Insights into the Regulation of Store-Operated Calcium Entry

by

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Abstract

Calcium (Ca^{2+}) ions serve a crucial role in numerous intracellular signaling pathways, controlling physiological functions as diverse as cell proliferation, immune system function, and muscular contraction. As such, the cell has evolved many different mechanisms to precisely control the movement of Ca^{2+} ions into and out of various cellular compartments. One such pathway is called store-operated Ca^{2+} entry (SOCE), which couples an initial depletion of endoplasmic reticulum (ER) luminal Ca^{2+} stores with Ca^{2+} influx across the plasma membrane into the cytoplasm. SOCE is controlled by two major proteins, STIM1 and Orai1. STIM1 senses ER luminal Ca^{2+} levels and transduces this signal across the ER membrane by migrating to subplasmalemmal punctae, where it activates Orai1, a plasma membrane Ca^{2+} channel that allows Ca^{2+} flux across the plasma membrane. SOCE is of key importance to human health; as such, a more complete understanding of how the cell controls SOCE will yield deeper insights into how the pathway might be manipulated for therapeutic purposes.

The research in this thesis focuses on novel cellular mechanisms to control SOCE, specifically focusing on protein-protein interactions of STIM1. We used a variety of biochemical and cell biological techniques to discover novel protein binding partners of STIM1 and to elucidate the functional consequences of these interactions. First, we discovered that ERp57, an ER luminal oxidoreductase, binds to the luminal domain of STIM1. ERp57 binding is dependent upon two highly conserved cysteine residues

within STIM1 and this binding serves to inhibit the initiation of SOCE. We hypothesize that ERp57-STIM1 interactions may act as a brake upon SOCE to prevent its overactivation. Importantly, the triggering of SOCE is known to occur within the ER lumen, and our results were the first published description of a protein-protein interaction within the ER luminal domain of STIM1. Second, we discovered that the cytoplasmic domain of STIM1 is subject to calpain cleavage. This cleavage occurs in healthy cells to regulate the abundance of STIM1: inhibition of calpain cleavage increases STIM1 levels. Additionally, STIM1 cleavage is upregulated during programmed cell death. We hypothesize that the interplay between STIM1 and calpains is a mechanism to control the cellular abundance of STIM1 and that perturbations of this mechanism may lead to dysregulation of STIM1 protein levels. Finally, we discovered that a previously described STIM1-calnexin interaction is of key importance in immune cells. Calnexin-deficient T cells exhibit severely impaired SOCE, which may be due to impaired movement of STIM1 to subplasmalemmal punctae or increased susceptibility to calpain cleavage. Calnexin's effects on SOCE seem to be specific to immune cells, suggesting that the calnexin-STIM1 interaction could be one mechanism by which the immune system strengthens SOCE, where a strong, sustained Ca^{2+} influx is so important. Overall, the research presented in this thesis describes three unique protein binding partners of STIM1 and explains how each interaction serves to regulate SOCE. Any alterations in these interactions may underlie dysregulated SOCE in disease; conversely, interventions targeting these protein binding partners could serve as methods to manipulate SOCE for therapeutic outcomes.

Preface

The literature review presented in chapter 1 of this thesis is my original work.

Chapter 2 of this thesis has been published as Prins, D., Groenendyk, J., Touret, N., and Michalak, M, 2011, "Modulation of STIM1 and capacitative Ca^{2+} entry by the endoplasmic reticulum luminal oxidoreductase ERp57", *EMBO Rep.* 12: 1182-8. J. Groenendyk performed the experiments and analyzed the data presented in Figure 2-1B. I performed all other experiments and data analysis, and also prepared and edited the manuscript. M. Michalak was the supervisory author and was involved in manuscript preparation.

The data and analysis presented in chapters 3 and 4 are my original work, as is the concluding analysis presented in chapter 5.

Acknowledgments

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The members of the Michalak laboratory have been an invaluable resource throughout my graduate career, both in terms of research and in my personal life. It has been a great comfort to know I always have such talented and generous people around me, always willing to help out with an experiment that still won't work after a dozen trials or with data that seem to defy explanation. I will consider myself lucky if my future research environments are even half as collegial.

My supervisory committee members, Dr Larry Fliegel and Dr Nicolas Touret, have been an invaluable resource in guiding my research and thesis preparation. I would like to thank them both for helping to supervise my research over the years, for our scientific collaborations, and for being so willing to provide numerous references. In addition, I thank Dr Gary Eitzen and Dr Jonathan Lytton for taking the time to serve on my PhD defense committee.

I also gratefully acknowledge the funding agencies that have granted me awards over my graduate career- Alberta Innovates- Health Solutions (formerly Alberta Heritage Foundation for Medical Research) and the Canadian Institutes of Health Research. It is a great honor to have received such prestigious awards.

No person is an island and so I want to acknowledge my family and friends who have supported me during my research career. To my parents, Phil & Judy, I want to say how much I appreciate the support I've received over the years, whether it was a temporary loan, a home-cooked meal, or career and life advice. I owe so much of who I am today to your example. To my brothers and sisters, it is difficult to imagine five siblings have more divergent career paths than the five of us, but I've always been able to count on you for encouragement and distraction in the form of Nintendo 64 and Ticket to Ride. To my friends, both inside of and outside of the world of science, I appreciate that you have always been there to help me not dwell on the bad days of research and celebrate the good ones.

Finally, I would be remiss not to thank Tom Brady and the Super Bowl XLIX champion New England Patriots for inspiring me thoughout my graduate career and the occasionally arduous thesis preparation process.

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List of Abbreviations

BiP	Immunoglobulin binding protein
CaM	Calmodulin
CaN	Calcineurin
CAPN	Calpain
CAST	Calpastatin
CFP	Cyan fluorescent protein
CIF	Calcium influx factor
CNX	Calnexin
CRAC	Calcium-release activated channel
CRACM1	Calcium release activated channel modulator
CRACR2A	Calcium release activated channel regulator 2A
CRT	Calreticulin
DNA	Deoxyribonucleic acid
EB1	End binding protein 1
ECCE	Excitation coupled calcium entry
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
ERp57	Endoplasmic reticulum protein of 57 kDa
FRET	Forster resonance energy transfer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GRP94	Glucose-regulated protein of 94 kDa
GST	Glutathione S-transferase
IP_3	Inositol trisphosphate
IP_3R	Inositol trisphosphate receptor
IRE1	Inositol responsive element 1

Lck	Lymphocyte-specific protein tyrosine kinase
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MW	Molecular weight
NCX	Sodium-calcium exchanger
NFAT	Nuclear factor of activated T cells
Ni-NTA	Nickel nitrilotriacetic acid
NK	Natural killer
NMR	Nuclear magnetic resonance
NSCLC	Non-small cell lung cancer
Orai1	Calcium release activated channel 1
P0	Myelin protein zero
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly ADP ribose polymerase
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PMCA	Plasma membrane calcium ATPase
PMP22	Peripheral myelin protein 22
POST	Partner of STIM1
RNA	Ribonucleic acid
SAM	Sterile alpha motif
SARAF	SOCE-associated regulatory factor
SDS	Sodium dodecyl sulfate
SERCA	Sarco/endoplasmic reticulum calcium ATPase
siRNA	Small interfering ribonucleic acid
SOAR	STIM1-Orai1 activating region
SOCE	Store-operated calcium entry
SOCI	Store-operated calcium influx
STAT3	Signal transducer and activator of transcription 3

STIM1	Stromal interaction molecule 1
TAM	Tubular aggregate myopathy
TG	Thapsigargin
TM	Transmembrane
TRP	Transient receptor potential
TRPC	Transient receptor potential, canonical
VSMC	Vascular smooth muscle cells
YFP	Yellow fluorescent protein

CHAPTER ONE

Introduction

1.1 The endoplasmic reticulum and calcium

The endoplasmic reticulum is an organelle consisting of interconnected tubules that is responsible for diverse functions, including synthesis of transmembrane and secretory proteins as well as quality control of their folding[1,2]. Most notably for this thesis, it is the major Ca^{2+} storage organelle within the cell[3,4], with a total Ca^{2+} concentration on the order of 1-3 mM and a free Ca^{2+} concentration of approximately 200-400 μM[4,5]. The majority of Ca^{2+} within the ER lumen is buffered by a variety of luminal Ca^{2+}-binding proteins[6]. Calcium (Ca^{2+}) ions serve an integral role in cellular function, especially as a second messenger for many cellular signaling processes. As such, maintaining ER Ca^{2+} homeostasis is key to the proper functioning of numerous Ca^{2+} signaling pathways, and so the movement of Ca^{2+} into and out of the ER is of key importance, as is depicted in Figure 1-1. Ca^{2+} can be released from the ER by several regulated pathways; for example, the inositol trisphosphate receptor (IP_3R) releases Ca^{2+} from the ER into the cytoplasm in response to the binding of IP_3[7]. The reverse of this process, Ca^{2+} uptake into the ER, is primarily the responsibility of the sarco/endoplasmic reticulum Ca^{2+} ATPase SERCA[8]. Ca^{2+} can also be moved out of the cytoplasm into the extracellular milieu by plasma membrane proteins such as the sodium-calcium exchanger (NCX) and the plasma membrane Ca^{2+} ATPase (PMCA)[8]. Elevations in cytoplasmic Ca^{2+} concentrations can trigger numerous signaling pathways, including control of the immune system, muscular contraction, and fertilization[9]. As Ca^{2+} signaling can control so many different cellular pathways- as varied as regulation of gene transcription, muscular contraction, and control of immune function- it is absolutely essential that the cell carefully regulate Ca^{2+} movement, both spatially and temporally. Some signaling pathways require a strong Ca^{2+} signal, both in amplitude and duration, and this strong Ca^{2+} signaling is frequently the responsibility of a pathway known by various names, including store-operated calcium influx (SOCI), calcium-release activated channels (CRAC), and store-operated calcium entry (SOCE). While there are slight different between the exact

meaning of these terms, for the purposes of these thesis, the term SOCE will be preferred.

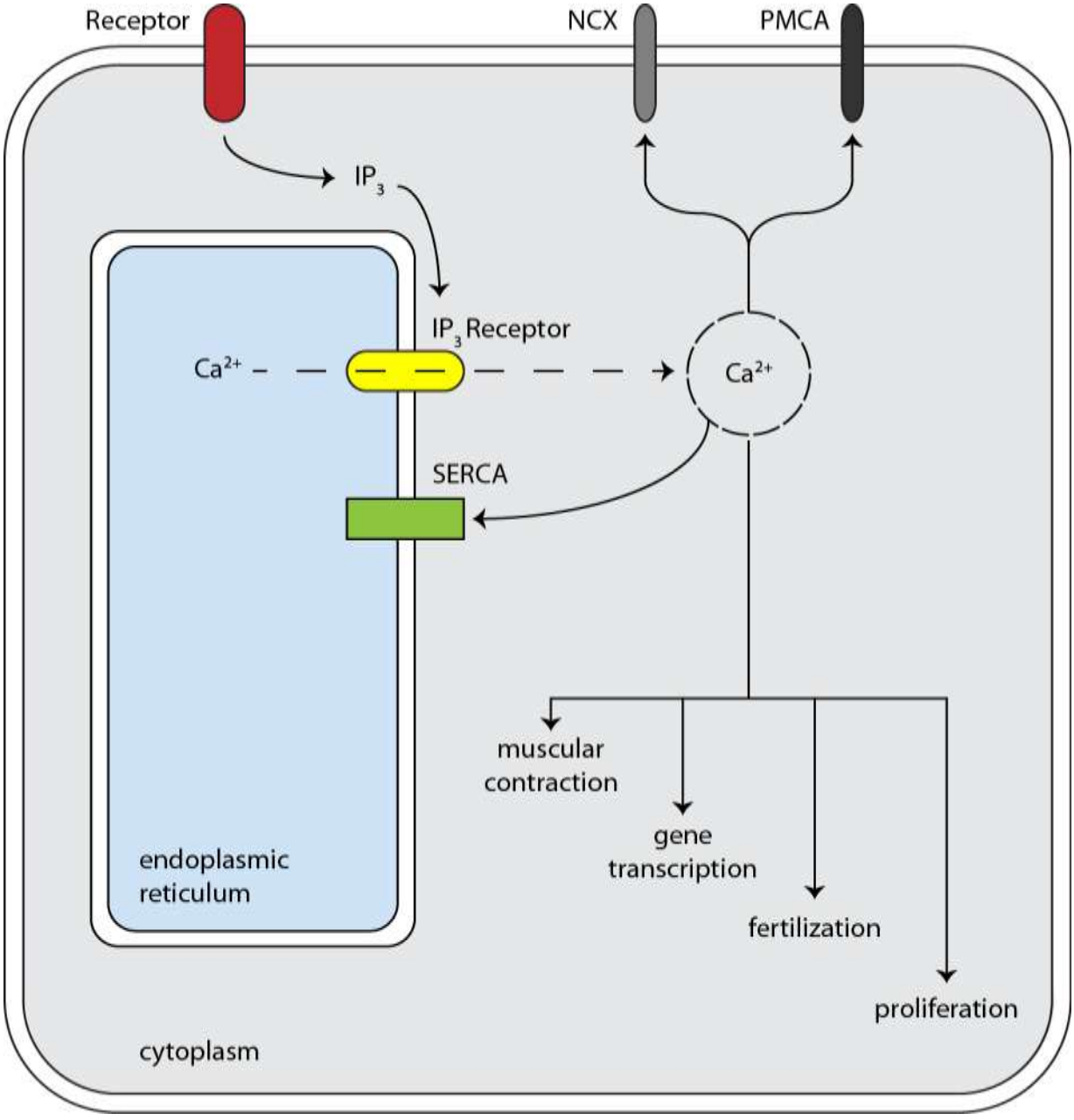
Receptor
NCX
PMCA
IP3
IP3 Receptor
Ca2+
Ca2+
SERCA
muscular contraction
gene transcription
fertilization
proliferation
endoplasmic reticulum
cytoplasm

Figure 1-1. Intracellular calcium dynamics. A condensed view of some pathways taken by Ca^{2+} ions in the cells is presented. At top left, a signal is received by a plasma membrane receptor, leading to production of inositol trisphosphate (IP_3). IP_3 binds to the IP_3 receptor on the endoplasmic reticulum, allowing for Ca^{2+} release from the ER lumen into the cytoplasm. Cytoplasmic Ca^{2+} can trigger various downstream signaling pathways, including muscular contraction, gene transcription, fertilization, and proliferation. In order to terminate the Ca^{2+} signal, Ca^{2+} ions can either be taken up into the ER via the actions of SERCA or it can be moved across the plasma membrane into the extracellular milieu via the actions of NCX and PMCA.

1.2 Calcium storage and buffering

As mentioned previously, the ER serves as the principal intracellular Ca^{2+} store, with a total concentration on the order of 1 mM and a free concentration in the range of 200 μM[5]. The remainder of ER Ca^{2+} is buffered by a variety of proteins, which will be discussed briefly. One of the most important ER Ca^{2+} buffers is a 46 kDa protein called calreticulin, which serves as a chaperone to ensure correct folding of glycoproteins and is also responsible for buffering up to half of ER Ca^{2+} in non-muscle cells[10,11]. Structurally, Ca^{2+}-binding is found within the C-domain of calreticulin, which contains abundant negatively charged amino acid residues[11]. Calreticulin is an absolutely essential protein: its absence is embryonic lethal due to impaired cardiogenesis, characterized by thin ventricular walls and aberrant myofibrillogenesis[12]. This defect can be traced back to calreticulin's role within Ca^{2+} signaling, as calreticulin-deficient cells showed no nuclear translocation of nuclear factor of activated T-cells (NFAT), which is normally activated in response to the Ca^{2+}-dependent phosphatase calcineurin[12]. Expression of constitutively active calcineurin rescued cardiogenesis[13]. Overexpression of calreticulin increased readily mobilizable ER Ca^{2+} stores[14], and, when targeted to the mouse heart, overexpression of calreticulin was lethal due to heart block[10]. Interestingly, recent results linked a series of mutations in the C-terminus of calreticulin with a family of cancers called myeloproliferative neoplasms. All these mutations were predicted to disrupt the amino acid sequence of calreticulin's C-terminus; as this would almost certainly affect calreticulin's Ca^{2+} signaling abilities, these results suggest a link between aberrant Ca^{2+} control and cancer progression[15].

BiP (immunoglobulin binding protein) is another ER luminal protein with dual functions in both control of protein folding and Ca^{2+} buffering[16,17]. It is thought to buffer approximately one quarter of ER luminal Ca^{2+}[17]. GRP94 (glucose-regulated protein of 94 kDa), also residing within the ER lumen, binds Ca^{2+} at up to fifteen sites of variable affinity[18,19,20]. Protein disulfide isomerase (PDI) is an ER luminal protein; as its name

suggests, it is responsible for isomerization of disulfide bonds. However, it also plays a role in Ca^{2+} buffering[19,21,22], and, intriguingly, its PDI activity may be Ca^{2+}-regulated[23]. Overall, it is clear that the importance of the ER as an intracellular Ca^{2+} storage organelle is reflected in the variety of proteins cells possess to buffer this Ca^{2+}. It is interesting to note the close relationship between Ca^{2+} binding proteins and protein chaperones, pointing to an inextricable link between these two essential functions of the ER.

2.1 Store-operated calcium entry

The process of SOCE has been known in the calcium community for almost thirty years, being first described by Putney in 1986. He and his colleagues described a mechanism that coupled the loss of Ca^{2+} from the ER into the cytoplasm (emblematic of many Ca2+ signaling pathways) with Ca^{2+} influx across the plasma membrane into the cytoplasm[24]. However, the molecular identities of the proteins mediating this process escaped identification for another twenty years. In 2005, a protein called stromal interaction molecule 1 (STIM1) was shown to be required for SOCE[25,26], followed by the identification of calcium release activated channel (Orai) family proteins in 2006[27]. The subsequent decade of research elucidated the intricate choreography of these two proteins. Though STIM1 and Orai1 are necessary and sufficient for SOCE to occur[28], the literature has also described the involvement of numerous other players. A cartoon version of the process of SOCE is depicted in Figure 1-2.

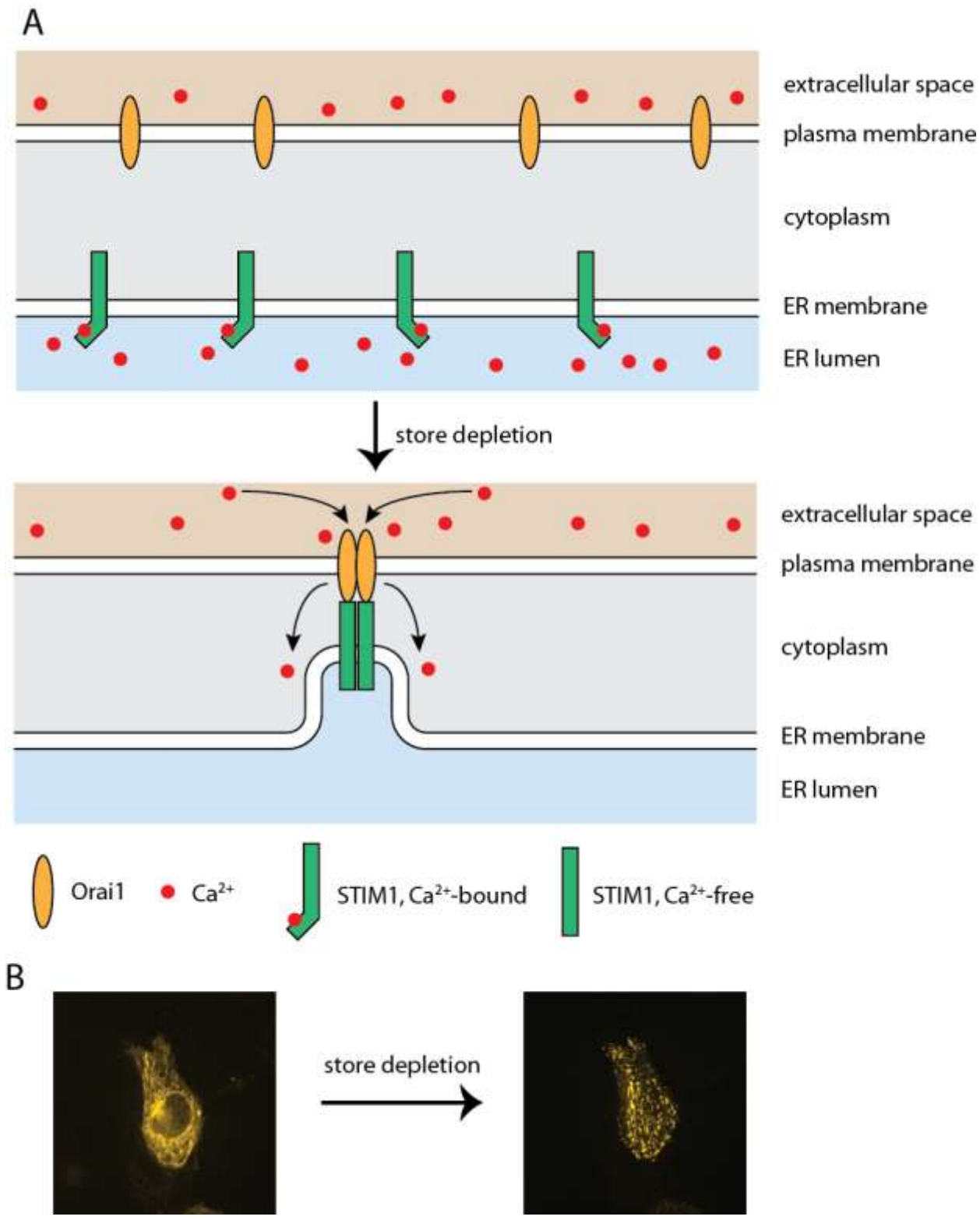
A
extracellular space
plasma membrane
cytoplasm
ER membrane
ER lumen
store depletion
extracellular space
plasma membrane
cytoplasm
ER membrane
ER lumen
Orai1
Ca2+
STIM1, Ca2+-bound
STIM1, Ca2+-free
B
store depletion

Figure 1-2. Store-operated calcium entry. (A) A cartoon depicting SOCE shows, at top, a resting cell, where STIM1 is bound to Ca^{2+} and held diffusely throughout the endoplasmic reticulum membrane, away from the plasma membrane. At bottom, after store depletion, Ca^{2+}-free STIM1 homooligomerizes, translocates to subplasmalemmal punctae, and binds to Orai1. This STIM1-Orai1 binding activates the Ca^{2+} channel activity of Orai1, allowing Ca^{2+} to enter the cell. (B) A cell expressing YFP-tagged STIM1 shows YFP-STIM1 at rest (left panel, diffusely distributed throughout ER) and after store depletion (right panel, within subplasmalemmal punctae).

2.2 STIM1

STIM1 was first identified as playing a role in SOCE via two RNA interference screens, one in *Drosophila* cells[25] and one in human cells[26]. Interestingly, STIM1 was first identified in literature (under the name SIM, or stromal interaction molecule) as a plasma membrane molecule capable of interacting with pre-B cells[29]. Publications on STIM1 were sparse until its identification as a constituent of SOCE, but further research in this period showed it to have a close homologue (named STIM2)[30], found that STIM1 could act as a tumor suppressor gene[31,32], and continued to localize it as a plasma membrane protein[31,33]. However, in describing STIM1's role in SOCE, Liou et al. instead found that a YFP-tagged STIM1 construct co-localized with endoplasmic reticulum markers and that antibodies against YFP (which would be in the putative extracellular domain, were STIM1 found in the plasma membrane) did not recognize any protein in intact cells. The authors further discovered that YFP-STIM1 was diffusely distributed throughout the ER when ER Ca^{2+} stores were replete, but quickly moved to subplasmalemmal punctae after Ca^{2+} store depletion[26]. Roos et al., working in both *Drosophila* and human cells, corroborated the importance of STIM1 in SOCE and showed STIM1 suppression impaired SOCE, via both cytoplasmic Ca^{2+} and patch-clamp experiment[25]. Though the subsequent decade of research has added much to our understanding of the nuances of STIM1 function and control, the extent to which these early papers presaged the accepted model of a decade later is remarkable.

Structurally, STIM1 is a 90 kDa single-pass transmembrane protein that is resident in both the ER and the plasma membrane (as shown in Figure 1-3). Its N-terminus is within the ER lumen and is characterized by several important domains, most notably a Ca^{2+}-binding EF-hand and a sterile α-motif (SAM)[30]. STIM1 then possesses a membrane-spanning domain and a long cytoplasmic region, which contains coiled-coil domains and regions rich in serine, proline, and lysine residues[34]. As the Ca^{2+}-binding EF-hand is within the ER lumen, it is perhaps unsurprising that this serves as the Ca^{2+} sensing

feature of STIM1: mutations within the EF-hand region were shown to eliminate STIM1's sensitivity to Ca^{2+} and render the protein constitutively active[26]. This EF-hand folds cooperatively with the SAM, yielding a domain called the 'EF-SAM motif' that is responsible for the initiation of SOCE. Structural work using nuclear magnetic resonance (NMR) showed that when cells are at rest and ER Ca^{2+} stores are replete, the EF-SAM motif folds in a manner so as to shield a series of hydrophobic residues from the ER lumen. When ER Ca^{2+} stores are depleted, Ca^{2+} necessarily dissociates from the EF hand; this in turn yields a conformational change in the EF-SAM motif that exposes hydrophobic residues. Exposure of these residues promotes the homo-oligomerization of the luminal domains of STIM1, which is the triggering event of SOCE[35]. Indeed, when the luminal domain of STIM1 is replaced with protein domains whose homo-oligomerization can be pharmacologically forced, it has been shown that this oligomerization event is necessary and sufficient to initiate SOCE[36]. If the luminal domain of STIM1 is instead deleted, these STIM1 mutant proteins are unable to translocate to subplasmalemmal punctae[37]. After aggregation of the luminal domains of STIM1, the interactions between multiple copies of STIM1 are strengthened by the cytoplasmic, coiled-coil domains[38]. In the Ca^{2+}-bound condition, STIM1 exists as an inactive dimer, as shown by the presence of Förster resonance energy transfer (FRET) signal between YFP-STIM1 and CFP-STIM1 even at rest[39,40]. Thus, the homo-oligomerization triggered by Ca^{2+} dissociation manifests itself as the creation of higher order oligomers.

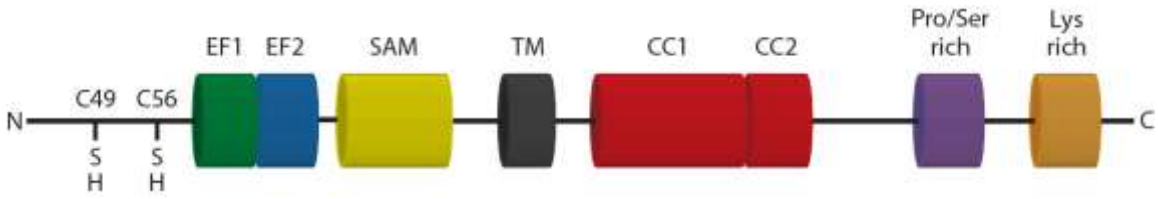

Figure 1-3. STIM1. A cartoon depicting structural features of STIM1 is shown. The luminal domain of STIM1 is characterized by two highly conserved cysteine residues, Ca^{2+}-binding EF-hands, and a sterile α-motif (SAM). The EF hands and SAM fold cooperatively into what is called the EF-SAM motif, which is responsible for triggering SOCE. STIM1 has a single transmembrane domain, several coiled-coil (CC) regions, and Pro/Ser and Lys-rich regions. The coiled-coil domain of STIM1 further mediate its homooligomerization.

After oligomerization, STIM1 clusters migrate to subplasmalemmal punctae, which are portions of the ER that are closely apposed to the plasma membrane[26]. Oligomerization precedes translocation: evidence of oligomerization can be seen within five seconds of store depletion, while formation of punctae takes on the order of 40 seconds[40]. Furthermore, STIM1 lacking its lysine-rich region can oligomerize as efficiently as its wild-type counterpart, but is completely unable to migrate to punctae[40]. This lysine-rich, highly basic region of STIM1 may be required because of its interaction with negatively charged phospholipids at the plasma membrane[41]. Thus, it is possible to distinguish the oligomerization and translocation of STIM1 as two separate steps within SOCE.

The movement of STIM1 has been well-studied. When ER Ca^{2+} stores are full, STIM1 moves constitutively throughout the ER in a microtubule-dependent manner[37], which requires STIM1 binding to the microtubule plus-end tracking protein EB1[42] via a SxIP motif[43]. During SOCE, STIM1 moves to distinct subplasmalemmal punctae that seem to appear at pre-determinated locations: repetitive stimulation of cells yields punctae at the same locations[44,45]. The formation of these punctae does not rely on microtubules, as disruption of microtubule dynamics prevented neither the formation of punctae nor SOCE itself[42]. Ultrastructural analysis showed STIM1 within three distinct subcompartments of the ER, termed the pre-cortical ER, cortical ER, and thin cortical ER[46]. Pre-cortical ER elements may correlate with regions of the ER where STIM1 is moving in conjunction with microtubules, while the cortical and thin cortical ER regions may arise in response to ER Ca^{2+} depletion and thus correspond to STIM1-enriched punctae[46]. Intriguingly, the cortical ER excludes ER proteins such as BiP and GFP-KDEL, suggesting that this subdomain may have Ca^{2+} signaling as its sole responsibility, and hence plays no role in other ER processes such as protein folding[46].

Clustering of STIM1 within recruits Orai1 to ER-plasma membrane junctions (at rest, Orai1 is diffusely distributed throughout the plasma membrane)[47]. STIM1 and Orai1 have been shown to directly interact, as determined via FRET signal between STIM1-

YFP and Orai1-CFP[39]. The identification of the region of STIM1 that couples with Orai1 has been targeted by several different groups and consensus finds this region within the CC2 and CC3 domains of STIM1, with the specific amino acid residues variously reported as 344-442[48], 233-474[49], 342-448[50], and 339-444[51]. This region has been given various names, but here I will refer to it as SOAR (STIM-Orai activating region), as it was christened in one early report[48]. Expression of SOAR alone in cells is sufficient to open the Orai1 Ca^{2+} channel[48], and SOAR and Orai1 interact *in vitro*, suggesting no requirement for any other protein[50]. A crystal structure of SOAR revealed that it exists as a dimer and suggested that at rest, the active site is masked via interactions with the CC1 domain of STIM1, a potential auto-inhibitory mechanism to prevent premature activation of Orai1[52]. Indeed, further studies using intramolecular FRET[53] and Tb^{3+}-acceptor energy transfer[54] contributed to a model whereby Ca^{2+} dissociation from the EF-hand causes a conformational shift that favors intermolecular CC1-CC1 interactions over intramolecular CC1-SOAR interactions. The unlocking of the SOAR domain favors the extension of the cytoplasmic domain of STIM1, permitting SOAR-Orai interactions and thus SOCE[53,54].

