INVOLVEMENT OF GAB1 IN MÜLLER CELL PROLIFERATION

by

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To my family and friends…

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ABSTRACT

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Müller cells, the major type of glial cells in the retina, are reactivated in response to most retinal injuries and diseases. In fish the reactivated Müller cells proliferate and transdifferentiate to make up for the lost neurons. Although in mammals, neurogenesis is not evident *in situ*, Müller cell proliferation can be stimulated by exogenous growth factors such as FGF2 through the activation of the Ras/ERK pathway. Previous work from our laboratory suggests that SIK2 is involved in a negative feedback mechanism of FGF2-induced Ras/ERK pathway. We propose that this process involves Gab1 phosphorylation by SIK2 at S266, which disrupts interactions of Gab1 with binding partners and downregulate proliferation. In this study, to provide further support for the role of Gab1 in this mechanism, SIK2 or Gab1 silenced MIO-M1 cells were generated by shRNA approach along with S266A mutant Gab1 transfected MIO-M1 cell line. The effects of these modulations on ERK activation and cell proliferation was investigated. The duration of ERK activation increased in both SIK2 downregulated and S266A mutant Gab1 expressing cells and higher level of proliferation was observed. Gab1 silencing led to a dramatic increase in amplitude of ERK phosphorylation in the initial phase of ERK activation but did not alter the duration of the signaling. These findings raise the possibility of dual role for Gab1 where it switches from suppressor to amplifier during the course of FGF2-induced ERK signaling.

ÖZET

GAB1'IN MÜLLER HÜCRELERİNİN PROLİFERASYONUNA ETKİSİ

Retina dokusundaki ana glia olan Müller hücreleri hemen tüm kalıtsal ve travmaya bağlı retinal hastalık koşullarında reaktif gliosis olarak adlandırılan bir dizi değişiklik gösterirler. Balıklarda bu değişikler proliferasyon ve transdifferansiyasyon içeren bir süreçle yeni ve işlevsel nöron oluşumu ile sonlanmaktadır. Memelilerde *in situ* nörogeneze işaret eden bulgular olmamasına karşın, FGF2 gibi büyüme faktörleri ile Müller hücre proliferasyonunun Ras/ERK yolağı yoluyla uyarılabildiği ve bazı nöronal markörlerin anlatımının tetiklendiği gösterilmiştir.

Laboratuvarımızda yapılan önceki çalışmalar SIK2'nin, FGF2'ye bağlı Ras/ERK aktivasyonunu negatif regüle eden bir geribildirim mekanizmasının elemanı olma olasılığını gündeme getirmiştir. Bu bağlamda SIK2'nin, Gab1'i S266 amino asitinden fosforlayarak partnerleri ile bağlanmasını etkileyerek Müller hücrelerinin proliferasyonuna ket vurduğu önerilmektedir. Bu çalışmada, SIK2 veya Gab1 anlatımı baskılanmış ve S266A mutant Gab1 ile transfekte edilmiş MIO-M1 hücreleri üretilerek, bu modülasyonların FGF2'ye bağlı ERK aktivasyonu ve hücre proliferasyonu üzerindeki etkisi irdelenmiştir. Bulgularımız ERK aktivasyonunun süresinin hem SIK2 baskılanmış hem de S266A mutant Gab1 proteini üreten hücrelerde arttığını, ve hücrelerin daha yüksek oranda prolifere olduğunu göstermiştir. Gab1'ın baskılanması, ERK aktivasyonunun ilk aşamasında ERK fosforilasyonunda büyük bir artışa neden olmuş, ancak ERK aktivasyonunun süresini değiştirmemiştir. Bulgularımız, Gab1'in rolünün FGF2 kaynaklı ERK aktivasyonu sırasında baskılayıcıdan amplifikatöre geçtiğini düşündürmektedir.

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LIST OF SYMBOLS

kDa	Kilodalton
mg	Milligram
min	Minutes
ml	Milliliter
mm	Millimeter
M	Molar
mM	Millimolar
ng	Nanogram
nm	Nanometer
rpm	Rotations Per Minute
sec	Seconds
xg	Times Gravity
µg	Microgram
µl	Microliter

LIST OF ACRONYMS/ABBREVIATIONS

AMPK	AMP-activated Protein Kinase
APS	Ammonium Persulfate
ATP	Adenosine Triphosphate
BCA	Bicinchronic Acid
BDNF	Brain-derived Neurotrophic Factor
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
cAMP	Cyclic Adenosine 5'-monophosphate
Cbl	Casitas B-lineage Lymphoma
CBP	CREB Binding Protein
cDNA	Complementary DNA
ChREBP	Carbohydrate Responsive Element-Binding Protein
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CO_2	Carbon Dioxide
CRALBP	Cellular Retinaldehyde-Binding Protein
CREB	cAMP Response Element-binding Protein
CRTC	c-AMP-regulated Transcriptional Coactivator
DAPI	Diaminophenylindolamine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
Dok	Downstream of Tyrosine Kinase
DOS	Daughter of Sevenless
ECM	Extracellular Matrix

EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Regulated Kinase
EtOH	Ethyl Alcohol
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
Frs2	Fibroblast Growth Factor Receptor Substrate
Gab	Grb2-associated Binder
GAP	GTPase Activating Protein
GDNF	Glial Cell-derived Neurotrophic Factor
Grb2	Growth Factor Receptor-bound Protein 2
GTP	Guanosine-triphosphate
H_2O	Water
HBS	HEPES Buffered Saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HGF	Hepatocyte Growth Factor
HRP	Horse Radish Peroxidase
HS	Heparan Sulphate
HSPG	Heparan Sulphate Proteoglycans
IgG	Immunoglobulin G
IRS	Insulin Receptor Substrate
JNK	C-Jun N-Terminal Kinase
KSR	Kinase suppressor of Ras
LB	Luria Broth
LIF	Leukemia İnhibitory Factor
LKB1	Liver Kinase B1
MAPK	Mitogen-activated Protein Kinase
MBD	c-Met-binding Domain

$MgCl_2$	Magnesium Chloride
MIO-M1	Moorfields Institute of Ophthalmology – Müller 1
MKP	MAPK Phosphatase
MP	Milk Powder
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
NGF	Nerve Growth Factor
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pen/Str	Penicillin/Streptomycin
PH	Plekstrin Homology
PI3K	Phosphoinositide 3-kinase
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLCγ	Phospholipase C-gamma
PTB	Phosphotyrosine Binding
PVDF	Polyvinyl Difluoride
Raf	Rapidly Accelerated Fibrosarcoma
RNA	Ribonucleic Acid
RTK	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
Sef	Similar expression to FGF
Ser	Serine
SH	Src Homology Domain
Shc	Src Homology 2 Domain Containing Transforming Protein

Shp2	SH2 Domain Containing Phosphatase 2
shRNA	Small Hairpin RNA
SIK	Salt Inducible Kinase
Soc	Suppressor of Clear
SOS	Son of Sevenless
Spry	Sprouty
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TEMED	Tetramethylethylenediamine
TGF	Transforming Growth Factor
Thr	Threonine
TORC2	Transducer of Regulated CREB Protein 2
UBA	Ubiquitin-associated Domain
VEGF	Vascular Endothelial Growth Factor
WB	Western Blot
wt	Wild-type

1. INTRODUCTION

1.1. Müller Glia

Müller cells are the major type of glial cells in the retina. Even though their cell bodies reside in the inner nuclear layer (INL), their extensions span all retinal layers and form extensive contact with the neurons. These cells play important roles in maintaining homeostasis and supporting survival and function of the retinal neurons (Goldman, 2014).

In the adult retina, Müller glia stores glycogen and provides lactate/pyruvate to the neurons for their oxidative metabolism (Poitry *et al.*, 2000). They are critically important in maintenance of K^+ and water homeostasis (Newman, 1996). They also contribute to protection against oxidative stress by scavenging free radicals (Pow and Crook, 1995). By uptake and recycling of neurotransmitters and providing trophic factors, Müller glia are important for neuronal functioning and for the prevention of neurotoxicity (Matsui *et al.*, 1999). Müller cells are vital in regulation of blood flow and in formation and maintenance of blood-retinal barrier (Tout *et al.*, 1993).

Müller cells provide various neurotropic factors such as Ciliary Neurotrophic Factor (CNTF) (Cao *et al.*, 1997), Leukemia Inhibitory Factor (LIF) (Neophytou *et al.*, 1997), Nerve Growth Factor (NGF) (Chakrabarti *et al.*, 1990) Brain-Derived Neurotrophic Factor (BDNF) (Seki *et al.*, 2005) and Fibroblast Growth Factors (FGFs) (Bugra and Hicks, 1997). Vasoactive substrates such as Vascular Endothelial Growth Factor (VEGF) (Eichler *et al.*, 2000), Platelet-Derived Growth Factor (PDGF) (Eichler *et al.*, 2004) are also synthesized and released by Müller glia.

Investigation of Müller cell functions *in vitro* have been challenging because of difficulties in producing pure cell population and their tendency to rapidly differentiate in

culture. A spontaneously immortalized Müller cell line MIO-M1 (Moorfields/Institute of Ophthalmology-Müller 1) that retains the characteristics of differentiated Müller cells in culture. They express well known Müller glial cell markers, such as Cellular Retinaldehyde Binding Protein (CRALBP), Epithelial Growth Factor Receptor (EGFR), vimentin and retinal stem cell markers including Sox2, Pax6, Chx10 and Notch1 *in vitro* (Limb *et al.*, 2002).

1.1.1. Müller Cell Gliosis

In response to pathological changes of the retina, such as damage, ischemia, retinal detachment, diabetic retinopathy, glaucoma and macular degeneration, Müller cells become reactivated. In zebrafish, Müller glia is reactivated in response to injury and generates a proliferating progenitor cell population that can differentiate into all retinal cell types and restore vision (Goldman, 2014). In postnatal chicks, in response to injury, Müller glia proliferation and a limited amount of regeneration is seen (Fischer and Reh, 2003). Even though, under pathological conditions Müller cell gliosis occurs in mammals, it does not result in regeneration of neurons (Hamon *et al.*, 2016).

Mammalian reactive gliosis is characterized by different morphological, biochemical and physiological alterations depending on severity and type of insult, and may have both protective and destructive effects on retinal neurons (Bringmann *et al.*, 2010). Early after injury, reactive Müller gliosis is considered as an attempt to keep the tissue from additional damage by releasing antioxidants and neurotrophic factors such as FGF2, CNTF, glial cell derived neurotrophic factor (GDNF). Consistent with the growth factor upregulation, activation of Extracellular signal-regulated kinase (ERK1/2) is observed in animal models of several retinopathies (Geller *et al.*, 2001). Under sustained insult and in severe cases, they may go through dedifferentiation contributing to neuronal cell death and have an additive effect on loss and dysfunction of neurons. Müller cells may also re-enter to the proliferation cycle leading to glial scar formation. Glial scars lead to the expression of inhibitory molecules on the surface of the cells, obstructing regular tissue repair and neuronal protection (Fawcett and Asher, 1999).

The molecular mechanisms underlying Müller cell gliosis are yet not well understood. In rodent species, proliferation and dedifferentiation of Müller cells and a small amount of neuronal regeneration can be stimulated by exogenous growth factors such as FGFs *in vivo* and *ex vivo* (Karl *et al.*, 2008; Löffler *et al.*, 2015). *In vitro* studies suggest that FGF2-induced signaling is critical for the proliferation of primary Müller cell cultures and MIO-M1 cell line (Hollborn *et al.*, 2004). In retinal detachment experiments, the phosphorylation of the FGF receptors and activation of ERK1/2 was observed within minutes as an indication of FGF2 release in Müller cells of the retina (Geller *et al.*, 2001)

1.2. Fibroblast Growth Factors

FGFs belong to a large family of proteins with 23 members that share sequence and structure similarity (Hui *et al.*, 2018). FGF family consists of secreted signaling proteins that signal to receptor tyrosine kinases (RTKs) and intracellular non-signaling proteins (iFGFs, also called FGF homologous factors (FHFs)), which act as cofactors for voltage gated sodium channels (Figure 1.1) iFGFs are not secreted and do not have any identified interaction with Fibroblast Growth Factor Receptors (FGFRs). Secreted FGFs function as autocrine or paracrine fashion (called canonical or paracrine FGFs) however, three members of secreted FGFs act as endocrine factors called endocrine FGFs. Canonical FGFs are expressed in nearly all tissues and control essential processes such cell proliferation, migration, differentiation, survival and metabolism. Endocrine FGFs are involved in regulation of phosphate, carbohydrate and lipid metabolism in addition to their canonical functions (Ornitz and Itoh, 2015).

Canonical FGFs use heparan sulfate (HS) proteoglycans (HSPGs) or heparin as their cofactor to form dimers with their receptors and act locally because of their high affinity for HSPGs. Endocrine FGFs have low affinity for HSPG and use Klotho family co-receptors instead (Xue *et al.*, 2018).

