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Calcium Signaling in Cell Compartments

- the Importance of Sphingosine Kinase 1 and ORP5/8

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ABSTRAKT

För att upprätthålla liv använder cellerna i en organism intrikata signaleringsräckor för regleringen av fysiologiska processer inom enskilda celler och mellan olika celler och vävnaderna som utgör en organism. Reglering av detta slag kallas cellsignalering. En av de mest centrala molekylerna i cellsignaleringen är kalciumjonen, Ca^{2+}. Många centrala biologiska processer, såsom muskelkontraktion, befruktning, cellproliferation, cellmigration, cellulär energiproduktion och celldöd, moduleras av Ca^{2+}-signalering. Dysfunktionell Ca^{2+}-signalering kan bidra till utvecklingen av olika sjukdomstillstånd såsom cancer och neurologiska sjukdomar.

I de publikationer som utgör basen för denna avhandling studerade vi hur tre proteiner av intresse påverkar regleringen av Ca^{2+}-hantering i specifika cellulära domäner, främst mitokondrier och caveoler. Mitokondrier är organeller som är centrala för cellulär energiproduktion och bidrar även till andra viktiga processer som Ca^{2+}-signalering och celldöd. Caveoler är små inbuktningar av cellmembranet och deltar i regleringen av viktiga cellulära funktioner. Caveoler fungerar till exempel som orkestratorer av cellsignalering. För att studera Ca^{2+}-signalering i cellmembranets domän nära caveoler skapade och karakteriserade vi en ny Ca^{2+}-indikator kopplad till det caveolära proteinet caveolin-1.

I dessa undersökningar fann vi att överuttryck av ett lipidkinasprotein, sfingosinkinas 1 (SK1), resulterar i ökad agonist-inducerad frigivning av Ca^{2+} från det endoplasmatiska retiklet (cellens huvudsakliga Ca^{2+}-förråd), samtidigt som det ökar Ca^{2+}-koncentrationen i mitokondriernas matrix och vid caveolerna. De SK1-inducerade Ca^{2+}-effekterna kan också vara bidragande faktorer i regleringen av cellandning och migration, som båda vi fann vara förstärkta av SK1-överuttryck. Dessa fynd bekräftar de tidigare indikationerna på att SK1 är involverad i processer relaterade till cancerfysiologi, möjligen genom Ca^{2+}-medierade processer.

Därtill fann vi att överuttryck av de oxysterolbindande proteinerna 5 respektive 8 (ORP5/8) ökar Ca^{2+}-koncentrationen i mitokondriernas matrix och i de caveolära regionerna i cellmembranet. Vi fann också att överuttryck av både ORP5 och ORP8 ökade cellproliferation. ORP5/8 är lokaliserade vid, och reglerar, membrankontakterna, och signaleringen mellan det endoplasmatiska retiklet, mitokondrierna och cellmembranet. Våra resultat som visar ORP5/8-medierade effekter i Ca^{2+}-signalering i mitokondriernas matrix och vid de caveolära cellmembrandomänerna belyser således den fysiologiska rollen som ORP5/8-proteinerna spelar som reglerare av signaleringen vid membrankontakterna i dessa intracellulära domäner.

Sammantaget visar dessa resultat att SK1- och ORP5/8-proteiner är involverade i regleringen av intracellulära Ca^{2+}-signaler vid specifika cellulära domäner.

ABSTRACT

To maintain life, the cells within an organism employ intricate signaling pathways to regulate physiological processes within single cells and between the various cells and tissues that constitute an organism. Regulation of this kind is termed cell signaling. One of the key molecules in cell signaling is the calcium ion, Ca^{2+}. Many central biological processes, such as muscle contraction, fertilization, cell proliferation, cell migration, cellular energy production and cell death, are modulated by Ca^{2+} signaling. Dysfunctional Ca^{2+} signaling may contribute to the development of various disease conditions such as cancer and neurological disease.

In the original publications of this thesis, we studied how three proteins of interest affect the regulation of Ca^{2+} handling in specific cellular compartments, mainly the mitochondria and the caveolae. The mitochondria are organelles that are key in cellular energy production and contribute also to other important processes such as Ca^{2+} signaling and cell death. The caveolae are small invaginations of the cell membrane with important functions, for instance as orchestrators of cell signaling events. To study the Ca^{2+} signaling events at the caveolar domain of the cell membrane, we created and characterized a novel Ca^{2+} indicator linked to the key caveolar protein, caveolin-1.

We found that overexpression of a lipid kinase protein, sphingosine kinase 1 (SK1), results in increased agonist-induced release of Ca^{2+} from the endoplasmic reticulum, while also increasing the Ca^{2+} concentration in the mitochondrial matrix and at the caveolae. The SK1-induced Ca^{2+} effects may also be contributing factors in the regulation of cell respiration and migration, both of which we found to be augmented by SK1 overexpression. These findings corroborate the previous indications of SK1 being involved in processes related to cancer physiology, possibly through Ca^{2+} mediated processes.

In addition, we found that overexpression of the oxysterol-binding proteins 5 and 8 (ORP5/8), respectively, increases the Ca^{2+} concentration in the mitochondrial matrix and at the caveolar regions of the cell membrane. We found that overexpression of ORP5 and ORP8 also increased cell proliferation. ORP5/8 are known to reside at and regulate the membrane contacts and signaling between the endoplasmic reticulum (the main Ca^{2+} store of the cell) and the mitochondria and the cell membrane. Our results showing ORP5/8-mediated Ca^{2+} signaling in the mitochondrial matrix and at caveolar cell membrane domains thus further elucidate the physiological role of the ORP5/8 proteins as regulators of signaling events at the membrane contact sites of these intracellular compartments.

Taken together, our results show that SK1 and ORP5/8 proteins are involved in the regulation intracellular Ca^{2+} signaling at specific cellular compartments.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

1. Pulli I*, Blom T*, Löf C, Magnusson M, Rimessi A, Pinton P, Törnquist K. (2015) A novel chimeric aequorin fused with caveolin-1 reveals a sphingosine kinase 1-regulated Ca2+ microdomain in the caveolar compartment, Biochim. Biophys. Acta 1853 (2015) 2173-2182, http://dx.doi.org/10.1016/j.bbamcr.2015.04.005

2. Pulli I, Löf C, Blom T, Asghar MY, Lassila T, Bäck N, K.-L. Lin, Nyström JH, Kemppainen K, Toivola DM, Dufour E, Sanz A, Cooper HM, Parys JB, Törnquist K. Sphingosine kinase 1 overexpression induces mitofusin-2 fragmentation and alters mitochondrial matrix Ca2+ handling in HeLa cells. Biochim. Biophys. Acta Mol. Cell Res. 2019 Sep;1866(9):1475-1486. https://doi.org/10.1016/j.bbamcr.2019.06.006

3. Pulli I, Lassila T, Pan G, Yan D, Olkkonen V, Törnquist K. (2018) Oxysterol-binding protein related-proteins (ORPs) 5 and 8 regulate calcium signaling at specific cell compartments. Cell Calcium 72: 62-69. https://doi.org/10.1016/j.ceca.2018.03.001

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AUTHOR CONTRIBUTIONS

Publication 1. The author designed the study together with CL, TB and KT. The author conducted the majority of the Ca^{2+} measurement experiments. The author wrote the manuscript with contributions from TB, CL and KT.

Publication 2. The author designed the study together with KT. The author conducted the Ca^{2+} measurement experiments. The author conducted the western blot experiments, the calpain activity assays as well as the proliferation and migration assays with contributions from co-authors. The author wrote the manuscript with contribution from KT.

Publication 3. The author designed the study together with KT and VO. The author conducted the Ca^{2+} measurement, proliferation and confocal microscopy experiments. The author wrote the manuscript with contribution from KT and VO.

LIST OF PUBLICATIONS NOT INCLUDED IN THE THESIS

Combot Y, Salo VT, Chadeuf G, Hölttä M, Ven K, Pulli I, Ducheix S, Pecqueur C, Renoult O, Lak B, Li S, Karhinen L, Belevich I, Le May C, Rieusset J, Le Lay S, Croyal M, Tayeb KS, Vihinen H, Jokitalo E, Törnquist K, Vigouroux C, Cariou B, Magré J, Larhlimi A, Ikonen E, Prieur X. (2022) Seipin localizes at endoplasmic-reticulum-mitochondria contact sites to control mitochondrial calcium import and metabolism in adipocytes. Cell Rep. 2022 Jan 11;38(2):110213. doi: 10.1016/j.celrep.2021.110213.

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Sukumaran P, Löf C, Pulli I, Viitanen T, Törnquist K. (2013) Significance of transient receptor potential canonical 2 (TRPC2) channel in the regulation of rat thyroid FRTL-5 cell proliferation, migration, adhesion and invasion. Mol Cell Endocrinol. 374: 10-21

Sukumaran P, Löf C, Viitanen T, Kankaanpää P, Kemppainen K, Pulli I, Näsman J, Törnquist K. (2012) Canonical transient receptor potential channel 2 (TRPC2) as a major regulator of calcium homeostasis in rat thyroid FRTL-5 cells: Importance of PKC-delta and STIM2. J. Biol Chem. 287: 44345-60

Löf C, Sukumaran P, Viitanen T, Vainio M, Kemppainen K, Pulli I, Näsman J, Kukkonen JP, Törnquist K. (2012) Communication Between the Calcium and cAMP Pathways Regulate the Expression of the TSH Receptor: TRPC2 in the Center of Action. Mol Endocrinol. 26: 2046-5

ABBREVIATIONS

AC	adenylyl cyclase
Aeq	aequorin
ATP	adenosine triphosphate
BACE	β-site APP cleaving enzyme 1
Ca^{2+}	calcium
CaM	calmodulin
CaMK	calcium/calmodulin dependent protein kinase
cav1	caveolin 1
$Ca_v3.2$	T-type calcium channel
CBP	calcium-binding protein
CDase	ceramidase
CerS	ceramide synthase
CIB1	calcium and integrin-binding protein 1
CIB2	calcium and integrin-binding protein 2
CICR	calcium-induced calcium release
CMT disease	Charcot-Marie-Tooth disease
CN	calcineurin
CRAC	calcium-release activated calcium channel
DAG	diacylglycerol
EGTA	ethylene glycol-bis β-aminoethyl ether
EMRE	essential MCU regulator
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GAP	GTPase activating protein
GDP	guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
HBSS	Hepes-buffered saline solution
HR1	heptad repeat 1 domain
HRP	horseradish peroxidase
IMM	inner mitochondrial membrane
IMS	intramembrane space
IP_3	inositol trisphosphate
LBS	Laemmli sample buffer
LETM1	leucine zipper and EF-hand containing transmembrane protein 1
MAM	mitochondria-associated ER membrane
MCS	membrane contact sites
MCU	mitochondrial calcium uniporter
MCUR	MCU regulator 1
MFN2	mitofusin 2
MICU	mitochondrial calcium uptake protein
MβCD	methyl-β-cyclodextrin

NCLX	mitochondrial sodium-calcium exchanger
NCX	sodium calcium exchanger
OMM	outer mitochondrial membrane
ORP5	oxysterol-binding protein-related protein 5
ORP8	oxysterol-binding protein-related protein 8
PC2	polycystin 2
PH domain	pleckstrin homology domain
$PI(3,5)P_2$	phosphatidylinositol 3,5-bisphosphate
PI3K	phosphoinositide 3-kinase
PI4P	phosphatidylinositol-4-phosphate
PIP_2	phosphatidylinositol-4,5-biphosphate
PKC	protein kinase C
PLC	phospholipase C
PM	plasma membrane
PMCA	plasma membrane Ca2+ ATPase
ROCE	receptor-operated calcium entry
RTK	receptor tyrosine kinase
RyR	ryanodine receptor
S1P	sphingosine 1-phosphate
S1Ppase	S1P phosphatase
S1PR	S1P receptor
SAM	sterile alpha-motif
SERCA	sarco-endoplasmic reticulum ATPase
SK1	sphingosine kinase 1
SK2	sphingosine kinase 2
SNAP25-Aeq	SNAP25-tagged aequorin
SOCE	store-operated calcium channel
SPH	sphingosine
SPNS2	spinster 2
SR	sarcoplasmic reticulum
STIM	stromal interaction molecule
TMRE	tetramethylrhodamine ethyl ester
TPC	two-pore channel
TRP channel	transient receptor potential channel
UCP	uncoupling protein
VDAC	voltage-dependent anion channel
VOCC	voltage-operated calcium channel

Introduction

The calcium ion (Ca^{2+}) is involved in key biological processes throughout the kingdoms of life. Therefore, the Ca^{2+} ion is considered as a universal second messenger, and the Ca^{2+} signaling pathway is involved in the modulation of most, if not all, of the major cellular functions such as cell proliferation, migration, energy production and cell death. Consequently, dysregulated Ca^{2+} signaling is a major factor in various pathological conditions such as cancer and various neurological diseases. Multiple molecular signaling pathways, as well as various cell organelles and specific cell compartments, are involved in the regulation of cellular Ca^{2+} homeostasis, Ca^{2+} signaling events, and the downstream effects of Ca^{2+} signaling.

Of note, lipid signaling and lipid regulation are involved in Ca^{2+} signaling and organelle function. In this thesis work, the organelle and compartment-specific regulation of Ca^{2+} signaling by proteins involved in lipid signaling and regulation was studied. The proteins of interest were sphingosine kinase 1 (SK1) and oxysterol-binding protein-related proteins 5 and 8 (ORP5/8). SK1 is a lipid kinase that catalyzes the formation of sphingosine 1-phosphate (S1P), a lipid with well-known signaling functions. S1P mainly acts through five G-protein-coupled S1P-receptors, most of which are known to be involved in the activation Ca^{2+} signaling pathways. Further, SK1/S1P and Ca^{2+} signaling pathways may function in an inter-regulated fashion, modulating each other through feedback mechanisms, as Ca^{2+} has been found to be a co-factor in the activation of SK1. ORP5 and 8 are proteins that are involved in lipid transfer and contact regulation between the endoplasmic reticulum (ER) and the plasma membrane (PM). In addition, ORP5/8 have been shown to exert their function also at the ER-mitochondria interaction sites. Importantly, ORP5/8 bind to the PM and regulate the levels of the phospholipid phosphatidylinositol-4,5-biphosphate (PIP_2) in the PM. Importantly, PIP_2 is the direct precursor of inositol trisphosphate (IP_3), a second messenger that binds to the IP_3-receptors to activate Ca^{2+} release from the main intracellular Ca^{2+} store, the ER. ORP5/8 are emerging as important regulator proteins at the membrane contact sites (MCS), such as the mitochondria-associated ER-membranes (MAMs). MAM contact sites, and the molecular interactions within the MAMs, have been shown to be of key importance in the regulation of Ca^{2+} signaling events between the organelles.

To study the Ca^{2+} concentrations in specific cell compartments and organelles, the genetically targeted Ca^{2+} indicator protein aequorin was employed. Aequorins targeted to the cytosol, to the mitochondria, to the ER, and to the PM were previously constructed by other researchers. For this study, a novel caveolin-1-tagged aequorin was created and validated. SK1 was found to regulate Ca^{2+} microdomains at the caveolin-1-enriched PM sub-compartments, as well as in the mitochondrial matrix and in the ER. The SK1-induced increase in the caveolar Ca^{2+} microdomain was mainly attributed to the autocrine effect

of S1P on its receptors that have been shown to localize to caveolae. SK1 overexpression was shown to induce mitofusin-2 (MFN2) fragmentation, likely through increased calpain protease activity. MFN2 promotes mitochondrial fusion and modulates ER-mitochondria MAM contacts, with indications to mitochondrial Ca^{2+} regulation. The expression of putative calpain-cleaved MFN2 fragments resembled the effects of SK1-overexpression on mitochondrial Ca^{2+}. Inhibition of the S1P-receptors did not ablate the SK1-mediated increase of mitochondrial Ca^{2+}. Thus, the observed MFN2 fragmentation may contribute to the SK1-induced increase in the mitochondrial matrix Ca^{2+}.

Overexpression of ORP5/8 was shown to augment the mitochondrial and the caveolar Ca^{2+} upon agonist stimulation whereas cytoplasmic Ca^{2+} was not affected. Down-regulation of ORP5/8 by small interfering RNA was without an effect. ORP5 overexpression augmented the IP_3 production, whereas ORP8 overexpression did not. The observed changes in Ca^{2+} handling were thus attributed to the increased IP_3 (mediated by ORP5) as well as to the previously reported modulatory functions of ORP5 and 8 at the MCS between the PM and the ER as well as at the MAM contact sites.

The effect of SK1 or ORP5/8 overexpression on cell physiological parameters such as proliferation, migration and respiration were also studied. SK1 overexpression augmented respiration and cell migration which are known downstream effects of mitochondrial Ca^{2+}. ORP5/8 overexpression led to increased cell proliferation, also in agreement with the characterized Ca^{2+} phenotype. These findings give new mechanistic insight to the role of SK1 as an oncogene and show a novel role for ORP5 and 8 as regulators of Ca^{2+} signaling in specific cell compartments.

1 REVIEW OF THE LITERATURE

1.1 Calcium signaling and cell compartments – an overview

A feature characteristic to all organisms is the ability to maintain ion gradients over the cell and organelle membranes to facilitate bioenergetic processes and signal transduction [1–3]. The calcium ion (Ca^{2+}), often referred to as a universal second messenger, regulates profound functions in virtually all life forms ranging from prokaryotes to uni- and multicellular eukaryotes, i.e. fungi, plants and animals [4–6]. After well over a hundred years of research, the importance of Ca^{2+} in human physiology and disease is well established [7]. Ca^{2+} has

widespread functions including the regulation of processes such as fertilization, muscle contraction, neuronal function, cell proliferation and migration, energy production, cell signaling, secretion, gene expression and induction of programmed cell death. To orchestrate these essential cell physiological events in a controlled manner, Ca^{2+} ion fluxes are tightly regulated by various protein molecules, such as Ca^{2+} pumps, channels, exchangers and Ca^{2+} binding proteins [3,8]. The signaling function of Ca^{2+} is mediated by coordinated induction of changes in the intracellular cytoplasmic Ca^{2+} concentration, as well as in the intraorganellar Ca^{2+} concentrations.

In a basal, resting condition, cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is kept at a low level (in the nanomolar range) whereas $[Ca^{2+}]$ in the extracellular milieu is high (in the millimolar range). Importantly, Ca^{2+} can be stored at high micromolar concentrations in the intracellular compartments such as the endoplasmic reticulum (ER) and the sarcoplasmic reticulum (SR). Mitochondria are also able to take up Ca^{2+} in the micromolar range, play important roles in Ca^{2+} handling and signaling, and may also exert Ca^{2+} buffering functions. In addition, also other organelles such as the Golgi apparatus and the lysosomes may store intracellular Ca^{2+} and be involved in Ca^{2+} signaling events. The control of cytoplasmic Ca^{2+} is mainly achieved by active adenosine trisphosphate (ATP)-dependent transport either out of the cell through the plasma membrane Ca^{2+} ATPases (PMCA) or by active uptake of Ca^{2+} through the sarco-endoplasmic reticulum ATPase (SERCA) to the ER (Fig 1) [5,7,9]. Thus, cells spend a considerable amount of energy to maintain the Ca^{2+} ion gradient across the cell membrane and the extracellular milieu, as well as across the overall cytoplasm and the organelle membranes. The flow of Ca^{2+}, driven by the electrochemical gradient and activated by the opening of Ca^{2+}-permeable ion channels, is utilized by a multitude of cellular signaling pathways to regulate the various effects that have been attributed to Ca^{2+} signaling [3]. In the sections below, the essential components of Ca^{2+} signaling that are of key interest in the scope of the original publications in this thesis are reviewed, and the components of general importance in Ca^{2+} signaling regulation are briefly discussed.

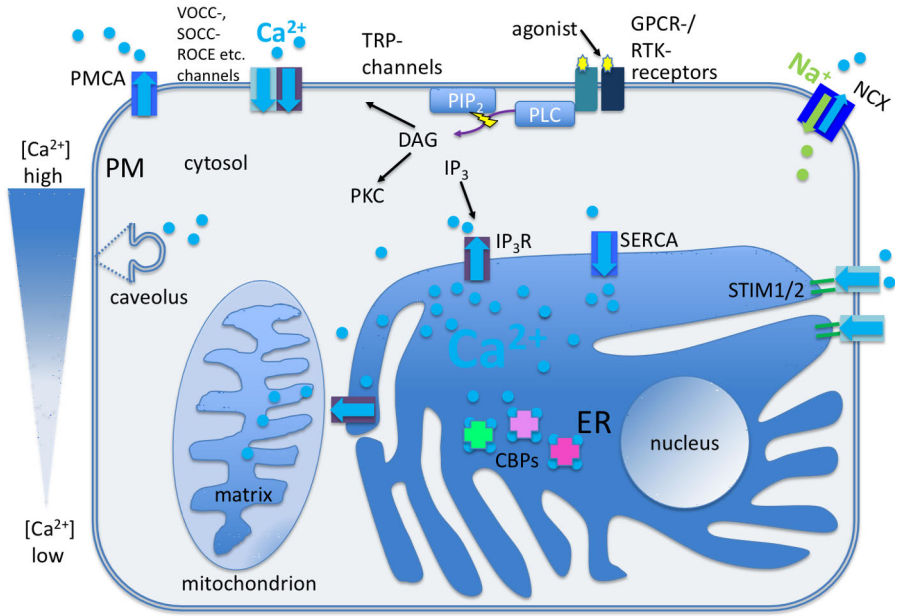

Figure 1. Generalized schematics of the fundamental components of intracellular Ca^{2+} regulation. The plasma membrane (PM) harbors the PM Ca^{2+} ATPase (PMCA) Ca^{2+} pumps, that actively transport Ca^{2+} from the cytoplasm to the extracellular space to maintain the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]$) low. Exchanger molecules, such as the sodium (Na^+)-Ca^{2+} exchanger NCX, also modulate the levels of cytoplasmic $[Ca^{2+}]$. PM Ca^{2+}-channels, such as the voltage-operated (VOCC), store-operated (SOCC) and receptor-operated (ROCE) calcium channels, as well as the ion channels of the transient receptor potential (TRP) family regulate the Ca^{2+} flux to the cytosol. The endoplasmic reticulum (ER) is the main intracellular Ca^{2+} storage. The sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCA-pumps) take up cytosolic Ca^{2+} to the ER. Inositol trisphosphate receptors (IP_3R) are ubiquitous Ca^{2+} release channels in the ER. Multiple PM-residing receptors (G-protein-coupled; GPCR, receptor tyrosine kinase; RTK) can be activated by agonists (e.g. histamine) and couple to phospholipase C (PLC) that cleaves phosphoinositol bisphosphate (PIP_2) to form the two second messenger molecules, diacylglycerol (DAG) and IP_3. DAG activates protein kinase C and ion channels in the PM, whereas IP_3 stimulates the opening of the IP_3 receptor channels (IP_3R) that reside in the ER membranes. Ca^{2+}-binding proteins (CBPs) modulate ER Ca^{2+} homeostasis and act as chaperones. Mitochondrial matrix readily accumulates Ca^{2+} and thus mitochondria are important regulators and buffers of intracellular Ca^{2+}. The activity of some of the mitochondrial enzymes involved in the oxidative phosphorylation is Ca^{2+} dependent. Caveolae harbor Ca^{2+} channels and multiple molecules and receptors involved in Ca^{2+} signaling. Ca^{2+} microdomains, i.e. domains of high $[Ca^{2+}]$ may emerge in the vicinity of IP_3 receptor channel upon activation, or near to the PM ion channels regulating entry of Ca^{2+}. Organelles and cellular compartments are involved in the regulation of Ca^{2+} microdomains: for example, mitochondria-ER interaction may modulate the IP3-sensitive Ca^{2+} pathway, and SOCE regulation can be modulated by mitochondrial Ca^{2+} uptake.

1.2 Ca^{2+} as a second messenger

In order to develop and function, the cells within an organism need to communicate through intricate physical and chemical mechanisms, including (inter)regulation by contact-dependent proteins, hormonal cues and various signal transmitter molecules that act on receptors residing in the plasma membrane (PM) of cells [10,11]. To modulate cell function, the extracellular signals are translated to intracellular signaling cascades that consist of protein molecules and small second messenger molecules, including cyclic nucleotides, lipid molecules such as inositol trisphosphate (IP_3) and sphingosine 1-phosphate (S1P), and, importantly, the Ca^{2+} ion [5]. Multiple PM-residing and intracellular receptors are known to activate intracellular Ca^{2+} fluxes through various cell signaling pathways. To exert its second messenger functions, Ca^{2+} binds to and changes the conformation and function of a variety of target proteins in the cell. The binding of Ca^{2+} may have a direct effect on the activity of the target protein, or Ca^{2+} may facilitate the interaction of proteins, thus affecting their function [3,7,8]. Importantly, Ca^{2+} signaling, being the key regulatory component involved in virtually all central cell physiological events and functions, has widespread implications for multiple disease-related cell physiological processes.

1.2.1 Ca^{2+}-binding proteins

The various Ca^{2+}-binding proteins (CBPs) bind Ca^{2+} ions with different affinities and through different motifs and domains. CBPs include Ca^{2+} transporter molecules, Ca^{2+} buffering proteins that are involved in the regulation of the Ca^{2+} concentration in the cytoplasm or within cell organelles, Ca^{2+} sensor proteins that relay Ca^{2+}-mediated signals, as well as enzymes that are directly or indirectly activated by Ca^{2+} binding [12,13]. The major classes of Ca^{2+}-binding sites of the CBPs are the EF-hand domains and the C2 domains, the former being the more ubiquitous one [13]. EF-hand domain harboring proteins include calcineurin, calmodulin and calpain proteases, whereas the key Ca^{2+} regulated enzymes, protein kinase C (PKC) and phospholipase C (PLC), contain C2 domains [12].

Proteins such as calreticulin and calnexin bind Ca^{2+} and act as Ca^{2+} buffering proteins to facilitate the Ca^{2+} storage function within the ER [12]. Perhaps one of the most studied mediators of Ca^{2+} signaling is calmodulin (CaM) that has been shown to bind to multiple target proteins to modulate their activity. The prominent binding targets of CaM are the protein phosphatase calcineurin (CN) and the Ca^{2+}/calmodulin-dependent protein kinase isoforms, CaMKI–IV. CN is a central regulator of the NFAT transcription factor and the CaM kinases phosphorylate multiple target proteins to modulate their function [5]. Interestingly, recent peptide binding prediction assays have recognized over 500 candidate targets with which CN may interact through short linear motifs, thus greatly expanding the potential CN interactome of the presently identified 50 CN

substrates [14]. CaM-kinases have been shown to phosphorylate multiple target proteins to regulate a wide variety of cellular events such as apoptosis, vesicle mobilization, gene transcription and (Ca^{2+}) ion channel activity [15].

Calpains are a class of proteases, and fifteen human calpain-encoding genes have been identified. The calpain protease protein family comprises the μ- and m-calpains which are referred to as the conventional calpains, while the other calpains are referred to as unconventional ones [16,17]. Importantly, calpain-mediated proteolytic processing differs from the proteasomal degradation in that the calpain products, i.e. protein fragments, may retain some of the original functionality or acquire an altered function [16,18]. Dysregulation of calpain is involved in multiple conditions including cancer and neurological disease [19,20].

PLC and PKC are central enzymes in the regulation and mediation of Ca^{2+} signaling events. PLC is a major regulator of the lipid second messengers diacylglycerol (DAG) and IP_3, which are involved in the regulation of intracellular Ca^{2+}, and thus PLC is an important modulator of Ca^{2+} signaling events [21].
PKCs are a large family of protein kinases with various downstream targets and cellular effects. The activity of the conventional PKCs is modulated by DAG and Ca^{2+}, and PKCs serve a feedback function in Ca^{2+} regulation through multiple proteins, e.g. by being involved in the activation of the PMCA-mediated Ca^{2+} extrusion from the cytoplasm to the extracellular space, and by inhibiting PLC [22].

The PLC-mediated Ca^{2+} signaling pathway is described in section 1.4.1. The PKC protein family with the associated main cellular functions are briefly discussed in section 1.4.2.

The Ca^{2+} channels and exchangers that are the most relevant to this study are described in sections below.

1.3 Regulation of Ca^{2+} at the cell membrane

The plasma membrane (PM) lipid bilayer as such is impermeable to Ca^{2+} ions. Therefore, the controlled flux of Ca^{2+} through the various PM-resident proteins is a major determinant of cytoplasmic Ca^{2+} levels. The previously mentioned PMCA pumps are the chief executioners of the active Ca^{2+} efflux from the cytoplasm to the extracellular milieu. Antiporter molecules such as the Na^+/Ca^{2+} exchanger (NCX) contribute to ion homeostasis [5]. Further, multiple types of PM ion channels with either specific or unspecific permeability for the Ca^{2+} ion regulate the influx of Ca^{2+}, and also other ion species, to the cytoplasm. These channels have different modes of activation. Ligand-gated ion channels function at the same time as the receptor and as the conducting channel; these include for

instance the ATP-sensitive P2X-receptors and the glutamate-sensitive NMDA-receptors.

Many of the transient receptor potential (TRP) -channels are activated by a downstream signal from a receptor that is separate from the channel itself, for instance through the GPCR-mediated PLC activation and its downstream signaling molecules [3,5,23]. This form of Ca^{2+} flux is sometimes termed receptor-operated Ca^{2+} entry, or ROCE [24,25]. Channels can also be activated by other factors such as changes in the membrane potential, temperature, as well as through mechanical stimuli such as stretching of the PM [3,5]. Also, Ca^{2+} channels at the PM can be activated as a response, and as a compensation, to the emptying of the intracellular Ca^{2+} stores, resulting in store-operated Ca^{2+} entry (SOCE) [26]. Interestingly, the PM is compartmentalized, having defined domains with e.g. specific lipid and protein composition. For instance, the PM harbors cholesterol-rich invaginated structures called caveolae, where specific lipid species and multiple signaling molecules, receptors and channels are localized, including those involved in Ca^{2+} regulation [27]. Caveolae are discussed in more detail in section 1.5.3.

1.4 Regulation of Ca^{2+} release and uptake of the (sarco)endoplasmic reticulum

The endoplasmic reticulum (ER) is the major intracellular Ca^{2+} store in most cell types [13]. In muscle cells, the sarcoplasmic reticulum (SR) serves the function of the ER [28]. Both reticula harbor the SR/ER Ca^{2+} ATPases, the SERCA-pumps, which by employing ATP hydrolysis effectively transfer Ca^{2+} from the cytoplasm to the lumen of the SR/ER to maintain the basal cytosolic Ca^{2+} at a low level. The active Ca^{2+} transport results in that the resting Ca^{2+} concentration within the SR/ER is at a high micromolar level (~400-500 μM) [29,30]. To effectively achieve the Ca^{2+} storage function, multiple Ca^{2+} buffering CBP-proteins reside inside the SR/ER (Fig. 1). These include calnexin, calsequesterin, calreticulin and BiP, that also act as chaperones to aid correct protein folding taking place within the organelle [3,5,12,13]. An important property of the ER-resident Ca^{2+} binding buffer proteins is that they are usually able to bind Ca^{2+} with high capacity but with low affinity which allows for rapid release of Ca^{2+} upon stimulus [13].

The ER contains Ca^{2+} release channels, such as the IP_3R, that can be triggered by a multitude of extracellular signals through the various PM-resident G-protein-coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs) to release ER Ca^{2+} through the phospholipase C -induced IP_3 signaling (please see the next section). In addition, ryanodine receptors, with three identified isoforms (RyR1-3), are key Ca^{2+} release channels of the SR/ER, mainly expressed in skeletal (RyR1) and cardiac muscle (RyR2 or brain (RyR3) [31]. RyR opening may be regulated by voltage-dependent Ca^{2+} channels, ions such as Ca^{2+} (Ca^{2+} induced

Ca^{2+} release) or Mg^{+}, proteins such as protein kinase A or calmodulin, and by agonists such as ATP or caffeine [31,32].

Importantly, reduction in the endoplasmic Ca^{2+} concentration triggers the so called store-operated Ca^{2+} entry (SOCE) pathway to replenish and maintain the ER Ca^{2+} content. The SOCE pathway discussed in more detail in section 1.4.4.

1.4.1 Phospholipase C/inositol trisphosphate -pathway

As mentioned above, many PM-residing GPCRs and RTKs relay agonist-induced signaling by inducing the release of ER Ca^{2+}, or by activating PM-resident ion channels such as the channels of the transient receptor potential (TRP) family [3]. Upon agonist binding to the receptors of the GPCR or RTK family, multiple downstream signaling events may be triggered, including the activation of phospholipase C (PLC). PLC is a central molecule for intracellular Ca^{2+} regulation. Upon activation, PLC translocates to the PM and cleaves the PM-residing PIP_2, thus contributing to the regulation of the levels of PIP_2 in the PM. A variety of proteins, including PLC, bind to, or are activated by, PM PIP_2. PIP_2 is also a precursor for PIP_3 that serves other signaling functions [33].

Importantly, PLC mediated cleavage of PIP_2 forms the two second messenger molecules, diacylglycerol (DAG) and inositol trisphosphate (IP_3), that are central molecular components involved in the of intracellular Ca^{2+} regulation. DAG is known to activate, for instance, PKC, and DAG has also been shown to regulate some of the PM-residing TPRC-channels [33]. IP_3 binds to and activates the ER-residing IP_3-receptor isoforms I-III, leading to rapid Ca^{2+} release to the cytosol from the ER Ca^{2+} store [34]. The IP_3-receptor pathway is described in more detail in a specific chapter, in section 1.4.3.

In total, thirteen different PLC-isoforms and isozymes have been identified and characterized (i.e. PLC-ß1-4; PLC-γ1-2; PLC-δ1, -δ3, -δ4; PLC-ε, PLC-ζ, PLC-η). The different isoforms are coupled to different upstream signaling pathways and receptors. For instance, the isoform PLC-ß has been shown to be activated by the GPCRs whereas the isoform PLC-γ has been shown to be regulated by the RTKs [3,5,21,33,35] (Fig. 1). The PLC-ß1-4 isoforms show different expression patterns in a tissue-specific manner and can be activated downstream from the GPCRs [35].

In the scope of this thesis, the S1P receptors, which are GPCRs that mediate their effects through activated G-proteins (see section 1.6.2 on S1P receptors), are of main interest with respect to PLC activation. The focus is therefore on the isoform PLC-ß. The four identified isozymes of the PLC-ß can each be activated by the G-protein $G\alpha_q$. The PLC-ß isozymes show different levels of activation upon binding of $G\alpha_q$, the isoforms PLC-ß1 and -ß3 being robustly activated while

the isoforms PLC-ß2 and - ß4 are activated to a lesser extent [36]. In addition, PLC-ß 1-3 are activated by the G-protein complex $G_{ßγ}$. Further, all PLC-ß isoforms are activated by Ca^{2+}, and Ca^{2+} may thus act as a synergistic amplifier of the PLC-ß-mediated signaling. Multiple different or complementary models for the specific molecular mechanisms for PLC activation by the $G_{α}$ and $G_{ßγ}$ proteins, respectively, have been proposed [33,36,37]. Interestingly, the PLC-ß proteins also act as GTPase activating proteins (GAP), inducing the GTP hydrolysis and subsequent inactivation of the $Gα_{q}$, specifically. Hence, the G_{q}-mediated activation of PLC-ß is rapidly reversed by the negative feedback through GAP activity. PLC-ß proteins may also be activated through phosphorylation by various protein kinases, and e.g. by association with the small GTPase, Rac [33,36,37].

1.4.2 Protein kinase C family

The PKC family of proteins are ubiquitously expressed, closely related enzymes that function as serine-threonine protein kinases, and, importantly, the activation of the PKC signaling pathway is closely linked to the components of the (activated) PLC-mediated Ca^{2+} pathway [38]. The PKCs have been identified as key regulators of multiple cellular processes, such as tumorigenesis, apoptosis, cell cycle regulation, differentiation, proliferation, angiogenesis and cell survival [39,40]. Not surprisingly, as the PKCs are involved in the regulation of a wide variety of cell physiological events, they have also been found to play a major role in multiple disease conditions such as cancer, stroke, neurodegenerative conditions, autoimmune disorders, heart failure and psychiatric illness [39].

The PKC proteins comprehend a multifunctional family including at least ten different isoforms and splice variants of the PKC isoenzymes that have been identified to date. The PKCs are further categorized as classical, novel and atypical PKCs by their mode of activation. The classical PKCs (cPKCs) were initially found to be regulated by Ca^{2+} binding, DAG and phorbol esters, whereas in contrast, the novel PKCs (nPKCs) that were characterized later on were found not to require Ca^{2+} binding for their activation and are thus modulated by DAG and phorbol esters only. The atypical PKCs' (aPKCs) activation is independent of both DAG and Ca^{2+} binding [38,40]. Further, the classical PKCs comprise the isoforms termed PKCα, PKCβI, PKCβII and PKCγ, the novel PKCs comprise the isoforms termed PKCδ, PKCε, PKCη and PKCθ, and, finally, the atypical PKCs are termed PKCζ and PKCι. [40].

