SIK2 INVOLVEMENT IN DOWNREGULATION OF FGF SIGNALING THROUGH GAB1 AND RAF-1

by

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ABSTRACT

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Proliferative response of Müller cells to FGF2 is propagated via Ras/MAPK pathway, and involves rapid and transient ERK1/2 activation. Results from our laboratory implicate SIK2 in the regulation of FGF2 signaling via serine/threonine phosphorylation of pathway elements Gab1 and Raf1, on Ser^{266} and Ser^{621} respectively. We propose that these phosphorylations hamper interaction with the partners to activate downstream events. In this study to test the hypothesis, Ser^{266} on Gab1 and Ser^{621} on Raf-1 were mutated to alanine via site-directed mutagenesis. As Ser^{621} has been described as an autophosphorylation site, Raf-1 was also rendered kinase inactive. Wild type and mutant proteins expressed in HEK 293T cells were purified by immunoprecipitation and were used for *in vitro* kinase assay. Results obtained from *in vitro* kinase assays indicated that the mutations obliterated the phosphorylation by SIK2, thus verified that SIK2 targets these serine residues. Co-immunoprecipitation studies revealed that S266A mutation increases FGF dependent Gab1-Shp2 binding, no differences were evident in Grb2-Gab1 interaction. Therefore, it is conceivable that the Ser^{266} phosphorylation by SIK2 is important in transient nature of Gab1 interaction with Shp2 and might regulate FGF-dependent ERK activation. The mutation led to modest enhancement of FGF dependent proliferation in MIO-M1 cells.

ÖZET

SIK2'NİN GAB1 ve RAF-1 ÜZERİNDEN FGF SİNYAL YOLAĞININ NEGATİF REGÜLASYONUNA KATILIMI

Müller hücrelerinin FGF2'ye karşı proliferasyon cevabı Ras/MAPK yolağı üzerinden, hızlı ve geçici ERK 1/2 aktivasyonu ile oluşturulmaktadır. Laboratuvarımızdan çıkan sonuçlar SIK2'nin, yolak elemanlarından Gab1 ve Raf-1 proteinlerini sırasıyla Ser^{266} ve Ser^{621} üzerinden fosforlaması ile FGF2 yolağını regüle ettiğini düşündürmektedir. Negatif regülasyon sürecinde gerçekleşecek olayların aktivasyonu için bu fosforlanmaların bağlanma partnerleri ile olan etkileşimleri engellediğini önermekteyiz. Bu hipotezi doğrulamak adına bu çalışma kapsamında, *in vitro* mutagenez yöntemi ile Gab1 üzerindeki Ser^{266} ve Raf-1 üzerindeki Ser^{621} amino asitleri alanine dönüştürülmüştür. 621. serin amino asidinin otofosfarilasyon noktası olması nedeniyle, Raf-1 proteini kinaz inaktif kılınmıştır. HEK293T hücrelerinde anlatımları sağlanan yabanıl ve mutant proteinler, *in vitro* kinaz deneyinde kullanılmak üzere immünçökeltme yöntemi ile izole edilmiştir. *İn vitro* kinaz deneyinden elde edilen sonuçlar bu mutasyonların SIK2 tarafından gerçekleştirilen fosforilasyonları engellediğini göstermiştir, böylece SIK2'nin bu serin amino asitlerini hedef aldığı doğrulanmıştır. Bağlanma partnerleri ile beraber immünçökeltme çalışmaları S266A mutasyonunun FGF uyarımına bağlı Gab1-Shp2 etkileşimini arttığına işaret ederken, Gab1-Grb2 etkileşiminde ise belirgin bir fark görülmemiştir. Bu veriler, SIK2 tarafından gerçekleştirilen Ser^{266} fosforlanmasının Gab1 proteininin Shp2'ye geçici bağlanmasında önemli olduğu ve FGF'e bağlı ERK aktivasyonunu regüle ettiği düşündürmektedir. Bu mutasyonun MIO-M1 hücrelerinin FGF'e bağlı proliferasyonunu bir miktar arttırdığı görülmüştür. Verilerimiz SIK2'nin FGF2 yolağını Gab1 ve Raf-1 proteinlerini, sırasıyla Ser^{266} ve Ser^{621} üzerinden fosforlaması ile regüle ettiği önerisini desteklemektedir.

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LIST OF ACRONYMS/ ABBREVIATIONS

A-loop	Activation Loop
AB	Acid Box
ACTH	Adenocorticotropic Hormone
AICAR	Activator 5-amino-4-imidazolecarboxamide
AMP	Adenosine Monophosphate
AMPK	AMP-activated Kinase
APS	Amonium Persulfate
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
BDNF	Brain-derived Neurotrophic Factor
bp	Base Pair
BSA	Bovine Serum Albumine
$CaCl_2$	Calcium Chloride
cAMP	Cyclic Adenosine 5'-monophosphate
cDNA	Complementary DNA
Cbl	Casitas B-lineage Lymphoma
C. Elegans	Caenorhabditis Elegans
CR	Conserved Region
CRD	Cysteine Rich Domain
CRE	cAMP-response Element
CREB	CRE Binding Protein
ChREBP	Carbohydrate Response Element-binding Protein
Co-IP	Co-immunoprecipitation
CO_2	Carbondioxide
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate

Dos	Daughter of Sevenless
DTT	Dithiothreitol
EBC	Extraction Buffer C
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ERK	Extracellular Regulated Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FBS	Fetal Bovine Serum
Frs2	Fibroblast Growth Factor Receptor Substrate
Gab	Grb2-asssociated Binder
GABA	Gamma –aminobutyric Acid
GFP	Green Fluorescent Protein
Grb2	Growth Factor Receptor-Bound Protein
GST	Glutathione S Transferase
H_2O	Water
HBS	HEPES Balanced Salt
HDAC	Class IIa Histone Deacetylase
HEK	Human Embryonic Kidney Cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HRP	Horse Radish Peroxidase
HSPG	Heparan Sulfate 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	Kilobase
kDa	Kilodalton
KI	Kinase Inactive

LB	Luria Broth
LKB1	Liver Kinase B 1
mA	Miliamper
MAPK	Mitogen-activated Protein Kinase
MEK	MAPK/ERK Kinase
MIO-M1	Moorfields/Institute of Opthalmology-Müller 1
mg	Miligram
$MgCl_2$	Magnesium Chloride
min.	Minutes
MKP	MAPK Phosphatase
ml	Mililiter
mm	Millimeter
mM	Milimolar
MP	Milk Powder
NaCl	Sodium Chloride
NGF	Nerve Growth Factor
Nm	Nanometer
ng	Nanogram
OD	Optical Density
ORF	Open Reading Frame
PAGE	Polyacylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pen/Strep	Penicillin/Streptomycin
PFA	Paraformaldehyde
PH	Plekstrin Homology
PI	Phosphatidylinositol-4,5-diphosphate
PI3K	Phosphoinositide 3-kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLCγ	Phospholipase C-gamma,
PME	Phosphatase 2A/phosphatase Methylesterase
pSer	Phospho Serine

PTB	Phosphotyrosine Binding
PVDF	Polyvinyl Difluoride
Raf	Rapidly Accelerated Fibrosarcoma
Ras	Rat Sarcoma
RBD	Ras Binding Domain
RGC	Retinal Ganglion Cells
RNA	Ribonucleic Acid
RPE	Retina Pigmented Epithelium
rpm	Rotations Per Minute
RTK	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS- polyacrylamide Gel Electrophoresis
sec.	Seconds
Sef	Similar Expression of FGF Genes
Ser	Serine
SH	Src Homology Domain
Shc	Src Homology 2 Domain Containing Transforming Protein
Shp2	SH2-domain Containing Phosphatase 2
SIK	Salt Inducible Kinase
SNF	Sucrose Nonfermenting
SNH	Sucrose Nonfermenting Homology
Soc	Suppressor of Clear
Sos	Son of Sevenless
SREBP	Sterol Regulatory Element Binding Proteins
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TEMED	Tetramethylethylenediamine
TORC2	Transducer of Regulated CREB Activity 2
TWEEN	Polysorbate
UBA	Ubiqitin-associated Domain
WB	Western Blot
WT	Wild-type
X-Gal	Bromo-chloro-indolyl-galactopyranoside

1. INTRODUCTION

1.1. Müller Cells

Major glial cells of the mammalian neural retina are the Müller cells (Bringmann *et al*., 2006). They span the entire thickness of the retina, interact with most of the retinal neurons and thus form an anatomical link between retinal neurons and the exchange compartments of the retina, like blood vessels, vitreous body and subretinal space (Bringmann *et al.*, 2009a). They contribute to maintain structural integrity, homeostasis and information processing. (Bringmann and Reichenbach, 2001). They also have important roles in the energy metabolism of the tissue; glutamate and GABA removal and recycling, modulation of neuronal excitability, regulation of extracellular space volume, extracellular pH in the retina and provide growth factors for neuronal survival (Tsacopoulos and Magistretti, 1996; Newman and Reichenbach, 1996; Bringmann *et al.*, 2009a). In many retinal injury cases and diseases, including ischemia, glaucoma, age-related macular degeneration and diabetic retinopathy, Müller glial cells are activated (Bringmann *et al.*, 2009b). Activated Müller cells release neurotrophic factors including FGF2, BDNF, IGF-1 and NGF which are thought to induce survival and proliferation of neurons and glial cells. However, in the long term proliferating Müller cells often result in glial scar formation and retinal detachment and finally blindness (Bringmann and Wiedemann, 2009). A small population of Müller cells preserve ability to undergo dedifferentiation to progenitor like cells and transdifferentiation to neurons (Fischer and Reh, 2001; Karl and Reh, 2010; Lawrence *et al*., 2007; Fischer and Bongini, 2010).

MIO-M1 cells are established as a spontaneously immortalized cell line derived from the Müller glia of a postmortem human retina, it has been shown that MIO-M1 cells express both Müller glial markers as well as stem cell markers (Limb *et al.*, 2002; Lawrence *et al*., 2007). Similar to primary Müller cells in culture, FGF2 and FGF9 induce proliferation via activation of ERK1/2 (Hollborn *et al*., 2004, Kinkl *et al.*, 2001; Çınaroğlu, 2005).

1.2. Fibroblast Growth Factors

Fibroblast growth factors (FGFs) 1 and 2 are among the first growth factors that have been identified (Armelin, 1973; Gospodarowicz, 1974). In 1986 FGF2 was cloned and characterized (Abraham *et al.*, 1986). Today 22 members of the family are known in the human genome. The FGFs are conserved from nematodes to humans (Ornitz *et al.*, 2001). Phylogenetic relationships classify mammalian FGFs into seven subfamilies (Itoh and Ornitz, 2004) (Figure 1.1).

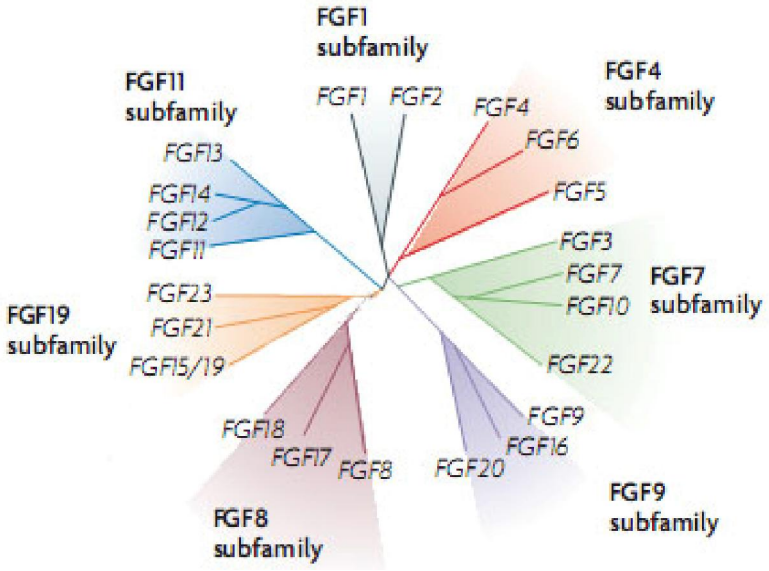

Figure 1.1. FGF subfamilies. Seven FGF subfamilies are shown according to their phylogenetic relationships. Human FGF19 is the orthologue of mouse FGF15 (Modified from Mason, 2007).

All members have conserved gene structure and the size of the coding region of FGF genes ranges from under 5 kb (FGF3 and FGF4) to over 100 kb (FGF12). The molecular weight of FGF proteins ranges between 17 to 34 kDa. The protein structure is similar among the family members with the internal 120 amino acid core region where 28 are highly conserved and 6 are identical (Ornitz, 2000). Ten of the amino acids are especially important in their interaction with FGF receptors (FGFR) (Plotnikov *et al.*, 2000). FGFs may be subjected to post-translational modification and alternative splicing

resulting in variable affinities for their receptors and thus affect their function (Olsen *et al.*, 2006).

Many FGFs have signal peptide sequences at their amino terminal leading to secretion. However, FGF 1, 2, 9, 16 and 20 lack the signal peptide and are secreted via an alternate path (Powers *et al.*, 2000). FGFs 11-14 are not secreted and do not function as FGFR ligands but they are localized to the cell nucleus (Goldfarb, 2005).

FGFs in different cellular contexts may elicit proliferation, differentiation or survival responses. They have important roles in processes such as angiogenesis, tissue repair and embryonic development of various organs including the nervous system (Ornitz *et al.*, 2001; Lamb and Harland, 1995; Reuss *et al.*, 2003). *In vitro* studies suggest that they play critical roles in axon guidance and synapse formation (Shirasaki *et al*., 2006; Dai and Peng, 1995). FGF 19, 21 and 23 were also found to be functioning as hormones that regulate bile, fatty acid, phosphate and glucose metabolisms in target organs (Kurosu *et al.*, 2007). They have poor heparin sulphate proteoglycan affinity; via binding of a transmembrane protein Klotho, they were shown to signal through FGFRs.

FGF expression is observed in developing eye and in adult retina. FGF 1, 2 and 9 are found to be mainly expressed in adult mammalin retina (Bugra *et al.*, 1994; Bugra and Hicks., 1997; Fischer *et al*., 2004). FGFs 1, 2 and 9 are found to have proliferative effect on Müller cells (Mascarelli *et al.*, 1991; Çınaroğlu *et al*., 2005). FGF8 plays a role in differentiation of retinal pigment epithelium cells into retinal neurons and its effect in induction of lens formation has been established (Vogel-Höpker *et al*., 2000). FGF15 expression is found in retinal progenitor cells at early stages of development and in ganglion and amacrine cells at later stages (Kurose *et al*., 2004). In early mouse embryo, FGF9 ectopic expression in retinal pigment epithelial cells causes transdifferentiation of these cells (Zhao and Overbeek; 1999). FGF1, FGF2, FGF9 have been shown to promote retinal ganglion cell survival *in vitro* (Desire *et al*., 1998; Kinkl *et al*., 2003), whereas FGF1, FGF2, FGF5, FGF18 and FGF19 are shown to have roles in photoreceptor survival (Faktorovich *et al*., 1990; Green *et al*., 2001; Siffroi- Fernandez *et al*., 2008).

1.3. Fibroblast Growth Factor Receptors

The FGF signaling is transduced through the transmembrane receptor tyrosine kinases (RTKs) with the aid of heparin (Ornitz and Itoh, 2001). FGFR family consists of 4 members, FGFR 1-4, with 55%-72% homology at protein level. FGF receptors share a common structure with three IgG like domains, an acid box (AB) and kinase domain (Figure 1.2). Alternative splicing of Ig III domain generates many FGFR isoforms (Figure 1.3), with different ligand affinities and contribute to differential responses obtained by different FGFs (Powers *et al*., 2000).

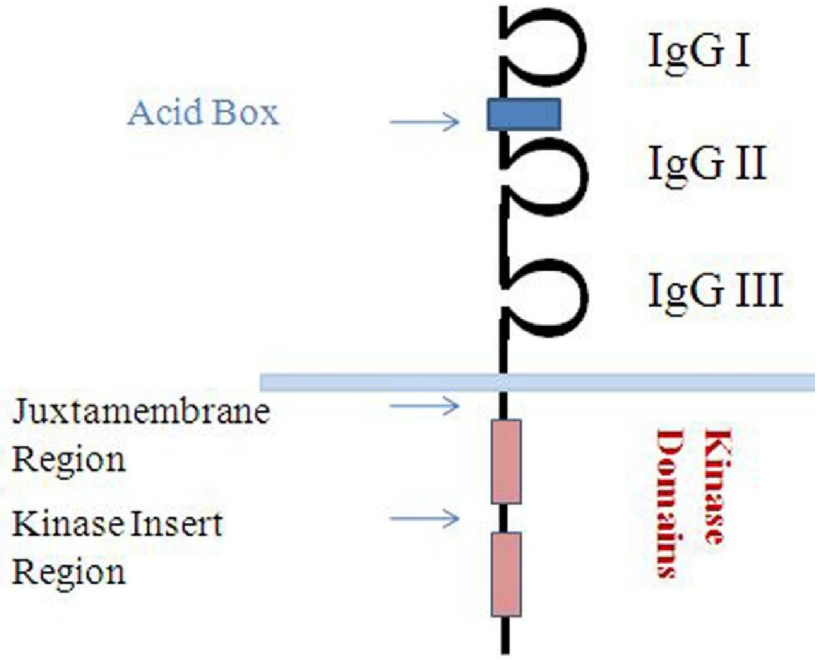

Figure 1.2. Structure of the fibroblast growth factor receptors. The extracellular domains of FGFRs consist of three IgG like loops (IgG I-III) and an acid box, located between IgG I and IgG II which is composed of seven to eight acidic residues.

