



Novel cancer-related regulatory targets for the signalling sphingolipids sphingosine-1-phosphate and sphingosylphosphorylcholine

Kati Kemppainen

Cell Biology
Faculty of Science and Engineering
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Supervised by

Professor Kid Törnquist, PhD
Åbo Akademi University
Tykistökatu 6, Biocity
20520 Turku, Finland
ktornqvi@abo.fi

Reviewed by

Professor Vesa Olkkonen, PhD
Minerva Foundation Institute for Medical Research
Tukholmankatu 8, Biomedicum 2U
00290 Helsinki, Finland
vesa.olkkonen@helsinki.fi

and

Professor Ilpo Vattulainen, DTech
University of Helsinki
POB 64
00014 University of Helsinki, Finland
ilpo.vattulainen@tut.fi

Opponent

Academy professor Elina Ikonen; MD, PhD
University of Helsinki
Haartmaninkatu 8, Biomedicum
00290 Helsinki, Finland
elina.ikonen@helsinki.fi

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Abstract

The signalling sphingolipid sphingosine-1-phosphate (S1P) is necessary for development of the immune system and vasculature and on a cellular level regulates migration, proliferation and survival. Due to these traits S1P has an important role in cancer biology. It is considered a primarily cancer-promoting factor and the enzyme which produces it, sphingosine kinase (SphK), is often over-expressed in tumours. S1P is naturally present in the blood, lymph, tissue fluids and cell cytoplasm and functions through its cell surface receptors (S1P₁₋₅) and as an intracellular second messenger. Sphingosylphosphorylcholine (SPC) is closely related to S1P and has similar regulatory functions but has not been extensively studied. Both S1P and SPC are able to evoke either stimulatory or inhibitory effects on cancer cells depending on the context. The aim of this thesis work was to study novel regulatory targets of S1P and SPC, which mediate the effects of S1P/SPC signalling on cancer cell behaviour. The investigated targets are the transcription factor hypoxia-inducible factor 1 (HIF-1), the intermediate filament protein vimentin and components of the Hippo signalling pathway. HIF-1 has a central role in cancer biology, as it regulates a multitude of cancer-related genes and is potently activated by intratumoural hypoxia through stabilization of the regulatory subunit HIF-1 α . Tumours typically harbour high HIF-1 α levels and HIF-1, in turn, facilitates tumour angiogenesis and metastasis and regulates cancer cell metabolism. We found S1P to induce follicular thyroid cancer cell migration in normal oxygen conditions by increasing HIF-1 α synthesis and stability and subsequently HIF-1 activity. Vimentin is a central regulator of cell motility and is also commonly over-expressed in cancers. Vimentin filaments form a cytoskeletal network in mesenchymal cells as well as epithelial cancer cells which have gone through epithelial-mesenchymal transition (EMT). Vimentin is heavily involved in cancer cell invasion and gives tumours metastatic potential. We saw both S1P and SPC induce phosphorylation of vimentin monomers and reorganization of the vimentin filament network in breast and anaplastic thyroid cancer cells. We also found vimentin to mediate the anti-migratory effect of S1P/SPC on these cells. The Hippo pathway is a novel signalling cascade which controls cancer-related processes such as cellular proliferation and survival in response to various extracellular signals. The core of the pathway consists of the transcriptional regulators YAP and TAZ, which activate predominantly cancer-promoting genes, and the tumour suppressive kinases Lats1 and Lats2 which inhibit YAP/TAZ. Increased YAP expression and activity has been reported for a wide variety of cancers. We found SPC to regulate Hippo signalling in breast cancer cells in a two-fold manner through effects on phosphorylation status, activity and/or expression of YAP and Lats2. In conclusion, this thesis reveals new details of the signalling function of S1P and SPC and regulation of the central oncogenic factors HIF-1 and vimentin as well as the novel cancer-related pathway Hippo.

List of original publications and manuscripts

This thesis is based on the following two original publications and one submitted manuscript. They are referred to in the text with their Roman numerals.

- I. Kalhori V*, **Kemppainen K***, Asghar MY, Bergelin N, Jaakkola P, Törnquist K, 2013. Sphingosine 1-phosphate as a regulator of hypoxia-induced factor 1 α in thyroid follicular carcinoma cells. Plos One 8, e66189.
- II. Hyder CL*, **Kemppainen K***, Isoniemi KO, Imanishi SY, Goto H, Inagaki M, Fazeli E, Eriksson JE, Törnquist K, 2015. Sphingolipids inhibit vimentin-dependent cell migration. J Cell Sci 128, 2057-69.
- III. **Kemppainen K**, Wentus N, Laiho A, Törnquist K, 2016. Sphingosylphosphorylcholine regulates the Hippo signalling pathway in a dual manner. Submitted manuscript.

* Equal contribution

Publication I has been previously included in the thesis of V. Kalhori and a manuscript of publication II in the thesis of C.L. Hyder. In project I, both V. Kalhori and K. Kemppainen designed experiments and analyzed and interpreted data. Both also did Western blotting, immunoprecipitation and Boyden chamber migration experiments. V. Kalhori performed the luciferase assays and K. Kemppainen the qPCR and proliferation experiments. K. Kemppainen wrote the manuscript with help from V. Kalhori. In project II, C. L. Hyder and K. Kemppainen together designed experiments and interpreted results. C.L. Hyder performed live-cell migration imaging, tracking and confocal imaging with help from K. Kemppainen. K. Kemppainen did the Western blotting, Boyden chamber migration and qPCR experiments. C. L. Hyder and K. Kemppainen co-wrote the article. In project III, K. Kemppainen designed experiments, analyzed and interpreted data and wrote the article manuscript. K. Kemppainen performed the large majority of the Western blotting, quantitative and qualitative PCR and proliferation experiments.

Abbreviations

ABC	ATP-binding cassette
AC	adenylate cyclase
AMOT	angiomin
APC	adenomatous polyposis coli
ATX	autotaxin
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
Cer	ceramide
CerS	ceramide synthase
CHO	Chinese hamster ovary
CNS	central nervous system
COX-2	cyclo-oxygenase 2
CTGF	connective tissue growth factor
CYR61	cysteine-rich angiogenic inducer 61
DAG	diacylglycerol
ECM	extracellular matrix
EDG	endothelial differentiation gene
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF-4E	eukaryotic translation initiation factor 4E
EMT	epithelial-mesenchymal transition
ER(+)/(-)	oestrogen receptor positive/negative
ERK	extracellular signal-regulated kinase
ERM	ezrin-radixin-moesin
FA	focal adhesion
FAK	focal adhesion kinase
FBS	foetal bovine serum
FDA	United States Food and Drug Administration
FIH-1	factor inhibiting HIF-1
FN1	fibronectin 1
FRAP	fluorescence recovery after photobleaching
GCase	glucosylceramidase
GCS	glucosylceramide synthase
GEF	guanine nucleotide exchange factor
GFAP	glial fibrillary protein
GlcCer	glucosylceramide
GPCR	G protein-coupled receptor
GSK3 β	glycogen synthase kinase 3 β
HDAC	histone deacetylase

Abbreviations

HDL	high-density lipoprotein
HIF	hypoxia-inducible factor
HRE	hypoxia-response element
Hsp90	heat-shock protein 90
ICAM-1	intercellular adhesion molecule-1
IF	intermediate filament
IGF1R	insulin-like growth factor receptor 1
IP	immunoprecipitation
IP ₃	inositol 1,4,5-trisphosphate
K _i	dissociation constant (inhibitor)
KO	knockout
Lats	large tumour suppressor
LC-MS	liquid chromatography–mass spectrometry
LDL	low-density lipoprotein
LPA	lysophosphatidic acid
LPP	lipid phosphate phosphatase
LSB	Laemmli sample buffer
LS-FBS	lipid stripped foetal bovine serum
Luc	luciferase
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MET	mesenchymal-epithelial transition
MMP	matrix metalloproteinase
Mob1	Mps One binder 1
MS	mass spectrometry, multiple sclerosis
Mst	mammalian Ste20-like protein kinase
mTOR	mammalian target of rapamycin
NF2	neurofibromin 2
NPD	Niemann-Pick disease
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFA	paraformaldehyde
PGE ₂	prostaglandin E ₂
PHD	prolyl hydroxylase
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PIP ₃	phosphatidylinositol 3,4,5-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G

Abbreviations

PLC	phospholipase C
PLD	phospholipase D
PMSF	phenylmethanesulfonyl fluoride
PP2A	protein phosphatase 2A
p70S6K	p70S6 kinase
PTEN	phosphatase and tensin homolog
PTM	post-translational modification
pVHL	von Hippel Lindau protein
qPCR	real-time quantitative PCR
RACK1	receptor of activated protein kinase C 1
RCC	renal cell carcinoma
ROCK	Rho-associated kinase
ROS	reactive oxygen species
RT	room temperature
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
S1P	sphingosine-1-phosphate
S1P ₁₋₅	sphingosine-1-phosphate receptor 1-5
S1PR	sphingosine-1-phosphate receptor
SM	sphingomyelin
SMase	sphingomyelinase
SMS	sphingomyelin synthase
SPC	sphingosylphosphorylcholine
SPP	S1P phosphatase
SPNS2	spinster-2
Sph	sphingosine
SphK	sphingosine kinase
STAT3	signal transducer and activator of transcription-3
TAZ	transcriptional co-activator with PDZ-binding motif
TGF	transforming growth factor
TNF	tumour necrosis factor
TRAF2	tumour-necrosis factor receptor-associated factor 2
TRPC1	transient receptor potential channel 1
ULF	unit-length filament
VASP	vasodilator-stimulated phosphoprotein
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VGLL4	Vestigial-like family member 4
YAP	Yes-associated protein
4E-BP1	eIF-4E binding protein 1

Table of contents

Abstract	3
List of original publications and manuscripts.....	4
Abbreviations	5
Table of contents.....	8
Introduction.....	11
Review of the literature	13
1 Characteristics of cancer cells	13
1.1 Invasion precedes metastasis.....	13
2 Sphingosine-1-phosphate and sphingosylphosphorylcholine are versatile cancer-related signalling molecules	15
2.1 The sphingolipid family.....	15
2.1.1 Sphingolipid structure and metabolism.....	15
2.2 S1P has a central role in tumour biology.....	18
2.2.1 Increased SphK1 expression and activity is common in tumours.....	18
2.2.2 S1P in the blood and tissues.....	19
2.2.3 S1P and the immune system.....	19
2.2.4 S1P regulates angiogenesis.....	20
2.2.5 Sphingosine kinases (SphK1/2)	20
2.2.6 S1P transport.....	22
2.2.7 S1P receptors.....	22
2.2.8 Intracellular S1P	28
2.2.9 S1P signalling as a drug target.....	28
2.3 SPC is an enigmatic signalling lipid	30
2.3.1 SPC mimics S1P in many ways but has also S1P-independent effects.....	30
2.3.2 Sphingomyelin deacylase produces SPC and autotaxin converts it to S1P.....	31
2.3.3 SPC receptors	32
2.3.4 Intracellular SPC	32
3 The oxygen-sensitive transcription factor HIF-1 promotes tumour vascularization and controls cancer cell metabolism	32
3.1 Regulation of HIF-1 α expression and HIF-1 activity	33
3.1.1 Stability of HIF-1 α	33
3.1.2 Synthesis of HIF-1 α	34
3.1.3 FIH-1	34
3.1.4 Cancer-related signalling changes stimulate HIF-1 α expression	35
3.2 HIF-1 promotes migration, invasion and metastasis.....	35
3.3 HIF-1 and angiogenesis.....	36
3.4 Role of HIF-1 in glucose metabolism	36
3.5 HIF-2 and HIF-3	37
3.5.1 HIF-2	37

3.5.2	HIF-3	37
4	The intermediate filament protein vimentin mediates cell motility and gives tumours metastatic potential	37
4.1	Intermediate filaments	37
4.2	Vimentin	38
4.2.1	Regulation of vimentin expression.....	39
4.2.2	Vimentin filament assembly and signalling function is regulated by PTMs	40
4.2.3	Vimentin regulates migration through interaction with the actin cytoskeleton and focal adhesions.....	40
4.2.4	Vimentin mediates cell signalling.....	41
5	The Hippo signalling pathway is a novel cascade of tumour suppressive and oncogenic factors.....	42
5.1	Hippo pathway components	42
5.2	Hippo pathway function	43
5.3	Regulation of the Hippo pathway.....	44
5.3.1	GPCRs	44
5.3.2	Growth factor and cytokine signalling	45
5.3.3	Cell-cell contacts.....	45
5.3.4	Hippo signalling is involved in mechanosensing.....	46
5.4	Hippo signalling and cancer.....	47
5.4.1	Tumour suppressor function of YAP	48
	Aims	49
	Experimental procedures	50
	Cell culture (I, II, III)	50
	Western blotting (I, II, III)	50
	Real-time quantitative PCR (qPCR) (I, II, III)	50
	Qualitative end-point PCR (III).....	51
	Migration (I, II).....	51
	³ H-thymidine incorporation (I, III).....	52
	Transfections (I, II, III).....	52
	Luciferase assay (I)	52
	Immunoprecipitation (IP) (I).....	53
	Mass spectrometry (II)	53
	Immunofluorescence (II)	53
	Cell shape (II).....	54
	Fluorescence recovery after photobleaching (II)	54
	Statistical analysis (I, II, III)	54
	Results and discussion.....	55
1	S1P regulates thyroid cancer cell migration through HIF-1 (I).....	55
1.1	S1P increases HIF-1 activity by up-regulating HIF-1 α through S1P ₃	55
1.2	S1P induces signalling involved in HIF-1 α translation	55

Table of contents

1.3	S1P also increases HIF-1 α stability	56
1.4	S1P-induced migration is mediated by HIF-1	56
1.5	S1P and HIF-1 signalling communicate in many ways.....	57
2	S1P and SPC regulate vimentin-dependent cancer cell migration (II)	58
2.1	S1P and SPC induce re-organization of vimentin	58
2.2	S1P and SPC stimulate vimentin S71 phosphorylation	59
2.3	The S1P ₂ -Rho-ROCK pathway mediates S1P/SPC-induced effects on vimentin	59
2.4	pS71-Vim mediates S1P/SPC-induced inhibition of migration.....	60
2.5	Actin may be involved in the S1P/SPC-induced and vimentin-mediated effects on migration	60
3	SPC regulates the Hippo signalling pathway (III).....	61
3.1	SPC inhibits MDA-MB-435S proliferation and up-regulates Lats2	61
3.2	SPC transiently activates YAP	62
3.3	SPC regulates Lats2, YAP and proliferation via S1P ₂	62
3.4	Hippo signalling does not mediate the anti-proliferative SPC response.....	64
	Concluding remarks.....	66
	Acknowledgements.....	67
	References.....	69
	Original publications and manuscripts.....	91

Introduction

Sphingolipids (Figure 2) are perhaps best known as structural components of cellular membranes, but many members of this lipid class are heavily involved in cell signalling. Membrane sphingolipids such as sphingomyelin, ceramide and glycosphingolipids control membrane fluidity and take part in functional membrane domains which modulate the local concentration and interactions of receptors and other signalling mediators. These lipids can also regulate the function of membrane-associated proteins by binding to them directly and with high specificity. Additionally, certain glycosphingolipids act as receptors for extracellular ligands. Other members of the sphingolipid family are amphiphilic enough to diffuse into the cytoplasm and tissue fluids instead of being restricted to membranes. These sphingolipid species can function as ligands for plasma membrane receptors and as intracellular second messengers. Sphingosine-1-phosphate (S1P) is a signalling molecule with a central role in development, tissue homeostasis as well as disease states. An important step in S1P research was the identification of five G protein-coupled receptors (GPCRs) as high affinity S1P receptors (S1P₁₋₅) which mediate much of S1P signalling. These receptors couple to the G proteins G_{i/o}, G_{q/11} and G_{12/13} with different combinations and preference and consequently activate central cell signalling pathways. Additionally, in the last few years several intracellular binding partners have been identified as direct regulatory targets of S1P. S1P signalling controls proliferation, motility and survival on a cellular level and processes such as angiogenesis and inflammation on a systemic level. These processes are central for cancer formation and progression and, accordingly, S1P is considered a central cancer-related factor. As a general rule, expression of sphingosine kinase (SphK) and consequent production of S1P promote tumour growth and protect cancer cells from apoptosis. In contrast, ceramide typically has an anti-proliferative and apoptotic effect and the balance between these two sphingolipids, the so-called sphingolipid rheostat, determines cell fate. Accordingly, compounds targeting S1P, SphK or the S1P receptors are being developed for use in cancer therapy. Sphingosylphosphorylcholine (SPC) shares many of the attributes of S1P but has evoked much less interest and details of its receptor-mediated or intracellular signalling function have not been well characterized. SPC does not always signal as S1P, but it is able to activate the S1P receptors and can be enzymatically converted to S1P. Though S1P is in general considered a tumour-promoting factor, S1P and SPC can have a potent inhibitory effect on cancer cells depending on the context, for example the receptor expression profile of the target cell. Since S1P is studied as a potential target for chemotherapy but can in some cases attenuate tumour development instead of promoting it and as SPC is also able to induce S1P signalling, the signalling pathways and molecular mechanisms mediating S1P/SPC effects on cancer cell behaviour require further studies.

In this thesis, new connections of S1P or SPC to the central cancer-related regulators hypoxia-inducible factor 1 (HIF-1) and vimentin as well as the novel signalling pathway Hippo are revealed. HIF-1 is an oxygen-sensitive transcription factor which regulates a vast array of cancer-related genes involved in angiogenesis, invasion and metastasis, proliferation and survival as well as changes in glucose metabolism. HIF-1 expression and activity are potently

induced in low oxygen conditions through stabilization of the regulatory subunit HIF-1 α and increased HIF-1 activity helps cells and tissues cope with and alleviate hypoxic stress. Tumour cells typically have high HIF-1 α levels due to intratumoural hypoxia but also up-regulation of HIF-1 α through typical cancer-related cell signalling. SphK1 has been shown to mediate hypoxia-related stabilization of HIF-1 α and HIF-1, in turn, promotes SphK1 gene expression. Our work showed that S1P promotes ML-1 follicular thyroid cancer cell migration in normal oxygen conditions by increasing HIF-1 α synthesis and stability and consequently HIF-1 activity. S1P up-regulates HIF-1 α through the S1P₃ receptor and the PI3K-Akt and MAPK pathways, which converge to activate the translational regulators p70S6K and eIF-4E. The intermediate filament protein vimentin forms a cytoskeletal network which gives cells shape and elasticity, interacts with other cytoskeletal structures, takes part in intracellular trafficking and has a central role in migration. Vimentin is expressed in mesenchymal cells and typically non-motile epithelial cells become migratory through the process of epithelial-mesenchymal transition (EMT), which involves a strong induction of vimentin expression. Accordingly, vimentin is implicated in promoting metastasis and is often highly expressed in tumour cells. We found both S1P and SPC to induce vimentin monomer phosphorylation and network reorganization and to regulate migration in MDA-MB-435S breast cancer cells and C643 anaplastic thyroid cancer cells. We showed that S1P and SPC induce phosphorylation of vimentin on S71 through the S1P₂-Rho-ROCK pathway and that this modification mediates the anti-migratory effect of S1P and SPC. The Hippo signalling pathway is a novel signalling cascade which controls tissue growth during development and in response to numerous external cues including extracellular receptor ligands, cell-cell contacts and mechanical stress. The pathway consists of tumour suppressive and oncogenic components, most importantly the kinases large tumour suppressor 1/2 (Lats1/2) and the transcriptional regulators YAP and TAZ. YAP/TAZ stimulate growth-promoting genes, are inhibited by Lats1/2 and their expression and activity is often elevated in cancers. S1P has previously been shown to induce YAP activity and we found SPC to have a two-fold effect in MDA-MB-435S cells. Through S1P₂ receptor activation SPC initially causes S127-dephosphorylation, up-regulation and activation of YAP. Later Lats2 expression and S127-YAP phosphorylation are increased and YAP target genes down-regulated. However, the SPC-induced effects on Hippo signalling did not mediate the anti-proliferative effect of SPC.

These studies shed new light on four cancer-related systems: signalling sphingolipids, HIF-1, the vimentin cytoskeleton and the Hippo pathway. Importantly, the thesis provides an example of how the effect of S1P and SPC on cancer cells is context-dependent, as S1P induces migration through S1P₃ and HIF-1 in one system and inhibits motility via S1P₂ and vimentin in another. The thesis work further underlines the importance of sphingolipid signalling in tumour biology and reveals new details of how these molecules exert their effects.

Review of the literature

1 Characteristics of cancer cells

Cancer is first and foremost a disease of excessive tissue growth. Cancer formation is a stepwise process where cells accumulate tumour-promoting properties and form increasingly malignant growths. Cancer cells are able to proliferate indefinitely and without the necessity for external growth-stimulating signals. They have resistance against growth-inhibiting and apoptotic signals and are able to survive and divide despite accumulation of genomic defects. In fact, their genomic instability further promotes tumour progression. Cancer cells facilitate oxygen and nutrient supply to the tumour by promoting angiogenesis in the surrounding tissue and within the tumour and alter their own glucose metabolism to promote growth. They are able to escape destruction by the immune system and are stimulated by pro-inflammatory factors. In the case of malignant tumours cancer cells have the ability to invade surrounding tissues, to form new colonies and to translocate to distant sites of the body via blood and lymph (Hanahan and Weinberg, 2011). The first steps of cancer formation typically involve genetic changes in tumour suppressor genes and proto-oncogenes which regulate cell proliferation, survival or DNA repair. For instance, the central tumour suppressor p53 whose activity is lost or reduced in the majority of cancers regulates all of these processes (Sigal and Rotter, 2000). The proto-oncogenes Ras and Myc in contrast promote proliferation and survival, are converted to oncogenes through genetic alterations which lead to their constitutive activation or expression and are able to transform normal cells into cancer cells (Meyer and Penn, 2008; Pylayeva-Gupta et al., 2011).

Though deregulated tissue growth is the starting point and central characteristic of solid tumours, a deciding factor of cancer lethality is the occurrence of metastasis. The cells of a benign primary tumour are confined within their original tissue and are not able to cross the extracellular matrix and invade surrounding tissues. These types of tumours can be surgically removed without leaving tumour cells behind. In contrast, the cells of a malignant, metastatic tumour become disseminated into neighbouring and distant tissues, can stay in a dormant state for long periods of time and cause relapse long after the patient has been deemed cancer-free. Additionally, the aggressive nature of metastases makes them typically more damaging to their surrounding tissue than primary tumours. Subsequently, benign tumours cause only 10% of cancer-related deaths while the remaining 90% are due to metastatic cancers (Gupta and Massagué, 2006; Weinberg, 2013a).

1.1 Invasion precedes metastasis

Metastasis requires a cancer cell to adapt a migratory phenotype and to invade the surrounding tissue. Cell motility is normal during embryonic development but in the adult body the large majority of cells are non-motile, with the exception of cells of the immune system and cells taking part in repair of damaged tissue. Cell migration involves changes in actin cytoskeleton which is assembled on the leading edge of the cell and disassembled at the trailing edge. At the leading edge actin assembly causes protrusions of the plasma membrane,

lamellipodia which pull the cell forward and filopodia which probe the environment to control the direction of movement (Bisi et al., 2013; Mattila and Lappalainen, 2008; Weinberg, 2013a). Cells may move for instance towards a gradient of a soluble substance (chemotaxis) or towards a substance associated with the extracellular matrix (ECM, haptotaxis) (Haeger et al., 2015). Adhesion of the cell to adjacent cells and the ECM plays an important part. A moving cell cannot be stuck in its bearings but on the other hand some level of adhesion and friction is needed to enable translocation of the cell body. Adhesion is mediated by integrins, cadherins and multiprotein structures which span the plasma membrane and interact with the ECM and adjacent cells. Of central importance are focal adhesions (FA) which connect the ECM to the actin cytoskeleton through integrins and mediate the traction needed for cell movement (Case and Waterman, 2015). The small G proteins of the Rho superfamily (Rho, Rac, Cdc42) control the actin cytoskeleton and cell movement in response to cell signalling. Classically, Rho promotes adhesion and attenuates migration whereas Rac and Cdc42 promote migration by inducing formation of filopodia and lamellipodia. However, in reality the interplay between these G proteins is complex (Burrige and Wennerberg, 2004; Rottner and Stradal, 2011; Sadok and Marshall, 2014). Though the difference between migration and invasion is not often clear-cut, invasion is typically used to describe movement in a three-dimensional environment and through the ECM. This typically requires breakdown of the ECM by proteases secreted by the cancer cell itself, but also by recruited cells of the immune system and surrounding tissues (Kessenbrock et al., 2010).

