

INVESTIGATION OF POTENTIAL ERK-DEPENDENT
PHOSPHORYLATION SITES ON SIK2

by

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To my family and friends

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ABSTRACT

INVESTIGATION OF POTENTIAL ERK-DEPENDENT PHOSPHORYLATION SITES ON SIK2

The previous studies from our laboratory suggested that the serine/threonine kinase SIK2 is involved in a feedback mechanism downregulating the FGF-dependent MAPK pathway. As it has been shown that the activity of SIK2 is rapidly increasing with concomitant threonine phosphorylation in response to transient FGF2 stimulation in Müller cells and that ERK directly phosphorylates SIK2 *in vitro*, ERK is proposed to be the activatory kinase of SIK2 in this regulation scheme. This study revealed that FRS2 and SOS1 are also present in the same signaling complex with SIK2 in FGF2 treated Müller cells, supporting our hypothesis. In this study, we focused on the investigation of the potential ERK-dependent phosphorylation sites on SIK2. For this purpose, the two putative ERK target residues T758 and T863 on SIK2 sequence that were identified by bioinformatic analysis were mutated to alanine residues by site-directed mutagenesis. The kinase inactive SIK2 was used for the generation of these mutants to distinguish phosphorylation by ERK from the autophosphorylation activity of SIK2 itself in further assays. KI-SIK2 and the mutant versions of KI-SIK2 were expressed in HEK 293T cells and purified by immunoprecipitation. Purified SIK2 samples were used in *in vitro* kinase assay with active ERK2. As the mutant forms of KI-SIK2 were phosphorylated at the same level with the KI-SIK2, the results of the *in vitro* kinase experiment indicated that neither of these threonine residues (T758 and T863) are targeted by ERK2, raising the possibility of non-canonical motifs as ERK targets. Thus, mass spectrometry analysis was also carried out to identify the ERK-dependent phosphorylation sites on SIK2. However, due to low coverage of SIK2 sequence, no informative data was obtained. In the context of this study, as a first step for the further investigation of Gab1-SIK2 interactions, bacterial expression constructs of these proteins were generated.

ÖZET

SIK2 ÜZERİNDEKİ ERK'E BAĞLI OLASI FOSFORİLASYON BÖLGELERİNİN İNCELENMESİ

Laboratuvarımızda daha önce yapılan çalışmalar, bir serin/treonin kinaz olan SIK2'nin FGF'e bağlı MAPK yolağının negatif regüle eden bir geri-besleme mekanizmasında rol aldığını önermektedir. SIK2'nin FGF2 ile kısa süreli uyarımda Müller hücrelerindeki hızlı aktivite ve treonin fosforilasyon artışının ve *in vitro* olarak ERK'in SIK2'yi direk olarak fosforlamasının gösterilmiş olması nedeniyle, ERK'in SIK2'nin aktive edici kinazı olduğu düşünülmektedir. Bu çalışma, hipotezimizi destekleyerek, FRS2 ve SOS1'in de SIK2'nin FGF2 stimüle edilen Müller hücrelerinde etkileştiği sinyal kompleksinde bulunduğunu göstermiştir. İkinci aşamada SIK2 üzerindeki ERK'e bağlı potansiyel fosforilasyon bölgelerinin incelenmesi üzerine yoğunlaşmıştır. Bu amaçla SIK2 üzerindeki olası ERK hedef amino asitleri biyoinformatik yaklaşımla belirlenmiş ve bu aday amino asitler T758 ve T863 *in vitro* mutagenез yöntemi ile alanine değiştirilmiştir. Deneylerde ERK tarafından gerçekleştirilen fosforilasyonu SIK2'nin otofosforilasyon aktivitesinden ayırmak için bu mutasyonları üretirken kinaz inaktif SIK2 kullanılmıştır. HEK 293T hücrelerinde ürettirilen KI-SIK2 ve KI-SIK2'nin mutant versiyonları immünopresipitasyon ile izole edilmiştir. İzole edilen SIK2 örnekleri aktif ERK2 ile birlikte *in vitro* kinaz deneylerinde kullanılmıştır. Mutant KI-SIK2'ler ile KI-SIK2'nin ERK fosforilasyon düzeyleri arasında önemli bir fark olmadığını göstermiş olan veriler T758 ve T863 amino asitlerinin ERK'in hedef noktaları olmadığına işaret etmektedir. Olası atipik ERK motiflerinin tanımlanabilmesi amacıyla kütle spektrometrisi analizi de yapılmış, ancak SIK2'nin kapsama seviyesindeki düşüklükten dolayı sonuç alınamamıştır. Ancak, bu kapsama seviyesini %35-40 dolaylarına yükseltmesini beklediğimiz, fosfo-peptitlerin belirlenmesine imkan verecek, yeni bir kütle spektrometrisi deneyi yapılmaktadır. Bu çalışmalara ek olarak SIK2-Gab1 ilişkisinin daha derinlemesine incelenmesi yolunda ilk adım olarak bu proteinlerin bakteriyel anlatım yapıları da üretilmiştir.

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LIST OF ACRONYMS/ABBREVIATIONS

AMPK	AMP-activated Protein Kinase
APS	Ammonium Persulfate
ATP	Adenosine Triphosphate
BCA	Bicinchronic Acid
BMP	Bromophenolblue
bp	Base Pair
BSA	Bovine Serum Albumin
CaCl ₂	Calcium Chloride
cAMP	Cyclic Adenosine 5'-monophosphate
cDNA	Complementary DNA
CO ₂	Carbon Dioxide
Co-IP	Co-immunoprecipitation
CREB	cAMP Response Element-binding Protein
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
Dos	Daughter of Sevenless
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ERK	Extracellular Regulated Kinase
EtOH	Ethyl Alcohol
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
Frs2	Fibroblast Growth Factor Receptor Substrate
Gab	Grb2-associated Binder
GFP	Green Fluorescent Protein
Grb	Growth Factor Receptor-bound Protein
H ₂ O	Water

HBS	HEPES Buffered Saline
HEK	Human Embryonic Kidney Cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HRP	Horse Radish Peroxidase
HSPG	Heparan Sulphate Proteoglycans
IGF	Insulin-Like Growth Factor
IgG	Immunoglobulin G
IP	Immunoprecipitation
IP3	Inositol-1,4,5-triphosphate
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IRS	Insulin Receptor Substrate
IVK	In Vitro Kinase
Kb	Kilo-base
kDa	Kilodalton
KI	Kinase Inactive
LB	Luria Broth
LKB1	Liver Kinase B1
MAPK	Mitogen-activated Protein Kinase
MEK	MAPK/ERK Kinase
mg	Miligram
MgCl ₂	Magnesium Chloride
Min	Minutes
MIO-M1	Moorfields Institute of Ophthalmology – Müller 1
MKP	MAPK Phosphatase
ml	Mililiter
mm	Millimeter
mM	Milimolar
MP	Milk Powder
NaCl	Sodium Chloride
ng	Nanogram
NGF	Nerve Growth Factor
nm	Nanometer
OD	Optical Density
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
Pen/Str	Penicillin/Streptomycin
PH	Plecstrin Homology Domain
PI	Phosphatidylinositol-4,5-diphosphate
PI3K	Phosphoinositide 3-kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC γ	Phospholipase C-gamma
pSer	Phospho-Serine
PTB	Phosphotyrosine Binding
PVDF	Polyvinyl Difluoride
Raf	Rapidly Accelerated Fibrosarcoma
RNA	Ribonucleic Acid
rpm	Rotations Per Minute
RTK	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
Sec	Seconds
Ser	Serine
SH	Src Homology Domain
Shc	Src Homology 2 Domain Containing Transforming Protein
Shp2	SH2 Domain Containing Phosphatase 2
SIK	Salt Inducible Kinase
SOC	Suppressor of Clear
SOS	Son of Sevenless
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TEMED	Tetramethylethylenediamine
TORC2	Transducer of Regulated CREB Protein 2
U	Unit
UBA	Ubiquitin-associated Domain
WB	Western Blot
WT	Wild-type

1. INTRODUCTION

1.1. Fibroblast Growth Factors

Fibroblast growth factors (FGFs) are secreted protein ligands which act as growth factors both in a paracrine and an endocrine fashion. Originally identified as proteins capable of promoting fibroblast proliferation, later they have been shown to be widely expressed in various types of cells and tissues with pleiotropic effects (Itoh, 2007). FGFs have been shown to play important roles in regulation of a variety of fundamental biological processes including cell proliferation, survival, migration, differentiation, wound healing and angiogenesis (Turner and Grose, 2010). They exert their functions through binding the membrane bound FGF receptors (FGFRs), which are tyrosine kinase receptors that get activated upon ligand binding.

There are 23 types of FGFs present in the FGF family; 22 of which are observed in humans (there is no *FGF15* gene in human). 18 members of this family are ligands for FGFRs, while four family members do not bind with FGFRs and act as FGF homologous factors (FGF11, FGF12, FGF13, and FGF14). FGFs are divided into 7 subfamilies based on their homology (Figure 1.1) (Itoh, 2007). Molecular sizes of FGFs in vertebrates vary from 17 to 34 kDa, and all FGFs share a conserved sequence of 120 amino acids that has a 16%-65% identity (Eswarakumar *et al.*, 2005). Most of the FGFs carry a signal peptide sequence at the N-terminal leading to secretion except FGF1, 2, 9, 16 and 20; which are secreted via an alternative pathway (Powers *et al.*, 2000).

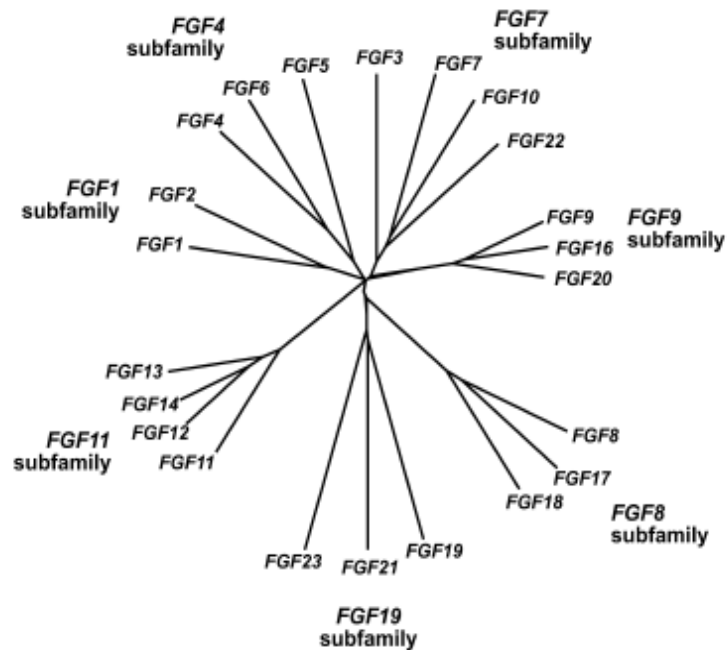


Figure 1.1. Evolutionary relationships among the human *FGF* gene family (Itoh, 2007).

FGFs are heparin binding proteins and they act in concert with heparins and heparan sulfate proteoglycans (HSPGs) to activate FGFRs and induce cellular responses. There are 4 known FGFRs (FGFR1-4) that show 55% to 72 % homology at protein level. These receptors have three extracellular immunoglobulin-like (Ig-like) domains (IgI, IgII and IgIII) an acid box (AB) domain between IgI and IgII, a transmembrane (TM) domain and an intracellular tyrosine kinase (TK) domain (Figure 1.2). Variance in these domains through alternative splicing provides isoforms of FGFRs with different ligand specificities and differential cellular responses (Powers *et al.*, 2000). A fifth receptor called FGFR5 (also called FGFR1) that can bind FGFs but lack the TK domain is thought to be a negative regulator of the signaling (Wiedemann and Trueb, 2000).

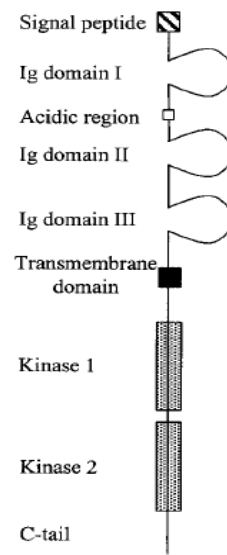


Figure 1.2. General structure of the fibroblast growth factor receptors (FGFRs) (Powers *et al.*, 2000).

High expression of FGFs can also be observed in both developing and adult eye. A number of FGF family members act as morphogens during the formation of optic vesicle and differentiation of retinal neurons (Martinez-Morales *et al.*, 2004). FGF1, 2 and 9 are being expressed abundantly in the mammalian retina and they have been shown to have a proliferative effect on Müller Glia cells (Buğra *et al.*, 1994; Buğra and Hicks, 1997; Fischer *et al.*, 2004; Çınaroğlu *et al.*, 2005).

1.2. FGF Signaling

Like most of the receptor tyrosine kinases (RTKs), FGF signaling is initiated by the ligand binding and receptor dimerization. FGF ligands, which are generally sequestered in the extracellular matrix (ECM) by HSPGs, are released from the ECM by heparinases, proteases or specific FGF-binding proteins and consequently, the free FGFs bind to cell surface HSPGs. The ligand-receptor interaction is stabilized with the help of cell surface HSPGs (Powers *et al.*, 2000). FGFR dimerization leads to conformational changes that activate intracellular TK domains and transphosphorylation of the TK domains and the C-terminal tail. These phosphorylated residues serve as docking sites for the adaptor proteins that carry Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains, which in turn

are activated by direct phosphorylation by the FGFR and trigger canonical signaling events (Eswarakumar *et al.*, 2005).

There are three major pathways activated through FGFRs: Mitogen-activated protein kinase (Ras/MAPK) pathway, PI3 kinase/Akt pathway and PLC γ pathway (Yun *et al.*, 2010) (Figure 1.3). Ras/MAPK and PI3K/Akt pathways are initiated by the docking protein FRS2 that is activated by the FGFR, whereas PLC γ interacts with the receptor to initiate the downstream PLC γ signaling (Goetz and Mohammadi, 2013). There also exists minor pathways downstream of the FGF signaling, such as v-crk sarcoma viral oncogene homolog (Src) signaling pathway and v-crk sarcoma virus CT10 oncogene homolog (Crk) mediated pathway (Powers *et al.*, 2000).

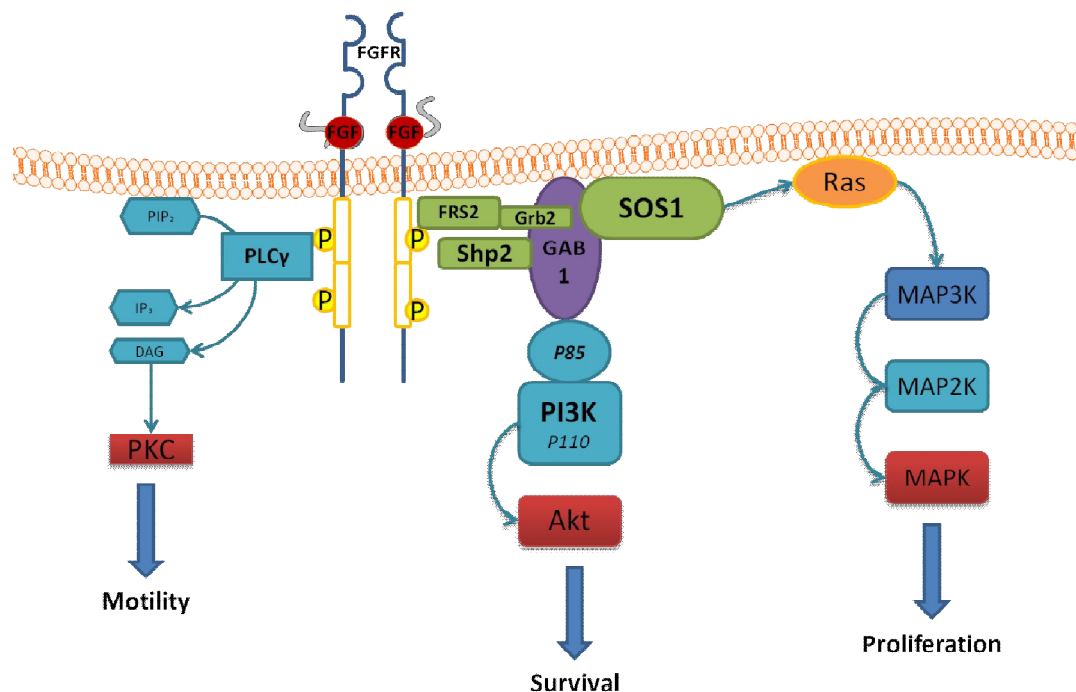


Figure 1.3. The three major FGF signaling pathways; the PLC γ pathway, the PI3 kinase/Akt pathway and the Ras/MAPK pathway.

Even though cellular response resulting from each of the FGF pathways may vary depending on the cellular contexts; Ras/MAPK pathway predominantly drives cells to proliferation, PI3K/Akt pathway mostly contributes to cell survival and PLC γ pathway regulates cell motility (Goetz and Mohammadi, 2013).

1.2.1. MAPK Pathways

MAPK pathway is a signaling cascade placed downstream of the RTKs that transmits the signal by sequential phosphorylation of the kinases of the pathway. The MAPK pathway is highly conserved evolutionarily and it is critical in regulation of various basic cellular processes such as proliferation, differentiation, survival and apoptosis (Pearson *et al.*, 2001). The extracellular signal-regulated kinase (ERK) family, the p38 kinase family and the c-Jun N-terminal kinase (JNK) family are the three main protein families that constitute the main effectors of the three major MAPK pathways (Meister *et al.*, 2013). The MAPK cascade itself is made up of three kinases that are called “three-tiers” and give the pathway its name: MAPK, MAPK kinase (MAP2K) and MAPK kinase kinase (MAP3K) (right arm on Figure 1.3). The focus of this study was on FGF-dependent Ras/MAPK (Ras/ERK) signaling.