FGF2, also called basic FGF or bFGF, is a member of FGF1 subfamily (represented with red in Figure 1.1), which lacks the secretory signal peptides but is exported by direct

translocation through the membrane (Ornitz and Itoh, 2015). FGF2 binds to FGFR1 and FGFR2 with high affinity (Su *et al.*, 2014). During CNS development, FGF2 plays a role in cell growth, differentiation, migration and survival. It was also shown to promote survival in both neural and non-neuronal cells and protect neurons from injury (O'Driscoll *et al.*, 2007).

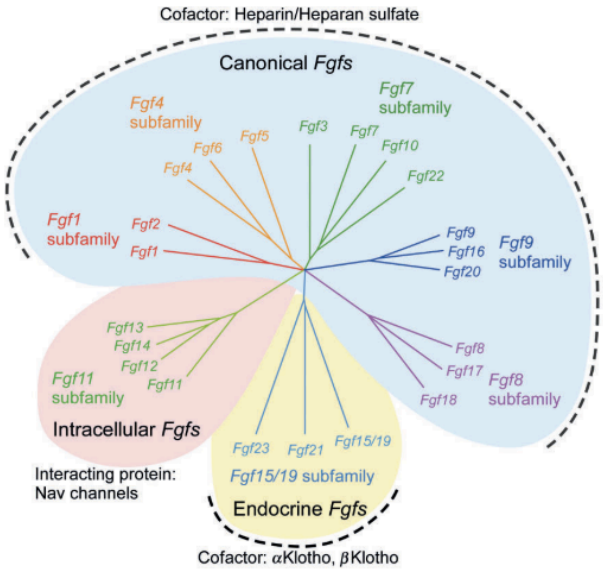

Figure 1.1 Classification of Fibroblast Growth Factors and their co-factors (Ornitz and Itoh, 2015)

Numerous FGF family members are expressed in both developing and adult retina. FGF2, along with FGF1 and FGF9, is shown to be expressed in postnatal mammalian retina and promote proliferation of Müller cells, which is mostly dependent on ERK activation (Bugra *et al.*, 1993; Bugra and Hicks, 1997; Cinaroglu *et al.*, 2005; Hollborn *et al.*, 2004).

1.3. Fibroblast Growth Factor Receptors

FGFs induce cellular responses by binding to FGFRs, which are transmembrane tyrosine kinase receptors that mediate signal transduction via either HSPG or Klotho-dependent pathways. There are five types of FGFRs including FGFR1, FGFR2, FGFR3, FGFR4 and

FGFR5 (also called FGFRL1). FGFR1-4 all have an intracellular split tyrosine kinase (TK1 and TK2) domain which is absent in FGFRL1 (Hui *et al.*, 2018; Xue *et al.*, 2018). FGFRL1 can bind to FGFs but it is thought to have a negative regulatory effect on the signaling since it lacks TK domain (Wiedemann and Trueb, 2000). FGFRs also contain three extracellular immunoglobulin-like (Ig-like) domains (D1, D2 and D3), an acid box and a single transmembrane helix. D2 and D3 domains form the ligand-binding pocket. Acid box is required for interaction between FGFRs and HSPGs (Tiong *et al.*, 2013).

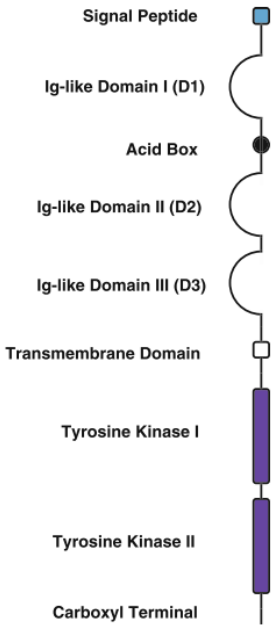

Figure 1.2 General structure of FGFRs (Tiong *et al.*, 2013)

FGFRs can bind to different FGFs with similar affinity and each FGF can bind to different FGFRs. Multiple isoforms of the receptors can be produced by the alternative splicing of mRNA of each FGFR gene or enzymatic hydrolysis on the cell surface (Hui *et al.*, 2018).

1.4. FGF/FGFR Signal Transduction Pathways

HPSGs are mandatory cofactors in canonical FGF signaling. They can interact with both FGFs and FGFRs and increase the affinity of FGF-FGFR dimer by simultaneously binding to FGF and D2 domain of FGFR (Ornitz and Itoh, 2015). Differences in primary sequences at HS binding sites of FGFs and FGFRs results in formation of distinct interactions with tissue-specific HPSGs. Different tissues can produce different HS chains with distinct sulphation patterns and lengths, which also influences FGF activity (Belov and Mohammadi, 2013). Formation of trimeric FGF-HSPG-FGFR complex leads to conformational changes in FGFR resulting in receptor activation. This allows autophosphorylation of different tyrosine residues in the cytoplasmic portion of the receptor creating docking sites for downstream adaptor proteins containing src homology 2 (SH2), src homology 3 (SH3) and phosphotyrosine binding (PTB) domains (Brooks *et al.*, 2012; Ornitz and Itoh, 2015). In addition to their role in facilitating FGF-FGFR dimerization, as a component of the extracellular matrix (ECM), HSPGs also function in storage of FGFs, modulate FGF diffusion through tissues and stabilize FGFs against degradation (Beenken and Mohammadi, 2013).

FGFR activation results in activation of several intracellular signaling cascades. The main pathways activated through the FGFRs are Ras/Mitogen-activated protein kinase (Ras/MAPK), phosphoinositide 3 kinase (PI3K/Akt), the phospholipase C gamma (PLCγ)/Ca^{2+} pathway (Figure 1.3). Several other pathways can also be activated through FGF signaling, including Src family kinases, Shc- and Crk-mediated pathways, and Janus kinase (JAK)/signal transducer and activator of transcription-dependent (STAT) signaling (Georgiou and Gkretsi, 2018). The MAPK signaling pathway is a three-layer signaling cascade. There are three major MAPK cascades, named according to their MAPK component, which are ERK1/2, Jun N-terminal kinases (JNK1-3), p38-MAPK. Even though all of these cascades are regulated by growth factor signaling, growth factors such as FGF2 are the main regulators of the Ras/ERK1/2 cascade (Katz *et al.*, 2007).

The outcome of Ras/ERK pathway is mainly cell proliferation, but it can also lead to cell cycle arrest, differentiation or other cellular responses. PI3K/Akt pathway promotes cell survival and PLCγ pathway mediates cell migration and differentiation and it is thought to influence Ras/ERK and PI3K/Akt pathways (Goetz and Mohammadi, 2013).

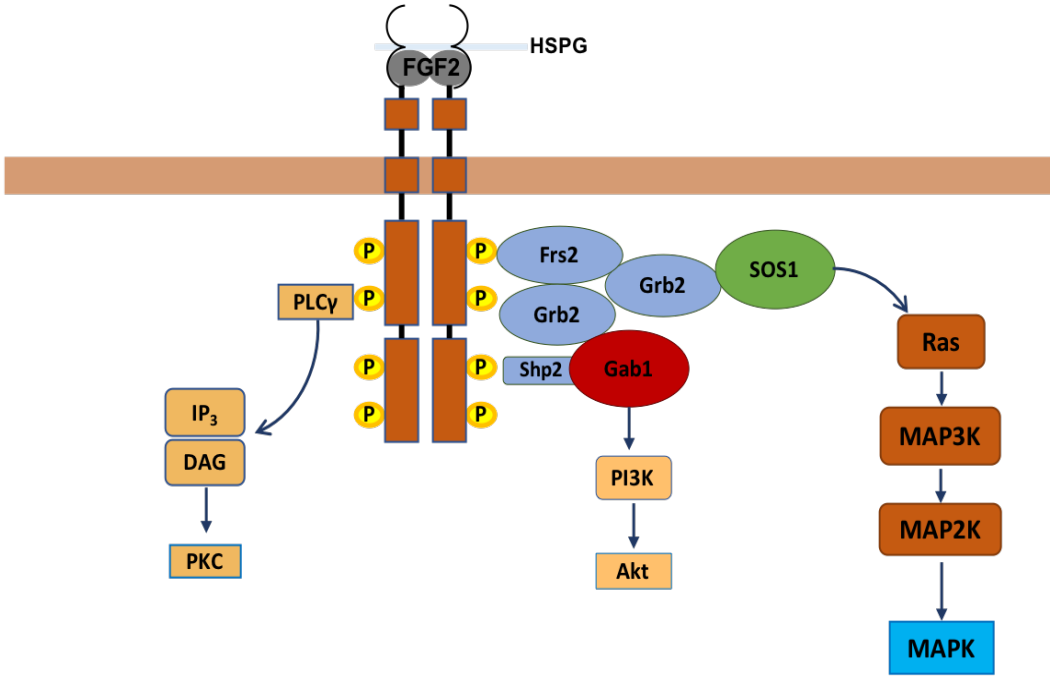

Figure 1.3 Major Fibroblast Growth Factor signaling pathways (Modified from Mason, 2007)

1.4.1. FGF-induced Ras/ERK Pathway

Upon activation by FGF stimulation, phosphorylated tyrosine residues of the receptor create docking sites for adaptor proteins. FGFR substrate 2 (Frs2) is a key adaptor protein, which binds to the FGFRs through its PTB domain. FGFRs phosphorylate C-terminal of Frs2 on multiple tyrosine residues which serve as docking sites for SH2 domains of growth factor receptor-bound 2 (Grb2) and the tyrosine kinase Shp2. Grb2 interacts with the guanine nucleotide exchange factor, Son of Sevenless 1 (SOS1) through its N-terminal SH3 domain and the docking protein Grb2-associated binding protein 1 (Gab1) through its C-terminal SH3 domain. SOS1 promotes the exchange of GDP with GTP on Ras, a small G protein.

Alternatively, recruitment of the Grb2/SOS1 complex can be mediated by the adaptor protein Shc. GTP-bound active Ras then binds and activates Raf (MAP3K protein). Active Raf phosphorylates serine residues in the activation loop of MEK1/2 (MAP2K protein), which in turn phosphorylates ERK1/2 (MAPK protein) at both threonine and tyrosine residues in its activation loop. Activated ERK either phosphorylate cytosolic targets or translocate to the nucleus to phosphorylate certain transcription factors, such as Elk1, c-Jun, ATF2. These factors regulate expression of specific genes to direct various cellular responses such as proliferation, differentiation, and survival (Katz *et al.*, 2007; Turner and Grose, 2010).

1.5. Regulation of FGF-induced Ras/ERK Pathway

Since FGF/FGFR signaling and the Ras/ERK pathway are involved in numerous critical cellular processes, they must be tightly regulated by positive and negative feedback mechanisms at each step of the cascade and their malfunction can result in several pathological events such as developmental disorders and tumorigenesis (Tsang and Dawid, 2004).

Two critical upstream regulators of ERK, SOS1 and Raf, are also substrates of ERK itself. Direct phosphorylation of SOS1 by ERK results in dissociation of Grb2/SOS1 complex, hence reduces Ras activation. In addition, Raf phosphorylation by ERK leads to downregulation of Raf kinase activity, in turn reduces MEK and ERK activation (Lemmon and Schlessinger, 2010). Active ERK was also shown to inhibit signaling by phosphorylating Frs2 on specific threonine residues which results in a decrease in tyrosine phosphorylation of Frs2 inhibiting recruitment of Grb2 to the receptor (Lax *et al.*, 2002).

The Ras/ERK and PI3K/Akt pathways can also regulate each other. Several components of PI3K/Akt pathway were shown to regulate the Ras/ERK pathway. For example, MEK was proposed to be affected by Rho family proteins downstream of PI3K. Moreover, PI3K was suggested to inhibit Ras/ERK pathway by directly interacting with Ras (Wortzel and Seger, 2011).

The late phase of ERK inactivation is protein synthesis dependent. Some of the genes, whose transcription regulated by FGFR pathway, are involved in this slower negative feedback regulation. These include Sprouty (Spry) proteins, Similar expression to FGF (Sef) and MAPK phosphatases (MKP1-3) (Tsang and Dawid, 2004). Spry proteins were shown to function by binding to SH2 domain of Grb2 and preventing recruitment of the Grb2/SOS1 complex to Frs2 or Shp2 therefore inhibits SOS1 mediated Ras activation. Tyrosine phosphorylation was shown to be required for inhibitory activity of Spry proteins, which occurs in a time-dependent manner upon FGF stimulation. Maximum tyrosine phosphorylation of Spry proteins was observed after 15 minutes of FGF stimulation in C2C12 and NIH3T3 cell lines and ERK inhibition was seen between 15 and 60 min. of stimulation (Hanafusa *et al.*, 2002). They can also directly bind to Raf and block ERK activation (Turner and Grose, 2010). Sef proteins function at multiple levels of the cascade. It has a transmembrane form that can interact with FGFRs and block all downstream signaling pathways (Tsang and Dawid, 2004). Sef-b isoform, which is localized in the cytoplasm, acts as a spatial regulator of the signaling by blocking dissociation of ERK from MEK, thus inhibits its nuclear activity by preventing its translocation to the nucleus but does not suppress its cytosolic activity (Torii *et al.*, 2004). MKP1-3 negatively regulate FGF signaling by specifically inactivating ERK. MKP3 dephosphorylates tyrosine and threonine residues on activation motif of ERK in the cytoplasm and MKP1 and MKP2 in the nucleus (Pouysségur and Lenormand, 2003). Cbl, a ubiquitin ligase, interacts with phosphorylated Frs2 and Grb2 leading to ubiquitination and degradation of FGFR and Frs2 (Ornitz and Itoh, 2015). Moreover, Cbl binding to Grb2 was shown to disrupt Grb2/SOS1 interaction resulting in inhibition of Ras activation (Dikic and Giordano, 2003). The cell-surface molecules Neural Cell Adhesion Molecule (N-CAM) and N-cadherin, and Sprouty-related Ena/vasodilator-stimulated phosphoprotein homology 1-domain-containing protein (SPRED1) are some other molecules that can attenuate ERK signaling (Brooks *et al.*, 2012).

