for 5 minutes at maximum speed. After centrifuge, the supernatant containing cytoplasmic extract was collected to a clean pre-chilled tube for later use. The insoluble (pellet) fraction produced above was resuspended in ice-cold NER. The tube was mixed thoroughly on the highest setting for 15 seconds and placed on ice. In every 10 minutes, the sample was continued vortexing for 15 seconds for a total of 40 minutes. Immediately, the sample was centrifuged at maximum speed ($\sim 16,000 \times g$) in a microcentrifuge for 10 minutes

and supernatant containing nuclear extract was transferred to a clean pre-chilled tube for later use.

2.2.2.3 Protein quantification and lysate preparation

Protein concentration was measured by the bicinchoninic acid (BCA) protein assay (Pierce) or Bradford Reagent Kit (Bio-Rad, Berkeley, CA, USA). 1 ul of each protein samples were normally added into 1000 ul of Bradford buffer and incubated at room temperature for 5 min. Absorbance readings of the prepared samples were then were measured at 595 nm using a Beckman DU[®] 530 Life Science UV/Visible spectrophotometer (Harlow Scientific, Arlington, MA). After measurement, the concentration of the unknown samples was determined based on standard absorbance value. An equal amount of protein in all samples was processed and lysates were heated with 5x sodium dodecyl sulfates (SDS) sample buffer at 95 °C for 5 min before they were loaded to 10% SDS-PAGE.

2.2.2.4 SDS-polyacrylamide gel electrophoresis and western blotting

Different percentages of acrylamide resolving and stacking gels were prepared manually as required for indicated proteins. Samples (20 ug) and rainbow markers (Thermo Scientific, Leicestershire, UK) were loaded onto the gels. Subsequently, electrophoresis was run at 80V for 30 min and changed to 120V for 2 hours in 1x running buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Samples were then transferred to Hybond ECL super nitrocellulose membranes (GE Healthcare). Subsequently the membranes were blocked in TBS containing 0.1% (v/v) Tween20 and 5% (w/v) non-fat milk for 1 h. The primary antibodies were probed with membranes overnight at 4 °C. The membranes were then washed three times in TBS/Tween for 15 min following incubation with HRP-conjugated secondary antibodies (1:3000 dilution) for 60 min. The membranes were then washed three times again and were detected with Enhanced chemiluminescence (ECL). Films were developed using a Konica SRX-1001A X-ray developer. Alternatively, membranes were incubated with IRDye Donkey anti-mouse or Donkey anti-rabbit secondary antibodies for 60 min and visualized by Odyssey Fc Imaging System (LI-COR).

2.2.3 RNA extraction and RT-qPCR

2.2.3.1 RNA extraction from cells

RNA extraction in this work was performed using RNeasy Mini Kit from Qiagen (Crawley, UK). Briefly, cells from 6-well plate were firstly washed three times with PBS, which was aspirated straight after wash. 300 µl of RLT buffer was added directly to the cells in the plate and mixed well. Lysate in RLT was then transferred into a QIAshredder spin column placed in a 2 ml collection tube, which was centrifuged at full speed for 2 min. Subsequently, an equal volume of 70% ethanol 300 µl was added to the homogenized lysate, and mixed well by pipetting. Next, the mix from previous step was transferred to an RNeasy spin column placed in a 2 ml collection tube, which was centrifuged at 10,000 rpm for 15s. The flow-through was discarded. 700 µl RW1 buffer was added directly to the RNease spin column and centrifuged at 10,000 rpm for 15s to wash the spin column membrane. 500 µl RPE buffer was then added to the RNease spin column and centrifuged at 10,000 rpm for 15s to wash the spin column membrane. Another 500 µl RPE buffer was added to the RNeasy spin column and centrifuged at 10,000 rpm for 2 min to wash the spin column membrane. All the flow-through above was abandoned. Lastly, the RNeasy spin column was placed in an autoclaved collection tube and 30 µl RNase-free water was added to the RNeasy spin column to elute RNA.

2.2.3.2 cDNA synthesis

The reverse transcription of total RNA to cDNA was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies Ltd, Paisley, UK). In this assay, 1 µg total RNA was used for all the samples acquired. To synthesize single-stranded cDNA from total RNA, a master mix containing 2 µl 10x RT buffer, 1 µl dNTP Mix, 2 µl 10x RT random primers, 1 µl MultiScribe™ reverse transcriptase and 1 µl RNase inhibitor was prepared per sample. Next, 1 µg total RNA with water was added to the master mix up to 20 µl per reaction. To perform reverse transcription, the reactions were loaded into the thermal cycler on the following conditions: step 1 25 °C for 10 min; step 2 37 °C for 120 min; step 3 85 °C for 5 min; step 4 4 °C ∞. When the reactions were finished, the cDNA samples were collected and diluted 1 in 10 with water for the next experiment.

2.2.3.3 Quantitative real-time PCR

RT-qPCR assays were performed to study the expression of genes of interest. For each reaction 5 µl of cDNA from the previous reverse transcription was used together with 10 µl

TaqMan[®] Universal PCR Master Mix (Applied Biosystems, Life Technologies Ltd, Paisley, UK), 1 µl of indicated TaqMan[®] probe and 4 µl of water. All the reactions were done in Fast Optical 96-well reaction plate (Applied Biosystems) in triplicate. Before putting on the machine, the plate was sealed with the Optical Adhesive Cover Starter Kit (Applied Biosystems) and centrifuged (2,000 x g) for 30 seconds at 4°C. RT-qPCR analysis was performed on a 7900HT Thermocycler (Applied Biosystems) on the program as follows: stage 1, 10 min at 95°C; stage 2, 20 s at 95°C and 20 s at 60°C for 40 cycles. Finally, the results were recorded and analysed accordingly.

2.2.4 RNA interference

For RNA interference experiment, all the certified siRNAs including control siRNA were purchased from Qiagen (Crawley, UK) and confirmed silencing efficiency in preliminary experiments. To do RNAi transfection, 5×10^5 cells were seeded onto 60 mm dishes for overnight to attach. On the transfection day, 20-40 nM of indicated siRNAs or siControl were pipetted into neat medium or Opti-MEM[®] without additives and left at room temperature for 10 min. After the first incubation, certain amount of HiPerFect Transfection Reagent was added straight to the complex and pipetted gently to mix well and kept at room temperature for another 15 min to allow them to form a master mix. This complex was then added drop-wise onto the cells, and the plates were swirled to ensure distribution of the transfection complexes evenly. Immediately, the cells were put back to incubators under normal growth condition for indicated time. Each transfection was performed at least three times for the experiments described in this work.

For RNAi screen of tyrosine kinases signalling pathways, a certified library of siRNAs containing at least a pool of 2 siRNAs against each individual tyrosine kinases has been obtained from Qiagen. All siRNAs were initially confirmed knockdown efficiency by RT-qPCR and western blotting before final screen. In the following experiments, MCF7 cells were primarily cultured in SILAC light/heavy medium for at least 7 cell divisions. After that, 1×10^6 cells were seeded into 100 mm dishes to attach overnight and were then transfected with validated siRNAs targeting TKs for 72 hours. On the last day, cells were washed with ice-cold PBS three times and collected in the pre-chilled tubes. Next, proteins were extracted and samples were proceeded to LC-MS/MS analyses for identification and quantification.

2.2.5 Plasmids transformation and DNA extraction

Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen, UK) were used for plasmids transformation. Briefly, 50µl of cells were gently aliquoted into pre-chilled 1.5 ml Eppendorf tubes mixing with 100ng of plasmid DNA. Then put on ice for 30 minutes followed by a 45 seconds heat shock at 42°C and subsequently cooled on ice for 2 minutes. After adding 600µl of S.O.C medium (Invitrogen, UK), the transformed cells were incubated at 37°C for 1 hour at 200 rpm and were subsequently spread on pre-warmed LB-agar kanamycin or ampicillin plates. Plates were incubated upside down overnight at 37°C. QIAGEN Plasmid Maxi Kits (QIAGEN, UK) was used to extract plasmid DNA according to the manufacturer's protocol. The acquired plasmid DNA was then diluted in water for future use.

2.2.6 Luciferase reporter assay

8×10^4 /well MCF7 cells were seeded into 24-well plate and transfection was performed using FuGENE® HD transfection reagent according to manufacturer's instructions (Roche, UK). Cells were transfected with different p53 constructs [349], and pCMV6-KSR1 or pCMV6-vector and together with renilla luciferase reporter vector (pRL-TK). Cell lysates were collected after 24 hours transfection and firefly and renilla luciferase activities were measured by the Dual-Glo™ Luciferase Assay kit as manufacturer's protocols described. Briefly, 50 µl of passive buffer was firstly added into each well and incubated at room temperature for 30 min. After incubation, cell lysis was transferred into white 96-well plate. Immediately, 50 µl of Dual-Glo® Luciferase Reagent was added into each well, mixed and left for 10 min at room temperature. Once incubation done, the plate was put on a reader to measure the firefly luminescence. Next, another 50 µl of Dual-Glo® Stop & Glo® Reagent was added each well, mixed and left for 10 min at room temperature. Renilla luminescence was subsequently measured in the same plate. The transcriptional activity of various p53 constructs were determined by firefly luciferase activity and normalized against renilla luciferase activity which was served as control for transfection efficiency.

2.2.7 Cell proliferation assay

Sulphorhodamine B (SRB) assay was performed to determine the growth of breast cancer cell lines in 96-well plates. After siRNA knockdown of KSR1 at indicated time points, plates were collected for the following protocol. Cells were fixed by adding 100 µl/well of ice-cold 40% trichloroacetic acid (TCA) to each culture for 1h and incubated in the fridge. Plates were then

washed x5 times in running tap water (allow the water to fill wells indirectly). Cells were stained with 100 μ l of 0.4% (W/V) SRB (Sigma S-9012) in 1% acetic acid for 30mins and plates were washed 5 times in 1% acetic acid and left to air dry overnight. On the day of reading plates, bound dye was solubilized by adding 100 μ l of 10mM tris base to all the wells. Then plates were measured at 492 nm on Tecan microplate reader.

2.2.8 Neddylation assay

MCF7 cells plated on 15 cm dishes were transfected using FuGENE[®] HD with 8 μ g pcDNA3-HA-NEDD8 (Addgene), 8 μ g pCMV6-KSR1 or 8 μ g pCMV6-Vector constructs as indicated. After 24 hours, cells were lysed in Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma Aldrich) supplemented with protease inhibitors. Total protein was quantified by the BCA assay (Pierce). 2 μ g Mouse IgG or p53 (DO-1) was pre-incubated with protein agarose beads for 2 hours to form IP matrix complex (ImmunoCruz[™] IP/WB Optima C System, Santa Cruz). 2 mg protein lysate was added into the beads and was incubated on a rotator overnight at 4 °C. Beads were washed with RIPA buffer for three times and were heated in SDS loading buffer. Neddylated p53 were detected by western blot using p53-specific DO-1 antibody or NEDD8 antibody (Cell Signalling) as described before [350, 351].

2.2.9 Ubiquitination assay

Cells were transfected with pCMV6-KSR1 or pCMV6-Vector constructs together with Myc-ubiquitin using FuGENE[®] HD for 48 hours and treated with MG132 for the last 6 hours [352]. Cells were lysed and total protein was quantified as described above. Rabbit IgG or BRCA1 antibody was pre-incubated with protein agarose beads for 2 hours to form IP matrix complex (ImmunoCruz[™] IP/WB Optima B System, Santa Cruz). 2 mg protein lysate was added into the beads and was incubated on a rotator overnight at 4 °C. Beads were then washed with RIPA buffer for three times and were heated in SDS loading buffer. Endogenous ubiquitinated BRCA1 was detected using anti-Myc antibody by western blot.

2.2.10 Soft agar assay

Agarose in PBS were prepared, autoclaved before use. For each experiment, MCF7-KSR1 or MCF7-vector cells after different treatments or transfections were harvested by trypsin, spun down and resuspended in warm medium with G418. Cells were counted and seeded in 0.3% agar on top of 0.6% agar in six-well plate in triplicates. Every 3 days, full DMEM medium

with G418 (500 µg/ml) was replenished on the top layer. After incubation for 2 weeks, colonies were counted in at least 3 random fields per well and photographed.

2.2.11 Three-dimensional overlay culture in Matrigel

Matrigel (BD Biosciences) was thawed on ice overnight at 4 °C and 70 µl was added into each of the wells of the eight-well glass slide chambers (Thermo Scientific), and spread evenly in the well to form a 1 mm thick bed. Matrigel was left to solidify at 37 °C for 15 min. Next, on the day of experiment, cells (5×10^3 /well) after different treatments or transfections were plated in medium containing 2% Matrigel and allowed to grow in a 5% CO₂ humidified incubator at 37 °C for 7 days. The assay medium containing 2% Matrigel was replaced every 3 days. Axiovert 100 MetaMorph microscope was used to image colonies in Matrigel.

2.2.12 Single nucleotide polymorphisms and genotyping

In those patients for whom adequate tissue was available, candidate KSR1 polymorphisms were chosen with the assistance of the Ensembl program using two main criteria: first, that the polymorphism has some degree of likelihood to alter the function of the gene in a biological relevant manner. Intronic or exonic polymorphisms can change gene transcription levels by alternative splicing or by affecting binding of a transcription factor. Second, that the frequency of the polymorphism is sufficient enough that its impact in clinical outcome would be meaningful on a population level (> 10% allele frequency). Genomic DNA was extracted from micro-dissected tissue specimens using the QIAamp kit (Qiagen). Two KSR1 polymorphisms (rs2241906 and rs1075952) were tested using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique.

2.2.13 Protein immunoprecipitation

5×10^6 cells were plated on 15 cm dishes to allow attachment overnight. On the next day, cells were transfected using Lipofectamine[®] 2000 with certain amount of indicated plasmid constructs according to experiment design. After 24 hours, cells were washed three times with ice-cold PBS, which was aspirated after last wash. Cells were lysed in IP buffer (50 mM HEPES, pH 7.4; 1% Triton X-100; 150 mM NaCl; 2 mM EDTA, pH 8; 10 % Glycerol) supplemented with fresh protease and phosphatase inhibitors for 15 min. Directly, cells were then scraped and transferred to a pre-chilled tube for centrifuge at 13,000 rpm for 10 min at 4 °C. Supernatant was subsequently transferred to a pre-chilled tube. Total protein was quantified and a minimum of 2 mg protein was used for each IP. 2-4 µg of indicated

antibodies or IgG were normally pre-incubated with protein agarose beads for 4 hours to form IP matrix complex (ImmunoCruz™ IP/WB Optima System, Santa Cruz). After incubation, the beads containing IP matrix complex were washed three times with IP buffer. 2 mg protein lysate was added into the beads and was incubated on a rotator overnight at 4 °C. On the following day, beads were washed three times with IP buffer for three times and supernatant was removed. Beads were then added with SDS loading buffer and boiled for 10 min and centrifuged at 13,000 rpm for 5 min. In the end of step, samples were loaded and western blotting for the appropriate proteins was performed.

2.2.14 Immunofluorescence staining

5 x 10⁵ MCF7 cells were seeded on glass coverslips in 6 cm dishes to attach. After overnight, cells were transfected with either pCMV6-KSR1 or pCMV6-Vector using FuGENE® HD. After 24 hours, medium was removed and cells on the coverslips were washed three times with ice-cold PBS. Next, cells were fixed in 4% w/v paraformaldehyde at 37 °C for 15 min and immediately permeabilized with 0.1% Triton-X for 10 min. Subsequently, cells were incubated in immune-fluorescent blocking buffer (10% AB-serum in PBS) for 1h, followed by incubation with p53 antibody (DO-1) for 1h at room temperature. Once the incubation was done, cells were washed with PBS and coverslips were incubated for 45 min at 37 °C with anti-mouse-IgG Alexa Fluor® -555 antibody (Invitrogen). DNA was visualized by DAPI staining. Cells were examined on an Axiovert-200 laser scanning inverted microscope (Zeiss) as previously described [174].

2.2.15 Genotyping and the determination of LOH

We analysed DNA samples - including leucocytes, tumour, and paired circulating cell-free DNA (cfDNA) analysed in a - previous study of 65 breast cancer patients [353]. Briefly, DNA samples were hybridized on Affymetrix SNP 6.0 arrays following the Genome-Wide Human SNP Nsp/Sty 6.0 protocol. Raw data CEL files underwent background correction before normalized by quantile normalization. Genotype calls for SNPs were made using Birdseed v2 (Broad Institute, <http://www.broadinstitute.org/mpg/birdsuite/index.html>), with loss of heterozygosity (LOH) being determined on a paired basis for cfDNA and primary tumour (matched to leucocyte DNA) using a max probability of 0.98 and genotype error of 0.02. Genomic decay was set to nought. This thresholding allowed for LOH to be called using either individual or multiple SNP markers within the genomic interval containing *KSR1* (60 SNP markers span just *KSR1*).

2.2.16 Tumour tissue microarray

TMAAs containing > 1000 primary operable breast cancer cases from the previously validated Nottingham Tenovus Primary Breast Carcinoma Series were employed [174, 346-348]. This well-characterized resource contains information on patients' clinical and pathological data including histological tumour type, primary tumour size, lymph node status, histological grade and data on other breast cancer relevant biomarkers. These include ER α , PgR, ERBB2, BRCA1, Chk1, cytokeratins (CKs; 5/6, 7/8, 18), Ki67 and E-cadherin. The immunoreactivity and scoring were defined in this series as previously described [354]. Patients within the good prognosis group (Nottingham Prognostic Index (NPI) ≤ 3.4) did not receive adjuvant therapy. Hormonal therapy was prescribed to patients with ER α positive tumours and NPI scores > 3.4 (moderate and poor prognostic groups). Pre-menopausal patients within the moderate and poor prognosis groups were candidates for chemotherapy. Conversely, postmenopausal patients with moderate or poor NPI and ER α positive were offered hormonal therapy, while ER α negative patients received chemotherapy. Data collected included overall survival (OS), disease-free survival (DFS). Clinical data were maintained on a prospective basis with a median follow-up of 124 months (range 1 to 233).

2.2.17 Immunohistochemistry

Anti-KSR1 rabbit polyclonal antibody (Santa Cruz, Heidelberg, Germany) was optimized to a working concentration of 2 μ g on full-face excisional tissue sections. Subsequently, breast cancer TMA (n = 945) cases comprising 4 μ m thick formalin fixed paraffin embedded tissue cores were immuno-stained with anti-KSR1 on the Leica BOND-MAX automated system using manufacturer's instructions. Hit-induced epitope retrieval was performed in citrate buffer (ER1) for 5 mins. Detection was achieved using the Polymer Detection kit (Leica Microsystems Inc., USA). Determination of the optimal cut-offs were performed using X-tile bioinformatics software (Yale University, USA). Cytoplasmic staining was scored based on intensity ranging from 0 to +2; 0 = null, +1 = intermediate and +2 = high level of staining intensity. Scoring for each tissue core on the TMAAs was performed by two independent investigators. High resolution digital imaging (NanoZomer, Hamamatsu Photonics, Welwyn Garden City, UK) at 20x magnification with a web-based interface (Distiller, SlidePath Ltd., Dublin, Ireland) was used. All cases were scored without knowledge of the clinic-pathologic or outcome data.

2.2.18 *in vivo* tumourigenicity assay in breast cancer xenografts in nude mice

Female 7-week-old, nu/nu-BALB/c athymic nude mice were acclimated in the animal house one week before experimentation. Before cells injection, a 0.72mg 17- β Estradiol 60-day release pellet was implanted subcutaneously (Innovative Research of America) into each mouse. MCF7-vector or MCF7-KSR1 cells were harvested by trypsin and spun down at 1000 rpm for 5 min. Subsequently, 5×10^6 cells were resuspended in the total volume of 100 μ L PBS supplemented with 1 mg/ml of G-418 solution. Nude mice were anaesthetized with 4% isoflurane and their body temperature was maintained stable by laying them on a thermal pad. Cells were then injected subcutaneously into the flank of the animals by groups (n=9, per group). Tumour growth was analysed by caliper measurements every three days and calculated using the formula $1/2 \text{ length (mm)} \times \text{width (mm)}^2$. After the mice were sacrificed, primary tumours were excised and formalin fixed. Samples were paraffin embedded, cut at 3 μ m and IHC stained for histological evaluation of target proteins expression. This study was conducted under the UK Home Office Project License.

2.2.19 Proteome analysis (SILAC)

2.2.19.1 SILAC cell culture

SILAC dialysed calf serum and custom DMEM mediums containing either unlabelled 12C6,14N4-arginine (Arg) and 12C6-lysine (Lys) (R0K0 -‘light’) or labelled 13C6-Arg and 2D-Lys (R6K4 -‘medium’), or labelled 13C6,15N4-Arg and 13C6-Lys (R10K8 -‘heavy’) were purchased from Dundee cell products (Dundee, UK). MCF7 cells were grown in these custom DMEM mediums along with 10% dialysed FCS and 1% of antibiotics (Penicillin and Streptomycin). All the cells that were grown for at least seven to eight passages to allow stable labelling were used for this experiment.

2.2.19.2 Protein digestion and peptide fractionation

Equal amounts of protein from unlabelled and labelled samples were combined prior to protein digestion. Briefly, samples were reduced in 10 mM DTT and alkylated in 50 mM Iodoacetamide prior to boiling in loading buffer, and then separated by one-dimensional SDS-PAGE (4-12% Bis-Tris Novex mini-gel, Invitrogen) and visualized by colloidal Coomassie staining (Novex, Invitrogen). The entire protein gel lanes were excised and cut into 10 slices each. Every gel slice was subjected to in-gel digestion with trypsin overnight at 37 $^{\circ}$ C. The

resulting tryptic peptides were extracted by formic acid (1%) and acetonitrile, lyophilized in a speedvac and resuspended in 1% formic acid.

2.2.19.3 Protein digestion and phosphopeptide enrichment

FASP Procedure was performed as previously described [355]. 500 μ l FASP 1 (8 M urea, 20 mM DDT in 100 mM Tris/HCL pH 8.5) was added to ~2 mg protein lysate to dilute SDS concentration and transferred to a Vivacon 500, 30k MWCO HY filter (Sartorius Stedim Biotech, VN01H22). The sample was buffer exchanged using FASP1 several times by spinning the tube at 7000g to remove detergents. The protein lysate was concentrated by centrifugation and diluted in FASP 2 (100 mM Tris/HCl pH 8.5) ready for trypsin digestion. The sample was reduced using 50mM fresh IAA in FASP 2 in the dark for 30 min. Lysates were spun down to remove excess IAA and buffer exchanged into FASP 3 (100mM TEAB (triethyl ammonium bicarbonate)). Trypsin was dissolved in FASP 3 to give a 1:200 enzyme to protein ratio and added in a volume of at least 100 μ l for 4-6 hours. This was repeated with fresh trypsin for a further overnight incubation. Lysates were spun down and washed with 0.5M NaCl and 150 μ l 10% TFA added to reduce the pH. A standard desalting procedure was used [356].

Hydrophilic Interaction Chromatography (HILIC) fractionation: A TSKgel Amide-80 separation column with a TSKgel Amide guard column was used for HILIC separation of FASP peptides. Buffer A: 80 % ACN, 0.1 % Formic Acid & Buffer B: 0.1 % Formic Acid was used for the gradient at a flow rate of 0.6 ml/min. The sample was separated into 45 fractions, collected 2mins/vial and dried using a speed vac.

TiO₂ Enrichment was performed as previously described [356]. TiO₂ beads were washed & re-suspended in Buffer B at 50 μ g/ μ L and added to tubes to give 1 mg per tube. The sample was re-suspended in loading buffer, added to beads & incubated at RT for 20 min. After washes using buffers A & B samples were eluted using aliquots of 0.5% NH₄OH. Elutions were pooled & 10 μ l of 20 % FA added to adjust the pH. Samples were dried and re-suspended in 1% FA.

2.2.19.4 Mass spectrometry methods

Trypsin-digested peptides were separated using an Ultimate 3000 RSLC (Thermo Scientific) nanoflow LC system. On average 0.5 μ g was loaded with a constant flow of 5 μ l/min onto an Acclaim PepMap100 nanoViper C18 trap column (100 μ m inner-diameter, 2 cm; Thermo

Scientific). After trap enrichment, peptides were eluted onto an Acclaim PepMap RSLC nanoViper, C18 column (75 μ m, 15 cm; Thermo Scientific) with a linear gradient of 2-40% solvent B (80% acetonitrile with 0.08% formic acid) over 65 min with a constant flow of 300 nl/min. The HPLC system was coupled to a linear ion trap Orbitrap hybrid mass spectrometer (LTQ-Orbitrap Velos, Thermo Scientific) via a nano electrospray ion source (Thermo Scientific). The spray voltage was set to 1.2 kV, and the temperature of the heated capillary was set to 250 °C. Full-scan MS survey spectra (m/z 335-1800) in profile mode were acquired in the Orbitrap with a resolution of 60,000 after accumulation of 1,000,000 ions. The fifteen most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision-induced dissociation (normalized collision energy, 35%; activation Q, 0.250; and activation time, 10 ms) in the LTQ after the accumulation of 10,000 ions. Maximal filling times were 1,000 ms for the full scans and 150 ms for the MS/MS scans. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. The lock mass option was enabled for survey scans to improve mass accuracy. Data were acquired using the Xcalibur software.

2.2.19.5 Proteome quantification

The raw mass spectrometric data files obtained for each experiment were collated into a single quantitated data set using MaxQuant [357] and the Andromeda search engine software [358]. Enzyme specificity was set to that of trypsin, allowing for cleavage N-terminal to proline residues and between aspartic acid and proline residues. Other parameters used were: (i) variable modifications, methionine oxidation, protein N-acetylation, gln $\rightarrow$ pyro-glu; (ii) fixed modifications, cysteine carbamidomethylation; (iii) database: target-decoy human MaxQuant (ipi.HUMAN.v3.68); (iv) heavy labels: R6K4 and R10K8; (v) MS/MS tolerance: FTMS- 10ppm , ITMS- 0.6 Da; (vi) maximum peptide length, 6; (vii) maximum missed cleavages, 2; (viii) maximum of labelled amino acids, 3; and (ix) false discovery rate, 1%. Peptide ratios were calculated for each arginine- and/or lysine-containing peptide as the peak area of labelled arginine/lysine divided by the peak area of non-labelled arginine/lysine for each single-scan mass spectrum. Peptide ratios for all arginine- and lysine-containing peptides sequenced for each protein were averaged. Data are normalised using 1/median ratio value for each identified protein group per labelled sample.

2.2.19.6 Bioinformatic analysis

All the bioinformatic analysis was completed with R. Specifically, the hierarchical clustering of SILAC proteomics data upon silencing TKs was performed based on calculated distances by R's hclust function [359]. The complete linkage method which aims to find similar clusters based on overall cluster measure was used. For the correlation heatmap, we calculated and plotted pairwise distances between tyrosine kinases according to their protein quantification signatures. The distance metric was conducted using the centred Pearson correlation [360]. To visualize the TKs-modulated proteomics, the heatmap of quantified values showing the overall pattern of regulation was presented. Individual protein was plotted in either red for down regulated proteins, or white for non-differential and non-identified, or blue for up-regulated proteins ($\log_2$ fold change of 0.7 as the cut-off for down regulation and 1.3 as the threshold value for up-regulation). The hyper-geometric test was used to identify gene terms that are enriched in the de-regulated genes for each cluster. For each identified cluster, GO analysis was performed in the three domains: biological process (BP) cellular component (CC) and molecular function (MF) [361, 362].

2.2.20 Statistical analysis

Statistical analysis for TMAs was performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Analysis of categorical variables was performed with the appropriate statistical test. Survival curves were analysed using the Kaplan-Meier method with significance determined by the Log rank test; multivariate analysis was performed by Cox hazards regression analyses. DFS was defined as the period from the date of initial diagnosis to the date of the first documented relapse or death, and OS was defined as the time from the initial diagnosis to death. DFS time was censored at the date of last follow-up if patients were still relapse-free and alive, and OS was censored at the time when patients were alive. A forward stepwise Cox regression model was conducted to select baseline patient demographic and tumour characteristics to be included in the multivariate analyses of 2 KSR1 polymorphisms and clinical outcome. Chi-square tests were used to examine the associations between tumour characteristics and KSR1 polymorphisms. Tests were 2-sided at a 0.05 significance level.

CHAPTER 3:

RESULTS

3.1 KSR1 regulates p53 transcriptional activity through deleted in breast cancer 1 (DBC1) modulation

3.1.1 Phospho-proteomic analysis of KSR1-regulated proteins using SILAC

To elucidate the KSR1-related signalling pathways in breast cancer, a quantitative proteomic analysis (SILAC-based) was performed to obtain a profile of KSR1-regulated phospho-proteins.

3.1.1.1 Strategy of studying KSR1-regulated phospho-proteome in breast cancer

In this work, we used MCF7 cells to study the KSR1-regulated phospho-proteome in breast cancer. MCF7 cells were first cultured and passaged in either R0K0 ‘light’ medium, containing unlabelled $^{12}\text{C}_6$, $^{14}\text{N}_4$ -arginine (Arg) and $^{12}\text{C}_6$ -lysine (Lys) amino acids, or in R10K8 ‘heavy’ medium, containing labelled $^{13}\text{C}_6$ $^{15}\text{N}_4$ -Arg and $^{13}\text{C}_6$ $^{15}\text{N}_2$ -Lys for at least 7 cell divisions to allow labels to incorporate. Following, labelled cells were transfected with either the pCMV6 vector construct (R0K0, control) or with a pCMV6-KSR1 plasmid (R10K8) that encodes for the full length KSR1. After 24h, cells were washed with ice-cold PBS and proteins were extracted, mixed 1:1, separated on SDS-PAGE, trypsin-digested and fractionated. The samples were then analysed and identified through specific peptides or phospho-peptides by LC-MS/MS using MaxQuant software [357]. A SILAC-based workflow is presented in **Figure 12**.

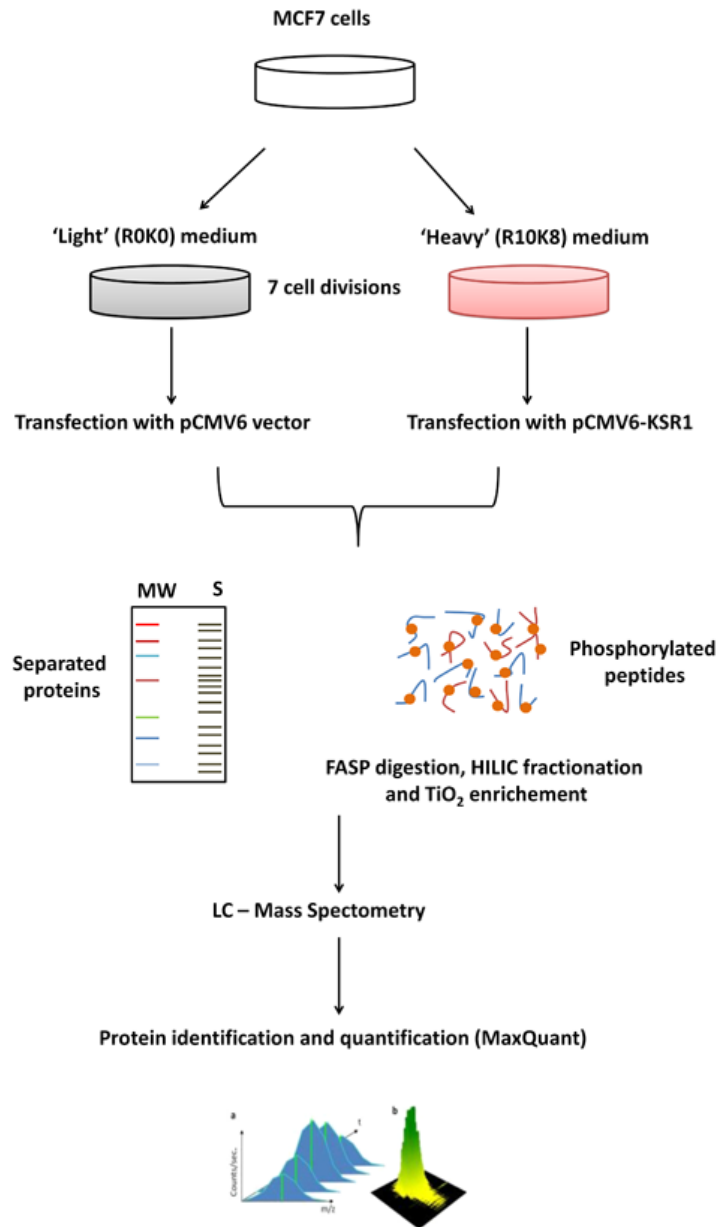


Figure 12 Strategy of studying KSR1-regulated phospho-proteomics in breast cancer.

MCF7 cells were cultured and passaged in either R0K0 'light' medium or in R10K8 'heavy' medium for at least 7 cell divisions. Labelled cells were then transfected with either the pCMV6 vector construct (R0K0, control) or with a pCMV6-KSR1 plasmid (R10K8) encoding the full length KSR1. After 24h, proteins were extracted, mixed 1:1, separated on SDS-PAGE, trypsin-digested, fractionated and analysed by LC-MS/MS using MaxQuant software.

3.1.1.2 Profile of KSR1-modulated phospho-proteomics in MCF7

In the KSR1-regulated proteome analysis, a total of 2504 proteins were identified by SILAC and 2032 of them were quantified (false discovery rate <1%). Moreover, 1409 phosphopeptides from 891 phospho-proteins were identified by SILAC and 1165 phosphopeptides from 812 phospho-proteins were quantified. After normalisation, the phosphorylation vs total protein level ratios between control and KSR1-overexpressed samples were determined accordingly. From our analysis, phosphorylation changes of 379 potential sites corresponding to 240 proteins were observed, as in some cases more than one potential phosphorylation sites have been identified for one protein. Amongst these modulated sites, 341 phosphoserine (pSer), 37 phosphothreonine (pThr), and 1 phosphotyrosine (pTyr) sites were reported.

Interestingly, only 3 out of the 379 identified phospho-sites were induced more than 50% while most of them (233 out of 379) were actually decreased (<50%) upon KSR1-overexpression in MCF7 cells. A scatter plot representing the value from total and phosphorylated proteins was generated to demonstrate the log₂ normalised ‘total proteins’ versus the log₂ ‘phosphorylated proteins’ ratios (**Figure 13**).

3.1.2 Gene ontology analysis of KSR1 regulated phospho-proteins in breast cancer

Gene Ontology (GO) analysis was implemented using the ‘Panther’ tool [363] to classify the KSR1-regulated phospho-proteome based on i) biological process, ii) molecular function, and iii) cellular component (**Figure 14**). Significant enrichments were identified for GO molecular functions terms related to binding (GO: 0005488), catalytic activity (GO: 0003824), structural molecule activity (GO: 0005198), as well as transcription regulator (GO: 0003824) and enzyme regulator (GO: 0030234) activities. While identifying biological processes modulated in this phospho-proteomics analysis, cell cycle (GO: 0007049) and communication (GO: 0007154) along with metabolic (GO: 0008152), cellular (GO: 009987) and transport (GO: 0006810) processes were significantly represented. Finally, in relation to the localisation of the identified KSR1-regulated phospho-proteins, a similar distribution between membrane/cytoplasmic and nuclear cell components were observed. To the best of our knowledge, this is the first ontology analysis of KSR1 regulated phospho-protein profile in breast cancer, which will provide new insights for others to study its functions in signalling pathways other than the canonical Ras-Raf-MAPKs cascades.

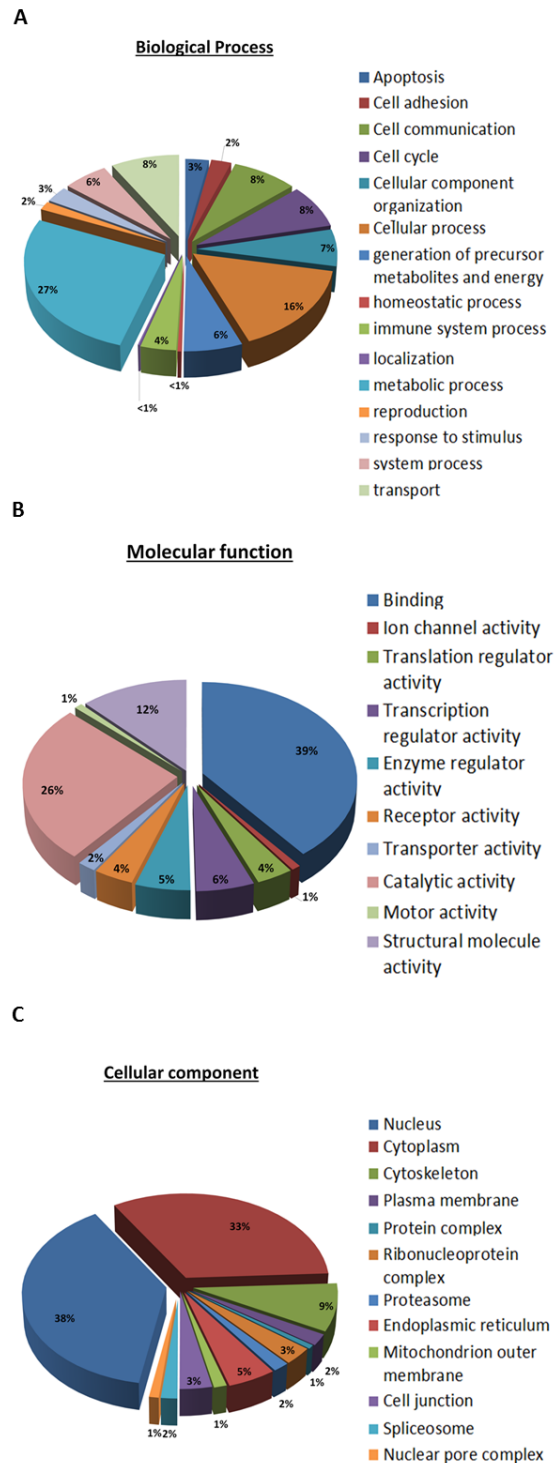


Figure 14 Gene Ontology (GO) classification of the KSR1-regulated phospho-proteome in MCF7 cells.

GO analysis was performed by the 'Panther' tool based on biological function (A), molecular process (B) and cellular component (C).

3.1.3 Phospho-proteomics analysis shows a decrease of phospho-DBC1 after KSR1 overexpression

The identified KSR1-regulated phospho-proteome provided us a number of interesting targets that might play a role in breast cancer. To further utilize the data we acquired from the SILAC analysis, we demonstrated that some of these phospho-proteins have been previously reported to be important in the development of breast cancer. For instance, androgen-induced proliferation inhibitor (APRIN), a protein phosphorylated by ATM and ATR kinases, has been documented as a BRCA2-interacting protein essential for genome integrity and a predictor of clinical outcome in response to chemotherapy in breast cancer [364, 365]. Rho GTPase activating protein 35 (ARHGAP35) has been shown to be important in RhoA inactivation, Ras activation and promotion of breast cancer growth and migration upon its phosphorylation by breast tumour kinase (Brk) [366]. Phospho-Hsp27 was correlated with HER-2/neu status and lymph node positivity in breast cancer, and was also connected to cancer invasiveness and drug resistance [367, 368]. Histone deacetylase 1 (HDAC1) /Sin3A complex phosphorylation has been correlated to ER α expression and hormonal therapy resistance in breast cancer cells [369]. These links suggest an important role of KSR1 and its regulated phospho-proteome in breast cancer.

Interestingly, phospho-DBC1 was one of the most significantly KSR1-regulated phosphorylated proteins (**Figure 13**). DBC1 has been shown previously to play an important role in p53 activation via SIRT1 and its phosphorylation is essential in this process [370, 371]. Considering the importance of p53 in breast cancer, we then studied the potential association between KSR1 and p53 activity through DBC1.

3.1.4 KSR1 regulates p53 transcriptional activity

Firstly, we sought to investigate the effects of KSR1 on p53 transcriptional activity. To do so, we performed luciferase reporter assays using various p53-dependent gene promoter constructs (including p53-R2, p53-AIP1, p53-IGFBP3 and p53-CYCLIN G1). MCF7 cells were transfected with either pCMV6 or pCMV6-KSR1 plasmids together with different p53-dependent promoter constructs. After 24 hours, cells were treated with either DMSO or etoposide, which can induce p53 activity. Interestingly, luciferase assays showed that the activity of all four different p53-regulated genes was significantly repressed upon KSR1 overexpression, indicating an inhibitory role of KSR1 on p53 activation (basal levels and after etoposide treatment; **Figure 15A**). On the other hand, KSR1 silencing led to a clear increase

in the activity of p53-dependent promoter genes (**Figure 15B**). These results support a potential involvement of KSR1 in regulating p53 transcriptional activity.

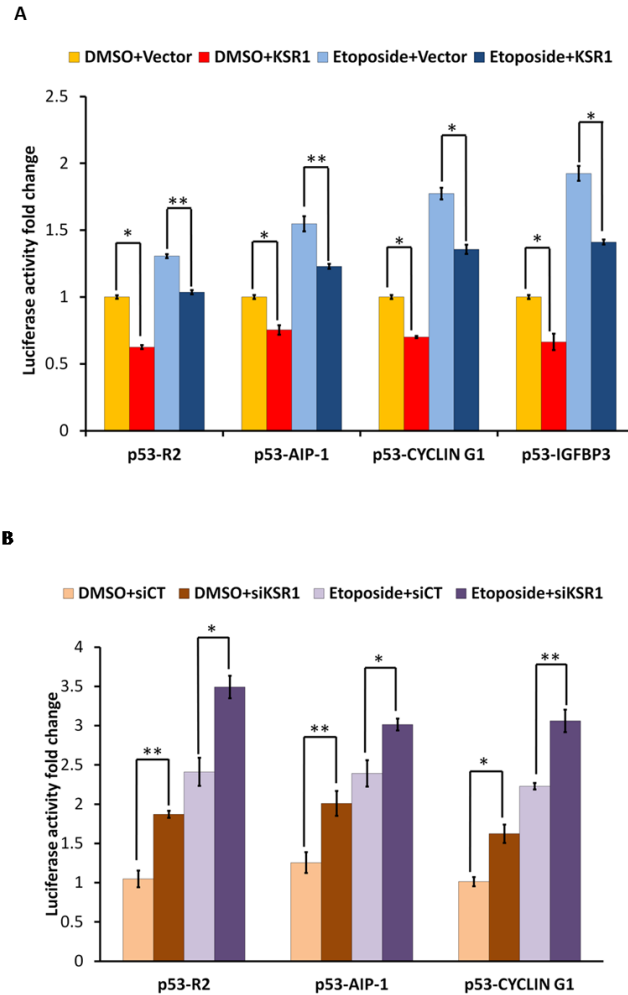


Figure 15 Effect of KSR1 on p53 transcriptional activity in the presence or absence of etoposide by luciferase assays.

A. MCF7 cells were transiently co-transfected with either pCMV6 (vector) or pCMV6-KSR1 plasmids in the presence of four individual p53-dependent promoter constructs expressing firefly luciferase genes (p53-R2, p53-AIP1, p53-CYCLIN G1, and p53-IGFBP3) following DMSO or etoposide (40μM) treatment for 3h. **B.** MCF7 cells were transfected with control siRNA (siCT) or siKSR1 for 48 hours, followed by transfection of three p53-dependent promoter constructs expressing firefly luciferase genes (p53-R2, p53-AIP1, and p53-CYCLIN G1) for additional 24 hours. DMSO or etoposide (40μM) were subsequently added. Firefly/ Renilla luciferase activity was measured and renilla luciferase activity was used to normalize transfection efficiency. The normalized luciferase activity of empty vector is set as 1. Results shown are the average of at least three independent experiments and error bars represent SD. (* $P < 0.05$, ** $P < 0.01$).

3.1.5 KSR1 does not affect p53 mRNA, protein, subcellular localisation and neddylation levels

To further examine the potential mechanism resulting in the effects of KSR1 overexpression on p53 transcriptional activity, we first checked whether KSR1 affects the gene (mRNA) and protein expressions of p53. RT-qPCR and western blotting analyses reported no significant changes in the p53 mRNA and protein levels respectively after KSR1 transient overexpression in MCF7 cells (**Figures 16A and 16B**). Similarly, no change in p53 protein levels was observed in MCF7 stably overexpressing KSR1 cells comparing with MCF7 parental cells (**Figure 16B**).

As it is known that nuclear localisation of p53 is essential for its transcriptional activity, immunofluorescence staining of p53 was therefore performed to observe its potential translocation alteration upon KSR1 overexpression. As shown in **Figure 17**, immunofluorescence revealed that there was no cytoplasmic/nuclear translocation of p53 upon KSR1 transient overexpression. To further confirm our results, a subcellular protein fractionation assay was performed showing that p53 did not translocate from the cytoplasm to the nucleus after overexpression of KSR1 (**Figure 18A**).

Moreover, it has been well studied that neddylation of p53 can inhibit its transcriptional activity [350, 351]. We performed a neddylation assay on p53. To do so, MCF7 cells were co-transfected with HA-NEDD8 and pCMV6 vector or pCMV6-KSR1 plasmids as indicated. After 24 hours, cells were harvested and protein was extracted. p53 was then immunoprecipitated using a p53 specific antibody and the neddylated-p53 was detected by immunoblotting using anti-NEDD8 and anti-p53 antibodies. However, results from the neddylation assay showed no significant changes following KSR1 overexpression ruling out neddylation as the cause and suggesting other alternative mechanisms might be responsible for the observed decrease in p53 activity (**Figure 18B**).

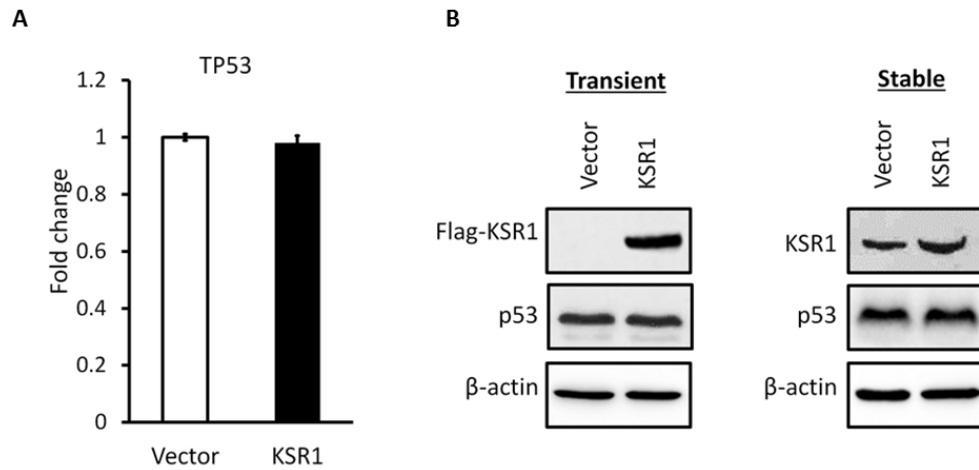


Figure 16 Effect of KSR1 overexpression on p53 mRNA and total protein levels.