While the vast majority of research has focused on STIM1, a closely related homologue, STIM2, is present in humans[30]. STIM2 shares high homology with STIM1, particularly with respect to its N-terminal domain and first two coiled-coil regions, but is notably different in regions downstream from these CC regions. STIM2 has been shown to have a higher Ca^{2+} dissociation constant than does STIM1 (ca. 400 μM for STIM2 vs ca. 200 μM for STIM1), suggesting it is more sensitive to ER luminal Ca^{2+} concentration[55]. STIM2 suppression reduced Ca^{2+} concentrations in both the ER lumen and the cytoplasm, giving it a role in the maintenance of Ca^{2+} homeostasis[55]. However, the research on STIM2 to date has not achieved a consensus as to its roles within SOCE: in some cell types, STIM2 contributes to SOCE while in other cell types STIM2 expression instead inhibits SOCE, as reviewed by Lopez et al[56]. STIM2 expression levels are highly tissue-dependent and is particularly abundant in neurons, where STIM2 and not STIM1 seems

to be the pre-dominant isoform. As such, in mouse neurons, STIM2 but not STIM1 is the key protein mediating SOCE[57]. Overall, the literature to date on STIM2 suggests that it plays a minor role in SOCE as compared to its better studied counterpart, STIM1. STIM2's significance may be limited to certain roles (such as maintenance of basal cytoplasmic calcium levels) or certain cell types.

In addition to the canonical STIM1, a new splice variant of STIM1 was discovered in 2011 and named STIM1L[58]. STIM1L is longer than canonical STIM1 at 115 kDa and is of especial importance in muscular tissue, where it interacts with actin to form permanent STIM1L clusters that co-localize with Orai1 even in cells where ER Ca^{2+} stores are replete[58]. These constitutive STIM1L clusters are required to support repetitive Ca^{2+} release that underlies physiological muscular contraction[58]. Experiments expressing STIM1L in fibroblasts did not show these permanent STIM1L clusters, indicating that the actin-mediated clustering is likely specific to muscle tissue[59]. Furthermore, STIM1L was unable to remodel the previously described cortical ER but was still able to mediate SOCE, suggesting remodeling of the cortical ER is not necessary for SOCE to occur[59].

Though STIM1 and Orai1 alone are sufficient to recapitulate SOCE in liposomes[28], many other proteins have been described to have roles in controlling SOCE. It is somewhat surprising that there is a dearth of ER luminal proteins that have been shown to bind to STIM1 and/or regulate SOCE; as the initiating step of SOCE occurs within the ER lumen, this would seem to be a logical way for the cell to increase its control over SOCE. Nevertheless, our group has described the binding of ERp57 to STIM1[60], which will be discussed in greater detail later in this thesis. SOCE is also regulated by several ER transmembrane proteins. As an example, STIM1 associates with SERCA2 (sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase) at punctae in a store-dependent manner, which could indicate a mechanism to link Ca^{2+} entry into the cytoplasm with uptake into the ER so as to refill ER Ca^{2+} stores[61,62]. Interactions between calnexin and STIM1 have been described in the literature, but not studied extensively[63]; some novel data on interactions

between calnexin and STIM1 will be presented later in this thesis. A new binding partner of STIM1 was identified in 2011 and given the imaginative name of POST (partner of STIM1)[64]. POST binds STIM1 more tightly post-store depletion and also recruits a variety of other calcium-handling proteins, including SERCA and PMCA (plasma membrane Ca^{2+} ATPase), two proteins that will typically remove Ca^{2+} from the cytoplasm (to the ER and extracellular milieu respectively). Interestingly, POST was shown to inhibit PMCA activity, which suggests that it may act to coordinate many different Ca2+ handling pathways in order to closely control the movement of Ca2+ within and throughout the cell[64]. The ER transmembrane protein junctate also binds STIM1 and Orai1 and is proposed to play a role in defining the ER-plasma membrane appositions that are at the heart of calcium entry[65]. SARAF (SOCE-associated regulatory factor) is a ER transmembrane protein thought to bind to STIM1 to facilitate slow, Ca2+-dependent inactivation of SOCE; this mechanism exists to prevent overloading of Ca2+ into cells, which can be deleterious[66].

STIM1, Orai1, and SOCE are known to be bound by, and controlled by, many cytoplasmic proteins. The protein calcium-release activated channel regulator 2A (CRACR2A) is found within the cytoplasm, binds Ca^{2+}, and forms a ternary complex with STIM1 and Orai1; this complex is thought to stabilize the binding of STIM1 and Orai1[67]. Calmodulin (CaM) binds to the cytoplasmic domain of STIM1[68] and is involved in fast Ca^{2+}-dependent inactivation of SOCE[69]; though this may be due to CaM acting upon Orai channels, not STIM1 itself[70]. A recent report indicates that calcineurin (CaN), a calcium-calmodulin regulated phosphatase, may dephosphorylate STIM1 as a mechanism to regulate SOCE in endothelial cells[71]. Cytoplasmic Homer proteins can enhance an interaction between STIM1 and $Ca_V1.2$ channels[72], which are voltage-activated Ca^{2+} channels whose gating is suppressed by STIM1 binding[73]. Septins, GTP-binding filamentous proteins, are associated with the plasma membrane and promote the proper organization of membrane domains required for STIM1-Orai1 association. Suppression of septin expression impairs communication between STIM1 and Orai1 and

thus downregulates SOCE[74]. A plasma membrane anchored protein called Golli can inhibit SOCE in T cells[75] via interactions with STIM1[76].

2.3 Post-translational modification of STIM1

As with so many eukaryotic proteins, STIM1 is a target for a variety of post-translational modifications, including phosphorylation, glycosylation, ubiquitination, and addition of S-linked glutathione. One of the earliest reports on STIM1 described it to be a phosphoprotein[33]; the subsequent years of research into STIM1 phosphorylation have shown it to be phosphorylated at many different sites by many different kinases for many different reasons. During meoisis, STIM1 is blocked from clustering to initiate SOCE; though this block correlates with hyperphosphorylation of STIM1, the available evidence suggests that phosphorylation does not cause this block[77]. By contrast, during mitosis, STIM1 is again inactivated with respect to SOCE and is again phosphorylated, but here preventing phosphorylation is sufficient to restore SOCE, suggesting that phosphorylation is in fact sufficient to inhibit SOCE during mitosis[78]. Further investigation revealed that STIM1 phosphorylation is required to properly exclude the ER from the mitotic spindle via termination of the interaction between STIM1 and EB1[79]. A subsequent report showed that STIM1 is phosphorylated by extracellular signal regulated kinases 1 and 2 (ERK1/2) and this phosphorylation enhances the interaction between STIM1 and Orai1 and hence, SOCE[80]. These phosphorylation sites are close to the TRIP motif that mediates the binding between STIM1 and EB1, with phosphorylation serving to disrupt the interaction between STIM1 and EB1, a key step in the initiation of SOCE[81], echoing earlier reports of phosphorylation disrupting the STIM1-EB1 interaction[79]. In addition, ERK phosphorylation of STIM1 specifically at Ser575 is critical for the promotion of myogenesis by STIM1[82]. Resveratrol, a natural plant product found within red wines, has had numerous health benefits attributed to its functions; intriguingly, it was recently reported to inhibit SOCE in HEK293 by disrupting the ability of ERK1/2 to phosphorylate STIM1[83]. 17β-estradiol also inhibits

STIM1 phosphorylation and SOCE in human bronchial epithelial cells[84]. ERK1/2 phosphorylation can be triggered by epidermal growth factor (EGF) and promotes *in vitro* migration of human endometrial adenocarcinoma cells[85]. Within platelets, STIM1 phosphorylation at as of yet unidentified tyrosine residues may occur within a few seconds of store depletion and act to enhance the binding between STIM1 and Orai1[86]. Overall, as with so many aspects of store-operated calcium entry, the data concerning phosphorylation of STIM1 indicate that phospho-regulation of this pathway is likely to be highly dependent on circumstances, particularly timing. During mitosis and meoisis, STIM1 phosphorylation correlates with and, for mitosis, is required to inhibit SOCE. By contrast, in many non-mitotic/meoitic cell types, STIM1 phosphorylation, particularly by ERK1/2, has been shown to enhance SOCE.

STIM1 has two N-linked glycosylation sites within its N-terminal domain; early reports on STIM1 indicated it is indeed a glycoprotein[31]. STIM1's binding to calnexin, a lectin-like chaperone, is not glycosylation-dependent[63]. Several mutational analyses of the importance of N-linked glycosylation have been performed, with occasionally contradictory results. Mutation of both asparagine 131 and 171 to glutamine (N131Q/N171Q) was shown to have no effect on SOCE currents in HEK293 cells[87]. Conversely, in the neuronal cell line NG115, that same STIM1 mutant was shown to be unable to initiate Ca^{2+} entry, though the authors of this paper were working from the now disfavored calcium influx factor (CIF) model, in which SOCE was hypothesized to be mediated by a diffusible cytoplasmic second messenger rather than direct binding of STIM1 and Orai1[88]. A more recent study showed that in HEK293 cells, the N131Q/N171Q mutant was ineffective in initiating SOCE, while a N131D/N171Q mutant demonstrated highly upregulated SOCE currents. The authors suggest that the N131D mutation better mimics the natural conformation of the STIM1 luminal domain; thus, prevention of glycosylation does not necessarily impair STIM1 function but may in fact predispose STIM1 to homooliogerize, thereby increasing function[89]. STIM1 has been shown to be ubiquitinated, but the authors of this study concluded that STIM1 was not

subject to proteasomal degradation[90]. STIM1 is also modified via S-linked glutathione at Cys56[91]. In conclusion, STIM1 is subject to numerous post-translational modifications under different cellular conditions. These modifications can be activating or inhibitory towards the process of SOCE, indicating that post-translational modification is one of many strategies the cell uses to control the essential process of SOCE.

2.4 Orai family proteins

The second key component of SOCE, Orai1 (and its homologues Orai2 and Orai3), was discovered in 2006. A genome-wide siRNA screen in *Drosophila* cells identified a protein named CRAC modulator 1 (CRACM1) as playing a key role in SOCE[92]. CRACM1 was later shown to be the plasma membrane Ca^{2+} channel itself and named Orai1. Though the protein's full name is calcium-released activated channel, the term Orai has a more creative provenance: it comes from Greek mythology, where the Orai are the keepers of the gates of heaven[27]. Similarly to STIM1, Orai1 is known to homooligomerize, where the functional Orai1 channel is thought to exist as a hexamer, as shown via a crystal structure of *Drosophila* Orai[93], confirming earlier observations of detergent-solubilized Orai1 eluting from size exclusion chromatography at the approximate MW of a hexamer[50]. Atomic force microscopy measurements confirmed the hexameric state of Orai1 and indicated that STIM1 binds Orai1 with sixfold symmetry[94]. The Ca^{2+} channel pore is lined with residues from transmembrane helix 1 (TM1) of Orai1[95,96]; of these residues, E106 is critical for ensuring high Ca^{2+} selectivity and thus its mutation noticeably affects Orai1 gating and permeability[97,98]. The Orai crystal structure confirms that the E106 residues serve as a selectivity filter for Ca^{2+}[93].

Interactions between STIM1 and Orai1 have been pinpointed to two sites within Orai1, one in the N-terminus[50] and one in the C-terminus[28,50]. The interactions within the C-terminus have been more completely described and rely on coiled-coil interactions between a helical domain within Orai1[93] and coiled coil domains within the SOAR of

STIM1 itself[39,99]. The C-terminal helical domain within Orai1 is very similar to a helical domain within STIM1, leading to a hypothesis that at rest, the SOAR is bound to an autoinhibitory region of STIM1. Upon activation, the C-terminus of Orai1 competes away the binding of this inhibitory helix, allowing for productive STIM1-Orai1 interactions[100,101]. Interactions between STIM1 and Orai1, within both the N- and C-termini of Orai1, are required for the trapping of the Orai1 protein as well as its gating[102]. Evidence suggests that STIM1 first binds to the Orai1 C-terminus, followed by docking of STIM1 onto the N-terminus of Orai1[103]. At rest, the Orai1 pore is occluded by a short non-polar segment of Orai1, but STIM1 binding induces a conformational shift that removes this barrier from the pore, allowing for ion flux[104]. A mutation in Orai1, R91W, has been shown to abrogate CRAC channel activity[27]; this lack of channel activity is due to impaired channel gating due to an inability of Orai1 to undergo this conformational change in response to STIM1 binding[104].

The other two Orai family members, Orai2 and Orai3, are noticeably less well studied than Orai1; indeed, there is a review whose title refers to Orai2, Orai3, and STIM2 as the "neglected CRAC proteins"[105]. Orai2 is widely expressed throughout various mouse tissues[106] and has similar CRAC channel activity to Orai1[105]. However, its contribution to endogenous SOCE remains unclear. In murine cells, overexpression of STIM1 and Orai1 noticeably upregulated SOCE, but STIM1/Orai2 overexpression had no such effect. Moreover, siRNA-mediated knockdown of Orai2 protein had no effect on endogenous SOCE currents in HEK293 cells, pointing against a role for Orai2 in classical SOCE pathways[106]. The Orai1 R91W mutation completely abrogates SOCE in T cells, suggesting that endogenous Orai2 is insufficient to recapitulate Ca2+ entry, though this could be due to a dominant negative effect of the Orai1 mutant on Orai2 multimer channels[27]. Orai3 can also function as a CRAC channel, but has some slightly different properties to those of Orai1 and Orai2[105]. In HEK293 cells, siRNA targeting Orai3 was able to reduce endogenous SOCE, suggesting that Orai1 and Orai3 may both be required for proper Ca2+ entry[106]. Overall, the role of Orai2 seems to be minimal in most

situations, but Orai3 may be of importance in particular cell types and systems, as will be discussed in the following sections.

2.5 TRPC ion channels

While the overwhelming body of evidence suggests that Orai family proteins, particularly Orai1, are both sufficient and necessary for SOCE, there is some evidence that other ion channel proteins may be involved. The transient receptor potential (TRP) proteins, especially the canonical TRP (TRPC) subfamily, are of particular interest. TRP proteins are ion channels; they are non-selective, allowing entry of both monovalent and divalent cations, in contrast to Orai family members, which are highly selective for Ca^{2+} ions[107]. Some authors have shown that STIM1 can interact directly with and gate TRPC1[108], with this interaction mediated by electrostatic interactions between K684/685 of STIM1 and D639/640 of TRPC1[109]. This contrasts with STIM1-Orai1 interactions, which do not depend upon electrostatic interactions[93,109]. Furthermore, there are reports of TRPC1 and Orai1 binding to each other within the plasma membrane in a complex that can be activated in two different ways. The TRPC1/Orai1 complex can allow for Ca^{2+} entry in a receptor-activated, diacylglycerol-mediated manner (i.e. non-store operated) or in response to activation by STIM1 (i.e. store-operated)[110]. Differential activation of the TRPC1/Orai1 complex may also depend on its location within the membrane: store-operated Ca^{2+} entry may occur within lipid rafts, while receptor-operated Ca^{2+} entry does not require lipid rafts[111,112]. However, a subsequent report showed that TRPC channel activation depended only upon phospholipase C-dependent mechanisms, with no role for STIM1[113]. These contradictory reports could have several different explanations, including different cell types and expression levels of the various proteins involved. As such, the controversy over a role for TRPC channels within SOCE has not been completely resolved.

3.1 SOCE in health and disease

Store-operated calcium entry is central to numerous physiological processes; as such, its absence or misregulation have been implicated in many disease states. The clinical relevance of SOCE is underscored by the numerous correlations between aberrant SOCE and human disease. These findings largely sort into four physiological systems, each of which will be discussed in greater detail:

(i) immunity and immunodeficiencies;

(ii) blood clotting and platelet function;

(iii) muscular function and development; and

(iv) within numerous cancers, particularly concerning metastatic behavior.

Loss-of-function mutations within STIM1 and Orai1 are known to have deleterious effects on immune system function and can manifest as immunodeficiency. Conversely, mutations that enhance SOCE tend to have their largest effects on other blood cells, including platelets, as well as muscles. Finally, upregulation or downregulation of STIM1 and Orai1 have been identified in a plethora of cancers, suggesting that changes in the abundance of these proteins, and the consequent changes in SOCE, can have important effects on cancer development. STIM1, Orai1, and SOCE have recently been implicated in a fifth disease, pancreatitis: here, Orai1-mediated cytoplasmic Ca^{2+} entry was shown to contribute to pancreatitis, and Orai1 blockers had a protective effect[114]. This discovery is very recent, and as of this writing, only one paper has been published on the involvement of SOCE with pancreatic disease.

3.2 SOCE in the immune system

The classical system for studying SOCE is within immune cells, and so it is no surprise that one of the earliest demonstrations of its importance in human health came within this system. An insertion within the gene for STIM1, causing a frameshift and premature truncation of the protein, has been implicated in immunodeficiencies, where SOCE is

non-functional[115]. A splice site mutation within the *STIM1* gene was shown to eliminate STIM1 mRNA and protein expression in B cells, leading to a fatal susceptibility to Kaposi sarcoma[116]. A homozygous point mutation within STIM1, R429C, impaired SOCE activity in T cells and was associated with poor natural killer cell function[117]. This defect in immune cell function was later traced to a structural defect within STIM1 that prevents the oligomerization of the cytoplasmic domain of STIM1 and blocks its binding to Orai1[118]. Similarly, mutations to the *Orai1* gene that destabilize either the mRNA or protein lead to minimal expression of Orai1, absent SOCE, and immunodeficiency[119]. A point mutation within Orai1, R91W, abolishes SOCE but has no effect on Orai1 mRNA and protein levels; this mutation is thought to instead affect gating of the CRAC channel[27]. In all reported cases, heterozygosity with respect to mutant Orai1 has no clinical phenotype, suggesting a single copy of the *Orai1* gene is sufficient to maintain immune system function[119]. To date, no reports of mutations within the genes encoding STIM2, Orai2, or Orai3 have been reported as causing disease, underlying the importance of STIM1 and Orai1 within SOCE and immune function.

In general, SOCE has been shown to be dispensible with respect to T cell development: the overall numbers of T cells and their distribution into various subsets are generally unaffected by the absence of STIM1[120,121] or Orai1[119,122]. Nevertheless, T cell proliferation and function are severely impaired whenever SOCE is dysfunctional. Both STIM1 and STIM2 are important for proper functioning of T cells, where STIM1 is crucial during the initial Ca^{2+} influx while STIM2 maintains high Ca^{2+} influx[55,120], critical for translocation of nuclear factor of activated T cells (NFAT) to the nucleus[123]. T cells from human patients with STIM1 mutations were less able to proliferate in response to stimulation with an anti-CD3 antibody[115]. In mice, T cell specific deletion of both STIM1 and STIM2 impaired the development and function of Treg ($Foxp3^+$) cells[120]. Natural killer (NK) cells from patients who lack STIM1 or Orai1 have impaired SOCE and demonstrate impaired cytokine synthesis and failed exocytosis of cytotoxic granules[117,124]. Overall, the roles of STIM1, Orai1, and SOCE within the immune system are crucial not at the development

stage of immune cells, but rather at the activation stage. Impaired SOCE affects the ability of cells to properly function within the immune system, manifesting as a clinical phenotype of immunodeficiency and autoimmunity.

3.3 SOCE and platelet function

Ca^{2+} serves as a key second messenger within platelets and its mobilization is controlled by STIM1 and SOCE. Some of the earliest evidence of STIM1's importance within platelets came from a mouse model expressing a constitutively active mutant of STIM1, D84G. Homozygosity for this mutation was embryonic lethal due to excessive bleeding, while heterozygous mice exhibited thrombocytopenia due to preactivation of platelets[125]. Both platelets and their precursor cells, megakaryocytes, have high levels of STIM1 and Orai1 mRNA and exhibit CRAC currents with similar characteristics to the currents measured in immune cells[126]. Mouse genetic models have also been used to investigate the roles of SOCE within platelet function. As STIM1 and Orai1 deletion lead to perinatal lethality, many of these studies used bone marrow chimeric mice, where bone marrow from a knockout mouse is injected into an irradiated recipient mouse[127]. Orai1-deficient bone marrow chimeric mice demonstrated impaired SOCE, as expected; this deficiency also resulted in defective platelet activation and affected thrombus formation *in vivo*[127]. As such, mice whose platelets lack Orai1 were resistant to arterial thrombosis and ischemic brain infarction[127]. A similar study used a mutated form of Orai1, R93W, that is known to cause immunodeficiency in human patients[27], and generated chimeric mice expressing only mutant Orai1 in blood cells. Platelets from these mice exhibited no SOCE, but showed no deficiencies with respect to *in vitro* thrombosis[128]. STIM1-deficient mice also demonstrated perinatal lethality in approximately 70% of mice; platelets from the survivors showed severely abrogated SOCE[129]. STIM1-deficient bone marrow chimeric mice had deficiences in arterial thrombosis and thus, ischemic brain infarction[129]; these results were very similar to what was seen with Orai1-deficient bone marrow chimeric mice. In mice where STIM1 was

specifically deleted in megakaryocyte-platelet lineages, platelets showed impaired SOCE but had no problems with *ex vivo* thrombus formation, though formation of arterial thrombi was impaired[130].

Though the data concerning SOCE and platelets do not always align completely, it is reasonable to conclude that SOCE is important for platelet function at least to some extent. The work done in mouse model systems was validated by the discovery of novel STIM1 mutations within Stormorken Syndrome, an extremely rare genetic disease characterized by increased bleeding, miosis, and tubular aggregate myopathy. These results verify that problems with SOCE also have an effect on blood clotting in humans[131,132]. Furthermore, STIM1 mutations have been associated with York platelet syndrome, which is associated with thrombocytopenia (lowered platelet counts) and myopathy[133]. Interestingly, an R304W mutation in STIM1 was identified in both Stormorken syndrome[132] and York platelet syndrome[133]; why this same mutation is associated with two different (albeit similar) syndromes is as of yet unclear. While there may be variability in its importance depending on injury type and assay used, the central role of SOCE in platelets warrants the concept of STIM1 and Orai1 as anti-platelet targets. As one preliminary example, pharmacological inhibitors of SOCE can block Ca^{2+} entry in platelets and, when administered to mice, can partially block thrombus formation and hence ischemic brain infarction in a murine model of stroke[134].

3.4 SOCE and muscle

The final physiological system that will be discussed in this introduction is muscle-specifically, the key roles of STIM1 and SOCE in proper muscular function. Proper cycling of Ca^{2+} is central to muscular contraction, and dysregulation of any of the pathways controlling Ca^{2+} homeostasis can manifest as muscle disease[135]. One of the earliest indications that SOCE was key for muscular development and function came from a STIM1-deficient mouse, which exhibited perinatal lethality due to myopathy[136].

Without STIM1, impaired SOCE results in impaired muscle differentiation *in vitro* and *in vivo*. Mice that were heterozygous for STIM1 did not show skeletal myopathy, but had contractile defects under conditions of high muscle usage[136]. The role of excitation-coupled Ca^{2+} entry (ECCE) is well-known in muscle cells; however, STIM1 knockdown and Orai1 mutations had no effect on ECCE, suggesting that ECCE and SOCE are separate biological pathways[137]. During their change from a contractile phenotype to a proliferative phenotype, arterial isolated myocytes display a marked increase in STIM1 and Orai proteins, with a resultant increase in SOCE[138]. Vascular smooth muscle cells (VSMC) showed similar results: as freshly isolated cells were cultured, STIM1 and Orai1 were upregulated with consequent higher SOCE[139]. Knockdown of STIM1 or Orai1 could inhibit VSMC proliferation and migration *in vitro*, supporting a key role for SOCE in the development of these cells[139,140]. Subsequent investigations confirmed and extended these results into animal models: injury of rat or mouse arteries led to an upregulation of STIM1 and Orai1 protein within VSMC[141]. Moreover, siRNA-mediated knockdown of STIM1 could abrogate VSMC proliferation *in vivo*, as assayed by monitoring the development of neointima in injured rat carotid arteries[140,141,142]. STIM1 and Orai1 also have roles in cardiomyocytes, regulating both normal[143] and hypertrophic growth[143,144]. In animal models, artificially increasing STIM1 levels led to higher levels of hypertrophic growth both *in vitro* and *in vivo*; conversely, lowering of STIM1 levels prevented cardiac hypertrophy[145,146]. Intriguingly, the development of cardiac hypertrophy has also been shown to correlate with the formation of a macromolecular complex containing STIM1, Orai1, and Orai3, with Orai3 being responsible for voltage-independent Ca^{2+} entry in these cells[147].

The crucial roles of STIM1, Orai1, and SOCE in correct muscular development and function were first discovered in cell lines and animal models, but the correlation was strengthened by additional findings in humans. Tubular aggregate myopathy (TAM) is a muscular disease characterized by regular arrays of membrane tubules that can arise from genetic causes or as a secondary consequence of other muscular diseases, such as

alcohol-induced myopathy. An initial report of four separate mutations in STIM1 were found in four families with members who suffered from TAM; all four mutations were inherited in a dominant autosomal fashion. The four mutations- D84G, H109N, H109R, H72Q- were all found within the EF-SAM domain of STIM1 and all resulted in constitutive activation of STIM1[148]. Further studies of TAM patients confirmed the H109R mutation and discovered a novel I115F mutation to cause TAM[149]. A larger scale study discovered four new mutations within the EF-SAM domain (N80T, L96V, F108I, F108L) to cause TAM, bringing the total to nine[150]. Of note is the fact that all nine mutations are found within a relatively small region of the EF-SAM domain, suggesting that this is a mutational hotspot within the *STIM1* gene. As well, all nine mutations result in constitutive clustering of STIM1, suggesting that all these mutants have the same mechanism of action- i.e. inability to properly sense ER Ca^{2+} levels. A previously discussed gain-of-function mutation in STIM1, R304W, has been identified to cause Stormorken Syndrome, which has TAM as one of its effects[131,132,151]. A gain-of-function mutation in Orai1, P245L, also causes TAM, but interestingly has no effect on any hemotological systems[151]. Two further TAM-associated Orai1 mutations, G98S and L138F, were discovered in 2014; both cause constitutive activation of SOCE[152]. Interestingly, glycine 98 is found within TM1 of Orai1 and its mutation has previously been reported to alter Orai1 channel activity[103,153], providing a parallel between basic laboratory discoveries and clinical findings.

Overall, the connections between STIM1 hyperactivation and TAM are clear, though some questions still remain. STIM1 and Orai1 loss-of-function mutations have their greatest effects in the immune system, but the studies on STIM1 and Orai1 gain-of-function mutations have not described any alterations to immune function (though the authors did not explicitly describe any investigations into the immune system). Similarly, while some activating mutations cause only TAM, others have wider effects and cause Stormorken syndrome, which includes not only TAM but also

thromocytopenia and miosis. The mechanisms behind these differences have yet to be completely elucidated.

3.5 SOCE and cancer

The roles of STIM1 and SOCE within cancer have been studied over the past decades; indeed, even before STIM1 was known to play a role within SOCE, deletion of STIM1 was shown to be associated with human rhabdomyosarcoma[32]. These results suggested STIM1 may serve as a tumor suppressor, a hypothesis supported by newer results indicating that impaired SOCE is associated with resistance to apoptosis in prostate[154] and colon cancer cells[155]. However, the body of evidence available to date instead overwhelmingly suggests that in the vast majority of cancers, STIM1, Orai1, and SOCE behave to increase cancer progression and metastasis. For example, in human and rat glioblastoma cell lines, SOCE seems to promote cancer survival, as knockdown of STIM1 or Orai1 was associated with lower tumor cell proliferation and increased apoptosis[156]. A genome wide study of human glioblastoma patients showed higher expression of the *STIM2* gene, which could, in theory, be related to increased cancer cell proliferation[157]. Samples from human glioblastoma multiforme revealed a significant upregulation of SOCE and that knockdown of STIM1 or Orai1 could reduce the invasive behavior of these cancer cells[158,159]. There is some evidence that increased STIM1-Orai1 mediated SOCE is associated with increased metastasis of lung cancer, as to the brain[160]. Non-small cell lung cancer (NSCLC) biopsies show increased levels of Orai3, and inhibition or knockdown or Orai3 could reduce cell proliferation *in vitro*, suggesting a key role for Orai3 in regulation of NSCLC growth[161]. Within colorectal cancer, STIM1 protein levels are higher in cancerous tissue than surrounding, healthy tissue. Moreover, high STIM1 levels correlate with increased metastatic behavior and poorer overall survival[162,163]. Highly metastatic melanoma samples show increased STIM1 and Orai1 protein and higher SOCE[164,165]; knockdown of STIM1 levels reduced the ability of melanoma cells to metastasize to lungs in mouse xenograft models[164]. Upregulation of

STIM1 and Orai1 contribute to cisplatin resistance in ovary carcinoma cells[166]; similarly, elevations in the abundance of STIM1 and Orai1 in pancreatic adenocarcinoma cells lines increase the resistance of these cells to apoptosis in response to chemotherapy[167]. Orai1 levels are elevated in clear cell renal cell carcinoma; the consequent increase in SOCE promotes cell migration and proliferation[168]. Lentiviral downregulation of STIM1 at the mRNA and protein levels reduced the proliferation of hypopharyngeal carcinoma cells and promoted their apoptosis[169].

Currently, the most intensively studied links between SOCE and human cancer are found within cervical and breast cancers. In human patients with cervical cancer, STIM1 protein levels were elevated in tumor cells as compared to nonneoplastic tissue in 71% of patients[170]. Furthermore, elevated STIM1 levels are associated with increased tumor size, a higher likelihood of metastasis, and a poorer clinical outcome[170]. An investigation of breast cancer patients also revealed a role for SOCE: breast cancer cell lines had elevated levels of Orai1, and a higher STIM1:STIM2 ratio was associated with the poorest prognoses[171]. Interestingly, there is also evidence for the involvement of Orai3 in breast cancer, as Orai3 mRNA levels were increased in breast cancer tissue as compared to healthy tissue and contributed to the survival and proliferation of cancerous but not nonneoplastic cells[172]. The connection between Orai3 and cell proliferation is mediated by the c-myc pathway[173], and knockdown of Orai3 via shRNA impeded the growth of breast cancer cell line-derived tumors *in vivo*[174]. This shift from Orai1- to Orai3-dependence may be related to differences between estrogen receptor positive and negative cancers. Estrogen receptor positive cancers use Orai3 to mediate SOCE, while their estrogen receptor negative counterparts use the canonical Orai1 instead[175]. Indeed, knockdown of estrogen receptor α reduces Orai3, suggesting that ERα regulates Orai3 expression, likely at the mRNA level[174]. This preference of ER+ breast cancer for Orai3 over Orai1 suggests that an Orai3-specific inhibitor would show great promise in the treatment of ER+ breast cancer[174]. Indeed, use of a polymersome to deliver siRNA targeting Orai3 was able to impede the growth of T47D cancer cell lines but leave non-

cancerous cells untouched[176], a promising sign of future developments targeting Orai3. This shift from Orai1 to Orai3 may not be exclusive to breast cancer: in prostate cancer, Orai3 expression is increased, and Orai3 heteromerizes with Orai1 to form a novel, store-independent Orai1/Orai3 channel that promotes proliferation[177].

Despite the importance of Orai3 in ER+ breast cancer, the importance of Orai1 cannot be overlooked. Knockdown of either STIM1 or Orai1 in the breast cancer cell line MDA-MB-231 reduced cell migration *in vitro* and metastasis of these cells to secondary tumors *in vivo*[178]. Intriguingly, metastatic behavior could also be reduced pharmacologically via the non-selective SOCE blocker SKF96365[178]. Conversely, in the breast cancer cell line MCF-7, Orai1 mediates Ca^{2+} influx in a store-independent manner. In an unusual finding, the secretory pathway Ca^{2+} ATPase 2 (SPCA2), which is highly upregulated in cancerous tissue, was shown to activate Orai1, allowing for constitutive Ca^{2+} signaling that promoted proliferation and anchor-independent growth[179]. Other results in MCF-7 cells showed that the endothelial-mesenchymal transition is associated with downregulation of Oct4, causing an upregulation of STIM1, Orai1, and hence, SOCE. Increased SOCE leads to higher migration of MCF-7 cells, while knockdown of STIM1 impairs both migration and EMT[180].