Most metastatic cancer cells utilize the mesenchymal invasion type where individual cells enter surrounding tissue after ECM digestion. Other types include collective invasion where a group of cells passes the ECM together, and amoeboid invasion where a cell is able to squeeze through the intact ECM (Friedl and Wolf, 2010; Hanahan and Weinberg, 2011). The vast majority of metastatic lethal cancers originate from epithelial tissue where normal cells are well organized and non-motile (Figure 1). They lose this phenotype and gain the ability for mesenchymal invasion through the process of epithelial-mesenchymal transition (EMT) which is heavily implicated in making epithelial tumours metastatic. During EMT an epithelial cell's gene expression profile and subsequently shape, adhesion, motility, polarity and susceptibility to cell death are altered to resemble that of a mesenchymal cell. EMT, and the reverse process of mesenchymal-epithelial transition (MET), are normal phenomena during embryonic development and wound healing where epithelial cells must temporarily be motile. The cytoskeleton of epithelial cells is mainly formed by keratin intermediate filaments and they attach to adjacent epithelial cells with the help of E-cadherin. During EMT, expression of these proteins is lost and replaced with N-cadherin and vimentin, the principal intermediate filament of mesenchymal cells (discussed in 4). Additionally, the post-EMT cells express and secrete the ECM protein fibronectin. The change from keratin to vimentin promotes motility and N-cadherin mediates binding to N-cadherin-expressing stromal cells and therefore facilitates invasion into the surrounding tissue. EMT is induced by typical cancer-related signalling changes as well as hypoxia (discussed in 3) which promote activation of EMT-related

genes and suppression of E-cadherin through the so-called EMT transcription factors (EMT-TFs) (Berx et al., 2007; Nieto, 2011; Weinberg, 2013a; Yang and Weinberg, 2008).

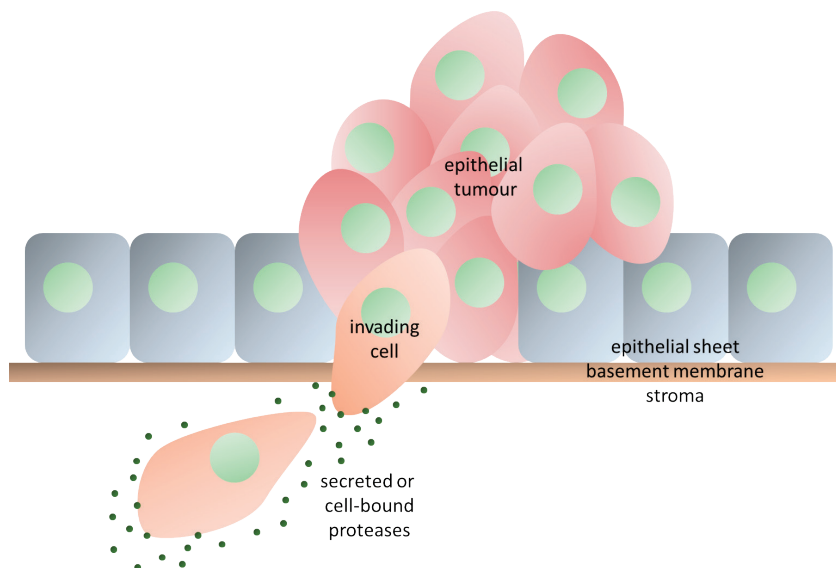


FIGURE 1: The majority of metastatic tumours are of epithelial origin. Normal epithelial cells form a well-organized sheet where cells are polar and non-motile. Invading cells of a malignant epithelial tumour lose these properties and gain the ability to cross the extracellular matrix (basement membrane) and invade the underlying tissue (stroma). The process of epithelial-mesenchymal transition is associated with this change in phenotype.

2 Sphingosine-1-phosphate and sphingosylphosphorylcholine are versatile cancer-related signalling molecules

2.1 The sphingolipid family

2.1.1 Sphingolipid structure and metabolism

Sphingolipids are a group of molecules which contain a sphingoid base backbone: an 18-carbon aliphatic amino alcohol chain containing hydroxyl groups at carbons C1 and C3, an amine at C2 and a double bond between C4-C5. This most basic sphingolipid structure belongs to sphingosine (Sph). The different sphingolipid species can be enzymatically converted to one another in a series of reversible reactions (Figure 2). Entry into the sphingolipid pool happens via *de novo* synthesis of ceramide (Cer) and exit through degradation of sphingosine-1-phosphate (S1P). *De novo* synthesis starts with condensation of serine and palmitoyl-CoA to 3-ketodihydrosphingosine catalysed by serine palmitoyltransferase (Gault et al., 2010; Hannun and Obeid, 2008). This enzyme may also use other amino acids than serine as substrates, mainly glycine or alanine, and this leads to production of toxic deoxy- and deoxymethylsphingolipids related to disease states (Penno et al., 2010; Zuellig et al., 2014). 3-ketohydrosphingosine reductase reduces 3-ketodihydrosphingosine to dihydrosphingosine

(dhSph, sphinganine) which is in turn *N*-acylated on the C2 amine group by ceramide synthase to form dihydroceramide (dhCer). There are six different ceramide synthases (CerS1-6) with different preferences for acyl chain length. Dihydroceramide is desaturated to ceramide by dihydroceramide Δ 4-saturase, forming the C4-C5 double bond typical of sphingolipids. The newly synthesized ceramide may be used by the cell as such or converted into sphingosine or sphingomyelin (SM), phosphorylated to form ceramide-1-phosphate (C1P) or glycosylated to form glycosphingolipids (Gault et al., 2010; Hannun and Obeid, 2008).

Deacylation of ceramide by a ceramidase (CDase) produces sphingosine. There are three types of ceramidases (acid, neutral, alkaline) with different pH optima and localization. Sphingosine can in turn be phosphorylated on the C1 hydroxyl by sphingosine kinase (SphK1/2) to produce sphingosine-1-phosphate (S1P). S1P can either be reverted back to sphingosine by specific S1P phosphatases (SPP1/2) or general lipid phosphate phosphatases (LPP1-3) or degraded by S1P lyase to produce phosphoethanolamine and hexadecanal. Sphingosine can be *N*-acylated to form ceramide by the same ceramide synthases involved in *de novo* synthesis. Ceramide can also be phosphorylated at the C1 hydroxyl by ceramide kinase (CerK) to produce ceramide-1-phosphate. Sphingomyelin is produced when sphingomyelin synthase (SMS) attaches a phosphocholine group to the C1 hydroxyl of ceramide. Sphingomyelinases (SMase) in turn catalyse removal of the phosphocholine and conversion of sphingomyelin back to ceramide. Since ceramide and sphingomyelin contain an acyl chain they both comprise a group of different molecular species whose acyl chains vary in length and saturation level. The sphingomyelinases, like ceramidases, are grouped into acid, neutral and alkaline based on their pH optima and also localize to different cellular compartments (Gault et al., 2010; Hannun and Obeid, 2008). The metabolism of sphingosylphosphorylcholine (SPC) has not been extensively studied but it can be produced from sphingomyelin through deacylation by sphingomyelin deacylase and it is converted to S1P by autotaxin (ATX) which catalyses removal of the choline group (Clair et al., 2003; Murata et al., 1996).

Glycosphingolipids are derivatives of ceramide which contain a sugar head group attached to the C1 hydroxyl of the sphingosine backbone. The simplest forms glucosylceramide (GlcCer) and galactosylceramide (GalCer) contain a singular glucose or galactose moiety and form the glycosphingolipid group of cerebrosides. They are produced by the glycosyltransferase enzymes glucosylceramide synthase (GCS) and galactosylceramide synthase (GalCerS) and converted back to ceramide by glucosylceramidase (GCase) and galactosylceramidase, respectively. Cerebrosides can be further converted to more complex glycosphingolipids by various glycosyltransferases. The group of globosides contain more than one sugar moiety in their head group while gangliosides have one or several sialic acid groups attached to the glucosyl or galactosyl of a cerebroside (Gault et al., 2010; Hannun and Obeid, 2008; Xu et al., 2010).

Sphingomyelin, ceramide and glycosphingolipids are restricted to membranes due to their hydrophobic sphingosine and acyl chains and require transfer proteins or vesicles in order to move from membrane to membrane. However, these lipids are not only structural building

blocks but also participate in cell signalling. As components of functional membrane domains they affect localization and interaction of receptors and other signalling molecules. They can also themselves bind target proteins with high specificity and even function as receptors for extracellular ligands (Contreras et al., 2012; Lingwood, 2011; Lingwood and Simons, 2010; Stancevic and Kolesnick, 2010). In contrast, the single chain sphingolipids are able to diffuse into the cytoplasm and tissue fluids. Sphingosine is mostly associated with membranes but S1P and SPC, which contain a hydrophilic head group, are more water soluble (García-Pacios et al., 2009; Hannun and Obeid, 2008). The interconversion of different sphingolipid species to one another facilitates maintenance of homeostasis and decreases the risk of excess accumulation of any one member of the family. Sphingolipidoses are lipid storage diseases which develop when membrane sphingolipids do accumulate in cellular compartments due to defects in enzymes responsible for their metabolism. The most famous of such diseases are Niemann-Pick disease (NPD) type A and B where defects in acid sphingomyelinase function cause accumulation of sphingomyelin in the lysosomes. Type A NPD, which is caused by a complete lack of acid sphingomyelinase activity, leads to death in early childhood (Sabourdy et al., 2008). Glycosphingolipids are abundant in the nervous system and involved in neuronal development and function. Consequently, abnormalities in their metabolism cause several neurodegenerative disorders (Xu et al., 2010; Yu et al., 2009).

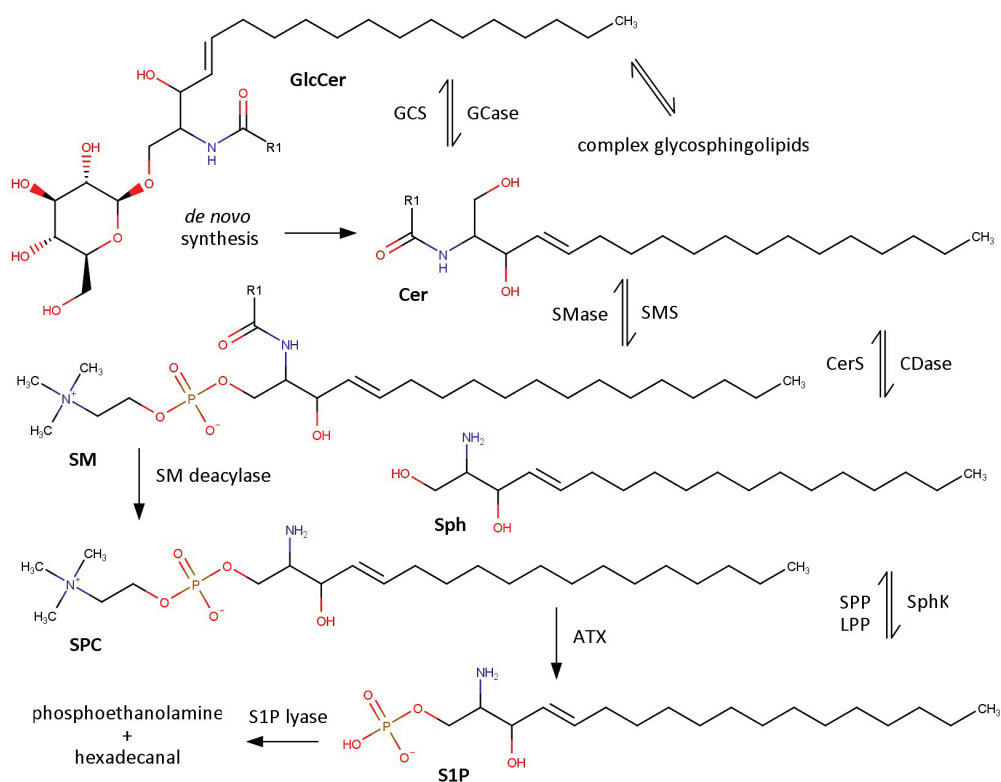


FIGURE 2: Sphingolipid structure and metabolism (created with MarvinSketch).

2.2 S1P has a central role in tumour biology

2.2.1 Increased SphK1 expression and activity is common in tumours

Sphingosine kinase 1 (SphK1) is considered oncogenic and for a good reason. Elevated SphK1 levels have been detected in a wide variety of cancers, including cancers of the breast, thyroid, lung, stomach, colon and rectum, ovaries and uterus, kidney, skin, blood, brain, head and neck, salivary gland and prostate. Importantly, high SphK1 expression in tumours is also related to metastasis, chemoresistance, short disease recurrence times and patient mortality (Albinet et al., 2014; Bayerl et al., 2008; Facchinetti et al., 2010; French et al., 2003; Guan et al., 2011; Johnson et al., 2005; Kawamori et al., 2006; Li et al., 2008a; Li et al., 2009a; Liu et al., 2010a; Long et al., 2015; Malavaud et al., 2010; Ohotski et al., 2012; Ruckhäberle et al., 2008; Van Brocklyn et al., 2005; Watson et al., 2010). The tumour-promoting action of SphK1-S1P has also been demonstrated in several studies conducted with animal models where inhibition, knockdown or deletion of SphK1 attenuates tumour growth (French et al., 2006; Kohno et al., 2006; Ponnusamy et al., 2012; Shirai et al., 2011; Sinha et al., 2011). SphK1 also gives xenograft tumours and cancer cells resistance against radiotherapy and common chemotherapy agents such as tamoxifen, imatinib and cisplatin (Baran et al., 2007; Matula et al., 2015; Salas et al., 2011; Sinha et al., 2011; Watson et al., 2010). Exogenous expression of SphK1 has been shown to induce malignant transformation of normal cells through activation of H-Ras (Xia et al., 2000) and SphK1 has been implicated in maintaining the Warburg effect where the glucose metabolism of cancer cells is altered to facilitate growth and survival (Watson et al., 2013). SphK1-S1P also mediates communication of tumours with their microenvironment. Melanoma tumours with high SphK1 expression secrete S1P and induce differentiation of neighbouring fibroblasts into myofibroblasts which have elevated SphK1 expression and whose subsequent S1P secretion promotes metastasis of the nearby melanoma cells (Albinet et al., 2014). In thyroid cancer cells exogenous SphK1 over-expression promotes cell motility via autocrine signalling (Bergelin et al., 2009). Though there is vast evidence implicating SphK1 in tumour growth and metastasis it is not considered a classical oncogene as it is rarely mutated in cancers. Instead, cancer cells are said to have a non-oncogene addiction to SphK1 where S1P is in many ways beneficial for cancer cells but not the cause of cancer initiation (Vadas et al., 2008). S1P in general promotes cell proliferation and survival and forms a so-called sphingolipid rheostat with tumour suppressive ceramide.

2.2.1.1 The sphingolipid rheostat

The sphingolipid rheostat is a concept in which the balance between different sphingolipid species with opposite effects on cell proliferation and survival determines cell fate (Newton et al., 2015). On one side of the balance are the anti-proliferative and pro-apoptotic ceramide and sphingosine and on the other side the generally pro-proliferative and anti-apoptotic S1P. Ceramide regulates cell signalling by binding signalling proteins at cellular membranes or through functional membrane domains such as ceramide-rich platforms which affect localization of receptors and other signalling molecules (Hannun and Obeid, 2008; Stancevic and Kolesnick, 2010). For instance, ceramide stimulates pro-apoptotic members (Bad, Bid,

Bax) and inhibits anti-apoptotic members (Bcl-2, Mcl-1) of the Bcl-2 family through regulation of kinases, phosphatases and proteases which target these proteins (Heinrich et al., 2004; Nica et al., 2008; Ruvolo et al., 1999; Xin and Deng, 2006; Zundel and Giaccia, 1998). In contrast, S1P signalling down-regulates several pro-apoptotic Bcl-2 members (Bad, Bax, Bim) and up-regulates anti-apoptotic ones (Bcl-2, Mcl-1) (Avery et al., 2008; Li et al., 2008b; Limaye et al., 2005). The sphingolipid rheostat has a role in current cancer therapy. Both chemo- and radiotherapy induce cellular stress responses which induce ceramide production and ceramide-mediated apoptosis while resistance to therapy often involves an ability to resist ceramide accumulation (Beckham et al., 2013). As mentioned earlier, SphK1 and S1P in contrast are involved in resistance against cancer therapeutics. Accordingly, cancer cells susceptible to chemotherapy typically have a higher Cer/S1P ratio than drug-resistant cells (Baran et al., 2007; Sobue et al., 2008). However, though the rheostat concept is valid as a general rule all S1P signalling is not cancer-promoting and ceramide species with certain acyl chain lengths have been found to promote proliferation and survival of cancer cells (Hartmann et al., 2012; Senkal et al., 2010).

2.2.2 S1P in the blood and tissues

S1P is released into the bloodstream mainly by erythrocytes but also platelets and vascular endothelial cells (Bode et al., 2010; Venkataraman et al., 2008). The high S1P level in erythrocytes is based on their lack of the S1P-degrading enzymes S1P lyase and S1P phosphatase (Ito et al., 2007), while platelets also lack S1P lyase and additionally have high SphK activity (Yatomi et al., 1997). The S1P concentration of human blood has been measured in numerous studies using mass spectrometry techniques and the concentration of S1P in healthy subjects ranges from approximately 200 nM up to 2 μ M (Bode et al., 2010; Frej et al., 2015; Hammad et al., 2010; Karuna et al., 2011; Murata et al., 2000a, 2000b; Scherer et al., 2009; Schmidt et al., 2006). The S1P level is clearly higher in serum than in plasma in studies comparing the two which is due to release of S1P from activated platelets (Yatomi et al., 1997). S1P circulating in the blood is bound to carrier proteins: approximately 70% to apolipoproteins (60% HDL, 10% LDL) and 30% to albumin (Aoki et al., 2005; Murata et al., 2000a). Due to its high production in cells of the blood and vasculature, S1P levels are highest in the blood, lower in lymph and lower still in the interstitial fluid which surrounds cells in tissues (Hla et al., 2008). All cell types are able to secrete S1P but in the case of other than hematopoietic and vascular cells, the released S1P is confined into the interstitial space and functions in an autocrine and paracrine manner activating S1P receptors on the secreting cell itself and adjacent cells. Though the SphK1-S1P axis in general is cancer-promoting, in prostate cancer patients a relation between low blood S1P levels and patient mortality was found. Apparently this was due to decreased expression of SphK1 in erythrocytes which may be involved in prostate cancer-related anaemia (Nunes et al., 2012).

2.2.3 S1P and the immune system

S1P has a central role in both development and maintenance of the immune system and as a mediator of immune responses. This regulation is mediated by the S1P gradient present

between blood, lymph and tissues. The gradient promotes egress of lymphocytes into the blood and lymph from the bone marrow, thymus and secondary lymphoid organs. Lymphocytes detect the gradient through their S1P₁ receptors and migrate towards S1P. Locally and transiently S1P levels may rise in response to local platelet activation at sites of injury or inflammation. Inflammation promotes vascular permeability and leakage of S1P into tissues which further induces S1P-related pro-inflammatory signalling (Hla et al., 2008). The pro-inflammatory cytokine TNF α promotes SphK1 activity and S1P regulates expression or activity of numerous pro-inflammatory factors such as NF- κ B, interleukins, cyclo-oxygenase 2 (COX-2), the COX-2 product prostaglandin E₂ (PGE₂) and intercellular adhesion molecule-1 (ICAM-1) (Alvarez et al., 2010; Galvani et al., 2015; Kitano et al., 2006; Lee et al., 2010; Lin et al., 2007; Liu et al., 2015a; Pettus et al., 2003; Spiegel and Milstien, 2011; Xia et al., 1998). NF- κ B, COX-2 and PGE₂ are also potent cancer-related factors which promote cell proliferation (Ben-Neriah and Karin, 2011; Wang and Dubois, 2010; Weinberg, 2013b) and consequently S1P has a role in cancer-promoting sustained inflammation. For example, S1P is involved in chronic inflammation of the colon (colitis) which promotes the formation of colorectal cancer (Liang et al., 2013; Snider et al., 2009). S1P signalling is implicated in the development of other autoimmune diseases as well. For example, inhibition of S1P production or transport in mice protects them from not only colitis but also allergic asthma, arthritis and autoimmune encephalopathy, which is a model for multiple sclerosis (Baker et al., 2010; Donoviel et al., 2015; Lai et al., 2008a, 2008b; Price et al., 2013).

2.2.4 S1P regulates angiogenesis

SphK1/2 and S1P₁ knockout mice have revealed that S1P signalling is necessary for angiogenesis during embryonic development. Embryos of these mice show defects in their vasculature and are not viable (Liu et al., 2000a; Mizugishi et al., 2005). Consistently, S1P signalling also promotes tumour angiogenesis. S1P-neutralizing antibodies have been shown to reduce angiogenesis in mouse eyes and xenograft tumours (O'Brien et al., 2009; Visentin et al., 2006). S1P₁ expression is increased in tumour blood vessels and its inhibition or knockdown decreases vascularization (Chae et al., 2004; LaMontagne et al., 2006; Patmanathan et al., 2015). The main regulator of blood vessel formation is vascular endothelial growth factor (VEGF), and S1P and VEGF signalling communicate in many ways. In bladder cancer cells VEGF stimulates SphK1 activity through VEGF receptor 2 (VEGFR2) and S1P in turn mediates VEGF-induced Ras activation (Wu et al., 2003b). In ML-1 thyroid cancer cells S1P promotes VEGF secretion, VEGFR2 expression and VEGFR2 activity while VEGFR2 promotes S1P₁ expression and mediates S1P-induced migration. VEGFR2 and S1P₁ also form a complex which mediates both S1P- and VEGF-induced signalling and cell motility (Balthasar et al., 2008; Bergelin et al., 2010).

2.2.5 Sphingosine kinases (SphK1/2)

As mentioned, sphingosine kinases phosphorylate sphingosine on the C1 hydroxyl to produce S1P. The two sphingosine kinase isoforms, SphK1 and SphK2, differ in their structure, localization, substrate specificity, tissue distribution and expression pattern during embryonic

development (Liu et al., 2000b; Melendez et al., 2000; Pitson et al., 2000). Despite these differences, the isoforms seem to have a high level of redundancy as knockout of neither SphK1 nor SphK2 causes a severe phenotype whereas double knockout mice are embryonic lethal. The double knockout embryos die due to defects in development of the nervous system and vasculature (Allende et al., 2004; Mizugishi et al., 2005). Both SphK1 and SphK2 have constitutive basal activity but they are further activated by e.g. post-translational modifications which affect both their catalytic activity and localization (Chan and Pitson, 2013).

