Studies of interactions of STIM1 and GPCRs with their binding partners

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2015

How, J. G. Z. (2015). Studies of interactions of STIM1 and GPCRs with their binding partners.
Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/65666>

<https://doi.org/10.32657/10356/65666>

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With Their Binding Partners**

**HOW GUAN ZHONG, JONATHAN
SCHOOL OF BIOLOGICAL SCIENCES**

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HOW GUAN ZHONG JONATHAN

SCHOOL OF BIOLOGICAL SCIENCES

**A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy**

2015

Acknowledgements

I would like to thank my supervisors, Professor Susana Geifman Shochat and Professor Said Eshaghi for all their guidance and support. I am truly grateful for the opportunity to work under them on this project. Professor Susana and Professor Said were both inspirational role models to me throughout my PhD journey.

I would like to thank Professor Ray Stevens and the members of the GPCR network for giving me the opportunity to work with G-protein coupled receptors and also providing me with valuable advice during my one year stint at The Scripps Research Institute, San Diego, California, USA.

I would like to thank the following friends and colleagues for their unstinting support and contributions to my project: Prof Pär Nordlund, Dr Yin Hoe Yau, Dr Albert Guskov, Dr Tobias Cornvik, Dr Agnes Jong, Dr Ignacio Asial, Dr Teo Hsiang Ling, Dr Andreas Larsson, Ms Chuah York Wiewo, Ms Zhang Ai, Ms Newsha Sahaf, Ms Aline Reynaud, Ms Vicky Lu, Ms Nurhuda Nordin, Ms Margaret Phillips, Mr Xia Yu, Ms Cori Renshaw, Ms Lucy Pan, Ms Chen Ying Hua, Ms Gladys Hoh, Ms Jeslin Puah, Ms Kit Yeung, Mr Daniel Sim, Ms Kelly Hew, Ms Cheng Yue Xiang and Mr Max Wu. Thank you all for a wonderful and memorable experience.

I would like to thank my thesis advisory committee members: Professor Koh Cheng Gee and Professor Curt Davey for all their helpful suggestions throughout these years.

I would like to thank my family, who has always given me their full support no matter the circumstances. Last but not least, I would like to thank God, without whom I would not be alive today, let alone be able to participate in this project. Thank you.

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Abbreviations

A2aR	Adenosine A _{2A} Receptor
BRIL	Apocytochrome b562RIL
CAD	Channel Activating Domain
CC1	First Coiled-Coil Domain
CC2	Second Coiled-Coil Domain
CC3	Third Coiled-Coil Domain
CRAC	Calcium Release-Activated Calcium
DAG	Diacyl-Glycerol
DDM	Detergent Dodecyl Maltoside
ER	Endoplasmic Reticulum
GDP	Guanosine Diphosphate
GPCRs	G-Protein Coupled Receptors
GTP	Guanosine Triphosphate
His-Tag	Hexa-histidine Tag
IP3	Inositol-1,4,5-Triphosphate
LIC	Ligation Independent Cloning
MSP	Membrane Scaffold Protein
NTA	Nitrilotriacetic Acid
PDB	Protein Data Bank
PIP2	Phosphatidylinositol 4,5-Bisphosphate

RU	Resonance Unit
SAM	Sterile- α Motif
SCID	Severe Combined Immunodeficiency
SEC	Size Exclusion Chromatography
SOCE	Store Operated Ca^{2+} Entry
SPR	Surface Plasmon Resonance
STIM1	Stromal Interacting Molecule 1
TEV	Tobacco Etch Virus
T_m	Melting Temperature

Abstract

Membrane proteins are responsible for cellular functions such as cell signalling and ion transport. Almost half of all modern medicinal drugs have membrane proteins as their targets. In this thesis, focus is on two transmembrane proteins: Adenosine A_{2A} receptor (A2aR) as a model for G-protein coupled receptors (GPCRs) and stromal interacting molecule 1 (STIM1). GPCRs interact with extracellular compounds that activate cellular signalling pathways, leading to the generation of a large variety of cellular responses. Down the signalling pathway is the calcium release-activated calcium (CRAC) channel that consists of the ER embedded STIM1 and the plasma membrane embedded Orai1. Together, these proteins are involved in the regulation of intracellular Ca²⁺ levels that are essential for immune system response.