The regulation of PKC activation and inactivation is modulated by multiple pathways. As a first step towards PKC activation, the enzyme needs to go through a maturation process involving phosphorylation by phosphoinositide-dependent kinase 1, followed by autophosphorylation events. Only after the

maturation process the PKC enzyme is stabile and gains the ability to become activated by the increase in cytoplasmic Ca^{2+} and DAG levels, respectively. The inactive maturated PKCs are mostly residing in the cytoplasm, and upon activation, the PKCs may translocate to the cell or organelle membranes to bind and phosphorylate their target proteins, thus initiating downstream signaling events [40]. Thus, the activation of PKC is following the dynamics of the intracellular Ca^{2+} signal, wheres the inactivation of PKC is following the dynamics of DAG breakdown [38,39].

The different PKCs couple to multiple central upstream and downstream signaling pathways and the many effects of the PKC isozymes may be cell-type specific and context-dependent, with some of the PKC isoforms having opposing effects e.g. in the regulation of cell survival, proliferation and apoptosis [40].

1.4.3 IP_3 receptors

Inositol trisphosphate (IP_3) receptors are the central regulators of the activation of Ca^{2+} efflux from the ER. As mentioned in the above chapter, the IP_3 receptor protein family comprises three closely related isoforms, the IP_3R I-III. The respective isoforms are ubiquitously expressed and the expression profiles of the isoforms overlap, but certain tissues may also show specific and limited IP_3R sub-type expression [41]. IP_3 receptor gating is mainly achieved by the receptor's ligand IP_3, but IP_3Rs also harbor Ca^{2+} binding sites which modulate the channel activity in a Ca^{2+} concentration dependent manner. Hence, Ca^{2+} can function as a regulator of the IP_3Rs, providing an important feedback pathway for tuning the ER Ca^{2+} release through the IP_3Rs. Importantly, Ca^{2+} binding to the IP_3R may be an activating (at low $[Ca^{2+}]_{cyt}$) or an inhibiting (at high $[Ca^{2+}]_{cyt}$) factor for the channel opening [42,43]. Of note, IP_3R stimulation and opening by Ca^{2+} is termed Ca^{2+} induced Ca^{2+} release, or CICR [44]. Further, it has been postulated that the IP_3 receptors may be modulated through proteolysis by calpains, resulting in fragmented but functional IP_3 receptor proteins with altered function and Ca^{2+} flux dynamics [18].

The cytoplasmic Ca^{2+} pattern, as induced by the generation of IP_3 and modulated by CICR, differs depending on the extent of IP_3 stimulation [9]. Bootman et al [45] have investigated the hierarchy of cytoplasmic Ca^{2+} events in terms of the kinetics and amplitude of the cytoplasmic Ca^{2+} signal, and defined three categories of the cytoplasmic Ca^{2+} pattern, i.e. Ca^{2+} "blips", "puffs and "waves". As identified by Bootman et al [45] and reviewed by Taylor et al ([44]) the different hierarchical events reflect the degree of IP_3 receptor stimulation levels and the kinetics and amplitude of the resulting cytoplasmic Ca^{2+} signal. In this scheme, the Ca^{2+} blib resembles the opening of a single IP_3 receptor, which can be followed by larger IP_3 and CICR induced Ca^{2+} release event, a Ca^{2+} puff, near to the specific sub-population of IP_3 receptors that are being activated upon a

modest stimulation [44]. Further, during a greater agonist stimulation, the flux of cytoplasmic Ca^{2+} can move throughout the volume of the cell cytoplasm as a Ca^{2+} wave. In addition, Ca^{2+} oscillations of repeatedly increasing and decreasing cytoplasmic Ca^{2+} concentrations may arise depending on the extent of agonist stimulation and IP_3 receptor population expressed by the cell. In addition, the cytoplasmic Ca^{2+} oscillations may be modulated by the above-mentioned concentration-dependent feedback activation or inhibition of the IP_3R by Ca^{2+} [42,44,46]. Notably, also the SOCE pathways along with the mechanisms regulating mitochondrial Ca^{2+} uptake are of importance in the modulation of the cytoplasmic Ca^{2+} oscillations [9,47], and functional mitochondrial Ca^{2+} uptake capacity along with undisturbed mitochondrial membrane potential have shown to be required for sustained cytoplasmic oscillations [48]. Cytoplasmic Ca^{2+} oscillations may also be relayed to the mitochondria in the form of sequential Ca^{2+} influx and efflux to and from the mitochondrial matrix [9,49]. In addition, cytoplasmic Ca^{2+} signal events, such as the Ca^{2+} waves or puffs, have been shown to be reflected in the cell nuclear compartment showing distinct Ca^{2+} kinetics [50]. Of note, nuclear Ca^{2+} has been shown to regulate nuclear target proteins, protein kinases or transcription factors [51].

Interestingly, a recent study by Bartok et al shows that the IP_3 receptor isoforms 1-3 are also involved in regulating the contacts and coupling between ER and mitochondria. In this study, the regulation of the ER-mitochondria contact sites by the IP_3 receptors was shown to be independent of the Ca^{2+} signaling function of the IP_3 receptors, thus proposing a novel functional role for the IP_3 receptors as modulators of organelle interaction. Further, it was shown in the same study that the IP_3 receptor 2 is the most efficient of the IP_3 receptor isoforms in transferring Ca^{2+} from the ER to the mitochondria [52].

Thus, IP_3 receptors are central regulators of cell physiology through controlling cellular Ca^{2+} signaling in a complex fashion with a multitude of cellular outcomes depending on the cell physiological context, or tissue. Hence, and not surprisingly given the widespread functions of the IP_3 receptor isoforms, there is an extensive body of evidence emerging, showing the involvement of IP_3 receptor mutations in a plethora of disease, such as neurological disease and cancer [41].

1.4.4 Store-operated calcium entry, SOCE

The depletion of the ER Ca^{2+}, that occurs for instance during agonist-stimulation and the consequent activation of the IP_3Rs, is compensated by Ca^{2+} entry through PM-residing channels in a process called the store-operated Ca^{2+} entry, SOCE. Essential to the initiation of SOCE are the transmembrane proteins, stromal interaction molecules (STIM1 & 2), that reside in the ER membrane and act as Ca^{2+} sensor proteins in the ER lumen [53]. SOCE is facilitated by the collectively

named Ca^{2+}-release-activated Ca^{2+} (CRAC) channels or, alternatively, store-operated Ca^{2+} channels (SOCCs). The prominent CRAC/SOCC channel-forming molecules in the PM are the Orai1-3 proteins. In addition, multiple channels of the canonical TRP-family (TRPC) have been shown to conduct Ca^{2+} during SOCE. Also, TRPC1 and Orai1 may form functional complexes to mediate the SOCE influx [54]. The STIM proteins contain a Ca^{2+}-binding EF-hand domain and a sterile alpha-motif (SAM) domain that together regulate the Ca^{2+} sensing of the STIMs. The dissociation of Ca^{2+} from the EF domains causes STIMs to oligomerize and to form puncta structures in the ER that reside close to the PM. The oligomerized STIMs activate the CRAC/SOCC channels upon depletion of ER Ca^{2+}. STIM1 and STIM2 have different activation kinetics, which is attributed to the differences in the SAM and the EF hand domains. STIM2 is sensitive to relatively small changes in ER luminal Ca^{2+} and is hence keeping control of the basal ER Ca^{2+}. In contrast, STIM1 is activated upon robust reduction in ER luminal Ca^{2+} levels. [24,26,53,55–59]. STIM1 oligomerization leads to clustering of the Orai channels at ER-PM puncta thus forming a microdomain for Ca^{2+} influx. The activation of the Orai channels by STIM1 is thought to be achieved by interactions between the Orai C-terminal residues and the conserved STIM1 domain, SOAR [55,59]. Interestingly, and relevant to the topic of this thesis, also other intracellular compartments than the ER play a role here, as caveolae have been shown to harbor SOCE components and mitochondrial Ca^{2+} uptake is required for functional SOCE [27,60]. Of note, dysregulated SOCE is an important factor in cancer cell migration and proliferation [61] as well as in conditions such as neurodegenerative disease [62] and cardiac hypertrophy [63].

1.5 Compartmentalized Ca^{2+} signaling and Ca^{2+} microdomains: mitochondria and caveolae in the spotlight

Due to the multiple molecular mechanisms involved in the orchestration of intracellular Ca^{2+} regulation, as well as due to the many biological effects Ca^{2+} directly or indirectly accomplishes in the cell, the Ca^{2+} signaling events within the cell are known to take many different shapes and forms. That is, intracellular Ca^{2+} handling is compartmentalized at multiple different levels within the cytoplasmic region and, importantly, within specific cell organelles, and Ca^{2+} signals are context-dependent and there is great variation in the magnitude and spatiotemporal nature of the Ca^{2+} signaling events. For instance, the cytoplasmic Ca^{2+} signal, i.e. the elevated Ca^{2+} concentration emerging upon a stimulation, can cover the whole of the cytoplasm, or be spatially restricted to a limited proportion of the cytoplasm [44,64]. Local cytoplasmic Ca^{2+} signaling events, or Ca^{2+} microdomains, of this kind may arise for instance near an IP_3 receptor channel opening upon a modest receptor stimulation by the agonist, or at the vicinity of ion channels regulating Ca^{2+} entry at the PM [9,44]. The emergence and nature of the cytoplasmic Ca^{2+} microdomains may also be orchestrated by

organelles and cellular compartments. For instance, mitochondrial Ca^{2+} uptake and mitochondrial apposition to the ER contributes in the regulation of the local events, such as the IP_3-mediated Ca^{2+}-release, and mitochondrial Ca^{2+} also contributes to SOCE regulation [9,65,66]. In addition, the PM is compartmentalized and harbors regions with distinct lipid and protein compositions, e.g. the flask-shaped membrane formations called caveolae that coordinate signaling events, including Ca^{2+} [67].

The mitochondrial and caveolar compartments, and their role in Ca^{2+} handling, were at the focus of this thesis, and are therefore respectively described below.

In addition, also other organelles, such as the lysosomes, have been shown to be important actors in the regulation of intracellular Ca^{2+} homeostasis, and are therefore briefly described.

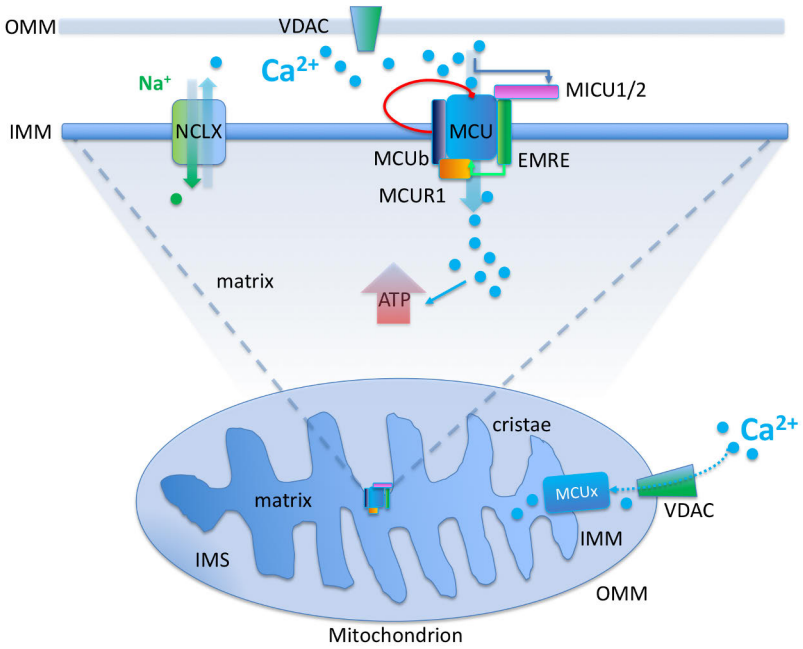

Figure 2. Overview of mitochondrial calcium (Ca^{2+}) handling. The outer mitochondrial membrane (OMM) is permeable to Ca^{2+}, facilitated by the non-specific voltage-dependent anion channel (VDAC), through which Ca^{2+} enters the intermembrane space (IMS). The inner mitochondrial membrane (IMM) harbors the mitochondrial calcium uniporter complex (MCUx) that regulates the influx of Ca^{2+} to the mitochondrial matrix. The MCU complex consists of the pore-forming mitochondrial calcium uniporter (MCU) and the regulatory subunits. MCUb-isoform is a negative regulator (red blunt arrow) of MCU whereas EMRE (essential MCU regulator) positively modulates (green arrow) MCU. The MCU regulator 1 (MCUR1) expression is required for MCU-mediated Ca^{2+} uptake. The mitochondrial calcium uptake proteins (MICU1/2) function as sensors of Ca^{2+} and modulate the Ca^{2+} uptake threshold of MCU. NCLX is the mitochondrial Na^{+}/Ca^{2+} exchanger. Ca^{2+} is an essential component in the regulation of mitochondrial adenosine trisphosphate (ATP) production. Adapted from [9].

1.5.1 Mitochondrial Ca^{2+}

Mitochondria, the energy-producing cell organelles, consist of two membranes, namely the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). Between these membranes is the intramembrane space (IMS). The IMM envelopes the mitochondrial matrix where the main cellular energy unit, ATP, is being produced through oxidative phosphorylation. The OMM is permeable to Ca^{2+}, the permeability of which is attributed to the non-selective voltage-dependent anion channel (VDAC) that resides in the OMM. VDAC is permeable to ions and small molecules. Importantly, VDAC also allows for ATP translocation from the mitochondria to the cytoplasm [9,68–70].

Mitochondria have been known to take up Ca^{2+} for decades [71] but the components and molecular mechanism of mitochondrial matrix Ca^{2+} uptake regulation, and some of the downstream effects, have been elucidated during the past decade. The early mitochondrial Ca^{2+} uptake studies highlighted the role of Ca^{2+} as a regulator the mitochondrial permeability transition, a process leading to the release of mitochondrial content, e.g. cytochrome c, to the cytoplasm, a process which initiates the process of cell death [72]. Importantly, Ca^{2+} has been found to be essential for the regulation of mitochondrial matrix enzymes that are involved in the ATP production by oxidative phosphorylation, and Ca^{2+} is thus involved in regulating the central elements of cellular energy metabolism [73–75]. Also, a recent study has highlighted the role of cytoplasmic Ca^{2+} in the regulation of oxidative phosphorylation [76]. In addition to the essential role as a regulator of mitochondrial energy metabolism, mitochondrial Ca^{2+} regulation also has been shown to exert widespread physiological and pathophysiological effects ranging from regulating skeletal muscle function to contributing to postmortem proteolysis and tenderness in beef cattle, and playing key roles in multiple neurodegenerative processes and disease conditions, contributing to multiple aspects of oncogenesis, and beyond [77–83].

1.5.2 Regulation of mitochondrial Ca^{2+}

The main protein component regulating mitochondrial Ca^{2+} uptake and Ca^{2+} conductance is the mitochondrial Ca^{2+} uniporter (MCU). The molecular identities of the uniporter MCU, and its isoform MCUb, were recently characterized [9,84–86]. MCU activity has been found to be modulated by several interacting molecules: MCUR1 (MCU regulator 1), MICU1-3 (mitochondrial Ca^{2+} uptake protein) and EMRE (essential MCU regulator). The characterization of these regulatory proteins have greatly expanded the knowledge on the regulatory toolkit of mitochondrial matrix Ca^{2+} handling [71,84,85,87–92]. Further, the molecular identity of the mitochondrial Na^+/Ca^{2+} exchanger NCLX was recently resolved and thus NCLX was confirmed to be a major regulator of mitochondrial matrix Ca^{2+} [93]. Interstingly, the NCLX may also operate in reverse mode, transporting Ca^{2+} from the IMS to the matrix [49]. The current view is that mitochondrial Ca^{2+} uptake is mainly achieved in a coordinated fashion by multiple different interacting proteins, the so called MCU complex (Fig. 2). The MCU complex consists of the pore-forming MCU and its regulatory subunits mentioned above.

The proteins regulating MCU may exert negative or positive regulation of the mitochondrial matrix Ca^{2+} uptake capacity. The MCUb-isoform has been characterized as a negative regulator of MCU, whereas EMRE expression and EMRE binding to MCU is needed for effective mitochondrial Ca^{2+} uptake [87,94]. Interestingly, the expression pattern of EMRE, and EMRE binding stoichiometry to MCU, were recently characterized in mouse tissues. In this study, it was found

that EMRE expression varies between the different tissue types, and, in accordance, the stoichiometry of MCU-EMRE binding also shows tissue specificity. This finding may in part elucidate the possible mechanism for the variable mitochondrial Ca^{2+} uptake dynamics that have been found in different tissue and cell types [95]. Further, it has been shown that MICU1 and MICU2 reside in the IMS and act as Ca^{2+} sensors to regulate the threshold of Ca^{2+} uptake through the MCU [87,94]. It has been shown that MICU1/2 form heterodimers and they both contain two EF-hand domains that bind Ca^{2+} [9,90]. The dimeric MICU1/2 has been postulated to interact with EMRE to regulate MCU activity [96]. Different models for the mode of MCU regulation by the gatekeeper MICU proteins have been reported. For instance, MICU1 has been shown to inhibit Ca^{2+} entry to mitochondria through the MCU at low cytoplasmic Ca^{2+}, whereas upon exposure to high cytoplasmic Ca^{2+} concentration, MICU1 augments MCU-mediated Ca^{2+} uptake. [9,87,94,97,98]. Of note, different tissues have been shown to have varying degrees of expression of the three MICU proteins, and thus, along with the above-mentioned EMRE protein level variation in tissues, their expression pattern is likely contributing to the tissue-specific differences in mitochondrial matrix Ca^{2+} uptake capacity [91,99].

In addition, other proteins that have been indicated to contribute to the regulation of mitochondrial matrix Ca^{2+} include the mitochondrial uncoupling proteins 2 and 3 (UCP2/3), mitochondrially localized TRPC3, and the leucine zipper EF-hand- containing transmembrane protein 1 (LETM1) that has been shown to act as a Ca^{2+}/H^{+} exchanger [9,100,101].

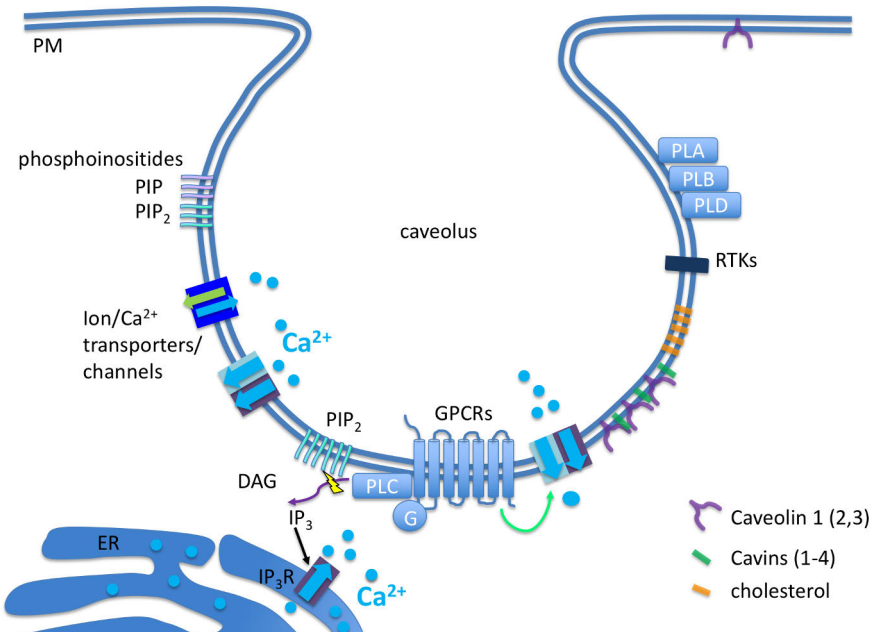

Figure 3. Plasma membrane (PM) invaginations, caveolae, harbor molecules, receptors and channels important for Ca^{2+} signaling. Caveolar compartments are cholesterol-rich and the main structural proteins of caveolae are the caveolins 1-3 and the cavins 1-4. Phosphoinositides such as phosphatidylinositol phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate PIP_2, as well as phospholipase proteins A, B, C and D reside in the caveolae. Ion and Ca^{2+} transporters and channels are associated with the caveolae. Receptor tyrosine kinase (RTK) and G-protein-coupled (GPCR) receptors localize to caveola. These receptors couple to phospholipase C (PLC) that cleaves PIP_2, thus forming inositol trisphosphate (IP_3) that binds to the endoplasmic reticulum (ER) IP_3-receptors to stimulate Ca^{2+} release from the organelle. Caveolae and ER may be in close apposition, thus forming a possible IP_3 and Ca^{2+} signaling microdomain [102]. Figure adapted from [27,103].

1.5.3 Ca^{2+} at the caveolar sub-compartments of the plasma membrane

Caveolae are omega-shaped, approximately 60 to 80 nanometer wide invaginations of the PM. The caveolae are considered to act mainly as signaling hubs where also multiple transporter proteins and ion channels have been found to be localized at. In addition, the caveolae have also been attributed functions as being involved in cellular mechanosensing processes and also contributing to mechanoprotection functions at the cell membrane [67,104]. Caveolae and caveolae-associated molecules are also found to be involved in the formation and regulation of endocytic vesicles which are involved in mediating intracellular vesicular transport.

The major identified constituents of caveolae are the caveolin 1-3 and cavin 1-4 proteins, respectively. The caveolin proteins are considered to be the main structural proteins of caveolae, whereas cavins are thought to contribute to the structure and regulation of caveolae formation. Of note, also other protein components that are possibly associated with the formation of caveolae have been identified [105,106]. The caveolar compartment of the PM has been shown to harbor a characteristic lipid composition, showing for instance accumulated levels of cholesterol and certain glycosphingolipid species, and thus differing from the overall PM also in this respect.

Functionally, the caveolins are integral proteins of the PM. In general terms, caveolin-1 and caveolin-3 are considered as the most abundant and constitute key components of caveolae. Caveolin proteins are able to form oligomers and have the capacity to bind to cholesterol. Further, the cavin proteins have also been shown to bind to lipids, such as PIP_2. Intriguingly, the amount of observed caveolae structures varies greatly between different tissues and cell types. For instance, the PM of skeletal muscle cells harbors caveolae at a high density whereas other cell types may be completely devoid of caveolae. In agreement, the caveolin protein expression levels highly vary in a tissue-specific manner [103,107]. While the various aspects of the molecular structure of caveolae are still to be elucidated, the current literature indicates that oligomerized caveolin proteins are the essential elements for the caveolae formation, and that the cavin proteins together with the caveolins contribute to the structure and stability, as well as to the functional modulation of the caveolae [107–109].

Importantly, caveolae and the related proteins have been linked to the progression of various diseases, including oncogenesis in humans [103,110]. For instance, cavins 1-3 have been shown to act as tumor suppressors, whereas the oncogenic effect of caveolin-1 expression is context-dependent [103,111].

Interestingly, multiple protein and lipid molecules that are either directly or indirectly involved in the regulation of intracellular Ca^{2+} have been shown to be localized to the caveolar sub-compartments of the PM. These molecules include TPRC channels, the PM Na^{+}/Ca^{2+} exchanger (NCX), the GPCR and RTK receptor proteins, the lipid PIP_2, and PLC that cleaves PIP_2 to form IP_3 [27]. Further, caveolin-1 expression has been shown to regulate cytoplasmic and mitochondrial Ca^{2+} uptake in a H-Ras-dependent tumor model [111]. Also, it has been shown that the IP_3-receptors and the ER may be closely positioned with the caveolae [102,112]. In addition, it has been recently shown that caveolin-1 is needed for proper localization of IP3R3 to the apical domain in cholangiocytes, and for the regulation of IP3R3 Ca^{2+} mediated signaling in these cells [113]. However, direct measurements of caveolar Ca^{2+} are limited. It has been shown that caveolae are preferred sites for SOCE as compared to the overall PM, and that caveolar intracellular vesicles may act as sources for ATP-mediated Ca^{2+}

release in endothelial cells [114,115]. Further, we have shown by employing a novel luminescent aequorin-based caveolin-1 -tagged Ca^{2+} reporter probe that caveolae may act as specific sub-compartments of the PM where specific Ca^{2+} (micro)domains may arise.

In this study, we found that sphingosine kinase 1 (SK1) overexpression, likely in a concerted fashion with the altered downstream sphingolipid-mediated signaling, is involved in the modulation of Ca^{2+} signaling specifically at the caveolar (micro)domains of the PM, while not affecting the Ca^{2+} signaling at the overall sub-PM domain (see Results section of this thesis and publication 1, ref. [116]).

1.5.4 The involvement of (endo)lysosomes in intracellular Ca^{2+} regulation

Lysosomes are dynamic organelles that are key in the management and clearance of cellular debris through proteolytic processes taking place in the lumen of the acidic organelle. To achieve this, lysosomes harbor a range of functions facilitating the pathways necessary for the function of the organelle. The components of these pathways and functions include structural membrane proteins, proteins that act as transporters or channels, along with regulatory proteins that are facilitating membrane vesicle trafficking functions such as the coordinated fusion and fission events of the endosomes and lysosomes, i.e. the endo-lysosomal pathway. In addition, signaling functions that are important for the cellular metabolic housekeeping and homeostasis have been attributed to the lysosomes. [117] Interestingly, lysosomes have been identified to have the capacity to store Ca^{2+} ions, and lysosomes have been proposed to play a role in regulating Ca^{2+} signaling in cells through direct lysosome-associated activatable membrane channels, and through interactions with the ER and ER Ca^{2+} release [118,119].

Notably, the functions of the endo-lysosomal two-pore channels (TPCs), shown to be directly involved in the lysosomal Ca^{2+} regulation, have been elucidated relatively recently. TPCs are currently considered as the key components that regulate Ca^{2+} release from the lysosomes. Intriguingly, TPCs are cation channels with a dual nature to their function, as their ion conductance depends on which of the two identified stimulatory pathways is activating the channel. That is, TPCs have been identified to become activated by nicotinic acid adenine dinucleotide phosphate, NAADP, which through a NAADP receptor associated with the TPC, activates a non-selective Ca^{2+} flux through the TPC channel. In addition, the TPCs have been shown to be activated by phosphatidylinositol 3,5-bisphosphate, $PI(3,5)P_2$. In contrast to the NAADP-mediated lysosomal Ca^{2+} flux, TPC stimulation by $PI(3,5)P_2$ takes place through direct binding and activates a selective Na^+ flux [119]. TRP channels of the mucolipin family have been

indicated as contributors to the lysosomal Ca^{2+} release [118]. It has been shown that endocytic processes and the following release of endosomal Ca^{2+} to the cytoplasm may facilitate Ca^{2+} uptake of cells [120].

Interestingly, lysosomes have been shown to interact through close membrane contacts or associations with the ER and mitochondria, with implications for inter-regulation of Ca^{2+} signaling by these organelles. Importantly, the Ca^{2+} pathways of the ER-mitochondria-lysosome axis have been shown to modulate key cell physiological processes such as autophagy and apoptosis [121]. It has been postulated that lysosomal Ca^{2+} refilling takes place at membrane contact sites between ER and the lysosomes [122].

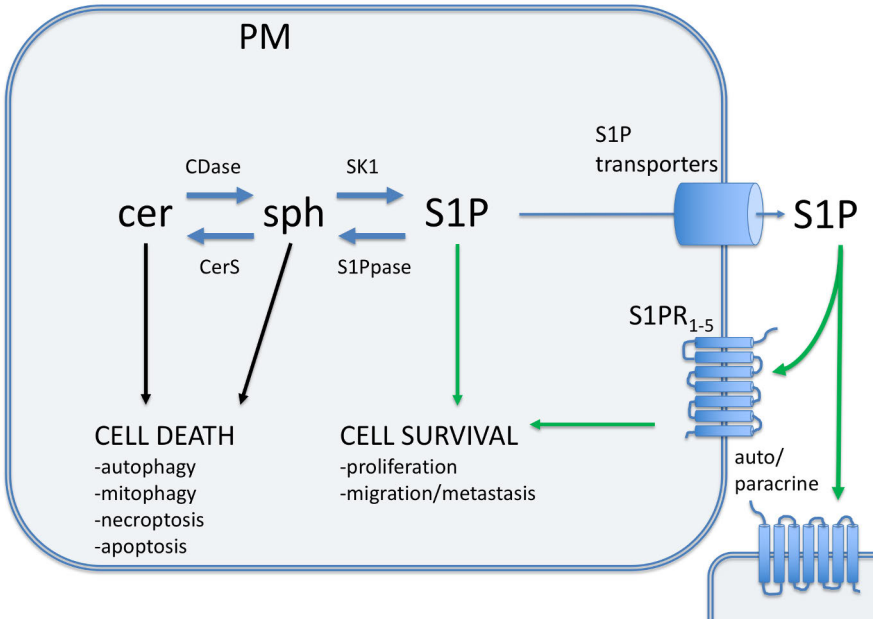

Figure 4. Processing and effects of the central sphingolipids ceramide (cer), sphingosine (sph) and sphingosine 1-phosphate (S1P). Ceramide is either synthesized de novo or converted from sphingosine by ceramide synthases (CerS). Sphingosine is generated from ceramide by ceramidase (CDase) and further phosphorylated to sphingosine 1-phosphate by sphingosine kinase 1 (SK1). S1P phosphatase (S1Ppase) dephosphorylates S1P back to sphingosine. S1P is transferred out from the cells through the plasma membrane (PM)-residing transporters for auto/paracrine activation of the G-protein-coupled S1P-receptors ($S1PR_{1-5}$). Ceramide and sphingosine promote cell death pathways whereas S1P augments cell survival and migration. Modified from [123,124].

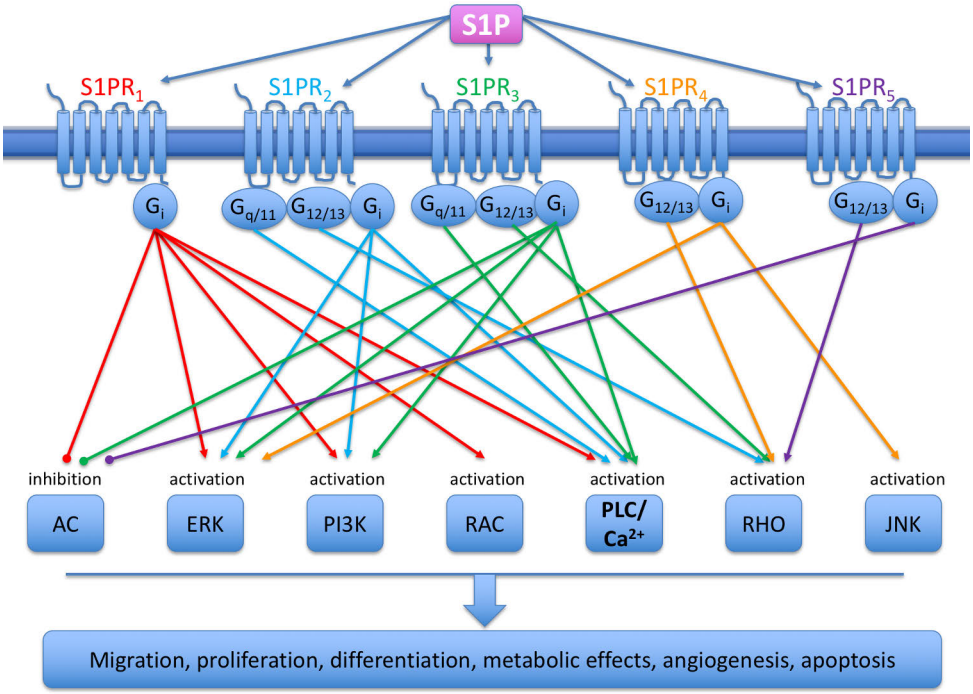

Figure 5. Sphingosine 1-phosphate receptors ($S1PR_{1\text{-}5}$) and some of their downstream targets. S1P-receptors couple to different G-proteins (G_i; $G_{q/11}$; $G_{12/13}$) and thus activate or inhibit different downstream targets such as adenylyl cyclase (AC), extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K), Rho and Rac guanosine trisphosphate (GTP)ases, and phospholipase C/Ca^{2+} signaling. Some of the downstream cellular effects are listed. Modified from [125–127].

1.6 Sphingolipid signaling and Sphingosine kinases

Sphingolipids serve important cellular functions as integrated constituents of the PM and organelle membranes, and, importantly, as signaling molecules [123]. The central sphingolipids that regulate cell function include ceramide, sphingosine and sphingosine 1-phosphate (S1P) (S1P metabolism is summarized in Fig. 4). Sphingosine is formed by ceramidases through deacetylation of ceramide, which is generated de novo or through hydrolysis of sphingomyelin [124]. Sphingosine is phosphorylated by one of the two identified sphingosine kinases (SK1 and SK2) to form the lipid S1P, which has been found to contribute to several important signaling functions. Phosphorylation of sphingosine to form S1P by SK1/2 is ATP dependent, and the SK isoforms contain domains that are conserved, including the ATP-binding domain. Also, various splice-variant forms for both SK1 and SK2 have been identified. Mouse model studies have shown that SK1/2 double knock-out results in embryonic lethality in these animals. Interestingly, the SK1 and SK2 are able to compensate for the knock-out of one of the kinases in mice, thus showing mechanistic redundancy

as these animals are observed as phenotypically normal [128]. However, the two kinase isoforms have been identified to serve distinct cellular functions and have different cellular localization patterns. For instance, SK1 has been found to be mostly cytosolic but translocation of the kinase may occur for instance to the nucleus and endosomes, and, importantly, to the cell membrane [129], whereas SK2 may show localization to mitochondria, to the endoplasmic reticulum and to the cell nucleus as well [128].

Importantly, the S1P generated by SK1/2 in the intracellular space can be transported to the extracellular milieu through specific transporter molecules residing at the PM. Some of the proteins of the ATP-binding cassette transporter family, as well as the spinster 2 (SPNS2) protein, have been identified as mediators of S1P transport to the extracellular space [130,131].

1.6.1 Cellular effects of sphingolipid signaling and S1P

In a generalized view, increased levels of ceramide and sphingosine are considered to promote cell death, whereas increase of S1P augments e.g. cell proliferation and migration. Thus, the SKs are in control of the balance of these sphingolipids that have opposing effects on the cell fate regulation (Fig. 4). This balance, and the downstream cellular effects on e.g. cell fate regulation, of the key sphingolipid species is sometimes termed as the "sphingolipid rheostat" [123]. Consequently, the SK1/S1P signaling axis is an important factor in the oncogenic processes [123,124] as well as in neurodegenerative disease such as multiple sclerosis and the Charcot-Marie-Tooth disease [132–135]. S1P has been identified to have direct binding to intracellular target proteins, contributing to the modulation of their function. To date, five PM-residing G-protein coupled S1P-receptors have been identified and characterized to mediate the extracellular singaling route of S1P [136]. The intracellular interaction targets of S1P include for instance prohibitin 2 (a mitochondrial regulatory protein for respiratory function), and it has been shown that SK2 depletion leads to decreased mitochondrial respiration. In addition, S1P binds to TNF receptor-associated factor 2 to regulate nuclear factor kappa B -mediated cell signaling [128,136]. Further, S1P modulates histone deacetylase 1/2 function which results in altered epigenetic control of target genes c-Fos and p21 [137]. Interestingly, it was shown in mice that S1P also modulates amyloid beta peptide metabolism by affecting the function of the β-site APP cleaving enzyme 1, BACE1, thus indicating potential relevance of intracellular S1P signaling in Alzheimer's disease [138]. S1P has also been shown to directly modulate the peroxisome proliferator-activated receptor gamma to regulate the downstream gene expression thereof [128]. Thus, intracellular S1P signaling plays a role in multiple cellular functions such as modulation of angiogenesis, mitochondrial function and respiration, as well as gene regulation [124].