There are also positive regulators of Ras/ERK pathway. Kinase suppressor of Ras (KSR), a scaffold protein, interacts with Raf, MEK and ERK, hence facilitates signal transmission and leads to a rapid ERK activation (Morrison, 2001). Another positive regulator of ERK signaling is a leucine-rich-repeat transmembrane protein (FLRT) called XFLRT3,

which regulates the signaling either directly or by recruiting cytoplasmic cofactors that increase activation of the pathway (Böttcher *et al.*, 2004).

1.6. Gab Family

Gab proteins, which include Gab1-3 in mammals, DOS (Daughter Of Sevenless) in Drosophila *melanogaster*, and Soc1 (Suppressor-Of Clear) in Caenorhabditis *elegans*, belongs to a family of scaffolding proteins that are closely related to insulin receptor substrates (IRS1-3), Frs2, Downstream of tyrosine kinase (Dok), Dok-related (Dok-R) and Linker for activation of T cells (LAT) (Liu and Rohrschneider, 2002). Gab1 was originally identified and cloned as a Grb2 SH3 domain binding protein. Gab2 was isolated as a Shp2 binding partner and Gab3 was found based on sequence similarity with Gab1/2. Gab3 is particularly expressed in lymphoid tissue, while Gab1 and Gab2 are ubiquitously expressed (Gu and Neel, 2003).

The Gab family proteins are 50 to 100 kDa in metazoans. They contain a PH domain in the N-terminal region that interact with phosphatidylinositol lipids in the plasma membrane, tyrosine and serine/threonine phosphorylation sites and several proline-rich sequences (PXXP) which provide binding sites for SH2, SH3 and PTB domain containing proteins (Wang *et al.*, 2015). In addition to these domains, Gab1 contains a unique phosphotyrosine recognition domain called c-Met-binding domain (MBD), which mediates direct interaction with the HGF receptor c-Met (Schaeper *et al.*, 2000) (Figure 1.4).

1.6.1. Gab1

Gab1-deficient mice die in utero and display similar phenotypes with HGF, PDGF or EGF signaling defective mice with concomitant decrease in ERK activation (Itoh *et al.*, 2000). Inhibition of Gab1 expression leads to decreased cell proliferation and survival in intrahepatic cholangiocarcinoma cells (Sang *et al.*, 2015). Gab1 overexpression in NIH3T3 cells was demonstrated to enhance growth factor responses (Holgado-Madruga *et al.*, 1996). Shao *et al.* (2018) showed that Gab1 silencing attenuates ERK and Akt activation and significantly

suppresses proliferation in human glioma cells. In contrast, Gab1 was suggested as a negative regulator of the thymus-independent antigen-2 (TI-2) response of marginal zone B cells (Itoh *et al.*, 2002). Moreover, EGF induced proliferation was inhibited by Gab1 overexpression during neoplastic progression in Syrian hamster embryo cell culture (Kameda *et al.*, 2001). There have been some reports stating that Gab1 is dispensable for ERK signaling. Li *et al.* (2014) showed that Frs2 and Shp2 mediate FGF signaling in lens development independent of Gab and knockout of Gab1/2 does not disrupt FGF signaling *in vitro* and lens development *in vivo*. Lamothe *et al.* (2004) also demonstrated that Gab1 is required for FGF1-induced PI3K and Akt activation while modulation of Gab1 does not affect FGF1-induced ERK stimulation.

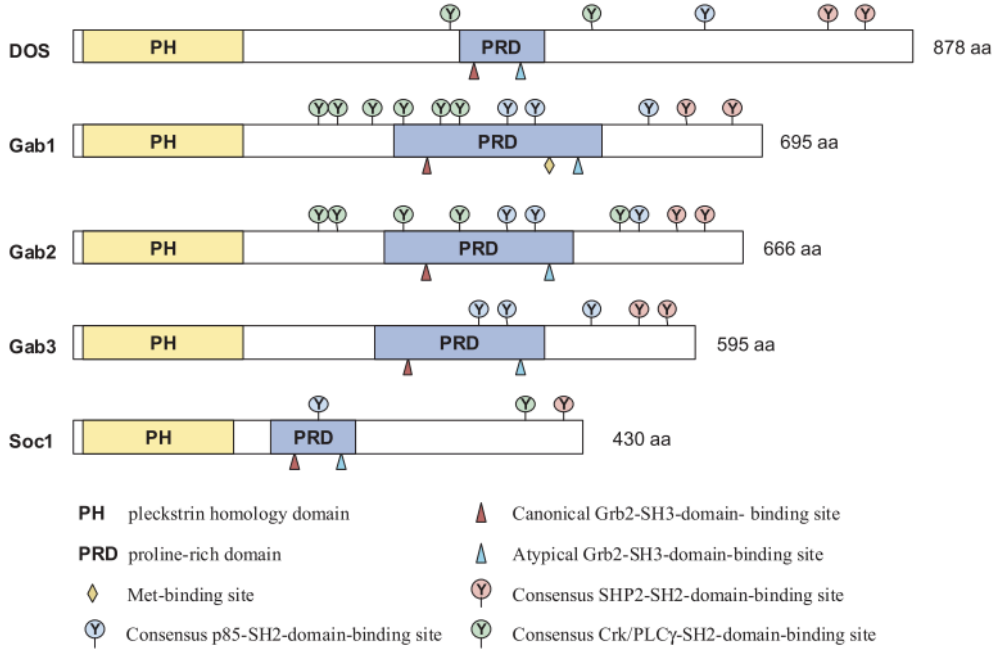

Figure 1.4 Schematic domain structures of Gab family proteins (Liu and Rohrschneider, 2002)

Gab1 can be recruited to the signaling complexes through direct or indirect mechanisms. Gab1 is directly recruited to tyrosine phosphorylated c-Met through MBD domain. Other RTKs, such as EGFR, indirectly recruit Gab1 via Grb2. C-terminal SH3 domain of Grb2 interacts with proline rich domain of Gab1. Grb2 targets the Grb2/Gab1 complex to receptors through its SH2 domain (Wang *et al.*, 2015). As mentioned before, Grb2 also interacts with SOS1 through its

N-terminal SH3 domain to initiate Ras activation. Even though Grb2 contains two distinct non-overlapping SOS1 and Gab1 binding sites, the binding of SOS1 to N-terminal SH3 domain of Grb2 causes allosteric conformational changes and blocks the binding of Gab1 to the C-terminal SH3 domain and vice versa, leading to the formation of two distinct pools of Grb2/SOS1 and Grb2/Gab1 complexes (Mcdonald *et al.*, 2014). In addition, other downstream partners of Grb2 such as the Cbl ubiquitin ligase, which can bind to both C-terminal and N-terminal SH3 domains of Grb2 (Wong *et al.*, 2002), might also modulate its access to SOS1 and Gab1 in an allosteric and competitive manner (Mcdonald *et al.*, 2014).

In case of some receptors including FGFR, another tyrosine phosphorylated adaptor protein is required for recruitment of Grb2/Gab1 complex to the receptor. Upon receptor activation, Frs2 undergoes tyrosine phosphorylation and binds to Grb2, which recruits Gab1. In NGF Receptor (NGFR) signaling, Grb2/Gab1 complex is recruited to receptor via Shc. In case of some other receptors, the PH domain of Gab1 also plays a role in recruitment of Gab1 near activated receptors. For example, Gab1 is initially recruited to EGFR via Grb2 then it becomes tyrosine phosphorylated and interacts with PI3K, leading to generation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3). Interaction of the PH domain of Gab1 with PIP_3 results in its retention near the receptor and more sustained downstream signaling (Gu and Neel, 2003). Once recruited to the vicinity of RTK, Gab1 is tyrosine phosphorylated, as such provides docking sites for SH2 containing signaling molecules such as PLCγ, p85 subunit of PI3K, Shc, Shp2, RasGAP and Crk proteins (Rajala *et al.*, 2013).

Gab1/Shp2 and Gab1/PI3K interactions are thought to be required for ERK and Akt activation, respectively (Schaeper *et al.*, 2000). Gab1 was shown to contribute to FGF dependent ERK activation through Shp2 in chondrocytes (Krejci *et al.*, 2007). Disruption of this interaction results in apoptosis by simultaneous inhibition of Ras/ERK and PI3K/Akt pathways in adenocarcinoma cells (Wang *et al.*, 2018). Gab1 Y627 and Y659 phosphorylation results in binding of tyrosine phosphatase Shp2, this interaction relieves an allosteric inhibition and a strong Shp2 activation occurs. Shp2 was shown to dephosphorylate Gab1 on RasGAP binding sites resulting in the release of RasGAP from the complex hence, increases the Ras activation

(Montagner *et al.* 2005). EGF-induced Ras activation was shown to be diminished in mouse embryonic fibroblast cells expressing Gab1 mutant, which cannot bind Shp2 (Fedele *et al.* 2018). In most signaling pathways, Gab1/Shp2 interaction is not essential for Ras/ERK activation, but required for full Ras/ERK activation (Schaeper *et al.* 2000). Shp2 was also shown to regulate the extent and magnitude of Gab1/PI3K interaction by dephosphorylating the PI3K binding sites on Gab1 in response to some RTKs. It is important to mention that Shp2 depletion also decreases Grb2/Gab1 interaction and Shp2 was suggested to regulate (directly or indirectly) the activity of the kinase which is responsible for phosphorylation of Grb2 binding sites of Gab1 (Zhang *et al.*, 2002).

In the retinal context, Shp2 was proposed to mediate ERK signaling in Müller glia during postnatal development and adult tissue. Shp2 deletion was shown to specifically disrupt ERK phosphorylation resulting in cell death and degeneration, while Akt phosphorylation was not affected in the neural retina. Shp2 depletion was also proposed to compromise the capability of Müller cells to release trophic factors to protect neurons from environmental stress under normal conditions, which is resembling neuroprotective response of Müller cells (Cai *et al.*, 2011).

While site specific tyrosine phosphorylations of Gab1 is activating, serine/threonine phosphorylations is mostly associated with negative regulation of Gab1 signaling (Wöhrle *et al.*, 2009). Gab1 has 47 potential serine/threonine phosphorylation sites for numerous protein kinases such as PKA, PKC and MAPKs. Hyperphosphorylation of Gab1 on serine/threonine residues was proposed to result in a conformational change which conceals its tyrosine phosphorylation sites hence, prevents its interaction with partners (Gual *et al.*, 2001).

Two threonine and four serine residues (T312, S381, S454, T476, S581, S597) within consensus sequence of MAPK phosphorylation sites are associated with ERK, four of which (S454, T476, S581, S597) are located near canonical PI3K binding sites, and their phosphorylation by ERK results in reduced Akt activation in response to insulin and EGF (Lehr *et al.*, 2004). ERK was also shown to phosphorylate Gab1 on MBD domain upon Met activation, leading to increased Gab1/PI3K association (Gu and Neel, 2003). Yu *et al.* (2002) demonstrated

that ERK phosphorylation enhances HGF-induced Gab1/Shp2 interaction but inhibits EGF-induced Gab1/Shp2 interaction. They suggest that, since Gab1 regulates ERK and Akt activation, phosphorylation of Gab1 by ERK can create a positive or negative feedback loop depending on the type of receptor and growth factor. Previous studies in our laboratory shows that SIK2, which is activated upon ERK activation, can also phosphorylate Gab1 on S266 residue disrupting its interaction with Shp2 and Grb2, and this phosphorylation is proposed to create a negative feedback loop for FGF2-induced ERK pathway (Küser, 2006; Yılmaz-Sert, 2011).

1.7. Salt-Inducible Kinase Family

The salt-inducible kinases (SIKs) are serine/threonine kinases that belong to a sucrose-nonfermenting-1 protein kinase/AMP-activated protein kinase (SNF1/AMPK) super family. There are three SIKs identified in mammals (SIK1-3), all contain an N-terminal catalytic domain with activation loop, followed by a Ubiquitin-associated (UBA) domain. Liver Kinase B1 (LKB1) is an activating kinase of SIKs targeting the activation loop (Walkinshaw *et al.*, 2013). All SIK isoforms also contain C-terminal Protein Kinase A (PKA) phosphorylation sites adjacent to an arginine-lysine-rich (RK-rich) region. This RK-rich region acts as a non-canonical nuclear localization signal. Phosphorylation of SIKs by PKA was shown to disrupt its catalytic activity and cause nuclear export of the protein (Bright *et al.*, 2009; Katoh *et al.*, 2004; Sonntag *et al.*, 2018).

