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INVOLVEMENT OF SIK2 IN FGF2 SIGNAL REGULATION VIA RAF-1
&
GENERATION OF MUTANTS FOR IDENTIFICATION OF PHOSPHORYLATION SITES ON SIK2 BY ERK

by

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Dedicated to my family and friends, especially Arman, Sinem and Edibe...

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ABSTRACT

INVOLVEMENT OF SIK2 IN FGF2 SIGNAL REGULATION VIA RAF-1 & GENERATION OF MUTANTS FOR IDENTIFICATION OF PHOSPHORYLATION SITES ON SIK2 BY ERK

Müller cell proliferation is induced by FGF2 signaling via Ras/MAPK pathway through rapid and transient ERK1/2 activation. Salt inducible kinase 2 (SIK2), a serine/threonine kinase, is a novel regulator in this context. Results from our laboratory implicate SIK2 in downregulation of FGF2 signaling through phosphorylation of adaptor protein, Gab1 on Ser266. SIK2 also phosphorylates another pathway element, Raf-1 on Ser621. In this study, we hypotesized that SIK2 phosphorylation of Raf-1 downregulates its activity and activation of downstream elements in FGF2 signaling. In this context modulation of Raf-1 phosphorylation on Ser621 was investigated in FGF2 treated control and SIK2 silenced Müller cells in a time course from 0 to 120 minutes. Raf-1 activity was analyzed by in vitro kinase assay using kinase inactive MEK as Raf-1 substrate. Our data revealed that Raf-1 phosphorylation on Ser621 reaches maximum level at 10 minutes and the minimum level at 60 minutes of FGF2 treatment. Raf-1 activity is the highest in SIK2 silenced cells at 10 minutes and close to basal level at 60 minutes. These data are in accordance with SIK2 activation profile. It can be concluded that SIK2 inhibits Raf-1 activity by phosphorylating it on Ser621 at 10 minutes when SIK2 has the maximum activity. Raf-1 phosphorylation status and activity approximates to basal levels at 60 minutes where SIK2 activity is at minimum level. In the second part of the study, phosphorylation of SIK2 by ERK was investigated through generation of SIK2 mutants. Our previous data shows that ERK phosphorylates SIK2 on two candidate threonines as 758 and 863 based on ERK phosphorylation motif, (S/T)P adjacent to FxFP motif (DED domain). This phosphorylation results in activation and modulation of SIK2. As a first step in identification of target residues, T863A SIK2 mutant was generated.

ÖZET

SIK2 PROTEİNİNİN RAF-1 PROTEİNİ ARACILIĞIYLA FGF2 SİNYAL YÖNETİMİNDEKİ GÖREVİ & SIK2 PROTEİNİNİN ERK PROTEİNİ TARAFINDAN FOSFORLANMA MOTİFLERİNİN BELİRLENMESİ İÇİN MUTANT ÜRETİMİ

Müller hücreleri çoğalımı Ras/MAPK yolağı üzerinden FGF2 sinyaliyle hızlı ve kısa süreli ERK1/2 aktivasyonuyla indüklenmektedir. Bir serin/treonin kinaz olan tuzla indüklenebilir kinaz 2 (SIK2), bu çerçevede yeni bir düzenleyicidir. Laboratuvar sonuçlarımız göstermiştir ki, SIK2 yolak üyesi Gab1'i Ser266 üzerinden fosforile ederek FGF2 sinyal iletimini baskılamada görev almaktadır. Ayrıca SIK2 bir diğer yolak üyesi olan Raf-1'i Ser621 üzerinden fosforile etmektedir. Bu araştırmada, Raf-1'in SIK2 tarafından fosforilasyonunun Raf-1 ve aşağı yönlü elemanlarının aktivitesi üzerinde negatif etkisi bulunduğunu öne sürülmektedir. Bunun analizi için, FGF2 ile uyarılmış kontrol ve SIK2 susturulmuş Müller hücrelerinde Raf-1 'in Ser621 üzerindeki 0 ile 120 dakika arasındaki zaman diliminde fosforilasyon düzeni incelenmiştir. Ayrıca, Raf-1 aktivitesi, Raf-1 substratı olarak kinaz inaktif MEK kullanılarak in vitro kinaz tayini ile analiz edilmiştir. Verilerimiz önermektedir ki, FGF2 uyarımının 10. dakikasında Raf-1'in SIK2 tarafından Ser 621 üzerindeki fosforilasyonu en yüksek seviyedeyken; 60. dakikada en düşük seviyededir. SIK2'nin susturulmuş olduğu hücrelerde Raf-1 aktivitesi 10. dakikada en yüksek seviyededir ve 60. dakikada bazal düzeye yakındır. Bu veriler SIK2'nin aktivasyon profili ile uyumludur. Bu durumda, 10. dakikada en yüksek aktivite seviyesine ulaşan SIK2'nin Raf-1 aktivitesini Ser621'i fosforile ederek baskıladığı sonucuna varılabilir. Raf-1 fosforilasyon durumu ve aktivitesi SIK2'nin en düşük aktivite düzeyinde olduğu 60. dakikada bazal düzeye yaklaşmaktadır. Araştırmanın ikinci kısmında, SIK2 mutantları oluşturularak, ERK tarafından SIK2'nin fosforilasyonu araştırılmıştır. Önceki verilerimize göre; ERK SIK2'yi FxFP motifine birleşik (S/T)P (DED domain) olan ERK fosforilasyon motifine dayanarak belirlenen 758 ve 863 numaralı 2 aday treonin üzerinden fosforile etmektedir. Bu fosforilasyon SIK2'nin aktivasyonu ve modülasyonu ile sonuçlanmaktadır. Hedef amino asitlerin belirlenmesinde ilk adım olarak, T863A SIK2 mutantı üretilmiştir.

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LIST OF SYMBOLS

^{o}C	Celcius degree
V	Volt
v	Volume
w	Weight
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µl	Microliter

LIST OF ACRONYMS/ ABBREVIATIONS

A	Acid box
ACTH	Adenocorticotropic hormone
AFX	Acute-lymphocytic-leukaemia-1 fusion gene from chromosome X
Akt	Murine thymoma viral oncogene homolog
AMPK	Adenosine monophosphate activated kinase
APS	Amonium Persulfate
ATP	Adenosine triphosphate
Bad	Bcl-2-associated death promoter
Bag	Bcl2-associated athanogene
BCA Assay	Bicinchoninic acid assay
Bcl-2	B-cell lymphoma 2
BCR/ABL	Breakpoint cluster region/ Abelson
BDNF	Brain derived neurotrophic factor
bp	Base pair
BSA	Bovine Serum Albumin
c-Myc	Cellular homolog of the retroviral v-myconcogene
Ca	Calcium
cAMP	Cyclic adenosine 5'-monophosphate
Cbl	Casitas B-lineage Lymphoma
CNK	Connector enhancer of kinase suppressor of Ras
CR	Conserved regions
CRALBP	Cellular retinaldehyde-binding protein
CRD	Zinc finger/cysteine rich domain
CRE	c-AMP responsive element
CREB	CRE binding protein
CO_2	Carbondioxide
DAG	Diacylglycerol
dd H_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E.*coli*	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene glycol tetraacetic acid
EGFR	Epidermal growth factor receptor
Elk-1	E twenty-six (ETS)-like transcription factor 1
ERK	Extracellular Regulated Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FKHR	Forkhead transcription factor
Frs2	Fibroblast growth factor receptor substrate
GAB1	Grb2-associated binder 1
GDNF	Glial cell line-derived neurotrophic factor
GFP	Green Fluorescent Protein
Glu	Glutamine
Grb2	Growth factor receptor-bound protein
GS	Glutamine synthetase
H_2O	Water
HB	Heparin-binding site
HCl	Hydrochloric acid
HDAC-5	Histone deacetylase-5
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
HSPG	Heparan sulfate proteoglycans
IgG	Immunoglobulin G
IP	Immunoprecipitation
IP3	Inositol-1,4,5-triphosphate
IRS	Insulin receptor substrate proteins
IVK	*In vitro* kinase

JNK	c-Jun N terminal kinase
kb	Kilobase
KCl	Potassium chloride
kDa	Kilodalton
KI	Kinase inactive
KSR	Kinase suppressor of Ras
LB	Luria Bertani Broth
LEOPARD2	LEOPARD syndrome type 2
LKB1	Liver kinase B 1
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
MKP1	MAPK phosphatase 1
ml	Mililiter
mm	Millimeter
mM	Milimolar
NaCl	Sodium Chloride
NF-kB	Nuclear Factor-KappaB
NS5	Noonan syndrome type 5
ng	Nanogram
NGF	IGF-1 nerve growth factor
NLS	Nuclear localization signal
Nm	Nanometer
NT3	Neurotrophin-3
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
Pax6	Paired Box Gene 6
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction

PC12	Pheochromocytoma
Pen/Strep	Penicillin/Streptomycin
pH	Power of hydrogen
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphotidylinositol-4,5-diphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLCγ	Phospholipase C-gamma
PMSF	Phenylmethylsulphonylfluoride
PTB	Phosphotyrosine binding
PVDF	Polyvinylidene fluoride
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
RBD	Ras-binding domain
RKIP	Raf kinase inhibitor protein
RNA	Ribonucleic acid
ROCK	Rho-associated *protein* kinase
rpm	Rotations per Minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAPK	Stress activated protein kinase
scr	Scrambled
SDS	Sodium Dedocyl Sulfate
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
sec.	Seconds
Ser	Serine
SH	Src homology domain
SHP2	SH2-domain containing phosphatase 2
SIK	Salt inducible kinase
SNF	Sucrose-nonfermenting-1 protein kinase
Sos	Son of sevenless
Sox2	Sex determining region Y box 2
SP	Cleavable secreted signal sequence

SP*	Uncleaved bipartite secreted signal sequence
Spry	Sprouty
Srebp1-c	Sterol regulatory element binding proteins
STAT	Signal transducer and activator of transcription
SUR8	Suppressor of Ras-8
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TEMED	Tetramethylethylenediamine
TGF	Transforming Growth Factor
Thr	Threonine
TK	Tyrosine kinases
TM	Transmembrane domain
TORC2	Transducer of regulated CREB activity
Trk	TRK1-transforming tyrosine kinase protein
TWEEN	Polysorbate
UBA	Ubiquitin-associated domain
Val	Valine
VEGF	Vascular endothelial growth factor
WB	Western Blot
x g	Times Gravity

1. INTRODUCTION

1.1. Retina

Vision starts by passage of a ray of light through the cornea, anterior chamber, the pupil, the lens and the vitreous, finally focusing on the retina. The retina is originated from central nervous system during embryonic development. Retina is composed of retinal pigment epithelium, the choroid and the sclera and is a layered structure of neuronal and supporting cells, converting light energy into nerve impulses to be processed in the visual cortex (Abramoff *et al.*, 2011; Figure 1.1).

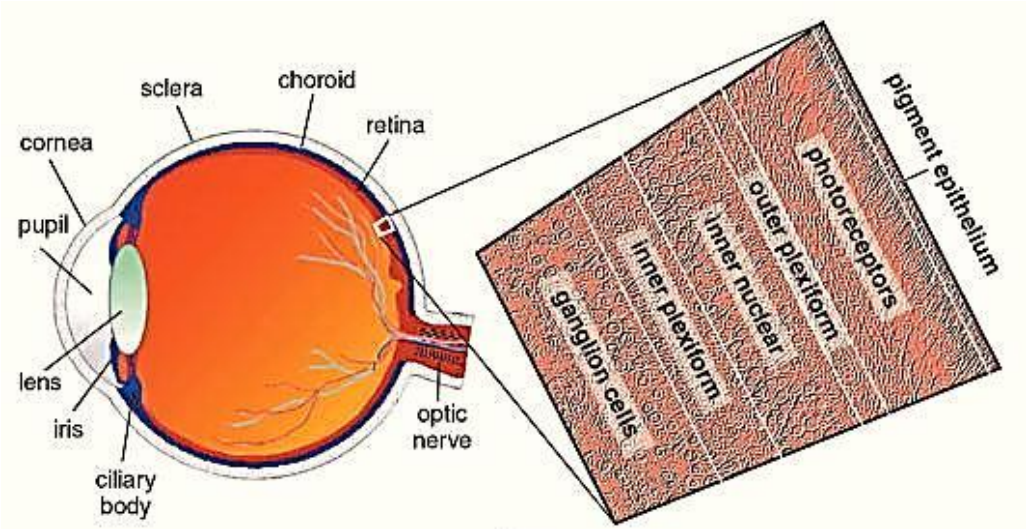

Figure 1.1. Schematic representation of retina (Modified from Abràmoff *et al.*, 2010).

Conversion of absorbed light energy to a change in membrane potential through the phototransduction cascade is done by two types of photoreceptor cells: rods and cones. Rods are responsible for detection of dim light and cones are instrumental in perception of color and daytime vision (den Hollander, 2010). In addition to rods and cones, there are four types of neuronal cells in retina: amacrine, horizontal, bipolar and ganglion cells. Amacrine cells take part in modulation between bipolar and ganglion cells via a particular type of neurotransmitter whereas horizontal cells are instrumental between ganglion cells

and receptors. Bipolar cells connect receptors and ganglion cells which form part of optic nerve (Newman and Reichenbach, 1996; Figure 1.2).

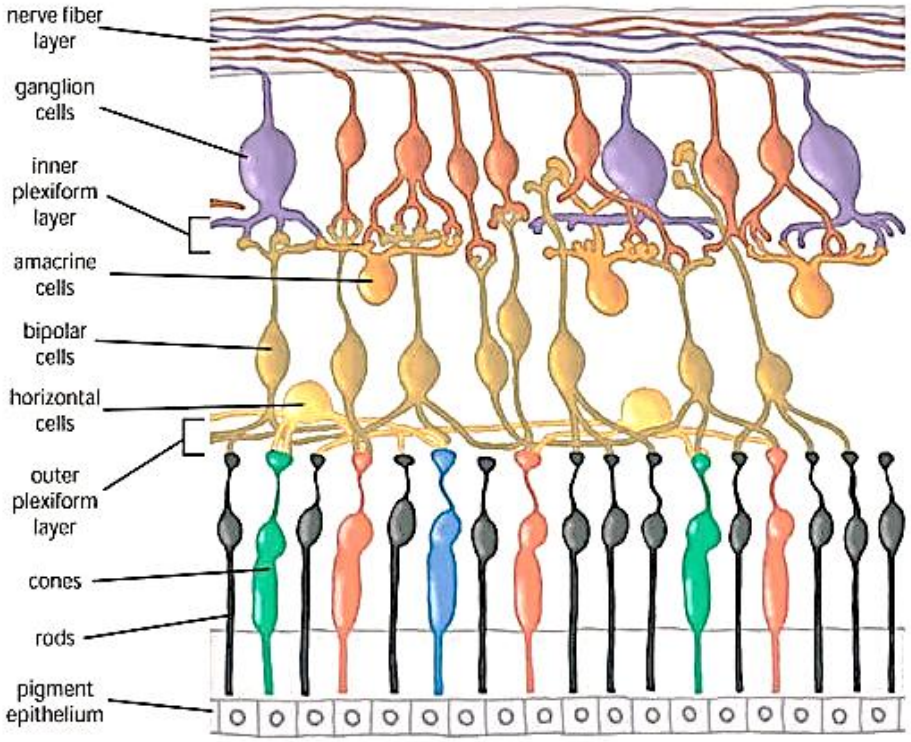

Figure 1.2. Schematic representation of retinal cells (Modified from Abràmoff *et al.*, 2010).

The mammalian retina contains three types of glial cells; the microglial and two types of macroglial cells (astrocytes and Müller cells). Microglial cells are resident immune cells derived from blood and located in the innermost retinal layers. They play important functions in host defense mechanisms against microorganisms, in inflammation and tissue repair (Bringmann *et al*, 2006). Astrocytes have critical roles during retinal vascularization (Dyer *et al*, 2000).

1.2. Müller cells

Müller cells are the principal glial cells spanning the entire thickness of retina. The human retina has approximately 8 to 10 million Müller cells. Each one interacts a column of retinal neurons symbiotically by providing the physical link to the exchange compartments of the retina, like blood vessels, vitreous body and subretinal space. Müller cells support their associated neurons metabolically and functionally by secretion of gliotransmitters and other trophic substances, neurotransmitter recycling and removal of waste which has an important role in neuronal protection and survival (Figure 1.3; Bringmann *et al.*, 2006).