MCF7 cells were transiently transfected with pCMV6 or pCMV6-KSR1 plasmids for 24 hours. Subsequently, relative mRNA levels of *TP53* and p53 total protein were measured by qRT-PCR (**A**) and western blotting (**B**), respectively. Gene expression level from cells transfected with pCMV6 vector was set as 1. Results shown are the average of at least three independent experiments. Similarly, in MCF7 stably overexpressing KSR1 cells, p53 total protein was evaluated by western blotting (**B**). Blots shown are representatives of at least three independent experiments.

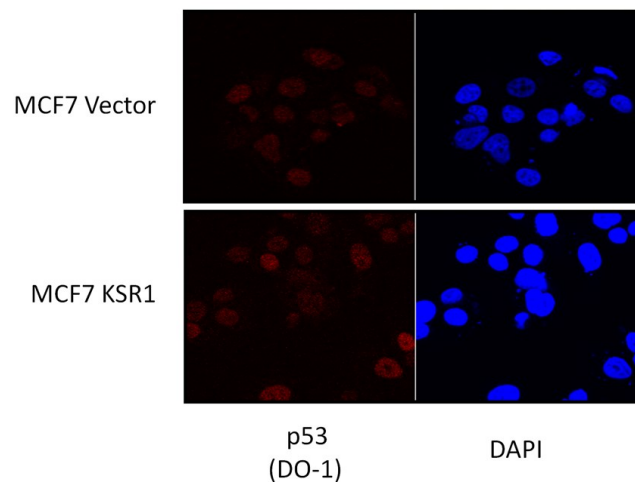


Figure 17 Immunofluorescence staining of p53 after KSR1 overexpression.

MCF7 cells were transfected with either pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. p53 was then detected with an anti-p53 antibody and the nucleus was stained with DAPI. Representative pictures of three independent experiments are shown.

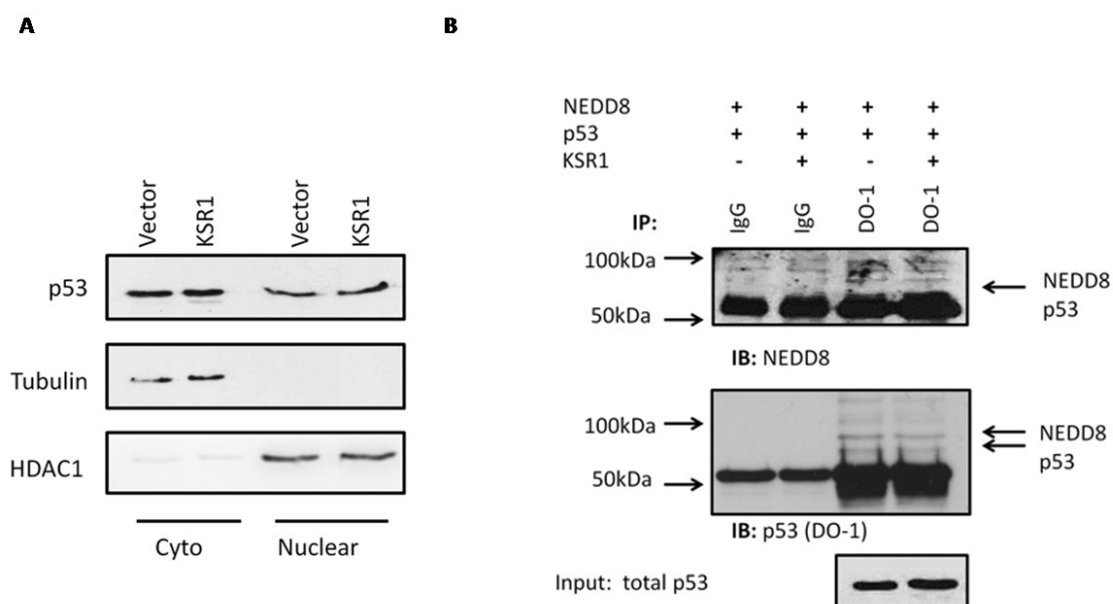


Figure 18 Effect of KSR1 overexpression on p53 subcellular localisation and neddylation.

A. Subcellular fractionation assays were performed after 24 hours transfection with either pCMV6 vector or pCMV6-KSR1 plasmids in MCF7. Tubulin and HDAC1 expression served as positive control for cytoplasmic and nuclear proteins respectively. Blots shown are representatives of at least three independent experiments. **B.** Neddylation assay on p53 after KSR1 overexpression. MCF7 cells were co-transfected with HA-NEDD8 and pCMV6 vector or pCMV6-KSR1 plasmids as indicated. p53 was immunoprecipitated using a p53 specific antibody (DO-1) and the neddylated-p53 was detected by immunoblotting using anti-NEDD8 and anti-p53 specific antibodies. Blots shown are representatives of at least three independent experiments.

3.1.6 KSR1 decreases p53 acetylation by reducing phosphorylation of DBC1

Post-translational modifications of p53 such as phosphorylation and acetylation are important for p53 activity in response to genotoxic stress [372, 373]. We therefore decided to investigate whether KSR1 has any effects on p53 post-translational modifications. We examined the effects of KSR1 on phospho-p53 and acetylated-p53. MCF7 cells were transfected with pCMV6-vector or pCMV6-KSR1 plasmids for 24 hours and then treated with either DMSO or etoposide, to induce genotoxic stress. As shown in **Figure 19A**, the phosphorylation levels of p53 (Ser15) did not change upon KSR1 overexpression in the presence or absence of etoposide. However, the level of acetylated-p53 was reduced after KSR1 overexpression upon etoposide treatment (**Figure 19A**).

It is already well established that SIRT1 is a NAD-dependent p53 deacetylase that regulates p53 activation [373-375]. Additionally, DBC1 can interact with and negatively regulate SIRT1 lead to an increase in p53 acetylation [370, 371]. Taking this potential link into consideration, we sought to study the effects of KSR1 on these two proteins.

Our western blotting results showed that KSR1 up-regulation did not affect the total protein levels of either SIRT1 or DBC1 **Figure 19A**. Based on the SILAC data showing the effects of KSR1 on the phosphorylation of DBC1 and the known relation between DBC1 and SIRT1, we checked the effect of KSR1 on DBC1 phosphorylation *in vitro*. Western blotting demonstrated that KSR1 overexpression resulted in a decrease of phospho-DBC1 (Thr454) in both basal and etoposide-induced conditions (**Figure 19A**). On the other hand, KSR1 silencing caused an increase in acetylation of p53 upon etoposide treatment (**Figure 19B**). Moreover, knockdown of KSR1 did not change the total proteins of SIRT1 and DBC1, but increased DBC1 phosphorylation at Thr454 (**Figure 19B**). These *in vitro* data further confirm our SILAC results and also suggest a new role of KSR1 in regulating p53 acetylation through phospho-DBC1 and SIRT1, providing an explanation for its effect on p53 transcriptional activation.

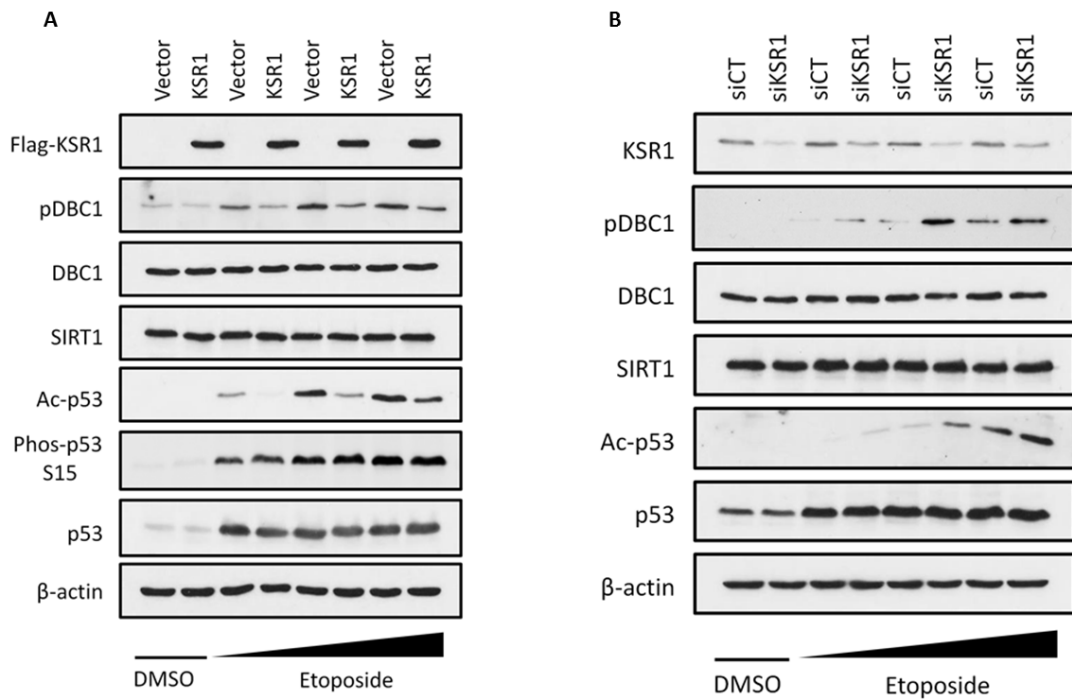


Figure 19 Effect of KSR1 on DBC1 phosphorylation.

A. Effects on p53 acetylation and phosphorylation of DBC1 after KSR1 overexpression followed by etoposide treatment. MCF7 cells were transiently transfected with pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. Subsequently, cells were treated with various concentration of etoposide (20, 40, 80 μ M, 3h). Total SIRT1, total DBC1, p53 phosphorylation, p53 acetylation and DBC1 phosphorylation were assessed by western blotting with specific antibodies as indicated. **B.** Effects on p53 acetylation and phosphorylation of DBC1 after KSR1 silencing followed by a titration of etoposide treatment. MCF7 cells were transfected with control siRNA (siCT) or siKSR1 for 72 hours followed by etoposide treatment (20, 40, 80 μ M, 3h). Total SIRT1, total DBC1, p53 acetylation and DBC1 phosphorylation were assessed by western blotting with specific antibodies as indicated.

3.1.7 The effects of KSR1 on p53 acetylation is dependent on DBC1 and requires its intact kinase domain

To further validate that the effect of KSR1 on p53 acetylation is dependent on DBC1, we examined the effects of KSR1 on acetylated p53 after DBC1 silencing upon etoposide treatment in MCF7 cells. As shown in **Figure 20A**, western blotting results demonstrated that acetylated p53 was consistently elevated after KSR1 was depleted, whereas the effects of KSR1 silencing on p53 acetylation disappeared after silencing DBC1 upon etoposide treatment. This suggests that KSR1 regulation of p53 acetylation requires DBC1, as depletion of DBC1 weakens the effect of KSR1 on acetylated p53.

Next, we examined whether the effect of KSR1 on DBC1 requires its catalytic domain. To do so, we generated a KSR1 mutant (KSR1/R502M) that comprises a key amino acid mutation (arginine to methionine) within its kinase domain resulting in impaired catalytic activity [179]. As shown in **Figure 20B**, wild type KSR1 consistently caused a decrease of phosphorylated DBC1, whereas mutant KSR1/R502M demonstrated no effect on pDBC1 levels compared to the control. This data indicates that an intact catalytic domain of KSR1 is indispensable for regulating DBC1 phosphorylation.

Finally, it has been reported that genotoxic stress induces DBC1 phosphorylation, which creates binding sites for SIRT1 and strengthens the interaction between SIRT1 and DBC1 [376, 377], which consequently weakens the deacetylase activity of SIRT1 on p53. Based on this, we then hypothesized that KSR1 could affect the SIRT1-DBC1 interaction induced by genotoxic stress. As shown in **Figure 20C**, co-immunoprecipitation revealed that the interaction between SIRT1 and DBC1 was decreased after KSR1 overexpression upon etoposide treatment in MCF7. This supports our hypothesis and also suggests that the reduced interaction between SIRT1 and DBC1 by KSR1 overexpression potentially enables SIRT1 to bind with p53 and promote deacetylase activity of SIRT1 on p53 acetylation.

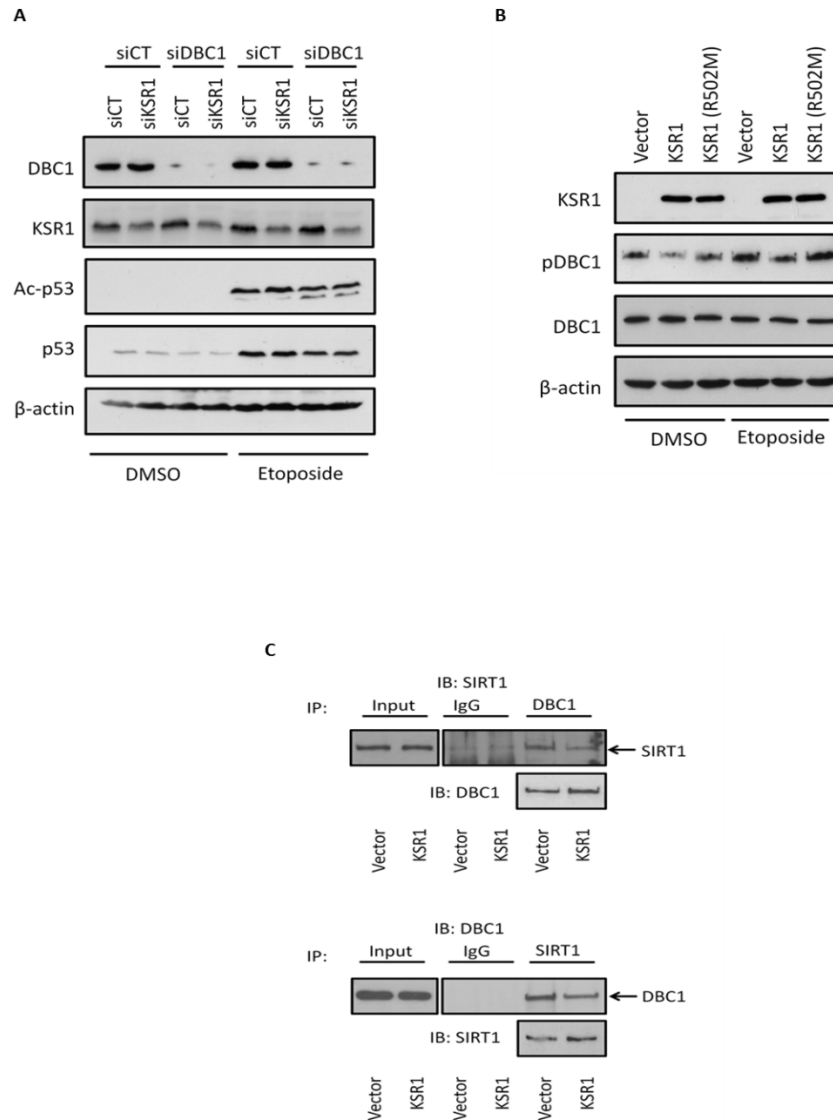


Figure 20 Mechanism of KSR1-regulated p53 acetylation.

A. Effect of KSR1 on p53 acetylation is mediated through DBC1. MCF7 cells were treated with control siRNA (siCT) or siKSR1 in concordant with siCT or siDBC1 for 72 hours followed by etoposide treatment (40 μ M, 3h). Acetylated p53, DBC1 and KSR1 protein levels were assessed by western blotting with specific antibodies as indicated. **B.** Effect of KSR1 on DBC1 phosphorylation is dependent on its intact kinase domain. MCF7 cells were transiently transfected with vector, wild type KSR1 or mutant KSR1 (R502M) plasmids for 24 hours followed by etoposide treatment (40 μ M, 3h). DBC1 phosphorylation was measured by western blotting with specific antibody. **C.** Interaction of DBC1 and SIRT1 after KSR1 overexpression with etoposide treatment by immunoprecipitation. MCF7 cells were transiently transfected with pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. Subsequently, cells were treated with etoposide (40 μ M, 3h). The interactions between SIRT1 and DBC1 were detected by immunoprecipitation (IP) of SIRT1 or DBC1 followed by immunoblotting with DBC1 and SIRT1 antibodies respectively. Blots shown are representatives of at least three independent experiments.

3.1.8 KSR1 is important for breast cancer proliferation

We next studied whether KSR1 has any effects on breast cancer growth *in vitro*. Cell proliferation assay (SRB assay) was implemented to examine its effect on four different breast cancer cell lines. As shown in **Figure 21**, KSR1 silencing markedly inhibited cell growth in all four breast cancer cell lines including MCF7, ZR75-1, SKBR3 and MDA231 after six days in comparison to control siRNA. This result demonstrates that KSR1 is important for breast cancer proliferation *in vitro*.

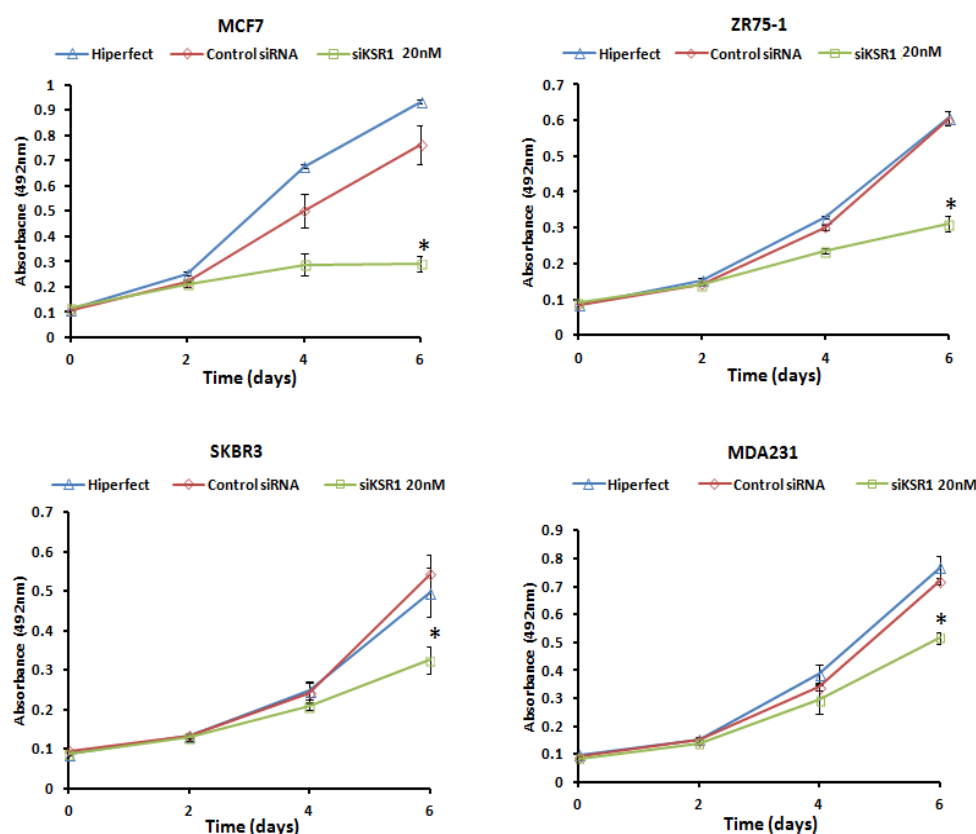


Figure 21 Effect of KSR1 silencing on breast cancer cell proliferation *in vitro*.

SRB assay was performed to examine its effects on cell proliferation. Briefly, MCF7, ZR75-1, SKBR3 and MDA231 cells were transfected with 20 nM of either siKSR1 or ‘non-targeting’ siRNA (Control siRNA) or vehicle (Hiperfect) for a time course to 6 days. At the end of experiment, absorbance at 492nm was measured and analysed. Error bars represent SD of 3 experiments each in quintuplicates (* $P < 0.05$, compared with control siRNA at day 6 (Student’s t test)).

3.1.9 Discussion

KSR1 was initially recognized as a novel protein kinase evolutionarily conserved in *Drosophila* and *Caenorhabditis elegans* acting between Ras and Raf in the Ras-Raf-MAPKs signalling pathway [107-109]. Owing to a mutation in the lysine residue in the catalytic domain important for the kinase activity, mammalian KSR1, however, has been widely denoted as a pseudokinase [43]. The scaffolding function of KSR1 was then revealed shortly after. Murine KSR1 (mKSR1) was shown to be able to interact with activated Ras to enhance the activity of MAPK kinases resulting in a stimulation of *Xenopus* oocyte maturation and cellular transformation [111]. Meanwhile, KSR1's role as an active kinase was also described. One group observed that upon TNF α and ceramide activation, KSR1 autophosphorylation was increased and KSR1 was capable of phosphorylating and activating Raf-1 [114]. Therefore, increasing evidence suggests that KSR1 can function as an active kinase as well as a scaffold protein [137, 146]. Additionally, KSR1 has been previously indicated as an oncogene in Ras-dependent cancers, such as pancreatic and lung carcinomas [127, 166]. Its biological functions and associated signalling pathways implicated in breast cancer have remained largely undefined.

In this work, a global comparative proteomic analysis using SILAC was performed to reveal the modulated phospho-proteome by KSR1 in MCF7 breast cancer cells. A number of new KSR1-regulated proteins as well as KSR1-involved signalling networks were identified, apart from the classical Ras-Raf-MAPKs pathway. Interestingly, our phospho-proteomic analysis showed that the majority (233 out of 379) of the identified phospho-sites were actually decreased upon KSR1 overexpression comparing to a small number of increased phosphorylation (3 out of the 379). This potentially suggests that KSR1 might function primarily as a scaffold protein not a kinase at least in this context. Alternatively, some negative feedback loops might exist in this system. However, none of the previous identified proteins or phospho-proteins including MEK and ERK in the canonical Ras-Raf-MAPKs pathways have been reported in our study. The possible explanation could be that the Ras-mutations are very rare in breast cancer [378]. Moreover, phospho-profiling of the major members of the Ras-Raf-MAPKs cascades after KSR1 overexpression/silencing had no significant effects on the phosphorylation levels of the main components in the Ras-Raf-MAPKs signalling pathway (results shown in section 3.2.3).

Furthermore, the KSR1-modulated phospho-proteins identified in this study provided us with a comprehensive picture of KSR1-involved signalling networks. Bioinformatic analysis

demonstrated the involvement of KSR1 in multiple biological processes and its capability of modulating many molecular functions, including cell cycle, metabolism and apoptosis. Importantly, some of the identified phospho-proteins have been indicated to play an essential role in the tumourigenesis and development of breast cancer. For instance, APRIN, a protein phosphorylated by ATM and ATR, was shown as a BRCA2-interacting protein important for genome integrity and correlated with chemotherapy in breast cancer [364, 365]. Moreover, ARHGAP35 phosphorylation was reported to play a part in breast cancer proliferation and migration [366]. Phospho-Hsp27 was related to invasiveness and drug resistance in breast cancer [367, 368]. Most interestingly, the KSR1-regulated phospho-proteomic analysis has established phospho-DBC1, which is a potential link to the modulation of p53 transcriptional activity by KSR1 in this work.

DBC1 was previously identified as a major player involved in regulating p53 acetylation [370, 371], which is crucial for activation of p53 transcriptional activity [373]. Thus, our work further studied the effect of KSR1 on p53 transcriptional activity, as well as the potential mechanism on KSR1-related regulation of p53 transcriptional activity, including through p53 acetylation. Luciferase assays using several different p53-dependent gene promoter constructs displayed that p53 transcriptional activity was inhibited after KSR1 overexpression in the absence or presence of etoposide, whereas the opposite results were detected upon knockdown of KSR1, indicating a regulatory role of KSR1 in p53 activity. Mechanistic studies revealed that overexpression of KSR1 did not affect p53 mRNA and total protein levels, or p53 subcellular localization, phosphorylation and neddylation, which are all potentially important in the regulation of p53 activity.

Interestingly, p53 acetylation was reduced after KSR1 overexpression, whereas knockdown of KSR1 caused an increase in p53 acetylation in the absence or presence of etoposide, indicating an involvement of KSR1 in regulating p53 acetylation. It has already been known that DBC1 can directly interact with and negatively regulate SIRT1, a NAD-dependent p53 deacetylase, leading to elevated p53 acetylation [370, 371]. In addition, upon genotoxic stress, phospho-DBC1 is able to produce binding sites and increase the interaction between SIRT1 and DBC1 complex, which successively abolishes the deacetylase activity of SIRT1 on p53 [376, 377]. Consistently, we revealed that KSR1 overexpression reduces the phosphorylation levels of DBC1 in the non-stressed context as well as in the presence of etoposide. On the other hand, KSR1 silencing increases levels of phosphorylated DBC1. These results provide us with an explanation of the observed effects of KSR1 on p53 acetylation and activity

suggesting that the action of KSR1 on p53 activation is through DBC1. Finally, co-immunoprecipitation results demonstrated that overexpression of KSR1 mitigates the SIRT1-DBC1 interaction, which therefore enables SIRT1 to bind more with p53 triggering a reduction of p53 acetylation. Our data presented is in agreement with previous reports and together with our SILAC analyses allows KSR1 to incorporate into an established signalling network with DBC1 and SIRT1 in modulating p53 activity.

However, more work remains needed to illuminate the mechanism of KSR1 on regulating DBC1 phosphorylation. The recent work from Zannini *et al.* demonstrated that ATM/ATR is competent to directly phosphorylate DBC1 on Thr454 upon DNA damage. Subsequently, phosphorylated DBC1 interacted with SIRT1 and suppressed its activity, leading to the dissociation of the SIRT1-p53 complex and eventually an increase in p53 acetylation [377]. Additionally, another group showed that protein kinase A and AMP-activated protein kinase can initiate the separation of SIRT1 from its endogenous inhibitor DBC1, hence regulating downstream effects [379]. Therefore, alternative mechanisms including whether the action of KSR1 on DBC1 phosphorylation is through ATM/ATR kinases await further exploration.

Taken together, the first SILAC analyses of phospho-proteome modulated by KSR1 highlight its contribution in numerous biological and molecular processes as well as cellular signalling pathways in breast cancer. The characterization of novel KSR1-regulated proteins will provide new insights into KSR1-modulated networks involved in breast cancer. In the present study, a model is proposed to define the function of KSR1 in the known DBC1-SIRT1-p53 network (**Figure 22**). When KSR1 is at basal levels in cancer cells, phosphorylated DBC1 directly binds and acts together with SIRT1 and inhibits the deacetylase activity of SIRT1, which results in an increase of p53 activation initiating gene transcription. On the other hand, in KSR1-transduced cells, DBC1 phosphorylation is reduced, which destabilises the direct interaction between DBC1 and SIRT1. This in turn enables SIRT1 to diminish p53's acetylated state, which consequently impedes transcriptional activity of p53. Our study sheds new light on the function of KSR1 in p53 regulation and reveals an interesting mechanism for its contribution in breast cancer development.

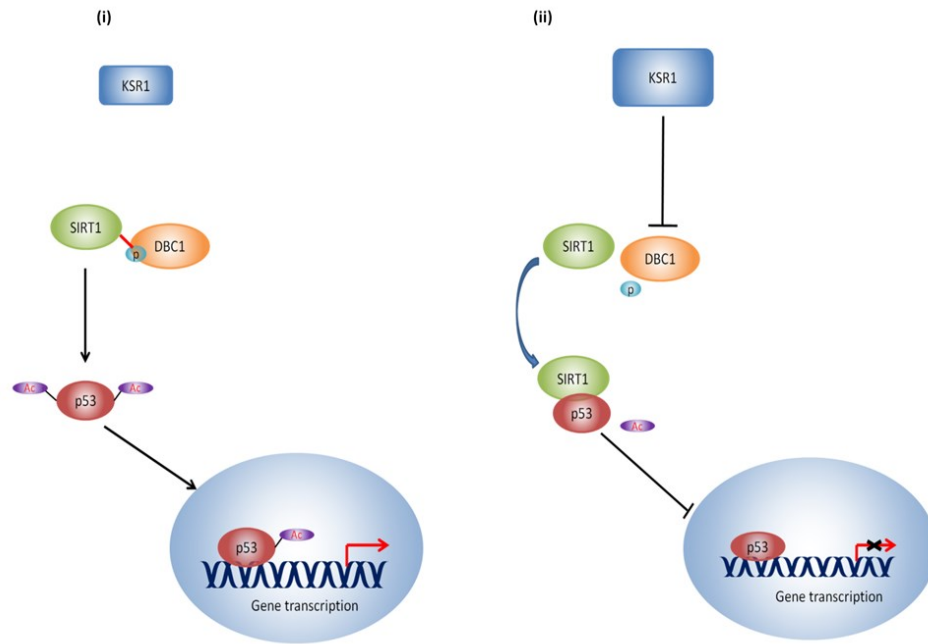


Figure 22 Schematic model illustrating the role of KSR1 on p53 transcriptional activity in breast cancer cells.

i) In cancer cells with basal level of KSR1 phosphorylated DBC1 directly interacts with SIRT1 and reduces its deacetylase activity resulting in p53 activation and gene expression initiation. **ii)** In KSR1-transduced cells, KSR1 suppresses DBC1 phosphorylation, which undermines the direct interaction between DBC1 and SIRT1. This in turns allows SIRT to decrease p53 acetylation state, thus inhibiting p53 transcriptional activity.

3.2 KSR1 regulates BRCA1 degradation and inhibits tumour growth

3.2.1 KSR1 is an independent predictor of overall survival in breast cancer

3.2.1.1 KSR1 expression in breast cancer patients

To determine the clinical significance of KSR1 in breast cancer patients, we employed TMAs containing > 1000 primary operable breast cancer cases from the previously validated Nottingham Tenovus Primary Breast Carcinoma Series [174, 346-348]. This well-characterized cohort encloses detailed information on patients' clinical and pathological data and immunoreactivity and scoring were characterised in this series as previously described [354]. Cytoplasmic KSR1 abundance in breast cancer tissues was determined by immunohistochemistry (**Figure 23**).

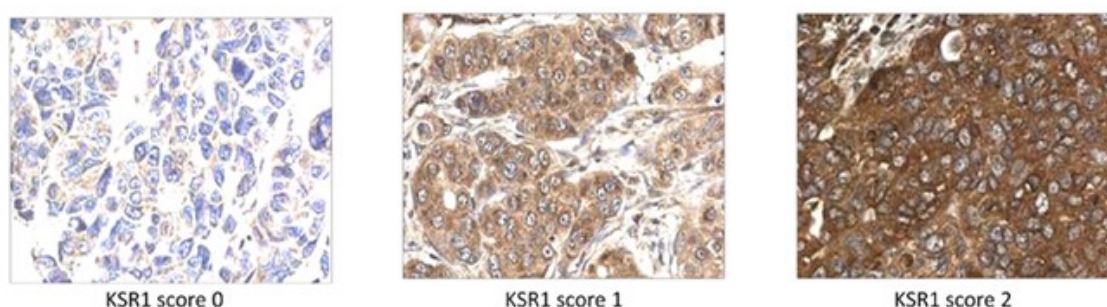


Figure 23 KSR1 expression in breast cancer tissues by IHC.

Immunohistochemistry staining (IHC) of paraffin embedded breast cancer tissues for KSR1 expression. Representative images of staining intensities: i) negative, ii) 1+ and iii) 2+. Images were adopted from the high resolution digital imaging (NanoZomer, Hamamatsu Photonics, Welwyn Garden City, UK) with a web-based interface (Distiller, Slide-Path Ltd., Dublin, Ireland) Magnification, x 100.

3.2.1.2 Clinical significance of KSR1 expression in the European cohort

KSR1 staining was scored based on intensity ranging from 0 to +2; 0 = null, +1 = intermediate and +2 = high level of staining intensity, which was performed by two independent investigators. Kaplan-Meier survival curves showed that high KSR1 levels were significantly associated with longer overall survival ($P = 0.012$; **Figure 24A**) and an increased disease free survival ($P = 0.014$; **Figure 24B**) in > 20 years follow-up. KSR1 expression was not significantly associated with an improved response to endocrine therapy ($n = 272$, $P = 0.064$; **Figure 24C**) and adjuvant chemotherapy ($n = 155$, $P = 0.096$; **Figure 24D**), although results indicated a trend of high KSR1 expression towards improved response to endocrine adjuvant chemotherapy.

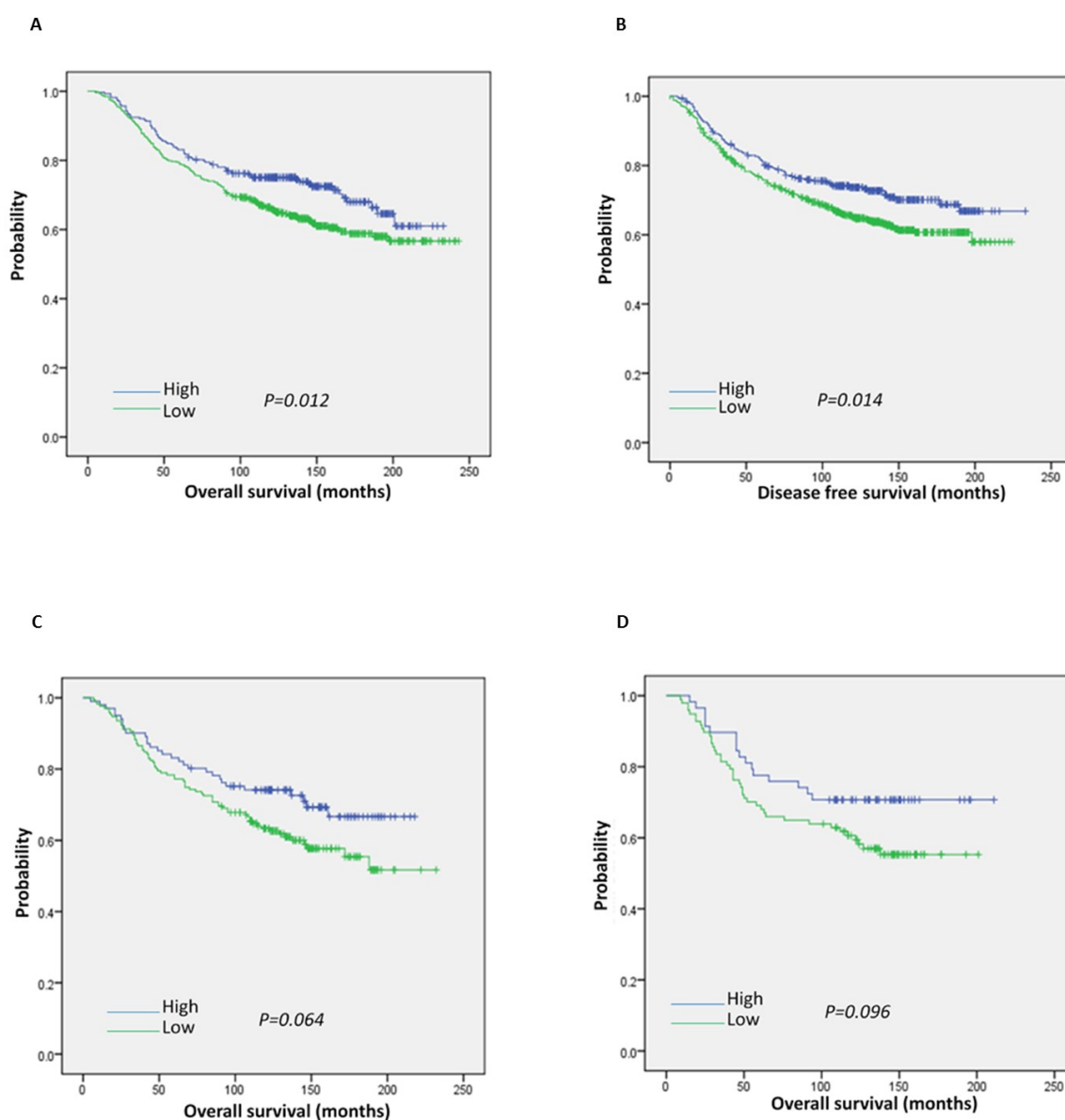


Figure 24 Clinical significance of KSR1 in breast cancer.

A. Kaplan-Meier survival curves for KSR1 expression levels with overall survival (High = 278, Low = 527; $P = 0.012$) and **B.** disease free survival (High = 325, Low = 620; $P = 0.012$) in a 240 months follow-up. **C.** Kaplan-Meier overall survival curves for KSR1 expression levels with response to endocrine therapy (High = 101, Low = 171; $P = 0.064$) and, **D.** to chemotherapy (High = 58, Low = 97; $P = 0.096$) in a 240 months follow-up.

Multivariate Cox-proportional hazards analysis revealed KSR1 as an independent prognostic factor (**Table 10**). Furthermore, KSR1 expression was also examined in the available paired adjacent normal breast (NB) and invasive breast carcinoma (BC) tissues from the same patients by western blotting. Consistently, KSR1 abundance is decreased in invasive BC compared to NB (**Figure 25**). We also identified two intronic KSR1 polymorphisms (*rs2241906* and *rs1075952*) but they were not correlated with either disease free or overall survival (**Figure 26**).

Variable	OS			DFS		
	Hazard ratio	95% CI	<i>P</i> value	Hazard ratio	95% CI	<i>P</i> value
Stage ^a	1.846	1.562-2.180	<0.001	1.893	1.585-2.260	<0.001
Grade ^b	1.755	1.435-2.146	<0.001	2.222	1.747-2.825	<0.001
Size>1.5 cm ^c	1.825	1.246-2.674	0.002	2.026	1.310-3.134	0.002
KSR1 status	1.543	1.189-2.001	0.001	1.541	1.166-2.037	0.002

a Fitted as linear term, i.e., Increase in risk for change in lymph node stage of one unit

b Fitted as linear term, i.e., Increase in risk for change in grade of one unit

c Compared with tumour size ≤ 1.5 cm

Table 10. Cox-proportional hazards analysis for predictors (model including KSR1 expression) of OS and DFS.

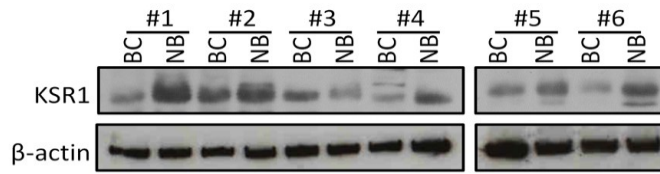


Figure 25 KSR1 expression in the paired adjacent normal breast and invasive breast cancer tissues.

Pairs of tissues from six patients were collected and western blotting was performed to examine the KSR1 expression using specific anti-KSR antibody. Representative images from three independent experiments are shown here.

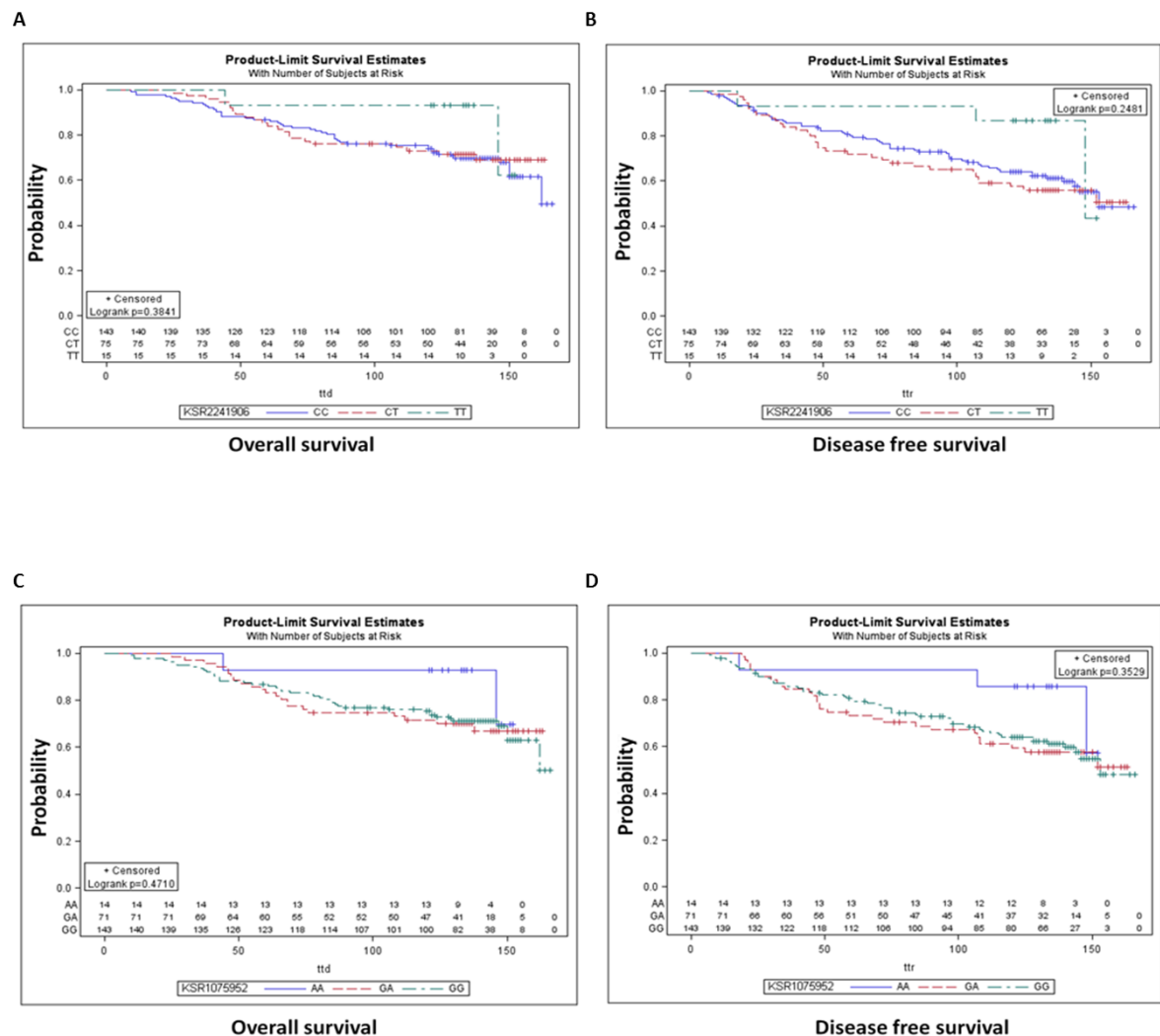


Figure 26 Correlations of KSR1 polymorphisms with overall survival and disease free survival in breast cancer patients.

Kaplan-Meier curves demonstrate the association between two KSR1 polymorphisms: **A.** rs2241906 and overall survival ($P = 0.3841$) and **B.** disease-free survival ($P = 0.2481$). **C.** rs1075952 and overall survival ($P = 0.4710$) and **D.** disease-free survival ($P = 0.3529$).

3.2.1.4 Correlations between KSR1 expression and tumour biomarkers in European breast cancer patients

To investigate patients' characteristics, tumour biomarkers and their correlations with KSR1 expression in European breast cancer patients, we utilised a well-characterized resource in this cohort [174]. It contains information on patients' clinical and pathological data including histological tumour type, primary tumour size, lymph node status, histological grade and data on other breast cancer relevant biomarkers. These include ER α , PgR, ERBB2, BRCA1, Chk1, cytokeratins (CKs; 5/6, 7/8, 18), Ki67 and E-cadherin.

Our results showed that there were no significant associations of KSR1 with standard breast cancer histopathologic biomarkers including tumour stage, grade and size (**Table 11**); KSR1 levels were also not associated with the expression of hormone receptors ER α and PgR, or ERBB2). However, high KSR1 was associated with high BRCA1 ($P = 0.002$), BARD1 ($P < 0.001$) and Chk1 ($P < 0.001$) expression (**Table 11**).

Variable/biomarker	KSR1 expression		P value
	Weak (%)	Strong (%)	
Tumour Stage			
1	370(59.7)	191(58.2)	0.787
2	194(31.3)	103(31.4)	
3	56(9.0)	34(10.4)	
Grade			
1	95(15.3)	42(12.8)	0.544
2	199(32.0)	104(31.7)	
3	328(52.7)	182(55.5)	
Tumour Size			
<1.5cm	141(22.7)	71(21.6)	0.719
=>1.5cm	481(77.3)	257(78.4)	
Age			
<50	197(31.6)	120(36.6)	0.119
=>50	427(68.4)	208(63.4)	
Vascular Invasion			
Definitive	217(34.9)	115(35.6)	0.973
Negative	326(52.4)	168(52.0)	
Probable	79(12.7)	40(12.4)	
Tumour type			
Invasive Ductal	376(61.3)	215(67.0)	0.18
Other	237(38.7)	106(33.1)	
ER Status			
Negative	167(27.5)	103(32.2)	0.149
Positive	440(72.5)	217(67.7)	
PgR Status			
Negative	258(43.1)	144(45.9)	0.42
Positive	341(56.9)	170(54.1)	
ERBB2 Status			
Negative	521(85.4)	273(84.3)	0.639
Positive	89(14.6)	51(15.7)	
Triple Negative Status			
Non-Triple	493(81.9)	249(77.8)	0.137
Triple Negative	109(18.1)	71(22.2)	
Cytokeratin 5/6			
Negative	504(83.3)	260(81.8)	0.555
Positive	101(16.7)	58(18.2)	
Cytokeratin 14			
Negative	522(86.4)	273(86.7)	0.919
Positive	82(13.6)	42(13.3)	
BRCA1			
0	100(20.0)	40(15.2)	0.002
1	222(44.4)	96(36.4)	
2	178(35.6)	128(48.5)	
BARD1			
Negative	502(80.3)	227(69.6)	<0.001
Positive	123(19.7)	99(30.4)	
Chk1			
Negative	460(90.4)	210(77.8)	<0.001
Positive	49(9.6)	60(22.2)	
Ki67			
0	175(42.0)	79(36.1)	0.149
1	242(58.0)	140(63.9)	

Table 11. Patients' characteristics and tumour biomarkers and correlations with KSR1 expression in European breast cancer patients

3.2.1.5 Oncomine analysis of *KSR1* gene expression between normal breast tissues versus breast carcinoma.

Oncomine analysis has been widely utilised to characterise gene expression using large microarray datasets in cancers [375]. Our searches for *KSR1* gene expression based on 11 datasets revealed four [380-383] with a significant p value ($P < 0.05$) and gene ranks in the top 18% amongst all differentially expressed genes. In all of these cases, *KSR1* was consistently decreased in invasive cancers compared to normal breast tissue (**Figures 27A-D**). Moreover, in the dataset of Finak *et al.*, which contained the largest sample number among these datasets, a similar pattern of *BRCA1* and *BARD1* expressions decreasing in invasive carcinoma was also observed, further supporting the correlation between *KSR1* with *BRCA1* and *BARD1* (**Figure 27E and 27F**).