One of the best studied SIK substrates is c-AMP-regulated transcriptional co-activators (CRTCs). Phosphorylation of these proteins by SIKs is crucial for regulation of their localization and activity. CRTCs are sequestered in cytoplasm via interacting with cytoplasmic 14-3-3 chaperones upon phosphorylation by SIKs. cAMP can inhibit SIKs by PKA-mediated phosphorylation and results in dephosphorylation of the SIK substrates and their translocation to nucleus where they regulate gene expression (Wein *et al.*, 2018).

SIK1 was the first SIK protein identified in myocardium and isolated from adrenal glands of high-salt fed rat (Wang *et al.*, 1999). The other members of SIK family were discovered by sequence homology with SIK1 gene (Katoh *et al.*, 2004). SIK family proteins show their effects in a tissue specific manner. SIK1 is highly expressed in adrenal glands, brain, skeletal muscles, testes and in lower amounts in liver, hearth and adipose tissue. SIK2 is mainly expressed in metabolic tissues such as adipose tissue, liver and brain and SIK3 is expressed ubiquitously (Katoh *et al.*, 2004). SIK2 expression was also shown in vertebrate retina (Özmen, 2006; Küser *et al.*, 2013) and MIO-M1 cells in our laboratory (Küser, 2006).

1.7.1. Salt-Inducible Kinase 2

Salt-Inducible Kinase 2 (SIK2) was first identified in adipose tissue. Its expression was shown in 3T3-L1 cells at early stages of adipogenesis suggesting its importance in adipocyte differentiation. The first discovered substrate of SIK2 was IRS1, an important regulator of the insulin signaling pathway, which is phosphorylated on its S794 residue by SIK2 (Katoh *et al.*, 2004). Expression and activity of SIK2 were increased in the white adipose tissue of diabetic mice indicating involvement of SIK2 in type 2 diabetes development (Horike *et al.*, 2003).

SIK2, like all SIKs, contains an N-terminal kinase domain, UBA domain and RK-rich region (Katoh *et al.*, 2004). Horike *et al.* (2003) demonstrated that mutation of lysine 49 (K49) residue of SIK2 perturbed its kinase activity indicating K29 residue is critical for its kinase activity. LKB1 phosphorylate SIK2 at T175 in the kinase domain resulting in SIK2 activation (Berdeaux, 2011). While SIK1 and SIK3 have two PKA sites, SIK2 has four PKA phosphorylation sites (S343, S358, T484, S587), which serve as docking sites for cytoplasmic 14-3-3 chaperones upon phosphorylation. SIK2/14-3-3 association blocks the ability of SIK2 to phosphorylate its substrates (Sonntag *et al.*, 2018). In addition to LKB1 and PKA, Akt was also shown to directly phosphorylate SIK2 on S358 upon insulin stimulation (Berdeaux, 2011) (Figure 1.5).

Figure 1.5 Schematic representation of domain structure of mouse SIK2 (Berdeaux, 2011)

The cAMP-response element binding protein (CREB)-specific coactivator 2 (TORC2) was shown to be an SIK2 substrate in pancreatic islet and liver cells. Elevation of plasma glucose and gut hormone levels leads to inhibition of SIK2 by PKA activity resulting in hypo-phosphorylation of TORC2 and its translocation to the nucleus where it interacts with CREB to upregulate CREB-dependent gene expression such as insulin (Screaton *et al.*, 2004). In addition to gluconeogenesis, SIK2 is involved in liver lipogenesis and ketogenesis. Phosphorylation of p300 at inhibitory S89 residue by SIK2 represses acetylation of carbohydrate-responsive element-binding protein (ChREBP) and p300-PPARα interaction resulting in suppressed lipogenesis and ketogenesis, respectively (Bricambert *et al.*, 2010; Zhang *et al.*, 2016). SIK2 was also shown to restrict the formation of regulatory macrophages by reducing the production of anti-inflammatory IL-10 and increasing the production pro-inflammatory cytokines such as TNFα, IL-6 and IL-12 (Yang *et al.*, 2013; Wein *et al.*, 2018).

SIK2 is expressed in prostate cancer cells and its depletion inhibits cell growth and promotes cell cycle arrest and apoptosis (Bon *et al.*, 2015). SIK2 overexpression induces metastasis and its depletion prevents metastasis in ovarian cancer *in vivo* (Miranda *et al.*, 2016). SIK2 was also shown as a potential tumor suppressor in breast cancer via inhibition of the Ras/ERK and PI3K/Akt pathways concomitantly (Zohrap *et al.*, 2018). Küser-Abali *et al.*, (2013) demonstrated that SIK2 negatively modulates insulin-dependent Müller glia survival via Akt phosphorylation and contributes to hyperglycemic cell death *in vitro*.

Previously in our laboratory, a C-terminal fragment of SIK2 was obtained in a yeast two hybrid screening of a retinal cDNA library using the cytoplasmic domain of the FGFR2 as bait (Özcan, 2003). Full length SIK2 cDNA was then cloned from retinal tissue and the existence of

three alternatively spliced transcripts was shown. Even though there are three isoforms, they seem to encode only two different proteins with a small variance at their C-terminal (Uysal, 2005).

SIK2 expression was seen in all retinal layers (Küser-Abali *et al.*, 2013; Özmen, 2006). Its phosphorylation and activity were shown to change in an FGF2 dependent manner in MIO-M1 cell line (Candaş, 2007). Küser (2011) showed that SIK2 silencing increases FGF2-induced ERK activation as well as cell proliferation while its overexpression leads to a decrease in active ERK levels. ERK was identified as an upstream kinase of SIK2 indicating a possibility of a negative feedback loop in the pathway (Ejder, 2011). In addition, Gab1 was identified as an SIK2 substrate and S266 residue of Gab1 was shown as an SIK2 phosphorylation site (Küser, 2006; Yılmaz-Sert, 2011). This phosphorylation was shown to disrupt interaction of Gab1 with Grb2 and Shp2 (Yılmaz-Sert 2011) and SIK2 silencing increases tyrosine phosphorylation of Gab1 and its interaction with Grb2, Shp2 and PI3K (Küser 2011).

In the light of these findings, we propose that SIK2 is phosphorylated upon ERK activation via FGF2 induction and phosphorylates Gab1 at S266 decreasing Gab1 partner interactions, which results in a negative feedback loop for the Ras/ERK signaling. A schematic representation of the proposed model for involvement of SIK2 and Gab1 in the FGF-induced Ras/ERK pathway is given in Figure 1.6

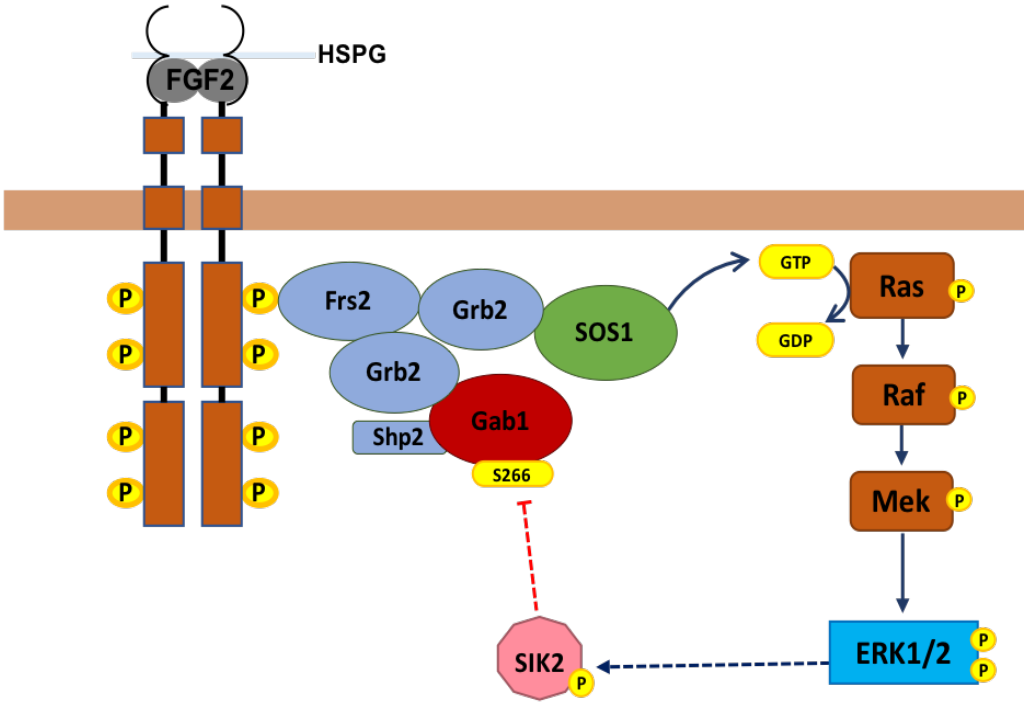

Figure 1.6 Proposed model for the role of SIK2 and Gab1

2. PURPOSE

Previous studies in our laboratory indicate that SIK2 is involved in a negative feedback mechanism of the FGF-induced ERK pathway resulting in reduced Müller cell proliferation. We propose that a rapid and transient SIK2 activation occurs as a result of FGF2-induced ERK activation. Activated SIK2 phosphorylates the docking protein Gab1 on Serine 266 (Figure 1.6) and hampers interaction of Gab1 with Shp2 and Grb2 leading to downregulation of the pathway, hence the proliferation (Küser, 2011; Yılmaz-Sert, 2011).

The aim of this project is to verify and to further study the involvement of Gab1 in this mechanism by investigating the effects of Gab1 and SIK2 modulations on ERK activation and proliferation in MIO-M1 cell line.

In this context,

- MIO-M1 cells lines where Gab1 and SIK2 where stably downregulated were created by shRNA approach.

- A stable MIO-M1 cell line expressing S266A mutant Gab1 was generated.

- ERK phosphorylation and proliferation profiles of these cell lines were investigated in response to FGF2-induction.

3. MATERIALS AND METHODS

3.1. Chemicals, Plastic and Glassware

All chemicals were purchased from Sigma-Aldrich (USA), AppliChem (USA), or Thermo Fisher Scientific (USA). Plastic products that are used in this study were obtained from TPP (Switzerland), Capp (Denmark) or Axygen (USA). All cell culture products were purchased from Invitrogen (USA) unless stated otherwise in the text. Glassware, tips and tubes and solutions were sterilized by autoclaving at 121 °C for 20 minutes.

3.2. Plasmid Isolation

Previously prepared and verified pCMV6-Entry Myc-DDK Gab1 containing bacterial colony (Yılmaz-Sert 2011) was grown overnight in LB medium containing neomycin at 37°C with shaking at 225 rpm. Plasmids were then isolated using MidiPrep kit (Qiagen, USA) as instructed by the manufacturer. Briefly, cells were lysed, and chromosomal DNA was denatured under strong alkaline conditions. Chromosomal DNA and cell debris were removed by centrifugation at 10000 g for 10 minutes. Supernatant was applied to the QIAprep spin columns where DNA binds to the silica membrane, impurities were removed by washing with wash buffer containing ethanol. The plasmid DNA was eluted with dH_2O, its concentration and purity were determined by measuring optical density at 260 nm with the NanoDrop Spectrophotometer. Plasmids were kept at -20°C for later use.

3.3. Cell Culture

Spontaneously immortalized human Müller glia cell line (MIO-M1) was provided by Professor Astrid Limb from Institute of Ophthalmology, University College London. Buffers and solutions used in cell culture are given in Table 3.1.

MIO-M1 cells were maintained in complete medium at 37°C under 5% CO_2. After they reached the confluence, the plates were washed with PBS then the cells were treated with Trypsin-EDTA solution for 5 minutes at 37°C with 5% CO_2 and scraped by using a plastic scraper. The cells were centrifuged at 500 g for 5 minutes and the pellet was resuspended in complete medium and split into three plates. For stocking, cells were resuspended in freezing medium and kept at -150°C until use.

Table 3.1. Chemical list used in this study

Complete Medium	Dulbecco's Modified Eagle Medium (DMEM) 10% Fetal Bovine Serum (FBS) 0.1% Penicillin/Streptomycin
Freezing Medium	70% DMEM 20% FBS 10% Dimethyl Sulfoxide (DMSO)
10X Trypsin-EDTA Solution	2.5% Trypsin 7mM Ethylenediaminetetraacetic Acid (EDTA) 0.9% NaCl diluted with Phosphate Buffered Saline (PBS)

For growth factor treatment, cells were seeded on 6-well cell culture plates and allowed to grow to about 70% confluence. The cells were washed with PBS and starved in DMEM containing 0.1% Penicillin/Streptomycin overnight, subsequently they were treated with 1 ng/ml FGF2 (Peprotech, UK) and 10 µg/ml heparin for varying times (0-60 minutes) at 37°C. At the end of the treatment period, cells were washed with PBS immediately, were lysed in 750 µl Laemli buffer (Table 3.2) per well and scraped with a plastic scraper. The cell lysates were transferred into centrifuge tubes and sonicated for 30 seconds at 40 kHz, the suspensions were boiled for 10 minutes at 95°C, centrifuged at 13000 rpm for 10 minutes. Lysates were either used directly or stored at -20°C for later use.