The nature of these membrane proteins has made their expression and purification difficult. In this study, methods were introduced which helped to improve the stability and expression of both GPCR and STIM1 with minimal mutation of residues. A2aR was incorporated into nanodiscs to improve its stability. Expression assays were performed in conjunction with thermo-stability and oligomerization assays to identify the regions of STIM1 necessary for good expression and stability. This was followed by the optimization of SPR binding assays and the determination of the affinity values for the interaction between (a) A2aR and their ligands; and (b) STIM1 and Orai1 N- and C- termini.

Additionally, the first coiled-coil domain of STIM1 was found to be involved in the interaction of STIM1 with Orai1, suggesting its potential role as a docking site for Orai1. The kinetic constants of the interaction between A2aR and its agonists were determined. The SPR assays developed within this project will be useful for the analysis of interactions between other GPCRs and their binders.

1. Introduction

1.1 Introduction to Membrane Proteins

A biological membrane serves as a permeability barrier that establishes distinct compartments and prevents random mixing of its components. Every eukaryotic cell is enclosed by a membrane phospholipid bilayer which contains a diverse range of proteins with different functions. These proteins are referred to as membrane proteins and they are essential for cell function and viability.

Structural membrane proteins are attached to micro-filaments of the cytoskeleton that contributes to cell structural integrity and stability. Receptor proteins located at the plasma membrane serve as an important connection, linking processes in the cell's external environment with its internal environment. Transport proteins embedded in the membrane are important for the maintenance of concentrations of metabolites and ions in the cells which is necessary to provide building blocks and for the activation and regulation of cellular processes. The adhesive properties of some membrane proteins located at the cell surface give cells the ability to identify, signal and interact with other cells [1, 2]. Membrane proteins can be classified as either integral or peripheral membrane proteins. A typical biological membrane contains a combination of both types (Figure 1).

Figure 1. Illustration of the difference between integral membrane protein and peripheral membrane protein. The integral membrane protein is coloured gold while the peripheral membrane protein is coloured orange.

Peripheral membrane proteins are proteins that do not integrate into the hydrophobic core of the phospholipid bilayer. They are attached to the membrane interactions with either other integral membrane proteins or the polar head groups of the phospholipid bilayer. Peripheral membrane proteins are not permanently attached to the membrane, giving way to reversible attachment mechanisms needed for certain cell signalling purposes [3].

Integral membrane proteins typically have one or more regions embedded within the phospholipid bilayer. The structure of these domains commonly consists of α -helices and/or multiple β -strands. They may contain additional hydrophilic domains that extend outwards into the aqueous medium at both sides of the membrane bilayer. These domains are usually sites for interaction with other biological molecules such as intracellular proteins and external stimuli. Integral membrane proteins make up a significant portion of all proteins present in the human genome and are essential for cell survival and function [4, 5].

Altered activity of the integral membrane proteins may result in severe consequences such as cancer [6], neurodegenerative diseases [7] and immunodeficiency diseases [8]. Among the top 50 pharmaceutical drugs that are in the market, more than half of them target integral membrane proteins [9-11]. Despite their relative importance for the development of pharmaceutical drugs, less than 5% of all structures deposited in the PDB are of integral membrane proteins [12]. This is because it is difficult to isolate and purify membrane proteins in large quantities, which is necessary for *in vitro* studies [13].

1.2 G-Protein Coupled Receptors

1.2.1 Characteristics of G-Protein Coupled Receptors

GPCRs are the largest class of membrane proteins present in mammals including humans. From analysis of the human genome, there are more than 800 different GPCRs having structural similarity, yet having largely different functions and characteristics [14]. All GPCR proteins contain a distinctive structure motif of seven transmembrane (7TM) helices with an intracellular carboxyl terminus and an extracellular amino terminus [15-17].