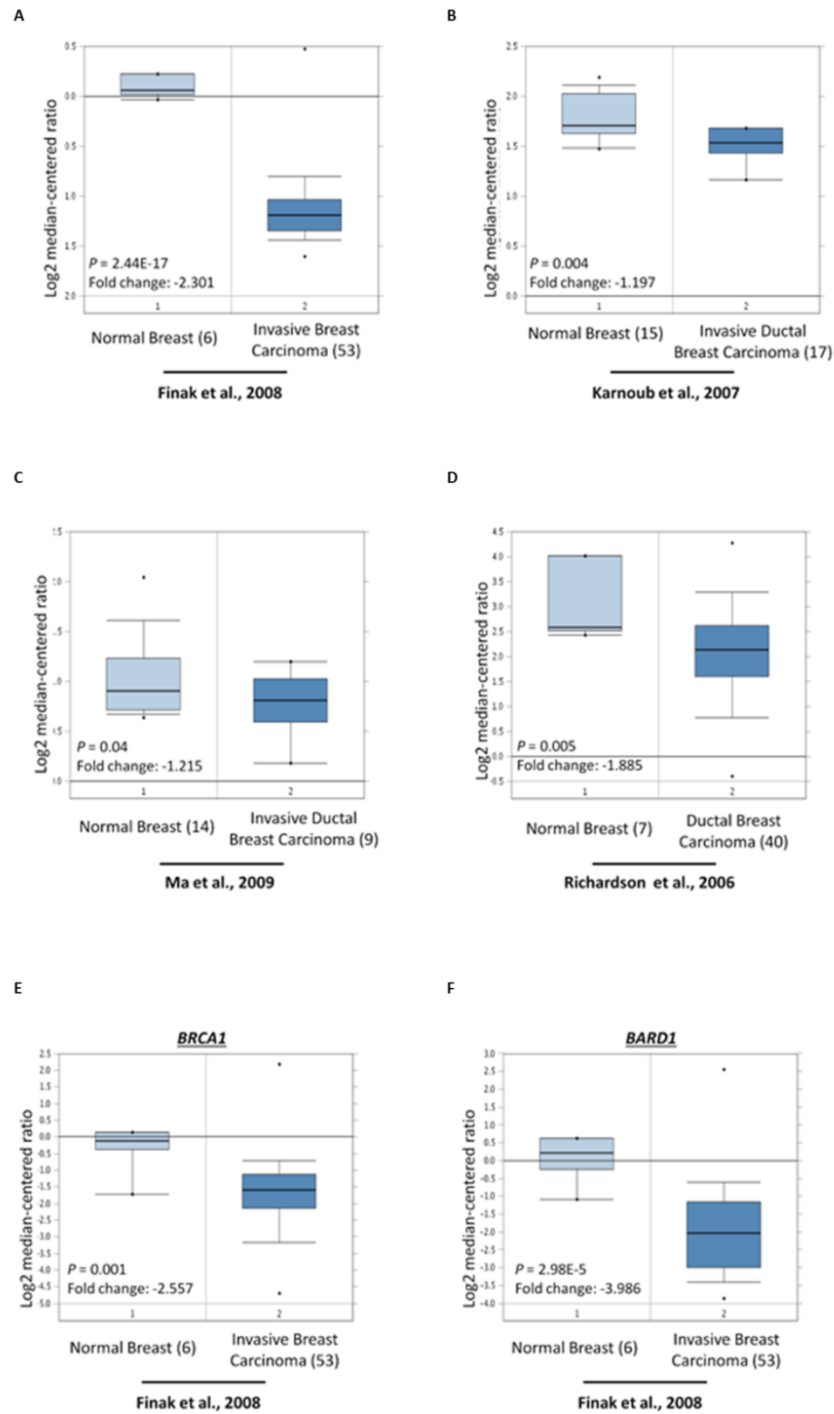


Figure 27 OncoPrint data-mining analysis of *KSR1* expression in different datasets.

KSR1 expression was decreased in invasive ductal breast cancers compared to normal breast tissue in Finak et al (A), Karnoub et al (B), Ma et al (C) and Richardson et al (D). In Finak et al dataset, a similar pattern of reduced *BRCA1* (E) and *BARD1* (F) mRNA levels was also observed in breast carcinoma in comparison to normal breast tissues.

3.2.1.6 Kaplan Meier Plotter analysis supports the good prognostic outcome of *KSR1*

Using public available microarray data from n=2878 breast cancer patients, we further validated the correlations between *KSR1* expression and clinical outcome. The positive prognostic effect of *KSR1* expression was supported by Kaplan Meier Plotter analysis (www.kmplot.com) [384], which showed that high *KSR1* abundance correlates with a better disease free survival using microarray data from n=2878 breast cancer patients (**Figure 28**).

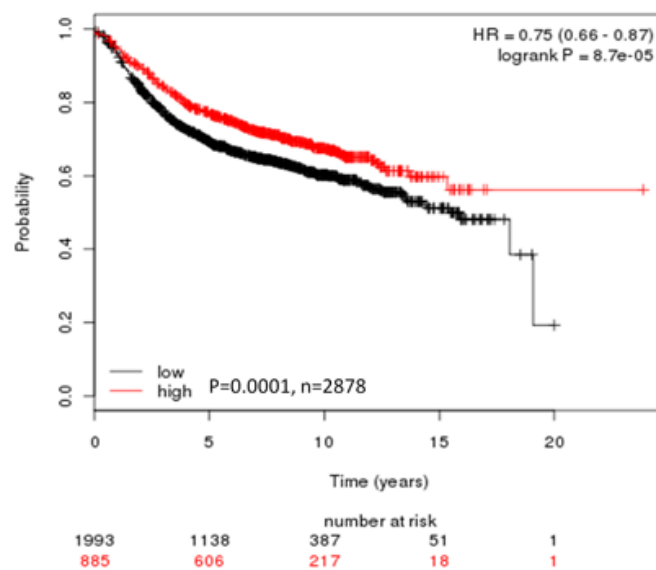


Figure 28 Kaplan Meier Plotter analysis of *KSR1* expression in breast cancer.

Kaplan-Meier survival plots demonstrating the good prognostic effect of *KSR1* overexpression correlates with a longer disease free survival in breast cancer patients gene samples (n=2878, P=0.0001).

3.2.2 The *KSR1* gene interval shows loss of heterozygosity (LOH) in primary breast tumour DNA

The Oncomine analysis revealed that *KSR1* gene expression is reduced in multiple breast cancer datasets which suggested that this gene may be silenced during breast cancer progression. Therefore, we examined SNP and copy number in the *KSR1* gene interval in paired tumour and plasma of patients with breast cancer (n=65) who were on follow-up [353], and identified LOH at *KSR1* in 35% of the primary tumour DNA samples. Moreover, in 10% of these patients matched LOH was also detectable in paired circulating cell-free DNA (cfDNA) samples taken at an average of 6 and 9 years after surgery and treatment respectively. In the rest of these patients, there was heterogeneity of LOH in cfDNA (**Figure 29**). We also examined concomitant LOH in *BRCA1* and *KSR1* in an independent cohort of 28 patient samples and found the following: 1). Concomitant LOH in 32% (9/28); 2). *KSR1* LOH only in 21% (6/28); 3). *BRCA1* LOH only in 4% (1/28); 4). LOH in neither genes in 43% (12/28). Thus, *KSR1* loss can occur independently of loss of *BRCA1*.

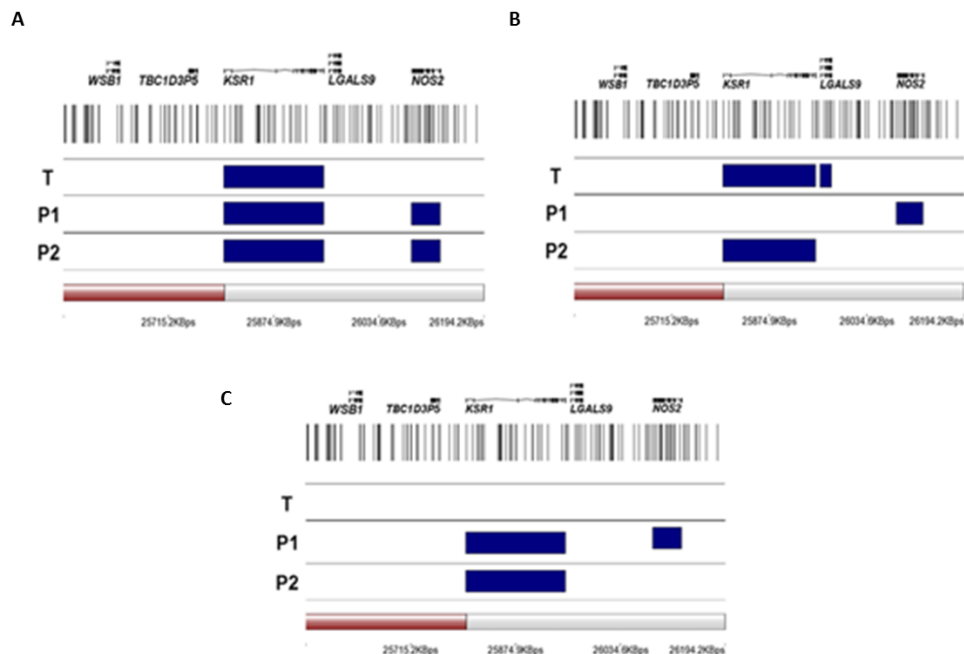


Figure 29 Loss of heterozygosity (LOH) reported in *KSR1* and surrounding genes in primary breast tumour and circulating cfDNA.

LOH was derived on a paired basis (matched to normal leucocyte DNA). In individual patients, common LOH was reported in: **A.** primary tumour DNA and matched 6- and 9-year cfDNA samples; **B.** primary tumour DNA and just 9-year cfDNA sample; and **C.** just the 6- and 9-year cfDNA samples. T, primary tumour DNA; P1, 6-year cfDNA sample; P2, 9-year cfDNA sample. Genomic positions of SNP markers from the chip are represented by vertical bars.

3.2.3 KSR1 is not a positive regulator of the canonical Ras-Raf-MAPKs pathway in breast cancer

3.2.3.1 KSR1 is ubiquitously expressed in breast cancer cell lines

To define the role of KSR1 *in vitro*, we examined its mRNA and protein expression levels in a panel of breast cancer cell lines and MCF10A non-tumourigenic human mammary gland cell. As shown in **Figure 30A**, *KSR1* gene is expressed in all the breast cell lines we have tested, although its expression varies individually. This variability in expression pattern was also observed in its protein expression levels (**Figure 30B**). Additionally, in certain cell lines such as BT474 and MDA453, the mRNA and protein expression levels are not well correlated, a potential indicator of post-transcriptional regulation.

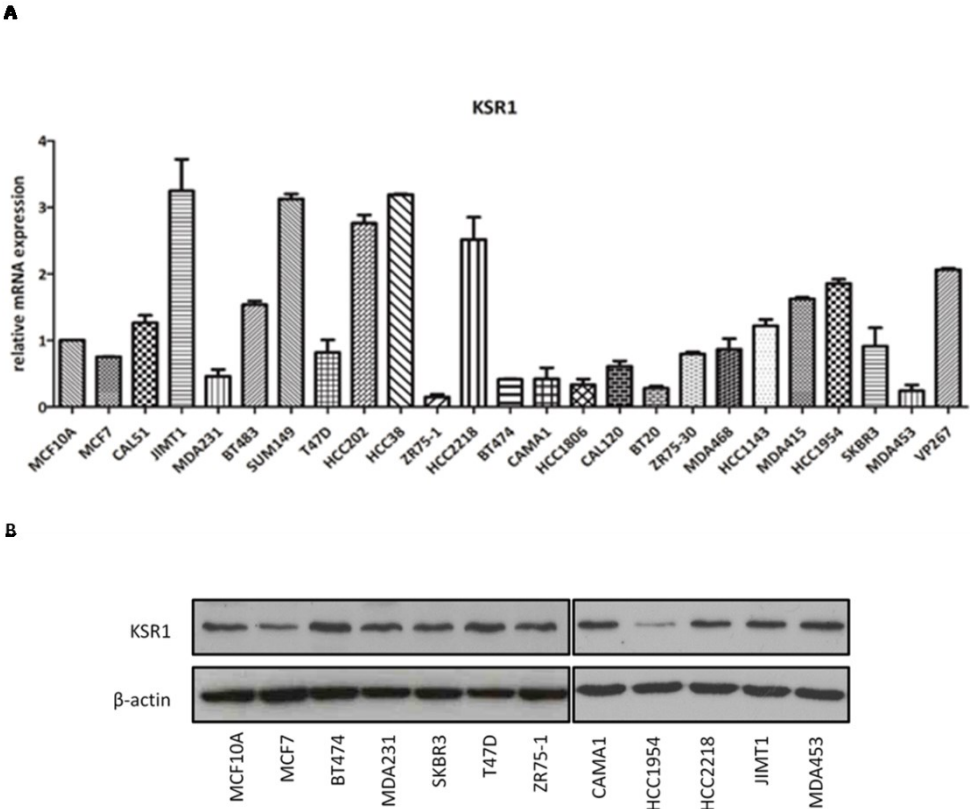


Figure 30 Expressions of KSR1 mRNA and protein in mammary epithelial cell MCF10A and 24 breast cancer cell lines.

A. KSR1 mRNA levels were measured by RT-qPCR. KSR1 expression in MCF10A is set as 1 and all other 24 cell lines are normalized relative to MCF10A mRNA levels. Error bars represent SD of 3 experiments, each in triplicate. **B.** KSR1 protein expressions were detected by western blotting using anti-KSR1 antibody. Representative blot of KSR1 expression is shown. Experiments were repeated at least three times.

3.2.3.2 KSR1 is not a positive regulator of the canonical Ras-Raf-MAPKs pathway in breast cancer

The clinical significance of KSR1 in breast cancer suggests a tumour suppressive role, which contradicts its documented oncogenic function in other cancer types. Moreover, KSR1 has been well established as a positive regulator of the Ras-Raf-MAPKs pathway playing an oncogenic role in Ras-transformed cancers such as pancreatic, lung and skin cancer [43]. We sought to study its effects on the main players in Ras-Raf-MAPKs cascades, including BRAf, CRAf, MEK1/2 and ERK1/2.

First of all, we overexpressed KSR1 in a breast cancer cell line MCF7 in the presence of serum. As shown in **Figure 31A**, our western blotting results showed that total and phospho-protein levels of BRAf, CRAf, MEK1/2 and ERK1/2 remained unaltered after KSR1 overexpression. We further confirmed this observation in another two breast cancer cells including T47D and ZR75-1 (**Figure 31B and 31C**). We then went on to check the effects of knockdown KSR1 in this context. Similarly, no significant changes in total and phospho-protein levels of BRAf, CRAf, MEK1/2 and ERK1/2 were observed upon KSR1 silencing in these cell lines (**Figure 31**).

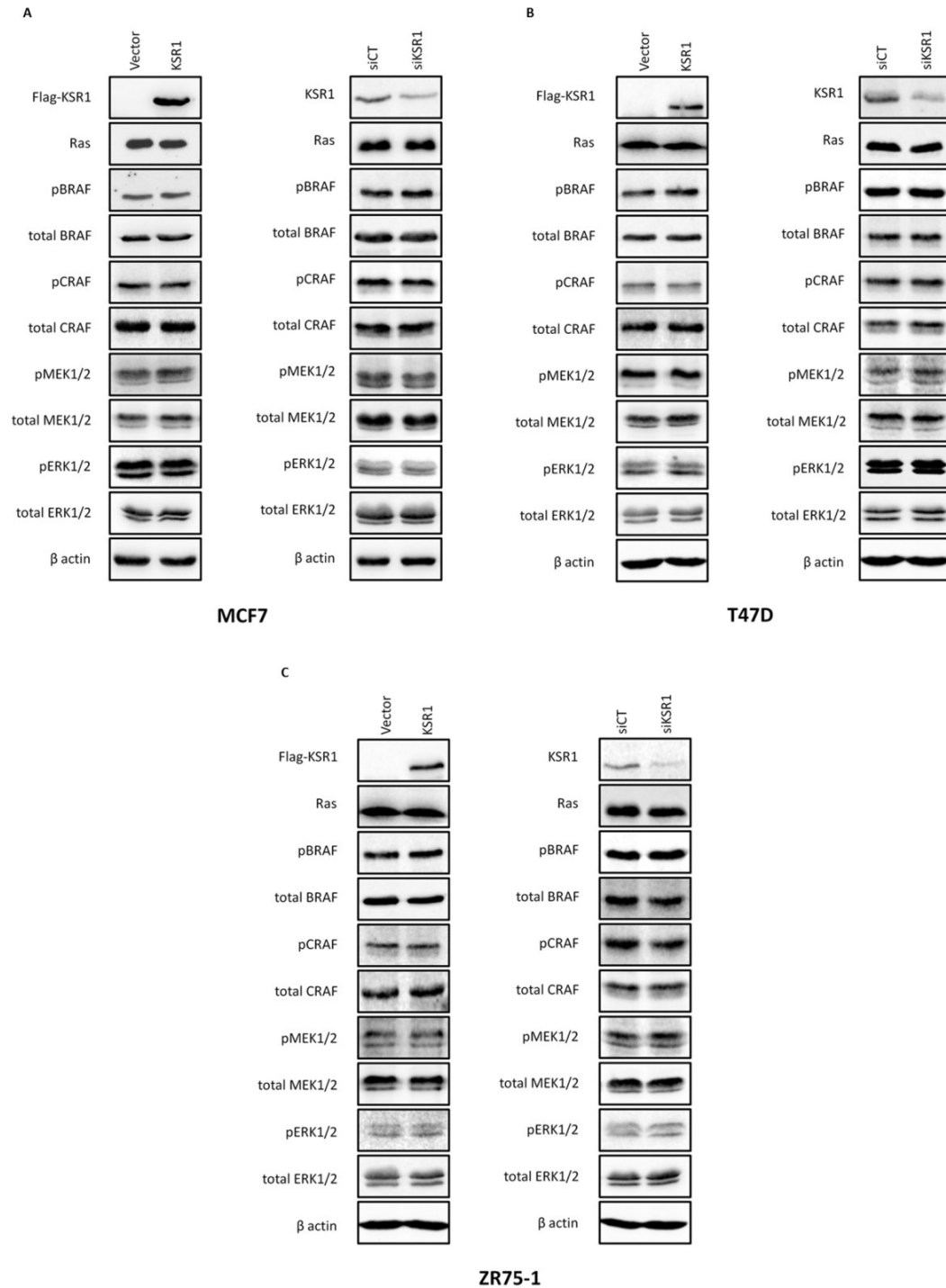


Figure 31 Effect of KSR1 on activation of canonical Ras-Raf-MAPKs pathway in breast cancer.

For KSR1 overexpression, breast cancer cell lines including MCF7 (A), T47D (B) and ZR75-1 (C) were transiently transfected with either pCMV6-KSR1 or pCMV6-vector for 24 hours in full medium. For KSR1 silencing, cells were transfected with either control siRNA (siCT) or siKSR1 for 72 hours. Subsequently, the protein expression levels of KSR1, Ras, pBraf, total Braf, pCraf, total CRAf, pMEK1/2, total MEK1/2, pERK1/2 and total ERK1/2 were assessed by western blotting using specific antibodies as indicated. Representative blots are shown here. Experiments were repeated at least three times.

To further address whether KSR1 has an effect on Ras-Raf-MAPKs signalling under stimulus conditions, MCF7 cells were treated with EGF at various time points (0, 3, 5, 10 mins) after KSR1 overexpression. The western blotting results showed that no significant changes were observed in pERK1/2 protein levels upon KSR1 overexpression, confirming that KSR1 is not a positive regulator after EGF-stimulus (**Figure 32**).

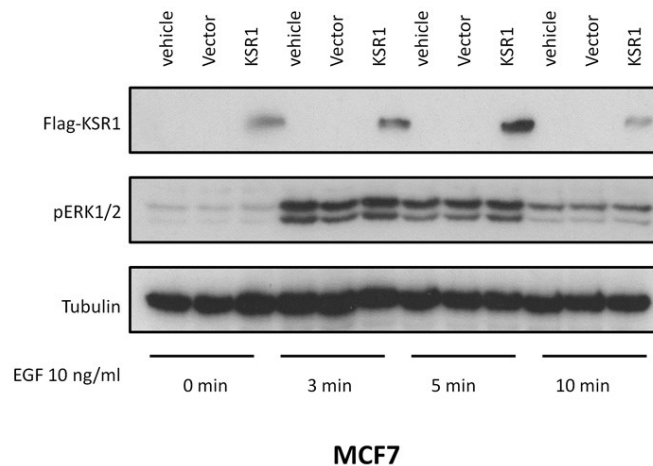


Figure 32 Effect of KSR1 on activation of ERK upon EGF treatment.

MCF7 cells were stripped for 72 hours before transfected with lipofectamine 2000 (vehicle), pCMV6-vector or pCMV6-KSR1. After 24 hours of transfection, cells were treated with EGF (10 ng/ml) and collected at the indicated time points (0, 3, 5 and 10 mins). The protein levels of Flag-KSR1, pERK1/2 and Tubulin were determined by western blotting by specific antibodies as indicated. Representative blots are shown here. Experiments were repeated at least three times.

3.2.4 KSR1 overexpression inhibits tumour formation and growth *in vitro* and *in vivo*

Given the fact that Ras-mutations are rare in breast cancer, our data so far suggest that the biological functions of KSR1 are different to and independent of the canonical Ras-Raf-MAPKs signalling in this setting. We next assessed whether KSR1 has a tumour suppressive effect on breast cancer formation *in vitro* and *in vivo* as suggested by its clinical correlations.

3.2.4.1 Generation of KSR1 stably overexpressing breast cancer cell lines

In order to study the role of KSR1 in breast cancer growth and colony formation, we generated a breast cancer cell line stably overexpressing KSR1 (MCF7-KSR1) through G418 selection as described in methods. Single clones were selected and KSR1 overexpression was confirmed by western blotting (**Figure 33A**).

3.2.4.2 KSR1 overexpression inhibits tumour formation *in vitro*

Firstly, 3D Matrigel-overlay assay allowing integration of the extracellular matrix signalling components was performed to study the tumourigenic effect of KSR1 using the selected clones from MCF7 cells as described above. Overexpression of KSR1 significantly reduced the size of the large and loose aggregates compared to the parental MCF7 cells (**Figure 33B**).

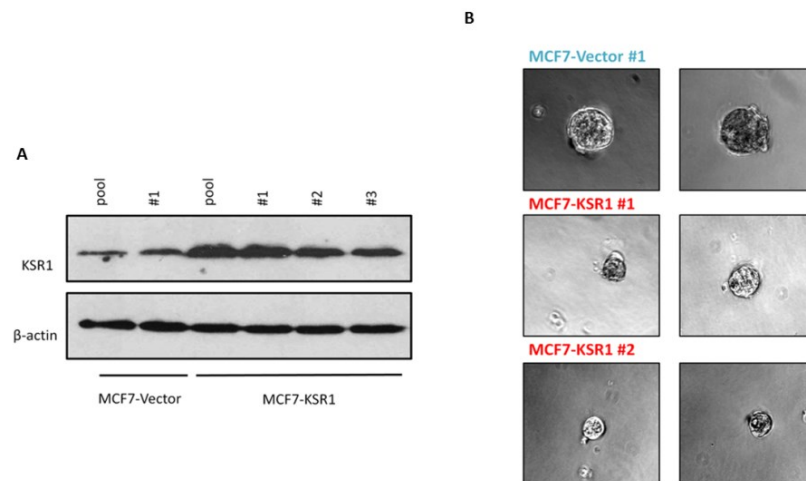


Figure 33 Validation of MCF7 stably overexpressing KSR1 cell lines and its effect on tumourigenesis in 3D matrigel assay.

A. MCF7-KSR1 and MCF7-vector stable cells were generated by transfection of either pCMV6-KSR1 or pCMV6-vector into MCF7 cells and single clones were selected in the presence of G418 (1 mg/ml). KSR1 overexpression was confirmed by western blotting. Representative blots are shown here. **B.** 3D matrigel assay was performed as described above using different MCF7-KSR1 clones and representative images from three independent experiments are shown here.

We next performed a soft agar assay to observe the tumour colony formation upon KSR overexpression. MCF7-KSR1 cells stably overexpressing KSR1 were seeded in medium with soft agar and cultured for 14 days. At the end of experiment, cell colonies were imaged, counted and colony sizes were measured. As shown in **Figure 34A**, MCF7-KSR1 cells derived much fewer and smaller colonies compared to MCF7-vector cells. Subsequent analysis also confirmed this finding (**Figure 34B**, $P < 0.001$).

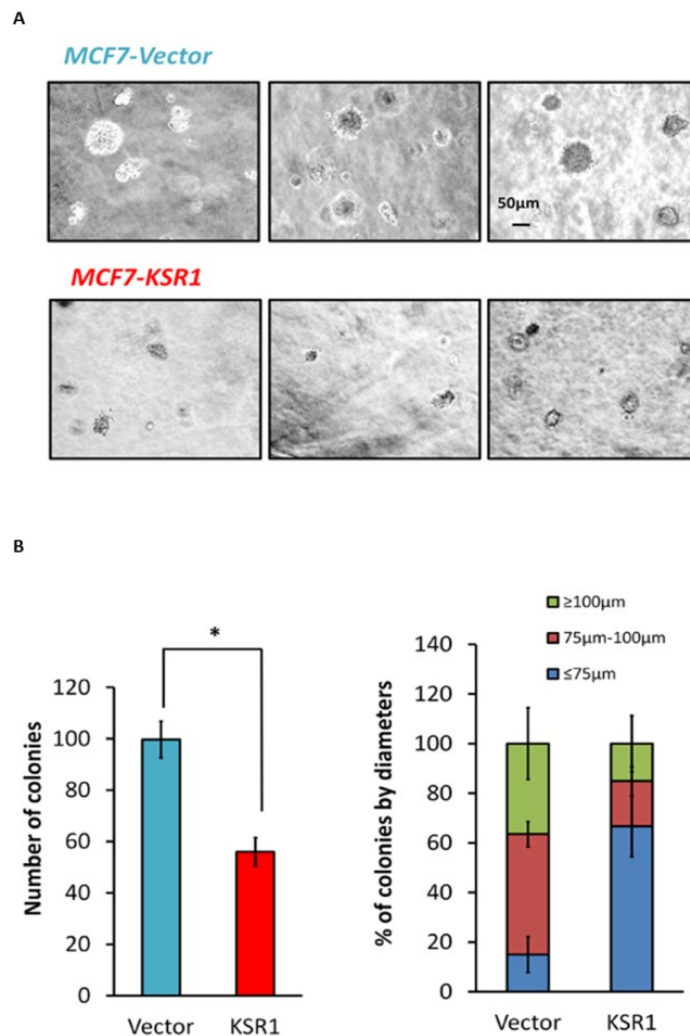


Figure 34 Effect of KSR1 on colony formation in soft agar assay.

A. MCF7-vector or MCF7-KSR1 cells were incubated for 14 days in soft agar. Optical sections of representative colonies are shown. Scale bar, 50 μm . **B.** Colonies from soft agar assays were counted in 5 random fields per well. Diameters of colonies were measured and grouped as (≤ 75 μm , 75-100 μm and 100 μm). Bar chart shows data of three independent experiments, mean $\pm$ SD ($*P < 0.001$, by Student's t-test).

To further prove this finding in other breast cancer cells, we generated another breast cancer cell line stably overexpressing KSR1 (MDA231-KSR1) and exerted the same 3D Matrigel-overlay assay. Similar results were obtained showing that KSR1 overexpression cells formed smaller size acini compared to parental MDA231 cells (**Figure 35**).

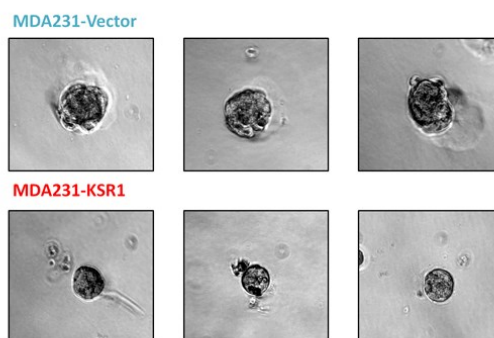


Figure 35 Effect of KSR1 on tumourigenesis in MDA231 cells by 3D matrigel assay. 3D matrigel assay was performed as above using stably overexpressing KSR1 (MDA231-KSR1) and MDA231-vector. Representative images from three independent experiments are shown here.

3.2.4.3 KSR1 overexpression inhibits tumour growth *in vivo*

We next examined the action of KSR1 overexpression on tumour growth in breast cancer xenograft mouse model *in vivo*. To do so, we subcutaneously injected MCF7 stably overexpressing cells MCF7-KSR1 or MCF7-parental cells into BALB/c nude mice. The tumour volumes of breast cancer xenografts overexpressing KSR1 were markedly reduced compared with the control group (**Figure 36A and 36B**). Immunohistochemistry analysis of mouse tumour tissues showed that the Ki67 proliferation index was strongly decreased in MCF7-KSR1 mice with higher KSR1 expression, lending further support to our hypothesis (**Figure 36C**). Collectively, our *in vitro* and *in vivo* results demonstrate the anti-proliferative effect of KSR1 in breast cancer, supporting our clinical results.

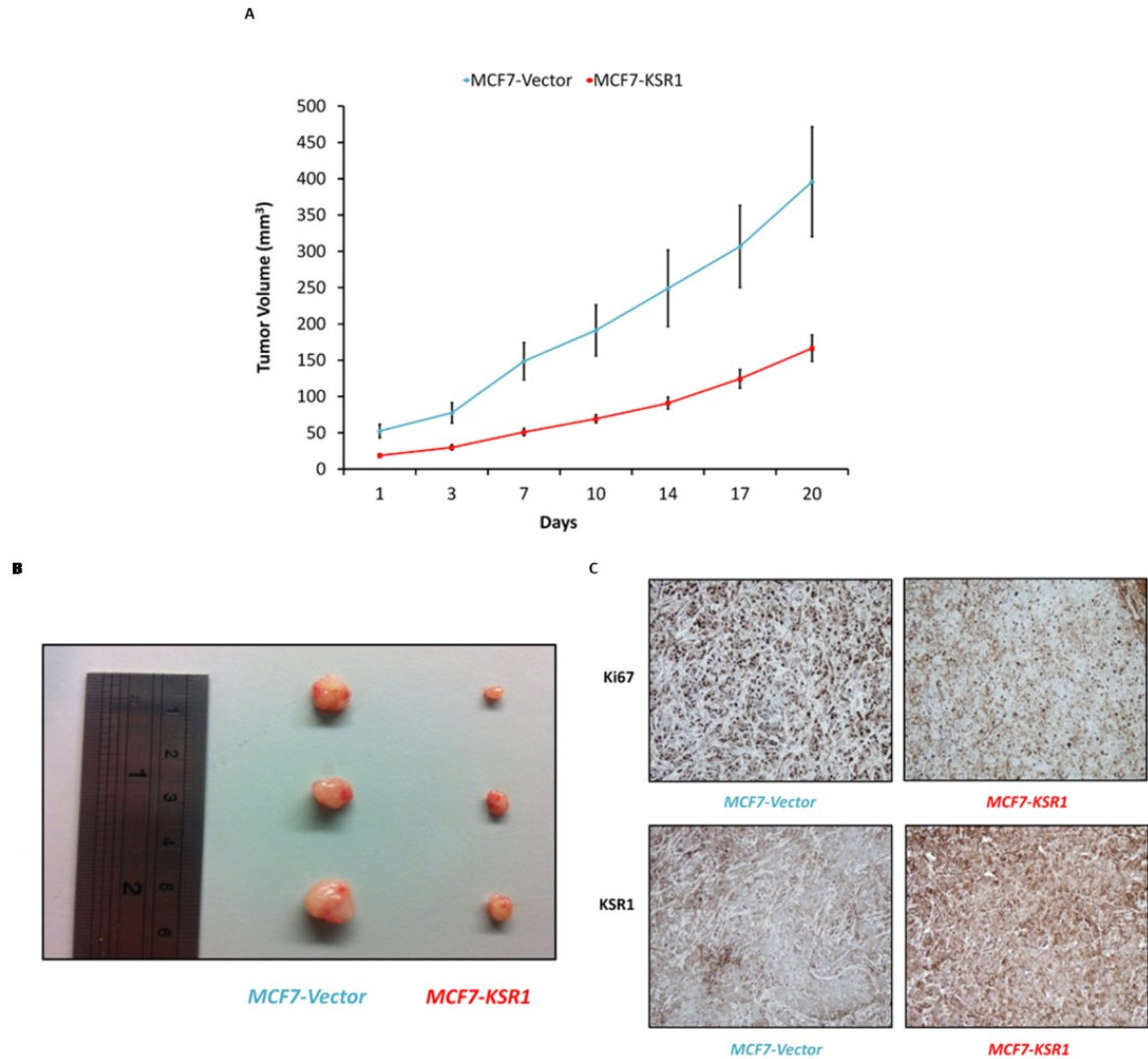


Figure 36 Effect of KSR1 overexpression on tumour growth *in vivo*.

A. Breast cancer xenograft model in BALB/c nude mice. 5×10^6 MCF7-vector or MCF7-KSR1 cells were injected subcutaneously in the flanks of nude mice ($n = 9$). Tumour volume was measured every 3 days using callipers. Growth curves shown are mean tumour $\pm$ SD. **B.** Representative set of xenografts derived from MCF7-vector or MCF7-KSR1 cells are shown. **C.** Representative IHC analyses for KSR1 and Ki67 on paraffin-embedded section of xenografts derived from MCF7-parental or MCF7-KSR1 cells.

3.2.5 KSR1 stabilizes BRCA1 by reducing BRCA1 ubiquitination and inhibits tumour growth through BRCA1

Based on the positive correlation between KSR1 and BRCA1 expressions in the clinical samples and the tumour growth inhibitory role of KSR1, we then examined the effect of KSR1 on BRCA1 protein and whether its tumour suppressive action is BRCA1-dependent.

3.2.5.1 KSR1 affects the protein abundance of BRCA1 but not the mRNA levels

We first studied the effect of KSR1 on BRCA1 mRNA expression. As shown in **Figure 37A**, overexpression of KSR1 in MCF7 cells had no effect on BRCA1 mRNA levels after 24 hours transient transfection. However, our western blotting showed that KSR1 overexpression increased BRCA1 protein abundance after 24 and 48 hours of transfection (**Figure 37B**). We then checked whether KSR1 has the same effect in other breast cancer cell lines. MDA231 and ZR75-1 cells were transfected with pCMV6-vector or pCMV6-KSR1 for 24 hours. Consistently, similar results showed that BRCA1 protein expression was elevated after KSR1 up-regulation in both MDA231 and ZR75-1 cells (**Figure 37C**).

We then went on to check the effect of KSR1 silencing on BRCA1 protein expression. Firstly, MCF7 cells were transfected with sicontrol (siCT) or siKSR1 for 72 hours. Western blotting was subsequently performed to measure the protein abundance of BRCA1. As shown in **Figure 37D**, KSR1 silencing resulted in a decrease of BRCA1 protein. Likewise, consistent results were observed in other two breast cancer cell lines, MDA231 and ZR75-1, which showed that BRCA1 protein was down-regulated upon knockdown of KSR1 (**Figure 37E**). These data support that KSR1 affects the protein abundance of BRCA1 but not its mRNA levels.

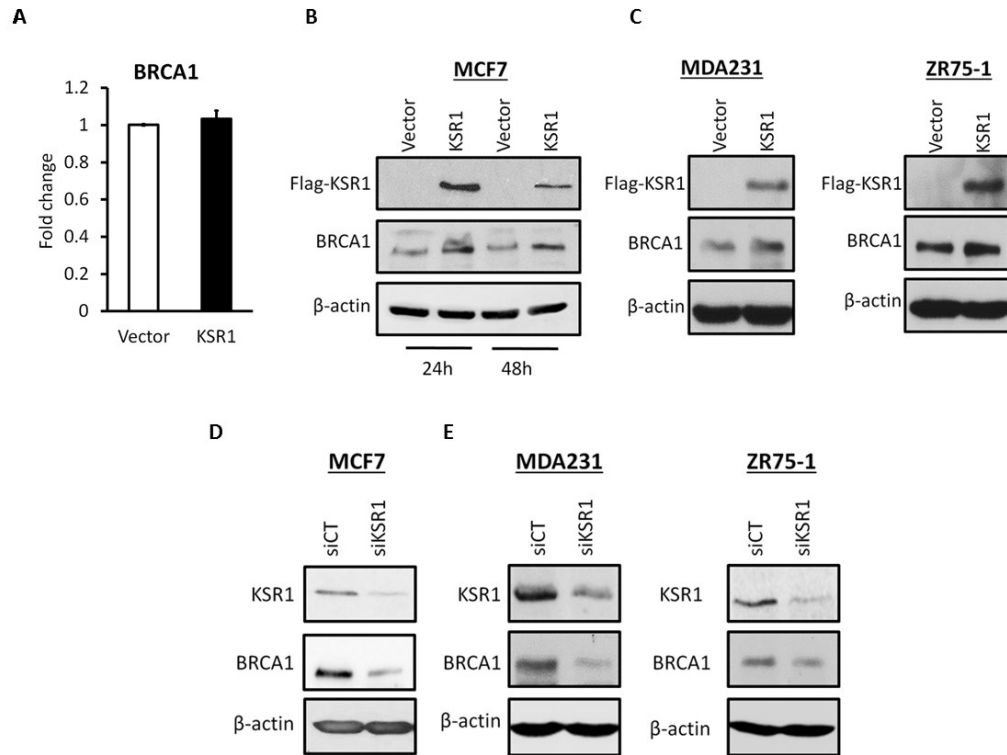


Figure 37 Effect of KSR1 on BRCA1 mRNA/protein levels.

A. MCF7 cells were transfected with either pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. RNA was extracted and RT-qPCR was performed to measure *BRCA1* gene expression. **B.** MCF7 cells were transfected with either pCMV6 vector or pCMV6-KSR1 plasmids for 24 or 48 hours. The protein expression levels of BRCA1 were assessed by western blotting using indicated antibodies. **C.** MDA231 or ZR75-1 cells were transiently transfected with either pCMV6 or pCMV6-KSR1 plasmids for 24 hours. The protein expression levels of BRCA1 were assessed by western blotting. **D.** For knock-down of KSR1, MCF7 cells were transfected with either control siRNA (siCT) or siKSR1 for 72 hours. BRCA1 abundance was assessed by western blotting. **E.** MDA231 or ZR75-1 cells were transfected with either control siRNA (siCT) or siKSR1 for 72 hours. BRCA1 abundance was assessed by western blotting. Representative blots are shown here. Experiments were repeated at least three times.

3.2.5.2 KSR1 stabilizes BRCA1 through reducing BRCA1 ubiquitination

Based on our results above showing that KSR1 affects the protein expression of BRCA1, we next decided to examine whether KSR1 is involved in BRCA1 degradation. MCF7 cells were transfected with either pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours followed by treatment with different proteasome inhibitors MG115 or MG132 (10 μ M, 6h), which can impede BRCA1 degradation through its ubiquitination. Subsequently, BRCA1 protein expression was measured by western blotting. Our results showed that the effect of KSR1 overexpression on BRCA1 protein levels was compromised in the presence of MG115 and MG132, as the fold change of BRCA1 abundance in the absence of proteasome inhibitors was 2.4 and subsequently declined to 1.5 (MG132) or 1.6 (MG115) (**Figure 38**). This indicated that KSR1 might affect BRCA1 stability partly by regulating BRCA1 degradation and ubiquitination, although an alternative mechanism other than protection from ubiquitin-mediated degradation is possible.

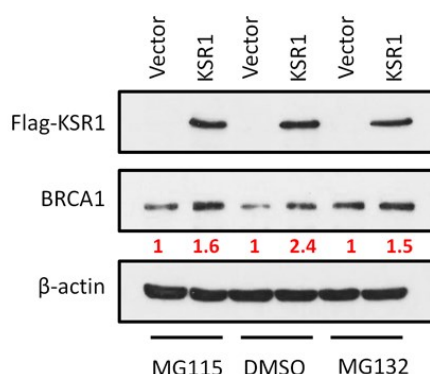


Figure 38 Effect of KSR1 overexpression on BRCA1 protein levels in the presence of proteasome inhibitors.

MCF7 cells were transiently transfected with pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours, followed by treatment with MG115 or MG132 (10 μ M, 6h). BRCA1 protein levels were then assessed by western blotting with specific antibodies as indicated. Representative blots are shown here. Experiments were repeated at least three times.

Therefore, we sought to investigate the effect of KSR1 overexpression on BRCA1 ubiquitination. As shown in **Figure 39A**, a ubiquitination assay revealed that ubiquitinated BRCA1 was reduced upon overexpression of KSR1 in MCF7 cells. In order to show whether this happens in other breast cancer cells, we performed the same ubiquitination assay in MDA231 cells where similar result was also observed (**Figure 39B**). In summary, our data

demonstrates that KSR1 appears to stabilize BRCA1 through reducing ubiquitination of BRCA1.

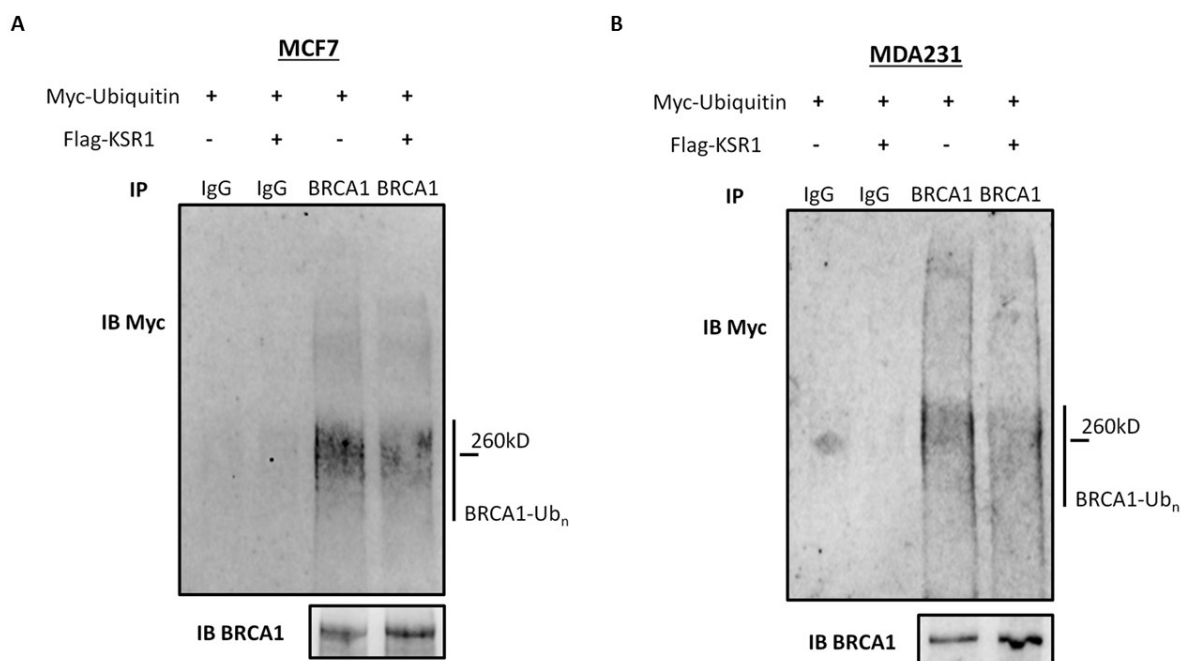


Figure 39 Effect of KSR1 on BRCA1 ubiquitination.

A. MCF7 or **B.** MDA231 cells were transfected with either pCMV6 vector or pCMV6-KSR1 plasmids together with Myc-Ubiquitin as indicated for 48 hours and followed by treatment with MG132 for 6 hours. Subsequently, protein samples were extracted and immunoprecipitation was then performed with IgG or specific anti-BRCA1 antibody. Western blotting was implemented to measure the Myc-tagged ubiquitinated BRCA1 using anti-Myc antibody. Representative blots are shown here. Experiments were repeated at least three times.

3.2.5.3 The inhibitory effect of KSR1 on tumour growth is through BRCA1

Based on the fact that BRCA1 is a well-established tumour suppressor in breast cancer and the described link herein between KSR1 and BRCA1, we suspected that KSR1 acts as an inhibitory factor through BRCA1 in this setting. Therefore, we examined whether the tumour suppressive action of KSR1 is dependent on BRCA1. *In vitro* 3D Matrigel and soft agar assays were performed to examine tumour formation after different combinations of KSR1 and BRCA1 expression levels.

As shown in **Figure 40A (upper panel)** and **Figure 40C**, 3D Matrigel assay showed that comparing with MCF7-vector cells, MCF7-KSR1 cells formed much smaller size acini (**lane 1 vs 3**), which is consistent to our previous finding. Furthermore, the tumour inhibitory effect

of KSR1 was abolished when BRCA1 protein was silenced, as 3D Matrigel demonstrated that the colonies recuperated in this setting (**lane 3 vs 4**). Similarly, in the soft agar assay (**Figure 40B, lower panel; Figure 40D**), our results revealed that the size of colonies derived from MCF7-KSR1 cells was significantly smaller than the counterparts formed by MCF7-vector cells. Moreover, when BRCA1 protein was deleted, the colonies generated by MCF7-KSR1 cells were recovered (**lane 3 vs 4**). These results strongly support our hypothesis that the inhibitory effect of KSR1 on breast tumour growth is through BRCA1.