1.6.2 Sphingosine 1-phosphate receptors

As mentioned in the previous chapter, the extracellular signaling functions of S1P are mainly mediated through the dedicated S1P-binding, PM-residing G-protein-coupled receptors (GPCRs). To date, five S1P receptors, termed SIPR1-5, have been characterized. Most of the S1PRs have also been found to couple to Ca^{2+} signaling events through the respective downstream signaling cascades (Fig. 5) [125]. Mechanistically, the S1P receptor activation resembles the classical GPCR activation, e.g. the binding of the ligand S1P triggers a change of conformation in the receptor protein, and the conformational change facilitates the exchange of guanosine diphosphate (GDP) to guanosine triphosphate GTP) on the G-protein subunit Gα, leading to the dissociation of the heterotrimeric G-protein complex Gαβγ from the receptor. Further, the Gα subunit dissociates from the subunit complex Gβγ. Both the monomeric Gα and the dimeric Gβγ subunits are thus activated and can contribute to various downstream signaling effects. In addition, multiple isoforms of the Gα subunit have been characterized, e.g. the stimulatory Gαs protein, the inhibitory Gαi protein and, importantly, the Gq group of proteins (e.g. Ga11) which are known to be PLC-activating and thus directly triggering intracellular Ca^{2+} release through the IP_3 mediated pathway [126,139].

The S1PRs have been characterized to contribute to the regulation of multiple downstream signaling pathways, modulating various cell physiological processes such as cell migration and proliferation [124,140], with important implications for disease conditions such as cancer, osteoporosis, atherosclerosis, diabetes and degenerative conditions including Parkinson's and Alzheimer's disease, as well as multiple sclerosis and Huntington's disease [133,141,142]. Some of the S1P-mediated cell physiological effects are summarized in Fig. 5.

1.6.3 Inter-regulation of Ca^{2+} signaling and the Sphingosine Kinase/S1P axis

As mentioned above, the activity of SK1 and SK2 regulate the production of S1P, and the site of S1P production may be affected by the intracellular localization of the two SK isoforms. When activated, SK1 localizes primarily to the PM where the substrate of the kinase, sphingosine, is found [140]. The SK1 PM translocation has been shown to be phosphorylation-dependent [143] and for instance the extracellular regulated kinase 1/2 (ERK1/2) has been shown to phosphorylate SK1 and to regulate its localization to the PM [144]. Calmodulin inhibition has been shown to block the PM translocation of SK1 [145]. Of note, it has been shown that calcium and calmodulin are involved in the regulation of ERK1/2 signaling [146] indicating the possibility that Ca^{2+} through ERK1/2 signaling may modulate SK1 translocation and activity. Indeed, by employing

computational modeling, it has been postulated that the interplay and feedback regulation of ERK1/2 and Ca^{2+} signaling along with the activation of SK1 are important components in the modulation of vascular endothelial growth factor receptor 2 (VEGFR2) signaling [147].

Interestingly, the Ca^{2+} and integrin binding protein, CIB1, which is a myristoylated protein with a Ca^{2+}-myristoyl switch function, has been shown to regulate the PM localization of SK1 [148,149]. Hence, CIB1 directly contributes to the Ca^{2+}-dependent activation of SK1. Of note, CIB2 which lacks the Ca^{2+}-myristoyl switch function, is able to bind SK1 at the same domain as CIB1, but this binding results in an inhibitory effect to SK1 translocation and activation [150]. Importantly, it has been shown that SK1 translocation to the PM increases both the amount of S1P being produced, and its release to the extracellular milieu [143,148]. As mentioned above, because the polar nature of S1P it cannot passively cross through the PM bilayer and thus active transport of S1P through the PM been shown to take place [123,131,151]. Interestingly, in rat platelets, it has been shown that the cellular extrusion of S1P is activated by Ca^{2+} [152]. The interplay and feedback between Ca^{2+} signaling, the SK1 activity, the S1P extrusion, and the auto/paracrine activation of S1P receptors, have been studied and it has been indicated that S1P may modulate Ca^{2+} through yet uncharacterized intracellular means [128,153–155]. Thus, the SK1/S1P and Ca^{2+} signaling axis may be inter-regulated, and the possible and indicated schemes are summarized in Fig. 6.

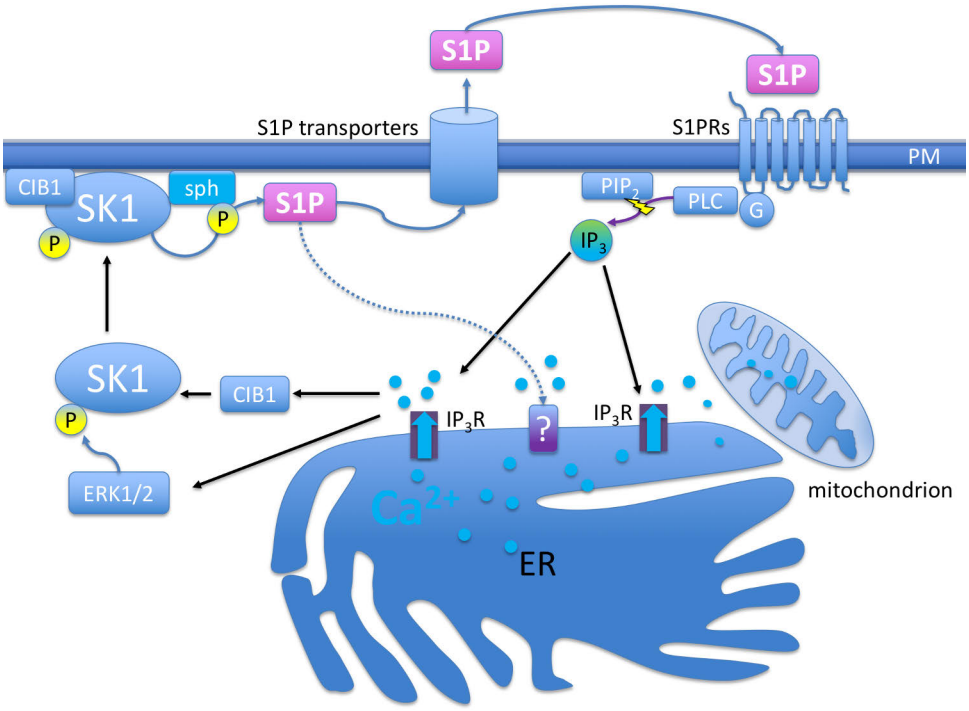

Figure 6. Inter-regulation of sphingosine kinase 1 (SK1) and Ca^{2+}. Membrane translocation and activation of SK1 are regulated by Ca^{2+} through extracellular signal-regulated kinase ½ (ERK1/2) -mediated phosphorylation (P) and through interactions with the Ca^{2+}/integrin-binding protein 1 (CIB1). Activated SK1 phosphorylates sphingosine (sph) to form sphingosine 1-phosphate (S1P). Plasma membrane (PM) -residing transporters move S1P to the extracellular space for auto- and paracrine activation of the G-protein coupled S1P-receptors (S1PRs), most of which couple to the phospholipase C (PLC) pathway. Activated PLC cleaves (phosphatidylinositol-4,5-biphosphate) PIP_2 to form inositol trisphosphate (IP_3), which stimulates the IP_3-receptors and the subsequent release of Ca^{2+} from the endoplasmic reticulum (ER). The released Ca^{2+} can be taken up by the mitochondria. S1P may induce the realease of intracellularly stored Ca^{2+} through unknown mechanisms (indicated with '?'). Adapted from Pulli et al, ref. [125].

1.7 Oxysterol-binding protein related-proteins 5 and 8 (ORP5/8) as regulators of lipids and Ca^{2+} at membrane contact sites

1.7.1 Cell and organelle membranes

The cell membrane and the membranes encapsulating the different cell organelles are essential for maintaining the processes of life [1]. The lipid composition of the different membrane types varies, and the lipid composition is also a key determinant of cell and organelle membrane function. Cell and organelle membranes are composed of various lipid species, and the specific ratios of different lipid species may greatly affect the membrane properties. Also, cell and organelle membranes may be compartmentalized, e.g. the membranes may harbor sites where certain lipid species may be organized in a specific composition with specific functions, and thus the lipid distribution within cell and organelle membranes is regulated by several mechanisms. Membranes are also compartmentalized in regard to their protein content and the protein and lipid distribution and interactions may affect the properties and biological function of the membrane, and in part modulate the function of the membrane receptors and channels [104,156,157].

To regulate the cell and organelle membranes' composition and function, lipid transfer from the site of lipid synthesis to the site where the lipid is to exert its function is essential. Further, the cellular lipid landscape is highly dynamic as the membrane's lipid composition is regulated by, and involved in regulating, various cell singaling events [156,158]. To achieve the specific lipid composition for a specific organelle, the lipid synthesis and transport between the different organelles is tightly controlled [156]. In this process, lipid-binding proteins are essential. Lipid-binding proteins are a class of proteins with a hydrophobic motif that allows the binding and transport of lipids in the cytoplasm from a membrane to another [159].

1.7.2 Oxysterol-binding related-proteins and their role as membrane lipid regulators and sensors

The protein family of oxysterol-binding protein related-proteins (ORPs) have been characterized as lipid-binding proteins [156]. Twelve mammalian genes have been identified to encode for ORPs with various cellular functions ascribed to them. The different members of the ORP family of proteins are mainly thought to function as lipid sensors and lipid transfer proteins, facilitating the movement of lipids within the cell and between the organelles. For instance, ORPs have been found to facilitate lipid transfer between the ER and the plasma membrane,

thus contributing to the cellular lipid dynamics. Importantly, ORP-mediated events regulating lipid composition or lipid dynamics also couple to a multitude of cellular events, including the modulation of protein interaction and activity, thus having the potential to regulate cell signaling events and pathways [156,160].

The different members of the ORP family of proteins have been shown to regulate the transport of lipids in a non-vesicular fashion [161,162]. ORP proteins have been found to ligate multiple lipid species, such as cholesterol, phosphoserine, phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol-4-phosphate (PI4P) [156,163,164].

1.7.3 Oxysterol-binding related-proteins 5/8 function at the membrane contact sites

Lipid transfer and other forms of communication between the cell and the different organelle membranes take place at the membrane contact sites (MCS), areas of close apposition and tight membrane interaction. MCS have been found to exist, for instance, between ER and mitochondria, ER and the PM, mitochondria and the PM as well as within the two mitochondrial membranes [165]. MCS play an important role in contributing to lipid transfer and facilitating communication between organelles by different mechanisms, including signaling through second messengers such as the Ca^{2+} ion [104]. Proteins of the ORP family have been shown to exert lipid transport functions at the MCS between specific cellular organelles and compartments, such as the junctions of the mitochondria and the ER as well as at the PM-ER contact sites [158,163,164,166,167].

Importantly, the ORP5 and ORP8 proteins which were the subjects of this study, have been found to contribute to the regulation of lipid localization in the different cellular compartments such as the PM, ER, mitochondria and lysosomes, and at the close appositions or contact sites of these organelles or compartments [168]. For instance, ORP5/8 have been found to contribute to countertransport of phosphatidylinositol-4-phosphate (PI4P) and phosphatidylserine at the PM-ER MCS [166]. In addition, ORP5/8 have been shown to bind to and regulate the transfer of PIP_2 from the PM to the ER [169].

Taken together, the ORP5/8 have been shown to function in a compartmentalized fashion to regulate local events at the membrane junctions of organelles, with important implications to oncogenic processes.

2 METHODS

2.1 Cell culture, plasmids and transfections

HeLa human cervical carcinoma cell lines were employed as the main cell model in this study. Further, human follicular thyroid cancer cell line, FTC-133, as well as the human thyroid cancer cell line ML-1 were used. The cells were grown in a humidified and thermostatic incubator with a controlled atmosphere (37 °C, 5% CO2). The HeLa and the ML-1 cells were grown in DMEM medium (#D6046, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics (Life Sciences). The FTC-133 cells were cultured in DMEM and F12 (Ham's nutrient) medium (1:1) which was supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin antibiotics. Plasmid transfections were conducted by using the transfection reagent TurboFect (#R0531, Thermo Scientific) and the transfection media, OptiMEM (#31985-070, Life Technologies). Alternatively, the electroporation transfection method was employed; 20 µg of the desired plasmid construct were suspended with approximately 4 million cells in 400 µL OptiMEM transfection medium. The electroporation conditions were set at 240 V and 500 µF. The plasmid carrying the ORP8 PH domain deletion was generated in a construct in pEGFP-C1 (Invitrogen) by amplifying the amino terminal fragment encoding aa residues 1-106 (Met-Val) with a SacI site at its 5' end and a SpeI site at its 3' end, and a another fragment downstream of the PH domain, beginning at Ser226, with a SpeI site at its 5' end and an EcoRI site at its 3' end. The fragments were ligated into pEGFP-ORP8 cut with SacI and EcoRI, resulting in a PH domain deletion construct lacking aa residues Ile107-Ser225. The sequence of the construct was before use confirmed by dideoxy sequencing. The plasmid constructs for aequorin expression and for expression of wild-type Orp5 and Orp8 were as described previously [30,116,170] Transduced HeLa mock cells or HeLa cells stably expressing sphingosine kinase 1 were created as described in [116].

2.2 Aequorin-based calcium measurements

As the primary method of this thesis work, the genetically targeted Ca^{2+} indicator protein, aequorin, was employed. Aequorin is a 22 kilodalton luminescent protein activated by Ca^{2+} ions. Upon Ca^{2+} binding, the prosthetic group of aequorin, coelenterazine, is oxidized in an irreversible reaction to produce photons (Fig. 7b'). Recombinant aequorin can be targeted to intracellular compartments by fusing the aequorin protein with a specific localization sequence. The aequorin constructs targeted to the cytoplasm, to the mitochondrial matrix, to the endoplasmic reticulum and to the plasma membrane were previously described [30]. The caveolin-1-aequorin construct

that targets to the caveolin-1-enriched microdomains of the plasma membrane was described and validated in Publication 1 of this thesis (ref. [116]). The advantage of aequorin-based calcium measurements is its specificity for a given cell compartment (Fig. 7), and its high signal-to-noise ratio.

The detailed aequorin measurement protocol is reviewed in [30] and described in the original publications of this thesis [116,170,171]. Briefly, the cells were plated on to 12- or 96-well plates and transfected with the desired aequorin plasmid constructs. The following day the aequorin was reconstituted with the molecules' prosthetic group coelenterazine. For this, the cells were incubated with 5 μM wild-type coelenterazine or coelenterazine n, respectively, in HEPES-buffered saline solution (HBSS; 118 mM NaCl, 4.6 mM KCl, 10 mM glucose, 20 mM HEPES, pH 7.4). Samples that were transfected with aequorins targeted to the compartments with low basal Ca^{2+} levels, i.e. the cytoplasm or the mitochondria, were incubated in HBSS supplemented with 1 mM $CaCl_2$ and wild-type coelenterazine. However, when aequorins targeted to the PM or to the caveolae were employed, the incubation with wild-type coelenterazine was conducted in HBSS supplemented with the Ca^{2+} chelator EGTA (150 μM; ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid). The Ca^{2+} concentration near the PM may reach high levels and hence the extracellular Ca^{2+} was chelated to avoid the oxidation of coelenterazine during the reconstituting incubation. To reconstitute the ER-targeting aequorin, the ER was pre-depleted of Ca^{2+} by treating the cells with the ionophore ionomycin in HBSS containing EGTA. Then, the cells were incubated for 3-4 hours at 4 ºC in HBSS supplemented with EGTA and the less reactive coelenterazine n as described in [30]. The luminescence recordings were conducted as described before either by employing a luminometry setup that was built in-house, or by utilizing a plate reader (HIDEX Corp., Turku, Finland) [170].

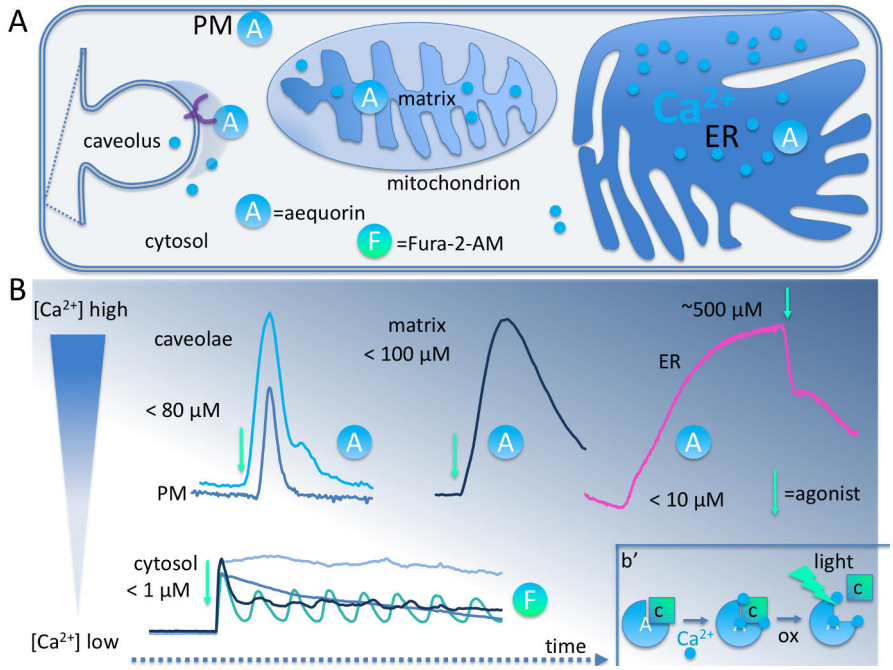

Figure 7. Calcium (Ca^{2+}) measurements employing the luminescent recombinant protein aequorin and the fluorescent Ca^{2+} indicator Fura-2-AM. A) Aequorin can be genetically modified to target to and to report Ca^{2+}concentrations from subcellular compartments e.g. the caveolae, the plasma membrane (PM), the mitochondria and the endoplasmic reticulum (ER). Wild-type aequorin localizes to the cytoplasm. The ratiometric fluorescent Ca^{2+} indicatior Fura-2 is tagged with an acetoxymethyl (AM)-group to facilitate PM permeability. Cytoplasmic esterases then cleave off the AM-group and Fura-2 is trapped in the cytoplasm, thus reporting the changes in Ca^{2+} in this compartment. B) The cytoplasmic Ca^{2+} flux and oscillations are representative from a four-cell colony of MCF7 breast carcinoma cells where the individual cells show different and distinct cytoplasmic Ca^{2+} kinetics upon stimulation with 10 µM adenosine trisphosphate (ATP) as indicated with the green arrow. The caveolar, PM, mitochondrial and ER Ca^{2+} fluxes are representative from HeLa cells stimulated with 100 µM histamine. b') Aequorin forms a complex with its prosthetic group coelenterazine (c). Upon Ca^{2+}-binding coelenterazine is oxidized (ox) and light is produced in a luminescence reaction.

2.3 Ca^{2+} measurements employing Fura-2-AM

The fluorescent, ratiometric Ca^{2+} indicator Fura-2-AM (Invitrogen) was employed as an alternative method for cytoplasmic Ca^{2+} measurements when cytosol-targeting aequorin was not applicable, e.g. for measurements of longer duration than what cytoplasmic aequorin allows for. The measurements

employing Fura-2-AM were conducted as described in the original publications of this thesis [116,170,171]. Briefly, the cells were plated either on to poly-L-lysine coated 35 mm glass coverslips or onto white plastic 96-well plates. Then, the cells were incubated with 2 μM Fura-2-AM in HBSS buffer in the dark and at room temperature for 30 minutes, followed by three washes and a further incubation in HBSS for 15 minutes. Then, after a brief wash, the cells were subjected to the fluorescence recordings. The fluorescent light was collected either by utilizing a microscopy setup or by a plate reader (HIDEX Corp, Turku, Finland), respectively. The excitation filters were set at 340 and 380 nanometers, respectively, and the emission was read at 505 nanometers. The ratio of the 340 nm and 380 nm excitations was calculated and used as a relative measure of intracellular cytoplasmic Ca^{2+}.

2.4 Western blot

Western blot was conducted as described in [116,170,171]. Briefly, to prepare the samples the cells were plated on to the suitable cell culture plates (100 mm, 35 mm, 12-well or 96-well plates depending on the experiment) followed by a desired treatment or transfection. To collect the cell lysates, the cells were briefly washed with cold PBS, and then subjected to direct lysis in Laemmli sample buffer (LBS). The samples were then subjected to SDS-PAGE and transferred by wet transfer to a nitrocellulose membrane. The membranes were incubated overnight with the primary antibodies, followed by a 1-hour incubation with the horseradish peroxidase (HRP) –conjugated secondary antibodies. Thereafter, the protein bands were visualized by chemiluminescence. The antibodies were as reported in the original publications 1-3.

2.5 Cell migration

The experiments to measure cell migration were conducted as described in [171] and [172]. Briefly, the cells were detached from the culture plates, suspended in a serum-free medium and placed in to uncoated Transwell (Corning) membrane inserts to allow for migration for 12 hours towards the chemoattractant, i.e. cell culture medium containing fetal bovine serum. The insert membranes were processed to allow for counting the migrated cells, and the acquired cell counts were used for statistical analysis.

2.6 Cell proliferation

Thymidine incorporation assay was employed to measure the proliferation of cells as described in [170]. In brief, the cells were seeded on to 35-millimeter

culture plates, and, after 24 hours, transfected with the desired plasmid vectors. The cells were allowed to grow for 44 hours and then treated for four hours with ^{3}H-thymidine at the final concentration of 0.4 μCi/ml. Thereafter the degree of thymidine incorporation was assessed by a measure of radioactivity by employing a liquid scintillation counter (Wallac 1410).

2.7 Immunocytochemistry and confocal microscopy

To conduct confocal microscopy imaging experiments, standard protocols for sample preparation and for visualizing the proteins of interest were employed as described in publications 1-3 [116,170,171]. In brief, the cells were plated on to poly-L-lysine coated coverslips and thereafter transfected with the desired plasmid constructs or subjected to the desired treatment, respectively. To visualize the proteins of interest we employed plasmid constructs to express the respective proteins with a fluorescent tag and immunocytochemical approaches where desired primary and secondary fluorescent antibodies were used for protein detection. Thereafter, the samples were fixed, mounted and imaged using confocal microscopes at the Turku Bioscience Cell Imaging and Cytometry core facility.

2.8 Electron microscopy

Electron microscopy experiments were carried out as described in [171].

2.9 Cellular oxygen consumption measurements

A 96-well Agilent Seahorse XF Cell Mito Stress Test Kit assay was employed to measure the oxygen consumption of cells by following the protocol provided by the manufacturer and as described in [171].

2.10 Calpain activity assay

The calpain activity was measured as described in the manufacturers protocol and as indicated in [171].

2.11 Mitochondrial membrane potential measurements

The fluorescent probe TMRE (tetramethylrhodamine ethyl ester) is sensitive to the mitochondrial membrane potential [173], and the accumulation of TMRE in the mitochondrial matrix, as investigated by fluorometric analysis, was used as a measure of mitochondrial membrane potential. The experiments were conducted as described in [171]. Briefly, the cells were plated on to 24-well plates and treated with TMRE for 20 minutes to allow the positively charged TMRE to be accumulated in the mitochondrial matrix. The cells were thereafter thoroughly washed, and the fluorescence was measured by using a HIDEX Sense plate reader (HIDEX, Turku, Finland). For normalizing the results a colorimetric crystal violet assay was employed as a control for cell count per sample.

2.12 Measurement of IP_3 production

The IP_3 measurements were conducted by employing a HitHunter IP3 Fluorescence Polarization Assay Kit (DiscoverRx Tech, Fremont, CA, USA) as described in [170]. To induce IP_3 production in the cells through the activation of the G-protein coupled signaling pathway, histamine was used as an agonist.

2.13 Statistics

Statistical analysis was done by using Student's t-test (unpaired) when two means were compared or alternatively by using One-Way Anova and Tukey's post-hoc test when more than two means were compared. The GraphPad Prism program (Versions 5, 6 and 7; GraphPad Software Inc., San Diego, CA) was used for analyzing the data. P-values below 0.05 were considered as statistically significant.

3 HYPOTHESIS AND SPECIFIC AIMS

Hypotheses for this thesis were:

- Sphingosine kinase 1 modulates Ca^{2+} signaling in cancer cells in a compartment-specific manner through a presently unknown mechanism. Deciphering the molecular details underlying the compartment-specific Ca^{2+} signaling can and will be used to further characterize the importance of SK1 in cancer cell physiology in terms of increased cell migration and mitochondrial respiration.

- ORP5/8 regulate Ca^{2+} signaling through a presently unknown mechanism and altered Ca^{2+} signalling pathways are involved in ORP5/8-mediated changes in cancer cell physiology, such as increased proliferation.

The specific aims of this study were to:

- Elucidate the role of SK1 in cancer cell calcium dynamics in mitochondria, caveolae, ER and plasma membrane.

- Define the molecular basis for SK1-induced generation of calcium microdomains in cancer cell compartments

- Characterize the significance and mechanisms of ORP5/8 in regulating the Ca^{2+} signalling in cancer cell compartments, especially at the PM and caveolae as well as in the mitochondrial compartment

- Investigate whether SK1 and ORP5/8 regulate the migration, proliferation and energy metabolism of cancer cells through compartmentalized Ca^{2+} signaling

4 RESULTS AND DISCUSSION

4.1 Sphingosine kinase 1 and compartmentalized Ca^{2+} signaling

Recapitulating the above sections, SK1/S1P axis and Ca^{2+} signaling pathways are affecting multiple aspects of cell physiology and contributing to numerous disease conditions. Importantly, and directly relating to the work in this thesis, Ca^{2+} and SK1/S1P signaling pathways are closely linked and have been indicated to be inter-regulated e.g. through feedback mechanisms where intracellular Ca^{2+} may be involved in SK1 activation, leading to increased S1P production and export to the extracellular space [125]. In particular, S1P has been previously shown to regulate Ca^{2+} signaling through binding to the PM-residing G-protein-coupled S1P-recepors (thus triggering the downstream PLC and Ca^{2+} signaling events), as well as through yet unknown intracellular mechanisms [125,174]. Further, overexpression of the S1P-receptors 2 and 3 has been shown to augment cytoplasmic and mitochondrial Ca^{2+} upon S1P-stimulation and to lead to diminished basal ER Ca^{2+} storage capacity [175]. Importantly, signaling by the sphingolipid species, S1P in particular, has been shown to be linked to the various aspects regulating mitochondrial fission and fusion dynamics [176].

Taken together, these indications suggest an important role for SK1 in the regulation of intracellular Ca^{2+}.

However, to our knowledge, the effect of SK1 overexpression on Ca^{2+} regulation in cell compartments, namely at the PM, at the caveolar microdomain of the PM, in the mitochondrial matrix and in the ER lumen had not been previously specifically characterized.

In the publications 1 and 2 of this thesis, SK1 overexpression (and S1P signaling) were shown to regulate Ca^{2+} dynamics specifically at the caveolae, in the mitochondrial matrix and in the ER. These results are summarized and discussed below.

4.2 Sphingosine kinase 1 and the caveolar Ca^{2+} microdomain

It has been postulated that the caveolae may act as “signaling hubs”, or microdomains, where various signaling molecules and signaling events, including those related to Ca^{2+} signaling, converge [27,67,103]. However, the methods for investigating Ca^{2+} at the caveolar region of the PM, or in caveolar vesicles, have been limited to two fluorescence-based approaches reported by

Isshiki et al (ref. [114,115]), and to indirect methods [177,178]. In publication 1, we created, characterized and validated the novel caveolae-targeting Ca^{2+} reporter caveolin-1-aequorin (cav1-Aeq) that specifically reports the Ca^{2+} concentrations of this microdomain at the cytoplasmic side of the PM. Our results are in agreement with the previous findings by Isshiki et al [114] showing that caveolar compartments can function as sites for Ca^{2+} signaling events. By employing the cav1-Aeq, and the previously described aequorin probes targeting the PM and the cytosol [30], as well as by conducting measurements by using the cytoplasmic fluorescent Ca^{2+} indicator Fura-2-AM, we characterized the effect of SK1 expression on Ca^{2+} signaling at the PM, at the caveolae and in the cytosol of HeLa cervical cancer cells and ML1 thyroid cancer cells. We found that SK1 expression modulates Ca^{2+} signaling specifically at the caveolar compartment of the PM. These results are summarized and discussed in the sections 4.2.1 and 4.2.2.

4.2.1 Validation of the caveolae-targeting Ca^{2+} indicator, caveolin-1-aequorin

To validate the cav1-Aeq, we first showed that the cav1-Aeq localized similarly with the endogenous caveolin-1 to the detergent-resistant membrane fraction, whereas the PM-targeting SNAP25-tagged aequorin (SNAP25-Aeq) did not (publication 1; Fig 1A). Then, by employing immunofluorescence, we showed that, when overexpressed, the cav1-Aeq formed caveolae-resembling puncta whereas the SNAP25-aeq was evenly distributed throughout the PM (publication 1; Fig 1B, Suppl Fig 1). The cav1-Aeq reported significantly higher Ca^{2+} concentrations as compared to the SNAP25-Aeq upon histamine stimulation. Co-expression of caveolin-1 with the SNAP25-Aeq did not augment the Ca^{2+} concentrations reported by the SNAP25-Aeq, showing importantly that caveolin-1 expression per se was not causing the increase in histamine-induced Ca^{2+} flux (publication 1; Fig 1C, D). The membrane lipid cholesterol is enriched at the lipid raft / caveolar compartment of the PM and required for the formation of caveolae [103] Genetically tagged caveolin-1 may localize improperly [67]. To validate the PM localization of overexpressed caveolin-1, we treated the cells with methyl-β-cyclodextrin (MβCD) that depletes PM cholesterol and disrupts caveolae structures. In this condition, as evidenced by immunofluorescence, the caveolin-1-EGFP-puncta dispersed indicating that overexpressed caveolin-1 forms structures in the PM that resemble caveolae (publication 1; Fig 2A, B). The histamine-induced Ca^{2+} flux as reported by cav1-Aeq was significantly blunted by MβCD treatment whereas the PM Ca^{2+} was affected to a lesser extent. Cytoplasmic Ca^{2+} was not affected by MβCD (publication 1; Fig 2C). Further, caveolin-1-RFP-enriched structures were shown by TIRF microscopy to localize to close apposition with the ER tubules (publication 1; Fig 2D). Collectively these data established cav1-Aeq as novel Ca^{2+} indicator tool, reporting Ca^{2+} from the

caveolar sub-compartments of the PM. See Fig 7. for a schematic presentation of the different Ca^{2+} indicators used in the publications of this thesis.

4.2.2 Sphingosine kinase 1 overexpression increases Ca^{2+} at the caveolae

A biphasic experiment was conducted to study the effect of SK1 overexpression during histamine-induced Ca^{2+} release as well as during receptor-operated Ca^{2+} entry (ROCE) at the PM, at the caveola and in the cytosol. In the first phase of the experiment, the cells were challenged with 100 μM histamine in the presence of the extracellular Ca^{2+} chelator EGTA. In this condition, Ca^{2+} is released from the intracellular IP_3-sensitive stores without any Ca^{2+} entry from the extracellular space. In the second phase of the experiment, Ca^{2+} was added back to the extracellular milieu in the presence of the agonist histamine, thus inducing ROCE. We found that SK1 overexpression was without an effect on PM Ca^{2+} levels during both phases of the experiment (publication 1; Fig 3A-C). Cytoplasmic Ca^{2+} was slightly but significantly increased during ROCE when SK1 was overexpressed (publication 1; Fig 3D-F). Interestingly, SK1 overexpression led to a marked increase in caveolar Ca^{2+} during both phases of the experiment (publication 1; Fig 4). Thus, the SK1-induced large increase in Ca^{2+} influx during ROCE at the caveolar compartment was reflected as a small increase in the overall cytoplasmic Ca^{2+} levels, whereas the Ca^{2+} concentration at the immediate vicinity of the overall PM was not significantly affected.

Further, it was shown that blocking of the S1P-receptors 1-3 with antagonists did not reverse the effect of SK1 during the first phase of the experiment (ER Ca^{2+} release) but did block the SK1-induced increase in ROCE (publication 1; Fig 4). Treatment of the the wild-type cells with S1P, as compared to the control-treated cells, augmented ROCE, but not Ca^{2+} release, at the caveolae (publication 1; Fig 5A, B). Further, SK1 inhibition, which impairs S1P production and thus suppresses the auto/paracrine S1P signaling, led to diminished caveolar Ca^{2+} during ROCE. These results indicate that the S1P-receptors are involved in the regulation of ROCE at the caveolar sub-compartments of the PM. This is in line with previous findings that indicate S1P-receptor signaling to be coordinated at the caveolae [179,180]. However, because of the lack of specifically binding antibodies, we were unable to directly investigate the localization of S1P-receptors in this study. Future studies by employing for instance fluorescently tagged S1P-receptor constructs and/or specific anti-S1PR antibodies in conjunction with caveolae markers suited for super-resolution imaging would be warranted to be able to determine the possible caveolar localization of S1P receptors.

Interestingly, as mentioned above, the SK1-mediated increase in the histamine-induced Ca^{2+} release was insensitive to the S1P-receptor 1-3 agonist treatments,

indicating that in this condition, the extracellular stimulation of the S1P receptors by S1P might not play a role. The finding that the S1PR agonists were without an effect in the above condition may be, in part, explained by taking together several previous observations indicating possible mechanisms by which SK1 might regulate Ca^{2+} at the caveolar compartment during agonist stimulation without the involvement of PM-residing S1P receptors. For instance, it has been shown that SK1 translocates to the PM in a Ca^{2+}-dependent fashion, and, importantly, that SK1 may be targeted to the caveolin-rich lipid-raft microdomains of the PM [148,181], indicating that during histamine-induced Ca^{2+} signaling, SK1 might show activity at the caveolar region. In addition, PLC, IP_3-receptors and the ER membranes may be found in close apposition to the caveolin-rich PM domains, thus forming a possible hotspot for the generation of augmented Ca^{2+} signaling at this specific membrane contact site [21,27,182] where SK1 may also localize to. Importantly, S1P may directly induce Ca^{2+} release from the intracellular stores [182]. Further, the local Ca^{2+} concentrations at the immediate vicinity of the activated IP_3-receptors has been shown to transiently reach very high (100 micromolar) levels as compared to the resting cytoplasmic Ca^{2+} concentration, and, importantly the IP_3-receptors may transactivate adjacent IP_3-receptors through the so called Ca^{2+}-induced Ca^{2+}-release (CICR) [34].

Thus, it may be hypothesized that together these converging elements, including the possible S1P-induced Ca^{2+} release, the following CICR, and the possible localization of SK1 to the caveolae along with the previously indicated apposition of ER and IP_3 receptors to the caveolae, may contribute to the augmented caveolar Ca^{2+} during histamine stimulation which was observed in the SK1-overexpressing cells.

Further, during the writing of this thesis it was shown that, in smooth muscle cells, a T-type Ca^{2+} channel ($Ca_v3.2$) colocalizes with caveolin-1, and that caveolin-1 expression is needed for the $Ca_v3.2$-mediated Ca^{2+} influx to activate the SR/ER-residing RyR channels and the subsequent ER Ca^{2+} efflux. It was postulated that caveolae are required for the generation of a high enough $Ca_v3.2$-mediated Ca^{2+} microdomain positioned close to the ER to activate the RyRs by Ca^{2+} binding to these channels [183]. Interestingly, RyRs, that are predominantly expressed by muscle and excitable cells, have been shown to be expressed also in HeLa cells [184]. HeLa cells were used as the cell model in all of the publications in this thesis. Thus, it is an interesting possibility to consider that also RyRs might act e.g. as a boosting factor, for instance through increased CICR activation and increased ER Ca^{2+} efflux through these channels, and thus RyRs might in part contribute to the SK1-mediated increase in the local caveolar Ca^{2+} microdomain.

In addition, SK1-downregulation was shown to inhibit store-operated Ca^{2+} entry (SOCE) specifically at the caveolae, whereas SK1-overexpression was without an

effect in this condition (publication 1; Fig 7). This finding may be explained by the previous observations showing that inhibition of SK1 leads to accumulation of the kinase substrate, sphingosine, which in turn may block PM Ca^{2+} channels, including voltage-operated Ca^{2+} channels [185] and regulate processes that are involved in the regulation of SOCE e.g. by inhibiting the association of STIM1 and Ora1 and by inhibiting the store-operated Ca^{2+} current [186–188].

Interestingly, Ca^{2+} and caveolae, respectively, are important regulators of cytoskeletal components and focal adhesions contributing to cell migration in the context of tumor cells [61,103,157]. Therefore, it is worthwhile mentioning here that we later showed (publication 2; Fig 5D) SK1-overxpression to augment also mitochondrial matrix Ca^{2+} uptake and to increase cell migration. Thus, follow-up investigations focusing on the possible SK1/Ca^{2+}-mediated local cytoskeletal re-arrangements near the caveolar compartments would be interesting to conduct. In addition, the control of mitochondrial Ca^{2+} uptake has been shown to function as a regulatory mechanism in cytoskeletal rearrangement during cell migration [189] thus raising the possibility that our findings showing augmented mitochondrial matrix and caveolar Ca^{2+} concentration by SK1 overexpression may potentially be converging factors together contributing to the migratory potential of cells. The main findings and potential mechanisms of publication 1 are summarized in Fig 8.

