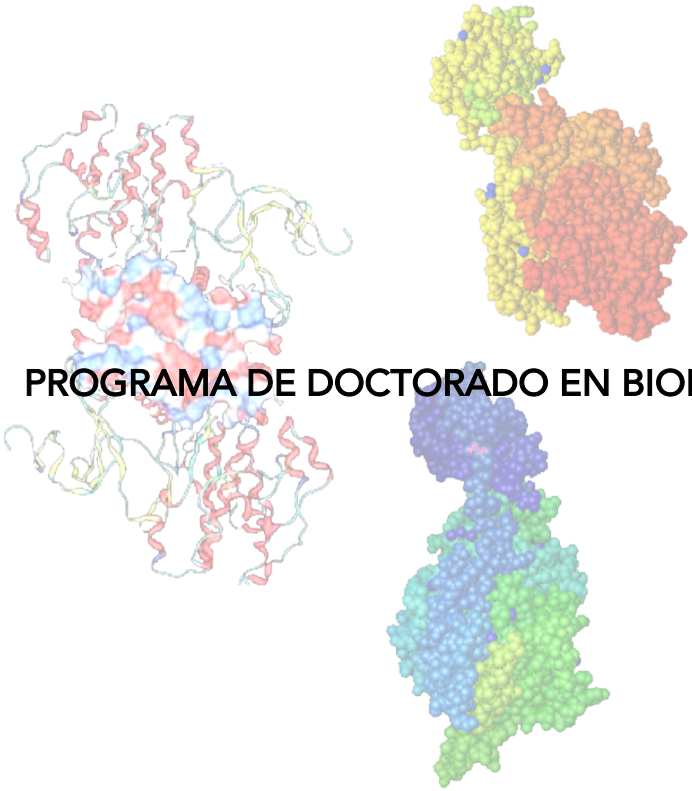


PhD THESIS

PROGRAMA DE DOCTORADO EN BIOLOGÍA MOLECULAR Y BIOMEDICINA

Optimization of ERK signalling by transphosphorylation across different scaffold protein species: implication in cancer therapeutics

---

Optimización de las señales de ERK mediante transfosforilación entre diferentes proteínas scaffold: implicaciones en terapia antitumoral

ANA MARTÍN VEGA | Santander, 2020

Dirigida por: **PIERO CRESPO**

UC UNIVERSIDAD DE CANTABRIA

ESCUELA DE DOCTORADO DE LA UNIVERSIDAD DE CANTABRIA

UNIVERSIDAD DE CANTABRIA

PROGRAMA DE DOCTORADO EN BIOLOGÍA MOLECULAR Y BIOMEDICINA

TESIS DOCTORAL

Optimización de las señales de ERK mediante transfosforilación entre diferentes proteínas scaffold: implicaciones en terapia antitumoral

PhD THESIS

Optimization of ERK signalling by transphosphorylation across different scaffold protein species: implication in cancer therapeutics

Realizada por: **Ana Martín Vega**

Dirigida por: **Piero Crespo**

Escuela de Doctorado de la Universidad de Cantabria **Santander, 2020**

El Dr. PIERO CRESPO BARAJA, Profesor de Investigación del Consejo Superior de Investigaciones Científicas (CSIC) en el laboratorio de Regulación espacial de las señales RAS/ERK en cáncer en el departamento de Señalización celular y molecular ubicado en el Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC) y como Tutor/Director de esta Tesis

CERTIFICA:

Que ANA MARTÍN VEGA ha realizado bajo su dirección el presente trabajo de Tesis Doctoral en el Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC) titulado:

Optimización de las señales de ERK mediante transfosforilación entre diferentes proteínas scaffold: implicaciones en terapia antitumoral

---

Optimization of ERK signalling by transphosphorylation across different scaffold protein species: implication in cancer therapeutics

Que considera que dicho trabajo se encuentra terminado y reúne los requisitos necesarios para su presentación como Memoria de Doctorado al objeto de poder optar al grado de Doctor en Biología Molecular y Biomedicina por la Universidad de Cantabria.

Y para que conste y surta los efectos oportunos, expide el presente certificado en Santander, en Enero de 2020.

Fdo.: Piero Crespo

La presente tesis doctoral titulada “Optimization of ERK signalling by transphosphorylation across different scaffold protein species: implication in cancer therapeutics” ha sido realizada en el Instituto de Biomedicna y Biotecnología de Cantabria (IBBTEC) en el laboratorio de Regulación espacial de las señales RAS/ERK en cáncer gracias a la ayuda del Programa de personal investigador en formación predoctoral en el área de biomedicina, biotecnología y ciencias de la salud de la Universidad de Cantabria (BOC 30.09.2015) y a los proyectos financiados por:

Durante el presente trabajo Ana Martín Vega ha realizado una estancia predoctoral de 3 meses en el laboratorio del Dr. David Engelberg en el instituto Alexander Silberman of Life Science en la Universidad Hebrea de Jerusalén, Israel, gracias a la financiación para estancias predoctorales de la Universidad de Cantabria

A mi familia

RESUMEN

RESUMEN

1. INTRODUCCIÓN

La ruta de señalización RAS/ERK desempeña un papel esencial en el control de la proliferación, diferenciación y supervivencia celular en condiciones fisiológicas. Los fallos en la regulación de dicha ruta contribuyen significativamente a la transformación celular y están incuestionablemente involucrados en la progresión tumoral, además de en otras patologías. De hecho, aproximadamente el 40% del total de los tumores humanos son debidos a la desregulación de la señalización a través de dicha cascada, causada por mutaciones activadoras en las distintas proteínas que la componen. Este porcentaje varía en función del tipo de cáncer, pudiendo alcanzar el 90%. En el caso del melanoma, un 60% de los casos están originados por la desregulación de las señales de ERK. Por estas razones, durante las últimas décadas, esta ruta ha sido el sujeto de intensas investigaciones, con el propósito de identificar componentes susceptibles de ser utilizados como dianas terapéuticas en el tratamiento del cáncer.

ERK1/2, las quinasas efectoras de esta vía de señalización, se activan en respuesta a diferentes estímulos intra o extracelulares a través de módulos de señalización compuestos por diferentes quinasas citoplásmicas. Las señales de ERK se regulan, entre otros mecanismos, por proteínas de andamiaje o "scaffolds". Dichas proteínas ensamblan simultáneamente al menos dos componentes de la cascada en un complejo multi-enzimático estable, mediante el cual se regulan la intensidad, amplitud y duración de las señales. Una característica importante de las proteínas scaffold es que la concentración óptima guarda una estricta estequiometría con la concentración de las quinasas que constituyen esta ruta de señalización. Por esta razón, cualquier alteración en sus niveles de expresión tendrá profundas consecuencias en la activación de ERK y, en última instancia, en las respuestas biológicas reguladas por esta ruta. Además, las proteínas scaffold desempeñan un papel central como reguladores espaciales de las señales de ERK. A este respecto, dependiendo de la localización subcelular de la que emanan las señales de RAS, determinados scaffolds especifican qué sustratos son susceptibles de ser fosforilados/activados por ERK.

Debido a la importancia de las proteínas scaffold en la regulación de esta cascada de señalización, se ha especulado con su posible implicación en la aparición de resistencia a terapias y con su potencial como dianas antitumorales. Una proteína scaffold con presunta capacidad como diana antineoplásica es IQGAP, ya que una cantidad significativa de melanomas exhiben considerables alteraciones en sus niveles de expresión. Por otro lado, la proteína KSR1 destaca como diana terapéutica debido a su inherente participación en la tumorogénesis mediada por RAS oncogénico. En consecuencia, recientemente se ha desarrollado el primer inhibidor de KSR, APS-2-79, que a la vez es también la primera molécula dirigida contra una proteína scaffold. Sin embargo, esta molécula, a pesar de estabilizar a KSR en su estado inactivo e impedir la activación de MEK dependiente de este scaffold, presenta efectos inhibitorios más bien modestos tanto en la supervivencia celular como en la activación de ERK mediada por KSR.

Una posible explicación a este fenómeno podría surgir de la interacción directa entre proteínas scaffold. A pesar de las funciones específicas y de regulación de ERK dependiente de la sublocalización de cada scaffold, se ha descrito que algunas de estas proteínas pueden asociarse entre sí. Por ello, hemos considerado la hipótesis de que estos macro-complejos scaffold-scaffold podrían constituir un nivel adicional de regulación para las señales de ERK y servir como nodos de integración para señales en respuesta a estímulos y para la diversificación subsiguiente de respuestas celulares específicas, en función de la afinidad por ERK que exhiben las distintas proteínas scaffold. Entender el mecanismo de regulación de ERK que supondría la coordinación entre proteínas scaffold podría facilitarnos nuevos medios para manipular las señales aberrantes de ERK con fines terapéuticos.

2. OBJETIVOS

Los objetivos planteados en esta tesis son:

- Evaluar APS-2-79 como inhibidor de la fosforilación/activación de ERK mediada por KSR y sus consecuencias biológicas.

- Investigar si las interacciones de KSR con otra/s proteína/s scaffold son la razón de la ineficacia de APS-2-79 en el bloqueo de las señales mediadas por KSR.
- Investigar si las diferentes proteínas scaffold exhiben diferentes afinidades por ERK.

3. MATERIALES Y MÉTODOS

Para evaluar el efecto apoptótico de la sobreexpresión de KSR o del tratamiento de líneas BRAF y NRAS mutantes de melanoma con APS-2-79 y compararlo con el tratamiento con PLX4032 o con la depleción de KSR se realizó un ensayo de detección de Anexina V mediante citometría de flujo.

Para estudiar el mecanismo de interacción de KSR1 con ERK fosforilado/activo se utilizaron mutantes incapaces de unirse a ERK (KSR1 ASAP), a MEK (KSR1 C809Y), deficientes para la homodimerización (KSR1 R615H) y se generó por mutagénesis dirigida una proteína KSR1 doble mutante, incompetente para la interacción con MEK y la homodimerización (KSR1 R615H/C809Y). La interacción entre estas construcciones y ERK activo se analizó, de forma directa, mediante ensayos de co-inmunoprecipitación y ensayos de ligación por proximidad (PLA) mediante microscopía de fluorescencia; o, de forma indirecta, mediante ensayos de activación de fosfolipasa A_2 citosólica por ERK dependiente de KSR1, a partir de cuantificación de ácido araquidónico tritiado liberado al medio extracelular.

Se generaron líneas estables de MEFs KSR1 -/- expresando las construcciones mutantes de KSR1 previamente mencionadas donde se analizó el efecto del silenciamiento de IQGAP1 sobre la activación de ERK dependiente de KSR y se valoró la capacidad de proliferación mediante contaje de células con la cámara de Neubauer.

La capacidad proliferativa de líneas representativas de melanoma BRAF o NRAS mutante tras el silenciamiento mediante siRNA contra las proteínas scaffold KSR1 e IQGAP1 fue evaluada a partir del análisis de la actividad metabólica a través de la técnica del Alamar Blue.

Con el fin de determinar la afinidad entre proteínas scaffold y ERK, se calcularon las constantes de disociación (Kd) a partir de la purificación de MP1, KSR1 (dominio de unión a ERK) e IQGAP1 (dominio WW de unión a ERK) fusionadas con GST con ERK2 generado *in vitro* marcado radioactivamente en metionina con ^{35}S.

4. RESULTADOS Y DISCUSIÓN

Observamos que el inhibidor de KSR APS-2-79 no mostró efectos en la supervivencia celular a pesar de bloquear la heterodimerización RAF-KSR, impidiendo así el cambio conformacional que esta interacción provoca en KSR exponiendo los sitios de activación de MEK unido a KSR. Con el objetivo de dilucidar si este resultado se correlaciona con la función de KSR, se silenció KSR en una línea de melanoma BRAF mutante y en otra NRAS mutante, y se observó que en esta última el silenciamiento tiene un claro efecto apoptótico, mientras que la inhibición de KSR no lo reflejaba. Por otro lado, se comparó el efecto del APS-2-79 con la depleción de KSR sobre los niveles totales de ERK activo en células HEK293T donde se advirtió que el efecto inhibitorio de la activación de ERK era significativamente mayor en respuesta a la depleción que a la inhibición de KSR. Asimismo, no se apreció disminución en los niveles de fosforilación de ERK dependiente de KSR originada por el inhibidor.

El hecho de que la inhibición de KSR por APS-2-79 y su depleción no tengan el mismo efecto sobre la activación de ERK y sobre sus respuestas biológicas nos llevó a analizar más en profundidad la regulación de ERK mediada por KSR. En el curso de estos experimentos observamos que una proteína KSR1 mutante incapaz de unir MEK (KSR1 C809Y) se comportaba de la misma manera que la proteína WT en cuanto a la activación de ERK y sus efectos apoptóticos en células de melanoma NRAS mutante (SKMEL2) y en células no tumorales (HEK293T). A consecuencia de este resultado inesperado, se observó que el mutante deficiente para la unión de MEK se comporta como el WT debido a que mantiene la capacidad de unirse a ERK activo. A este respecto, nuestra hipótesis de asociación entre scaffolds y/o la descrita homodimerización de KSR, y la consiguiente interacción entre sus quinasas, podría ofrecer una explicación, pero no era descartable que MEK libre en el

citoplasma pudiera ser el responsable de esta fosforilación. Por ello se generó un KSR1 doble mutante deficiente para la homodimerización y la interacción con MEK. En células HEK293T dicho mutante perdía su capacidad de unirse a ERK fosforilado, lo que invalidaba la posibilidad de que MEK libre fuera el causante de la activación de ERK unido a KSR. Con la finalidad de explicar este fenómeno, se descubrió que el scaffold IQGAP1 se unía a KSR. De esta manera, se vio que la sobreexpresión de IQGAP1 rescataba la activación de ERK mediada por el KSR doble mutante, lo que claramente apuntaba a la existencia de transactivación entre las quinasas unidas a scaffolds capaces de asociarse. Hemos denominado este nuevo mecanismo transfosforilación.

Hemos estudiado con más detalle la interacción entre estas dos proteínas scaffold, como resultado hemos delimitado la región C-terminal de IQGAP1 como la parte de la proteína capaz de unirse a KSR. Por su parte, en KSR1 el motivo de interacción con IQGAP1 está definido entre los residuos 402 y 521.

En relación a estos resultados, hemos visto que un mutante de IQGAP1 deficiente para unir MEK (IQGAP1 ΔIQ), al igual que KSR1 C809Y, mantiene la capacidad de incorporar ERK activo. Estos resultados apuntan a la posibilidad de que la transfosforilación sea recíproca entre scaffolds asociados, pero aún necesitamos experimentación adicional para confirmar que es KSR1 y no otro scaffold el responsable de esta transfosforilación entre diferentes scaffolds.

En cualquier caso, la cooperación entre scaffolds podría implicar beneficios en el flujo de la señalización de ERK en situaciones en las cuales la concentración de alguna de las quinasas de la ruta sea limitante. En este caso se favorecería la activación del pool de sustratos fosforilados por ERK unido a ese scaffold hacia el que muestre mayor afinidad. En este contexto, las diferencias en la afinidad por ERK mostradas por los distintos scaffolds tendrían un papel importante. Con este fin, se han analizado las constantes de disociación de ERK2 con las proteínas scaffold MP1, KSR1 e IQGAP1, siendo MP1 el de mayor e IQGAP1 el de menor afinidad. Estas diferencias en afinidad están reflejadas en la cinética de interacción con ERK en diferentes condiciones de estimulación.

En definitiva, considerando a) la capacidad de scaffolds para homodimerizar; b) la posibilidad de que distintos scaffolds heterodimericen y c) las distintas afinidades hacia ERK que muestran distintos tipos de scaffolds, que las señales de ERK se transmitan a través de un scaffold u otro dependería de: 1) la afinidad de homodimerización de cada scaffold; 2) la afinidad de heterodimerización entre distintos scaffolds y, por último, 3) de las constantes de disociación de cada scaffold hacia ERK.

5. CONCLUSIONS

- APS-2-79 es ineficaz como inhibidor de la oncogénesis inducida por la ruta RAS/ERK. Posiblemente como consecuencia de su incapacidad para interferir en la fosforilación de ERK dependiente de KSR.
- Las proteínas scaffold KSR1 e IQGAP1 deficientes para unir MEK pueden incorporar ERK fosforilado a través de transfosforilación.
- En el caso de KSR1, la transfosforilación es llevada a cabo, no por MEK libre, sino por MEK unido a la otra molécula de KSR que forma el homodímero o a una molécula de IQGAP1 formando un herodímero.
- KSR1 se une a IQGAP1. Tal interacción ocurre a través de la región CA4 de KSR1 y de la región C-terminal de IQGAP1, y es altamente dependiente de la estequiometría entre KSR1 e IQGAP1.
- La ineficiencia de APS-2-79 para inhibir la fosforilación de ERK unido a KSR es una consecuencia de su incapacidad para prevenir la transfosforilación, probablemente porque este inhibidor no puede interferir en la interacción entre KSR1 e IQGAP1.
- Las proteínas scaffold MP1, KSR1 e IQGAP1 exhiben diferentes afinidades por ERK. Estas diferencias dictan la cinética de interacción de ERK con los distintos scaffolds en respuesta a estimulación.

-

INDEX

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABBREVIATIONS

aa	Amino acid
ab	antibody
Ala (A)	Alanine
Amp	Ampicillin
ATP	Adenosine 5'-triphosphate
APS	Ammonium Persulfate
Arg (R)	Arginine
ASAP	Ala-Ser-Ala-Pro
ATP	Adenosine Triphosphate
bGH	Bovine growth hormone
BSA	Bovine Serum Albumin
BMK	Big MAPK
ºC	Degree Celsius
CA	Conserved Area
CC-SAM	coiled-coil fused to a Sterile □ motif
CD-domain	Common docking domain
CDK	Cycline dependent kinase
CHD	Calponin Homology Domain
CMGC group	CDKs, MAP kinases, GSKs and CDK-like kinases
CMV	Cytomegalovirus
CRD	cysteine-rich domain
C-terminal	Carboxy-terminal
cPLA$_2$	cytosolic Phospholipase A$_2$

Cpm	Counts per minute
CRM1	Chromosomal Maintenance 1
C-TAK1	cdc25C associated protein kinase
DBP	DEF binding pocket
ddw	distilled deionized water
DEF	Docking site for ERK FXF
DMEM	Dulbecco´s Modified Eagle Medium
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
dsDNA	double-stranded DNA
DTT	Dithiothreitol
DUSP	Dual specificity phosphatase
EBD	ERK binding domain
ECL	Enhanced Chemiluminescence
ED	Exchange docking site
EDTA	Ethylenediaminetetraacetic acid
EF-1α	Elongation factor-1 alpha
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene glicol tetraacetic acid
Elk-1	ETS Like-1 protein
EMA	European Medicines Agency
ERK	Extracellular signal-regulated kinase
FADH	Flavin Adenin Dinucleotide
FAK	Focal Adhesion Kinase

FDA	Food and Drug Administration
FGF	Fibroblast growth factor
g	Gram
GAB1	Grb2-associated-binder 1
GAP	GTPase-Activating Protein
GDP	Guanosine diphosphate
GEF	Guanine-Nucleotide-Exchange factors
Gly (G)	Glycine
Glu (E)	Glutamic acid
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
GRD	GAP Related Domain
GSK	Glycogen synthase kinase
GST	Glutatione S-transferase
GTP	Guanosine triphosphate
HCl	Hydrochloric Acid
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HGF	Hepatocyte growth factor
His (H)	Histidine
H-RAS	Harvey-Rat Sarcoma
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
H_2O_2	Hydrogen Peroxide
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinase

Kd	Dissociation constant
KO	Knock out
Ile (I)	Isoleucine
IQGAP	IQ motif containing GTPase-activating protein
IMP	Impedes Mitogenic signal Propagation
IPTG	Isopropyl β-D-thiogalactoside
K-RAS	Kirsten-Rat Sarcoma
Kan	Kanamycin
Kb	Kilo base
kDa	Kilo Dalton
KSR	Kinase suppressor of RAS
L	Litre
LB	Lysogeny broth medium
LMB	Leptomycin B
LPA	Lysophosphatidic acid
m	Milli
M	Molar
µ	Micro
MAPK	Mitogen activated protein kinase
MEK	Mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) kinase
MEFs	Mice Embryonic Fibroblasts
min	Minute
MKB	MAPK binding sequence
MKP	MAPK phosphatase
MORG1	MAPK organizer 1

MP1	MEK Partner 1
n	Nano
NADPH	Nicotinamide adenine dinucleotide phosphate
N-RAS	Neuroblastoma-Rat Sarcoma
NT	Non-transfected
N-terminal	Amino terminal
NES	Nuclear Export Signal
NLS	Nuclear Localization Sequence
NLK	Nemo-Like Kinase
NP40	Nonidet-40
O/N	Over-Night
P	Phosphate group
PAGE	Polyacrylamide gel electrophoresis
PAK1	p21 activated kinase
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDE4	Phosphodiesterase type 4
PDGF	Platelet derived growth factor
PEI	Polyethylenimine
Phe (F)	Phenilalanine
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein Kinase C
PLA	Proximity Ligation Assay
PLCε	Phospholipase C ε
PP	Protein phosphatase

PPMTase	Prenylated protein methtyl transferase
Pro (P)	Proline
PTP	Protein tyrosine phosphatase
RAC-1	Ras-related C3 botulinum toxin substrate 1
RAF	Rapidly accelerated fibrosarcoma
RAS	Rat sarcoma
RASSF1	Ras Association Domain Family Member 1
RBD	Ras Binding Domain
RGCT	Ras GAP C-terminus
RIN	Ras interaction/interference
ROCK	Rho-Rho-associated coiled coil-containing protein kinase
RSK	ribosomal s6 kinase
RTK	Receptor Tyrosine Kinase
rpm	Revolutions per minute
S	Sulfur
SAPK	Stress-Activated Protein Kinase
SDS	Sodium dodecyl sulfate
SEF	Similar expression to FGF protein
Ser (S)	Serine
SH-2	Src Homology 2
shRNA	Short hairpin RNA
siRNA	Small Interfering RNA
SOS	Son of sevenless
SRPK1	Serine/arginine protein kinase 1
S/T PP	Serine/Threonine protein phosphatase

STEP	Striatum enriched Phosphatase
SV40	Simian Virus 40
s	Second
TAD	Transactivation domain
TAE	Tris-acetate-EDTA
TBS-T	Tris Buffered Saline-Tween
TEMED	Tetramethylethylenediamine
Thr (T)	Threonine
TIAM1	T-cell lymphoma invasion and metastasis 1
TL	Total Lysates
TLK	Tyrosine-like Kinase
Tm	Temperature of melting
TPL2	Tumor progression locus 2
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
V	Volts
Val (V)	Valine
v/v	Volume to volume
WB	Western Blot
WT	Wild type
w/v	Weight to volumen

1. INTRODUCTION

1.1. SIGNAL TRANSDUCTION

Signal transduction is one of the main mechanisms whereby cell fate is regulated. On a general basis, and under physiological conditions, cells do not govern their functions autonomously, but mainly in response to stimuli generated by the organism, that will tell cells when to and for how long to undergo key processes such as: proliferation, differentiation, and apoptosis, among many other cell-type specific functions. These stimuli are mainly conveyed in the form of soluble proteins, known as growth factors that are secreted to the bloodstream and distributed throughout the organism. Growth factors are aimed at defined target cells, characterized by expressing membrane receptors, highly specific for the growth factors in question. These receptors are mostly of the tyrosine kinase and of the G-protein-coupled type. The binding of the ligands to their cognate membrane receptors triggers receptor activation and the subsequent switching-on of cytoplasmic signalling routes, through which information is relayed to the inside of the cell, where biochemical effectors and genetic programs are activated in order to execute the orders initially carried by the growth factors, in the form of biological responses (Dedrick Jordan, Landau and Iyengar, 2000). Activated membrane receptors convey their signals to the inside of the cells through multiple signal transduction pathways, mainly mediated both by lipidic secondary messengers and/or cascades involving protein phosphorylations and protein-protein interactions, the latter include the pathways mediated by Mitogen-Activated Protein Kinases (MAPKs hereafter).

1.2. PROTEIN KINASES

Approximately 90% of the proteins expressed in cultured human cells are phosphorylated (Sharma *et al.*, 2014). Furthermore, the sequencing of the human genome has unveiled that at least 2% of the known human genes encode for protein kinases. Today the human *kinome* is composed of 535 protein kinases which highlights the importance of protein phosphorylation in biochemical

processes *(Fig. 1.2)*. In fact, over 85% of the *kinome* has been reported to be dysregulated in diseases or developmental disorders (Manning *et al.*, 2002; Lahiry *et al.*, 2010; Creixell *et al.*, 2018; Wilson *et al.*, 2018). The phosphorylation status of a given protein results from the equilibrium between the addition and the removal of phosphate groups. Phosphate addition is carried out by protein kinases, these catalyze the reversible transfer of a phosphate group, generally obtained from the ATP γ phosphate, to the hydroxyl group of tyrosine, threonine or serine residues. The removal of phosphate groups is undertaken by protein phosphatases that, through dephosphorylation, cleave phosphate groups from previously phosphorylated residues *(Fig. 1.1)*. Phosphorylation is a critical process for the modulation of protein properties such as catalytic activity, protein stability, protein-protein interactions, subcellular localization, etc. (Hanks and Hunter, 1995; Johnson and Lapadat, 2002). In this way, aberrant phosphorylation as a consequence of the unregulated activity of either protein kinases or phosphatases imply, in most cases, physiological disorders and serious pathologies such as cancer, among many other maladies (Hanahan and Weinberg, 2000; Low and Zhang, 2016; Flores *et al.*, 2019).

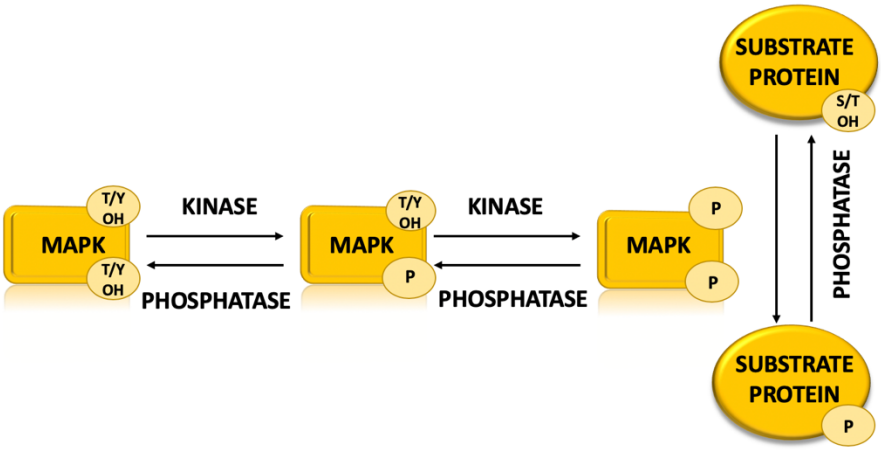


Figure 1.1. Schematic representation of the activation of a signalling route and the enzymatic reaction of substrates by reversible phosphorylation*. MAPKs are activated upon threonine (T) and tyrosine (Y) phosphorylation. Specific phosphatases are necessary for deactivation of the cascade by removing one or two phosphate groups (P) from the aforementioned residues. Once activated, MAPKs are able to phosphorylate serine (S) or threonine (T) residues in substrate proteins, that are returned to a basal state by phosphatases upon dephosphorylation.*

1.3. MAP KINASES

Signalling pathways mediated by MAPKs are among the best studied and well characterized. MAPKs are included within the CMGC group of serine-threonine kinases, which is composed of 9 highly-conserved families, including important kinases such as the Glycogen synthase 3 family; the cell cycle CDKs (cyclin dependent kinases) and other kinases involved in splicing and metabolic control such as SRPK1 (Colwill *et al.*, 1996; Jope and Johnson, 2004; Kannan and Neuwald, 2004) *(Fig. 1.2).*

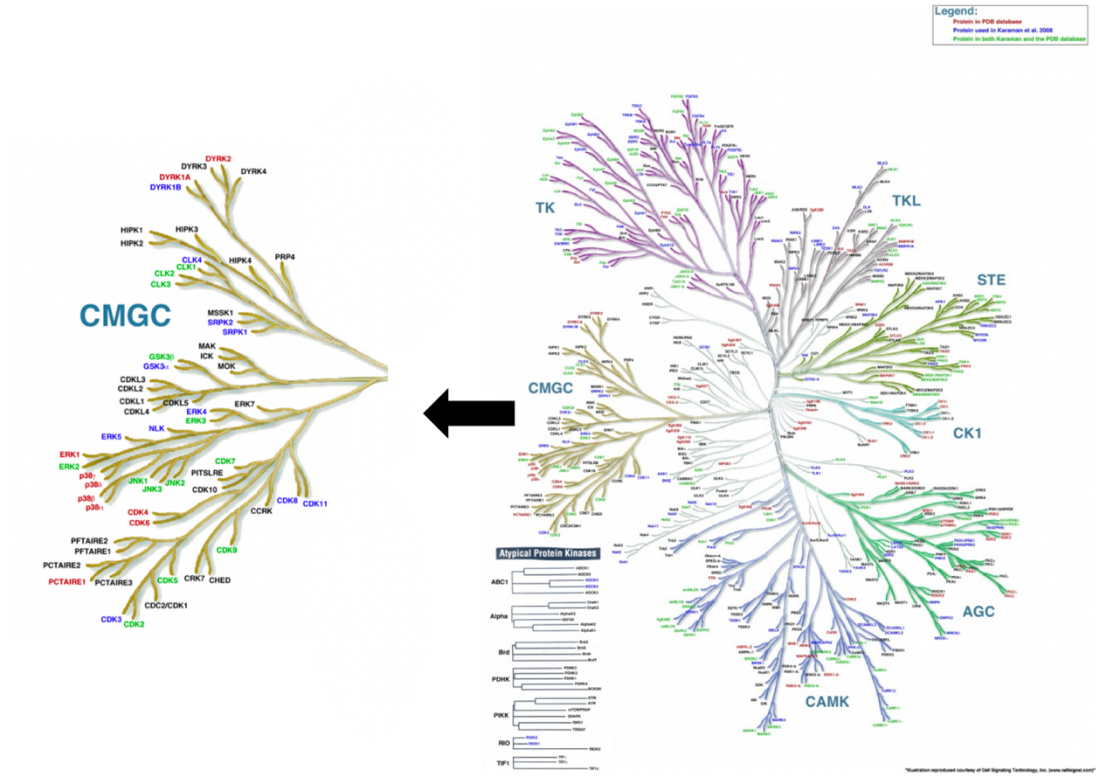


Figure 1.2. Phylogenetic tree of human protein kinases: Kinome. *Organization of the 535 human genes bearing a protein kinase domain into seven groups, plus a group composed of more divergent atypical protein kinases (down-left). The part of the dendrogram where CMGC group of serine-threonine kinases is located is amplified on the left. (Figure adapted from* (Chartier *et al.*, 2013)*).*

The original term MAPK has two origins: it comes from the ability to phosphorylate microtubule associated protein 2 (MAP-2 Protein Kinase), detected in Extracellularly-Regulated Kinase 2 (ERK2) which was the first MAPK identified (Ray and Sturgill, 1988); and also from the

finding that ERK2 was activated in response to stimulation by mitogenic growth factors (Mitogen-Activated Protein Kinase) (Rossomando *et al.*, 1989). As such, the name MAPKs was initially restricted to the ERK1/2 family. However, today the term has been extended to describe a whole family, comprising not only ERKs, but also a series of similar kinases discovered afterwards, such as: Stress-Activated Protein Kinases (SAPKs)/Jun-N-terminal Kinases (JNKs); p38 family kinases and ERK5/Big MAPKs (BMK) (Cargnello and Roux, 2011).

MAPKs constitute a protein family highly conserved throughout evolution, from fungi and plants to animals (Widmann *et al.*, 1999). Structurewise, the different MAPKs contain eleven subdomains in the catalytic region with a 30% identity. Thus, the structure and the catalytic properties of the proteins are very similar among the different families (Taylor *et al.*, 1993). To date, 20 different MAPKs have been identified in mammalians, encoded by 10 different genes. In addition, there are three atypical MAPKs: ERK3, ERK4 and ERK7 (English *et al.*, 1999; Coulombe and Meloche, 2007) *(Fig. 1.3)*.

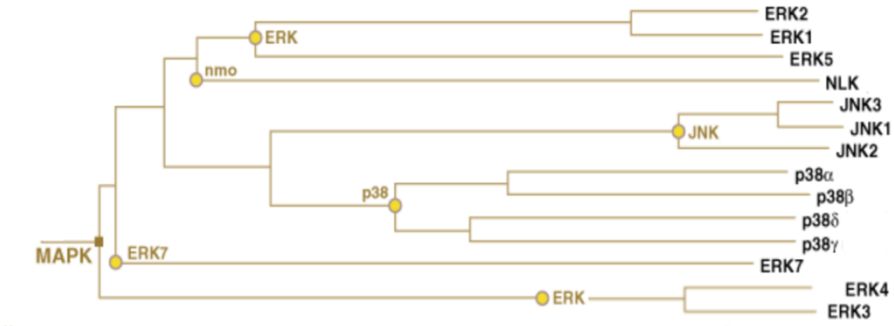


Figure 1.3. MAPKs family cladogram.

1.3.1. Identification and classification of MAPKs

As mentioned previously, there are four MAPKs families, even though they share many characteristics, they also harbour distinctive features.

1.3.1.1. Extracellular-Regulated Kinases (ERKs)

ERK1 was the first MAPK identified in mammalians, as a kinase activated in response to growth factors, one of the main characteristics of this subfamily (Ray and Sturgill, 1988). The identification of ERK2 by Cobb's group followed soon after (Boulton and Cobb, 1991). ERKs are activated by most mitogenic agonists including phorbol esters, cytokines and hormones. However, they are also switched-on by many other types of stimuli including differentiation and survival-promoting factors (Eblen, 2018). This family, in addition to p44 ERK1 and p42 ERK2, also includes the splicing isoforms ERK1b (Yung *et al.*, 2000), ERK1c (Aebersold *et al.*, 2004) and ERK2b (Gonzalez *et al.*, 1992). Like all MAPKs, ERKs are activated by the phosphorylation of a motif present in the activation loop of the protein. This motif is the TXY motif, where the X amino acid is specific for each of the MAPKs subfamilies. In the case of ERKs it corresponds to Thr-Glu-Tyr (TEY).

1.3.1.2. JNKs (c-Jun N-terminal Kinases)

Also known as SAPKs (Stress Activated Protein Kinases) since they are activated in response to signals generated by environmental and oxidative stress. They are also activated by cytokines and to a lesser extent by mitogenic stimuli. The proteins encoded by the JNK1, JNK2 and JNK3 genes belong to this family. This include over ten isoforms per gen resulting from alternative splicing (Kyriakis and Avruch, 2001). Their phosphorylation motif is Thr-Pro-Tyr (TPY). JNKs play major roles in inflammatory processes (Zeke *et al.*, 2016).

1.3.1.3. p38 MAPK

Also known as CSBP, mHOG1, RK and SAPK2. Like JNKs this family is also activated in response to stress stimuli such as osmotic and thermal shock (Han *et al.*, 1994; Rouse *et al.*, 1994). This subfamily includes four isoforms encoded by four different genes: p38α (Han *et al.*, 1993; Freshney *et al.*, 1994), p38β (Jiang *et al.*, 1996), p38γ (Li *et al.*, 1996), p38δ (Jiang *et al.*, 1997). In addition, there are isoforms generated by alternative splicing such as CSBP1 (Lee *et al.*, 1994), Exip (Sudo *et*

al., 2002) and Mxi2 (Zervos *et al.*, 1995) derived from p38α. Its phosphorylation motif is Thr-Gly-Tyr (TGY). Physiologically they are mainly activated by inflammatory cytokines, and therefore play major roles in inflammatory processes. They are also deeply involved in the regulation of cell survival and apoptosis and in the control of immune responses. As such, they have an important role in human maladies such as asthma and autoimmune diseases (Johnson and Lapadat, 2002). The majority of stimuli which activate p38 also activate JNKs.

1.3.1.4. ERK5/BMK1 (Big Mitogen-Activated Protein Kinase 1)

This MAPK was discovered in 1995 (Lee, Ulevitch and Han, 1995). Its main distinctive feature is its size, whereas most MAPKs have a molecular weight around 40-50 KDa, BMK1 is 115 KDa due to its unique C-terminus. There are 3 isoforms generated by alternative splicing named ERK5a, ERK5b and ERK5c (Yan *et al.*, 2001) and it is mainly activated by mitogens and cellular stress (Kamakura, Moriguchi and Nishida, 1999). Like ERK1 and 2 its phosphorylation motif is Thr-Glu-Tyr (TEY), however unlike ERK1 and 2 its phosphorylation is no necessary for its nuclear translocation because in its C-terminus it harbours a nuclear localization signal (Buschbeck and Ullrich, 2005).

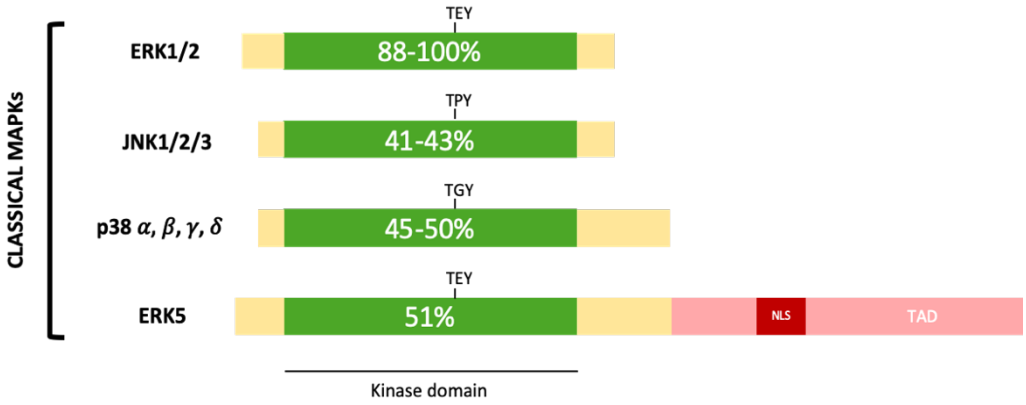


Figure 1.4. Schematic representation of the classical human MAPKs. *MAPKs contain a Ser/Thr kinase domain (in green) flanked by the N- and C-terminal regions of different lengths. The phosphorylation motif is represented within the kinase domain by the amino acid abbreviation, as well as the homology percentage in regard to ERK1. Different additional domains are also present in ERK5, including a transactivation domain (TAD) and a nuclear localization sequence (NLS). (Figure adapted from* (Cargnello and Roux, 2011)*).*

In addition to the above mentioned classical MAPKs, other MAPKs, referred to as atypical, have been described, such is the case for ERK4-3, NLK (Nemo-Like Kinase) and ERK7/8 (Coulombe and Meloche, 2007).

1.3.2. MAPKs cascades

Signal transduction pathways that utilize MAPKs are highly conserved across evolution. Indeed, the assembly of the first MAPK pathway was unravelled in yeast. In this organism MAPK cascades regulate key biological processes such as mating, budding, cell wall biosynthesis and osmoregulation (Pearson *et al.*, 2001). The main difference between MAPKs pathways in yeast and in mammalian cells is that in yeast the response to specific stimuli is undertaken by a unique MAPK route, whereas in mammalians a given stimulus can activate multiple MAPKs simultaneously (Raman and Cobb, 2003).

All MAPKs are activated by a cascade of phosphorylation events that occur in sequential tiers and involve different types of upstream-activating kinases. The first tier is composed of Mitogen-Activated Protein Kinase Kinase Kinases (MAPKKKs), these are in general serine/threonine kinases. In turn, MAPKKKs phosphorylate and activate Mitogen-Activated Protein Kinase Kinases (MAPKKs), in all cases MAPKKs are dual-specificity protein kinases capable of phosphorylating their substrates in tyrosine and threonine residues. MAPKKs are the direct activators of MAPKs which are the last tier of the cascade. All MAPKs pathways are activated at their origin by small GTPases of the Ras superfamily (Chartier *et al.*, 2013).

The MAPKKK also named MEKK or MKKK are the first echelon of the MAPKs signalling cascades, these are serine/threonine kinases generally activated by phosphorylation, in response to the activation of small GTPases such as RAS, in the case of ERK1, 2 and 5, and Rho GTPases in the case of JNKs and p38 (Gutkind, 1998). Their activation is generally triggered by agonist stimulation of membrane receptors, even though, they can also be activated by internal stimuli such as

oxidative stress, DNA damage, etc (Chen and Thorner, 2007). There is a great structural diversity among MAPKKKs. Such variability is generally a consequence of the presence of different regulatory domains, which dictate the response of each MAPKKK to specific stimuli. The main MAPKKKs families are shown in figure 1.5. MAPKKKs are the direct upstream activators of MAPKKs (Raman and Cobb, 2003).

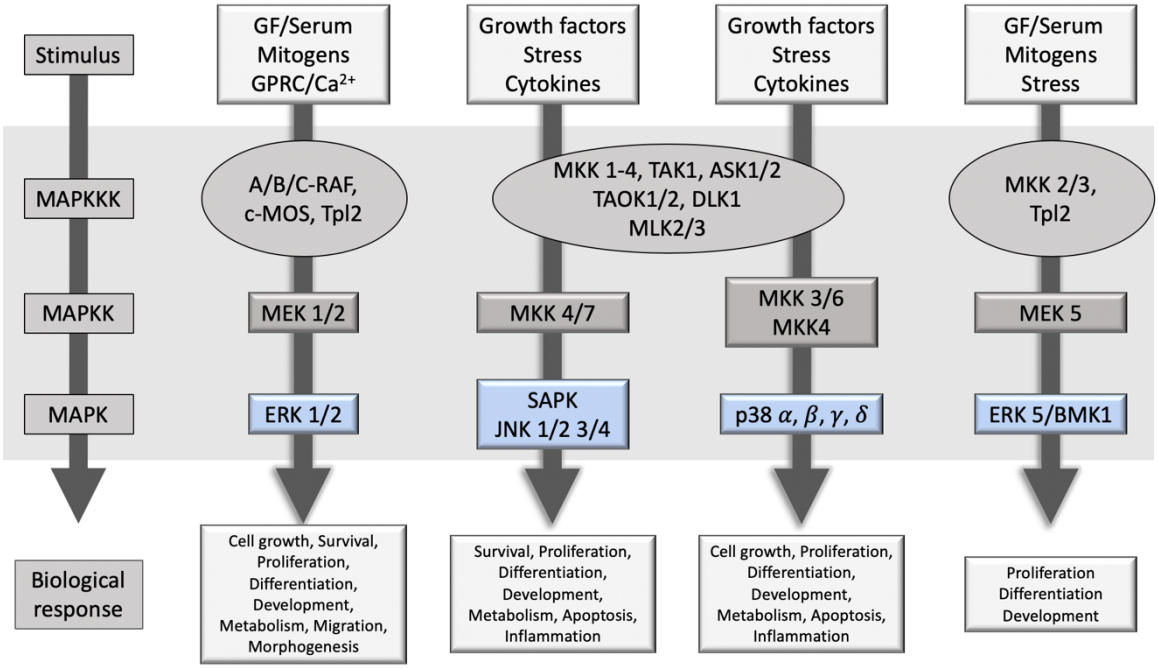


Figure 1.5. A simplified and general scheme of the four classical MAPK three-tiered signalling pathways. *The first tier represents the specific stimuli; ERK1/2, JNK, p38 and ERK5 MAPKs multi-enzyme cascades shown are described in the text and the last tier illustrate the biological outcomes of each pathway. GF=Growth factor.*

MAPKKs include MEKs and MKKs, and make up the next step of the cascades. MAPKKs are enzymes with dual specificity as they can phosphorylate threonine and tyrosine residues. In spite of their ability to phosphorylate the lateral chains of aliphatic and aromatic amino acids, they exhibit a high substrate specificity and the number of their substrates is very limited. In such a way that, each MAPKK can phosphorylate only one or just a few MAPKs. MAPKKs specificity for MAPKs is determined by the phosphorylation motif, present in each MAPK family either TEY, TGY or TPY. Up to date, only 7 MAPKKs have been described, not including some alternative splicing isoforms: MEK1 and 2 or MKK1/2 are those responsible for activating ERK1 and 2; MKK3 and MKK6 activate p38 (Raingeaud *et al.*, 1996), whereas MKK4 is capable of phosphorylating both p38 and JNKs

(Dérijard *et al.*, 1995). MKK7 is the upstream activator for JNKs (Fleming *et al.*, 2000) and, finally, MKK5 is highly specific for ERK5 (Zhou, Bao and Dixon, 1995).

As already mentioned MAPKs are activated as a consequence of the conformational changes following the phosphorylation of the TXY motif in the activation loop. This double phosphorylation is essential to unleash their enzymatic activity (Robbins *et al.*, 1993). MAPKs possess a highly characteristic tridimensional structure, they are globular proteins characterized by two lobes, a small N-terminal lobe (N-lobe) rich in beta-sheets and two alfa helices; and a C-terminal lobe mainly made up of α-helices, the active site is located between both lobes (Goldsmith and Cobb, 1994). The N-terminal lobe creates a pocket structure where the contacts with the ATP adenine ring take place. The C-terminal lobe includes the catalytic region typical of kinases and it comprises the magnesium binding site and the phosphorylation motif within the activation loop. Phosphorylation of the TXY motif causes a conformational reorganization of the loop which allows binding to the phospho-acceptor region of the substrates generating a functional catalytic center (Huse and Kuriyan, 2002) *(Fig. 1.6).* MAPKs belong to the functional family of proline-directed kinases. As such they phosphorylate serine or threonine residues immediately followed or preceded by a proline (P+1/-1) (Chang and Karin, 2001).

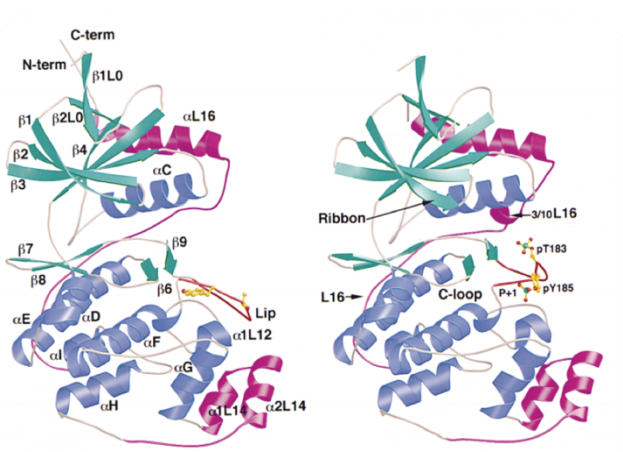


Figure 1.6. ERK2 structure in its non-phosphorylated and inactive state (left) and its phosphorylated and active state (right). *ERK2 is bi-phosphorylated on a threonine (pTyr-185) and a tyrosine (pTyr-183) residue within the phosphorylation lip (red). This phosphorylation provokes a conformational change in the lip and neighbouring structures (Figure obtained from* (Canagarajah *et al.*, 1997)*).*

1.4. THE ERK CASCADE: THE RAS/ERK PATHWAY.

Undoubtedly one of the best characterized signalling pathways is the one that connects RAS stimulation to the switching-on of the cascade which brings about ERK activation *(Fig. 1.7)*. This route is central in key biological processes such as the regulation of cellular proliferation, differentiation, survival, migration and many cell-type specific functions, in addition to multiple developmental processes. Furthermore, the unregulated function of this signalling route is involved in multiple developmental alterations and serious adulthood pathologies including cancer (Kim and Choi, 2010, 2015).