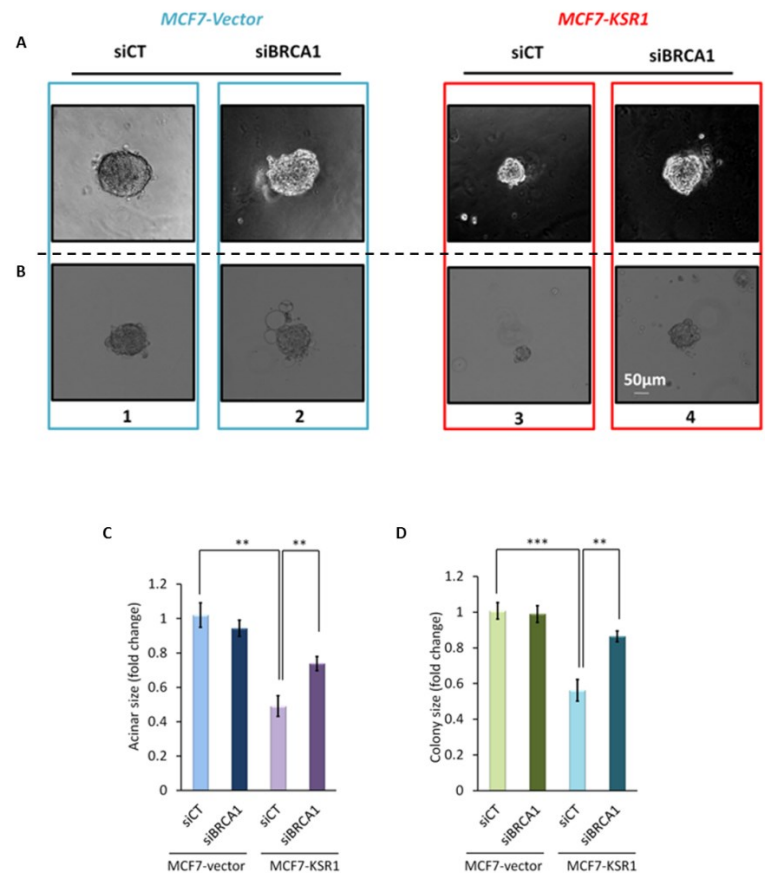


Figure 40 BRCA1 is required for the tumour suppressive effect of KSR1.

A. 3-D Matrigel overlay assay (**upper panel**). MCF7-vector or MCF7-KSR1 stable cells were transfected with either siCT or siBRCA1 for 72 hours and then embedded in Matrigel and cultured for 6 or 7 days in 10% FCS DMEM supplemented with 2% Matrigel. Phase contrast images of representative acini structures were taken in the end of the experiment. Scale bar, 50 μ m. **B.** Soft agar growth assay (**lower panel**). After 72 hours transfection with either siCT or siBRCA1, MCF7-parental or MCF7-KSR1 cells were incubated for 14 days in soft agar. Optical sections of representative colonies are shown. Scale bar, 50 μ m. **C.** Acini or **D.** Colonies (~30 per condition) were measured by Axiovert 100 MetaMorph as described before and normalized to MCF7-vector siCT group. Bar chart shows data of three independent experiments, mean $\pm$ SD (** $P < 0.01$ and *** $P < 0.001$; P value determined by Student's t-test).

3.2.6 KSR1 regulates BRCA1 stability via elevated BARD1 abundance and increased BRCA1-BARD1 interaction

3.2.6.1 KSR1 affects the protein abundance of BARD1 not its mRNA level

To further explore the mechanism through which KSR1 regulates BRCA1 expression, the positive association between KSR1 and BARD1 provided a potential link in the present study. It is already known that BRCA1 forms a heterodimeric complex with BARD1 that promotes stabilization by reducing its ubiquitination [385, 386]. We therefore studied the effect of KSR1 on BARD1 mRNA and protein expression levels. We first studied the effect of KSR1 on *BARD1* mRNA expression. Our RT-qPCR results showed that KSR1 overexpression does not affect the mRNA level of *BARD1* (**Figure 41A**). Interestingly, western blotting revealed that up-regulation of KSR1 increases BARD1 protein levels in MCF7 (**Figure 41B**). Following, we wanted to confirm whether KSR1 has the similar effect on BARD1 expression in other breast cancer cell lines. Therefore, MDA231 and ZR75-1 cells were transfected with pCMV6-vector or pCMV6-KSR1 for 24 hours and western blotting was performed to determine the protein expression levels of BARD1. As anticipated, consistent results were observed from MDA231 and ZR75-1 cells showing that BARD1 protein expression was increased after KSR1 up-regulation (**Figure 41C**).

We next decided to examine the effect of KSR1 knockdown on BARD1 protein expression. As shown in **Figure 41D**, BARD1 protein expression was down-regulated upon KSR1 silencing in MCF7 cells. As expected in MDA231 and ZR75-1 cells, same results by western blotting showed that BARD1 abundance was also decreased after KSR1 silencing (**Figure 41E**). These data support that KSR1 affects the protein abundance of BARD1 not mRNA level.

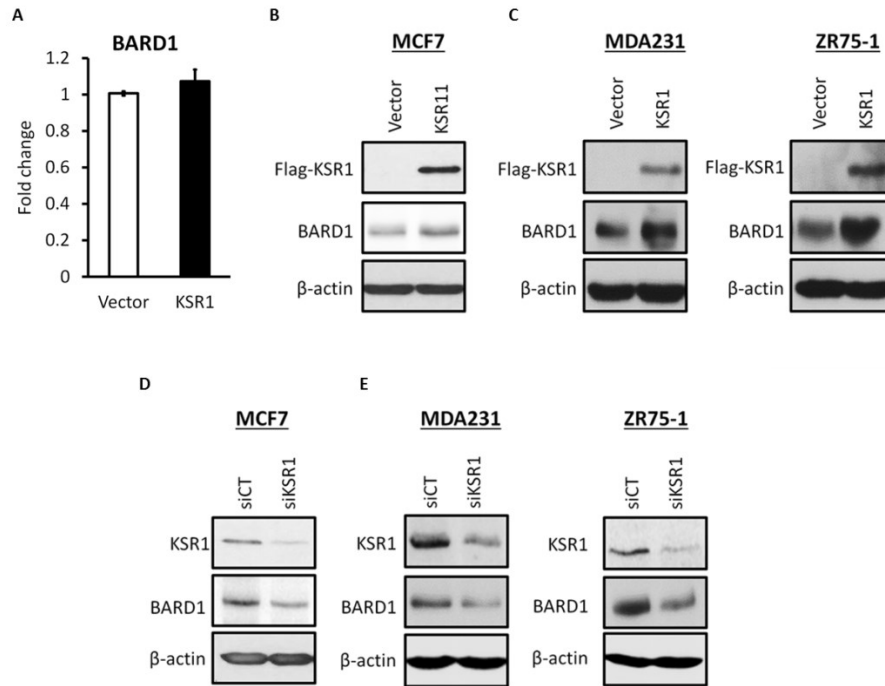


Figure 41 Effect of KSR1 on BARD1 mRNA/protein levels.

A. MCF7 cells were transfected with either pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. RNA was extracted and RT-qPCR was performed to measure *BARD1* gene expression. **B.** MCF7 cells were transfected with either pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. The protein expression levels of BARD1 were assessed by western blotting using indicated antibodies. **C.** MDA231 or ZR75-1 cells were transiently transfected with either pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. The protein expression levels of BARD1 were assessed by western blotting. **D.** For knock-down of KSR1, MCF7 cells were transfected with either control siRNA (siCT) or siKSR1 for 72 hours. BARD1 abundance was assessed by western blotting. **E.** MDA231 or ZR75-1 cells were transfected with either control siRNA (siCT) or siKSR1 for 72 hours. BARD1 abundance was assessed by western blotting. Representative blots are shown here. Experiments were repeated at least three times.

3.2.6.2 The effect of KSR1 on BRCA1 expression is partly through BARD1

To investigate whether the effect of KSR1 on BRCA1 regulation is mediated through BARD1, we decided to silence BARD1 following overexpression of KSR1 to examine the total BRCA1 protein levels. To do so, MCF7 cells were transfected with control siRNA (siCT) or siBARD1 for 48 hours followed by KSR1 transient transfection with pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. As previously reported, silencing of BARD1 resulted in a decrease of BRCA1 expression (**Figure 42A, lane 1 vs 3**). Moreover, KSR1 overexpression after BARD1 deletion did not restore the up-regulated BRCA1 levels in MCF7 (**Figure 42A, lane 2 vs 4**), indicating a requirement of BARD1 in the regulation of BRCA1 by KSR1. However, an alternative mechanism of BRCA1 up-regulation by KSR1

other than through BARD1, such as a potential cooperative action of KSR1 and BARD1 or another unidentified protein involved, cannot be ruled out, as BRCA1 expression was still elevated albeit much less in the BARD1-knockdown cells (**Figure 42A, lane 3 vs 4**). We then went on to check whether this is a more general effect of KSR1 in other breast cancer cell lines. As above, ZR75-1 and MDA231 cells were used to confirm the effects of KSR1 on BRCA1 regulation is through BARD1. The same protocols were performed to measure the BRCA1 protein levels after KSR1 overexpression in BARD1 silenced cells. Consistently, the above observation was confirmed in ZR75-1 and MDA231 cells as well (**Figure 42B and 39C**).

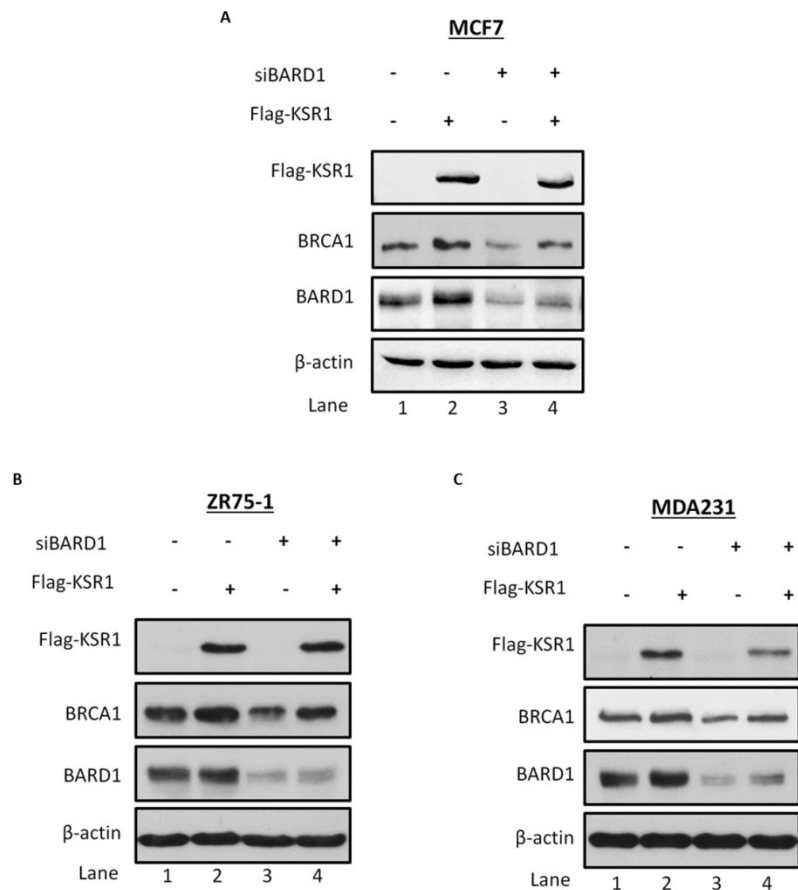


Figure 42 Effect of KSR1 overexpression on BRCA1 protein levels after BARD1 silencing.

A. MCF7 cells were transfected with control siRNA (siCT) or siBARD1 for 48 hours followed by KSR1 transient transfection with pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. The total levels of BRCA1 and BARD1 were assessed by western blotting using indicated antibodies. **B.** ZR75-1 cells or **C.** MDA231 cells were transfected with control siRNA (siCT) or siBARD1 for 48 hours followed by KSR1 transient transfection with pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. The expressions of BRCA1 and BARD1 were measured by western blotting. Representative blots are shown here. Experiments were repeated at least three times.

3.2.6.3 The interaction between BRCA1 and BARD1 is increased upon KSR1 overexpression

We then speculated as to whether KSR1 could directly interact with BRCA1 or BARD1. To determine a direct interaction between KSR1 with BRCA1 or BARD1, anti-KSR1 immunocomplexes were precipitated to examine its capability of co-precipitating endogenous BRCA1 or BARD1 in MCF7 cells. Western blotting of the immunocomplexes with anti-BRCA1 antibody showed that KSR1 did not co-precipitate with BRCA1. However, western blotting of the immunocomplexes with anti-BARD1 antibody showed that KSR1 co-precipitated with endogenous BARD1 (**Figure 43A**). On the other hand, western blotting analysis of anti-BARD1 immunocomplexes showed that BARD1 was capable of co-precipitating with KSR1, as well as BRCA1, which has already been demonstrated previously (**Figure 43B**). These results suggest that KSR1 interacts with BARD1 but not with BRCA1.

Next, we sought to explore the effect of KSR1 on BRCA1-BARD1 complex formation. Anti-BRCA1 immunocomplexes were precipitated to evaluate the interaction between BRCA1 and BARD1. Western blotting revealed that the interaction between BRCA1 and BARD1 was increased after KSR1 overexpression (**Figure 43C**). We could therefore conclude that KSR1 increases BARD1 abundance resulting in elevated BRCA1 and enhanced steady state level of the BRCA1-BARD1 dimer.

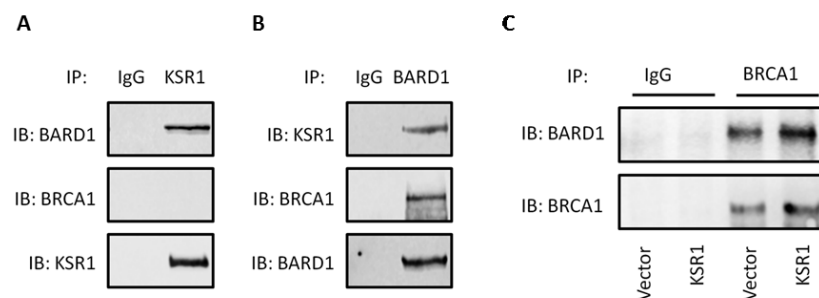


Figure 43 Effect of KSR1 on BRCA1 and BARD1 interaction.

A. Cell lysates from MCF7 were immunoprecipitated (IP) with an anti-KSR1 antibody. Subsequently, western blotting was performed to examine the interaction between KSR1 with BRCA1 and BARD1 with specific antibodies indicated. **B.** Cell lysates from MCF7 were immunoprecipitated (IP) with an anti-BARD1 antibody and western blotting was exerted to confirm the interaction between KSR1 and BARD1 with the indicated antibodies. **C.** Effects of KSR1 overexpression on the interaction between BRCA1 and BARD1. MCF7 cells were transiently transfected with pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. The interactions between BRCA1 and BARD1 were detected by immunoprecipitation (IP) of BRCA1 followed by western blotting with an anti-BARD1 antibody. Representative blots are shown here. Experiments were repeated at least three times.

3.2.6.4 BARD1-BRCA1 nuclear abundance is increased upon KSR1 overexpression

We then examined whether KSR1 can increase BARD1-BRCA1 nuclear abundance, which is linked to BRCA1 activation and essential for the tumour suppressive function of BRCA1. To do so, we performed cell fractionation assays. MCF7 cells were transfected with either pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. Subsequently, cytoplasmic and nuclear proteins were isolated following manufacturer's protocol. Our results showed that KSR1 overexpression resulted in an increase of nuclear accumulation of both BARD1 and BRCA1 (**Figure 44**), which can consequently result in an enhancement of BRCA1 activity.

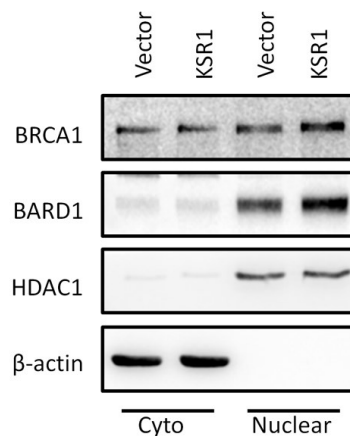


Figure 44 Effect of KSR1 on subcellular localization of BRCA1 and BARD1.

MCF7 cells were transiently transfected with pCMV6 vector or pCMV6-KSR1 plasmids for 24 hours. Protein fractions from the nucleus and cytoplasm were extracted as described in Materials and Methods. HDAC1 and β -actin expressions served as positive normalising control for nuclear and cytoplasmic proteins respectively. All blots shown are representatives of at least three independent experiments.

3.2.7 Discussion

Scaffolding proteins play an essential role in the assembly of multiprotein complexes that initiate signal transduction in various intracellular compartments; correct complex organization determines key cellular activities including cell proliferation, differentiation and apoptosis [387, 388]. KSR1 was originally identified more than 15 years ago as a novel protein kinase implicated in the Ras-Raf pathway; defects in KSR1 inhibit activated Ras, without affecting the pathways induced by activated Raf [107-109]. Interestingly, it appears that the expression levels of KSR1 can determine its function. Specifically, low levels of KSR1 enhance Ras-induced activity, whereas high KSR1 expression can suppress the Ras pathway [127]. Emerging evidence suggests that KSR1 possesses dual activity as active kinase phosphorylating Raf-1 and MEK1 [144-146] and as a scaffold protein that can also form dimers with Raf modulating its activity [137, 139]. Taken into consideration of its role in Ras-Raf-MAPKs signalling, most work have focused on the contribution of KSR1 in Ras-dependent tumours, including skin [162], pancreatic [166] and lung carcinomas [167]. Its clinical significance and biologic functions have not been comprehensively studied in breast cancer.

Here, we establish a role of KSR1 as an independent prognostic biomarker in breast cancer. High KSR1 expression correlates with a better overall and disease free survival in a large patient cohort (n=1000) with 20 years follow-up. In addition, a trend approaching statistical significance associating KSR1 expression with response to endocrine and chemotherapy treatments was observed perhaps suggesting a predictive role, which requires further validation. This observation, however, is supported by a previous report, in which Kim et al showed that KSR1 overexpression in MCF7 cells increased CDDP sensitivity and apoptosis [168]. Our present study is the first in-depth report to demonstrate a protective role for KSR1 in cancer, as thus far it has been linked to tumour induction in pancreatic and lung cancer [166], indicative of contrasting and tissue specific mechanisms of function in different biologic contexts. In addition, we also showed that KSR1 positively correlates with BRCA1 and BARD1 expression in breast cancer tissues in this cohort. In agreement with our clinical observations, Oncomine analysis revealed that KSR1 gene expression is reduced in four different breast cancer datasets, while as expected it is up-regulated in lung and colorectal cancers probably due to abnormally activated/mutated Ras signalling pathways. However, disparities still exist in different breast cancer datasets, as we recently reported that KSR1 mRNA expression is increased in breast carcinoma in one TCGA dataset contradicting to our

findings in this work [389]. One possible reason is that it is difficult to compare between datasets owing to sometimes small sample sizes as well as the heterogeneity of samples. Another important aspect is that a correlation between KSR1 with BARD1 and BRCA1 was identified in the Finak's dataset in this study, but not in the previous TCGA dataset. This suggests that the tumour suppressive role of KSR1 might require a cooperative action of BARD1 and BRCA1 as we suggest herein. Moreover, a large microarray data set analysis (n=2878) further supported the good prognostic effect of KSR1 overexpression.

We also identified a high frequency of LOH (35%) at the *KSR1* gene interval in primary tumour DNA samples analysed as part of a previous study [353] suggesting a tumour suppressor role for KSR1 in some breast cancers. Additionally, we found LOH at *KSR1* in 10% of matched cfDNA samples taken an average of 6 and 9 years after surgery and treatment respectively. In some cases, we also identified LOH that was unique potentially suggesting the emergence of new tumour clone harbouring a loss of *KSR1*. This finding also suggests that *KSR1* loss can occur independently of loss of *BRCA1*, highlighting its value in cancer screening.

We then explored whether the action of KSR1 is through its involvement in the canonical Ras-Raf-MAPKs pathway in breast cancer. In doing so, we examined a phospho-profile on the main members of this pathway upon overexpression/silencing KSR1 in several breast cancer cell lines. KSR1 has no significant effects on pBRaf, pCRaf, pMEK1/2 and pERK1/2 in both full serum condition and EGF-induced treatment. This finding suggests that the function of KSR1 in breast cancer might not be connected to this pathway, also supported by the fact that Ras mutation is very rare in breast cancer.

Using cells stably overexpressing KSR1, we examined the biological role of KSR1 *in vitro* and *in vivo*. The physiologically relevant 3D-Matrigel overlay assay, which integrates signals from the extracellular matrix was performed, and showed a decrease in the number and size of tumour acini formed by KSR1 overexpressing MCF7 cells. To further address the effect of KSR1 on the tumourigenic potential of breast cancer cells, we performed a soft agar assay, where KSR1-MCF7 cells colony formation was significantly compromised. Moreover, results from mice xenografts revealed that KSR1 up-regulation markedly inhibits tumour growth, supporting a tumour suppressive role of KSR1 in breast cancer. These results add complexity to the role of KSR1 in breast tumourigenesis, as another study of ours recently showed that KSR1 knockdown inhibits the proliferation of breast cancer cell lines including MCF7 (result section 3.1.8) [389]. The discrepancies might be due to the scaffolding function

of KSR1 itself, as while at basal levels scaffold concentration enhances signal transmission, a further increase or decrease beyond an optimal level can inhibit or even reverse the original effect [390]. Consistently, this fact is also strongly supported by other studies that demonstrated that either high or low KSR1 expression exhibits inhibitory effects resulting in regulation of the proliferative potential of cells [120, 122, 126, 127].

The correlation between KSR1 and BRCA1 in breast cancer tissues underscores the potential role of KSR1 in tumour suppression. Our *in vitro* results showing increased BRCA1 levels after KSR1 overexpression are in accordance with the clinical findings. Our results revealed that KSR1 increases BRCA1 protein through reducing its ubiquitination. Of note, this study showed that the tumour suppressive action of KSR1 is BRCA1 dependent as the oncogenic ability of breast cancer cells overexpressing KSR1 recovered when BRCA1 is depleted. The positive association between KSR1 and BARD1 from clinical samples also indicated a link in the potential KSR1-BRCA1-BARD1 complexes. Mechanistically, we also determined that KSR1 does not affect the mRNA levels of BARD1 but increases BARD1 protein, which has been previously reported to form a heterodimer with BRCA1 thereby stabilising it by reducing its ubiquitination [386]. Moreover, the BRCA1-BARD1 interaction is elevated upon KSR1 overexpression, resulting in BRCA1-BARD1 translocation to the nucleus, which is essential for tumour inhibition in breast cancer, as previously described [391].

The effect of KSR1 on BRCA1 is modulated partly by BARD1, as KSR1 overexpression increases BRCA1 less strongly if BARD1 is silenced, indicating a need of BARD1 in the regulation of BRCA1 by KSR1. However, alternative mechanisms of BRCA1 up-regulation by KSR1 other than through BARD1, such as a potential cooperative action of KSR1 and BARD1, cannot be ruled out, as BRCA1 expression was still elevated albeit to a lesser degree in the BARD1-knockdown cells. Our recent work showing that KSR1 inhibits phosphorylation of DBC1, which was previously identified as a transcriptional repressor for BRCA1, suggests an alternative mechanism controlling the effect of KSR1 on BRCA1 [389, 392]. Interestingly, in this same study, we reported that KSR1 negatively regulates p53 activity through modulation of DBC1 (result section 3.1) [389]. Given that DBC1 is a well-studied positive regulator of p53 as well as a repressor for BRCA1, it potentially links KSR1 into a network involving p53 and BRCA1, which merits further investigation. Up to now, we have already shown that KSR1 decreases p53 activity, whereas it increases BRCA1 expression. A rational explanation to the discrepancies between the previous study and the current one regarding the tumour suppressive function of KSR1 in carcinogenesis might be

that BRCA1 can compensate for loss of p53, as it has been previously shown [393]. Therefore, this allows KSR1 to employ its inhibitory effects through BRCA1 up-regulation. However, further investigation is needed to address the underlying mechanisms. In addition, we have shown that KSR1 interacts directly with BARD1 but not BRCA1. Whether KSR1 regulates BARD1 through phosphorylation or other alternative functions also requires exploration.

Taken together, we present that patients with breast cancer with high KSR1 showed better disease free- and overall survival. Moreover, KSR1 interacts with BARD1 and regulates BRCA1 ubiquitination through elevated BARD1 expression and increased BRCA1-BARD1 interaction. This in turn allows the nuclear import of BRCA1-BARD1 complex leading to increased BRCA1 activity, and lends mechanistic support to the tumour suppressive function of KSR1 in breast cancer (**Figure 45**).

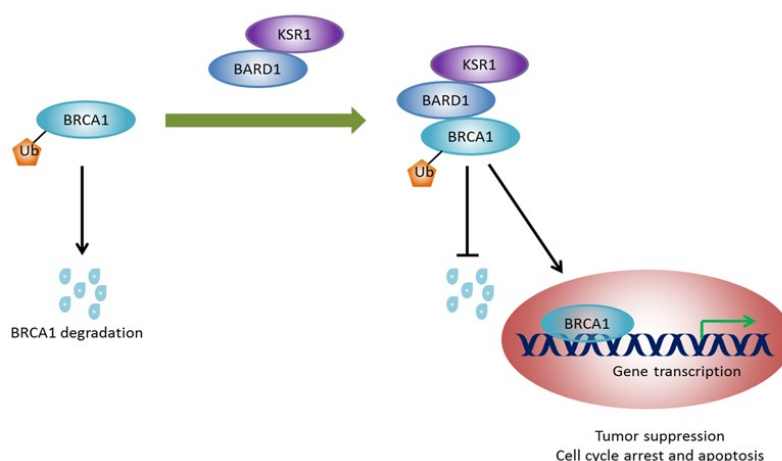


Figure 45 Proposed model of BRCA1 regulation by KSR1.

KSR1 acts as a scaffold protein that interacts with BARD1, which is capable of forming a heterodimer with BRCA1 and stabilising each other. KSR1 regulates BRCA1 degradation, partly through elevated BARD1 expression and increased BRCA1-BARD1 interaction. This in turn results in increased translocation of BRCA1 to the nucleus where it functions as a tumour suppressive protein through regulating cell cycle arrest and apoptosis.

3.3 Global mapping of TKs signalling by combined use of RNAi and quantitative proteomics

3.3.1 Strategy for identification of TKs signalling by combined use of quantitative proteomics and RNAi

The purpose in this study was to perform a global TKs-signalling mapping by using a high throughput RNAi screen combined with SILAC-based quantitative proteomics in breast cancer cells. In doing so, we were hoping to identify an in-depth proteomic signature for each TK investigated herein. Our approach employed to achieve this aim is shown in **Figure 46**.

Briefly, step 1 was to determine the expression profile of all 90 TKs using RT-qPCR and then set a minimum expression threshold to allow candidates to proceed to the next step. In step 2, a certified RNAi library composed of 2 siRNAs against TKs was used to silence each member of the TKs identified from the previous stage. Two follow-up validation assays by RT-qPCR and western blotting were performed to confirm the knockdown efficiency. In step 3, MCF7 cells were primarily cultured in SILAC either R0K0 ‘light’ medium, containing unlabelled $^{12}\text{C}_6$, $^{14}\text{N}_4$ -arginine (Arg) and $^{12}\text{C}_6$ -lysine (Lys) amino acids, or R6K4 ‘medium’, R10K8 ‘heavy’ medium, containing labelled $^{13}\text{C}_6$, $^{15}\text{N}_4$ -Arg and $^{13}\text{C}_6$ -Lys for at least 7 cell divisions and were then transfected with validated siRNAs targeting the 65 TKs that are expressed in MCF7 cells (CT value <34.5). Subsequently, protein samples were taken and LC-MS/MS protein identification and quantification analyses were exerted. Finally, bioinformatic analysis was performed to evaluate changes in signalling dynamics and eventually propose a new classification for the TKs family based on similarities in their regulated proteomes.

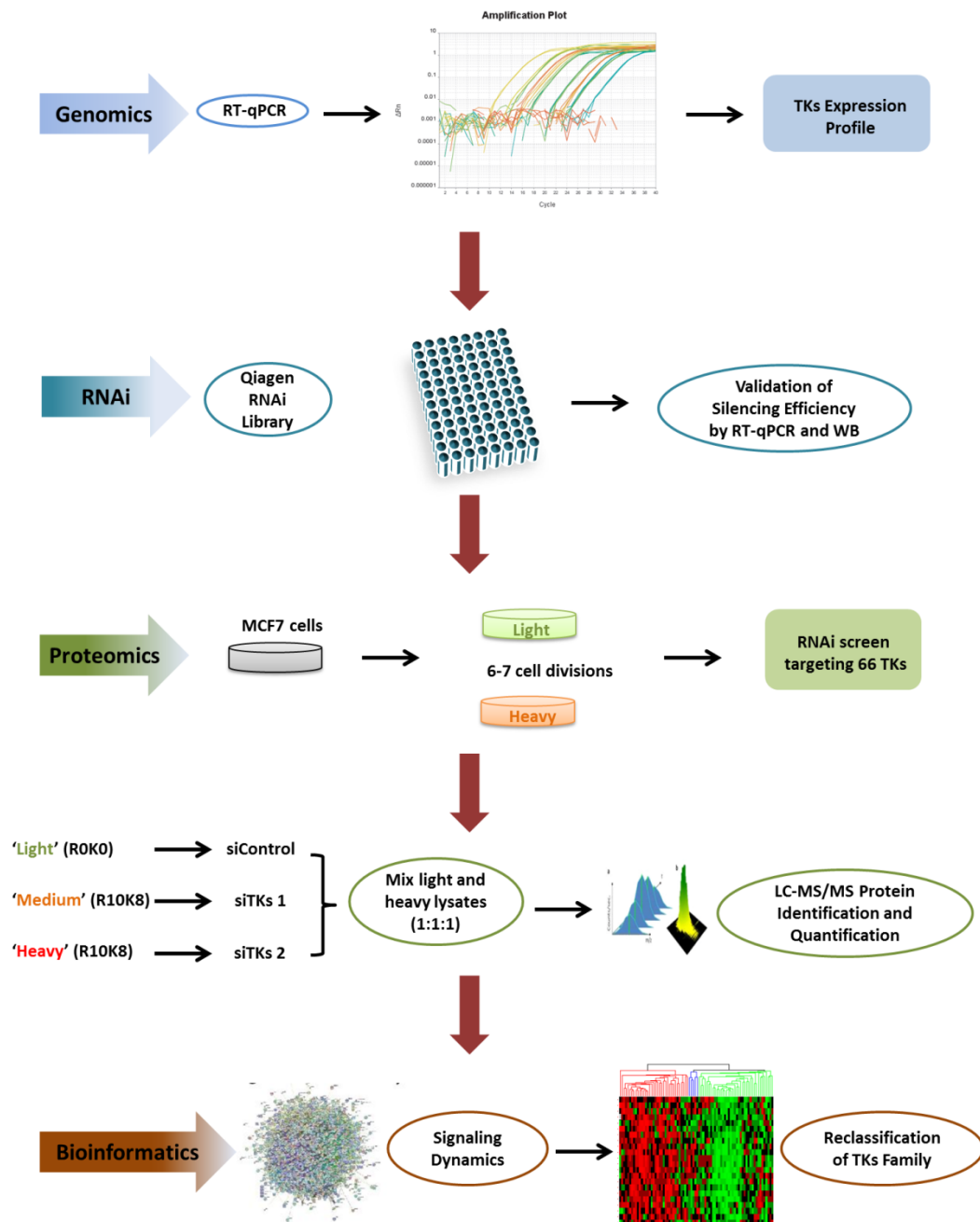


Figure 46 Strategy for identification of TKs signalling by combined use of quantitative proteomics and RNAi.

The expression profile of all 90 TKs was examined using RT-qPCR in MCF7 cells. A certified RNAi library composed of 2 siRNAs against TKs was used to silence each member of the TKs identified from the previous stage. SILAC proteomic analysis was then performed after silencing each verified tyrosine kinase in MCF7 cells. Bioinformatic analyses were subsequently used to reclassify the family of TKs.

3.3.2 Expression profile of TKs family in MCF7 breast cancer cells

First of all, we decided to examine the expression profile of all members of TKs family in MCF7 breast cancer cells. To do so, a quantitative RT-qPCR analysis was performed to determine the endogenous expression levels of all 90 TKs including 58 Receptor Tyrosine Kinases (RTKs) and 32 Cytoplasmic Tyrosine Kinases (CTKs) (**Figure 47**). We set as cut-off a threshold cycle (CT) value of <34.5, above which the data has a high chance of resulting from cross contamination or amplification of fluorescent artifacts (using GAPDH as endogenous control housekeeping gene with a CT value of ~17). Based on this analysis, 66 TKs validated are expressed at variable levels with CT values ranging from 21.5 to 34.5 (**Figure 48**).

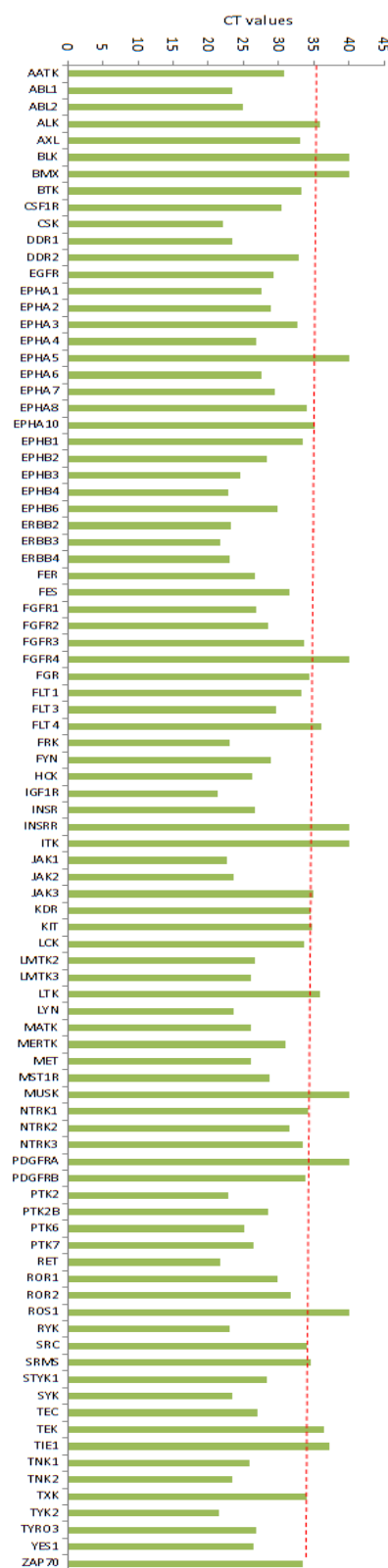


Figure 47 Expression profile of TKs family in MCF7 breast cancer cells.

A quantitative RT-qPCR analysis was performed to determine the endogenous expression levels of all 90 TKs in MCF7. CT values of all 90 TKs are presented here. A cut-off threshold cycle (CT) value was set as 34.5, above which the data can be interpreted as results of cross contamination or amplification of fluorescent artifacts. RT-qPCR assay was done two times in four replicates each time.

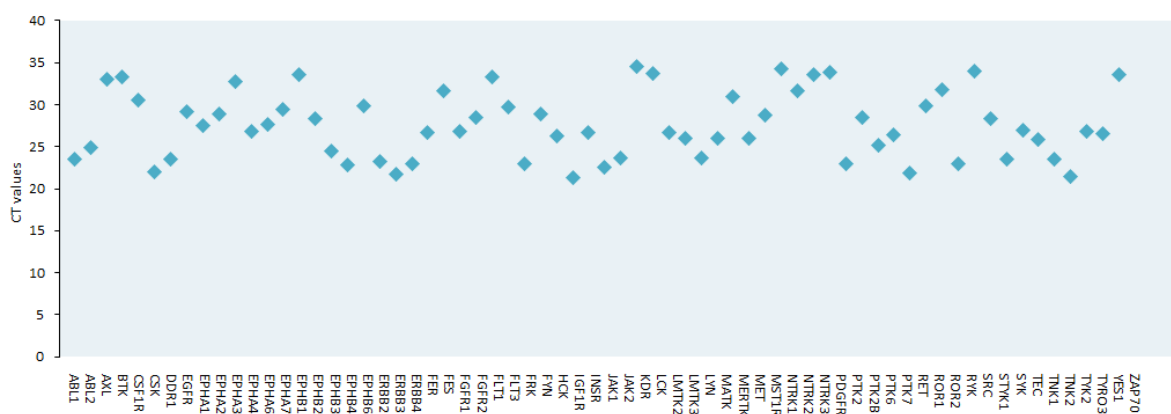


Figure 48 Expression levels of targeted TKs in this study.

66 validated TKs are expressed at variable levels with a CT value ranging from 21.5 to 34.5, all of which were targeted using siRNA for the next proteomic analysis.

3.3.3 A high-throughput RNAi screen of TKs family in MCF7 breast cancer cells

We continued to silence each individual TK expressed in MCF7 cells, using a siRNA library composed of 2 siRNAs/targeted gene. MCF7 cells were transfected for 72h, followed by RT-qPCR to verify the knockdown efficiency of the siRNAs. 65 out of 66 TKs were successfully silenced with a decrease of $\geq 70\%$ at the mRNA levels (**Figure 49**). We also validated the siRNA silencing efficacy by performing western blotting analysis on several of the TKs (**Figure 50**).

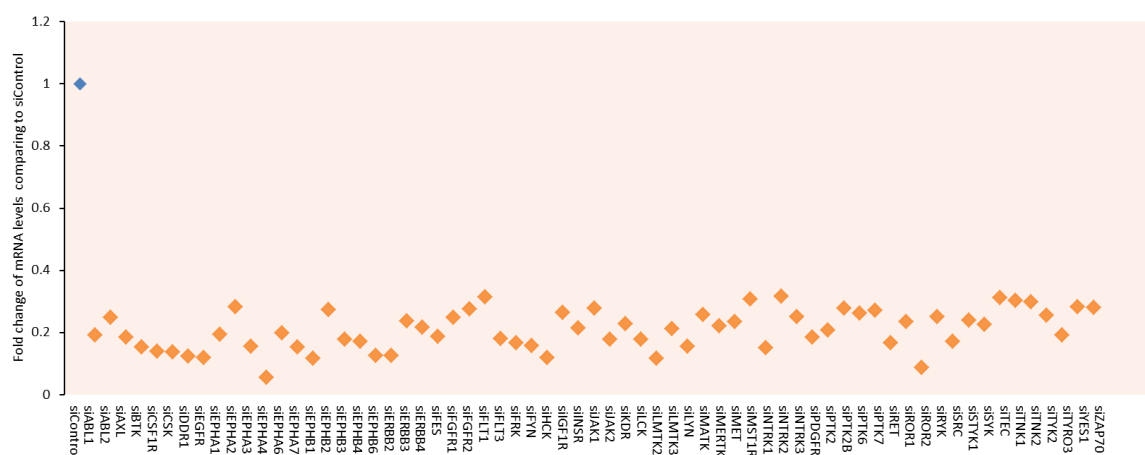


Figure 49 Validation of siRNAs targeting TKs using RT-qPCR.

MCF7 cells were transfected with an siRNA library composed of 2 siRNAs/targeted gene against 65 TKs for 72 hours. RT-qPCR was then performed to verify the knockdown efficiency of the siRNAs. Fold changes of mRNA levels comparing to siControl (blue) are presented here.

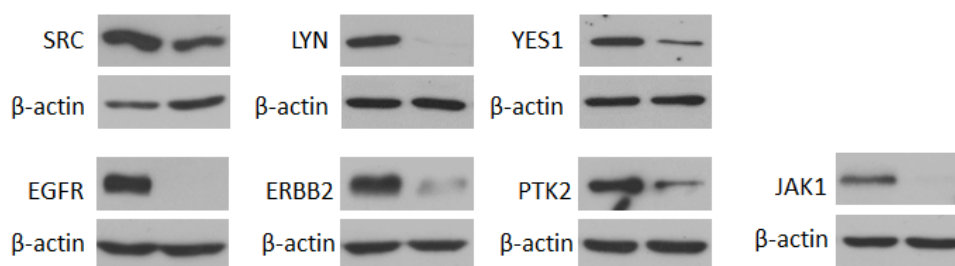


Figure 50 Validation of siRNAs targeting TKs using western blotting.

MCF7 cells were transfected with siRNAs for 72 hours. Western blotting was then performed to verify the knockdown efficiency of the siRNAs on 7 randomly chosen TKs. All blots shown are representatives of at least three independent experiments.

3.3.4 Identification of TKs regulated proteomes in MCF7

Considering the documented role and involvement of TKs in cancer signalling [13] and the fact that till now there has been a focus on only a few of them, we performed a quantitative proteomic analysis of the breast cancer tyrosine kinome after silencing each one of them to elucidate the TKs-regulated protein networks.

To do so, we used the SILAC approach which uses metabolic labelling into cellular proteomes during normal biological processes, introducing natural “light”, “medium” or “heavy” labelled amino acids into synthesized proteins. When light and heavy cell populations are mixed and digested to peptides, proteins are identified by liquid chromatography tandem MS (LC-MS/MS). This thus enables direct and accurate comparison of changes in protein abundance while concurrently allowing for the identification of modulated proteome upon silencing each tyrosine kinase in the heavy SILAC state [394].

Specifically, MCF7 cells were grown for 7 cell divisions in either R0K0 ‘light’ medium, containing unlabelled $^{12}\text{C}_6$, $^{14}\text{N}_4$ -arginine (Arg) and $^{12}\text{C}_6$ -lysine (Lys) amino acids, or R6K4 ‘medium’, R10K8 ‘heavy’ medium, containing labelled $^{13}\text{C}_6$, $^{15}\text{N}_4$ -Arg and $^{13}\text{C}_6$ -Lys. Following, R0K0, R6K4 and R10K8 labelled cells were transfected with either control siRNA or a pool of 2 validated siRNAs targeting two individual TKs out of 65 for 72 hours, respectively. Total protein lysates were prepared, mixed 1:1:1, digested, fractionated, subjected to LC-MS/MS and analysed using MaxQuant [357]. From ~ 27,000 nonredundant peptide sequences, a SILAC-based proteome comprising more than 2,000 distinguishable and unambiguously identified proteins with a minimum of two peptides (minimal peptide length 7 aa) was assembled in this study.

In all SILAC proteomics analyses after silencing 65 individual TKs, approximately 2,000 proteins were identified in each dataset with either 1 peptide or a minimum of 2 peptides, both with a false discovery rate (FDR) of 1%. Out of these proteins, more than 1,500 were quantified and their distribution according to fold changes was determined. The $\log_2$ ratios distribution shows a constant incorporation of the label and the excellent quantitative precision of the experiment. A representative summary of key features of one SILAC analysis after silencing ABL2 and its $\log_2$ ratios distribution is shown here (**Table 12** and **Figure 51**).

High Confidence Quantitative Proteome: Size and Features	siABL2/siControl
Proteins (with unique peptides > 0 + nonunique >1)	1907
Proteins (unique > 1, ratio counts >2)	1607
Identified nonredundant peptides	26914
Quantifiable peptide ratios	26881
False positive rate (FPR) peptides	1%
False positive rate (FPR) proteins	1%
Minimal peptide length (aa)	7
Up-regulated (Ratio significance B <0.05)	81
Down-regulated (Ratio significance B < 0.05)	103

Table 12. Key features from the SILAC analyse of siABL2/siControl.

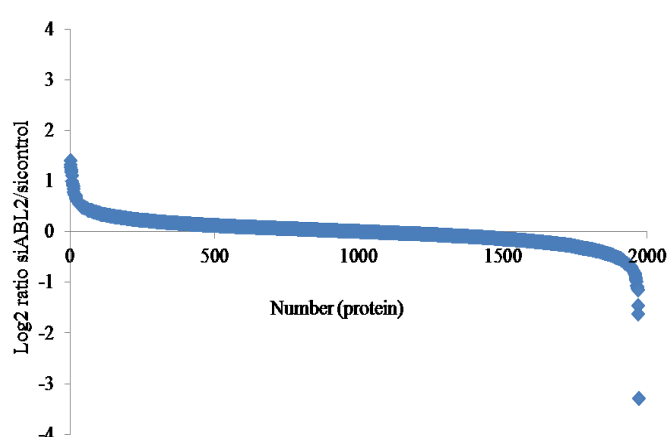


Figure 51 $\log_2$ ratios distribution of siABL2 vs siControl from SILAC quantitative proteomics.

$\log_2$ normalized H/L ratios (siABL2/siControl) distribution with a standard deviation of 0.2294.

3.3.5 Global proteomic portrait of TKs signalling

3.3.5.1 Proteomic alteration after knockdown of TKs

A SILAC ratio representing the fold change of protein abundance after individually silencing 65 TKs was compared and normalized to control group (siControl). After normalization, values of fold changes are all above 0, with value 1 showing that the expression levels of the specific proteins are not altered after TKs silencing. To acquire a portrait of the TKs-regulated proteome, the overall pattern of regulation is shown in the heatmap of quantified values. To improve resolution and concentrate the plot in the most significantly enriched region, approximately 1000 identified proteins were presented. For each knockdown (rows), the quantified values are plotted in red for down-regulated, white for no-change and non-identified and blue for up-regulated proteins (**Figure 52**).

