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The Role and Regulation of Sphingosine Kinases in Cancer Biology

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Mohamad Adada

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Mohamad Adada

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend

acceptance of this dissertation.

Lina Obeid, M.D. – Dissertation Advisor
Dean of Research, Professor of Medicine, School of Medicine

Jian Cao , M.D. – Chairperson of Defense
Associate Professor of Research Medicine, School of Medicine

Yusuf Hannun, M.D.
Director of Stony Brook Cancer Center, Vice Dean of Cancer Medicine, Joel Kenny Professor of Medicne, School of Medicine

Kenneth Shroyer, M.D, Ph.D
Marvin Kuschner Professor and Chair, Department of Pathology, School of Medicine

Vincent Yang, M.D, Ph.D
Simons Chair of Medicine, Professor of Medicine, School of Medicine

Michael Pearl, M.D.
Director of Gynecologic Oncology, Professor of Medicine, Department of Obstetrics, Gynecology, and Reproductive Medicne

This dissertation is accepted by the Graduate School

Charles Taber
Dean of the Graduate School

Abstract of the Dissertation

The Role and Regulation of Sphingosine Kinases in Cancer Biology

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Doctor of Philosophy

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Sphingolipids have emerged as bioactive constituents of eukaryotic cells. The bioactive sphingolipid ceramide mediates cellular senescence, apoptosis and cell cycle arrest. Acted upon by ceramidase, it is transformed to sphingosine that in turn gets phosphorylated by sphingosine kinases (SK1 and SK2) to generate sphingosine-1-phosphate (S1P). This lipid, on the contrary, mediates cellular proliferation, mitogenesis, inflammation, angiogenesis, and cancer metastasis through its actions on specific G-protein coupled receptors (S1PR1-5). ERM (ezrin, radixin, and moesin) proteins are a group of adaptor molecules linking the cortical actin cytoskeleton to the plasma membrane, and are emerging as critical regulators of cancer metastasis and progression via regulation of cell morphology and motility. Recently, we have identified S1P as an acute and potent ERM activator (via phosphorylation) through its action on its receptor S1PR2. We have also demonstrated that S1P-mediated filopodia formation, a first step in cell invasion, is through ERM activation. Growth factors are known activators of ERM proteins; however, it is not known

if it involves the newly described S1P/S1PR2 axis, as well as upstream metabolites of the sphingolipid pathway. We demonstrate that SK2, and not SK1, is essential and sufficient in EGF-mediated ERM phosphorylation. Surprisingly, and for the first time, we prove that this event, although strictly dependent on S1PR2 activation, does not require extracellular S1P secretion; therefore introducing a potential novel model of autocrine/intracrine action of S1P that still involves its GPCR receptors. We also establish Spns2 as an essential S1P transporter that is required for S1PR2 activation and subsequent ERM phosphorylation. In addition, we rule out the role of previously established ezrin kinases, and involve the checkpoint kinases, CHK1 and CHK2, as novel mediators of ERM activation downstream of S1PR2; therefore, emphasizing the newly emerging concept stating that a cell need to stop proliferation for it to invade. Finally, we identify SK2, S1PR2 and CHKs as new and potent targets in the pathway of EGF-driven invasion. In fact, inhibiting SK2, S1PR2 or CHKs eradicates EGF-mediated lamellipodia formation, adhesion and extracellular matrix invasion. In conclusion, this body of work uncovers new mechanistic insights for EGF-mediated invasion. More importantly, it sets the stage for novel alternative therapeutic targets that could be of utmost importance especially in patients that become resistant to current EGFR-tyrosine kinase inhibitors.

DEDICATION

Dear Mom,

Every experiment was done by your morals,

Every word was engraved with your love,

All my knowledge resulted from your sacrifices,

All my rationale was inspired by your wisdom,

And all my conclusions were accompanied by your smile.

May God protects you, and keeps you as my guardian angel forever.

Yours Truly,

Hammoudi

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LIST OF ABBREVIATIONS

ABC	AATP Binding Cassette
BBB	Blood Brain Barrier;
bCDase	Bacterial Ceramidase
brms1	breast carcinoma metastasis suppressor 1
bSMase	Bacterial Sphingomyelinase
C1P	Ceramide-1-Phosphate
CAPP	Ceramide Activated Protein Phosphatase
CD	Cluster of Differentiation
CerS	Ceramide Synthase
CFTR	Cystic Fibrosis Transmembrane Receptor
CML	Chronic Myolegenic Leukemia
COX	Cyclo-oxygenase
CTGF	Connective Tissue Growth Factor;
DAG	Diacylglycerol
EC	Endothelial Cells
EDG	Endothelial Differentiation Gene
EGF	Epithelial Growth Factor
ERK	Extracellular-signal Regulated Kinases
ERM	Ezrin-Radixin-Moesin
GEF	Guanine Exchange Factor
GIST	Gastro-Intestinal Stromal Tumors
GPCR	G-Protein Coupled Receptor
HIV	Human Immunodeficiency Virus
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
IL	Interleukin
IκB	Inhibitor of NF-κB
L1CAM	L1 Cell Adhesion Molecule
LPA	Lysophosphatidic Acid
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinases
MCP-1	Monocyte Chemoattractant Protein-1
MMP	Matrix Metallo-Proteinase
MYPT	Myosin Phosphatase Targeting Protein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHE	Sodium Hydrogen Exchanger
NHERF	Sodium Hydrogen Exchanger Regulatory Factor

NK	Natural Killer Cell
NOS	Nitrous Oxide Synthase
NPT2a	Sodium Phosphate co-Transporter 2a
PI3K	Phosphatidylinositide 3-kinases
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PKH	Phosphoinositide-dependent protein kinase (PDK) Kinase Homolog
PLC	Phospholipase C
PMA	Phorbol Myristate Acetate
PP	Protein Phosphatase
PRL-3	Phosphatase of Regenerating Liver-3
PSGL-1	P-Selectin Glycoprotein Ligand-1
PTEN	Phosphatase and Tensin homolog
ROCK	Rho-associated protein kinase
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-Phosphate Receptor
SDF-1	Stroma Derived Factor 1 alpha
SK	Sphingosine Kinase
SNARE	Soluble NSF Attachment Protein Receptor
SPT	Serine Palmitoyl Transferase
SRE	Serum Response Element
STAT3	Signal Transducer and Activator of Transcription 3
TNF	Tumor Necrosis Factor
UV	Ultraviolet Light
WBC	White Blood Cells

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This thesis would have been impossible without the help and support of so many people. I would like to use this opportunity to thank them one by one, as each experiment in this thesis is a witness for their love, care, and assistance.

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Second, I would like to thank Lina and Yusuf for being my second American family. I am greatly thankful for Lina who took a bet on me, and accepted me in her lab, knowing that I had zero experience. She wasn't only an exceptional mentor that guided me with my science, but she was a caring "mom" that gave me advice and support in all life matters. And Yusuf, behind all your critical and harsh comments, there was a valuable lesson learned and a kind "dad"

hidden. Both of you are great inspirations that I will always look up to, and I hope that someday I can achieve what an exceptional career you had. I know that I am only one student among many you mentored, but I hope I made you proud.

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Finally, I would like to thank all my committee members, first for accepting to be part of my Ph.D. career knowing their busy schedule, and second for all their valuable suggestions that boosted my research and widened my scope of thinking. I would also like to thank the American University of Beirut and Stony Brook University, since both of them provided me with a formidable environment to work and grow in.

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Induction Through p38 MAPK but Independently of NF-κB Activation. *J Biol Chem.* **2013** Aug 9.

2. **Adada M**, Canals D, Hannun YA, Obeid LM. Sphingosine-1-phosphate receptor 2. *FEBS J*. **2013** Jul 24. doi: 10.1111/febs.12446.
3. **Adada M**, Canals D, Hannun YA, Obeid LM. Sphingolipid regulation of ezrin, radixin, and moesin proteins family: Implications for cell dynamics. *Biochim Biophys Acta*. **2013** Jul 12. doi: pii: S1388-1981(13)00140-6. 10.1016/j.bbalip.2013.07.002.
4. Orr Gandy KA*, **Adada M ***, Canals D, Carroll B, Roddy P, Hannun YA, Obeid LM. Epidermal growth factor-induced cellular invasion requires sphingosine-1-phosphate/sphingosine-1-phosphate 2 receptor-mediated ezrin activation. *FASEB J.* **2013** Aug;27(8):3155-66. doi: 10.1096/fj.13-228460.

The co-authors listed in the above publications directed and supervised the research that forms the basis for this thesis or dissertation. Permission has been granted for their use by their respective publishers. Copies of these permissions are provided on the ProQuest/UMI website.

Sincerely,

Mohamad Adada

Vita, Publications and/or Fields of Study

MOHAMAD MOUNIR ADADA
605 Stonegate Way,
Middle Island, NY 11953
1 (843) 814-3734
Mohamad.Adada@stonybrookmedicine.edu

Education:

MD/PHD student **2011 to present**
SUNY, Stony Brook University, school of medicine, 4th year PHD
Mentor: Lina Obeid, M.D, Dean of Research, Stony Brook University

United States Medical Licensing Examination, Step 1 exam **2011**
Passed in Charleston, South Carolina, Score: 256 (99th percentile)

Medicine 2 **2010**
American University of Beirut, Lebanon, Dean's Honor List, rank 2 out of 90

Medicine 1 **2009**
American University of Beirut, Lebanon, Dean's Honor List, rank 10 of 92

B.S. Biology **2008**
American University of Beirut, Lebanon, Dean's Honor List

Lebanese Baccalaureate **2005**
Life Sciences, Honor List

First Author Publications:

1. **Adada M,** Canals D, Kelkar A, Hernandez M, Donaldson J, Hannun YA, Obeid LM. Intracellular SK2-derived S1P mediates EGF-induced ERM phosphorylation and cancer cell invasion. *Ready for submission*.

2. **Adada M**, Kelkar A, Tan K, Liu M, Obeid LM, Hannun YA, Canals D. A novel role for checkpoint kinases, Chk1 and Chk2, in S1P signaling and cancer cell invasion. *In preparation*.

3. **Adada M**, Orr-Gandy KA, Snider AJ, Canals D, Hannun YA, Obeid LM, Clarke CJ. Sphingosine Kinase 1 (SK1) regulates Tumor Necrosis Factor (TNF)-mediated RANTES

Induction Through p38 MAPK but Independently of NF-κB Activation. *J Biol Chem*. **2013** Aug 9.

4. **Adada M**, Canals D, Hannun YA, Obeid LM. Sphingosine-1-phosphate receptor 2. *FEBS J*. **2013** Jul 24. doi: 10.1111/febs.12446.

5. **Adada M**, Canals D, Hannun YA, Obeid LM. Sphingolipid regulation of ezrin, radixin, and moesin proteins family: Implications for cell dynamics. *Biochim Biophys Acta*. **2013** Jul 12. doi: pii: S1388-1981(13)00140-6. 10.1016/j.bbalip.2013.07.002.

6. Orr Gandy KA*, **Adada M** *, Canals D, Carroll B, Roddy P, Hannun YA, Obeid LM. Epidermal growth factor-induced cellular invasion requires sphingosine-1-phosphate/sphingosine-1-phosphate 2 receptor-mediated ezrin activation. *FASEB J*. **2013** Aug;27(8):3155-66. doi: 10.1096/fj.13-228460.

*** These authors contributed equally to the work**

Co-Author Publications:

7. Salama MF, Carroll B, **Adada M**, Pulkoski-Gross M, Hannun YA, Obeid LM. A novel role of sphingosine kinase-1 in the invasion and angiogenesis of VHL mutant clear cell renal cell carcinoma. FASEB J. **2015** Mar 24. pii: fj.15-270413

8. Perry DM, Newcomb B, **Adada M**, Wu BX, Roddy P, Kitatani K, Siskind L, Obeid LM, Hannun YA. Defining a role for acid sphingomyelinase in the p38/interleukin-6 pathway. J Biol Chem. **2014** Aug 8;289(32):22401-12. doi: 10.1074/jbc.M114.589648.

9. El Osta M, Liu M, **Adada M**, Senkal CE, Idkowiak-Baldys J, Obeid LM, Clarke CJ, Hannun YA. Sustained PKCβII activity confers oncogenic properties in a phospholipase D- and mTOR-dependent manner. FASEB J. **2014** Jan;28(1):495-505. doi: 10.1096/fj.13-230557.

10. Gandy KA, Canals D, **Adada M**, Wada M, Roddy P, Snider AJ, Hannun YA, Obeid LM. Sphingosine 1-phosphate induces filopodia formation through S1PR2 activation of ERM proteins. *Biochem J*. **2013** Feb 1;449(3):661-72. doi: 10.1042/BJ20120213.

Meetings and Presentations:

- 8th Institute of Chemical Biology and Drug Discovery Annual Symposium 2014
Student Poster Presentation, Stony Brook, NY

- 7th International Ceramide Conference **2013**
Student Oral Presentation, Montauk, NY

- 13th International Conference: Bioactive lipids in Cancer, Inflammation and Related Diseases **2013**
Student Poster Presentation (two posters), San Juan, Puerto Rico.

- 29th Annual National MD/PHD student conference **2013**
Student Poster Presentation, Keystone, CO

- Molecular Cell Biology Student Symposium **2013**
Student Oral Presentation, Stony Brook, NY

- FEBS Advanced Course in Lipid Signaling and Cancer **2012**
Student Poster Presentation, Vico Equense, Italy.

Awards:

- Winner of the Distinguished Travel Award **2014**
  presented by Stony Brook University graduate school as a recognition of the applicant's outstanding scholarship and the impact of his work both within their field and academia.

- Travel Award for the 13th International Conference: Bioactive lipids **2013**
  in Cancer, Inflammation and Related Diseases.

- Finalist for the Santosh Nigam Memorial "Outstanding Young Scientist" **2013**
  Award.

- 2nd best poster presentation in the 13th International Conference: **2013**
  Bioactive lipids in Cancer, Inflammation and Related Diseases.

- Travel Award for the 7th International Ceramide Conference. **2013**

- Best Student Oral presentation in the 7th International Ceramide **2013**
  Conference.

- 1st Award for an Outstanding Student's Poster Presentation in the **2007**
  2007 IBSAR Forum in the celebration of the International Biodiversity Day at the American University of Beirut (AUB).

Research and Academic Experience:

Upper Gastrointestinal Bleeding in patients using NSAIDS **2009**
With Dr. Kassem Barada, Internal Medicine, Endoscopy Unit.

My work focused on getting information from the AUBMC medical records to fill out forms concerning previous medical history, drugs used including anticoagulants, GI problems and the endoscopies performed including any therapeutic treatment.

Second assistant in the Operation Room of Otolaryngology Department **2009**
Prague Motol Teaching Hospital, supervised by Dr. Betka, Chairperson

I participated in many surgeries of the Head and Neck on daily bases including the removal of tumors of the thyroid, oropharynx, nasopharynx, metastasis of the neck tumors and many others. I got the opportunity to travel abroad as I am a member of the International Federation of Medical Students Associations (IFMSA).

The mechanisms of anti-inflammatory action of Gallotannin **2008**
The roles of anti-inflammatory drugs as anti-cancer drugs and vice versa

Under the supervision of the Chairman of the Biology department, I was involved in the research she was conducting on Gallotannin, a product found in tea. We were investigating its anti-inflammatory effect in cancerous and normal cells, in vitro and in vivo (mice). I got familiar with many laboratory techniques including ELISA, PCR, Electrophoreses, flow cytometry, cell culture, cell counting, and many others. Furthermore, I have been tested orally on weekly basis for my knowledge and at the end of the semester I submitted a report on which I was excellently evaluated by getting a grade of 95/100.

X-rays technician **2007**
Clinical Assistant in Orthodontics Department at AUBMC Supervised by Dr. Joseph Ghafari, Head of Department

I worked for more than 1 year in the Orthodontics department at AUBMC as an X-Ray technician, where I got used to take X-Rays for the patients' teeth, jaw and hands (adults and children). This work was done under the supervision of the Head of Department Dr. Joseph Ghafari.

Rhinoplasty, Face Lifts, in Otolaryngology Department of AUBMC **2007**
With Dr. Nabil Fuleihan, Chairperson

1.5 years of experience in searching for articles on the internet using AUB databases and

libraries concerning these topics.

Extracurricular activities:

Member of the New York Road Runners (NYRR)

Member of The folk Dance Festival at AUB for two consecutive Years

Red Cross official trainee in First Aid

Spoken and Written Languages:

Arabic, Native Language
French, Second Language
English, Third Language
Spanish, Fourth Language

Chapter 1

Sphingolipid regulation of ezrin, radixin, and moesin protein families: implications for cell dynamics

Abstract

A key but poorly studied domain of sphingolipid functions encompasses endocytosis, exocytosis, cellular trafficking, and cell movement. Recently, the ezrin, radixin and moesin (ERM) family of proteins emerged as novel potent targets regulated by sphingolipids. ERMs are structural proteins linking the actin cytoskeleton to the plasma membrane, also forming a scaffold for signaling pathways that are used for cell proliferation, migration, and invasion, and cell division. Opposing functions of the bioactive sphingolipids ceramide and sphingosine-1-phosphate (S1P), contribute to ERM regulation. S1P robustly activates whereas ceramide potently deactivates ERM via phosphorylation/dephosphorylation, respectively. This recent dimension of cytoskeletal regulation by sphingolipids opens up new avenues to target cell dynamics, and provides further understanding of some of the unexplained biological effects mediated by sphingolipids. In addition, these studies are providing novel inroads into defining basic mechanisms of regulation and action of bioactive sphingolipids. This chapter describes the current understanding of sphingolipid regulation of the cytoskeleton, it also describes the biologies in which ERM proteins have been involved, and finally how these two large fields have started to converge.

1.1. Introduction

Long thought to be chiefly structural and metabolic molecules, sphingolipids have emerged as bioactive constituents of eukaryotic cells. They are involved in many vital cellular processes and functions and are generated in response to many extracellular inducers such as UV light, chemotherapy, growth factors, and cytokines (*1*). The bioactive sphingolipid ceramide mediates cellular senescence, apoptosis and cell cycle arrest. Its metabolite, S1P, mediates cellular proliferation, mitogenesis, inflammation, angiogenesis, and cancer metastasis (*1*). This model of sphingolipid-mediated biologies highlights the opposing roles that can be exerted by the different bioactive sphingolipids(*2*).

This chapter focuses on a special area in sphingolipid biology, specifically their pleiotropic effects on the actin cytoskeleton and their contribution to cell shape. Such effects are crucial for mediating cellular changes observed in endocytosis, exocytosis, cellular trafficking, and filopodia and lamellipodia formation, processes required for the maintenance of cell polarity and induction of cell migration (*3*) . Here we focus on the ERM family and the emerging intimate connections to sphingolipids.

ERM (ezrin, radixin, and moesin) proteins are a group of adaptor molecules linking the cortical actin cytoskeleton to the plasma membrane (*4*). They are considered to be core proteins with which different molecules interact to facilitate signal transduction between the cell and the extracellular matrix (*5*). ERMs are essential for dictating cellular morphology, a role made apparent by their ability to modulate formation of specialized cellular structures such as filopodia, lamellipodia, and other membrane protrusions in which ERMs exist in abundance. Also, ERMs have important roles in cell biological processes such as cell polarity, adhesion,

migration, and invasion. Therefore, ERMs are emerging as critical regulators of cancer metastasis and progression (*6-11*). ERM proteins exist in two conformational states, which dictate their function: an inactive or closed state in which the N-terminal domain (FERM) and the C-terminal domain (C-ERMAD) interact, and an open or active state in which phosphorylation of the C-terminal threonine residue (ezrin Thr-567, radixin Thr-564, and moesin Thr-558) disrupts this interaction. When in the closed state, ERMs are cytosolic, and when in the open/active state, the FERM domain interacts with the plasma membrane and the C-ERMAD is linked to the actin cytoskeleton (*4*). Other phosphorylation sites exist, but these have different functions as will be discussed later.

After providing a brief summary of sphingolipid metabolism and its regulation of cell morphology, this chapter will address two main areas. First, it will address ERM in-depth to educate the reader about the diverse functions of ERMs. Next, this chapter will expand on the connections established between sphingolipid metabolic products and their regulation of ERM functions.

1.2. The sphingolipid pathway

Sphingolipid synthesis is a complex interconnected set of reactions that yield abundant bioactive intermediate products. *De novo* sphingolipid synthesis that occurs in the endoplasmic reticulum, begins with a condensation reaction between serine and palmitoyl CoA mediated by serine palmitoyl transferase (SPT) to form 3-keto-dihydrosphingosine, which is directly reduced to dihydrosphingosine (*12*). This product is acted upon by dihydroceramide synthase (also known as Lass or CerS), to form dihydroceramide, which is subsequently desaturated, generating ceramide (*13*). Ceramide occupies a central position in this pathway, and it is a target for diverse

enzymes to yield hundreds of biosynthetic pathway products. Multiple sugar residues can be added to ceramide to yield complex glycosphingolipids through the initial action of glucosyl- or galactosyl- ceramide synthases, the first steps in glycosphingolipid synthesis (*14*). Alternately, sphingomyelin can be generated by the addition of a phosphocholine headgroup, a step catalyzed by sphingomyelin synthases (*15*). Ceramide can also be phosphorylated by ceramide kinase to form ceramide-1-phosphate (*16*).

Sphingolipid catabolism is accomplished by multiple enzymes that drive the production of intermediate bioactive lipids and shift the balance among them. Sphingomyelin is acted upon by 4-6 sphingomyelinases (acid, 1-3 neutral, alkaline, and one that is mitochondrial), which differ in their cellular localization (*17*). Complex glycosphingolipids are gradually degraded by hydrolases to form glucosylceramide and galactosylceramide, which serve as substrates for specific glucosidases and galactosidases (*18, 19*).

Finally, ceramide is metabolized to sphingosine through the action of five known ceramidases (acid, neutral, and three alkaline) that also differ in their cellular localization and tissue distribution (*20*). Sphingosine, in turn, participates in the formation of ceramide through the salvage pathway via multiple ceramide synthases, or it can be phosphorylated by two known sphingosine kinase isoforms, SK1 and SK2, to form sphingosoine-1-phosphate (S1P), one of the most studied sphingolipid molecules (*21*). S1P can either be dephosphorylated back to sphingosine by S1P phosphatases (*22*), or it can be cleaved irreversibly to ethanolamine phosphate and hexadecanal by S1P lyase, which represents the only exit point of the sphingolipid pathway (*23*). **Scheme 1** provides a schematic illustration of the sphingolipid metabolic pathway described above.

1.3 Sphingolipids and cellular morphology

Sphingolipids are important for membrane and cell shape integrity because of their structural characteristics, and their involvement in the activation/inhibition of signaling pathways that alter cellular morphology.

1.3.1. Sphingolipids in endocytosis and exocytosis

Endocytosis and exocytosis require major cytoskeletal and membranous rearrangements that are initiated by membrane-invagination and conclude with vesicle formation. Endocytosis is dramatically altered in yeast carrying mutations in lcb1p, a component of SPT, which catalyzes the first step of *de novo* lipid synthesis (*24*). Such an effect is mediated by a deregulation of actin dynamics (*25*), an effect that can be reversed to the normal phenotype by overexpression of protein kinase C1 and PKH1 or PKH2 (orthologue of mammalian protein-phosphoinositide dependent protein kinase 1, PDK1) (*26, 27*). These studies suggest a possible regulatory role for sphingolipids with these enzymes, modifying cellular structures. In contrast, membrane fusion between a donor vesicle and the plasma membrane during exocytosis requires the presence of membrane-associated proteins, SNAREs. These proteins are encoded by SNC genes, and are altered in the mutant yeast strain *M42A snc2delta* that has defective exocytosis. Interestingly, this defect is restored by overexpression of the sphingoid base phosphate lyase, which diminishes cellular dihydrosphingosine-1-phosphate (*28*). In addition, a mutation in S1P lyase in *Drosophila* gives rise to cellular migration defects, specifically affecting F-actin distribution (*29, 30*). Also, ceramidase has been reported to be essential in exocytosis in yeast and *Drosophila*. Yeast defective in YPC1, which encodes a ceramidase, have defective vesicular trafficking from the

Golgi apparatus to the cell exterior (*31*). Moreover, *Drosophila* with a mutated neutral ceramidase, also have defects in neuronal synaptic vesicular fusion and cellular trafficking (*32*).

Finally, neutral sphingomyelinase has been recently reported to play a crucial role in exosomes secretion (*33, 34*). Exosomes are extracellular vesicles derived from the endocytic compartment and carry molecules involved in paracrine signaling. Overexpression of this enzyme increases the secretion of miRNA-loaded exosomes, which causes increased angiogenesis and metastasis (*33*).

1.3.2. Sphingolipids and plasma membrane protrusions

S1P is a potent bioactive sphingolipid that exerts its function either through intracellular interactions, or by being transported to cell exterior via the ABC transporter family (*35*) and/or the spinster-2-transporter (*36*), where it acts on G-protein coupled receptors (S1P receptors; S1PR). Five S1PRs have been identified (S1P1–5). S1P receptors differ with respect to tissue distribution and with respect to the G-proteins to which they are coupled (*37*). S1P is a well-known chemoattractant and a modulator of cell migration, exerting its functions by acting on receptors that, in turn, couple to the Rho and Rac family of small G proteins (*3*). Activation of the Rac pathway polarizes the cell, and gives rise to the production of lamellipodia along with focal adhesion contacts with the extracellular matrix. Activation of the Rho pathway, in contrast, causes cell detachment and stress-fiber formation. Alternating and concerted activation of these two different pathways ultimately lead to cell migration and forward movement (*38*). S1PR1 has been coupled to Rac activation and cell movement, whereas S1PR2 has been linked to Rho activation and Rac inhibition, thereby inhibiting cell movement (*37*). Detailed effects of S1P

receptors on cell movement are beyond the scope of this chapter, and have been adequately reviewed elsewhere as well as in chapter 2 (*39*).

1.4. Ezrin activation and role in biology

Ezrin activation is a multistep process requiring the phosphorylation of the threonine 567 residue. Multiple cellular stimuli are known to induce ezrin phosphorylation, which has a well-defined biological significance. **Table 1** offers a current list of all kinases known to induce ERM phosphorylation with respect to the stimulus applied and the biology involved. The Table also contains details about the specific cell line in which the mechanism has been studied. As previously mentioned, ERM phosphorylation is not limited to the activating threonine 567 residue, but it involves several other residues key for cell signaling (also described in **Table 1**). The Table represents a reference for studying relationships between the ERM family of proteins and sphingolipids, which will be discussed in more detail later. A discussion of different functions affected by ERM phosphorylation is offered below.

1.4.1. Ezrin in normal physiology

ERM proteins are essential for normal physiologic human body functions. They have been studied in the nervous, digestive, endocrine, and genitourinary system, and they have been studied for their role in cell division.

First, the interaction between ezrin and the actin cytoskeleton is required to maintain a normal cell shape (*40*). Phosphorylation of ezrin, or the expression of the phosphomimetic T567D, causes lamellipodia formation along with microvillar projections in LLC-PK1 epithelial

cells (*41*). Ezrin phosphorylation also produces an elongated cellular morphology and an enrichment of the actin cortical network in HeLa and NIH 3T3 cells (*42*). Furthermore, cellular detachment and inhibition of cell adhesion also causes ezrin phosphorylation and induction of microvilli formation in HEK293 cells (*43*).

Next, ERM protein activation is needed to maintain a healthy reproductive system. Ezrin phosphorylation is needed for sperm capacitation, which is necessary for producing functional sperm (*44*). After conception, ERM proteins guide cellular mitosis (*45*) required for proper blastocyst morphogenesis (*46*) and germ layer formation during gastrulation (*47*).

In the nervous system, phosphorylation of ERM proteins is essential for promotion of axonal outgrowth and regeneration. This effect has been related to the activation and recruitment of ERM proteins downstream of neural cell adhesion molecule L1, which creates a highly active cytoskeletal area and a new branch protrusion (*48*). ERM have also been reported to cause this effect through interaction with the deleted-in-colorectal-cancer (DCC) receptor, which subsequently activates a growth cascade (*49*). Furthermore, phosphorylation of ERMs has been shown to regulate dopamine transporter activity in the nucleus accumbens (*50*), and this may be involved in pre-synaptic trafficking (*51*). Interestingly, creating an artificial milieu with excessive ERM phosphorylation caused neural growth retardation (*52*).

In the digestive system, ERM proteins are required for stomach acid secretion through their regulation of acid pump positioning. In fact, expression of the phosphomimetic mutant T567D changes the polarity of gastric parietal cells (*53*), causing internal cellular membranes to be inserted in the basal vesicles instead of into the apical ones (*54*). Cargo proteins such as the H/K-ATPase transporter insert into the basolateral plasma membrane along with long, microvillar projections, effectively halting acid secretion (*53*). PKA-mediated ezrin

phosphorylation on the serine 66 residue is essential for positioning the acid pump on the apical membrane (*55*) through its interaction with an adaptor protein—the WW domain-containing oxido-reductase protein (*56*).

Ezrin activation has been linked to the sodium hydrogen exchanger isoforms 1 and 3 (NHE1, NHE3). In fact, ezrin has been reported to bind to NHE1, and, together, these two proteins form a scaffold for AKT activation and cellular proliferation (*57*). This signaling role is essential after cardiovascular ischemia for the maintenance of left ventricular function, prompting its hypertrophy (*58*). In addition, overexpression of ezrin in kidney cells increases the surface expression of NHE3, which is important for maintenance of body sodium homeostasis (*59*). Moreover, ezrin affects phosphate balance through its interaction with the parathyroid hormone receptor and sodium hydrogen exchanger regulatory factor 1 (NHERF-1) that in turn regulates apical expression of the sodium-phosphate co-transporter 2a (NPT2a) (*60*).

Finally, ERM proteins are needed for a functional endocrine system. Ezrin phosphorylation is essential for insulin secretion from pancreatic cells. In fact, ezrin activation is necessary for insulin granule trafficking and membrane docking (*61*). Ezrin deactivation is observed in diabetic mice (*61*), and in organs affected by diabetic complications such as the kidney (*62*). Actually, advanced glycation end products bind the end terminal of ezrin in the kidney, subsequently inhibiting its activation and thus kidney tubule formation (*62*).

1.4.2. Ezrin in immunity and inflammation

Increased ezrin phosphorylation and activation is reported to occur in many inflammatory diseases such a rheumatoid arthritis (*63, 64*), systemic lupus erythematosus (*65*), pulmonary

fibrosis (*66*) and traumatic brain injury (*67*). Ezrin activation in the immune response is thought to be important for inflammation and immunity, either by influencing cellular movement, cell shape, and/or cell survival, which is discussed below.

1.4.2.1. Leukocyte recruitment

ERM proteins have been described to be needed for all phases of leukocyte recruitment to sites of inflammation. Capture of free circulating leukocytes is the first step in this process, and this is mediated by p-selectin glycoprotein ligand-1 (PSGL-1). The deletion of the ERM binding sequence in the cytoplasmic tail of PSGL-1 dramatically impairs leukocyte tethering, slow rolling on the activated endothelium, and promotes defective MAPK activation (*68*). Furthermore, ERM proteins have been shown to link PSGL-1 to Syk kinase (*69*). Generation of a moesin mutant that could not attach to Syk abrogated serum response element (SRE) transcriptional activity that is usually induced upon PSGL-1 stimulation (*69*). In contrast, transgenic mice expressing ezrin phosphomimetic protein (T567E) had defective T lymphocyte migration across the endothelium but no disturbances with the slow rolling mechanism mentioned above. Such transgenic mice had decreased lymphocytes within lymph nodes along with increased cellular adhesion and decreased cellular migration rates (*70*). White blood cells (WBC) undergo cytoskeletal polarization once outside the blood vessel for proper migration. In B cells, once phosphorylated, ERM proteins translocate to the front of the cell's lamellipodia to participate in forward movement (*71*). In concordance with this activity, phosphorylation of ERM proteins in NK cells facilitates their polarization and their migration towards chemoattractants (*72*). In T lymphocytes however, overexpression of the phosphomimetic T567D ezrin was reported to widen the uropod on the posterior aspect of the cell, enhancing its migration ability. ERM proteins are hypothesized to establish the "posteriority" of a cell (*73*).

Other groups reported that ERM proteins, specifically moesin, redistribute adhesion molecules to the posterior of the migrating lymphocytes to increase ligand accessibility (*74*). Certainly, ERM proteins are involved in modulating WBC movement, but the mechanism by which this occurs remains unclear.

1.4.2.2. Endothelial permeability

ERM proteins have variable effects on endothelial permeability and this is dependent on the activation stimulus. While ERM depletion abolished S1P's protective effect on the endothelium that is usually manifested by increased adherens junctions and decreased endothelial permeability (*75*) (this point will be explained later), individual depletion of ERM proteins markedly attenuated membrane leakiness caused by 2-methoxyestradiol anti-proliferative treatment (*76*), TNF-alpha treatment (*77*) and the accumulation of advanced glycation end products in diabetes (*78*). Therefore, the effect of ERM proteins on endothelial integrity may be related to different downstream targets with which they associate upon the application of different stimuli.

1.4.2.3. Infections

ERM phosphorylation is caused by diverse microorganisms such as chlamydia (*79*), HIV (*80, 81*), *Listeria monocytogenes* (*82*), and *E. coli* (*83, 84*). This activation appears to be necessary for infectivity or pathogenesis via different mechanisms. Using a dominant -negative ezrin that cannot be phosphorylated, chlamydial infection was attenuated due to diminished entry into host cells (*79*). Also, *Listeria's* ability to spread across cells without activating the immune system depends on induction of cellular protrusions, which are compromised upon ERM depletion. (*82*). Enteropathogenic and enterohemorrhagic *E. coli* recruit and activate ezrin at their site of attachment on intestinal epithelial cells (*84*), disrupting the epithelial tight junctions

which is manifested by diarrhea and gastric bleeding (*83*). Interestingly, this effect is observed specifically in pathogenic strains of *E. coli*; non-pathogenic strains do not activate ezrin (*83*). Finally, HIV's effect on ezrin activation depends on the component of HIV studied. Viral protein R (Vpr) decreases ezrin activity that is normally associated with AKT activation, and thus cell survival (*80*). In contrast, the envelope glycoprotein gp120 combined with IL-2, causes a sustained ezrin phosphorylation that is associated with increased cell surface expression of CD95 (also named FAS receptor), which causes increased T lymphocyte susceptibility to apoptosis (*81*).

1.4.3. Ezrin in malignancies

1.4.3.1. ERM expression and phosphorylation status

ERM proteins have been widely implicated in tumor growth and metastasis; they affect cellular modeling and signaling pathways involved in cell survival and proliferation. Ezrin was found to increase migration and metastasis in dog models of osteosarcoma (*85*). Ezrin overexpression in myxofibrosarcoma was associated with a higher histological grade of pathology and cancer stage (*86*). In fact, ezrin predicted decreased metastasis-free survival (*86*). Its overexpression was also correlated with worse outcomes in gastro-intestinal-stromal-tumors (GIST) (*87*), esophageal cancers (*88*) and ovarian epithelial carcinomas (*89*). Whether ezrin overexpression alone drives a malignant phenotype, or whether over-activation is the culprit, studies show that overexpression of phospho-mimetic ezrin is sufficient to confer metastatic capacity in mouse xenografts of hepatocellular carcinoma (*90*). Also, estrogen has been shown to

increase cell migration and invasion of T47-D breast cancer cells by inducing ezrin phosphorylation (*91*). This is also true for dinitrosopiperazine (a known carcinogen) which enhances cell motility, invasion, and metastasis of nasopharyngeal carcinoma through ezrin activation (*92*). However other studies suggest that for successful metastatic progression, ezrin must undergo a dynamic recycling between a phosphorylated and dephosphorylated state. For example, ezrin is only found to be phosphorylated just after osteosarcoma seeding in the lung parenchyma and then later during metastatic progression (*93*). Stable overexpression of either a phosphomimetic ezrin or a non-phosphorylatable form in osteosarcoma blocks primary tumor growth and metastasis (*94*). In fact, cells expressing the T567A mutant undergo significant apoptosis once they arrive within the lung. This apoptosis arises from the cells' inability to handle the metabolic stress of a different milieu. Evidence for this is provided by upregulation of genes involved in cellular metabolism and cellular energy (*94*). Thus, a new perspective is emerging relating to the importance of ezrin as an essential molecule allowing cell metabolic flexibility in the face of environmental stressors. Another way by which cell manage these stressors is through ezrin dependent activation of the MAPK pathway (*95*). Although an abundance of literature implicates ezrin phosphorylation as a driver of tumor progression and metastasis, other data contradict this. High-grade metastatic osteosarcoma was correlated with dephosphorylated cytoplasmic ezrin rather than an active isoform (*96*). In addition, phosphatase of regenerating liver-3 (PRL-3) was shown to exert its function by promoting tumor development via ezrin dephosphorylation on thr567 (*97*). Also, Raf-1 oncogene transfection into a mouse hepatic cell line deactivated ezrin (*98*). Furthermore, knocking down ezrin in human colonic epithelial cells increased their matrix adhesion and their spreading, thus their invasive capabilities (*99*). Finally, knocking down ezrin prevents cisplatin-induced apoptosis in human

colon cancer cells (*100*). In conclusion, further studies are needed to elucidate the exact role of ERM activation on cancer progression as it might vary depending on the cancer grade and stage.

1.4.3.2. Downstream effectors of ERM

ERM-promotion of cell invasion and metastasis is mediated by several downstream effectors, depending on which residue of ezrin is phosphorylated. Generally, phosphorylation of tyrosine residues activates a signaling cascade that subsequently activates the PI3K/AKT pathway as seen in breast cancer (*101*), whereas phosphorylation of threonine 567 promotes cell invasion by modulating cell shape (as seen in melanoma cells that form elongated microvilli) (*102*). The tyrosine kinase src can phosphorylate ezrin, and thus promote several outcomes. An ezrin mutant that cannot be phosphorylated by this kinase abolishes src-induced anchorage-independent growth and cell invasion (*103*). This has been confirmed in another study, which described cooperatively between src and ezrin in destabilizing cell-cell contacts and promoting scattering of mammary carcinoma cells (*104*). Furthermore, the pro-metastatic effects of androgen on prostate cancer cells occur through permissive effects on anchorage-independent growth also mediated by ezrin (*105*). A possible explanation for how ezrin promotes this type of growth is postulated in a study in which shRNA was used against ezrin in breast cancer (*106*). E-cadherin expression was dramatically increased upon ezrin knockdown, implicating this adhesion molecule as a downstream effector of ezrin. Matrix metalloproteinases (MMPs) have also been incriminated as an effector arm for ezrin. Podocalyxin, an anti-adhesive transmembrane protein involved in the development of aggressive forms of breast and prostate cancer, leads to increased MMP expression via interaction with ezrin (*107*). Similarly, expression of a dominant-negative form of ezrin reduced MMP expression and invasiveness of human malignant glioma cells (*108*). Another downstream effector of ezrin is the NF-κB

pathway, which is required for colorectal cancer metastasis to the liver (*109*). Ezrin is essential for iκB phosphorylation in response to engagement of the L1 cell adhesion molecule (L1CAM) (*109*).

1.4.3.3. Upstream effectors of ERM

Many pathways have been shown to regulate ezrin expression and/or phosphorylation. The EGF signaling cascade can increase ezrin expression, and thus cellular invasion and metastasis in MDA-231 (*110*). Similarly, fos oncogene-mediated malignant transformation of Rat-1 fibroblasts involves an increase in ezrin expression and ezrin hyperphosphorylation (*7*). Inhibiting ezrin function in these transformed cells abolishes fos-mediated membrane ruffling and cell motility (*7*). Furthermore, GIST harbors a mutation in the KIT oncogene that activates ezrin at different residues to promote tumor progression (*111*).

1.5. Ezrin de-activation

1.5.1. Deactivators

Little is known about ezrin threonine 567 dephosphorylation. As mentioned before, dephosphorylation of threonine 567 inactivates ezrin. Ezrin has a PIP2 binding site on its N-terminus that is required for its association with the plasma membrane. This site as well as other plasma membrane protein binding sites remain hidden by interacting with the c-terminus site. However, even when dephosphorylated, the two states of ezrin are in equilibrium: interacting with the plasma membrane and resting in the cytosol. Inactive ezrin remains either in a monomeric closed conformation or it forms dimers. Inactivation of ezrin is related to loss of cell

attachment, loss of plasma membrane protrusions (filopodia, microvilli, etc) and enhancement of cell invasion, and cell migration. Few ezrin inactivators have been described, and these mainly affect PIP2 at the plasma membrane. Thus, when plasma membrane PIP2 is hydrolyzed, ezrin is quickly dephosphorylated. Another more recent mechanism offered to explain ezrin deactivation involves ceramide production at the plasma membrane, a concept that is expanded in the next section.

Ezrin is well-studied in lymphocytes, mainly T cells and NK cells. It plays an important role in lymphocyte polarization, immunological synapses, receptor signaling and T-cell activation (*112*). In these cells, ezrin function is tightly regulated by tyrosine phosphorylation, which is stimulated by ligation of CD3 and CD4 receptors. General activation of ezrin is also stimulated by the CD43 receptor. Other lymphocyte receptors have been reported to play an important role in ezrin biochemistry, such CD95, CD44, CD81 or I-CAMs. A mechanism of ezrin inactivation in lymphocytes is via ezrin cleavage by calpain (*113*). The first described deactivator signal for ezrin was a cytokine, stroma derived factor 1-alpha (SDF-1 alpha, or CXCL12). Within seconds after SDF-1 treatment of human peripheral blood T cells, threonine 567 was dephosphorylated, and microvillie were lost. This effect was blocked by the phosphatase inhibitor calyculin A, although no phosphatase was defined in that study (*114*). Next, the small GTPase Rac1 was suggested to mediate the SDF-1a effect on microvilli collapse and ezrin dephosphorylation. Accordingly, a constitutively active Rac1 mutant produced ezrin dephosphorylation, and a dominant-negative mutant blocked cytokine-induced ezrin dephosphorylation (*115*). Moreover, the SDF-1a effect was blocked by PLC inhibitors, suggesting that hydrolysis of PIP2 was required for SDF-1 effects; the phosphomimetic mutant of ezrin (T567D) was released to the cytosol after PIP2 hydrolysis (*116*). In naïve T cells, both

chemokines CXCL12 and CCL21 triggered ezrin dephosphorylation, reducing cell attachment and enhancing cell migration.

In other cell lines, dephosphorylation of the ezrin threonine 567 occurred in response to confluence in endothelial cells(*77*), and in response to chemotherapeutics in breast cancer cell lines (*117*).

1.5.2. Phosphatases

Several phosphatases have been reported to be involved in dephosphorylation of ezrin at Threonine 567, and these include myosin phosphatase (PP1b-MYPT1) (*118*), PP2A, PP2C, PRL-3 (a tyrosine phosphatase reported to have dual activity) (*97*) and PP1a (*119*). This diversity of phosphatase involvement suggests that regulation of ezrin dephosphorylation depends on the cell type and the nature of the stimulus applied.

Different strategies have been developed to study the functional and phenotypic consequences of ezrin dephosphorylation . The most common tool for this is expression of a phospho-incompetent mutant, in which threonine 567 has been substituted with an alanine residue. Knockdown of ezrin is another approach. Recently, two small molecule inhibitors have been reported to induce ezrin dephosphorylation, as well as inhibit cell migration and invasion of osteosarcoma cells (*120*).

1.6. Ezrin and sphingolipids

1.6.1. Ceramide

The first study to describe a connection between ezrin status and sphingolipids was from our group in 2008. We noted that cisplatin caused loss of plasma membrane protrusions in MCF-7 tumor breast cancer cells, which was accompanied by loss of ezrin association to the plasma membrane, ezrin dephosphorylation, and co-localization of ezrin with cortical actin. These cytoskeletal rearrangements coincided with the loss of sphingomyelin and the generation of ceramide. In fact, cisplatin activated the acid sphingomyelinase in a PKC delta-dependent mechanism, causing its translocation to the plasma membrane. Exogenous ceramide, or recombinant bacterial sphingomyelinase from *Bacillus cereus* (bSMase) treatment for 1 h produced the same phenotype as cisplatin treatment, suggesting that ceramide chiefly triggered ezrin inactivation. This study also involved a ceramide activated protein phosphatase (CAPP) as a downstream effector of ceramide that is required for ezrin dephosphorylation (*117*). In a follow up study, HeLa cells were shown to undergo ezrin dephosphorylation after bSMase treatment, and this effect was more dramatic. As early as 2 min after treatment (25 mU bSMase), complete ERM dephosphorylation occurred (*121*). To elucidate which sphingolipid was required for ezrin dephosphorylation, the sphingolipid pathway from ceramide was dissected using a combination of several recombinant enzymes and SK inhibitors. Thus, treatment with bSMase dephosphorylated ezrin in a dose-dependent manner, localizing ezrin to the cytoplasm. Purified recombinant *Loxosceles* sphingomyelinase D, which generates ceramide 1-phosphate (C1P) instead of ceramide, proved that ceramide, and not decreased sphingomyelin or C1P production, was required for ezrin deactivation (*121*). Interestingly, regulation of ezrin dephosphorylation in HeLa cells after ceramide generation proved to be the result of another CAPP phosphatase; pretreatment with okadaic acid, and knockdown of PP2A did not block bSMase effects. In HeLa cells, the only serine/threonine phosphatase responsible for bSMase-induced ezrin

dephosphorylation was PP1a (*119*). This result was verified using pharmacologic inhibitors, siRNA technology, and PP1 catalytically inactive mutants (*119*). Interestingly, bSMase treatment neither altered PIP2 levels nor localization as shown with PLCδ-PH domain constructs, which specifically bind PIP2. Those data suggested that ceramide-induced ezrin dephosphorylation did not involve PIP2 loss. In contrast, PIP2 hydrolysis caused ezrin dephosphorylation in a PP1a-independent manner. Activation of PP1a by ceramide seemed necessary for ezrin dephosphorylation, and did not contribute to regulating plasma membrane attachment. This was confirmed by expressing the N-terminal fragment of ezrin, which could bind PIP2 but lacked the C-terminal region containing threonine 567, which could not be dephosphorylated in response to ceramide generation. This "ceramide-PP1a" axis also suggested a novel mechanism for ERM protein regulation: it not only did not involve PIP2, but it was independent of PP1b-MYPT1, which has been shown to regulate basal levels of ERM phosphorylation (*119*).

1.6.2. S1P

Recent experiments indicate that generation of high levels of S1P with the use of purified bacterial recombinant ceramidase (bCDase) after bSMase treatment was accompanied by ezrin hyperphosphorylation. In fact, we reported that S1P production and decreased ceramide was responsible for this dramatic effect. Thus, ceramide and S1P play opposing roles in ezrin regulation (*121*). Subsequent studies by our group showed that exogenously added or endogenously produced S1P resulted in a time- and dose-dependent increase in ERM phosphorylation as early as 30 seconds after treatment. S1P induction of ezrin phosphorylation seemed to be a universal effect; it was observed in several cell lines including MDA, MCF7, A549, HEK, and MEF (*122*). Dose-response experiments reveal that nanomolar concentrations

could trigger the maximum S1P response on ezrin phosphorylation, suggesting a receptor-mediated effect. Indeed, it was shown that this phosphorylation required the activation of S1PR2 exclusively on HeLa cell surfaces, and blocking all other receptors maintained ezrin activation (*122*). This was shown using pharmacologic inhibitors, and also siRNA and genetic knockout approaches. S1P-induced ezrin phosphorylation was required for filopodia formation; using dominant-negative non-phosphorylatable ezrin mutant abolished this S1P-mediated effect (*122*). The fact that S1P mediated ezrin phosphorylation inhibited cell invasion is contradictory to what is currently known of this bioactive sphingolipid, probably because S1P works on difference receptors. How this system is coordinated is unclear. The significance of this pathway was further explored by *Djanybek* and colleagues in human pulmonary endothelial cells (EC) in studies of EC barrier function (*75*). S1P protected EC barrier integrity by remodeling the actin cytoskeleton, increasing focal adhesion, and adherent junction formation. In this work, S1P was confirmed to activate ezrin and moesin at nanomolar concentrations within a few minutes of treatment. With pharmacological inhibitors, multiple PKC isoforms were shown to be responsible for ezrin and moesin (but not radixin) phosphorylation in response to S1P (in addition to P38 MAPK and Rac1). However, direct interactions with ERMs or other approaches involving these protein kinases were not proposed (*75*). Radixin was not phosphorylated after S1P stimulation, but it was shown to be required for S1P's effect on ezrin and moesin activation (*75*). A summary of sphingolipids modulation of ERM activity is illustrated in **scheme 2**.

1.7. Conclusion

This chapter describes novel mechanisms by which different bioactive sphingolipids can differentially modulate the cell cytoskeleton and cell behavior. They can mediate acute changes via activating or deactivating ERM proteins. On one end, ceramide causes acute ERM de-activation by recruiting protein phosphatase 1 alpha; and at the other end, S1P causes acute and potent ERM activation by a yet un-identified kinase that is discussed in chapters 5 and 6.

Until now, the scientific literature reviewed herein described experiments that used sphingolipids exogenously applied to the cell. Although this approach is limited, it does offer discovery of the basic regulation of this novel pathway. Recently, using a known activator of the endogenous SK/S1P pathway, epidermal growth factor (EGF), we found that EGF induced ERM phosphorylation in an SK/S1P-dependent manner. Furthermore, S1P generated upon EGF treatment acted on the same previously described receptor (S1PR2) to mediate this activation. This has been confirmed with siRNA technology as well as pharmacologic inhibitors. Interestingly, EGF induced lamellipodia formation and such invasive behavior was partly dependent on ERM activation through the SK2/S1P/S1PR2 pathway (*123*). This pathway is fully explored in chapters 3 and 4. Such a discovery will open new avenues for novel cancer treatments; EGF-dependent cancers can now be targeted from different angles. Because SK2, S1PR2, and ezrin inhibitors are commercially available, they can be tested for cancer prevention, or for slowing the progression of established cancers and metastases.

Table 1 lists ERM activators with well-defined biologies. Possibly, these stimuli act through the SK2/S1P/S1PR2 pathway to mediate this activation and the corresponding biology.

A number of these functions have already been described to be sphingolipid-related. Thus, these data warrant additional investigations.

Chapter 2

Sphingosine-1-phosphate receptor 2: A Review

Abstract

Sphingosine-1-phosphate (S1P) is a potent bioactive sphingolipid involved in cell proliferation, angiogenesis, inflammation, and malignant transformation among other functions. S1P acts either directly on intracellular targets or activates G-protein coupled receptors, specifically five S1P receptors (S1PRs). The identified S1PRs differ in cellular and tissue distribution, and each is coupled to specific G-proteins, which mediate unique functions. In this chapter, we describe functional characteristics of all five receptors, emphasizing S1PR2, which is critical in the immune, nervous, metabolic, cardiovascular, musculoskeletal, and renal systems. This chapter also describes the role of this receptor in tumor growth and metastasis, and suggests potential therapeutic avenues that exploit S1PR2.

2.1. Introduction

Lipids not only provide structure to the cell membrane, but they also exhibit bioactive roles, such as interaction with and activation of different pathways (e.g., phosphatidylinositol 4, 5-bisphosphate [PIP2], diacylglycerol [DAG], lysophosphatidic acid [LPA], and several sphingolipids). The sphingolipid family is comprised of multiple members: sphingomyelins and glycosphingolipids, which are major components of biological membranes. Other sphingolipids, such as ceramide, ceramide 1-phosphate (C1P), sphingosine, and sphingosine 1-phosphate (S1P) are often generated from the large pool of membrane sphingolipids in response to cellular signaling, and they contribute to the determination of cell fate.

Sphingolipid biosynthesis (see (*1, 124*)) normally occurs by condensation of coenzyme A-activated palmitoyl group transfer and L-serine to form 3-ketosphinganine. More recently, other amino acid-based sphingolipids have been described such as L-alanine, or L-glycine, to form 1-deoxy- or 1-deoxy-methyl-derivatives, respectively (*125, 126*). 3-ketosphinganine is reduced to sphinganine, and *N*-acylated with different fatty acids to generate dihydroceramides. Desaturation of the alkyl chain results in *de novo* synthesis of ceramide, which is considered to be a central component of sphingolipid metabolism. Complex sphingolipids, such as sphingomyelin and glycosphingolipids are generated from ceramide (*127*), which can be cleaved to form sphingosine, or phosphorylated, yielding ceramide 1-phosphate. Sphingosine can also be phosphorylated to form S1P, one of the most studied sphingolipids due to its bioactive roles in cellular biology and physiology (cellular proliferation, inflammation, migration, and angiogenesis).

Intracellular and extracellular S1P are under tight control by several enzymes.

Specifically, hydrolysis of complex sphingolipids is controlled by sphingomyelinases and glycosidases. Subsequently, ceramidases can hydrolyze ceramide to produce sphingosine, a direct precursor of S1P by the action of sphingosine kinases, SK(*128*)). S1P is also regulated by enzymes responsible for its degradation (S1P phosphatases, and S1P lyase). S1P's biological roles are mediated either directly by intracellular-targets (*37*), or by the action of five different transmembrane G-protein coupled receptors (S1PR1-5,(*129*)), which belong to the endothelial differentiation gene (EDG) family of receptors. S1P receptors participate in cellular responses based on the cell type and downstream available effectors. **Scheme 3** offers a depiction of the sphingolipid metabolic pathway.

In this chapter, the functional roles of S1P receptors are described, prefaced with a brief history of their discovery. S1PR1 and S1PR3 have been extensively studied, and will only be briefly discussed here. Next, S1PR4 and S1PR5, which are less well characterized, will be discussed more comprehensively. The main focus of this chapter will be on the S1PR2 receptor: specifically its normal physiologic functions, and its role in pathophysiology and in disease. Issues and apparent controversies surrounding the S1PR2 receptor will also be discussed.

2.2. S1P transporters

Before delving into S1PR activation, an understanding is needed of how S1P relocates to the cell exterior to activate its receptors in an autocrine or paracrine manner. Unlike sphingosine, S1P cannot freely traverse the lipid bilayer to leave the cell(*1*). Its polar nature prevents this; thus, it requires a specific transport mechanism. To date, two mechanisms have been proposed for S1P transport out of the cell. First, several members of the ABC (ATP-binding cassette) family of transporters have been thought to participate in this translocation (*130, 131*). CFTR

(Cystic fibrosis transmembrane receptor) has been implicated in S1P transport as well as LPA (lysophosphatidic acid) and dihydro-S1P in C127/CFTR cells (*131*). ABCC1, on the other hand, has been described in mast cells, and its inhibition affected the migratory capabilities of mast cells during inflammation (*130*). The second mechanism proposed is through the newly identified spinster-2 transporter in vascular endothelial cells. Mice lacking this protein have 60% less circulating S1P, and they have defective lymphocyte egress (*36*).

2.3. S1P receptors

Before 1995, S1P-mediated actions on cellular processes such as proliferation, cell movement, and intracellular calcium levels were thought to be primarily related to its intracellular second messenger effects. Also during that year—and thereafter—evidence accumulated that this sphingolipid acts on G-Protein coupled receptors (GPCRs). Goodemote *el al*. noted that some S1P-specific effects in Swiss 3T3 fibroblasts, such as DNA synthesis and calcium release, were inhibited by Pertussis toxin (*132*). One year later, Van Koppen and co-workers, reported that this toxin inhibited S1P-mediated increases in intracellular calcium and adenylyl cyclase inhibition (*133*). These effects were observed in a myriad of cell types (*133*) and suggested that S1P can act extracellularly at G-protein coupled receptors. S1PR1, previously known as EDG-1, was cloned in the 1990s as an early inducible gene in endothelial cells in response to Phorbol Myristate Acetate (PMA). S1PR1 induction gives rise to endothelial differentiation of capillary-like tubular structures (*134*). The ligand for S1PR1 remained unidentified for 8 years after the receptor had been cloned, until Lee et al. identified S1P to be a potent (kd=8.1 nM) and specific ligand for this receptor (*135*). The second S1PR was cloned in

rat aortic smooth muscle cells in 1993, referred to as S1PR2 (also known as EDG-5) (*136*). S1PR3 (EDG-3), was cloned three years later by Yamaguchi and colleagues (*137*). S1PR4 (EDG-6), was cloned in 1998 from human and murine dendritic cells (*138*). Interestingly, S1P was reported to bind each of these receptors with high specificity, and with similar high affinities (Kd = S1PR2: 27 nM; S1PR3: 23 nM; and S1PR4: 63 nM) (*139*).

The tissue and cellular distributions differ for each of these receptors, and these differences are summarized in **Table 2**. These factors, along with differences in G proteins to which each of receptor is coupled, account for differences in biological functions associated with the receptors, a topic discussed in the next section.

2.4. Signaling pathways associated with S1PRs

S1PRs can be coupled to different G proteins depending on the cell type where the receptor is located, and the specific receptor involved. Thus, activation of different pathways and subsequent engagement of different biological responses occurs in response to receptor activation. Whereas S1PR1 has been reported only to be linked to GaI, S1PR2 and S1PR3 have been reported to be coupled to different G proteins, depending on the cell type and the stimulus that activates the receptor. Thus, G protein associations for S1PR2 and S1PR3 include GaI, Ga12/13, and GaQ. In contrast, S1PR4 and S1PR5 can only activate GaI and Ga12/13 (*39*). Each of these G-proteins activates diverse pathways involved in cell proliferation, differentiation, and cell motility and morphology. GaI recruitment can, in turn, activate several pathways, among which is the small GTPase Rac, a well-known regulator of the cellular cytoskeleton and a promoter of cell migration. GaI can also enhance cell survival and inhibition of apoptosis by

activating the AKT pathway. Cell survival is supported by way of coupling to the GTPase Ras, which subsequently activates the ERK pathway (*37*). GaQ recruitment typically activates phospholipase C, which, in turn, promotes cell survival via several mechanisms through calcium release and activation of PKC and down stream targets, among which is the NF-kB pathway (*39*). Ga12/13 recruitment, in contrast, activates a critical GTPase involved in cellular dynamic modulation: Rho GTPase. The main functional consequences of Rho activation are inhibition of migration, compromised endothelial barrier integrity, and smooth muscle contraction (*140*). Examination of each G protein coupled to S1P receptors, and the different pathways that can be activated by each individual G protein, suggests diverse potential combinations and responses arising from receptor activation. This does not even account for the activation of several receptors within a single cell.