Overall, the studies of STIM, Orai family members, and SOCE in cancers have shown that protein levels, and hence strength of SOCE, are dysregulated in a wide variety of cancer types. In the vast majority thereof, protein constituents of SOCE are upregulated, with resultant increases in cell proliferation and survival. A recent study confirmed that Orai1 and Orai3 expression increased the proliferation of HEK293 cells *in vitro*. Interestingly, this study also demonstrated that this upregulation of proliferative behavior occurred even without external Ca^{2+} or with non-Ca^{2+} conducting mutants of Orai1/Orai3[181]. These results raise the possibility that Orai proteins may not be acting solely as Ca^{2+} channels, but may play other roles in the promotion of cell proliferation.

In conclusion, the STIM1-Orai1-SOCE axis is indispensible in a variety of physiological systems as diverse as immunity, muscular contraction, and cancer metastasis; thus, the cell must carefully regulate the process of SOCE. If SOCE is weakened or eliminated, proper immune system function cannot be supported and muscular proliferation is impaired, demonstrating that a certain amount of SOCE is required for proper organismal function. Conversely, excessive SOCE can itself lead to muscular abnormalities including tubular aggregate myopathy, and is additionally associated with increased growth and metastasis of numerous different types of cancers. The consequent importance of maintaining SOCE at a careful balance is reflected in the numerous mechanisms the cell has evolved to finetune cellular control of SOCE, including post-translational modification of STIM1 and protein-protein interactions between STIM1 and numerous binding partners.

SOCE is an attractive target for development of novel therapeutics because of its centrality in several different disease states. Importantly, several preliminary studies have already validated manipulation of SOCE *in vivo* as being both useful and practical. As an example, pharmacological block of SOCE can inhibit platelet activation, yielding a protective effect with respect to ischemic brain damage during stroke[134]. Moreover, reducing SOCE via siRNA targeting of either STIM1 and Orai1[178] or Orai3[176] can effectively reduce the metastatic potential of cancerous cells in animal models. It is therefore clear there exists great potential for effective and realistically deliverable therapies based on targeting SOCE. A deeper understanding of the cellular mechanisms the cell uses to control SOCE can only assist in the development of new methods to artificially manipulate SOCE where it is dysregulated in human disease.

4.1 Research Objective

The available evidence clearly shows that SOCE is an indispensible pathway for numerous physiological systems and that perturbations in SOCE function can have severely deleterious effects on human health. The research question this thesis seeks to address is **what mechanisms does the cell use to control store-operated calcium entry?** A survey of the literature shows numerous described protein binding partners of STIM1 and Orai1. My research aimed to discover novel STIM1 binding partners and describe the mechanisms by which they exert control over SOCE.

4.2 Research Hypothesis

STIM1 is a transmembrane protein of the endoplasmic reticulum membrane; as such, it has a luminal domain, a transmembrane domain, and a cytoplasmic domain. My research hypothesis is that **STIM1 may be controlled via protein-protein interactions within any or all of its luminal, transmembrane, and cytoplasmic domains.** To address this hypothesis, I consider three protein-protein interactions in turn. First, we discovered a novel interaction of STIM1 and ERp57 within the ER lumen. Chapter 2 describes this finding, explains its mechanism of action, and shows the functional consequences. Second, we discovered calpain cleavage of STIM1 within its cytoplasmic domain. Chapter 3 describes this finding, its mechanism, and the consequent effects on STIM1 and SOCE. Finally, we confirm a previously described interaction between STIM1 and the transmembrane protein calnexin. Chapter 4 shows the importance of this interaction within T cells and offers some evidence as to the mechanism of action.

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CHAPTER TWO

Modulation of STIM1 and capacitative Ca^{2+} entry by the endoplasmic reticulum luminal oxidoreductase ERp57

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Author contributions:

J.G. designed and performed experiments (Analysis of interactions between ERp57 and the STIM1 luminal domain, Figure 2-1B).

Abstract

STIM1 is an ER membrane Ca^{2+} sensor responsible for activation of store-operated Ca^{2+} influx. We discovered that STIM1 oligomerization and store-operated Ca^{2+} entry are modulated by the ER oxidoreductase ERp57. ERp57 interacts with the ER luminal domain of STIM1 with this interaction involving two conserved cysteine residues, C^{49} and C^{56}. Store-operated Ca^{2+} entry is accelerated in the absence of ERp57 and inhibited in C^{49} and C^{56} mutants of STIM1. We show that ERp57, *via* ER luminal interaction with STIM1, plays a modulatory role in capacitative Ca^{2+} entry. This is the first demonstration of a protein involved in ER intraluminal regulation of STIM1.

Introduction

Intracellular Ca^{2+} is an important second messenger in processes as varied as muscular contraction, gene transcription, and apoptosis. Ca^{2+} is released from the ER into the cytoplasm and the loss of ER Ca^{2+} stores necessitates refilling *via* store-operated Ca^{2+} entry (SOC)[182,183], mediated by STIM1, a transmembrane ER protein that senses the Ca^{2+} filling state of the ER[25], and Orai1, a plasma membrane Ca^{2+} channel that, upon stimulation by STIM1, allows Ca^{2+} to enter the cell from the extracellular medium[184]. STIM1 has an N-terminal, ER luminal portion containing a Ca^{2+}-sensing EF-hand[34]. At rest, Ca^{2+} is bound to STIM1's EF-hand in the lumen of ER and the protein is held as an inactive dimer[37,38,183]. In response to Ca^{2+} depletion in the ER, Ca^{2+} dissociates from STIM1, leading to oligomerization of STIM1 and its translocation to subplasmalemmal punctae[25,38,39,50,185]. While STIM1 and Orai1 alone can reconstitute SOC activity, other associated proteins may be involved including SERCA[186,187], EB1[42], and CRACR2A[188].

Here, we discovered that ERp57, an ER luminal oxidoreductase modulates STIM1 function in the lumen of the ER. ERp57 bound to STIM1, dependingon the presence of two conserved cysteine residues (C^{49} and C^{56}) in the ER luminal domain of STIM1. SOC was accelerated in the absence of ERp57 and inhibited in C^{49} and C^{56} mutants of STIM1. This is the first demonstration of a protein involved in ER intraluminal modulation of STIM1.

Materials & Methods

Expression and purification of recombinant proteins

cDNA encoding the ER luminal domain of STIM1 (amino acids 23-213) was generated by PCR-driven amplification of a Stim1pCMV6-XL5 plasmid. The following DNA primers were used: forward primer, 5'-TTT AAA CTC GAG CTC AGC CAT AGT CAC AGT GAG AAG-3', and reverse primer, 5'-GGG CCC GAA TTC CCA TGA AGT CCT TGG GTG-3'. The cDNA product was cloned into pBAD/gIII *E. coli* expression vector yielding a plasmid encoding ER luminal domain of STIM1 designated pBAD-STIM1$_{ER}$. Recombinant STIM1 ER luminal domain protein was expressed in *E. coli* and purified from cell lysates using Ni-NTA affinity column chromatography[189]. Recombinant mouse calreticulin, calnexin (soluble calnexin), ERp57 and GST were expressed in *E. coli* and purified[189]. Cardiac calsequestrin was purified from dog hearts[190]. Protein PDI was purified from bovine liver[191].

Surface plasmon resonance (BIACore) analysis

Surface plasmon resonance measurements were carried out using BIACore[192]. Sensor chips were coupled with the recombinant ER luminal domain of STIM1 in 10 mM sodium acetate buffer, pH 4, at a flow rate of 5 ml/min, followed by blocking with 1 M ethanolamine, pH 8.5, for 7 min[192]. The binding of purified ER luminal proteins, soluble calnexin and GST was measured in a flow buffer containing 20 mM Tris, pH 7.0, 135 mM KCl, 0.05% Tween-20, 200 μM phenylmethylsulfonyl fluoride, 100 μM benzamidine, and a mixture of protease inhibitors[190]. Binding was carried out at 20°C at a flow rate of 30 ml/min.

Plasmid DNA and site specific mutagenesis

YFP-STIM1 plasmid was a generous gifts from N. Demaurex (University of Geneva)[193]. C^{49}A and C^{56}A mutations were introduced using the QuikChange Site-Directed Mutagenesis Kit. Primers used were: for C^{49}A mutation, 5'-CCG AAG CAG AGT TTG

CCC GAA TTG CAA GCC CCT GTG CC-3' and its gene complement; and for the $C^{56}A$ mutation, 5'-CGA ATT GAC AAG CCC CTG GCC CAC AGT GAG GAT GAG AAGC-3' and its gene complement. ERp57-CFP was constructed by PCR-driven amplification of cDNA encoding mouse ERp57 using a forward primer 5'-CCG GGT ACC ATG GCC TCC GAC GTG CTA-3' and a reverse primer 5'-CCG GAA TTC TTA GAG ATC CTC CTG TGC-3'. The PCR product was ligated into a pcDNAZeo-SS-CFP plasmid. Orai1-dsRed was constructed from two plasmids, Orai1-YFP (from N. Demaurex, University of Geneva) and a dsRed vector (from J. Krebs, University of Gottingen). Orai1-YFP and dsRed plasmids were cut with AgeI and NheI restriction enzymes to generate DNA encoding Orai1 and DNA corresponding to the dsRed vector, respectively. Insert and vector were ligated to generate Orai1-dsRed expression vector. The nucleotide sequences of DNA used in this study was confirmed by nucleotide sequence analysis.

Cell culture and immunoblotting

Generation and characterization of *ERp57*$^{-/-}$ cells were described earlier[194]. To generate *STIM1*$^{-/-}$ cells we first created *STIM1*$^{-/-}$ mice using the gene trap technique. Gene trapping with the trap vector pGT1TMpfs was used to generate *STIM1* gene disrupted embryonic stem cells from the Gene Trap Resource (BayGenomics)[194]. *STIM1*$^{-/-}$ embryos were harvested at E9.5 followed by the isolation of MEFs[195]. All animal experimental procedures were approved by the Animal Welfare Program at the Research Ethics Office, University of Alberta, and conformed to relevant regulatory standards.

Cells were transfected with YFP-STIM1 or YFP-STIM1-$C^{49/56}A$ using the Neon transfection system as per the manufacturer's protocol. Cells were harvested 16-24 hours post-transfection into RiPA lysis buffer containing 50 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate. Lysates were prepared in non-reducing sample buffer containing 10% glycerol, 2% SDS, 65 mM Tris, and 0.005 mg/ml bromophenol blue and separated *via* SDS-PAGE[196] either in the presence (reducing conditions) or absence (non-reducing conditions) of 1% β-mercaptoethanol.

Ca^{2+} flux measurements

For measurements of cytoplasmic Ca^{2+} concentrations, Ca^{2+}-containing and Ca^{2+}-free buffers were used, composed of 143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 20 mM Hepes pH 7.4, and 0.1% glucose, with or without 1 mM $CaCl_2$. Ca^{2+} measurements on cells in suspension were carried out using Fura2-acetoxymethyl ester (Fura2-AM)[195]. Measurements were carried out using a Photon Technology International instrument with excitation wavelengths of 340 nm and 380 nm and emission wavelength of 510 nm[195,197]. Ca2+ concentrations were calculated via two calibration steps: (1) a maximum value obtained by addition of 10 mM $CaCl_2$ and 10 μM ionomycin and (2) a minimum value obtained by addition of 33 mM EGTA, 25 mM Tris, and 0.42% Triton X-100.

For single cell Ca^{2+} imaging, cells were transfected with expression plasmids encoding YFP-STIM1 or YFP-STIM1-$C^{49/56}$A and Orai1-dsRed using Neon. Post-transfection (24-48 hrs), cells were incubated with 2 μM Fura2-AM in Ca^{2+}-containing buffer for 30 min at room temperature, followed by incubation in Ca^{2+}-containing buffer without Fura2-AM for 15 min. Cover slips were then washed three times with Ca^{2+}-free/EGTA buffer (1 mM EGTA) and incubated with Ca^{2+}-free/EGTA buffer for imaging. Cells were imaged using a 20x objective at excitation wavelengths of 340 nm and 380 nm and emission wavelength of 510 nm. Cells were treated with 1 μM thapsigargin, 4 mM $CaCl_2$ (yielding a final concentration of free Ca^{2+} of 3 mM), and 4.4 mM $CaCl_2$ with 10 μM ionomycin.

Confocal microscopy and FRET analysis

Cells were transfected with expression plasmids encoding YFP-STIM1, YFP-STIM1-$C^{49/56}$A, ERp57-CFP, or combinations thereof using the Neon transfection system following the manufacturer's protocols. Fluorescent imaging was carried out 24-48 hrs post-transfection on a confocal laser scanning microscope (Olympus Imaging) using a 60x oil immersion lens.

FRET was quantified using the protocol developed by the Youvan laboratory[198]. Briefly, bleed-through of YFP-STIM1 and ERp57-CFP into the FRET channel was measured in

singly transfected cells. The bleed-through values were used to correct raw FRET measurements in doubly transfected cells, as calculated by Volocity software (Improvision, Waltham, MA). To quantitate FRET and account for variations in expression levels of YFP-STIM1 and ERp57-CFP, N_{FRET} was calculated using the method of Xia and Liu[199].

siRNA transfections

STIM1 knockdown was performed using a pre-designed siRNA (Ambion, 151019) targeting STIM1 mRNA and a negative siRNA control (Ambion). ERp57-deficient cells were transfected with siRNA using DharmaFECT 1 (Dharmacon). Ca^{2+} measurements and protein collection were performed 48 hours post-transfection.

Results

ERp57, an ER resident oxidoreductase, interacts with the luminal domain of STIM1

Our initial goal was to identify molecules that bind to the ER luminal domain of STIM1, so we used surface plasmon resonance (BIACore) to screen for ER resident proteins that may interact with STIM1 within the ER lumen. The recombinant ER luminal domain of STIM1, encompassing the EF-hand and SAM domains (Fig. 2-1A), was immobilized to BIACore sensor chips to test whether ER luminal resident proteins including ERp57, protein disulfide isomerase (PDI), calreticulin, calsequestrin, and the ER luminal domain of calnexin, bound to STIM1. Of all tested proteins, only ERp57 bound to the immobilized STIM1 luminal domain (Fig. 2-1B). We concluded that the oxidoreductase ERp57 interacts *in vitro* with the STIM1 ER luminal domain.

ERp57 is a 58-kDa thiol oxidoreductase and a member of the PDI-like family[200] that promotes disulfide bond formation and isomerisation[201]. ERp57 is widely expressed in mouse tissues and has been most studied for its role in quality control in the secretory pathway[200]. The protein is a required component of the peptide loading complex of MHC Class I molecules[202], affects STAT3 signalling[194], and modulates SERCA function[203].

To further analyse the interaction between ERp57 and STIM1, we carried out fluorescence resonance energy transfer (FRET) analysis. We generated *STIM1*$^{-/-}$ mice and mouse embryonic fibroblasts (MEF) as well as *ERp57*$^{-/-}$ cell lines. *STIM1*$^{-/-}$ MEFs were transfected with expression vectors encoding YFP-tagged STIM1 (YFP-STIM1) and CFP-tagged ERp57 (ERp57-CFP). In resting cells, YFP-STIM1 was distributed throughout the ER and co-localized with ERp57-CFP (Fig. 2-1C, *iii*). Co-expression of YFP-STIM1 and ERp57-CFP produced a robust FRET signal under resting conditions (Fig. 2-1C, *iv*). FRET signal remained present after thapsigargin-induced store depletion (Fig. 2-2). These observations supported our *in vitro* observations (Fig. 2-1B) and indicated that ERp57 and STIM1 form complexes *in vivo* and *in vitro*.

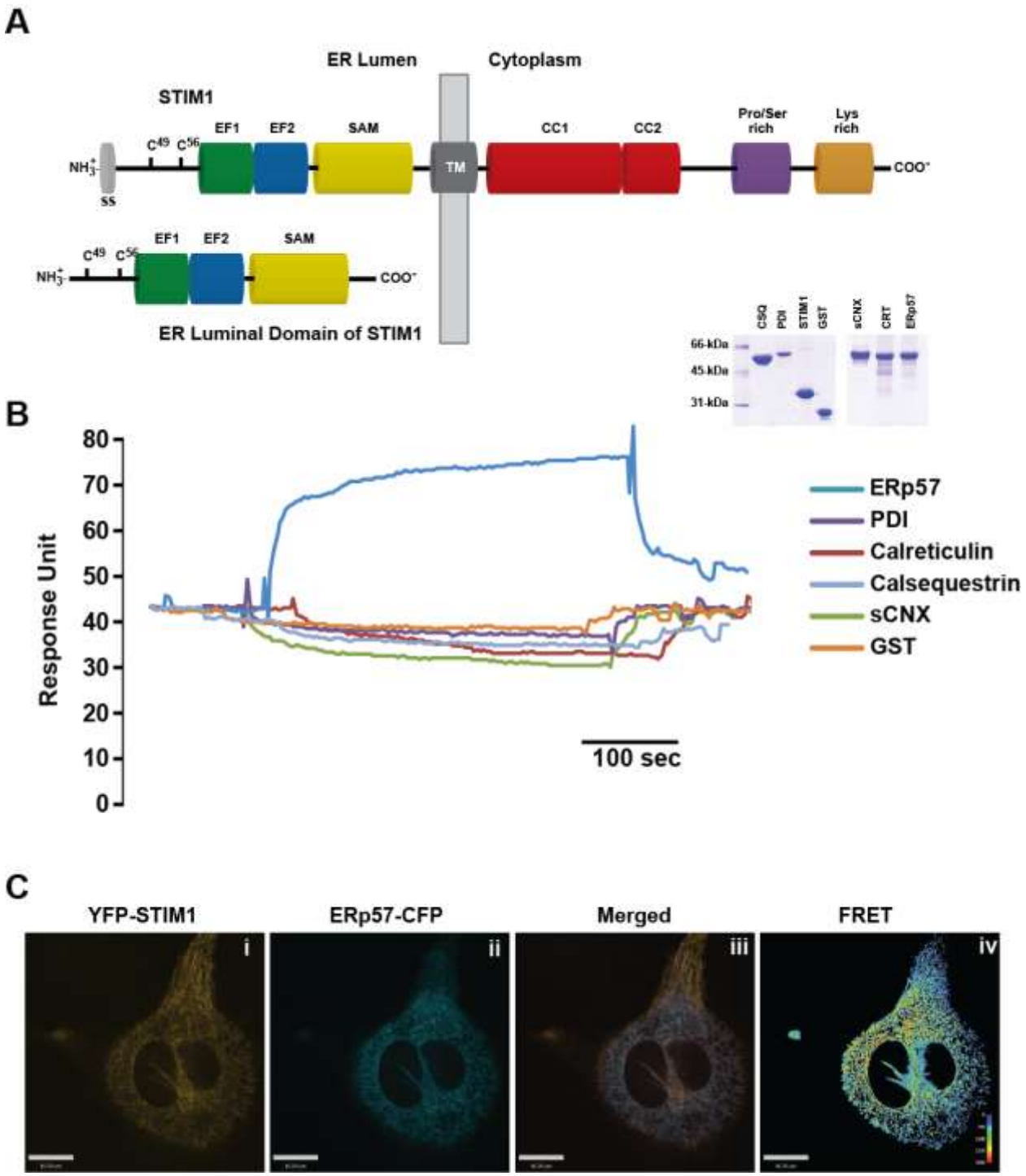

A
ER Lumen
Cytoplasm
STIM1
EF1
EF2
SAM
TM
CC1
CC2
Pro/Ser rich
Lys rich
C49
C56
NH3+
SS
COO-
ER Luminal Domain of STIM1
CSQ
PDI
STIM1
GST
sCNX
CRT
ERp57
66-kDa
45-kDa
31-kDa
B
Response Unit
80
70
60
50
40
30
20
10
0
100 sec
ERp57
PDI
Calreticulin
Calsequestrin
sCNX
GST
C
YFP-STIM1
ERp57-CFP
Merged
FRET
i
ii
iii
iv

Figure 2-1. Analysis of interactions between ERp57 and the STIM1 ER luminal domain. (A) A linear model of STIM1 and the ER luminal domain of STIM1. The ER luminal domain of STIM1 (amino acid residues 23-213) was used for BIACore analysis. The location of the two conserved cysteines (C^{49} and C^{56}) is indicated. ER luminal and cytoplasmic domains of the protein are also indicated: containing EF1 and EF2, EF-hand domains 1 and 2; SAM, sterile-α motif domain; TM, transmembrane domain; and CC1 and CC2, coiled-coil domains. The locations of proline/serine and lysine rich domains are also indicated. (B) ERp57 was injected over immobilized recombinant STIM1 ER luminal domain (amino acid residues 23-213). The ER luminal domain of STIM1 was coupled to a BIACore chip and exposed to different ER luminal proteins as ligands. Insert, SDS-PAGE of recombinant proteins used for BIACore analysis. CSQ, calsequestrin; PDI, protein disulfide isomerase; GST, glutathione S-transferase; CRT, calreticulin; sCNX, soluble calnexin. (C) *STIM1*$^{-/-}$ cells were transfected with expression vectors encoding YFP-STIM1 and ERp57-CFP. Each panel shows YFP signal (i), CFP signal (ii), a merger of YFP and CFP signals (iii), and FRET signal (iv). Scale bar=16 μm. Bleedthrough values were 0.05 for YFP and 0.6 for CFP.

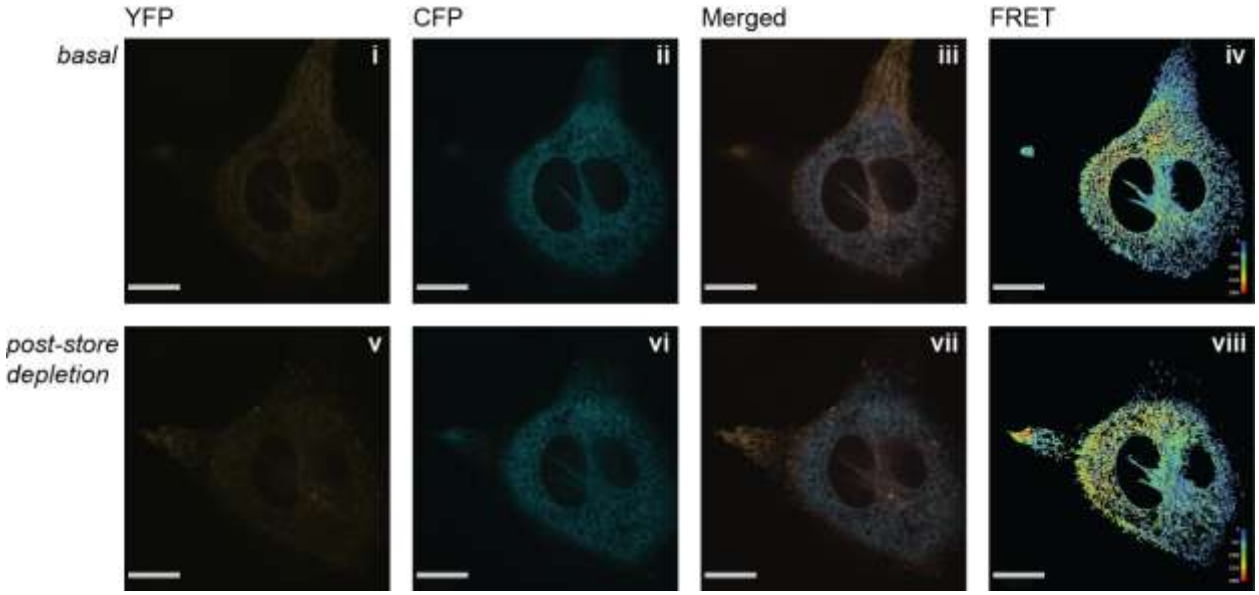


Figure 2-2. FRET before and after store depletion. *STIM1*$^{-/-}$ cells co-expressing YFP-STIM1 and ERp57-CFP were treated with 2 μM thapsigargin to induce store depletion.

Enhanced store-operated Ca^{2+} entry (SOC) in the absence of ERp57

We next tested if binding of ERp57 to STIM1 had an effect on the ability of STIM1 to activate SOC. To do this, we used ERp57-deficient MEFs and Fura2 imaging of intracellular Ca^{2+} dynamics[194]. We used thapsigargin, a SERCA inhibitor, to deplete ER Ca^{2+} stores, followed by addition of extracellular Ca^{2+} to initiate SOC. As expected, there was robust SOC in wild-type cells while *STIM1*$^{-/-}$ MEFs showed minimal SOC (Fig. 2-3A,B) (extent of SOC: wild-type: 233 ± 10 nM, n=3; *STIM1*$^{-/-}$: 140 ± 6 nM, n=4; p=0.004). In contrast, *ERp57*$^{-/-}$ MEFs exhibited enhanced SOC when compared to wild-type cells (extent of SOC: wild-type: 233 ± 10 nM, n=3; *ERp57*$^{-/-}$: 336 ± 22 nM, n=3; p=0.02) (Fig. 2-3A,B), indicating that STIM1-dependent activation of SOC was enhanced in the absence of ERp57. Wild-type and *ERp57*$^{-/-}$ cells had comparable rates of basal Ca^{2+} entry (i.e. without store depletion), further supporting a role for ERp57 in SOC (data not shown). Next, we expressed recombinant ERp57 in *ERp57*$^{-/-}$ cells[194] to test for the specificity of the ERp57 effect on SOC. *ERp57*$^{-/-}$ cells expressing recombinant ERp57 showed significantly reduced SOC a similar extent as seen in wild-type cells (extent of SOC: *ERp57*$^{-/-}$: 336 ± 22 nM, n=3; *ERp57*$^{-/-}$ + ERp57: 232 ± 26 nM, n=3; p=0.05) (Fig. 2-3A,B), demonstrating the specificity of the effect of ERp57 on SOC. *ERp57*$^{-/-}$ cells had lower thapsigargin-releasable ER Ca^{2+} stores than did wild-type cells (wild-type: 427 ± 26 nM, n=3; *ERp57*$^{-/-}$: 283 ± 1 nM, n=3; p=0.03) (Fig. 2-3A, Fig. 2-4). However, expression of recombinant ERp57 did not increase ER Ca^{2+} stores (*ERp57*$^{-/-}$: 283 ± 1 nM; *ERp57*$^{-/-}$ + ERp57: 281 ± 14 nM) (Fig. 2-3A, Fig. 2-4) but did inhibit SOC, verifying a direct role for ERp57 on STIM1 rather than an effect on the size of ER Ca^{2+} stores.

Triggering of SOC is intimately linked to the oligomerization and movement of STIM1 to punctae[35]. Therefore, we examined whether increased SOC in the absence of ERp57 was accompanied with STIM1 punctae formation. *ERp57*$^{-/-}$ cells were transfected with a YFP-STIM1 expression vector followed by confocal microscopy. In wild-type cells under resting conditions, YFP-STIM1 was primarily localized in a reticular ER-like distribution (Fig. 2-3C, *i*). In *ERp57*$^{-/-}$ cells, in addition to reticular distribution of YFP-STIM1, we also

observed a number of constitutively formed STIM1 containing punctae (Fig. 2-3C, *ii*, *arrows*). This was not observed in *ERp57*$^{-/-}$ cells expressing recombinant ERp57 (Fig. 2-3C, *iii*). To further investigate how ERp57 regulates STIM1 movement to punctae, we compared cells expressing YFP-STIM1 alone with cells co-expressing YFP-STIM1 and ERp57-CFP (Fig. 2-3D), where cells also expressing ERp57-CFP represent a condition with high levels of ERp57 expression. When YFP-STIM1 was expressed without ERp57 over-expression, punctae were visible 3 minutes after store depletion (Fig. 2-3D, *ii*) and translocation to punctae was complete by 6 minutes post-store depletion (Fig. 2-3D, *iii*). In contrast, in the presence of excess ERp57, YFP-STIM1 translocation to punctae was significantly slowed: the first punctae were not visible until after 6 minutes after store depletion (Fig. 2-3D, *vii*), with large amounts of YFP-STIM1 remaining away from punctae even after 10 minutes post-store depletion (Fig. 2-3D, *viii*). These results indicated that ERp57 expression inhibited the movement of STIM1 to punctae.