Initial modulator of the FGF dependent Ras/MAPK pathway is the docking protein FGF receptor substrate 2 (FRS2) that binds to the phosphorylated tyrosine residues on the cytoplasmic domain of FGFR via its PTB domains and become tyrosine phosphorylated (Gotoh, 2008). Phosphorylated FRS2 recruits the signaling complex proteins by interacting with the SH2 domain of the adapter protein growth factor receptor-bound protein 2 (Grb2). Grb2 interacts with the other signaling complex proteins the docking protein Grb2-associated binding protein 1 (Gab1) and the guanine exchange factor Son of Sevenless (SOS) through its SH3 domains (Eswarakumar *et al.*, 2005). FRS2 also recruits the tyrosine phosphatase Src homology phosphatase 2 (Shp2), which gets activated upon tyrosine phosphorylation and promote strong activation of Ras/MAPK pathway (Mohi and Neel, 2007).

The link between RTKs and the MAP kinases is mainly provided by the GTPase Ras (Ras-dependent MAPK activation). Ras is activated by the guanine exchange factor SOS. The GTP-bound active Ras can then bind to the first tier of the phosphorylation cascade, Raf (MAP3K), recruiting it to the plasma membrane and increasing its activity. Activated Raf stimulates MEK1/2 (MAP2K) by phosphorylating at Ser218 and Ser 222 residues (Kyriakis *et al.*, 1992). MEK1 and MEK2 are dual specificity kinases that share very high sequence homology. MEK1/2 phosphorylate ERK1/2 (MAPK) at both threonine and

tyrosine residues of the TXY motifs (Dhanasekaran and Reddy, 1998). When ERK1 and 2 get activated, they either phosphorylate certain target molecules in cytosol or translocate to the nucleus and phosphorylate nuclear substrates; including transcription factors Elk-1, c-Jun and ATF2 (Yang *et al.*, 2013). These factors consequently, trigger various cellular processes including transmission of cytokine production, proliferation and survival.

1.3. Regulation of FGF-dependent Ras/MAPK Pathway

Since FGF signaling and MAPK pathway have key roles in several critical cellular processes, their tight control is of high importance in different tissues and different developmental stages. In most cases, FGFs stimulate proliferation, survival and migration. However, in certain cellular conditions, FGF signaling drives cells to differentiation and/or cell cycle arrest (Turner and Grose, 2010). The great variance in the cellular responses to FGF results in stem from the regulation mechanisms at each step of the signaling cascade, depending on the physiological context of the cell or the tissue. General mechanisms for the regulation of FGF signaling and MAPK pathway include changes on receptor protein levels, negative feedback loops, phosphorylation and dephosphorylation events and alterations in duration and cellular compartmentalization of the pathway components (Ebisuya *et al.*, 2005).

One of the known inhibitors of the FGF signaling is the proto-oncogenic protein Casitas b-lineage Lymphoma (Cbl). Cbl works by targeting activated tyrosine kinase receptors through ubiquitylation and leading to the degradation or recycling of the receptors (Thien and Langdon, 2001). MAPK phosphatase family proteins are also negative modulators of FGF signaling. For instance, MAPK phosphatase-3 (MKP3) dephosphorylates pThr and pTyr on TXY activation motifs on ERK1 and ERK2 and thus inhibits MAPK pathway (Zhao and Zhang, 2001).

ERK1 and ERK2 have been shown to downregulate signaling by phosphorylating FRS2 on multiple threonine residues, leading to a decrease in tyrosine phosphorylation that inhibits the recruitment of Grb2 to the plasma membrane (Lax *et al.*, 2002). Another example of a negative feedback mechanism to downregulate Ras/MAPK signaling is through the protein Sprouty. Sprouty is activated upon growth factor stimulation through

tyrosine phosphorylation, and inhibits signal transmission either by binding to the adapter protein Grb2 and inhibiting its interaction with SOS or blocking the activation of ERK (Hanafusa *et al.*, 2002). Similar expression to FGF genes (Sef) is another inhibitor of the MAPK pathway. Sef blocks dissociation of the activated ERK from MEK; which inhibits nuclear activity of ERK by its preventing its translocation to the nucleus. However, Sef inhibition of ERK does not suppress its cytosolic activity (Torii *et al.*, 2004).

Positive regulators of the MAPK pathway also exist, constituted mostly by the scaffold proteins. Kinase suppressor of Ras (KSR) is a scaffold protein that binds with Raf, MEK and ERK, thereby facilitating transduction and leading to a faster ERK activation (Morrison, 2001; Therrien *et al.*, 1996). XFLRT3 is a leucine-rich-repeat transmembrane protein and another positive modulator of FGF signaling (Böttcher *et al.*, 2004).

1.4. Gab Family

Gab protein family consists of large scaffold/docking proteins which are found to be 50 to 100 kDa in metazoans (Liu and Rohrschneider, 2002). Homologs of Gab; *Daughter of Sevenless* (DOS) and *Suppressor of Clear* (SOC) were first discovered as regulators of RTK pathways in genetic screens of *Drosophila* and *Caenorhabditis* (Herbst *et al.*, 1996; Schultzman *et al.*, 2001). Gab proteins have been reported to have regulatory roles in downstream of many growth factors, such as epidermal growth factors (EGF), FGFs, hepatocyte growth factors (HGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and nerve growth factor (NGF) (Liu and Rohrschneider, 2002; Lemarié and Lehoux, 2011).

There are three Gab proteins identified in human; namely Gab1, Gab2 and Gab3. Gab proteins have similar structural patterns and critical domains. All Gab proteins share a pleckstrin homology (PH) domain at their N-termini, a central proline-rich region and several phosphorylated tyrosine residues that may serve as binding sites for SH3 domain containing proteins (Wöhrle *et al.*, 2009). The N-terminal PH domain of Gab proteins recognizes phosphoinositides, mostly the membrane-bound $PI_{3,4,5}P_3$, and helps to concentrate Gab proteins near the cell membrane and the receptors (Wöhrle *et al.*, 2009).

Recruitment of Gab proteins to the signaling complexes happens through several different mechanisms. Grb2 is an adapter protein that binds to the proline-rich regions on Gab proteins through its C-terminal SH3 domain and enables indirect recruitment of Gab proteins to the active receptor sites (Lock *et al.*, 2002). Recruitment of Gab1 in FGF signaling includes the docking protein FRS2 in addition to Grb2. Direct interaction of FRS2 with the activated FGF receptor provides binding sites for SH2 domain of Grb2, which at the same time binds to Gab1 with its SH3 domain (Kouhara *et al.*, 1997). Grb2 recruits Gab1 to the active FGFR complex where Gab1 gets tyrosine phosphorylated and transmits the signal to downstream effectors (Pawson, 2007).

Tyrosine phosphorylation has a significant role in the control of Gab-dependent regulation of signaling events. Individual protein tyrosine kinases (PTKs) phosphorylate distinct tyrosine residues on Gab that leads to changes in tyrosine phosphorylation pattern (Wöhrle *et al.*, 2009). This way, Gab proteins provide docking sites for different proteins in different cellular conditions and act as a hub for the cellular signaling systems.

1.4.1. Gab1

Gab1 is a 105 kDa scaffold/docking protein. Similar to other Gab family proteins, Gab1 gets tyrosine phosphorylated upon stimulation through a variety of RTKs. Tyrosine phosphorylated Gab1 binds to several signaling molecules to propagate the stimulus including Shc, Shp2, PLC γ , p85 subunit of PI3K and Crk family proteins (Rajala *et al.*, 2013). Interaction of Gab1 with Grb2 is established through SH3 domain of Grb2, in a phospho-tyrosine independent manner (Lewitzky *et al.*, 2001).

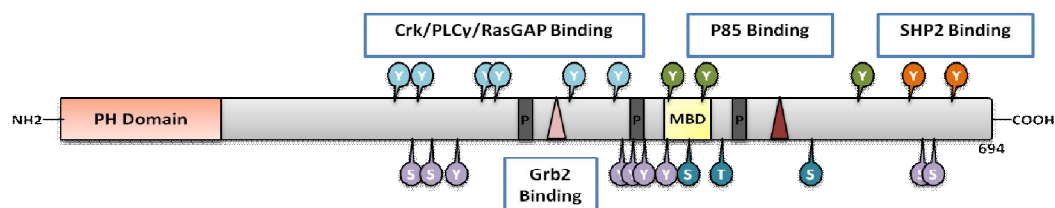


Figure 1.4. Schematic representation of critical domains and residues on human Gab1.

Numbers represent the aminoacid numbers.

Studies in our laboratory, indicated Gab1 as a target of SIK2 in a negative feedback regulation of FGF dependent MAPK pathway (Küser, 2011). Ser²⁶⁶ residue on Gab1 was shown to be the target phosphorylation site for SIK2 via site-directed mutagenesis and in vitro phosphorylation experiments (Yılmaz Sert, 2011).

1.5. ERK

The MAPK family includes highly conserved serine/threonine protein kinases that modulate diverse cellular processes including proliferation, differentiation and survival (Torii *et al.*, 2006). Ubiquitously expressed 42 and 44 kDa proteins ERK1 and ERK2 are the two main members of the MAPK family (Yung *et al.*, 2000). These two kinases are 84% identical and share most of their functions, and therefore referred as ERK1/2 in general. The dual-kinases MEK1 and MEK2 are the main activatory kinases for ERK1/2, which phosphorylate both threonine 202-185 and tyrosine 204-187 residues in human, respectively (Roskoski Jr., 2012). Upon activation, ERKs can phosphorylate their vast array of substrates in the cytoplasm or in the nucleus. Transcriptions factors such as Ets-1/2, c-Jun, c-Fos, p53 and Elk1 that are crucial for modulation of proliferation and oncogenic transformation; and cytosolic substrates such as MAPK-activated protein kinases (MAPKAPKs) that transmit the signal to a distinct subset of cytosolic and nuclear substrates, and the focal adhesion protein Paxillin that participates in regulation of cellular morphology and motility can be listed among ERKs' substrates (Shaul and Seger, 2006; Roux and Blenis, 2004; Ku and Meier, 2000) (Figure1.5).

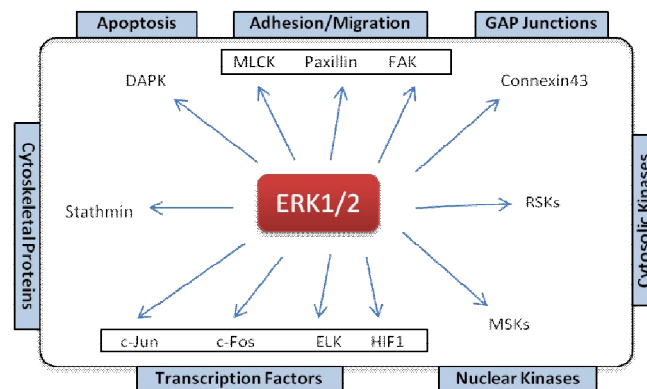


Figure 1.5. The diversity of ERK1/2 substrates and their functions (modified from Ramos, 2008).

ERKs are proline-directed protein kinases that phosphorylate serine or threonine residues followed by a proline residue ([S/T]P motif) on their substrates (Davis, 1993). ERK interacting proteins (substrates, activators or inhibitors) contain particular docking sites such as DEJL domain (Docking site for ERK and JNK, LXL; group of basic residues followed by LXL motif) and DEF domain (Docking site for ERK or; FXFP motif) (Fantz *et al.*, 2001). ERK proteins do not carry a nuclear localization signal (NLS) sequence, and are thought to be transported into the nucleus by importin-dependent pathways (Matsubayashi *et al.*, 2001).

Bioinformatic tools predicted the presence of putative ERK docking and phosphorylation consensus sites on SIK2. Previous cell culture and in vitro phosphorylation studies indicated ERK2 as an activatory kinase of SIK2 via threonine phosphorylation (Ejder, 2011).

1.6. Salt-Inducible Kinase (SIK) Family

The salt-inducible kinases (SIKs) are serine/threonine kinases that are members of the AMP-activated protein kinase (AMPK) super family. Three SIK isoforms exist in mammals; namely SIK1, SIK2 and SIK3 (Katoh *et al.*, 2004). SIK1 was originally isolated from adrenal glands of high-salt diet-fed rats (Wang *et al.*, 1999). The other isoforms, SIK2 and SIK3, were discovered by homology with *Sik1* (Katoh *et al.*, 2004). The SIK proteins share a serine/threonine kinase domain at their N-termini, a protein kinase A (PKA) phosphorylation site at their C-termini, and a central sucrose-nonfermenting 1 (SNF-1) kinase homology domain (Okamoto *et al.*, 2004). They show their effects in tissue-specific manner.

SIK1 is expressed abundantly in adrenal glands, and in lower levels in brain, pituitary, ovary and lung (Feldman *et al.*, 2000). SIK2 is expressed in adipose tissue and at a lower level in the testes; while SIK3 is expressed ubiquitously (Katoh *et al.*, 2004). SIK2 was also shown to be expressed in vertebrate retina and cultured Müller glia cells (Küser-Abalı *et al.*, 2013).

A well-known regulation mechanism for SIK proteins is through phosphorylation of the T-Loop by Liver Kinase B 1(LKB1). The T-loop phosphorylation site of SIK1 and SIK3 binds to the adaptor protein 14-3-3 that regulates the activity and localization of SIKs (Al-Hakim *et al.*, 2005). A recent study in adipocytes reported that SIK2 is phosphorylated on Ser³⁵⁸ upon cAMP stimulation that enables 14-3-3 binding (Henriksson *et al.*, 2012).

1.6.1. Salt-Inducible Kinase 2 (SIK2)

SIK2, also called QIK (Qin-induced kinase), is a 120kDa serine/threonine kinase that was first discovered in adipocytes (Horike *et al.*, 2003). Expression of SIK2 is seen mostly in metabolic tissues such as liver, adipose tissue and brain; the expression has been observed to a lower level in testis and retina as well (Okamoto *et al.*, 2004; Horike *et al.*, 2003; Özcan, 2003).

SIK2 has a kinase domain located near N-terminal, a ubiquitin-associated (UBA) domain and an RK-rich region that is important for intracellular localization (Horike *et al.*, 2003; Katoh *et al.*, 2004). Lysine 49 (K49) located in the kinase domain is a critical residue for kinase activity of SIK2, as it has been shown that K49M mutant of SIK2 has no kinase activity (Horike *et al.*, 2003). LKB1, an upstream activator of most AMPKs, activates SIK2 by phosphorylating Thr¹⁷⁵ (Lizcano *et al.*, 2004). PKA is another well-known upstream kinase of SIK2. The targets of PKA phosphorylation on SIK2 are Ser³⁵⁸ and Ser⁵⁸⁷ (Henriksson *et al.*, 2012; Horike *et al.*, 2003). In Henriksson *et al.*, it has been shown that the phosphorylation of Ser³⁵⁸ is required for 14-3-3 binding to SIK2, rather than Ser⁵⁸⁷, and that it inhibits nuclear activity of SIK2 by preventing its nuclear translocation (Henriksson *et al.*, 2012). The critical domains and residues on SIK2 sequence is shown in Figure 1.6.

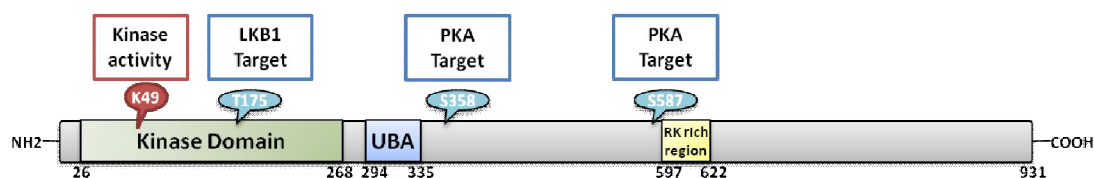


Figure 1.6. Schematic representation of critical domains and residues on human SIK2.

Numbers represent the aminoacid numbers.

SIK2 is involved in insulin metabolism as the insulin receptor substrate-1 (IRS1) being one of the best characterized substrates of SIK2. Human IRS1 is phosphorylated on Ser⁷⁹⁴ by SIK2 (Horike *et al.*, 2003). SIK2 inhibits the cAMP response element-binding protein (CREB) dependent gene expression by phosphorylating the CREB activator protein transducer of regulated CREB protein 2 (TORC2) and sequester it in the cytoplasm (Screaton *et al.*, 2004).

In studies from our laboratory, SIK2 was obtained in a yeast two hybrid screening of a retina cDNA library using the cytoplasmic part of the FGFR2 as the bait (Özcan, 2003). SIK2 has been shown to be expressed in all layers of retina and its subcellular localization, phosphorylation status and activity were shown to change in MIO-M1 cells (A Müller glia cell line) in a FGF2 dependent manner (Özcan, 2003; Özmen, 2006; Candaş, 2007; Küser, 2011). The activity of SIK2 was transiently upregulated in FGF2-treated MIO-M1 cells, which peaked at 10 minutes of treatment showing a similar activity pattern with ERK (Küser, 2011). Overexpression of SIK2 in MIO-M1 cells leads to a decrease in the FGF-dependent ERK activity, while silencing of SIK2 in these cells increased the duration of activity of ERK and also FGF2-dependent cell proliferation (Küser, 2011). These results indicated the possibility of a negative feedback mechanism involving SIK2 acting on the FGF signaling.

The FGF signaling elements Grb2, Gab1 and Raf1 are carrying putative SIK2 phosphorylation motifs. However, only Gab1 is shown to be a substrate of SIK2, and phosphorylated on its Ser²⁶⁶ by SIK2 (Yılmaz Sert, 2011). Also, SIK2 was shown to be phosphorylated on threonine residues by ERK2 in vitro kinase assays (Ejder, 2011).

In the light of these findings, we propose that SIK2 is activated upon FGF2 treatment via getting phosphorylated by ERK2 and inhibits the pathway creating a feedback loop by phosphorylating Gab1 to decrease the interaction of Gab1 with other signaling complex components. Proposed model for involvement of SIK2 in the negative regulation of the FGF-dependent Ras/MAPK pathway is presented in Figure 1.7.