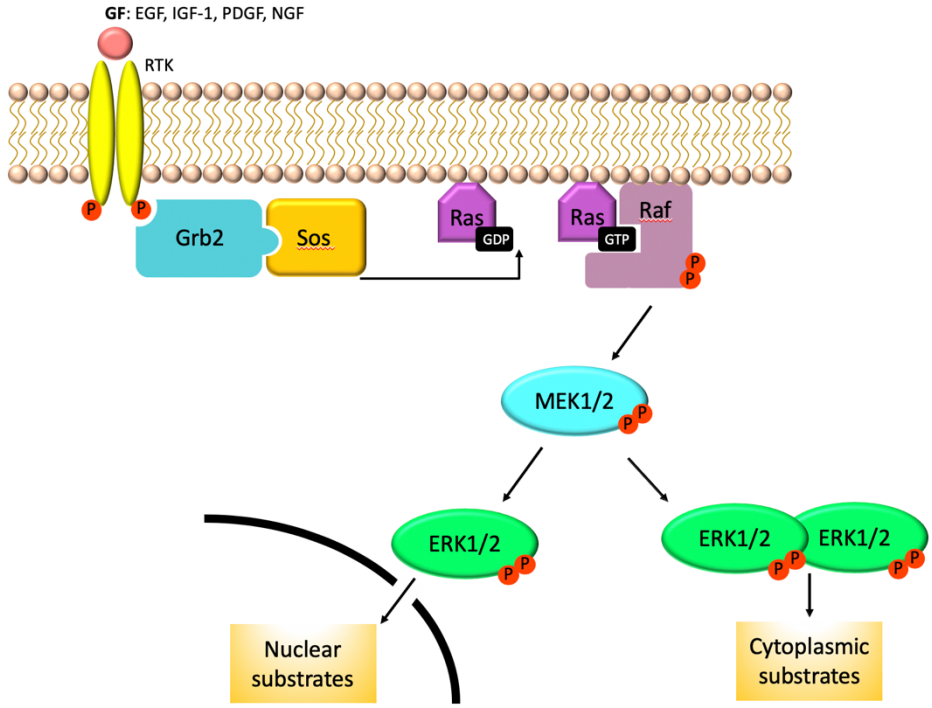


Figure 1.7. RAS/ERK pathway activation by sequential phosphorylations*. Upon activation by ligand binding, receptor tyrosine kinase autophosphorylates at multiple tyrosine residues on its cytoplasmic domain. This brings about the recruitment of the SH2 domain containing adaptor protein Grb-2, which recruits the guanine nucleotide exchange factor (GEF) SOS. SOS provokes the release of GDP from Ras which subsequently binds to GTP (active state). GTP-RAS recruits RAF to the membrane where it becomes activated. RAF phosphorylates two serine residues in the activation loop of MEK1/2. MEK is a dual specificity kinase that phosphorylates ERK on both threonine and tyrosine residues in the TEY motif of its activation loop. Activated ERK can phosphorylate a huge number of substrates at the cytoplasm in its dimeric form or it can translocate into the nucleus as a monomer where it activates transcription factors among other nuclear substrates. P=phosphate.*

The RAS/ERK pathway is generally switched-on by external agonists acting on membrane receptors, usually of the tyrosine kinase type (Hubbard and Miller, 2007; Lemmon and Schlessinger, 2010), though not excluding G protein-coupled receptors and cytokine receptors (Gutkind, 1998; Jain *et al.*, 2018; Watson *et al.*, 2018). Engagement of the membrane receptors brings about the activation of Guanine nucleotides Exchange Factors (GEFs) by different mechanisms, depending on the receptor type (Zhang *et al.*, 2005). GEFs acting on RAS are generally those of the SOS (Son Of Sevenless) and RasGRF families. In the first case, they are mainly activated by tyrosine kinase receptors, whereas, the latter usually respond to the activation of G protein-coupled receptors (Mckay and Morrison, 2007; Fernández-Medarde and Santos, 2011; Rojas, Oliva and Santos, 2011).

GEFs catalyze nucleotide exchange on RAS family GTPases (Stanley and Thomas, 2016). Under resting conditions, RAS is bound to GDP, GEFs promote the expulsion of GDP and its exchange for GTP, that is more abundant in the cytoplasm. GTP binding induces a structural change on RAS to its active conformation, in which it can interact with its effector molecules. To date, seven *bona fide* RAS effector have been described including: PI3K, RalGDS, RIN, TIAM-1, PLCε, RASSF1 and RAF family kinases, ARAF, BRAF and CRAF (Marshall, 1996; Mott and Owen, 2015; Nakhaeizadeh *et al.*, 2016). RAF kinases are the MAPKKKs that will initiate the activation of the ERK cascade. The exact mechanism whereby RAF family kinases are activated is still unclear, though it is known that it requires the interaction with GTP-bound Ras at the plasma membrane (Nussinov, Tsai and Jang, 2019); the phosphorylation of tyrosine residues by SRC family tyrosine kinases (Bunda *et al.*, 2014) and the interaction with HSP90 chaperones and 14-3-3 scaffold proteins (Fischer *et al.*, 2008; Mitra *et al.*, 2016). RAF activity is also subject to regulation by negative feedback loops generated via MEK- and ERK-mediated phosphorylation (Chong, Lee and Guan, 2001; Lake, Corrêa and Müller, 2016).

Upon activation, RAF kinases will phosphorylate MAPKK of the MEK (MAPK ERK Kinase) family, composed of two members with high homology, MEK1 and MEK2 (Kocieniewski and Lipniacki, 2013). MEKs are activated by serine phosphorylation (S218 and S222 in MEK1) within their

activation loop that contains the motif S-X-A-X-S/T typical of all MAPKKs. Apart from RAF, MEK can also be phosphorylated by TPL-2, MOS and MEKK1 in addition to p21 PAK1 (Salmeron *et al.*, 1996; Hagemann, Troppmair and Rapp, 1999; Pearson *et al.*, 2001). As such all of these kinases can also be considered MAPKKKs. In a similar fashion to RAF, MEK can also be regulated by negative feedback loops. Specifically, by the retro-phosphorylation in Threonine 292 by ERKs (Eblen *et al.*, 2004). MEK phosphorylation unleashes its dual specificity kinase activity exerted on its hitherto only known substrates ERK1 and 2 (Liu *et al.*, 2018).

In addition to its role as a direct ERK1/2 upstream activator, MEK1 also functions as an ERKs cytoplasmic anchor (Fukuda, Gotoh and Nishida, 1997). Under resting conditions, ERKs are located in the cytoplasm bound to several anchoring proteins, including MEK1 (Lidke *et al.*, 2010; Whitmarsh, 2011). Upon phosphorylation, MEK-ERK interaction is broken and ERKs are free to diffuse to multiple cellular locations (Caunt and McArdle, 2010). Furthermore, upon activation, MEK1 translocates to the nucleus, but it is rapidly exported to the cytoplasm guided by the nuclear export signal (NES) located in its N-terminus, and in so doing, it takes ERK back to the cytoplasm (Fukuda, Gotoh and Nishida, 1997; Adachi, Fukuda and Nishida, 2000). This mechanism requires the participation of shuttling proteins such as CRM-1 that is inhibited by leptomycin B (LMB) whose treatment prevents ERK nucleo-cytoplasmic shuttling (Fukuda *et al.*, 1997; Kudo *et al.*, 1998).

1.4.1. ERK activity

As mentioned before, ERK1 and 2 are the only known MEK substrates. ERKs appear throughout the evolutionary scale, originating as a primitive ERK that yields ERK1 and ERK2 by gene duplication, with the advent of amniote (Li, Liu and Zhang, 2011; Buscà *et al.*, 2015). In mammalians ERK1 and ERK2 are ubiquitously expressed and share a 90% homology in their amino acid sequence (Boulton *et al.*, 1991). In agreement with their high homology, they appear to be redundant in their functions (Lloyd, 2006; Lefloch, Pouysségur and Lenormand, 2008; Voisin *et al.*, 2010; Saba-El-Leil, Frémin and Meloche, 2016). ERK1 knock-out mice are perfectly viable (Pagès *et al.*, 1999; Mazzucchelli *et*

al., 2002; Nekrasova *et al.*, 2005), whereas ERK2-defective mice are embryonic lethal (Hatano *et al.*, 2003; Saba-El-Leil *et al.*, 2003). However, it has been recently shown that overexpression of ERK1 can overcome ERK2 deficiency during development, indicating that both proteins share similar functions (Frémin *et al.*, 2015).

ERK phosphorylation by MEK entails different regions within ERK, both in its N- and C-terminus, such as the regions spamming amino acids 19-25 and 320-321 (Rubinfeld, Hanoch and Seger, 1999; Eblen *et al.*, 2001) and also require the interaction between MEK D-domain and ERK CD-domain which induces a conformational change on ERK structure exposing the canonical sites Thr183 and Tyr185 (in ERK2) for phosphorylation. Phosphorylation by MEK enhance ERK kinase activity by 5 or 6 orders of magnitude above its basal activity (Zhang *et al.*, 1994). Upon phosphorylation, the G-helix in the C-lobe is involved in the formation of a pocket structure surrounded by hydrophobic residues that constitute the region that recognizes the proline in the substrates, enabling their phosphorylation (Clark-Lewis, Sanghera and Pelech, 1991).

Upon phosphorylation ERKs dimerize. These are ERK1 or ERK2 homodimers, heterodimers are unstable. Even though ERK can also dimerize when unphosphorylated, its Kd is 3,000 times below the value corresponding to the dimer formed by phosphorylated ERK (Khokhlatchev *et al.*, 1998). The role of ERK dimerization is unclear, it has been proposed that it is relevant for the sustainability of ERK activity levels (Philipova and Whitaker, 2005) and it was initially believed to play a role on nuclear translocation (Khokhlatchev *et al.*, 1998). However, previous data from our laboratory demonstrated that ERK dimers are formed, and most remain, at the cytoplasm associated to scaffold proteins (Casar, Pinto and Crespo, 2008). In such a way that scaffold proteins serve as ERK dimerization platforms where ERK dimers are assembled, forming complexes competent for phosphorylating ERK cytoplasmic substrates. Contrarily to the cytoplasm, phosphorylated ERK enters the nucleus mainly as monomers (Casar, Pinto and Crespo, 2009).

Phosphorylated ERK will distribute throughout the cell. As previously mentioned, about 50% of the ERK molecules will remain at the cytoplasm, in dimeric form, and a similar fraction will translocate to the nucleus as monomers. The mechanism whereby ERKs translocate to the nucleus is not yet fully understood and, apparently, it can take place by different modes. It must be noticed that ERK does not possess a nuclear localization signal (NLS) (Maik-Rachline, Hacohen-Lev-Ran and Seger, 2019). ERK can diffuse freely and energy-independently into the nucleus (Yazicioglu *et al.*, 2007). In this respect, ERK has been shown to directly bind to the FXFG sequences of nucleoporins, at the nuclear pore complex (Whitehurst *et al.*, 2002). However, the main mechanism whereby activated ERK enters the nucleus requires the participation of nuclear shuttles, such as Importin 7. In *Drosophila*, this nuclear shuttle has been shown to interact with ERK and transport it to the nucleus (Lorenzen *et al.*, 2001). Another nuclear shuttle is the p38α isoform, Mxi2, capable of binding to ERK and promoting its nuclear accumulation, by a mechanism that requires direct interaction between both proteins and also Mxi2 binding to nucleoporins (Casar *et al.*, 2007). The duration of ERK residence at the nucleus is a critical factor for its biological activity and it depends, both, on its interaction with nuclear anchors, such as the phosphatase DUSP5 (Mandl, Slack and Keyse, 2005; Rushworth *et al.*, 2014), and on its binding to proteins that will mediate in its translocation back to the cytoplasm, such as MEK (Adachi, Fukuda and Nishida, 2000; Michailovici *et al.*, 2014; Maik-Rachline, Hacohen-Lev-Ran and Seger, 2019).

1.4.2. ERK substrates

Up to date, 500 proteins have been described as substrates subject to ERK phosphorylation (Yang *et al.*, 2019; Smorodinsky-Atias, Soudah and Engelberg, 2020). About half of these are nuclear proteins and the rest are distributed through extranuclear regions. Many types of proteins have been identified as ERK substrates, including: membrane receptors, signalling molecules, kinases, metabolic enzymes, transcriptions factors, DNA binding proteins, structural components, etc. (Yoon and Seger, 2006). As previously mentioned, all ERK substrates are phosphorylated in the motif Ser/Pro P+/-1, however, the presence of such motif does not necessary imply that it will be

phosphorylated by ERK. What defines an ERK-interacting protein, including substrates, is the presence of defined docking domains:

-D domain: it is present in the vast majority of ERK-interacting proteins, including substrates and other types of proteins such as: regulatory proteins and its activator MEK. This domain is composed of two or more basic amino acids followed by a series of hydrophobic residues (Leu, Iso or Val) [(K/R)2-3-X1-6-ϕL,I,V-X-ϕL,I,V] (Kallunki *et al.*, 1994). This domain is not specific for ERK as it also serves as a docking site for other MAPKs such as p38 and JNK. It was initially described in the c-Jun transcription factor and was lately identified in many other transcription factors, phosphatases and scaffold proteins (Sharrocks, Yang and Galanis, 2000). It provides a high affinity binding site for ERK and other MAPKs. D domains interact with ERK CD domain, a region rich in acidic and hydrophobic residues of which D316 and D319 are critical (Tanoue *et al.*, 2000; Lee *et al.*, 2004), in such a way that binding of the D domain to the CD domain involves both electrostatic and hydrophobic interactions.

-DEF domain (Docking site for ERK; FXFP): It comprises the sequence Phe-X-Phe-Pro. This sequence is generally found close to the phosphorylation site, including residues 185 to 261 next to the activation loop. Unlike the CD-domain, it is not present in all ERK substrates, suggesting that it serves as a high specificity binding motif (Sharrocks, Yang and Galanis, 2000; Fantz *et al.*, 2001). The DEF domain binds to the DEF motif Binding Pocket (DBP) present in ERK. The DBP is found in the hydrophobic cleft known as the insert region, that is only accessible when ERK is phosphorylated. DEF domains have been identified in transcription factors (c-Fos and ELK-1), scaffold proteins (KSR) and phosphatases (MKP1 and DUSP4) (Jacobs *et al.*, 1999). Even though it is preferentially utilized by ERK, under some circumstances other proteins can also bind to this motif. There are some ERK substrates which harbour both docking domains, such is the case for the transcription factor ELK-1 (Yang *et al.*, 1998; Fantz *et al.*, 2001).

Since they are mostly mediated by the same types of binding motifs, ERKs interactions with substrates, upstream activators and regulatory proteins are generally mutually exclusive. As such, a mechanism of action has been proposed, according to which ERK activation by MEK will occur in the first place, after which substrate phosphorylation will ensue and ERK inactivation by phosphatases will follow (Tanoue *et al.*, 2001).

1.4.3. ERK regulation

It is nothing but logical that a protein playing such important roles in signal transduction is subject to a tight regulation, both in its activation and its inactivation. Indeed, both the duration and the amplitude of ERK-mediated signals are the resultant of the equilibrium reached between the activating and inactivating processes.

1.4.3.1. ERK regulation by phosphatases

ERK inactivation takes place mainly by the cleavage of the phosphate group in one or both of its phosphorylated residues. This is undertaken by protein phosphatases that can be classified according to their ability to dephosphorylate such residues (Yao and Seger, 2005). These include:

Ser/Thr phosphatases (PPs): these are protein phosphatases with the ability to eliminate the phosphate group from the threonine in the TEY motif. The main phosphatases involved in ERK dephosphorylation belonging to this class are the PP1A and PP1B family phosphatases. These phosphatases can dephosphorylate ERK, both, in the nucleus and the cytoplasm. They are highly promiscuous and undertake the dephosphorylation of multiple proteins (Alessi *et al.*, 1995; Sun and Wang, 2012).

Tyr phosphatases (PTPs): these are enzymes capable of removing the phosphate group from the tyrosine in the TEY motif. Most of these belong to the PTP-SL (Step like PTP), STEP (striatum enriched phosphatase) and HePTP (hematopoietic PTP) families. Most of these are membrane

bound phosphatases and they mainly participate in cytoplasmic processes (Pulido, Zúñiga and Ullrich, 1998; Toledano-Katchalski *et al.*, 2003).

Dual Specificity phosphatases (DUSPs): also named MKPs (MAPK phosphatases). These are protein phosphatases with the ability to dephosphorylate both, threonine and tyrosine residues in their substrates. They are highly specific for MAPKs, this specificity is dictated by their N-terminus, where they harbour a sequence of hydrophobic residues named MKB (MAPK binding) which mediates in the specific interaction with MAPKs *(Fig. 1.8)* (Theodosiou and Ashworth, 2002). Some of them like MKP3 bind to ERK under resting conditions, functioning as a cytoplasmic anchor. Their activity can be induced, both by growth and stress signals. MKPs are the main inactivators of MAPKs and they act both at the cytoplasm and the nucleus. Some MKPs display a high specificity for a given MAPK family, whereas others are more promiscuous. For example, MKP1 can dephosphorylate ERK, JNKs and p38, whereas MKP3 is highly specific for ERK (Sun *et al.*, 1993).

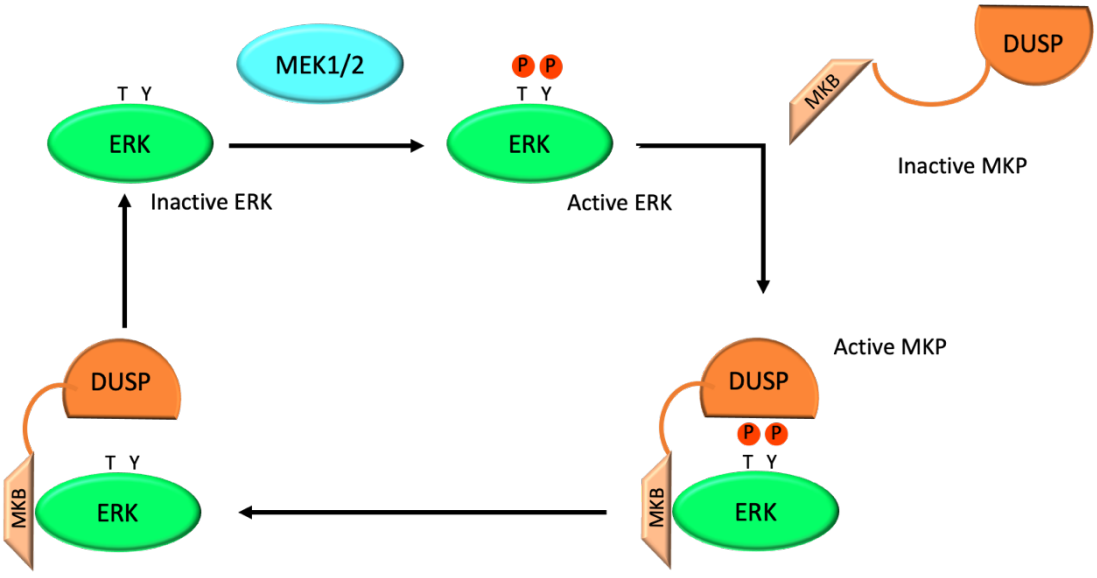


Figure 1.8. Dual Specificity phosphatases (DUSPs) mechanism. *These phosphatases are able to dephosphorylate both, threonine and tyrosine residues in their substrates. In their N-terminus they possess the MAPK binding (MKB) sequence which binds to the MAPK substrate and the C-terminus dephosphorylates the Threonine (T) and Tyrosine (Y) residues*

1.4.3.2. ERK regulation by feedback loops

Positive and negative feedback loops are among the complex molecular mechanisms whereby ERK signals are fine-tuned (Shin *et al.*, 2009). Once phosphorylated, ERK is able to feed back on its own activation pathway at different levels, both inducing (positive feedback loops) or inhibiting (negative feedback loops) *(Fig. 1.9)* the signal flux which ultimately shapes the duration and the intensity of its own signals and, as a consequence, its biological outcomes (Kolch, 2005), modulated up or down by the feedback loops.

Among the ERK negative feedback loops that restrain signal flux and restore the basal activation state of the pathway, are those that target RAF, MEK and SOS.

An important negative feedback loop is aimed at the guanine nucleotide exchange factor SOS, which is responsible of RAS activation. SOS phosphorylation by ERK at residues S1132, S1167, S1178 and S1193 prevents its interaction with Grb-2, blocking SOS translocation to the plasma membrane (Chen *et al.*, 1996; Corbalan-Garcia *et al.*, 1996). Also phosphorylation at S1134 and S1161 is undertaken by serine/threonine kinase RSK2, whose activation at the cytoplasm is ERK-dependent (Porfiri and McCormick, 1996; Shin *et al.*, 2009; Kamioka *et al.*, 2010). These phosphorylations generate a binding site for 14-3-3 proteins localizing SOS at the cytoplasm and preventing its interaction with RAS (Douville and Downward, 1997; Saha *et al.*, 2012).

In the case of RAF, ERK phosphorylates it at six sites: S29 and S43 in its N-terminus, S642 at its C-terminus and S289, S296 and S301 at the flexible hinge between the regulatory and catalytic domains. This hyperphosphorylation impairs RAF interaction with RAS and enhances RAF dephosphorylation by the phosphatase PP2A (Dougherty *et al.*, 2005; Hekman *et al.*, 2005).

Regarding the negative feedback loop exerted over MEK, activated ERK phosphorylates MEK at Thr292 (Eblen *et al.*, 2004) and Thr212 (Sundberg-Smith *et al.*, 2005). The phosphorylation at T292 impedes MEK activation by PAK1 essential to enhance MEK-CRAF interaction, as a consequence RAF-induced phosphorylation of MEK at its activating sites S218 and S222 is

attenuated (Slack-Davis *et al.*, 2003) leading to a drop on MEK activity that, ultimately, decreases ERK activation.

An alternative, indirect negative feedback system is the ERK-mediated transcriptional upregulation of phosphatases such as DUSP1, DUSP2, DUSP5 and DUSP9 (Owens and Keyse, 2007). In addition, ERK phosphorylates DUSP1 stabilizing the protein and increasing its half-life (Brondello *et al.*, 1997; Brondello, Pouysségur and McKenzie, 1999). The functional difference between these two processes, is that whereas posttranslational modifications, such as phosphorylation, have an immediate effect, the consequences of *de novo* synthesis of phosphatases are felt in the long run.

The main positive feedback loops mediated by ERK impinge on RAF and DUSP6. Just as ERK can inhibit RAF by multiple phosphorylations, some of these phosphorylation sites such as S289, S296 and S302 can have an enhancing effect on RAF activity (Balan *et al.*, 2006). Likewise, ERK-induced phosphorylation of the cytosolic phosphatase DUSP6 (MKP3) at S159 and S197 targets

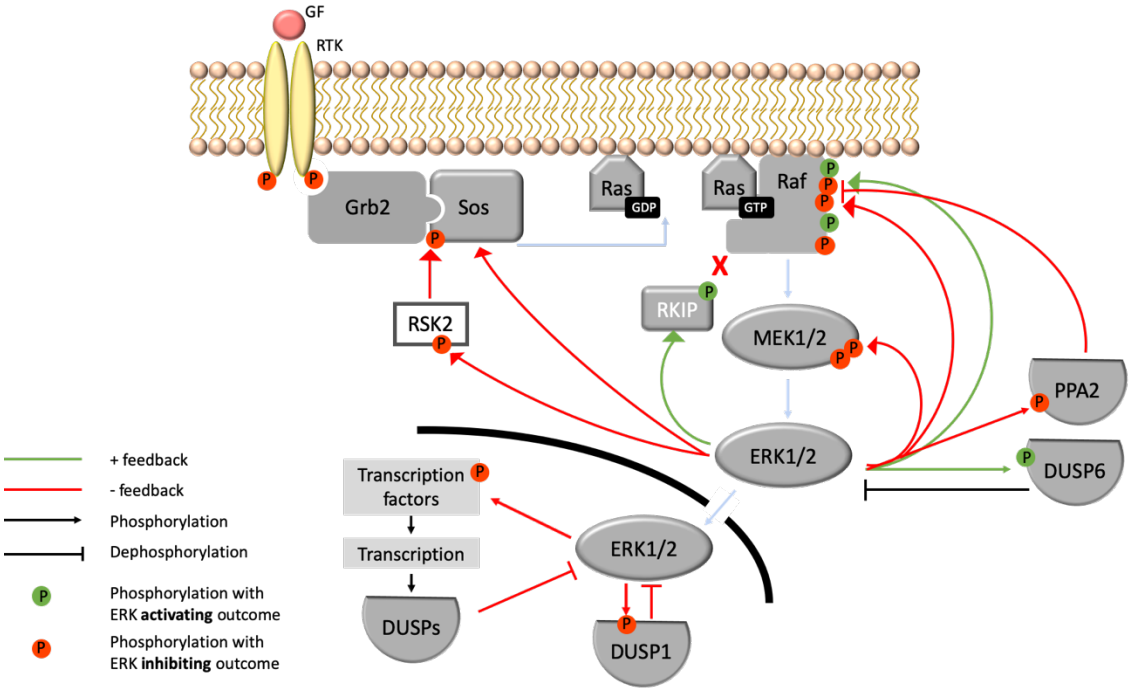


Figure 1.9. The Ras/ERK pathway feedback regulation mechanisms. *Activated ERK triggers positive and negative regulatory feedback loops: the main negative feedback loops act on SOS, RAF and MEK, while the major positive feedback loops inactivate DUSP6 and RKIP and activate RAF.*

DUSP6 for degradation in the proteasome (Marchetti *et al.*, 2005), thereby retarding ERK dephosphorylation. Finally, another positive feedback loop is directed at RKIP, which is a negative regulator of the interaction between MEK1 and CRAF. ERK phosphorylation inhibits RKIP kinase activity, and by that means its inhibitory function is prevented and the signal flux along the pathway is increased (Yeung *et al.*, 1999, 2000; Shin *et al.*, 2009).

1.5. SCAFFOLD PROTEINS

It is striking that the RAS/ERK pathway is activated in response to multiple stimuli to generate a broad variety of biochemical and biological responses, something difficult to reconcile with a pathway initially envisioned as a linear signalling route. Thus, it is conceivable that additional levels of regulation could confer variability to ERK signals. ERKs signal output is not shaped exclusively as a consequence of the phosphorylation events that take place within the different echelons of the pathway. In addition to the kinases included in the different tiers, and the phosphorylation-mediated regulatory mechanisms described above, past evidence has discovered the participation of other types of regulatory proteins that add further levels of control to the flow of signals. A group of such proteins known as Scaffold Proteins serve this purpose by fine-tuning amplitude and intensity and conferring spatial selectivity to ERKs signals (Dhanasekaran *et al.*, 2007).

As of today, the accepted requisite for a protein to be considered as a “scaffold”, is its ability to bind to at least two members of the signalling cascade simultaneously, forming a complex with functional stability *(Fig. 1.10)*. The first scaffold protein serving this role in a MAPK cascade was Ste5, identified in the budding yeast *Saccharomyces pombe*. It was found that Ste5 stabilized the complex formed by Fus3 (MAPK), Ste7 (MAPKK) and Ste11 (MAPKKK) at the tips of mating projections, following stimulation with mating pheromones (Choi *et al.*, 1994; Yablonski, Marbach

and Levitzki, 1996). Since then, scaffold proteins have also been identified in mammalian cells and, to date, the list of proteins that qualify as scaffolds for the RAS/ERK pathway has grown up to 15 members. Intriguingly, in spite of their common role, there is not a significant sequence homology among these proteins, and they do not resemble Ste5, for which hitherto no mammalian homologue has been identified (Kolch, 2005; Dhanasekaran *et al.*, 2007). Even though MAPKs scaffold proteins remain enigmatic on many aspects, it is clear that their functions are more complex than just serving as hubs for the assembly of the kinases (Witzel, Maddison and Blüthgen, 2012).

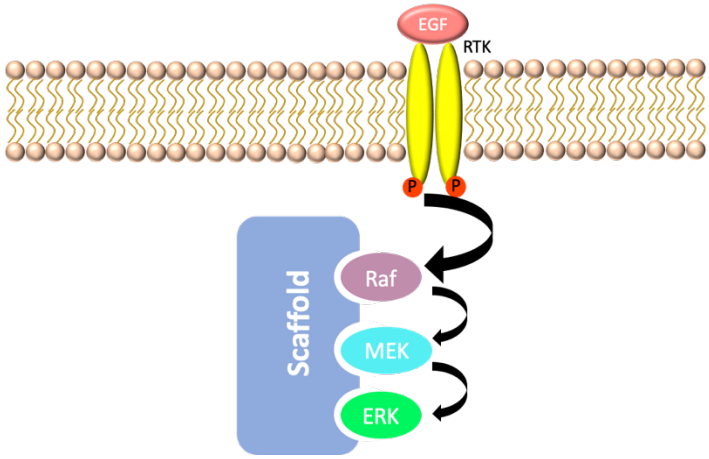


Figure 1.10. Illustration of a scaffold protein*. By definition, a protein can be considered a scaffold when it is able to bind simultaneously, at least, two members of the cascade forming a functional stable complex.*

Structurally, it is believed that scaffold proteins optimize signalling by clustering enzymes and substrates, thereby increasing their effective concentrations. In addition to positioning these proteins in optimal orientation relative to each other, thereby facilitating phospho-transfer reactions *(Fig. 1.11 A)* (Scott, Haystead and Haystead, 1995; Levchenko, Bruck and Sternberg, 2000). Furthermore, other proteins or small molecules can associate to scaffold proteins working as allosteric regulators. Such binding provokes a conformational change in the scaffold inducing or inhibiting signal transduction along the pathway *(Fig. 1.11 B).* For example, in the case of KSR it has been shown that its overexpression enhances RAF activation, as a consequence of its kinase-

homology domain directly binding to RAF, thereby inducing its kinase activity (Rajakulendran *et al.*, 2009). In the same vein, KSR interaction with RAF in cis, evokes a conformational switch on MEK, resulting in the exposure of its activation loop making it available for phosphorylation by RAF in trans (Brennan *et al.*, 2011).

Another mechanism whereby scaffold proteins optimize MAPKs signalling is by shielding the components of the cascade from dephosphorylation, by preventing their interaction with soluble phosphatases *(Fig. 1.11 C)* (Levchenko, Bruck and Sternberg, 2000). However, this notion is somewhat controversial as it questions whether they promote or impede signal amplification. Conceptually, since free kinases can phosphorylate multiple substrates, it is believed that the signal is amplified exponentially along the pathway. This implies that when proteins are locked onto a scaffold, a kinase can only phosphorylate its accompanying substrate, preventing signal amplification. However, in an environment with a high concentration of phosphatases, in which signalling based on freely diffusing kinases will be strongly inhibited, scaffolding will achieve a "local" concentration effect and, as a consequence, signal amplification; as it will increase the chances for a successful encounter between active kinases in spite of the surrounding high levels of deactivating phosphatases (Locasale, Shaw and Chakraborty, 2007).

In addition to the aforementioned functions, it is now clearly established that scaffold proteins are essential determinants of ERK signals spatial regulation, by orchestrating ERKs activity in a sublocalization-specific fashion *(Fig. 1.11 D)*. Hitherto all of the proteins that have been identified as ERK scaffolds are extranuclear proteins. In agreement, a broad collection of studies demonstrate that scaffold proteins participate in ERK signalling at multiple extranuclear sites: KSR1 regulates ERKs signals generated at the plasma membrane in lipid rafts domains (Matheny *et al.*, 2004). MP1 works primarily at endosomes (Teis, Wunderlich and Huber, 2002), SEF regulates ERKs functions at the Golgi complex (Torii *et al.*, 2004) and Paxillin mainly at focal adhesions (Ishibe *et al.*, 2003). Furthermore, it has been demonstrated that spatial selectivity provided by scaffold proteins is an essential factor for defining ERKs substrate specificity. In this respect, it has been shown that the

type of membrane from which ERK is being activated by RAS incoming signals, dictates which substrates are amenable for phosphorylation, and this is achieved by the intervention of defined scaffolds proteins (Casar *et al.*, 2009).

The molecular mechanism whereby scaffold proteins determine ERK substrate specificity has been recently unravelled, and it is based on the scaffold proteins capacity for promoting the formation of ERK dimers. The interaction of scaffolds with ERK in dimeric form makes possible that one ERK monomer binds to the scaffold and the other to the pertinent substrate (Casar, Pinto and Crespo, 2008). Therefore, scaffold proteins function as ERK dimerization platforms thereby facilitating the assembly of the ERK enzymatic complexes competent for the activation of its cytoplasmic substrates *(Fig. 1.11 E)*.

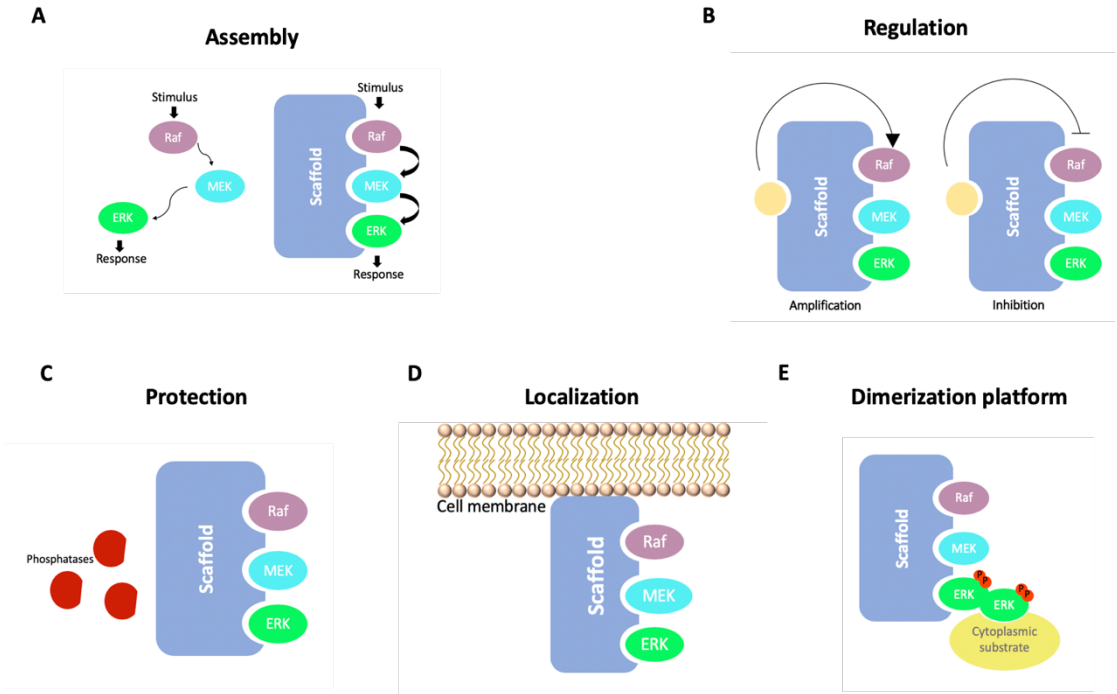


Figure 1.11. Scaffold protein functions. *Scaffold proteins serve as an assembly platform for the kinases orienting them and facilitating phospho-transfer reactions (**A**). They have a regulatory function when a small molecule binds to them it can induce or inhibit signal transduction (**B**). Scaffolds protect kinases from dephosphorylation by soluble phosphatases and from signal interferences from other pathways (**C**). Scaffolds confer spatial regulation for ERK signals due to the specific subcellular localization (**D**). And they function as dimerization platforms for ERK dimers, where they can activate cytoplasmic substrates (**E**).*

In support of this notion, results from our lab have shown that ERKs cytoplasmic substrates like cPLA2, RSK1 and PDE4, specifically associate to ERK in dimeric form (Casar, Pinto and Crespo, 2008, 2009; Herrero *et al.*, 2015). In agreement with these findings, it had been previously shown that the overexpression of scaffolds like KSR1, β-arrestin and SEF enhanced ERK activity at the cytoplasm (Sugimoto *et al.*, 1998; DeFea, Zalevsky, *et al.*, 2000; Tohgo *et al.*, 2002; Torii *et al.*, 2004) and, at the same time, it downregulated ERK nuclear events. At the nucleus, as previously mentioned, ERK activity is mainly undertaken in monomeric form (Casar, Pinto and Crespo, 2008).

A central notion concerning scaffold proteins, supported by a wide array of data, is that scaffolds function at an optimal concentration that yields maximum signal output. As such, scaffold proteins promote a bell-shaped MAPK activation kinetics. As a consequence, sub-optimal MAPK activation takes place when the scaffolds concentration is low; therefore, there are not enough scaffolds to productively assemble all of the available signalling cascade constituents. An excessive scaffold concentration has the same effect as MAPKs, MAPKKs and MAPKKKs are scattered in incomplete complexes. This phenomenon has been called "combinatorial inhibition" and "prozone effect" *(Fig. 1.12)* (Levchenko, Bruck and Sternberg, 2000; Heinrich, Neel and Rapoport, 2002).

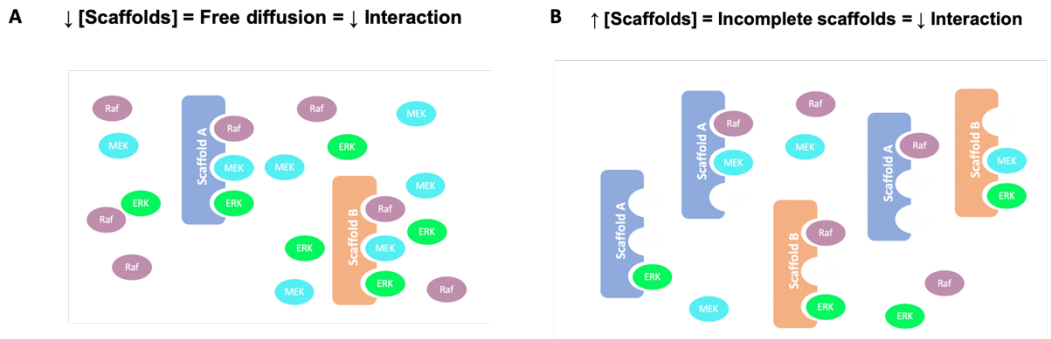


Figure 1.12. A change on scaffold proteins expression reduces ERK signalling. *Scaffolds expression levels are in an optimal stoichiometric proportion with MEK and ERK in order to maximize signalling. Due to this fact, a low expression of scaffold proteins results in a free diffusion of the kinases, decreasing the interaction between them (**A**). And, on the other hand, a high concentration of scaffold proteins produces the dispersion of the kinases in incomplete scaffolds (**B**).*

Following this rationale, it can be envisioned that the regulation of scaffold concentrations could be an efficient mode for regulating MAPKs signal output. In theory, maximum MAPK signal output would be achieved only when scaffolds concentrations are optimal. Therefore, up- or downregulating scaffolds expression could be an effective way for attenuating MAPK signalling. It is noteworthy, that the expression of the majority of scaffold proteins, in most cell types, is quite stable and not subject to immediate changes in response to external stimuli that govern MAPKs activation. This does not invalidate that alterations on scaffolds levels, when they happen, can result in profound, long-term consequences on MAPKs biological outcomes, in some cases contributing to pathological processes. In this respect, there is ample data showing that some ERK scaffold proteins display altered expression in some types of tumours (www.oncomine.com; www.cBioportal.com).

1.5.1. Coordination among scaffold proteins

As of today, about 15 scaffold proteins have been described for the RAS/ERK pathway, and the available data suggests that they function independently from each other. However, this is difficult to reconcile with results showing that altering the expression of anyone of them, affects much more profoundly ERK signals intensity than should be expected from interfering with just one of the fifteen scaffolds. For example, when KSR1 levels are downregulated by gene knock-out or siRNA, over an 80% drop on ERK activation levels is detected (Nguyen *et al.*, 2002; Lozano *et al.*, 2003). This is also the case for IQGAP1 (Roy, Li and Sacks, 2004; Jameson *et al.*, 2013), and for MP1 (Sharma *et al.*, 2005; Teis *et al.*, 2006). Overall, these data are hard to reconcile with the notion that scaffold proteins function independently.

In light of these observations it could be hypothesized that scaffold proteins act in conjunction, influencing the activity of each other (Casar and Crespo, 2016). In this respect, it is quite evident how up- or downregulation on the expression levels of any scaffold could affect others, since they compete for the same pool of kinases. For example, reducing the levels of a given scaffold would,

on one hand, reduce signalling through its own pathway and, on the other hand, could enhance signalling as mediated by another scaffold, by increasing the number of available free kinases, thereby increasing the amount of complete complexes for the second scaffold *(Fig. 1.13)*.

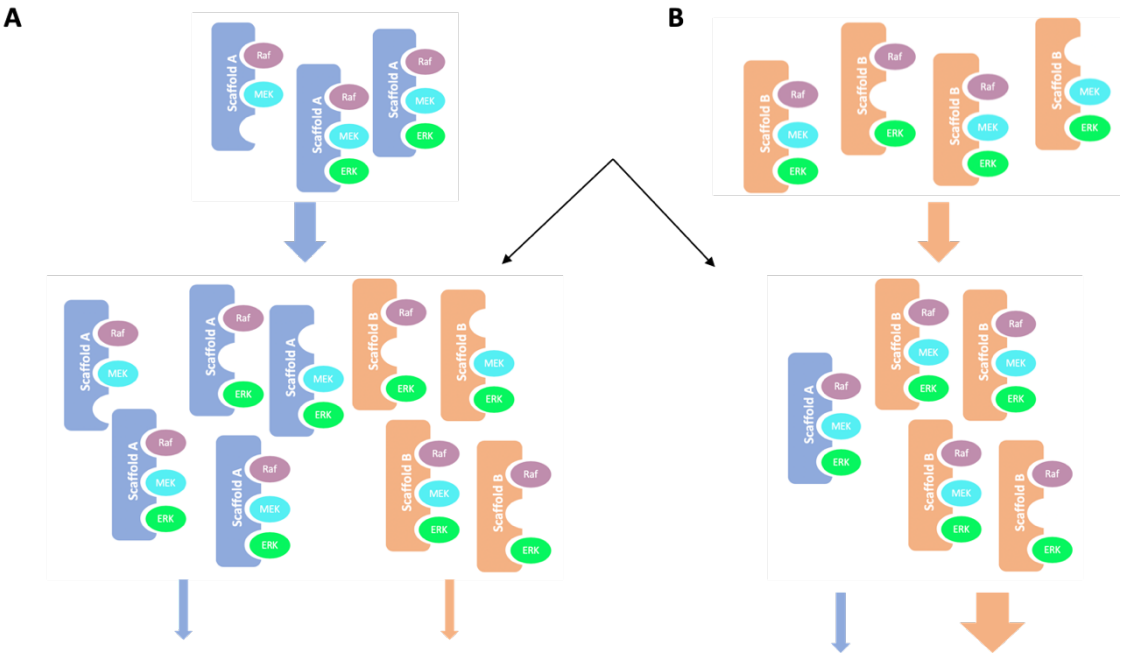


Figure 1.13. A change on the expression of a single scaffold can drive to a change in the signalling through other scaffolds. *The overexpression of a given scaffold can attenuate signalling through it or through other scaffolds due to the dispersion of the kinases, for which they are competing, in incomplete scaffolds (**A**). While its depletion can result in an increasement in the signalling through another scaffold decreasing its own signals, due to a higher amount of available kinases for the other scaffold (**B**).*

Furthermore, even though it is believed that scaffold proteins function autonomously from each other, regulating ERK signals at specific sub-localizations as induced by defined stimuli, it cannot be discarded that they also act in a coordinated fashion to some extent. In this respect, recent data clearly demonstrated that some scaffold proteins can associate among themselves. It is not unprecedented that protein serving regulatory functions such as docking and scaffold proteins interact among themselves forming macro-complexes (Pan *et al.*, 2012). With regards to

scaffold proteins of the RAS/ERK pathway, there have been described interactions between: MP1 and MORG1 (Vomastek *et al.*, 2004); IQGAP1 and MP1 (Schiefermeier *et al.*, 2014); paxillin and GAB1 (Ren *et al.*, 2004); and IQGAP1 and β-arrestin2 (Feigin *et al.*, 2014). Even though the functional meaning of these associations is largely unknown and the processes and mechanisms regulating interactions among scaffolds are completely unveiled, in some cases they appear to have important biological implications. As an example, during cellular migration IQGAP1 binding to MP1 plays a critical role in the regulation of focal adhesion dynamics (Schiefermeier *et al.*, 2014).

1.5.2. Scaffold Proteins Species

1.5.2.1. SEF

Initially discovered in zebrafish as an inhibitor of the RAS/ERK pathway following FGF stimulation. SEF can bind to MEK activated mutants, inhibiting the dissociation of the MEK-ERK complex and blocking ERK nuclear translocation without affecting its cytoplasmatic activity (Fürthauer *et al.*, 2002; Tsang *et al.*, 2002). Unlike most scaffolds, SEF is not a soluble protein, it is associated to membrane systems particularly the Golgi complex. SEF overexpression can inhibit ERK nuclear signal (Preger *et al.*, 2004; Torii *et al.*, 2004). The details of SEF interaction with ERK and other constituents of the RAS/ERK pathway have not been described. Extranuclearly, SEF intervenes in $cPLA_2$ activation by ERK induced by RAS signals coming from the Golgi complex (Casar *et al.*, 2009). With respect to its biological relevance ERK retention at the cytoplasm by SEF inhibits the differentiation of PC12 cells induced by NGF or FGF2 (Xiong *et al.*, 2003). SEF 2 is downregulated in a broad number of human tumors (Zisman-Rozen *et al.*, 2007). Its downregulation positively correlates with highly invasive metastatic prostate cancer (Darby *et al.*, 2006). In prostate cancer cells, SEF inhibits FGF tumorigenic effects and has been considered a potential tumor suppressor (Darby *et al.*, 2009).

1.5.2.2. PAXILLIN

A 68 kDa protein initially described as a substrate for tyrosine kinases and localized in focal adhesions (Glenney and Zokas, 1989; Turner, Glenney and Burridge, 1990). There, upon FGF stimulation, paxillin is tyrosine phosphorylated and associates with ERK. It also binds to MEK in a constitutive fashion and to RAF following HGF treatment. ERK binds to paxillin through an FXF domain and paxillin Y118 is essential for this interaction, in fact, mutation of this residue prevents ERK binding and the scattering of epithelial cells provoked by HGF. It has been shown that HGF promotes ERK-induced phosphorylation of paxillin at S83, mutation of this residue blocks the interaction between paxillin and FAK and the subsequent activation of Rac (Ishibe *et al.*, 2003). As such the ERK-paxillin complex is considered as the main regulator of FAK and RAC stimulation at focal adhesions, implying this association on lamellipodia formation and focal adhesion recycling, both needed for migratory responses in tumour cells (Ishibe *et al.*, 2004).

1.5.2.3. β-ARRESTIN

β-arrestin family is composed of two isoforms, β-arrestin-1 and β-arrestin-2, of around 46 kDa, ubiquitously expressed in mammalian tissues (Luttrell and Lefkowitz, 2002). Both play a critical role on GPCR signalling. It is long known that these proteins act as blockers of the signals that emanate from GPCRs, by uncoupling the receptors and their associated G proteins (Lefkowitz and Shenoy, 2005; Moore, Milano and Benovic, 2007). In addition to receptor desensitation, β-arrestins are also related to trafficking, recycling and degradation of GPCRs upon stimulation by agonists (Moore, Milano and Benovic, 2007).

In addition to its activation by tyrosine kinase receptors, the RAS/ERK pathway is also switched-on by GPCRs, this activation can take place either through G proteins or through β-arrestin (Wei *et al.*, 2003). The kinetics and molecular consequences of ERK activation as triggered by either mechanism are different: in HEK293 cells, that express the angiotensin II type receptor, G-protein activation results in an acute ERK activity peak and the distribution of ERK both in the nucleus and

the cytoplasm (Wei *et al.*, 2003). Contrarily, the signal mediated by β-arrestin, yields a prolonged ERK activation and prevents ERK nuclear localization. β-arrestin-dependent effects on ERK activation as induced by GPCRs are related to β-arrestin role as a scaffold protein. In this respect, protein complexes formed by RAS/ERK pathway components and β-arrestin had been detected in response to GPCR stimulation (Ahn *et al.*, 2004). This could explain the retarded kinetics and the blockage of ERK nuclear translocation provoked by β-arrestin, acting as a cytoplasmic anchor and a spatial regulator for ERK, retaining it at early endosomes (DeFea, Vaughn, *et al.*, 2000; Tohgo *et al.*, 2002).

1.5.2.4. DYSTROGLYCAN

It is a dimeric protein made up of two glycosylated isoforms generated by the same gene (Weir and Muschler, 2003). The alpha subunit is extracellular and the beta subunit is a type 1 transmembrane protein that binds α-dystroglycan C-terminus extracellularly and to acting intracellularly (Bozzi *et al.*, 2009). The cytoplasmic region of β-dystroglycan has putative ERK binding regions. Specifically, the ELM motif (Moore and Winder, 2010). Both activated MEK and ERK have been shown to interact with this region, though, surprisingly, dystroglycan interaction with MEK and with ERK differs in subcellular localization: whereas active ERK co-localizes with dystroglycan in focal adhesions, activated MEK co-localizes mainly in ruffles (Spence *et al.*, 2004). The reason for this is uncertain but it may be related to dystroglycan antagonist role in ERK activation by integrins, where dystroglycan would intervene separating MEK from ERK (Ferletta *et al.*, 2003).

1.5.2.5. MORG1

It is a 35 kDa protein belonging to the WD40 family. It was initially described as a binding partner for MP1 (Vomastek *et al.*, 2004). Lately, it has been shown to be part of a protein macro-

complex that also includes p14 and p18 and it is particularly enriched at endosomes, where it participates in vesicular trafficking during cellular migration processes (Schiefermeier *et al.*, 2014). While included in this complex, MORG1 has been found to associate to CRAF, BRAF, MEK1/2 and ERK1/2. MORG1 overexpression enhances ERK activation in response to serum, whereas its silencing attenuates this response. MORG1 has also been shown to mediate in ERK response to phorbol esters and lysophosphatidic acid (LPA). But it does not participate on ERK activation by EGF suggesting that MORG1 is implicated on signal transduction from G protein-coupled receptors but not RTKs (Vomastek *et al.*, 2004; Kolch, 2005). Interestingly, MORG1 knockout mice are embryonically lethal due to defective vascularization. And MORG1 expression is reduced in ischemic human brains, suggesting that MORG1 may play an important role in vasculogenesis (Haase *et al.*, 2009).