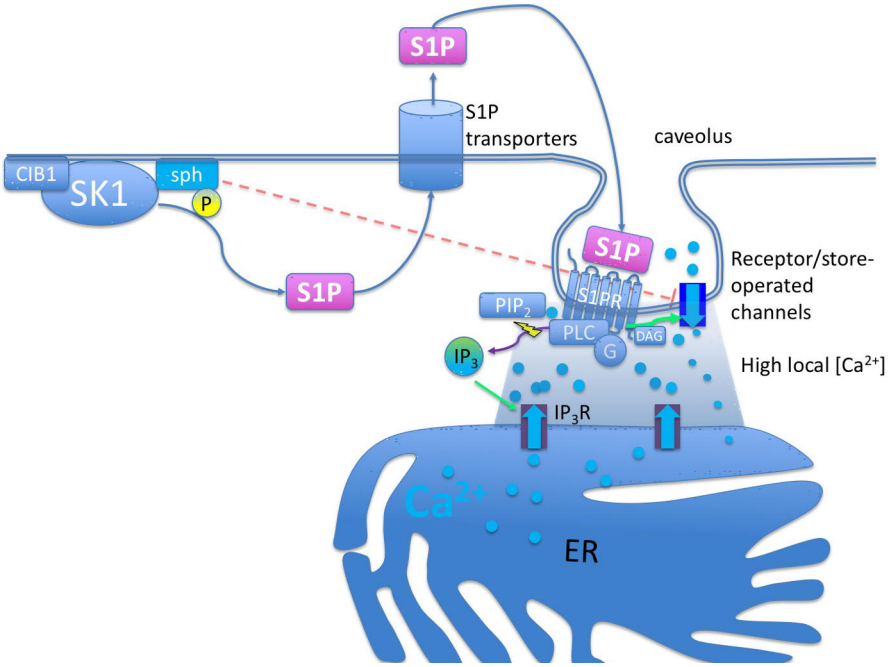

Figure 8. Graphical summary and a proposed model for the findings in publication 1. Sphingosine kinase 1 (SK1) overexpression augments Ca^{2+} signaling at the caveolar plasma membrane microdomain. The translocation of activated SK1 to the plasma membrane is facilitated by calcium and integrin-binding protein 1 (CIB1). SK1 phosphorylates (P) sphingosine to form sphingosine 1-phosphate (S1P), and S1P is transported from the cytoplasm to the extracellular space through S1P transporters for auto- or paracrine activation of the G-protein coupled S1P receptors (S1PRs). Downstream of the S1PR stimulation are the activation of the phospholipase C (PLC) and the activation of receptor-operated ion channels. Activated PLC cleaves phosphatidylinositol 4,5-bisphosphate to inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to the IP_3-receptor (IP_3R) Ca^{2+} channels, resulting in the release of luminal endoplasmic reticulum (ER) Ca^{2+} to the cytoplasm. SK1 overexpression results in the generation of high local Ca^{2+} concentration near to the caveolar sub-compartments of the plasma membrane.

4.3 Sphingosine kinase 1 modulates mitochondrial Ca^{2+}

Sphingolipid signaling and mitochondrial matrix Ca^{2+} regulation, respectively, have been identified to contribute to multiple converging cell physiological processes, such as the regulation of cell proliferation and migration, cellular energy metabolism and cell death [124,128,140,189,190]. In addition, the dynamic fission and fusion processes that the mitochondria and the mitochondrial network constantly undergo are in part regulated by sphingolipids and S1P [176]. The regulation of mitochondrial Ca^{2+} and the numerous downstream cell physiological and pathophysiological effects have

been widely studied [9,77,191–194]. However, information on SK1-mediated mitochondrial effects is limited. For example, sphingolipid metabolism has been found to be localized to the mitochondria, and sphingolipids are known to regulate mitochondrial functions such as mitophagy. In addition, increased mitochondrial ceramide and sphingosine are involved in the initiation of cell death [195]. On the other hand, inhibition of S1P production by a sphingosine kinase inhibitor compound disrupts mitochondrial respiration and ATP production, and promotes cell death in pancreatic β-cells [196]. SK2 has been shown to associate with the mitochondria. For instance, SK2-generated, mitochondrially produced S1P positively regulates mitochondrial respiration [197] and is involved in promoting cardiac cell survival [198]. Interestingly, according to the author's current knowledge, the sole evidence for SK1 to be localized to the mitochondria is from a very recent study (Kim & Sieburth, 2018, ref [199]), showing SK1 to regulate the mitochondrial unfolded protein response, and, intriguingly, indicating that SK1 overexpression induces mitochondrial fragmentation in the muscle cells of the nematode C. elegans. In the second publication of this thesis the role of SK1 as a regulator of IP_3-mediated release of endoplasmic reticulum lumen Ca^{2+} and, especially, as a regulator of mitochondrial matrix Ca^{2+} concentration, was characterized. The results are summarized and discussed in the sections below.

4.3.1 Overexpression of SK1 augments IP_3-mediated ER Ca^{2+} release and mitochondrial Ca^{2+} uptake

This project was initiated by the finding that when overexpressed in HeLa cells, SK1 led to increased mitochondrial matrix Ca^{2+} during histamine stimulation, both in the absence and presence of extracellular Ca^{2+} (publication 2; Fig 1B, C). It is important to note here that in the absence of extracellular Ca^{2+} the main source for cytoplasmic and mitochondrial Ca^{2+} upon IP_3-coupled histamine stimulation is the ER. Interestingly, overexpression of SK1 also induced an increase in the histamine-induced, IP_3-mediated release of ER Ca^{2+} in the absence of extracellular Ca^{2+} (publication 2; Fig 1A). The augmented ER Ca^{2+} release induced by SK1 overexpression was an intriguing observation, as our previous findings revealed cytoplasmic Ca^{2+} not to be affected by SK1-overexpression upon histamine-stimulation when extracellular Ca^{2+} was chelated (publication 1; Fig 3D). Together, these initial findings thus indicated strongly that an SK1-mediated organelle-level microdomain of Ca^{2+} might exist specifically in the ER lumen and in the mitochondrial matrix, and/or at the close contact sites between the organelles, the ER-mitochondria interface.

The following initial working hypotheses were formed and tested:

A) SK1 overexpression modulates the intrinsic mitochondrial uptake capacity through altering the MCU activity through one of the components of the MCU

complex, or alternatively through modulating NCLX protein expression or activity, leading to increased mitochondrial Ca^{2+} uptake. Mechanistically, a converging effect was also found to possibly augment the SK1-induced increase in mitochondrial Ca2+ uptake: it was further hypothesized that the increased mitochondrial Ca^{2+} buffering capacity might lead to decreased Ca^{2+}-mediated autoinhibition of the IP_3-receptors ([44,200]) through increased clearance of Ca^{2+} by mitochondria at the vicinity of the IP_3-receptors, further motivated by the previous findings showing mitochondria to effectively take up Ca^{2+} upon IP_3-receptor stimulation at the specific microdomain [201];

B) mitochondria-ER interactions, and hence the transfer of Ca^{2+} between the organelles, is altered, leading to the observed SK1-induced mitochondrial Ca^{2+} phenotype.

To test whether the intrinsic mitochondria Ca^{2+} uptake capacity was affected by SK1-overexpression, the mitochondrial Ca^{2+} uptake was studied in permeabilized cells. In this setup, the PM was rendered permeable to Ca^{2+} and other solutes by digitonin treatment. Then, the permeabilized cells were challenged with 4 μM free Ca^{2+} in an intracellular buffer, and mitochondrial Ca^{2+} uptake was monitored. In this condition, SK1-overexpression did not affect the mitochondrial Ca^{2+} uptake (publication 2; Fig 2A), indicating that the expression of the molecules directly involved in the mitochondrial Ca^{2+} handling might not be affected. Indeed, western blot experiments showed that none of the tested components of the MCU complex, nor the NCLX, showed altered expression upon SK1-overexpression (publication 2; Suppl. Fig 1).

Interestingly, when mitochondrial Ca^{2+} was monitored upon treating the (intact) cells in the absence of extracellular Ca^{2+} with the Ca^{2+} ionophore ionomycin, we found that SK1-overexpressing cells accumulated more Ca^{2+} in the mitochondria as compared to the mock-transduced control cells (publication 2; Fig 2B). Again, cytoplasmic Ca^{2+} was unaffected in the same experimental condition (publication 2; Fig 2D), indicating that the effect of SK1-overexpression was specific to mitochondria. The ionomycin stimulation experiment was repeated in a condition where the cells were briefly incubated with the cytoplasmic Ca^{2+} chelator BAPTA-AM. A short BAPTA-AM treatment has been previously shown to partially chelate cytoplasmic Ca^{2+} [202]. In this condition, the SK1-overexpressing cells showed a large mitochondrial Ca^{2+} peak, whereas mitochondrial Ca^{2+} uptake in the mock-cells was largely inhibited (publication 2; Fig 2C). Upon ionomycin treatment in these conditions, the main source for Ca^{2+} release is the ER, and BAPTA-AM rapidly chelates any free cytoplasmic Ca^{2+}. Thus, these results, and in particular the results derived from the BAPTA-AM experiment, indicated that SK1 overexpression might somehow augment the arrangement of the ER-mitochondria contacts and thus facilitate effective "Ca^{2+} shunting" between these organelles. Alternatively, or in addition to increased ER-mitochondrial contacts, the mitochondrial network integrity might have

been affected by SK1 overexpression. For instance, the mitochondrial protease AFG3L2 has been shown to modulate the mitochondrial dynamics, thus affecting the Ca^{2+} uptake capacity of the mitochondrial network as a whole [203,204]. However, AFG3L2 expression was not altered in SK1-overexpressing cells (publication 2; Suppl. Fig 1).

Mitofusin 2 (MFN2) is a key regulator of mitochondrial fusion and ER-mitochondria contacts with implications to mitochondrial Ca^{2+} regulation [205]. Hence, we investigated by western blotting whether MFN2 expression was altered by SK1 overexpression. Interestingly, we found that the full-length MFN2 protein band at the predicted 80 kilodalton mark was unchanged, but instead observed an additional, approximately 50 kilodalton band in the SK1-overexpressing cell lysates (publication 2; Fig 3A). Cleavage of MFN2 has been shown to be mediated by the protease calpain [206], and in agreement we observed increased calpain activity in SK1-overexpressing cells (publication 2; Fig 3B). Further, we conducted a calpain cleavage prediction analysis on the MFN2 sequence (www.calpain.org, based on ref [207]), and found that the most likely location for calpain-mediated cleavage would occur between histidine 467 and arginine 468 of the full-length MFN2 protein. The resulting N-terminal fragment resembles the approximately 50 kilodalton band observed in SK1-overexpressing cell lysates.

Further, we generated truncated N- and C-terminal MFN2 plasmid constructs resembling those that would result from the predicted calpain-mediated cleavage (publication 2; Fig 3C). When expressed in wild-type HeLa cells, these plasmid constructs induced an increase in mitochondrial Ca^{2+} concentration, closely resembling the results obtained from SK1-overexpressing HeLa cells (publication 2; Fig 3D). MFN2-derivated short peptides and N- or C terminal domain-truncated MFN2 have been shown to retain biological activity and to modulate mitochondrial function, such as respiration [208–210]. Calpain is found to be activated through the S1P-mediated pathways [211,212]. Hence, SK1-overexpression may through the activation of calpain and the resulting MFN2-fragments, modulate mitochondrial function and mitochondrial Ca^{2+} uptake.

Next, to gain further insight on the mitochondrial Ca^{2+} handling dynamics, we measured the mitochondrial Ca^{2+} concentration by employing an experimental protocol where cells were challenged with repeated and increasing agonist concentrations. We found that SK1-overexpression augmented mitochondrial Ca^{2+} uptake upon all agonist concentrations tested, and throughout the experiment (publication 2; Fig 4A). Also, the mitochondrial Ca^{2+} level remained at a higher level after agonist stimulation in the SK1-overexpressing cells as compared to the mock-transduced cells. This indicated that the Ca^{2+} extrusion kinetics might be affected by SK1-overexpression. As mentioned previously, the expression level of the protein NCLX that exchanges Ca^{2+} mitochondrial matrix

to Na^+ and hence regulates Ca^{2+} extrusion, was unchanged (publication 2; Suppl. Fig 1). Nevertheless, NCLX activity can be positively modulated e.g. by protein kinase A (PKA)- mediated phosphorylation [213]. Interestingly, we observed that pharmacological inhibition of the NCLX activity augmented the mitochondrial Ca^{2+} levels in mock-transduced cells, while having no effect on the SK1-overexpressing cells (publication 2; Fig 4B), bringing mock and hSK1 expressing cells' mitochondrial matrix Ca^{2+} concentration to a similar level in this condition. Thus, this experiment indicated that the basal activity of NCLX was likely inhibited in SK1-overexpressing cells, thus contributing to the increased mitochondrial matrix Ca^{2+} levels that were observed upon SK1 overexpression. We examined the possibility that NCLX activity would be inhibited by SK1-overexpression through the lack of PKA-mediated phosphorylation by treating the mock- and SK1-overexpressing cells with the PKA-activating drug, forskolin. We found that the forskolin-treatment was without an effect (data not shown). Nevertheless, it has been recently reported that MFN2 contributes to the modulation of NCLX activity [49]. Hence, it is possible that SK1-overexpression causes increased mitochondrial matrix Ca^{2+} uptake through a dual pathway where: 1) MFN2 fragmentation possibly modulates the local mitochondrial dynamics and the ER-mitochondria contact sites and the related molecular interactions related to Ca^{2+} transfer at these specific interfaces and/or 2) where MFN2 fragments through an unknown mechanism contribute to the inhibition of the NCLX, contributing to accumulated mitochondrial matrix Ca^{2+}.

As the SK1-induced MFN2 fragmentation indicated that the mitochondrial network and/or ER-mitochondria interactions would be altered, we conducted electron microscopy studies to look at these parameters. We found surprisingly that the mitochondrial surface area and the physical ER-mitochondria contacts, as visualized by electron microscopy, were not altered upon SK1-overexpession (publication 2; Fig 5A). Thus, this suggests that the MFN2 fragments might modulate local events at the ER-mitochondria interface, facilitating the mitochondrial Ca^{2+} uptake, without affecting the extent of direct physical contacts between the membranes of these organelles. Importantly, we found that the oxygen consumption in SK1-overexpressing cells was increased, as reported by a Seahorse respiration assay (publication 2: Fig 5B). This finding is in line with the various reports showing mitochondrial Ca^{2+} to augment respiration, and affecting the modulation of ATP production [9,73,214,215]. Further, we found that the SK1 overexpression was without an effect on cell proliferation, whereas migration was significantly augmented (publication 2; Fig 5C, D). The latter finding is thus in line with the previous studies linking mitochondrial Ca^{2+} handling to the migratory potential of cells [189,216]. Further, our previous findings have showed that SK1-overexpression augments Ca^{2+} at the caveolar microdomain (publication 1: Fig 4). Therefore, altered caveolar Ca^{2+} signaling might be a co-factor in the SK1-induced migration, as caveolae are important coordinators of Ca^{2+} signaling, also affecting cytoskeletal and focal adhesion remodeling as well as cell migration capacity [61,103,157].

4.3.2 Discussion and further implications for SK1-mediated effects on MFN2 and mitochondrial Ca^{2+}

Some intriguing novel data on the role of MFN2, and its various observed mutated forms, in disease and Ca^{2+} signaling have been published after the acceptance of publication 2, raising further possibilities to hypothesize on the underlying mechanisms for the observations regarding the role of SK1 and MFN2 fragmentation in mitochondrial Ca^{2+} handling.

For instance, very recent findings show that downregulation of MFN2 results in decreased cytoplasmic Ca^{2+} oscillation frequency in pre-osteoclast cells [217]. Interestingly, early on to our studies regarding SK1 and Ca^{2+} regulation, we observed reduced histamine-induced cytoplasmic Ca^{2+} oscillations in HeLa cells when SK1 was overexpressed (Pulli & Törnquist, unpublished data; Fig. 9). However, we were unable to elucidate the precise mechanism for the reduced amount of cytoplasmic Ca^{2+} oscillations at the time when these observations were made. The above novel results ([217]) may thus offer a possible mechanism in which SK1 overexpression, through modulation of MFN2, affects the cytoplasmic Ca^{2+} oscillatory pattern, further corroborated by the previous findings on the importance of mitochondria in cytoplasmic Ca^{2+} oscillation regulation [9,48,218]. Further, in regard to the methodology used in this study, that is, the luminescence measurements using aequorin-based organelle-targeted indicators, an interesting question remains to be answered. More specifically, data collection from the aequorin-based measurements is conducted from a cell population, i.e. a total luminescence of the whole cell population sample is recorded, as the low light output of aequorin does not allow for single-cell imaging experiments to be conducted, which is a limitation of the technique. Therefore, while being robust, reproducible and allowing for a high signal-to-noise ratio, data at the level of individual cells' mitochondrial Ca^{2+} handling cannot be produced with the aequorin-based method that was used in this study. As a result, the exact effect of the SK1 overexpression on the detailed (spatio)temporal dynamics of mitochondrial Ca^{2+} handling at the level of single cells, or mitochondrial populations or mitochondrial networks within a single cell, is not known. That is, from the mitochondrial aequorin measurements conducted in this study one cannot determine e.g.:

- whether the mitochondrial matrix Ca^{2+} oscillation pattern is affected by SK1 or;
- whether the effect on matrix Ca^{2+} level is a more temporally sustained one throughout the mitochondrial network (i.e. no oscillation, but a sustained increase);
- whether there are Ca^{2+} 'hotspots' within the mitochondrial network, for instance at the ER-mitochondria or PM-mitochondria contact sites that are specifically affected by SK1 overexpression.

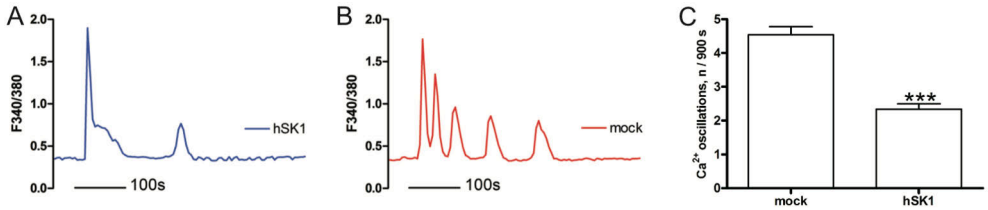

Figure 9. Sphingosine kinase 1 modulates histamine induced Ca^{2+} oscillations in HeLa cells. HeLa cells expressing the SK1 or the mock vector, respectively, were treated with 10 µM histamine to induce intracellular Ca^{2+} oscillations in the presence of 150 µM EGTA in the extracellular buffer. N=83 cells (mock), N=64 cells (hSK1); ***P=0.01, the bar diagrams show the average oscillation counts and the error bars show S.E.M.

Especially given the implications from the observed alterations in cytoplasmic Ca^{2+} oscillations upon SK1 overexpression, it would be of interest to employ methods that allow for fluorescent Ca^{2+} recordings from individual cells' mitochondria to investigate the mitochondrial matrix Ca^{2+} handling dynamics in more detail. In addition, these experiments could provide insight to any spatial alterations in the Ca^{2+} handling of the mitochondrial networks within individual cells that might take arise as a result of the SK1 overexpression.

Intriguingly, findings by Samanta et al [49] show that cytoplasmic Ca^{2+} oscillations are relayed to the mitochondrial matrix by NCLX reverse activity, and that this process is found to be modulated by MFN2. Previously, Hernández-SanMiguel and co-workers [219] have shown that inhibition of NCLX by the antagonist molecule CGP37157 results in a diminished cytoplasmic Ca^{2+} oscillation rate in HeLa cells. Very interestingly, in the same study ([219]) it is shown that NCLX-inhibition augments ER Ca^{2+} release upon histamine stimulation, closely resembling the findings in Fig. 1A in publication 2, and further, that mitochondrial Ca^{2+} uptake is augmented by CGP37157 treatment in a similar fashion as induced by SK1-overexpression (Fig. 1C, publication 2). Thus, these observations and previous results support the hypothesis that NCLX may be involved in the modulation of mitochondrial Ca^{2+} by SK1-overexpression as reported in publication 2.

In our study (publication 2; [171]), we observed that SK1 overexpression increases mitochondrial Ca^{2+} uptake and oxygen consumption, and, that, at the same time, the mitochondrial membrane potential was significantly reduced. These effects were in part attributed to the possible downstream effects induced by the observed fragmentation of MFN2 when SK1 was upregulated. However, the observation that mitochondrial Ca^{2+} is increased upon SK1 expression, while the membrane potential is reduced in the same condition, is not easily addressed, as mitochondrial membrane potential is considered to be the driving force for mitochondrial Ca^{2+} uptake [220,221]. Previous studies have shown that ablation

of MFN2 reduces mitochondrial respiration and membrane potential [222]. There is clear evidence showing that MFN2 knock-down increases the association of the mitochondria and ER membranes, contributing to increased susceptibility for the initiation of the Ca^{2+}-induced mitochondrial apoptotic pathway. In the same study, it was shown that MFN2 knock-down did not alter respiration or ATP production in mouse embryonic fibroblast cells [223]. Recently, it was shown in rat aortic muscle cells that MFN2 knock-down induces an increase in the distance between mitochondria and sarcoplasmic reticulum. Further, MFN2 knock-down was shown to reduce the rate of mitochondrial Ca^{2+} uptake and the rate of clearance of cytoplasmic Ca^{2+} upon agonist stimulation. In addition, it was shown that MFN2 knock-down caused a reduction in the mitochondrial membrane potential and cell proliferation, while the overexpression of MFN2 had the opposite effect to these parameters [224].

An interesting example of the complex nature of the regulation of mitochondrial function by MFN2 comes from a recent study by Wolf and co-workers ([225]). In this study, the authors show that a specific point mutation of MFN2, which is indicated in the progression of the Charcot-Marie-Tooth (CMT) disease subtype 2A2A, causes, to some extent, fragmentation of the mitochondrial network but is nevertheless without a phenotype with regard to mitochondrial respiration, membrane potential and ATP production in a basal condition. However, upon exposing the cells to mild oxidative stress, the expression of the specific point mutant of MFN2 shows increased mitochondrial respiration whereas ATP production is decreased in these cells [225].

In addition, the structural properties of MFN2 homodimerization and heterodimerization with MFN1, along with the properties regarding the fusogenic capacity of CMT2A-related MFN2 mutant proteins, was recently characterized [226]. Interestingly, MFN2 was found to form homodimers through the GTPase domain even after the hydrolysis of GTP, which was not the case for MFN1 dimerization. Further, Li et al [226] proposed two alternative models for a mechanism causing the CMT2A phenotype, depending on the effect of the specific mutation on the fusion and dimerization capacity of MFN2. The two models proposed by Li et al are; 1) a mutation completely inhibits MFN2 dimerization; or 2), a mutation allows for MFN2 dimerization but nevertheless results in diminished mitochondrial fusion. Effectively, both of the above alternative scenarios result in defects of mitochondrial function, causing CMT2A disease progression. The experimental data and the two models presented by Li et al are interesting as they show alternative pathways for how defects in MFN2 protein function and interaction may cause downstream effects affecting the mitochondria and downstream processes. In a similar fashion, the hypothesis presented in the publication 2 of this thesis, proposes a model where the SK1-induced MFN2 fragments may bind to and modulate the full-length MFN2 affecting function and protein interaction properties of MFN2, resulting in downstream mitochondrial effects. On this note, it would be extremely

interesting to characterize the tentative binding of the MFN2 fragments to the full-length MFN2 molecule, as postulated in publication 2 of this thesis, using similar approaches as e.g. in the publication by Li et al by employing protein binding-assays and purified proteins in vitro.

Another recent and very interesting study by Kuo et al showed that knocking down the protein polycystin 2 (PC2) resulted in increased MFN2 expression, leading to diminished cytoplasmic Ca^{2+} and increased mitochondrial Ca^{2+} concentration upon ATP stimulation in LLC-PK1 cells. The effect of PC2 knock-down, augmenting mitochondrial Ca^{2+}, was attributed to a proposed model where PC2 in a coordinated fashion with the IP-receptor 3 and MFN2 proteins regulates the Ca^{2+} fluxes at the MAM contacts [227]. Of note, mutations in the polycystin 1 and 2 proteins are implicated in the development of polycystic kidney disease [228]. Interestingly, Kuo et al also showed, very much similarly to what we show in Publication 2; Fig 7A, that the basal oxygen consumption was increased in the cells where mitochondrial Ca^{2+} uptake was augmented through the proposed PC2-MFN2-pathway. Further, it has been shown by employing a rat disease model for polycystic kidney disease, that S1P levels were accumulated in these animals, and that the immunomodulatory drug FTY720, by targeting S1P metabolism, alleviates disease phenotype [229]. With these implications, it might be of interest to further characterize whether there may be a common mechanism for kidney disease involving mitochondrial Ca^{2+} signaling, PC2 and sphingolipid metabolism [228,230]. Thus, multiple studies indicate an important role for MFN2 and its related regulatory pathways, such as Ca^{2+} and sphingolipid signaling, both upstream and downstream of the protein. It will be of utmost interest to conduct follow-up studies further elucidating the role of these processes in regulating mitochondrial function in health and disease. The main findings and a tentative mechanistic model of publication 2 are summarized in Fig. 10.

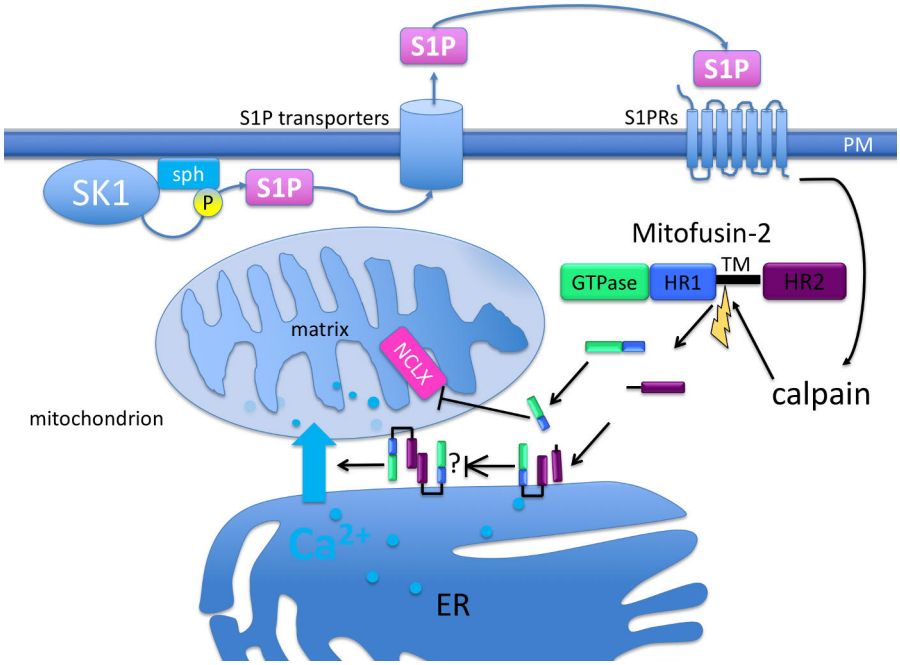

Figure 10. Graphical summary and a proposed model for the findings in publication 2. Sphingosine kinase 1 (SK1) phosphorylates (P) sphingosine to sphingosine 1-phosphate (S1P), which is transported to the extracellular space through S1P transporters. S1P binds to its G-protein coupled receptors (S1PRs) and, upon SK1-overexpression, calpain is activated. Mitofusin-2 (MFN2) is cleaved by calpain. The resulting MFN2 fragments may retain intact active domains, e.g. GTPase domain or coiled-coil heptad repeat (HR1) domain, that may bind to full-length MFN2, thus modulating the activity of the protein. Alternatively, or concomitantly, the cleavage of MFN2 may through an unknown mechanism inhibit the activity of the mitochondrial Na^+/Ca^{2+} exchanger, NCLX, blocking the mitochondrial Ca^{2+} extrusion, thus leading to accumulation of mitochondrial matrix Ca^{2+} as observed upon overexpression of SK1.

4.4 Summary on the SK1-mediated regulation of mitochondrial and caveolar Ca^{2+}

Taken together, we found that SK1-overexpression augmented significantly the mitochondrial Ca^{2+} uptake, the ER-calcium release, and the Ca^{2+} concentration at the local caveolar microdomain of the PM during agonist stimulation. The observed SK1-induced alterations in local Ca^{2+} handling were reflected in the form of altered cell physiological processes, such as augmented mitochondrial respiration and increased cell migration [116,171]. SK1 was thus characterized as a novel modulatory component of compartment-specific Ca^{2+} signaling in HeLa cells, with implications for downstream regulation of central

(patho)physiological cellular processes, providing additional insight to the complex nature of the interplay between sphingolipid and Ca^{2+} ion signaling [125].

4.5 ORP5/8 regulate Ca^{2+} in specific cell compartments

At the time of designing the ORP5/8 study of this thesis (publication 3), the information on ORP proteins' relevance for Ca^{2+} signaling was limited. To date, however, several studies have shown that multiple proteins of the ORP family are linked to, or may regulate, Ca^{2+} signaling events [170,231–235].

Of specific interest in the context of this study, and while forming the initial hypothesis, were the existing observations, also mentioned in the above introductory sections, that ORP 5/8 had been shown to localize to the MCS of the ER and the mitochondria, as well as to the ER-PM MCS, and that ORP5/8 had been shown to interact with phosphatidylinositol-4,5-bisphosphate (PIP_2) in the PM, along with the finding that ORP5/8 may modulate mitochondrial respiration and morphology [164].

Therefore, given that PIP_2 is the direct precursor of IP_3, the key second messenger responsible for Ca^{2+}-release from the intracellular Ca^{2+} stores [5,158,163,164,166] and that the MCS are emerging as important regulatory "hot-spots" of cell signalling events, including Ca^{2+} signalling [158] we asked whether the expression of ORP5/8 would play a role in modulating Ca^{2+} signalling at the specific MCS or cell compartments of mitochondria and caveolae, or in the cell cytoplasm in general.

4.5.1 ORP5/8 regulate Ca^{2+} in mitochondria and at the caveolae

Based on the indications above, we set out to study the possible role of ORP5/8 in modulating compartmentalized intracellular Ca^{2+}. We investigated whether ORP5 or ORP8 overexpression, respectively, would affect Ca^{2+} signalling specifically at the caveolae, in the mitochondrial matrix or in the overall cytoplasm. For this, we employed the genetically engineered aequorin Ca^{2+} probes to interrogate the mitochondrial matrix and caveolar Ca^{2+} concentrations.

Indeed, our results show that ORP5 overexpression leads to increased mitochondrial matrix Ca^{2+} during the histamine-induced GPCR-mediated Ca^{2+} flux activation in the presence of extracellular Ca^{2+} while overexpression of ORP8 in this condition did not affect the mitochondrial matrix Ca^{2+} concentration (publication 3; Fig 1A). To characterize further whether ORP8 overexpression might play a role in the regulation of the mitochondria matrix Ca^{2+} flux, we

employed a different experimental setting omitting extracellular Ca^{2+} by EGTA chelation. Interestingly, when the extracellular Ca^{2+} was chelated, and the source of Ca^{2+} thus were the intracellular stores, ORP8 overexpression indeed significantly increased mitochondrial Ca^{2+} uptake during histamine stimulation (publication 3; Fig 1B). Intriguingly, downregulation of ORP5 and 8, either respectively or while both proteins were downregulated at the same time, did not have any effect on the agonist-induced uptake of Ca^{2+} to the mitochondrial matrix (publication 3; Fig 2A). The overall cytoplasmic, histamine-induced Ca^{2+} flux was not affected by ORP5/8 overexpression (publication 3, Fig 1.).

In addition, we found that ORP5 and 8 overexpression, respectively, increased Ca^{2+} concentration at the caveolar subdomains of the PM during histamine stimulation. (publication 3, Fig 3).

Downregulation of either ORP5 or ORP8, or downregulation of both proteins at the same time, did not alter Ca^{2+} levels in the mitochondria or in the overall cytoplasm (publication 3, Fig 2).

Interestingly, ORP5/8 overexpression was found also to significantly increase cell proliferation (publication 3, Fig 6).

Of note, we employed a mutant ORP8 plasmid construct deficient of the pleckstrin homology (PH) domain that binds to the PIP_2 in the PM to study the importance of the PIP_2-binding capacity of ORP8 for Ca^{2+} regulation. Interestingly, and in contrast to the increased Ca^{2+} in caveolae upon overexpression of wild-type ORP8, the overexpression of the mutant ORP8 did not affect caveolar Ca^{2+} handling during histamine stimulation (Fig. 11, unpublished data, Olkkonen V, Törnquist K, Pulli I). This finding indicates that a functional PH domain for PIP_2 binding is a requirement for OPR8 to exert its modulatory function in regulating caveolar Ca^{2+}.

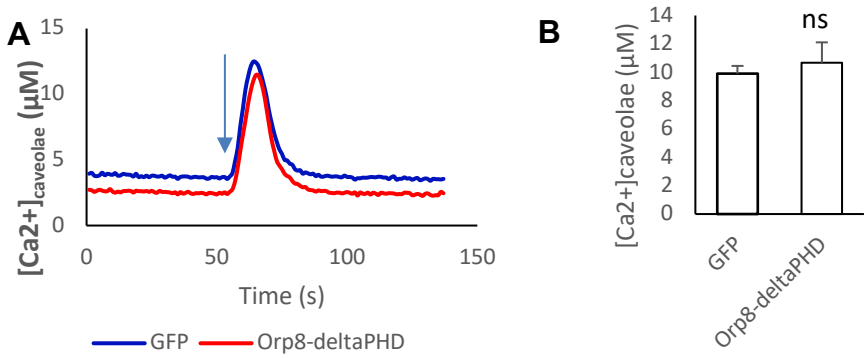

Figure 11. Transient expression of mutant ORP8, deficient of the pleckstrin homology domain (PHD) function (Orp8-deltaPHD), does not affect the histamine induced Ca^{2+} flux in the caveolar microdomain of HeLa cells as compared to the control cells (GFP). A) Representative traces of the experiment. B) The bar diagrams show the average with S.E.M. N=3, ns=not significant, blue arrow=100 µM histamine stimulation. The histamine stimulation was done in the presence of 150 µM EGTA and following the same protocol as described in Pulli *et al* 2018 for the same condition [170].

4.5.2 Implications for the ORP5/8 -mediated modulation of Ca^{2+} signaling events

Our results above show that ORP5/8 are involved in the regulation of Ca^{2+} signaling events at specific MCS sites, possibly through modulating the molecular or inter-organellear interactions at the ER-mitochondria and at the ER-caveolae domains. These findings have possible indications in the context of previous findings regarding ORP-proteins and, especially, ORP5/8. For instance, it has been found that ORP5/8 depletion causes aberrations in mitochondrial respiration [164]. This finding is interesting in the context of our findings, and may be reflecting the observed ORP5/8-induced increase in mitochondrial matrix Ca^{2+} concentration, as mitochondrial matrix Ca^{2+} concentration and also the physical apposition of mitochondria to the intracellular Ca^{2+} stores, have been characterized as key factors determining the activity of mitochondrial oxidative energy production processes [73,236,237].

Interestingly, previous results show that upregulation of another ORP-protein family member, ORP4L, facilitates PLCß3 activation and increases IP3 production in T-cell acute lymphoblastic leukemia cells, resulting in augmented cytoplasmic Ca^{2+} signals [238]. In line with this finding, we showed that ORP5 overexpression increased IP3 production upon PLC activation in histamine-stimulated HeLa cells, whereas overexpression of ORP8 was without an effect in this respect. However, in our study, we identified no significant alterations in the

histamine-induced cytoplasmic Ca^{2+} fluxes in ORP5/8 overexpressing HeLa cells. The lack of an effect in terms of global cytoplasmic Ca^{2+} handling may highlight the importance of ORP5/8 in the compartment-specific regulation of intracellular Ca^{2+} signalling at the caveolar and mitochondrial sites. The ORP5-induced increase in the observed local Ca^{2+} concentrations may in part be attributed to the increased IP_3 production, while the ORP8-induced increase in mitochondrial and caveolar Ca^{2+} is likely due to other factors.

In addition, and again in contrast to our findings showing that ORP5/8 overexpression increased HeLa cell proliferation, ORP8 overexpression has been shown to inhibit cell growth in non-small cell lung cancer cell lines. In these cells, the basal ORP8 expression was found to be low, while overexpression of ORP8 resulted in the release of cytochrome c from the mitochondria, leading to apoptotic cell death [239]. In this study, Li et al did not however investigate whether ORP8 overexpression would modulate Ca^{2+} levels in the mitochondria of the tested cell lines. As the mitochondrial apoptotic pathway resulting from the release of cytochrome c may be initiated by Ca^{2+} overloading in the mitochondrial matrix [240], and as Ca^{2+} regulation at the (ER-)mitochondria compartment may be of importance in terms of oncogenesis, apoptosis and emerging chemotherapy approaches [241], it would be interesting to investigate whether mitochondrial Ca^{2+} is a factor in the ORP8-mediated apoptotic process in non-small lung cancer cells.