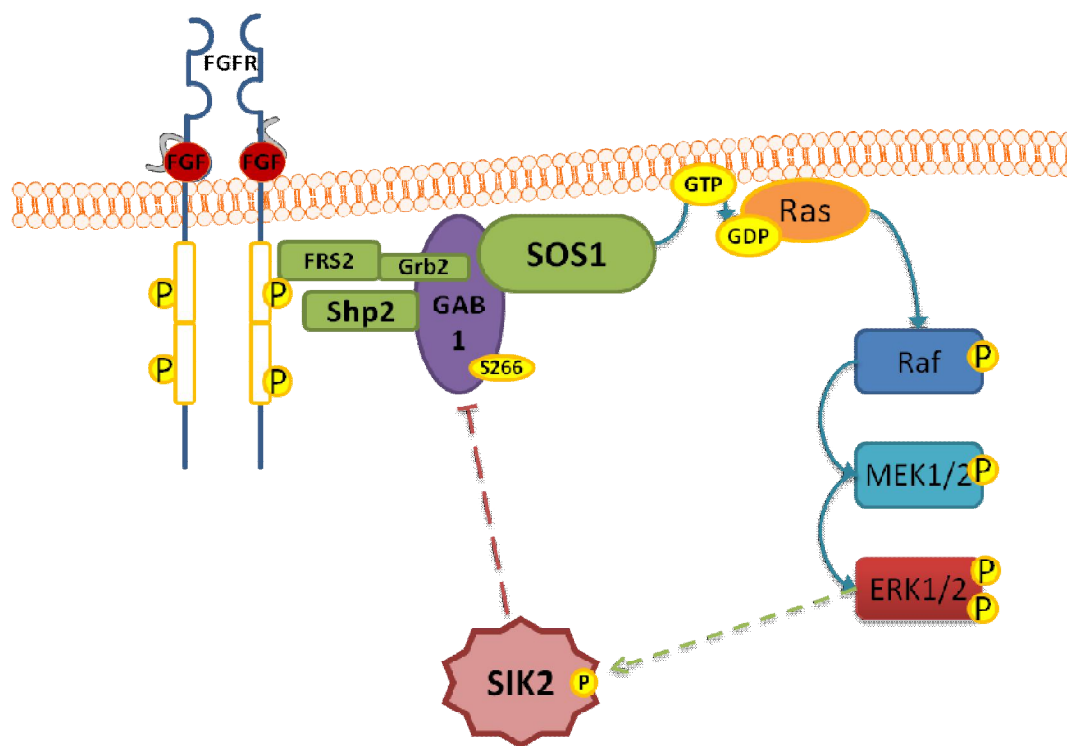


Figure 1.7. Model for involvement of SIK2 in a negative feedback mechanism regulating FGF-dependent MAPK pathway.

2. PURPOSE

The working hypothesis of our laboratory suggests that SIK2 is a part of a negative feedback mechanism acting on the FGF-dependent MAPK pathway, via directly activation by ERK and phosphorylating the docking protein Gab1 on S266.

To verify and further support this hypothesis it was aimed to:

- Verify the presence of FRS2 and SOS1 in the signaling complex that SIK2 is interacting with upon FGF2 stimulation, by Co-IP experiments.
- Generate mutants of SIK2 for the predicted ERK phosphorylation sites.
- Test these mutants by in vitro kinase assays for phosphorylation by ERK.
- Analyze SIK2 with mass spectrometry to find potential ERK phosphorylation sites.
- Generate bacterial expression vectors for SIK2 and Gab1 to further study the interaction between these proteins.

3. MATERIALS

3.1. Cell Lines

Spontaneously immortalized human Müller glia cell line (MIO-M1) was kindly provided by Professor Astrid Limb from University College London, Institute of Ophthalmology, London, United Kingdom. Human embryonic kidney 297T cells (HEK 297T) were kindly provided by Professor Nesrin Özören from Boğaziçi University, İstanbul, Turkey.

3.2. Chemicals, Plastic and Glassware

All chemicals used in this study were purchased from Sigma-Aldrich (USA), AppliChem (Germany) and Merck (Germany). Plastics from TPP (Switzerland) and Capp (Denmark); and tips and tubes from Axygen (USA) were used throughout the study. Glassware, tips and tubes were sterilized by autoclaving at 121 °C for 20 min before use.

3.3. Buffers and Solutions

Buffers and solutions used in this study and their compositions are given in Table 3.1 through Table 3.7. Bacterial culture mediums were used after sterilization by autoclaving at 121 °C for 20 min.

3.3.1. Bacterial Culture

Table 3.1. Materials used in bacterial culture.

LB Medium	1% Tryptone 1% NaCl 0.5% Yeast Extract
LB Agar	LB Medium 1.5% Agar

Table 3.1. Materials used in bacterial culture (cont.).

Terrific Broth	1.2% Tryptone 2.4% Yeast Extract 0.8% Glycerol Solution 10% Potassium Phosphate Solution (added after sterilization)
Potassium Phosphate Solution	170 mM KH ₂ PO ₄ 720 mM K ₂ HPO ₄
Glycerol Solution	50% Glycerol in dH ₂ O
SOC Medium	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 20 mM MgSO ₄ 0.4% Glucose (added after sterilization)

Table 3.2. List of the antibiotics used and their working concentrations.

Ampicillin (1000X) (Merck, Germany)	50 mg/ml in dH ₂ O
Kanamycin (1000X) (Fisher Scientific, USA)	30 mg/ml in dH ₂ O
Chloramphenicol (1000X) (Sigma, USA)	34 mg/ml in EtOH
Gentamicin (1000X) (Sigma, USA)	10 mg/ml in dH ₂ O

3.3.2. Cell Culture

Table 3.3. Materials used in cell culture.

Dulbecco's Modified Eagle Medium (DMEM)	Gibco Invitrogen, USA
Fetal Bovine Serum (FBS)	Gibco Invitrogen, USA
Penicillin/Streptomycin	Gibco Invitrogen, USA
MEM Non-Essential Amino Acids (NEAA) 100X	PAN-Biotech, Germany
10X Trypsin-EDTA Solution	2.5% Trypsin 7 mM EDTA 0.9% NaCl

Table 3.3. Materials used in cell culture (cont.).

2X HBS (HEPES-buffered saline)	50 mM HEPES 280 mM NaCl 1.5 mM Na ₂ HPO ₄ (pH 7.05)
DMSO	AppliChem, Germany
PBS 10X	Gibco Invitrogen, USA

3.3.3. Protein Isolation, Immunoprecipitation and *In Vitro* Kinase Assay

Table 3.4. Materials for protein isolation, immunoprecipitation and *in vitro* kinase assay.

Immunoprecipitation (IP) Lysis Buffer	50 mM Tris-HCl (pH 8.0) 150 mM NaCl 1% NP40
Co-immunoprecipitation (Co-IP) Lysis Buffer	20 mM Tris-HCl (pH8.0) 125 mM NaCl 0.5% NP-40 2 mM Na ₂ EDTA
Bacterial Cell Lysis Buffer	50 mM Tris-HCL (pH8.0) 150 mM NaCl 0.5% NP-40
2X Kinase Buffer	25 mM HEPES 150 mM NaCL 10 mM MgCl ₂ 2 mM MnCl ₂
Mg/Mn ATP Cocktail	100 µM ATP 75 mM MnCl ₂ 75 mM MgCl ₂ in Kinase buffer
Protein A Agarose Beads	Roche, Germany
Protease Inhibitor Cocktail	Roche, Germany
Phosphatase Inhibitor Solution	Roche, Germany
PQ Paper Developer	Ilford, UK
Rapid Fixer	Ilford, UK

3.3.4. SDS-PAGE and Western Blotting

Table 3.5. Materials used in SDS-PAGE and western blot.

10% SDS-Polyacrylamide Gel (Running Gel)	10% Acrylamide:Bisacrylamide (37.5:1) 375 mM Tris-HCl (pH 8.8) 0.1% Sodium Dodecyl Sulfate (SDS) 0.1% Ammonium Persulfate (APS) 0.1% Tetramethylethylenediamine (TEMED)
8% SDS-Polyacrylamide Gel (Running Gel)	8% Acrylamide:Bisacrylamide (37.5:1) 375 mM Tris-HCl (pH 8.8) 0.1% SDS 0.1% APS 0.1% TEMED
5% SDS-Polyacrylamide Gel (Stacking Gel)	5% Acrylamide:Bisacrylamide (37.5:1) 125 mM Tris-HCl (pH 6.8) 0.1% SDS 0.1% APS 0.1% TEMED
6X Protein Sample Buffer	300 mM Tris-HCl (pH 6.8) 12 mM EDTA 60% glycerol 12% SDS 6% β -mercaptoethanol 0.04% BPB
Running Buffer	25 mM Tris-HCl 250 mM Glycine 0.2% SDS
Transfer Buffer	25 mM Tris-HCl 200mM Glycine 15% Methanol
10X TBS	1 M Tris-Cl (pH 7.5) 1.5 M NaCl
TBS Tween (TBS-T)	0.1% Tween-20 in TBS
Sodium Dodecyl Sulfate (SDS)	10% solution

Table 3.5. Materials used in SDS-PAGE and western blot (cont.).

Ammonium Persulfate (APS)	10% solution
Stripping Solution	62.5 mM Tris-HCl (pH 6.8) 2% SDS 0.7% β -mercaptoethanol
PageRuler™ Prestained Protein Ladder	Thermo Scientific, USA
Polyvinyl Difluoride (PVDF) Membrane	Roche, Germany
Methanol	Sigma-Aldrich, USA
Bovine Serum Albumin Fraction (BSA)	AppliChem, Germany
Skim Milk Powder (MP)	Sigma-Aldrich, USA
SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Scientific, USA
ImmunoCruz Western Blotting Luminol Reagent	Santa Cruz Biotechnology, USA

3.3.5. Molecular Cloning and Site-Directed Mutagenesis

Table 3.6. Materials used in cloning and site-directed mutagenesis reactions.

Restriction Enzymes	New England Biolabs, USA
T4 DNA Ligase	New England Biolabs, USA
Q5 High Fidelity DNA Polymerase	New England Biolabs, USA
T4 Polynucleotide Kinase (PNK)	New England Biolabs, USA

3.3.6. Agarose Gel Electrophoresis

Table 3.7. Materials used in agarose gel electrophoresis.

50X Tris Acetic Acid EDTA (TAE) Buffer	2 M Tris 1 M Acetate 50 mM EDTA
6X DNA Gel Loading Dye	Thermo Scientific, USA
1 kb DNA Ladder	Thermo Scientific, USA
Ethidium Bromide (EtBr)	Merck, Germany

3.4. Fine Chemicals

3.4.1. Primers

Primers used in this study were synthesized at Macrogen Europe (The Netherlands).

Table 3.8. List of primers used for site-directed mutagenesis of SIK2.

Primer Name	Mutagenesis	Primer Sequence (5'-3')	Tm (°C)
New_SIK2_T758A_Fw	T763A	CCGCCAGGAGGCTCCACCGCC	73.1
New_SIK2_T758A_Rev	T763A	GGAAGGGGCAGCTGCTGACTTG	67.7
New_SIK2_T863A_Fw	T863A	AGACTATCCCGCTCCCTGTCAGT ATCCTG	73.3
New_SIK2_T863A_Rev	T863A	GGCGCAGGGGAAGCAGCG	65.3

Table 3.9. List of primers used in gateway cloning of Gab1 and SIK2.

Primer Name	Primer Sequence (5'-3')	Tm (°C)
Gab1_Gw_For	AAAAAGCAGGCTTCGAAGGAGATAGAACCAT GA GCGGTGGTGAA	79.9
Gab1_Gw_Rev	AGAAAGCTGGGTCTTAAACCTTATCGTCGTCA TCCTTG	75.7
Gway_FL-SIK2_Fw	AAAAAGCAGGCTTCGAAGGAGATAGAACCAT GGTCATGGCGGATGG	81.6
Gway_FL-SIK2_Rev	AGAAAGCTGGGTCATTCACCAGGACATACCCG TT	75.3
Gway_2piecesSIK2_Fw	AAAAAGCAGGCTTCGAAGGAGATAGAACCAT GGTTGCCAAGGCACAGACT	82.2
Gway_2piecesSIK2_Rev	AGAAAGCTGGGTCCTGTGCCTTGGCAACTGTT	75.1
Gway_2piecesSIK2_Fw	AAAAAGCAGGCTTCGAAGGAGATAGAACCAT GGTTGCCAAGGCACAGACT	82.2
Gway_2piecesSIK2_Rev	AGAAAGCTGGGTCCTGTGCCTTGGCAACTGTT	75.1

Table 3.9. List of primers used in gateway cloning of Gab1 and SIK2 (cont.).

attB1_adapter_primer	GGGGACAAGTTTGTACAAAAAAGCAGGCT	69.0
attB2_adapter_primer	GGGGACCACTTTGTACAAGAAAGCTGGGT	71.9

Table 3.10. List of primers used for sequencing.

Primer Name	Primer Sequence (5'-3')	Tm (°C)
Gab1_human_for	AGAGCAAACATGGAATGAATGGC	61.1
Gab1_human_rev	CGATGTCCCAGATGGGGTATTA	62.1
Gab1_for2_human	ATTCCAGCAGTTTTACAGAACCAA	60.1
Gab1_rev2_human	TCACAGTCTGCAACAGGAACT	59.4
GFP-SIK2_for	ATCACATGGTCCTGCTGGAGTT	62.1
SIK2_human_for	CTGGACATCTGGAGTATGGG	60.5
SIK2_human_rev	GAATCTTCCTTCCAGAACCCT	59.4
SIK2_For2_human	TATGATATGGGGTCTGTTCAGAG	61.1
SIK2_For3_human	CTCTTTCTTCAGAAGCAGTCTCAA	61.8
SIK2_Rev2_human	CTGAAGATGTTGTCTAAATGCTACAA	61.6
T7promoter	TAATACGACTCACTATAGGG	45.6
SV40-pArev	CCTCTACAAATGTGGTATGG	51.5
CMV-F	CGCAAATGGGCGGTAGGCGTG	72.0

3.4.2. Plasmids

Table 3.11. List of plasmids used in this study.

pCMV6-Entry Myc-DDK Gab1	Origene, USA
pEGFP-C3 SIK2	Retina Lab
pEGFP-C3 KI SIK2 (K49M)	Retina Lab
pET-DEST42	Invitrogen, USA
pDEST 14	Invitrogen, USA
pDONR 207	Plant Lab, Boğaziçi University
pEGFP-C3	Retina Lab

3.4.3. Antibodies

Table 3.12. List of antibodies used in this study.

Antibody	Host	Application	WB Dilution	Blocking Solution	Company
SIK2	Rabbit	WB	1/1000	5% BSA in TBST	Cell Signaling
GFP	Rabbit	IP, WB	1/2500	5% MP in TBST	Abcam
β -Actin-HRP	Mouse	WB	1/1000	1% MP in TBST	Santa Cruz
Gab1	Rabbit	WB	1/1000	1% MP in TBST	Santa Cruz
Gab1	Rabbit	IP, WB	1/1000	5% BSA in TBST	Abcam
Myc-Tag	Mouse	WB	1/1000	5% MP in TBST	Cell Signaling
DYDDDDK Tag Antibody (Anti-FLAG)	Rabbit	IP, WB	1/1000	5% BSA in TBST	Cell Signaling
His probe	Mouse	IP, WB	1/1000	5% BSA in TBST	Santa Cruz
Normal rabbit IgG	Rabbit	IP	-	-	Cell Signaling
FRS2	Rabbit	WB	1/1000	1% MP in TBST	Santa Cruz
SOS1	Rabbit	WB	1/1000	3% MP in TBST	Upstate
Grb2	Mouse	WB	1/1000	3% MP in PBS	Upstate
Sh-ptp2 (Shp2)	Rabbit	WB	1/1000	1% MP in TBST	Santa Cruz
Phosphothreonine	Rabbit	WB	1/1000	5% BSA in TBST	Invitrogen

Table 3.12. List of antibodies used in this study (cont.).

Anti-rabbit HRP	Goat	WB	1/5000	Blocking solution of primary antibody	Santa Cruz
Anti-mouse HRP	Goat	WB	1/5000	Blocking solution of primary antibody	Santa Cruz

3.5. Kits

Table 3.13. List of kits used in this thesis.

Pierce™ BCA Protein Assay Kit	Thermo Scientific, USA
Universal Kinase Activity Kit	R&D Systems, USA
Zyppy Plasmid Miniprep Kit	Zymo Research, USA
DNA Clean & Concentrator™ -5	Zymo Research, USA
Zymoclean™ Gel DNA Recovery Kit	Zymo Research, USA
Nucleobond® Xtra Maxi	Macherey-Nagel, Germany
QIAGEN® Plasmid Midi Kit	Qiagen, USA
QIAGEN® Plasmid Maxi Kit	Qiagen, USA

3.6. Equipment

Table 3.14. List of equipments used in this thesis.

Equipment	Brand and Model
Autoclave	CV-EL 12L Tabletop, CertoClav, Austria Midas 55, Priorclave, UK Model ASB260T, Astell, UK Swiftlock SecureTouch Colour, Astell, UK
Balances	ME54 Analytical Balance, Mettler Toledo, Sweden UW6200H Electronic Balance, Shimadzu, Japan
Carbon-dioxide (CO ₂) Tank	Genç Karbon, Turkey

Table 3.14. List of equipments used in this thesis (cont.).

