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Feedback regulation of Gab2-dependent signaling by the Ras/MAPK pathway

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Thèse présentée à la Faculté de Médecine
en vue de l'obtention du grade de Philosophiae Doctor (Ph.D.)
en Biologie Moléculaire
option Biologie des Systèmes

Dec., 2014

Résumé

La voie de signalisation des Récepteurs Tyrosine Kinase (RTK) occupe un rôle central dans la régulation de la croissance cellulaire, la prolifération, la différentiation et la motilité. Une régulation anormale des RTKs mène à plusieurs maladies humaines telles que le cancer du sein, la seconde cause de mortalité chez les femmes à cause de l'amplification et la mutation fréquente de la protéine tyrosine kinase HER2 (ERBB2). Grb2-associated binder (Gab) 2 est une protéine adaptatrice qui agit en aval de plusieurs RTKs, y compris HER2, pour réguler de multiples voies de signalisation. En réponse à la stimulation par de nombreux facteurs de croissances et cytokines, Gab2 est recruté à la membrane plasmique où il potentialise l'activation des voies de signalisation Ras/mitogen-activated protein kinase (MAPK) et PI3K (phosphatidylinositol-3-kinase)/Akt (protein kinase B). En plus d'occuper un rôle essentiel durant le développement du système hématopoïétique, Gab2 est souvent amplifié dans les cancers, notamment le cancer du sein et les mélanomes. Cependant, les mécanismes moléculaires qui régulent le fonctionnement de Gab2 sont peu connus.

Il est établi que lors de l'activation des RTKs, Gab2 est phosphorylé au niveau de plusieurs résidus Tyrosine, menant à l'association de différentes protéines comme p85 et Shp2. En plus de la phosphorylation en Tyrosine, notre laboratoire ainsi que d'autres groupes de recherche avons identifié que Gab2 est aussi phosphorylé au niveau de résidus Ser/Thr suite à l'activation de la voie de signalisation MAPK. Cependant, la régulation des fonctions de Gab2 par ces modifications post-traductionnelles est encore peu connue. Dans le but de comprendre comment Gab2 est régulé par la voie de signalisation MAPK, nous avons utilisé différentes approches. Dans la première partie de ma thèse, nous avons déterminé un nouveau mécanisme démontrant que la voie de signalisation Ras/MAPK, par le biais des protéines kinases RSK (p90 ribosomal S6 kinase), phosphoryle Gab2. Ce phénomène se produit à la fois *in vivo* et *in vitro* au niveau de trois résidus Ser/Thr conservés. Des mutations au niveau de ces sites de phosphorylation entrainent le recrutement de Shp2 menant à l'augmentation de la motilité cellulaire, ce qui suggère que les protéines RSK restreignent les fonctions dépendantes de Gab2. Ce phénomène est le résultat de la participation de RSK dans la boucle de rétroaction négative de la voie de signalisation MAPK. Dans la seconde partie de ma thèse, nous avons démontré que les protéines ERK1/2 phosphorylent Gab2 au niveau de plusieurs résidus

pS/T-P à la fois *in vitro* et *in vivo*, entrainant l'inhibition du recrutement de p85. De plus, nous avons établi pour la première fois que Gab2 interagit physiquement avec ERK1/2 dans des cellules lors de l'activation de la voie de signalisation MAPK. Par ailleurs, nous avons montré un nouveau domaine d'attache du module ERK1/2 sur Gab2. Des mutations sur les résidus essentiels de ce domaine d'attache n'entrainent pas seulement la dissociation de ERK1/2 avec Gab2, mais diminuent également la phosphorylation dépendante de ERK1/2 sur Gab2.

Ainsi, nos données montrent que la voie de signalisation MAPK régule les fonctions de la protéine Gab2 par le biais des kinases RSK et ERK1/2. Cette thèse élabore par ailleurs un schéma complet des fonctions de Gab2 dépendantes de la voie de signalisation MAPK dans le développement de nombreux cancers.

Mots clés: Gab2; MAPK; RSK; ERK1/2; D-domain; phosphorylation; Shp2; p85;

Abstract

Receptor tyrosine kinase (RTK) signaling plays an essential role in modulating cell growth, proliferation, differentiation and motility. Abnormal regulation of RTKs results in many human diseases, including breast cancer, the second leading cause of cancer mortality in women by the frequent amplification and mutation of the *HER2* (*ERBB2*) tyrosine kinase. The Grb2-associated binder (Gab) 2 is an adaptor protein that acts downstream of several RTKs, including HER2, to regulate multiple signaling pathways. In response to the stimulation of a number of growth factors and cytokines, Gab2 is recruited to the plasma membrane, where it potentiates the activation of the Ras/mitogen-activated protein kinase (MAPK) and PI3K (phosphatidylinositol-3-kinase)/Akt (protein kinase B) pathways. In addition to playing an important role in the hematopoietic system during development, *GAB2* is often amplified in cancers including breast cancer and melanoma, however, little is known about the molecular mechanisms that regulate Gab2 function.

It has been well established that upon RTKs activation, Gab2 becomes phosphorylated on several Tyr residues leading to diverse adaptor protein associations, such as p85 and Shp2. Aside from the tyrosine phosphorylation, our lab and other groups noticed that Gab2 is also phosphorylated on Ser/Thr residues upon activation of MAPK signaling. However, less is known about this post-translational modification in the regulation of Gab2 functions. In order to understand how Gab2 is regulated by the MAPK pathway, we used different approaches. In the first part of my thesis, we determined a new mechanism by which the Ras/MAPK pathway through RSK (p90 ribosomal S6 kinase) phosphorylated Gab2 on three conserved Ser/Thr residues, both *in vivo* and *in vitro*. Mutation of these phosphorylation sites promoted Shp2 recruitment leading to increased cell motility, suggesting that RSK restricts Gab2-dependent functions as a result of participation in the negative feedback loop of MAPK signaling. In the second part of the thesis, we found that ERK1/2 phosphorylated Gab2 on several potential pS/T-P residues, both *in vivo* and *in vitro*, resulting in inhibited p85 recruitment. In addition, to the best of our knowledge, we established for the first time that Gab2 physically interacted with ERK1/2 in cells upon activation of the MAPK pathway. Furthermore, we revealed a novel ERK1/2 docking domain in Gab2. Mutation of the essential residues in this docking domain not only disrupted ERK1/2-Gab2 interaction, but also

diminished ERK1/2-dependent phosphorylation on Gab2.

Taken together, our data showed that the MAPK pathway regulates Gab2 functions through both RSK- and ERK1/2-dependent manners. Given that Gab2 is overexpressed in several cancers, this thesis decodes a complete figure of modulating actions of Gab2 by MAPK signaling in cancer development.

Keywords: Gab2; MAPK; RSK; ERK1/2; D-domain; phosphorylation; Shp2; p85;

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List of Abbreviations

5-FU	fluorouracil
AGC family	protein kinases A, G and C
Akt	protein kinase B
AMPK	AMP-activated protein kinase
Apaf-1	apoptotic protease activating factor-1
APC	anaphase-promoting complex
Arf6	ADP-ribosylation factor 6
BAC	bacterial artificial chromosome array
Bad	Bcl-2-associated death promoter
Bim	BCL2-like 11, apoptosis facilitator
BMMCs	bone marrow-derived mast cells
BRCA1	breast cancer gene 1
Bub1	budding uninhibited by benzimidazoles 1
CAMK	Ca^{2+} /calmodulin-dependent protein kinase
CB	cysteine cathepsin B
CBP	CREB binding protein
CD domain	common docking domain
CD36	a member of the class B scavenger receptor family
Cdc25	cell division cycle 25
CDKs	cyclin-dependent kinases
CGH	comparative genomic hybridization
Chk1	checkpoint kinase 1
CKI	cyclin-dependent kinase inhibitor
CLS	Coffin-Lowry syndrome
c-Met	hepatocyte growth factor receptor
CREB	cAMP response element-binding protein

Csw	Corkscrew; Shp-2 ortholog in Drosophila
CTKD	carboxyl-terminal kinase domain
CXCL1/2	C-X-C motif chemokine 1/2
DAPK	death-associated protein kinase
DCIS	ductal carcinomas in situ
DEF domain	docking site for ERK, FXF
E2F	E2 factor family of transcription factors
ECM	extracellular matrix
EGFR	epithermal growth factor receptor
eIF2	eukaryotic initiation factor 2
eIF4E	eukaryotic translation initiation factor 4E
ELISA	enzyme-linked immunosorbent assay
ELK1	ETS domain-containing protein
Emi2	early mitotic inhibitor 2
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
ERα	estrogen receptor α
ES	embryonic stem
EST	expressed sequence tag
FcεRI	lgE receptor
FGF	fibroblast growth factor
FN1	fibronectin 1
FOXO3a	forkhead box O 3a
Gab2	Grb2-associated binder 2
Gadd45	growth arrest and DNA-damage inducible
GAP	GTPase-activating protein

Grb2	growth factor receptor-bound protein 2
HIF-α	hypoxia-inducible factor 1-alpha
HRG	heregulin
IBC	invasive breast carcinoma
IEG	immediate early genes
IGF-1	insulin-like growth factor
IHC	immunohistochemical
IL	interleukin
JIP1	JNK-interaction protein 1
JNK	c-Jun N-terminal kinases
Ki67	a marker for proliferation rate
KIBRA	kidney and brain expressed protein
KIM	kinase interaction motif
KSR	suppressor of Ras
Lyn	a non-receptor tyrosine-protein kinase
Mad1	mitotic arrest-deficient protein
MAP2	microtubule-associated protein-2
MAPK	mitogen-activated protein kinase
MAPKAPKs	MAPK activated protein kinases
MARK	MAP-regulation kinase/microtubule affinity regulating kinase
MBD	c-Met-binding domain
Mcl1	myeloid cell leukemia sequence 1
MDCK	Madin-Darby canine kidney
MET	mesenchymal-epithelial transition
Mi	microphthalmia
mIMCD-3	mouse inner medullary collecting duct
miRNAs	microRNAs

MK2/3	MAPK-activated protein kinase 2/3
MKP	MAP kinase phosphatase
MLC	myosin light chains
MLCK	myosin light chain kinase
MMP-12	macrophage metalloelastase
MMP-9	matrix metallopeptidase 9
MNKs	MAPK-interacting kinases
MP1	MEK partner1
MSKs	mitogen- and stress-activated kinases
MSP	macrophage-stimulating protein
MST1R	macrophage-stimulating protein receptor
NES	nuclear export signals
NLK	Nemo-like kinase
NLS	nuclear localization sequences
nNOS	neuronal nitric oxide synthase
NPC	nuclear pore complex
NTKD	amino-terminal kinase domain
NTS	nuclear transport signal
P	phosphate
p21	WAF1/CIP1
p85	regulatory subunit of PI3K
PAK1	p21-activated kinase-1
PDCD4	programmed cell death protein 4
PDGF	platelet-derived growth factor
PDK1	3-phosphoinositide-dependent protein kinase-1
PFS	progression-free survival
PH domain	PIP3-containing domain

PI	phosphatidylinositol
PI3K	phosphatidylinositol-3-kinase
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKA	protein kinase A
PLC	phospholipase C
PP	protein phosphatase
PP2A	protein phosphatase-2A
PR	progesterone receptor
PSPL	positional scanning peptide library
PTB	phosphotyrosine binding
PTEN	phosphatase and tensin homolog
PTP	protein-tyrosine phosphatase
PUMA	p53 upregulated modulator of apoptosis
RA	rheumatoid arthritis
Raf	regulator of α-fetoprotein
RANK	receptor activation of NF-κB
Raptor	regulatory associated protein of mTOR
RB1	retinoblastoma-associated
RBL	rat basophilic leukemia
Rheb	Ras homolog enriched in brain
ROCK II	Rho kinase II
Ron	recepteur d'origine nantais
RSK	p90 ribosomal S6 kinase
RTK	receptor tyrosine kinase
S100A8/9	protein S100-A8/A9
S6K	S6 kinase
SCF	stem cell factor

SDF-1	stromal cell-derived factor-1
SGK	serum-and glucocorticoid-induced protein kinase
SH	Src-homology
Shc	Src homology and collagen
Shp2	SH2 domain-containing protein tyrosine phosphatase
SNP	single nucleotide polymorphism
SOS	guanylnucleotide exchange factor
SRF	serum response factor
STAT3	signal transducer and activator of transcription 3
Stk/Ron	receptor tyrosine kinase
TGFβ	transforming growth factor beta
TKIs	tyrosine kinase inhibitors
TLR	toll-like receptor
TNF	tumor necrosis factor
TSP-1	thrombospondin-1
VASP	vasodilator-stimulated phospho-protein
VEGF	vascular endothelial growth factor
WT	wild-type
XPC	xeroderma pigmentosum C

Dedicated to my father and mother

Acknowledgements

There are many people who have accompanied and assisted me along the way, including families, friends and colleagues. To them, I owe a big debt of gratitude. Firstly, I would like to express my foremost and deepest sincere gratitude to my supervisor Dr. Philippe Roux, with whom I started my scientific career. Thanks for his recruitment and giving me an opportunity to do excellent cutting edge research. Having him on my side is a very rewarding experience. Throughout my Ph.D. studies, he provided unremitting support, guidance and encouragement. I am also especially thankful to him for his friendly attitude and help which make me overcome all difficulties throughout these years. As a mentor, he is a wise man and always gave me valuable suggestions and guidance; as a person, he is a kindly human being, and always cares about my life in Canada since I am far away from my country. I feel proud and grateful to work in the Roux lab and I will cherish this tremendous experience throughout my life.

I would also like to thank the members of my advisory committee, Drs. Sylvain Meloche and Marc Servant, for their support and invaluable comments throughout the past five years. Moreover, I would like to thank Dr. Sébastien Carréno for his helpful technical advice and support.

I would also like to thank all the members of the Roux lab, past and present, Drs. Joseph Tcherkezian, Jacob Galan and Yves Romeo for their invaluable advice, support and comments. I especially feel indebted to thank Dr. Geneviève Lavoie, who assisted me throughout these years. In addition, I am also grateful to Louis-André Julien, Marie Cargnello, Jérome Roger, Antoine Méant, Justine Paradis, Roméo Blanc, Farah Dandachi and Yasaman Nouhi for support and help in the lab.

My gratitude to the Institute for Research in Immunology and Cancer (IRIC) for providing me with such an excellent platforms to support cutting-edge scientific research. Also, I would like to thank the Molecular Biology Program, including Pascale LeThérizien and Vivianne Jodoin for supporting my studies, and giving me many chances to present my work in conferences. I am also very grateful to the China Scholarship Council (CSC) for

sponsoring my Ph.D. studies abroad. Thanks to the Canadian Institutes of Health Research (CIHR) and other sources of funding for supporting my work.

Lastly, I would like to thank my husband Junkai for his love, patience and support throughout my Ph.D. studies. Thanks to my dear friends, Ting Jin, Yi Fang, Yang Liu, Ming Sun, Peng Wang, Wei Yuan, Yifei Yan and Jayshree Khanikar for all their friendship during my studies here. Most importantly, I would like to express my gratitude to my father, mother and brother. Without their unconditional love and support, this thesis would not have been possible.

Contribution of Co-authors

Chapter 2-Gab2 phosphorylation by RSK inhibits Shp2 recruitment and cell motility.

Zhang X, Lavoie G, Fort L, Huttlin EL, Tcherkezian J, Galan JA, Gu H, Gygi SP, Carreno S, Roux PP.
(Mol Cell Biol. 2013 Apr; 33(8):1657-70.)

Zhang X. participated in the conception of the project, experiment design, analysis of results and preparation of the manuscript. Lavoie G. participated in the analysis of the interaction between phosphorylated Gab2 by RSK and Shp2 (Figure 2.6A and F). Lavoie G. and Fort L. participated in the analysis of cell migration (Figure 2.8A). Huttlin EL. and Gygi SP. performed the mass spectrometric analysis (Figure 2.3E-F). Tcherkezian J. participated in the generation and quantification of confocal images (Figure 2.7A and C). Gu H. provided the $Gab2^{-/-}$ MEF cells (Figure 2.6F). Carreno S. participated in the data analysis of cell motility (Figure 2.8D). Galan JA. generated data that was not include in the manuscript. Roux PP. supervised the project, analyzed the results and prepared the article.

Chapter 3-ERK1/2 participate in a negative feedback loop that limits Gab2 function in response to growth factors.

Zhang X, Lavoie G, Meant A, Cargnello M, Roux PP.
(The manuscript is in preparation)

Zhang X. participated in the conception of the project, experiment design, analysis of the results and preparation of the manuscript. Lavoie G. participated in the *in vitro* kinase assay of Gab2 by ERK1, measuring the interaction between Gab2 and ERK1 CD-domain mutant as well as phosphorylation level of different Gab2 mutants (Figure 3.2F, 3.4A and 3.5B). Meant A. participated in the analysis of the interaction between Gab2 and p85 upon stimulation of MAPK signaling (Figure 3.6A). Cargnello M. participated in the λ-phosphatase experiment and preparation of article (Figure 3.1B). Roux PP. supervised the project, analyzed the results and prepared the article.

Chapter 1 Introduction and Literature Review

1.0 General Introduction

Breast cancer is the second leading cause of cancer mortality in women and accounts for 6.8% of all cancer deaths in 2014 *(data from American Cancer Society)*. In most breast cancers, ductal carcinomas *in situ* (DCIS) are precursors, and their progression to invasive breast carcinoma (IBC) is a representative feature of tumor aggressiveness (Tamimi *et al.*, 2008). Classifications of tumor grades, stages and molecular subtypes of breast cancers are helpful to define the cancer-related types and target diagnosis. In general, by using several immunohistochemical (IHC) markers, including the estrogen (ER)/progesterone(PR) receptor, HER2/neu (a member of the epidermal growth factor receptor family) and Ki67 (a marker for proliferation rate), breast cancers are predominately classified into four subtypes, including luminal A, luminal B, the triple negative basal-like and HER2 types (Carey *et al.*, 2006). To date, around 10~15% of breast cancer patients have been determined with aberrant regulation of HER2 by overexpression or mutation, which leads to the upregulation of multiple intracellular signaling pathways, such as MAPK (mitogen-activated protein kinase) signaling (Kurokawa *et al.*, 2000).

Aside from being involved in breast cancers, MAPK signaling has also been implicated in the development of several human diseases, including inflammatory diseases and severe bone disorders (Lawrence *et al.*, 2008). Given that the MAPK pathway, through specific downstream effectors, leads to diverse biological events such as cell survival, proliferation and motility, the determination of novel substrates of MAPK signaling, likely substrates of ERK (extracellular signal-regulated kinase) and RSK (p90 ribosomal S6 kinase), will reveal new therapeutic targets for the treatment of human diseases. While ERK and RSK often co-regulate common protein substrates, they both phosphorylate specific sequences within target proteins. Whereas ERK phosphorylates substrates on the Ser/Thr-Pro residues that often require protein-protein interactions, RSK is a basophilic kinase that phosphorylates substrates on Arg-Xxx-Xxx-pSer/Thr consensus sequences (Cargnello and Roux, 2011).

Gab2 (Grb2-associated-binding protein 2) has been identified as an “amplifier” in signal transduction from receptors to multiple downstream signaling pathways, including MAPK and PI3K (phosphatidylinositol-3-kinase) signaling, resulting in increased cell proliferation and migration (Liu and Rohrschneider, 2002). Intriguingly, Gab2 in turn can act as a substrate

of the MAPK and PI3K pathways, indicating that Gab2 is involved in feedback loops within these signaling pathways (Lynch and Daly, 2002; Arnaud *et al.*, 2004). In many instances, MAPK and PI3K signaling-mediated Gab2 phosphorylation on Ser/Thr residues result in altered cell proliferation and migration (Lynch and Daly, 2002; Arnaud *et al.*, 2004). Given that the *Gab2* gene is located on chromosome 11q14.1, a region frequently amplified in breast cancer (Bocanegra *et al.*, 2009), the understanding of how Gab2 is regulated at the molecular level will be useful for the treatment of breast cancer.

1.1 Characteristics of carcinogenesis

The behavior of a normal cell is governed by precise molecular mechanisms that respond to the external environment. Changes in the extracellular environment are often transduced to intracellular signaling cascades through specific receptor systems, such as ion channel-linked receptors, enzyme-linked receptors and G protein-coupled receptors. These receptor systems stimulate many different signaling pathways that are tightly regulated and act as an integrated network, driving cells to divide, differentiate, proliferate, undergo apoptosis or migrate (Hille, 1994; Delcourt *et al.*, 2007). During these processes, monitoring programs, such as cell cycle checkpoints, are executed to ensure cells are behaving properly (Topham and Taylor, 2013). However, a series of diseases such as cancer can occur when the monitoring programs in an individual cell are deregulated, and the cell behaves inappropriately. Cancer often originates from heritable genetic alterations or epigenetic changes within normal tissue, which results in the accumulation of genomic alterations that convert the cell's behavior from normal to malignant. For instance, gene silencing, gene amplification and gene mutation affect the structure or function of the genome, and are possible inducers of cancer.

Intriguingly, based on the common characteristics of carcinogenesis, Hanahan and Weinberg in 2011 proposed a paradigm in which a normal cell acquires six hallmark capabilities to become malignant, fostered by genome instability (Hanahan and Weinberg, 2011). These six hallmarks include: (I) sustaining growth-stimulatory signals; (II) downregulating growth-inhibitory signals; (III) resisting cell death; (IV) enabling replicative immortality; (V) inducing angiogenesis; and (VI) activating invasion and metastasis (Hanahan and Weinberg, 2011). This description provides a solid foundation for a better understanding

of the molecular mechanisms related to cancer biology. In the following sections, the basic characteristics of the tumor are summarized.

1.1.1 Sustaining proliferation and evading growth suppressors

Normal tissues properly secrete and release growth-stimulatory signal molecules inducing cell proliferation. Tumor cells often upregulate these growth-promoting signals, which sustain chronic proliferation. Several mechanisms underlie the upregulation of these enabling signals. Firstly, aberrant regulation of receptor proteins on the cell surface by overexpression or mutation promotes cell proliferation. It may either allow a cell to hyper-respond to the low concentration of circulating growth factors or cause ligand-independent receptor activation by spontaneous receptor dimerization, such as occurs with the members of the receptor tyrosine kinase family (Foley *et al.*, 2010). In addition, autocrine or paracrine signaling can affect cancer cell proliferation. In the former pathway, a cancer cell produces its own growth factors, such as interleukin (IL)-6, to stimulate its own proliferation (Gao *et al.*, 2007); in the latter pathway, cell growth is dependent on cross-communication between different cell types. For instance, stromal cells adjacent to carcinoma tissue secrete stromal cell-derived factor-1 (SDF-1) for cancer cell proliferation (Kojima *et al.*, 2010). Moreover, alteration of components of intracellular signaling cascades, such as MAPK and PI3K signaling, is another mechanism underlying cancer cell proliferation. For instance, either Ras/Raf (regulator of α-fetoprotein) mutation or PTEN (phosphatase and tensin homolog) deletion leading to the upregulation of Ras/MAPK (mitogen-activated protein kinase) and PI3K (phosphatidylinositol-3-kinase)/Akt (protein kinase B) signaling have been observed in certain human tumors (Hollestelle *et al.*, 2007; Hollander *et al.*, 2011).

Aside from sustaining growth-promoting signaling, tumor cells also have to circumvent powerful monitoring programs that negatively regulate cell proliferation, such as tumor suppressors. Two of the famous tumor suppressors are *RB1* (retinoblastoma-associated) and *p53*, which act as central nodes in regulation of cell proliferation, senescence and apoptosis (Sherr and McCormick, 2002). The first tumor suppressor gene, *RB1*, was identified in 1988 by studies of retinoblastoma, a rare childhood eye tumor (Dunn *et al.*, 1988). When receiving physiological growth-inhibitory signals from the outside of a cell, such as transforming growth factor beta (TGF-β) or contact inhibition, pRb, the phosphorylation product of Rb,

interacts with E2F (the E2 factor family of transcription factors) leading to the inhibition of the cell cycle-related gene expressions, such as cyclinB (Zhang *et al.*, 1999). Moreover, oncogene expression, DNA damage, or cellular stress can induce p53 stabilization and activation. If the DNA is excessively damaged, or if the levels of growth-stimulatory signals, glucose or oxygenation tend to be high, p53 arrests cell at different phases of the cell cycle until these abnormal events have been successfully addressed (Zilfou and Lowe, 2009). Otherwise, the p53 pathway promotes cellular apoptosis, through activation of the caspase proteases, such as caspase 9, or release apoptogenic factors such as pro-apoptotic proteins of the Bcl-2 family from mitochondria (Schuler and Green, 2001). In order to circumvent these anti-growth factors, cancer cells become insensitive to these factors by defection or the lack of some growth-inhibitory proteins related to the cell cycle or cell survival, such as $p21^{Cip1}$, $p27^{Kip1}$, $p16^{INK4A}$, pRb, Smad3, or p53 (Grady, 2005; Schwartz and Shah, 2005).

1.1.2 Angiogenesis formation in the primary tumor site

Angiogenesis is a normal physiological process in which new blood vessels sprout from pre-existing vessels. The function of angiogenesis is to provide sustenance to support the active cells. Whereas the development of the vasculature in embryogenesis involves vasculogenesis and angiogenesis, the normal vasculature is quiescent in healthy adults (Patan, 2004). On certain physiological conditions, angiogenesis is transiently "turned on", such as tissue regeneration. However, in cancer development, an "angiogenic switch" always remains on and is hyperactivated leading to a continued sprouting of new vessels to supply nutrients for the neoplastic growth. In carcinogenesis, angiogenesis leads to excessive vessel branching in solid tumors. Because these blood vessels have an erratic blood flow, they become distorted and enlarged with endothelial cell fast proliferation and resistance to apoptosis (Ucuzian *et al.*, 2010).

Angiogenesis is a complex process that is modulated either by angiogenic stimulatory factors or inhibitory factors. One such angiogenic inducer is the vascular endothelial growth factor (VEGF) (Shweiki *et al.*, 1992). Hypoxia and oncogene-induced signals, such as Ras proto-oncogenes, drive angiogenesis leading to VEGF secretion (Rak *et al.*, 1995; Forsythe *et al.*, 1996). Upon VEGF binding to its specific cell-surface receptor, the VEGFR-mediated downstream signaling pathways are activated leading to increased vascular endothelial cell

proliferation and migration.

Angiogenesis is also a well organized stepwise process beginning with extracellular matrix (ECM) degradation by ECM degrading proteases, such as matrix metallopeptidase 9 (MMP-9), which are secreted and released from VEGF-induced endothelial cells (Ghosh *et al.*, 2012). Next, the migrated endothelial cells undergo proliferation and differentiation to form new blood vessels; Finally, endothelial cells form a new basement membrane and produce growth factors such as platelet-derived growth factor (PDGF) to ensure the stability of the new blood vessel (Appelmann *et al.*, 2009). In contrast, the angiogenesis process is counterbalanced by angiogenic inhibitory factors. For instance, thrombospondin-1 (TSP-1) is a critical antagonistic molecule of VEGF that blocks cell survival signals and drives cell apoptosis (Kaur *et al.*, 2010). It has been shown that TSP-1 regulates cell survival by directly modulating the formation of the CD36 (a member of the class B scavenger receptor family)/CD47/intergrins/VEGFR complex (Neufeld *et al.*, 1999; Rodriguez-Manzaneque *et al.*, 2001; Lawler and Lawler, 2012). Together, these findings suggest that the understanding of angiogenic stimulatory and inhibitory factors may pave the way for developing novel therapeutic approaches and anti-cancer drug innovations.

1.1.3 Invasion and metastasis

Aside from uncontrolled proliferation, cancer cells progressing from epithelial tissues to higher malignancy carcinomas require alterations of cell shape and de-attachment from the adjacent cells or the extracellular matrix (ECM). In addition, cancer metastases formation in the distant organs away from the primary site represents aggressive cancers and accounts for 90% of cancer deaths (Mehlen and Puisieux, 2006). Metastasis is a well-organized process and consists of several consecutive steps: (I) local invasion by carcinomas; (II) migration into adjacent blood or lymphatic vessels; (III) trafficking in the lymphatic and hematogenous systems; and (IV) colonization of distant tissue.

The epithelial-mesenchymal transition (EMT) enables cancer cells to become invasive

One of the acquisitions of tumor cell invasion is the EMT program execution, which allows cells to survive, invade and disseminate. Activation of the EMT program is either transient or permanent depending on the cancer cell type and stage.

The EMT is induced by several signals, such as Wnt signaling and TGF-β signals, correlating with the alteration of adherence molecules (Taki *et al.*, 2003; Xu *et al.*, 2009). Among these adherence molecules, E-cadherin plays the predominant role in EMT progression, being responsible for maintaining cells in a proper position and assembling epithelial cell sheets. Decreased E-cadherin expression promotes cell migration and invasion, whereas increased expression of E-cadherin allows cells to resist metastasis (Onder *et al.*, 2008). Several transcription factors, such as Snail, Slug, Twist and Zeb1/2, affect EMT program execution (Cano *et al.*, 2000; Bolos *et al.*, 2003; Vesuna *et al.*, 2008; Lau *et al.*, 2013). For example, Snail, Slug, Twist and Zeb1/2 negatively regulate E-cadherin expression in several cancer cell lines, resulting in reduced adherence junctions and alterations of cell morphologies from polygonal/epithelial to spindly/fibroblastic.

Paracrine signaling from stromal cells promote tumor invasion and metastasis

Many studies indicate that tumorigenesis is not solely a cell-autonomous behavior, and cannot be understood simply by analysis of the genomes of tumor cells. Alterations in the microenvironment, such as the cross-communication between stromal and tumor tissues (referred to as paracrine signaling) is required for cell invasion and metastasis. The stromal cell-derived factors influence the adjacent target cancer cell. For instance, macrophages in the tumor periphery produce matrix-degrading proteases such as the macrophage metalloelastase (MMP-12) and the cysteine cathepsin B (CB), leading to cancer cell invasion by degrading the cell matrix membrane (Dong *et al.*, 1997; Vu and Werb, 2000; Vasiljeva *et al.*, 2006). Notably, paracrine signaling is different from endocrine signaling. In the former, the secreted molecules cannot be diffused too far, and only reach the adjacent target cells; in the latter, the secreted hormones are distributed widely throughout the body by the blood circulation (Molecular Biology of The cell. 4th edition).

In addition to tumor metastasis, chemoresistance which causes the failure of cancer treatment is affected by paracrine signaling. A network of endothelial-carcinoma-myeloid signaling interactions provides a mechanism linking chemoresistance and metastasis (Acharyya *et al.*, 2012). Although chemotherapeutic agents kill cancer cells, under certain conditions they also induce TNF-α production by endothelial cells and other stromal cells. In turn, TNF-α acts on cancer cells and increases NF-κB activity, leading to the

chemotherapeutic resistance of cancer cells by upregulating of the CXCL1/2 (C-X-C motif chemokine 1/2)-S100A8/9 (protein S100-A8/A9) loop (Acharyya *et al.*, 2012).

Formation of metastatic colonies in anatomical sites

Cancer metastasis consists of two steps: (I) tumor cells migrate from a primary site to distant organs and; (II) they settle down in foreign tissues. Colonization is a complex process and also a challenging step of tumor metastasis. Indeed, some disseminated cancer cells hardly survive in an external microenvironment. Hence, each disseminated cancer cell needs its own set of solutions for colonizing a particular organ (Gupta *et al.*, 2005). On the one hand, disseminated cancer cells colonize foreign tissues through self-seeding, which are supported by stromal cells adjacent to the primary tumor. On the other hand, after residing in the distant tissue, cancer cells no longer benefit from EMT-inducing signals from primary tumors. In the absence of such signals, cancer cells may make this new home a noninvasive stage, and undergo the mesenchymal to epithelial (MET) process, which is the reverse of EMT, leading to new cancer colonies similar to the primary tumor (Elshamy and Duhe, 2013). Although these findings suggest the possible mechanisms regarding the metastases, the explanations likely remain simplistic. The precise mechanisms by which disseminated cancer cells settle down in secondary sites require in-depth analysis.

1.2 Breast cancer and receptor tyrosine kinase

1.2.1 Grades, stages and types of breast cancer

Tumor grade is a classification based on the morphological similarities between tumor and normal cells. In general, the more the morphological similarities between tumor cells and normal cells, the lower the tumor grade tends to be; the faster the growth rate of tumor cells, the higher the tumor grade tends to be. Based on these two factors, there are three tumor grades: (I) Grade 1 or low grade: the tumor cells look the most similar to normal cells, high differentiation and are slow-growing; (II) Grade 2 or intermediate/moderate grade: the tumor cells look different from normal cells and grow faster; and (III) Grade 3 or high grade: the tumor cells look very abnormal, poorly-differentiated and are fast-growing *(http://ww5.komen.org/)*.

In addition to tumor grade, staging of cancer is another key indicator in cancer prognosis. Cancer stage is different from tumor grade because it refers to many factors, such as a primary

tumor location, a tumor size, lymph node involvement, and metastasis formation in a secondary organ (Gannon *et al.*, 2013). Among these, the high capabilities of invasion and metastasis are the most harmful factors in breast cancer prognosis. While colon carcinomas are likely to invade the liver, breast cancers promiscuously form metastases in many organs throughout the body, including lungs, bones, liver, and the brain. In the invasion-metastasis cascade of breast cancer, invasive ductal cancer begins in the milk ducts, spreads into the surrounding breast tissue, and invades the rest of the body through the lymph nodes and blood circulation. Given that the ability of cancer cells invasion from the primary tumor into surrounding tissues and blood vessels is different, ductal carcinoma *in situ* (DCIS, intraductal carcinoma) is described as non-invasive breast cancer (stage 0), and invasion of tumor cells into distant lymph nodes and other organs is classified as stage IV. In general, the higher the stage of invasive breast cancer, the poorer is the prognosis of survival.

The classification of breast cancer characteristics can be helpful to define the cancer-related types and diagnosis in the future. By using molecular and genetic tools, a complete profile of each subtype of tumor cell can be determined. Hormone receptor status (estrogen receptor or progesterone receptor), HER2/neu status and proliferation rate (Ki67) are the three major testing standards. Based on these factors, breast cancers are classified into four major molecular subtypes: (1) luminal A; (2) luminal B; (3) triple negative basal-like type and; (4) HER2 type (Tamimi *et al.*, 2008). Additional details of the four cancer subtypes are shown in the Table 1. Notably, not all breast cancer types are included in this classification, such as apocrine molecular type (looks like normal breast cells but characterized by androgen receptor expression) and claudin-low type (Prat *et al.*, 2010), indicating the complexity of breast cancer types.

Luminal tumor cells. Most breast cancers belong to luminal tumors, which normally start in the inner (luminal) cells of the mammary ducts. Luminal tumors consist of two types: luminal A and luminal B, both of which tend to be hormone receptors-positive (ER+/PR+). In some cases, luminal A tumors also tend to be HER2/neu-negative (HER2-) with low expression of Ki67, indicating that this type of cancer has a lower-growth rate (Sun *et al.*, 2014). Most of the luminal A tumor cells are classified as tumor grade 1 or 2, and likely to have a good prognosis with high survival-rates. However, approximately 15% of luminal A tumors have p53 mutations leading to a poor prognosis (Carey *et al.*, 2006). Unlike luminal A-type, luminal B-type tumors are positive for HER2+ and/or Ki67 and 30% of them have p53 mutations. The survival rates of luminal B patients are fairly high but not as good as those with luminal A tumors.

Table I Molecular subtypes of breast cancer

Subtype	Molecular marker	Prevalence (approximate)
Luminal A	ER+ and/or PR+, HER2-, low Ki67	40%
Luminal B	ER+ and/or PR+, HER2+ (or HER2- with high Ki67)	20%
Triple negative/basal-like	ER-, PR-, HER2-	15-20%
HER2 type	ER-, PR-, HER2+ (HER2-)	10-15%
Note: ER: estrogen receptor; PR: progesterone receptor; HER2: neu receptor; Ki67: a cellular marker for proliferation		

(Data adapted from http://ww5.komen.org/BreastCancer)

Triple negative/basal–like tumor cells. This subtype of tumor cells is hormone receptors negative (ER-, PR-), and HER2 negative (HER2-). Most triple negative tumors are also basal-like tumors because of a similar feature to the outer cells of the mammary ducts. In patients, about 15~20% of breast cancer are triple negative or basal-like tumors, and most basal-like tumors have p53 mutations (Badve *et al.*, 2010). Compared to luminal cancer subtypes, the triple negative and the basal-like tumors are often more aggressive and have a

poor prognosis. The *BRCA1* (breast cancer gene 1) gene is the most studied gene in triple negative cancer. *BRCA1* encodes a tumor suppressor protein BRCA1, which is involved in DNA damage repair and transcriptional response to DNA damage by regulating the transcription of several genes, including *p21*(WAF1/CIP1), *Gadd45* (growth arrest and DNA-damage inducible), *Cyclin B1*, *XPC* (xeroderma pigmentosum C), *14-3-3σ* (Somasundaram, 2003). It has been shown that *BRCA1* mutations lead to carcinogenesis by inducing DNA damage and genome instability (Foulkes and Shuen, 2013). However, the mechanisms by which *BRCA1* mutations are likely to promote carcinogenesis in estrogen-responsive tissues, such as ovary and breast, remain elusive (Wang and Di, 2014).

HER2 type. Around 10~15% of breast cancers fit this molecular profile, and 75% of HER2-type cancers have the *p53* gene mutations. However, the HER2 type does not completely have HER2+ status because 30% of these cancers are also HER negative (HER2-). There are four common characteristics in HER2 type cancers, including ER-, PR-, lymph node involvement and higher tumor grade (Kim *et al.*, 2006). The classification of breast tumor types helps to estimate the diagnosis and plan the best treatment for patients, and also provides information for developing novel therapies.

1.2.2 Treatments of breast cancer

Based on patients' conditions and stages of cancer progression, different treatments are available. Local and systemic therapies are the two major ways for treating cancers. The former therapy is to remove tumors at the original site without affecting the rest of the body, including surgery and radiation therapies. The latter treatment involves administering drugs via the bloodstream and lymph cycles, including chemotherapy, hormone therapy, and targeted therapy *(http://www.cancer.org/).*

Chemotherapy is a treatment with drugs, which can reach cancer cells in most part of the body via the bloodstream. Given that cancer cells divide more quickly than normal cells, the purpose of these drugs is to attack cells with higher division rates. However, some cells in the body such as bone marrow cells also divide quickly. Hence, chemo drugs are prone to attack these normal cells as well, leading to severe side effects (Crozier *et al.*, 2014). The most common used chemotherapy drugs are anthracyclines, taxanes, and the combination of fluorouracil (5-FU) and cyclophosphamide.

Hormone therapy. Around 67% of breast cancers are the hormone receptor-positive type, which contain hormone receptors for estrogen and/or progesterone. The aim of hormone therapy is to inhibit tumor growth by interrupting the estrogen-mediated pathway. However, this kind of treatment only works in hormone receptor-positive breast cancers (ER+/PR+) rather than hormone receptor-negative tumors (Mustonen *et al.*, 2014). Tamoxifen, fulvestrant, and toremifene are common drugs for this treatment.

Targeted therapy. Since alterations of some related genes in tumorigenesis have been explored, scientists have been able to develop specific drugs based on their target proteins. Unlike chemo drugs, these drugs individually act on target proteins and have fewer side effects. One of the representatives is HER2 inhibitors (Li and Li, 2014). HER2/neu is overexpressed in 10~15% of breast cancer cell surface and makes them more aggressive and highly metastatic. Alternatively, mTOR inhibitor and vascular endothelial growth factor (VEGF) inhibitor are also used in cancer treatment. Examples regarding targeted therapy are shown in Table II.

Table II Drugs for targeted therapy in breast cancers

Drug name	Target protein	Functions	Side effects
Trastuzumab (Herceptin) Lapatinib (Tykerb) Pertuzumab	HER2	Decreasing the tumor growth and stimulating the immune system to attack the cancer cells.	Relatively mild.
Everolimus	Blocking mTOR	Preventing cancer cells from growing and developing new blood vessels.	Increase blood lipids, blood sugars and risk of infection.
Bevacizumab	Against vascular endothelial growth factor (VEGF)	Preventing new blood vessel formation in tumors.	Much more severe side effects with high-blood pressure.

1.2.3 ErbB2/HER2 in breast cancer

1.2.3.1 EGFR family

The epithermal growth factor receptor (EGFR) family is a major class of the receptor tyrosine kinase (RTK) superfamily, consisting of four members: EGFR (HER1), HER2 (ErbB2), HER3, and HER4 (Foley *et al.*, 2010). HER receptors are transmembrane glycoproteins containing a highly conserved extracellular domain, and an intracellular ATP-binding kinase domain (Figure 1.1) (Yarden, 2001). To date, several ligands binding to different HER receptors (HER1, HER3, HER4) have been identified. Upon binding to ligands, HER receptors form dimers followed by intracellular kinase domain phosphorylation and adaptor protein recruitments, leading to the activation of several downstream signaling pathways. Unlike other isoforms, HER2 is the only isoform of the EGFR family whose ligand is still unknown. However, HER2/HER3 heterodimerization, leading to the auto-phosphorylation of HER2, is the most relevant to HER2-dependent carcinogenesis. Abnormal regulation of HER2 by overexpression and mutation has been indicated in around 10~15% of breast cancers.

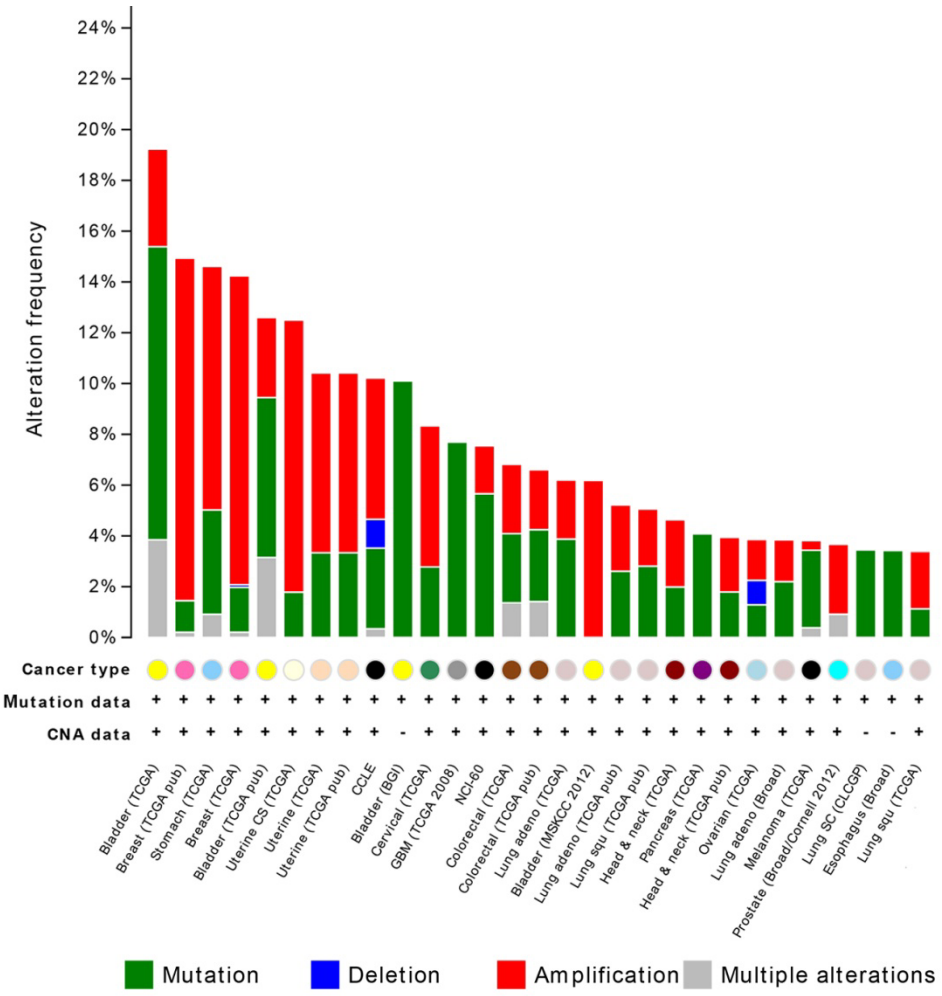

Figure 1.1 Cross-cancer alteration summary for the *ERBB2* gene.

It has been found that the *ERBB2* gene is located on human chromosome 17 (17q12), which is amplified in multiple cancers. Amplification and mutation of *ERBB2* are the two major causes in ErbB2/HER2-induced carcinogenesis. In breast cancer, the *ERBB2* gene set is amplified in 13.5% of 482 cases. In addition, 6.3% of 127 cases in bladder urothelial carcinoma carried mutations in the *ERBB2* gene set, indicating that the differences of *ERBB2* gene alterations depend on the cancer types. *(Adapted from http://www.cbioportal.org, Cerami et al., Cancer Discov. 2012 & Gao et al. Sci. Signal. 2013.)*

1.2.3.2 Activation mechanisms of HER2 receptor

As indicated in Figure 1.2, the alternation of the *ERBB2/HER2* gene set occurs not only in breast cancer but also in other cancers, such as bladder cancer. De-regulation of the

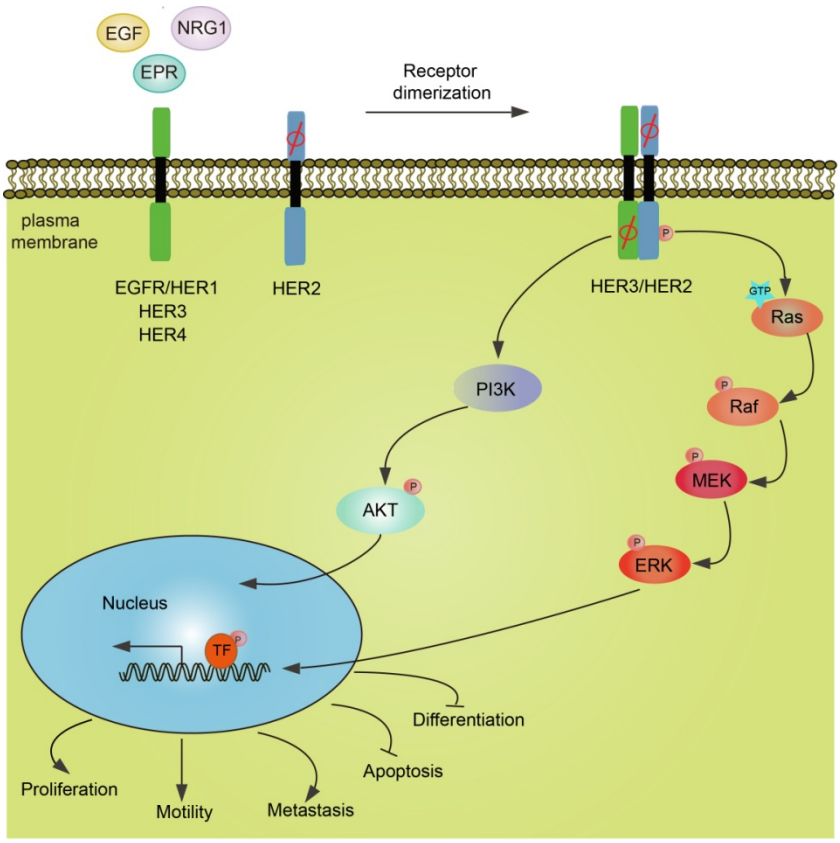

Figure 1.2 Schematic representation of HER receptors-mediated downstream signaling pathways.

Upon ligands binding, HER receptors dimerize in the plasma membrane. Notably, among these receptors, no ligands of HER2 have been determined, and the intracellular kinase domain of HER3 is impaired. But the dimerization of receptors (HER2/HER3) could induce phosphorylation of tyrosine residues in the cytoplasmic domain and thereby drives amplification of the MAPK and the PI3K pathways. Diverse signaling pathways converge in the nucleus. For instance, ERK1/2 translocate into the nucleus and phosphorylate some transcription factors, leading to cell proliferation, motility, and metastasis. Alternatively, cell apoptosis and cell differentiation are inhibited.

ErbB2/HER2 receptor by overexpression or mutation induces hyper-activation of the ligand-independent receptor through spontaneous receptor dimerization or allow a cell to hyper-respond to a low concentration of circulating growth factors (Yarden, 2001). Being upstream (inducer) of the RTK signaling cascade, activated HER2 receptor drives several downstream effector activations and amplifies signal transduction, leading cells to proliferate, migrate and invade. Based on the crucial function of HER2 in tumorigenesis, the understanding of the mechanisms of how HER2 functions is essential for cancer therapy innovation.

After receptor dimerization, tyrosine residues of RTKs in the cytoplasmic domain become auto-phosphorylated leading to HER2-mediated complex formation. Src-homology (SH) 2 domains present in binding partners play crucial roles in HER2 association. One such example is p85, which binds to the pYXXM motif of HER2, through its SH2 domain. Moreover, Grb2 and phospholipase C (PLC) through their SH2 domains associate with specific phospho-tyrosine sites in HER2 (Yamazaki *et al.*, 2002). Phosphotyrosine binding (PTB) domains presented in some adaptor proteins are also responsible for HER2 interactions. For instance, Shc through PTB domains interacts with the NPXpY motif in HER2 (Batzer *et al.*, 1995).

Even though the HER2-associated adaptor proteins Grb2 and p85 lack catalytic activities, they can still amplify the activation of HER2-mediated signaling cascades through binding to diverse enzymes such as the guanylnucleotide exchange factor (SOS) and the phosphatidylinositol-4,5-bisphosphate 3-kinase (p110), separately. It has been shown that Grb2 through its SH3 domains constitutively binds to SOS. Upon recruitment to the activated receptor, Grb2 acts as a bridge linking activated receptor to the downstream signaling apparatus (Margolis and Skolnik, 1994). Alternatively, activated HER2 induces PI3K/Akt signaling activation through p85 recruitment (De Luca *et al.*, 2012). p85 is a regulatory subunit of PI3K, which constitutively binds to the catalytic subunit of PI3K (p110). Upon binding to the receptor, p85 PI3K recruits p110 PI3K to the plasma membrane where p110 promotes phosphatidylinositol (3,4,5)-trisphosphate (PIP3) production leading to the activation of Akt (Hu *et al.*, 1992).

1.3 The Ras/MAPK/RSK signaling pathway

1.3.1 Background: Mechanisms of MAPK cascade activation

Mitogen-activated protein kinases (MAPKs) are Ser/Thr kinases that transmit extracellular ligand-binding events to intracellular signaling transduction cascades, resulting in diverse cellular functions, such as cell growth, proliferation, survival and differentiation. In mammals, 14 MAPKs have been identified and divided into 2 types: conventional MAPKs and atypical MAPKs (Figure 1.3). Conventional MAPKs consist of four groups: the extracellular signal-regulated kinase1/2 (ERK1/2), p38 isoforms (α, β, γ and δ), c-Jun N-terminal kinases1/2/3 (JNK1/2/3), and ERK5 (Cargnello and Roux, 2011). Atypical MAPKs comprise ERK3/4, ERK7, and Nemo-like kinase (NLK), which have not been well documented.

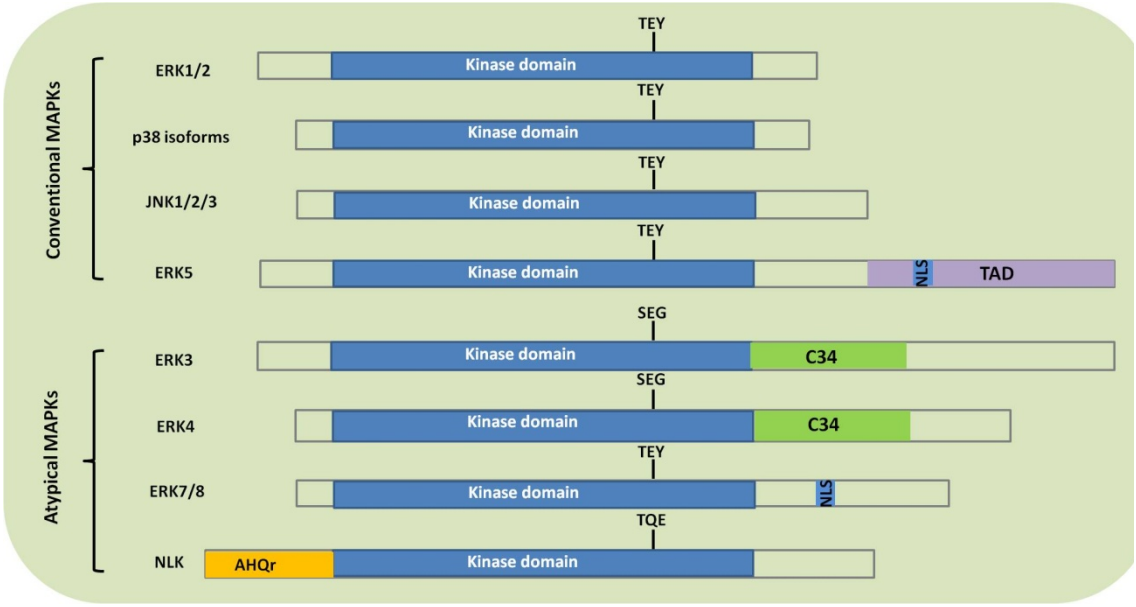

Figure 1.3 Schematic representation of two classifications of MAPKs: conventional MAPKs and atypical MAPKs.

All MAPKs contain the kinase domain in the middle, as shown in blue. Additional regions as indicated: TAD: a transactivation domain; C34: a region conserved in ERK3 and ERK4; AHQr: a domain rich in Ala, His, and Glu. Notably, two putative nuclear localization sequences (NLS) are present in ERK5 and ERK7/8. *(Adapted from Microbio.l Mol. Biol. Rev., 2011 Mar;75(1):50-83)*

Each conventional MAPK cascade consists of at least three conserved, successively acting kinases: a MAP kinase kinase kinase (MAP3K or MEKK), a MAP kinase kinase (MAP2K or MEK) and a MAP kinase (MAPK). The MAP3K is often activated by small GTP-binding proteins of the Ras/Rho family, such as Ras or Rap (Chong *et al.*, 2003). Phosphorylated MAP3K emits a signal to MAP2K, a dual-specificity kinase, which phosphorylates both Thr and Tyr residues present in the activation loop of most MAPKs. Once phosphorylated and activated, the MAPK phosphorylates numerous substrates either in the cytoplasm or the nucleus (Deschenes-Simard *et al.*, 2014).