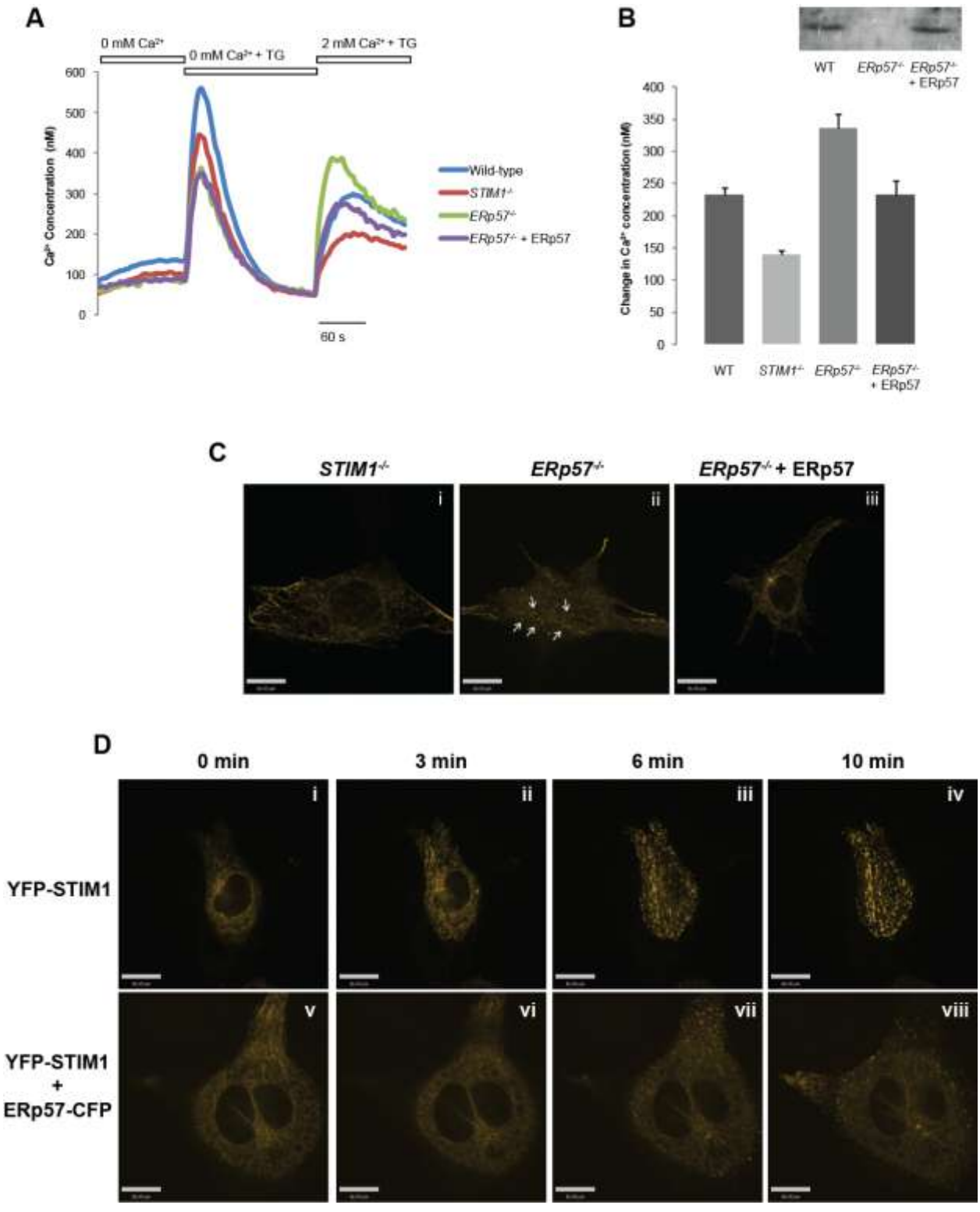

A
0 mM Ca2+
0 mM Ca2+ + TG
2 mM Ca2+ + TG
Ca2+ Concentration (nM)
600
500
400
300
200
100
0
60 s
Wild-type
STIM1-/-
ERp57-/-
ERp57-/- + ERp57
B
WT
ERp57-/-
ERp57-/- + ERp57
Change in Ca2+ concentration (nM)
400
350
300
250
200
150
100
50
0
WT
STIM1-/-
ERp57-/-
ERp57-/- + ERp57
C
STIM1-/-
ERp57-/-
ERp57-/- + ERp57
i
ii
iii
D
0 min
3 min
6 min
10 min
YFP-STIM1
YFP-STIM1 + ERp57-CFP
i
ii
iii
iv
v
vi
vii
viii

Figure 2-3. Store-operated Ca^{2+} entry in *ERp57*$^{-/-}$ cells. (A) Representative traces of Fura2 fluorescence in wild-type (*wt*), STIM1-deficient cells (*STIM1*$^{-/-}$), ERp57-deficient cells (*ERp57*$^{-/-}$), and ERp57-deficient cells expressing recombinant ERp57 (*ERp57*$^{-/-}$ +ERp57). ER store Ca^{2+} depletion was induced with 2 μM thapsigargin (TG) followed by activation of store operated Ca^{2+} entry (SOC) with the addition of 2 mM Ca^{2+}. Ca^{2+} concentrations were calculated via calibration steps as described in Methods. (B) Maximum extent of SOC for wild-type cells, *STIM1*$^{-/-}$, *ERp57*$^{-/-}$ and *ERp57*$^{-/-}$ cells expressing recombinant ERp57. Insert, Western blot analysis of wild-type (*wt*), *ERp57*$^{-/-}$ and *ERp57*$^{-/-}$ cells expressing recombinant ERp57 probed with anti-ERp57 antibodies. (C) YFP-STIM1 punctae formation in *STIM1*$^{-/-}$ cells, *ERp57*$^{-/-}$ cells and *ERp57*$^{-/-}$ cells expressing recombinant ERp57. Under resting conditions (*basal*), YFP-STIM1 displays a reticular pattern when expressed in *STIM*$^{-/-}$ cells (*i*). Under resting conditions in *ERp57*$^{-/-}$ cells, in addition to reticular distribution of YFP-STIM1, there was also a number of punctae forming as indicated by the arrows (*ii*) with this abolished in *ERp57*$^{-/-}$ cells expressing recombinant ERp57 (*iii*). Thapsigargin-dependent depletion of Ca^{2+} stores induced YFP-STIM1 relocation to subplasmalemmal punctae in all cells investigated (*iv, v, vi*). Scale bar=16 μm. (D) YFP-STIM1 punctae formation in *STIM1*$^{-/-}$ cells without (*i-iv*) or with (*v-viii*) concomitant expression of ERp57-CFP. Three minutes after thapsigargin-induced depletion of Ca^{2+} stores, there was evidence of punctae formation in cells without ERp57-CFP but none in cells over-expressing ERp57-CFP (compare *ii* & *vi*). After six minutes, extensive punctae can be seen in cells without ERp57-CFP (*iii*) but minimal punctae are present in cells over-expressing ERp57-CFP (*vii*). Even ten minutes after store depletion, most YFP-STIM1 signal remains non-punctate in cells over-expressing ERp57-CFP (*viii*).

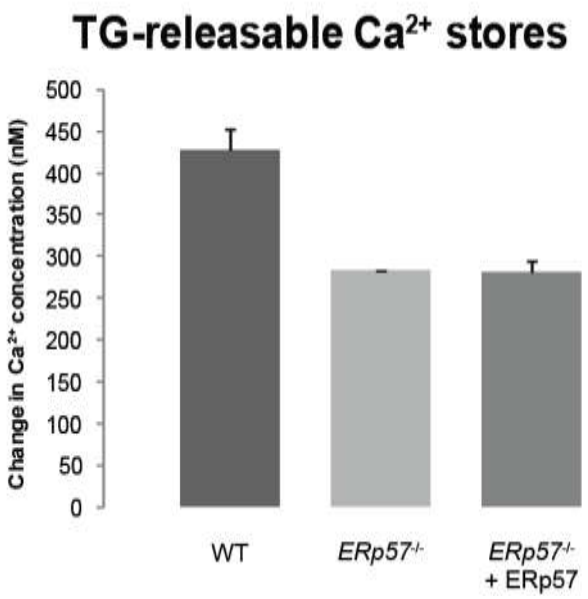


Figure 2-4. Thapsigargin-releasable Ca^{2+} stores and SOC in the absence and presence of ERp57. Fura2 measurements of wild-type, *ERp57*$^{-/-}$, and *ERp57*$^{-/-}$ + ERp57 cells were performed to measure thapsigargin-releasable ER Ca^{2+} stores. ERp57-deficient cells had lowered ER Ca^{2+} content than wild-type cells (WT: 427 ± 26 nM n = 3; *ERp57*$^{-/-}$: 283 ± 1 nM, n = 3; p = 0.03) that could not be rescued by expression of recombinant ERp57 (*ERp57*$^{-/-}$: 283 ± 1 nM, n = 3; *ERp57*$^{-/-}$ + ERp57: 256 ± 24 nM, n = 4; p = 0.4).

To further verify that altered Ca^{2+} dynamics in ERp57-deficient cells were related to SOC, we used small interfering RNA (siRNA) to knock down STIM1 and performed Ca^{2+} measurements (Fig. 2-5A,B). Knockdown of STIM1 significantly reduced the extent of SOC in *ERp57*$^{-/-}$ cells (wild-type: 315±41 nM; STIM1 knockdown: 135±7 nM, p=0.048). Transfection with non-STIM1 targeting siRNA had no significant effect on SOC (wild-type: 315±41 nM; negative siRNA: 305±36 nM, p=0.849). This further supported a role for ERp57 in SOC. Western blotting confirmed that STIM1 levels were lower in STIM1 knockdown cells (Fig. 2-5C). Altered SOC in *ERp57*$^{-/-}$ cells is not solely attributable to different ER Ca^{2+} store content, substantiating a direct role for ERp57 binding to STIM1 (Fig. 2-4). We concluded that ERp57, *via* binding to STIM1 in the lumen of ER, may serve as a brake on the initiation of SOC.

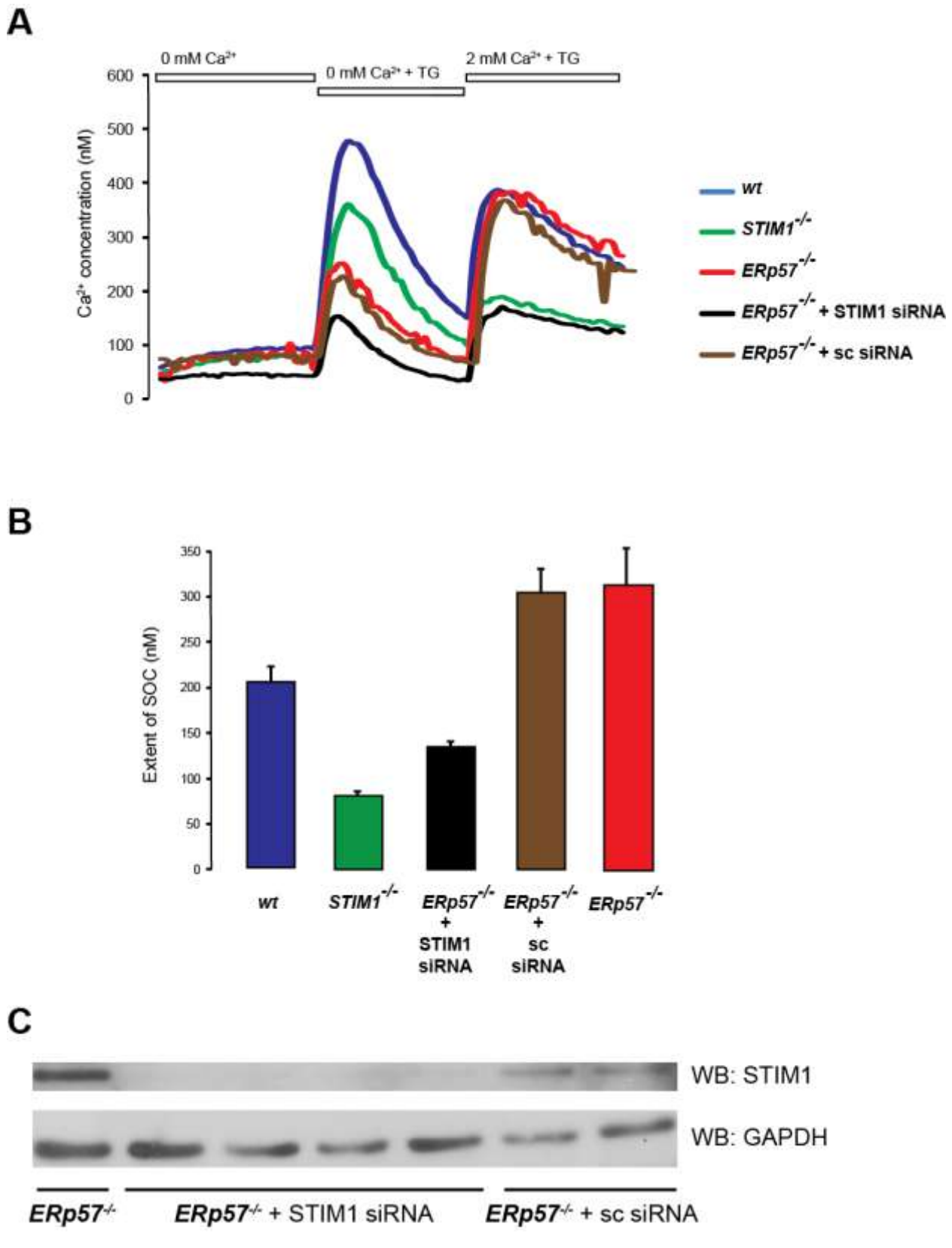
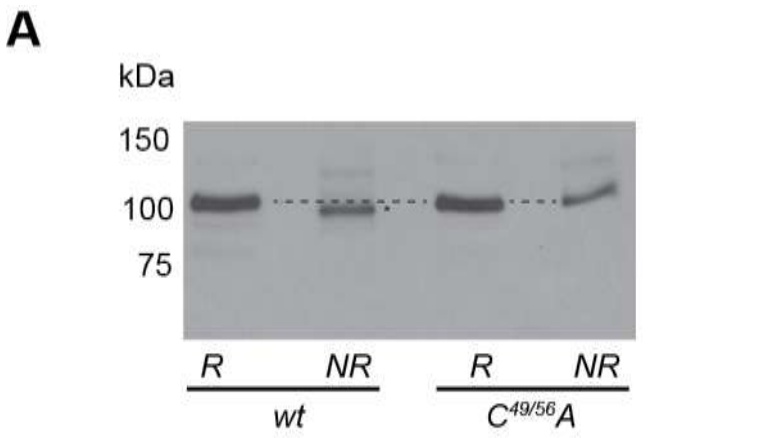
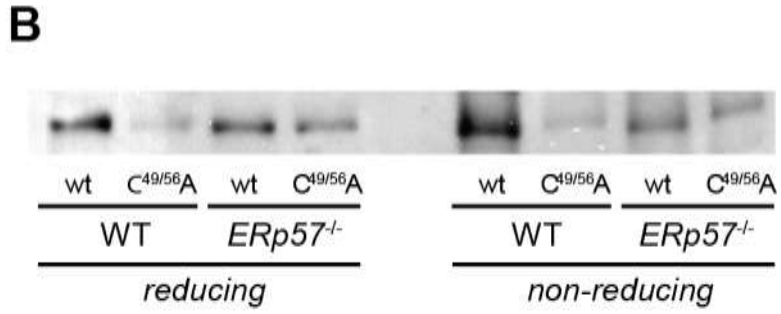

A
0 mM Ca2+
0 mM Ca2+ + TG
2 mM Ca2+ + TG
Ca2+ concentration (nM)
600
500
400
300
200
100
0
wt
STIM1-/-
ERp57-/-
ERp57-/- + STIM1 siRNA
ERp57-/- + sc siRNA
B
Extent of SOC (nM)
350
300
250
200
150
100
50
0
wt
STIM1-/-
ERp57-/- + STIM1 siRNA
ERp57-/- + sc siRNA
ERp57-/-
C
WB: STIM1
WB: GAPDH
ERp57-/-
ERp57-/- + STIM1 siRNA
ERp57-/- + sc siRNA

Figure 2-5. Store-operated Ca^{2+} entry in *ERp57*$^{-/-}$ cells with knockdown of STIM1. (A) Representative traces of Fura2 fluorescence in wild-type (*wt*) cells, *ERp57*$^{-/-}$ cells, and in *ERp57*$^{-/-}$ cells transfected with siRNA targeting STIM1, or scrambled siRNA (*sc siRNA*). ER store Ca^{2+} depletion was induced with 2 μM thapsigargin (TG) followed by activation of store operated Ca^{2+} entry (SOC) with the addition of 2 mM Ca^{2+}. Ca^{2+} concentrations were calculated via calibration steps as described in Methods. (B) Extent of SOC in wild-type, *ERp57*$^{-/-}$ cells, and *ERp57*$^{-/-}$ cells transfected with siRNA targeting STIM1, or scrambled siRNA. (C) Western blot analysis of *ERp57*$^{-/-}$ cells treated with siRNA using anti-STIM1 and anti-GAPDH antibodies (loading control).

ER luminal cysteines of STIM1 form a disulfide bond

ERp57 is an ER luminal oxidoreductase, therefore we identified two highly conserved cysteine amino acid residues (C^{49} and C^{56}) upstream of the EF-hand Ca^{2+} binding domain as potential ERp57 binding sites. We carried out site-specific mutagenesis of these residues and SDS-PAGE analysis of STIM1 and STIM1 mutants under reducing and non-reducing conditions to examine whether C^{49} and C^{56} form a intramolecular disulfide bond in the ER luminal domain of STIM1. Under non-reducing conditions, wild-type YFP-STIM1 migrated more slowly than under reducing conditions supporting the presence of an intramolecular disulfide bond in STIM1 (Fig. 2-6A). In contrast, the YFP-STIM1-$C^{49/56}$A mutant showed the same mobility in SDS-PAGE (Fig. 2-6A), indicating that ER luminal C^{49} and C^{56} form a disulfide bond in STIM1. Analysis of YFP-STIM1 expressed in *ERp57*$^{-/-}$ cells indicated that ERp57 was not necessary for disulfide bond formation (Fig. 2-6B). Similar experiments using lysates from wild-type and *ERp57*$^{-/-}$ cells expressing Orai1-YFP showed no shifts in apparent size, pointing against a role for ERp57 on Orai1 itself (Fig. 2-6C).


A
kDa
150
100
75
R
NR
R
NR
wt
C49/56A


B
wt
C49/56A
wt
C49/56A
wt
C49/56A
wt
C49/56A
WT
ERp57-/-
WT
ERp57-/-
reducing
non-reducing

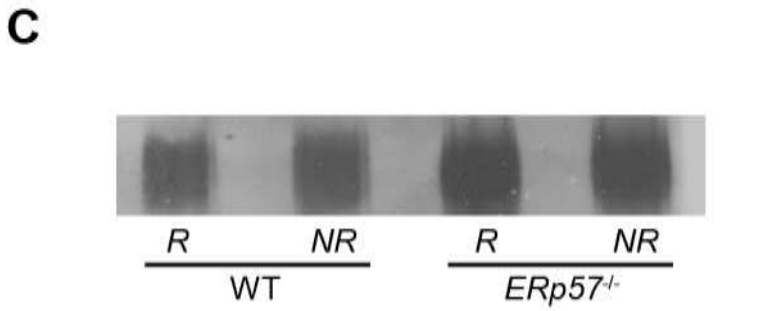

C
R
NR
R
NR
WT
ERp57-/-

Figure 2-6. Analysis of STIM1 and Orai1 under reducing and non-reducing conditions. (A,B) Lysates from wild-type and *ERp57*$^{-/-}$ cells expressing YFP-STIM1 or YFP-STIM1-C$^{49/56}$A were separated via SDS-PAGE under reducing and non-reducing conditions. A characteristic shift in apparent size between reducing and non-reducing conditions is seen for YFP-STIM1 but not YFP-STIM1-C$^{49/56}$A. Importantly, this shift is present in both wild-type and *ERp57*$^{-/-}$ cells, indicating that the luminal disulfide bond of STIM1 exists even in the absence of ERp57. (C) Lysates from wild-type and *ERp57*$^{-/-}$ cells expressing Orai1-YFP were separated via SDS-PAGE under reducing and non-reducing conditions. No changes in mobility are seen in ERp57-deficient cells, suggesting that the absence of ERp57 has no effect on the conformation and oxidation state of Orai1.

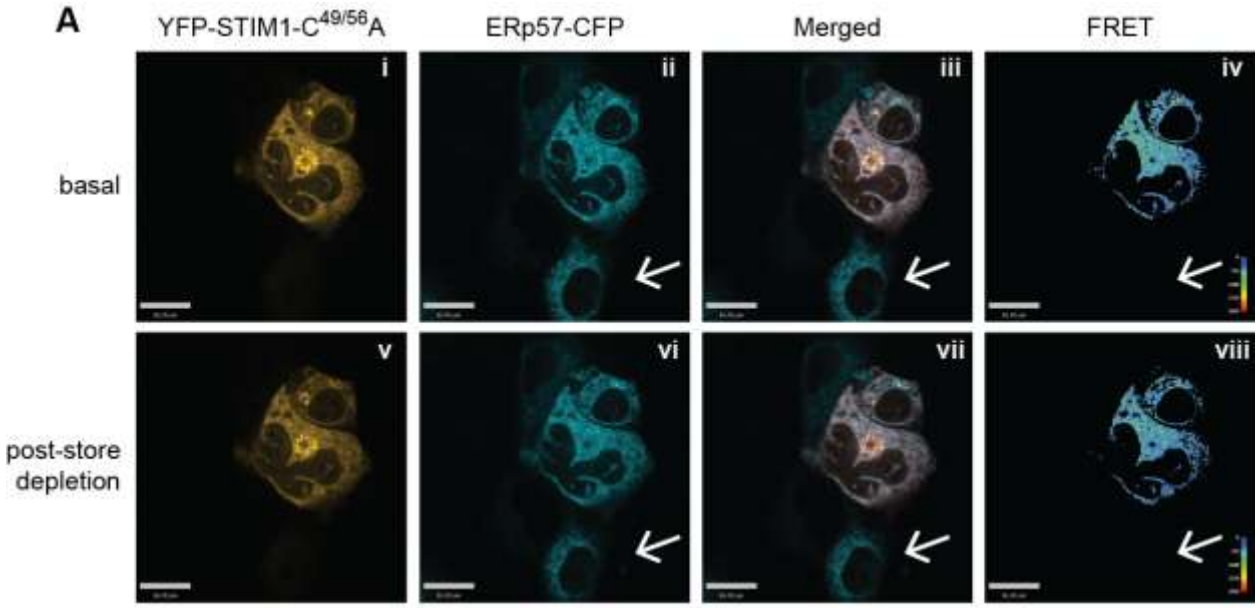
ER luminal cysteines of STIM1 are required for ERp57 binding and STIM1 oligomerization

To test the role of STIM1 ER luminal domains and luminal cysteines, we used our YFP-STIM1-$C^{49/56}A$ mutant for analysis of punctae formation, FRET interactions between YFP-STIM1 and ERp57-CFP, and SOC activity. The YFP-STIM1-$C^{49/56}A$ mutant expressed in *STIM1*$^{-/-}$ cells showed ER-like reticular localization with no visible punctae (Fig. 2-7A, *i*). Importantly, thapsigargin-dependent Ca^{2+} store depletion did not result in oligomerization and punctae formation by the YFP-STIM1-$C^{49/56}A$ mutant (Fig. 2-7A, *v, post-store deletion*). We concluded that ER luminal C^{49} and C^{56} play a role in STIM1 subplasmalemmal punctae localization.

We next examined whether STIM1 ER luminal cysteines are involved in ERp57 binding to STIM1. *STIM1*$^{-/-}$ cells were co-transfected with expression vectors encoding YFP-STIM1-$C^{49/56}A$ and ERp57-CFP followed by FRET analysis. Figure 2-7A shows that YFP-STIM1-$C^{49/56}A$ and ERp57-CFP produced robust FRET at rest (Fig. 2-7A, *iv* and *viii*), though the signal was at a lower level than that observed between ERp57-CFP and wild-type YFP-STIM1. Although ER luminal C^{49} and C^{56} may play a role in ERp57 binding to STIM1, the FRET signal was unaffected by depletion of ER Ca^{2+} stores (Fig. 2-7A, *post-store depletion*). When *ERp57*$^{-/-}$ cells were co-transfected with the same expression vectors, we again observed robust FRET signal between YFP-STIM1 and ERp57-CFP (Fig. 2-8A, *i-iv*) and reduced FRET between YFP-STIM1-$C^{49/56}A$ and ERp57-CFP (Fig. 2-8A, *v-viii*).

To quantitate, we calculated N_{FRET} values for FRET between ERp57-CFP and YFP-STIM1 or YFP-STIM1-$C^{49/56}A$. We used the method of Xia and Liu[199] to account for differences in relative expression of YFP and CFP fluorophores. N_{FRET} was significantly reduced (33%) with YFP-STIM1-$C^{49/56}A$ as compared to wild-type YFP-STIM1 (N_{FRET} for wild-type: 0.274±0.011, n=70; N_{FRET} for $C^{49/56}A$: 0.182±0.007, n=64; $p=9.02\times10^{-8}$) (Fig. 2-7B). Expression of ERp57-CFP and YFP-STIM1 or YFP-STIM1-$C^{49/56}A$ in *ERp57*$^{-/-}$ cells gave similar results, with a decrease in N_{FRET} by 28% for YFP-STIM1-$C^{49/56}A$, significant at $p = 1 \times 10^{-6}$

(Fig. 2-8B). We concluded that STIM1 ER luminal cysteines were involved in binding to ERp57.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YFP-STIM1-C49/56A
ERp57-CFP
Merged
FRET
basal
post-store depletion
i
ii
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v
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viii

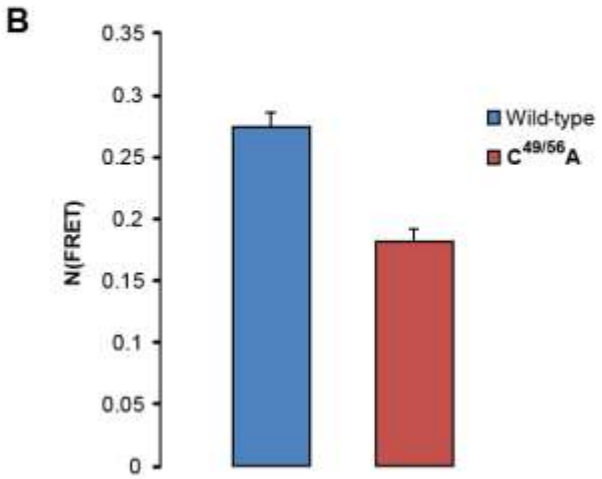
B
N(FRET)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Wild-type
C49/56A

Figure 2-7. Fluorescence resonance energy transfer (FRET) analysis of STIM1 mutants. (A) *STIM1*$^{-/-}$ cells were transfected with expression vectors encoding YFP-STIM1-C$^{49/56}$A and ERp57-CFP. Upper panel (*basal*) and lower panel (*post-store depletion*) show distribution of YFP-STIM1-C$^{49/56}$A and ERp57-CFP in resting cells and thapsigargin treated cells. Each panel shows YFP signal (*i, v*), CFP signal (*ii, vi*), a merger of YFP and CFP signals (*iii, vii*), and FRET signal (*iv, viii*). *i, ii, iii, iv*, FRET between ERp57-CFP and YFP-STIM1-C$^{49/56}$A in resting cells. *v, vi, vii, viii*, FRET between ERp57-CFP and YFP-STIM1-C$^{49/56}$A in thapsigargin-treated cells. The arrows indicate the cell with expressing ERp57-CFP only with no FRET, showing that mathematical corrections ruled out false positives. Scale bar=16 μm. (B) N_{FRET} values for FRET between ERp57-CFP and either YFP-STIM1 or YFP-STIM1-C$^{49/56}$A. N_{FRET} was significantly lower with STIM1-C$^{49/56}$A as compared to wild-type STIM1 (N_{FRET} for wild-type: 0.274±0.011, n=70; N_{FRET} for C$^{49/56}$A: 0.182±0.007, n=64; p = 9.02x10^{-8}). Bleedthrough values were 0.05 for YFP and 0.6 for CFP.

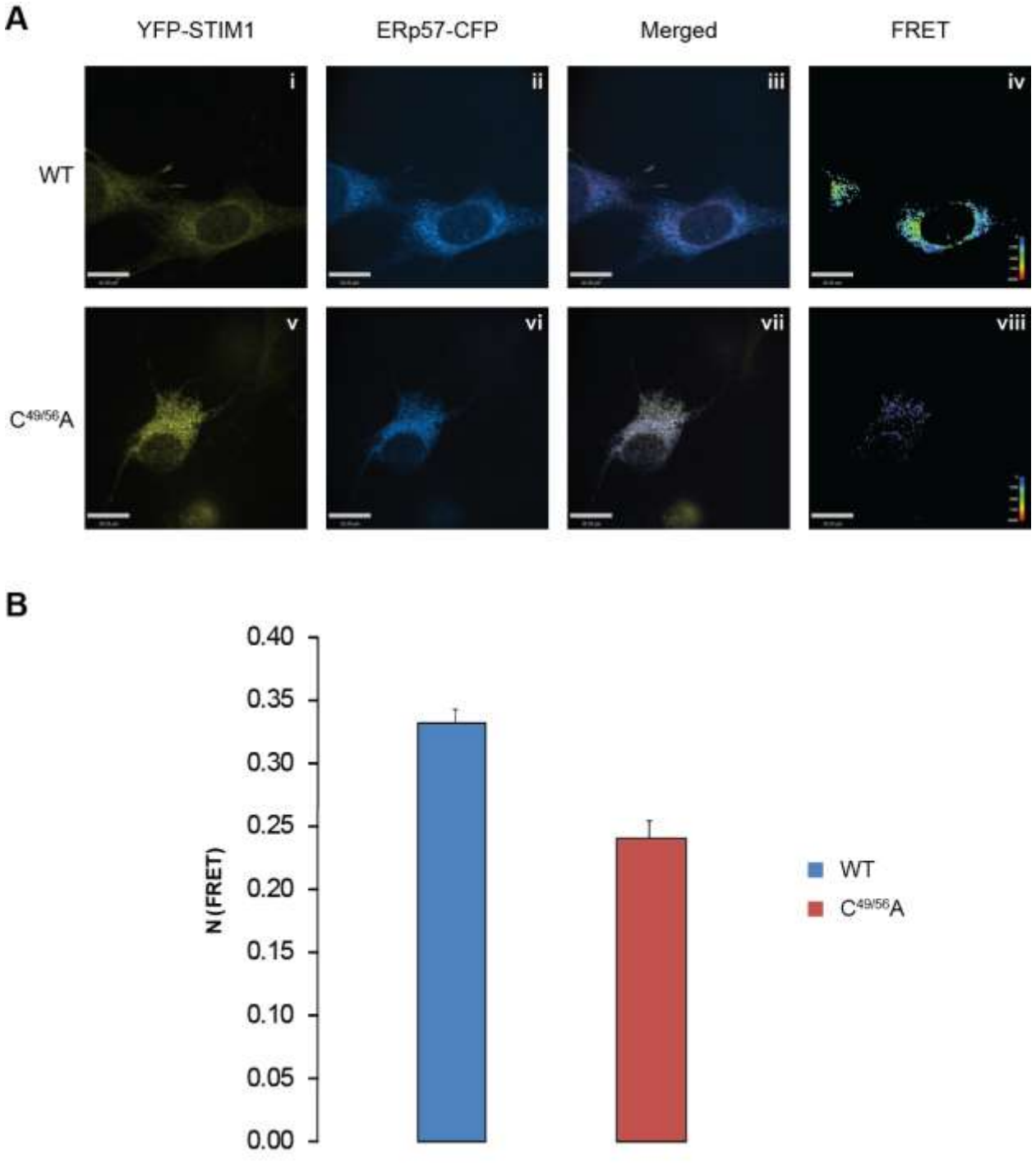
A
YFP-STIM1
ERp57-CFP
Merged
FRET
WT
C49/56A
i
ii
iii
iv
v
vi
vii
viii
B
N (FRET)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
WT
C49/56A

Figure 2-8. Fluorescence resonance energy transfer. (A) *ERp57*$^{-/-}$ cells were co-transfected with vectors encoding ERp57-CFP and either YFP-STIM1 or YFP-STIM1-$C^{49/56}A$. Panels show YFP signal, CFP signal, merge, and FRET for YFP-STIM1 (*i, ii, iii, iv*) and YFP-STIM1-$C^{49/56}A$ (*v, vi, vii, viii*). (B) Values for YFP, CFP, and FRET were obtained via confocal microscopy and used to calculate N_{FRET} values. N_{FRET} was significantly lower with YFP-STIM1-$C^{49/56}A$ than with wild-type YFP-STIM1 (N_{FRET} for wild-type: 0.332 ± 0.003, $n = 22$; N_{FRET} for $C^{49/56}A$: 0.240 ± 0.005, $n = 25$; $p = 1 \times 10^{-5}$).

STIM1 ER luminal cysteines are critical for SOC activation

Finally, we tested if mutations of cysteine amino acid residues affected SOC. *STIM1*$^{-/-}$ cells were co-transfected with expression vectors encoding Orai1-dsRed and YFP-STIM1 or YFP-STIM1-$C^{49/56}A$. Cells were treated with thapsigargin to empty ER Ca^{2+} stores followed by addition of Ca^{2+} to initiate SOC. Cells expressing YFP-STIM1 (or YFP-STIM1-$C^{49/56}A$) and Orai1-dsRed were identified *via* fluorescence microscopy followed by monitoring of Fura2 fluorescence. Figure 2-9 shows robust SOC observed in cells co-expressing wild-type YFP-STIM1 and Orai1-dsRed. In cells co-expressing YFP-STIM1-$C^{49/56}A$ and Orai1-dsRed, the extent of SOC was severely inhibited, as with *STIM1*$^{-/-}$ cells expressing Orai1-dsRed alone (Fig. 2-9), indicating that C^{49} and C^{56} of STIM1 were critical for the activation of SOC. In summary, site-specific mutation of C^{49} and C^{56} not only reduced ERp57 binding but also made STIM1 unable to translocate to punctae or to stimulate SOC activity.