3.4. Generation of S266A-Gab1 MIO-M1 cell line with $CaPO_4$ Transfection

One day before transfection, $3x10^6$ cells were seeded on 10 cm culture dishes and allowed to grow overnight. Before transfection, medium was changed with fresh complete medium containing 25 μM chloroquine. Sixteen μg of pCMV6-Entry Myc-DDK Gab1 plasmid and 0.25 M $CaCl_2$ were mixed in dH_2O with a total volume of 400 μl then incubated for 5 minutes at room temperature (RT). Plasmid DNA mixture was added onto 400 μl 2X HBS buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.05) and gently mixed. The mixture was incubated at RT for 10 minutes to allow co-precipitation of DNA/calcium phosphate. Subsequently, the mixture was added onto the cells drop-wise and incubated in complete medium for 8 hours. The medium was replaced with fresh growth medium to remove chloroquine and incubated for 36 hours, split into 5 plates and allowed to grow overnight. Medium containing 750 μg/ml of Geneticin was replenished every two days. Individual colonies, once became visible, were selected using cloning cylinders and taken into 6-well plates. The cells were allowed to grow in geneticin containing medium for two weeks. The stably transfected clones were picked and S266A-Gab1 expression was confirmed by western blotting using cMyc-tag antibody.

3.5. Generation of SIK2 and Gab1 Silenced Stable Cell Lines

Lentiviral particles containing shSIK2 and shGab1 constructs and scrambled shRNA (Scr shRNA) particles were purchased from Santa Cruz Biotechnology (USA).

The cells were seeded in 10 cm plates ($2x10^5$ per plate) and allowed to grow overnight. The next day, cells were infected by adding 20 μl of shSIK2, shGab1 or Scr shRNA containing lentiviral particles and after 24 hours, the medium was replenished. To select stable clones, cells were split into 5 plates. Medium containing 1 μg/ml puromycin (Sigma, USA) was replenished every other day for 2 weeks. Puromycin resistant colonies were selected using cloning cylinders and grown in 10 cm plates. Downregulation was confirmed by western blotting with the appropriate antibody.

3.6. Protein Extraction and Bicinchoninic Acid (BCA) Assay

For protein extraction, cells were washed with PBS (Gibco Invitrogen, USA), incubated with Trypsin-EDTA solution for 5 minutes at 37°C then scraped and centrifuged at 500 g for 5 minutes at 4°C. Pellets were resuspended in ice-cold lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1mM EDTA, 1% Triton X-100) containing protease and phosphatase inhibitor cocktails. The suspension was incubated on ice for 30 min on a rocking platform and lysed using MagnaLyser homogenizor (Roche, Germany) with ceramic beads (Roche, Germany) at 6500 rpm for 30 seconds. Cell debris was removed by centrifugation at 13200 rpm at 4°C for 30 minutes. The supernatant containing the protein extract was taken into a fresh tube and kept at -20°C as cell lysate. The protein concentrations were measured by using Pierce BCA Protein Assay Kit (Thermo Scientific, USA).

Unknown samples and bovine serum albumin (BSA) dilutions ranging from 0.025 to 2 mg/ml were prepared in a 96-well plate and mixed with 200 µl of 50:1 diluted BCA Working Solution as instructed by the manufacturer. After 30 minutes of incubation at 37°C, absorbance measured at 595 nm using Plate Reader (VersaMax, Molecular Devices, USA). Unknown sample concentrations were extrapolated using the standard curve.

3.7. Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Compositions of the buffers and solutions used in western blotting are given in Table 3.2. Total protein extracts resolved on 10% or 8% polyacrylamide gels with 5% stacking gel. After BCA assay, the lysates were boiled in Laemli buffer at 95°C for 5 minutes, then loaded to the gels in appropriate concentrations depending on the experiment. The cells seeded in 6-well plates were directly collected and lysed in 2X Laemli buffer, boiled at 95°C for 10 minutes and centrifuged at 13000 rpm for 10 minutes prior to the loading. The gels were run at 80-110 V. PageRuler™ Prestained Protein Ladder (Thermo Scientific, USA) was used to determine sizes of proteins.

The samples resolved on polyacrylamide gels were electro-blotted to Polyvinyl Difluoride (PVDF) membranes (Roche, Germany). The transfer was performed at 100 V in transfer buffer at 4°C while stirring constantly for a time period required for protein to be transferred depending on the size of protein (1 minute per 1 kDa).

After the transfer, for equilibration, PVDF membranes were washed three times for 5 minutes each with TBST. Then, they were blocked on a rocking platform for 1 hour at RT in 5% skimmed milk powder (MP) in TBST (Sigma-Aldrich, USA). After blocking, the membranes were washed with TBST to remove excess milk powder solution, incubated with the appropriate primary antibody solution on a rocking platform at 4°C overnight. Antibody dilutions and compositions of the solutions are given in Table 3.3. Subsequently, membranes were washed three times for 5 minutes each with TBST to remove unbound antibodies. The membranes were incubated with appropriate HRP-conjugated secondary antibody for one hour at RT, followed by washing in TBST.

ImmunoCruz Western Blotting Reagent (Santa Cruz, USA) or SuperSignal West Femto Maximum Sensitivity Substrate (Santa Cruz, USA) was used for band detection. Appropriate amount of luminol mixture was added on the membranes and they were visualized on the G-Box SynGene imaging system.

For stripping, the membranes were incubated with stripping solution (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.7% β-mercaptoethanol) at 55°C for 30 minutes and washed three times for 5 minutes each with TBST. Then blocked and incubated with primary antibody as described above.

Table 3.2. Buffers and Solutions used for Western Blotting

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

Table 3.3. Antibodies used in this study

Antibody	Host	Application	Dilution	Blocking Solution	Company
SIK2	Rabbit	WB	1/1000	5% BSA in TBST	Cell Signaling
β-Actin-HRP	Mouse	WB	1/1000	5% BSA in TBST	Santa Cruz
Gab1	Rabbit	WB	1/1000	5% BSA in TBST	Santa Cruz
Myc-Tag	Rabbit	WB	1/1000	5% BSA in TBST	Cell Signaling
Anti-rabbit HRP	Mouse	WB	1/5000	1% MP in TBST	Santa Cruz
Anti-BrdU	Mouse	ICC	1/500	3% BSA in PBST	Roche
Alexa Fluor 488	Mouse	ICC	1/1000	3% BSA in PBST	Invitrogen

3.8. Cell Proliferation Assay

Prior to proliferation assay, cells were seeded on 24 well plates with round coverslips and treated with 1 ng/ml FGF2 and 10 μg/ml heparin for 24 hours as described before. BrdU powder (Roche, Germany) was dissolved in dH_2O. The BrdU solution (with a final concentration of 10 μM/ml) was added to the culture medium 5 hours before fixation with 4% PFA in PBS for 10 minutes. Following the fixation, the cells were treated with 4 M HCl for 15 minutes to denature DNA, washed in PBS containing 0.1% Tween-20 (PBST) to bring the pH of the medium above 6.5. The cells were incubated in blocking solution (3% BSA in PBST) for 1 hour at RT. After this point, the experiment was carried out in a dark environment. At the end of the blocking, the cells were incubated in BrdU antibody dilution in the blocking solution overnight at 4°C. After washing with PBST three times for 5 minutes each, the cells were incubated in Alexa Fluor 488 conjugated secondary antibody for 1 hour at RT. To visualize the nuclei, the cells were incubated with DAPI for 5 minutes, washed with PBS three times for 5 minutes each and observed under Axio Observer Z1 Inverted Microscope (Zeiss, USA), at least 500 cells were counted in randomly selected areas.

4. RESULTS

Previous work from our laboratory suggests that SIK2 is involved in a negative feedback regulation of the FGF-induced ERK pathway leading to reduced proliferation in Müller cell context (Küser 2011). We propose that FGF2-induced ERK activation results in rapid and transient activation of SIK2, in turn SIK2 phosphorylates Gab1 on Serine 266 leading to the downregulation of the cascade (Küser 2011; Yılmaz-Sert 2011). To verify these earlier findings and to provide further support for our hypothesis, we generated SIK2 and Gab1 silenced MIO-M1 cell lines, as well as a line that expresses S266A mutant Gab1. These cell lines were induced with FGF2 and the effect of the modulations on ERK activation profile and MIO-M1 proliferation were investigated.

4.1. Generation of Stable SIK2, Gab1 downregulated and S266A mutant Gab1 expressing MIO-M1 cell lines

MIO-M1 cells were transduced with shSIK2, shGab1 or Scr shRNA containing lentiviral particles and the stably transduced cells were puromycin selected. The antibiotic resistant single cells were propagated and analyzed for downregulation of SIK2 or Gab1 by western blotting, where anti-β-actin was used as the loading control.

There was a significant decrease in SIK2 protein expression in shSIK2 C18 and shSIK2 C19 clones (in the order of 60% and 40%, respectively) as shown in Figure 4.1a. Gab1 protein levels were not affected in these clones (data not shown).

Gab1 expression was downregulated in the order of 55% in shGab1 C5 and 32% in shGab1 C6 (Figure 4.1b), hence these clones were used in the later studies. SIK2 protein levels remained unaffected in these clones (data not shown).

Transduction with Scr shRNA caused no significant change of neither in SIK2 nor Gab1 levels compared to wild type MIO-M1 cells (Figure 4.1).

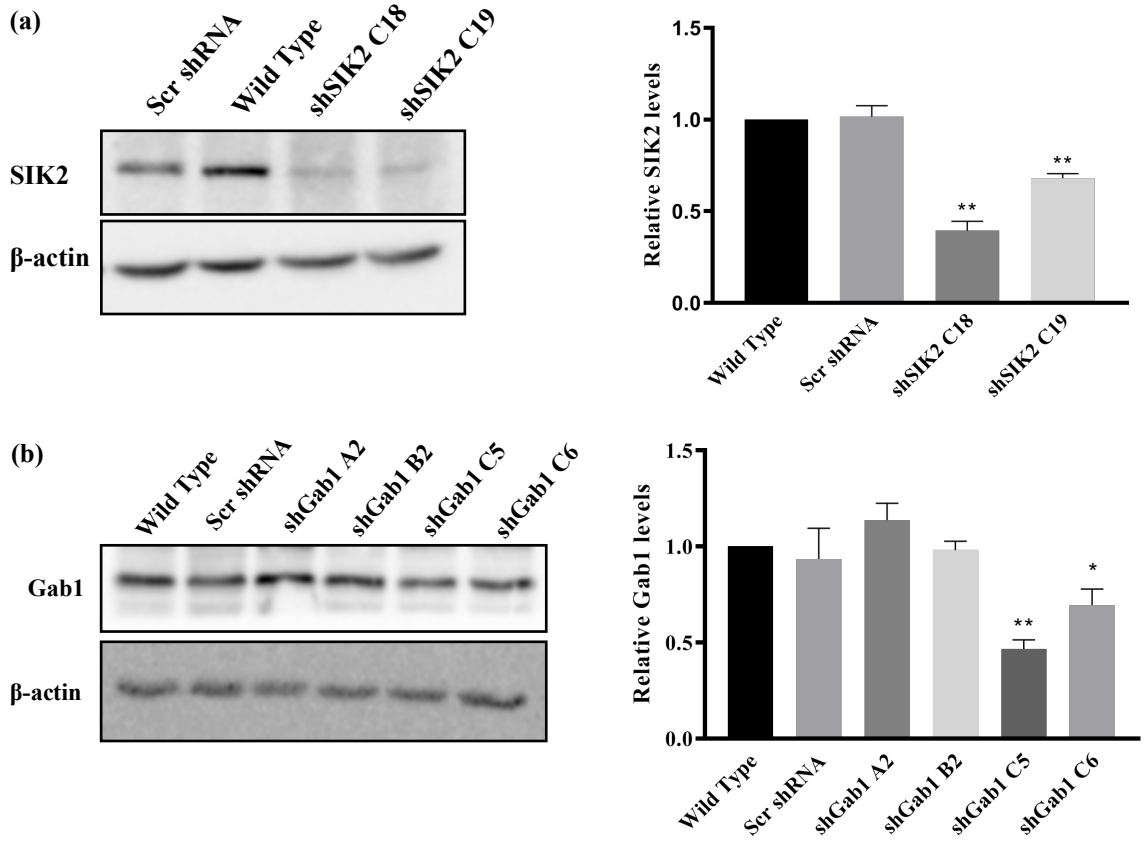

Figure 4.1 Downregulation of SIK2 and Gab1 expression. Protein extracts were prepared from MIO-M1 cells transduced with viral particles carrying scrambled or gene specific shRNA sequences. Silencing was verified by immunoblotting with anti-SIK2 and anti-Gab1 *P< 0.05 ** P < 0.01 compared to the scrambled control and wild type.

S266A Gab1 mutant was expressed as cMyc-Gab1 fusion protein. The tag is 1.2 kDa, thus the expected size of the fusion protein has similar apparent molecular weight, around 100 kDa, as the endogenous Gab1. The selection of the successful transformants were based on the detection of 100 kDa band with anti-c-Myc Tag on western blots, where no counterpart was

detectable in the wild type cells. The screening indicated that the two of the colonies express the fusion protein. When these membranes were stripped and probed with anti-Gab1 antibody, endogenous 100 kDa Gab1 protein expression were evident in all samples. Endogenous Myc protein, 55 kDa, was used as loading control (Figure 4.2).