2.5. S1P receptors: Roles in health and disease

The body of work relating to the roles of the different S1PRs in normal physiology and in disease continues to grow. Important insights were elucidated about the physiologic roles of individual S1P receptors from S1P receptor knockout mice that revealed functional redundancy of several receptors with the exception of S1PR1. Knockout of S1PR1 in mice was embryonically lethal, giving rise to major vascular abnormalities. **Table 3** summarizes known phenotypic abnormalities, if any, for each of S1P receptor knockout mouse strains. When these knockout mice, or when cells arising from these mice, were subjected to stress, the necessity of these receptors was clear. Various pathophysiological conditions in which these receptors are important are briefly presented below.

2.5.1. S1PR1

Other than being essential for life (noted in S1PR1 knockout mouse embryos), the S1PR1 receptor gained scientific interest due to its involvement in multiple physiological functions. This receptor is essential for T- and B-lymphocyte egress from lymphoid tissue (*141*), and is differentially regulated during immune response development. S1PR1 up-regulation allows T cells to migrate from the thymus to the lymph nodes, where the receptor gets down-regulated to mediate T-cell retention in secondary lymphoid organs (*141*). The inhibition of this receptor using the immunosuppressant drug FTY720 has proven to be beneficial for patients with relapse-remitting multiple sclerosis (*142*). This compound is a sphingosine analogue that binds all S1PRs with the exception of S1PR2 after being phosphorylated by SK2. FTY720-mediated activation of S1PR1 leads to its ubiquitin-dependent degradation, which causes lymphocytes sequestration and thus immunosuppression (*143, 144*). It is currently used in the clinic, and is known as Fingolimod (*142*).

In addition S1PR1 (along with S1PR3) is not only responsible for proper angiogenesis, but also is needed for lymphangiogenesis through the phospholipase C/calcium pathway (*145*). Moreover, S1PR1 is critical for mediating S1P signaling in pro-inflammatory pathways such as COX-2 activation in rheumatoid arthritis synoviocytes (*146*), and for ICAM-1 expression in human endothelial cells (*147*). Finally, S1PR1 is reported to be a pro-tumorigenic receptor, promoting cancer cell migration and invasion in addition to tumor neo-vascularization. For example, in Wilms tumor, S1P-mediated exacerbations in malignancy occur through S1PR1 activation of PI3K and the pro-migratory Rac pathway (*148*). Also, S1PR1 knockdown *in vivo* dramatically inhibited tumor growth of implanted Lewis lung carcinoma cells by inhibiting new blood vessel formation within the growing tumor mass (*149*).

2.5.2. S1PR3

Studies that address the functional capabilities of S1PR3 alone have been historically scarce; only now is research being reported about this receptor. Several published observations suggest that S1PR3-mediated functions occur in co-ordination with S1PR1 or S1PR2. By itself, S1PR3 was revealed to have several important functions. Its expression in dendritic cells is essential for switching immune reactions to T helper cell TH1 responses. This immune conversion was evident when dendritic cells deficient in this receptor were implanted into wild type mice. The receptor activated the TH2/IL-4 response and provided renal protection against ischemic reperfusion injury in the mice (*150*). Moreover, S1PR3 is up-regulated in astrocytes in multiple sclerosis (*151, 152*), which is necessary for maintaining astrocyte activation, although the biological significance of this has yet to be determined: it could be detrimental or beneficial for disease progression (*152*). Due to the tight association of the S1PR3 receptor in some aspects of inflammation, S1PR3 has been proposed to be a potential biomarker to measure acute lung injury. S1PR3 is shed into the blood during sepsis, lowering blood vessel resistance while increasing vessel permeability (*153*). In cardiovascular disease, S1PR3 seems to be essential for macrophage recruitment to atherosclerotic lesion sites as well as promoting secretion of tumor necrosis factor (TNF) and monocyte chemoattractant protein-1 (MCP-1). In contrast, S1PR3 exerts an anti-proliferative and anti-migratory effect on smooth muscle cells after an arterial insult (*154*). In addition, S1P-mediated decreases in coronary blood flow are strictly caused by S1PR3 agonism. In fact, S1PR3 engagement leads to coronary smooth muscle contraction via increases in intracellular calcium and Rho activation (*155*). The role of S1PR3 in tumorogenesis has also been addressed: S1PR3 signaling was found to be closely associated with EGF signaling. In lung cancer, S1PR3 increased expression of the EGF receptor in a Rho-dependent manner leading to increased proliferation and anchorage-independent growth (*156*). In breast

cancer, however, S1PR3 transactivates the EGF receptor in response to estrogen stimulation, and thus amplifies its signaling. In both cases, S1PR3 could be a potential target for cancer therapy (*157*).

2.5.3. S1PR4

S1PR4 is mainly expressed in the hematopoietic system, thus most functions attributed to it are described for that particular system. Physiological roles for the receptor are unclear at this time. However, the few studies in the literature offer the following observations about the receptor. First overexpression of this receptor in CHO cells induced cell rounding and stress-fiber formation via coupling with Gi and Ga12/13. Overexpression of S1PR4 in Jurkat T cells, in contrast, induced cell motility even in the absence of exogenous S1P (*158*). Later studies disproved the role of S1PR4 in affecting T cell motility in mice. T cells expressing only S1PR4, but not S1PR1, failed to migrate not only towards S1P, but also toward several chemotactic stimuli (*159*). S1PR4, however, was essential in promoting inhibitory effects of S1P in T cells. S1PR4 inhibited T cell proliferation, switching the cells to an inhibitory phenotype that secretes interleukin (IL)-10, but not IL-2, IL-4 or interferon (IFN)-gamma (*159*). Next, a recent study using S1PR4 knockout mice by Schulze *et al*. suggested that S1PR4 had only a modest effect on T-cell function (*160*). Rather, the main defect in the knockout mouse was found within dendritic cell differentiation and cytokine secretion. Specifically, dendritic cells could no longer switch T cells to the Th17 phenotype, thereby switching the immune response to a TH2 response (*160*). Finally, S1PR4 inhibition may have a therapeutic value: S1PR4 knockout mice were protected from dextran sulfate-induced colitis (*160*).

2.5.4. S1PR5

S1PR5 has been studied in a limited fashion, chiefly investigated in the brain and within NK cells, where it is highly expressed. Some studies also implicate S1PR5 in tumorigenesis. In the brain, S1PR5 expression is limited to oligodendrocytes and their precursors. The functional consequences of S1PR5 activation in these cells depend on their developmental stage. The S1PR5 receptor mediates process retraction (*161*) and impedes migration of immature oligodendrocytes (*162*) through a Rho kinase-dependent pathway whereas S1PR5 mediates survival of mature oligodendrocytes, through an AKT-dependent pathway (*161*). S1PR5 is gaining attention due to the high expression of S1PR5 in NK cells and its essential role in NK egress into the lymphatic system (*163*) (See **Table 3**). The Duane mouse, an animal strain with a mutation in the Tbx21 gene encoding the T-bet transcription factor, is similar in phenotype to the S1PR5 knockout mice. The Duane mouse was reported to have dramatically fewer S1PR5 transcripts and proteins, and T-bet was shown to induce S1PR5 expression by binding to its promoter region (*164*). Furthermore, S1PR5 is reported to be key for maintaining blood brain barrier (BBB) integrity as well as its immunological quiescence (*165*). In fact, S1PR5 increases BBB tight junctions, decreasing its permeability. S1PR5 also lowers monocyte transendothelial migration into the brain parenchyma, which decreases inflammation (*165*). Also, S1PR5 can decrease inflammation by inhibiting NF-kb activation, and subsequent cytokine secretion (*165*). Finally, studies to investigate the effect of S1PR5 signaling in tumor development are scarce and conflicting. Autophagy, a mechanism by which some cancers thrive during conditions of scarce nutrient supply, is induced by S1P in prostate cancer cells in an S1PR5-dependent manner (*166*). In contrast, S1PR5 inhibits migration and proliferation of esophageal cancer cells, and, in fact, these cancerous cells may down-regulate the S1PR5 receptor to escape cellular control (suggested by Hu et al. (*167*)). Further studies are needed to elucidate the function of this

receptor in malignancy because it may represent a potential target for chemotherapy.

2.6. Roles of S1PR2

2.6.1. S1PR2 in mediating different cellular functions and pathologies

2.6.1.1. Endothelial cell functions

S1PR2 has been implicated in increasing the capillary paracellular permeability. In fact, S1PR2 stimulation causes stress-fiber formation and disrupts adherens junctions between endothelial cells (*168*). This effect has been attributed to the ability of S1PR2 to couple to the Rho-ROCK pathway which will, in turn, activate phosphatase PTEN, thereby inhibiting the PI-3 kinase pathway (*168*). TNF- and lipopolysaccharide (LPS)-induced swelling and edema have been reported to arise from increased dermal microvasculature permeability attributed to increased S1PR2 expression (*169*). S1PR2 is also known to modify endothelial function with respect to wound healing: S1PR2 expression increases in senescent endothelial cells as reported by Lu and colleagues (*170*). This increased expression is responsible for malfunctions of endothelial cells associated with aging, such as impaired wound healing due to decreased tube formation and impaired endothelial cell migratory capabilities (*170*). These impairments were reversed by the expression of a dominant-negative PTEN (*171*), which confirms this phosphatase as a downstream target of S1PR2 in endothelial cells. S1PR2 is also up-regulated by hypoxic stress in the retina, ultimately leading to defective neovascularization (*172*). In fact, a component of this retinopathy is due to inhibition of eNOS expression and induction of COX-2 by S1PR2 (*172*). This validates S1PR2 as a regulator of the inflammatory response, a topic that will be explored in greater detail later in this review.

2.6.1.2. Metabolic functions

Evidence is emerging that S1PR2 functions in the liver and pancreas that suggests important metabolic dimensions for this receptor. S1PR2 blockade has been involved in protection against streptozocin-induced diabetes in mice. In fact, S1PR2-/- mice were protected from pancreatic beta cells apoptosis, had higher insulin, and lower glucose (*173*). It has also been described to be the receptor for conjugated bile acids in the liver (*174*). Their ligation to this receptor was shown to regulate multiple hepatic metabolic pathways including glucose control, bile acid synthesis, and lipid metabolism through the activation of AKT and ERK1/2 pathways. Interestingly, these actions are mediated through S1PR2 activation (*174*). Actions of S1PR2 on hepatocytes are not only limited to regulating their metabolic functions, but also extend to regulation of hepatocyte regeneration after injury (*175*). In fact, the liver of S1PR2-/- mice was less fibrotic and had increased regeneration after hepatic injection with the model hepatotoxicant, carbon tetrachloride. Thus, targeting this receptor in patients suffering from early stages of liver cirrhosis may be a future experimental goal.

2.6.1.3. Allergy and immunity

The role of S1PR2 in anaphylaxis is unclear. One laboratory has reported that it is essential for mast cell activation and degranulation in response to FceRI cross-linking, and that its activation leads to release of histamine, the chemokine CCL2, and TNF, which aggravates anaphylactic shock (*176*). Another group reported that S1PR2 signaling contributed to histamine clearance, and actually shortened the duration of shock (*177*). Such discrepancies have uncertain origins, but an agonist or an antagonist for treatment of type 1 allergic reactions may warrant further study. S1PR2 functions are not limited to mast cells, but they extend to macrophages. Whereas S1P has been shown to mediate lymphocyte egress and migration through S1PR1, S1P inhibits macrophage migration to the inflammation site via S1PR2 activation (*178*). This

response is partly achieved by cAMP production, which activates PKA (*178*). In contrast, S1P enhances phagocytosis of *Cryptococcus neoformans* by macrophages via S1PR2-mediated expression of FC gamma receptors 2 and 3 (*179*).

2.6.1.4. Musclar functions

S1PR2 studies in muscle cells are extensive. This receptor has been implicated in muscle cell chemotaxis (*180*), proliferation (*147, 181, 182*), differentiation (*182, 183*) and contraction (*184, 185*). Early studies of S1PR2 in muscle cells were reported in 2002 (*186*). These findings revealed that, along with S1PR3, S1PR2 is responsible for the calcium peak observed in myoblasts after S1P treatment (*186*), but no definitive mechanism was proposed to explain this. In contrast, investigators reported that S1P inhibited migration of vascular smooth muscle cells via action on S1PR2 (*180*). The mechanism behind this was reported to be coupling of the receptor to Gq and G12/13 for the purpose of activating small GTPase Rho and inhibiting Rac, leading to cell migration arrest (*180*). This activity is not limited to smooth muscle cells, but also occurs in other cell types, which are discussed later. Increased smooth muscle proliferation and migration is usually observed in neo-intimal lesions, giving rise to blood vessel blockade and thrombosis. Therefore, S1PR2 activity was thought to be beneficial for the prevention of formation and growth of these lesions due to Rho-dependent reduced arterial smooth muscle cell proliferation and migration (*147, 181*). S1PR2 can also induce the transcription of smooth muscle differentiating genes, such as alpha-actin, by promoting binding of serum response factor to the promoter of these genes (*181*). The study of mechanism of RhoA activation by S1PR2 revealed that a RhoA-specific guanine exchange factor (GEF), referred to as leukemia-associated Rho GEF (LARG), activated RhoA, to promote expression of smooth muscle differentiating genes (*183*). The role of S1PR2 in skeletal and muscle stem cells (satellite cells) was different

from its role in vascular smooth muscles. In fact, S1P-mediated muscle regeneration and proliferation after injury was shown to depend on S1PR2 signaling (*182*). Moreover, S1PR2 activated signal transducer and activator of transcription 3 (STAT3), which subsequently repressed cell cycle inhibitors p21 and p27 (*182*). Finally, although S1PR2 stimulation caused smooth muscle contraction (*184, 185*), the mechanism by which this happens depends on the tissue of origin. For example, in bronchial smooth muscle cells, S1P/S1PR2-mediated bronchoconstriction occurs through the Rho/ROCK pathway (*184*), whereas in mesenteric vascular smooth muscles, S1P/S1PR2-mediated vasoconstriction occurs via Rho-independent activation of the p38-MAPKinase pathway (*185*).

2.6.1.5. Neuronal functions

Blockade of S1PR2 by a specific inhibitor, JTE013, was revealed to significantly augment the migration of neural progenitor cells toward areas of brain infarction (*187*). This was proven in *in vitro* models of cell migration as well as in *in vivo* mouse models, in which JTE013 was administered to brain ventricles (*187*). Thus, JTE013 may be a therapeutic target for stroke patients. Subsequent studies using S1PR2-/- mice are intriguing; S1PR2 in the brain is chiefly expressed in the hippocampus, and its absence caused extensive gliosis in this area. Thus, the mice were not only susceptible to lethal seizures, but also they suffered from CNS functional impairments such as deficits in spatial working memory and increased anxiety (*188*).

2.6.1.6. Kidney functions

The role of S1PR2 in the kidney is uncertain, as well. In the context of diabetic nephropathy, S1PR2 is up-regulated in mesengial cells compared to normal rats, leading to stimulation of fibronectin synthesis and accumulation in the mesengium, which is a hallmark of

this disease (*189*). S1PR2 in mesengial cells is tightly coupled to the ERK-MAP kinase pathway which is essential for fibronectin up-regulation (*189*). In contrast, S1PR2 has been shown to exacerbate ischemia-reperfusion (IR) injury in renal proximal tubules (*190*). For the first time, studies revealed that S1PR2 inhibition induces SK1 expression and S1P secretion (*190*). Furthermore, the protective effects of this antagonism were thought to be due to increased S1P production and its interaction with S1PR1, rather than arising from S1PR2 alone (*190*). These data were the first experiments to use the novel S1PR2 agonist, SID46371153 (*190*).

2.6.2. S1PR2 in Cancer

2.6.2.1. S1PR2 as an anti-cancerous receptor

Most scientific evidence supports an anti-migratory effect of S1PR2, and it is also thought to be anti-tumorigenic, inhibiting cells from undergoing distant metastasis. Early reports regarding S1PR2 and tumor formation emerged in 2003, specifically in a mouse melanoma model (*191, 192*). Melanoma B16F10 cells chiefly express S1PR2, and their migration was enhanced by receptor blockade with JTE013. Interestingly, S1PR2 not only inhibited migration via activation of RhoA, but also by simultaneously inhibiting the pro-migratory G protein Rac in response to S1P (*192*). To further explore the role of this G-protein coupled receptor in metastasis, S1P pre-treatment of these melanoma cells dramatically decreased lung metastasis in an S1PR2 dependent manner in tail-vein injection mouse models (*191*). The physiologic role of such a system warrants investigation; S1P is abundant in plasma, and its presence does not inhibit melanoma metastasis, as pre-treatment with S1P did. The anti-tumorigenic actions of S1PR2 were further explored in glioblastomas (*140*). The anti-migratory phenotype of S1PR2 was discerned in glioblastoma cell lines that express this receptor by way of activation of RhoA and its downstream effector, ROCK, without the need for inhibiting Rac signaling (*140*).

Interestingly, the anti-tumorigenic capabilities of S1PR2 are not limited to its anti-migratory effect, but are also related to its ability to inhibit proliferation and growth as observed in Wilms tumor (*193*). In fact, S1P treatment of WiT49 cells prompted expression of the anti-proliferative connective tissue growth factor (CTGF) in an S1PR2-dependent manner. These data represent some of the few studies to prove that S1PR2 can induce gene expression though the ROCK/c-jun axis (*193*). Later reports showed that S1PR2 KO mice develop diffuse large B cell lymphomas at advanced ages (*194*), thereby identifying a novel role for S1PR2 in B cell germinal-centered hematopoiesis. Interestingly, a quarter of patients with such a malignancy were found to harbor a mutation in S1PR2 (*194*). Taken together, this set of data suggests important anti-tumor functions of S1PR2.

2.6.2.2. S1PR2 as a pro-cancerous receptor

The first report implicating S1PR2 as a pro-cancerous receptor dates back to 2000 when studies were conducted in hepatoma and Jurkat cells (*195*). Co-transfection of S1PR2 and S1PR3 in these cells led to S1P-mediated cellular proliferation and inhibition of apoptosis. These effects were attributed to activation of the ERK/MAPKinase pathway, along with the immediate initiation of c-jun and c-fos transcription in a Rho-dependent manner (*195*). Pro-tumorigenic effects of this receptor are not only linked to its ability to affect cell movement, but also its effects on protein stability as observed in chronic myeloid leukemia (*196*). In fact, S1PR2 inactivates the phosphatase PP2A, which normally dephosphorylates the constitutively active bcr-abl, and tags it for proteosomal degradation. Reports support the idea that S1PR2 may constitute a novel target in chronic myelogenous leukemia (CML) patients who were resistant to imatinib treatment (*196*). Finally, S1PR2 was shown to enhance tumorigenesis by down-regulating tumor suppressor proteins as reported in bladder cancer (*197*). Although detailed

mechanistic studies of such down-regulation were not addressed, inhibition of S1PR2 dramatically increased tumor suppressor breast carcinoma metastasis suppressor 1 (brms1), leading to a decrease in prostate cancer growth and lung metastasis (*197*).

Lastly, our group has very recently identified a novel signaling function that is specific for S1PR2. We showed that S1PR2 is essential for the activation of the ezrin radixin moesin (ERM) family of proteins in response to S1P and epithelial growth factor (EGF). ERM proteins are adaptor molecules linking the actin cytoskeleton with the plasma membrane, and they have been implicated in filopodia and lamellipodia formation, thus cancer invasion. Inhibition of this receptor in HeLa cells caused a dramatic decrease in the EGF-induced invasion in an ERM dependent manner. The identification of S1PR2 as one of the effector arms in the EGF-induced malignancies could constitute an important target for therapy (*123*).

In conclusion, the effects of S1PR2 on tumor growth and progression are cell-type specific. Possibly, the specific G protein coupled to that receptor may dictate its biologic functions. Therefore, inhibition or stimulation of S1PR2 can prove to be beneficial or harmful, depending on the tumor being studied. Contrasting data presented previously suggest that several questions remain unanswered. One such question is whether JTE013 in patients with leukemia will predispose them to develop secondary types of cancer such as Wilms tumor or lymphomas.

2.6.3. S1PR2 agonist and antagonist

JTE013 is the most used S1PR2 antagonist. It was synthesized at the Central Pharmaceutical Institute in Japan during 2001 (*198*). It is a pyrazolopyridine derivative, that was shown to antagonize the binding of radiolabeled S1P in CHO cells overexpressing S1PR2 (*199*). The specificity of JTE013 to S1PR2 is debatable. This compound did not inhibit S1P-induced

calcium mobilization in HCT4 cells overexpressing S1PR2, but also in those overexpressing S1PR4 (*200*). Its action on S1PR4 have also been eluded in MDA-MB-453 cells. These cells express endogenous S1PR2 and S1PR4. JTE-013 inhibited S1P-induced ERK phosphorylation in these cells, whereas the use of S1PR2 siRNA did not (*200*). Furthermore, JTE013 was still effective in abolishing S1P-induced vasoconstriction in S1PR2 knockout mice (*199*). Therefore, the use of JTE013 as the only method to prove S1PR2-mediated events is not sufficient, and could explain the discrepancies in the literature regarding this receptor.

On the other hand, SID46371153 is the first novel reported S1PR2 agonist (*201*). It was discovered after a high throughput screening of thousands of compounds in 2007. Its specificity for S1PR2 was demonstrated using CHO cells overexpressing S1PR2 and the cAMP response element-beta lactamase (CRE-BLA) reporter construct (*201*). It has an EC50 of 0.72 μM (*201*). This compound has only been used once so far in-vivo studies assessing ischemic-reperfusion injury in kidneys as mentioned above (*190*).

2.7. Conclusion

S1PR2 has diverse functions and has been implicated in many organ-system pathologies. However, a simple single conclusion cannot be drawn about the receptor with respect to any organ or system. The literature contains conflicting data that diverge to the degree that we cannot generalize much about the receptor and its effects. Unique data reported in the literature can arise from the use of different cell lines, even if the cells are from the same organ system. Also, S1PR2 in each cell line may be coupled to a different G protein, and therefore a different signaling pathway. Also, the use of S1P receptor inhibitors for studies may have various specificities, with multiple non-specific functions. Thus, investigations should be consistent with

respect to cells used for studies. For example, they should probably be derived from S1PR2 -/- mice. In addition, S1P receptors have redundant functions as evidenced by studies in knockout mice. Thus data should be assessed carefully when receptor inhibition studies reveal no discernible effects. Future assessments are needed to clarify downstream effects of S1PR2.

Now equipped with an in-depth knowledge regarding bioactive sphingolipids, the ERM family of proteins and the different S1P receptors, the reader is ready to dive into the following three chapters. Chapter 3 will evaluate the role of the different S1P receptors, and specifically S1PR2 in epidermal growth factor-induced ERM activation and cancer cell invasion. Next, chapter 4 will dissect the mechanism of S1P generation following EGF treatment and its implications on S1P signaling and ERM regulation. Finally, chapter 5 will assess the kinase responsible for ERM phosphorylation downstream of the sphingolipid pathway.

Chapter 3

Epidermal growth factor-induced cellular invasion requires sphingosine 1-phosphate/sphingosine 1-phosphate 2 receptor-mediated ezrin activation

Abstract

ERM (Ezrin, Radixin and Moesin) proteins link cortical actin to the plasma membrane and coordinate cellular events that require cytoskeletal rearrangement, including cell division, migration and invasion. While ERM proteins are involved in many important cellular events, the mechanisms regulating their function are not completely understood. Our lab previously identified reciprocal roles for the sphingolipids ceramide and sphingosine 1-phosphate (S1P) in the regulation of ERM proteins. We recently showed ceramide-induced activation of PP1α leads to dephosphorylation and inactivation of ERM proteins, while S1P results in phosphorylation and activation of ERM proteins. Following these findings, we aimed to examine known inducers of the SK/S1P pathway and evaluate their ability to regulate ERM proteins. We examined EGF, a known inducer of the SK/S1P pathway, for its ability to regulate the ERM family of proteins. We found that EGF induces ERM c-terminal threonine phosphorylation via activation of the SK/S1P pathway, as this was prevented by siRNA knock down or pharmacological inhibition of SK. Using pharmacological as well as genetic knock down approaches, we determined that EGF induces ERM phosphorylation via activation of S1PR2. Additionally, EGF led to cell polarization in the form of lamellipodia and this occurred through a mechanism involving S1PR2-mediated phosphorylation of ezrin T567. EGF-induced cellular invasion was also found to be dependent on S1PR2-induced T567 ezrin phosphorylation, such that S1PR2 antagonist, JTE-013, and expression of a dominant negative ezrin mutant prevented cellular invasion towards EGF. In this chapter, a novel mechanism of EGF-stimulated invasion is unveiled, whereby S1P-mediated activation of S1PR2 and phosphorylation of ezrin T567 is required.

3.1. Introduction

Ezrin, Radixin and Moesin (ERM) make up a portion of the ERM family of proteins which are involved in linking cortical actin to the plasma membrane, and are therefore involved in the many cellular processes that coordinated changes in cytoskeletal architecture (*202*). ERM proteins also function as signaling platforms, acting as a scaffold for the necessary arrangement of effector molecules (*202*). ERM proteins have been implicated in numerous cellular functions including spindle formation and cytokinesis (*203*), migration (*202*) and invasion (*202*). Given the pro-survival and proliferative events in which the ERM family of proteins is involved, it is not surprising that deregulation of ERM proteins have been related to many cancers, even serving as a potential biomarker and prognostic indicator of disease(*204*). Targeting the ERM family of proteins and the mechanisms that regulate their activity may serve as a novel therapeutic modality for the treatment of various cancers and other proliferative diseases.

The conformation of ERM proteins dictates their activity, such that when the N- and C-termini are folded onto one another, the protein is inactive and therefore unable to coordinate changes in the cytoskeleton (*205*). On the other hand, ERM proteins are activated when phosphorylated on the C-terminal threonine residue (Ezrin T567, Radixin T564 and Moesin T558) following binding to plasma membrane phospatidylinositol 4, 5 bisphosphate (PIP2) (*206*). While the N-terminus of ERM proteins binds the plasma membrane, the phosphorylated C-terminus binds F-actin (*207*), thereby linking changes in actin to changes in the plasma membrane (*207*). Many ligands are known to induce the phosphorylation of ERM proteins including Epidermal Growth Factor (EGF) and Platelet Derived Growth Factor (PDGF) (*208*). Recently, however, novel mechanisms of ERM regulation by sphingolipids were identified in our lab (*206, 209, 210*). ERM proteins were first found to be negatively regulated by cisplatin-

mediated activation of acid sphingomeylinase (*211*) and subsequent production of ceramide. More recently, our lab has identified a novel PIP2-independent mechanism of ERM regulation; whereby ERM proteins are dephosphorylated via ceramide-mediated activation of PP1α (*206*). Moreover, work from our lab identified sphingosine 1-phosphate as an inducer of ERM phosphorylation and activation (*209, 210*). Following these findings, we became interested in S1P regulation of ERM proteins and therefore examined known inducers of the SK/S1P pathway for their ability to regulate phosphorylation and subsequent activation of ERM proteins.

Sphingosine kinase (SK), the enzyme responsible for the production of the ERM-activating lipid S1P, undergoes regulation by numerous extracellular ligands, including EGF (*212*), such that EGF has been shown to activate both SK isoforms, SK1 and SK2, (*212, 213*) resulting in production of S1P and activation of S1P cell-surface receptors (S1PR1-S1PR5) (*214*). In this chapter, we examine the role of the SK/S1P pathway in EGF-mediated ERM phosphorylation. We found that EGF induces phosphorylation of ERM proteins and pharmacological inhibition or siRNA knockdown of SK1 and SK2 inhibited this. Also, S1P signaling through S1PR2 was found to be necessary for EGF-induced ERM phosphorylation. Cellular invasion induced by EGF was also dependent on SK/S1P/S1PR2 activation of ERM proteins. A novel mechanism of EGF-induced invasion is presented, and it involves sphingolipid regulation of ERM proteins. It also clarifies the role for S1PR2 in the regulation of cellular invasion. These findings highlight the importance of the SK/S1P pathway as a secondary messaging system and reveal a potential therapeutic target for the treatment of cancers that are or have become resistant to EGF-targeted treatments, such as that which often occurs in Non-Small Cell Lung Carcinomas (NSCLC) (*215, 216*).

3.2. Materials and Methods

3.2.1 Materials

High glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), rhodamine-phalloidin penicillin-streptomycin, and Superscript III First-Strand Synthesis kit were purchased from Invitrogen (Carlsbald, CA). Essentially fatty acid free bovine serum albumin (BSA) and monoclonal anti-β-actin antibody were from Sigma-Aldrich (St. Louis, MO). D-*erythro*-sphingosine 1-phosphate (S1P) and D-*erythro*-17C sphingosine (17C-Sph) were from Avanti polar lipids (Alabaster, AL). Anti-phospho ERM antibody was from Cell Signaling Technology (Danvers, MA). Anti VSV-G and HRP-labeled secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Chemiluminescence kit was from ThermoScientific (Suwanee, GA). Draq5 and 488-conjugated secondary antibody were purchased from Alexis Biochemicals (San Diego, CA). SYBR Green was purchased from Bio-Rad (Hercules, CA). JTE-013, BML-241, VPC23019 and SKi-II (4-[4-(4-chloro-phenyl)-thiazol-2-ylamino]-phenol) (*217*) were purchased from Cayman Chemical (Ann Arbor, MI). S1P2R-GFP plasmid construct was purchased from Origene (RG210163, Rockville, MD), SKX (amidine-based SK inhibitor) was a kind gift from Dr. Kevin Lynch and Dr. Andrew Bolt of SphynKx Therapeutics (Charlottesville, VA) (*218*). Tumor Invasion System 24-multiwell, Calcein AM fluorescent dye, and human recombinant EGF were from BD Biosciences.

3.2.2. Cell lines and Culture Conditions

HeLa cells were originally purchased from American Type Culture Collection (ATCC, Manassas, VA). and maintained as described previously (*209*). Briefly, DMEM was supplemented with 1% penicillin-streptomycin and 10% FBS and cells were incubated in standard culture conditions: 37˚C, and 5% CO_2. When serum free medium was used, 0.1% BSA,

1% penicillin-streptomycin and 10mM HEPES were supplemented to DMEM. In all cases, before conducting treatments, cells were serum starved overnight (16-18h).

3.2.3. Immunoblotting

Immunoblotting was performed as previously described (*209, 210*). Briefly, after removal of media, cells were directly lysed in buffer containing 1% (w/v) SDS. Next, cells were sonicated and boiled before proteins were separated via SDS-PAGE (4-15%, Tris-HCl) using the Bio-Rad Criterion system. Proteins were transferred to nitrocellulose membranes and blocked for at least 1 h with 5% nonfat milk in PBS/0.1% Tween 20 (PBS-T). Membranes were incubated with primary antibody diluted 1:000 pERM or 1:3000 β-actin at 4°C overnight. Secondary antibody incubation occurred for 1 hour at room temperature at a 1:5000 dilution. Visualization was carried out per manufacturer protocol.

3.2.4. RNA Interference

Gene silencing was carried out as described previously, with minor changes (*209*). Using siRNA purchased from Qiagen (Valencia, CA), directed against human SK1, SK2 and S1PR2: SK1, 5′-AAGGGCAAGGCCTTGCAGCTC-3′; SK2, Hs_SPHK2_5 FlexiTube siRNA SI00288561 (FlexiTube siRNA, experimentally verified, Qiagen); S1PR2, Hs_EDG5_6 FlexiTube siRNA SI02663227 (FlexiTube siRNA, experimentally verified, Qiagen). Scrambled siRNA was used as a negative control: SCR, Negative control siRNA (1027130, Qiagen). All-Star siRNA was also used a negative control and was obtained from Qiagen, Hela cells were seeded at 30% confluence in DMEM with 10% FBS. One day later, siRNA transfection was carried with the corresponding siRNA at final concentration of 20nM using Oligofectamine

transfection reagent from Invitrogen (Grand Island, NY) and following the manufacturer protocol. 36 hours later, Hela cells were serum starved overnight. All treatments were carried out 48 hours following siRNA transfection.

3.2.5. Quantitative Real Time Reverse Transcription-PCR

Cells were washed with ice-cold PBS, then directly lysed with the lysis buffer provided in the RNA mini-easy kit from Qiagen. RNA extraction was carried out as per the manufacturer protocol. RNA was then quantified using Thermo Scientific NanoDrop. 1ug of RNA was converted into cDNA using SuperScript III First-Strand Synthesis Systems and following Invitrogen protocol. The cDNA was diluted (1:15) and 5ul was used per 25ul reaction. Each 25 μl real-time RT–PCR contained a ratio of 12.5:0.5:0.5:6.5 [SYBR Green/10 μM F (forward) primer/10 μM R (reverse) primer/distilled water]. Using Applied Biosystems® 7500 Real-Time PCR System, PCR reactions conditions were carried out as described previously by our group (*209*)The following primers sequences were used to detect expression: human β-actin F, 5′-ATTGGCAATGAGCGGTTCC-3′; human β-actin R, 5′-GGTAGTTTCGTGGCCACA-3′; hSK1 F, 5′-CTGGCAGCTTCCTTGAACCAT-3′; hSK1 R, 5′-TGTGCAGAGACAGCAGGTTCA-3′; hSK2 F, 5′-TTGCTCAACTGCTCACTG-3′; hSK2 R, 5′-AGACAGGAAGGAGAAACAG-3′. Using Q-Gene software, β-actin was used to normalize the obtained threshold cycle values that were shown as mean normalized expression.

3.2.6. C17-Sphingosine Labeling

Hela cells were plated at 150,000 cells/60mm dish. Once approximately 75% confluent, cells were serum starved overnight 16-18h. Following starvation, cells were pretreated for 1h with vehicle or 0.3 μM. 1 μM C17 sphingosine was added and allowed to equilibrate for 20

minutes. 10ng/mL EGF was added for 2 minutes and media was removed and the reaction was stopped with lipid extraction buffer and sent for analysis at the MUSC shared Lipidomics core facility.

3.2.7. Immunofluorescence and Confocal Microscopy

Laser-scanning confocal and Immunofluorescence microscopy analysis were carried out as previously described (*209*). Grown on poly-d-lysine coated confocal dishes (MatTek Corporation), cells were deprived of serum prior to treatments. Cells were fixed, permeabilized, blocked then exposed to primary antibodies (1:500) and secondary antibodies, according to Manufacturer's protocol. Using a LSM510 META confocal microscope (Carl Zeiss, Inc.) and Leica, SP8 confocal microcopy system, photo images were obtained and analyzed using LSM Image Browser Software (www.Zeiss.com) and Leica software respectively.

3.2.8. Plasmid Constructs and Transient Transfections

S1P2R-GFP plasmid Full-length ezrin cDNA, VSV-G tag pCB6 plasmids has been previously described (*209, 211*). Ezrin point mutations were generated as previously described (*209*). Cells, growing on 35 mm dishes or 100 mm dishes, were transfected with 1-3μg of pCB6-WT Ezrin, pCB6-TA Ezrin or S1P2R-GFP plasmid DNA using Lipofectamine (pCB6 constructs) (Invitrogen) or Effectene (pEGFP construct) (Qiagen) transfection reagent according to the manufacturer's instructions.

3.2.9. Cellular Invasion Assays

Cell invasion studies were carried out using the BD Biosciences Tumor Invasion System (345166) according to manufacturer's protocol, with minor changes as previously

described (*209*). Briefly, following serum starvation cells were removed from culture dish and resuspended at 200,000 cells/mL 750µL appropriate media or media plus chemo attractant was placed in the well of a 24-well plate, followed by 500µL cell suspension in SF media, plus or minus 5µM JTE013, in the apical chamber of the trans well insert. Cells were allowed to invade under normal cell culture conditions for 48h. After 48h, invading cells were stained with Calcein AM and then visualized under fluorescence microscopy total invading cells were counted using NIH Image J software.

3.3. Results

3.3.1. EGF induces ERM phosphorylation in a sphingosine kinase-dependent manner

EGF is known to induce ERM phosphorylation, and in order to determine if this occurs in a SK/S1P-dependent manner, phospho-ERM responses to varying doses and times of EGF treatment were characterized **(Fig. 1)**. While using S1P as a positive control for the induction of ERM phosphorylation (*209, 219*), we established the range of EGF concentrations that activate ERM proteins by treating cells with increasing doses of EGF for 5 minutes. As little as 1ng/mL EGF was able to evoke ERM phosphorylation which increased further with increasing doses of EGF, up to 100ng/mL **(Fig. 1)**. In addition to examining the doses of EGF that phosphorylate and activate ERM proteins, we examined the temporal regulation of ERM proteins by EGF **(Fig. 1)**. HeLa cells treated with 10ng/mL EGF displayed acute phosphorylation of ERM proteins beginning as early as 0.5 minutes and decreasing by approximately 15 minutes **(Fig. 1)**. These data verify previous reports that EGF regulates ERM proteins through threonine 567 phosphorylation (*208, 220, 221*).

Next, in order to assess the role of SK in EGF-mediated ERM phosphorylation, both genetic and pharmacological approaches were used. First, the effect of siRNA-mediated sphingosine kinase down regulation on EGF-induced phospho-ERM was determined. HeLa cells were pretreated with siRNA targeting SK1, SK2 or both SK1 and SK2 and knockdown confirmed **(Fig. S1)** (*209*) (*222*). Following 48 hours of sphingosine kinase knockdown, cells were treated with 10ng/mL EGF for 2 minutes and ERM C-terminal phosphorylation was evaluated via western blot **(Fig. 2A).** Cells treated with control All Star siRNA displayed robust ERM phosphorylation following 5 minutes of EGF treatment. In contrast, knockdown of SK2 and not SK1, diminished EGF-induced phosphor-ERM. Furthermore, double knockdown of SK1 and SK2 was comparable to the SK2 knockdown alone **(Fig. 2A)**. These data suggest that SK2 is required for EGF-induced ERM phosphorylation.

To further consolidate the role of SK in EGF activation of ERM proteins, two pharmacological inhibitors of SK were employed; SKX (*218*), an amidine-based, SK inhibitor and SKi-II (*217*), a non-lipid, small molecule inhibitor of SK. HeLa cells were pretreated with DMSO vehicle or SK inhibitor for 1 hour, prior to addition of 10ng/mL EGF for 2 minutes and ERM phosphorylation was examined **(Fig. 2B, 2C).** The EGF-mediated phospho-ERM response remained intact in cells subjected to DMSO vehicle treatment **(Fig. 2B, 2C)**. In contrast to DMSO vehicle-treated cells, cells pretreated with 0.1μM SKX or 10μM SKi-II did not elicit phospho-ERM responses following EGF treatment **(Fig. 2B, 2C)**. These findings, taken together, provide evidence solidifying a role for SK in EGF-induced ERM phosphorylation.

3.3.2. EGF activates the SK/S1P pathway

Based on the data above, it became important to determine if EGF-induced ERM phosphorylation requires activation of SK and subsequent S1P signaling. With the purpose of determining if EGF induces activation of SK and consequent S1P production, 17C-sphingosine (sphingosine with a 17-carbon backbone) labeling was utilized (*223*) **(Fig. 3 A)**. HeLa cells were first exposed to 17C-sphingosine, and then treated with 10ng/mL EGF. Next, HPLC/MS was used to measure incorporation of 17C-sphingosine into 17C-S1P. Media from cells treated with EGF contained twice the levels of 17C-S1P than did media from non-EGF-treated cells, supporting previous reports of EGF-mediated SK activation and S1P production (*218*). Next, to determine if EGF-induced S1P generation was the result of SK activation, a pharmacological inhibitor of SK was employed. The presence of 17C-S1P in the media from cells treated with SKX alone was undetectable; furthermore, addition of EGF to SKX-treated cells did not induce 17C-S1P generation. These data corroborate reports that EGF activates the SK/S1P pathway (*212, 224, 225*) in HeLa cells. With the aim of examining the physiological impact of EGF-generated extracellular S1P, S1P receptor internalization was used as a tool. Using confocal microscopy, GFP-tagged S1PR2 receptor localization was observed primarily at the plasma membrane in untreated cells **(Fig. 3B)** or cells treated with SK inhibitor, SKi-II **(Fig. 3C)**, or S1PR2 antagonist JTE-013 **(Fig. 3D)** alone. On the other hand, cells treated with 5 μM sphingosine **(Fig. 3E)** or 100nM S1P **(Fig. 3F)**, exhibited GFP-S1PR2 redistribution to punctate intracellular spots, which is indicative of receptor internalization occurring in response to activation. Treating HeLa cells with EGF also resulted in S1P receptor internalization **(Fig. 3H),** suggesting that S1P in the media following EGF treatment is physiologically relevant and sufficient to activate S1P cell-surface receptors. Finally, to determine if EGF-mediated S1P receptor internalization was SK-dependent, and to rule out other possible EGF effects, cells were

pretreated with SKi-II prior to EGF or sphingosine treatment and GFP-tagged S1P receptor localization was assessed **(Fig. 3G, 3I)**. Inhibition of SK obstructed sphingosine- and EGF-induced S1P receptor internalization **(Fig. 3G, 3I)**, consolidating evidence that EGF-mediated S1P-receptor internalization is SK-dependent. Additionally, cells were pretreated with S1PR2 antagonist and EGF-induced S1PR2 internalization was evaluated **(Fig. 3J)**. As expected, JTE-013 was able to inhibit EGF-induced receptor internalization **(Fig. 3J)**. These data demonstrate that EGF-induced extracellular S1P is biologically significant and capable of acting on cell surface S1P receptors, in particular S1PR2. Collectively these results suggest that EGF induces activation of the SK/S1P pathway, and indicates that activation of the SK/S1P pathway may be necessary for some EGF-mediated events.

3.3.3. EGF induces ERM phosphorylation and translocation to lamellipodia via S1PR2

Having previously reported that S1P-mediated ERM phosphorylation occurs in a S1P2 receptor-dependent manner (*209*), we were interested in determining the role of S1P receptors in EGF-induced activation of ERM proteins. To investigate the role of S1P receptor signaling in EGF-stimulated ERM activation, gene silencing techniques as well as receptor-specific antagonists were used. HeLa cells were treated with siRNA targeting the S1PR2 receptor and the effect on EGF-stimulated phospho-ERM was evaluated **(Fig. 4)**. Cells exposed to scrambled control siRNA exhibited robust ERM phosphorylation in response to both S1P and EGF treatment **(Fig. 4A, 4B)** In contrast, S1PR2 knockdown significantly inhibited ERM phosphorylation following both S1P and EGF treatment, suggesting that S1PR2 plays a pivotal role in EGF-induced ERM phosphorylation.

To further solidify the role of S1PR2 in EGF-mediated phospho-ERM, pharmacological inhibitors were used and EGF-stimulated ERM phosphorylation evaluated **(Fig. 4B)**. Cells receiving vehicle treatment maintained the ability to activate ERM proteins following treatment with EGF **(Fig. 4B)**; likewise pretreatment with antagonist for S1PR1 (VPC23019) or S1PR3 (VPC23019, BML241) had no effect on EGF-induced ERM phosphorylation **(Fig. 4B)**, indicating that S1PR1 and S1PR3 are not involved in the activation of ERM proteins by EGF. Next, cells were pretreated with S1PR2-specific antagonist, JTE013, and the effects on EGF-mediated ERM phosphorylation were assessed. Pretreating HeLa cells for 1 hour with 5μM JTE013 completely inhibited the ability of EGF to induce phosphorylation of ERM proteins **(Fig. 4C)**. These data provide genetic and pharmacological evidence supporting a major role for S1PR2 in EGF-mediated C-terminal phosphorylation of ERM proteins.

Previous work from our lab showed that S1P-mediated cytoskeletal changes were dependent on ezrin T567 phosphorylation (*209*); moreover, others have shown that ezrin T567 phosphorylation is required for cytoskeletal rearrangement events brought about by EGF (*208*), such that C-terminal ezrin phosphorylation is required for EGF-induced lamellipodia formation (*208*). In order to determine the cytoskeletal changes which occur in response to EGF treatment, immunofluorescence microscopy was used to evaluate phospho-ERM localization as well as morphological changes following the addition of EGF. As visualized in **Figure 5A** with pERM (green) and F-actin (red), HeLa cells that received no treatment exhibited low levels of phosphorylated ERM that exists intracellularly in a punctate pattern. Treatment with 100nM S1P resulted in increased levels of pERM which localized to newly formed filopodial protrusions, consistent with our previous findings **(Fig. 5A, 5B)** (*209*). In accordance with other reports, treating cells with EGF resulted in phospho-ERM-dependent translocation to newly formed

lamellipodia which was accompanied by nuclear retraction **(Fig. 5C)** (*226*). Having established that EGF induction of ERM phosphorylation occurs via SK and S1PR2, we were interested in determining the mechanism by which EGF-induces lamellipodia formation and cellular polarization occurs. Given the role SK and S1PR2 play in S1P-induced pERM-dependent filopodia formation (*209*) and their role in EGF-induced ERM phosphorylation, S1PR2-specific antagonist JTE013 was used and EGF-mediated phospho-ERM and lamellipodia formation evaluated **(Fig. 5D)**. HeLa cells pretreated with S1PR2 inhibitor, followed by treatment with EGF, displayed no increase in phospho-ERM levels indicated by the lack of green staining, and were unable to undergo cell polarization, evidenced by lack of lamellipodia formation and nuclear retraction **(Fig. 5D).** These findings suggest that S1PR2 signaling is necessary for EGF-induced cellular polarization. Next, and in order to evaluate the role of ezrin T567 phosphorylation in EGF-mediated cytoskeletal changes, VSV-G-tagged wild type (WT) and non-phosphorylatable (TA) ezrin plasmid constructs were used and cytoskeletal architecture evaluated following EGF treatment **(Fig. 6)**. Cells overexpressing WT ezrin display cytoskeletal protrusions, even in the absence of treatment, which is visualized with total ezrin (VSV-G) in green and F-actin in red **(Fig. 6A)**. Treatment of WT-ezrin-expressing cells with S1P only slightly enhanced cytoskeletal protrusions compared to that of non-treated cells **(Fig. 6B)**, while treatment with EGF induced lamellipodia formation, as expected **(Fig. 6C)**. Cells overexpressing TA-ezrin displayed no cytoskeletal protrusions in the absence of treatment **(Fig. 6D)**; moreover, S1P was unable to provoke filopodia formation in TA-ezrin-expressing cells (Fig. 6E), just as EGF was unable to induce lamellipodia formation and cellular polarization **(Fig. 6F)**, indicating that T567 phosphorylation of ezrin is required for EGF-induced cytoskeletal rearrangement.

Collectively, these data indicate that EGF induces phospho-ERM, as well as lamellipodia formation and cellular polarization, in a S1PR2-dependent manner.

3.3.4. EGF-mediated cell invasion occurs via S1PR2-dependent phosphorylation of ezrin T567

ERM protein activation and EGF have both been related to metastasis; thus, in order to assess the biological significance of EGF-mediated ERM phosphorylation and its potential role in metastasis, cell invasion assays were performed. Using trans-well chambers, invasion was measured as a read-out of metastatic behavior. Non-transfected HeLa cells or HeLa cells expressing VSV-G-tagged wild type (WT) ezrin or non-phosphorylatable ezrin mutant (TA) were assessed for their ability to invade through matrigel. In the presence or absence of S1PR2 antagonist, cells were allowed to invade toward complete media (CM) or 50ng/mL EGF (EGF) for 48 hours **(Fig. 7)**. Non-transfected and WT ezrin-expressing HeLa cells exhibited invasion toward complete media with WT ezrin-expressing cells invading more than non-transfected cells, while cells overexpressing the non-phosphorylatable TA ezrin mutant- invaded less than non-transfected HeLa cells **(Fig 7)**, suggesting that ezrin T567 phosphorylation plays a pivotal role in cellular invasion. Albeit at lower levels, cells invaded toward EGF in a pattern similar to that of complete media with WT-expressing cells invading the most, followed by normal HeLa cells then TA-expressing cells, indicating that EGF-induced cell invasion is mediated by ERM proteins and specifically by phosphorylation of ezrin T567 **(Fig. 7)**. To determine the role of S1PR2 in EGF-mediated cell invasion, cells were plated in the presence of S1PR2 inhibitor and invasion towards EGF was evaluated. Inhibition of S1PR2 resulted in inhibition of EGF-stimulated invasion, inhibiting invasion by at least 50% under all three ezrin conditions

(endogenous ezrin expressing, $p<0.05$; WT-ezrin overexpressing, $p<0.05$, and TA-ezrin overexpressing, $p=0.055$ cells) **(Fig. 7)**. In concert with the previously presented data, these findings suggest that EGF-mediated cell invasion occurs through S1PR2-dependent phosphorylation of ezrin T567 and may reveal novel targets for the development of therapeutics for pathologies that are driven by EGF, including many cancers.

3.4. Discussion

Here a novel mechanism of EGF-induced cell invasion which requires S1P-mediated activation of ERM proteins has been identified. EGF led to stimulation of the SK/S1P pathway such that treatment with EGF resulted in elevated levels of S1P, activation of S1PR2 and ERM-dependent cell invasion. S1PR2-mediated C-terminal threonine phosphorylation of ezrin was required for EGF-induced lamellipodia formation and consequent cellular invasion. Taken together, these results have important implications for ERM regulation, for the role of the SK/S1P pathway and ERM in EGF-mediated events, particularly those involved in cancer promotion, and for the role of S1PR2 in cellular invasion, which has been controversial as discussed in chapter 2.

3.4.1. EGF regulates ERM proteins through the SK/S1P pathway

The effects of EGF on cell growth and proliferation have been well studied and perhaps best studied, are the roles of EGF in the initiation, maintenance and progression of cancer (*227, 228*). EGF contributes to cancer development and metastasis by promoting cell motility and invasion which both require specific, coordinated changes in the actin cytoskeleton (*229*). EGF promotes lamellipodia formation dependent on direct phosphorylation of ezrin by Nck-interacting kinase (NIK) in breast adenocarcinoma cells (*208*) and regulates invasion through

ezrin tyrosine phosphorylation in ovarian cancer (*89*). In line with previous studies, we found that cells expressing a non-phosphorylatable ezrin mutant were unable to form lamellipodia or invade in response to EGF, suggesting EGF-stimulated cytoskeletal rearrangement and invasion are dependent on ezrin phosphorylation at the C-terminal threonine.

The sphingosine kinase/sphingosine 1-phosphate pathway is a secondary messaging system in response to numerous ligands, including EGF. EGF has been shown to induce acute activation, as well as late transcriptional up-regulation of SK1, both of which were required for EGF-mediated MCF7 breast cancer cell growth and motility (*230*). EGF has also been shown to activate the SK/S1P pathway and provoke migration of MB-MDA-453 via ERK-mediated phosphorylation and activation of SK2 (*231*). Although SKX is reported to be specific for SK1 inhibition, it was still able to inhibit EGF-induced ERM phosphorylation unlike SK1 siRNA. This can be due to several reasons. As mentioned before, this inhibitor causes ceramide accumulation that our group showed before is able to dephosphorylate ezrin by activating PP1A. Another reason could be its accumulation in Hela cells making it act on SK2 due to its higher concentration. In conclusion, the S1P/S1PR2 pathway is definitely involved in EGF-induced ezrin phosphorylation, and detailing the specific iso-enzyme responsible for this effect would be the subject of chapter 4. By studying EGF-activation of ERM proteins, we were able to implicate the SK/S1P pathway in yet another EGF-mediated biology. In our study, EGF induced phospho-ERM through a mechanism involving the SK/S1P pathway, such that S1P generation and S1P-receptor internalization following EGF treatment were found to be SK-dependent. These findings are in agreement with previous work from our lab indicating that exogenously added S1P induced cytoskeletal rearrangement via activation of ERM proteins (*209*), as well as with work by Dudek, *et. al.* implicating S1P-mediated phosphorylation of ERM proteins in lung endothelial

barrier function (*232*). These data provide evidence that EGF leads to ERM phosphorylation via activation of the SK/S1P pathway and suggest that SK/S1P may participate in other EGF-mediated events, including changes in cellular architecture. Our results give merit to the idea of targeting the SK/S1P pathway in cancers with primary or acquired resistance to EGF/EGFR-targeted therapies such as occurs in non-small cell lung carcinomas (NSCLC) (*216*) and breast cancers as will be discussed in chapter 6 (*233*).

3.4.2. EGF induces cellular invasion via S1PR2-dependent activation of ERM proteins

One of the many direct connections between EGF and S1P is highlighted in cancer cell migration and invasion. EGF induces invasion of breast cancer cells through CD44-mediated expression of matrix metalloproteinase 9 (MMP); interestingly, CD44 is an ERM-binding protein also known to coordinate actin-based cytoskeletal reorganization (*234*). Like EGF, S1P induces expression of MMP9, leading to MCF10A breast cancer cell invasion (*234*). Also in support of a role for the SK/S1P signaling pathway in the invasive behavior of some cancers are reports of glioma cell invasion via S1PR2 and up-regulation of the metastatic inducer uPA, plasminogen activator (*235*). Supporting the aforementioned findings, the SK/S1P pathway was found to be downstream of EGF and upstream of ERM activation and invasion. Stimulation of cells with EGF led to cellular invasion through extracellular matrix, which may have occurred through a mechanism involving matrix metalloproteases; however, while ezrin is intimately involved in metastasis no correlations with MMPs have been made. It will be interesting to explore the potential connection between ezrin and MMPs. While S1PR1 and S1PR3 have been already related to cell migration and invasion, S1PR2 has opposing effects (*236*). In this work, a novel

mechanism of EGF-stimulated invasion is unveiled, whereby S1P-mediated activation of S1PR2 and phosphorylation of ERM proteins are required. Importantly, we do not discard that other S1P receptors become activated following EGF treatment; however, we do propose that receptor localization is key in determining the invasive or non-invasive result of S1PR2 activation.

3.4.3. The Role of S1PR2 in Cell Migration and Invasion

There is evidence supporting a major role for S1P and S1P receptors in the promotion of cancer cell migration and invasion; however, many cancer-promoting functions of the SK/S1P pathway rely on S1P receptors other than S1PR2; for example, S1PR1 mediates hepatocellular carcinoma metastasis (*237*) and S1PR3 promotes MCF10A breast cancer cell invasion (*234*). Contrary to the role of S1PR1 and S1PR3 in cell migration and invasion, S1PR2 has been shown to inhibit migration and invasion. S1PR2 has been reported to negatively regulate nephroblastoma tumor growth and progression (*238*), as well as prevent migration of hematopoietic stem cells (*239*) and invasion of cervical cancer cells (*209*). Despite evidence supporting inhibitory roles for S1PR2 in cancer, there is also evidence supporting a role for S1PR2 in cancer promotion. While S1PR2 inhibits migration of glioma cells, it instead enhances invasion (*240, 241*). Our findings provide additional evidence supporting a positive role for S1PR2 in cancer, such that inhibition of S1PR2 impeded EGF-stimulated cell invasion. The mechanisms or circumstances encompassing the role of S1PR2 in cancer prevention or promotion are not very well understood; therefore we put forth the idea that S1PR2 cellular localization dictates its pro- or anti-invasive functions.

Induction and regulation of cell polarization, the differential cellular compartmentalization of proteins and signals (*242*), is an overlapping function shared by EGF

and ezrin. While EGF signals for cells to undergo polarization, ERM proteins carry out this function directly. EGF induces front-rear polarity and directional migration in epidermal keratinocytes (*243*); moreover, EGF maintains an evolutionarily conserved role in organizing the dorso-ventral axis of *Drosophila* by establishing ovum polarity during oogenesis (*244-246*). In our study, EGF induced lamellipodia formation and cell invasion, both of which were dependent on activation of ezrin, suggesting that EGF and ERM function coordinately to regulate cell polarity under these conditions. In support of our findings, ERM proteins are known to participate in the establishment of cell polarity such that, mice devoid of ezrin exhibit improper development and homeostasis of the intestine, a result of disrupted apical integrity of the gut epithelium (*247*). While the role of the SK/S1P pathway in polarization is not well understood, there are reports of SK/S1PR2 localizing to the leading edge of migrating cells (*248*), reinforcing our observation that EGF-mediated cell polarization is S1PR2-dependent. Further investigation into ERM-mediated regulation of S1PR2 localization following EGF treatment is necessary; however, we suggest that S1PR2 may undergo specific cellular localization in response to EGF, leading to the asymmetrical activation of ERM proteins which drives cell migration and invasion. Perhaps ligand-induced polarization of cells leads to localized S1P production and signaling driving directional cell migration or invasion. In stark contrast, treating cells with exogenous S1P, in the absence of cellular polarization, inhibits migration and invasion due to global activation of ERM proteins around the entire periphery of the cell. Our data show that EGF induces cell polarity, in the form of lamellipodia, and mediates cell invasion, via induction of ERM C-terminal threonine phosphorylation, through a mechanism involving SK and S1PR2.

3.4.4. Implications

The findings from this chapter have significant implications to cancer biology. The EGF and SK/S1PR2 signaling pathways, along with ERM proteins, encompass important prognostic indicators of disease, especially for cancer. Components of the EGF signaling pathway currently function as markers of cancer grade and gauge treatment responsiveness and prognosis for numerous forms of cancer (*249, 250*). Likewise, SK/S1P pathway components are used as markers of disease including cardiovascular disease, inflammatory disease, and cancer (*251, 252*). Not surprisingly, ERM proteins have great potential to be used as prognostic indicators offering information about cancer severity and metastatic potential. For example, up-regulation of ERM proteins, along with down-regulation of E-cadherin, correlates with invasion *in vitro* (*253, 254*) and is associated with lymph node metastasis *in vivo* (*253, 255, 256*). Given the role that S1PR2 plays in EGF-mediated, phospho-ERM-dependent cell invasion, it may serve as a potential prognosticator and/or biomarker of EGF and SK/S1P-mediated diseases, often occurring in cancer.