In some respects, atypical MAPKs behave differently from conventional MAPKs. Compared to the three-tiered conventional MAPK signaling cascades, it seems that atypical

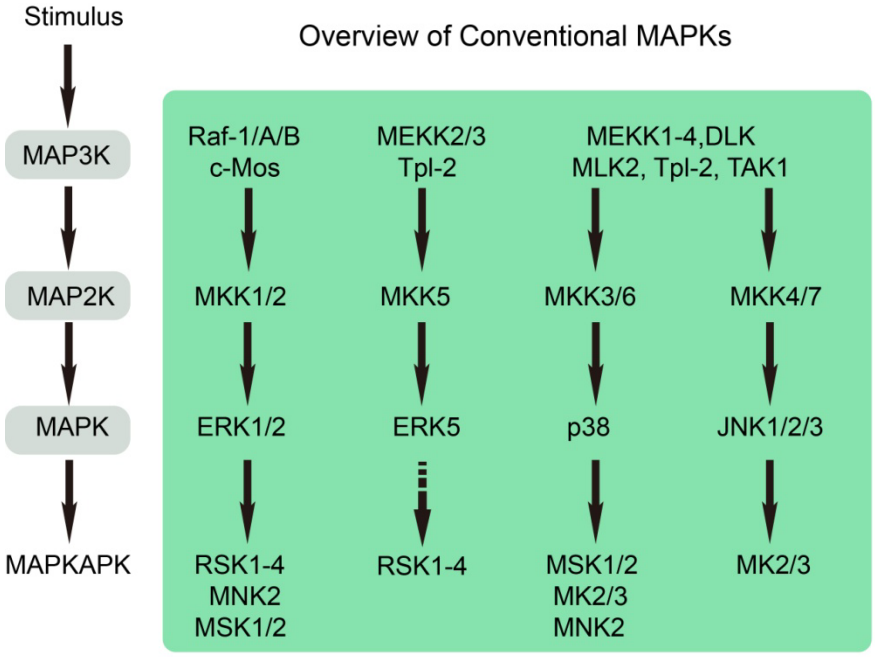

Figure 1.4 Activation of the conventional MAPK-mediated signaling pathways results in the phosphorylation of MAPKAPKs.

MAPK signaling cascades are triggered upon stimulation by the diverse growth factors, cytokines and cellular stress. MAPKAPKs include five subgroups: RSK, MNK, MSK, MK2/3 and MK5. Each MAPKAPK is phosphorylated by its respective upstream kinase. *(Adapted from Journal of Molecular Signaling 2012 7:9* and *from Microbiol. Mol. Biol. Rev., 2011 Mar; 75(1):50-83.)*

MAPKs are organized into two-tiered kinase cascades. In addition, atypical MAPKs have no conserved T-X-Y motif in their activation loop (Figure 1.3). It appears that these atypical MAPKs have only one residue in the kinase domain to be phosphorylated. For instance, ERK3 and ERK4 have an S-E-G motif, while NLK contains a T-Q-E motif (Deleris *et al.*, 2010; Ishitani *et al.*, 2010). Although ERK7 has a T-X-Y motif in its kinase domain, it is auto-phosphorylated by ERK7 rather than an upstream MAP2K (Abe *et al.*, 2001).

Accumulating evidence has shown the involvement of scaffold proteins, which hold the components of three-tiered kinase cascades together and keep the kinase close to its substrate, which is essential for maintaining the magnitude and duration in the conventional MAPKs signaling pathway. For example, in the ERK1/2 signaling cascade, the kinase suppressor of Ras (KSR) is responsible for the assembly of Raf, MEK1/2 and ERK1/2, and the MEK partner1 (MP1) holds MEK1/2 and ERK1/2 together (Therrien *et al.*, 1995; Sharma *et al.*, 2005). In JNK signaling, the JNK-interaction protein 1 (JIP1) holds MLK, MKK7 and JNK1-3 together (Yasuda *et al.*, 1999). In addition, β–arrestin 2 serves as a scaffold protein for both the ERK1/2 and JNK pathways. In the former signaling pathway, β–arrestin 2 directly interacts with Raf and ERK1/2 but indirectly binds to MEK1/2; in the latter signaling cascade, β–arrestin 2 assembles ASK1, MKK4 and JNK3 together (Tohgo *et al.*, 2002; Guo and Whitmarsh, 2008).

Once phosphorylated and activated, MAP kinases phosphorylate substrates on Ser/Thr-Pro residues, which is a common characteristic of conventional and atypical MAPKs. The diverse consequences of MAPK activation in an individual cell are eventually determined by the specific substrates involved in the different cellular processes. Most notably, among these substrates, phosphorylation of the MAPK activated protein kinases (MAPKAPKs) is the additional enzymatic and amplification step of MAPK signaling. As indicated in Figure 1.4, MAPKAPKs include the p90 ribosomal S6 kinase (RSKs), mitogen- and stress-activated kinase (MSKs), MAPK-interacting kinases (MNKs), MAPK-activated protein kinase 2/3 (MK2/3), and MK5 (Chung *et al.*, 1991; Cargnello and Roux, 2011).

1.3.2 The ERK1/2 module

1.3.2.1 Identification of ERK1/2 and their physiological functions

The cDNAs of ERK1 and ERK2 were cloned in the early 1990s, and their products were originally found to be phosphorylated on Tyr and Thr residues upon growth factor stimulation (Boulton *et al.*, 1990; Boulton *et al.*, 1991). The amino acid identity between human ERK1 and ERK2 is 84%. Given the high similarity of sequences between ERK1 and ERK2, these two proteins are traditionally designated as ERK1/2. ERK1 and ERK2 are co-expressed widely in many cells and tissues, with high levels in the brain, skeletal muscle, thymus and heart (Boulton *et al.*, 1991). Whereas the activation of ERK1 and ERK2 are induced in parallel upon stimulation, few reports indicated that each ERK isoform could also be selectively phosphorylated (Papkoff *et al.*, 1994). Moreover, other studies have shown that activation of ERK1 and ERK2 result in different biological events. For instance, in MCF-10A cells, Shin *et al.* observed that ERK2 (not ERK1) was required in the Ras-induced EMT process (Shin *et al.*, 2010). Consistent with these results, it was also found that *erk1*-knockout mice (*erk1*$^{-/-}$) could survive with a defect in thymocyte development, whereas *erk2*-null mice (*erk2*$^{-/-}$) were embryonic lethal due to the severely impaired development of the placental vasculature (Pages *et al.*, 1999). Why did *erk1*$^{-/-}$ mice and *erk2*$^{-/-}$ mice have such different phenotypes? Were they due to the different isoform functions or expression levels? These were addressed by Lefloch *et al.* in a series of gene ablation experiments (Lefloch *et al.*, 2008). It was shown that only a single *erk* allele expressed in mouse, regardless of *erk1* or *erk2*, resulted in a lethal phenotype rather than the mice with two single alleles separate from *erk1* and *erk2*, indicating that a particular dosage of *erk* is necessary for mouse survival. Given that ERK2 expression is higher than ERK1 in most cells, the unfavorable outcome of *erk2* knockout mice may be due to the diminished total ERK content instead of its unique biological functions. Even though several studies have reported different pathological roles of ERK1 and ERK2, these two isoforms are still considered to possess the same functions in most biological processes.

1.3.2.2 Activation mechanisms and inhibitors of ERK1/2

ERK1/2 are core components in the Ras/MAPK pathway, which regulate multiple biological processes such as cell proliferation, differentiation and migration. ERK1/2 are activated in response to different growth factors, cytokines, osmotic stress, hormones and microtubule disorganization (Xing *et al.*, 1998; Conway *et al.*, 1999; Naor *et al.*, 2000; Komis

et al., 2011). The activation of ERK1/2 is caused mainly by a receptor on the plasma membrane, such as receptor tyrosine kinases (RTKs) or heterotrimeric G protein-coupled receptors (Porter and Vaillancourt, 1998). For instance, upon a ligand binding, tyrosine kinase receptors (RTKs) are activated by trans-phosphorylation on tyrosine residues in the intracellular kinase domain. The subsequent trans-phosphorylation of RTKs leads to the recruitment of various adaptor proteins, such as Src homology and collagen (Shc), growth factor receptor-bound protein 2 (Grb2) and phospholipase C-γ (PLCγ), which contain Src homology 2 (SH2) domains or phosphotyrosine-binding (PTB) domains. One of the well-characterized mechanisms of RTK-induced Ras activation is mediated by the Grb2-SOS complex. Upon RTK activation, the guanine-exchange factor SOS is recruited to the plasma membrane from the cytosol by constitutive association with Grb2 (Pruett *et al.*, 1995). Membrane-bound SOS promotes Ras activity by catalyzing GDP/GTP exchange. Once bound to GTP and activated, two "switch domains" in Ras shift enabling Ras to interact with several downstream effectors, such as Raf. Furthermore, this physical association leads to Raf phosphorylation. Activated Raf transmits signals by phosphorylating the downstream effectors MEK1/2. MEK1/2 are dual-specificity protein kinases since they can phosphorylate substrates on both Thr and Tyr residues. As the only substrate of MEK1/2, ERK1/2 are phosphorylated by MEK1/2 on the Thr-Glu-Tyr motif, which is required for ERK1/2 activation. This phosphorylation also induces an allosteric conformational change in ERK1/2, leading to MEK1/2 dissociation. Once phosphorylated and activated, ERK1/2 either translocate into the nucleus or stay in the cytoplasm, where they can phosphorylate many substrates such as transcription factors or cytoskeletal proteins.

Appropriate regulation of Ras/Raf activity is essential for the proper maintenance of cell proliferation. Abnormal regulation of Ras/Raf leads to the hyperactivation of the MAPK pathway and cause many human diseases such as cancer. Many studies have already shown that mutation of *KRAS* or *BRAF* occurs in a wide range of cancers, including pancreatic, colorectal cancer, biliary cancer, melanoma, thyroid cancer and ovarian cancer (Brose *et al.*, 2002; Lee *et al.*, 2003). Given its diverse modes of action in cancer development, a specific blockade of the ERK1/2 cascade by inhibitors represents an attractive target in cancer therapy. Since ERK1/2 are the only substrates of MEK1/2, MEK1/2 inhibitors are used to characterize

ERK1/2 function in a wide range of biological contexts. Unlike other kinase inhibitors, most of the MEK1/2 inhibitors are non-competitive ATP molecules such as PD184352 and U0126, which interact more strongly with an inactive form of MEK rather than an active form (Favata *et al.*, 1998; Allen *et al.*, 2003). This interaction blocks conformational transition of MEK1/2 and thus prevents their enzymatic activities.

One of the conundrums in cancer treatment is that a few cancer cells often survive and are resistant to chemotherapy, leading to a failure of the treatments. For example, B-Raf inhibitors can cause drug resistance in patients by re-activating MAPK signaling. Therefore, the combination of B-Raf and MEK inhibitors in cancer treatment could be more efficient than either alone. For instance, co-treatment of trametinib (the first MEK inhibitor in clinical treatment) and dabrafenib (a $BRAF^{V600E}$ inhibitor with ATP competitor), which was approved by the FDA in January of 2014, sustains melanoma patients progression-free survival (PFS) up to 9.4 months, whereas the average PFS of only trametinib-treated patients is 5.8 months (Menzies and Long, 2014).

1.3.2.3 Docking interactions from ERK1/2 to substrates

In an individual cell, various molecules, independently or coordinately, fulfill their specific actions at a particular time and place. To avoid unfavorable outcomes, these molecules must be precisely arranged and regulated. As such, appropriate partner selection becomes one of the fundamental mechanisms to keep the high efficiency and fidelity of signal transduction. To date, several mechanisms underlying the precise signal transmission in the ERK-MAPK cascade have been determined. One such example is the docking interaction, which is achieved by the common docking domain (the CD domain) in ERK1/2 (Tanoue *et al.*, 2001). It has been shown that the CD domain, which locates outside of the catalytic region of MAPKs and consists of negatively charged (acidic residues) and hydrophobic amino acids, is crucial for forming electrostatic and hydrophobic interactions with substrates (Tanoue *et al.*, 2001). In addition, it has been found that the ED/TT motif in ERK1/2 forms a docking grove with the CD domain, and thus participates in the association with partner molecules (Tanoue *et al.*, 2001).

A docking interaction is unlike a transient enzyme-substrate interaction, which occurs in

the active center. Indeed, it takes place outside of the kinase domain. For instance, MEK1 interacts with ERK1/2 through the N-terminus of MEK1/2, outside of its kinase domain. RSK binds to ERK1/2 through the C-terminus of RSK, away from its kinase domain. Given that one of the predominant functions of docking interactions is to maintain the specificity of kinase-substrate interactions, ERK2 associates with its specific phosphatase MKP-3 through a docking interaction (Camps *et al.*, 1998), while JNK similarly interacts with another phosphatase, MKP-7 (Tanoue *et al.*, 2001).

Consistently, it has been shown that ERK1/2 associate with their substrates through two independently specialized docking domains present in substrates: a D-domain and a DEF-domain (Tanoue and Nishida, 2003). Notably, some substrates possess one domain or both domains, while others have neither domain. Since the docking interaction plays an important role in maintaining the fidelity and efficiency of the ERK-MAPK signaling pathway, the main features of docking interactions are discussed below.

D-domain. The classical consensus docking motif, D-domain, comprises a core of positively charged amino acids followed by hydrophobic amino acids: $(R/K)_{2-3}$-X_{2-6}-Φ-X-Φ (Fantz *et al.*, 2001) (Figure 1.5A). To date, it has been found that the D-domain exists widely in ERK1/2 activators, inactivators as well as the substrates, such as MEK1/2 (ERK1/2 activators), MKP-1 and STEP (ERK1/2 inactivators) and ELK1 (ERK1/2 substrates) (Tanoue and Nishida, 2003). When the positively charged amino acids or the hydrophobic amino acids are substituted by neutral ones, both the interaction between ERK1/2 and the substrates and the efficiency of the enzymatic reactions are severely decreased, indicating that the D-domain is required for MAPK-mediated transfer of phosphate group to protein substrates.

KIM domain. The KIM (kinase interaction motif) domain is a subgroup of D-domains, which contains a hydrophobic amino acid followed by two positively charged amino acids, likely Lys or Arg. As indicated in Figure 1.5, RSK binds ERK to via its KIM domain, which consists of **L/V**XX**RR/KK**XXXXX**L**. KIM domains are often found in the MAPKAPKs family members. However, MK5 is an exceptional because a region responsible for ERK3/4 interactions does not match neither the classical D-domain, nor the KIM domain (Kant *et al.*, 2006).

DEF domain. The second major docking motif in MAPK substrates is referred to as the DEF domain (docking site for ERK, FXF). The DEF domain is characterized as an F/YXF/YP consensus motif. It appears that the DEF domain exists in certain MAPK substrates, such as transcription factors Elk1, c-FOS, Lin1, but not in MAPKAPKs (Jacobs *et al.*, 1998; Murphy *et al.*, 2002; Burkhard *et al.*, 2010). In addition to ERK1/2, p38α also binds to the DEF domain of substrates, such as SAP-1(transcription factor) (Galanis *et al.*, 2001).

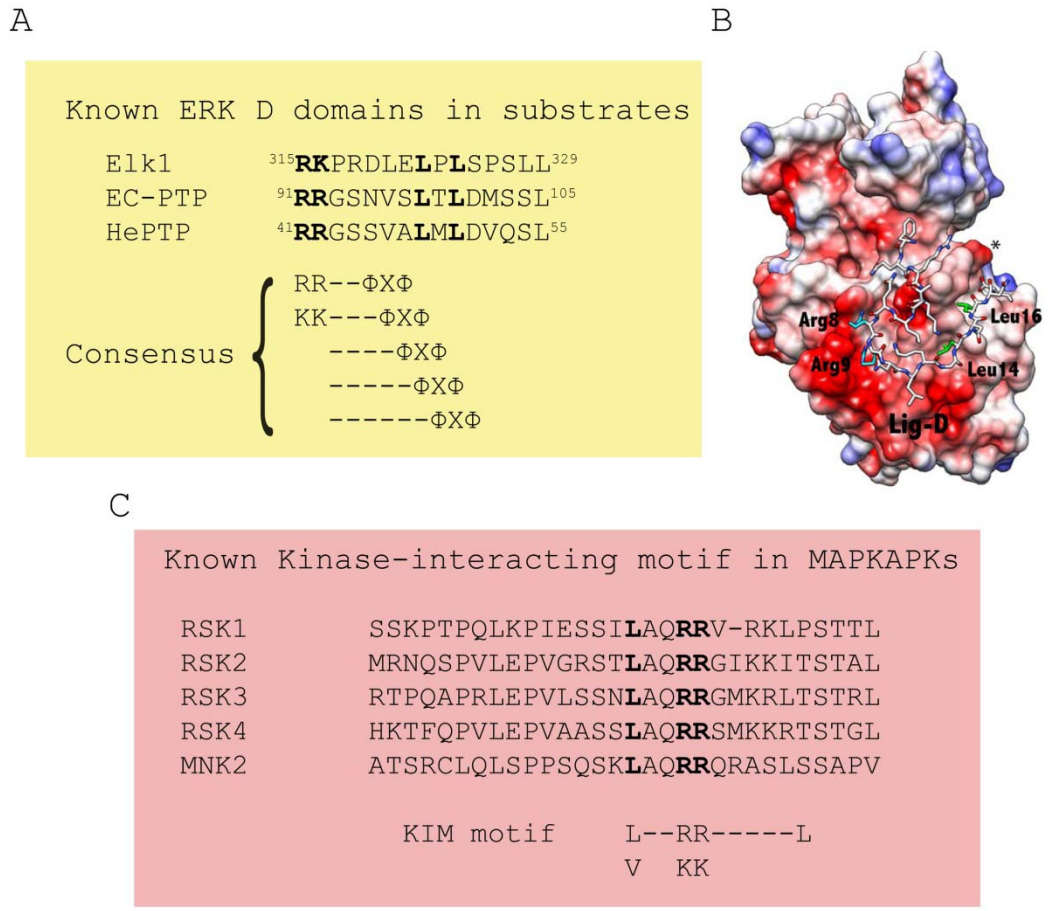

Figure 1.5 Alignment of MAPK-binding domains in substrates and molecular model of Lig-D bound to ERK2.

A. Known ERK1/2 D-domains in substrates. The D-domain consists of positively charged amino acids followed by hydrophobic amino acids: $(R/K)_{2-3}$-X_{2-6}-Ψ-X-Ψ. This motif has been found to be conserved in different substrates such as Elk1, EC-PTP and HePTP; B. Molecular structural model of D-domain peptide ligand bound to ERK2. Lig-D (a peptide with FQRKTLQ**RR**NLKG**L**N**L**NL) bound to the DRS of ERK2. The two hydrophobic Leu of Lig-D bind to hydrophobic sites of the CD-domain in ERK2. *(Adapted from PLoS One. 2011 Apr 11;6(4):e18594)(Lee et al., 2011).* C. KIM, a D-domain like sequence in MAPKAPKs is responsible for ERK/MAPKAPKs interaction.

1.3.2.4 Subcellular localization of the ERK1/2 cascade and its biological functions

The ERK1/2 module converts signals from extracellular stimuli into intracellular

responses, and induces different biological events, such as cell proliferation, cell motility and cell differentiation. However, one conundrum remains to be answered: how activation of the same kinases ERK1/2 induces such different biological events. Accumulating evidence has demonstrated that the duration, magnitude and compartmentalization of activated ERK1/2 can affect cell fates. Current models suggest that there are five potential mechanisms of ERK1/2-mediated diverse biological functions: (i) difference in the duration and strength of ERK activity; (ii) interaction with various scaffold proteins; (iii) localization in the different compartments of the cell; (iv) regulation of a wide range of substrates; and (v) cross-regulation between diverse signaling pathways (Wortzel and Seger, 2011). In the following section, the first four mechanisms are described on the basis of ERK1/2 localization in different subcellular compartments. Since the last one is more crucial for clinical research and drug development, it will be addressed in more detail in an independent section below.

1.3.2.4.1 ERK1/2 in the nucleus and cell proliferation

Although most ERK1/2 molecules are located in the cytoplasm of quiescent cells, they are able to shuttle between the cytoplasm and the nucleus. Upon stimulation by serum, Chen *et al.* observed that a large proportion of ERK1/2 translocated into the nucleus within 10 to 20 minutes (Chen *et al.*, 1992). Unlike classical mechanism, it was initially thought that ERK1/2 contain neither an NLS (nuclear localization signals) nor an NES (nuclear export signals), and cannot freely diffuse into the nucleus via nuclear membrane pores because only small molecules and proteins can penetrate (up to 40 kDa) this barrier (Nardozzi *et al.*, 2010). Binding of ERK1/2 to the NPC (nuclear pore complex) directed by energy-dependent transport machinery was one of the previous explanations (Ranganathan *et al.*, 2006). In 2008, a novel nuclear transport signal (NTS) consisting of the Ser-Pro-Ser residues was identified in ERK1/2 (Chuderland *et al.*, 2008). Phosphorylation of this sequence leads to ERK1/2 tightly binding to importin7 and further translocation into the nucleus (Lorenzen *et al.*, 2001; Chuderland *et al.*, 2008). Most notably, translocation of MEK1 and SMAD3 into the nucleus also is mediated by this residue (Chuderland *et al.*, 2008).

The nuclear translocation of ERK1/2 plays a crucial function in a wide range of cellular processes. Robinson *et al.* demonstrated that ERK2 was required for differentiation in PC-12 cells and transformation in NIH-3T3 cells, respectively (Robinson *et al.*, 1998). More

interestingly, after maintaining ERK1/2 in the cytoplasm by expression a catalytically inactive form of cytoplasmic MAP kinase phosphatase (MKP-3/Pyst-1), Brunet *et al.* observed that Elk1-dependent gene transcription was strongly inhibited as well as cell cycle re-entry (Brunet *et al.*, 1999). Whereas ERK1/2 activity and their ability to phosphorylate substrates in the cytoplasm, such as RSK, were not altered, suggesting that the nuclear localization of ERK1/2 is required for cell cycle-related gene transcriptions and cell division (Brunet *et al.*, 1999).

As mentioned above, ERK1/2 phosphorylate a number of substrates, most of which are in the nuclear. In general, the consequences of ERK1/2 acting on the nucleus substrates are: (1) modulation of transcription factor activities: positively or negatively (i.e., Elk-1, Erf-1); (2) impacting chromatin remodeling (i.e., PARP-1 regulation); (3) promoting substrate nuclear translocation (i.e., NUP50) (Babu *et al.*, 2000; Le Gallic *et al.*, 2004; Cohen-Armon, 2007; Kosako *et al.*, 2009). One of the best characterized actions of ERK1/2 in the nucleus is that they are involved in cell cycle progression as well as cell proliferation. On the one hand, ERK1/2 modulate the activity of the transcription factor Elk-1, which controls the expression of immediate early genes (IEG), such as c-Fos. Alternatively, ERK1/2 directly phosphorylate c-Fos leading to its stabilization. Stabilized c-Fos is involved in transcriptionally active AP-1 complex formation through interaction with c-Jun (Wang and Prywes, 2000). It has been shown that AP-1 is an essential transcription factor that controls the expression of *cyclinD1*, which interacts with cyclin-dependent kinases(CDKs) and allows G1/S transition (Shaulian and Karin, 2001). In addition, ERK1/2 modulate several mediators of apoptosis in the nucleus. FOXO3a (Forkhead box O 3a) is a transcription factor upregulating several tumor suppressor gene expressions, such as *Bim (BCL2-like 11, apoptosis facilitator)* and *PUMA (p53 upregulated modulator of apoptosis)*. It was found that ERK1/2 phosphorylated FOXO3a, thereby promoting MDM2-dependent FOXO3a degradation and leading to robustly upregulated cell proliferation (Yang *et al.*, 2008).

1.3.2.4.2 ERK1/2 in the plasma membrane and regulation of cytoskeletal elements

The first step in transmitting signals from extracellular stimuli into intracellular targets occurs at the plasma membrane. In response to stimuli, many components of the signaling

cascades shuttle at the plasma membrane where they receive inputs in response to extracellular signals. In this process, scaffold proteins play crucial functions during signal transduction, such as KSR (kinase suppressor of Ras) and 14-3-3 (Therrien *et al.*, 1995; Fanger *et al.*, 1998). KSR was the first identified scaffold protein in the ERK1/2 cascade. In quiescent cells, KSR constitutively binds to the inactivated MEK1/2. Meanwhile, KSR also associates with 14-3-3 via two phosphoserine residues ($Ser^{297/392}$) and thereby retains KSR in the cytoplasm (Xing *et al.*, 1997). Upon activation by agonists, protein phosphatase-2A (PP2A) dephosphorylates KSR leading to 14-3-3 disassociation and KSR tethering at the plasma membrane, where it forms a complex with Raf initiating the activation of MEK1/2. Furthermore, activated MEK1/2 phosphorylate ERK1/2, which in turn modulate a multitude of biological functions, including cell proliferation.

ERK1/2 participate in the regulation of cell spreading and cell migration by modulating cytoskeletal components. ERK1/2 can bind either directly or indirectly to cytoskeletal elements. It was shown that ERK1/2 not only directly bind to actin or microtubules, but also indirectly interact with them through association with the actin-binding protein calponin (Appel and Morgan, 2010). In addition, it was found that activated ERK1/2 phosphorylate myosin light chain kinase (MLCK), which in turn phosphorylates myosin light chains (MLC) leading to actin-based cell motility (Klemke *et al.*, 1997).

Scaffold proteins are also involved in the mechanism by which ERK1/2 affect cell migration through regulating cytoskeletal components. Given that scaffold proteins tether the components of the signaling cascade to a particular cellular location, it seems that the involvement of scaffold proteins is an additional way to ensure the fidelity and magnitude of the ERK1/2 cascade. As a scaffold protein, Ras GTPase-activating-like protein IQGAP1 interacts with B-Raf, MEK1/2 and ERK1/2, and recruits them to actin filaments, resulting in cell proliferation (Jadeski *et al.*, 2008). Another scaffold protein, LSP1, enhances ERK1/2 signals leading to inhibited cell apoptosis as a result of tethering MEK1 and ERK2 to peripheral actin filaments (Huang *et al.*, 1997). Moreover, paxillin, a focal adhesion-associated adaptor protein, associates with ERK1/2 and guides them to the cytoskeleton. In mIMCD-3 (mouse inner medullary collecting duct) cells, it was found that activated Raf and MEK bound to paxillin and further induced activation of ERK1/2 by

cell-matrix interactions upon HGF stimulation (Ishibe *et al.*, 2003). Disruption of ERK/paxillin interaction decreased HGF-induced cell spreading and branching, indicating that the association of these two proteins is crucial for HGF-induced epithelial morphogenesis. Consistent with these results, it has been shown that the depletion of paxillin downregulates ERK1/2 activity in human oral squamous carcinoma HSC-4 cells, suggesting that paxillin is indispensable in the ERK-MAPK signaling cascade (Takino *et al.*, 2010).

1.3.2.4.3 ERK1/2 in endosomes and signal transduction

The predominant functions of endosomes are to sort molecules and proteins for degradation, re-cycling, and signal transduction. Previous studies have shown that endocytosis and endosomes are crucial for proper receptor-signal transduction via the ERK1/2 module (Lavoie *et al.*, 2002). More specifically, endocytosis of the GPCR β2-adrenergic receptor is necessary for ERK1/2 activity, which occurs in the early endosomes (Lavoie *et al.*, 2002). In this process, scaffold proteins, such as β-arrestin, are indispensable for ERK1/2 activity. For instance, β-arrestin associates with ERK1/2 and directs them to clathrin-coated endosomes upon stimulation (Zhang *et al.*, 1997). This irreversible association between activated ERK1/2 and β-arrestin retains ERK1/2 in the cytoplasm, where they phosphorylate cytoplasmic substrates instead of nuclear target proteins. Aside from β-arrestin, another scaffold protein MP1 (MEK-1 partner) facilitates ERK1/2 localization in endosomes (Schaeffer *et al.*, 1998). Intriguingly, MP1 preferentially associates with specific MEK or ERK isoforms. In late endosomes, MP1 interacts with MEK1 and ERK1 rather than MEK2 and ERK2. It was shown that MP1 interaction with p14, an adaptor protein, enhanced ERK1-induced cell proliferation (Teis *et al.*, 2006). Downregulation or upregulation of p14/MP1 leads to reduced or increased activation of the ERK1/2-cascade. An alternative view suggests that ERK1/2 regulate endosomal trafficking through ADP-ribosylation factor 6 (Arf6) GTPase-regulated pathway, rather than the clathrin-dependent mechanisms. It was shown that inhibition of MEK1/2 by U0126 prevents Arf6 cargo expansion, correlating with accumulation of ERK1/2, MEK and KSR in the Arf6 recycling compartment (Robertson *et al.*, 2006).

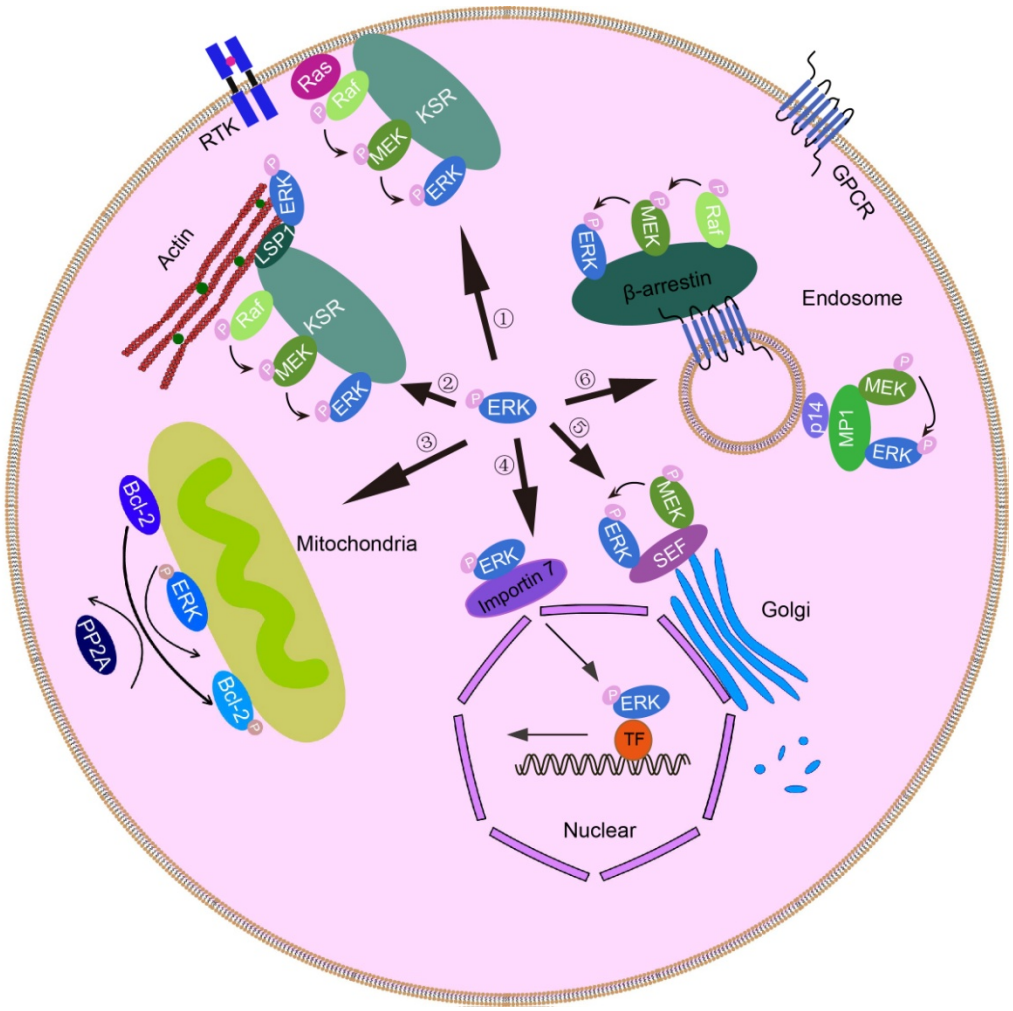

Figure 1.6 Simplified overview of phosphorylated ERK1/2 (p-ERK1/2) and their substrates localization in distinct cellular compartments.

① Upon RTK activation, ERK1/2 is phosphorylated by the Ras/Raf/MEK cascade, which is present at the plasma membrane; ② p-ERK1/2 bind to cytoskeletal elements through a scaffold protein termed LSP1; ③ Some p-ERK1/2 translocate to the mitochondria, where they phosphorylate the anti-apoptotic regulator Bcl-2; ④ Most p-ERK1/2 shuttle into the nucleus and affect transcription factor activities; ⑤ p-ERK1/2 binds to Sef, leading to ERK1/2 retention in the cytoplasm; ⑥ p-ERK is involved in GPCR recycling (more detail in the text). *(Adapted from Genes Cancer. 2011 Mar; 2(3):195-209)(Yu et al., 2011)*

Although a report indicates that ERK1/2 are involved in endosomal protein trafficking, it appears that one of the predominant functions of the endosome is to participate in the regulation of ERK1/2 activity and direct them to their proper substrates in the cytoplasm.

Notably, in the RTK and GPCR-mediated signaling pathways, a different set of scaffold/anchor proteins cooperate with ERK1/2 to maintain the specificity and efficiency of intracellular signal transduction.

1.3.2.4.4 ERK1/2 in other cellular compartments

In contrast to phosphorylation-dependent events, most functions within the Golgi apparatus involve glycosylation and protein trafficking. GRASP55 is responsible for Golgi cisternal stacking and cell migration and can be targeted by ERK1/2 (Jesch *et al.*, 2001; Wei and Seemann, 2009). It was shown that GRASP55 was phosphorylated by ERK1/2 at Ser^{277} leading to the unstacking of Golgi cisternae, which is crucial for polarization of the Golgi, centrosome orientation and cell migration. Thus, these results indicate that ERK1/2 play a key role in the modulation of Golgi remodeling. In addition, the process of Golgi fragmentation, which is considered as a "mitotic checkpoint", is regulated by the ERK1/2 cascade (Colanzi and Corda, 2007). Previous reports indicated that MEK1/2 inhibitors were able to inhibit Golgi fragmentation, suggesting that the ERK1/2 cascade is involved in the process of Golgi fragmentation. However, neither active ERK1/2 nor MEK1/2 have been detected in the Golgi. Furthermore, Inbal *et al.* demonstrated that monophosphorylation of the ERK1 splice variant ERK1c accumulated in the late G2 and mitosis of synchronized cells (Shaul and Seger, 2006). Since ERK1c was activated by MEK1b, depletion of either MEK1b or ERK1c had the same function in the reduction of mitotic Golgi fragmentation and preventing mitotic progression. Therefore, these results demonstrate that the activity of the MEK1b-ERK1c cascade is crucial for Golgi fragmentation during mitosis. Moreover, scaffold proteins are required in Golgi-mediated ERK1/2 signaling cascades. One such scaffold protein is Sef1, which is located on the outer surface of the Golgi and interacts with inactivated ERK1/2 in resting cells (Torii *et al.*, 2004). Upon stimulation, Sef1 associates irreversibly with activated MEK1/2, and inhibits activated ERK1/2 nuclear translocation. Silencing of Sef1 leads to the accumulation of ERK1/2 in the nucleus and the upregulation of ERK1/2-mediated gene transcription, such as *ELK1* (Torii *et al.*, 2004). Taken together, the MEK1/2-Sef1-ERK1/2 complex on the outer surface of Golgi apparatus allows activated ERK1/2 to remain in the cytoplasm instead of the nucleus.

The function of the mitochondria is to integrate signals from the environment/cellular

stimuli leading to a wide range of biological events, such as energy production, apoptosis (programmed cell death) and cellular metabolism. By using pharmacological inhibitors of MEK1/2, it was found that the ERK1/2 cascade was able to modulate mitochondrial functions such as cell survival (Erhardt *et al.*, 1999). In cisplatin-treated cells, retention of ERK1/2 in mitochondria inhibits oxidative phosphorylation leading to the upregulation of caspase-3 activity and cell apoptosis (Nowak, 2002). Moreover, ERK1/2 colocalize and phosphorylate the anti-apoptotic mitochondrial protein Bcl-2, thereby suppressing cell apoptosis. Notably, this process can be reversed by serine/threonine phosphatase 2 (PP2A) (Breitschopf *et al.*, 2000).

Localization of ERK1/2 to the mitochondria is important for developmental brain activities (Alonso *et al.*, 2004). In mice model, it was shown that the peak level of ERK1/2 in mitochondria was at from stage E19 to P2, then decreased from P3 to adulthood (Alonso *et al.*, 2004; Wortzel and Seger, 2011), whereas the maximal presence of ERK1/2 in the nuclear occurred after stage P3. Given that the level of nuclear ERK1/2 is variable during brain development and ERK1/2 localization in mitochondria is sensitive to redox regulation (Chu *et al.*, 2004), these results suggest that ERK1/2 nuclear localization induced by mitochondrial energetic and redox status is crucial for cell differentiation and proliferation. In addition, it was shown that activated ERK1/2 in mitochondria protects cancer cells from death (Rasola *et al.*, 2010). However, how do ERK1/2 localize to the mitochondria, and which transporter protein is involved remain unknown. Although there is no convincing mechanism for ERK1/2 translocation into mitochondria, proteomic study by Soledad *et al.* demonstrated that VDAC1 might serve as a transporter protein to facilitate ERK1/2 localization to the mitochondria (Galli *et al.*, 2009).

1.3.2.5 Regulation of the ERK1/2 cascade by feedback loops

Activation of the Ras/MAPK pathway leads to different cellular outcomes. For example, activation of the same protein kinases ERK1/2, can elicit different cellular responses, such as cell proliferation *vs.* cell differentiation. This cannot be simply explained by the specificity of cell type, because even in the same cell type ERK1/2 activation results in distinct outcomes in response to different agonists. Accumulating evidence is demonstrating that the duration and the magnitude of ERK1/2 activity in response to different agonists lead to discrete biological

events. Modulation of Ras/MAPK signaling by feedback loops might be one of the explanations for these discrepancies (Mendoza *et al.*, 2011). Given that oncogenes upstream of the MAPK pathway, such as *K-Ras* and *B-Raf*, lead to the hyperactivation of ERK1/2 module and cause diseases such as cancers, a understanding of the regulatory mechanisms of the Ras/MAPK pathway is necessary for better targeting ERK1/2 activity in disease.

Negative feedback regulation within the Ras/MAPK signaling pathway

Negative feedback loops occur in the same signaling pathway, in which downstream effectors inhibit their own upstream inducers. For instance, in the MAPK pathway, ERK1/2 inhibit their own upstream activator Ras by directly phosphorylating SOS and resulting in Grb2 dissociation (Dong *et al.*, 1996). ERK1/2 were also found to directly phosphorylate Raf on C-terminal SPKTP sequence leading to reduced biological activity (Brummer *et al.*, 2003). One possible reason is that phosphorylated Raf by ERK1/2 dissociates from the plasma membrane and also its upstream activators. In addition, ERK1/2 induce the expression of certain Ser/Thr phosphatase proteins, such as the MAPK phosphatase MKP1, that in turn dephosphorylate and inactivate ERK1/2 (Wang *et al.*, 2006). Moreover, it was shown that inhibition of RSK activity using pharmacological inhibitors increases ERK1/2 activity in certain types of cells, suggesting that RSK negatively regulates ERK1/2 activity (Saha *et al.*, 2012). One conceivable explanation is that RSK modestly upregulates MAPK activation by phosphorylating SOS, leading to 14-3-3 association (Saha *et al.*, 2012).

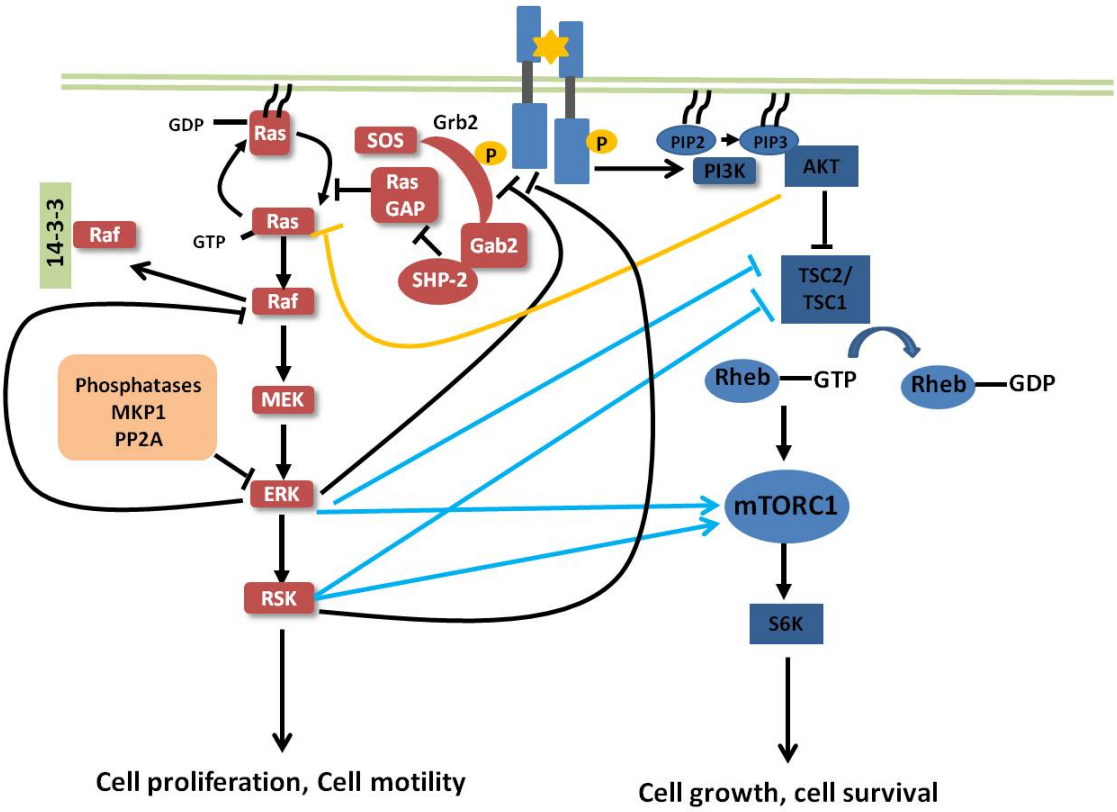

Figure 1.7 Schematic representation of the cross-talk between the Ras/MAPK and PI3K/mTOR signaling pathways.

Activated growth factor receptors trigger the parallel activation of the Ras/MAPK and PI3K/mTOR signaling pathways. Upon stimulation, the SOS/Grb2 complex is tethered to the plasma membrane and acts as a positive regulator by promoting GTP binding to Ras. ERK1/2 and RSK create feedback loops by phosphorylating SOS. In order to prevent hyperactivation of the MAPK pathway, ERK1/2-modulated phosphatases such as MKP1, inactivate ERK1/2. Moreover, ERK1/2 directly phosphorylate Raf and inhibit Raf activity with an unknown mechanism. Akt also phosphorylates Raf leading to downregulated MAPK activity. Alternatively, the MAPK pathway positively regulates mTORC1 activity. On the one hand, ERK1/2 and RSK indirectly regulate mTOR signaling by limiting TSC2 activity. On the other hand, ERK1/2 and RSK directly regulate mTORC1 activity by phosphorylating Raptor. (more detail in the text).

Cross-inhibition or -activation between the Ras/MAPK pathway and other signaling cascades

Inhibition of MAPK signaling activation by several signaling cascades. The PI3K-Akt-mTOR pathway negatively regulates MAPK signaling by phosphorylating Raf.

Upon IGF1 stimulation, it was observed that Akt phosphorylated inhibitory sites in the N-terminal of Raf leading to downregulated Raf and ERK1/2 activities (Moelling *et al.*, 2002). However, this inhibitory effect could be counteracted by PP1 or PP2 (PP: protein phosphatase) (Abraham *et al.*, 2000). Moreover, Salazar *et al.* demonstrated that p85 negatively regulated the Ras/MAPK pathway upon FGF stimulation (Salazar *et al.*, 2009). Using the yeast two-hybrid system, they confirmed that p85 directly interacted with FGFR3. Upon FGF stimulation, depletion of p85β in multiple myeloma cells promoted ERK1/2 activity, suggesting that p85-FGFR3 association acts as a negative regulator of ERK1/2 activity. More intriguingly, mTORC1 has been found to negatively regulate activation of the MAPK pathway through stabilization of Grb10 with an unknown mechanism (Yu *et al.*, 2011). PKA (protein kinase A) also involves in the regulation of MAPK activity. In response to cAMP agonists, phosphorylation of Raf by PKA promotes 14-3-3 interaction, leading to Ras and MEK1/2 dissociation (Dumaz and Marais, 2003).

Cross-activation between the Ras/MAPK and PI3K/Akt/mTORC1 pathways. The MAPK signaling cascade positively regulates mTORC1 activity at several nodes of the PI3K/Akt/mTOR pathway. On the one hand, Ras directly binds to the catalytic subunit p110 of PI3K and enhances Akt activity, independently of the regulatory subunit p85 of PI3K (Gupta *et al.*, 2007). On the other hand, ERK1/2 and RSK cooperate to upregulate mTORC1 activity by phosphorylating TSC2, a GTPase-activating protein (GAP) toward Rheb (Ras homolog enriched in brain) (Roux *et al.*, 2004; Ma *et al.*, 2005). Moreover, ERK1/2 and RSK directly regulate mTORC1 activity by phosphorylating Raptor, leading to PI3K-independent 4E-BP phosphorylation (Carriere *et al.*, 2008; Carriere *et al.*, 2010).

Implication of pathway cross-talk in tumor development and cancer treatments

During tumor progression from benign nevi to a melanoma, cross-inhibition between the Ras/MAPK and PI3K/Akt pathways plays an important role. In benign lesions, the first mutation of Ras or Raf induces such high and sustained activation of ERK1/2, resulting in cell cycle arrest and cell senescence. However, secondary mutations occurring in the PI3K/Akt pathway reduce over-activation of the Ras/MAPK pathway (Cheung *et al.*, 2008). Eventually, cooperation of the Ras/MAPK and PI3K/Akt pathways induces cell transformation and promotes tumor progression.

Understanding cross-talk between the Ras/MAPK and PI3K/Akt pathways has a significant implication in clinical research. For instance, given that treatment of melanoma cells which contain a $BRAF^{V600E}$ mutation induces drug resistance through re-activation of the P13K/Akt pathway, co-inhibition of both signaling pathways has been proposed (Chen *et al.*, 2012). Interestingly, these findings have been successfully characterized by studies in the mouse, whereby co-treatment with dual inhibitors of both the Ras/MAPK and PI3K/Akt signaling is found to be most effective in the inhibition of prostate and lung tumor growth (Kinkade *et al.*, 2008; Meng *et al.*, 2010). Although intense work is required in future to fully characterize the various modes of cross-talk between signaling pathways after a drug is administered, this work is helpful to expand our knowledge in cancer intervention.

1.3.3 MAPK–activated protein kinase RSK (90 kDa ribosomal S6 kinase)

1.3.3.1 Discovery of RSKs, their tissue expression and physiological functions

Understanding the regulated phosphorylation of rpS6, a ribosomal protein, has become an attractive research topic during the 1970s (Gressner and Wool, 1974). Given that phosphorylated rpS6 was initially thought to participate in protein synthesis by regulating binding of mRNA to 40S ribosomal subunits (Burkhard and Traugh, 1983), several research groups endeavored to identify kinases involved in rpS6 phosphorylation. In 1985, an intracellular kinase activity that phosphorylated rpS6 was purified from unfertilized *Xenopus laevis* eggs (Erikson and Maller, 1985). Initially, this activity was termed ribosomal S6K (S6 kinase). In 1988, two protein kinases, ribosomal S6 kinase I and ribosomal S6 kinase II, were identified by biochemical purification from egg extracts of *Xenopus laevis*, resulting in the cloning of two cDNAs encoding highly homologous proteins which were renamed p90 RSKs or RSKs (Jones *et al.*, 1988; Romeo *et al.*, 2011). Upon insulin stimulation, it was found that S6KII (or p90 RSKs or RSKs) was phosphorylated by MAP2 (microtubule-associated protein-2) kinase (renamed ERK2) (Sturgill *et al.*, 1988), providing the first evidence that S6KII (or p90 RSKs or RSKs) was phosphorylated by the ERK/MAPK pathway.

The RSK family consists of four isoforms: RSK1, RSK2, RSK3 and RSK4. Whereas RSK1-3 mRNAs are widely expressed in every mouse and human tissue tested, the abundance of RSK4 mRNA is relatively low (Moller *et al.*, 1994; Zeniou *et al.*, 2002). More specifically, while RSK1 mRNA is mostly expressed in the lung, kidney and pancreas, RSK2 and RSK3 mRNAs are predominately found in skeletal muscle, heart and pancreas (Zeniou *et al.*, 2002). Although RSK4 mRNA is lower compared to that for RSK1-3, it can still be detected in certain tissues, including brain, heart cerebellum, kidney and skeletal muscle, rather than lung, liver, pancreas and adipose tissue (Dummler *et al.*, 2005). To date, RSK1-4 knockout mice have been generated, and they are all viable and fertile. Although no obvious phenotypes are observed in RSK1- and RSK3-deficient mice, RSK2 knockout mice display impaired learning and cognitive functions (Poirier *et al.*, 2007). It is also found that RSK2 knockout mice are ~14% shorter and have ~10% less weight compared to wild-type (WT) mice. Most interestingly, insulin treatment and exercise remarkably increase ERK1/2 phosphorylation in skeletal muscle of RSK2 knockout mice (~2-fold compared to WT mice),

indicating that RSK2 participates in the negative feedback regulation of ERK1/2-cascade in skeletal muscle (Dufresne *et al.*, 2001). Since the physiological function of human RSK2 is highlighted because mutations in *Rps6ka3* is a cause of CLS (Coffin–Lowry syndrome), an X-linked mental retardation syndrome (Kesler *et al.*, 2007), the studies from RSK2 knockout mice would be helpful to prevent Coffin–Lowry syndrome.

1.3.3.2 Structural features of RSKs

The amino acid identity between the human RSK isoforms is around 80%, and the most divergent regions between them are the N- and C-terminal sequences (Figure 1.8). Notably, RSKs are most likely fusion proteins and composed of two different kinase domains (Fisher and Blenis, 1996): (1) the carboxyl-terminal kinase domain (CTKD) belongs to the CAMK family (Ca^{2+} /calmodulin-dependent protein kinase), including AMPK (AMP-activated protein kinase), MARK (MAP-regulation kinase/microtubule affinity regulating kinase), and DAPK (death-associated protein kinase). The role of the CTKD is to receive upstream signals from ERK1/2 and transmit them to the N-terminus of RSK. To date, no substrate of the CTKD has been determined aside from RSK itself; (2) the amino-terminal kinase domain (NTKD) belongs to the AGC family (protein kinases A, G and C) including Akt (protein kinase B), SGK (serum-and glucocorticoid-induced protein kinase) and S6K1/2. The function of the NTKD in RSK is to phosphorylate downstream substrates. Between the CTKD and NTKD there is a linker region of around 100 amino acids that, consists of a hydrophobic motif and a turn motif. The predominant function of this middle linker region is to transduce a signal from the CTKD to NTKD.

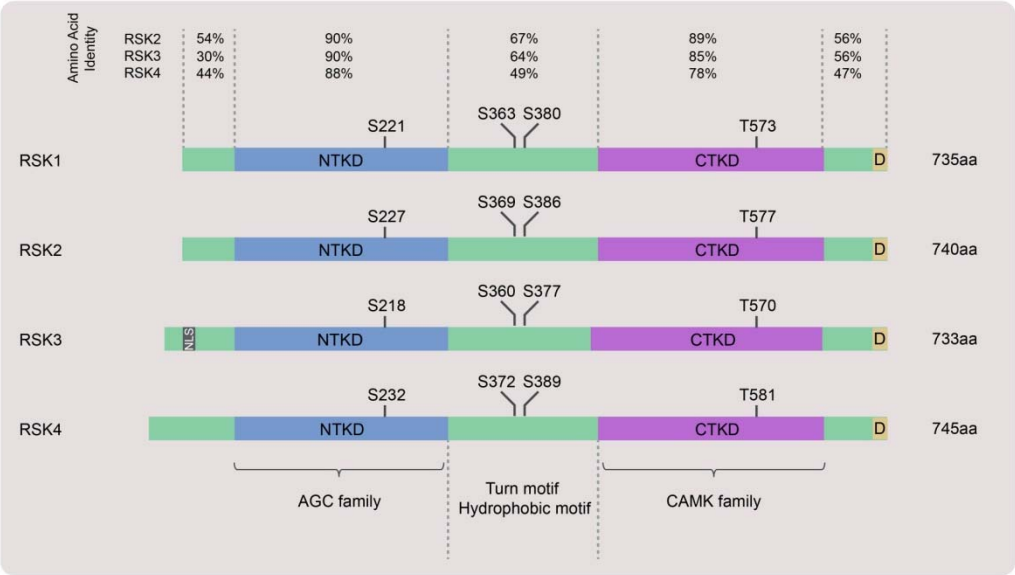

Figure 1.8 Schematic representation of structural features in RSK isoforms.

All RSK isoforms consist of the NTKD, the middle-linker region and the CTKD. Among these, the NTKD belongs to the AGC family and the CTKD belongs to the CAMK family. Only RSK3 contains a putative NLS (nuclear localization sequence), but its biological function remains elusive. *(Adapted from Microbiol Mol Biol Rev. 2011 Mar;75(1):50-83* and *Cancer Research 2013 Sep 1;73(17):5301-8)*

As indicated above, the CTKD of RSK is responsible for receiving an output signal from ERK (Fisher and Blenis, 1996). ERK catalyzes downstream effectors through interaction with the D-domain or DEF-domain present in substrates. As a substrate of ERK, RSK also follows this rule. However, unlike classical docking domains, the ERK binding region in RSK does not match the well-characterized consensus sequences of either D-domains or DEF-domains. Instead, it fits a KIM (kinase interaction motif), a D-domain-related motif that consists of a hydrophobic residue with two adjacent positively charged lysine or arginine residues (Leu-Xaa_2-Lys/Arg- Lys/Arg-Xaa_5-Leu) (Gavin and Nebreda, 1999). Aside from the KIM domain, a PDZ domain-binding motif (Ser-Thr-Xaa-Leu) in the C-terminal tail of all four RSK isoforms has been found to be functional and may help recruiting RSK to its targets (Thomas *et al.*, 2005).