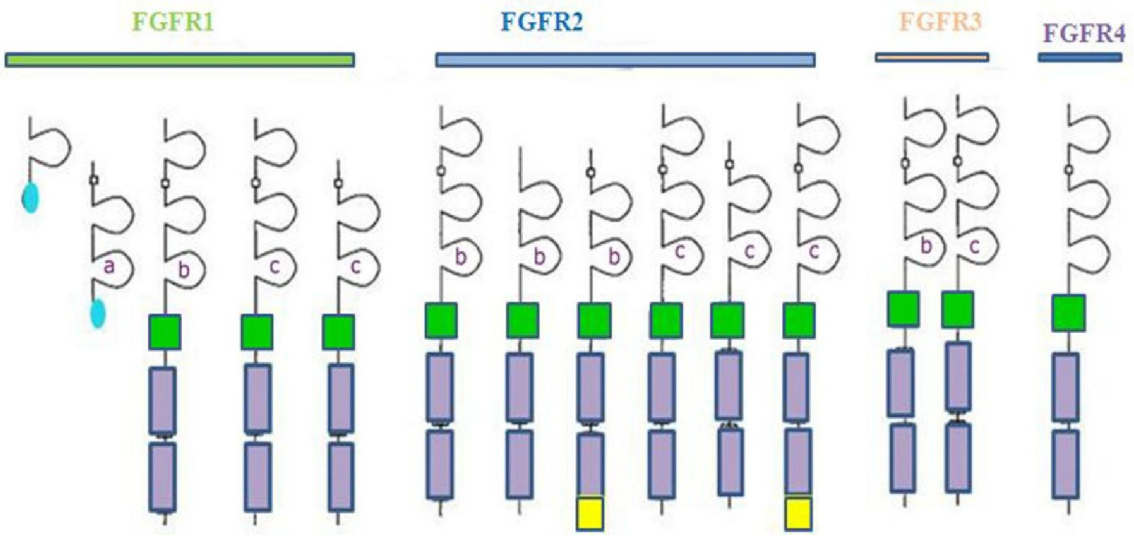

Figure 1.3. FGFR alternative splicing variants. The exons coding for IgIII domains are a, b, c. Blue box: truncation, green box: transmembrane domain, purple box: tyrosine kinase domains, yellow box: alternative C-terminal (Modified from Powers *et al*., 2000).

FGFR1 and FGFR2 knock-out mice die at early stages due to severe developmental defects (Deng *et al*., 1997; Arman *et al*., 1998). FGFR3 and FGFR4 knock-out mice were viable but have many developmental defects (Weinstein *et al*., 1998).

In vertebrates, all FGF receptors are expressed in the retina (Kinkl *et al*., 2002). It has been shown that all FGFR1, FGFR2 and FGFR3 isoforms are expressed in Müller cells (Çınaroğlu *et al*., 2005).

1.4. FGF Receptor Dimerization and Activation

Like many RTKs, FGFRs transmit the extracellular signal through dimerization and trans-tyrosine phosphorylation (Schlessinger, 1988). Heparin or heparin sulphate proteoglycans (HSPGs) are required for FGF-FGFR interaction and increase the half-life of FGF-FGFR complex (Yayon *et al*., 1991; Ornitz and Itoh, 2001; Harmer *et al*., 2004).

Ligand-dependent dimerization creates seven phosphotyrosine sites in the cytoplasmic tail of FGFR1; Tyr^{463} in the juxtamembrane domain, Tyr^{583} and Tyr^{585} in the kinase insert domain, Tyr^{653} and Tyr^{654} in the activation loop of the kinase domain, Tyr^{730}

and Tyr^{766} in the C-terminal tail. All these sites are also conserved in FGFR2 (Furdui *et al.*, 2006; Mohammadi *et al.*, 1996; Lundin *et al.*, 2003). Phosphorylated tyrosine residues create docking sites for intracellular signaling molecules containing Src-homology-2 (SH2) domains (Pawson, 1995) leading to the activation of alternative signaling pathways.

1.5. FGF Signaling Pathways

There are three major pathways that are activated by FGFRs (Figure 1.4): Phospholipase-C-γ (PLCγ)/Ca^{+2}, phosphoinositide 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog (Akt) and rat sarcoma (Ras)/mitogen activated kinase-like protein (MAPK) pathways (Mason, 2007). Besides these 3 main pathways, there are alternate pathways such as v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (Src) signaling pathway or v-crk sarcoma virus CT10 oncogene homolog (Crk) mediated pathway (Mason, 2007). Through its SH2 domain, Crk binds to activated FGFR and form Crk-Src homology 2 domain containing transforming protein (Shc)-Son of Sevenless (Sos) complex, this complex might regulate activation of p38 and Jun kinases and cytoskeletal rearrangement. The mechanism of Src pathway activation is not yet clear (Wong *et al.*, 2002; Dailey *et al.*, 2005).

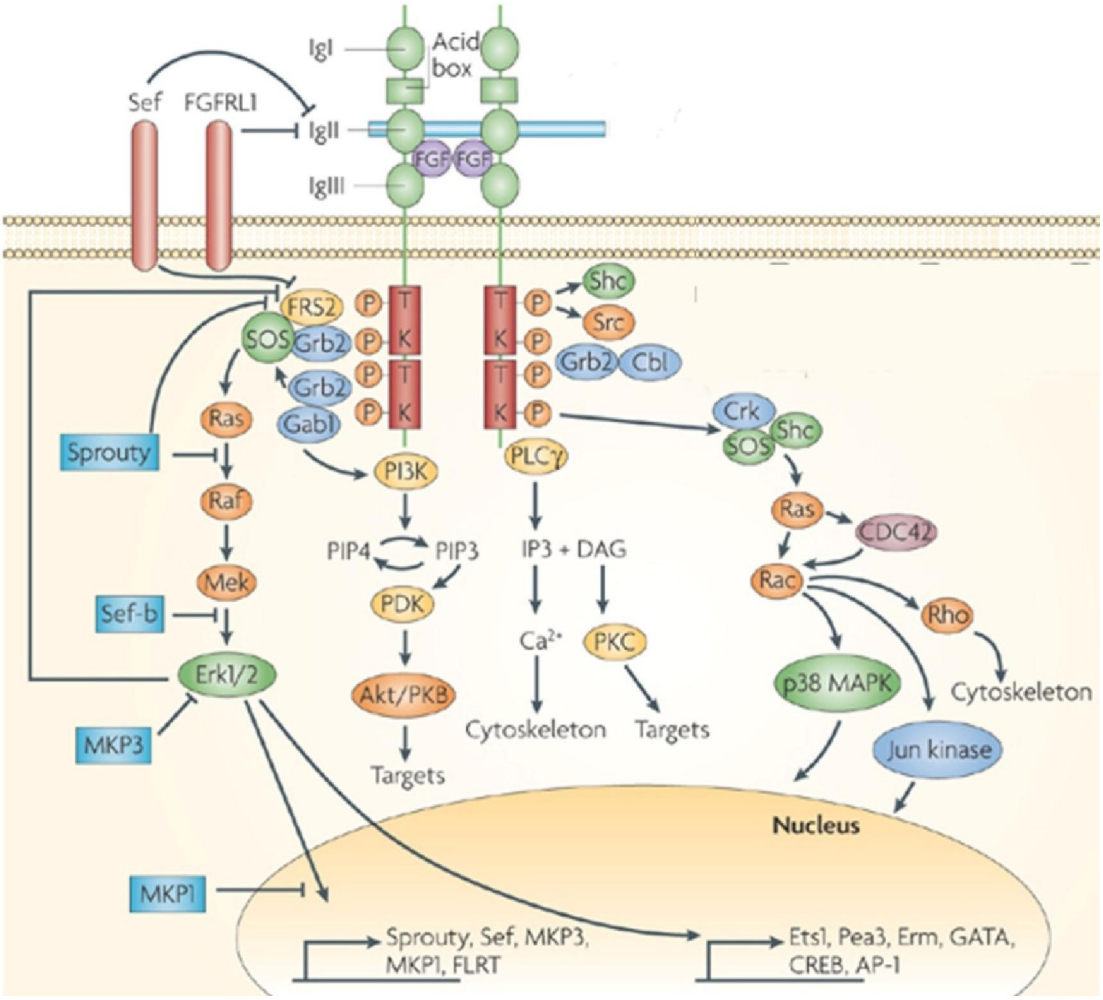

Figure 1.4. FGF Signaling Pathways. Three main pathways activated by FGFs are Ras/MAPK pathway, PI3K/AKT pathway and PLCγ/Ca^{+2} pathway (Modified from Mason, 2007).

1.5.1. PLCγ/Ca^{+2} signaling pathway

PLCγ is the first protein in the pathway that is activated by autophosphorylated FGFRs. Autophosphorylation of Tyr^{766} in carboxyl tail of FGFRs is required for PLCγ binding (Maffucci *et al.*, 2009; Mohammadi *et al.*, 1991). Activated PLCγ then hydrolyzes phosphatidylinositol-4,5-diphosphate (PI) to diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). DAG activates protein kinase C (PKC), whereas IP3 triggers the release of calcium ions from intracellular compartments (Burgess *et al.*, 1990; Mohammadi *et al.*, 1991). Activated protein kinase C reinforces the activation of the MAPK pathway by phosphorylating rapidly accelerated fibrosarcoma-1 (Raf-1) (Huang *et al.*, 1995).

1.4.1. PI3 kinase/Akt pathway

When FGFRs are autophosphorylated, there are three mechanisms that PI3K/Akt pathway is activated. In the first one, phosphorylated tyrosine residues recruit SH2 domain containing PI3K regulatory subunit, p85 (Salazar *et al.,* 2009). The second mechanism relies on "Grb2 Associated Binder1 (Gab1)-Fibroblast Growth Receptor Substrate 2 (Frs2)-Growth Factor Receptor Bound Protein 2 (Grb2)" complex to recruit p85 subunit of PI3K to membrane (Ong *et al.,* 2001). The last mechanism depends on recruitment of PI3K to cell membrane by activated Ras (Rodriguez-Viciana *et al*., 1994). Activated PI3K then activates Akt, which results in phosphorylation and inactivation of apoptotic proteins (Schlessinger, 2000).

1.4.2. Ras/MAPK pathway

Activated FGFR recruits Frs2 and activates it (Kouhara *et al.,* 1997), this creates docking sites for Grb2, Sos and SH2 domain containing protein tyrosine phosphatase 2 (Shp2). Tyr^{436} phosphorylation of Frs2 is required for efficient Shp2 recruitment, whereas Tyr^{196} phosphorylation functions as a docking site for Grb2-Sos complexes (Kouhara *et al.*, 1997; Hadari *et al.,* 1998; Eswarakumar *et al*., 2005). This complex then interacts with small G-protein Ras and activates it, activated protein recruits Raf, which becomes a target of several kinases. Activated Raf in turn kinases MAPK/ERK Kinase (MEK), followed by Extracellular Signal Regulated Kinase (ERK) activation. This activation allows phosphorylation of target transcription factors and results in changes in gene expression pattern (Kouhara *et al.*, 1997).

1.6. Negative Regulation of Ras/MAPK Pathway

Since FGF pathway is involved in critical cell fate decisions, its control is vital for the cell. A tight regulation of FGF signaling is achieved by several mechanisms. One of the mechanisms for tight regulation of FGF signaling is through receptor downregulation by Casitas B-lineage Lymphoma (Cbl) binding. Cbl is recruited via Grb2 binding which results in ubiquitination and subsequent degradation of FGFR and Frs2 (Wong *et al.*, 2002).

Second mechanism involved in downregulation of FGF signaling requires inhibition through phosphorylation and dephosphorylation events. The most well studied proteins in this context are Sprouty, MAPK phosphatase 3 (MKP3), MAPK phosphatase 1, (MKP-1), and Similar expression of FGF genes (Sef) family members. It has been shown that four members of Sprouty have differential preferences in terms of FGFR isoforms and inhibit Ras/MAPK pathway at the level of Raf activation (Yusoff *et al*., 2002). Sprouty may also compete with Sos1 for Grb2 binding in the pathway (Lao *et al*., 2006). Sef proteins inhibit MEK phosphorylation of ERK; overexpression of Sef results in a decrease in Frs2 and FGFR1 phosphorylation levels and FGF induced cell proliferation (Kovalenko *et al.,* 2003). As their name imply, MAPK phosphatases work on dephosphorylation of ERK 1/2 on phosphotyrosine and phosphothreonine residues (Farooq and Zhou, 2004).

Finally, ERK in a negative feedback loop downregulates Ras/MAPK signaling (Lax *et al.,* 2002; Gotoh, 2008). ERK1 and ERK2 have been shown to phosphorylate Frs2 on eight canonical ERK phosphorylation residues when activated by FGFs, thus inhibit the recruitment of Grb2 through Frs2.

1.7. Gab Family

Gab proteins belong to a family of scaffold proteins (Gu and Neel, 2003). Gab1 was first isolated from a human glial tumor expression library (Holgado-Madruga *et al*., 1996); later Gab2 and Gab3 were identified based on sequence similarities. They range in size from 586 to 695 amino acids (Gu *et al.,* 1998; Wolf *et al.,* 2002). C. Elegans homolog is Suppressor of Clear 1 (Soc1), Drosophila homolog Daughter of sevenless (Dos) was first identified as a potential substrate of Corkscrew, Drosophila homolog of Shp2 (Herbst *et al.*, 1996).

Gab proteins are involved in several signaling pathways stimulated by many ligands (Table 1.1) such as epidermal growth factor (EGF), insulin, interleukins, interferons, erythropoietin and thrombopoietin (Liu and Rohrschneider., 2002).

Table 1.1. List of pathways involving Gab1 (Modified from Liu and Rohrschneider, 2002).

Receptor	Ligand
Epidermal growth factor receptor	Epidermal growth factor
Insulin receptor	Insulin
Met	Hepatocyte growth factor
Fetal liver kinase receptor 2	Fetal liver kinase 2 ligand
Stem cell growth factor receptor	Stem cell factor
Neurotrophic tyrosine kinase receptor	Nerve growth factor
Platelet-derived growth factor receptor	Platelet-derived growth factor
B cell receptor	Immunoglobin M
T cell receptor	Cluster of differentiation 3ε
Interleukin 2 receptor	Interleukin 2
Interleukin 3 receptor	Interleukin 3
Interleukin 15 receptor	Interleukin 15
Interleukin 6 signal transducer	Interleukin 6
Interpheron γ receptor	Interpheron γ
Interpheron α receptor	Interpheron α
Granulocyte-colony stimulating factor receptor	Granulocyte-colony stimulating factor
Granulocyte-macrophage colony stimulating factor receptor	Granulocyte-macrophage colony stimulating factor
Erythropoietin receptor	Erythropoietin
Thrombopoietin receptor	Thrombopoietin
Prolactin receptor	Prolactin

Domains that are shared between Gab family members are N-terminal Pleckstrin Homology (PH) domain, proline rich region that is centrally located and also multiple tyrosine phosphorylation sites that allow Gab1 to interact with several proteins' SH2 domains (Rodrigues *et al.,* 2000) (Figure 1.5). PH domains are required for Gab recruitment to the membrane, where it binds phosphatidylinositol-3,4,5-triphosphate (PIP3) (Lamothe *et al*., 2004). Proline rich region of Gab proteins facilitate interaction with SH3 domain containing proteins such as Grb2 (Feng *et al.,* 1994). A recent study has revealed

that one molecule of Sos1 binding to the SH3 domain of Grb2, allosterically favors Gab1 binding to SH3 domain of Grb2 in a tertiary complex (Mcdonald *et al*., 2010).

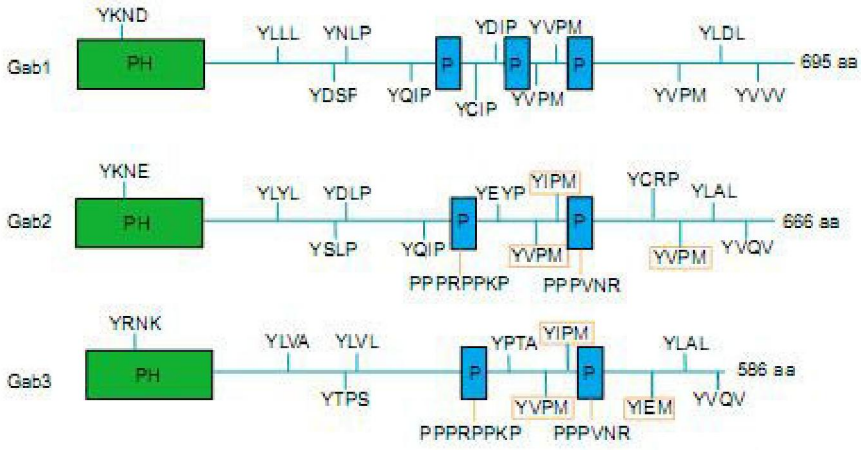

Figure 1.5. Gab family. Green boxes represent plecstrin homology domain, blue boxes indicate proline rich region, tyrosine residues that are phosphorylated are indicated in the figure (Modified from Gu and Neel, 2003).

Shp2 and p85 subunit of PI3K are well defined interaction partners of Gab. The interaction between Gab-p85 is required for activating Akt pathway in response to FGF stimulation (Ong *et al*., 2001). The tyrosines that are located closer to C terminal constitute the binding motif (YXXV/I/L) of Shp2 (Liu and Rohrschneider, 2002). When Tyr^{627} and Tyr^{659} are phosphorylated, Shp2-Gab1 binding is enhanced (Cunnick *et al*., 2001).

Schaeper *et al.* (2007) have shown that Gab1-Shp2 binding is crucial for Met-receptor directed placental development and cell migration. Bard-Chapeau *et al.* (2006) generated Gab1 and Shp2 knock-out mice and have shown that in both cases ERK1/2 levels were decreasing and there were defects in liver regeneration. Similarly, Gab1 deficiency in Shp2 binding resulted in a decrease in ERK1/2 activation levels with respect to control under insulin-like growth factor stimulation (Koyama *et al*., 2008).

1.8. Raf Family

Raf is as a serine/threonine protein kinase and is a well known proto-oncogene. The family of Raf consists of three members; A-Raf, B-Raf/RafB1and C-Raf/Raf-1 (Figure 1.6). Raf-1 was first isolated from murine sarcoma virus in 1984 (Mark and Rapp, 1984). All three isoforms share three conserved regions (CR) with distinct functions; two of CRs are located in amino-terminus; CR1 contains Ras binding domain (RBD) that is required for Raf activation and a cysteine rich domain (CRD) which is involved in Ras binding as well as interaction with the kinase domain for autoinhibition (Chong*et al*., 2003). CR2 is a serine/threonine rich region, these residues when phosphorylated lead to inactivation. The last CR domain, CR3, spans the kinase domain, it is located near carboxyl-terminus, phosphorylation sites on this domain are crucial for the activity of Raf-1 (Chong *et al.*, 2003; Wellbrock *et al.*, 2004).