2.2.5.1 SphK1

Of the two sphingosine kinase isoforms SphK1 has been much more extensively studied and, as discussed earlier, it is considered a significant regulator of tumour biology. It is primarily a cytosolic protein but has also been found in the nucleus, as an exoenzyme in the extracellular space and associated with the plasma membrane where the substrate sphingosine is readily available. Localization at the plasma membrane through increased affinity for membrane phospholipids is often involved in increased S1P production by SphK1. Phosphorylation of SphK1 on Ser225 by ERK1/2 increases both catalytic activity and plasma membrane translocation and mediates the oncogenic function of SphK1 (Lidington et al., 2009; Pitson et al., 2003, 2005; Stahelin et al., 2005). On the other hand, activation of the proto-oncogene K-Ras or $G\alpha_q$ proteins induce SphK1 plasma membrane localization and increased S1P production independently of Ser225 (ter Braak et al., 2009; Gault et al., 2012). Calcium also regulates SphK1 plasma membrane translocation through calcium- and integrin-binding protein 1 (CIBP1) and calmodulin which are stimulated by Ca^{2+} binding to interact with SphK1 (Jarman et al., 2010; Sutherland et al., 2006). SphK1 binds to the membrane phospholipid phosphatidic acid (PA) and therefore increased PA production by phospholipase D increases SphK1 membrane localization without modification of the protein itself (Delon et al., 2004). The Src family tyrosine kinases Fyn and Lyn have been shown to bind and activate SphK1 in mast cells but this does not involve tyrosine phosphorylation of SphK1 (Olivera et al., 2006; Urtz et al., 2004). Other stimulatory binding partners of SphK1 include δ -catenin and eukaryotic elongation factor 1A (eEF1A) (Fujita et al., 2004; Leclercq et al., 2008). Additionally, hypoxia-induced production of reactive oxygen species (ROS) activates SphK1 by a yet unknown mechanism and hypoxia increases SphK1 gene expression through hypoxia-inducible factor (HIF-1/2) activity (Ader et al., 2008, 2009; Anelli et al., 2008; Schwalm et al., 2008). SphK1 gene expression is also induced by the oncogenic transcription factor AP-1 whose activity S1P receptors stimulate in a positive feedback loop (Huang et al., 2014a; Paugh et al., 2009; Van Brocklyn et al., 2005).

2.2.5.2 SphK2

In comparison to SphK1, SphK2 contains a longer N-terminus and a central proline-rich segment specific to SphK2. The extended N-terminus contains a nuclear localization sequence (NLS) and the proline-rich region a nuclear export sequence (NES). Consequently, SphK2 is primarily a nuclear enzyme but can also be found in the cytosol and in stress conditions in the

ER (Neubauer and Pitson, 2013). The area of Ser225 which is important for SphK1 activation is not intact in SphK2 but ERK1/2 still activates SphK2, possibly through phosphorylation of another serine residue (Hait et al., 2007). Many of the above-mentioned SphK1 binding partners also interact with and activate SphK2. Due to differences in their sphingosine-binding pockets, SphK2 is more promiscuous than SphK1 and able to efficiently phosphorylate substrates such as phytosphingosine and the sphingosine-analogue FTY720 (Liu et al., 2000b; Paugh et al., 2003). Unlike SphK1, SphK2 contains a BH3 domain which enables it to function as a BH3-only protein and bind and inhibit anti-apoptotic Bcl-2 family members such as Bcl-xL (Liu et al., 2003). SphK2 also stimulates the pro-apoptotic Bcl-2 protein Bak (Chipuk et al., 2012). Accordingly, SphK2 was initially deemed pro-apoptotic. However, in many studies a pro-survival, pro-proliferative and/or migratory effect as well as a role in chemoresistance similar to SphK1 have been shown (Neubauer and Pitson, 2013). In some cases SphK2 knockdown has inhibited cancer cell proliferation and migration even more potently than knockdown of SphK1 (Van Brocklyn et al., 2005; Gao and Smith, 2011). Additionally, nuclear S1P produced by SphK2 regulates proto-oncogene (c-Myc, c-Fos) and tumour suppressor (p21) gene expression via histone deacetylases (HDAC1/2) and promotes indefinite cancer cell proliferation and tumour growth by binding and stabilizing the catalytic telomerase subunit hTERT (Panneer Selvam et al., 2015). Recently, SphK2, but not SphK1, was shown to mediate EGF-induced cancer cell invasion via activation of the ezrin-radixin-moesin (ERM) proteins which control interaction of the actin cytoskeleton and the plasma membrane (Adada et al., 2015).

2.2.6 S1P transport

The majority of S1P functions outside of the vascular system occur in an autocrine or paracrine manner through S1P receptors. For this purpose, S1P produced inside the cell must be exported outside. SphK1 often produces S1P at the inner surface of the plasma membrane which already facilitates S1P transport. But due to its hydrophilic nature, S1P cannot freely traverse the plasma membrane and must be actively transported by transporter proteins. Initially several members of the ATP-binding cassette (ABC) family were shown to transport S1P across the plasma membrane (Mitra et al., 2006; Sato et al., 2007; Takabe et al., 2010) and later the spinster-2 (SPNS2) protein has been identified as a S1P transporter (Fukuhara et al., 2012; Hisano et al., 2011; Osborne et al., 2008). While the ABC transporters target a wide variety of different molecules, SPNS2 seems to be specific to phosphorylated sphingoid bases. Importantly, SPNS2 has been shown to control plasma S1P levels and formation of the S1P gradient needed for lymphocyte egress (Fukuhara et al., 2012). It is also able to transport the S1P-analogue p-FTY720 (Hisano et al., 2011). FTY720 in turn has been shown to regulate expression of the ABC family member ABCA1 (Blom et al., 2010).

2.2.7 S1P receptors

Five high affinity transmembrane receptors have been identified for S1P (S1P₁₋₅). They are G protein-coupled receptors (GPCRs) and belong to the endothelial differentiation gene (EDG) family of lysophospholipid receptors. The EDG family also includes receptors for

lysophosphatidic acid (LPA) and several orphan receptors (OGR1, GPR4, G2A, GPR12, GPR3, GPR6) many of which are modulated by S1P and/or SPC (Meyer zu Heringdorf and Jakobs, 2007).

2.2.7.1 G protein-coupled receptors

GPCRs contain seven hydrophobic transmembrane alpha-helices (TM1-7) connected by extra- and intracellular loops, an extracellular N-terminus and an intracellular C-terminus. The intracellular part of the receptor is in contact with a heterotrimeric G protein complex ($G\alpha\beta\gamma$). Ligand binding causes a conformational change which enables the receptor to function as a guanine nucleotide exchange factor (GEF) for the $G\alpha$ subunit and facilitate $G\alpha$ -GTP binding. This in turn causes dissociation of the $G\alpha\beta\gamma$ complex into $G\alpha$ and $G\beta\gamma$. These subunits go on to modulate separate signalling pathways. Four groups of $G\alpha$ proteins couple to GPCRs: $G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$. $G\alpha_{i/o}$ inhibits production of the second messenger cAMP by the enzyme adenylate cyclase (AC) and subsequently cAMP-related activity of protein kinase A (PKA) whereas $G\alpha_s$ stimulates AC, increases cAMP levels and causes PKA activation. $G\alpha_{q/11}$ activates phospholipase C (PLC) which catalyses the cleavage of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into membrane-bound diacylglycerol (DAG) and free inositol 1,4,5-trisphosphate (IP_3). IP_3 diffuses into the cytoplasm, activates IP_3 receptors on the ER and consequently induces release of Ca^{2+} into the cytosol from intracellular stores. Ca^{2+} and/or DAG in turn activate different protein kinase C (PKC) isoforms. $G\alpha_{12/13}$ activates the small G protein Rho. The dimeric $G\beta\gamma$ subunits also have a signalling function and can activate the MAPK and PI3K-Akt pathways as well as PLC. $G\beta\gamma$ -dependent signalling has been attributed especially to $G\beta\gamma$ subunits associated with $G\alpha_{i/o}$ (Smrcka, 2008; Wettschureck and Offermanns, 2005).

2.2.7.2 The different S1P receptors have both similar and opposing signalling functions

The signalling induced by extracellular S1P depends on the S1P receptor composition of the cell, the availability of coupled G proteins as well as receptor dimerization. The S1P receptors couple to $G_{i/o}$, $G_{q/11}$ or $G_{12/13}$ in different combinations and with different preference (Figure 3). Accordingly, the receptors have similar and opposite as well as cumulative effects on cell signalling. The most striking difference is related to migration as receptors $S1P_1$ and $S1P_3$ predominantly induce migration through $G_{i/o}$ -mediated Rac activation whereas $S1P_2$ typically inhibits migration via $G_{12/13}$ -Rho (Taha et al., 2004). S1P receptors form homodimers and heterodimers with each other and other EDG family members and also interact with receptor tyrosine kinases (Pyne and Pyne, 2008; Zaslavsky et al., 2006). In order to quench S1P signalling, activated S1P receptors are internalized and either recycled back to the plasma membrane or degraded. As shown for $S1P_1$, ligand binding, internalization and degradation of the receptors are regulated by post-translational modifications such as phosphorylation, ubiquitination, palmitoylation and glycosylation (Kohno et al., 2002; O'Sullivan and Dev, 2013; Ohno et al., 2009; Oo et al., 2007, 2011; Watterson et al., 2002).

Studies concerning the mechanism of S1P-S1PR binding have also mainly been conducted with S1P₁. Based on early structure predictions of S1P₁ a so called dual-binding model of S1P-S1PR interaction was proposed where the phospho group of S1P binds one site while the hydrophobic, aliphatic sphingosine chain binds a hydrophobic binding pocket. The hydrophobic binding pocket is not entirely conserved between the different S1P receptors which explains their different affinities for S1P and related ligands (Fujiwara et al., 2007; O'Sullivan and Dev, 2013; Parrill et al., 2000). After the 3D crystallization of S1P₁ it was proposed that while S1P comes into contact with the receptor in the extracellular space it will then bind to the hydrophobic binding pocket by means of lateral diffusion within the plasma membrane. Comparison of binding properties of different ligands points to the amino group at C2 and the hydroxyl at C3 to be essential for S1P-S1P₁ binding (Hanson et al., 2012; Parrill et al., 2012).

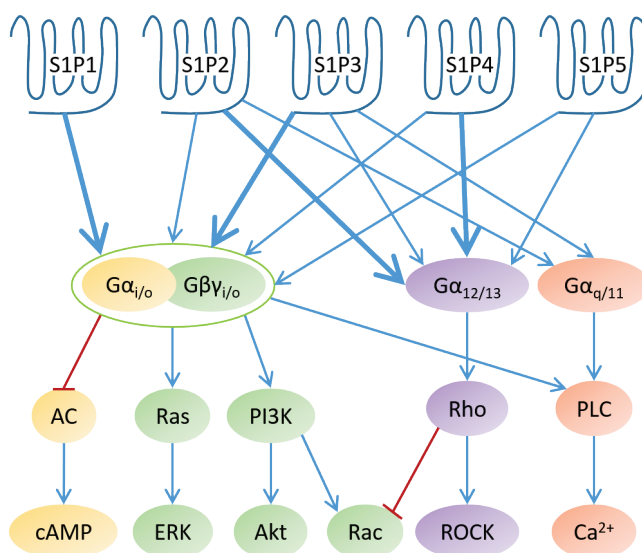


FIGURE 3: An overview of the classical signalling evoked by S1P receptors. Bold arrows indicate preferential coupling. Modified from Taha et al. (2004).

2.2.7.2.1 S1P₁ (EDG1)

S1P₁ was the first S1P receptor to be identified and it is also the most extensively studied and best characterized S1P receptor. It is ubiquitously expressed and only couples to G_{i/o}, which has made study of its function straightforward in comparison to the other S1P receptors. As mentioned, S1P₁ is a central regulator of angiogenesis both during normal development and cancer progression. S1P₁-knockout mice are embryonic lethal due to defects in vascular development (Liu et al., 2000a), S1P₁ is up-regulated in tumour vessels and knockdown or inhibition of S1P₁ attenuates tumour vascularization (Chae et al., 2004; LaMontagne et al., 2006). As S1P₁ only couples to G_{i/o} it promotes migration via PI3K-Rac activation (Kölsch et al., 2008; Taha et al., 2004). For instance, in the kidney cancer Wilms tumour and in S1P₁-transfected Chinese hamster ovary (CHO) cells, S1P₁ was shown to stimulate chemotaxis via

both Rac and PI3K (Li et al., 2009b; Okamoto et al., 2000a) and in xenografted melanoma cells S1P₁ mediated metastasis via Rac (Yamaguchi et al., 2003). S1P₁ promotes proliferation through ERK1/2 (Pyne and Pyne, 2010) and has been shown to facilitate cancer cell survival through both ERK1/2 and PI3K-Akt signalling and regulation of Bcl-2 family members (Bim, Mcl-1) (Rutherford et al., 2013)

S1P₁ mediates much of the effects of S1P in the immune system. During lymphocyte egress, lymphocytes use S1P₁ to detect S1P in their surroundings and move towards it (Matloubian et al., 2004). S1P₁ also mediates regulation of inflammatory factors by S1P. For instance, S1P₁ promotes inflammation in the synovial tissue of rheumatoid arthritis patients by increasing COX-2 expression and subsequently PGE₂ production (Kitano et al., 2006). On the other hand, S1P₁ has an anti-inflammatory effect in chondrocytes, cells of the cartilage, which are also associated with arthritis (Moon et al., 2012). Also in endothelial cells S1P₁ activity may either promote or attenuate inflammation by regulating expression of ICAM-1 and subsequently the interaction of leukocytes and endothelial cells (Galvani et al., 2015; Lin et al., 2007). S1P-S1P₁ and the oncogenic transcriptional activator signal transducer and activator of transcription-3 (STAT3) form a pro-inflammatory and tumour-promoting positive feedback loop. S1P-S1P₁ signalling stimulates STAT3 activity and STAT3 in turn increases gene expression of S1P₁ as well as the pro-inflammatory cytokine IL-6 (Lee et al., 2010). Because of its central role in the immune system, S1P₁ inhibitors are immunosuppressive. One such compound, FTY720, is currently in clinical use as a drug for multiple sclerosis (MS) and will be discussed in more detail later.

2.2.7.2.2 S1P₂ (EGD5)

S1P₂ is a ubiquitous receptor which couples preferably to G_{12/13} but also to G_{i/o} and G_{q/11}. Through G_{α12/13} S1P₂ activates the small G protein Rho which makes it a predominantly anti-migratory receptor. Rho inhibits migration either through activation of Rho-associated kinase (ROCK) or inhibition of Rac. For instance, S1P₂-Rho-mediated inhibition of migration involves ROCK activation in glioblastoma cells (Lepley et al., 2005) and Rac inhibition in melanoma, vascular smooth muscle and S1P₂-transfected CHO cells (Arikawa et al., 2003; Okamoto et al., 2000; Takashima et al., 2008; Yamaguchi et al., 2003). Through Rho-ROCK S1P₂ promotes formation of stress fibers which are contractile actin filament bundles involved in adhesion and inhibition of migration (Lepley et al., 2005; Sanchez et al., 2007). Production of phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) at the plasma membrane by PI3K promotes Rac activity and S1P₂-Rho has been shown to stimulate phosphatase and tensin homolog (PTEN) which counteracts PI3K signalling by dephosphorylating PIP₃ (Kölsch et al., 2008; Sanchez et al., 2005). In contrast to S1P₁ and S1P₃, which promote lymphocyte migration and egress, S1P₂ attenuates macrophage migration and trafficking to sites of inflammation and this involves decreased Akt phosphorylation (Michaud et al., 2010). S1P-induced vascular permeability also involves S1P₂-Rho-mediated PI3K inhibition in endothelial cells (Sanchez et al., 2007). Despite the classical anti-migratory role of S1P₂ there are also opposite examples. As mentioned earlier, SphK2 was recently shown to mediate EGF-induced migration of HeLa

cells through activation of ERM proteins. This pro-migratory effect also involves S1P₂ activation (Adada et al., 2015; Orr Gandy et al., 2013). In oesophageal cancer cells S1P₂ was found to mediate TGFβ-induced migration and invasion (Miller et al., 2008). S1P₂ activity has also been shown to increase the metastatic potential of bladder cancer cells by negatively regulating breast cancer metastasis-suppressor 1 (Brms1) which is an anti-metastatic tumour suppressor (Ponnusamy et al., 2012). In glioma cells S1P₂ has been shown to on the one hand inhibit cell motility through Rho-ROCK signalling and stress fibre formation but on the other hand facilitate invasion via effects on adhesion (Young and Van Brocklyn, 2007).

S1P₂ also regulates function of the immune system, liver, kidneys, central nervous system (CNS), vasculature and muscles (Adada et al., 2013). S1P₂-knockout mice are viable but become deaf due to loss of sensory hair cells and show some changes in their cardiovascular system and regeneration of the liver and skeletal muscle (Germinario et al., 2012; Kono et al., 2004; Lorenz et al., 2007; Means and Brown, 2009; Serriere-Lanneau et al., 2007). Depending on their genetic background, some S1P₂-KO mice experience severe seizures due to changes in CNS function (Akahoshi et al., 2011; MacLennan et al., 2001). With age the S1P₂-KO mice develop B-cell lymphomas and loss of S1P₂ expression has also been reported for 26% of human B-cell lymphomas (Cattoretti et al., 2009). S1P₂ regulates proliferation and has an inhibitory effect in Wilms tumour cells and in cultured ER(-) breast cancer cells (Li et al., 2008c; Ohotski et al., 2014). Remarkably, in the latter case the anti-proliferative effect was mediated by translocation of S1P₂ to the nucleus. Interestingly, bile salts have been shown to bind to and activate S1P₂ in the liver. Through S1P₂ bile salts induce ERK1/2 and Akt signalling, regulate hepatic gene expression and up-regulate SphK2. S1P₂-KO and SphK2-KO mice develop fatty livers easier than wild type mice which reveals their role in regulation of fatty acid metabolism in the liver (Nagahashi et al., 2015; Studer et al., 2012). Furthermore, in cholangiocarcinoma, a rare malignant cancer of the bile ducts, S1P₂ promotes pro-inflammatory NF-κB and COX2/PGE₂ signalling and tumour growth (Liu et al., 2015a). Another cancer-promoting role for S1P₂ has been shown in chronic myeloid leukaemia (CML) where S1P₂ promotes Bcr-Abl stability and imatinib resistance through PP2A inhibition (Salas et al., 2011).

2.2.7.2.3 S1P3 (EDG3)

S1P₃ is the third ubiquitous S1P receptor. It couples to G_{i/o}, G_{q/11} and G_{12/13} but prefers G_{i/o}, which makes its function similar to that of S1P₁ (Taha et al., 2004). Like S1P₁, S1P₃ also has a role in regulation of lymphocyte function, inflammation and the vasculature and is primarily a pro-migratory receptor. For instance, S1P₃ together with S1P₁ was shown to be elevated in astrocytes found in multiple sclerosis lesions or grown under pro-inflammatory conditions (Van Doorn et al., 2010). In S1P₃-transfected CHO cells S1P₃ induces migration through PI3K and Rac similarly to S1P₁. However, the coupling of S1P₃ to G_{q/11} and G_{12/13} expectedly causes differences between effects of the two receptors. Knockout of S1P₃ in mice was not embryonic lethal and caused no clear changes in phenotype (Ishii et al., 2001). If G_{i/o} signalling is blocked S1P₃ signals through G_{12/13} similarly to S1P₂ (Sugimoto et al., 2003) and S1P-induced vascular contraction mediated by S1P₃ involves activation of Rho and increased intracellular Ca²⁺

instead of typical $G_{i/o}$ -related signalling (Murakami et al., 2010). In relation to cancer, S1P₃ has been shown to promote EGF signalling by either up-regulating EGFR or trans-activating it in lung and breast cancer cells (Hsu et al., 2012; Sukocheva et al., 2006). In ER(+) breast cancer patients high tumour S1P₃ expression, together with SphK1 and S1P₁, is related to poor prognosis, shorter disease recurrence times and tamoxifen resistance (Watson et al., 2010).

2.2.7.2.4 S1P₄ (EDG6)

S1P₄ has not been studied as much as the ubiquitous S1P₁₋₃. S1P₄ expression has been detected in lymphoid and hematopoietic tissue and in the lung (Gräler et al., 1998) and it couples preferably to $G_{12/13}$ but also to $G_{i/o}$. In S1P₄-transfected CHO cells, S1P₄ mediates Rho activation, cell rounding and stress fibre formation (Gräler et al., 2003). S1P₄ is involved in S1P responses in the immune system as it has been in many ways implicated in regulation of T lymphocyte function, including their proliferation, migration, differentiation and cytokine production. On the one hand, S1P₄ has been shown to have an immunosuppressive effect through inhibition of T cell proliferation and cytokine secretion, but on the other hand the loss of S1P₄ protected S1P₄-KO mice against colitis (Schulze et al., 2011; Wang et al., 2005). In hematopoietic tissue S1P₄ mediates the normal development of platelet-producing megakaryocytes in the bone marrow (Golfier et al., 2010). In relation to cancer, in ER(-) breast cancer high S1P₄ expression is associated with short disease recurrence times and poor survival due to stimulation of HER2-ERK1/2 signalling (Ohotski et al., 2012). Interestingly, S1P₄ may promote progression of ER(-) breast cancer by preventing S1P₂ from translocating from the plasma membrane into the nucleus to inhibit cell growth. This growth-stimulating effect of S1P₄ is induced by SphK2-produced S1P and may involve heterodimerization of S1P₄ and S1P₂ (Ohotski et al., 2014). S1P₄ also regulates apoptosis, though this has been shown in normal myoblasts and resulted in muscle tissue degeneration (Cencetti et al., 2013).

2.2.7.2.5 S1P₅ (EDG8)

Like S1P₄, S1P₅ has not been extensively studied. Studies concerning S1P₅ have mostly been conducted in cells of the brain and leukocytes but its mRNA expression has been detected also in the spleen, lung, placenta, aorta and foetal tissues (Im et al., 2001). S1P₅ couples to $G_{i/o}$ and $G_{12/13}$ and like the other S1P receptors it is also implicated in regulation of the immune system and inflammation. S1P₅ mediates natural killer cell egress from the thymus and trafficking to sites of inflammation, as revealed by S1P₅-knockout mice and so-called Duane mice which exhibit very low S1P₅ expression (Jenne et al., 2009; Walzer et al., 2007). In brain endothelial cells S1P₅ maintains an immunquiescent state by inhibiting NF- κ B-dependent expression of pro-inflammatory factors. S1P₅ activity of these cells is involved in formation and maintenance of the blood brain barrier and makes the barrier less permeable (van Doorn et al., 2012). Interestingly, S1P₅ may mediate trafficking of monocytes from the bone marrow to the periphery of the body independently of S1P (Debien et al., 2013). In relation to cancer, S1P₅ has been identified as a prognostic factor whose high expression level in glioblastoma samples correlates with poor patient survival (Quint et al., 2014). S1P₅ mediates S1P-induced autophagy in prostate cancer cells. Autophagy may either attenuate cancer progression or

help tumours survive starvation and in these cells autophagy has a pro-survival role (Chang et al., 2009; Huang et al., 2014b). On the other hand, S1P₅ has been shown to inhibit proliferation and migration of oesophageal cancer cells (Hu et al., 2010). S1P₅ can be localized to the centrosomes as well as the plasma membrane and centrosomal S1P₅ may be a target for intracellular S1P as SphK1/2 co-localize at the centrosomes with S1P₅. The G_{i/o} activity provided by S1P₅ may be involved in regulation of microtubules and the mitotic spindle during cell division (Gillies et al., 2009).