1.3.3.3 Activation mechanisms and inhibitors of RSK

Activation of RSK is a result of sequential phosphorylation of four critical sites (Ser^{221},

Ser^{363}, Ser^{380} and Thr^{573} in human RSK1), that are highly conserved in all RSK isoforms (Figure 1.9). Given that sequential phosphorylation is necessary for RSK activation, the model of RSK activation is reviewed in the following.

The C-terminal end of RSK: interacts with ERK1/2 and promotes RSK phosphorylation on Thr^{573}

As an initial step upon mitogen stimulation, the interaction between ERK1/2 and the KIM domain in the C-terminus of RSK is required for activation. Following ERK1/2 docking, Thr^{573} in the activation loop segment of the CTKD is phosphorylated, leading to the auto-phosphorylation of RSK at other residues (Smith *et al.*, 1999). Moreover, phosphorylation on Thr^{573} may play an additional function in RSK translocation to the plasma

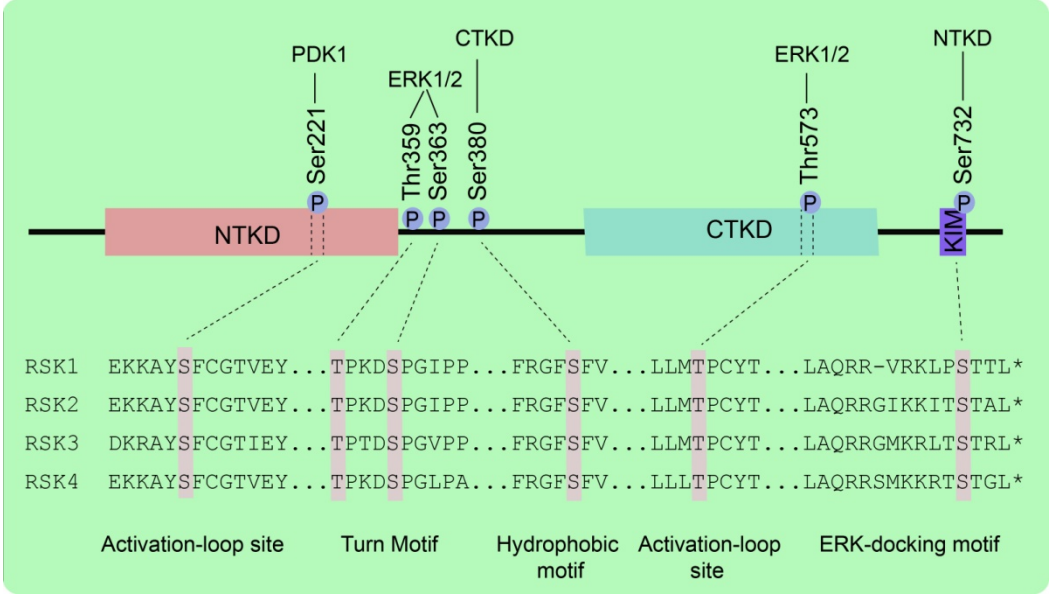

Figure 1.9 Schematic representation of human RSK1 kinase domains and phosphorylation sites.

RSK1 consists of two kinase domains: the N-terminal and the C-terminal kinase domains, which are linked by a region of 100 aa. The KIM domain is located on the C-terminus, which is responsible for ERK1/2 docking to RSK. There are at least six phosphorylation sites in RSK1, which are all conserved from RSK1 to RSK4, suggesting the significance of these sites in evolution. Phospho-Ser/Thr residues are modulated by different kinases, including ERK1/2, PDK1, CTKD and NTKD, which are displayed in the above figure. *(Adapted from Biochem J. 2012 Jan 15;441(2):553-69.)*

membrane (Richards *et al.*, 2001).

The Middle-linker region of RSK: "the turn motif" and "the hydrophobic motif"

The "hydrophobic" and "turn" motifs, which contain Thr^{359}/Ser^{363} and Ser^{380} sites respectively, are located in the linker region between the CTKD and NTKD (Vik and Ryder, 1997; Richards *et al.*, 1999; Newton, 2003). Whereas Thr^{359}/Ser^{363} are phosphorylated by ERK1/2 directly, phosphorylation of Ser^{380} is a result of ERK1/2-dependent phosphorylation on Thr^{573} in the CTKD (Richards *et al.*, 1999; Newton, 2003). Although the consequence of Ser^{363} and Ser^{380} phosphorylation is to enhance NTKD enzymatic activity, the mechanism by which Ser^{363} enhances NTKD activity remains unclear. One possible function of the turn motif is to modulate heat shock protein bindings, thereby ensuring the correct folding of AGC kinases, such as PKC (Gao and Newton, 2002). Phosphorylation of Ser^{380} by the RSK CTKD provides a docking site for PDK1 that further phosphorylates and activates the NTKD of RSK (Frodin *et al.*, 2000). Moreover, aside from the CTKD, Ser^{380} may also be phosphorylated by other kinases, such as the NTKD and other heterologous kinases (Vik and Ryder, 1997).

The N-terminus of RSK: Complete RSK activation by phosphorylation of Ser^{221}

Phosphorylation of Ser^{221} is the last step in completing RSK activation. 3-phosphoinositide-dependent protein kinase-1 (PDK1) belongs to the AGC family, which includes AKT, S6K and SGK (Arencibia *et al.*, 2013). The fact that RSK1-3 cannot be activated in PDK1-deficient cells suggests that recruitment of PDK1 to the hydrophobic motif of RSK is required for Ser^{221} phosphorylation (Williams *et al.*, 2000). Although structurally, the PH domain in PDK1 is crucial for PDK1-dependent Akt1 activation, it appears that the PH domain is unnecessary for RSK activation (Bayascas *et al.*, 2008). Additionally, PI3K inhibitors have no effect on RSK activity, indicating that PDK1 modulating RSK activity is independent of the PI3K/Akt pathway (Williams *et al.*, 2000). Collectively, these results suggest that PDK1 is indispensable in mitogen-induced RSK1-3 activity, independently of the PIP_3–containing domain (PH domain) and the PI3K pathway. Notably, it seems that RSK4 is excluded from the PDK1-dependent activation mechanism, but the reasons for this are currently unknown.

Phosphorylation of Ser^{221} stabilizes the NTKD in an active conformation, leading to the

synergistic full activation of RSK. Meanwhile, phosphorylation of Ser^{732} at the C-terminus of RSK by the NTKD releases ERK1/2 and allows translocation of activated RSK into different cellular compartments, where it can act on particular substrates (Roux *et al.*, 2003). The substrates of RSK have the following two common phospho-motifs, which consist of minimum sequences: (i) an Arg/Lys-Xaa-Arg-Xaa-Xaa-pSer/Thr motif; (ii) an Arg-Xaa-pSer/Thr-Xaa-Pro motif. Notably, this consensus motif can also be recognized by other AGC kinases, such as Akt, indicating the redundant functions of AGC kinases on the same substrate, such as PDCD4 (programmed cell death protein 4) (Palamarchuk *et al.*, 2005; Galan *et al.*, 2014).

RSK pharmacological inhibitors. To date, two types of RSK inhibitors have been generated on the basis of the different kinase domains: (i) a CTKD inhibitor: FMK is an irreversible inhibitor that covalently modifies the CTKD of RSK1, 2 and 4 isoforms with an *in vitro* IC_{50} (half maximal inhibitory concentration) of 15 nM (Cohen *et al.*, 2005); (ii) NTKD inhibitors: these are ATP-competitive inhibitors of the RSK NTKD ATP-interacting sequence, including SL0101, BI-D1870, LJH685 and LJI308 (Smith *et al.*, 2005; Sapkota *et al.*, 2007; Aronchik *et al.*, 2014). SL0101 is a natural product, purified from the plant *Fosteronia refracta* with an *in vitro* EC_{50} (half maximal effective concentration) of 50 nM and IC_{50} of 90 nM. It was shown that 10 μM of BI-D1870 was enough to inhibit phosphorylation of RSK substrates in cells. LJH685 and LJI308 are two recently identified RSK inhibitors. It appears that these two inhibitors also prefer to associate with the NTKD of RSK. A study determined that suppression of RSK by LJH685 and LJI308 resulted in reduced anchorage-independent cell growth (Aronchik *et al.*, 2014).

1.3.3.4 Subcellular localization and RSK-mediated biological functions

To date, few studies have been published regarding the subcellular localization of RSK isoforms in cells. In resting cells, RSK1-4 are usually distributed in the cytoplasm. Upon stimulation, a large proportion of RSK1-3 translocates into the nucleus whereas RSK4 remains in the cytoplasm (Chen *et al.*, 1992; Zhao *et al.*, 1995). Interestingly, it was shown that RSK1 accumulated transiently at the plasma membrane within a few minutes of stimulation, indicating that this protein might require additional signals for activation before entering the nucleus (Richards *et al.*, 2001). The mechanisms by which RSK accumulates in

the nucleus remain unclear. One possible explanation is that RSK interacts with some adaptor proteins, which either translocate or antagonize them into the nucleus. For instance, PEA-15, a small death effector domain protein, binds to RSK2 and inhibits its translocation into the nucleus (Vaidyanathan and Ramos, 2003). RSK3 is the only isoform that contains a putative nuclear localization sequence (NLS). However, the functionality of this region has never been tested.

Given that the mechanisms regarding RSK localization remain elusive and very few studies have been reported, RSK-mediated biological functions have always been determined through their specific target proteins under different conditions. In the following section, the predominant functions of the RSK family will be discussed.

1.3.3.4.1 Regulation of transcription factor activities

RSK plays an important function in ERK1/2-regulated gene transcription. On the one hand, being a downstream amplifier of ERK1/2, RSK receives input signals from ERK1/2 and transmits them to the substrates. On the other hand, ERK and RSK coordinate together to regulate their common substrates. In MDCK (Madin-Darby canine kidney) cells, it was shown that around 20% of mRNAs controlled by ERK1/2 were also regulated by RSK (Doehn *et al.*, 2009). One possible mechanism is that some ERK1/2-regulated transcription factors can also be regulated by RSK, such as ELK1 (ETS domain-containing protein) (De Cesare *et al.*, 1998; Babu *et al.*, 2000).

RSK modulates a number of transcription factors, which are either the IEG (immediate-early gene) products or involved in the IEG response. It has been shown that RSK directly modulates c-Fos activity or its expression by phosphorylating or regulation of its transcription factor activities, such as the ELK1 (ETS domain-containing protein)/SRF (serum response factor) complex, and CREB (cAMP response element-binding protein) (Chen *et al.*, 1993; De Cesare *et al.*, 1998). For instance, De Cesare D *et al.* demonstrated that both phosphorylation of CREB and c-Fos were downregulated in fibroblast cell lines that were established from a CLS (Coffin-Lowry syndrome) patient without functional RSK2 (De Cesare *et al.*, 1998). Intriguingly, RSK is not the only kinase responsible for CREB phosphorylation. A study showed that MSK1 was also required for CREB phosphorylation in

mouse embryonic stem (ES) cells (Arthur and Cohen, 2000). Given that mitogens, such as PDGF (platelet-derived growth factor) and IGF-1 (insulin-like growth factor), induce CREB phosphorylation in the absence of RSK2, this suggests that other kinases, likely MSK1, cooperate with RSK to modulate CREB activity upon mitogenic stimulation (Arthur and Cohen, 2000; Bruning *et al.*, 2000). In addition to directly modulating transcription factors, some studies suggested that activated RSK may bind to the transcriptional co-activators CBP (CREB binding protein) and p300, and cooperate with them to modulate transcription factor activities, including c-Fos, CREB, ERα, ER81, and NF-κB (Nakajima *et al.*, 1996).

Studies have shown that RSK is crucial for the regulation of growth-related gene transcription initiation (Wu and Janknecht, 2002; Zhao *et al.*, 2003; Yamnik and Holz, 2009). Growth factor-induced RSK1 directly phosphorylates the estrogen receptor α (ERα) and further increases ERα-dependent transcription (Yamnik and Holz, 2009). Upon serum stimulation, RSK2 phosphorylates transcription initiation factor TIF-1A, which is required for RNA polymerase I transcription and rRNA synthesis (Zhao *et al.*, 2003). RSK phosphorylates ER81, an ETS transcription factor, and increases ER81-dependent transcription (Wu and Janknecht, 2002). In malignant melanoma, RSK also phosphorylates another transcription factor Mi (microphthalmia) (Richards *et al.*, 2001).

Aside from controlling growth-related gene transcription, RSK also regulates inflammatory response-related gene transcriptions, such as NF-κB. In the presence of the hormone angiotensin II, It was found that activation of RSK increased NF-κB activity in the vascular smooth muscle cells (Doyon and Servant, 2010). Furthermore, Priscilla D. *et al*. oberseved that the MAPK/RSK pathway phosphorylats the T-loop of IKKβ, leading to its full activation. Given that IKKβ phosphorylates and mediates the degradation of IκB, an inhibitory moleculer of NF-κB, these results indicate that RSK impacts NF-κB activity through regualtion of the IKK complex.

1.3.3.4.2 Regulation of cell cycle progression and cell proliferation

One of the well-characterized functions of RSK is to regulate cell cycle progression and cell proliferation. It was found that depletion of RSK1/2 inhibited melanoma cell proliferation and also tumor growth in mice (Romeo *et al.*, 2012). Another piece of evidence with regard to

the importance of RSK in cell proliferation is that inhibition of RSK by the pharmacological inhibitor SL0101 prevents cell cycle progression in the breast cancer cell line MCF-7 (Smith *et al.*, 2005). Consistent with these results, data from EST (expressed sequence tag) sources indicate that expression of RSK1 in tumors is higher compared to normal tissues (Romeo *et al.*, 2011). However, RSK2 shows no obvious preference between human malignancies and normal tissues. By contrast, RSK3 and RSK4 were found in lower abundance in tumor cells. These evidence indicate that RSK isoforms may have different functions.

Regulation of G1/S progression. RSK controls cell proliferation through phosphorylating downstream effectors which are mediators of the cell cycle. In G1/S progression, it has been shown that RSK either indirectly or directly regulates cyclinD1 or CKI (cyclin-dependent kinase inhibitor) activity. For instance, it was shown that RSK phosphorylated c-Fos at Ser^{362}, a transcription factor that directly up-regulates cyclinD1 expression (Chen *et al.*, 1993). $p27^{kip1}$ is an inhibitor of CDK2 activity in G1 phase progression. It was found that RSK phosphorylated $p27^{kip1}$ at Thr^{198} and thus promoted 14-3-3 association resulting in a blockage of $p27^{kip1}$ translocation into the nucleus (Fujita *et al.*, 2003). In addition, RSK has some indirect modes of actions in controlling cell cycle progression. GSK3 is a Ser/Thr kinase and acts as a negative regulator in tumor progression. The canonical GSK3 substrate is the oncogene β-Catenin. Phosphorylation of β-Catenin by GSK3 leads to its recognition by β-TrCP, an ubiquitin E3-ligase, and its degradation by the proteasome (Hart *et al.*, 1999). Besides this canonical inhibitory mechanism of GSK3 in tumorgenesis, it was found that RSK phosphorylated GSK3 abrogated GSK3's inhibitory function on c-Myc and cyclinD1, leading to G1/S progression (De Mesquita *et al.*, 2001).

Regulation of G2/M progression and metaphase-to-anaphase transition. RSK1 has been found to play a crucial role in accelerating G2/M transition and promoting cell proliferation (Nam *et al.*, 2008). Upon HGF stimulation or in the presence of an active form of the HGF receptor M1268T, either the MEK inhibitor U0126 or a kinase-dead RSK can delay cell cycle progression, suggesting that RSK is required for G2/M transition (Nam *et al.*, 2008). RSK-promoted cell cycle progression may be due to the involvement of RSK in the regulation of checkpoint-related proteins such as Chk1 (checkpoint kinase 1) and Cdc25 (cell division cycle 25) (Ray-David *et al.*, 2012). Chk1 is a G2/M checkpoint kinase, which

prevents cells entering into mitosis by phosphorylating Cdc25, a phosphatase activating the Cdk/cyclins by dephosphorylating Wee1. In melanoma cell lines, hyperactivated N-Ras or B-Raf by mutations lead to constitutive phosphorylation of Chk1 on Ser^{280}, an inhibitory site promoting Chk1 ubiquitination (Ray-David *et al.*, 2012). Interestingly, upon DNA-damaging agent treatments, a study found that inhibition of RSK upregulated Chk1 activity correlating with downregulation of phosphorylation of Ser^{280}, indicating that the Ras/MAPK pathway modulates Chk1 action in response to DNA damage (Ray-David *et al.*, 2012). Furthermore, it was observed that while activation of RSK impaired the G2/M DNA damage checkpoint through phosphorylation of Chk1, inhibition of RSK sensitized melanoma cells to DNA-damaging agents. These results suggest that RSK inhibitors could be used to modulate chemosensitivity, which is one of the major issues in melanoma treatment. Another potential mechanism for RSK modulating G2/M transition is through direct phosphorylation of Cdc25 (Wang *et al.*, 2010). In the human HEK293 and PC-3MM2 human prostate cancer cells, phosphorylation of Cdc25A and Cdc25B by RSK accelerates cell cycle progression entering M phase (Wu *et al.*, 2013). Suppression of RSK-dependent phosphorylation of Cdc25 abrogates G2/M transition. Furthermore, the high-phosphorylation status of RSK on the Thr^{359}/Ser^{363} residues in mitotic cells indicates that RSK is more active in these cells compared to interphase cells (Wu *et al.*, 2013). RSK is also involved in the spindle assembly checkpoint by phosphorylating Mad1 (mitotic arrest-deficient protein) and promoting its degradation, resulting in increased Myc-induced cell proliferation and transformation (Zhu *et al.*, 2008). Moreover, RSK participates in metaphase II by phosphorylating and regulating mediators of APC (anaphase-promoting complex) inhibition, such as Bub1 (budding uninhibited by benzimidazoles 1) and Emi2 (early mitotic inhibitor 2) (Tunquist *et al.*, 2002)**.**

Compared to RSK1 and RSK2, less is known about RSK3/4. Recently, it was reported that the expression level of RSK3 in ovarian cancer was reduced compared to normal ovarian epithelium (Bignone *et al.*, 2007). Overexpression of RSK3 leads to G1 phase arrest in UCI101 cells (ovarian cancer cell lines) (Bignone *et al.*, 2007). However, the mechanisms by which RSK3 negatively regulates cell cycle progression still remain unclear. RSK4 is the most enigmatic member with the lowest expression level in the RSK family. In breast cancer cell lines, it was found that exogenous expression of RSK4 inhibited cell proliferation as a

result of G0/G1 arrest, indicating that RSK4 is a negative regulator of cell proliferation (Thakur *et al.*, 2007). RSK4 is also involved in p53-dependent cell cycle arrest and oncogene-induced senescence (Lopez-Vicente *et al.*, 2009). Given that RSK3 and RSK4 have different functions compared to RSK1 and RSK2, further studies are needed to better understand their underlying mechanisms.

1.3.3.4.3 Regulation of protein synthesis and cell growth

RpS6 is a component of the 40S ribosomal subunit, and phosphorylation of rpS6 is thought to participate in regulation of cell size and glucose homeostasis (Meyuhas and Dreazen, 2009). Although S6Ks are the major kinases responsible for rpS6 phosphorylation, many reports have indicated that RSK is also involved in rpS6 phosphorylation leading to protein synthesis (Roux *et al.*, 2007). In $S6K1^{-/-}$ $S6K2^{-/-}$ double knockout mice, it was found that the absence of S6K1/2 resulted in an almost complete loss of rpS6 phosphorylation (Pende *et al.*, 2004). However, low levels of rpS6 phosphorylation could still be observed. Moreover, it was found that rpS6 was phosphorylated upon the activation of oncogene- or agonist-induced activation of the MAPK pathway when S6K1/2 were inactive (Pende *et al.*, 2004). To date, accumulating evidence has shown that S6K1/2 phosphorylate rpS6 on Ser^{235}, Ser^{236}, Ser^{240} and Ser^{244}, whereas the MAPK pathway via RSK phosphorylates rpS6 on Ser^{235} and Ser^{236} (Roux *et al.*, 2007). Collectively, these findings suggest that both RSK and S6K1/2 are required for phosphorylation of rpS6 leading to the assembly of the cap-binding complex and cap-dependent translation.

Indirect regulation of mTORC1 activity. One potential mechanism of RSK participating in mRNA translation is through modulating the activity of mTORC1, an essential regulator of ribosome biogenesis, mRNA translation and cell growth. It has been shown that RSK directly phosphorylates TSC2, a negative regulator of mTORC1 activity by inhibiting Rheb GTPase activity (Roux *et al.*, 2004). Alternatively, RSK phosphorylates Raptor (the regulatory associated protein of mTOR), resulting in increased mTORC1 activity (Carriere *et al.*, 2008).

Regulation of translation-initiation factors (mTOR-independent manner). RSK regulates translation-initiation factors in both indirect and direct manners. GSK3β is a

negative regulator of protein synthesis by inhibition of eIF2B, a small G-protein responsible for the exchange of guanine nucleotides on translation initiation factor 2 (eIF2). It was found that RSK phosphorylated GSK3β on Ser^{9}, thereby releasing its inhibitory function on eIF2B and promoting mRNA translation (Welsh *et al.*, 1998). Aside from this indirect mode of action on mRNA translation, RSK directly phosphorylates the translation initiation factor eIF4B (Shahbazian *et al.*, 2006). RSK phosphorylation of eIF4B promotes its recruitment to the translation-initiation complex, thereby enhancing the translation of mRNAs encoding proteins which participate in cell growth and survival. Moreover, upon typeIII IFN receptor activation, it has been determined that RSK affects mRNA translation initiation by regulating the phosphorylation of 4E-BP on Thr^{37}/Thr^{46} (Kroczynska *et al.*, 2010), an eukaryotic translation initiation factor 4E (eIF4E)-binding protein, thereby releasing eIF4E and initiating mRNA translation.

1.3.3.4.4 Regulation of cell survival

RSK1/2 promote cell survival. It has been shown that RSK2 promotes cell survival through regulating CREB-dependent gene transcriptions, such as *Bcl-2*, *Bcl-xL* and *Mcl1* (myeloid cell leukemia sequence 1) (Xing *et al.*, 1996). On the other hand, RSK protects cell against death through direct phosphorylation and inactivation of pro-apoptotic proteins, such as Bad (Bcl-2-associated death promoter) (Tan *et al.*, 1999). More recently, RSK was found to enhance cell survival by modulating the transcription factor NF-κB (Peng *et al.*, 2010). In addition, it was shown that RSK1 and RSK2 inactivated the tumor suppressor DAPK (death-associated protein kinase) by phosphorylating it on Ser^{289}, resulting in enhanced cell survival (Anjum *et al.*, 2005).

Mitochondria-mediated cell apoptosis is a canonical apoptotic pathway. During this process, cytochrome-c release results in caspase enzyme activation, thereby inducing cell apoptosis (Jiang and Wang, 2004). Kim *et al.* observed that activation of the MAPK pathway led to the suppression of cytochrome-c-promoted caspase activation (Kim *et al.*, 2012). Furthermore, they found that this regulation was modulated by RSK-phosphorylated Apaf-1 (apoptotic protease activating factor-1). Given that RSK and 14-3-3 have the same consensus phospho-motif: RXXpS/T, it was hypothesized that RSK-induced Apaf-1 phosphorylation led to 14-3-3ε association and further prevented cytochrome-c release. Alternatively, RSK can

also regulate caspase activities, independently of cytochrome-c. For instance, it was shown that inhibition of the MAPK/RSK pathway decreased stellate cell survival as a result of alteration of the MAPK-RSK-C/EBP (CCAAT/enhancer binding protein)-(β)FLIPL (a critical inhibitor of caspase 8)-caspase 8 signaling pathway (Buck and Chojkier, 2007).

1.3.3.4.5 Regulation of cell migration

Inhibition of tumor invasion and metastasis by RSK pharmacological inhibitors suggests that RSK is a therapeutic target for the treatment of disseminated cancers (Sulzmaier and Ramos, 2013). The first evidence of RSK1/2 involvement in cell migration was the identification of the cytoskeleton-associated protein filamin A as a novel substrate of RSK. More specifically, RSK phosphorylated filamin A on Ser^{2152}, the site identified previously as a PAK1 (p21-activated kinase-1) phosphorylation site and required for membrane ruffling (Woo *et al.*, 2004). More recently, it was found that RSK phosphorylated KIBRA (kidney and brain expressed protein), a new regulator in the Hippo signaling pathway, leading to upregulated cell migration (Yang *et al.*, 2013). In MDA-MB-231 breast cancer cells, Yang *et al.* determined that RSK and ERK1/2 phosphorylated KIBRA on Ser^{548} and Thr^{929}/Ser^{947}, respectively. Moreover, another report indicated that RSK modulated cell movement through mediating the expression of Fascin-1, a protein involved in cell movement by association with actin filaments (Li *et al.*, 2013). By analyzing primary human tumor tissue samples from head and neck squamous carcinoma patients, Li *et al.* observed that silencing of RSK2 not only disrupted filopodia formation and cell invasion but also significantly reduced Fascin-1 expression, indicating that RSK2 is a potential upstream regulator of Fascin-1 (Li *et al.*, 2013). Furthermore, it was indicated that RSK-induced Fascin-1 expression depended on CREB activity. Taken together, these results indicate that the RSK2-CREB-Fascin-1 pathway is a novel mechanism by which RSK regulates cell invasion (Li *et al.*, 2013). Moreover, RSK phosphorylates VASP (vasodilator-stimulated phospho-protein) on Thr^{278}, leading to actin polymerization and cell migration (Doppler and Storz, 2014).

Intriguingly, it was found that RSK2 was also involved in macrophage-stimulating protein (MSP)-induced EMT (Ma *et al.*, 2011). Upon MSP stimulation, Ron (recepteur d'origine nantais), a receptor tyrosine kinase also referred to as MST1R (macrophage-stimulating protein receptor), is activated. It was observed that the treatment of

MSP promoted RSK2 translocation into the nucleus, relying on both Ron and ERK1/2 activities. By using the RSK inhibitor SL0101, Ma *et al.* observed that RSK activation was completely blocked as well as the MSP-induced spindle-like cell morphology and cell migration (Ma *et al.*, 2011). Moreover, whereas deletion of RSK2 inhibited MSP-induced EMT, overexpression of RSK2 in HT-29 cells (a human colorectal adenocarcinoma cell line barely expressing RSK2) led to the recovery of the EMT-like phenotype. These results suggest that RSK2 is a crucial regulator in MSP-induced EMT, and inhibition of RSK2 activity can be a potential therapeutic target in Ron-mediated cancer cell migration and invasion.

1.3.3.4.6 Other substrates and functions

Recently, an advanced mass spectrometry approach and a genome-wide screen have been adapted to identify novel substrates of RSK. Recently, by using the global quantitative phosphoproteomic screen, our group firstly characterized RSK-dependent phosphorylation events in melanoma (Galan *et al.*, 2014). Also, by using the genome-wide RNAi screen, the Haber group identified a number of RSK substrates in human MCF-10A mammary epithelial cells, which involve in a wide range of intracellular events, such as signal transduction, metabolism, cellular trafficking (Smolen *et al.*, 2010). In addition to classical functions in cell proliferation, growth, survival and migration, RSK has been determined to be involved in other cellular processes. For example, RSK2 knockout mice have impaired abilities of learning and coordination, suggesting that RSK2 is involved in neuron activity (Dufresne *et al.*, 2001). Moreover, it was found that RSK2 phosphorylated nNOS (neuronal nitric oxide synthase) on Ser^{847} in rat hippocampal neurons and cerebellar granule cells, leading to inhibited nNOS activity in the brain (Song *et al.*, 2007).

In addition, several lines of evidence suggest that RSK participates in the negative feedback loop of the Ras/MAPK signaling pathway. First, inhibition of RSK by the pharmacological inhibitor BI-D1870 increases ERK1/2 activity in certain cell types (Saha *et al.*, 2012). Second, consistent with this result, by *in vivo* experiments with RSK2 knockout mice, it was also found that ERK1/2 activity was increased in skeletal muscle tissue. One of the conceivable explanations is that RSK phosphorylates SOS, leading to Grb2 disassociation and thereby inhibiting ERK1/2 activity (Saha *et al.*, 2012). Taken together, these findings

suggest that one of RSK functions may be to prevent hyperactivity of the ERK1/2 cascade.

1.4 The Gab/Dos family of docking proteins

RTK signaling plays a crucial role in modulating cell growth, proliferation, differentiation and motility. Aberrant regulation of RTKs by mutation or overexpression results in many human diseases, such as cancer, inflammatory diseases and severe bone disorders (Lemmon and Schlessinger, 2010). Upon extracellular stimulation by a ligand, RTKs typically form stabilized dimers, which result in the *trans*-phosphorylation of tyrosine residues in the cytoplasmic domains. The tyrosine phosphorylated residues on the receptors in turn provide several binding sites for multiple signal relay proteins, such as those containing SH2 and PTB domains, including PLCγ and adaptor proteins (Jorissen *et al.*, 2003). These phospho-dependent binding domains enable the association of adaptor proteins to receptors in order to serve as platforms for the initiation of downstream signaling. Although adaptor proteins often lack intrinsic catalytic activities, such as Grb2-associated binder (Gab) proteins, Grb2 and p85, they typically interact with one or more enzymes to amplify upstream signals. In the following section, I will focus on how RTKs signal through the Gab family of adaptor proteins.

1.4.1 Discovery of Gab proteins and their biological functions in mice

The Gab family consists of five members, including Gab1-3 in vertebrates, Dos in *Drosophila* and Soc-1 in *C. elegans*. Gab1 was the first identified Gab protein, which was characterized as a Grb2 SH3-domain binding protein (Holgado-Madruga *et al.*, 1996). In 1998, Gu *et al.* cloned Gab2 as a binding protein and substrate of the SH2-domain containing tyrosine phosphatase Shp2 (Gu *et al.*, 1998). Gab3 was later identified due to its sequence similarity to Gab1 and Gab2 (Wolf *et al.*, 2002). In *Drosophila*, Dos was determined as a potential substrate of Csw (Corkscrew; Shp-2 ortholog in *Drosophila*) (Herbst *et al.*, 1996). Soc-1 was identified from a screen for suppressors of Egl-15 (a fibroblast growth factor receptor in *C.elegans*) signaling (Schutzman *et al.*, 2001). Whereas *GAB1* is localized to human chromosome 4, *GAB2* and *GAB3* are located on chromosomes 11 and X, respectively (Yamada *et al.*, 2001; Seiffert *et al.*, 2003). In the mouse, Gab1 and Gab2 are expressed ubiquitously, but expression of Gab3 is mainly found in the hematopoietic system (Yamada *et al.*, 2001; Seiffert *et al.*, 2003).

All three mouse Gab knockouts have been generated and resulted in very different phenotypes. Deletion of *GAB1* in the mouse results in embryonic lethality between 12.5 and 18.5 days with defects in heart, placenta, liver, skin and muscle developments (Itoh *et al.*, 2000). Given that the phenotypes of mice that lack signals from EGF, PDGF, HGF and gp130 pathways are similar to the ones observed in the *GAB1*-deficient mice (Itoh *et al.*, 2000; Sachs *et al.*, 2000), these results suggest that the phenotype of $Gab1^{-/-}$ mice may stem from the combined deficiencies in these signals. In contrast to *GAB1*-deficient mice, *GAB2*-deficient mice are viable but defective in the mast cell lineage (Nishida *et al.*, 2002). It was shown that *GAB2* knockout ($Gab2^{-/-}$) mice displayed decreased mast cell numbers in different tissues, such as stomach and skin (Nishida *et al.*, 2002). Moreover, Gab2-deficient mast cells grow poorly and show impaired Kit (a receptor tyrosine kinase)-induced signals. *GAB2*-deficient mice also have defects in IgE-induced allergic responses (Gu *et al.*, 2001). *GAB3*-deficient mice have been generated, but no detectable defects have been observed in these animals (Seiffert *et al.*, 2003).

1.4.2 Activation mechanisms of Gab protein-dependent signaling

1.4.2.1 Structural features of Gab proteins

Although the biological functions of Gab family members are different and the overall amino acid identity between mouse Gab1-3 is only 38~42%, Gab proteins still have a similar topology (Figure 1.10). Each Gab protein contains an N-terminal pleckstrin homology (PH) domain, multiple proline-rich domains (PXXP), and several potential tyrosine and serine/threonine phosphorylation sites (Gu and Neel, 2003). In humans, the PH domains found in Gab1~Gab3 have 62-76% amino acid identity, suggesting that these domains perform a similar function between Gab isoforms (Wohrle *et al.*, 2009). While the PH domains bind to membrane-associated phospholipids and mediate the membrane localization of Gab proteins, the proline-rich domains of Gab proteins constitutively associate with SH3 domains-containing proteins, such as Grb2 (Zhao *et al.*, 1999). Upon RTK activation, Grb2 brings Gab proteins to the plasma membrane, where they become tyrosine phosphorylated and, in turn, mediates SH2 domain-containing protein associations, such as Shp2 (the SH2 domain-containing protein tyrosine phosphatase 2) and p85 (regulatory subunit of PI3K) (Wohrle *et al.*, 2009). Given the importance and complexity of Gab-mediated signals, more

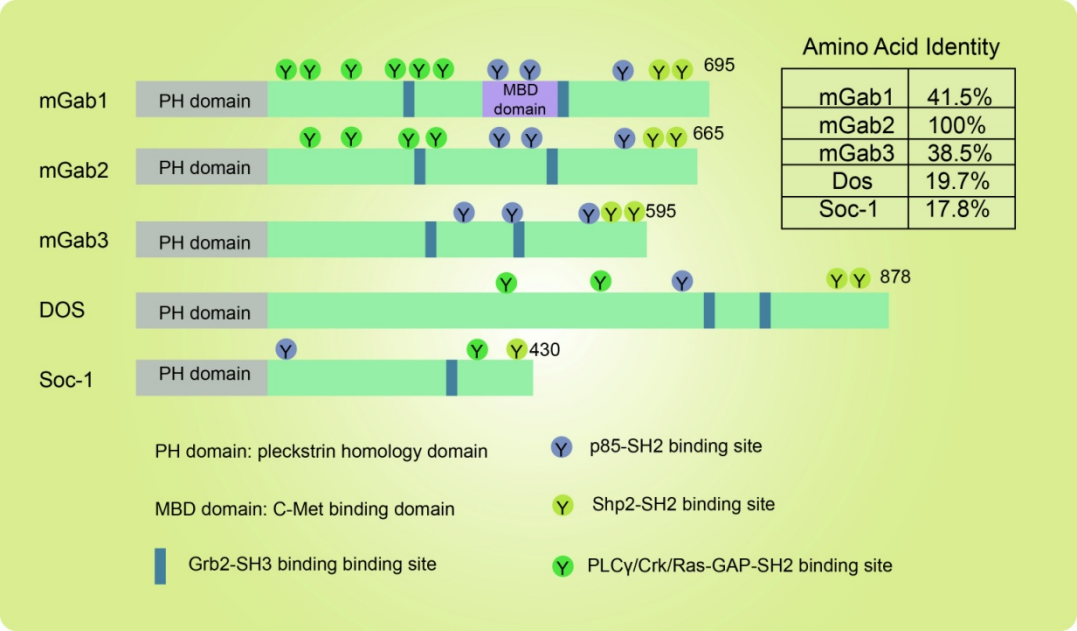

Figure 1.10 Schematic representation of structural features of Gab proteins.

The Gab family consists of five members, including Gab1-3 in vertebrates, Dos in *Drosophila* and Soc-1 in *C. elegans*. As the amino acid identity between mGab1 and mGab2 is only 41.5%, it suggests that Gab1 may have different biological functions from Gab2. All Gab proteins consist of several proline-rich domains responsible for SH3 domain-containing protein associations, such as Grb2. Meanwhile, several potential phosphotyrosine residues in Gab proteins provide binding sites for SH2 or PTB domain-containing protein. Notably, only Gab1 has the MBD domain which is responsible for Gab1's direct binding to the C-met receptor (HGF receptor) *(Adapted from Int J Inflam. 2013 2013:141068)(Nakaoka and Komuro, 2013).*

details about how Gab proteins participate in the activation of intracellular signaling pathways will be described in the following section.

1.4.2.2 Role of the PH domain

PH domains are found in a wide range of proteins that are involved in diverse cellular processes, including intracellular trafficking, cellular signaling transduction and cytoskeletal remodeling (Rebecchi and Scarlata, 1998). The three-dimensional structure of the PH domains has been determined to be highly conserved, suggesting that these domains have important functions. Structurally, PH domains consist of ~120 amino acids and share a

common core fold formed from two near orthogonal beta sheets (β1/β2 loop) (Rebecchi and Scarlata, 1998). Within these domains, there is a well-defined binding site for the headgroup of phosphoinositides. Despite the remarkable conservation in their secondary structure, variations can be found in the loops that enable an individual PH domain to have binding affinities for specific phosphoinositides, such as PI-4,5-P_2, PI-3,4-P_2 or PI-3,4,5-P_3 (PI: phosphatidylinositol; P: phosphate) (Chen *et al.*, 1997). Furthermore, their association with phosphoinositides and localization on different cellular compartment membranes allow PH-domain-containing proteins to transmit signals from lipid messengers to downstream targets (Rebecchi and Scarlata, 1998).

The role of the PH domains in mediating Gab proteins localization has been well characterized. The Park group observed that truncation of the PH domain impaired Gab1's translocation from the cytoplasm to the plasma membrane upon HGF stimulation (Maroun *et al.*, 1999). They also found that this translocation of Gab1 was required for transducing HGF-induced intracellular signaling (Maroun *et al.*, 1999). Upon EGF stimulation, the PH domain of Gab1 was shown to bind specifically to PI-3,4,5-P_3 in the plasma membrane, where it enhanced EGFR signaling (Rodrigues *et al.*, 2000). Overexpression of the Gab1 PH domain was dominant-negative and abolished activation of the EGFR/PI3K pathway, suggesting further that Gab1 is the primary mediator transducing a signal from EGFR to PI3 kinase (Rodrigues *et al.*, 2000). In the case of Gab2, upon fibroblast growth factor (FGF) stimulation, deletion of the PH domain impaired its localization at the plasma membrane, abolished tyrosine phosphorylation and also p85 recruitment leading to decreased Akt activity (Mao and Lee, 2005). Taken together, these findings suggest that the PH domain is crucial for localization of Gab proteins on the plasma membrane where receptors are activated.

1.4.2.3 Role of the proline-rich domains in Gab2

The SH3 domain was first identified as conserved residues in several tyrosine kinases such as Src and Abl (Pawson and Schlessingert, 1993). They are small peptide sequences of approximately 60 amino acids. Proteins having SH3 domains are likely to constitutively interact with proline-rich sequence (PXXP)-containing proteins (Alexandropoulos *et al.*, 1995). One such an example is Grb2 through its SH3 domains constitutive association with two proline-rich domains in Gab2. Grb2 is the predominant upstream regulator of Gab2. Upon

stimulation by diverse growth factors and cytokines, such as EGF and IL-2, Grb2 recruits Gab2 to the receptors at the plasma membrane where Gab2 becomes tyrosine phosphorylated and thus acts on its membrane-associated substrates such as Shp2. However, some receptors that lack Grb2 binding sites cannot directly bind to the Grb2/Gab2 complex, such as the β chain of IL-2 and IL-3 receptors (Gu *et al.*, 2000). In these signaling pathways, adaptor proteins, such as Shc, are required to provide an additional link between the Grb2/Gab2 complex and tyrosine-phosphorylated receptors (Gu *et al.*, 2000).

1.4.2.4 Tyrosine phosphorylated Gab2 mediates Gab2-dependent complex formation and sustains its downstream signaling

Upon RTKs activation, Gab2 becomes tyrosine phosphorylated and, in turn, provides numerous binding sites to different SH2- or PTB-domain-containing proteins such as Shp2, p85, PLCγ, RANK, Crk, Shc and SHIP (Gu *et al.*, 1998; Bone and Welham, 2000; Wada *et al.*, 2005; Mao *et al.*, 2006). More details regarding how Gab2 interacts with binding partners leading to the activation of downstream pathways will be described in following.

Roles in Shp2-dependent multiple signaling pathways

Gab2 contains two conserved tyrosine motifs consisting of I/V/LX**Y**XXI/V/L responsible for its association with Shp2 (Gu *et al.*, 1998). Under basal condition, the phosphatase activity of Shp2 is very low due to allosteric inhibition of the PTP (protein-tyrosine phosphatase) domain by its N-terminal SH2 domains (Dance *et al.*, 2008). Following association of SH2 domains to Gab1/2, the PTP domain of Shp2 is released leading to the dephosphorylation of downstream targets, such as RasGAP (Agazie and Hayman, 2003; Montagner *et al.*, 2005).

The best-characterized function of the Gab2-Shp2 axis is its involvement in the activation of the Ras/MAPK pathway. Phosphorylation of Gab2 on tyrosine residues 614 and 643 is required for Shp2 SH2 domains recruitment (Gu *et al.*, 1998). Mutation of $Tyr^{614/643}$ to Phe in Gab2 not only prevents Shp2 association but also inhibits the downstream signaling, such as ERK1/2 phosphorylation. Co-expression of ErbB2 and Gab2 with a Shp2 binding mutant (ΔShp2) impairs cell proliferation as well as ErbB2-induced breast tumor growth in mice (Bentires-Alj *et al.*, 2006).

Aside from its involvement in the MAPK pathway, the Gab2-Shp2 complex also

participates in the Rac/JNK pathway in mast cells (Yu *et al.*, 2006). In response to stem cell factor (SCF) stimulation, deletion of Gab2 ($Gab2^{-/-}$) in mast cells inhibits cell proliferation, indicating that Gab2 is required for SCF-induced mast cell proliferation. Furthermore, it was found that disruption of Shp2 binding to Gab2 decreased SCF-mediated Rac/JNK signaling. Taken together, these results demonstrate that the Gab2-Shp2 complex positively regulates signal transduction from the SCF receptor (Kit) to the Rac/JNK pathway leading to mast cell proliferation.

Role in the PI3K/Akt pathway

Another important downstream signaling event mediated by Gab2 is the PI3K/Akt pathway. Interaction of the p85 subunit of PI3K with phosphorylated Tyr^{452}, Tyr^{476} and Tyr^{584} of Gab2 results in the upregulation of Akt activity (Wang *et al.*, 2011). Recently, many reports have indicated that Gab2 acts through the PI3K/Akt pathway to promote ovarian cancer formation (Wang *et al.*, 2011). Using an *in vivo* multiplexed transformation assay, Dunn *et al.* found that amplification of *GAB2* strongly induced immortalized ovarian epithelial cell transformation (Dunn *et al.*, 2014). Furthermore, they determined that p85 recruitment was necessary for Gab2-induced cell transformation, suggesting that the PI3K pathway is crucial for Gab2-mediated ovarian cancer development. Consistent with these results, it was shown that inhibitors of PI3K, LY294002 and GDC-0941, or an mTOR inhibitor, rapamycin, abrogated Gab2-induced cell migration and invasion. One possible mechanism for the Gab2-induced ovarian cancer cell migration is through the PI3K-upregulated Zeb1 expression, a transcription factor related to the EMT, leading to downregulated E-cadherin expression (Wang *et al.*, 2011).

Although many studies established that p85 recruitment is essential for Gab2-mediated PI3K/Akt signaling, others argued that p85 association is dispensable. To address this, using the MCF-10A cell line as model for understanding the molecular mechanisms of breast cancer progression, Bentires-Alj *et al.* demonstrated that co-expression of Gab2 with HER2 resulted in an invasive phenotype independently of p85 recruitment (Bentires-Alj *et al.*, 2006). Given that Shp2 dissociation from Gab2 inhibited the acinar growth of epithelial mammary cells as well as Akt activity, it appears that Shp2 recruitment is more crucial than p85 binding in biological contexts (Brummer *et al.*, 2006). However, the molecular mechanism regarding this

regulation is ill defined. It was shown that Gab2 recruited Shp2 to an inhibitory site of Lyn (a non-receptor tyrosine-protein kinase), leading to its activation (Futami *et al.*, 2011). Given that Lyn is a predominant Src kinase in myeloid cells and Src induces PI3K to phosphorylate Akt, it is possible that the Gab2/Shp2 axis may regulate Akt activity through the Shp2/Src/Akt pathway.

Other Gab2-dependent signaling pathways

Gab2-STAT3 signaling. Gab2 has an important function in the hematopoietic system. It was found that $Gab2^{-/-}$ mice are viable and fertile, but the number of circulating mast cells is severely decreased (Itoh *et al.*, 2007). For this reason, many research groups endeavored to understand the roles of Gab2 in mast cell growth and development. As such, several new PTB domain containing proteins have been identified. One of these is STAT3 (signal transducer and activator of transcription 3). In primary hematopoietic cells, it was found that upon activation of Stk/Ron (receptor tyrosine kinase), Gab2 directly interacted with STAT3 resulting in hematopoietic cell transformation (Ni *et al.*, 2007). Given that STAT3 is one of the major transcription factors in the regulation of mast cell growth (Sonnenblick *et al.*, 2004), it is possible that Gab2 association with STAT3 in mast cells results in accelerated cell growth.

Gab2-SHIP signaling. Studies have shown that Gab2 is required in FcεRI (lgE receptor) induced mast cell aggregation (Gu *et al.*, 2001; Xie *et al.*, 2002). In rat basophilic leukemia RBL-2H3 cells, FcεRI clustering induced translocation of Gab2 to the plasma membrane, where it became tyrosine phosphorylated. Then, tyrosine phosphorylated Gab2 associated with SHIP, an SH2-containing inositol 5'-phosphatase (Leung and Bolland, 2007). Given that the predominant function of SHIP is to convert PI(3,4,5)P3 to PI(3,4)P2, this raises the possibility that SHIP acts as a negative modulator in FcεRI/Gab2-mediated pathways. Indeed, it was shown that upon FcεRI engagement, SHIP interacted with Gab2 leading to the inhibition of microtubule polymerization (Leung and Bolland, 2007). Because microtubules participate in mast cell degranulation (Draber *et al.*, 2012), these results suggest that FcεRI-mediated mast cell degranulation is inhibited by SHIP recruited to Gab2.

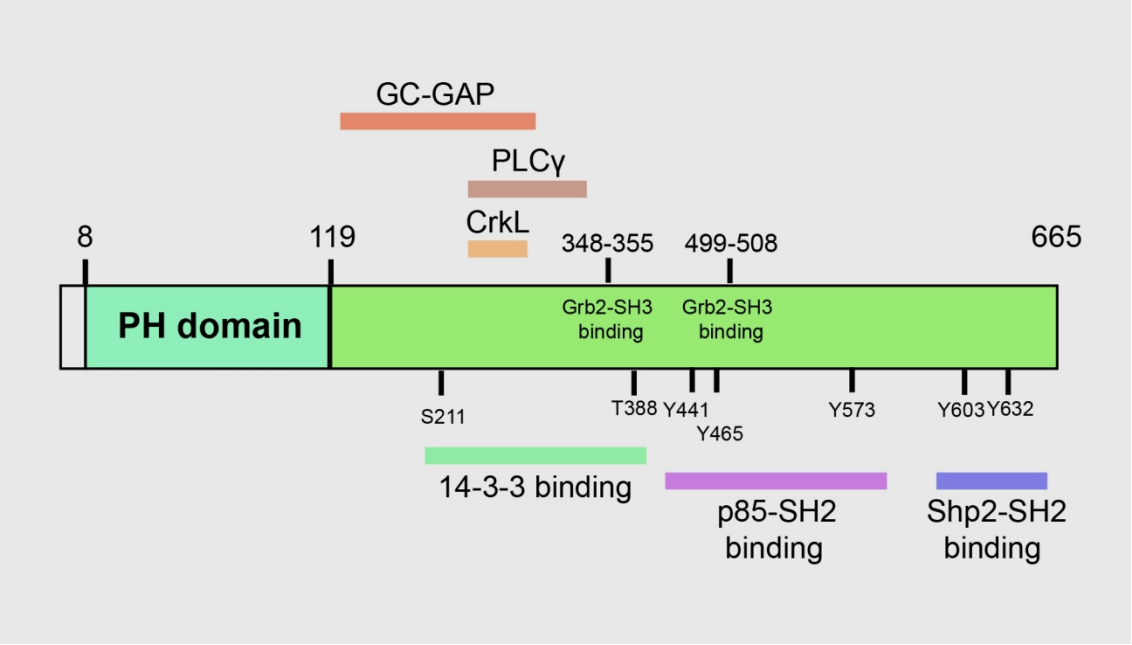

Figure 1.11 Schematic representation of various binding proteins recruited to Gab2.

Both phospho-Tyr and -Ser/Thr residues mediate Gab2-dependent complex formation. For instance, the former induces direct binding of Shp2 and p85 to Gab2, and the latter is responsible for 14-3-3 recruitment. Other proteins can also associate directly with Gab2, such as GC-GAP, CrkL and PLCγ.

PLCγ-Gab2-RANK-NF-κB signaling. PLCγ2 (phosphoinositide phospholipase C gamma) is important for early osteoclast development (Mao *et al.*, 2006). It was found that activated PLCγ2-induced Gab2 recruitment was necessary for osteoclastogenesis (Mao *et al.*, 2006). More specifically, PLCγ associated with Gab2 induced its tyrosine phosphorylation, resulting in RANK (receptor activation of NF-κB) recruitment that, in turn, activated the NF-κB pathway, Lacking of NF-κB has been found to cause severe osteopetrosis in mice, a disease with impaired osteoclastic bone resorption, indicating this signaling pathway is crucial for generating mature osteoclasts (Franzoso *et al.*, 1997).

1.4.2.5 Other binding partners to Gab2

14-3-3. Aside from phosphotyrosine residues, some phospho-serine/threonine residues on Gab2 also provide binding sites for protein interactions. One such Gab2 binding protein is

14-3-3, a small adaptor protein of only 29kDa that frequently acts as a bridge between two molecules, resulting in the assembly of a complex and also the stabilization of its conformational state (Bridges and Moorhead, 2005). Upon EGF stimulation, phosphorylation of Gab2 on Ser^{210} and Thr^{391} promotes its interaction with 14-3-3 (Brummer *et al.*, 2008). Binding of 14-3-3 was shown to terminate Gab2-dependent signaling by sequestering Grb2, disassociating it from Gab2 and thus preventing Gab2 recruitment to the receptors.

GC-GAP. By using a yeast two-hybrid approach with hGab2 (120–587) as bait, Zhao *et al.* identified that GC-GAP, a GTPase-activating protein (GAP) of the Rho family, is a novel binding partner of Gab2 (Zhao *et al.*, 2003). Expression of GC-GAP in HEK293T cells resulted in reduced activity of Rac1 and Cdc42 rather than RhoA (Zhao *et al.*, 2003). Moreover, deletion of GC-GAP in C6 astroglioma cells inhibited cell proliferation. On the basis of these findings, it was suggested that Gab2 interacts with GC-GAP leading to decreased activity of Rac1 and Cdc42. Given that the expression of GC-GAP is specific in the nervous system, it also suggests that the Gab2/GC-GAP complex may play an important role in neural/glial cell proliferation.

Shc and CrkL. Gab2 also interacts with some proteins whose functional significance is less established. In some cases, the Gab2/Grb2 complex lacks the capability to directly interact with receptors, such as the β chain of IL-2 and IL-3 receptors. In this situation, tyrosine phosphorylation of a small adaptor protein termed Shc plays a crucial role in Gab2/Grb2 recruitment to the receptors (Gu *et al.*, 2000). CrkL is a Crk-like protein and participates in Bcr-Abl tyrosine kinase induced fibroblast transformation (Senechal *et al.*, 1996). By using a modified yeast two-hybrid system with the catalytic Lyn tyrosine kinase (belonging to the Src family), Crouin *et al.* found that Gab2 directly interacted with CrkL through phosphorylated Tyr^{266} and Tyr^{293} of Gab2 (Crouin *et al.*, 2001). However, the biological function of the Gab2/CrkL interaction has not been fully determined.

1.4.3 Role of Gab2 in tumorigenesis

Genetic and epigenetic changes are the two major causes of oncogene-induced carcinogenesis (You and Jones, 2012). Amplification of chromosome 11q13-14.1 is often found in human malignancies (Johnson *et al.*, 2008). In 1995, amplification of the *CCND1* gene, that encodes the cell cycle regulatory protein cyclinD1, was first identified on human

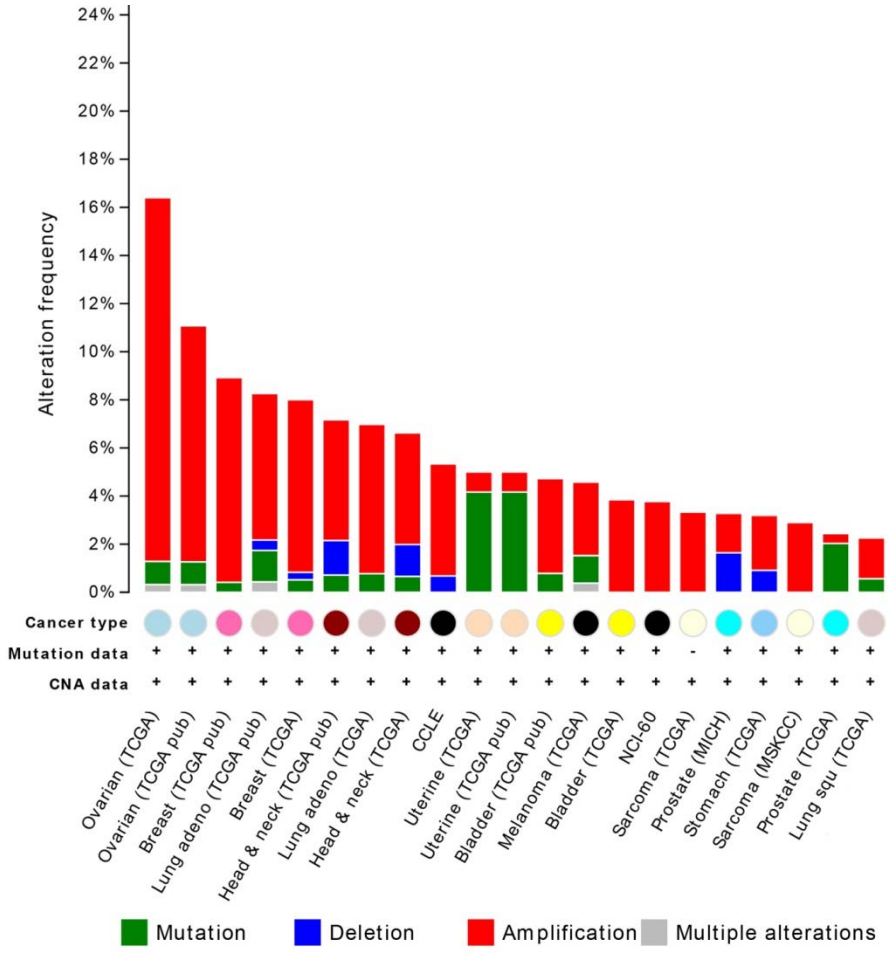

Figure 1.12 Cross-cancer alteration summary for the *GAB2* gene.