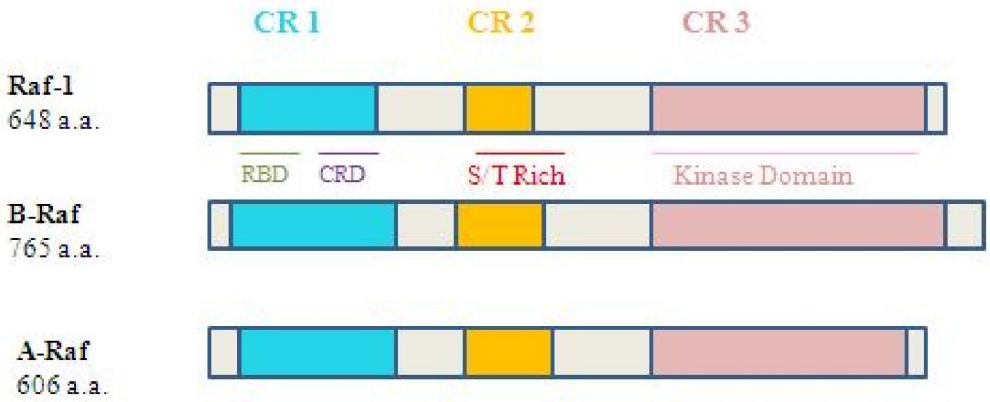

Figure 1.6. Raf family. Raf family has 3 conserved regions; CR1, CR2 and CR3. CR-1 has Ras binding domains RBD and CRD, CR2 is composed of several serine and threonine residues whereas CR3 is the catalytic kinase domain (Modified from Chong *et al.*, 2003).

Raf-1 has a complex mechanism of activation (Figure 1.7). In basal state, Raf-1 is in closed conformation through 14-3-3 binding facilitated by phosphorylated Ser^{259} and Ser^{621} residues. Dephosphorylation of Ser^{259} releases 14-3-3 interaction and allows Ras binding, thus Raf-1 recruitment to the membrane (Yip-Schnedider *et al.*, 2000). Membrane recruited Raf-1 is then phosphorylated on several serine/threonine residues in the activation loop, which is followed by its dimerization and a shift to highly active conformational state. This

state is stabilized by 14-3-3 binding via $pSer^{621}$. $pSer^{621}$ is subjected to phosphorylation by many kinases; Raf-1 itself, Protein kinase A (PKA) and Adenosine monohosphate-activated protein kinase (AMPK), this phosphorylation is shown to have negative effect on Raf-1 activity (Mischak *et al.,* 1996; Sprenkle *et al.,* 1997). This dual role of $pSer^{621}$ is not well understood. Full kinase activation and phosphorylated state allows Raf-1 to interact with MEK (Zhu *et al*., 2005; Zang and Guan, 2000). Deactivation of Raf-1 starts with phosphatase recruitment and dephosphorylation of Ser^{338}. Dephosphorylation allows it to interact with ERK that results in further inactivation (Dougherty *et al*., 2005).

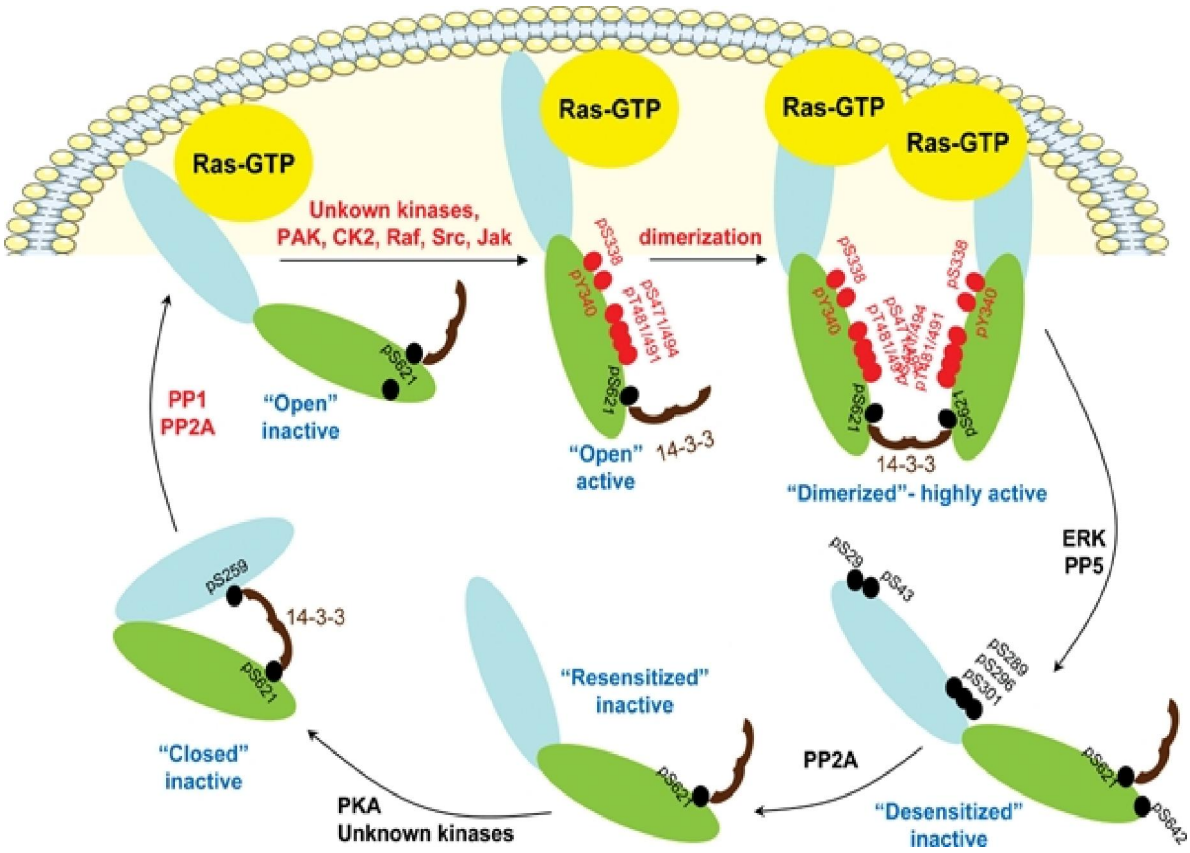

Figure 1.7. Activation/deactivation cycle of Raf1. Raf-1 activity is tightly controlled by phosphorylation and dephosphorylation events (Matallanas *et al*., 2011).

In malignant melanomas, 66% of the cases have been shown to have a mutation on B-Raf and lower frequency of mutations have been identified in other cancer types (Davies *et al.*, 2002). Most frequent B-Raf mutation is an activating mutation V600E that leads the kinase to fold in an active conformation. This mutated B-RAF stimulates sustained and strong activation of ERK and thus trigger melanocyte proliferation and clonal expansion

(Wellbrock and Hurlstone, 2010). A-Raf activation cycle is similar to Raf-1 but the binding affinities are lower (Matallanas *et al*., 2011). It is known that, all three Raf kinases can activate MEK1/2, which in turn activates ERK1/2. A-Raf is weakly activated by Ras and it is not a strong MEK activator (Marais *et al*., 1997). The biological function is still under investigation.

1.9. Salt-Inducible Kinase Family

The salt inducible kinase **(**SIK) family, which consists of 3 serine/threonine protein kinases, belongs to a sucrose-nonfermenting-1 protein kinase (SNF)/ AMPK super family (Bright *et al*., 2009). SIK1 was first isolated from adrenal glands of high-salt diet-fed rats (Wang *et al.*, 1999). Homology search on human and mouse genomes identified two SIK1 related genes (Figure 1.8), named SIK2 and SIK3 (Katoh *et al*., 2004). Both SIK2 and SIK3 genes were found on chromosome 11, while the SIK1 gene is located on chromosome 21 (Katoh *et al.*, 2004).

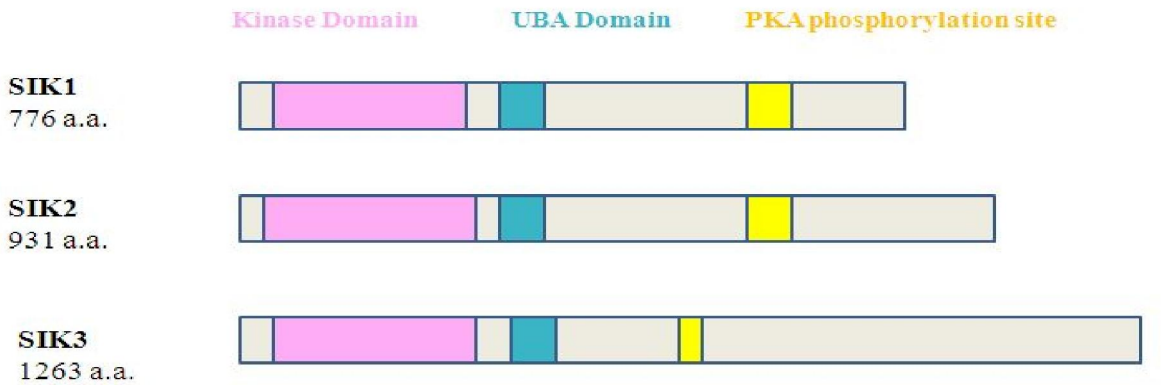

Figure 1.8. Salt-inducible kinase family. SIK family has three isoforms with 3 conserved domains. They have a conserved kinase domain at N-terminal, a SNF homology domain as well as a PKA phosphorylation site at C-terminal end.

The family of AMPKs, including SIKs have flexible activation loops (A-loop) near their substrate binding pocket. When this loop is phosphorylated, a structural change in catalytic site enhanced kinase activity. Findings have shown that tumor suppressor LKB1 phosphorylates threonine residues in A-loop of SIKs (Lizcano *et al.*, 2004) and this phosphorylation creates a binding site for 14-3-3 protein which then results in a stable

active conformation and affects its localization (Al-Hakim *et al.,* 2005, Takemori *et al.,* 2008).

1.9.1. Salt-Inducible Kinase 1 and 3

The SIK1 gene encodes a protein containing 766 amino acids. It has a nuclear import/export domain that spans between amino acids 567-612 (Katoh *et al.,* 2002). In addition, many kinase regulatory sites have been identified; cAMP dependent kinase at Serine-577, calmodulin kinase site at Threonine-322, LKB1 site at Threonine-182 and an autophosphorylation site at Serine-186 (Horike *et al.,* 2002, Jaleel *et al*., 2005; Hashimoto *et al.,* 2008).

SIK1 functions in the regulation of cAMP responsive element-binding (CREB) dependent gene expression. Lin *et al*. (2001) are the first ones to suggest SIK1 repression causes steroidogenic gene expression upon adenocorticotropic hormone (ACTH) stimulation. Later, Doi *et al*. (2002) showed that SIK1 phosphorylates the basic leucine zipper domain of transcription factor CREB in the nucleus and causes repression of target genes. PKA can phosphorylate SIK1 on Ser^{577} and results in its translocation to the cytoplasm (Figure 1.9), thus controls CREB phosphorylation (Takemori *et al*., 2002). Recently, it was found that SIK1 regulates lipogenic gene expression by phosphorylating and inactivating nuclear sterol regulatory element-binding protein-1 (SREBP-1) in liver (Yoon *et al.,* 2009). Another target of SIK1 is class IIa histone deacetylase-5 (HDAC-5), phosphorylation promotes its nuclear export (Cheng *et al*., 2011) and inhibits its deacetylase activity. Since HDAC inhibition is related with neuroprotective mechanisms, SIK1 is proposed as a neuroprotector. SIK1 was also suggested to take part in the regulation of intracellular sodium levels (Sjöström *et al.,* 2007) via phosphorylation of phosphatase 2A/phosphatase methylesterase-1 (PME-1).

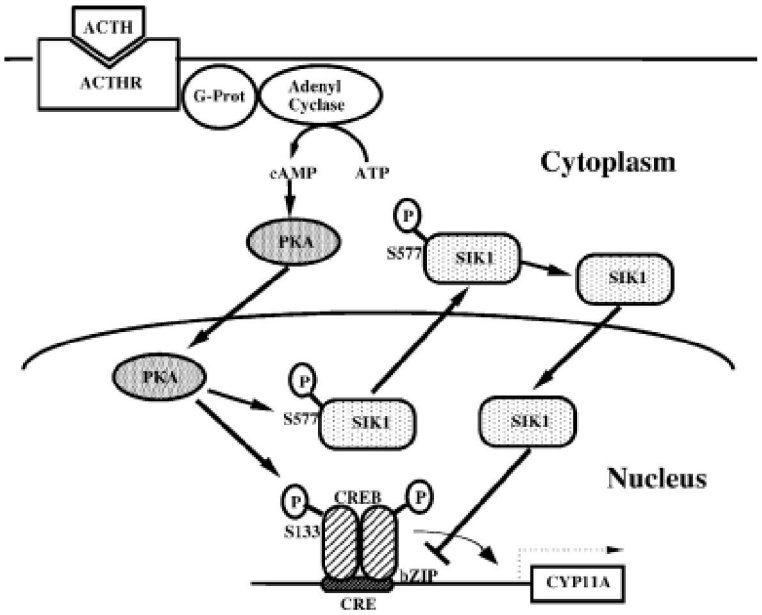

Figure 1.9. The role of SIK1 in repression of ACTH stimulated steroidogenic gene expression. PKA phosphorylation of SIK1 results in its shuttling between nucleus and cytoplasm. (Katoh *et al*., 2004)

SIK3 is composed of 1263 amino acids (Katoh *et al*., 2004). It has a kinase domain between the residues 8-259, a UBA domain between the residues 283-336 and finally a phosphorylation domain between 486-518. LKB1 phosphorylates SIK3 on the kinase domain, on Thr^{163} (Lizcano *et al*., 2004). Northern blot analyses revealed that SIK3 is ubiquitously expressed (Okamoto *et al*., 2004). In *Drosophila*, downregulation of SIK3 ortholog CG15072 leads to similar effects as the downregulation of LKB1, resulting in mitotic defects such as spindle and chromosome abnormalities (Bettencourt-Dias *et al*., 2004). SIK3 was found to be activated directly by Akt in response to insulin revealing a role in energy balance in *Drosophila* (Wang *et al*., 2011).

SIK1 and SIK3 roles in cancer were suggested recently. SIK1 has been identified as a regulator of p53 dependent anoikis, apoptosis induced by cell detachment (Cheng *et al*., 2009). Loss of SIK1 has been shown to result in spread and survival of micrometastases. Elevated SIK3 levels are found in ovarian cancer cases (Charoenfuprasert *et al*., 2011), it was shown that SIK3 overexpression results in enhanced cell proliferation and c-Src was found to be downstream signaling mediator of SIK3.

1.9.2. Salt-Inducible Kinase 2

SIK2 is initially found as abundantly expressed in both human and mouse adipose tissues and SIK2 mRNA is rapidly induced during adipogenesis (Horike *et al*., 2003). Also SIK2 expression has been observed in testis and in developing and adult retina. (Horike *et al.*, 2003; Özcan, 2003; Özmen, 2006)

SIK2 is a 931 amino acid protein and the kinase domain is located between the amino acids 20-271. It has a ubiqitin-associated domain (UBA) between the residues 293-346, a phosphorylation domain between the residues 577-623. The isoforms of SIKs have a relatively high degree of amino acid similarity. The similarities between SIK1 with SIK2, SIK2 with SIK3 in kinase domains are 70% and 73%; in UBA domains 70% and 40%; in phosphorylation domains 73% and 33% respectively (Katoh *et al*., 2004).

One of the first identified kinases of SIK2 is LKB1, which phosphorylates SIK2 at Thr^{172} that is located on the activation loop (Lizcano *et al*., 2004). The phosphorylation of this residue renders the kinase 30 fold more active. Second one is PKA which phosphorylates SIK2 on Ser^{587}. This phosphorylation leads to translocation of SIK2 to the cytoplasm (Horike *et al*., 2003).

Several SIK2 roles in metabolism have been suggested. In human adipocytes it phosphorylates IRS1 on Ser^{794} (rat Ser^{789}) and modulates the efficiency of insulin signaling pathway, since SIK2 activity found elevated in diabetic rats it was suggested to participate in development of insulin resistance (Horike *et al.*, 2003; Katoh *et al.*, 2004). In adipocytes SIK2 was identified to respond low energy levels in activator 5-amino-4-imidazolecarboxamide (AICAR) responsive manner, replacing AMPK in AICAR mediated glucose uptake (Du *et al.*, 2008). Screaton *et al.* (2004) have reported that SIK2 phosphorylates Transducer of Regulated CREB Activity 2 (TORC2), CREB co-activator. The resulting phospho-TORC2 is sequestered in the cytoplasm via interaction with 14-3-3 protein (Figure 1.10). Dentin *et al.* (2007) have shown that SIK2 is phosphorylated by Akt in response to insulin and activated, thus regulate TORC2 phosphorylation. *Drosophila* studies agree with the previous results and showed RNAi mediated knockdown of SIK2 enhanced TORC activity and results in a resistance to starvation (Wang *et al*., 2008). It was

found that in cortical neurons SIK2 degradation results in elevation of TORC1 activity, increase of CREB-dependent gene expression and this prevents neural death after oxygen-glucose deprivation (Sasaki *et al.*, 2011). Bricambert *et al.* (2010) showed that SIK2 can phosphorylate histone acetyltransferase p300 and regulate hepatic lipogenesis by inhibiting acetylation of carbohydrate-responsive element–binding protein (ChREBP).

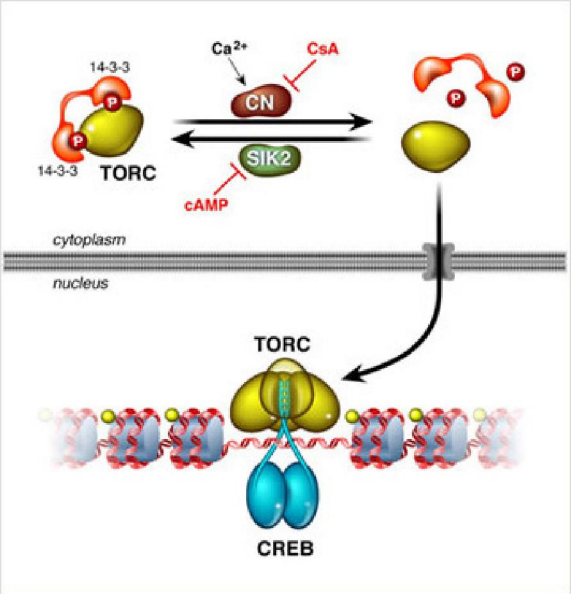

Figure 1.10. Role of SIK2 in TORC2:CREB mediated gene transcription. SIK2 phosphorylates TORC2 which results in its translocation from the nucleus. (Screaton *et al.*, 2004)

Recently, non-metabolic functions of SIK2 are reported. A centrosome linker protein c-Nap1 was found to be phosphorylated by SIK2, initiating centrosome separation in interphase (Ahmed *et al.*, 2010). In diffuse large B-cell lymphoma (DLBCL), overexpression of SIK2 and amplification of SIK2 was found (Nagel *et al.*, 2010).