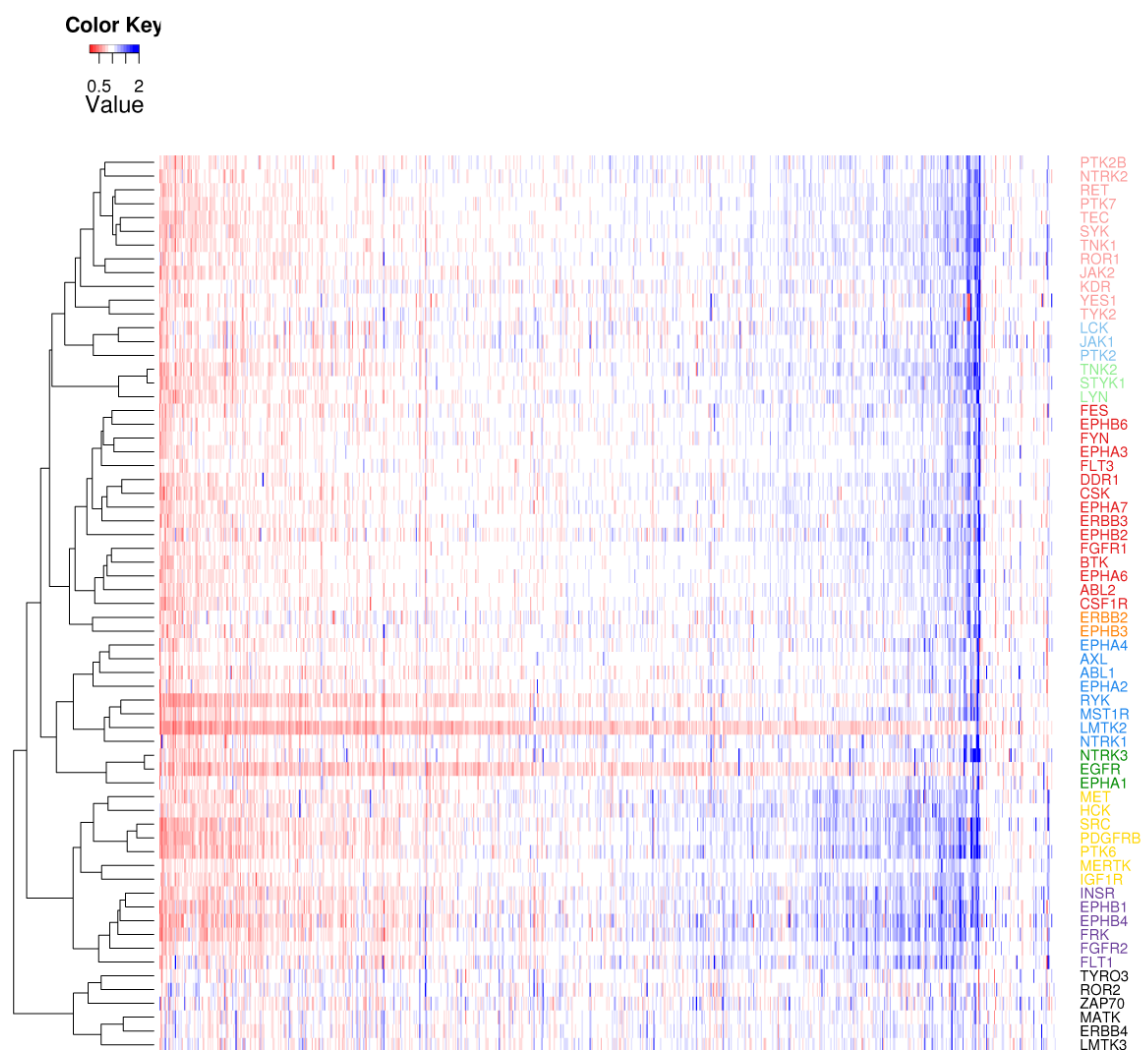


Figure 52 Heatmap of quantified proteins after silencing TKs.

The overall pattern of regulation is shown in the heatmap of quantified values. After normalized to siControl, values of fold changes are all above 0, with value 1 showing that the expression levels of the specific protein are not altered after silencing TKs. For each knockout (rows) the quantified value for an identified protein is plotted in red for down regulated proteins (below 1), white for non-differential and non-identified and blue for up-regulated proteins (above 1). The row labels indicate the knock out experiment and the colours correspond to the clusters described below.

3.3.5.2 Identification of 10 distinctive clusters of TKs

We then calculated and plotted pairwise distances based on the quantifications of TKs-regulated proteomic signatures using centred Pearson correlation. In the correlation heatmap showing the distance metric between all kinases in our study, the smaller distances are displayed in purple corresponding to similar signatures, whereas the longer distances are in green (**Figure 53**). Based on the calculated distances, we performed hierarchical clustering of 65 TKs using R's hclust function. The complete linkage method that aims to find similar clusters based on overall cluster measure was used. Ten distinctive clusters were obtained and

the complete dendrogram is shown with the labels coloured for these clusters (**Figure 54**). The number of TKs in the clusters is plotted against the clusters and the colour coding of the clusters is used throughout to identify the analysis relevant to the corresponding clusters (**Figure 55**).

We thus characterized 10 novel clusters according to the similarity of each TKs-regulated proteome. Cluster 1 contains 8 members: ABL1, AXL, EPHA2, EPHA4, LMTK2, MST1R, NTRK1, RYK; Cluster 2 is the biggest family with 15 members, including ABL2, BTK, CSF1R, CSK, DDR1, EPHA3, EPHA6, EPHA7, EPHB2, EPHB6, ERBB3, FES, FGFR1, FLT3, FYN; Cluster 3 comprises 3 members, which are EGFR, EPHA1, NTRK3; Cluster 4 has 6 members, namely EPHB1, EPHB4, FGFR2, FLT1, FRK, INSR; Cluster 5 is the smallest family with only 2 members: EPHB3, ERBB2; Cluster 6 consists of 6 members, including ERBB4, LMTK3, MATK, ROR2, TYRO3, ZAP70; Cluster 7 contains 7 TKs, which are HCK, IGF1R, MERTK, MET, PDGFRB, PTK6, SRC; Cluster 8 comprises JAK1, LCK, PTK2; Cluster 9 is the second largest group with 12 TKs, consisting of JAK2, KDR, NTRK2, PTK2B, PTK7, RET, ROR1, SYK, TEC, TNK1, TYK2, YES1; Cluster 10 has 3 members, which are LYN, STYK1, TNK2.

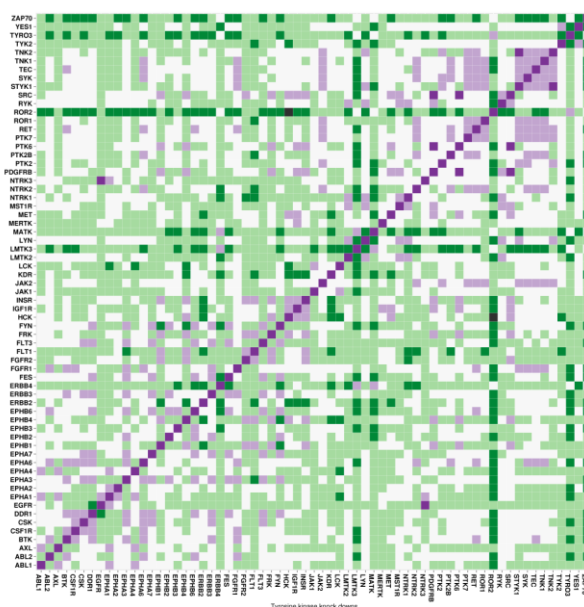


Figure 53 Correlation heatmap of the distance metric between all TKs in this study.

Pairwise distances were calculated and plotted according to the quantifications of TKs-regulated proteomic signatures using centred Pearson correlation. In the correlation heatmap showing the distance metric between all kinases in our study, the smaller distances are displayed in purple, whereas the longer distances are in green.

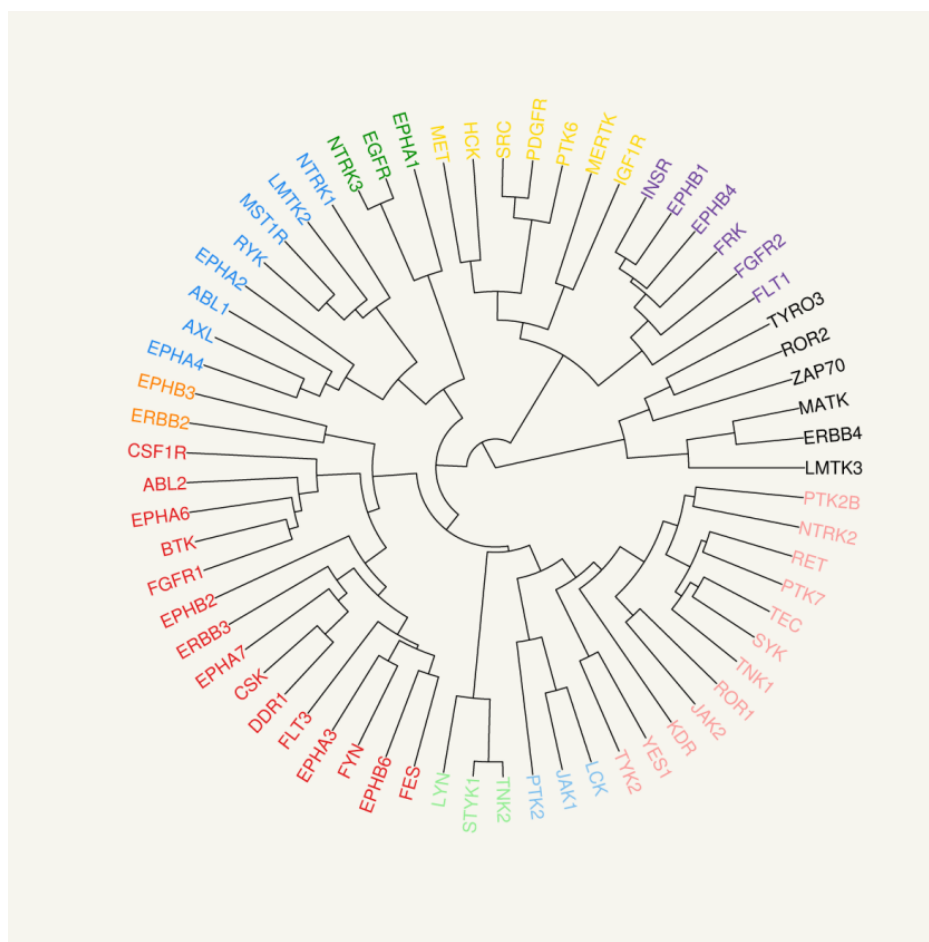


Figure 54 Hierarchical clustering of the 65 TKs expressed in MCF7.

Hierarchical clustering of the 65 TKs was performed using R's hclust function. The complete linkage method which aims to identify similar clusters based on overall cluster measure was used. 10 distinctive clusters were obtained and the complete dendrogram is shown with the labels coloured for these clusters.

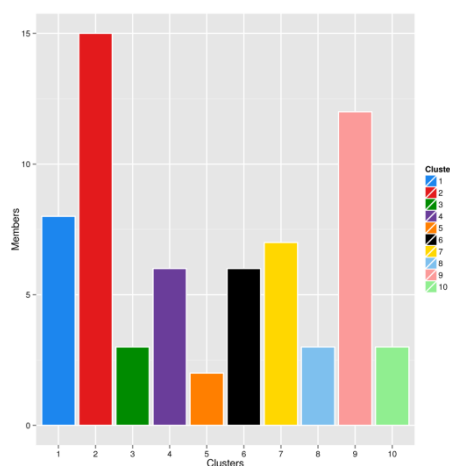


Figure 55 Number of TKs in each identified cluster.

Number of TKs in the chosen clusters is plotted against the clusters. The colour coding of the clusters is used throughout to identify the analysis relevant to the corresponding clusters.

3.3.5.3 Characterization of a proteomic signature and functional portrait for each cluster

We next investigated the TKs-regulated proteomic changes within each classified cluster in more detail to delineate the proteomic signature of each cluster. Using normalized fold changes of each protein quantified, we identified proteins significantly affected after knockdown of TKs in each cluster. Specifically, we used 0.7 as the cut-off for down regulation and 1.3 as the threshold value for up-regulation. All the subsequent analyses were conducted based on these cut-off values. To characterize the top hits within each cluster, we firstly filtered the proteins by requiring that they should be identified in at least 30% of the TKs in the cluster. We then selected up-regulated proteins that have a mean cluster value of more than 1.3 and down-regulated proteins with a mean value of less than 0.7. Counts of the most significantly regulated proteins in each cluster are shown in **Figure 56**. It appears that more proteins were up-regulated than down-regulated, which is an interesting discovery requiring further study (A full list of proteins significantly regulated in each cluster is not presented here due to its large size and limited space).

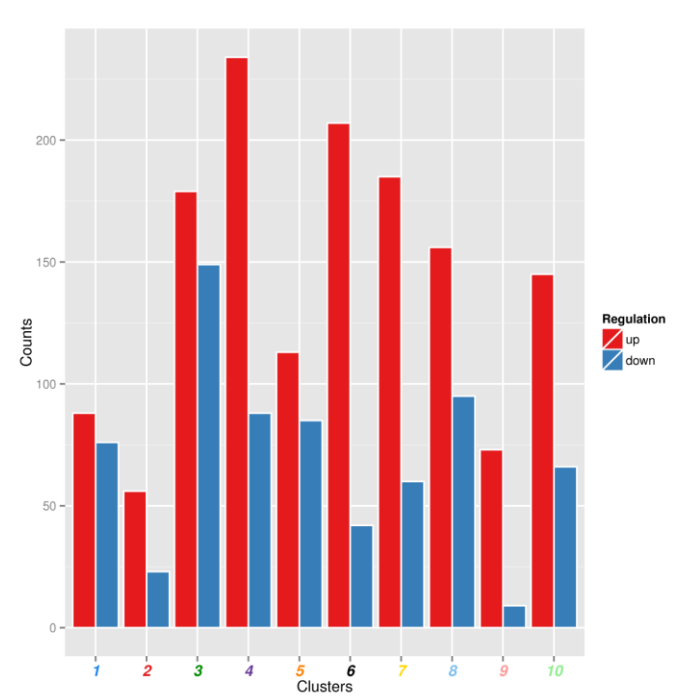


Figure 56 Number of proteins significantly up or down-regulated in each identified cluster.

For each cluster, we firstly filtered the proteins by requiring that they should be identified in 30% of the experiments in the cluster. We then selected the up-regulated proteins that have a mean cluster value of more than 1.3 (Red). Similarly, down-regulated proteins have a mean value of less than 0.7 (Blue). x-axis shows 10 different clusters and y-axis indicates the counts.

In the proteomic profiles of all 10 classified clusters, there is a unique signature of proteins and associated pathways regulated by the TKs/cluster, although a few proteins were recognized in more than one cluster. To gain insight on the biological processes distinctively modulated by each cluster, we identified the top GO categories to which the differential proteins in each cluster belong. We ran the GO analysis on cellular components (CC), biological processes (BP) and molecular function (MF). Overall, the identified proteins are scattered in a variety of cellular components, including extracellular matrix, cell membrane, cytoplasm, nuclei, synapse and other organelles. They are involved in a wide range of cellular activities such as immune system process, reproduction, metabolic process, growth, cell communication, development, cell cycle, transcription and cell adhesion, whose deregulation can contribute to oncogenesis (**Figure 57**).

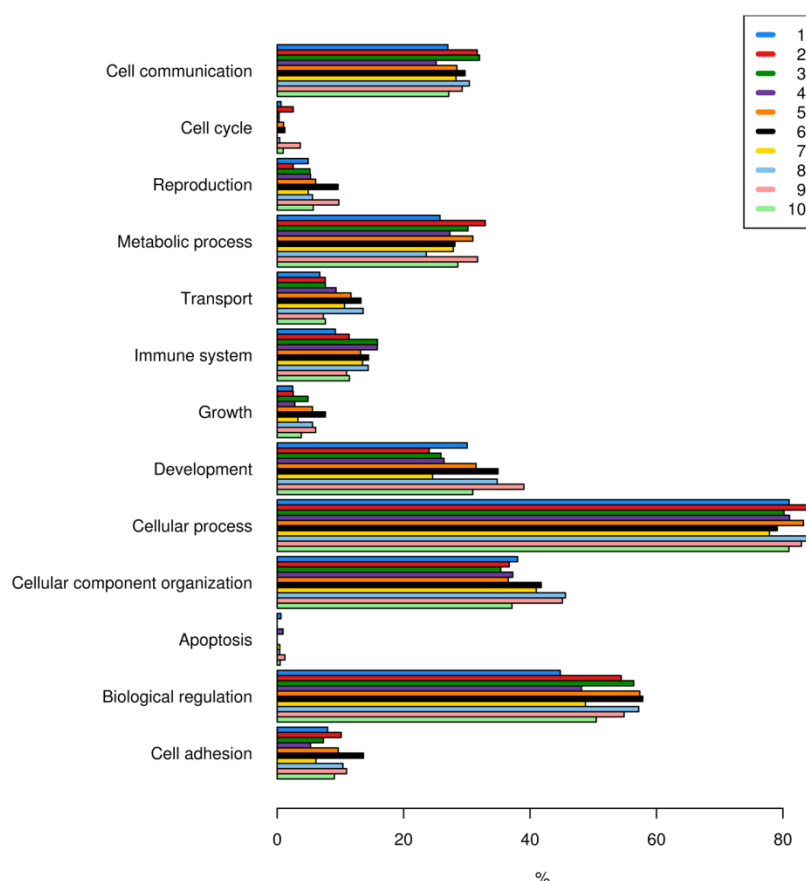


Figure 57 Functional portrait of protein-related biological processes in each cluster.

A functional profile of top GO biological processes that the up- and downregulated proteins belong to is presented. x-axis shows the percentage of hits in each cluster that belong to a GO biological process term. The colour coding and the number for each cluster are indicated as above.

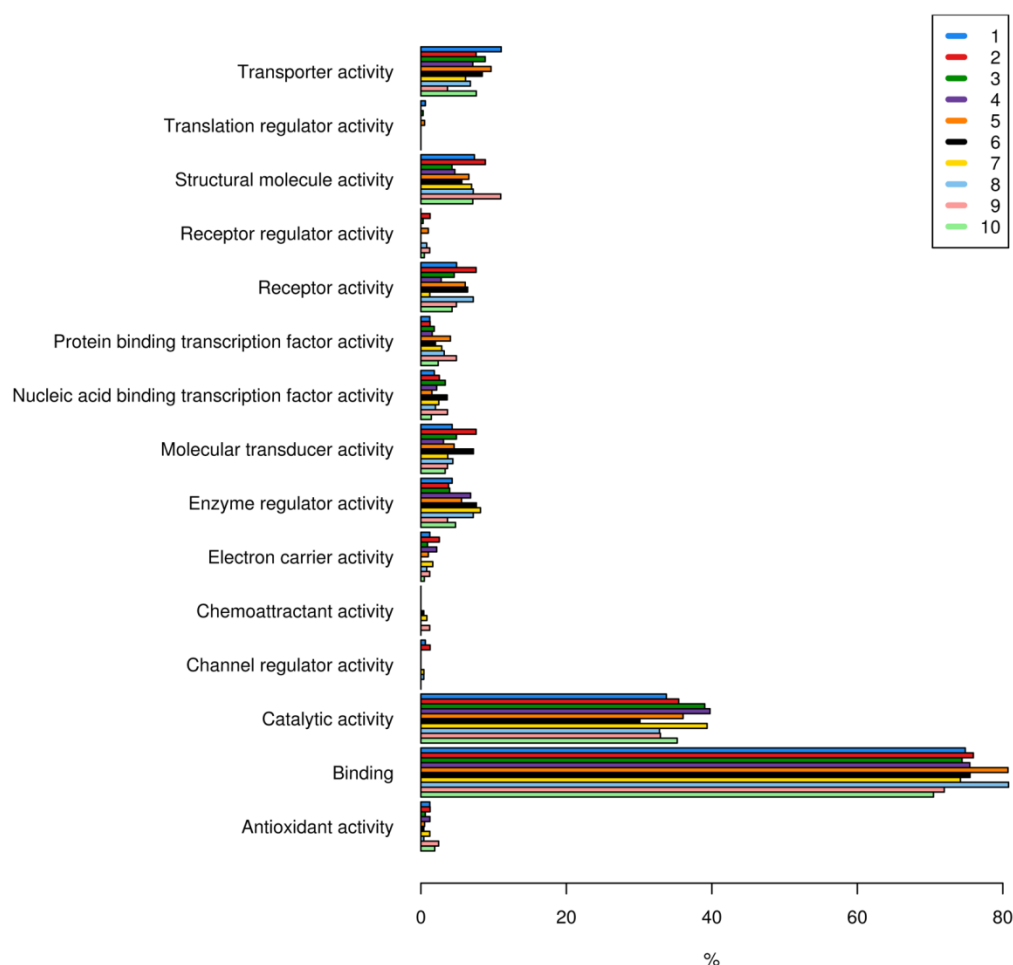


Figure 58 Functional portrait of protein-related molecular functions in each cluster.

A functional profile of top GO molecular functions that the up- and downregulated proteins belong to is presented. x-axis shows the percentage of hits in each cluster that belong to a GO molecular function term. The colour coding and the number for each cluster are indicated as above.

The GO analysis of molecular functions demonstrated a large percentage of proteins with binding, catalytic, structural and enzyme regulatory activities within each cluster (**Figure 58**). This supports the established role of TKs as important modulators in signal transduction via functional and physical protein-protein interactions. We thus sought to integrate the GO analyses with the STRING database to delineate the enrichment of functional protein-protein interaction networks in each cluster. First we identified GO terms that were overrepresented in the differential proteins of each cluster. We then depicted the STRING network for the differential proteins within an overrepresented GO term. Colour-coded networks were generated to distinguish up- (green) or down-regulation (red) of proteins. As expected,

comprehensively integrated sub-networks emerged in a functional linked manner. The sub-networks from each cluster uncovered an exclusive feature of functional connections contributed by the regulated proteomes and also highlighted the complexities in TKs-modulated cellular signalling.

Representatives of the most enriched functional networks within each cluster are displayed (**Figure 59**): in cluster 1, spliceosome assembly and anion transport; in cluster 2, RNA processing and catabolic process; in cluster 3, immune response; in cluster 4, metabolic process; vehicle-mediated transportation, endocytosis; in cluster 5, regulation of apoptotic signalling pathway and catabolic process; in cluster 6, cell adhesion; cellular component; cell cycle; in cluster 7, small molecule metabolic process, cellular component organization; in cluster 8, DNA replication initiation; organelle organization; in cluster 9, DNA-dependent transcription; cellular response to stress; in cluster 10, response to reactive oxygen species; cellular amino acid metabolic process. However, similarity is also present across some clusters, suggesting the fact that TKs in different clusters can regulate the same pathways and compensate for each other. For example, metabolic process is overrepresented in both cluster 4 and 7, as these two clusters are indeed very close in distance in our dendrogram of hierarchical clustering (**Figure 54**).

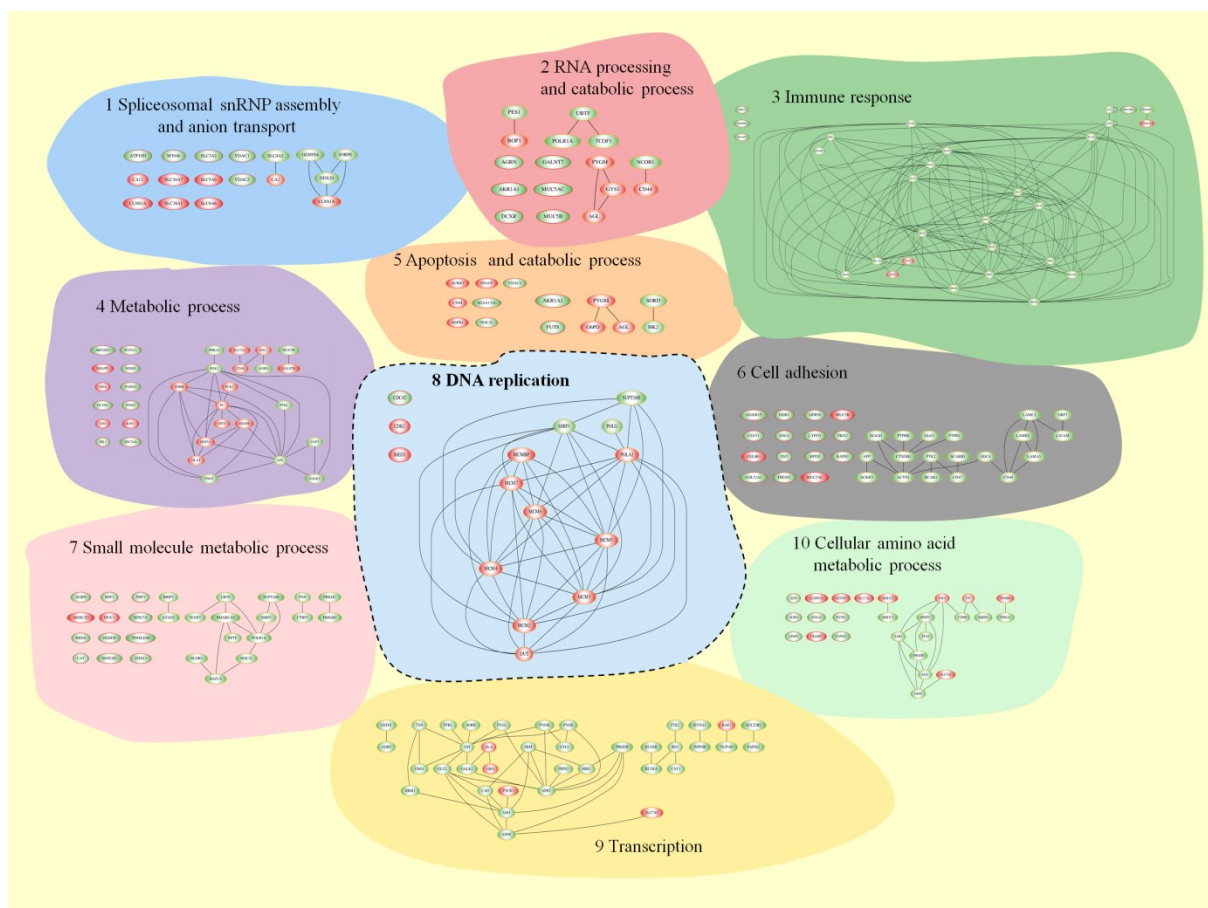


Figure 59 Representatives of defined functional networks in each classified TK cluster.

The functional networks were generated using GO analysis combined with the STRING platform. Proteins in green are up-regulated, whereas red indicates down-regulation. Arrows show the interactions between connected proteins. Representative defined functional networks associated with their clusters are shown here. The colour coding and the number for each cluster are indicated as above.

Instead of structural homology, our classification highlights the similarity in biological function and signalling network shared among TKs in different clusters. Significantly, this clustering verifies current knowledge of the recognized functions of TKs, and also indicates the potential involvement of TKs in undocumented biological processes, which merits further investigation. One good example is manifested in cluster 8, which comprises JAK1, LCK, and PTK2. In this study, the bioinformatics analysis revealed a key characteristic of cluster 8 that is its contribution to DNA replication initiation and elongation during cell cycle through regulating mini-chromosome maintenance (MCM) proteins (MCM2-7) (**Figure 59**), whose deregulation is associated with genomic instability and cancer [395-398]. Our SILAC proteomic data showed that, upon silencing the three kinases in cluster 8, protein levels of all MCM members were decreased, which would subsequently lead to an inhibition in

uncontrolled DNA replication in cancer. Ultimately, silencing TKs can block sustained replicative immortality of tumour cells. This finding is consistent with the current perceptions of TKs, that they are oncogenic and good therapeutic targets in cancer. In addition to the role in DNA replication, several lines of evidence from previous literature have hinted at a putative network involving JAK1, LCK and PTK2 together with MCMs in regulating transcription. Specifically, MCM5 protein, as well as other members of the MCM family, has been shown to be essential for transcriptional activation mediated by STATs [399, 400], whose activity can be regulated by JAK1, PTK2 and LCK. Above all, the signalling network related to MCMs and STATs, which is coordinated by JAK1, PTK2 and LCK, can modulate DNA replication and transcription, as well as other processes including proliferation, apoptosis, migration and cell cycle [302].

Collectively, we performed for the first time a global unbiased analysis of the TKs-regulated proteomes in breast cancer. Our study identified 10 new distinctive clusters and characterized a unique proteomic signature and functional portrait in each cluster. The biological relevance of our proteomic study in decoding the TKs-regulated proteomes supports the essential role of TKs in regulating all aspects of cellular activities, underlining TKs as good therapeutic targets in cancer. Further exploration of the data will surely improve our understanding of signalling in TKs and might identify potential targets for tumour treatments.

3.3.6 Discussion

TKs play an essential role in regulating various intracellular signal-transduction pathways involving almost every aspect of cell activity, including proliferation, survival, differentiation, apoptosis, metabolism, angiogenesis, immune surveillance and motility [13]. Perturbation of TK signalling affects their kinase activities, which contributes to various human diseases, including multiple malignancies [13, 56]. Given their importance in cellular processes and their involvement in tumourigenesis, enormous efforts have been devoted to illustrate their structural characteristics, biological implications and associated signalling pathways. However, most of the early studies have focused on elucidating the function of individual member within the TK family in cancer. Less than half of the 90 TKs described so far have been thoroughly studied and in these cases a global functional analysis and understanding of their regulated-proteome is still lacking [197]. Therefore, a complete proteomic portrait and mapping of the TK signalling pathways could produce novel insights and a more complete functional picture albeit accompanied by certain technical challenges [401]. With the recent progress in global comparative quantitative proteomic analysis, a large-scale, robust and confident identification of biochemical networks implicated in cancer has become feasible [402].

SILAC represents one of the most popular quantitative methods that have been extensively employed in proteomic research [403]. Pioneering studies on genome-wide interaction in the yeast proteome demonstrated that protein-protein interactions can determine eukaryotic cellular machines [404, 405]. Functional mapping of protein interactions in the ERBB family also emphasized an important role of protein-protein interaction in contributing to the oncogenic potential of tyrosine kinases [406]. Global phosphoproteome analysis was applied to identify and quantify the dynamics in signalling networks upon EGF stimulus delineating an integrative picture of cellular regulation [407]. In addition, loss-of-function studies using genome-wide RNAi screens have been successfully developed to identify molecular targets as well as key regulators in biological processes in cancer [408-410].

Here, we describe a global mapping of TKs-regulated proteome and their associated signalling using a high throughput RNAi screen combined with SILAC-based quantitative proteomics in breast cancer cells MCF7. First, the expression profile of all 90 known TKs was determined by RT-qPCR in MCF7 cells. Our results showed that 66 TKs with CT values ranging from 21.5 to 34.5 are present. A RNAi library targeting against 65 out of 66 identified TKs was verified to produce good knockdown efficiency by RT-qPCR and western blotting.

Subsequently, a quantitative proteomic analysis using SILAC was performed after silencing 65 TKs individually in MCF7 to identify the TKs-regulated proteomes and to quantify their expression changes, thus elucidating their involvement in multiple signalling networks.

Our SILAC proteomic analyses showed that around 2,000 proteins were identified in each dataset after silencing each of 65 TKs. Among these proteins, more than 1,500 were quantified and the distribution of their log₂ ratios showed a constant incorporation of the label and an excellent quantitative precision. Silencing of individual TKs, despite their high homology in their kinase domains and phylogenetic relationship, results in a diverse proteomic landscape. However, some of the protein changes observed might be due to off-target siRNA effects (**Figure 52**). This could be tested by separately analysing two (or more) siRNAs for some of the more interesting TKs. The massive amount of data allowed us to carry out further bioinformatics analysis to gain a broad picture of the TKs-modulated proteomes. A heatmap of quantified protein expression levels was generated to visualize the overall regulation pattern of the altered proteomic architecture upon silencing all 65 TKs. From the acquired heatmap, similarities in modulated proteomes after knockdown of TKs could be observed. We therefore calculated correlation distances based on the quantifications of TKs-regulated proteomic signatures and performed hierarchical clustering of 65 TKs accordingly. As a result, a new classification of TKs, which contains 10 distinctive clusters, was established. This new grouping based on similarity in regulated proteomes differs with the canonical classification of TKs in the human kinome, although in certain cases some TKs are categorized into the same group by the two clustering, such as EPHA2 and EPHA4, EPHB1 and EPHB4. The number of TKs in each of the 10 clusters varies, with 15 in the largest group and only 2 in the smallest one. This variance might indicate that some TKs in the bigger families tend to function together in regulating related signalling pathways, but other TKs in the smaller groups are likely to act alone with less connection to fellow TKs.

We further explored the proteomic changes within each cluster more thoroughly and identified the most up- and down-regulated proteins in each group. Our analysis uncovered numerous significantly modulated proteins, among which more proteins were actually overexpressed than down-regulated. The mechanisms of these indirect downstream effects require further investigation. Importantly, from the detailed proteomic profiles of the 10 classified clusters, a distinctive signature of regulated proteins in each group was perceived. To fully characterize the modulated proteomes and to gain insights on the biological functional portrait belonging to each cluster, GO categories enriched in each group were

compared between the clusters. In general, the identified proteins appear widely distributed across different cellular compartments, including the extracellular matrix, cell membrane, cytoplasm, nuclei, synapse and other organelles. Moreover, our analysis showed that these proteins are implicated in numerous biological processes, including immune system process, reproduction, metabolic process, cell growth, developmental process, cell cycle, transcription and cell adhesion, many of whose aberrant regulation can lead to tumourigenesis.

Our results also verified the fundamental role of TKs as crucial players in cellular signalling relay through functional and physical interactions with their substrates, supported by the GO analysis revealing a prominent fraction of proteins with binding, catalytic, structural and enzyme regulatory activities within each cluster. Further combined analyses using GO biological processes together with the STRING database demonstrated a clear picture of comprehensively integrated functional protein-protein interaction networks assembled by the TKs-regulated proteomes. Unique features were uncovered for each cluster and are presented by defined TKs-regulated functional networks, for example immune response predominating in cluster 3, metabolic process in cluster 4, regulation of apoptosis in cluster 5 and DNA replication in cluster 8. Conversely, some clusters are featured by their involvement in similar biological processes as shown by the overrepresentation of metabolic processes in cluster 4 and 7, pointing to the presence of functional similarity in certain clusters. This further supports the notion that TKs in different clusters can contribute to the activation of the same pathways and compensate for each other through cross-talk between cellular signalling.

In strong support of the acquired TKs-regulated proteomic data, our classification underscores the similarity in biological function shared by TKs and signalling network coordinated by TKs within each unique cluster. Meanwhile, our clustering confirms the known role of TKs in cell activities and implies the participation of TKs in undocumented biological processes that await further investigation. For example, in cluster 8, functional analysis of the proteomes modulated by JAK1, LCK and PTK2 establishes the extensive regulation of processes related to DNA replication through governing MCM proteins (MCM2-7). MCM2-7 has been shown to play an important role in DNA replication initiation and elongation during cell cycle and perturbation of MCM proteins can result in genomic instability and cancer [395-398]. In our study, silencing of kinases in cluster 8 caused reduced protein levels of all MCM proteins. This might result in an inhibition of sustained replicative immortality of tumour cells, which supports current opinions that TKs are tumourigenic and can represent therapeutic targets in cancer. Furthermore, previous studies have documented that the MCM members are central

for transcriptional activation mediated by STATs [399, 400], which are downstream effectors of JAK1, PTK2 and LCK. A potential signalling network involving JAK1, LCK and PTK2 with MCMs is therefore indicated to play a role in regulating transcription. So far, we highlight a functional protein-protein interaction coordinated by JAK1, LCK and PTK2, which is implicated in modulation of multiple cellular processes in oncogenesis.

However, to fully interpret the massive amount of data from SILAC proteomics, further *in vitro* and *in vivo* studies are needed to investigate the most enriched functional networks in each cluster, particularly those associated to cancer development. Moreover, it would be of interest to characterize the TKs-regulated phospho-proteomic profile using a similar strategy by SILAC and RNAi screen as phosphorylating substrates is one of the most important activities executed by TKs. Notably, global proteomic analysis that can complement genomic studies can help us study cellular regulation such as protein interaction information, and can uncover novel mechanisms contributing to tumour progression. Indeed, one recent study has quantified phospho-proteomic changes in skin cancer development using *in vivo* SILAC revealing a deregulated PAK4-PKC/SRC network during mouse skin carcinogenesis [411]. Another group has presented the protein interaction landscape of the human CMGC kinase family discovering a set of protein interactions genetically linked to various human diseases, including cancer [412]. Surely, our research will benefit greatly from the global-scale proteomics integrated with functional analysis carried out by other groups in future.

In summary, the experimental approach through combined use of SILAC and RNAi screen in this study shows high accuracies for the proteomics quantification and important advantages for interpretation of the results. The global comparative proteomic analysis identifies a unique classification of TKs, which is purely based on the similarity in their regulated proteomes, presenting a different angle to understand TKs. Our results provide new insights to the current understanding of TKs families, and particularly highlight the resemblance in biological processes shared among various members from different groups. This key characteristic strongly supports the fact that TKs can compensate for each other to maintain functional activity upon disruption and can contribute to the same downstream signalling in tumour initiation and development. Importantly, it offers an explanation to the observed resistance to the TKs-targeted inhibitors considering the plasticity and reciprocity between TKs. Our study clearly emphasizes a rationale for the development of a combination of multi-targeted TKs as antitumour drugs. Furthermore, we uncover various biochemical

networks that will help us further understand and elucidate the cross-talk between cellular signalling pathways implicated in breast cancer.

CHAPTER 4:

DISCUSSION

4.1 The role of KSR1 in breast cancer

KSR1, initially described as a novel protein kinase in the Ras-Raf pathway [107-109], is an essential scaffolding protein that co-ordinates the assembly of the MAPK complex, consisting of MEK and ERK [144, 145, 413]. Mammalian KSR1 has been extensively referred to as a pseudokinase, due to the fact that it lacks a key catalytic residue in its kinase domain [43]. However, a great body of evidence suggests that KSR1 functions as an active kinase as well as a scaffold protein.

Given the role of KSR1 in the Ras-Raf-MAPKs cascade, intensive efforts have focused on Ras-dependent cancers. For instance, recent studies reported that KSR1 regulates the proliferative and oncogenic potential of cells and inhibition of KSR1 abrogates Ras-dependent pancreatic cancer [127, 166]. Similarly, KSR1 is required for tumour formation in a skin cancer mouse model [162]. Previous studies indicated that KSR1 is important in breast cancer metastasis and depletion of KSR1 reduced tumour formation in Ras-dependent mammary tissue in mice [125, 151]. As Ras mutations are rare in breast cancer, the involvement of KSR1 may not depend on the canonical Ras-Raf-MAPKs pathway [378]. Moreover, its biologic function in this setting needs further exploration, as do its major partner proteins and pathways including those connected to p53 and BRCA1, which are implicated here.

4.1.1 KSR1 in p53 regulation

The p53 tumour suppressor is a well-established central player in cell growth, cell cycle arrest, apoptosis and cellular response to genotoxic stress [414, 415]. Its transcriptional activity is highly regulated by post-transcriptional modifications including acetylation [372, 373]. Previous data has demonstrated that DBC1 directly interacts and negatively regulates the deacetylase SIRT1 resulting in an increase of p53 acetylation [370, 371]. Furthermore, phosphorylation of DBC1 is necessary for its interaction with SIRT1 while phosphorylated DBC1 inhibits the activity of SIRT1 in response to DNA damage [376, 377].

4.1.1.1 Summary of findings

In the present study, we reveal a KSR1-regulated phospho-proteome profile in breast cancer cells using a comparable quantitative proteomic approach, SILAC. Numerous new KSR1-regulated proteins and its associated pathways were identified outlining a comprehensive picture of KSR1-involved signalling networks. Bioinformatic analysis indicated a role of KSR1 in various biological processes, including cell cycle, metabolism and apoptosis. A

handful of the characterized phospho-proteins, such as APRIN [364, 365], ARHGAP35 [366], Hsp27 [367, 368], have been shown to play an important role in breast cancer initiation, development and migration. Most interestingly, the KSR1-regulated phospho-proteomic analysis has identified phospho-DBC1, which is a connection of KSR1 to the modulation of p53 transcriptional activity.

Subsequently, we investigated the effect of KSR1 on p53 transcriptional activity, as well as the potential mechanism on KSR1-related regulation of p53 transcriptional activity, including through p53 acetylation, which is indispensable for p53 activity. Using different p53-dependent gene promoter constructs, luciferase assays demonstrated that p53 transcriptional activity is inhibited after KSR1 overexpression and is increased upon depletion of KSR1. We then revealed that KSR1 overexpression results in a reduction of p53 acetylation and knockdown of KSR1 causes an increase in p53 acetylation. Mechanistic studies showed that KSR1 overexpression reduces the phosphorylation levels of DBC1 and silencing increases phosphorylated DBC1. Moreover, Co-IP results demonstrated that overexpression of KSR1 diminishes the SIRT1-DBC1 interaction, which allows SIRT1 to interact with p53, thus reducing p53 acetylation. These findings highlight the action of KSR1 on p53 activation is through DBC1.

4.1.1.2 Future direction

As we have shown that KSR1 might regulate p53 activity through phosphorylation of DBC1, future work will focus on addressing the mechanism of KSR1 on regulating DBC1 phosphorylation. To do so, it would be necessary to study:

- whether KSR1 can directly interact with DBC1, therefore somehow inhibiting the phosphorylation of DBC1 by associated kinases;
- whether the action of KSR1 on DBC1 phosphorylation is through ATM/ATR kinases, as one group recently has demonstrated that ATM/ATR is competent to directly phosphorylate DBC1 on Thr454 upon DNA damage, inducing the detachment of the SIRT1-p53 complex and ultimately an increase in p53 acetylation [377];
- another alternative mechanism through PKA and AMP-activated protein kinase, since it has been revealed that these kinases can trigger the separation of SIRT1 from its endogenous inhibitor DBC1, hence affecting downstream effects [379];

4.1.1.3 Conclusion

In conclusion, we present the first SILAC analyse of KSR1-modulated phospho-proteome establishing its involvement in multiple important cellular processes in breast cancer. Furthermore, we identify a role of KSR1 in the regulation of p53 transactional activity by reducing p53 acetylation via the modulation of DBC1-SIRT1 interaction.

4.1.2 KSR1 in BRCA1 regulation

BRCA1 tumour suppressor gene mutations contribute to a proportion of hereditary breast and ovarian cancers [416]. *BRCA1* forms a heterodimer with *BARD1* protein, which was discovered in a yeast two-hybrid screen as a binding partner of *BRCA1* [391]. Subsequent work revealed that *BARD1* interacts with and stabilises *BRCA1* by inhibiting its ubiquitination [385, 386]. This heterodimer has ubiquitin ligase activity and regulates numerous cellular functions including DNA repair, apoptosis, cell cycle progression and gene transcription, essentially contributing to genomic stability and tumour suppression [417].

4.1.2.1 Summary of findings

In this work, using tumour tissue microarrays in a large patient cohort ($n > 1000$) with 20 years follow-up, we determined that breast cancer patients with high *KSR1* displayed better disease free- and overall survival, results also supported by Oncomine analyses, microarray data ($n = 2878$) and genomic data from paired tumour and cfDNA samples revealing loss of heterozygosity. *KSR1* expression is associated with high *BRCA1*, high *BARD1* and *Chk1* levels in breast cancer specimen. In paired normal/breast cancer tissues, higher *KSR1* was detected in normal tissues, whereas *KSR1* is ubiquitously expressed in a panel of breast cancer cell lines. Phospho-profiling of major components of the canonical Ras-Raf-MAPKs pathway, including Raf, MEK and ERK, showed no significant changes after *KSR1* overexpression or silencing.

Furthermore, breast cancer cells overexpressing *KSR1* formed fewer and smaller size colonies compared to the parental ones. Likewise, the growth of xenograft tumours overexpressing *KSR1* was significantly slower than the control group. Mechanistically, it appears that the tumour suppressive action of *KSR1* is *BRCA1*-dependent shown by *in vitro* 3D-matrigel and soft-agar assays. Consistent with the positive correlation between *KSR1* and *BRCA1* in clinical samples, our *in vitro* results showed *KSR1* overexpression increases *BRCA1* protein levels through decreasing *BRCA1* ubiquitination. Additionally, based on the fact that *BARD1* has been previously reported to form a heterodimer with *BRCA1* thereby stabilising it by reducing its ubiquitination [386], we then assessed the effect of *KSR1* on *BARD1* and whether the action of *KSR1* on *BRCA1* is through *BARD1*. We revealed that *KSR1* has no effect on the mRNA levels of *BARD1* but up-regulates *BARD1* protein abundance, and the *BRCA1*-*BARD1* interaction is increased upon *KSR1* overexpression. Importantly, the effect of *KSR1* on *BRCA1* is partly through *BARD1*, as *KSR1* overexpression increases *BRCA1*

less strongly when BARD1 is depleted. These findings integrate KSR1 into a complicated signalling network involving BRCA1-BARD1 complex in breast cancer.

4.1.2.2 Future direction

In light of the results presented in this work, numerous issues remain to be addressed to fully understand the role of KSR1 in regulating BRCA1 and the associated tumour suppressive effect.

We have established that high KSR1 expression correlates with better overall and disease free survival in European patients. Thus, whether the associations between KSR1 expression and clinical outcomes are the same in Asian patients merits further exploration. It would be necessary to determine this in other cohorts, such as a Singapore cohort from our collaborators [348].

We have also shown that the effect of KSR1 on BRCA1 is partially mediated via BARD1. Therefore, other alternative mechanisms regarding KSR1's effect on BRCA1 need to be investigated. Moreover, our results have demonstrated that KSR1 increases protein levels of BARD1 and directly interacts with BARD1. However, the detailed mechanism underlying the action of KSR1 on BARD1 is still not clear. It would be essential to assess:

- whether KSR1 has any effect on the cell cycle proteins CDK2 and cyclin E1, as it has been shown that overexpression of CDK2 and cyclin E1 can reduce the abundance of BRCA1 and BARD1, and enhance their export to the cytoplasm [418];
- the mechanism of how KSR1 regulates BARD1 protein expression. Furthermore, as phosphorylation of BARD1 is critical for the function of BRCA1/BARD1 complex [419], the effect of KSR1 on BARD1 phosphorylation is worthwhile examining.

In addition, our work has reported a positive correlation between KSR1 and Chk1 in patient tissues. As previously shown that BRCA1 regulates Chk1, Wee1 and 14-3-3 proteins to control the G2/M checkpoint [420], it would be interesting to study whether KSR1 contributes to cell cycle control through BRCA1 and Chk1.

Finally, we have revealed that KSR1 decreases p53 activity, and at the same time it increases BRCA1 expression. Based on the literature, it seems that BRCA1 can compensate for loss of p53 [393]. It would be necessary to explore:

- the effect of KSR1 on BRCA1 transcriptional activity by a luciferase assay using BRCA1-dependent gene constructs;

- whether KSR1 cooperates with DBC1 to affect BRCA1 activity and the BRCA1-p53 signalling, given that DBC1 is a well-studied positive regulator of p53 as well as a repressor for BRCA1 [389, 392].

4.1.2.3 Conclusion

Collectively, our study shows that KSR1 may be a valuable prognostic marker in breast cancer. The mechanistic findings demonstrate that KSR1 regulates breast cancer development through the BRCA1-BARD1 complex, and consequently affects the *in vitro* and *in vivo* oncogenic potential of breast cancer cells, highlighting a new role of KSR1 in tumourigenesis.