GPCRs can also be found in the genomes of archaea, bacteria, yeast, plants, nematodes and other invertebrate groups indicating that the family of GPCRs may have an early evolutionary origin [18]. Approximately 5% of all cell proteins interact with GPCRs and when combined, their genes make up approximately 1% of the mammalian total genome [19].

GPCRs are defined based on two requirements. First, they must have a total of seven transmembrane helices of around 25 to 35 consecutive residues that contain a high degree of hydrophobicity with the transmembrane helices spanning the entire plasma membrane in a counter-clockwise manner [14]. Second, they must have the ability to interact with intracellular G-proteins. The binding of the agonists to GPCRs changes the conformation of the 7TM helix pockets, resulting in the conformational change of the receptors' intracellular domains. This structural change encourages the specific binding of GPCRs with their respective heterotrimeric G-proteins. Based on sequence similarity of the 7TM segments, GPCRs can be classified into five sub-families: adhesion, taste/frizzled, glutamate, rhodopsin and secretin [14].

1.2.2 GPCR Coupling with G-Proteins and Other Proteins

There is much diversity in the functional coupling of G-proteins with GPCRs. G-proteins belong to a family of guanosine nucleotide-binding proteins responsible for relaying signals from external stimuli into the cell. G-proteins operate like molecular switches and their functions are regulated by factors that affect their ability to bind and hydrolyse guanosine triphosphate (GTP) into guanosine diphosphate (GDP). When GTP is bound, a G-protein is active or 'on' and when GDP is bound, it is considered inactive or 'off'.

The family of G-proteins comprises two distinct classes. The first class functions as monomeric small GTPases while the second class functions as heterotrimeric G-protein complexes. It is this second class that is activated by GPCRs. They consist of three subunits: alpha (α), beta (β) and gamma (γ), of which the β and γ subunits may form a stable dimeric complex which is referred to as the $\beta\gamma$ -complex [20].

The binding and subsequent activation of G-proteins will result in a number of alternative signalling pathways through their interaction with other proteins. Studies have shown that there are more than 23 different types of human $G\alpha$ -proteins that are known to couple with GPCRs [19, 21]. $G\alpha$ -subunits are known to form heterotrimeric complexes with $G\beta$ - and $G\gamma$ - subunits. There are at least five different types of $G\beta$ -subunits and eleven types of $G\gamma$ -subunits [19].

GPCR activation causes the exchange of GDP into GTP within the active site of the $G\alpha$ -subunit. GTP binding leads to the breaking up of the G-protein heterotrimeric complex, allowing the $G\alpha$ -, $G\beta$ - and the $G\gamma$ - subunits to interact with other intracellular effectors such as enzymes and ion channels [19, 22]. The $G\alpha$ -subunit hydrolyses the bound GTP back into GDP, assuming its inactive conformation and increases its binding affinity towards the $G\beta$ - and $G\gamma$ - subunits to re-form the G-protein heterotrimeric complex (Figure 2).

The binding of ligands to GPCRs triggers not only the activation of G-proteins but also leads to the binding of other cellular proteins like kinases and arrestins to GPCRs which results in the deactivation of the GPCRs. Desensitization of

activated GPCRs at the cell surface is important as it prevents uncontrolled signalling.

GPCRs are deactivated as the result of phosphorylation of the receptors [23, 24] by GPCR kinases (GRKs) [25] and the binding of arrestins to the phosphorylated GPCRs. The arrestin family comprises four members: visual arrestin, β -arrestin1, β -arrestin2 and cone arrestin [26]. The binding of β -arrestins to the phosphorylated receptor leads to faster receptor desensitization.

Figure 2. Activation cycle of a GPCR. When an agonist (red) binds to the GPCR and activates it, a conformational change occurs resulting in the exchange of GDP into GTP on the $G\alpha$ -subunit. This exchange causes the dissociation of the G-protein ($G\alpha G\beta G\gamma$ complex) which allows the subunits to bind to other effectors and induce signalling. The hydrolysis of GTP back to GDP located at the $G\alpha$ -subunit deactivates the GPCR which returns to its resting state.