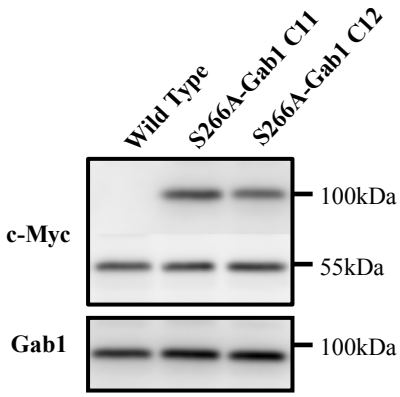

Figure 4.2 c-Myc-Gab1 fusion protein expression in vector transfected and untransfected MIO-M1 cells. Protein extracts were prepared from wild type or S266A mutant Gab1 transfected MIO-M1 cells and immunoblotted with anti-c-Myc Tag and anti-Gab1 antibodies.

4.2. FGF2-induced ERK Phosphorylation and Proliferation in wild type and Scr shRNA transduced MIO-M1 cell lines

When wild type MIO-M1 cells were exposed to FGF2, within 10 minutes of induction pERK levels increased 1.78-fold, then gradually dropped to the 0.53-fold of the basal levels at 60 minutes post induction (Figure 4.3). These results are consistent with the previous results from our laboratory (Cinaroglu *et al.* 2005; Küser 2011). Three time points were chosen for the further studies: 0 (uninduced/basal), 10 and 60 minutes of induction, per highest and the lowest pERK levels.

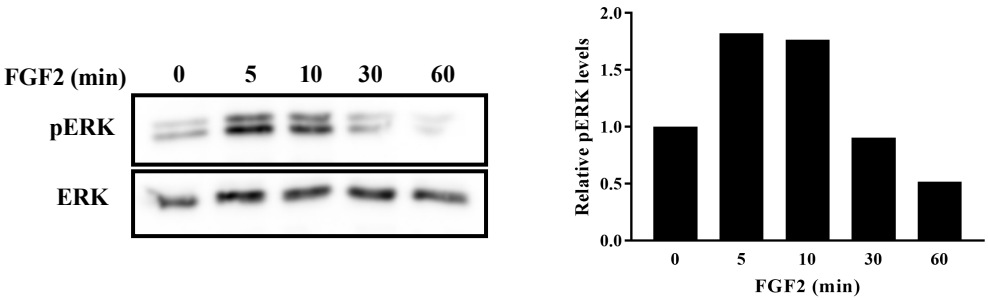

Figure 4.3 FGF2-dependent ERK phosphorylation profile in MIO-M1 cells. Serum starved MIO-M1 cells were treated with FGF2 for the indicated durations. Western blot analysis was performed by using anti-pERK and anti-ERK antibodies.

In both wild type and the Scr shRNA transduced MIO-M1 cells, 10 min FGF2 induction led to about a 1.75-fold increase in pERK levels, which then dropped to around 0.56-fold of the untreated samples (Figure 4.4).

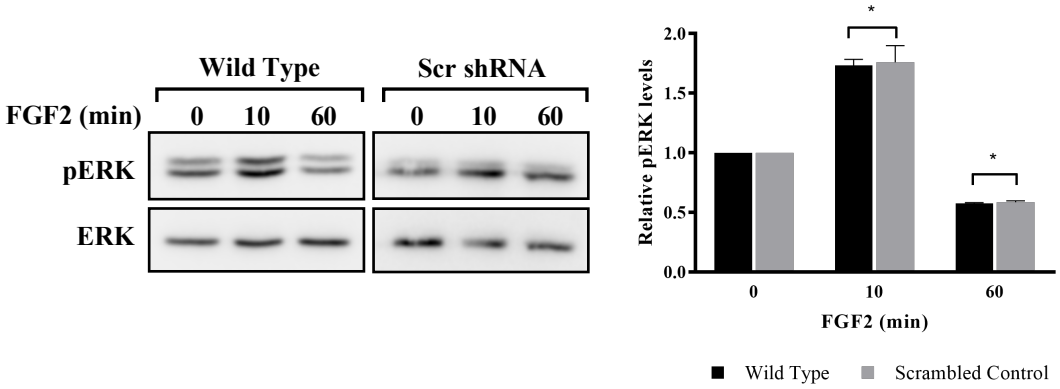

Figure 4.4 FGF2-induced ERK activation in wild type and Scr shRNA carrying cells. Western blot analyses were carried out using anti-pERK and anti-ERK antibodies. Graphic representation of relative pERK band intensities normalized to that of ERK in the same sample shown in the right panel. *P >0.1

Similarly, 24 hours of FGF2 treatment resulted in a 2-fold increase in the fraction of proliferating cells (Figure 4.5) in both wild type and the scrambled control cells.

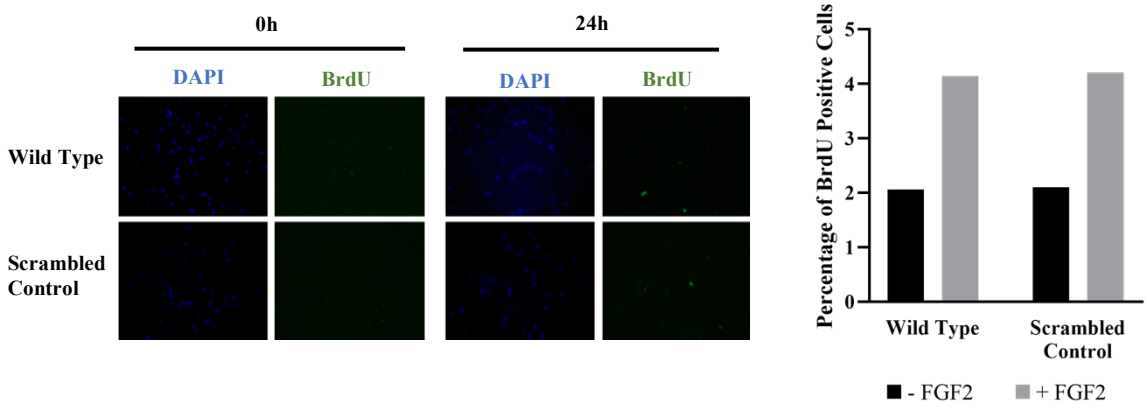

Figure 4.5 FGF2-induced proliferation profile of the wild type and Scr shRNA transduced MIO-M1 cells. Representative images of proliferating cells detected with BrdU incorporation (green) and cell nuclei stained with DAPI (blue). Graphic representation of the number of the BrdU-positive cells normalized to that of DAPI stained nuclei shown in the right panel.

Since wild type and Scr shRNA transduced cells showed no significant difference in FGF2-induced ERK phosphorylation and proliferation profiles, the latter was (the Scr shRNA transduced cells) used as the control line in the experiments involving downregulation.

4.3. Studies using MIO-M1 Cells with Downregulated SIK2 Expression

4.3.1. Effect of SIK2 Silencing on FGF2-induced ERK Phosphorylation

In both the scrambled control and SIK2 silenced cells, pERK levels increased about 1.8-fold after 10 minutes of FGF2 treatment (Figure 4.6). At the end of 60 minutes of treatment, pERK levels dropped to around 0.56-fold of basal in the scrambled control sample, in shSIK2 C18 the reduction reached only to the basal levels. In shSIK2 C19 cells, active ERK levels were slightly below basal but still about 1.5-fold higher than the Scr shRNA controls. Thus, there was

a significant difference between pERK levels of shSIK2 C18 and C19, which show 60% and 40% SIK2 downregulation, respectively. These results suggest that SIK2 silencing delays attenuation of ERK activation in a concentration dependent manner.

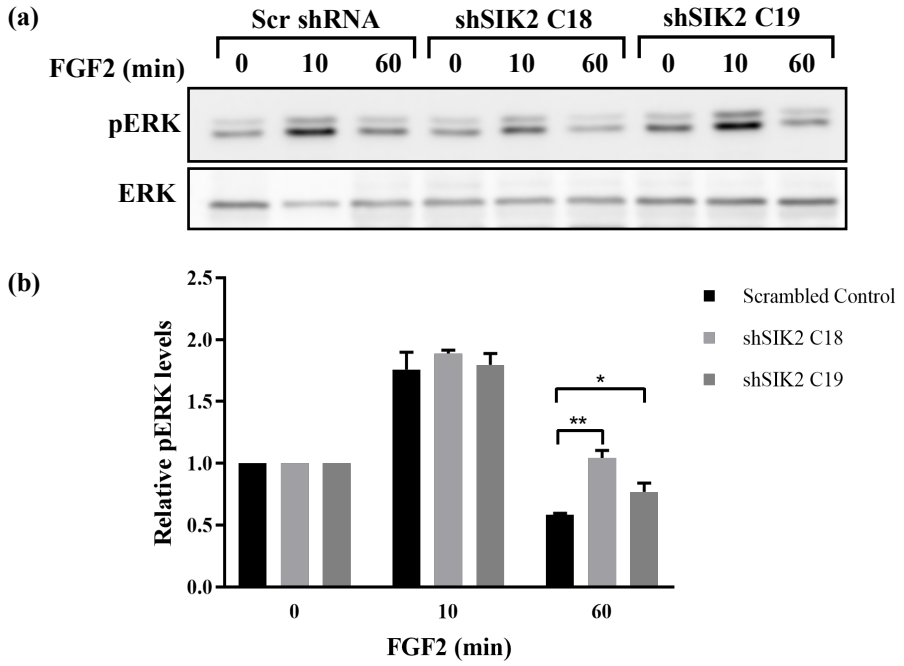

Figure 4.6 FGF2-induced ERK phosphorylation levels in SIK2 downregulated and the scrambled control cells. (a) Western blot analysis was performed with anti-pERK and anti-ERK was used as the loading control. (b) Graphic representation of relative pERK band intensities normalized to that of ERK band intensities. *P < 0.05, **P<0.01

4.3.2. Effect of SIK2 Silencing on FGF2-induced Cell Proliferation

The number of proliferating cells in SIK2 silenced clones was 2-fold of the scrambled control even in the absence of FGF2. When both cell lines were exposed to FGF2, the number of proliferating cells increased 2-fold in the scrambled control and 4.5-fold in SIK2 silenced clones compared to uninduced state (Figure 4.7).

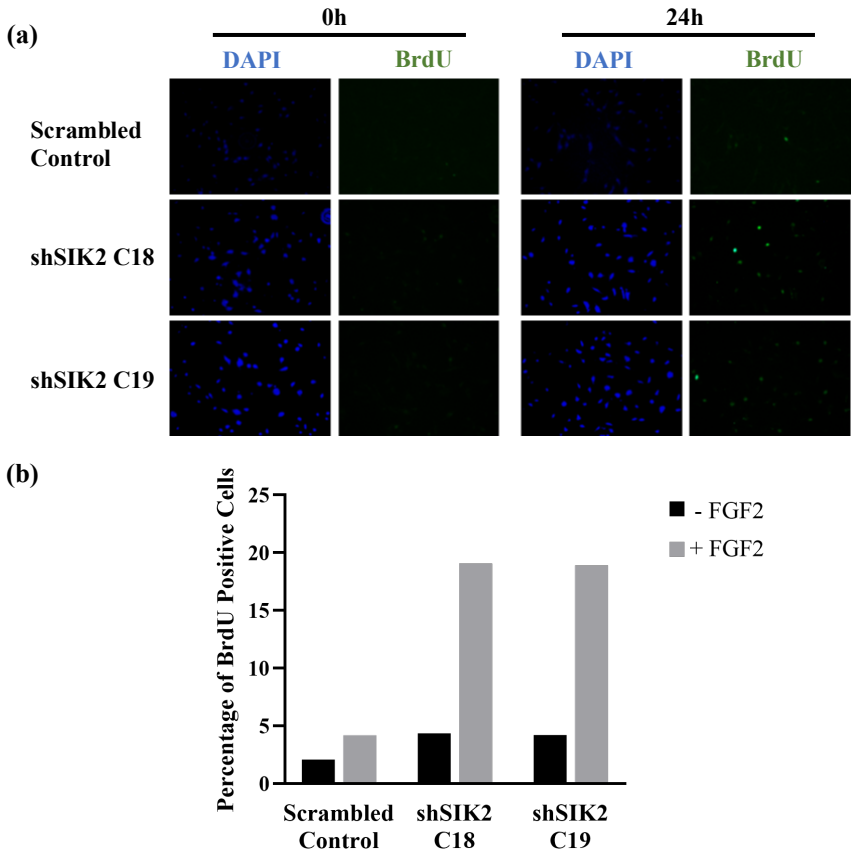

Figure 4.7 FGF2-induced proliferation profile of the Scr shRNA or shSIK2 transduced MIO-M1 cells. (a) Representative images of proliferating cells detected with BrdU incorporation (green) and cell nuclei stained with DAPI (blue) (b) Graphic representation of the number of the BrdU-positive cells normalized to that of DAPI stained nuclei in the same samples.