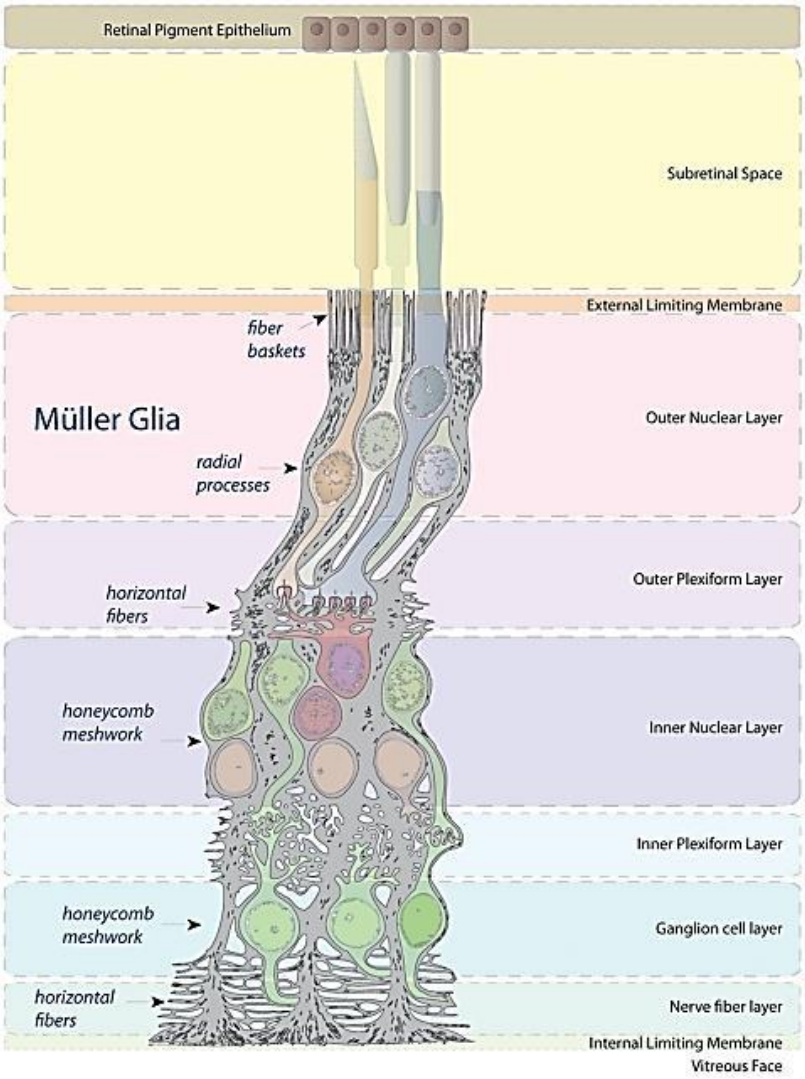

Figure 1.3. Müller glial cells in the retina (Modified from Klimczak *et al.*, 2009).

They are also crucial in structural stabilization, regulation of extracellular space volume, pH, ion and water homeostasis in retina, formation of blood – retinal barrier and modulation of inflammatory and immune responses (Bringmann *et al.*, 2009; Bringmann and Wiedemann, 2012).

Müller cells are primarily dependent on glycolysis for ATP production with high rate of lactate formation. Moreover, Müller cells show resistance to anoxia or absence of glucose, presumably to spare oxygen for retinal neurons which have high metabolic rates for energy and oxygen consumption under normal physiological conditions (Winkler *et al.*, 2000).

In vascularized retinae, Müller cells are also postulated to have roles in control of angiogenesis, and the regulation of retinal blood flow by synthesis of vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ; Bringmann *et al.*, 2006).

In the retinal defense against free radicals, Müller cells synthesize the tripeptide glutathione from glutamate, cysteine, and glycine (Pow and Crook, 1995; Reichelt *et al.*, 1997; Schütte and Werner, 1998). Reduced glutathione takes role in detoxification of reactive oxygen species and free radicals (Dringen *et al.*, 2000). Glutamate is converted to glutamine by glutamine synthetase (GS) in Müller cells. Glutamine is recycled to neurons for maintenance of excitatory synapses. pH is regulated by means of carbonic anhydrase and balancing of potassium ions (Barnett and Pow, 2000).

One of the most important features of Müller cells is to provide trophic factors for neuronal survival and neuritogenesis. (Tsacopoulos and Magistretti, 1996; Newman and Reichenbach, 1996; Bringmann *et al.*, 2009; Garcia *et al.*, 2002). Upon activation, they secrete neurotrophic factors such as basic fibroblast growth factor (FGF2; Bringmann *et al.* 2012) , brain derived neurotrophic factor (BDNF), IGF-1 nerve growth factor (NGF; Brightmann *et al.*, 2006), neurotrophin-3 (NT3, Taylor *et al.*, 2003) and their high-affinity Trk (Wahlin *et al.*, 2000 and Oku *et al.*, 2002) or low-affinity p75 (Garcia and Vecino, 2003) receptors.

In addition, the glial cell line-derived neurotrophic factor (GDNF) upregulates FGF2 through phosphorylation of ERK1/2 in Müller cells, enhancing photoreceptor survival (Brightmann *et al.*, 2012). FGF2 also contributes to differentiation, proliferation and neuroprotection (Meyer-Franke *et al.*, 1995, Bugra *et al.*,1997, Harada *et al.*, 2000, Peterson *et al.*, 2000 and Kawasaki *et al.*, 2000).

When retina is exposed to injury or disease such as ischemia, glaucoma, age-related macular degeneration and diabetic retinopathy, Müller cells are activated which is proposed to enhance retinal survival (Bringmann *et al.*, 2009). Nevertheless, long term activation may result in glial scar formation and retinal detachment and even blindness (Bringmann and Wiedemann, 2009).

Müller cells are differentiated at day 8-10 from precursor cells during retinal development (Barnett and Pow, 2000). It has been proposed that in adult retina, a small population of mature Müller cells maintain ability to dedifferentiate and transdifferentiate into neuron-like cells (Fischer and Reh, 2001; Karl and Reh, 2010; Fischer and Bongini, 2010).

Moorfiels Institute of Ophthalmology-Müller 1 (MIO-M1) cell line is a spontaneously immortalized human Müller cells isolated from postmortem human retina, expressing both Müller glial markers such as CRALBP, EGFR, vimentin and gluthamine synthase as well as stem cell markers including Sox2, Pax6 and Notch1 and can be induced to transdifferentiate (Limb *et al.*, 2002; Lawrence *et al.*, 2007). In line with Müller cells in primary culture, their proliferation can be induced by FGF2 and FGF9 via activation of ERK1/2 (Hollborn *et al.*, 2004; Kinkl *et al.*, 2001; Çınaroglu, 2005).

1.3. Fibroblast Growth Factors

Fibroblast growth factor (FGF) family is composed of 23 identified polypeptide members, present in species ranging from nematodes to humans. Twenty two of the members are present in mammals (FGF1- FGF23) with sizes ranging from 17 to 34 kilodaltons (kDa; FGF15 had not been identified in humans). These members can be

classified in three groups by course of action as intracellular, canonical and hormone-like and into 7 subfamilies by phylogenetic relationships (Figure 1.4.; Itoh and Ornitz, 2011).

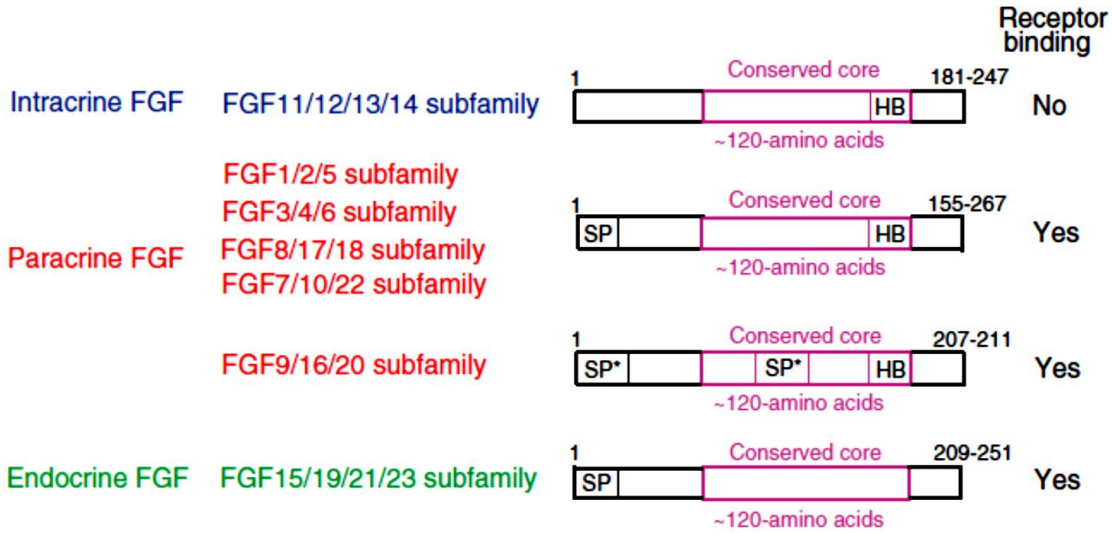

Figure 1.4. Evolutionary relationships and structures of human FGF gene family. HB: heparin-binding site SP: cleavable secreted signal sequence, SP*: uncleaved bipartite secreted signal sequence (Modified from Itoh and Ornitz, 2011).

FGFs show high conservation in both gene structure and aminoacid sequence in vertebrates. The internal core domain is composed of 120 aminoacids of which 28 are conserved and 6 are identical. Intacrine FGFs (FGF11- 14) do not bind and activate FGF receptors (FGFRs) though they have the conserved core (Itoh, *et al.*, 2004; Itoh and Ornitz, 2011).

Endocrine FGF families (FGF 15, 19, 21, 23) are dependent on transmembrane klotho proteins to relay signals to various pathways upon specific interactions (Goetz, 2007) and take part in regulation of cholesterol, glucose, vitamin and phosphate homeostasis.

Paracrine FGF families have important roles in organogenesis and tissue patterning (Fu *et al.*, 2004; Kharitonenekov *et al.*, 2005; Tomlinson *et al.*, 2002). Especially, expression of FGF1, 2, 3, 8, 9 and 17 are important in eye development and homeostasis in

mature retina of vertebrates (Vogel-Hopker *et al.,* 2000; Walshe and Mason, 2003). FGF3, 4, 8, 15, 17, and 19 are expressed only in the course of embryonic development (Ornitz *et al.,* 2001).

Since FGF signal peptide sequences are at the amino terminal, they are generally destined to be secreted. FGF 1, 2, 9, 16 and 20 which don't contain the signal peptide and use an alternate pathway for secretion (Powers *et al.,* 2000). FGF2 and FGF3 also have nuclear localization signal (Powell and Klagsbrun, 1991).

FGFs have a high affinity for heparin sulfate proteoglycans and their interaction is essential for activation FGF receptors (FGFRs; Zhu X *et al.,* 1991, Plotnikov *et al.,* 1999). Furthermore, their affinities for receptors and thus, the pathways they are involved can be varied by mechanisms such as alternative splicing and post-translational modification (Olsen *et al.,* 2006).

FGFs are dimerized upon binding to FGFRs which induces receptor transphosphorylation and several potential signaling pathways are activated. Best studied ones are RAS-RAF-MAPK, PI3K-AKT, STAT and PLCγ (Itoh and Ornitz, 2011).

FGF1 and FGF2 were the first identified FGFs and they were purified from brain as mitogenic factors of fibroblasts in culture (Gospodarowicz and Moran, 1975). FGF1, 2 and 9 are expressed at high levels in adult mammalian retina (Bugra *et al.,* 1994; Bugra and Hicks., 1997; Fischer *et al.,* 2004). They also induce proliferation in Müller cells (Mascarelli *et al.,* 1991; Çınaroglu *et al.,* 2005), retinal ganglion cell survival *in vitro* (Desire *et al.,* 1998; Kinkl *et al.,* 2003) and photoreceptor survival (Siffroi-Fernandez *et al.,* 2008).

FGF2 is involved in initiation and mediation of neural differentiation in chick retina (Pittack *et al.,* 1997). In mature chick retina, FGF2 evokes a proliferative response in Müller cells, followed by transdifferentiation into neurons via sustained MAPK activation (Fischer *et al.,* 2002; Fischer *et al.,* 2009; Lawrence *et al.,* 2007).

1.4. Fibroblast Growth Factor Receptors

There are four FGFRs that have been identified, FGFR 1-4, sharing 55%-72% homology at protein level in mouse and human. FGFR genes encode for receptor tyrosine kinases which have a common structure of three immunoglobulin (IgG) like domains as extracellular binding domain, transmembrane domain and intracellular tyrosine kinase domain (Figure 1.5; Ornitz and Itoh, 2011). The specificity and affinity of extracellular ligand binding is determined by alternative splicing of Ig III domain by creating isoforms which leads to variety of responses (Powers *et al.*, 2000). Other than domains, FGFRs also share a homology for acid box of seven to eight acidic residues located between Ig-I and Ig-II; and signal peptide in their structure (Figure 1.5).

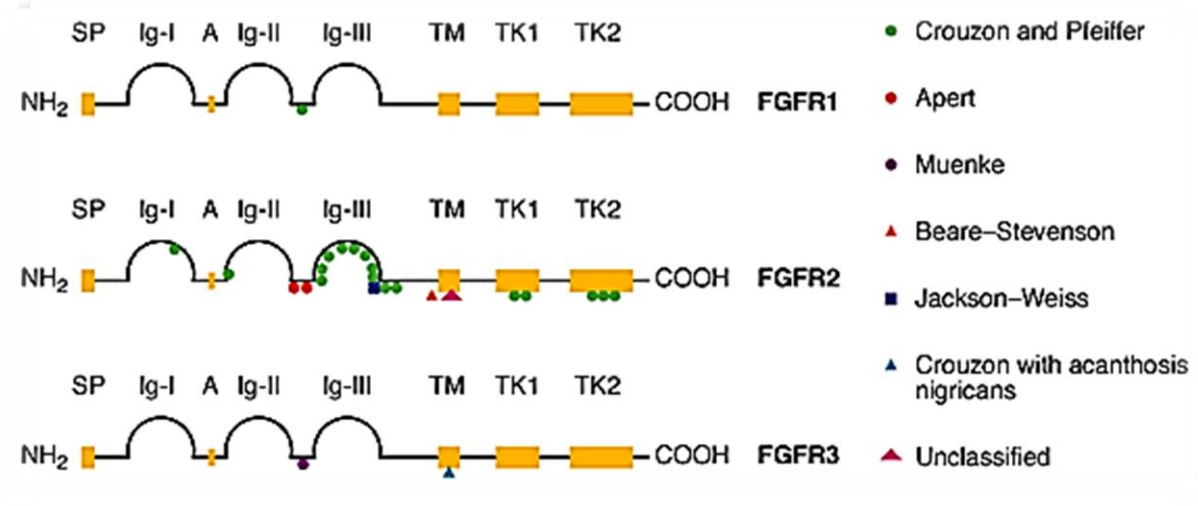

Figure 1.5. Schematic of the FGFR structure. SP stands for signal peptide, A for acid box, TM for transmembrane domain and TK for tyrosine kinases. The symbols show mutations leading to syndromes (Modified from Bonaventure and El Ghouzzi, 2003).

Mutations in the structure of FGFRs may cause many defects and diseases such as Crouzon and Pfeiffer syndrome, Jackson –Weiss syndrome and Muenke syndrome (Bonaventure and El Ghouzzi, 2003). In case of knockout of FGFR1 and FGFR2, severe developmental defects occur and mice with these knockouts die at early stages (Deng *et al.*, 1997; Arman *et al.*, 1998).

In vertebrate retina, expression of all FGFRs is observed (Kinkl *et al.*, 2002). Especially FGFR1, FGFR2 and FGFR3 isoforms are expressed in Müller cells (Çınaroglu *et al.*, 2005).

1.5. Cell Signaling via FGF Receptors

Cell signaling via FGFRs is transduced by binding of extracellular ligands, dimerization of FGFRs and trans-tyrosine phosphorylation (Schlessinger, 1988). Heparin or heparin sulphate proteoglycans (HSPGs) are important in initiation and stabilization of the interaction of FGF and FGFR (Ornitz and Itoh, 2011; Harmer *et al.*, 2004).