1.2.3 Ligand Binding to GPCRs

The structural and functional similarities of GPCRs are in stark contrast with the diversity of their natural ligands [27]. There are many types of ligands that interact with and activate GPCRs. These ligands include peptides, ions, amines, odorants, proteins, lipids and nucleotides [28]. The binding of these ligands results in the activation of their receptors, generating intracellular responses that are essential to many signalling processes in the cell [29, 30].

A single neurotransmitter is able to activate the production of multiple secondary messengers. The diversity of signalling responses in the cell by a single neurotransmitter can be attributed to the different GPCR sub-types such as serotonin; β -adrenergic or adenosine receptors which have different G-protein coupling specificities. Furthermore, alternative splicing of receptor genes and RNA editing results in the formation of multiple isoforms of the same GPCR but with totally different biochemical properties [31, 32].

The binding of agonists to GPCRs activates intracellular G-proteins and is an essential step for the subsequent signaling cascade process within the cell. Likewise, antagonists block the binding of agonists to GPCRs, inhibiting the agonist function. The role of GPCRs in signal transduction is essential for most physiological processes in mammals. This includes basic senses such as sight, smell and taste. The critical importance of GPCRs in the function of a wide variety of cellular processes makes them important therapeutic targets [33, 34].

The human genome codes for around 800 GPCRs. However, presently only approximately 50 are being targeted for pharmacological drug discovery [35]. The large discrepancy highlights the need to expand this number for the discovery of better drugs to treat diseases. One major reason for this shortfall of information is that for many GPCRs in the human genome the natural ligand has not been identified. These receptors are called orphan GPCRs. De-orphanization of GPCRs is critical for the understanding of their functions.

1.2.4 Adenosine A_{2A} Receptor: Structure and Mechanism

A_{2A}R is part of the rhodopsin family of GPCRs and its activation is important for various physiological processes like cerebral blood flow [36], basal ganglia functions [37], regulation of pain [38], sleep [39] and respiration [40]. A_{2A}R is activated by the attachment of adenosine which leads to the activation of adenylyl cyclase which induces the synthesis of intracellular cyclic adenosine monophosphate (cAMP) [41].

Naturally occurring methylxanthines, such as caffeine, was observed to be an antagonist of A_{2A}R [41]. Humans drinking large quantities of coffee, a rich natural source of caffeine, were found to have lower incidence of Parkinson's disease [42]. This effect is linked to caffeine's interaction with A_{2A}R which controls the locomotor behaviour in basal ganglia [43, 44]. Development of more selective compounds for A_{2A}R will lead to better therapeutic treatments for various diseases such as Huntington disease [45], Parkinson's disease [46], asthma [47], seizures [48] and many other neurological diseases [49].

The structure of A_{2A}R was elucidated at a resolution of 2.6 Å by co-crystallizing A_{2A}R with the antagonist ZM 241385, a derivative of caffeine [41]. It was observed that the binding pocket of A_{2A}R contains four disulphide bonds that facilitate the binding of antagonist to A_{2A}R. Removal of these disulphide bonds resulted in the loss of antagonist binding to A_{2A}R [50]. The structure of the active state of A_{2A}R bound to the agonist UK 432097 was obtained at a resolution of 2.7 Å [50]. The agonist UK 432097 is a derivative of adenosine and is developed as a drug candidate for chronic obstructive pulmonary disease treatment [51]. Comparison of the active and inactive state of the A_{2A}R showed that core adenine interactions between the ligands and the receptor were conserved. For the active A_{2A}R, there was a substantial rearrangement of the transmembrane helical bundle core which showed a seesaw-like movement of helix VII around the agonist UK 432097 [50].

The conformational differences observed between the A_{2A}R-agonist and A_{2A}R-antagonist complexes are similar to the differences observed in the light activated rhodopsin/opsin system [52] and the β_2 -adrenergic receptors [53]. The resemblance in the structural rearrangements of A_{2A}R, opsin and β_2 -adrenergic

receptors suggests the presence of common features for the activation mechanism in GPCRs. In these GPCRs, the attachment of agonist to the receptors resulted in large scale movement of the transmembrane helices which are required for G-protein activation [50]. The binding of antagonist to the GPCRs does not induce large conformational changes to the seven transmembrane helices resulting in no activation of the G-protein [41].