4.2 Proteomic analysis of TKs signalling in breast cancer

TKs are important regulators in multiple intracellular pathways related to most aspects of cell activities, including proliferation, survival, differentiation, apoptosis, metabolism and motility [13]. Deregulation of TK signalling leads to alteration of their kinase activity, which contributes to the development and progression of human diseases, particularly cancer [13, 56]. Considering their pivotal role in cellular signalling and essential involvement in oncogenesis, extensive studies on addressing the structural characteristics and biological functions have focused on certain members of the TK family, such as the EGFR subgroup. Additionally, in some cases, TKs have become an important group of drug targets and therapies against TKs have improved the clinical outcome of patients in the past decades [56, 218]. However, up to now, still approximately half of the 90 TKs are poorly investigated and a global functional analysis to elucidate their regulated-proteome and related signalling processes is very much needed [197]. A global mapping of the TKs-regulated proteomes with an integrated functional exploration will shed new light on our understanding of this important family in the human kinome.

4.2.1 Summary of findings

In this work, we established an approach using a high throughput RNAi screen combined with SILAC-based quantitative proteomics to decode the TKs-regulated proteome and their associated signalling in MCF7 breast cancer cells. We first determined the expression profile of all 90 TKs by RT-qPCR and our results revealed that 66 TKs are expressed in MCF7. Among the 66 identified TKs, we were able to successfully silence 65 of them verified by RT-qPCR showing a decrease of at least 70% at mRNA levels. A good silencing efficacy was also confirmed by western blotting on several of these TKs. Next, a SILAC-based quantitative proteomic analysis to accurately identify the TKs-regulated proteomes and to quantify their expression alterations was carried out upon silencing 65 TKs individually in MCF7. Our SILAC proteomic analyses identified 2,000 proteins modulated and quantified 1,500 out of them after each individual silencing of the 65 TKs. Intriguingly, in spite of structural homology, silencing of each TKs results in diverse proteomic landscapes and the massive data acquired from SILAC-based analysis enabled further bioinformatics exploration to obtain a fuller picture of TKs-modulated proteomes and their associated functional networks.

We then produced a heatmap of quantified protein expression levels to visualize the overall regulation pattern of altered proteomic portrait upon silencing all 65 TKs. As presented in the

heatmap, similarity was observed in the altered expression levels of the proteomes leading to hierarchical clustering of the 65 TKs. Subsequently, we characterized a new classification of TKs arising from the calculated correlation distances, which represent the similarity in regulated proteomes. A total of 10 distinctive clusters were established and the analysis revealed that the number of TKs within each cluster varies, with 15 in the largest group and 2 in the smallest one. Although our clustering method is completely different from the way used for the well-established TKs classification, which is based on sequence relatedness of catalytic domains, some TKs are grouped into a same cluster by the two classifications, such as EPHA2 and EPHA4, EPHB1 and EPHB4. This supports a degree of consistency between the two approaches of clustering TKs, but also indicates that some TKs probably should not be classified together in the first place, considering the marked differences in regulated proteomes and their associated biological function.

Further analysis identified the most up- and down-regulated proteins in the 10 classified clusters, which showed a distinctive signature of regulated proteins in each group. In-depth GO analysis was performed to elucidate the biological functional portrait related to each cluster. Consistent to what has already known about TKs-modulated molecules, our results demonstrated that the proteins regulated by TKs are distributed into a variety of cellular compartments, including the extracellular matrix, cell membrane, cytoplasm, nucleus, synapse and other organelles. Moreover, these proteins are important in a wide range of biological processes, including immune system process, reproduction, metabolic process, cell growth, developmental process, cell cycle and cell adhesion, whose perturbation can contribute to cancer initiation and development. We also discovered a major population of proteins with binding, catalytic, structural and enzyme regulatory activities in each cluster highlighting the influential contribution of TKs in coordinating signal transduction through phosphorylation and direct interaction. We then integrated GO analysis with the STRING database to evaluate the functional networks assembled by the TKs-regulated proteomes. Distinctive features displayed by specific functional networks were uncovered in each cluster. Interestingly, in some cases, same biological processes were shown to be enriched in different clusters, such as metabolic processes in cluster 4 and 7, possibly indicating a functional compensation through cross-talk by TKs from different clusters. Finally, our classification highlights the similarity in TKs-regulated biological function and their associated signalling network within each cluster, and supports their recognized role in a wide range of cellular processes, while hinting at their participation in undetermined signalling pathways that require further exploration.

4.2.2 Future direction

In this study, the SILAC-based proteomics has accurately identified and quantified a substantial part of the proteome upon silencing 65 TKs in breast cancer. Following bioinformatics analysis has identified 10 unique clusters and illustrated multiple defined functional networks within each cluster. Up to now, all the initial analyses have been completed, and future work will aim to:

- validate altered expression levels of specific candidates, such as the MCM proteins in cluster 8;
- investigate the oncogenic potential of the identified TKs-regulated signalling network in breast cancer, including the JAK1, LCK and PTK2 associated-MCMs cascade;
- determine the clinical significance of the established clusters in breast cancer patients using online free-access database such as KM Plotter and Oncomine platform.

4.2.3 Conclusion

In conclusion, we performed a global unbiased SILAC-based proteomic analysis to identify the TKs-regulated proteomes in breast cancer. More than a thousand proteins were detected and quantified, showing diverse landscapes of modulated proteins upon silencing 65 individual TKs. Based on the similarity in their proteomic changes, we presented 10 new distinctive clusters from the 65 TKs and ultimately characterized a unique proteomic signature and functional portrait in each cluster. Our defined functional analysis of the TKs-regulated proteomes supports a fundamental involvement of TKs in every aspect of key biological processes. Our established database in this study is a useful resource for the initial inspection of the function of your TK of interest and its associated signalling networks. Furthermore, it provides researchers with many potential targets that make up cellular processes of known importance in breast cancer.

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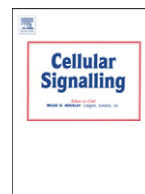
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APPENDICES



Review

The role of pseudokinases in cancer

Hua Zhang, Andrew Photiou, Arnhild Grothey, Justin Stebbing, Georgios Giamas*

Department of Cancer and Surgery, Imperial College, Hammersmith Hospital Campus, Du Cane Road, London, W12 0NN, UK

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ABSTRACT

Kinases play a critical role in regulating many cellular functions including development, differentiation and proliferation. To date, over 518 proteins with kinase activity, comprising ~2–3% of total cellular proteins, have been identified from within the human kinome. Interestingly, approximately 10% of kinases are categorised as pseudokinases since they lack one or more conserved catalytic residues within their kinase domain and were originally thought to have no enzymatic activity. Recently, there has been strong evidence to suggest that some pseudokinases can not only function as scaffold proteins, but may also possess kinase activity leading to modulation of cell signalling pathways. Altered activity of these pseudokinases can result in impaired cellular function, particularly in malignancies. In this review we are discussing recent evidence that apart from a scaffolding role, pseudokinases also orchestrate cellular processes as active kinases *per se* in signalling pathways of malignant cells.

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Contents

1. Introduction	1174
2. Pseudokinases: evolutionary counterparts of protein kinases?	1174
3. Pseudokinases with activity	1175
3.1. KSR1	1175
3.1.1. Dual activity of KSR1: scaffold protein and protein kinase	1175
3.1.2. KSR1 in cancers: a potential therapeutic target?	1175
3.1.3. Recent advances on KSR1 functions	1176
3.2. HER3	1176
3.2.1. HER3 and cancer	1177
3.3. CASK	1177
3.3.1. CASK, and its role in cell proliferation and cancer	1177
3.4. ILK	1178
3.4.1. ILK and cancer	1178
3.5. JAKs and cancer	1178
4. Pseudokinases that exert allosteric regulation and scaffolding function	1179
4.1. STRAD α	1179
4.1.1. STRAD α and cancer	1179
4.2. Tribbles family	1179
4.2.1. Trb1, trb2 and cancer	1179

Abbreviations: ALL, Acute Lymphoblastic Leukaemia; AML, Acute Myeloid Leukaemia; AMPK, 5'-Adenosine Monophosphate Activated Protein Kinase; ATF4, Activating Transcriptional Factor 4; ATP, Adenosine-5'-Triphosphate; BTICs, Brain Tumour-Initiating Cells; C/EBP α , CCAAT/Enhancer-Binding Protein-A; CASK, Ca²⁺/Calmodulin-Dependent Serine Protein Kinase; CCK4, Colon Carcinoma Kinase 4; C-TAK1, Cdc25C-Associated Kinase 1; EGF, Epidermal Growth Factor; EGFR, EGFR Receptor; ER α , Oestrogen Receptor A; FOXO3a, Forkhead Box O3a; GSK3, Glycogen Synthase Kinase 3; HAT, Histone Acetyltransferase; HER3 (erbB3), Receptor Tyrosine Protein Kinase 3; IL-9, Interleukin-9; ILK, Integrin-Linked Kinase; JAK, Janus Tyrosine Kinase; JNK1, C-Jun Amino Terminal Kinase-1; KSR1, Kinase Suppressor of Ras1; LIM, Lin11, Isl-1 and Mec-3; LKB1, Liver Kinase B1; LOH, Loss of Heterozygosity; MAGUK, Membrane Associated Guanylate Kinase; MAPK, Mitogen-Activated Protein Kinase; MO25 α , Mouse Protein 25 A; MT1-MMP, Membrane Type-1 Matrix Metalloproteinase; mTOR, Mammalian Target of Rapamycin; PI3K, Phosphatidylinositol-3-OH Kinase; PIKK, Phosphatidylinositol 3-Kinase-Related Kinase; PJS, Peutz-Jeghers Syndrome; PKA, Protein Kinase A; PP2A, Protein Phosphatase 2A; PRD, PIKK-Regulatory Domain; RTK7, Receptor Tyrosine Kinase 7; STATs, Signal Transducers and Activators of Transcription; STRAD α , STE-20-Related Adaptors A; TFF1, Trefoil Factor 1; TGF- β , Transforming Growth Factor-B; TNF α , Tumour Necrosis Factor A; Trb, Tribbles Homolog; TRRAP, Transformation/Transcription Domain-Associated Protein; Tyk2, Tyrosine Kinase 2; VRK-3, Vaccinia-Related Kinase 3.

* Corresponding author.

E-mail address: g.giamas@imperial.ac.uk (G. Giamas).

4.2.2.	Trb3 and cancer	1180
4.3.	TRRAP	1180
4.3.1.	TRRAP and cancer	1180
4.4.	CCK4 and cancer	1181
4.5.	VRK-3	1181
4.5.1.	VRK-3 and cancer	1181
5.	Conclusions and perspectives	1181
	Acknowledgements	1181
	References	1181

1. Introduction

The human kinome accounts for approximately 2% of all genes [1,2] and protein kinases are essential for regulating many cellular functions in both the physiological and pathological context. Kinases have conserved residues necessary for their catalytic activity, by transferring phosphate groups from adenosine-5'-triphosphate (ATP) to specific serine, threonine or tyrosine residue in target proteins. Phosphorylation leads to activation or inhibition, depending on the target substrate, usually resulting in downstream effects involving other proteins. However nearly 10% of known kinases lack one or more of these residues and are classified as pseudokinases therefore becoming catalytically inactive or lacking necessary binding domains. Pseudokinases are randomly distributed on the phylogenetic tree of kinases suggesting that they may have evolved from diverse active kinases. The initial belief that pseudokinases are vestigial remnants of active kinases that primarily act as scaffold proteins is changing. Some of them demonstrate kinase activity while others play critical roles as activators of their specific targets. Aberrant regulation of pseudokinases is implicated in the cause and development of a variety of diseases. In this review, we identify which pseudokinases have been associated with cancer and discuss recent important advances in uncovering their potential roles as therapeutic targets.

2. Pseudokinases: evolutionary counterparts of protein kinases?

The term “pseudokinase” originated from the concept that these protein kinases lack one or more conserved residues that are crucial for enzymatic activities compared to classic protein kinases such

as protein kinase A (PKA), and therefore they are not be able to phosphorylate different substrates. Based on sequence analysis, Manning and colleagues [1] showed that approximately 50 protein kinases in the human kinome are classified as pseudokinases, which require the intact three motifs: i) VAIK (Val-Ala-Ile-Lys), ii) HRD (His-Arg-Asp) and iii) DFG (Asp-Phe-Gly) for ATP and peptide binding (Fig. 1) (reviewed by Boudeau and Alessi, Zeqiraj and Aalten [3–5]). Interestingly, it appears that these pseudokinases are widely located in every branch in the phylogenetic tree, which implies from evolutionary perspective that they might function similarly to active protein kinases. Recent structural investigations have provided further evidence to support this hypothesis. For example, the WNK kinase lacking the key lysine (K) residue in the VAIK motif, within the catalytic subdomain II was shown that it can function actively by using another neighbouring lysine in the β strand 2 domain [6,7]. Ca^{2+} /calmodulin-dependent serine protein kinase (CASK) is another good example where despite the absence of key enzymatic residues in the DFG motif, which is indispensable for Mg^{2+} binding, it can still implement itself in a constitutively active conformation, capable of autophosphorylation and for phosphorylating specific substrate such as the synaptic protein neuexin-1. Unlike other kinases that require Mg^{2+} for their function, CASK works independently of Mg^{2+} , which surprisingly inhibits its catalytic activity [8,9]. The elucidation of the crystal structure of the receptor tyrosine protein kinase HER3 (erbB3) challenged the concept that it was an inactive pseudokinase, since it was lacking the intact conserved motif HRD, which is required for phosphorylation. In fact, in order to promote phosphate transfer, HER3 can adopt itself in an active conformation through a unique mechanism by its kinase-defective domain [10,11].

No matter what functions these pseudokinases perform either as scaffold proteins, allosteric regulators or active kinases, emerging

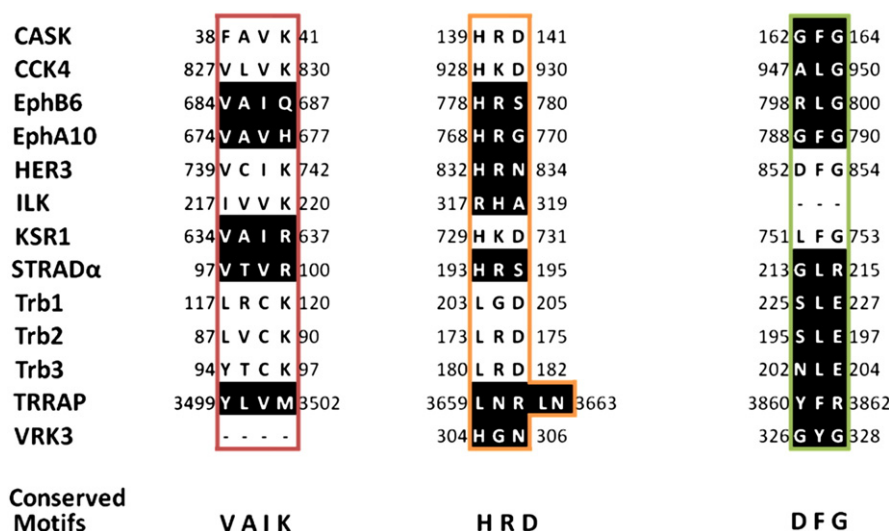


Fig. 1. Multiple sequence alignment of pseudokinases comparing the three conserved motifs (VAIK, HRD and DFG), located within the catalytic domain, which are essential for enzymatic activity. Highlighted in black are the indicated missing motifs for each pseudokinase.

Table 1
Catalytic activity and functions of pseudokinases involved in cancer.

Pseudokinases	Kinase family	Activity	Substrates	Function	Types of cancer	References
CASK	CAMK	Yes	Neurexin-1	Not known	Oesophageal, acute lymphoblastic leukaemia, squamous cell carcinomas	[91–93]
CCK4	TK	Not known	N/A	Promotes tumour proliferation and invasion	Colon, melanoma and AML	[208,209,211,213]
EphB6	TK	Not known	N/A	Suppresses tumour invasion and metastasis	Neuroblastoma, non-small cell lung, melanoma and breast	[225–232] and reviewed in [233]
EphA10	TK	Not known	N/A	Potentially promotes tumour metastasis	Prostate and breast	[234,235] and reviewed in [233]
HER3	TK	Yes	N/A	Promotes tumour proliferation, invasion and metastasis	Breast, non-small cell lung, melanoma, ovarian and colorectal	[53,54,68,69,73,76,77,79,80] and reviewed in [67]
ILK	TKL	Controversial	β 1 Integrin, AKT, MLC, GSK3 β and others	Promotes/suppresses tumour proliferation, invasion and migration	Prostate, colon, gastric, ovarian, melanoma, breast and rhabdomyosarcoma	Reviewed in [95,96,99,119,236]
KSR1	TKL	Controversial	Raf, MEK, ERK	Promote tumour proliferation	Breast, pancreatic and non-small cell lung	[31–34]
STRAD α	STE	Not known	N/A	Indirect tumour suppressor activity	Breast, colon, pancreatic, stomach, ovary	[157–159]
Trb1	CAMK	Not known	N/A	Induces leukemogenesis	AML	[167–170]
Trb2	CAMK	Not known	N/A	Induces leukemogenesis and promotes tumour proliferation	AML, melanoma, non-small cell lung	[167,171,172,175,176]
Trb3	CAMK	Not known	N/A	Promotes tumour proliferation, invasion and migration	Colon, oesophageal, breast and lung	[177–179]
TRRAP	Atypical protein kinase	Not known	N/A	Involved in tumorigenesis and cell proliferation	Breast, malignant melanoma, glioblastoma multiforme, pancreatic	[199,201,202,206,207]
VRK3	CK1	Not known	N/A	Not known	Colon	[224]

evidence has shown that they are involved in cell proliferation, differentiation, apoptosis and metastasis (Table 1).

3. Pseudokinases with activity

3.1. KSR1

Kinase suppressor of ras1 (KSR1) was first identified more than 15 years ago as a novel protein kinase functioning either downstream of Ras or on a parallel pathway to Raf in *Drosophila* and *Caenorhabditis elegans* by genetic screenings, showing that defects in KSR1 inhibited activated Ras, but did not affect the pathways induced by activated Raf [12,13]. KSR1 was demonstrated to be able to translocate from the cytoplasm to the plasma membrane to form a complex involving Raf-1, MEK1 and 14-3-3 in the presence of activated Ras. In turn, this led to the activation of Raf-1 without requiring kinase activity of KSR1, which indicated the central role as a modulator in the mitogen-activated protein (MAP) kinase pathway [14–16]. KSR1 contains five conserved areas named CA1–CA5. CA1 is located in the N-terminus and is unique to KSR1; CA2 is a proline-rich domain with unidentified functions; CA3 is a cysteine-rich sequence mediating membrane recruitment of KSR1 [16]; CA4 is a serine/threonine-rich region with an FXFP motif that binds ERK [14,17]; CA5 is kinase-like domain lacking the conserved lysine residue in subdomain II that is required for phosphorylation [18,19].

3.1.1. Dual activity of KSR1: scaffold protein and protein kinase

Several groups showed that murine KSR1 (mKSR1) can bind to MEK-1/2 and ERK but not Ras or Raf, and that mKSR1 in fact functions as a scaffold protein suppressing the activity of MAPKs after different stimulations such as growth factors and activated Ras [20–23]. Phosphorylation sites at Ser297 and Ser392 of KSR1 were shown to regulate binding to 14-3-3 proteins in serum-free conditions and the function of KSR1 depended on its expression levels. More specifically, at low levels, KSR1 appeared to increase Ras-induced activity, but inhibited the Ras pathway when expressed at higher levels [24]. This was confirmed in KSR1^(−/−) mouse embryo fibroblasts, expressing a range of KSR1 concentrations. With low KSR1 expression, ERK activation was enhanced with a concomitant increase in cell growth

and elevated RasV12 oncogenic transformation. Conversely with higher KSR1 expression all these activities were significantly reduced [25,26].

The structural similarity between KSR1 and the Raf kinase family and its implications in cancer generated much interest in trying to elucidate its potential kinase activity. TNF α and ceramide were firstly reported to be capable of inducing KSR1 autophosphorylation and phosphorylating Raf-1 at Thr269 both *in vitro* and *in vivo* [20]. The same group showed that epidermal growth factor (EGF) acts as an activator to increase the kinase activity of KSR1 in a two-stage *in vitro* assay. Phosphorylation was only possible with an intact kinase domain, as deletions at the C- and N-terminal domains rendered the domain inactive. Meanwhile, its kinase ability did not rely on binding to other kinases, such as MEK [21,22,27]. Furthermore, threonine phosphorylation of Raf-1 by tumour necrosis factor α (TNF α) activation is regulated by KSR1. When mouse colon cells were transfected to express a dominant-negative kinase-inactive KSR1, TNF α treatment reduced survival and increased apoptosis, supporting the essential role of KSR1 in cell survival pathways in intestinal epithelial cells [23,27,28].

Further insights on the function of KSR1 other than its kinase activity have been gained, particularly as a scaffold protein in the Ras–Raf–MAPKs signalling pathways. In quiescent cells, phosphorylation of KSR1 at Ser297 and Ser392 by Cdc25C-associated kinase 1 (C-TAK1) is required for KSR1 to remain in the cytoplasm through binding to 14-3-3. Upon growth factor stimulation, KSR1 is dephosphorylated by protein phosphatase 2A (PP2A) resulting in the release of 14-3-3. This exposes the CA3 domain of KSR1 leading to cell membrane translocation, where a complex is formed with activated Ras, Raf-1 and MEK facilitating downstream MAPKs signalling (Fig. 2) [24,29,30].

3.1.2. KSR1 in cancers: a potential therapeutic target?

Studies are beginning to elucidate the function of KSR1 regarding the pivotal role of Ras–Raf–MAPKs module in cancer. In a Ras-dependent breast cancer model, tumour inhibition was observed in KSR1^(−/−) mice where KSR1 deficiency led to the loss of MEK and ERK complexes [31]. Similarly, in a skin cancer mouse model Tg.AC (v-Ha-ras mutated at codons 12 and 59), KSR1 was necessary for

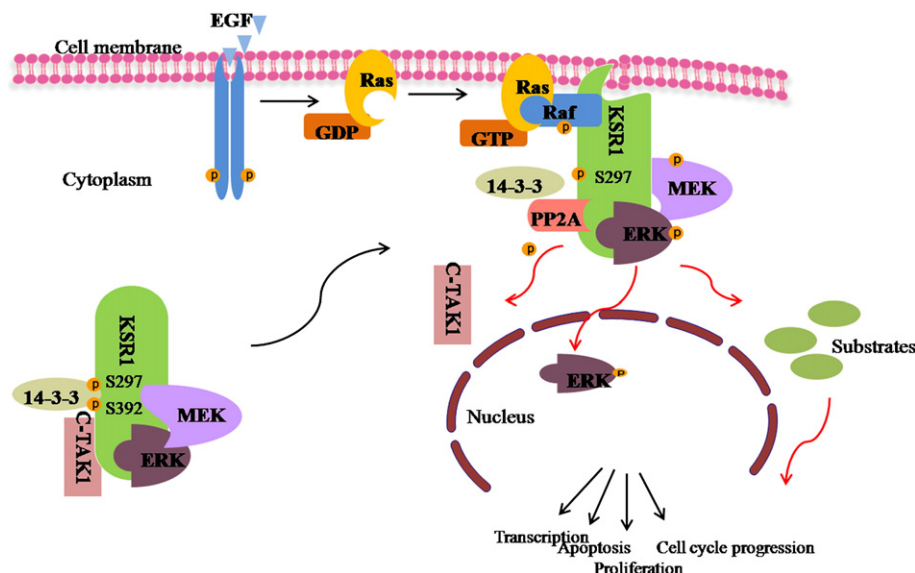


Fig. 2. Schematic model for the function of KSR1. In inactive cells, phosphorylation of KSR1 at Ser297 and Ser392 by C-TAK1 allows it binding to 14-3-3, which maintains KSR1 in the cytoplasm. KSR1 also constitutively interacts with MEK and ERK. Upon stimulation by growth factors such as EGF, KSR1 is dephosphorylated by PP2A at S392 resulting in the release of 14-3-3 and exposure of the CA3 domain on KSR1. This leads to cell membrane translocation where KSR1 forms an active complex with phosphorylated Raf, MEK and ERK. The activated ERK can in turn phosphorylate various substrates in the cytoplasm and nucleus regulating proliferation, cell cycle and apoptosis.

the formation of tumours, which also indicated KSR1 as a potential therapeutic target in Ras-related cancers [32]. Continuous infusion of KSR1 phosphorothioate antisense oligonucleotides into human PANC-1 pancreatic and A549 non-small-cell lung carcinoma xenografts in nude mice produced significant PANC-1 tumour shrinkage and induced inhibition of A549 lung metastasis [33,34]. Furthermore, KSR1 downregulation using antisense oligonucleotide sensitised ionising radiation treatment in epidermal growth factor receptor (EGFR)-dependent A431 and oncogenic K-Ras-driven A549 human lung carcinomas *in vivo* [35]. Overexpression of wild-type KSR1 into MCF-7 breast cancer cells increased cisplatin-related ERK activity and enhanced cisplatin sensitivity [36]. A follow-up screen using a collection of cancer cell lines and the NCI60 anticancer drugs also confirmed a correlation between KSR1 expression and drug sensitivity [37].

KSR1 was also shown to interact with metastasis suppressor Nm23-H1 in breast cancer cells. Nm23-H1 can phosphorylate KSR1 and promote its degradation resulting in decreased activities in the ERK pathway [38,39]. In addition, KSR1 was necessary for cells to recover from DNA damage in response to various agents such as mitomycin C [40]. Recently, by performing a whole human kinome screening using RNA interference, we identified that KSR1 is a novel regulator of oestrogen receptor α (ER α) function. Knockdown of KSR1 resulted in downregulation of known ER α regulated genes such as *TFF1* suggesting that KSR1 might play an important role in ER α -dependent breast cancer [41].

3.1.3. Recent advances on KSR1 functions

There is still some argument whether KSR1 is an active kinase or just a scaffold protein functioning in the Ras-Raf-MAPKs pathway. Recent evidence suggests that KSR1 may possess dual activity. By using purified and recombinant wild-type KSR1, two different groups demonstrated that wild-type KSR1 was capable of directly phosphorylating Raf-1 and MEK1, whereas mutated KSR1 was without activity [42,43]. A further study on the catalytic activity of KSR1 was reported by Hu et al. This group generated a mutant KSR1 construct (A587F) by adding a bulky phenylalanine at the ATP binding pocket of KSR1 to impair ATP binding. This mutant still maintained the closed active conformation required for scaffold function allowing it to interact with C-Raf and MEK constitutively. However, only the wild-type

KSR1 was able to phosphorylate MEK induced by C-Raf, which strongly indicates that enzymatic activity of KSR1 might be specific to certain substrates in a defined context [44].

Other studies have supported the belief that KSR1 can directly form a side-to-side dimer with Raf to regulate the activation of Raf without the need of its catalytic activity. A potential dimer formation was suggested based on crystal structure analysis of the conserved residues within KSR1 and Raf. In addition, Raf activation was impaired by residue mutations in the dimer interface of KSR1 [45]. Interestingly, McKay et al. found that some Raf inhibitors were preferentially capable of inducing KSR1/B-Raf dimerisation compared to C-Raf/B-Raf dimerisation, on the condition that they all had a complete dimer interface. Furthermore, KSR1/B-Raf interaction abrogated the activation of the ERK cascade pathway [46].

Collectively, these findings re-emphasise the crucial role of KSR1 in modulating Ras-Raf-MAPKs signalling functioning both as protein kinase and scaffolding protein.

3.2. HER3

HER3 belongs to the human epidermal growth factor receptor family of receptor tyrosine kinases, which also include EGFR, HER2 and HER4 isoforms. A number of growth factors bind extracellularly to these receptors resulting in the formation of dimers leading to the activation of downstream signalling pathways resulting in cell proliferation and differentiation, whereas, abnormal activation contributes to pathogenesis of many cancers [47,48]. Among these diverse homodimer and heterodimer complexes, HER2-HER3 is shown to be the most active form [49]. The uniqueness of HER3 is that it has long been shown to be an inactive kinase because of amino acid substitutions within the conserved kinase domain compared to the other members in this family. Thus, HER3 can function allosterically as a catalytic partner in order to activate the other family members [50,51].

A recent study showed that the kinase domain of HER3 was able to bind ATP and to trans-autophosphorylate its intracellular region by forming a heterodimer between the intact intracellular kinase domain and the kinase domain [10]. However, compared to other EGFR family members, the kinase activity of HER3 was significantly decreased and no phosphorylation could be demonstrated when

using exogenous peptides as substrates. Moreover, Shi and colleagues demonstrated that although HER3 lacked several key conserved residues, it might employ a distinctive mechanism to transfer the phosphate group by using the existing kinase-like conformation instead of the catalytic base aspartate [10,11]. A further study suggested that although the kinase activity of HER3 is far lower than the other family members, it may still be adequate to activate phosphatidylinositol-3-OH kinase (PI3K)/AKT pathway and contribute to the resistance of tyrosine kinase inhibitors (TKIs) [52]. Considering that HER3 may play a critical role in overcoming the resistance to EGFR or HER2 inhibitors in cancers, more detailed functional and mechanistic studies are needed to focus on the interaction between family members and identification of unique substrates.

3.2.1. HER3 and cancer

All members of the HER family are believed to be involved in human cancers, including breast, skin, colon, lung and brain. Although HER3 has not been proved to be an independent oncogene, the allosteric function of HER3 as a dimer partner is widely implicated in tumour development.

Much interest was generated after the HER3 gene was cloned and its expression was detected in primary breast carcinomas. Its role in breast cancer has been intensively studied [53,54]. The association between HER2 and HER3 expression in mammary carcinoma cells after heregulin activation indicated that cancerous transformation required them to function together [55,56]. Elevated HER2 expression and activity were not sufficient to drive tumour cell proliferation, without forming a heterodimer with HER3 [57]. Consistent with this finding, knockdown of HER3 inhibited HER2 tyrosine phosphorylation, decreased breast cancer cell proliferation and colony formation and reduced HER2-mediated tamoxifen resistance by suppressing PI3K/Akt signalling [58]. In addition, HER3 was required in oestrogen-induced breast cancer cell growth and activated HER2 tyrosine kinase, Akt, and mitogen-activated protein kinase signalling [59]. Moreover, activation of the HER3-dependent pathway increased intravasation and lung metastasis in orthotopic breast cancer models [60]. By analysing paired patient specimens, increased HER3 expression and somatic mutations in EGFR, HRAS, KRAS, NRAS or PIK3CA were observed compared to primary breast tumours [61]. HER3 also played a critical role in HER2-mediated paclitaxel resistance. In both transient and stable HER3 overexpression models, cell growth inhibition and apoptosis induced by paclitaxel were greatly reduced in breast cancer cells overexpressing HER2, but not in cells with upregulated EGFR. Mechanistic studies suggested that amplified HER3 contributed to HER2-mediated paclitaxel resistance by upregulating Survivin via the PI3K/Akt/mTOR signalling pathway [62]. Upregulation of HER3 was believed to play a pivotal role to compensate for cell growth signals after treated with PI3K or EGFR inhibitors [63,64]. In addition, Garrett and Cook et al. showed that a combination of HER2, HER3 and PI3K inhibitors may provide a better outcome in HER2 and PI3K dependent tumours, as knockdown of HER3 by either siRNA or specific antibodies improved the response to lapatinib in breast cancer cells both *in vitro* and *in vivo*. Furthermore, ablation of both HER2 and HER3 produced a greater inhibition of PI3K/Akt activity compared to single inhibitor [65,66].

Further studies demonstrated that HER3 was involved in EGFR dependent non-small cell lung cancer, mainly through the ability of somatic mutant EGFR to phosphorylate kinase-defective HER3, leading to activation of PI3K/AKT cell growth pathways [67]. Although patients treated with EGFR inhibitors, such as gefitinib or erlotinib, initially showed a good response, acquired resistance and tumour relapse due to secondary mutations soon developed [68–72]. Recently, a novel mechanism for gefitinib resistance involving HER3 was reported. Engelman et al. described that upregulated proto-oncogene MET can trigger the activation of HER3 downstream pathways that contributes to gefitinib resistance in lung cancer [73,74].

Apart from the central role of HER3 in cell survival and mediation of drug resistance, it was also shown that phosphorylated HER3 is significantly increased in lung cancer brain metastases relative to primary tumours, supporting its possible role in invasion and distant metastasis [75].

HER3 has also been shown to be upregulated in other types of malignancies, including melanoma, ovarian and colorectal cancers. Elevated HER3 correlated with poor prognosis in melanoma and colorectal cancers [76–78] while HER3 overexpression was associated with decreased survival in patients with ovarian cancer [79]. Blockade of HER3 suppressed melanoma cell proliferation, migration, and invasion *in vitro* [76]. A neuregulin (NRG1)/HER3 autocrine signal-transducing loop was found to play an important role in ovarian cancers, and use of either siRNA against HER3 or a monoclonal antibody targeting HER3 significantly reduced ovarian cancer cells proliferation *in vitro* and inhibited tumour growth *in vivo* [80]. Although some questions have been clarified on functions of HER3 in tumour proliferation, invasion and metastasis, further understanding will help us to develop novel therapies.

3.3. CASK

Calcium/calmodulin-dependent serine protein kinase (CASK) is a pseudokinase belonging to the membrane-associated guanylate kinase protein family (MAGUK) that function as multiple domain adaptor (scaffolding) proteins [81]. CASK (LIN2) was originally reported in *C. elegans* and in *Drosophila* (Caki) [82,83]. CASK is one of the 10 subfamilies consisting of a PDZ domain, a catalytically inactive guanylate kinase (GUK) domain and a Src Homology 3 (SH3) domain [84] and acts as a scaffold protein playing a role in protein organisation. In mammals, it is located at cell junctions and synapses and is thought to play a critical role in brain development and central nervous system function [85,86]. It can bind to more than 20 cellular proteins in different subcellular regions of neurons and form protein complexes for the interaction with the transmembrane protein neuroligin at pre-synaptic sites leading to downstream events [87]. Mutations lead to brain malformation, mental retardation and neonatal lethality [88]. In a single study, it was demonstrated that CASK functions as an active protein kinase that phosphorylates neuroligins providing further evidence to suggest that at least some “pseudokinases” retain kinase activity in addition to scaffold properties [8].

3.3.1. CASK, and its role in cell proliferation and cancer

To date, there are only a few studies that associate CASK with cancer, although it has been implemented in the regulation of cell proliferation. Human CASK was reported to bind inhibitor of DNA binding 1 (Id1) via its kinase domain to inhibit cell growth of ECV304 cells. Over-expression of human CASK resulted in a reduced rate of cell growth while a reduction of CASK levels using siRNA led to an increase of cell proliferation. CASK was also shown to regulate p21^{waf1/cip1} at the mRNA and protein level [89]. In another study, Ojeh et al. showed that CASK is able to regulate proliferation and adhesion of epidermal keratinocytes. CASK is localised in nuclei of basal keratinocytes in newborn rodent skin and developing hair follicles. Knockdown of CASK led to increased proliferation in cultured keratinocytes and in skin raft cultures [90].

In one study, matched normal tissues were compared to human oesophageal carcinoma samples taken from patients that did not receive treatment. CASK and its target gene Reelin were significantly up-regulated in human oesophageal carcinoma at the mRNA and protein level. In contrast, mRNA expression patterns of CASK in gastric and colon carcinomas were up-regulated in approximately 50% of cases but Reelin mRNA levels did not differ from matched normal tissue. The interaction between CASK and Reelin appeared to differ

in organs and it was speculated that CASK may have a role to play in oesophageal cancer [91].

A differential expression of CASK in high-risk non-relapsed *versus* relapsed non-determinant chromosomal translocation (NDCT) acute lymphoblastic leukaemia patients has been reported. Gene expression levels were 3 times higher in non-relapsed *versus* relapsed NDCT patients [92].

Finally, using laser capture dissection and gene expression profiling, CASK was found to be elevated in squamous cell carcinomas samples when compared to either adenocarcinomas, small cell lung cancers or normal lung tissue [93].

3.4. ILK

Integrin-linked kinase (ILK), a serine/threonine kinase, was originally discovered using a yeast two-hybrid screening system and was found to interact with the cytoplasmic domain of $\beta 1$ integrin [94]. ILK has an N-terminal ankyrin repeat domain, a pleckstrin-homology (PH)-like domain and a C-terminal catalytic domain [95]. The ankyrin repeat domain binds to LIM (Lin11, Isl-1 and Mec-3) found in scaffold protein domains of the interestingly novel cysteine–histidine rich protein PINCH family. The C-terminal domain reacts with parvin forming the ILK–PINCH–Parvin (IPP) complex, which forms a scaffold with other factors [96]. ILK has an important role in cell adhesion by linking integrin to the actin cytoskeleton [96,97].

The argument as to whether ILK is a pseudokinase or it possesses kinase activity has recently been discussed by Hannigan et al., as there has been some debate in recent years relating to its true function [98]. In some studies, no substrates were identified for ILK and it was considered to be a pseudokinase as it lacks the HRD and DFG motifs in its kinase domain [96]. Furthermore, the crystal structure of the kinase domain, differs from those of active kinases [97]. However, as shown by Hannigan et al., ILK has been shown to phosphorylate a variety of different substrates, including glycogen synthase kinase 3 (GSK3) and AKT [99]. Taking everything into consideration, it is most plausible that ILK is a true kinase and therefore requires re-classification, while it is likely to have a novel mode of activity resulting from changes to its catalytic domain.

ILK has many important functions in signal transduction pathways associated with cell survival, migration, cell adhesion and extracellular matrix, differentiation, cell survival and angiogenesis and is essential for embryonic development and tissue homeostasis, as deregulation leads to abnormal tissue development [95].

3.4.1. ILK and cancer

ILK was originally described as a putative oncogene [100]. There is little doubt that the expression of ILK is strongly associated with tumour progression in the clinical context and unequivocally shown to be associated with tumorigenesis in cell lines and tumour models [101–104]. In a prostate cancer model, inhibition of ILK reduced the rate of growth and decreased the metastatic potential of DU145 cells [105]. ILK siRNA has also been shown to inhibit cell attachment, growth and invasion of gastric cancer *in vitro* and *in vivo* models, possibly through interference with tumour angiogenesis processes, since vascular endothelial growth factor (VEGF) was decreased after silencing [101]. Similar observations were made in human bladder cancer cell lines [106,107] and melanoma [108].

In human malignancies, overexpression of ILK correlates with progressive disease. In tumour tissue, ILK protein levels were increased in many different human cancers including melanoma, colon, gastric, non small cell lung, thyroid, pancreatic, ovarian, prostate, and primitive neuroectodermal tumours, as well as mesothelioma, Ewing sarcoma and medulloblastoma [95,99]. For example, in a paired normal:tumour tissue analysis of human astrocytoma, positive staining was found in 208 of 228 histological specimens and ILK was

significantly overexpressed at the mRNA level. Moreover, overexpression of ILK was associated high grade tumours and shorter patient survival [109]. ILK was shown to phosphorylate AKT leading to the activation of anti-apoptotic pathways [110–112]. Immunohistochemistry analysis showed that ILK and phosphorylated AKT protein levels were overexpressed in laryngeal squamous cell carcinomas, although no correlation was found with stage of disease [113]. Similarly, ILK overexpression and AKT activation were found in hepatocellular carcinoma, where no correlation was found between expression levels and patient survival [114,115].

In ovarian carcinoma, ILK expression was correlated with tumour grade and expression was absent in normal ovarian epithelium [116]. Likewise, no ILK was detected in normal bronchial epithelium, while it was expressed in human basal cell carcinoma [117] as well as in 68.5% of squamous cell carcinoma and 61.5% adenocarcinoma cases respectively [118]. ILK has also been reported to upregulate the transcription factor Twist1 which is associated with several aggressive forms of breast, prostate, gastric and oesophageal cancers [118].

Interestingly, observations in paediatric rhabdomyosarcoma were different to those for other malignancies. In these cases, ILK was found to act as a tumour suppressor protein and this was linked to overexpression of c-jun amino terminal kinase-1 (JNK1) and not to any ILK mutations. Conversely in alveolar rhabdomyosarcoma, ILK acts as a proto-oncogene [119,120].

In conclusion, since the involvement of ILK in cancer has been clearly demonstrated, inhibition of ILK may provide a possible novel cancer treatment. To this end, several small molecule ILK inhibitors have been evaluated as possible anticancer agents [95,99,121,122].

3.5. JAKs and cancer

The Janus tyrosine kinase (Jaks) family has four members (Jak1, Jak2, Jak3 and Tyrosine kinase 2 (Tyk2)). This family is composed of seven Jak homology (JH) domains, named JH1–JH7 from the carboxyl to amino terminus. Interestingly, JH1 is a typical tyrosine kinase domain whereas the neighbouring JH2 region is a pseudokinase domain serving regulatory functions of Jaks activity through binding to JH1 [123–125]. Because of the differing domains, it is difficult to classify these as pseudokinases. Jaks normally interact with cytokine receptors on the cell surface becoming active and can in turn phosphorylate the cytoplasmic domain of the receptors. This results in the recruitment of downstream signalling molecules such as signal transducers and activators of transcription (STATs) whose activation is widely implicated in many cancers [126–129].

Jak members are highly associated with hematopoietic malignancies. For example, a gain-of-function mutation (V617F) in the JH2 domain of Jak2 correlates with myeloproliferative neoplasias [130–133]. Jak1 mutations were also reported in patients with acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) [134,135]. In one study, approximate 18% of patients with the T cell precursor ALL were shown to have Jak1 mutations, which associates with poor response to therapy and overall prognosis. Three gain-of-function mutations identified were implicated in contributing to interleukin-9 (IL-9) independent resistance to apoptosis of T cell lymphoma BW5147 cells [134]. In addition, Jak3 pathway genes were shown to be elevated in B-lineage acute lymphoblastic leukaemia and the increased expression of these genes correlates with steroid resistance and relapse [136].

Jak family members have also been suggested to play a role in solid tumour growth. In breast cancer, Jaks were shown to interact with Src tyrosine kinase contributing in constitutive activation of Stat3. Inhibition of Jaks by tyrosine kinase selective inhibitors resulted in reduced growth and increased programmed cell death in breast cancer cells [137]. In prostate cancer, Jaks inhibitor, tyrphostin AG490, can suppress Stat3 activation and inhibit the growth of human prostate cancer cells. This indicates that Jaks–Stat3 pathway

is crucial in the survival and proliferation of prostate cancer cells [138]. Furthermore, erythropoietin was capable of activating Jak2–Stat5 signalling leading to metastasis in head and neck squamous cell carcinoma [139]. The Jaks–Stats pathway is also involved in lung cancer. Gao et al. showed that in EGFR constitutively activated human lung adenocarcinoma-derived cells, a pan-Jaks inhibitor (P6) significantly reduced Stat3 phosphorylation. Additionally, P6 suppressed tumorigenesis of human lung adenocarcinoma cells *in vitro* and *in vivo*. Results from this study conclude that Stat3 phosphorylation in lung adenocarcinoma is dependent upon the IL-6/gp130/Jaks pathway [140].

Numerous reviews on the role of Jaks in cancer have been published recently [141–146]. Involvement of Jak kinases in the development of malignancies has resulted in a great number of inhibitors currently being evaluated in clinical trials [147–149].

4. Pseudokinases that exert allosteric regulation and scaffolding function

4.1. STRAD α

STE-20-related adaptor α and β (STRAD α and STRAD β) isoforms (also known as STK5 and STK6), which belong to the STE20 protein kinase family, also lack kinase activity [3]. These pseudokinases are upstream components in signal-transduction pathways controlling a wide range of biological processes. The function of STRAD α is to allosterically activate the tumour suppressor Liver Kinase B1 (LKB1) in a complex with mouse protein 25 α (MO25 α) scaffolding protein, resulting in the activation of 5'-adenosine monophosphate activated protein kinase (AMPK) family members. Similarly, the STRAD β and MO25 β isoforms also stabilise LKB1 [150,151]. Activation of AMPK leads to the regulation of cellular energy status, fatty acid synthesis, cell polarity and proliferation. The kinase domain structure of STRAD α consists of N and C lobes with a cleft between them making up the active site. The activation loop is found in the C terminal lobe and it is phosphorylated at specific sites when the kinase is in an active state, allowing substrate binding. In turn, LKB1 is activated by binding to STRAD α thus not relying on activation by phosphorylation. Direct interaction of their kinase domains strongly suggests an allosteric mechanism of activation [152]. ATP and MO25 α binding to STRAD α changes its kinase domain to an active-like kinase conformation that is characterised by extending its A-loop, allowing STRAD α to bind LKB1. This results in the kinase domain of LKB1 to adopt an active kinase conformation, which is further stabilised by the binding of MO25 α to the A-loop of LKB1 [153]. Moreover, its cellular distribution is determined by STRAD α and MO25 α . Re-localization of LKB1 is induced by STRAD α from the nucleus to the cytoplasm [154]. Mutant forms of STRAD α that are unable to bind MO25 α and ATP fail to activate LKB1 [155,156].

4.1.1. STRAD α and cancer

Peutz–Jeghers Syndrome (PJS) is a rare autosomal dominant disorder where patients have greater chance of developing colon, breast, pancreatic, stomach, and ovarian cancers [157]. PJS is characterised by a predisposition to polyps and hyperpigmentation of the buccal mucosa [158]. Most PJS can be linked to pathogenic LKB1 germline mutations. It was hypothesised that aberrant STRAD α activity may lead to similar effects as those resulting from LKB1 inactivation. Loss of heterozygosity (LOH) analysis was performed in 42 PJS associated tumours (sporadic lung, colon, gastric, and ovarian adenocarcinomas) using eight microsatellite markers on chromosome 17, including p53, BRCA1, and STRAD α . Loss of STRAD α marker near the locus was seen in 13 of 29 patients, which included gastric adenocarcinomas. Specific LOH of the STRAD α marker was found in only 4 cases and no somatic mutations were identified. Furthermore, no germline STRAD α mutations were found in 10 patients with PJS and family members without

LKB1 germline mutation. The authors concluded that STRAD α is unlikely to be a tumour suppressor. Alhopuro et al. reported that germline mutations in STRAD α were not the cause of PJS [159]. Single amino acid substitution mutants of LKB1 isolated from human cancers have lost the ability to interact with STRAD α and were unable to induce G1 cell cycle arrest when overexpressed in cells, indicating the importance of the STRAD α –LKB1 interaction [160,161].