It has been found that the *GAB2* gene is located on human chromosome 11 (11q14.1), which is altered in diverse malignancies such as breast cancer, ovarian cancer and melanoma. As displayed in the above figure, *GAB2* is often found amplified in numerous cancers. Amplification of the *GAB2* gene occurs in 8.5% of 482 breast tumor cases, and 16.1% of 311 Ovarian Serous Cystadenocarcinoma cases. This suggests that *GAB2* is a potential oncogene in these cancers. (*Adapted from http://www.cbioportal.org, Cerami et.al., Cancer Discov. 2012 & Gao et. al., Sci. Signal. 2013.)*

chromosome 11q13 (Adelaide *et al.*, 1995). Given that human chromosome 11q13-14.1 is a large region (around 9~10Mb), this raises the possibility that other potential proto-oncogenes also co-amplify with *CCND1*. To date, it has been determined that a number of genes, including *EMS1, PAK1*, *RSF1* and *GAB2*, are located in this region and act as potential drivers of tumorigenesis (Brown *et al.*, 2008).

Compared to other isoforms of the Gab family, it appears that Gab2 is more closely related to cancer. As shown in 1.12, the alteration frequency of *GAB2* is due to amplification rather than mutation, especially in ovarian cancer, breast cancer and lung cancer. Although *CCND1* and *GAB2* are located on the same chromosome, there is no evidence to indicate that focal amplification of *GAB2 (11q14.1)* is correlated with *CCND1 (11q13.2)* in breast cancers (Bocanegra *et al.*, 2009). Aside from breast cancers, 11% of human metastatic melanomas harbor *GAB2* amplification (Horst *et al.*, 2009). Given that overexpression of Gab2 has been observed in several cancers including ovarian cancer, breast cancer, leukemia and melanoma (Bocanegra *et al.*, 2009; Horst *et al.*, 2009), it raises the possibility that *GAB2* is a potential proto-oncogene.

1.4.3.1 Gab2 in breast cancer

Breast cancer is the second leading cause of cancer mortality in women in the United States, and it has a poor prognosis. The contribution of Gab2 to breast cancer development has been well studied. Silencing of Gab2 in several breast cancer cell lines, such as SUM52, SUM44PE and MDA468, inhibits cell proliferation, survival and invasion (Bocanegra *et al.*, 2009). Deregulation of *ERBB2* (*HER2*) is one of the primary causes of breast cancer, which was determined many years ago (Schechter *et al.*, 1984). Recently, studies have revealed that Gab2 is required in ErbB2-overexpressed cancer cell lines (Bocanegra *et al.*, 2009). For instance, in the breast cancer cell line BT474 with ErbB2 overexpression, it was shown that knockdown of Gab2 not only inhibited the activities of downstream effectors, such as ERK1/2 and Akt but also reduced cell proliferation (Bocanegra *et al.*, 2009).

Understanding how *GAB2* is regulated at the transcriptional level has attracted much attention. This can be answered by studies concerning microRNAs (miRNAs). These small non-coding RNA molecules (21~25 nucleotides) are natural products, that are partially complementary to one or more messenger RNA (mRNA) molecules in the 3'-untranslated region and inhibit gene expression by mRNA cleavage, deadenylation, and translational repression (Ling *et al.*, 2013). By using *in situ* hybridization screening for the let-7 family of miRNA, one study has shown that let-7g is the only one in the let-7 family related to lymph node metastasis and the poor survival rate of breast cancer patients (Qian *et al.*, 2011). Downregulation of let-7g expression promoted rapid metastasis of non-metastatic mammary

carcinoma cells, leading to increased expression levels of Gab2, fibronectin 1 (FN1), matrix metalloproteinases (MMPs) as well as upregulated ERK1/2 activity (Qian *et al.*, 2011). These studies suggest that *GAB2* may be a new target of let-7g miRNA, and provide a new insight into Gab2 regulation in breast cancer.

Although considered as an "amplifier" in Neu/ErbB2-induced tumorigenesis, overexpression of Gab2 alone is insufficient to induce tumor in mice. To address this, the human MCF-10A mammary epithelial cell is widely used as a cell model for better understanding the underlying mechanisms of Gab2 in breast cancer development. It was shown that Gab2 cooperation with other oncogenes, including ErbB2 and Src, was necessary for mammary epithelial cell transformation (Bentires-Alj *et al.*, 2006; Bennett *et al.*, 2008). Furthermore, Ke *et al.* determined that ErbB2-mediated tyrosine phosphorylation of Gab2 was required for the Gab2-induced invasive phenotype (Ke *et al.*, 2007). Consistent with these results, overexpression of ErbB2 with or without Gab2 was found to induce mammary carcinogenesis in mice, whereas overexpression of Gab2 alone was insufficient (Bentires-Alj *et al.*, 2006). Notably, the tumor-free period in mice was found to be dependent on Gab2 expression levels. The more Gab2 was expressed, the shorter the tumor-free period was in these mice (Bentires-Alj *et al.*, 2006). Therefore, these results suggest that the contribution of Gab2 to tumor development depends on its upstream inducers and is also related to its expression level. Aside from being downstream of RTKs, Gab2 is also a substrate of the non-receptor kinase c-Src (Bennett *et al.*, 2008). Coexpression of c-Src and Gab2 in MCF-10A cells promoted EGF-independent acinar growth. Consistent with this result, Gab2 cooperation with an activated form of c-Src Y527F enhanced MCF-10A cells to a more dispersed phenotype. However, p85 recruitment is dispensable in this process. In addition, it appears that activation of Src-STAT3 signaling is another potential mechanism behind the Gab2/Src-induced invasive phenotype (Mira *et al.*, 2009).

Although Gab2-induced cell motility has been well characterized, the mechanisms by which Gab2 is involved remain elusive. Some studies have indicated that Gab2-mediated cell motility is regulated by RhoA activity (Herrera Abreu *et al.*, 2010). RhoA belongs to the Rho GTPase family, which is involved in several cellular functions, such as cytoskeleton dynamics, cell polarity, and cell movement (Schwartz, 2004). Overexpression of Gab2 in MCF-10A

cells results in decreased RhoA activity, less cell spreading, and more migration (Herrera Abreu *et al.*, 2010). Given that constitutive expression of active RhoA restores stress fibers and focal adhesions, it suggests that Gab2 is upstream of RhoA. In addition, Shp2 recruitment to Gab2 also modulates cell spreading, but the underlying mechanisms remain unclear. Together, these studies indicate new mechanisms by which Gab2 regulates cell motility, and shed light on the role of Gab2 in disease treatments.

1.4.3.2 Gab2 in melanoma

Genetic alterations of *BRAF*, *NRAS*, and *KIT* by mutation frequently occur in metastatic melanoma patients leading to hyperactivation of the MAPK and PI3K pathways. In order to identify the genome-wide copy number of *GAB2*, bacterial artificial chromosome (BAC) array comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) arrays were adapted (Horst *et al.*, 2009). Based on these approaches, it was shown that the gene copy level of *GAB2* in metastatic melanoma tissues and melanoma cell lines was higher compared to melanocytic *nevi*, as well as melanoma cell lines with low metastatic potential, indicating that Gab2 acts as a diagnostic marker in melanoma. As the amplification level of the *GAB2* gene correlates with protein expression levels, knockdown of *GAB2* in metastatic melanoma cell lines was found to result in decreased cell invasion, whereas overexpression of Gab2 led to tumor growth and metastasis (Horst *et al.*, 2009).

In melanocytes, Gab2 cooperates with Ras^{G12V} to promote tumor development. Yang *et al.* observed that Gab2 accelerated tumor angiogenesis in *NRAS*-driven melanoma as a result of upregulation of HIF-1α (hypoxia-inducible factor 1-alpha) and VEGF (Yang *et al.*, 2012). However, the molecular mechanisms with regard to these findings are ill-defined. Ras^{G12V} cannot induce Gab2 tyrosine phosphorylation; instead, it promotes Ser/Thr phosphorylation on Gab2. Compared to the well characterized mechanisms of tyrosine phosphorylated Gab2, less is known about Ser/Thr phosphorylation. Therefore, more in-depth studies are needed to better understand how Ser/Thr phosphorylation modulates Gab2 function.

1.4.3.3 Gab2 in leukemia

Bcr-Abl is an oncoprotein associated with chronic myelogenous leukemia (CML) that results from a translocation between chromosomes 9 and 22 (Chu *et al.*, 2007). This fusion

tyrosine kinase is hyperactivated even at basal level owing to its auto-phosphorylation on Tyr^{177}. Phosphorylated Bcr-Abl associates with the Grb2/Gab2 complex, and thus initiates the activation of diverse downstream signaling pathways: Ras/MAPK, PI3K/Akt, and JAK/STAT (Sattler *et al.*, 2002).

Tyrosine phosphorylated Gab2 by Bcr-Abl induces Shp2 recruitment. Shp2 is a tyrosine phosphatase that behaves as an oncoprotein in tumorigenesis. In an inactive state, Shp2 is auto-inhibited since its SH2 domains bind to its phosphatase domain (PTP) resulting in a blockage of substrates to PTP (Li *et al.*, 2012). Upon activation, SH2 domains of Shp2 bind to some tyrosine phosphorylated proteins such as Gab2, leading to PTP accessible to substrates. Being a scaffold protein, Gab2 serves as a platform for protein-protein interactions and amplifies signal transduction. Interaction between Gab2 and Shp2 enhances signals from upstream of Ras and tranduces them to downstream effectors, such as ERK1/2 and STAT5 (Scherr *et al.*, 2006). In Bcr-Abl-mediated CML, knockdown of *GAB2*, *SHP2*, or *STAT5* inhibits cell proliferation and colony formation (Scherr *et al.*, 2006).

Tyrosine kinase inhibitors (TKIs), such as imatinib and nilotinib, have been used in CML treatment for years. However, certain TKIs can cause unexpected upregulation of downstream signals, leading to drug resistance in CML treatment. One such example is a LYN (tyrosine-protein kinase) inhibitor that prolongs tyrosine phosphorylation of Gab2 and Bcr-Abl (Wu *et al.*, 2008). Silencing of Gab2 results in increased TKI sensitivity in CML cells, suggesting that Gab2 participates in protecting CML cells from a variety of Bcr-Abl inhibitors (Wohrle *et al.*, 2012). In addition, Ding *et al*. found that nilotinib was able to induce activation of the PI3K pathway (Ding *et al.*, 2013). These results suggest that a combination of TKI inhibitors and PI3K/mTOR inhibitors would be a new strategy in the treatment of TKI-resistant BCR-ABL positive leukemia.

1.4.4 Feedback regulation of Gab2 function

The roles of Gab2 in cancer cell proliferation and invasion suggest that Gab2 is a potential therapeutic target for cancer treatment. Tyrosine phosphorylation is crucial for Gab2-dependent functions. Tyrosine-phosphorylated residues in Gab2 mediate its interaction with several adaptor proteins, such as Shp2 and p85, resulting in activation of the MAPK and the PI3K pathways, respectively. In addition, some studies suggest that other

post-translational modulations, such as Ser/Thr phosphorylation, can also regulate Gab2-dependent functions. Details regarding this regulation will be discussed below.

Negative regulation by the PI3K/Akt pathway

Gab2 acts as a central node in transducing signals from receptors to downstream targets. Upon stimulation with Heregulin (HRG), tyrosine-phosphorylated Gab2 enhances activation of the PI3K pathway. Inhibition of Akt activation by a PI3K inhibitor, wortmannin, increases tyrosine phosphorylation of Gab2, suggesting that Akt negatively regulates Gab2-dependent signaling (Lynch and Daly, 2002). Furthermore, Gab2 is identified as a substrate of Akt, which phosphorylates Gab2 on Ser^{159}. Mutation of Ser^{159} to unphosphorylatable Ala not only increases Gab2 tyrosine phosphorylation, but also promotes Shc and ErbB2 binding to Gab2, leading to the transformation of NIH 3T3 fibroblast cells (Lynch and Daly, 2002).

Feedback regulation of Gab proteins by MAPK signaling

In the Gab family, Gab1 was the first member identified as an ERK1/2 substrate (Roshan *et al.*, 1999). In 1999, Gab1 was found to associate with activated ERK2 upon EGF or HGF stimulation (Roshan *et al.*, 1999). It was also observed that the MBD (c-Met–binding domain), a domain directly involved in Gab1's association with c-Met (hepatocyte growth factor receptor HGFR), was also responsible for the ERK1/2 interaction (Roshan *et al.*, 1999). Later, several ERK1/2 phosphorylation sites in Gab1 were determined by a mass spectrometry approach (Lehr *et al.*, 2004). Among these sites, mutation of Ser^{552} to Ala blocked Gab1 localization at the plasma membrane (Eulenfeld and Schaper, 2009). Interestingly, it appears that phosphorylation of Gab1 by ERK1/2 has different functions in response to diverse forms of stimulation. For instance, whereas ERK1/2 phosphorylation of Gab1 upon EGF stimulation inhibits p85 recruitment (Yu *et al.*, 2002), HGF-induced ERK1/2 phosphorylation of Gab1 increases p85 interaction and its downstream effectors activity, such as Akt (Yu *et al.*, 2001). Therefore, the roles of ERK1/2 in regulating Gab1 functions remain controversial, and more in-depth studies are required to fully understand the intricate mechanism by which Gab1 is regulated in response to different forms of stimuli.

Similar to Gab1, several Ser/Thr phosphorylation sites in Gab2 have been identified, suggesting that Gab2 is a potential target for Ser/Thr protein kinases (Halbach *et al.*, 2013). It

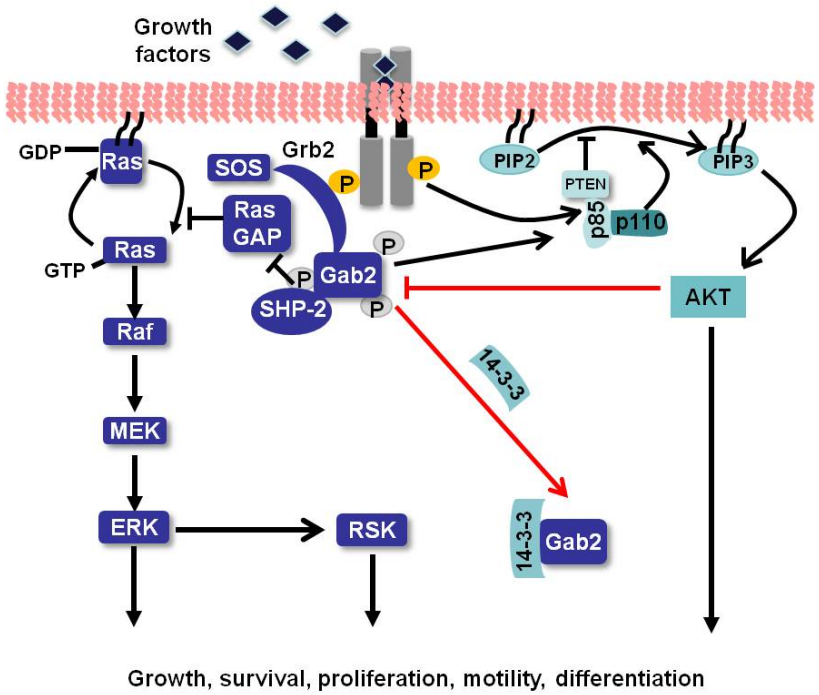

Figure 1.13 Schematic representation of feedback regulation of Gab2-dependent signaling. Upon RTKs activation, Gab2 is phosphorylated at tyrosine residues, leading to increased activation of the Shp2/MAPK and p85/PI3K pathways. However, phosphorylation of Gab2 by Akt on Ser^{159} attenuates Gab2-dependent signaling. Moreover, phospho-Ser/Thr residues mediate Gab2/14-3-3 interaction, which in turn terminates Gab2-dependent biological functions, such as cell transformation.

was shown that inhibition of the MAPK pathway upregulates Gab2 tyrosine phosphorylation, indicating that the MAPK pathway negatively regulates Gab2 function (Arnaud *et al.*, 2004). Mutation of the ERK-mediated phosphorylation site, Ser^{623}, to unphosphorylatable Ala in Gab2 was shown to sustain Shp-2 recruitment concomitantly with a decrease in STAT5 activity.

Role of 14-3-3 in terminating Gab2 function

14-3-3 proteins were the first known phospho-serine/threonine-binding proteins (Freeman and Morrison, 2011). It has been found that 14-3-3 proteins are widely expressed in all eukaryotic cells. Binding of 14-3-3 to a number of proteins are involved in diverse biological events including cell proliferation, cell migration, and the epithelial-to-mesenchymal transition (EMT) (Freeman and Morrison, 2011). In general, 14-3-3 proteins bind to many

target proteins through two phospho-Ser/Thr modes: (1) an RXpS/TXP motif (mode I); (2) an RXXpS/T motif (mode II). Besides, certain ligands binding to 14-3-3 proteins is through the C-terminal binding consensus (mode III). Intriguingly, it was found that around 0.6% of the human proteome had 14-3-3 proteins engaged, suggesting that 14-3-3 proteins are key regulatory components in modulating normal growth and development in human (Jin *et al.*, 2004). Gab2 was identified as a binding protein to 14-3-3 upon EGF stimulation. Phosphorylation on Ser^{210} and Thr^{391} in Gab2 was found to be crucial for the Gab2/14-3-3 interaction. Mutations of Ser^{210} and Thr^{391} to Ala in Gab2 enhanced Grb2 recruitment resulted in MCF-10A cell proliferation and transformation (Brummer *et al.*, 2008). Meanwhile, constitutive 14-3-3 binding to Gab2 led to impaired Gab2-dependent biological functions such as cell proliferation, cell transformation and cell motility (Herrera Abreu *et al.*, 2010).

Taken together, these findings provide important information on the feedback regulation of Gab2-dependent functions. Based on the essential roles of Gab2 in the receptor-mediated signaling pathways, these findings also expand our knowledge and pave the way to develop novel therapeutics for cancer treatment.

Implication of feedforward and feedback loops in diseases

Pathway cross-talk affects intracellular signal transduction, leading to decreased or increased target protein activities. Understanding feedforward and feedback loops has a significant implication in disease treatments, including cancer and inflammatory diseases. On certain conditions, such as drug treatments, pathway cross-talk results in upregulated compensatory signaling cascades. For instance, disruption of a feedforward loop of VEGF-VEGFR-Akt-mTOR-VEGF by an inhibitor (ZD6474) of VEGFR2 switches tumor cells from an angiogenic to a proliferative phenotype with feedback activation of the IRS/MAPK signaling cascade (Chatterjee *et al.*, 2013). Hence, co-treatment of lung cancer patients with both VEGFR2 (ZD6474) and MEK1/2 (PD0325901) inhibitors is found to be more effective to inhibit tumor growth than either of them alone (Chatterjee *et al.*, 2013). In addition, it has been found that inhibition of either the PI3K/Akt/mTOR or Ras/MAPK pathway leads to upregulation of its compensatory signaling cascade, allowing tumor cells to survive and evade apoptosis (Carracedo *et al.*, 2008; Turke *et al.*, 2012). Co-inhibition of both the PI3K/Akt/mTOR and Ras/MAPK pathways counteracts this feedback regulation and is

more efficient in cancer treatments. Interestingly, the balance between feedforward and feedback inhibition also plays a key role in normal regulation of macrophage activation. Whereas TLR (toll-like receptor) induces production of an inflammatory cytokine TNF (tumor necrosis factor) and an anti-inflammatory molecule IL-10 at the same time, IL-10, in turn, inhibits TNF secretion in a STAT3-dependent manner (Hu *et al.*, 2008). Given that dysregulation of the balance between these feedforward and feedback loops leads to autoimmune diseases, such as high level of IL-10 inducing rheumatoid arthritis (RA), it is suggested that perturbation of IL-10 production would be a potential therapeutic target for this disease (Cush *et al.*, 1995).

1.5 Rationale and objectives

1.5.1 Rationale

GAB2 is located on chromosome 11q14.1, a region frequently amplified in breast cancers, melanomas, ovarian cancers and leukemia. As a scaffold protein, Gab2 serves as a platform that assembles multiple signal relay proteins together. In response to the stimulation of several growth factors and cytokines, Gab2 becomes phosphorylated by activated RTKs on Tyr residues, and thus associates with diverse adaptor molecules, such as Shp2 and p85, leading to the activation of MAPK and PI3K signaling, respectively. To date, the essential role of tyrosine-phosphorylated Gab2 in signal transduction has been well characterized. However, we noted that agonists also promote Gab2 phosphorylation on Ser/Thr residues independently of tyrosine phosphorylation, but the roles of these phosphorylation events, how they are regulated, and by which protein kinase(s) remains elusive. Therefore, the primary goal of my Ph.D. thesis was to determine how Gab2 is regulated at the molecular level by MAPK signaling, and how these events regulate Gab2 function, especially in the context of breast cancer cell growth, proliferation and migration.

1.5.2 Objectives

There are three main objectives to this thesis:

1. Given that the exact kinases in the MAPK pathway involved in phosphorylation of Gab2 are ill-defined, we intend to demonstrate that the MAPK pathway, through both ERK1/2 and RSK, phosphorylates Gab2 on several Ser/Thr residues.

2. Less is known about the molecular mechanisms by which MAPK signaling regulate Gab2 functions. In this objective, we will characterize that RSK and ERK1/2-mediated feedback loops have different roles on stabilizing the Gab2-mediated complex.

3. To further determine the physiological functions of these two feedback loops, we developed an *in vitro* cell model to fully understand the different impacts of phosphorylated Gab2 by RSK and ERK1/2 signaling.

Preface to Chapter 2

Accumulating evidence has shown that Gab2 is overexpressed in several malignancies, such as breast cancer, melanoma, ovarian cancers and leukemia. Although the contribution of tyrosine phosphorylated Gab2 to signal transduction has been well characterized, it is still unclear how Gab2 is regulated at the molecular level. Upon stimulation by agonists, our group has found that activation of the Ras/MAPK pathway induces an electrophoretic mobility shift in Gab2. This result suggests that phosphorylation a protein kinase within this pathway directly regulate Gab2 phosphorylation. In this study, we determined that Gab2 is a novel substrate of the MAPK-activated protein kinase RSK by using pharmacological inhibitors and RNA interference. Using quantitative mass spectrometry and through the generation of unphosphorylatable mutants, we further defined that RSK phosphorylates Gab2 on Ser^{160}, Ser^{211} and Ser^{620}. Expression of an unphosphorylatable Gab2 mutant (S3A) in MCF-10A cells resulted in an invasive-like phenotype and increased cell motility, suggesting that RSK participates in a negative feedback loop to inhibit Gab2-dependent function. We found that Shp2 recruitment to Gab2 was affected by RSK-mediated phosphorylation, implicating RSK in the regulation of Gab2-Shp2 signaling.

Chapter 2 Gab2 phosphorylation by RSK inhibits Shp2 recruitment and cell motility

Mol Cell Biol. 2013 Apr; 33(8):1657-70. doi: 10.1128/MCB.01353-12. Epub 2013 Feb 11.

Gab2 Phosphorylation by RSK Inhibits Shp2 Recruitment and Cell Motility

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ABSTRACT

The scaffolding adapter protein Gab2 (Grb2-associated binder) participates in the signaling response evoked by various growth factors and cytokines. Gab2 is overexpressed in several human malignancies, including breast cancer, and was shown to promote mammary epithelial cell migration. The role of Gab2 in the activation of different signaling pathways is well documented, but less is known regarding the feedback mechanisms responsible for its inactivation. We now demonstrate that activation of the Ras/mitogen-activated protein kinase (MAPK) pathway promotes Gab2 phosphorylation on basic consensus motifs. More specifically, we show that RSK (p90 ribosomal S6 kinase) phosphorylates Gab2 on three conserved residues, both *in vivo* and *in vitro*. Mutation of these phosphorylation sites does not alter Gab2 binding to Grb2, but instead, we show that Gab2 phosphorylation inhibits the recruitment of the tyrosine phosphatase Shp2 in response to growth factors. Expression of an unphosphorylatable Gab2 mutant in mammary epithelial cells promotes an invasion-like phenotype and increases cell motility. Taken together, these results suggest that RSK is part of a negative-feedback loop that restricts Gab2-dependent epithelial cell motility. On the basis of the widespread role of Gab2 in receptor signaling, these findings also suggest that RSK plays a regulatory function in diverse receptor systems.

INTRODUCTION

The binding of growth factors to receptor tyrosine kinases (RTKs) regulates a number of biological processes, such as cell cycle progression, migration, metabolism, survival, and differentiation (1). Upon activation, RTKs recruit several docking adapter proteins that play a key role in transducing extracellular signals to downstream signaling cascades, such as the Ras/mitogen-activated protein kinase (MAPK) pathway (2, 3). The adapter protein Grb2 (growth factor receptor-bound protein 2) links the receptors to the MAPK pathway by binding to the Ras-specific guanine nucleotide exchange factor (GEF) son of sevenless (SOS). Recruitment of SOS to the plasma membrane leads to the activation of Ras, which results in the sequential phosphorylation and activation of Raf, MEK1/2, and extracellular signal-regulated kinase 1/2 (ERK1/2) protein kinases (4). Once activated, ERK1/2 phosphorylate numerous cytoplasmic and nuclear substrates, including the p90 ribosomal S6 kinase (RSK) family of Ser/Thr kinases (5, 6), which often collaborate with ERK1/2 to regulate cell growth, survival, and proliferation.

In addition to SOS, Grb2 associates with a number of docking adapter proteins that modulate RTK signaling (7). Members of the Grb2-associated binder (Gab) family of proteins function downstream of a variety of RTKs and comprise three vertebrate members (Gab1 to Gab3) (8, 9). These proteins mainly function as RTK signal transducers that activate pathways involved in cell growth, proliferation, and motility. Gab proteins contain several highly conserved regions, including an N-terminal pleckstrin homology (PH) domain, a central proline-rich domain, and multiple phosphotyrosine residues (9). While the PH domain binds membrane-associated phospholipids, the proline-rich domain contains several PXXP motifs that serve as docking sites for Src homology 3 (SH3) domain-containing proteins, including Grb2 (10). The Gab proteins harbor several tyrosine residues that become phosphorylated upon RTK activation. Many of these phosphorylated tyrosines are capable of interacting with Src homology 2 (SH2) domain-containing proteins, including the protein tyrosine phosphatase Shp2 and the p85 subunit of phosphoinositide 3-kinase (PI3K). Recruitment of these proteins leads to the activation and potentiation of the Ras/MAPK and PI3K/Akt pathways, respectively (9).

The Gab proteins perform important functions in normal physiology, and certain Gab

isoforms also contribute to human malignancies (11). The GAB2 gene is frequently amplified in human cancer and was identified to be a potential oncogene in breast and ovarian cancers, as well as leukemia and melanoma (12). While Gab2 appears to be insufficient to transform primary mammary epithelial cells, it was shown to cooperate with ErbB2 (Neu or HER2) to potentiate tumorigenic signaling (13–15). Gab2 seems to contribute to a metastatic phenotype in breast cancer, as its overexpression in human mammary epithelial cells results in increased proliferation, invasiveness, and motility (13–15). The mechanisms by which Gab2 contributes to breast cancer are not fully understood, but Shp2 recruitment and the subsequent activationof the Ras/MAPK pathway were shown to be required (14). Moreover, recent evidence indicates that Gab2 regulates cytoskeletal organization and mammary epithelial cell motility through the recruitment of Shp2 (16).

The main role of Gab2 is to activate downstream signaling cascades via tyrosine phosphorylation and SH2 domain interactions, such as with Shp2. Conversely, Gab2 phosphorylation on Ser/Thr residues was previously reported to play inhibitory roles. Akt was shown to regulate the phosphorylation of Ser159, resulting in reduced ErbB2-mediated tyrosine phosphorylation through unknown mechanisms (17). ERK1/2 also phosphorylates Gab2 on Ser613, which was found to modulate Shp2 recruitment in response to interleukin-2 (IL-2) (18). More recently, phosphorylation of Gab2 on Ser210 and Thr391 by an unknown protein kinase was shown to promote 14-3-3 binding, resulting in reduced Grb2 binding and tyrosine phosphorylation (19). In the current study, we describe the regulation of Gab2 phosphorylation on Ser/Thr residues in response to the Ras/MAPK pathway. Our results indicate that RSK directly phosphorylates Gab2 on three serine residues, both *in vivo* and *in vitro*. We show that RSK-mediated Gab2 phosphorylation inhibits Shp2 recruitment, suggesting that RSK mediates a negative-feedback loop that attenuates Gab2-dependent functions, including cell motility.

MATERIALS AND METHODS

DNA constructs and recombinant proteins. The plasmids encoding hemagglutinin (HA)-tagged murine Gab1 and Gab2 were provided by Morag Park (McGill University, Canada) and Isabelle Royal (University of Montreal, Montreal, Quebec, Canada), respectively, and described previously (20, 21). The vectors encoding constitutively active forms of Ras (G12V) and MEK1 (MEK-DD) and the inactive form of Ras (S17N) were described previously (22, 23). All HA-tagged RSK1 constructs were described previously (24). To subclone murine Gab1 and Gab2 into pcDNA3.0-6myc, HA-tagged Gab1 and Gab2 were amplified by PCR using these primers: primers Gab1-sense (5`-GCTTAGAATTCTATGAGCGGCGGCGAAGTGG-3`) and Gab1-antisense (5`-GCATAGAATTCCTACTTCACATTCTTGGTGGGTG-3`) and primers Gab2-sense (5`-GCTTACTCGAGTATGAGCGGCGGCGGCGGCGACGACGT-3`) and Gab2-antisense (5`-GCATACTCGAGTCATTACAGCTTGGCACCCTTGGAAG-3`), respectively. All murine Gab2 mutants were generated using the QuikChange methodology (Stratagene, La Jolla, CA). To subclone murine Gab2 into pBabe-puro and produce retroviral particles used in the generation of stable cell lines, Myc-tagged wild-type (wt) and mutant Gab2 were amplified by PCR using these primers: Gab2-sense (5`-GCTTAGGATCCATTTAAAGCTATGGAGCAAAAGC-3`) and Gab2-antisense (5`-GCATAGGATCCTCATTACAGCTTGGCACCCTTGGAAG-3`).

Antibodies. Antibodies targeted against Arg/Lys-X-X-pSer/Thr (RXXpS/T; X is any amino acid) and Arg/Lys-X-X-pSer/Thr-X-Pro (RXXpS/TXP) consensus sequences, Gab2, RSK1 to RSK3, phospho-Akt (S473), Akt, phospho-Gab2 (S159), p85, ERK1/2, phospho-ERK1/2 (T202/Y204), phospho-RSK (S380), and Shp2 were purchased from Cell Signaling Technologies (Beverly, MA). The Shp2 antibody used for immunoprecipitation was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-Myc and anti-HA monoclonal antibodies were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). All secondary horseradish peroxidase (HRP)-conjugated antibodies used for immunoblotting were purchased from Chemicon (Temecula, CA).

Cell culture and transfection. HEK293 and mouse embryonic fibroblasts (MEFs) were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose

supplemented with 10% fetal bovine serum (FBS) and antibiotics. Wild-type and Gab2-deficient MEFs were described elsewhere (25). MCF-10A cells were cultured in DMEM–F-12 medium with growth medium (supplemented with 5% [vol/vol] horse serum [Invitrogen], 20 ng/ml human recombinant epidermal growth factor [EGF; R&D Systems, Minneapolis, MN], 0.5 μg/ml hydrocortisone [Sigma], 100 ng/ml cholera toxin [Sigma], 10 μg/ml bovine insulin [Sigma], 50 U/ml penicillin G [Invitrogen], and 50 μg/ml streptomycin sulfate [Invitrogen]). Gab2-deficient MEFs and MCF-10A stable cell lines were generated using pBabe-puro-derived retroviral particles, and expressing cells were selected using puromycin (2 μg/ml). HEK293 cells were transfected by calcium phosphate precipitation as previously described (26). Cells were grown for 24 h after transfection and serum starved using serum-free DMEM where indicated for 16 to 18 h. Starved cells were pretreated with PD184352 (10 μM), U0126 (20 μM), or BI-D1870 (10 μM) (Biomol, Plymouth Meeting, PA), where indicated, and stimulated with FBS (10%), phorbol myristate acetate (PMA; 25 to 100 ng/ml), or EGF (25 ng/ml) before being harvested. Unless indicated otherwise, all drugs and growth factors were purchased from Invitrogen (Burlington, Ontario, Canada). The proliferation rate was measured by the colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay.

RNA interferences. For small interfering RNA (siRNA)-mediated knockdown of RSK1 and RSK2, validated 21-nucleotide cRNAs with symmetrical 2-nucleotide overhangs were obtained from Qiagen. HEK293 cells were transfected using calcium phosphate and 50 nM siRNA per dish. At 24 h following transfection, cells were serum starved overnight before being harvested.

Immunoprecipitations and immunoblotting. Cell lysates were prepared as previously described. Briefly, cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in CLB (10 mM K_3PO_4, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 50 mM -glycerophosphate, 0.5% Nonidet P-40, 0.1% Brij 35, 0.1% deoxycholic acid, 1 mM sodium orthovanadate [Na_3VO_4], 1 mM phenylmethylsulfonyl fluoride, and a cOmplete protease inhibitor cocktail tablet [Roche]). For immunoprecipitations, cell lysates were incubated with the indicated antibodies for 2 h, followed by a 1-h incubation with protein A–Sepharose CL-4B beads (GE Healthcare). Unless they were used for kinase assays, immunoprecipitates

were washed three times in lysis buffer and beads were eluted and boiled in 2×reducing sample buffer (5×buffer is 60mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue). Eluates and total cell lysates were subjected to 8 to 10% SDS-PAGE, and resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes for immunoblotting. Densitometry analysis was conducted using identical areas for each lane of a given blot and inverted histograms in the Adobe Photoshop CS6 package. After subtracting the background, Shp2 levels were normalized to the levels of immunoprecipitated Gab2.

Protein phosphotransferase assays. For RSK1 assays, transfected HA-tagged wt or kinase-inactive (kinase-deficient [kd]) RSK1 (K112/464R) was immunoprecipitated from cells lysed in BLB buffer, as previously described (26). Immunoprecipitates were washed thrice in BLB and twice in kinase buffer (25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM β-glycerophosphate). Kinase assays were performed with immunopurified full-length Myc-tagged wt Gab2 or the S160/211/620A mutant (from here on termed the S3A mutant) as the substrates under linear assay conditions. Assays were performed for 10 min at 30°C in kinase buffer supplemented with 5 μCi [λ-^{32}P]ATP per reaction. All samples were subjected to SDS-PAGE, and incorporation of radioactive phosphate (^{32}P) was determined by autoradiography using a Fuji PhosphorImager with Multi-Gauge (version 3.0) software. The data presented are representative of at least three independent experiments.

Digestion, TMT labeling, and mass spectrometry analysis. Following SDS-PAGE separation with Coomassie staining, bands corresponding to Gab2 were excised and digested in gel with sequencing-grade trypsin (Promega, Madison, WI) as described previously (27). Labeling with sixplex iobaric tandem mass tag (TMT^6) reagents (Thermo Scientific) was accomplished as published previously with minimal modifications (28, 29). Labeled peptides were then mixed and underwent C_{18} solid-phase extraction as described previously (30). Finally, combined samples were resuspended in 5% acetonitrile–5% formic acid for mass spectrometry analysis. All liquid chromatography (LC)-mass spectrometry (MS) experiments were performed on an LTQ-Velos-Orbitrap column (Thermo Fisher Scientific) equipped with a Famos autosampler (LC Packings) and an Agilent 1100 binary high-pressure liquid chromatography pump (Agilent Technologies) essentially as described previously (29). The

LTQ-Velos-Orbitrap was operated in the data-dependent mode and collected high-resolution Orbitrap MS/MS spectra after higher-energy C-trap dissociation for the top 10 most intense ions following each survey scan collected in the Orbitrap.

Peptide, protein, and phosphorylation site identification. Following acquisition, individual MS/MS spectra were assigned to peptides using the Sequest program (31). To maximize phosphorylation site identifications, a two-stage database searching strategy was employed. In the first stage, all MS/MS spectra were searched against a database containing all protein sequences from the human International Protein Index database (version 3.6) in the forward and reverse orientations as well as sequences of common contaminants. Initial searches were performed with the following parameters: 25-ppm precursor ion tolerance; 0.02-Da product ion tolerance; fully tryptic digestion with up to two missed cleavages; static modifications Cys alkylation (57.021464) and TMT labeling of Lys and peptide N termini (+229.162932); and dynamic modifications Met oxidation (+15.994915) and phosphorylation of Ser, Thr, and Tyr (+79.966330). The target-decoy approach (32) was then used to distinguish correct and incorrect peptide identifications using linear discriminant analysis based on several parameters, including Xcorr, dCn`, peptide length, precursor ion mass error, numbers of missed cleavages, peptide length, and charge state (33). After filtering to an initial 1% peptide-level false detection rate (FDR), peptides were then assembled into proteins, and proteins were scored and filtered to a final protein FDR of 1% (33).

In the second stage, relaxed parameters were used to match additional MS/MS spectra against a filtered database. Sequest was again employed, but this time the database was filtered to include only forward and reverse sequences for proteins that were identified in stage 1. Stage 2 search parameters included a 3.1-Da precursor ion tolerance; a 0.02-Da product ion tolerance; no enzyme specificity; static modifications Cys alkylation (+57.021464) and TMT labeling of Lys and peptide N termini (+229.162932); and dynamic modifications Met oxidation (+15.994915) and phosphorylation of Ser, Thr, and Tyr (+79.966330). The resulting peptides were again filtered via linear discriminant analysis, but this time, the number of tryptic ends was included as an additional feature and peptide mass errors were corrected to account for occasional incorrect monoisotopic mass assignments. To evaluate phosphorylation site localization, all phosphopeptides matching Gab2 were scored using the

Ascore algorithm (34) and peptides were grouped according to the phosphorylation sites that they contained. A minimum Ascore of 13 was required for phosphorylation site localization ($P < 0.05$), and phosphorylation site assignments were manually validated to ensure reliability.

Phosphorylation site quantification. Relative quantification of each peptide was accomplished on the basis of the intensities observed for all six reporter ions from high-resolution Orbitrap MS/MS spectra, after correcting for batch-specific isotopic enrichments of each TMT reagent. Each peptide was required to have a minimum isolation specificity of 0.75 (29) and a summed reporter ion intensity of at least 500 with no more than four missing reporter ions. Individual sites were quantified on the basis of the summed reporter ion intensities for all matching peptides. Nonphosphorylated peptides matching Gab2 were combined to estimate unmodified protein abundance. Quantitative profiles for all phosphorylation sites were normalized to account for slight changes in Gab2 abundance. Finally, analysis of variance (ANOVA) was used to identify statistically significant, site-specific changes in protein phosphorylation. Within each experiment, all P values were adjusted to account for multiple-hypothesis testing via the method of Hochberg and Benjamini (35).

Epifluorescence microscopy. For immunofluorescence analyses, 5×10^4 MCF-10A cells were seeded in 12-well plates containing coverslips. Twenty-four hours later, cells were washed twice in PBS and fixed in 3.7% formaldehyde for 10 min at room temperature. Cells were washed twice in PBS, permeabilized for 5 min in PBS containing 0.2% Triton X-100, and blocked with PBS containing 0.1% bovine serum albumin for 30 min. Cells were incubated for 2 h with anti-Myc antibodies, washed twice with PBS, and incubated for 1 h with a secondary Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen), Texas Red-phalloidin, and DAPI (4`, 6-diamidino-2-phenylindole) diluted in PBS. Images were acquired on a Zeiss Axio Imager Z1 wide-field fluorescence microscope using a ×40 oil-immersion objective.

Proliferation assays. For proliferation assays, MCF-10A cells were grown in medium supplemented with 10% FBS. The relative number of viable cells was measured every 24 h during four consecutive days using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-*2H*-tetrazolium,

inner salt (MTS), cell proliferation assay from Promega, as shown elsewhere (36). The absorbance was measured at 490 nm using a Tecan GENios Plus microplate reader, and the results displayed represent the mean of triplicates ± standard error (SE).

Cell migration assays. For the Transwell assays, MCF-10A cells stably expressing wt Gab2 or the S3A mutant were starved overnight, and 4×10^4 cells suspended in 100 µl of EGF-free medium were seeded in the top chamber of polyethylene terephthalate Transwells (24-well insert; pore size, 8 m; Becton, Dickinson). Cells were allowed to migrate toward 20 ng/ml EGF for 24 h at 37°C in the presence of dimethyl sulfoxide or PD184352 (1 µM), fixed with 10% Formalin, and stained with hematoxylin-eosin (H&E). Cells that did not migrate (top side of the filter) were wiped with a cotton swab. Filters were scanned, and migrated cells were automatically counted using the VisiomorphDP tool (Visiopharm).

For the live-cell-imaging wound-healing assays, an Oris cell migration assay kit (Platipus Technologies) was used. Cells were seeded at 100% confluence (1×10^4 cells) into 96 wells containing a round disk in the center, for creation of the detection zone, and allowed to adhere overnight. Disks were then removed, allowing cells to migrate to the center of the well. Cells were filmed at 37°C with a DeltaVision microscope (Olympus U Plan S-Apo×20/0.75 numerical aperture) using softWoRx software equipped with a camera (CoolSNAP HQ2). Images were acquired every 20 min for 24 h. Tracking was performed on phase-contrast image series using ImageJ software (http://rsb.info.nih.gov/ij/).

RESULTS

Identification of Gab2 as a substrate of the Ras/MAPK pathway.

To determine if the Ras/MAPK pathway promotes Gab2 phosphorylation on basic consensus motifs, we used two phosphorylation site-specific antibodies directed against similar consensus sequences: Arg/Lys-X-X-pSer/Thr (RXXpS/T) and Arg/Lys-X-XpSer/Thr-X-Pro (RXXpS/TXP). These consensus sequences are often found in substrates of AGC family kinases, such as Akt, RSK, and S6K1 (37). HEK293 cells transfected with Myc-tagged mouse Gab2 were serum starved overnight and stimulated with different agonists. Immunoprecipitated Gab2 was then analyzed for phosphorylation by immunoblotting using the anti-RXXpS/T and anti-RXXpS/TXP antibodies. With this approach, we found that treatment of cells with agonists of the Ras/MAPK pathway, including the phorbol ester PMA, epidermal growth factor (EGF), and serum (10%), led to the phosphorylation of Gab2 on basic consensus motifs (Fig. 2.1A). Gab2 phosphorylation correlated with the phosphorylation of both ERK1/2 (T202/Y204) and RSK (S380), suggesting that this pathway converges on Gab2 to promote its phosphorylation. The involvement of the Ras/MAPK pathway was further confirmed using constitutively activated (G12V) and dominant-negative (S17N) forms of H-Ras. We found that expression of RasG12V strongly stimulated Gab2 phosphorylation in the absence of serum or growth factors (Fig. 2.1B), indicating that Ras-dependent signaling is sufficient to promote Gab2 phosphorylation. To determine if the Ras/MAPK pathway specifically targets the Gab2 isoform, we performed a similar analysis of Gab1 and Gab3, which display ~38% amino acid identity with Gab2. While we found that Gab3 is not sufficiently soluble to allow efficient immunoprecipitation, our results indicated that Gab1 is not significantly phosphorylated on basic consensus sites compared to the level of phosphorylation of Gab2 (Fig. 2.1C). To determine if endogenous Gab2 is also regulated by the Ras/MAPK pathway, we used the RBL-2H3 leukemic cell line, which was previously shown to express high levels of Gab2 (38). Importantly, we found that stimulation of the Ras/MAPK pathway in these cells using dinitrophenyl produced a robust increase in endogenous Gab2 phosphorylation on basic consensus sites (Fig. 2.1D). Together, these findings demonstrate that growth factors and mitogens promote the specific phosphorylation of Gab2 through a pathway that likely involves ERK1/2 signaling.

RSK phosphorylates Gab2 at basic consensus motifs *in vivo* and *in vitro*.

RSK is the most likely basophilic protein kinase operating downstream of the MAPK pathway (5). To test its potential involvement in the regulation of Gab2 phosphorylation, we used a MEK1/2 inhibitor (PD184352), which prevents ERK1/2 from activating RSK, as well as an RSK inhibitor (BI-D1870) to directly block its activity (Fig. 2.2A). We found that treatment of cells with PD184352 or BI-D1870 strongly prevented Gab2 phosphorylation in response to PMA stimulation (Fig. 2.2B), suggesting that Gab2 is an RSK substrate in cells. The involvement of ERK/RSK signaling was further confirmed by expressing a constitutively activated form of MEK1 (MEK-DD; S212/218D), which was found to be sufficient to promote Gab2 phosphorylation in serum-starved cells (Fig. 2.2C). We found that treatment of these cells with PD184532 or BI-D1870 completely prevented Gab2 phosphorylation induced by MEK-DD expression, suggesting that RSK is the predominant basophilic kinase regulating Gab2 phosphorylation downstream of the MAPK pathway. Consistent with this, we found that knockdown of RSK1 and RSK2 significantly reduced Gab2 phosphorylation induced by expression of MEK-DD (Fig. 2.2D) or PMA stimulation (Fig. 2.2E), indicating that RSK1 and RSK2 are required for the phosphorylation of Gab2 in cells.

To determine if RSK directly phosphorylates Gab2, we performed *in vitro* kinase assays with purified proteins and [λ-^{32}P]ATP. HEK293 cells were transiently transfected with wt or kinase-deficient (K112/464R) HA-tagged RSK1, and purified RSK1 from unstimulated or PMA-treated cells was incubated in a reaction buffer with full-length Myc-Gab2 immunopurified from serum-starved cells. Although low levels of ^{32}P label incorporation were detected in purified Gab2 incubated with unstimulated RSK1, we found that activated RSK1 robustly increased ^{32}P label incorporation (~12-fold) in purified Gab2 (Fig. 2.2F). The phosphotransferase activity of RSK1 was found to be necessary for this effect, as the kinase-deficient form of RSK1, which retained some ability to autophosphorylate, did not have significantly increased ^{32}P label incorporation in Gab2. Taken together, our results indicate that RSK directly promotes Gab2 phosphorylation in vivo and in vitro in response to Ras/MAPK pathway activation.

Identification of Ser160, Ser211, and Ser620 as RSK-dependent phosphorylation sites.

To identify the RSK-dependent phosphorylation sites in Gab2, we analyzed the sequence surrounding all Ser/Thr residues for similarities to phosphorylation sites in known substrates of RSK (5). We located six potential consensus phosphorylation sites (RXXpS/T), consisting of Ser160, Ser211, Thr256, Thr388, Ser434, and Ser620 (according to mouse Gab2 numbering). To determine whether RSK phosphorylates these sites in cells, each one was individually mutated to an unphosphorylatable alanine residue and Gab2 phosphorylation was assessed using phosphorylation site-specific antibodies against basic consensus sequences. While mutation of Thr256, Thr388, and Ser434 did not affect Gab2 phosphorylation, we found that mutation of Ser160, Ser211, and Ser620 partly prevented Gab2 phosphorylation induced by PMA stimulation, as detected by the anti-RXXpS/TXP or the anti-RXXpS/T antibodies (Fig. 2.3A). These results suggested that these three serine residues are directly phosphorylated by RSK in cells.

To further validate the link between Gab2 phosphorylation and RSK, we made use of a phosphorylation site-specific antibody raised against Ser160 (Ser159 in human Gab2), a site previously shown to be regulated by Akt (17). HEK293 cells were transfected with wt or kinase-deficient RSK1 (K112/464R), and endogenous Gab2 phosphorylation at Ser160 was assessed by immunoblotting on total cell lysates. Compared to control cells, we found that expression of wt RSK1 specifically increased Gab2 phosphorylation at Ser160 after PMA stimulation (Fig. 2.3B), suggesting that Gab2 is a substrate of RSK in cells. Expression of wt RSK1 induced Gab2 phosphorylation even in the absence of serum and PMA stimulation, consistent with the idea that expression of the wt protein increases basal RSK activity (Fig. 2.3B). RSK1 phosphotransferase activity was found to be required for stimulating Gab2 phosphorylation, as an RSK1 mutant with inactivating mutations in both kinase domains (K112/464R) did not increase Gab2 phosphorylation to a level over the level already stimulated by endogenous RSK activity (Fig. 2.3B). In order to validate that ERK/RSK signaling is required for Gab2 phosphorylation at Ser160 in cells, we pretreated cells with MEK1/2 inhibitors (PD184352, U0126) or an RSK inhibitor (BI-D1870) prior to stimulation with PMA. We found that both MEK1/2 and RSK inhibitors almost completely abrogated PMA-induced Gab2 phosphorylation at Ser160 (Fig. 2.3C), suggesting that RSK activity is

required for Gab2 phosphorylation in cells. Residual levels of Ser160 phosphorylation may be due to other basophilic kinases, such as Akt, which was previously shown to phosphorylate this site in Gab2 (17).

While there are no phosphorylation site-specific antibodies against Ser211 and Ser620, we sought to confirm that these RSKdependent phosphorylation sites are modulated in cells using a quantitative mass spectrometry (MS)-based approach. HEK293 cells were transfected with Myc-tagged Gab2, serum starved overnight, and pretreated with vehicle or the MEK1/2 inhibitor PD184352, prior to being stimulated with PMA (Fig. 2.3D). Alternatively, cells were cotransfected with an empty vector or constitutively activated (G12V) or dominant-negative (S17N) Ras for 48 h prior to serum starvation and cell lysis (Fig. 2.3D). Immunoprecipitated Gab2 was then isolated via SDS-PAGE, digested in-gel with trypsin, and labeled with 6-plex isobaric tandem mass tags (TMT^6), as done previously (28). Samples were then combined and analyzed by liquid chromatography-assisted tandem MS, and the relative abundances of all identified phosphopeptides were measured across experimental conditions (Fig. 2.3D). Using this approach, we found that two phosphopeptides containing basic consensus sequences showed significant changes (ANOVA; FDR<5%) between the activated (PMA or RasG12V) and inactive (PD184352 or RasS17N) conditions (Fig. 2.3E and F, insets). These phosphopeptides were found to contain Ser211 and Ser620, and identification of these residues as being phosphorylated was obtained by MS/MS sequencing, as depicted by the annotated high-resolution MS/MS spectra (Fig. 2.3E and F). While previous studies have reported Ser160 as being phosphorylated in cells, we were not able to identify this phosphopeptide in the current study, likely due to the large size of the tryptic peptide containing Ser160 (44 amino acids). Together, our data indicate that RSK regulates the phosphorylation of Ser160, Ser211, and Ser620.

Next, we combined the three RSK-dependent phosphorylation sites identified as described above into a single Gab2 molecule (S3A) and determined its phosphorylation levels in cells treated with agonists of the Ras/MAPK pathway. Importantly, we found that mutation of the three sites completely abrogated Gab2 phosphorylation at basic consensus motifs in response to PMA (Fig. 2.4A) and EGF (Fig. 2.4B) stimulation, as well as MEK-DD expression (Fig. 2.4C), indicating that Ser160/211/620 are the predominant RSK-dependent

sites in Gab2. We performed *in vitro* kinase assays to confirm that RSK predominantly phosphorylates Ser160/211/620 in vitro. Importantly, we found that RSK-mediated ^{32}P label incorporation was strongly reduced when mutant Gab2 (S3A) was used as the substrate (Fig. 2.4D), indicating that RSK primarily promotes Gab2 phosphorylation on Ser160/211/620. Some level of phosphorylation was also detected with the Gab2 S3A mutant, suggesting that RSK may target additional residues *in vitro*. While Ser160 and Ser211 are located between the PH domain and the first Grb2-SH3 domain-binding site, it is interesting to note that Ser620 lies between the two Shp2-SH2 domain-binding sites (Tyr603, and Tyr632) (Fig. 2.4E), suggesting that RSK may modulate Shp2 recruitment in response to growth factors. All three phosphorylation sites are evolutionarily conserved along with the basic residues forming the consensus motif for RSK phosphorylation (Fig. 2.4F), suggesting that they play important functions.

The Ras/MAPK pathway modulates Shp2 recruitment in an RSK-dependent manner.

To address whether RSK-mediated Gab2 phosphorylation modulates its interaction with protein partners, we analyzed Gab2 immunoprecipitates from HEK293 cells stimulated with EGF for different times. As expected, we identified Grb2 within Gab2 immunoprecipitates but did not find that their interaction was modulated by either EGF or inhibitor treatments (Fig. 2.5A), consistent with the fact that this interaction is mediated via SH3 domains (10). Interestingly, we found that the EGF-dependent recruitment of Shp2 was significantly enhanced in cells exposed to PD184352, suggesting that the Ras/MAPK pathway negatively regulates Shp2 recruitment (Fig. 2.5A). To further address the role of Gab2 phosphorylation in the recruitment of Shp2, we pretreated HEK293 cells with PMA to promote Gab2 phosphorylation at basic consensus motifs (as for Fig. 2.1A) and then exposed cells to EGF for 5 or 10 min prior to Gab2 immunoprecipitation. Whereas PMA treatment by itself did not promote Shp2 recruitment (Fig. 2.5B, first two lanes), we found that pretreatment of cells with PMA severely decreased the ability of Gab2 to associate with Shp2 in response to EGF stimulation (Fig. 2.5B). Consistent with a role for the MAPK pathway, we found that inhibition of MEK1/2 using PD184352 rescued the inhibitory effect of PMA on Shp2 recruitment (Fig. 2.5B, last two lanes). The role of MEK1/2-dependent signaling was further addressed by expressing a constitutively activated form of MEK1 (MEK-DD) prior to EGF

stimulation. Using this approach, we found that the constitutive activation of the MAPK pathway decreased Gab2-mediated recruitment of Shp2 in response to EGF stimulation (Fig. 2.5C). To determine if Shp2 recruitment to Gab2 was regulated in an RSK dependent manner, we pretreated HEK293 cells with the RSK inhibitor BI-D1870 prior to a time course of EGF stimulation. Importantly, we found that inhibition of endogenous RSK activity increased Shp2 recruitment induced by EGF treatment (Fig. 2.5D), indicating that the Ras/MAPK pathway negatively regulates Gab2-mediated Shp2 recruitment in an RSK-dependent manner. Notably, inhibition of RSK activity using BI-D1870 was found to increase ERK1/2 phosphorylation in response to EGF stimulation, consistent with a previous report showing that RSK negatively regulates ERK1/2 signaling by directly phosphorylating SOS1 (39).