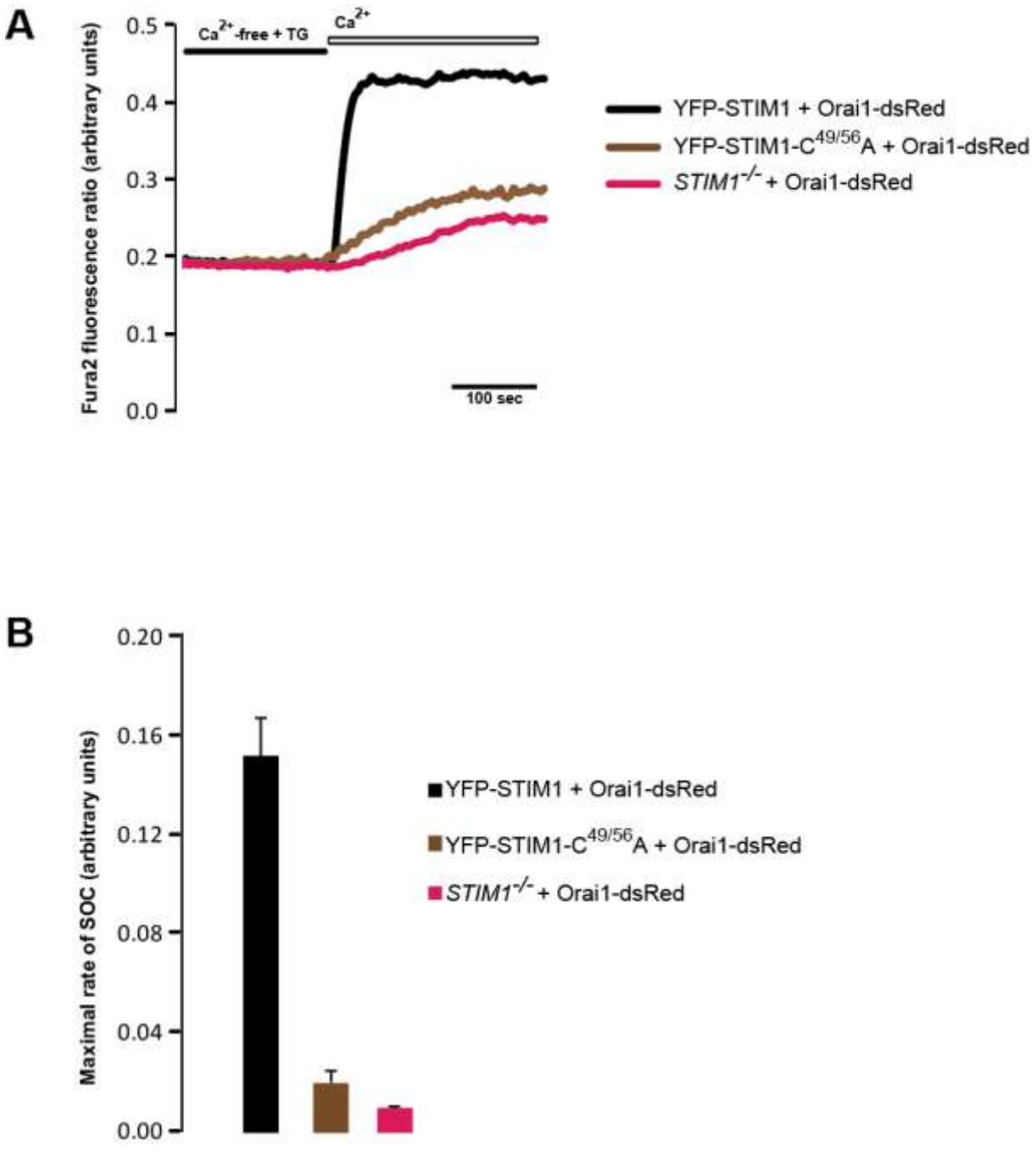
A
Ca2+-free + TG
Ca2+
Fura2 fluorescence ratio (arbitrary units)
0.5
0.4
0.3
0.2
0.1
0.0
100 sec
YFP-STIM1 + Orai1-dsRed
YFP-STIM1-C49/56A + Orai1-dsRed
STIM1-/- + Orai1-dsRed
B
Maximal rate of SOC (arbitrary units)
0.20
0.16
0.12
0.08
0.04
0.00
YFP-STIM1 + Orai1-dsRed
YFP-STIM1-C49/56A + Orai1-dsRed
STIM1-/- + Orai1-dsRed

Figure 2-9. Store-operated Ca^{2+} entry (SOC) in cells expressing STIM1 mutants. (A) Fura2 fluorescence in STIM1-deficient cells (*STIM1*$^{-/-}$), and STIM1-deficient cells expressing YFP-STIM1 or YFP-STIM1-$C^{49/56}A$. Cells were treated with 2 μM thapsigargin followed by addition of 2 mM Ca^{2+}. Fura2 ratios were calibrated by addition of excess Ca^{2+} and ionomycin to obtain a maximum Fura2 ratio, which was arbitrarily set to equal 1 in all cases. (B) Maximum rate of SOC achieved after Ca^{2+} addition in *STIM1*$^{-/-}$ cells and *STIM1*$^{-/-}$ cells expressing YFP-STIM1 and YFP-STIM1-$C^{49/56}A$.

Discussion

In this study, we have identified two unique aspects of STIM1 function that are separate but overlapping.We discovered that ERp57, an ER resident oxidoreductase, interacts with the ER luminal domain of STIM1 and that this interaction involves two conserved cysteines (C^{49} and C^{56}), forming a disulfide bridge upstream from the EF-hand domain. The mutation of these residues to eliminate the disulfide bond rendered STIM1 incapable of proper punctae translocation. As the conserved C^{49} and C^{56} are located in close proximity to the EF-hand Ca^{2+} sensor in STIM1, these results suggested that the conformation of the disulfide-containing region of STIM1 may affect that of the EF hand (and vice versa). Indeed, extensive *in vitro* data demonstrates that the oligomerization behaviour of STIM1 is highly dependent on the degree of folding and unfolding of its EF-SAM motif, as modulated by Ca^{2+}[35,204,205].

While this work was in preparation for publication, Hawkins *et al*.[206] reported that oxidative stress promotes STIM1 redistribution and punctae formation. Upon induction of oxidative stress, STIM1 ER luminal C^{56} undergoes S-glutathionylation, resulting in decreased Ca^{2+} binding to STIM1, its oligomerization, and SOC activation[206]. In contrast, we did not observe constitutive Ca^{2+} entry in cells expressing STIM1-$C^{49/56}$A (Fig. 6). The discrepancy between this study and Hawkins *et al.* may represent differences in cellular systems used and/or differences in the way store-operated Ca^{2+} was measured, but both studies strongly support the notion that STIM1 is regulated by redox state. Importantly, if the cysteine residues of STIM1 are modified, for example by S-glutathionylation, it may not bind to ERp57. Modification of cysteine residues may be one way that STIM1, upon oxidative stress, escapes ERp57-dependent inhibition.

Interestingly, Ca^{2+} release *via* inositol (1,4,5)-triphosphophosphate receptor is inhibited by another ER resident oxidoreductase, ERp44[207]. We showed that ERp57 plays a role in Ca^{2+} homeostasis by affecting STIM1 function and consequently SOC, suggesting that ER resident oxidoreductases are critical regulators of ER Ca^{2+} homeostasis. The ER is the intersection of many signalling pathways and communication between different cellular

organelles[194,207,208,209,210]. Ca^{2+} is an important signalling molecule in the lumen of the ER [183,211] and regulation of STIM1 by ERp57 may be involved in converging Ca^{2+}-dependent information from the lumen of the ER to the cytoplasm.

Acknowledgments

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CHAPTER THREE

STIM1 is cleaved by calpains

Abstract

Store-operated calcium entry (SOCE) is a pathway that moves Ca^{2+} across the plasma membrane and is mediated by two major proteins, STIM1 and Orai1. Here, we discovered that the cytoplasmic domain of STIM1 is a target for calpains, a family of calcium-activated proteases. We found that calpain cleavage of STIM1 serves to control its cellular abundance and was noticeably increased under conditions of cellular stress and apoptosis. Dysregulation of STIM1 levels has been reported to have human disease consequences and our results suggest a mechanism for controlling STIM1 abundance.

Introduction

Ca^{2+} ions serve as versatile intracellular messengers; as such, the cell has evolved various pathways to properly control the movement of Ca^{2+} ions across various membranes and the accumulation of ions within cellular organelles. One such pathway is store-operated calcium entry (SOCE), which couples depletion of ER luminal Ca^{2+} stores with Ca^{2+} influx across the plasma membrane[24]. SOCE is mediated by two key proteins: stromal interaction molecule 1 (STIM1), an ER transmembrane protein serving as a Ca^{2+} store sensor[25,26]; and calcium release-activated calcium channel (Orai) family membrane proteins, which serve as plasma membrane Ca^{2+} channels[27]. Structurally, STIM1 can be separated into several domains: a luminal domain, containing a Ca^{2+}-binding EF hand and a sterile-α-motif (SAM)[30]; a transmembrane domain; and a cytoplasmic domain, containing several coiled-coil domains, regions rich in serines and prolines, and a region rich in lysines[34]. Initiation of SOCE occurs via Ca^{2+} dissociation from the luminal domain of STIM1, leading to homooligomerization of STIM1[205]. The cytoplasmic coiled-coil domains of STIM1 are thought to further mediate the oligomerization of STIM1 as well as mediate direct contact between STIM1 and Orai1[49]. Additionally, the cytoplasmic domain is implicated in controlling the stability and turnover of STIM1; STIM1 lacking its cytoplasmic domain is noticeably less stable than is wild-type STIM1[63]. STIM1 and SOCE have been implicated in several different disease states: non-functional STIM1 is associated with severe combined immunodeficiency[115], while increased STIM1 levels and SOCE have been linked to increased metastatic behavior in breast cancer[178].

Calpains are a family of Ca^{2+}-sensitive, neutral pH proteases; there are fourteen known isoforms in humans, with the best-characterized and most ubiquitously expressed being CAPN1 and CAPN2[212]. These both exist as heterodimers, consisting of a large catalytic subunit and a small regulatory subunit, CAPN4, common to CAPN1 and CAPN2[212]; deletion of the gene encoding CAPN4, *CAPNS4*, renders both CAPN1 and CAPN2

catalytically inactive[213]. While calpain cleavage is known as a highly regulated event, it does not occur at a rigidly defined site within a protein's amino acid sequence, but instead is dependent upon the primary sequence as well as secondary[214] and three-dimensional structure[215].

In this study, we describe cleavage of STIM1 both under endogenous conditions and in response to cell stress events. When calpain activity was inhibited, STIM1 cleavage was significantly reduced. Cleavage took place over a time course of hours to days, suggesting it does not occur at sufficient speed as to serve as a direct regulator of the process of SOCE. Instead, we conclude that STIM1 is targeted for calpain cleavage as a mechanism to regulate the steady state levels of STIM1 within the cell. Furthermore, STIM1's susceptibility to calpain cleavage leads to enhanced STIM1 degradation during the progression of apoptosis.

Materials & Methods

Protein synthesis, cell culture, SDS-PAGE, and immunoblotting

STIM1C protein was synthesized as described previously[216]: cDNA encoding the cytoplasmic domain of STIM1 (residues 271-685) was generated via PCR-driven amplification using a STIM1-pCMV6-XL5 plasmid as template. The cDNA product was cloned into a pBAD/gIII *E. coli* expression vector; recombinant STIM1C was expressed in *E. coli* and purified from lysates using Ni-NTA affinity column chromatography[189].

Generation and characterization of *STIM1*$^{-/-}$ cells were described earlier[60]. Cells were transfected with expression vectors encoding YFP-STIM1 and/or CAST using the Neon transfection system (Invitrogen). Protein concentration was estimated using a Lowry-based protein assay. SDS polyacrylamide gel electrophoresis, transfer to nitrocellulose, and immunoblotting were carried out as described previously[60]. Antibodies used were: GFP (Dr. Luc Berthiaume, University of Alberta); STIM1 and CRT (generated in the laboratory); GAPDH, γ-tubulin, and β-tubulin (Pierce Antibodies); calnexin (Enzo Life Sciences); and PARP (Cell Signaling Technology).

Cytoplasmic Ca^{2+} measurements

Cytoplasmic Ca^{2+} concentrations in bulk cell populations were measured using Fura2-acetoxymethyl ester (Fura2-AM) using previously described protocols[11]. Measurements were conducted using a Photon Technology International instrument with excitation wavelengths of 340 nm/380 nm and an emission wavelength of 510 nm. Single cell cytoplasmic Ca^{2+} concentrations were measured using a previously described protocol[60]. A perfusion system was used to alternately expose the cells to Ca^{2+}-free buffer (+1 mM EGTA) and Ca^{2+}-containing buffer (+ 2 mM $CaCl_2$).

Results

The cytoplasmic domain of STIM1 is a target for proteolytic cleavage

During experiments involving immunoblots probed for N-terminally tagged YFP-STIM1, we noticed the presence of protein bands at a lower molecular weight than full-length YFP-STIM1 that were still recognized by an antibody against YFP. We hypothesized that these YFP-STIM1 fragments were produced by the regulated proteolytic cleavage of STIM1 followed by proteasomal degradation. The sizes of the fragments and their maintenance of the N-terminal YFP tag were consistent with cleavage of STIM1 within its long cytoplasmic domain. We therefore treated *STIM1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) expressing N-terminally tagged YFP-STIM1 with MG132, a proteasomal inhibitor. Indeed, we observed an increase in the levels of YFP-STIM1 fragments, suggesting that STIM1 is a target for regulated cleavage (Fig 3-1A). We repeated this experiment in wild-type cells and probed for native STIM1 using an antibody against the N-terminus of STIM1. We found very similar results: treatment of cells with MG132 led to increased levels of lower molecular weight fragments of STIM1 (Fig 3-1B).

We next aimed to identify the protease or proteases responsible for the cleavage of STIM1. We considered several different potential proteases, including calpains. We used several online algorithms to scan the amino acid sequence of STIM1 for predicted calpain cleavage sites, with results summarized in Table 3-1. As these algorithms identified numerous sites within STIM1 that could potentially be cleaved by calpains, we decided to further investigate calpains.

We first developed a STIM1 expression construct we named STIM1C, which encoded all the cytoplasmic amino acid residues of STIM1. We expressed this protein in, and purified it from, *E. coli*, followed by incubation with commercially purchased calpain

protein in the presence of calcium to test for cleavage of STIM1C. Even this simple *in vitro* system was able to recapitulate cleavage of STIM1C, serving as a first piece of evidence that the *in vivo* cleavage of STIM1 may be due to the activity of calpains (Fig 3-1C).

To further pinpoint a role for calpains, we obtained cells deficient in the regulatory subunit of calpains (*CAPN4*$^{-/-}$ cells) and their wild-type counterparts and transfected these cells with YFP-STIM1. We found that accumulation of YFP-STIM1 fragments was markedly reduced in calpain-deficient cells (Fig 3-1D). Finally, we co-expressed YFP-STIM1 and calpastatin (CAST), a protein inhibitor of calpains, in the same cells and treated these cells with MG132. Again, inhibition of calpains reduced the cleavage of YFP-STIM1 (Fig 3-1E). We therefore concluded that the cleavage of STIM1 is by calpains.

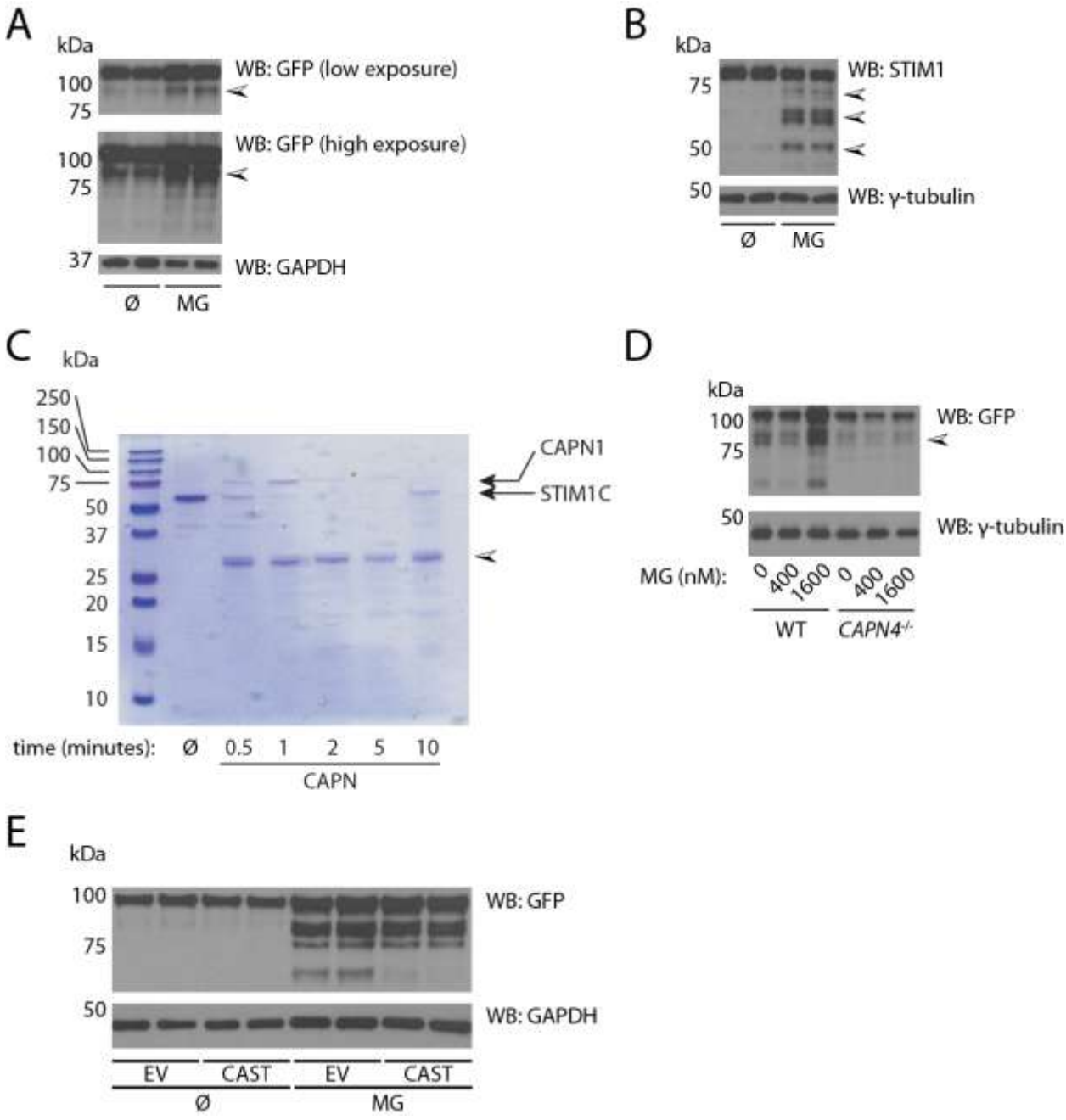

A
kDa
100
75
WB: GFP (low exposure)
100
75
WB: GFP (high exposure)
37
WB: GAPDH
Ø
MG
B
kDa
75
50
WB: STIM1
50
WB: γ-tubulin
Ø
MG
C
kDa
250
150
100
75
50
37
25
20
15
10
CAPN1
STIM1C
time (minutes):
Ø
0.5
1
2
5
10
CAPN
D
kDa
100
75
WB: GFP
50
WB: γ-tubulin
MG (nM):
0
400
1600
0
400
1600
WT
CAPN4-/-
E
kDa
100
75
WB: GFP
50
WB: GAPDH
EV
CAST
EV
CAST
Ø
MG

Figure 3-1. STIM1 is cleaved by calpains. (A) *STIM1*$^{-/-}$ MEFs were transfected with a vector encoding YFP-STIM1 and left untreated or treated with 500 nM MG132 (MG). Lysates were separated via SDS-PAGE, and immunoblotted for GFP and GAPDH (loading control). MG132 treatment increased levels of lower molecular fragments of YFP-STIM1 (indicated by arrowheads). (B) Wild-type MEFs were left untreated or treated with 500 nM MG132 (MG). MG132 treatment increased abundance of lower molecular weight STIM1 fragments (indicated by arrowheads). γ-tubulin serves as a loading control. (C) STIM1C, purified from *E. coli*, was incubated with calpain protein in the presence of calcium for time points up to 10 minutes as indicated in the figure. Reactions were stopped by addition of sample loading buffer, after which samples were separated via SDS-PAGE and stained with Coomassie blue. Addition of CAPN1 led to cleavage of STIM1C almost immediately, as shown by diminished levels of full-length STIM1C and the presence of new bands at approximately half the MW of full-length STIM1C (indicated by arrowhead). (D) Wild-type and and *CAPN4*$^{-/-}$ fibroblasts were transfected with a vector encoding YFP-STIM1 and left untreated, or treated with MG132 (400 nM or 1600 nM), as indicated. Lysates were separated via SDS-PAGE and probed with antibodies against GFP. Immunoblotting for GFP shows some cleavage of YFP-STIM1 under all conditions (indicated by arrowhead), with a marked increase at the higher MG132 concentration. Conversely, much less cleavage is seen in the absence of CAPN4, even when treated with 1600 nM MG132. γ-tubulin serves as a loading control. (E) *STIM1*$^{-/-}$ MEFs were transfected with a vector encoding YFP-STIM1 and either empty vector or a vector encoding human calpastatin (CAST). Cells were then left untreated or treated with 500 nM MG132 (MG). Lysates were separated via SDS-PAGE and probed with an antibody against GFP. Co-expression of CAST inhibits production of lower molecular weight YFP-STIM1 fragments. Western blot against GAPDH serves as a loading control.

Algorithm	Score (arbitrary units)	Cleavage site (after)	Sequence
SitePrediction, CAPN1	1269 1212 445	637 420 496	RAL**Q**AS SEV**T**AA PSL**Q**SS
SitePrediction, CAPN2	630 424 346	496 584 637	PSL**Q**SS KEL**R**EG RAL**Q**AS
http://calpain.org	0.14 0.14	637 584	RAL**Q**AS KEL**R**EG

Table 3-1. Predicted calpain cleavage sites within STIM1. Online algorithms (SitePrediction: http://www.dmbr.ugent.be/prx/bioit2-public/SitePrediction/index.php; http://calpain.org) and the sequence of human STIM1 (NCBI RefSeq NM_003156.3) were used to predict potential calpain cleavage sites within STIM1.

STIM1 cleavage does not modulate store-operated calcium entry

After observing that calpains cleave STIM1, we aimed to determine its physiological significance. Our hypothesis was that calpain cleavage of STIM1 may serve as a shutdown mechanism for SOCE, with Ca^{2+} entry via Orai1 activating calpains to cleave STIM1, neutralizing STIM1 activation of Orai1 and thus terminating SOCE. We therefore measured SOCE in wild-type and calpain-deficient cells, using a standard Fura2 protocol. Cells were treated with thapsigargin (to deplete ER Ca^{2+} stores, stimulating STIM1 movement to punctae) followed by external $CaCl_2$ (to initiate SOCE). Ca^{2+} elevations due to thapsigargin treatment were very similar between wild-type and calpain 4-deficient MEFs, suggesting that calpain deficiency has no effect on the amount of Ca^{2+} stored within the ER (Fig 3-2A). After Ca^{2+} addback, SOCE was significantly higher in the absence of calpains than in wild-type cells, both in terms of peak Ca^{2+} level reached and the rate of Ca^{2+} entry (Fig 3-2B). However, SOCE was still shut down in the absence of calpains; indeed, more quickly than in wild-type cells.

We next designed an experiment to monitor multiple cycles of SOCE to more precisely determine if calpain cleavage of STIM1 acts to modulate SOCE. We grew cells (wild-type or calpain 4 deficient) on glass cover slips, loaded cells with Fura 2, and monitored Fura2 fluorescence using a microscope equipped with a perfusion system, allowing us to flow various buffers (with EGTA or with $CaCl_2$) over the cells. By changing back and forth from calcium-free to calcium-containing buffers, we could measure multiple cycles of SOCE in individual cells. If STIM1 is being cleaved immediately after SOCE, we hypothesized that each successive calcium entry would be lower. However, in wild-type cells, we did not see a deterioration of SOCE over time: each cycle of calcium entry and exit was largely identical to the preceding cycle in both wild-type and calpain-deficient cells (Fig 3-2C). Calpain-deficient cells exhibited significantly higher and faster SOCE as well as faster calcium clearance from the cytoplasm, as was seen in bulk cell Fura2 measurements.

As a final experiment to determine if calpain cleavage of STIM1 might act during SOCE in order to modulate SOCE directly, we measured STIM1 cleavage over the time frame of two hours. We added thapsigargin to cells for time points from 0 to 120 minutes and looked for the characteristic STIM1 banding pattern. However, no accumulation of lower molecular weight STIM1 fragments was observed (Fig 3-2D), suggesting that STIM1 cleavage does not occur within this limited time frame. Along with our previous results looking at SOCE directly, this supported a model in which STIM1 cleavage does not act to directly modulate SOCE.

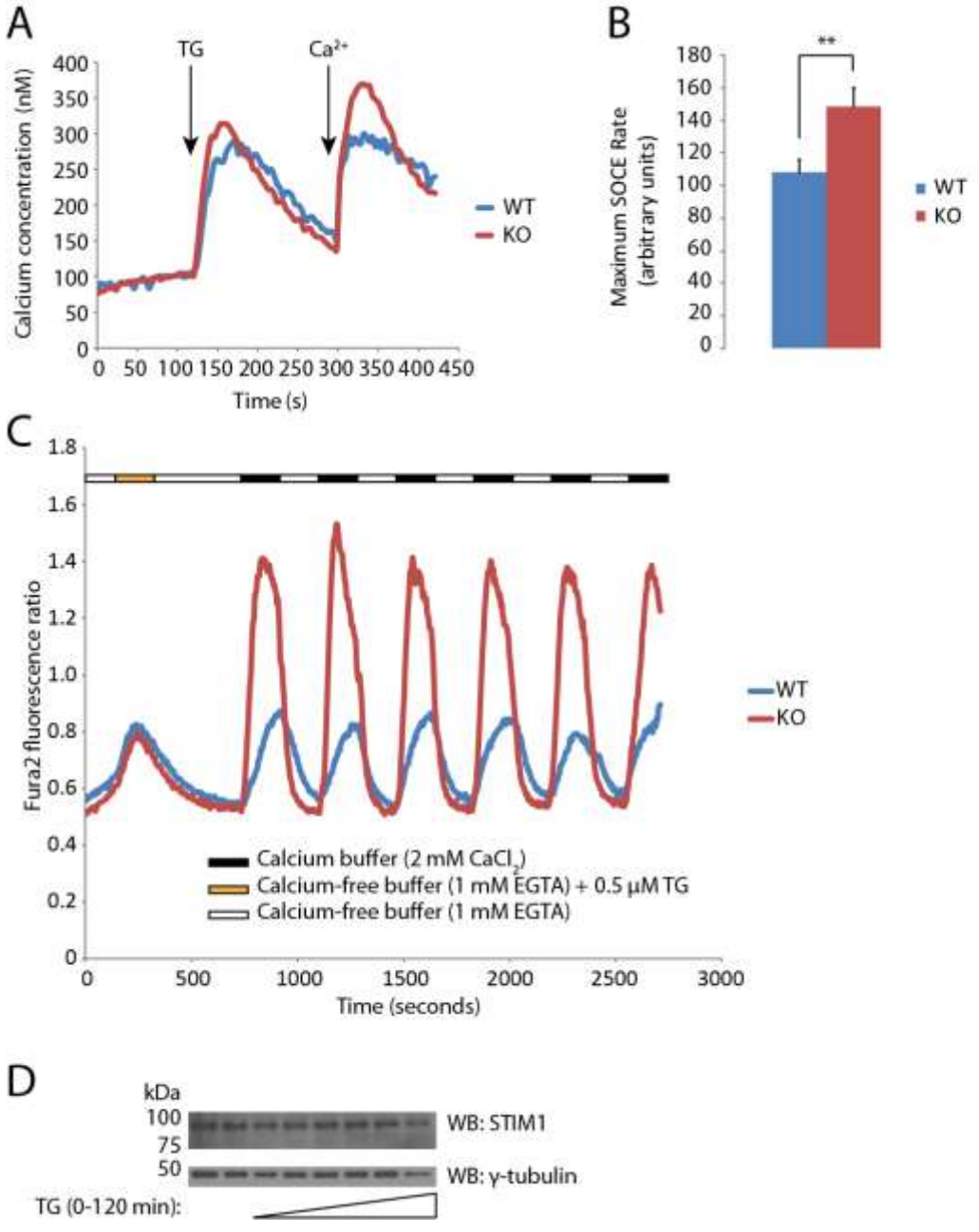

A
TG
Ca2+
Calcium concentration (nM)
Time (s)
WT
KO
B
Maximum SOCE Rate (arbitrary units)
**
WT
KO
C
Fura2 fluorescence ratio
Time (seconds)
Calcium buffer (2 mM CaCl2)
Calcium-free buffer (1 mM EGTA) + 0.5 µM TG
Calcium-free buffer (1 mM EGTA)
WT
KO
D
kDa
100
75
50
WB: STIM1
WB: γ-tubulin
TG (0-120 min):

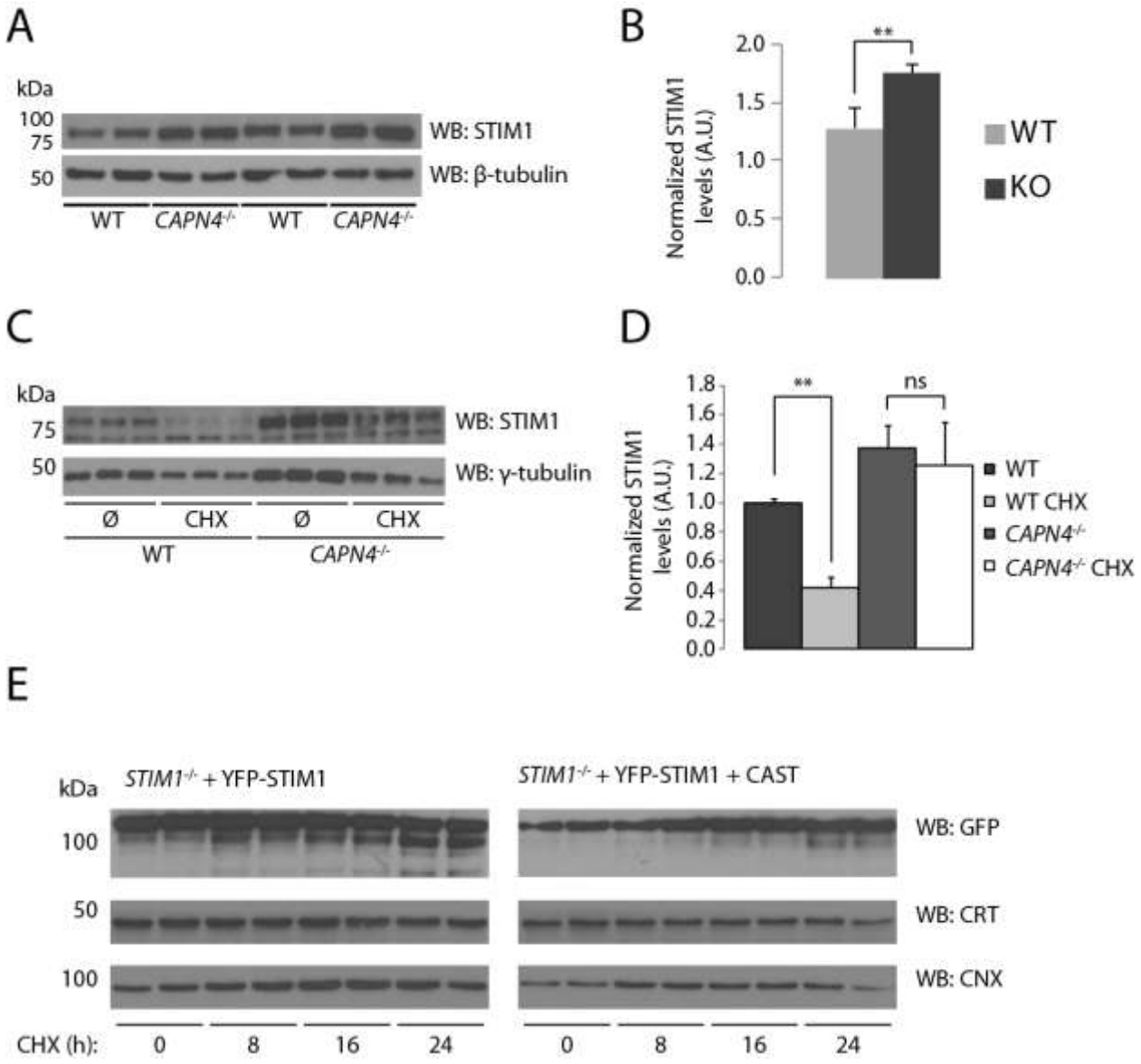
Figure 3-2. Calpain affects store-operated calcium entry. (A) Wild-type and *CAPN4*$^{-/-}$ fibroblasts were loaded with Fura2-AM dye to measure cytoplasmic calcium. Cells were treated with 1 μM thapsigargin (TG) to deplete ER Ca^{2+} stores and then 2 mM external $CaCl_2$ was added (Ca^{2+}) to induce SOCE. *CAPN4*$^{-/-}$ fibroblasts had significantly higher and faster store-operated calcium entry than did wild-type cells. Traces represent an average of 6 independent biological replicates. (B) Maximum rates of store-operated calcium entry (SOCE) were calculated based on data in Fig 2A. *CAPN4*$^{-/-}$ cells had significantly faster calcium entry than did wild-type cells (WT: 108±8; KO: 148±12; p=0.007) (C) Wild-type and *CAPN4*$^{-/-}$ fibroblasts were grown on glass cover slips and loaded with Fura2-AM dye. As shown in the figure, after treatment with thapsigargin, cells were subjected to Ca^{2+}-containing and Ca^{2+}-free buffers in succession in order to measure multiple cycles of SOCE. No significant diminishment of SOCE was seen over multiple cycles. Traces represent an average of 8 cells from 3 separate experiments. (D) Wild-type cells were treated with 1 μM thapsigargin for time points up to 120 minutes, as indicated. Lysates were separated via SDS-PAGE and probed for STIM1 and γ-tubulin as a loading control. No STIM1 cleavage was seen over this time period.