Chapter 4

Intracellular SK2-derived S1P mediates EGF-induced ERM phosphorylation and cancer cell invasion

Abstract:

The bioactive sphingolipid sphingosine-1-phosphate (S1P) mediates cellular proliferation, mitogenesis, inflammation, and angiogenesis. These biologies are mediated through S1P-binding to specific G protein-coupled receptors (S1PR1-5), and some other less well-characterized intracellular targets. ERM (ezrin, radixin, and moesin) proteins, a family of adaptor molecules linking the cortical actin cytoskeleton to the plasma membrane, are emerging as critical regulators of cancer invasion via regulation of cell morphology and motility. Recently, we identified S1P as an acute ERM activator (via phosphorylation) through its action on S1PR2. In this work, we dissect the mechanism of S1P generation downstream of EGF leading to ERM phosphorylation and cancer invasion. We demonstrate that SK2, and not SK1, is essential and sufficient in EGF-mediated ERM phosphorylation. Furthermore, we provide evidence that SK2 is necessary and sufficient to mediate EGF-induced invasion. Surprisingly, and for the first time, we find that this event, although dependent on S1PR2 activation, does not generate and does not require extracellular S1P secretion; therefore introducing a potential novel model of autocrine/intracrine action of S1P that still involves its GPCR receptors. These results define new mechanistic insights for EGF-mediated invasion and novel actions of SK2, therefore setting the stage for novel targets in the treatment of growth factor-driven malignancies.

4.1. Introduction

Sphingolipids are a class of molecules that have important functions in maintaining membrane structure and integrity (*1*). With the identification that sphingosine exerts regulatory roles on protein kinase C (*257*), sphingolipids have emerged as important bioactive molecules that exert regulatory functions on important signaling pathways; therefore, affecting a myriad of cellular processes, including cell survival, proliferation, motility, inflammation and differentiation (*124*). An interlinked network of metabolizing enzymes tightly regulates sphingolipids levels. Of particular interest are the sphingosine kinases (SKs) SK1 and SK2, not only because they can be modulated by external stimuli (TNF, IL-1 and PDGF), but also changes in their activity have significant effects on the levels of their product sphingosine-1-phosphate (S1P) (*1*). SK1 has been by far the most studied isoform, and there is a large body of literature supporting its role in promoting cell proliferation, regulating inflammation, as well as driving neoplastic transformation and carcinogenesis. This enzyme has been well targeted by several pharmacological inhibitors that proved effective in reducing tumor growth, metastasis and inflammation (*258*) although results with some high potency inhibitors recently developed have questioned some of these roles (*259*).

On the other hand, SK2 has been less well studied. Human SK2 is localized on chromosome 19, with peak expression during later stages of embryonic development especially in the liver and kidneys (*260*). It possesses an N-terminal region that is absent in SK1, making this isoform significantly larger (*260*). It is shown that it possesses several unique phosphorylation sites on its N-terminus that are believed to regulate the activity of the catalytic subunit (*261*) . This isoform also possesses a central proline-rich sequence that coincides with

the substrate-binding site, making this enzyme more promiscuous in its substrate specificity (*262*).

SK2 plays an important role in modulating inflammation. Whereas SK1 has been mostly proposed to promote inflammation, the role of SK2 has been less clear. Knocking down SK2 in a murine mouse model of arthritis exacerbated the disease and increased cytokines production (*263*). A similar effect on cytokine production was seen in a xenograft murine model using SK2-/- MCF7 cells (*264*). On the other hand, inhibition of SK2, albeit with low potency inhibitors of unclear specificity, ameliorated the disease progression of murine inflammatory bowel disease models such as ulcerative colitis (*265*) and Crohn's disease (*266*). The studies on SK2 extend to its described roles in tumorogenesis. Early studies have implicated SK2 in pro-apoptotic functions owing to its BH3 domain (*267*), or via regulation of cytochrome c release from mitochondria following TNF stimulation, using siRNA technology in mouse embryonic fibroblasts (*268*). More recently, it has also been implicated in inducing cell cycle arrest (*269*). On the other hand, more recent studies have emerged demonstrating a pro-tumorigenic role for SK2. For example, it has been shown that SK2-derived S1P exacerbates colon cancer by acting as an antagonist to the retinoic acid receptor b, and that its overexpression reversed ATRA-induced growth inhibition (*270*). Furthermore, the pharmacologic inhibition of SK2 has been proven successful in a multitude of malignancies including hepatocellular carcinoma (*271*), renal cell carcinoma, and pancreatic adenocarcinomas (*272*). More importantly, SK2 has been shown to play a crucial role in promoting EGF-driven chemotaxis of MDA-MB-453 breast cancer cells using siRNA and overexpression techniques (*273*), however the mechanism of SK2-induced motility has not been described. In addition, SK2's effect on adhesion and invasion, the two other crucial steps involved in cancer progression, is still unknown.

ERM (ezrin, radixin, and moesin) proteins are a family of molecules that link the plasma membrane to the cortical actin cytoskeleton in a well-regulated manner that consists of a two-step process (*4*). In its inactive conformation, the protein is closed due to the interaction between the N-terminal domain (FERM) and the C-terminal domain (C-ERMAD). This conformation locks the protein in the cytosol. After binding phosphatidylinositol 4,5-bisphosphate (PIP2), the phosphorylation of a C-terminal threonine residue (ezrin Thr567) creates a steric hindrance between the N-terminus and the C-terminus opening up the protein. Now exposed, the FERM domain binds to the plasma membrane, while the actin binding site on C-ERMAD is readily available to bind cortical actin (*4*). Their localization near the plasma membrane allows them to provide a stage where different molecules can interact (such as CD95 (*274*), CD44 (*275*), CD43 (*276*), NHE1 (*277*)), facilitating signal transduction between the extracellular matrix and the cytosol. Furthermore, these proteins play an important role in dictating cell shape (*7*) and motility (*6, 9, 11*) as they are heavily enriched in specialized cellular protrusions such as filopodia, and lamellopodia. That being said, they have emerged as important regulators of cancer progression by enhancing adhesion, and invasion of malignant cells, leading to metastasis (*278*).

More recently, our group identified sphingolipids as regulators of ERM proteins through both ceramide and S1P. Whereas ceramide generation on the plasma membrane caused rapid ERM dephosphorylation (*121*) via activation of protein phosphatase 1 alpha (PP1a) (*119*), S1P treatment resulted in an acute and potent ERM activation that was dependent on S1PR2 signaling (*279*). In addition, we have shown in chapter 3 that EGF-mediated ERM activation, and subsequent lamellopodia formation and invasion is dependent on the S1P/SP1R2 axis (*122*). However, several questions remain unsolved including the mechanism of S1P generation

following EGF stimulation and its site of action. Answering these questions will unveil new targets in the pathway of EGF-driven invasion; also, it will uncover new modes of actions for the bioactive sphingolipid S1P.

In this chapter, we have explored the mechanism by which SK regulates ERM phosphorylation and its downstream biologies following EGF treatment. Using cervical cancer HeLa cells as a model system, we demonstrate that SK2 and not SK1 is essential for EGF-mediated ERM phosphorylation. In addition, increased intracellular S1P production achieved by overexpression of either SK2 or the ceramidase ACER2 is sufficient in promoting ERM activation. Moreover, we identify SK2 as a novel and potent target in the pathway of EGF-driven invasion. As such, down regulation of SK2 prevents EGF-mediated adhesion and subsequent extracellular matrix invasion. We also show that SK2 overexpression increases EGF-mediated adhesion and invasion via activation of the ERM proteins. Surprisingly, and for the first time, we demonstrate that this event, although dependent on S1PR2 activation, does not require extracellular S1P secretion, defining a new model for intracellular S1P signaling. We identify Spns2 as a potential transporter of S1P from the cytosolic side to the vicinity of S1PR2. Taken together, these studies define a new role for SK2 that depends on production of S1P, and an intracellular action for S1P on the S1PR2 with a critical role in regulating growth factor-induced invasion.

4.2. Materials and Methods

4.2.1. Materials

High glucose DMEM, FBS, Lipofectamine 2000, Lipofectamine RNAiMAX, Superscript III first strand synthesis kit, 488 and 647-conjugated secondary antibodies were purchased from Life Technologies. Mononclonal anti-b-actin antibody were from Sigma-Aldrish. Anti-pERM antibody, anti-EGFR, anti-ErbB2, anti-pERK antibodies, and EGF were from Cell Signaling Technology (Danvers, MA). Anti-total Ezrin, protein A/G agarose, and HRP-labeled secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). BCA kit, Pierce ECL and Supersignal West Dura chemiluminescent Substrate were from Thermo Scientific (Suwanee, GA). Anti-SK2 antibody was from Abcam. PF-543, Ski-II, Erlotininb, ErbB2 inhibitor, Lapatinib, EMD476485, Bisindoylmaleimide I, G06976, EMD124017, U016, and JTE-013 were from EMD Millipore. Sphingomab was obtained from Lpath Incorporated (SanDiego, CA).

4.2.2. Cell Culture

HeLa cells were purchased form the American Type Culture Collection, ATCC (Manassas, VA). Cells were grown in DMEM supplemented with 10% FBS under standard cell culture conditions: 37^{o}C and 5% CO_2. When starved, DMEM without FBS was added for at least 4 hours. Cells were tested for mycoplasma infection once per month.

4.2.3. RNA interference

Gene silencing was carried out using siRNA directed against human SK2: Hs_SPHK2_5 FlexiTube siRNA SI00288561 (Qiagen), human ErbB1: Hs_EGFR_11 FlexiTube siRNA SI02660147 (Qiagen), human ErbB2: Hs_ERBB2_14 FlexiTube siRNA SI02223571 (Qiagen) and with Qiagen all-star siRNA as a negative control. Transfections were carried out using Lipofectamine RNAiMAX according to the manufacturer's protocol (Life Technologies) .

4.2.4. Plasmid constructs and DNA transfection

Full length untagged SK2, transcript variant 1 (SC113181) was obtained from Origene (Rockville, MD). The MYC tag was introduced at the C-terminus of SK2 by sub-cloning the WT SK2 obtained from Origene into a pcDNA3.1(-)myc/his/Amp vector using Xho/HindIII flanked primers. Single mutations on WT SK2-MYC were generated using Quick Change Site-Directed Mutagenesis kit form Stratagene (La Jolla, CA). The sequences of all plasmids were confirmed in the DNA Sequencing Facility (Stony Brook, NY). Cells growing in 12-well plates were transfected with 1mg of plasmid DNA using Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's protocol.

4.2.5. Immunoprecipitation and immunoblotting

For immunoprecipitation studies, cells were treated with phosphate buffer saline (PBS) or EGF (10 ng/ml). The reaction was stopped by adding 3.75% formaldehyde in DMEM. Cells were then lysed using a buffer that contains 20nM Tris-HCl,, 100 mM NaCl, 1% Triton-X-100, 1mM EDTA, protease and phosphatase inhibitors from Sigma. Cell lysates were then sonicated, centrifuged at 10000g for 10 min to remove cell debris. Cells were then pre-cleared for 1 hour using Protein A/G agarose beads in a circular rotator at 4^{o}C. Antibodies (1mg/500mg lysate) were then incubated with the supernatants overnight at 4^{o}C. Immune complexes were then precipitated by centrifugation at 12000 rpm for 1 min, washed 3 times, than 2X Laemmli buffer was added. Proteins were separated via SDS-PAGE (4-20% Tris-HCl) using the Bio-Rad criterion system. Proteins were then transferred into a nitrocellulose membrane, blocked for 1

hour with 5% milk in PBS with 0.1% Tween. Primary antibodies were then added to the membranes at4^{o}C overnight. The membranes were then incubated with the secondary antibodies at room temperature for 1 hour. Proteins were visualized by chemiluminescence after several washes with PBS-T.

4.2.6. Immunofluorescence and confocal microscopy

Laser microscopy and immunofluorescence were carried using similar techniques as previously published. Briefly, HeLa cells plated on 35 mm confocal dishes, were fixed with warmed paraformaldehyde solution (3.7% in PBS) for 20 minutes. Cells were then washed 3 times with PBS, them permeabilized with 0.1% Triton-X in PBS for 15 minutes. Cells were then blocked with 6% human serum in PBS for 20 minutes at room temperature. Cells were then incubated with the primary antibodies diluted at 1:100 in 6% human serum overnight at 4^{o}C. Cells were then washed three times and then incubated for 1 hour with the secondary antibodies in the dark. DAPI was then added, and cells were examined using the Leica SP8 confocal microscopy system (Leica Microsystems, Wetzlar, Germany).

4.2.7. C17-Sphingosine labeling

Cells were plated at 500,000 cell/60-mm dish. Once 80% confluent, cell were washed with serum free DMEM, than starved overnight. Next, cells were treated with 250 nM C17 sphingosine for 30 minutes before stimulating with EGF for 30 seconds or 5 minutes. Cells were then directly lysed with 2:3 70% isopropanol/ethyl acetate after media removal. Extracted lipids were then sent to Stony Brook University 73rlotinib73 core facility for analysis.

4.2.8. Cellular adhesion and invasion assays

For cell adhesion, 12-well plates were coated with fibronectin according to the manufacturer protocol. After 4 hours of starvation, cells were trypsinized and plated on the fibronectin-precoated dishes for 12 hours. Media was then washed twice with PBS; attached cells were then quantified by either an MTT assay according to the manufacturer protocol (Life Technologies) or by performing a western blotting against actin. As for cell invasion, it was carried out using the Corning Tumor Invasion system, and following the manufacturer's protocol with minor modifications as previously described.

4.3. Results

4.3.1. EGF-induced ERM phosphorylation is ErbB1 dependent

EGF exerts its functions by binding the EGF receptor (EGFR) also known as ErbB1, which subsequently forms a homo-dimer with another ErbB1 or a heterodimer with ErbB2 also known as Her2 (*280*). The homo-dimer regulates a myriad of signaling pathways that are different than those regulated by the heterodimer owing to the different structure of ErbB2; thus the heterogeneity of EGF responses (*281*) . To identify the branch of the EGF pathway involved in ERM phosphorylation, Erlotinib an inhibitor of ErbB1, an ErbB2 inhibitor, and Lapatinib, well known inhibitor of both ErbB1 and ErbB2 were used. As can be seen, inhibition of ErbB1 by Erlotinib diminished ERM phosphorylation following EGF treatment **(Fig. 8A)**. On the contrary, while inhibition of ErbB2 decreased its autophosphorylation and activation, it had no effect on phospho-ERM levels **(Fig. 8A)**. As expected, inhibiting both receptors by Lapatinib abrogated ERM activation **(Fig. 8A)**. Importantly, Erlotinib and Lapatinib did not affect ERM phosphorylation following S1P treatment, ruling out the possibility that these inhibitors might be affecting the signaling pathway downstream of S1P **(Fig. 8A)**. To further verify that ERM

phosphorylation is strictly ErbB1 dependent, siRNA against EGFR and Her2 was used, and more than 90% knockdown was achieved **(Fig. 8B)**. Again, ERM activation by EGF was only affected by EGFR knockdown **(Fig. 8B)**. Together, these results indicate that EGF-mediated ERM phosphorylation is strictly ErbB1 dependent in HeLa cells.

4.3.2. SK2, and not SK1, mediates ERM phosphorylation upon EGF stimulation

We previously reported that S1P is a potent inducer of ERM phosphorylation and that sphingosine led to ERM phosphorylation through its SK1 dependent phosphorylation to S1P (*279*). More recently we showed that SK activity is required for ERM phosphorylation upon EGF stimulation (*122*). Nonetheless, the specific SK involved in mediating EGF action remained unknown. To address this question, a newly established compound (PF453) was utilized to acutely inhibit SK1 in cells stimulated with Sph and EGF **(Fig. 9A, 9B)**. As expected, inhibition of SK1 significantly decreased ERM phosphorylation upon sphingosine treatment (which we previously showed was SK1 dependent (*279*))**(Fig. 9A, 9B);** however, PF543 did not affect EGF-mediated ERM phosphorylation compared to vehicle treated cells **(Fig. 9A, 9B)**. The role of SK1 in EGF-induced ERM phosphorylation was also ruled out by knocking it down using siRNA, also showing no effect on ERM phosphorylation **(Fig. S2)**. Next and to test the role of SK2, it was knocked down in HeLa cells, and the effects on SK2 mRNA **(Fig. S3)** and ERM phosphorylation were analyzed **(Fig. 9C, 9D)**. As can be seen, SK2 siRNA-treated cells showed more than 80% knockdown of SK2 mRNA compared with all-Star (Astar) negative control treated cells **(Fig.S3)**. SK2 knockdown significantly diminished ERM phosphorylation in response to EGF compared to Astar treated cells **(Fig. 9C, 9D)**. Importantly, the changes in ERM

phosphorylation seen by western blot were also observed on fixed cells by confocal microscopy **(Fig. 9E)**, thus documenting the occurrence of these effects in cells. To show that the effects of blocking SK2 were due to the effects on S1P production, S1P was added to samples pre-treated with ABC294640, a known SK2 specific inhibitor (*282*). Notably, this inhibitor significantly diminished EGF-mediated ERM phosphorylation **(Fig. 9F, 9G)**. On the other hand, SK2 inhibition did not show a statistically significant decrease in ERM phosphorylation upon S1P treatment **(Fig. 9F, 9G)**. Taken all together, these results strongly suggest that SK2, and not SK1, is essential for EGF-mediated ERM phosphorylation.

4.3.3. Overexpression of SK2 is sufficient to promote ERM phosphorylation

Once SK2 emerged as necessary for EGF signaling to ERM activation, it became important to determine if SK2 was sufficient to promote ERM phosphorylation. To this end, human wild type MYC-tagged SK2b was transiently transfected into HeLa cells. SK2b is the longer isoform of SK2 that is widely expressed in human tissues. SK2 transfection into cells resulted in baseline increases in ERM phosphorylation in the absence of EGF stimulation **(Fig. 10A, 10B)**. Moreover, EGF stimulation of SK2-transfected cells showed a statistically significant increase in ERM phosphorylation compared to mock and vector transfected cells that were stimulated with EGF **(Fig. 10A, 10B)**. To further validate that the catalytic activity of SK2, i.e. S1P production, is necessary for ERM phosphorylation, we generated the human catalytically inactive SK2 G213E (*273*). Mutating the glycine residue at position 213 in the catalytic site into glutamic acid had been previously described to abolish SK2 catalytic activity (*273*). Of note, G213E SK2-expressing cells failed to demonstrate the increase in ERM phosphorylation in un-stimulated cells, and did not enhance the EGF response **(Fig. 10A, 10B)**. Taken together, these

results indicate that overexpression of WT, but not the catalytically inactive SK2 (G213E) is sufficient to increase ERM phosphorylation. In addition, it has been previously reported that EGF induces phosphorylation of SK2 at S351 and T578, which increases its activity (*283*). Importantly, overexpressing the phosphomimetic SK2 (S387D, T614D) did not only increase basal ERM phosphorylation compared to WT SK2 transfected cells, but it also blunted the EGF response **(Fig. 10C).** On the other hand, overexpressing the non-phosphorylatable form of SK2 (S387A, T614A) did not show any increase in basal phospho-ERM levels **(Fig. 10C)**. Nonetheless, cells transfected with the latter construct still responded to EGF, although to a much lesser extent than the WT SK2 transfected cells **(Fig. 10C)**. This could be due to either the presence of the endogenous SK2, or the regulation of SK2 activity by EGF at other undiscovered sites. Collectively, these results demonstrate that SK2 is sufficient to induce ERM phosphorylation, as well as they highlight the importance of its activation by EGF.

4.3.4. EGF-mediated cell adhesion and subsequent invasion occurs in a SK2-dependent phosphorylation of ezrin T567

ERM proteins and EGF have both been reported to play an important role in cell adhesion, migration and invasion (*284*) . We have previously demonstrated that EGF-mediated lamellipodia formation and cell invasion occur via S1PR2 (*122*). It is also known that EGF mediated cellular migration is dependent on SK2 in MDA-MB-453 breast cancer cells (*273*). Therefore, it became crucial to determine whether SK2 plays an essential role in EGF-induced cellular adhesion and invasion, and whether it is ERM dependent. Using Fibronectin coated plates, non-transfected HeLa cells or HeLa cells expressing VSV-G-tagged WT ezrin or the dominant negative non-phosphorylatable ezrin mutant (TA) were allowed to adhere for 12 hours

in serum-free media. WT-ezrin and Astar double transfected cells showed a significant baseline higher adhesion levels than non-transfected cells, while cells overexpressing the non-phosphorylatable TA-ezrin mutant adhered significantly less than non-transfected and WT ezrin overexpressing cells **(Fig. 11A)**. This result consolidates the role of T567-ezrin phosphorylation in cellular adhesion. A1s reported in the literature, EGF treatment significantly increases cellular adhesion in non-transfected cells. Importantly, EGF did not increase the adhesion of TA-ezrin transfected cells, indicating that EGF-mediated cellular adhesion is mostly mediated by ezrin T567 phosphorylation **(Fig. 11A)**. To determine the role of SK2 in EGF-mediated cellular adhesion, this enzyme was knocked down before overexpressing the WT ezrin and the TA-ezrin mutant. SK2 knock-down significantly decreased baseline and EGF-mediated cell adhesion under all 3 conditions **(Fig. 11A)**. In addition to the data presented above, these results suggest that EGF-mediated cell adhesion occurs through SK2-dependent phosphorylation of ezrin T567.

It was previously showed that EGF mediated cell-invasion is greatly dependent on ezrin phosphorylation at T567 residue (*122*). Since adhesion is the first step necessary for subsequent cell invasion, the role of SK2 in the latter process was assessed by performing invasion assays using transwell chambers coated with Matrigel **(Fig. 11B)**. EGF treatment caused a 3-fold increase in cellular invasion in Astar transfected cells. Knocking down SK2 significantly decreased cellular invasion basally, and upon EGF treatment **(Fig. 11B)**. In concert with the results shown above, these data suggest that EGF-mediated cell adhesion and subsequent invasion occur through SK2-dependent phosphorylation of ezrin T567. To further assess the role of SK2 and its product S1P in cellular adhesion, we transfected HeLa cells with the phosphomimetic mutant SK2 (S387D, T614D) and the non-phosphorylatable form SK2 (S387A, T614A) before allowing them to adhere. SK2 (S387D, T614D) overexpressing cells showed 15%

higher basal adherence levels compared to WT SK2 overexpressing cells, while SK2 (S387A, T614A) overexpressing cells adhered significantly less **(Fig. 11C)**. Having proven that SK2 is partially sufficient in promoting cellular adhesion, its implication on invasion was then tested. Notably, HeLa cells overexpressing WT SK2 showed 2-fold higher basal and EGF-induced invasion than vector-transfected cells **(Fig. 11D)**. Collectively, these results indicate that SK2 is essential and partially sufficient in promoting cellular adhesion and invasion in an ezrin dependent manner.

4.3.5. S1P production by ACER2 and SK2 is sufficient to promote ERM phosphorylation

ACER2, also known as alkaline ceramidase 2, belongs to the family of alkaline ceramidases that de-acylate ceramide, thus generating sphingosine. It localizes to the ER and Golgi, and its overexpression leads to increased intracellular sphingosine and S1P levels (*20*). To evaluate the mechanism of S1P production in response to EGF, cells stably overexpressing ACER2 were used. ACER2-overexpressing cells showed 15-fold increase in ACER2 mRNA **(Fig. S4A)** and an increase in intracellular S1P levels **(Fig. S4B)**, confirming previously published results. ACER2 stable cells showed a significant baseline increase in ERM phosphorylation compared to vector stable cells, which increased further upon EGF treatment **(Fig. 12A)**. To rule out a chronic adaptive phenotype from the stable cell lines, HeLa cells were transiently transfected with ACER2. As found for the stable cell line, HeLa cells transiently overexpressing ACER2 also had a significant basal increase in ERM phosphorylation that was accentuated by EGF treatment compared to vector transfected cells **(Fig. 12B)**. Functionally, HeLa cells showed a significant increase in ERM phosphorylation upon ACER2 overexpression

that was heightened after EGF treatment when examined under confocal microscopy **(Fig. 12C)**. To test whether the higher phospho-ERM levels are due to increased S1P levels, the non-specific SK inhibitor Ski-II, the SK1 specific inhibitor PF-543 (*259*), and the SK2 specific inhibitor ABC294640 (*282*) were used. Ski-II and ABC294640 significantly decreased ERM phosphorylation upon EGF treatment not only in vector overexpressing cells, but also in ACER2 stable cells **(Fig. 12D, 12E, 12F)**. Notably, SK1 inhibition by PF543 did not affect the ERM response **(Fig. 12D, 12E, 12F).** These results suggest that, upon EGF treatment, sphingosine that is generated by ACER2 is specifically channeled to SK2 for S1P conversion, and subsequent ERM phosphorylation. To further validate that these effects are mediated by S1P, the specific S1PR2 antagonist JTE-013 was utilized. As expected, treatment with this receptor antagonist significantly diminished EGF-induced ERM phosphorylation in vector as well as in ACER2 stable cells **(Fig. 12D, 12E, 12F)**, consolidating previous findings that establish S1PR2 as an essential player in ERM activation downstream of EGF (*122*). Collectively, these results suggest that ACER2 and SK2 are sufficient to promote ERM phosphorylation.

4.3.6. EGF-mediated ERM phosphorylation occurs by intracellular S1P generation and not by extracellular S1P secretion

EGF has been shown to activate both SK1 and SK2 (*273*). However, as shown before, only SK2 is required for ERM phosphorylation upon EGF treatment, suggesting that the SK response to EGF towards ERM phosphorylation is somewhat compartmentalized. SK1 has been located to the plasma membrane, and it generates intracellular as well as extracellular S1P. The topology of SK2 and S1P generated from SK2 are more controversial. Due to the different locations of SK1 and SK2, and since both are reported to be activated by EGF, we focused in the

study of a possible different location of their product, S1P. First, the localization of S1P production was assessed post EGF treatment. For this purpose, we utilized C17-sphingosine (C17-Sph), an exogenously-utilized sphingosine for mass spectrometry-based measurements of cellular S1P generation (*285*). HeLa cells were first pre-treated with C17-Sph, and then simulated with EGF. Next, HPLC/MS was used to measure C17-S1P in the media and cells. EGF treatment caused a 40% increase in intracellular C17-S1P production that peaked in 30 seconds **(Fig. 13A)**. However, there was no change in the C17-S1P levels in the media upon EGF stimulation **(Fig. 13A)**. These results suggested that EGF strictly increases intracellular and not extracellular S1P levels. The effects of sphingosine stimulation on S1P production were also assessed **(Fig. 13B)**. Treatment of HeLa cells with sphingosine (5mM) significantly increased intracellular as well as extracellular S1P levels **(Fig. 13B)**.

To study the implications of these observations on ERM phosphorylation, the newly described S1P-neutralizing antibody Sphingomab was utilized (*286*). Pre-treatment of HeLa cells with Sphingomab abolished the effects of exogenously applied S1P on ERM phosphorylation compared to vehicle treated cells **(Fig. 13C)**. This demonstrates the efficacy of this neutralizing antibody. Likewise, neutralizing extracellular-S1P significantly decreased ERM phosphorylation upon treatment with sphingosine (5mM) **(Fig. 13C)**. However, HeLa cells stimulated with EGF to induce ERM phosphorylation were not blocked using Sphingomab. These results suggest that sphingosine treatment results in extracellular S1P production that is necessary for ERM phosphorylation whereas EGF stimulation results in intracellular S1P production, which will induce ERM phosphorylation likely without being secreted extracellulary.

To further distinguish autocrine/intracrine from paracrine actions of EGF via S1P, we used conditioned media from cells treated with Sph or EGF since Sph should produce extracellular S1P whereas EGF does not. To avoid the effects of carryover of Sph and EGF in the conditioned media, the 'recipient' cells were treated with a SK and EGFR inhibitor, respectively; as shown above that pre-treatment of cells with PF543 inhibits sphingosine-induced ERM phosphorylation and treatment with EGFR inhibitor Erlotinib inhibits the actions of EGF. As expected, addition of the conditioned medium extracted from sphingosine-treated cells increased ERM phosphorylation of HeLa cells that were pre-treated with the SK1 inhibitor **(Fig. 13D)**. This can only be explained by the presence of S1P in the conditioned media that was able to bypass SK1 inhibition **(Fig. 13D)**. On the other hand, addition of the conditioned medium extracted from EGF-treated cells did not affect phospho-ERM levels of HeLa cells that were pre-treated with Erlotinib **(Fig. 13D)**. This provides further evidence that EGF-mediated ERM phosphorylation does not require extracellular S1P generation. Taken together, these results suggest that ERM phosphorylation in response to EGF treatment occurs via an intracrine S1P signaling mechanism.

4.3.7. SPNS2 is required to deliver intracellular S1P to S1PR2

Since EGF-mediated ERM phosphorylation does not require extracellular S1P secretion yet is S1PR2 dependent, it became imperative to determine the mechanism of S1P transport to this receptor. This is a key step to explore, since it is very unlikely for intracellular S1P to reach its receptor (oriented extra-cytoplasmically even in intracellular compartments) passively due to topological restraints. First, the activation status of S1PR2 was checked upon neutralizing extracellular S1P. To this end, S1PR2-GFP internalization was assessed upon treatment with EGF, sphingosine, and S1P in the presence or absence of Sphingomab. As expected,

Sphingomab completely abolished S1PR2 internalization induced by S1P compared to vehicle treated cells, further validating the efficacy of this antibody in binding extracellular S1P **(Fig. 14A)**. In addition, it greatly reduced S1PR2 internalization induced by sphingosine, yet had no effect on EGF-induced S1PR2 internalization **(Fig. 14A)**, thus strongly corroborating the notion that SK2-derived S1P activates S1PR2 without extracellular secretion. Next, it became essential to determine how SK2-derived S1P reaches its receptor. Upon cellular fractionation and confocal microscopy, we found that SK2 is mostly present in cellular membranes including ER, golgi, endosomes and plasma membrane (data not shown) further confirming previously published results. The crystal structure of S1PR1 revealed that its S1P binding site is on it lateral surface, and that SK-derived S1P reaches this site via lateral diffusion on the outer leaflet of the plasma membrane (*287, 288*). Although the crystal structure of S1PR2 is not yet resolved, we were able to model it by superimposition with S1PR1 **(Fig. 14B, 14C)**. There was great similarity between S1PR1 and S1PR2 binding pocket, where both are present on the lateral surface of the receptor **(Fig. 14B, 14C)**. Furthermore, it has been previously shown that SK2 resides on the cytosolic side of cellular membranes (*289*); thus there must be a transport mechanism that delivers S1P produced on the cytosolic side to the outer membranous leaflets for S1PR2 activation. SPNS2, a lipid transporter, has been recently shown to be involved in S1P export *in-vitro* (*290*) as well as *in-vivo* (*36*). Hence, its role in EGF-mediated ERM activation was tested. Spns2 was knocked down in HeLa cells, and the effects on Spns2 mRNA and ERM phosphorylation were analyzed **(Fig. 14D)**. As can be seen, Spns2 siRNA treated cells showed more than 95% knockdown of Spns2 mRNA compared with all-Star (Astar) negative control treated cells **(Fig. S5)**. Spns2 knockdown significantly diminished ERM phosphorylation in response to EGF and sphingosine compared to Astar treated cells **(Fig. 14D)**. These results indicate that Spns2 is partly required

for both sphingosine and EGF-mediated ERM phosphorylation. Next, to test for the role of Spns2 in S1PR2 activation, we knocked it down in S1PR2-GFP overexpressing HeLa cells and checked for receptor internalization. Cells treated with Spns2 siRNA showed a partial reduction in EGF and sphingosine-induced S1PR2 internalization compared to Astar treated cells **(Fig. 14E)**. Therefore, these results demonstrate that Spns2 is required to transport S1P (generated from sphingosine by SK1) across the plasma membranes, and to transport S1P (generated by SK2 in response to EGF), possibly across intracellular membranes to access S1PR2 and lead to ERM phosphorylation.

4.4. Discussion

In the current chapter, we describe a new mechanism for EGF-driven cancer invasion by SK2-deriven intracellular S1P and the activation of the ERM (ezrin, radixin and moesin) family of proteins. In short, EGF stimulates ERM activation in a ErbB1 and SK2 dependent manner. This is followed by intracellular S1P production that is then exported to the exo-cytoplasmic leaflet of endogenous membranes, where it docks on S1PR2. Once active, this receptor triggers a signaling cascade leading to ERM phosphorylation, which in turn increases cellular adhesion and subsequent invasion **(Scheme 4)**. This mechanism is different, but probably complementary, to previously reported mechanisms of EGF-induced cellular invasion, by either inducing the MAPK pathway, and/or triggering the activation of the PI3K cascade. These results have implications to several fields including novel functions pertaining to intracellular S1P signaling, a novel role for the spns2/S1PR2 axis in cancer cell motility, revealing a novel SK2-specific function, and defining new targets for therapy in growth factor-driven cancers.

4.4.1 A novel and specific SK2 function

The first significant finding corresponds to establishing a specific and novel function downstream of SK2 related to cell adhesion and cell invasion. There is a marked paucity in the literature about biological functions that are mediated by a specific SK isoform. Although both SKs have been reported to be activated by EGF (*273, 291*), we report, in this context, only SK2 as being necessary for ERM phosphorylation in response to EGF. This is an important finding as it ascribes a completely novel function that is specific for SK2. Very few pathways have been described to be specifically mediated by SK2. These include the sequestration of the pro-survival BCL-XL protein via its BH3 domain, the activation of BAK, and the inhibition of HDAC1/2 in the nucleus. In addition, most animal studies using SK2 inhibitors fail to relate the phenotype observed to SK2 inhibition alone. This is the first report assigning a mechanistic function for SK2; that is ERM activation.

4.4.2. The role of SK2 in growth factor mediated adhesion and invasion

The results of this study also support the emerging evidence on the pro-cancerous roles of SK2. Previously, SK2 has been suggested to increase the migration of MDA-MB-453 cells towards EGF (*273*). Nonetheless, increased cell motility is not enough to confer a malignant cellular behavior since other processes, such adhesion and invasion, are crucial. In this study, we provide additional evidence of the tumorigenic role of SK2, as it is not only essential, but also sufficient to augment cellular adhesion and subsequent invasion. Furthermore, there is a paucity of studies that explore the mechanisms exploited by SK2 to confer malignancy. In this study, we found that the ERM family of proteins is a potent downstream effector conducting the pro-tumorigenic functions of SK2. These proteins have been increasingly involved in cancer metastasis, and are being suggested as biomarkers for tumor aggressiveness. Therefore, targeting

SK2 could constitute a novel strategy to turn-off the activity of these proteins; ultimately lessening the cancer cells' ability for adhesion and invasion.

4.4.3. A novel intracellular S1P signaling model

More importantly, this study defines a new mechanism for intracellular S1P signaling. We demonstrated that EGF-mediated ERM phosphorylation does not necessitate the secretion of S1P extracellularly using several lines of evidence. Based on these results, it becomes clear that the fate of S1P is dictated by the enzyme producing it, as well as its localization. Whereas SK1-derived S1P (that is generated after sphingosine treatment) requires extracellular secretion for ERM phosphorylation, SK2-derived S1P is either maintained in the intracellular milieu or in the plasma membrane. Strikingly, S1PR2 activation was still required to mediate ERM activation. This raised the question of how this intracellular lipid can encounter its receptor. S1PR2 and SK2 are membranous proteins that are continuously trafficking between the different endosomal compartments, a phenomenon that would facilitate their interaction. Indeed, blocking intracellular trafficking by hypertonic sucrose abolished ERM-induced phosphorylation by EGF **(Fig. S6)**. In addition, the possibility that S1PR2 might be signaling while localized intracellularly is very likely. This will not be the first S1P receptor described to have intracellular functions, as recently S1PR5 has been shown to localize to centrosomes along with both SKs to regulate cell division (*292*). On the other hand, one might argue that EGFR might cause S1PR2 internalization and activation irrespective of SK activation as has been previously shown with S1PR1 and PDGF (*293*). However, in our hands, knocking down SK2 significantly decreased S1PR2 activation (data not shown) thus ruling out this hypothesis. Collectively, these results describe a new mechanism for intracellular S1P signaling. Whilst it is thought that intracellular

S1P mediates its functions by binding exclusively to specific proteins such as TRAF2 and HDACs, we provide an alternative model for intracellular S1P that still requires S1P receptors signaling. While this model is still nascent, it can explain the scarcity of known intracellular targets for S1P.

The current study highlights a novel function for the newly identified S1P transporter Spns2. This transporter has been shown to play an essential role in lymphocyte trafficking (*294*) and cardiac development (*295*). Although this transporter is a major regulator of S1P levels, and therefore cell motility, its role in cancer progression has been understudied as well as controversial. One study showed that knocking down this transporter increase intracellular S1P levels and consequently migration of lung cancer cells (*290*). In contrast, another study showed that spns2 is an essential player in promoting colon cancer following inflammation (*296*). The controversy may be in part due to the dependency of tumor motility on S1P receptors. Whereas migration of lung cancer cells seems to be receptor independent (*290*), colon cancer development is dependent on S1PR1 activation (*296*). This is similar to our study, where cervical cancer motility is dependent on S1PR2 signaling, and spns2 is an essential player in transporting S1P to its receptor. Besides, we are aware that the effect of spns2 knockdown on EGF-induced ERM phosphorylation and S1PR2 internalization are partial, but this could be due to a complimentary role that is played by other S1P transporters such as ABC transporters (*297*). In fact, inhibiting these transporters by Probenecid or MK-571 partially inhibited EGF-induced ERM activation **(Fig. S7)**. Therefore, it could be that S1P transport in HeLa cells is not exclusively mediated by Spns2, but other several carriers could also play a role, including the ABC family of proteins.

Moreover, since both sphingosine and EGF treatment require Spns2, and therefore S1P transport, it became essential to explain the reason how S1P generated upon sphingosine treatment make it to the extracellular milieu, whereas that generated upon EGF treatment remains inside the cell. This could mostly be due to differences in SKs localization. Our group, as well as many others, have shown that upon activation, SK1 is localized to the plasma membrane, where it phosphorylates sphingosine. Spns2 and the ABC proteins transport S1P that is produced on the inner leaflet of the plasma membrane to the extracellular milieu. On the other hand, SK2 does not localize to the plasma membrane, and is mainly present on the intracellular membranes, mainly in the ER, Golgi and endosomes **(Fig. S8)**. In addition, it has been previously demonstrated that SK2 produces S1P on the cytosolic side of these intracellular vesicles. We suspect that Spns2 will then import S1P to the inside of these vesicles, which will then fuse with others that contain S1PR2 either intracellularly or on the plasma membrane, where activation of this receptor occurs. This activation is then also responsible for enhancing endocytosis of the receptor. This model is supported by the essential role of intracellular trafficking that we proved essential for ERM activation **(Fig. S6)**. Further testing is required to validate this hypothesis, and is being currently investigated in our lab.

4.4.4. The Spns2/S1PR2 connection

The Spns2/S1PR2/ERM axis as defined in this chapter seems to be evolutionary connected. In fact, we note that mice deficient in Spns2, S1PR2, or radixin each develop progressive hearing loss (*298-300*). Spns2 and S1PR2 knockout mice show degeneration of the sensory hair cells in the inner ear (*298, 300*). This is thought to be caused by abnormally dilated and branched stria vascularis with irregular cortical actin patterns. Furthermore, radixin knockout

mice develop progressive degeneration of the steriocilia present on hair cells leading to deafness (*299*). On the other hand, the Spns2/S1PR2 axis has also been well established in Zebrafish as it plays an essential role in vertebrate heart morphogenesis (*301, 302*). Spns2 and S1PR2 deletion causes the zebrafish to develop cardia bifida, a cardiac malformation characterized by the development of two hearts; it was attributed to a defect in the migration of myocardial precursors to the midline. Importantly, the addition of S1P to Spns2 deficient embryos restored normal heart development in a S1PR2 dependent manner (*301*). These observations strengthen the current results implicating the Spns2/S1PR2 axis in cell movement. Moreover, these two genetic mutants also share the same abnormal phenotype that is characterized by the appearance of tail blisters in zebrafish (*301*). In conclusion, this body of work solidifies the role of the Spns2/S1PR2 axis in cellular movement, however in a different context that is related to cancer cell motility.

In conclusion, these results delineate a novel pathway for growth factor-driven invasion, which is dependent on intracellular S1P signaling under EGF stimulation. This pathway could have tremendous implications in the treatment of EGF aggravated tumors, as it provide novel therapeutic targets. This is especially important for patients that become refractory to current treatment modalities due to acquired mutations in the EGF receptor.

Chapter 5

A novel role for checkpoint kinases, Chk1 and Chk2, in S1P signaling and cancer cell invasion

5.1. Introduction

ERM (ezrin, radixin, moesin) constitute a family of proteins that links the plasma membrane with the cortical actin cytoskeleton (*4*). They are mostly found in distinct cellular structures such as filopodia, lamellopodia and invadiopodia (*5*). Thus, they play an important role in regulating the cell shape, polarity and motility (*6, 7, 9, 10*). They are frequently altered in tumors, giving cancerous cells the advantages of survival, adhesion, migration and invasion, therefore promoting malignancy (*278*).

When inactive, the N-terminal domain (FERM) and the C-terminal domain (C-ERMAD) interact, therefore locking the protein in the cytosol. Upon phosphorylation on a C-terminal threonine residue (ezrin Thr-567, radixin Thr-564 and moesin Thr-558), steric hindrance is created between the N and C termini, causing their dissociation. The C-ERMAD domains will then bind to the cortical actin, and the FERM domain will bind to the plasma membrane in a phosphatidylinositol 4,5-bisphosphate (PIP_2) dependent manner (*284*).

Several kinases have been proposed to phosphorylate ERM proteins and activate them. This depends on a multitude of factors including the stimulus inducing activation, as well as the specific cell line involved. Examples of these kinases include the ROCK (Rho-associated protein kinase) (*49*), GRK2 (G protein coupled receptor kinase) (*303*), AKT2 (or protein kinase B) (*304*), NIK (Nck-interacting kinase) (*305*) and LOK (lymphocyte-oriented kinase) (*306*). A full list of these kinases is provided in **table 1** (chapter 1).

Protein kinase Cs (PKCs) have been by far the most kinases implicated in ERM phosphorylation in a variety of cell lines (PC12 (*50*), NPC (*92*), osteosarcoma (*85, 93*), HPAAEC (*76*), LNCaP-FGC (*307*), fibrosarcoma (*308*)) as well as downstream of many

inducers (amphetamine (*50*), dinotoso-piperazine (*92*), glutamate (*51*), estradiol (*76*), androgen (*307*), parathyroid hormone (*309*)). In addition, PKCs have been also shown to mediate several ERM functional biologies that are not limited to migration (*308*) and invasion (*85, 307*), but extend to endocytosis (*309*), differentiation (*310*), and synaptic plasticity (*50*).

Sphingolipids play an important role in regulating the cellular cytoskeleton as well as cell migration and invasion (*284*). Specifically, our group has recently showed that sphingosine-1-phosphate is a potent and acute inducer of ERM phosphorylation via activation of S1PR2 (*122*). S1P-mediated ERM activation induced filopodia formation. In addition, S1P production downstream of EGF stimulation triggered lamellopodia formation, as well cellular adhesion and invasion in an ERM dependent manner as shown in chapter 3 and 4 (*122*). There is ample of literature showing that the bioactive lipid S1P can activate several proteins, including serine/threonine kinases, suggesting the presence of S1P-activated kinase downstream of S1PR2 that is responsible for ERM phosphorylation.

In this study, we aim to identify the kinase responsible for ERM phosphorylation downstream of S1P stimulation in HeLa cells. We rule out all the previously known ERM kinases as possible mediators of ERM activation downstream of S1PR2 in HeLa cells, including PKCs. In addition, we demonstrate that checkpoint kinases, Chk1 and Chk2, are involved in mediating ERM phosphorylation after S1P treatment. We also implicate these kinases in a novel biological function that is cellular invasion; besides their recognized roles in controlling the cell cycle; thus further corroborating the new emerging concept stating that a cell should stop its proliferation phase for it to invade.

5.2. Material and Methods

5.2.1. Materials

High glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Oligofectamine, Lipofectamine siRNA max and Lipofectamine 2000were purchased from Life Technologies (Norwalk, CT). Essentially fatty acid-free bovine serum albumin (BSA), and monoclonal anti-β-actin antibody were from Sigma-Aldrich (St. Louis, MO). Anti-pERM, anti-Total ezrin, anti-PKCα, anti-PKCβII, anti-ChK1 and anti-Chk2 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-PKCε, Anti-PKCδ, Anti-PKCη, and Anti-PKCθ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Chemiluminesence kit and BCA kit were from ThermoScientific (Suwanee, GA). AZD7762 (3- [(aminocarbonyl)amino]- 5- (3- fluorophenyl)- N- (3S)- 3- piperidinyl- 2- thiophenecarboxamide) was from Cayman Chemicals (Ann Arbor, MI). MK-8776 was purchased from Selleckchem (Houston, TX). Chk2 inhibitor 2 was purchased form Sigma-Aldrich (St. Louis, MO). G06976 was purchased from. Bisindolylmaleimide I was purchased from. Sphingosine-1-phosphate was purchased from Avanti (Miami, FL)

5.2.2. Cell culture and siRNA

HeLa were purchased from American Type Culture Collection (ATCC, Manassas, VA). DMEM was supplemented with 10% FBS, and cells were grown at 37^0C and 5% CO_2. In all cases, before S1P treatment, cells were starved with serum free DMEM for at least 4 hours. Gene knockdown was carried out using siRNA directed against GRK2, NIK, LOK, PKCα, PKCβII, PKCε, PKCθ, PKCη, PKCδ, Chk1 and Chk2. Qiagen all-star siRNA was used a negative control. Transfections for PKCs siRNAs were carried out using Lipoctamine siRNA max, and transfections for Chks siRNAs were carried out using Oligofectamine according to the manufacturer's protocol.

5.2.3. Immunoblotting

Cells were directly lysed in 0.5% SDS in H_2O, then sonicated on ice. Protein quantification was done using the BCA kit and according to the manufacturer's protocol. Normalization was performed and Laemlli loading buffer was added to a final concentration of 2X. Samples were than boiled for 10 minutes, and proteins were separated via SDS-PAGE using the Life Technologies NuPAGE gels. Proteins were transferred to nitrocellulose membranes and blocked for 1 hour in 5% milk in PBST/0.1% Tween. Primary antibodies were than added at a dilution of 1:1000 or 1:20000 for β-actin, and left on the membranes at 4^0C overnight. Secondary antibodies were than added at a dilution of 1:5000 for 1 hour at room temperature, before visualization by chemiluminescence.

5.2.4. Plasmid constructs and DNA transfection

WT PKCα , CA PKCα δ (ID:21233), DN PKCα, WT PKCβ, CA PKCβ δ (ID:16383), WT PKCε δ (ID:21240), CA PKCε δ (ID:21241), DN PKCε δ (ID:212143), WT PKCη δ (ID:21244), CA PKCη δ (ID:21245), DN PKCη δ (ID:21246), WT PKCδ (ID:16386), CA PKCδ δ (ID:16387) or DN PKCδ δ (ID:16389), and WT Chk1-Flag (ID: 22894) DNA constructs were obtained from Addgene (Cambridge, MA). Chk2 (Myc-DDK-tagged), transcript variant1 was purchased from Origene (Rockville, MD). All plasmid sequences were confirmed in the DNA Sequncing Facility (Stony Brook University, NY). Transient overexpressions were carried out using Lipofectamine 2000 (Life Technologies), and according to the manufacturer's protocol.

5.2.5. Cellular invasion assays

Cell invasion studies were performed using the Corning Tumor Invasion System, and according to the manufacturer's protocol. Briefly, HeLa cells were treated with AStar, Chk1, Chk2, or both siRNA for 48 hours. Cells were then starved for 4 hours prior to trypsinization. Cells were counted, and equal numbers were loaded on the upper chamber of the 24-well transwell plate. DMEM with or without serum was added to the lower chamber. Cells were allowed to invade for 24 hours, before staining the invading cells with the fluorescent dye, Calcein AM (Life Technologies). Fluorescence was then assessed on a Spectramax M5 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA).

5.3. Results

5.3.1. GRK2, NIK, LOK, ROCK and MAPK are not essential for S1P-mediated ERM phosphorylation

In order to find the specific kinase phosphorylating the ERM proteins downstream of S1P, we started by screening for all the kinases that had been previously reported to mediate ERM phosphorylation on the Threonine 567 residue. G-protein coupled receptor kinase 2 (GRK2) has been found to phosphorylate the ERM proteins in response to wound closure (*303*), angiotensin-II (*311*) and acetylcholine (*312*) in MDCK cells, kidney Podocytes and Hep2 cells respectively. siRNA was used to knock down GRK2 in HeLa cells, and the effects on ERM phosphorylation was analyzed. As can be seen, a significant knockdown of GRK2 protein was observed in siRNA-treated cells compared to all-Star (AStar) negative controls **(Fig. 15A)**. Notably, ERM phosphorylation was not affected by GRK2 knockdown upon treatment with increasing doses of S1P (1nM, 10nM, 100nM, 1uM) **(Fig. 15A)**. These results indicate that GRK2 is not involved in ERM phosphorylation in HeLa cells upon S1P stimulation. Next, the role of the members of the STE20/P21-activated kinases family (PAK): Nck-interacting kinase

(NIK) and lymphocyte-orienting kinase (LOK) was assessed. Importantly, NIK had been also reported to phosphorylate the ERM proteins downstream of EGF and PDGF in MTLn3 cells (*305*). Although a significant knockdown of NIK mRNA was achieved upon treatment with NIK siRNA (data not shown), there was no effect on ERM activation upon S1P stimulation compared to AStar treated cells **(Fig. 15B)**. These results rule out a possible role for NIK in this system. Furthermore, knocking down LOK, which was shown to regulate lymphocytes motility via direct ERM phosphorylation (*306*), did not affect ERM activation following S1P stimulation **(Fig. 15C)**. Next, the role of ROCK was assessed, as this enzyme had been widely implicated in ERM phosphorylation downstream of many stimuli including but not limited to estrogen (*91*), netrin (*49*), LPA (*313*), cisplatin (*100*)and TNF (*314*). To this end, Y27, RK13 and H11, well-known pharmacological inhibitors of ROCK were utilized. Strikingly, none of these inhibitors decreased ERM phosphorylation after S1P treatment **(Fig. 15D)**. The p38 MAPK pathway had also been reported to mediate ERM phosphorylation in response to TNF (*63*) and advanced-glycation-end-products (*78*) in synoviocytes and endothelial cells respectively. The MEK inhibitor, U013, was used to check if this pathway plays a role in ERM activation. As can be seen, U013 had no effect on ERM phosphorylation following S1P stimulation **(Fig. 15E)**. Collectively, these results indicate that the previously reported ERM kinases, GRK2, NIK, LOK, ROCK and p38 MAPK are not required for ERM phosphorylation downstream of S1P in HeLa cells.

5.3.2. Classical and novel PKCs are not required for S1P-mediated ERM phosphorylation

Classical and novel PKCs have been reported to mediate ERM phosphorylation downstream of many stimuli that include amphetamine (*50*), dinitrosopiperazine (*92*) , 2-methoxy-estradiol (*76*), glutamate (*51*), latent membrane protein-1 (*6*), hypotonicity (*315*), androgen (*307*) and parathyroid hormone (*309*). Furthermore, ERM activation by PKCs has been functionally attributed to the invasive behavior of osteosarcoma (*85, 93*) and fibosarcoma (*308*) and the differentiation of intestinal epithelial cells (*310*). Owing to the fact that PKCs have been heavily implicated in ERM phosphorylation in many cellular contexts along with diverse functional consequences, their role in our system was studied extensively. First, G06976 (G0) and Bisindoylmaleimide I (Bis), two known pharmacological inhibitors for classical PKCs (PKC α and β) and all PKCs respectively, were utilized. Importantly, G0 and Bis treatment resulted in a significant decrease in ERM phosphorylation upon S1P stimulation, with Bis having the most substantial effect **(Fig. 16A)**. These results suggest that PKCs might play a role in ERM phosphorylation downstream of S1P. Since these two inhibitors are known to have multiple off-target effects, we resorted to siRNA technology. As can be seen, a significant knockdown of PKC α, β, δ, η, ε and θ was observed in siRNA-treated cells compared to AStar negative controls **(Fig. S9)**. Unexpectedly, Knocking down classical PKCs, PKCα and PKCβ, did not affect ERM phosphorylation following S1P treatment **(Fig. 16B)**. Similarly, knocking down novel PKCs, individually **(Fig. 16C)**, in combination **(Fig. 16D)** or all of them **(Fig. 16D)**, did not decrease ERM activation. Because knocking down PKCs did not mirror the inhibitors data above, it can be inferred that both Bis and G0' effect on ERM phosphorylation is a non-specific, or the knock down of PKCs was not optimal. To rule out the latter explanation, and since it is reported that PKCs can maintain full functional abilities even at lower protein levels; we resorted to the overexpression of constitutionally active (CA) and dominant negative (DN) PKCs

constructs. HeLa cells transiently transfected with the WT, CA or DN forms of PKC α **(Fig. 16E)**, βII **(Fig. 16E),** δ **(Fig. 16H)**, η **(Fig. 16G),** and ε **(Fig. 16F)** had the same levels of basal as well as S1P-induced ERM phosphorylation compared to mock transfected cells. In conclusion, these results exclude the role of classical and novel PKCs in ERM phosphorylation in HeLa cells, and confirm that G0 and Bis are non-specifically inhibiting a yet unidentified ERM-kinase. .

5.3.3. CHK1 and CHK2 kinases are required in mediating S1P-induced ERM phosphorylation

Besides targeting classical PKCs, G06976 has been widely used as a checkpoint kinases (Chks) inhibitor. These proteins are a family of two specific serine/threonine kinases known as Chk1 and Chk2. They have been extensively involved in regulating the cell cycle as well as the DNA damage response. Chk1 is overexpressed in multiple tumors including colon, breast and liver, and its levels correlated with tumor grade and progression. These kinases have been targeted by many pharmacological inhibitors that are being tested in clinical trials, due to their potential therapeutic role in inhibiting tumor progression. Thus, we hypothesized that Chk kinases are involved in regulating the pro-tumorigenic S1P/ERM pathway. To test this hypothesis, siRNA technology was the first method used. HeLa cells treated with either Chk1 and/or Chk2 siRNA show almost complete protein knockdown compared to AStar treated cells **(Fig. 17A)**. Importantly, knocking down Chk1 or chk2 causes a modest but a significant decrease in S1P-mediated ERM phosphorylation **(Fig. 17A, 17B)**. On the other hand, cells treated with both Chk1 and Chk2 siRNA had more than 50% decrease in ERM activation **(Fig. 17A, 17B)**.

These results suggest that Checkpoint kinases are required for ERM phosphorylation upon S1P treatment. In addition, the role of Chk2 kinase in ERM phosphorylation was explored in HeLa cells knocked out from Chk2 using CRISPR technology **(Fig. 17C)**. Decreased Chk2 levels correlated with lower S1P-induced phospho-ERM levels, therefore verifying the siRNA results. For further validation, Chk2 inhibitor 2, MK-8776 and AZD7762 known pharmacological inhibitors for Chk2, Chk1 and both respectively, were used. HeLa cells pretreated with Chk2 inhibitor 2 **(Fig. 17D)**, MK-8776 **(Fig. 17E)**, and Azd7762 **(Fig. 17F)** show a significant decrease in S1P-induced ERM phosphorylation compared to vehicle treated cells. Collectively, these results indicate that checkpoint kinases, Chk1 and Chk2 are essential in S1P-mediated ERM phosphorylation. Because some tumors exhibit higher Chk kinases levels, which correlated with their aggressiveness, we wanted to test if overexpressing these proteins is sufficient to induce ERM activation. To this end, HeLa cells were transiently transfected with WT Chk1-FLAG or WT Chk2-myc overexpressing vectors for 24 hours. Cells overexpressing CHK1 had the same baseline phospho ERM levels as vector transfected cells **(Fig. 17G)**. Nevertheless, cells expressing CHK2 had higher baseline phospho ERM levels compared to vector transfected cells **(Fig. 17G)**. These results indicate that Chk2 and not Chk1 is sufficient to induce ERM activation. In conclusion, Chk1 and Chk2 play an important role in the S1P/ERM axis, yet their role as direct ERM kinases is still to be determined.

5.3.4. Knocking down Chk1 and Chk2 decreases cell invasion

ERM activation has been shown to modulate the cell shape and promote cell igration and invasion. Since checkpoint kinases are essential in ERM phosphorylation (**Fig. 17)**, it became crucial to determine their role in cellular motility. To this end, an invasion assay using transwell chambers coated with Matrigel was conducted **(Fig. 18)**. HeLa cells transfected with Chk1

siRNA exhibited a 3-fold decrease in invasion compared to AStar transfected cells **(Fig. 18)**. In addition, HeLa cells treated with Chk2 siRNA also showed a decrease in invasion, however to a lesser extent than Chk1 treated cells **(Fig. 18)**. Importantly, knocking down both checkpoint kinases had an addictive effect with a significant decrease in cellular invasion **(Fig. 18).** Collectively, these results indicate that checkpoint kinases play an essential role in the invasion of the extracellular matrix in HeLa cells.

5.4. Discussion

In this chapter, we identify a new role for checkpoint kinases, Chk1 and Chk2, in the acute regulation of ERM (ezrin radixin, moesin) proteins by S1P and S1PR2. Thus, S1PR2 activation induces ERM phosphorylation through Chk1 and Chk2, therefore increasing cellular invasion. This mechanism is distinct from the previously reported mechanisms of ERM regulation by PKCs, and other serine/threonine kinases that had been also ruled out in this system. These results have implications for the regulation of ERM proteins by novel kinases, and the function of checkpoint kinases in cellular invasion, in addition to their established role in the DNA damage response and cell cycle progression.