Next, we wanted to determine whether Gab2 phosphorylation on Ser160/211/620 was directly responsible for the reduction in Shp2 recruitment. To assess this, HEK293 cells were transfected with wt Gab2 or the Gab2 S3A mutant (S160/211/620A), treated with EGF over a time course, and Gab2 immunoprecipitated. As shown in Fig. 2.6A, we found that the Gab2 S3A mutant more efficiently recruited Shp2 in response to EGF treatment, suggesting that RSK-dependent phosphorylation of Ser160/211/620 regulates Shp2 binding. This effect appeared to be specific to Shp2, as no modulations were observed in the recruitment of p85 in response to EGF stimulation (Fig. 2.6B). The increase induced by the Gab2 S3A mutant was quantified by densitometry and found to be significantly different from that induced by wt Gab2 at the 5-, 10-, and 30-min time points (Fig. 2.6C). Interestingly, we also found that expression of the Gab2 S3A mutant potentiated ERK1/2 phosphorylation in response to EGF stimulation (Fig. 2.6A), consistent with the established role of Shp2 as a positive regulator of Ras/MAPK signaling. We also determined whether constitutive activation of the MAPK pathway using MEK-DD could similarly negatively regulate Shp2 recruitment to Gab2. Whereas MEK-DD expression strongly suppressed Shp2 recruitment to wt Gab2, we found that the Gab2 S3A mutant was mostly insensitive to the activation of the Ras/MAPK pathway (Fig. 2.6D). To further validate these findings, we made use of Gab2-deficient MEFs (25) to stably express Myc-tagged wt Gab2 or the Gab2 S3A mutant in a background without endogenous Gab2 expression (Fig. 2.6E). To determine the ability of exogenous Gab2 to recruit Shp2, we analyzed the levels of Myc-tagged Gab2 within endogenous Shp2

immunoprecipitates. Compared to wt Gab2, we found that the Gab2 S3A mutant recruited more Shp2 in response to EGF stimulation (Fig. 2.6F). We also determined which of the three RSK-dependent phosphorylation sites participated in the regulation of Shp2 binding and found that mutation of Ser211 or Ser620 was sufficient to increase Shp2 recruitment in response to EGF stimulation (Fig. 2.6G). Together, these results provide robust evidence that RSK-mediated phosphorylation of Gab2 on Ser211 and Ser620 impedes Shp2 recruitment and function in response to growth factors.

RSK-mediated Gab2 phosphorylation impairs mammary epithelial cell migration.

Gab2-mediated Shp2 recruitment has previously been shown to promote the migration of mammary epithelial cells (14, 16). We thus used this biological system to address the function of RSK-mediated Gab2 phosphorylation. We generated stable MCF-10A cells, which express very low endogenous levels of Gab2, to express an empty vector, wt Gab2, or the Gab2 S3A mutant. Upon generation of pooled populations of stably expressing cells, we readily observed a change in morphology between parental and Gab2-expressing cells (Fig. 2.7A). This change in morphology induced by the expression of wt Gab2 was previously shown to correlate with enhanced cell motility (16). Interestingly, we also observed a change in morphology between cells expressing wt Gab2 and the S3A mutant (Fig. 2.7A), with the latter having a more elongated cell shape reminiscent of mesenchymal morphology. Immunofluorescence experiments indicated that wt Gab2 and the S3A mutant were expressed at similar levels, which was confirmed by immunoblotting the total cell lysates of all three cell lines (Fig. 2.7B). The differences in morphology between the three stable cell lines were quantified, and the S3A Gab2-expressing cells were found to be 2 and 7 times more likely to have an elongated shape than wt Gab2 and empty vector-expressing cells, respectively (Fig. 2.7C). We also performed an assay to verify whether Shp2 recruitment was modulated in MCF-10A cells and found that the Gab2 S3A mutant recruited more Shp2 than wt Gab2 in response to EGF stimulation (Fig. 2.7D).

Based on the known role of Gab2 in cell motility, we determined whether Gab2 phosphorylation affected MCF-10A cell migration using a Transwell assay. While we confirmed previous data by showing that expression of wt Gab2 increases MCF-10A migration (16), we found that cells expressing the Gab2 S3A mutant exhibited a significant

increase in cell migration (~1.5-fold compared to wt Gab2-expressing cells) (Fig. 2.8A and B). As shown in Fig. 2.8C, we confirmed that this effect was not a result of increased cell proliferation. To further characterize this apparent gain of function of mutant Gab2, we performed live-cell-imaging wound healing assays by tracking migrating cells. Quantifications of cell paths revealed that MCF-10A cells expressing the Gab2 S3A mutant exhibited a robust increase in displacement from the point of origin (Fig. 2.8D). Indeed, the speed of movement of Gab2 S3A expressing cells was found to be ~1.6-fold higher than that of wt Gab2-expressing cells, reaching 0.45 m/min (Fig. 2.8E). As shown in Fig. 2.7B and D, we found that cells expressing the Gab2 S3A mutant displayed increased ERK1/2 phosphorylation, consistent with the role of Shp2 in the regulation of the Ras/MAPK pathway. To determine whether this pathway was partly responsible for the observed increased motility of cells expressing the Gab2 S3A mutant, we treated cells with the MEK1/2 inhibitor PD184352 during image acquisitions. As shown in Fig. 2.8E, we found that PD184352 treatment abrogated the increased motility of Gab2 S3A-expressing cells, consistent with a role of the Ras/MAPK pathway. Taken together, our results indicate that RSK-mediated phosphorylation of Gab2 inhibits cell elongation and Gab2-dependent migration of mammary epithelial cells. These findings suggest that RSK-mediated regulation of Gab2 function represents a novel negative-feedback pathway that restricts cell migration occurring in response to Ras/MAPK signaling (Fig. 2.9).

DISCUSSION

The role of the Gab2 adapter protein has been widely investigated in hematopoietic cells and in cancer progression, where it functions to regulate the activation of several signaling pathways, including Ras/MAPK and PI3K/Akt. In this study, we demonstrate in different cell types that Gab2 phosphorylation is regulated by the Ras/MAPK pathway in an RSK-dependent manner (Fig. 2.1 to 2.2). Using phosphomotif antibodies, mass spectrometry, and site-directed mutagenesis, we found that RSK phosphorylates Gab2 on three serine residues which fall into the RXXpS/T consensus sequence (Ser160, Ser211, Ser620), both *in vivo* and *in vitro* (Fig. 2.3 to 2.4). Of note, we found that the closely related Gab1 isoform is not significantly phosphorylated by RSK in response to growth factors (Fig. 2.1C), which is indicative of the specificity of the regulation of Gab2. Using both gain- and loss-of-function approaches, we showed that RSK-mediated Gab2 phosphorylation decreases Shp2 binding in response to EGF stimulation (Fig. 2.5 to 2.7), suggesting that RSK negatively regulates the Gab2-Shp2 axis. Using mammary epithelial cells that stably express exogenous Gab2 alleles, we found that Gab2 phosphorylation on Ser160/211/620 inhibits cell motility (Fig. 2.8), suggesting a model whereby RSK limits Gab2-dependent cell migration upon activation of the Ras/MAPK pathway. Based on the widespread role of Gab2 in receptor signaling, our results suggest that RSK may regulate Gab2 function in response to many types of growth factors and cytokines.

Whereas Gab2 tyrosine phosphorylation and activation of downstream signaling pathways are well characterized, relatively little is known about the mechanisms involved in Gab2 inactivation by Ser/Thr phosphorylation. Our data demonstrate that the Ras/MAPK pathway and, more specifically, RSK phosphorylate Gab2 on three serine residues to inhibit Shp2 recruitment. A previous report has shown that Akt phosphorylates Gab2 on Ser159 (Ser160 is the homologous site in mouse Gab2) and thereby reduces Gab2 tyrosine phosphorylation via unknown mechanisms (17). While Ser159 is one of the three phosphorylation sites that we found to be regulated by RSK, we did not observe that Ser159 phosphorylation modulates Gab2 tyrosine phosphorylation in response to EGF stimulation. More recently, Gab2 was shown to be phosphorylated on two additional residues (Ser210 and Thr391, according to the human numbering) in response to growth factors, but the actual

protein kinase involved in these phosphorylation events remains elusive (19). Interestingly, phosphorylation of these sites was shown to mediate 14-3-3 binding and to inhibit Grb2 binding, but again, the mechanisms involved have not been determined. While we found that Ser210 (Ser211 is the homologous site in mouse Gab2) is regulated by RSK (Fig. 2.3), we did not observe that RSK-mediated phosphorylation of Gab2 altered Grb2 or p85 binding (Fig. 2.5A and 6B). Our results also indicate that Thr391 is not regulated by RSK, consistent with the idea that RSK is mostly a serine kinase and that very few substrates of RSK have been shown to be phosphorylated on threonine residues (5).

We found that RSK phosphorylates a novel site in Gab2 (Ser620), which is located between the two SH2 domain-binding sites for Shp2 (Tyr603 and Tyr632) (Fig. 2.3 and 2.4). Our results indicate that RSK-mediated phosphorylation of Gab2 inhibits Shp2 recruitment, suggesting that phosphorylated Ser620 hinders Shp2 interaction to the SH2 domain-binding sites. Another possibility is that phosphorylation of Ser160/211/620 causes a conformational change in Gab2 that reduces its affinity for Shp2, but more experimentation will be required to fully understand the molecular events taking place upon RSK-mediated phosphorylation. Interestingly, ERK1/2 was also shown to modulate Shp2 recruitment in response to IL-2 stimulation (18). ERK1/2 was found to phosphorylate Ser613, which is also located in close proximity to the SH2 domain-binding motif of Gab2. Although we have not been able to identify phosphorylated Ser613 in our MS experiments, these results suggest that RSK may collaborate with ERK1/2 to inhibit Shp2 recruitment in response to agonists of the Ras/MAPK pathway. Based on the fact that RSK-mediated phosphorylation of Gab2 does not perturb recruitment of the p85 subunit of PI3K or Grb2 binding, our results suggest that Gab2 phosphorylation on Ser160/211/620 specifically regulates Shp2 recruitment. Our findings indicate that RSK is part of a negative feedback loop that restricts Gab2-Shp2 signaling, similar to the recently described loop involving RSK and SOS1 (39). Based on the established role of Shp2 in the regulation of Ras/MAPK signaling (40), our results highlight a second RSK-dependent inhibitory loop that negatively regulates ERK1/2 signaling.

The mechanisms by which Gab2 contributes to breast cancer are not fully understood, but several studies implicated Shp2 in Gab2-dependent cell proliferation and motility (14, 16). Similar to our findings, expression of Gab2 in MCF-10A cells has been shown to promote a

change in morphology and cell motility (16), providing an excellent system to analyze the impact of Gab2 phosphorylation on its function. We found that mutation of Ser160/211/620 potentiated the effect of wt Gab2 on cell motility, which is consistent with the important role that Shp2 plays in cytoskeletal organization and cell migration (16). In addition to regulating the Ras/MAPK pathway, Shp2 has recently been shown to modulate RhoA activation (16, 41). Indeed, the recruitment of Shp2 to the plasma membrane was found to promote the dephosphorylation of Rho kinase II (ROCKII), resulting in modulation of RhoA induced cell rounding (41). Based on the known roles of the Ras/MAPK pathway in cell motility in diverse systems (42), these findings indicate that RSK likely regulates the involvement of Shp2 in both of these pathways to promote a change in cell shape and cell motility.

In summary, we have identified a novel mechanism by which growth factors regulate Gab2 function. Based on the involvement of Gab2 in the pathogenesis of breast cancer, our results provide important information about the complexity of its regulation. Our findings also have potential clinical implication, as RSK1 and RSK2 have been shown to be upregulated in both breast and prostate cancers (43). While these two RSK isoforms likely play important proproliferative functions (5, 6), our results indicate that they are also involved in the negative regulation of RTK signaling.

ACKNOWLEDGMENTS

We deeply appreciate all the members of our laboratories for their insightful discussions and comments on the manuscript and the data. We also specifically acknowledge Chris Jahns (HMS Taplin Biological Mass Spectrometry Facility) for assistance with preparation of in-gel digests.

This work was supported by grants from the Canadian Institutes of Health Research (CIHR; MOP123408), the Canadian Cancer Society Research Institute (700878), and the Cancer Research Society (F121153) (to P.P.R), as well as the United States National Institutes of Health (to S.P.G.; HG3456) and the CIHR (to S.C.; MOP89877). P. P. Roux holds the Canada Research Chair in Signal Transduction and Proteomics, and S. Carreno holds a Chercheur Boursier award from the Fonds de Recherche du Québec en Santé (FRQS). X. Zhang holds a China Scholarship Council studentship. J. A. Galan holds a postdoctoral fellowship from the Canadian Institutes of Health Research (CIHR). IRIC core facilities are supported in part by the FRQS.

We have no conflict of interest to declare.

REFERENCES

1. **Lemmon MA, Schlessinger J.** 2010. Cell signaling by receptor tyrosine kinases. Cell 141:1117–1134.
2. **Murphy LO, Blenis J.** 2006. MAPK signal specificity: the right place at the right time. Trends Biochem. Sci. 31:268 –275.
3. **Roux PP, Blenis J.** 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol. Mol. Biol. Rev. 68:320 –344.
4. **Cargnello M, Roux PP.** 2011. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol. Mol. Biol. Rev. 75:50–83.
5. **Romeo Y, Zhang X, Roux PP.** 2012. Regulation and function of the RSK family of protein kinases. Biochem. J. 441:553–569.
6. **Carriere A, Ray H, Blenis J, Roux PP.** 2008. The RSK factors of activating the Ras/MAPK signaling cascade. Front. Biosci. 13:4258–4275.
7. **Brummer T, Schmitz-Peiffer C, Daly RJ.** 2010. Docking proteins. FEBS J. 277:4356–4369.
8. **Gu H, Neel BG.** 2003. The "Gab" in signal transduction. Trends Cell Biol. 13:122–130.
9. **Wohrle FU, Daly RJ, Brummer T.** 2009. Function, regulation and pathological roles of the Gab/DOS docking proteins. Cell Commun. Signal 7:22. doi:10.1186/1478-811X-7-22.
10. **Wohrle FU, Daly RJ, Brummer T.** 2009. How to Grb2 a Gab. Structure 17:779 –781.
11. **Nishida K, Hirano T.** 2003. The role of Gab family scaffolding adapter proteins in the signal transduction of cytokine and growth factor receptors. Cancer Sci. 94:1029 –1033.
12. **Celebi JT, Adams SJ, Aydin IT.** 2012. GAB2—a scaffolding protein in cancer. Mol. Cancer Res. 10:1265–1270.
13. **Brummer T, Schramek D, Hayes VM, Bennett HL, Caldon CE, Musgrove EA, Daly RJ.** 2006. Increased proliferation and altered growth factor dependence of human

mammary epithelial cells overexpressing the Gab2 docking protein. J. Biol. Chem. 281:626–637.

14. **Bentires-Alj M, Gil SG, Chan R, Wang ZC, Wang Y, Imanaka N, Harris LN, Richardson A, Neel BG, Gu H.** 2006. A role for the scaffolding adapter GAB2 in breast cancer. Nat. Med. 12:114 –121.
15. **Bocanegra M, Bergamaschi A, Kim YH, Miller MA, Rajput AB, Kao J, Langerod A, Han W, Noh DY, Jeffrey SS, Huntsman DG, Borresen-Dale AL, Pollack JR.** 2010. Focal amplification and oncogene dependency of GAB2 in breast cancer. Oncogene 29:774 –779.
16. **Herrera Abreu MT, Hughes WE, Mele K, Lyons RJ, Rickwood D, Browne BC, Bennett HL, Vallotton P, Brummer T, Daly RJ.** 2011. Gab2 regulates cytoskeletal organization and migration of mammary epithelial cells by modulating RhoA activation. Mol. Biol. Cell 22:105–116.
17. **Lynch DK, Daly RJ.** 2002. PKB-mediated negative feedback tightly regulates mitogenic signalling via Gab2. EMBO J. 21:72– 82.
18. **Arnaud M, Crouin C, Deon C, Loyaux D, Bertoglio J.** 2004. Phosphorylation of Grb2-associated binder 2 on serine 623 by ERK MAPK regulates its association with the phosphatase SHP-2 and decreases STAT5 activation. J. Immunol. 173:3962–3971.
19. **Brummer T, Larance M, Herrera Abreu MT, Lyons RJ, Timpson P, Emmerich CH, Fleuren ED, Lehrbach GM, Schramek D, Guilhaus M, James DE, Daly RJ.** 2008. Phosphorylation-dependent binding of 14-3-3 terminates signalling by the Gab2 docking protein. EMBO J. 27:2305–2316.
20. **Caron C, Spring K, Laramee M, Chabot C, Cloutier M, Gu H, Royal I.** 2009. Non-redundant roles of the Gab1 and Gab2 scaffolding adapters in VEGF-mediated signalling, migration, and survival of endothelial cells. Cell Signal. 21:943–953.
21. **Lock LS, Maroun CR, Naujokas MA, Park M.** 2002. Distinct recruitment and function of Gab1 and Gab2 in Met receptor-mediated epithelial morphogenesis. Mol.

Biol. Cell 13:2132–2146.

22. **Romeo Y, Moreau J, Zindy PJ, Saba-El-Leil M, Lavoie G, Dandachi F, Baptissart M, Borden KL, Meloche S, Roux PP.** 16 July 2012. RSK regulates activated BRAF signalling to mTORC1 and promotes melanoma growth. Oncogene [Epub ahead of print.] doi:10.1038/onc.2012.312.
23. **Carriere A, Romeo Y, Acosta-Jaquez HA, Moreau J, Bonneil E, Thibault P, Fingar DC, Roux PP.** 2011. ERK1/2 phosphorylate Raptor to promote Ras-dependent activation of mTORcomplex 1 (mTORC1). J. Biol. Chem. 286:567–577.
24. **Carriere A, Cargnello M, Julien LA, Gao H, Bonneil E, Thibault P, Roux PP.** 2008. Oncogenic MAPK signaling stimulates mTORC1 activity by promoting RSK-mediated raptor phosphorylation. Curr. Biol. 18:1269–1277.
25. **Gu H, Saito K, Klaman LD, Shen J, Fleming T, Wang Y, Pratt JC, Lin G, Lim B, Kinet JP, Neel BG.** 2001. Essential role for Gab2 in the allergic response. Nature 412:186 –190.
26. **Roux PP, Ballif BA, Anjum R, Gygi SP, Blenis J.** 2004. Tumor promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc. Natl. Acad. Sci. U. S. A. 101:13489 –13494.
27. **Shevchenko A, Wilm M, Vorm O, Mann M.** 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68:850–858.
28. **Cargnello M, Tcherkezian J, Dorn JF, Huttlin EL, Maddox PS, Gygi SP, Roux PP.** 2012. 4E-T phosphorylation by JNK promotes stress-dependent P-body assembly. Mol. Cell. Biol. 32:4572– 4584.
29. **Ting L, Rad R, Gygi SP, Haas W.** 2011. MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. Nat. Methods 8:937–940.
30. **Rappsilber J, Ishihama Y, Mann M.** 2003. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal. Chem. 75:663– 670.

31. **Yates JR, III, Eng JK, McCormack AL, Schieltz D.** 1995. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. Anal. Chem. 67:1426 –1436.

32. **Elias JE, Gygi SP.** 2007. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat.Methods 4:207–214.

33. **Huttlin EL, Jedrychowski MP, Elias JE, Goswami T, Rad R, Beausoleil SA, Villen J, Haas W, Sowa ME, Gygi SP.** 2010. A tissue-specific atlas of mouse protein phosphorylation and expression. Cell 143: 1174 –1189.

34. **Beausoleil SA, Villen J, Gerber SA, Rush J, Gygi SP.** 2006. A probabilitybased approach for high-throughput protein phosphorylation analysis and site localization. Nat. Biotechnol. 24:1285–1292.

35. **Hochberg Y, Benjamini Y.** 1990. More powerful procedures for multiple significance testing. Stat. Med. 9:811– 818.

36. **Julien LA, Carriere A, Moreau J, Roux PP.** 2010. mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling. Mol. Cell. Biol. 30:908 –921.

37. **Alessi DR, Caudwell FB, Andjelkovic M, Hemmings BA, Cohen P.** 1996. Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. FEBS Lett. 399:333–338.

38. **Huang F, Tong X, Deng H, Fu L, Zhang R.** 2008. Inhibition of the antigen-induced activation of RBL-2H3 cells by Gab2 siRNA. Cell. Mol. Immunol. 5:433– 438.

39. **Saha M, Carriere A, Cheerathodi M, Zhang X, Lavoie G, Rush J, Roux PP, Ballif BA.** 2012. RSK phosphorylates SOS1 creating 14-3-3-docking sites and negatively regulating MAPK activation. Biochem. J. 447:159–166.

40. **Neel BG, Gu H, Pao L.** 2003. The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. Trends Biochem. Sci. 28:284 –293.

41. **Lee HH, Chang ZF.** 2008. Regulation of RhoA-dependent ROCKII activation by Shp2.

J. Cell Biol. 181:999 –1012.

42. **Mendoza MC, Er EE, Zhang W, Ballif BA, Elliott HL, Danuser G, Blenis J.** 2011. ERK-MAPK drives lamellipodia protrusion by activating the WAVE2 regulatory complex. Mol. Cell 41:661– 671.
43. **Romeo Y, Roux PP.** 2011. Paving the way for targeting RSK in cancer. Expert Opin. Ther. Targets 15:5–9.

FIGURES

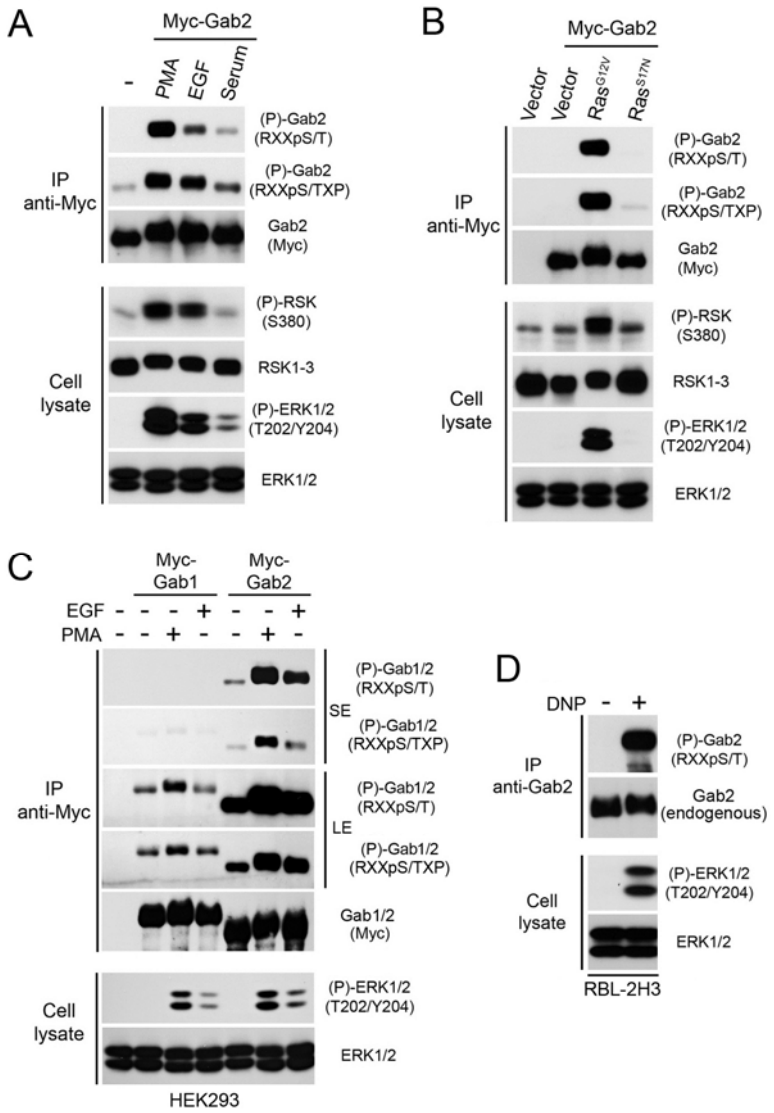

Figure 2.1 Identification of Gab2 as a target of Ras/MAPK signaling.

(A and C) HEK293 cells were transfected with an empty vector or Myc-tagged Gab1 or Gab2, serum starved overnight, and stimulated for 30 min with PMA (100 ng/ml) or 10 min with EGF (25 ng/ml) or fetal bovine serum (10%). Immunoprecipitated (IP) Myc-Gab1 or Myc-Gab2 was then assayed for phosphorylation with phosphomotif antibodies that recognize the RXXpS/T and RXXpS/TXP consensus motifs. Phosphorylated and total levels of RSK and ERK1/2 were assayed by immunoblotting on total cell lysates. SE, short exposure; LE, long exposure. (B) As for panel A, except that HEK293 cells were cotransfected with Myc-Gab2 and a constitutively active (G12V) or inactive (S17N) form of Ras. (D) RBL-2H3 cells were incubated overnight with anti-2,4-dinitrophenol IgE and stimulated with 2,4-dinitrophenol for 10 min prior to endogenous Gab2 immunoprecipitation. Phosphorylation of endogenous Gab2 was assayed using a phosphomotif antibody that recognizes RXXpS/T sequences.

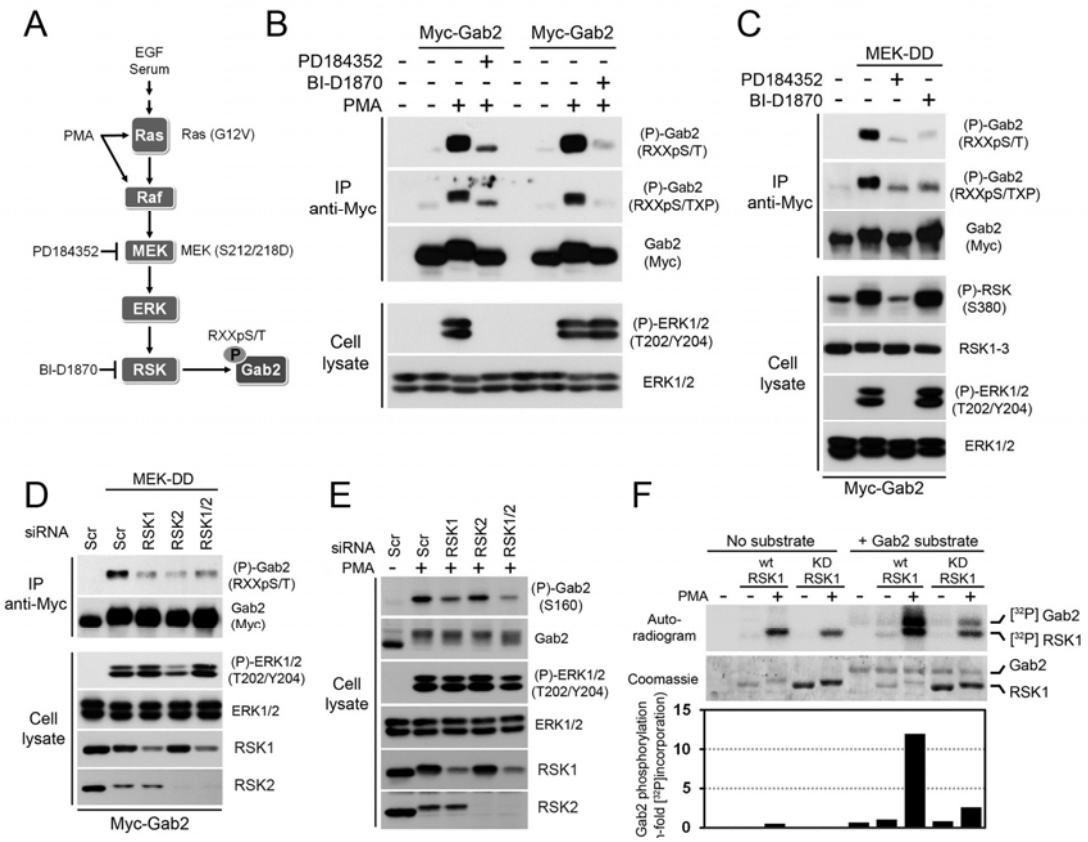

Figure 2.2 Activation of the Ras/MAPK pathway induces RSK-dependent phosphorylation of Gab2.

(A) Schematic representation of the agonists and pharmacological inhibitors used in this study. (B) HEK293 cells were transfected with an empty vector or Myc-tagged Gab2 and serum starved overnight. Cells were pretreated with PD184352 (10 μM) or BI-D1870 (10 μM) for 30 min prior to stimulation with PMA (100 ng/ml) for 30 min. Immunoprecipitated Myc-Gab2 was then assayed for phosphorylation using phosphomotif antibodies that recognize the RXXpS/T and RXXpS/TXP consensus sequences. (C) As for panel B, except that cells were also cotransfected with a constitutively activated form of MEK1 (MEK-DD). (D) HEK293 cells were transfected with Flag-tagged MEK-DD and either a scrambled siRNA or siRNAs targeting RSK1 and/or RSK2. Cells were serum starved overnight, and immunoprecipitated Myc-Gab2 was assayed for phosphorylation using a phosphomotif antibody that recognizes the RXXpS/T consensus sequence. (E) As for panel D, except that cells were stimulated with PMAinstead of being transfected with MEK-DD. In addition, total cell lysates were immunoblotted with a phosphorylation site-specific antibody against Ser160. (F) Wild-type and kinase-deficient RSK1 (K112/464R) were immunoprecipitated from HEK293 cells stimulated with PMA (100 ng/ml), and the resulting immunoprecipitates were incubated in a kinase reaction with [γ-^{32}P]ATP and immunopurified full-length Myc-Gab2. The resulting samples were run on SDS-polyacrylamide gels and analyzed by exposing dried film on an autoradiogram. Levels of both RSK1 and Gab2 are shown on the Coomassie-stained gel.

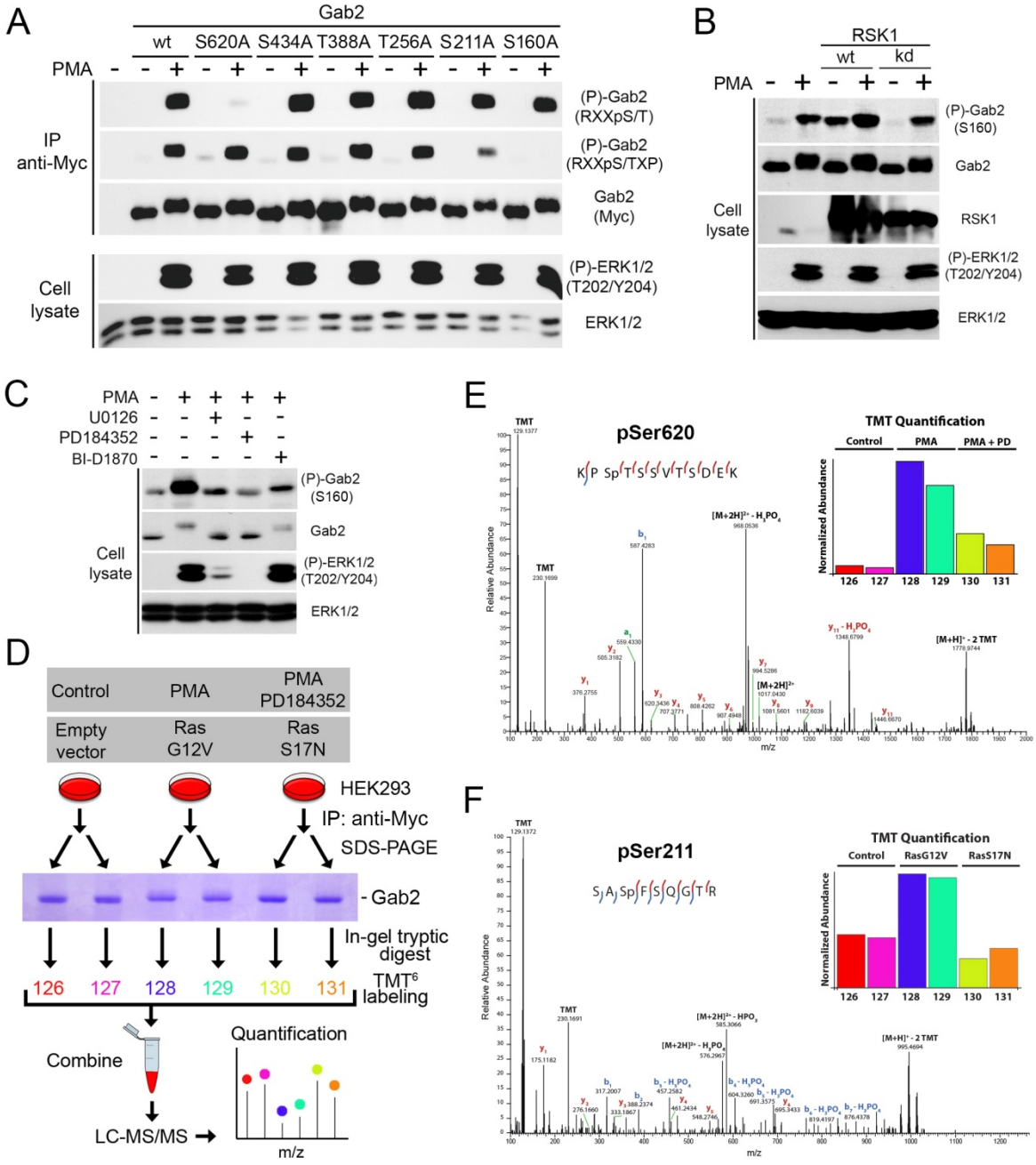

Figure 2.3 Identification of Ser160, Ser211, and Ser620 as being regulated by Ras/MAPK signaling. (A) HEK293 cells were transfected with wt Gab2 or potential RSK phosphorylation site mutants (S160A, S211A, T256A, T388A, S434A, and S620A), serum starved overnight, and stimulated with PMA (100 ng/ml) for 30 min. Immunoprecipitated Myc-Gab2 was then assayed for phosphorylation with phosphomotif antibodies that recognize the RXXpS/T and RXXpS/TXP consensus motifs. (B) HEK293 cells were transfected with wt or a kinase-inactive form of RSK1, serum starved overnight, and stimulated with PMA (25 ng/ml) for 15 min. Endogenous Gab2 phosphorylation at Ser160 was determined with a

phosphorylation site-specific antibody. (C) As for panel B, except that phosphorylation of endogenous Gab2 at Ser160 was monitored in HEK293 cells pretreated with UO126 (20μM), PD184352 (10 μM), or BI-D1870 (10 μM) for 30 min prior to PMA stimulation. (D) Phosphorylation of murine Gab2 was confirmed via high-resolution MS/MS sequencing. Gab2 was immunoprecipitated in biological duplicate from cells following either mock treatment, treatment with PMA, or treatment with PMA and PD184352. In a separate experiment, cells were either transfected with an empty vector or different activated (G12V) and inactivated (S17N) alleles of Ras. After subsequent SDS-PAGE separation, bands corresponding to Gab2 were excised and proteins were digested in gel with trypsin followed by TMT labeling for relative quantitation. (E) The spectrum identifies a tryptic peptide bearing phosphorylation at Ser620, as well as TMT labels on both lysine side chains and the peptide N terminus; localization of the phosphorylation site is indicated by the presence of the y9 ion, which matches its theoretical mass to approximately 1 ppm. (Inset) Relative abundance of Ser620 across the six samples normalized with respect to overall Gab2 abundance. PD, PD184352. (F) The spectrum identifies a tryptic peptide bearing two TMT labels as well as phosphorylation of Ser211. Phosphorylation site localization is confirmed by the y5 and y6 ions and b1 and b2 ions, as well as the b3 and b4 ions with subsequent neutral loss of H_3PO_4. (Inset) Relative levels of Gab2 phosphorylation at Ser211, after normalization on the basis of overall Gab2 abundance. Red and blue lines within the peptide sequences indicate y and b ions, respectively.

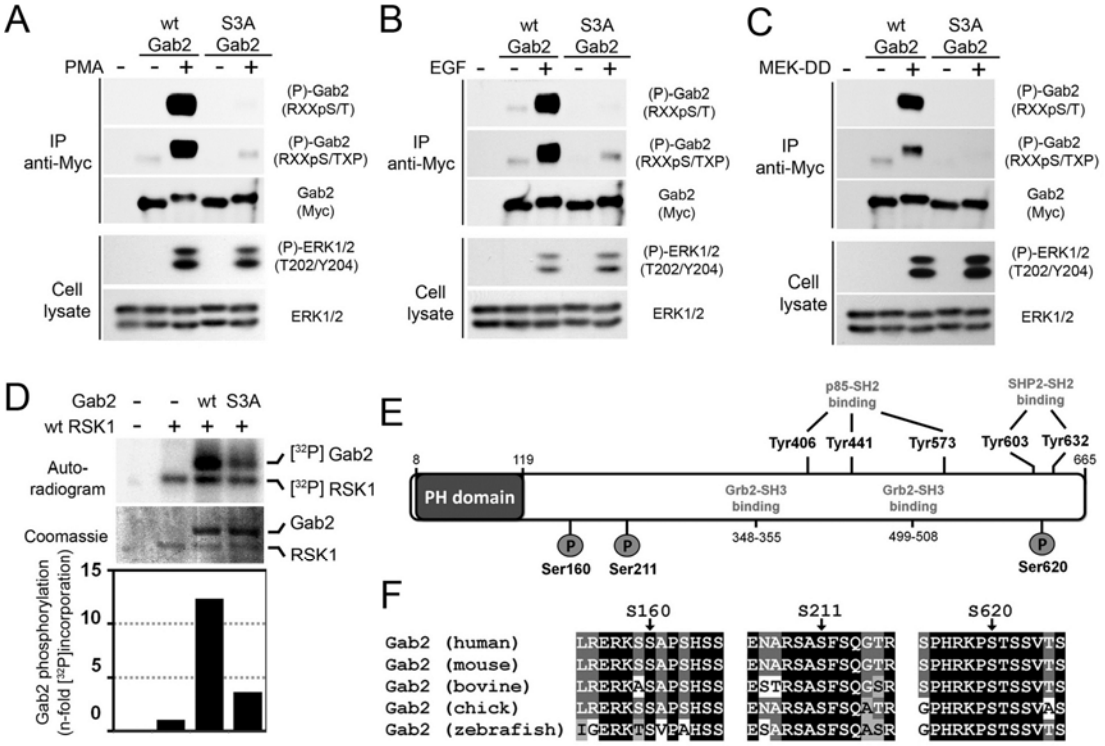

Figure 2.4 RSK predominantly phosphorylates Ser160, Ser211, and Ser620 in cells and *in vitro*.

(A) HEK293 cells were transfected with Myc-tagged wt Gab2 or a triple mutant with all three serine residues converted to alanines (S160/211/620A [S3A]), serum starved overnight, and stimulated with PMA (100 ng/ml) for 30 min. Immunoprecipitated Myc-Gab2 was then assayed for phosphorylation with phosphomotif antibodies that recognize the RXXpS/T and RXXpS/TXP consensus motifs. (B) As for panel A, except that cells were treated with EGF (25 ng/ml) for 10 min. (C) As for panel A, except that cells were transfected with MEK-DD prior to serum starvation and cell lysis. (D) Wild-type RSK1 was immunoprecipitated from HEK293 cells stimulated with PMA (100 ng/ml), and the resulting immunoprecipitates were incubated in a kinase reaction with [γ-^{32}P]ATP and immunopurified wt or S3A Myc-Gab2. The resulting samples were run on SDS-polyacrylamide gels and analyzed by exposing dried film on an autoradiogram. Levels of both RSK1 and Gab2 are shown on the Coomassie-stained gel. (E) Schematic representation of Gab2 with known phosphorylation binding sites. The three phosphorylation sites identified in this study are shown with circles labeled P. (F) Alignment of Gab2 amino acid sequences from different vertebrate species which demonstrate evolutionary conservation of all three phosphorylation sites.

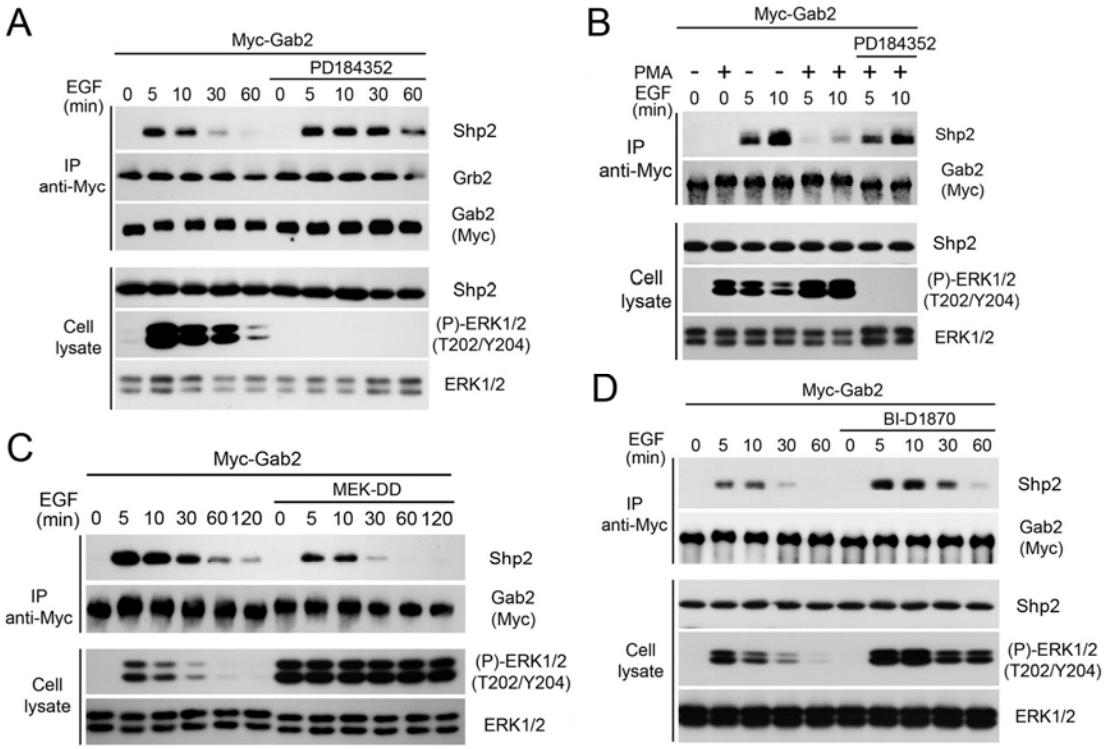

Figure 2.5 The Ras/MAPK pathway modulates Shp2 recruitment in an RSK-dependent manner.

(A) HEK293 cells were transfected with Myc-Gab2, serum starved overnight, pretreated with PD184352 (10 μM), and stimulated with EGF (25 ng/ml) over a time course. Associated endogenous Grb2 and Shp2 within Myc-Gab2 immunoprecipitates were assayed by immunoblotting. (B) HEK293 cells were transfected with Myc-tagged Gab2, serum starved overnight, and pretreated with PD184352 (10 μM) and/or PMA (100 ng/ml) for 30 min, prior to treatment with EGF (25 ng/ml) for 5 or 10 min. Associated Shp2 was assayed as described for panel A. (C) As for panel A, except that cells were cotransfected with MEK-DD, serum starved overnight, and stimulated with EGF over a time course. (D) As for panel A, except that cells were pretreated with the RSK inhibitor BI-D1870 (10 μM) for 30 min prior to EGF stimulation.

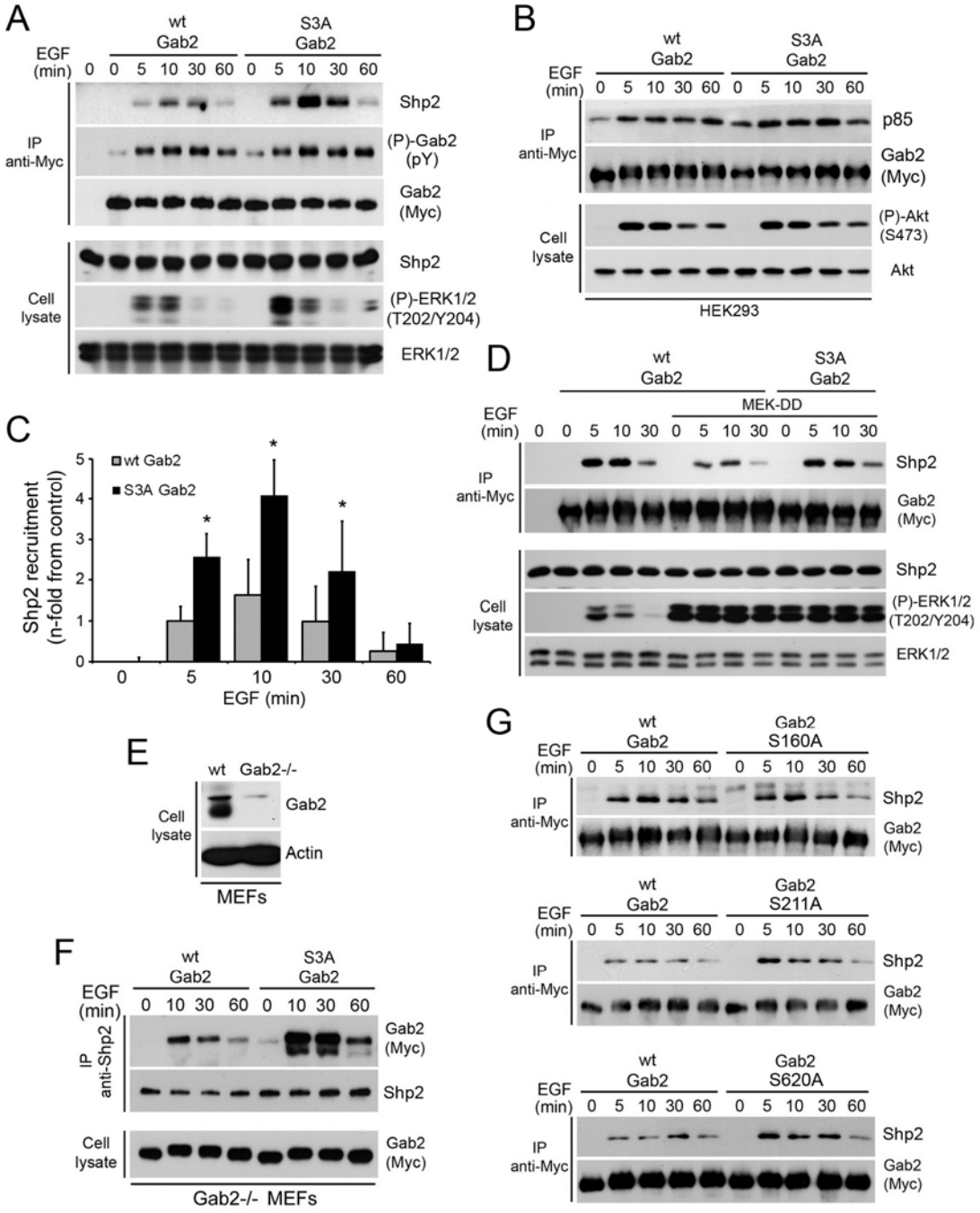

Figure 2.6 A Gab2 mutant that cannot be phosphorylated on Ser160/211/620 promotes Shp2 recruitment.

(A) HEK293 cells were transfected with wt Myc-Gab2 or the S3A mutant, serum starved overnight, and stimulated with EGF (25 ng/ml) over a time course. Associated Shp2 within Gab2 immunoprecipitates was evaluated by immunoblotting. (B) HEK293 cells were transfected with wt Myc-Gab2 or the S3A mutant, serum starved overnight, and stimulated with EGF (25 ng/ml) over a time course. Associated p85 was

evaluated within Gab2 immunoprecipitates by immunoblotting, as well as Akt phosphorylation at Ser473. (C) The bar graph shows the relative Shp2 recruitment quantified by densitometry (as described in Materials and Methods) from three independent experiments similar to the one whose results are shown in panel A. The data are expressed as the mean fold increase in Shp2 recruitment comparing immunoprecipitations with wt Gab2 and the S3A mutant. Results are means SEMs. One-tailed unequal variance Student's t test P values are indicated for a comparison of the means of the wt and mutant (S3A) Gab2 levels. *, $P<0.01$. (D) HEK293 cells were cotransfected with wt Myc-Gab2 or the S3A mutant, with or without MEK-DD, serum starved overnight, and stimulated with EGF (25 ng/ml) over a time course. Shp2 recruitment was evaluated as described for panel A. (E) Wild-type and Gab2-deficient MEFs were evaluated for the presence of endogenous Gab2 by immunoblotting on total cell lysates. (F) Gab2-deficient MEFs stably expressing wt Gab2 or the S3A mutant were seeded at a similar density and serum starved overnight, prior to EGF (25 ng/ml) stimulation over a time course. Associated Myc-Gab2 was assayed within endogenous Shp2 immunoprecipitates by immunoblotting. (G) HEK293 cells were transfected with wt Myc-Gab2 or each of the Gab2 mutants (S160A, S211A, or S620A), serum starved overnight, and stimulated with EGF (25 ng/ml) over a time course. Associated Shp2 was evaluatedwithin Gab2 immunoprecipitates by immunoblotting.

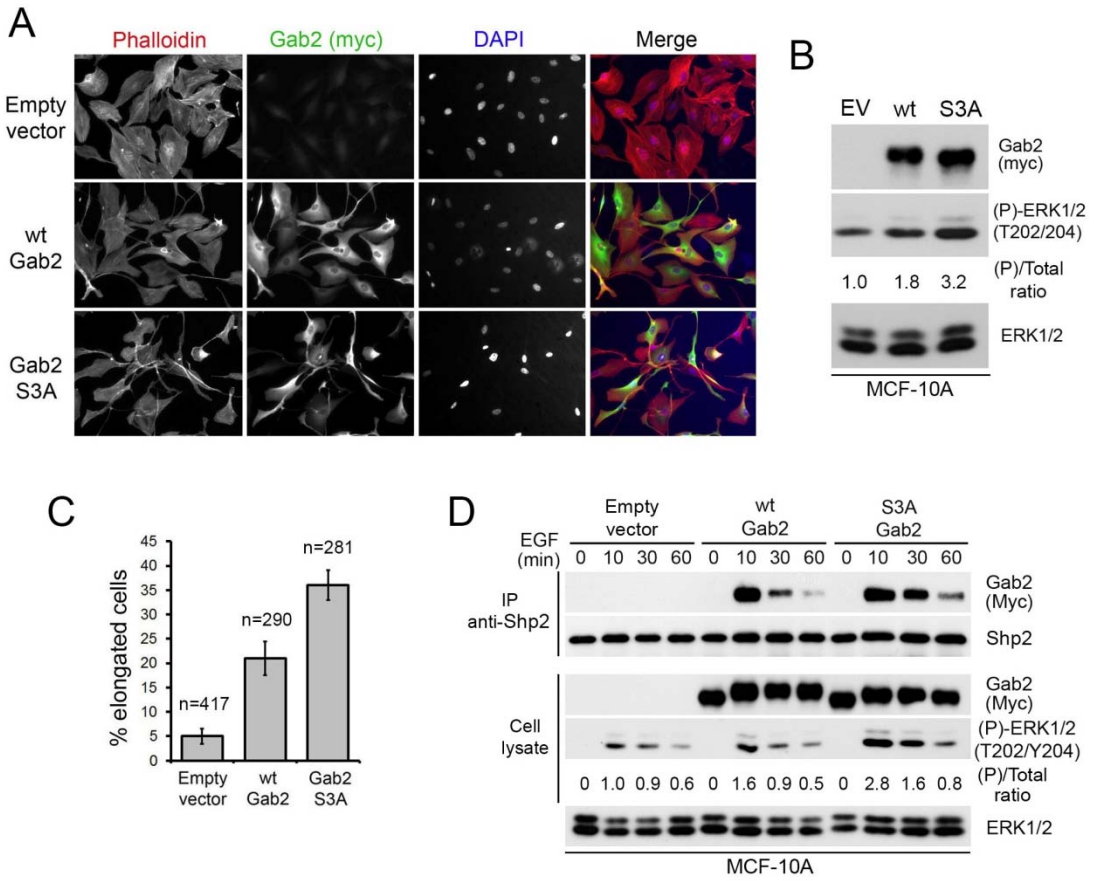

Figure 2.7 Stable expression of the Gab2 S3A mutant alters MCF-10A cell morphology.

(A) Confocal images of growing MCF-10A cells stably expressing an empty vector, wt Gab2, or the S3A mutant. Cells were stained with phalloidin to visualize the actin cytoskeleton, anti-Myc to monitor Gab2 expression, and DAPI to visualize nuclei. (B) Stable MCF-10A cells were lysed and immunoblotted for Gab2 (anti-Myc) to show equal expression levels between wt Gab2 and the S3A mutant, as well as phosphorylated ERK1/2 (T202/Y204) to show increased activation in Gab2 S3A-expressing cells. The ratio of ERK1/2 phosphorylation over total ERK1/2 levels was calculated on the basis of densitometric values. EV, empty vector. (C) Quantification of elongated cells from immunostaining experiments similar to the one shown in panel A. (D) MCF-10A cells stably expressing an empty vector, wt Myc-Gab2, or the S3A mutant were serum starved overnight and stimulated with EGF (25 ng/ml) over a time course. Associated Myc-Gab2 within endogenous Shp2 immunoprecipitates was evaluated by immunoblotting. The ratio of ERK1/2 phosphorylation over total ERK1/2 levels was calculated on the basis of densitometric values.