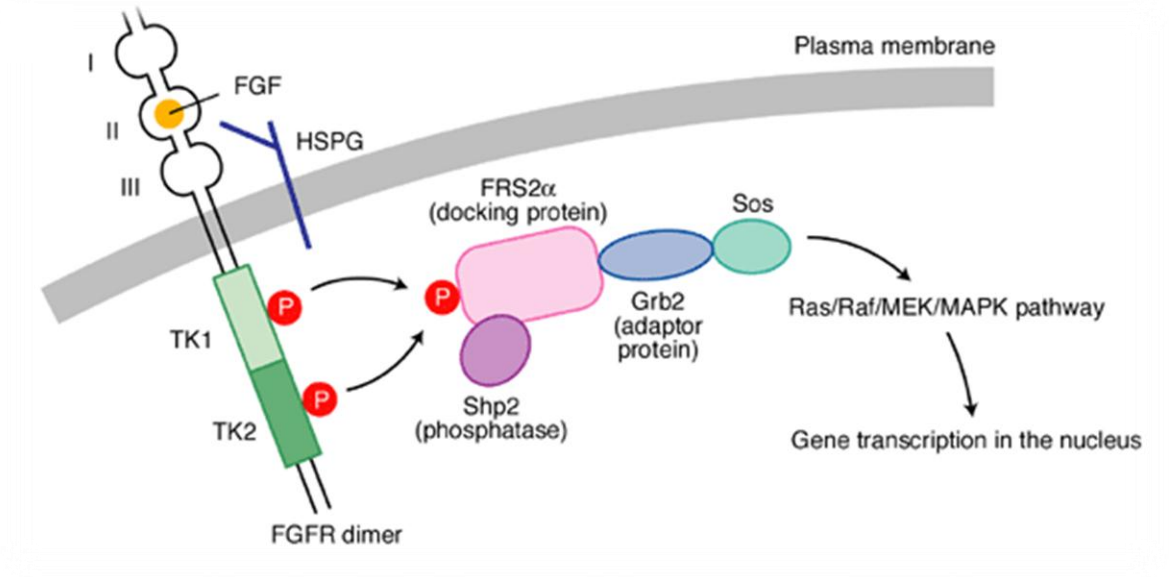

Figure 1.6. Structure and function of FGFRs (Modified from Bonaventure and El Ghouzzi, 2003).

When the ligand binds to FGFRs, the receptors dimerize and undergo an activatory conformational shift leading to autophosphorylation of tyrosine kinase domains and the C-terminal tail. Phosphorylated tyrosines serve as docking sites for SH2 (src homology 2), SH3 (src homology 3) and phosphotyrosine binding (PTB) domains of adaptor proteins, relaying the signal (Figure 1.6). There are seven conserved tyrosine residues in FGFRs and

variances in phosphorylation pattern leads to activation of alternate pathways (Kristen *et al.*, 2001).

There are three main alternate pathways in FGF signaling (Figure 1.7): 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).

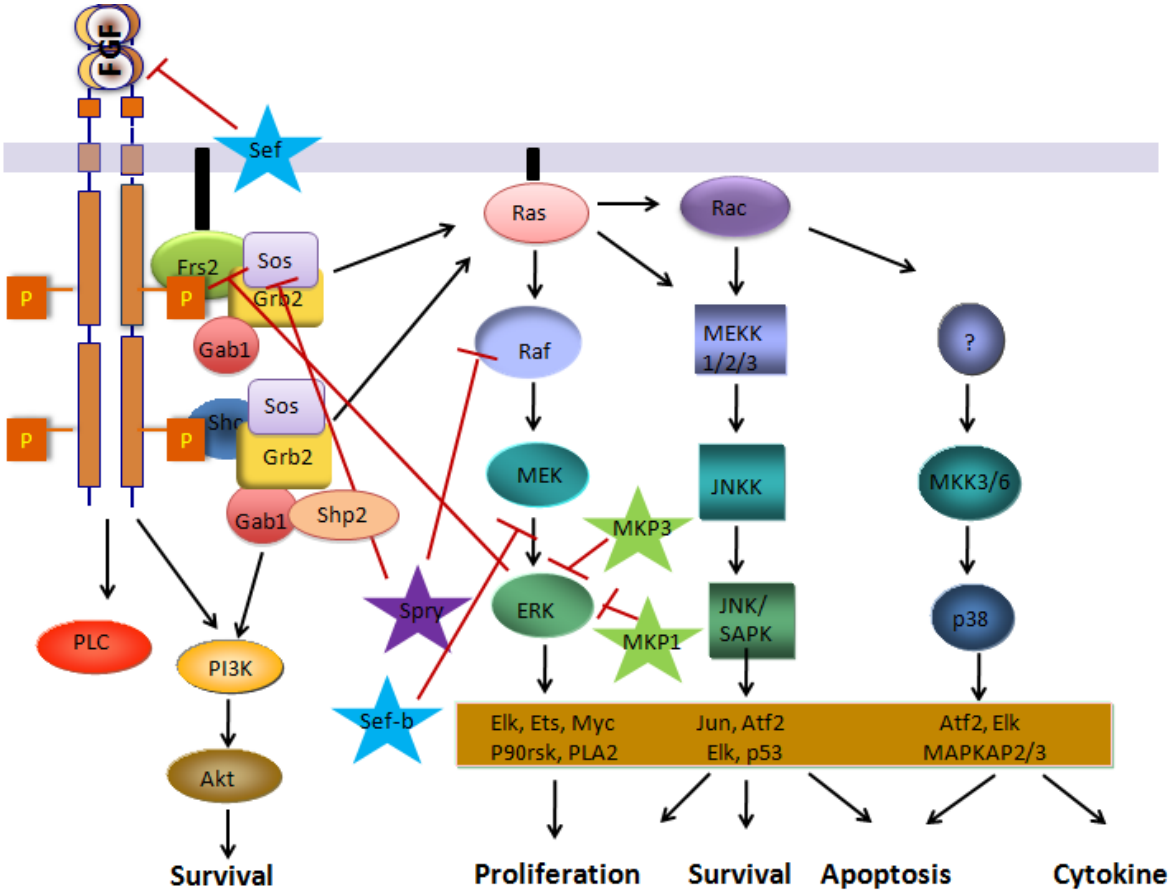

Figure 1.7. Three main pathway elements in FGF signaling: PLCγ/Ca2+ pathway, Ras/MAPK pathway, and PI3K/Akt pathway (Modified from Küser, 2011).

1.5.1. PLCγ/Ca2+ pathway

Phospholipase C γ is a known substrate of all FGFRs. Following binding of ligand, Tyrosine 766 on the receptor is autophosphorylated and creates a docking site for SH2 domain of PLCγ (Maffucci *et al.*, 2009; Mohammadi *et al.*, 1991). Upon activation, PLCγ

hydrolyzes phosphotidylinositol-4,5-diphosphate (PIP2) to inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 stimulates Ca^{2+} release from intracellular compartments and DAG activates protein kinase C (PKC; Burgess *et al.*, 1990; Mohammadi *et al.*, 1991). PKC, in turn, phosphorylates rapidly accelerated fibrosarcoma-1 (Raf-1), activating the Ras/MAPK pathway (Huang *et al.*, 1995).

1.5.2. PI3 kinase/Akt pathway

This pathway is one of the main players in blockage of apoptosis and FGF dependent cell survival (Hawkins *et al.*, 1997). Upon activation of FGFRs by autophosphorylation, there three mechanisms that are involved in activation of PI3K. In two of the mechanisms, PI3K-regulatory subunit p85 binds to phosphorylated tyrosine on FGFR or transferred to the membrane via recruitment of SH2 domain of p85 by Grb2 Associated Binder1 (Gab1)-Fibroblast Growth Receptor Substrate 2 (Frs2)-Growth Factor Receptor Bound Protein 2 (Grb2; Salazar *et al.*, 2009; Ong *et al.*, 2001). Alternatively, PI3K catalytic subunit p110 is brought to membrane by activated Ras (Rodriguez *et al.*, 1994). Active PI3K is involved in activation of Akt, directly or indirectly, which in turn, inactivates propapoptotic proteins, Bad and Caspase 9 by phosphorylation as well as sequestering forkhead transcription factors such as FKHRL1, FKHR, and AFX by binding of 14-3-3 protein to the phospho serine residues (Hadari *et al.*, 2001; Schlessinger, 2000) leading to cell survival or proliferation.

1.5.3. Ras/MAPK Pathway

Proliferation and differentiation events through FGF signaling are mainly evoked by Ras/MAPK pathway. Juxtamembrane region of activated FGFR interacts with Frs2, which in turn is phosphorylated on tyrosines especially on 436 and 196, thus forming docking sites for the Grb2-Sos complex and Shp2 (Kouhara *et al.*, 1997; Hadari *et al.*, 1998; Eswarakumar *et al.*, 2005). Sos, a guanine nucleotide exchange factor, when recruited to membrane, activates small G-protein Ras which in turn, activates Raf. Raf is a serine-threonine kinase and activates MAPK/ERK Kinase (MEK) which phosphorylates and activates Extracellular Signal Regulated Kinase (ERK) on its threonine and tyrosine residues (Kouhara *et al.*, 1997). ERK, upon activation, phosphorylates target transcription

factors such as Elk-1 and c-Myc, leading to upregulation in gene expression and cell proliferation (Yang *et al.,* 2004). Gab-1 is involved in Ras activation by Shp association. It has been proposed that when activation of ERK is transient, it results in proliferation of MIO-M1 cells (Marshall, 1994; Yamada and Yoshimura, 2002; Çınaroğlu *et al.,* 2005). p38 and c-Jun N terminal kinase/stress activated protein kinase (JNK/SAPK), members of the MAPK family, are involved in cytokine production and cytokine stimulated cellular proliferation and survival (Allen *et al.,* 2000, Crawley *et al.,* 1997).

1.5.4. Negative Regulation of FGF Signaling Pathway

FGF signaling pathway is strictly controlled by many regulators. Sprouty (Spry), Sef, MAPK phosphatase 1 (MKP1) and MAPK phosphatase 3 (MKP3) proteins are negative feedback regulators of FGF induced Ras/ERK pathway. Expressions of these inhibitors are enhanced by ERK signaling (Tsang and Dawid, 2004). Spry acts at the level of Raf by inhibiting binding of Raf to MEK. Spry also competitively binds to Sos1 negatively regulating Grb2 recruitment (Yusoff *et al.,* 2002; Lao *et al.,* 2006). Sef inhibits phosphorylation of ERK by MEK (Kovalenko *et al.,* 2003). MKP dephosphorylates ERK1/2 phosphotyrosine and phosphothreonine residues (Farooq and Zhou, 2004). ERK itself is involved in a negative feedback mechanism by phosphorylation Frs2 and inhibition of recruitment of Grb2 (Lax *et al.,* 2002; Gotoh, 2008).

FGFR and Frs2 are ubiquitinated and subsequently degraded by binding of ubiquitin ligase Casitas B-lineage Lymphoma (Cbl; Wong *et al.,* 2002).

1.6.Raf Protein Family

Raf is a serine- threonine kinase which participate in the RAS- ERK signal relaying cascade (Kolch, 2000), thus is important in cell growth and differentiation and a well known oncogene. Raf is regulated by many growth factors, such as FGF, epidermal growth factor, platelet- derived growth factor, erythropoietin, and insulin (MacDonald *et al.,* 1993). Raf family members are cellular homologues of retroviral oncogenes discovered in 1983. The murine sarcoma virus 3611 positively regulates fibro-sarcoma induction in newborn MSF/N mice, giving Raf its name: rapidly accelerated fibrosarcoma. Mammals

have three Raf proteins: Raf-1 (c-Raf), A-Raf and B-Raf. Raf-1 was discovered in 1985, A-Raf in 1986 and B-Raf in 1988 (Roskoski, 2010). Raf- 1 is ubiquitously expressed but A-Raf and B-Raf have more restricted expression profiles. All isoforms have Ras as the upstream activator and MEK as the downstream substrate (Kolch, 2000). Structurally, they share three conserved regions, Ras-binding domain (RBD) which is required for Raf activation, the zinc finger/cysteine rich domain (CRD) which is involved in Ras binding and interaction with the kinase domain for autoinhibition and the kinase domain (Figure 1.8; Leicht *et al*., 2007). CR2 is serine/threonine rich region instrumental in inactivation upon phosphorylation and CR3 involves kinase domain which have target phosphorylation sites for regulation of Raf (Chong *et al.*, 2003; Wellbrock *et al.*, 2004).

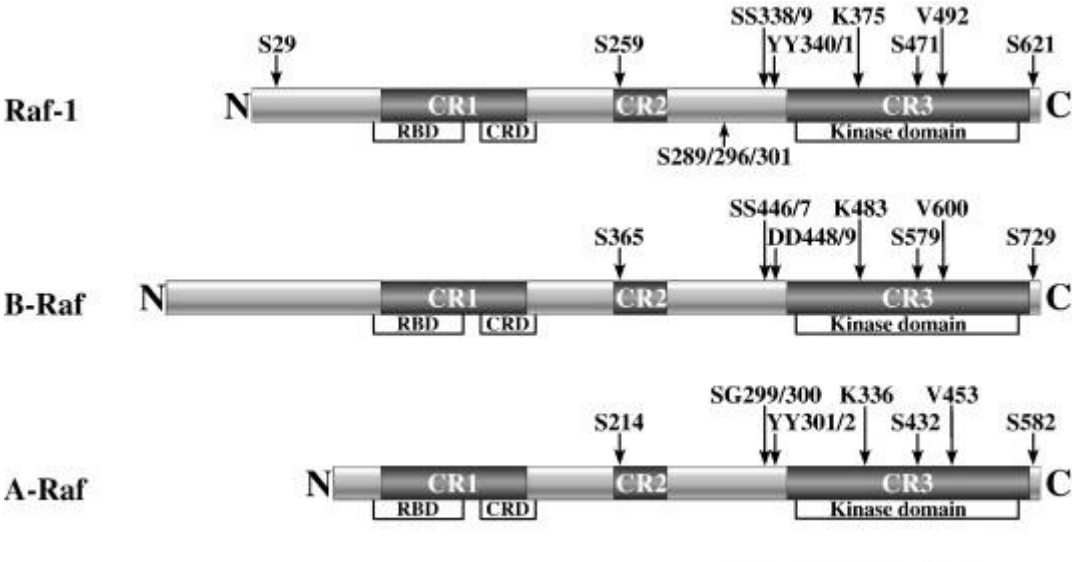

Figure 1.8. Structure of Raf protein family. (CR: conserved regions, RBD: Ras-binding domain, CRD: the zinc finger/cysteine rich domain Modified from Leicht *et al*, 2007).

1.6.1. A-Raf

The A-Raf gene is located on X chromosome in humans and translates into a 68 kDa protein.. Knockout studies resulted in neurological and gastrointestinal defects leading to postnatal lethality. The exact role of A-Raf has not been determined yet; however in A-Raf knockout mice, ERK activation has been observed indicating other members of Raf family

compansate. A role in stimulation of the growth of hematopoietic cells has been proposed (Steelman, 2004).

By mass spectrometry, 35 phosphorylation sites have been identified on A-Raf. Ser214 and Ser582 have been inferred as 14-3-3 protein binding sites for regulation its activity. It was also shown that Ser432 in the catalytic loop is required for kinase activity and for MEK1/2 binding (Roskoski, 2010).

A-Raf is the weakest Raf kinase; unlike B-Raf or Raf-1 it is not implicated in cell cycle progression and proliferation (Chang and McCubrey, 2001; Shelton *et al.*, 2004).

1.6.2. B-Raf

The B-Raf gene is located on chromosome 7q34, encoding a 94 kDa protein. It is highly expressed in the testes and neuronal tissue and subjected to alternative splicing. Knockout studies resulted in intrauterine death due to vascular hemorrhage with enlarged blood vessels and increased apoptosis of differentiated endothelial cells (Steelman, 2004; Kolch, 2000). Overexpression of B-Raf inhibits caspase activity and leads to decreased apoptosis.

B-Raf is the strongest isoform of Raf its activation is regulated by Akt and the serum glucocorticoid-regulated kinase (SGK; Steelman, 2004). Phosphorylation of Thr599 and Ser602 in the activation segment along with Ser365 in the CR2 region and Ser729 near the C-terminus are required for its full activation. It has also specific ERK target sites resulting in feedback inhibition.