5.4.1 A novel role for Chk1 and Chk2 that is independent of DNA damage

Chk1 and Chk2 are two structurally different serine/threonine kinases but with overlapping functions (*316*). They perform multiple redundant roles as it becomes evident by many of their common substrates including Mdm2, p53, Cdc25A and Cdc25C. They are activated in response to diverse genotoxic stressors, which depending on their type, time frame

and cellular context will lead to activation of distinct cellular pathways. These include induction of 1) transcription of the stress-induced program, 2) DNA repair, 3) cell cycle arrest, and/or 4) apoptosis (*316*). The first significant finding of this study is the discovery of a completely novel pathway that is regulated by these proteins, therefore extending their scope of actions. In fact, this is the first report that links the regulation of ERM proteins to checkpoint kinases.

In addition, this is one of very few reports that ascribe a checkpoint kinase function that is independent of DNA damage. In fact, Chk1 has been shown to act as a positive regulator of EGFR signaling (*317*). Knocking down Chk1 inhibited Mig-6 (a tumor suppressor gene product) phosphorylation at Ser251, which increased Mig-6 inhibitory activity against EGFR. As a result, Chk1 depletion decreased EGF-induced EGFR activation and cell growth (*317*). Another study also implicates Chk2 with the EGF response, yet inversely. In fact, EGF stimulation of HNSCC cells (UT-SCC-26A) resulted in a dose dependent increase in Chk2 levels (up to 10ng/mL), which altered growth and proliferation (*318*). Nevertheless, this correlation of Chk2 with cell growth has been recently challenged. In short, a new study has shown that Chk2 bind to ERK1/2 and inhibits their actions. Thus, expressing a dominant negative form of Chk2 increase the pro-oncogenic activity of ERK1/2 (*319*). Although our study ascribes a pro-tumorigenic role for Chk2, the causes of these discrepancies regarding the role of Chk2 in tumorogenesis are still unknown.

5.4.2. New Mechanisms for Chk1 and Chk2 regulation

This chapter sheds the light on a potential new mechanism for Chk1 and Chk2 regulation. Following DNA damage, Chk2 is phosphorylated by ATM on Threonine 68 in response to double-strand DNA breaks. This in turn promotes homodimerization of Chk2, which causes

autophosphorylation of additional residues on its C-terminal (T387, T383), thus allowing full activation (*320*). Chk1 on the other hand is activated by phosphorylation on Serine 317 and Serine 345 by ATR (*316*). Interestingly, S1P treatment does not affect the phosphorylation of any of these sites (data not shown). Therefore, there might be other post-translational modifications that are induced by S1P, and that do not affect Chk1 or Chk2 activity. In fact, Chk1 and Chk2 possess several other phosphorylation sites, that don't affect their kinase activity levels, nevertheless have other functional consequences. For example, EGF induces Chk1 phosphorylation at Serine 280 via the PI3K pathway, which will tether Chk1 in the cytoplasm to mediate DNA-damage independent functions (*317*). Alternatively, Chk1 and Chk2 could provide the platform for other kinases that are essential for ERM phosphorylation, yet require the presence of these checkpoint proteins for their functions. Further studies are ongoing in our laboratory to determine the mechanism of Chk1/2 regulation by S1PR2.

5.4.3. Chk1 and Chk2 regulation of cellular invasion

Another important contribution of this report is that it defines a new biological function for Chk1 and Chk2 that has not been studied before that is cellular invasion. As previously mentioned, knocking down checkpoint kinases decreased cellular invasion; hence, defining two druggable targets that might be useful in tumor therapy. In fact, Chk1 inhibitors are currently being tested in clinical trials for cancer treatment in combination with chemotherapy (*321*). The rationale behind it lies in that Chk1 inhibition will eliminate an essential cell cycle checkpoint that is required for DNA repair. Therefore, cells won't be able to repair DNA damage induced by chemotherapy. Accumulated mutations will then lead to cell death. This drug combination will thus increase chemotherapy effectiveness and decrease its side effects (*322*). These clinical trials had limited successes due to the toxicity associated with these inhibitors as well as due to their

low bioavailability (*316*). Our results indicate that Chk1 and Chk2 inhibitors might be sufficient by themselves in decreasing cellular invasion without the need of concomitant chemotherapy. However, further studies are needed to test this hypothesis.

5.4.4. The inverse relationship between cell cycle control and cellular invasion

Finally, a critical area of investigation would be to test for the status of the cell cycle in our system (HeLa cells) upon knocking down the checkpoint kinases and in the absence of genotoxic stressors. In fact, our preliminary results indicate that Chk1 absence (induced by siRNA) decreases the proportion of cells in the G1 phase, and increases the proportion of cells in the G2/M phase (data not shown). At a first glance, it seems that Chk1 absence triggers the cells to undergo mitosis, yet decreases their invasion capability. This corroborates the newly emerging concept, which ascertains that for a cell to invade, it is required to stop its proliferation cycle (*323-325*). This theorem needs further investigation in our system, as an increase in the G2/M phase could also mean a cell cycle arrest in that phase. Studies further exploring this concept are ongoing in our laboratory.

In conclusion, our results define a distinctive mechanism for ERM phosphorylation downstream of S1P stimulation, which is independent of previously described mechanisms. Importantly, Chk1 and Chk2 seemed to be involved in ERM activation, and subsequent cellular invasion, and independently of DNA damage. Taken together, these results suggest that these two kinases could be considered as attractive therapeutic targets for a multitude of cancers without the need to use contemporaneous chemotherapy.

Chapter 6

Discussion and Future Directions

6.1. Determine the mechanism of SK2 regulation by EGF

Sphingolipid metabolizing enzymes, especially SKs, have been extensively reported to be regulated by growth factors such as EGF, TGF (*326*), PDGF (*327*), and IGF (*328*). SK1 is the most studied enzyme isoform. Upon stimulation, SK1 gets phosphorylated by ERK1/2 on serine 351 and threonine 578, which causes its transport by CIB1 (calcium and integrin-binding protein 1) to the plasma membrane, where it initiates its oncogenic signaling (*329*). Alternatively, SK1 has also been found to be phosphorylated, and activated by phospholipase D. The regulation of SK2 however is more challenging, and is still to be determined.

Given the time frame for growth factors responses (1-2 minutes), it rules out transcriptional and translational mechanisms of SK2 activation. SK2 is localized in many cellular structures including the ER, Golgi, endosomes, nucleus, cytoplasm and plasma membranes (*261*). Upon performing cellular fractionations, we have found MYC-tagged SK2b expressed in HeLa cells localized mainly in cellular membranes (ER, Golgi, Endosomes) with a small fraction in the cytosol **(Fig. S8)**. Interestingly, EGF did not cause SK2 translocation to a different compartment **(Fig. S8)**, thus ruling out allosteric mechanisms. Therefore, other regulatory mechanisms must be taking place that would be crucial to pursue in the future.

First, it will be essential to identify the missing players between ErbB1 and SK2 as will be described in section 6.1.1. In addition, since overexpressing the SK2 mutants (SK2 S387A, T614A) did not abolish ezrin phosphorylation upon EGF treatment **(Fig. 10)**, we suspect additional layers of SK2 regulation. Therefore, it will be noteworthy to determine if other posttranslational mechanisms are taking place in an independent line of study as will be described in section 6.1.2.

6.1.1 Identify the kinase mediating SK2 activation

We already started our investigation in this subject. It has been previously reported that EGF induces phosphorylation of SK2 at S351 and T578 by ERK1/2 (as part of the MAPK pathway), which increases SK2 activity (*283*). Therefore, the MEK inhibitor U0126 was used to test the role of the p38 MAPK in ERM activation. Unexpectedly, ERK inhibition did not affect ERM phosphorylation after EGF stimulation **(Fig. 19)**; To further pursue this goal, we continued our search by exploring multiple kinases that have been reported to play an essential role downstream of ErbB1 including but not limited to PKA, PKCs and AKT. We elected to test the effect of these three kinases on ERM phosphorylation as it has been predicted that SK2 possesses several putative phosphorylation sites for them (*330*). To this end, we utilized EMD476485, Bisindoyl-maleimide I, GÖ6976 and EMD124017 to inhibit PKA, all PKCs, classical PKCs and AKT respectively. Of interest, the inhibition of PKA and AKT did not affect ERM phosphorylation following EGF nor S1P treatment **(Fig. 19)**. Although the inhibition of PKCs drastically reduced ERM phosphorylation after EGF stimulation, it also inhibited S1P-mediated ERM activation, suggesting that Bisindoyl-maleimide I and GÖ6976 might be affecting the pathway downstream of S1P production and not the one upstream of SK2, as already discussed in chapter 5 **(Fig. 19)**. In summary, none of these kinases ap mediate SK2 activation downstream of the EGF/EGFR pathway. These results suggest that there is a novel mechanism for SK2 regulation by EGFR.

The best way to proceed exploring this kinase is by searching for SK2 binding partners on immunoprecipitated SK2. This strategy would clearly identify a novel SK2 kinase, as all previously described partners have been ruled out.

6.1.2. Identify SK2 post-translational modifications upon EGF treatment

This goal is best achieved by using proteomic approaches on immunoprecipitated protein (SK2). Once identified, overexpressing phosphorylation mutants is crucial to check the role of these modifications on SK2 activity. Alternatively, tyrosine phosphorylation, and to a lesser extent serine/threonine phosphorylation, can be assessed on immunoprecipitated SK2 by western blot using anti phospho-tyrosine and anti phospho-serine antibodies. This method is crude however, and will not be as sensitive as mass spectrometry.

6.2. Identify the ERM kinase and the signaling pathway between S1PR2 and the ERM family of proteins.

EGF and S1P are known to induce tumor progression by promoting cell migration and invasion. As previously mentioned, ezrin is known to be an essential component for cell invasion as demonstrated in several animal models of metastasis. EGF, as well as its downstream effector S1P, are two robust ezrin activators that mediate this action by activating S1PR2. In chapter 5, we have investigated the mechanisms of ERM phosphorylation downstream of S1PR2. We ruled out all the possible kinases that have been previously shown to phosphorylate ezrin including PKCs, GRK2, AKT, ROCK, p38 MAPK, LOK and NIK. We have also determined that checkpoint kinases, Chk1 and Chk2 are essential components downstream of S1PR2 that mediate ezrin activation. However, we did not assess weather they can directly bind ERM proteins to phosphorylate them or if they are indirectly required. This point will be elaborated upon in section 6.2.1. In addition, the signaling pathway downstream of S1PR2 and leading to

ERM phosphorylation is still unknown and requires further investigations as will be discussed in section 6.2.2.

6.2.1. Determine the direct ERM kinase downstream of S1PR2

First, we wanted to check if checkpoint kinases are the direct ERM kinases. To this end, several immunoprecipitation studies were performed. Pulling down endogenous CHK2 did not result in the co-immunoprecipitation of ezrin **(Fig. 20A)**. Since the CHK2-ezrin interaction is transient, and could be missed by regular immunoprecipitation studies, this experiment was repeated but with prior cellular cross-linking with 3.7% formaldehyde. No interaction between Chk2 and ezrin was noted as well **(Fig. 20B)**. Furthermore, another approach was undertaken and that is more aggressive. The system was forced by overexpressing ezrin that is VSVG tagged, cells were then cross-linked with formaldehyde, then an ezrin pull down with VSVG antibody was performed, followed by probing for Chk1 or Chk2 **(Fig. 20C)**. Similarly, there was no interaction between checkpoint kinases and ezrin. Collectively, these results suggest that Chks are involved in ERM phosphorylation downstream of S1PR2, yet are not the direct kinases. Therefore, in order to identify the direct ERM kinase, we are currently pursuing two different approaches:

6.2.1.1. Mass spectrometry

In this approach, we will overexpress VSVG-ezrin in HeLa cells, then perform cellular cross-linking followed by immunoprecipitation with magnetic beads, that are in turn cross linked with the VSVG antibody. After several attempts, we obtained the optimal conditions for this experiment as can be seen in **figure 21**, in preparation to perform it at a big scale. This will allow

us to identify all ezrin binding partners by mass spectrometry. These results should in turn narrow the pool of possible kinases that might act on ezrin for its phosphorylation.

6.2.1.2. In vitro kinase assays

This assay consists of incubating the kinase identified by mass spectrometry with ATP and ezrin, and check if it will induce ezrin phosphorylation. This will provide direct evidence that the kinase identified above, is able to phosphorylate ezrin in vitro. We started by purifying large amounts of ezrin protein in preparation for this assay. As such, human His-tagged ezrin was cloned in a pET28 plasmid. It was successfully expressed in the soluble fraction of pLEMO bacteria that were induced by IPTG and grown at 22 degrees **(Fig. 22A)**. Ezrin was then purified **(Fig. 22D)** by Fast Protein Liquid Chromatography (FPLC) using two columns: the first one is a His-trap column **(Fig. 22B)**, and the second one is a size exclusion Superdex200 column **(Fig. 22C)** that is used for additional purification.

6.2.2. Identify the signaling pathway downstream of S1PR2 that leads to ERM phosphorylation.

S1P mediates its effects by activating its G-protein coupled receptors (S1PR1-5) on the cell surface (reviewed in chapter 2), or by binding to less characterized intracellular targets. We have shown that S1P mediates ERM phosphorylation via activation of S1PR2 specifically. Yet the signaling mechanism that is described downstream of this receptor is highly diverse and cell-line dependent. S1PR2 is coupled to different G proteins ($G\alpha_i$, $G\alpha_{12/13}$, $G\alpha_q$), and each $G\alpha$ protein can activate several pathways, including activation of small GTPases (mainly RhoA, Cdc42 and Rac), which have been involved in cell motility. Identifying the specific GTPase

involved in ERM phosphorylation will be the first step in deciphering the pathway downstream of S1PR2. In fact, we initiated the experiments that will lead to identify this pathway. As a first step, ML141, CCG-1423, and Rac1 inhibitor II, well described pharmacological inhibitors for Cdc42, RhoA and Rac1 respectively were used. As shown in **figure 23**, ERM phosphorylation was significantly blunted following S1P stimulation upon Cdc42 and RhoA inhibition, while it was not affected upon Rac1 inhibition, suggesting that RhoA and Cdc42 are possible mediators of S1P functions in this system. These results need to be further confirmed by expression of the dominant positive and negative forms of these GTPases, as the inhibitors used might have off-target effects.

Furthermore, as part of the search for the kinase, and since the effect of Bis and Go on ERM phosphorylation was not associated with PKC inhibition, other known targets of these compounds were explored. Indeed, Bis had been described to be a potent inhibitor of GSK3 with an IC50 of 360 nM *in vitro* and 5 μM in total cell lysates of rat adipocytes (*331, 332*). This indicates that Bis' effects on ERM phosphorylation could be due to GSK3 inhibition. To test this hypothesis, we first used a previously described pharmacological inhibitor of GSK3, Bromo-hydroxy-imino-biindolinylidene-one (BIO) that blocks its ATP binding site (*333*). BIO-treated HeLa cells show a significant decrease in ERM phosphorylation following S1P treatment compared to vehicle treated cells **(Fig. 24A)**. These findings suggest that GSK-3 might be involved in this pathway. To confirm these results, siRNA to knockdown GSK3α or both isoforms of GSK3 α and β were used. GSK3α-siRNA and GSK3 α/β-siRNA treated cells show a significant decrease in ERM phosphorylation after S1P treatment compared to Mock-treated cells with more than 90% GSK3 knockdown efficiency **(Fig. 24B)**. These results indicate that GSK3 is indeed required for S1P-mediated ERM phosphorylation. To further confirm the role of

GSK3 in this pathway, wild type (WT) GSK3β was overexpressed along with the previously described constitutively active form of GSK3β that has a mutation at its serine S9 converting it to alanine (S9A) (*334*). HeLa cells expressing WT GSK3β exhibit higher baseline and S1P-induced phospho-ERM levels compared to mock transfected cells under confocal microscopy **(Fig. 24C)**. Importantly, HeLa cells expressing GSK3β S9A show markedly higher ERM phosphorylation levels at baseline and upon S1P stimulation **(Fig. 24C)**. These results uncover a novel role for GSK3 that proved to be essential and sufficient in promoting ERM activation.

Besides PKCs and GSK3, Bis had been also reported to inhibit PKA and AKT. Therefore, we opted to check the role of these two kinases in ERM phosphorylation for completion. To this end, EMD476485 and EMD124017, pharmacological inhibitors for PKA and AKT respectively were used. Notably, inhibiting PKA or AKT did not affect ERM phosphorylation upon S1P stimulation compared to vehicle treated cells **(Fig. 25A)**. On the contrary, and as seen previously, GSK3 inhibition by BIO significantly reduced ERM activation **(Fig. 25A)**. Strikingly however, inhibiting all these three enzymes with their respective inhibitors rescued ERM phosphorylation, and alleviated the inhibitory effect of the GSK3 inhibitor BIO **(Fig. 25A)**. These results indicate that GSK3 is not the direct kinase phosphorylating the ERM proteins; rather it exerts an inhibitory effect on either AKT or PKA, which in turn negatively regulates ERM phosphorylation. Therefore, it became important to assess which compound, the PKA or the AKT inhibitor, alleviated the effect of the GSK3 inhibitor. Inhibiting GSK3 and PKA together was comparable to GSK3 alone **(Fig. 25C).** However, inhibiting GSK3 and AKT relieved the inhibitory effect of the GSK3 inhibitor, and restored ERM phosphorylation **(Fig. 25C)**. Consequently, these results suggest that the kinase phosphorylating ERM proteins falls under a double inhibitory pathway: GSK3 inhibits AKT that in turn inhibits the ERM kinase. In

other words, inhibiting GSK3 by BIO relieved the inhibition on AKT that can therefore exhibit its inhibitory role on the kinase phosphorylating the ERM proteins.

Previous studies have demonstrated that Rac1 is a potent inhibitor of RhoA (*335*). Furthermore, AKT is a well-known activator of Rac1 (*336-338*). These connections led us to hypothesize that Rac1 mediates the double inhibitory mechanism exerted on the kinase phosphorylating the ERM proteins. To test this hypothesis, Rac1 and GSK3 were inhibited simultaneously using their respective pharmacological inhibitors, and then ERM phosphorylation was checked. As seen before, Rac1 inhibition with increasing doses of Rac1 inhibitor II did not affect ERM phosphorylation opposite to GSK3 inhibition that abolishes it **(Fig. 26)**. Unexpectedly, adding Rac1 inhibitor 2 along with the GSK3 inhibitor BIO caused a gradual recovery in ERM phosphorylation **(Fig. 26)**. These results suggest that GSK3 inhibits Rac1, which exerts a tonic inhibition of ERM phosphorylation. In addition, we have shown earlier that GSK3 is upstream of AKT **(Fig. 25),** and it is known that AKT activates Rac1, which in turn inhibits RhoA. Collectively, these results indicates that upon GSK3 inhibition, the AKT activity is recovered, Rac1 is active, and is able to inhibit RhoA that is necessary for ERM phosphorylation. This pathway is illustrated in **scheme 5**.

The signaling pathway described above is still nascent and requires further testing and verification. Nonetheless, it introduces a novel role for GSK3 and AKT as effector proteins downstream of S1PR2 signaling. However, several questions remain unsolved. How are the checkpoint kinases, Chk1 and Chk2, regulated by this pathway, and where do they fit? S1P causes phosphorylation of GSK3β and thus its inactivation, so how GSK3 inhibits AKT while inactive? Does S1P cause it to switch substrate preference form β-catenin to AKT? Does S1PR3 play any role in this pathway?

6.3. Determine the role of the SK2/S1PR2 pathway in EGFR mutant cell lines

We have showed that EGF mediated ERM phosphorylation is strictly dependent on ErbB1 receptor, also known as EGFR. Cervical cancer development is highly dependent on EGF signaling, that become more accentuated upon metastasis due to overexpression of the EGF receptor (*339*). In fact, we have checked the SK2/ERM axis validity in a metastatic cervical cancer cell line, MS571. Indeed, MS571 cells showed higher EGFR protein expression levels compared to HeLa cells. Furthermore, SK2 knockdown significantly decreased ERM activation in these cells **(Fig. 27A)**. These results suggest that EGF-mediated ERM phosphorylation is highly dependent on the SK2/S1PR2 axis in cells expressing the wild type EGF receptor.

Therefore, it would be very interesting to check if this axis could be generalized to a wider array of tumors that depend on the EGF pathway for their development and progression. We have started investigating this hypothesis by switching to non-small cell lung cancer, as it is highly dependent on EGFR signaling.

Lung cancer cell is the leading cause of cancer related death worldwide with a dismal 5-year survival rate of 15% (*340*). Although the most effective strategy for treatment is surgery, yet most patients present with an advanced disease stage that would render them ineligible for that option (*340*). Chemotherapy is the standard care of treatment in patients with advanced lung cancer. However, resistance to chemotherapy develops quickly, dramatically decreasing patients' survival. Resistance has been attributed to increased tumor proliferation rates and decreased apoptosis; this is secondary to accentuated EGF signaling resulting from gain of function mutations in the EGF receptor (as seen in 10% of patients). Nonetheless, these patients showed a

substantial response rate to newly developed EGFR tyrosine kinase inhibitors including Erlotinib and Gefitinib (*341*). This can be explained by the so called "oncogene addiction theory", whereby cells harboring these mutations become exquisitely sensitive to the growth inhibitory actions of these drugs. However, tumor recurrence is almost certain after one year of treatment with EGFR targeted therapies, due to the development of secondary mutations that decrease the effectiveness of these drugs (*341*). Having said that, it becomes imperative to test the applicability of the SK2/S1PR2/ERM axis not only in the context of wild type EGFR expressing tumors, but also in tumors that express mutated forms of this receptor. This is really important, because it provides additional druggable targets in patients that become resistant to chemotherapy, as well as to tyrosine kinase inhibitors.

6.3.1. Determine the role of the SK2/S1PR2 axis in lung cancer cells expressing wild type EGF receptor

We started exploring this system by using H292 non-small cell lung carcinoma cell line that is known to express wild type EGFR. To check if ERM phosphorylation is S1P dependent in this system, these cells were treated with 100 nM S1P. Expectedly, there was a marked increase in ERM phosphorylation **(Fig 27B.)**. In addition, ERM phosphorylation induced by EGF treatment was significantly reduced upon the inhibition of both sphingosine kinases with the non-specific SK inhibitor Ski-II **(Fig. 27C)**, as well as upon knocking them down with SK1 and SK2 siRNAs **(Fig. 27D)**. However, inhibiting S1PR2 in this cell line did not affect ERM phosphorylation upon EGF treatment. Therefore, it seems that EGF-mediated ERM phosphorylation is indeed SK dependent, yet does not require S1PR2. This could be due to several reasons. The S1P receptors expression profile in this cell line is unknown, and it could be

that S1PR2 is not expressed, or that S1P-mediated ERM phosphorylation is dependent on another receptor. Alternatively, ERM activation in this system could be strictly dependent on intracellular S1P production and S1P receptor independent. All these possibilities are to be further explored in the future as they can provide further insight into the applicability of the SK/S1PR2 axis in lung cancer cells.

6.3.2. Define the role of the SK2/S1PR2 axis in lung cancer cells harboring mutated forms of the EGF receptor.

In order to determine if ERM phosphorylation in lung cancer cells with mutated EGFR is dependent on the SK/S1PR2 axis, we used HCC827 and H1975 cells lung cancer cells. HCC827 cells have an acquired mutation in the EGFR tyrosine kinase domain (E746 - A750 deletion), which destabilizes its auto-inhibitory confirmation, thus rendering it constitutively active; these cells are extremely sensitive to Erlotinib treatment (*342*). H1975 cells, on the other hand, are resistant to Erlotinib, because they possess a secondary point mutation substituting methionine for threonine on codon 790 (T790 mutation) in addition to their primary activating mutation L858R. The secondary T790 mutation causes a structural disturbance that hinders the binding of tyrosine kinase inhibitors (*342*).

Interestingly, HCC827 and H1975 cells had higher basal pERM levels than H292 cells, suggesting increased EGFR signaling **(Fig. 27B)**. However, HCC827 and H1975 cells differed in their response to S1P. While HCC827 cells showed marked increase in ERM phosphorylation upon S1P treatment, H1975 cells had a minimal response **(Fig. 27A)** Furthermore, both of these cell lines failed to respond to EGF treatment since pERM levels remained unchanged **(Fig. 27C)** These results might insinuate that the EGF signaling pathway is already maximal in the context

of a constitutively active EGF receptor. To investigate whether the sphingolipid metabolism is perturbed in these cells, endogenous S1P levels were measured. In fact, cells that harbor mutated EGF receptors (HCC827 and H1975) have considerably higher S1P levels than cells with wild type EGFR (H292) **(Fig. 27E)**; therefore suggesting higher sphingosine kinase activity levels in cells with constitutively active EGFR. Surprisingly, inhibiting SK1 and SK2 in these two cell lines did not affect pERM levels **(Fig. 27D)**.

In conclusion, it become apparent that the SK2/S1PR2/ERM axis holds true in the context of wildtype EGF receptor only. We have demonstrated the validity of this pathway in multiple tumors including cervical cancer (HeLa cells and MS751) and lung cancer (H292). Conversely, this axis doesn't apply in the context of mutated EGFR signaling, since ERM activation was independent of SK activity. It appears that alterations in the EGFR status cause cancer cells to switch to alternative mechanisms that will activate ERM proteins. These mechanisms are worth exploring, as they might provide further insights into mutants EGFR signaling. Consequently, this will open new avenues in finding novel druggable targets that would be of utmost importance in the treatment of patients resistant to current therapeutic modalities.

6.4. Determine the role of the S1P/S1PR2/Ezrin axis in *in-vivo* mouse models of metastasis.

The role of ERM proteins, especially ezrin, in tumor metastasis has been demonstrated in several studies. For example, there was a significant decrease in the distant foci of metastasis of primary osteosarcoma upon expression of the dominant negative form of ezrin (*343*). However,

the role of ezrin in S1P and growth factor-induced tumors has not been evaluated *in-vivo*. Thus, it is essential to check if the SK2/S1PR2 axis is applicable *in-vivo* as a pre-requisite for prospective translational research. Results presented in previous chapters as well as in previous published data, we showed that ezrin is essential for S1P-induced filopodia, and EGF-induced lamellopodia formation in HeLa cells. We have also showed that growth factor-induced cellular adhesion and subsequent invasion is dependent on SK2 and S1PR2-mediated ERM activation using pharmacological inhibitors as well as knockdown techniques. Given these promising results *in-vitro*, it become crucial to establish the validity of the SK2/S1PR2/ezrin pathway *in-vivo*. If this pathway is applicable in animals, it would open the doors for new druggable targets that would be crucial to test in growth factor-driven tumors in humans. These points should be addressed by performing several *in-vivo* studies that are described in the following sections.

6.4.1. Describe and analyze the SK2/S1PR2/ezrin axis upon cancer metastasis *in-vivo*

Since EGF plays a crucial role in breast cancer development and lung metastasis, the Polyoma middle-T antigen mouse model of mammary carcinogenesis (PyMT) would constitute a suitable starting point. The role of the sphingolipid pathway in this model is still unknown. In addition, the mechanism of EGF-induced lung metastasis is also lacking. Our previous results indicate that the SK2/S1PR2/ezrin axis could be perturbed and might play a role in this system. Preliminary results show that S1P levels are higher in the blood and tumors of PyMT mice compared to wild type mice **(Fig. 28B)**. In addition, PyMT mice have higher S1PR2 and Ezrin levels in their mammary tissue compared to WT mice **(Fig. 28A)**. These promising data could be supplemented by performing additional experiments including:

a. Assess SK2 protein expression and activity levels in the PyMT tumor cells.

b. Determine the phospho-ERM levels in the tumor cells as well as in their vicinity.

If a perturbation of the SK2/S1PR2/ezrin axis was confirmed in this breast cancer model, it will constitute a first step towards performing further experiments to assess the usefulness of SK2 and S1PR2 as druggable targets.

6.4.2. Determine the role of SK2 and S1PR2 in cancer metastasis *in-vivo*.

To evaluate the role of the SK2/S1PR2/ezrin axis in growth factor driven metastasis *in-vivo*, we will make use of ABC294640 and JTE013, two known pharmacological inhibitors for SK2 and S1PR2, respectively. JTE013 has been used before for up to two weeks in mice via gavage, at 30 mg JTE013/kg mouse, and resulted in shrinkage of the primary tumor (*344*). In our case, we would like to study the effect of S1PR2 in breast tumor metastasis in PyMT mice. Therefore, it is best to start administering the compound at 10 weeks of age, when the primary breast tumors have been established, yet not metastasized. The treatment will continue, daily, till 16 weeks of age when lung metastases are usually developed. Mice will then be euthanized, and lung metastasis will be measured. Tumor tissues should also be evaluated for pERM levels to test if JTE013 decreases ERM activation in vivo. The same approach will also apply for the SK2 inhibitor, AC294640, with slight modifications. This compound will also be administered orally due to its excellent bioavailability, but at a dose of 100mg/kg-body weigh as previously shown (*282*).

6.4.3. Resolve the role of the SK2/S1PR2/ezrin axis in cancer cell invasion *in-vivo.*

The experiments suggested above will only determine the role of SK2 and S1PR2 in cancer metastasis without addressing their mechanism of action. Therefore to study the mechanism by which SK2 and S1PR2 affect lungs' implantation, we suggest using B16F10 mice melanoma cells, as they exclusively express S1PR2. We already generated B16F10 cells stably overexpressing GFP. This will allow for easier monitoring of tumor cells progression and development by performing in vivo imaging (using the Maestro Imager that can detect GFP expressing cells) following tail vein injections. We have also verified the S1PR2/ezrin axis in these cells, since S1PR2 inhibition abolished ERM activation. To demonstrate whether SK2 and S1PR2 modulation of cell invasion *in-vivo* occurs via ezrin regulation, we suggest the following experiment. We will knock down SK2 or S1PR2 in the cells that will be used for the tail vein injections. Next, we will co-express WT ezrin or dominant negative ezrin (T567A) in cells that are pretreated with SK2 or S1PR2 shRNAs. This will allow us to prove that SK2 and S1PR2-mediated invasion occurs via ezrin modulation. Therefore, we expect that knocking down SK2 or S1PR2 will reduce the number of lung metastatic lesions, similar to T567A transfected cells. On the other hand, we expect that overexpressing a WT or constitutively active form of ezrin (T567D) will overcome SK2 or S1PR2 KD effect on invasion. Expected results are summarized in **table 4**.

Figures

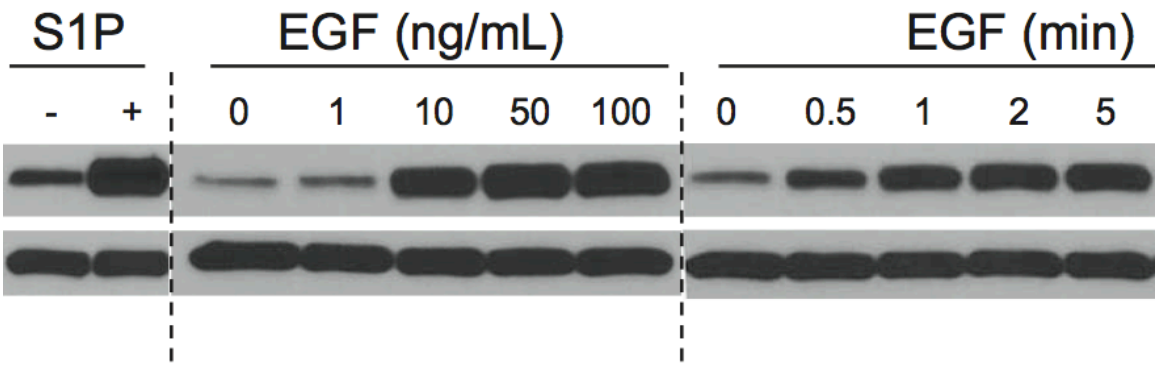


Figure 1: EGF-induced ERM phosphorylation.

HeLa cells were serum starved overnight and treated with 100nM S1P for 5 minutes, indicated doses of EGF (ng/mL) for 5 minutes, or 10ng/mL EGF for the indicated times (minutes). Phospho-ERM (pERM) and actin levels were analyzed via western blot; blots representative, n=3

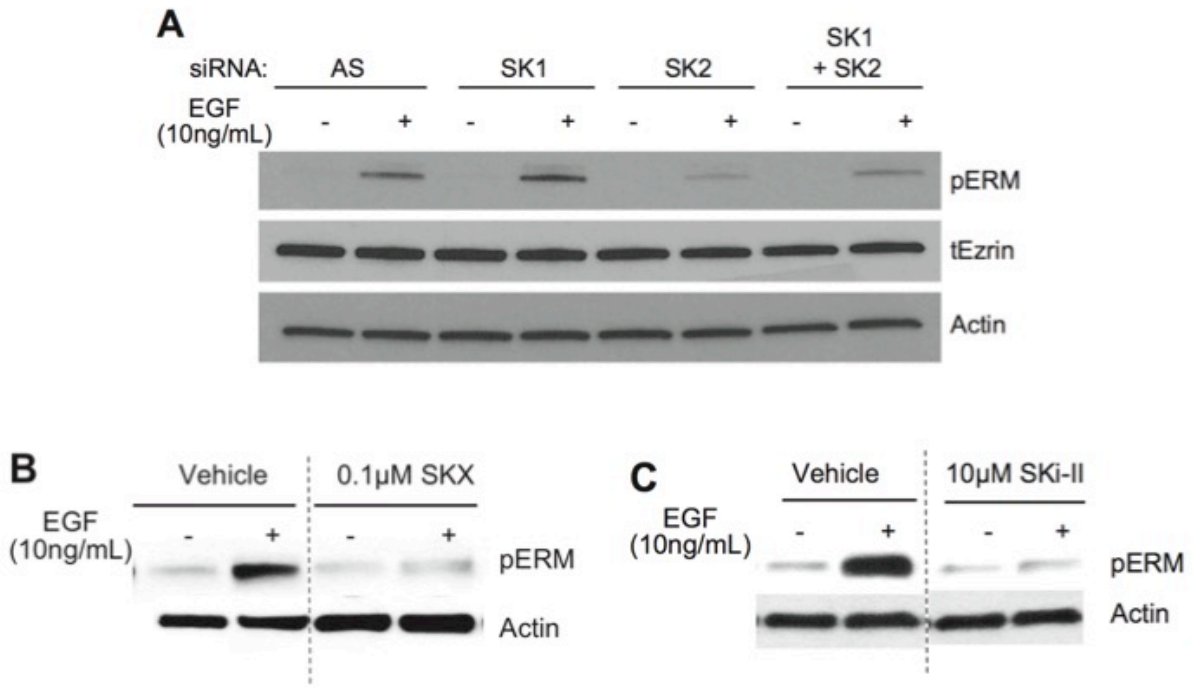


Figure 2: Involvement of sphingosine kinase in EGF-mediated phospho- ERM.

A) HeLa cells were treated with 20 nM SK1, SK2, or SK1 plus SK2 siRNA for 48 hours. After 48 hours of siRNA, cells were serum starved then treated with 10ng/mL EGF for 5 minutes. B, C) HeLa cells were serum starved 16-18 hours. Following 1 hour pretreatment with DMSO vehicle, B) 0.1μM SKX or C) 10μM SKi-II, cells were treated with 10ng/mL EGF for 5 minutes. Phospho-ERM and actin levels were analyzed via western blot; blots representative, n=3.

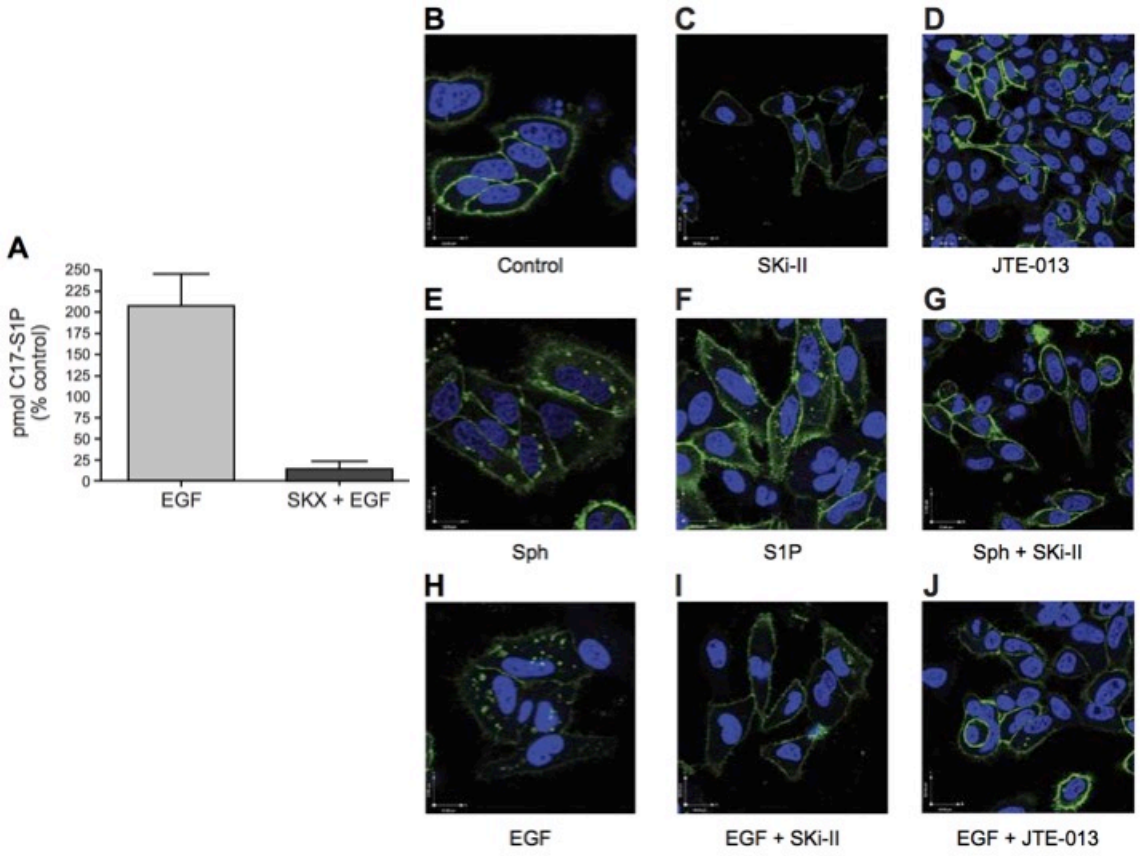


Figure 3: EGF generates S1P and transactivates S1P receptors.

A) HeLa cells were pretreated for 1h with DMSO vehicle or 0.1μM SKX and 1μM C17-sph was added for 15 minutes. Following 15 minutes C17-sph labeling, 10ng/mL EGF was added for 2 minutes. Media was analyzed via mass spectroscopy for C17-S1P levels. $n \geq 3$, B-J) Hela cells were transfected with S1PR2 receptor tagged with GFP for 24 hours. Following transfection, cells were serum starved overnight, then C, G, I) pre-treated for 6 hours with 10 uM SKI or D, J) for 1 hour with 5 uM JTE-013. After that cells were treated with either E, G) 5 uM sphingosine (Sph) or F) 100 nM sphingosine 1-phosphate (S1P) or H, I, J) 100 ng/ml EGF for 5 minutes. Cells were then fixed, and nuclei were stained with DAPI. Images were taken using SP8 Leica confocal microscope. Images are representative of at least 4 independent experiments.

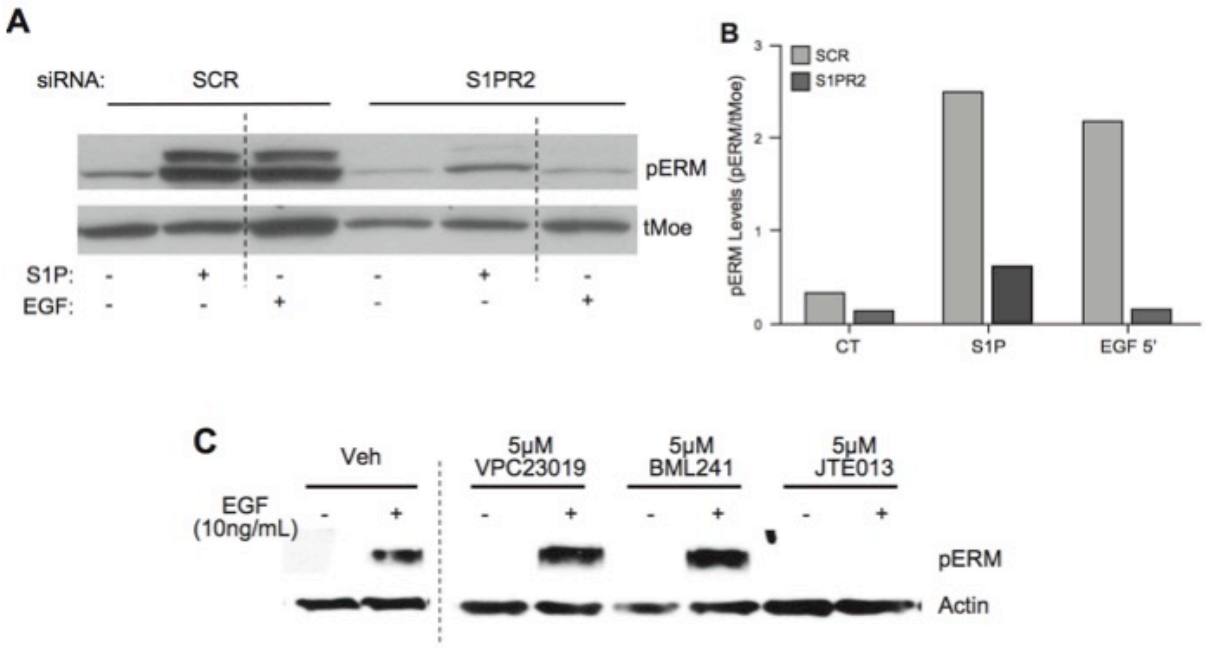


Figure 4: Effect of S1P receptor modulation on EGF-mediated phospho-ERM.

A) HeLa cells were treated with 20 nM S1R2 siRNA for 48 hours. After 48 hours of siRNA, cells were serum starved then treated with 10ng/mL EGF for 5 minutes, n=2. B) pERM levels in A were quantified using Image J software. C) HeLa cells were serum starved 16-18 hours. Following 1 hour pretreatment with DMSO vehicle or indicated S1P receptor antagonists, cells were treated with 10ng/mL EGF for 5 minutes. Phospho-ERM and actin levels were analyzed via western blot; blots representative, n=5.

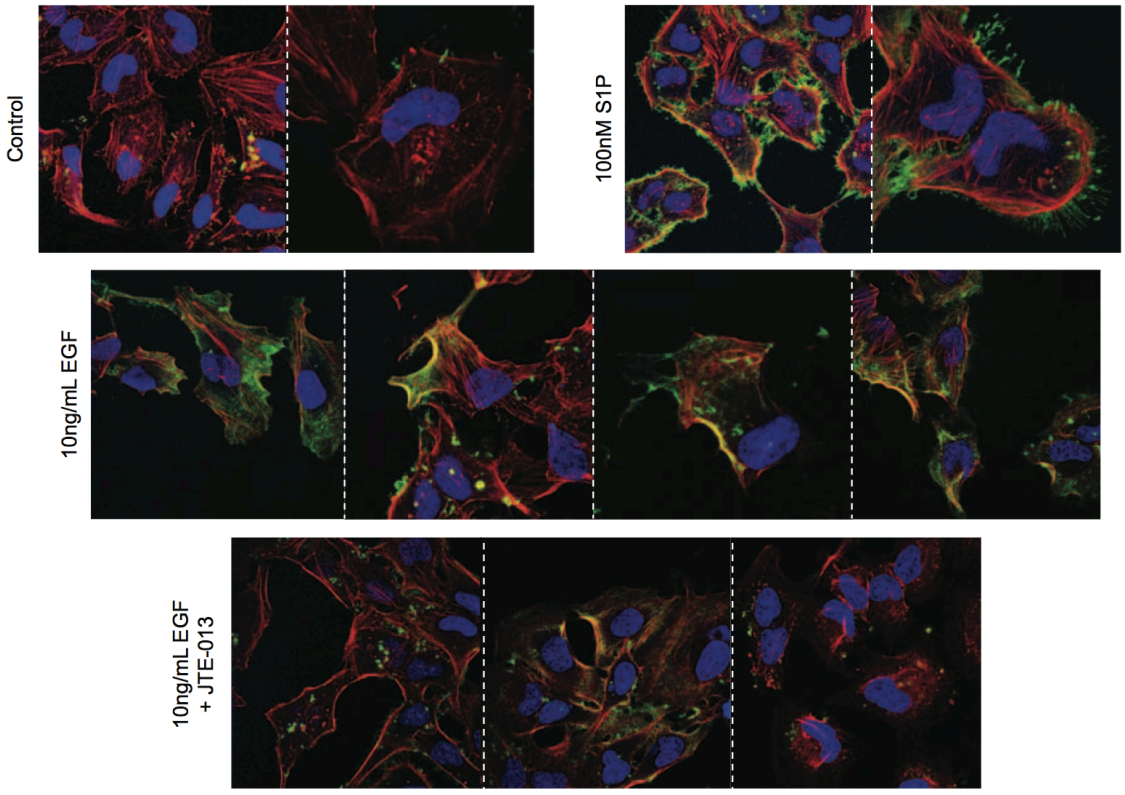


Figure 5: EGF-mediated phospho-ERM localization to lamellipodia occurs via S1PR2.

HeLa cells were serum deprived and received A) no treatment, or were treated with B) 100 nM S1P for 5 minutes, C) 10ng/mL EGF for 5 minutes or D) 1 hour 5μM JTE-013, followed by 10ng/mL EGF for 5 minutes. Cells were fixed and stained for phospho-ERM (green). Phospho-ERM, Nuclei (Draq5, blue) and F-actin (phalloidin, red) were visualized using confocal microscopy. Images are representative of at least two independent experiments.

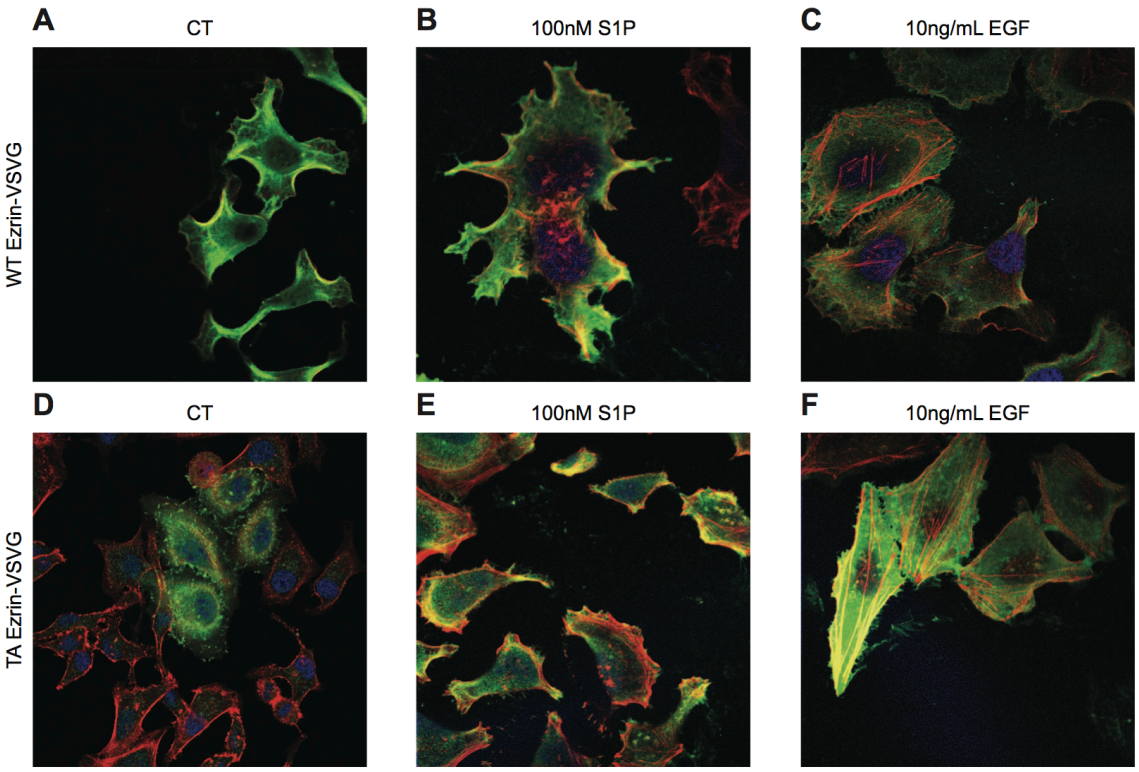


Figure 6: Ezrin T567 phosphorylation is required for EGF-mediated cytoskeletal changes.

HeLa cells were transfected with A, B, C) VSV-G-tagged wild type ezrin or D, E, F) VSV-G-tagged nonphoshorylatable ezrin mutant T567A. Following transfection, cells were serum starved and received A, D) no treatment B, E) 100 nM S1P treatment for 5 minutes or C, F) 10ng/mL EGF for 5 minutes .Cells were fixed and VSV-G (green), nuclei (DRAQ5, blue) and F-actin (phalloidin, red) were visualized using laser-scanning confocal microscopy. Images are representative of at least 2 independent experiments.

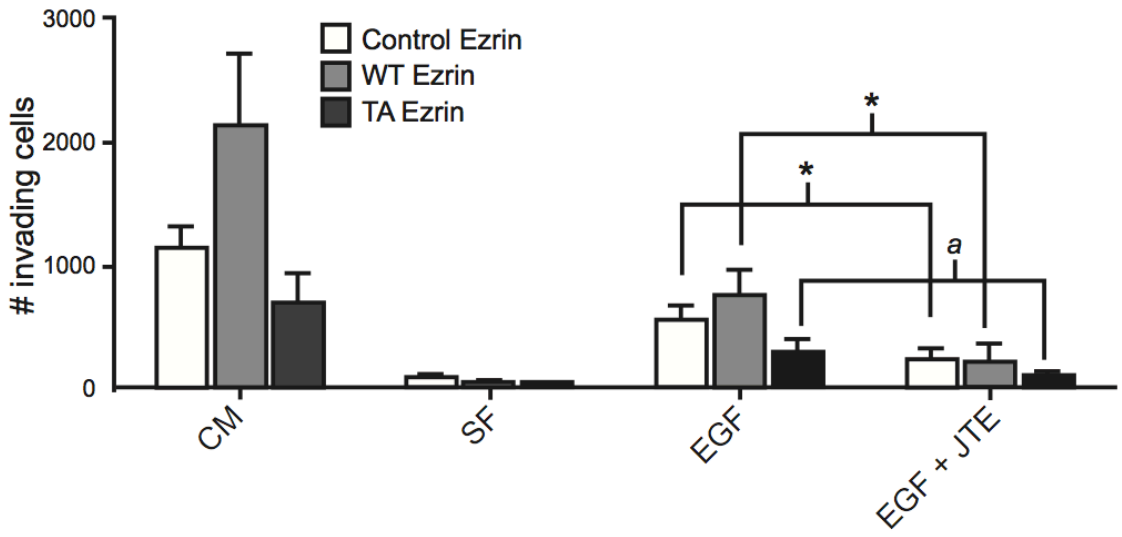


Figure 7: EGF-induces invasion by S1PR2 -dependent ezrin T567 phosphorylation.

Non-transfected HeLa cells, cells overexpressing WT ezrin, or TA ezrin were serum starved 4-6 hours and plated, in the presence or absence of 5μM JTE-013, in the apical chamber of matrigel-coated trans well inserts and allowed to invade for 48 hours towards complete media (CM) or 50ng/mL EGF (EGF). Invading cells were stained with fluorescent dye, photographed and counted. Statistical analysis was performed using a student's t-test (*, $p<0.05$; *a*, $p=0.055$), $n \geq 3$

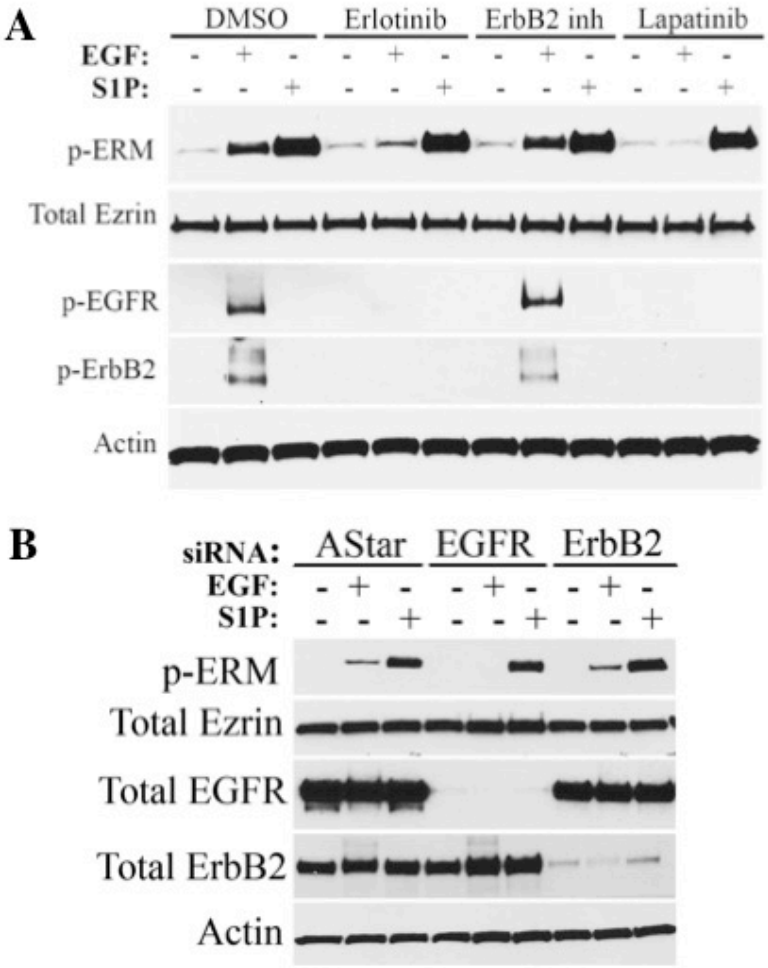


Figure 8: EGF-induced ERM phosphorylation is ErbB1 dependent.

A) HeLa cells were pre-treated with DMSO, Erlotinib (100nM), ErbB2 inhibitor (10μM), or Lapatinib (10μM) for 1 hour prior to treatment with EGF (10ng/ml) or S1P (100nM) for 5 minutes. pERM. pEGFR and pErbB2 levels were then assessed by immunoblotting. B) HeLa cells were treated with AStar, EGFR, or ErbB2 siRNA for for 48 hours. Cells were then starved for 4 hours prior to treatment with EGF (10ng/ml) for 5 minutes. pERM, total EGFR and total ErbB2 levels were then assessed by immunoblotting.

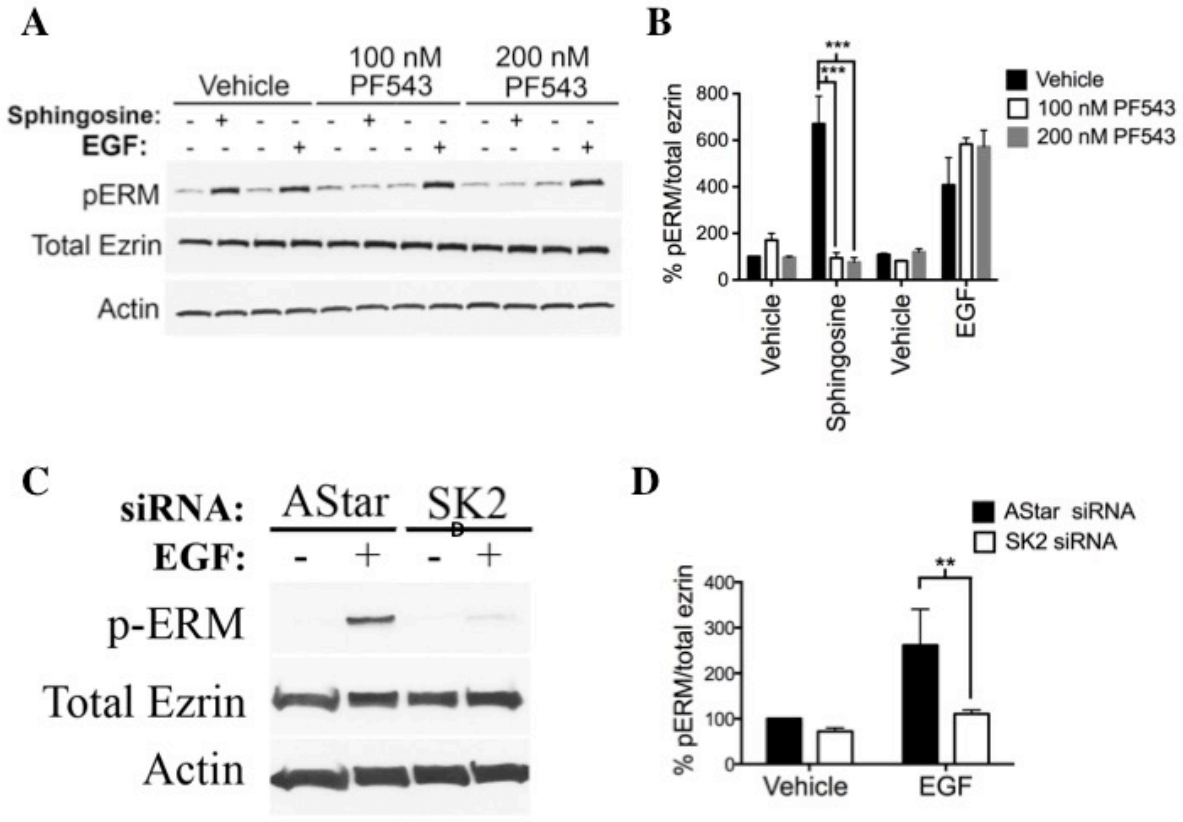


Figure 9: SK2 and not SK1 is essential for EGF-mediated ERM phosphorylation.