In a similar fashion, low basal ORP8 expression was found in hepatocellular cancer cells, while overexpression of ORP8 resulted in a marked ER stress response, followed by apoptosis through the Fas/Fas ligand -mediated apoptotic pathway [242]. As a Ca^{2+} is a factor in ER stress, linking ER stress also to mitochondrial Ca^{2+} overloading and apoptosis [9], it would be intriguing to investigate whether Ca^{2+}-regulated cell stress and apoptosis pathways would function as a co-factor in the ORP8-mediated cell death in hepatocellular cancer cells observed by Zhong et al [242].

In summary, the novel observations presented in this thesis regarding the ORP5/8-mediated modulation of compartment-specific Ca^{2+} signaling events may have intriguing implications for the ORP5/8 proteins in the regulation of a wide range of cell physiological processes such as mitochondrial respiration, cell proliferation, oncogenesis and cell death. The main results and a potential model for the findings in publication 3 are summarized in Fig 12.

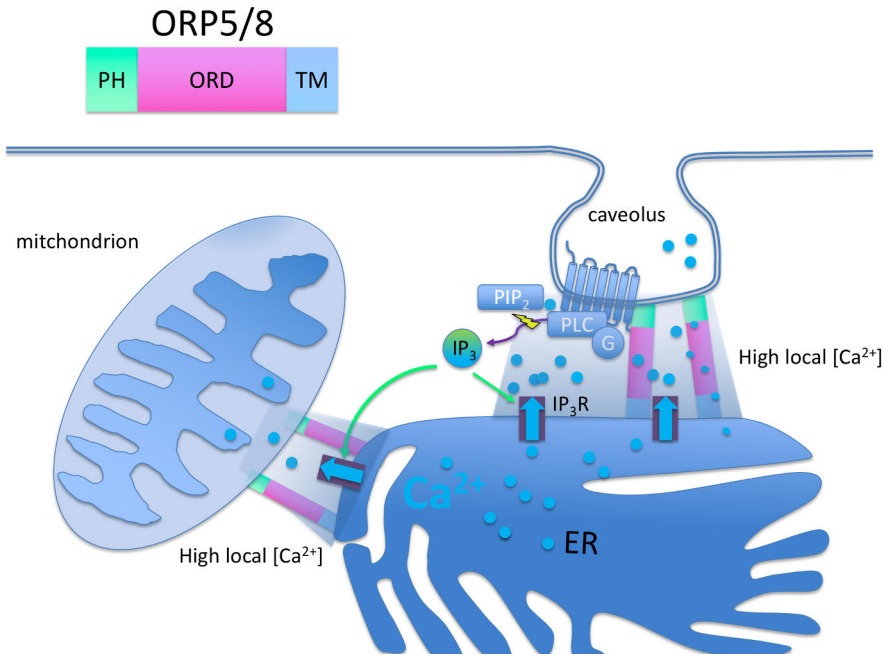

Figure 12. Graphical summary and a proposed model for the findings in publication 3. Oxysterol-binding protein-related proteins 5 and 8 (ORP5/8) localize to the membrane contact sites between the endoplasmic reticulum (ER) and mitochondria, and ER and the plasma membrane (PM), respectively. Overexpression of ORP5 or 8 augments mitochondrial Ca^{2+} uptake, as well as increases the Ca^{2+} concentration at the caveolin-rich PM sub-compartments during G-protein coupled receptor stimulation.

5 CONCLUSIONS

The main findings of this thesis are:

1. Sphingosine kinase 1 (SK1) regulates cellular Ca^{2+} signaling in a compartment-specific manner
2. In terms of Ca^{2+} signaling, overexpression of SK1 in HeLa cells leads to:
    a. increased Ca^{2+} at the caveolar sub-compartment of the plasma membrane upon agonist-induced Ca^{2+} release from the intracellular stores and during receptor-operate Ca^{2+} entry (summarized in Fig. 8)
    b. increased mitochondrial matrix Ca^{2+} during agonist-induced Ca^{2+} release (summarized in Fig. 10)
3. SK1 overexpression augments cell migration and cell respiration, possibly through the altered Ca^{2+} handling in the specific cell compartments
4. ORP5/8 overexpression regulates Ca^{2+} in the mitochondrial matrix and at the caveolar compartment (summarized in Fig. 12)
5. ORP5/8 overexpression augments cell proliferation, possibly through the altered Ca^{2+} handling in the specific cell compartments

Taken together, by employing a novel aequorin-based Ca^{2+} indicator targeted to the caveolae, and using SK1 overexpression in HeLa cells as a cell physiological model, the work in this thesis provides new insight for Ca^{2+} regulation in this specific caveolar compartment of the PM. In addition, this thesis provides indications for a tentative pathway by which SK1 overexpression may through calpain-mediated modulation of MFN2, and possibly through modulation of NCLX activity, regulate mitochondrial matrix Ca^{2+} level and cellular respiration. In addition, the findings in this thesis corroborate the relevance of ORP5/8 as regulators of cellular events at membrane contact sites, and, specifically, as a novel finding, provides data indicating that ORP5/8 participate in the regulation of caveolar and mitochondrial matrix Ca^{2+}.

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APPENDIX: ORIGINAL PUBLICATIONS

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A novel chimeric aequorin fused with caveolin-1 reveals a sphingosine kinase 1-regulated Ca^{2+} microdomain in the caveolar compartment☆

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ABSTRACT

Caveolae are plasma membrane invaginations enriched in sterols and sphingolipids. Sphingosine kinase 1 (SK1) is an oncogenic protein that converts sphingosine to sphingosine 1-phosphate (S1P), which is a messenger molecule involved in calcium signaling. Caveolae contain calcium responsive proteins, but the effects of SK1 or S1P on caveolar calcium signaling have not been investigated. We generated a Caveolin-1–Aequorin fusion protein (Cav1–Aeq) that can be employed for monitoring the local calcium concentration at the caveolae ($[Ca^{2+}]$cav). In HeLa cells, Cav1–Aeq reported different $[Ca^{2+}]$ as compared to the plasma membrane $[Ca^{2+}]$ in general (reported by SNAP25–Aeq) or as compared to the cytosolic $[Ca^{2+}]$ (reported by cyt-Aeq). The Ca^{2+} signals detected by Cav1–Aeq were significantly attenuated when the caveolar structures were disrupted by methyl-β-cyclodextrin, suggesting that the caveolae are specific targets for Ca^{2+} signaling. HeLa cells overexpressing SK1 showed increased $[Ca^{2+}]$cav during histamine-induced Ca^{2+} mobilization in the absence of extracellular Ca^{2+} as well as during receptor-operated Ca^{2+} entry (ROCE). The SK1-induced increase in $[Ca^{2+}]$cav during ROCE was reverted by S1P receptor antagonists. In accordance, pharmacologic inhibition of SK1 reduced the $[Ca^{2+}]$cav during ROCE. S1P treatment stimulated the $[Ca^{2+}]$cav upon ROCE. The Ca^{2+} responses at the plasma membrane in general were not affected by SK1 expression. In summary, our results show that SK1/S1P-signaling regulates Ca^{2+} signals at the caveolae. This article is part of a Special Issue entitled: 13th European Symposium on Calcium.

1. Introduction

Sphingosine kinase 1 (SK1) is an oncogenic protein that converts sphingosine to the lipid messenger molecule sphingosine-1-phosphate (S1P). Sphingosine and its metabolic precursor ceramide are considered to be pro-apoptotic lipids whereas S1P is involved in promoting cell survival. SK1/S1P signaling acts through direct intracellular mechanisms as well as through extracellular stimulation of specific G-protein coupled S1P receptors (S1PR1-5), some of which are known to couple to phospholipase C (PLC) [1,2]. Activation of PLC results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into two central Ca^{2+} signaling molecules, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3).

Members of the transient receptor potential canonical channels (TRPC1-7) are activated upon DAG-binding [3], thus facilitating receptor-operated Ca^{2+} entry (ROCE) and S1P has been shown to be involved in this process [3,4]. In accordance, S1P treatment activates TRPC2 in rat thyroid FRTL-5 cells [5]. IP3 is a major regulator of the intracellular endoplasmic reticulum (ER) Ca^{2+} store and acts by ligating the IP3 receptor (IP3R) Ca^{2+} channels in the ER membrane [6]. There is also evidence for S1P to directly mediate the release of Ca^{2+} from the intracellular stores [7]. However, the role of SK1 and S1P in regulating Ca^{2+} signaling in specific cellular sub-compartments is poorly characterized.

Since the development of organelle-specific Ca^{2+} reporters, it has become increasingly evident that highly localized Ca^{2+} signals regulate various cell physiological phenomena. For instance, recent reports have highlighted the importance of mitochondrial Ca^{2+} handling in multiple (patho)physiological situations, and the long-sought molecular identity of the mitochondrial Ca^{2+} uniporter (MCU) complex is now emerging [8–11]. Also, the importance of the interplay between the major Ca^{2+} store, the endoplasmic reticulum (ER), and the mitochondria is being characterized [12,13]. Furthermore, the lysosomal compartments are involved in cellular Ca^{2+} handling [14].

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In addition to the Ca^{2+} domains in membrane enclosed organelles, local micro domains of cytosolic Ca^{2+} play a role in cell physiological events. Such Ca^{2+} micro domains may for instance arise near channels and pumps involved in Ca^{2+} handling near the plasma membrane (PM) or the ER [15,16]. Many components of the cellular Ca^{2+} handling reside in the PM. These include the plasma membrane Ca^{2+} ATPases (PMCAs), ion channels of the transient receptor potential (TRP) family, and the Orai channels with functions ranging from maintaining the Ca^{2+} homeostasis to regulation of cell proliferation and migration. PM and ER have been shown to interact to regulate Ca^{2+} fluxes. For instance, the stromal interaction molecules (STIMs) that reside in the ER and sense the luminal Ca^{2+} concentrations of the organelle can couple to the Orai and TRP canonical (TRPC) channels to activate store-operated calcium entry (SOCE) [17,18].

To organize cellular signaling, the lipid and protein composition of the PM is compartmentalized through cytoskeletal and integral membrane protein scaffolding [19]. Caveolae are invaginated cholesterol-rich compartments of the PM which are involved in facilitating and organizing cellular signals. Caveolin proteins (caveolin1–3) are the main structural components of caveolae, caveolin-1 being the most abundantly expressed isoform in most tissues. Caveolae have numerous proposed functions including the regulation of local Ca^{2+} signaling as well as contact coordination at the PM, ER and mitochondrial interfaces. Multiple molecules that are related to Ca^{2+} signaling have been shown to localize to caveolae, including G-protein coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), ion channels (e.g. TRPC1 and 4) as well as PIP2, PLC and IP3R. Also scaffolding functions for caveolin-1 mediating the activity of TRPC1 upon SOCE have been proposed [20–22].

Depending on the cellular context, SK1 may translocate to the caveolin-1 enriched domains in PM upon activation [23]. This PM translocation of SK1 is in part mediated by Ca^{2+} [24] and may facilitate S1P secretion to the cell exterior, leading to autocrine S1P signaling [5, 24–26]. Since caveolae are versatile Ca^{2+} signaling domains with implicated functions for all of the major cellular Ca^{2+} handling mechanisms (i.e. ER Ca^{2+}-release through the IP3R channels and Ca^{2+}-uptake through ROCE and SOCE), we wanted to investigate whether SK1-mediated signaling would act locally at the caveolae to coordinate some of these central Ca^{2+} signaling events.

Experimental methods for measuring Ca^{2+} at the caveolae are relatively limited. Ca^{2+} signaling molecules interacting with caveolae-associated structures are to some extent characterized, but experimental data for specific caveolar Ca^{2+} fluxes is scarce. To our knowledge, two approaches to investigate Ca^{2+} signaling related to caveolae have been reported. By employing targeted fluorescent Ca^{2+} reporters and FRET, Isshiki et al. [27,28] have shown that caveolae are the preferred plasma membrane domains for SOCE, and that the internalized caveolin-enclosed vesicles may act as sealed compartments that are able to release Ca^{2+} in an IP3R-dependent manner.

Here, we characterize a novel chimeric Ca^{2+} reporter protein comprising of the Asp119Ala-mutated aequorin [29] and caveolin-1 (designated here as Cav1–Aeq) that specifically reports Ca^{2+} signals from a caveolar microdomain. These Ca^{2+} signals are registered at the cytoplasmic subcellular domain near to the caveolae and are distinct from the Ca^{2+} signals from the overall near PM Ca^{2+} compartment. By employing this approach, we show novel roles for the oncogenic protein SK1 in controlling the caveolar Ca^{2+} microdomain upon IP3R stimulation, ROCE and SOCE.

2. Materials and methods

2.1. Cell culture, transduction and transfection

HeLa cells were cultured in DMEM (#D5546, Sigma-Aldrich) supplemented with 10% FBS, 1% penicillin–streptomycin and 1% L-glutamine (Life Technologies) at 37 °C and 5% CO_2. The ML1 thyroid cancer cells that were used in a supplementary experiment were grown in the same medium as described here for HeLa cells. To overexpress SK1, we used a previously described lentiviral expression vector [5]. Transduction with the SK1 overexpression vector was performed by incubating the cells with polybrene (8 mg/ml) along with the viral particles at multiplicity of infection 10 for 6 h. Control cells were created by mock-vector transduction. The lentiviral particles for SK1 shRNA (sc-156038-V), as well as the control vectors (sc-108080), were acquired from Santa Cruz Biotechnology and the cells were transduced by incubating the cells for 12 h with polybrene (8 mg/ml) along with the lentiviral particles at multiplicity of infection 30. After 24 h the cells were subjected to continuous selection with 0.5 mg/ml puromycin (Life Technologies). Plasmid transfection mixtures were prepared by using TurboFect transfection reagent (#R0531, Thermo Scientific), OptiMEM media (#31985-070, Life Technologies) and 1 μg/ml plasmid (final concentration). The transfections were conducted in the same cell culture conditions as described above.

2.2. Construction of the caveolin-1–aequorin plasmid

For measurements in the caveolar compartment we created a plasmid chimera with the sequences for caveolin-1 and the previously described Asp119Ala-mutated aequorin (mut-Aeq) [29]. Among other targeting sequences (see Materials and methods, Section 2.6 for additional references), this mut-Aeq has been previously characterized for the plasma membrane targeting SNAP25–Aeq chimera [29]. The Cav1–Aeq was generated by amplifying Cav-1 using the primers (5-CGGGGT ACCATGTCTGGGGGAAATAC-3; forward, 5-GGCGAATTCTATTTCTTTCT GCAAGTTGAT-3; reverse). The PCR fragment was subcloned into a pSC-A vector. The fragment coding Cav-1 was excised by KpnI and EcoRI digestion, and the fragment encoding Cav-1 was ligated into pcDNA3 to generate pcDNA3-Cav1. The mut-Aeq fragment was cut from a pSC-A plasmid using EcoRI, and ligated into the pcDNA3-Cav1, to generate the pcDNA3-Cav1–Aeq encoding a chimeric protein with the N-terminus of mut-Aeq fused to the C-terminus of caveolin-1. N-terminal modifications to the mut-Aeq have been shown not to affect the luminescence production of the chimeric proteins [30].

2.3. Separation of detergent resistant membrane fractions on Optiprep gradient

All steps were performed at +4 °C. Cells were washed 2× in PBS, harvested and pelleted by centrifugation for 5 min at 2500 rpm. The cells were homogenized in 250 μl TNE buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Triton-X 100, 10% sucrose, 1 mM DTT and protease inhibitor cocktail). The lysate was blended with 480 μl 60% Optiprep and mixed. The lysate was transferred to an ultracentrifuge tube, and TNE buffer with decreasing percent Optiprep were layered on top. The samples were centrifuged for 4 h, 40,000 rpm using a Beckman Coulter centrifuge with a SW 60Ti rotor after which the fractions were collected in Eppendorf tubes.

2.4. Western blot

For western blotting, the cells were grown to 70% confluence, followed by three washes with phosphate buffered saline (PBS) on ice. The cells were then lysed by addition of 3x Laemmli sample buffer (LSB), where after the lysates were boiled for 5 min. Then, the proteins were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were then blocked with 5% milk in TBS (Tris-buffered saline, 150 mM NaCl, 20 mM Tris base, pH 7.5) with 0.1% Tween 20 (Sigma-Aldrich) and incubated overnight with the desired primary antibody at +4 °C. The primary antibodies were diluted 1:1000 in PBS containing 0.5% bovine serum albumin (BSA). Then, the membranes were washed and incubated with the secondary, horseradish peroxidase (HRP) conjugated antibodies (1:3000 dilution in

TBST + 5% milk). The proteins were then detected by chemiluminescence using Western Lightning Plus-ECL substrate (Perkin Elmer). The sphingosine kinase 1 antibody was from Cell Signaling and the HSC70 antibody was from Enzo life sciences. The caveolin-1 antibody was from Santa Cruz and the anti-HA antibody from Sigma-Aldrich.

2.5. Immunocytochemistry and microscopy

For immunocytochemistry, the cells were grown on poly-L-lysine coated coverslips and, when targeted fluorescent plasmids were used, transfected with the desired plasmid constructs. Then, the cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 15 min, followed by three washes and a 5-minute treatment with 1% Triton-X in PBS. Next, the cells were treated with 1% BSA in PBS for 1 h followed by three washes in PBS. Then, the cells were treated overnight at +4 °C with the desired primary antibody. After this, the cells were washed, treated with the secondary antibodies and mounted using Mowiol mounting medium. The primary antibodies used were caveolin-1 (#sc-894, Santa Cruz) and anti-HA (#H9658, Sigma-Aldrich). The AlexaFluor secondary antibodies were from Life Technologies. The ER-GFP was described before in [31] and the plasmids encoding RFP and EGFP tagged CAV1 were from Addgene (plasmids #14434 and #27704, deposited by Ari Helenius, described in [32]). TIRF microscopy was performed with a Nikon Eclipse Ti-E microscope using a 100×/1.49 NA oil immersion objective. ER-GFP and Cav1-RFP were excited by the 488 nm and 561 nm laser lines, respectively. The calculated imaging depth was ~100 nm, and the microscope was controlled with NIS-Elements Advanced Research 3.1 (Nikon). Widefield microscopy was done using the same microscope with epifluorescence settings and a 40×/0.75 objective. Confocal microscopy was done on a Leica TCS SP2 attached to a DM RXA2 microscope using a 63×/0.90 NA water immersion objective.

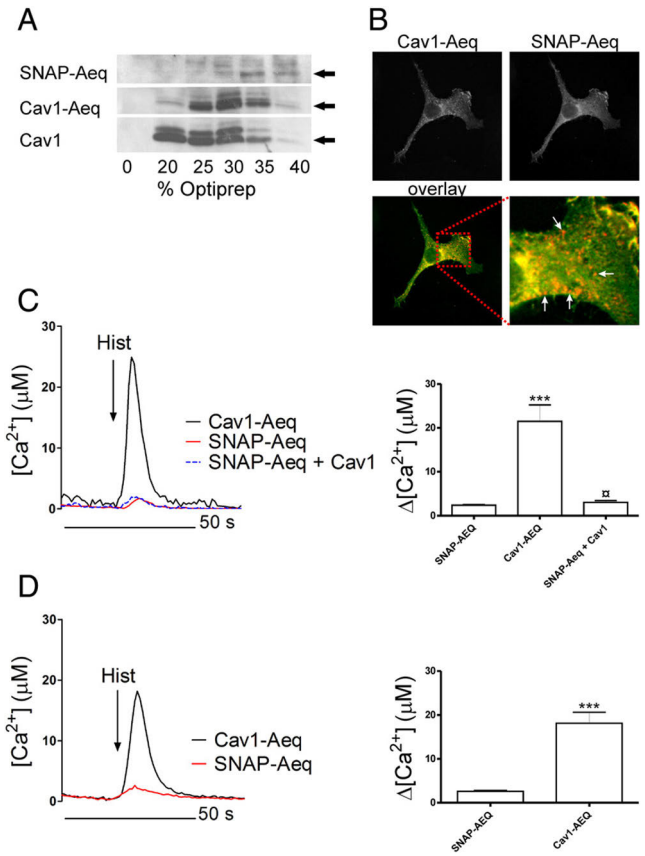

Fig. 1. Caveolae sense Ca^{2+}-signals differently from the plasma membrane. (A) HeLa cells overexpressing Caveolin-1–Aequorin (Cav1–Aeq) and SNAP25–Aequorin (SNAP–Aeq) were harvested and detergent resistant and soluble fractions were separated on an Optiprep gradient by ultracentrifugation. The gradient fractions were collected and the detergent resistance of the indicated proteins was addressed by western blotting. (B) Confocal images showing the distribution of Cav1–Aeq (pseudo color red, indicated by white arrows in the magnification) versus SNAP–AEQ (pseudo color green). (C) HeLa cells transfected with Cav1–Aeq, SNAP–Aeq or SNAP–Aeq + Cav1–EGFP were stimulated with 100 μM histamine in Ca^{2+}-free conditions. Representative traces are shown. The bar diagrams show the average change in $[Ca^{2+}]$. The error bar depicts S.E.M., n = 4–20, ***$P < 0.001$ (SNAP–AEQ compared to Cav1–AEQ), ¤$P < 0.01$ (Cav1–AEQ compared to SNAP–Aeq + Cav1) (D) HeLa cells were stimulated with 100 μM histamine in Ca^{2+}-containing (1 mM) conditions. Representative traces are shown. The bar diagrams show the average change in $[Ca^{2+}]$. The error bar depicts S.E.M., n = 12–13, ***$P < 0.001$.

2.6. Aequorin-based Ca^{2+} measurements

Measurements of subplasmalemmal [Ca^{2+}] and cytosolic [Ca^{2+}] were conducted by employing the SNAP25-fused aequorin or the non-targeted aequorin (cyt-Aeq), respectively, as described in [29]. Cav1–Aeq was employed for the measurements of [Ca^{2+}]cav. The general experimental setup was as described in [33]. Briefly, one hundred thousand cells per well were seeded on 12-well plates containing poly-L-lysine (Sigma-Aldrich) coated round 13 mm coverslips. The following day, the cells were transfected with the desired plasmid constructs as described in Section 2.1. Twenty-four hours after transfection the cells were washed three times with Ca^{2+} free HEPES-buffered saline solution (HBSS: 118 mM NaCl, 4.6 mM KCl, 10 mM glucose and 20 mM HEPES) supplemented with 150 μM EGTA. Thereafter, the cells were incubated for 1 h in HBSS containing 150 μM EGTA and 5 μM wild-type coelenterazine (#s053, Synchem). To record the luminescence the cells were transferred to a purpose-built chamber and perfused with the desired solutions at 37 °C. The sampling rate of the luminescence recordings was set to one measurement per second. The intracellular Ca^{2+} stores were replenished by incubating the cells in HBSS containing 1 mM Ca^{2+} for 2–3 min before starting the experimental treatments. The maximal luminescence of each sample was obtained by permeating the cells with 100 μM digitonin (Sigma-Aldrich) in HBSS containing 10 mM Ca^{2+}. The results were then calibrated using the calibration values for Asp119Ala-mutated aequorins as described in [29,34–39]. Histamine was from Sigma-Aldrich, the sphingosine kinase inhibitor

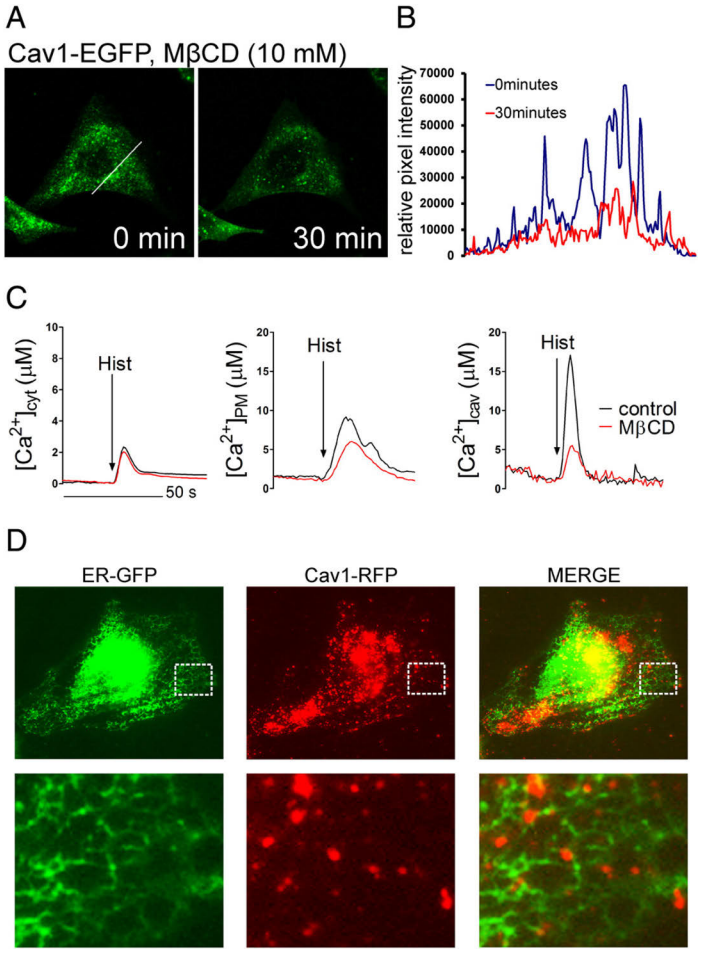

Fig. 2. Disrupting caveolae by depleting plasma membrane cholesterol leads to an attenuation of the Ca^{2+}-signal sensed by Cav1–Aeq. (A) Hela cells overexpressing Cav1–EGFP were imaged by confocal microscopy before and after treatment with 10 mM MβCD for 30 min. (B) Cav1–EGFP intensity along the line displayed in (A), before and after MβCD treatment. (C) HeLa cells transfected with cytosolic Aeq (cyt-Aeq), SNAP25–Aeq or cav1–Aeq were treated with or without 10 mM MβCD for 30 min, and Ca^{2+} transients at the bulk cytosol, at the plasma membrane (PM) or at the caveolae, respectively, were measured upon stimulation with 100 μM Histamine in the presence of 1 mM Ca^{2+}. Representative traces of 4–10 separate experiments are shown. Please note the different scale in cytosolic measurements. (D) TIRF microscopy pictures of HeLa cells expressing Cav1–RFP and ER-GFP. The lower panels are magnifications from the area within the rectangles (dotted line) in the upper panels.

was from Calbiochem (#567731), JTE013 was from Tocris (#2391) and VPC23019 was from Avanti Polar Lipids (#857360P). Sphingosine 1-phosphate (S1P) was from Enzo Life Sciences. JTE013 and sphingosine kinase inhibitor were dissolved in DMSO. VPC23019 was dissolved according to the compound datasheet (95 parts of 3% fatty acid free bovine serum albumin and 5 parts acidified DMSO). S1P was dissolved in HBSS buffer containing 4 mg/mL fatty acid free bovine serum albumin. The solvents served as controls.

2.7. Ca^{2+} measurements using Fura-2 AM

For Ca^{2+} experiments where Fura-2 AM (Life Technologies) was employed, the cells were grown on 25 mm poly-L-lysine coated coverslips, washed three times in HBSS and incubated for 30 min with 2 μM Fura-2 AM at room temperature (RT). Then the cells were washed and incubated for 15 min at RT. The coverslips were then transferred to a Sykes-Moore gasket which was mounted on a custom-made holder for perfusions. The excitation filters were set to 340 and 380 nanometers (nm), respectively, and the emission was recorded at 510 nm. The filters were controlled by a Lambda 10-2 device (Sutter Instruments, USA) and the excitation light was produced by an XBO 75 W/2 xenon lamp. To collect the images, we used an inverted Zeiss Axiovert 35 microscope with a Hamamatsu ORCA2 camera. The recordings were operated with an Axon Imaging Workbench software (version 6.0, Axon Instruments, USA). The recording frequency was set to one image per second and the fluorescence ratio (F_{340}/F_{380}) at each measured time point was used to evaluate the changes in cytosolic Ca^{2+}.

2.8. Statistics

Statistical analysis of the data was conducted by unpaired Student's t-test when two variables were compared or by One-Way Anova with Tukey's post-hoc test when three or more variables were compared. P-values below 0.05 were considered as statistically significant. The results are shown with S.E.M.

3. Results

3.1. Caveolin-1-aequorin chimera reports Ca^{2+} signals specifically from the caveolar microdomain

Caveolae are rich in sphingolipids and cholesterol and form distinct "raft-like" domains in the PM. The caveolae are associated with proteins central to Ca^{2+} signaling cascades [40–43] and thus likely play an important role in coordinating Ca^{2+} signaling events. In order to study Ca^{2+} signaling in caveolae, we generated a novel chimeric protein of caveolin-1 and aequorin (Cav1–Aeq). First, we validated the correct subcellular localization of Cav1–Aeq and its usefulness as reporter of caveolar Ca^{2+} signals in HeLa cells.

Cav1–Aeq partitioned similarly to endogenous caveolin-1 to the detergent resistant membrane fractions on an Optiprep gradient, while the general PM reporter SNAP–Aeq was found mainly in the soluble fraction (Fig. 1A). Immunofluorescence staining showed that Cav1–Aeq separated into small domains on the cell surface (Fig. 1B) in a similar fashion as Cav1-RFP (Supplementary Fig. 1), while SNAP–Aeq was distributed uniformly on the PM (Fig. 1B), as reported previously [34]. The caveolar Ca^{2+} responses ($[Ca^{2+}]$cav) reported by Cav1–Aeq differed substantially from the Ca^{2+} responses in the PM ($[Ca^{2+}]$PM) reported by SNAP25–aequorin chimera (SNAP–Aeq). When intracellular Ca^{2+} stores were mobilized by histamine stimulation in Ca^{2+} free medium, the peak of $[Ca^{2+}]$cav was higher than $[Ca^{2+}]$PM. Importantly, $[Ca^{2+}]$PM was not affected by caveolin-1 expression (Fig. 1C). Also, $[Ca^{2+}]$cav was higher than $[Ca^{2+}]$PM upon histamine stimulation in a Ca^{2+}-containing buffer (Fig. 1D).

To further verify that Cav1–Aeq reports Ca^{2+} transients in caveolar domains, we disrupted the caveolar structures by removing PM cholesterol using methyl-β-cyclodextrin (MβCD) (Fig. 2A, B). The MβCD treatment did not affect the cytosolic Ca^{2+} response upon histamine treatment suggesting that MβCD does not interfere with the mobilization of intracellular Ca^{2+} stores (Fig. 2C, left panel, the average $\Delta[Ca^{2+}] \pm$ S.E.M. were 2.24 ± 0.14 (control) and 1.84 ± 0.11 (MβCD), $P > 0.05$). The $[Ca^{2+}]$PM was reduced by approximately 40% (Fig. 2C, middle panel, the average $\Delta[Ca^{2+}] \pm$ S.E.M. were 8.5 ± 0.6 (control) and 5.1 ± 0.27 (MβCD), $P < 0.01$) which may be due to the cholesterol-dependence of SNAP-25 for effective PM localization [44]. However, the Ca^{2+} transient reported by the dispersed Cav1–Aeq in MβCD treated cells was blunted by approximately 80% (Fig. 2C, right panel, the average $\Delta[Ca^{2+}] \pm$ S.E.M. were 14.3 ± 1.9 (control) and 2.7 ± 1.1 (MβCD), $P < 0.001$), indicating that the Ca^{2+} signaling is

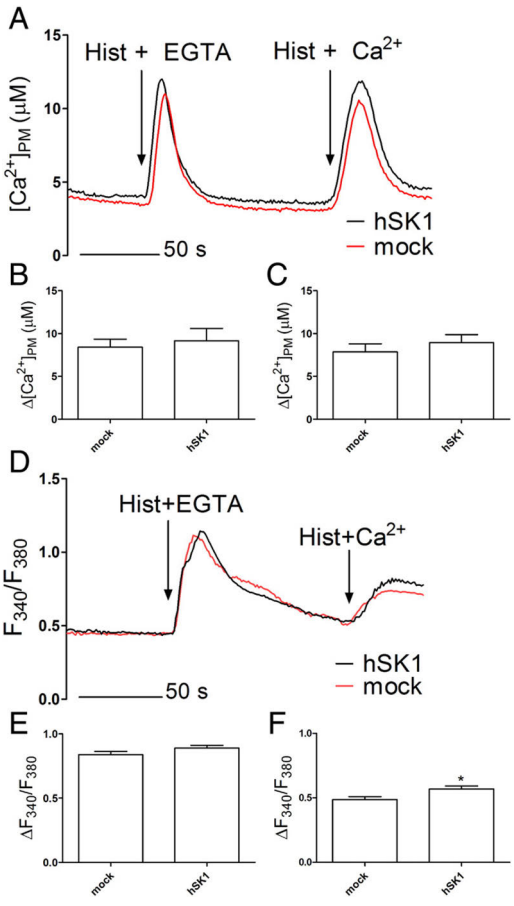

Fig. 3. The effect of SK1-overexpression on $[Ca^{2+}]$ at the plasma membrane as reported by SNAP25–aequorin (SNAP–Aeq) and in the cytosol as reported by Fura-2 AM. (A) Representative traces showing $[Ca^{2+}]$PM during histamine (100 μM) stimulation in presence of EGTA (150 μM), and Ca^{2+} (1 mM) re-addition (ROCE). Bar diagrams show the mean with S.E.M. indicating the change in $[Ca^{2+}]$PM during histamine-stimulation (B), and during Ca^{2+} re-addition (C), n = 5. (D) Average traces showing the Fura-2 AM fluorescence ratio (F_{340}/F_{380}) upon the same experimental conditions as in (A). The bar diagrams show the mean with S.E.M. indicating the change in the fluorescence ratio ($\Delta F_{340}/F_{380}$) during histamine stimulation in the presence of EGTA (E) and during Ca^{2+} re-addition (F), n = 6, *$P < 0.05$.

directed towards properly assembled caveolae and not the caveolin-1 protein by itself.

In HeLa cells, histamine is known to mobilize Ca^{2+} from the ER by activating the PLC/IP3/IP3R pathway [45]. Interestingly, TIRF microscopy showed that ER tubules in close proximity to the PM tend to associate with caveolae (Fig. 2D). These results establish that Cav1–Aeq is a useful probe for measuring caveolar Ca^{2+} transients. Moreover, the histamine-induced Ca^{2+} mobilization seems to be concentrated in the vicinity of caveolae.

3.2. Sphingosine kinase 1 regulates $[Ca^{2+}]$ specifically at caveolar microdomains

As caveolae are enriched in sphingomyelin [46] and have been reported to associate with receptors for the sphingomyelin metabolite S1P [47], we next utilized the Cav1–Aeq probe to test whether sphingosine kinase 1/S1P induced Ca^{2+} signaling is integrated at caveolae.

To investigate the importance of SK1 in HeLa cells, we created cell lines where SK1 is either overexpressed (hSK1 cells) or downregulated (shSK1 cells) with the respective control cell lines (mock and shC cells). The SK1 protein levels were approximately 4-fold higher in the hSK1 as compared to the mock control cells (Supplementary Fig. 2). Since caveolae are rich in sphingolipids and caveolae have shown to contain multiple components of the Ca^{2+} signaling machinery, we wanted to investigate whether SK1 could be involved in Ca^{2+} signaling locally at the caveolar sub-compartment of the PM. To test this hypothesis, we employed Cav1–Aeq reporter and compared the caveolar Ca^{2+} signals to the overall PM Ca^{2+} signals by utilizing the previously characterized SNAP–Aeq [29].

First, we performed experiments where the cells were stimulated with 100 μM histamine in presence of 150 μM EGTA, which was followed by Ca^{2+} 1 mM re-addition (induction of ROCE). During the both phases of this experiment, the $[Ca^{2+}]$PM response remained unchanged upon SK1 overexpression, compared with control cells (Fig. 3A, B, C). The cytosolic Ca^{2+} responses ($[Ca^{2+}]_i$), as reported by the Ca^{2+} indicator Fura-2 AM, were unaffected by SK1 overexpression during 100 μM histamine stimulation in the presence of 150 μM EGTA (Fig. 3D & E) and slightly but significantly increased upon ROCE (Fig. 3D & F). However, we found that $[Ca^{2+}]$cav was increased by SK1 overexpression during both phases of the experiment, as compared with control cells. Furthermore, the SK1-induced increase in $[Ca^{2+}]$cav during ROCE was sensitive to the S1PR inhibitors JTE013 (10 μM) and VPC23019 (1 μM) (Fig. 4). Interestingly, stable SK1 overexpression in ML1 thyroid cancer cells showed increased $[Ca^{2+}]$cav during 40 μM ATP stimulation in the presence of 150 μM EGTA but not during the 1 mM Ca^{2+} re-addition (Supplementary Fig. 3). Also, please note the effect of the control solutions (see Materials and methods, Section 2.6) on $[Ca^{2+}]$cav as evidenced by comparison of Fig. 4 and Supplementary Fig. 4.