Centrifuges	Allegra X-22R Centrifuge, Beckman Coulter, USA B5 Centrifuge B. Braun Biotech, Germany Centrifuge 5415R, Eppendorf, USA J2-21 Centrifuge, Beckman Coulter, USA Mini Centrifuge 17307-05, Cole Parmer, USA MiniSpin Plus, Eppendorf, USA
Documentation Systems	Chromato-Vue Transilluminator, UVP, USA G-Box, Syngene, UK Gel Doc XR System, Bio-Rad, USA Stella, Raytest, Germany
Electrophoresis Equipment	Owl™ EasyCast™ B1A, Thermo Fisher Scientific, USA Mini Protean 3 Cell, Bio-Rad, USA Mini Protean Tetra Cell, Bio-Rad, USA
Freezers	-152°C Ultra Low Freezer, Sanyo, Japan 2021D deep-freezer (-20°C), Arçelik, Turkey MDF-135 Medical Freezer (-35°C), Sanyo, Japan Forma ULT Freezer(-86°C), Thermo Fisher Scientific, USA
Haemocytometer	Improved Neubauer, , Weber Scientific, UK
Heat Block	DRI-Block DB-2A, Techne, UK
Heating Magnetic Stirrers	MS-H-S, Scilogex, USA RCT Basic, Ika, Germany
Hybridization Oven	Shake n' Stack, Hybaid, UK
Ice Machine	Chip Type Ice Flaker, Brema, Italy
Incubators	Forma Orbital Shaker Incubator Model 420, Thermo Fisher Scientific, USA Forma Series II Water Jacket CO ² Incubator, Thermo Fisher Scientific, USA
Laminar Flow Cabinet	Class II A Tezsan, Turkey Class II B Tezsan, Turkey
Micropipettes	Gilson, France
Microscopes	Axio Observer Z1 Inverted Microscope, Zeiss, USA CKX41 Inverted Microscope, Olympus Life Science, UK
Microwave Oven	MD55I Arçelik, Turkey
pH meter	HI-83141, Hannah Instruments, UK

Table 3.14. List of equipments used in this thesis (cont.).

Pipette Controller	Pipetting Aid, Gilson, USA
Plate Reader	VersaMax, Molecular Devices, USA
Power Supply	Power Pac Basic, Bio-Rad, USA
Refrigerators	2082C, Arçelik, Turkey 4030T, Arçelik, Turkey
Shakers	Adjustable Rocker Shaker Single Platform, Cole-Parmer, USA
Sonicator	HD 2070 Sonopuls, Bandelin, Germany
Spectrophotometers	NanoDrop ND-1000, Thermo, USA
Thermocyclers	C1000 Thermal Cycler, Bio-Rad, USA Peltier-Based Thermal Cycler SCM 96G, Sacem Life Technologies, Turkey TC-312 Thermal Cycler, Techne, UK
Vortex	Vortex Mixer VM20, Chiltern Scientific, UK
Water Bath	Precision™, Thermo Fisher Scientific, USA
Water Purification System	Ultra Pure Water Purification System, WA-TECH, Germany

4. METHODS

4.1. Site-Directed Mutagenesis

Site-directed mutagenesis experiments were performed in this study to create single base substitutions on the template plasmid, kinase inactive SIK2 DNA (pEGFP C3 KI SIK2).

4.1.1. Primer Design

The primer pairs were designed so that 5' ends of the two primers could anneal back-to-back and only the forward primer carried the desired mutation in the center of the primer. Lengths of the primers were between 30-35 bases and at least 10 complementary nucleotides were left on both sides of the mutations.

4.1.2. 5'-Phosphorylation of Primers

Primers to be used in mutagenesis PCR were phosphorylated at their 5' ends prior to use. The phosphorylation reaction was carried out in T4 ligase buffer and included 300 pmol of the oligonucleotides, 1 U of T4 PNK in a final volume of 50 μ L. The reaction was allowed to proceed at 37°C for 30 min. T4 PNK was inactivated by incubating the reaction mixture at 65°C for 20 min. The reaction products were either used directly in the mutagenesis PCR reaction or stored at -20°C.

4.1.3. Generation of Mutation

Mutations were generated using PCR-based mutagenesis technique. The PCR mix composed of ~25 ng template plasmid, 0.5 μ M of each 5' phosphorylated primer, 0.2 mM dNTP mix (from 10mM stock), 10 μ L 5X Q5 DNA Polymerase Buffer and dH₂O to a final volume of 50 μ L. Finally, 1 U Q5 High-Fidelity DNA Polymerase was added and each reaction was cycled using the parameters given in Table 4.1 below.

Table 4.1. The PCR cycle used in mutagenesis reactions.

Step	Cycle	Temperature	Time
1	1	98°C	1 minute
2	18	98°C	15 seconds
		72°C	3 minutes (for ~7400 bp extension)
3	1	72°C	5 minutes

Following the PCR reaction, 5 μ L of each reaction was run on an agarose gel to check the PCR products and the rest of the samples were digested by using 1 μ L of Dpn I enzyme at 37°C for 1 hour in order to remove the parental DNA. The PCR products were isolated using DNA Clean & Concentrator Kit (Zymo Research, USA) and eluted in 10 μ L of elution buffer. Four μ L of the purified products were subjected to overnight ligation at room temperature. Following the ligation, 5 μ L of the products were transformed into 50 μ L of TOP10 competent cells. The cells were spread on LB agar plates containing the appropriate antibiotic and grown overnight at 37°C.

Positive colonies were selected by colony PCR followed by gel electrophoresis. Plasmids isolated from the selected colonies were sent for sequencing to verify the mutation.

4.2. Gateway Cloning

Gateway[®] cloning system (Invitrogen, USA) was used in this study to produce bacterial expression vectors for full length Gab1 and different SIK2 constructs (1-1120 WT, 1-1120 KI and 1060-2791 WT). In all cloning experiments, pDONRTM207 plasmid was used as the donor vector to produce the entry clones. Two vectors that contain T7 lac promoters for high-level and IPTG-inducible expression of the gene of interest were used as destination vectors: namely pDESTTM14 and pET-DEST42. The latter includes a C-terminal 6xHis tag, while the former has no tag.

4.2.1. Adapter Sequence Addition

To be able to clone a desired DNA fragment via Gateway[®] cloning, the attB1 and attB2 adapter sequences are needed to be incorporated at the ends of the fragment by PCR. Since, attB1 and attB2 adapters are too long to be added in one PCR reaction, two sequential PCR reactions were performed. Primers to be used for this purpose were designed according to the instructions in Gateway[®] Technology user guide.

The first PCR was prepared using primer pairs that have first 12 bp of the attB1 and attB2 adapters (Table 3.9), together with the gene specific regions. The forward primers also contained the Shine-Dalgarno and Kozak consensus sequences to allow protein expression. In the first step annealing temperature was calculated for just the gene specific region. The cycle used in the primary PCR is given in the Table 4.2 below. The primary PCR product was run on 0.8% agarose gel and gel-extracted to be used in secondary PCR.

In the second PCR, attB1 and attB2 specific primers were used and the annealing temperature was calculated for the whole primer sequence.

Table 4.2. The cycle used for primary PCR in Gateway[®] cloning.

Step	Cycle	Temperature	Time
1	1	98°C	30 seconds
2	6	98°C	10 seconds
		66°C	30 seconds
		72°C	1 minute
3	24	98°C	30 seconds
		72°C	1 minute
4	1	72°C	2 minute

The secondary PCR reaction was cycled using the cycling parameters given in Table 4.3. The PCR product was again gel-extracted after running on 0.8% agarose gel, to be used in BP cloning reaction. Agarose gel extractions were performed using the Zymoclean Gel DNA Recovery Kit (Zymo Research, USA).

Table 4.3. The cycle used for secondary PCR in Gateway® cloning.

Step	Cycle	Temperature	Time
1	1	98°C	30 seconds
2	6	98°C	10 seconds
		45°C	30 seconds
		72°C	1 minute
3	24	98°C	30 seconds
		72°C	1 minute
4	1	72°C	2 minute

4.2.2. Vector Insertion with BP and LR Cloning

The PCR product flanked by adapter attB sites was inserted in to the donor vector pDONR207 containing attP1 and attP2 sites using BP clonase enzyme. Transfer of the insert fragment from the donor vector (called entry vector after BP cloning) to destination vectors that carry attR sites was conducted via LR clonase. The clonase enzymes recognize specific attachment sites (attB and attP or attL and attR) and allow recombination between them, switching the flanking DNA fragments while securing the direction of the fragment. A basic schematic representation of Gateway® cloning is given in Figure 4.1 below.

Both BP and LR cloning reactions were carried out in 10 µl total volume according to the instructions in Gateway® Technology user guide. After 1 hour incubation at 25° C, reactions were halted by addition of 2 µl of Proteinase K solution, followed by 10 minutes incubation at 37° C. Generated recombinant vectors were transformed into TOP10 competent *E. coli*. Positive clones were selected first by using appropriate antibiotic, and then by colony PCR and DNA sequencing.

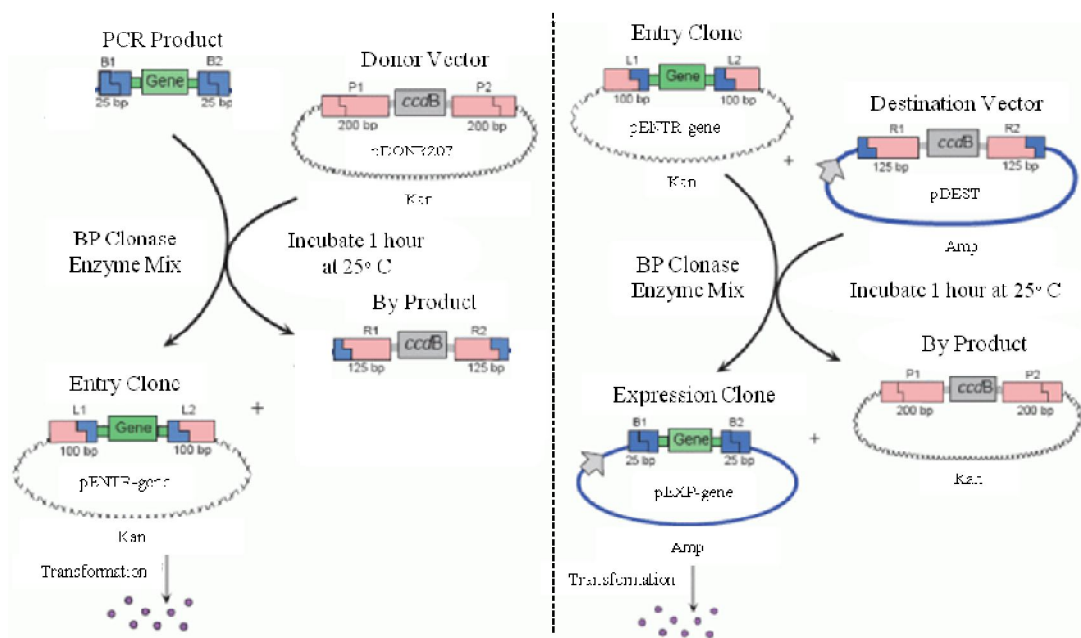


Figure 4.1. Gateway[®] cloning system diagram (modified from bioinforx.com).

4.3. Bacterial Culture

4.3.1. Bacterial Transformation and Plasmid Isolation

For bacterial transformation, 50 µl of TOP10 chemically competent cells were gently mixed with 10 to 50 ng of a plasmid or 5 to 10 µl of a ligation mix, and placed on ice for 30 minutes. Cells were then heat shocked by placing into a 42°C water bath for 90 seconds and taking back on ice for 5 minutes. Next, 500 µl of SOC medium was added on the cells, and the cells were incubated at 37°C for 1 hour with rotation for recovery. After recovery, 50 to 100 µl of grown bacteria was spread on 10 cm LB agar plate containing the appropriate antibiotic and incubated at 37°C overnight. Individual colonies were picked from the LB plate and cultured in 5 ml liquid LB medium (with appropriate antibiotic) overnight at 37°C with rotation. Plasmid DNAs were isolated using the Zippy Plasmid Miniprep Kit and were stored at -20°C.

4.3.2. Colony PCR

Colony PCR was used in this work to be able to select positive bacterial colonies from overnight cultured plates following site-directed mutagenesis and Gateway cloning experiments. Selected colonies were picked with a pipette tip and the tips were dipped into microcentrifuge tubes containing 10 μ l of distilled water, to be used as templates in the PCR reaction; then the tips were placed in tubes with 5 ml liquid LB solution for bacterial growth.

Following the PCR reaction, 20 μ l of PCR products were run on 1% agarose gel for the detection of positive colonies. The ones that yield expected sized products were selected and the corresponding samples in LB solutions were incubated at 37°C overnight.

4.3.3. Bacterial Stock Preparation

To prepare glycerol stocks of each selected positive clone from cloning experiments, 500 μ l of the overnight culture aliquots in liquid LB were gently mixed with 500 μ l of 50% glycerol in a 2 ml microcentrifuge tube. The glycerol stock was frozen and stored at -80 °C.

4.3.4. Recombinant Protein Expression in E.Coli

A starter culture was grown overnight by inoculating bacteria from a Rosetta DE3 strain *E.coli* glycerol stock that was constructed after gateway cloning with a micropipette tip, in 5 ml of LB medium containing appropriate antibiotic. Three ml of overnight grown culture was transferred into an erlenmeyer flask that contains fresh 150 ml TB (Terrific Broth) with the appropriate antibiotic (1/50 starter culture to medium ratio). The culture was grown at 37°C with rotation, while checking OD₆₀₀ every 20-30 minutes. IPTG induction was done when OD₆₀₀ of the culture reached the 0.6-0.8 interval, by adding 1 mM IPTG to the culture flask. After 4 hours of induction, bacteria were collected by centrifugation at 5000 g for 10 min and then either directly subjected to cell lysis or stored at -80 °C.

4.3.5. Bacterial Cell Lysate Preparation

Bacterial cell pellet was dissolved in lysis buffer (4ml buffer per 1 gr of pellet). The suspension was sonicated 10 times for 30 seconds each at 90% power amplitude on ice, with 20-30 second pauses in between to prevent heating. Sonicated sample was centrifuged at 10000g and 4 °C for 30 minutes; the pellet was again dissolved in lysis buffer and sonicated as described before. Concentrations of both the supernatants and the pellet suspensions were measured by using Pierce BCA Protein Assay Kit and recombinant protein expression was checked by SDS-PAGE and western blotting. Samples were stored at -80 °C.

4.4. Eukaryotic Cell Culture

4.4.1. Maintenance of MIO-M1 and HEK 293T Cells

MIO-M1 cells were maintained in DMEM with 10% FBS and 0.1% Pen/Str. HEK 293T cells were maintained in DMEM with 10% FBS, 1% NAEE and 0.1% Pen/Str. All cells were grown in the 37°C incubator with 5% CO₂ and were split or frozen as a stock when they reached high confluency. For splitting, cells were washed with PBS, treated with trypsin-EDTA solution for 5min at 37°C and scraped. Scraped cells were collected by centrifugation at 500g for 5 min, resuspended with their growth medium and divided into 3 plates. For stock preparation, MIO-M1 cell pellets were resuspended with 1 ml of their freezing medium consisting of 70% DMEM, 20% FBS and 10% DMSO; and HEK 293T cell pellets were resuspended with their freezing medium consisting of 50% DMEM, 40% FBS and 10% DMSO. Resuspended pellets were kept at -20 °C overnight, and transferred to -80 °C the day after for long term storage.

4.4.2. CaPO₄ Transfection of MIO-M1 and HEK 293T Cells

The day before transfection, 5x10⁶ log-phase cells were seeded on a 10 cm culture dish and incubated overnight under normal growth conditions. Right before the transfection, cells were replenished with fresh growth medium with 25 µM chloroquine. In a microcentrifuge tube 10 µg of desired plasmid was diluted with dH₂O to a final volume

of 438 μ l and 62 μ l 2M CaCl_2 was added. Subsequently, 500 μ l 2X HBS solution was added to the mixture in a drop-wise fashion and mixed by pipetting thoroughly. The mixture was incubated at room temperature for 10 min to allow DNA/calcium phosphate co-precipitate. After incubation, the mixture was added onto the cells by dripping slowly and evenly, and cells were incubated at normal growth conditions. The medium was replaced with fresh growth medium after 8 h, to remove chloroquine. GFP expression, if used as a control or fusion tag, was detected after 24 h of transfection. Cells were collected after 24-48 h of transfection for protein extraction.

4.4.3. FGF2 Treatment of MIO-M1 Cells

Cells to be treated with FGF2 were washed with PBS and starved overnight in starvation medium (DMEM with 0.1% pen/str). If the treatment was to be applied on transfected cells, starvation was started at 32 h after transfection. Right before treatment, cells were washed with PBS. The cells were incubated with DMEM containing 1 ng/ml FGF2 and 100 μ g/ml heparin for 10 min at 37 °C. PBS was used instead of FGF2 for the control samples. After 10 minutes of treatment, cells were washed with ice-cold PBS and scraped off the plate using a scraper in ice-cold PBS containing protease and phosphatase inhibitor cocktails. Cell suspensions were collected into cold centrifuge tubes and pelleted by centrifugation. Cell pellets were either used directly or stored at -80 °C for later use.

4.5. Protein Extraction from Eukaryotic Cells

Pelleted cells were resuspended with appropriate ice-cold lysis buffer (Table 3.4) containing protease and phosphatase inhibitor cocktails. The cell suspension was sonicated six times for five seconds on ice and incubated again on ice for 1 h on a rocking platform. Cell debris was removed by centrifuging the suspension for 30 min at 13,200 rpm at 4 °C. The supernatant was kept as the cell lysate and transferred into a fresh tube, while the pellet was discarded. The protein concentration was measured using Pierce BCA Protein Assay Kit.

4.6. Immunoprecipitation

For the immunoprecipitation procedure, protein A agarose beads were equilibrated before use by washing three times with IP lysis buffer containing protease and phosphatase inhibitor cocktails. For pre-clearing; cell lysate was containing 1 mg protein was incubated on ice for 1 hour on a rocking platform with 30 μ l of protein A agarose beads only; in order to eliminate non-specific binding to beads. The mixture was centrifuged and the cell lysate was collected in fresh microcentrifuge tube, while the beads were discarded.

Next, 40 μ l of washed protein A agarose beads were incubated with 1 μ g of specific antibody in IP lysis buffer for 4 hours at 4 °C. After the incubation, the antibody-bead mixture was centrifuged and the supernatant was discarded. The pre-cleared lysate was added on the bead-antibody complex and incubated at 4 °C for 2 hours. Beads were collected by centrifugation at the end of incubation and washed with IP lysis buffer 3 times at 4 °C. For western blot analysis, beads were mixed with equal volume of 2X SDS protein sample buffer and boiled at 95 °C to elute immunoprecipitated protein.