1.5.2.6. MP1

MP1 is a 13.5 kDa protein initially identified as a MEK1 binding partner (Schaeffer *et al.*, 1998). MP1 interacts with MEK1 and ERK1, but not ERK2, though other authors suggest that MP1 can bind inactive ERK1 and 2 (Brahma and Dalby, 2007). And its overexpression can potentiate ERK1 activation. As previously mentioned for MORG1, MP1 forms a macro-complex with p14 at late endosomes, where MEK1 and ERK1 are recruited (Teis *et al.*, 2006; Schiefermeier *et al.*, 2014). Downregulating the expression of MP1 or p14 attenuates the duration MEK1/ERK1 signals, suggesting that MP1-p14 could be involved in prolonging ERK1 activation (Teis, Wunderlich and Huber, 2002). MP1 is involved on PAK1-dependent ERK activation that takes place during cell adhesion and colony dissemination. During colony dissemination stimulated by fibronectin MP1 is required for PAK1-evoked MEK phosphorylation and the subsequent ERK1 activation. Likewise, MP1-mediated MEK/ERK activation is required for the suppression of ROCK (Rho-Rho-associated coiled coil-containing protein kinase) signalling, an essential requisite for cellular adhesion. On the other hand, in the same process, MP1 is dispensable for ERK activation by PDGF. This data suggests

that MP1 plays a role in the activation of the ERK cascade in response to adhesion to fibronectin while isolating it from PDGF-generated signals (Pullikuth *et al.*, 2005).

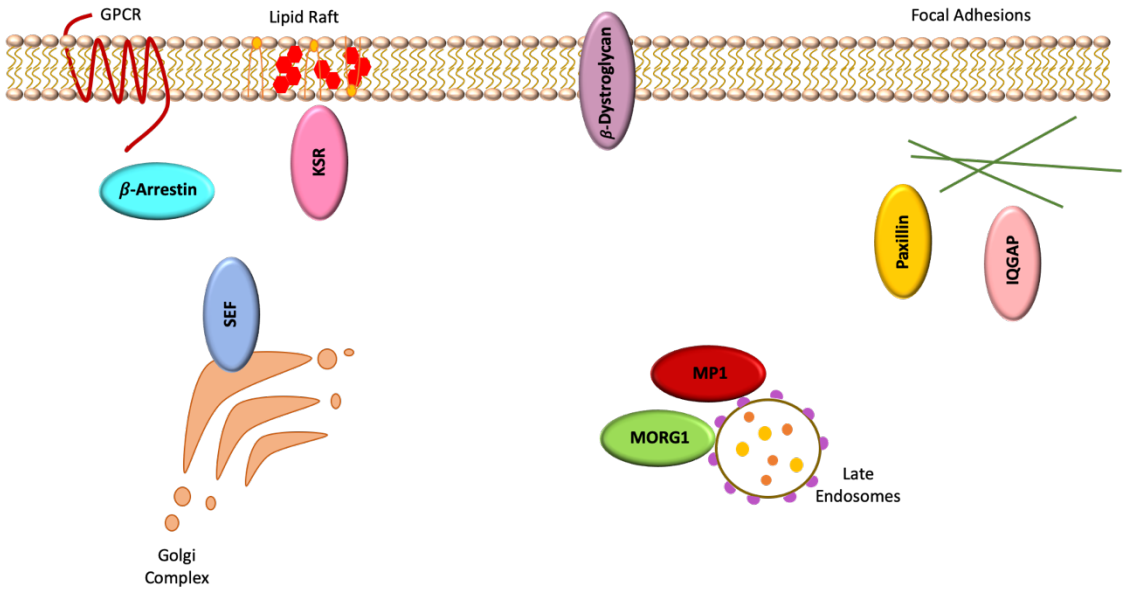


Figure 1.14. Scaffold proteins subcellular localization. *Scaffold proteins are involved in the spatial regulation of ERK signals from different sublocalizations of the cell: β-arrestins regulate RAS signals arising from G protein-coupled receptors; KSR from lipid rafts; β-dystroglycan at the plasma membrane; paxillin and IQGAP from focal adhesions; MORG1 and MP1 from late endosomes; and Sef from the Golgi complex.*

1.6. KSR

The aforementioned scaffold Ste5 was described in 1994 by Choi and collaborators as a novel component in the yeast pheromone response pathway. Ste5 is able to link simultaneously Ste11, Ste7 and FUS3 forming a functional activating complex, that facilitates two sequential phosphorylation reactions (Choi *et al.*, 1994).

A year later, in 1995, a protein was discovered by a genetic screen for downstream effectors of RAS performed, simultaneously, in *Drosophila virilis/Drosophila melanogaster* (Therrien *et al.*, 1995) and *Caenorhabditis elegans* (Kornfeld, Hom and Horvitz, 1995; Sundaram and Han, 1995). This protein was recognized as the most likely equivalent of the yeast MAPK scaffold protein Ste5 in mammalians. The novel locus identified, SR3-1 (Suppressor of RAS1 3-1), encoded a protein closely related to RAF family, both belonging to the TKL group of kinases *(Fig. 1.15)* (Manning *et al.*, 2002). The phenotypes of the KSR1 loss of function mutants were able to inhibit RAS signals.

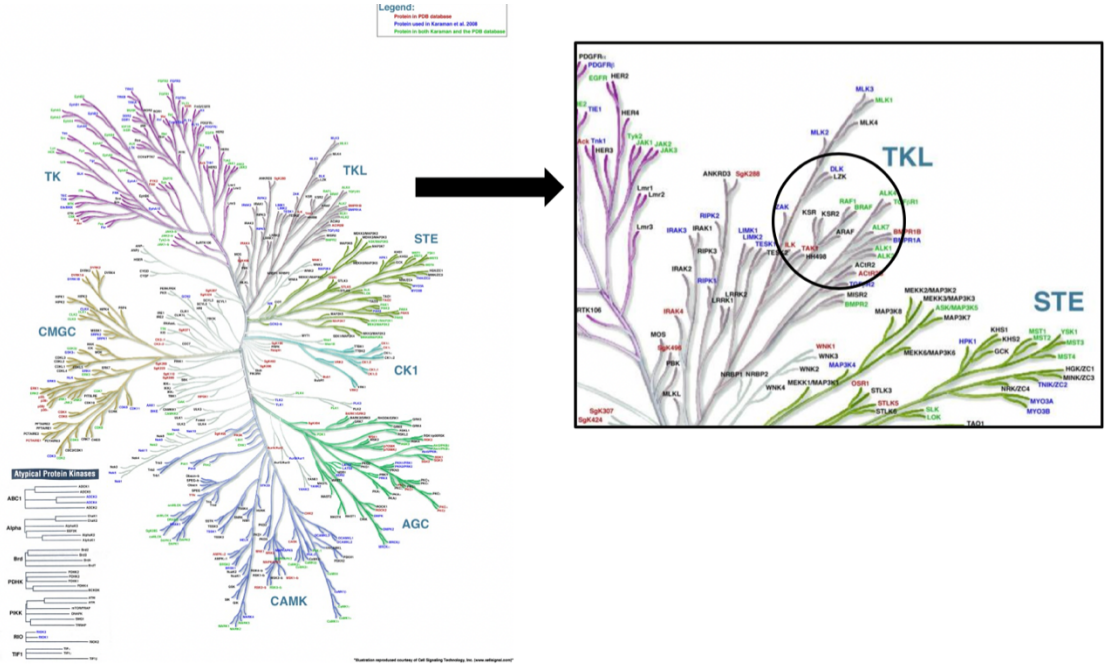


Figure 1.15. KSR location in the phylogenetic tree of human protein kinases. *The part of the dendrogram where the Tyrosine kinase like (TKL) group is found is amplified on the right and the RAF/KSR kinase family is highlighted with a black circle. Figure adapted from Manning et al., 2002.*

Because of this SR3-1 was renamed Kinase Suppressor of RAS (KSR) (Kornfeld, Hom and Horvitz, 1995; Therrien *et al.*, 1995).

Even though KSR was originally described in *Drosophila* and *C. elegans*, a homologous protein was also isolated in mouse and human, this suggested that KSR had an evolutionary conserved function (Therrien *et al.*, 1995). Indeed, while most of the studies regarding its biochemistry and biology have been performed using the murine protein, in most cases the results are extrapolable to lower organisms.

The KSR family is made up of two members, KSR1 (Therrien *et al.*, 1995) and KSR2 (Channavajhala *et al.*, 2003). Both of them are 105 kDa proteins composed by 5 **C**onserved **A**reas: CA1-CA5 (Therrien *et al.*, 1995) *(Fig. 1.16)*. CA1 to CA4 comprise the N-terminal region and constitute the regulatory domain, while the CA5 is found at the C-terminus and harbours the pseudokinase region. CA1 is the only region with not known homology, this domain is part of a coiled-coil fused to a sterile α-motif (CC-SAM) implicated in KSR localization at membrane ruffles. CA1, together with CA5, participates in BRAF binding in a CC-SAM-independent manner (McKay, Ritt and Morrison, 2009; Koveal *et al.*, 2012). CA2 is rich in proline residues and it is believed to function as SH3 binding domain. The CA3, a cysteine-rich domain (CRD), is a lipid binding domain similar to those of PKC and CRAF and it is implicated in KSR subcellular localization, being essential to target KSR to the plasma membrane following RAS activation (Driedger and Blumberg, 1980; Michaud *et al.*, 1997; Zhou *et al.*, 2002; Clapéron and Therrien, 2007). CA4 is a region rich in serine/threonine residues and includes the FXFP docking motif where ERK binds to (Therrien *et al.*, 1996; Jacobs *et al.*, 1999; Fantz *et al.*, 2001). Lastly, the CA5 region contains the MEK-binding (Denouel-Galy *et al.*, 1998) and the RAF-binding (Xing, Kornfeld and Muslin, 1997) domains; and the eleven subdomains typical of all protein kinases (Hanks, Quinn and Hunter, 1988; Hanks and Hunter, 1995).

However, even if KSR had kinase activity it is not clear whether it would be a Tyr or a Ser/Thr

kinase, as the sequence YI(L)APE in subdomain VIII, conserved in all known Ser/Thr kinases, is found in all KSR genes cloned as far; but in *C. elegans* and *D. melanogaster* the sequence HKDLR characteristic of Tyr kinases, is also present. Whereas *Drosophila* and *C. elegans* KSR possesses the same catalytic sequence as conventional protein kinases (VAV**K**), in mammals, KSR has an arginine in subdomain II of the CA5 catalytic domain instead of the highly evolutionary conserved lysine among the kinases (Clapéron and Therrien, 2007). This lysine residue is required to orient ATP (Weinmaster, Zoller and Pawson, 1986), and no other residue at this position seems to replace it (Hanks, Quinn and Hunter, 1988). The mutation of the intact lysine in *Drosophila* and *C. elegans* does not compromise KSR function, supporting RAF activation and increasing ERK signalling (Stewart *et al.*, 1999; Roy *et al.*, 2002). These results are consistent with a noncatalytic role of KSR. For this reason, KSR has been classified as a pseudokinase (Boudeau *et al.*, 2006; Hu *et al.*, 2011; Eyers and Murphy, 2013; Zhang *et al.*, 2013). However, it has been a central point of controversy since KSR discovery. Indeed, while some studies have reported a residual kinase activity (Zhang *et al.*, 1997; Brennan *et al.*, 2011; Goettel *et al.*, 2011; Hu *et al.*, 2011), others have failed to detect it (Michaud *et al.*, 1997; Stewart *et al.*, 1999; Roy *et al.*, 2002).

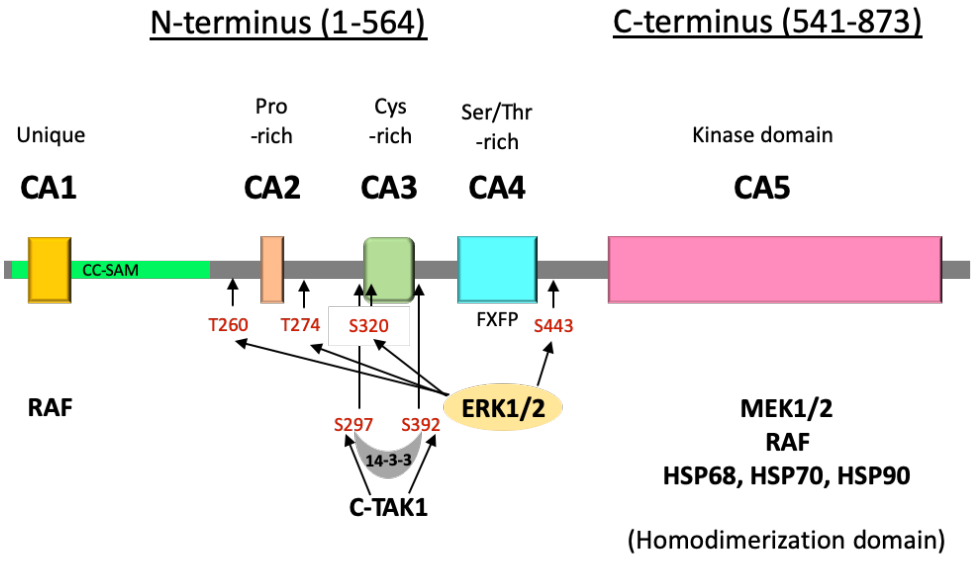


Figure 1.16. Schematic depiction of KSR1. *The KSR family proteins are structurally formed by five conserved domains: a domain unique to the KSR proteins (CA1), a proline-rich region (CA2), a cysteine-rich domain (CA3), a serine/threonine-rich region (CA4), and a putative kinase domain (CA5). The main interacting proteins are shown below their corresponding interaction domain. Arrows originating in the proteins that, in addition to interact, phosphorylate KSR indicate phosphorylation sites.*

In vivo KSR1 is a phosphoprotein. Up to twelve serine/threonine phosphorylation sites have been identified, even though some of the kinases responsible for these phosphorylations are unknown. It is known that ERK can phosphorylate KSR at T260, T274, S320 and S443 *(Fig. 1.16)* in response to growth factor stimulation or Ras activation (Cacace *et al.*, 1999; Volle *et al.*, 1999; McKay, Ritt and Morrison, 2009). Even though the functional significance of these phosphorylations is still unclear, some studies suggest that the phosphorylation of these sites by ERK contribute to BRAF-KSR1 dissociation and relocates KSR1 from the plasma membrane to the cytoplasm (McKay and Morrison, 2007). Phosphorylation of KSR1 on S297 and S392 by the kinase C-TAK1 creates 14-3-3 binding sites whose dephosphorylation by PPA2 is required to localize KSR to triton resistant microdomains (lipid rafts) (Cacace *et al.*, 1999; Müller *et al.*, 2001; Ory *et al.*, 2003). Also, KSR phosphorylation has been associated with its nucleo-cytoplasmic shuttling (Brennan *et al.*, 2002). However, KSR nuclear localization is controversial.

Both KSR1 and KSR2 have a cytoplasmic localization, however, upon stimulation with serum, EGF or LPA, among other agonists, and RAS activation, KSR rapidly translocates to the plasma membrane, by means of the CA3 region, where it interacts with RAF ensuing MEK phosphorylation. To date, KSR has not been found to interact with RAS. Within the plasma membrane, KSR exhibits selectivity towards defined microdomains, responding preferentially to signals that emanate from lipid rafts. It has been shown that interaction with the E3 ligase IMP (Impedes Mitogenic signal Propagation) promotes the recruitment of KSR to triton-resistant structures that sequester KSR1 and block ERK activation (Matheny *et al.*, 2004; Chen, Lewis and White, 2008). RAS activation catalyzes IMP proteasome degradation, facilitating KSR-mediated ERK activation (Matheny and White, 2006). In this respect, results from our group have shown that KSR selectively couples RAS signals from lipid rafts to the activation of $cPLA_2$ by ERK (Casar, Pinto and Crespo, 2009).

In cells, KSR is part of a macromolecular complex over 1,000 kDa that also includes the components of the ERK cascade; heat shock proteins such as HSP90, HSP70 and HSP68; proteins belonging to the 14-3-3 family (Cacace *et al.*, 1999; Stewart *et al.*, 1999); $\beta\gamma$ subunits from

heterotrimeric G proteins (Bell *et al.*, 1999); and other proteins of unknown function (p33, p34, p36 and p60) (Stewart *et al.*, 1999). It is unclear if KSR binds directly to all of these proteins, but its role as a scaffold protein for the ERK cascade is beyond doubt. The initial studies in this respect showed that KSR binds to MEK constitutively, whereas binding both to RAF and to ERK occurs in response to RAS activation, following agonist stimulation (Therrien *et al.*, 1996; Xing, Kornfeld and Muslin, 1997; Denouel-Galy *et al.*, 1998; Yu *et al.*, 1998).

KSR proteins exhibit a significant degree of homology with RAF family kinases (Manning *et al.*, 2002), both in amino acid sequence and in the domain distribution in its tertiary structure (Therrien *et al.*, 1995; Clapéron and Therrien, 2007). RAF proteins are known to homodimerize and heterodimerize among family members, likewise RAF proteins, in particular BRAF, also heterodimerize with KSR. KSR heterodimerization with RAF induces the formation of side-to-side heterodimers believed to trigger RAF activation. Furthermore, it is described that KSR and RAF share an identical dimer interface (Rajakulendran *et al.*, 2009; Verlande *et al.*, 2018). Apart from KSR-RAF interaction through the C-terminus, selective heterodimerization of RAF with KSR1 occurs through direct contacts of N-terminal regulatory region of each protein, involving the coiled-coil-sterile alpha motif (CCSAM) in KSR (Koveal *et al.*, 2012). Interestingly, MEK binding to the CA5 domain of KSR1 promotes KSR heterodimerization with RAF. As a result of KSR-RAF dimerization, KSR acts as an allosteric activator of RAF enhancing its catalytic activity, in addition to its function as a scaffold, by which it connects RAF with its substrate MEK (Rajakulendran *et al.*, 2009). On the other hand, KSR dimerization with RAF triggers a conformational change in KSR as a result of which MEK activation loop is exposed facilitating its phosphorylation. Following the model proposed by Brennan and collaborators in 2011 (Brennan *et al.*, 2011), once KSR and BRAF form a heterodimer, the MEK activating sites, T218 and T222, are unmasked to be phosphorylated by another catalytic active RAF molecule in *trans (Fig. 1.17)*.

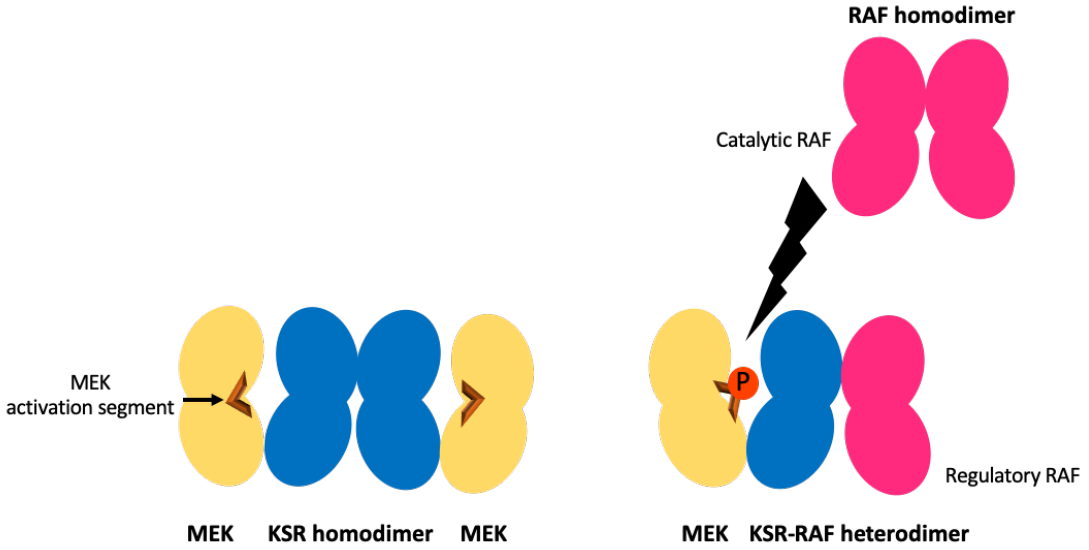


Figure 1.17. Schematic representation of RAF-mediated activation of MEK. *When KSR homodimerize in a side-to-side fashion, the activation segment of the MEK molecule interacting face-to-face remains inaccessible (left). On the other hand, the side-to-side heterodimerization with RAF regulatory molecule provokes the conformational change in MEK exposing the activation segment. A catalytic RAF molecule, different from the one heterodimerizing with KSR, will phosphorylate MEK in the activating sites.*

In a similar fashion to what occurs with RAF proteins, KSR1 can also homodimerize, forming side-to-side dimers as well. KSR1 homodimerization occurs through its C-terminus (Matheny and White, 2009; Rajakulendran *et al.*, 2009). In *D. melanogaster* KSR arginine 732 (Arg 615 in mouse) plays a critical role in this process, since its mutation to histidine prevents KSR1 dimerization (Douziech *et al.*, 2006). KSR2 also forms homodimers forming a side-to-side interface specifically dependent on R718 (Rajakulendran *et al.*, 2009). The mutation of this site blocks RAS activity suggesting that KSR dimerization may play an important role on RAS signals. However, how dimerization affects KSR role as a regulator of RAS/ERK signals and its biological implications are unknown hitherto.

1.6.1. KSR biological roles

Most of what we know about KSR physiological and biological roles comes from animal studies. Seminal studies in *Drosophila* demonstrated that loss of KSR suppresses RAS signalling and prevented the rough eye phenotype induced by constitutive RAS activity (Therrien *et al.*, 1995). In

the same vein, loss of function mutations in *C. elegans* KSR1 suppressed the multiple vulva phenotype (Kornfeld, Hom and Horvitz, 1995; Sundaram and Han, 1995; Yoder *et al.*, 2004). In mammalian KSR1 knock out mice are viable and fertile with no overt abnormalities. Of note, KSR1 -/- mice resemble EGF receptor -/- mice in defective hair follicles suggesting that KSR1 may participate downstream from EGF signals. Probably the most significant biological consequence of KSR1 loss in mice, is their resistance to RAS-dependent tumour formation. In this respect, carcinogens that induce mammary and skin tumours, by promoting H-RAS mutations, are markedly reduced in KSR1 -/- mice (Hansen *et al.*, 1997; Nguyen *et al.*, 2002; Lozano *et al.*, 2003). These data clearly point to KSR1 as a potential target for antineoplastic therapeutic intervention. Contrarily to KSR1, KSR2 -/- mice have reduced fertility and become spontaneously obese; adaptive thermogenesis, metabolic rate and leptin sensitivity are compromised in these mice. In agreement, humans harbouring KSR2 mutations present early onset obesity (Revelli *et al.*, 2011; Pearce *et al.*, 2013; Henry *et al.*, 2014). Interestingly, whereas KSR1 is ubiquitously expressed (Giblett *et al.*, 2002; Nguyen *et al.*, 2002), KSR2 is almost exclusively found in brain and pituitary. Brain specific disruption of KSR2 is enough to cause obesity in mice (Guo *et al.*, 2017).

1.7. IQGAP1

IQ motif-containing GTPase Activating Protein (IQGAP) 1, a 1657-amino acid protein, was first identified in 1994 by Weissbach and collaborators in a screen to reveal novel matrix metalloproteases (Weissbach *et al.*, 1994). But it was not described as scaffold protein of the Ras/ERK pathway until 2004 (Roy, Li and Sacks, 2004). IQGAPs are a family of multidomain proteins highly conserved throughout evolution. In mammals there are 3 members IQGAP1, IQGAP2 (Brill *et al.*, 1996; McCallum, Wu and Cerione, 1996), and IQGAP3 (S. Wang *et al.*, 2007) which share a 58% homology with IQGAP1. All of them encode proteins of 190 kDa, of these, IQGAP1 is the best studied. IQGAP proteins exhibit different tissue expression patterns: whereas IQGAP1 is ubiquitous

(Weissbach *et al.*, 1994), IQGAP2 is mainly expressed in liver and in the gastro-intestinal and urogenital track (Schmidt *et al.*, 2003; Cupit *et al.*, 2004) and IQGAP3 can be found mainly in brain, lung and testicles (Nojima *et al.*, 2008). Regarding its subcellular localization IQGAP1 is a cytoplasmic protein and it is mainly associated to the cytoskeleton, being markedly enriched in zones of cell-to-cell contacts (Li *et al.*, 1999). Conversely, IQGAP2 and IQGAP3 distribution is not very well described, but they have been observed in the nucleus and in cell-to-cell junctions, respectively, as well as diffused in the cytoplasm, depending on the cellular context (Zhou *et al.*, 2003; Chew *et al.*, 2005).

IQGAP1 harbours different structural domains that participate in distinct molecular processes. At its N-terminus, it harbours a Calponin Homology Domain (CHD), this domain participates in the interaction with the cytoskeleton, in particular with F-actin (Mateer *et al.*, 2004) and it is also known to bind calmodulin/Ca^{2+} under particular circumstances (Ho *et al.*, 1999). Further downstream, there is a WW domain with two conserved Trp residues (W). WW domains are known to bind to proline-rich regions in the binding partners. IQGAP WW domain is peculiar in the sense that its main binding partners, ERK1/2, lack such regions (Roy, Li and Sacks, 2004). IQGAP also harbours an IQ domain that binds to its typical partners such as calmodulin (Li and Sacks, 2003), in addition, IQGAP utilizes this domain to bind to EGF receptor (McNulty *et al.*, 2011), MEK (Roy, Li and Sacks, 2005), BRAF (Ren, Li and Sacks, 2007) and the small GTPase RAP1 (Jeong *et al.*, 2007). The IQ motif is also important as it mediates the formation of IQGAP dimers and oligomers (Ren *et al.*, 2005). At its C-terminus there is the GAP Related Domain (GRD) which defines IQGAP as a GTPase activating protein (GAP) for Rho GTPases such as Cdc42 and RAC1 (Owen *et al.*, 2008; Kurella *et al.*, 2009).

Despite their family name and structural similarity to GAPs, IQGAPs have not yet been demonstrated to exhibit GAP activity (Brill *et al.*, 1996; Hart *et al.*, 1996). Noticeably, in this GAP domain the conserved arginine is replaced by a threonine, the lack of this residue allows IQGAP1 to stabilize small GTPases in their GTP state. IQGAPs bind to the Rho family GTPases RAC1 and Cdc42, and this association inhibits GTP hydrolysis by these GTPases, stabilizing their GTP-bound state

(Hart *et al.*, 1996; Swart-Mataraza, Li and Sacks, 2002). At its distal C-terminus, there is a RGCT (Ras GAP C-terminus) domain, this region is unique to IQGAP and it binds diverse targets, for example phosphoinositides, this phosphpoinositide binding region differs from all previously identified phosphoinositide binding regions described in other proteins (Choi *et al.*, 2013) *(Fig. 1.18)*. Overall, the unique binding capabilities of IQGAPs involve these proteins in the formation of multiple signalling complexes to regulate a broad variety of biochemical routes and cellular processes.

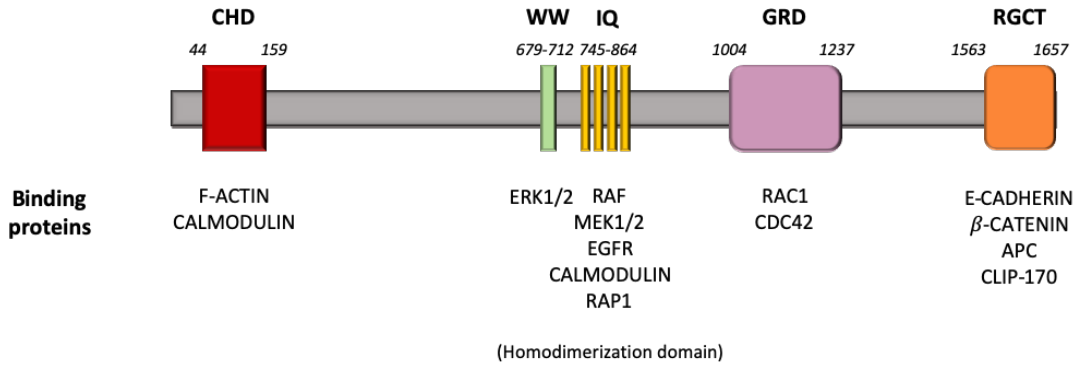


Figure 1.18. Schematic depiction of IQGAP1. *IQGAP1 structure consists in 5 domains: CHD (Calponin homology domain); WW, ERK interaction domain with two conserved Trp (W) residues; IQ domain MEK interaction domain with 4 repeats of isoleucine- and glutamine-containing motifs (IQ) motif; GRD (RAS-GAP related domain); and RGCT (RasGAP C-terminus). The numbers above indicate the position of the limiting amino acid residues of each domain. Some of the most important IQGAP1 interacting proteins are indicated below.*

Among IQGAP1 biochemical functions is its role as a scaffold for the RAS/ERK pathway (Roy, Li and Sacks, 2004). As mentioned before, IQGAP1 can bind both MEK and ERK (Roy, Li and Sacks, 2005), in addition, it can also interact with BRAF and modulate its functions (Ren, Li and Sacks, 2008), particularly in response to EGF signals and CD44 engagement (Bourguignon *et al.*, 2005). EGF stimulation evokes the binding of the different components of the ERK cascade to IQGAP1. Noticeably, IQGAP1-bound BRAF has higher kinase activity than free BRAF, though it is unclear if binding to IQGAP1 promotes this BRAF high activity or, contrarily, if highly activated BRAF has a preference for binding to IQGAP (Ren, Li and Sacks, 2008). Upon EGF stimulation, IQGAP1 binding to MEK1 increases, while binding to MEK2 decreases. In this respect, it has been suggested that MEK1 binding to IQGAP1 promotes proliferation whereas binding to MEK2 induces differentiation

(Ussar and Voss, 2004). Contrarily to MEK and BRAF, ERK1/2 binding to IQGAP is constitutive (Roy, Li and Sacks, 2004). The isoform IQGAP3, in addition to interacting with the aforementioned ERK cascade components, also interacts with H-RAS (Nojima *et al.*, 2008), whereas IQGAP1 interacts with K-RAS (Matsunaga *et al.*, 2014). Though these interactions only occur when IQGAP1 or IQGAP3 are overexpressed (Morgan *et al.*, 2019) With respect to IQGAP1 participation on EGF signalling, IQGAP1 has been shown to directly bind to EGFR and to modulate its activation (McNulty *et al.*, 2011). Interestingly, IQGAP1 serves as the scaffold protein that promotes EGFR phosphorylation by ERK when RAS signals emanate from lipid raft domains (Casar *et al.*, 2009). Regarding its role as a scaffold protein, IQGAP1 can form complexes with other ERK pathway scaffolds such as β-arrestin-2 (Alemayehu *et al.*, 2013) and MP1 (Schiefermeier *et al.*, 2014), even though, to date, the purpose of these interactions is unknown.

The best-known role for IQGAP1 is in the control of cell migration, as a regulator of the cytoskeleton. Specifically, IQGAP1 has a major role in the regulation of focal adhesions, a requirement for cell motility (Noritake *et al.*, 2005; Watanabe, Wang and Kaibuchi, 2015). Stimuli that promote the formation of focal adhesions, such as PDGF, stimulate the assembly, at these sites, of complexes containing IQGAP1 and the focal adhesion proteins vinculin and paxillin (Kohno *et al.*, 2013). During migration, a coordinated assembly and disassembly of focal adhesions is necessary. MP1 plays a role in this process by scaffolding MEK and ERK to late endosomes via p14. Knock down of MP1 impairs migration by the formation of abnormal focal adhesions that accumulate IQGAP1, suggesting that the interaction between IQGAP1, MP1 and the ERK cascade plays a major role in this process (Schiefermeier *et al.*, 2014).

IQGAP1 also participates in signalling from integrins and the regulation of the leading edge in migrating cells. In these, IQGAP1 is detected at the leading edge and promotes cell migration in a RAC1- and Cdc42-dependent fashion. In response to integrin engagement, IQGAP1 forms a complex with filamin A to recruit RAC1GAP, which inactivates RAC1. Decreased expression of IQGAP1,

filamin or RAC1GAP results in uncontrolled formation of membrane protrusions, resulting in impaired directional cell migration (Jacquemet *et al.*, 2013).

F-actin crosslinking is a key step in the regulation of cytoskeleton processes such as the formation of focal adhesions and filopodia, essential for cellular migration (Watanabe *et al.*, 2004). IQGAP1 is known to bind F-actin and participates in F-actin crosslinking stimulated by high Cdc42 activity, stabilized by IQGAP1 (Fukata *et al.*, 1997, 2002). Interestingly, the participation of IQGAP1 in this process is highly dependent on its complexity: IQGAP1 monomers cannot crosslink F-actin, as a consequence of which adherent junctions are destabilized. Contrarily, IQGAP1 dimers, formed in response to Cdc42 activity, are competent for F-actin crosslinking and the generation of strong adherent junctions (LeCour *et al.*, 2016).

1.8. THE RAS/ERK PATHWAY: IMPLICATIONS IN CANCER

The RAS/ERK pathway is unquestionable linked to the origin and progression of malignant tumours in mammals, particularly in humans. About 40% of human malignant tumours harbour activating mutations in some component of this signalling route (Karnoub and Weinberg, 2008; Baines, Xu and Der, 2011). However, this figure as important as it is, fades when we consider the incidence of RAS/ERK pathway mutations in specific types of tumours, that, in some cases, can raise up to 95% (Waters and Der, no date; Jones *et al.*, 2008).

RAS is the component that appears mutated with the highest frequency, about 27% of all human tumours (Prior, Lewis and Mattos, 2012; Hobbs, Der and Rossman, 2016). The incidence of RAS mutations varies significantly depending on the organ; ranging from: 90% in pancreas (Hruban, Wilentz and Kern, 2000), 50% colon (Serebriiskii *et al.*, 2019), 50% thyroid (Lemoine *et al.*, 1989), 25% lung (Naidoo and Drilon, 2016) and 20% melanoma (Fedorenko, Gibney and Smalley, 2013), to highly infrequent in tumours such as prostate (Moul *et al.*, 1992), mammary (von Lintig *et al.*, 2000) and brain (Knobbe, Reifenberger and Reifenberger, 2004). RAS mutations also vary significantly

depending on the isoform. The RAS isoform that appears most frequently mutated in cancer is KRAS which accounts for about 85% of the cases; followed by NRAS (11%) and HRAS (4%) (Hobbs, Der and Rossman, 2016; Kodaz *et al.*, 2017). In addition to activating point mutations, RAS oncogenes appear frequently overexpressed in many types of tumours, something that contributes to their transforming potential (Bai *et al.*, 2017). Also, loss of heterozygosity for the wild type allele is common in human tumours as well (Osaka *et al.*, 1997).

RAS mutations primarily appear in codons 12, 13 and 61 (Prior, Lewis and Mattos, 2012). These mutations render RAS proteins constitutively active, by locking them in a permanent GTP-bound state, as their GTPase activity becomes insensitive to the enhancing effect of GAP proteins (Simanshu, Nissley and Mccormick, 2017). As a result of RAS constitutive activation, its effector pathways are constantly engaged conveying aberrant signals to the interior of the cell (Rajalingam *et al.*, 2007).

RAF family serine/threonine kinases also appear mutated in a large number of cancer cases; about 10% of all tumours. Almost invariably, the mutated isoform is BRAF (Davies *et al.*, 2002). As before, the frequency of BRAF mutations increases enormously when we consider specific types of tumours. As such, nearly 60% of melanoma cases harbour mutant BRAF (Ascierto *et al.*, 2012), and high incidence of BRAF mutations is also found in colorectal (10%) (Costigan and Dong, 2020), thyroid (45%) (Cohen *et al.*, 2003)and lung tumours (2%) (Leonetti *et al.*, 2018). Similarly to the RAS case, BRAF genes also appear amplified and/or overexpressed in many tumours. Over 80% of the BRAF mutations that appear in cancer involve a glutamic acid to valine substitution at codon 600. This mutation increases BRAF kinase activity several orders of magnitude and makes it largely RAS-independent in its activity (White *et al.*, 2018).

Unlike RAS and RAF, mutations in MEK are highly infrequent in human tumours. Only a few have been described, particularly in melanoma. These are mainly point mutations and truncations that enhance MEK kinase activity, but their frequency is insignificant (Nikolaev *et al.*, 2012; Maust,

Whitehead and Sebolt-Leopold, 2018). MEK overexpression has also been described in a few melanoma cases (Huynh *et al.*, 2003). Likewise, mutations in ERK are notably unusual (Jaiswal *et al.*, 2018).

A remarkable feature of the RAS/ERK pathway mutations is that they occur in a mutually exclusive fashion (Gorden *et al.*, 2003; Platz *et al.*, 2008). Tumours in which mutations in more than one component of the RAS/ERK pathway overlap are extremely rare. This clearly indicates that the constitutive activation of just one component of the signalling route is sufficient to convey transforming signals. Aberrant RAS/ERK signals, irrespective of being a consequence of RAS or BRAF mutations have been shown, in multiple cellular and animal models, to induce cellular transformation (McCubrey *et al.*, 2007). Likewise, these signals are well known to trigger, or at least to facilitate, most features characteristic of tumour progression, such as: blockade of differentiation; promotion of cell survival and/or inhibition of apoptosis; cell migration; degradation of the surrounding extracellular matrix and local invasion; intravasation; survival in the bloodstream, extravasation; colonization of distant tissues and secondary metastatic growth (McCubrey *et al.*, 2007; Roskoski, 2012; Gimple and Wang, 2019).

Similarly to the main players in the RAS/ERK pathway, scaffold proteins have also been shown to participate in neoplastic processes, such is the case of IQGAP1 (White, Brown and Sacks, 2009). IQGAP1 levels are normally elevated in many tumour types, including colon, lung, gastric and brain tumours such as oligodendroglioma, similar results have been observed in cultured cells. Indeed, IQGAP1 has been proposed as an oncogene since its overexpression can stimulate cell proliferation and favour transformation of human epithelial cells (Johnson, Sharma and Henderson, 2009). Likewise, overexpressed IQGAP1 has been utilized as a model of prostate cancer (White, Brown and Sacks, 2009). In the same vein, downregulating IQGAP1 levels in MCF7 cells using siRNAs diminishes cell migration, proliferation and prevents tumour formation in xenografted mice (Mataraza *et al.*, 2003; Jadeski *et al.*, 2008).

In tumours, no mutations with known functional consequences have been identified in IQGAP1, though the gene is amplified in some tumours (Sugimoto et al. 2001). IQGAP1 was identified as being overexpressed in lung, endometrial, ovarian, gastric, colon, and breast cancer, as well as hepatocellular carcinoma (HCC) and melanoma (Johnson et al. 2009). In gastric cancer and HCC, IQGAP1 increased in protein and mRNA levels while IQGAP2 mRNA levels were down regulated (Morris et al. 2005, Xia et al. 2014). For some authors, this suggests that IQGAP1 acts as an oncogene, and IQGAP2 as a possible tumour suppressor.

1.8.1. Inhibitors of the RAS/ERK pathway

In light of the unquestionable relevance of RAS/ERK pathway in the upbringing of cancer, it is hardly surprising that this route has attracted huge attention as a therapeutic target. And colossal efforts have been devoted both by industry and academia to find a molecular target and optimal drugs to curtail aberrant signalling through this pathway. Ever since the discovery in 1982 of RAS involvement in human cancer, multiple strategies have aimed at the RAS molecule as a potential antitumor target (Saxena *et al.*, 2008; Cox and Der, 2010). Initial moves aimed at reverting oncogenic RAS GTP-bound state were unsuccessful, mainly as a consequence of RAS picomolar range affinity towards GTP. Thus, RAS was considered for a long time as a undruggable target (Cox *et al.*, 2014; Mörchen *et al.*, 2019). This shifted the focus towards RAS posttranslational modifications, as a means to inhibit RAS localization to the plasma membrane, an essential requisite for RAS activity (Arozarena, Calvo and Crespo, 2011).

Farnesyl-transferase inhibitors, which block RAS farnesylation was highly successful in mouse models(Buss and Marsters, 1995), however, these results were not reproduced in clinical trials (Appels, 2005; Bagchi *et al.*, 2018), largely because KRAS and NRAS evade these inhibitors as they can be also geranyl-geranylated (Wang and Casey, 2016). Another strategy has been based on the inhibition of the prenylated protein methtyl transferase (PPMTase) which is involved in the methylation of RAS proteins C-terminus (Marom *et al.*, 1995). However, its inhibitor, salirasib, has

had modest success in first clinical trials (Rotblat *et al.*, 2008). More recently, the attention has been reverted to targeting RAS activity and new drugs have appeared aimed at oncogenic KRAS G12C that can revert its preference to GDP. These are presently under clinical evaluation (Stephen *et al.*, 2014; Ostrem and Shokat, 2016).

Parallel strategies have focused on the kinases downstream from RAS. In the early 90s PD098059 appeared as the first of such inhibitors, targeting MEK1/2 (Dudley *et al.*, 1995). However, pharmacological limitations (not be sufficiently soluble and bioavailable) precluded its further development, even though its mechanism of action, a non-ATP competitive allosteric inhibitor, set the tempo for the development of new inhibitors. PD184352 followed as the first MEK inhibitor with clear antitumor effect in animal models (Sebolt-Leopold *et al.*, 1999). This was the lead molecule from which new generations of derivatives have evolved and escalated to the final stages of clinical evaluation (Wang *et al.*, 2007). These include trametinib and cobimetinib, approved both by the FDA and the EMA for the treatment of BRAF mutant melanoma (Wu and Park, 2015; Cheng and Tian, 2017).

The discovery at the turn of the century of oncogenic mutation in BRAF in a significant number of human tumours has let to great developments in this realm. Sorafenib was the first BRAF inhibitor to be approved, however, it displayed modest clinical efficacy in BRAF mutant tumours and it is now used in some types of cancer like kidney due to its inhibitory effect over other kinases (Strumberg, 2005; Eisen *et al.*, 2006). Structure-based initiatives unveiled compounds that specifically bound to BRAF V600E. Included among these, was Vemurafenib (PLX4032) (Bollag *et al.*, 2012), to be followed by Dabrafenib (GSK2118436) (Rheault *et al.*, 2013). These ATP-competitive inhibitors are highly selective for this BRAF-mutant form and have been approved by FDA and EMA for their use against BRAF-mutant melanoma. Both of them have yielded significant clinical efficacy, both with respect to overall survival and disease-free progression. However, their efficacy is short-lived as drug resistance appears almost invariably after about one year, leading to a fatal end (Samatar and Poulikakos, 2014).

In the last few years compounds targeting ERK have made their appearance. The first of these, FR180204 (Ohori *et al.*, 2005) did not progress as a consequence of pharmacological shortcomings, but served as a guide for structure-based studies that have led to the development of VTX11E (Aronov *et al.*, 2009) and its derivative ulixertinib (BVD523) (Germann *et al.*, 2017). Both of these compounds are reversible ATP-competitive inhibitors and are now undergoing phase I clinical trials, and have shown efficacy in NRAS- and BRAF-mutant tumours. Another ERK inhibitor, SCH772984 (Wong *et al.*, 2014), has been shown to inhibit the phosphorylation and, subsequently, the activation of ERK, and its derivative MK8353 (Boga *et al.*, 2018) is currently in phase I trials, after promising results in pre-clinical models using BRAF-mutant tumours. Alternative strategies aiming at ERK have yielded other types of inhibitors such as DEL-22379 targeting ERK dimerization. This compound has shown a remarkable efficiency with mild toxicity in pre-clinical models both of BRAF- and RAS- mutant tumours (Herrero *et al.*, 2015).

1.8.2. KSR as an antitumoral target

Due to KSR1 relevance as an orchestrator of the RAS/ERK pathway, and in light of the resistance to tumorigenesis displayed by KSR1 KO mice, targeting KSR1 is an attractive strategy for attacking RAS-driven tumours. The absence of deleterious effects on KSR1 KO mice suggests a reduced toxicity if KSR1 was inhibited in patients. In this line, the inhibition of KSR1 expression in xenografts of RAS-driven pancreatic tumours, by continuous infusion of phosphothioate anti-sense oligonucleotides causes the regression of the tumours without overt toxicity in mice (Zhang *et al.*, 2008).

KSR mutational analyses showed that KSR mutations adjacent to its ATP binding pocket suppressed transforming RAS signals (Kornfeld, Hom and Horvitz, 1995; Sundaram and Han, 1995; Therrien *et al.*, 1995), suggesting that targeting of the ATP binding cleft may be a therapeutic option. In this line a small molecule APS-2-79 binds to and stabilizes KSR in an inactive conformation interfering with KSR-RAF heterodimerization, preventing KSR-bound MEK phosphorylation and

inhibiting oncogenic RAS signalling. However, in spite of its conceptual validity, APS-2-79 has exhibited only modest inhibitory effect on the viability of RAS-mutant and does not affect RAF-mutant tumour cells. On the other hand, it did exhibit a synergistic effect with MEK inhibitors in RAS-mutant cells suggesting a potential use in combinatorial therapy (Dhawan, Scopton and Dar, 2016; Neilsen *et al.*, 2017).

APS-2-79 modest effects do not, however, question KSR validity as a therapeutic target in cancer. But rather raise doubts whether the mechanism of action of this drug, a stabilizer of KSR inactive conformation, is the appropriate strategy for approaching the inhibition of this scaffold protein. The notion of KSR as a valid antitumor target is clearly endorsed by the phenotype exhibited by KSR-null mice, as mentioned above (Lozano *et al.*, 2003). Furthermore, as we have previously demonstrated, preventing ERK dimerization has been shown to be a valid antineoplastic strategy that has yielded effective antitumor drugs, at least in preclinical settings (Herrero *et al.*, 2015). Since it has been demonstrated that KSR, like other scaffolds, functions as an ERK dimerization platform, another way of interfering with ERK dimerization would be by preventing either: i) ERK interaction with KSR or ii) KSR-bound ERK phosphorylation; something that could be avoided by blocking KSR-MEK interaction. Thus, KSR-ERK and/or KSR-MEK interactions constitute attractive targets for the generation of novel types of scaffold inhibitors. Unravelling which of these two interactions is the best suited as a molecular target is an open question.

2. OBJECTIVES

As mentioned in the introduction, scaffold proteins are, at least conceptually, attractive antineoplastic targets. However, the only small molecule developed thus far as a scaffold inhibitor, APS-2-79 targeting KSR, has shown somewhat disappointing effects in preclinical models (Dhawan, Scopton and Dar, 2016). One possibility is that even though APS-2-79 is blocking KSR in its inactive conformation it fails to prevent the phosphorylation of KSR-bound ERK, something that was not ruled out in the original publication. Thus, one of the aims of this thesis is to unravel whether this is the case.

In this respect, since it has been shown that scaffold proteins can interact among themselves (Ren *et al.*, 2004; Vomastek *et al.*, 2004; Feigin *et al.*, 2014; Schiefermeier *et al.*, 2014), another of our aims will be to investigate if KSR interaction with some other scaffold protein/s can explain APS-2-79 inefficacy as a blocker of KSR-mediated signals.

Undisclosing a mechanistic interaction between scaffold proteins of different species would add and additional level of regulation for the phosphorylation/activation of scaffold-bound ERK. It is presumable that this process would be highly dependent on the affinity that each of the scaffold species exhibit for ERK. It is noteworthy that the affinity for ERK has not been measured for most, if not all, of the scaffold proteins described hitherto. So, another of our objectives will be to investigate whether different scaffold species exhibit different affinities for ERK.

Objective 1. To evaluate APS-2-79 as an inhibitor of KSR-mediated ERK phosphorylation/activation and of its biological consequences.

Objective 2. To investigate if KSR interactions with some other scaffold protein/s underlie in APS-2-79 inefficacy as a blocker of KSR-mediated signals.

Objective 3. To investigate whether different scaffold species exhibit different affinities for ERK.