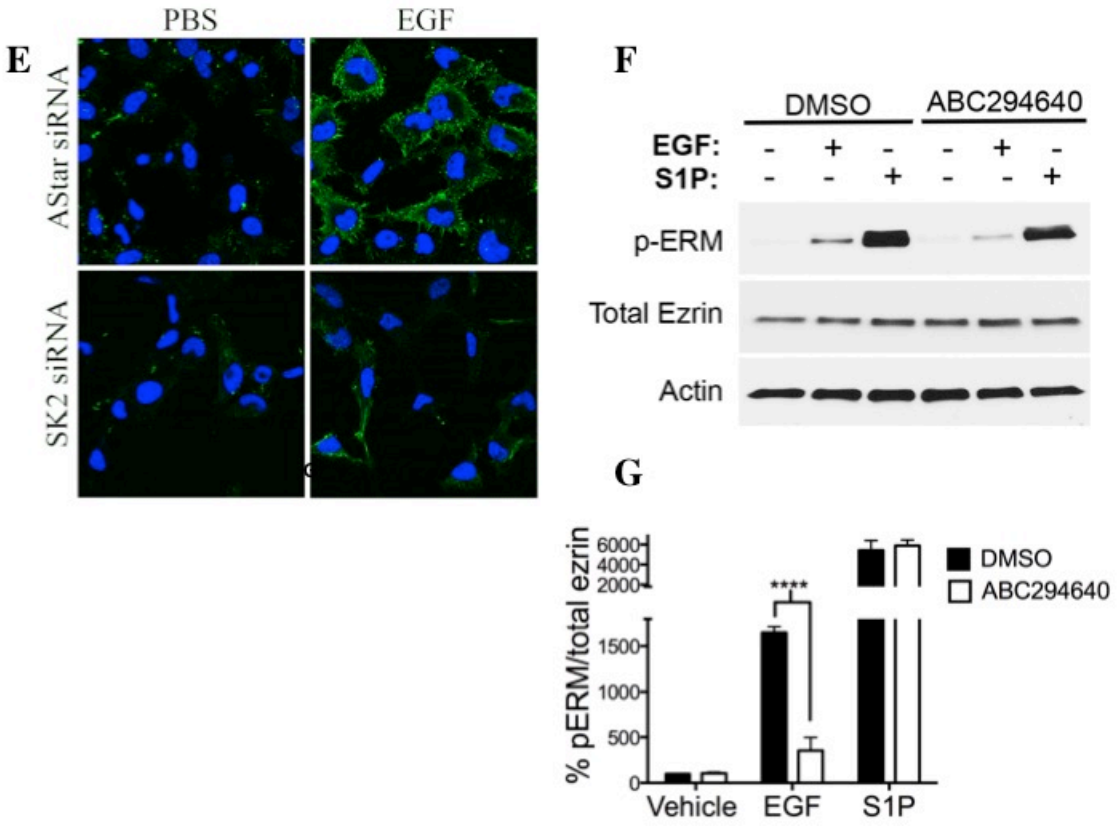


Figure 9 (continued): SK2 and not SK1 is essential for EGF-mediated ERM phosphorylation.

A) HeLa cells were pre-treated with 100nM or 200 nM of PF543 for 1 hour prior to stimulation with EGF (10ng/ml) or sphingosine (5mM) for 5 minutes. pERM levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls. C) HeLa cells were treated with AStar or SK2 siRNA for 48 hours. Cells were then starved for 4 hours prior to treatment with EGF (10ng/ml) for 5 minutes. pERM levels were then assessed by immunoblotting (C) and confocal microscopy (E), where the green color corresponds to pERM levels labeled with Alexa488 fluorophore antibody, and the blue color corresponds to DAPI staining the nuclei (E). Total ezrin and actin were also included as loading controls (C). F) HeLa cells were pre-treated with DMSO or 10μM ABC294640 for 1 hour prior to stimulation with EGF (10ng/ml) or S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls. B), D), G), Quantification of the ratio of pERM/total ezrin in A, C, and F, respectively was performed using Image J software.

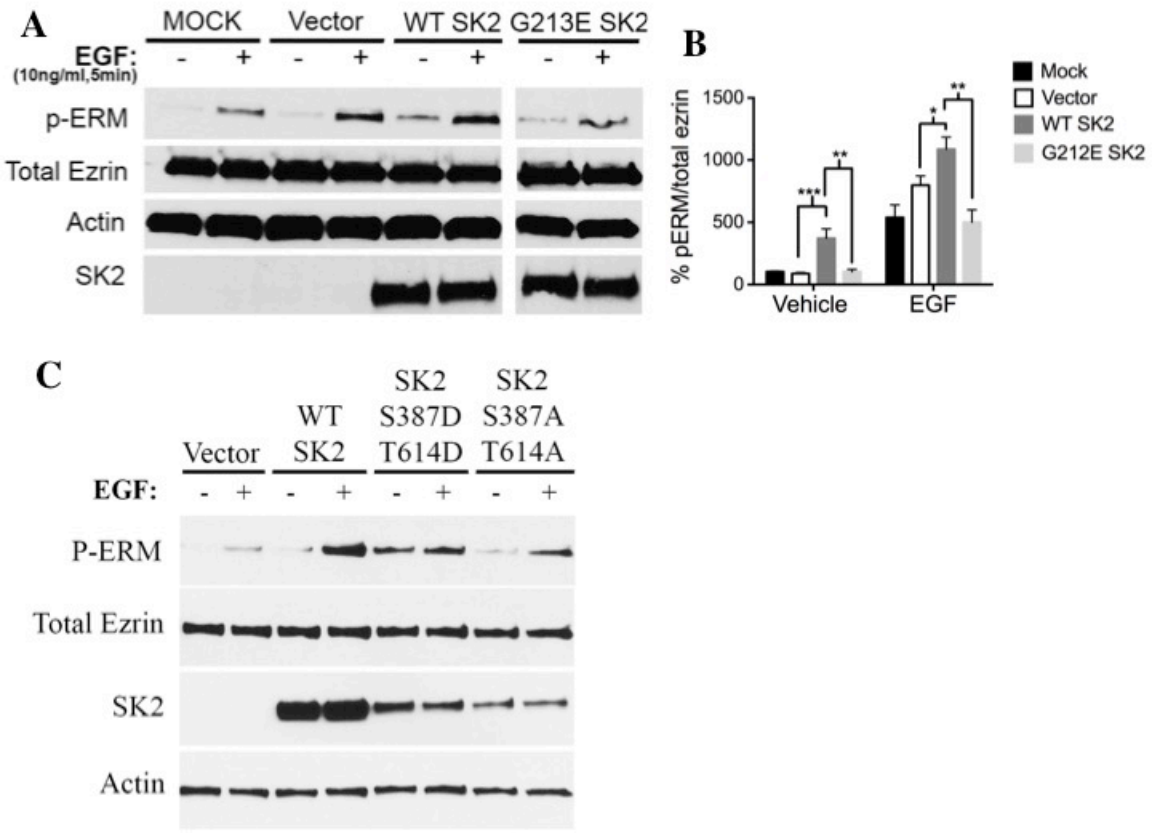


Figure 10: SK2 is sufficient to promote ERM activation.

A) HeLa cells were transfected with vector, WT SK2 and G213E SK2 DNA for 24 hours. Cells were then starved for 4 hours prior to treatment with EGF (10ng/ml) for 5 minutes. pERM and total SK2 levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls. B) Quantification of the ratio of pERM/total ezrin in A was performed using Image J software. C) HeLa cells were transfected with vector, WT SK2, S387D;T614D SK2, and S387A;T614A SK2 DNA for 24 hours. Cells were then starved for 4 hours prior to treatment with EGF (10ng/ml) for 5 minutes. pERM and total SK2 levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls.

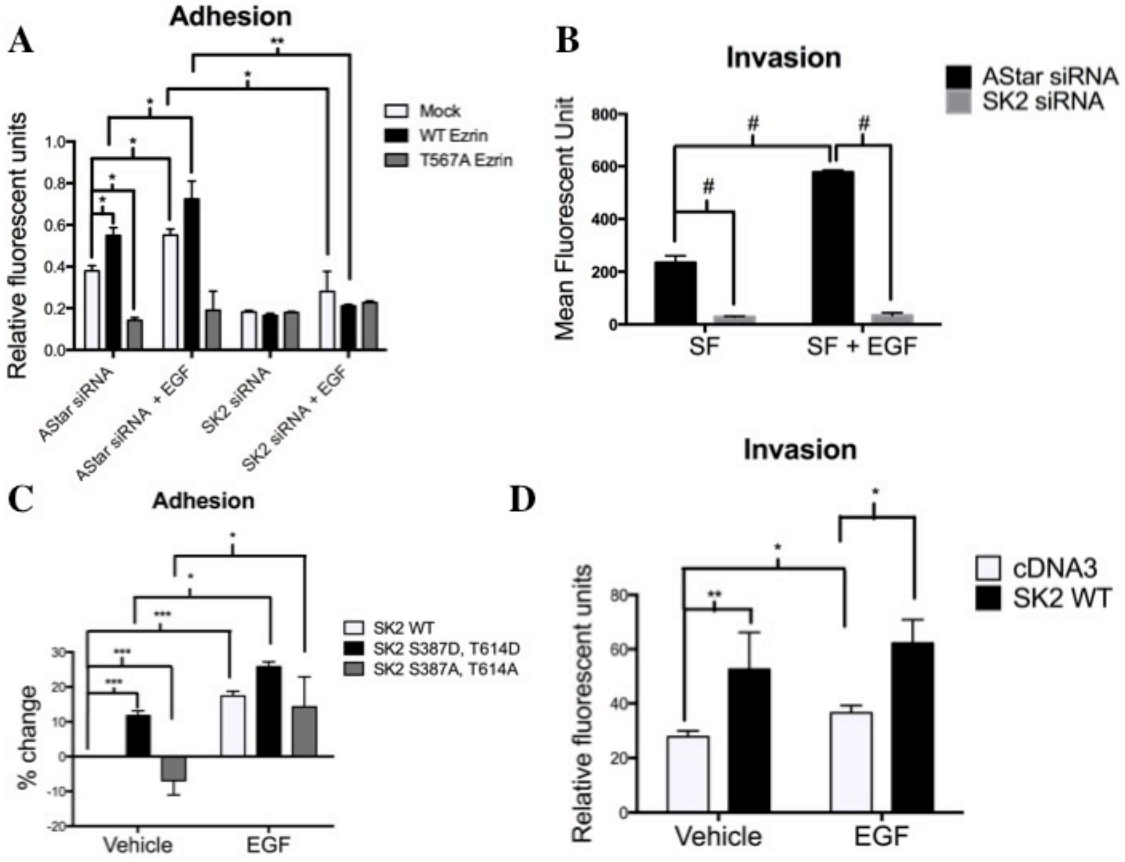


Figure 11: SK2 is required and sufficient for cell adhesion and invasion toward EGF.

A) HeLa cells were treated with AStar or SK2 siRNA for 24 hours. Cells were then transfected with MOCK, WT ezrin or T56A ezrin DNA for another 24 hours. Cells were then starved for 4 hours, trypsinized and plated on fibronectin-coated plates. Cells were then treated with vehicle (PBS) or EGF (10ng/ml) for 6-12 hours. MTT assay was then performed, and absorbance measured as a quantification of cell number. B) HeLa cells were treated with AStar or SK2 siRNA for 48 hours. Cells were then starved for 4 hours prior to their plating in the apical chamber of matrigel-coated transwell inserts, and allowed to invade for 24 hours toward serum-free or EGF supplemented media. Invading cells were then stained with 4 ug/ul Calcein AM, and absorbance was red in a plate reader as described in materials and methods section. C) HeLa cells were transfected with WT SK2, S387D;T614D SK2, and S387A;T614A SK2 DNA for 24 hours. Cell adhesion was then assessed as described in A. D) HeLa cells overexpressing cDNA3 or WT SK2 were plated in the apical chamber of matrigel-coated transwell inserts, and allowed to invade for 24 hours toward serum-free or EGF supplemented media. Invaded cells were then quantified as previously described.

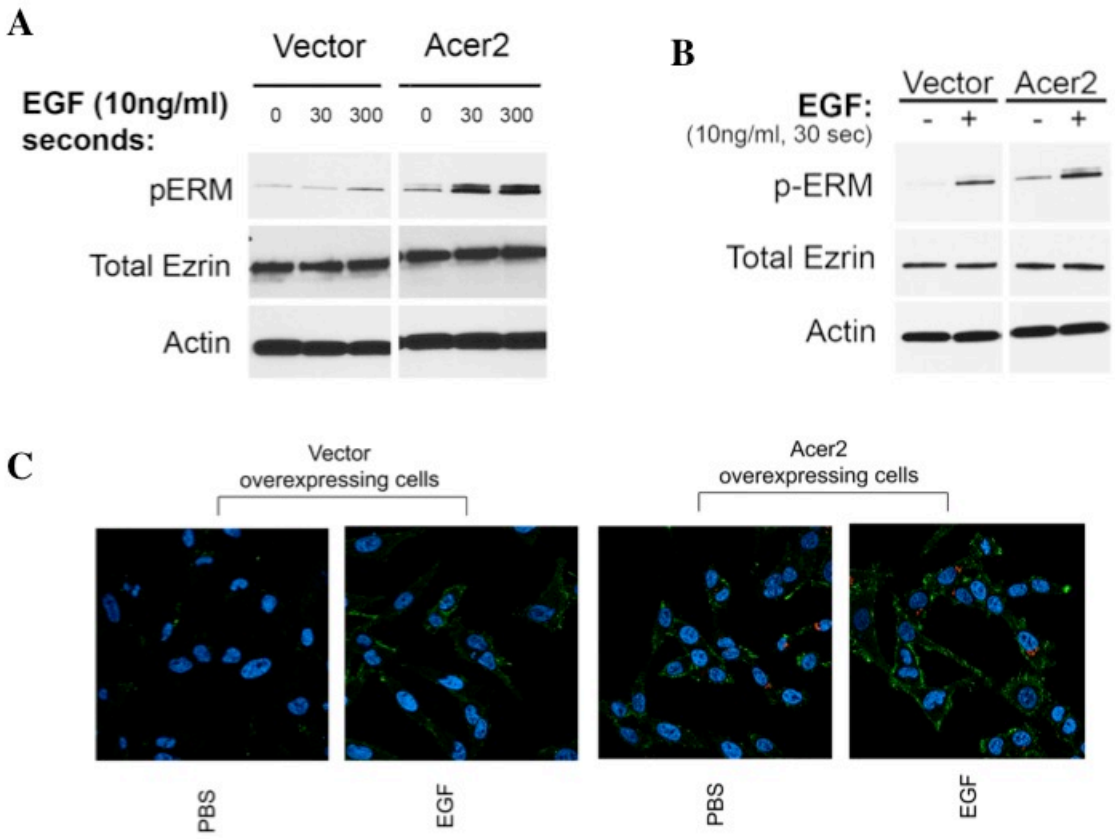


Figure 12: Increased intracellular S1P production is sufficient to promote ERM activation.

A) Ethanol or tetracycline (5ng/ml) was added to Hela-ACER2-TET-ON and HeLa-Vector control cells for 12 hours. These cells were then treated with EGF (10ng/ml) for either 30 sec or 5 min. pERM levels were then assessed by immunoblotting (A) and by confocal microscopy (C). The green color corresponds to pERM levels labeled with Alexa488 fluorophore antibody, and the blue color corresponds to DAPI staining the nuclei (C). B) HeLa cells were transiently transfected with either vector control or ACER2 overexpressing DNA for 24 hours. Cells were then starved for 4 hours prior to treatment with EGF (10ng/ml) for 5 minutes. pERM levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls.

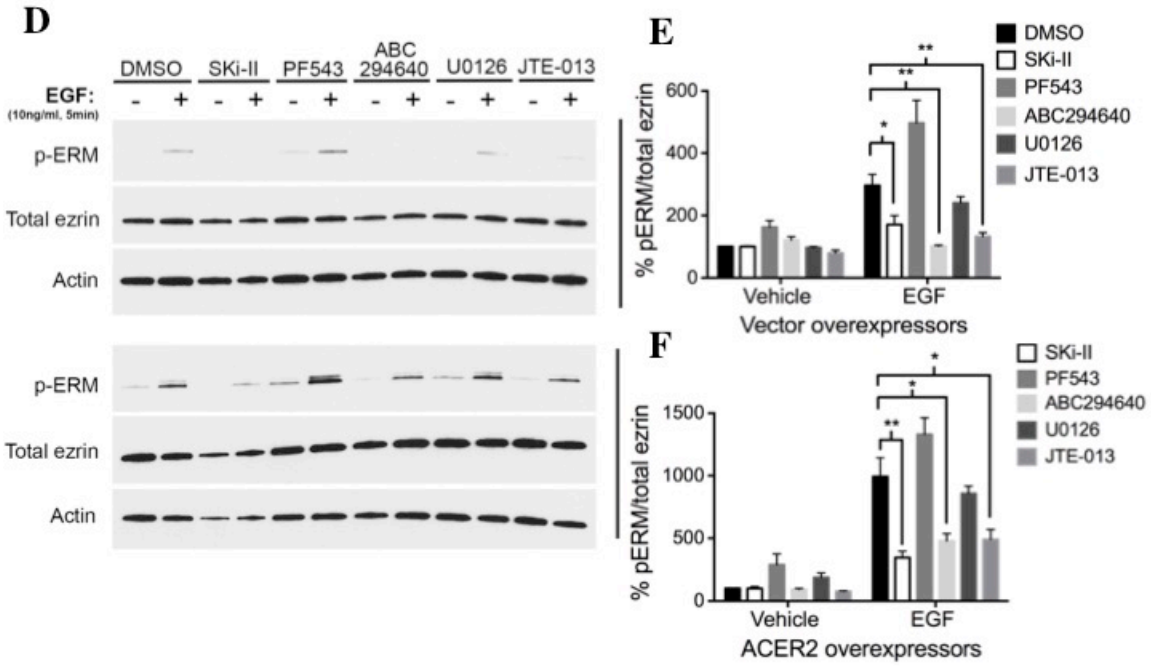


Figure 12 (continued): Increased intracellular S1P production is sufficient to promote ERM activation.

D) Hela cells stably overexpressing vector or ACER2 were starved overnight. These cells were then pre-treated with either Ski-II (1μM), PF543 (100nM), ABC294640(10μM), U0126(10μM), or JTE-013(5μM) prior to stimulation with EGF (10ng/ml) for 5 minutes. E), F) Quantification of the ratio of pERM/total ezrin in D was performed using Image J software.

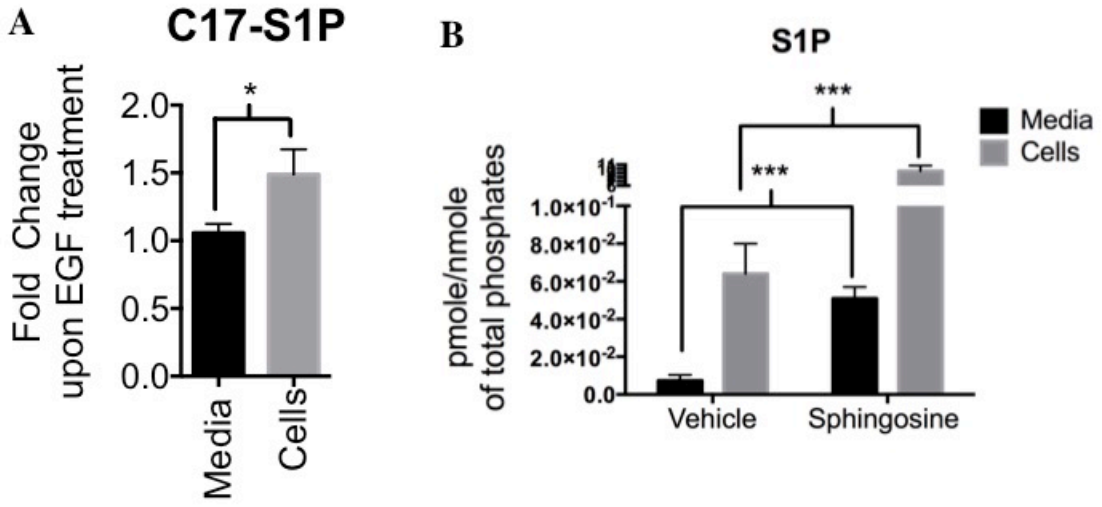


Figure 13: ERM phosphorylation does not require extracellular S1P production.

A) HeLa were pre-labeled for 30 min with 250 nM C17-Sph. EGF (10ng/ml) was then added for 30 sec. C17-S1P levels from media and cells were then analyzed via mass spectroscopy. B) HeLa cells were starved overnight, then treated with 5 mM sphingosine for 5 minutes. Endogenous S1P levels from media and cells were then analyzed via mass spectroscopy

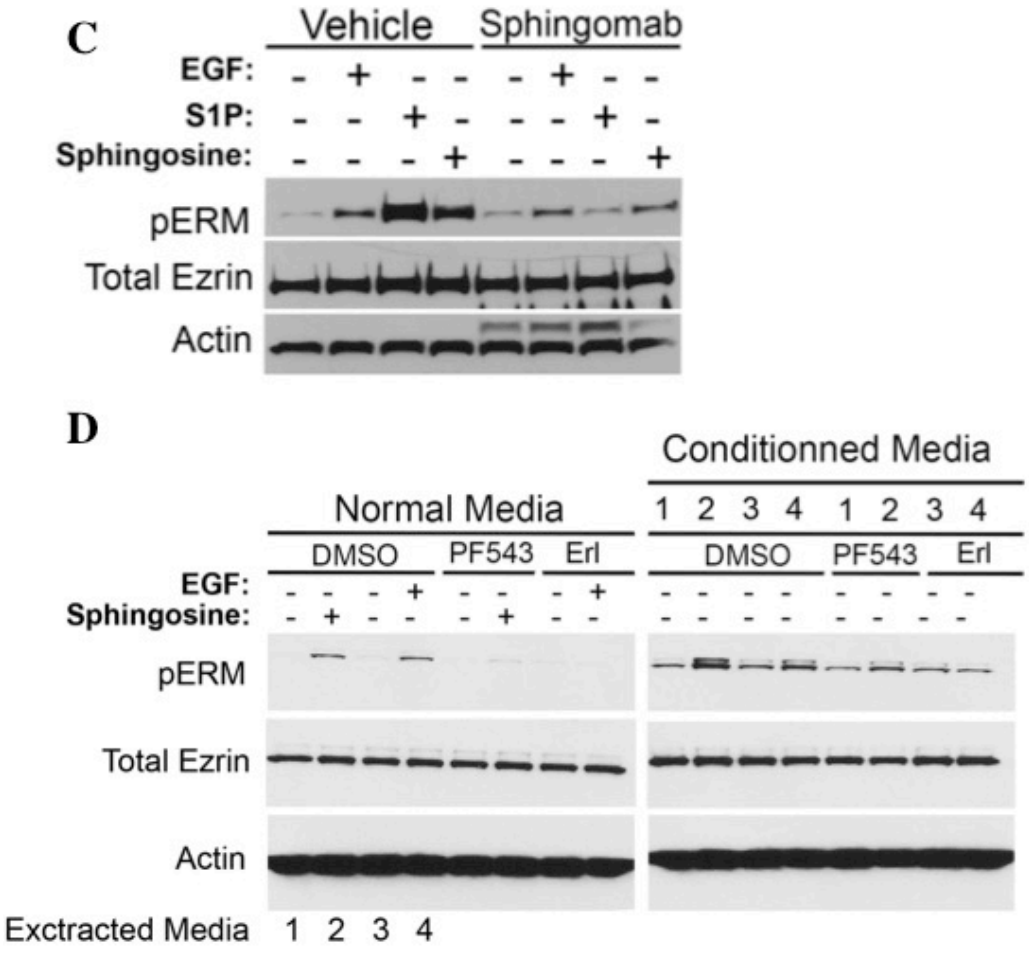


Figure 13 (continued): ERM phosphorylation does not require extracellular S1P production.

C) HeLa cells were pretreated for 1 hour with either PBS or 50 mg/well Sphingomab prior to treatment with EGF (10ng/ml), or sphingosine (5μM), or S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls. D) HeLa cells were pre-treated with DMSO, PF543 (100nM), or Erlotinib (Erl) (100nM) for 1 hour, then followed by treatment with either sphingosine (5mM) or EGF (10ng/ml) for 2 minutes. Media from DMSO pre-treated cells, labeled 1-4, was then added on top of HeLa cells pre-treated with DMSO, PF543 (100nM), or Erlotinib (Erl) (100nM) for 1 hour. pERM levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls.

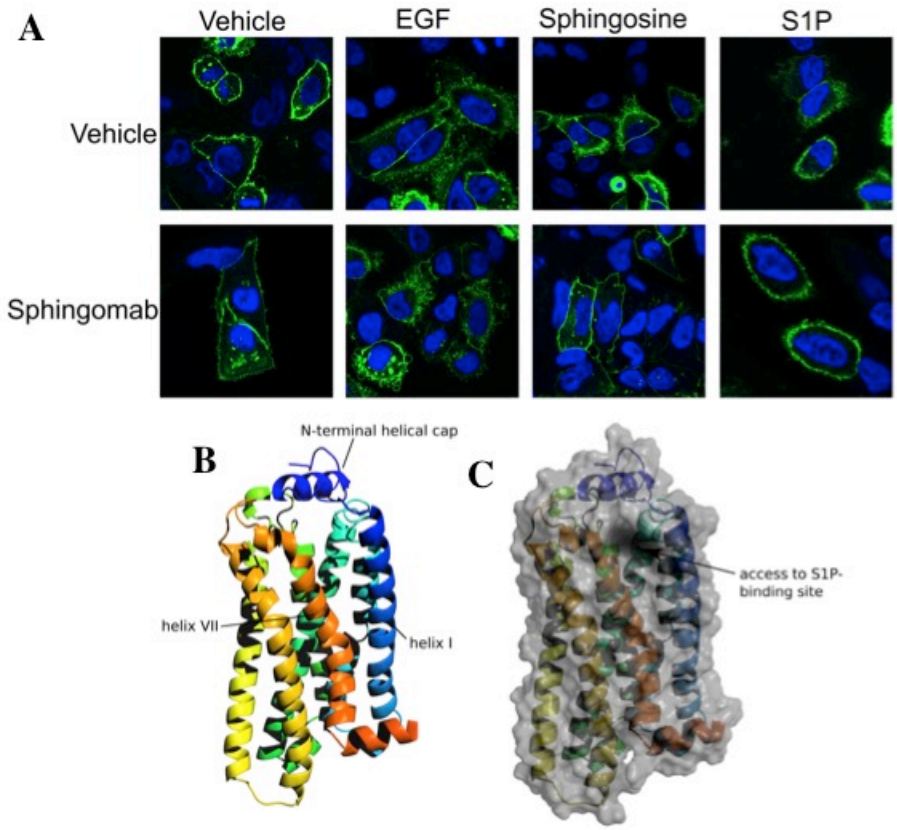


Figure 14: Spns2 is partially required for EGF-mediated ERM phosphorylation and S1PR2 internalization.

A) HeLa cells were transfected with S1PR2-GFP for 24 hours. Following transfection, cells were starved for 4 hours, and then pre-treated for 1 hour with PBS or Sphingomab. Cells were treated with 100 nM S1P, 10nM EGF, or 5mM sphingosine. Cells were than fixed, and nuclei were stained with DAPI (blue). Images were taken using SP8 Leica confocal microscope. B) C) Structure model for S1PR2 generated using I-TASSER server, using top threading templates A2A adenosine receptor (PDB 3EML), S1PR1 (PDB 3V2Y), 5-HT receptor (PDB 4IAQ), and beta1-adrenergic receptor (PDB 3ZPQ). All normalized Z-scores > 2.0, indicating good alignment between query protein and template. Model exhibits a C-score of –0.7, estimated TM-score of 0.62±0.14, and estimated RMSD of 8.1±4.4Å. Cartoon representation of S1PR2 model (B). Cartoon with surface overlay, demonstrating the proposed point of access for S1P from the membrane into its receptor-binding site (C).

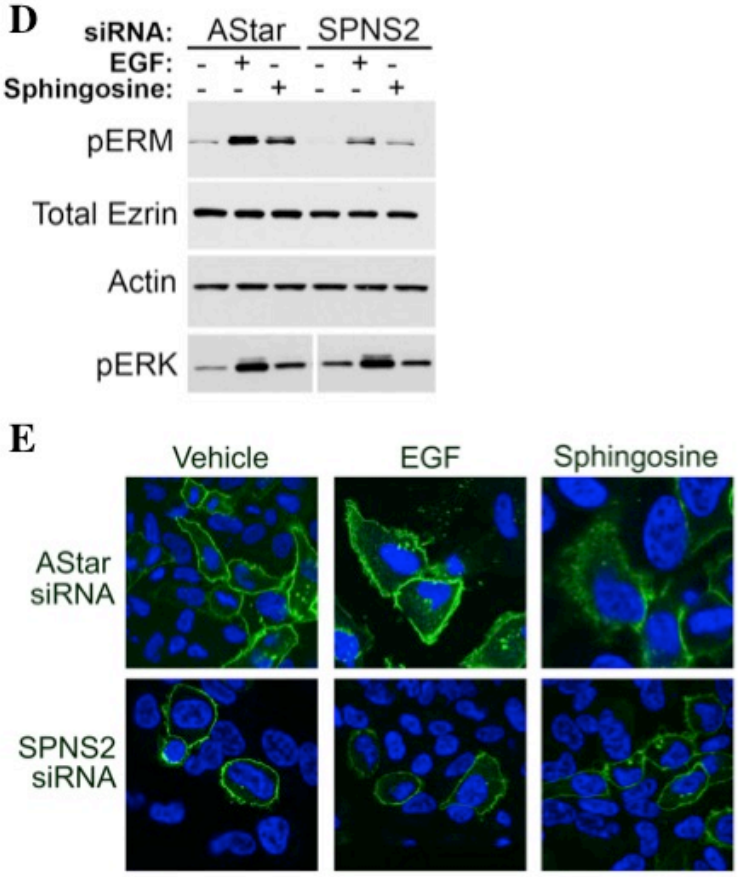


Figure 14 (continued): Spns2 is partially required for EGF-mediated ERM phosphorylation and S1PR2 internalization. D) HeLa cells were treated with AStar or Spns2 siRNA for 48 hours. Cells were then starved for 4 hours prior to treatment with EGF (10ng/ml) or sphingosine (5μM) for 5 minutes. pERM levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls. E) HeLa cells were treated with AStar or Spns2 siRNA for 48 hours. HeLa cells were then transfected with S1PR2-GFP, 24 hours following siRNA transfection. Cells were starved for 4 hours, and then treated with 10nM EGF, or 5mM sphingosine. Cells were than fixed, and nuclei were stained with DAPI (blue). Images were taken using SP8 Leica confocal microscope.

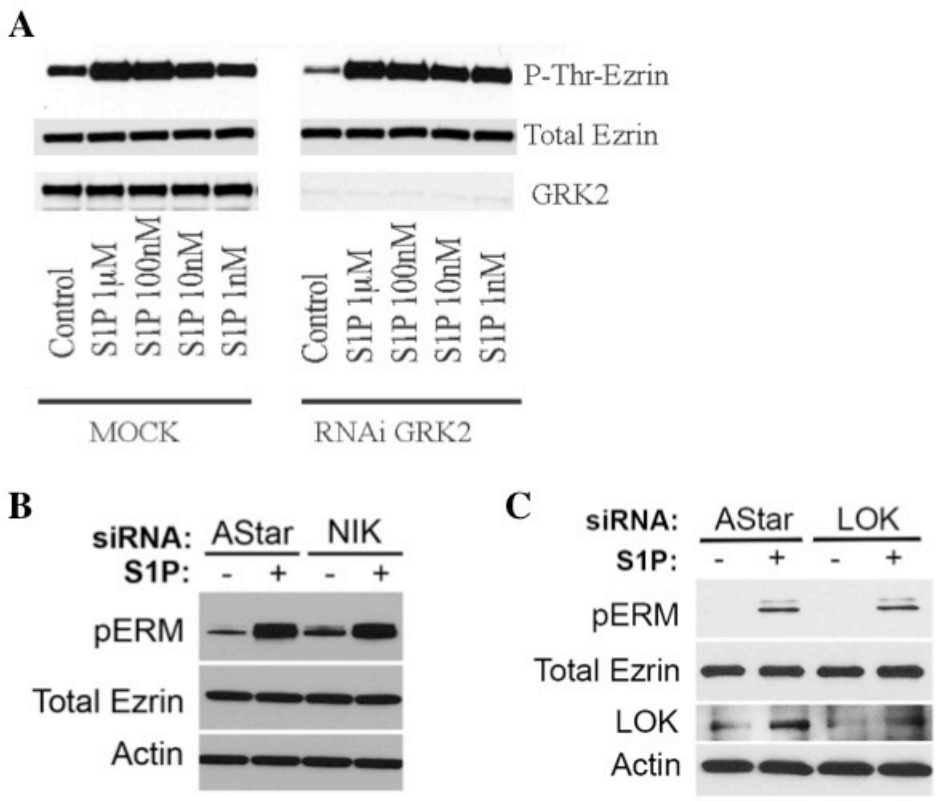


Figure 15: Previously identified ERM kinases are not involved in ERM phosphorylation downstream of S1P.

A) HeLa cells were treated with AStar or GRK2 siRNA for 48 hours. Cells were then starved for 4 hours prior to treatment with increasing doses of S1P (1nM, 10nM, 100nM, 1μM) for 5 minutes. pERM levels were then assessed by immunoblotting. GRK2 levels were included to verify knockdown. B) HeLa cells were treated with AStar or NIK siRNA for 48 hours. Cells were then starved for 4 hours prior to treatment with 100nM S1P for 5 minutes. pERM levels were then assessed by immunoblotting. C) HeLa cells were treated with AStar or LOK siRNA for 48 hours. Cells were then starved for 4 hours prior to treatment with 100nM S1P for 5 minutes. pERM levels were then assessed by immunoblotting. LOK levels were included to verify knockdown. Total ezrin and actin were also included as loading controls.

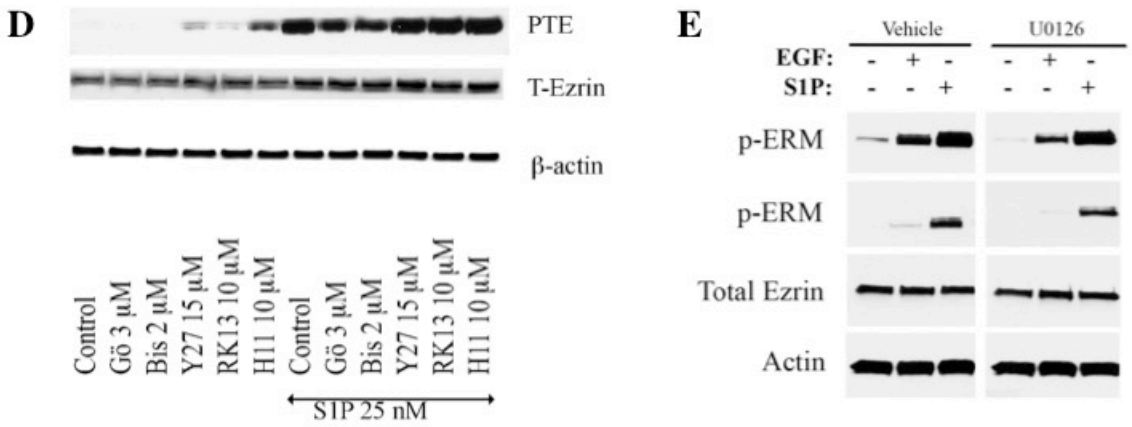


Figure 15 (continued): Previously identified ERM kinases are not involved in ERM phosphorylation downstream of S1P.

D) HeLa cells were pre-treated with DMSO, 3μM G0, 2μM Bis, 15μM Y27, 10μM RK13 or 10μM H11 for 1 hour prior to stimulation with S1P (25nM) for 5 minutes. pERM levels were then assessed by immunoblotting. E) HeLa cells were pre-treated with DMSO or 10μM U0126 for 1 hour prior to stimulation with EGF (10ng/ml) or S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls.

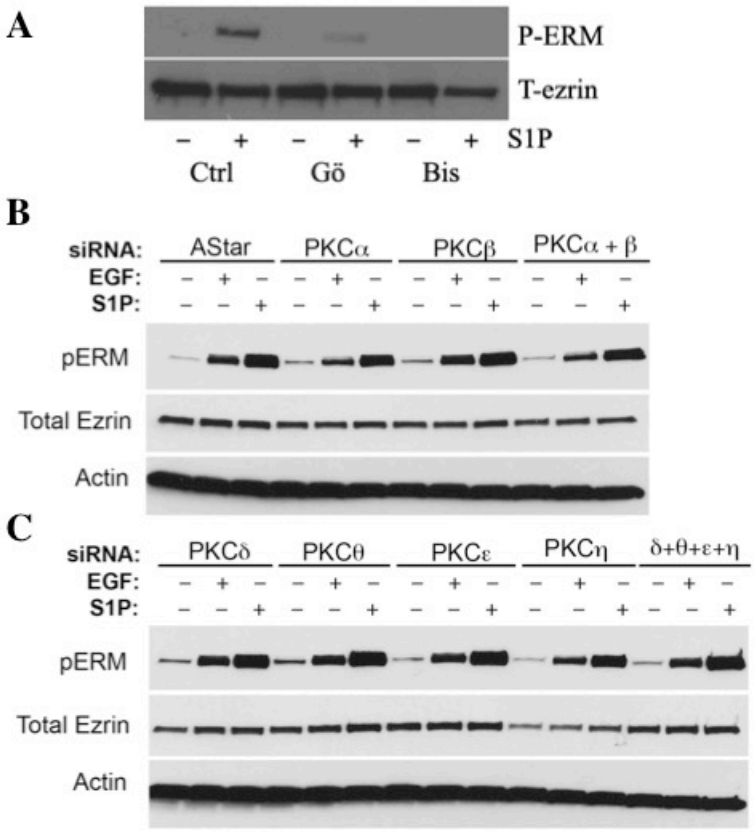


Figure 16: Classical and novel PKCs are not required for ERM phosphorylation induced by S1P treatment.

A) HeLa cells were pre-treated with DMSO, 3μM G0 or 2μM Bis for 1 hour prior to stimulation with S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. B) C) HeLa cells were treated with AStar, PKCα, PKCβ, PKCδ, PKCθ, PKCε or PKCη for 48 hours. Cells were then starved for 4 hours prior to treatment with 100nM S1P for 5 minutes. pERM levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls.

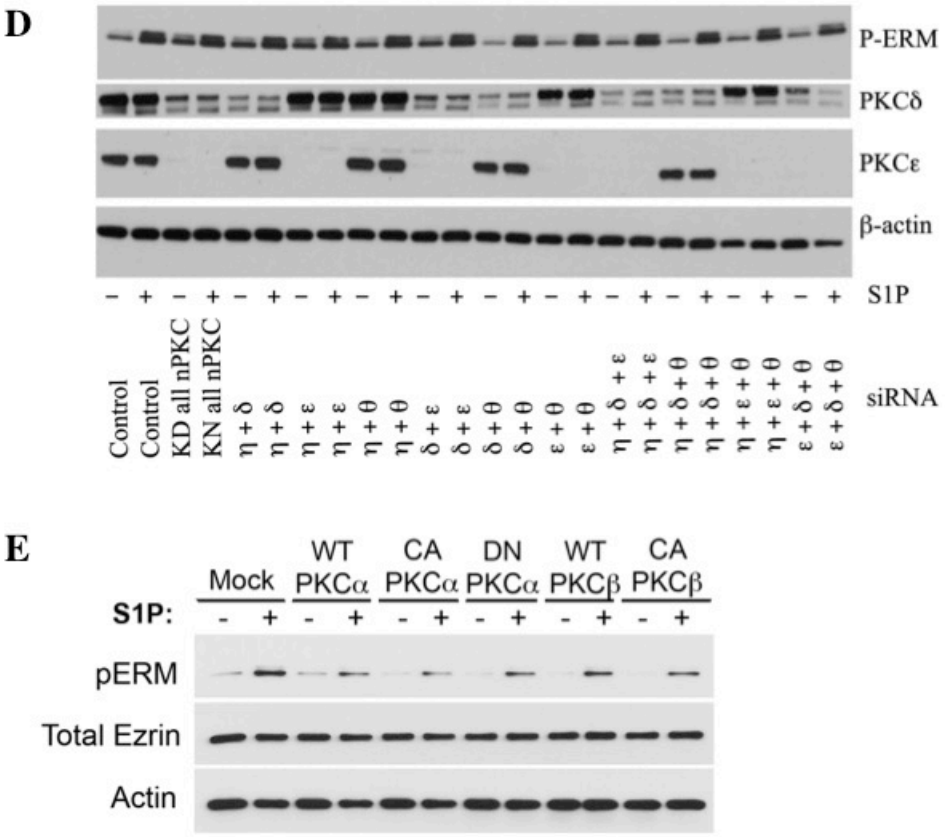


Figure 16 (continued): Classical and novel PKCs are not required for ERM phosphorylation induced by S1P treatment.

D) HeLa cells were treated with AStar, or various combinations of novel PKCs siRNA for 48 hours. Cells were then starved for 4 hours prior to treatment with 100nM S1P for 5 minutes. pERM levels were then assessed by immunoblotting. E) HeLa cells were transfected with Mock, WT PKCα, CA PKCα, DN PKCα, WT PKCβ, or CA PKCβ DNA for 24 hours. Cells were then starved for 4 hours prior to treatment with 100nM S1P for 5 minutes. pERM were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls.

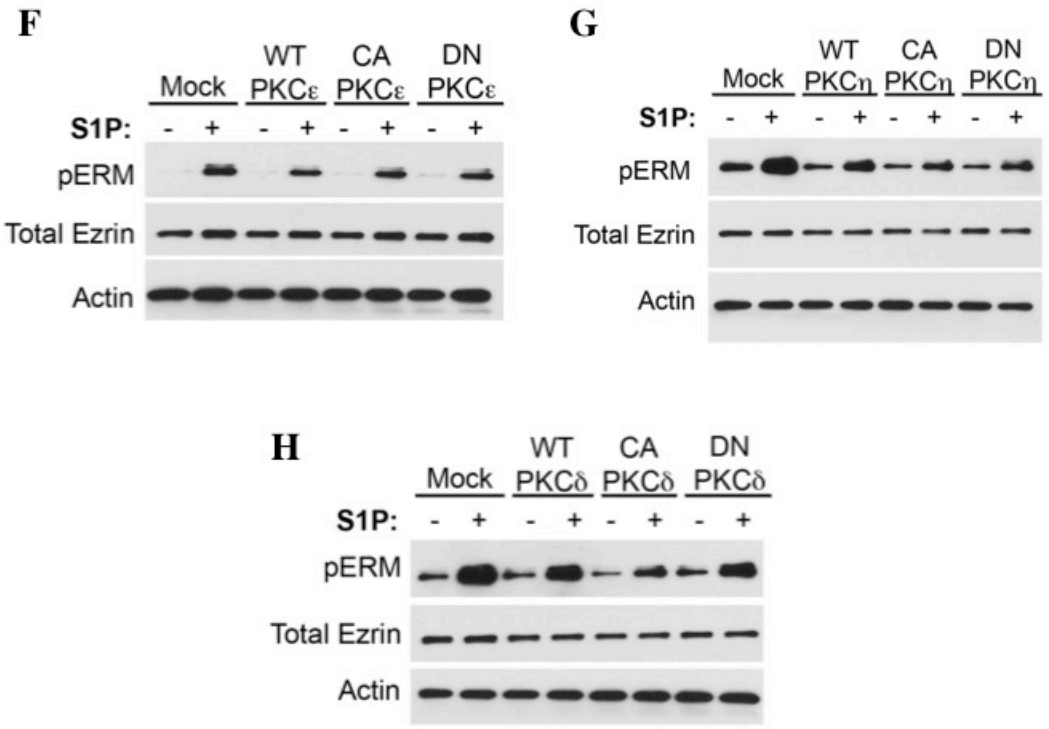


Figure 16 (continued): Classical and novel PKCs are not required for ERM phosphorylation induced by S1P treatment.

F) G) H) HeLa cells were transfected with Mock, WT PKCε, CA PKCε, DN PKCε, WT PKCη, CA PKCη, DN PKCη, WT PKCδ, CA PKCδ or DN PKCδ DNA for 24 hours. Cells were then starved for 4 hours prior to treatment with 100nM S1P for 5 minutes. pERM were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls.

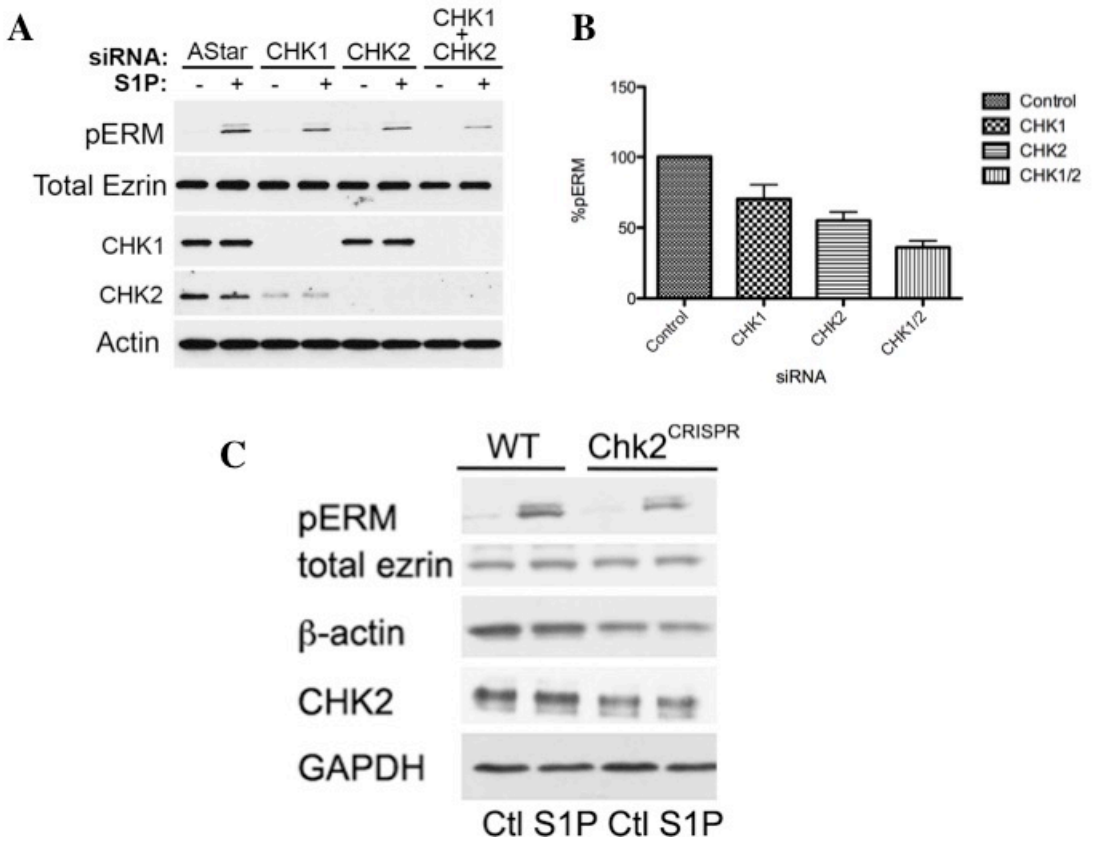


Figure 17: Chk1 and Chk2 are essential for S1P-induced ERM activation.

A) HeLa cells were treated with AStar, Chk1 siRNA, Chk2 siRNA, or both for 48 hours. Cells were then starved for 4 hours prior to treatment with 100nM S1P for 5 minutes. pERM levels were then assessed by immunoblotting. Chk1 and Chk2 levels were included to verify knockdown. B) Quantification of the ratio of pERM/total ezrin in A was performed using Image J software. C) WT or polyclonal HeLa cells knocked out of CHK2 using CRISPR technology were starved for 4 hours prior to treatment with 100nM S1P for 5 minutes. pERM levels were then assessed by immunoblotting. Chk2 levels were included to verify knock out. Total ezrin, GAPDH and actin were also included as loading controls.

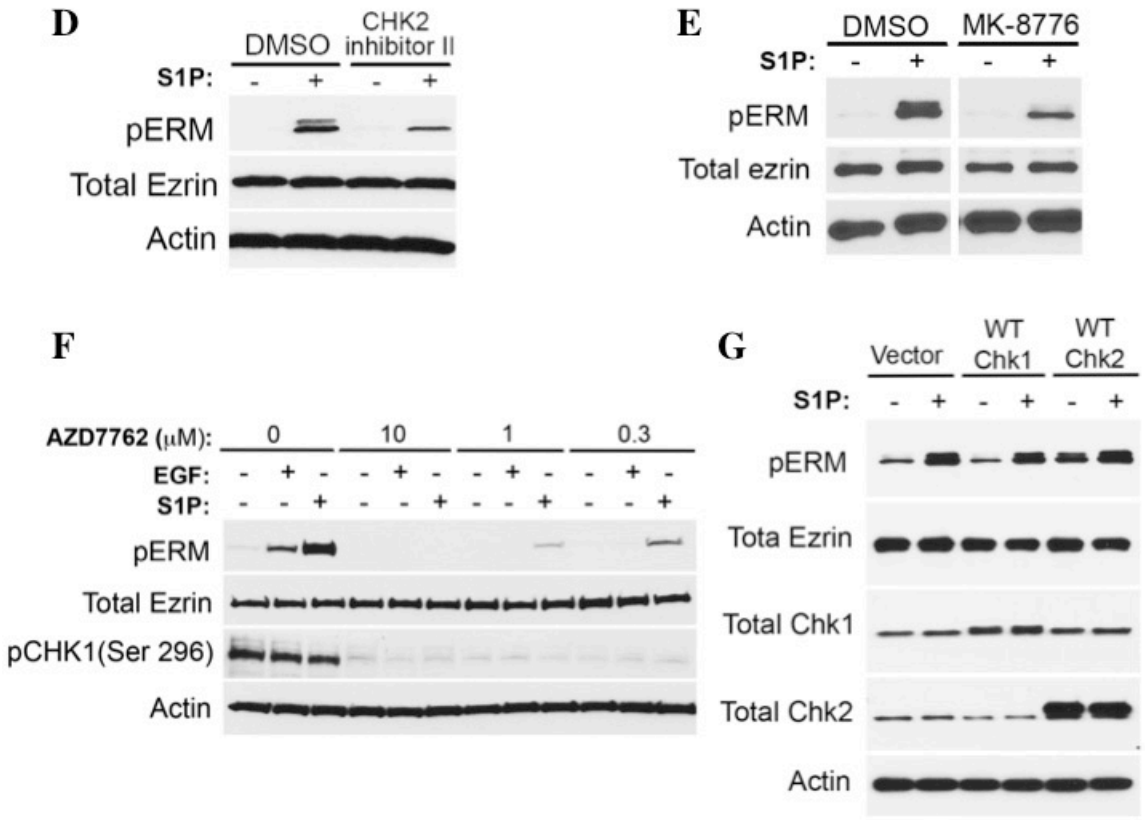


Figure 17 (continued): Chk1 and Chk2 are essential for S1P-induced ERM activation.

D) HeLa cells were pre-treated with DMSO or 10μM Chk2 inhibitor 2 for 1 hour prior to stimulation with S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. E) HeLa cells were pre-treated with DMSO or 10μM MK-8776 for 1 hour prior to stimulation with S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. F) HeLa cells were pre-treated with DMSO or AZD7762 (0.3,1, or 10μM) for 1 hour prior to stimulation with EGF (10ng/ml) or S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. P-CHK1(Ser296) levels were also assessed to check for inhibitor efficacy. G) HeLa cells were transfected with empty vector, WT Chk1 or WT Chk2 DNA for 24 hours. Cells were then starved for 4 hours prior to treatment with S1P (100nM) for 5 minutes. pERM were then assessed by immunoblotting. Total Chk1 and Chk2 levels were also assessed to check for transfection efficiency. Total ezrin and actin were also included as loading controls.

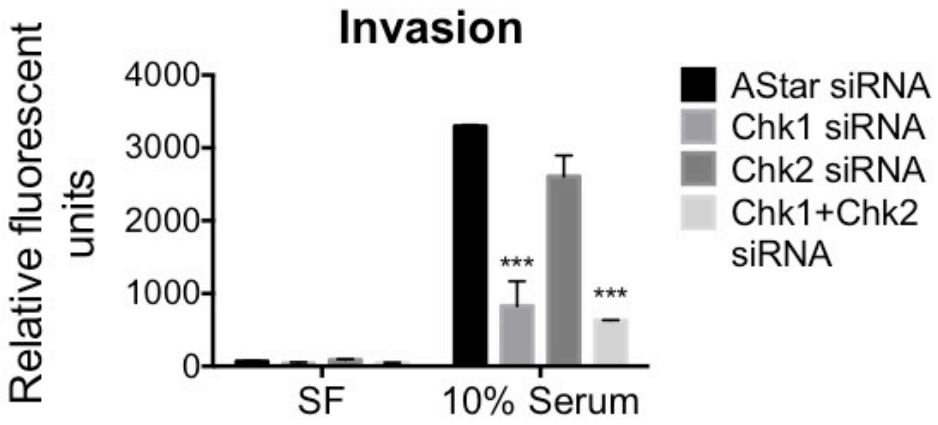


Figure 18: Chk1 and Chk2 are required for HeLa cells invasion of the ECM.

HeLa cells were treated with AStar, Chk1 siRNA, Chk2 siRNA, or both for 48 hours. Cell were then starved for 4 hours prior to their plating in the apical chamber of matrigel-coated transwell inserts, and allowed to invade for 24 hours toward serum-free or serum supplemented media. Invading cells were then stained with 4 ug/ul Calcein AM, and absorbance was red in a plate reader as indicated in materials and methods section.

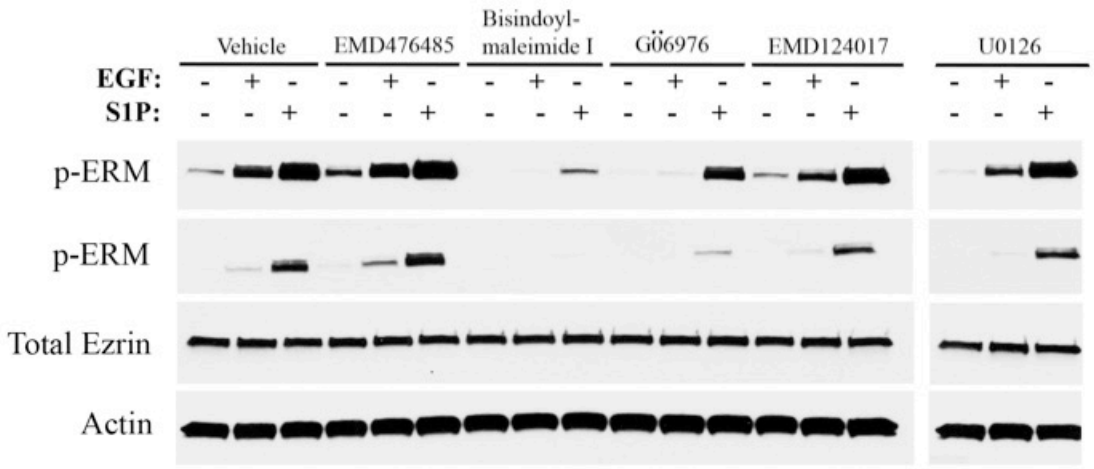


Figure 19: ERK, PKA, AKT, and PKCs are not required for EGF-induced ERM phosphorylation.

HeLa cells were pre-treated with DMSO (vehicle), EMD476485 (10μM), Bis (2μM), G0976 (3μM), EMD124017 (10μM) or U0126 (10μM) for 1 hour prior to stimulation with EGF (10ng/mL) or S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting and represented by two different exposures. Total ezrin and actin were also included as loading controls.

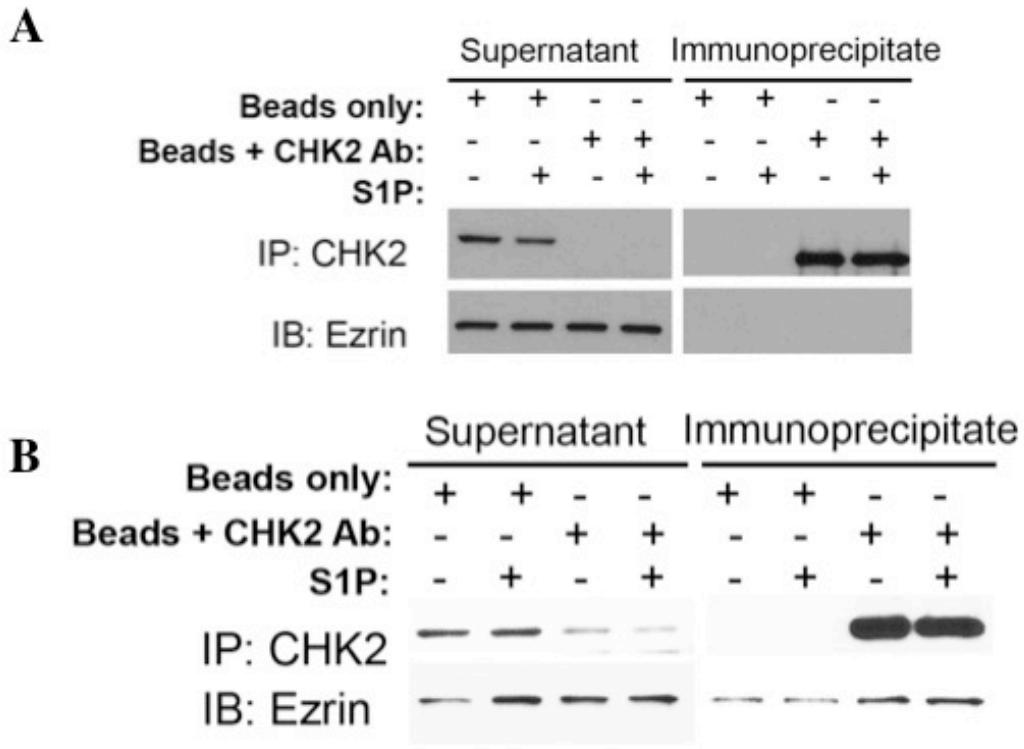


Figure 20: Chk1 and Chk2 are not the direct kinases phosphorylating ezrin upon S1P treatment.