4.7. Co-immunoprecipitation

For co-immunoprecipitation (Co-IP) experiment, protein extraction was performed as described before with the exception of using the Co-IP lysis buffer instead of IP lysis buffer and excluding the sonication step.

4.8. In Vitro Kinase Assay

Twenty μ l of agarose the beads of the immunoprecipitated protein was used as the substrate in the in vitro kinase assays. Beads were washed with 200 μ l kinase buffer twice. The reaction was set up on agarose beads using the kinase buffer, 5 μ l ATP cocktail containing 100 μ M ATP and 1 μ Ci [γ ³²P] ATP (3000 Ci/mmol) and 0.1 μ g recombinant active ERK2 (R&D Systems, USA) in a total volume of 25 μ l. The kinase reaction was run at 30 °C for 30 minutes.

At the end of 30 minutes, the reaction was terminated by adding 6X SDS sample buffer. The IVK samples were boiled at 95 °C for 5 minutes and then they were centrifuged. After centrifugation, the beads were discarded and reaction mixtures were run in 10% SDS-PAGE. The gel was first rinsed with water and then fixed in fixation solution for 15 min. The fixed gel was then dried at 80 °C for 1 hour under vacuum. The dried gel was exposed to X-ray film (Amersham, USA) for varying times at -80 °C. The film was immersed in the developer solution (Ilford, UK) for 1-2 minutes until bands were detected and then washed with distilled water and fixed in fixation solution (Ilford, UK) for 5 minutes. X-ray film was scanned for analysis.

4.9. Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For SDS-PAGE analysis, protein samples were mixed with SDS sample buffer and boiled at 95 °C for 5 minutes. The denatured protein samples were loaded into the wells of the polyacrylamide gel. The electrophoresis was run at 80 V until samples entered the resolving gel and then the voltage was set to 110 V. In order to apply coomassie blue staining after electrophoresis, the polyacrylamide gel was first washed distilled water and then incubated in coomassie fixation solution that consists of 40% methanol and 10% acetic acid for 1 hour on a rocking platform. Next, the gel was incubated in staining solution for 30 minutes, which was prepared by dissolving 0.005% coomassie brilliant blue R250 in fixation solution. Finally, the gel was washed with washing buffer, which contains 10% methanol and 7% acetic acid, until the protein bands were clear enough. The washing buffer was changed occasionally with fresh buffer throughout the washing process.

4.10. Western Blot

For western blotting, samples resolved on polyacrylamide gels were electro-blotted to PVDF membranes. The transfer was performed at 100 V in transfer buffer where an ice pack placed and the stirred constantly, for a time period that depends on the size of the protein to be transferred (e.g. ~100 min for a 100 kDa protein).

After the transfer, the membrane was washed with TBS-T for three times for five minutes each, and blocked for one hour at room temperature on a rocking platform, using a

blocking solution depending on the antibody being used (Table 3.13). After blocking, the membrane was incubated with the appropriate primary antibody solution at dilutions indicated at Table 3.13 (the antibody diluted in the blocking solution) on a shaker at 4°C overnight. The next day, unbound antibodies were removed by washing the membrane with TBS-T for three times, for five minutes each. The membrane was incubated next with the appropriate HRP-tagged secondary antibody solution for one hour at room-temperature, and at the end was washed three times with TBS-T, each for 5 minutes.

ImmunoCruz Western Blotting Reagent (Santa Cruz, USA) or SuperSignal West Femto Maximum Sensitivity Substrate was used for band detection. Luminol mixture was applied on the membrane and the membrane was visualized on the imaging system Stella.

4.11. Preparation of Samples for Mass Spectrometry Analysis

5x10⁶ HEK 293T cells seeded onto 10 cm plates were transfected with pEGFP-C3 KI-SIK2 and harvested 24h after transfection. For the preparation of each mass spectrometry (MS) sample, cells from five 10 cm plates were pooled. KI-SIK2 was isolated from the cell lysates by immunoprecipitation using 30 µl protein agarose beads and the α -GFP antibody. Transfections, the lysate preparation and the immunoprecipitation were performed as described before.

The immunoprecipitated beads were washed two times with 100 µl kinase buffer. In vitro kinase reaction was prepared that includes the 30 µl of immunoprecipitated KI-SIK2 beads, 200 µM ATP, 0.1 µg recombinant active ERK2 (R&D Systems, USA) that were mixed with the kinase buffer in 50 µl total volume. The reaction was allowed to proceed at 30 °C for 30 minutes. The beads were next washed with 100 µl of the kinase buffer two times and sent to the MS analysis.

5. RESULTS

5.1. Interaction of SIK2 with FGF2 Signaling Complex Proteins

In order to check the interactions between SIK2 and the critical components of the signaling complex in FGF2-dependent MAPK pathway, Co-IP experiments were carried out in Müller Cells. SIK2 was probed from 10 minutes FGF2-treated and untreated MIO-M1 cells and the Co-IP samples were analyzed by western blot.

Result of the western blot analysis is given in Figure 5.1. Immunoprecipitated SIK2 signal is detected around 120 kDa in both samples (Figure 5.1a). In western blots of the same Co-IP samples, FGF signaling complex proteins SOS1, Gab1, Frs2, Shp2 and Grb2 were also detected, with their specific antibodies (Figure 5.1b-f). With FGF2 treatment, there was an increase in the interaction of SIK2 with SOS1, Shp2 and Grb2; while Gab1 and FRS2 levels did not seem to change in an FGF2-dependent manner.

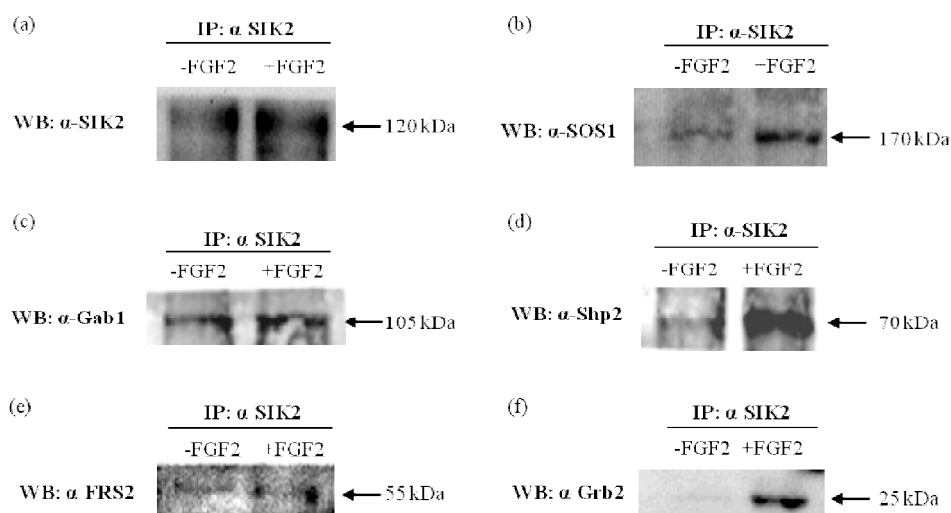


Figure 5.1. Association of SIK2 with the critical components of the FGF2-dependent MAPK pathway. SIK2 was immunoprecipitated by using α-SIK2 antibody from lysates of MIO-M1 cells treated with FGF2 for 10 minutes or left untreated. The IP samples were fractioned on SDS-PAGE. Presence of SIK2 (a), SOS1 (b), Gab1 (c), Shp2 (d), FRS2 (e) and Grb2 (f) were analyzed by western blotting using the specific antibodies.

These results confirm the previous findings of our lab indicating the presence of SIK2 with the Gab1, Grb2 and Shp2 in the same signaling complex (Küser, 2011). Our results also revealed that FRS2 and SOS1 are parts of the same signalosome with SIK2, and supports the proposed involvement of SIK2 modulation of Ras/MAPK arm of the FGF pathway (Küser, 2011).

5.2. Identification of the ERK2-dependent Phosphorylation Sites on SIK2

Our previous studies suggested ERK as an activatory kinase of SIK2 (Ejder, 2011). Therefore, we aimed to examine ERK-dependent phosphorylation sites on SIK2.

We used the ELM (Eukaryotic Linear Motif) and NetPhos 3.1 proteomics tools to scan the full length SIK2 sequence for motif prediction. The ELM (<http://elm.eu.org>) tool predicted two potential MAPK docking motifs, FSFP (FxFP) at aminoacids 399-402 and RTKGILEL ([R/K]xxxx#x#, # is a hydrophobic residue) at aminoacids 607-614 (Table 5.1). Furthermore, multiple motifs for ERK-dependent serine/threonine phosphorylation (Proline-directed phosphorylation, [S/T]P motifs) were predicted via both the ELM and Phospho 3.1 tools. Since our previous studies indicated that transient FGF2 stimulation in MIO-M1 cells affected SIK2 threonine phosphorylation via ERK2 (Ejder, 2011), the two putative threonine sites, T758 and T863, were picked as candidate target sites for ERK2 phosphorylation on SIK2 (Table 5.1).

Table 5.1. Possible ERK2 interaction and phosphorylation motifs on SIK2.

Site	Peptide Sequence	Motif	Description
399-402	FSFP	FxFP	MAPK-docking motif, FxFP motif
607-614	RTKGILEL	(R/K)xxxx#x#, # is a hydrophobic residue	MAPK-docking motif, D-motif or kinase interaction motif (KIM)
T758	PRQE(T)PPPS	...(S/T)P...	Proline-directed kinase phosphorylation site
T863	PDYP(T)PCQY	...(S/T)P...	Proline-directed kinase phosphorylation site

5.2.1. Site-directed Mutagenesis of KI-SIK2

To test whether T758 and T863 residues are ERK targets, site-directed mutagenesis approach was employed to produce point mutations at these sites. Since autophosphorylation activity of SIK2 would interfere with the phosphorylation analysis, these mutations were carried out on kinase inactive (KI, K49M mutant) SIK2. pEGFP C3 KI SIK2 eukaryotic expression plasmid was used to generate ERK mutants of KI SIK2; namely T758A mutant, T863A mutant and T758A T863A double-mutant (DM). Threonine to alanine conversions in each mutant KI SIK2 form was confirmed by DNA sequencing (Figure 5.2, 5.3, 5.4 and 5.5). Reverse sequences were inverted for clarity.

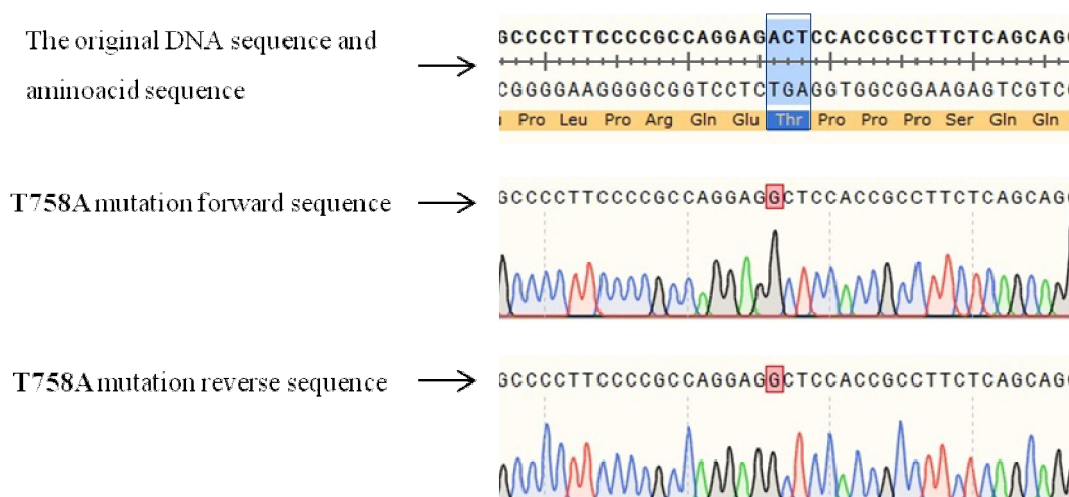


Figure 5.2. Verification of T758A KI SIK2 site-directed mutagenesis product. Partial sequencing result showing T758 region is given together with the corresponding wild type SIK2 DNA and amino acid sequences. The mutation site is highlighted.

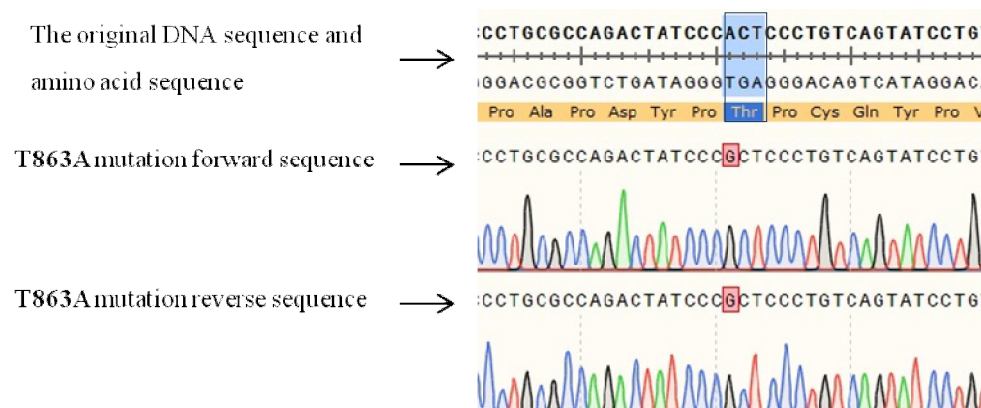


Figure 5.3. Verification of T863A KI SIK2 site-directed mutagenesis product. Partial sequencing result showing T863 region is given together with the corresponding wild type SIK2 DNA and amino acid sequences. The mutation site is highlighted.

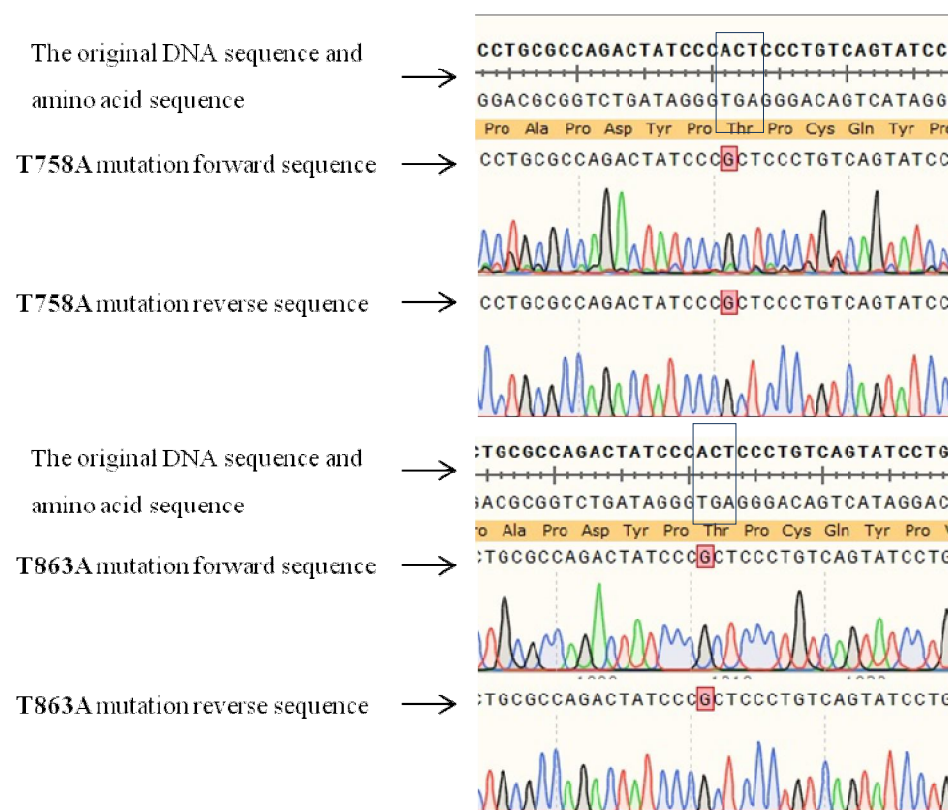


Figure 5.4. Verification of DM (Double Mutant) KI SIK2 site-directed mutagenesis product. Partial sequencing results showing T758 (upper panel) and T863 (lower panel) regions are given with the corresponding wild type SIK2 DNA and amino acid sequences. The mutation sites are indicated.

5.2.2. Expression of Mutants of KI-SIK2 in HEK 293T Cells and Their Isolation

The pEGFP C3 vectors carrying KI SIK2 and its mutants (T758A, T863A and DM) were transfected separately into HEK 293T cells. Empty pEGFP C3 vector transfected HEK 293T cells were used as a control sample. GFP expressions were observed under fluorescence microscope 24 h after transfection for each sample (Figure 5.5). Expression of GFP-tagged exogenous proteins was verified by western blotting using α -SIK2 antibody (Figure 5.6a). In the control mock transfected sample, only the endogenous SIK2 band was observed (the lower bands, ~120 kDa), while other samples also revealed the GFP-fused SIK2 proteins (the upper bands, ~150 kDa).

To be able to utilize the mutant GFP-tagged SIK2 proteins in *in vitro* kinase assays, lysates of transfected cells were subjected to immunoprecipitation (IP) using α -GFP antibody. One fifth of each IP sample was reserved for western blotting to verify the IP, and the rest was used in the *in vitro* kinase experiments. Western blot analysis of the immunoprecipitation samples using α -SIK2 antibody revealed that the exogenously expressed SIK2 isoforms (~150kDa) were isolated (Figure 5.6b). The lower bands in this blot are unidentified, however they are not thought to be representing endogenous SIK2 as the sizes should have been smaller in that case.