2.2.8 Intracellular S1P

The first regulatory targets of intracellular S1P were identified in 2009 when S1P produced by SphK2 in the nucleus was shown to directly bind nuclear histone deacetylases (HDAC1/2). HDAC1/2-mediated deacetylation of histones epigenetically represses gene expression. SphK2 and S1P together bind to and inhibit HDAC1/2 which leads to decreased acetylation of histone H3 and subsequent increased gene expression from certain promoters, including those controlling p21 and c-Fos (Hait et al., 2009). In the following years several other intracellular targets have been found. Tumour-necrosis factor receptor-associated factor 2 (TRAF2), an E3 ubiquitin ligase, binds both SphK1 and S1P and requires S1P for its K63-linked ubiquitin ligase activity. TRAF2 in turn stimulates NF- κ B activity by facilitating degradation of its inhibitor I κ B α (Alvarez et al., 2010). Similarly, SphK1 is able to form a complex with the apoptosis inhibitor cIAP2 and S1P induces its K63-linked ubiquitin ligase activity also (Harikumar et al., 2014). S1P produced by SphK2 at the mitochondria binds the mitochondrial protein prohibitin 2 (PHB2) to facilitate mitochondrial respiration (Strub et al., 2011). Recently, SphK2-produced S1P was shown to bind hTERT, the catalytic subunit of telomerase, and increase its stability (Panneer Selvam et al., 2015). Moreover, as mentioned above, S1P₅ localizes to the centrosomes together with SphK1/2 and S1P₂ is able to translocate to the nucleus and these receptors may well be further targets for intracellular S1P (Gillies et al., 2009; Ohotski et al., 2014).

2.2.9 S1P signalling as a drug target

The central role of S1P signalling in the normal function of the immune system and vasculature as well as cancer progression makes it an attractive target for therapy. Since inhibition or knockdown of SphK1, the S1P receptors and S1P transporters or neutralization of S1P with antibodies typically alleviates the malignant phenotype of cancer cell lines or xenograft tumours, compounds targeting S1P and the proteins mediating S1P signalling have been studied as potential cancer drugs. However, the only S1P-related drug approved by the United States Food and Drug Administration (FDA) and currently in clinical use is an immunosuppressant.

2.2.9.1 S1P₁ targeting compounds

To date the only FDA-approved S1P-related drug is the immunosuppressant FTY720 (fingolimod, Gilenya) which is used in the treatment of multiple sclerosis (Brinkmann et al., 2010; Strader et al., 2011). It is a sphingosine analogue which enters the cell and is then phosphorylated by SphK2 to form the S1P analogue and S1P receptor antagonist p-FTY720 (Figure 4) (Billich et al., 2003; Paugh et al., 2003). Its function as an immunosuppressant is

based on degradation of S1P₁ and subsequent prevention of lymphocyte egress. After being transported out of the cell p-FTY720 binds S1P₁ with higher affinity than S1P itself and after consequent internalization the receptor is degraded instead of being recycled (Brinkmann et al., 2010; Strader et al., 2011). FTY720 has been suggested to have drug potential outside of the immune system as well. For instance, it was suggested to have an atheroprotective effect through regulation of cholesterol homeostasis in human macrophages (Blom et al., 2010). Importantly, FTY720 and/or p-FTY720 has shown anti-cancer properties such as inhibition of tumour growth, vascularization and metastasis in animal models as well as stimulation of apoptosis specifically in cancer cells (LaMontagne et al., 2006; Patmanathan et al., 2015). As p-FTY720 also targets S1P₃₋₅, and possibly even S1P₂ (Sobel et al., 2015), derivatives of FTY720 are being continuously developed. Several other S1P₁-targeting compounds are in clinical trials for use as immunomodulators, including ponesimod (Actelion), siponimod (Novartis Pharmaceuticals) and ceralifimod (Merck Serono). These compounds are more selective towards S1P₁ and have improved pharmacokinetic properties in comparison to FTY720 (D'Ambrosio et al., 2016; Krösser et al., 2015; Selmaj et al., 2013).

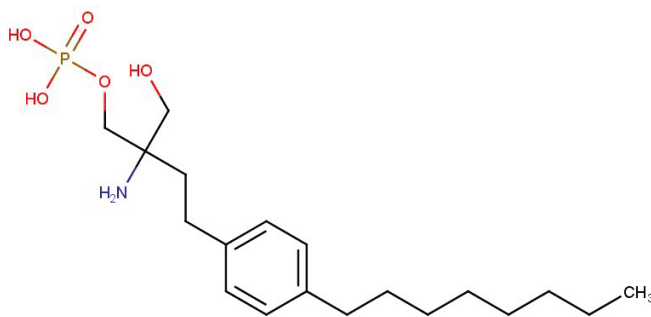


FIGURE 4: Structure of p-FTY720 (created with MarvinSketch).

2.2.9.2 SphK inhibitors

As discussed earlier, over-expression and increased activity of SphK1 is a common feature in human cancers and consequently there have been high hopes for the efficacy of SphK inhibitors in cancer therapy. Numerous SphK inhibitors have been identified and shown to have an anti-cancer effect in mice studies or cancer cell lines (Pyne and Pyne, 2010). The pan SphK inhibitor safinagol was the first SphK inhibitor to enter clinical trials. It has been tested in phase I clinical trials in combination with cisplatin on patients with solid tumours and was shown to decrease plasma S1P levels (Dickson et al., 2011). The isoflavone phenoxodiol has been shown to inhibit SphK1 and induce ceramide accumulation and apoptosis in osteosarcoma cells and xenograft tumours when administered in combination with doxorubicin (Yao et al., 2012). It has been in phase II/III clinical trials for treatment of ovarian cancer (MEI Pharma, Inc.) but showed no clinical efficacy in the phase III study (Fotopoulou et al., 2014). The SphK2 inhibitor ABC294640 was shown to induce apoptosis of multiple myeloma (MM) cells and decrease myeloma tumour growth in mouse models and it is in phase I/II clinical trials for treatment of B-cell lymphoma and solid tumours (RedHill Biopharma

Limited). A problem with many SphK inhibitors is a lack of potency (K_i in the μM range) or target specificity. The most potent SphK inhibitor known to date is the novel compound PF-543 (K_i 3.6 nM) which is also highly selective for SphK1 (Schnute et al., 2012). Surprisingly, in the study characterizing PF-543 it did not attenuate proliferation or survival of a panel of cancer cell lines. There are also other new and potent SphK inhibitors which have shown no effect on survival of cultured cancer cells or growth of xenograft tumours (Kharel et al., 2011; Rex et al., 2013). However, in a very recent study PF-543 did inhibit xenograft tumour growth and strongly promoted survival of affected mice (Ju et al., 2016). In conclusion, though SphK inhibitors have potential as cancer drugs they may not be as effective as hoped.

2.2.9.3 S1P antibodies

One strategy of attenuating S1P signalling is to physically sequester S1P itself with the help of an antibody. Sphingomab is a murine S1P-binding antibody which has been humanized for potential clinical use. In xenograft mice studies sphingomab has halted tumour progression and decreased metastasis by neutralizing blood S1P and recently it was shown to sensitize hypoxic tumours to chemotherapy by normalizing tumour vasculature and facilitating the delivery of other chemotherapeutic agents (Ader et al., 2015; Ponnusamy et al., 2012; Visentin et al., 2006). The humanized form, sonpimizumab, has been in phase I/II clinical trials for treatment of renal cell carcinoma or solid tumours in general (as ASONEP) and age-related macular degeneration (as iSONEP) (Lpath Inc).

2.3 SPC is an enigmatic signalling lipid

While S1P signalling has been quite heavily studied and much of its metabolism and receptor-mediated signalling has been well characterized this is not the case for SPC. SPC metabolism has mostly been studied in relation to disease states where its levels are significantly elevated and research concerning its signalling function has been thwarted by dramatic setbacks in SPC receptor research.

2.3.1 SPC mimics S1P in many ways but has also S1P-independent effects

Like S1P, SPC is also a normal constituent of human blood and SPC concentrations of 50 nM and 130 nM have been measured from plasma and serum, respectively (Liliom et al., 2001). That is, SPC is present in the blood at a clearly lower level than S1P. Though the source of blood SPC is not clear, the elevated serum SPC level suggests that it may be released by platelets similarly to S1P. SPC also circulates in the blood bound to lipoproteins (Sachinidis et al., 1999). SPC is a low-affinity agonist for the S1P receptors and regulates many of the same signalling pathways and cellular processes as S1P. But differences have also been found in studies comparing the two lipids. For instance, in vascular smooth muscle cells SPC was shown to potently induce secretion of the pro-inflammatory cytokine TNF α while S1P was without an effect (Nixon et al., 2008). In the same cells both S1P and SPC induced a calcium response and activation of the transcription factor cAMP response element-binding protein (CREB) but did so through different mechanisms and signalling intermediates. While S1P activated ERK1/2

and caused release of Ca^{2+} from intracellular stores, SPC stimulated p38 and induced influx of extracellular Ca^{2+} (Mathieson and Nixon, 2006).

Both stimulatory and inhibitory effects on cell proliferation, migration and survival have been reported for SPC. In an early study SPC was identified as a mitogen in a panel of untransformed and transformed cell lines and the strongest pro-proliferative effect was seen with untransformed contact-inhibited cells (Desai and Spiegel, 1991). SPC was also found to promote wound healing in mice through increased proliferation of various cell types including keratinocytes, fibroblasts and endothelial cells (Sun et al., 1996). On the other hand, SPC was found to inhibit proliferation of human ovarian and pancreatic cancer cells (Xu et al., 1995; Yamada et al., 1997). In pancreatic cancer cells SPC also induces elasticity and invasion through re-organization of the keratin cytoskeleton (Beil et al., 2003). A pro-migratory effect has been shown also in dendritic cells, vascular smooth muscle cells and endothelial cells (Boguslawski et al., 2000; Lee et al., 2006; Piao et al., 2005; Wang et al., 2009). SPC has been shown to protect non-small cell lung cancer cells, cardiomyocytes and vascular endothelial cells from apoptosis by inducing autophagy (Ge et al., 2011; Yue et al., 2014, 2015) but was found to promote apoptosis in prostate cancer cells lines, umbilical vein endothelial cells and neuronal cells (Jeon et al., 2007; Konno et al., 2007; Mulders et al., 2007). These studies show that similarly to S1P, the effect of SPC is highly context-dependent.

2.3.2 Sphingomyelin deacylase produces SPC and autotaxin converts it to S1P

Increased SPC levels in comparison to healthy subjects have been found in ascites fluids of ovarian cancer patients (Xiao et al., 2001), skin of atopic dermatitis patients (Hara et al., 2000), cerebrospinal fluid of subarachnoid haemorrhage (SAH) patients (Kurokawa et al., 2009) and brain, liver and spleen of Niemann-Pick disease type A patients (Rodriguez-Lafrasse and Vanier, 1999). The only enzyme found to produce SPC is sphingomyelin deacylase which breaks sphingomyelin into SPC and free fatty acid. Sphingomyelin deacylase was discovered due to its action in atopic dermatitis where its elevated expression causes ceramide-deficiency and subsequent skin symptoms through competition with the ceramide-producing enzyme sphingomyelinase for their common substrate sphingomyelin (Hara et al., 2000; Imokawa, 2009; Murata et al., 1996). It would seem logical that the increased SPC level in tissues of NPD-A patients is due to an excess of sphingomyelin combined with no competition from sphingomyelinase. Autotaxin is the only enzyme known to degrade SPC. It has phospholipase D activity towards lysophospholipids and has mainly been studied because it produces the signalling lipid lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC) through removal of the choline group. Similarly, it removes the choline of SPC to produce S1P (Clair et al., 2003). Autotaxin is an exoenzyme present in the blood and serum autotaxin has been shown to metabolize added SPC into S1P. Though normal physiological levels of SPC could not cause significant changes in total blood S1P level, autotaxin does apparently regulate SPC concentrations in the blood (Ohkawa et al., 2015). Autotaxin has been implicated in many ways in cancer biology, though this research has concentrated on its role in LPA signalling (Houben and Moolenaar, 2011).

2.3.3 SPC receptors

Specific SPC receptors have not been unambiguously identified. In the early 2000s the G protein-coupled receptors OGR1 and GPR4 were identified as high affinity SPC receptors and G2A as a low affinity SPC receptor. However, later they were found to function as proton sensors which react to pH changes and the original publications concerning their function as SPC receptors were retracted (Ludwig et al., 2003; Murakami et al., 2004; Nixon et al., 2008; Seuwen et al., 2006). These developments decreased interest for SPC research. It has been suggested that these receptors are regulated by both SPC and protons and that SPC can modulate their pH-related function, as SPC was shown able to inhibit proton-induced activity of OGR1 (Meyer zu Heringdorf and Jakobs, 2007; Mogi et al., 2005). Orphan receptors GPR3, GPR6 and GPR12 are EDG family members, which have high constitutive activity but can be further activated by SPC or S1P. GPR12 especially has been shown to bind SPC with high affinity (Ignatov et al., 2003; Uhlenbrock et al., 2002; Yang et al., 2012).

2.3.4 Intracellular SPC

As mentioned, both S1P and SPC are able to induce changes in cytosolic Ca²⁺ levels and regulate Ca²⁺ signalling. SPC has been shown to directly bind to purified proteins related to Ca²⁺ signalling in *in vitro* experiments. SPC is able to bind the Ca²⁺ sensor protein calmodulin (CaM) and inhibit its interaction with Ca²⁺ (Kovacs and Liliom, 2008; Kovacs et al., 2010a). SPC can also directly bind the ryanodine receptor (RyR) Ca²⁺ channels present at the sarco/endoplasmic reticulum and modulate their activity and interaction with CaM (Kovacs et al., 2010a, 2010b). These proteins may be targets for intracellular SPC *in vivo*.

3 The oxygen-sensitive transcription factor HIF-1 promotes tumour vascularization and controls cancer cell metabolism

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor which senses the cellular oxygen level and protects cells against hypoxic stress. Hundreds of genes are directly activated by HIF-1 binding and many more are indirectly under its regulation. The identified target genes regulate processes such as angiogenesis, erythropoiesis, glucose metabolism, cellular proliferation, migration, invasion and survival (Semenza, 2003, 2012a). HIF-1 has a central role in tumour biology as numerous HIF-1 target genes are cancer-promoting, hypoxia is a common feature of solid tumours and HIF-1 activity is also promoted by many cancer-related signalling pathways. An elevated HIF-1 level has been reported for tumours in cancers of the bladder, brain, breast, cervix, colon, endometrium, head and neck, lung, oropharynx, oesophagus, ovaries, pancreas, prostate, rectum, skin, stomach, thyroid and uterus and HIF-1 expression is often associated with metastasis and high patient mortality (Burrows et al., 2010; Semenza, 2010). Furthermore, HIF-1 promotes chemoresistance and many cancer drugs currently in use, such as imatinib, doxorubicin and trastuzumab, have been shown to inhibit HIF-1 activity (Semenza, 2012b; Unruh et al., 2003).

3.1 Regulation of HIF-1 α expression and HIF-1 activity

HIF-1 is found in all metazoans (Loenarz et al., 2011). It is comprised of two subunits, HIF-1 α and HIF-1 β , which contain a basic helix-loop-helix-PAS (bHLH-PAS) domain enabling their dimerization and subsequent DNA binding (Wang et al., 1995). On target gene promoters HIF-1 binds to the sequence 5'-(A/G)CGTG-3' consequently called a hypoxia-response element (HRE) (Semenza et al., 1996a; Wenger et al., 2005). However, this is not the only factor determining HIF-1 association as not all genomic loci containing a HRE bind HIF-1. Interestingly, while HIF-1 activates genes via direct binding it induces gene suppression indirectly through other target genes (Mole et al., 2009). HIF-1 α is a regulatory subunit whose expression level is the main determinant of HIF-1 activity while HIF-1 β is constitutively expressed (Semenza et al., 1996b).

3.1.1 Stability of HIF-1 α

Hypoxia-induced HIF-1 activity is based on robust stabilization of the HIF-1 α protein (Figure 5). In normoxic conditions HIF-1 α is prolyl hydroxylated by prolyl hydroxylases (PHD1-3) on two proline residues (P402, P564) which reside in the so-called oxygen-dependent degradation (ODD) domains. As a result HIF-1 α is bound by the E3 ubiquitin ligase von Hippel Lindau protein (pVHL), ubiquitinated and degraded in the proteasomes. The stabilization of HIF-1 α in hypoxia is based on the fact that PHDs require oxygen as a substrate for HIF-1 α hydroxylation (Cockman et al., 2000; Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Maxwell et al., 1999; Tanimoto et al., 2000; Yu et al., 2001). HIF-1 α stability is also regulated by other mechanisms than oxygen-dependent prolyl hydroxylation. Glycogen synthase kinase 3 β (GSK3 β) is able to phosphorylate HIF-1 α and subsequently promote its ubiquitination and degradation (Cassavaugh et al., 2011; Flügel et al., 2007). PI3K-Akt signalling inhibits GSK3 β through Akt-mediated phosphorylation and therefore causes HIF-1 α stabilization. As the PI3K-Akt pathway is stimulated by hypoxia, the PI3K-Akt-GSK3 β axis also mediates hypoxia-induced HIF-1 α stabilization (Beitner-Johnson et al., 2001; Chen et al., 2001; Cross et al., 1995; Mottet et al., 2003). Interestingly, SphK1 has been shown to mediate hypoxia-induced PI3K-Akt activation and consequent GSK3 β -dependent stabilization of HIF-1 α . SphK1 activity is induced through an unknown mechanism by reactive oxygen species (ROS) formed in hypoxic conditions (Ader et al., 2008). HIF-1 α stability is additionally regulated independently of oxygen by receptor of activated protein kinase C 1 (RACK1) and the chaperone heat-shock protein 90 (Hsp90) which compete for binding to the same site on HIF-1 α . RACK1-binding marks HIF-1 α for ubiquitination and subsequent proteasomal degradation whereas Hsp90 prevents this (Liu et al., 2007).

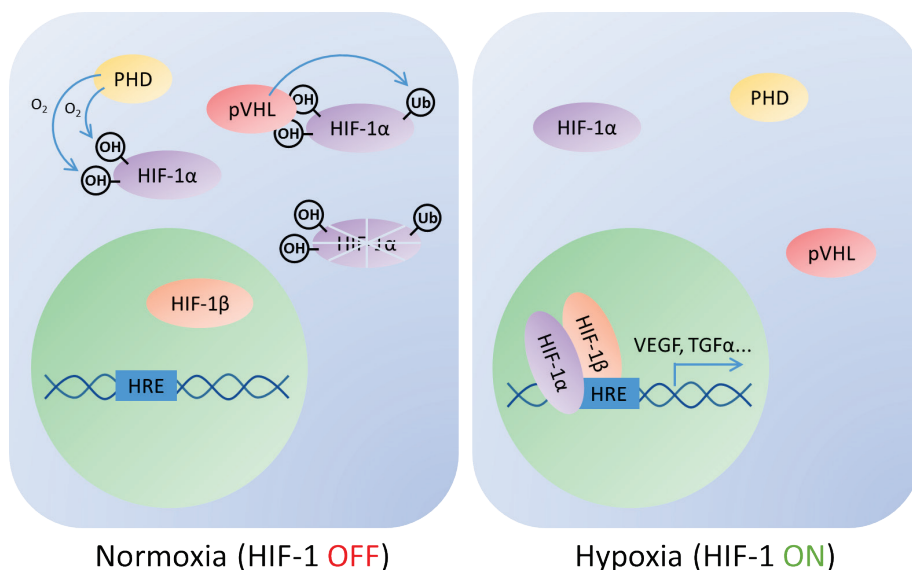


FIGURE 5: Classical oxygen-dependent HIF-1 α degradation. In normoxia prolyl hydroxylases (PHD) hydroxylate HIF-1 α on P402 and P564. This attracts the von Hippel Lindau protein to bind HIF-1 α and promote its ubiquitination and degradation. In the absence of oxygen (hypoxia) HIF-1 α is not hydroxylated and degraded and together with HIF-1 β forms the HIF-1 transcription factor, which promotes transcription from promoters containing the hypoxia-response element (HRE) sequence.

3.1.2 Synthesis of HIF-1 α

HIF-1 α protein synthesis is regulated by both the PI3K-Akt-mTOR and MAPK pathways which converge to activate p70S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E (eIF-4E) to increase translation of HIF-1 α mRNA (Fukuda et al., 2002; Herbert et al., 2000; Laughner et al., 2001; Pyronnet et al., 1999; Semenza, 2003; Shi et al., 2002; Waskiewicz et al., 1999; Zhong et al., 2000). p70S6K stimulates translation by phosphorylating ribosomal protein S6 which is a component of the ribosomal subunit 40S (Dufner and Thomas, 1999). eIF-4E is the 5'-cap binding subunit of the eIF-4F translation initiation complex which facilitates translation of capped mRNAs. The eIF-4E binding protein 1 (4E-BP1) is an inhibitor of eIF-4E which prevents its association with the translation initiation complex (Gingras et al., 1999). Both mTOR and ERK phosphorylate p70S6K to stimulate it and 4E-BP1 to dissociate it from eIF-4E (Fukuda et al., 2002; Herbert et al., 2000; Laughner et al., 2001; Shi et al., 2002; Zhong et al., 2000). Additionally, the MAPK pathway induces MAPK-interacting kinase (MNK) to directly phosphorylate eIF-4E which promotes its association with capped mRNA and other components of the eIF-4F complex (Pyronnet et al., 1999; Waskiewicz et al., 1999).

3.1.3 FIH-1

HIF-1 activity is additionally regulated by hydroxylation of HIF-1 α on the asparagine N803 by factor inhibiting HIF-1 (FIH-1). Hydroxylation of this residue inhibits HIF-1 activity through decreased binding of HIF-1 α to the co-activator proteins CBP and p300 which bind HIF-1 in the

nucleus to facilitate its transcription factor function. FIH-1-induced hydroxylation is oxygen-dependent and therefore also attenuates HIF-1 function in normoxia (Freedman et al., 2002; Hewitson et al., 2002; Lando et al., 2002a, 2002b; Mahon et al., 2001).

3.1.4 Cancer-related signalling changes stimulate HIF-1 α expression

HIF-1 α expression in cancer cells is not caused only by intratumoural hypoxia but also signalling-related changes, which elevate HIF-1 α levels even in non-hypoxic cancer cells. For example, loss of the central tumour suppressor p53 promotes HIF-1 activity. Phosphorylation by p53 leads to HIF-1 α degradation mediated by the E3 ubiquitin ligase Mdm2 and p53 also attenuates HIF-1 transcription factor function by competing with it for the co-activator p300 (Ravi et al., 2000; Schmid et al., 2004). Since the PI3K-Akt pathway induces both stability and synthesis of HIF-1 α , loss of the tumour suppressor and PI3K inhibitor PTEN promotes HIF-1 α expression (Zundel et al., 2000). Excessive activity of receptor tyrosine kinases such as EGFR and IGF1R, which activate both PI3K-Akt and MAPK signalling, similarly up-regulate HIF-1 α (Fukuda et al., 2002; Zhong et al., 2000). The oncogenic Bcr-Abl fusion protein, which is associated with chronic myeloid leukaemia (CML) and caused by the so-called Philadelphia translocation of chromosomes 9 and 22, also promotes HIF-1 α expression through PI3K (Mayerhofer et al., 2002). Subsequently, HIF-1 is needed for CML stem cell maintenance and it mediates imatinib-resistance of CML cells by promoting glycolysis over aerobic glucose metabolism (Zhang et al., 2012; Zhao et al., 2010b).