A) HeLa cells were starved for 4 hours prior to treatment with S1P (10nM, 5 minutes). Cells were then lysed. Chk2 was then immunoprecipitated using the anti-Chk2 antibody, and co-immunoprecipitation with ezrin was analyzed by blotting for total ezrin. B) HeLa cells were starved for 4 hours prior to treatment with S1P (10nM, 5 minutes). Cells were then fixed with 3.75% formaldehyde and lysed. Chk2 was then immunoprecipitated using the anti-Chk2 antibody, and co-immunoprecipitation with ezrin was analyzed by blotting for total ezrin.

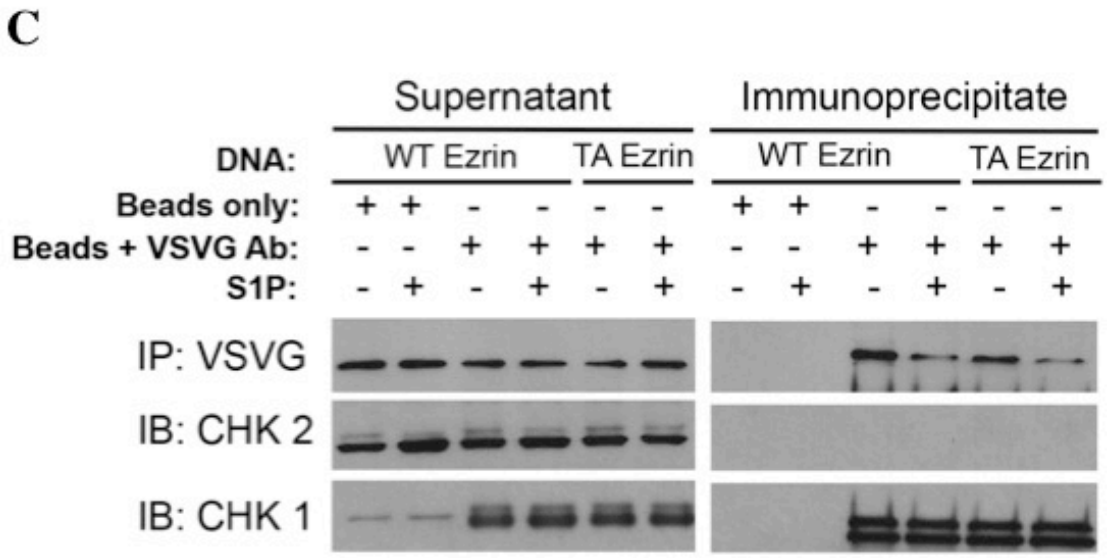


Figure 20 (continued): Chk1 and Chk2 are not the direct kinases phosphorylating ezrin upon S1P treatment.

C) HeLa cells were transfected with WT ezrin or T564A ezrin (TA ezrin) for 24 hours. Cells were then starved for 4 hours prior to treatment with S1P (10nM, 5 minutes). Cells were then fixed with 3.75% formaldehyde and lysed. Ezrin was then immunoprecipitated using the anti-VSVG antibody, and co-immunoprecipitation with Chk1 and Chk2 was analyzed by blotting for total Chk1 and total Chk2 antibodies.

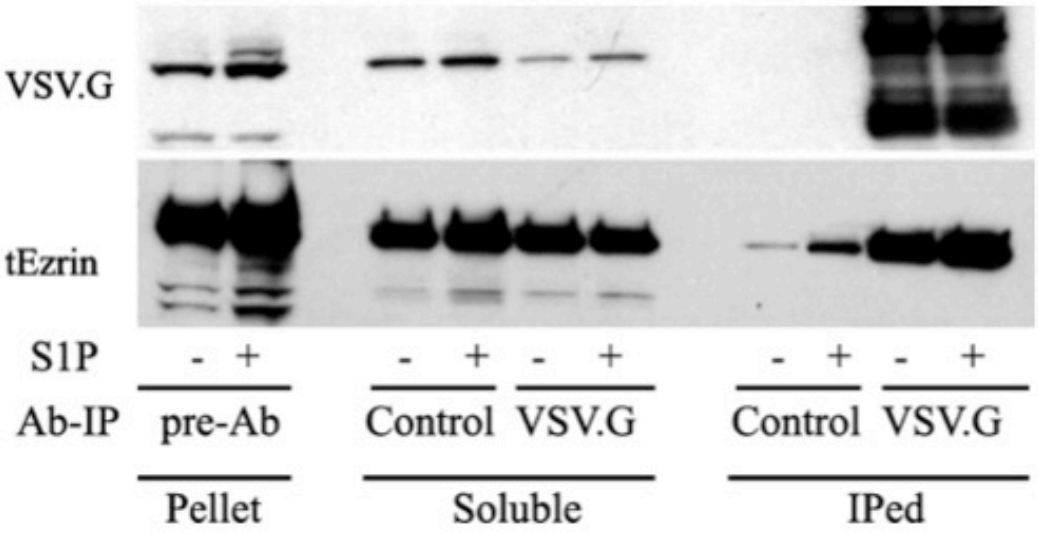


Figure 21: Optimization of ezrin-VSVG pull down using magnetic beads.

HeLa cells were transfected with WT ezrin tagged with VSVG for 24 hours. Cells were then starved for 4 hours prior to treatment with S1P (10nM, 5 minutes). Cells were then fixed with 3.75% formaldehyde and lysed. Ezrin was then immunoprecipitated using the anti-VSVG antibody. Ezrin pull-down efficiency was then analyzed by probing with VSVG and total ezrin antibody in the cell debris, cytosol and pellet.

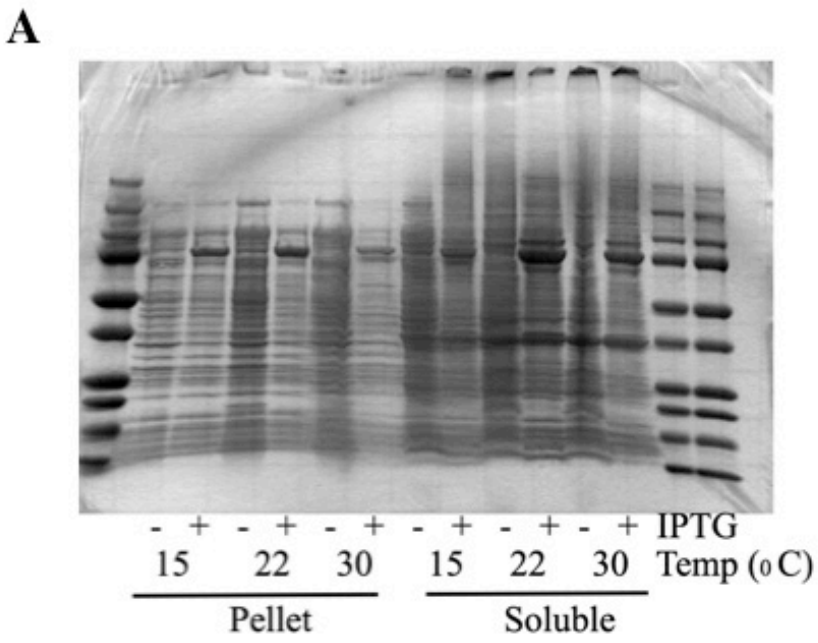


Figure 22: Ezrin purification process.

A) BL21 cells transfected with pET28-HIS-ezrin-VSV.G were cultured without (-) or induced with 1mM IPTG (+) at 15, 22 or 30^0C overnight. Cells were collected, and the insoluble (pellet) and soluble fractions were analyzed by Coomassie staining. Expression of soluble recombinant ezrin (80KDa) was optimal at 22^0C.

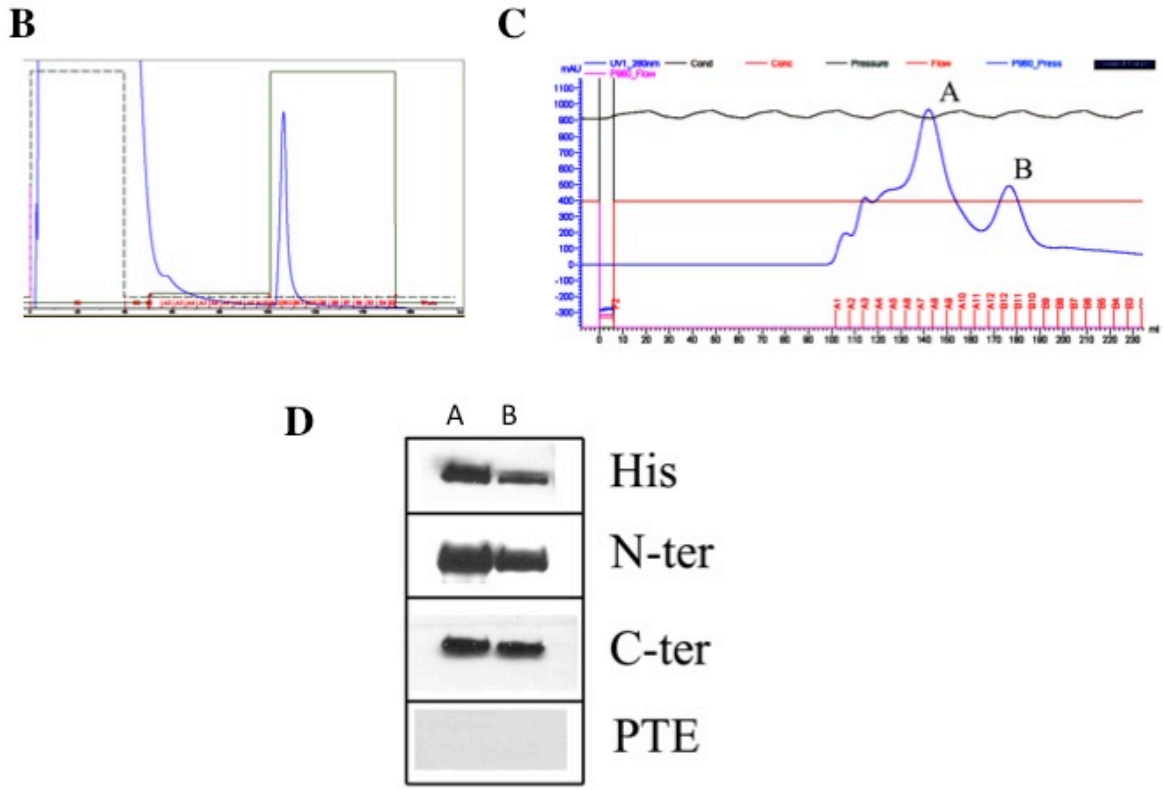


Figure 22 (continued): Ezrin purification process.

B) Four liters of bacterial culture were induced with IPTG, and the soluble cell lysate was applied to a HIS-TRAP column. Unbound proteins were washed using PBS buffer, and HIS-ezrin was eluted using 250mM imidazole. C) Collected protein was then applied to a Sephadex 200 size exclusion column, and ezrin was collected as dimer A and monomer B forms. D) The integrity of the protein was shown by western blotting using a His-antibody, and a N-terminal and C-terminal ezrin antibodies.

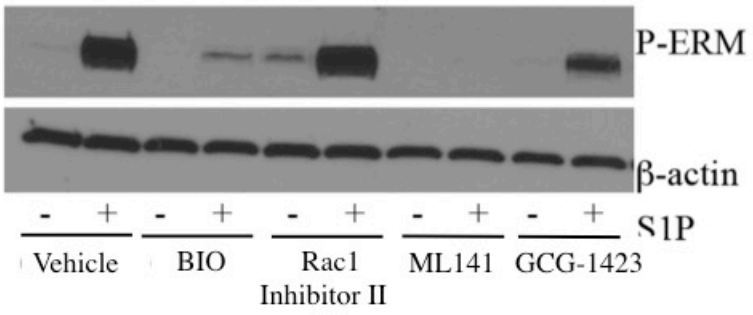


Figure 23: The small GTPases Cdc42 and RhoA are essential for ERM activation downstream of S1PR2.

HeLa cells were pre-treated with DMSO (vehicle), ML141 (10μM), CCG-1423 (10μM), and Rac1 inhibitor II (10μM) for 1 hour prior to stimulation with EGF (10ng/mL) or S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. Actin was also included as a loading control.

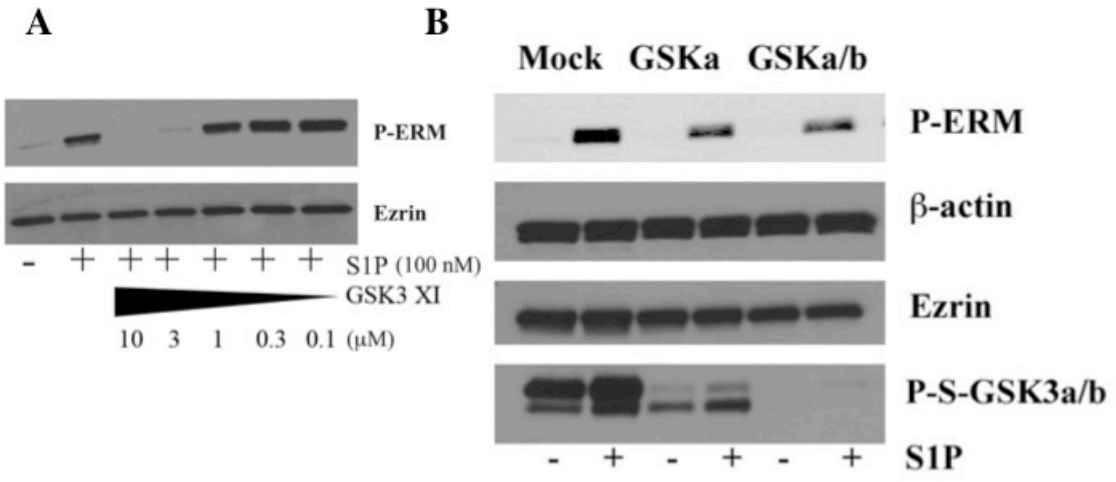


Figure 24: GSK3 beta is essential and sufficient in inducing ERM phosphorylation.

A) HeLa cells were incubated with DMSO (vehicle), or increasing concentrations of the GSK3 inhibitor BIO (0.1-10 μM) for 1 hour prior to stimulation with S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. B) HeLa cells were treated with Mock, GSK3α siRNA or GSK3α/β siRNA for 48 hours. Cells were then starved for 4 hours prior to treatment with 100nM S1P for 5 minutes. pERM levels were then assessed by immunoblotting. Phospho-GSK3α/β levels were included to verify knockdown. Total ezrin and actin were also included as loading controls.

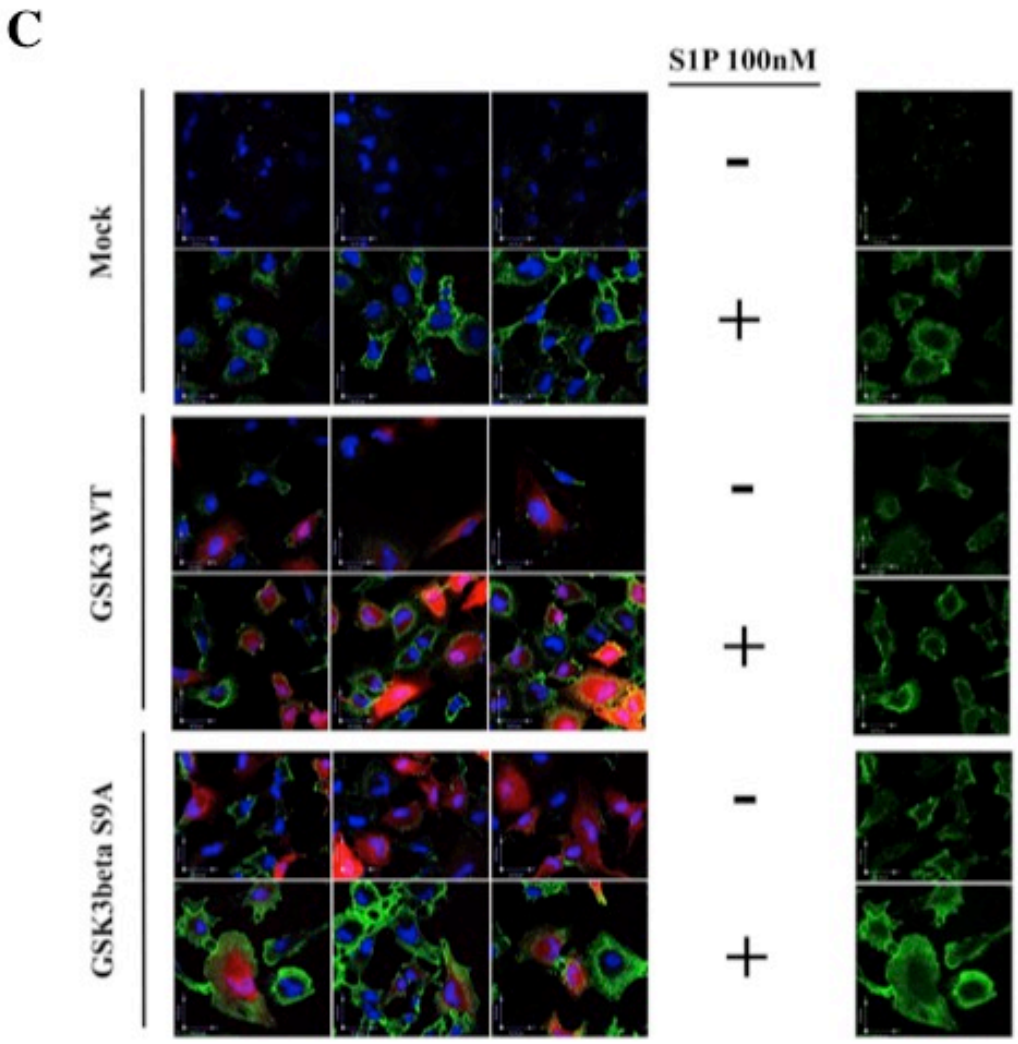


Figure 24 (continued): GSK3 beta is essential and sufficient in inducing ERM phosphorylation.

C) HeLa cells were transiently transfected with either mock control, GSK3 WT or GSK3β S9A overexpressing DNA for 24 hours. Cells were then starved for 4 hours prior to treatment with S1P (100nM) for 5 minutes. Cells were than fixed, and nuclei were stained with DAPI (blue), pERM (green) and GSK3 (red). Images were taken using SP8 Leica confocal microscope.

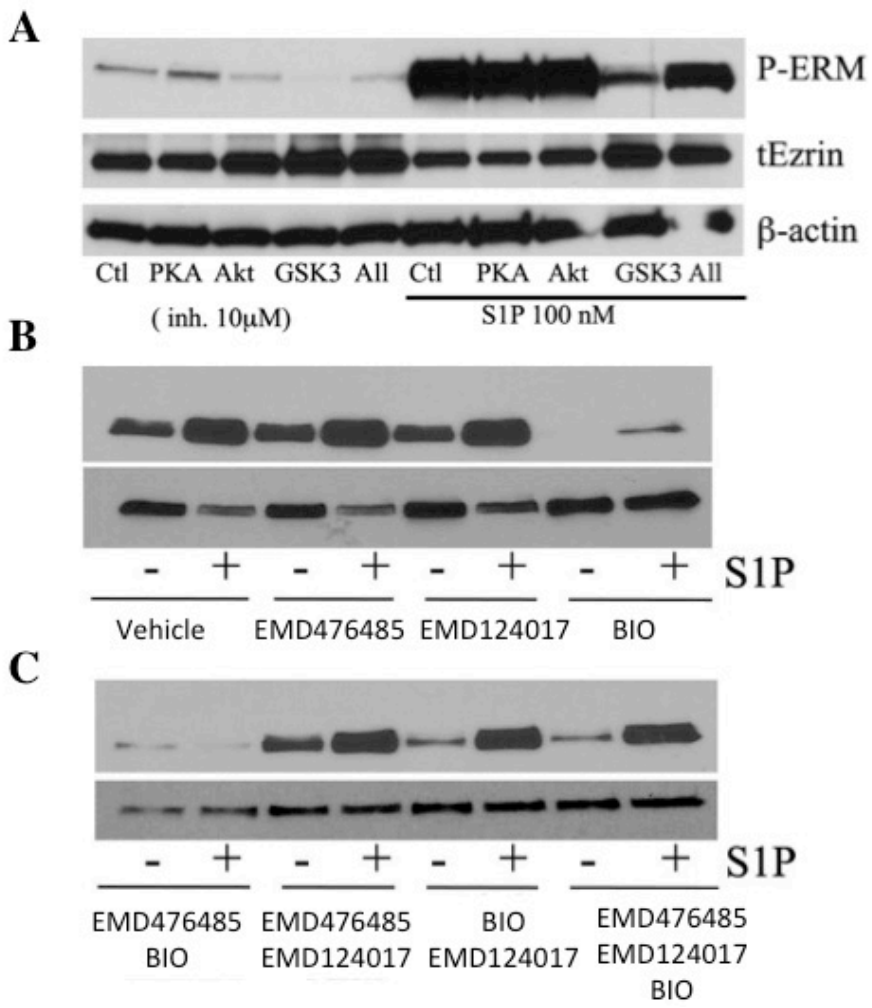


Figure 25: Concomitant AKT and GSK3 inhibition restores ERM phosphorylation.

A) B) C) HeLa cells were pre-treated with DMSO (vehicle), EMD476485 (10μM), EMD124017 (10μM), BIO (10μM) or different combinations for 1 hour prior to stimulation with S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls.

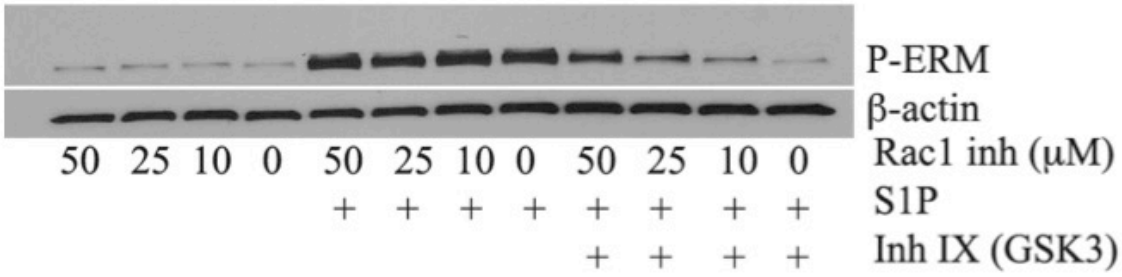


Figure 26: Inhibition of Rac1 and GSK3 rescues ERM activation.

HeLa cells were incubated with DMSO (vehicle), or increasing concentrations of the Rac1 inhibitor II (10, 25, 50μM) in the absence or presence of the GSK3 inhibitor BIO (10 μM) for 1 hour prior to stimulation with S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. Actin was also included as a loading control.

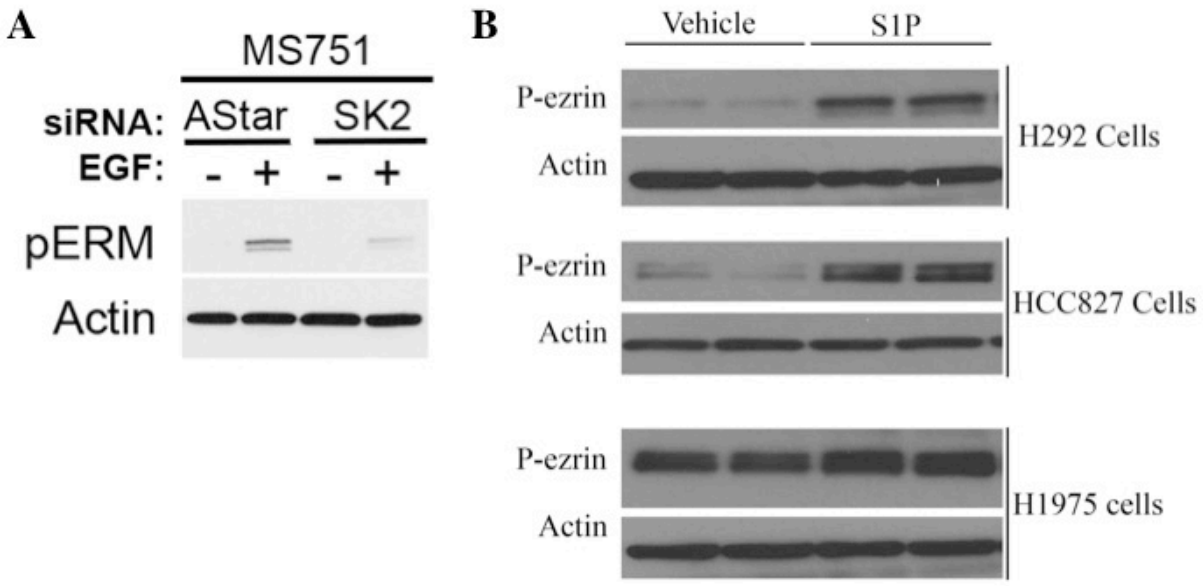


Figure 27: Role of the SK2/S1P axis in ERM phosphorylation tested in multiple cell lines.

A) Metastatic cervical cancer cells MS751 were treated with AStar or SK2 siRNA for 48 hours. Cells were then starved for 4 hours prior to treatment with EGF (10ng/ml) for 5 minutes. pERM levels were then assessed by immunoblotting. B) Lung cancer cells H292, HCC827, and H1975 were starved overnight, prior to stimulation with 100nM S1P for 5 minutes. pERM levels were then assessed by immunoblotting. Results are shown in duplicates. Actin was also included as a loading control.

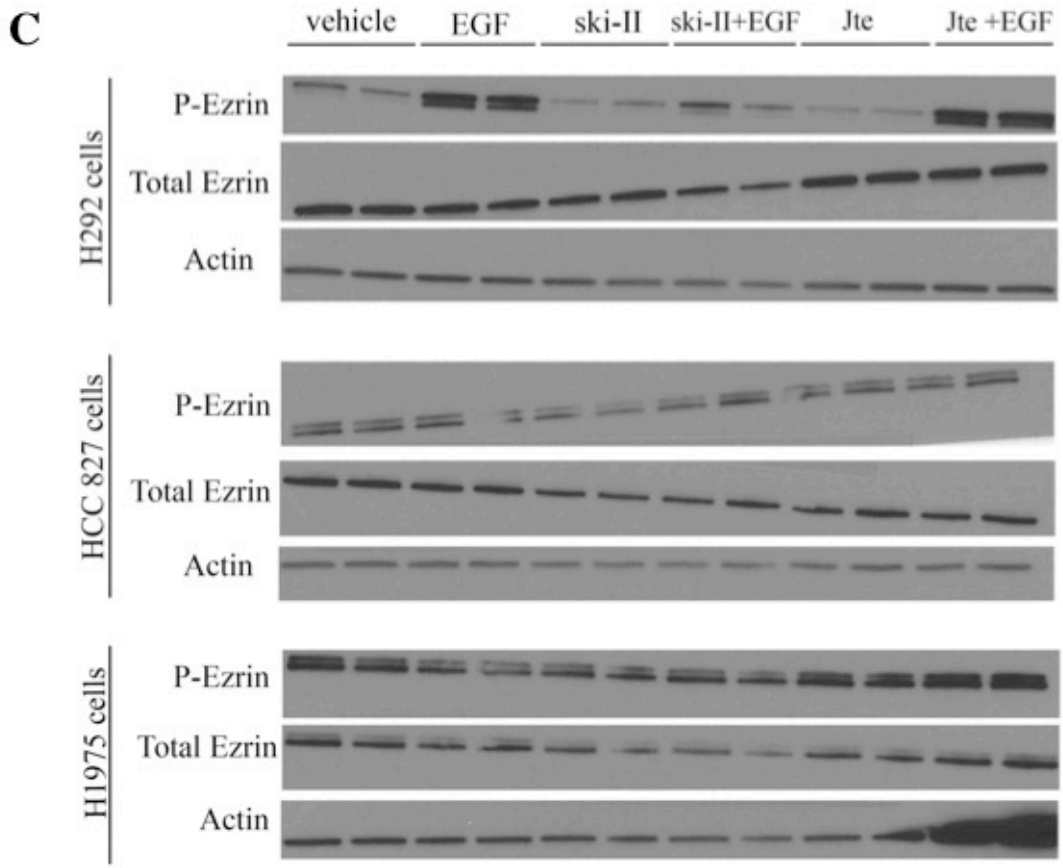


Figure 27 (continued): Role of the SK2/S1P axis in ERM phosphorylation tested in multiple cell lines.

C) H292, HCC827, and H1975 cells were starved overnight, prior to pre-treatment with vehicle, Ski-II or JTE013 for 1 hour. Cells were then stimulated with 10 ng/mL EGF for 5 minutes. pERM levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls.

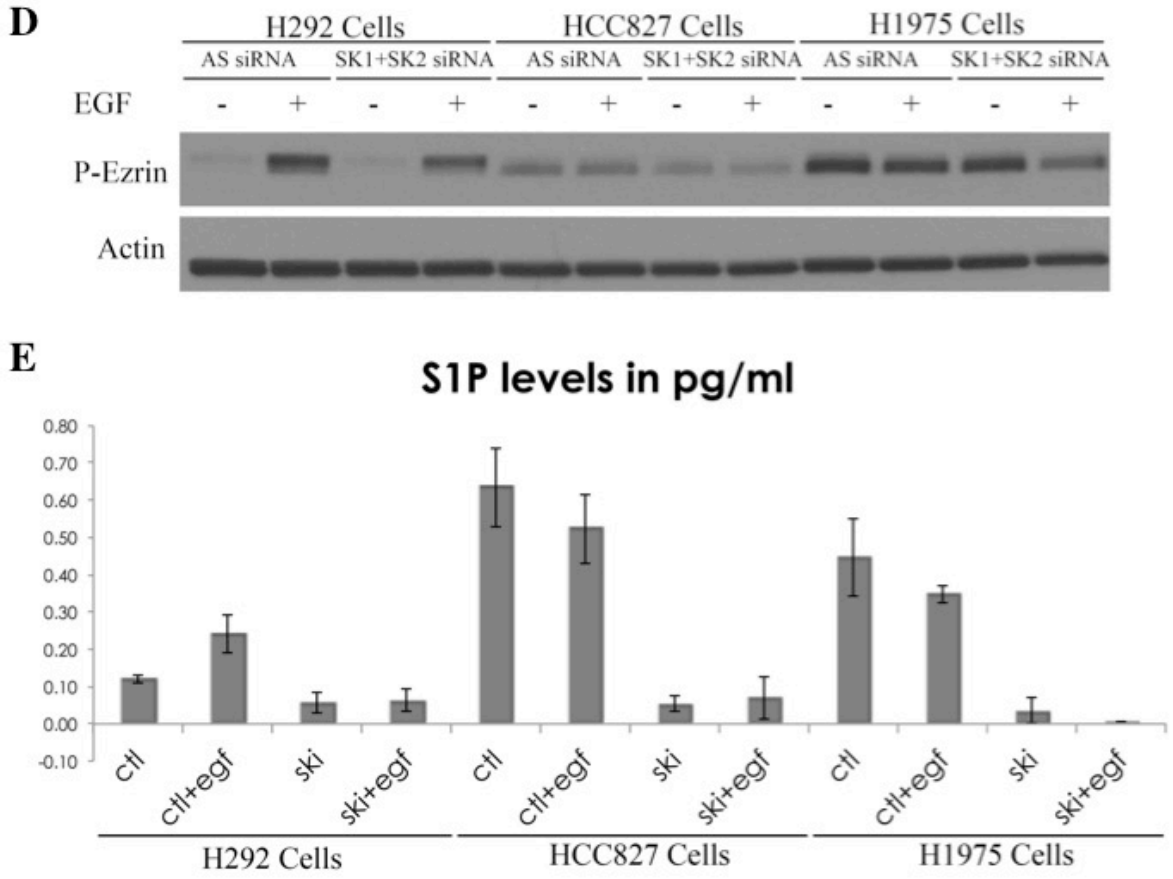


Figure 27 (continued): Role of the SK2/S1P axis in ERM phosphorylation tested in multiple cell lines.

D) H292, HCC827, and H1975 cells were treated with AStar or SK1 and SK2 siRNA for 48 hours. Cells were then starved for 4 hours prior to treatment with EGF (10ng/ml) for 5 minutes. pERM levels were then assessed by immunoblotting. E) H292, HCC827, and H1975 cells were starved overnight, prior to pre-treatment with vehicle or Ski-II for 1 hour. Cells were then stimulated with 10 ng/mL EGF for 5 minutes. Endogenous S1P levels from cells were then analyzed via mass spectroscopy.

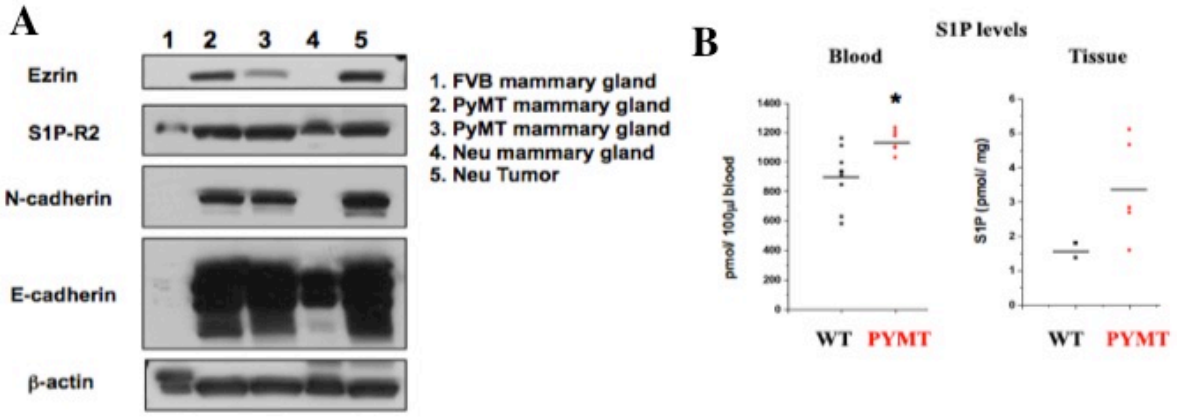


Figure 28: S1P/S1PR2/ezrin axis is overexpressed in PYMT and Neu breast cancer mouse models.

A) Western blotting of non-cancerous (lanes 1 and 4) tissue and cancer tissue of PYMT (2 and 3) and Neu (5) mice. Ezrin, S1PR2, and other typical cancer overexpressed proteins are detected in cancer tissue but not in normal tissue. Samples were normalized by protein content. B) S1P levels in blood and tissue in WT and PYMT mice. PYMT mice showed increased levels in both samples.

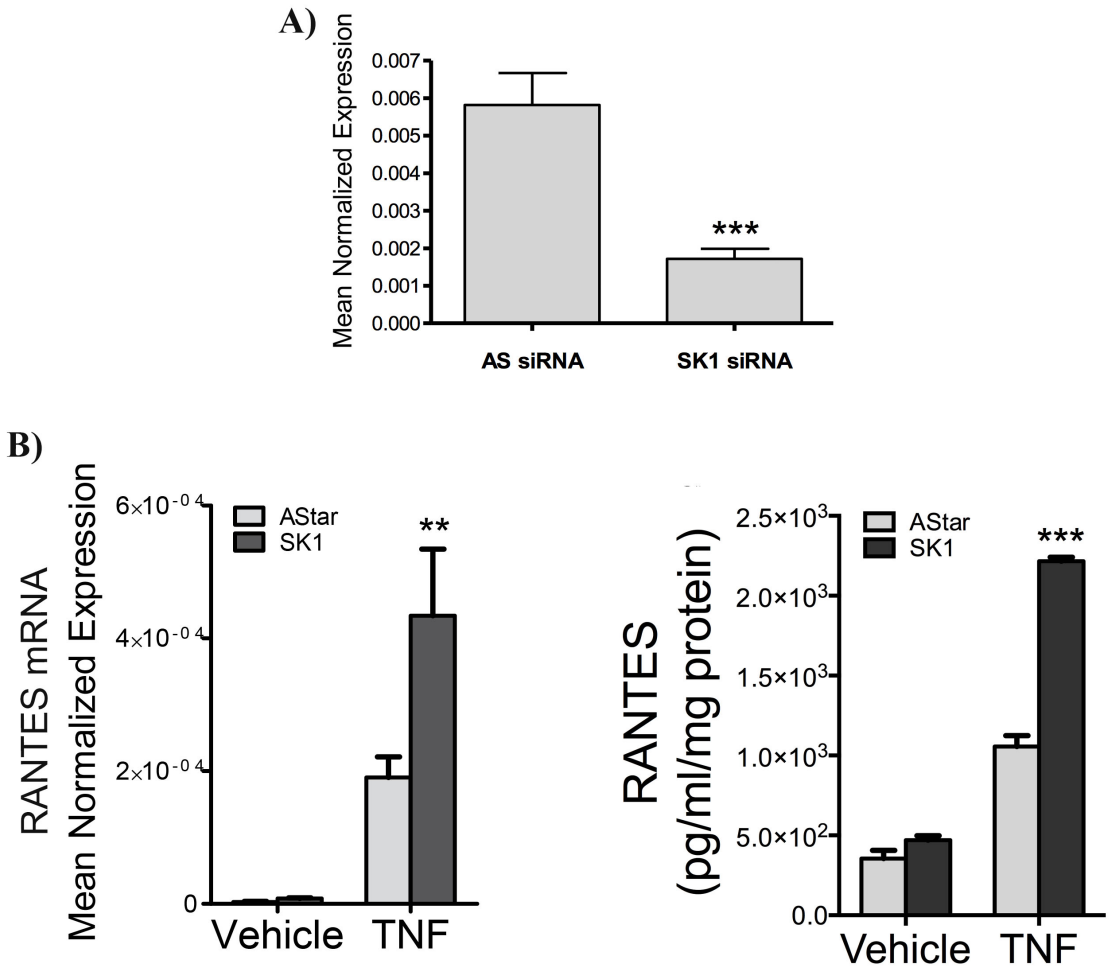


Figure 29: Loss of SK1 significantly enhances RANTES induction.

A) HeLa cells were treated with 20nM Astar or SK1 siRNA for 48 h and SK1 mRNA levels were assessed by qRT-PCR n=3. B) HeLa cells were treated with 20nM Astar or SK1 siRNA for 48h, treated with vehicle (PBS) or TNF (20ng/ml) for 24h and the levels of RANTES mRNA assessed by qRT-PCR. Data represent mean ± SEM from 6 independent experiments. C) HeLa cells were treated with 20nM Astar or SK1 siRNA for 48h, treated with vehicle (PBS) or TNF (20ng/ml) for 24h in serum-free media, and RANTES levels in the media was analyzed by ELISA. Data represent mean ± SEM of 2 independent experiments performed in duplicate. (** $p<0.01$, *** $p<0.001$)

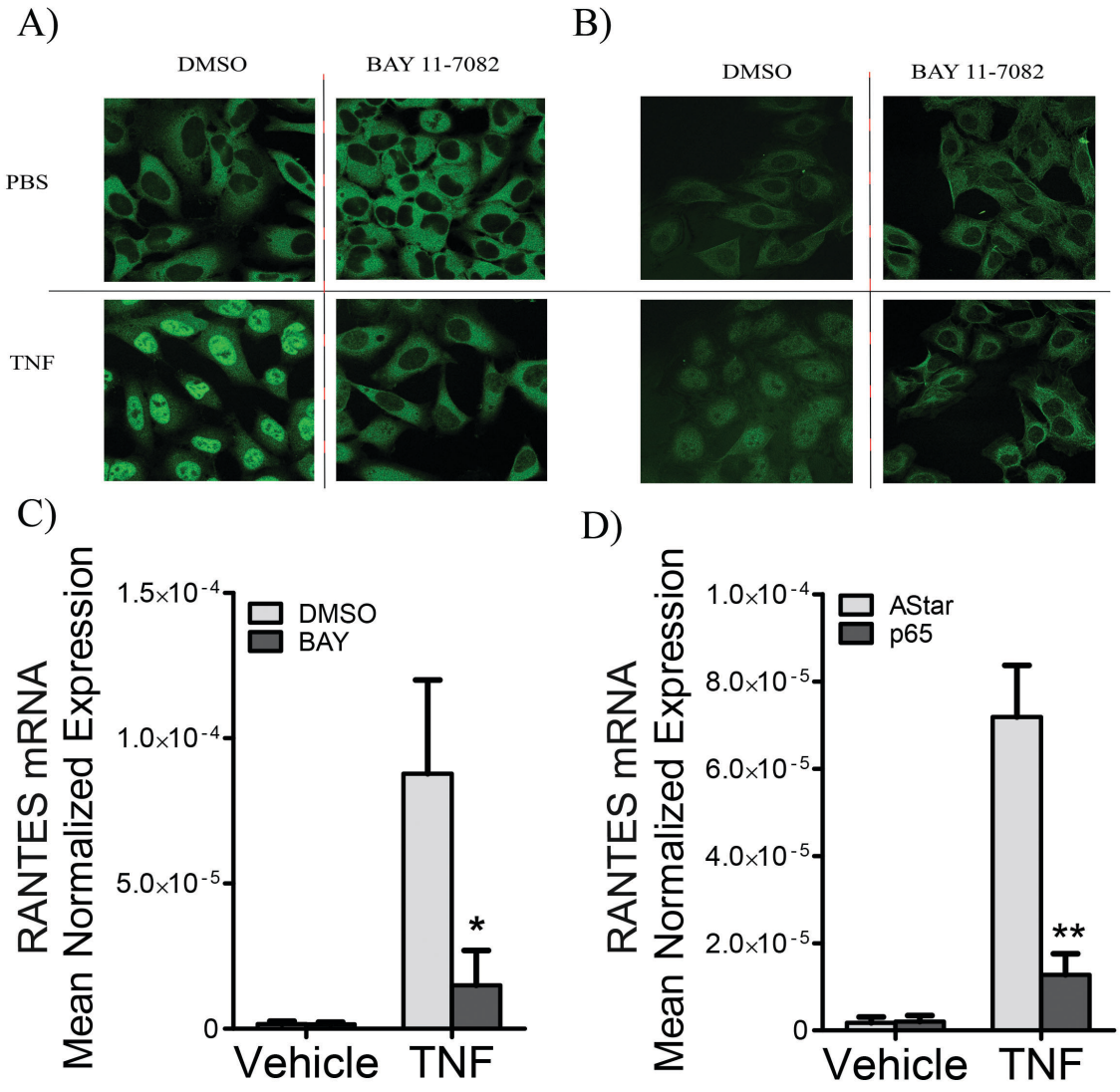


Figure 30: RANTES induction is highly NF-kB dependent.

HeLa cells were pre-treated with either DMSO or 5μM BAY for 30 min prior to treatment with vehicle (PBS) or TNF (20ng/ml) for 30 min. Cells were fixed and incubated with (A) anti-p65 or (B) anti-p50 antibodies. A secondary antibody with a 488 fluorophore was utilized, and cells were visualized by confocal microscopy. C) HeLa cells were treated with vehicle (DMSO) or BAY (5μM) for 30 min prior to stimulation with PBS or TNF (20ng/ml) for 24h. RNA was extracted and RANTES mRNA levels were assessed by qRT-PCR, Data represent mean ± SEM of 3 independent experiments. D) HeLa cells were treated with 20nM Astar or p65 siRNA for 48h, treated with vehicle (PBS) or TNF (20ng/ml) for 24h and the levels of RANTES mRNA assessed by qRT-PCR. Data represent mean ± SEM, of 3 independent experiments. (*$p<0.05$, ** $p<0.01$).

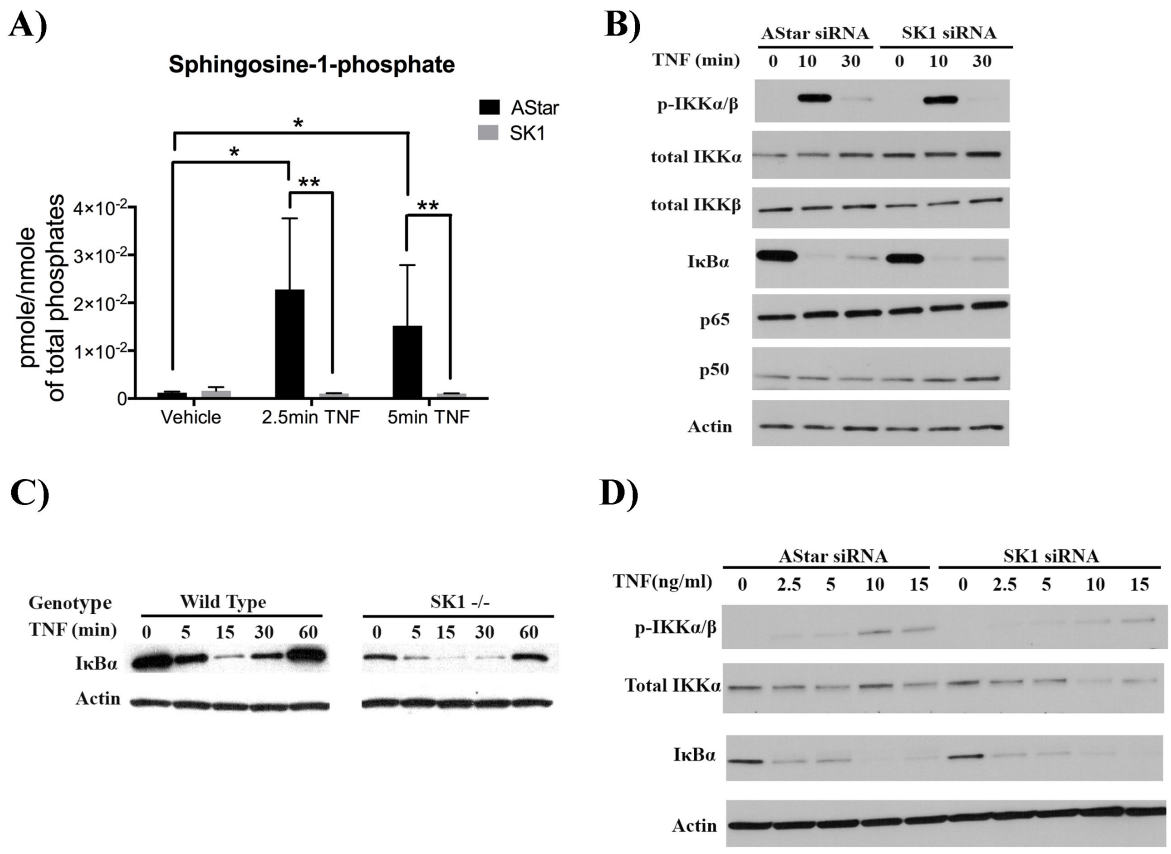


Figure 31: SK1 is dispensable for TNF-mediated activation of NF-κB.

A) HeLa cells were treated with 20nM Astar or SK1 siRNA for 48h prior to treatment with TNF (20ng/ml) or PBS for 2.5 and 10 min. Cellular lipids were directly extracted in organic solvent extraction and S1P levels were analyzed by tandem LC/MS mass spectrometry. Lipid levels were normalized to nmol of total. Data represent mean ± SEM, of 2 independent experiments performed in duplicate. B) HeLa cells were treated with 20nM Astar or SK1 siRNA for 48h prior to treatment with TNF (20ng/ml) for 0, 10 or 30 min. Early downstream signals of TNF including IKKα/β phosphorylation, IκBα degradation were examined via immunoblot. Total levels of NF-κB subunits p65 and p50 were also analyzed. Actin was included as loading control.. Each blot is representative of 3 independent experiments. C) MEFs derived from WT or $SK1^{-/-}$ mice were treated with 4nM TNF for 0, 5, 15, 30 or 60 minutes, and IκBα levels were assessed by immunoblot with actin as a loading control; blots are representative of 3 independent experiments. D) HeLa cells were treated with 20nM Astar or SK1 siRNA for 48h prior to treatment with various TNF doses as indicated for 10 min 2.5, 5, 10, or 15 ng/ml TNF were added for 10 min. IKKα/β phosphorylation and IκBα degradation were examined via by immunoblot with actin as loading controls. Each blot is representative of 3 independent experiments. (*$p<0.05$, ** $p<0.01$)

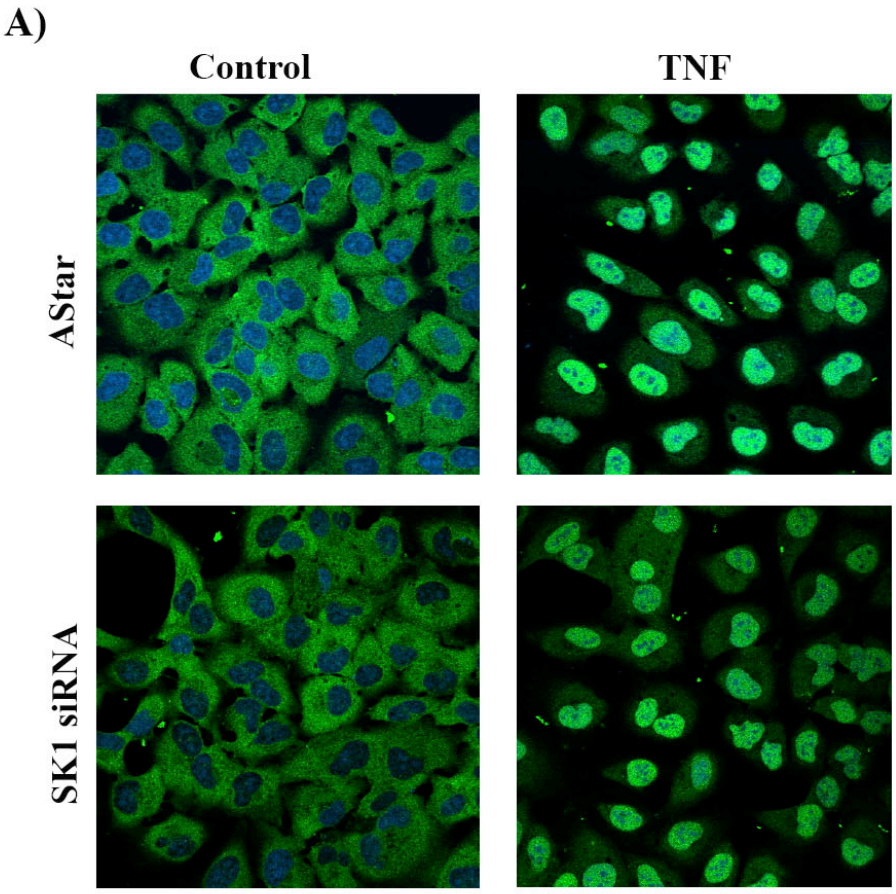


Figure 32: SK1 is not required for TNF induced nuclear translocation of p65/RelA.

A) HeLa cells were treated with 20nM Astar or SK1 siRNA for 48h prior to treatment with vehicle (PBS) or TNF (20ng/ml) for 30 min. Cells were fixed, permeabilized, and probed with p65 antibody. p65 localization was examined by confocal microscopy.

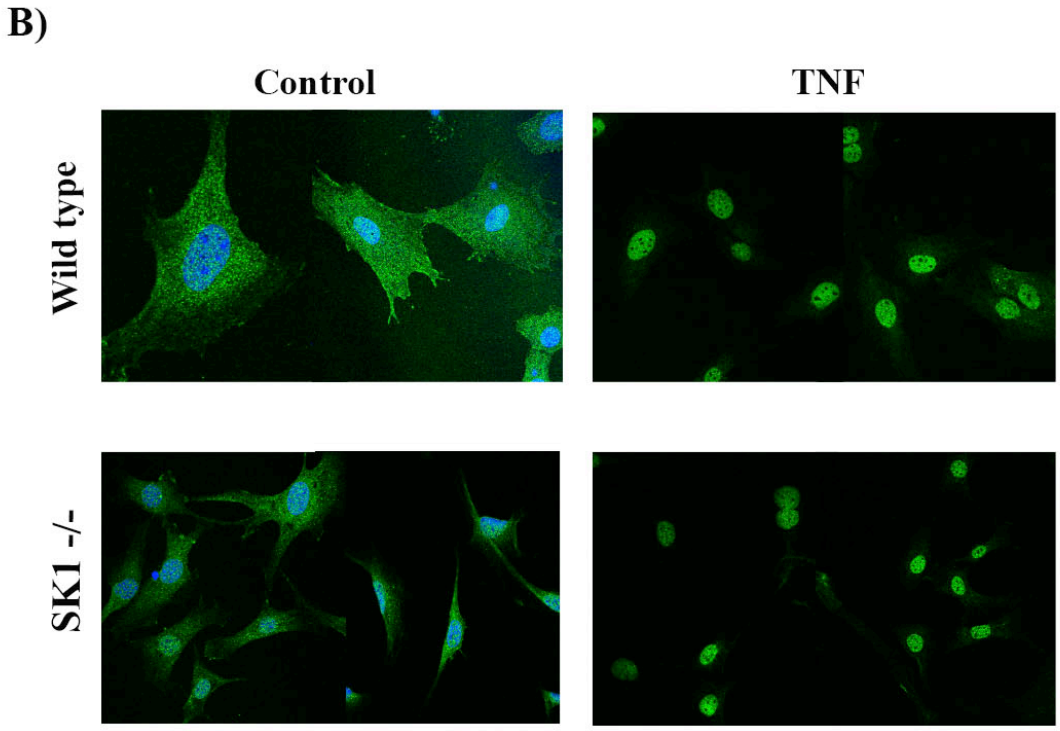


Figure 32 (continued): SK1 is not required for TNF induced nuclear translocation of p65/RelA.

B) WT or $SK1^{-/-}$ MEFs were exposed to TNF (50ng/ml) for 30 min. Cells were fixed, permeabilized and probed with p65 antibody. p65 localization was examined using confocal microscopy. Images are representative of at least 5 fields taken from at least 2 independent experiments.

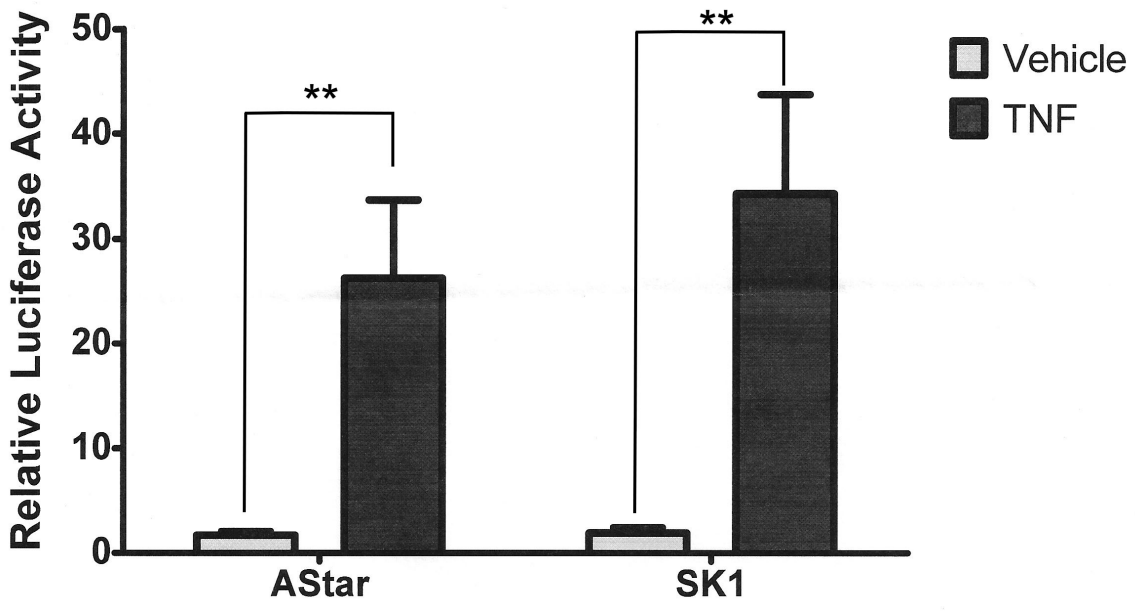


Figure 33: SK1 is not required for transcriptional NF-κB activity following TNF treatment.

HeLa cells were treated with 20nM Astar or SK1 siRNA for 24h prior to co-transfection with LacZ and NF-κB-Luciferase constructs. After 6 hours of transfection, cells were treated with vehicle (PBS) or TNF (20ng/ml) for 18h. Luciferase and galactosidase activities were extracted and assayed as described in 'Experimental Procedures" and measured luciferase activity was normalized to measured galactosidase activity. Data are presented as mean ± SEM of 3 independent experiments.