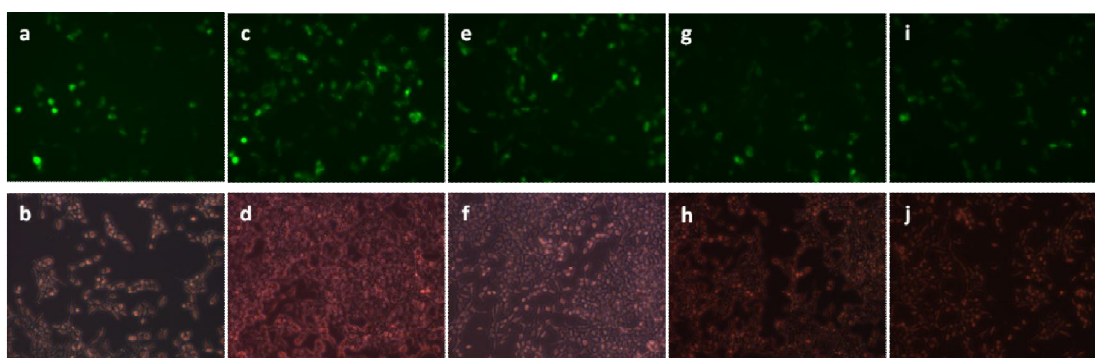


Figure 5.5. Transfection of HEK293T cells with EGFP-SIK2 KI and its mutant forms. Cells expressing EGFP-SIK2 KI (a), T758A (c), T863A (e), DM EGFP-SIK2 KI (g) and only EGFP (i) were analyzed 24 h post-transfection under the fluorescence microscope.

Corresponding bright field images are given (b, d, f, h and j).

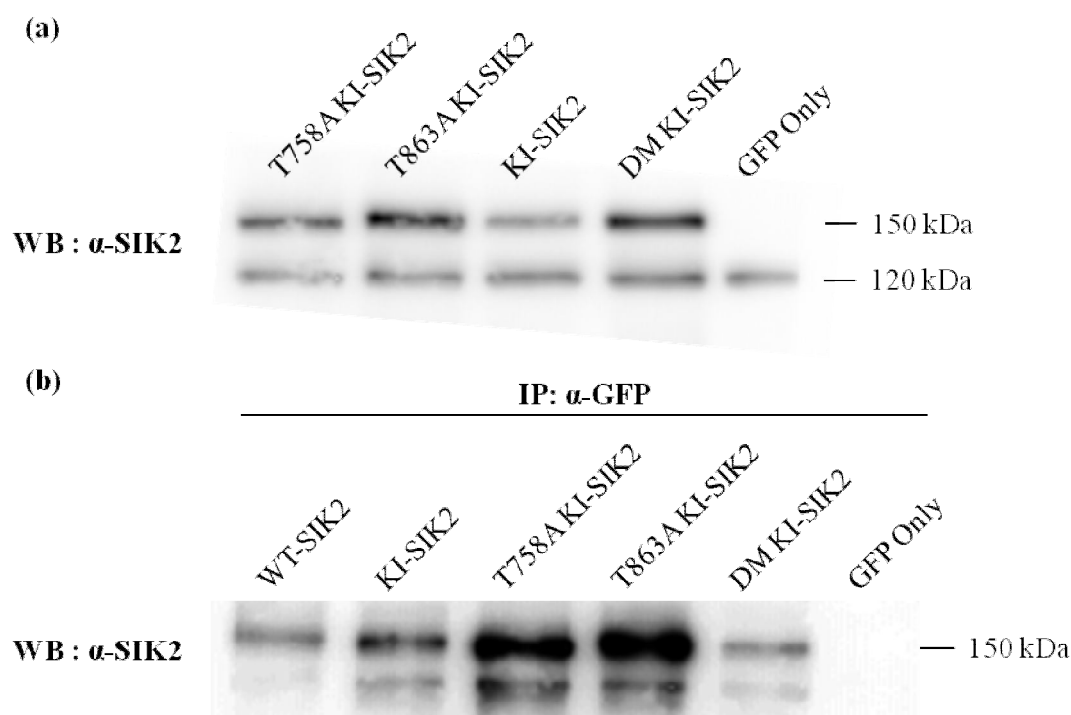


Figure 5.6. Expression and isolation of the mutant SIK2 forms. Western blot analysis of cell lysates (a) and immunoprecipitation samples (b) belonging to mutant and wild-type KI SIK2 samples and WT-SIK2 and only GFP controls. Samples were resolved on 8% SDS-PAGE and western blots were probed with α -SIK2 antibody.

5.2.3. Phosphorylation of Mutants of KI SIK2 by ERK2

In vitro kinase experiments were done in the presence of [γ - ^{32} P]-ATP using constitutively active ERK2 (commercially purchased from R&D Systems, USA). The immunoprecipitated KI-SIK2, T758A, T863A and DM samples were separately used as substrates. Immunoprecipitated WT-SIK2 was used as an assay control in these experiments due to its autophosphorylation activity, and the immunoprecipitates from cells transfected with empty vector were used as the negative control. The lack of kinase activity of K49M SIK2 (KI-SIK2) was previously shown by Küser, 2011 and Ejder, 2011 (Figure 5.7a).

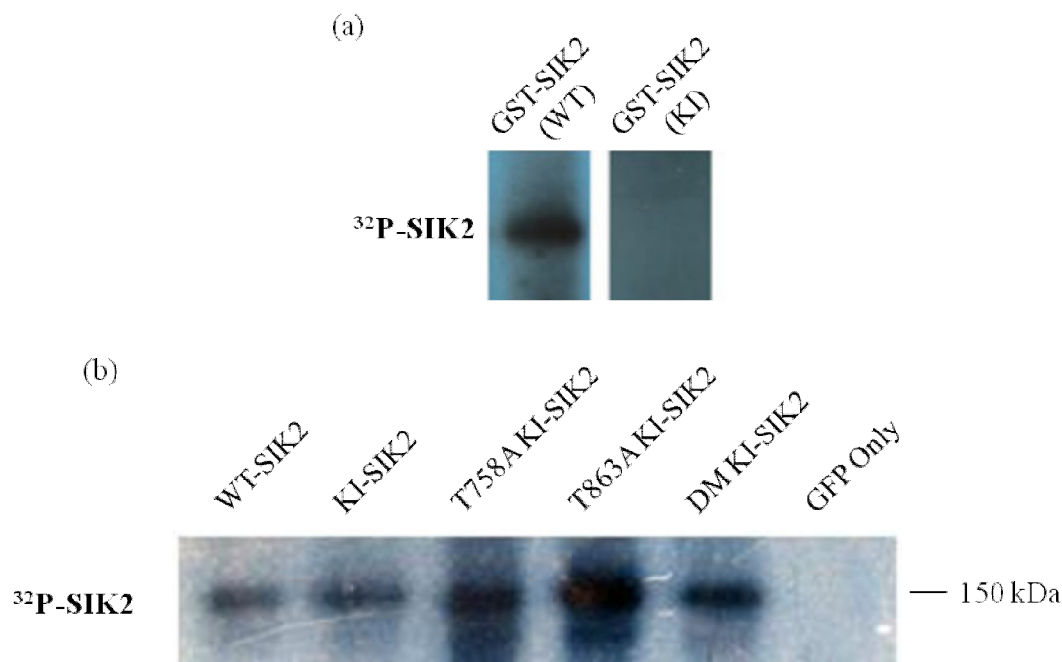


Figure 5.7. Analysis of phosphorylation of SIK2 by ERK2. In vitro kinase assays were carried out (a) using GST-WT SIK2 and GST-KI SIK2 (Küser, 2011) autophosphorylation signals were evaluated. (b) Phosphorylation activity of ERK2 on KI-SIK2, mutant forms of KI-SIK2 (T758A, T863A and DM) and GFP only control sample; and autophosphorylation activity of WT-SIK2 was assayed.

In contrary to our expectations, both mutant forms of SIK2 and the double mutant form were phosphorylated by ERK2 (Figure 5.7b). When relative phosphorylation levels were calculated by normalizing the band intensities in the autoradiograph to the respective controls in the WB, mutant forms of KI-SIK2 seemed to be phosphorylated by ERK2 almost at the same level with the KI-SIK2. Hence, it was concluded that both T758 and T863 residues on SIK2 sequence are not ERK target sites.

5.2.4. Mass Spectrometry Analysis of KI-SIK2

Mass spectrometry (MS) technique was also utilized for the purpose of detecting ERK-directed phosphorylation sites on SIK2. To prepare samples for MS, GFP KI SIK2 was expressed in HEK 293T cells and isolated by immunoprecipitation with α -GFP

antibody. The immunoprecipitated KI-SIK2 was divided into two samples for two kinase reactions that only contains cold ATP. One reaction included CA-ERK2 while the other had no CA-ERK2 for comparison. On bead samples were washed with kinase buffer and sent to Dr. Nurhan Özlü's laboratory at Koç University for MS analysis. There, the samples were resolved on a precast 10% polyacrylamide gel and the gel was stained. The two bands detected around 150 kDa were excised and subjected to the MS analysis (Figure 5.8). MS was carried out using Thermo Scientific Q Exactive Quadrupole-Orbitrap Mass Spectrometer with Nano Liquid Chromatography (n-LC MS/MS). The results of the mass spectrometry analysis are given in Tables 5.2, 5.3, 5.4 and 5.5.

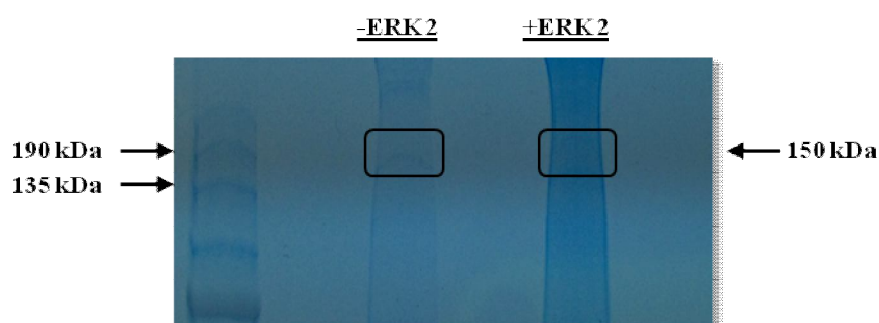


Figure 5.8. SDS-PAGE fractionation of mass spectrometry samples. IVK samples of (+) ERK and (-) ERK were fractionated in 10% SDS-PAGE and the proteins were detected by coomassie staining before mass spectrometry analysis.

In mass spectrometry analysis; “coverage” shows the default percentage of the protein sequence covered by identified peptides, “PSMs” (peptide spectrum matches) shows the total number of identified peptide sequences for the protein including those redundantly identified and “AAs” gives the default sequence length of the protein. Table 5.2 and Table 5.3 gives the list of proteins detected in the IVK samples of -ERK and +ERK, respectively. Only the proteins with coverage level higher than 0.75 were listed and only one protein is included from one protein family.

It is expected to have coverage levels at least around 40% for the target protein in IP samples. Although, peptides of SIK2 were detected in both IVK samples; the coverage level of SIK2 was too low in both samples (5.62 and 4.43), and the level was very similar

to the other proteins detected in the samples. The peptide sequences of SIK2 identified in MS analysis of two IVK reaction samples are listed in Table 5.4 and Table 5.3, respectively. The coverage of SIK2 was very low to identify phosphopeptides, for which we could only detect 6 and 4 peptides in the two samples respectively. Therefore, we were not able to find any phosphorylation sites in this analysis.

Table 5.2. Mass spectrometry analysis of immunoprecipitated kinase inactive SIK2.

Description	Coverage	Unique Peptides	Peptides	PSMs	AAs	MW (kDa)
Keratin, type I cytoskeletal	12.5	2	7	13	584	58.8
Serine/threonine-protein kinase SIK2	5.62	2	6	6	926	103.8
Tubulin alpha-1C chain	2.23	1	1	1	449	49.9
ATP-dependent RNA helicase A	1.57	2	2	3	1270	140.9
Eu-translation initiation factor 3 subunit B	1.11	1	1	1	814	92.4
Scaffold attachment factor B1	0.98	1	1	1	915	102.6

Table 5.3. Mass spectrometry analysis of immunoprecipitated kinase inactive SIK2 phosphorylated by constitutively active ERK2.

Description	Coverage	Unique Peptides	Peptides	PSMs	AAs	MW (kDa)
Keratin, type I cytoskeletal	4.79	0	3	3	584	58.8
Serine/threonine-protein kinase SIK2	4.43	0	4	4	926	103.8
Carbohydrate sulfotransferase 10	1.97	0	1	2	356	42.2
SAM and SH3 domain-containing protein 1	1.76	0	1	1	1247	136.6
PDZ domain-containing protein 2	1.37	0	2	2	2839	301.5
Bile salt export pump	1.14	0	1	1	1321	146.3
Sterol regulatory element-binding protein 1	0.78	0	1	2	893	95.8

Table 5.4. SIK2 peptides identified in mass spectrometry analysis of immunoprecipitated kinase inactive SIK2.

Sequence	PSMs	Proteins	Protein Groups	Modifications	Exp Value A4	Missed Cleavages
DIMLANQP SPR	1	1	1	-	0.002242129	0
RPSTIAEQ TVAK	1	1	1	-	0.114384323	0
GILELNK	1	1	1	-	0.006867705	0
AQNTCQL YCK	1	1	1	C5(Carbamidomethyl) C9(Carbamidomethyl)	0.036640254	0
AQNTCQL YCKEPPR	1	1	1	C5(Carbamidomethyl) C9(Carbamidomethyl)	1.928816699	1
LYQVMET K	1	1	1	-	1.47530528	0

Table 5.5. SIK2 peptides identified in mass spectrometry analysis of immunoprecipitated kinase inactive SIK2 phosphorylated by constitutively active ERK2.

Sequence	PSMs	Proteins	Protein Groups	Modifications	Exp Value A4	Missed Cleavages
AQNTCQL YCK	1	1	1	C5(Carbamidomethyl) C9(Carbamidomethyl)	0.0647081	0
SSFPVEQR	1	1	1	-	0.1350649	0
QSLETQYL QHR	1	1	1	-	0.3509533	0
RPSTIAEQT VAK	1	1	1	-	2.2623975	0

5.3. Generation of Gab1 and SIK2 Bacterial Expression Vectors

As the previous studies in our lab indicated the docking protein Gab1 as a substrate of SIK2, Gab1 is our prominent candidate to be the target for SIK2 dependent negative regulation of FGF signaling. To be able study the biochemical interactions between the two proteins, their bacterial expression vectors were produced.

5.3.1. Cloning of Gab1 and SIK2 Genes via Gateway® Technology

Gateway® cloning system was employed to generate bacterial expression vectors for Gab1 and WT and KI forms of SIK2, which enables cloning of the desired DNA sequence into a “Gateway destination vector” with use of “Gateway att” sites. Using the Gateway® cloning technique, Gab1 and SIK2 cDNA sequences were subcloned from pEGFP WT SIK2, pEGFP KI SIK2 and pCMV-Entry Myc-DDK (C-terminal Myc and Flag tag) Gab1 plasmids. pET-DEST42 was selected for SIK2 as the destination vector, for C-terminal 6xHis-tagged expression of the gene in bacteria. Gab1 was cloned together with its C-terminal Myc-DDK tag into the pDEST 14 vector for bacterial expression, which provides no tags on its own.

Our attempts to clone full length SIK2 into the pET-DEST42 vector have failed. Therefore, we decided to clone the N-terminal and the C-terminal halves of SIK2 separately into this bacterial expression vector; with the two pieces of SIK2 were being SIK2 (1-1107 bases) and SIK2 (1093-2778 bases). Since the kinase domain of SIK2 resides in the N-terminal part, kinase-inactive (KI) mutant was cloned only for the N-term SIK2 (1-1107).

At the final step of the cloning, LR clonase reaction samples were transformed into *E. coli* and positive colonies were selected via colony PCR reactions using attB1 and attB2 adapter primers (Figure 5.11). Selected plasmids were sequenced for further verification, mutation-free clones were determined and plasmid DNA of each verified clone was isolated. Positive plasmid DNAs were transformed into Rosetta DE3 pLysS competent cells to use for eukaryotic protein expression and later stored as glycerol stocks.

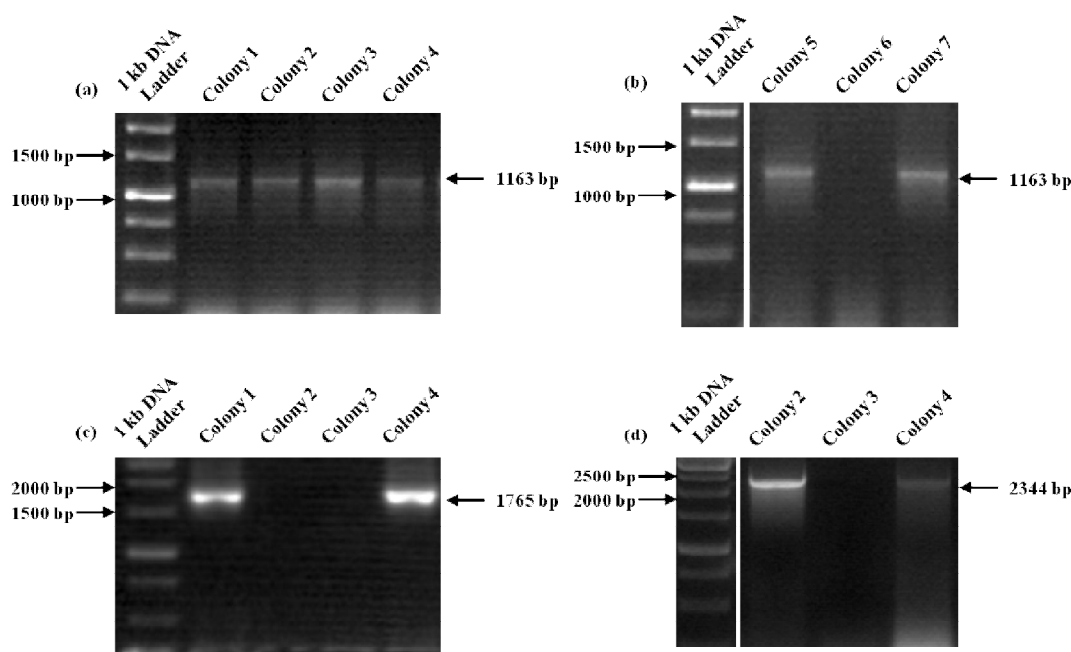


Figure 5.9. Agarose gel electrophoresis results for colony PCR verifications of *E. coli* transformant colonies. Colony PCR was performed after transformation of the bacteria with the Gateway LR cloning reactions of N-term WT SIK2 (1-1149) (a), N-term KI SIK2 (1-1149) (b), C-term SIK2 (1149-2820) (c) and WT Gab1 (d).