To study the possibility that extracellular S1P regulates the caveolar Ca^{2+} microdomain, we acutely treated HeLa cells with 700 nM S1P and conducted caveolar Ca^{2+} measurements with the same experimental protocol as in Fig. 3. Interestingly, we found that acute S1P treatment was without an effect during the histamine-induced Ca^{2+}-release from the intracellular Ca^{2+} stores (Fig. 5A), whereas upon ROCE the uptake of Ca^{2+} via caveolae was augmented by S1P (Fig. 5A & B). We next asked whether pharmacological inhibition of SK1 might have an effect. For this purpose, we treated HeLa cells with a SK inhibitor (SKi; 10 μM for 1 h), followed by the same experimental procedures as in Fig. 3. SKi, or the vehicle DMSO, were present throughout the whole experiment. This experiment showed that SKi blocked the Ca^{2+}-uptake during ROCE (Fig. 5C & D), whereas Ca^{2+}-release upon histamine

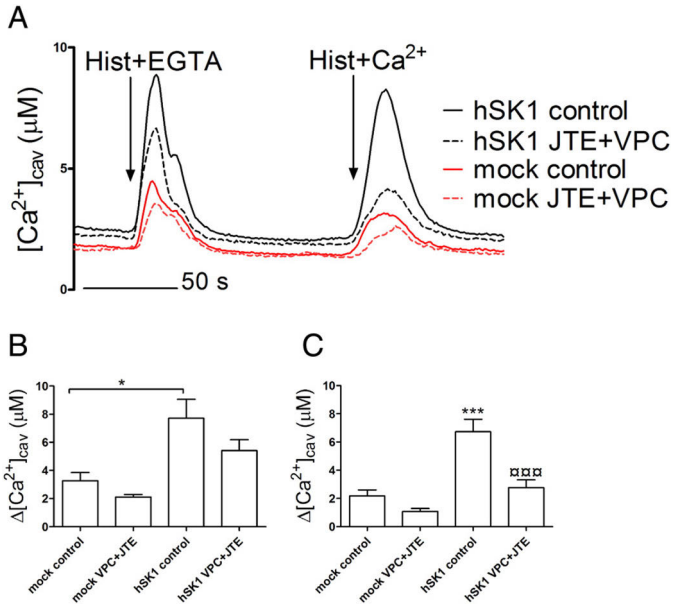

Fig. 4. Overexpression of SK1 induces an increase in $[Ca^{2+}]$cav during histamine-stimulated release of endoplasmic Ca^{2+}, as well as during receptor-operated Ca^{2+} entry (ROCE). The cells were pretreated for 1 h with either 1 μM VPC and 10 μM JTE in combination, or with the control solutions. Following the pre-incubation, the compounds were present throughout the experiments. A) Representative traces of experiments showing $[Ca^{2+}]$cav during histamine (100 μM) stimulation in presence of EGTA (150 μM), and Ca^{2+} (1 mM) re-addition (ROCE). B) Quantification of the change in $[Ca^{2+}]$ at the caveolae during histamine-induced Ca^{2+} mobilization in the absence of extracellular Ca^{2+}. C) Quantification of the change in $[Ca^{2+}]$cav during Ca^{2+} re-addition in the presence of histamine. The bar diagrams show the mean with S.E.M., n = 7–10, *P < 0.05, ***P < 0.001 (mock control vs hSK1 control), ¤¤ ¤P < 0.001 (hSK1 control vs hSK1 VPC + JTE).

stimulation in extracellular Ca^{2+} free condition was not significantly changed (Fig. 5C). The $[Ca^{2+}]$PM was unaffected by S1P treatment whereas the SKi treatment diminished the Ca^{2+} during ROCE (Supplementary Fig. 5).

To indirectly assess whether SK1 induces the increase in $[Ca^{2+}]$cav through increased physical interactions between the ER and caveolae, we performed an experiment where the ER Ca^{2+} is passively released by ionomycin (5 μM) treatment in the absence of extracellular Ca^{2+}. In this case, SK1 did not affect $[Ca^{2+}]$cav (Fig. 6) indicating that the physical ER-caveolae contact sites remain unaltered upon SK1 overexpression.

Sphingosine, the substrate for SK1, is known to block the interactions between STIM1 and Orai1 at the cell membrane, thus attenuating store-operated Ca^{2+} entry in the cells [48]. Therefore, we performed thapsigargin (TG, 1 μM) induced store-operated Ca^{2+} entry (SOCE) experiments. Overexpression of SK1 did not affect the $[Ca^{2+}]$ at the caveolae or at the PM during SOCE (Fig. 7), whereas cells with SK1 downregulation (Supplementary Fig. 2) displayed slightly reduced $[Ca^{2+}]$cav upon SOCE, leaving the $[Ca^{2+}]$PM fluxes unchanged (Fig. 7). Cellular sphingosine has been shown to accumulate upon SK1 downregulation [49]. Therefore, we find it possible that sphingosine levels are increased in our shSK1 HeLa cells, which might facilitate the sphingosine-induced inhibition of SOC channels, and reduced SOCE at the caveolae.

4. Discussion

Caveolin-1 expression has been associated to regulation of cancer progression in a multifaceted and context-dependent manner. Caveolin-1 has both anti-tumorigenic as well as cancer-promoting qualities [20,50, 51]. In this work, we describe a novel probe for investigating Ca^{2+} signaling at the cytoplasmic face of the caveolae. Further, we establish that with our approach it is possible to detect truly distinguishable Ca^{2+} microdomains at the caveolae that functionally differ from the overall PM Ca^{2+} domain (Figs. 1 & 2).

By comparing overall PM Ca^{2+} levels to those reported by Cav1–Aeq we show that specific Ca^{2+} signaling events are taking place locally at the caveolae upon altered expression levels of the oncogenic protein SK1. These findings represent interesting examples for the local Ca^{2+} microdomain signaling that has been proposed to be coordinated at the caveolae or directly controlled via scaffolding functions of caveolin-1.

Deranged regulation of Ca^{2+} handling is an emerging concept in cancer biology affecting many aspects of cancer cell physiology such as proliferation, cellular migration and deregulated apoptotic pathways. These physiological processes are in part regulated by intricate Ca^{2+} signaling events that are controlled by a multitude of specialized Ca^{2+} channels, Ca^{2+} pumps and exchangers, Ca^{2+} binding proteins, as well as cellular organelles and compartments [52,53]. In this context, our findings on the SK1-mediated caveolar Ca^{2+} microdomain may have several functional implications.

As we demonstrate here, overexpression of SK1 induces an increase in $[Ca^{2+}]$cav upon the histamine-stimulated, IP3-receptor mediated Ca^{2+}-release when extracellular Ca^{2+} is chelated, while the $[Ca^{2+}]$PM remains unaltered. This effect of SK1 was not blocked by S1PR1-3 antagonism indicating that autocrine S1P signaling might not play a major role here.

However, IP3Rs and ER may be found in close association to caveolae [21], and S1P has been shown to directly release Ca^{2+} from the ER [54]. Also, SK1 is known to be activated and translocated in a Ca^{2+}-dependent manner [24] and Serine225 phosphorylation of SK1 guides the activated kinase to caveolin-1 enriched lipid raft domains of the PM [23]. Thus, it may be feasible to motivate this SK1-induced increase in $[Ca^{2+}]$cav by a Ca^{2+}-activated local S1P production. Further, local Ca^{2+}-induced Ca^{2+} release (CICR) [6] might play a role in amplifying the SK1-induced $[Ca^{2+}]$cav.

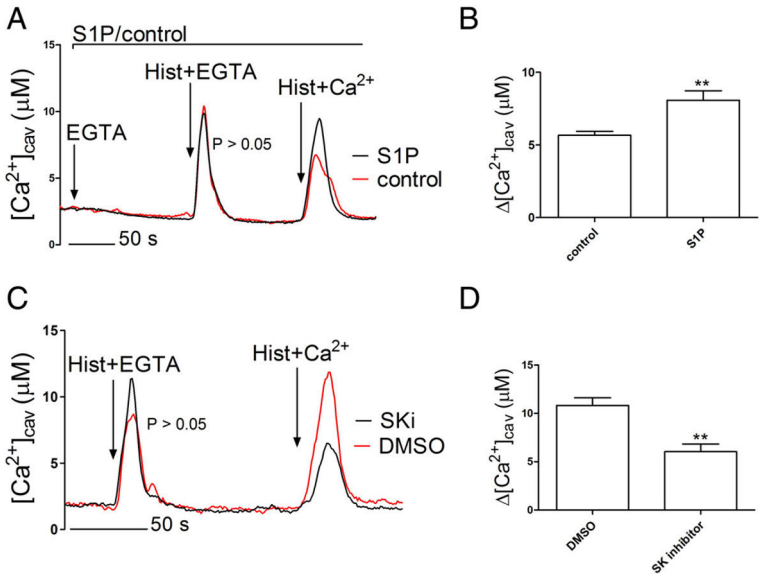

Fig. 5. Sphingosine 1-phosphate (S1P) treatment increases Ca^{2+} at the caveolar compartment during receptor-operated Ca^{2+} entry whereas SK inhibitor (SKi) blocks ROCE. A) Representative traces of an experiment where the cells were acutely treated with 700 nM S1P as indicated in the figure, followed by consecutive perfusions with histamine (150 μM) in the presence of 150 μM EGTA and 1 mM Ca^{2+}, respectively. B) Quantification of the Ca^{2+} addition in (A), showing the mean with S.E.M., $^{**}p < 0.01$, $n = 5$. C) Representative traces from an experiment where the cells were pre-treated for 60 min with 10 μM SKi and stimulated with histamine in the presence of 150 μM EGTA, followed by 1 mM Ca^{2+} re-addition. SKi or DMSO was present throughout the whole experiment. D) Quantification of the Ca^{2+} re-addition in (C) showing the mean with S.E.M., $^{**}p < 0.01$, $n = 5$ for DMSO, $n = 7$ for SK inhibitor.

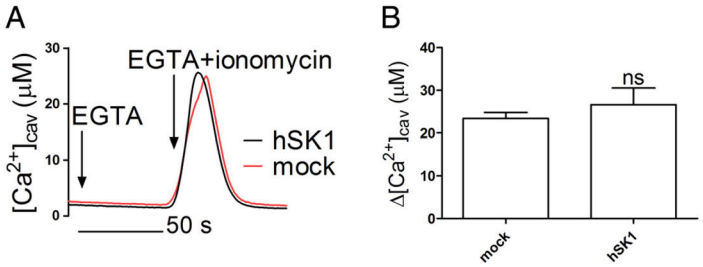

Fig. 6. Ionomycin treatment in presence of EGTA does not show a SK1-induced caveolar microdomain. The cells were perfused with HBSS containing 150 μM EGTA followed by perfusion with EGTA and 5 μM ionomycin. Representative traces (A) and bar diagrams indicating the mean with S.E.M. (B) are shown, n = 3, ns = not significant.

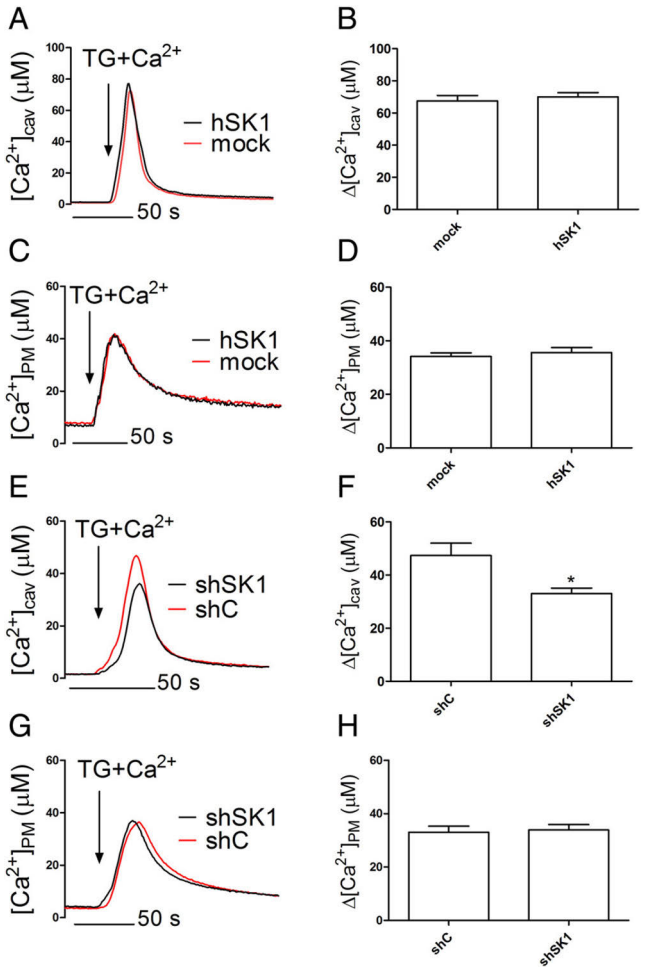

Fig. 7. Store-operated Ca^{2+} entry (SOCE) at the caveolae and at the plasma membrane upon SK1 overexpression and downregulation, respectively. The cells were pre-treated with 1 μM thapsigargin (TG) in HBSS containing EGTA (150 μM) for 4 min. Then, the cells were perfused with 1 mM Ca^{2+} in the presence of 1 μM TG. SOCE was not affected by SK1 overexpression at the caveolae (A, B) nor at the PM (C, D), whereas knock-down of SK1 resulted in diminished SOCE specifically at the caveolae (E, F) but not at the overall PM (G, H). The bar diagrams indicate the means with S.E.M., n = 3–6, *P < 0.05.

Also, we show that caveolar ROCE is increased upon SK1 overexpression and this effect of SK1 is reversed by antagonizing the S1PR1-3. Further, we demonstrate that S1P treatment mimics SK1 overexpression by increasing $[Ca^{2+}]$cav upon ROCE, whereas SK1 inhibition attenuates ROCE in the caveolar domain. It has been shown that S1PR1 is localized to the caveolar fraction [47] and as noted above, SK1 translocates to caveolin-1 enriched lipid rafts upon activation. The increased ROCE through caveolae could thus be explained by autocrine S1P signaling, where S1P receptor activation leads to generation of DAG to promote ROC channel opening [55] upon histamine stimulation locally at the caveolae.

Interestingly, SK1 seems to affect Ca^{2+} mobilization and ROCE via two partially independent signaling pathways. The SK1 effect on ROCE is highly dependent on S1PR1-3 and can be reproduced by acute addition of S1P and antagonized by SK1-inhibition. In contrast, the effect of SK1 on histamine-induced Ca^{2+} mobilization is largely independent of S1PR1-3 and is not significantly affected by short term treatment with S1P or by acute inhibition of SK1. These results suggest that SK1 affects Ca^{2+} mobilization by a process requiring long term adaptation of the cells, while ROCE can be regulated on short notice.

Further, our results may have implications for the oncogenic actions of SK1. As SK1 activity and translocation are controlled by Ca^{2+}, it is possible that aberrant SK1 overexpression could lead to deranged amplification of other cellular Ca^{2+} signals through over-activated autocrine or intracellular S1P signaling. This would, in turn, lead to aberrant activation of ROCE or increased local Ca^{2+} release from the intracellular stores. SK1 induced increase in $[Ca^{2+}]$cav, during both IP3-receptor stimulation and ROCE, might then have importance for oncogenic processes such as deregulated cytoskeletal and focal adhesion assembly, leading to altered migratory capacity of the cells [53].

Taken together, we describe here a novel tool for Ca^{2+} measurement that reports Ca^{2+} concentrations from the caveolar domain of the PM. By employing this method, we show for the first time the importance of SK1 for regulating local Ca^{2+} signaling at this PM microdomain.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.04.005.

Disclosure of conflicts of interest

The authors declare that there are no conflicts of interest.

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Sphingosine kinase 1 overexpression induces MFN2 fragmentation and alters mitochondrial matrix Ca^{2+} handling in HeLa cells

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ABSTRACT

Sphingosine kinase 1 (SK1) converts sphingosine to the bioactive lipid sphingosine 1-phosphate (S1P). S1P binds to G-protein-coupled receptors ($S1PR_{1-5}$) to regulate cellular events, including Ca^{2+} signaling. The SK1/S1P axis and Ca^{2+} signaling both play important roles in health and disease. In this respect, Ca^{2+} microdomains at the mitochondria-associated endoplasmic reticulum (ER) membranes (MAMs) are of importance in oncogenesis. Mitofusin 2 (MFN2) modulates ER-mitochondria contacts, and dysregulation of MFN2 is associated with malignancies. We show that overexpression of SK1 augments agonist-induced Ca^{2+} release from the ER resulting in increased mitochondrial matrix Ca^{2+}. Also, overexpression of SK1 induces MFN2 fragmentation, likely through increased calpain activity. Further, expressing putative calpain-cleaved MFN2 N- and C-terminal fragments increases mitochondrial matrix Ca^{2+} during agonist stimulation, mimicking the SK1 overexpression in cells. Moreover, SK1 overexpression enhances cellular respiration and cell migration. Thus, SK1 regulates MFN2 fragmentation resulting in increased mitochondrial Ca^{2+} and downstream cellular effects.

1. Introduction

Sphingosine kinase 1 (SK1) is a lipid kinase involved in multiple pathologies, including cancer and severe neurological conditions [1,2]. SK1 acts by converting sphingosine into the signaling lipid sphingosine 1-phosphate (S1P), which is exported to the extracellular space for auto/paracrine stimulation of the G-protein coupled S1P receptors 1–5 ($S1PR_{1-5}$). S1P has been shown to have direct intracellular effects, including modulation of intracellular calcium (Ca^{2+}) signaling. Also, there is evidence for the inter-regulation of SK1/S1P and Ca^{2+} signaling, as SK1 activation is in part modulated by Ca^{2+} [3,4]. Being a ubiquitous second messenger, Ca^{2+} regulates a wide variety of cellular functions, such as proliferation, migration, respiration, neuronal function and cell death [5–8]. Aberrant intracellular Ca^{2+} signaling may thus lead to pathological effects. The regulation of Ca^{2+} signaling is tightly controlled and compartmentalized. The main intracellular Ca^{2+} store is the endoplasmic reticulum (ER). Also, mitochondria are important players in intracellular Ca^{2+} handling, as they readily take up Ca^{2+} and can act as Ca^{2+} buffers or sinks [9,10]. Importantly, Ca^{2+} is a key regulator of mitochondrial enzymes involved in ATP production [7]. On the other hand, mitochondrial Ca^{2+} overload may lead to programmed cell death [11]. Interactions between the ER and the mitochondria are regulated at specific membrane contact sites between the two organelles. The sites of the ER-membranes that are involved in this inter-organellar regulation are thus named mitochondria-associated membranes, or MAMs. For instance, lipid transfer and Ca^{2+} flux are coordinated at the MAM sites [12,13]. Several proteins, including mitofusin 2 (MFN2), have been found to reside at and to regulate the dynamics of the MAM contacts [13,14]. Interestingly, opposing findings concerning the effect of MFN2 expression/depletion on ER-mitochondria tethering and mitochondrial Ca^{2+} signaling have been reported [14–20].

The concept and importance of compartmentalized intracellular Ca^{2+} signaling is actively studied with much emphasis put on the ER-

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mitochondria interactions and mitochondrial Ca^{2+} uptake [21,22]. Interestingly, S1P signaling is linked to mitochondrial biology. It has been reported that S1P produced by the sphingosine kinase 2 (SK2) isoform modulates mitochondrial respiration and antagonizes the opening of the mitochondrial permeability transition pore (mPTP) in ischemic heart tissue [23,24]. Also, S1P treatment (0.5–2 mg/kg) after experimental heart ischemia/reperfusion in rats suppresses the mPTP opening and thus maintains the mitochondrial membrane potential, which blunts the ischemic reperfusion injury [25]. Further, SK1 has been recently shown to localize to mitochondria to activate the mitochondrial unfolded protein response in *C. elegans* [26]. Also, inhibition of SK1 through reduced S1P levels induces mitochondrial dysfunction in pancreatic β-cells [27]. As mentioned earlier, S1P has been shown to affect Ca^{2+} handling through unknown intracellular mechanisms as well as through the G-protein coupled S1P-receptors. Upon activation, the $S1PR_{1-4}$ couple to phospholipase C (PLC) to induce inositol trisphosphate (IP_3) generation which is followed by Ca^{2+} release from the ER through the IP_3-receptors [3]. Mitochondrial uptake of Ca^{2+} that is released from the ER is coordinated at the MAM contact sites [28]. Further, altered SK1/S1P signaling as well as disrupted Ca^{2+} signaling in the mitochondrial matrix and at the MAM sites, respectively, have strong oncogenic implications [1,11,22,29]. Taken together, these observations led us to test whether SK1 overexpression would affect Ca^{2+} handling at the ER-mitochondria compartments. For this, we used a previously established HeLa cell model [30] overexpressing human SK1 (hSK1), and genetically targeted aequorins for the Ca^{2+} measurements [31]. We previously showed that the $[Ca^{2+}]$ in caveolin-1 (Cav-1)-enriched microdomains was augmented by hSK1 overexpression as reported by the luminescent chimeric Cav-1-aequorin [30]. However, SK1 overexpression did not significantly increase the cytoplasmic $[Ca^{2+}]$ in HeLa cells during histamine-induced Ca^{2+} release [30]. Hence, in the present study, we characterized the effects of SK1 overexpression on ER lumen ($[Ca^{2+}]_{ER}$) and mitochondrial matrix Ca^{2+} concentrations ($[Ca^{2+}]_{mit}$).

2. Results

2.1. SK1 augments ER Ca^{2+} release and mitochondrial Ca^{2+} uptake

First, we examined mitochondrial matrix and luminal ER Ca^{2+} concentrations in mock-transduced and SK1-overexpressing (hSK1) HeLa cells. The HeLa cell lines were as described in [30], and the SK1 overexpression is shown in Supplementary Fig. 1. The cells were challenged with the IP_3-generating agonist histamine (100 μM) in the presence of the Ca^{2+} chelator EGTA (150 μM) in the extracellular buffer. EGTA was added directly prior to the agonist stimulation to avoid unwanted emptying of the intracellular Ca^{2+} stores, and importantly, to eliminate extracellular Ca^{2+} as a source for cytoplasmic Ca^{2+} entry during agonist stimulation. In this experimental setting, both the release of ER Ca^{2+} as well as the Ca^{2+} uptake to the mitochondrial matrix were significantly augmented in SK1-overexpressing cells (Fig. 1A, B). $[Ca^{2+}]_{mit}$ was significantly higher in hSK1 cells upon histamine stimulation also when 1 mM Ca^{2+} was present in the extracellular buffer solution (Fig. 1C). These results indicate that the increased ER-derived Ca^{2+} is effectively shunted to the mitochondria in SK1-overexpressing cells. This is further highlighted by the previous results from our group [30] that showed no SK1-induced changes in the cytoplasmic Ca^{2+}. Of note, SK1-overexpression did not lead to marked changes in the IP_3-receptor protein levels, indicating that the increased release of ER Ca^{2+} during histamine stimulation was not due to altered IP_3R expression (Suppl. Fig. 1). Importantly, also transient SK1-overexpression in both HeLa and FTC-133 cell lines led to increased mitochondrial Ca^{2+} uptake upon agonist stimulation (Suppl. Fig. 2A, B; and see Suppl. Fig. 5).

We then examined the possibility that SK1 overexpression would affect the mitochondrial Ca^{2+} uptake *per se*. For this, the cells were permeabilized by digitonin treatment in the presence of an intracellular buffer (IB, supplemented with ATP) containing EGTA to chelate Ca^{2+} [31]. After plasma membrane permeabilization, the cells were perfused with IB containing 4 μM free Ca^{2+} to allow for mitochondrial Ca^{2+} uptake [31]. In this setting, SK1 overexpression had no effect on $[Ca^{2+}]_{mit}$ as compared to the mock-transduced cells (Fig. 2A). This finding is in line with the observation that SK1 overexpression did not markedly alter the expression levels of the proteins involved in mitochondrial matrix Ca^{2+} handling that were tested (MCU [32], MICU1 [33], MCUR1 [34], NCLX [35], AFG3L2 [36], OPA1 [37], Suppl. Fig. 1). Next, we treated mock-transduced and SK1-overexpressing cells with the ionophore ionomycin in the presence of EGTA. In this experiment, the intracellular Ca^{2+} released by the ionomycin treatment accumulated to a greater extent in the matrix of SK1-overexpressing cell mitochondria (Fig. 2B). The effect of SK1 overexpression was more pronounced when the cells were briefly incubated with the cytoplasmic Ca^{2+} chelator BAPTA-AM prior to the ionomycin treatment (Fig. 2C). Importantly, cytoplasmic calcium was unaffected in SK1-overexpressing cells upon ionomycin treatment in the presence of EGTA (Fig. 2D). These data thus suggest that SK1-overexpression augments the shunting of intracellularly stored Ca^{2+} to the mitochondria, whereas the intrinsic mitochondrial Ca^{2+} uptake capacity in permeabilized cells is not altered by SK1.

2.2. SK1 overexpression induces cleavage of mitofusin-2

As we did not see any changes in the mitochondrial inner membrane Ca^{2+} handling proteins, we asked whether SK1 affects the expression of mitofusin-2 (MFN2), an essential regulator of mitochondrial fusion and mitochondria-ER interactions at the MAM sites [38]. We found that the levels of full-length MFN2 (~80 kDa) were not altered, but observed that the SK1-overexpressing cells showed a distinct protein fragment recognized by the N-terminal targeting anti-MFN2 antibody (Fig. 3A). Intriguingly, calpain has been reported to induce MFN2 fragmentation [39] and calpain is activated by S1P and an S1P analogue [40,41]. In agreement with this, we observed an increase in calpain activity in SK1-overexpressing cells (Fig. 3B). Of note, calpain activity was increased, and a MFN2-fragment was identified, in HeLa and FTC-133 cells upon transient SK1 overexpression. Further, long-term SK1-inhibitor treatment reduced MFN2 fragment expression levels (Suppl. Fig. 3). MFN2 contains multiple putative calpain cleavage sites with the highest score predicted for cleavage between the His467 and Arg468 residues (prediction based on ref. [42] and conducted with the online tools at www.calpain.org). Hence, we generated plasmid constructs to express MFN2 fragments that would result from calpain-mediated cleavage at the putative His467-Arg468 site (Fig. 3C). Of note, the resulting fragments include intact active domains of the full-length protein: the N-terminal fragment harbors the GTPase, coiled-coil heptad repeat (HR1) and proline rich (PR) domains, whereas the transmembrane (TM) and HR2 domains reside in the C-terminal fragment (see Fig. 3C and ref. [14] for a schematic representation). Interestingly, the expression of both the N- and C-terminal MFN2 fragments, respectively, was found to augment the mitochondrial Ca^{2+} uptake (Fig. 3D), and similar results were obtained when the FTC-133 cell line was used (Suppl. Fig. 2C). In contrast, cytoplasmic Ca^{2+} was unaffected by MFN2 fragment expression (Fig. 3E). These results indicate that the MFN2 fragments are biologically active and are able to modulate mitochondrial physiology in a manner that is coupled to mitochondrial Ca^{2+} uptake. Importantly, this is in agreement with the previous reports that have shown MFN2 fragment/mutant expression or MFN2-derived cell permeable short peptides to be biologically active and to affect mitochondrial function [43–45].

2.3. Mitochondrial Na^+/Ca^{2+} exchanger activity is reduced by SK1 overexpression

The mitochondrial Na^+/Ca^{2+} exchanger protein (NCLX) regulates

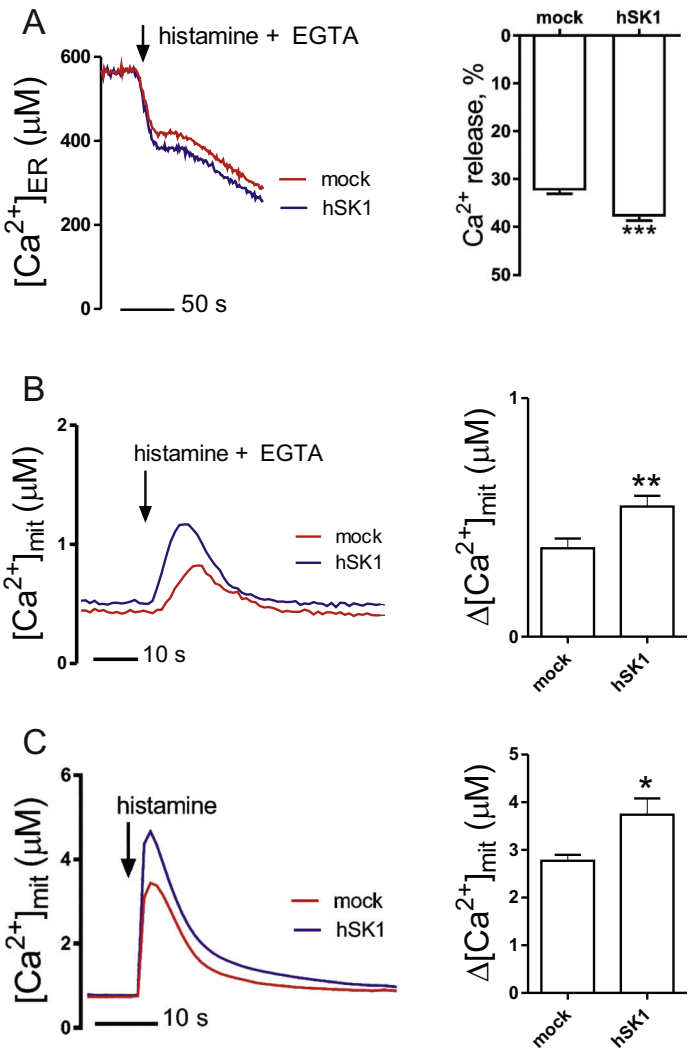

Fig. 1. The effect of SK1 overexpression on ER Ca^{2+} release and mitochondrial Ca^{2+} uptake. A) Histamine-induced Ca^{2+} release from the ER is greater in SK1-overexpressing HeLa cells than in the mock-transduced controls. Targeted aequorins as described in Section 4.2 were employed for Ca^{2+} measurements in these compartments. The baseline ER Ca^{2+} of the cells was recorded in an HBSS buffer containing 1 mM Ca^{2+}. Thereafter, the cells were perfused with HBSS containing 100 μM histamine and 150 μM EGTA when indicated by the arrow to induce a rapid IP_3-mediated Ca^{2+} release from the ER. Representative traces and quantification with S.E.M of the percental ER Ca^{2+} release are shown, N = 8 for mock, N = 9 for hSK1, ***P < 0.001. B) Mitochondrial Ca^{2+} was measured upon 100 μM histamine treatment in the presence of 150 μM EGTA in the buffer. Representative traces and quantification of the change (Δ, delta) in $[Ca^{2+}]_{mit}$ from basal to maximal stimulated concentration are shown, N = 11, **P < 0.01. C) Mitochondrial Ca^{2+} was measured after 100 μM histamine treatment in the presence of 1 mM Ca^{2+} in the buffer. Representative traces and quantification of $\Delta[Ca^{2+}]_{mit}$ are shown, N = 7, *P < 0.01.

the Ca^{2+} efflux from the mitochondrial matrix in a Na^+-dependent manner, and consequently, NCLX-inhibition leads to accumulation of Ca^{2+} in the matrix [35,46]. Interestingly, the involvement of MFN2 in the regulation of mitochondrial matrix Ca^{2+} by modulating the activity of NCLX has been reported [47]. We conducted mitochondrial Ca^{2+} recordings during increasing agonist concentrations to study the kinetics of $[Ca^{2+}]_{mit}$ upon repeated increases in intracellular $[Ca^{2+}]$. In this experiment, SK1-overexpressing cells again showed increased $[Ca^{2+}]_{mit}$ compared to the mock-transduced cells (Fig. 4A). To assess the possible involvement of NCLX, the cells were treated with the NCLX inhibitor CGP-37157 (CGP). Blocking NCLX did not significantly affect the $[Ca^{2+}]_{mit}$ in cells overexpressing SK1, whereas the mock-transduced cells showed an increase in $[Ca^{2+}]_{mit}$ when NCLX was inhibited (Fig. 4B). This experiment suggests that the basal NCLX activity is low in SK1-overexpressing cells, contributing, at least in part, to the accumulation of Ca^{2+} in the mitochondrial matrix.

2.4. Overexpression of SK1 or the MFN2 N- and C-terminal fragments has no effect on mitochondrial-ER networks

As mitochondria-ER dynamics regulates Ca^{2+} signaling, we set out to investigate whether SK1 or MFN2 N- and C-terminal fragment overexpression, respectively, would have an effect on these organelles. First, SK1 overexpression showed no significant effects on the appearance of the ER-mitochondria networks as visualized by immunofluorescence and confocal microscopy (Fig. 5A). The expression of MFN2 N- and C- terminal fragments did not have an effect on the mitochondrial network, whereas full-length MFN2 expression resulted in

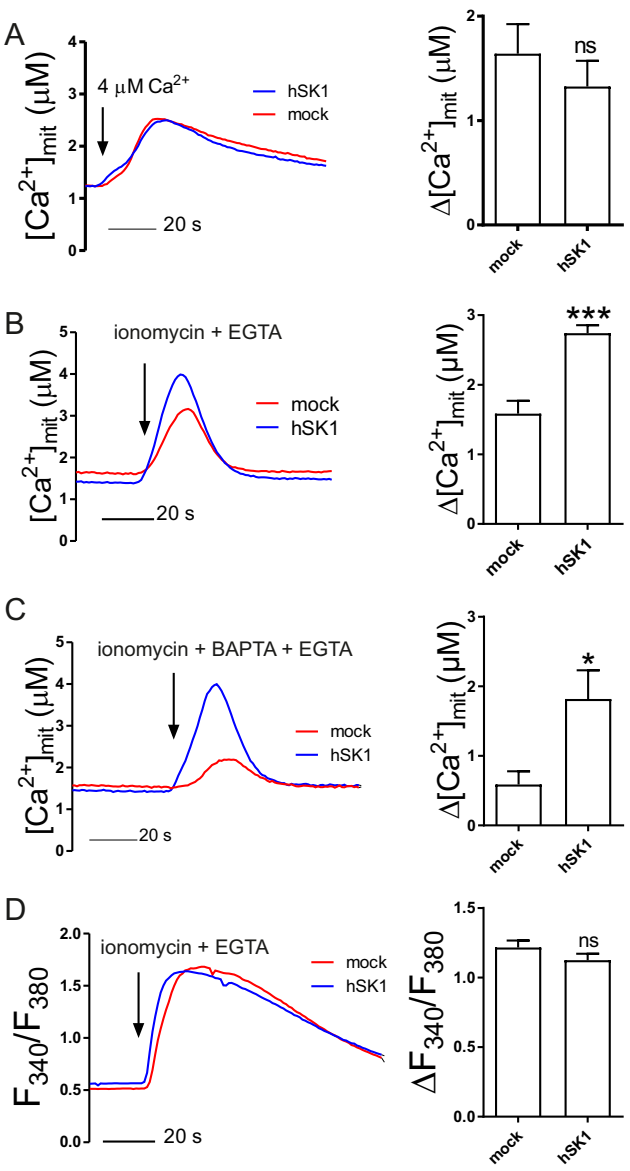

Fig. 2. Effect of SK1 overexpression on mitochondrial Ca^{2+} in permeabilized cells during Ca^{2+} addition as well as in intact cells during ionomycin treatment. Mitochondria-targeted aequorin was employed in the experiment shown in panels A–C, whereas the Ca^{2+} indicator Fura-2-AM was used for the measurements shown in panel D, see Section 4.2. A) To permeabilize the plasma membrane, the cells were treated with 25 μM digitonin in an intracellular buffer (IB, see the text and ref. [31]) in the presence of EGTA. Then, the cells were perfused with IB containing 4 μM free Ca^{2+} to allow for mitochondrial Ca^{2+} uptake (as described in [31]. In this setting, no significant differences between hSK1 and mock-transduced cells could be detected, N = 9 for mock, N = 8 for hSK1. B) The cells were treated with 5 μM ionomycin in the presence of 150 μM EGTA in the extracellular buffer when indicated by the arrow. $[Ca^{2+}]_{mit}$ was significantly higher in SK1-overexpressing cells, N = 4 for mock, N = 5 for hSK1. C) The cells were pretreated with BAPTA-AM for 2.5 min prior to treating the cells with 5 μM ionomycin in the presence of 150 μM EGTA. The brief BAPTA-AM treatment resulted in significant reduction in ionomycin-induced $[Ca^{2+}]_{mit}$ in the mock-transduced cells as compared to the SK1-overexpressing cells, N = 5 for mock, N = 6 for hSK1. D) Cytoplasmic Ca^{2+} was not affected by SK1 overexpression during 5 μM ionomycin treatment in the presence of 150 μM EGTA in the extracellular buffer, N = 4. The bar diagrams show the average of the change (Δ) from basal to maximal $[Ca^{2+}]_{mit}$ recorded during each experiment. The error bar shows S.E.M. * = $P < 0.05$, *** = $P < 0.001$, ns = not significant.

the previously reported mitochondrial clustering [44,48] (Fig. 5B). Of note, SK1-GFP did not colocalize with the mitochondria (Fig. 5C). Further, we performed electron microscopy to analyze the mitochondria-ER network. In agreement with the results obtained by confocal microscopy, the mitochondrial surface area or the mitochondria-ER contacts were not affected by SK1 overexpression as evidenced by the electron microscopy approach (Fig. 6).