Table 1.2. Known substrates of SIK2 and their phosphorylation motifs. Phosphorylated residues are indicated with red.

SIK2 phosphorylation motif: (L/I) [(B)X or X(B)] (S/T)X**(S/T)**XXXL	
Ser^{794} on IRS1	L R L S T S S G R L
Ser^{171} on TORC2	L N R T S S D S A L
Ser^{2392} on CNAP1	L H H S L S H S L L
Ser^{89} on P300	L L R S G S S P N L

1.9.2.1. SIK2 in FGF pathway**:** SIK2 was identified in a yeast-two hybrid screen of a retinal cDNA library as an FGFR2 interactor and subsequently SIK2 expression was observed in developing and adult retina (Özcan, 2003; Özmen 2006). Sequence analysis of full length retinal SIK2 transcript revealed the presence of SH2 and SH3 domains, lending further support of its involvement in FGFR pathway.

Further experiments showed that phosphorylation status, SIK2 activity and cellular localization are changed with FGF2 treatment in Müller cells (Küser, unpublished data; Candaş, 2007). Through canonical SIK2 phosphorylation motif search, three members of FGF pathway Grb2, Gab1 and A-Raf were revealed as potential targets. SIK2 was able to kinase Gab1 and A-Raf *in vitro* (Küser, 2006). On going studies in our laboratory indicate an increase in ERK 1/2 phosphorylation levels and proliferation rates in MIO-M1 cells when SIK2 is knocked down (Küser, unpublished data). We hypothesize that SIK2 is activated in response to FGF2 and phosphorylates Gab1 and Raf-1 to decrease the binding with other signaling intermediates, thereby creating a negative feedback loop (Figure 1.11).

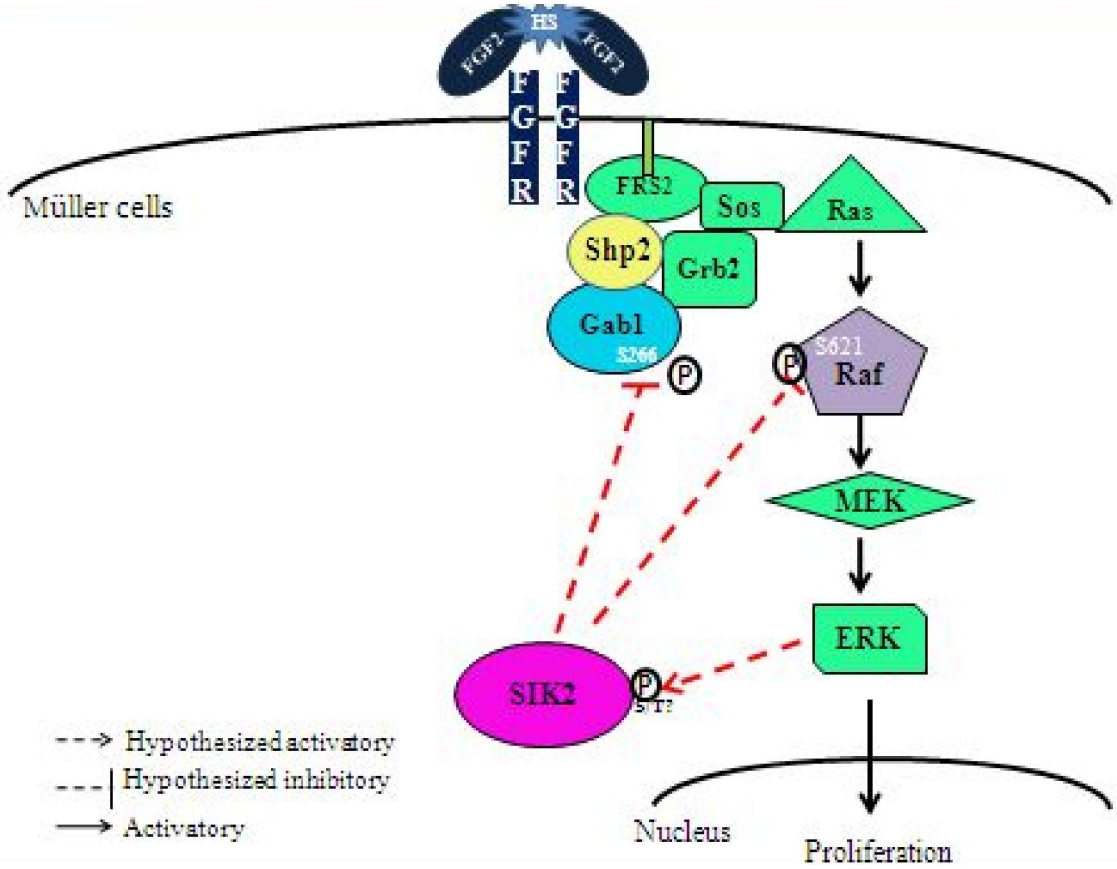

Figure 1.11. Working Model of SIK2 involvement in FGF signaling

2. PURPOSE

Çınaroğlu (2005) has proposed that rapid and transient FGF2 dependent ERK 1/2 activation observed in Müller cells might be a result of negative feedback mechanisms involving changes in the phosphorylation status of intermediate signaling molecules. Our working hypothesis suggests that SIK2 might be a part of negative regulation of FGF pathway through phosphorylation of Gab1 on Ser^{266} and Raf-1 on Ser^{621}.

In order to test this hypothesis, we aimed in this study:

- To generate Gab1 and Raf-1 mutants and to verify SIK2 phosphorylation sites of Gab1 as Ser^{266} and Raf-1 as Ser^{621} *in vitro*.
- To analyze the effect of phosphorylation of Gab1-Ser^{266} on interactions with Gab1 binding partners Shp2 and Grb2 in response to FGF2.
- Finally to investigate the effect of Ser^{266} phosphorylation of Gab1 on FGF2 dependent proliferation of MIO-M1 cells.

3. MATERIALS

3.1. Cell Lines

Spontaneously immortalized human Müller glia cell line (MIO-M1) was a kind gift of Astrid Limb from University College London, Institute of Ophthalmology, London. Human embryonic kidney 293T cells (HEK 293T) were kindly provided by Nesrin Özören from Boğaziçi University, İstanbul.

3.2. Chemicals, Plastic and Glassware

All chemicals were purchased from Merck (Germany) or Sigma Aldrich (USA), all cell culture products were purchased from Invitrogen (USA) unless stated otherwise in text. Plastic products were purchased from TPP (Switzerland) and Greiner (USA). Sterilization of solutions and all glassware is done by autoclaving at 121^{o}C for 20 minutes.

3.3. Buffers and Solutions

Buffer and solution compositions are given in Table 3.1 through Table 3.5.

Table 3.1. Solutions for Bacterial Transformation.

Luria Broth (LB) Medium	10 g Tryptone 5 g Yeast Extract 5 g NaCl to 1000 ml with H_2O

Table 3.1. Solutions for Bacterial Transformation (continued).

NZ amine and Yeast Extract (NZY^+) Broth	10 g Casein enzymatic hydrolysate (NZ amine) 5 g Yeast Extract 5 g NaCl 12.5 mM $MgCl_2$ 12.5 mM $MgSO_4$ 0.4 % Glucose to 1000 ml with H_2O
LB Agar	1000 ml LB Medium 20 g Agar

Table 3.2. Buffers and solutions for Cell Culture and assays.

Complete Medium for MIO-M1 Cells	Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) 0.1% Penicillin/Streptomycin
Complete Medium for HEK 293T Cells	DMEM supplemented with 10% FBS 0.1% Penicillin/Streptomycin 1% Non-essential Amino Acid (Biochrom, Germany)
10X Trypsin-EDTA Solution	2.5% Trypsin 7 mM Ethylenediaminetetraacetic acid **(**EDTA) 0.9% NaCl diluted with 1X Phosphate Buffered Saline (PBS)
Freezing Medium for MIO-M1 Cells	70% DMEM 20% FBS 10% Dimethyl Sulfoxide (DMSO)
Freezing Medium for HEK 293T Cells	50% DMEM 40% FBS 10% DMSO

Table 3.2. Buffers and solutions for Cell Culture and assays (continued).

FGF2 Solution	DMEM supplemented with 1 ng/ml FGF2 100 µg/ml heparin
2X HBS Buffer	1.6 g NaCl 0.074 g KCl 0.027 g Na_2HPO_4 0.2 g Glucose 5 ml 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to 100 ml with dd H_2O Adjust pH 7.05
4% PFA	4% Paraformaldehyde (PFA) in 1X PBS
Blocking Solution for Proliferation Assay	5% donkey serum 0.1% Triton X-100 In 1X PBS

Table 3.3. Buffers and solutions for Western Blot analysis.

10% SDS-polyacrylamide gel (running gel)	10% Acrylamide:bisacrylamide (37.5:1) 375 mM Tris-HCl (pH 8.8) 0.1% Sodium dodecyl sulphate (SDS) 0.1% Ammonium persulphate (APS) 0.1% N,N,N',N',-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

Table 3.3. Buffers and solutions for Western Blot analysis (continued).

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	2% SDS 80 mM Tris.HCl (pH 6.8) 20% Glycerol 10% β-mercaptoethanol 0.005% Bromophenol Blue
Runing Buffer	25 mM Tris.HCl 250 mM Glycine 0.2% SDS
Transfer Buffer	25 mM Tris.HCl 200 mM Glycine 15% Methanol
Tris Buffered Saline with Tween 20 (TBST)	20 mM Tris.HCl (pH 8.0) 150 mM NaCl 0.1% Tween 20
Coomassie Blue Solution	50% Methanol 10% Acetic Acid 0.05% Coomassie R250
Fixing Solution	50% Methanol 7% Acetic Acid
Destaining Solution	5% Methanol 7% Acetic Acid
Stripping Solution	62.5 mM Tris.HCl (pH 6.8) 2% SDS 0.7% β-mercaptoethanol

Table 3.4. Buffers for Protein Isolation.

Immnunoprecipitation (IP) Lysis Bufer	20 mM Tris-Cl (pH 7.5) 150 mM NaCl 1 mM Na_2EDTA 1% Triton X-100
Co-immunoprecipitation (Co-IP) Lysis Buffer	20 mM Tris-Cl (pH 8.0) 125 mM NaCl 0.5% NP-40 2 mM Na_2EDTA

Table 3.5 Buffers and solutions for Kinase Assay.

2X Kinase Buffer	25 mM HEPES (pH 7.5) 150 mM NaCl 10 mM $MgCl_2$ 2 mM $MnCl_2$
Mg/Mn ATP Cocktail	1X Kinase Buffer supplemented with 100 µM cold Adenosine triphosphate (ATP) 75 mM $MnCl_2$ 75 mM $MgCl_2$

3.4. Plasmids

Myc-DDK-tagged open reading frame (ORF) clone of Homo sapiens Raf-1 and Myc-DDK-tagged ORF clone of Homo sapiens GAB1, transcript variant 1 was purchased from Origene (Maryland ,USA). Gab1 cDNA clone has 2175 base pairs whereas Raf-1 cDNA clone is composed of 1947 base pairs. cDNAs inserted in pCMV6-ENTRY vector has cMyc and Flag tags at their N-terminal (Figure 3.1). pEGFP-N3 plasmid encoding a brighter variant of green fluorescent protein (GFP) was used.

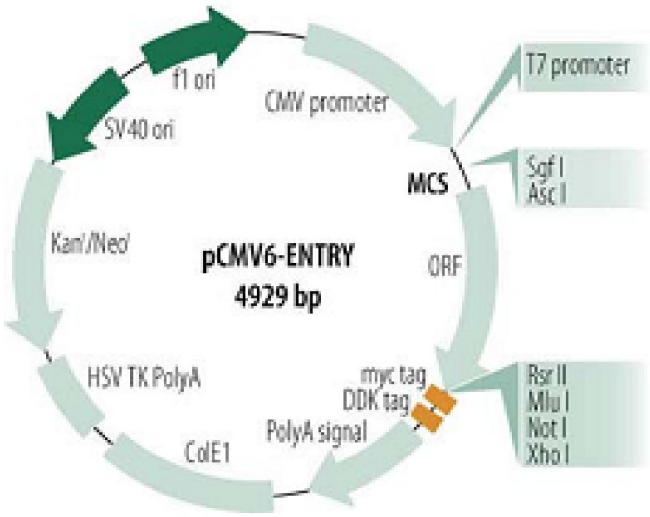

Figure 3.1. Plasmid map of pCMV6-ENTRY (Origene, USA).

3.5. Primers

Primers for sited-directed mutagenesis are designed and they are synthesized at Iontek (Istanbul, Turkey).

Table 3.6. Primers used for sited-directed mutagenesis.

Gene	**Mutagenesis**	**Primer Sequence (5'-3')**	**Tm (^{o}C)**
GAB1	S266A	Forward: CTGCCCAGGAGTTAT**G**CCCATGATGTTTTAC	79.6
		Reverse: GTAAAACATCATGGG**C**ATAACTCCTGGGCAG	
RAF1	S621A	Forward: GATCAACCGGAGCGCT**G**CCGAGCCATCCTTGC	87.3
		Reverse: GCAAGGATGGCTCGG**C**AGCGCTCCGGTTGATC	
RAF1	K375M	Forward: GGAGATGTTGCAGTAA**T**GATCCTAAAGGTTGTC	78.4
		Reverse: GACAACCTTTAGGATC**A**TTACTGCAACATCTCC	

Table 3.7. List of primers used for sequencing.

Gene	**Primer Sequence (5'-3')**		**Tm (^{o}C)**
GAB1 (7-582 bp)	Forward:	GGTGGTGAAGTGGTCTGCTC	60.72
	Reverse:	GGGCTTCTTGCTTTGACAGT	59.48
GAB1 (500-1054 bp)	Forward:	CCACCACACCTGGAAACTCT	59.43
	Reverse:	TTCCACAGGAGATCGGTCAT	57.3
GAB1 (1032-1625 bp)	Forward:	GCCACCGAAACCACATCC	62.36
	Reverse:	AAACCATGGGGTTTGAGTCTT	59.72
GAB1 (1611-2172 bp)	Forward:	CAAACCCCATGGTTTAGAGC	59.43
	Reverse:	TTTCACACTCTTCGCTGGC	60.13
RAF1 (16-415 bp)	Forward:	GGAGCTTGGAAGACGATCAG	59.95
	Reverse:	GTGTTGTGAGGGGAACATGA	59.37
RAF1 (339-930 bp)	Forward:	TTGGAATACTGATGCTGCGT	59.30
	Reverse:	GGTTTTCGGCTGTGACCAG	61.65
RAF1 (880-1570 bp)	Forward:	TCCAGTAGCCCCAACAATCT	59.55
	Reverse:	GGTTGTTATCCTGCATTCGG	60.33
RAF1 (1466-1920 bp)	Forward:	TGGCAACAGTAAAGTCACGC	59.91
	Reverse:	GGTCAGCGTGCAAGCATT	61.00

Table 3.8. Antibodies used.

Antibody	Application	Dilution	Blocking Solution	Source	Supplier
Anti-cMyc tag	Western Blotting	1:1000	5% milk powder (MP) in TBS	mouse	Cell Signaling (USA)
	Immuno-precipitation	1:1000			
	Cell Proliferation Assay	1:1000	5% donkey serum 1% Triton X-100 in PBS		
Anti-GFP	Immuno-precipitation	1:1000		rabbit	Abcam (UK)
Anti-SIK2	Western Blotting	1:1500	5% MP 1% Bovine Serum Albumin (BSA) in TBST	rabbit	Novus Biologicals (USA)
Anti-phospho Raf1-Ser621	Western Blotting	1: 1000	5% BSA in TBST	rabbit	Thermo Scientific (USA)
Anti-Gab1	Western Blotting	1:1000	1% MP in TBST	rabbit	Santa Cruz (USA)
Anti-Shp2	Western Blotting	1:1000	1% MP in TBST	rabbit	Santa Cruz (USA)
Anti-Grb2	Western Blotting	1:500	3% MP in PBS	mouse	Upstate (USA)
Anti-Ki67	Cell Proliferation Assay	1: 1500	5% donkey serum 1% Triton X-100 in 1X PBS	rabbit	Leica Biosystems (Germany)
Anti-rabbit Alexa 546	Cell Proliferation Assay	1: 1000	5% donkey serum 1% Triton X-100 in 1X PBS	donkey	Invitrogen (USA)
Anti-mouse Alexa 488	Cell Proliferation Assay	1:250	5% donkey serum 1% Triton X-100 in 1X PBS	donkey	Invitrogen (USA)
Anti-mouse IgG-HRP	Western Blotting	1:5000	In blocking solution of primary antibody	goat	Santa Cruz (USA)
Anti-rabbit IgG-HRP	Western Blotting	1:5000	In blocking solution of primary antibody	goat	Santa Cruz (USA)

3.6. Kits

Kits used in this study are listed in Table 3.9.

Table 3.9. Kits used.

Kit	**Supplier**
QuikChange XL Site-Directed Mutagenesis Kit	Stratagene (USA)
QIAprep Spin Miniprep kit	Qiagen (Germany)
Purelink HiPure Plasmid Filter Maxiprep Kit	Invitrogen (Germany)
Bicinchoninic acid (BCA) assay kit	Thermo Scientific (USA)

3.7. Equipment

Table 3.10. Equipments used.