5.3.2. Expression of Gab1 and SIK2 Constructs in Rosetta Cells

Generated Flag-Gab1 and different His-tagged SIK2 constructs were expressed in Rosetta cells by IPTG induction. For comparison, an aliquot of bacteria cells were taken from each culture before induction (No-IPTG control). The pellets collected after cell lysis were also kept to check if the recombinant proteins form insoluble precipitates as inclusion bodies. All of the samples were tested for recombinant protein expression with SDS-PAGE followed by coomassie staining and western blotting.

Flag-tagged Gab1 expression was monitored using DYKDDDDK-tag (Flag-tag) and α -Gab1 antibodies. Both western blots and the coomassie stainings gave a band for Gab1 in the cell extract of pellet at around 110 kDa, which was absent in no-IPTG samples (Figure 5.12). WT N-term SIK2, KI N-term SIK2 and C-term SIK2 expressions

were monitored with His-probe antibody. N-term SIK2 isoforms gave a band around 80 kDa in both coomassie staining and western blot (Figure 5.13 and 5.14), while C-term SIK2 isoform gave a band around 95 kDa in both methods (Figure 5.15). The results revealed also that all of the recombinantly expressed His-tagged SIK2s and Flag-tagged Gab1 were insoluble and forming inclusion bodies and therefore, extracts from the pellets should be used in further experiments.

To study the SIK2-Gab1 interaction, these recombinant proteins were planned to be used in pull-down assays. However, due to technical problems, a successful pull-down assay could not be achieved.

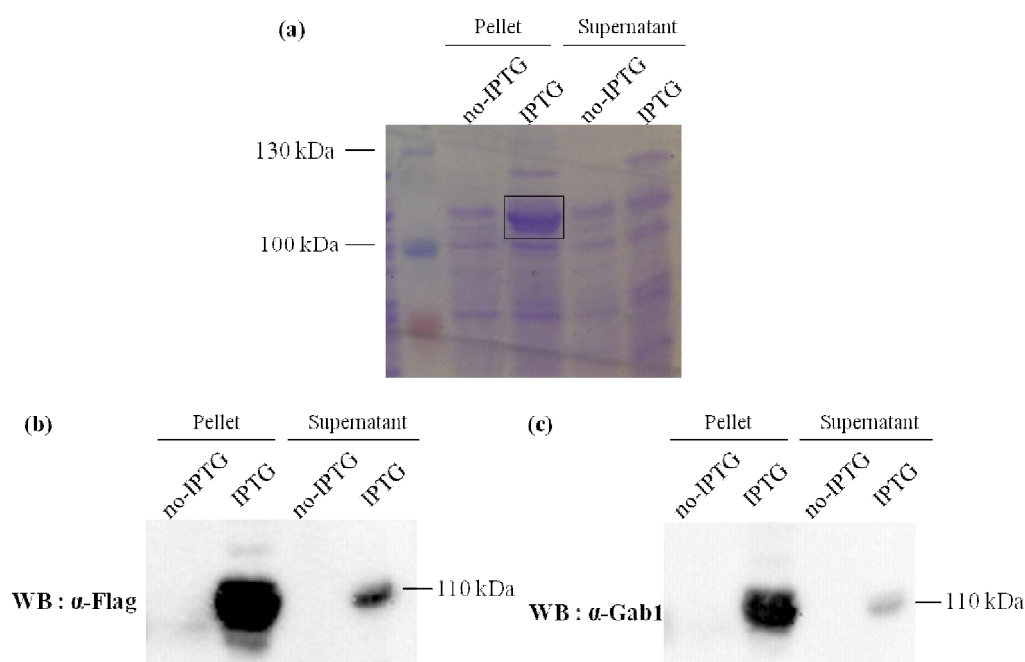


Figure 5.10. Control of Flag-Gab1 expression in Rosetta cells. Samples after bacterial lysis were fractioned by SDS-PAGE and analyzed by coomassie staining (a) and western blotting with α -Flag (b) and α -Gab1 antibody (c).

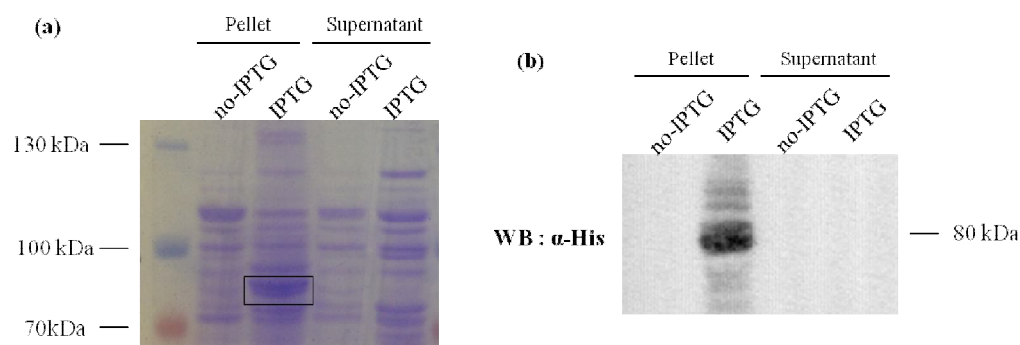


Figure 5.11. Control of N-term WT-SIK2 expression in Rosetta cells. Samples after bacterial lysis were fractioned by SDS-PAGE and analyzed by coomassie staining (a) and western blotting with α -His (b).

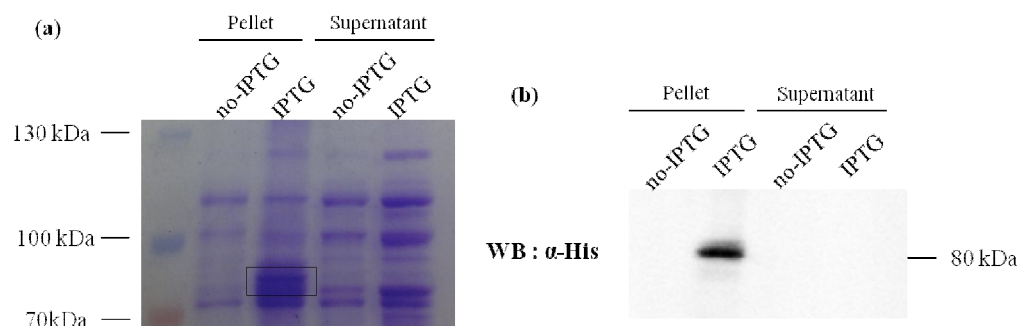


Figure 5.12. Control of N-term KI-SIK2 expression in Rosetta cells. Samples after bacterial lysis were fractioned by SDS-PAGE and analyzed by coomassie staining (a) and western blotting with α -His (b).

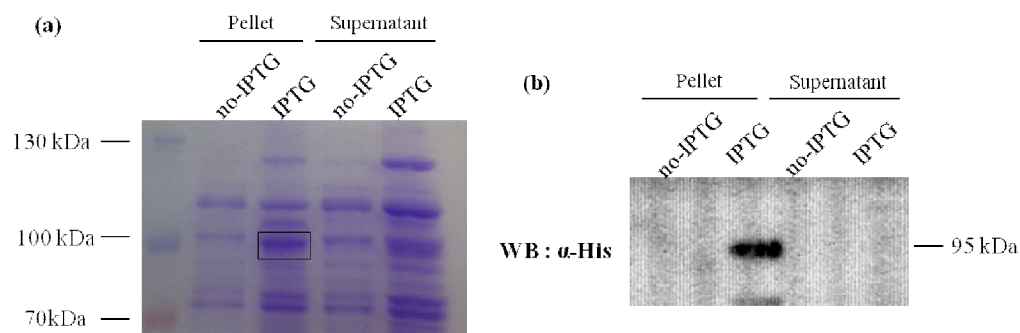


Figure 5.13. Control of C-term SIK2 expression in Rosetta cells. Samples after bacterial lysis were fractioned by SDS-PAGE and analyzed by coomassie staining (a) and western blotting with α -His (b).

6. DISCUSSION

FGFs are known as one of the key regulators of various significant cellular processes including proliferation, differentiation, survival and migration; and thus have effects on many physiological functions such as angiogenesis, wound healing and maintaining tissue homeostasis (Yun *et al.*, 2010; Li *et al.*, 2015). As the very complex signaling network downstream of the FGFRs allow positive or negative modulation of the signaling at many different levels, any deterioration in FGF signaling can cause serious diseases including developmental disorders and cancer (Korsensky and Ron, 2016). Therefore, identifying unique modulators and understanding their regulation mechanisms is critical for understanding the roles of FGF signaling in different physiological contexts and possibly for development of novel translational approaches.

In our laboratory, the serine/threonine kinase SIK2 was first obtained from a yeast two hybrid screening of a retinal cDNA library using FGFR cytoplasmic domain as the bait (Özcan, 2003). Further studies presented SIK2 as a potential player in a feedback mechanism down-regulating FGF signaling (Figure 1.7) (Küser, 2011; Yılmaz, 2011; Ejder, 2011). Moreover, extracellular signal-regulated kinase (ERK), the effector kinase in the FGF-dependent Ras/MAPK pathway, was proposed to be the activatory kinase of SIK2 upon FGF2 stimulation (Küser, 2011; Ejder, 2011).

In this study, Co-IP experiments were carried out using FGF2-treated Müller Glia cells to further characterize the SIK2 interaction partners. Our results suggest SIK2 interaction with Gab1 and FRS2 is likely to be constitutive, while SOS1, Grb2 and Shp2 appear to be recruited to the signalosome in an FGF2 dependent manner (Figure 5.1)

Gab1, Grb2 and Shp2 proteins are also involved in various other signaling events including G-protein coupled receptor signaling, cytokine signaling and signaling under other receptor tyrosine kinases (Wöhrle *et al.*, 2009; Belov and Mohammadi, 2012; Lauriol *et al.*, 2015). FRS2 is considered as the main modulator in the initiation of FGF signaling and though to be constitutively associated with FGFR. The current paradigm suggests that upon FGFR activation, FRS2 is phosphorylated on several tyrosine residues, four of which

create docking sites for Grb2-SOS1 complex and two of them for Shp2 binding (Gotoh, 2008). The guanine exchange factor SOS1 is critically important for activation of Ras and subsequent MAPK signaling (Pierre *et al.*, 2011). Though the Co-IP results does not give a direct clue about the mechanism how SIK2 is involved in the regulation of FGF signaling, the results strengthen the possibility of SIK2 being an element of FGF signaling complex and supports our hypothesis of SIK2-dependent Ras/MAPK pathway regulation (Figure 1.7).

As mentioned earlier, ERK is known to be an important signaling protein that is activated by a phosphorylation cascade, and becomes central to regulation of a wide array of cellular events (Wortzel and Seger, 2011); in the context of Müller cells ERK activation mainly associated with proliferation (Çınaroğlu, 2005). Earlier results from our group indicated ERK-dependent threonine phosphorylation of SIK2 *in vitro* (Ejder, 2011; Küser, 2011) and raised the possibility of SIK2 involvement in negative feedback regulation of Ras/MAPK pathway. Therefore, studying the ERK-SIK2 interaction further is crucial for better understanding of such a regulation mechanism and carries a great promise for finding the potential cellular roles of SIK2. Bearing these in mind, in this study, we aimed to investigate potential ERK-dependent phosphorylation sites on SIK2.

Phosphorylation is an important post-translational modification on proteins that mediate various cellular activities in the contexts of different signaling pathways (von Stechow *et al.*, 2015). Therefore, before going on with the functional studies, it is a crucial task to study the phospho-proteins and kinase-substrate interactions and gain knowledge about the phosphorylation sites on proteins signaling mechanisms in pathways. A general approach when studying phosphorylation sites is determining candidate residues on the substrate by using bioinformatic tools and mutating these residues to look for a change in the phosphorylation pattern of the mutant forms of the substrate in biochemical assays. Following this approach, for the verification of the candidate phosphorylation sites on SIK2, the identified residues were mutated, and the isolated mutant proteins were checked for a phenotypic change in *in vitro* phosphorylation reactions.

Bioinformatic analysis on amino acid sequence of human SIK2 indicated the presence of potential docking sites for ERK between amino acids 399-402 and 607-614,

and consensus target sites for ERK phosphorylation at threonine residues 758 and 863 (Table 5.1). Putative serine residues were neglected as there was no upregulation in serine phosphorylation level of SIK2 in FGF treated Müller cells (Ejder, 2011). Threonine residues 758 and 863 were determined as the candidate ERK-dependent phosphorylation sites on SIK2.

Candidate target sites T758 and T863 on SIK2 were mutated to methionine residues and mutant forms of SIK2 were analyzed to see whether they are phosphorylated by ERK2 by *in vitro* phosphorylation experiments using ^{32}P -ATP followed by SDS-PAGE. The analysis indicated that mutant versions of KI-SIK2; T758A KI-SIK2, T863A KI-SIK2 and DM KI-SIK2 underwent similar amount of phosphorylation with KI-SIK2 due to ERK2 activity (Figure 5.7 and 5.8) and thus it was inferred that both of these residues are not targeted by ERK. Therefore, we hypothesize that there might be non-consensus target sites for ERK phosphorylation on the SIK2 sequence.

Even though this mutagenesis approach that is based on the predictions from bioinformatic tools is quite reliable as it directly gives information about the effect of a change in certain residues on phosphorylation of the substrate, a drawback with this method could be that the bioinformatics make predictions in reference to the linear amino acid sequence of the substrate instead of its three dimensional structure. Consequently, another explanation for the failure of our methodology might be that the tertiary structure of SIK2 masks the putative target sites suggested by the bioinformatic analysis, or that the distance between the candidate sites and the docking sites is too long for ERK to operate in the three dimensional conformation. In this study we focused on canonical S/TP motif as potential ERK phosphorylation sites. It is also possible that on SIK2, ERK might be phosphorylating non-canonical target motifs as in the case for ETV3 (Carlson *et al.*, 2011).

To identify the possible non-canonical target sites, mass spectrometry (MS) technique was utilized. Two *in vitro* phosphorylation reactions; one including KI-SIK2 and active ERK2 and the other one including only KI-SIK2 were subjected to MS analysis. However, as the coverage levels were too low for both samples to detect any functional sites in the MS analysis, no phospho-peptides could be detected (Table 5.4 and 5.5). Low coverage levels indicate that the amount of the substrate, SIK2, was not sufficient in the IP

samples. The coverage value for the MS analysis of these samples should be elevated to 35-40% levels for healthy identification of specific peptides. Therefore, an MS analysis using around 20-fold more input is underway.

In summary, this study has shown that SIK2 is interacting with the FGF signaling complex proteins Gab1, Grb2, Shp2, FRS2 and SOS1 in Müller cells. Presence of FRS2 and SOS1 in this complex further supports our working model that places SIK2 in a negative feedback mechanism acting on FGF-dependent MAPK signaling. The ERK phosphorylation of SIK2 does not occur through the proposed consensus ERK target residues T758 and T863, but it happens through another threonine site or sites.

REFERENCES

- Al-Hakim, A. K., O. Göransson, M. Deak, R. Toth, D. G. Campbell, N. Morrice, A. R. Prescott and D. R. Alessi, 2005, “14-3-3 cooperates with LKB1 to regulate the activity and localization of QSK and SIK.”, *Journal of cell science*, 118(Pt 23), pp. 5661–73. doi: 10.1242/jcs.02670.
- Belov, A., and M. Mohammadi, 2013, "Molecular Mechanisms of Fibroblast Growth Factor Signaling in Physiology and Pathology", *Cold Spring Harb Perspect Biol*, 5(6), p. a015958. doi: 10.1101/cshperspect.a015958.Molecular.
- BioInfoRx, *BsSeqTools Ultimate Molecular Cloning Guides – Gateway Cloning*, 2016, http://bioinforx.com/lms1/bxseqtools/ultimate-molecular-cloning-guides/help_cloning_gw.php, accessed at July 2016.
- Böttcher, R. T., N. Pollet, H. Delius and C. Niehrs, 2004, “The transmembrane protein XFLRT3 forms a complex with FGF receptors and promotes FGF signalling”, *Nat Cell Biol*. Nature Publishing Group, 6(1), pp. 38–44.
- Bugra, K. and D. Hicks, 1997, “Acidic and basic fibroblast growth factor messenger RNA and protein show increased expression in adult compared to developing normal and dystrophic rat retina”, *Journal of Molecular Neuroscience*, 9(1), pp. 13–25. doi: 10.1007/BF02789391.
- Bugra, K., L. Olivier, E. Jacquemin, M. Laurent, Y. Courtois and D. Hicks, 1994, “Acidic Fibroblast Growth Factor is Expressed Abundantly by Photoreceptors within the Developing and Mature Rat Retina”, *European Journal of Neuroscience*, 6(6), pp. 1062–1062. doi: 10.1111/j.1460-9568.1994.tb00602.x.
- Carlson, S. M., C. R. Chouinard, A. Labadorf, C. J. Lam, K. Schmelzle, E. Fraenkel and F. M. White, 2011, "Large-scale Discovery of ERK2 Substrates Identifies ERK-Mediated Transcriptional Regulation by ETV3", *Science signaling*, 4(196), p.

rs11-rs11. doi: 10.1126/scisignal.2002010.

Candaş, D., 2007, *SIK2 Is a Potential Mediator of Cross-talk Between FGF and PKA Pathways*, M.S. Thesis, Boğaziçi University.

Cinaroglu, A., Y. Ozmen, A. Ozdemir, F. Ozcan, C. Ergorul, P. Cayirlioglu, D. Hicks and K. Bugra, 2005, "Expression and possible function of fibroblast growth factor 9 (FGF9, and its cognate receptors FGFR2 and FGFR3 in postnatal and adult retina.", *Journal of neuroscience research*, 79(3), pp. 329–39. doi: 10.1002/jnr.20363.