3. MATERIALS AND METHODS

3.1. DNA MANIPULATION AND ANALYSIS

3.1.1. Plasmidic DNA purification from bacterial cultures

The purification of plasmidic DNA was carried out from bacterial cultures derived from bacterial competent cells, DH5α, an *Escherichia coli* strain modified to maximize transformation efficiency. The transformed bacteria were inoculated in 400 ml (maxiprep) or 5 ml (miniprep) of Luria-Bertani Broth (LB) culture medium with their specific resistance antibiotic provided by the plasmid, usually ampicillin or kanamycin at a concentration of 50 µg/ml. They grew shaking overnight (O/N) at 140 rpm in a 37ºC incubator. In order to harvest the bacterial cells, the cultures were centrifuged at 4ºC at 6000 rmp for 15 minutes (maxiprep) or at 4000 rpm for 5 minutes (miniprep). In the case of maxiprep (Qiagen Plasmid Maxi Kit), the pellet was resuspended in 10 ml of resuspension buffer (50mM Tris/HCl pH 8, 10Mm EDTA, 10 µg/ml RNase A). After this, cells were lysed with 10 ml of lysis buffer (200mM NaOH y 1% SDS) mixed by inverting 6 times and it was incubated with the buffer 5 minutes at room temperature and a neutralization solution was added (3M CH_3COOH pH 5,5) and it was incubated during 5 minutes. Then, the mix was centrifuged at 12000 rpm for 5 minutes. The precipitated material contains genomic DNA, proteins, cell debris, and SDS and the supernatant, where the plasmidic DNA is, was filtrated in a properly equilibrated anion-exchange Qiagen column by gravity flow. After two washes the DNA was eluted with 5 ml of elution buffer and 10 ml of isopropanol were added to precipitate the DNA. This mix was centrifuged at 10000 rpm 30 minutes at 4ºC, and then washed with 1 ml of ethanol 70%. Once dried was resuspended in 300 µl of distilled deionized water (ddw).

In the case of bacterial cultures of lower scale (5 ml volume), the bacterial culture was processed by GeneJET Plasmid Miniprep Kit (Thermo Fisher). In this procedure the harvested bacteria were resuspended by adding 250 µL of Resuspension Solution and vortexed, 250 µL of Lysis Solution was then used to lyse bacteria and incubated 5 minutes at room temperature and 350 µL of Neutralization Solution was added and centrifuged at 13000 rpm for 5 minutes. The supernatant

was then transferred to a Thermo Scientific GeneJET Spin Column and centrifuged at 13000 rpm for 1 minute. After that, columns were washed twice with 500 µL of washing buffer. To elute the purified DNA 40 µL of Elution Buffer were added to the column and incubated 10 minutes. Finally, it was centrifuged for 2 minutes at 10000 rpm and the flow-through containing the plasmidic DNA was collected.

The plasmidic DNA obtained was analyzed in the NanoDrop to check the yield and quality. And, in parallel, it was loaded in an 8% agarose gel electrophoresis, run at 80 V in TAE buffer (0.09 M Tris-acetate, 2 mM EDTA) and visualized by staining with SYBER safe (Invitrogen). A loading buffer with bromophenol blue to monitor the progress of the electrophoresis was added to the DNA sample. And, finally, it was stored at -20ºC until subsequent uses to perform transfection or cloning.

3.1.2. Plasmid description

All the plasmid *(Table 3.1)*, as well as iRNA *(Table 3.2)*, utilized in the experiments of this thesis are described in the following table.

PLASMID	***DESCRIPTION***
pCEFL	Mammal expression vector. EF-1α Promoter/ bGH poly-A Plasmid used as a control to equalize the amount of DNA to transfect.
pCMV FLAG KSR 1 WT	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the scaffold protein KSR1 fused to N-Terminal FLAG epitope. Source: J. Lozano (Joneson *et al.*, 1998)
pCMV FLAG KSR1 C809Y	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the KSR1 mutant unable to bind MEK fused to N-Terminal FLAG epitope. Source: J. Lozano

pCMV FLAG KSR1 ASAP	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the KSR1 mutant unable to bind ERK fused to N-Terminal FLAG epitope. Source: J. Lozano
pCMV FLAG KSR1 R615H	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the KSR1 mutant unable to homodimerize fused to N-Terminal FLAG epitope. Generated by site-directed mutagenesis in this thesis.
pCMV FLAG KSR1 R615H C809Y	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the KSR1 mutant unable to homodimerize and bind MEK fused to N-Terminal FLAG epitope. Generated by site-directed mutagenesis in this thesis.
pCMV FLAG KSR1 176	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the KSR1 deletion mutant including aa from 1 to 176 fused to N-Terminal FLAG epitope. Source: J. Lozano
pCMV FLAG KSR1 305	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the KSR1 deletion mutant including aa from 1 to 305 fused to N-Terminal FLAG epitope. Source: J. Lozano
pCMV FLAG KSR1 402	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the KSR1 deletion mutant including aa from 1 to 402 fused to N-Terminal FLAG epitope. Source: J. Lozano
pCMV FLAG KSR1 521	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the KSR1 deletion mutant including aa from 1 to 521 fused to N-Terminal FLAG epitope. Source: J. Lozano
pCMV FLAG KSR1 ΔN	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the KSR1 C-terminal region including aa from 541 to 873 fused to N-Terminal FLAG epitope. Source: J. Lozano
pBHEN	Mammal expression vector. SV40 Promoter/ poly-A

	pBABE retroviral vector with modified polilinker (HindII-BamHI-EcoRI-NotI). Plasmid used as a control to equalize the amount of DNA to transfect. Source: A. Herrero
pBHEN FLAG KSR1 WT	Mammal expression vector. SV40 Promoter/ poly-A Encodes the scaffold protein KSR1 fused to N-Terminal FLAG epitope. Generated by subcloning from pCMV FLAG KSR1 WT.
pBHEN FLAG KSR1 ASAP	Mammal expression vector. SV40 Promoter/ poly-A Encodes the KSR1 mutant unable to bind ERK fused to N-Terminal FLAG epitope. Generated by subcloning from pCMV FLAG KSR1 ASAP.
pBHEN FLAG KSR1 C809Y	Mammal expression vector. SV40 Promoter/ poly-A Encodes the KSR1 mutant unable to bind MEK fused to N-Terminal FLAG epitope. Generated by subcloning from pCMV FLAG KSR1 C809Y.
pBHEN FLAG KSR1 R615H	Mammal expression vector. SV40 Promoter/ poly-A Encodes the KSR1 mutant unable to homodimerize fused to N-Terminal FLAG epitope. Generated by subcloning from pCMV FLAG KSR1 R615H.
pBHEN FLAG KSR1 R615H C809Y	Mammal expression vector. SV40 Promoter/ poly-A Encodes the KSR1 mutant unable to homodimerize and bind MEK fused to N-Terminal FLAG epitope. Generated by subcloning from pCMV FLAG KSR1 R615H/C809Y.
pBHEN FLAG KSR1 WT	Mammal expression vector. EF-1α Promoter/ bGH poly-A Encodes the scaffold protein KSR1 fused to N-Terminal FLAG epitope. Generated by subcloning from pCMV FLAG KSR1 WT.
pCEFL FLAG KSR1 ASAP	Mammal expression vector. EF-1α Promoter/ bGH poly-A Encodes the KSR1 mutant unable to bind ERK fused to N-Terminal FLAG epitope. Generated by subcloning from pCMV FLAG KSR1 ASAP.

pCEFL FLAG KSR1 C809Y	Mammal expression vector. EF-1α Promoter/ bGH poly-A Encodes the KSR1 mutant unable to bind MEK fused to N-Terminal FLAG epitope. Generated by subcloning from pCMV FLAG KSR1 C809Y.
pCEFL FLAG KSR1 R615H	Mammal expression vector. EF-1α Promoter/ bGH poly-A Encodes the KSR1 mutant unable to homodimerize fused to N-Terminal FLAG epitope. Generated by subcloning from pCMV FLAG KSR1 R615H.
pCEFL FLAG KSR1 R615H C809Y	Mammal expression vector. EF-1α Promoter/ bGH poly-A Encodes the KSR1 mutant unable to homodimerize and bind MEK fused to N-Terminal FLAG epitope. Generated by subcloning from pCMV FLAG KSR1 R615H/C809Y.
pCDNA3 Ksr1 GLU	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the scaffold protein KSR1 fused to C-Terminal GLU (PYO) epitope. Source: W. J. Fantl (Yu *et al.*, 1998)
pEF-BOS MYC IQGAP1	Mammal expression vector. EF-1α Promoter Encodes the scaffold protein IQGAP1 fused to N-terminal MYC epitope. Source: K. Kaibuchi
pCDNA3 IQGAP1 WW	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the scaffold protein IQGAP1 deletion mutant lacking WW domain (aa missing 643-744), unable to bind ERK, fused to N-Terminal Myc epitope. Source: D. Sacks
pCDNA3 IQGAP1 ⊿IQ	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the scaffold protein IQGAP1 deletion mutant lacking IQ domain (aa missing 699-905), unable to bind MEK, fused to N-Terminal Myc epitope. Source: D. Sacks
pCDNA3 IQGAP1 ⊿CHD	Mammal expression vector. CMV promoter/ bGH poly-A

	Encodes the scaffold protein IQGAP1 deletion mutant lacking CHD domain (aa missing 37-265) fused to N-Terminal Myc epitope. Source: D. Sacks
pCDNA3 IQGAP1 N1	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the first half (aa 1-431) of the N-terminal region of the scaffold protein IQGAP1, fused to N-Terminal Myc epitope. Source: D. Sacks
pCDNA3 IQGAP1 N2	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the second half (aa 432-863) of the N-terminal region of the scaffold protein IQGAP1, fused to N-Terminal Myc epitope. Source: D. Sacks
pCDNA3 IQGAP1 N	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the N-terminal region (aa 1-863) of the scaffold protein IQGAP1, fused to N-Terminal Myc epitope. Source: D. Sacks
pCDNA3 IQGAP1 C	Mammal expression vector. CMV promoter/ bGH poly-A Encodes the C-terminal region (aa 864-1657) of the scaffold protein IQGAP1, fused to N-Terminal Myc epitope. Source: D. Sacks
pGEX 4T-3 KSR1 ERK BD	Bacterial expression vector. Tac Promoter Encodes GST fused to ERK binding domain of KSR1 (aa: 901-1203). Source: A. Herrero
pGEX HA IQGAP1 WW	Bacterial expression vector. Tac Promoter Encodes GST fused to ERK binding domain of IQGAP1. Source: M. Morante
pGEX 4T MP1	Bacterial expression vector. Tac Promoter Encodes GST fused to MP1. Source: L. Huber
pNpT 7-5 His ERK2	Bacterial expression vector. T7 Promoter

Encodes N-terminal His-tagged ERK2. Source: M. Cobb

Table 3.1. Description of the plasmids used for mammal and bacterial expression

iRNA	Company
siRNA against KSR1 (human) 10 µM	sc-35762, Santa Cruz Biotechnology
siRNA against IQGAP1 (human) 10 µM	sc-35700, Santa Cruz Biotechnology
shRNA against KSR1 (human)	TRCN 006226, TRCN 006227, TRCN 006229, TRCN 006230 XM 290793, Sigma-Aldrich
shRNA against KSR2 (human)	TRCN 007062, TRCN 335901, TRCN 199619, TRCN 199136, TRCN 195374 NM 173593, Sigma-Aldrich
shRNA against IQGAP1 (human)	TRCN 47485, TRCN 47487, TRCN 298928, TRCN 298930, TRCN 298931 Sigma-Aldrich

Table 3.2. iRNAs used to knock down scaffold proteins

3.1.3. Plasmid cloning

For subcloning the different constructs of pCMV Flag KSR1 mentioned above *(Table 3.1)* into the pBHEN vector, the restriction sequences were introduced amplifying the gene of interest (Flag-KSR1 WT; ASAP; C809Y; R615H; R615H/C809Y) by PCR using primers targeting the 5' and 3' of the gene. In this case the restriction sites EcoRI (5') and NotI (3') were introduced by PCR *(Table 3.3)* using the following primers:

***EcoRI* Flag KSR1:** 5' GGT GGT *GAATTC* ATG GAC TAC AAG GAC GAT

KSR1 *NotI*: 3' TGC TTC *GCGGCCGC* CTA CAT CTT TGG ATT ACC

- The PCR reaction to perform the amplification of the fragments was as follow:

10 µl of 5X reaction buffer

1 µl of dsDNA template (50 ng/µl)

2.5 µl of each oligonucleotide primer (10 µM)

1 µl of dNTP mix (10 mM)

ddH_2O to a final volume of 50 µl

Then add 0.5 µl of Phusion High-Fidelity DNA Polymerase (2 U/µL, ThermoFisher) on ice.

- The parameters to run the PCR were:

Step	Cycles	Temperature	Time
Denaturing	1 X	98ºC	30 seconds
Denaturing	30 X	98 ºC	30 seconds
Primers Annealing		68 ºC	30 seconds
Extending		72 ºC	2 min

Elongation	1 X	72 ºC	10 min
Hold	1 X	4ºC	∞

Table 3.3. Cloning PCR conditions

PCR product was digested with the restriction enzymes specific for the inserted restriction sites following the manufacturer's protocol (ThermoFisher). DNA fragments were separated by agarose gel electrophoresis at 0.8% w/v. Agarose gels were prepared by dissolving agarose (% w/v) in 1X Tris acetic acid EDTA (TAE) buffer. SYBR safe (Invitrogen) was added to allow visualization of DNA under UV lights. DNA samples were mixed with DNA loading buffer (0.005% (w/v) bromophenol blue (Sigma) and 30% of glycerol (Sigma) and run at 80-100 V. 1Kb DNA ladder was used as molecular weight marker. DNA band, corresponding to the desired fragment, was excised and purified from agarose gel using Thermofisher column, according to the manufacturer's instructions. Once vector and DNA insert were purified, ligation mediated by T4 DNA ligase (Promega) was performed. Ligation reaction was carried out O/N at 22 ºC, in a final volume of 20 µL using ligation buffer (300 mM Tris-HCl, pH 7.8, 100 mM $MgCl_2$, 10 mM ATP, 100 mM DTT). Finally, 4-6 µL of ligation mix were transformed into DH5α Escherichia coli competent cells (Invitrogen). 2 µl of the ligation product was added over the competent bacteria, they were kept on ice for 30 minutes. The mix was exposed to a heat shock for 1 minute and, finally, to 5 minutes on ice. The transformation product was incubated during 1 hour at 37ºC in SOC medium antibiotic-free, to allow the expression of the resistance antibiotic gene. Transformed bacterial were then seeded in LB-agar plates made dissolving 1.5% of agar and 50 µg/mL of ampicillin for selection. Some colonies were inoculated into LB media O/N and purified for verification by digestion and sequencing.

On the other hand, the subcloning of the aforementioned genes (KSR1 WT; ASAP; C809Y; R615H; R615H/C809Y) into pCEFL was carried out by digestion of the insert from the pBHEN generated constructs, as well as pCEFL vector, by EcoRI and NotI restriction enzymes. The DNA

fragments were run in an agarose gel, cut, purified and ligated to the new pCEFL vector following the same process previously described.

3.1.4. Site-directed mutagenesis

The QuickChange II site-directed mutagenesis kit (Agilent Technologies) was used to introduce point mutations, to replace a given aminoacid. The procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers, both containing the desired mutation. To design these primers, we considered a length between 25 and 45 bases with a melting temperature (Tm) ≥78°C.

The following formula was used for estimating the T_m of the primers:

$T_m = 81.5 + 0.41(\%GC) - (675/N) - \%mismatch$

For calculating T_m:

- *N* is the primer length in bases
- values for **%GC** and **% mismatch** are whole numbers

Both of the mutagenic primers must contain the desired mutation preferably in the middle and anneal to the same sequence on opposite strands of the plasmid. The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

The primers (Hylabs) used to introduce the point mutation R615H in pCMV Flag KSR1 and in pCMV Flag KSR1 C809Y were:

Mutation R615H: R>H = CGG>CAT

Oligonucleotide 5'-3': GAACTACCGGCAGACG**CAT**CATGAGAACGTGGTGC

Oligonucleotide 3'-5': GCACCACGTTCTCATG**ATG**CGTCTGCCGGTAGTTC

Length: 35 nucleotides

Mismatch=2 (5.71%)

GC=20 (57.14%)

Tm= **79.93**

- The PCR reaction to perform the mutagenesis was as follow:

5 µl of 10X reaction buffer

1.5 µl of dsDNA template (100 ng/µl)

1.2 µl of each oligonucleotide primer (10 µM)

1 µl of dNTP mix (10 mM)

ddH_2O to a final volume of 50 µl

Then add 1 µl of PfuUltra HF DNA polymerase (2.5 U/µl) on ice

- The parameters to run the PCR for **KSR1** mutants were:

Step	Cycles	Temperature	Time
Denaturing	1 X	95ºC	2 minutes
Denaturing	30 X	95ºC	30 seconds
Annealing primers		60ºC	2 minutes
Extending		65ºC	10 minutes
Elongation	1 X	72ºC	20 minutes

Hold	1 X	4ºC	∞

Table 3.4. PCR conditions to introduce the point mutation R615H into pCMV Flag KSR1 and pCMV Flag KSR1C809Y

The PCR product was placed on ice. Then, 1 µl of the *Dpn* I restriction enzyme (target sequence: 5´-Gm^{6}ATC-3´) (10 U/µl) was added directly to each amplification reaction and it was incubated 1 hour and 30 minutes at 37ºC in order to digest the parental methylated DNA template to select for mutation-containing synthesized DNA. The last step was to transform the 10 µl of PCR product into competent bacteria by heat shock: Incubate DNA and bacteria 30 minutes on ice, 1 minute heat shock at 42ºC and 5 minutes on ice to close again the bacterial wall. Add 200 µl of culture medium without antibiotic selection and place the tubes at 37ºC for 1 hour and 30 minutes. Then, the transformation product was added to LB agar plates with the specific antibiotic selection and leave them growing O/N at 37ºC. The day after, some colonies were grown O/N on LB medium and the DNA extracted by miniprep was analyzed by sequencing.

3.2. TISSUE CULTURE

3.2.1. Cell lines

The cell lines utilized during this thesis are listed in the following table *(3.5)*.

CELL LINE	**DESCRIPTION**
HEK 293T	Epithelial cells derived from Human Embryo Kidney. Immortalized with SV40 T-antigen
HeLa	Epithelial cells derived from human cervical carcinoma.

MEFs	Mice Embryonic fibroblasts.
MEFs KSR1 -/-	Mice Embryonic fibroblasts. KSR1 Knock Out. (Nguyen *et al.*, 2002) Source: J. Lozano
A375P	Epithelial human metastatic melanoma cells. B-RAF mutant
501-MEL	Epithelial human metastatic melanoma cells. B-RAF mutant
SKMEL28	Epithelial human metastatic melanoma cells. B-RAF mutant
SKMEL2	Epithelial human metastatic melanoma cells. N-Ras mutant
MEL-JUSO	Epithelial human metastatic melanoma cells. N-Ras mutant
CJM	Epithelial human metastatic melanoma cells. N-Ras mutant

Table 3.5. Cell lines used in this thesis.

Culture Medium: These cell lines were grown in Dulbecco´s Modified Eagle Medium (DMEM) (Thermo Fisher) supplemented with 10% Fetal Bovine Serum (Gibco) and 1% Penicillin-Streptomycin (10000 U/mL) (Thermo Fisher).

Basal medium: DMEM culture mediium without Fetal Bovine Serum.

All cells were grown at a 37ºC and 5% CO_2.

Throughout this thesis:

EGF (Epidermal Growth Factor) has been used to stimulate cells at 50 ng/ml for 5 minutes.

The KSR inhibitor APS-2-79 (Med-Chem Express) was administered to HEK293T and to melanoma cells at 5 µM for from 2 hours, O/N or 48 hours, depending on the experiment.

The MEK inhibitor Vemurafenib (Selleck Chemicals) (PLX4032) was used in melanoma cells at 10 µM for 48 hours.

3.2.2. Mammalian cell transfection

3.2.2.1. Polyethylenimine (PEI)

Polyethylenimine (PEI) condenses DNA into positively charged particles that bind to anionic cell surfaces. Consequently, the DNA:PEI complex is endocytosed by the cells and the DNA is released into the cytoplasm (Longo et al., 2013).

HEK293T cells were split into p60 plates 24 hours before transfection because the cells are actively proliferating and, in this way, it is easier that DNA reaches the nucleus. The cells are transfected when they are at around 60% of confluence. PEI (1 mg/ml) (Polysciences, Inc.) is used in a ratio 1:4 (DNA:PEI). 8 µg of PEI were added to 500 µL of Opti-MEM medium (Gibco) and 2 µg of DNA was diluted in the same tube. The mix was vortexed and spinned down and incubated 15 minutes at room temperature. Before adding the mix to the cells, they were washed with 1X PBS (Phosphate-Buffered Saline), and 10% FBS DMEM was added to a final volume of 2.5 ml. For an optimal expression the cells were not harvested before 48 hours post-transfection.

3.2.2.2. Lipofectamine LTX

Lipofectamine contains lipids that can form liposomes in an aqueous environment which catches the DNA plasmids. The cationic liposomes form a complex with negatively charged DNA to overcome the electrostatic repulsion of the cell membrane. (Cardarelly et al., 2016)

HeLa cells were transfected using Lipofectamine LTX (Invitrogen. Thermo Fisher). Cells were split 24 hours before transfection to reach 80% of confluence at transfection. 8 µL of LTX was diluted

in 250 µL Opti-MEM medium (Gibco. Thermo Fisher). Then, 2 µg of DNA was diluted in another Eppendorf tube containing 250 µL Opti-MEM medium and 4 µL of PLUS Reagent was added. After 5 minutes at room temperature, the content of the DNA tube was added to the Lipofectamine LTX Reagent tube and was incubated at room temperature for 10 minutes. Meanwhile, the cells were washed with 1X PBS and the DNA-lipid complex was added to the cells. Next day, the medium was changed and cells were incubated for 24 hours more before collecting them.

3.2.2.3. Lipofectamine 2000

A375P melanoma cells were transfected following an adapted protocol of Lipofectamine 2000 (Invitrogen. Thermo Fisher). Two Eppendorf tubes with 500 µL of Opti-MEM medium were prepared, in one of them 2 µg of DNA was added and, in the other one, 8 µL of Lipofectamine 2000 reagent (ratio 1:4). They were incubated separately at room temperature for 5 minutes. Then, they were mixed, vortexed and incubated for 30 minutes at room temperature. The cells were washed with 1X PBS before adding the transfection mixture and, once added, the cells were incubating with this 1 ml mixture for 30-45 minutes at 37ºC, 5% CO_2. After this incubation, 10% FBS DMEM was added to a total volume of 3 ml. For an optimal expression the cells were harvested at least 48 hours post-transfection.

3.2.2.4. Lipofectamine 3000

The SKMEL2 melanoma cells transfection was done with Lipofectamine 3000 (Invitrogen. Thermo Fisher). 2 µg of DNA plus 14 µL of P3000 reagent were diluted in 250 µL of Opti-MEM medium and 7 µL of Lipofectamine 3000 reagent were added to another tube with 250 µL of Opti-MEM medium. They were incubated separately for 5 minutes at room temperature and, then, the content of the DNA tube was added to the one with Lipofectamine. They were incubated for 10 minutes and added to the cells previously washed with 1X PBS. 2 ml of 10% FBS DMEM were added. For an optimal expression, the cells should not be harvested before 48 hours post-transfection.

3.2.2.5. Lipofectamine RNAiMAX

The small interfering RNA (siRNA) against scaffold proteins were transfected with Lipofectamine RNAiMAX (Invitrogen. Thermo Fisher). 10 µL of 10 µM siRNA were diluted in 250 µL of Opti-MEM medium and in other tube with 250 µL of Opti-MEM medium 10 µL of Lipofectamine RNAiMAX were added. They were incubated separately for 5 minutes at room temperature and, then, they were mixed and vortexed and incubated together for 10 minutes at room temperature. After this incubation, the mix was added to the cells after a wash with 1X PBS. For an optimal knocking down, cells should not be collected before 48 hours post-transfection.

3.2.2.6. Nucleofection

Electroporation is a physical transfection method that permeabilizes the cell membrane by applying an electrical pulse and moves molecules via the electrical field into the cell.

Mice embryonic fibroblasts (MEFs) were transfected by nucleofection. Around 8 millions of cells were washed with 1X PBS and trypsinized to detach them. They were centrifuged at 1500 rpm for 5 minutes and the pellet was resuspended in 400 µL of electroporation solution (Ingenio® Electroporation Kit. Mirus). The resuspended cells were split in four Eppendorf tubes (100 µL per tube) and around 2-3 µg of the corresponding DNA were added. They were gently mixed avoiding bubbles and transferred to an electroporation cuvette. The cells in suspension were electroporated by an electrical pulse, duration and voltage of the pulse is detailed by the MEFs specific program (A 023) in the nucleofector (Lonza). After the pulse, the nucleofected cells were resuspended in 800 µL of 10% FBS DMEM and 200 µL were seeded in each well of a T24 plate. For an optimal result the cells need 48 hours of expression after transfection.

3.2.2.7. Stable cell lines generation

2 µg of the Flag-tagged plasmids constructs *(Table 3.1)*: pHBEN KSR1 WT, pHBEN KSR1 C809Y, pHBEN KSR1 R615H and pHBEN KSR1 R61H/C809Y were transfected in the parental cell line MEFs KSR1 -/- following the aforementioned lipofectamine 3000 transfection method. The pHBEN vector contains an antibiotic-resistance gene for puromycin which functions as a drug-resistance positive marker in the transfectants. The selection antibiotic, puromycin, was added at the optimal

concentration of 1 µg/ml replacing the media with selection antibiotic every 2 days up to a week selecting the transfecting cells. The ectopic expression of each cell subline was verified by western blot, using either Flag or KSR1 antibodies.

3.2.3. Cell proliferation assay

This assay was used to determine the effect of the silencing of IQGAP1 and KSR1 scaffold proteins in B-RAF and N-Ras melanoma cell lines. Therefore, 24 hours post- transfection with the different shRNAs, the cells were counted by Neubabuer chamber or Nucleocounter (method based on propidium iodide staining), and 6000 cells were plated per well in three 96-well plates, one for each time point (24, 48 and 72 hours) and three replicates per condition. At the estimated time, 10 µL of room temperature AlamarBlue Reagent was added and incubated in the dark at 37ºC and the absorbance was read every 30 minutes from one hour after the reagent was added.

The AlamarBlue Cell Viability Reagent (Thermo Fisher), used to perform proliferation assays, has as an active component a non-toxic, cell-permeable non-fluorescent blue compound called Resazourin. This molecule can be reduced by several mitochondrial reductases (NADPH, NADH, FADH) as it acts as an intermediate electron acceptor in the electron transport chain and as well by cytochromes and other enzymes as flavin reductase or NAD(P)H: quinone oxidoreductase. This indicates viability and metabolic activity and, indirectly, number of cells by medium colour change to a red highly fluorescent compound that is Resorufin. In consequence, this change of colour can be measure using absorbance-based plate readers using 600 nm as a reference wavelength and monitoring reagent absorbance at 570 nm.

3.2.4. Apoptosis assay

A million cells were plated per T6 plate well. 24 hours later, the cells were transfected with the corresponding shRNAs, one well was transfected with an empty vector as a negative control, in other well APS-2-79 (5 μM; 48 h) (Med-Chem Express) was added. And, in another one, Staurosporine (1 μM) (Sigma) was added for 12 hours as positive control. In parallel, the same transfections were carried out in p60 plates to check the expression or silencing.

48 hours post- transfection the medium was collected into a 5 ml Eppendorf tube, 250 μl of 10X trypsin were added in each well and the cells were resuspended with the same medium previously collected. The cells were collected by centrifugation at 800 rpm for 5 minutes at 4ºC. They were washed with 1 ml of filtrated 3 mM EDTA PBS and centrifuged again. The pellet was resuspended in 300 μl of binding buffer (BB) (10X BB: HEPES 0.1 M pH 7.4, NaCl 1.4 M, $CaCl_2$ 25 mM) and placed in cytometry tubes. Then, 1 μl of FITC Annexin V (BD Pharmagen) and 10 μl of FBS were added to avoid unspecific interactions. The mix was incubated 30 minutes in dark at 4ºC. After incubation, one wash with 1 ml 3 mM EDTA PBS was done and the cells were collected by centrifugation and resuspended in 250 μl of 3 mM EDTA PBS to do the flow cytometry. Apoptosis rate was determined in MACSQuant VYB (Miltenyi Biotec) and the results were analyzed with Flow Logic software (Miltenyi Biotec).

To analyze the apoptotic effect of pCMV Flag KSR1 WT and pCMV Flag KSR1 C809Y overexpression SKMEL2 cells were plated in a T6 plate (1 million cells per well) and transfected with increasing amounts (0.5, 1 and 2 μg) of the corresponding DNA's following the Lipofectamine 3000 transfection protocol. 48 hours post-transfection the apoptosis was assessed by Annexin V + as previously described.

In the case of APS apoptotic effect analysis, A375, SKMEL28 and 501-Mel BRAF-mutant melanoma cell lines and SKMEL2, CJM and Mel-Juso NRAS-mutant melanoma cells were plated in a T6 plate (1 million cells per well). These cells were treated with APS (5 μM 48 hours), PLX4032 (10

μM 48 hours) as RAS/ERK pathway specific inhibitor or Staurosporine (0.5 μM 48 hours) as a positive control of apoptosis. The Annexin V + analysis was carried out as mentioned above.

3.3. PROTEIN ANALYSES

3.3.1. SDS-PAGE and western blotting

Cell plates were collected on ice, the culture medium was removed and the cells were washed in cold 1X PBS and harvested in 200-500 μl of lysis buffer. Then, to remove cell debris and DNA and keep the proteins, the lysates were centrifuged at 13000 rpm for 10 minutes at 4ºC. After that, the supernatant was collected into a new tube and proteins samples were quantified according to the absorbance at 620 nm wavelength using the Bradford Method. A Bovine Serum Albumin (BSA) standard curve was used to calibrate unknown concentrations. 5X laemli loading buffer was added to around 30 μg of protein and the mix was boiled at 95ºC for 5 minutes.

Proteins were resolved in sodium dodecyl sulfate (SDS)- poliacrylamide gel electrophoresis (PAGE). SDS-gel was composed of a stacking part and a resolving part. The vertical electrophoresis was performed in a Mini-Protean Bio-Rad device with running buffer. Then, proteins were electrophoretically transferred to Nitrocellulose membranes (Thermo Fisher) at 400 mA constant amperage (1 minute for each 1 kDa of the protein) at 4ºC in transfer solution. Membranes were then blocked in Tris Buffered Saline-Tween (TBS-T) containing 4% BSA (blocking solution) for 1 hour shaking at room temperature. Blots were incubated from 1 hour at room temperature to O/N at 4ºC (depending on the antibody affinity) with the different antibodies *(Table 3.6)* prepared in blocking solution. The blots were washed 3 times for 10 minutes with TBS-T and incubated for 1 hour shaking at room temperature with anti-rabbit Immunoglobulin (Ig) (Bio-Rad) or anti-mouse Ig (Bio-Rad) secondary antibodies conjugated with peroxidase (1:10000) in 2% milk (GE Healthcare)

TBS-T. After that, membranes were washed (3 x 10') with TBS-T and the proteins were detected by chemiluminescence with an enhanced chemiluminescent system (ECL) and an autoradiography with Konica films was performed to develop the blots.

Buffers used:

- **Lysis buffer**: 20 mM HEPES pH 7.5, 10 mM EGTA, 40 mM β-Glycerophosphate, 1% NP40, 2.5 mM $MgCl_2$, 1 mM $NaVO_4$, 1 mM DTT and protease inhibitors: 10 µg/ml of aprotinin and 10 µg/ml of leupeptin.
- **5X laemli loading buffer:** 100 mM Tris pH 6.8, 4% SDS, 20% glycerol, 20 mM DTT and 0.005% bromophenol blue.
- **Poliacrylamide gels**:
  - Stacking gel part: 4% acrylamide, 125 mM Tris-HCl pH 6.8, 0.4% SDS, 0.1% Ammonium Persulfate (APS) and 0.1% Tetramethylethylenediamine (TEMED) in H_2O.
  - Resolving gel part: acrylamide (percentage depends on the molecular weight of the protein), 375 mM Tris-HCl pH 8.8, 0.4% SDS, 0.1% APS and 0.1% TEMED in H_2O.
- **Running buffer**: 25 mM Trizma base, 192 mM Glycine, 0,1% SDS.
- **Transfer buffer**: 25 mM Trizma base and 192 mM Glycine.
- **Tris Buffered Saline-Tween (TBS-T):** 20 mM Tris, pH 7.4, 137 mM NaCl and 0.05% tween.

Enhanced chemiluminescent system (ECL):

Solution 1: 1M TRIS HCl pH 8.5, 90 mM Coumaric Acid, 250 mM Luminol.

Solution 2: 1M TRIS HCl pH 8.5, 30% H_2O_2 (Hydrogen Peroxide).

ANTIBODIES

ANTIBODY	SPECIFICITY	DILUTION	SOURCE
Flag	Mouse monoclonal	1:5000 (WB) IP: 0.5 µg	F1804, Sigma

		1:150 (IF)	
Glu-Glu (Pyo)	Rabbit policlonal	1:1000 (WB)	AB3788, Millipore
ERK2	Mouse monoclonal	1:1000 (WB)	sc-1647, Santa Cruz
MAPK (ERK1/2)	Rabbit policlonal	1:1000 (WB)	Cell Signalling 4695S
p-ERK1/2 (Tyr 204)	Mouse monoclonal	1:1000 (WB)	sc-7383, Santa Cruz
Activated MAP Kinase (Diphosphorylated ERK-1/2: Thr183 and Tyr185)	Mouse monoclonal	IP: 0.5 µg	M9692, Sigma
Activated MAP Kinase (Diphosphorylated ERK-1/2: Thr183 and Tyr185)	Mouse monoclonal	1:1000 (WB) 1:100 (IF)	M8159, Sigma
p-MEK1/2 (S217/S221)	Rabbit policlonal	1:3000 (WB)	9154S, Cell Signalling
MEK	Rabbit policlonal	1:500 (WB)	8727S, Cell Signalling
α-Tubulin	Mouse monoclonal	1:5000 (WB)	T5168, Sigma
Myc tag	Mouse monoclonal	1:1000 (WB)	06-549, Millipore
Myc tag	Mouse monoclonal	IP: 1 µg	(9E10) MA1-980, Thermo Fisher
KSR1	Mouse monoclonal	IP: 1 µg	sc-515924, Santa Cruz
KSR1	Rabbit policlonal	1:500 (WB)	(EPR2421Y) ab68483, Abcam
IQGAP 1	Mouse monoclonal	1:1000 (WB)	610611, BD Bioscience

IQGAP 1	Mouse monoclonal	IP: 1 µg	sc-376021 Santa Cruz
Anti-Mouse-HRP (Mouse IgG)	Goat	1:10000	170-5047, Bio-Rad
Anti-Rabbit-HRP (Rabbit IgG)	Goat	1:10000	170-5046, Bio-Rad
Alexa Fluor 488 (Mouse IgG)	Goat	1:300	A-11034, ThermoFisher
Alexa Fluor 594 (Rabbit IgG)	Goat	1:800	A-11032, ThermoFisher

Table 3.6. Antibodies used for western blot, immunoprecipitation and immunofluorescence analysis.

3.3.2. Protein interaction by co-immunoprecipitation assay

Previous to collecting the cells, they were washed with cold PBS. The total lysates were centrifuged at 13000 rpm at 4ºC for 10 minutes. The cleared lysates were quantified and around 30 µg of protein from the total lysate were separated and loading buffer Laemli 5X was added. 0.5-1 µg of the specific antibody for immunoprecipitation was added to 300 µg of protein, and it was incubated rocking at 4ºC from 2 hours to O/N. After this, 20 µl of protein G-Sepharose 4B (GE Healthcare, 17-0756-01) were added to the lysates with antibody and it was incubated 20 minutes at 4ºC shaking. The protein G is going to bind the immunoglobulins which allows to precipitate the immunocomplexes (protein-antibody) by centrifugation. The next step was washing the beads once with lysis buffer and twice with cold 1X PBS 1% NP-40. Finally, the beads were resuspended in 20 µl of loading buffer Laemli 2.5 X and boiled 5 minutes at 95ºC. The protein interaction was analyzed by SDS-PAGE as previously described.

3.3.3. Protein interaction by proximity ligation assay + immunoflorescence

Proximity Ligation Assay (PLA) (Duolink®) is a technology which allows to detect *in situ* protein interaction. For these purpose two primary antibodies, raised in different species, specific for each protein of interest are used. These primary antibodies are detected by secondary antibodies (PLA probes) bound to a short single strand DNA sequence. When both probes are close enough (<40 nm), the DNA strands are able to hybridize forming a rolling circle DNA (ligation reaction). During the amplification reaction, concatemeric product of this nucleotide sequence were synthesized at the same time that a fluorophore, detected at 594 nm wave length, is intercalated. The signal is easily observed in the microscope as red fluorescent spots in the cellular localization where the analyzed proteins are interacting.

The transfected HeLa cells were grown to subconfluence in a glass of 10 mm of diameter. The cells were washed with 1X PBS and were fixed with 4% paraformaldehyde in 1X PBS during 10 minutes at room temperature. Later they were washed twice with 1X PBS during 5 minutes, followed by one wash with 0.1 M glycine and two with 1X PBS. Subsequently, they were permeabilized during 10 minutes with a solution composed by 0.1 M glycine, 0.5% Triton X-100 in PBS, followed by three washes with 1X PBS for 5 minutes. Then, the cells were blocked during 15 minutes by adding one drop over each glass of 3% BSA, 0.01% Triton X-100 in 1X PBS. The primary antibodies were prepared in blocking solution in a dilution from 1:75 to 1:200 depending on the antibody specificity, they were also added as a drop over the glass and incubated for 1 hour in a humidity chamber to prevent drying. During the incubation the MINUS and PLUS PLA probe solution was prepared in a ratio 1:3 with blocking buffer and the mix was incubated for 20 minutes at room temperature. After the primary antibody incubation, the cells were washed twice 5 minutes with buffer A (0.15 M NaCl, 0.01 M TRIS base, 0.05 % Tween 20 pH 7.4 filtered) and then a drop of PLA probe solution was added per glass and the humidity chamber was left incubating at 37ºC for 1 hour. During incubation the ligation solution (ligation buffer diluted 1:5 and ligase 1:40 in ultrapure water) was prepared, adding the ligase just before to use. One drop of ligation solution was added

over the glasses after two 5-minutes washes with buffer A and it was incubated at 37ºC for 30 minutes. After ligation, the cells were washed twice with buffer A, followed by the addition of the amplification solution (amplification buffer diluted 1:5 and polymerase 1:80 in ultrapure water). The amplification step lasts 100 minutes at 37ºC. After the incubation period the cells were washed twice for 10 minutes with buffer B (0.1 M NaCl, 0.2 M TRIS base, TRIS HCl pH 7.5) and they were left at 4ºC O/N. The day after, a secondary antibody (conjugated with a fluorophore), specific for the primary antibody which recognizes the transfected protein, was added for 1 hour in the humidity chamber and washed twice with 1X PBS. Finally, the glasses were set over a slide in mounting media with DAPI and sealed with clear nail polish.

The cells were examined by fluorescence microscopy (photomicroscope Axiophot, Carl Zeiss). The images were processed using Image J software.

3.3.4. Cytosolic Phospholipase A_2 activation assay

MEFs KSR1 -/- were nucleofected, as previously described, with the different KSR1 constructions. 48 hours post-transfection the cells were deprived of serum and 1 µCi/ml tritiated arachidonic acid (Perkin Elmer) was added to the medium in order to be incorporated into the plasma membrane. After 18 hours of incubation, the cells were washed twice with fatty acids- free DMEM, 5mM Hepes pH 7,5, 0.2% BSA, the medium was replaced by this and EGF was added (50 ng/ml, 2 hours) where it corresponded. 2 hours later, 500 µl of the medium were taken and mixed with 2 ml of scintillation liquid in a counting vial. The radioactive emission was measured by scintillation counter.

In this way, the marked arachidonic acid released to the medium is a phospholipase A_2 activation indicator.

3.3.5. GST and pull-down assays

- **Obtaining recombinant proteins for *in vitro* protein binding assays**

The gene fusion system GST (Glutatione S-tranferase) is a useful method to purify proteins from *E. coli* as fusion with GST from *Schistosoma japonicum*. The pGEX plasmids are deigned to obtain high inducible expression levels of whole genes or genes fragments.

The recombinant fusion proteins (pGEX KSR1 ERK BD, pGEX IQGAP1 WW and pGEX MP1) were transformed in *E. coli*, and, then, inoculated in 50 ml of LB medium with the corresponding antibiotic resistance O/N at 37ºC. The day after, this inoculum was diluted in 400 ml of LB medium and it was grown for 4 hours at 37ºC. Induction of the recombinant proteins was carried out by adding to the culture 0.2 mM of IPTG (isopropil-β-D- tiogalactopiranoside, Sigma) shaking at 37ºC for 3 hours. The bacteria were collected by centrifugation at 6000 rpm for 10 minutes and the pellet was resuspended in 10 ml of 1X PBS, 1% NP-40, 10 µg/ml aprotinine and 10 µg/ml leupeptine. They were sonicated on ice at 80% amplitude, 0.9 cycles for 7 minutes. The extract from sonication was centrifuged at 3500 rpm for 30 minutes at 4ºC, and 500 µl de Glutation-Sepharose 4B (GE) were added to the supernatant in order to precipitate the GST protein. The mix was incubated rocking at 4ºC for 3 hours. The resin Glutation-Sepharose 4B bound to the proteins was washed three times with cold washing buffer, twice with cold 1X PBS and one more time with MLB buffer: 25 mM Hepes pH 7,5, 150 mM NaCl, 1% Nonidet-P40, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1mM EDTA, and 1mM sodium ortovanadate. It can be stored at 4ºC for up to 10 days. The quantification of GST was done using a BSA standard concentrations by SDS-PAGE dyed with Coomasie brilliant blue (Sigma)

- **Pull down in vitro assay**

The GST purified proteins were incubated with total lysates of HEK293T cells lysed in MLB buffer during 2 hours in a roter at 4ºC. After incubation, the precipitated Glutation-Sepharose beads were washed twice with cold 1X PBS, twice with cold 1X PBS 1% NP-40 and, finally, twice with MLB buffer. The beads were resuspended in loading buffer 2X Laemli and loaded in a 12% SDS-PAGE, as described above.

3.3.6. K_D determinations by *in vitro* binding assays

The first step was to generate ^{35}S radiolabelled ERK2 in rabbit reticulocytes (TNT® Coupled Reticulocyte Lysate Systems. Promega). To synthesize this radiolabelled ERK2, in L-methionine residues (Perkin Elmer Cat.# NEG709A), it was prepared the following reaction *(Table 3.7)*:

Components	Volume
TnT® Rabbit Reticulocyte Lysate	12.5 µl
TnT® Reaction Buffer	1 µl
TnT® RNA Polymerase T7	0.5 µl
Amino Acid Mixture, minus Methionine, 1 mM	0.5 µl
[^{35}S]-Methionine (>1,000Ci/mmol at 10mCi/ml)	1 µl
Ribonuclease Inhibitor (40 U/µl)	0.5 µl
DNA template(s)	1 µg
Nuclease-Free Water to a final volume of	25 µl

Table 3.7. ERK2 [^{35}S]-Methionine radiolabelling reaction.

This mixture reaction was incubated in a water bath at 30ºC for 90 minutes. During this period of time, the transcription of the DNA and the translation of the protein occurred, in this way the product is [^{35}S]-L-methionine- ERK2.

Then, increasing concentrations of the purified GST-proteins (0, 20, 40, 100, 200 nM) were incubated with a fixed amount of [^{35}S]-L-methionine- ERK2 (20 nM) at 30ºC for 2 hours. Once the binding reaction is done, 20 µl of loading buffer Laemli 2X were added and the proteins were resolved by SDS-PAGE. The [^{35}S] L-methionine- ERK2 was quantified by autoradiogram with the ImageJ software and by scintillation counter adding 2 µl of the reaction to 3 ml of scintillation liquid.

The K_D values were calculated with the software GraphPad Prism.

3.4. BIOINFORMATIC ANALYSES

- Statistical analysis: Data was processed and analyzed using the **GraphPad Prism 7** Software (GraphPad Software, Inc., San Diego, CA).

In bar graphs data is given as Mean ± SEM and Two tailed unpaired Student's t-test was used to determine differences between data sets and significance (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001).

The dissociation constant (K_D values) from binding assays were calculated using Dissociation-One phase exponential decay analysis (non-linear regression).

- The western blot analyses and confocal images processing was carried out and analyzed using **Fiji-Image-J** Software.

- The apoptosis results were analyzed by **Flow Logic** Software (Miltenyi Biotec).

- The bibliography was sorted by **Mendeley** reference management Software.

4. RESULTS

4.1. APS-2-79 biological and biochemical effects in connection with its role as a KSR inhibitor.

4.1.1. Effects of APS-2-79 and KSR disruption on cell viability.

APS-2-79 has been described as a KSR inhibitor (Dhawan, Scopton and Dar, 2016), however, the original publication showed that APS-2-79 displayed no effect at all on the cell viability of BRAF-mutant cells such as SKMEL239 and A375; and only modest effects on RAS-mutant cell lines such as HCT116 and A549. To further extend these findings we analyzed the effects of APS-2-79 on cell viability, on a broader panel of BRAF- and NRAS-mutant melanoma lineages, including A375, SKMEL28 and 501-MEL BRAF-mutant and SKMEL2, CJM and MEL-JUSO NRAS-mutant cell lines. As

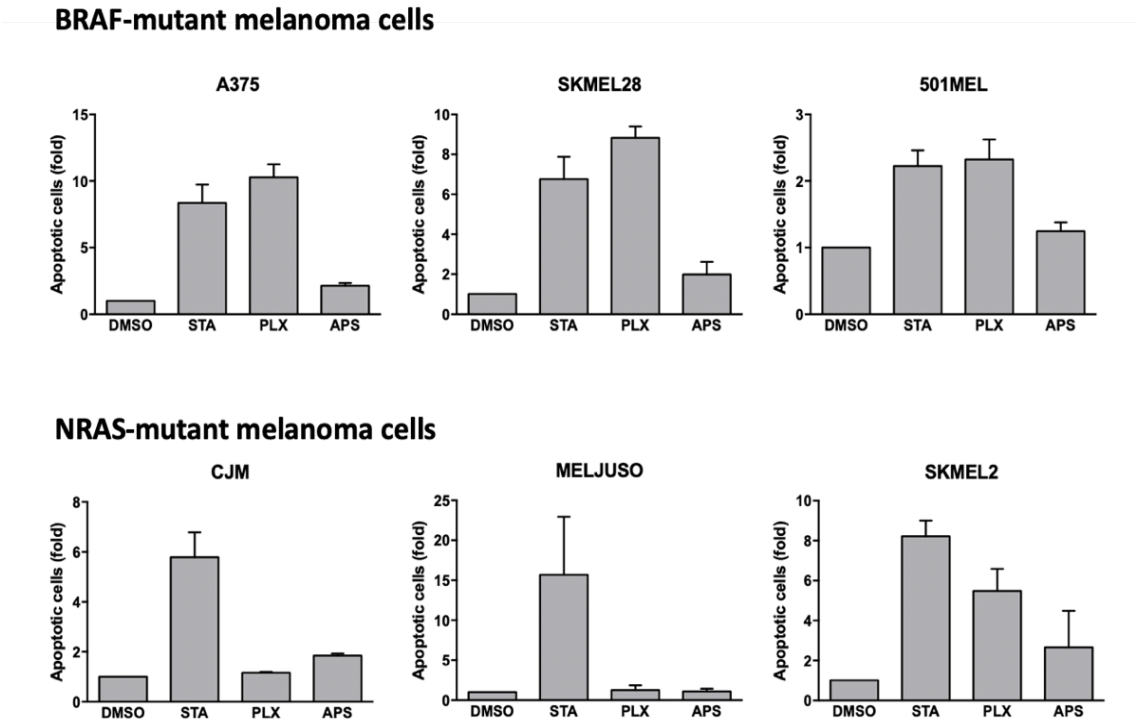


Figure 4.1. Induction of apoptosis by APS-2-79 in tumour cell lines harbouring RAS/ERK pathway oncogenes. *The shown BRAF- and NRAS-mutant cell lines were treated for 48 h with APS-2-79 (5 μM), PLX4032 (10 μM) or staurosporine (0.5 μM). Apoptosis was evaluated by annexin V detection using MACSQuant cytometer and results were analyzed with FlowLogic software. The graph shows annexin V positive cells. Results show Mean ± SEM of three independent experiments normalized to the levels of control (DMSO treated) cells.*

shown in figure 4.1, APS-2-79 displayed no significant apoptotic effects compared to staurosporine, as an unspecific apoptosis inducer, and to PLX4032 that specifically kills BRAF-mutant cell lines.

These findings reproduced the results exhibited in the original publication.