B-Raf is deregulated in many of cancer types. Mutations are usually found in the activation segment or in the glycine-rich loop, rendering prolonged active state of the kinase. Val600Glu mutation is the most frequent mutation of B-Raf, favoring active conformation (Roskoski, 2000; Muller *et al*, 2008).

1.6.3. C-Raf (Raf-1)

The Raf-1 gene is located on chromosome 3p25, encoding a 74 kDa protein. Raf-1 protein is ubiquitously expressed in adult tissues. It takes part in apoptosis by inactivating Bad by phosphorylation as well as interacting with Bcl-2 and Bag in BCR/ABL-expressing cells. One of the most important roles of Raf-1 is the involvement in Ras /MAPK pathway leading to cell proliferation and cell cycle progression (Steelman, 2004). It is also involved in activatory phosphorylation of adenylyl cyclases and NF-kB. It inhibits signal relaying proteins such as ROCK2 which have roles in motility. Raf-1 activity is required for wound healing (Chen *et al*, 2001; Ding *et al*, 2004; Jin *et al*, 2005).

Defective Raf-1 activity may result in Noonan syndrome type 5 (NS5) which have symptoms like dysmorphic facial features, short stature, hypertelorism, cardiac anomalies, deafness, motor delay, and a bleeding diathesis; and LEOPARD syndrome type 2 (LEOPARD2) which is characterized by lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormalities of genitalia, retardation of growth, and deafness (Pandit *et al*, 2007; Longoni *et al*, 2010).

1.6.4. Regulation of Raf-1

Raf-1 is involved in dimer formation, both homodimers and heterodimers by Ser621 and residues occurring within the aC helix of C-Raf. If heterodimer consists of B-Raf and C-Raf, it is more active than either homodimer (Roskoski, 2010).

Raf-1 activity is controlled by many kinases, phosphatases and scaffold proteins in a complex way (Figure 1.9, 1.10 and 1.11). There are minimally thirteen phophorylation sites implicated in regulation of Raf-1 activity (McCubrey *et al*, 2006).

According to a recent model in the basal conditions, 14-3-3 protein stays bound to Ser259 and Ser621 phosphorylated Raf-1, keeping it in a closed state. Upon dephosphorylation of Ser259 by phosphatases such as protein phosphatase 2A (PP2A), 14-3-3 is disassociated from Raf-1 and becomes available for Ras binding, thus recruited to the membrane (Yip-Schnedider *et al.*, 2000; Dhillon and Kolch, 2002).

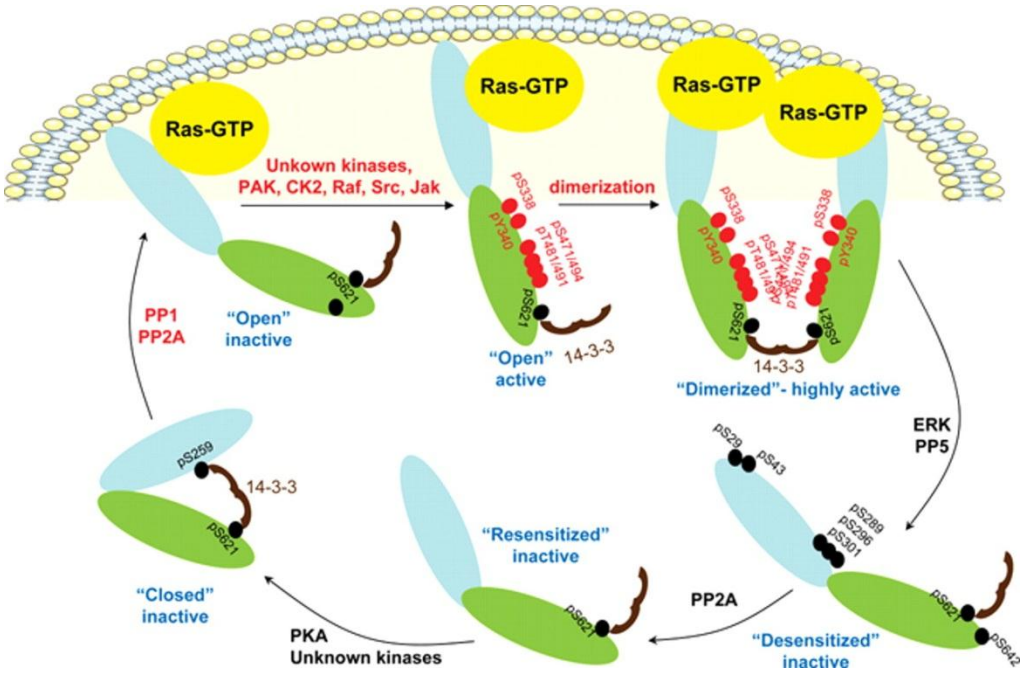

Figure 1.9. Regulation of Raf-1 by kinases and phosphatases (Modified from Matallanas *et al*, 2011).

Subsequently, Raf-1 is phosphorylated on S338, Y340 and Y341 in the activation loop leading to dimerization and Raf-1 assumes active conformation (McCubrey *et al*, 2006). 14-3-3 protein binds to pSer-621, rendering stabilization of this active state. Phosphorylation on Ser621 has a dual role and may lead to negative regulation of Raf-1 activity. Many kinases such as Raf-1 itself, Protein kinase A (PKA) and Adenosine monophosphate activated protein kinase (AMPK) are involved in phosphorylation of this residue (Mischak *et al.*, 1996; Sprenkle *et al.*, 1997). In its active state, Raf-1 interacts and phosphorylates MEK (Zhu *et al.*, 2005). Dephosphorylation of Ser338 results in initiation of deactivation of Raf-1 and subsequent ERK interaction leads to further inactivation (Dougherty *et al.*, 2005).

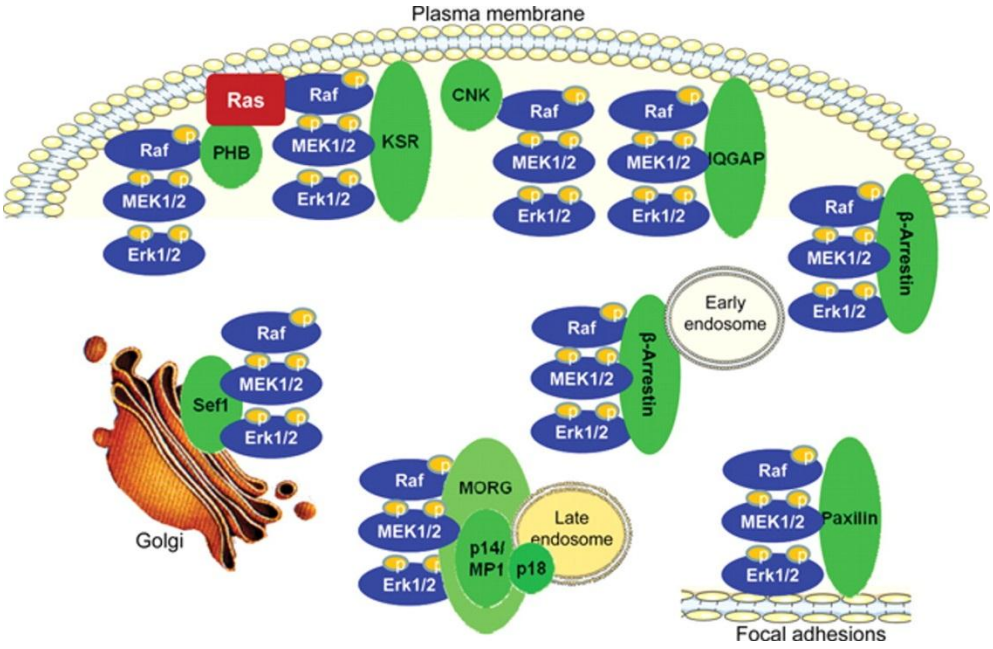

Figure 1.10. Regulation of Raf-1 by scaffold proteins (Modified from Matallanas *et al*, 2011).

Scaffold proteins such as CNK, KSR, RKIP, SUR8 also have roles in Raf-1 regulation. RKIP prevents MEK1 activation by Raf-1. SUR8 is involved in Ras and Raf-1 activation. CNK has been identified as enhancer of Raf signaling in Drosophila. KSR (kinase suppressor of Ras), serves as a scaffolding protein for Raf. It binds to MEK and ERK constitutively, however binding to Raf-1 is only realized at the cell membrane, enhancing coupling of Raf-1 to MEK (Kolch, 2000; Figure 1.10).

As a general rule, Raf-1 is inhibited by itself, Akt and PKA and activated by Pak and Ras (Figure 1.11). Activated Raf-1 phosphorylates and activates MEK1/2, which in turn activates ERK1/2 leading to upregulation of transcription factors.

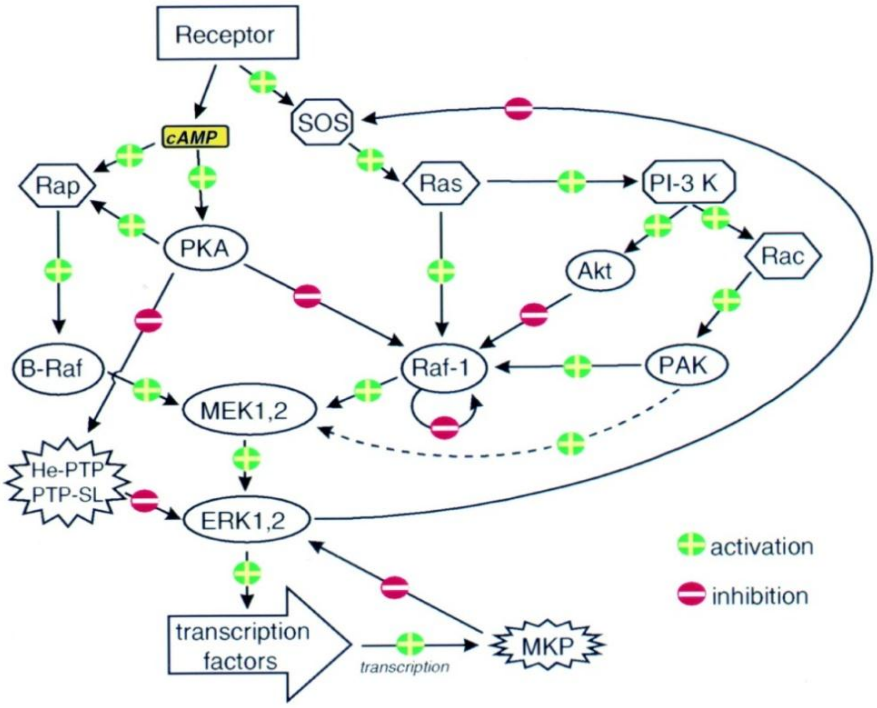

Figure 1.11. Regulatory components of Raf-1 signaling (Kolch, 2000).

1.7.MEK

MEK family is composed of five genes, MEK 1-5. Structurally, MEK proteins have an amino-terminal negative regulatory domain and a carboxy-terminal MAP kinase-binding domain that plays role in binding and activation of ERKs. MEK1 is a tyrosine and serine/threonine dual specificity protein kinase (Alessi *et al.*, 1994). MEK1 consists of 393 amino acids and is 44 kDa. Highest expression profile is detected in adult brain and it is also modestly expressed during embryonic development. Knockout studies showed that MEK silencing results in death due to placental vascularization problems. Constitutively active MEK is involved in promotion of senescence and p53 activity while inhibiting NF-kB transcription and p38 MAPK activity (Steelman, 2004).

MEK activity is positively regulated by phosphorylation on serine residues in the catalytic domain by Raf-1 and activates ERK in the cascade. MEK may also be activated by PKA (McCubrey *et al.*, 2006).

1.8.ERK

The ERK family is composed of four groups: ERK, Jun amino-terminal kinases (JNK1/2/3), $p38^{MAPK}$ and ERK5. ERK1/2 are the most studied members for their role in Ras/ MAPK pathway. ERK1 and ERK2 genes encode for 42 and 44 kDa proteins, respectively. ERK 1/2 proteins are serine/threonine kinases and they are activated by phosphorylation of threonine 182 and Tyrosine 184 by MEK1/2. Their activities are downregulated by phosphatases. Upon activation, ERK can phosphorylate more than 100 substrates leading to diverse outcomes (Yoon *et al.*, 2006). These diverse roles can be exemplified as cell cycle progression, proliferation, cytokinesis, control of transcription, differentiation, senescence, cell death, migration, GAP junction formation, cell adhesion, actin and microtubule networks (Lloyd, 2006). Recently, it has been postulated that ERK1 inhibits ERK2 expression. ERK1 has role in cognitive brain functions whereas ERK2 expression is associated with cell proliferation.

Phosphorylation triggers dimerization of ERK which is involved in maintenance and nuclear localization of the protein. ERKs favorably phosphorylate serine/threonine residues followed by a proline (S/T-P; McCubrey *et al.*, 2006). The D-domain on ERK recognizes DEJL motif and the DEF domain recognizes FxTP motif in ERK substrates (Biondi *et al.*, 2003; Yoon *et al.*, 2006). The DEF domain is instrumental in phosphorylation of S/T-P residues adjacent to the sequence FxFP (Kallunki *et al.*, 1994; Dimitri *et al.*, 2005).

ERKs can also phosphorylate transcription factors such as Ets-1, c-Jun and c-Myc directly and nuclear factor immunoglobulin k chain enhancer-B cell (NF-kB) indirectly (McCubrey *et al.*, 2006). A new substrate of ERK has been identified as salt inducible kinase 2 (SIK2) which is a member of AMP-Activated and Related Protein Kinase family (Ejder, 2011).

1.9.Salt Inducible Kinase Family

Salt inducible kinases (SIK) are composed of three serine/threonine protein kinase isoforms and belong to the family of sucrose-nonfermenting-1 protein kinase (SNF)/ AMPK super family of 14 kinases (Bright *et al.*, 2009). They differ from other family members in that they are not sensitive to glycogen and they function as single subunit enzymes (Hardie *et al.*, 2004).

SIK1 was first identified in mycardium and isolated from adrenal glands of rats fed with high salt diet (Wang *et al.,* 1999; Ruiz *et al.*, 1994). Other isoforms were identified by homology search in human genome. SIK2 and SIK3 are located on chromosome 11 while SIK1 resides on chromosome 21 (Katoh *et al.*, 2004).

SIK proteins share an N-terminal kinase domain with flexible activation loops (A-loop) near their substrate binding pockets. A loops undergo a structural change upon phosphorylation resulting in increase in kinase activity and change in localization (Lizcano *et al.,* 2004; Al-Hakim *et al.*, 2005).

1.9.1. SIK1

The SIK1 gene encodes a protein of 766 amino acids with a nuclear export domain between residues 567-612 (Katoh *et al.,* 2002). In mammals, highest expression of SIK1 is in adrenal glands, brain, testes and skeletal muscles and in small amounts adipose tissue, liver and heart (Horike *et al.*, 2003).

SIK1 is subjected to regulation by many kinases such as PKA 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 has role in cAMP responsive element-binding (CREB) dependent PKA pathway upon adenocorticotropic hormone (ACTH) stimulation. SIK1 phosphorylates

transcription factor CREB in the nucleus and negatively regulates its targets including steroidogenic genes. PKA phosphorylates SIK1 on Ser 577 and causes its nuclear export, relieving repression on CREB and transcription of its targets (Takemori *et al.*, 2002; Doi *et al.*, 2002; Figure 1.12). SIK1 is also associated with CREB-TORC2 pathway under fasting conditions. It phosphorylates TORC2 and causes its nuclear exclusion (Koo *et al.*, 2006).