Autoclave	Model MAC-601 (Eyela, Japan) Model ASB260T (Astell, UK)
Balances	Electronic Balance VA124 (Gec Avery,USA) DTBH 210 (Sartorius, Germany)
Blotting apparatus	Mini Trans-Blot Cell (Bio-Rad, USA)
Carbon-dioxide tank	2091 (Habaş, Turkey)
Centrifuges	Centrifuge 5415R (Eppendorf, USA) MiniSpin (Eppendorf, USA) Allegra X-22R Centrifuge (Beckman Coulter, USA) ProFuge, 10K (Strategene, USA) Centrifuge B5 (B.Braun B. Int., Germany) J2-MC Centrifuge (Beckman Coulter, USA) J2-21 Centrifuge (Beckman Coulter, USA)
Deep-freezers	-20^0C (2021D) (Arçelik, Turkey) -20^0C (2021D) (Sanyo, Japan) -70^0C Freezer (Harris, UK) -86^0C ULT Freezer (ThermoForma, USA)

Table 3.10. Equipments used in this study (continued).

Documentation System	Gel Doc XR System (Bio-Rad, USA)
Electrophoresis Equipment	Mini-PROTEAN 3 Cell (Bio-Rad, USA)
Gel dryer	Model 583 Gel Dryer (Bio-Rad, USA)
Heat Blocks	DRI-Block DB-2A (Techne, UK) Thermal Reactor, (Hybaid, UK)
Hybridization Oven	Shake'n'Stack (Hybaid, UK)
Ice machine	AF20 (Scotsman Inc., Italy)
Incubator	Forma Series II Water Jacket (Thermo Scientific, USA)
Laminal flow cabinet	Class II A (Tezsan, Turkey) Class II B (Tezsan, Turkey)
Magnetic Stirrers	M221 (Elekro-mag, Turkey) Clifton Hotplate Magnetic Stirrer (HS31, UK)
Micropipettes	Gilson (France)
Microscopes	CM110 Inverted Microscope (Prior, UK) Axio Observer Z1 Inverted Mic. (Zeiss, USA)
Microwave owen	M1733N (Samsung, Malaysia)
pH meter	SB70P (Symphony, USA)
Pipettor	Pipetting Aid (Gilson, USA)
Power supply	Power Pac Basic (Bio-Rad, Italy)
Protein Visualization	Stella (Raytest, Germany)
Refrigerators	2082C (Arçelik, Turkey) 4030T (Arçelik, Turkey)
Shakers	GyroMini Nutating Mixer (Labnet, USA) Adjustable Rocker (Cole-Parmer, USA)
Software	AxioVision Rel 4.6 SP1 (Carl Zeiss, USA) Xstella (Raytest, Germany) Quantity One (Bio-Rad, Italy)
Sonicator	HD 2070 Sonopuls (Bandelin, Germany)

Table 3.10. Equipments used in this study (continued).

Spectrophotometer	NanoDrop ND-1000 (Thermo, USA)
Thermocycler	MyCycler (Bio-Rad, USA)
Vacuum pump	Hydrotech (Bio-Rad, USA)
Vortex	Vortexmixer VM20 (Chiltern Scientific, UK)
Water bath	TE-10A (Techne, UK)
Water purification system	WA-TECH Ultra Pure Water Purification System (WA-TECH, Germany)

4. METHODS

4.1. Site-Directed Mutagenesis

4.1.1. Primer Design

The primers with 31-33 bp for in vitro mutagenesis were designed to have the mutated base situated in the middle. The GC content was minimum of 40%. The sequences and Tm values are listed in Table 3.6.

4.1.2. Generation of Mutation

Mutagenesis was generated by following the instructions of the manufacturer. To synthesize two complimentary oligonucleotides containing the desired mutation, thermal cycling reaction was carried out using 10 ng plasmid, 125 ng of each primer, 1µl dNTP mix, 3 µl QuikSolution, 5 µl 10X reaction buffer in a total volume of 50 µl. dNTP mix, QuikSolution and 10X reaction buffer were included in the kit. Finally 2.5 U *PfuUltra* HF DNA Polymerase was added and each reaction was cycled using the cycling parameters outlined in Table 4.1. For control, pWhitescript control plasmid was used.

Following temperature cycling, the samples were incubated on ice for 2 minutes, spinned shortly and parental DNA removed by *Dpn I* digestion (10 U) at 37^{o}C for 1 hour.

Table 4.1. Thermal Cycling Parameters for Mutagenesis

Step	Cycle	Temperature	Time
1	1	95^{o}C	1 minute
2	18	95^{o}C	50 seconds
		60^{o}C	50 seconds
		68^{o}C	7 minutes
3	1	68^{o}C	7 minutes

4.1.3. Transformation of XL10-Gold Ultracompetent Cells with Mutated Plasmids

Forty five µl of XL10-Gold ultracompetent cells that are provided with mutagenesis kit were thawed slowly on ice, 2 µl XL10 Gold β-mercaptoethanol mix was added, after 10 minutes of incubation, 2 µl *Dpn I* treated DNA were added. Subsequent to gentle mixing incubation on ice continued for addtional 30 minutes, cells were heat-pulsed at 42^{o}C water bath for 30 seconds exactly and returned on on ice for 2 minutes. 500 µl of preheated NZY^{+} broth was added on each sample, the cells were allowed to grow for 1 hour at 37^{o}C and shaking at 250 rpm. For transformation efficiency control, pUC18 plasmid was used. The cells were spread on LB-agar plates containing 80 µg/ml X-Gal, 20 mM IPTG and 25 µg/ml Kanamycin for pCMV6-ENTRY vectors or 100 µg/ml ampicillin for control plasmids and incubated at 37^{o}C for 16 hours.

4.1.4. Plasmid Isolation and DNA Sequencing

Following the blue-white colony screening, selected white colonies were grown in LB medium containing 25 µg/ml kanamycin at 37^{o}C for 16 hours with shaking at 250 rpm. Plasmids were isolated using MiniPrep kit or Purelink HiPure Maxiprep, according to manufacturers' instructions. Briefly, bacterial cells were lysed under alkaline conditions and detergents with RNase. In the second step, the lysate is neutralized to allow renaturation of plasmid DNA, cell debris and chromosomal DNA were removed by centrifugation. The supernatant was applied to silica gel columns, where plasmid DNA would bind under high salt conditions. In the last step, plasmid DNA was eluted with TE buffer. Concentration of the eluted plasmid DNA was determined by measuring optical density at 260 nm with the NanoDrop Spectrophotometer. To verify the generation of mutagenesis, plasmid DNA was sent for sequencing. The plasmid DNA was stored at -20°C for further studies.

4.2. Cell Culture

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

MIO-M1 cells were maintained in DMEM with Glutamax supplemented with 10 % fetal bovine serum and 0.1 % penicillin/streptomycin. Human embryonic kidney cell line, HEK 293T, was maintained in DMEM with Glutamax, 10 % fetal bovine serum, 0.1 % penicillin/streptomycin and 1 % non-essential amino acids. Both cell lines were grown at 37^0C and under 5% CO_2. When the plates reach confluency; cells were washed with 1X PBS, treated with trypsin solution for 5 minutes at 37^0C and scraped. Cells were collected by centrifugation at 500 g for 5 minutes, resuspended in complete medium and divided into 3 plates. For stock preparation, cells were resuspended in freezing medium as in Table 3.2.

4.2.2. Transfection of MIO-M1 and HEK 293T Cells

For transfection; $5x10^6$ HEK 293T cells or $4.5x10^6$ MIO-M1 cells were seeded on cell culture plates with 10 cm diameter and incubated overnight at 37^0C, 5% $C0_2$ with 10 ml complete medium. On the next day, chloroquine with a final concentration of 25 µM was added on each plate. 10 µg of desired plasmid and 2 µg of pEGFP-N3 vector were added to a microcentrifuge tube with a final volume of 438 µl dH_2O. Sixty two µl of 2 M $CaCl_2$ was added and the mixes were let to stand for 5 minutes at room temperature. DNA mixtures were added slowly on 500 µl 2x HBS buffer in a new microfuge tube while blowing air inside. The tubes were incubated at room temperature for 10 minutes to precipitate the DNA molecules with PO_4^{-3} ions. After incubation, the mixture was added to the cell media and incubated at 37^0C, 5% CO_2 for 8 hours. The medium was replaced with fresh complete medium to remove chloroquine. In order to validate transfection, GFP expression was observed at 48 hours of transfection with fluorescent microscopy.

4.2.3. FGF2 Treatment of MIO-M1 Cells

32 hours after transfection, cells were washed with 1X PBS twice and starved with DMEM with Glutamax including 0.1 % penicillin/streptomycin for 16 hours. Subsequently, they were incubated with 1 ng/ml FGF2 and 100 µg/ml heparin in DMEM

with Glutamax for 10 minutes at 37^{o}C. Control samples received 1X PBS instead of FGF2. After 10 minutes of incubation, plates were washed with ice cold 1X PBS and scraped on ice with ice-cold 1X PBS including protease and phosphatase inhibitor cocktails (Roche, Germany). Cells were collected into microfuge tubes and pelleted by centrifugation at 13.200 rpm for 5 minutes at 4^{o}C. Pelleted cells were directly processed or stored at -80^{o}C for later analysis.

4.3. Immunoprecipitation

Pelleted cells were resuspended with 1ml ice cold immunoprecipitation lysis buffer including protease and phosphatase inhibitor cocktails. The cells were sonicated for 5 seconds in 3 rounds and incubated on ice for 1 hour with gentle mixing occasionally. Cell debris was removed by centrifugation 20 minutes at 13.200 rpm at 4^{o}C. In paralelle 30 µl Protein G or Protein A agarose beads (Roche, Germany) were washed with 1 ml immunoprecipitation lysis buffer three times. After 20 minutes centrifugation, the cell lysates were transferred on the washed agarose beads and incubated with gentle rotation at 4^{o}C for 30 minutes. The mixture was then centrifuged for 1.5 minutes at 13.200 rpm at 4^{o}C and the pre-cleared lysate was removed to a new microfuge tube. Aliquots were taken for western blot analysis. For determination of protein concentration, BCA assay was performed. Lysate concentration was adjusted to 1 mg/ml and GFP/cMyc tag antibody (1:1000) was added and rotated overnight at 4^{o}C. Next day, 50 µl Protein A/G agarose beads equilibrated with the lysis buffer as before were added to the samples and incubated with gentle rotation for 3 hours at 4^{o}C. The beads were collected, washed with ice cold lysis buffer including protease and phosphatase inhibitor cocktails for 1 minute at 13.200 rpm at 4^{o}C, 3 times. Finally, beads were washed with ice-cold 1X kinase buffer and resuspended with 50 µl 1X kinase buffer. One forth of resuspended beads were taken for western blot analysis boiled at 95^{o}C for 5 minutes and centrifuged for 5 minutes before loading for SDS-PAGE analysis. The rest is kept at -80^{o}C until kinase assays.

4.4. Co-immunoprecipitation

Co-immunoprecipitation protocol was adapted from Comai (2003). Cells were pelleted as described before. The pellets were resuspended with 250 µl ice cold Co-IP lysis buffer supplemented with protease and phosphatase inhibitor cocktails and then rotated on a rocking platform for 2.5 hours at 4^{o}C. To remove cell debris, samples were centrifuged at 13.200 rpm at 4^{o}C for 20 minutes. The supernatants were transferred to microfuge tubes with 25 µl Protein G agarose beads (Roche, Germany) which were washed with Co-IP lysis buffer buffer 3 times beforehand. Lysate and the beads were rotated at 4^{o}C for 20 minutes, precleared lysates were then taken into new microfuge tubes. Protein concentration was determined by BCA protein assay. Lysate concentrations were adjusted to 1 mg/ml and lysate was incubated with cMyc tag antibody overnight at 4^{o}C with gentle rotation on a rocking platform. Next morning, 50 µl Protein G agarose beads were washed with Co-IP lysis buffer three times and incubated with lysate-antibody mix for 3 hours rotation at 4^{o}C. The beads were then washed with ice-cold EBC buffer including protease and phosphatase inhibitors three times. Finally bead-protein conjugate were collected and resuspended with 25 µl 2X SDS sample buffer, boiled at 95^{o}C for 5 minutes and samples were centrifuged for 5 minutes before loading for SDS-PAGE analysis.

4.5. BCA Assay

For determination of protein concentration BCA assay was used according to manufacturer's instructions. In brief; BSA dilutions ranging from 0.025 to 2 mg/ml were prepared with BCA mix and assayed alongside with the unknown samples in BCA mix. Based on the colorimetric reading at 562 nm by a spectrophotometer, a standard curve was formed from which the unknown sample concentration was expolated.

4.6. SDS-PAGE and Western Blotting

Lysate was fractionated on polyacrylamide gels, 8% or 10% according to the expected size of the proteins under study. The gels were then run in running buffer at 80V until samples pass stacking gel, then run at 120 V. For western blotting, the samples that resolved on polyacrylamide gels were electroblotted to polyvinyl difluoride (PVDF)

membranes (Roche, Germany) in transfer buffer at 100 V for 1.5-2 hours. The membrane was washed with TBST solution and incubated with blocking solution for 1-1.5 hours, at room temperature. Later, the membrane was incubated with primary antibody dilution overnight at 4^{o}C with gentle rotation. Antibody blocking and diluting solutions, and dilution ratios are given at Table 3.8. Next day, membrane was washed with TBST for 5 minutes and 3 times. Following incubation with Horseradish Peroxidase (HRP) conjugated secondary antibody diluted in blocking solution for 1 hour at room temperature, membrane was again washed with TBST 3 times, 5 minutes each. Subsequently, ImmunoCruz Western Blotting Luminol Reagent (Santa Cruz, USA) was applied on the membrane and bands were visualized on the imaging system, Stella, with various exposure times.

In order to analyze the same membrane with different antibodies; bound antibodies were removed by incubating the membrane with pre-warmed stripping solution for 30 minutes at 50^{o}C with gentle rotation. Then the membranes were washed with TBST for 10 minutes and three times. Finally, they were incubated with blocking solution of choice.

For Coomassie Blue staining; after transfer was completed, polyacrylamide gel was incubated with fixing solution for 1 hour and then incubated in Coomassie Blue solution overnight. Next day, gel was taken in destaining solution for several minutes until the bands start to visualize clearly.

4.7. In Vitro Kinase Assay

In vitro phosphorylation assay protocol was followed from Küser's M.Sc. thesis (Küser, 2006). Briefly, 1/4th of cMyc-Gab1 fusion proteins adsorbed with agarose beads were used as substrate and 1/8th GFP fused SIK2 on beads were used as the kinase. The reaction was carried out in 1X kinase buffer and 1 µl radioactive ATP cocktail containing 100 µM cold ATP, 1 µCi [γ^{32}P]ATP (3000 Ci/mmol) at 30^{o}C for 30 minutes. In negative controls SIK2 was omitted. Autophosphorylation of SIK2 and GST-IRS1 phosphorylation by SIK2 was used as positive control.

The reactions were terminated with the addition of 6X SDS sample buffer. Samples were boiled at 95^{o}C for 5 minutes, centrifuged for 5 minutes at 13.2K rpm and then were

loaded on 8% polyacrylamide gel for fractionation. Gel was washed with distilled water for 10 minutes, dried at 80^{o}C for 1 hour under vacuum. Dried gel was exposed to X-ray film (Amersham, USA) for various times at –80^{o}C. The film was developed in developing solution (Kodak, USA) until bands start to visualize, for 0.5-2 minutes. The film was washed with tap water and fixed in fixative solution (Kodak, USA) for 5 minutes.

For Raf1 *in vitro* phosphorylation assay; reaction was set as described above except radioisotope was omitted. As control, only kinase inactive Raf1 protein and only kinase inactive S621A mutated Raf1 were used. The reactions were initiated with the addition of 1 µl 10 mM cold ATP and incubated at 30^{o}C for 45 minutes. With the addition of 6X sample buffer and boiling at 95^{o}C for 5 minutes, reactions were terminated. Centrifuged samples were loaded to 8% polyacrylamide gels and continued with Western blotting as described under 4.6. SDS-PAGE and Western Blotting, with SIK2 antibody and phospho- Raf1 specific for serine 621 antibody.

4.8. Cell Proliferation Assay

Three and a half million MIO-M1 cells were seeded on 10 cm tissue culture plates with round coverslips. The cells were transfected with cMyc tagged wild-type Gab1 or S266A mutated Gab1 as described in Section 4.2.2. Thirty two hours later, cells were starved overnight. Next day, half of the coverslips were taken to 24 well plate and treated with FGF2 solution for 2 days. For control, rest of the coverslips were transferred to 24 well plates and allowed to grow in the absence of FGF2.

After 2 days, cells were washed for 5 minutes and incubated with 4% PFA in 1XPBS for 10 minutes. Following 3 washes with PBS for 10 minutes each, cells were incubated with 150 µl blocking solution 1 hour at room temperature. Then the cells were incubated with 150 µl cMyc tag antibody dilution overnight at 4^{o}C. Cells were washed with PBS for 5 minutes and incubated with Ki-67 antibody for 1 hour at room temperature according to Table 3.8. Then the cells were washed with 1XPBS for 10 minutes for 3 times. Afterwards, cells were incubated with Alexa Fluor 546 conjugated donkey anti-rabbit antibody and Alexa Fluor 488 conjugated donkey anti-mouse antibody in blocking solution at room temperature for 1 hour in dark. Cells were washed with 1XPBS for 5 minutes followed by

DAPI incubation for 5 minutes. Cells were washed with 1XPBS for 10 minutes, for 3 times and observed under fluorescent microscope. For quanitification; Ki-67 positive nuclei were counted among cMyc tag antibody labeled cells. Transfection efficiency was calculated by counting cMyc tag labeled cells and nuclei stained with DAPI. Experiments were repeated for three times, at least 300 cells were counted for each experiment.

5. RESULTS

5.1. Site-directed Mutagenesis of Gab1 and Raf-1

The serine residue in SIK2 phosphorylation motif (L/I)[(B)X or X(B)] (S/T)X(S/T)XXXL found on Raf-1 and Gab1 sequences were proposed as the target site of SIK2 kinase activity (Table 5.1). Therefore, this residue in each sequence was mutated to the amino acid alanine by a single nucleotide change, T to G, the 796th nucleotide on Gab1 and the 1861st nucleotide on Raf-1 by site-directed mutagenesis approach. Since Raf-1 autophosphorylates this site as well, the protein was rendered kinase inactive by mutating the lysine residue at position 375 to methionine, the ATP binding site, via substitution of A at nucleotide position 1124 to T.