As APS-2-79 displayed no effect on cell viability, we investigated if this result correlated with KSR function. To this end, we compared the effects of the drug to those resulting from KSR depletion by shRNAs, in two representative cell lines. Since there are two KSR isoforms, KSR1 and KSR2, and both of them are targeted by APS-2-79, we analysed the depletion of both proteins separately and in combination. As shown in figure 4.2 neither KSR depletion nor APS-2-79 treatment had any effect on cell viability in the BRAF-mutant cell line A375P, as evaluated by the amount of annexin V positive cells.

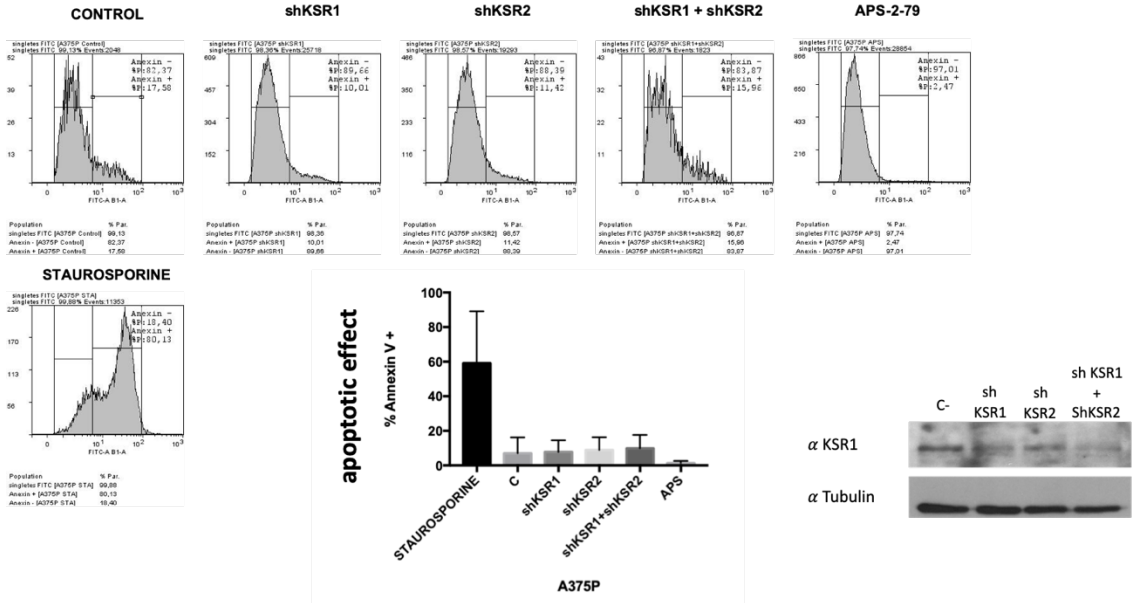


Figure 4.2. APS-2-79 treatment and KSR1/2 ablation have no apoptotic effect in A375P. *Cells were transfected with the corresponding shRNAs (0.5 μg each), with an empty vector as a negative control (C) or treated with APS-2-79 (5 μM; 48 h). Staurosporine treatment (1 μM, 16 h) was used as a positive control. Apoptosis was evaluated 48 h post-transfection by annexin V detection using MACSQuant cytometer and results were analyzed with FlowLogic software. The graph shows the percentages of annexin V positive cells obtained from the respective histograms. Results show Mean ± SEM of three independent experiments. The silencing of KSR1 was verified by western blot 48 h after transfection (**bottom right**) KSR2 expression was not monitored due to the unacceptable quality of all commercially-available antibodies for KSR2.*

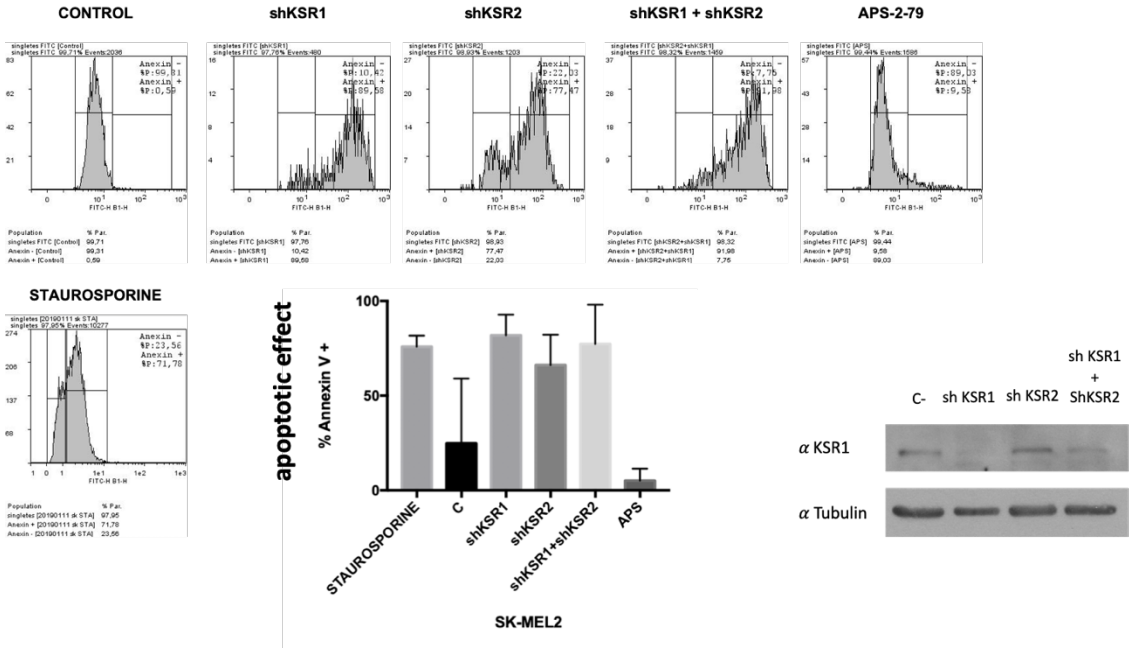


Figure 4.3*. *APS-2-79 does not mirror the apoptotic effect of KSR1/2 ablation. *SKMEL2 cells were transfected with the corresponding shRNAs (KSR1 or KSR2 or KSR1 + KSR2), with an empty vector as a negative control (C) or treated with APS-2-79 (5 μM; 48 h). Staurosporine treatment was used as a positive control. Apoptosis was evaluated 48 h post-transfection by annexin V detection using MACSQuant cytometer and results were analyzed with FlowLogic software. The graph shows the percentages of annexin V positive extracted from the histograms. Results show Mean ± SEM (N=3). The silencing of KSR1 and KSR2 was checked by western blot 48 h after transfection (**bottom right**) KSR2 expression was not monitored due to the unacceptable quality of all commercially-available antibodies for KSR2.*

However, in SKMEL2 NRAS-mutant melanoma cells, the silencing of each KSR isoform, separately or at the same time, resulted in a high proportion of Annexin V positive cells, reflecting a pronounced apoptotic effect. Surprisingly, this was not the case when cells where treated with the KSR inhibitor APS-2-79 *(Fig.4.3)*, indicating that KSR inhibition, as exerted by APS-2-79, did not have the same effect as KSR depletion.

4.1.2. Effect of KSR1 inhibition/depletion on ERK activation.

In the same vein, similarly to the effect observed on apoptosis, it was found that treatment with APS-2-79 did not affect total ERK phosphorylation as profoundly as KSR1 depletion *(Fig.4. 4)*.

This could be concluded by the observation that the drop on ERK phosphorylation levels in response to treatment with APS-2-79 was significantly lower than that resulting from shRNA-mediated suppression of KSR1 expression. In spite of the fact that APS-2-79 treatment affects all the cells, whereas the effect of KSR1 suppression is restricted to a smaller fraction of cells, i.e. those that

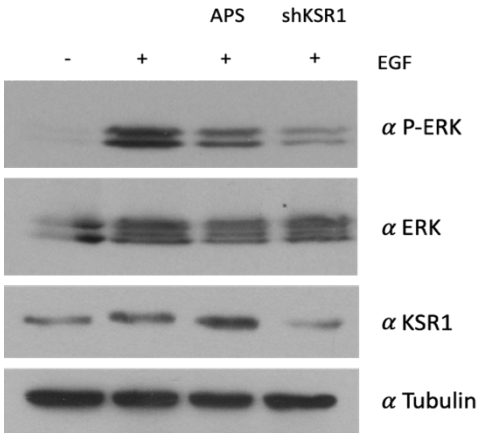


Figure 4.4. Divergent effects of KSR1 depletion and KSR1 inhibition on ERK activation. *HEK293T cells were treated with APS-2-79 (5 μM; 2 h) or transfected with shRNAs against KSR1 for 48 h and ERK activation was analyzed after stimulation with EGF (50 ng/ml; 5') where shown (+) after 18 h starvation.*

uptake the shRNAs.

Moreover, when analyzing the effect of APS-2-79 on the fraction of phosphorylated ERK bound to KSR1 following EGF stimulation, coimmunoprecipitation assays demonstrated that such levels were

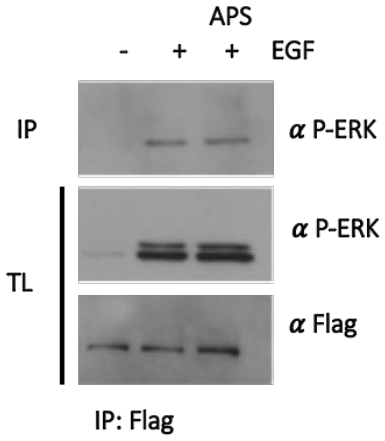


Figure 4.5. APS-2-79 does not affect KSR1-bound phosphorylated ERK levels. *HEK293T cells were transfected with Flag-tagged KSR1 (2 μg) and stimulated with EGF (50 ng/ml; 5') where shown (+), after APS-2-79 treatment (5 μM; 2 h) where indicated. Phosphorylated ERK associated to KSR1 was determined by western blotting after anti-Flag immunoprecipitation. IP= Immunoprecipitation. TL=Total Lysates.*

unaltered by APS-2-79 in comparison to untreated cells *(Fig. 4.5)*.

Overall, these results revealed APS-2-79 deficiency as an inhibitor of KSR-mediated ERK activation and as an inhibitor of neoplasia driven by RAS/ERK pathway oncogenes such as NRAS and BRAF.

4.2. Analyses on the regulation of ERK activity and functions by KSR1

4.2.1. Effect of KSR1 overexpression on ERK activation and apoptosis.

It was intriguing that KSR inhibition, as executed by APS-2-79, did not have evident effects on apoptosis, whereas down-regulation of KSR expression did. Since KSR is a scaffold protein and these are known to exert their activity following the "pro-zone effect" (Burack and Shaw, 2000; Ha, Kim and Ferrell, 2016), we reasoned that any fluctuation on KSR expression levels could have similar consequences. Therefore, in SKMEL2 cells, we tested how KSR overexpression affected cell viability. To this end, we transfected increasing concentrations of KSR1 WT. As an additional control, we also tested the effect of a KSR1 mutant, defective for binding MEK (C809Y). It was found that the overexpression of ectopic KSR1 WT induced apoptosis in a dose-dependent fashion following a bell-shaped kinetics. Most surprisingly, even though KSR1 C809Y was expected to be non-functional because of its inability to bind MEK, it had an effect on apoptosis similar to that observed with the WT protein *(Fig. 4.6)*. Likewise, the overexpression of both KSR1 forms had a similar effect on ERK activation, also following a bell-like effect, causing a slight reduction on ERK activation levels as their concentration increased *(Fig. 4.7)*.

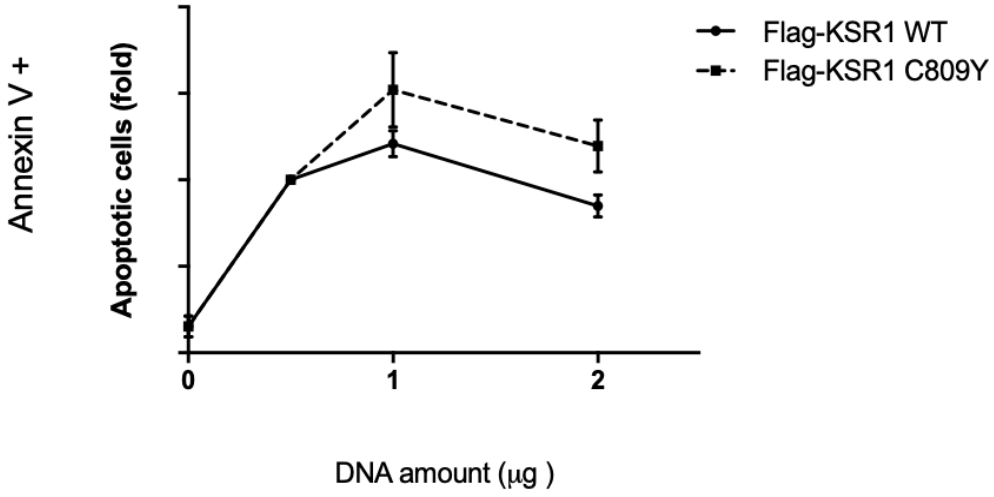


Figure 4.6. Effect of KSR1 WT and C809Y overexpression on apoptosis. *Apoptosis was evaluated in SKMEL2 melanoma cells, 48 h post-transfection by annexin V detection, using MACSQuant cytometer. The results were analyzed with FlowLogic software. Data shows mean ± SEM of three independent experiments, normalized to the negative control (cells transfected with an empty vector).*

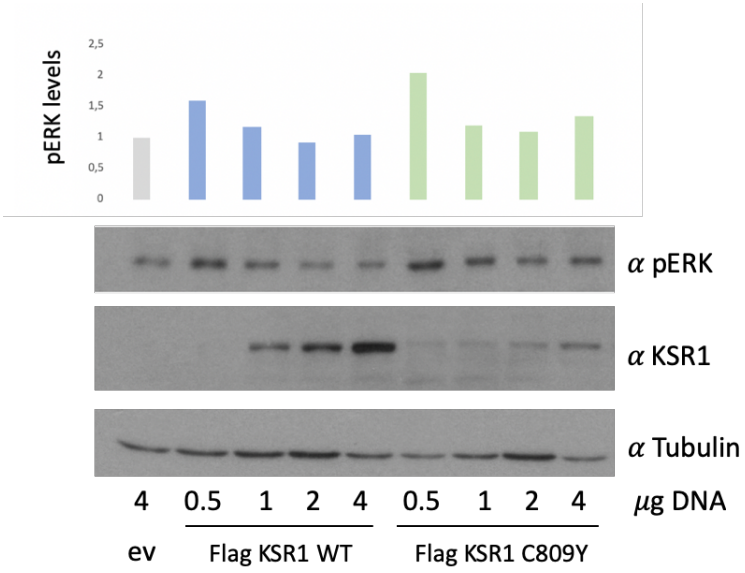


Figure 4.7. Effect of KSR1 WT and C809Y overexpression on ERK activation. *SKMEL2 cells were transfected with increasing concentrations (0.5-4 μg) of the indicated Flag-tagged KSR1 constructs. Phosphorylated ERK levels were monitored by western blotting and quantitated by ImageJ. Data shows a representative experiment of two. ev=empty vector*

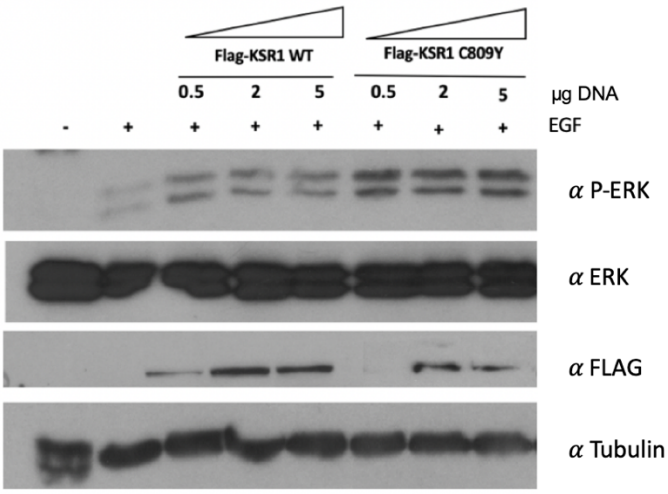


Figure 4.8. Overexpression of MEK-binding deficient KSR1 potentiates ERK activation. *ERK activation levels were analyzed by western blot in HEK293T cells transfected with increasing concentrations (0.5-5 µg) of the shown Flag-tagged KSR1 constructs, upon EGF stimulation (50 ng/ml; 5') (+) after 18h starvation.*

In light of this unexpected result, we also analyzed the effect of the aforementioned KSR1 MEK-binding mutant on ERK activation in HEK293T cells. In these cells, KSR1 overexpression did not induce apoptosis (data not shown). Once again, the C809Y mutant behaved similarly to WT KSR1, only that in this case the overexpression of both KSR1 forms enhanced ERK phosphorylation *(Fig. 4.8)*, even more pronouncedly in the case of KSR1 C809Y.

It was very surprising that the C809Y mutant, incapable of binding MEK, always behaved similarly to WT KSR1, regardless of the fact that, in theory, its inability to bind MEK should render it defective for conveying RAF-ERK signals. In order to gain further insights into this conundrum, we analyzed the competence of the KSR1 C809Y mutant for conveying signals that lead to ERK activation. Remarkably, and most surprisingly, it was found that this mutant, in spite of its inability to bind MEK, incorporated phosphorylated ERK as effectively as the WT KSR1 upon EGF stimulation

(Fig. 4.9).

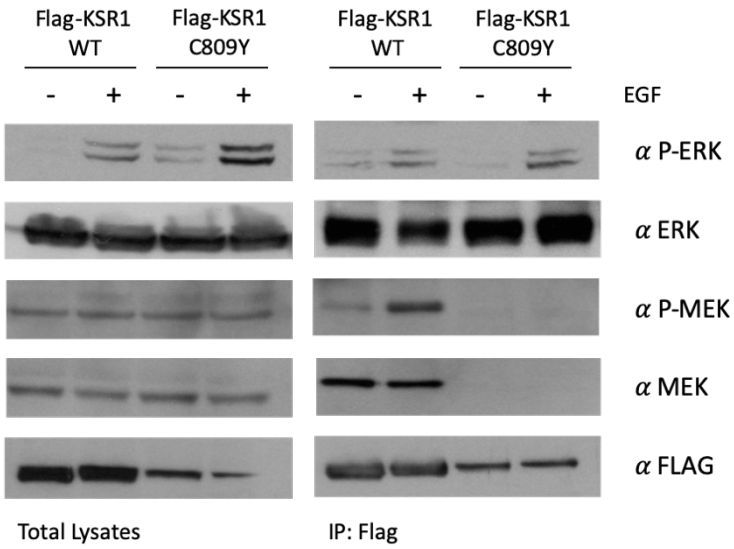


Figure 4.9. KSR1 C809Y mutant, unable to bind MEK, incorporates phosphorylated ERK. *HEK293T cells were transfected with the indicated Flag-tagged KSR1 constructs (2 μg) and stimulated with EGF (50 ng/ml; 5') where shown (+) after 18 h starvation. ERK and MEK associated to KSR1 were determined by co-immunoprecipitation upon anti-Flag immunoprecipitation (IP: Flag) and subsequent western blotting for the indicated proteins.*

The results obtained by co-immunoprecipitation analyses were further ascertained *in vivo*, by means of proximity ligation assays (PLA) performed in HeLa cells. By this method, it was verified that ectopically-expressed KSR1 C809Y could associate to endogenous ERK, and that this interaction mainly took place at the cytoplasm *(Fig. 4.10)*.

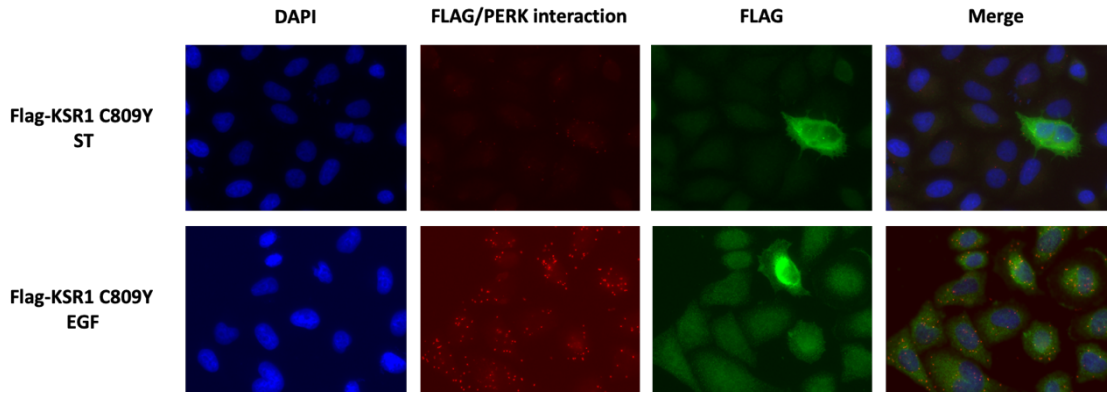


Figure 4.10. KSR1 C809Y mutant binds to phosphorylated ERK in vivo. *HeLa cells were transfected with KSR1 C809Y (2 μg) and Proximity Ligation Assays (PLA) were performed in cells starved for 18 h and after treatment with EGF (50 ng/ml; 5') as shown. Interaction between overexpressed Flag KSR1 C809Y (green) and endogenous, phosphorylated ERK is shown by red fluorescence. Nuclei are stained in blue.*

These results demonstrated that a MEK-binding defective KSR1 is competent for conveying upstream signals that bring about the phosphorylation/activation of its bound ERK. In addition, these data open the possibility that some other protein could be complementing KSR1 C809Y deficiency for binding MEK, in order to induce the phosphorylation of its bound ERK.

4.2.2. Transphosphorylation among KSR homodimers.

Previous data has demonstrated that KSR proteins have the ability to homodimerize (Rajakulendran *et al.*, 2009). Indeed, we have observed that the KSR1 C809Y mutant retains this ability *(Fig 4.11)*.

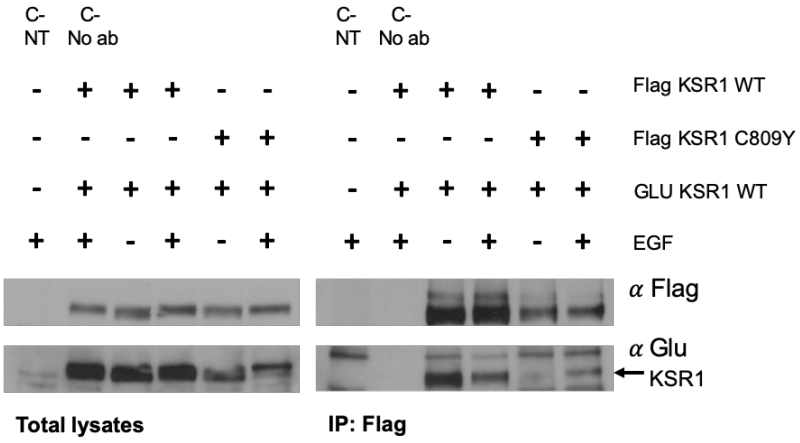


Figure 4.11. KSR1 C809Y maintains its homodimerization capacity*. KSR1 homodimerization was tested by a co-immunoprecipitation assay in HEK293T cells transfected with Glu- and Flag-tagged KSR1 WT and Flag-tagged KSR1 C809Y (1.5 μg each) upon EGF stimulation (50 ng/ml; 5') where indicated (+). C- NT= untransfected cells. C- no ab= lysates incubated only with beads.*

In light of these previous data, it was possible that the presence of phosphorylated ERK bound to KSR1 C809Y could be the consequence of ERK being phosphorylated in *trans* by a MEK molecule bound to another KSR1 WT protein, forming a homodimer with KSR1 C809Y. A second possibility existed: in the sense that ERK bound to KSR1 C809Y could be phosphorylated by free, soluble MEK.

To put these hypotheses to test, two KSR1 mutants were generated: on one hand, a mutant unable to homodimerize, due to the disruptive point mutation R615H, that affects the KSR-KSR interaction interface (Stewart *et al.*, 1999). And, on the other hand, a double mutant which, in addition to the R615H mutation, harboured the aforementioned C809Y mutation which prevents KSR1 interaction with MEK.

In these mutants, we analyzed their ability to bind phosphorylated ERK. It was found that the R615H dimerization-deficient mutant retained its ability to bind phosphorylated ERK, probably as a consequence of the RAF-ERK signal flowing through itself. However, no phosphorylated ERK was found associated to the MEK-, dimerization-defective C809Y, R615H double mutant *(Fig. 4.12)*.

This result suggested that, indeed, ERK phosphorylation within KSR1 C809Y was being

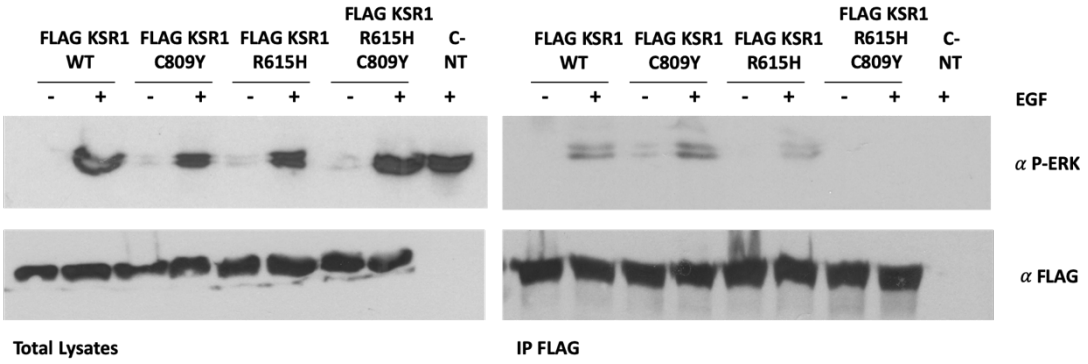


Figure 4.12. KSR1 MEK-, dimerization- defective C809Y, R615H double mutant fails to bind phosphorylated ERK. *KSR1 interaction with phosphorylated ERK was analyzed by a co-immunoprecipitation assay in HEK293T cells transfected with the indicated Flag-tagged KSR1 constructs (2 μg each), in starved cells (-) or upon EGF stimulation (50 ng/ml; 5') where indicated (+). C- = untransfected cells. TL= total lysates.*

supported by a trans-acting MEK, bound to another homodimerizing KSR1 molecule, and excluded the possibility that free, cytoplasmic MEK was the responsible for such phosphorylation. In light of these data, we have termed this mechanism "transphosphorylation".

The results obtained by co-immunoprecipitation assays were, once again, validated *in vivo*, by Proximity ligation assays performed in HeLa cells. These ascertained that KSR1 dimerization-

defective but not the double mutant could bind to endogenous, phosphorylated ERK at the cytoplasm. As an additional control we transfected cells with a KSR1 mutant incapable of binding ERK (KSR1 ASAP) which, as expected, also failed to bind endogenous, phosphorylated ERK *(Fig. 4.13)*.

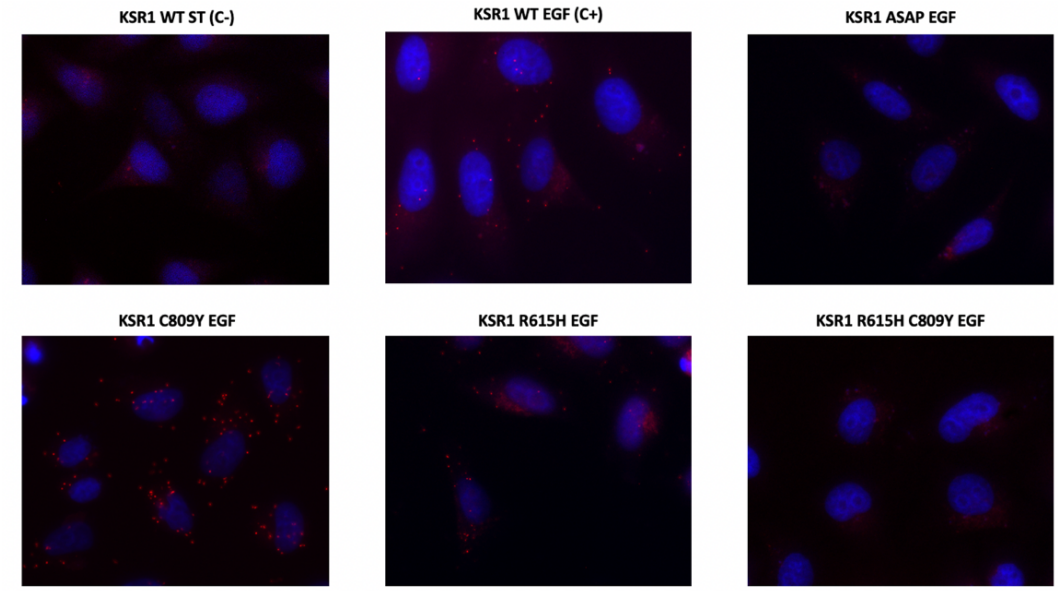


Figure 4.13. KSR1 C809Y, R615H mutant fails to bind phosphorylated ERK in vivo. *HeLa cells were transfected with the indicated KSR1 constructs (2 μg) and Proximity Ligation Assays (PLA) were performed in cells starved for 18 h (ST) and after treatment with EGF (50 ng/ml; 5') as shown. Interaction between overexpressed Flag KSR1 constructs and endogenous, phosphorylated ERK is shown by red fluorescence. Nuclei are stained in blue.*

In order to confirm our transphosphorylation hypothesis, we used another approach: it has been described that cytosolic phospholipase A_2 (PLA_2) is activated by ERK when its signals are specifically scaffolded by KSR1 (Casar *et al.*, 2009). Therefore, we tested if the KSR1 C809Y mutant, was capable of mediating on PLA_2 activation. In order to do this, ectopic KSR1 WT and the C809Y mutant were overexpressed in KSR1 -/- MEFs, to determine the extent to which EGF-induced PLA_2 activation could be rescued by the overexpressed proteins. In this assay we also included the KSR1 mutant defective for binding ERK (ASAP). In agreement with the previous results, it was found that both, WT and C809Y KSR1 behaved in an identical fashion, facilitating a potent EGF-induced PLA_2

activation. Conversely, the control ASAP mutant was incapable of supporting PLA_2 activation, as expected from its inability to bind ERK *(Fig. 4.14)*.

Remarkably, in this case the ability of C809Y KSR1 for activating PLA_2 could not be explained through its transphosphorylation by an endogenous KSR molecule because these are absent in KSR1 -/- fibroblasts. So, the possibility existed that other scaffold protein species could also participate in the transphosphorylation process by heterodimerizing with KSR1 C809Y.

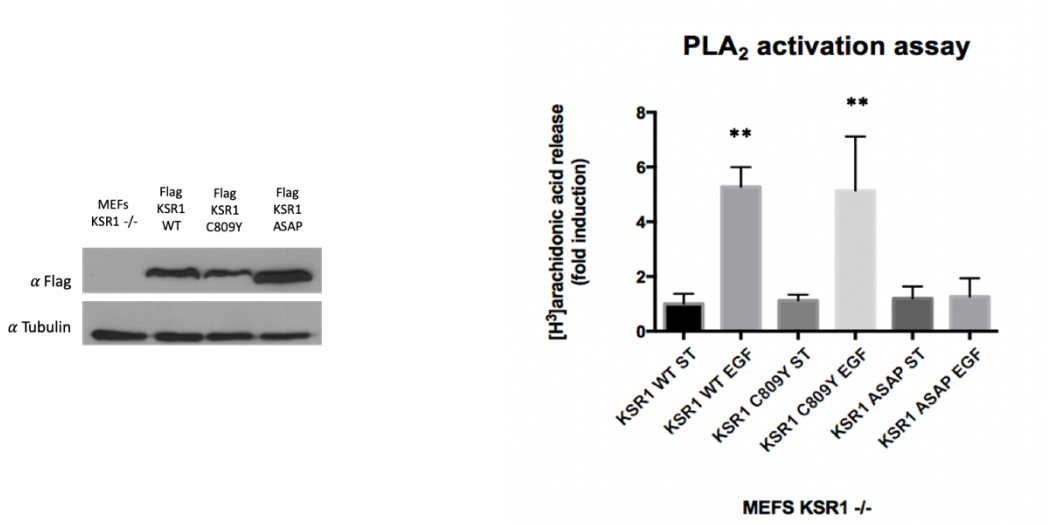


Figure 4.14. MEK-binding deficient KSR1 rescues the activation of cPLA₂ in KSR1 -/- MEFs. *cPLA2 activity was measured by radiolabelled arachidonic acid release. In KSR1 -/- MEFs transfected with the indicated KSR1 constructs (2 μg each) under starvation conditions (ST) or upon EGF stimulation (50 ng/ml; 5'). **Right panel:** The graph represents the activation fold of each condition normalized to the value of KSR1 WT (ST). Results show Mean ± SEM (N=2) using two-tailed unpaired Student T-Test (**p<0.01). **Left panel:** KSR1 expression levels were detected by western blot.*

To further substantiate this point, we also monitored the ability of the different KSR1 constructs for matching the differences on proliferative potential displayed by WT and KSR1 -/- fibroblasts. As shown in figure 4.15, KSR1 -/- fibroblasts proliferate at a faster rate than their WT counterparts. Their proliferative capacity was returned to WT levels by the ectopic expression of

KSR1 WT or by the mutant forms defective for binding MEK (C809Y) and for homodimerization (R615H) and also by the MEK-, dimerization- defective C809Y, R615H double mutant. This result

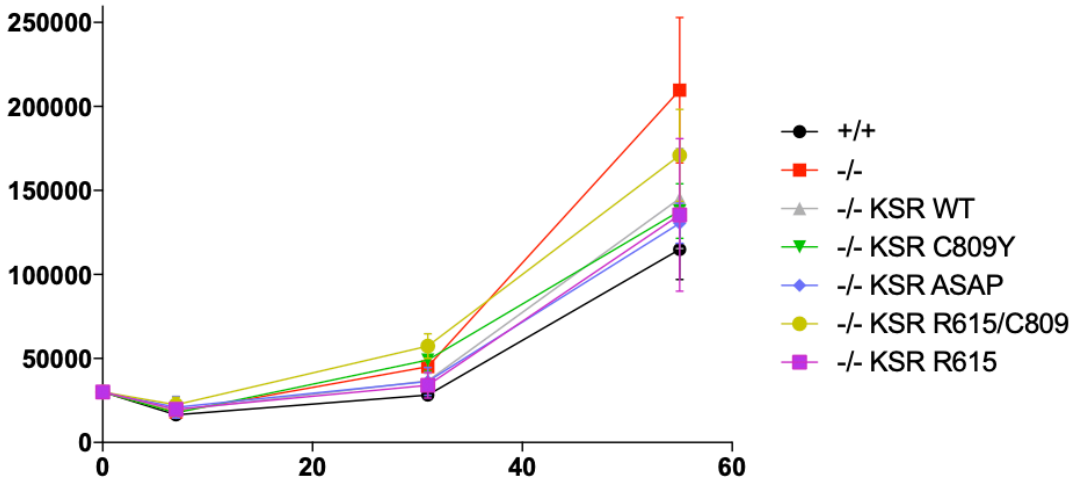


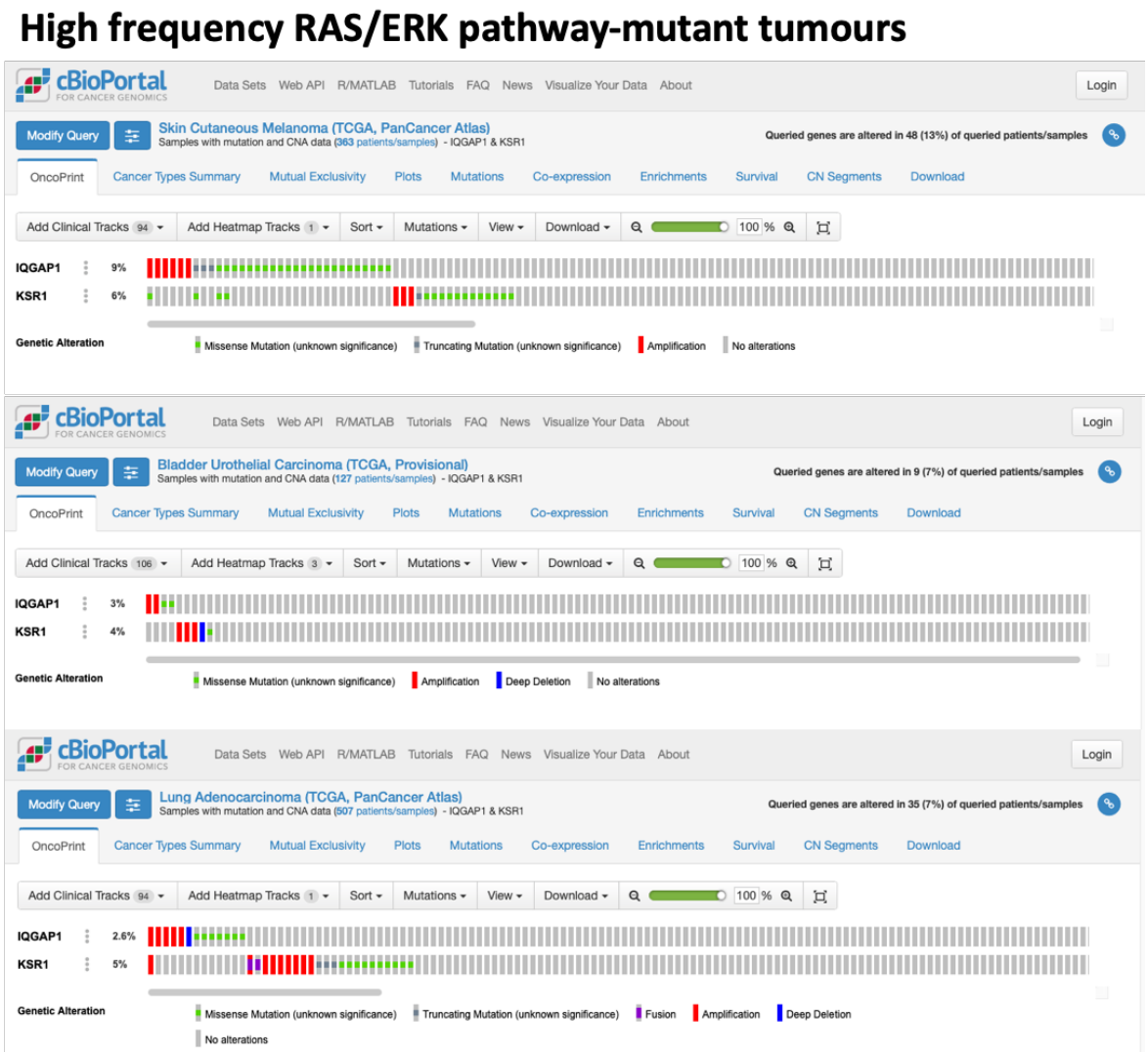
Figure 4.15. KSR1 MEK-, dimerization-defective double mutant restores normal proliferation in KSR1 -/- MEFs. *Proliferation was analyzed in KSR1 WT (+/+) and -/- MEFs stably expressing the indicated KSR1 constructs. Results show Mean ± SEM (N=3).*

was, again, suggestive of some other type of scaffold being capable of supporting the activity of the KSR1 C809Y, R615H double mutant, via transphosphorylation.

4.2.3. IQGAP1 as a candidate for supporting transphosphorylation.

Previous publications have unveiled that scaffold proteins are capable of interacting among themselves, for example, MORG1 has been shown to interact with MP1 (Vomastek *et al.*, 2004). Likewise, IQGAP1 can interact with MP1 and also β-arrestin2 (Feigin *et al.*, 2014). So, the possibility existed that some other scaffold species could also be intervening on KSR-bound ERK transphosphorylation. With the aim of identifying other scaffolds supporting ERK transphosphorylation in KSR, a mass spectrometry was carried out to unveil proteins which associate to KSR1. These analyses identified IQGAP1 as a KSR1-interacting protein (data not shown).

Furthermore, in light of this finding we analysed tumour data bases looking for potential correlations between KSR1 and IQGAP1 expression and mutational status patterns. Remarkably, in tumours where IQGAP1 and KSR1 exhibited genetic alterations, these occurred in a mutually exclusive fashion. This was the case in tumours where mutations in the RAS/ERK pathway are common, such as melanoma, lung adenocarcinoma and bladder carcinoma; but, also, in some types of tumours where RAS/ERK pathway mutations are infrequent such as prostate adenocarcinoma and breast carcinoma *(Fig 4.16)*. The fact that genetic alterations in two proteins are mutually exclusive suggest that such proteins may be participating in the same signalling route. For example, such is the case with BRAF and RAS mutations (Alsina *et al.*, 2003).



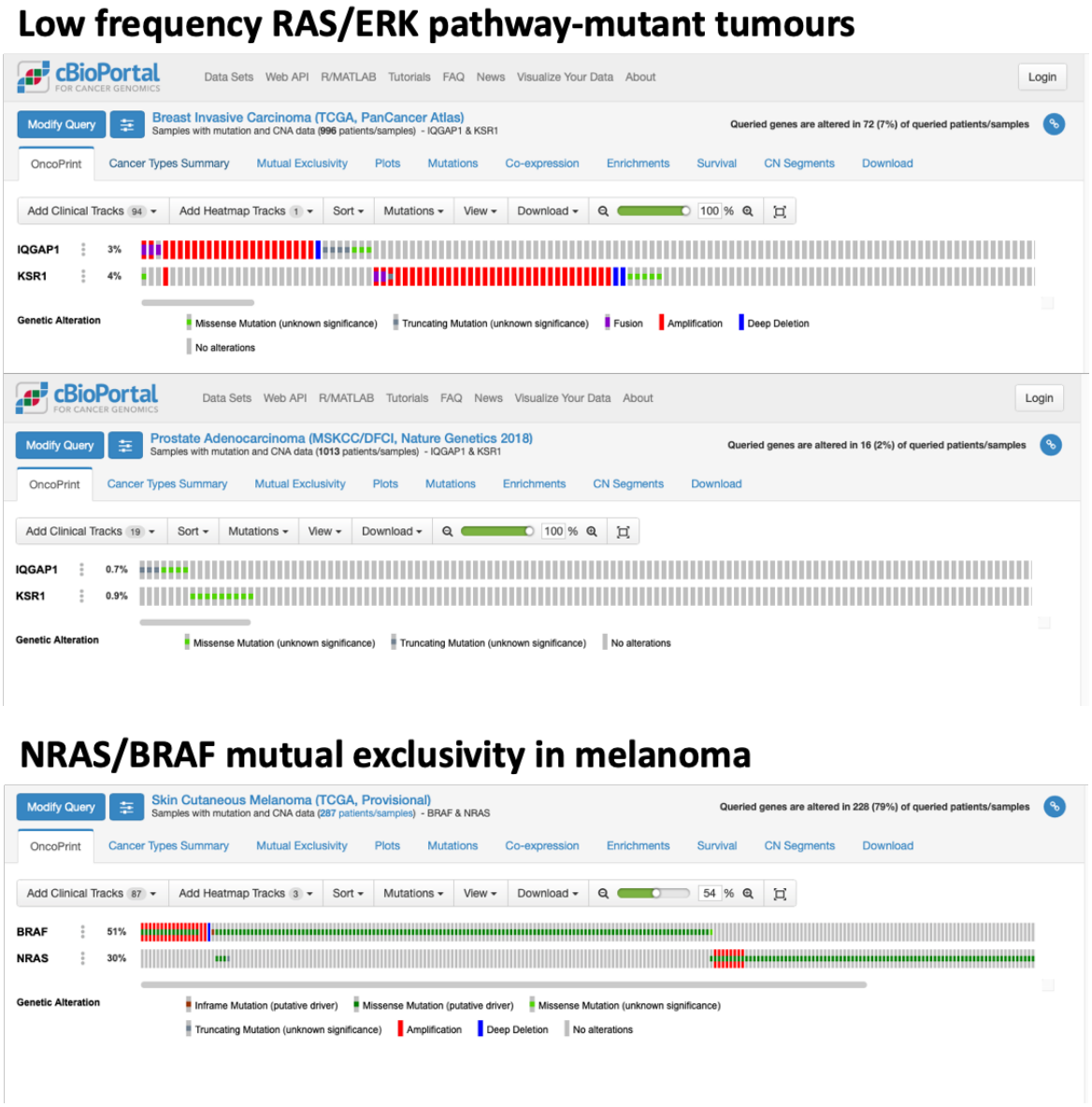


Figure 4.16. Mutual exclusivity of KSR1 and IQGAP1 genetic alterations in tumours. *Oncocharts obtained from the TCGA database for the genetic alterations in KSR1 and IQGAP1 genes, detected in the indicated types of tumours. The distribution of NRAS and BRAF mutations in melanoma is also shown as a typical mutual exclusivity pattern.*

To further substantiate these observations, in the previously utilized melanoma cell lines *(Fig. 2 and 3)* we evaluated whether depletion of IQGAP1 had similar effects to those found for KSR1 down-regulation. Indeed, it was observed that the proliferation of BRAF-mutant A375P cells was unaffected by the depletion of either scaffold; whereas the proliferation of NRAS-mutant SKMEL2 cells was attenuated to similar extents by the down-regulation of KSR1 or of IQGAP1 *(Fig 4.17)*, once again suggestive that KSR1 and IQGAP1 could be participating in the regulation of the same processes in the same cellular settings.

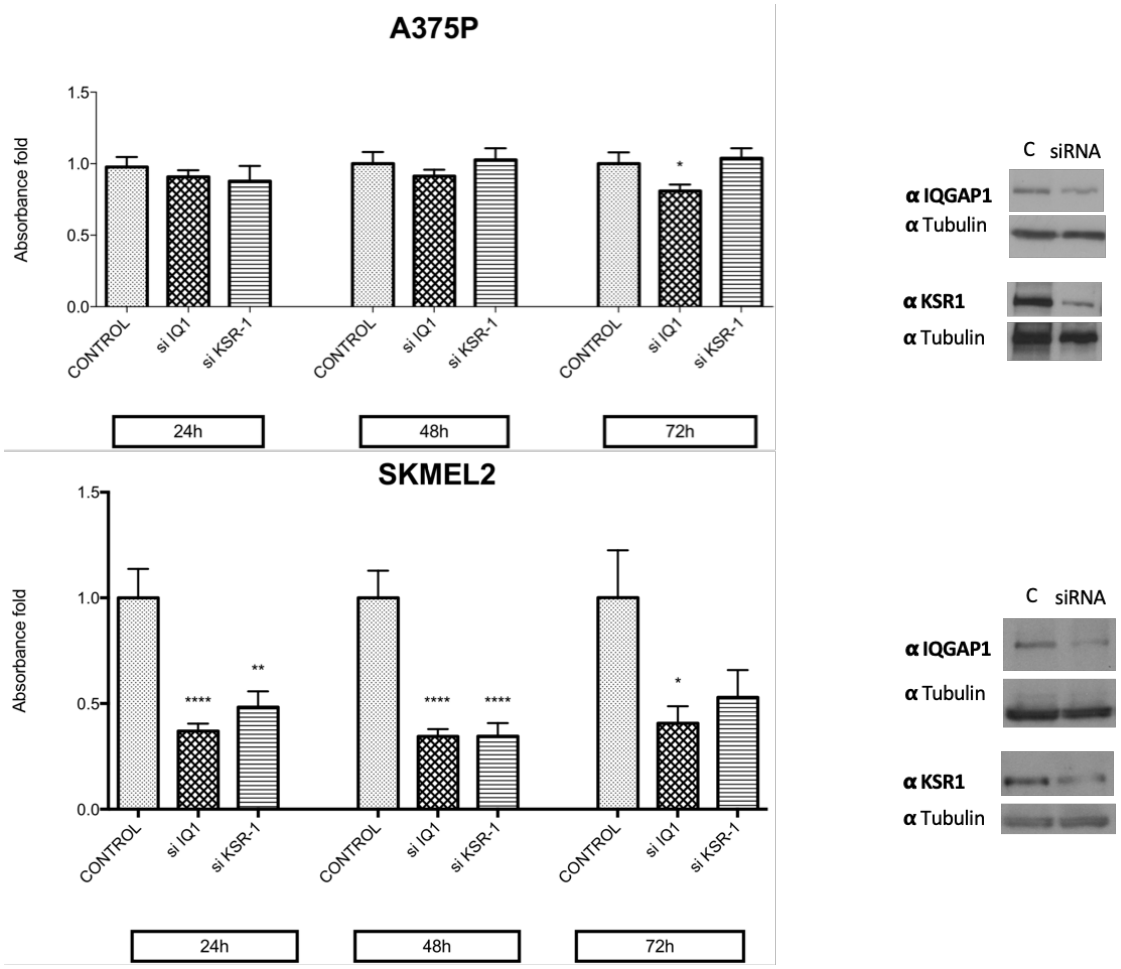


Figure 4.17. KSR1 and IQGAP1 down-regulation affect proliferation of NRAS- but not BRAF-mutant cells. *Proliferation was evaluated in the indicated cells after transfection of siRNAS (2 μg each) for the indicated scaffolds. The graph represents the rate of Alamar Blue reduction at the indicated time point, normalized to the value of the untreated control cells. Results show Mean ± SEM (N=3) using two-tailed unpaired Student T-Test (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001).* ***Right panels:*** *KSR1 and IQGAP1 expression levels were detected by western blot.*

It was of the utmost interest to verify if KSR1 and IQGAP1 physically interacted. In order to put this notion to test, we performed co-immunoprecipitation assays in HEK293T cells, in which it was found that endogenous IQGAP1 and KSR1 readily co-immunoprecipitated upon the immunoprecipitation of either protein *(Fig. 4.18)*, demonstrating that, indeed, both scaffold proteins can associate in the context of entirely physiological settings.