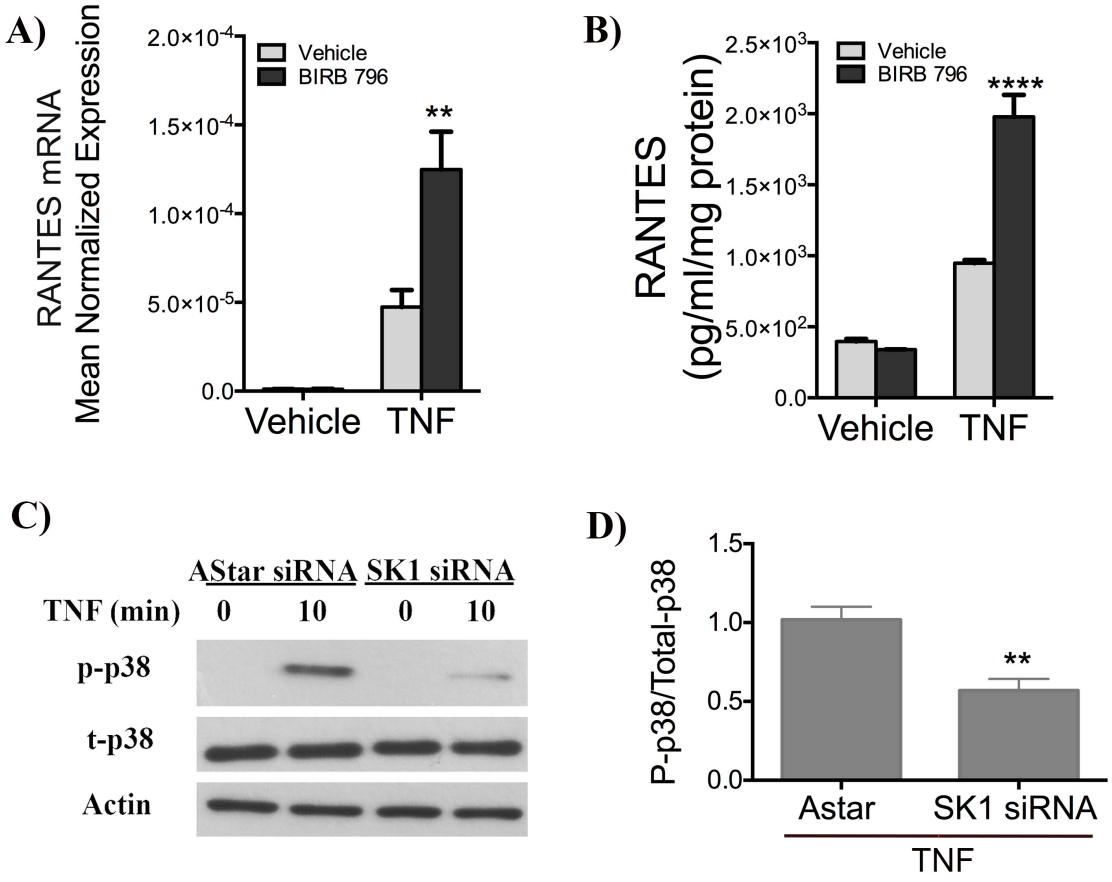


Figure 34: Inhibition of p38 MAPK enhances RANTES induction.

A) HeLa cells were treated with vehicle (DMSO) or the p38 MAPK inhibitor BIRB796 (10μM) for 1h prior to treatment with PBS or TNF (20ng/ml) for 24h. Subsequently, RANTES mRNA levels were assessed by qRT-PCR. Data is presented as mean ± SEM of 3 independent experiments. B) HeLa cells in serum-free media were treated with vehicle (DMSO) or the p38 MAPK inhibitor BIRB796 (10μM) for 1h prior to treatment with PBS or TNF (20ng/ml) for 24h.. RANTES protein levels in the media were assessed by ELISA. Data are shown asmean ± SEM from 2 independent experiments performed in duplicate. C) HeLa cells were treated with 20nM Astar or SK1 siRNA for 48h prior to treatment with vehicle (PBS) or TNF (20ng/ml) for 10 min.. Phospho-p38 and total p38 levels were assessed by immunoblotting with actin as loading. Data is representative of 3 independent experiments. D) The ratio of phopho-p38/total p38 in TNF-stimulated samples was quantified using ImageJ Software. Data are shown as mean ± SEM from three independent experiments. (**p<0.01, **** p<0.0001)

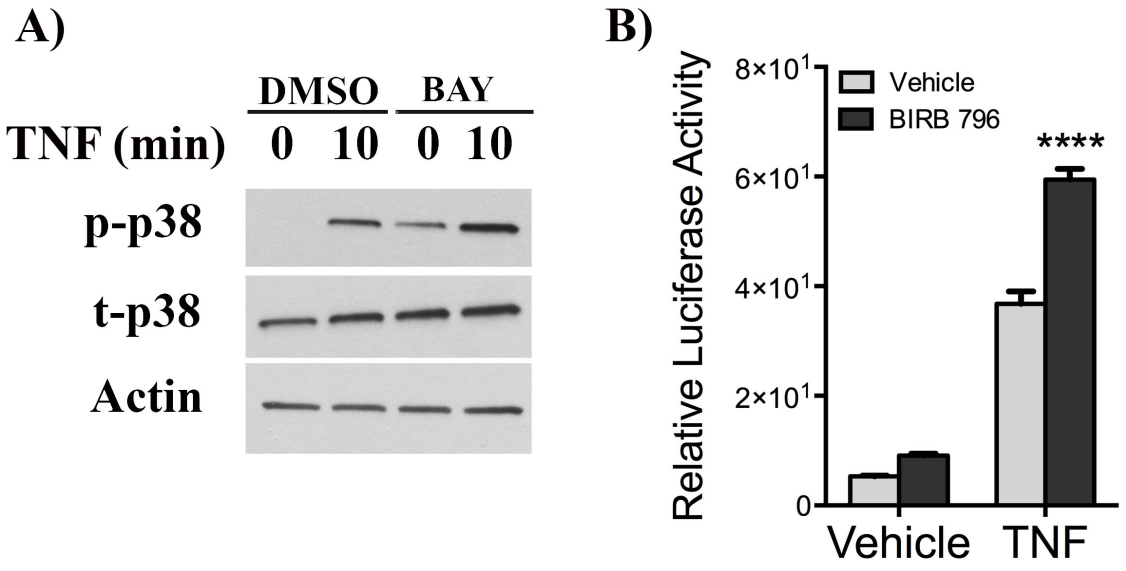


Figure 35: p38 MAPK and NF-κB pathways crosstalk in the TNF response.

A) HeLa cells were treated with DMSO or BAY (5uM) for 30 min prior to stimulation with vehicle (PBS) or , TNF (20ng/ml) for 10 min. Phospho-p38 and total p38 levels were assessed by immunoblotting with actin as loading control. Data are shown as mean ± SEM from three independent experiments B) HeLa cells were co-transfected with LacZ and NF-κB-Luciferase constructs for 6 hours, pre-incubated with vehicle (DMSO) or BIRB796 (10μM) for 1h prior to stimulation with PBS or TNF (20ng/ml) for 18h, Luciferase and galactosidase activities were extracted and assayed as described in 'Experimental Procedures" and measured luciferase activity was normalized to measured galactosidase activity. Data are presented as mean ± SEM of 3 independent experiments**.** (**** $p<0.0001$)

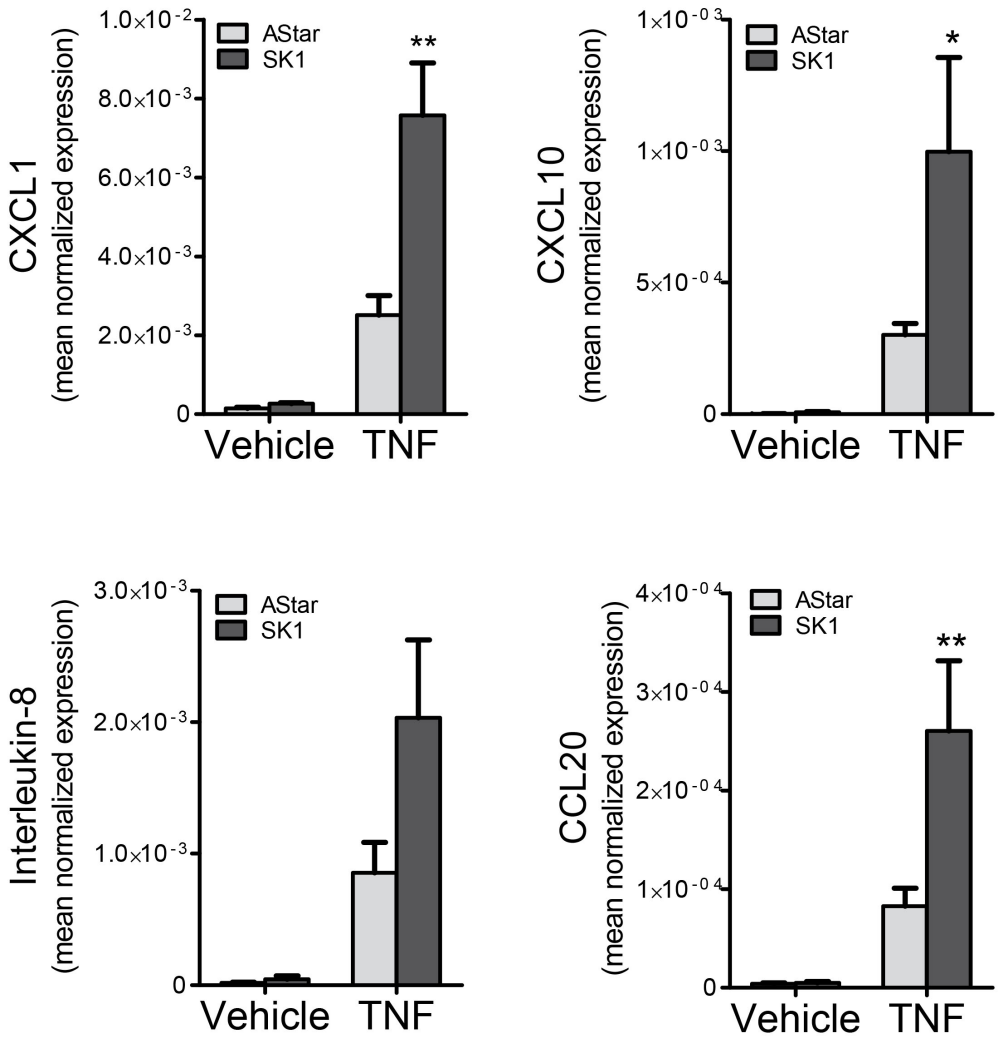


Figure 36: Loss of SK1 enhances the levels of multiple chemokines and cytokines.

HeLa cells were treated with 20nM Astar or SK1 siRNA for 48h prior to treatment with vehicle (PBS) or TNF (20ng/ml) for 24h. RNA was extracted and the levels of CXCL1, CXCL10, IL-8 and CCL20 mRNA were assessed by qRT-PCR. Data are presented as mean ± SEM from 3 independent experiments. (*$p<0.05$, ** $p<0.01$)

Supplemental Figures

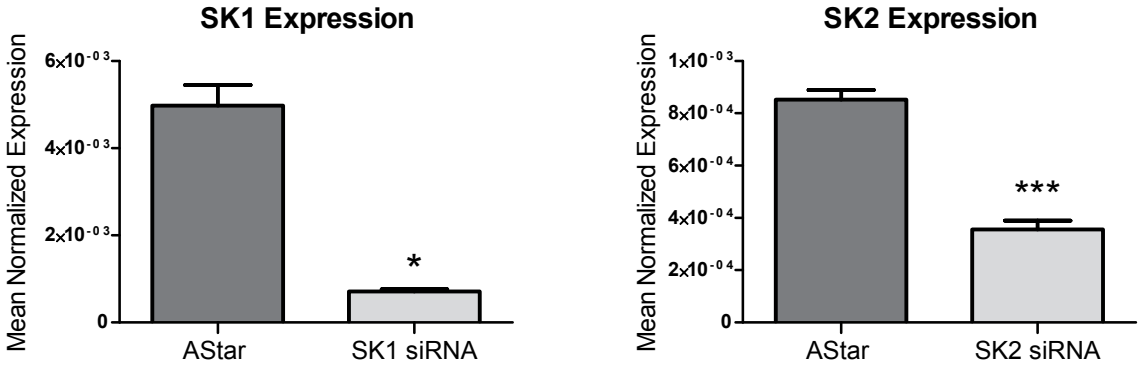


Figure S1: Verification of SK1 and SK2 knockdown.

HeLa cells were treated with 20nM AStar, SK1 siRNA or SK2 siRNA for 48 hours, and SK1 mRNA and SK2 mRNA were assessed by qRT-PCR.

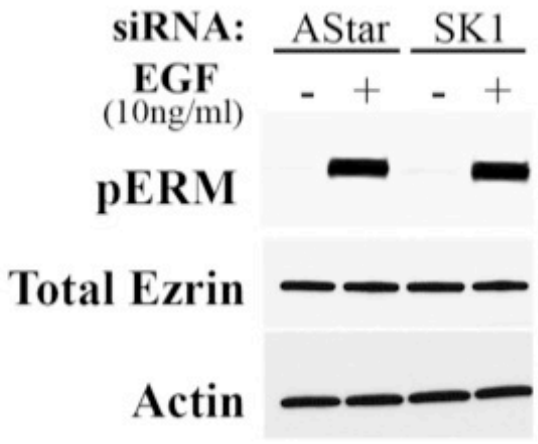


Figure S2: SK1 is not involved in EGF-induced ERM phosphorylation.

HeLa cells were treated with AStar or SK1 siRNA for 48 hours. Cells were then starved for 4 hours prior to treatment with EGF (10ng/ml) for 5 minutes. pERM levels were then assessed by immunoblotting. Total ezrin and actin were also included as loading controls.

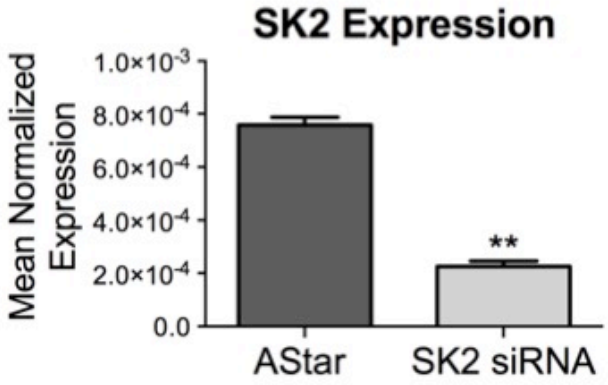


Figure S3: Verification of SK2 knockdown.

HeLa cells were treated with 20nM AStar or SK2 siRNA for 48 hours, and SK2 mRNA were assessed by qRT-PCR.

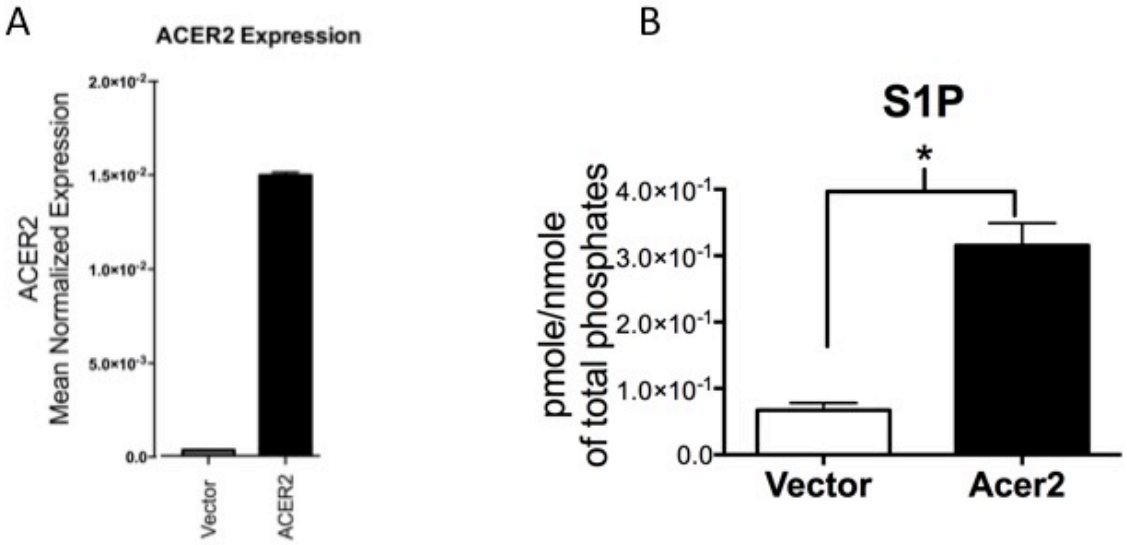


Figure S4: ACER2 and S1P levels in ACER2 overexpressing cells.

A) mRNA of HeLa cells overexpressing empty vector or ACER2 were assessed by qRT-PCR. B) Cellular lipids were directly extracted in organic solvents from HeLa cells overexpressing empty vector or ACER2. S1P levels were analyzed by tandem LC/MS mass spectrometry.

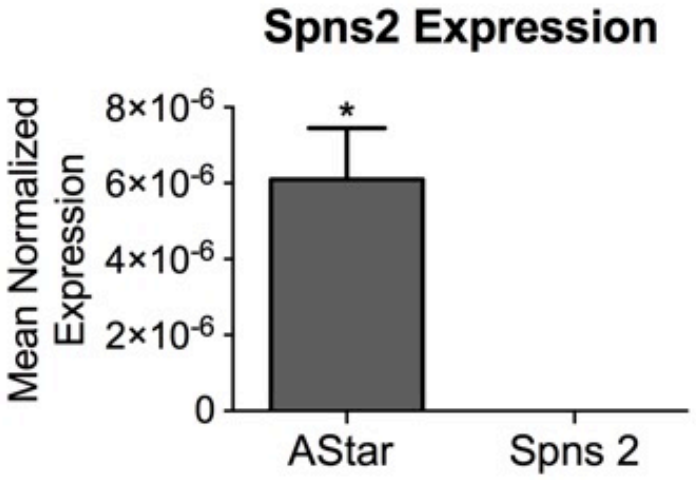


Figure S5: Verification of Spns2 Knockdown.

HeLa cells were treated with 20nM AStar or Spns2 siRNA for 48 hours, and Spns2 mRNA were assessed by qRT-PCR.

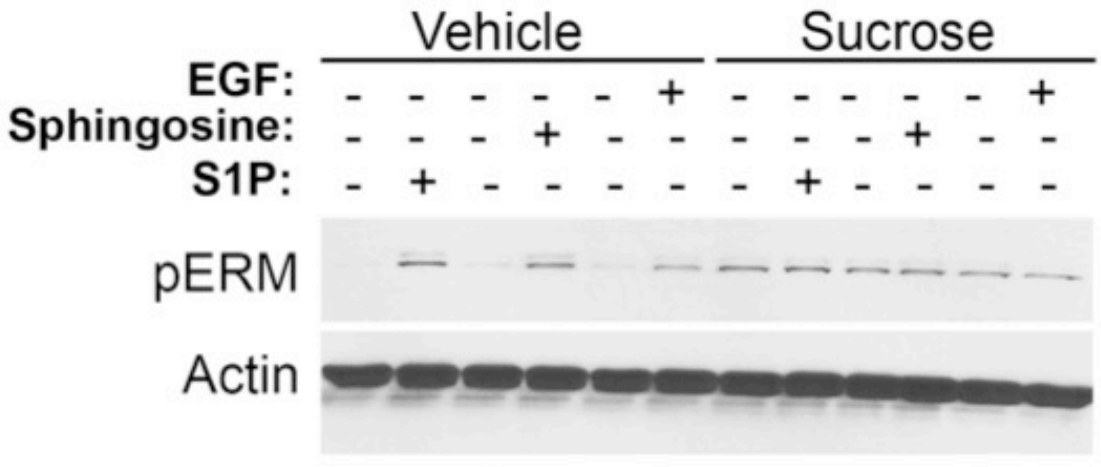


Figure S6: Cellular trafficking is necessary for EGF-induced ERM phosphorylation.

HeLa cells were pretreated for half an hour with either PBS or 300 mM sucrose prior to treatment with EGF (10ng/ml), or sphingosine (5μM), or S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. Actin was also included as a loading control.

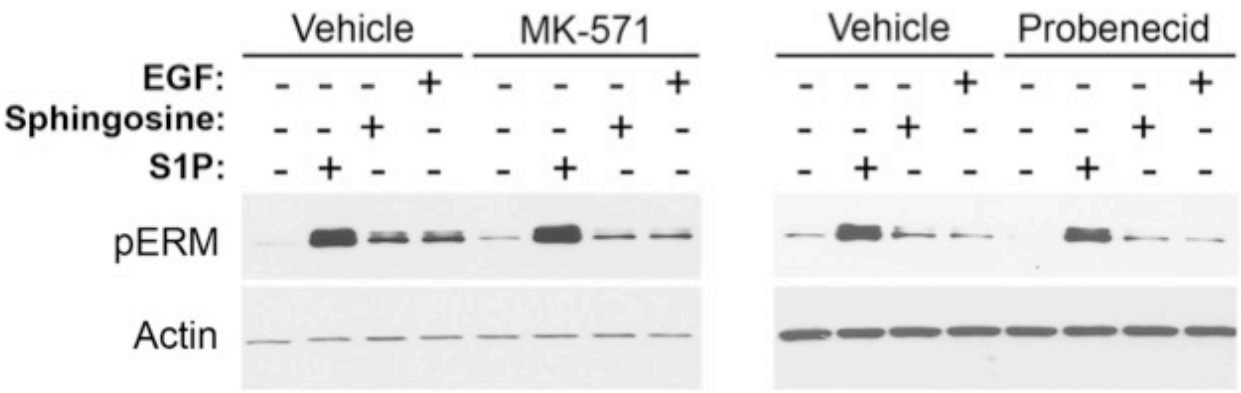


Figure S7: ABC family of transporters is partially required for EGF-induced ERM phosphorylation.

HeLa cells were pretreated for an hour with either vehicle (H2O for MK-571 and methanol for Probenecid) or 10 μM MK-571 or 10 μM Probenecid prior to treatment with EGF (10ng/ml), or sphingosine (5μM), or S1P (100nM) for 5 minutes. pERM levels were then assessed by immunoblotting. Actin was also included as a loading control.

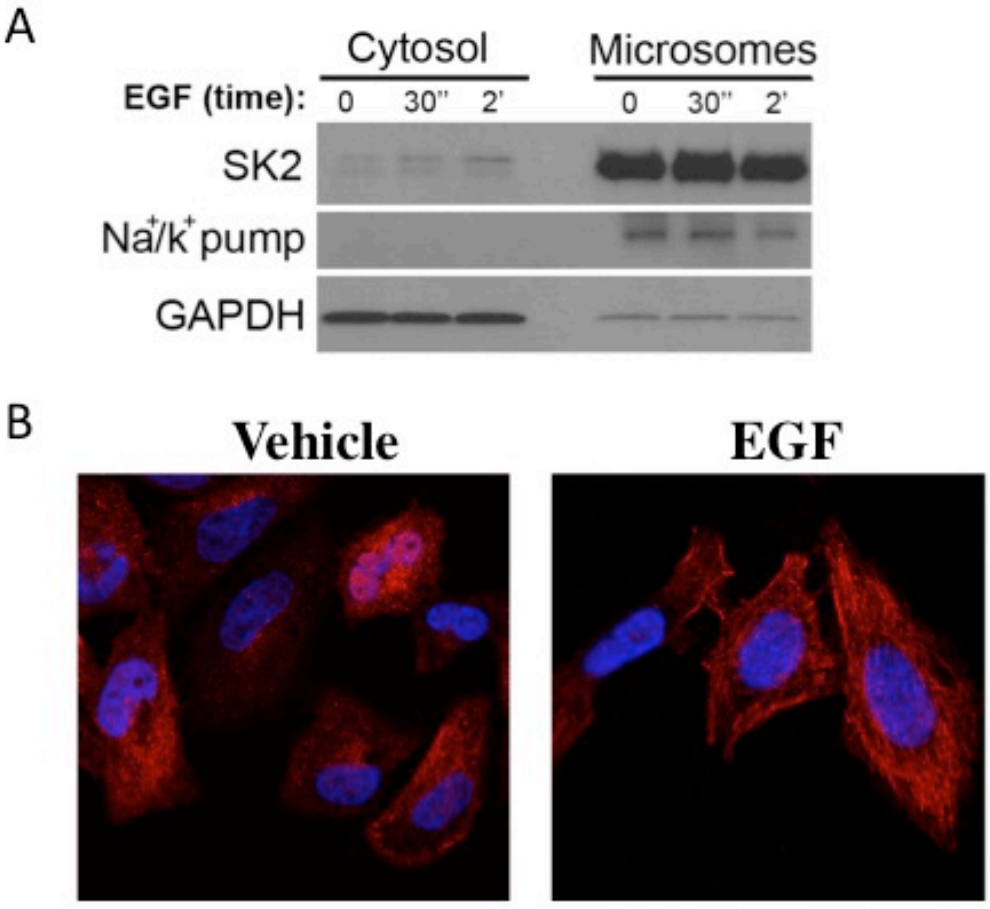


Figure S8: SK2 is a membranous protein that does not localize to the plasma membrane.

A) HeLa cells were transiently transfected with SK2-MYC for 24 hours. Cells were then starved, and treated with 10 ng/mL EGF for 30 seconds and 2 minutes. SK2 localization was then assessed by performing cellular fractionation followed by western blotting. Na^+/K^+ pump was used as a marker for membranous fractions, and GAPDH was used as a cytosolic marker. B) HeLa cells overexpressing SK2-MYC were fixed, and SK2 localization was visualized using confocal microscopy. SK2 was shown in *red,* and the nuclei stained with DAPI were shown in *blue*.

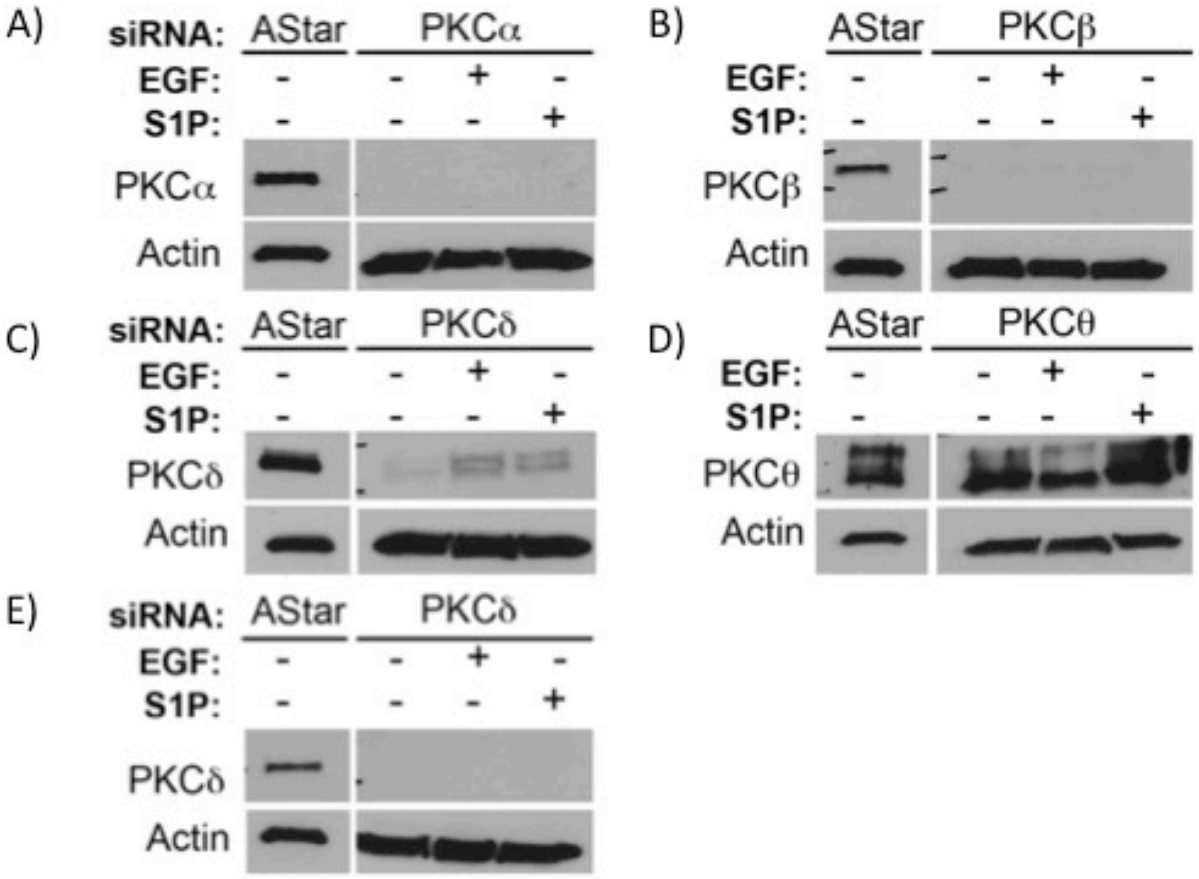


Figure S9: Verification of PKCs knockdown.

HeLa cells were treated with AStar siRNA, PKCa siRNA, PKCbII siRNA, PKCd siRNA, PKCq siRNA, PKCe siRNA for 48 hours. Cells were then starved for 4 hours prior to treatment with S1P (100 nM) or EGF (10 ng/mL) for 5 minutes. PKCa, PKCbII, PKC d, PKCq and PKCe levels were then assessed by western blotting to verify knockdown.

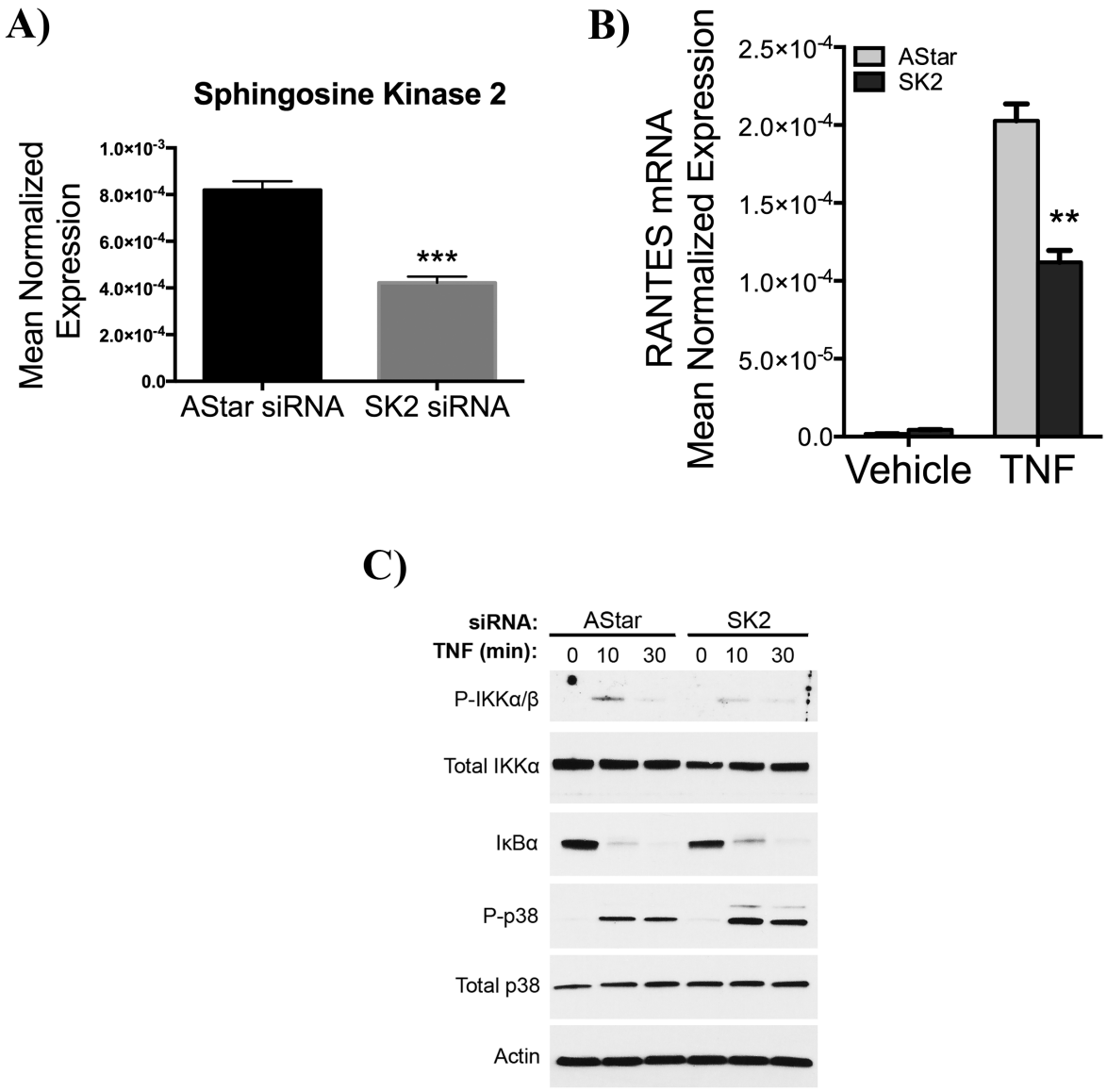


Figure S10: Effect of SK2 on RANTES induction, acute NF-kB activation and p38 MAPK phosphorylation.

A) 48 hours after treatment with either Astar or SK2 siRNA, Hela cells were treated with 20 ng/ml TNF for 24 hours and the levels of RANTES mRNA assessed by realtime-RT-PCR. Data represent mean ± SEM from 3 independent experiments. B) HeLa cells were exposed to AStar siRNA or SK2 siRNA for 48 hours then exposed to 20 ng/mL TNF for 0, 10 or 30 minutes. Early downstream signals of TNF including IKKα/β phosphorylation, IκBα degradation were examined via western blot. Phospho-p38 was also assessed. Actin and total protein were included as loading controls. Each blot is representative of n=2 independent experiments.

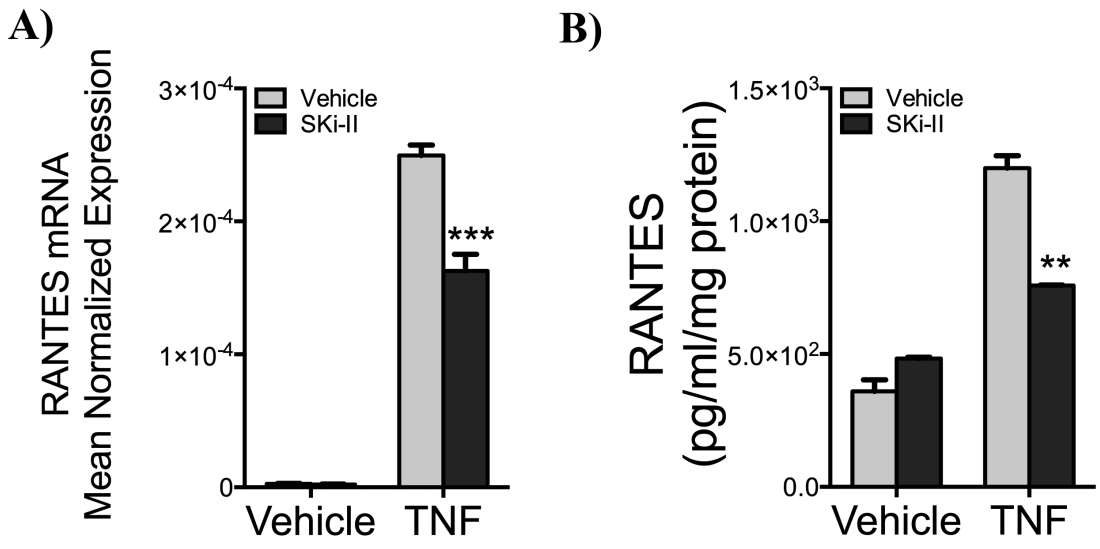


Figure S11: Effect of the sphingosine kinase inhibitor Ski-II on RANTES mRNA and protein levels.

A) Hela cells were incubated with the 10uM of the sphingosine kinase inhibitor Ski-II overnight or its vehicle DMSO, then treated with PBS or 20ng/ml TNF for 24 hours. RANTES mRNA levels were assessed by real time-RT-PCR. This data represents mean ± SEM of n=3 independent experiments. B) RANTES protein levels were determined in HeLa cells treated 10uM of the sphingosine kinase inhibitor Ski-II overnight or its vehicle DMSO, prior to 24 hours treatment with PBS (vehicle) or TNF (20ng/ml). Data represent mean ± SEM, of 2 independent experiments done in duplicates.

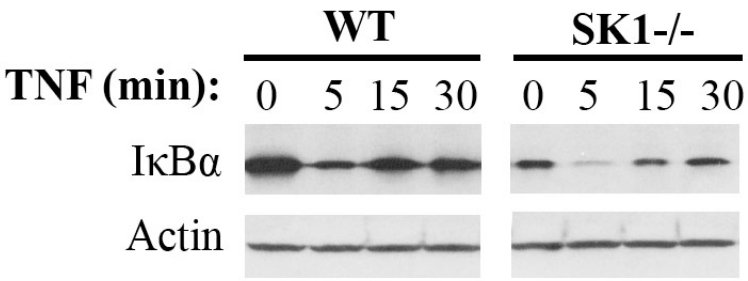


Figure S12: SK1 is not necessary for TNF-mediated activation of NF-κB in MEFs derived from a different clone than those presented before.

MEFs derived from WT or SK1$^{-/-}$ mice were treated with 4nM TNF for 0, 5, 15, or 30 minutes, and IκBα levels were assessed via western blot with actin as a loading control; blots are representative of n=3 independent experiments.

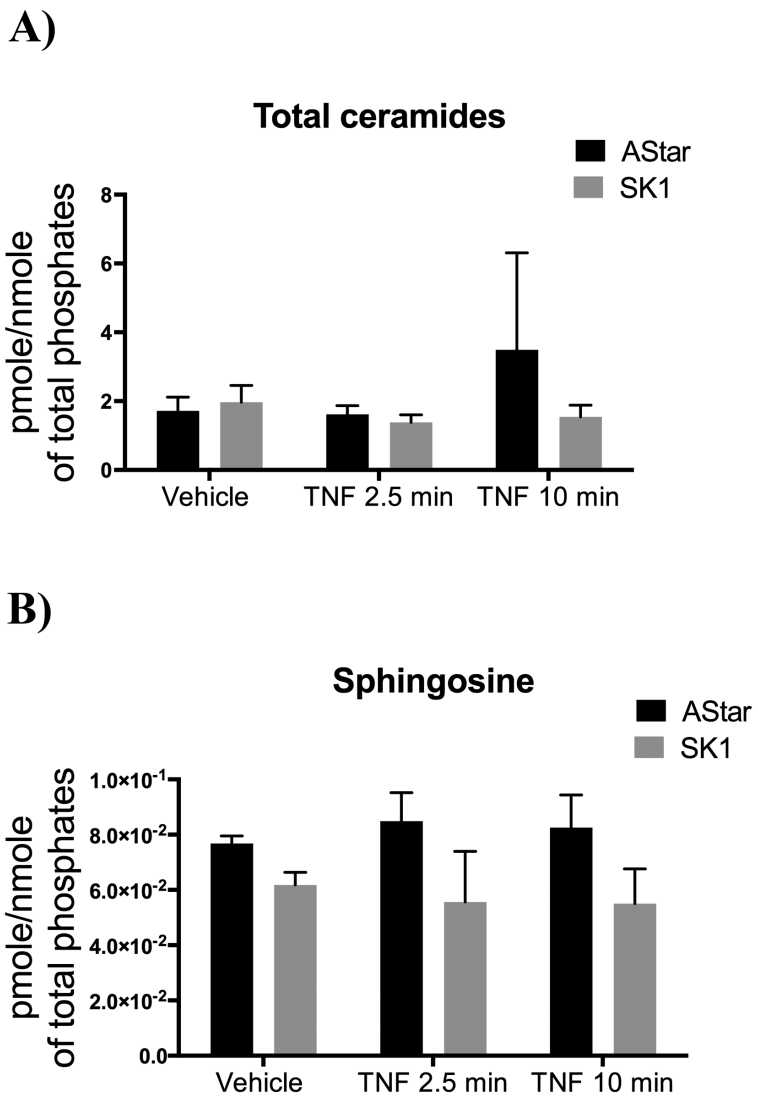
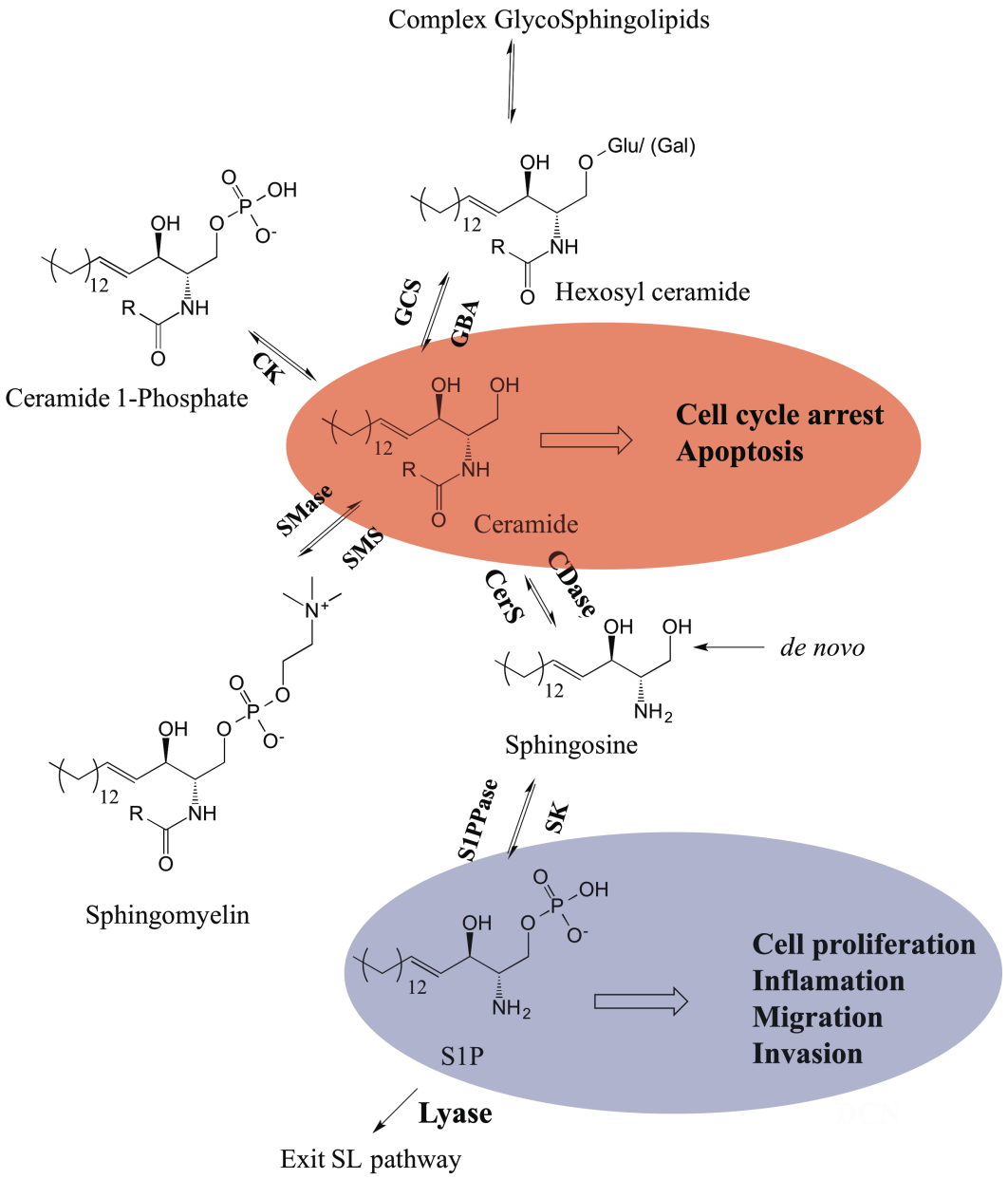


Figure S13: Effect of TNF on total ceramides and sphingosine in HeLa cells lacking SK1.

A) Total ceramides levels and B) sphingosine level were determined in HeLa cells treated with 20 nM control AStar siRNA or SK1 siRNA for 48 hours prior to 2.5 min and 10 minutes treatment with PBS (vehicle) or TNF (20 ng/ml). Cellular lipids were determined following direct organic solvent extraction and were normalized to nmol of total phosphates as described in the methods section of the manuscript. Data represent mean ± SEM, of 2 independent experiments done in duplicates.

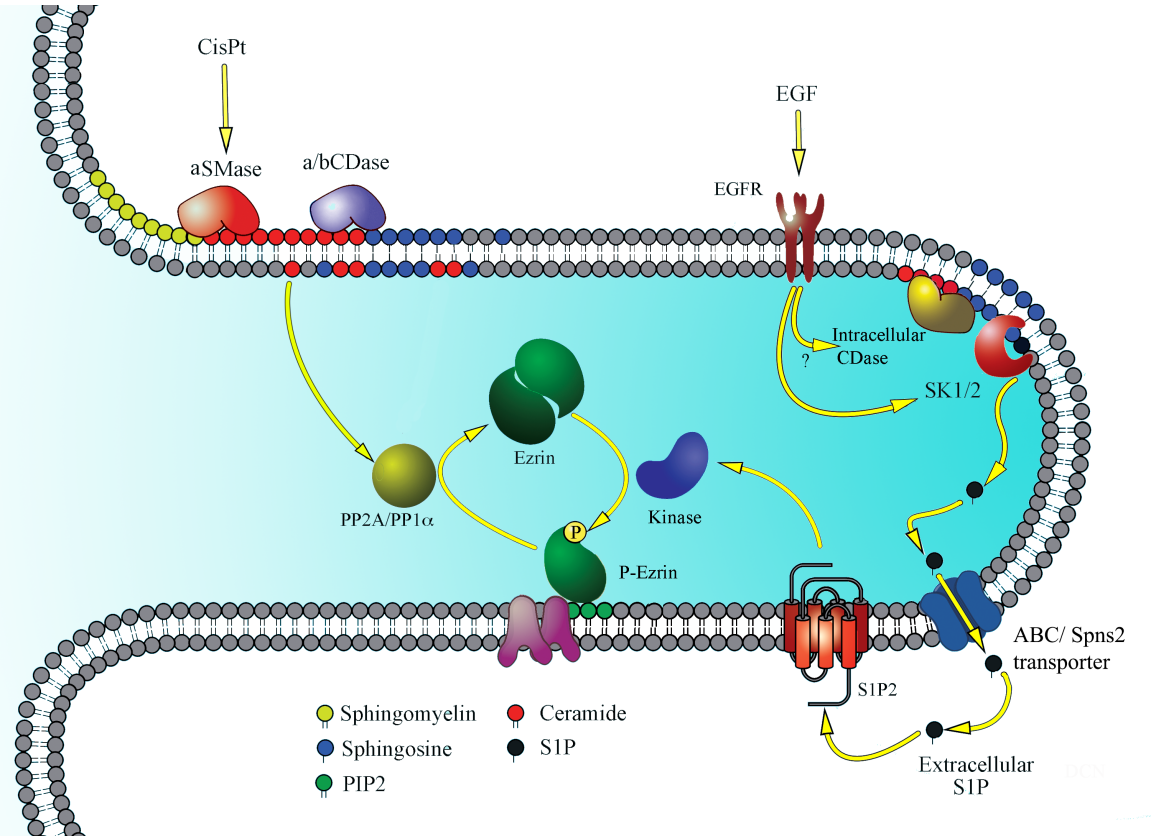
Schemes



Scheme 1: Sphingolipid metabolic pathway.

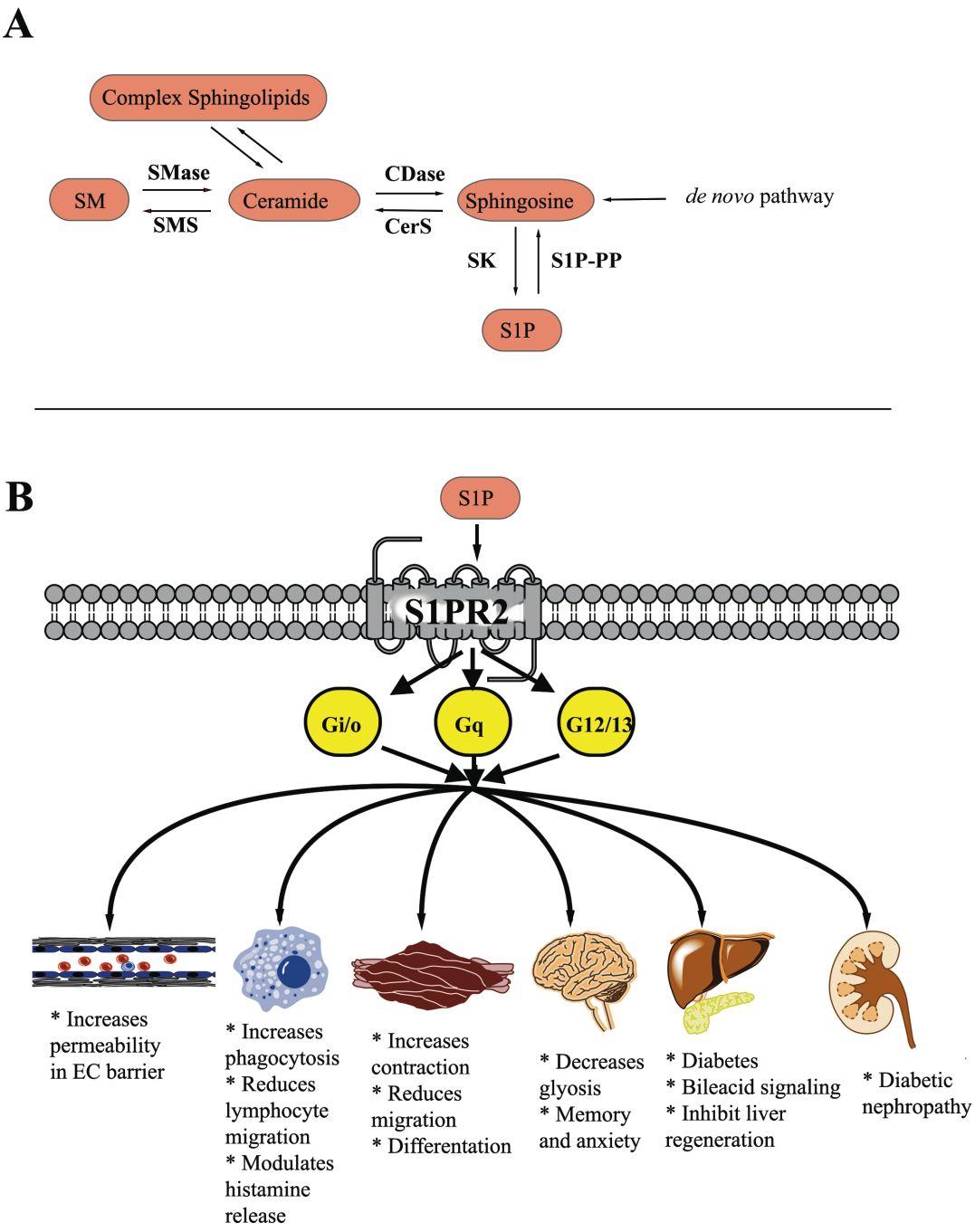
This figure illustrates the sphingolipid metabolic pathway described in the text along with the structure of each product. It emphasizes the opposing roles played by two major bioactive products of sphingolipid metabolism, ceramide and sphingosine-1-phosphate. While ceramide is known to play a role in cell cycle arrest and apoptosis, S1P is known to be involved in promoting cell proliferation, inflammation, migration, and invasion.

Glu (Glucose), Gal (Galactose), GCS (Glucosylceramide Synthase), GBA (Glucocerebrosidase), CK (ceramide kinase), SMS (sphingomyelin synthase), SMase (sphingomyelinase), CDase (Ceramidase), CerS (Ceramide Synthase), SK (sphingosine kinase), S1PPase (S1P Phosphatase).



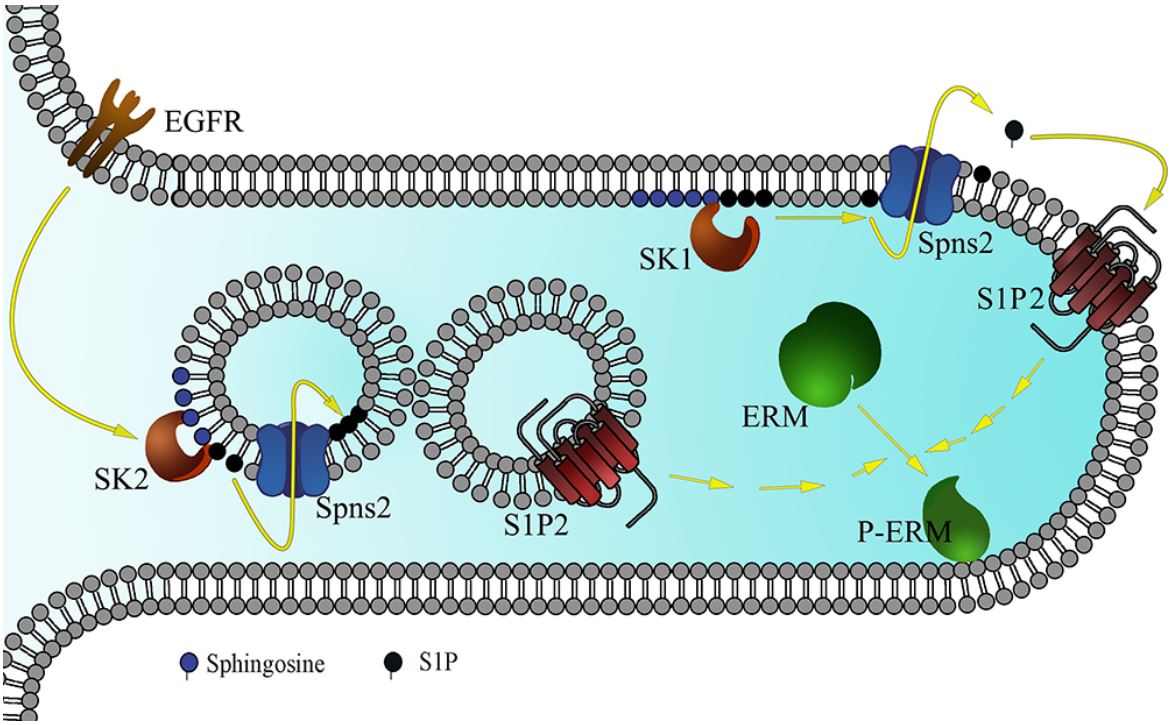
Scheme 2: Sphingolipid regulation of ERM activation.

This figure illustrates the mechanism of ERM regulation by sphingolipids on the tip of a cellular extension. Cisplatin causes activation of the acid sphingomyelinase (aSMase), which causes ceramide production. Ceramide activates protein phosphatase 2A (PP2A) to cause ezrin dephosphorylation and detachment from the cell membrane. Alternatively, the use of bacterial sphingomyelinase causes activation of protein phosphatase 1 alpha (PP1a) to cause ezrin dephosphorylation. In contrast, S1P, generated after bacterial ceramidase use (bCDase) or after EGF treatment translocates to the extracellular space using one of the described transporters such as the ABC transporter to activate sphingosine-1-phosphate receptor 2 (S1P2). S1P2 signaling activates a yet unknown kinase to cause ezrin phosphorylation and binding to the plasma membrane using phosphatidylinositol biphosphate (PIP2).



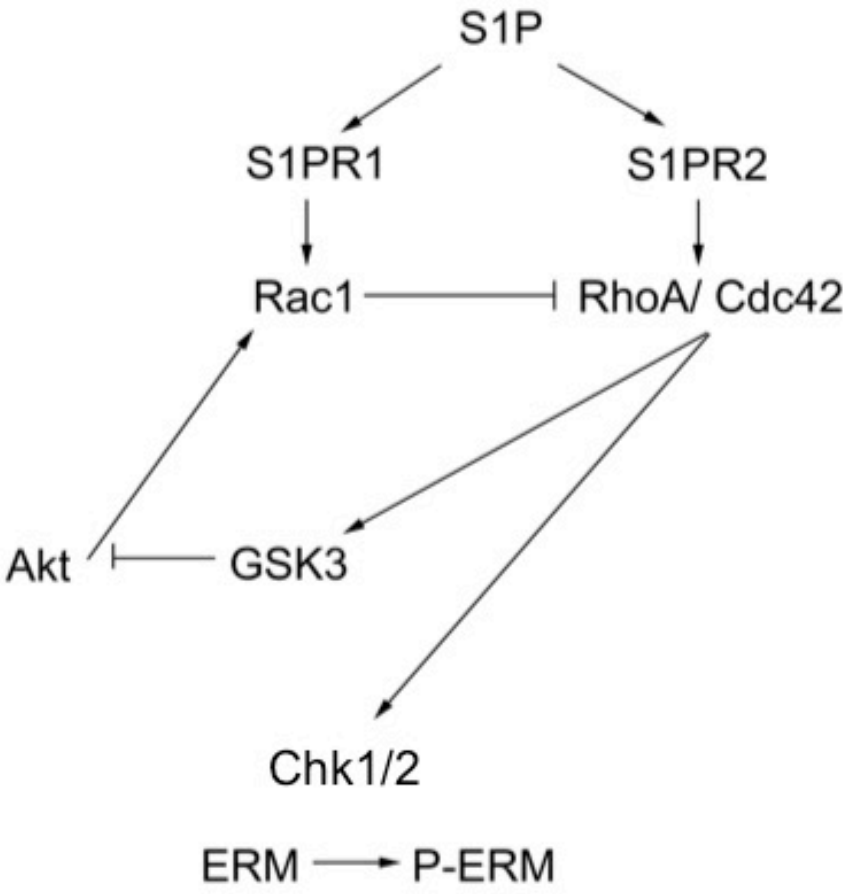
Scheme 3:

A) Schematic representation of the sphingolipids metabolic pathway. B) The different biological functions downstream of S1PR2.