Cinaroglu, E. A., 2005, *Müller Cells as Potential Targets of FGF9 in Rodent Retina*, Ph.D. Thesis, Boğaziçi University

Davis, R., 1993, "The mitogen-activated protein kinase signal transduction pathway", *J. Biol. Chem.*, 268(20), pp. 14553–14556.

Dhanasekaran, N. and E. Premkumar Reddy, 1998, "Signaling by dual specificity kinases.", *Oncogene*, 17(11) Reviews, pp. 1447–1455. doi: 10.1038/sj.onc.1202251.

Ebisuya, M., K. Kondoh and E. Nishida, 2005, "The duration, magnitude and compartmentalization of ERK MAP kinase activity: mechanisms for providing signaling specificity.", *Journal of cell science*, 118(Pt 14), pp. 2997–3002. doi: 10.1242/jcs.02505.

Ejder, F., 2011, *ERK, AKT and PKA as Regulatory Kinases of SIK2 in FGF Signaling in Müller Cells*, M.S. Thesis, Boğaziçi University.

Eswarakumar, V. P., I. Lax and J. Schlessinger, 2016, "Cellular signaling by fibroblast growth factor receptors", *Cytokine and Growth Factor Reviews*. Elsevier, 16(2), pp. 139–149. doi: 10.1016/j.cytogfr.2005.01.001.

- Fantz, D. A., D. Jacobs, D. Glossip and K. Kornfeld, 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.
- Feldman, J. D., L. Vician, M. Crispino, W. Hoe, M. Baudry and H. R. Herschman, 2002, "The Salt-Inducible Kinase, SIK, Is Induced by Depolarization in Brain", *Journal of Neurochemistry*, 74(6), pp. 2227–2238. doi: 10.1046/j.1471-4159.2000.0742227.x.
- Fischer, A. J., G. Omar, J. Eubanks, C. R. McGuire, B. D. Dierks and T. Reh, 2004, "Different aspects of gliosis in retinal Muller glia can be induced by CNTF, insulin, and FGF2 in the absence of damage.", *Molecular vision*, 10(December), pp. 973–986.
- Goetz, R. and M. Mohammadi, 2013, "Exploring mechanisms of FGF signalling through the lens of structural biology", *Nat Rev Mol Cell Biol.* Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 14(3), pp. 166–180.
- Gotoh, N., 2008, "Regulation of growth factor signaling by FRS2 family docking/scaffold adaptor proteins", *Cancer Science*, 99(7), pp. 1319–1325. doi: 10.1111/j.1349-7006.2008.00840.x.
- Hanafusa, H., S. Torii, T. Yasunaga and E. Nishida, 2002, "Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway", *Nat Cell Biol.* Nature Publishing Group, 4(11), pp. 850–858.
- Henriksson, E., H. A. Jones, K. Patel, M. Pegg, N. Morrice, K. Sakamoto and O. Göransson, 2012, "The AMPK-related kinase SIK2 is regulated by cAMP via phosphorylation at Ser(358, in adipocytes", *Biochemical Journal*. Portland Press Ltd., 444(Pt 3), pp. 503–514. doi: 10.1042/BJ20111932.

- Herbst, R., P. M. Carroll, J. D. Allard, J. Schilling, T. Raabe and M. A. Simon, 1996, “Daughter of sevenless is a substrate of the phosphotyrosine phosphatase corkscrew and functions during sevenless signaling”, *Cell*, 85(6), pp. 899–909. doi: 10.1016/S0092-8674(00)81273-8.
- Horike, N., H. Takemori, Y. Katoh, J. Doi, L. Min, T. Asano, X. J. Sun, H. Yamamoto, S. Kasayama, M. Muraoka, Y. Nonaka and M. Okamoto, 2003, “Adipose-specific expression, phosphorylation of Ser794 in insulin receptor substrate-1, and activation in diabetic animals of salt-inducible kinase-2”, *Journal of Biological Chemistry*, 278(20), pp. 18440–18447. doi: 10.1074/jbc.M211770200.
- Itoh, N., 2007, “The Fgf families in humans, mice, and zebrafish: their evolutionary processes and roles in development, metabolism, and disease”, *Biological & Pharmaceutical Bulletin*, 30(10), pp. 1819–1825. doi: 10.1248/bpb.30.1819.
- Katoh, Y., H. Takemori, N. Horike, J. Doi, M. Muraoka, L. Min and M. Okamoto, 2004, “Salt-inducible kinase (SIK, isoforms: their involvement in steroidogenesis and adipogenesis”, *Molecular and Cellular Endocrinology*, 217(1–2), pp. 109–112.
- Korsensky, L. and D. Ron, 2016, “Regulation of FGF signaling: Recent insights from studying positive and negative modulators”, *Seminars in Cell & Developmental Biology*, 53, pp. 101–114.
- Kouhara, H., Y. R. Hadari, T. Spivak-Kroizman, J. Schilling, D. Bar-Sagi, I. Lax and J. Schlessinger, 1997, “A Lipid-Anchored Grb2-Binding Protein That Links FGF-Receptor Activation to the Ras/MAPK Signaling Pathway”, *Cell*. Elsevier, 89(5), pp. 693–702. doi: 10.1016/S0092-8674(00)80252-4.
- Ku, H. and K. E. Meier, 2000, “Phosphorylation of paxillin via the ERK mitogen-activated protein kinase cascade in EL4 thymoma cells”, *Journal of Biological Chemistry*, 275(15), pp. 11333–11340. doi: 10.1074/jbc.275.15.11333.

- Küser, G., 2006, *Identification of Candidate Substrates of SIK2 in Vitro*, M.S. Thesis, Boğaziçi University.
- Küser, G., 2011, *SIK2: A Key Player in FGF2-Induced Proliferation and Insulin Induced Survival/Hyperglycemia-Dependent Apoptosis in Müller Cells*, Ph.D. Thesis, Boğaziçi University.
- Küser-Abalı, G., F. Ozcan, A. Ugurlu, A. Uysal, S. H. Fuss and K. Bugra-Bilge, 2013, “SIK2 Is Involved in the Negative Modulation of Insulin-Dependent Müller Cell Survival and Implicated in Hyperglycemia-Induced Cell Death SIK2 Negatively Regulates Insulin/Akt Pathway”, *Investigative Ophthalmology & Visual Science*, 54(5), pp. 3526–3537.
- Kyriakis, J. M., H. App, X. Zhang, P. Banerjee, D. L. Brautigan, U. R. Rapp and J. Avruch, 1992, “Raf-1 activates MAP kinase-kinase”, *Nature*, 358(6385), pp. 417–421.
- Lauriol, J., F. Jaffre and M. I. Kontaridis, 2015, "The Role of the Protein Tyrosine Phosphatase SHP2 in Cardiac Development and Disease", *Seminars in cell & developmental biology*, 0, pp. 73–81. doi: 10.1016/j.semcdb.2014.09.013.
- Lax, I., A. Wong, B. Lamothe, A. Lee, A. Frost, J. Hawes and J. Schlessinger, 2002, “The docking protein FRS2alpha controls a MAP kinase-mediated negative feedback mechanism for signaling by FGF receptors”, *Molecular Cell*, 10(4), pp. 709–719. doi: 10.1016/S1097-2765(02)00689-5.
- Lemarié, C. A. and S. Lehoux, 2011, “The gift of Gab1 (Grb-2-associated binder 1)", *Arteriosclerosis, Thrombosis, and Vascular Biology*, 31(5), pp. 956–957. doi: 10.1161/ATVBAHA.111.225987.
- Lewitzky, M., C. Kardinal, N. H. Gehring, E. K. Schmidt, B. Konkol, M. Eulitz, W. Birchmeier, U. Schaeper and S. M. Feller, 2001, “The C-terminal SH3 domain of the adapter protein Grb2 binds with high affinity to sequences in Gab1 and SLP-

- 76 which lack the SH3-typical P-x-x-P core motif.”, *Oncogene*, 20(9), pp. 1052–1062. doi: 10.1038/sj.onc.1204202.
- Li, X., C. Wang, J. Xiao, W. L. McKeehan and F. Wang, 2015, "Fibroblast growth factors, old kids on the new block", *Seminars in Cell and Developmental Biology*. Elsevier Ltd, 53, pp. 155–167. doi: 10.1016/j.semcdb.2015.12.014.
- Liu, Y. and L. R. Rohrschneider, 2002, “The gift of Gab”, *FEBS Letters*, 515(1–3), pp. 1–7. doi: 10.1016/S0014-5793(02)02425-0.
- Lizcano, J. M., O. Göransson, R. Toth, M. Deak, N. A. Morrice, J. Boudeau, S. A. Hawley, L. Udd, T. P. Mäkelä, D. G. Hardie and D. R. Alessi, 2004, “LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1”, *The EMBO Journal*. Nature Publishing Group, 23(4), pp. 833–843. doi: 10.1038/sj.emboj.7600110.
- Lock, L. S., C. R. Maroun, M. A. Naujokas and M. Park, 2002, “Distinct Recruitment and Function of Gab1 and Gab2 in Met Receptor-mediated Epithelial Morphogenesis”, *Molecular Biology of the Cell*. Edited by J. S. Brugge. The American Society for Cell Biology, 13(6), pp. 2132–2146. doi: 10.1091/mbc.02-02-0031.
- Martinez-Morales, J. R., F. Del Bene, G. Nica, M. Hammerschmidt, P. Bovolenta and J. Wittbrodt, 2005, “Differentiation of the vertebrate retina is coordinated by an FGF signaling center”, *Developmental Cell*, 8(4), pp. 565–574. doi: 10.1016/j.devcel.2005.01.022.
- Matsubayashi, Y., M. Fukuda and E. Nishida, 2001, “Evidence for Existence of a Nuclear Pore Complex-mediated, Cytosol-independent Pathway of Nuclear Translocation of ERK MAP Kinase in Permeabilized Cells”, *Journal of Biological Chemistry*, 276(45), pp. 41755–41760. doi: 10.1074/jbc.M106012200.
- Meister, M., A. Tomasovic, A. Banning and R. Tikkanen, 2013, “Mitogen-activated

- protein (MAP) kinase scaffolding proteins: A recount”, *International Journal of Molecular Sciences*, 14(3), pp. 4854–4884. doi: 10.3390/ijms14034854.
- Mohi, M. G. and B. G. Neel, 2007, “The role of Shp2 (PTPN11) in cancer”, *Current Opinion in Genetics & Development*, 17(1), pp. 23–30.
- Morrison, D. K., 2001, “KSR: a MAPK scaffold of the Ras pathway?”, *Journal of cell science*, 114(Pt 9), pp. 1609–1612.
- Okamoto, M., H. Takemori and Y. Katoh, 2004, “Salt-inducible kinase in steroidogenesis and adipogenesis”, *Trends in Endocrinology & Metabolism*. Elsevier, 15(1), pp. 21–26. doi: 10.1016/j.tem.2003.11.002.
- Özcan, F., 2003, *Identification of Putative Serin/Threonine Kinase Implicated in FGF Signal Transduction and Its Compatibility with an FGF Pathway Simulation Model*, Ph.D. Thesis, Boğaziçi University.
- Özmen, Y., 2006, *SIK2 Expression in Retinal Cells and Its Possible Involvement Along with PKA in FGF9 Signal Transduction*, Ph.D. Thesis, Boğaziçi University.
- Pawson, T., 2007, “Dynamic control of signaling by modular adaptor proteins”, *Current Opinion in Cell Biology*, 19(2), pp. 112–116.
- Pearson, G., F. Robinson, T. Beers Gibson, B. Xu, M. Karandikar, K. Berman and M. H. Cobb, 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.
- Pierre, S., A. S. Bats and X. Coumoul, 2011, "Understanding SOS (Son of Sevenless)", *Biochemical Pharmacology*, 82(9), pp. 1049–1056. doi: 10.1016/j.bcp.2011.07.072.
- Powers, C. J., S. W. McLeskey and A. Wellstein, 2000, “Fibroblast growth factors, their

- receptors and signaling”, *Endocrine-Related Cancer*, 7(3), pp. 165–197. doi: 10.1677/erc.0.0070165.
- Rajala, A., A. K. Dilly and R. V. S. Rajala, 2013, “Protein tyrosine phosphatase-1B regulates the tyrosine phosphorylation of the adapter Grb2-associated binder 1 (Gab1) in the retina”, *Cell Communication and Signaling*, 11(1), pp. 1–15. doi: 10.1186/1478-811X-11-20.
- Ramos, J. W., 2008, “The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells”, *The International Journal of Biochemistry & Cell Biology*, 40(12), pp. 2707–2719.
- Roskoski Jr., R., 2012, “ERK1/2 MAP kinases: Structure, function, and regulation”, *Pharmacological Research*, 66(2), pp. 105–143.
- Roux, P. P. and J. Blenis, 2004, “ERK and p38 MAPK-Activated Protein Kinases: a Family of Protein Kinases with Diverse Biological Functions”, *Microbiology and Molecular Biology Reviews*. American Society for Microbiology, 68(2), pp. 320–344. doi: 10.1128/MMBR.68.2.320-344.2004.
- Schutzman, J. L., C. Z. Borland, J. C. Newman, M. K. Robinson, M. Kokel and M. J. Stern, 2001, “The *Caenorhabditis elegans* EGL-15 Signaling Pathway Implicates a DOS-Like Multisubstrate Adaptor Protein in Fibroblast Growth Factor Signal Transduction”, *Molecular and Cellular Biology*. American Society for Microbiology, 21(23), pp. 8104–8116. doi: 10.1128/MCB.21.23.8104-8116.2001.
- Screaton, R. A., M. D. Conkright, Y. Katoh, J. L. Best, G. Canettieri, S. Jeffries, E. Guzman, S. Niessen, J. R. Yates III, H. Takemori, M. Okamoto and M. Montminy, 2004, “The CREB Coactivator TORC2 Functions as a Calcium- and cAMP-Sensitive Coincidence Detector”, *Cell*. Elsevier, 119(1), pp. 61–74. doi: 10.1016/j.cell.2004.09.015.
- Shaul, Y. D. and R. Seger, 2006, “ERK1c regulates Golgi fragmentation during mitosis”,

Journal of Cell Biology, 172(6), pp. 885–897. doi: 10.1083/jcb.200509063.

Therrien, M., N. R. Michaud, G. M. Rubin and D. K. Morrison, 1996, “KSR modulates signal propagation within the MAPK cascade”, *Genes and Development*, 10(21), pp. 2684–2695. doi: 10.1101/gad.10.21.2684.

Thien, C. B. F. and W. Y. Langdon, 2001, “Cbl: many adaptations to regulate protein tyrosine kinases”, *Nat Rev Mol Cell Biol.* Nature Publishing Group, 2(4), pp. 294–307.

Torii, S., K. Nakayama, T. Yamamoto and E. Nishida, 2004, “Regulatory Mechanisms and Function of ERK MAP Kinases”, *The Journal of Biochemistry*, 136(5), pp. 557–561. doi: 10.1093/jb/mvh159.

Torii, S., T. Yamamoto, Y. Tsuchiya and E. Nishida, 2006, “ERK MAP kinase in G cell cycle progression and cancer.”, *Cancer science*, 97(8) pp. 697–702. doi: 10.1111/j.1349-7006.2006.00244.x.

Turner, N. and R. Grose, 2010, “Fibroblast growth factor signalling: from development to cancer”, *Nat Rev Cancer.* Nature Publishing Group, 10(2), pp. 116–129.

von Stechow, L., C. Francavilla and J.V. Olsen, 2015, "Recent findings and technological advances in phosphoproteomics for cells and tissues", *Expert Review of Proteomics.* Informa Healthcare, 12(5), pp. 469–487. doi: 10.1586/14789450.2015.1078730.

Wang, Z., H. Takemori, S. K. Halder, Y. Nonaka and M. Okamoto, 1999, “Cloning of a novel kinase (SIK) of the SNF1/AMPK family from high salt diet-treated rat adrenal 1”, *FEBS Letters*, 453(1–2), pp. 135–139. doi: 10.1016/S0014-5793(99)00708-5.

Wiedemann, M. and B. Trueb, 2000, “Characterization of a Novel Protein (FGFRL1) from Human Cartilage Related to FGF Receptors”, *Genomics*, 69(2), pp. 275–279.

- Wöhrle, F. U., R. J. Daly and T. Brummer, 2009, “Function, regulation and pathological roles of the Gab/DOS docking proteins”, *Cell Communication and Signaling: CCS*. BioMed Central, 7, p. 22. doi: 10.1186/1478-811X-7-22.
- Wortzel, I. and R. Seger, 2011, “The ERK Cascade: Distinct Functions within Various Subcellular Organelles.”, *Genes & Cancer*, 2(3), pp. 195–209. doi: 10.1177/1947601911407328.
- Yang, S.-H., A. D. Sharrocks and A. J. Whitmarsh, 2013, “MAP kinase signalling cascades and transcriptional regulation”, *Gene*, 513(1), pp. 1–13.
- Yılmaz Sert, Y., 2011, *SIK2 Involvement in Downregulation of FGF Signaling through Gab1 and Raf-1*, M.S. Thesis, Boğaziçi University
- Yun, Y.-R., J. E. Won, E. Jeon, S. Lee, W. Kang, H. Jo, J.-H. Jang, U. S. Shin and H.-W. Kim, 2010, “Fibroblast growth factors: biology, function, and application for tissue regeneration.”, *Journal of tissue engineering*, 2010, p. 218142. doi: 10.4061/2010/218142.
- Yung, Y., Z. Yao, T. Hanoch and R. Seger, 2000, “ERK1b, a 46-kDa ERK isoform that is differentially regulated by MEK”, *Journal of Biological Chemistry*, 275(21), pp. 15799–15808. doi: 10.1074/jbc.M910060199.
- Zhao, Y. and Z.-Y. Zhang, 2001, “The Mechanism of Dephosphorylation of Extracellular Signal-regulated Kinase 2 by Mitogen-activated Protein Kinase Phosphatase 3”, *Journal of Biological Chemistry*, 276(34), pp. 32382–32391. doi: 10.1074/jbc.M103369200.