3.2 HIF-1 promotes migration, invasion and metastasis

Numerous HIF-1 target genes stimulate cell motility, facilitate invasion and subsequently promote metastasis. For instance, HIF-1 regulates many EMT-related genes either directly or indirectly. Vimentin and fibronectin 1 (FN1) are direct HIF-1 target genes while E-cadherin is indirectly repressed by HIF-1 through up-regulation of the EMT transcription factors Zeb1/2 (Krishnamachary et al., 2006). Several other HIF-1 targets are also associated with invasion and/or metastasis (Semenza, 2003, 2010). The matrix metalloproteinases 2 and 14 (MMP2/14), urokinase-type plasminogen-activator receptor (uPAR) and cathepsin D facilitate invasion through digestion of the ECM (Semenza, 2003). While FN1 is itself an ECM glycoprotein it is involved in promoting MMP secretion, invasion and metastasis of cancer cells (Chen et al., 2010; Shibata et al., 1997). Lysyl oxidase (LOX) in turn is an exoenzyme which remodels the ECM by inducing lysine crosslinking of collagen and other extracellular proteins and subsequently facilitates invasion and metastatic growth (Erler et al., 2006). Autocrine motility factor (AMF) is a cytokine secreted by tumour cells which activates plasma membrane receptors and promotes migration through PI3K signalling (Funasaka and Raz, 2007). The chemokine receptor CXCR4, which often has low expression in healthy tissue but is over-expressed in a vast amount of cancers, promotes intra- and extravasation of cancer cells and their organ-specific metastasis to sites such as bone, lung, liver and brain which express its ligand CXCL12 (Sun et al., 2010).

3.3 HIF-1 and angiogenesis

During embryonic development HIF-1 regulates formation of the entire oxygen circulatory system (heart, vasculature, red blood cells) and consequently HIF-1 α knockout mice are embryonic lethal (Iyer et al., 1998; Semenza, 2003, 2012b). In relation to cancer, the ability to induce blood vessel formation is an important determinant for tumour growth and malignancy. The inner parts of a growing tumour mass eventually start suffering from a shortage of oxygen and nutrients leading to cell death. The subsequently accumulating HIF-1 induces gene expression of numerous pro-angiogenic genes, vascular endothelial growth factor (VEGF) and its receptors VEGFR1/2 being the most important. Interestingly, while VEGF and VEGFR1 are direct HIF-1 target genes, VEGFR2 expression is indirectly induced by hypoxia (Gerber et al., 1997). The tumour cells secrete VEGF into the surrounding tissue along with MMPs which degrade the ECM and enable VEGF to reach nearby endothelial cells. The process where hypoxic cells acquire this ability to induce angiogenesis is called the angiogenic switch. Hypoxic tumours are typically more aggressive and malignant than ones with better oxygenation due to their persistent HIF-1 activity. And even though HIF-1 induces tumour angiogenesis, this does not often effectively alleviate hypoxia as the tumour-related blood vessels are typically deformed. They do still promote tumour growth and also facilitate metastasis through intravasation of cancer cells into the blood stream (Deryugina and Quigley, 2015; Liao and Johnson, 2007; Semenza, 2010; Weinberg, 2013c).

3.4 Role of HIF-1 in glucose metabolism

HIF-1 is a central regulator of a cell's glucose metabolism. Normal cells living in normal oxygen conditions use glycolysis followed by mitochondrial, oxygen-dependent glucose metabolism to produce ATP. However, when faced with hypoxic conditions cells are resorted to using glycolysis only. Remarkably, cancer cells use this oxygen-independent mode of energy production regardless of oxygen availability, a phenomenon called the Warburg effect (Semenza, 2013). HIF-1 activates a multitude of genes involved in this metabolic transition, including glycolytic enzymes, inhibitors of mitochondrial function and glucose transporters. Increased glucose transport and a high level of glycolysis facilitate the rapid proliferation of cancer cells as they can use glycolytic intermediates for biosynthesis of cellular components (Lunt and Vander Heiden, 2011). Glycolysis produces lactate and HIF-1 also up-regulates genes involved in export of lactate and protons. This leads to acidification of the extracellular space and alkalization of the cytosol which in turn facilitates invasion, proliferation and survival (Chiche et al., 2010). Inducing the transition to glycolysis may be the original role of HIF-1 since it is expressed even in the simplest metazoan species which lack a cardiovascular system (Loenarz et al., 2011). Interestingly, it seems that the reason for this metabolic transition is not that the cells don't have access to enough oxygen substrate, but that in hypoxia the electron transfer involved in oxidative glucose metabolism is inefficient and leads to cytotoxic accumulation of reactive oxygen species (ROS). Embryonic fibroblasts from HIF-1 α -knockout mice (KO-MEFs) actually produce more ATP than wild-type MEFs but die due to accumulation of ROS (Kim et al., 2006; Zhang et al., 2008).

3.5 HIF-2 and HIF-3

HIF-2 α and HIF-3 α are HIF-1 α paralogues whose stability is also regulated by PHDs and oxygen availability. They pair with the same HIF-1 β subunit as HIF-1 α , to form HIF-2 and HIF-3, and also function as transcriptional regulators.

3.5.1 HIF-2

Unlike the ubiquitous HIF-1 α , HIF-2 α is only expressed in certain cell types and only in vertebrates. It regulates many of the same biological processes as HIF-1. HIF-1 and HIF-2 share some of their target genes but others are specific to either one. For instance, regulation of glycolytic enzymes is typically HIF-1-specific and independent of HIF-2 (Hu et al., 2003). In many cases HIF-1 and HIF-2 have opposite regulatory effects and also negatively regulate each other. For example, the balance between HIF-1 α and HIF-2 α expression has been shown to regulate arterial pressure and blood flow (Cowburn et al., 2013). In renal clear cell carcinoma (RCC) where loss of pVHL is common it is elevated HIF-2 which is associated with tumour progression and poor prognosis whereas HIF-1 attenuates oncogenic HIF-2 function (Raval et al., 2005; Salama et al., 2015a). On the other hand, studies with colon and liver cancer tumours have shown an opposite effect where HIF-1 mediates cancer-promoting processes and HIF-2 inhibits them (Imamura et al., 2009; Sun et al., 2013). Additionally, when both HIF-1 α and HIF-2 α are expressed in the same cell, HIF-1 may still be alone responsible for regulation of transcription. Interestingly, HIF-2 has been shown to up-regulate SphK1 in both hypoxia and in pVHL-deficient RCC cells (Anelli et al., 2008; Salama et al., 2015b).

3.5.2 HIF-3

Studies concerning HIF-3 are scarce but in general it is considered an inhibitor of HIF-1/2. HIF-3 α can directly bind to HIF-2 α and prevent it from binding DNA and promoting gene expression and it also competes with HIF-1/2 α for their common subunit partner HIF-1 β . Different HIF-3 α splice variants differ in this respect and also in their tissue distribution and expression pattern during embryonic development. HIF-3 α is also up-regulated by HIF-1 in a negative feedback loop and therefore functions to quench activated HIF-1 signalling. However, HIF-3 is not solely an inhibitor of HIF1/2 but functions also itself as a transcription factor. Its target genes are partly HIF-3-specific and partly common with HIF-1 (Makino et al., 2002; Maynard et al., 2003, 2007; Zhang et al., 2014a).

4 The intermediate filament protein vimentin mediates cell motility and gives tumours metastatic potential

4.1 Intermediate filaments

The cytoskeleton of eukaryotic cells is formed of three different types of protein filaments capable of forming structural networks: microfilaments, intermediate filaments (IFs) and microtubules. Microfilaments are the thinnest with a diameter of approximately 7 nm, microtubules are the thickest with a diameter of 25 nm while intermediate filaments typically have a diameter of 10 nm. While microfilaments consist solely of actin and microtubules of α -

and β -tubulin, the IFs comprise of approximately 70 different proteins. Their expression is often specific to cell type and/or developmental stage and they are classified into six groups based on sequence differences (type I-VI). The IF cytoskeleton gives the cell shape, protects it from mechanical stresses, controls cell motility and adhesion and takes part in trafficking of vesicles and organelles (Chung et al., 2013; Styers et al., 2005).

An IF protein monomer consists of a non-helical N-terminal head domain, a long central α -helical rod domain and a non-helical C-terminal tail domain. The rod domain is quite conserved among the IF family members, while the head and tail domains differ in length and sequence. Monomers bind each other in a parallel fashion by their rod domains, which coil around each other to form a coiled-coil dimer. These dimers are not always homodimers formed by one IF protein and some IF proteins are only able to form heterodimers with other members of the family. Dimers pair in an anti-parallel and staggered fashion to form tetramers and eight tetramers come together to form a so-called unit-length filament (ULF). ULFs in turn polymerize end-to-end to create a larger filament structure (Figure 6) (Dave and Bayless, 2014; Goldman et al., 2008). Finally, the individual filaments together constitute a cytoskeletal network. Crosslinking proteins such as plectin and other plakin family members connect IF filaments to one another as well as to microfilaments and microtubules (Wiche et al., 2015). The filament structure described above gives the IF cytoskeleton elasticity and enables it to protect the cell against mechanical stress. In contrast, the structure of microfilaments and microtubules where actin monomers or α/β -tubulin dimers attach to each other end-to-end makes these filaments susceptible to breakage (Goldman et al., 2008; Wagner et al., 2007).

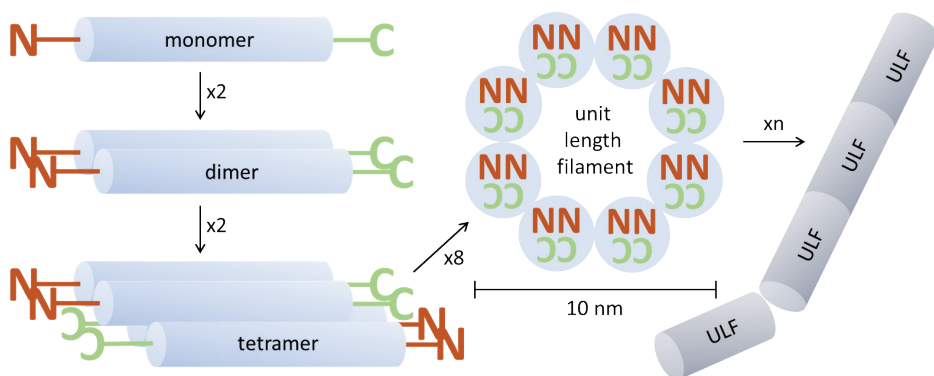


FIGURE 6: Intermediate filament assembly. Monomers form parallel dimers, dimers form anti-parallel and staggered tetramers, tetramers form unit length filaments (ULF) which have the typical 10 nm diameter of intermediate filaments and ULFs join end-to-end to form longer filament structures. Modified from Dave and Bayless (2014).

4.2 Vimentin

Vimentin is a type III intermediate filament along with desmin, glial fibrillary acidic protein (GFAP) and peripherin. Vimentin is expressed in mesenchymal cells such as fibroblasts, endothelial cells and smooth muscle cells which are derived from the mesodermal layer of the

embryo. The other type III proteins are specific to either muscles (desmin) or the nervous system (GFAP, peripherin). Vimentin is present mostly in homodimers but it also forms heterodimers with type III-IV IFs. Vimentin is a central mediator of cellular migration and invasion and a vast amount of evidence links it to cancer progression. High vimentin expression is typical in a wide array of cancers and in metastases, and vimentin expression correlates with metastatic potential and poor prognosis (Satelli and Li, 2011). Elevated vimentin levels have been reported for a vast array of cancers including those of the breast, cervix, colon and rectum, endometrium, kidney, liver, lung, lymphatic tissue, oesophagus, pancreas, prostate, skin, stomach and thyroid (Dauphin et al., 2013; Satelli and Li, 2011; Toiyama et al., 2013). As discussed earlier, epithelial cells gain both vimentin expression and motility through the process of EMT which is an important step in the malignant progression of epithelial tumours. For example, most breast cancers originate from epithelial tissue and in breast cancer cells lines vimentin levels correlate with migratory and invasive potential (Gilles et al., 2003). Importantly, vimentin is not just a marker for post-EMT cells as it has also been shown to actively mediate the transition through regulation of other EMT-related factors (Ivaska, 2011; Virtakoivu et al., 2015). Accordingly, vimentin regulates migration physically as a component of the cytoskeleton, but also by controlling migration-related signalling. Though vimentin knockout mice don't suffer from any severe symptoms they have defects in wound healing. Cells lacking vimentin show impaired migration and invasion and changes in their actin cytoskeleton and focal adhesions (Eckes et al., 1998, 2000; Nieminen et al., 2006; Schiffers et al., 2000; Terzi et al., 1997).

4.2.1 Regulation of vimentin expression

Both during development and EMT vimentin expression is regulated through gene expression. Accordingly, the vimentin gene is under the control of several regulatory elements and transcription factors. Transcriptional activators inducing vimentin expression are typically cancer-promoting and include AP-1, β -catenin/TCF (activated by Wnt signalling), HIF-1, NF- κ B, Smad proteins (induced by TGF β) and PEA3 (Chen et al., 1996; Gilles et al., 2003; Krishnamachary et al., 2003; Min et al., 2008; Rees et al., 2006; Rittling et al., 1989; Wu et al., 2007). For example, β -catenin is associated with the high vimentin expression of migratory and invasive breast cancer cell lines (Gilles et al., 2003). Many of the above-mentioned factors and their upstream signalling pathways also stimulate the EMT transcription factors (EMT-TFs; Snail, Slug, Twist, Zeb), which repress E-cadherin gene expression and are central for the induction of EMT. Vimentin expression is also regulated by epigenetic methylation of the vimentin gene. Aberrant methylation has been reported for several cancer types and has been studied especially in relation to colorectal cancer (Chen et al., 2005; Kitamura et al., 2011; Li et al., 2014; Shirahata and Hibi, 2014; Shirahata et al., 2009; Ulirsch et al., 2013). Additionally, several micro-RNAs regulating vimentin mRNA and protein levels have been identified. For instance, in renal cell carcinoma (RCC) high vimentin expression is associated with low levels of miR-138 (Yamasaki et al., 2012) and in breast cancer low expression of vimentin-targeting miR-30a correlates with metastasis and poor prognosis (Cheng et al., 2012). Stability of the vimentin protein is regulated by proteases such as calpain and caspases though even the

cleaved vimentin fragments often have a signalling function as discussed later. Gigaxonin is an E3 ubiquitin ligase adaptor protein which targets vimentin and other IF proteins for proteasomal degradation (Mahammad et al., 2013).

4.2.2 Vimentin filament assembly and signalling function are regulated by PTMs

Vimentin function is regulated mainly through post-translational modifications (PTMs), most notably serine phosphorylations but also sumoylation, acetylation, citrullination, prenylation or glycosylation as well as cleavage by proteases. Vimentin is a known substrate for several kinases and over 20 phosphorylation sites on vimentin have been identified, though the functional significance of many of them is not known. Vimentin-phosphorylating kinases include Akt, Aurora-B kinase, CaM kinase II, cyclin-dependent kinase 1 (Cdk-1), p21-activated kinase (PAK), PKA, PKC, polo-like kinase 1 (PLK-1) and ROCK. They predominantly target the N-terminal head domain of a vimentin monomer and to a lesser extent the C-terminal tail. Phosphorylation regulates for instance filament assembly, disassembly and reorganization, segregation of the filament network during cell division, vimentin-dependent migration and vesicle trafficking and the function of vimentin as a signalling mediator. Phosphorylation of the N-terminal head domain typically promotes filament disassembly as phosphorylation pushes the monomers in a dimer farther away causing dissociation of the filament structure (Dave and Bayless, 2014; Eriksson et al., 2004; Goto et al., 2002; Ivaska et al., 2005; Tzivion et al., 2000; Yamaguchi et al., 2005; Zhu et al., 2011). Phosphatases, including PP2A, also target vimentin to counteract the phosphorylation-mediated effects (Turowski et al., 1999). As mentioned, several proteases are able to cleave vimentin. The calcium-dependent protease calpain cleaves vimentin monomers at several sites on the N-terminal head domain, creating a pool of soluble vimentin fragments which function as pro-angiogenic signalling molecules (Dave and Bayless, 2014; Kwak et al., 2012). During apoptosis caspases cleave vimentin resulting in disruption of the vimentin cytoskeleton but also production of a pro-apoptotic vimentin fragment which intensifies the cell death signal (Byun et al., 2001). Phosphorylation of vimentin by Akt1 protects vimentin from caspase-mediated degradation and vimentin may therefore be involved in the pro-survival function of PI3K-Akt signalling (Zhu et al., 2011).

4.2.3 Vimentin regulates migration through interaction with the actin cytoskeleton and focal adhesions

Interaction of vimentin with the actin cytoskeleton and protein structures involved in cell adhesion has a central role in vimentin-dependent migration. As discussed previously, lamellipodia are actin-rich cell protrusions on the leading edge of a migrating cell and central mediators of cell movement while focal adhesions control cell adhesion. Lamellipodia formation requires local disassembly of vimentin filaments in response to phosphorylation. When vimentin organization is disrupted, cells lose their polarity and form lamellipodia over the entire cell perimeter and subsequently migrate less (Helfand et al., 2011). Vimentin filaments attach to the intracellular protein components of focal adhesions and vimentin also controls focal adhesion organization, size and turnover (Mendez et al., 2010; Tsuruta and Jones, 2003). Accordingly, vimentin knockout MEFs and vimentin-null endothelial cells have

smaller and more separated focal adhesions than wild type cells (Eckes et al., 1998, 2000). Vimentin also forms a complex with focal adhesion kinase (FAK) and regulates its expression. FAK is a component of the focal adhesion protein complexes and its kinase activity is induced in response to integrin-mediated cues. Interestingly, in endothelial cells S1P together with growth factors has been shown to induce endothelial sprout formation and angiogenesis through increased vimentin expression and complex formation with FAK (Dave et al., 2013). As mentioned, integrins are components of focal adhesions. They are transmembrane proteins which interact with the ECM and adjacent cells, mediate adhesion and induce cell signalling. Vimentin regulates integrin expression on the cell surface and migration-related integrin recycling and trafficking within the cell (Ivaska et al., 2005; Nieminen et al., 2006).

4.2.4 Vimentin mediates cell signalling

Vimentin was initially thought to function only as a structural element of cells but has since been shown to be heavily involved in cell signalling, often modulating signalling pathways involved in cell motility. Also, vimentin expression is not only induced by EMT but vimentin itself promotes EMT. Recently, vimentin was shown to function as a scaffold for interaction of the EMT transcription factor Slug and ERK which promotes phosphorylation and activation of Slug by ERK (Virtakoivu et al., 2015). Similarly, vimentin is a scaffold for vasodilator-stimulated phosphoprotein (VASP) and protein kinase G (PKG) and therefore promotes VASP phosphorylation by PKG. Additionally, binding to vimentin is essential for localization of VASP at focal adhesions and VASP-mediated endothelial cell migration (Lund et al., 2010). Vimentin can also prevent interaction between signalling mediators. Scrib is a scaffold protein which is on the one hand involved in epithelial cell polarity and migration and on the other hand functions as a tumour suppressor. Vimentin facilitates directed cell migration by interacting with Scrib and protecting it from proteasomal degradation (Phua et al., 2009). Binding of phosphorylated vimentin to 14-3-3 proteins decreases their interaction with other binding partners (Tzivion et al., 2000). And, in the sciatic nerve soluble vimentin fragments have been shown to promote ERK1/2 signalling by binding to phosphorylated ERK1/2 and protecting them from dephosphorylation through steric hindrance (Perlson et al., 2005, 2006). The calpain-produced vimentin fragments mentioned earlier bind and activate the matrix metalloproteinase MT1-MMP, which in turn mediates ECM degradation, endothelial sprouting and angiogenesis. Interestingly, S1P is one of the factors able to induce this calpain-mediated fragmentation of vimentin (Kwak et al., 2012). Vimentin also regulates gene expression but the mechanism is not clear. Vimentin promotes transcription of p21 (Mergui et al., 2010) and the tyrosine kinase Axl which mediates EMT-related cancer cell migration (Vuoriluoto et al., 2011). Vimentin can localize to the nucleus, bind DNA with its N-terminus and affect chromatin organization (Hartig et al., 1998; Traub et al., 1992), but it is not known if vimentin directly functions as a transcriptional regulator. Vimentin has also been implicated as a regulator of micro-RNA expression. Recently, vimentin was shown to down-regulate expression of a group of phospholipase D (PLD)-targeting tumour suppressive micro-RNAs in breast cancer cells and as a result promote their metastatic capacity (Fite and Gomez-Cambronero, 2016).

5 The Hippo signalling pathway is a novel cascade of tumour suppressive and oncogenic factors

5.1 Hippo pathway components

In mammals, the Hippo pathway regulates cellular proliferation, migration, survival and differentiation necessary for both normal embryonic development and tissue homeostasis. Therefore, deregulation of the pathway is involved in tumorigenesis and cancer progression. The signalling pathway consists of a tumour suppressive kinase cascade and oncogenic transcriptional regulators, which the kinases inhibit. Additionally, scaffold and co-activator proteins help bring the different components together and promote the kinase activity. The kinase cascade consists of mammalian Ste20-like protein kinases 1 and 2 (Mst1/2) and large tumour suppressor kinases 1 and 2 (Lats1/2). Activated Mst1/2 phosphorylates and activates Lats1/2. The substrates and inhibitory targets of Lats1/2 are the closely related Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ). While non-phosphorylated YAP and TAZ are free to translocate to the nucleus to activate target genes, after phosphorylation by Lats1/2 they are sequestered in the cytoplasm through binding to 14-3-3 proteins and/or degraded (Moroishi et al., 2015a).

The Hippo pathway was originally identified in the fly (*Drosophila melanogaster*) where it regulates organ size. The name of the pathway comes from the fly Mst1/2 orthologue, named Hippo due to the overgrowth phenotype caused by its gene deletion (Harvey et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003a). The fly Lats1/2 orthologue was named Warts (Wts) due to the wart-like growths caused by its loss (Justice et al., 1995). While deletion of the other Hippo pathway components led to excess growth, deletion of the YAP/TAZ orthologue Yorkie prevented these effects (Huang et al., 2005). This led to the discovery that the upstream kinases regulate transcription via Yorkie, or YAP/TAZ in mammals. It should be noted that the term “Hippo signalling” is used not only in the context of the canonical pathway leading from Mst1/2 through Lats1/2 to YAP/TAZ (Figure 7) but often also for function of the kinase module only, function and regulation of YAP/TAZ independently of Mst1/2-Lats1/2 as well as Lats1/2 signalling not induced by Mst1/2.

The auxiliary components of the pathway include Mps One binder 1 (Mob1, more specifically Mob1A/B) and Sav1/WW45. Activation of Lats1/2 requires two phosphorylation events: phosphorylation of the hydrophobic motif by Mst1/2 and autophosphorylation of the T-loop by Lats1/2 itself. Activated Mst1/2 phosphorylates Mob1 and promotes its binding to Lats1/2 and Mob1 in turn induces Lats1/2 autophosphorylation (Praskova et al., 2008). This mechanism of activation is typical of the NDR subclass of the AGC kinase family which Lats1/2 belongs to (Hergovich and Hemmings, 2009). Mst1/2 also phosphorylates the scaffold protein Sav1/WW45 which brings Mst1/2 and Lats1/2 together and therefore stimulates their activity (Harvey et al., 2003; Wu et al., 2003a). Hippo signalling is of central importance during development as revealed by the embryonic lethality of Mst1/2 double knockout, Lats2 knockout, YAP knockout and Mob1A/B double knockout in mice (Morin-Kensicki et al., 2006; Nishio et al., 2012; Song et al., 2010; Yabuta et al., 2007).