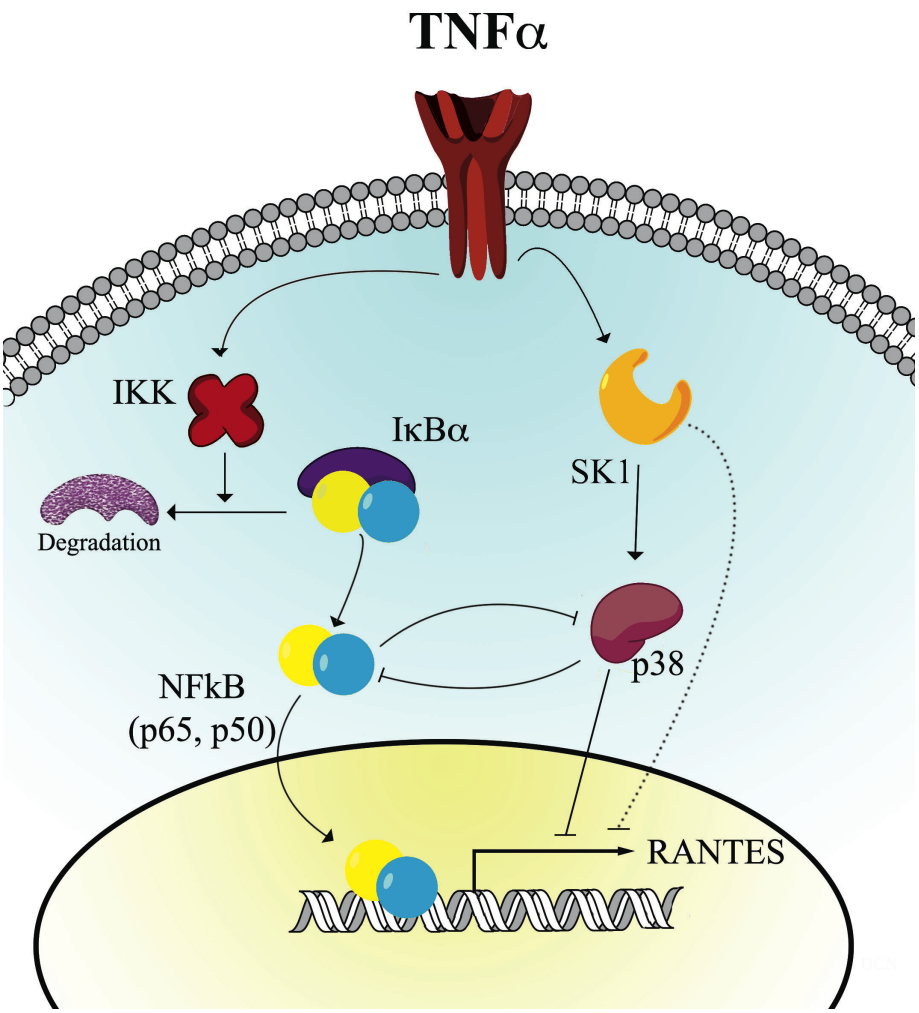


Scheme 4: Schematic representation of the proposed model for EGF-induced ERM phosphorylation.

EGF treatment activates SK2 localized on the cytosolic side of intracellular vesicles. This will lead to S1P being produced and transported by Spns2 (as well as other ABC transporters) to the inner side of these vesicles. These vesicles will then fuse with S1PR2 containing vesicles or with the plasma membrane. This is followed by S1PR2 activation and ERM phosphorylation, and filopodia formation as shown in this cellular extrusion. On the other hand, sphingosine treatment causes S1P production by SK1 localized on the plasma membrane. S1P will then be exported to the extracellular milieu by Spns2, leading to S1PR2 activation and ERM phosphorylation.



Scheme 5: Summary of the signaling pathway downstream of S1P leading to ERM phosphorylation.



Scheme 6: Proposed model of RANTES regulation by SK1.

TNF stimulation leads to activation of SK1 and S1P generation that, in turn, mediates activation of p38 MAPK. Activation of this pathway suppresses RANTES transcription through an as yet unknown repressor factor. Concurrently, TNF activates the NF-κB pathway independently of SK1, which causes transcriptional upregulation of RANTES expression and a simultaneous inhibition of p38 MAPK activation. It should also be noted that we cannot rule out the possibility that SK1 has effects on RANTES transcription independently of the p38 MAPK pathway.

Tables

P-site	Kinase	Stimulus	Cell line	Function	Ref
Thr567	PKC	Amphetamine	PC12 cells	Synaptic plasticity	(*50*)
		Dinitro-sopiperazin	NPC cells	Motility, invasion, metastasis	(*92*)
		N/A	Osteoscarcoma cells	Migration and invasion	(*85, 93*)
		2-methoxy-estradiol	HPAEC cells	Hyper-permeability	(*76*)
		Glutamate	Hippocampal cells	Filopodia and synaptic boutons formation	(*51*)
		Latent membrane protein-1 expression	NPC cells	Motility and invasion	(*6*)
		N/A	Intestinal epithelial cells	Epithelial differentiation	(*310*)
		Hypotonicity	Collecting duct epithelial cells	Cytoskeletal remodeling	(*315*)
		Androgen	LNCaP-FGC	Invasion	(*307*)
		Parathyroid Hormone	Oposum Kidney cells	Endocytosis	(*309*)
		N/A	2C4 Fibrosarcoma	Migration	(*308*)
	Rho/ROCK	Netrin	Cortical Neurones	Axonal growth	(*49*)
		Estrogen	T47-D Breast cancer cells	Migration and invasion	(*91*)
		LPA	Ovarian Cancer	Migration and metastasis	(*313*)
		N/A	Hepatoma cells	Invasion	(*90*)
		Cisplatin	HT29 cells	Apoptosis	(*100*)
		N/A	NPC cells	Metastasis	(*345*)
		Fas receptor activation	Jurkat T cells	Apoptosis	(*346*)
		TNF	Endothelial cells	Inhibition of proliferation	(*314*)
		Net and Dbl overexpression	NIH3T3 fibroblasts	Malignant transformation	(*347*)
	P38 MAPK	TNF	Synoviocytes	Proliferation	(*63*)
		Advanced glycation end products	Endothelial cells	Hyper-permeability	(*78*)
	GRK-2	Wound closure	MDCK cells	Migration	(*303*)
		Angiotensin-II	Kidney	Actin turnover	(*311*)

			Podocytes	and migration	
		Acetylcholine	Hep2 and HEK293 cells	Membrane ruffles formation and internalization	(*312*)
	NIK	EGF, PDGF	MTLn3 cells	Lamellipodia formation	(*305*)
	AKT2	Glucose	Caco-2BBe cells	Translocation of NHE-3 to plasma membrane	(*304*)
	LRRK2	N/A	Primary Neurons	Retardation of growth and increased filopodia	(*52*)
	Mst-4	N/A	W4 cell line	Apical brush border formation	(*348*)
	LOK	N/A	Lymphocytes	Decreased migration and polarization	(*306*)
	Slik	Mitosis initiation	Drosophila S2 cells	Cortical microtubular stability	(*45*)
	MRCK	Cdc42 overexpression	NIH3T3 fibroblasts	Filopodia Formation	(*349*)
Tyr145 and/or 343 and/or 477	**Syk**	CD81 engagement	B lymphocytes	Cell re-organization and signaling	(*350*)
	Flt3	Kit overexpression	Pro-erythroblasts	Apoptosis resistance and proliferation	(*351*)
	Src	EGF	A431 cells	Spreading and proliferation	(*352*)
		ICAM1 ligation	Endothelial cells	N/A	(*353*)
		N/A	Hek239	Association with KBTBD2	(*354*)
	Lck	N/A	Jurkat T cells	Signaling during T cell activation	(*355*)
Thr235	**Cdk5**	Overexpression of Retinoblastoma	Osteosarcoma	Cell senescence and flat cell morphology	(*356-358*)
Ser66	**PKA**	Histamine	Gastric Parietal cells	Acid secretion	(*55, 56*)

Table 1: The kinases known to phosphorylate ERM proteins and their functional consequences. (N.B. 1 — The ERM column refers to the specific ERM member that is phosphorylated by the corresponding kinase, 2 — the numbering of the phosphorylation sites reflects their position in the original ERM sequence; this might slightly differ in the original manuscripts as some authors use their tagged constructs for numbering).

S1PR	Human chromosomal location	Distribution		Reference
		Tissue	Cellular	
S1PR1 (EDG1)	1p21	Most tissues, highest in CD19+ B cells and cerebellum	Plasma membrane, caveolae, cytoplasmic vesicles, nucleus, perinuclear region	(*39, 359*)
S1PR2 (EDG5)	19p13.2	Most tissues	Plasma membrane, cytoplasm	(*39*)
S1PR3 (EDG3)	9q22.1-q22.2	Highest in heart, lung, spleen, kidney, intestine, diaphragm	Plasma membrane	(*39, 198*)
S1PR4 (EDG6)	19p13.3	Highly expressed in lymphoid tissues and blood cells, especially CD19+ B cells, and lung	N/A	(*39, 360*)
S1PR5 (EDG8)	19p13.2	Mostly in Brain and skin and NK cells	N/A	(*360, 361*)

Table 2: Distribution of the different S1P receptors

S1PR KO embryo	Lethality	Phenotype	Ref
S1PR1 -/-	Normal up to E11.5 All die before E14.5	• Abnormal yolk sacs, edematous, lack blood • Normal vasculature • Intra-embryonic bleeding • Underdeveloped, rounded limbs • Defective blood vessel maturation due to defective vascular smooth muscle migration and enforcement of vessels walls	(*362*)
S1PR2 -/-	Normal at birth 14% die between week 3 and 7 due to lethal seizures	• Sporadic and spontaneous seizures between week 3 and 7 • Increased excitability of the pyramidal cortical neurons • Decreased renal and mesenteric vascular resistance • Deaf by one month of age due to vasculature disturbances within the stria vascularis	(*298, 363, 364*)
S1PR3 -/-	Normal Smaller litter size	• Normal	(*365*)
S1PR4 -/-	Normal	• 9% of megakaryocytes have aberrant non-grained cytoplasm • Aberrant terminal megakaryocyte maturation but not affecting bleeding time	(*366*)
S1PR5 -/-	Normal	• Aberrant NK cell homing and migration to site of inflammation • Lack of Ly6C- patrolling monocytes in the periphery	(*361, 367*)

Table 3: Lethality and phenotype of the S1P receptors knockout mice.

Expected lung metastatic lesions	Scrambled control shRNA	SK2 shRNA or S1PR2 shRNA
Co-expression (none)	+++	+
Co-expression ezrin T567A	+	+
Co-expression ezrin WT or T567D	++++++	+++++

Table 4: Expected metastatic lesions resulting from B16F10 tail vein injections.

	Fold increase upon TNF treatment with respect to Astar non-treated cells	
Cytokine/Chemokine	**Astar**	**SK1 siRNA**
ADIPOQ	1.52	1.75
BMP2	1.52	-1.13
BMP4	-1.33	-2.28
BMP6	1.51	1.74
BMP7	-1.33	3.55
C5	-1.26	-2.21
CCL1	-1.32	3.38
CCL11	3.04	7.05
CCL13	3.00	3.45
CCL17	-2.63	1.76
CCL18	3.00	6.95
CCL19	-1.33	1.75
CCL2	3.00	3.50
CCL20	11.99	27.63
CCL21	1.52	1.71
CCL22	6.08	6.91
CCL24	3.00	3.48
CCL3	1.50	1.74
CCL5	49.32	113.61
CCL7	1.51	3.45
CCL8	1.51	3.48
CD40LG	-1.33	1.75
CNTF	3.02	1.75
CSF1	3.04	7.05
CSF2	3.02	7.00
CSF3	5.91	3.45
CX3CL1	1.51	1.70
CXCL1	48.30	110.51
CXCL10	193.21	890.21
CXCL11	96.60	442.03
CXCL12	-2.69	1.74
CXCL13	1.52	-1.14
CXCL16	3.02	13.72
CXCL2	23.98	55.64
CXCL5	1.49	-1.15
CXCL9	1.52	1.74
FASLG	23.98	3.48
GPI	1.51	-1.17
IFNA2	1.51	1.76
IFNG	1.55	1.78
IL10	1.52	1.73
IL11	3.02	3.48
IL12A	3.00	3.45
IL12B	3.02	-1.16
IL13	3.04	1.76

IL15	1.50	1.71
IL16	1.50	1.74
IL17A	-1.33	-1.15
IL17F	2.98	1.73
IL18	1.51	1.74
IL1A	12.08	27.82
IL1B	6.00	13.81
IL1RN	6.04	13.72
IL2	1.49	1.71
IL21	-2.60	1.73
IL22	1.54	7.20
IL23A	3.04	3.45
IL24	1.52	3.53
IL27	-1.34	1.71
IL3	1.51	1.75
IL4	-1.32	-1.14
IL5	1.51	1.73
IL6	12.16	14.10
IL7	3.06	7.00
IL8	97.95	225.66
IL9	-1.33	1.71
LIF	3.04	3.50
LTA	1.52	-1.13
LTB	3.00	6.86
MIF	1.50	1.71
MSTN	3.30	3.50
NODAL	1.52	-4.93
OSM	1.50	-1.16
PPBP	-1.33	-1.13
SPP1	-1.33	1.73
TGFB2	1.50	1.74
THPO	1.49	1.74
TNF	12.08	27.82
TNFRSF11B	2.98	3.48
TNFSF10	3.02	13.81
TNFSF11	3.06	3.48
TNFSF13B	5.95	27.44
VEGFA	1.51	1.75
XCL1	5.87	-1.25
ACTB	-1.29	-1.13
B2M	1.53	1.75
GAPDH	-1.35	-2.33
HPRT1	1.50	1.73
RPLP0	-1.32	-1.15
HGDC	2.90	-1.18

Table 5: Selected Cytokines and Chemokines fold changes upon TNF treatment of the array done on AStar or SK1 siRNA transfected cells.

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Appendix

Sphingosine Kinase 1 (SK1) regulates Tumor Necrosis Factor (TNF)-mediated RANTES Induction Through p38 MAPK but Independently of NF-κB Activation

Synopsis

This chapter focuses on the role played by SK1 in inflammation and the regulation of the chemokine response in tumor cells. Although it is distinct from the previous chapters, as it does not deal with the ERM proteins, yet it highlights the opposing functions played by SK1 versus SK2.

Abstract

Sphingosine kinase 1 (SK1) produces the pro-survival sphingolipid sphingosine 1-phosphate (S1P) and has been implicated in inflammation, proliferation and angiogenesis. Recent studies identified TRAF2 as an S1P target, implicating SK1 in activation of the NF-κB pathway, but the functional consequences of this connection on gene expression are unknown. Here, we find that loss of SK1 potentiates induction of the chemokine RANTES (also known as CCL5) in HeLa cells stimulated with tumor necrosis factor-alpha (TNF) despite RANTES induction being highly dependent on the NF-κB pathway. Additionally, we find that SK1 is not required for TNF-induced IKK phosphorylation, IkB degradation, nuclear translocation of NF-κB subunits and transcriptional NF-κB activity. In contrast, loss of SK1 prevented TNF-induced phosphorylation of p38 MAPK and inhibition of p38 MAPK, like SK1 knockdown, also potentiates RANTES induction. Finally, in addition to RANTES, loss of SK1 also potentiated the induction of multiple chemokines and cytokines in the TNF response. Taken together, these data identify a potential and novel anti-inflammatory function of SK1 in which chemokine levels are suppressed through SK1-mediated activation of p38 MAPK. Furthermore, in this system, activation of NF-κB is dissociated from SK1 suggesting that the interaction between these pathways may be more complex than currently thought.

A.1 Introduction

Originally considered structural molecules, sphingolipids (SPLs) have emerged as biological mediators in a variety of cellular processes (*1*). As multiple SPLs have bioactive functions, their levels are under tight control by a complex, interlinked network of enzymes. The sphingosine kinases (SK) serve as a key point in the sphingolipid pathway by converting pro-apoptotic sphingosine to pro-survival sphingosine 1-phosphate (S1P) and considerable research has implicated both SKs and S1P in a variety of pathologies including cardiovascular diseases (*368*), ischemia and reperfusion injuries in the brain and kidneys (*190, 369*), inflammatory diseases, and cancer (*370*). Thus, SKs have emerged as potential therapeutic targets.

Currently, two isoforms of SK are known - SK1 and SK2 - with distinct cellular localizations. SK1 is primarily cytoplasmic and translocates to the plasma membrane upon activation (*371*) while SK2 can be found in the endoplasmic reticulum, nucleus and mitochondria (*372*). The generation of S1P by SK1 can exert its signaling effects through two routes. In the first, S1P is exported to the outer leaflet via transporter proteins (*36, 373*), where it can act on any of five identified G-protein coupled receptors (S1PR1-S1PR5) (*39*). Alternatively, S1P is also thought to function as an intracellular signaling molecule acting directly on intracellular targets. One recently identified S1P target is TRAF2 (*374*), an important mediator of NF-κB activation in response to the cytokine tumor necrosis factor (TNF). However, the functional consequences of this proposed connection between SK1 and NF-κB in regulating TNF-mediated gene expression have yet to be fully explored.

TNF is a key player in inflammation and is a major target of therapy in diseases such as rheumatoid arthritis, inflammatory bowel disease, and asthma amongst others. In the inflammatory response, TNF plays a role in the recruitment of circulating immune cells to sites of inflammation by upregulating adhesion molecules (ICAM, VCAM) and inducing the secretion of chemokines such as IL-8, MCP1 and CCL5 (RANTES). Considerable research has shown that TNF mediates pro-survival and pro-inflammatory pathways through activation of NF-κB, c-jun, Ras/ERK, and Akt. However, activation of sphingolipid metabolism is also well established as a central part of TNF signaling (*375-377*). Indeed, recent studies from our laboratory and others have implicated bioactive sphingolipids as important mediators of pro-inflammatory signaling by TNF (*378, 379*); however, the roles of specific enzymes of SPL metabolism and the relevant bioactive lipid mediators are not clear. Defining the specific roles of individual enzymes and specific lipid mediators is not only critical for understanding these pathways, but is also important for delineating candidate therapeutic targets and defining downstream mechanisms.

RANTES induces the chemotaxis of multiple immune cells (*380*), and is secreted by many cell types including fibroblasts (*381*), epithelial cells (*382*), and vascular smooth muscle cells (*383*). High levels of RANTES are associated with several pathologies including atherosclerosis (*384*), various cancers (*385, 386*), diabetes (*387*), glomerulonephritis (*388*), and rheumatoid arthritis (*389*). In contrast, failure to secrete RANTES impairs many T cell functions such as the clearance of viral infections (*390*). Regulation of RANTES is thought to occur primarily at the transcriptional level, as its mRNA is reported to be extremely stable (*391*). However, transcriptional regulation of RANTES is both stimulus and cell-type dependent. In the TNF response, many transcription factors have been implicated in RANTES induction including

NF-κB and IRF1 (*392, 393*), and AP1 and NF-AT in airway epithelial and smooth muscle cells respectively (*394*). Moreover, upstream of transcription factors, other signaling cascades have been implicated in RANTES regulation such as JNK (*395*), ERK (*396*), and p38-MAPK (*397*). More recently, our group identified SPLs as regulators of RANTES through the acid sphingomyelinase/acid ceramidase pathway (*379*). Strikingly, our data indicated that loss of SK1 significantly enhanced RANTES induction. However, the mechanism by which this was mediated was unclear.

Here, we have explored the mechanism by which SK1 regulates RANTES induction. Using HeLa cells as a model system, we find that TNF induction of RANTES is strongly NF-κB dependent yet loss of SK1 strongly up-regulates RANTES independently of effects on NF-kappa B activation. In contrast, SK1 is required for activation of p38 MAPK, and inhibiting p38 MAPK also leads to induction of RANTES, thus suggesting that p38 MAPK is the mediator of SK1 effects on suppression of RANTES. Finally, the effect of SK1 knockdown on RANTES is part of a broader response with several other chemokines responding in a similar manner. Taken together, we conclude that SK1 negatively regulates RANTES induction through activation of p38 MAPK in TNF-stimulated HeLa cells and suggests that it could play a modulatory effect on the immune system that may not necessarily be pro-inflammatory. Moreover, SK1 was dispensable for TNF-induced activation of the NF-κB pathway in this system.

A.2. Material and Methods

A.2.1. Materials

High glucose Dulbecco's modified Eagle's medium (DMEM), RPMI medium-1640, fetal bovine serum (FBS), penicillin-streptomycin, Oligofectamine, and Superscript III First-Strand Synthesis kit were purchased from Invitrogen (Carlsbald, CA). Essentially fatty acid-free bovine serum albumin (BSA), and monoclonal anti-β-actin antibody were from Sigma-Aldrich (St. Louis, MO). Anti-phospho-IKKα/β, anti-total IKKα, anti-total IKKβ, anti-phospho-IκBα, anti-total IκBα, anti-p65 and anti-p50 antibodies were from Cell Signaling Technology (Danvers, MA). Anti-phospho-p38 was from Promega (Madison, WI). Anti-total p38 and HRP-labeled secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Chemiluminescence kit and BCA kit was from ThermoScientific (Suwanee, GA). Draq5 and 488-conjugated secondary antibody were purchased from Alexis Biochemicals (San Diego, CA). SYBR Green and iTAQ mastermixes were purchased from Bio-Rad (Hercules, CA). Human and mouse TNF-α were purchased from PeproTech (Rocky Hill, NJ). SKi-II (4-[4-(4-chloro-phenyl)-thia-zol-2-ylamino]-phenol) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

A.2.2. Cell Culture and siRNA

HeLa and A549 cells were originally purchased from American Type Culture Collection (ATCC, Manassas, VA). DMEM and RPMI were supplemented with 1% penicillin-streptomycin and 10% FBS, and cells were incubated in standard culture conditions: 37˚C, and 5% CO_2. When serum free medium was used, 0.1% BSA, 1% penicillin-streptomycin and 10mM HEPES were

supplemented to DMEM or RPMI. In all cases before treatment with TNF, media were changed on cells to fresh new media. Gene silencing was carried out using siRNA directed against human SK1 (target sequence 5′-AAGGGCAAGGCCTTGCAGCTC-3) and human SK2: Hs_SPHK2_5 FlexiTube siRNA SI00288561 (Qiagen) and with Qiagen all-star siRNA as a negative control. The siRNA directed against p65 was a validated predesigned sequence from Invitrogen. Transfections were carried out using Oligofectamine (Invitrogen) according to the manufacturer's protocol. For siRNA experiments, HeLa cells were seeded into 60 mm plates at ~200K cells per dish and treated with 20nM for 48h prior to stimulation

A.2.3. Generation of Mouse Embryonic Fibroblasts (MEFs)

WT and SK1-/- fibroblasts were obtained as previously described (*398*). MEFs were generated from SK1+/- littermate matings in a C57BL6.129S background. MEFs were isolated from E13.5 embryos. Cells were maintained in high glucose DMEM supplemented with 10% FBS in standard culture conditions. For genotyping, genomic DNA was extracted using DNeasy Blood and Tissue kit (Qiagen), and 2µl of DNA was combined with 1µl each of either SK1 primer 1 and SK1 primer 2 or SK1 primer 1 and the NEO primer. This was combined with 21µl of PCR Platinum SuperMix (Invitrogen) for a total reaction volume of 25µl for PCR. The following primers (10µM) from Integrated DNA Technologies (Coralville, IA) were used for the detection of the wild-type or knockout SK1: SK1 primer 1 5′-GCAGTGACAAA AGCTGCCGAATGCTGATG-3′. PCR was performed on a Biometra Thermocycler T3000 with the following reaction conditions: 95 °C for 0.5 min; 55 °C for 0.5 min; 72 °C for 0.5 min for 40 cycles.

A.2.4. Immunoblotting

Cells were directly lysed in RIPA buffer (purchased as 10x concentrate from Cell Signaling technologies) and sonicated. Protein levels were estimated by BCA assay (Pierce), proteins were normalized, and Laemmli buffer was added accordingly. Samples were boiled for 5-10 min, and proteins were separated via SDS-PAGE (4-15%, Tris-HCl) using the Bio-Rad Criterion system. Proteins were transferred to nitrocellulose membranes and blocked for at least 1 h with 5% nonfat milk in PBS/0.1% Tween 20 (PBS-T). Membranes were incubated with primary antibodies diluted 1:1000 or 1:3000 β-actin at 4°C overnight. Secondary antibody incubation occurred for 1 hour at room temperature at a 1:5000 dilution. Proteins were visualized by enhanced chemiluminescence (Pierce).

A.2.5. Quantitative (Real-time) Reverse Transcriptase-PCR (qRT-PCR)

Extraction of RNA from HeLa cells was performed using Qiagen RNeasy kits in combination with Qiashredders (Qiagen). RNA (1ug) was converted to cDNA using SuperScript III First-Strand Synthesis Systems (Invitrogen), and cDNA was diluted 1:10 with molecular biology grade water (Sigma). Each 25 µL RT-PCR contained a ratio of 12.5: 0.5: 0.5: 6.5 (SYBR

Green: reverse primer, 10uM: forward primer, 10 μM: dH_2O). Using a Bio-Rad iCycler, reactions detecting expression of human genes were carried out as previously described (*379*). Briefly, following 3 minutes at 95ºC, 40 cycles of 10s melt at 95 °C, 45s annealing at 55 °C, and 45s extension at 68 °C were carried out. The following primers sequences were used to detect expression: human β-actin forward (hβ-actin F), 5'-ATTGGCAATGAGCGGTTCC-3' ; hβ-actin reverse (hβ-actin R), 5'-GGTAGTTTCGTGGCCACA-3; hSK1 F, 5'-CTGGCAGCTTCCTTGAACCAT-3'; hSK1 R, 5'-TGTGCAGAGACAGCAGGTTCA-3'; hRANTES F, 5'-GCTGTCATCCTCATTGCTACTG -3'; hRANTES R, 5'-TGGTGTAGAAATACTCCTTGATGTG-3'; mRANTES F 5'-ACTCCCTGCTGCTTTGCCTAC-3', mRANTES R 5'-ACTTGCTGGTGTAGAAATACT-3'; hCXCL1 F, 5'-CGCCCAAACCGAAGTCAT AGCC-3'; hCXCL1 R 5'-TTCCTCCCTTCTGGTCAGTTG-3'; mCXCL1 F 5'-ACCG AAGTCATAGCCACACTCAAGAATG-3'; mCXCL1 R 5'-TCTCCGTTACTTGGG GACACCTTTTAGC-3'; hCCL20 F 5'-AGCAACTTTGACTGCTGTCTTGG-3'; hCCL20 R 5'-AGGAGACGCACAATATATTTCACCC-3'; mCCL20 F 5'-GCGTCTGCTCTTCCTTGCTTTG-3', mCCL20 R 5'-GTCGTAGTTGCTTGCTGCTTCTG; mβ-actin F), 5'-CGGGACCTCACAGACTACCTC-3'; mβ-actin R, 5'-AACCGCTCGTTGCCAATA-3'. Taqman primer/probe mixes for p65, CCXL10 and IL-8 primers were purchased from Invitrogen and were used according to manufacturer protocol. Using Q-Gene software, threshold cycle (C_t) values were normalized to β-actin and displayed as mean normalized expression.

A.2.6. Measurement of RANTES levels in media

The ELISA kit for human RANTES was obtained from R&D Systems (Minneapolis, MN). Measurement of secreted RANTES in serum-free DMEM was done according to the manufacturer's protocol. RANTES levels were normalized to the total amount of proteins determined by BCA assay.

A.2.7. Immunofluorescence and Confocal Microscopy

Laser-scanning confocal and immunofluorescence microscopy analysis were carried out as previously described with minor modifications (*121*). HeLa cells were grown on poly-d-lysine coated 35mm confocal dishes (MatTek Corporation). The following day, cells were deprived of serum overnight prior to being exposed to treatments. Cells were fixed using 3.7% paraformaldehyde, washed with 1X PBS and permeabilized with 0.1% Triton X-100. Cells were blocked in 2% human serum and exposed to primary antibodies (1:500) in 2% serum overnight. Incubation with secondary antibody was performed according to the manufacturer's protocol. Using a LSM510 META confocal microscope (Carl Zeiss, Inc.), photo images were obtained, then analyzed using free downloadable LSM Image Browser Software (www.Zeiss.com).

A.2.8. Cellular Sphingolipids Extraction and Analysis

This procedure was performed as previously described (*121*). Briefly, adherent cells were directly lysed with two ml of 2:3 70% isopropanol/ethyl acetate. After addition of internal standards to the tubes, the samples were centrifuged at 2000xg. The upper phase was transferred to a new glass tube and a new round of extraction was performed on the remaining volume. After combining the two extracts, sphingolipids and inorganic phosphates were measured. Sphingolipid species were identified on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectroscopy. Sphingolipid species were normalized to total lipid phosphates present in the cells after a Bligh and Dyer extraction.

A.2.9. Luciferase assay

Transcriptional activity of NF-κB was measured using an NF-kB consensus sequence upstream of the luciferase reporter gene (Stratagene). Briefly, HeLa cells were plated in 60mm dishes and co-transfected with NF-κB-luciferase (2mg) and constitutively-expressing galactosidase (0.5mg) (Invitrogen) using Xtremegene 9 (Roche) according to the manufacturer's protocol. After 6h of transfection, media were replaced for 1h prior to incubation with inhibitors and stimulation with TNF as indicated. For siRNA experiments, cells were treated with AStar or SK1 siRNA for 48h prior to transfection with reporter constructs. Following stimulation, luciferase activity was assessed using the Luciferase reporter kit (Stratagene) according to the manufacturer's protocol. Galactosidase activity was assayed using the High Sensitive B-galactosidase kit (Stratagene) according to the manufacturer's protocol. Measured luciferase activity (NF-κB-dependent) was normalized to measured galactosidase activity (constitutive).

A.2.10. Statistical Analysis

Data are represented as mean ± SEM. Unpaired student's t-test and 2-way ANOVA with Bonferroni post-test statistical analyses were performed using Prism/GraphPad software.

A.3. RESULTS

A.3.1. SK1 knockdown enhances TNF-induced RANTES in HeLa cells

We previously reported an increase in RANTES induction upon loss of SK1 in MCF7 and MEFs (*379*). However, as MCF7 cells undergo cell death in response to TNF and MEFs are not particularly responsive to TNF, we elected to find an alternative model system. To this end, HeLa cells were selected as an inflammatory model that can withstand TNF in the absence of translational inhibitors. In order to validate the previous observations, siRNA was used to knock down SK1, and the effects on RANTES and SK1 mRNA were analyzed **(Fig. 29)**. As can be seen, a significant knockdown of SK1 mRNA was observed in siRNA-treated cells compared to all-Star (AStar) negative controls with greater than 80% knockdown **(Fig. 29A)**. Notably, a small but statistically insignificant increase in SK2 mRNA was also observed (data not shown). Importantly, TNF induced RANTES mRNA expression in both AStar and SK1 siRNA treated cells. Strikingly, SK1 knockdown significantly enhanced RANTES induction in response to TNF

with no statistically significant effect on unstimulated levels **(Fig. 29B)** Importantly, the changes in RANTES mRNA were also reflected in levels of RANTES protein in the media **(Fig, 29C)**. These results replicate our previous findings in MCF7 cells and MEFs, and they confirm the utility of HeLa cells as a model system to explore regulation of RANTES by SK1.

A.3.2. SK2 knockdown and inhibition diminish RANTES production

Our previous study suggested that sphingosine was important for RANTES induction in response to TNF. Thus, it was a possibility that the observed effects may not be specifically due to the loss of SK1. To confirm this, the effects of SK2 siRNA on TNF-stimulated RANTES induction were determined. As can be seen, SK2 significantly decreased SK2 mRNA levels **(Fig. S10A**) but had minimal effects on SK1 expression (data not shown). As with SK1, loss of SK2 had no significant effect on basal RANTES but, surprisingly, in TNF-stimulated cells, SK2 siRNA significantly decreased RANTES induction **(Fig S10B)**. Thus, the specific loss of SK1 but not SK2 enhances RANTES production in TNF-stimulated HeLa cells.

The differential effects of SK1 and SK2 prompted us to determine if pharmacological inhibitors of SKs produced the same effect as loss of SK1. For this, the generic SK inhibitor SKi-II was utilized. As can be seen, SKi-II had no effect on basal RANTES mRNA or protein yet caused a small but significant decrease in RANTES mRNA and protein levels **(Fig. S11A, S11B)** in TNF-stimulated cells. Recent studies have suggested that SKi-II preferentially inhibits SK2 (*201*), and therefore these results are consistent with the observed effect of SK2 siRNA.

A.3.3. RANTES induction in response to TNF is NF-κB dependent

Several transcription factors have been shown to regulate RANTES transcription including STAT3 (*383, 399*), and NF-κB (*400, 401*) depending on the cell type and stimulus. Accordingly, to examine SK1 regulation of RANTES, it was first important to determine the transcription factor primarily responsible for RANTES induction in HeLa cells in response to TNF. To this end, we utilized BAY 11-7082, a well-known inhibitor of NF-κB activation, which acts by irreversible inhibition of IκB-α phosphorylation (*402*). To test the efficiency of this inhibitor, localization of the NF-κB subunits p65/RelA and p50/ NF-κB was assessed by immunofluorescence and confocal microscopy **(Fig. 30)**. As can be seen in control samples, p65 and p50 primarily reside in the cytosol basally and translocate to the nucleus upon TNF stimulation. Importantly, BAY treatment resulted in a dramatic decrease in nuclear translocation of p65 **(Fig. 30A)** and p50 **(Fig. 30B),** thus confirming its efficacy. Next, the effect of BAY treatment on RANTES induction was assessed by RT-PCR. The data demonstrate that basal levels of RANTES were largely unaffected by BAY treatment. Notably, TNF induction of RANTES was inhibited by more than 80% **(Fig. 30C)**. To further verify that RANTES is NF-KB dependent in the current system, we used siRNA against p65 and achieved greater than 80% knockdown (data not shown). Again, TNF induction of RANTES was similarly abolished **(Fig. 30D)**. Together, these results indicate that RANTES induction in response to TNF is largely NF-KB dependent in HeLa cells.

A.3.4. TNF induced-acute activation of NF-kB is SK1 and S1P-independent

Previous research has reported regulation of the NF-κB pathway by SK1 (*403*) with subsequent studies suggesting this occurs through S1P-mediated inhibition of TRAF2 (*374, 403*). However, as the effects of SK1 on RANTES (knock down increases RANTES levels) are contradictory to the role of NF-κB in induction of RANTES, it became critical to determine any possible interactions between SK1 and the well known TNF signaling cascade leading to NF-κB activation (*404*). However, it was first necessary to determine if TNF increased S1P levels within the time frame of early NF-κB pathway activation and if this was through SK1 **(Fig. 31)**. For this, HeLa cells were transfected with AStar or SK1 siRNA and subsequently stimulated with TNF for 2.5 or 10min prior to lipid extraction. These times were selected to correlate with early activation of NF-kB pathway as described previously (11)(*405*). As can be seen, HeLa cells treated with AStar siRNA show a significant increase in S1P production at both 2.5 min (approx. 20-fold) and 10 min of TNF stimulation (approx. 15-fold; **Fig. 31A**). Strikingly, these increases were completely abrogated in HeLa cells treated with SK1 siRNA **(Fig. 31A)** Thus, acute TNF stimulation leads to an increase in cellular S1P levels that is wholly dependent on SK1.

Having confirmed this, the role of SK1 and, by extension, S1P in regulating key components of the TNF signaling pathway were next evaluated **(Fig. 31)**. One of the earliest events in this cascade is phosphorylation of the IκB-kinases (IKK). As can be seen, HeLa cells treated with control AStar siRNA exhibited robust increases in phosphorylated IKKα/β (pIKKα/β) at 10 minutes of TNF treatment, returning to basal levels by 30 minutes **(Fig. 31B)**. Notably, cells treated with SK1 siRNA also exhibited robust phosphorylation of IKK proteins in response to TNF, which temporally mirrored the control siRNA-treated cells **(Fig. 31B)**. Importantly, there was no difference in total levels of IKKα and IKKβ in the presence or absence of TNF or SK1 siRNA **(Fig. 31B)**.

The next major step downstream of IKK phosphorylation and activation is the phosphorylation and degradation of IκB-α. Consistent with activation of IKKs, cells treated with AStar siRNA or SK1 siRNA showed robust IκBα phosphorylation in response to TNF treatment, which was followed by IκB-α degradation **(Fig. 31B)**. Furthermore, robust degradation of IkB in response to TNF was also observed in A549 cells – a distinct model of TNF-induced inflammatory signaling (data not shown).

The above results suggest that TNF activation of the NF-κB cascade is not affected by loss of SK1 in A549 or HeLa cells. However, to further evaluate this by genetic means, IκB-a degradation was examined in mouse embryonic fibroblasts (MEFs) derived from WT and $SK1^{-/-}$ mice **(Fig. 31C)**. Similar to the results with knock down of SK1 in HeLa cells, IκB-α degradation upon TNF treatment was unaffected in SK1 null fibroblasts **(Fig. 31C)**. WT cells treated with TNF displayed IκB-α degradation beginning at 5 minutes, which returned to basal levels following 1 hour of treatment **(Fig. 31C)**. $SK1^{-/-}$ fibroblasts, although expressing less total IκBα, exhibited the same pattern of IκB-α degradation as did WT. To confirm that this was not due to clone specific effects, these observations were confirmed in a different clone of WT and SK1 MEFs **(Fig. S12)**. As expected, despite lower basal levels of IκB-a, there was no significant difference in the pattern of IκB-a degradation was seen in $SK1^{-/-}$ fibroblasts compared to WT

cells **(Fig. S12).** These results are in contradistinction to those of Alvarez et al (*374*), who showed that the NF-κB cascade is totally halted in $SK1^{-/-}$ MEFs upon TNF stimulation.

Finally, to be sure that the dose of TNF used is not overwhelming the response thus masking any differences that could otherwise be seen, a dose response with TNF in HeLa cells treated with AStar or SK1 siRNA was performed, and IKK phosphorylation and IκBα degradation were assessed. Even at very low doses, no differences were seen between the control and the SK1 knock down cells **(Fig. 31D).** Taken together, these results demonstrate that SK1 is not required for this early signaling event.

A.3.5. Loss of SK2 does not affect NF-κB pathway activation

As loss of SK2 resulted in a decrease in RANTES, we also considered that SK2 might affect NF-κB activation in this system. As can be seen, loss of SK2 somewhat decreased total IKK levels, yet IKK phosphorylation remained intact at both time points compared to AStar treated cells **(Fig. S10C)**. Moreover, IκB-α degradation was comparable in SK2 treated cells compared to AStar controls **(Fig. S10C)**. Collectively, these results show that SK2 is also not required for acute activation of the NF-κB cascade.

A.3.6. Nuclear Translocation of NF-κB subunits does not require SK1

Although the early activation of the NF-kB pathway by TNF appears to be independent of SK1, it is also possible that subsequent steps – such as translocation of NF-κB subunits to the nucleus, an essential step for NF-κB-mediated gene expression (*406*) may require SK1. Initially, the levels of the NF-κB subunits p50/NFkB1 and p65/RelA were assessed in the presence and absence of SK1 **(Fig. 31)** with results indicating no significant difference. Accordingly, the translocation of both subunits to the nucleus was assessed by immunofluorescence and confocal microscopy **(Fig. 32)**. As seen in Figure 4A, the p65 subunit localized to the cytoplasm under basal conditions and upon treatment with TNF translocated to the nucleus (blue, Draq5) **(Fig. 32A)**. Strikingly, this occurred in both AStar and SK1-siRNA treated cells. To confirm this, TNF-mediated p65 nuclear translocation was assessed in MEFs derived from WT or $SK1^{-/-}$ mice. Importantly, as with siRNA-treated HeLa cells, TNF induced comparable translocation of p65 to the nucleus in both WT and $SK1^{-/-}$ cells **(Fig. 32B)**. Taken together, these results indicate that SK1 is not required for nuclear translocation of NF-kB subunits in response to TNF.

A.3.7. SK1 is not required for TNF-mediated activation of NF-kB reporter activity

Although nuclear translocation of NF-kB subunits remained intact, it is also possible that loss of SK1 may affect NF-κB-mediated transcription following translocation of NF-κB to the nucleus (e.g. promoter binding, subunit phosphorylation, or interactions with other factors). To probe this, an NF-κB luciferase reporter assay was performed **(Fig. 33)**. The results demonstrated that TNF strongly increases NF-κB transcriptional activity (25-fold over vehicle) in negative

control siRNA-transfected cells. Strikingly, loss of SK1 had no significant effect on basal reporter activity and did not prevent TNF-mediated NF-κB activity. Indeed, loss of SK1 tended to increase NF-κB reporter activity compared to negative control cells although this did not reach statistical significance **(Fig 33)**. Taken together with the results above, this indicates that SK1 is not required for NF-κB-mediated gene transcription in response to TNF.

A.3.8. p38 MAPK requires SK1 to regulate RANTES expression

As SK1 did not appear to significantly affect the NF-κB pathway, it was necessary to consider alternative pathways that might regulate RANTES expression in an SK1-dependent manner. The p38 MAPK pathway is a well-known component of TNF signaling (*407, 408*) and can also be regulated by SK1 (*409-411*). Furthermore, this pathway can also affect chemokine expression, although the effects on RANTES vary depending on the cell type and stimulus (*397, 412, 413*) Consequently, as a first step, the role of p38 MAPK in RANTES regulation was determined utilizing the p38 MAPK inhibitor BIRB 796 (*414*). As shown in **Figure 34A**, inhibition of p38 MAPK resulted in a significant increase in both RANTES mRNA and protein levels upon TNF stimulation (2.5-fold compared to vehicle) **(Fig. 34A. B)**.

As this result strongly mimics the effect of SK1 loss on RANTES levels **(Fig. 29B, C)**, it next became important to assess the effect of SK1 knockdown on p38 MAPK activation. Following 10 minutes of TNF stimulation, phosphorylation of p38 MAPK was notably increased in control cells. Strikingly, TNF activation of p38 MAPK was significantly blunted following treatment with SK1 siRNA **(Fig. 34C)**. Quantification of the levels of phospho-p38 shows a significant decrease in p38 MAPK activation **(Fig. 34D)**. These results suggest that induction of p38 MAPK by TNF serves as a negative regulator of induction of RANTES. Moreover, the results suggest that SK1 is required for full activation of p38 MAPK in TNF-stimulated HeLa cells.

Finally, as loss of SK2 suppressed RANTES and appeared to be independent of NF-kB activation, we also speculated that loss of SK2 might also affect p38 MAPK. Interestingly, cells treated with SK2 siRNA showed significantly higher p38 MAPK phosphorylation compared to AStar-treated cells **(Fig. S10B).** Together with the above results, this provides further evidence that p38 MAPK activation attenuates RANTES production in TNF-stimulated HeLa cells.

A.3.9. Interactions of the p38 MAPK and NF-KB pathways

Previous studies have suggested that p38 MAPK and NF-κB may function as complementary pathways in response to some stimuli (*415, 416*). Here, as SK1 appears to be acting through regulation of p38 MAPK but independent of the NF-kB pathway, it was important to determine if these pathways are functioning independently of each other. To determine the role of the NF-κB pathway in the p38 MAPK pathway, the effect of the NF-kB inhibitor BAY 11-7082 on p38 MAPK phosphorylation was assessed. Strikingly, BAY treatment resulted in a significant increase in the phosphorylation of p38 MAPK both at baseline and following TNF stimulation **(Fig. 35A)**. Similarly, following pretreatment with the p38 MAPK inhibitor BIRB

796, there was a significant increase in the transcriptional activity of NF-κB following TNF treatment **(Fig. 35B**). Therefore, in Hela cells the p38 MAPK pathway and the NF-κB pathway exert dual inhibition on each other following TNF treatment.

A.3.10. SK1 knockdown affects multiple cytokines and chemokines in a manner similar to the effects on RANTES

Results thus far suggest that SK1 regulates RANTES independently of NF-κB, despite this pathway being a major regulatory factor for RANTES in HeLa cells. Moreover, the observation that loss of SK1 enhanced RANTES induction is somewhat counterintuitive as SK1 is thought to be pro-inflammatory (*1*). This led us to question what are the effects of SK1 on other chemokines and cytokines beyond RANTES. To this end, a quantitative PCR array that evaluates the expression of 84 key-secreted cytokines and chemokines central to the immune response was utilized. As expected, RANTES levels were enhanced in SK1 siRNA cells treated with TNF, consistent with the preceding results. Interestingly, SK1 knockdown dramatically increased TNF induction of multiple other chemokines including CXCL1, CCL20, CXCL10, and the interleukins IL-8 and IL-6 when compared to TNF-stimulated negative control siRNA cells **(Table 5)**. Moreover, real-time PCR analysis of some of these mediators with independent primers confirmed the array results indicating that a loss of SK1 significantly enhanced TNF induction of numerous chemokines in HeLa cells including CXCL1, CXCL10, IL-8 and CCL20 **(Fig 36)**. Collectively, these results suggest that SK1 has a broad role in regulation of TNF-mediated chemokine transcription and, together with the results above, suggest that SK1 might be a negative regulator of NF-κB activation in the coordination of the TNF-induced inflammatory response in HeLa cells.

A.4. DISCUSSION

Sphingosine kinase 1 (SK1) is a key enzyme of sphingolipid metabolism, regulating the levels of the pro-growth lipid S1P with the pro-apoptotic lipids ceramide and sphingosine. In the current study, we report that loss of SK1 significantly increases levels of the pro-inflammatory chemokine RANTES in TNF-stimulated HeLa cells, and that SK1 exerts these effects through regulation of the p38 MAPK pathway. Notably, SK1 was not required for TNF-stimulated activation, nuclear translocation or transcriptional activity of NF-κB subunits. Finally, we also show that loss of SK1 has similar effects on multiple chemokines. These results suggest that SK1 can have an anti-inflammatory role in the TNF response that mechanistically occurs through p38 MAPK.

A.4.1. SK1 regulation of RANTES

RANTES promotes the chemotaxis of multiple cell types to sites of inflammation and is essential for the physiological inflammatory response. Here, we find that a loss of SK1 in a TNF-stimulated model of inflammation markedly increases RANTES mRNA and protein,

corroborating and extending our previous study (*379*). Importantly, this was specific to SK1 as loss of SK2 produced the opposite effect, attenuating RANTES induction by TNF. Notably, this latter effect differs from our previous observations in MEFs and MCF-7 cells (*379*). This suggests cell-specific roles for SK2 in the TNF response and, consistent with this, the effects of TNF on acute S1P levels were wholly SK1-dependent. Thus, the effect of SK2 could be due to basal effects on cellular sphingolipids rather than a specific signaling role in the TNF response. Studies exploring this further are ongoing in our laboratory.

As RANTES mRNA is reported to be extremely stable (*391*), efforts to further explore SK1-RANTES connections focused on transcriptional regulation. Although regulated by multiple transcription factors such as STAT3 (*383*), NF-AT, AP-1 (*394*), RANTES induction in the current system was highly NF-κB-dependent **(Fig. 30)**. Despite this, SK1 does not appear to affect RANTES through the NF-κB pathway; indeed, loss of SK1 was able to enhance RANTES levels even in the presence of NF-κB inhibitors (data not shown), thereby identifying a novel pathway by which SK1 regulates gene expression. As RANTES is primarily regulated at the transcriptional level, we can surmise that the effects of SK1 must also occur transcriptionally, potentially through transcription factors downstream of the p38 MAPK pathway. Alternatively, this may occur through non-promoter mediated effects such as regulation of histone deacetylases (HDAC). Indeed, as SK2 is reported to increase gene transcription through inhibiting HDACs (*269*), we initially speculated that loss of SK1 could increase substrate availability for SK2, thereby upregulating RANTES through prolonged inhibition of HDACs. However, when we explored this possibility, we found that the HDAC inhibitor trichostatin A significantly blunted RANTES induction in our system (data not shown). Clearly, the transcriptional mechanisms by which SK1 affects RANTES require further study.

A.4.2. SKi-II and the RANTES response

Despite the use of genetic tools, assessing the effects of SK inhibitors on RANTES induction was also important yet, unexpectedly, experiments with the generic inhibitor SKi-II showed an inhibition of RANTES induction. A primary reason for this relates to specificity of the SKi-II inhibitor for SK isoforms as recently published data reports that this inhibitor more favorably targets SK2 rather than SK1 (*201*). Consistent with this, results with this inhibitor more closely match the effects of SK2 siRNA in the HeLa system **(Fig. S10B)**. A second, related reason, relates to the more general possibility of off target effects – indeed, SKi-II is reported to also inhibit PI3K, ERK and PKCα (*417*). In the absence of strong specific SK1 inhibitors, we acknowledge this as a limitation of the current study. The testing of newer, more specific SK1 inhibitors remains an on-going endeavor in our lab; moreover, as SK1 is considered a strong therapeutic target for the treatment of various pathologies, future studies determining if post-translational inhibition of SK1 can adversely induce chemokines are of paramount importance.

A.4.3. SK1 and the NF-kB pathway

A major finding of this study, mechanistically, is that SK1 and SK1-derived S1P are completely dispensable for activation of the NF-κB pathway and, in fact, can enhance gene expression

mediated by this pathway. This is shown by a number of lines of evidence. Firstly, TNF induced an acute increase in S1P levels that was completely prevented by loss of SK1. Second, loss of SK1 did not prevent phosphorylation of IKK, IκB or the subsequent degradation of IκB in response to TNF. Importantly, this was not an effect of siRNA or cell type as similar effects were observed in $SK1^{-/-}$ fibroblasts as well as in A549 cells. Third, loss of SK1 did not have effects further downstream in the NF-κB pathway as total levels of p65 and p50 were unchanged, and their translocation into the nucleus remained intact (also observed in both siRNA treated cells and $SK1^{-/-}$ fibroblasts). Finally, transcriptional NF-κB activity, as assessed by a luciferase reporter assay, remained intact and, indeed, was slightly increased (albeit not significantly) following loss of SK1. This was also true for endogenous genes, with loss of SK1 enhancing the induction of the highly NF-κB-dependent RANTES, rather than inhibiting it as would be expected if NF-κB activation were compromised. Furthermore, loss of SK1 had similar effects even in the presence of NF-kB pathway inhibition (data not shown). Taken together, this clearly suggests an NF-κB-independent mechanism for SK1 and, by extension, SK1-derived S1P in this system. Strikingly, this sharply contrasts with previous studies reporting SK1 as essential for NF-κB activation through effects on TRAF2 activation and subsequent activation of IKK and degradation of IκB (*374*) .The reasons for this discrepancy are unclear. It cannot be explained by the doses of TNF utilized, as similar effects were observed at a wide range of TNF doses **(Fig. 31C)**. It is also possible that SK1 requirement for NF-κB activation is cell type specific; however, comparable effects were seen across three different cell types including MEFs that were previously reported to require SK1 for activation of the TNF cascade (*374*). A primary possibility to consider was that TNF did not induce an acute generation of S1P in these systems – given that S1P was reported to be key for activation of TRAF2 and subsequent IκB degradation. However, as seen above **(Fig. 31A)**, this was not the case as TNF induced a clear increase in intracellular S1P that was wholly SK1-dependent. Looking beyond sphingolipids, an alternative reason somewhat related to cell type, is that TRAF5 could compensate for TRAF2 (presuming that is inactive in the absence of S1P) in the current system. Consistent with this, NF-κB activation remains intact in TRAF2 knockout mice due to TRAF5 compensation, whereas only double knockout mice for TRAF2 and TRAF5 have defective NF-κB activation (*418*). Although exploring this possibility is beyond the scope of the current study, our data nonetheless suggest that SK1 and intracellular S1P regulation of NF-κB and gene transcription in the cytokine response may be more complex than currently thought.

A.4.4. SK1 regulation of p38 MAPK activation and the interconnection with the NF-κB pathway

The role of p38 MAPK in the inflammatory response is well documented, and it has been reported to affect the levels of multiple cytokines and chemokines including IL-12, IL-1B, IL10, and IL-6 (*419, 420*); notably, though, this regulation is both cell type- and stimulus-dependent. Here, in TNF-stimulated HeLa cells, inhibition of p38 MAPK resulted in a marked upregulation of RANTES mRNA and protein, results that were concordant with a study in smooth muscle cells stimulated with LPS and IFN-Y (*397*). Consequently, the fact that SK1 knockdown significantly reduces p38 MAPK activity strongly suggests that the effects of SK1 on RANTES are mediated by activation of the p38 MAPK pathway. Corroborating evidence for this inhibitory role of p38 MAPK on RANTES was also provided by experiments with SK2 siRNA. As with

loss of SK1, the effects of SK2 siRNA on RANTES appear to be largely independent of the NF-κB pathway yet led to augmented phosphorylation of p38 MAPK.

The p38 MAPK pathway cross talks with several other pathways such as the NF-κB, Akt and Wnt/β-catenin pathways (*421*). Most reports state that p38 MAPK can positively regulate NF-KB activity albeit through various mechanisms (*422-424*). In contrast, inhibition of p38 MAPK in our system caused a significant increase in NF-κB activity in HeLa cells. This is consistent with two previous studies in IL-1-stimulated HeLa and HEK293 cells (*425*), and in KB epithelial cells (*426*). Although TNF is reported to activate p38 MAPK independently of the NF- κB pathway (*427*), it was also important to determine if there was a reciprocal cross-talk between NF-κB and p38 MAPK in the current system. Indeed, inhibition of the NF-κB pathway also enhanced p38 MAPK phosphorylation both basally and upon stimulation with TNF, although it should be noted that the magnitude of p38 MAPK activation was comparable. Nonetheless, this does raise the possibility that the reduced induction of RANTES seen upon NF-κB inhibition is a result of dual mechanisms; namely, both decreased NF-κB-dependent transcription and an active suppression of RANTES mediated by p38 MAPK. These mechanisms, and their relationship to SK1, are currently under further investigation in our laboratory. The interconnections and the complexity of SK1 regulation of the RANTES response are summarized in **Scheme 6**.

A.4.5. The sphingolipid regulator of RANTES.

The current study also returns us to the question of the identity of specific sphingolipid regulator(s) of RANTES induction in the TNF response. In our previous study, data suggested that sphingosine derived from secretory sphingomyelinase (S-SMase) and acid ceramidase was important for RANTES induction in MCF-7 cells, and this was further supported by the enhancing effects of SK1 and SK2 siRNA on RANTES induction in that system (16). In contrast, in HeLa cells, no significant increases in ceramide or sphingosine were observed upon acute TNF stimulation. Moreover, no differences in either lipid was observed in SK1 siRNA-treated cells suggesting that enhancement of RANTES upon loss of SK1 is not through a general increase in sphingosine **(Fig S13)**.

As noted above, the time frame of S1P generation and its coincidence with p38 MAPK activation leads us to conclude that likely the effects of SK1 in this system are mediated through S1P-activation of p38 MAPK activation. That being said, all lipid measurements here were at very short time points to detect S1P production as documented in Alvarez et al (*374*) and Pettus et al (*405*). Thus, we cannot explicitly rule out a role for ceramide or sphingosine in regulating RANTES production at later time points. Indeed, in our previous study, TNF-induced increases in S-SMase activity and ceramides occurred at later times (>12h) of stimulation (*379*). In addition, although we could not detect any changes in total sphingosine or ceramide levels at early time points, we also cannot rule out the possibility of local changes in lipid levels occurring in specific cellular compartments that may impact RANTES induction. Finally, it is a distinct possibility that multiple sphingolipids can regulate RANTES induction through distinct mechanisms. Clearly, given the importance of RANTES in physiological inflammation, and its role in a variety of pathologies, further research in this area could yield a number of potential therapeutic targets.

A.4.6. SK1 regulation of the chemokine response

Beyond RANTES, the loss of SK1 also had a broader effect on inflammatory cytokine and chemokine production in tumor cells, having a comparable effect on CCL20, CXCL1, and CXCL10 amongst others. Importantly, this offers additional evidence arguing against a role for SK1 in regulating NF-κB in this system; induction of CCL20 and CXCL10 is also partially NF-kB-dependent whereas CXCL1 induction is NF-κB independent (data not shown). Although the biological consequences of SK1 loss and subsequent chemokine upregulation are unclear, many possibilities are suggested by their known roles. For example, CXCL10 overexpression leads to anti-tumor effects by blocking angiogenesis as seen in cervical carcinoma (*428*). Notably, SK1 is reported to be strongly pro-angiogenic (*429*); thus, regulation of CXCL10 may be a potential mechanism by which inhibiting SK1 blocks angiogenesis. CCL20 expression in the colon recruits dendritic cells to combat infections (*430*), important for recognition of cancer cells on one hand, but also the source of an exacerbated inflammatory response that would set the ground for cancer; thus, targeting SK1 in this context could be a double-edged sword. Finally, CXCL1 has been considered an oncogene in human melanoma (*431*) and so targeting SK1 in this context might exacerbate its oncogenic effects. Collectively, the upregulation of any of these chemokines in the tumor environment could be detrimental to cancer patients or could promote tumor progression in some cancers. Consequently, although studies have suggested that SK1 is an attractive therapeutic target for many cancers (*1*), further research is clearly required, to ensure that undesirable side effects which could potentially promote tumor progression, do not occur.

A.4.7. Conclusions

In summary, the current study suggests a novel anti-inflammatory function of SK1 and intracellular S1P in the regulation of chemokine production in the TNF response. Importantly, SK1 seemed to exert its effects through modulation of the p38 MAPK pathway, and was not required for activation of the canonical NF-kB pathway; indeed, NF-κB activity was somewhat enhanced by loss of SK1. Taken together, this suggests a novel mechanism downstream of p38 MAPK by which SK1 can negatively regulate gene expression in the TNF response. As SK1 is considered an attractive therapeutic target for many cancers and other disorders, a cautious approach may be required to ensure that SK1 inhibition does not trigger a similar pro-inflammatory chemokine response. Indeed, such an effect could have deleterious effects on disease progression and patient health.