In another study, miR-451 was reported to regulate the LKB1/AMPK pathway in glial tumour cells. It was proposed as a mechanism for enabling tumour cells to adapt to conditions of hypoxia and low energy levels conditions [162]. When glucose levels were not in limited supply, elevated miR-451 levels led to reduced LKB1/AMPK pathway activation, allowing cell growth, increasing mTOR activity and reducing apoptosis. When glucose levels were depleted, miR-451 levels declined allowing activation of AMPK by LKB1-mediated phosphorylation. This promoted cell survival in response to metabolic stress and activated pathways involved in glioma motility. Overexpression of LKB1 caused G1 arrest in Hela cells and the G361 melanoma cell line [163], whereas mutated LKB1 failed to do this allowing proliferation to proceed. Several shorter STRAD α transcripts were identified in colorectal cancer cell lines but no full length transcripts were found. Nevertheless, binding and activation of LKB1 was still possible *in vitro* [158]. Mutations of LKB1 in cancer patients showed that some mutations prevented binding to STRAD α [160]. Aberrant activity of LKB1 is associated with carcinogenesis through deregulation of cell cycle, of which STRAD α and MO25 α co-factors are essential for its normal function.

4.2. Tribbles family

Tribbles was first shown as a regulator in coordinating mitosis and morphogenesis during *Drosophila* development. Through promoting the degradation of Cdc25/String, tribbles blocked mitosis at a critical point to control cell cycle progression [164–166]. There are three mammalian trb isoforms (trb1, trb2 and trb3) homologues to the *Drosophila* tribbles and they all share the highly conserved kinase domain, which lacks catalytic residues.

Despite their conserved structure, their actions differ; trb1 and trb2 are mainly involved in the development of leukaemia, while trb3 is upregulated in non-haematopoietic malignancies. Dedhia et al. generated mice using hematopoietic stem cells expressing all three tribbles isoforms separately. Only those overexpressing trb1 or trb2 developed AML. In addition, this data also showed that trb1 and trb2, but not trb3, have the ability of promoting the degradation of CCAAT/enhancer-binding protein- α (C/EBP α) by resulting in their differential functions in leukemogenesis [167].

4.2.1. Trb1, trb2 and cancer

Trb1 is overexpressed in AML and proven to interact with Homeobox proteins Hox-A9 and Meis1 (Hoxa9/Meis1), which have been demonstrated to induce AML in mice by Hoxa9/Meis1-transduced bone marrow cells [168,169]. Moreover, trb1 appears to be involved in the MAPKs pathway by binding to MEK1 via its C-terminal ILLHPWF motif contributing to myeloid leukemogenesis [170]. Trb2 was described as an oncogene contributing to pathogenesis of AML in mice via suppressing C/EBP α activity, a key transcriptional regulator of differentiation and frequently found mutated in AML while Trb2 expression was also elevated in a group of AML patients [171]. Recently, Trb2 was also suggested to cooperate with HoxA9 and Meis1 to accelerate the process from hematopoietic progenitors to AML [172,173]. More recently, an *in vitro* and *in vivo* structural function analysis revealed that the trb2 kinase domain and the C-terminal constitutive photomorphogenesis 1 (COP1)-binding domain are indispensable in trb2 induced leukaemia for the activity of binding and degrading C/EBP α [174]. Further studies are still needed to establish the functions of trb1 and trb2 in leukemogenesis.

In solid tumours, *trb2* was shown to be overexpressed in malignant melanoma, but not in other types of skin cancer using a cancer-profiling array. *Trb2* expression levels were associated with cytoplasmic localization of Forkhead box O3a (FOXO3a). Decreased *trb2* reduced cell proliferation and colony formation and impaired wound healing. In a melanoma xenograft model, tumour growth was significantly inhibited after *trb2* knockdown [175]. Increased expression of *trb2* was also identified in primary human lung tumours and in non-small cell lung cancer cells and a further study suggested that *trb2* triggered lung tumorigenesis through degradation of C/EBP α [176].

4.2.2. *Trb3* and cancer

Trb3 is overexpressed in a variety of human cancers, including lung, colon and breast tumours and by interacting with activating transcriptional factor 4 (ATF4), it can reduce ATF4 levels to slow down the cell cycle, thereby enabling tumour cells to adjust to hypoxic conditions [177]. It has also been suggested that *trb3* is associated with CtBP-interacting protein (CtIP), a transcriptional factor implicated in malignancy where *trb3* mRNA expression was increased in certain tumour tissues [178]. A recent study showed that *trb3* can induce tumour cell migration and invasion through its kinase domain interacting with SMAD3 to regulate transforming growth factor- β (TGF- β)-SMAD-mediated pathway [179].

4.3. TRRAP

Transformation/transcription domain-associated protein (TRRAP) is a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family that shares a high homology to the PI3Ks [180,181]. TRRAP was originally discovered in 1998 where it was found to interact with c-Myc and E2F oncoproteins [182]. In humans it is one of six family members, which also include mammalian target of rapamycin (mTOR), ataxia-telangiectasia mutated (ATM), ataxia- and Rad3-related (ATR), DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and suppressor of morphogenesis in genitalia (SMG-1) [183].

PIKK family possesses a conserved kinase domain regulated by two C-terminal regions known as PIKK-regulatory domain (PRD) and FRAP-ATM-TRRAP-C-terminal (FATC). TRRAP protein encompasses 4 domains: the FRAP-ATM-TRRAP (FAT) domain, the kinase domain (KD), the PRD and the FATC domain [181].

TRRAP has a bipartite nuclear localization signal and is predominantly found in the nucleus. The function of the FAT domain has not been elucidated whereas the FATC domain is necessary for kinase activity within this PIKK family. TRRAP differs from other members because it lacks the conserved amino acids required for ATP binding and catalytic activity [184].

TRRAP has been reported to act as a scaffold protein for numerous regulatory factors including GCN5, p53, BRCA1, E2F and c-Myc [185,186]. It acts as a mediator, being part of several different histone acetyltransferase (HAT) complexes [184,186–189]. In turn these complexes act as a platform for different regulatory factors. Transcriptional profiling revealed that TRRAP knockdown affected numerous signalling pathways and cell cycle components. In one study TRRAP was associated with repair of DNA double-strand breaks [186]. Collectively, TRRAP plays a role in forming part of the complex necessary for c-Myc oncogene activation and is involved in the regulation of tumour suppressor gene p53.

4.3.1. TRRAP and cancer

TRRAP has been identified as a transcriptional cofactor of c-Myc [190] playing an important role in cell cycle regulation [191]. Deregulated function of TRRAP can lead to aberrant c-Myc function resulting in abnormal cell proliferation. Normally, c-Myc

recruits TRRAP and the acetyltransferase enzyme GCN5, by interacting with its N-terminal activation/transformation domain. There is strong evidence showing that TRRAP is a co-activator of c-Myc as preventing its recruitment leads to downregulation of c-Myc-modulated genes [192]. In a gene inducible mouse model, null mutation of TRRAP resulted in peri-implantation lethality, and cell proliferation was inhibited because cells failed to complete cytokinesis [193]. Studies have shown that TRRAP is required for cellular transformation by c-Myc, where c-Myc overexpression leads to deregulated cell growth and tumorigenesis [182,194,195]. TRRAP was reported as a target of the adenovirus E1A oncoprotein and to be important for oncogenic transformation mediated by viral oncoproteins [196,197]. TRRAP has also been demonstrated to act as a docking platform for p53 and as part of a multiprotein complex enabling p53 to directly activate MDM2 expression [198].

In breast cancer, 70% of cases are ER α positive, where activated receptor, bound by its ligand (oestrogen) drives cell proliferation. Antisense TRRAP RNA inhibited oestrogen dependent breast cell growth. It was found that 3 LXXLL motifs located around the middle of the TRRAP protein normally bind to the phosphorylated surface of ER α as well as other nuclear receptors [199].

Genetic mutations in the C-terminal transactivation domain of BRCA1, as found in breast and ovarian cancer patients, caused the loss of physical interaction between BRCA1 and TRRAP and significantly reduced the co-activation of BRCA1 transactivation function by hGCN5/TRRAP [188,200].

TRRAP has also been studied in malignant melanoma. Whole-exon DNA sequencing of 14 matched normal and metastatic melanoma tumours from patients, who were not previously treated, showed somatic mutations in TRRAP. The recurring mutation in TRRAP was a cytosine to thymine change at position 2165 of the gene transcript, resulting in a serine to phenylalanine substitution at amino acid residue 722 of the protein. Knockdown of TRRAP had minimal effect on the survival of cells expressing wild-type TRRAP but substantially increased apoptosis rates of melanoma lines carrying mutant TRRAP [201].

Glioblastoma multiforme is a highly aggressive form of brain cancer which is believed to stem from poorly differentiated brain tumour-initiating cells (BTICs). Several genes whose silencing induced differentiation of these cells were discovered using a kinome-wide RNA interference screen. Knockdown using TRRAP siRNA induced differentiation of cultured BTICs, sensitised the cells to apoptotic stimuli, negatively affected cell cycle progression and significantly inhibited tumour formation upon intracranial BTIC implantation into mice. These findings show that TRRAP was maintaining BTICs in a more tumorigenic state [202].

Deregulation of the Wnt signalling pathway is found in many cancers leading to overexpression of oncogenes such as c-Myc. Loss of TRRAP leads to hyperactivation of the Wnt pathway possibly leading to tumorigenesis [203]. The L11 ribosomal protein activates p53 by inhibiting oncoprotein MDM2 and c-Myc, leading to inhibition of cell cycle progression. Overexpression of L11 inhibits c-Myc-induced transcription and cell proliferation, while reduction of endogenous L11 increases c-Myc activities. L11 competes with co-activator TRRAP for binding to c-Myc [204,205].

A comparative genomic hybridization of 22 human pancreatic cancer cell lines, using cDNA microarrays measuring 26,000 human genes, showed TRRAP to be significantly amplified [206]. In a further study, 44 surgically resected pancreatic adenocarcinomas were assessed using array-based comparative genomic hybridization. TRRAP at 7q22.1 was found to be amplified and overexpressed in 36% of cases [207]. From these two studies TRRAP was proposed as a putative oncogene in pancreatic cancer. Disrupting the function of scaffolding protein TRRAP can change signalling activities involved in cancer cell proliferation.

4.4. CCK4 and cancer

Colon carcinoma kinase 4 (CCK4 or receptor tyrosine kinase 7, RTK7) was originally isolated from colon carcinoma tissue but not normal colon tissues. Sequence analysis identified CCK4 as an inactive kinase belonging to the RTK family proteins [208]. A subsequent study showed that CCK4 was also expressed in melanoma cell lines and tumour biopsies. However, levels of CCK4 expression were significantly reduced or lost in advanced melanoma which indicated that it might have a role in tumour metastasis [209]. A recent study suggested that purified extracellular domain of CCK4 can undermine the activity of endogenous CCK4 resulting in inhibition of vascular endothelial growth factor (VEGF)-induced tube formation, cell migration and angiogenesis of endothelial cells, which emphasise its important role in metastatic processes [210]. Moreover, in a study using an immunophenotyping screen of a large group of patients treated for haematologic malignancies, CCK4 was shown to be expressed in AML. A significantly lower leukaemia-free survival and greater resistance to anthracycline-based frontline therapy was reported for CCK4 positive AML patients. Notably, this study suggested that CCK4 could be an independent prognostic factor of survival in certain sub-populations of patients [211]. CCK4 was also involved in membrane type-1 matrix metalloproteinase (MT1-MMP)-PTK7 axis, implicating its role in both cancer cell invasion and normal embryogenesis in vertebrates [212]. Knockdown of CCK4 in colon cancer cell line HCT116 inhibited proliferation and increased apoptosis in a caspase-10 dependent pathway [213]. These findings indicate that CCK4 may be a novel therapeutic target in malignancies.

4.5. VRK-3

Vaccinia-related kinase 3 (VRK-3) is one of three members (including VRK-1 and VRK-2) belonging to the vaccinia related kinase (VRK) family, which is a novel family of serine/threonine kinases possessing a high homology to vaccinia virus B1R kinase [214,215]. Structural studies have shown that only VRK-3 has an inactive pseudokinase domain due to amino acids changes within its binding site not allowing ATP attachment [216]. The NH₂-terminal region of the VRK-3 contains a bipartite nuclear localization signal, enabling it to migrate to the nucleus [214]. VRK-3 has been shown to activate vaccinia H1-related (VHR) phosphatase and can regulate the activity of ERK [217,218]. In one study, VRK-3 was associated with phosphatase activity in neuronal cells and after neurotoxic damage [219]. Further work is needed to identify the biological effects of this pseudokinase.

4.5.1. VRK-3 and cancer

Both VRK-1 and VRK-2 have been linked to malignancies [220–223]. However, to our knowledge only one study so far has shown VRK-3 to be associated with cancer. Differential expression of VRK-3 was identified in colon cancer for the first time using a comparative kinome screen. In this study it was found that VRK-3 was significantly downregulated at the transcript level in both adenoma and adenocarcinomas when compared to normal colon tissue [224]. Any association between VRK-3 and malignancy requires further evaluation.

5. Conclusions and perspectives

Our knowledge of this enigmatic family of protein kinases has increased in recent years. To date, approximately 10% of these proteins have been classified as pseudokinases. Their initial classification was largely based on sequence analysis and the discovery that pseudokinases lacked at least one highly conserved amino acid within their kinase domain. Based on our current knowledge, it appears that pseudokinases can have different regulatory roles and functions. Some provide a 'molecular platform', steering enzymes and their

substrates into critical positions allowing cellular reactions to proceed, while others are able to allosterically activate kinases. In addition, some pseudokinases, although they have evolved "inactive" kinase domains, still exhibit catalytic activity by various unique mechanisms, indicating they are evolutionary counterparts of protein kinases. New experimental facts will allow a more precise classification of pseudokinases in the future.

New insights gained from structure analysis of WNK, CASK and HER3 suggest that it would be interesting to re-evaluate the remaining pseudokinases to delineate possible novel mechanisms in regulating phosphorylation. Thus, uncovering their three-dimensional structure will improve our understanding of their intramolecular interactions, their involvement in signal transduction and possibly this will result in re-classifying some of them into active protein kinases. Re-instating known active domains within pseudokinases may shed more light on their functions. In addition, further evidence on elucidating the pathways they participate in and the exact role of proteins they cooperate with may reshape our perspectives on their biological functions.

Herein, we review the literature on various pseudokinases, including KSR1, HER3, CASK, ILK, JAKs, STRAD α , the Tribbles family, TRRAP, CCK4, VRK-3, accounting approximately for 30% of all known pseudokinases, whose role and/or involvement in cancer has already been reported. Irrespective of their mechanism of action, either as scaffold proteins, allosteric activators or as 'partly' active kinases, they have already been shown to play a critical role in human diseases including cancer. Deciphering an interacting signalling network for individual pseudokinase will provide us with potentially novel prognostic tumour markers and targets for cancer therapy and shed new light on addressing cancer-drug resistance in future.

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The dual function of KSR1: a pseudokinase and beyond

Hua Zhang¹, Chuay Yeng Koo, Justin Stebbing and Georgios Giamas¹

Department of Surgery and Cancer, Division of Cancer, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, U.K.

Abstract

Protein kinases play a pivotal role in regulating many aspects of biological processes, including development, differentiation and cell death. Within the kinome, 48 kinases (~10%) are classified as pseudokinases owing to the fact that they lack at least one conserved catalytic residue in their kinase domain. However, emerging evidence suggest that some pseudokinases, even without the ability to phosphorylate substrates, are regulators of multiple cellular signalling pathways. Among these is KSR1 (kinase suppressor of Ras 1), which was initially identified as a novel kinase in the Ras/Raf pathway. Subsequent studies showed that KSR1 mainly functions as a platform to assemble different cellular components thereby facilitating signal transduction. In the present article, we discuss recent findings regarding KSR1, indicating that it has dual activity as an active kinase as well as a pseudokinase/scaffolding protein. Moreover, the biological functions of KSR1 in human disorders, notably in malignancies, are also reviewed.

Introduction

KSR (kinase suppressor of Ras) 1 was originally discovered more than 15 years ago as a novel protein kinase evolutionarily conserved in *Drosophila* and *Caenorhabditis elegans* functioning between Ras and Raf in the Ras signalling pathway [1,2]. However, mammalian KSR1 has been extensively referred to as a pseudokinase, because of the mutation in the lysine residue in the catalytic domain, which is required for its kinase activity. Subsequently, the role of KSR1 as a scaffolding protein was revealed. Murine KSR1 was first reported to co-operate with activated Ras to facilitate MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase] and MAPK activation, thereby promoting *Xenopus* oocyte maturation and cellular transformation [3]. In addition, KSR1 was observed to translocate from the cytoplasm to the plasma membrane in the presence of activated Ras, where it forms a complex involving Raf-1, MEK1 and 14-3-3 protein. This in turn led to the activation of Raf-1, which is independent of its enzymatic activity, highlighting its role as a scaffold in the MAPK pathway [4,5]. Meanwhile, the notion of KSR1 as an active kinase was described from the finding that TNF α (tumour necrosis factor α) and ceramide were shown to significantly increase KSR1 autophosphorylation and its capacity to phosphorylate and activate Raf-1 [6]. These data thus support the dual activity of KSR1 as an active kinase as well as a scaffold protein in the Ras/Raf/MAPK pathway.

Key words: cancer, kinase, kinase suppressor of Ras 1 (KSR1), phosphorylation, pseudokinase.

Abbreviations used: AMPK, AMP-activated protein kinase; CC, coiled coil; C-TAK1, Cdc25C-associated kinase 1; EGF, epidermal growth factor; ER α , oestrogen receptor α ; ERK, extracellular-signal-regulated kinase; IFN γ , interferon γ ; iNOS, inducible nitric oxide synthase; KSR, kinase suppressor of Ras; MAPK, mitogen-activated protein kinase; MARK2, microtubule affinity-regulating kinase 2; MEF, mouse embryonic fibroblast; MEK, MAPK/ERK kinase; mTOR, mammalian target of rapamycin; NK, natural killer; PPAR γ , peroxisome-proliferator-activated receptor γ ; PP2A, protein phosphatase 2A; SAM, sterile α -motif; TNF α , tumour necrosis factor α .
¹Correspondence may be addressed to either of these authors (email h.zhang10@imperial.ac.uk or g.giamas@imperial.ac.uk).

In the present article, we discuss the recent advances in unravelling its structure and function, especially in cancers.

Structure of KSR1

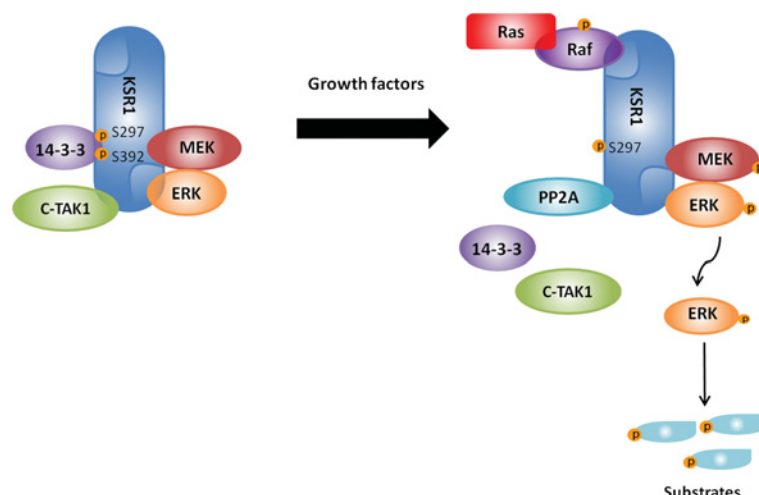
The KSR family members, namely KSR1 and KSR2, are conserved from invertebrates to mammals. From sequence comparison, the similarity of amino acids between KSR1 and KSR2 is approximately 61%. KSR1 is closely related to the Raf kinase family containing five conserved areas named CA1–CA5. The CA1 domain is located in the N-terminus encompassing 40 amino acids which are exclusive to KSR1, but absent from KSR2; CA2 is a proline-rich domain with undetermined function; CA3 is a cysteine-rich atypical C1 motif mediating its membrane recruitment with phospholipids [5]; CA4 is a serine/threonine-rich region with an FXFP (Phe-Xaa-Phe-Pro) motif that interacts with ERK [3,7]; and CA5 is a putative kinase domain in which the conserved lysine residue required for phosphorylation is lacking [7]. One recent study identified another domain composed of a CC (coiled coil) and a SAM (sterile α -motif) in KSR1. In fact, by binding directly to micelles and bicelles, the CC–SAM domain guided KSR1 to certain sites at the plasma membrane upon growth factor stimulus. Furthermore, NMR spectroscopy and *in vitro* assays demonstrated that the helix α of the CC motif is essential for modulation of membrane binding, indicating that, combined with the atypical C1 domain, the CC–SAM domain is indispensable for KSR1 cellular translocation [8].

KSR1 as a scaffold protein

The scaffolding function of KSR1 has been well described in numerous cellular contexts in various conditions, although different binding partners or even contradictory downstream

Figure 1 | KSR1 regulation in the canonical Ras/Raf/MAPK pathway

In non-stimulated cells, KSR1 is sequestered in the cytosol through 14-3-3 protein binding after phosphorylation by C-TAK1 at Ser²⁹⁷ and Ser³⁹². Meanwhile, KSR1 constitutively interacts with MEK and ERK. Upon growth factor stimulation, activated Ras triggers the dephosphorylation of KSR1 at Ser³⁹² by PP2A, leading to the release of 14-3-3 protein from its binding sites. This in turn allows KSR1 to translocate to the cell membrane, where KSR1 forms a complex with Raf, MEK and ERK. KSR1 thus potentially enhances the phosphorylation of Raf, MEK and ERK, facilitating the upstream signalling transduction as well as regulating multiple cellular functions by activation of various substrates.



effects have been reported, compared with the initial notion as a positive regulator in MAPK pathway. KSR1 was shown to co-operate with MEK1/2, but not Ras or Raf-1, and this interaction resulted in an inhibition of proliferation of embryonic neuroretina cells induced by Ras [9]. Another group described that KSR1 can interact directly with MEK-1 and ERK, and expression of KSR1 in COS-7 cells inhibited serum-stimulated MAPK activation [10]. KSR1 was also able to suppress EGF (epidermal growth factor) and Ras-induced phosphorylation of ternary complex factors, which are substrates of MAPK [11]. In addition, phosphorylation of KSR1 at Ser²⁹⁷ and Ser³⁹² was shown to affect its binding capacity to 14-3-3 protein in serum-starved medium, whereas MAPK was able to phosphorylate KSR1 at Thr²⁶⁰, Thr²⁷⁴ and Ser⁴⁴³ depending on activated Ras [12]. The scaffolding role of KSR1 is supported further by its effects on the Ras/Raf/MAPK pathway depending on the levels of its expression. Indeed, at a higher expression, KSR1 appeared to decrease Ras-induced activity, but increased the Ras pathway when expression was lower [12]. A further study in *Ksr1*^{-/-} mouse embryo fibroblasts confirmed this observation when titrating the levels of KSR1 expression. With low KSR1 expression, ERK activity was enhanced as well as cell growth and RasV12 oncogenic transformation. Likewise, all of these effects were markedly reduced with higher KSR1 expression [13,14].

More studies have revealed a dynamic interplay showing that KSR1 is acting as an allosteric regulator in the Ras/Raf/MAPK signalling cascade. In non-stimulated cells, C-TAK1 (Cdc25C-associated kinase 1) phosphorylates KSR1 at Ser³⁹², which creates the binding site for 14-3-3 protein, resulting in sequestration of KSR1 in the

cytosol, where it constitutively interacts with MEK and ERK. Upon growth factor stimulation, activated Ras triggers the dephosphorylation of KSR1 at Ser³⁹² by PP2A (protein phosphatase 2A), leading to the release of 14-3-3 protein from its binding sites. This in turn allows KSR1 to translocate to the cell membrane, where KSR1 forms a complex with Raf, MEK and ERK. KSR1 thus potentially enhances the phosphorylation of Raf, MEK and ERK, facilitates the upstream signalling transduction as well as phosphorylation of multiple substrates in the cytoplasm and nucleus, and, through this manner, regulates proliferation, cell cycle and apoptosis [15–17] (Figure 1).

Recent advances in KSR1 structure demonstrated further its involvement in multiple scaffold complexes. Upon growth factor stimulation, a functional CA1 region of KSR1 was reported to be necessary for the assembly of a ternary complex with B-Raf and MEK, resulting in activation of MEK and ERK. This in turn allows ERK to phosphorylate KSR1 and B-Raf on several feedback serine/threonine phosphorylation sites, which leads to the dissociation of KSR1 from the plasma membrane [18]. Rajakulendran et al. [19] showed that, by forming side-to-side KSR1–Raf heterodimers, KSR1 can regulate Raf activation directly, despite its lack of catalytic function. Moreover, mutations in the dimer interface of KSR1 suppressed the activity of Raf [19]. The case for the scaffolding role of KSR1 is supported further by the observation that Raf inhibitors can trigger KSR1–B-Raf complex formation, dependent on conserved dimer interface residues in each partner [20]. In addition, this study demonstrates that KSR1 competes with C-Raf for inhibitor-induced dimerization to B-Raf, thereby modulating downstream ERK signalling [20].

KSR1 as an active kinase

Mounting evidence supports the concept of KSR1 as an active kinase, despite suggestions of an incomplete catalytic domain. Initial *in vitro* kinase assays reported that different concentrations of natural ceramide induced KSR1 autophosphorylation and transactivated Raf-1 at Thr²⁶⁹ [6]. Afterwards, EGF was shown to be able to stimulate the kinase activity of KSR1 in a two-stage kinase assay. In addition, only full-length KSR1 was capable of signalling c-Raf-1-dependent activity, but not kinase-inactive and C- and N-terminal deletion mutants [21]. The same group proposed further that phosphorylation of c-Raf-1 on Thr²⁶⁹ by KSR1 is required for optimal activation in response to EGF stimulation. The kinase activity of KSR1 appears to act independently of KSR1-binding to MEK [22,23]. Direct phosphorylation of Raf-1 by KSR1 is essential for TNF α -induced ERK1/2 activation in intestinal epithelial cells, revealing a protective role of KSR1 in inflammation process, which requires its regulatory kinase activity [24,25].

New biochemical techniques such as protein purification and molecular modelling have enabled further aspects of KSR1 function to be probed, generating new perspectives on its catalytic functions. By using a specific monoclonal antibody against phospho-Raf-1 (Thr²⁶⁹), the purified KSR1 was shown to be capable of phosphorylating BSA-conjugated Raf-1 peptide [26]. Consistently, recombinant wild-type KSR1, but not kinase-inactive KSR1, can autophosphorylate its serine residues and directly activate MEK1 through phosphorylation, regulating cell survival in response to TNF α [27]. Furthermore, in order to distinguish between the scaffold and kinase function of KSR1, Hu et al. [28] generated a KSR1 mutant construct (A587F) by adding a bulky phenylalanine residue at the ATP-binding pocket of KSR1 to impair ATP binding. However, this mutant can still maintain a closed active conformation, but is unable to interact with ATP. Indeed, the KSR1 mutant was not able to phosphorylate MEK, although it can constitutively bind to MEK as a scaffold. On the other hand, the wild-type KSR1 was shown to phosphorylate MEK induced by c-Raf, indicating a requirement of an active kinase activity in this process [28]. Crystal structure studies of the kinase domain of KSR2 illustrated that the side-to-side interaction between KSR2 and MEK1 is through their respective activation segments and C-lobe α G helices. Upon ATP binding to its catalytic site, KSR2 was shown to phosphorylate MEK1 by *in vitro* kinase assays and chemical genetics [29].

Taken together, these findings support dual function of KSR1 as a scaffolding protein and active kinase in orchestrating the Ras/Raf/MAPK signalling cascade.

The roles of KSR1 in various biological processes

KSR1 in cancers: a therapeutic target?

As KSR1 plays an essential role in the Ras/Raf/MAPK module, which is one of the well-known oncogenic

pathways, studies are beginning to explicate its biological characteristics in different cancers. In a v-Ha-Ras-mediated skin cancer mouse model, KSR1 was shown to contribute to tumorigenesis through the Raf-1/MAPK cascade [30]. Subsequent work from the same group reported that targeting KSR1 by continuous infusion of phosphorothioate antisense ODNs (oligodeoxynucleotides) reduced tumour growth of K-Ras-dependent human PANC-1 pancreatic and A549 non-small-cell lung carcinoma xenografts in nude mice, suggesting inhibition of KSR1 as a potential therapeutic target in Ras-dependent malignancies [31]. Similarly, the introduction of KSR1 into *Ksr1*^{-/-} MEFs (mouse embryonic fibroblasts) induced cell proliferative and oncogenic potential, and removal of KSR1 inhibited Ras(V12)-dependent transformation [13]. In addition, KSR1 was involved in cell sensitivity to cisplatin-induced apoptosis. Specifically, in comparison with wild-type MEFs, KSR1 depletion in MEFs correlated with reduced ERK activation by cisplatin and elevated resistance to cisplatin-stimulated apoptosis. Moreover, transduction of KSR1 into *Ksr1*^{-/-} MEFs and MCF7 cells increased ERK activation and sensitivity to cisplatin [32]. A further screen to characterize KSR1 expression on drug sensitivity using a collection of cancer cell lines and the NCI60 anticancer drugs suggested an important role for KSR1 in defining cellular sensitivity [33]. In human acute myeloid leukaemia cells, KSR1 was revealed to be down-regulated by the oncoprotein Cot1, thus providing extended information of its involvement in non-solid tumours [34]. Metastasis suppressor Nm23-H1 can bind directly to KSR1 and phosphorylate KSR1 at Ser³⁹², and can therefore facilitate its degradation as a result of decreased ERK activation [35,36]. KSR1 was also required for cell-cycle reinitiation in response to DNA-damage agents such as mitomycin C [37]. Additionally, through modulation of PGC1 α [PPAR γ (peroxisome-proliferator-activated receptor γ) co-activator 1 α] and ERR α (oestrogen-related receptor α), KSR1 was demonstrated to induce oncogenic Ras-dependent anchorage-independent growth [38].

The inactivation of KSR1 in *Myc* (v-Myc myelocytomatosis viral oncogene homologue)-overexpressing mice led to an enhancement in B-cell apoptosis as well as an impediment in the onset of B-cell tumorigenesis, suggesting further that KSR1 modulates the co-operation of Ras/MAPK signalling pathway and Myc to drive oncogenic transformation [39]. Of note, a recent study has revealed that KSR2 controls tumour metabolism and cell growth in an AMPK (AMP-activated protein kinase)-dependent manner [40]. The forced expression of KSR2 in *Ksr1*-null MEFs resulted in augmented proliferation and induction of anchorage-independent growth, whereas the introduction of AMPK restores the transformed phenotype and tumour metabolic activities upon KSR2 depletion. Interestingly, our kinome screen using RNAi on identifying novel regulators of ER α (oestrogen receptor α) revealed a potential contribution of KSR1 to ER α -dependent breast cancer [41]. These results indicate a significant role of KSR1 in various cancers and it might be a potential therapeutic target in Ras-dependent cancers.

KSR1 in immune regulation

KSR1 is an important regulator of immune function. *Ksr1*-null mice displayed impairment in MEK and ERK activities, thus resulting in a marked reduction in T-cell proliferation [42]. A recent study has established a relationship between MAPK and mTOR (mammalian target of rapamycin) pathways through the involvement of KSR1 in T-cells. Although KSR1 modulates mTORC1 (mTOR complex 1) activity, KSR1 deficiency has no obvious effects in mTOR-dependent T-cell differentiation [43]. As for NK (natural killer) cells, it was shown that the loss of KSR1 in NK cells perturbed NK cell cytolytic capacity. Upon T-cell activation, KSR1 was recruited to NK lytic synapse that in turn regulated the localization of active ERK to the synapse [44]. Several studies have documented the protective role of KSR1 against cytokine-induced apoptosis such as TNF α in intestinal inflammatory conditions [25,45]. *Ksr1*^{-/-} *Il10*^{-/-} mice were shown to be susceptible to an early manifestation of colitis [46]. Additionally, elevated expression of IFN γ (interferon γ) in T-cells was detected in the double-knockout mice and the inhibition of IFN γ was able to reduce the extent of severity in colitis. KSR1 has also been shown to be a protective factor against bacterial infection. In fact, *Ksr1*-deficient mice were highly vulnerable to pulmonary *Pseudomonas aeruginosa* infection, compared with wild-type mice. Upon infection, KSR1 was capable of iNOS (inducible nitric oxide synthase) and Hsp90 (heat-shock protein 90) recruitment resulting in increased iNOS activity and NO release to eliminate bacteria [47]. These findings underscore an important role of KSR1 in immune responses.

KSR1/2 in metabolism

Both KSR members are involved in the maintenance of cellular metabolism in which metabolic abnormalities were evident in *Ksr1*- and *Ksr2*-deficient mice. Phenotypically, *Ksr1*^{-/-} mice exhibited adipocyte hypertrophy, whereas *Ksr2*^{-/-} counterparts were obese and glucose-intolerant [14,48]. The initiation of adipocyte differentiation relied indirectly on suitable amounts of KSR1 that orchestrated the temporal co-ordination of Raf/MEK/ERK and RSK (ribosomal protein S6 kinase) signalling, and, in this manner, controlled the activities of adipogenic transcription factors such as C/EBP β (CCAAT/enhancer binding protein β) and PPAR γ [14]. A key study by Costanzo-Garvey et al. [48] has also revealed a direct interaction between the two KSR isoforms and AMPK, a prominent energy sensor for metabolic processes. The disruption of this interaction prevented AMPK activation and phosphorylation that is imperative to induce fatty acid oxidation and regulate glucose uptake. Apart from the role of KSR2 in insulin homeostasis, KSR1 has also been recently implicated in regulating glucose tolerance and insulin sensitivity via the interplay between KSR1 and MARK2 (microtubule affinity-regulating kinase 2) [49]. The mode of action in which MARK2 acts as a negative modulator in insulin sensitivity relies on the direct phosphorylation on Ser³⁹² of KSR1 [49].

Collectively, these studies have underlined the significance of KSR1/2 in regulating cellular metabolism and in energy homeostasis, and KSR1/2 could be potential therapeutic targets in metabolic disorders, such as obesity.

Closing remarks

Comprehensive insights regarding the structural and functional studies of KSR1 have started to redefine our previous knowledge, as it is becoming obvious now that the original classification of KSR1 as a pseudokinase may not be representative of its exact biological behaviour. Advances made in the last few years have changed our perspectives in the functions of KSR1, whereas a more complicated conception of KSR1 as a scaffold protein and an active kinase is established. Nevertheless, numerous gaps remain to be filled in order to understand the complexity of KSR1 biological actions. First of all, physiological substrates of KSR1 and its catalytic activity need to be identified further and validated in differential cellular contexts, as well as its subcellular localization where it performs certain functions. Moreover, as a scaffold protein, it is crucial to determine its binding partners in dynamic scenarios since the subsequent interaction will eventually contribute to various biological outcomes. Finally, the inquiry as to whether KSR1 is an attractive therapeutic target in malignancies that are confined to be Ras-dependent tumours requires further clarification.

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SILAC-based phosphoproteomics reveals an inhibitory role of KSR1 in p53 transcriptional activity via modulation of DBC1

H Zhang¹, Y Xu¹, A Filipovic¹, L C Lit^{1,2}, C-Y Koo¹, J Stebbing¹ and G Giamas^{*,1}

¹Division of Cancer, Department of Surgery and Cancer, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London, W12 0NN, UK and ²Department of Physiology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia

Background: We have previously identified kinase suppressor of ras-1 (KSR1) as a potential regulatory gene in breast cancer. KSR1, originally described as a novel protein kinase, has a role in activation of mitogen-activated protein kinases. Emerging evidence has shown that KSR1 may have dual functions as an active kinase as well as a scaffold facilitating multiprotein complex assembly. Although efforts have been made to study the role of KSR1 in certain tumour types, its involvement in breast cancer remains unknown.

Methods: A quantitative mass spectrometry analysis using stable isotope labelling of amino acids in cell culture (SILAC) was implemented to identify KSR1-regulated phosphoproteins in breast cancer. *In vitro* luciferase assays, co-immunoprecipitation as well as western blotting experiments were performed to further study the function of KSR1 in breast cancer.

Results: Of significance, proteomic analysis reveals that KSR1 overexpression decreases deleted in breast cancer-1 (DBC1) phosphorylation. Furthermore, we show that KSR1 decreases the transcriptional activity of p53 by reducing the phosphorylation of DBC1, which leads to a reduced interaction of DBC1 with sirtuin-1 (SIRT1); this in turn enables SIRT1 to deacetylate p53.

Conclusion: Our findings integrate KSR1 into a network involving DBC1 and SIRT1, which results in the regulation of p53 acetylation and its transcriptional activity.

Kinase suppressor of ras-1 (KSR1), initially described more than 15 years ago as a novel protein kinase in the Ras-Raf pathway (Kornfeld *et al*, 1995; Therrien *et al*, 1995) is an essential scaffolding protein that co-ordinates the assembly of the mitogen-activated protein kinase (MAPK) complex, consisting of the MAPK kinase MEK and the extracellular signal-regulated kinase (ERK) to facilitate activation of MEK and thus ERK (Kolesnick and Xing, 2004; Zafrullah *et al*, 2009; Goettel *et al*, 2011). KSR1 has been extensively referred to as a pseudokinase, because of its lack of a key catalytic residue (Zhang *et al*, 2012). However, emerging evidence suggests that KSR1 functions as an active kinase as well as a scaffold protein. Using recombinant wild-type KSR1, different groups have shown that KSR1 is capable of

directly phosphorylating Raf-1 and MEK1 (Zafrullah *et al*, 2009; Goettel *et al*, 2011; Hu *et al*, 2011). On the other hand, crystal structure analysis suggests a direct interaction between KSR and Raf that enables KSR1 to regulate Raf activation independent of its catalytic activity (Rajakulendran *et al*, 2009). Such findings add complexity to the simple view of ERK spatio-temporal pathway control and identify pseudokinases such as KSR1 as potential targets.

Given the role of KSR1 in the Ras–Raf–MAPKs cascade, intensive efforts have focused on Ras-dependent cancers. For instance, recent studies reported that KSR1 regulates the proliferative and oncogenic potential of cells and inhibition of KSR1 abrogates Ras-dependent pancreatic cancer growth (Xing *et al*, 2003; Kortum and Lewis, 2004). Similarly, KSR1 is required

*Correspondence: Dr G Giamas; E-mail: g.giamas@imperial.ac.uk

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for tumour formation in a skin cancer mouse model (Lozano *et al*, 2003). As Ras mutations are rare in breast cancer, the involvement of KSR1 may not depend on the canonical Ras–Raf–MAPKs pathway. Using a short interfering RNA (siRNA) kinome screen, we identified KSR1 as a novel regulator of the transcriptional activity of oestrogen receptor alpha (ER α ; Giamas *et al*, 2011). Moreover, we show that KSR1 expression is significantly correlated with overall and disease-free survival in patients with breast cancer (our unpublished data). However, its biologic functions in this setting have remained unexplored, as are its major partner proteins and pathways including those connected to p53, which we implicate here.

The p53 tumour suppressor is well known to have a central role in cell growth arrest, apoptosis and cellular response to genotoxic stress (Levine, 1997; Vogelstein *et al*, 2000). Its transcriptional activity is highly regulated by post-transcriptional modifications including acetylation (Brooks and Gu, 2003; Tang *et al*, 2008). Previous data demonstrate that deleted in breast cancer 1 (DBC1) directly interacts and negatively regulates the deacetylase SIRT1 resulting in an increase of p53 acetylation (Kim *et al*, 2008; Zhao *et al*, 2008). Furthermore, phosphorylation of DBC1 is necessary for its interaction with SIRT1, while it inhibits the activity of SIRT1 in response to DNA damage (Yuan *et al*, 2012; Zannini *et al*, 2012).

Herein, we present a KSR1-regulated phosphoproteomic profile in breast cancer cells using a stable isotope labelling of amino acids in cell culture (SILAC) approach. Furthermore, we identify a role of KSR1 in the regulation of p53 transactional activity by reducing its acetylation via the modulation of DBC1–SIRT1 interaction.

MATERIALS AND METHODS

SILAC cell culture. SILAC dialysed calf serum and custom Dulbecco's modified Eagle's medium (DMEM) mediums containing either unlabelled [$^{12}\text{C}_6$, $^{14}\text{N}_4$]-arginine (Arg) and [$^{12}\text{C}_6$]-lysine (Lys) (R0K0 –'light') or labelled [$^{13}\text{C}_6$, $^{15}\text{N}_4$]-Arg and [$^{13}\text{C}_6$]-Lys (R10K8 –'heavy') were purchased from Dundee Cell Products (Dundee, UK). MCF7 cells were grown in these custom DMEM mediums along with 10% dialysed fetal calf serum (FCS) and 1% of antibiotics (penicillin and streptomycin). The cells that were grown for at least seven passages were used for this experiment.

Protein digestion and peptide fractionation. Equal amounts of protein from unlabelled and labelled samples were combined before protein digestion. Briefly, samples were reduced in 10 mM dithiothreitol (DTT) and alkylated in 50 mM iodoacetamide before boiling in loading buffer, and then separated by one-dimensional sodium dodecyl sulphate (SDS)–PAGE 4–12% Bis-Tris Novex mini-gel from Invitrogen (Paisley, UK) and visualised by colloidal Coomassie staining from Invitrogen. The entire protein gel lanes were excised and cut into 10 slices each. Every gel slice was subjected to in-gel digestion with trypsin overnight at 37 °C. The resulting tryptic peptides were extracted by formic acid (1%) and acetonitrile, lyophilised in a speedvac and resuspended in 1% formic acid.

Protein digestion and phosphopeptide enrichment. FASP procedure was performed as previously described (Wisniewski *et al*, 2009). In all, 500 μl FASP 1 (8 M urea, 20 mM DDT in 100 mM Tris/HCl pH 8.5) was added to $\sim 2\text{ mg}$ protein lysate to dilute SDS concentration and transferred to a Vivacon 500, 30k MWCO HY filter from Sartorius Stedim Biotech (Epsom, UK). The sample was buffer exchanged using FASP 1 several times by spinning the tube at 7000 g to remove detergents. The protein lysate was concentrated by centrifugation and diluted in FASP 2 (100 mM Tris/HCl pH 8.5) ready for trypsin digestion. The sample was reduced using 50 mM fresh IAA in FASP 2 in the dark for 30 min. Lysates were spun down to remove excess IAA and buffer exchanged into FASP 3 (100 mM triethyl ammonium bicarbonate). Trypsin was dissolved in FASP 3 to give a 1:200 enzyme to protein ratio and added in a

volume of at least 100 μl for 4–6 h. This was repeated with fresh trypsin for a further overnight incubation. Lysates were spun down and washed with 0.5 M NaCl and 150 μl 10% TFA added to reduce the pH. A standard desalting procedure was used (Thingholm *et al*, 2006).

Hydrophilic interaction chromatography (HILIC) fractionation:

A TSKgel Amide-80 separation column with a TSKgel Amide guard column was used for HILIC separation of FASP peptides. Buffer A: 80% ACN, 0.1% formic acid and buffer B: 0.1% formic acid was used for the gradient at a flow rate of 0.6 mL min^{-1} . The sample was separated into 45 fractions, collected 2 mins per vial and dried using a speed vac.

TiO $_2$ enrichment was performed as previously described (Thingholm *et al*, 2006). TiO $_2$ beads were washed and re-suspended in buffer B at 50 $\mu\text{g } \mu\text{l}^{-1}$ and added to tubes to give 1 mg per tube. The sample was re-suspended in loading buffer, added to beads and incubated at RT for 20 min. After washing, using buffers A and B samples were eluted using aliquots of 0.5% NH $_4\text{OH}$. Elutions were pooled and 10 μl of 20% FA added to adjust the pH. Samples were dried and re-suspended in 1% FA.

Mass spectrometry methods. Trypsin-digested peptides were separated using an Ultimate 3000 RSLC nanoflow LC system from Thermo Scientific (Cramlington, UK). On average 0.5 μg was loaded with a constant flow of 5 $\mu\text{l min}^{-1}$ onto an Acclaim PepMap100 nanoViper C18 trap column (100 μm inner-diameter, 2 cm; Thermo Scientific). After trap enrichment, peptides were eluted onto an Acclaim PepMap RSLC nanoViper, C18 column (75 μm , 15 cm; Thermo Scientific) with a linear gradient of 2–40% solvent B (80% acetonitrile with 0.08% formic acid) over 65 min with a constant flow of 300 nL min^{-1} . The HPLC system was coupled to a linear ion trap Orbitrap hybrid mass spectrometer (LTQ-Orbitrap Velos, Thermo Scientific) via a nano electrospray ion source (Thermo Scientific). The spray voltage was set to 1.2 kV, and the temperature of the heated capillary was set to 250 °C. Full-scan MS survey spectra (m/z 335–1800) in profile mode were acquired in the Orbitrap with a resolution of 60 000 after accumulation of 1 000 000 ions. The 15 most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision-induced dissociation (normalised collision energy, 35%; activation Q, 0.250; and activation time, 10 ms) in the LTQ after the accumulation of 10 000 ions. Maximal filling times were 1000 ms for the full scans and 150 ms for the MS/MS scans. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. The lock mass option was enabled for survey scans to improve mass accuracy. Data were acquired using the Xcalibur software from Thermo Scientific.

Quantification and bioinformatics analysis. The raw mass spectrometric data files obtained for each experiment were collated into a single quantitated data set using MaxQuant (Cox and Mann, 2008) and the Andromeda search engine software (Cox *et al*, 2011). Enzyme specificity was set to that of trypsin, allowing for cleavage N-terminal to proline residues and between aspartic acid and proline residues. Other parameters used were: (i) variable modifications, methionine oxidation, protein N-acetylation, gln $\rightarrow$ pyro-glu; (ii) fixed modifications, cysteine carbamidomethylation; (iii) database: target-decoy human MaxQuant (ipi.HUMAN.v3.68); (iv) heavy labels: R6K4 and R10K8; (v) MS/MS tolerance: FTMS – 10 p.p.m., ITMS – 0.6 Da; (vi) maximum peptide length, 6; (vii) maximum missed cleavages, 2; (viii) maximum of labelled amino acids, 3; and (ix) false discovery rate, 1%. Peptide ratios were calculated for each arginine-containing and/or lysine-containing peptide as the peak area of labelled arginine/lysine divided by the peak area of non-labelled arginine/lysine for each single-scan mass spectrum. Peptide ratios for all

arginine-containing and lysine-containing peptides sequenced for each protein were averaged. Data are normalised using 1/median ratio value for each identified protein group per labelled sample.