Table 5.1. SIK2 phosphorylation motifs on Gab1 and Raf-1. Serine residues indicated with red are the predicted phosphorylation sites for SIK2.

SIK2 phosphorylation motif: (L/I)[(B)X or X(B)] (S/T)X(S/T)XXXL			
Protein Name	**Motif**	**Position**	**SwissProt No.**
Gab1	LPRSYSHDVL	266	Q13480
Raf-1	INRSASEPSL	621	P04049

Following the site-directed mutagenesis, cells were grown on LB agar plates in the presence of kanamycin, IPTG and X-Gal. Transformation efficiency was calculated to be 98%. Individual blue colonies were selected and grown in LB medium, plasmids were isolated. The entire coding sequences of Gab1 and Raf-1 clones were determined by DNA sequencing using the primers listed in Table 3.7. Sequencing results confirmed the generations of targeted mutations in all cases (Figure 5.1 and Figure 5.2). No other mutations than the expected ones were detected.

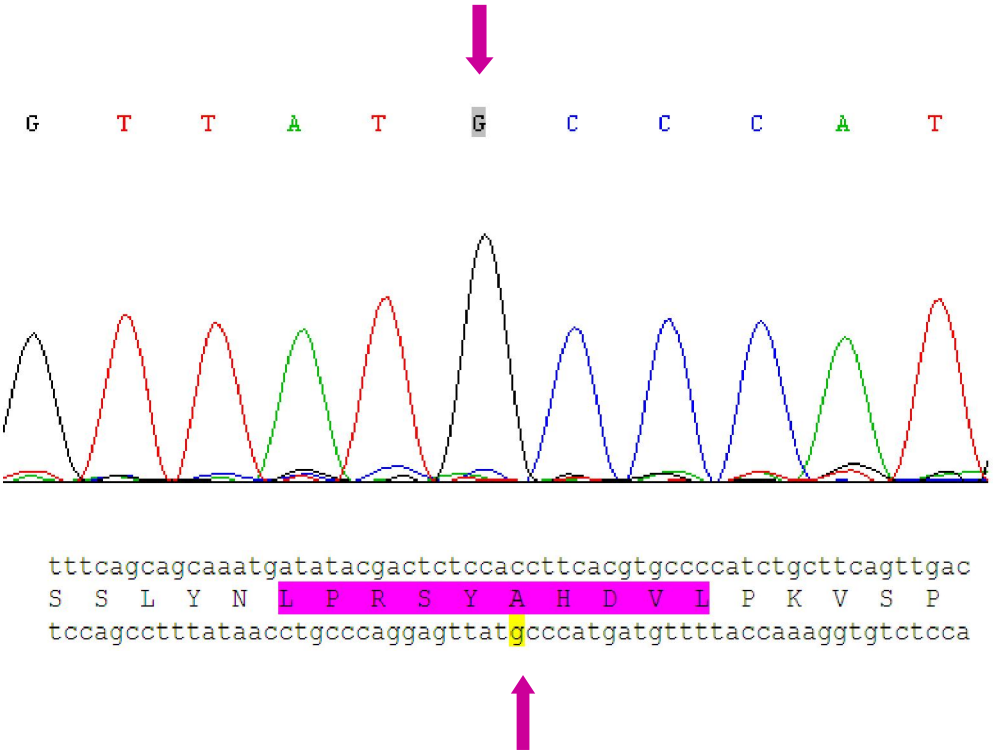

Figure 5.1. Verification of Gab1 *in vitro* mutagenesis products. The targeted residue is indicated with an arrow and the SIK2 phosphorylation motif with magenta highlighting.

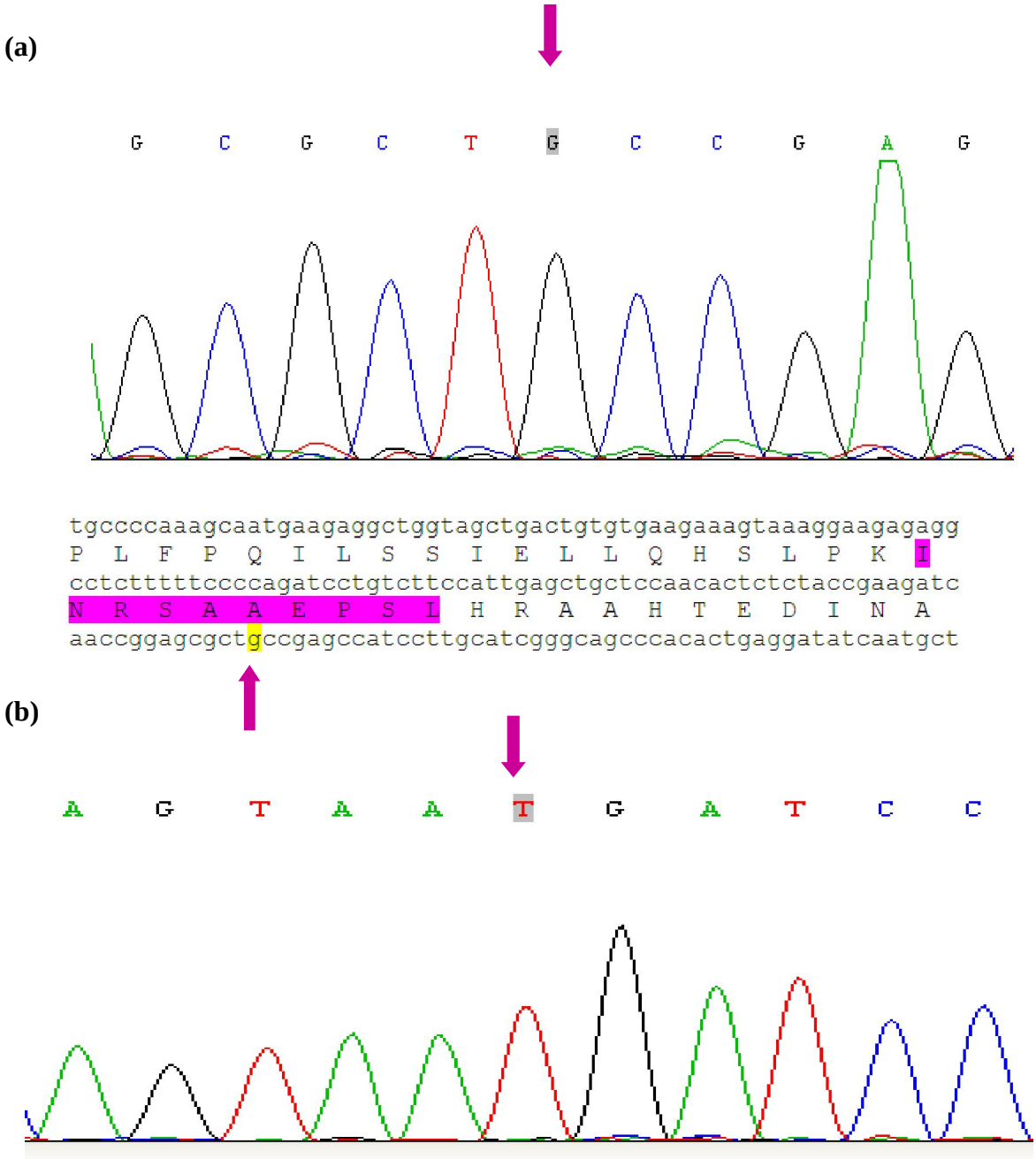

Figure 5.2. Verification of Raf-1 *in vitro* mutagenesis products. (a) Targeted residue within SIK2 phosphorylation motif, highlighted as magenta, is indicated with arrow. (b) Mutagenesis at 1124th nucleotide for Raf-1 kinase inactive is indicated with arrow.

5.2. Expression and Isolation of Gab1 and Raf-1 Proteins

Ten micrograms of pCMV6-Entry vectors carrying Gab1, S266A-Gab1, KI-Raf-1 or KI- S621A-Raf-1 cDNAs were co-transfected with 2 µg of pEGFP-N3 plasmids to HEK293T cells. Another group of cells were transfected with 12 µg of GFP-SIK2 plasmids. After 48 hours in culture, evaluation of GFP expression under a fluorescent microscope indicated a transfection efficiency of 70%. (Figure 5.4, Figure 5.5 and Figure 5.6)

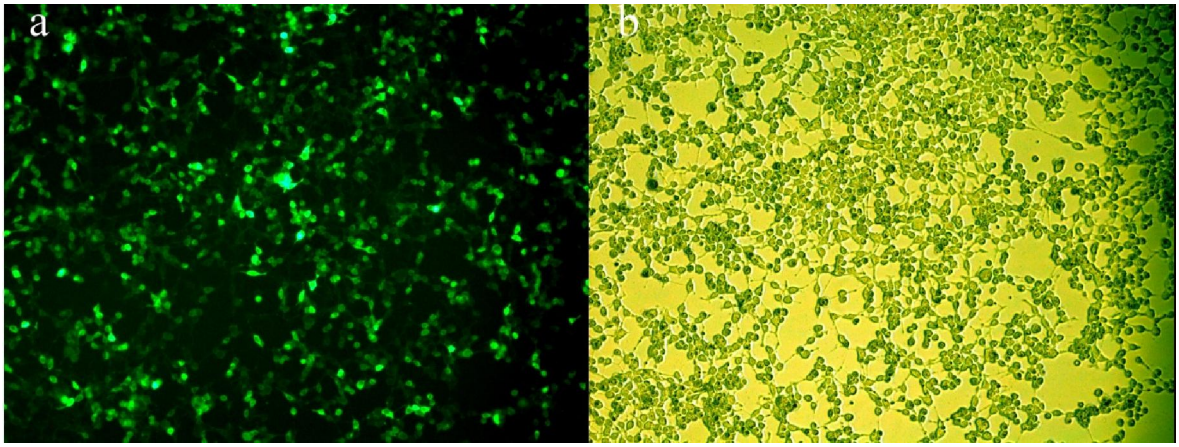

Figure 5.3. Transfection of HEK293T cells with GFP-SIK2. Five million cells were transfected with 12 µg GFP-SIK2 and allowed to grow for 48 hr. (a) Expression was analyzed under fluorescent microscope. (b) Corresponding bright field image.

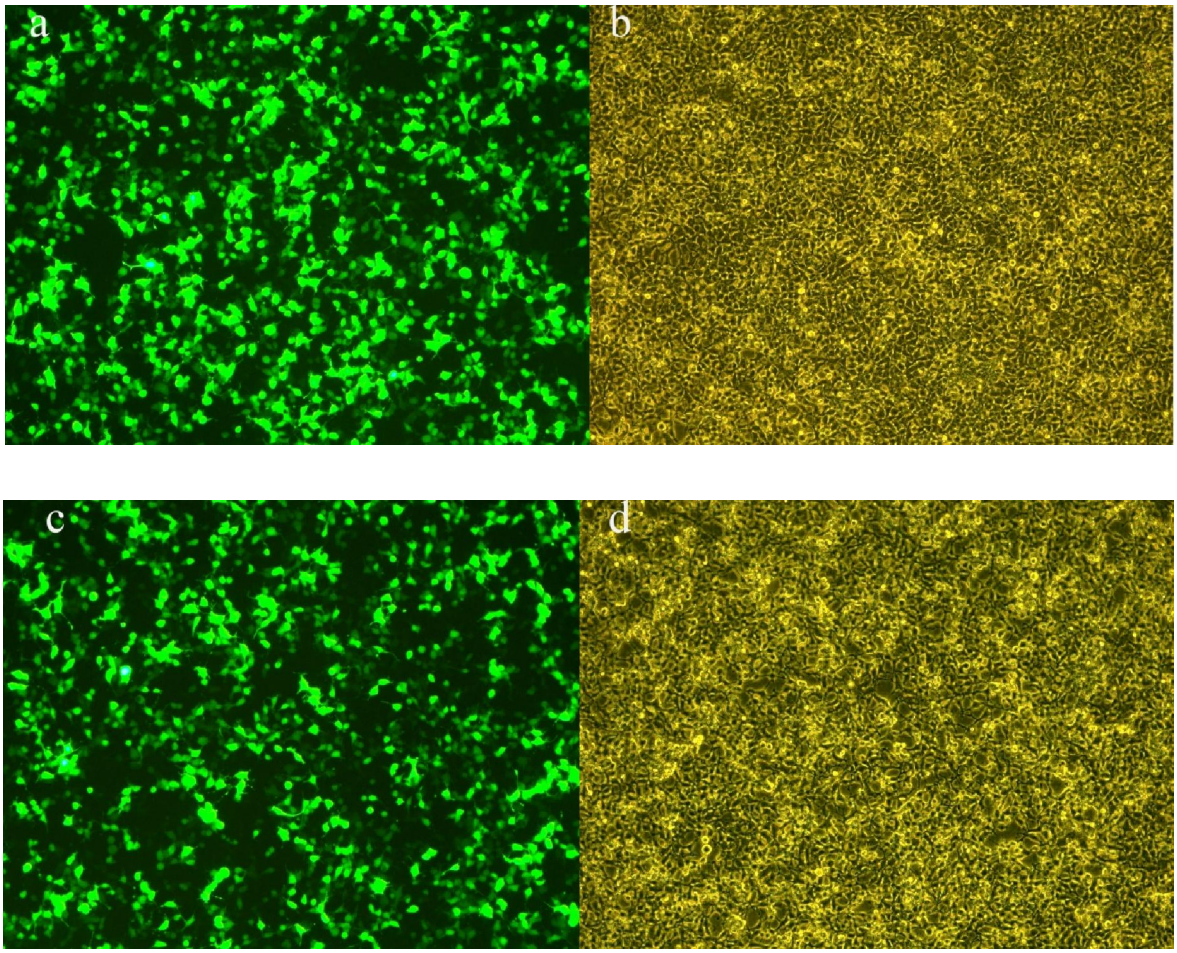

Figure 5.4. Transfection of HEK293T cells with Gab1. HEK293T cells expressing GFP and WT-Gab1 (a) or S266A-Gab1 (c) proteins were analyzed 48 hr post-transfection under the fluorescent microscope. (b, d) Corresponding brightfield images are given.

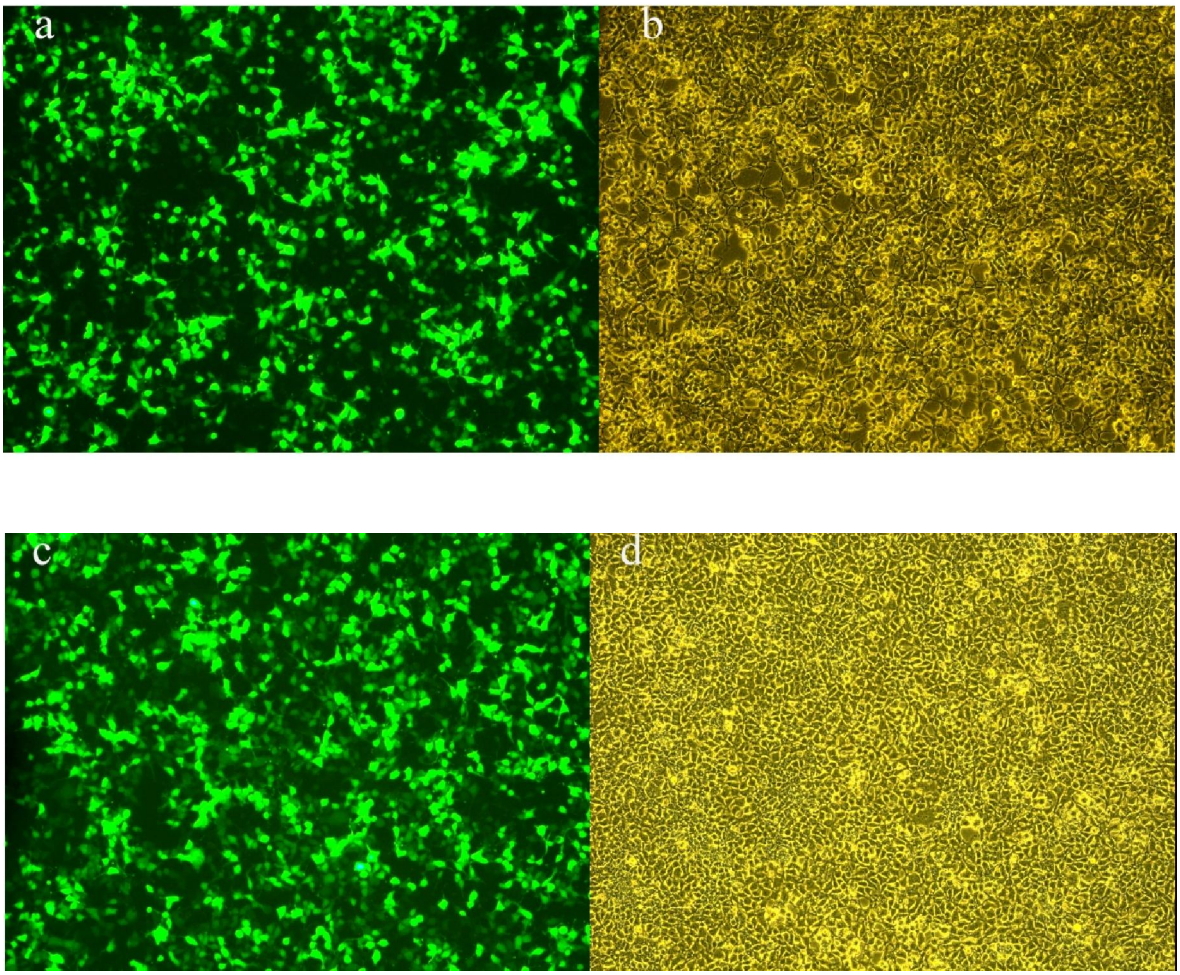

Figure 5.5. Transfection of HEK293T cells with Raf-1. HEK cells expressing GFP and KI-Raf-1 (a) or with KI-S621A-Raf-1 proteins (c) were analyzed 48 hr post-transfection under the fluorescent microscope. (b, d) Corresponding brightfield images are given.