This data shows that SIK2 silencing led to an increase in proliferation both FGF2 induced and uninduced cases compared to the control and suggests that SIK2 has a negative effect on MIO-M1 cell proliferation.

4.4. Studies using MIO-M1 Cells Expressing S266A Mutated Gab1

4.4.1. Effect of S266A mutation on FGF2-induced ERK Phosphorylation

When wild type and S266A-Gab1 expressing cells were treated with FGF2 for 10 minutes, pERK levels increased about 1.7-fold. Active ERK levels in S266A-Gab1 expressing cells failed to drop below basal level and remained about 2-fold higher than wild type at the end of 60 minutes of FGF2 exposure (Figure 4.8).

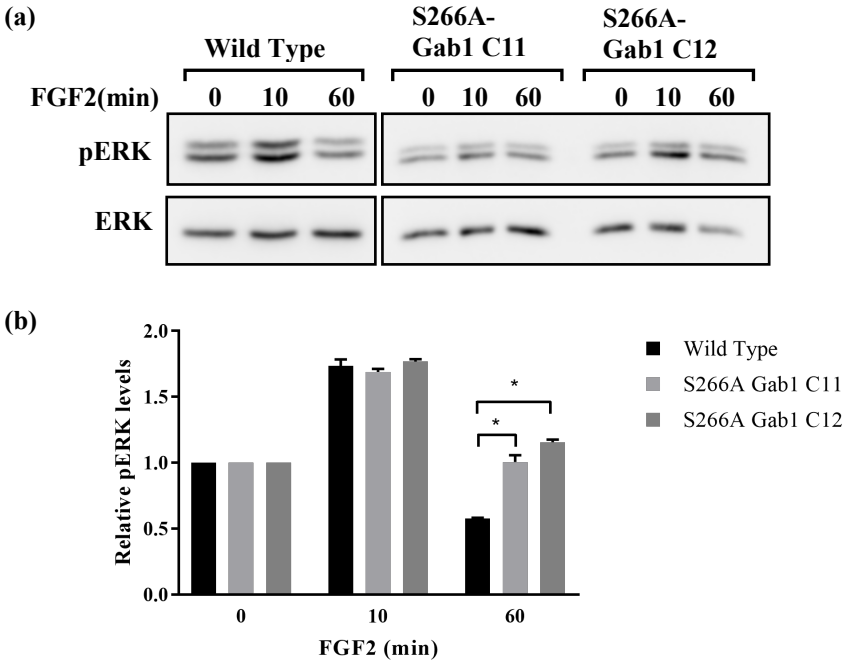

Figure 4.8 FGF2-induced ERK phosphorylation levels in S266A mutant Gab1 carrying and wild type MIO-M1 cells. (a) Protein extracts were subjected to western blot analysis using anti-pERK and anti-ERK antibodies. (b) Graphic representation of relative pERK band intensities normalized to that of ERK in the same samples. *P<0.01

In agreement with our hypothesis, SIK2 silencing and S266A mutation of Gab1 appear to reduce attenuation of ERK phosphorylation in the MIO-M1 cell line.

4.4.2. Effect of S266A mutation on FGF2-induced Cell Proliferation

In the uninduced state, the number of proliferating cells in both of the S266A-Gab1 transfected clones was 2-fold of the wild type (Figure 4.9). At the end of the treatment period, the number of proliferating cells increased 2-fold in wild type cells, this increase was about 4-fold in the S266A mutant Gab1 carrying clones.

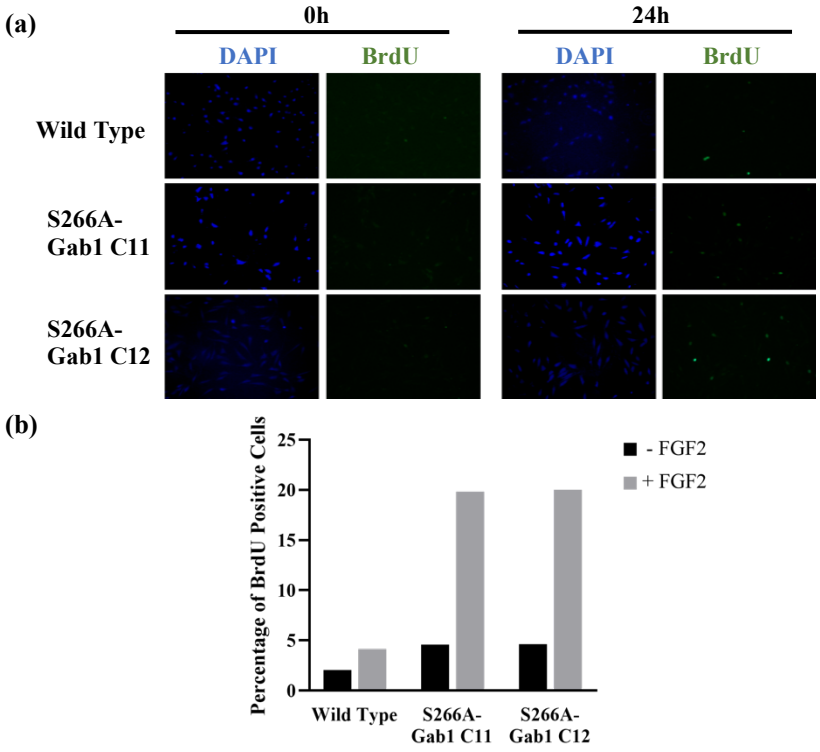

Figure 4.9 FGF2-induced proliferation profile of the wild type and S266A-Gab1 transfected MIO-M1 cells. The cells were treated with FGF2 for 24 hours. (a) Proliferating cells detected with BrdU incorporation (green) and cell nuclei stained with DAPI (blue) (b) Representation of the number of the BrdU-positive cells normalized to that of DAPI stained nuclei.

The results suggest that similar to SIK2 silencing, S266A mutation of Gab1 negatively affects the proliferation of MIO-M1 cells. It is conceivable that phosphorylation of Gab1 at S266 by SIK2 is an important factor in negative regulation of FGF2-induced proliferation.

4.5. Studies with Gab1 silenced MIO-M1 Cells

4.5.1. Effect of Gab1 Downregulation on FGF2-induced ERK Phosphorylation

In the scrambled control cells, pERK levels increased about 1.77-fold at 10 minutes and dropped to around 0.6-fold of basal levels at 60 minutes of induction. However, in shGab1 C5 cells with 55% downregulation of Gab1 expression, pERK levels increased about 4-fold at 10 minutes post induction and returned to basal levels at 60 minutes of treatment (Figure 4.10). In case of shGab1 C6, where Gab1 expression was reduced 32%, there was about 2-fold increase in pERK levels after 10 minutes of treatment. pERK levels dropped to around 0.6-fold of basal similar to the scrambled control after 60 minutes of treatment.

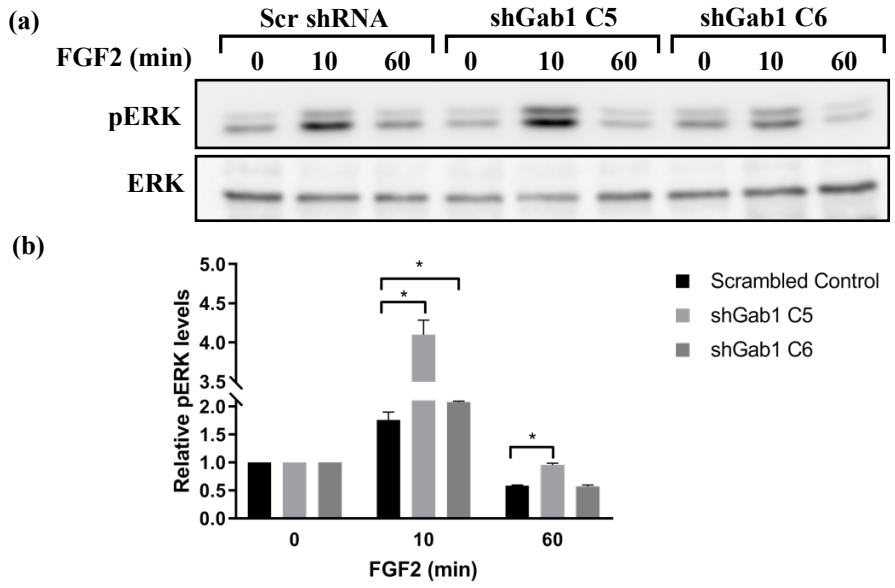

Figure 4.10 FGF2-induced ERK phosphorylation levels in Gab1 downregulated and Scr shRNA transduced control cells. Protein extracts were subjected to western blot analysis using anti-pERK and anti-ERK antibodies. *P<0.01

Considering downregulation levels of Gab1 expression in these two clones, the silencing of Gab1 increased FGF2-induced ERK activation in a concentration dependent manner.

4.5.2. Effect of Gab1 Downregulation on FGF2-induced Cell Proliferation

Without FGF2 induction, the number of BrdU positive cells was 2% of the total cell population in both Gab1 silenced and the scrambled control cells. After FGF2 induction, the number of BrdU incorporated cells increased 2-fold in the scrambled control cells, this increase was 3.5-fold in the Gab1 silenced clones (Figure 4.11).

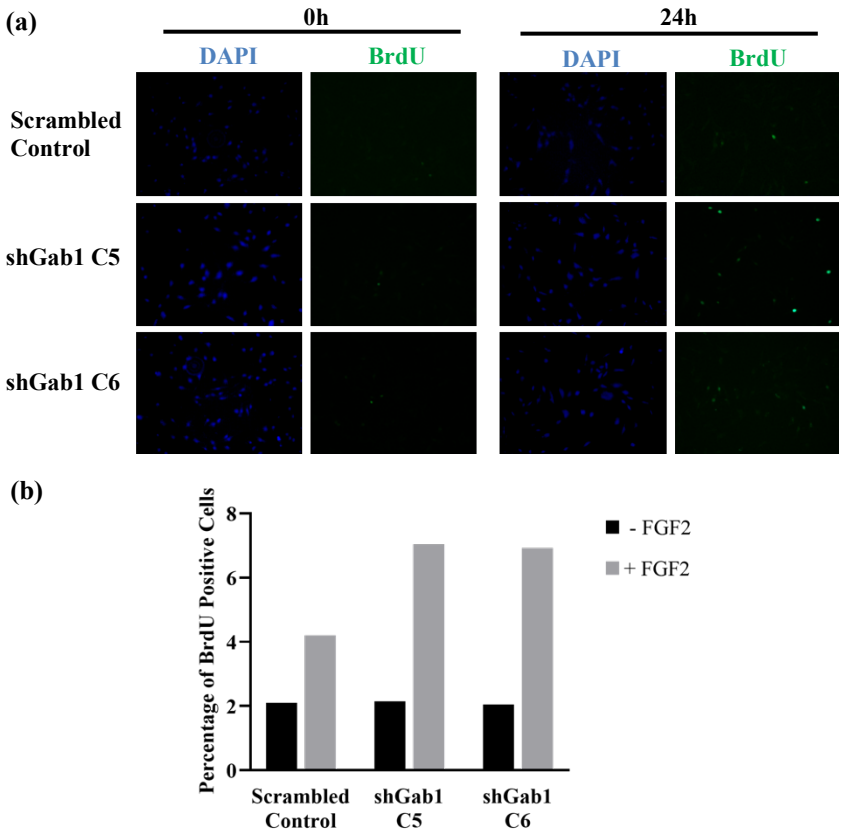

Figure 4.11 FGF2-induced proliferation profile of the Scr shRNA or shGab1 transduced MIO-M1 cells. (a) Representative images of proliferating cells detected with BrdU incorporation (green) and cell nuclei stained with DAPI (blue) (b) Graphic representation of the number of the BrdU-positive cells normalized to that of DAPI stained nuclei in the same samples.

Taken together, ERK activation and the proliferation profiles of SIK2 and Gab1 modulated and the control MIO-M1 cells lend support to the negative regulatory role of SIK2 on FGF2-induced Müller cell proliferation via regulation of ERK pathway through S266 phosphorylation of Gab1.

5. DISCUSSION

Müller cells are the main glial cells of the retina which maintain tissue homeostasis, and provide support to survival and functioning of retinal neurons (Goldman, 2014). In response to most retinal injuries and diseases Müller cells undergo a series of changes collectively referred as glial reactivation (Bringmann *et al.*, 2010). In zebrafish, upon injury a group of activated Müller glia transdifferentiate to progenitor cells that regenerate all major retinal cells and restore vision (Goldman, 2014). Injury-induced proliferation of Müller glia and small amount of regeneration also occurs in postnatal chicks (Fischer and Reh, 2003). In mammals, under pathological conditions, Müller cells undergo reactive gliosis, which results in proliferation and expression of genes associated with retinal stem cells, however no *in situ* neurogenesis has been observed (Hamon *et al.*, 2016). Müller cell proliferation, dedifferentiation, and limited amount of regeneration can be stimulated by exogenous growth factors such as FGFs in rodent species *in vivo* and *ex vivo* (Karl *et al.*, 2008; Löffler *et al.*, 2015). FGF2 is one of the factors that can stimulate the Ras/ERK pathway, which is required for Müller cell proliferation and transdifferentiation (Fischer *et al.*, 2009; Geller *et al.*, 2001; Lawrence *et al.*, 2007). Further analysis of FGF2-induced Ras/ERK pathway and its regulation in Müller cells is crucial for understanding its role in retinal regeneration and may lead to discoveries of novel targets for therapeutic use.