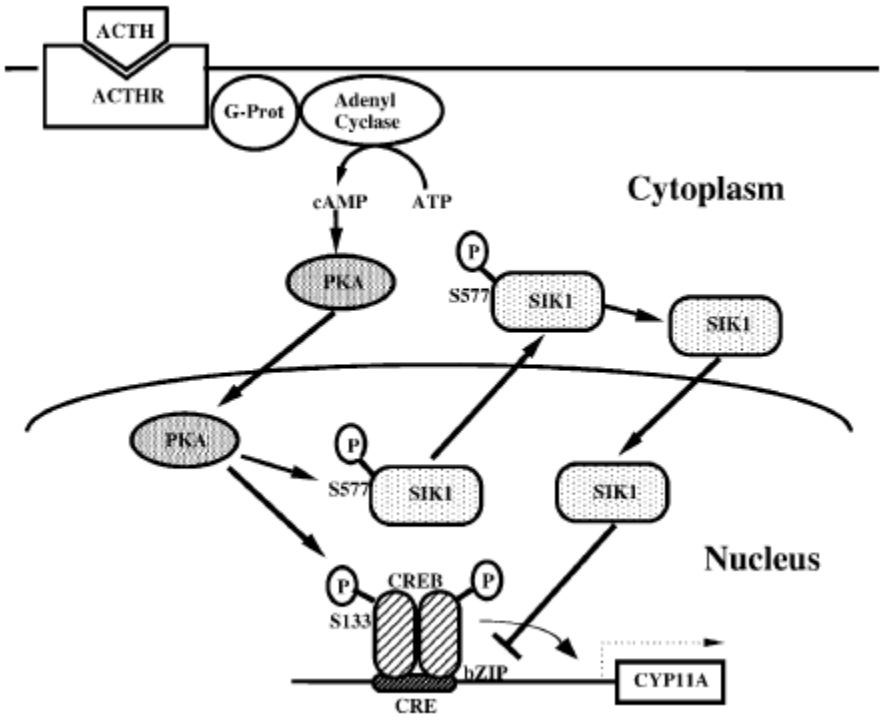

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Figure 1.12. SIK1 induced steroidogenic gene repression model. SIK1 represses CREB in the nucleus and exported to cytoplasm upon phosphorylation by PKA (Okamoto *et al.*, 2004).

Nuclear export of SIK1 is also facilitated by LKB1 which phosphorylates SIK1 on Thr182, causing an activatory conformation change and binding of 14-3-3 chaperone protein (Lizcano *et al.*, 2004; Al-Hakim *et al.*, 2005).

SIK1 is involved in lipogenesis and it negatively regulates it by phosphorylation of Sterol regulatory element binding proteins (Srebp1-c) on multiple serine residues in liver (Yoon *et al.*, 2009). It also phosphorylates class IIa histone deacetylase-5 (HDAC-5),

inhibiting its deacetylase activity and causing nuclear export. SIK1 also participates in survival of neurons in cerebral ischemia (Cheng *et al.*, 2011).

1.9.2. SIK2

SIK2 was identified in adipose tissue of mice and predicted as 103 kDa (Horike *et al.*, 2003). It is generally expressed in metabolic tissues such as adipose tissue, liver and brain (Okomoto, 2004). SIK2 expression is detected also in testis and adult retina (Horike *et al.*, 2003; Özcan, 2003; Özmen, 2006). It is involved in melanogenesis in skin, neuronal survival after ischemia in brain and centrosome separation in ovarian cancer (Horike *et al.*, 2010; Sasaki *et al.*, 2011; Ahmed *et al.*, 2010).

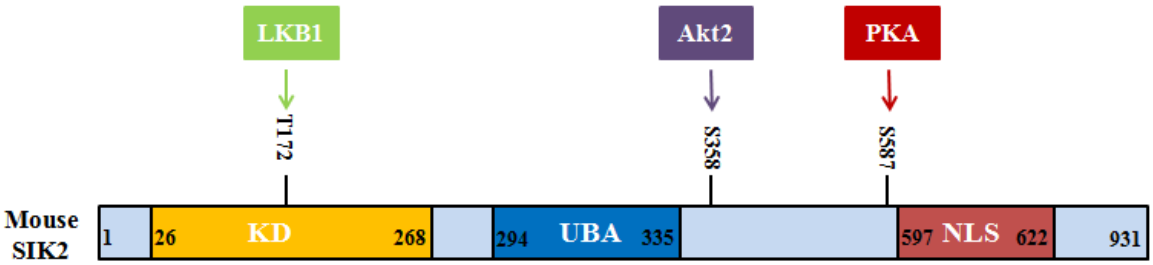


Figure 1.13. Structure and phosphorylation sites of mouse SIK2 protein (Küser, 2011).

Human SIK2 is composed of 931 amino acids. Kinase domain is located between residues 20-271, ubiqitin-associated domain (UBA) between residues 293-346 and phosphorylation domain between the residues 577-623. SIK1 and SIK2 share homology up to 73 % for domain structure (Katoh *et al.*, 2004). Lysine 49 in the ATP-binding loop is required for SIK2 kinase activity, when this site is mutated, SIK2 is rendered kinase inactive (Horike *et al.*, 2003). LKB1 phosphorylates SIK2 on threonine 172 in the activation loop, enhancing its activity. Mutations in this residue result in constitutively active SIK2 (Lizcano *et al.*, 2004). UBA domain interacts with the kinase domain of SIK2, promoting a conformational change to enable phosphorylation by LKB1: STRAD: MO25 complex (Jaleel *et al.*, 2006). Also PKA phosphorylates SIK2 between 577-623 amino acid residues neighbouring nuclear localization signal (NLS) which leads to nuclear export of SIK2. Rat SIK2 bears 94% overall identity resemblance to mouse and 89.3% to human SIK2 (Horike *et al.*, 2003; Uysal, 2005).

The first identified substrate of SIK2 is IRS1 which regulates the insulin pathway. Phosphorylation of IRS on Ser 789 by SIK2, results in downregulation of insulin signaling in adipocytes suggesting role of SIK2 in development of type 2 diabetes (Horike *et al.*, 2003; Gual *et al.*, 2005). SIK2 is also involved in primary cortical neuron survival through the CREB-TORC1 in case of ischemic injury along with G1/S transition (Sasaki *et al.*, 2011; Ahmed *et al.*, 2010).

In addition to IRS1, Gab-1, TORC2, P300 and Raf-1 are found to be SIK2 substrates through presence of consensus SIK2 phosphorylation motif, (I/L)[(B)X or X(B)](S/T)X(S/T)XXX(I/L), where B represents basic residues. Identification of Gab-1 and Raf-1 as novel substrates suggested role of SIK2 in FGF pathway. (Screaton *et al.*, 2004; Küser, 2006; Küser, 2011).

SIK2 was obtained in a yeast two-hybrid screen of a retinal cDNA library in which the cytoplasmic part of FGFR2 was used as bait (Özcan, 2003). Localization, phosphorylation status and activity of SIK2 were modulated by FGF2 in MIO-M1 cells (Özmen, 2006; Candaş, 2007). SIK2 is expressed in all retinal layers and have three alternatively spliced transcripts leading to two cognitive peptides (Özcan, 2003; Özmen, 2006). The involvement of SIK2 was investigated in FGF pathway through overexpression and downregulation studies. Based on the presence of canonical SIK2 phosphorylation motif, Grb2, Gab1 and Raf-1 were suggested as substrates of SIK2. Also interacting members of the FGF2 pathway were identified in a time dependent manner in MIO-M1 cells (Özcan, 2003; Özmen, 2006; Candaş, 2007; Küser, 2006; Küser, 2011). It was also shown that SIK2 phosphorylates Raf-1 on Ser 621 (Yılmaz Sert, 2011). SIK2 has ERK phosphorylation motif (S/T)P residues adjacent to FxFP motif, raising the possibility of Threonine 758, 863 and Serine 379, 775, 891 residues being targets for this dual kinase. SIK2 and ERK phosphorylation kinetics are similar in that their activity reaches maxima within 10 min FGF2 induction (Küser, 2011). It is shown that ERK is an upstream kinase of SIK2. When SIK2 phosphorylation profile was analyzed through ERK inhibition by MEK inhibitor, threonine levels have changed significantly while serine phosphorylation profile was stable indicating that SIK2 phosphorylates ERK on either Threonine 758 or Threonine 863 or both (Ejder, 2011).

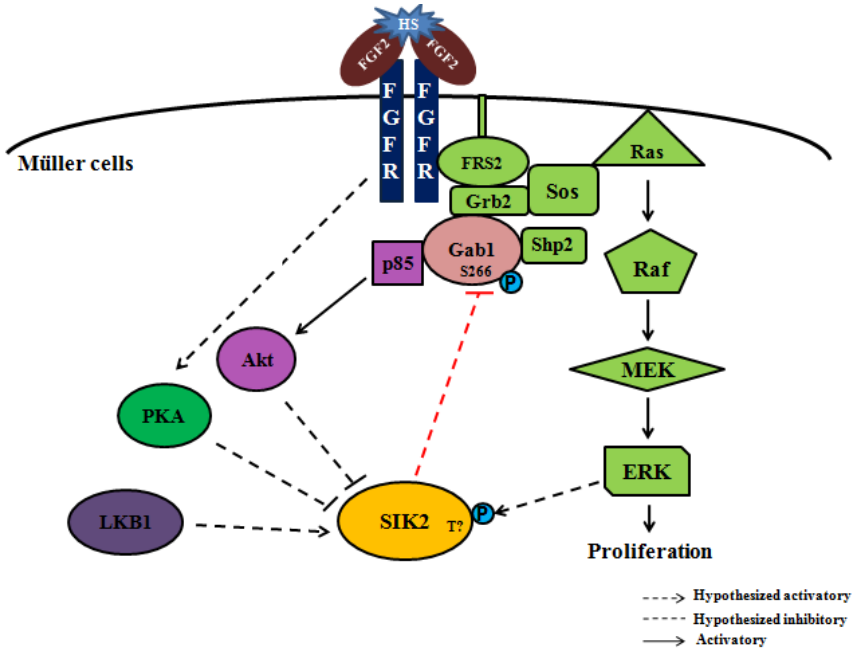


Figure 1.14. The working model of SIK2 involvement in FGF2 pathway (Küser, 2011).

Based on these findings the working model (Fig 1.14.) proposes that SIK2 is activated by ERK phosphorylation in response to FGF2 and phosphorylates Gab1 and Raf-1, downregulating their interaction with their partners, thereby creating a negative feedback loop (Yılmaz Sert, 2011; Küser, 2011).

1.9.3. SIK3

SIK3 is composed of 1263 amino acids and found to be ubiquitously expressed in human, mouse and rat (Okamoto *et al.*, 2004; Katoh *et al.*, 2004). Knockdown studies in Drosophila resulted in mitotic defects such as spindle and chromosome abnormalities (Bettencourt-Dias *et al.*, 2004). SIK3 is phosphorylated by LKB1 on Threonine 163 in kinase domain (Lizcano *et al.*, 2004). It is also activated in response to insulin suggesting a role in energy metabolism (Wang *et al.*, 2011). Overexpression of SIK3 has been associated with ovarian cancer cases for it enhances cell proliferation (Charoenfuprasert *et al.*, 2011).

2. PURPOSE

Studies carried out in our laboratory indicated that SIK2 is involved in the control of FGF dependent Müller cell proliferation via downregulation of Ras/ERK pathway (Küser, 2011). SIK2 was shown to phosphorylate the pathway element Gab-1 on Ser 266 and that this kinasing negatively affects Gab-1 interaction with its partners (Küser, 2006; Yılmaz Sert, 2011). Parallel studies indicated that serine 621 Raf-1 is also a potential SIK2 target in this pathway (Küser, 2006; Yılmaz Sert, 2011) and FGF-dependent SIK2 activity is modulated by Erk (Ejder, 2011).

In the first part of this work we aimed to further investigate the putative role of SIK2 in negative regulation FGF2 signaling through Raf-1. To this end we analyzed changes in FGF2-dependent temporal profile of Raf-1 phosphorylation on serine 621 and Raf-1 activation in the context of Müller cells where SIK2 expression was downregulated.

In the second part of this study, the starting aim was to identify Erk target residues on SIK2. On SIK2 there exists two candidate threonine residues (758 and 863) where ERK phosphorylation motif, (S/T)P adjacent to FxFP (DED domain. We were able to generate SIK2 T863A mutant and express them for *in vitro* kinase studies.

3. MATERIALS

3.1. Cell Lines

Spontaneously immortalized human Müller glia cell line (MIO-M1) was a kind gift from Prof. Astrid Limb from University College London, Institute of Ophthalmology, London, UK.

3.2. Chemicals, Plastics and Glassware

All chemicals used in this study were obtained from Sigma Aldrich (USA) and all cell culture products were purchased from Invitrogen (USA), unless stated otherwise in text. Plastic products were purchased from TPP (Switzerland) and Greiner (USA). All solutions, plastic and glassware were sterilized by autoclave at 121^0 C for 20 minutes before use where possible.

3.3. Buffers and Solutions

Buffer and solution compositions are given in Table 3.1 through Table 3.9.

3.3.1. Cell Culture

Table 3.1. Cell Culture Materials.

Complete Medium for MIO-M1	DMEM with Glutamax Supplemented with: 10% Fetal bovine serum (FBS) 0,1% Penicillin/ Streptomycin
Freezing Medium	20% FBS 70 % DMEM GlutaMAX 10 % Dimethyl sulfoxide (DMSO)

Table 3.1. Cell Culture Materials (cont.).

FGF2 Supplemented Medium	DMEM supplemented with 1 ng/ml FGF2 10 μg/ml heparin
Phosphate Buffered Saline (PBS)	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4 supplemented with Phosphatase Inhibitor Cocktail and Protease Inhibitor Cocktail
10X Trypsin-EDTA Solution	2.5% Trypsin 7 mM EDTA 0.9% NaCl diluted with PBS

3.3.2 Plasmid Isolation

Table 3.2. Plasmid Isolation Materials.

TE Buffer	10 mM Tris-HCl (pH 7.5) 1 mM EDTA

3.3.3. Protein Isolation

Table 3.3. Protein Isolation Materials.

Protease Inhibitor Cocktail	10 μg/ml leupeptin 5μg/ml aprotinin (Roche, Germany)

Table 3.3. Protein Isolation Materials (cont.).

Phosphatase Inhibitor Cocktail	1 mM Na_3VO_4 1 mM phenylmethylsulphonylfluoride (PMSF) 20 mM NaF (Roche,Germany)
Cell Lysis Buffer	20 mM Tris-HCl (pH 7.5) 150 mM NaCl 1 mM Na_2EDTA 1 mM EGTA 1% Triton X-100 2.5 mM sodium pyrophosphate 1 mM beta-glycerophosphate 1 mM Na_3VO_4 1 µg/ml leupeptin

3.3.4. Western Blot

Table 3.4. Western Blot Materials.

Acrylamide:Bisacrylamide (37.5:1) :	29.22 g acrylamide 0.78 g N`N`-bis-methylene-Acrylamide in dd H_2O Final volume 100 ml
Ammonium Persulfate	10% APS (w/v) in dd H_2O
SDS (Sodium dodecyl sulfate)	10% SDS (w/v) in dd H_2O
Blocking Solution For Raf-1	3% non-fat milk powder in TBST
Blocking Solution For pRaf (Ser 621)	3% Bovine Serum Albumin (BSA) in TBST

Table 3.4. Western Blot Materials (cont.).

Coomassie Brilliant Blue Staining	125 ml Ethanol 100% 0.625 g Coomassie Brilliant Blue 25 ml Acetic acid 100 ml dd H_2O
Coomassie Blue Destaining Solution	37.5 ml Acetic acid 25 ml Ethanol 100% 437 ml dd H_2O
Polyviniyl difluoride (PVDF) Membrane	Roche,Germany
Protein Marker : PageRuler^{TM} Plus Prestained Protein Ladder (10-170 kDa)	Thermo Fisher Scientific Inc, USA
Resolving Gel (10%)	10% Acrylamide:Bisacrylamide (37.5:1) 1.5 M Tris.HCl pH 8.8 0.1% SDS 0.1% APS 0.1 % TEMED
Stacking Gel (5%)	5% Acrylamide: Bisacrylamide (37.5:1) 1 M Tris-HCl (pH 6.8) 0.1 % SDS 0.1% APS 0.1 % TEMED
6x Sample Buffer	1.2 g SDS 0.9 g Dithiothreitol (DTT) 6 mg bromophenol blue 4.7 ml glycerol 1.2 ml Tris 0.5 M pH 6.8 2.1 ml dd H_2O

Table 3.4. Western Blot Materials (cont.).