Calpains cleave STIM1 to control its steady state levels

Intriguingly, we did observe that calpain-deficient cells had higher store-operated calcium entry than did their wild-type counterparts (Fig 3-2A, B). We sought another explanation for this difference, hypothesizing that calpain cleavage of STIM1 may maintain its equilibrium levels. When we compared levels of STIM1 in wild-type and calpain-deficient cells, we found STIM1 to be noticeably more abundant in the absence of calpains (Fig 3-3A, 3B), which would account for increased SOCE.

We next compared the levels of STIM1 in the presence and absence of calpain activity via treatment with cycloheximide to halt protein translation. Incubation of wild-type cells with cycloheximide for 24 hours shows a significant decrease in STIM1 protein levels, while calpain-deficient cells have a noticeably smaller decrease in STIM1 protein levels. When normalized for γ-tubulin levels, STIM1 levels decrease by approximately 60% in wild-type cells but only 10% in calpain-deficient cells, suggesting that calpains control the major part of STIM1 turnover (Fig 3-3C, 3-3D). We performed further cycloheximide chase experiments over various time points (0, 8, 16, and 24 hours) in cells expressing either YFP-STIM1 alone or YFP-STIM1 and CAST together. Consistently with our results for native STIM1, YFP-STIM1 levels showed a gradual diminishment over the time course of cycloheximide treatment with concurrent accumulation of lower molecular weight cleavage products. Co-expression of CAST was able to reduce the loss of YFP-STIM1 levels over cycloheximide treatment and thus led to lower levels of YFP-STIM1 cleavage products (Fig 3-3E). We also monitored levels of two other ER proteins, calreticulin (CRT) and calnexin (CNX) to see if inhibition of calpains affected their half-lives. The degradation of these proteins was unaffected by CAST; therefore, we concluded that the higher abundance of STIM1 in the absence of calpain activity is at least somewhat specific to STIM1. Similar results were seen when comparing loss of native STIM1 in wild-type cells and cells expressing calpastatin (Fig 3-3F).

Finally, we aimed to determine if expression of calpastatin can increase STIM1 abundance, as our hypothesis would expect. We therefore transfected wild-type cells with a plasmid encoding zeocin alone or the zeocin plasmid in combination with a calpastatin plasmid, with the intention of establishing stable cells. After using 400 μg/mL of zeocin to select for cells that were successfully transfected, we maintained cells in culture in the presence of 200 μg/mL for ten days, then took cell lysates to measure STIM1 abundance (Fig 3-3G). We found that expression of calpastatin led to an increase of approximately 40% in STIM1 levels ($p = 0.01$) (Fig 3-3G, 3-3H). Therefore, we concluded that one reason calpains act upon STIM1 is as a mechanism for the regulation of its steady state levels.


A
kDa
100
75
50
WB: STIM1
WB: β-tubulin
WT
CAPN4-/-
WT
CAPN4-/-
B
Normalized STIM1 levels (A.U.)
2.0
1.5
1.0
0.5
0.0
**
WT
KO
C
kDa
75
50
WB: STIM1
WB: γ-tubulin
Ø
CHX
Ø
CHX
WT
CAPN4-/-
D
Normalized STIM1 levels (A.U.)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
ns
WT
WT CHX
CAPN4-/-
CAPN4-/- CHX
E
kDa
STIM1-/- + YFP-STIM1
STIM1-/- + YFP-STIM1 + CAST
100
50
100
WB: GFP
WB: CRT
WB: CNX
CHX (h):
0
8
16
24
0
8
16
24

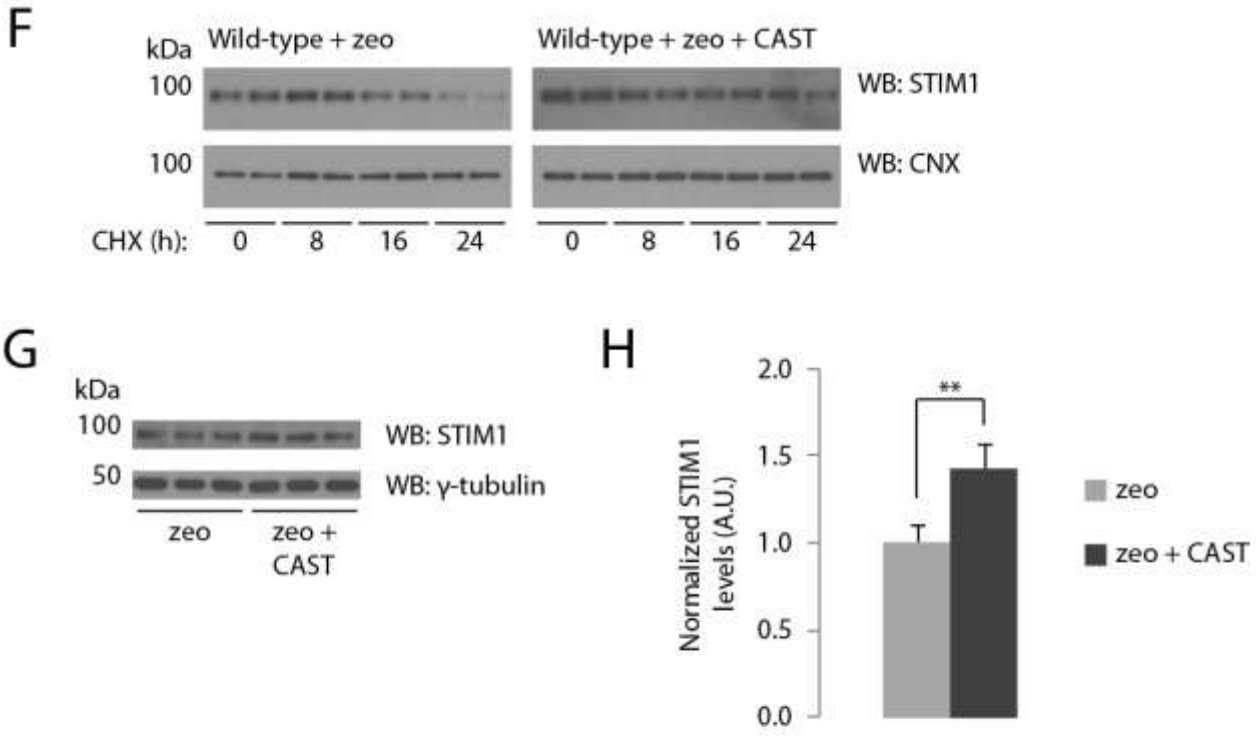

F
kDa
Wild-type + zeo
Wild-type + zeo + CAST
100
WB: STIM1
100
WB: CNX
CHX (h):
0
8
16
24
0
8
16
24
G
kDa
100
WB: STIM1
50
WB: γ-tubulin
zeo
zeo + CAST
H
Normalized STIM1 levels (A.U.)
2.0
1.5
1.0
0.5
0.0
**
zeo
zeo + CAST

Figure 3-3. STIM1 turnover is controlled by calpains. (A) Immunoblots probing for STIM1 in lysates from wild-type and *CAPN4*$^{-/-}$ fibroblasts show elevated levels of STIM1 in the absence of calpain activity. (B) Quantification of data in (A) ** indicates statistical significance at $p < 0.01$. (C) Wild-type and *CAPN4*$^{-/-}$ fibroblasts were left untreated or treated with 30 μg/mL cycloheximide to inhibit protein synthesis, allowing for monitoring of protein turnover. While STIM1 levels diminished after cycloheximide treatment in wild-type cells, STIM1 levels remained unchanged in *CAPN4*$^{-/-}$ cells. (D) Quantification of data in (C) ** indicates statistical significance at $p < 0.01$ (WT vs. WT CHX). ns indicates non-statistical signifigance ($p = 0.6$). (E) *STIM1*$^{-/-}$ MEFs were transfected with YFP-STIM1 alone or YFP-STIM1 and CAST, then subjected to cycloheximide treatment for 8, 16, or 24 hours. Immunoblots for GFP show that presence of lower molecular weight fragments of YFP-STIM1 is increased over time as YFP-STIM1 is cleaved. Co-expression of CAST noticeably decreased the presence of these lower molecular weight fragments. Immunoblots for calreticulin (CRT) and calnexin (CNX) are included to demonstrate that the turnover of these proteins is unaffected by expression of CAST. (F) Wild-type MEFs were transfected with zeocin plasmid alone or zeocin and calpastatin plasmids, then subjected to cycloheximide treatment for 8, 16, or 24 hours. Immunoblots for STIM1 show that full-length STIM1 levels decrease over cycloheximide treatment time course, but this decrease can be ameliorated by expression of CAST. Immunoblots for calnexin (CNX) are included to demonstrate that its turnover is unaffected by CAST. (G) Wild-type MEFs were transfected with zeocin plasmid alone or zeocin and calpastatin plasmids. Lysates demonstrate that expression of calpastatin increased STIM1 levels by approximately 40%. (H) Quantification of data in (G). ** indicates statistical significance at $p < 0.01$.

STIM1 cleavage is enhanced in apoptotic cells

We next tested a third hypothesis: that STIM1 cleavage may occur during the progression of apoptosis. We observed that treating cells with MG132 overnight often led to their detachment from cell culture dishes, an event that correlates with the onset of apoptosis. Furthermore, in addition to inhibiting the proteasome, MG132 has been shown to stimulate apoptosis via activation of c-Jun N-terminal kinase (JNK1)[217]. To investigate this, we repeated our previous experiments, but separately collected adherent and non-adherent cells, using cleavage of poly ADP ribose polymerase (PARP) as a marker for apoptosis. YFP-STIM1 cleavage was significantly enhanced in apoptotic cells and could be inhibited by the calpain inhibitor PD150606, indicating a role for calpains. PD150606 treatment did not affect PARP cleavage (Fig 3-4A). We observed similar results with native STIM1 cleavage: a characteristic pattern of lower molecular weight bands representing STIM1 cleavage products in cells treated with drugs to induce apoptosis (Fig 3-4B). Much like YFP-STIM1, the degradation of native STIM1 was particularly enhanced in non-adherent, late apoptotic cells and could be inhibited by PD150606.

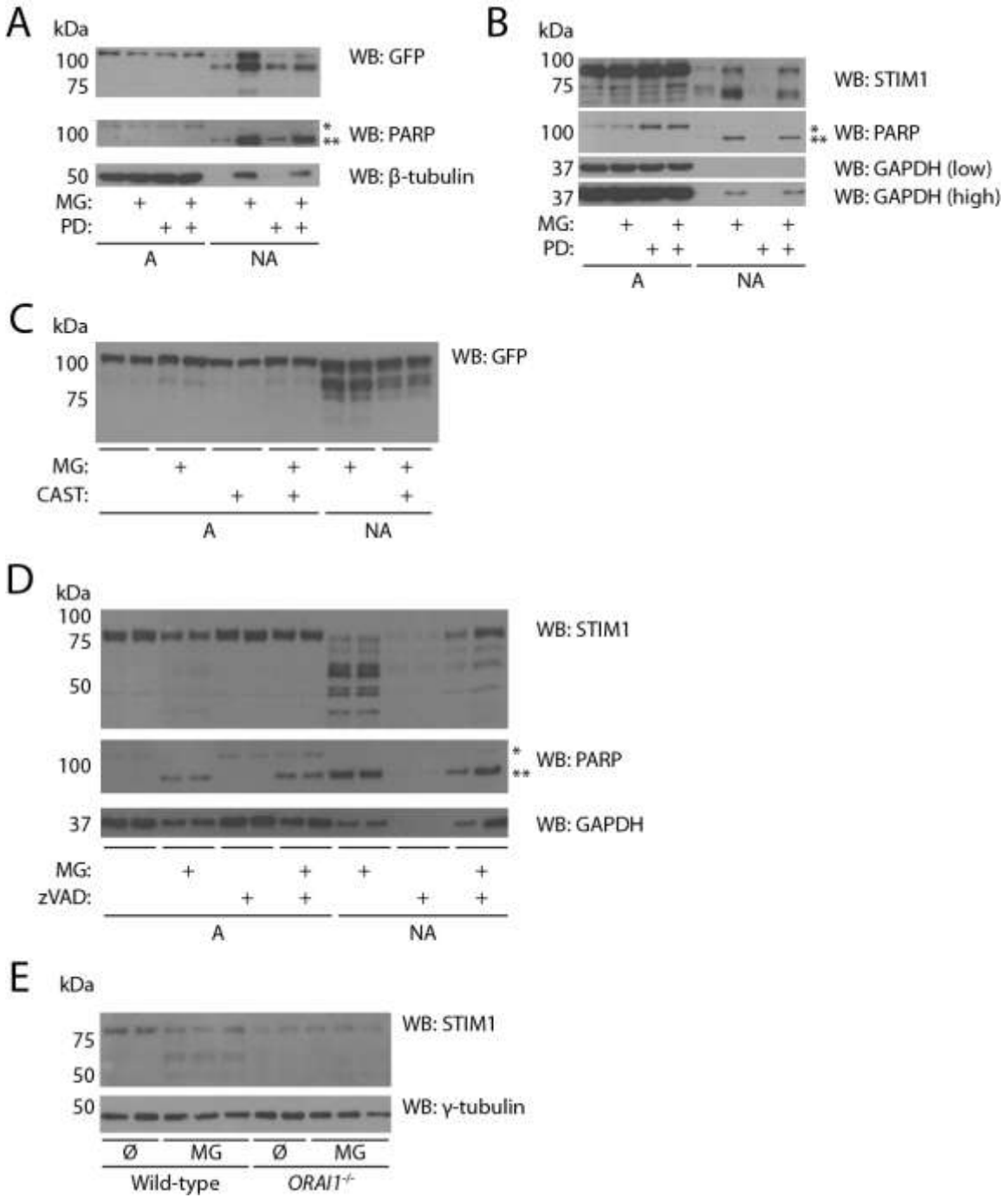
We next transfected cells with a vector encoding YFP-STIM1 alone or vectors encoding YFP-STIM1 and calpastatin, treated with MG132, and collected adherent and non-adherent cells separately. As shown previously, non-adherent cells showed much higher levels of YFP-STIM1 cleavage than did adherent cells. Non-adherent cells also expressing calpastatin showed noticeably lower accumulation of YFP-STIM1 lower molecular weight fragments, indicating that these cleavage fragments are in fact due to calpain activity (Fig 3-4C).

Caspases are another family of proteases activated during apoptosis; additionally, there is evidence of crosstalk between caspase and calpain pathways. For example, during apoptosis in HL-60 cells, caspase activation occurs upstream of calpain activation and

blocking caspase activity can therefore prevent the triggering of calpains[218]. We first used the online algorithm Cascleave to search for caspase cleavage sites, but found no caspase cleavage sites within full-length STIM1, pointing against direct cleavage by caspases[219]. We therefore used zVAD-FMK-OMe, a cell-permeable general caspase inhibitor, in conjunction with MG132 to look for any effect on the cleavage of STIM1. We found that inhibition of caspases significantly reduced the production of lower molecular weight STIM1 cleavage products (Fig 3-4D). As STIM1 has no caspase cleavage sites within its sequence, we did not favor a model where caspases themselves cleave STIM1. Rather, we concluded that calpains were acting downstream of caspases, and therefore caspase inhibition prevented calpain activation, thus abrogating STIM1 cleavage.

Orai1 is required for cleavage of STIM1

Calpains are calcium-activated; therefore, we asked how the cell elevates cytoplasmic Ca^{2+} levels in order to stimulate STIM1 cleavage. A logical candidate was Orai1, as it is intimately involved with STIM1 in controlling cytoplasmic Ca^{2+}. We obtained Orai1-deficient cells and repeated our previous experiments comparing cleavage of native STIM1 in cells with or without Orai1. We found that after overnight treatment with MG132, Orai1-deficient cells showed no accumulation of lower molecular weight STIM1 fragments (Fig 3-4E).


A
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WB: GFP
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*
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WB: PARP
50
WB: β-tubulin
MG:
PD:
A
NA
B
kDa
100
75
WB: STIM1
100
*
**
WB: PARP
37
WB: GAPDH (low)
37
WB: GAPDH (high)
MG:
PD:
A
NA
C
kDa
100
75
WB: GFP
MG:
CAST:
A
NA
D
kDa
100
75
50
WB: STIM1
100
*
**
WB: PARP
37
WB: GAPDH
MG:
zVAD:
A
NA
E
kDa
75
50
WB: STIM1
50
WB: γ-tubulin
Ø
MG
Ø
MG
Wild-type
ORAI1-/-

Figure 3-4. Calpain cleavage is elevated in apoptotic cells. (A) *STIM1*$^{-/-}$ MEFs were transfected with a vector encoding YFP-STIM1 and then treated with 500 nM MG132, 50 μM PD150606, or both, as indicated. After 18 hours of treatment, lysates from adherent (A) and non-adherent (NA) cells were harvested independently, separated via SDS-PAGE, and immunoblotted as indicated. For PARP, (*) represents full-length PARP, a marker of non-apoptotic cells; (**) represents cleaved PARP, a marker of apoptotic cells. With respect to YFP-STIM1 cleavage, noticeably higher cleavage is seen in non-adherent, apoptotic cells as compared to adherent, non-apoptotic cells. This cleavage can be reduced by co-treatment with PD150606, a calpain inhibitor. (B) Wild-type MEFs were treated with 500 nM MG132, 50 μM PD150606, or both, as indicated. After 18 hours of treatment, lysates from adherent (A) and non-adherent (NA) cells were harvested independently, separated via SDS-PAGE, and immunoblotted as indicated. For PARP, (*) represents full-length PARP, a marker of non-apoptotic cells; (**) represents cleaved PARP, a marker of apoptotic cells. STIM1 cleavage is higher in non-adherent, apoptotic cells as compared to adherent, non-apoptotic cells. Similarly to results with YFP-STIM1, this cleavage can be reduced by inhibition of calpains. (C) *STIM1*$^{-/-}$ MEFs were transfected with YFP-STIM1 alone or YFP-STIM1 and calpastatin. Cells were then left untreated or treated with 500 nM MG132, as indicated; 16 hours after treatment, adherent (A) and non-adherent (NA) cells were harvested separately. CAST expression reduced YFP-STIM1 cleavage; this effect was particularly noticeable in non-adherent, apoptotic cells. (D) Wild-type cells were treated with 500 nM MG132 and/or 25 μM zVAD-FMK-OMe, as indicated; 16 hours after treatment, adherent (A) and non-adherent (NA) cells were harvested separately. zVAD-FMK-OMe treatment was able to reduce STIM1 cleavage; this effect was particularly noticeable in non-adherent, apoptotic cells. (E) Wild-type and *ORAI1*$^{-/-}$ MEFs were left untreated or treated with 500 nM MG132, as indicated. After 18 hours of treatment, lysates were harvested, separated via SDS-PAGE, and immunoblotted as indicated. While wild-type cells showed cleavage of STIM1 in response to MG132 treatment, no such cleavage was seen in Orai-deficient cells.

Conclusions

Here, we discovered that STIM1 is a target for calpain, a Ca^{2+}-dependent protease. Our results point to a role of calpain cleavage of STIM1 in controlling the equilibrium levels of STIM1 and within apoptosis. A previous report indicated that STIM1 had a half-life of approximately 19 hours and that its stability was dependent on the presence of its cytoplasmic, coiled-coil domains[63]. We therefore hypothesized that calpain cleavage of STIM1 within its cytoplasmic domain may destabilize STIM1, promoting its turnover. Indeed, in cells lacking calpain activity, resting STIM1 levels were elevated as compared to wild-type cells. This also manifested in changes in SOCE: an increase in the amount of STIM1 was reflected in an increase in SOCE. Furthermore, inhibition of protein synthesis via cycloheximide led to a decrease in the abundance of STIM1 in wild-type cells, but this decrease could be significantly ameliorated by inhibition of calpains with calpastatin, indicating that the previously described turnover of STIM1 can be largely attributed to calpains. Finally, long-term expression of calpastatin in wild-type cells was able to increase STIM1 levels as compared to cells without calpastatin, further supporting our assertion that calpain cleavage of STIM1 is a mechanism by which the cell can control STIM1 levels.

In light of the fact that STIM1 cleavage was enhanced after extended drug treatments to induce cell stress, we considered a role for apoptosis in controlling STIM1 cleavage. STIM1 cleavage was elevated in non-adherent cells undergoing the late stages of apoptosis, suggesting that the degradation of STIM1 occurs concomitantly with apoptosis. Despite possessing no caspase cleavage sites, treating cells with the pan-caspase inhibitor zVAD-FMK-OMe significantly reduced STIM1 cleavage in response to MG132 treatment. We believe this result was due to previously described cross-talk between caspases and calpains, where caspase activation is known to be upstream of calpain activation during the progression of apoptosis[218]. In further support, calpastatin was a potent inhibitor of STIM1 cleavage though, to our knowledge, it has never been

described as an inhibitor of caspases. In fact, caspases have been shown to cleave calpastatin itself[220]. We also found that STIM1 cleavage was severely reduced in the absence of Orai1, implicating Orai1 as the source of Ca^{2+} required to activate calpains.

STIM1 is a key constituent of intracellular Ca^{2+} signaling and has been a target of intensive research due to its roles in numerous pathways implicated in disease[221]. Artificially lowering the levels of STIM1 has been shown to reduce breast cancer metastasis[178], while increased levels of STIM1 are observed in models of cardiac hypertrophy[145]; therefore, an understanding of how the cell controls STIM1 abundance is essential for a more complete appreciation of the roles of STIM1 and SOCE in human disease. Here, we show that calpain activity serves as a mechanism to control endogenous STIM1 levels. We further show that STIM1 is a target for regulated cleavage during the progression of apoptosis, with high levels of STIM1 cleavage observed in cells undergoing the latest stages of apoptosis. Overall, we suggest that the interplay between STIM1 and calpains may play a heretofore unknown role in the control of SOCE.

Acknowledgments

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10.1038/embor.2011.173.

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10.1161/CIRCULATIONAHA.111.031229 (2011).

CHAPTER FOUR

Calnexin modulates store-operated calcium entry in T cells

Abstract

Store-operated calcium entry (SOCE) is a key cell signaling pathway within systems as diverse as muscular development and immunity. SOCE couples loss of Ca^{2+} from the endoplasmic reticulum (ER) lumen with Ca^{2+} transit across the plasma membrane into the cytoplasm, where it can regulate signaling pathways or be taken up into the ER. It is mediated by two proteins, STIM1 and Orai1, whose involvement in the process is both necessary and sufficient. However, SOCE is regulated by a host of other factors, including protein-lipid interactions, post-translational modifications, and protein-protein interactions. Here, we show a role in SOCE for the ER transmembrane protein calnexin, which is best known for its role as a chaperone, where it ensures the correct folding of its client proteins. We find that calnexin also plays a role in the regulation of SOCE, specifically within T lymphocytes. Loss of calnexin has a deleterious effect on SOCE, and this may be due to impaired movement of STIM1 to subplasmalemmal punctae. Thus, calnexin may be considered one of many STIM1 interacting partners that act to regulate SOCE.

Introduction

In addition to its essential role in the regulation of Ca^{2+} signaling, the ER is also responsible for the proper synthesis and folding of numerous proteins, particularly transmembrane proteins and those destined for secretion, which may be up to a third of all cellular proteins[222]. To ensure proteins are properly folded, the cell has evolved a class of proteins known as chaperones, which are responsible for assisting in the correct folding of substrate proteins. Two of the most important chaperones are the closely related lectin chaperones calnexin and calreticulin; most proteins that enter the ER will associate with calnexin and/or calreticulin[1]. Both calnexin and calreticulin are lectin-like chaperones: they recognize glycoproteins and assist in their proper folding. The two proteins are highly homologous, but while calreticulin is a soluble, ER luminal protein, calnexin possesses not only an ER luminal domain but also a transmembrane domain and a cytoplasmic C-tail[1]. The differences in the structures of calnexin and calreticulin are reflected in their different functions: while both regulate protein folding, calreticulin also plays a key role in Ca^{2+} signaling[12,14]. Indeed, calreticulin deficiency is embryonic lethal due to deleterious effects on Ca^{2+}-dependent cardiac development[12]. Conversely, calnexin deficiency is not embryonic lethal but instead manifests itself in neurological disorders due to impaired myelin sheath formation[223], which can be traced to misfolding of the crucial myelin glycoproteins P0 and PMP22[224]. A well-studied client protein of calnexin is the major histocompatibility complex class I (MHC class I) molecule, which is a protein responsible for presenting protein fragments to T cell within the immune system[225]. Interestingly, calnexin has also been described to bind STIM1; this binding was not glycosylation-dependent, pointing against the interaction solely representing chaperone activity[63]. However, the consequences of the STIM1-calnexin binding event have not been described in the literature. We therefore decided to investigate the STIM1-calnexin interaction in greater detail.

Materials and Methods

Immunoprecipitation, SDS-PAGE, and immunoblotting

Immunoprecipitation experiments were carried out following a previously described protocol[226]. In brief, cells were lysed in via a Hepes-based buffer (50 mM Hepes pH 7.4, 200 mM NaCl, 2% Chaps) to isolate proteins, followed by pre-clearing of lysates with 10% Protein A-Sepharose in buffer. The pre-cleared lysate was incubated overnight with antibodies against the protein of interest, followed by addition of 10% Protein A-Sepharose to immunoprecipitate protein complexes. Protein A-Sepharose beads were washed four times, then proteins were eluted via addition of sample loading buffer. Samples were separated via SDS-PAGE and immunoblotted as previously described[60].

Generation of T lymphocyte-specific CNX$^{-/-}$ mice

To generate mice with calnexin deleted specifically in T lymphocytes, we turned to the Cre recombinase LoxP system. Specifically, we used a mouse strain in which exons 3 and 4 of the calnexin gene are flanked by LoxP sites, which was a generous gift from Dr Masahito Ikawa[227]. This strain was crossed with a mouse strain expressing Cre recombinase under the control of the *Lck* (lymphocyte protein tyrosine kinase) promoter, which is primarily expressed in T lymphocytes[228]. Hence, mice homozygous for the calnexin-LoxP transgene also expressing Cre recombinase in T lymphocytes will have excision of a portion of the calnexin gene, enabling for T lymphocyte-specific deletion of calnexin. Mice with T lymphocyte-specific deletion of calnexin can be monitored via PCR-based genotyping for the Cre recombinase gene and via immunoblotting for calnexin protein in freshly isolated T cells.

Isolation of T cells

Isolation of T cells was based on the method of Kruisbeek[229]. After sacrifice of mouse, the thymus was removed and placed on ice. To isolate T cells from the thymus, 1 mL of Advanced RPMI media (Advanced RPMI + 10% heat-inactivated fetal bovine serum +

1% L-glutamine + 1% penicillin/streptomycin) was added. The thymus was dissected with a surgical blade to release cells into the media, after which the media and thymus pieces were passed through a 70 μm clearance nylon filter, which was then rinsed with more media. Cells were then pelleted for 6 minutes at 1200 rpm, washed with fresh media, pelleted for 6 minutes at 1200 rpm, and resuspended in ACK buffer (15.5 mM NH_4Cl, 1 mM $KHCO_3$, 10 mM EDTA) to lyse red blood cells. Fresh media was added to halt lysis, after which the remaining cells, which are largely T cells, were pelleted again.

Cytoplasmic Ca^{2+} measurements

Cytoplasmic Ca^{2+} measurements were performed as described previously[60]. Freshly isolated T cells were loaded with Fura2-AM dye, resuspended in Ca^{2+}-free buffer (143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 0.1% glucose, 1 mM EGTA, 20 mM Hepes-NaOH pH 7.4) and then placed in a fluorometer (Protein Technologies International). Cells were treated with 1 μM thapsigargin to deplete ER Ca^{2+} stores, followed by addition of 2 mM $CaCl_2$ to induce store-operated Ca^{2+} entry. Cells were variably excited at 340 nm and 380 nm, with excitation at 510 nm. Calibration steps were performed to enable conversion of raw 340/380 ratios into cytoplasmic Ca^{2+} concentrations.