2.5. SK1 does not affect proliferation but increases cell respiration and migration

Finally, we set out to characterize other possible physiological effects of SK1 overexpression. As Ca^{2+} is an important regulator of metabolism and mitochondrial ATP production, we measured cellular respiration. We found that SK1 overexpression slightly but significantly increased the basal oxygen consumption in HeLa cells (Fig. 7A). Further, mitochondrial Ca^{2+} is of importance in the regulation of cancer

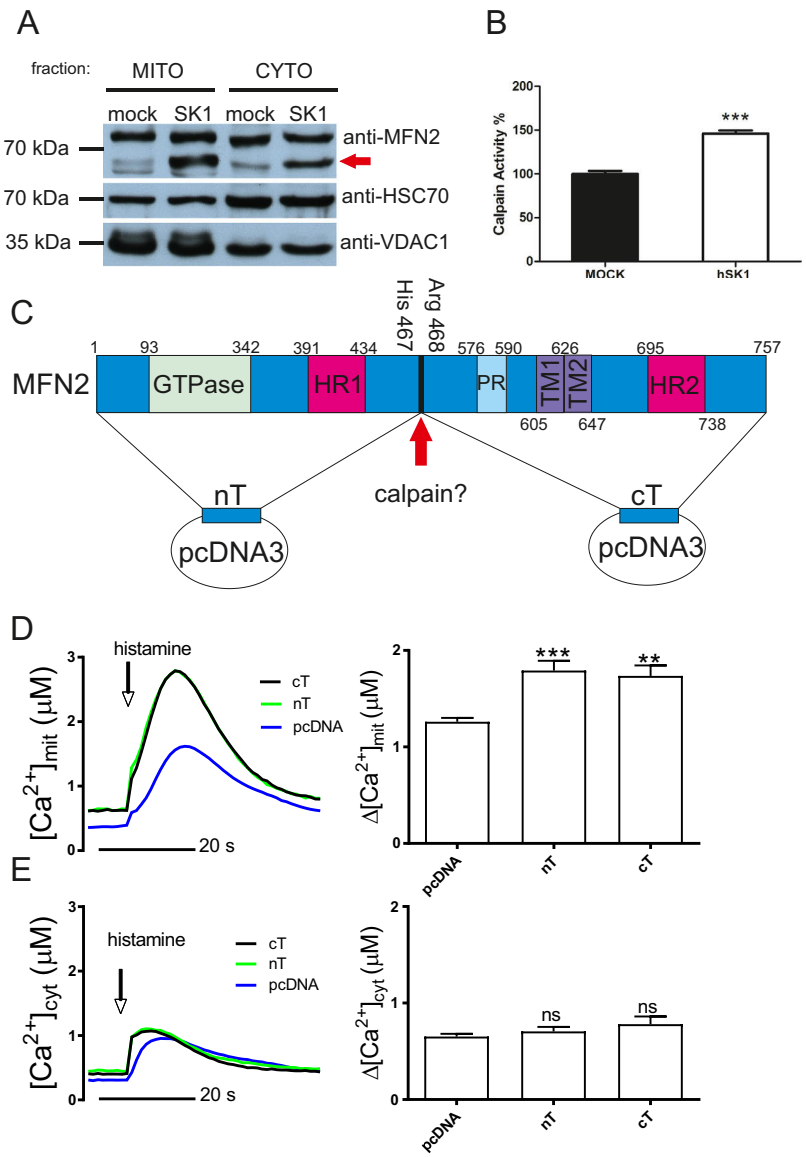

Fig. 3. Mitofusin-2 is fragmented in SK1-overexpressing cells, and transient MFN2 fragment expression increases mitochondrial Ca^{2+}. Mitochondria-targeted, or non-targeted (cytoplasmic) aequorin was employed in the Ca^{2+} experiments shown. A) Western blot analysis revealed the presence of a fragment of MFN2 protein in lysates of SK1-overexpressing cells. The fragmented MFN2, indicated by the red arrow, was present both in the mitochondrial fraction and in the whole cell lysate of cells overexpressing SK1. HSC70 and the mitochondrial outer membrane protein, VDAC1, served as loading controls. B) Calpain activity was increased in cells overexpressing SK1 (%, mock-cells normalized to 100%). N = 5, ***P < 0.001. C) Schematic presentation of MFN2 with the functional domains, adapted from [14]. The predicted calpain cleavage site is between Arginine 468 and Histidine 467 of MFN2. Plasmid expression vectors (pcDNA3) carrying the N- and C-terminal parts of MFN2 that would result after calpain cleavage at the indicated site (red arrow) were created. GTPase = GTPase domain; HR1, HR2 = heptad-repeat domains; PR = proline-rich domain; TM1, TM2 = transmembrane domains. D) Expression of the N- and C-terminal plasmid constructs augmented mitochondrial Ca^{2+} uptake, whereas cytoplasmic Ca^{2+} was unaffected in these conditions (E), N = 14 in D & E. The change in Ca^{2+} concentration from basal to maximal stimulated concentration is quantified in the bar diagrams. The error bars show the S.E.M. **P < 0.01, ***P < 0.001, ns = not significant.

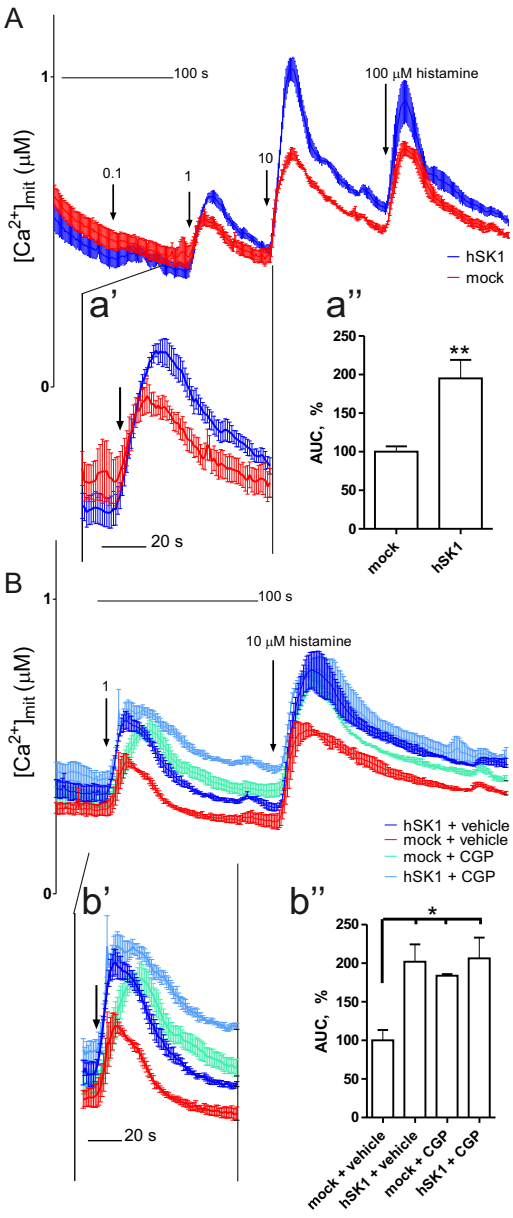

Fig. 4. SK1-overexpressing cell mitochondria accumulate Ca^{2+} more effectively than the mock-transduced control cells during stimulation with increasing histamine concentrations, and inhibition of the mitochondrial Na^+/Ca^{2+} exchanger NCLX augments mitochondrial Ca^{2+} retention in the mock-transduced cells. Mitochondria-targeted aequorin was employed in the Ca^{2+} experiments shown. A) SK1-overexpressing HeLa cells were treated with increasing histamine concentrations and mitochondrial matrix Ca^{2+} was measured. SK1 overexpression augmented $[Ca^{2+}]_{mit}$ during all stimulations. Area under curve (AUC) analysis was conducted on the selected timeframe of the experiment (a′) and quantified in (a″), N = 4. B) Inhibition of the mitochondrial Na^+/Ca^{2+} exchanger NCLX by CGP-37157 (CGP; 10 μM, 1 h) increases the accumulation of mitochondrial matrix Ca^{2+} in mock-transduced cells to a level resembling that of SK1-overexpressing cells. The AUC was analysed between the experimental time points shown in (b′) and the acquired values were analysed (b″), N = 4 vehicle-treated mock and hSK1, N = 3 for CGP-treated mock and hSK1. The average AUC from the shown time points is quantified in the bar diagrams and the error bars depict S.E.M. *P < 0.05, **P < 0.01.

cell proliferation [49]. However, SK1-overexpression did not affect proliferation (Fig. 7B). Finally, we observed an SK1-induced increase in cell migration (Fig. 7C). This finding is in line with the various mechanisms reported by which mitochondrial Ca^{2+} may regulate cell migration [50], and with the widely reported effects of SK1/S1P/S1P-receptor signaling on cancer cell migration and invasion [1,51,52].

3. Discussion

Aberrant sphingosine kinase 1/2 (SK1/2) expression and deregulated S1P signaling have been previously associated with severe conditions such as cancer, cardiovascular pathologies, inflammation, diabetes and neurological disorders [1,29]. Here, we show that SK1 overexpression modulates several important physiological parameters in HeLa cervical cancer cells. First, we show that SK1 overexpression significantly augments the IP_3-induced release of ER Ca^{2+}, and that the released Ca^{2+} is taken up by the mitochondria. The mechanisms underlying SK1-induced ER Ca^{2+} release and the following mitochondrial Ca^{2+} uptake could be many. Firstly, SK1 activation is modulated by Ca^{2+} [3]. Histamine-induced Ca^{2+} flux to the cytoplasm may thus activate SK1 leading to S1P production, which would amplify the IP_3 production in SK1-overexpressing cells through auto/paracrine stimulation of the G-protein coupled S1P-receptors. However, short-term blocking of the S1P receptors did not specifically abolish the effect of SK1 overexpression on mitochondrial Ca^{2+} (Suppl. Fig. 4A). Hence, the autocrine/paracrine S1P signaling loop does not play a major role here. S1P has been reported to have direct intracellular effects, including the release of Ca^{2+} from intracellular stores [3,53]. However, treatment with SK1 inhibitor (SKi, 10 μM, 1 h) did not abolish the effects of stable SK1 overexpression on mitochondrial Ca^{2+} (Suppl. Fig. 4B). Thus, instead of acute S1P-mediated effects, these findings indicated that SK1 overexpression has a long-term effect on ER-mitochondrial Ca^{2+} handling.

The robust increase in the $[Ca^{2+}]_{mit}$ induced by SK1 overexpression initially suggested that some of the recently characterized components of the mitochondrial calcium uniporter (MCU) might be affected [10,42]. However, no changes in the expression levels of the tested MCU components were found. In agreement with this, the intrinsic mitochondrial Ca^{2+} uptake capacity was not affected by SK1 overexpression. To further elucidate the nature of the SK1-mediated increase in $[Ca^{2+}]_{mit}$, we first treated the cells with ionomycin in the presence of the extracellular Ca^{2+} chelator EGTA (to induce rapid depletion of stored intracellular Ca^{2+}). In these conditions, SK1-overexpressing cells show higher $[Ca^{2+}]_{mit}$ than the mock-transduced cells. We then included a short incubation with the intracellular Ca^{2+} buffer BAPTA-AM in this experimental protocol [54]. The brief BAPTA-AM treatment blunted the ionomycin-induced mitochondrial Ca^{2+} uptake in mock-transduced cells, whereas the mitochondria in SK1-overexpressing cells were able to effectively accumulate Ca^{2+}. The results from the ionomycin treatments thus indicate an altered interaction between the mitochondria and the intracellular Ca^{2+} deposits (mainly the ER) in SK1-overexpressing cells. In addition, the previous finding that cytoplasmic Ca^{2+} was unaffected by SK1 overexpression upon ionomycin treatment (ref. [30]) corroborates the interpretation that SK1 somehow affects the Ca^{2+} flux between the ER and mitochondria.

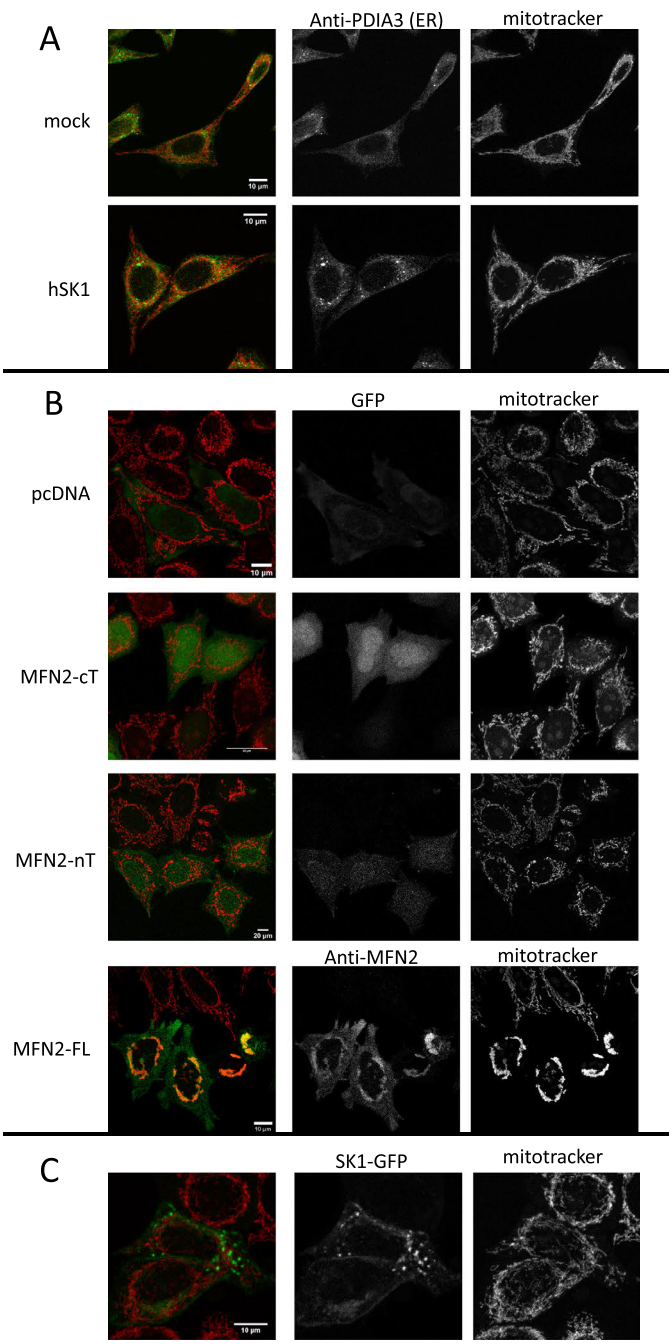

Fig. 5. Analysis of mitochondrial and ER network, as well as SK1 localization by employing confocal microscopy analysis. A) SK1-overexpression does not alter the mitochondria-ER network in HeLa cells. The panels show composite (left), anti-PDIA3 stained (ER-marker, middle) and mitotracker stained (right) representative confocal microscopy images. B) Control transfection (pcDNA) or MFN-2 C- or N-terminal (MFN2-cT; MFN2-nT) fragment overexpression do not alter the mitochondrial network, whereas full-length MFN-2 (MFN2-FL) overexpression leads to the previously reported clustering of mitochondria [44,48]. Co-expression of GFP with the pcDNA, MFN2-cT and MFN2-nT was employed to indicate the transfected cells. Mitochondrial network was visualized by mitotracker. Overexpressed MFN2 was detected by the anti-MFN2 antibody. C) SK1 does not localize to mitochondria in HeLa cells as evidenced by SK1-GFP and mitotracker.

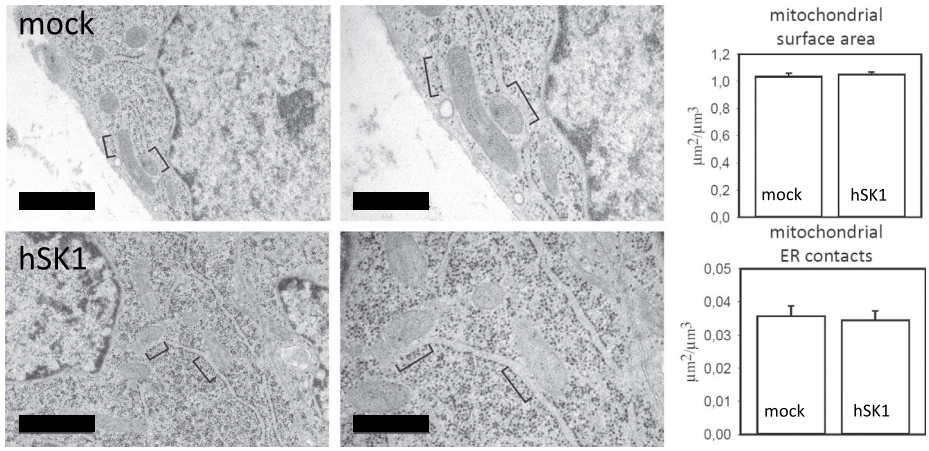

Fig. 6. Assessment of mitochondrial surface area and ER-mitochondrial contacts by electron microscopy. Areas of mitochondria-ER contact (brackets, at higher magnification at right) are equivalent in mock and hSK1 cells. The bar diagrams show the total surface area of mitochondria (µm2/µm3 of cytoplasm) and mitochondrial surface area in contact with the ER (µm2/µm3 of cytoplasm), respectively. Scale bar = 1 µm (left) and 500 nm (right). N = two independent experiment with 60 mock cell profiles and 60 hSK1 cell profiles analysed, respectively.

The results indicated that SK1 might modulate the ER-mitochondria interactions. However, confocal or electron microscopy revealed no changes in the ER-mitochondria organelle network or contacts, or mitochondrial surface area, upon SK1 overexpression. Nevertheless, we found that SK1 induces fragmentation of the MFN2 protein, which is a positive regulator of mitochondrial fusion, and, in addition, regulates the ER-mitochondria dynamics [14]. As mentioned before, whether MFN2 promotes or hinders the ER-mitochondria tethering is debated [14–20]. However, there is well-established evidence that MFN2 plays an important role in mitochondrial physiology [14]. Our results show that the expression of putative calpain-cleaved MFN2 N- and C- terminal fragments augments $[Ca^{2+}]_{mit}$ upon agonist stimulation. Intriguingly, a truncated, non-fusogenic form of MFN2 has been previously shown to have an effect on mitochondrial metabolism, and MFN2 fragments lacking the transmembrane domain re-establish mitochondrial targeting and activity when co-expressed with the C-terminal and the transmembrane domain MFN2 fragments [43,44]. Thus, MFN2 fragments are able to interact and retain (some degree) of their functionality. Also, short peptide fragments of MFN2 coiled-coil heptad repeat region (HR)-domains have been shown to bind to and modulate MFN2 functions [45]. Thus, protein fragments and/or peptide derivatives of MFN2 have been shown by others, and by us in the present study, to be biologically active. Further, glutamate-induced calpain activation has been shown to induce MFN2 degradation [39]. These findings are in line with our results showing an SK1-induced calpain activation and MFN2 fragmentation. Interestingly, calpain may localize to caveolae and we have previously shown that SK1-overexpression augments Ca^{2+} signaling at the caveolar sub-compartment without affecting the overall cytoplasmic Ca^{2+} [30,55]. As mentioned above, it is debated whether MFN2 antagonizes or augments mitochondrial Ca^{2+} [14–16]. Our results suggest that MFN2 fragment expression increases mitochondrial Ca^{2+}, but whether this effect is through inhibitory or

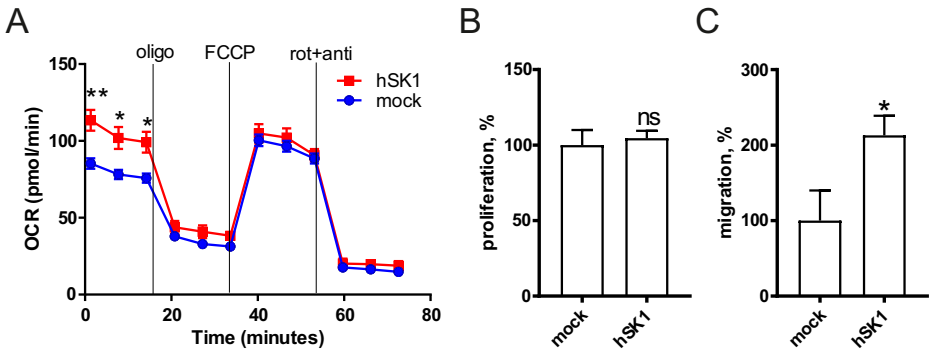

Fig. 7. Assessment of cell respiration, proliferation and migration. A) The basal oxygen consumption rate (OCR) in HeLa cells is augmented by SK1 overexpression. Oligo = 1 µM oligomycin, FCCP = 1 µM Trifluoromethoxy carbonylcyanide phenylhydrazone, rot = 0.5 µM rotenone, anti = 0.5 µM antimycin A. N = 5–6. B) Proliferation, as reported by a thymidine incorporation assay, is not affected by SK1 overexpression. *N* = 3. C) SK1 overexpression increases cell migration as reported by a Transwell migration assay. The cells were allowed to migrate for 12 h. N = 3. The bar diagrams show the average values with S.E.M, *P < 0.05, **P < 0.01.

activating interactions with endogenous MFN2, or through other effects, remains to be clarified.

Charcot-Marie-Tooth (CMT) disease is a neuropathy with multiple known contributing factors leading to the disease phenotype [56]. MFN2 mutations are associated with CMT type 2A (CMT2A) [57]. Interestingly, as evidenced by a *Drosophila* model, mutant MFN2 expression causes a CMT2A-resembling phenotype through excessive mitochondrial fusion or fission, respectively, depending on the site of mutation [58]. Further, S1P lyase deficiency is found in CMT patients, leading to increased S1P plasma levels [59]. SK1 overexpression might resemble S1P lyase deficiency as S1P levels are expected to increase in both conditions [1]. Also, disturbed Ca^{2+} homeostasis and mitochondrial dysfunction has been observed in a CMT model [60]. Given the recent pre-clinical advances in targeting mutant MFN2 in CMT2A models [57], and the fact that S1P signaling can be pharmacologically targeted [61], it would be interesting to test whether MFN2 is affected in patients with S1P lyase dysfunction.

MFN2 has been shown to modulate mitochondrial Ca^{2+} through affecting the activity of the mitochondrial matrix Ca^{2+}/Na^{+} exchanger NCLX in conditions where mitochondrial membrane potential is reduced [47]. Intriguingly, we found that mitochondrial membrane potential was reduced in SK1-overexpressing cells (Suppl. Fig. 3D). We observed that inhibition of NCLX did not significantly affect the mitochondrial Ca^{2+} dynamics in SK1-overexpressing cells, whereas the mock-transduced control cells showed increased accumulation of mitochondrial Ca^{2+} when extrusion of Ca^{2+} through NCLX was blocked. Also, it has been very recently reported that reduced mitochondrial membrane potential inhibits NCLX activity leading to accumulation of $[Ca^{2+}]_{mit}$ ([62]; NCLX regulation reviewed in [63]). It is therefore possible that altered NCLX activity in SK1-overexpressing cells might, in part, account for the observed increase in $[Ca^{2+}]_{mit}$.

The SK1-induced alterations in ER-mitochondrial Ca^{2+} handling may be of relevance in regard to the oncogenic functions of SK1, since the importance of Ca^{2+} signaling at the MAM sites in cancer is well-acknowledged [11,64]. The SK1-induced increase in mitochondrial matrix Ca^{2+} may affect cell energetics as $[Ca^{2+}]_{mit}$ regulates ATP production [10,65]. Further, mitochondrial Ca^{2+} has been shown to modulate cell migration [50]. Interestingly, we observed an SK1-mediated increase in cell respiration and migration, which is in agreement with the effects of altered $[Ca^{2+}]_{mit}$. The involvement of MFN2 in the regulation of mitochondrial Ca^{2+} is mechanistically complex and partially unclear as evidenced by multiple previous studies ([47], reviewed in [14]). Our data suggests that SK1, through fragmentation of MFN2 and possibly through the modulation of NCLX activity causes alterations in the mitochondrial Ca^{2+} handling. Further studies are needed to clarify the mechanistic details of MFN2 and mitochondrial Ca^{2+} dynamics. Taken together, our results show augmented mitochondrial Ca^{2+} uptake upon SK1 overexpression, with implications for the regulation of cell respiration and migration, providing novel insights to the oncogenic role of SK1 (schematic representation; Fig. 8).

4. Materials and methods

4.1. Cell culture, plasmids and transfections

HeLa [30] and human follicular thyroid cancer FTC-133 [66] cell lines were used in this study. The HeLa cells were grown in a humidified and thermostatic incubator with a controlled atmosphere (37 °C, 5% CO_2) in DMEM medium (#D6046, Sigma-Aldrich) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Sciences). The FTC-133 cells were cultured in DMEM and F12 (Ham's nutrient) medium (1:1) supplemented with 10% FBS, 1% P/S and 2 mM L-glutamine. Plasmid transfections were carried out by employing TurboFect (#R0531, Thermo Scientific) transfection reagent and OptiMEM media (#31985-070, Life Technologies), or by electroporation (4,000,000 cells with 20 μg of the desired plasmid suspended in 400 μL OptiMEM; 240 V; 500 μF). The stable SK1-overexpressing and mock-transduced HeLa cell lines, respectively, were created as described previously [30]. SK1-EGFP plasmid (SK1-GFP) was as described previously [67,68] the GFP plasmid (EGFP) was as in [69], and the GFP plasmid used in Fig. 5 was from Clontech (EGFP, #6085-1). Full length human MFN2, NT and CT fragments were cloned from human Nthy-ori 3.1 cell cDNA and inserted into pcDNA3 with *Hin*dIII and BamHI. All constructs were verified by Sanger sequencing. Primer sequences used are available upon request. Aequorin plasmids were as described in [31].

4.2. Ca^{2+} measurements

For compartment-specific Ca^{2+} measurements, we used luminescent aequorin chimeras [31] genetically targeted to the sarco/endoplasmic reticulum (mutated aequorin), to the mitochondrial matrix (wild-type aequorin) and to the cytoplasm (wild-type, no targeting sequence). These measurements were conducted as described previously by employing an in-house built luminometry setup or by utilizing a plate reader [31,69]. Briefly, the cells were plated either on to 12-well plates (100,000 cells per well) containing poly-L-lysine coated 13 mm coverslips, or, when employing a HIDEX Sense plate reader (HIDEX corp., Turku, Finland), on to 96-well plates (10,000 cells per well). The cells were transfected with the desired plasmid constructs (1 μg plasmid/ml medium, final total plasmid concentration on each well). Twenty-four hours post-transfection the cells were incubated in HBSS buffer (118 mM NaCl, 4.6 mM KCl, 10 mM glucose, 20 mM HEPES, pH 7.4) supplemented with 5 μM coelenterazine and 1 mM $CaCl_2$ (mitochondrial and cytoplasmic measurements) or 5 μM coelenterazine *n* along with 100 μM EGTA and 1 mM ionomycin (endo/sarcoplasmic measurements). Mitochondrial aequorin measurements in permeabilized cells were conducted exactly as described by Bonora et al. [31]. The cytoplasmic fluorescent Ca^{2+} indicator Fura-2-AM (Life Technologies) was employed as described in [69].

4.3. Western blot and antibodies

Western blotting was conducted as described previously [30]. Briefly, cells well were seeded onto 100 mm or 35 mm cell culture plates. At approximately 70% confluence, the cells were washed with phosphate buffered saline solution (PBS) and then harvested in a lysis buffer (10 mM Tris, 150 mM NaCl, 7 mM EDTA, 0.5% NP-40, 0.2 mM PMSF, and 0.5 μg/ml leupeptin, pH 7.7). The mitochondrial isolation was conducted as described in [70]. The lysates were supplemented with Laemmli sample buffer (LSB) and boiled for 5 min. The proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane by wet transfer. The membranes were incubated overnight with the primary antibodies and then with the horseradish peroxidase (HRP) –conjugated secondary antibodies. The protein bands were detected by chemiluminescence (WesternLightning, PerkinElmer, USA). The antibodies were: Anti-SK1 (Cell Signaling, #32975 [Suppl. Fig. 1] and #12071 [Suppl. Fig. 5]), Anti-Mitofusin-2 (M6319, Sigma-Aldrich), Anti-MCU antibody (HPA016480, MERCK), Pan-IP3R antibody IP3R1/2/3 H300 (sc-28613, Santa Cruz Biotechnology), Anti-CBARA1/MICU1 antibody (ab102830, Abcam), anti-GFP antibody (ab290, Abcam), AFG3L2 Polyclonal (#14631-1-AP, Proteintech), anti-OPA1, anti-MCUR1, Anti-mouse IgG HRP-linked secondary, Anti-Rat IgG HRP-linked secondary antibodies were from Cell Signaling Technology. Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (#1706515, Biorad). Anti-HSC70 (ADI-SPA-815, Enzo Life Sciences) and anti-VDAC1 (MABN504, Merck) antibodies served as loading controls for whole cell and mitochondrial lysates, respectively. The anti-NCLX antibody [35] was a kind gift from professor Israel Sekler. The anti-IP3R1 (rbt03) antibody was described in [71]. In addition, antibodies that were used only for immunocytochemistry were: Anti-protein disulfide-isomerase A3

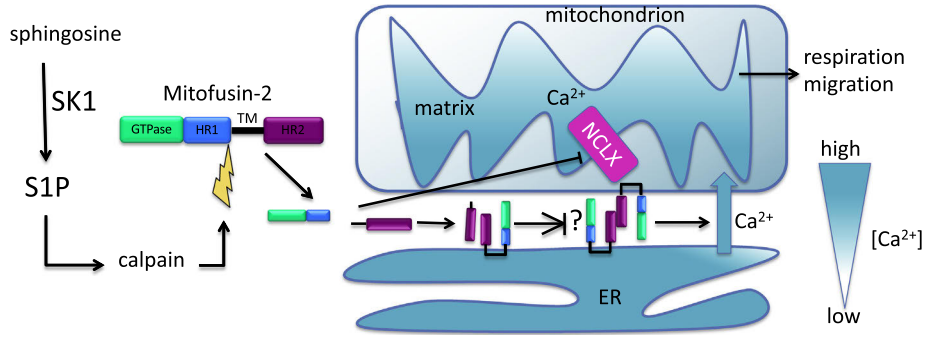

Fig. 8. Schematic representation of the proposed model. Sphingosine kinase 1 (SK1) phosphorylates sphingosine to form sphingosine 1-phosphate (S1P), which promotes calpain activation and consequent cleavage of mitofusin-2 (MFN2) at histidine 467. The cleaved fragments of MFN2 contain the functional GTPase and heptad-repeat 1 (HR1) domains, as well as the transmembrane (TM) and heptad-repeat 2 (HR2) domains, respectively. The MFN2 fragmentation modulates mitochondria-endoplasmic reticulum (ER) dynamics and inhibits the mitochondrial sodium-calcium (Ca^{2+}) exchanger NCLX, leading to increased mitochondrial matrix Ca^{2+} concentration ([Ca^{2+}]). Then, mitochondrial Ca^{2+} augments respiration and cell migration.

(PDIA3, AMAB90988, Sigma), Alexa Fluor 488 conjugate (A-11017 Mouse, A-11008 Rabbit, Life Technologies).

4.4. Cell migration and proliferation

Corning Transwell membrane inserts were employed to study cell migration according to a protocol modified from [72]. Briefly, 75,000 cells were collected in serum-free medium and allowed to migrate through uncoated Transwell membrane inserts for 12 h. Fetal bovine serum (FBS) was utilized as a chemoattractant. Then, the membranes were processed as described in [72] and the migrated cells were manually counted. The normalized cell counts were statistically analysed. Cell proliferation was measured by a thymidine incorporation assay at 48 h after seeding the cells as previously described in ref. [69].

4.5. Electron microscopy

For electron microscopy cells were fixed with 2.5% glutaraladehyde in 0.1 M cacodylate buffer, scraped and pelleted in gelatin, postfixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide, dehydrated and embedded in an Epon resin. Ultrathin sections were poststained with uranyl acetate and lead citrate and 30 cell profiles from each culture were systematically sampled and photographed at 6000× with a Jeol JEM-1400 electron microscope equipped with a Gatan Orius SC 1000B bottom mounted CCD-camera. The pictures were viewed at a final magnification of 160,000×. A regular line grid was overlaid and the surface to volume ratios of mitochondrial outer surface membrane and mitochondrial membrane in close contact with the ER were determined according to the formula $S/V = 4c/lh$, where c is the number of times the lines intersected the surface of interest, h is the number of times the end points of the lines fell on cytoplasm and l is the length of a test line [73]. The graphs represent the results from 2 experiments ($n = 2$, in all 60 mock cell profiles, 60 hSK1 cell profiles).

4.6. Calpain activity assay

A calpain activity assay kit (#ab65308, Abcam, Cambridge, MA, USA) was employed. The assay was performed according to the manufacturer's instructions as described previously [66]. Briefly, 2,000,000 cells were grown overnight on 100-mm cell culture plates. The next day, the cells were detached and washed three times with PBS. Fluorescence was measured using a Hidex sense microplate reader instrument (HIDEX Corp, Turku, Finland) with the excitation light set at 400 nm and emitted light collected at 505 nm.

4.7. Cellular oxygen consumption measurements

For the oxygen consumption measurements, 10,000 HeLa cells per well were seeded onto a 96-well Agilent Seahorse XF Cell Mito Stress Test Kit plate and the measurements were conducted as defined in the manufacturer's protocol. To study the different mitochondrial respiration pathways, the cells were sequentially challenged with 1 µM oligomycin, 1 µM Trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) and 0.5 µM rotenone + 0.5 µM antimycin A, respectively.

4.8. Immunocytochemistry

The cells were seeded on 35 mm coverslips in 6-well plates the day before transfection. Cells were cultured and transfected as described in Section 4.1. Sixteen hours after transfection, the cells were incubated with MitoTracker-Red (MitoTracker® Red FM, M22425, Thermo) for 20 min, then fixed with 4% paraformaldehyde for 10 min at room temperature and rinsed in PBS three times. Permeabilization and block was performed in PBS containing 5% FBS and 0.3% (*v*/v) Triton X-100 for 5 min at room temperature. The cells were then incubated with appropriate primary antibodies (as listed below) for 90 min at 37 °C in the dark. After rinsing, the cells were incubated with corresponding secondary antibody for 1 h at 37 °C in the dark. All antibodies were diluted in blocking solution (5% FBS). The cells were then washed three times with PBS for 15 min and mounted with Vectashield Mounting Media with DAPI (H-1200, Vector Laboratories). Cells were imaged with 3i (Intelligent Imaging Innovations) CSU-W1 Confocal Spinning Disk Microscope (Yokogawa Corporation of America) equipped with Hamamatsu sCMOS Orca Flash 4 (Hamamatsu Photonics), using 63× oil immersion. Also used was Zeiss LSM 780 Confocal Microscope (Zeiss, Germany) with a 63× water immersion objective. The excitation/emission wavelengths (nm) used were: green (GFP), 498/516; and red (DsRed), 558/583. Image acquisition was performed with SlideBook v 6.0 and Zeiss Zen, respectively. Image processing was done using Fiji (ImageJ plugin collection) [74].

4.9. Mitochondrial membrane potential measurements

The cells were plated on to 24-well plates and treated on the next day with tetramethylrhodamine ethyl ester perchlorate (TMRE) for 20 min in the cell culture conditions as described in 4.1. The control

cells were treated with 100 μM FCCP 10 min prior to TMRE treatment. Thereafter, the cells were briefly washed in PBS containing 0.2% bovine serum albumin, and the TMRE fluorescence was read by utilizing a plate reader (HIDEX Sense, Hidex, Finland). Values obtained from a colorimetric crystal violet assay [75] was used to normalize the acquired TMRE fluorescence values.