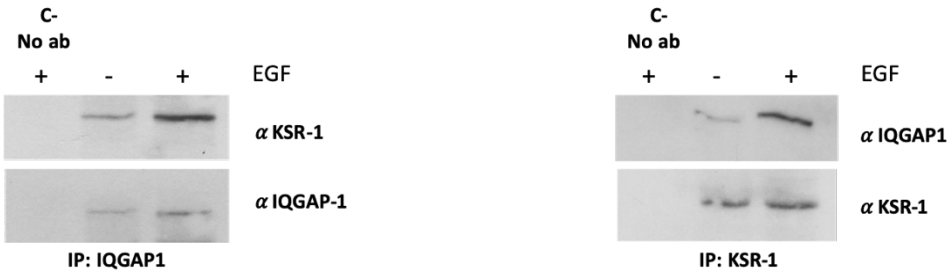


Figure 4.18. IQGAP1 and KSR1 interaction. Left: *immunoprecipitation of endogenous IQGAP1.* ***Right:*** *immunoprecipitation of endogenous KSR1, in HEK293T cells serum-starved for 18 h (-) or EGF-stimulated (50 ng/ml; 5). C- no ab= lysates incubated only with beads.*

In line with the previous result showing that KSR1 and IQGAP1 can bind to each other, a possible explanation for the data shown in figure 4.14, demonstrating the ability of KSR1 C809Y to activate PLA_2 in the absence of endogenous KSR (since KSR1 -/- MEFs do not express KSR2 either (Fernandez, Henry and Lewis, 2012)), stemmed from the observation that in such cells, IQGAP1 was overexpressed *(Fig. 4.19)*. This suggested that in KSR1 -/- fibroblasts IQGAP1 could substitute endogenous KSR as a "transphosphorylating" partner for the ectopic KSR1 C809Y mutant.

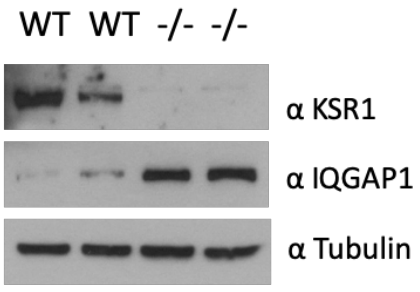


Figure 4.19. IQGAP1 is overexpressed in the absence of KSR1 in MEFs*. The levels of endogenous IQGAP1 and KSR1 were analyzed by western blotting in both MEFs WT and KSR1-less (-/-)*

To further substantiate this notion, we tested whether the different KSR1 mutants were capable of binding to IQGAP1. Noticeably, both the MEK-binding and the homodimerization-deficient KSR1 mutants were capable of binding to IQGAP1, both under starvation and EGF-stimulated conditions though to a slightly lesser extent. However, it was quite remarkable that the KSR1 C809Y, R615H double mutant could interact with IQGAP1 under starvation conditions, but such interaction was broken upon EGF stimulation *(Fig. 4.20)*.

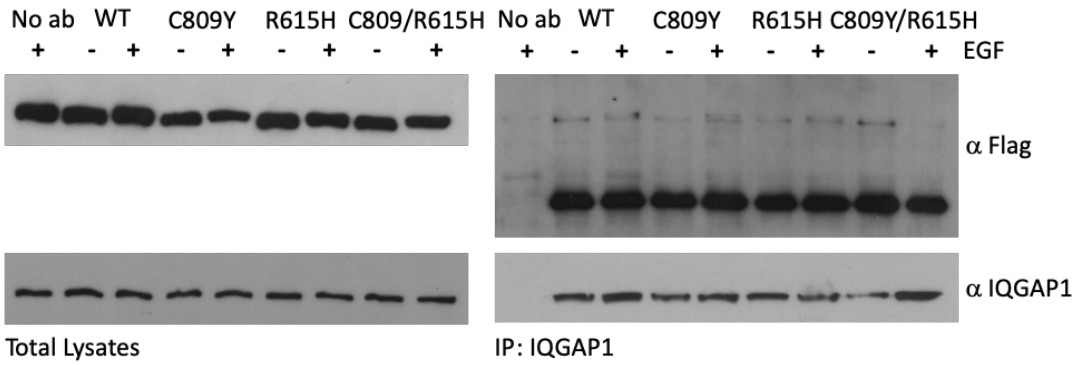


Figure 4.20. IQGAP1 interaction with KSR1 mutants. *IQGAP1 interaction with the different KSR1 mutants was analyzed by a co-immunoprecipitation assay in HEK293T cells co-transfected with the indicated Flag-tagged KSR1 constructs (1.5 μg each), upon EGF stimulation (50 ng/ml; 5') where indicated (+). No ab= lysates incubated only with beads.*

It was necessary to understand the reason why in HEK293T the KSR1 double mutant C809Y, R615H could not incorporate phosphorylated ERK, in spite of the fact that these cells express IQGAP1. We hypothesized that this could be a matter of the stoichiometry governing KSR1-IQGAP1 interaction. In such way that in HEK293T cells the levels of IQGAP1 are not sufficiently high to produce a significant transphosphorylation on overexpressed KSR1 C809Y, R615H, which exhibits a reduced capacity to bind IQGAP1 under stimulation, in comparison to the endogenous WT KSR1. As such, the possibility existed that the overexpression of IQGAP1 could bring about KSR1 C809Y, R615H transphosphorylation in HEK293T cells. To put this notion under test, we repeated the previous experiment *(Fig. 1.12)* in the presence of overexpressed, ectopic IQGAP1. Under these premises, it was found that KSR1 C809Y, R615H could bind to IQGAP1 and readily incorporated phosphorylated ERK upon EGF stimulation *(Fig. 4.21)*, demonstrating that, under optimal

stoichiometric proportions, IQGAP1 could associate to the ectopic KSR1 MEK-, dimerization-defective C809Y, R615H double mutant and transphosphorylate its bound ERK.

In light on the above data, it was necessary to verify that IQGAP1 was, indeed, the scaffold

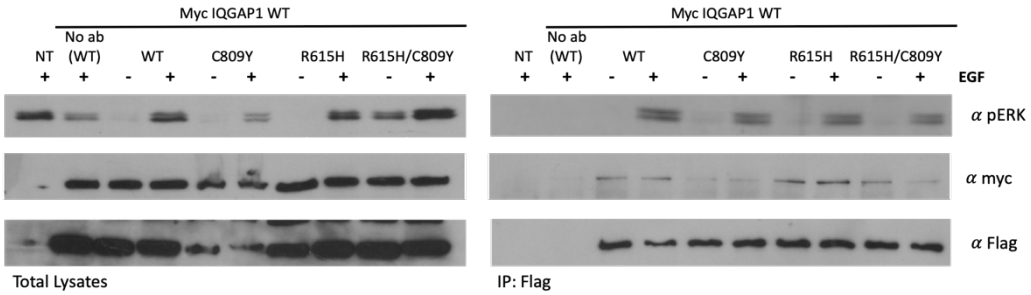


Figure 4.21. IQGAP1 rescues KSR1 double mutant ability for interacting with activated ERK. *KSR1 interaction with phosphorylated ERK was analyzed by a co-immunoprecipitation assay in HEK293T cells transfected with the indicated Flag-tagged KSR1 constructs (1.5 μg each), in addition to Myc-tagged IQGAP1 (1.5 μg), in starved cells (-) or upon EGF stimulation (50 ng/ml; 5') where indicated (+). NT = untransfected cells. No ab= lysates incubated only with beads*

responsible for supporting KSR1 C809Y activity in KSR1 -/- MEFs. To this end, in these cells, we downregulated IQGAP1 levels using shRNAs and analysed its impact on the ability of the different KSR1 mutant forms for incorporating phosphorylated ERK. It was found that IQGAP1 depletion did not have any effect on phosphorylated ERK levels bound to WT KSR1, as this protein would not require IQGAP1 for phosphorylating its bound ERK. However, it significantly reduced the levels of phospho-ERK associated to the C809Y and R615H mutant forms and completely abolished it in the case of the double mutant *(Fig. 4.22)*. We posit that in the case of the C809Y mutant the drop on its phospho-ERK levels would be a consequence of the depletion of IQGAP1, its essential transphosphorylation partner. In the case of R615H mutant, this form would be capable of signalling either by itself, as a monomer, or by IQGAP1-mediated heterodimerization/transphosphorylation. Downregulation of IQGAP1 would prevent the transphosphorylation component thereby reducing its phospho-ERK bound levels by almost 50%.

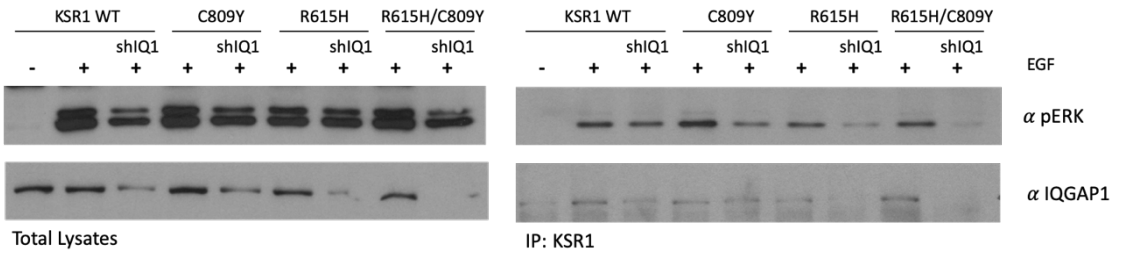


Figure 4.22. IQGAP1 depletion prevents phosphorylatied ERK binding to KSR1 mutants. *KSR1 interaction with phosphorylated ERK was analyzed by a co-immunoprecipitation assay in MEFs KSR1 -/- stably expressing KSR1 constructs and transfected with shRNA against IQGAP1 where indicated (shIQ1) and upon EGF stimulation (50 ng/ml; 5') (+).*

Overall, these results demonstrate that IQGAP1 can associate to KSR1 and to KSR1 mutant forms incapable of binding to MEK and of homodimerizing. Furthermore, they reveal that IQGAP1 can complement KSR1 MEK-binding mutant forms deficiency for phosphorylating their bound ERK, unveiling IQGAP1 as a KSR1 transphosphorylation partner.

4.2.4. Identification of the IQGAP1-binding motif in KSR1

Since KSR1 is a multidomain protein, it was important to identify the region/s whereby KSR1 was interacting with IQGAP1. To this end, we used a series of deletion mutants spanning through the whole KSR1 molecule (Fig. 25), and determined which of these harboured the ability to bind to IQGAP1 by co-immunoprecipitation assays. The results of these analyses suggest that KSR1 mainly binds to IQGAP1 through the CA4 region spanning aminoacids 402-521, though not through the FXFP domain, included in this region, as the ASAP mutant (Phe > Ala substitution in the FXFP domain) retains its ability to bind IQGAP1 *(Fig. 4.23)*.

However, our results are suggestive that other KSR1 regions may also be participating in binding to IQGAP1. For instance, it is remarkable that the KSR1 construct 1-521, which lacks the CA5 region, binds with much greater efficiency that the WT and the ASAP constructs which, in addition to the CA4, harbour the CA5 region. This could imply that the CA5 region could, to some extent, attenuate binding to IQGAP1. In this respect, the KSR1 double mutant C809Y/R615H,

harbouring mutations within the CA5 region, binds to IQGAP1 with less efficiency *(Fig. 4.20)*, also suggestive of the CA5 domain regulating to some extent the binding to IQGAP1.

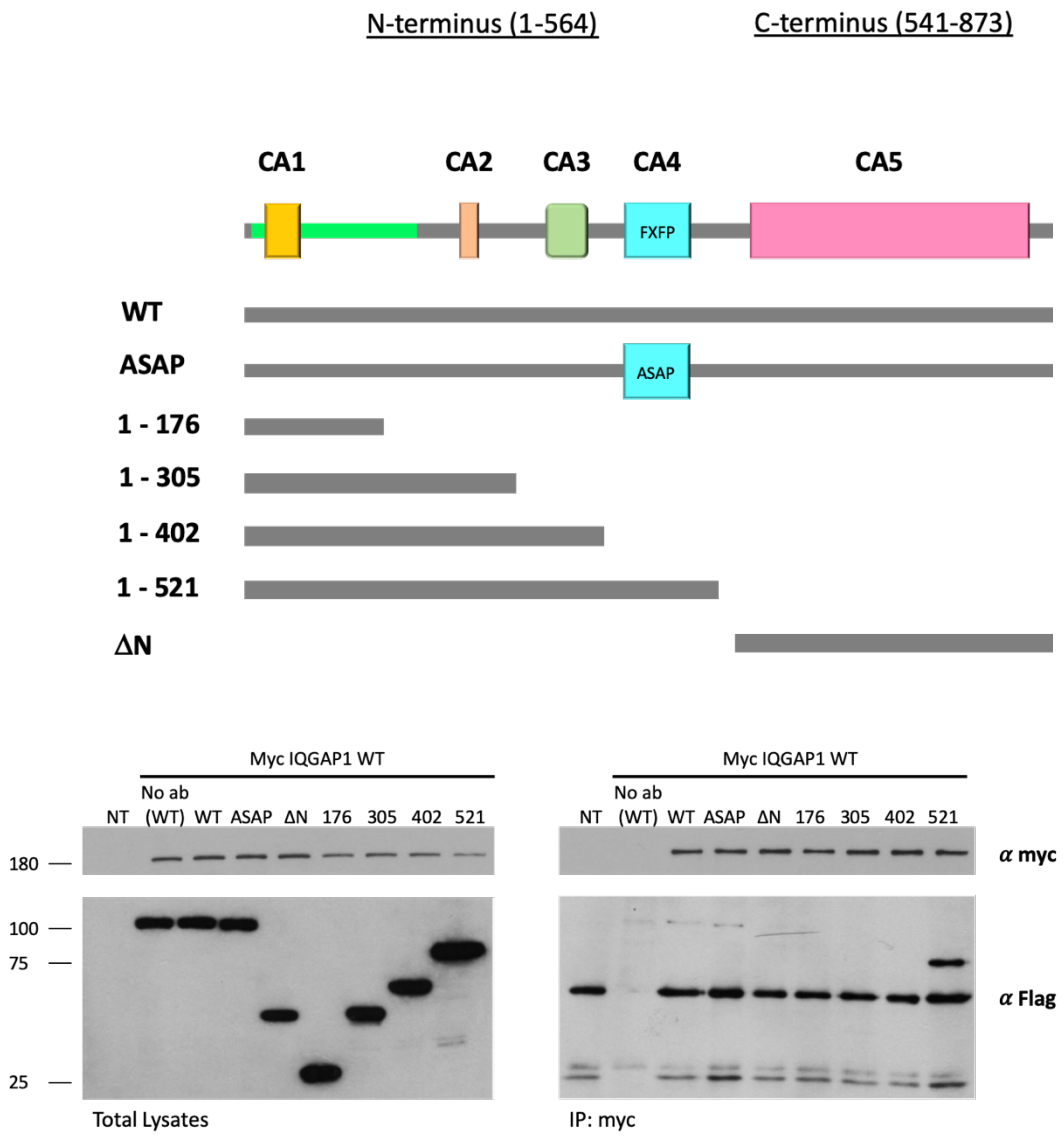


Figure 4.23. KSR1 IQGAP1-binding domain is localized between amino acids 402-521. Top panel: *Depiction of the KSR1 deletion mutants utilized.* ***Lower panel:*** *HEK293T cells were transfected with the indicated Flag-tagged KSR1 constructs, in addition to Myc-tagged IQGAP1 (1.5 μg each) and stimulated with EGF (50 ng/ml; 5') after 18 h starvation. The KSR1 constructs associated to IQGAP1 were determined by co-immunoprecipitation upon anti-Myc immunoprecipitation and subsequent anti-Flag western blotting.*

4.2.5. Identification of the KSR1-binding motif in IQGAP1

Since IQGAP1 is also a multidomain protein, in order to identify which IQGAP1 region is mediating on its interaction with KSR1 we utilized the same strategy as above, and used a battery of IQGAP1 deletion mutants for the different domains present in the protein. By co-immunoprecipitation assays, it was found that all those constructs that contain the C-terminal region spanning from aa 864-1657, which include the GRD and the RGCT domains, could bind to KSR1. While construct devoid of this region could not *(Fig. 4.23)*.

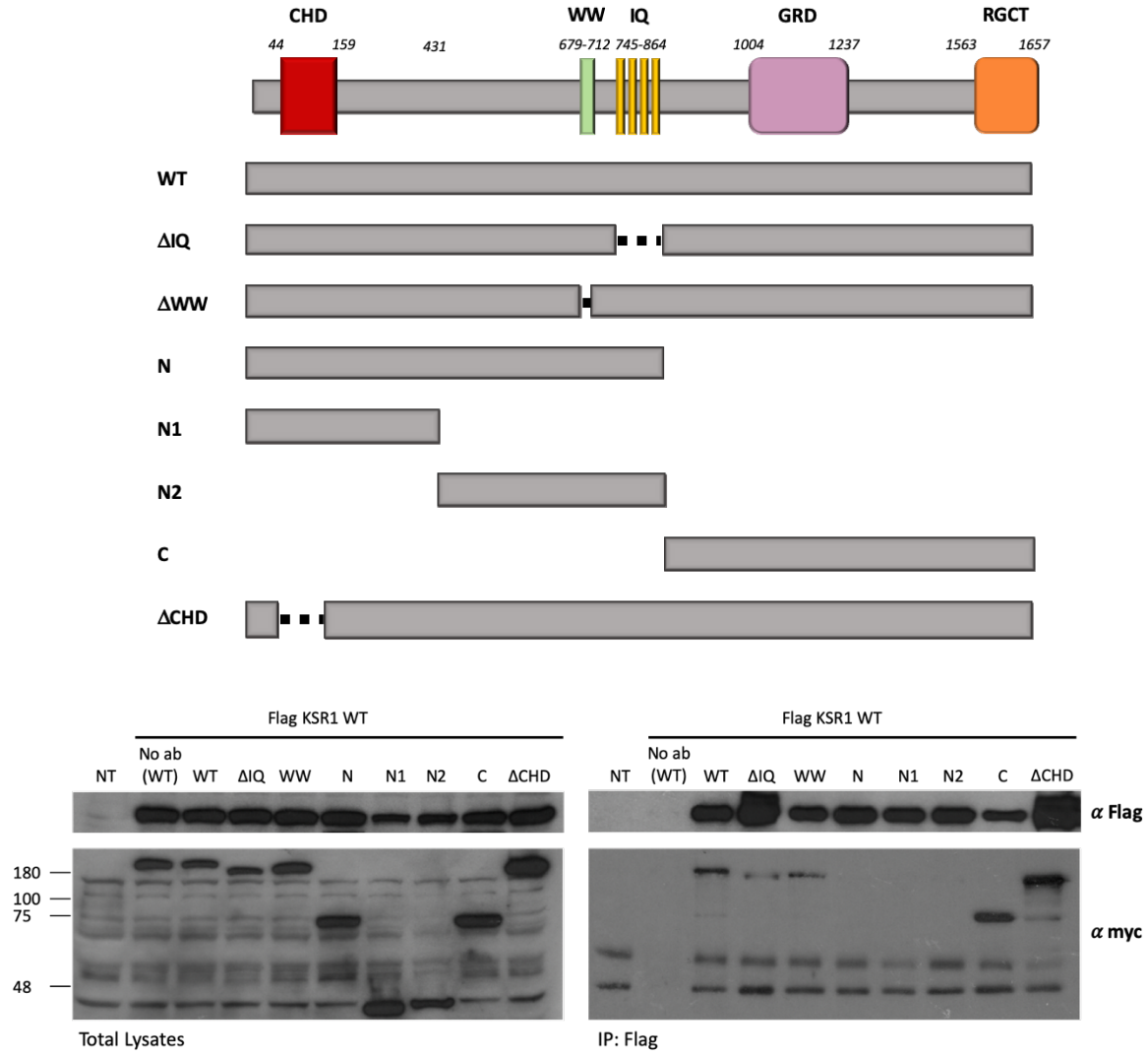


***Figure 4.23. IQGAP1 KSR1-binding domain is localized the C-terminal region**. **Top panel:** Depiction of the IQGAP1 deletion mutants utilized**. Lower panel:** HEK293T cells were transfected with the indicated Myc-tagged IQGAP1 constructs, in addition to Flag-tagged KSR1 (2 μg) and stimulated with EGF (50 ng/ml; 5') after 18 h starvation. The IQGAP1 constructs associated to KSR1 were determined by co-immunoprecipitation upon anti-Flag immunoprecipitation and subsequent anti-Myc western blotting.*

4.2.6. The transphosphorylation could be reciprocal between KSR1 and IQGAP1

Since we have observed that ERK bound to KSR1 could be transphosphorylated by another scaffold such as IQGAP1, we asked if IQGAP1 was subject to the same phenomenon. In a similar fashion to the previous experiments performed with KSR1, we used an IQGAP1 mutant defective for binding MEK (ΔIQ) and analyzed its ability to bind phosphorylated ERK. It was found that, indeed, IQGAP1 defective for binding MEK was capable of associating to phosphorylated ERK *(Fig. 4.24)*. This result demonstrated that IQGAP1 could also be transphosphorylated by some yet unidentified scaffold protein, which could be another IQGAP1 molecule, through homodimerization, or KSR1 via heterodimerization.

4.2.7. Effects of APS-2-79 on transphosphorylation

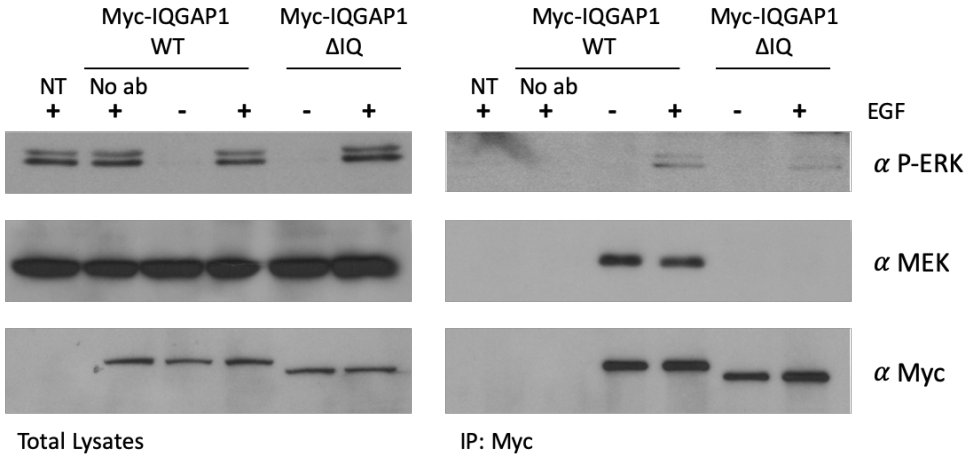


Figure 4.24. ERK transphosphorylation in IQGAP1 ΔIQ mutant, unable to bind MEK. *HEK293T cells were transfected with the indicated Myc-tagged IQGAP1 constructs (2 μg) and stimulated with EGF (50 ng/ml; 5') where shown (+) after 18 h starvation. Phosphorylated ERK associated to IQGAP1 was determined by co-immunoprecipitation upon anti-Myc immunoprecipitation (IP: Myc) and subsequent western blotting for the indicated proteins.*

As demonstrated in our aforementioned experiments, APS-2-79 is highly inefficient for preventing KSR1-bound ERK phosphorylation, in spite of locking KSR1 in its inactive form and preventing the phosphorylation of KSR1-bound MEK (Dhawan, Scopton and Dar, 2016). In light of our discovery of KSR1 transphosphorylation by IQGAP1, a possible explanation could be that APS-2-79 would not prevent KSR1-IQGAP1 heterodimerization and, as a consequence, the

transphosphorylation of KSR1-bound ERK by IQGAP1-bound MEK. To put this notion under test, we analysed whether APS-2-79 treatment could impede KSR1-IQGAP1 heterodimerization. For this we performed co-immunoprecipitation experiments in HEK293T cells treated with the drug and found that APS-2-79 did not affect KSR1-IQGAP1 interaction whatsoever *(Fig. 4.25)*.

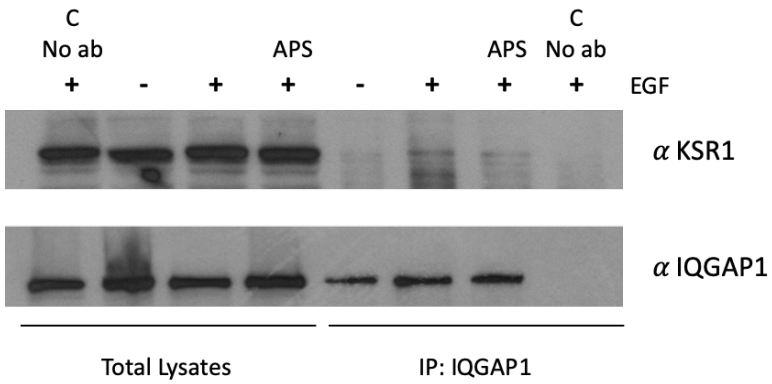


Figure 4.25. APS does not affect KSR1-IQGAP1 interaction. *HEK293T cells were* treated with APS-2-79 (5 μM; 48 h) *and stimulated with EGF (50 ng/ml; 5') where shown (+) after 18 h starvation. Endogenous KSR1 associated to IQGAP1 was determined by co-immunoprecipitation upon anti-IQGAP1 immunoprecipitation (IP: IQGAP1) and subsequent western blotting for the indicated proteins.*

4.3. Analyses of scaffolds-ERK affinity

Previous data demonstrates: 1) That IQGAP1 and KSR1 can intervene in different ERK-mediated biochemical processes, both with respect to the subcellular localizations where they act and the substrates whose activation they regulate (Casar *et al.*, 2009). and 2) The data presented herein, shows that the flux of RAS/ERK pathway signals through IQGAP1 and KSR1 can take place by direct phosphorylation of their respective cascade components and also through transphosphorylation between each other's components. Thus, the decision direct phosphorylation vs transphosphorylation could have important consequences regarding the spectrum of substrates being subject to ERK-mediated activation. Therefore, it is very likely that the

bifurcation phosphorylation vs transphosphorylation, is a tightly regulated step, that may tilt one way or the other depending on the circumstances.

An essential factor in the prevalence of either the direct phosphorylation or of the transphosphorylation component could be highly dependent on the differences in affinity towards ERK exhibited by each of the participating scaffolds, in addition to the affinity whereby the different scaffolds bind to each other. Surprisingly, and in spite of the fact that scaffold proteins have been around for over a decade, the literature is, to the best of our knowledge, entirely devoid of measurements of scaffold-ERK affinities for all of the ERK scaffold proteins identified thus far. To fill this gap, and as an initial step towards understanding the above postulate, we aimed at unravelling if different scaffolds species exhibit differences in their affinity for ERK, towards finding its implications in transphosphorylation.

4.3.1. Calculation of the dissociation constant (Kd) values for MP1, KSR1 and IQGAP1 interaction with ERK2.

To analyze if distinct scaffold proteins exhibit differences on their affinity for ERK, we performed *in vitro* binding assays to determine the dissociation constant (K_d) values for the interactions between the aforementioned scaffold proteins and ERK2. To this end, we generated constructs expressing GST fusion proteins for the ERK-binding domains of KSR1 (EBD) and IQGAP1

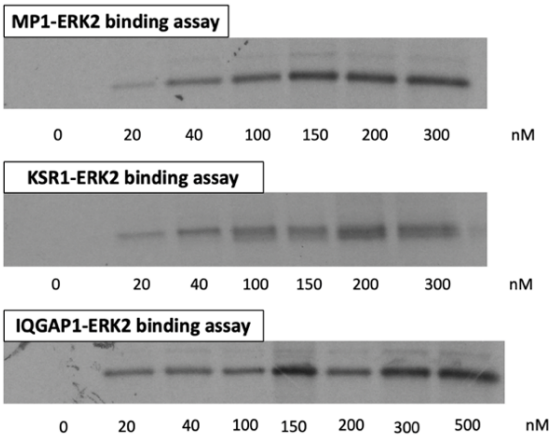


Figure 4.26. Representative autoradiographs corresponding to MP1-, KSR1- and IQGAP1-ERK2 bindings. *Increasing concentrations of radiolabelled ERK2 were incubated with a fixed amount (20 nM) of purified MP1, KSR1 and IQGAP1*

(WW), or the MP1 whole protein. A fixed concentration of the GST-scaffold proteins bound to glutathione-sepharose beads, was incubated with increasing concentrations of in vitro-synthesized methionine S^{35} radiolabelled ERK2, up to saturation, and the binding constants for the different scaffolds were calculated after quantification by autoradiography *(Fig .4.26)*. The results obtained *(Fig. 4.27)* demonstrated that the distinct scaffold proteins exhibit different affinities towards ERK2 with MP1 displaying the greatest affinity (K_d= 0.1359 μM) followed by KSR1 (K_d= 0.1511 μM) and IQGAP1 (K_d= 0.2681 μM) showing the lowest.

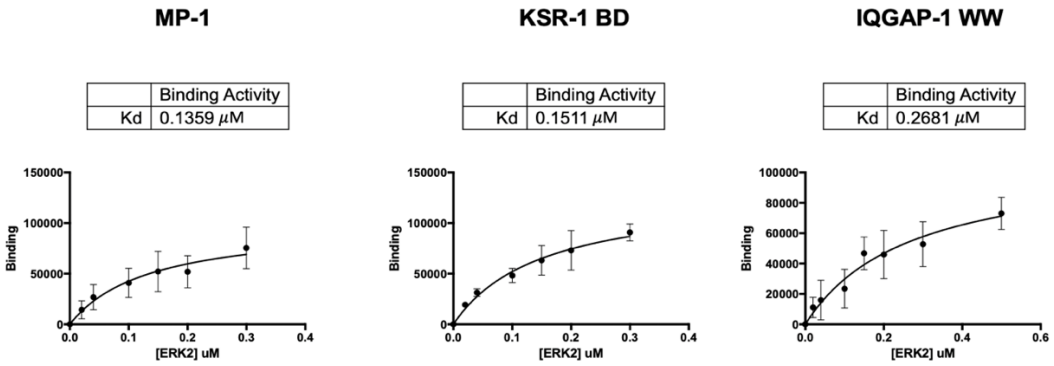


Figure 4.27. Quantification of the dissociation constants (K_d) for ERK2 interaction with the indicated scaffold proteins. *Kd values were calculated by Dissociation-One phase exponential decay analysis (non-linear regression) analysis using GraphPad Prism 7 software. Results show Mean ± SEM of 4 independent experiments.*

4.3.2. In vivo binding ascertains KSR1 and IQGAP1 different affinities towards ERK.

We wanted to ascertain if the differences on scaffold-ERK K_d's determined in vitro also held in vivo. In order to do so, by co-immunoprecipitation assays we analyzed ERK binding to the different scaffolds upon dose-response analyses using increasing concentrations of EGF *(Fig.4.28)*. It was

found that KSR1 co-immunoprecipitated with phosphorylated ERK under minimal stimulation, whereas IQGAP1 binding was only evident upon stimulation with 10 ng/ml of EGF, suggesting that KSR1 binds to ERK with a greater affinity than IQGAP1. Unfortunately, there are no good

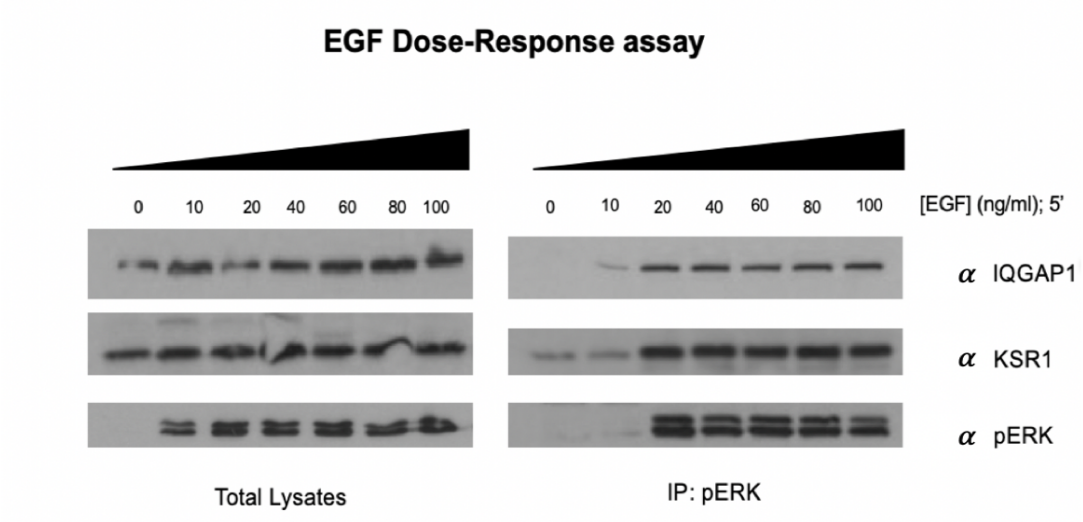


Figure 4.28. KSR1 binds ERK2 with higher affinity than IQGAP1. *HEK293T cells were stimulated with increasing concentrations of EGF during 5 minutes and phosphor-ERK was immunoprecipitated in order to analyze co-immunoprecipitated KSR1 and IQGAP1 levels.*

commercially available antibodies for MP1, so we could not perform the same assays for this scaffold.

Finally, we evaluated how KSR1 and IQGAP1 interacted with ERK following its activation/phosphorylation kinetics in response to EGF stimulation. For this purpose, HEK293T cells were stimulated with EGF for different times and the amount of scaffold proteins associated to ERK were monitored through the activation period. Once again, it was found that KSR1 could bind to ERK under basal conditions, when no IQGAP1-ERK association was detected, this only became apparent after 2 min stimulation. Interestingly, IQGAP1 binding to ERK coincided with a drop on ERK-bound KSR levels, that peaked again once IQGAP1 had dissociated from ERK *(Fig. 4.29)*. This result could suggest that, under some circumstances KSR1 and IQGAP1 could be competing for the same pool of ERK.

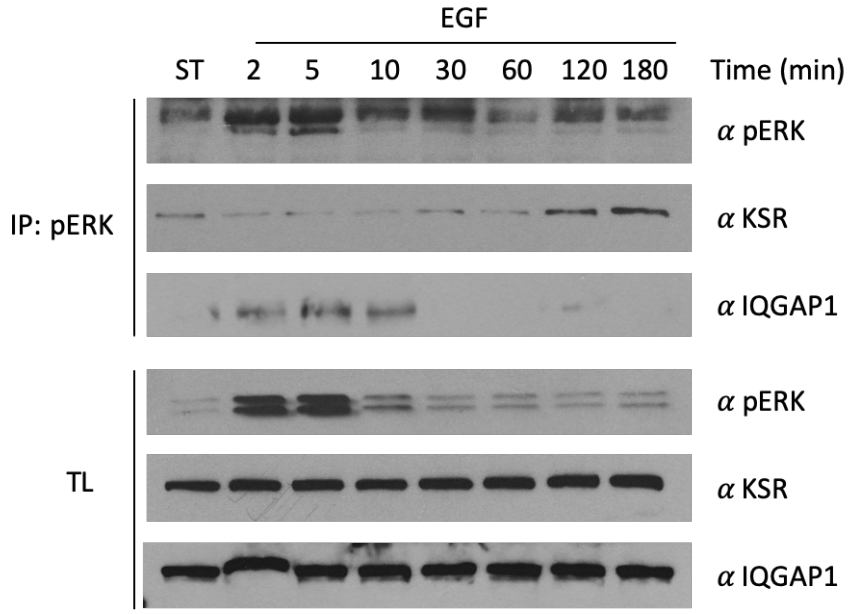


Figure 4.29. Kinetics of ERK interaction with IQGAP1 and KSR1 upon EGF stimulation. *HEK293T cells were stimulated with EGF (50 ng/ml) for the indicated times after 18 h starvation. The scaffold proteins associated to ERK were determined by co-immunoprecipitation with phosphorylated ERK and subsequent western blotting for the indicated proteins.*

5. DISCUSSION

For the past decades the quest for inhibitors of the RAS/ERK pathway has attracted enormous efforts. As the strategies for targeting the kinase activities of the different components of the pathway have become almost exhausted, attention has been placed on regulatory molecules of different types including scaffold proteins. As mentioned in the introduction, due to their mode of action, scaffold proteins offer attractive possibilities as therapeutic targets, in order to curtail aberrant RAS/ERK signalling (Calvo, Agudo-Ibáñez and Crespo, 2010; Langeberg and Scott, 2015; Zaballos *et al.*, 2019). This novel concept for antitumor drugs has already yielded the first compound at the preclinical stage: APS-2-79. APS-2-79 has emerged in a screening guided by KSR mutations that selectively suppress oncogenic but not WT RAS signalling. This small molecule apparently stabilizes KSR in an inactive state previously unknown. As a consequence, APS-2-79 locks KSR in a structural conformation that prevents RAF heterodimerization and precludes the conformational changes necessary for the phosphorylation and activation of KSR-bound MEK, thereby preventing ERK phosphorylation/activation (Dhawan, Scopton and Dar, 2016).

However, in spite of its theoretical efficacy, as reported in the original publication, the antiproliferative/antiapoptotic effects of APS-2-79 on tumour cell lines harbouring RAS/ERK pathway oncogenes are, at best, modest. APS-2-79 does not work at all as monotherapy, and only displays some efficacy in combination with MEK inhibitors in RAS- but not BRAF-mutant cell lines (Dhawan, Scopton and Dar, 2016). Indeed, we have extended this initial observation in a broader panel of BRAF- and NRAS-mutant melanoma cell lines and ascertained the inefficacy of this compound for inducing apoptosis. Moreover, it is quite surprising that the original publication did not thoroughly address APS-2-79 effects on ERK phosphorylation, both total and KSR-bound.

Herein we have filled in this gap and have found that APS-2-79 has little, if any, effect on ERK phosphorylation, both with respect to its total levels and the KSR-bound fraction. This is in sharp contrast with the consequences of downregulating KSR expression levels. In this respect, previous publications have shown that ablation of KSR1 yields mice with a remarkable resistance to RAS-induced carcinogenesis (Lozano *et al.*, 2003). In the same vein, herein we have demonstrated that

downregulating KSR levels, using shRNAs, induces a potent apoptotic response in melanoma cell lines harbouring oncogenic NRAS but not BRAF. This result hints for the necessity of KSR in RAS oncogenic signalling and on its dispensability for oncogenic BRAF functions. This is in line with previous publications showing that KSR can antagonize mutant BRAF oncogenic signalling via heterodimerization (Mckay, Freeman and Morrison, 2011; Mckay, Ritt and Morrison, 2011), and suggest that BRAF does not require KSR for effectively activating ERK. In addition, we also demonstrate that KSR downregulation, unlike APS-2-79 treatment, markedly diminishes total ERK activation levels. In light of these data we can conclude that APS-2-79 is a highly ineffective inhibitor of aberrant RAS/ERK signals and its pathological consequences.

The question remains as to why APS-2-79, in spite of maintaining KSR in its inactive conformation, does not phenocopy KSR down-regulation, neither in its biochemical consequences, with respect to ERK phosphorylation, nor in its biological results. In this respect we have observed that both KSR downregulation and overexpression affect ERK phosphorylation levels in NRAS-mutant cells and, probably as a consequence, induce apoptosis. Regarding KSR overexpression, downregulation of ERK phosphorylation and apoptosis induction follow a bell-shaped kinetics, as would be expected from a scaffold protein.

In the course of these experiments we utilized the mutant form KSR1 C809Y, impaired for binding MEK (Stewart *et al.*, 1999). While we were expecting that this mutant behaved in a dominant inhibitory fashion or, at least, would not have any effect, quite surprisingly we have observed that this mutant behaves similarly to KSR1 WT, both with respect to ERK activation and to apoptosis induction. In our quest to understand the mechanisms underlying in this unexpected behaviour we have found that KSR1 C809Y, in spite of its inability to bind MEK can incorporate phosphorylated ERK under EGF stimulation, as demonstrated by biochemical and cell biology analyses. This finding is in full agreement with a previous report showing that KSR1 C809Y can support RAS-induced senescence in KSR1 -/- MEFs as efficiently as WT KSR1 (Kortum *et al.*, 2006).

All together, these data demonstrate that KSR1 C809Y is an active protein that can efficiently convey ERK signals.

Previously, we had hypothesized the possibility that a direct interaction between two scaffold molecules could compensate for one of the molecules deficiency for binding MEK, via cross-phosphorylation. Whereby the ERK molecule on the deficient scaffold could be activated by MEK bound to the other scaffold (Casar and Crespo, 2016). In light of previous publications demonstrating that KSR1 can homodimerize (Rajakulendran *et al.*, 2009) we have tested whether such homodimerization could explain the presence of phosphorylated ERK in KSR1 C809Y. Indeed, the introduction of the mutation R615H in KSR1 C809Y, which precludes its homodimerization, prevents it from incorporating phosphorylated ERK. It is noteworthy that in the previous publication (Kortum *et al.*, 2006) the ability of KSR1 C809Y for mediating in RAS-induced senescence, was interpreted as KSR1 C809Y-bound ERK being phosphorylated by soluble MEK. Our results showing that KSR1 C809Y/R615H does not bind phosphorylated ERK speaks against this possibility, as ERK bound to this mutant should also be subject to phosphorylation by soluble MEK. Alternatively, it points to what we have coined as “transphosphorylation” as the mechanism responsible for such process.

Transphosphorylation implies that scaffold proteins can interact among themselves, something demonstrated by KSR1 heterodimerization, and that such association can make possible the phosphorylation of the different kinase tiers by upstream kinases bound to the associated scaffolds.

We have observed that KSR1 C809Y can intervene on ERK-regulated processes, such as PLA_2 activation, in cellular contexts devoid of endogenous KSR molecules such as KSR1 -/- MEFs. Something that would invalidate the possibility of KSR1 C809Y being transphosphorylated by a homodimerizing, endogenous KSR oligomer. In this realm, we have observed that the deficiency for endogenous KSR can be compensated by another scaffold protein species: IQGAP1. Indeed, we

have unveiled that KSR1 and IQGAP1 can directly associate. This result adds KSR1 to the list of scaffold proteins species previously shown to bind IQGAP1, namely: MP1 (Schiefermeier *et al.*, 2014) and β-arrestin2 (Alemayehu *et al.*, 2013; Feigin *et al.*, 2014).

Furthermore, we have mapped IQGAP1 KSR1-binding region in its C-terminus; and KSR1 IQGAP1-binding region in its CA4 region, close to the FXFP motif whereby it binds to ERK, even though it cannot be discarded that other regions in KSR1, such as the CA5 region, also contribute to some extent to IQGAP1 binding in a negative way, as KSR1 forms which include this region bind with lower efficiency. In this respect, our results suggest that IQGAP1-KSR1 binding is subject to a stringent stoichiometric relationship. We have found that in HEK293T cells, which express endogenous IQGAP1, KSR1 R615H/C809Y does not bind to IQGAP1 upon EGF stimulation, probably due to its reduced affinity when compared to KSR1 WT. However, it does when IQGAP1 is overexpressed. To explain this observation, we posit that under stimulation IQGAP1 could have greater affinity, either for itself through homodimerization (Ren *et al.*, 2005) or for other partners, than for KSR1 R615H/C809Y. This binding deficiency would be compensated by high IQGAP1 levels resulting from overexpression.

In this respect, we demonstrate that IQGAP1 can function as the transphosphorylating partner on KSR1 R615H/C809Y, since in parental HEK293T cells this mutant does not incorporate phosphorylated ERK, but it does so when IQGAP1 is overexpressed. In addition, the unquestionable proof that points to IQGAP1 being capable of mediating transphosphorylation on KSR1 comes from our very preliminary data showing that IQGAP1 mutant forms deficient for binding MEK or for interacting with KSR1 cannot promote KSR1 binding to phosphorylated ERK. This result would exclude the possibility of IQGAP1 inducing ERK phosphorylation by other means.

Overall, our results support the notion that cross phosphorylation can occur across different scaffold species. In this respect, we also present preliminary results demonstrating that IQGAP1 deficient for binding MEK can incorporate phosphorylated ERK. Even though further

experimentation will be necessary to identify KSR1 as the responsible for such an effect, if this was the case it would mean that transphosphorylation can happen in both directions. However, at this moment, we cannot exclude that some other scaffold protein is responsible for transphosphorylating IQGAP1.

Whatever the case, our data clearly show that KSR1 and IQGAP1 can physically associate and both interact in the regulation of ERK activation. Not excluding the possibility that both scaffolds can act on their own, our results provide strong evidence to support that both proteins could be acting in combination for the regulation of yet unknown cellular and biological processes. In this respect, the data that we have gathered from the TCGA tumour data repository shows that genetic alterations in IQGAP1 and KSR1 occur in a mutually exclusive fashion, both in tumours with high and low frequency of RAS/ERK pathway oncogenic mutations. As it is well-known, in tumours, the mutually exclusive mutational pattern is characteristic of genes/proteins involved in the same processes. The best example for this is the non-overlaping occurrence of BRAF and NRAS mutations (Gorden *et al.*, 2003). Further supporting this notion, we show that IQGAP1 downregulation is as effective as that of KSR1 for preventing proliferation in NRAS-mutant cells, while neither of them affect the growth of BRAF-mutant cells.

We have previously shown that IQGAP1 and KSR1 regulate the activation of different substrates by ERK (Casar *et al.*, 2009). By these scaffolds acting in a coordinated fashion, such as via transphosphorylation, it is possible that the spectrum of ERK substrates is broadened. In agreement with this notion, it has been shown that MORG1 participates in ERK activation induced by serum but not by EGF (Vomastek *et al.*, 2004), whereas MP1 mediates in EGF- (Teis, Wunderlich and Huber, 2002) but not serum-induced signalling (Sharma *et al.*, 2005). Considering these cases, if the pools of ERK substrates defined by MORG1 and MP1 were different, EGF or serum stimulation would yield activation of a narrow repertoire of substrates. Contrarily, if transphosphorylation also occurred between MORG1 and MP1 it would facilitate the whole spectrum of substrates both to serum- and to EGF-induced ERK signals.

The available data, though scarce, appears to hint for the existence of higher-order associations among regulatory proteins, in which the involvement, probably in a coordinated fashion, of different scaffolds, could provide an additional degree of complexity to the already highly complex regulation of signal flux through the RAS-ERK pathway. In this respect, and based on our current knowledge showing that different scaffold species engage distinct pools of substrates (Casar, Pinto and Crespo, 2009), it can be envisioned that complexes made up of different scaffolds, competent for cooperating among themselves, may constitute a novel type of regulatory node, whereby distinct incoming signals are integrated and outgoing signals are diversified with respect to substrate usage (Casar and Crespo, 2016).

Our results demonstrate for the first time the cooperation of two scaffold species in the regulation of a signalling pathway. Such cooperativity between scaffold species would open the possibility that scaffolds missing one or more kinases could associate in *trans* with other incomplete scaffolds of other species to allow signal flux. This coordination between different scaffolds would allow the complementation and compensation of each other's deficiencies. As such, incomplete scaffold proteins, theoretically incompetent in their role, as is the case for our KSR1 and IQGAP1 MEK-binding mutants, would be fit for signalling, as we demonstrate. In this fashion, signal transmission could take place under circumstances where different scaffold species would fail if acting on their own. Cooperation of this nature would be particularly beneficial in cases where the collaborating scaffolds display different affinities for a kinase whose concentration is limiting.

It is noteworthy that the affinity for ERK has not been measured for most, if not all, of the scaffold proteins described hitherto. In this respect, we have filled in this gap by measuring the affinities towards ERK displayed by KSR1, IQGAP1 and MP1. Our data demonstrates that, indeed, these different scaffold species bind to ERK with different affinities, MP1 showing the highest and IQGAP1 the lowest affinity. In agreement, we also showed for KSR1 and IQGAP1 that this differences in Kd dictate the kinetics of the interaction of these scaffold proteins with ERK under stimulation in vivo.