Subcellular fractionation

Subcellular fractionation was performed largely as previously described[230], with slight modifications. In brief, isolated T cells were washed with PBS and resuspended in homogenization buffer (10 mM Hepes-NaOH pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA). Cells were lysed via 25 passages through a ball bearing homogenizer (Isobiotec) with a 4 micron clearance, followed by centrifugation at 800x g for 10 minutes to pellet nuclei and cell debris. The supernatant was then layered onto an eight-step gradient (25%, 22%, 19%, 16%, 13%, 10%, 7%, and 4% iodixanol in homogenization buffer). The gradient was centrifuged for 6 hours at 150,000g, after which 12 fractions were harvested from the top of the gradient. Proteins were precipitated with 90%

acetone, washed with 100% ethanol, and resuspended in sample buffer (20% glycerol, 60 mM Tris pH 6.8, 2% SDS) for analysis via immunoblotting.

Results

STIM1 interacts with calnexin

Binding of STIM1 and calnexin (CNX) has been described previously by another group[63]. To confirm this we carried out co-immunoprecipitation of STIM1 and CNX in various fibroblast cell lines. Immunoprecipitation with antibodies against STIM1 successfully pulled down calnexin in quantities much higher than did beads alone (no antibody added) (Fig 4-1). We did not investigate the co-immunoprecipitation of STIM1 and CNX in great detail, as this finding has already been described and validated in the literature.

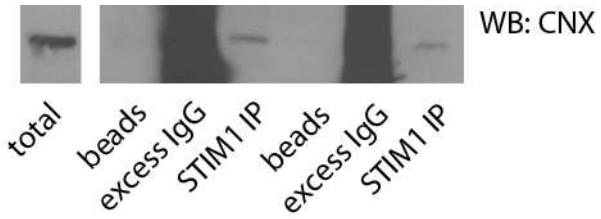

Figure 4-1. Co-immunoprecipitation of STIM1 and calnexin. Wild-type fibroblasts were lysed and incubated with antibodies against STIM1. Immunoprecipitates were subjected to SDS-PAGE and probed with antibodies against calnexin. STIM1 immunoprecipitation also pulled down calnexin, confirming an interaction between the two proteins; Protein A beads alone were unable to pull down calnexin.

Store-operated calcium entry is largely unaffected in CNX$^{-/-}$ fibroblasts

We next aimed to determine if CNX, presumably via interaction with STIM1, may have an effect on SOCE, as this process is the key responsibility of STIM1. To do so, we used calnexin-deficient fibroblasts and their wild-type counterparts, cell lines whose derivation was described earlier[231]. We used Fura2 to measure cytoplasmic Ca^{2+} levels in response to addition of thapsigargin (to deplete ER Ca^{2+} stores) and external Ca^{2+} (to initiate SOCE). Interestingly, thapsigargin-releasable Ca^{2+} stores were slightly but noticeably higher in *CNX$^{-/-}$* cells than in their wild-type counterparts, suggesting that calnexin-deficient cells may have higher ER luminal Ca^{2+} stores (Fig 4-2). This could also represent a higher ER Ca^{2+} leak: passive ER Ca^{2+} leak has been shown to occur via the translocon-ribosome complex[232], and calnexin is known to associate with this complex[233]. Our major interest was in the process of SOCE, and here we saw no significant difference between wild-type and calnexin-deficient fibroblasts. This suggested that the absence of calnexin in fibroblasts leaves SOCE unaffected.

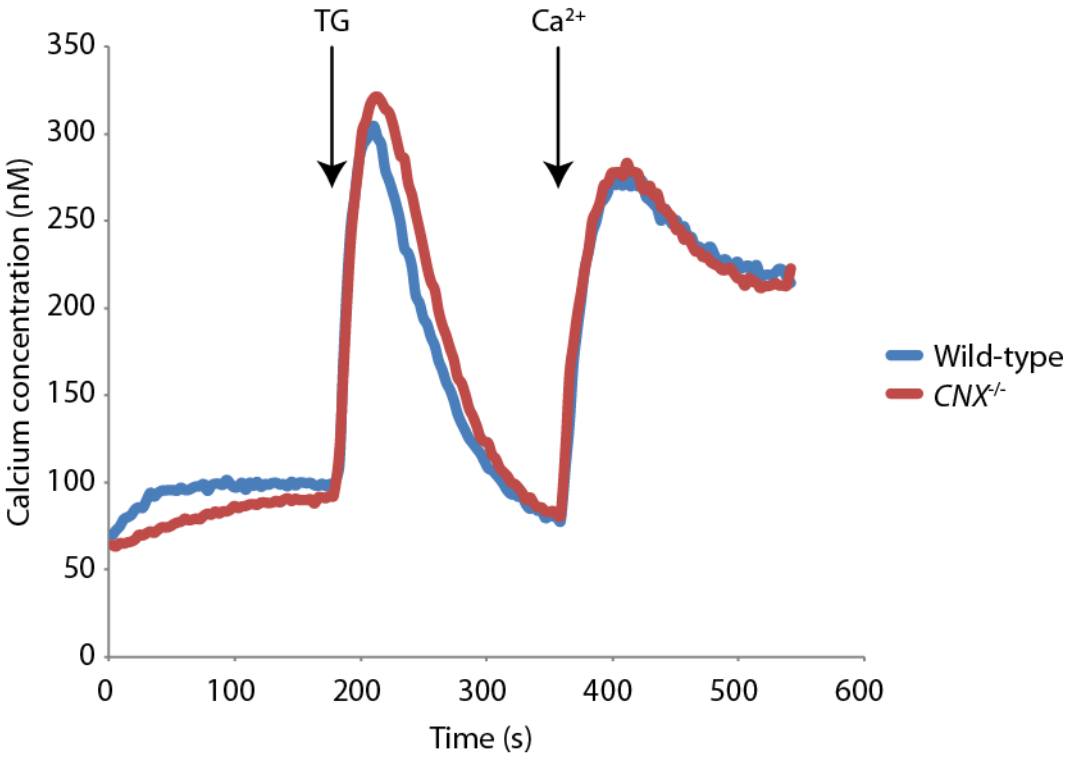

Figure 4-2. Calnexin deficiency does not alter store-operated calcium entry (SOCE) in fibroblastss. Fura2 Ca^{2+} measurements of wild-type and *CNX*$^{-/-}$ fibroblasts. Wild-type and calnexin-deficient fibroblasts were loaded with Fura2-AM dye to measure cytoplasmic Ca^{2+}, then treated with 1 μM thapsigargin to deplete ER Ca^{2+} stores, followed by 2 mM Ca^{2+} to induce SOCE. While calnexin-deficient fibroblasts show higher thapsigargin-releasable Ca^{2+} stores, the SOCE phase is virtually indistinguishable between the two cell lines. Traces represent an average of 3 separate biological replicates.

Store-operated calcium entry is impaired in CNX$^{-/-}$ T cells

Next we extended our experiments into T cells. We were interested in T cells specifically because calnexin has previously been shown to play a key role within the immune system: for example, calnexin participates in the loading of antigens onto the major histocompatibility complex (MHC) class I loading complex within the lumen of the ER[234]. Furthermore, calnexin is known to associate with various subunits of the T cell receptor, suggesting that any perturbations in calnexin levels or functions may have deleterious effects on T cell function[235]. Finally, currently unpublished results from our laboratory suggested that calnexin deficiency may be protective against certain autoimmune diseases. For these reasons, we developed a mouse based on the Cre recombinase system, wherein the exons 3 and 4 of the calnexin gene were flanked by loxP sites to allow for deletion of this part of the gene in any cells where Cre recombinase was expressed. To specifically delete the calnexin gene in T lymphocytes, we crossed calnexin-loxP mice with mice expressing Cre recombinase under the control of the endogenous *Lck* (lymphocyte protein tyrosine kinase) promoter, which is expressed in T lymphocytes[228]. Hence, these mice should have the calnexin gene deleted in all T lymphocytes. Mice with thymus-specific calnexin deficiency were identified via PCR-based genotyping for the Cre recombinase gene; deletion of calnexin was confirmed via immunoblotting.

After isolation of T cells from freshly dissected thymuses, we performed Fura2-based cytoplasmic Ca^{2+} measurements. To maximize viability and health of these T cells, all experiments were performed T cells within an hour of their isolation. We observed that T cells lacking calnexin had slightly higher thapsigargin-releasable Ca^{2+} stores than did their wild-type counterparts, similar to the difference observed in fibroblasts (Fig 4-3A). However, in contrast to fibroblasts, we did observe a sharp difference in SOCE: calnexin deficiency in T cells severely impaired SOCE, both in terms of initial speed of Ca^{2+} entry and in extent. Also worth noting is that in wild-type T cells, SOCE was much stronger than in wild-type fibroblasts (Fig 4-3B, Fig 4-3C). These data correspond well with the

literature consensus that it is within the immune system that SOCE plays one of its most crucial roles[221]. Furthermore, these results confirm that calnexin itself is of especial importance within the proper functioning of the immune system. Overall, we concluded that lack of calnexin impairs SOCE, with this effect seemingly specific to T cells, at least as compared to fibroblasts.

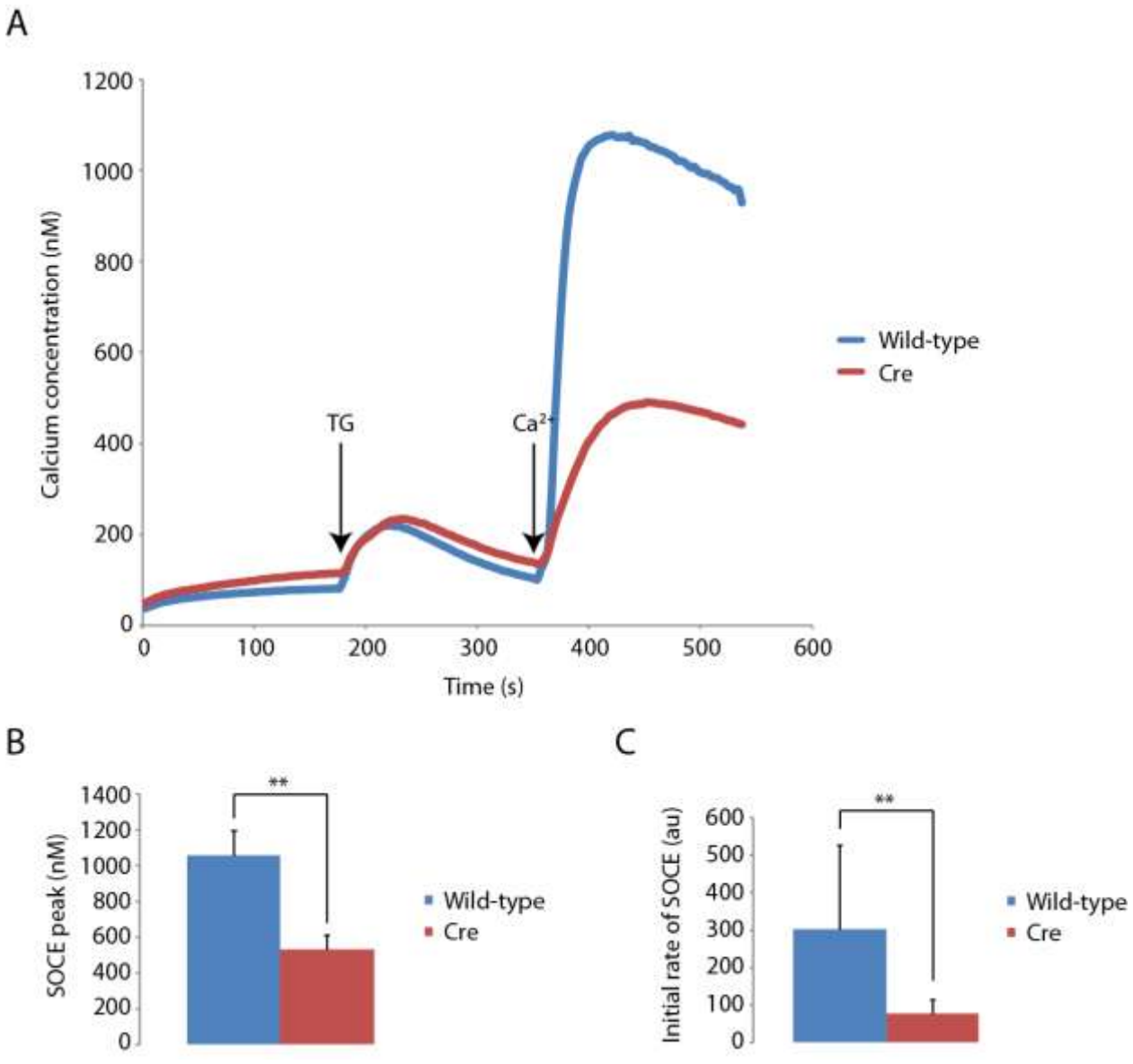
A
1200
1000
800
600
400
200
0
Calcium concentration (nM)
TG
Ca2+
0
100
200
300
400
500
600
Time (s)
Wild-type
Cre
B
1400
1200
1000
800
600
400
200
0
SOCE peak (nM)
**
Wild-type
Cre
C
600
500
400
300
200
100
0
Initial rate of SOCE (au)
**
Wild-type
Cre

Figure 4-3. Store-operated calcium entry (SOCE) is impaired in calnexin-deficient T cells of thymic origin. (A) Fura2 was used to measure cytoplasmic Ca^{2+} concentration in wild-type and Cre-expressing T cells of thymic origin. The wild-type trace represents an average of 10 experiments, while the Cre trace represents an average of 7 experiments. TG indicates addition of 1 μM thapsigargin to deplete ER Ca^{2+} stores, while Ca^{2+} indicates addition of 2 mM free Ca^{2+} to induce SOCE. SOCE was noticeably higher in wild-type cells than in Cre-expressing cells. (B) Quantification of the SOCE peak shows that the height of the SOCE peak was significantly higher in wild-type cells than in Cre-expressing cells (WT, 1057 ± 140 nM; Cre, 531 ± 80 nM; $p = 0.004$). (C) Quantification of the initial rate of SOCE shows that the initial rate of calcium entry was significantly higher in wild-type cells than in Cre-expressing cells (WT, 302 ± 224; Cre, 77 ± 38; $p = 0.004$).

Calnexin deficiency does not affect STIM1 or Orai1 levels

As calnexin is well-described as a chaperone, we first considered the possibility that calnexin may regulate the stability of STIM1. In light of our discovery that the cellular abundance of STIM1 is dependent upon the activity of calpains (as described in Chapter 3 of this thesis), we hypothesized that the absence of calnexin may lead to an improperly folded STIM1 that is more susceptible to calpain cleavage, thus lowering STIM1 levels and hence, SOCE. To investigate this, we took lysates from wild-type and Cre T cells, separated via SDS-PAGE, and immunoblotted to monitor levels of calnexin (to determine efficiency of calnexin depletion), STIM1, and Orai1, with an immunoblot against β-tubulin serving as a loading control. Overall, we observed very efficient deletion of calnexin in the Cre-expressing T cells, with minimal levels of residual calnexin (Fig 4-4). STIM1 levels were largely consistent across all samples, with no significant variations in amounts of full-length STIM1 or STIM1 degradation products. These results point against a role for calnexin in regulating STIM1 stability or susceptibility to calpain cleavage. Similarly, while Orai1 levels demonstrated a larger variation across the eight samples investigated, there was no noticeable pattern to these variations. From these data, we therefore concluded that calnexin deficiency has no effect on the abundance or stability of the two key proteins mediating SOCE, STIM1 and Orai1. We thus sought a different explanation for the changes in SOCE observed in calnexin-depleted T cells.

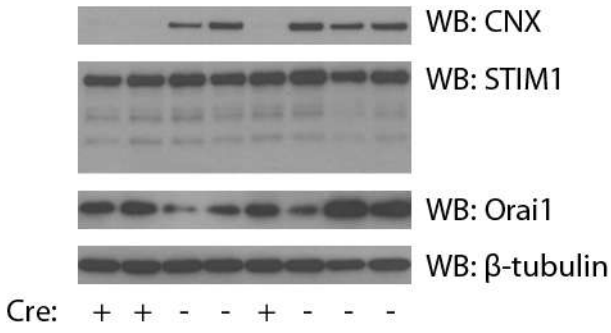

Figure 4-4. STIM1 and Orai1 levels are unaffected by the presence of calnexin. Immunoblotting with an antibody against calnexin showed almost complete loss of calnexin in Cre-expressing T cells of thymic origin. Immunoblotting for STIM1 and Orai1 showed similar levels of STIM1 and Orai1 when comparing wild-type and Cre-expressing T cells, pointing against a role for calnexin in regulating the levels of these proteins. β-tubulin serves as a loading control.

STIM1 redistributes in response to ER Ca^{2+} store depletion

It is well established that in response to thapsigargin-induced ER Ca^{2+} store depletion, STIM1 redistributes throughout the ER membrane into subplasmalemmal punctae[26]. We therefore asked if impaired SOCE in calnexin-deficient T cells might be due to deficiencies in the movement of STIM1 to these subplasmalemmal punctae. To investigate this question, we decided to use subcellular fractionation of freshly isolated T cells to monitor the distribution of STIM1 with and without thapsigargin treatment. To our knowledge, use of this technique to investigate SOCE has yet to be described in the literature. We discovered a slight but reliable redistribution of STIM1 in response to 20 minutes of treatment with thapsigargin, with an enrichment of STIM1 into lower fractions, particularly fractions 11 and 12, as well as fraction 5 (Fig 4-5A, B). These data are quantified in Fig 4-5C, showing that STIM1 levels, as a percentage of the total, are higher in these fraction (5, 11, and 12) after thapsigargin treatment, with a corresponding reduction in STIM1 levels in the intermediate fractions. We therefore hypothesize that these fractions may represent the STIM1 punctae formed in response to store depletion.

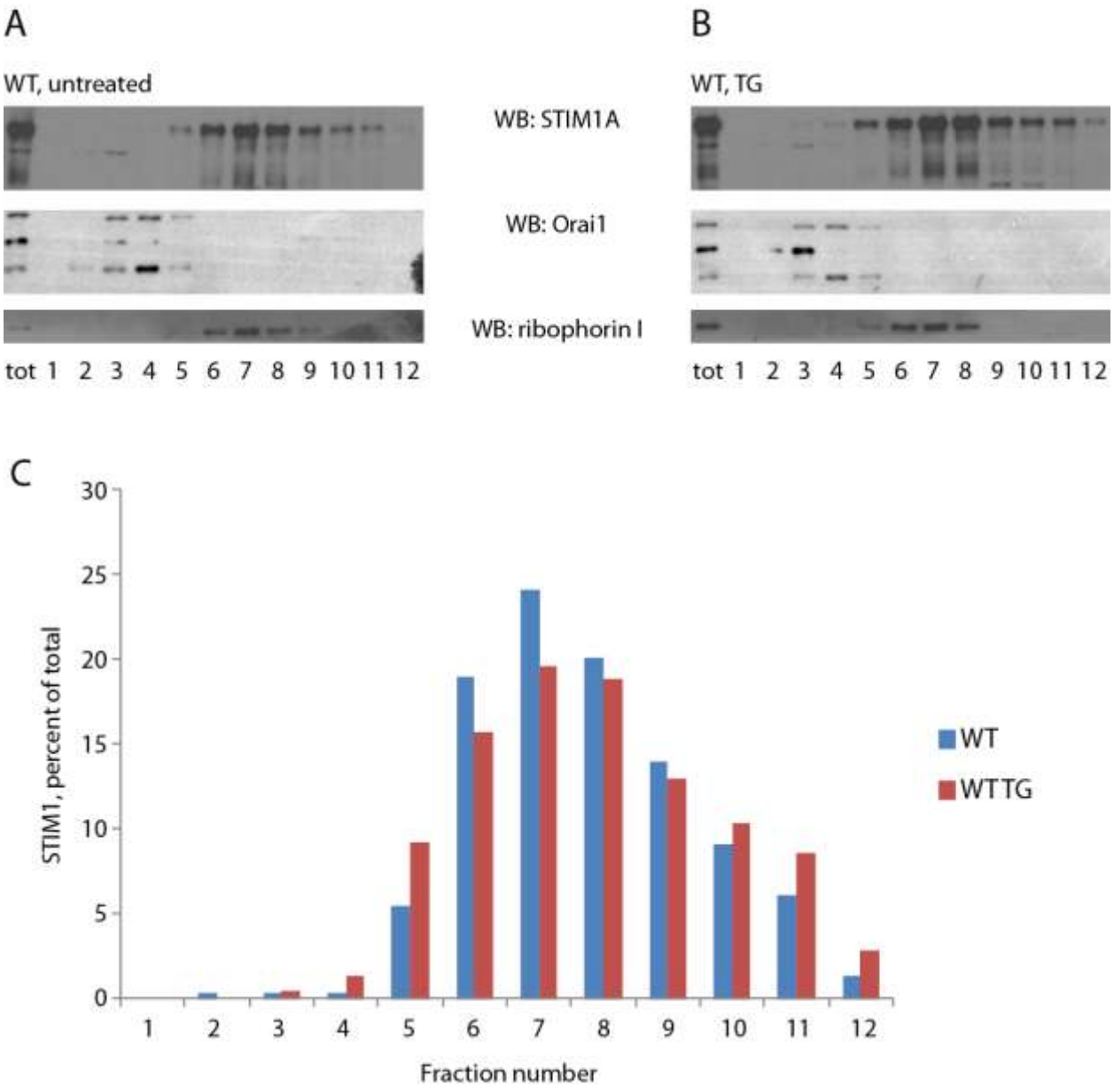
A
WT, untreated
WB: STIM1A
WB: Orai1
WB: ribophorin I
tot 1 2 3 4 5 6 7 8 9 10 11 12
B
WT, TG
tot 1 2 3 4 5 6 7 8 9 10 11 12
C
STIM1, percent of total
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12
Fraction number
WT
WT TG

Figure 4-5. STIM1 redistribution in response to store depletion can be visualized via subcellular fractionation. (A,B) Subcellular fractionation of freshly isolated wild-type cells shows that STIM1 distribution is altered after thapsigargin treatment to deplete ER Ca^{2+} stores. In particular, STIM1 is enriched in the two lowest (heaviest) fractions. 'tot' represents a sample of the total (i.e. pre-fractionation). Orai1 is largely found in the lightest fractions (2-5), which correspond to the plasma membrane. Ribophorin I is an ER marker; it does not redistribute to fraction 11 & 12 after store depletion, suggesting the movement of STIM1 is at least somewhat specific. One representative blot of two independent experiments is shown. (C) Quantification of STIM1 levels in A and B.

STIM1 redistribution is impaired in calnexin-reduced T cells

We next compared the distribution of STIM1, as assayed via subcellular fractionation, in wild-type and calnexin-reduced T cells. Wild-type and calnexin-reduced T cells were isolated from thymuses, left untreated or treated with thapsigargin, and subjected to subcellular fractionation as described above. Interestingly, the redistribution of STIM1 into fractions 11 and 12 that we observed in wild-type T cells was noticeably reduced in cre recombinase positive T cells (Fig 4-6A, B). These data are quantified in Fig 6C. In these cells, the reduction in calnexin protein levels was incomplete and some residual calnexin remained (data not shown). However, the amount of calnexin present was lower than in wild-type T cells, showing that the cre recombinase technique was at least able to reduce calnexin levels. Overall, these results indicate that subcellular fractionation can be used to probe the movement of proteins throughout the ER membrane as a part of SOCE. Furthermore, our findings suggest that STIM1 movement in T cells may be partially reliant upon the presence of calnexin: with lowered levels of calnexin or in its absence, STIM1 redistribution within a subcellular fractionation is impaired. We hypothesize that this impaired relocalization of STIM1 may underlie the deficiencies in SOCE observed in calnexin-depleted T cells. Figure 7 compares the abundance of STIM1 in the heaviest fractions in all four fractionation experiments, focusing on fractions 11 and 12.

Interestingly, the subcellular fractionations also present another intriguing finding. We earlier found that STIM1 cleavage by calpains is equivalent in unstimulated wild-type and calnexin-depleted T cells (Fig 4-4). Comparing wild-type T cells with or without store depletion, we also observe very little differencee in STIM1 calpain cleavage products (Fig 4-5A, B). However, comparing calnexin-reduced T cells with or without store depletion, there was a noticeable increase in the abundance of lower molecular weight STIM1 fragments in response to thapsigargin treatment (Fig 4-6A, B). This finding is in sharp contrast to the results presented in Chapter 3 of this thesis, which indicate that in MEFs, thapsigargin treatment of up to 2 hours does not induce STIM1

cleavage (Fig 3-2D). In this case, a mere 20 minutes of store depletion is sufficient to significantly increase the cleavage of STIM1 in T cells lacking calnexin. While there exist differences in the procedures in these two experiments, the minimal cleavage seen in wild-type T cells suggests these results are not an artefact resulting from the subcellular fractionation protocol. Instead, we suggest that calpain cleavage of STIM1 may be elevated in T cells, with calnexin somehow preventing this cleavage event. It is possible that increased cleavage of STIM1 may underlie the lowered levels of SOCE in calnexin-depleted T cells, at least to some extent.

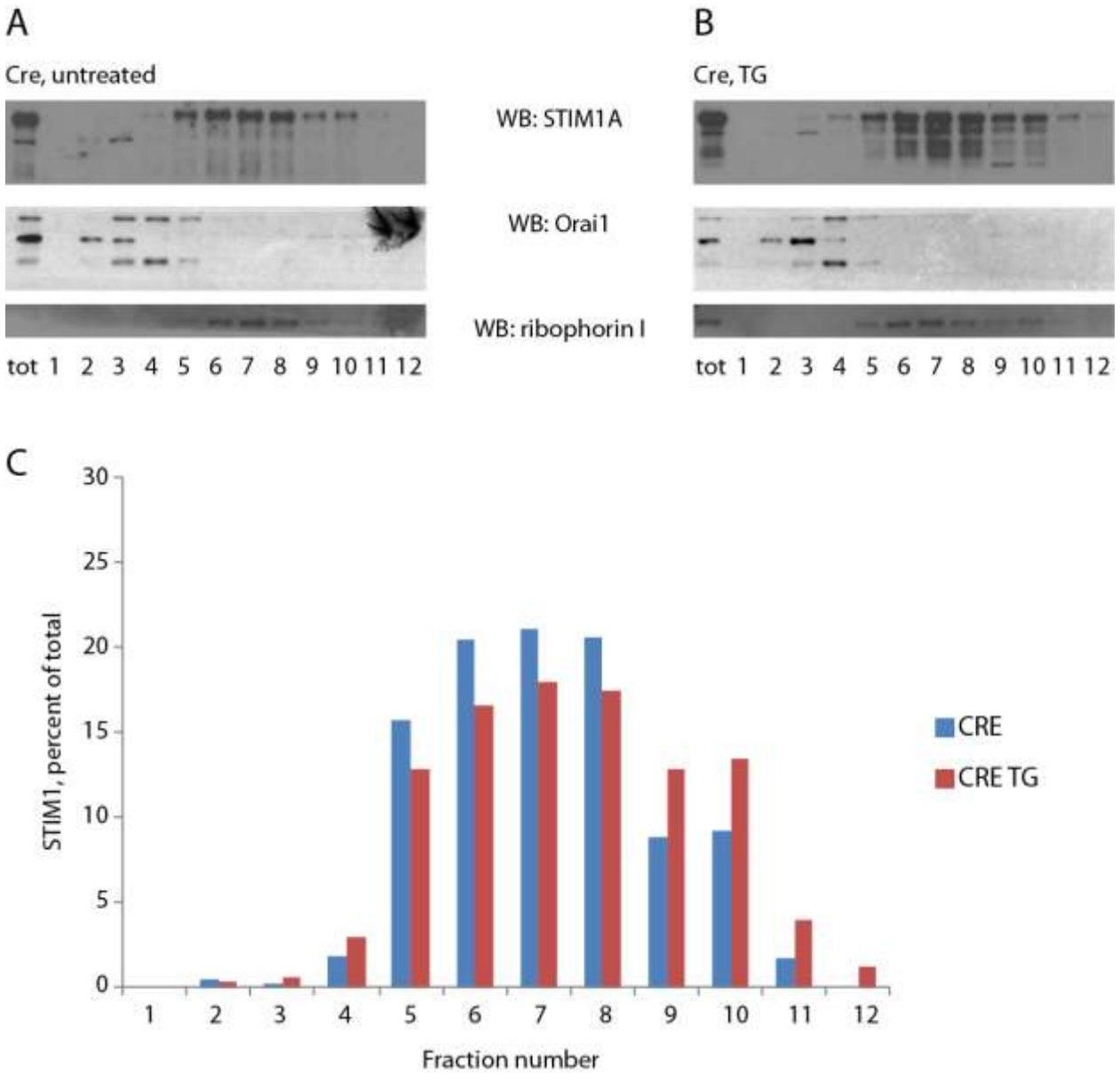
A
Cre, untreated
B
Cre, TG
WB: STIM1A
WB: Orai1
WB: ribophorin I
tot 1 2 3 4 5 6 7 8 9 10 11 12
tot 1 2 3 4 5 6 7 8 9 10 11 12
C
STIM1, percent of total
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12
Fraction number
CRE
CRE TG

Figure 4-6. STIM1 redistribution is impaired in calnexin-depleted T cells. (A,B) Subcellular fractionation of freshly isolated Cre recombinase positive T cells shows that thapsigargin treatment is less able to induce STIM1 redistribution than in wild-type cells. 'tot' represents a sample of the total (i.e. pre-fractionation). Orai1 is largely found in the lightest fractions (2-5), which correspond to the plasma membrane. Ribophorin I is an ER marker. One representative blot of two independent experiments is shown. (C) Quantification of STIM1 levels in A and B.