4.10. Statistics

Statistical analysis was conducted by unpaired Student's *t*-test when two means were compared or by One-Way Anova with Tukey's post-hoc test when three or more means were compared. The GraphPad Prism 6 program (GraphPad Software Inc., San Diego, CA) was employed for the statistical analyses. A *P*-value below 0.05 was considered statistically significant.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2019.06.006.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Oxysterol-binding protein related-proteins (ORPs) 5 and 8 regulate calcium signaling at specific cell compartments

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ABSTRACT

Oxysterol-binding protein related-protein 5 and 8 (ORP5/8) localize to the membrane contact sites (MCS) of the endoplasmic reticulum (ER) and the mitochondria, as well as to the ER-plasma membrane (PM) MCS. The MCS are emerging as important regulators of cell signaling events, including calcium (Ca^{2+}) signaling. ORP5/8 have been shown to interact with phosphatidylinositol-4,5-bisphosphate (PIP_2) in the PM, and to modulate mitochondrial respiration and morphology. PIP_2 is the direct precursor of inositol trisphosphate (IP_3), a key second messenger responsible for Ca^{2+}-release from the intracellular Ca^{2+} stores. Further, mitochondrial respiration is linked to Ca^{2+} transfer from the ER to the mitochondria. Hence, we asked whether ORP5/8 would affect Ca^{2+} signaling in these cell compartments, and employed genetically engineered aequorin Ca^{2+} probes to investigate the effect of ORP5/8 in the regulation of mitochondrial and caveolar Ca^{2+}. Our results show that ORP5/8 overexpression leads to increased mitochondrial matrix Ca^{2+} as well as to increased Ca^{2+} concentration at the caveolar subdomains of the PM during histamine stimulation, while having no effect on the cytoplasmic Ca^{2+}. Also, we found that ORP5/8 overexpression increases cell proliferation. Our results show that ORP5/8 regulate Ca^{2+} signaling at specific MCS foci. These local ORP5/8-mediated Ca^{2+} signaling events are likely to play roles in processes such as mitochondrial respiration and cell proliferation.

1. Introduction

Members of the oxysterol-binding protein related-protein (OSBP-related, or ORP) family are involved in the regulation of lipid transport at membrane contact sites (MCS) [1]. MCS are crucial for inter-organellar communication and lipid transfer as well as for the regulation of second messenger molecules, including the calcium ion (Ca^{2+}) [2]. ORPs, including ORP5 and ORP8 (ORP5/8), are involved in oncogenesis, possibly through the regulation of membrane lipids and the related downstream signaling events [3]. ORP5/8 have been characterized as countertransporters of phosphatidylinositol-4-phosphate (PI4P) and phosphatidylserine at the interface of the endoplasmic reticulum (ER) and the plasma membrane (PM) [4]. ORP5/8 were also shown to bind to phosphatidylinositol-4,5-bisphosphate (PIP_2) and modulate its transport from the PM to the ER [5]. Also, it was shown that ORP8 may not bind to P14P and PIP_2 with high affinity [6]. Interestingly, it was recently reported that ORP8 is not strongly associated with the PM in resting conditions but becomes recruited to the PM upon induction of PIP_2 production [7]. Moreover, ORP5/8 were recently found to localize at the ER-mitochondria contact sites where they modulate mitochondrial morphology and function [8]. Approximately 80% of the ORP5 and ORP8 sequences overlap, they both insert into the ER membrane through a C-terminal transmembrane (TM) domain, contain a lipid transfer domain (ORD) and interact with the PM through a pleckstrin homology (PH) domain [5,9]. The mitochondrial interactions of ORP5/8 depend on the ORD domain that binds to the mitochondrial outer membrane protein PTPIP51 [8].

Ca^{2+} is a ubiquitous second messenger that regulates several cellular functions including proliferation, migration and respiration. Intracellular Ca^{2+} concentrations are tightly regulated and multiple organelles and cellular compartments are involved in the handling and sensing of Ca^{2+} signals. Ca^{2+} is stored in the ER from where it can be released to the cytosol upon different stimuli, such as activation of the inositol trisphosphate receptor (IP_3R) [10]. ER Ca^{2+} release can be directed towards the mitochondria through the molecular interactions at contact sites designated as mitochondria-associated membranes

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(MAMs). Mitochondria take up Ca^{2+} through the mitochondrial calcium uniporter (MCU) to activate the respiratory enzymes, whereas mitochondrial Ca^{2+} overloading can lead to apoptosis [11,12]. MCU is tightly controlled by accessory proteins, the threshold Ca^{2+} concentration for MCU activation is high, and hence, ER-mitochondria tethering is essential for the generation of high Ca^{2+} microdomains that allow for MCU activation [12–17]. Also, local, spatiotemporally restricted Ca^{2+} signaling events may take place at cytoplasmic leaflets of the PM and at the lipid-rich PM compartments, caveolae, where many molecules related to Ca^{2+} signaling, such as PIP_2, have been shown to localize [18,19]. Importantly, MAMs and caveolae-ER contacts are implicated in oncogenesis, and Ca^{2+} transfer trough the IP_3R to the mitochondria is essential for cancer cell metabolism [20,21]. Further, ORP5/8 reside at the MAMs, which have been recognized to play a role in cardiovascular pathophysiology in part through disturbed mitochondrial Ca^{2+} signaling [22]. Also, the possible involvement of ORP5/8 in Ca^{2+} signaling has been recently reviewed [23].

Considering that ORP5/8 are involved in the physical interactions and lipid transfer between the ER, the PM and the mitochondria, we hypothesized that this might also affect the Ca^{2+} handling at these membrane interfaces. Further, ORP5/8 modulate the PM levels of PIP_2 [5], which is the substrate for phospholipase C (PLC) that hydrolyzes PIP_2 to IP_3 and diacylglycerol (DAG). IP_3 binds to and activates the IP_3R in the ER, and DAG activates many of the PM Ca^{2+} channels. The ORP5/8 mediated modulation of PIP_2 transport might thus affect intracellular Ca^{2+} signaling. Therefore, we set out to characterize the importance of ORP5/8 in regulating Ca^{2+} specifically in the mitochondria, at the caveolar compartments of the PM, and in the overall cytoplasm.

2. Methods

2.1. Cell culture and plasmid and siRNA transfections

HeLa cells were used in all experiments of this study. The cells were routinely cultured in DMEM (#D6046, Sigma-Aldrich) medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies). The cells were grown in a humidified incubator at 37 °C and 5% CO_2. The GFP-ORP5 expression plasmid is described in [8]. The human ORP8 ORF was inserted in the XbaI site of pEGFP-C1 (Clontech/TakaraBio, Mountain View, CA). ORP5 and −8 specific Silencer® Select siRNAs (s377 and s41692, respectively) and a non-targeting control siRNA (Cat n:o 4390846) were purchased from ThermoFisher Scientific (Waltham, MA). HeLa cells were transfected for 24 h with the GFP-ORP5 or −8 plasmids, or plain pEGFP-C1 as a negative control. TurboFect (#R0531, Thermo Scientific) transfection reagent and OptiMEM media (#31985-070, Life Technologies) were used for all plasmid transfections. siRNA transfections were carried out using HiPerfect transfection reagent (Qiaqen).

2.2. Intracellular calcium measurements by employing aequorin

The luminescent Ca^{2+} binding recombinant protein, aequorin, was employed to measure Ca^{2+} concentrations in the cytoplasm, in the mitochondria, and at the caveolae as previously described in [24,25]. Briefly, 5000–10,000 HeLa cells were seeded on to a 96-well cell culture plate for measurements when a plate reader setup was employed (HIDEX Sense plate reader, HIDEX corp., Turku, Finland). Alternatively, when employing an in-house built single-tube luminometer setup with a perfusion chamber, 75 000–100,000 HeLa cells were seeded on to 13-mm poly-L-lysine coated coverslips in a 12-well plate. The cells were grown overnight and transfected with the desired aequorin plasmids. The measurements were conducted on the following day. Before every measurement, the cells were incubated with 5 μM coelenterazine (SynChem) for one hour in HEPES-buffered saline solution (HBSS: 118 mM NaCl, 4.6 mM KCl, 10 mM glucose, 20 mM HEPES with 1 mM CaCl2 or, alternatively, 150 μM EGTA, when Ca^{2+} free buffer was used). The cells were stimulated with 100 μM histamine in Ca^{2+} containing or in Ca^{2+} free HBSS depending on the experimental setup. The cells were primed for store-operated calcium entry (SOCE) by treating the cells with 1 μM thapsigargin (TG) for five minutes in the presence of 150 μM EGTA whereafter the SOCE was induced by perfusing the cells with HBSS buffer containing 1 μM TG and 1 mM Ca^{2+}. At the end of each experiment, the cells were lysed with 100 μM digitonin in the presence of 10 mM Ca^{2+} to obtain the maximal light production values of the sample. The obtained luminescence raw data values were analysed and transformed as described in [24]. The cytoplasmic and mitochondria targeting aequorin constructs were a kind gift from professor Paolo Pinton (University of Ferrara, Italy).

2.3. Calcium measurements by employing fura-2 AM

For Fura-2 AM measurements 5000–10,000 cells were seeded on to 96-well plates. The cells were grown overnight and transfected with the desired constructs. On the following day, they were washed twice with HBSS and incubated with Fura-2 AM (2 μM, Life Technologies) for 30 min at room temperature. Then, the cells were washed twice and incubated for 15 min in HBSS at room temperature, washed twice and transferred to the HIDEX plate reader. The excitation filters were set to 340 and 380 nanometers, respectively, and the emission was read at 510 nanometers. The fluorescence signal was recorded at one second intervals and the ratios of the two excitation wavelengths were calculated for each time point. These values were used for analysis of the data.

2.4. Western blot analysis

Cell lysates were prepared by washing the cells three times with PBS, whereafter Laemmli sample buffer was added to the samples. The lysates were boiled for 5 min and separated by SDS-PAGE, whereafter the proteins were transferred to a nitrocellulose membrane. The membranes were subjected to the desired primary or secondary antibodies in a phosphate buffered saline (PBS) solution containing 1% bovine serum albumin. The ORP5 antibodies were from Sigma-Aldrich and the ORP8 antibodies are described in [26]. HSC70 antibody was purchased from Enzo life sciences. The dilutions for the primary antibodies were 1:1000 and 1:3000 for the secondary antibodies (anti-rabbit HRP-conjugated antibody, BioRad; anti-rat HRP-conjugated antibody, Cell Signaling Technology). The protein bands were visualized by chemiluminescence (ECL, Perkin Elmer). Anti-Xpress and Anti-Xpress-HRP antibodies (Invitrogen) were employed for the detection of epitope-tagged ORP5/8 constructs that were used in the IP_3 production measurements.

2.5. Measurement of IP_3 production

IP_3 was measured using the HitHunter IP_3 Fluorescence Polarization Assay Kits (DiscoverRx Tech, Fremont, CA, USA). Briefly, 10,000 cells transfected with pcDNA4 HisMax C, ORP5 or ORP8 vector in 96-well plates (Corning, USA) were treated with 10 μM histamine for the designated times, and the cellular reaction was terminated by adding 0.2 N perchloric acid. The plate was shaken at 650 r.p.m. for 5 min. Then 20 ul mixture buffer from 96-well plates were tansferred into black 384-well plates (Greiner, Germany). The IP_3 tracer was subsequently added to each well, and the IP_3 binding protein was added to the plate. The polarized fluorescence from the IP_3 tracer was read on a Microplate Reader (CLARIOstar, BMG LABTECH). The IP_3 concentration was calculated from the IP_3 standard curve.

2.6. Proliferation assay

Fifty-thousand HeLa cells were plated on 6-well plates and grown

overnight. Thereafter the cells were transfected with plasmid constructs carrying GFP, GFP-ORP5 or GFP-ORP8, respectively. The cells were grown for 48 h and subjected to ^{3}H-thymidine (0.4 μCi/ml) for the final four hours. Thereafter the cells were washed three times in ice-cold PBS on ice, followed by a 10-min incubation in 5% trichloric acetic acid, and then incubated for 10 min with 0.1 M NaOH. Then the suspension was mixed 1:5 with Optiphase Hisafe 3 scintillation liquid (PerkinElmer). Radioactivity was measured using a Wallac 1410 liquid scintillation counter. ^{3}H-thymidine was from PerkinElmer.

2.7. Confocal microscopy

The cells were plated on poly-L-lysine coated coverslips, grown overnight and transfected with plasmid constructs carrying GFP, GFP-ORP5 or GFP-ORP8, respectively. The cells were then grown overnight, washed in PBS, fixed using 4% paraformaldehyde and mounted with Mowiol mounting medium. Microscopy was conducted by employing a Leica SP5 (Wetzlar, Germany) confocal microscope.

2.8. Statistics

The results are shown as the mean ± S.E.M. Statistical analysis of the data was conducted by unpaired Student's *t*-test when two means were compared or by One-Way Anova with Tukey's post-hoc test when three or more means were compared. The GraphPad Prism 5 program (GraphPad Software Inc., San Diego, CA) was used for the statistical analyses. A P-value below 0.05 was considered statistically significant.

3. Results

To assess the importance of ORP5/8 in regulating Ca^{2+} in intracellular compartments, we employed the luminescent calcium indicator protein, aequorin. Upon transient expression, wild-type aequorin localizes to the cytosol, but aequorin has been genetically engineered to target different cell compartments such as the mitochondria and the caveolae [24,25]. First, we set out to measure cytoplasmic and mitochondrial Ca^{2+} upon overexpression of GFP-ORP5 or −ORP8 in the presence of extracellular Ca^{2+}. To induce the intracellular Ca^{2+} fluxes we used the G-protein coupled receptor (GPCR) agonist, histamine. Our results show that the cytoplasmic Ca^{2+} was not affected in cells overexpressing either ORP5 or ORP8 when stimulated with 100 μM histamine, whereas ORP5 overexpression resulted in increased mitochondrial Ca^{2+} uptake upon histamine stimulation (Fig. 1A). To further explore the possible involvement of ORP8 in the ER-mitochondria Ca^{2+} flux, we excluded the extracellular Ca^{2+} from the experimental setting. In this condition, ORP8 overexpression significantly increased mitochondrial Ca^{2+} uptake upon stimulation with histamine (Fig. 1B). Treating the cells with 10 μM BAPTA-AM for 1 h completely abolished the mitochondrial Ca^{2+} responses (Supplementary Fig. 1A). Overexpression of the GFP-ORP5/8 constructs was confirmed by microscopy (Fig. 1C). However, knock-down of ORP5 and −8 separately or simultaneously did not affect the stimulation-evoked mitochondrial Ca^{2+} uptake (Fig. 2A). Efficient knock-down of ORP5/8 was confirmed by western blotting (Fig. 2B)

As mentioned above, ORP5 and −8 bind to PIP_2 and are localized to the ER-PM contact sites, and caveolae are enriched in PIP_2 [5,19]. Therefore, we tested whether the caveolar microdomain of Ca^{2+} might be affected by ORP5/8 overexpression or silencing. We found that ORP8 overexpression slightly but significantly increased the histamine-induced Ca^{2+} concentration at the caveolae ($[Ca^{2+}]_{cav}$) in the presence of extracellular Ca^{2+} (Fig. 3A) This effect was more pronounced in the absence of extracellular Ca^{2+} (Fig. 3B). Also ORP5 overexpression significantly augmented the histamine-induced $[Ca^{2+}]_{cav}$ (Fig. 3C). In contrast, knockdown of ORP5/8 did not affect $[Ca^{2+}]_{cav}$ (Fig. 3D).

Caveolae may harbour molecules that are involved in the regulation of the store-operated Ca^{2+} entry (SOCE) [18]. Hence, we investigated whether ORP5/8 would affect $[Ca^{2+}]_{cav}$ during thapsigargin (TG)-induced SOCE. We found that overexpression of ORP5 or −8 was without an effect on SOCE at the caveolar microdomain (Fig. 4A&B). Interestingly, cytoplasmic Ca^{2+} was slightly but significantly inhibited by ORP5 overexpression during TG-induced SOCE (Fig. 4C). Further, ORP5 overexpression inhibited the cytosolic Ca^{2+} flux in an experimental setting where Ca^{2+} was present in the extracellular milieu during the TG treatment (Supplementary Fig. 1B).

Even though we could not detect an effect of ORP5 or −8 on the agonist-induced cytoplasmic Ca^{2+}, we found it interesting to test whether the overexpression of these proteins might modify the release of IP_3 by PLCs. Therefore, the cellular IP_3 concentration was measured upon histamine stimulation. We found that ORP5 overexpression significantly augments IP_3 production whereas ORP8 overexpression was without an effect (Fig. 5).

ORP proteins have been implicated in the control of cell proliferation. It has been shown that ORP4 silencing halts cell proliferation and that ORP4L modulates proliferation through Ca^{2+} and the nuclear factor of activated T cells (NFAT) pathway [27,28]. Also, it is well established that intracellular Ca^{2+} plays key roles in controlling cell proliferation [10]. Hence, we used the ^{3}H-thymidine incorporation assay to quantify the proliferation of GFP, GFP-ORP5 or GFP-ORP8 transfected HeLa cells. Interestingly, we found that the ORP5/8 overexpressing cells showed a significant increase in proliferation as compared to the GFP-transfected controls (Fig. 6).

4. Discussion

In this study we present evidence that elevated cellular levels of the OSBP-related proteins ORP5 or −8 modify Ca^{2+} homeostasis in an organelle-specific fashion. Experiments with caveolae- or mitochondria-targeted aequorin Ca^{2+} probes demonstrated that while neither ORP5 nor −8 overexpression had a significant effect on the cytoplasmic Ca^{2+} concentration in histamine-stimulated cells, ORP5 and −8 elevated the Ca^{2+} concentrations both in the mitochondrial matrix as well as at the caveolar sub-compartments of the PM. The effect of ORP5 on mitochondrial Ca^{2+} was more pronounced than that of ORP8, the effect of which was only detectable when extracellular Ca^{2+} was chelated. Modulation of mitochondrial calcium by ORP5/8 is consistent with the reported disturbance of mitochondrial respiratory function observed in ORP5/8 depleted cells [8], considering that mitochondrial calcium, as well as the proximity of mitochondria to the intracellular Ca^{2+} stores, are crucial for the activity of key mitochondrial machineries responsible for oxidative energy production [21,29,30].

In contrast to what has been reported for ORP4L in T-cell acute lymphoblastic leukemia cells [31], we detected no significant changes in the cytoplasmic Ca^{2+} levels during histamine-stimulation in cells overexpressing ORP5/8. This finding underlines the role of ORP5/8 as regulators and coordinators of local Ca^{2+} signaling events specifically at the MCS at the ER-mitochondria and at the ER-PM/caveolae interfaces. Previously, ORP4L has been reported to augment IP_3 production in T-cells [31]. In our study, we found that ORP5 overexpression leads to increased cellular IP_3 levels during histamine stimulation, whereas ORP8 overexpression did not affect the histamine-induced IP_3 generation. Hence, ORP5 may modulate the detected local Ca^{2+} signaling events in part through IP_3-dependent mechanisms whereas the effect of ORP8 is attributed to other factors. We find it possible that ORP5 overexpression augments mitochondrial Ca^{2+} uptake in part through increased apposition of ER and mitochondria, and that the resulting increase in mitochondrial Ca^{2+} buffering capacity masks the cytoplasmic Ca^{2+} effects that would otherwise be expected due to the ORP5-mediated increase in the IP_3 production. Further, we speculate that ORP5-mediated interactions at the ER-caveolae MCS may allow for the generation of local caveolar Ca^{2+} hotspots at these sites even in the presence of high mitochondrial Ca^{2+} buffering.

As noted above, the effect of ORP8 overexpression on mitochondrial

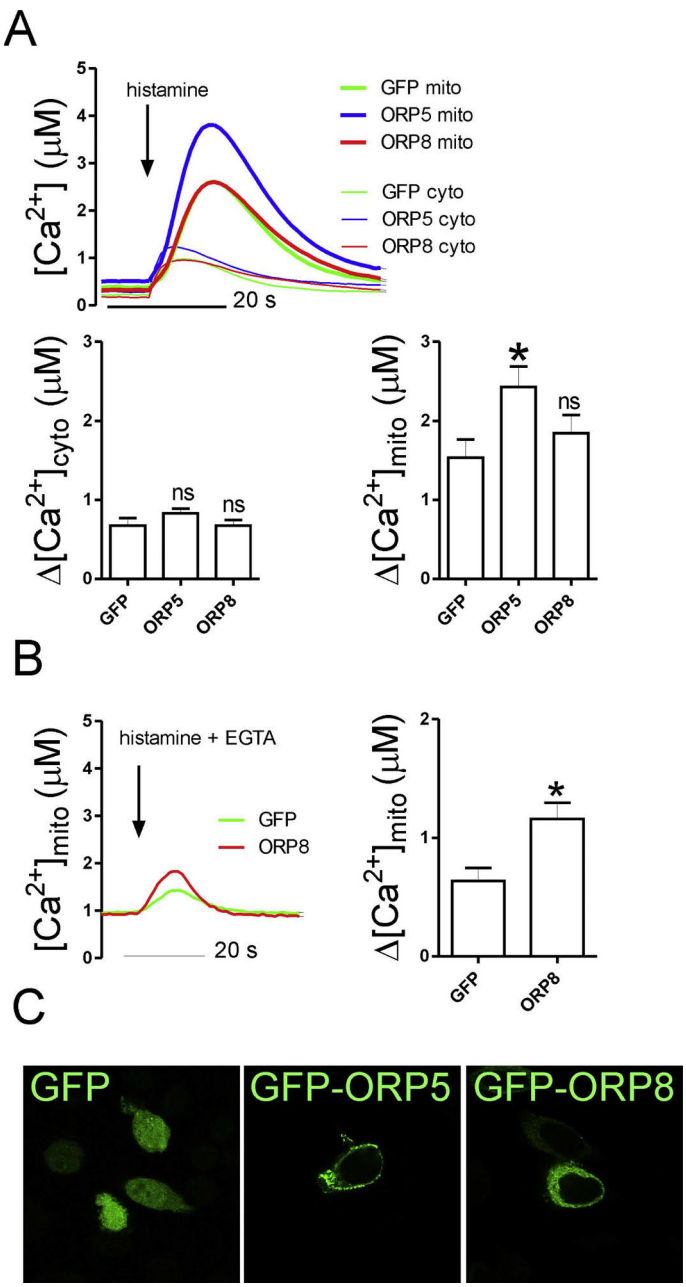

Fig. 1. Mitochondrial, but not cytosolic, $[Ca^{2+}]$ is increased by ORP5/8 overexpression upon 100 μM histamine stimulation. A) Representative traces and quantification of cytoplasmic and mitochondrial Ca^{2+} fluxes during histamine stimulation in the presence of 1 mM Ca^{2+}. B) Mitochondrial Ca^{2+} flux during histamine stimulation in the presence of 150 μM EGTA. C) Confocal microscopy images showing HeLa cells expressing GFP, GFP-ORP5 and GFP-ORP8 constructs, respectively. The bars represent the mean ± SEM of the change in the calcium concentration during the experiment (maximal value – basal value). *, $p < 0.05$; ns = not significant. N = 3–6.

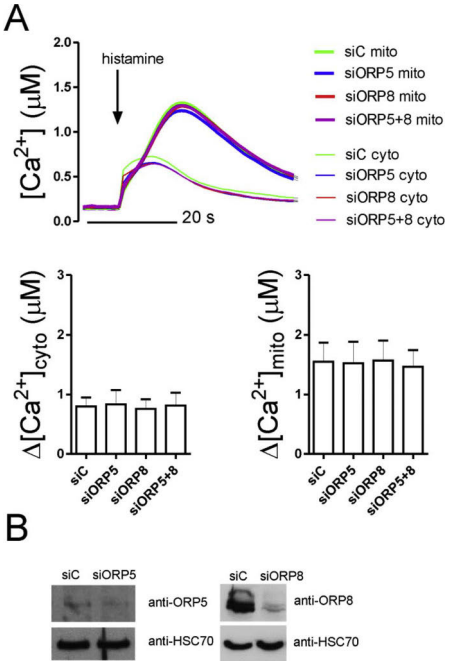

Fig. 2. Downregulation of ORP5/8 does not affect mitochondrial calcium upon 100 μM histamine treatment. A) HeLa cells transfected with control siRNA or siRNAs targeting ORP5, ORP8 or ORP5 and −8 were stimulated with 100 μM histamine in the presence of 1 mM Ca^{2+}. The bars represent the mean ± SEM of the change in the calcium concentration during the experiment (maximal value – basal value). N = 3. B) Representative western blot images showing the effect of ORP5 (left panel) and ORP8 (right panel) targeting siRNAs as compared to the control siRNAs (siC).

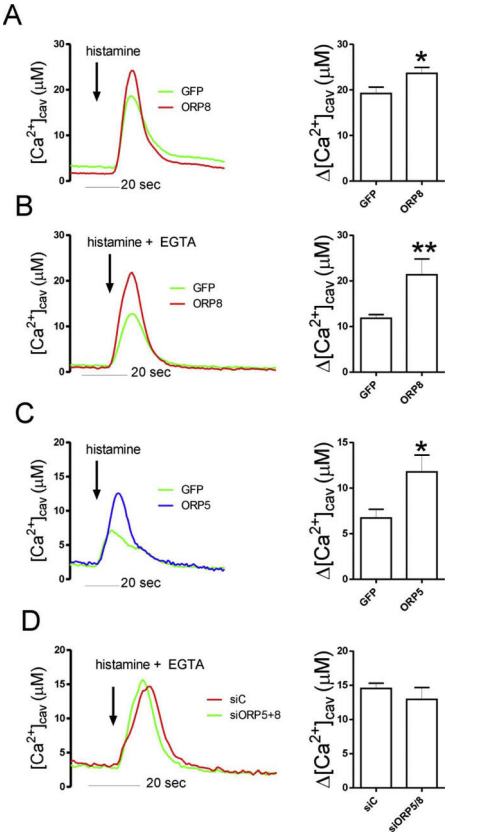

Fig. 3. Histamine-induced Ca^{2+} flux at the caveolar micro-domain is augmented by ORP5/8 overexpression whereas knock-down of ORP5/8 was without an effect. A) ORP8 overexpression increases $[Ca^{2+}]_{cav}$ during 100 μM histamine-stimulation in the presence of 1 mM Ca^{2+}. B) The effect of ORP8 overexpression on $[Ca^{2+}]_{cav}$ is more pronounced in the presence of 150 μM EGTA. C) ORP5 overexpression augments $[Ca^{2+}]_{cav}$ during histamine-stimulation in the presence of 1 mM Ca^{2+}. D) Simultaneous knock-down of ORP5 and −8 is without an effect on $[Ca^{2+}]_{cav}$ during histamine-stimulation in the presence of 150 μM EGTA. The traces are representative and the bars represent the mean ± SEM of the change in the calcium concentration during the experiment (maximal value – basal value). *, $p < 0.05$; **, $p < 0.01$. N = 3-8.

calcium was only detectable in the absence of extracellular Ca^{2+}. This may be explained by the previous finding that ORP8 is more evenly distributed throughout the ER membranes whereas ORP5 is more specifically localized to the MAM MCS [4,8]. Thus, the effect of ORP8 overexpression on mitochondrial Ca^{2+} uptake was unmasked when the extracellular Ca^{2+} was chelated and the IP_3-releaseable ER Ca^{2+} was the primary source of Ca^{2+}. The data suggests that ORP5/8, and ORP5 in particular, has the ability to organize and expand an ER-mitochondrial membrane contact domain at which Ca^{2+} transport is active. The physical association of ORP5/8 with PTPIP51 is consistent with this notion, considering that the mitochondrial outer membrane protein PTPIP51 in a complex with the ER protein VAPB is also reported to control the ER-mitochondria associations [8,32]. Further, ORP5/8 are involved in the modulation of mitochondrial morphology and the integrity of the mitochondrial network, key aspects which are known to affect the regulation of mitochondrial Ca^{2+} [8,33].

It has been reported that ER and PM can form junctions at the caveolae [34]. Also, the interaction of IP_3R1 and −3 with the Ca^{2+}-conducting transient receptor potential canonical cation channels 1 and 3 (TRPC1, −3), respectively, is coordinated at the caveolae [35,36]. Further, caveolae are considered as important signaling hubs that control a wide range of cellular events such as migration, adhesion and invasion, and these processes are modulated by Ca^{2+} signals [18,34]. Our results showing that ORP5/8 modulate Ca^{2+} at the caveolae may thus in part offer new mechanistic insight to the previously reported ORP5/8-mediated regulation of invasion and migration [3,37].

We did not observe disturbances in mitochondrial or caveolar calcium upon siRNA-mediated knock-down of ORP5, −8 or both. This is consistent with the previous observation that inter-organelle MCSs rarely depend on a single tethering component. For example, in yeast the abolishment of the ER-PM contact sites required the genetic disruption of six MCS components [38]. We also find it possible that knocking down the ORPs involved in the vital MCS calcium transport processes will lead to compensatory responses in order to maintain organelle Ca^{2+} homeostasis, which may lead to a dampening of the effect of ORP5/8 knock-down on mitochondrial or caveolar Ca^{2+}. Also, the partial knock-down of ORP5/8 proteins achieved by siRNA treatment may not be sufficient to induce significant effects in the robust Ca^{2+} fluxes that were studied. Further, we found that ORP5/8 overexpression did not affect SOCE at the caveolar microdomain of the plasma membrane. However, cytoplasmic Ca^{2+} was slightly reduced in ORP5 overexpressing cells during TG-induced SOCE. Interestingly,SOCE proteins STIM1 and Orai1 are translocated from the PIP_2-

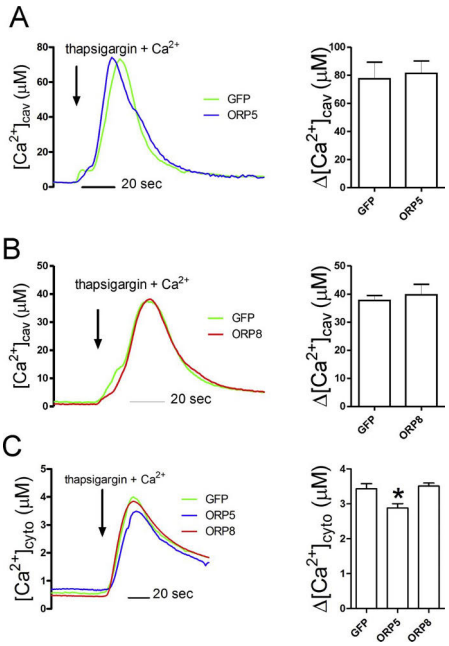

Fig. 4. Store-operated calcium entry at the caveolar sub-compartment is not affected by ORP5 (A) or ORP8 (B) overexpression whereas cytoplasmic Ca^{2+} is reduced by ORP5 overexpression (C). The cells were treated for 5 min with 1 μM TG in HBSS buffer containing 150 μM EGTA and then perfused, as indicated by the arrows, with HBSS buffer containing 1 μM TG and 1 mM Ca^{2+} to induce SOCE. The traces are representative and the bars represent the mean ± SEM of the change in the calcium concentration during the experiment (maximal value – basal value). N = 4–8, *P < 0.05.

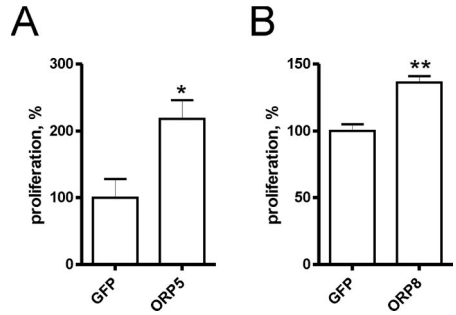

Fig. 6. Overexpression of ORP5 (A) or ORP8 (B) increases cell proliferation. The proliferation of Hela cells transfected for 48 h with plain GFP (control) or the GFP-ORP constructs was determined by ^{3}H-thymidine incorporation during 4 h. The bars represent the mean ± SEM of 3–4 experiments. *p < 0.05, **p < 0.01.

rich PM domains to the PIP_2-poor PM domains upon ER Ca^{2+} depletion, whereas ORP5/8, and especially ORP5, interact with PIP_2 at the PM [5,7,39]. Intriguingly, we find that ORP5 augments histamine-induced Ca^{2+} fluxes whereas cytoplasmic Ca^{2+} during SOCE is reduced upon ORP5 overexpression. These results are not contradictory as histamine-induced Ca^{2+} fluxes and SOCE recruit different proteins and signaling pathways, even if these pathways are interconnected (e.g. SOCE following the IP_3-mediated Ca^{2+} release). Hence, there are kinetic differences in the activation of the histamine-induced Ca^{2+} fluxes and SOCE, SOCE being activated through STIM and ORAI oligomerization in response to the IP_3-mediated ER emptying [40,41].

We also found that ORP5/8 overexpression increases the proliferation of HeLa cells. This is in line with the similar findings regarding ORP4L [28]. Further, ORP5 overexpression is associated with tumor cell invasion [3] and proliferation (unpublished observations disclosed in ref. [3] and published during the revision of the present article [42]). In contrast to our finding, ORP8 has been linked to inhibition of cell growth in gastric cancer cells through induction of ER stress and to initiation of apoptosis in hepatocellular cancer cells through the Fas-pathway [43,44]. Interestingly, Ca^{2+} is essential for the initiation of Fas-mediated apoptosis [45], and ER stress, as well as the regulation of apoptotic events are associated with ER-mitochondria Ca^{2+} transfer [46]. On the other hand, Ca^{2+} handling at the ER-mitochondria interfaces is involved in the regulation of energy metabolism and cell proliferation [47]. Therefore, we find it possible that ORP8-mediated changes in mitochondrial Ca^{2+} homeostasis may modulate cell fate in a context-dependent manner.

In conclusion, our results provide evidence for the importance of ORP5/8 in regulating Ca^{2+} in the mitochondrial matrix as well as at the caveolar sub-compartments of the PM. These findings corroborate the role of ORP5/8 as MCS proteins facilitating organelle interactions at specific membrane interfaces.

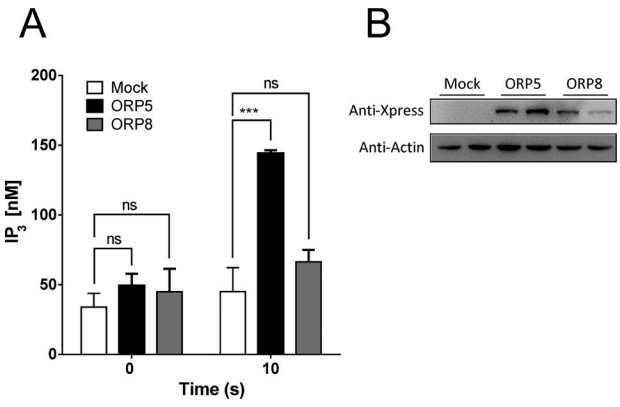

Fig. 5. Overexpression of ORP5 increases cellular IP_3 levels during histamine stimulation whereas ORP8 overexpression is without an effect. The IP_3 concentration was measured from unstimulated cells (time point 0), and at 10 s after histamine stimulation (A). The expression of epitope-tagged recombinant ORP5 and ORP8 was confirmed by western blot using anti-Xpress epitope antibodies (B). ***P < 0.001, N = 3.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ceca.2018.03.001.

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Ilari Pulli

Calcium Signaling in Cell Compartments

- the Importance of Sphingosine Kinase 1 and ORP5/8

To maintain life, the cells within an organism employ intricate signaling pathways to regulate physiological processes within single cells and between the various cells and tissues that constitute an organism. Regulation of this kind is termed cell signaling. One of the key molecules in cell signaling is the calcium ion, Ca^{2+}. Many central biological processes, such as muscle contraction, fertilization, cell proliferation, cell migration, cellular energy production and cell death, are modulated by Ca^{2+} signaling. Dysfunctional Ca^{2+} signaling may contribute to the development of various disease conditions such as cancer and neurological disease. In this thesis, Ca^{2+} handling in specific cellular compartments, mainly the mitochondria and the caveolae, was studied. The mitochondria are organelles that are key in cellular energy production, that contribute also to other important processes such as Ca^{2+} signaling and cell death. The caveolae are small invaginations of the cell membrane with important functions, for instance as orchestrators of cell signaling events. The results of this thesis show that the proteins that were the key subjects of this study, sphingosine kinase 1 and oxysterol-binding protein 5 and 8, respectively, are involved in the regulation of intracellular Ca^{2+} signaling at specific cellular compartments – the mitochondria and the caveolae.

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