The Ras/ERK pathway is strictly regulated by positive signals and the counter acting attenuation mechanisms (Brightman and Fell 2000; Katz *et al.*, 2007). The findings from our laboratory suggests that in Müller cell context SIK2 negatively regulates FGF2-induced ERK activation and cell proliferation via the docking protein Gab1 (Küser, 2011). We propose that FGF2-induced ERK activation results in SIK2 phosphorylation, in turn active SIK2 phosphorylates Gab1 at S266 creating a negative feedback loop for the pathway, leading to the downregulation of MIO-M1 cell proliferation (Ejder, 2011; Küser, 2006; Yılmaz-Sert, 2011). Though the role of Gab1 in this pathway is yet not well understood, Gab1/Shp2 interaction is

thought to be required for full ERK1/2 activation (Schaeper *et al.*, 2000). Disruption of this interaction by serine/threonine phosphorylation of Gab1 was reported to sequester its tyrosine phosphorylation sites, and lead to the downregulation of EGF-induced ERK activation (Yu *et al.*, 2002). Consistent with these reports, Gab1 phosphorylation at S266 by SIK2 shown to disrupt Gab1/Shp2 and Grb2/Gab1 interaction (Küser, 2006; Yılmaz-Sert, 2011), and SIK2 silencing was shown to increase tyrosine phosphorylation of Gab1 and its interaction with Shp2 and Grb2 (Küser, 2011). In this study we extended these findings to further investigate the role of Gab1 in our proposed mechanism.

In the first part of the study, we confirmed the previous results on FGF2-induced ERK activation profile of MIO-M1 cells without any modulation of SIK2 and Gab1 expression, and determined the working time-points for later experiments. Subsequently, the effects of SIK2 and Gab1 silencing on the ERK activation profile were investigated by using shSIK2, shGab1 or Scr shRNA transduced MIO-M1 cell lines. To understand the role of S266 phosphorylation of Gab1 by SIK2, S266A mutant Gab1 transfected stable clones were generated and analyzed under the same experimental setup. Along with ERK activation, the effects of these modulations on FGF2-induced MIO-M1 proliferation profile were studied.

FGF2 induction of wild type MIO-M1 cells resulted in a rapid and transient ERK activation. We have seen that active ERK levels peaked at 10 min. and dropped below basal at 60 min. post induction, consistent with previous data from our laboratory (Çınaroğlu, 2005; Küser, 2011). FGF2-induced Ras/ERK pathway activation is considered as the main proliferation pathway in various cell lines (Yun *et al.*, 2010), including MIO-M1 cell line (Hollborn *et al.*, 2004). In agreement with these studies and previous data from our laboratory (Küser, 2011; Yılmaz-Sert, 2011), we have seen that transient FGF2-induced ERK activation results in proliferation in MIO-M1 cells.

Next, we confirmed the negative regulatory effect of SIK2 on FGF2-induced ERK activation and proliferation using cells expressing shSIK2. SIK2 silencing led to no significant difference at 10 min. of treatment compared to the scrambled control, but at 60 min. of treatment

pERK levels were significantly higher in SIK2 silenced cells than the scrambled control. These results indicate that SIK2 does not affect the amplitude of initial ERK phosphorylation but decreases the duration of ERK activation after initiation of the signaling. As expected from the ERK activation data, SIK2 silencing led to a dramatic increase in basal and FGF2-induced cell proliferation compared to the scrambled control. In agreement with Küser (2011), these data suggests that SIK2 has an inhibitory effect on FGF2-induced MIO-M1 cell proliferation through regulation of ERK signaling.

As mentioned above, SIK2 was shown to phosphorylate Gab1 at its S266 residue (Küser, 2006; Yılmaz-Sert, 2011). Yılmaz-Sert (2011) studied the effect of S266 phosphorylation on cell proliferation by using a S266A mutant Gab1 transfected MIO-M1 cells, but ERK activation was not studied in these cells. Here, we investigated the effect of S266A mutation on ERK activation for the first time by using S266A mutant Gab1 expressing MIO-M1 cell lines. S266A mutation did not affect the peak of ERK phosphorylation at 10 min. of FGF2 induction but pERK levels remained significantly higher than wild type after 60 min. of induction. Similarity of the ERK activation profile in S266A mutant and SIK2 silenced cells suggests that S266 phosphorylation by SIK2 is required for ERK attenuation. Previous proliferation studies in our laboratory conducted on MIO-M1 cells transiently transfected with S266A mutant Gab1 carrying vector was inconclusive (Yılmaz-Sert, 2011). Although our proliferation experiments lack statistical analysis, S266A mutation led to an increase in proliferation with respect to wild type in uninduced and FGF2-induced cells, reminiscent of the proliferation data obtained from SIK2 silenced cells. These findings together with SIK2 silencing data support the hypothesis that SIK2 negatively regulates FGF2-induced Ras/ERK pathway by phosphorylating Gab1 at S266, therefore reduces cell proliferation.

In the last part of the study, we examined effect of Gab1 silencing on FGF2-induced ERK activation. Because SIK2 negatively regulates the pathway through Gab1, we would expect Gab1 silencing to inhibit ERK activation. Surprisingly, Gab1 silencing led to an increase in ERK activation compared to the scrambled control at the first 10 min. of induction, suggesting that Gab1 may have a role in suppressing overactivation of ERK in the initial phase of FGF2-

induced ERK activation. Since Gab1 was shown to inhibit Ras activity by binding to RasGAP (Montagner *et al.*, 2005), it is possible that in the absence of Gab1, sustained Ras activation leads to ERK overactivation. This can also explain why ERK activation in 55% Gab1 downregulated cells was significantly higher than 32% Gab1 downregulated cells. SIK2 silencing and S266A mutation data is supporting this idea. Gab1/Shp2 interaction is required to extent the duration of ERK activation after the initiation phase by releasing RasGAP from Gab1 (Kiyatkin *et al.*, 2006) and the strongest Gab1/Shp2 interaction was seen between 10 and 60 min. of FGF2 induction (Krejci *et al.*, 2007). Therefore, it is conceivable that the negative effect of S266 phosphorylation by SIK2 comes into play at this phase. Studies showing that expression of Gab1 mutant which cannot bind Shp2, diminishes Ras activation, also support our hypothesis (Fedele *et al.*, 2018).

Another answer to these puzzling results may stem from the changes in the interaction of Grb2 with Gab1 and SOS1. Küser (2011) showed that the highest level of interaction between Grb2 and Gab1 occurs at 10 min. of FGF2 induction and this interaction decreases between 10 and 60 min. of induction. Although Grb2 contains two different binding sites for Gab1 and SOS1, the binding of one was shown to restrict access to the other, resulting in the formation of two distinct pools of Grb2/Gab1 and Grb2/SOS1 complexes (Mcdonald *et al.*, 2014). When Gab1 expression is downregulated, this may increase the number of Grb2/SOS1 complexes at 10 min. of treatment leading to overactivation of ERK. The interaction of Grb2 with SOS1 in these cells should be studied to verify this.

Interestingly, the active ERK levels significantly decreased at 60 min. of induction in both Gab1 silenced clones. As the ERK activation profiles at 10 min. of induction in Gab1 silenced cells indicate a suppressor role for Gab1, we did not expect to see this decrease at 60 min. However, active ERK levels decreased 4-fold from 10 min. to 60 min. in both Gab1 silenced clones and the scrambled control, suggesting that Gab1 silencing does not alter the duration of the signaling but only increases the amplitude of initial ERK activation. These results led us to consider the possibility of a temporal switch in the role of Gab1 partner interactions during the course of the Ras/ERK signaling. It is possible that Gab1 is playing a negative

regulatory role on ERK activation in the first 10 min. of FGF2 induction by binding to RasGAP and inhibiting Ras activation, but between 10 and 60 min of induction it may positively affect the ERK activation by binding and activating Shp2, a known amplifier of ERK activation (Araki *et al.*, 2003; Bunda *et al.*, 2015). Interaction of Shp2 with docking proteins such as Gab1 and IRS via its SH2 domain is shown to be necessary for its catalytic activity (Fedele *et al.* 2018; Tsutsumi *et al.*, 2018). Therefore, it would be intriguing to test activation profile of Shp2 and its interaction with binding partners such as Grb2, SOS1 and Ras in Gab1 silenced cells.

Interestingly, Cbl ubiquitin ligase, which functions in late phase of ERK attenuation, was shown to compete with SOS1 and Gab1 for Grb2 binding (Dikic and Giordano, 2003). Although downregulation of Gab1 might increase the number of Grb2/SOS1 complexes, Cbl may sequester Grb2 preventing its interaction with SOS1 hence, inhibits Ras activation, leading to ERK attenuation (Dikic and Giordano, 2003). Grb2/Cbl interaction can also explain the negative effect of S266 phosphorylation of Gab1 by SIK2 between 10 and 60 min. of FGF2 induction. Because this phosphorylation disrupts Grb2/Gab1 interaction (Küser, 2011; Yılmaz-Sert, 2011), it would increase the number of Grb2 proteins that can bind Cbl, resulting in ubiquitination and degradation of FGFR and Frs2 (Ornitz and Itoh, 2015). Therefore, studying Grb2/Cbl interaction profile between 10 and 60 min. of FGF2 treatment in our cell lines is important to confirm these postulations.

Another explanation for these results could be the presence of other compensatory mechanisms that are responsible for the attenuation of ERK phosphorylation. For example, Spry proteins function by either inhibiting Grb2/SOS1 complex formation or directly binding to Raf to block ERK activation and their maximum inhibitory activity was shown to be between 15 and 60 min. of FGF induction (Hanafusa *et al.*, 2002; Turner and Grose, 2010), which can explain the decrease in ERK activation in Gab1 silenced cells at 60 min. of FGF2 treatment.

Next, we examined the effect of Gab1 on proliferation in Müller cell context. The proliferation levels of both Gab1 silenced cells were similar to each other and higher than the control upon 24 hours of FGF2 induction. However, the proliferation level of Gab1 silenced

cells was less than SIK2 silenced and S266A mutant Gab1 expressing cells in response to FGF2 treatment. In addition, Gab1 silencing did not affect the basal level of proliferation while SIK2 silencing and S266A mutation led to an increase even in the uninduced cells. Although the duration of ERK activation was not affected by Gab1 silencing, the increase in proliferation in Gab1 silenced cells may be a result of the increase in the amplitude of the initial ERK activation. Since SIK2 functions through Gab1 to negatively regulate proliferation, it is also possible that downregulation of proliferation by SIK2 activity is decreased in the absence of Gab1. Additional studies on ERK activation profile and interaction profiles of other proteins in the Ras/ERK signaling such as Shp2, Grb2, SOS1 and Ras during this 24 h period can shed light to the overall mechanism of FGF2-induced proliferation in Müller cell context.

In summary, we propose that the role of Gab1 switches from suppressor to amplifier depending on its interactions with partners during the course of FGF2-induced ERK signaling. We suggest that Gab1 plays a negative regulatory role in the first 10 min. of ERK activation via RasGAP interaction (Montagner *et al.*, 2005). By interacting with Grb2, Gab1 also balances the number of Grb2/SOS1 complexes (Mcdonald *et al.*, 2014), hence the amplitude of ERK activation. After the initiation of signaling, Gab1 binds and activates Shp2 (Fedele *et al.*, 2018), hence, contributes to positive regulation of the signaling from 10 to 60 min. of induction. Phosphorylation of Gab1 by SIK2 at S266 decreases Gab1/Shp2 interaction (Küser, 2011; Yılmaz-Sert, 2011) and downregulates ERK signaling between 10 and 60 min. of induction. This phosphorylation also decreases Grb2/Gab1 interaction (Küser, 2011; Yılmaz-Sert, 2011) and possibly leads to an increase in Cbl activity resulting in downregulation of the signaling between these time points. Interactions of Gab1, Shp2 and Grb2 with each other and the other components of the signaling pathway such as SOS1, Ras and Cbl between 10 and 60 min. of induction should also be tested for a better understanding on the role of interaction of these partners in this context.

Our proliferation studies show that both SIK2 and Gab1 silencing, and S266A mutation increases FGF2-induced MIO-M1 cell proliferation compared to wild type and the scrambled control suggesting that both Gab1 and SIK2 is required for fine-tuning of cell proliferation.

Since these proliferation studies were conducted after 24 h of FGF2 stimulation, it is hard to temporally connect ERK activation data with proliferation. Therefore, studying ERK activation and partner interactions in a longer time interval can also be beneficial to understand the mechanism underlying the regulation of Müller cell proliferation. Even though further proliferation studies on these cells are necessary for statistical analysis, these results are consistent with our hypothesis stating that FGF2-induced ERK activation leads to SIK2 phosphorylation, subsequently, active SIK2 phosphorylates Gab1 at S266 and blocks the interaction of Gab1 with binding partners therefore, creates a negative feedback loop that downregulates MIO-M1 cell proliferation.

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