Transfer Buffer (10X)	72 g Glycine 15,15 g Tris-Base 500 ml dd H_2O
Transfer Buffer (1X)	100 ml 10X Transfer Buffer 150 ml Methanol 750 ml dd H_2O
Tris-Glycine Buffer (5X) (Running Buffer)	7,55 g Tris-Base 47 g Glycine 50 ml 10 % SDS 450 ml dd H_2O
Tris buffered saline (TBS) (10X)	24,2 g. Tris-Base 87,66 g. NaCl 1000 ml dd H_2O (pH 8.0)
Tris buffered saline-tween (TBST) (1X)	50 ml 10X TBS 450 ml dd H_2O 500 µl Tween 20
Stripping Solution	62.5 mM Tris-HCl, pH 6.8 2% SDS 0.7% β-Mercaptoethanol
Western Blot Luminol Reagent	Santa Cruz Biotechnology, Inc., USA

3.3.5 Immunoprecipitation

Table 3.5. Immunoprecipitation Materials.

Protein A Matrix	Roche, Germany
Pierce Protein A Plus Agarose	Thermo Fisher Scientific Inc, USA

3.3.6 *In vitro* Kinase Assay

Table 3.6. *In vitro* Kinase Assay Materials.

[γ32P]ATP (3000 Ci/mmol)	Institute of Isotopes Co.Ltd,Hungary
2X Kinase Buffer	25 mM HEPES (pH 7.5) 150 mM NaCl 10 mM MgCl2 2 mM MnCl2
Adenosine Tri Phosphate (ATP) cocktail	1X Kinase Buffer supplemented with 100 μM cold Adenosine triphosphate (ATP) 75 mM MnCl2 75 mM MgCl2
Fixing Solution	50% Methanol 7% Acetic Acid
X-ray Amersham Hyperfilm	GE Healthcare, USA
Developer Solution	Kodak, USA
Fixative Solution	Kodak, USA

3.3.7 Bacterial Transformation

Table 3.7. Solutions for Bacterial Transformation.

NZY Broth^{+}	10g NZ amine (casein hydrolysate) 5g yeast extract 5g NaCl In deionized H_2O to a final volume of 1l Adjust to pH 7.5 using 5M NaOH Autoclave Add the following filter sterilized supplements before use: 12.5 ml of 1 M $MgCl_2$ 12.5 ml of 1M $MgSO_4$ 20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose)
LB Agar (per liter)	10 g NaCl 10 g tryptone 5 g yeast extract 20 g agar Add deionized H_2O to a final volume of 1 liter Adjust pH to 7.0 with 5 N NaOH Autoclave Pour into petri dishes (25 ml / 100 mm plate)
LB Ampicillin Agar (per Liter)	1 liter of LB agar Autoclave and cool to 55^0C Add 100 mg/l of filter sterilized ampicillin Pour into petri dishes (25 ml / 100 mm plate)

3.4. Fine Chemicals

3.4.1. Antibodies

Table 3.8. Antibodies Used in Western Blot and Immunoprecipitation.

Antibody	**Source**	**Company**	**Dilution Used**	**Usage**
Anti-rabbit Immunoglobulin G (IgG)	Goat	Santa Cruz, USA	1:5000	WB
Anti Raf-1	Rabbit	Millipore, (Merck), Germany	1:1000	WB, IP
Anti pRaf-1 (Ser621)	Rabbit	Abcam, UK	1:5000	WB

3.4.2. Peptides

Human kinase inactive MEK1 (K97R) peptide was purchased from Millipore (Merck, Germany) and used for *in vitro* kinase assay.

3.4.3. Primers

Table 3.9. Primers Used for Site Directed Mutagenesis and Sequencing.

Gene	Primer Sequence (5'- 3')	Tm (°C)
SIK2 (T863A) Forward	CCCCTGCGCCAGACTATCCCGCTCCCTGTCAGTATCCTGTGG	64.2

Table 3.9. Primers Used for Site Directed Mutagenesis and Sequencing (cont.).

SIK2 (T863A) Reverse	CCACAGGATACTGACAGGGAGCGGGATAGTCTGGCGCAGGGG	64.2
SSIK2-U3 (2462-2481)	TCCTTACAGTTCTCCTATCAG	50.6

3.4.3. Plasmids

pEGFP C3 SIK2 KI (K49M) plasmid was used throughout this study (Küser, 2011).

3.5. Kits

3.5.1. Plasmid Isolation

Table 3.10. Plasmid Isolation Kits.

Genopure Plasmid Midi Kit	Roche, Germany.
QIAGEN Plasmid Isolation MiniPrep Kit	QIAGEN, USA.

3.5.2. BCA assay kit

Table 3.11. BCA Assay Materials.

BCA protein assay	Thermo Scientific Inc., USA

3.5.3. Site- Directed Mutagenesis

Table 3.12. QuikChange® II XL Site- Directed Mutagenesis Kit.

QuikChange® II XL Site- Directed Mutagenesis Kit	Stratagene, USA

3.6. Equipments

Table 3.13. Equipments Used in This Study.

Equipments	**Models**
Autoclave	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Balances	Electronic Balance VA 124, Gec Avery,USA DTBH 210, Sartorius, GERMANY
Blotting apparatus	Mini Trans-Blot Cell (Bio-Rad, USA)
Carbon dioxide tank	2091, Habaş, TURKEY
Centrifuges	ProFuge, 10K, Strategene, USA Mini Centrifuge 17307-05, Cole Parmer, US Genofuge 16M, Techne, UK Centurion K40R, UK Centrifuge B5,B.Braun B. Int. ,GERMANY Centrifuge 5415R, Microfuge tube, USA J2-MC Centrifuge, Beckman Coulter, USA J2-21 Centrifuge, Beckman Coulter, USA
Cold room	Birikim Elektrik Soğutma, Turkey
Deep Freezers	2021D (-20^{0}C), Arçelik, Turkey -70^{0}C Freezer, Harris, UK -86^{0}C ULT Freezer, ThermoForma, USA
Documentation System	Gel Doc XR System, Bio Doc, ITALY

Table 3.13. Equipments Used in This Study (cont.).

Electrophoretic Equipments	Mini-PROTEAN 3 Cell, BIO-RAD, USA Easi-cast system, Hybaid, UK
Gel dryer	Model 583 Gel Dryer (Bio-Rad, USA)
Heat blocks	DRI-Block DB-2A, Techne, UK StableTemp Dry Bath Incubator, Cole Parmer (USA)
Hybridization Oven	Shake'n Stack, Hybaid, UK
Ice Machine	Scotsman Inc., AF20, ITALY
Incubator	Hepa Class II Forma Series, Thermo Electron
Laminar flow cabinet	Class II A Tezsan, TURKEY Class II B Tezsan, TURKEY
Magnetic Stirrer	M221 Elekro-mag, TURKEY Clifton Hotplate Magnetic Stirrer, HS31, UK
Micropipettes	Gilson, FRANCE
Microscopes	CM110 Inverted Microscope, Prior, UK Zeiss, Axio Observer Z1 Inverted Mic., USA
NanoDrop ND-1000	Thermo Scientific Inc., USA
pH meter	WTW, GERMANY
Pipettor	Pipetus-akku, Hirscmann Labogerate, GERMANY
Power Supplies	EC135-90, Thermo Electron Corporation Power Pac Universal, BIO-RAD, USA
Protein Visualization	Stella, Raytest, Germany
Refrigerators	2082C, Arçelik, TURKEY 4030T, Arçelik, TURKEY

Table 3.6. Equipments Used in This Study (cont.).

Sealer	Vacuplus FS400A, Electric Petra, GERMANY
Shakers	VIB Orbital Shaker, InterMed, DENMARK Lab-Line Universal Oscillating Shaker, 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)
Spectrophotometer	CE5502, Cecil, UK
Thermocycler	MyCycler (Bio-Rad, USA)
Vacuum pump	Hydrotech (Bio-Rad, USA)
Water Purification	WA-TECH Ultra Pure Water Purification System (WATECH, Germany)
X-Ray Film Cassettes	24X30 IMS, ITALY 24X 30 DIA-X, GERMANY

4. METHODS

4.1. Cell Culture

4.1.1. Maintenance of MIO-M1 Cells

Spontaneously immortalized MIO-M1 Müller glia cells were maintained in DMEM Glutamax with glutamine, supplemented with 10% FBS and 0.1% penicillin/streptomycin. Cells were grown at 37°C and under 5% CO2. When the cells are near confluence, they were washed with PBS and treated with 0.05% trypsin solution (0.05% trypsin in PBS) for 5 minutes at 37°C. After incubation, equal volume of complete medium is added to the plates and the cells were scraped. Cells were collected by centrifugation at 2000 x g for 5 minutes and after resuspension in complete medium they were divided into three plates twice a week. For stock preparation, cells were resuspended in freezing medium. The cells are kept at -20°C and transferred to be kept at -80°C, the next day.

4.1.2. Treatment of MIO-M1 Cells

Cells were seeded on 10 cm tissue culture dishes and were allowed to grow until subconfluency. The cells were washed with PBS and starved in DMEM with 0.1% penicillin/streptomycin overnight. Subsequently, they were treated with 1 ng/ml FGF2 and 10 μg/ml heparin for 0 minutes (negative control), 5, 10, 30, 60 and 120 minutes. Following treatment, cells were immediately washed with ice-cold PBS supplemented with protease and phosphatase inhibitor cocktails (The cells were then scraped in with ice-cold 1x PBS supplemented with protease and phosphatase inhibitor cocktails, collected into microfuge tubes, pelleted with centrifugation at13.200 rpm for 5 minutes at 4°C. Pelleted cells were directly lysed or stored at -80^{0}C until used after removal of residual supernatant.

4.1.3. Cell Lysis

Following FGF2 treatment, MIO-M1 cell pellets were resuspended in 1 ml ice cold lysis buffer including protease and phosphatase inhibitor cocktails on ice for 1 hour with gentle mixing occasionally. The cells were then sonicated for 5 seconds, 3 rounds. Cell debris was removed by centrifugation 20 minutes at 13.200 rpm at 4°C. Cell lysates were processed immediately or stored in -80°C.

4.2. Immunoprecipitation

In cell lysates, protein concentrations were determined with BCA Assay. Protein A agarose beads (25µl) were washed three times on ice with 0.5- 1 ml PBS including protease and phosphatase inhibitor cocktails. Equal quantities of lysates (400 µg) were transferred on the washed agarose beads and were incubated with gentle rotation at 4°C for 30 minutes. Subsequently, lysates were centrifuged for 3 minutes at 13 200 rpm at 4°C, the beads were discarded and the precleared lysates were used for immunoprecipitation. To washed 50 µl Protein A agarose beads, 2-4 µg Raf-1 antibody was added in 500 µl PBS and incubated with gentle rotation for 1 hour at 4°C. After incubation, the tubes containing the antibody were centrifuged for 1.5 minutes at 13 200 rpm at 4°C and washed with lysis buffer including protease and phosphatase inhibitor cocktails three times at 4°C. The precleared lysates were added to the tubes containing antibody conjugated beads and the mixture was incubated for 1 hour with gentle rotation at 4°C. Subsequently, the beads were collected and washed with PBS as above. Finally, beads were washed with ice-cold kinase buffer and resuspended in 50 µl kinase buffer. Half of resuspended beads (25 µl) were taken for western blot analysis and the rest is kept at -80°C to be used in *in vitro* kinase assay.

4.3. SDS-PAGE and Western Blot

A fraction of cell lysates obtained following FGF2 treatment (25µl) were mixed with 6X SDS protein sample buffer and boiled for five minutes at 95°C in a heat block. Samples were transferred to ice for 1 minute and spinned down briefly before loading. PageRuler™

Plus Prestained Protein Ladder (10-170 kDa) was used for estimation of molecular weight of the bands. The samples were run on 10% polyacrylamide gel in Running Buffer at 80 V till passing the line of stacking gel, then the voltage was switched to 120 V. After the samples were fractionated on polyacrylamide gels, they were electroblotted to PVDF membranes in Transfer Buffer containing methanol at 100 V for 1 hour at 4°C or with ice. Following transfer, the membrane was washed with TBST for 10 minutes for removal of methanol. The membrane was incubated in appropriate blocking solution (Table 3.3.4) for 1 hour at room temperature with gentle shaking.

After blocking, the membrane was washed with TBST for 5 minutes three times. The membrane was incubated overnight in blocking solution containing Raf-1antibody at 4°C. Subsequently, the membrane was washed with TBST three times for 5 minutes to remove unbound antibody and incubated with HRP conjugated secondary antibody at room temperature for 1 hour with gentle shaking. The membrane was washed with TBST as above. Western blot luminol reagent was applied onto the membrane for 1 minute and exposed to chemoluminescence detection in XStella for 10 minutes with 1 minute intervals. Image J programme was used to analyze the protein bands.

Subsequently the membrane was stripped by placing in 15 ml preheated (50°C) Stripping Solution for 35 minutes with gentle rotation in hybridization oven after washing in TBST. Following stripping, the membrane was washed with TBST as above and proceeded with blocking and re-probing with Ser621-pRaf-1 antibody.

For analysis, phosphorylated band intensity detected with Ser621- pRaf-1was compared to the total band intensity detected with Raf-1 antibody.

For checking the efficiency of transfer process, the polyacrylamide gel was subjected to Coomasie Blue staining for 30 minutes after transfer to PVDF membrane and destained overnight in Destaining Solution at room temperature with gentle shaking.

4.4. *In vitro* Kinase Assay

The *in vitro* autophosphorylation assay and kinase assays performed as described by Feldman *et al.*,(2000). Immunoprecipitated Raf-1 protein (25µl) received 1 µl inactive MEK-1 (K97R) (500 ng) and 1 µl of kinase cocktail containing 100 µM cold ATP (1 µCi [γ^{32}P] ATP 3000 Ci/mmol (Isotop, Hungary) and kinase buffer.

The kinase reaction was allowed to proceed for 30 minutes at 30°C and terminated by the addition of 6X SDS protein sample buffer. The samples were boiled at 95°C for 5 minutes, taken on ice and the beads were removed by means of centrifugation for 5 minutes at 13200 rpm at room temperature. The reaction mixtures were run in 10 percent SDS-PAGE and the gel was fixed for 10 minutes in Fixing Solution. It was subjected to Coomassie Staining for 20 minutes and destained in Destaining solution at room temperature overnight. After capturing the imaging pattern in XStella, the gel was dried and exposed to Amersham Hyperfilm for varying times at -80^{o}C. The bands were visualized by immersing the film in Developer Solution varying times from 30 seconds to 2 min. The gel was rinsed with water and placed in Fixative Solution for 5 min. The film was air-dried and scanned for analysis.

4.5. Site Directed Mutagenesis

4.5.1. Site Directed Mutagenesis (QuikChange®)

pEGFP C3 SIK2 KI (K49M) plasmid (produced by Gamze Kuser from Retina Lab) was subjected to site directed mutagenesis by primers carrying the mutation. Threonine 863 was converted to alanine by QuikChange® II XL Site- Directed Mutagenesis Kit, Stratagene (USA) according to manufacturers' instructions with primers in Table.3.4.2. The cycling parameters were modified according to the plasmid length (7.4 kb) as in Table 4.1.

Table 4.1. Cycling Parameters for the QuikChange II Site-Directed Mutagenesis.

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12- 18	95°C	30 seconds
		55°C	1 minute
		68°C	7 minutes

To test the efficiency of mutant plasmid generation, pWhitescript control plasmid was also included in the PCR according to the instructions in the kit.

After PCR reaction, samples were digested with DpnI enzyme at 37°C for 1 hour to digest the parental strands.

4.5.2. Bacterial Transformation and Selection of Colonies

50 µl DH5α *Escherichia coli* competent cells received 2 µl of mutated plasmids (300ng). pUC18 control plasmid was used as a control. The reactions were incubated on ice for 30 minutes; heat pulsed for 30 seconds at 42°C and placed on ice for 2 minutes. Preheated 500 µl NZY^+ Broth was added to each tube and they were incubated at 37°C with shaking at 225 rpm for 1 hour. Onto LB agar plates, 10 mM IPTG (100µl) and 2% X-gal (100µl) were spread 30 minutes prior to plating the transformations. Grown bacterial culture (250 µl) was plated on LB plates containing 100 µg/ml ampicillin (for control reactions) or 25 µg/ml kanamycin (for selection of mutated plasmids) and grown overnight at 37^0C.