5.2 Function of the Hippo pathway

Lats1/2 target HXRXXS motifs on their substrate proteins and phosphorylate numerous serine residues on both YAP and TAZ. However, only two of these modifications have a known functional outcome. Phosphorylation of YAP on S127 or TAZ on S89 lead to their binding to 14-3-3 proteins and sequestration in the cytoplasm while phosphorylation of YAP on S381 or TAZ on S311 promotes their degradation (Hao et al., 2008a; Hergovich, 2013; Lei et al., 2008; Liu et al., 2010b; B. Zhao et al., 2010; Zhao et al., 2007a). YAP phosphorylated on S381 is further phosphorylated by casein kinase 1 (CK1) and this recruits the E3 ubiquitin ligase SCF^{β-TRCP} to ubiquitinate YAP and cause its proteasomal degradation (Zhao et al., 2010a). In addition to direct phosphorylation, Lats1/2 regulates YAP/TAZ also indirectly through the scaffold protein angiominin (AMOT). Several different research groups have shown using different cell types that AMOT binds YAP/TAZ and inhibits them through sequestration in the cytoplasm. Phosphorylation of AMOT by Lats1/2 was found to facilitate this inhibitory interaction (Chan et al., 2011; Lucci et al., 2013; Oka et al., 2012; Zhao et al., 2011). However, it has also been shown that in hepatic cells an AMOT splicing variant (AMOT-p130) is able to induce YAP/TAZ nuclear accumulation and act as a co-activator of YAP-TEAD-mediated transcription to promote proliferation (Yi et al., 2013). In addition to targeting YAP/TAZ directly or through AMOT, Lats2 functions as a tumour suppressor also via p53. Lats2 binds to and stabilizes p53 by preventing its ubiquitination by the Mdm2 E3 ubiquitin ligase. p53 in turn up-regulates transcription of the Lats2 gene in a positive feedback loop (Aylon et al., 2006).

The most commonly mentioned YAP-TEAD target genes are connective tissue growth factor (CTGF, CCN2) and cysteine-rich angiogenic inducer 61 (CYR61, CCN1) which belong to the CCN intercellular signalling protein family. They are secreted into the ECM and have a regulatory role in proliferation, survival, differentiation, adhesion, migration and angiogenesis and their aberrant expression is associated with cancer (Chen and Lau, 2009). YAP and TAZ do not themselves contain DNA binding motifs and function by enhancing transcription mediated mainly by the TEAD family of transcription factors but also the Smad proteins (Alarcón et al., 2009; Varelas et al., 2010a) and the ErbB4 cytoplasmic fragment (Komuro et al., 2003). Recently YAP/TAZ were shown to function as co-repressors of transcription in addition to their canonical co-activator role and inhibit expression of tumour suppressive and pro-apoptotic genes (Kim et al., 2015). YAP and TAZ are mostly functionally redundant but there are some exceptions. For example, only YAP has been implicated as a tumour suppressor in haematopoietic cells, as discussed later, and in the breast only TAZ is involved in lineage switching between luminal and basal mammary epithelial cells (Skibinski et al., 2014). While YAP knockout is embryonic lethal in mice, TAZ knockout mice are viable but develop kidney disease (Hossain et al., 2007).

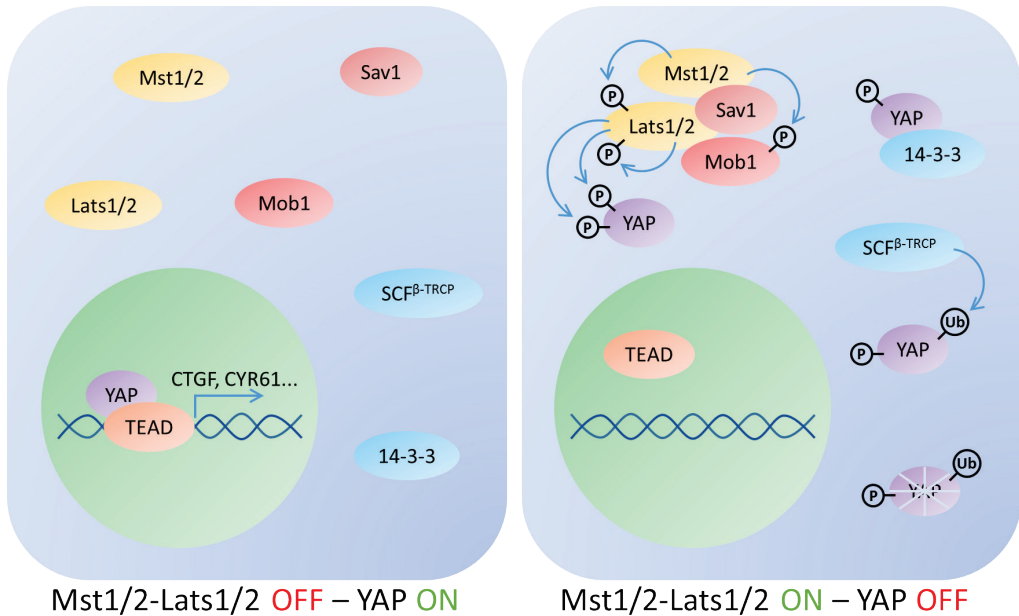


FIGURE 7: The classical Hippo pathway. When the Mst1/2-Lats1/2 kinase cascade is inactive YAP (and TAZ) is free to translocate to the nucleus, bind TEAD transcription factors and stimulate transcription of YAP target genes. Activated Mst1/2 directly phosphorylates Lats1/2 and promotes its autophosphorylation through Mob1. Sav1 functions as a scaffold for the kinase cascade. Phosphorylated active Lats1/2 in turn phosphorylates YAP on S127 and S381. S127 phosphorylation causes sequestration of YAP by 14-3-3 proteins while S381 phosphorylation promotes ubiquitination by SCF^{β-TRCP} and degradation of YAP.

5.3 Regulation of the Hippo pathway

5.3.1 GPCRs

In 2012 two studies identified the GPCRs of LPA and S1P as regulators of Hippo signalling (Miller et al., 2012; Yu et al., 2012). Yu et al. (2012) studied the effects of specific G proteins on the pathway and found that G_s activates Lats1/2 through its classical cAMP-PKA pathway and subsequently inhibits YAP/TAZ, while $G_{12/13}$ and to a lesser extent also $G_{i/o}$ and $G_{q/11}$ inhibit Lats1/2 and activate YAP/TAZ. Both of the studies identified activation of Rho and actin polymerization as mediators of $G_{12/13}$ -induced YAP/TAZ activation. $G_{q/11}$ activation can also through Rho and the actin cytoskeleton but independently of Mst1/2-Lats1/2 lead to decreased interaction of AMOT and YAP, freeing YAP to translocate to the nucleus. Here $G_{q/11}$ signals through activation of the Rho-GEF TRIO instead of its canonical PLC signalling (Feng et al., 2014). In uveal melanoma, where stimulatory $G_{q/11}$ mutations are common, YAP is an important oncogenic factor mediating tumour growth (Feng et al., 2014; Yu et al., 2014). Additionally, YAP/TAZ are able to regulate Mst1/2-Lats1/2 in a negative feedback loop by inducing mRNA expression of both Lats2 and the Mst1/2 activator neurofibromin 2 (NF2)

(Moroishi et al., 2015b). In this case Lats1/2 induction and subsequent YAP/TAZ inhibition can be mediated by the G proteins $G_{12/13}$, $G_{q/11}$ and $G_{i/o}$ which initially stimulate YAP/TAZ function.

5.3.2 Growth factor and cytokine signalling

EGF-EGFR signalling has been shown to activate YAP/TAZ through both the PI3K and MAPK pathways. Activation of PI3K leads to direct binding of PDK1 to the Mst1/2-Lats1/2 complex and their dissociation (Fan et al., 2013), while Ras-MAPK signalling promotes binding of the inhibitory Wilms tumour protein 1 interacting protein (WTIP) to Lats1/2 (Reddy and Irvine, 2013). YAP/TAZ are also able to induce EGF signalling in a positive feedback loop through their target gene amphiregulin (AREG) which is an EGFR ligand (Zhang et al., 2009). TGF β and Hippo signalling also interact in several ways. TGF β induces transcription through the Smad transcription factors and promotes binding of YAP/TAZ to Smad complexes. YAP/TAZ can either promote nuclear localization and activity of Smad proteins or sequester them in the cytoplasm depending on cell density (Grannas et al., 2015; Varelas et al., 2008, 2010a). Smad proteins are also regulated by the cytokine family of bone morphogenetic proteins (BMP) and TAZ has been shown to induce expression of BMP4 (Lai and Yang, 2013). Recently several members of the MAP kinase kinase kinase kinase (MAP4K) family were shown to activate Lats1/2 through direct phosphorylation of its hydrophobic motif which is classically targeted by Mst1/2 (Meng et al., 2015; Zheng et al., 2015).

YAP/TAZ are associated with the oncogenic Wnt/ β -catenin pathway in many ways. The β -catenin destruction complex binds both β -catenin and YAP/TAZ. This interaction causes degradation of β -catenin and sequesters YAP/TAZ in the cytoplasm. Interestingly, YAP/TAZ promote β -catenin degradation by recruiting the SCF $^{\beta}$ -TRCP ubiquitin ligase to the complex. Binding of Wnt proteins to their receptors causes release of both β -catenin and YAP/TAZ from the destruction complex, freeing them to translocate to the nucleus and activate target genes (Azzolin et al., 2014). Subsequently, activated β -catenin induces YAP expression (Konsavage et al., 2012). Adenomatous polyposis coli (APC) is a component of the β -catenin destruction complex. It has been shown to function as a scaffold to facilitate Sav1-Lats1 binding and therefore inhibit YAP/TAZ also independently of the destruction complex. YAP/TAZ are mediators of the tumourigenesis related to loss of APC and YAP/TAZ activity is typically high in cancers where APC is lost or Wnt signalling is constitutively active (Cai et al., 2015).

5.3.3 Cell-cell contacts

Hippo signalling has been revealed as a central mediator of contact inhibition. The Hippo pathway balances the growth-promoting signals of growth factors and the inhibitory signals of cell-cell contacts. In situations of high cell density, such as in the middle of a cell colony or a tumour, a higher growth factor concentration is needed to promote proliferation than in cases of low cell density, such as a wound or edges of a colony (Gumbiner and Kim, 2014). TGF β -induced and Smad-mediated transcription is facilitated by YAP/TAZ in cells living in low density conditions whereas in dense colonies YAP/TAZ prevent nuclear localization of Smad (Grannas et al., 2015; Varelas et al., 2008, 2010a). Cell density cues are mediated to the Hippo pathway by adherens junctions, desmosomes and tight junctions which are multiprotein

complexes spanning the plasma membrane and attaching to respective structures on adjacent cells. Epithelial polarity complexes (Par, Scribble, Crumbs) are involved in establishing the polarity of epithelial cells in response to cell and ECM contacts and interact with the above-mentioned cell junctions. They are also present in non-epithelial cells and regulate Mst1/2-Lats1/2 and YAP activity. NF2 and Kibra are examples of proteins which bind to both the cell junction proteins and Mst1/2-Lats1/2 and activate them in response to cell-cell interaction. AMOT, which was discussed earlier and can directly bind YAP, is a tight junction protein (Gumbiner and Kim, 2014; Kim et al., 2011a).

The term “cell competition” is used to describe the competition and interaction between different cell populations within the same compartment and the resulting effects on cell proliferation and survival. On the one hand a rapidly growing cell population makes room for itself by causing apoptosis of neighbouring cells but on the other hand a slow-growing cell population is able to attenuate the more proliferative phenotype of adjacent cells or even eliminate over-proliferative cells. The Hippo pathway has a central role in this phenomenon, which has been studied mostly in *Drosophila* and in relation to organ development, but has been implicated also in mammals and cancer biology. Cancer cells and the surrounding normal cells comprise adjacent cell populations with different growth characteristics (Baker and Li, 2008). In an example from *Drosophila*, an inhibitory mutation of the tumour suppressor Scrib in the fly imaginal discs causes hyper-activation of Yorkie and an over-proliferative phenotype when all cells of the population are Scrib(-) mutants. However, if normal cells are also present in the imaginal discs they prevent excess proliferation of Scrib(-) cells by causing Yorkie inhibition through cell-cell communication (Chen et al., 2012). Hippo signalling also itself regulates communication between cells by controlling expression of Notch pathway components. The Notch receptors and their ligands are present on cell surfaces and activation of the receptors classically requires contact between adjacent cells. The receptor Notch2 and the ligand Jagged1 are YAP-TEAD target genes and mediate the pro-proliferative effect of YAP (Tschaharganeh et al., 2013; Yimlamai et al., 2014). YAP up-regulates Jagged1 in hepatocellular carcinoma (HCC) cells and their expression levels correlate in tumour samples from HCC and colorectal cancers (Tschaharganeh et al., 2013).

5.3.4 Hippo signalling is involved in mechanosensing

As mentioned, regulation of Hippo signalling by GPCRs is mediated by the small G protein Rho and changes in the actin cytoskeleton. Rho activation induces formation of filamentous actin (F-actin) from globular actin (G-actin) and this causes activation of YAP/TAZ either via Lats1/2 inhibition or independently of Lats1/2 (Miller et al., 2012; Yu et al., 2012). Cell shape, mechanical stress and elasticity/stiffness of the ECM also regulate Hippo signalling via the actin cytoskeleton. Single cells forced to grow on a small surface area in a rounded shape have lower YAP/TAZ activity than cells, which are able to spread more. Cells trapped inside the ECM in a small volume and in a round cell shape show lower YAP/TAZ activity than less confined cells, which can spread themselves. Mechanical stretching of a cell monolayer also induces YAP/TAZ. Additionally, single cells growing on hard surfaces are more spread, experience more

tension and have more nuclear YAP/TAZ than cells with a rounder cell shape growing on soft surfaces. Apparently, cells growing on stiff ECM have elevated YAP/TAZ activity and cells growing within a stiff ECM in a 3D setting also have high YAP/TAZ activity as long as they are able to remodel the ECM in order to spread (Aragona et al., 2013; Benham-Pyle et al., 2015; Dupont, 2015; Dupont et al., 2011). The ECM of breast cancer tumours is typically stiffer than that of healthy tissue and this property also drives proliferation, invasion and cancer progression. It has been suggested that YAP/TAZ activity is involved in this stiffness-related malignant phenotype (Acerbi et al., 2015; Schedin and Keely, 2011). It is not well known how mechanosensory or cell signalling cues are mediated through actin to Hippo components. As mentioned, actin polymerization can induce YAP activity by decreasing inhibitory binding of AMOT to YAP. AMOT is an F-actin binding protein and the mechanism here is that polymerized actin competes with YAP for AMOT binding (Mana-Capelli et al., 2014).

5.4 Hippo signalling and cancer

As the Hippo pathway controls cell growth and survival its aberrant function can facilitate cancer formation and progression through excess YAP/TAZ activity and concomitant increased expression of their pro-proliferative, pro-migratory and anti-apoptotic target genes. High nuclear localization and activity of YAP has been reported for various solid tumours including breast, colorectal, liver, lung, ovarian and skin cancer, and YAP activity is also associated with poor prognosis (Feng et al., 2014; Konsavage et al., 2012; Steinhardt et al., 2008; Xu et al., 2009; Yu et al., 2014). The YAP gene locus resides on chromosome 11q22 whose amplification is common in human cancers (Overholtzer et al., 2006). Mutations in Hippo pathway components are rare but there are some instances of Hippo-related mutations associated with cancer. Lats2 and NF2 are mutated in malignant mesothelioma (Bianchi et al., 1995; Murakami et al., 2011). As its name implies, NF2 is also mutated in neurofibromatosis, a condition which causes tumours of the nervous system (Petrilli and Fernández-Valle, 2015). As mentioned earlier, YAP activity is associated with uveal melanoma where the GNAQ and GNA11 genes (which encode G_q and G₁₁) are constitutively active (Feng et al., 2014; Yu et al., 2014). Inactivation of YAP/TAZ in cancer cell lines or xenograft tumours inhibits cellular migration and EMT occurrence, decreases cancer stem cell characteristics, makes cells more susceptible to anchorage-dependent cell death (anoikis) and ultimately reduces metastasis. In contrast, over-expression of YAP/TAZ in normal cells induces EMT and transforms them into malignant cells with cancer stem cell properties and metastatic ability. Accordingly, YAP has been shown to mediate K-Ras-dependent tumour formation. Hippo signalling also mediates transformation and tumorigenesis induced by tumour viruses (Kaposi's sarcoma-associated virus, simian virus 40, Merkel cell polyomavirus). They inhibit Mst1/2-Lats1/2 and activate YAP/TAZ either via GPCR signalling or through NF2 inhibition (Liu et al., 2015b; Nguyen et al., 2014).

Some Hippo pathway targeting compounds have been studied from the viewpoint of cancer therapy. Verteporfin is a drug in clinical use as a photosensitizer in photodynamic therapy used to destroy defective blood vessels in the eye. It is also an inhibitor of YAP and has been shown

to inhibit growth of several cancer cell types (Ciamporcero et al., 2015; Garcia-Rendueles et al., 2015; Liu-Chittenden et al., 2012; Slemmons et al., 2015; Yu et al., 2014). Statins are a group of drugs used to lower blood cholesterol levels and they have also been shown to inhibit the nuclear localization of YAP/TAZ (Sorrentino et al., 2014). Recently, it was shown that the cancer drug dasatinib inhibits YAP/TAZ in cancer cells and that a combination of statins and dasatinib has a cumulative inhibitory effect on YAP/TAZ (Taccioli et al., 2015). Vestigial-like family member 4 (VGLL4) is a tumour suppressor, which inhibits YAP activity by competing with YAP for TEAD binding. A VGLL4-mimicking peptide has been developed and shown to inhibit growth of xenograft gastric cancer tumours (Jiao et al., 2014; Zhang et al., 2014b).

5.4.1 Tumour suppressor function of YAP

Though YAP is predominantly a potent pro-carcinogenic factor it has been shown to function as a tumour suppressor in some contexts. YAP is able to inhibit the proto-oncogene β -catenin by recruiting the SCF ^{β -TRCP} ubiquitin ligase to the β -catenin destruction complex and inducing β -catenin degradation, by sequestering β -catenin in the cytoplasm and by preventing its interaction with other binding partners (Azzolin et al., 2014; Imajo et al., 2012). In haematological cancers YAP has a tumour suppressor function through induction of apoptosis. DNA damage leads to nuclear translocation of the ABL1 tyrosine kinase. ABL1 in turn phosphorylates nuclear YAP and causes it to complex with the tumour suppressive transcription factor p73. The ABL1-YAP-p73 complex then induces expression of pro-apoptotic genes (Bax, PUMA, PIG3). In contrast to other cancer types, in haematological cancers YAP expression is typically low due to deletions affecting the YAP locus and lack of YAP hinders DNA damage-induced apoptosis of these cancer cells (Cottini et al., 2014; Levy et al., 2008).

Aims

The aim of this thesis work was to investigate novel regulatory targets for S1P and SPC signalling which may mediate the cancer-related function of these sphingolipids. In more detail, the goals of the studies included in the thesis were to:

- i. Find target molecules, which are affected by S1P or SPC treatment of cancer cells. These may be molecules, which have implications of being S1P/SPC targets but have not been studied extensively or in the context of cancer.
- ii. Determine whether the target molecule mediates the effect S1P/SPC has on cancer cell behaviour.
- iii. Study the signalling leading from sphingolipid to target molecule.

Experimental procedures

Cell culture (I, II, III)

The human follicular thyroid cancer cell lines ML-1 and FTC-133 were from Dr. Johann Schönberger (University of Regensburg, Germany) and Banca Biologica e Cell Factory, National Institute for Cancer Research (Genova, Italy), respectively. MDA-MB-435S human breast cancer cells were from Dr. Hans-Peter Altevogt (German Cancer Research Center (DKFZ), Heidelberg, Germany) and C643 human anaplastic thyroid cancer cells from Dr. Nils-Erik Heldin (Karolinska Institutet, Stockholm, Sweden). Wild type and vimentin-knockout immortalized mouse embryonic fibroblast (MEF) cells were isolated by Dr. John Eriksson's lab. MDA-MB-435S cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% non-essential amino acids (NEAA), 1% L-glutamine and 1% penicillin-streptomycin. All other cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin. All cells were cultured in a humidified chamber at 37°C and 5% CO₂. Before experiments with sphingolipids cells were lipid-starved overnight in medium containing 5% lipid-stripped FBS (LS-FBS) instead of normal FBS. LS-FBS was prepared with activated charcoal (5%) and dextran (0.5%). They were washed twice with water and then mixed with FBS. The mixture was incubated in a shaker (>60 rounds per minute, 1 h, 37°C) and the charcoal and dextran were removed from the FBS through centrifugation (1125 g, 20 min) and sterile filtration.

Western blotting (I, II, III)

Cell lysates were prepared either with a NP-40 lysis buffer (10 mM Tris, 150 mM NaCl, 7 mM EDTA, 0.5% NP-40, 0.2 mM PMSF, 0.5 mg/ml leupeptin, pH 7.7) or as whole cell lysates directly in Laemmli sample buffer (LSB). When using lysis buffer, the lysates were cleared from cell debris with centrifugation (13 000 g, 15 min, 7°C) and concentrations of the lysates were determined with a bicinchoninic acid (BCA) assay before addition of LSB and boiling. Samples were loaded on 6-12% acrylic amid gels for SDS-PAGE (120 V, 90 min) and proteins were then transferred onto nitrocellulose membranes (100 V, 60 min). The membranes were blocked with 5% milk (RT, 60 min) before incubation in primary antibody (RT, 1 h or 7°C, overnight) and then secondary antibody (RT, 1 h). β -actin or Hsc70 were used as loading controls. Proteins were visualized on X-ray film with enhanced chemiluminescence (ECL). Densitometry of the protein bands was performed with the ImageJ software (<http://rsbweb.nih.gov/ij/>).

Real-time quantitative PCR (qPCR) (I, II, III)

Total RNA samples were prepared either with the Aurum Total RNA Mini kit (Bio-Rad; Hercules, CA, USA) or with TRI reagent (Sigma-Aldrich; St. Louis, MO, USA). RNA concentrations were measured either with the RiboGreen fluorescent dye or with a NanoDrop spectrophotometre (both from Thermo Fisher Scientific; Waltham, MA, USA). RNA purity was checked spectrophotometrically by measuring the absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} . cDNA was prepared with reverse transcriptase PCR from equal amounts of RNA, with either SuperScript III or RevertAid reverse transcriptase (both from Thermo Fisher Scientific). A non-

template control (NTC) cDNA sample and a reverse transcriptase negative cDNA sample with a mixture of RNAs as template were prepared as negative controls. Primers were designed with either Universal Probe Library (UPL) Assay Design Center (www.roche-applied-science.com) or NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/). qPCR reactions were prepared with Absolute qPCR ROX Mix (Thermo Fisher Scientific) or KAPA Probe Fast qPCR Mix (Kapa Biosystems; Boston, MA, USA) containing the DNA polymerase, nucleotides, reference dye and reaction buffer, or KAPA SYBR Fast qPCR Mix (Kapa Biosystems) which additionally contains the SYBR Green DNA dye. The qPCR runs were performed with either the 7900HT Fast Sequence Detection System or StepOnePlus Real-Time PCR System (both from Thermo Fisher Scientific), either Taqman probes (UPL; Roche; Basel, Switzerland) or SYBR Green DNA dye was used for fluorescence signal production and either the relative standard curve method or the comparative threshold cycle method was used to determine relative gene expression. GAPDH, β -actin, HPRT or PBGD was used as a reference gene.

Qualitative end-point PCR (III)

RNA samples were prepared as for qPCR. To make sure that genomic DNA contamination did not cause false positives, the RNA samples were treated with DNase and a reverse transcriptase negative cDNA reaction was prepared from every RNA sample and used side-by-side with the respective reverse transcriptase positive cDNA sample. PCR reactions were prepared with JumpStart *Taq* DNA polymerase (Sigma-Aldrich) and thermal cycling was performed with a PTC-200 Thermal cycler (MJ Research; Waltham, MA, USA). PCR products were run on a 1% agarose gel containing ethidium bromide (1 $\mu\text{g}/\text{ml}$) and DNA bands were visualized with the AlphaImager HP system (Protein Simple; Santa Clara, CA, USA). GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific) was used as a DNA size marker.