1.2.5 Characterization of Ligand Interaction with A2aR using SPR

For a chemical reaction between two compounds A and B, the equation can be written as follows:

Where AB is the product of the reaction, k_{on} is the association rate constant and k_{off} is the dissociation rate constant.

The mass action law states that if a system is in equilibrium at a given temperature, the rate of the reaction is proportional to the molar concentration of the products [54]. Equilibrium of the reaction is reached when:

$$[A] \cdot [B] \cdot k_{on} = [AB] \cdot k_{off}$$

Where [A], [B] and [AB] represents the molar concentration of the compounds, A and B, and the product formed, AB.

The equilibrium constant for the dissociation, K_D , is derived when the forward (k_{off}) and backwards (k_{on}) reactions are equal:

$$K_D = \frac{k_{off}}{k_{on}} = \frac{[A] \cdot [B]}{[AB]}$$

The equilibrium constant, K_D , has the molar unit M, which corresponds to the concentration of [A] when 50% of B is bound to A. For a reaction between a ligand and a protein, the equilibrium constant, K_D , can also be described as the binding affinity that reflect how tightly the ligand binds to the protein. Smaller values of K_D imply a higher affinity between ligand and protein.

The determination of the binding affinity of ligand-receptor interactions for A2aR was conducted using radio-labeled ligands [41, 50, 55]. Radio-labeled assays are typically limited by the types of radio-labeled ligands that are

available. Additionally, radio-labeled ligands are hazardous to work with and are typically costly and hard to synthesize.

Determination of the affinity between A2aR and its ligands can also be performed using surface plasmon resonance (SPR) based biosensors. The key advantage of SPR over the radio-labelled studies is that the materials used in the SPR assays are label-free and the kinetic constants can be determined. With the use of SPR biosensors, Myszka and colleagues have successfully determined the kinetics of antagonistic ligands binding to a thermostable A2aR [56].

SPR is an optical phenomenon that occurs when polarized light strikes onto a metal surface placed in the interface between two different media under total internal reflection conditions. When a light beam enters a semi-circle prism, the light bends towards the plane of the interface as it loses speed when passing from a denser medium to a less dense medium. Total internal reflection (TIR) occurs when the incident angle is greater than the critical angle, resulting in the incoming light being entirely reflected within the prism.

When polarized light strikes the surface at a certain angle known as the resonance angle, collective oscillations of free electrons or plasmons resonate with the light, resulting in the absorption of light at that angle. The resonance angle is determined by observing a dip in the intensity of the refracted light (Figure 3). The refractive index changes are monitored in real time resulting in the generation of sensorgrams (Figure 4). Kinetic constants are obtained from these binding sensorgrams by fitting the experimental data to a mathematical model of the interaction process. The constants appear as parameters in the mathematical model, and the fitting procedure finds the values for these parameters that best fit the experimental data. The affinity is derived from the kinetic constants. Using Biacore biosensors, it is possible to obtain both the association and dissociation rates of an interaction within a certain limit (association rate, k_a , limit: 10^3 to $3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$; dissociation rate, k_d , limit: 10^{-5} to 1 s^{-1}).

Figure 3. Pictorial representation of how SPR is used to detect binding events on a sensor chip surface. Ligands are immobilized onto a metal surface which can be coated for example with carboxymethyl dextran. When an analyte binds to the ligand, there is a change in the resonance angle and the photo-detector will detect a decreased intensity of the reflected light at the resonance angle.

Figure 4. An SPR sensorgram. The baseline is determined from the signal obtained with running buffer. Upon binding of the ligand to the surface, an increase in resonance units is observed and plotted as a function of time. Equilibrium occurs when the rate of association is equal to the rate of dissociation. When the ligand injection is stopped and the buffer runs over the surface again, the ligand separates from the surface resulting in a dissociation curve. If dissociation is slow a regeneration solution is added to remove any bound analyte returning the surface to pre-injection response levels.