In light of all these data, it can be envisioned that the flux of ERK signals through two scaffold species that can interact and transphosphorylate each other, will be dependent on, at least, five Kds (Fig): if KdAB is higher than KdAA or KdBB signalling will flow through scaffolds A and B independently. However, if, on the contrary, A or B display more affinity for each other than for themselves heterodimerization and transphosphorylation will occur. If this happens, within the scaffolds heterodimer there would be a prevalence for the signal flowing either through A or B depending on the Kd that each scaffold protein displays for ERK. Out of this it can be deduced that the whole process would be highly complex and subject to tight regulation, since it is conceivable that the previously mentioned affinities could vary depending on structural changes triggered by different stimuli. Likewise, changes on scaffold concentrations could also influence significantly the flux of signals one way or another.

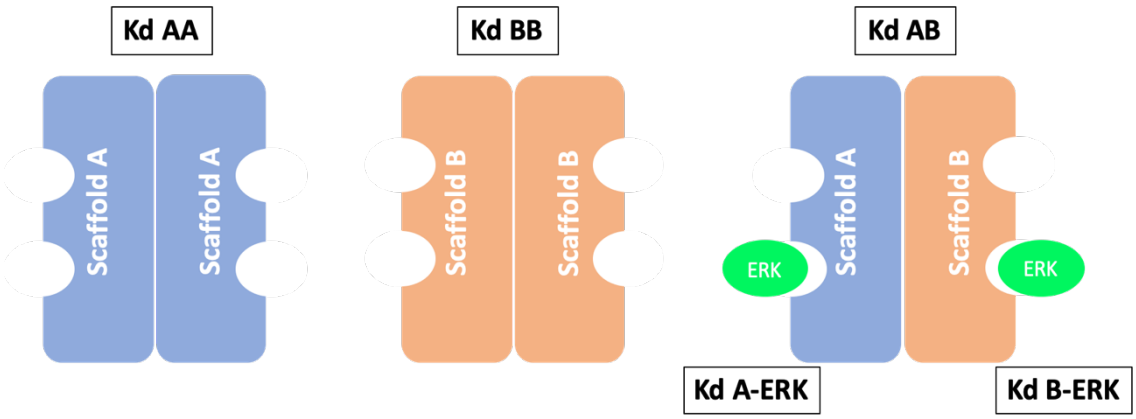


Figure 5.1. The flux of ERK signals through two scaffold species able to interact and transphosphorylate each other, will be dependent on, at least, five Kds. *The triggered ERK-dependent response would eventually depend on the affinity of self-association of each scaffold, the affinity between each other and the affinity towards ERK of each of them.*

Both the original publication and our present data, show that APS-2-79 is highly inefficient as an inhibitor of KSR1-mediated ERK signals and its oncogenic consequences. In spite of the fact that APS-2-79 locks KSR in an inactive conformation effectively preventing KSR-bound MEK phosphorylation. The results that we present herein could provide an explanation for APS-2-79 failure. We demonstrate that APS-2-79 cannot prevent KSR1-IQGAP1 interaction and IQGAP1-mediated transphosphorylation of KSR1-bound ERK. As such this drug, even though it can inhibit

the activation of the KSR-bound upper tiers of the ERK cascade, it cannot prevent ERK phosphorylation. Indeed, in the original publication it is shown that APS-2-79 markedly inhibits MEK but not ERK phosphorylation (Dhawan, Scopton and Dar, 2016).

APS-2-79 inefficiency does not discard KSR1 as a promising antitumoral target. Indeed, in agreement with previous publications (Lozano *et al.*, 2003; Llobet *et al.*, 2011; Li *et al.*, 2013; Stebbing *et al.*, 2015; Zhou *et al.*, 2016) we showed that KSR ablation has clear antineoplastic effects. This indicates that what is invalid is not the therapeutic target, but rather the mechanism of action of the drug. However, alternative mechanisms of action can be envisioned for preventing the flux of ERK signals through KSR. For example, drugs that physically block the interaction of KSR with either MEK or ERK. In this respect, the results presented herein are of great value, as we show that, as a consequence of transphosphorylation, drugs aimed at preventing KSR1 interaction with MEK would be inefficient because they would fail to impede KSR1-bound ERK transphosphorylation by IQGAP1-bound MEK. Therefore, our results clearly indicate that the most efficient mechanism for preventing KSR1-mediated RAS/ERK pathway oncogenic signals is by blocking the interaction between KSR1 and ERK.

6. CONCLUSIONS

1. APS-2-79 is highly inefficient as an inhibitor of RAS/ERK pathway mediated oncogenesis. Probably as a consequence of its inability to interfere with KSR-bound ERK phosphorylation.

2. Scaffold proteins such as KSR1 and IQGAP1 deficient for binding MEK can incorporate phosphorylated ERK.

3. In the case of KSR1, transphosphorylation is undertaken not by free MEK, but by MEK bound either to another homodimerizing KSR molecule, or to a heterodimerizing IQGAP1 molecule.

4. KSR1 binds to IQGAP1. Such interaction occurs through KSR1 CA4 region and IQGAP1 C-terminus, and it is highly dependent on KSR1-IQGAP1 stoichiometry.

5. APS-2-79 failure to inhibit KSR-bound ERK phosphorylation is a consequence of its inability to prevent its transphosphorylation, probably as this drug cannot interfere with KSR1-IQGAP1 interaction.

6. Scaffold proteins such as MP1, KSR1 and IQGAP1 exhibit different binding affinities towards ERK. These differences dictate kinetics of ERK interaction with the different scaffold species in response to stimulation.

7. BIBLIOGRAPHY

Adachi, M., Fukuda, M. and Nishida, E. (2000) 'Nuclear export of MAP kinase (ERK) involves a MAP kinase kinase (MEK)-dependent active transport mechanism', *Journal of Cell Biology*, 148(5), pp. 849–856. doi: 10.1083/jcb.148.5.849.

Aebersold, D. M. *et al.* (2004) 'Extracellular signal-regulated kinase 1c (ERK1c), a novel 42-kilodalton ERK, demonstrates unique modes of regulation, localization, and function.', *Molecular and cellular biology*, 24(22), pp. 10000–15. doi: 10.1128/MCB.24.22.10000-10015.2004.

Ahn, S. *et al.* (2004) 'Differential kinetic and spatial patterns of β-arrestin and G protein-mediated ERK activation by the angiotensin II receptor', *Journal of Biological Chemistry*, 279(34), pp. 35518–35525. doi: 10.1074/jbc.M405878200.

Alemayehu, M. *et al.* (2013) 'β-Arrestin2 Regulates Lysophosphatidic Acid-Induced Human Breast Tumor Cell Migration and Invasion via Rap1 and IQGAP1', *PLoS ONE*, 8(2). doi: 10.1371/journal.pone.0056174.

Alessi, D. R. *et al.* (1995) 'Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines.', *Current biology : CB*, 5(3), pp. 283–95. doi: 10.1016/s0960-9822(95)00059-5.

Alsina, J. *et al.* (2003) 'Detection of mutations in the mitogen-activated protein kinase pathway in human melanoma.', *Clinical cancer research : an official journal of the American Association for Cancer Research*, 9(17), pp. 6419–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14695143 (Accessed: 4 January 2020).

Appels, N. M. G. M. (2005) 'Development of Farnesyl Transferase Inhibitors: A Review', *The Oncologist*. Alphamed Press, 10(8), pp. 565–578. doi: 10.1634/theoncologist.10-8-565.

Aronov, A. M. *et al.* (2009) 'Structure-guided design of potent and selective pyrimidylpyrrole inhibitors of extracellular signal-regulated kinase (ERK) using conformational control', *Journal of Medicinal Chemistry*, 52(20), pp. 6362–6368. doi: 10.1021/jm900630q.

Arozarena, I., Calvo, F. and Crespo, P. (2011) 'Ras, an actor on many Stages: Posttranslational modifications, Localization, and Site-Specified Events', *Genes and Cancer*. SAGE Publications Inc., 2(3), pp. 182–194. doi: 10.1177/1947601911409213.

Ascierto, P. A. *et al.* (2012) 'The role of BRAF V600 mutation in melanoma', *Journal of Translational Medicine*. doi: 10.1186/1479-5876-10-85.

Bagchi, S. *et al.* (2018) 'Farnesyl Transferase Inhibitors as Potential Anticancer Agents', *Mini-Reviews in Medicinal Chemistry*. Bentham Science Publishers Ltd., 18(19), pp. 1611–1623. doi: 10.2174/1389557518666180801110342.

Bai, S. *et al.* (2017) 'Overexpression of wild-type p21Ras plays a prominent role in colorectal cancer', *International Journal of Molecular Medicine*. Spandidos Publications, 39(4), pp. 861–868. doi: 10.3892/ijmm.2017.2903.

Baines, A. T., Xu, D. and Der, C. J. (2011) 'Inhibition of Ras for cancer treatment: The search continues', *Future Medicinal Chemistry*, pp. 1787–1808. doi: 10.4155/fmc.11.121.

Balan, V. *et al.* (2006) 'Identification of novel in vivo Raf-1 phosphorylation sites mediating positive feedback Raf-1 regulation by extracellular signal-regulated kinase', *Molecular Biology of the Cell*, 17(3), pp. 1141–1153. doi: 10.1091/mbc.E04-12-1123.

Bell, B. *et al.* (1999) 'KSR-1 binds to G-protein βγ subunits and inhibits βγ-induced mitogen-activated protein kinase activation', *Journal of Biological Chemistry*, 274(12), pp. 7982–7986. doi: 10.1074/jbc.274.12.7982.

Boga, S. B. *et al.* (2018) 'MK-8353: Discovery of an Orally Bioavailable Dual Mechanism ERK Inhibitor for Oncology', *ACS Medicinal Chemistry Letters*. American Chemical Society, 9(7), pp. 761–767. doi: 10.1021/acsmedchemlett.8b00220.

Bollag, G. *et al.* (2012) 'Vemurafenib: The first drug approved for BRAF-mutant cancer', *Nature Reviews Drug Discovery*, pp. 873–886. doi: 10.1038/nrd3847.

Boudeau, J. *et al.* (2006) 'Emerging roles of pseudokinases', *Trends in Cell Biology*, 16(9), pp. 443–452. doi: 10.1016/j.tcb.2006.07.003.

Boulton, T. G. *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), pp. 663–675. doi: 10.1016/0092-8674(91)90098-J.

Boulton, T. G. and Cobb, M. H. (1991) 'Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies', *Molecular Biology of the Cell*. American Society for Cell Biology, 2(5), pp. 357–371. doi: 10.1091/mbc.2.5.357.

Bourguignon, L. Y. W. *et al.* (2005) 'Hyaluronan-CD44 interaction with IQGAP1 promotes Cdc42 and ERK signaling, leading to actin binding, Elk-1/estrogen receptor transcriptional activation, and ovarian cancer progression', *Journal of Biological Chemistry*, 280(12), pp. 11961–11972. doi: 10.1074/jbc.M411985200.

Bozzi, M. *et al.* (2009) 'Functional diversity of dystroglycan.', *Matrix biology : journal of the International Society for Matrix Biology*, 28(4), pp. 179–87. doi: 10.1016/j.matbio.2009.03.003.

Brahma, A. and Dalby, K. N. (2007) 'Regulation of protein phosphorylation within the MKK1-ERK2 complex by MP1 and the MP1•P14 heterodimer', *Archives of Biochemistry and Biophysics*, 460(1), pp. 85–91. doi: 10.1016/j.abb.2006.11.031.

Brennan, D. F. *et al.* (2011) 'A Raf-induced allosteric transition of KSR stimulates phosphorylation of MEK', *Nature*. Nature Publishing Group, 472(7343), pp. 366–369. doi: 10.1038/nature09860.

Brennan, J. A. *et al.* (2002) 'Phosphorylation regulates the nucleocytoplasmic distribution of kinase suppressor of Ras', *Journal of Biological Chemistry*, 277(7), pp. 5369–5377. doi: 10.1074/jbc.M109875200.

Brill, S. *et al.* (1996) 'The Ras GTPase-activating-protein-related human protein IQGAP2 harbors a potential actin binding domain and interacts with calmodulin and Rho family GTPases.', *Molecular and Cellular Biology*. American Society for Microbiology, 16(9), pp. 4869–4878. doi: 10.1128/mcb.16.9.4869.

Brondello, J. M. *et al.* (1997) 'The dual specificity mitogen-activated protein kinase phosphatase-1 and -2 are induced by the p42/p44(MAPK) cascade', *Journal of Biological Chemistry*, 272(2), pp. 1368–1376. doi: 10.1074/jbc.272.2.1368.

Brondello, J. M., Pouysségur, J. and McKenzie, F. R. (1999) 'Reduced MAP kinase phosphatase-1 degradation after p42/p44(MAPK)- dependent phosphorylation', *Science*, 286(5449), pp. 2514–2517. doi: 10.1126/science.286.5449.2514.

Bunda, S. *et al.* (2014) 'Src promotes GTPase activity of Ras via tyrosine 32 phosphorylation', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 111(36), pp. E3785–E3794. doi: 10.1073/pnas.1406559111.

Burack, W. R. and Shaw, A. S. (2000) 'Signal transduction: Hanging on a scaffold', *Current Opinion in Cell Biology*. Current Biology Ltd, pp. 211–216. doi: 10.1016/S0955-0674(99)00078-2.

Buscà, R. *et al.* (2015) 'ERK1 and ERK2 present functional redundancy in tetrapods despite higher evolution rate of ERK1', *BMC Evolutionary Biology*, 15(1), p. 179. doi: 10.1186/s12862-015-0450-x.

Buschbeck, M. and Ullrich, A. (2005) 'The unique C-terminal tail of the mitogen-activated protein kinase ERK5 regulates its activation and nuclear shuttling', *Journal of Biological Chemistry*, 280(4), pp. 2659–2667. doi: 10.1074/jbc.M412599200.

Buss, J. E. and Marsters, J. C. (1995) 'Farnesyl transferase inhibitors: the successes and surprises of a new class of potential cancer chemotherapeutics.', *Chemistry & biology*, 2(12), pp. 787–91. doi: 10.1016/1074-5521(95)90083-7.

Cacace, A. M. *et al.* (1999) 'Identification of Constitutive and Ras-Inducible Phosphorylation Sites of KSR: Implications for 14-3-3 Binding, Mitogen-Activated Protein Kinase Binding, and KSR Overexpression', *Molecular and Cellular Biology*. American Society for Microbiology, 19(1), pp. 229–240. doi: 10.1128/mcb.19.1.229.

Calvo, F., Agudo-Ibáñez, L. and Crespo, P. (2010) 'The Ras-ERK pathway: Understanding site-specific signaling provides hope of new anti-tumor therapies', *BioEssays*, 32(5), pp. 412–421. doi: 10.1002/bies.200900155.

Canagarajah, B. J. *et al.* (1997) 'Activation mechanism of the MAP kinase ERK2 by dual phosphorylation', *Cell*. Cell Press, 90(5), pp. 859–869. doi: 10.1016/S0092-8674(00)80351-7.

Cargnello, M. and Roux, P. P. (2011) 'Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases', *MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS*, 75(1), pp. 50–83. doi: 10.1128/MMBR.00031-10.

Casar, B. *et al.* (2007) 'Mxi2 promotes stimulus-independent ERK nuclear translocation', *EMBO Journal*, 26(3), pp. 635–646. doi: 10.1038/sj.emboj.7601523.

Casar, B. *et al.* (2009) 'Ras subcellular localization defines extracellular signal-regulated kinase 1 and 2 substrate specificity through distinct utilization of scaffold proteins.', *Molecular and cellular biology*. American Society for Microbiology (ASM), 29(5), pp. 1338–53. doi: 10.1128/MCB.01359-08.

Casar, B. and Crespo, P. (2016) 'ERK Signals: Scaffolding Scaffolds?', *Frontiers in Cell and Developmental Biology*. doi: 10.3389/fcell.2016.00049.

Casar, B., Pinto, A. and Crespo, P. (2008) 'Essential Role of ERK Dimers in the Activation of Cytoplasmic but Not Nuclear Substrates by ERK-Scaffold Complexes', *Molecular Cell*, 31(5), pp. 708–721. doi: 10.1016/j.molcel.2008.07.024.

Casar, B., Pinto, A. and Crespo, P. (2009) 'Cell Cycle ERK dimers and scaffold proteins: Unexpected partners for a forgotten (cytoplasmic) task'. doi: 10.4161/cc.8.7.8078.

Caunt, C. J. and McArdle, C. A. (2010) 'Stimulus-induced uncoupling of extracellular signal-regulated kinase phosphorylation from nuclear localization is dependent on docking domain interactions', *Journal of Cell Science*, 123(24), pp. 4310–4320. doi: 10.1242/jcs.076349.

Chang, L. and Karin, M. (2001) 'Mammalian MAP kinase signalling cascades', *Nature*, pp. 37–40. doi: 10.1038/35065000.

Channavajhala, P. L. *et al.* (2003) 'Identification of a Novel Human Kinase Supporter of Ras (hKSR-2) That Functions as a Negative Regulator of Cot (Tp12) Signaling', *Journal of Biological Chemistry*, 278(47), pp. 47089–47097. doi: 10.1074/jbc.M306002200.

Chartier, M. *et al.* (2013) 'Kinome Render: a stand-alone and web-accessible tool to annotate the human protein kinome tree.', *PeerJ*. PeerJ, Inc, 1, p. e126. doi: 10.7717/peerj.126.

Chen, C., Lewis, R. E. and White, M. A. (2008) 'IMP modulates KSR1-dependent multivalent complex formation to specify ERK1/2 pathway activation and response thresholds', *Journal of Biological Chemistry*, 283(19), pp. 12789–12796. doi: 10.1074/jbc.M709305200.

Chen, D. *et al.* (1996) 'SOS phosphorylation and disassociation of the Grb2-SOS complex by the ERK and JNK signaling pathways', *Journal of Biological Chemistry*, 271(11), pp. 6328–6332. doi: 10.1074/jbc.271.11.6328.

Chen, R. E. and Thorner, J. (2007) 'Function and regulation in MAPK signaling pathways: Lessons learned from the yeast Saccharomyces cerevisiae', *Biochimica et Biophysica Acta - Molecular Cell Research*, pp. 1311–1340. doi: 10.1016/j.bbamcr.2007.05.003.

Cheng, Y. and Tian, H. (2017) 'Current development status of MEK inhibitors', *Molecules*. MDPI AG, 22(10). doi: 10.3390/molecules22101551.

Chew, C. S. *et al.* (2005) 'IQGAPs are differentially expressed and regulated in polarized gastric epithelial cells', *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 288(2 51-2). doi: 10.1152/ajpgi.00290.2004.

Choi, K. Y. *et al.* (1994) 'Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in S. cerevisiae', *Cell*, 78(3), pp. 499–512. doi: 10.1016/0092-8674(94)90427-8.

Choi, S. *et al.* (2013) 'IQGAP1 is a novel phosphatidylinositol 4,5 bisphosphate effector in regulation of directional cell migration', *EMBO Journal*, 32(19), pp. 2617–2630. doi: 10.1038/emboj.2013.191.

Chong, H., Lee, J. and Guan, K. L. (2001) 'Positive and negative regulation of Raf kinase activity and function by phosphorylation', *EMBO Journal*, 20(14), pp. 3716–3727. doi: 10.1093/emboj/20.14.3716.

Clapéron, A. and Therrien, M. (2007) 'KSR and CNK: Two scaffolds regulating RAS-mediated RAF activation', *Oncogene*, pp. 3143–3158. doi: 10.1038/sj.onc.1210408.

Clark-Lewis, I., Sanghera, J. S. and Pelech, S. L. (1991) 'Definition of a consensus sequence for peptide substrate recognition by p44mpk, the meiosis-activated myelin basic protein kinase.', *The Journal of biological chemistry*, 266(23), pp. 15180–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1907971 (Accessed: 24 November 2019).

Cohen, Y. *et al.* (2003) 'BRAF mutation in papillary thyroid carcinoma', *Journal of the National Cancer Institute*. Oxford University Press, 95(8), pp. 625–627. doi: 10.1093/jnci/95.8.625.

Colwill, K. *et al.* (1996) 'SRPK1 and Clk/Sty protein kinases show distinct substrate specificities for serine/arginine-rich splicing factors', *Journal of Biological Chemistry*, 271(40), pp. 24569–24575. doi: 10.1074/jbc.271.40.24569.

Corbalan-Garcia, S. *et al.* (1996) 'Identification of the mitogen-activated protein kinase phosphorylation sites on human Sos1 that regulate interaction with Grb2.', *Molecular and Cellular Biology*. American Society for Microbiology, 16(10), pp. 5674–5682. doi: 10.1128/mcb.16.10.5674.

Costigan, D. C. and Dong, F. (2020) 'The extended spectrum of RAS-MAPK pathway mutations in colorectal cancer', *Genes, Chromosomes and Cancer*, 59(3), pp. 152–159. doi: 10.1002/gcc.22813.

Coulombe, P. and Meloche, S. (2007) 'Atypical mitogen-activated protein kinases: Structure, regulation and functions', *Biochimica et Biophysica Acta - Molecular Cell Research*, pp. 1376–1387. doi: 10.1016/j.bbamcr.2006.11.001.

Cox, A. D. *et al.* (2014) 'Drugging the undruggable RAS: Mission Possible?', *Nature Reviews Drug Discovery*. Nature Publishing Group, pp. 828–851. doi: 10.1038/nrd4389.

Cox, A. D. and Der, C. J. (2010) 'Ras history: The saga continues', *Small GTPases*. Taylor and Francis Inc., 1(1), pp. 2–27. doi: 10.4161/sgtp.1.1.12178.

Creixell, P. *et al.* (2018) 'Hierarchical Organization Endows the Kinase Domain with Regulatory

Plasticity', *Cell Systems*. Cell Press, 7(4), pp. 371-383.e4. doi: 10.1016/j.cels.2018.08.008.

Cupit, L. D. *et al.* (2004) 'Distinct PAR/IQGAP expression patterns durinq murine development: Implications for thrombin-associated cytoskeletal reorganization', *Mammalian Genome*, 15(8), pp. 618–629. doi: 10.1007/s00335-004-2370-8.

Darby, S. *et al.* (2006) 'Loss of Sef (similar expression to FGF) expression is associated with high grade and metastatic prostate cancer', *Oncogene*, 25(29), pp. 4122–4127. doi: 10.1038/sj.onc.1209428.

Darby, S. *et al.* (2009) 'Similar expression to FGF (Sef) inhibits fibroblast growth factor-induced tumourigenic behaviour in prostate cancer cells and is downregulated in aggressive clinical disease', *British Journal of Cancer*, 101(11), pp. 1891–1899. doi: 10.1038/sj.bjc.6605379.

Davies, H. *et al.* (2002) 'Mutations of the BRAF gene in human cancer', *Nature*, 417(6892), pp. 949–954. doi: 10.1038/nature00766.

Dedrick Jordan, J., Landau, E. M. and Iyengar, R. (2000) *Signaling Networks: The Origins of Cellular Multitasking*, *Cell*.

DeFea, K. A., Vaughn, Z. D., *et al.* (2000) 'The proliferative and antiapoptotic effects of substance P are facilitated by formation of a β-arrestin-dependent scaffolding complex', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 97(20), pp. 11086–11091. doi: 10.1073/pnas.190276697.

DeFea, K. A., Zalevsky, J., *et al.* (2000) 'β-Arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2', *Journal of Cell Biology*, 148(6), pp. 1267–1281. doi: 10.1083/jcb.148.6.1267.

Denouel-Galy, A. *et al.* (1998) 'Murine Ksr interacts with MEK and inhibits Ras-induced transformation', *Current Biology*, 8(1), pp. 46–55. doi: 10.1016/S0960-9822(98)70019-3.

Dérijard, B. *et al.* (1995) 'Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms', *Science*, 267(5198), pp. 682–685. doi: 10.1126/science.7839144.

Dhanasekaran, D. N. *et al.* (2007) 'Scaffold proteins of MAP-kinase modules', *Oncogene*, 26(22), pp. 3185–3202. doi: 10.1038/sj.onc.1210411.

Dhawan, N. S., Scopton, A. P. and Dar, A. C. (2016) 'Small molecule stabilization of the KSR inactive state antagonizes oncogenic Ras signalling', *Nature*. Nature Publishing Group, 537(7618), pp. 112–116. doi: 10.1038/nature19327.

Dougherty, M. K. *et al.* (2005) 'Regulation of Raf-1 by direct feedback phosphorylation', *Molecular Cell*, 17(2), pp. 215–224. doi: 10.1016/j.molcel.2004.11.055.

Douville, E. and Downward, J. (1997) 'EGF induced SOS phosphorylation in PC12 cells involves P90 RSK-2', *Oncogene*. Nature Publishing Group, 15(4), pp. 373–383. doi: 10.1038/sj.onc.1201214.

Douziech, M. *et al.* (2006) 'A KSR/CNK complex mediated by HYP, a novel SAM domain-containing protein, regulates RAS-dependent RAF activation in Drosophila', *Genes and Development*, 20(7), pp. 807–819. doi: 10.1101/gad.1390406.

Driedger, P. E. and Blumberg, P. M. (1980) 'Specific binding of phorbol ester tumor promoters', *Proceedings of the National Academy of Sciences of the United States of America*, 77(1), pp. 567–571. doi: 10.1073/pnas.77.1.567.

Dudley, D. T. *et al.* (1995) 'A synthetic inhibitor of the mitogen-activated protein kinase cascade', *Proceedings of the National Academy of Sciences of the United States of America*, 92(17), pp. 7686–7689. doi: 10.1073/pnas.92.17.7686.

Eblen, S. T. *et al.* (2001) 'Biochemical and Biological Functions of the N-Terminal, Noncatalytic Domain of Extracellular Signal-Regulated Kinase 2', *Molecular and Cellular Biology*. American Society for Microbiology, 21(1), pp. 249–259. doi: 10.1128/mcb.21.1.249-259.2001.

Eblen, S. T. *et al.* (2004) 'Mitogen-Activated Protein Kinase Feedback Phosphorylation Regulates MEK1 Complex Formation and Activation during Cellular Adhesion', *Molecular and Cellular Biology*. American Society for Microbiology, 24(6), pp. 2308–2317. doi: 10.1128/mcb.24.6.2308-2317.2004.

Eblen, S. T. (2018) 'Extracellular-Regulated Kinases: Signaling From Ras to ERK Substrates to Control Biological Outcomes', in *Advances in Cancer Research*. Academic Press Inc., pp. 99–142. doi: 10.1016/bs.acr.2018.02.004.

Eisen, T. *et al.* (2006) 'Sorafenib in advanced melanoma: A Phase II randomised discontinuation trial analysis', *British Journal of Cancer*, 95(5), pp. 581–586. doi: 10.1038/sj.bjc.6603291.

English, J. *et al.* (1999) 'New Insights into the Control of MAP Kinase Pathways', *Experimental Cell Research*, 253(1), pp. 255–270. doi: 10.1006/excr.1999.4687.

Eyers, P. A. and Murphy, J. M. (2013) 'Exploring Kinomes: Pseudokinases and beyond: Dawn of the dead: Protein pseudokinases signal new adventures in cell biology', *Biochemical Society Transactions*, 41(4), pp. 969–974. doi: 10.1042/BST20130115.

Fantz, D. A. *et al.* (2001) 'Docking Sites on Substrate Proteins Direct Extracellular Signal-regulated Kinase to Phosphorylate Specific Residues', *Journal of Biological Chemistry*, 276(29), pp. 27256–27265. doi: 10.1074/jbc.M102512200.

Fedorenko, I. V., Gibney, G. T. and Smalley, K. S. M. (2013) 'NRAS mutant melanoma: Biological behavior and future strategies for therapeutic management', *Oncogene*, pp. 3009–3018. doi: 10.1038/onc.2012.453.

Feigin, M. E. *et al.* (2014) 'G-protein-coupled receptor GPR161 is overexpressed in breast cancer and is a promoter of cell proliferation and invasion', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 111(11), pp. 4191–4196. doi: 10.1073/pnas.1320239111.

Ferletta, M. *et al.* (2003) 'Opposing roles of integrin alpha6Abeta1 and dystroglycan in laminin-mediated extracellular signal-regulated kinase activation.', *Molecular biology of the cell*, 14(5), pp. 2088–103. doi: 10.1091/mbc.e03-01-0852.

Fernández-Medarde, A. and Santos, E. (2011) 'The RasGrf family of mammalian guanine nucleotide exchange factors', *Biochimica et Biophysica Acta - Reviews on Cancer*, pp. 170–188. doi: 10.1016/j.bbcan.2010.11.001.

Fernandez, M. R., Henry, M. D. and Lewis, R. E. (2012) 'Kinase Suppressor of Ras 2 (KSR2) Regulates Tumor Cell Transformation via AMPK', *Molecular and Cellular Biology*. American Society for Microbiology, 32(18), pp. 3718–3731. doi: 10.1128/mcb.06754-11.

Fischer, A. *et al.* (2008) 'Regulation of RAF Activity by 14-3-3 Proteins RAF KINASES ASSOCIATE FUNCTIONALLY WITH BOTH HOMO-AND HETERODIMERIC FORMS OF 14-3-3 PROTEINS * □ S and the'. doi: 10.1074/jbc.M804795200.

Fleming, Y. *et al.* (2000) 'Synergistic activation of stress-activated protein kinase 1/c-Jun N-terminal kinase (SAPK1/JNK) isoforms by mitogen-activated protein kinase kinase 4 (MKK4) and MKK7', *Biochemical Journal*, 352(1), pp. 145–154. doi: 10.1042/0264-6021:3520145.

Flores, K. *et al.* (2019) 'At the Cutting Edge The Nuclear Translocation of Mitogen-Activated Protein Kinases: Molecular Mechanisms and Use as Novel Therapeutic Target Keywords MAPK · Beta-like importins · Nuclear translocation · ERK1/2 · JNK · p38 · Cancer · Inflammation Overview of the Composition and Regulation of the Mitogen-Activated Protein Kinase Cascades', *Neuroendocrinology*, 108, pp. 121–131. doi: 10.1159/000494085.

Frémin, C. *et al.* (2015) 'Functional redundancy of ERK1 and ERK2 MAP kinases during development', *Cell Reports*. Elsevier B.V., 12(6), pp. 913–921. doi: 10.1016/j.celrep.2015.07.011.

Freshney, N. W. *et al.* (1994) 'Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of hsp27', *Cell*, 78(6), pp. 1039–1049. doi: 10.1016/0092-8674(94)90278-X.

Fukata, M. *et al.* (1997) 'Regulation of cross-linking of actin filament by IQGAP1, a target for Cdc42', *Journal of Biological Chemistry*, 272(47), pp. 29579–29583. doi: 10.1074/jbc.272.47.29579.

Fukata, M. *et al.* (2002) 'Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170', *Cell*. Cell Press, 109(7), pp. 873–885. doi: 10.1016/S0092-8674(02)00800-0.

Fukuda, M. *et al.* (1997) 'CRM1 is responsible for intracellular transport mediated by the nuclear export signal', *Nature*, 390(6657), pp. 308–311. doi: 10.1038/36894.

Fukuda, M., Gotoh, Y. and Nishida, E. (1997) 'Interaction of MAP kinase with MAP kinase kinase: Its possible role in the control of nucleocytoplasmic transport of MAP kinase', *EMBO Journal*, 16(8), pp. 1901–1908. doi: 10.1093/emboj/16.8.1901.

Fürthauer, M. *et al.* (2002) 'Sef is a feedback-induced antagonist of RAs/MAPK-mediated FGF

signalling', *Nature Cell Biology*, 4(2), pp. 170–174. doi: 10.1038/ncb750.

Germann, U. A. *et al.* (2017) 'Targeting the MAPK signaling pathway in cancer: Promising preclinical activity with the novel selective ERK1/2 inhibitor BVD-523 (ulixertinib)', *Molecular Cancer Therapeutics*. American Association for Cancer Research Inc., 16(11), pp. 2351–2363. doi: 10.1158/1535-7163.MCT-17-0456.

Giblett, S. M. *et al.* (2002) 'Expression of kinase suppressor of Ras in the normal adult and embryonic mouse.', *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research*, 13(7), pp. 307–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12133899 (Accessed: 8 December 2019).

Gimple, R. C. and Wang, X. (2019) 'RAS: Striking at the Core of the Oncogenic Circuitry', *Frontiers in Oncology*. Frontiers Media S.A. doi: 10.3389/fonc.2019.00965.

Glenney, J. R. and Zokas, L. (1989) 'Novel tyrosine kinase substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton', *Journal of Cell Biology*, 108(6), pp. 2401–2408. doi: 10.1083/jcb.108.6.2401.

Goettel, J. A. *et al.* (2011) 'KSR1 is a functional protein kinase capable of serine autophosphorylation and direct phosphorylation of MEK1', *Experimental Cell Research*. Academic Press Inc., 317(4), pp. 452–463. doi: 10.1016/j.yexcr.2010.11.018.

Goldsmith, E. J. and Cobb, M. H. (1994) 'Protein kinases', *Current Opinion in Structural Biology*, 4(6), pp. 833–840. doi: 10.1016/0959-440X(94)90264-X.

Gonzalez, F. A. *et al.* (1992) 'Heterogeneous expression of four MAP kinase isoforms in human tissues', *FEBS Letters*, 304(2–3), pp. 170–178. doi: 10.1016/0014-5793(92)80612-K.

Gorden, A. *et al.* (2003) 'Analysis of BRAF and N-RAS mutations in metastatic melanoma tissues', *Cancer Research*, 63(14), pp. 3955–3957.

Guo, L. *et al.* (2017) 'Kinase Suppressor of Ras 2 (KSR2) expression in the brain regulates energy balance and glucose homeostasis', *Molecular Metabolism*. Elsevier GmbH, 6(2), pp. 194–205. doi: 10.1016/j.molmet.2016.12.004.

Gutkind, J. S. (1998) 'The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades', *Journal of Biological Chemistry*, pp. 1839–1842. doi: 10.1074/jbc.273.4.1839.

Ha, S. H., Kim, S. Y. and Ferrell, J. E. (2016) 'The Prozone Effect Accounts for the Paradoxical Function of the Cdk-Binding Protein Suc1/Cks', *Cell Reports*. Elsevier, 14(6), pp. 1408–1421. doi: 10.1016/j.celrep.2016.01.033.

Haase, D. *et al.* (2009) 'Reduced Morg1 expression in ischemic human brain', *Neuroscience Letters*, 455(1), pp. 46–50. doi: 10.1016/j.neulet.2009.03.048.

Hagemann, D., Troppmair, J. and Rapp, U. R. (1999) 'Cot protooncoprotein activates the dual specificity kinases MEK-1 and SEK-1 and induces differentiation of PC12 cells', *Oncogene*. Nature Publishing Group, 18(7), pp. 1391–1400. doi: 10.1038/sj.onc.1202431.

Han, J. *et al.* (1993) 'Endotoxin induces rapid protein tyrosine phosphorylation in 70Z/3 cells expressing CD14', *Journal of Biological Chemistry*, 268(33), pp. 25009–25014.

Han, J. *et al.* (1994) 'A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells', *Science*, 265(5173), pp. 808–811. doi: 10.1126/science.7914033.

Hanahan, D. and Weinberg, R. A. (2000) 'The Hallmarks of Cancer', *Cell*, 100(1), pp. 57–70. doi: 10.1016/S0092-8674(00)81683-9.

Hanks, S. K. and Hunter, T. (1995) 'The eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification', *FASEB Journal*, pp. 576–596. doi: 10.1096/fasebj.9.8.7768349.

Hanks, S. K., Quinn, A. M. and Hunter, T. (1988) 'The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains', *Science*, 241(4861), pp. 42–52. doi: 10.1126/science.3291115.

Hansen, L. A. *et al.* (1997) 'Genetically null mice reveal a central role for epidermal growth factor receptor in the differentiation of the hair follicle and normal hair development.', *The American journal of pathology*, 150(6), pp. 1959–75. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9176390 (Accessed: 12 December 2019).

Hart, M. J. *et al.* (1996) 'IQGAP1, a calmodulin-binding protein with a rasGAP-related domain, is a potential effector for cdc42Hs.', *The EMBO journal*, 15(12), pp. 2997–3005. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8670801 (Accessed: 14 December 2019).

Hatano, N. *et al.* (2003) 'Essential role for ERK2 mitogen-activated protein kinase in placental development', *Genes to Cells*, 8(11), pp. 847–856. doi: 10.1046/j.1365-2443.2003.00680.x.

Heinrich, R., Neel, B. G. and Rapoport, T. A. (2002) 'Mathematical models of protein kinase signal transduction.', *Molecular cell*. Elsevier, 9(5), pp. 957–70. doi: 10.1016/S1097-2765(02)00528-2.

Hekman, M. *et al.* (2005) 'Novel C-Raf phosphorylation sites: Serine 296 and 301 participate in Raf regulation', *FEBS Letters*, 579(2), pp. 464–468. doi: 10.1016/j.febslet.2004.11.105.

Henry, M. D. *et al.* (2014) 'Obesity-dependent dysregulation of glucose homeostasis in kinase suppressor of ras 2-/- mice', *Physiological Reports*. American Physiological Society, 2(7). doi: 10.14814/phy2.12053.

Herrero, A. *et al.* (2015) 'Small Molecule Inhibition of ERK Dimerization Prevents Tumorigenesis by RAS-ERK Pathway Oncogenes', *Cancer Cell*. Cell Press, 28(2), pp. 170–182. doi: 10.1016/j.ccell.2015.07.001.

Ho, Y. D. *et al.* (1999) 'IQGAP1 integrates Ca2+/calmodulin and Cdc42 signaling', *Journal of Biological Chemistry*, 274(1), pp. 464–470. doi: 10.1074/jbc.274.1.464.

Hobbs, G. A., Der, C. J. and Rossman, K. L. (2016) 'RAS isoforms and mutations in cancer at a glance', *Journal of Cell Science*. Company of Biologists Ltd, 129(7), pp. 1287–1292. doi: 10.1242/jcs.182873.

Hruban, R. H., Wilentz, R. E. and Kern, S. E. (2000) 'Genetic progression in the pancreatic ducts', *American Journal of Pathology*. American Society for Investigative Pathology Inc., pp. 1821–1825. doi: 10.1016/S0002-9440(10)65054-7.

Hu, J. *et al.* (2011) 'Mutation that blocks ATP binding creates a pseudokinase stabilizing the scaffolding function of kinase suppressor of Ras, CRAF and BRAF', *Proceedings of the National Academy of Sciences of the United States of America*, 108(15), pp. 6067–6072. doi: 10.1073/pnas.1102554108.

Hubbard, S. R. and Miller, W. T. (2007) 'Receptor tyrosine kinases: mechanisms of activation and signaling', *Current Opinion in Cell Biology*, pp. 117–123. doi: 10.1016/j.ceb.2007.02.010.

Huse, M. and Kuriyan, J. (2002) 'The conformational plasticity of protein kinases', *Cell*. Cell Press, pp. 275–282. doi: 10.1016/S0092-8674(02)00741-9.

Huynh, H. *et al.* (2003) 'Over-expression of the mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK in hepatocellular carcinoma: Its role in tumor progression and apoptosis', *BMC Gastroenterology*, 3. doi: 10.1186/1471-230X-3-19.

Ishibe, S. *et al.* (2003) 'Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis', *Molecular Cell*. Cell Press, 12(5), pp. 1275–1285. doi: 10.1016/S1097-2765(03)00406-4.

Ishibe, S. *et al.* (2004) 'Paxillin serves as an ERK-regulated scaffold for coordinating FAK and Rac activation in epithelial morphogenesis', *Molecular Cell*, 16(2), pp. 257–267. doi: 10.1016/j.molcel.2004.10.006.

Jacobs, D. *et al.* (1999) *Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase*, *Genes and Development*. Cold Spring Harbor Laboratory Press. doi: 10.1101/gad.13.2.163.

Jacquemet, G. *et al.* (2013) 'Rac1 is deactivated at integrin activation sites through an IQGAP1-filamin-A-RacGAP1 pathway', *Journal of Cell Science*, 126(18), pp. 4121–4135. doi: 10.1242/jcs.121988.

Jadeski, L. *et al.* (2008) 'IQGAP1 stimulates proliferation and enhances tumorigenesis of human breast epithelial cells', *Journal of Biological Chemistry*, 283(2), pp. 1008–1017. doi: 10.1074/jbc.M708466200.

Jain, R. *et al.* (2018) 'ERK Activation Pathways Downstream of GPCRs', in *International Review of Cell and Molecular Biology*. Elsevier Inc., pp. 79–109. doi: 10.1016/bs.ircmb.2018.02.003.

Jaiswal, B. S. *et al.* (2018) 'ERK Mutations and amplification confer resistance to ERK-inhibitor therapy', *Clinical Cancer Research*. American Association for Cancer Research Inc., 24(16), pp. 4044–4055. doi: 10.1158/1078-0432.CCR-17-3674.

Jameson, K. L. *et al.* (2013) 'IQGAP1 scaffold-kinase interaction blockade selectively targets RAS-MAP kinase-driven tumors.', *Nature medicine*, 19(5), pp. 626–630. doi: 10.1038/nm.3165.

Jeong, H. W. *et al.* (2007) 'IQGAP1 binds Rap1 and modulates its activity', *Journal of Biological Chemistry*, 282(28), pp. 20752–20762. doi: 10.1074/jbc.M700487200.

Jiang, Y. *et al.* (1996) 'Characterization of the structure and function of a new mitogen- activated protein kinase (p38β)', *Journal of Biological Chemistry*, 271(30), pp. 17920–17926. doi: 10.1074/jbc.271.30.17920.

Jiang, Y. *et al.* (1997) 'Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38δ', *Journal of Biological Chemistry*, 272(48), pp. 30122–30128. doi: 10.1074/jbc.272.48.30122.

Johnson, G. L. and Lapadat, R. (2002) 'Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases', *Science*, pp. 1911–1912. doi: 10.1126/science.1072682.

Johnson, M., Sharma, M. and Henderson, B. R. (2009) 'IQGAP1 regulation and roles in cancer', *Cellular Signalling*, pp. 1471–1478. doi: 10.1016/j.cellsig.2009.02.023.

Jones, S. *et al.* (2008) 'Core signaling pathways in human pancreatic cancers revealed by global genomic analyses', *Science*, 321(5897), pp. 1801–1806. doi: 10.1126/science.1164368.

Joneson, T. *et al.* (1998) *Kinase Suppressor of Ras Inhibits the Activation of Extracellular Ligand-regulated (ERK) Mitogen-activated Protein (MAP) Kinase by Growth Factors, Activated Ras, and Ras Effectors**. Available at: http://www.jbc.org/ (Accessed: 24 June 2019).

Jope, R. S. and Johnson, G. V. W. (2004) 'The glamour and gloom of glycogen synthase kinase-3', *Trends in Biochemical Sciences*. Elsevier Ltd, pp. 95–102. doi: 10.1016/j.tibs.2003.12.004.

Kallunki, T. *et al.* (1994) 'JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation', *Genes and Development*, 8(24), pp. 2996–3007. doi: 10.1101/gad.8.24.2996.

Kamakura, S., Moriguchi, T. and Nishida, E. (1999) 'Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus', *Journal of Biological Chemistry*, 274(37), pp. 26563–26571. doi: 10.1074/jbc.274.37.26563.

Kamioka, Y. *et al.* (2010) 'Multiple decisive phosphorylation sites for the negative feedback regulation of SOS1 via ERK', *Journal of Biological Chemistry*, 285(43), pp. 33540–33548. doi: 10.1074/jbc.M110.135517.

Kannan, N. and Neuwald, A. F. (2004) 'Evolutionary constraints associated with functional specificity of the CMGC protein kinases MAPK, CDK, GSK, SRPK, DYRK, and CK2α', *Protein Science*, 13(8), pp. 2059–2077. doi: 10.1110/ps.04637904.

Karnoub, A. E. and Weinberg, R. A. (2008) 'Ras oncogenes: Split personalities', *Nature Reviews*

Molecular Cell Biology, pp. 517–531. doi: 10.1038/nrm2438.

Khokhlatchev, A. V. *et al.* (1998) 'Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation', *Cell*. Cell Press, 93(4), pp. 605–615. doi: 10.1016/S0092-8674(00)81189-7.

Kim, E. K. and Choi, E. J. (2010) 'Pathological roles of MAPK signaling pathways in human diseases', *Biochimica et Biophysica Acta - Molecular Basis of Disease*, pp. 396–405. doi: 10.1016/j.bbadis.2009.12.009.

Kim, E. K. and Choi, E. J. (2015) 'Compromised MAPK signaling in human diseases: an update', *Archives of Toxicology*. Springer Verlag, pp. 867–882. doi: 10.1007/s00204-015-1472-2.

Knobbe, C. B., Reifenberger, J. and Reifenberger, G. (2004) 'Mutation analysis of the Ras pathway genes NRAS, HRAS, KRAS and BRAF in glioblastomas', *Acta Neuropathologica*, 108(6), pp. 467–470. doi: 10.1007/s00401-004-0929-9.

Kocieniewski, P. and Lipniacki, T. (2013) 'MEK1 and MEK2 differentially control the duration and amplitude of the ERK cascade response', *Physical Biology*, 10(3). doi: 10.1088/1478-3975/10/3/035006.

Kodaz, H. *et al.* (2017) 'Frequency of Ras Mutations (Kras, Nras, Hras) in Human Solid Cancer', *EJMO*, 1(1), pp. 1–7. doi: 10.14744/ejmo.2017.22931.

Kohno, T. *et al.* (2013) 'IQGAP1 links PDGF receptor-β signal to focal adhesions involved in vascular smooth muscle cell migration: Role in neointimal formation after vascular injury', *American Journal of Physiology - Cell Physiology*, 305(6). doi: 10.1152/ajpcell.00011.2013.

Kolch, W. (2005) 'Coordinating ERK/MAPK signalling through scaffolds and inhibitors', *Nature Reviews Molecular Cell Biology*, 6(11), pp. 827–837. doi: 10.1038/nrm1743.

Kornfeld, K., Hom, D. B. and Horvitz, H. R. (1995) 'The ksr-1 gene encodes a novel protein kinase involved in Ras-mediated signaling in C. elegans', *Cell*, 83(6), pp. 903–913. doi: 10.1016/0092-8674(95)90206-6.

Kortum, R. L. *et al.* (2006) 'The Molecular Scaffold Kinase Suppressor of Ras 1 Is a Modifier of RasV12-Induced and Replicative Senescence', *Molecular and Cellular Biology*, 26(6), pp. 2202–2214. doi: 10.1128/mcb.26.6.2202-2214.2006.

Koveal, D. *et al.* (2012) 'A CC-SAM, for coiled coil-sterile α motif, domain targets the scaffold KSR-1 to specific sites in the plasma membrane', *Science Signaling*, 5(255). doi: 10.1126/scisignal.2003289.

Kudo, N. *et al.* (1998) 'Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1', *Experimental Cell Research*. Academic Press Inc., 242(2), pp. 540–547. doi: 10.1006/excr.1998.4136.

Kurella, V. B. *et al.* (2009) 'Crystal structure of the GTPase-activating protein-related domain from IQGAP1', *Journal of Biological Chemistry*, 284(22), pp. 14857–14865. doi: 10.1074/jbc.M808974200.

Kyriakis, J. M. and Avruch, J. (2001) *Mammalian Mitogen-Activated Protein Kinase Signal Transduction Pathways Activated by Stress and Inflammation*. Available at: http://physrev.physiology.org (Accessed: 7 July 2019).

Lahiry, P. *et al.* (2010) 'Kinase mutations in human disease: Interpreting genotype-phenotype relationships', *Nature Reviews Genetics*, pp. 60–74. doi: 10.1038/nrg2707.

Lake, D., Corrêa, S. A. L. and Müller, J. (2016) 'Negative feedback regulation of the ERK1/2 MAPK pathway', *Cellular and Molecular Life Sciences*. Birkhauser Verlag AG, pp. 4397–4413. doi: 10.1007/s00018-016-2297-8.

Langeberg, L. K. and Scott, J. D. (2015) 'Signalling scaffolds and local organization of cellular behaviour', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, pp. 232–244. doi: 10.1038/nrm3966.

LeCour, L. *et al.* (2016) 'The Structural Basis for Cdc42-Induced Dimerization of IQGAPs', *Structure*. Cell Press, 24(9), pp. 1499–1508. doi: 10.1016/j.str.2016.06.016.

Lee, J. C. *et al.* (1994) 'A protein kinase involved in the regulation of inflammatory cytokine biosynthesis', *Nature*, 372(6508), pp. 739–746. doi: 10.1038/372739a0.

Lee, J. D., Ulevitch, R. J. and Han, J. (1995) 'Primary structure of BMK1: A new mammalian MAP kinase', *Biochemical and Biophysical Research Communications*, 213(2), pp. 715–724. doi: 10.1006/bbrc.1995.2189.