Migration (I, II)

Boyden chamber chemotaxis and haptotaxis experiments were performed with Costar Transwell permeable support inserts (Corning Inc.; Corning, NY, USA) with a 6.5 mm diameter and 8 μm pore size. In chemotaxis experiments the chamber membranes were coated with 5 $\mu\text{g}/\text{cm}^2$ collagen IV (I) (overnight, RT) or left uncoated (II). The membranes were reconstituted with lipid-stripped medium for at least 1 h prior to the experiment and 50 000-100 000 cells per insert were added into the upper chamber. The chemoattractant and test substances were added into the lower well (12-well plate well). In some experiments with ML-1 cells (I) only S1P (100 nM) was used as a chemoattractant, otherwise 10% LS-FBS was used as a chemoattractant in chemotaxis experiments (I, II). In haptotaxis experiments (I) a collagen IV coating on the outer surface of the Boyden chamber membrane was used as an attractant. Cells were allowed to migrate for 6-16 h. In overnight migration experiments (II) mitomycin C (1 $\mu\text{g}/\text{ml}$) was added to both chambers to prevent cell proliferation. Migration was stopped by removing the medium from the upper chamber, wiping non-migrated cells from the membrane with a cotton swab and fixing the cells with 2% paraformaldehyde (PFA, RT, 10 min). The cells were then stained with crystal violet (RT, 5 min) and the membranes rinsed two times with PBS and once with water. Migrated cells were counted at 40x magnification

from eight microscopic fields per chamber. In single cell random motility experiments cells plated in 24-well plates were imaged every 1 h for 20 h with a Cell-IQ instrument (Chip-Man Technologies; Tampere, Finland). From the resulting videos movement of individual cells was manually tracked for 20 cells per video with the ImageJ Manual Tracking plugin and vector plots were created with the Ibidi Chemotaxis and Migration Tool (www.ibidi.com). In wound healing experiments cells were plated in 96-well Essen Image Lock plates, wounds were made with an Essen WoundMaker and imaged with Incucyte ZOOM. Images were taken every 2 h for 24 h and wound closure was analyzed with the Incucyte Scratch Wound Analysis module (all from Essen Bioscience; Ann Arbor, MI, USA).

³H-thymidine incorporation (I, III)

Cells were plated on 35-mm plates either directly in LS-FBS medium (75 000 cells) or in normal medium (50 000 cells) followed by lipid starvation the next day. Duplicate or triplicate plates were prepared for each treatment. Cells were treated overnight with S1P or SPC and ³H-thymidine (4 μ Ci/ml) was added on the plates 4 hours before the end of the experiment. Finally, cells were washed three times with PBS, fixed with 5% trichloroacetic acid (TCA; RT, 10 min) and solubilized with 0.1 N NaOH (RT, 10 min). The NaOH solution was placed in a scintillation tube, scintillation liquid was added and radioactivity as counts per minute (cpm) was measured with a scintillation counter.

Transfections (I, II, III)

Transfection of small interfering RNA (siRNA) and plasmids was performed either with electroporation or transfection reagents. The Gene Pulser Xcell Electroporation System (Bio-Rad; 2 μ M siRNA, 20-25 μ g plasmid, 0.4 cm cuvette, 240 V, 975 μ F) or the Amaxa electroporation device (Lonza; Basel, Switzerland; according to the manufacturer's instructions) was used for electroporation. The transfection reagents used were Metafectene Pro (Biontex; Munich, Germany; 100-200 nM siRNA), N-TER (Sigma-Aldrich, 100 mM siRNA), HiPerfect (Qiagen; Venlo, Netherlands; 5-20 nM siRNA) and Lipofectamine (Thermo Fisher Scientific, according to the manufacturer's instructions). The siRNA sequences were either from the literature, commercial or designed with the Eurofins Genomics siRNA Design Service (www.eurofinsgenomics.eu/). Cells were used in experiments 48-72 h after transfection. mCherry-tagged vimentin plasmids (Vim^{WT}, Vim^{S71A}) were from Hong-Chen Chen (National Chung Hsing University, Taiwan) (Pan et al., 2011), Myc-tagged Lats2 plasmids (Lats2^{WT}, Lats2^{KD}) were from Dr. Norikazu Yabuta and Dr. Hiroshi Nojima (Osaka University, Japan) (Toji et al., 2004) and Flag-tagged YAP plasmids (YAP^{WT}, YAP^{S127A}) from Addgene (plasmids 17790 and 17791) (Komuro et al., 2003). Stable cell pools of mCherry-Vim expressing cells were made with geneticin selection and the cells were transfected again before experiments in order to maximize mCherry-Vim expression.

Luciferase assay (I)

Cells were transfected with a plasmid containing the firefly luciferase gene (Luc) under either a HRE promoter or a HIF-1-independent TK promoter. A plasmid containing the *Renilla*

reniformis luciferase gene under a highly active Ubi promoter was co-transfected into the cells to enable normalization of the firefly luciferase signal with *Renilla* luciferase luminescence. Cobalt chloride (CoCl₂) was used as a positive control for HRE activation and luminescence was measured with the DualGlo Luciferase Kit (Promega; Fitchburg, WI, USA). The HRE-Luc plasmid was from Addgene (plasmid 26731) (Emerling et al., 2008) and the TK-Luc and Ubi-*Renilla* constructs from Klaus Elenius (University of Turku) and Jukka Westermarck (University of Turku), respectively.

Immunoprecipitation (IP) (I)

Cells were lysed with an IP lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 0.2 mM PMSF, 0.5 mg/ml leupeptin). Protein concentrations were measured and the lysates adjusted to equal protein amount and volume (typically 0.5-1.0 µg/µl, 200-400 µl). The lysates were pre-cleaned with Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Inc.; Dallas, TX, USA; 20 µl, 1 h, 4°C) before addition of antibody or IgG control and incubation to enable antibody and target protein binding (2 µg, overnight, 4°C). Next Protein A/G PLUS-agarose beads were added and the samples were incubated to allow binding of beads and antibodies (40 µl, 2 h, 4°C). The beads were then washed five times with IP washing buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.1% NP-40) before boiling in LSB. Immunoprecipitation and co-immunoprecipitation of proteins was then examined with Western blotting.

Mass spectrometry (II)

Mass spectrometry was used to detect changes in post-translational modifications of the vimentin protein. Cell lysates were prepared with an SDS buffer, sonicated and boiled. Proteins were separated with SDS-PAGE, the gels were stained with Coomassie Blue and an intermediate filament preparation was used as a marker for vimentin migration. The vimentin-containing areas were cut from the gel, digested with trypsin and chromatography and mass spectrometry (LC-MS/MS) were performed with an EASY-nLC 1000 nanoflow liquid chromatograph coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific) as described elsewhere (Imanishi et al., 2007). Database search was done against the SwissProt (*Homo sapiens*) database and the reversed sequence decoy database using Mascot 2.4 (Matrix Science; London, UK) through the Proteome Discoverer 1.3 software (Thermo Fisher Scientific). For label-free quantification the Progenesis LC-MS 4.0 software (Nonlinear Dynamics; Newcastle upon Tyne, UK) was used.

Immunofluorescence (II)

Cells were grown on glass coverslips and after treatment fixed with 3.7% PFA (5 min, RT) and permeabilized with 0.1% Triton X-100 (RT). The coverslips were blocked with 10% goat serum or 1% bovine serum albumin (BSA; 1 h, RT), incubated in primary antibody (1 h, RT or overnight, 4°C), washed with PBS and incubated in Alexa-Fluor-conjugated secondary antibody. For visualization of actin the coverslips were incubated in Alexa-Fluor-conjugated phalloidin. Next the coverslips were washed twice with PBS and once with water and mounted with Mowiol. Imaging was performed with a Leica SP5 laser scanning confocal microscope

(Leica; Wetzlar, Germany) using a 60×/1.4 NA plan-apochromat oil immersion objective. In some experiments widefield images were attained with a Leica DMRE 20× magnification objective

Cell shape (II)

Cells were labelled with CFSE CellTracker dye. After sphingolipid treatment cells were fixed and mounted and whole cells were imaged with a Leica SP5 or Zeiss LSM 780 confocal microscope with a 20× air objective. Cell volume was analyzed with BioImageXD (Kankaanpää et al., 2012). Images were converted into 512 x 512 resolution and stacks were segmented to identify individual cells. Objects with less than 500 voxels were removed from the analysis. For measurement of the cell perimeter 3D image stacks were made into maximum projections and analysis was performed with CellProfiler (Carpenter et al., 2006).

Fluorescence recovery after photobleaching (II)

Cells were plated on 35-mm glass-bottomed dishes and transfected with the mCherry-Vim^{WT} plasmid. The fluorescent mCherry tag was utilized to measure vimentin filament turnover with FRAP. FRAP was performed with a Zeiss 780 confocal microscope with a 60× water immersion objective. Bar-shaped 2 µm-wide regions were bleached and recovery was imaged every 30 s for 15 min. Intensity of the bleached spot and control area was analyzed with ImageJ and fitting of the recovery curve and calculation of half-recovery and mobile fraction values was performed with the FCalc software. Analysis was performed for areas where individual filaments could be followed for the entire length of the experiment. For FRAP calculation one function fit and correction for bleaching were used. Recovery curve data was normalized by deducting background noise and normalizing all experiments to a 0-1 scale. The exponential one-phase association recovery curve was fitted with the GraphPad Prism software (GraphPad Prism 5; San Diego, CA, USA).

Statistical analysis (I, II, III)

Results are shown as mean ± SEM. For comparison of means either Student's *t*-test or one-way ANOVA with Dunnett's or Bonferroni's *post hoc* test was used. In half-life measurements curve fit with the one phase exponential decay equation was used. Statistical analysis was performed and graphs made with GraphPad Prism.

Results and discussion

1 S1P regulates thyroid cancer cell migration through HIF-1 (I)

The follicular thyroid cancer cell line ML-1 has been used by our research group in several studies concerning S1P and VEGF signalling. A physiological concentration (100 nM) of S1P had been shown to potently induce ML-1 migration (Balthasar et al., 2006). Additionally, S1P induced VEGF secretion in these cells and inhibition of VEGFR2 or neutralization of VEGF with an antibody attenuated their S1P-induced migration (Balthasar et al., 2008). As HIF-1 is a central regulator of VEGF signalling as well as migration and metastasis, we hypothesized that it might be involved in the pro-migratory S1P response.

1.1 S1P increases HIF-1 activity by up-regulating HIF-1 α through S1P₃

We found S1P to increase HIF-1 α protein levels in ML-1 and FTC-133 follicular thyroid carcinoma cells in normoxia (I, Fig 1A,B; S1A). S1P also increased HIF-1 transcription factor activity in ML-1 cells as it induced transcription from the HRE-containing promoter of a HRE-luciferase construct and up-regulated mRNA of the HIF-1 target genes VEGF-A, autocrine motility factor (AMF) and TGF α in a HIF-1 α -dependent manner (I, Fig 1D,E). We found S1P to regulate HIF-1 α through the S1P₃ receptor and its coupling to G_{i/o}. Inhibition or knockdown of S1P₃ and inhibition of G_{i/o} prevented the S1P response, whereas knockdown of S1P₁ and S1P₂ or stimulation of S1P₁ was without an effect on HIF-1 α (I, Fig 2A-D, S2). Additionally, knockdown of PKC β 1 attenuated S1P-induced HIF-1 α expression (I Fig 3E).

1.2 S1P induces signalling involved in HIF-1 α translation

We wanted to determine whether the mechanism of S1P-induced up-regulation of HIF-1 α was increased synthesis or stability. To address the question of whether HIF-1 α synthesis is induced we first checked HIF-1 α mRNA expression and found that S1P had no effect on HIF-1 α transcription during the time frame of HIF-1 α protein up-regulation (I, Fig S3). As discussed earlier, HIF-1 α synthesis is increased by activation of the translational regulators p70S6 kinase (p70S6K) and eukaryotic initiation factor 4E (eIF-4E) which lie downstream of the MAPK and PI3K-Akt-mTOR pathways (Fukuda et al., 2002; Herbert et al., 2000; Laughner et al., 2001; Semenza, 2003; Shi et al., 2002; Zhong et al., 2000). p70S6K and eIF-4E activity is stimulated by phosphorylation of Thr389 on p70S6K among other residues and Ser209 on eIF-4E (Dennis et al., 1998; Dufner and Thomas, 1999; Pyronnet et al., 1999; Waskiewicz et al., 1999). Additionally, eIF-4E is regulated by binding of the inhibitor protein 4E-BP1 whose phosphorylation on several residues, including Ser65, decreases its affinity for eIF-4E (Heesom et al., 2001; Niedzwiecka et al., 2002). We found S1P to increase phosphorylation of p70S6K, eIF-4E and 4E-BP1 on the mentioned residues and also to stimulate phosphorylation of mTOR (I, Fig 5A-G). Importantly, inhibition of p70S6K, MEK, PI3K or mTOR abolished S1P-induced HIF-1 α expression (I, Fig 3A-C, 5H). Also, the S1P-induced phosphorylation of the different pathway components was prevented by inhibition of the upstream molecules. Inhibition of not only PI3K but also MEK prevented mTOR phosphorylation, inhibition of MEK, PI3K or mTOR

prevented phosphorylation of eIF-4E and 4E-BP1 and inhibition of PI3K or mTOR prevented phosphorylation of p70S6K (I, Fig 5A-G). This data strongly points to S1P regulating HIF-1 α synthesis through a MEK/PI3K-mTOR-p70S6K/eIF-4E pathway.

1.3 S1P also increases HIF-1 α stability

Since the HIF-1 α level is classically mainly determined by PHD- and pVHL-dependent effects on protein stability, we studied this aspect also (Cockman et al., 2000; Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Maxwell et al., 1999; Tanimoto et al., 2000; Yu et al., 2001). In order to determine whether S1P affected HIF-1 α stability we measured half-life of HIF-1 α with the help of the translation inhibitor cycloheximide (Chx). We found that S1P-induced HIF-1 α ($t_{1/2}$ 3.0 min) is more stable than basal HIF-1 α ($t_{1/2}$ 0.4 min) but clearly less stable than hypoxia-induced ($t_{1/2}$ 9.7 min) or CoCl₂-induced ($t_{1/2}$ 41.5 min) HIF-1 α (I, Fig 4A). Therefore, S1P treatment does increase stability of HIF-1 α , though not nearly as potently as hypoxia, which is the classical inhibitor of HIF-1 α degradation, or CoCl₂, which is commonly used as a positive control for HIF-1 α expression due to its robust induction of HIF-1 α stability. Proteasome inhibition expectedly caused a massive increase in HIF-1 α level but also prevented any further effect by S1P (I, Fig S4A). When we checked the hydroxylation status of the two proline residues classically regulating HIF-1 α stability, we found that the OH-P564-HIF-1 α level rose in tandem with the total-HIF-1 α level, while the OH-P402-HIF-1 α level stayed unchanged (I, Fig S4B,C). Therefore, we hypothesized that decreased P402-hydroxylation might be responsible for S1P-induced HIF-1 α stabilization. Surprisingly, co-immunoprecipitation of pVHL and HIF-1 α showed that even S1P-induced HIF-1 α was bound to pVHL and, thus, the up-regulation of HIF-1 α was not due to decreased interaction with pVHL (I, Fig 4B). HIF-1 α stability is also regulated by binding of RACK1, which promotes HIF-1 α degradation, and Hsp90 which prevents the RACK1-HIF-1 α interaction (Liu et al., 2007). We conducted immunoprecipitation experiments to determine whether the binding of RACK1/Hsp90 to HIF-1 α mediates the S1P-induced increase of its stability but saw no co-immunoprecipitation of HIF-1 α with either protein. However, inhibition of Hsp90 did prevent S1P-induced HIF-1 α expression in addition to significantly decreasing the basal HIF-1 α level (I, Fig S4E), which implies that S1P may stabilize HIF-1 α via increased Hsp90 binding. The PI3K-Akt pathway stabilizes HIF-1 α by inhibiting GSK3 β -mediated phosphorylation and subsequent ubiquitination of HIF-1 α (Cassavaugh et al., 2011; Flügel et al., 2007). Since we showed PI3K to mediate the up-regulation of HIF-1 α (I, Fig 3A), it is possible that the Akt-GSK3 β axis is also involved in the increased stability of HIF-1 α .

1.4 S1P-induced migration is mediated by HIF-1

A central part of the project was to determine whether S1P-induced HIF-1 activity was involved in the S1P-induced chemotactic migration of ML-1 cells previously shown by our laboratory (Balthasar et al., 2006). We found that inhibition of HIF-1 prevented S1P-induced migration towards serum in Boyden chambers (I, Fig 6A). Additionally, knockdown of HIF-1 α attenuated basal migration towards serum and S1P-induced migration in a serum-free setting (I, Fig 6B,C). Also, inhibition of p70S6K prevented and knockdown of S1P₃ inhibited S1P-

induced migration towards serum (I, Fig 6D,E). Therefore, HIF-1 does mediate the pro-migratory effect of S1P in normoxic conditions. We also conducted experiments in hypoxic conditions. We saw a robust up-regulation of the pro-migratory receptor S1P₁ in hypoxia but surprisingly no increase in basal migration or any change in the potent pro-migratory S1P response (I, Fig 7A,B). This was the case in both chemotaxis experiments, where serum was used as a chemoattractant, and in haptotaxis experiments, where cells migrate towards the ECM protein collagen IV. Therefore, though HIF-1 is involved in S1P-induced ML-1 migration in normoxia, it is apparently not the main pro-migratory factor mediating the S1P response in either normoxia or hypoxia.

1.5 S1P and HIF-1 signalling communicate in many ways

During the early stages of our project, SphK1-S1P was revealed to mediate the hypoxia-induced stabilization of HIF-1 α through the Akt-GSK3 β pathway (Ader et al., 2008) and SphK1 was shown to be a HIF-1 target gene up-regulated during hypoxia (Anelli et al., 2008; Schwalm et al., 2008). While our project was ongoing, four studies found S1P able to regulate HIF-1 α expression in normoxic conditions. Two showed S1P to up-regulate HIF-1 α in white blood cells via increased mRNA expression (Herr et al., 2009; Srinivasan et al., 2008). One investigated S1P function in vascular biology and showed S1P to stabilize HIF-1 α in vascular endothelial and smooth muscle cells through S1P₂ (Michaud et al., 2009). One showed S1P to up-regulate the HIF-1 α protein in HepG2 liver carcinoma cells but no experiments regarding mechanism or signalling of S1P-induced HIF-1 α expression were conducted, as the emphasis of the study was on characterization of the S1P-derivative NHOBTD and its inhibitory effect on S1P responses (Kim et al., 2011b). Therefore, our publication was the first to study the connection of S1P and HIF-1 signalling in normoxic cancer cells in more depth. The significance of normoxic HIF-1 α regulation in relation to cancer is that it is a clearly oncogenic factor whose increased expression facilitates tumour progression.

During our project and since its publication our research group has continued to study ML-1 migration. We have shown that in these cells VEGFR2 and S1P₁ are able to form a signalling complex which activates ERK1/2 and mediates haptotaxis induced by both VEGF and S1P (Bergelin et al., 2010), that S1P promotes secretion of matrix metalloproteinases (MMP2/9) to facilitate ML-1 invasion and that the calcium channel TRPC1 regulates expression of S1P₃, VEGFR2, MMP2/9 as well as HIF-1 α and is, therefore, involved in S1P-induced ML-1 migration (Asghar et al., 2015). Interestingly, another group has recently also studied S1P-induced migration in ML-1 and FTC-133 cells and found that the S1P response was mediated by repression of the micro-RNA miR-17, up-regulation of its target PTK6 which is a cytoplasmic tyrosine kinase and subsequent ERK1/2 activation (Zhao and Li, 2015). This shows another mechanism for S1P-induced MAPK signalling which according to our study and other studies by our group mediates the pro-migratory S1P response.

After the publication of our study, it has been shown that S1P and a HIF-1-stabilizing PHD-inhibitor have a cumulative pro-angiogenic effect on fibroblasts and endothelial cells (Lim et al., 2013). In relation to hypoxia, exogenous S1P has been shown to alleviate the negative

effects of hypoxia-induced stress on murine splenocytes by increasing HIF-1 α stability (Chawla et al., 2014). Recently, blockage of S1P-S1PR signalling by the S1P-neutralizing antibody sphingomab was shown to attenuate hypoxia-induced HIF-1 α accumulation in several cancer cell lines. Sphingomab also decreased intratumoural hypoxia *in vivo* by normalizing tumour vasculature and subsequently sensitized hypoxic tumours to chemotherapy (Ader et al., 2015).

2 S1P and SPC regulate vimentin-dependent cancer cell migration (II)

Both S1P signalling and vimentin are central cancer-related regulators of cellular migration and invasion and involved in metastatic cancer progression. Previously, S1P had been shown able to affect vimentin organization (Sin et al., 1998) and SPC to promote migration of Panc-1 pancreatic cancer cells through phosphorylation and reorganization of keratin intermediate filaments (Beil et al., 2003). Therefore, we wanted to test if we could see S1P- or SPC-induced effects on vimentin in cancer cells whose migration these lipids regulate.

2.1 S1P and SPC induce re-organization of vimentin

In a preliminary experiment, we checked vimentin expression in a panel of cell lysates from different cancer cell lines. We saw no change in vimentin levels in whole cell lysates but fractionated lysates showed some differences. These samples had been made by lysing the cells in a NP-40 (0.5%) containing lysis buffer and then removing insoluble matter through centrifugation. Especially in the breast cancer cell line MDA-MB-435S and the anaplastic thyroid cancer cell line C643 the solubility, organization or localization of vimentin seemed to be affected as S1P and SPC caused a decrease in vimentin in the soluble fraction (Figure 8). When we used immunofluorescence to check the effect of S1P and SPC on the vimentin filament network, we saw a rapid reorganization with both lipids and cell lines. While S1P caused the vimentin filament network to collapse into the perinuclear space into a more compact structure, SPC caused the network to change into a more fuzzy appearance (II, Fig 2A, S1C). S1P and SPC also caused a rapid rounding of the cells and a decrease in cell perimeter (II, Fig 2B,C) but the effect on the vimentin network was still evident, especially in C643 cells which round up much less than MDA-MB-435S cells. Fluorescence recovery after photobleaching (FRAP) measurements with S1P revealed that it decreased vimentin filament turnover in MDA-MB-435S cells (II, Fig S2A,B), which is consistent with the organization change. Our research group had previously shown S1P to inhibit chemotactic migration of C643 cells in Boyden chambers (Asghar et al., 2012) and we found SPC to have the same effect. In MDA-MB-435S cells both S1P and SPC also potentially inhibited Boyden chamber migration (II, Fig 1A,B). Additionally, we found S1P and SPC to inhibit random movement of MDA-MB-435S cells in cell tracking experiments (II, Fig 4D, 5C) and wound healing of C643 cells (II, Fig 1C).

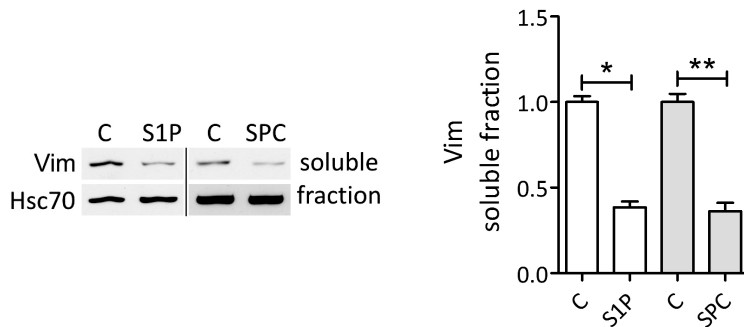


FIGURE 8: Preliminary experiments revealed S1P and SPC to induce a change in vimentin solubility or organization in MDA-MB-435S cells. Cells were treated with S1P (100 nM, 30 min) or SPC (10 μ M, 1 h) and lysed with a NP-40 lysis buffer. The lysates were cleared with centrifugation and vimentin protein expression in the supernatant (soluble fraction) was analysed by western blotting. Results are mean \pm SEM, n = 3-5; *P<0.05, **P<0.01; Student's *t*-test.