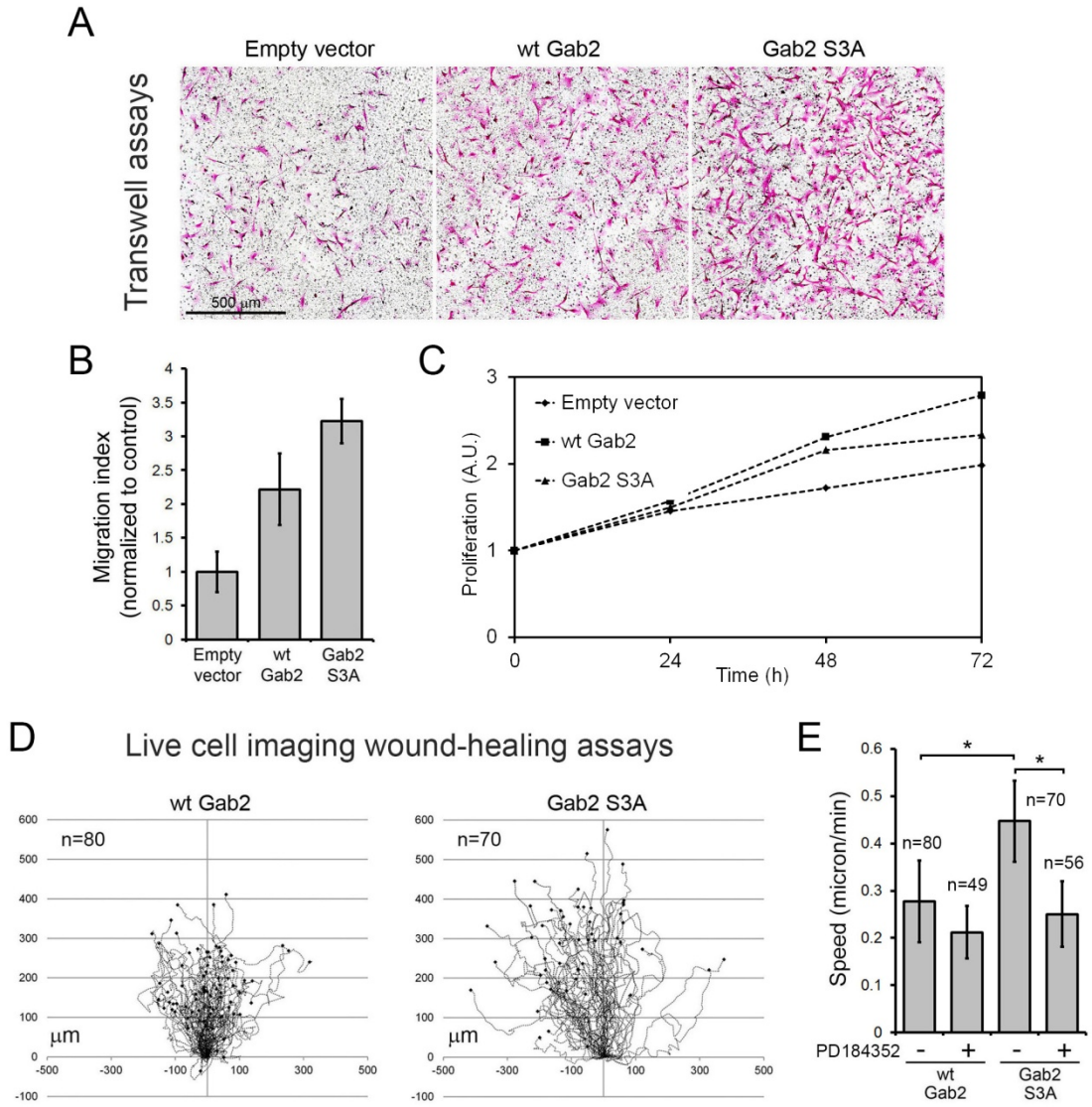

Figure 2.8 Stable expression of the Gab2 S3A mutant promotes MCF-10A cell migration.

(A) MCF-10A cells stably expressing an empty vector, wt Gab2, or the S3A mutant were seeded on top of Boyden chambers in EGF-free medium. Cells were allowed to migrate toward 20 ng/ml EGF for 24 h, fixed, and stained using H&E. (B) Filters processed as described for panel A were scanned, and migrated cells were automatically counted, as described in Materials and Methods. (C) Analysis of the proliferation of stable MCF-10A cells using the MTS assay over a time course of 72 h. The data are expressed as the mean fold increase in cell proliferation. A.U., absorbance units. (D) Live-cell-imaging wound-healing assays were performed using MCF-10A cells stably expressing wt Gab2 or the S3A mutant. Tree graphs show the length (μm), direction, and displacement (distance from the origin) of more than 70 cell paths. (E) Quantification of cell speed (μm/min) based on the total displacement calculated in wound-healing assays. An unpaired Student's t test was performed to determine that the differences between wt and S3A Gab2 conditions and the effect of PD184352 treatment were statistically significant (*, P <0.00001).

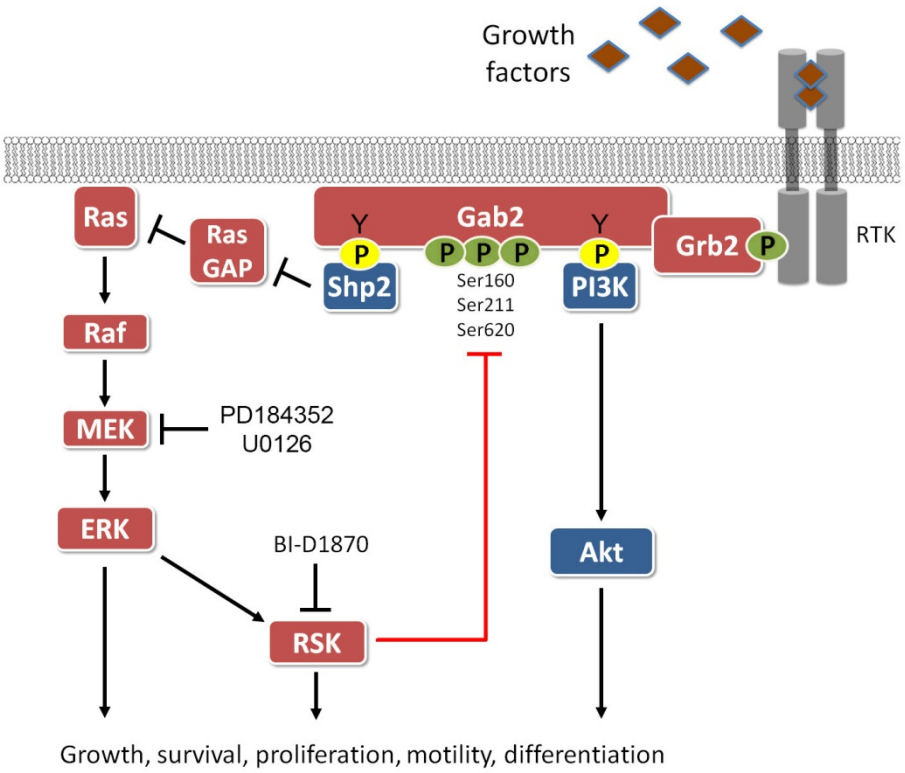

Figure 2.9 Schematic representation of Gab2 signaling and its negative regulation by RSK.

While Gab2 phosphorylation at tyrosine residues promotes Shp2 recruitment and activation of the Ras/MAPK pathway, RSK-mediated phosphorylation of Gab2 on Ser/Thr residues inhibits Shp2 binding and Shp2-dependent signaling. RSK-mediated phosphorylation of Gab2 likely plays a role in tightly regulating Gab2-dependent biological functions, such as the regulation of cell motility.

Preface to Chapter 3

Deregulation of ERK1/2 activity causes a number of human diseases, such as cancer, inflammatory diseases and severe bone disorders. Hence, understanding the contribution of ERK1/2 to these diseases has become an attractive research field. Indeed, this question can be addressed by the identification of ERK1/2 substrates, which participate in numerous biological events, such as cell proliferation, apoptosis, transformation and migration. Several years ago, Gab2 was identified as an ERK1/2 substrate, and overexpression of Gab2 has been found to promote cell proliferation and migration. However, one question concerning how ERK1/2 regulate Gab2 function still remains elusive. Using a combination of genetics and biochemistry tool, we asked (a) How ERK1/2 mediate Gab2 phosphorylation. (b) Whether these phosphorylation events affect Gab2 association with different binding partners, such as Shp2 and p85. (c) If mitogen-induced Gab2 phosphorylation by ERK1/2 affects its biological functions, such as cell proliferation and cell motility.

Chapter 3 ERK1/2 participate in a negative feedback loop that limits Gab2 function in response to growth factors

In preparation

ERK1/2 participate in a negative feedback loop that limits Gab2 function in response to growth factors

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Keywords: Gab2; MAPK; ERK1/2; D-domain; phosphorylation; PI3K.

ABSTRACT

The scaffolding adapter protein Gab2 (Grb2-associated binder) promotes cell proliferation, survival and motility by engaging several signaling pathways downstream of growth factor and cytokine receptors. The involvement of Gab2 in the activation of different signaling pathways is well documented, but very little is known about the molecular mechanisms that potentiate or attenuate its function. Here we show that Gab2 becomes phosphorylated on Ser/Thr-Pro residues upon stimulation of the Ras/MAPK pathway. More specifically, we demonstrate that the protein kinases ERK1 and ERK2 directly phosphorylate Gab2 *in vitro* and in cells. We find that ERK1/2 interact with Gab2 via a novel docking domain which appears to be important for subsequent Gab2 phosphorylation by these protein kinases. Using site-directed mutagenesis, we identified five ERK1/2-dependent phosphorylation sites in Gab2 that are conserved amongst vertebrate Gab2 orthologues. We show that mutation of ERK1/2-dependent phosphorylation sites promotes p85 recruitment, suggesting that the Ras/MAPK pathway negatively regulates Gab2-dependent signaling. Taken together, our results provide a novel regulatory mechanism by which ERK1/2 participates in a negative feedback loop that limits the signaling response of growth factor and cytokine receptors.

INTRODUCTION

The Ras/mitogen-activated protein kinase (MAPK) pathway plays a central role in transducing extracellular signals to intracellular target proteins involved in cell growth, survival and proliferation (1-4). As such, this pathway is deregulated in a variety of human disorders and diseases, including many types of cancer (5-7). Activation of the Ras/MAPK pathway is often initiated at the plasma membrane, where the binding of growth factors to receptor tyrosine kinases (RTKs) promotes the recruitment of several adaptor proteins, such as Grb2 (growth factor receptor-bound protein 2). Using its SH3 (Src homology 3) domains, Grb2 interacts with the Ras-specific guanine nucleotide exchange factor (GEF) SOS (son of sevenless) to promote the activation of Ras GTPases at the plasma membrane (8, 9). These events trigger the Ras-dependent activation of Raf, resulting in the sequential activation of MEK1/2 (MAPK/ERK kinase 1/2) and the MAPKs ERK1 and ERK2 (extracellular signal-regulated kinase 1/2) (10). ERK1/2 are proline-directed Ser/Thr kinases that phosphorylate substrate protein sequences containing, at minimum, a proline at the +1 position (S/T-P). Upon activation, ERK1/2 phosphorylate many cellular targets involved in cell growth, survival and proliferation (1, 3). ERK1/2-mediated phosphorylation is often facilitated by docking domains (D-domains) located in protein substrates (11), which consist of two or more basic residues and a cluster of hydrophobic residues separated by a short peptide linker (R/K-R/K-X_{2-6}-Φ -X-Φ, where Φ is a hydrophobic residue, such as Leu, Iso or Val, and X is any amino acids). ERK1/2 contain a common docking (CD) domain, which includes aspartate residues (D316 and D319 in ERK2), that mediate interactions with D-domains (12, 13).

In addition to SOS, Grb2 associates with many adaptor proteins, including Gab (Grb2-associated binding protein) family members (Gab1-3) (14, 15). Gab proteins are devoid of enzymatic activity, but act as signaling platforms downstream of several RTKs and non-TK receptors, such as cytokine and G protein-coupled receptors. The Gab proteins have a similar topology, including an N-terminal pleckstrin homology (PH) domain, multiple proline-rich domains, and several phosphotyrosine residues. While the PH domain binds membrane-associated phospholipids and mediates the rapid membrane localization of Gab proteins, the proline-rich domains (PXXP) of Gab proteins constitutively associate with the

SH3 domains of Grb2 (14). Grb2 binding also participates in plasma membrane localization, but this interaction appears to be more important for the long-term residency of Gab proteins in this cellular compartment (16). Upon recruitment to the plasma membrane, Gab proteins become tyrosine phosphorylated and interact with SH2 domain-containing proteins, such as Shp2 (SH2 domain-containing protein tyrosine phosphatase 2) and the p85 regulatory subunit of PI3K (phosphoinositide 3-kinase), which stimulate the Ras/MAPK and PI3K/Akt signaling pathways, respectively (14).

Amongst Gab family proteins, the Gab2 isoform has received much attention due to its emerging role in human cancer (17). The *GAB2* gene is amplified in 10-15% of ovarian (18) and breast (19) tumors, suggesting its potential contribution to tumorigenesis. Overexpression of Gab2 in human mammary epithelial cells was shown to increase cell proliferation and invasion, and these effects were associated with enhanced and/or more sustained activation of the Ras/MAPK and PI3K/Akt signaling pathways (20, 21). Gab2 was shown to collaborate with HER2 (ErbB2 or Neu) in promoting breast epithelial cell transformation, suggesting its role in signal amplification downstream of this RTK (22). The mechanisms by which Gab2 contributes to cancer development are ill-defined, but the effect of Gab2 on cell proliferation was shown to be dependent on intact Shp2 and Grb2 binding motifs, and was enhanced by its potential to recruit PI3K (20).

In addition to being positively regulated by tyrosine phosphorylation, Gab2 was shown to be phosphorylated on several Ser/Thr residues in response to agonists of the Ras/MAPK and PI3K/Akt pathways (23). Akt phosphorylates Gab2 on Ser159 (according to mouse numbering), which in turn abrogates its phosphorylation on tyrosine residues (23). Additional PI3K-dependent phosphorylation sites exist on Gab2 (Ser210 and Thr391), but the identity of the protein kinase involved in their regulation remains unknown (24). Nonetheless, these phosphorylation sites were shown to mediate 14-3-3 binding, which in turn disrupt Grb2 binding and Gab2 tyrosine phosphorylation (24). With regards to the Ras/MAPK pathway, one study has shown that ERK1/2 phosphorylates Gab2 on Ser612 and thereby inhibits Shp2 recruitment (25). More recently, the MAPK-activated protein kinase RSK (p90 ribosomal S6 kinase) was shown to phosphorylate Gab2 on three residues (Ser159, Ser210, Ser620) that also impair Shp2 recruitment (26), suggesting that ERK and RSK collaborate to inhibit Gab2

function. Evidence suggests that Gab2 is phosphorylated by ERK1/2 on multiple additional residues (25), but the identity of these phosphorylation sites as well as their functions remain unknown.

In the current study, we characterized Gab2 phosphorylation in response to agonists of the Ras/MAPK pathway and identified multiple Ser/Thr residues that are directly phosphorylated by ERK1/2 *in vitro* and in cells. We found that ERK1/2 directly interact with Gab2 through a D-domain located near one of the Grb2-SH3 binding domains. Mutation to ERK1/2-dependent phosphorylation sites resulted in increased Gab2 tyrosine phosphorylation and association with p85, suggesting that ERK1/2 negatively regulate Gab2 function.

MATERIALS AND METHODS

DNA constructs and recombinant proteins. The plasmids encoding hemagglutinin (HA)-tagged murine Gab1 and Gab2 were graciously provided by Drs. Morag Park (McGill University, Canada) and Isabelle Royal (University of Montreal, Canada), respectively, and described previously (27, 28). The vectors encoding constitutively active forms of Ras (G12V) and MEK1 (MEK-DD) and the inactive form of Ras (S17N) were described previously (29, 30). All HA-tagged ERK1 constructs were provided by Dr. Sylvain Meloche (IRIC, University of Montreal, Canada). To subclone murine Gab1 and Gab2 into pcDNA3.0-6myc, HA-tagged Gab1 and Gab2 were amplified by PCR and described previously (26). All murine Gab2 mutants were generated using the QuikChange methodology (Stratagene, La Jolla, CA). To subclone murine Gab2 into pBabe-puro and produce retroviral particles used in the generation of stable cell lines, myc-tagged wild-type (wt) and mutant Gab2 were amplified by PCR as described previously (26).

Antibodies. Antibodies targeted against pSer/Thr-Pro residues, Gab2, phospho-Akt (S473), Akt, p85, ERK1/2, phospho-ERK1/2 (T202/Y204), S6 and phospho-S6 (S235/236) were purchased from Cell Signaling Technologies (Beverly, MA). The Gab2 antibody used for immunoprecipitation and phospho-Tyr (PY99) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-Myc, anti-HA and anti-Tubulin monoclonal antibodies were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). All secondary horseradish peroxidase (HRP)-conjugated antibodies used for immunoblotting were purchased from Chemicon (Temecula, CA).

Cell culture and transfection. HEK293 cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose supplemented with 10% fetal bovine serum (FBS) and antibiotics. HEK293 cells were transfected by calcium phosphate precipitation as previously described (31). Cells were grown for 24h after transfection and serum-starved using serum-free DMEM where indicated for 16 to 18h. Starved cells were pretreated with PD184352 (10 μM), U0126 (20 μM), or BI-D1870 (10 μM) (Biomol, Plymouth Meeting, PA), where indicated, and stimulated with FBS (10%), phorbol 12-myristate 13-acetate (PMA; 100 ng/ml), or EGF (25 ng/ml) before being harvested. Unless

indicated otherwise, all drugs and growth factors were purchased from Invitrogen (Burlington, Ontario, Canada).

RNA interference. For small interfering RNA (siRNA)-mediated knockdown of ERK1 and ERK2, validated 21-nucleotide cRNAs with symmetrical two-nucleotide overhangs were obtained from Qiagen. HEK293 cells were transfected using calcium phosphate and 50 nM siRNA per dish. At 24 h following transfection, cells were serum starved overnight before being harvested.

Immunoprecipitations and immunoblotting. Cell lysates were prepared as previously described (31). Briefly, cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in BLB (10 mM K_3PO_4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 0.5% Nonidet P-40, 0.1% Brij 35, 0.1% deoxycholic acid, 1 mM sodium orthovanadate [Na_3VO_4], 1 mM phenylmethylsulfonyl fluoride, and a cOmplete protease inhibitor cocktail tablet [Roche]). For immunoprecipitations, cell lysates were incubated with the indicated antibodies for 2h, followed by a 1h incubation with protein A-Sepharose CL-4B beads (GE Healthcare). Unless they were used for kinase assays, immunoprecipitates were washed three times in lysis buffer and beads were eluted and boiled in 2×reducing sample buffer (5×buffer is 60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue). For λ-phosphatase assay, exogenous myc-Gab2 immunoprecipitates were washed twice in lysis buffer and incubated for 1h at 30°C with λ-phosphatase (New England BioLabs, Beverly, MA) in the supplied buffer. The reaction was stopped by adding 2×reducing sample buffer. Eluates and total cell lysates were subjected to 8 to 12% SDS-PAGE, and resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes for immunoblotting. The data presented are representative of at least three independent experiments.

Protein phosphotransferase assays. Recombinant activated ERK1 purchased from Millipore was used for kinase assays, which were performed with immunopurified full-length myc-tagged Gab2 or the S143/469/532/612/614A mutant (from here on termed the S5A mutant) as substrates, under linear assay conditions. Assays were performed for 20 min at 30°C in kinase buffer supplemented with ATP. The data presented are representative of at least three independent experiments.

RESULTS

Gab2 is phosphorylated in response to agonists of the Ras/MAPK pathway

To determine whether the Ras/MAPK or the PI3K/Akt pathways could alter the electrophoretic properties of Gab2, we analyzed endogenous Gab2 from HEK293 cells by SDS-PAGE. We found that strong agonists of the Ras/MAPK pathway, such as the phorbol ester PMA (phorbol 12-myristate 13-acetate) and EGF (epidermal growth factor), induced a robust electrophoretic shift in Gab2 which correlated with the phosphorylation of ERK1/2 (Fig. 3.1A). Conversely, treatment of cells with insulin or serum, which are potent agonists of the PI3K/Akt pathway, did not significantly alter Gab2 mobility on SDS-PAGE. To determine if the mobility shift of Gab2 was indeed due to protein phosphorylation, we immunoprecipitated transfected myc-tagged Gab2 from cells stimulated with PMA and treated immunoprecipitates with λ-phosphatase. With this approach, we found that phosphatase treatment completely abolished the mobility shift of Gab2 induced by PMA stimulation (Fig. 3.1B), demonstrating that stimulation of the Ras/MAPK pathway promotes direct Gab2 phosphorylation. Next, we sought to delineate the protein kinase(s) involved in Gab2 phosphorylation using pharmacological inhibitors of MEK1/2 (PD184352 and U0126) to prevent ERK1/2 activation (Fig. 3.1C). We also tested a RSK inhibitor (BI-D1870), as we have previously shown that this kinase directly phosphorylates Gab2 in response to agonists of the Ras/MAPK pathway (Fig. 3.1C). While RSK inhibition did not significantly affect the mobility shift of Gab2 induced by PMA, we found that treatment of cells with MEK1/2 inhibitors efficiently blocked Gab2 phosphorylation (Fig. 3.1D). As ERK1/2 are the only known MEK1/2 substrates, these results suggest that ERK1/2, or a kinase other than RSK downstream of ERK1/2, regulates Gab2 phosphorylation. Finally, to confirm the direct involvement of ERK1/2 in Gab2 phosphorylation, we used small interfering RNA (siRNA) mediated RNAi (RNA interference) to specifically reduce endogenous expression of ERK1 and ERK2. We found that depletion of endogenous ERK1 and ERK2 resulted in a strong inhibition of the mobility shift in Gab2 upon PMA stimulation (Fig. 3.1E), suggesting that endogenous ERK1/2 are required for Gab2 phosphorylation.

ERK1/2 phosphorylate Gab2 *in vitro* and in cells

To determine if Gab2 is a direct substrate of ERK1/2, which are proline-directed kinases (10), we took advantage of a phospho-motif antibody that recognizes potential ERK1/2 phosphorylation sites (pS/T-P), as done previously (32). HEK293 cells transfected with myc-tagged Gab2 were serum-starved overnight and stimulated over a time-course of EGF. Immunoprecipitated Gab2 was then analyzed for phosphorylation by immunoblotting using the anti-pS/T-P antibody. Using this approach, we found that EGF treatment rapidly stimulated Gab2 phosphorylation on the pS/T-P consensus motif, which correlated well with the phosphorylation status of ERK1/2 (Fig. 3.2A). The requirement for ERK1/2 activity was confirmed by showing that treatment of cells with MEK1/2 inhibitors (PD184352) completely abrogated Gab2 phosphorylation on the pS/T-P consensus motif (Fig. 3.2B). RSK was excluded from these effects by showing that two different pharmacological inhibitors (BI-D1870 and SL-0101) did not affect Gab2 phosphorylation on the pS/T-P consensus motif (Fig. 3.2C). To confirm the direct involvement of ERK1/2 in the regulation of Gab2 phosphorylation on the pS/T-P consensus motif, we used siRNAs to specifically knockdown endogenous ERK1 and/or ERK2 in HEK293 cells. As shown with the mobility shift (Fig. 3.1E), we found that knockdown of endogenous ERK1 and ERK2 significantly reduced Gab2 phosphorylation on the pS/T-P consensus motif (Fig. 3.2D), indicating that Gab2 is targeted by both ERK1 and ERK2. To determine whether endogenous Gab2 is also subject to a similar regulation, we immunoprecipitated endogenous Gab2 in HEK293 cells and detected its phosphorylation using the anti-pS/T-P antibody. Compared to unstimulated cells, we found that PMA stimulation increased endogenous Gab2 phosphorylation on the pS/T-P consensus motif (Fig. 3.2E). This effect was completely inhibited by the addition of a MEK1/2 inhibitor (PD184352), suggesting that ERK1/2 phosphorylates endogenous Gab2 in cells. Finally, to investigate whether Gab2 is a direct substrate of ERK1/2, we performed *in vitro* kinase assays using recombinant activated ERK1 and immunopurified wild-type Gab2 as substrate. While no phosphate incorporation was seen in purified Gab2 in the absence of ATP or recombinant ERK1, we found a significant induction of Gab2 phosphorylation in the presence of both ATP and recombinant ERK1 (Fig. 3.2F). Taken together, these results demonstrate that ERK1/2 directly phosphorylate Gab2 *in vitro* and in cells.

ERK1/2 are recruited to Gab2 in a MEK1/2-dependent manner

To determine if ERK1/2 physically interact with Gab2, HEK293 cells were transfected with myc-tagged Gab2 and immunoprecipitates were assayed for the presence of endogenous ERK1/2 over a time-course of EGF treatment. While Gab2 and ERK1/2 did not appear to interact in unstimulated cells, we found that EGF stimulation potently induced their association in cells (Fig. 3.3A). The interaction between Gab2 and ERK1/2 correlated very well with the levels of ERK1/2 phosphorylation, suggesting that ERK1/2 activation was necessary for its association with Gab2. To determine this, we pretreated cells with the MEK1/2 inhibitor PD184352 prior to PMA or EGF stimulation, and found that MEK1/2 inhibition completely abrogates ERK1/2 interaction (Fig. 3.3B). We confirmed the requirement for ERK1/2 activation by expressing an activated form of MEK1 (S212/218D), which resulted in the association of Gab2 and ERK1/2 in the absence of growth factors or serum (Fig. 3.3C). Having demonstrated that Gab2 interacts with ERK1/2 in response to MEK1/2 activation, we next determined whether ERK1/2 activity was required for this association. For this, myc-tagged Gab2 was cotransfected with wild-type ERK1 or an unphosphorylatable ERK1 mutant (T202A/Y204F; AEF). While exogenous wild-type ERK1 was found to interact with Gab2 in response to PMA stimulation (Fig. 3.3D), the AEF mutant of ERK1 did not, suggesting that ERK1/2 needs to be in an activated conformational state to associate with Gab2. We confirmed that endogenous Gab2 also interact with ERK1/2 in a stimulation-dependent manner, by looking at ERK1/2 association within endogenous Gab2 immunoprecipitates from HEK293 cells (Fig. 3.3E). The Gab1 isoform was previously shown to interact with ERK1/2 (33). We thus compared Gab1 and Gab2 in their ability to interact with ERK1/2 in response to stimulation of the Ras/MAPK pathway. While we found that Gab1 appeared to interact with ERK1/2 to some degree, we did not see evidence for a regulated interaction as shown for Gab2 (Fig. 3.3F). Together, these results demonstrate that Gab2, but not Gab1, specifically interacts with ERK1/2 in response to agonists of the Ras/MAPK pathway.

Identification of a D-domain in Gab2 involved in ERK1/2 binding

Next, we sought to determine whether Gab2 directly interacts with activated ERK1/2. ERK1/2 often associate with substrate proteins using specialized docking motifs, termed D-domains (34). These domains specifically interact with residues within the CD domain of ERK1/2, which includes aspartate residues (D328 in ERK1) (12, 13). To determine if the CD domain of ERK1 is involved in Gab2 binding, we performed co-immunoprepicipation experiments with wild-type Gab2 and wild-type ERK1 or the ERK1 D328N mutant (Fig. 3.4A). Compared to wild-type ERK1, we found that the ERK1 D328N mutant was impaired in its ability to associate with Gab2, suggesting that Gab2 possess a D-domain. Using the bioinformatics tool Scansite (35), we analyzed the Gab2 sequence for the presence of a potential D-domain. Notably, this search led to the high-confidence identification of a potential D-domain (score 0.377; percentile 0.031%) located between residues 510 and 524 of mouse Gab2 (**RK**A**K**PTP**L**D**L**RNNTV, where the important residues are shown in boldface). Sequence alignment revealed that this motif is conserved within vertebrate orthologues of Gab2, but appears to contain some substitutions in the Gab1 and Gab3 isoforms (Fig. 3.4B). To determine if this putative D-domain was functional, we individually mutated all charged residues within this motif and assayed Gab2 association to ERK1/2. As shown in Fig. 3.4C, we found that mutation of Arg510, Lys511, Lys513, Leu517 and Leu519 resulted in decreased association with ERK1/2. We also evaluated Gab2 phosphorylation on pS/T-P consensus motifs, and found that mutation of Leu517 and Leu519 had the greatest impact (Fig. 3.4C), consistent with the idea that protein-protein interaction mediated by hydrophobic residues usually results in tighter binding than salt bonds (36). Mutation of Leu506 had no effect on ERK1/2 association and Gab2 phosphorylation, demonstrating that this hydrophobic residue is not part of the newly identified D-domain. To determine the respective role of Leu517 and Leu519, we generated a double mutant (L517/519A) and analyzed its ability to interact with ERK1/2 and become phosphorylated by these protein kinases. As shown in Fig. 3.4D, we did not find additive effects of mutating these two residues, indicating that they are both core constituents of the D-domain (Fig. 3.4D). We also analyzed the ability of the Gab2 L517A mutant to interact with ERK1/2 during a time-course of EGF stimulation. As shown in Fig. 4E, we found that this mutant is impaired in ERK1/2 recruitment and also becomes less

efficiently phosphorylated by these enzymes. Having demonstrated that the D-domain in Gab2 is functional, we next determined whether Ile537 in Gab1, which corresponds to Leu519 in Gab2, was responsible for the reduced association of this isoform to ERK1/2. For this, we mutated Leu519 in Gab2 to Ile and determined the ability of this mutant to interact with ERK1/2 compared to wild-type Gab2. Notably, we found that the L519I mutant of Gab2 was impaired in its ability to interact with ERK1/2 (Fig. 3.4F), providing a potential explanation for the observed differences between Gab1 and Gab2. Together, these results indicate that ERK1/2 directly interact and phosphorylate Gab2 in response to stimulation of the Ras/MAPK pathway.

Identification of ERK1/2 phosphorylation sites in Gab2

To identify ERK1/2-dependent phosphorylation sites in Gab2, we analyzed the sequence surrounding all Ser/Thr-Pro residues for similarities to phosphorylation sites in known substrates of ERK1/2 (10). We located five potential phosphorylation sites, consisting of Ser143, Ser469, Ser532, Ser612, and Ser614 (according to the Gab2 mouse numbering), which were previously found in proteomics studies to be phosphorylated in cells (Fig. 3.5A) (37). Amongst these, Ser143, Ser469, Ser532, Ser612 were shown to be modulated by EGF stimulation by quantitative mass spectrometry (24). Given the lack of phosphorylation site-specific antibodies, we mutated all five residues to unphosphorylatable alanines, and assayed Gab2 phosphorylation in response to PMA stimulation. While the mutation of each individual site did not noticeably reduce overall Gab2 phosphorylation, the combination of multiple mutations resulted in reduced electrophoretic mobility and phosphorylation as assessed with the anti-pS/T-P antibody (Fig. 3.5B). Indeed, our results indicate that the S2A (S612/614A), S3A1 (S143/469/532A) and S3A2 (S532/612/614A) mutants of Gab2 had reduced electrophoretic mobility in response to PMA stimulation (Fig. 3.5B). The combination of all these mutations into a single Gab2 protein (S5A) appeared to almost completely prevent Gab2 phosphorylation in response to EGF (Fig. 3.5C), PMA (Fig. 3.5D) and MEK-DD expression (Fig. 3.5E), suggesting that these resides are the predominant ERK1/2 phosphorylation sites in Gab2. Furthermore, we performed *in vitro* kinase assays to confirm that ERK1/2 directly phosphorylate Ser143/469/532/612/614. As shown in Fig. 3.5F, we found that ERK1-mediated phosphate incorporation was robustly decreased in the Gab2

S5A mutant compared to wild-type Gab2, indicating that these residues are directly phosphorylated by Gab2. These phosphorylation sites, as well as the following proline residue, appear to be conserved amongst vertebrate species, suggesting that they play important biological functions (Fig. 3.5G). Notably, many of these sites appear to cluster around the p85-SH2 binding sites (Fig. 3.5A), suggesting that they may be involved a feedback regulation of p85 and/or Shp2 binding.

ERK1/2 negatively regulate Gab2-dependent signaling by restricting p85 recruitment

To assess whether ERK1/2-mediated Gab2 phosphorylation affects p85 recruitment to Gab2, we first analyzed immunoprecipitated Gab2 from HEK293 cells transfected with MEK-DD, to constitutively stimulate Gab2 Ser/Thr phosphorylation at ERK1/2 sites. In response to EGF stimulation, we found that cells expressing MEK-DD and having increased Gab2 phosphorylation on Ser/Thr residues showed reduced association between Gab2 and p85, as well as reduced Gab2 tyrosine phosphorylation (Fig. 3.6A). These results suggest that the Ras/MAPK pathway negatively regulates p85 recruitment to Gab2, possibly due to ERK1/2-dependent phosphorylation of Gab2. To test this, HEK293 cells transfected with wild-type Gab2 or the S5A Gab2 mutant were treated with EGF over a time course, prior to Gab2 immunoprecipitation. As shown in Fig. 3.6B, we found that p85 was more efficiently recruited to the Gab2 S5A mutant compared to wild-type Gab2 in response to EGF stimulation. These results suggest that ERK1/2-dependent phosphorylation of Gab2 on Ser143/469/532/612/614 negatively regulates p85 recruitment, possibly due to a local steric effect of Ser/Thr phosphorylation. These feedback regulatory mechanisms appear to be specific to p85, as we did not see differences between wild-type Gab2 and the S5A mutant in their ability to recruit and interact with Shp2 and Grb2, respectively. Taken together, these results suggest a model in which ERK1/2 negatively regulates Gab2 function by reducing p85 association.

DISCUSSION

While the physiological functions of Gab2 during mouse development and in human cancer have been largely elucidated, much less is known about the molecular mechanisms involved in its regulation. As for other Gab isoforms, the role and regulation of Gab2 tyrosine phosphorylation is relatively well understood and is usually associated with increased Gab2-dependent signaling in response to growth factors and cytokines. Conversely, Ser/Thr phosphorylation is usually associated with reduced Gab2-dependent signaling, but the protein kinases involved and the exact molecular functions of these phosphorylation events are only partially understood. Our study brings new evidence that ERK1/2 is an important binding partner of Gab2 that mediates its phosphorylation on multiple Ser/Thr residues. These phosphorylation events occur in a feedback paradigm where EGF stimulation promotes ERK1/2 signaling, but also following heterologous activation of ERK1/2, such as in response to phorbol esters and the expression of activated components of the Ras/MAPK pathway. These findings suggest that ERK1/2 may serve to limit Gab2 activation following stimulation, but also indicate that cancer cells harboring activating mutations in components of the Ras/MAPK pathway may have perturbed Gab2-dependent signaling. The fact that Gab2 participates in the signaling response of many types of receptors, including several RTKs and cytokine receptors, suggests that our results may be applicable to a large number of biological situations.

Our results indicate that ERK1/2-mediated Gab2 phosphorylation inhibits the recruitment of p85, possibly by affecting Gab2 tyrosine phosphorylation. The mechanism by which ERK1/2 may affect Gab2 tyrosine phosphorylation remains unknown, but our results suggest that Grb2 binding to Gab2 is not affected by ERK1/2-mediated phosphorylation. Previous reports have shown that Shp2 may dephosphorylate Gab2 on tyrosine residues (38), but our results indicate that Shp2 recruitment is also not affected by ERK1/2-mediated phosphorylation. This is in contrast to a previous study which indicated that ERK1/2-mediated Gab2 phosphorylation on Ser612 inhibits Shp2 recruitment (25). These discrepancies may be explained by results showing that the ERK1/2-activated protein kinase RSK directly phosphorylates Gab2 and thereby inhibits Shp2 recruitment (26). In this scenario, it may not be the direct action of ERK1/2 that affects Shp2 recruitment, but rather

the activation of RSK which depends on the Ras/MAPK pathway. Together, these results suggest that ERK1/2 and RSK may collaborate in the inhibition of Gab2 function by promoting site-specific phosphorylation events that prevents the recruitment of both Shp2 and p85. These results are consistent with the idea that the Ras/MAPK pathway participates in feedback loops that inhibits its own activation and that of the PI3K/Akt pathway, as shown in a number of studies using MEK1/2 inhibitors. Our results suggest that Gab2 may be an important signaling node that synthesizes upstream and downstream signals.

One of the interesting observations that arise from our work is the identification of a new ERK1/2-docking domain within Gab2 (**RK**AKPTP**L**D**L**), which appears to be required for efficient Gab2 phosphorylation by ERK1/2. While comparing Gab1 and Gab2, we found that growth factors specifically promote the recruitment of ERK1/2 to Gab2, suggesting that Gab1 does not have a functional ERK1/2-docking domain. Consistent with this, we found that the corresponding sequence in Gab1 contains an amino acid change that reduces the affinity to ERK1/2, which likely explains differences in ERK1/2 binding. Curiously, a previous study has reported that Gab1 interacts with ERK1/2 with a higher affinity than Gab2 (39). This interaction was shown to depend on the Met-binding domain (MBD) of Gab1, which interestingly contains part of the identified ERK1/2-docking domain. These discrepancies likely results from the type of bait proteins used in GST pull-downs (39), which for Gab2 consisted of a deletion mutant that only contained part of the ERK1/2-docking domain that we identified in this study. Together, these results suggest that ERK1/2 plays an important role in the regulation of Gab2 function, whereas the role of ERK1/2 in the regulation of Gab1 remains to be determined.

Based on its frequent amplification in breast cancer, Gab2 has been characterized as a potential oncogene that appears to collaborate with HER2 (21, 40). The deletion of Gab2 was shown to reduce HER2-mediated tumorigenesis (21), suggesting that Gab2 participates in the signaling response elicited by HER2. Our results suggest that ERK1/2 limits Gab2-dependent signaling by inhibiting its association with key binding partners. These findings suggest that mutation of ERK1/2 phosphorylation sites in Gab2 may increase its ability to collaborate with HER2, but more experiments will be required to directly address this. We have looked at databases of cancer somatic mutations, but did not find evidence of Gab2 mutation at ERK1/2

phosphorylation sites. Nonetheless, it is probable that ERK1/2-dependent phosphorylation of Gab2 may impede its ability to participate in HER2-mediated tumorigenesis.

In summary, our study provides evidence for a new mechanism by which ERK1/2 negatively regulate Gab2 function. We propose a model whereby the Ras/MAPK pathway has a dual role in determining the strength and duration of Gab2-dependent signaling, namely via ERK1/2- and RSK-mediated phosphorylation. Based on the involvement of Gab2 in several types of cancer, including breast cancer and melanoma, our results provide important information regarding the complexity of RTK signaling.

ACKNOWLEDGMENTS

We deeply appreciate all members of the Roux laboratory for their insightful discussions and comments on the manuscript and data. This work was supported by grants from the Canadian Institutes of Health Research (CIHR; MOP123408), the Canadian Cancer Society Research Institute (#700878), and the Cancer Research Society (F121153) (to P.P.R). P.P.R holds the Canada Research Chair in Signal Transduction and Proteomics, and X.Z. holds a China Scholarship Council studentship. M.C. holds a doctoral studentship from the Fonds de la Recherche du Québec en Santé (FRQS). IRIC core facilities are supported by the FRQS. We have no conflict of interests to declare.

REFERENCES

1. **Meloche S, Pouyssegur J.** 2007. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. Oncogene **26:**3227-3239.
2. **Rubinfeld H, Seger R.** 2005. The ERK cascade: a prototype of MAPK signaling. Mol Biotechnol **31:**151-174.
3. **Yoon S, Seger R.** 2006. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors **24:**21-44.
4. **Roux PP, Blenis J.** 2004. ERK and p38 MAPK-Activated Protein Kinases: a Family of Protein Kinases with Diverse Biological Functions. Microbiology and molecular biology reviews : MMBR **68:**320-344.
5. **Lawrence MC, Jivan A, Shao C, Duan L, Goad D, Zaganjor E, Osborne J, McGlynn K, Stippec S, Earnest S, Chen W, Cobb MH.** 2008. The roles of MAPKs in disease. Cell research **18:**436-442.
6. **Roux PP, Blenis J.** 2007. MAPK Signaling in Human Diseases, p. 135-149. *In* Srivastava R (ed.), Apoptosis, Cell Signaling, and Human Diseases: Molecular Mechanisms, vol. 2. Humana Press.
7. **Schubbert S, Shannon K, Bollag G.** 2007. Hyperactive Ras in developmental disorders and cancer. Nature reviews. Cancer **7:**295-308.
8. **Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA.** 1993. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature **363:**45-51.
9. **Rozakis-Adcock M, Fernley R, Wade J, Pawson T, Bowtell D.** 1993. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature **363:**83-85.
10. **Cargnello M, Roux PP.** 2011. Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. Microbiology and molecular biology reviews : MMBR **75:**50-83.
11. **Tanoue T, Nishida E.** 2003. Molecular recognitions in the MAP kinase cascades. Cellular signalling **15:**455-462.

12. **Zhou T, Sun L, Humphreys J, Goldsmith EJ.** 2006. Docking interactions induce exposure of activation loop in the MAP kinase ERK2. Structure **14:**1011-1019.
13. **Lee T, Hoofnagle AN, Kabuyama Y, Stroud J, Min X, Goldsmith EJ, Chen L, Resing KA, Ahn NG.** 2004. Docking motif interactions in MAP kinases revealed by hydrogen exchange mass spectrometry. Molecular cell **14:**43-55.
14. **Wohrle FU, Daly RJ, Brummer T.** 2009. Function, regulation and pathological roles of the Gab/DOS docking proteins. Cell Commun Signal **7:**22.
15. **Wohrle FU, Daly RJ, Brummer T.** 2009. How to Grb2 a Gab. Structure **17:**779-781.
16. **Sampaio C, Dance M, Montagner A, Edouard T, Malet N, Perret B, Yart A, Salles JP, Raynal P.** 2008. Signal strength dictates phosphoinositide 3-kinase contribution to Ras/extracellular signal-regulated kinase 1 and 2 activation via differential Gab1/Shp2 recruitment: consequences for resistance to epidermal growth factor receptor inhibition. Molecular and cellular biology **28:**587-600.
17. **Adams SJ, Aydin IT, Celebi JT.** 2012. GAB2--a scaffolding protein in cancer. Molecular cancer research : MCR **10:**1265-1270.
18. **Cancer Genome Atlas Research N.** 2011. Integrated genomic analyses of ovarian carcinoma. Nature **474:**609-615.
19. **Cancer Genome Atlas N.** 2012. Comprehensive molecular portraits of human breast tumours. Nature **490:**61-70.
20. **Brummer T, Schramek D, Hayes VM, Bennett HL, Caldon CE, Musgrove EA, Daly RJ.** 2006. Increased proliferation and altered growth factor dependence of human mammary epithelial cells overexpressing the Gab2 docking protein. The Journal of biological chemistry **281:**626-637.
21. **Bentires-Alj M, Gil SG, Chan R, Wang ZC, Wang Y, Imanaka N, Harris LN, Richardson A, Neel BG, Gu H.** 2006. A role for the scaffolding adapter GAB2 in breast cancer. Nature medicine **12:**114-121.
22. **Bocanegra M, Bergamaschi A, Kim YH, Miller MA, Rajput AB, Kao J, Langerod A, Han W, Noh DY, Jeffrey SS, Huntsman DG, Borresen-Dale AL, Pollack JR.** 2010. Focal amplification and oncogene dependency of GAB2 in breast cancer. Oncogene **29:**774-779.

23. **Lynch DK, Daly RJ.** 2002. PKB-mediated negative feedback tightly regulates mitogenic signalling via Gab2. The EMBO journal **21:**72-82.
24. **Brummer T, Larance M, Herrera Abreu MT, Lyons RJ, Timpson P, Emmerich CH, Fleuren ED, Lehrbach GM, Schramek D, Guilhaus M, James DE, Daly RJ.** 2008. Phosphorylation-dependent binding of 14-3-3 terminates signalling by the Gab2 docking protein. The EMBO journal **27:**2305-2316.
25. **Arnaud M, Crouin C, Deon C, Loyaux D, Bertoglio J.** 2004. Phosphorylation of Grb2-associated binder 2 on serine 623 by ERK MAPK regulates its association with the phosphatase SHP-2 and decreases STAT5 activation. J Immunol **173:**3962-3971.
26. **Zhang X, Lavoie G, Fort L, Huttlin EL, Tcherkezian J, Galan JA, Gu H, Gygi SP, Carreno S, Roux PP.** 2013. Gab2 phosphorylation by RSK inhibits Shp2 recruitment and cell motility. Molecular and cellular biology **33:**1657-1670.
27. **Caron C, Spring K, Laramee M, Chabot C, Cloutier M, Gu H, Royal I.** 2009. Non-redundant roles of the Gab1 and Gab2 scaffolding adapters in VEGF-mediated signalling, migration, and survival of endothelial cells. Cellular signalling **21:**943-953.
28. **Lock LS, Maroun CR, Naujokas MA, Park M.** 2002. Distinct recruitment and function of Gab1 and Gab2 in Met receptor-mediated epithelial morphogenesis. Molecular biology of the cell **13:**2132-2146.
29. **Carriere A, Cargnello M, Julien LA, Gao H, Bonneil E, Thibault P, Roux PP.** 2008. Oncogenic MAPK signaling stimulates mTORC1 activity by promoting RSK-mediated raptor phosphorylation. Current biology : CB **18:**1269-1277.
30. **Romeo Y, Moreau J, Zindy PJ, Saba-El-Leil M, Lavoie G, Dandachi F, Baptissart M, Borden KL, Meloche S, Roux PP.** 2013. RSK regulates activated BRAF signalling to mTORC1 and promotes melanoma growth. Oncogene **32:**2917-2926.
31. **Roux PP, Ballif BA, Anjum R, Gygi SP, Blenis J.** 2004. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proceedings of the National Academy of Sciences of the United States of America **101:**13489-13494.
32. **Carriere A, Romeo Y, Acosta-Jaquez HA, Moreau J, Bonneil E, Thibault P, Fingar DC, Roux PP.** 2011. ERK1/2 Phosphorylate Raptor to Promote Ras-dependent Activation of mTOR Complex 1 (mTORC1). The Journal of biological chemistry **286:**567-577.

33. **Osawa M, Itoh S, Ohta S, Huang Q, Berk BC, Marmarosh NL, Che W, Ding B, Yan C, Abe J.** 2004. ERK1/2 associates with the c-Met-binding domain of growth factor receptor-bound protein 2 (Grb2)-associated binder-1 (Gab1): role in ERK1/2 and early growth response factor-1 (Egr-1) nuclear accumulation. The Journal of biological chemistry **279:**29691-29699.
34. **Tanoue T, Adachi M, Moriguchi T, Nishida E.** 2000. A conserved docking motif in MAP kinases common to substrates, activators and regulators. Nature cell biology **2:**110-116.
35. **Obenauer JC, Cantley LC, Yaffe MB.** 2003. Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic acids research **31:**3635-3641.
36. **Ubersax JA, Ferrell JE, Jr.** 2007. Mechanisms of specificity in protein phosphorylation. Nature reviews. Molecular cell biology **8:**530-541.
37. **Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, Latham V, Sullivan M.** 2012. PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. Nucleic acids research **40:**D261-270.
38. **Gu H, Pratt JC, Burakoff SJ, Neel BG.** 1998. Cloning of p97/Gab2, the major SHP2-binding protein in hematopoietic cells, reveals a novel pathway for cytokine-induced gene activation. Molecular cell **2:**729-740.
39. **Roshan B, Kjelsberg C, Spokes K, Eldred A, Crovello CS, Cantley LG.** 1999. Activated ERK2 interacts with and phosphorylates the docking protein GAB1. The Journal of biological chemistry **274:**36362-36368.
40. **Celebi JT, Adams SJ, Aydin IT.** 2012. GAB2 - A Scaffolding Protein in Cancer. Molecular cancer research : MCR.

FIGURES

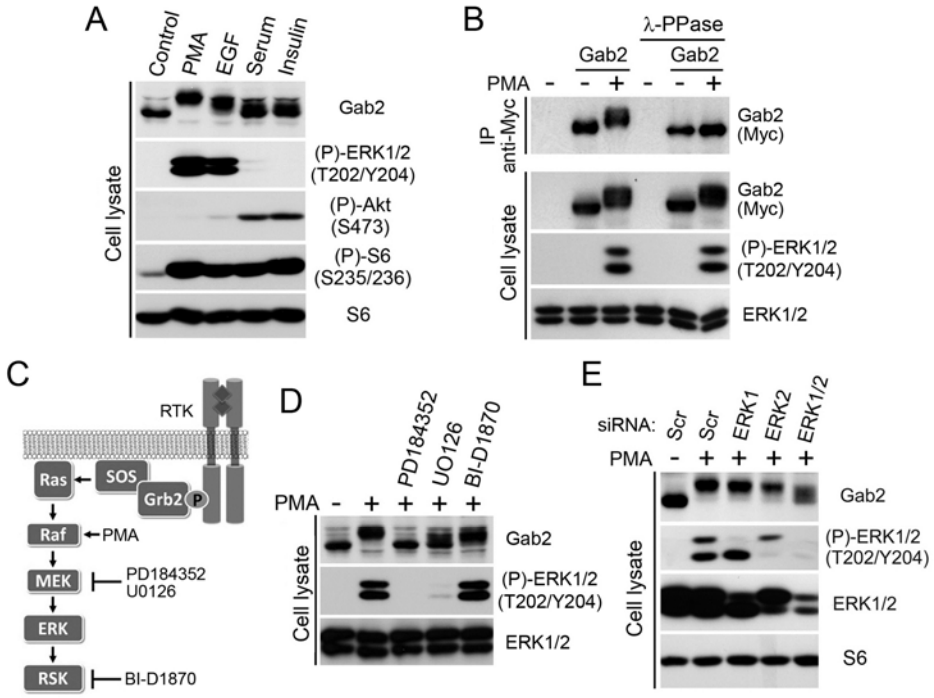

Figure 3.1 Gab2 is phosphorylated in response to agonists of the Ras/MAPK pathway.

(A) HEK293 cells were serum-starved overnight, and stimulated for 30 min with PMA (100 ng/ml; 30 min), EGF (25 ng/ml; 10 min), fetal bovine serum (10%; 15 min), or insulin (100 nM; 20 min). Total and phosphorylated forms of endogenous ERK1/2, Gab2, Akt and S6 were determined by immunoblotting. (B) HEK293 cells were transfected with myc-tagged murine Gab2, serum-starved overnight, and stimulated for with PMA (100 ng/ml) for 30 min. Immunoprecipitated (IP) Gab2 was then treated with 400 units λ-phosphatase for 60 min at 30 °C, and Gab2 phosphorylation was assayed by mobility shift assay. (C) Schematic representation of the agonists and pharmacological inhibitors used in this study. (D) HEK293 cells were serum-starved overnight, pretreated with PD184352 (10 μM), U0126 (20 μM), or BI-D1870 (10 μM) for 30 min prior to PMA (100 ng/ml) stimulation. Phosphorylation of endogenous Gab2 was assayed by mobility shift assay. (E) As for panel (D), except that HEK293 cells were transfected with a constitutively active form of MEK1 (MEK-DD) rather than being stimulated with PMA. (F) HEK293 cells were transfected with either a scrambled siRNA or siRNAs targeting ERK1 and/or ERK2, serum-starved overnight, and stimulated for 30 min with PMA (100 ng/ml). Again, phosphorylation of endogenous Gab2 was assayed by mobility shift assay.

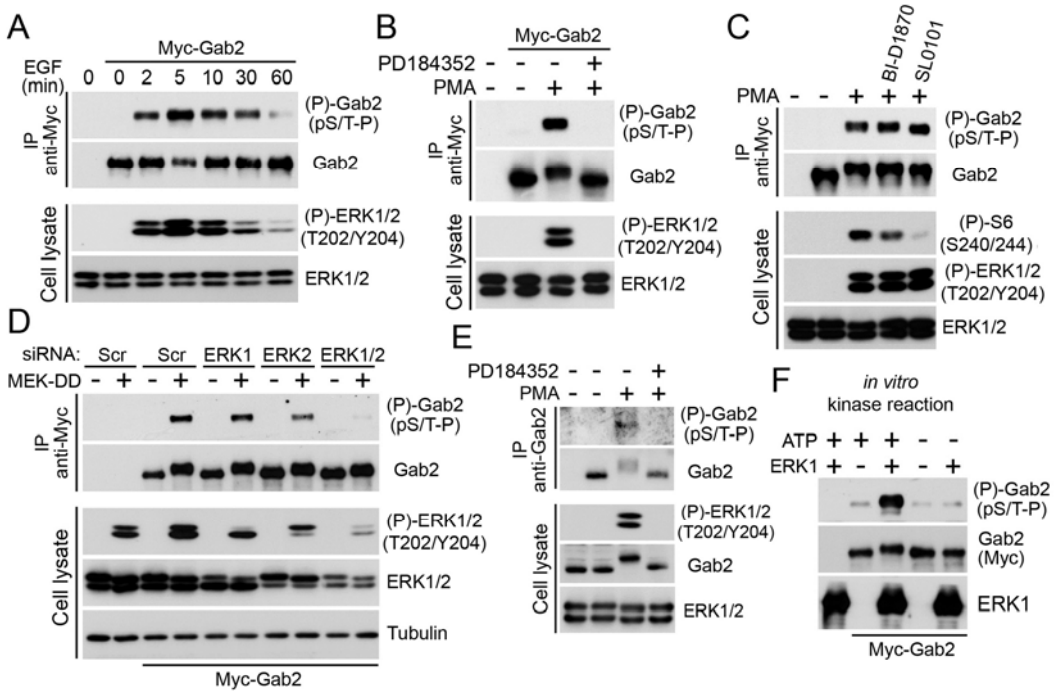

Figure 3.2 ERK1/2 phosphorylate Gab2 *in vitro* and in cells.

(A) HEK293 cells were transfected with myc-Gab2, serum-starved overnight, and stimulated with EGF (25 ng/ml) over a time course (0-60 min). Immunoprecipitated (IP) Gab2 was then assayed for phosphorylation with a phospho-motif antibody that recognizes the pS/T-P motif. Total and phosphorylated forms of ERK1/2 were assayed by immunoblotting on total cell lysates. (B and C) HEK293 cells were transfected with myc-Gab2 and serum-starved overnight. Cells were pre-treated with MEK1/2 (PD184352) or RSK (BI-D1870 and SL0101) inhibitors prior to stimulation with PMA (100 ng/ml) for 30 min. Immunoprecipitated (IP) Gab2 was assayed for phosphorylation using a phospho-motif antibody which recognizes the pS/T-P sequence. (D) HEK293 cells were transfected with flag-tagged MEK-DD and either a scrambled siRNA or siRNAs targeting ERK1 and/or ERK2. Cells were serum-starved overnight, and immunoprecipitated (IP) Gab2 was assayed for phosphorylation using a phospho-motif antibody which recognizes the pS/T-P sequence. (E) As for panel (A), except that endogenous Gab2 was immunoprecipitated. (F) Recombinant activated ERK1 was incubated with immunopurified wt Gab2 in a kinase reaction, and the resulting samples were immunoblotted for Gab2 phosphorylation using a phospho-motif antibody which recognizes the pS/T-P sequence.