Lee, T. *et al.* (2004) 'Docking motif interactions in Map kinases revealed by hydrogen exchange mass spectrometry', *Molecular Cell*, 14(1), pp. 43–55. doi: 10.1016/S1097-2765(04)00161-3.

Lefkowitz, R. J. and Shenoy, S. K. (2005) 'Transduction of receptor signals by β-arrestins', *Science*, pp. 512–517. doi: 10.1126/science.1109237.

Lefloch, R., Pouysségur, J. and Lenormand, P. (2008) 'Single and combined silencing of ERK1 and ERK2 reveals their positive contribution to growth signaling depending on their expression levels.', *Molecular and cellular biology*, 28(1), pp. 511–27. doi: 10.1128/MCB.00800-07.

Lemmon, M. A. and Schlessinger, J. (2010) 'Cell signaling by receptor tyrosine kinases', *Cell*, pp. 1117–1134. doi: 10.1016/j.cell.2010.06.011.

Lemoine, N. R. *et al.* (1989) 'High frequency of ras oncogene activation in all stages of human thyroid tumorigenesis.', *Oncogene*, 4(2), pp. 159–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2648253 (Accessed: 8 January 2020).

Leonetti, A. *et al.* (2018) 'BRAF in non-small cell lung cancer (NSCLC): Pickaxing another brick in the wall', *Cancer Treatment Reviews*. W.B. Saunders Ltd, pp. 82–94. doi: 10.1016/j.ctrv.2018.04.006.

Levchenko, A., Bruck, J. and Sternberg, P. W. (2000) 'Scaffold proteins may biphasically affect the

levels of mitogen-activated protein kinase signaling and reduce its threshold properties', *Proceedings of the National Academy of Sciences*, 97(11), pp. 5818–5823. doi: 10.1073/pnas.97.11.5818.

Li, B. *et al.* (2013) 'Terbinafine inhibits KSR1 and suppresses Raf-MEK-ERK signaling in oral squamous cell carcinoma cells', *Neoplasma*, 60(4), pp. 406–412. doi: 10.4149/neo_2013_052.

Li, M., Liu, J. and Zhang, C. (2011) 'Evolutionary History of the Vertebrate Mitogen Activated Protein Kinases Family', *PLoS ONE*. Edited by M. V. Matz, 6(10), p. e26999. doi: 10.1371/journal.pone.0026999.

Li, Z. *et al.* (1996) 'The primary structure of p38γ: A new member of p38 group of MAP kinases', *Biochemical and Biophysical Research Communications.* Academic Press Inc., 228(2), pp. 334–340. doi: 10.1006/bbrc.1996.1662.

Li, Z. *et al.* (1999) 'IQGAP1 and calmodulin modulate E-cadherin function', *Journal of Biological Chemistry*, 274(53), pp. 37885–37892. doi: 10.1074/jbc.274.53.37885.

Li, Z. and Sacks, D. B. (2003) 'Elucidation of the interaction of calmodulin with the IQ motifs of IQGAP1', *Journal of Biological Chemistry*, 278(6), pp. 4347–4352. doi: 10.1074/jbc.M208579200.

Lidke, D. S. *et al.* (2010) 'ERK Nuclear Translocation Is Dimerization-independent but Controlled by the Rate of Phosphorylation * □ S contains supplemental Tables S1 and S2, Figs. S1-S3, and Movies 1 and 2. Downloaded from', *THE JOURNAL OF BIOLOGICAL CHEMISTRY*, 285(5), pp. 3092–3102. doi: 10.1074/jbc.M109.064972.

Lin, L. L. *et al.* (1993) 'cPLA2 is phosphorylated and activated by MAP kinase', *Cell*, 72(2), pp. 269–278. doi: 10.1016/0092-8674(93)90666-E.

von Lintig, F. C. *et al.* (2000) 'Ras activation in human breast cancer', *Breast Cancer Research and Treatment*, 62(1), pp. 51–62. doi: 10.1023/A:1006491619920.

Liu, F. *et al.* (2018) 'Targeting ERK, an Achilles' Heel of the MAPK pathway, in cancer therapy', *Acta Pharmaceutica Sinica B*. Chinese Academy of Medical Sciences, pp. 552–562. doi: 10.1016/j.apsb.2018.01.008.

Llobet, D. *et al.* (2011) 'KSR1 is overexpressed in endometrial carcinoma and regulates proliferation and TRAIL-induced apoptosis by modulating FLIP levels', *American Journal of Pathology*. Elsevier Inc., 178(4), pp. 1529–1543. doi: 10.1016/j.ajpath.2010.12.041.

Lloyd, A. C. (2006) 'Distinct functions for ERKs?', *Journal of Biology*. doi: 10.1186/jbiol46.

Locasale, J. W., Shaw, A. S. and Chakraborty, A. K. (2007) 'Scaffold proteins confer diverse regulatory properties to protein kinase cascades.', *Proceedings of the National Academy of Sciences of the United States of America*, 104(33), pp. 13307–12. doi: 10.1073/pnas.0706311104.

Lorenzen, J. A. *et al.* (2001) 'Nuclear import of activated D-ERK by DIM-7, an importin family member encoded by the gene moleskin', *Development*, 128(8), pp. 1403–1414.

Low, H. B. and Zhang, Y. (2016) 'Regulatory roles of MAPK phosphatases in cancer', *Immune Network*. Korean Association of Immunologists, pp. 85–98. doi: 10.4110/in.2016.16.2.85.

Lozano, J. *et al.* (2003) 'Deficiency of kinase suppressor of Ras1 prevents oncogenic ras signaling in mice.', *Cancer research*, 63(14), pp. 4232–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12874031 (Accessed: 15 September 2019).

Luttrell, L. M. and Lefkowitz, R. J. (2002) 'The role of β-arrestins in the termination and transduction of G-protein-coupled receptor signals', *Journal of Cell Science*, 115(3), pp. 455–465.

Maik-Rachline, G., Hacohen-Lev-Ran, A. and Seger, R. (2019) 'Nuclear erk: Mechanism of translocation, substrates, and role in cancer', *International Journal of Molecular Sciences*. MDPI AG. doi: 10.3390/ijms20051194.

Mandl, M., Slack, D. N. and Keyse, S. M. (2005) 'Specific inactivation and nuclear anchoring of extracellular signal-regulated kinase 2 by the inducible dual-specificity protein phosphatase DUSP5.', *Molecular and cellular biology*, 25(5), pp. 1830–45. doi: 10.1128/MCB.25.5.1830-1845.2005.

Manning, G. *et al.* (2002) 'The protein kinase complement of the human genome', *Science*, 298(5600), pp. 1912–1934. doi: 10.1126/science.1075762.

Marchetti, S. *et al.* (2005) 'Extracellular signal-regulated kinases phosphorylate mitogen-activated protein kinase phosphatase 3/DUSP6 at serines 159 and 197, two sites critical for its proteasomal degradation.', *Molecular and cellular biology*, 25(2), pp. 854–64. doi: 10.1128/MCB.25.2.854-864.2005.

Marom, M. *et al.* (1995) 'Selective inhibition of ras-dependent cell growth by farnesylthiosalisylic acid', *Journal of Biological Chemistry*, 270(38), pp. 22263–22270. doi: 10.1074/jbc.270.38.22263.

Marshall, C. J. (1996) 'Ras effectors.', *Current opinion in cell biology*, 8(2), pp. 197–204. doi: 10.1016/s0955-0674(96)80066-4.

Mataraza, J. M. *et al.* (2003) 'IQGAP1 Promotes Cell Motility and Invasion', *Journal of Biological Chemistry*, 278(42), pp. 41237–41245. doi: 10.1074/jbc.M304838200.

Mateer, S. C. *et al.* (2004) 'Actin filament binding by a monomeric IQGAP1 fragment with a single calponin homology domain', *Cell Motility and the Cytoskeleton*, 58(4), pp. 231–241. doi: 10.1002/cm.20013.

Matheny, S. A. *et al.* (2004) 'Ras regulates assembly of mitogenic signalling complexes through the effector protein IMP', *Nature*. Nature Publishing Group, 427(6971), pp. 256–260. doi: 10.1038/nature02237.

Matheny, S. A. and White, M. A. (2006) 'Ras-Sensitive IMP Modulation of the Raf/MEK/ERK Cascade Through KSR1', *Methods in Enzymology*. Academic Press, pp. 237–247. doi: 10.1016/S0076-6879(05)07020-5.

Matheny, S. A. and White, M. A. (2009) 'Signaling threshold regulation by the Ras effector IMP',

Journal of Biological Chemistry, pp. 11007–11011. doi: 10.1074/jbc.R800082200.

Matsunaga, H. *et al.* (2014) 'IQGAP1 selectively interacts with K-Ras but not with H-Ras and modulates K-Ras function', *Biochemical and Biophysical Research Communications*, 444(3), pp. 360–364. doi: 10.1016/j.bbrc.2014.01.041.

Maust, J. D., Whitehead, C. E. and Sebolt-Leopold, J. S. (2018) 'Oncogenic mutants of MEK1: A trilogy unfolds', *Cancer Discovery*. American Association for Cancer Research Inc., 8(5), pp. 534–536. doi: 10.1158/2159-8290.CD-18-0192.

Mazzucchelli, C. *et al.* (2002) 'Knockout of ERK1 MAP kinase enhances synaptic plasticity in the striatum and facilitates striatal-mediated learning and memory', *Neuron*. Cell Press, 34(5), pp. 807–820. doi: 10.1016/S0896-6273(02)00716-X.

McCallum, S. J., Wu, W. J. and Cerione, R. A. (1996) 'Identification of a putative effector for Cdc42Hs with high sequence similarity to the RasGAP-related protein IQGAP1 and a Cdc42Hs binding partner with similarity to IQGAP2', *Journal of Biological Chemistry*, 271(36), pp. 21732–21737. doi: 10.1074/jbc.271.36.21732.

McCubrey, J. A. *et al.* (2007) 'Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance', *Biochimica et Biophysica Acta - Molecular Cell Research*, pp. 1263–1284. doi: 10.1016/j.bbamcr.2006.10.001.

Mckay, M. M., Freeman, A. K. and Morrison, D. K. (2011) 'Complexity in KSR function revealed by Raf inhibitor and KSR structure studies', *Small GTPases*. Taylor and Francis Inc. doi: 10.4161/sgtp.2.5.17740.

Mckay, M. M. and Morrison, D. K. (2007) 'Integrating signals from RTKs to ERK/MAPK', *Oncogene*, 26, pp. 3113–3121. doi: 10.1038/sj.onc.1210394.

McKay, M. M. and Morrison, D. K. (2007) 'Caspase-dependent cleavage disrupts the ERK cascade scaffolding function of KSR1', *Journal of Biological Chemistry*, 282(36), pp. 26225–26234. doi: 10.1074/jbc.M702692200.

Mckay, M. M., Ritt, D. A. and Morrison, D. K. (2011) 'Raf Inhibitor-induced KSR1/B-Raf Binding and Its Effects on ERK Cascade Signaling', *Curr Biol*, 21(7), pp. 563–568. doi: 10.1016/j.cub.2011.02.033.

McKay, M. M., Ritt, D. A. and Morrison, D. K. (2009) 'Signaling dynamics of the KSR1 scaffold complex', *Proceedings of the National Academy of Sciences of the United States of America*, 106(27), pp. 11022–11027. doi: 10.1073/pnas.0901590106.

McNulty, D. E. *et al.* (2011) 'MAPK scaffold IQGAP1 binds the EGF receptor and modulates its activation', *Journal of Biological Chemistry*, 286(17), pp. 15010–15021. doi: 10.1074/jbc.M111.227694.

Michailovici, I. *et al.* (2014) 'Nuclear to cytoplasmic shuttling of ERK promotes differentiation of muscle stem/progenitor cells', *Development (Cambridge)*. Company of Biologists Ltd, 141(13), pp. 2611–2620. doi: 10.1242/dev.107078.

Michaud, N. R. *et al.* (1997) 'KSR stimulates Raf-1 activity in a kinase-independent manner', *Proceedings of the National Academy of Sciences of the United States of America*, 94(24), pp. 12792–12796. doi: 10.1073/pnas.94.24.12792.

Mitra, S. *et al.* (2016) 'Bipartite role of heat shock protein 90 (Hsp90) keeps CRAF kinase poised for activation', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology Inc., 291(47), pp. 24579–24593. doi: 10.1074/jbc.M116.746420.

Moore, C. A. C., Milano, S. K. and Benovic, J. L. (2007) 'Regulation of Receptor Trafficking by GRKs and Arrestins', *Annual Review of Physiology*. Annual Reviews, 69(1), pp. 451–482. doi: 10.1146/annurev.physiol.69.022405.154712.

Moore, C. J. and Winder, S. J. (2010) 'Dystroglycan versatility in cell adhesion: A tale of multiple motifs', *Cell Communication and Signaling*. doi: 10.1186/1478-811X-8-3.

Mörchen, B. *et al.* (2019) 'Targeting the "undruggable" RAS - new strategies - new hope?', *Cancer Drug Resistance*. OAE Publishing Inc. doi: 10.20517/cdr.2019.21.

Morgan, C. J. *et al.* (2019) 'Endogenous IQGAP1 and IQGAP3 do not functionally interact with Ras', *Scientific Reports*. Nature Publishing Group, 9(1). doi: 10.1038/s41598-019-46677-9.

Mott, H. R. and Owen, D. (2015) 'Structures of Ras superfamily effector complexes: What have we learnt in two decades?', *Critical Reviews in Biochemistry and Molecular Biology*. Informa Healthcare, pp. 85–133. doi: 10.3109/10409238.2014.999191.

Moul, J. W. *et al.* (1992) 'Infrequent RAS oncogene mutations in human prostate cancer', *The Prostate*, 20(4), pp. 327–338. doi: 10.1002/pros.2990200407.

Müller, J. *et al.* (2001) 'C-TAK1 Regulates Ras Signaling by Phosphorylating the MAPK Scaffold, KSR1', *Molecular Cell*. Cell Press, 8(5), pp. 983–993. doi: 10.1016/S1097-2765(01)00383-5.

Naidoo, J. and Drilon, A. (2016) 'Kras-mutant lung cancers in the era of targeted therapy', *Advances in Experimental Medicine and Biology*. Springer New York LLC, 893, pp. 155–178. doi: 10.1007/978-3-319-24223-1_8.

Nakhaeizadeh, H. *et al.* (2016) 'The RAS-effector interface: Isoform-specific differences in the effector binding regions', *PLoS ONE*. Public Library of Science, 11(12). doi: 10.1371/journal.pone.0167145.

Neilsen, B. K. *et al.* (2017) 'KSR as a therapeutic target for Ras-dependent cancers', *Expert Opinion on Therapeutic Targets*. Taylor & Francis, 21(5), pp. 499–509. doi: 10.1080/14728222.2017.1311325.

Nekrasova, T. *et al.* (2005) 'ERK1-Deficient Mice Show Normal T Cell Effector Function and Are Highly Susceptible to Experimental Autoimmune Encephalomyelitis', *The Journal of Immunology*. The American Association of Immunologists, 175(4), pp. 2374–2380. doi: 10.4049/jimmunol.175.4.2374.

Nguyen, A. *et al.* (2002) 'Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-

activated protein kinase activation in vivo.', *Molecular and cellular biology*, 22(9), pp. 3035–45. doi: 10.1128/MCB.22.9.3035.

Nikolaev, S. I. *et al.* (2012) 'Exome sequencing identifies recurrent somatic MAP2K1 and MAP2K2 mutations in melanoma', *Nature Genetics*. Nature Publishing Group, 44(2), pp. 133–139. doi: 10.1038/ng.1026.

Nojima, H. *et al.* (2008) 'IQGAP3 regulates cell proliferation through the Ras/ERK signalling cascade', *Nature Cell Biology*, 10(8), pp. 971–978. doi: 10.1038/ncb1757.

Noritake, J. *et al.* (2005) 'IQGAP1: A key regulator of adhesion and migration', *Journal of Cell Science*, 118(10), pp. 2085–2092. doi: 10.1242/jcs.02379.

Nussinov, R., Tsai, C. and Jang, H. (2019) 'Does Ras Activate Raf and PI3K Allosterically?', *Frontiers in Oncology*, 9(November), pp. 1–10. doi: 10.3389/fonc.2019.01231.

Ohori, M. *et al.* (2005) 'Identification of a selective ERK inhibitor and structural determination of the inhibitor-ERK2 complex', *Biochemical and Biophysical Research Communications*, 336(1), pp. 357–363. doi: 10.1016/j.bbrc.2005.08.082.

Ory, S. *et al.* (2003) 'Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites.', *Current biology : CB*, 13(16), pp. 1356–64. doi: 10.1016/s0960-9822(03)00535-9.

Osaka, M. *et al.* (1997) 'Loss of heterozygosity at the N-ras locus in 7,12-dimethylbenz[a] anthracene-induced rat leukemia.', *Molecular carcinogenesis*, 18(4), pp. 206–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9142215 (Accessed: 10 January 2020).

Ostrem, J. M. L. and Shokat, K. M. (2016) 'Direct small-molecule inhibitors of KRAS: From structural insights to mechanism-based design', *Nature Reviews Drug Discovery*. Nature Publishing Group, pp. 771–785. doi: 10.1038/nrd.2016.139.

Owen, D. *et al.* (2008) 'The IQGAP1-Rac1 and IQGAP1-Cdc42 interactions: Interfaces differ between the complexes', *Journal of Biological Chemistry*, 283(3), pp. 1692–1704. doi: 10.1074/jbc.M707257200.

Owens, D. M. and Keyse, S. M. (2007) 'Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases', *Oncogene*, pp. 3203–3213. doi: 10.1038/sj.onc.1210412.

Pagès, G. *et al.* (1999) 'Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice', *Science*, 286(5443), pp. 1374–1377. doi: 10.1126/science.286.5443.1374.

Pan, C. Q. *et al.* (2012) 'Modularity and functional plasticity of scaffold proteins as p(l)acemakers in cell signaling', *Cellular Signalling*. Elsevier Inc., 24(11), pp. 2143–2165. doi: 10.1016/j.cellsig.2012.06.002.

Pearce, L. R. *et al.* (2013) 'KSR2 mutations are associated with obesity, insulin resistance, and impaired cellular fuel oxidation', *Cell*. Cell Press, 155(4), p. 765. doi: 10.1016/j.cell.2013.09.058.

Pearson, G. *et al.* (2001) 'Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions*', *Endocrine Reviews*. The Endocrine Society, 22(2), pp. 153–183. doi: 10.1210/edrv.22.2.0428.

Philipova, R. and Whitaker, M. (2005) 'Active ERK1 is dimerized in vivo: Bisphosphodimers generate peak kinase activity and monophosphodimers maintain basal ERK1 activity', *Journal of Cell Science*, 118(24), pp. 5767–5776. doi: 10.1242/jcs.02683.

Platz, A. *et al.* (2008) 'Human cutaneous melanoma; a review of NRAS and BRAF mutation frequencies in relation to histogenetic subclass and body site', *Molecular Oncology*, pp. 395–405. doi: 10.1016/j.molonc.2007.12.003.

Porfiri, E. and McCormick, F. (1996) 'Regulation of epidermal growth factor receptor signaling by phosphorylation of the Ras exchange factor hSOS1', *Journal of Biological Chemistry*, 271(10), pp. 5871–5877. doi: 10.1074/jbc.271.10.5871.

Preger, E. *et al.* (2004) 'Alternative splicing generates an isoform of the human Sef gene with altered subcellular localization and specificity', *Proceedings of the National Academy of Sciences of the United States of America*, 101(5), pp. 1229–1234. doi: 10.1073/pnas.0307952100.

Prior, I. A., Lewis, P. D. and Mattos, C. (2012) 'A comprehensive survey of ras mutations in cancer', *Cancer Research*, pp. 2457–2467. doi: 10.1158/0008-5472.CAN-11-2612.

Pulido, R., Zúñiga, Á. and Ullrich, A. (1998) 'PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif', *EMBO Journal*, 17(24), pp. 7337–7350. doi: 10.1093/emboj/17.24.7337.

Pullikuth, A. *et al.* (2005) 'The MEK1 scaffolding protein MP1 regulates cell spreading by integrating PAK1 and Rho signals.', *Molecular and cellular biology*, 25(12), pp. 5119–33. doi: 10.1128/MCB.25.12.5119-5133.2005.

Raingeaud, J. *et al.* (1996) 'MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway.', *Molecular and Cellular Biology*. American Society for Microbiology, 16(3), pp. 1247–1255. doi: 10.1128/mcb.16.3.1247.

Rajakulendran, T. *et al.* (2009) 'A dimerization-dependent mechanism drives RAF catalytic activation', *Nature*, 461(7263), pp. 542–545. doi: 10.1038/nature08314.

Rajalingam, K. *et al.* (2007) 'Ras oncogenes and their downstream targets', *Biochimica et Biophysica Acta - Molecular Cell Research*, pp. 1177–1195. doi: 10.1016/j.bbamcr.2007.01.012.

Raman, M. and Cobb, M. H. (2003) 'MAP Kinase Modules: Many Roads Home Dispatch', *Current Biology*, 13, pp. 886–888. doi: 10.1016/j.cub.2003.10.053.

Ray, L. B. and Sturgill, T. W. (1988) 'Characterization of insulin-stimulated microtubule-associated protein kinase. Rapid isolation and stabilization of a novel serine/threonine kinase from 3T3-L1 cells.', *The*

Journal of biological chemistry, 263(25), pp. 12721–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2842341 (Accessed: 21 November 2019).

Ren, J. G. *et al.* (2005) 'Self-association of IQGAP1: Characterization and functional sequelae', *Journal of Biological Chemistry*, 280(41), pp. 34548–34557. doi: 10.1074/jbc.M507321200.

Ren, J. G., Li, Z. and Sacks, D. B. (2007) 'IQGAP1 modulates activation of B-Raf', *Proceedings of the National Academy of Sciences of the United States of America*, 104(25), pp. 10465–10469. doi: 10.1073/pnas.0611308104.

Ren, J. G., Li, Z. and Sacks, D. B. (2008) 'IQGAP1 integrates Ca2+/calmodulin and B-raf signaling', *Journal of Biological Chemistry*, 283(34), pp. 22972–22982. doi: 10.1074/jbc.M804626200.

Ren, Y. *et al.* (2004) 'Roles of Gab1 and SHP2 in Paxillin Tyrosine Dephosphorylation and Src Activation in Response to Epidermal Growth Factor', *Journal of Biological Chemistry*, 279(9), pp. 8497–8505. doi: 10.1074/jbc.M312575200.

Revelli, J. P. *et al.* (2011) 'Profound obesity secondary to hyperphagia in mice lacking kinase suppressor of ras 2', *Obesity*, 19(5), pp. 1010–1018. doi: 10.1038/oby.2010.282.

Rheault, T. R. *et al.* (2013) 'Discovery of dabrafenib: A selective inhibitor of Raf Kinases with antitumor activity against B-Raf-driven tumors', *ACS Medicinal Chemistry Letters*, 4(3), pp. 358–362. doi: 10.1021/ml4000063.

Robbins, D. J. *et al.* (1993) 'Regulation and properties of extracellular signal-regulated protein kinases 1, 2, and 3.', *Journal of the American Society of Nephrology : JASN*, 4(5), pp. 1104–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8305637 (Accessed: 24 November 2019).

Rojas, J. M., Oliva, J. L. and Santos, E. (2011) 'Mammalian son of sevenless guanine nucleotide exchange factors: Old concepts and new perspectives', *Genes and Cancer*, pp. 298–305. doi: 10.1177/1947601911408078.

Roskoski, R. (2012) 'Invited review ERK1/2 MAP kinases: Structure, function, and regulation', *Pharmacological Research*, 66, pp. 105–143. doi: 10.1016/j.phrs.2012.04.005.

Rossomando, A. J. *et al.* (1989) *Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase (phorbol ester/epidermal growth factor/anti-phosphotyrosine antibody)*, *Biochemistry*.

Rotblat, B. *et al.* (2008) 'The Ras Inhibitor Farnesylthiosalicylic Acid (Salirasib) Disrupts The Spatiotemporal Localization Of Active Ras: A Potential Treatment For Cancer', *Methods in Enzymology*, pp. 467–489. doi: 10.1016/S0076-6879(07)00432-6.

Rouse, J. *et al.* (1994) 'A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins', *Cell*, 78(6), pp. 1027–1037.

Roy, F. *et al.* (2002) 'KSR is a scaffold required for activation of the ERK/MAPK module', *Genes and Development*, 16(4), pp. 427–438. doi: 10.1101/gad.962902.

Roy, M., Li, Z. and Sacks, D. B. (2004) 'IQGAP1 Binds ERK2 and Modulates Its Activity', *Journal of Biological Chemistry*, 279(17), pp. 17329–17337. doi: 10.1074/jbc.M308405200.

Roy, M., Li, Z. and Sacks, D. B. (2005) 'IQGAP1 Is a Scaffold for Mitogen-Activated Protein Kinase Signaling', *Molecular and Cellular Biology*, 25(18), pp. 7940–7952. doi: 10.1128/mcb.25.18.7940-7952.2005.

Rubinfeld, H., Hanoch, T. and Seger, R. (1999) 'Identification of a cytoplasmic-retention sequence in ERK2', *Journal of Biological Chemistry*, 274(43), pp. 30349–30352. doi: 10.1074/jbc.274.43.30349.

Rushworth, L. K. *et al.* (2014) 'Dual-specificity phosphatase 5 regulates nuclear ERK activity and suppresses skin cancer by inhibiting mutant Harvey-Ras (HRasQ61L)-driven SerpinB2 expression', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 111(51), pp. 18267–18272. doi: 10.1073/pnas.1420159112.

Saba-El-Leil, M. K. *et al.* (2003) 'An essential function of the mitogen-activated protein kinase Erk2 in mouse trophoblast development', *EMBO Reports*. Oxford University Press, 4(10), pp. 964–968. doi: 10.1038/sj.embor.embor939.

Saba-El-Leil, M. K., Frémin, C. and Meloche, S. (2016) 'Redundancy in the world of MAP kinases: All for one', *Frontiers in Cell and Developmental Biology*. Frontiers Media S.A. doi: 10.3389/fcell.2016.00067.

Saha, M. *et al.* (2012) 'RSK phosphorylates SOS1 creating 14-3-3-docking sites and negatively regulating MAPK activation', *Biochemical Journal*, 447(1), pp. 159–166. doi: 10.1042/BJ20120938.

Salmeron, A. *et al.* (1996) 'Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase.', *The EMBO journal*, 15(4), pp. 817–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8631303 (Accessed: 27 November 2019).

Samatar, A. A. and Poulikakos, P. I. (2014) 'Targeting RAS-ERK signalling in cancer: Promises and challenges', *Nature Reviews Drug Discovery*. Nature Publishing Group, pp. 928–942. doi: 10.1038/nrd4281.

Saxena, N. *et al.* (2008) 'RAS: Target for cancer therapy', *Cancer Investigation*, pp. 948–955. doi: 10.1080/07357900802087275.

Schaeffer, H. J. *et al.* (1998) 'MP1: A MEK binding partner that enhances enzymatic activation of the MAP kinase cascade', *Science*, 281(5383), pp. 1668–1671. doi: 10.1126/science.281.5383.1668.

Schiefermeier, N. *et al.* (2014) 'The late endosomal p14-MP1 (LAMTOR2/3) complex regulates focal adhesion dynamics during cell migration', *Journal of Cell Biology*. Rockefeller University Press, 205(4), pp. 525–540. doi: 10.1083/jcb.201310043.

Schmidt, V. A. *et al.* (2003) 'IQGAP2 functions as a GTP-dependent effector protein in thrombin-induced platelet cytoskeletal reorganization', *Blood*, 101(8), pp. 3021–3028. doi: 10.1182/blood-2002-09-

2807.

Scott, A., Haystead, C. M. and Haystead, T. A. (1995) 'Purification of a 12,020-dalton protein that enhances the activation of mitogen-activated protein (MAP) kinase by MAP kinase kinase.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 270(41), pp. 24540–7. doi: 10.1074/jbc.270.41.24540.

Sebolt-Leopold, J. S. *et al.* (1999) 'Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo', *Nature Medicine*, 5(7), pp. 810–816. doi: 10.1038/10533.

Serebriiskii, I. G. *et al.* (2019) 'Comprehensive characterization of RAS mutations in colon and rectal cancers in old and young patients', *Nature Communications*. Nature Publishing Group, 10(1). doi: 10.1038/s41467-019-11530-0.

Sharma, C. *et al.* (2005) 'MEK partner 1 (MP1): Regulation of oligomerization in MAP kinase signaling', *Journal of Cellular Biochemistry*, 94(4), pp. 708–719. doi: 10.1002/jcb.20344.

Sharma, K. *et al.* (2014) 'Ultradeep Human Phosphoproteome Reveals a Distinct Regulatory Nature of Tyr and Ser/Thr-Based Signaling', *Cell Reports*. Elsevier, 8(5), pp. 1583–1594. doi: 10.1016/j.celrep.2014.07.036.

Sharrocks, A. D., Yang, S. H. and Galanis, A. (2000) 'Docking domains and substrate-specificity determination for MAP kinases.', *Trends in biochemical sciences*, 25(9), pp. 448–53. doi: 10.1016/s0968-0004(00)01627-3.

Shin, S. Y. *et al.* (2009) 'Positive- and negative-feedback regulations coordinate the dynamic behavior of the Ras-Raf-MEK-ERK signal transduction pathway', *Journal of Cell Science*, 122(3), pp. 425–435. doi: 10.1242/jcs.036319.

Simanshu, D. K., Nissley, D. V and Mccormick, F. (2017) 'RAS Proteins and Their Regulators in Human Disease HHS Public Access', *Cell*, 170(1), pp. 17–33. doi: 10.1016/j.cell.2017.06.009.

Slack-Davis, J. K. *et al.* (2003) 'PAK1 phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation', *Journal of Cell Biology*, 162(2), pp. 281–291. doi: 10.1083/jcb.200212141.

Smorodinsky-Atias, Soudah and Engelberg (2020) 'Mutations That Confer Drug-Resistance, Oncogenicity and Intrinsic Activity on the ERK MAP Kinases—Current State of the Art', *Cells*, 9(1), p. 129. doi: 10.3390/cells9010129.

Spence, H. J. *et al.* (2004) 'Dystroglycan, a scaffold for the ERK-MAP kinase cascade', *EMBO Reports*, 5(5), pp. 484–489. doi: 10.1038/sj.embor.7400140.

Stanley, R. J. and Thomas, G. M. H. (2016) 'Activation of G Proteins by Guanine Nucleotide Exchange Factors Relies on GTPase Activity', *PloS one*, 11(3), p. e0151861. doi: 10.1371/journal.pone.0151861.

Stebbing, J. *et al.* (2015) 'KSR1 regulates BRCA1 degradation and inhibits breast cancer growth',

Oncogene. Nature Publishing Group, 34(16), pp. 2103–2114. doi: 10.1038/onc.2014.129.

Stephen, A. G. *et al.* (2014) 'Dragging ras back in the ring', *Cancer Cell*. Cell Press, pp. 272–281. doi: 10.1016/j.ccr.2014.02.017.

Stewart, S. *et al.* (1999) 'Kinase Suppressor of Ras Forms a Multiprotein Signaling Complex and Modulates MEK Localization', *Molecular and Cellular Biology*. American Society for Microbiology, 19(8), pp. 5523–5534. doi: 10.1128/mcb.19.8.5523.

Strumberg, D. (2005) 'Preclinical and clinical development of the oral multikinase inhibitor sorafenib in cancer treatment', *Drugs of Today*, pp. 773–784. doi: 10.1358/dot.2005.41.12.937959.

Sudo, T. *et al.* (2002) 'Exip, a new alternative splicing variant of p38α, can induce an earlier onset of apoptosis in HeLa cells', *Biochemical and Biophysical Research Communications*, 291(4), pp. 838–843. doi: 10.1006/bbrc.2002.6529.

Sugimoto, T. *et al.* (1998) 'The kinase suppressor of Ras (KSR) modulates growth factor and Ras signaling by uncoupling Elk-1 phosphorylation from MAP kinase activation', *EMBO Journal*, 17(6), pp. 1717–1727. doi: 10.1093/emboj/17.6.1717.

Sun, H. *et al.* (1993) 'MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo', *Cell*, 75(3), pp. 487–493. doi: 10.1016/0092-8674(93)90383-2.

Sun, H. and Wang, Y. (2012) 'Novel Ser/Thr protein phosphatases in cell death regulation', *Physiology*, pp. 43–52. doi: 10.1152/physiol.00034.2011.

Sundaram, M. and Han, M. (1995) 'The C. elegans ksr-1 gene encodes a novel raf-related kinase involved in Ras-mediated signal transduction', *Cell*, 83(6), pp. 889–901. doi: 10.1016/0092-8674(95)90205-8.

Sundberg-Smith, L. J. *et al.* (2005) 'Adhesion stimulates direct PAK1/ERK2 association and leads to ERK-dependent PAK1 Thr212 phosphorylation', *Journal of Biological Chemistry*, 280(3), pp. 2055–2064. doi: 10.1074/jbc.M406013200.

Swart-Mataraza, J. M., Li, Z. and Sacks, D. B. (2002) 'IQGAP1 is a component of Cdc42 signaling to the cytoskeleton', *Journal of Biological Chemistry*, 277(27), pp. 24753–24763. doi: 10.1074/jbc.M111165200.

Tanoue, T. *et al.* (2000) 'A conserved docking motif in MAP kinases common to substrates, activators and regulators', *Nature Cell Biology*, 2(2), pp. 110–116. doi: 10.1038/35000065.

Tanoue, T. *et al.* (2001) 'Identification of a docking groove on ERK and p38 MAP kinases that regulates the specificity of docking interactions', *EMBO Journal*, 20(3), pp. 466–479. doi: 10.1093/emboj/20.3.466.

Taylor, S. S. *et al.* (1993) 'A template for the protein kinase family', *Trends in Biochemical Sciences*, pp. 84–89. doi: 10.1016/0968-0004(93)80001-R.

Teis, D. *et al.* (2006) 'p14-MP1-MEK1 signaling regulates endosomal traffic and cellular proliferation during tissue homeostasis', *Journal of Cell Biology*, 175(6), pp. 861–868. doi: 10.1083/jcb.200607025.

Teis, D., Wunderlich, W. and Huber, L. A. (2002) 'Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction', *Developmental Cell*. Cell Press, 3(6), pp. 803–814. doi: 10.1016/S1534-5807(02)00364-7.

Theodosiou, A. and Ashworth, A. (2002) 'MAP kinase phosphatases', *Genome Biology*. doi: 10.1186/gb-2002-3-7-reviews3009.

Therrien, M. *et al.* (1995) 'KSR, a novel protein kinase required for RAS signal transduction', *Cell*, 83(6), pp. 879–888. doi: 10.1016/0092-8674(95)90204-X.

Therrien, M. *et al.* (1996) 'KSR modulates signal propagation within the MAPK cascade', *Genes and Development*, 10(21), pp. 2684–2695. doi: 10.1101/gad.10.21.2684.

Tohgo, A. *et al.* (2002) 'β-arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation', *Journal of Biological Chemistry*, 277(11), pp. 9429–9436. doi: 10.1074/jbc.M106457200.

Toledano-Katchalski, H. *et al.* (2003) 'Protein tyrosine phosphatase epsilon inhibits signaling by mitogen-activated protein kinases.', *Molecular cancer research : MCR*, 1(7), pp. 541–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12754301 (Accessed: 30 November 2019).

Torii, S. *et al.* (2004) 'Sef is a spatial regulator for Ras/MAP kinase signaling', *Developmental Cell*, 7(1), pp. 33–44. doi: 10.1016/j.devcel.2004.05.019.

Tsang, M. *et al.* (2002) 'Identification of sef, a novel modulator of FGF signalling', *Nature Cell Biology*, 4(2), pp. 165–169. doi: 10.1038/ncb749.

Turner, C. E., Glenney, J. R. and Burridge, K. (1990) 'Paxillin: A new vinculin-binding protein present in focal adhesions', *Journal of Cell Biology*. Rockefeller University Press, 111(3), pp. 1059–1068. doi: 10.1083/jcb.111.3.1059.

Ussar, S. and Voss, T. (2004) 'MEK1 and MEK2, different regulators of the G1/S transition', *Journal of Biological Chemistry*, 279(42), pp. 43861–43869. doi: 10.1074/jbc.M406240200.

Verlande, A. *et al.* (2018) 'Metabolic stress regulates ERK activity by controlling KSR-RAF heterodimerization e', 19(2), pp. 320–336. doi: 10.15252/embr.201744524.

Voisin, L. *et al.* (2010) 'Genetic Demonstration of a Redundant Role of Extracellular Signal-Regulated Kinase 1 (ERK1) and ERK2 Mitogen-Activated Protein Kinases in Promoting Fibroblast Proliferation', *Molecular and Cellular Biology*. American Society for Microbiology, 30(12), pp. 2918–2932. doi: 10.1128/mcb.00131-10.

Volle, D. J. *et al.* (1999) 'Phosphorylation of the kinase suppressor of Ras by associated kinases',

Biochemistry, 38(16), pp. 5130–5137. doi: 10.1021/bi983050d.

Vomastek, T. *et al.* (2004) 'Modular construction of a signaling scaffold: MORG1 interacts with components of the ERK cascade and links ERK signaling to specific agonists', *Proceedings of the National Academy of Sciences of the United States of America*, 101(18), pp. 6981–6986. doi: 10.1073/pnas.0305894101.

Wang, D. *et al.* (2007) 'Clinical experience of MEK inhibitors in cancer therapy', *Biochimica et Biophysica Acta - Molecular Cell Research*, pp. 1248–1255. doi: 10.1016/j.bbamcr.2006.11.009.

Wang, M. and Casey, P. J. (2016) 'Protein prenylation: Unique fats make their mark on biology', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, pp. 110–122. doi: 10.1038/nrm.2015.11.

Wang, S. *et al.* (2007) 'IQGAP3, a novel effector of Rac1 and Cdc42, regulates neurite outgrowth', *Journal of Cell Science*, 120(4), pp. 567–577. doi: 10.1242/jcs.03356.

Watanabe, T. *et al.* (2004) 'Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration', *Developmental Cell*, 7(6), pp. 871–883. doi: 10.1016/j.devcel.2004.10.017.

Watanabe, T., Wang, S. and Kaibuchi, K. (2015) 'IQGAPs as Key Regulators of Actin-cytoskeleton Dynamics Mini-review and Review', *Cell Structure and Function*, 40, pp. 69–77. Available at: https://www.jstage.jst.go.jp/article/csf/40/2/40_15003/_pdf/-char/en.

Waters, A. M. and Der, C. J. (no date) 'KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer'. doi: 10.1101/cshperspect.a031435.

Watson, U. *et al.* (2018) 'Spatiotemporal Modulation of ERK Activation by GPCRs', in *International Review of Cell and Molecular Biology*. Elsevier Inc., pp. 111–140. doi: 10.1016/bs.ircmb.2018.02.004.

Wei, H. *et al.* (2003) 'Independent β-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2', *Proceedings of the National Academy of Sciences of the United States of America*, 100(19), pp. 10782–10787. doi: 10.1073/pnas.1834556100.

Weinmaster, G., Zoller, M. J. and Pawson, T. (1986) 'A lysine in the ATP-binding site of P130gag-fps is essential for protein-tyrosine kinase activity.', *The EMBO Journal*. Wiley, 5(1), pp. 69–76. doi: 10.1002/j.1460-2075.1986.tb04179.x.

Weir, M. L. and Muschler, J. (2003) 'Dystroglycan: Emerging roles in mammary gland function', *Journal of Mammary Gland Biology and Neoplasia*, pp. 409–419. doi: 10.1023/B:JOMG.0000017428.38034.a7.

Weissbach, L. *et al.* (1994) 'Identification of a human rasGAP-related protein containing calmodulin-binding motifs.', *The Journal of biological chemistry*, 269(32), pp. 20517–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8051149 (Accessed: 19 November 2019).

White, C. D., Brown, M. D. and Sacks, D. B. (2009) 'IQGAPs in cancer: A family of scaffold proteins

underlying tumorigenesis', *FEBS Letters*, pp. 1817–1824. doi: 10.1016/j.febslet.2009.05.007.

White, R. *et al.* (2018) 'BRAF V600E Mutation in Multiple Primary Malignancies: A Hairy Affair', *Cureus*. Cureus, Inc. doi: 10.7759/cureus.3600.

Whitehurst, A. W. *et al.* (2002) 'ERK2 enters the nucleus by a carrier-independent mechanism', *Proceedings of the National Academy of Sciences of the United States of America*, 99(11), pp. 7496–7501. doi: 10.1073/pnas.112495999.

Whitmarsh, A. J. (2011) 'Casein Kinase 2 Sends Extracellular Signal-Regulated Kinase Nuclear', *Molecular and Cellular Biology*. American Society for Microbiology, 31(17), pp. 3512–3514. doi: 10.1128/mcb.05916-11.

Widmann, C. *et al.* (1999) 'Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human', *Physiological Reviews*. American Physiological Society, pp. 143–180. doi: 10.1152/physrev.1999.79.1.143.

Wilson, L. J. *et al.* (2018) 'Review New Perspectives, Opportunities, and Challenges in Exploring the Human Protein Kinome'. doi: 10.1158/0008-5472.CAN-17-2291.

Witzel, F., Maddison, L. and Blüthgen, N. (2012) 'How scaffolds shape MAPK signaling: what we know and opportunities for systems approaches', *Frontiers in Physiology*. Frontiers, 3, p. 475. doi: 10.3389/fphys.2012.00475.

Wong, D. J. *et al.* (2014) 'Antitumor activity of the ERK inhibitor SCH722984 against BRAF mutant, NRAS mutant and wild-type melanoma', *Molecular Cancer*, 13(1), p. 194. doi: 10.1186/1476-4598-13-194.

Wu, P. K. and Park, J. I. (2015) 'MEK1/2 Inhibitors: Molecular Activity and Resistance Mechanisms', *Seminars in Oncology*. W.B. Saunders, pp. 849–862. doi: 10.1053/j.seminoncol.2015.09.023.

Xing, H., Kornfeld, K. and Muslin, A. J. (1997) 'The protein kinase KSR interacts with 14-3-3 protein and Raf', *Current Biology*, 7(5), pp. 294–300. doi: 10.1016/S0960-9822(06)00152-7.

Xiong, S. *et al.* (2003) 'hSef Inhibits PC-12 Cell Differentiation by Interfering with Ras-Mitogen-activated Protein Kinase MAPK Signaling', *Journal of Biological Chemistry*, 278(50), pp. 50273–50282. doi: 10.1074/jbc.M306936200.

Yablonski, D., Marbach, I. and Levitzki, A. (1996) 'Dimerization of Ste5, a mitogen-activated protein kinase cascade scaffold protein, is required for signal transduction', *Proceedings of the National Academy of Sciences of the United States of America*, 93(24), pp. 13864–13869. doi: 10.1073/pnas.93.24.13864.

Yan, C. *et al.* (2001) 'Molecular Cloning of Mouse ERK5/BMK1 Splice Variants and Characterization of ERK5 Functional Domains', *Journal of Biological Chemistry*, 276(14), pp. 10870–10878. doi: 10.1074/jbc.M009286200.

Yang, L. *et al.* (2019) 'Comprehensive Analysis of ERK1/2 Substrates for Potential Combination

Immunotherapies', *Trends in Pharmacological Sciences*. Elsevier Ltd, pp. 897–910. doi: 10.1016/j.tips.2019.09.005.

Yang, S.-H. *et al.* (1998) 'The Elk-1 ETS-Domain Transcription Factor Contains a Mitogen-Activated Protein Kinase Targeting Motif', *Molecular and Cellular Biology*. American Society for Microbiology, 18(2), pp. 710–720. doi: 10.1128/mcb.18.2.710.

Yao, Z. and Seger, R. (2005) 'The Molecular Mechanism of MAPK / ERK Inactivation', *Current Genomics*. Bentham Science Publishers Ltd., 5(4), pp. 385–393. doi: 10.2174/1389202043349309.

Yazicioglu, M. N. *et al.* (2007) 'Mutations in ERK2 binding sites affect nuclear entry', *Journal of Biological Chemistry*, 282(39), pp. 28759–28767. doi: 10.1074/jbc.M703460200.

Yeung, K. *et al.* (1999) 'Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP', *Nature*, 401(6749), pp. 173–177. doi: 10.1038/43686.

Yeung, K. *et al.* (2000) 'Mechanism of Suppression of the Raf/MEK/Extracellular Signal-Regulated Kinase Pathway by the Raf Kinase Inhibitor Protein', *Molecular and Cellular Biology*. American Society for Microbiology, 20(9), pp. 3079–3085. doi: 10.1128/mcb.20.9.3079-3085.2000.

Yoder, J. H. *et al.* (2004) 'Modulation of KSR activity in Caenorhabditis elegans by Zn ions, PAR-1 kinase and PP2A phosphatase', *EMBO Journal*, 23(1), pp. 111–119. doi: 10.1038/sj.emboj.7600025.

Yoon, S. and Seger, R. (2006) 'The extracellular signal-regulated kinase: Multiple substrates regulate diverse cellular functions', *Growth Factors*. Informa UK Limited, 24(1), pp. 21–44. doi: 10.1080/02699050500284218.

Yu, W. *et al.* (1998) 'Regulation of the MAP kinase pathway by mammalian Ksr through direct interaction with MEK and ERK', *Current Biology*. Cell Press, 8(1), pp. 56–64. doi: 10.1016/S0960-9822(98)70020-X.

Yung, Y. *et al.* (2000) 'ERK1b, a 46-kDa ERK isoform that is differentially regulated by MEK', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology Inc., 275(21), pp. 15799–15808. doi: 10.1074/jbc.M910060199.

Zaballos, M. A. *et al.* (2019) 'Regulators of the RAS-ERK pathway as therapeutic targets in thyroid cancer', *Endocrine-Related Cancer*, 26(6), pp. R319–R344. doi: 10.1530/ERC-19-0098.

Zeke, A. *et al.* (2016) 'JNK Signaling: Regulation and Functions Based on Complex Protein-Protein Partnerships'. doi: 10.1128/MMBR.00043-14.

Zervos, A. S. *et al.* (1995) 'Mxi2, a mitogen-activated protein kinase that recognizes and phosphorylates Max protein', *Proceedings of the National Academy of Sciences of the United States of America*, 92(23), pp. 10531–10534. doi: 10.1073/pnas.92.23.10531.

Zhang, B. *et al.* (2005) 'Mechanism of the Guanine Nucleotide Exchange Reaction of Ras

GTPaseEvidence for a GTP/GDP Displacement Model', *Biochemistry*, 44(7), pp. 2566–2576. doi: 10.1021/bi048755w.

Zhang, F. *et al.* (1994) 'Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution', *Nature*, 367(6465), pp. 704–711. doi: 10.1038/367704a0.

Zhang, H. *et al.* (2013) 'The dual function of KSR1: A pseudokinase and beyond', *Biochemical Society Transactions*, 41(4), pp. 1078–1082. doi: 10.1042/BST20130042.

Zhang, J. *et al.* (2008) 'Downregulation of KSR1 in pancreatic cancer xenografts by antisense oligonucleotide correlates with tumor drug uptake', *Cancer Biology and Therapy*, 7(9), pp. 1492–1497.

Zhang, Y. *et al.* (1997) 'Kinase suppressor of Ras is ceramide-activated protein kinase', *Cell*. Cell Press, 89(1), pp. 63–72. doi: 10.1016/S0092-8674(00)80183-X.

Zhou, G., Bao, Z. Q. and Dixon, J. E. (1995) 'Components of a new human protein kinase signal transduction pathway', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology Inc., 270(21), pp. 12665–12669. doi: 10.1074/jbc.270.21.12665.

Zhou, L. *et al.* (2016) 'The scaffold protein KSR1, a novel therapeutic target for the treatment of Merlin-deficient tumors', *Oncogene*. Nature Publishing Group, 35(26), pp. 3443–3453. doi: 10.1038/onc.2015.404.

Zhou, M. *et al.* (2002) 'Solution structure and functional analysis of the cysteine-rich C1 domain of kinase suppressor of Ras (KSR)', *Journal of Molecular Biology*. Academic Press, 315(3), pp. 435–446. doi: 10.1006/jmbi.2001.5263.

Zhou, R. *et al.* (2003) 'Polarized distribution of IQGAP proteins in gastric parietal cells and their roles in regulated epithelial cell secretion.', *Molecular biology of the cell*, 14(3), pp. 1097–108. doi: 10.1091/mbc.e02-07-0425.

Zisman-Rozen, S. *et al.* (2007) 'Downregulation of Sef, an inhibitor of receptor tyrosine kinase signaling, is common to a variety of human carcinomas.', *Oncogene*, 26(41), pp. 6093–8. doi: 10.1038/sj.onc.1210424.