4.5.3. Plasmid Isolation

Primary cultures were set up by growing the selected white colonies in 5 ml NZY^+ Broth with 25µg/ml kanamycin overnight at 37°C with shaking at 200 rpm. For secondary cultures, 1 ml primary culture aliquots were added to 30 ml broth and grown at 37°C overnight. Plasmids were isolated using MiniPrep kit (QIAGEN, USA) or Genopure Midiprep kit (Roche, Germany), according to manufacturers' instructions.

Briefly, bacterial cells were lysed under alkaline conditions containing RNase. Then, the lysate is mixed with neutralization buffer to allow renaturation of plasmid DNA. Cell debris along with chromosomal DNA was removed by centrifugation at 13 200 rpm for 15 minutes. The supernatant was applied to silica gel columns for plasmid DNA to bind under high salt conditions and washed several times to remove residual impurities with wash buffer containing ethanol. Finally, plasmid DNA was eluted with TE Buffer. Eluted plasmids were analyzed in 1% agarose gel electrophoresis and the concentration was determined by measuring optical density at 260 nm using NanoDrop Spectrophotometer.

To confirm the mutation, plasmid DNA was sequenced by Genome Lab GeXP genetic analysis (GeXP DTCS DNA sequencing), (Beckman Coulter, USA). The plasmid DNA was stored at -20°C for further studies.

4.5.4 GeXP DTCS DNA Sequencing

The procedure was followed according to the manufacturer's instructions. For PCR reaction for sequencing, a master mix was prepared with 0.5 µl sequencing primer (10 pmol/µl) (U3 for T863A), 300 ng DNA (mutated plasmid), 4µl DTCS Master Mix® and High Pure H_2O (Sigma, USA) in a total of 10µl solution. Cycling parameters for sequencing PCR are given in Table 4.2.

Table 4.2. Cycling Parameters for PCR.

96°C	20 sec	30 cycles
50°C	20 sec	
60°C	4 min	
4°C	Overnight	

After the PCR is completed, 10 µl High Pure water was added to the samples. Fresh STOP solution (2 µl 3M sodium acetate, 2 µl 100mM Na-EDTA, 1 µl glycogen (20 mg/ml) was prepared and 5 µl was added to each sample. Subsequently, 60 µl cold absolute ethanol (- 20°C) was added and mixed thoroughly. The samples were centrifuged at 13200 rpm for 20 min at 4 °C. The supernatant was transferred to fresh tubes and the pellet was rinsed with 200 µl cold (- 20°C) 70% ethanol. The ethanol was discarded and the pellets were air dried. The samples were resuspended in 35 µl Genome Lab Sample Loading Solution and loaded to the capillaries.

4.6. Statistical Analysis

All statistical analyses were performed on Statistical Package for Social Sciences (SPSS) software version 20.0. Independent t-test was applied for normally distributed data on Raf-1 activity in *in vitro* kinase assay. Since the data on phosphorylation profile were normally distributed and variances were equal, one-way ANOVA test with LSD- post hoc multiple comparison was used to determine the levels of significance. The confidence interval of 95% was used as significance criterion in all tests. Error bars represent 1 standard error of the mean (SEM) in all charts.

5. RESULTS

5.1. Modulation of FGF-dependent Raf-1 activity by SIK2

In this study, the possible involvement of SIK2 on the regulation of Raf-1 by phosphorylation on serine 621 in the context of FGF 2 stimulation in Müller cells was investigated. It is hypothesized that this phosphorylation negatively regulates MAPK pathway.

5.1.1. Downregulation of SIK2 expression in Müller cells by lentiviral particles

To investigate the change in the phosphorylation levels of Raf-1 on serine 621, Müller cells with downregulated SIK2 expression was used and compared to the untransfected control Müller cells. Downregulation of SIK2 expression was performed by lentiviral particles carrying shSIK2 or scrambled RNA and stably transduced cells were selected with puromycin (Küser, 2011).

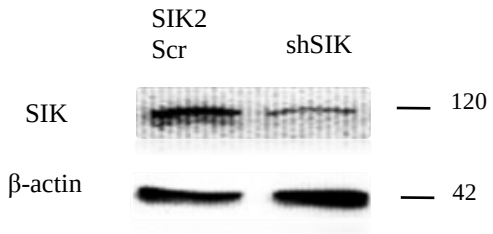


Figure 5.1. Downregulation of SIK2 expression with lentiviral particles**.** SIK2 expression was downregulated with shSIK2 compared to scrambled control. β- actin levels were used as loading controls (Küser, 2011).

SIK2 expression was downregulated 60% in shSIK2 carrying cells compared to scrambled RNA containing cells, which showed similar expression to uninfected cells (Figure 5.1). SIK3 expression profiles in these cells were not affected (data not shown).

5.1.2. Changes in FGF2-dependent Raf-1 phosphorylation on Serine 621 in control and SIK2 downregulated Müller cells

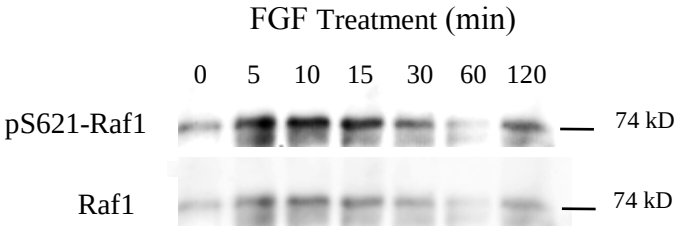
In order to investigate whether FGF2 induction is involved in serine phosphorylation of Raf-1, overnight serum-starved MIO-M1 cells were treated with 1 ng/ml FGF2 for 0, 5, 10, 30, 60 and 120 minutes. Lysates were prepared in the presence of phosphatase inhibitors. Cell lysates were fractionated on polyacrylamide gel and immunoblotted with pS621-Raf-1 antibody. For loading control, an antibody that recognizes all Raf-1 forms was used. The results of three independent experiments were plotted after normalization of pS621-Raf-1 band intensities to that of corresponding Raf-1 bands using ImageJ program (Figure 5.2).

In control Müller cells, FGF2 dependent Ser 621-pRaf-1 phosphorylation levels increased significantly, about 1.7 fold, within 5 minutes and progressively returned to basal levels by 60 minutes of treatment. These data are parallel with the activation profile of SIK2 where maximum activity was observed at 10 minutes and minimum activity at 60 minutes of FGF2 stimulation in the context of Müller cells (Küser, 2011) supporting the possibility of involvement of SIK2 for this phosphorylation pattern. The decrease in the phosphorylation was also significant between 30 and 60 minutes (1.8 fold). In this scheme, the highest phosphorylation levels were at 5 and 10 minutes and the lowest one was at 60 minutes followed by 120 minutes which was close to the basal level.

The cells where SIK2 was silenced, were treated with FGF2 for indicated times, lysates were prepared in the presence of phosphatase inhibitors. In these cells, we did not observe FGF2 dependent upregulation of pS621-Raf-1 levels at any time points examined (Figure 5.3). On the contrary, at 5 min post-induction there was a small but statistically significant drop in S621-Raf-1 levels. The difference between other time points were not significant and the profile stayed stable.

The difference between SIK2 silenced and control cells strongly suggests that SIK2 contributes to phosphorylation of Raf-1 on Serine 621.

a.



b.

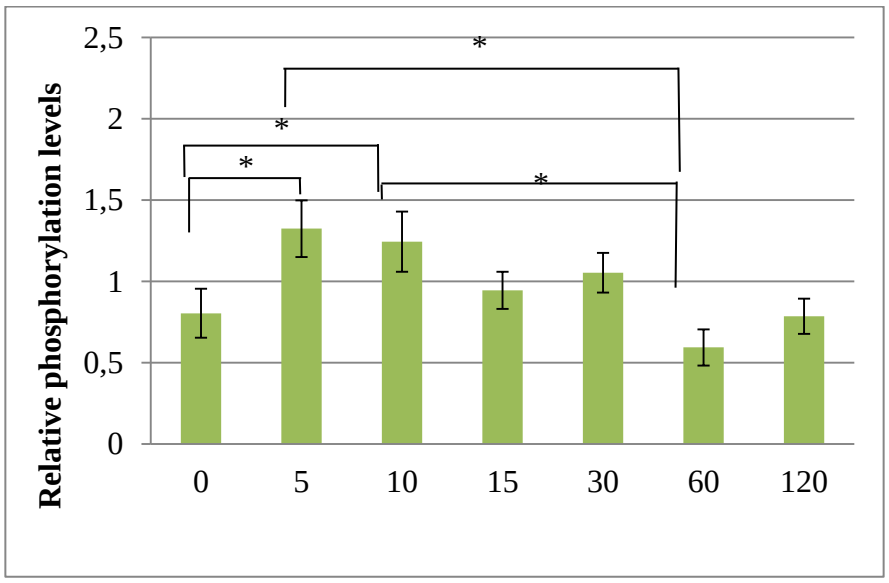
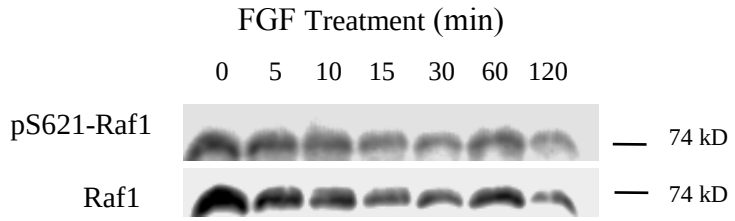


Figure 5.2. FGF2 Dependent Phosphorylation profile of Raf-1 on Serine 621 in control Müller cells. a. Western blot analysis was performed with anti-Ser 621-pRaf-1, anti-Raf-1 was the loading control. b. Graphic representation of relative Ser 621-pRaf-1 band intensities normalized to that of corresponding Raf-1. Experiment was repeated eleven times. * $p < 0.05$ significance at indicated time points.

a.



b.

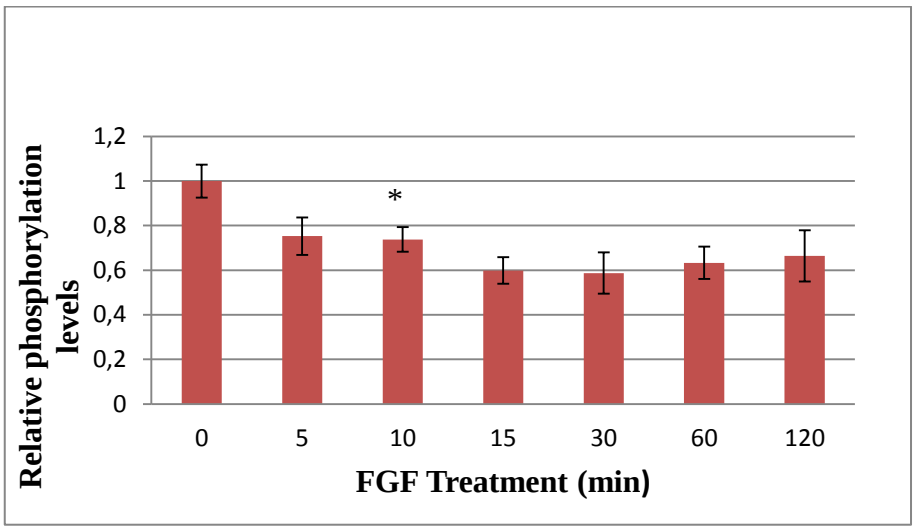


Figure 5.3. FGF2 Dependent Phosphorylation Profile of Raf-1 on Serine 621 in SIK2 Silenced Müller cells. a. Western blot analysis was performed with anti-Ser 621-pRaf-1, anti-Raf-1 was the loading control. b. Graphic representation of relative Ser 621-pRaf-1 band intensities normalized to that of corresponding Raf-1. Experiment was repeated three times.* $p<0.05$: level of significance of the time points in comparison to basal level.

5.1.3. Raf-1 activity profile in control and SIK2 silenced Müller cells

In order to investigate if SIK2 downregulation induces changes in the activation profile of Raf-1; Raf-1 was immunoprecipitated from the cells treated a with FGF2 for 0, 10 and 60 min and used in *in vitro* kinase assays in the presence of [^{32}P] γ-ATP, kinase inactive MEK1 protein was included as the Raf-1 substrate. Subsequently, the samples

were fractionated by SDS-PAGE and dried gels were subjected to autoradiography for varying times (Figure 5.4a).

For determination of Raf-1 activity profile, the results obtained from *in vitro* kinase assay were normalized with results from western blots of immunoprecipitated Raf-1of the aliquots of the same samples (Figure 5.4b).

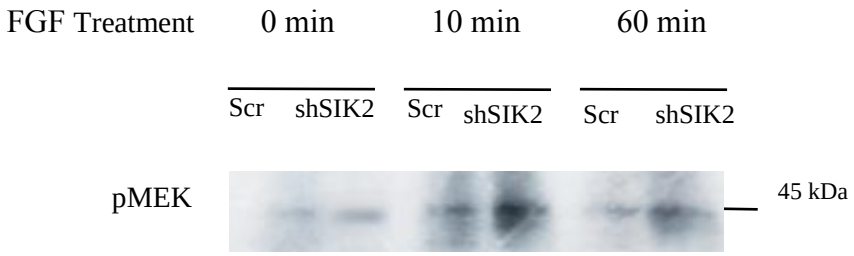
In Figure 5.4, * stands for p values of smaller than 0.05 which denotes the level of significance between designated time points and; control and SIK2 silenced cells. Raf-1 activity is significantly different at 0 and 10 minutes of FGF2 treatment in SIK2 silenced cells and also at 10 minutes between control and SIK2 silenced cells.

In control samples, a modest increase in MEK1 phosphorylation indicating Raf-1 activity was observed at 10 min of FGF2 treatment. At 60 min of FGF treatment, Raf-1 activity level was similar to that of the basal levels. The changes in MEK-1 phosphorylation within control samples were not statistically significant.

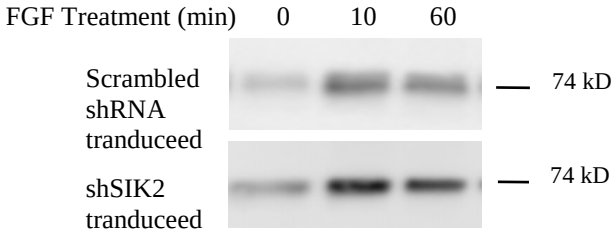
In SIK2 downregulated samples, the increase in MEK1 phosphorylation by 10 minutes was statistically significant compared to basal levels ($p<0.05$). The high activity of Raf-1 at 10 minutes was decreased by 60 minutes post FGF2 treatment.

When activation data in control cells were compared with data from SIK2 silenced cells, at 10 minute post FGF2 treatment, there was a significant difference ($p<0.05$). Activity in SIK2 silenced cells was higher, in parallel with the activation profile of SIK2. At 10 minutes, SIK2, at its most active mode, phosphorylates Raf-1 maximally on Ser 621 and negatively regulates its activity.

a.



b.



c.

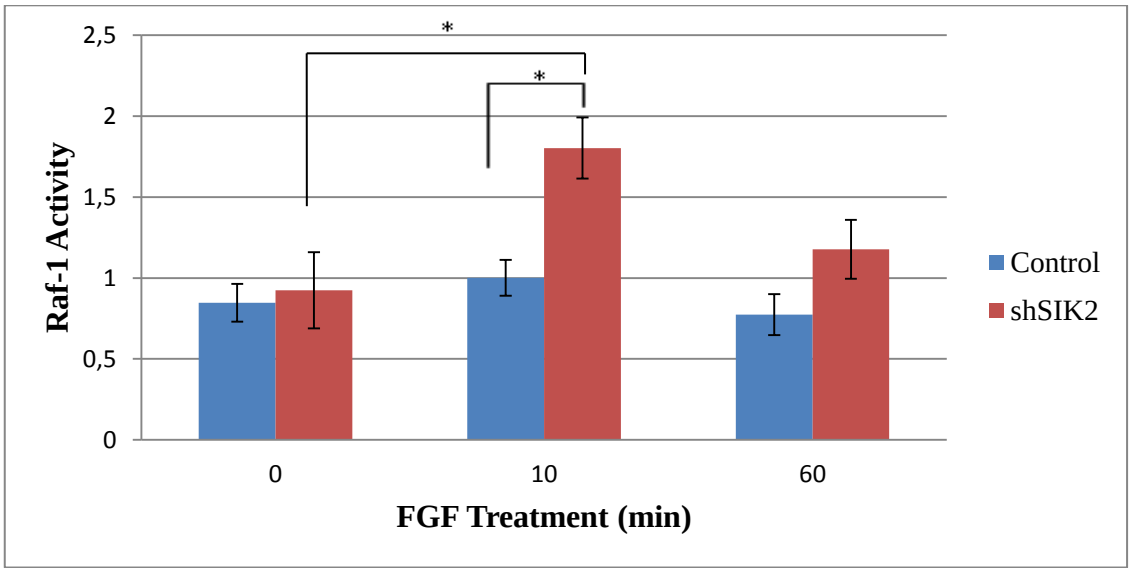


Figure 5.4. FGF2 dependent changes in Raf-1 activity with SIK2 silencing. a. Kinasing was detected by autoradiography with radiolabelled ATP and catalytically inactive MEK. b. Aliquotes of the same samples were subjected to WB for evaluation of Raf-1 *in vitro*. c. The band intensities in the autoradiograms were normalized to that of SIK2 in the corresponding samples analyzed by WB and graphically presented. * denotes $p<0.05$.