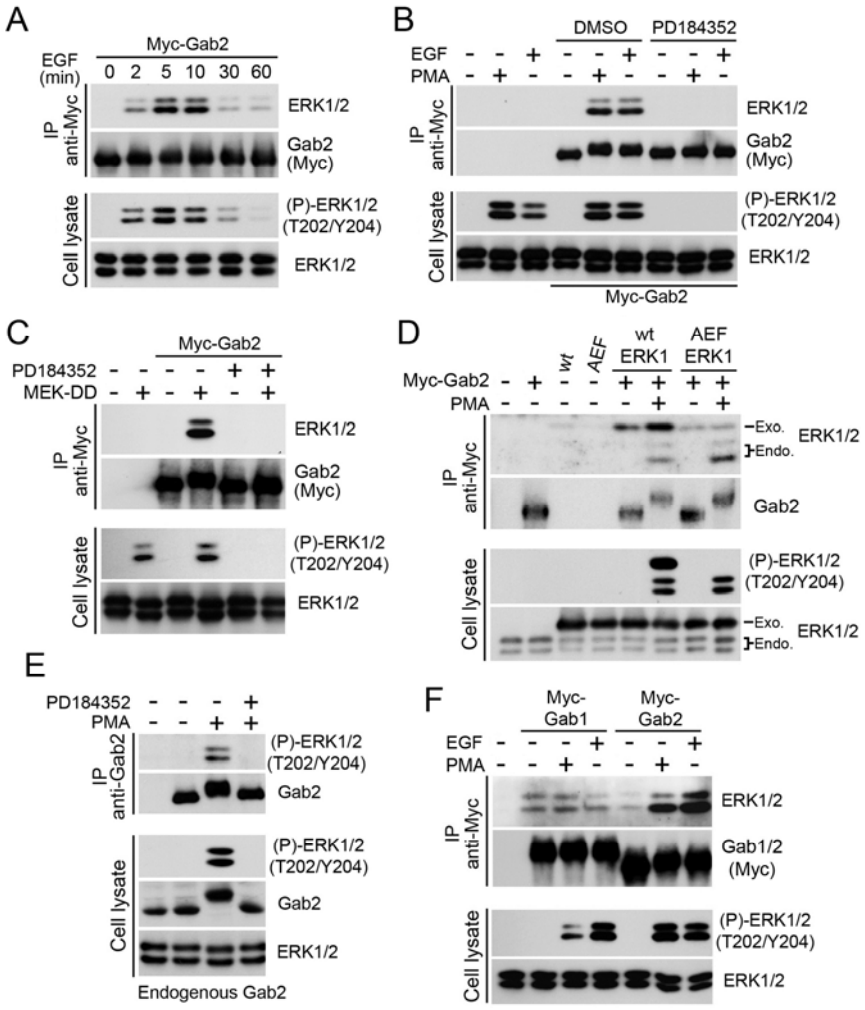

Figure 3.3 ERK1/2 interact with Gab2 in a MEK1/2-dependent manner.

(A) HEK293 cells were transfected with myc-Gab2, serum-starved overnight, and stimulated with EGF (25 ng/ml) over a time course. Associated endogenous ERK1/2 within myc-Gab2 immunoprecipitates was assayed by immunoblotting. (B) HEK293 cells were transfected with myc-Gab2, serum-starved overnight, pre-treated with PD184352 (10 μM) prior to stimulation with EGF (25 ng/ml) for 10 min, or PMA (100 ng/ml) for 30 min. The presence of endogenous ERK1/2 was assayed as described in (A). (C) As for panel (B), except that cells were transfected with MEK-DD instead of being stimulated with PMA. (D) HEK293 cells were co-transfected with myc-Gab2, and wt HA-ERK1 or the AEF (T202A/Y204F) mutant. Cells were serum-starved overnight and stimulated with PMA for 30 min. Associated exogenous ERK1within myc-Gab2 immunoprecipitates was assayed as described in (A). Total and phosphorylated forms of ERK1/2 (endogenous and exogenous) were assayed by immunoblotting on total cell lysates. (E) As for panel (B), except that endogenous Gab2 was immunoprecipitated, and the presence of endogenous phosphorylated ERK1/2 within Gab2 immunoprecipitates was assayed by immunoblotting. (F) HEK293 cells were transfected with myc-Gab1 or myc-Gab2, serum-starved overnight, and stimulated with EGF or PMA. Associated endogenous ERK1/2 was assayed as described for panel (A).

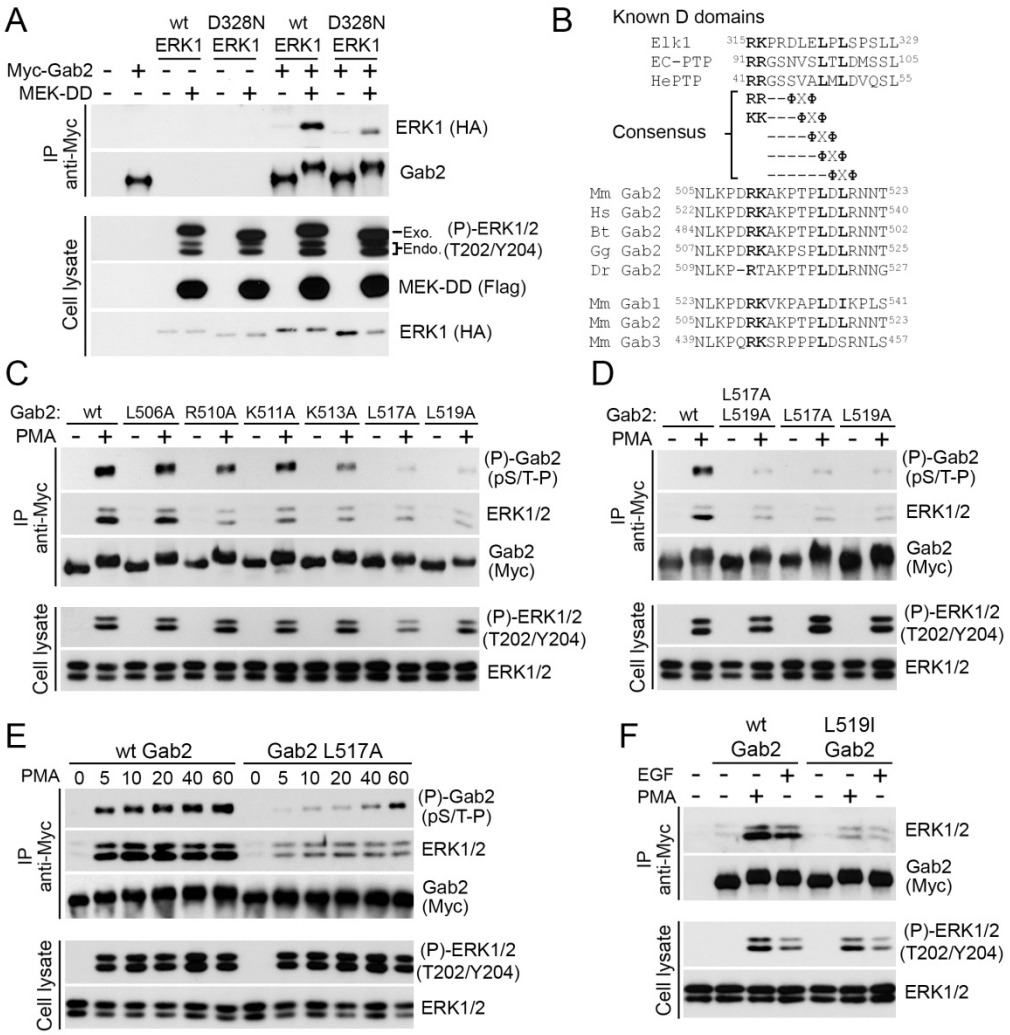

Figure 3.4 Identification of a D-domain in Gab2 involved in ERK1/2 binding.

(A) HEK293 cells were co-transfected with myc-Gab2, and wt HA-ERK1 or the D328N mutant (CD domain mutant), with or without MEK-DD, and serum-starved overnight. Associated exogenous ERK1 within myc-Gab2 immunoprecipitates was assayed by immunoblotting. Total and phosphorylated forms of ERK1/2 (endogenous and exogenous) were assayed by immunoblotting on total cell lysates. (B) Alignment of known D-domains with the potential D-domain found in Gab2 from different vertebrate species, as well as Gab1 and Gab3. (C) HEK293 cells were transfected with myc-Gab2, or potential ERK-binding site mutants (L506A, R510A, K511A, K513A, L517A, L519A), serum-starved overnight, and stimulated with PMA (100 ng/ml) for 30 min. Immunoprecipitated (IP) Gab2 was then assayed for phosphorylation with the phospho-motif antibody which recognizes the pS/T-P sequence, and also endogenous ERK1/2 association. (D) As for panel (C), except that cells were transfected with myc-Gab2, or potential ERK-binding site mutants (L517/519A, L517A, L519A). (E) HEK293 cells were transfected with myc-Gab2 or the Gab2 L517A mutant, serum-starved

overnight, and stimulated with PMA over a time over a time course. Immunoprecipitated (IP) Gab2 was then assayed as described for panel (C). (F) HEK293 cells were transfected with myc-Gab2 or the Gab2 L519I mutant (mimicking the putative Gab1 D-domain), serum-starved overnight, and stimulated with PMA or EGF. Associated exogenous ERK1/2 within myc-Gab2 immunoprecipitates were assayed by immunoblotting.

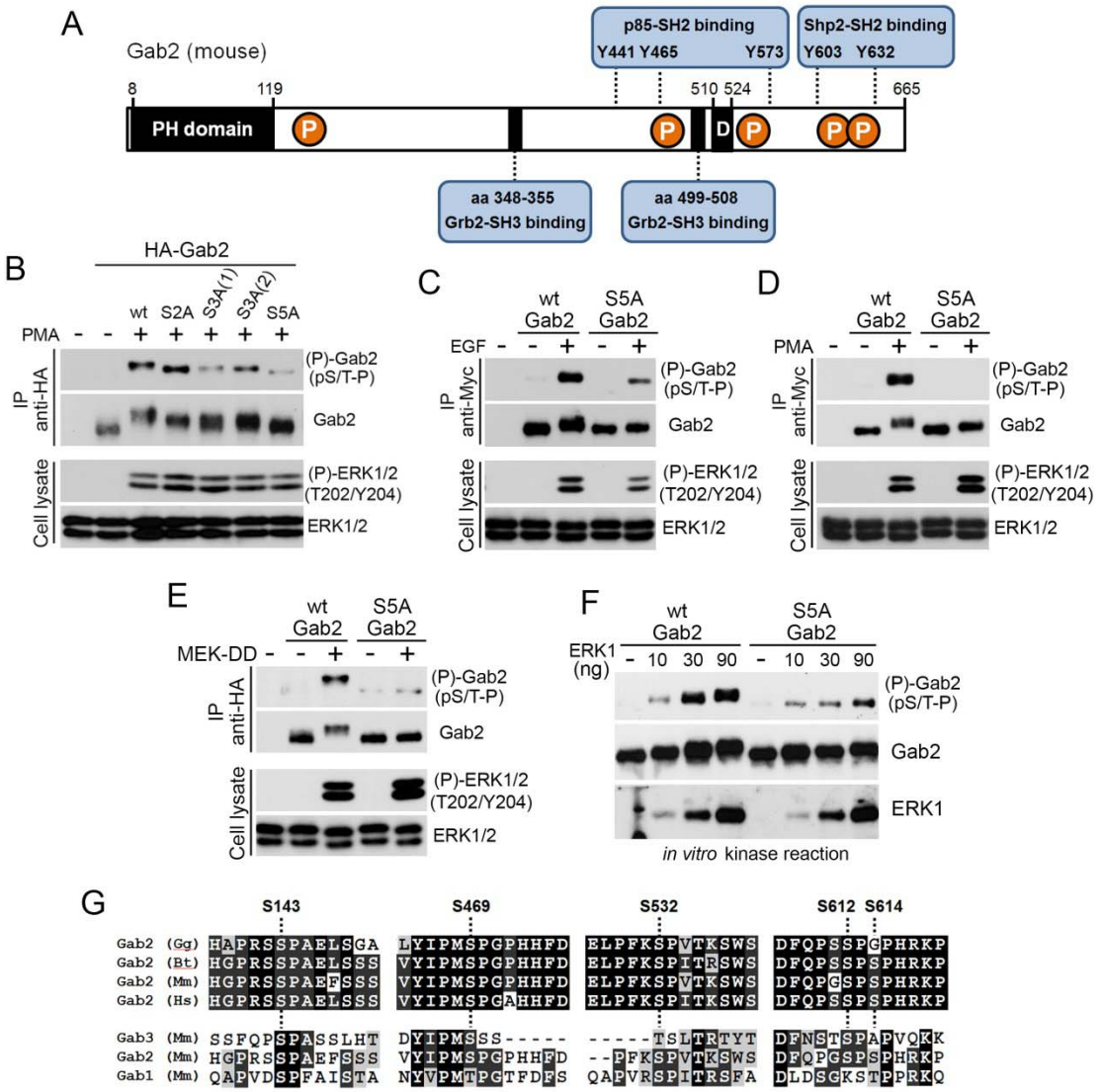

Figure 3.5 Identification of ERK1/2 phosphorylation sites in Gab2.

(A) Schematic representation of Gab2 with known tyrosine phosphorylation sites and domains. (B) HEK293 cells were transfected with myc-tagged wt Gab2 or various combination mutants, including S2A (S612/614A), S3A1 (S143/469/532A), S3A2 (S532/612/614A) and S5A (S143/469/532/612/614A). Cells were serum-starved overnight, and stimulated with PMA (100 ng/ml) for 20 min. Immunoprecipitated (IP) Gab2 was then assayed for phosphorylation with the phospho-motif antibody which recognizes pS/T-P sequences. (C) HEK293 cells were transfected with myc-tagged wt Gab2 or a quintuple mutant with all five serine residues converted to alanines S5A (S143/469/532/612/614A), serum-starved overnight, and stimulated with EGF (25 ng/ml) for 10 min. Immunoprecipitated (IP) Gab2 was then assayed for phosphorylation with the phospho-motif antibody. (D) As for panel (C), except that cells were treated with PMA (100 ng/ml) for 30 min. (E) As for panel (C), except that cells were transfected with MEK-DD prior to serum-starvation.

(F) Recombinant activated ERK1 was incubated with immunopurified wt Gab2 or the Gab2 S5A mutant in a kinase reaction, and the resulting samples were immunoblotted for Gab2 phosphorylation using the phospho-motif antibody. (G) Alignment of the five potential ERK1/2 phosphorylation sites in Gab2 from different vertebrate species and also from different Gab isoforms.

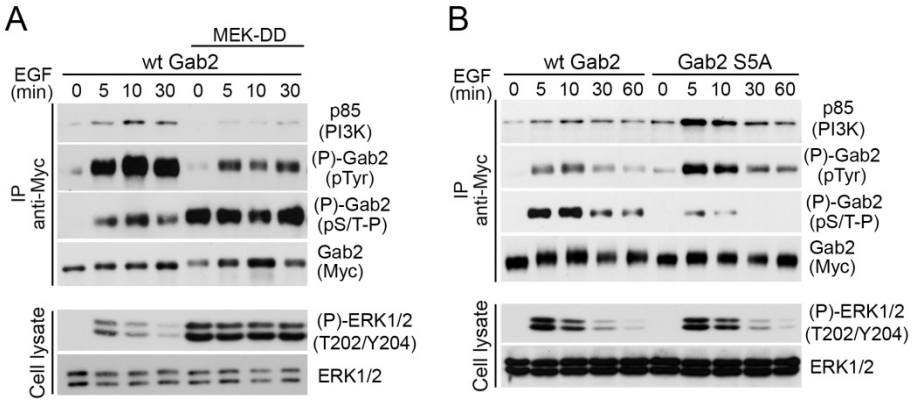

Figure 3.6 ERK1/2 negatively regulate Gab2-dependent signaling by reducing p85 recruitment.

(A) HEK293 cells were co-transfected with myc-Gab2, an empty vector or MEK-DD, serum-starved overnight, and treated with EGF (25 ng/ml) over a time course. Associated endogenous p85 within myc-Gab2 immunoprecipitates was assayed by immunoblotting. Total tyrosine phosphorylation and proline-directed serine/threonine phosphorylation in Gab2 were immunoblotted using the PY99 antibody and the pS/T-P phospho-motif antibodies, respectively. (B) As in panel (A), except that cells were transfected with wt myc-Gab2 or the unphosphorylatable Gab2 S5A mutant. Associated endogenous p85, total tyrosine phosphorylation, and the pS/T-P motif were assayed as described previously.

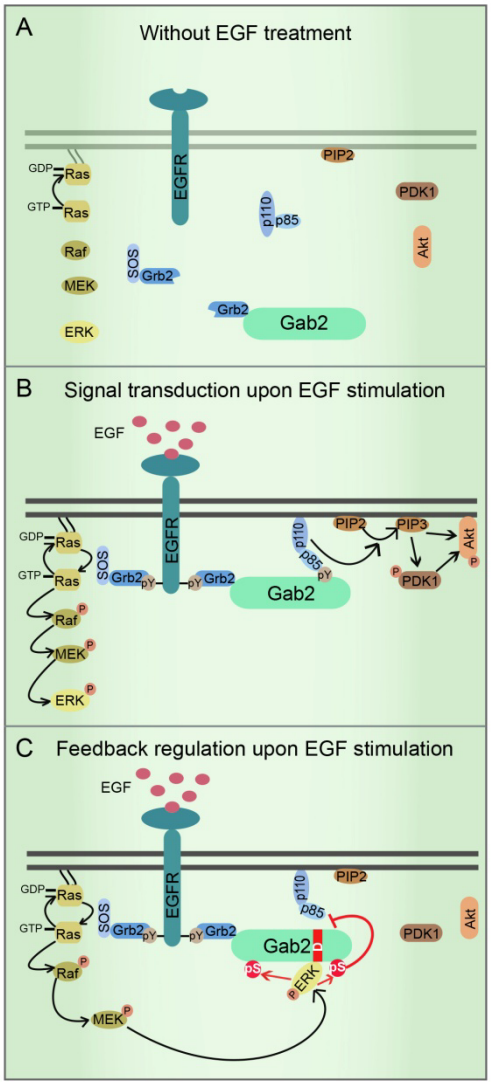

Figure 3.7 Schematic representation of Gab2 regulation by the Ras/MAPK pathway.

Schematic representation of the molecular mechanisms by which the Ras/MAPK pathway regulates Gab2-dependent signaling in response to EGF treatment. (A) Without EGF, Gab2 and its binding partners are cytoplasmic. (B) Upon activation of the EGF receptor, Gab2 is recruited to the plasma membrane where it becomes tyrosine phosphorylated and participates in the activation of the Ras/MAPK and PI3K/Akt signaling pathways. (C) Finally, activated ERK1/2 is recruited to Gab2 via its D-domain, which promotes the ERK1/2-dependent phosphorylation of Gab2 on sites that negatively regulate p85 recruitment.

Chapter 4 General Discussion

4.1 Regulation of Gab2 by RSK kinases

4.1.1 Phosphorylation of Gab2 by RSK impacts the recruitment of its binding partners

In this thesis, using different approaches, we identified Ser^{160}, Ser^{211} and Ser^{620} (according to mouse Gab2 numbering) as three phosphorylation sites in Gab2 with basic residues at the -5 and -3 positions. Among these sites, phosphorylation of Ser^{211} was suggested to be regulated by an unknown PI3K-dependent protein kinase (Brummer *et al.*, 2008), and Ser^{160} was previously found to be directly phosphorylated by Akt1 (Lynch and Daly, 2002). Although the ability to phosphorylate substrates on the basic consensus motif: R/K-X-R/K-X-X-pS/T is a common characteristic of the AGC family of kinases, some members have their own additional preferences for selecting substrates, such as Akt. It was shown that Akt additionally prefers to select substrates with Ser/Thr at -2 position and Phe at +1 position (Obata *et al.*, 2000). Moreover, maintaining a Pro residue at the +2 position to form a tight turn is a feature of Akt distinct from PKC, because the latter is likely to select basic or aromatic residues at this position (Datta *et al.*, 1997). Interestingly, although the substrate specificities of certain AGC kinases have been characterized, Akt and RSK are considered likely to target similar sequences. Several lines of evidence support this thought. First, using PSPL (positional scanning peptide library), Galan *et al.* recently showed that RSK not only selects substrates with Arg at -5 and -3 positions, but also prefers them to have Ser and Pro residues at the -2 and -1 positions, respectively, matching the optimized sequence targeted by Akt (Galan *et al.*, 2014). Second, numerous common substrates of Akt and RSK have been identified, such as PDCD4, TSC2, Bad and Chk1 (Datta *et al.*, 1997; Fang *et al.*, 1999; Inoki *et al.*, 2002; King *et al.*, 2004; Roux *et al.*, 2004; Palamarchuk *et al.*, 2005; Ray-David *et al.*, 2012; Galan *et al.*, 2014). Hence, the phospho-motif antibodies (RXXpS/T & RXXpS/TXP) for selecting Akt substrates can also be used to detect potential RSK substrates. Given that PMA and Ras^{G12V} (an activated form of Ras) do not significantly activate the PI3K/Akt pathway, we strongly believe that mitogen-induced phosphorylation of Gab2 recognized by these two phospho-motif antibodies is dependent on RSK (Chapter 2, Figure 2.1A and B). On the basis of our current study and a previous report which have clearly characterized that Gab2 acts as both upstream and a substrate of RSK and Akt, these

findings indicate that crosstalk between the Ras/MAPK and PI3K/Akt pathways converge on Gab2.

It has been characterized that Gab2 tyrosine phosphorylation by RTK creates numerous binding sites responsible for protein interactions, such as Shp2 and p85 (Gu and Neel, 2003). Ser^{620}, a novel phosphorylation site identified in our study, falls between the two Shp2-binding residues (Tyr^{603} and Tyr^{632}) (Chapter 2, Figure 2.4E). Although it is unclear how these two phospho-tyrosine residues modulate Gab2-Shp2 interaction allosterically, it is still possible that RSK-mediated phosphorylation may impede Shp2 recruitment to Gab2. Indeed, we show that the MAPK pathway through RSK restricts Shp2 recruitment to Gab2 in HEK293 and MCF-10A cells (Chapter 2, Figure 2.6A and 2.7D). Given that Shp2 is a membrane receptor-associated protein and that Gab2 transiently localizes at the plasma membrane upon EGF stimulation, it is possible that phosphorylation of Gab2 by RSK alters its localization at the plasma membrane, where Gab2-Shp2 interaction occurs. Moreover, since the RSK phosphorylation consensus motif (RXXpS/T) matches the minimum 14-3-3 binding motif (Galan *et al.*, 2014), it is possible that RSK-dependent Gab2 phosphorylation leads to the recruitment of 14-3-3 proteins. Given that phosphorylation of Ser^{211} has been characterized as a 14-3-3 binding site in Gab2 (Brummer *et al.*, 2008), it would be interesting to investigate whether other sites, likely Ser^{160} or Ser^{620}, are involved in Gab2/14-3-3 interaction. A previous study identified Ser^{160} as an Akt phosphorylation site, and mutation of Ser to Ala led to increased Gab2 tyrosine phosphorylation, as well as Shc and ErbB2 association (Lynch and Daly, 2002). However, we have not observed an increased tyrosine phosphorylation level in the Gab2 S160A mutant in our experimental system. The reason for these discrepancies may be due to the distinct agonists used in respective studies. Whereas EGF (an ErbB1 ligand) was used in our experiments, the previous report took advantage of Heregulin (HRG; ErbB3 and ErbB4 ligand) to stimulate cells. It is possible that different expression levels of receptors (ErbB1, ErbB3 and ErbB4) in respective cell types and their affinity to a specific ligand may lead to distinct downstream signal transduction.

4.1.2 Potential roles of phosphorylated Gab2 by RSK in signal transduction

Having demonstrated the crucial contribution of Gab2 to cancer development, several studies indicated that the Gab2-Shp2 axis is required for Gab2-induced cell proliferation and

motility (Bentires-Alj *et al.*, 2006; Brummer *et al.*, 2006; Herrera Abreu *et al.*, 2010). In MCF-10A cells co-expressing ErbB2 and Gab2, it was shown that disruption of Gab2-Shp2 association inhibited cell proliferation and migration (Bentires-Alj *et al.*, 2006). In line with these findings, we observed that expression of the unphosphorylatable Gab2 S3A (S160/211/620A) mutant which displays sustained association with Shp2, led to increased cell motility (Chapter 2, Figure 2.8D). Our data suggest that RSK attenuates Gab2-mediated cell motility through inhibition of Shp2 recruitment. Being one of the critical adaptor binder proteins to Gab2, Shp2 has been well characterized as an oncogene in tumor progression. Upon RTK activation, Shp2 is recruited to the plasma membrane, where it dephosphorylates p120-RasGAP, leading to the upregulation of the Ras-Raf-MEK1/2-ERK1/2 signaling cascade (Montagner *et al.*, 2005; Matozaki *et al.*, 2009). Our data fit this model, whereby sustained Shp2 recruitment to Gab2 S3A increases ERK1/2 activity. However, other potential molecular mechanisms concerning the Gab2-Shp2 axis regulation of the downstream signaling cascades cannot be excluded. It was shown that Gab2 cooperated with other proto-oncogenes, such as Src, to transform primary human MCF-10A mammary epithelial cells, leading to an invasive multiacinar phenotype in a three-dimensional culture system (Bennett *et al.*, 2008). However, it is unclear by which mechanism Gab2 is involved in Src signaling. One possible explanation is that Gab2 recruits Shp2 to an inhibitory site of Src. Indeed, it was found that Gab2-mediated Shp2 recruitment to Lyn (a predominant Src kinase in myeloid cells) increased Lyn activity by dephosphorylating its inhibitory site Tyr^{507} (Futami *et al.*, 2011). Another potential mechanism of RSK modulating Gab2 function in cell motility is through the reorganization of cytoskeleton. It has been shown that Gab2 modulates RhoA activity (Herrera Abreu *et al.*, 2010). Given that Shp2 regulates Rho kinase II (ROCK II) activity and further acts on the cytoskeleton, we can reasonably hypothesize that increased Shp2 recruitment to the Gab2 S3A mutant may promote cell motility by modulating RhoA/ROCK II activity (Lee and Chang, 2008). Based on these statements, measuring Shp2-modulated downstream effector activities in the Gab2 S3A mutant overexpressed MCF-10A cells, such as Src and ROCK II, would be helpful to expand our knowledge of RSK-modulated Gab2 actions on multiple downstream signaling cascades.

4.1.3 The potential role of Gab2 phosphorylation by RSK in primary mast cells

and the allergic response

The physiological function of Gab2 in the immune system has been studied using *GAB2* knockout mice. As we described in Chapter 1.4.1, *GAB2*-deficient mice are viable and fertile, but were found to have decreased circulating mast cells, suggesting that Gab2 is involved in the allergic response through transducing signals from c-Kit (stem cell factor receptor) to the downstream signaling cascades, such as the Ras/MAPK or Rac/JNK pathways (Nishida *et al.*, 2002; Yu *et al.*, 2006; Sharma *et al.*, 2014). Here, by using rat basophilic leukemia(RBL)-2H3 cells with high expression levels of Gab2, we show that endogenous Gab2 is phosphorylated by RSK upon dinitrophenyl treatment (Chapter 2, Figure 2.1D). Because our data showed that RSK through Gab2 participates in the negative feedback loop of MAPK signaling in HEK293 cells (Chapter 2, Figure 2.9), it is reasonable to think that RSK may also affect Kit signaling in mast cells. Experiments that are specifically performed in bone marrow-derived mast cells (BMMCs) purified *GAB2*-deficient mice, will be helpful to confirm this assumption. To do this, we can re-express Gab2 wild-type or S3A mutant (S159/211/620A) in the $Gab2^{-/-}$ BMMCs cells by retroviral vector infections. The contribution of Gab2 S3A mutant to Kit-induced signal transduction to downstream effectors, such as ERK1/2 or JNK activities, will be assayed upon stimulation of dose-dependent SCF (stem cell factor; Kit ligand) treatment. Moreover, given that RSK participates in the regulation of Gab2 functions and $Gab2^{-/-}$ mice have decreased circulating mast cells in a number of tissues, we can reasonably assume that RSK may play a role in an allergic response. Notably, different isoforms of RSK have distinct functions. Therefore, we expect that certain RSK isoforms may be involved in the regulation of mast cell proliferation and the allergic response. This hypothesis can be addressed by evaluating the allergic response in RSK1-3 knockout animals subjected to mast cell activation through antigen induced aggregation of FcεRI. Collectively, this work will be helpful to understand the potential role of RSK in the allergic response.

4.2 Regulation of Gab2 functions by MAP kinases

4.2.1 ERK1/2 bind to Gab2 and further regulate its phosphorylation

One of the fascinating observations deriving from our work is the identification a new ERK1/2-docking domain in Gab2. To increase the fidelity and duration of Ras/MAPK

signaling, ERK1/2 specifically bind to specific sequences in protein substrates, such as the D-domain (Roskoski, 2012). It was shown that hydrophobic interactions were crucial for activated ERK1/2(p-ERK1/2)-substrate binding to occur, which either attracts substrates surrounding the kinases or ensures the correct orientation of substrates in the kinase active site (Ubersax and Ferrell, 2007). Since our data demonstrated that Gab2 only interacts with activated ERK1/2 (p-ERK1/2) rather than the inactivated form, we reasonably thought that Gab2 association with p-ERK1/2 is modulated by hydrophobic contact. Although we lack the crystal structure support of Gab2, several lines of evidence suggest that the identified Gab2 D-domain forms a hydrophobic bond with activated ERK1/2. Firstly, in our co-immunoprecipitation assays, both ERK1/2 activity and ERK1/2 CD-domain are crucial for Gab2-ERK1/2 association, raising the possibility of physical interaction of ERK and Gab2 in cells (Chapter 3, Figure 3.3D and 3.4A). Secondly, mutation of each positively charged amino acid and hydrophobic amino acids to alanine residues within the Gab2 D-domain disrupts Gab2-ERK1/2 interaction (Chapter 3, Figure 3.4C), which is accompanied with a significant reduction of Gab2 phosphorylation. This result implicates that Gab2 directly interacts with ERK1/2 in cells. Most importantly, among these residues, mutation of the hydrophobic amino acids L517 and L519 have the greatest impact on ERK1/2 association, indicating an essential role for the L-X-L motif in Gab2. Indeed, the same crucial function of L-X-L has been characterized in the transcription factor Ets-1, in which a ERK1/2 docking site is also located on the C-terminus and regulates Ets-1 transcription activity (Seidel and Graves, 2002). Although Gab1 is relatively similar to Gab2, we were not able to observe Gab1-ERK1/2 interaction upon the activation of the MAPK pathway, indicating the specificity action of ERK1/2 on Gab2 (Chapter, Figure 3.3F). This result is controversial with a previous report that Gab1, rather than Gab2, associated with activated ERK2 (Roshan *et al.*, 1999). It was shown that Gab1 associated with ERK2 via its Met-binding domain (MBD), which interestingly contains part of the identified ERK1/2-docking domain. The reasons for these discrepancies are likely due to the respective assays, because we mostly monitored protein-protein interaction under physical conditions, rather than GST-pull downs using only MBD in Gab1 or the corresponding sequence in Gab2 as a bait protein.

4.2.2 Potential functions of ERK1/2-dependent phosphorylation sites in Gab2

Another outstanding question that arises from our work is the identification of potential ERK-phosphorylation sites in Gab2. To date, we cannot rule out which phosphorylation site in Gab2 is dependent on ERK1/2 interaction. Although several reports indicated that there are numerous proline-directed phospho-Ser/Thr sites in Gab2, including S143, S469, S532, S612, S614 (Arnaud *et al.*, 2004; Halbach *et al.*, 2013), no systematic analysis was performed to fully characterize the exact functions of these phosphorylation sites. Our work shows that ERK1/2 phosphorylate Gab2 on these five Ser/Thr-Pro residues both *in vitro* and in cells. A previous report showed that phosphorylation of Gab2 on Ser^{623} (Ser^{612} is the homologous site in mouse Gab2) by ERK1/2 inhibited Shp2 recruitment (Arnaud *et al.*, 2004). We did not observe that Gab2 phosphorylation on Ser143/469/532/612/614 had any effect on Shp2 recruitment in HEK293 cells. However, our previous report showed that phosphorylated Gab2 by RSK impeded Shp2 association (Zhang *et al.*, 2013). Given that RSK is regulated by ERK1/2, it raises the possibility that the previous observation of ERK1/2-modulated Shp2 recruitment is due to RSK. Most notably, among these five phosphorylation sites, S469 in Gab2 is located adjacent to one of the p85 binding sites (^{465}YXXM^{468}). To date, three tyrosine phosphorylation sites in Gab2 responsible for p85 recruitment have been established, including Tyr^{452}, Tyr^{476} and Tyr^{584} (Crouin *et al.*, 2001). Unlike Shp2 which preferentially associates with tyrosine residue 614 in Gab2 rather than tyrosine 643 (it is the same case in Gab1) (Rocchi *et al.*, 1998; Crouin *et al.*, 2001), all three tyrosine residues responsible for p85 binding have been confirmed to function equally (Crouin *et al.*, 2001). Moreover, Yu *et al.* found that ERK1/2 negatively regulated the p85 subunit of PI3K interaction with Gab1 upon EGF stimulation (Yu *et al.*, 2002). Although they did not characterize any ERK1/2-dependent phosphorylation sites in Gab1 involved in limiting p85 association, this result still raises the possibility that ERK1/2 affect p85-Gab1 interaction through certain pS/T-P sites. Based on these statements, we assume that phosphorylation of Gab2 by ERK1/2 on certain sites, likely S469, induces a conformational change in Gab that reduces its affinity for p85. Alternatively, Gab2 phosphorylation by ERK1/2 provokes steric hindrance for the recruitment of p85.

Is p85 a negative or positive regulator in the PI3K/Akt pathway?

A critical question we next sought to answer was how the feedback regulation of Gab2 mediated by ERK1/2 affected the activity of downstream effector proteins. Our results

indicate that Gab2 phosphorylation by ERK1/2 limits p85 recruitment. p85 is a regulatory subunit of PI3K and constitutively binds to the PI3K catalytic subunit p110, leading to increased PIP3 levels and Akt activation. However, accumulating evidence suggest that p85 should be considered carefully when analyzing the activity of Akt induced by PI3K. p85 appears to have dual functions in the activation of the catalytic subunit p110 of PI3K: (1) Stabilization of p110; (2) Inhibition of p110 lipid kinase activity (Yu *et al.*, 1998). Aside from having this dual function in regulating p110 activity, p85 also directly binds to the tumor suppressor PTEN and negatively regulates PIP3 lipid products, raising the possibility that more p85 recruitment to the Gab2 S5A mutant may in fact lead to a dysregulation of the balance action between the reciprocal actions of PTEN and p110 (Chagpar *et al.*, 2010). Moreover, it was shown that p85 acts as a negative regulator of the insulin-induced signaling cascade. In p85 knockout ($p85^{-/-}$) mice, Akt activity was found to be significantly increased in the presence of insulin, suggesting that cells depleted of p85 are more sensitive to insulin treatment (Terauchi *et al.*, 1999). Furthermore, Luo *et al*. found that p85 restricted insulin-induced signal transduction by associating with IRS1, resulting in sequestered p110 from potential p85/p110 complexes (Luo *et al.*, 2005). In addition, a recent study indicated that localization of p85 at the plasma membrane, rather than its expression level, is crucial for PI3K activity (Chiu *et al.*, 2014). Given the complexity of p85-mediated signal transduction, the measurement of PI3K activity by ELISA (enzyme-linked immunosorbent assay) in the Gab2 S5A mutant will be helpful in understanding the role of ERK1/2 in regulating Gab2 function. To address this, the PI3-kinase will be purified from cells by the immunoprecipitation method and further react with $PI(4,5)P_2$ substrate. After completing the PI3-Kinase reaction, the $PI(3,4,5)P_3$ product will be detected by ELISA as described in the protocol of PI3-Kinase Activity ELISA: Pico.

One possible effect of ERK1/2 on Gab2 function is to alter its PH domain-mediated localization at the plasma membrane. Ser^{532}, which was identified by our group as an ERK1/2 phosphorylation site in Gab2, was also characterized as an ERK1/2-modulated phosphorylation site in Gab1 (Ser^{551} in mouse Gab1). Most intriguingly, Eulenfeld *et al.* found that ERK1/2 phosphorylation of Gab1 on Ser^{551} was required for Gab1 residency at the plasma membrane, where Gab1-mediated protein interactions is initiated (Eulenfeld and

Schaper, 2009). Given the relatively high homology between Gab1 and Gab2, these findings raise the possibility that ERK1/2 may have the same effect on Gab2 PH-domain localization to the plasma membrane, leading to altered downstream effector activities. Nevertheless, future work will be required to identify the exact mechanisms by which ERK1/2 regulate Gab2 function, either through p85 recruitment or PH domain-mediated localization.

4.3 Other potential mechanisms of Gab2-mediated cell motility

While the involvement of Gab2 in cell motility has been characterized, the molecular mechanisms underlying this process still remain elusive. Our work has showed that increased ERK1/2 activity induced by an unphosphorylatable Gab2 S3A (S160/211/620A) mutant promoted MCF-10A cell motility. Aside from classical Gab2 downstream effectors, such as ERK1/2, which have been shown to play important roles in cell migration, others, such as members of the Rho family were also reported to play a role downstream of Gab2 (Herrera Abreu *et al.*, 2010). Hence, other potential mechanisms regarding how Gab2 promotes cell migration should be considered. At this point in time, identifying whether phosphorylated Gab2 by ERK1/2 participates in the activation of small Rho GTPases would be helpful to address this question. It has been shown that Gab2 directly binds to GC-GAP, a novel Rho family GTPases-activating protein which decreases Rac1 and Cdc42 activity (Zhao *et al.*, 2003). Given that Rac1 and Cdc42 modulate cytoskeletal reorganization and also filopodia and lamellipodia formation, this finding raises the possibility that ERK1/2-mediated phosphorylation of Gab2 may affect GC-GAP recruitment, leading to altered cell motility. Another possibility is that phosphorylation of Gab2 by ERK1/2 may regulate RhoA activity. This hypothesis is supported by a previous report that Gab2 negatively regulates RhoA activity resulting in enhanced cell migration and motility (Brummer *et al.*, 2008). Moreover, Gab2-induced cell motility may also involve the engagement of actin-nucleating protein N-WASP. It has been shown that Gab1 directly binds to WASP and enhances dorsal ruffle formation in MDCK cells (Abella *et al.*, 2010). Most notably, the Park group found that disruption of the Gab1-Shp2 interaction had no effect on dorsal ruffle formation, indicating that Gab1 modulates actin reorganization independently of the classical Gab1-Shp2-ERK1/2 cascade. Given the high similarity between Gab1 and Gab2 topology, it is probable that ERK1/2 may affect the ability of Gab2 to induce actin reorganization via N-WASP. More

work will be required to fully understand the exact mechanisms by which Gab2 promotes cell motility.

4.4 Deciphering the role of the MAPK pathway in the regulation of Gab2-dependent function

In this thesis, I systematically analyzed the feedback regulation of Gab2-dependent signaling by the MAPK pathway. Upon RTK activation, Gab2 becomes tyrosine phosphorylated and mediates the recruitment of different binding proteins, such as Shp2 and p85, which are involved in the amplification of downstream pathways. In addition, our data indicate that Gab2 is a substrate of the MAPK pathway. Indeed, our results suggest that post-translational modification of Gab2 occurs in both ERK1/2- and RSK-dependent manners. We suggest that while ERK1/2 associate and phosphorylate Gab2, and thereby decrease p85 recruitment, RSK negatively regulates MAPK signaling by restricting Shp2 recruitment.

Given that hyperactive RSK signaling has been implicated in a number of human cancers, including breast cancer and melanoma, it raises the possibility that RSK could be a potential therapeutic target for these cancers, which have inappropriate activation of the MAPK pathway. To address this question, in 2005, the Lannigan group developed the first RSK-specific inhibitor, SL0101 (Smith *et al.*, 2005). Furthermore, they found that this inhibitor selectively suppresses breast cancer cell growth (MCF-7 cells) rather than normal breast epithelial cells (MCF-10A cells) (Smith *et al.*, 2005). Based on these statements, it appears that administering RSK inhibitors to patients may be a potential powerful approach to treat cancers. However, some studies show that RSK inhibitors should be used with caution because they may upregulate ERK1/2 activity. One piece of evidence is that inhibition of RSK activity by pharmacological inhibitors upregulates ERK1/2 activity in certain types of cells (Saha *et al.*, 2012). Consistent with this result, it was found that ERK1/2 activity is increased in skeletal muscle tissue of RSK2 knockout mice (Dufresne *et al.*, 2001). To date, several possible molecular mechanisms have been characterized to explain this negative feedback regulation of MAPK signaling by RSK. In 2012, collaboration between the Ballif group and our group indicated that RSK phosphorylates the Ras-GEF SOS1, leading to Grb2 dissociation and thereby preventing ERK1/2 activity (Saha *et al.*, 2012). Moreover, my work suggests an alternative mechanism whereby RSK negatively regulates ERK1/2 activity by

restricting Gab2 functions. Since RSK negatively regulates ERK1/2 activity, it raises the possibility that RSK inhibitors may lead to cancer development by increasing ERK1/2 signaling. In this regard, drug combinations consisting of RSK and ERK1/2 inhibitors may be an effective cancer-targeted therapy. This proposal is reminiscent a clinical treatment for PTEN-deficient glioblastoma patients with both the mTORC1 and PI3K pathway inhibitors (Cloughesy *et al.*, 2008). Taken together, our findings expand the repertoire of actions mediated by Gab2, and also highlight the development of novel therapeutic approaches for cancer treatments.

Chapter 5 Conclusions and Perspectives

Gab2 has been characterized as a potential oncogene in breast cancer. Tyrosine phosphorylation of Gab2 by RTKs plays a crucial role in signal transduction from the receptor to the Ras/MAPK and PI3K/Akt pathways, leading to cell proliferation, migration and invasion. Interestingly, Gab2 is also found to be phosphorylated on Ser/Thr residues, but less is known about this post-translational modification. In this context, our work led to a better understanding of the mechanisms by which Gab2 is regulated by the MAPK pathway, which may help us in the design of novel therapeutic approaches for breast cancer.

The most fascinating finding arising from our work is the demonstration that the MAPK pathway plays a dual role in the regulation of Gab2 function. On the one hand, we found for the first time that Gab2 is a novel substrate of RSK. Gab2 phosphorylation by RSK restricts Shp2 recruitment and downregulates ERK1/2 activity. Using live-cell-imaging wound-healing assays with *in intro* cellular model, we have investigated the impact of RSK on Gab2-mediated biological functions. Our findings helped characterize a novel negative feedback loop, whereby RSK through Gab2 negatively regulates MAPK signaling, leading to decreased cell motility. Given the widespread role of RTKs in signal transduction, our work also suggests that RSK may play a regulatory function in diverse receptor systems. On the other hand, we determined a novel ERK1/2 docking domain in Gab2. This observation provides us with a more detailed understanding of how ERK1/2 regulate Gab2 function at the molecular level. Our study of the effect of the MAPK pathway on Gab2 allows us to show that phosphorylation of Gab2 by ERK1/2 inhibits p85 recruitment. Given the complex functions of p85 in the regulation of PI3K/Akt signaling, future studies are required to fully characterize whether phosphorylated Gab2 by ERK1/2 results in altered Akt activity as well as other possible downstream effectors, such as RhoA and Rac. Lastly, given that Gab2 is known to participate in HER2-mediated tumorigenesis, our results also suggest that phosphorylated Gab2 by ERK1/2 may affect its ability to collaborate with HER2 in breast cancer development, but more experiments will be required to directly address this.

Our study has important clinical implications because RSK has been established as a major effector of the Ras/MAPK pathway and is constitutively activated in a large number of human tumors. For this reason, RSK may be a potential therapeutic target in cancer treatment (Romeo *et al.*, 2012). However, based on our observations, we suggest that RSK inhibitors

should be used in with caution because RSK negatively regulates MAPK signaling through by modulating upstream inducers of Ras, such as SOS and Gab2 (Saha *et al.*, 2012; Zhang *et al.*, 2013). Given that RSK inhibitors may thus promote the hyperactivation of ERK1/2, it is possible that co-treatment using ERK1/2 and RSK inhibitors in cancer may be more effective than either of them alone.

Finally, given accumulating evidence showing that Gab2 is the key molecule in cancer, understanding how Gab2 is regulated at the molecular level will help us define its role in certain biological contexts. Based on our data, we show a more complete mechanistic illustration of Gab2 regulation by the MAPK pathway, which will allow us to control cancer progression in the future.

Bibliography

Abe, M. K., K. T. Kahle, et al. (2001). "ERK7 is an autoactivated member of the MAPK family." *J Biol Chem* 276(24): 21272-9.

Abella, J. V., R. Vaillancourt, et al. (2010). "The Gab1 scaffold regulates RTK-dependent dorsal ruffle formation through the adaptor Nck." *J Cell Sci* 123(Pt 8): 1306-19.

Abraham, D., K. Podar, et al. (2000). "Raf-1-associated protein phosphatase 2A as a positive regulator of kinase activation." *J Biol Chem* 275(29): 22300-4.

Acharyya, S., T. Oskarsson, et al. (2012). "A CXCL1 paracrine network links cancer chemoresistance and metastasis." *Cell* 150(1): 165-78.

Adelaide, J., G. Monges, et al. (1995). "Oesophageal cancer and amplification of the human cyclin D gene CCND1/PRAD1." *Br J Cancer* 71(1): 64-8.

Agazie, Y. M. and M. J. Hayman (2003). "Molecular mechanism for a role of SHP2 in epidermal growth factor receptor signaling." *Mol Cell Biol* 23(21): 7875-86.

Alexandropoulos, K., G. Cheng, et al. (1995). "Proline-rich sequences that bind to Src homology 3 domains with individual specificities." *Proc Natl Acad Sci U S A* 92(8): 3110-4.

Allen, L. F., J. Sebolt-Leopold, et al. (2003). "CI-1040 (PD184352), a targeted signal transduction inhibitor of MEK (MAPKK)." *Semin Oncol* 30(5 Suppl 16): 105-16.

Alonso, M., M. Melani, et al. (2004). "Mitochondrial extracellular signal-regulated kinases 1/2 (ERK1/2) are modulated during brain development." *J Neurochem* 89(1): 248-56.

Anjum, R., P. P. Roux, et al. (2005). "The tumor suppressor DAP kinase is a target of RSK-mediated survival signaling." *Curr Biol* 15(19): 1762-7.

Appel, S. and K. G. Morgan (2010). "Scaffolding proteins and non-proliferative functions of ERK1/2." *Commun Integr Biol* 3(4): 354-6.

Appelmann, I., R. Liersch, et al. (2009). "Angiogenesis inhibition in cancer therapy: platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF)

and their receptors: biological functions and role in malignancy." *Recent Results Cancer Res* 180: 51-81.

Arencibia, J. M., D. Pastor-Flores, et al. (2013). "AGC protein kinases: from structural mechanism of regulation to allosteric drug development for the treatment of human diseases." *Biochim Biophys Acta* 1834(7): 1302-21.

Arnaud, M., C. Crouin, et al. (2004). "Phosphorylation of Grb2-associated binder 2 on serine 623 by ERK MAPK regulates its association with the phosphatase SHP-2 and decreases STAT5 activation." *J Immunol* 173(6): 3962-71.

Aronchik, I., B. A. Appleton, et al. (2014). "Novel Potent and Selective Inhibitors of p90 Ribosomal S6 Kinase Reveal the Heterogeneity of RSK Function in MAPK Driven Cancers." *Mol Cancer Res*.

Arthur, J. S. and P. Cohen (2000). "MSK1 is required for CREB phosphorylation in response to mitogens in mouse embryonic stem cells." *FEBS Lett* 482(1-2): 44-8.

Babu, G. J., M. J. Lalli, et al. (2000). "Phosphorylation of elk-1 by MEK/ERK pathway is necessary for c-fos gene activation during cardiac myocyte hypertrophy." *J Mol Cell Cardiol* 32(8): 1447-57.

Badve, S., D. J. Dabbs, et al. (2010). "Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists." *Mod Pathol* 24(2): 157-67.

Batzer, A. G., P. Blaikie, et al. (1995). "The phosphotyrosine interaction domain of Shc binds an LXNPXY motif on the epidermal growth factor receptor." *Mol Cell Biol* 15(8): 4403-9.

Bayascas, J. R., S. Wullschleger, et al. (2008). "Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance." *Mol Cell Biol* 28(10): 3258-72.

Bennett, H. L., T. Brummer, et al. (2008). "Gab2 and Src co-operate in human mammary epithelial cells to promote growth factor independence and disruption of acinar

morphogenesis." *Oncogene* 27(19): 2693-704.

Bentires-Alj, M., S. G. Gil, et al. (2006). "A role for the scaffolding adapter GAB2 in breast cancer." *Nat Med* 12(1): 114-21.

Bignone, P. A., K. Y. Lee, et al. (2007). "RPS6KA2, a putative tumour suppressor gene at 6q27 in sporadic epithelial ovarian cancer." *Oncogene* 26(5): 683-700.

Bocanegra, M., A. Bergamaschi, et al. (2009). "Focal amplification and oncogene dependency of GAB2 in breast cancer." *Oncogene* 29(5): 774-9.

Bolos, V., H. Peinado, et al. (2003). "The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors." *J Cell Sci* 116(Pt 3): 499-511.

Bone, H. and M. J. Welham (2000). "Shc associates with the IL-3 receptor beta subunit, SHIP and Gab2 following IL-3 stimulation. Contribution of Shc PTB and SH2 domains." *Cell Signal* 12(3): 183-94.

Boulton, T. G., J. S. Gregory, et al. (1991). "Purification and properties of extracellular signal-regulated kinase 1, an insulin-stimulated microtubule-associated protein 2 kinase." *Biochemistry* 30(1): 278-86.

Boulton, T. G., S. H. Nye, et al. (1991). "ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF." *Cell* 65(4): 663-75.

Boulton, T. G., G. D. Yancopoulos, et al. (1990). "An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control." *Science* 249(4964): 64-7.

Breitschopf, K., J. Haendeler, et al. (2000). "Posttranslational modification of Bcl-2 facilitates its proteasome-dependent degradation: molecular characterization of the involved signaling pathway." *Mol Cell Biol* 20(5): 1886-96.

Bridges, D. and G. B. Moorhead (2005). "14-3-3 proteins: a number of functions for a numbered protein." *Sci STKE* 2005(296): re10.

Brose, M. S., P. Volpe, et al. (2002). "BRAF and RAS mutations in human lung cancer and

melanoma." *Cancer Res* 62(23): 6997-7000.

Brown, L. A., S. E. Kalloger, et al. (2008). "Amplification of 11q13 in ovarian carcinoma." *Genes Chromosomes Cancer* 47(6): 481-9.

Brummer, T., M. Larance, et al. (2008). "Phosphorylation-dependent binding of 14-3-3 terminates signalling by the Gab2 docking protein." *EMBO J* 27(17): 2305-16.

Brummer, T., H. Naegele, et al. (2003). "Identification of novel ERK-mediated feedback phosphorylation sites at the C-terminus of B-Raf." *Oncogene* 22(55): 8823-34.

Brummer, T., D. Schramek, et al. (2006). "Increased proliferation and altered growth factor dependence of human mammary epithelial cells overexpressing the Gab2 docking protein." *J Biol Chem* 281(1): 626-37.

Brunet, A., D. Roux, et al. (1999). "Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry." *EMBO J* 18(3): 664-74.

Bruning, J. C., J. A. Gillette, et al. (2000). "Ribosomal subunit kinase-2 is required for growth factor-stimulated transcription of the c-Fos gene." *Proc Natl Acad Sci U S A* 97(6): 2462-7.

Buck, M. and M. Chojkier (2007). "C/EBPbeta associates with caspase 8 complex proteins and modulates apoptosis in hepatic stellate cells." *J Clin Gastroenterol* 41 Suppl 3: S295-9.

Burkhard, K. A., F. Chen, et al. (2010). "Quantitative analysis of ERK2 interactions with substrate proteins: roles for kinase docking domains and activity in determining binding affinity." *J Biol Chem* 286(4): 2477-85.

Burkhard, S. J. and J. A. Traugh (1983). "Changes in ribosome function by cAMP-dependent and cAMP-independent phosphorylation of ribosomal protein S6." *J Biol Chem* 258(22): 14003-8.

Camps, M., A. Nichols, et al. (1998). "Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase." *Science* 280(5367): 1262-5.

Cano, A., M. A. Perez-Moreno, et al. (2000). "The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression." *Nat Cell Biol* 2(2): 76-83.

Carey, L. A., C. M. Perou, et al. (2006). "Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study." *JAMA* 295(21): 2492-502.

Cargnello, M. and P. P. Roux (2011). "Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases." *Microbiol Mol Biol Rev* 75(1): 50-83.

Carracedo, A., L. Ma, et al. (2008). "Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer." *J Clin Invest* 118(9): 3065-74.

Carriere, A., M. Cargnello, et al. (2008). "Oncogenic MAPK signaling stimulates mTORC1 activity by promoting RSK-mediated raptor phosphorylation." *Curr Biol* 18(17): 1269-77.

Carriere, A., Y. Romeo, et al. (2010). "ERK1/2 phosphorylate Raptor to promote Ras-dependent activation of mTOR complex 1 (mTORC1)." *J Biol Chem* 286(1): 567-77.

Chagpar, R. B., P. H. Links, et al. (2010). "Direct positive regulation of PTEN by the p85 subunit of phosphatidylinositol 3-kinase." *Proc Natl Acad Sci U S A* 107(12): 5471-6.

Chatterjee, S., L. C. Heukamp, et al. (2013). "Tumor VEGF:VEGFR2 autocrine feed-forward loop triggers angiogenesis in lung cancer." *J Clin Invest* 123(4): 1732-40.

Chen, B., C. Tardell, et al. (2012). "BRAFV600E negatively regulates the AKT pathway in melanoma cell lines." *PLoS One* 7(8): e42598.