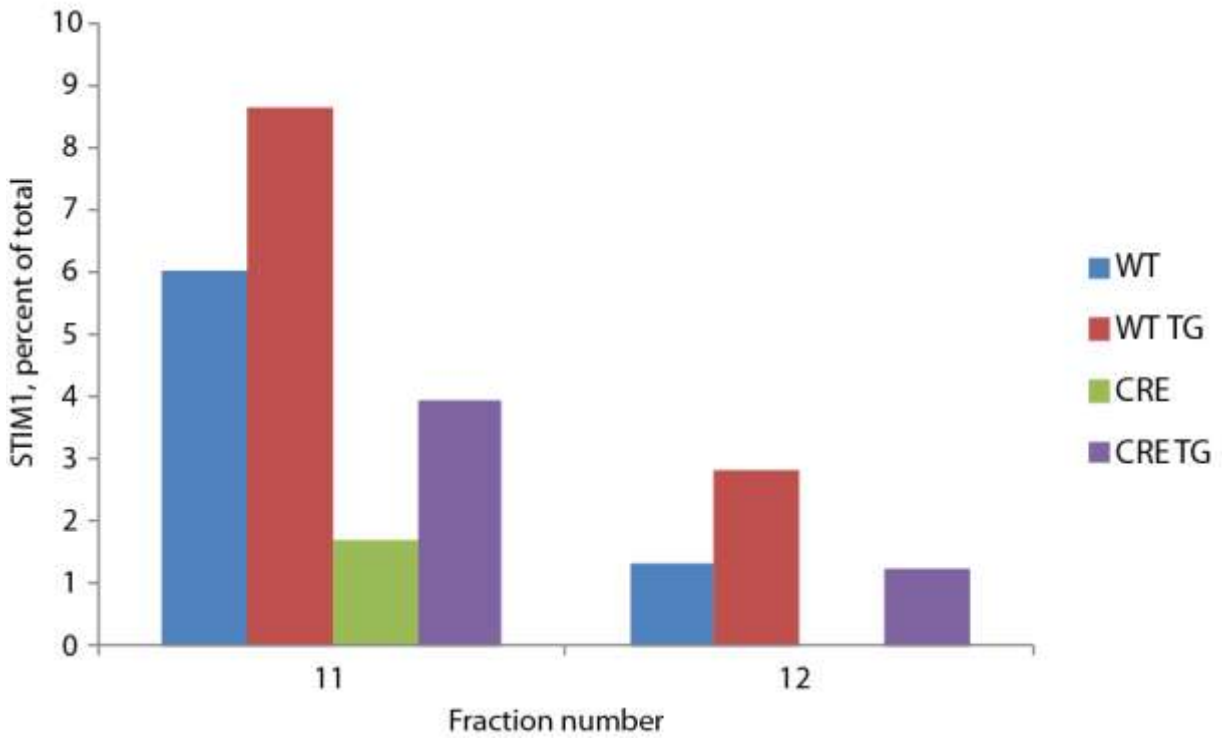

Figure 4-7. Comparison of STIM1 levels in heaviest fractions. When comparing STIM1 levels in all four fractions (WT, WT TG, CRE, and CRE TG), it is evident that the heaviest fractions contain noticeably more STIM1 in wild-type cells than in calnexin-depleted cells. Furthermore, thapsigargin treatment enhances this effect, suggesting that these heaviest fractions may correspond to STIM1 enriched subplasmalemmal punctae.

Conclusions

Overall, our preliminary investigation into the interaction between STIM1 and calnexin found several interesting results. First, we discovered that while STIM1 and calnexin bind each other in fibroblasts, the presence or absence of calnexin has no noticeable effect on SOCE in these cells. However, in sharp contrast, thymic T cells with calnexin levels depleted via Cre-mediated recombination showed a severe reduction in SOCE when calnexin was absent. Importantly, the extent and speed of SOCE were much higher in T cells than in fibroblasts, underscoring the important role SOCE plays in these immune cells. As calnexin-depleted T cells showed sharply abrogated SOCE, we concluded that calnexin is required for efficient SOCE within T cells but not fibroblasts. As these are the only two cell types we have yet studied, it is conceivable that the calnexin-STIM1 interaction may be important in many different cell types or it may be specific to T cells alone.

We next aimed to determine a mechanism whereby the absence of calnexin impairs SOCE. As calnexin has been extensively studied as a chaperone, we first looked at levels of STIM1 and Orai1 to see if calnexin deficiency affects their abundances. While there was a noticeable degree of variability in STIM1 and Orai1 levels amongst T cells isolated from different mice, there was no correlation between levels of calnexin and levels of SOCE-mediating proteins. Furthermore, while we have described the cleavage of STIM1 by cytoplasmic calpains, we did not notice any significant difference in the levels of STIM1 cleavage products when comparing unstimulated wild-type and calnexin-depleted T cells. These results point against a model in which calnexin is serving as a chaperone for STIM1 and/or Orai1, requiring a different mechanism by which calnexin may affect SOCE. We therefore exploited subcellular fractionation to monitor the movement of STIM1 within cellular membranes in response to store depletion. While our results are extremely preliminary, they are also encouraging: thapsigargin treatment leads to an enrichment of STIM1 within certain fractions that we hypothesize correlate to subplasmalemmal punctae. Furthermore, this STIM1 enrichment is reduced in

calnexin-reduced T cells. Taken together, these results suggest that within T cells, calnexin supports the movement of STIM1 to punctae, where STIM1 acts to initiate SOCE. Without calnexin, STIM1 movement is impaired, which is reflected in the lower peak and speed of SOCE in calnexin-depleted T cells.

A second intriguing hypothesis is that calnexin may somehow protect STIM1 from being cleaved by calpain proteins. We observe that in calnexin-reduced T cells, store depletion induces significant STIM1 cleavage after only 20 minutes. These findings are interesting in light of published results describing increased calpain activity during the activation of T cells. For example, in Jurkat T cells, calpains are essential for chemotaxis and migration[236]. Thus, a reasonable hypothesis would be as follows: T cell activation involves a significant upregulation in calpain activity. As STIM1 is susceptible to calpain cleavage (as shown in Chapter 3 of this thesis), this would lead to significant loss of STIM1 were there no mechanism to prevent calpains acting on STIM1. Instead, calnexin binding to STIM1 serves to somehow protect STIM1, for example, by masking the calpain cleavage site. Without calnexin present, calpains are free to cleave STIM1; this increased cleavage may be the reason or one of several reasons underlying impaired SOCE in the absence of calnexin in T cells.

As our data to date are incomplete, we must be cautious of overinterpretation. It seems clear that calnexin-deficiency has all but no effect on SOCE within fibroblasts, but a strongly negative effect on the same process within T cells. We have used subcellular fractionation to suggest that calnexin may affect SOCE by controlling the movement of STIM1 to punctae. However, to our knowledge, subcellular fractionation of T cells to monitor STIM1 movement during SOCE has never been described in the literature and thus we cannot be certain that the shifts of STIM1 from one fraction to another truly represent the movement of STIM1 to punctae. Use of another technique, such as immunostaining for STIM1 pre- and post-store depletion, would significantly strengthen our hypothesis. If this hypothesis proves to be true, a critical question still exists: why do T cells need calnexin for efficient SOCE while fibroblasts do not? As our

data are only preliminary, we cannot yet answer this question. The answer may lie within the different ensemble of proteins expressed in fibroblasts and T cells: perhaps calnexin is recruiting some T cell-specific protein to the SOCE complex. Alternatively, it is possible that variations in calpain expression and activity result in changes in STIM1 cleavage, thus affecting the amount of SOCE. While the precise mechanism is unclear, we believe our current data are exciting enough to warrant further investigation. It is interesting to note that calnexin has already been shown to play a key role in chaperoning several proteins within the immune system. Our results further underscore the importance of calnexin within immune function, but point to it playing an alternative, non-chaperone role.

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CHAPTER FIVE

Concluding Remarks

Conclusions

Store-operated calcium entry (SOCE) is a critical cellular process, playing a key role within physiological systems as varied as muscular contraction, the immune system, and cancer progression[221]. It has been the subject of strong interest for many years, and research into its mechanism and functions has only grown more intense in the decade following the identification of its two key molecular components, STIM1[25,26] and Orai1[27]. My research to date has focused on elucidating new mechanisms for the control of SOCE, as further insights into how the cell controls this pathway may explain how its regulation and dysregulation can contribute to human health and disease. A more complete understanding of the complexity of SOCE can only aid in development of novel strategies to target this pathway as a treatment of various human disease states. We have described three protein-protein interactions that allow the cell to exert a further degree of control over SOCE and its constituent protein STIM1:

(1) an interaction between STIM1 and ERp57;

(2) an interaction between STIM1 and calpains; and

(3) an interaction between STIM1 and calnexin.

Rather than using the final chapter to simply summarize the data and conclusions presented in the first four chapters of this thesis, I will attempt to contextualize these data and conclusions within the currently available literature. This will allow me to expand upon the relevance of my findings, as well as consider the potential wider ramifications, both within the field of SOCE and outside of it.

STIM1 is controlled from the ER lumen by ERp57

The first part of my research focused on the luminal regulation of SOCE, specifically honing in on the role of ERp57 binding to STIM1. Numerous lines of evidence demonstrate that SOCE is initiated from the ER lumen, where conformational shifts within STIM1 promote its homooligomerization[35]. This luminal oligomerization is both necessary and sufficient for the triggering of SOCE[36], and as such is of key interest in our understanding of SOCE is controlled. Thus, we aimed to find protein-protein interactions that could exert further control over the luminal events of SOCE, discovering a novel ERp57-STIM1 interaction[216]. To our knowledge, this was the first published description of STIM1 binding to a protein within the ER lumen. We discovered that ERp57 binding to STIM1 inhibits its movement to punctae, suggesting a potential braking mechanism by which the cell can control the timing and extent of SOCE. Cells lacking ERp57 demonstrated enhanced SOCE due to the absence of this brake, while overexpression of ERp57 could slow the movement of STIM1 to subplasmalemmal punctae. We further found a critical role for two highly conserved cysteine residues within the luminal domain of STIM1, Cys49 and Cys56. Not only do these residues serve as a binding site for ERp57, but they also form an intramolecular disulfide bond and are required for STIM1 initiation of SOCE. Interestingly, this disulfide bond still exists in the absence of ERp57, suggesting that the presence of ERp57 is not required for its synthesis. We speculate that this intramolecular disulfide bond may play a key structural role in STIM1. This is of particular interest in light of published studies investigating the structure of the luminal of STIM1 using NMR, as some of these studies used STIM1 constructs lacking these cysteine residues[35]. We suggest that a more complete understanding of the structure of STIM1 requires use of constructs encompassing all luminal residues, not only those within the EF-SAM motif.

Our results demonstrate binding between STIM1 and ERp57. A more speculative model postulates that additional proteins may be involved in a larger protein complex. For

example, STIM1 has been shown to bind to SERCA within subplasmalemmal punctae[61,62]; interestingly, ERp57 also binds SERCA to modulate its activity in a Ca^{2+}-dependent fashion[237]. A large scale screen for STIM1 binding partners identified calnexin[63], which is also known to bind ERp57[238]. Combining all these data leads to a model where calnexin, ERp57, SERCA, and STIM1 may combine to form a macromolecular complex. It is also possible that the association of proteins with this complex is dynamic: for example, ERp57 seems to be excluded from STIM1 punctae[216] (as is the ER luminal protein BiP[46]) while SERCA mostly associates with STIM1 within said punctae[61,62]. STIM1 binding to calnexin was discovered when Ca^{2+} stores were replete-- i.e. when STIM1 is distributed throughout the ER membrane-- but the dependence of the STIM1-calnexin interaction on the Ca^{2+} filling state of the ER was not investigated[63]. Further experiments would be required to confirm this hypothesis, and these associations could simply be coincidental. However, the potential existence of a large, multi-protein complex responsible for nuanced control of SOCE certainly warrants further consideration. Furthermore, it is intriguing to note that both ERp57 and calnexin are typically thought of as serving within ER chaperone pathways, controlling the correct folding of client proteins. Here, both ERp57 and calnexin seem to instead control ER-originating Ca^{2+} signaling pathways. There are many other examples of chaperones and related proteins binding to Ca^{2+} or regulating Ca^{2+} signaling, including calreticulin[10,11], BiP[19], and PDI[22]. Our results therefore fit well with an emerging model, in which the ER's dual functions in regulating both protein folding and Ca^{2+} signaling are not separate, but instead intertwined ever more closely.

A second larger implication of our work on ERp57 regulating STIM1 activity concerns the nature of ERp57 itself. As a member of the protein disulfide isomerase (PDI) family, ERp57 is typically considered to be responsible for isomerization of disulfide bonds within client protein to assist in attaining of the proper conformation[239]. However, our results suggest that, at least with respect to STIM1, ERp57 does not play a role in the formation or isomerization of a disulfide bond. Instead, we hypothesize that ERp57

exploits its natural proclivity for binding to disulfide bonds as a mechanism to bind to STIM1 directly. This protein-protein interaction allows for ERp57 to modulate STIM1 activity, thus exerting another layer of control over SOCE. We further argue that this concept-- a PDI-like protein acting not as an isomerase but instead as a modulator of cell signaling-- may not be limited to ERp57 and STIM1 but could instead point to a broader dogma. For example, the PDI family member ERp44 has been shown to bind to the inositol trisphosphate receptor (IP_3R) to modulate its activity[207]. Furthermore, our laboratory has recently discovered that a different PDI-like protein, Pdia6, regulates the activity of the ER stress related protein IRE1 (inositol responsive element 1)[240]. Thus, it is evident that PDI-like proteins can act in non-canonical ways: their cellular role is not limited to manipulation of disulfide bonds. Instead, they can exert control over cellular signaling pathways originating from the ER, including Ca^{2+} signaling[207,216] and ER stress[240]. These novel roles for members of the PDI family may partially explain why cells possess so many different PDI-like proteins -- twenty at last count[241]. Furthermore, it is intriguing to note that proteins are typically assigned to the PDI-like family based on possession of a thioredoxin-like motif. However, some members of this family do not possess the catalytic residues that would be required to behave as isomerases[241]. Additionally, calsequestrin, a sarcoplasmic reticulum luminal resident Ca^{2+}-binding protein, has been shown to have thioredoxin-like motifs within its structure[242]. However, calsequestrin has not been shown to act as a PDI, though it does regulate the activity of other proteins, such as the ryanodine receptor[243]. We therefore hypothesize that the thioredoxin-like motif may be a structural element that is particularly favored within in the ER/SR lumen. It may also serve as a module enabling protein-protein binding allowing thioredoxin-containing proteins to regulate cell signaling pathways involving the ER.

STIM1 is a target for calpains

After investigating luminal regulation of STIM1 and SOCE, we next considered regulation of the cytoplasmic domain of STIM1, specifically looking at STIM1's cleavage by calpain proteins. Initially, we observed that cells expressing STIM1 or YFP-tagged STIM1 frequently exhibited a protein band at a lower molecular weight than full-length STIM1 or YFP-STIM1. This protein band was still recognized by antibodies against either STIM1 or YFP, suggesting that it represents a different form of STIM1 that is somewhat shorter than the full-length protein. We hypothesized that this protein band exists due to cleavage of STIM1 by some regulated process, after which STIM1 may be targeted for proteasomal degradation. Indeed, treatment of cells with the proteasomal inhibitor MG132 increased the levels and number of lower molecular weight bands. The levels of these lower molecular weight bands could be reduced by inhibition of calpains, whether genetically (via deletion of the calpain regulatory subunit CAPN4 or expression of calpastatin, a protein inhibitor of calpains) or pharmacologically. We therefore concluded that STIM1 is cleaved by calpains within its cytoplasmic domain, after which STIM1 products are targeted for proteasomal degradation.

Our next goal was to determine the cellular significance of STIM1 cleavage via calpains. Ca^{2+} measurements showed that calpain cleavage did not act to directly regulate SOCE, nor did cleavage occur within the SOCE time frame (on the order of minutes). Thus, calpains are not acting to shut down SOCE. Instead, we found two major effects of STIM1's susceptibility to calpains. First, we found that calpains cleave STIM1 to regulate its steady state levels: cells lacking calpain activity or overexpressing calpastatin exhibited higher levels of STIM1 protein. This indicates that calpains are at least partially responsible for the natural turnover of STIM1. Second, we found that calpain cleavage of STIM1 is noticeably enhanced in cells undergoing the later stages of apoptosis. Furthermore, inhibition of caspases could reduce STIM1 cleavage, which we concluded was due to the previously described activation of calpains by caspases

during apoptosis[218]. Taken in summation, these results indicate that the cytoplasmic domain of STIM1 is cleaved by calpains. This cleavage occurs in healthy, unstressed cells, where it serves as a mechanism for the cell to regulate the abundance of STIM1. During apoptosis, calpains are typically highly activated, and so STIM1's propensity to be cleaved by calpains naturally leads to extensive degradation of STIM1.

The discovery that STIM1 is cleaved by calpains extends the current literature in several important ways. For example, experiments investigating the response of STIM1 and SOCE to oxidative stress showed STIM1 degradation products in response to treatment with hydrogen peroxide, though the authors did not discuss this finding in any detail[91]. Similarly, though we did not investigate the ubiquination state of STIM1 and its cleaved products, STIM1 has previously been shown to be ubiquitinated[90]. The finding that STIM1 is regulated by the ubiquitin-proteasome system strengthens our hypothesis that this system, in conjunction with calpains, serves to control STIM1 levels *in vivo*. Second, our finding demonstrates a novel method by which the cell can control SOCE: i.e. manipulation of the rate of degradation of STIM1. There is ample evidence that dysregulation of STIM1 levels can have deleterious effects, particularly with respect to cancer metastasis: as one example amongst many, increased STIM1 levels are associated with higher metastatic behavior in human colorectal cancer[162,163]. Thus, it is reasonable to hypothesize that impaired calpain activity in cancerous cells could lead to higher levels of STIM1, increasing metastatic potential. Curiously, most evidence concerning calpains and cancer suggests that highly metastatic tumor samples have increased calpain activity[244]. In our model, this would lead to increased cleavage and lower levels of STIM1-- however, most human cancer samples instead show elevated STIM1. The reason for the discrepancy is unclear: it is possible that STIM1 is somehow escaping recognition by calpains, or that STIM1 is upregulated at the mRNA level to such an extent that increased calpain cleavage is unable to compensate. Currently, these are only hypotheses: to confirm, we would need to perform experiments on cancer cell lines or tumor samples that have both calpain and STIM1 levels dysregulated.

Our current model of calpain cleavage of STIM1 suggests that it is inactivating and that cleaved STIM1 is unable to initiate SOCE. Calpain cleavage of STIM1 occurs within its cytoplasmic domain; this cytoplasmic domain contains numerous coiled-coil domains, the STIM1-Orai activating region (SOAR), and positively charged lysine residues that interact with negatively charged phosphoinositide head groups at the plasma membrane[34,41]. An examination of this structure suggests that cleaved STIM1 should be less active than full-length STIM1: any cleavage would almost certainly remove the lysine-rich region of STIM1, which is crucial for STIM1 recruitment to the plasma membrane[41]. Furthermore, cleavage within STIM1's coiled-coil domains or SOAR would also be predicted to impair STIM1 activity, whether through prevention of homooligomerization or abrogating STIM1-Orai1 interactions. Nevertheless, there exists the intriguing possibility that STIM1 cleavage may instead yield a fragment that is more active that its full-length counterpart. For example, the CC1 domain of STIM1 is thought to act in an auto-inhibitory fashion at rest: it binds to SOAR to prevent premature binding to, and activation of, Orai1[53,54]. If calpain cleavage of STIM1 were to cleave between the CC1 and CC2 domains of STIM1, the resulting fragment would contain all the residues required to activate SOCE and none of the auto-inhibitory or store-dependence of full-length STIM1. This fragment could theoretically activate Orai1 and thus represent a hyperactivator of SOCE. At this point, the potential for STIM1 cleavage to be activating is entirely speculative and we still favor a model in which the cleavage reduces or eliminates STIM1 function. When considering a complex, dynamic protein such as STIM1, there are many more ways in which cleavage can inactivate the protein than ways in which it can activate the protein. Furthermore, the hypothetical cleavage described above would result in a situation in which activation of Orai1 occurs entirely independently of ER Ca^{2+} store filling state. As SOCE is a pathway linking the concentration of Ca^{2+} within the ER lumen with Ca^{2+} influx across the plasma membrane, a model in which the two are decoupled should be considered skeptically.

Finally, after looking into calpain regulation of STIM1 levels, I began an investigation of the importance of calnexin binding to STIM1. This finding has been reported in the literature[63], and was also confirmed in our laboratory. Though the investigation into the relevance of calnexin-STIM1 binding remains incomplete, the preliminary results presented in this thesis suggest this binding may have functional consequences, particularly in the T cells in which SOCE is of such high importance. First, we demonstrated that calnexin deficiency has no significant effect on the strength of SOCE in fibroblasts, but we found severely impaired SOCE in freshly isolated T cells from mice with thymus-specific deletion of the gene encoding calnexin. SOCE was noticeably lower when considering both initial rate of Ca^{2+} entry and the highest cytoplasmic Ca^{2+} concentration attained after SOCE initiation. Therefore, it is clear that calnexin plays a role in enhancing SOCE in T cells, and as such its absence is deleterious to SOCE. As calnexin is known to chaperone numerous molecules within the immune system including T cell receptor α proteins[235] and aid in loading antigens onto MHC class I molecules[234], we first considered a role for calnexin in chaperoning STIM1 and Orai1. However, the abundance of both proteins was unchanged by depletion of calnexin in T cells; furthermore, at rest, STIM1 was equally susceptible to the previously described calpain cleavage. This suggests that calnexin deficiency does not lead to sufficient misfolding of STIM1 or Orai1 as to cause ER-associated degradation of either protein. We instead considered a different mechanism: the impaired movement of STIM1 to punctae in the absence of calnexin. Via subcellular fractionation, it is possible to monitor a shift of STIM1 in response to store depletion, which we assume to correlate to the shift of STIM1 from a diffuse ER reticular distribution to localization within distinct subplasmalemmal punctae. Interestingly, calnexin-depleted T cells showed a noticeable increase in STIM1 cleavage in response to thapsigargin treatment, while their wild-type counterparts did not. This suggests that in T cells, calnexin may somehow protect STIM1 from calpain cleavage.

A large amount of work remains to complete the investigation into how calnexin affects SOCE and STIM1, particularly in T cells. However, at this point, we can develop some admittedly speculative hypotheses as to the greater significance of our finding. First, the differences between SOCE infibroblasts and T cells are striking: SOCE is much higher in T cells than in fibroblasts. This finding is not unexpected: SOCE is of especial importance in T cells, and so its upregulation is no surprise. However, the discovery that calnexin deficiency affects SOCE much more strongly in T cells than in fibroblasts is illuminating. This finding suggests that there may be system- or cell-specific regulation of SOCE and that this regulation may be dependent upon proteins binding to STIM1 and Orai1. For example, with respect to calnexin, the moderate amount of SOCE seen in fibroblasts seems to be relatively indifferent to the presence or absence of calnexin. However, in T cells, a much higher amount of SOCE is seen in wild-type cells; this high SOCE cannot be supported without calnexin. Thus, the use of calnexin may be one method by which T cells are able to stimulate so much more calcium entry than are fibroblasts. However, our knowledge of how this could occur is far from complete. Potential hypotheses include calnexin aiding in the movement of STIM1 through the ER membrane to subplasmalemmal punctae; calnexin binding to STIM1 to alter its structure and thus its sensitivity to Ca^{2+}; calnexin protecting STIM1 from calpain cleavage; or calnexin serving as a scaffolding protein, enhancing the recruitment of Orai1 or some other SOCE constituent, such as CRACR2A[67] or septins[74]. The question of how this process differs between fibroblasts and T cells remains, however. It is conceivable that calnexin recruits some T cell-specific protein to STIM1 and that it is this T cell-specific protein that acts as an enhancer of SOCE. Finally, it is interesting to note that calnexin, like STIM1, is an ER transmembrane protein. We have already shown that STIM1 is regulated by protein-protein interactions within its luminal and cytoplasmic domains; the effects of calnexin on STIM1 activity suggest it may also be controlled via its transmembrane domain.

The broader implications of our work concern the role of calnexin within the immune system. Adding to the existing literature on calnexin's role in immune cells, preliminary, unpublished work from our laboratory suggests that calnexin deficiency, particularly within the immune system, is protective against experimental autoimmune encephalitis (EAE). This is an experimental animal model that is widely used to study demyelinating diseases such as multiple sclerosis (MS) and other T cell related autoimmune diseases. The finding that the absence of calnexin may affect autoimmunity may be at least partially explained by our discovery that calnexin deficiency abrogates SOCE. Without SOCE, T cells are activated less efficiently, and therefore the strength of autoimmunity would be predicted to be lesser. Further experiments would be needed to verify this hypothesis: were it correct, we would predict that the protective effect of calnexin deficiency would be eliminated by experimental treatments that sidestep the reliance of T cells on STIM1-mediated Ca^{2+} entry. For example, the ionophore ionomycin, which allows for Ca^{2+} entry into the cytoplasm irrespective of SOCE, would be expected to bypass calnexin's effects on STIM1 and SOCE, and hence allow for stimulation of T cells within autoimmunity.

While STIM1, Orai1, and SOCE are currently being intensively researched as therapeutic targets, there exist concerns about targeting this pathway as a treatment. Evidence from human patients shows that SOCE is required for several different systems, and its dysregulation leads to disease. However, dysregulation can and does occur in both directions: lowered or absent SOCE impairs immunity, while hyperactive SOCE contributes to cancer metastasis. Thus, we cannot disregard the possibility that any sort of SOCE-based therapeutic may have severe off-target effects. For example, a SOCE activator to boost the immune system might also increase SOCE within cancer cells, thus converting a benign, slow-growing tumor into a quickly proliferating, highly metastatic tumor. Conversely, any sort of SOCE blocker aimed at lowering cancer metastasis or stopping blood clots would be likely to also compromise the immune system. Any therapeutics based on SOCE must therefore be carefully targeted to the physiological

system that is affected. Consequently, a larger ramification of our work here is that it sheds lights upon some differences between how SOCE is regulated in different physiological systems, and may thus aid in developing SOCE modifiers that are specific to an individual system. For example, we have preliminary data suggesting that the STIM1-calnexin interaction may be of higher importance within immune cells. Were this to be borne out by further research, it would be feasible to suggest that this STIM1-calnexin axis as a worthwhile target for development of therapeutic that affect SOCE within the immune system only. As a hypothetical, a small molecule that enhances the STIM1-calnexin interaction might be able to activate the immune system-- useful for immunocompromised patients-- but not affect SOCE within cancerous or pre-cancerous cells, thus avoiding the unfortunate side effect of promotion of cancer metastasis. In theory, this principle might apply to other physiological systems, not solely the immune system. It may be that while STIM1 and Orai1 underpin SOCE within all cells, different types of cells use a different complement of proteins to further regulate and control the process. Discovering which proteins are involved in which systems could aid in creation of therapeutics that target SOCE specifically within certain systems, thus avoiding off-target effects. Additionally, these findings could help explain why SOCE-associated diseases display such a wide variety of phenotypes- as an example, certain activating mutations within STIM1 and Orai1 cause tubular aggregate myopathy but seem to leave immune function or blood clotting unperturbed[148,151,152]. Variable expression of proteins that regulate SOCE could go some away to explaining these apparent contradictions.

Conclusions

Calcium signaling is one of the most important mechanisms by which cells can communicate across membranes, particularly the ER membrane and the plasma membrane. Store-operated Ca^{2+} entry (SOCE) links the ER luminal Ca^{2+} store to Ca^{2+} movement across the plasma membrane and is of particular importance within the

immune system, muscular development, and cancer proliferation and metastasis. As such, SOCE and its constituent proteins STIM1 and Orai1 have been the subject of intense study over the past ten years. Research has focused on the structure of STIM1 and Orai1; post-translational modifications of both proteins; the interactions between STIM1, Orai1, and other proteins as well as lipids; and the role of SOCE within various physiological systems, both in health and in disease. The research presented in this thesis has added to the body of knowledge on protein-protein interactions of STIM1 that aid in the regulation and control of SOCE. First, we identified the ER luminal protein ERp57 as an inhibitor of STIM1 movement to punctae and hence, SOCE. We also discovered a key structural role for two highly conserved residues within the luminal domain of STIM1, expanding the field's understanding of the basic structural unit of luminal Ca^{2+} sensing. Second, we showed that calpains cleave STIM1 within its cytoplasmic domain to regulate its steady state levels. Dysregulated STIM1 levels underpin several different diseases, and we present a mechanism that may explain some of this variation. Finally, we presented preliminary data suggesting that the binding of calnexin to STIM1 may be an enhancer of SOCE, particularly within the immune system. These exciting results suggest system-specific regulation of SOCE, which may help elucidate how mutations within only two genes, *STIM1* and *Orai1*, can present with such a wide variety of phenotypes. Taken together, these data show that STIM1 is regulated via binding to other proteins within both its luminal and cytoplasmic domains. Figure 5-1 depicts some of the many binding partners of STIM1, with the protein-protein interactions discussed within this thesis highlighted in green. While STIM1 and Orai1 are both necessary and sufficient for SOCE to occur, a plethora of proteins can modulate the details of calcium entry, adding significant nuance to our understanding of SOCE. As shown by the many diseases associated with dysregulated SOCE, the incredible complexity of the protein-protein interactions that regulate STIM1 underscore how much interest the cell takes in tightly controlling this process.

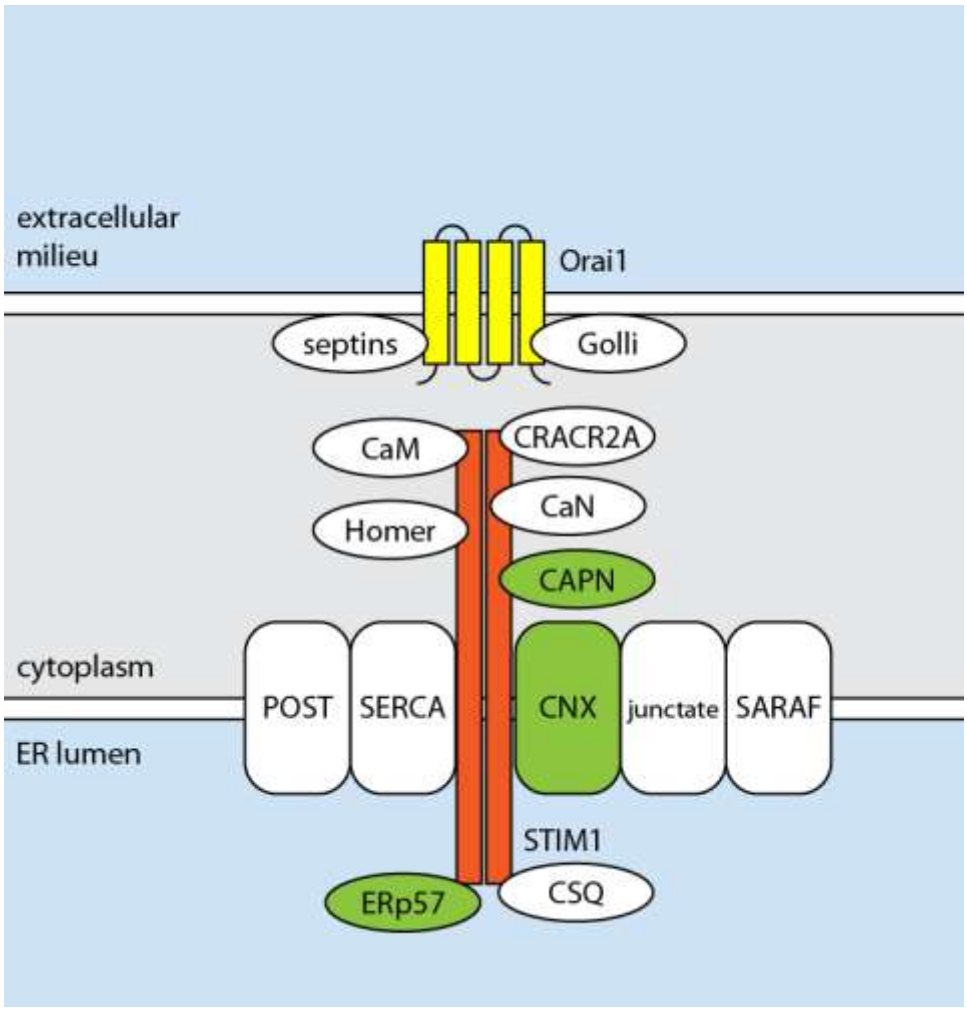

Figure 5-1. Protein-protein interactions within SOCE. While STIM1 and Orai1 are the two key players controlling SOCE, the pathway is modulated by numerous other proteins. The above figure depicts but some of these proteins; highlighted in green are the three proteins- ERp57, CAPN, and CNX- that were the focus of this thesis.

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