5.2. Generation of mutants for identification of phosphorylation sites by ERK on SIK2

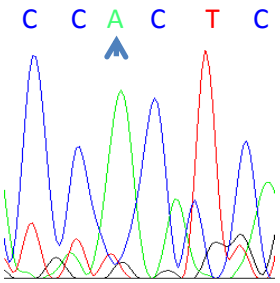
It has been shown that ERK phosphorylation motif (S/T)P adjacent to FxFP motif (DED domain) is present on SIK2 and and *in vitro* kinase results indicated ERK as an potential upstream kinase for SIK2. The threonine 758, 863 and serine 379, 775, 891 are the putative sites for this dual kinase on basis of phosphorylation motif (Ejder, 2011). Furthermore, SIK2 and ERK phosphorylation kinetics are similar in that their activity maxima are within 10 min with FGF2 induction (Küser, 2011).

In order to determine if these residues are ERK targets, Threonine 758 and 863 in pEGFP C3 SIK2 KI (K49M) plasmid were converted to Alanine by a single nucleotide change by *in vitro* mutagenesis. The mutated plasmids were amplified, isolated and sequenced with GeXP DTCS DNA Sequencing System.

The sequencing results indicated A to G (2587^{th} nucleotide) transition was successful (Figure 5.5). The mutagenesis of threonine 758 to alanine could not be confirmed due to technical problems.

After verification, mutated plasmids were transfected to MIO-M1 cells for GFP expression and the cells were collected and lysed at 48^{th} hour. SIK2 was immunoprecipitated from lysates (Figure 5.6) and *in vitro* kinase assay was carried out by using active ERK as SIK2 kinase. However, due to technical problems, an informative analysis was not possible.

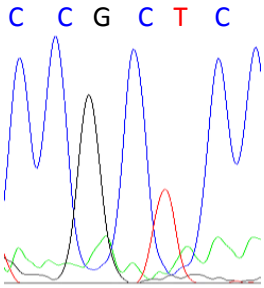
a.Wildtype SIK2 vector



2584 cccactccc

P T P

b. T863A Mutated Sample



2584 cccgctccc

P A P

Figure 5.5. Verification of K49M SIK2 *in vitro* mutagenesis products. a. 2587th nucleotide, adenine, targeted to be converted to guanine, is marked with blue arrow and with turquoise highlighting in the sequence. ERK phosphorylation motif is indicated with purple font color. b. Threonine 863 was converted to alanine by mutating A to G and highlighted with turquoise.

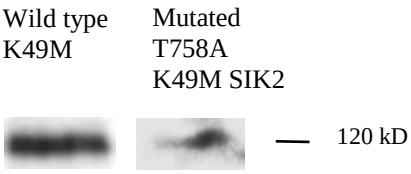


Figure 5.6. Immunoblot of immunoprecipitated wildtype and mutated SIK2 (T758A) protein. SIK2 was immunoprecipitated from lysates of control and transfected MIO-M1 cells.

6. DISCUSSION

Müller cells are the main glia of the retina, maintaining homeostasis and providing neuronal support (Bringmann *et al.*, 2011). FGF2 has been reported to be one of the key signaling molecules in Müller cell proliferation (Hicks and Courtois, 1992; Çınaroğlu and Buğra, 2005) and transdifferentiation (Karl and Reh, 2010; Ahmad *et al.*, 2011: Schmeer *et al.*, 2012; Fisher and Bongini, 2010).

In the initial studies, SIK2 was obtained in a yeast two-hybrid screen of a retinal cDNA library in which FGFR2 was used as bait (Özcan, 2003). Localization, phosphorylation status and activity of SIK2 were shown to be modulated in FGF2 dependent manner in MIO-M1 cells (Özmen, 2006; Candaş, 2007). The involvement of SIK2 was investigated through overexpression and downregulation studies. When SIK2 is overexpressed, Akt and ERK activity levels were significantly reduced, on the other hand, when it is downregulated, the intensity and the duration of ERK and Akt activity levels along with Muller cell proliferation rate have increased. This indicates a negative feedback mechanism by SIK2 on Ras/ERK and PI3K/Akt pathways in context of FGF2 signaling (Küser, 2011). Furthermore, it has been found that Gab-1 and Raf-1 are novel substrates of SIK2. It was proposed that SIK2 phoshorylation of Gab-1 on Ser 266, negatively regulates Gab-1 interaction with its partners and resulting in downregulation of the pathway (Yılmaz Sert, 2011; Küser, 2011). It was shown that SIK2 phosphorylates Raf-1 on Ser 621 (Yılmaz Sert, 2011).

In this study, the aim was to identify the FGF2 dependent kinetics of Raf-1 phosphorylation on Ser 621 by SIK2 and determine its effect on Raf-1 activity. In this context, MIO-M1 cell line in which SIK2 was downregulated with lentiviral particles carrying sh-SIK2 was used, the cells were treated with FGF2 for 0-120 minutes, Ser621-pRaf-1 levels were analysed by immunoblotting.

In naive cells , FGF2 dependent Raf-1 phosphorylation on Ser 621 showed nearly 50% increase over the basal levels within 5 minutes ($p<0.05$). The Ser621-pRaf-1 levels subsequently declined back to basal levels by 60 minutes. The data parallel the activation profile of SIK2, which reaches maximum activity at 10 minutes and basal activity was

restored by 60 minutes of FGF2 treatment (Küser, 2011). In the SIK2 downregulated cells at all time points examined, the phosphorylation Ser621-pRaf1 levels were significantly below basal levels ($p<0.05$). These observations support the hypothesis of SIK2 being an upstream kinase of Raf-1 in the context of FGF2 pathway.

To assess the effect of phosphorylation by SIK2 on Raf-1, in vitro kinase assays were carried out. Control and SIK2 silenced cells were treated with FGF2 for 0, 10 and 60 minutes, and immunopurified Raf-1 was used in *in vitro* kinase assays in the presence of [^{32}P] γ-ATP where kinase inactive MEK-1 was the substrate. Aliquotes of the samples were used in immunoblotting with anti-Raf-1 for normalization of autoradiography results. Raf-1 activity peaked at 10 minutes of FGF2 treatment in SIK2 silenced cells, which returned to basal after 60 minutes of growth factor exposure. The increase at 10 minutes (about two-fold) was statistically significant in comparison to the basal levels observed both in SIK2 silenced and control cells ($p<0.05$). When control and SIK2 silenced cells were compared, the difference at 10 minutes was statistically significant ($p<0.05$), paralleling the activation profile of SIK2. When these data are combined, it can be inferred that SIK2 phosphorylates Raf-1 on Ser 621 and negatively regulates its activity in an FGF2 dependent manner.

It has been shown that ERK is an upstream kinase of SIK2 and potential ERK phosphorylation motifs, (S/T)P adjacent to FxFP motif (DED domain), are present on SIK2 sequence. The candidate sites for ERK phosphorylation had been determined as threonine 758, 863 and serine 379, 775, 891. Through ERK inhibition by MEK inhibitor, SIK2 phosphorylation profile was analyzed and threonine levels have changed significantly (Ejder, 2011). Therefore, in this part of the study, it was intended to analyse whether these threonines are the target residues by *in vitro* mutagenesis.

To this end, pEGFP C3 SIK2 KI (K49M) plasmid this residue was replaced with alanine, the T863A mutation was verified by DNA sequencing. The mutant protein was expressed in *E.coli* and purified by immunoprecipitation. However, due to technical difficulties, the analysis and in vitro kinase assays were not informative. Threonine 758 was also included in this study but the mutation in the end product could not be confirmed due to technical difficulties in sequencing.

As mentioned earlier that we propose SIK2, which reaches maximum activity 10 minutes post FGF2 treatment, downregulates ERK activation through phosphorylation of Raf-1 It is also known that ERK is active in 5 minutes and in turn activates SIK2, creating a feedback loop. In order to further inspect the kinetics of this regulation, ERK activation status within this frame should be included.

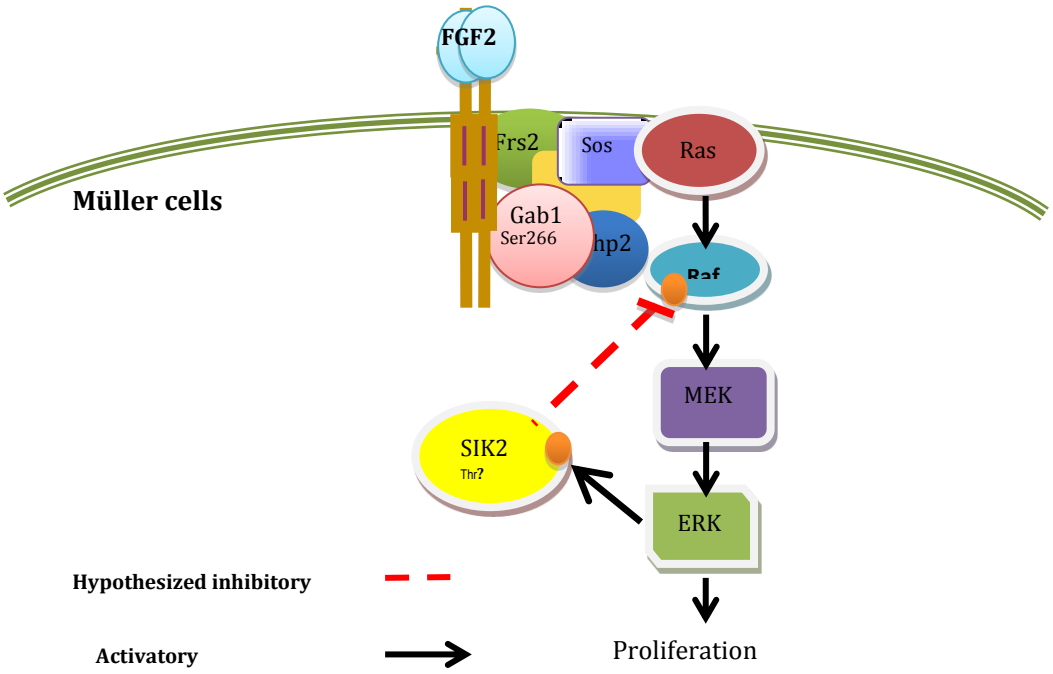


Figure 6.1. The working model of SIK2 involvement in FGF2 pathway (Küser, 2011).

The data obtained from this study is in line with our working model of Ras/ Erk pathway in FGF2 signaling (Küser, 2011). In this scheme, activated FGFR recruits Frs2, Sos, Grb2, Shp2 and Gab1, subsequently Ras, Raf, MEK and ERK are activated within 5 minutes. It is conceivable that ERK phosphorylates SIK2 on threonine 758 and/or 863 leading to its activation. SIK2, when fully active at 10 minutes, negatively regulates Ras/ERK cascade through phosphorylation of Gab-1 on Ser 266 and Raf-1 on Ser 621. SIK2 phosphorylation on Gab-1 results in dissociation of Grb2, Shp2 and p85 complex and phosphorylation of Raf-1 leads to downregulation ERK and Akt activation. When SIK2 is silenced, Müller cell proliferation rate increases as activation and duration of Ras/ERK

signaling increased. SIK2 is downregulated by Akt and PKA in FGF2 induced MIO-M1 cells (Ejder, 2011), indicating a very tight regulation of SIK2.

In this study, the effects of SIK2 silencing have been considered in comparison to control cells. To further understand the pathway regulation, the effects of SIK2 overexpression on Raf-1 activity and Ser 621 phosphorylation should also be investigated and compared with SIK2 silenced and control Müller cells. It is shown that SIK2 downregulates Ras-ERK pathway upon phosphorylation of Gab-1 on Ser 266 (Yılmaz Sert, 2011; Küser, 2011). In this study, it is also shown that SIK2 downregulates the same pathway upon phosphorylation of Raf-1 on Ser 621. To what extent SIK2 exerts its inhibitory effect on this pathway and the factors determining the choice of the targets should be investigated to a greater extent. To tackle this question, Gab-1 and SIK2 and also Raf-1 with SIK2 pull down assays can be designed and the timings of the regulation and activation status of these proteins can be examined.

As discussed before, Ser 621 is an important site for regulation of Raf-1 activity (Matallannas *et al*, 2011). Phosphorylation on Ser 621 is required for 14-3-3 protein binding which is an important component of Raf-1 regulation (Dhillon and Kolch, 2002). The bound 14-3-3 results in either closed inactive state or dimerized highly active state of Raf-1. In one case, it may bind both Ser 259 and 621, rendering Raf-1 in inactive state as the regulatory domain masks the catalytic domain. However binding of 14-3-3 protein to Ser 621 may also have an activatory role by preventing phosphorylated Ser 621 from interacting with the catalytic domain which leads to inhibition. For clarification of the role of 14-3-3 proteins in this scheme, pull down assays can be designed with Raf-1 under context of SIK2 silencing and overexpression with comparison to control cells. Therefore, the activation status of Raf-1 and the role of 14-3-3 proteins may be examined in more detail in this context.

The outcome of 14-3-3 binding is also dependent of many regulatory kinases that target Raf-1 on this residue such as PKA, AMPK and Raf-1, itself. Our results indicate SIK2 is also can phosphorylate Raf-1 at this residue. However, since many different kinases and phosphatases are involved in regulation of Raf-1 targeting Ser 621, the exact effect and kinetics of SIK2 phosphorylation on Raf-1 activity was not possible to

determine. For more accurate detailed scheme, kinase inactive form of Raf-1 with inhibitors of other regulatory kinases such as PKA and AMPK should be included in the analysis. PKA phosphorylates Raf-1 on Ser 43 and reduces its affinity for Ras and also on Ser 259 and 621, converting Raf-1 to catalytically inactive state (Dhillon and Kolch, 2002). PKA also phosphorylates SIK2 on Ser 587 and inactivates it (Muraoka *et al*, 2009). Since SIK2 is suggested as a negative regulator of Raf-1 via Ser 621, the timing and possible crosstalk mechanism of SIK2 with PKA should be further considered for a better picture of FGF2 signaling scheme.

Another hypothesis proposes that Raf-1, independent of its role as MEK kinase, may serve as a scaffolding protein, or it may have other substrates than MEK in the pathways that it is involved (Dhillon and Kolch, 2002). This hypothesis suggests that Raf-1 may still be able to exert effect in the pathway regardless of its ability to phosphorylate MEK. To further understand the FGF pathway, a knock in model with kinase negative Raf-1 may be used to see if Raf-1 can still have an effect on the pathway regulation.

Another known upstream kinase that phosphorylate Raf-1 on Ser 621 is AMPK, which belongs to the same super family with SIK2. ERK is shown to inhibit and reduce the phosphorylation of AMPK in cardiac cells but inhibition of ERK restores the activity (Du *et al*, 2008). Also, as a late event in the regulation scheme, AMPK inhibits ERK phosphorylation. AMPK and SIK2 may have same or similar interaction partners and participate in the regulation of the pathway in similar ways.

SIK2 and AMPK are unique in that they are also involved in many other processes such as energy metabolism implicating a crosstalk not only between the components of FGF pathway but also with the other metabolic pathways. Thus, SIK2 may play a dual role and possibly it might be a key component of crosstalk between energy metabolism and cell proliferation. Modulation of SIK2 activity in response to changing glucose concentrations may be important for the progression of FGF dependent proliferation, with possible contribution of AMPK . Thus, SIK2 may be involved in a more complex network of signaling pathways and instrumental in of integration of various stimuli in shaping cellular response than it was assumed before.

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