Before isolating the proteins expressed in HEK-293T cells, their presence in the lysates were confirmed by western blotting using anti-cMyc tag antibody. Control samples were transfected with pEGFP-N3 plasmid. The results (Figure 5.6) did not reveal any bands in the control lysates, whereas a 115 kDa band was observed in the lysates obtained from cMyc tagged Gab1 transfected cells and a 74 kDa band was evident in the lysates of Raf-1 transfected cells, as predicted for the fusion proteins. GFP antibody was used to detect the expression of GFP-SIK2 in transfected HEK293T cell lysates (Figure 5.7). The expected 150 kDa band corresponding to the fusion protein was observed. No bands were present at

150 kDa in control cell lysates, transfected with pEGFP-N3 only. Lysates were used in subsequent immunoprecipitation studies.

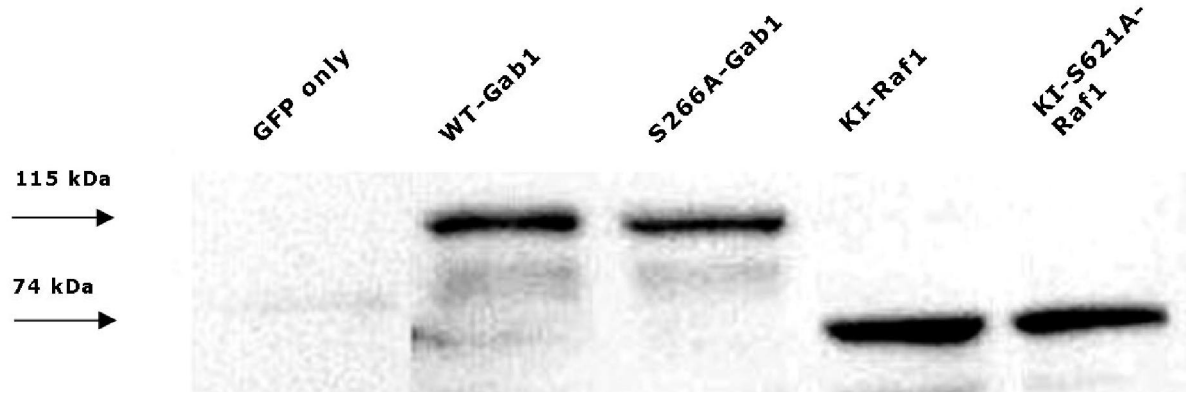

Figure 5.6. Expression of cMyc tagged Gab1 and Raf-1 proteins. Lysates from HEK cells were fractionated on 8% PAGE and analyzed by Western bloting using anti-cMyc antibody. The control samples were transfected with pEGFP-N3.

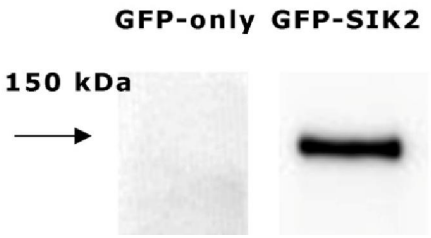

Figure 5.7. Expression of GFP tagged SIK2 protein. Lysates from HEK cells transfected with GFP-SIK2 were fractionated on 8% PAGE and analyzed by Western blotting using anti-GFP antibody. Control samples were transfected with pEGFP-N3 vector.

Proteins that were expressed in HEK293T cells were isolated by immunoprecipitation using antibodies specific for their tag sequences, anti-cMyc for Gab1 and Raf-1 proteins and anti-GFP antibody for SIK2. Following the isolation of proteins, aliquots were resolved on 8% SDS-PAGE gels and Western blotting using anti-cMyc tag antibody for Gab1 and Raf-1 and anti-SIK2 antibody for SIK2 was performed. In WT/S266A-Gab1 samples 115 kDa bands were observed (Figure 5.8.) as predicted. In KI-Raf-1 and KI-S621A Raf-1 samples, the expected 74 kDa bands were evident. Western blot analysis indicated that 150 kDa GFP-SIK2 protein was purified (Figure 5.8).

Figure 5.8. Immunoprecipitated WT/S266A-Gab1 and KI-Raf1/KI-S621A-Raf-1 proteins. One forth of antibody-bead conjugates were fractionated on 8% PAGE and Western blots were probed with anti-cMyc antibody.

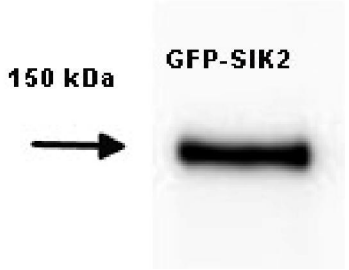

Figure 5.9. Western blot analysis of immunoprecipitated SIK2. One forth of antibody-bead conjugates were resolved on 8% PAGE and Western blots were probed with anti-SIK2 antibody.

5.3. Phosphorylation of Gab1 and Raf-1 by SIK2

Immunoprecipitated WT-Gab1 and S266A-Gab1 were used as substrates in *in-vitro* kinase assays in the presence of [γ ^{32}P] –ATP and GFP-SIK2 was used as the kinase. With SIK2 autophosphorylation activity, phosphorylation of the 150 kDa band represented an internal control in these assays. Activity of SIK2 was also evaluated using its known substrate IRS1. We used IRS1 fragment, expressed as a fusion protein with GST in

bacterial cells and purified by GST affinity chromatography. As negative controls, reactions including only WT-Gab1 or S266A-Gab1 were run.

Initially we verified the kinase activity of immunopurified SIK2 used in this series of experiments. As expected; the protein catalyzes the phosphorylation of IRS1 and exhibit autophosphorylation activity, as indicated by the presence of 47 kDa and 150 kDa bands on the autoradiogram, respectively (Figure 5.10a). In the kinase reactions carried in the absence of SIK2, neither WT-Gab1 nor S266A-Gab1 samples on the autoradiograms revealed signal (Figure 5.10b). When WT-Gab1 was included in the reactions along with SIK2, we observed a 150 kDa band corresponding to the autophosphorylated SIK2 and a 115 kDa band – the predicted size for the cMyc-Gab1 fusion protein. This 115 kD band was not detectable in samples where S266A-Gab1 was used as the substrate (Figure 5.10c). These results are in agreement with the earlier findings of our group suggesting Gab1 as SIK2 substrate (Küser, 2006) and indicate that the SIK2 target phosphorylation site is Ser^{266} on Gab1.

KI-Raf-1 phosphorylation analysis was also carried out *in vitro*. Since an antibody specific for phospho-Ser^{621} Raf-1 was available, *in vitro* kinase assays were performed in the presence of cold ATP and the products were analyzed by Western blotting. In the same samples input KI-Raf-1 and SIK2 levels were evaluated with anti-cMyc or SIK2 antibodies, respectively (Figure 5.11).

In the control samples, where only KI-Raf-1 was included, a slight signal at 74 kDa was observed, indicating the presence of a residual autophosphorylation activity (Figure 5.11a). The signal at this band was significantly enhanced in the presence of SIK2 in the assay (Figure 5.11b). In the reactions where S621A-Raf-1 mutants were used as substrate, no bands were detected with pSer ^{621}-Raf-1 specific antibody. These data support the idea that Ser^{621} on Raf-1 is the *in vitro* target of SIK2 (Figure 5.11b).

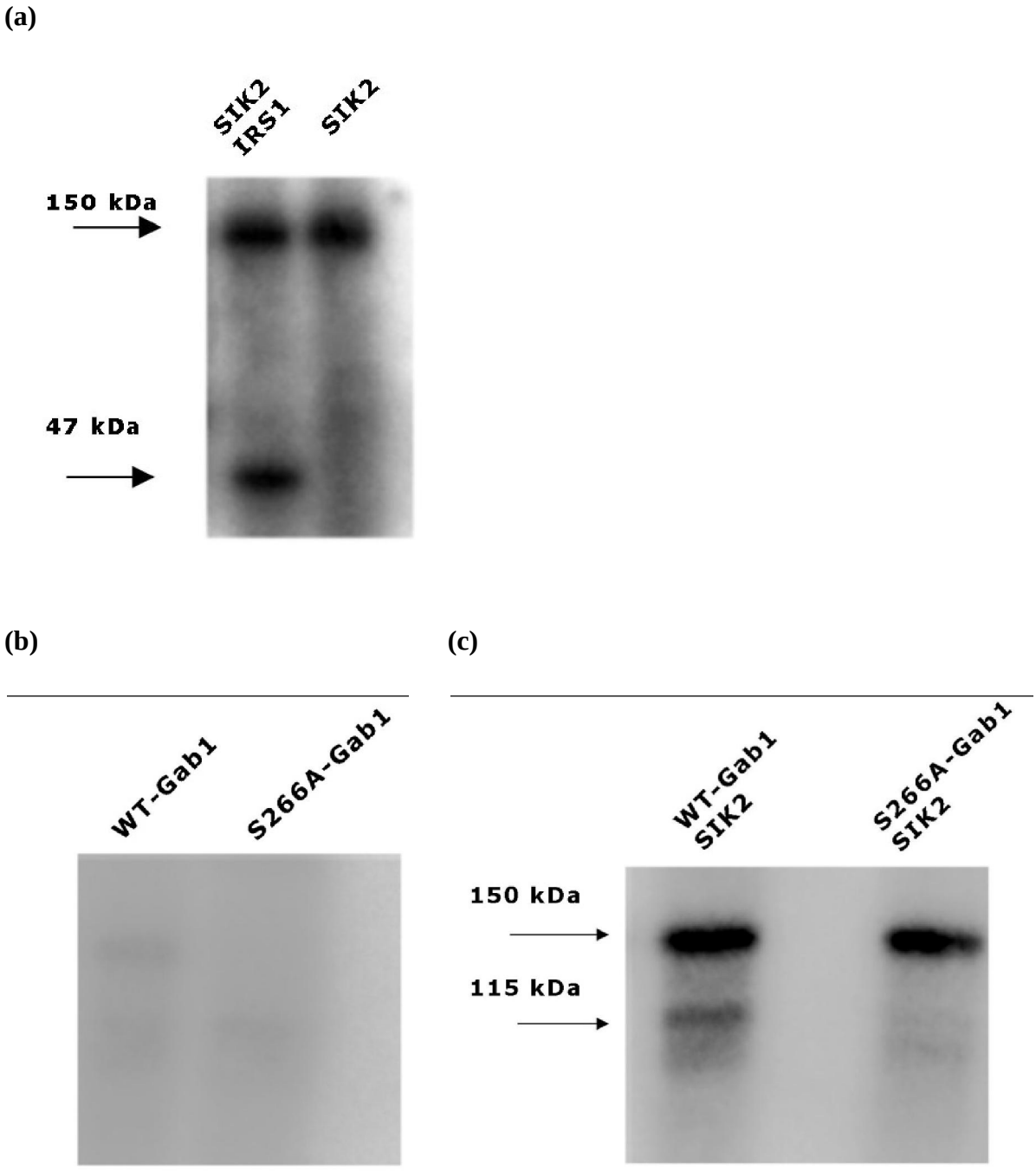

Figure 5.10. Analysis of Gab1 phosphorylation by SIK2. (a) SIK2 activity was assayed. (b) Samples including WT-Gab1 or S266A-Gab1 were devoid of SIK2. (c) The samples including WT-Gab1 or S266A-Gab1 and SIK2.

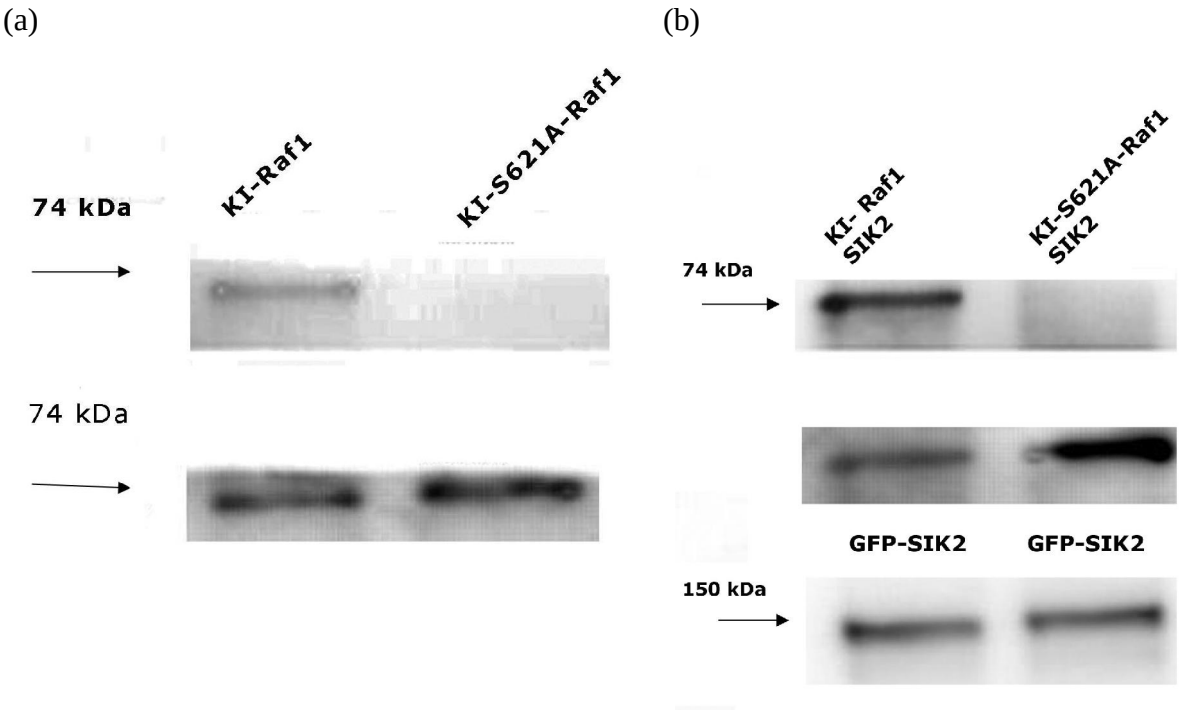

Figure 5.11. Analysis of Raf-1 phosphorylation by SIK2. (a) KI-Raf-1 autophosphorylation was shown in upper panel, input KI-Raf1 in lower panel. (b) KI-Raf-1 phosphorylation by SIK2 is shown in upper panel, input Raf-1 in middle panel, input SIK2 in lower panel.

5.4. Interaction of Gab1 with Its Partners

In order to analyze the effect of S266A mutation on the interaction of Gab1 with its binding partners in MAPK signaling, MIO-M1 cells transfected with either WT-Gab1 or S266A-Gab1 were used in co-immunoprecipitation studies with or without 10 minutes of FGF treatment. The immunoprecipitations were carried out by anti-cMyc tag antibody and the interactions were analyzed using anti-Shp2, anti-Grb2 and anti-SIK2 by Western blotting.

In the immunocomplexes formed from cell lysates, Gab1 was detected as a 115 kDa band (Figure 5.12). The probing of the membrane with Shp2 and Grb2 antibodies revealed 68 kDa and 25 kDa bands, respectively (Figure 5.12). Results indicate that 10 minutes of FGF2 treatment enhances the interaction of Gab1 with these partners. Similarly, the S266A mutation appeared to augment Shp2 levels in the complex in FGF dependent

manner. This supports the idea that SIK2 negatively affects the stability of the complex in response to FGF2 in MIO-M1 cells.

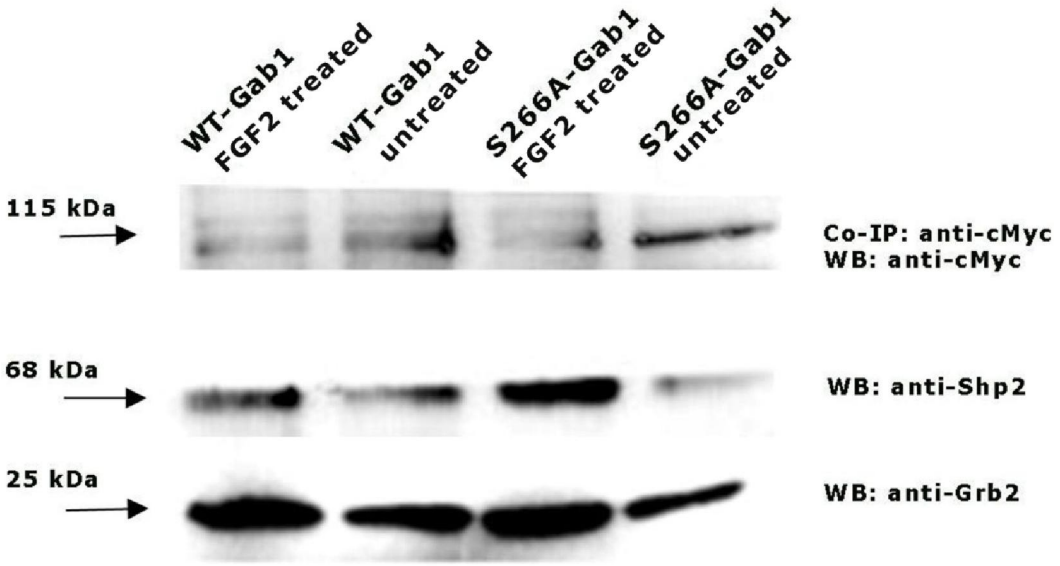

Figure 5.12. Interaction of Gab1 with binding partners. Samples were analyzed by Western blotting. Membranes were probed with anti-cMyc tag antibody (the upper panel), with anti-Shp2 antibody (the middle panel) or Grb2 antibody (lower panel).

5.5. Proliferation of WT-Gab1 or S266A-Gab1 Expressing MIO-M1 Cells

Transfected and overnight starved MIO-M1 cells were treated with 1 ng/ml FGF2 and 100 µg/ml heparin for 2 days, control cells received PBS and 100 µg/ml heparin. Then the cells were assayed for proliferation. Staining of cells with anti-cMyc tag antibody, followed by an Alexa-488 conjugated secondary antibody, indicated that transfection efficiencies were 90-94% in all samples. Anti-Ki-67 antibody, in conjunction with an Alexa 546 conjugated secondary antibody, was used to asses proliferation.

Results in Figure 5.13 were quantified as explained in section 4.18. Statistically significant increase in the proliferaton of WT-Gab1 or S266A-Gab1 transfected MIO-M1 cells upon FGF2 treatment was observed (Figure 5.15). FGF dependent proliferation

difference between WT-Gab1 and S266A-Gab1 expressing cells was not found to be significant according to the Student's T-test.