2.2 S1P and SPC stimulate vimentin S71 phosphorylation

Changes in vimentin structure or function are typically mediated by post-translational modifications (PTMs), most often phosphorylation (Eriksson et al., 2004). Because of the large number of known vimentin PTM sites a mass spectrometry screen was required to find residues and modifications affected by S1P/SPC-treatment. Several sites were modified in response to S1P or SPC in MDA-MB-435S cells, but the only residue with the same modification affected by both lipids in the same way was S71 whose phosphorylation was increased by both lipid treatments (II, Table S1, Fig 1D). We used Western blotting to validate the result and found both S1P and SPC to induce a strong, rapid increase in pS71-Vim levels (II, Fig 1E,F). We did a time series treatment with the lipids to study the kinetics of the phosphorylation and found that in both MDA-MB-435S and C643 cells the S1P response was more rapid and more transient, while the SPC response was slower but more persistent (II, Fig 3).

2.3 The S1P₂-Rho-ROCK pathway mediates S1P/SPC-induced effects on vimentin

As mentioned, S1P₂ is the classically anti-migratory S1P receptor which preferably couples to G_{12/13} and activates Rho and Rho-ROCK signalling (Adada et al., 2013; Lepley et al., 2005). Importantly, ROCK had been shown to mediate phosphorylation of S71-Vim in several previous studies (Goto et al., 1998; Inada et al., 1999; Nakamura et al., 2000; Yokoyama et al., 2005). Moreover, S1P treatment (1 μ M) had been shown to induce changes in both the vimentin cytoskeleton and ROCK activation in HeLa cells (Sin et al., 1998). Therefore, we hypothesized that S1P and SPC might regulate phosphorylation and organization of vimentin, as well as migration, through S1P₂ and Rho-ROCK in MDA-MB-435S and C643 cells. We found that inhibition or knockdown of S1P₂ either prevented or significantly attenuated S1P/SPC-induced S71-Vim phosphorylation (II, Fig 4A,B), as did inhibition of ROCK (II, Fig 5A). Inhibition of either S1P₂ or ROCK also prevented S1P/SPC-induced changes in the vimentin cytoskeleton (II, Fig 4C, 5B) and cell motility in single cell tracking experiments (II, Fig 4D,E; 5C,D). Furthermore, ROCK inhibition prevented the anti-migratory effect of S1P in C643 wound

healing experiments (II, Fig S3C-E). Additionally, we found that the S71 residue of vimentin resides within a consensus site for ROCK-binding. The consensus sequence is R/K-X-S/T or R/K-X-X-S/T (Amano et al., 1996), where X is any amino acid, and the vimentin protein sequence at S71 is VRLRS⁷¹SVPG.

2.4 pS71-Vim mediates S1P/SPC-induced inhibition of migration

Next we wanted to determine if vimentin actually mediates the anti-migratory effect of S1P and SPC. For this purpose, we used vimentin knockout MEF cells. When we conducted migration experiments with these cells, we saw a strong S1P/SPC-induced inhibition of migration in wild-type cells but no significant effect in Vim-KO cells (II, Fig 6B). Importantly, we saw an increase in pS71-Vim levels after S1P and SPC treatment in wild-type MEFs (II, Fig 6A). In order to determine whether pS71-Vim mediates the anti-migratory S1P/SPC response we used exogenous over-expression of a mCherry-tagged S71A mutant vimentin which cannot be phosphorylated on S71. Expression of S71A-Vim in MDA-MB-435S cells strongly attenuated the S1P response and practically prevented the effect of SPC in Boyden chamber chemotaxis experiments (II, Fig 6D). In the S1P experiments, expression of the mutant also increased basal migration, which points to S71-Vim constitutively attenuating MDA-MB-435S migration to some extent. These results strongly point to vimentin, and more specifically pS71-Vim, mediating the anti-migratory S1P/SPC response.

2.5 Actin may be involved in the S1P/SPC-induced and vimentin-mediated effects on migration

The S1P/SPC-induced anti-migratory effect is most likely mediated by changes in actin in addition to vimentin. As discussed earlier, actin dynamics are essential for cell movement (Rottner and Stradal, 2011). S1P and SPC induced a change in the actin cytoskeleton as well as the vimentin network, as indicated by cell rounding and immunofluorescence imaging. The actin cytoskeleton seems to be responsible for the cell rounding effect, as the myosin II inhibitor blebbistatin prevented S1P/SPC-induced rounding of MDA-MB-435S cells and the actin inhibitor cytochalasin D caused a complete cell rounding which the lipids could not further affect (results not shown). As vimentin regulates focal adhesions in many ways as well as lamellipodia formation (Helfand et al., 2011; Mendez et al., 2010; Tsuruta and Jones, 2003), the S1P/SPC-induced effects on vimentin may well modulate migration through effects on these actin-related structures.

Very little has been known of the connection between S1P or SPC signalling and vimentin. An early study investigating ROCK-mediated regulation of intermediate filament organization used S1P as a Rho-ROCK activator in HeLa cells and found S1P to induce vimentin network reorganization which closely resembles our findings (Sin et al., 1998). However, the details of this effect or its relation to migration were not addressed before our study nearly two decades later. S1P has been associated with EMT and subsequent vimentin expression. S1P mediates the abnormal level of EMT typical of the lung disease idiopathic pulmonary fibrosis (Milara et al., 2012) and recently SphK1 was linked to increased EMT and vimentin expression in colorectal cancer cells (Long et al., 2015). In endothelial cells, S1P together with growth factors

was shown to promote angiogenic sprouting through a mechanism where S1P and growth factors induce calpain-mediated cleavage of vimentin and S1P then facilitates translocation of a vimentin/MT1-MMP complex to the plasma membrane (Kwak et al., 2012).

SPC has not been previously linked to vimentin but several studies have investigated SPC-induced effects on keratin intermediate filaments. SPC induces phosphorylation of keratin monomers in pancreatic and gastric cancer cells and reorganization of the keratin network. This, in turn, increases elasticity of the cells and facilitates their motility and invasion (Beil et al., 2003; Busch et al., 2012; Park et al., 2011). We saw no keratin expression in MDA-MB-435S or C643 cells and it is a fascinating thought that perhaps SPC-induced effects on migration could be determined by the intermediate filament protein profile of the target cell and whether their phenotype is epithelial or mesenchymal. The Panc-1 cells used in most of the SPC/keratin studies have been reported to express vimentin in addition to keratin (Long et al., 2006), but effects of SPC on vimentin in these cells have not been studied. However, neither has the receptor mediating the SPC response been determined and, therefore, it is impossible to say whether the opposite effect on migration is due to keratin versus vimentin or cell-specific signalling induced by SPC.

3 SPC regulates the Hippo signalling pathway (III)

The Hippo pathway is a novel signalling cascade formed of tumour suppressors and oncogenes implicated especially in regulation of tissue growth and emerging as an important player in tumour biology. We investigated whether Hippo signalling mediates anti-proliferative SPC signalling.

3.1 SPC inhibits MDA-MB-435S proliferation and up-regulates Lats2

We found SPC to potently inhibit proliferation of MDA-MB-435S cells in a thymidine incorporation assay, while S1P had no significant effect (III, Fig 1A). A microarray that had been performed with SPC-treated MDA-MB-435S samples showed that one of the most clearly up-regulated genes was Lats2, a Hippo pathway component and a novel tumour suppressor. We verified the results with qPCR and Western blotting and saw SPC induce a strong up-regulation of Lats2 on both mRNA and protein levels (III, Fig 1B-D). S1P also increased Lats2 expression, but the mRNA and protein expression induced by S1P was much more transient compared to the SPC response, which was sustained for the entire 24-h period monitored (III, Fig 1B,C). Interestingly, these kinetics resemble those of S1P/SPC-induced S71-Vim phosphorylation, where also the S1P response was transient and the effect of SPC long-lasting (II, Fig 3). This is indicative of a more robust effect of SPC on MDA-MB-435S cells in comparison to S1P. As Lats2 functions as a tumour suppressor by catalysing inhibitory phosphorylation of YAP on S127 (Hao et al., 2008; Zhao et al., 2007), we determined whether SPC caused changes in phosphorylation of this residue. While we saw no effect in whole cell lysates (III, Fig 3A), the NP-40 (0.5%) soluble cytosolic fraction showed a robust up-regulation of S127-phosphorylation at time points subsequent to increased Lats2 expression (9-24 h; III, Fig 3B)). Importantly, SPC down-regulated the YAP target genes CTGF and Cyr61 in response to long (9

h) SPC treatment (III, Fig 3C), which implies an inhibitory effect on YAP activity. S1P caused no change in YAP phosphorylation in the same setting (III, Fig 3B), which we hypothesized to be due to the transient effect of S1P on Lats2.

3.2 SPC transiently activates YAP

While our project was ongoing, S1P and another bioactive lipid, lysophosphatidic acid (LPA), were shown to regulate the Hippo pathway. The lipids induced a rapid dephosphorylation, up-regulation and nuclear localization of YAP via Lats1/2 in HEK293A cells and YAP mediated the pro-proliferative effect of LPA in these cells (Miller et al., 2012; Yu et al., 2012). When we checked the acute effect of SPC and S1P on MDA-MB-435S cells, we saw the same response: a rapid dephosphorylation of S127-YAP and a simultaneous increase in total YAP protein (III, Fig 5A). Also, CTGF and Cyr61 mRNA expression was potently but transiently induced by SPC within 0.5-3 h of SPC treatment (III, Fig 5B), which also indicates YAP activation. The decrease in target gene expression kinetics is consistent with the time-frame of SPC-induced Lats2 up-regulation, which apparently quenches the initial activation of YAP. We did not check phosphorylation of S381-YAP, but the effect on YAP protein level may well be due to dephosphorylation of this residue which regulates YAP stability and is also targeted by Lats1/2 (Zhao et al., 2010a). Since YAP/TAZ have recently been shown to induce Lats2 gene transcription in a negative feedback loop (Moroishi et al., 2015b) and SPC caused an acute YAP activation followed by up-regulation of Lats2 mRNA, we hypothesized that perhaps SPC-induced YAP and Lats2 also comprised a feedback loop. However, knockdown of YAP or TAZ had no effect on the SPC-Lats2 response (III, Fig 6B). Also, over-expression of wild-type YAP or a constitutively active S127A mutant had no effect on basal or SPC-induced Lats2 expression (III, Fig 6C).

3.3 SPC regulates Lats2, YAP and proliferation via S1P₂

Early on in the project we tested whether the receptors S1P₁₋₃ mediate SPC-induced Lats2 expression. We found that inhibition of S1P₂, but not of S1P_{1/3} (Figure 9), prevented the up-regulation and knockdown of S1P₂ strongly attenuated it (III, Fig 2A,B). However, inhibition of Rho or ROCK did not affect the Lats2 response indicating that the G_{12/13}-Rho-ROCK pathway is not mediating the up-regulation (III, Fig 2C,D). S1P₂ has been shown to inhibit Akt through Rho-ROCK-PTEN or PKC δ activation (Du et al., 2010; Sanchez et al., 2005; Schüppel et al., 2008; Takuwa et al., 2011). We found both PI3K and Akt inhibition to up-regulate Lats2 in MDA-MB-435S cells (III, Fig S1B,C,E), whereas inhibition of PTEN had no effect (III, Fig S1F). PKC δ is a DAG-dependent PKC isoform and the DAG-analogue and PKC activator phorbol 12-myristate 13-acetate (PMA) did cause a Lats2 up-regulation with similar kinetics as SPC (Figure 9). However, inhibition of DAG-activated PKCs did not affect the fold change of SPC-induced Lats2 expression though it did strongly decrease basal Lats2 expression (Figure 9).

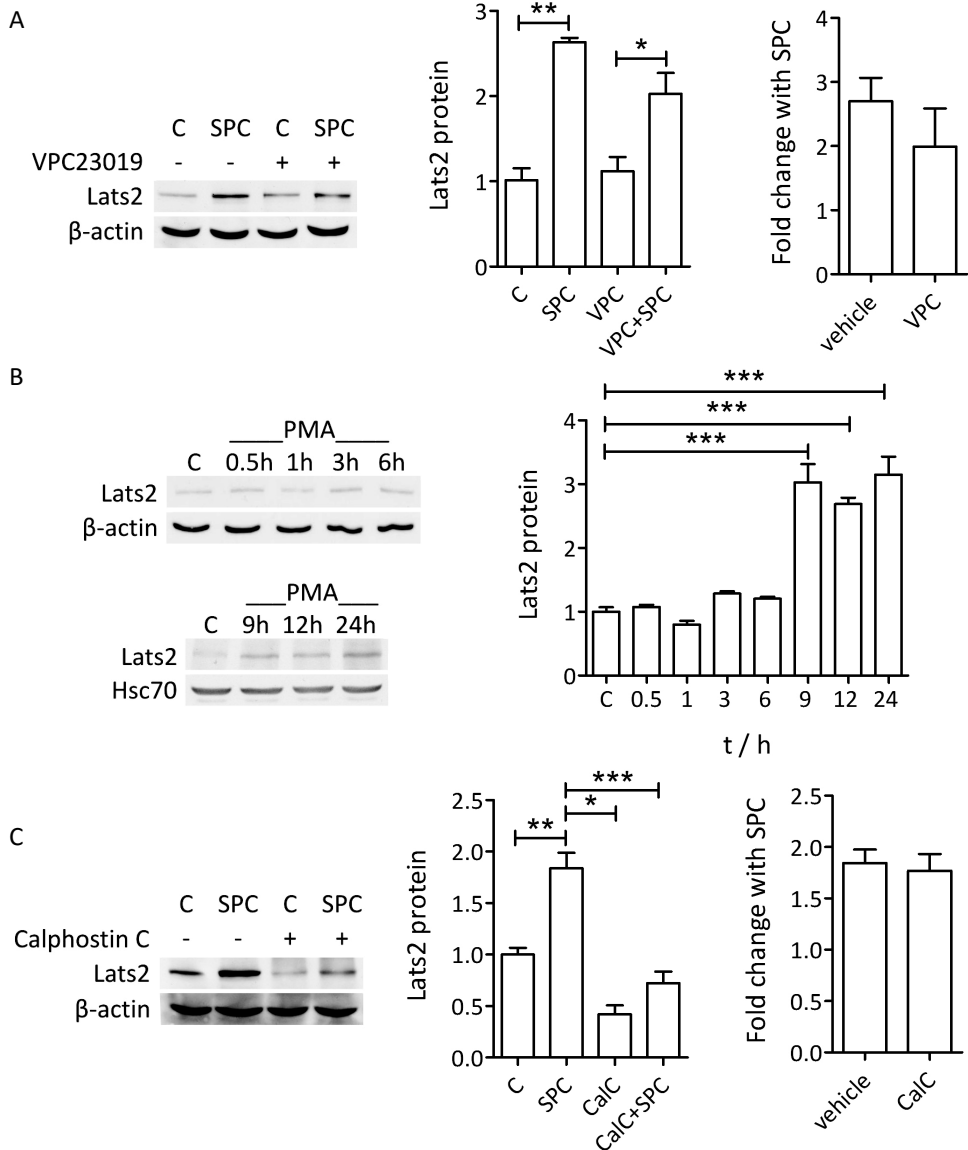


FIGURE 9: S1P_{1,3} do not mediate SPC-induced Lats2 expression in MDA-MB-435S cells and though DAG-dependent PKC activation up-regulates Lats2 similarly to SPC, DAG-dependent PKC isoforms do not mediate the SPC-evoked increase in Lats2. A) Cells were pre-treated with the S1P_{1,3} inhibitor VPC23019 (VPC; 1 μM, 1 h) and then treated with SPC (10 μM) for 9 h. Results are mean ± SEM, n = 3. B) Cells were treated with the DAG-analogue PMA (100 nM) for the indicated times. Results are mean ± SEM, n = 3. C) Cells were pre-treated with the PKC-DAG binding inhibitor Calphostin C (CaIC; 100 nM, 1 h) and then treated with SPC (10 μM) for 9 h. Results are mean ± SEM, n = 4. Lats2 protein expression was analyzed by western blotting. *P<0.05, **P<0.01; one-way ANOVA with Bonferroni's (A, C) or Dunnett's (B) *post hoc* test.

The studies revealing S1P as an inducer of YAP activity also showed that in their model systems the S1P response was mediated by S1P₂ and Rho, and in further studies LPA was shown to stimulate YAP via G₁₃-Rho-ROCK (Cai and Xu, 2013; Hwang et al., 2014; Miller et al., 2012; Yu et al., 2012). Surprisingly, knockdown of S1P₂ in MDA-MB-435S cells attenuated S1P-induced but not SPC-induced YAP dephosphorylation (III, Fig 5C). Also, inhibition of ROCK did not affect the SPC effect on YAP (III, Fig 5G). The surprising siS1P₂ result may reflect the more robust effect of SPC in MDA-MB-435S cells in general in comparison to S1P, as inhibition of S1P₂ did prevent the SPC-YAP response (III, Fig 5F). However, we also hypothesized that another receptor, perhaps one of the putative SPC receptors, might be mediating the SPC response. We used qualitative PCR to determine the S1P/SPC receptor profile of MDA-MB-435S cells and found none of the tested “SPC receptors” (OGR1, GPR4, G2A, GPR12) to be expressed. The only other receptor present in addition to S1P₂ was S1P₅ (III, Fig S2A). Interestingly, S1P₅ has been previously shown to mediate TGFβ₂-induced CTGF expression, but a connection to Hippo signalling was not investigated (Wünsche et al., 2015). S1P₂ seemed to be the highly predominant receptor of the two, based on both the amount of PCR product and amplification of the receptor genes in subsequent quantitative PCR where a S1P₂ signal was seen approximately eight cycles prior to S1P₅. Nonetheless, we conducted knockdown experiments with S1P₅ also and found it not to be involved in SPC-induced YAP dephosphorylation (III, Fig 5D,E). Based on this data, the SPC-induced effects on Hippo signalling are mediated by S1P₂ but not the Rho-ROCK pathway. We also determined whether S1P₂ or S1P₅ mediates the anti-proliferative effect of SPC and found that knockdown of S1P₂ caused a moderate but significant attenuation in the SPC response (III, Fig 6D). Therefore, our results suggest that the SPC-induced effects on both Hippo signalling and proliferation are mediated by S1P₂.

3.4 Hippo signalling does not mediate the anti-proliferative SPC response

In order to determine whether the SPC-induced changes in YAP and Lats2 are involved in the SPC-induced inhibition of MDA-MB-435S proliferation we used knockdown and over-expression of the different Hippo components. Knockdown of Lats2, Lats1 or both did not attenuate the anti-proliferative effect of SPC and neither did exogenous expression of a kinase-dead form of Lats2 (III, Fig 4A,B). Over-expression of the wild-type form of Lats2 also had no effect on proliferation (III Fig 4B), which is surprising but consistent with the fact that SPC-induced Lats2 up-regulation does not mediate changes in proliferation. Knockdown of either Lats2 or Lats1 significantly increased basal proliferation and double knockdown of both had a cumulative pro-proliferative effect (III, Fig 4A). This is further evidence for the tumour suppressive function of Lats1/2 but also for redundancy of these two isoforms. Apparently, the constitutive level of Lats1/2 expression in MDA-MB-435S cells is controlling proliferation, but perhaps availability and/or activity of other components in the signalling pathway is limiting the ability of increased Lats1/2 expression to halt proliferation. Activation of Lats2 requires phosphorylation and this may be lacking from the up-regulated protein. This is supported by the fact that the changes in YAP and its target genes following SPC-induced Lats2 expression are mild in comparison to the acute SPC response where YAP dephosphorylation is visible in whole cell lysates and CTGF shows a nearly tenfold up-regulation (III Fig 3, 5A,B).

Additionally, we used siRNA against YAP and TAZ and again saw no decrease in the fold change of SPC-induced inhibition of proliferation. Surprisingly, double knockdown of the two caused an increase in basal proliferation (III, Fig 6A). Though YAP functions as a tumour suppressor in some contexts (Imajo et al., 2012; Varelas et al., 2010b), it is unexpected that knockdown of both Lats1/2 and YAP/TAZ would have a similar effect in the same cells. However, we did not investigate this phenomenon further. In conclusion, though SPC has a potent effect on both proliferation and Hippo pathway components in MDA-MB-435S cells, Hippo signalling is not involved in the anti-proliferative SPC response. However, this study does expand on the GPCR- and lipid-related regulation of the Hippo pathway which is currently under intense investigation.

Concluding remarks

The three thesis projects have revealed new details of cancer-related sphingolipid signalling and regulation of HIF-1, vimentin and the Hippo pathway.

- i. S1P regulates synthesis and stability of HIF-1 α and subsequently the transcription factor activity of HIF-1 in normoxic conditions. The S1P₃ receptor, the PI3K-Akt and MAPK pathways and the translational regulators p70S6K and eIF-4E mediate this S1P response. HIF-1 in turn mediates the pro-migratory effect of S1P on ML-1 follicular thyroid cancer cells.
- ii. S1P and SPC are able to induce phosphorylation of vimentin monomers and re-organization of the vimentin filament network in MDA-MB-435S breast cancer and C643 anaplastic thyroid cancer cells. Phosphorylation of S71-vimentin is involved in the anti-migratory effect of S1P/SPC. The S1P/SPC response is mediated by the S1P₂-G_{12/13}-Rho-ROCK pathway.
- iii. SPC regulates the activity and/or expression of Hippo pathway components YAP and Lats2 via S1P₂. However, the SPC-induced effects on Hippo signalling do not mediate the anti-proliferative effect of SPC on MDA-MB-435S cells.

The thesis work as a whole is an example of the context-dependence of S1P signalling, the variety of molecules and pathways mediating the cancer-related function of this signalling lipid and also the significance of the lesser known bioactive sphingolipid SPC. The HIF-1 study supports the classical cancer-promoting role of S1P and the pro-migratory function of S1P₃. Importantly, it is also part of the now increasing evidence linking SphK-S1P and HIF-1 in cancer biology. In contrast, the vimentin study exemplifies the inhibitory effect of S1P and SPC on certain cancer cells, the classical anti-migratory function of S1P₂ and the ability of SPC to induce S1P-related signalling. Importantly, this project is the first to identify vimentin as a mediator of the anti-migratory function of S1P₂ and shows that S1P/SPC-S1P₂ signalling can be utilized to halt vimentin-dependent cancer cell motility. As SPC has been shown to promote migration of certain cancer cells through effects on keratin intermediate filaments, it would be of interest to study how EMT of keratin-expressing cells or MET of vimentin-expressing cells would affect their migratory response to SPC.

S1P-targeting compounds and especially SphK inhibitors are being developed for use in cancer therapy. However, in tumour cells which signal dominantly via S1P₂, decreased S1P levels may have an adverse effect through increased cell motility. Also, targeting SphK-S1P overlooks SPC and leaves it able to induce S1P-related signalling through the S1P receptors and also to function as an alternative source of S1P. Simultaneous targeting of both SphK and S1P_{1,3-5} would ideally tip the sphingolipid rheostat towards ceramide and cell death, inhibit the oncogenic function of intracellular S1P and promote activation of S1P₂ by SPC and the remaining S1P. As the immunosuppressant drug FTY720 inhibits both SphK1/2 and the S1P_{1,3-5} receptors and is being studied for potential repurposing as a cancer drug, it would be of interest to know if it has the ability to attenuate S1P-related HIF-1 signalling and promote S1P/SPC-induced effects on vimentin and the Hippo pathway.

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