Chen, R. H., C. Abate, et al. (1993). "Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase." *Proc Natl Acad Sci U S A* 90(23): 10952-6.

Chen, R. H., S. Corbalan-Garcia, et al. (1997). "The role of the PH domain in the signal-dependent membrane targeting of Sos." *EMBO J* 16(6): 1351-9.

Chen, R. H., C. Sarnecki, et al. (1992). "Nuclear localization and regulation of erk- and rsk-encoded protein kinases." *Mol Cell Biol* 12(3): 915-27.

Cheung, M., A. Sharma, et al. (2008). "Akt3 and mutant V600E B-Raf cooperate to promote early melanoma development." *Cancer Res* 68(9): 3429-39.

Chiu, Y. H., J. Y. Lee, et al. (2014). "BRD7, a Tumor Suppressor, Interacts with p85alpha and Regulates PI3K Activity." *Mol Cell* 54(1): 193-202.

Chong, H., H. G. Vikis, et al. (2003). "Mechanisms of regulating the Raf kinase family." *Cell Signal* 15(5): 463-9.

Chu, C. T., D. J. Levinthal, et al. (2004). "Oxidative neuronal injury. The dark side of ERK1/2." *Eur J Biochem* 271(11): 2060-6.

Chu, S., L. Li, et al. (2007). "BCR-tyrosine 177 plays an essential role in Ras and Akt activation and in human hematopoietic progenitor transformation in chronic myelogenous leukemia." *Cancer Res* 67(14): 7045-53.

Chuderland, D., A. Konson, et al. (2008). "Identification and characterization of a general nuclear translocation signal in signaling proteins." *Mol Cell* 31(6): 850-61.

Chung, J., R. H. Chen, et al. (1991). "Coordinate regulation of pp90rsk and a distinct protein-serine/threonine kinase activity that phosphorylates recombinant pp90rsk in vitro." *Mol Cell Biol* 11(4): 1868-74.

Cloughesy, T. F., K. Yoshimoto, et al. (2008). "Antitumor activity of rapamycin in a Phase I trial for patients with recurrent PTEN-deficient glioblastoma." *PLoS Med* 5(1): e8.

Cohen-Armon, M. (2007). "PARP-1 activation in the ERK signaling pathway." *Trends Pharmacol Sci* 28(11): 556-60.

Cohen, M. S., C. Zhang, et al. (2005). "Structural bioinformatics-based design of selective, irreversible kinase inhibitors." *Science* 308(5726): 1318-21.

Colanzi, A. and D. Corda (2007). "Mitosis controls the Golgi and the Golgi controls mitosis." *Curr Opin Cell Biol* 19(4): 386-93.

Conway, A. M., S. Rakhit, et al. (1999). "Platelet-derived-growth-factor stimulation of the p42/p44 mitogen-activated protein kinase pathway in airway smooth muscle: role of pertussis-toxin-sensitive G-proteins, c-Src tyrosine kinases and phosphoinositide 3-kinase." *Biochem J* 337 (Pt 2): 171-7.

Crouin, C., M. Arnaud, et al. (2001). "A yeast two-hybrid study of human p97/Gab2 interactions with its SH2 domain-containing binding partners." *FEBS Lett* 495(3): 148-53.

Crozier, J. A., A. Swaika, et al. (2014). "Adjuvant chemotherapy in breast cancer: To use or not to use, the anthracyclines." *World J Clin Oncol* 5(3): 529-38.

Cush, J. J., J. B. Splawski, et al. (1995). "Elevated interleukin-10 levels in patients with rheumatoid arthritis." *Arthritis Rheum* 38(1): 96-104.

Dance, M., A. Montagner, et al. (2008). "The molecular functions of Shp2 in the Ras/Mitogen-activated protein kinase (ERK1/2) pathway." *Cell Signal* 20(3): 453-9.

Datta, S. R., H. Dudek, et al. (1997). "Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery." *Cell* 91(2): 231-41.

De Cesare, D., S. Jacquot, et al. (1998). "Rsk-2 activity is necessary for epidermal growth factor-induced phosphorylation of CREB protein and transcription of c-fos gene." *Proc Natl Acad Sci U S A* 95(21): 12202-7.

De Luca, A., M. R. Maiello, et al. (2012). "The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: role in cancer pathogenesis and implications for therapeutic approaches." *Expert Opin Ther Targets* 16 Suppl 2: S17-27.

De Mesquita, D. D., Q. Zhan, et al. (2001). "p90-RSK and Akt may promote rapid phosphorylation/inactivation of glycogen synthase kinase 3 in chemoattractant-stimulated neutrophils." *FEBS Lett* 502(3): 84-8.

Delcourt, N., J. Bockaert, et al. (2007). "GPCR-jacking: from a new route in RTK signalling to a new concept in GPCR activation." *Trends Pharmacol Sci* 28(12): 602-7.

Deleris, P., M. Trost, et al. (2010). "Activation loop phosphorylation of ERK3/ERK4 by

group I p21-activated kinases (PAKs) defines a novel PAK-ERK3/4-MAPK-activated protein kinase 5 signaling pathway." *J Biol Chem* 286(8): 6470-8.

Deschenes-Simard, X., F. Kottakis, et al. (2014). "ERKs in cancer: friends or foes?" *Cancer Res* 74(2): 412-9.

Ding, J., J. Romani, et al. (2013). "Inhibition of PI3K/mTOR overcomes nilotinib resistance in BCR-ABL1 positive leukemia cells through translational down-regulation of MDM2." *PLoS One* 8(12): e83510.

Doehn, U., C. Hauge, et al. (2009). "RSK is a principal effector of the RAS-ERK pathway for eliciting a coordinate promotile/invasive gene program and phenotype in epithelial cells." *Mol Cell* 35(4): 511-22.

Dong, C., S. B. Waters, et al. (1996). "SOS phosphorylation and disassociation of the Grb2-SOS complex by the ERK and JNK signaling pathways." *J Biol Chem* 271(11): 6328-32.

Dong, Z., R. Kumar, et al. (1997). "Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma." *Cell* 88(6): 801-10.

Doppler, H. and P. Storz (2014). "Regulation of VASP by phosphorylation: consequences for cell migration." *Cell Adh Migr* 7(6): 482-6.

Doyon, P. and M. J. Servant (2010). "Tumor necrosis factor receptor-associated factor-6 and ribosomal S6 kinase intracellular pathways link the angiotensin II AT1 receptor to the phosphorylation and activation of the IkappaB kinase complex in vascular smooth muscle cells." *J Biol Chem* 285(40): 30708-18.

Draber, P., V. Sulimenko, et al. (2012). "Cytoskeleton in mast cell signaling." *Front Immunol* 3: 130.

Dufresne, S. D., C. Bjorbaek, et al. (2001). "Altered extracellular signal-regulated kinase signaling and glycogen metabolism in skeletal muscle from p90 ribosomal S6 kinase 2 knockout mice." *Mol Cell Biol* 21(1): 81-7.

Dumaz, N. and R. Marais (2003). "Protein kinase A blocks Raf-1 activity by stimulating

14-3-3 binding and blocking Raf-1 interaction with Ras." *J Biol Chem* 278(32): 29819-23.

Dummler, B. A., C. Hauge, et al. (2005). "Functional characterization of human RSK4, a new 90-kDa ribosomal S6 kinase, reveals constitutive activation in most cell types." *J Biol Chem* 280(14): 13304-14.

Dunn, G. P., H. W. Cheung, et al. (2014). "In vivo multiplexed interrogation of amplified genes identifies GAB2 as an ovarian cancer oncogene." *Proc Natl Acad Sci U S A* 111(3): 1102-7.

Dunn, J. M., R. A. Phillips, et al. (1988). "Identification of germline and somatic mutations affecting the retinoblastoma gene." *Science* 241(4874): 1797-800.

Elshamy, W. M. and R. J. Duhe (2013). "Overview: cellular plasticity, cancer stem cells and metastasis." *Cancer Lett* 341(1): 2-8.

Erhardt, P., E. J. Schremser, et al. (1999). "B-Raf inhibits programmed cell death downstream of cytochrome c release from mitochondria by activating the MEK/Erk pathway." *Mol Cell Biol* 19(8): 5308-15.

Erikson, E. and J. L. Maller (1985). "A protein kinase from Xenopus eggs specific for ribosomal protein S6." *Proc Natl Acad Sci U S A* 82(3): 742-6.

Eulenfeld, R. and F. Schaper (2009). "A new mechanism for the regulation of Gab1 recruitment to the plasma membrane." *J Cell Sci* 122(Pt 1): 55-64.

Fang, X., S. Yu, et al. (1999). "Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway." *Oncogene* 18(48): 6635-40.

Fanger, G. R., C. Widmann, et al. (1998). "14-3-3 proteins interact with specific MEK kinases." *J Biol Chem* 273(6): 3476-83.

Fantz, D. A., D. Jacobs, et al. (2001). "Docking sites on substrate proteins direct extracellular signal-regulated kinase to phosphorylate specific residues." *J Biol Chem* 276(29): 27256-65.

Favata, M. F., K. Y. Horiuchi, et al. (1998). "Identification of a novel inhibitor of

mitogen-activated protein kinase kinase." *J Biol Chem* 273(29): 18623-32.

Fisher, T. L. and J. Blenis (1996). "Evidence for two catalytically active kinase domains in pp90rsk." *Mol Cell Biol* 16(3): 1212-9.

Foley, J., N. K. Nickerson, et al. (2010). "EGFR signaling in breast cancer: bad to the bone." *Semin Cell Dev Biol* 21(9): 951-60.

Forsythe, J. A., B. H. Jiang, et al. (1996). "Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1." *Mol Cell Biol* 16(9): 4604-13.

Foulkes, W. D. and A. Y. Shuen (2013). "In brief: BRCA1 and BRCA2." *J Pathol* 230(4): 347-9.

Franzoso, G., L. Carlson, et al. (1997). "Requirement for NF-kappaB in osteoclast and B-cell development." *Genes Dev* 11(24): 3482-96.

Freeman, A. K. and D. K. Morrison (2011). "14-3-3 Proteins: diverse functions in cell proliferation and cancer progression." *Semin Cell Dev Biol* 22(7): 681-7.

Frodin, M., C. J. Jensen, et al. (2000). "A phosphoserine-regulated docking site in the protein kinase RSK2 that recruits and activates PDK1." *EMBO J* 19(12): 2924-34.

Fujita, N., S. Sato, et al. (2003). "Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinases promotes its binding to 14-3-3 and cytoplasmic localization." *J Biol Chem* 278(49): 49254-60.

Futami, M., Q. S. Zhu, et al. (2011). "G-CSF receptor activation of the Src kinase Lyn is mediated by Gab2 recruitment of the Shp2 phosphatase." *Blood* 118(4): 1077-86.

Galan, J. A., K. M. Geraghty, et al. (2014). "Phosphoproteomic analysis identifies the tumor suppressor PDCD4 as a RSK substrate negatively regulated by 14-3-3." *Proc Natl Acad Sci U S A* 111(29): E2918-27.

Galanis, A., S. H. Yang, et al. (2001). "Selective targeting of MAPKs to the ETS domain transcription factor SAP-1." *J Biol Chem* 276(2): 965-73.

Galli, S., O. Jahn, et al. (2009). "A new paradigm for MAPK: structural interactions of

hERK1 with mitochondria in HeLa cells." *PLoS One* 4(10): e7541.

Gannon, L. M., M. B. Cotter, et al. (2013). "The classification of invasive carcinoma of the breast." *Expert Rev Anticancer Ther* 13(8): 941-54.

Gao, S. P., K. G. Mark, et al. (2007). "Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas." *J Clin Invest* 117(12): 3846-56.

Gao, T. and A. C. Newton (2002). "The turn motif is a phosphorylation switch that regulates the binding of Hsp70 to protein kinase C." *J Biol Chem* 277(35): 31585-92.

Gavin, A. C. and A. R. Nebreda (1999). "A MAP kinase docking site is required for phosphorylation and activation of p90(rsk)/MAPKAP kinase-1." *Curr Biol* 9(5): 281-4.

Ghosh, S., M. Basu, et al. (2012). "ETS-1 protein regulates vascular endothelial growth factor-induced matrix metalloproteinase-9 and matrix metalloproteinase-13 expression in human ovarian carcinoma cell line SKOV-3." *J Biol Chem* 287(18): 15001-15.

Grady, W. M. (2005). "Transforming growth factor-beta, Smads, and cancer." *Clin Cancer Res* 11(9): 3151-4.

Gressner, A. M. and I. G. Wool (1974). "The phosphorylation of liver ribosomal proteins in vivo. Evidence that only a single small subunit protein (S6) is phosphorylated." *J Biol Chem* 249(21): 6917-25.

Gu, H., H. Maeda, et al. (2000). "New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway." *Mol Cell Biol* 20(19): 7109-20.

Gu, H. and B. G. Neel (2003). "The "Gab" in signal transduction." *Trends Cell Biol* 13(3): 122-30.

Gu, H., J. C. Pratt, et al. (1998). "Cloning of p97/Gab2, the major SHP2-binding protein in hematopoietic cells, reveals a novel pathway for cytokine-induced gene activation." *Mol Cell* 2(6): 729-40.

Gu, H., K. Saito, et al. (2001). "Essential role for Gab2 in the allergic response." *Nature* 412(6843): 186-90.

Guo, C. and A. J. Whitmarsh (2008). "The beta-arrestin-2 scaffold protein promotes c-Jun N-terminal kinase-3 activation by binding to its nonconserved N terminus." *J Biol Chem* 283(23): 15903-11.

Gupta, P. B., S. Mani, et al. (2005). "The evolving portrait of cancer metastasis." *Cold Spring Harb Symp Quant Biol* 70: 291-7.

Gupta, S., A. R. Ramjaun, et al. (2007). "Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice." *Cell* 129(5): 957-68.

Halbach, S., K. T. Rigbolt, et al. (2013). "Alterations of Gab2 signalling complexes in imatinib and dasatinib treated chronic myeloid leukaemia cells." *Cell Commun Signal* 11(1): 30.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." *Cell* 144(5): 646-74.

Hart, M., J. P. Concordet, et al. (1999). "The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell." *Curr Biol* 9(4): 207-10.

Herbst, R., P. M. Carroll, et al. (1996). "Daughter of sevenless is a substrate of the phosphotyrosine phosphatase Corkscrew and functions during sevenless signaling." *Cell* 85(6): 899-909.

Herrera Abreu, M. T., W. E. Hughes, et al. (2010). "Gab2 regulates cytoskeletal organization and migration of mammary epithelial cells by modulating RhoA activation." *Mol Biol Cell* 22(1): 105-16.

Hille, B. (1994). "Modulation of ion-channel function by G-protein-coupled receptors." *Trends Neurosci* 17(12): 531-6.

Holgado-Madruga, M., D. R. Emlet, et al. (1996). "A Grb2-associated docking protein in EGF- and insulin-receptor signalling." *Nature* 379(6565): 560-4.

Hollander, M. C., G. M. Blumenthal, et al. (2011). "PTEN loss in the continuum of common cancers, rare syndromes and mouse models." *Nat Rev Cancer* 11(4): 289-301.

Hollestelle, A., F. Elstrodt, et al. (2007). "Phosphatidylinositol-3-OH kinase or RAS pathway

mutations in human breast cancer cell lines." *Mol Cancer Res* 5(2): 195-201.

Horst, B., S. K. Gruvberger-Saal, et al. (2009). "Gab2-mediated signaling promotes melanoma metastasis." *Am J Pathol* 174(4): 1524-33.

Hu, P., B. Margolis, et al. (1992). "Interaction of phosphatidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors." *Mol Cell Biol* 12(3): 981-90.

Hu, X., S. D. Chakravarty, et al. (2008). "Regulation of interferon and Toll-like receptor signaling during macrophage activation by opposing feedforward and feedback inhibition mechanisms." *Immunol Rev* 226: 41-56.

Huang, C. K., L. Zhan, et al. (1997). "LSP1 is the major substrate for mitogen-activated protein kinase-activated protein kinase 2 in human neutrophils." *J Biol Chem* 272(1): 17-9.

Inoki, K., Y. Li, et al. (2002). "TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling." *Nat Cell Biol* 4(9): 648-57.

Ishibe, S., D. Joly, et al. (2003). "Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis." *Mol Cell* 12(5): 1275-85.

Ishitani, S., K. Inaba, et al. (2010). "Homodimerization of Nemo-like kinase is essential for activation and nuclear localization." *Mol Biol Cell* 22(2): 266-77.

Itoh, M., Y. Yoshida, et al. (2000). "Role of Gab1 in heart, placenta, and skin development and growth factor- and cytokine-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation." *Mol Cell Biol* 20(10): 3695-704.

Itoh, S., F. Yoshitake, et al. (2007). "Gab2 plays distinct roles in bone homeostasis at different time points." *J Bone Miner Metab* 25(2): 81-5.

Jacobs, D., G. J. Beitel, et al. (1998). "Gain-of-function mutations in the Caenorhabditis elegans lin-1 ETS gene identify a C-terminal regulatory domain phosphorylated by ERK MAP kinase." *Genetics* 149(4): 1809-22.

Jadeski, L., J. M. Mataraza, et al. (2008). "IQGAP1 stimulates proliferation and enhances tumorigenesis of human breast epithelial cells." *J Biol Chem* 283(2): 1008-17.

Jesch, S. A., T. S. Lewis, et al. (2001). "Mitotic phosphorylation of Golgi reassembly stacking protein 55 by mitogen-activated protein kinase ERK2." *Mol Biol Cell* 12(6): 1811-7.

Jiang, X. and X. Wang (2004). "Cytochrome C-mediated apoptosis." *Annu Rev Biochem* 73: 87-106.

Jin, J., F. D. Smith, et al. (2004). "Proteomic, functional, and domain-based analysis of in vivo 14-3-3 binding proteins involved in cytoskeletal regulation and cellular organization." *Curr Biol* 14(16): 1436-50.

Johnson, N., V. Speirs, et al. (2008). "A comparative study of genome-wide SNP, CGH microarray and protein expression analysis to explore genotypic and phenotypic mechanisms of acquired antiestrogen resistance in breast cancer." *Breast Cancer Res Treat* 111(1): 55-63.

Jones, S. W., E. Erikson, et al. (1988). "A Xenopus ribosomal protein S6 kinase has two apparent kinase domains that are each similar to distinct protein kinases." *Proc Natl Acad Sci U S A* 85(10): 3377-81.

Jorissen, R. N., F. Walker, et al. (2003). "Epidermal growth factor receptor: mechanisms of activation and signalling." *Exp Cell Res* 284(1): 31-53.

Kant, S., S. Schumacher, et al. (2006). "Characterization of the atypical MAPK ERK4 and its activation of the MAPK-activated protein kinase MK5." *J Biol Chem* 281(46): 35511-9.

Kaur, S., G. Martin-Manso, et al. (2010). "Thrombospondin-1 inhibits VEGF receptor-2 signaling by disrupting its association with CD47." *J Biol Chem* 285(50): 38923-32.

Ke, Y., D. Wu, et al. (2007). "Role of Gab2 in mammary tumorigenesis and metastasis." *Oncogene* 26(34): 4951-60.

Kesler, S. R., R. J. Simensen, et al. (2007). "Altered neurodevelopment associated with mutations of RSK2: a morphometric MRI study of Coffin-Lowry syndrome." *Neurogenetics* 8(2): 143-7.

Kim, J., A. B. Parrish, et al. (2012). "Rsk-mediated phosphorylation and 14-3-3varepsilon binding of Apaf-1 suppresses cytochrome c-induced apoptosis." *EMBO J* 31(5): 1279-92.

Kim, M. J., J. Y. Ro, et al. (2006). "Clinicopathologic significance of the basal-like subtype of breast cancer: a comparison with hormone receptor and Her2/neu-overexpressing phenotypes." *Hum Pathol* 37(9): 1217-26.

King, F. W., J. Skeen, et al. (2004). "Inhibition of Chk1 by activated PKB/Akt." *Cell Cycle* 3(5): 634-7.

Kinkade, C. W., M. Castillo-Martin, et al. (2008). "Targeting AKT/mTOR and ERK MAPK signaling inhibits hormone-refractory prostate cancer in a preclinical mouse model." *J Clin Invest* 118(9): 3051-64.

Klemke, R. L., S. Cai, et al. (1997). "Regulation of cell motility by mitogen-activated protein kinase." *J Cell Biol* 137(2): 481-92.

Kojima, Y., A. Acar, et al. (2010). "Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts." *Proc Natl Acad Sci U S A* 107(46): 20009-14.

Komis, G., P. Illes, et al. (2011). "Microtubules and mitogen-activated protein kinase signalling." *Curr Opin Plant Biol* 14(6): 650-7.

Kosako, H., N. Yamaguchi, et al. (2009). "Phosphoproteomics reveals new ERK MAP kinase targets and links ERK to nucleoporin-mediated nuclear transport." *Nat Struct Mol Biol* 16(10): 1026-35.

Kroczynska, B., S. Joshi, et al. (2010). "Regulatory effects of ribosomal S6 kinase 1 (RSK1) in IFNlambda signaling." *J Biol Chem* 286(2): 1147-56.

Kurokawa, H., A. E. Lenferink, et al. (2000). "Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells." *Cancer Res* 60(20): 5887-94.

Lau, M. T., W. K. So, et al. (2013). "Fibroblast growth factor 2 induces E-cadherin down-regulation via PI3K/Akt/mTOR and MAPK/ERK signaling in ovarian cancer cells." *PLoS One* 8(3): e59083.

Lavoie, C., J. F. Mercier, et al. (2002). "Beta 1/beta 2-adrenergic receptor heterodimerization regulates beta 2-adrenergic receptor internalization and ERK signaling efficacy." *J Biol Chem* 277(38): 35402-10.

Lawler, P. R. and J. Lawler (2012). "Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2." *Cold Spring Harb Perspect Med* 2(5): a006627.

Lawrence, M. C., A. Jivan, et al. (2008). "The roles of MAPKs in disease." *Cell Res* 18(4): 436-42.

Le Gallic, L., L. Virgilio, et al. (2004). "ERF nuclear shuttling, a continuous monitor of Erk activity that links it to cell cycle progression." *Mol Cell Biol* 24(3): 1206-18.

Lee, H. H. and Z. F. Chang (2008). "Regulation of RhoA-dependent ROCKII activation by Shp2." *J Cell Biol* 181(6): 999-1012.

Lee, S., M. Warthaka, et al. (2011). "A model of a MAPK*substrate complex in an active conformation: a computational and experimental approach." *PLoS One* 6(4): e18594.

Lee, S. H., J. W. Lee, et al. (2003). "BRAF and KRAS mutations in stomach cancer." *Oncogene* 22(44): 6942-5.

Lefloch, R., J. Pouyssegur, et al. (2008). "Single and combined silencing of ERK1 and ERK2 reveals their positive contribution to growth signaling depending on their expression levels." *Mol Cell Biol* 28(1): 511-27.

Lehr, S., J. Kotzka, et al. (2004). "Identification of major ERK-related phosphorylation sites in Gab1." *Biochemistry* 43(38): 12133-40.

Lemmon, M. A. and J. Schlessinger (2010). "Cell signaling by receptor tyrosine kinases." *Cell* 141(7): 1117-34.

Leung, W. H. and S. Bolland (2007). "The inositol 5'-phosphatase SHIP-2 negatively regulates IgE-induced mast cell degranulation and cytokine production." *J Immunol*

179(1): 95-102.

Li, D., L. Jin, et al. (2013). "The prometastatic ribosomal S6 kinase 2-cAMP response element-binding protein (RSK2-CREB) signaling pathway up-regulates the actin-binding protein fascin-1 to promote tumor metastasis." *J Biol Chem* 288(45): 32528-38.

Li, S., D. D. Hsu, et al. (2012). "Dual faces of SH2-containing protein-tyrosine phosphatase Shp2/PTPN11 in tumorigenesis." *Front Med* 6(3): 275-9.

Li, S. G. and L. Li (2014). "Targeted therapy in HER2-positive breast cancer." *Biomed Rep* 1(4): 499-505.

Ling, H., M. Fabbri, et al. (2013). "MicroRNAs and other non-coding RNAs as targets for anticancer drug development." *Nat Rev Drug Discov* 12(11): 847-65.

Liu, Y. and L. R. Rohrschneider (2002). "The gift of Gab." *FEBS Lett* 515(1-3): 1-7.

Lopez-Vicente, L., G. Armengol, et al. (2009). "Regulation of replicative and stress-induced senescence by RSK4, which is down-regulated in human tumors." *Clin Cancer Res* 15(14): 4546-53.

Lorenzen, J. A., S. E. Baker, et al. (2001). "Nuclear import of activated D-ERK by DIM-7, an importin family member encoded by the gene moleskin." *Development* 128(8): 1403-14.

Luo, J., S. J. Field, et al. (2005). "The p85 regulatory subunit of phosphoinositide 3-kinase down-regulates IRS-1 signaling via the formation of a sequestration complex." *J Cell Biol* 170(3): 455-64.

Lynch, D. K. and R. J. Daly (2002). "PKB-mediated negative feedback tightly regulates mitogenic signalling via Gab2." *EMBO J* 21(1-2): 72-82.

Ma, L., Z. Chen, et al. (2005). "Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis." *Cell* 121(2): 179-93.

Ma, Q., S. Guin, et al. (2011). "Ribosomal protein S6 kinase (RSK)-2 as a central effector molecule in RON receptor tyrosine kinase mediated epithelial to mesenchymal transition induced by macrophage-stimulating protein." *Mol Cancer* 10: 66.

Mao, D., H. Epple, et al. (2006). "PLCgamma2 regulates osteoclastogenesis via its interaction with ITAM proteins and GAB2." *J Clin Invest* 116(11): 2869-79.

Mao, Y. and A. W. Lee (2005). "A novel role for Gab2 in bFGF-mediated cell survival during retinoic acid-induced neuronal differentiation." *J Cell Biol* 170(2): 305-16.

Margolis, B. and E. Y. Skolnik (1994). "Activation of Ras by receptor tyrosine kinases." *J Am Soc Nephrol* 5(6): 1288-99.

Maroun, C. R., M. Holgado-Madruga, et al. (1999). "The Gab1 PH domain is required for localization of Gab1 at sites of cell-cell contact and epithelial morphogenesis downstream from the met receptor tyrosine kinase." *Mol Cell Biol* 19(3): 1784-99.

Matozaki, T., Y. Murata, et al. (2009). "Protein tyrosine phosphatase SHP-2: a proto-oncogene product that promotes Ras activation." *Cancer Sci* 100(10): 1786-93.

Mehlen, P. and A. Puisieux (2006). "Metastasis: a question of life or death." *Nat Rev Cancer* 6(6): 449-58.

Mendoza, M. C., E. E. Er, et al. (2011). "The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation." *Trends Biochem Sci* 36(6): 320-8.

Meng, J., B. Dai, et al. (2010). "Combination treatment with MEK and AKT inhibitors is more effective than each drug alone in human non-small cell lung cancer in vitro and in vivo." *PLoS One* 5(11): e14124.

Menzies, A. M. and G. V. Long (2014). "Dabrafenib and Trametinib, alone and in combination for BRAF-mutant metastatic melanoma." *Clin Cancer Res*.

Meyuhas, O. and A. Dreazen (2009). "Ribosomal protein S6 kinase from TOP mRNAs to cell size." *Prog Mol Biol Transl Sci* 90: 109-53.

Mira, A., C. Isella, et al. (2009). "The GAB2 signaling scaffold promotes anchorage independence and drives a transcriptional response associated with metastatic progression of breast cancer." *Oncogene* 28(50): 4444-55.

Moelling, K., K. Schad, et al. (2002). "Regulation of Raf-Akt Cross-talk." *J Biol Chem* 277(34): 31099-106.

Moller, D. E., C. H. Xia, et al. (1994). "Human rsk isoforms: cloning and characterization of tissue-specific expression." *Am J Physiol* 266(2 Pt 1): C351-9.

Montagner, A., A. Yart, et al. (2005). "A novel role for Gab1 and SHP2 in epidermal growth factor-induced Ras activation." *J Biol Chem* 280(7): 5350-60.

Murphy, L. O., S. Smith, et al. (2002). "Molecular interpretation of ERK signal duration by immediate early gene products." *Nat Cell Biol* 4(8): 556-64.

Mustonen, M. V., S. Pyrhonen, et al. (2014). "Toremifene in the treatment of breast cancer." *World J Clin Oncol* 5(3): 393-405.

Nakajima, T., A. Fukamizu, et al. (1996). "The signal-dependent coactivator CBP is a nuclear target for pp90RSK." *Cell* 86(3): 465-74.

Nakaoka, Y. and I. Komuro (2013). "Gab docking proteins in cardiovascular disease, cancer, and inflammation." *Int J Inflam* 2013: 141068.

Nam, H. J., S. Kim, et al. (2008). "The ERK-RSK1 activation by growth factors at G2 phase delays cell cycle progression and reduces mitotic aberrations." *Cell Signal* 20(7): 1349-58.

Naor, Z., O. Benard, et al. (2000). "Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor." *Trends Endocrinol Metab* 11(3): 91-9.

Nardozzi, J. D., K. Lott, et al. (2010). "Phosphorylation meets nuclear import: a review." *Cell Commun Signal* 8: 32.

Neufeld, G., T. Cohen, et al. (1999). "Vascular endothelial growth factor (VEGF) and its receptors." *FASEB J* 13(1): 9-22.

Newton, A. C. (2003). "Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm." *Biochem J* 370(Pt 2): 361-71.

Ni, S., C. Zhao, et al. (2007). "A novel Stat3 binding motif in Gab2 mediates transformation of primary hematopoietic cells by the Stk/Ron receptor tyrosine kinase in response to Friend virus infection." *Mol Cell Biol* 27(10): 3708-15.

Nishida, K., L. Wang, et al. (2002). "Requirement of Gab2 for mast cell development and KitL/c-Kit signaling." *Blood* 99(5): 1866-9.

Nowak, G. (2002). "Protein kinase C-alpha and ERK1/2 mediate mitochondrial dysfunction, decreases in active Na+ transport, and cisplatin-induced apoptosis in renal cells." *J Biol Chem* 277(45): 43377-88.

Obata, T., M. B. Yaffe, et al. (2000). "Peptide and protein library screening defines optimal substrate motifs for AKT/PKB." *J Biol Chem* 275(46): 36108-15.

Onder, T. T., P. B. Gupta, et al. (2008). "Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways." *Cancer Res* 68(10): 3645-54.

Pages, G., S. Guerin, et al. (1999). "Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice." *Science* 286(5443): 1374-7.

Palamarchuk, A., A. Efanov, et al. (2005). "Akt phosphorylates and regulates Pdcd4 tumor suppressor protein." *Cancer Res* 65(24): 11282-6.

Papkoff, J., R. H. Chen, et al. (1994). "p42 mitogen-activated protein kinase and p90 ribosomal S6 kinase are selectively phosphorylated and activated during thrombin-induced platelet activation and aggregation." *Mol Cell Biol* 14(1): 463-72.

Patan, S. (2004). "Vasculogenesis and angiogenesis." *Cancer Treat Res* 117: 3-32.

Pawson, T. and J. Schlessingert (1993). "SH2 and SH3 domains." *Curr Biol* 3(7): 434-42.

Pende, M., S. H. Um, et al. (2004). "S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway." *Mol Cell Biol* 24(8): 3112-24.

Peng, C., Y. Y. Cho, et al. (2010). "RSK2 mediates NF-{kappa}B activity through the phosphorylation of IkappaBalpha in the TNF-R1 pathway." *FASEB J* 24(9): 3490-9.

Poirier, R., S. Jacquot, et al. (2007). "Deletion of the Coffin-Lowry syndrome gene Rsk2 in mice is associated with impaired spatial learning and reduced control of exploratory behavior." *Behav Genet* 37(1): 31-50.

Porter, A. C. and R. R. Vaillancourt (1998). "Tyrosine kinase receptor-activated signal transduction pathways which lead to oncogenesis." *Oncogene* 17(11 Reviews): 1343-52.

Prat, A., J. S. Parker, et al. (2010). "Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer." *Breast Cancer Res* 12(5): R68.

Pruett, W., Y. Yuan, et al. (1995). "Association between GRB2/Sos and insulin receptor substrate 1 is not sufficient for activation of extracellular signal-regulated kinases by interleukin-4: implications for Ras activation by insulin." *Mol Cell Biol* 15(3): 1778-85.

Qian, P., Z. Zuo, et al. (2011). "Pivotal role of reduced let-7g expression in breast cancer invasion and metastasis." *Cancer Res* 71(20): 6463-74.

Rak, J., Y. Mitsuhashi, et al. (1995). "Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis." *Cancer Res* 55(20): 4575-80.

Ranganathan, A., M. N. Yazicioglu, et al. (2006). "The nuclear localization of ERK2 occurs by mechanisms both independent of and dependent on energy." *J Biol Chem* 281(23): 15645-52.

Rasola, A., M. Sciacovelli, et al. (2010). "Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition." *Proc Natl Acad Sci U S A* 107(2): 726-31.

Ray-David, H., Y. Romeo, et al. (2012). "RSK promotes G2 DNA damage checkpoint silencing and participates in melanoma chemoresistance." *Oncogene* 32(38): 4480-9.

Rebecchi, M. J. and S. Scarlata (1998). "Pleckstrin homology domains: a common fold with diverse functions." *Annu Rev Biophys Biomol Struct* 27: 503-28.

Richards, S. A., V. C. Dreisbach, et al. (2001). "Characterization of regulatory events associated with membrane targeting of p90 ribosomal S6 kinase 1." *Mol Cell Biol* 21(21): 7470-80.

Richards, S. A., J. Fu, et al. (1999). "Ribosomal S6 kinase 1 (RSK1) activation requires signals dependent on and independent of the MAP kinase ERK." *Curr Biol* 9(15):

810-20.

Robertson, S. E., S. R. Setty, et al. (2006). "Extracellular signal-regulated kinase regulates clathrin-independent endosomal trafficking." *Mol Biol Cell* 17(2): 645-57.

Robinson, M. J., S. A. Stippec, et al. (1998). "A constitutively active and nuclear form of the MAP kinase ERK2 is sufficient for neurite outgrowth and cell transformation." *Curr Biol* 8(21): 1141-50.

Rocchi, S., S. Tartare-Deckert, et al. (1998). "Determination of Gab1 (Grb2-associated binder-1) interaction with insulin receptor-signaling molecules." *Mol Endocrinol* 12(7): 914-23.

Rodrigues, G. A., M. Falasca, et al. (2000). "A novel positive feedback loop mediated by the docking protein Gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling." *Mol Cell Biol* 20(4): 1448-59.

Rodriguez-Manzaneque, J. C., T. F. Lane, et al. (2001). "Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor." *Proc Natl Acad Sci U S A* 98(22): 12485-90.

Romeo, Y., J. Moreau, et al. (2012). "RSK regulates activated BRAF signalling to mTORC1 and promotes melanoma growth." *Oncogene* 32(24): 2917-26.

Romeo, Y., X. Zhang, et al. (2011). "Regulation and function of the RSK family of protein kinases." *Biochem J* 441(2): 553-69.

Roshan, B., C. Kjelsberg, et al. (1999). "Activated ERK2 interacts with and phosphorylates the docking protein GAB1." *J Biol Chem* 274(51): 36362-8.

Roskoski, R., Jr. (2012). "ERK1/2 MAP kinases: structure, function, and regulation." *Pharmacol Res* 66(2): 105-43.

Roux, P. P., B. A. Ballif, et al. (2004). "Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase." *Proc Natl Acad Sci U S A* 101(37): 13489-94.

Roux, P. P., S. A. Richards, et al. (2003). "Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity." *Mol Cell Biol* 23(14): 4796-804.

Roux, P. P., D. Shahbazian, et al. (2007). "RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation." *J Biol Chem* 282(19): 14056-64.

Sachs, M., H. Brohmann, et al. (2000). "Essential role of Gab1 for signaling by the c-Met receptor in vivo." *J Cell Biol* 150(6): 1375-84.

Saha, M., A. Carriere, et al. (2012). "RSK phosphorylates SOS1 creating 14-3-3-docking sites and negatively regulating MAPK activation." *Biochem J* 447(1): 159-66.

Salazar, L., T. Kashiwada, et al. (2009). "A novel interaction between fibroblast growth factor receptor 3 and the p85 subunit of phosphoinositide 3-kinase: activation-dependent regulation of ERK by p85 in multiple myeloma cells." *Hum Mol Genet* 18(11): 1951-61.

Sapkota, G. P., L. Cummings, et al. (2007). "BI-D1870 is a specific inhibitor of the p90 RSK (ribosomal S6 kinase) isoforms in vitro and in vivo." *Biochem J* 401(1): 29-38.

Sattler, M., M. G. Mohi, et al. (2002). "Critical role for Gab2 in transformation by BCR/ABL." *Cancer Cell* 1(5): 479-92.

Schaeffer, H. J., A. D. Catling, et al. (1998). "MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade." *Science* 281(5383): 1668-71.

Schechter, A. L., D. F. Stern, et al. (1984). "The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen." *Nature* 312(5994): 513-6.

Scherr, M., A. Chaturvedi, et al. (2006). "Enhanced sensitivity to inhibition of SHP2, STAT5, and Gab2 expression in chronic myeloid leukemia (CML)." *Blood* 107(8): 3279-87.

Schuler, M. and D. R. Green (2001). "Mechanisms of p53-dependent apoptosis." *Biochem Soc Trans* 29(Pt 6): 684-8.

Schutzman, J. L., C. Z. Borland, et al. (2001). "The Caenorhabditis elegans EGL-15 signaling pathway implicates a DOS-like multisubstrate adaptor protein in fibroblast growth factor

signal transduction." *Mol Cell Biol* 21(23): 8104-16.

Schwartz, G. K. and M. A. Shah (2005). "Targeting the cell cycle: a new approach to cancer therapy." *J Clin Oncol* 23(36): 9408-21.

Schwartz, M. (2004). "Rho signalling at a glance." *J Cell Sci* 117(Pt 23): 5457-8.

Seidel, J. J. and B. J. Graves (2002). "An ERK2 docking site in the Pointed domain distinguishes a subset of ETS transcription factors." *Genes Dev* 16(1): 127-37.

Seiffert, M., J. M. Custodio, et al. (2003). "Gab3-deficient mice exhibit normal development and hematopoiesis and are immunocompetent." *Mol Cell Biol* 23(7): 2415-24.

Senechal, K., J. Halpern, et al. (1996). "The CRKL adaptor protein transforms fibroblasts and functions in transformation by the BCR-ABL oncogene." *J Biol Chem* 271(38): 23255-61.

Shahbazian, D., P. P. Roux, et al. (2006). "The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity." *EMBO J* 25(12): 2781-91.

Sharma, C., T. Vomastek, et al. (2005). "MEK partner 1 (MP1): regulation of oligomerization in MAP kinase signaling." *J Cell Biochem* 94(4): 708-19.

Sharma, N., S. Everingham, et al. (2014). "SHP2 phosphatase promotes mast cell chemotaxis toward stem cell factor via enhancing activation of the Lyn/Vav/Rac signaling axis." *J Immunol* 192(10): 4859-66.

Shaul, Y. D. and R. Seger (2006). "ERK1c regulates Golgi fragmentation during mitosis." *J Cell Biol* 172(6): 885-97.

Shaulian, E. and M. Karin (2001). "AP-1 in cell proliferation and survival." *Oncogene* 20(19): 2390-400.

Sherr, C. J. and F. McCormick (2002). "The RB and p53 pathways in cancer." *Cancer Cell* 2(2): 103-12.

Shin, S., C. A. Dimitri, et al. (2010). "ERK2 but not ERK1 induces epithelial-to-mesenchymal transformation via DEF motif-dependent signaling events." *Mol Cell* 38(1): 114-27.

Shweiki, D., A. Itin, et al. (1992). "Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis." *Nature* 359(6398): 843-5.

Smith, J. A., C. E. Poteet-Smith, et al. (1999). "Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK in vivo." *J Biol Chem* 274(5): 2893-8.

Smith, J. A., C. E. Poteet-Smith, et al. (2005). "Identification of the first specific inhibitor of p90 ribosomal S6 kinase (RSK) reveals an unexpected role for RSK in cancer cell proliferation." *Cancer Res* 65(3): 1027-34.

Smolen, G. A., J. Zhang, et al. (2010). "A genome-wide RNAi screen identifies multiple RSK-dependent regulators of cell migration." *Genes Dev* 24(23): 2654-65.

Somasundaram, K. (2003). "Breast cancer gene 1 (BRCA1): role in cell cycle regulation and DNA repair--perhaps through transcription." *J Cell Biochem* 88(6): 1084-91.

Song, T., K. Sugimoto, et al. (2007). "p90 RSK-1 associates with and inhibits neuronal nitric oxide synthase." *Biochem J* 401(2): 391-8.

Sonnenblick, A., C. Levy, et al. (2004). "Interplay between MITF, PIAS3, and STAT3 in mast cells and melanocytes." *Mol Cell Biol* 24(24): 10584-92.

Sturgill, T. W., L. B. Ray, et al. (1988). "Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II." *Nature* 334(6184): 715-8.

Sulzmaier, F. J. and J. W. Ramos (2013). "RSK isoforms in cancer cell invasion and metastasis." *Cancer Res* 73(20): 6099-105.

Sun, Y., G. Nie, et al. (2014). "Luminal breast cancer classification according to proliferative indices: clinicopathological characteristics and short-term survival analysis." *Med Oncol* 31(7): 55.

Taki, M., N. Kamata, et al. (2003). "Down-regulation of Wnt-4 and up-regulation of Wnt-5a expression by epithelial-mesenchymal transition in human squamous carcinoma cells." *Cancer Sci* 94(7): 593-7.

Takino, T., H. Tsuge, et al. (2010). "MT1-MMP promotes cell growth and ERK activation

through c-Src and paxillin in three-dimensional collagen matrix." *Biochem Biophys Res Commun* 396(4): 1042-7.

Tamimi, R. M., H. J. Baer, et al. (2008). "Comparison of molecular phenotypes of ductal carcinoma in situ and invasive breast cancer." *Breast Cancer Res* 10(4): R67.

Tan, Y., H. Ruan, et al. (1999). "p90(RSK) blocks bad-mediated cell death via a protein kinase C-dependent pathway." *J Biol Chem* 274(49): 34859-67.

Tanoue, T., R. Maeda, et al. (2001). "Identification of a docking groove on ERK and p38 MAP kinases that regulates the specificity of docking interactions." *EMBO J* 20(3): 466-79.

Tanoue, T. and E. Nishida (2003). "Molecular recognitions in the MAP kinase cascades." *Cell Signal* 15(5): 455-62.

Tanoue, T., T. Yamamoto, et al. (2001). "A Novel MAPK phosphatase MKP-7 acts preferentially on JNK/SAPK and p38 alpha and beta MAPKs." *J Biol Chem* 276(28): 26629-39.

Teis, D., N. Taub, et al. (2006). "p14-MP1-MEK1 signaling regulates endosomal traffic and cellular proliferation during tissue homeostasis." *J Cell Biol* 175(6): 861-8.

Terauchi, Y., Y. Tsuji, et al. (1999). "Increased insulin sensitivity and hypoglycaemia in mice lacking the p85 alpha subunit of phosphoinositide 3-kinase." *Nat Genet* 21(2): 230-5.

Thakur, A., K. W. Rahman, et al. (2007). "Aberrant expression of X-linked genes RbAp46, Rsk4, and Cldn2 in breast cancer." *Mol Cancer Res* 5(2): 171-81.

Therrien, M., H. C. Chang, et al. (1995). "KSR, a novel protein kinase required for RAS signal transduction." *Cell* 83(6): 879-88.

Thomas, G. M., G. R. Rumbaugh, et al. (2005). "Ribosomal S6 kinase 2 interacts with and phosphorylates PDZ domain-containing proteins and regulates AMPA receptor transmission." *Proc Natl Acad Sci U S A* 102(42): 15006-11.

Tohgo, A., K. L. Pierce, et al. (2002). "beta-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin

AT1a receptor stimulation." *J Biol Chem* 277(11): 9429-36.

Topham, C. H. and S. S. Taylor (2013). "Mitosis and apoptosis: how is the balance set?" *Curr Opin Cell Biol* 25(6): 780-5.

Torii, S., M. Kusakabe, et al. (2004). "Sef is a spatial regulator for Ras/MAP kinase signaling." *Dev Cell* 7(1): 33-44.

Tunquist, B. J., M. S. Schwab, et al. (2002). "The spindle checkpoint kinase bub1 and cyclin e/cdk2 both contribute to the establishment of meiotic metaphase arrest by cytostatic factor." *Curr Biol* 12(12): 1027-33.

Turke, A. B., Y. Song, et al. (2012). "MEK inhibition leads to PI3K/AKT activation by relieving a negative feedback on ERBB receptors." *Cancer Res* 72(13): 3228-37.

Ubersax, J. A. and J. E. Ferrell, Jr. (2007). "Mechanisms of specificity in protein phosphorylation." *Nat Rev Mol Cell Biol* 8(7): 530-41.

Ucuzian, A. A., A. A. Gassman, et al. (2010). "Molecular mediators of angiogenesis." *J Burn Care Res* 31(1): 158-75.

Vaidyanathan, H. and J. W. Ramos (2003). "RSK2 activity is regulated by its interaction with PEA-15." *J Biol Chem* 278(34): 32367-72.

Vasiljeva, O., A. Papazoglou, et al. (2006). "Tumor cell-derived and macrophage-derived cathepsin B promotes progression and lung metastasis of mammary cancer." *Cancer Res* 66(10): 5242-50.

Vesuna, F., P. van Diest, et al. (2008). "Twist is a transcriptional repressor of E-cadherin gene expression in breast cancer." *Biochem Biophys Res Commun* 367(2): 235-41.

Vik, T. A. and J. W. Ryder (1997). "Identification of serine 380 as the major site of autophosphorylation of Xenopus pp90rsk." *Biochem Biophys Res Commun* 235(2): 398-402.

Vu, T. H. and Z. Werb (2000). "Matrix metalloproteinases: effectors of development and normal physiology." *Genes Dev* 14(17): 2123-33.

Wada, T., T. Nakashima, et al. (2005). "The molecular scaffold Gab2 is a crucial component of RANK signaling and osteoclastogenesis." *Nat Med* 11(4): 394-9.

Wang, L. and L. J. Di (2014). "BRCA1 And Estrogen/Estrogen Receptor In Breast Cancer: Where They Interact?" *Int J Biol Sci* 10(5): 563-573.

Wang, R., S. Y. Jung, et al. (2010). "Direct roles of the signaling kinase RSK2 in Cdc25C activation during Xenopus oocyte maturation." *Proc Natl Acad Sci U S A* 107(46): 19885-90.

Wang, Y. and R. Prywes (2000). "Activation of the c-fos enhancer by the erk MAP kinase pathway through two sequence elements: the c-fos AP-1 and p62TCF sites." *Oncogene* 19(11): 1379-85.

Wang, Y., Q. Sheng, et al. (2011). "Gab2 regulates the migratory behaviors and E-cadherin expression via activation of the PI3K pathway in ovarian cancer cells." *Oncogene* 31(20): 2512-20.

Wang, Z., H. Yang, et al. (2006). "Phosphatase-mediated crosstalk control of ERK and p38 MAPK signaling in corneal epithelial cells." *Invest Ophthalmol Vis Sci* 47(12): 5267-75.

Wei, J. H. and J. Seemann (2009). "Remodeling of the Golgi structure by ERK signaling." *Commun Integr Biol* 2(1): 35-6.

Welsh, G. I., C. M. Miller, et al. (1998). "Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin." *FEBS Lett* 421(2): 125-30.

Williams, M. R., J. S. Arthur, et al. (2000). "The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells." *Curr Biol* 10(8): 439-48.

Wohrle, F. U., R. J. Daly, et al. (2009). "Function, regulation and pathological roles of the Gab/DOS docking proteins." *Cell Commun Signal* 7: 22.

Wohrle, F. U., S. Halbach, et al. (2012). "Gab2 signaling in chronic myeloid leukemia cells confers resistance to multiple Bcr-Abl inhibitors." *Leukemia* 27(1): 118-29.

Wolf, I., B. J. Jenkins, et al. (2002). "Gab3, a new DOS/Gab family member, facilitates macrophage differentiation." *Mol Cell Biol* 22(1): 231-44.

Woo, M. S., Y. Ohta, et al. (2004). "Ribosomal S6 kinase (RSK) regulates phosphorylation of filamin A on an important regulatory site." *Mol Cell Biol* 24(7): 3025-35.

Wortzel, I. and R. Seger (2011). "The ERK Cascade: Distinct Functions within Various Subcellular Organelles." *Genes Cancer* 2(3): 195-209.

Wu, C. F., S. Liu, et al. (2013). "RSK promotes G2/M transition through activating phosphorylation of Cdc25A and Cdc25B." *Oncogene*.

Wu, J. and R. Janknecht (2002). "Regulation of the ETS transcription factor ER81 by the 90-kDa ribosomal S6 kinase 1 and protein kinase A." *J Biol Chem* 277(45): 42669-79.

Wu, J., F. Meng, et al. (2008). "Lyn regulates BCR-ABL and Gab2 tyrosine phosphorylation and c-Cbl protein stability in imatinib-resistant chronic myelogenous leukemia cells." *Blood* 111(7): 3821-9.

Xie, Z. H., I. Ambudkar, et al. (2002). "The adapter molecule Gab2 regulates Fc epsilon RI-mediated signal transduction in mast cells." *J Immunol* 168(9): 4682-91.

Xing, H., K. Kornfeld, et al. (1997). "The protein kinase KSR interacts with 14-3-3 protein and Raf." *Curr Biol* 7(5): 294-300.

Xing, J., D. D. Ginty, et al. (1996). "Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase." *Science* 273(5277): 959-63.

Xing, J., J. M. Kornhauser, et al. (1998). "Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation." *Mol Cell Biol* 18(4): 1946-55.

Xu, J., S. Lamouille, et al. (2009). "TGF-beta-induced epithelial to mesenchymal transition." *Cell Res* 19(2): 156-72.

Yamada, K., K. Nishida, et al. (2001). "Comparative FISH mapping of Gab1 and Gab2 genes in human, mouse and rat." *Cytogenet Cell Genet* 94(1-2): 39-42.

Yamazaki, T., K. Zaal, et al. (2002). "Role of Grb2 in EGF-stimulated EGFR internalization." *J Cell Sci* 115(Pt 9): 1791-802.

Yamnik, R. L. and M. K. Holz (2009). "mTOR/S6K1 and MAPK/RSK signaling pathways coordinately regulate estrogen receptor alpha serine 167 phosphorylation." *FEBS Lett* 584(1): 124-8.

Yang, J. Y., C. S. Zong, et al. (2008). "ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation." *Nat Cell Biol* 10(2): 138-48.

Yang, S., M. Ji, et al. (2013). "Phosphorylation of KIBRA by the extracellular signal-regulated kinase (ERK)-ribosomal S6 kinase (RSK) cascade modulates cell proliferation and migration." *Cell Signal* 26(2): 343-51.

Yang, Y., J. Wu, et al. (2012). "GAB2 induces tumor angiogenesis in NRAS-driven melanoma." *Oncogene* 32(31): 3627-37.

Yarden, Y. (2001). "The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities." *Eur J Cancer* 37 Suppl 4: S3-8.

Yasuda, J., A. J. Whitmarsh, et al. (1999). "The JIP group of mitogen-activated protein kinase scaffold proteins." *Mol Cell Biol* 19(10): 7245-54.

You, J. S. and P. A. Jones (2012). "Cancer genetics and epigenetics: two sides of the same coin?" *Cancer Cell* 22(1): 9-20.

Yu, C. F., Z. X. Liu, et al. (2002). "ERK negatively regulates the epidermal growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase." *J Biol Chem* 277(22): 19382-8.

Yu, C. F., B. Roshan, et al. (2001). "ERK regulates the hepatocyte growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase." *J Biol Chem* 276(35): 32552-8.

Yu, J., Y. Zhang, et al. (1998). "Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit." *Mol Cell Biol* 18(3): 1379-87.

Yu, M., J. Luo, et al. (2006). "The scaffolding adapter Gab2, via Shp-2, regulates kit-evoked mast cell proliferation by activating the Rac/JNK pathway." *J Biol Chem* 281(39): 28615-26.

Yu, Y., S. O. Yoon, et al. (2011). "Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling." *Science* 332(6035): 1322-6.

Zeniou, M., T. Ding, et al. (2002). "Expression analysis of RSK gene family members: the RSK2 gene, mutated in Coffin-Lowry syndrome, is prominently expressed in brain structures essential for cognitive function and learning." *Hum Mol Genet* 11(23): 2929-40.

Zhang, H. S., A. A. Postigo, et al. (1999). "Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition." *Cell* 97(1): 53-61.

Zhang, J., L. S. Barak, et al. (1997). "A central role for beta-arrestins and clathrin-coated vesicle-mediated endocytosis in beta2-adrenergic receptor resensitization. Differential regulation of receptor resensitization in two distinct cell types." *J Biol Chem* 272(43): 27005-14.

Zhang, X., G. Lavoie, et al. (2013). "Gab2 phosphorylation by RSK inhibits Shp2 recruitment and cell motility." *Mol Cell Biol* 33(8): 1657-70.

Zhao, C., H. Ma, et al. (2003). "GC-GAP, a Rho family GTPase-activating protein that interacts with signaling adapters Gab1 and Gab2." *J Biol Chem* 278(36): 34641-53.

Zhao, C., D. H. Yu, et al. (1999). "Gab2, a new pleckstrin homology domain-containing adapter protein, acts to uncouple signaling from ERK kinase to Elk-1." *J Biol Chem* 274(28): 19649-54.

Zhao, J., X. Yuan, et al. (2003). "ERK-dependent phosphorylation of the transcription initiation factor TIF-IA is required for RNA polymerase I transcription and cell growth." *Mol Cell* 11(2): 405-13.

Zhao, Y., C. Bjorbaek, et al. (1995). "RSK3 encodes a novel pp90rsk isoform with a unique N-terminal sequence: growth factor-stimulated kinase function and nuclear translocation." *Mol Cell Biol* 15(8): 4353-63.

Zhu, J., J. Blenis, et al. (2008). "Activation of PI3K/Akt and MAPK pathways regulates Myc-mediated transcription by phosphorylating and promoting the degradation of Mad1." *Proc Natl Acad Sci U S A* 105(18): 6584-9.

Zilfou, J. T. and S. W. Lowe (2009). "Tumor suppressive functions of p53." *Cold Spring Harb Perspect Biol* 1(5): a001883.