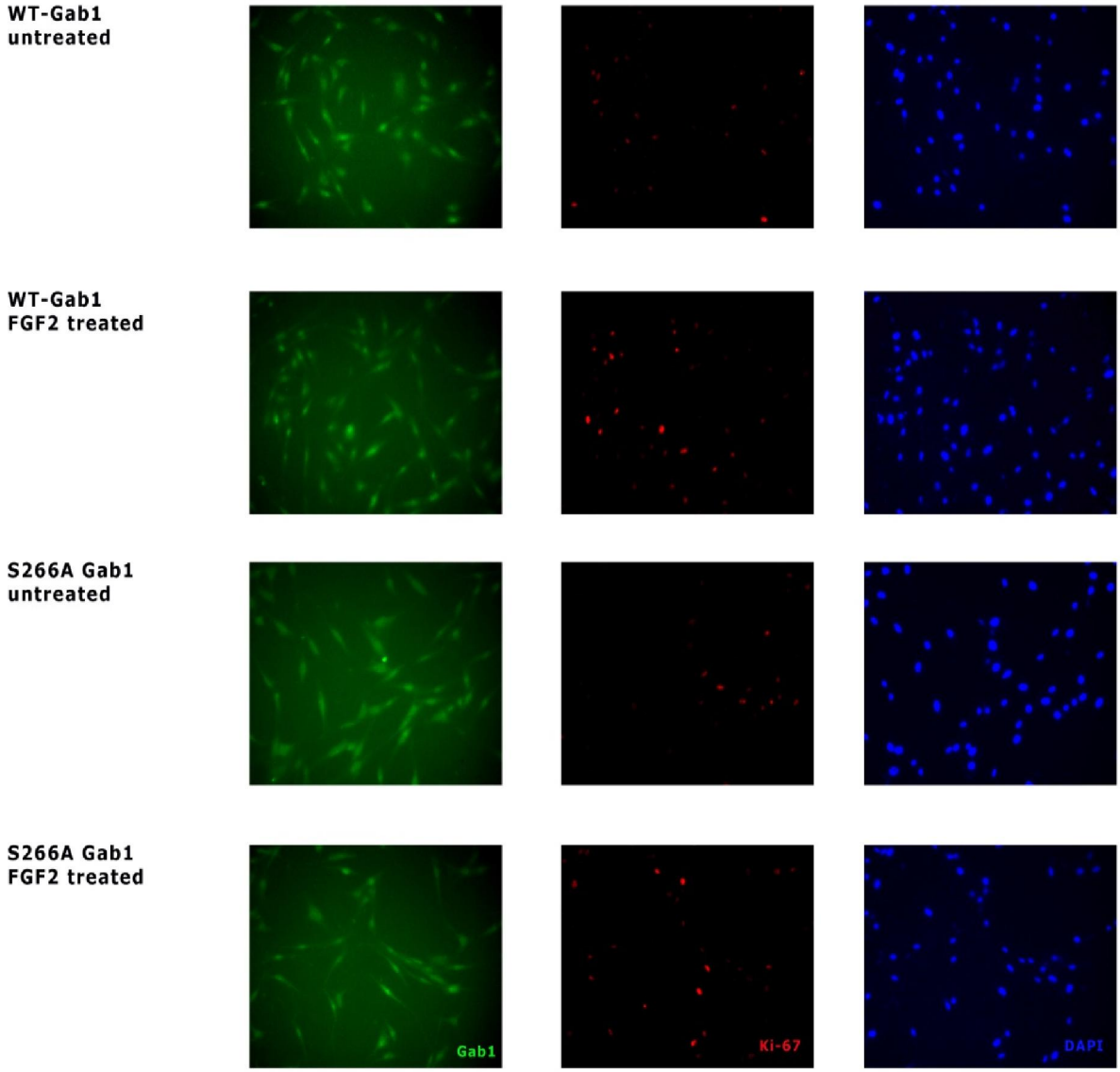

Figure 5.13. FGF dependent proliferation of MIO-M1 cells expressing WT-Gab1 or S266A-Gab1. Proliferation was followed with Ki-67 antibody staining. cMyc tagged Gab1 expressing cells were labeled with cMyc. Cell nuclei were stained with DAPI.

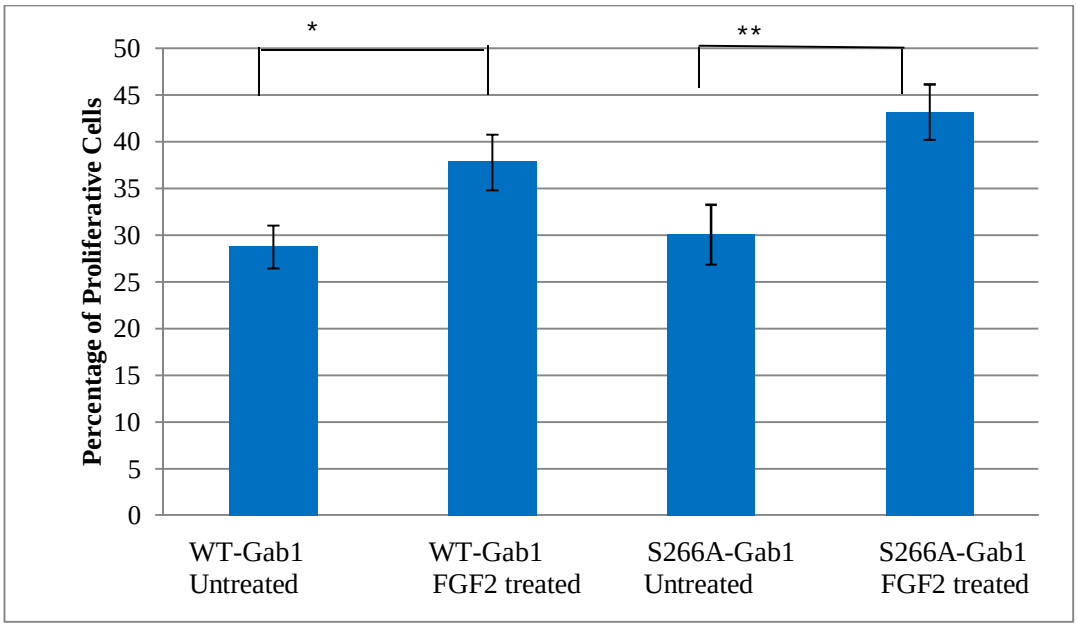

Figure 5.14. Quantification of MIO-M1 proliferation. Ki-67 expressing nuclei among cMyc tag antibody labeled cells were counted. Data was analyzed by Student's T-test.

* $p \leq 0.1$ ** $p \leq 0.05$.

6. DISCUSSION

Müller cells are the major glial cells of the retina and they have many roles involving maintenance of retinal integrity and neuronal survival (Bringmann and Reichenbach, 2001). Müller cell activation is observed in almost all retinal diseases and injury cases (Bringmann *et al*., 2006). This activation includes proliferation and the release of neurotrophic factors including FGF2, perhaps as an attempt to aid in neuronal survival. But often continued proliferation causes retinal detachment and might result in blindness in the long term (Bringmann *et al*., 2009b). One of the factors implicated in the process is FGF2, as it has been reported to be mitogenic for Müller cells *in vitro* (Mascarelli *et al.*, 1991; Çınaroğlu *et al.*, 2005) and exogenously applied factor has been shown to stimulate proliferation of Müller cells *in vivo* (Lewis *et al.*, 1992; Fischer *et al*., 2002). This cellular response involves rapid and transient ERK1/2 activation. This ERK activation profile suggests existence of strong negative feedback control mechanisms, likely to involve phosphorylation/dephosphorylation events. Studies from our laboratory raise the possibility that SIK2 is part of such regulation of FGF signal transduction.

SIK2 was isolated as a FGFR2 interactor and its activity, phosphorylation levels and cellular localization are found to be modulated in response to FGF2 (Özcan, 2003; Özmen 2006; Candaş, 2007; Küser, unpublished data). SH2 and SH3 domains are present on SIK2, which would allow interaction with other intermediates of the FGF pathway (Koch *et al.*, 1991). Through canonical SIK2 phosphorylation motif searches Gab1 and A-Raf were revealed as candidate substrates and further shown to be phosphorylated by SIK2 *in vitro* (Küser, 2006). Recently, it has been shown that ERK phosphorylation and proliferation were increased when SIK2 is suppressed in MIO-M1 cells (Küser, unpublished data). Based on these findings, we hypothesized that FGF activation of ERK might activate SIK2, activated SIK2 in turn phosphorylates Gab1 and Raf-1. We reason that reduced binding of Gab1 and Raf-1 with their partners creates a negative feedback loop for the FGF pathway.

In this context, we aimed at investigating whether SIK2 can target Ser^{266} on Gab1 and Ser^{621} on Raf-1 phosphorylations in FGF dependent manner. Therefore, these serine residues were mutated to alanine via site-directed mutagenesis. As Ser^{621} has been

described as an autophosphorylation site (Morrison *et al.,* 1993), to eliminate the masking of SIK2 kinase activity, Raf-1 was also rendered kinase inactive. Mutant and wild-type proteins expressed in HEK293T were obtained by immunoprecipitation to be used in *in vitro* kinase assay. We also studied the role of Ser^{266} mutation in interaction of Gab1 with its binding partners and the effect of this mutation on proliferation of MIO-M1 cells under FGF stimulation.

The double mutant form of Raf-1 was used to investigate SIK2 activity on this protein by *in vitro* kinase assay and Western blotting with a p-Ser621 specific antibody. A residual phosphorylation activity of KI-Raf-1 was observed in reactions involving the K375M mutation. However, in the presence of SIK2, KI-Raf-1 phosphorylation was increased significantly. Kinase-inactive Raf-1 proteins carrying the S621A mutation were phosphorylated neither by themselves nor by the presence of SIK2. These results support our hypothesis that Ser^{621} on Raf-1 is a substrate of SIK2. The importance of this residue has been discussed in many publications (Mischak *et al.,* 1996; Sprenkle *et al.,* 1997; Matallanas *et al.,* 2011). In this respect a particularly important interaction involves 14-3-3 binding, which depends on Ser^{621} phosphorylation and keeps Raf-1 in either a “closed inactive state” or a “dimerized highly active state”. The dual role of $pSer^{621}$ is controlled by many kinases such as Raf-1 itself, PKA and AMPK. We suggest that Raf-1 activation and stabilization is tightly controlled by the known kinases and the newly proposed upstream kinase SIK2. Involvement of other kinases makes it difficult to investigate the effect of SIK2 phosphorylation on Raf-1 *in vivo*. To gain further understanding of the SIK2-Raf-1 interaction, experiments with sh-SIK2 MIO-M1 cell line should be designed. Since Ser^{621} phosphorylation is recognized by the 14-3-3 protein, SIK2 phosphorylation might have a regulatory role for 14-3-3/Raf-1 binding. Thus, this binding should be further studied.

In vitro kinase experiments using WT-Gab1 verified our earlier findings that it can be phosphorylated by SIK2. S266A mutation on Gab1 obliterated the phosphorylation of the protein. In the reactions carried out in the absence of SIK2, neither WT-Gab1 nor S266A Gab1 was kinased. Thus, we conclude that the *in vitro* SIK2 target on Gab1 is Ser^{266}. Gab1 serine/threonine phosphorylations have been proposed to have negative regulatory role on RTK pathways. It has been previously shown that PKC, the serine/threonine kinase, can phosphorylate Gab1 *in vitro* (Gual *et al.,* 2001). This

phosphorylation proposed to lower tyrosine phosphorylation of Gab1, hampering its interaction with PI3K and downregulates HGF signaling. In another study, Lehr *et al.* (2004) have shown that ERK phosphorylation of Gab1 in response to insulin on several serine/threonine residues causes negative regulation of PI3K/Akt pathway. Likewise, our finding raises the possibility that Ser^{266} phosphorylation of Gab1 by SIK2 represents a mechanism of downregulation of FGF signaling.

Gab1, all mammalian forms, as well as its *Drosophila* ortholog DOS and its *C.elegans* ortholog Soc1, have been shown to interact with Shp2 (Liu and Rohrschneider, 2002; Sarmay *et al.*, 2006; Gu and Neel, 2003). A number of studies involving knock-out animals and mutant Shp2 or Gab1 proteins have underscored the importance of Gab1-Shp2 binding for ERK activation (Cai *et al.*, 2002; Bard-Chapeau *et al.*, 2006). Grb2 interaction is also important for membrane recruitment of Gab1 and allows receptor complex formation with Frs2, Shc or Sos1. Grb2 binding sites are conserved among all mammalian Gab proteins thus this indicates a common recruitment mechanism for Gab1. Gual *et al.*, (2001) have suggested that serine/threonine phosphorylation might change the three-dimensional structure of Gab1 where tyrosine phosphorylation sites are hidden, thus block its interaction with the partners. Therefore, we investigated the effect of Ser^{266} mutation on its interaction with the direct binding partners, Shp2 and Grb2. Our results showed that the Gab1-Shp2 interaction was enhanced with FGF treatment in comparison to the controls, agreeing with the literature (Ong *et al.*, 1997). We observed that there is a significant increase of Shp2 in the immunoprecipitates after 10 minutes of FGF treatment for mutant Gab1 with respect to wild-type. Therefore, it is conceivable that the mutation leads to sustained Gab1-Shp2 interaction. In this context, we suggest that SIK2 phosphorylation of Gab1 on Ser^{266} disrupting Gab1-Shp2 binding, might lead to downregulation of FGF-dependent ERK activation. Phosphorylation of this site did not create a change in binding of Grb2/Gab1, so we can assume that this site is not directly related with Grb2 interaction.

Next, the effect of $pSer^{266}$-Gab1 on proliferation of MIO-M1 cells under FGF2 induction was studied. Though, significant increases in proliferation of FGF treated cells were observed, higher proliferation rates were expected. The difference that FGF2 was expected to create on proliferation might be masked by Gab1 overexpression. Mitogenic effect of Gab1 overexpression has been pointed out in various studies (Chan *et al.*, 2010;

Kameda *et al*., 2006) Gab1 and Shp2 knock-outs resulted in lower ERK phosphorylation and suppressed hepatocyte proliferation (Bard-Chapeau *et al*., 2006). Given the importance of Gab1-Shp2 binding in proliferation (Felici *et al*., 2010), we expected to have a significant elevation of proliferation by S266A-Gab1 mutant. Contrary to our expectations, S266A did not result in a significant proliferation increase in comparison to WT. One of the reasons for this might be the involvement of a multitude of proteins in proliferation. Thus, the effect of only one protein with a single mutation might not be enough to create a significant change. Additionally, some other kinase activities might be involved that might balance this change. Besides, technical issues might be another reason. It is difficult to express exogenous proteins in MIO-M1 cells. A stable line expressing S266A-Gab1 might be used to see a significant effect in proliferation. Also repeating these assays using SIK2 silenced MIO-M1 cell lines would clarify the role of Ser^{266} phosphorylation on proliferation.

In summary, we have shown that SIK2 phosphorylates Gab1 on Ser^{266}, mutation of this site leads to enhanced Gab1-Shp2 binding, and modest increase in Müller cell proliferation. These findings support our working model suggesting Ser^{266}-Gab1 and Ser^{621}-Raf-1 phosphorylations by SIK2 is part of the negative feedback regulation of FGF2 pathway (Figure 6.1). This proposed negative feedback mechanism contributes to the better understanding of Müller cell activation and proliferation.

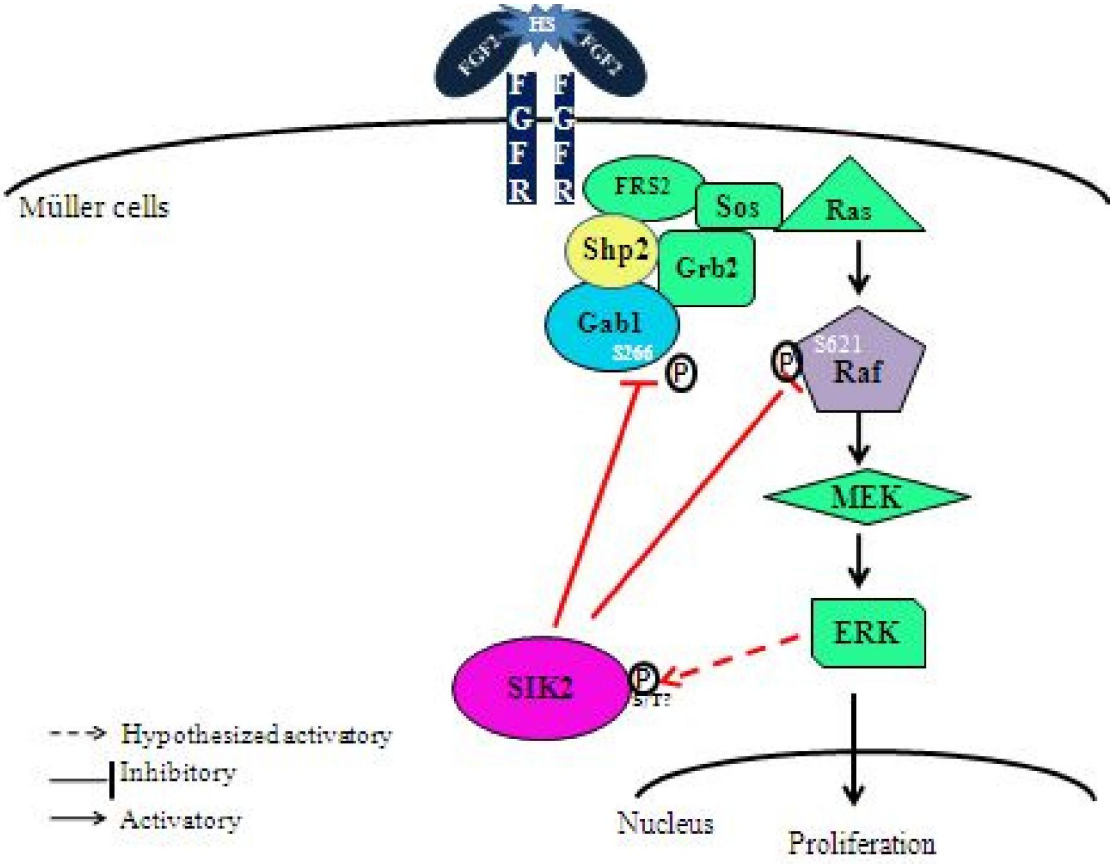

Figure 6.1. Proposed negative feedback mechanism of FGF pathway involving Gab1 and Raf-1 phosphorylations by SIK2.

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