Cell lines, reagents, antibodies and plasmids. MCF7, ZR75-1, SKBR3, MDA-MB-231 (MDA231) and p53^{+/+} HCT116 cells were maintained in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin/glutamine. All cells were incubated at 37 °C in humidified 5% CO₂. FuGENE HD transfection reagent was obtained from Roche (Burgess Hill, UK). siKSR1 were purchased from Qiagen (Crawley, UK) and verified. Plasmids containing human wild-type KSR1 (pCMV6-KSR1) and empty vector (pCMV6-vector) were obtained from OriGene (Cambridge, UK). Mutant KSR1 R502M was generated using QuikChange Site-Directed Mutagenesis Kit were from Stratagene (Stockport, UK) and confirmed by plasmid sequence. MCF7-parental and MCF7-KSR1 stable cells were generated by transfection of either pCMV6-vector or pCMV6-KSR1 in MCF7 cells and selected in the presence of G418 (1 mg ml⁻¹). KSR1 overexpression was confirmed by RT-qPCR and western blotting. Etoposide was purchased from Sigma Aldrich (Gillingham, UK). The Dual-Glo Luciferase Assay System was purchased from Promega (Southampton, UK). The following antibodies were used: KSR1 rabbit polyclonal from Cell Signaling (Hitchin, UK), anti-Flag mouse monoclonal (Sigma Aldrich), p53 mouse monoclonal DO-1 from Santa Cruz (Wiltshire, UK), acetylated-p53 and phospho-p53 Ser15 rabbit polyclonal (Cell Signaling), SIRT1 rabbit polyclonal (Santa Cruz), DBC1 and phospho-DBC1 Thr454 rabbit polyclonal (Cell Signaling) and β -actin mouse monoclonal from Abcam (Cambridge, UK). The following p53-target gene promoter-reporter constructs previously described (Vikhanskaya *et al.*, 2007) were a kind gift from Professor Kanaga Sabapathy: AIP-1-luciferase (luc), IGFBP3-luc, R2-luc and cyclinG1-luc. The renilla luciferase reporter vector (pRL-TK) was purchased from Promega.

Protein extraction and western blotting. NP40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% (v/v) glycerol, 1% NP40, 5 mM DTT, 1 mM EDTA, 1 mM EGTA, 50 μ M leupeptin and 30 μ M ml⁻¹ aprotinin) was used to extract whole-cell lysates. Cell pellets were mixed thoroughly with NP40 lysis buffer, and then incubated in ice for 15 min before centrifuging at 15 000 r.p.m. for 15 min at 4 °C. Nuclear and cytoplasmic extracts were prepared from whole-cell lysis using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Protein concentration was measured by the bicinchoninic acid (BCA) protein assay from Pierce (Epsom, UK). Lysates were heated with 5 $\times$ SDS sample buffer at 95 °C for 5 min before they were loaded to 10% SDS-PAGE. Samples were then transferred to Hybond ECL super nitrocellulose membranes from GE Healthcare (Little Chalfont, UK). Subsequently, the membranes were blocked in TBS containing 0.1% (v/v) Tween20 and 5% (w/v) non-fat milk for 1 h. The primary antibodies were probed with membranes overnight at 4 °C. The membranes were then washed three times in TBS/Tween for 15 min following incubation with HRP-conjugated secondary antibodies (1:3000 dilution) for 60 min. The membranes were then washed three times again and were detected with enhanced chemiluminescence (ECL). Films were developed using a Konica SRX-1001A X-ray developer (Banbury, UK). Alternatively, membranes were incubated with IRDye donkey anti-mouse or donkey anti-rabbit secondary antibodies for 60 min and visualised by Odyssey Fc Imaging System from LI-COR (Cambridge, UK).

RNA extraction and RT-qPCR. RNeasy kit (Qiagen) was used to isolate total RNA. Reverse transcription was performed using high-capacity cDNA reverse transcription kit from Applied Biosystems (Warrington, UK). RT-qPCR analysis was performed on a 7900HT Thermocycler (Applied Biosystems) using TaqMan mastermix and primers for TP53 and GAPDH cDNAs, purchased from Applied Biosystems.

Luciferase reporter assay. In all, 8×10^4 per well MCF7 cells were seeded into 24-well plate and transfection was performed using FuGENE HD transfection reagent according to the manufacturer's instructions. Cells were transfected with different p53 constructs, and pCMV6-KSR1 or pCMV6-vector and together with renilla luciferase reporter vector (pRL-TK). Cell lysates were collected after 24-h transfection and firefly and renilla luciferase activities were measured by the Dual-Glo Luciferase Assay kit as per the manufacturer's protocols described. The transcriptional activity of various p53 constructs were determined by firefly luciferase activity and normalised against renilla luciferase activity, which was served as control for transfection efficiency.

Immunofluorescence staining. MCF7 cells seeded on glass coverslips in 6 cm dishes were transfected with pCMV6-KSR1 or pCMV6-Vector using FuGENE HD. Cells were fixed in 4% w/v paraformaldehyde at 37 °C for 15 min, permeabilised with 0.1% Triton-X for 10 min and incubated in immune-fluorescent blocking buffer (10% AB-serum in PBS) for 1 h, followed by incubation with p53 antibody (DO-1). After washing with PBS, coverslips were incubated for 45 min at 37 °C with anti-mouse-IgG Alexa Fluor – 555 antibody (Invitrogen). DNA was visualised by DAPI staining. Cells were examined on an Axiovert-200 laser scanning inverted microscope from Zeiss (Welwyn Garden City, UK) as previously described (Giamas *et al.*, 2011).

Cell proliferation assay. Sulphorhodamine B (SRB) assay was performed to determine the growth of breast cancer cell lines in 96-well plates. After siRNA knockdown of KSR1 at indicated time points, plates were collected for the following protocol. Cells were fixed by adding 100 μ l per well of ice-cold 40% trichloroacetic acid (TCA) to each culture for 1 h and incubated in the fridge. Plates were then washed $\times$ 5 times in running tap water (allow the water to fill wells indirectly). Cells were stained with 100 μ l of 0.4% (w/v) SRB from Sigma Aldrich in 1% acetic acid for 30 min and plates were washed five times in 1% acetic acid and left to air dry overnight. On the day of plate reading, bound dye was solubilised by adding 100 μ l of 10 mM Tris base to all the wells and plates were measured at 492 nm using Tecan microplate reader (Reading, UK).

Neddylolation assay. MCF7 cells plated on 15 cm dishes were transfected using FuGENE HD with 8 μ g pcDNA3-HA-NEDD8 from Addgene (Cambridge, MA, USA), 8 μ g pCMV6-KSR1 or 8 μ g pCMV6-vector constructs as indicated. After 24 h, cells were lysed in radio-immunoprecipitation assay (RIPA) buffer (Sigma Aldrich) supplemented with protease inhibitors. Total protein was quantified by the BCA assay (Pierce). In all, 2 μ g mouse IgG or p53 (DO-1) was pre-incubated with protein agarose beads for 2 h to form IP matrix complex (ImmunoCruz IP/WB Optima C System, Santa Cruz). In total, 2 mg protein lysate was added into the beads and was incubated on a rotator overnight at 4 °C. Beads were washed with RIPA buffer for three times and were heated in SDS loading buffer. Neddylolated p53 were detected by western blot using p53-specific DO-1 antibody or NEDD8 antibody (Cell Signaling) as described before (Xirodimas *et al.*, 2004; Abida *et al.*, 2007).

Statistical analysis. Each experiment was repeated at least three times. Student's *t*-test was two-sided at a 0.05 significance level and performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

Phosphoproteomics analysis of KSR1-regulated proteins using SILAC. In order to obtain the KSR1-regulated phosphoproteome profile, a necessary step to understand its integrated signalling pathways in breast cancer, we first performed a quantitative proteomic analysis.

Here, MCF7 cells were maintained for seven cell divisions in either R0K0 'light' medium, containing unlabelled [¹²C₆,¹⁴N₄]-arginine (Arg) and [¹²C₆]-lysine (Lys) amino acids, or in R10K8 'heavy' medium, containing labelled [¹³C₆,¹⁵N₄]-Arg and [¹³C₆,¹⁵N₂]-Lys. Labelled cells were then transfected with either the pCMV6 vector (R0K0, control) or with a pCMV6-KSR1 plasmid (R10K8) that encodes for the full-length KSR1. After 24 h, proteins were extracted, mixed 1:1, separated on SDS-PAGE, trypsin-digested, fractionated and analysed by LC-MS/MS using MaxQuant software (Cox and Mann 2008; Figure 1A).

We identified a total of 2504 proteins out of which 2032 were quantified (false discovery rate <1%). Similarly, we found 1409 phosphopeptides from 891 phosphoproteins out of which 1165 phosphopeptides from 812 phosphoproteins were quantified. After normalisation, we determined the phosphorylation vs total protein level ratio between control and KSR1-overexpressed samples. Based on our data, we obtained information about the phosphorylation change of 379 potential sites that correspond to 240 proteins, as several proteins had more than one potential phosphorylation sites. Among these modulated sites, 341 phosphoserine (pS), 37 phosphothreonine (pT) and 1 phosphotyrosine (pY) sites were included (Supplementary Excel File 1).

Surprisingly, only 3 out of the 379 identified phospho-sites were induced >50% while most of them (233 out of 379) were actually

decreased (<50%) after KSR1 overexpression. These data partly support the characterisation of KSR1 as a pseudokinase, emphasising its primary role as a scaffold protein not a kinase. The values from total and phosphorylated proteins were plotted to create a graph showing the log₂ normalised 'total proteins' vs the log₂ 'phosphorylated proteins' ratios (Figure 1B).

Ontology analysis of differentially regulated KSR1 phosphoproteins.

We then performed gene ontology (GO) analysis and classification (using the 'Panther' software; Mi *et al*, 2013) to assign the KSR1-regulated phosphoproteome according to (i) molecular functions, (ii) biological processes and (iii) cellular components (Figure 1C). We found significant enrichment for GO molecular functions terms related to binding (GO: 0005488), catalytic activity (GO: 0003824), structural molecule activity (GO: 0005198), as well as transcription regulator (GO: 0003824) and enzyme regulator (GO: 0030234) activities. Our phosphoproteomics analysis facilitated the identification of biological processes associated with cell cycle (GO: 0007049) and communication (GO: 0007154) along with metabolic (GO: 0008152), cellular (GO: 009987) and transport (GO: 0006810) processes. Finally, regarding the localisation of the identified KSR1-regulated phosphoproteins, there was a similar distribution between membrane/cytoplasmic and nuclear cell components, resulting from the direct and indirect effects of

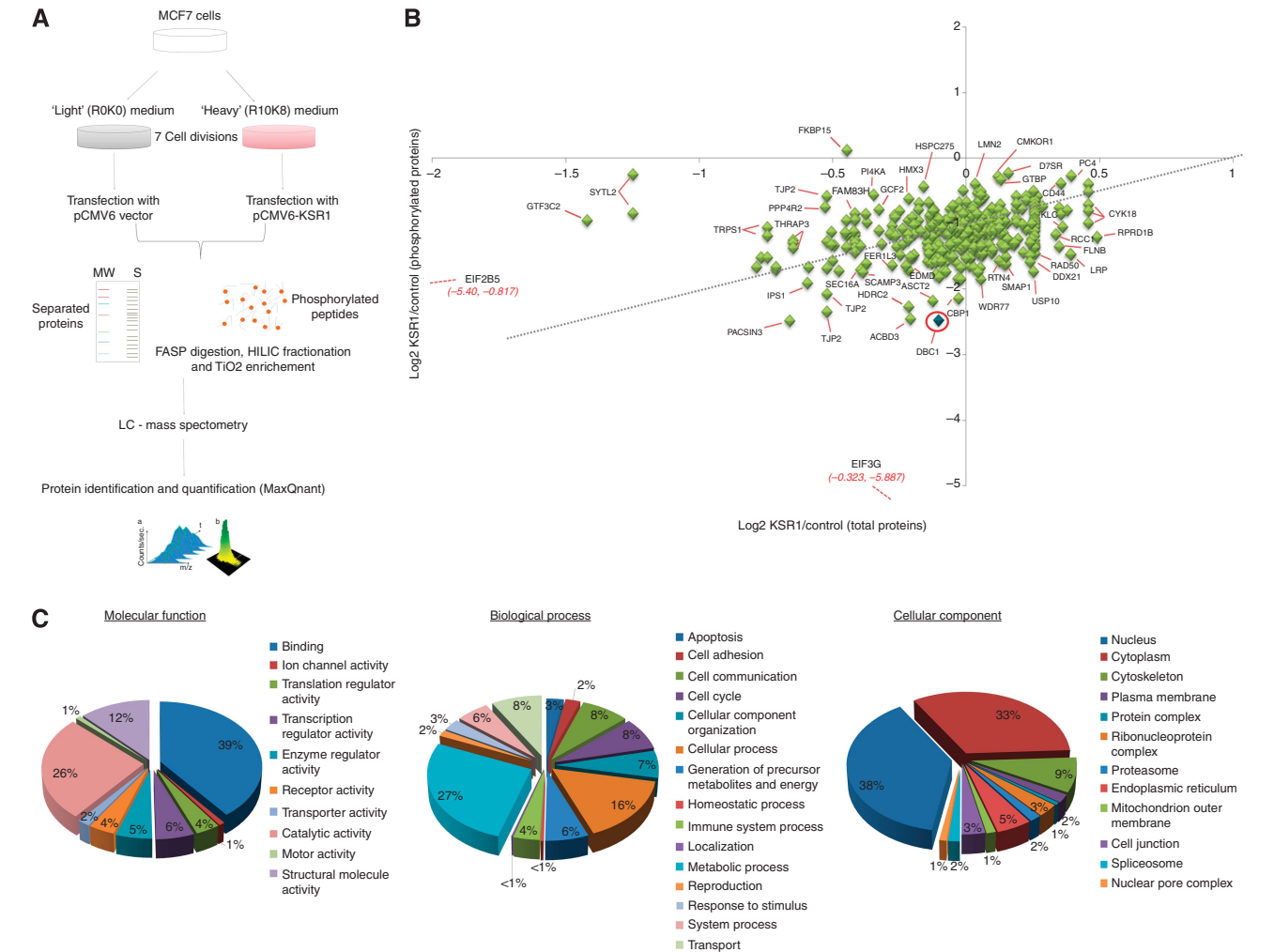


Figure 1. Identification of KSR1-regulated phosphoproteome in breast cancer cells. (A) Experimental schematic outline of SILAC experiment. (B) Scatter plot comparison of phosphosite ratios quantified from control vs KSR1-overexpressed MCF7 cells. (C) Gene ontology (GO) Classification of the KSR1-regulated phosphoproteome in MCF7 cells according to molecular functions, biological processes and cellular compartmentalisation.

KSR1 abundance. This is by far the first ontology analysis of KSR1-regulated phosphoprotein profile in cancer, which will for sure enable extensive study of its functions in signalling pathways other than the canonical Ras–Raf–MAPKs cascades.

Phosphoproteomics analysis reveals a downregulation of phospho-DBC1 by KSR1. Further examination of the identified KSR1-regulated phosphoproteins unveiled that some of these proteins have been previously implicated in the development of breast cancer. For instance, androgen-induced proliferation inhibitor (APRIN), a protein phosphorylated by ATM (ataxia telangiectasia-mutated) and ATR (ataxia telangiectasia and Rad3-related) kinases, was described as a BRCA2-interacting protein required for genome integrity and a predictor of outcome in response to chemotherapy in breast cancer (Matsuoka *et al*, 2007; Brough *et al*, 2012). Rho GTPase-activating protein 35 (ARHGAP35) was shown to be phosphorylated by breast tumour kinase (Brk) leading to RhoA inactivation, Ras activation and promotion of breast cancer growth and migration (Shen *et al*, 2008). Phosphorylation of Hsp27 was correlated with HER-2/neu status and lymph node positivity in breast cancer, and phosphorylated Hsp27 was also linked to invasiveness and drug resistance (Zhang *et al*, 2007; Fujita *et al*, 2011). Histone deacetylase 1/Sin3A complex phosphorylation has been linked to ER α expression and hormonal therapy resistance in breast cancer cells (De Amicis *et al*, 2011).

Interestingly, among the most significantly KSR1-regulated phosphorylated proteins, was DBC1, whose involvement in the regulation of p53 activity via SIRT1 has been described previously (Kim *et al*, 2008; Zhao *et al*, 2008). Taking into consideration the importance of p53 in breast cancer, we then assessed the connection between KSR1 and p53 activity *in vitro*.

KSR1 regulates the transcriptional activity of p53. We first examined the effects of KSR1 on p53 transcriptional activity by performing luciferase reporter assays using various p53-dependent gene promoter constructs (including p53-R2, p53-AIP1, p53-IGFBP3 and p53-CYCLIN G1). MCF7 cells were co-transfected with either pCMV6 or pCMV6-KSR1 plasmids and each individual p53-dependent promoter construct, in the presence or absence of etoposide, which can induce p53 activity. Interestingly, the luciferase activity of all four different p53-regulated genes was significantly repressed after overexpression of KSR1, suggesting an oppressive role of KSR1 on p53 activation (basal levels and after etoposide treatment; Figure 2A). As expected, silencing of KSR1

resulted in a marked increase in the activity of p53-dependent promoter genes (Figure 2B). These results suggest a potential involvement of KSR1 in p53 transcriptional activity regulation.

KSR1 does not affect the mRNA, protein, subcellular localisation and neddylation levels of p53. In order to investigate the potential mechanism resulting in the downregulation of p53 transcriptional activity, we initially evaluated the p53 gene and protein expression after KSR1 overexpression. RT-qPCR and western blotting analyses did not reveal any changes in the p53 mRNA and protein levels, respectively, after KSR1 transient overexpression in MCF7 cells (Figure 3A). Similarly, no change in p53 protein levels was observed in MCF7 stably overexpressing KSR1 cells comparing with MCF7 parental cells (Figure 3A). As the nuclear localisation of p53 is essential for its activity, we examined whether KSR1 could affect p53 compartmentalisation. Immunofluorescence and subcellular protein fractionation did not reveal any cytoplasmic translocation of p53 upon KSR1 transient overexpression (Figure 3B). Moreover, it has been recently reported that neddylation of p53 can inhibit its transcriptional activity (Xirodimas *et al*, 2004; Abida *et al*, 2007). However, our neddylation assay did not show any significant changes following KSR1 overexpression ruling out neddylation as the reason for the observed p53 decreased activity (Figure 3C).

KSR1 decreases p53 acetylation by reducing phosphorylation of DBC1 resulting in impaired DBC1–SIRT1 interaction. Post-translational modifications of p53 such as phosphorylation and acetylation are essential for p53 activity in response to genotoxic stress (Brooks and Gu, 2003; Tang *et al*, 2008). To examine effects of KSR1 on phospho-p53 and acetylated-p53, cells were transfected with pCMV6 or pCMV6-KSR1 plasmids in the presence of etoposide or doxorubicin. Although the phosphorylation levels of p53 (Ser15) did not change, interestingly the acetylated-p53 was reduced after KSR1 overexpression upon either etoposide (Figure 4A) or doxorubicin (Supplementary Figure 1) treatment. Similar results were confirmed in several other breast cancer cell lines (including ZR75-1 and SKBR3) and one colon cancer cell line HCT116 (Supplementary Figure 1).

It is already known that SIRT1 functions as an NAD-dependent p53 deacetylase that affects p53 activation (Vaziri *et al*, 2001; Tang *et al*, 2008). In addition, DBC1 can interact with and negatively regulate SIRT1 resulting in increased p53 acetylation (Kim *et al*, 2008; Zhao *et al*, 2008). Taking this potential link into

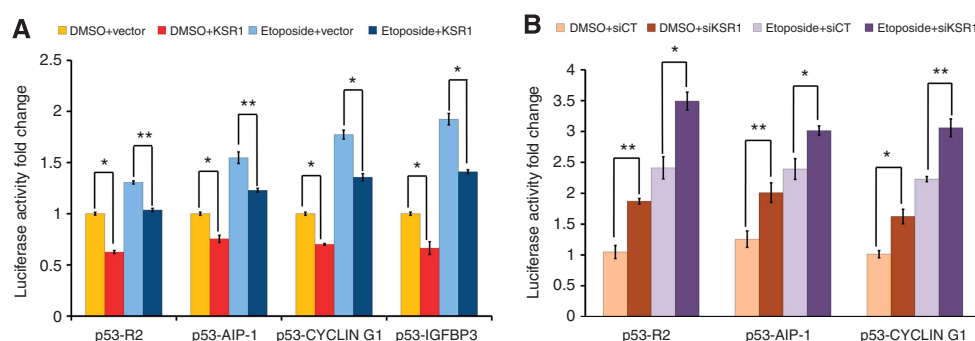


Figure 2. Effects of KSR1 on p53 transcriptional activity in the presence or absence of etoposide by luciferase assays. **(A)** MCF7 cells were transiently co-transfected with either pCMV6 (vector) or pCMV6-KSR1 plasmids in the presence of four individual p53-dependent promoter constructs expressing firefly luciferase genes (p53-R2, p53-AIP1, p53-CYCLIN G1 and p53-IGFBP3) following dimethylsulphoxide (DMSO) or etoposide (40 μ M) treatment for 3 h. **(B)** MCF7 cells were transfected with control siRNA (siCT) or siKSR1 for 48 h, followed by transfection of three p53-dependent promoter constructs expressing firefly luciferase genes (p53-R2, p53-AIP1 and p53-CYCLIN G1) for additional 24 h. DMSO or etoposide (40 μ M) were subsequently added as described above. Firefly luciferase activity was measured (renilla luciferase activity was used to normalise transfection efficiency). The normalised luciferase activity of empty vector is set as 1. Results shown are the average of at least three independent experiments and error bars represent s.d. Student's *t*-test was performed using SPSS 16.0 statistical software (SPSS Inc.). (**P* < 0.05, ***P* < 0.01).

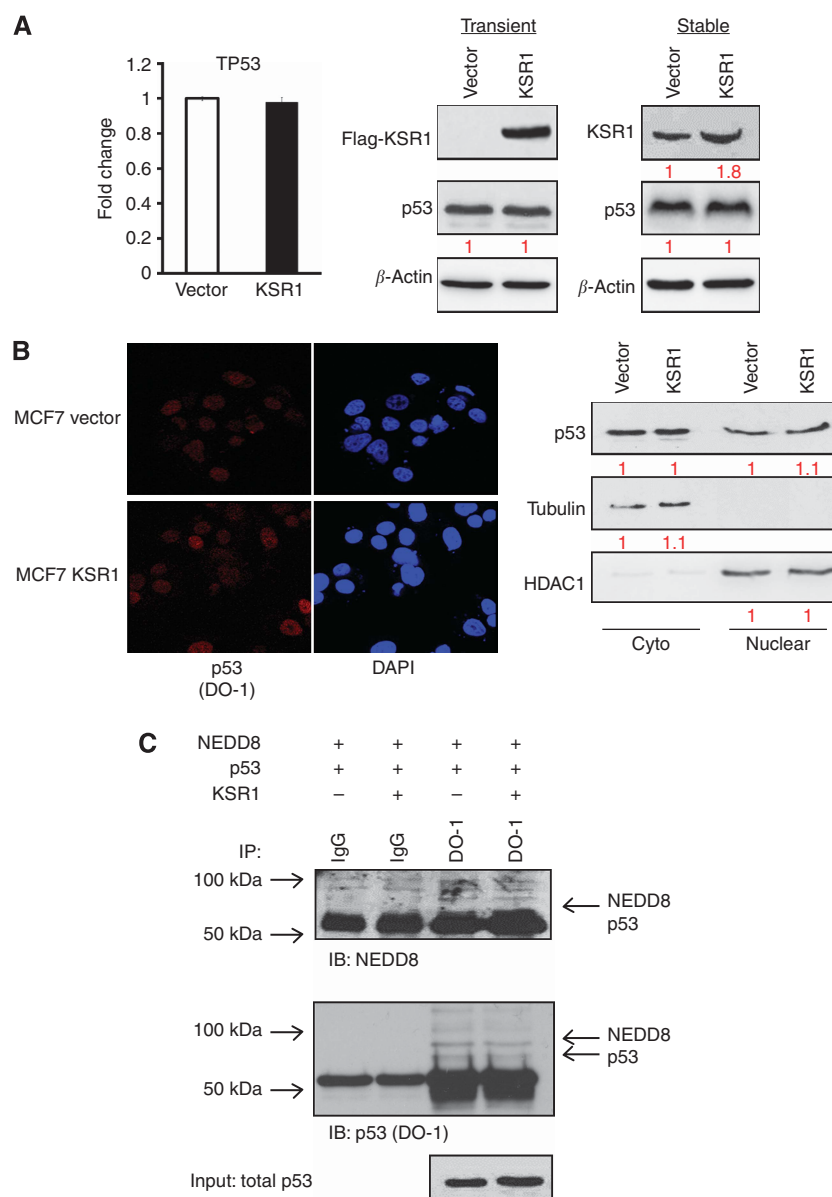


Figure 3. Effects of KSR1 on p53 mRNA, total protein and neddylation levels and on p53 subcellular localisation. **(A)** Effects on p53 mRNA and total protein levels after KSR1 overexpression. MCF7 cells were transiently transfected with pCMV6 or pCMV6-KSR1 plasmids for 24 h. Subsequently, relative mRNA levels of *TP53* and p53 total protein were measured by RT-qPCR and western blotting, respectively. Gene expression level from cells transfected with pCMV6 was set as 1. Results shown are the average of at least three independent experiments. Similarly, in MCF7 stably overexpressing KSR1 cells, p53 total protein was evaluated by western blot. Blots shown are representatives of at least three independent experiments. **(B)** Immunofluorescence staining of p53 cells after 24-h transfection with either pCMV6 or pCMV6-KSR1 plasmids in MCF7. p53 was detected with an anti-p53 antibody while the nucleus was stained with 4,6-diamidino-2-phenylindole (DAPI). Representative pictures of three independent experiments are shown. Subcellular fractionation assays were performed after 24-h transfection with either pCMV6 or pCMV6-KSR1 plasmids in MCF7. Tubulin and histone deacetylase 1 (HDAC1) expression served as positive normalising control for cytoplasmic and nuclear proteins respectively. Blots shown are representatives of at least three independent experiments. **(C)** Neddylation assay on p53 after KSR1 overexpression. MCF7 cells were co-transfected with HA-NEDD8 and pCMV6 or pCMV6-KSR1 plasmids as indicated. p53 was immunoprecipitated using a p53-specific antibody (DO-1) and the neddylated-p53 was detected by immunoblotting using anti-NEDD8 and anti-p53-specific antibodies. Blots shown are representatives of at least three independent experiments. Abbreviations: IgG = immunoglobulin G; IP = immunoprecipitation.

consideration, we decided to investigate the effects of KSR1 on these two proteins.

Total SIRT1 levels were not affected by KSR1 upregulation (Figure 4A). Based on the SILAC data regarding the effects of KSR1 on the phosphorylation of DBC1 and the association between DBC1 and SIRT1, we performed western blotting of phospho-DBC1 (Thr454) revealing a decrease in the phosphorylation levels in basal and etoposide-induced conditions

(Figure 4A). Consistently, KSR1 silencing rescued the abolished acetylation of p53 after etoposide treatment (Figure 4B). Moreover, knock-down of KSR1 did not alter the total proteins of SIRT1 and DBC1, but increased DBC1 phosphorylation at Thr454 (Figure 4B). These data further validate our SILAC results and allow KSR1 to integrate into a known network of p53 acetylation regulation involving phospho-DBC1 and SIRT1.

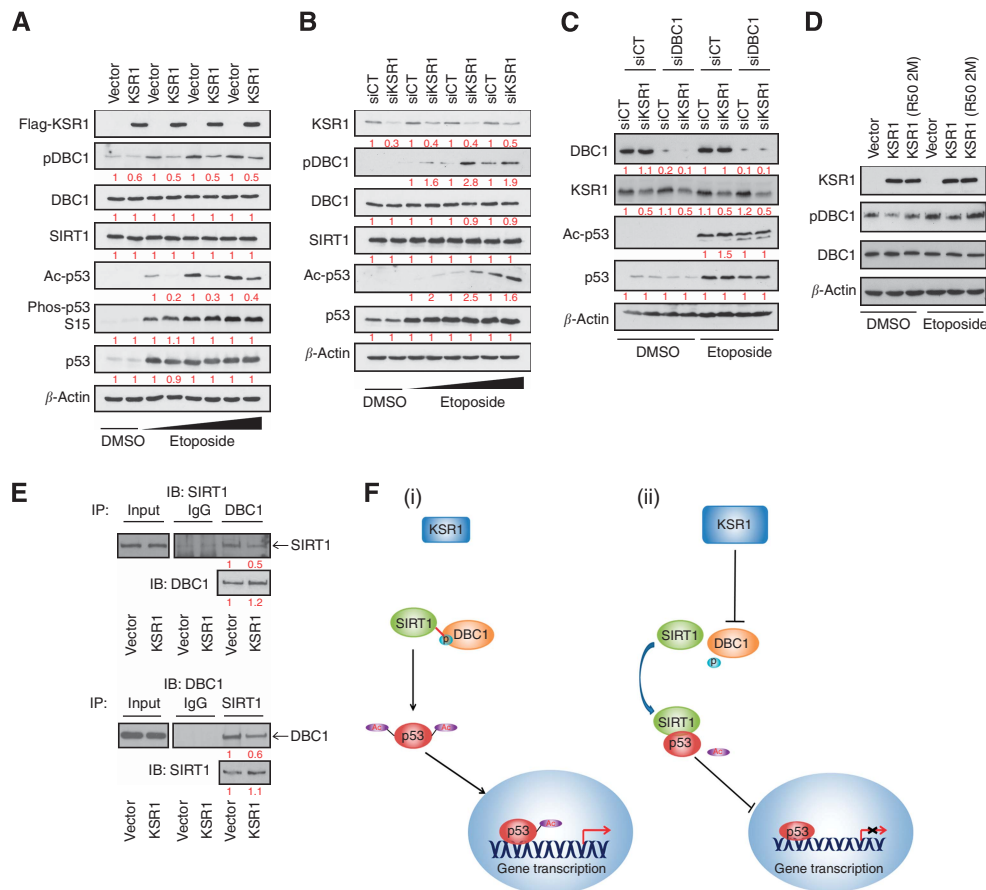


Figure 4. Mechanisms of KSR1-regulated p53 transcriptional activity. (A) Effects on p53 acetylation and phosphorylation of DBC1 after KSR1 overexpression followed by etoposide treatment. MCF7 cells were transiently transfected with pCMV6 (vector) or pCMV6-KSR1 plasmids for 24 h. Subsequently, cells were treated with various concentrations of etoposide (20, 40, 80 μ M, 3 h). p53 acetylation and DBC1 phosphorylation at Thr454 were assessed by immunoblotting with specific antibodies as indicated. (B) Effects on p53 acetylation and phosphorylation of DBC1 after KSR1 silencing followed by a titration of etoposide treatment. MCF7 cells were transfected with control siRNA (siCT) or siKSR1 for 72 h followed by etoposide treatment (20, 40, 80 μ M, 3 h). p53 acetylation and DBC1 phosphorylation at Thr454 were assessed by immunoblotting with specific antibodies as indicated. (C) Effect of KSR1 on p53 acetylation is through DBC1. MCF7 cells were transfected with control siRNA (siCT) or siKSR1 in concordance with siCT or siDBC1 for 72 h followed by etoposide treatment (40 μ M, 3 h). Acetylated p53, DBC1 and KSR1 protein levels were assessed by immunoblotting with specific antibodies as indicated. (D) Effect of KSR1 on DBC1 phosphorylation is dependent on its intact kinase domain. MCF7 cells were transiently transfected with vector, wild-type KSR1 or mutant KSR1 (R502M) plasmids for 24 h followed by etoposide treatment (40 μ M, 3 h). DBC1 phosphorylation was measured by immunoblotting with specific antibody. (E) Interaction of DBC1 and SIRT1 after KSR1 overexpression with etoposide treatment by immunoprecipitation (IP). MCF7 cells were transiently transfected with pCMV6 or pCMV6-KSR1 plasmids for 24 h. Subsequently, cells were treated with etoposide (40 μ M, 3 h). The interactions between SIRT1 and DBC1 were detected by IP of SIRT1 or DBC1 followed by immunoblotting with DBC1 and SIRT1 antibodies respectively. Blots shown are representatives of at least three independent experiments. Quantification of blots was analysed by ImageJ software (NIH, Bethesda, MD, USA). (F) Schematic model illustrating the role of KSR1 on p53 transcriptional activity in breast cancer cells with (i) basal or (ii) up-regulated levels of KSR1. Abbreviation: IgG = immunoglobulin G.

To further demonstrate that KSR1 regulates p53 acetylation through DBC1, we studied the effects of KSR1 on p53 acetylation after DBC1 knockdown upon etoposide treatment in MCF7 cells. As shown in Figure 4C, western blotting experiments revealed that acetylated p53 was consistently increased after silencing of KSR1, whereas no change in p53 acetylation was observed after knocking down both KSR1 and DBC1 upon etoposide treatment. This suggests that KSR1 regulates p53 acetylation through DBC1, as depleting DBC1 undermines the effect of KSR1 on acetylated p53.

Moreover, to examine whether the effect of KSR1 on DBC1 depends on its catalytic activity, we generated a KSR1 mutant (KSR1/R502M) that encompasses a key amino-acid mutation (arginine to methionine) within its kinase domain resulting in impaired catalytic activity (Laurent *et al*, 2004). Consistently, wild-type KSR1 decreased pDBC1, whereas mutant KSR1/R502M sustained pDBC1 levels in comparison with the control

(Figure 4D). This result suggests that an intact catalytic domain of KSR1 is essential for regulating DBC1 phosphorylation.

Phosphorylation of DBC1, induced by genotoxic stress, creates binding sites and enhances the interaction between SIRT1 and DBC1 (Yuan *et al*, 2012; Zannini *et al*, 2012), which subsequently undermines the deacetylase activity of SIRT1 on p53. For this reason, we tested whether KSR1 affects the stress-induced SIRT1–DBC1 interaction. As shown in Figure 4E, upon etoposide treatment, the interaction between SIRT1 and DBC1, determined by co-immunoprecipitation (IP), was undermined after KSR1 overexpression in MCF7, which allows SIRT1 to interact more with p53 resulting in declined p53 acetylation.

KSR1 is important for breast cancer proliferation and its expression is altered in breast cancer. To study whether KSR1 affects breast cancer growth *in vitro*, cell proliferation assay was

performed in four different breast cancer cell lines. As shown in Figure 5, knockdown of KSR1 significantly reduced breast cancer cell growth in MCF7, ZR75-1, SKBR3 and MDA231 after six days, showing an important role of KSR1 in breast cancer cell proliferation. This is also supported by further examination of KSR1 expression in breast tumour tissues. Oncomine analysis (Rhodes *et al.*, 2004) was conducted to study the levels of KSR1 in normal breast and cancer tissues from TCGA Breast database. Indeed, KSR1 abundance is varied between normal breast and various breast carcinomas, including invasive ductal breast carcinoma and invasive lobular breast carcinoma (Figure 6). Specifically, KSR1 expression is significantly upregulated in invasive ductal breast carcinoma, lobular breast carcinoma and invasive breast carcinoma.

DISCUSSION

KSR1 was originally identified as a novel protein kinase evolutionarily conserved in *Drosophila* and *Caenorhabditis elegans* functioning between Ras and Raf in the Ras–Raf–MAPKs signalling pathway (Kornfeld *et al.*, 1995; Therrien *et al.*, 1995). However, mammalian KSR1 has been extensively referred as a pseudokinase, because of the mutation in the lysine residue in the catalytic domain, which is essential for its kinase activity (Zhang *et al.*, 2012). Later, the role of KSR1 as a scaffolding protein was discovered. Murine KSR1 was first reported to cooperate with activated Ras to facilitate MAPK kinases activation thus stimulating *Xenopus* oocyte maturation and cellular transformation (Therrien *et al.*, 1996). At the same time, the idea of KSR1 as an active kinase was described from the finding that TNF- α and ceramide were shown to significantly increase KSR1 autophosphorylation and its capacity to phosphorylate and activate Raf-1 (Zhang *et al.*, 1997). Therefore, emerging evidence suggests dual function of KSR1 as an active kinase as well as a scaffolding protein

(Rajakulendran *et al.*, 2009; Hu *et al.*, 2011). Moreover, KSR1 has been previously shown as an oncogene in Ras-dependent cancers, such as pancreatic and lung carcinomas (Xing *et al.*, 2003; Kortum and Lewis, 2004). However, its biological functions and modulated signalling pathways in breast cancer have remained undefined.

In this study, we performed a global comparative proteomic analysis and for the first time reveal the complex KSR1-regulated phosphoproteome in MCF7 breast cancer cells. Various new KSR1-regulated proteins and signalling networks that KSR1 is involved in were identified. Notably, our phosphoproteomic analysis showed that the majority (233 out of 379) of the identified phospho-sites were actually reduced after KSR1 over-expression in comparison with a small number of increased phosphorylations (3 out of the 379), suggesting its primary role as a scaffold protein not a kinase at least in this context. Interestingly, none of the previously described proteins including MEK and ERK in the canonical Ras–Raf–MAPKs pathways have been identified in our study. The possible explanation could be that the Ras mutations are very rare in breast cancer (Adjei, 2001). Indeed, our recent work has also shown no significant alteration in phosphorylation of the main components in the Ras–Raf–MAPKs cascades upon overexpressing or silencing KSR1 (unpublished work). Moreover, the KSR1-regulated phosphoproteins identified in this study illustrated a much more extensive view of KSR1 involved biological functions, including cell cycle, metabolism and apoptosis. Notably, some phosphoproteins have been shown to have vital roles in multiple aspects of breast cancer. For example, APRIN was reported as a BRCA2-interacting protein essential for genome integrity and associated with chemotherapy response in breast cancer (Matsuoka *et al.*, 2007; Brough *et al.*, 2012), whereas ARHGAP35 was shown to be important in breast cancer growth and migration (Shen *et al.*, 2008). Phospho-Hsp27 was related to invasiveness and drug resistance in breast cancer (Zhang *et al.*, 2007; Fujita *et al.*, 2011). Most importantly, the KSR1-regulated phosphoproteomic

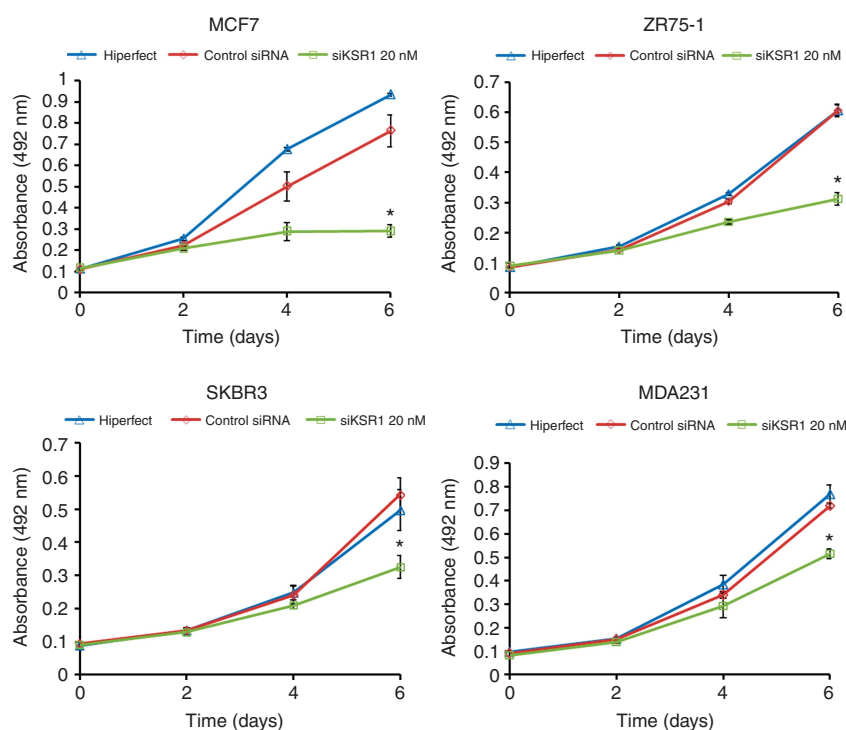


Figure 5. Effects of KSR1 silencing on breast cancer cell proliferation *in vitro*. SRB assays of MCF7, ZR75-1, SKBR3 and MDA231 cells after transfection with 20 nM of either siKSR1 or 'non-targeting' siRNA (control siRNA) or vehicle (Hiperfect) for 6 days. Error bars represent s.d. of three experiments each in quintuplicates (* $P < 0.05$, compared with control siRNA at day 6; Student's *t*-test).

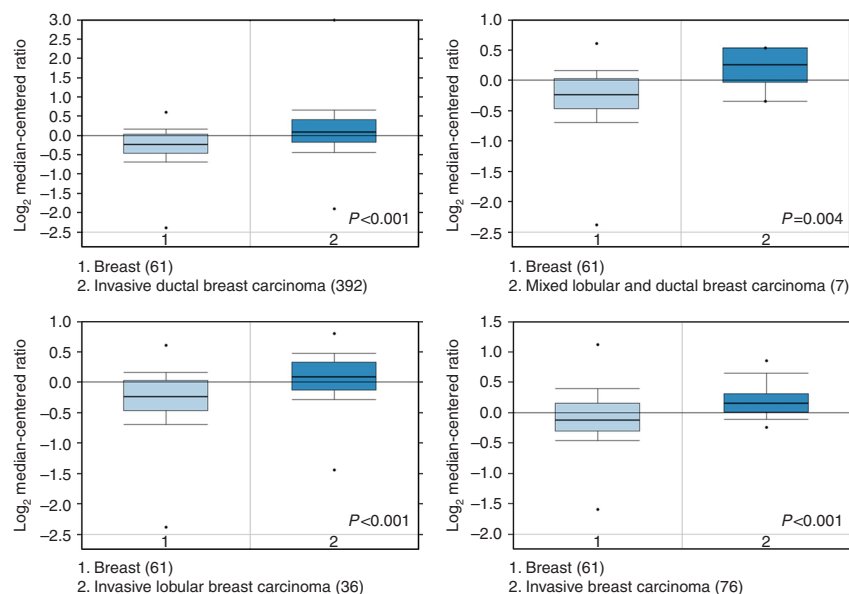


Figure 6. KSR1 expression is altered in breast cancer tissues. Oncomine analysis was performed to examine KSR1 expression in breast normal and cancer tissues using online TCGA microarray data (www.oncomine.org).

analysis has identified phospho-DBC1 as a potential connection to its modulation of p53 transcriptional activity here.

Deleted in breast cancer-1 was previously reported to be involved in regulating p53 acetylation (Kim *et al*, 2008; Zhao *et al*, 2008), which is indispensable for p53 transcriptional activity (Tang *et al*, 2008). Therefore, our work further investigated the effects of KSR1 on p53 transcriptional activity, as well as the potential mechanism on KSR1-mediated regulation of p53 transcriptional activity, including p53 acetylation. Luciferase assays using different p53-dependent gene promoter constructs showed that KSR1 overexpression suppressed p53 transcriptional activity in the absence or presence of etoposide, whereas the opposite results were observed after silencing of KSR1, indicating a regulatory role of KSR1 in p53 activity. Mechanistic study demonstrated that overexpression of KSR1 had no effect on p53 mRNA and total protein levels, as well as subcellular localisation, phosphorylation and neddylation, which are all potentially involved in p53 activity modulation.

As expected, KSR1 overexpression reduced the acetylation of p53, whereas silencing of KSR1 rescued the decreased p53 acetylation in the absence or presence of etoposide, suggesting that KSR1 is involved in regulating p53 acetylation. It has already been shown that DBC1 can interact with and negatively regulate SIRT1, a NAD-dependent p53 deacetylase, resulting in increased p53 acetylation (Kim *et al*, 2008; Zhao *et al*, 2008). Moreover, phospho-DBC1, induced by genotoxic stress, is competent to create binding sites and enhance the interaction between SIRT1 and DBC1, which subsequently undermines deacetylase activity of SIRT1 on p53 (Yuan *et al*, 2012; Zannini *et al*, 2012). Consistently, we demonstrated that KSR1 overexpression decreases the phospho-DBC1 levels, whereas KSR1 silencing increases DBC1 phosphorylation. This provides us an explanation to the observed effects of KSR1 on p53 acetylation and activity. Similar results were obtained after overexpression or silencing KSR1 in different breast cancer cell lines including ZR75-1 and SKBR3. Further co-IP experiments proved that overexpression of KSR1 attenuates the interaction of SIRT1–DBC1, which therefore allows SIRT1 to interact more with p53 resulting in decreased p53 acetylation. Our data herein are consistent with previous reports and in combination with our SILAC analyses enable KSR1 to integrate into a known network together with DBC1 and SIRT1 on regulation of p53 acetylation.

However, more evidence is still needed to elucidate the mechanism of KSR1 on modulating DBC1 phosphorylation. The work from Zannini *et al* (2012) demonstrated that ATM/ATR can directly phosphorylate DBC1 on Thr454 upon DNA damage. Phosphorylated DBC1 bound to and inhibited SIRT1, leading to the separation of the SIRT1–p53 complex and an increase of p53 acetylation. Moreover, another group indicated that protein kinase A and AMP-activated protein kinase was capable of inducing the dissociation of SIRT1 from its endogenous inhibitor DBC1, resulting in an alteration in downstream effects (Nin *et al*, 2012). Therefore, whether the effect of KSR1 on DBC1 phosphorylation is through ATM/ATR kinases or alternative pathways requires further investigation.

Collectively, our SILAC analyses of the KSR1-regulated phosphoproteome profile in cancer cells demonstrate its involvement in multiple biological and molecular processes as well as intricate signalling pathways. The identification of novel KSR1-regulated proteins will shed light on new KSR1-modulated signalling pathways implicated in breast cancer. In this work, we propose a model (Figure 4F) where in cancer cells with basal levels of KSR1, phosphorylated DBC1 directly interacts with SIRT1 and reduces its deacetylase activity resulting in p53 activation and gene expression initiation. However, in KSR1-transduced cells, KSR1 suppresses DBC1 phosphorylation, which undermines the direct interaction between DBC1 and SIRT1. This in turn allows SIRT to decrease p53 acetylation, which eventually inhibits p53 transcriptional activity. Our study provides a new insight on the role of KSR1 in p53 regulation and unveils an interesting mechanism for its function in breast cancer development.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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