1.2.6 Incorporating Membrane Proteins into Nanodiscs

Recent research has established the use of model membrane systems to improve the stability of membrane proteins. These membrane systems include detergent micelles, mixed detergent/lipid micelles, bicelles and liposomes that contribute to additional stability and purification of functional membrane proteins [57]. Phospholipid bilayer nanodiscs are a novel membrane system that removes the need for detergent and keeps the membrane proteins in a more native phospholipid bilayer environment [58].

A nanodisc consists of a membrane protein surrounded by a phospholipid bilayer that is encompassed by membrane scaffold proteins (MSP) (Figure 5). MSP are derived from apolipoprotein A-1 that is known to bind tightly to phospholipids thus maintaining the integrity of the lipoprotein structure [59]. The size of the MSP can be varied, and it restricts the size of the bilayer and causes nanodisc particle size distributions to be much more consistent and mono-dispersed as compared to other membrane systems such as liposomes and detergent micelles. Lipid compositions can also be varied during preparation, allowing the preparation of complex membrane proteins that typically require certain lipids for their activity and function.

Recent studies have shown that nanodiscs provide a better representation of the complex phase transition behaviour present in biological membranes when compared to other conventional model membranes [60, 61]. In the assembly process of nanodiscs, detergent-solubilized membrane proteins are first mixed with a ratio of phospholipids and MSP [62, 63]. The detergent is then removed from the solution by the use of hydrophobic adsorbents resulting in the formation of nanodiscs. The newly formed nanodiscs maintain membrane protein stability and functionality by providing a native-like phospholipid bilayer environment. The end result is that the membrane proteins enter into a detergent-free and membranous environment which is similar to its natural environment.

Most membrane protein systems require some type of phospholipids to maintain their activity which is not provided by detergent micelles. The introduction of different types of phospholipids like phosphocholine and

phosphatidylserine into the detergent micelles mixtures improves the stability and functionality of the target membrane proteins [58, 64, 65]. As mentioned before, nanodisc technology introduces a way to study membrane proteins in an almost native membrane environment. Thus far, a series of membrane proteins such as cytochrome P450 [65-67], bacteriorhodopsin [58, 68] and bacterial chemoreceptors [69] have been successfully incorporated into nanodiscs and used in structural and functional studies.

Figure 5. Model diagram depicting the composition of a nanodisc. The membrane protein is surrounded by lipids; membrane scaffold proteins surround the lipid bilayer and maintain the nanodisc structure.

1.2.7 Objectives of the GPCR Study

In drug discovery, the affinity values between the drug and its target are commonly used to screen and identify potential drug targets [70, 71]. However, the affinity values are sometimes insufficient for the identification of drugs with high efficacy [72]. In physiological situations, the effect produced by a drug-target complex is correlated to the lifetime of the complex [70, 73]. The longer the drug is bound to the receptor, the longer the drug effect lasts [73]. The dissociation rate, k_{off} , of the interaction between the drug and its target is correlated to the drug efficacy [74-76]. Drugs that have a fast dissociation rate with their targets are best suited for treatments that have mechanism-based toxicity in which a prolonged effect will lead to adverse reactions [77]. Likewise, drugs that have a slow dissociation rate are best suited for treatments where a prolonged drug effect is necessary and is not affected by mechanism-based toxicity [77]. Drugs targeting GPCRs account for about 50% of all prescription pharmaceuticals on the market making GPCR an important drug target [78].

In this study, A2aR was used as a model system for the determination of the kinetic constants of the interaction between a GPCR and its ligands. Despite considerable advancements in the field of A2aR expression [79, 80], structural studies [41, 50] and the determination of binding affinity values for agonist-A2aR interactions [56], there have been no reports on the kinetic constants of these interactions. In the SPR study involving A2aR, thermo-stabilized mutants of A2aR were used and only the kinetics of antagonist-A2aR interactions were reported [56, 81]. The benefit of using thermo-stabilized mutants is their higher stability compared to its wild type form. However, the drawback is that the functionality of a thermo-stabilized receptor may be compromised after mutations.

This study aims to develop a strategy to investigate the kinetics of both antagonist and agonist interactions with A2aR by means of SPR with minimal receptor modification. The native non-thermo-stabilised A2aR will be incorporated into nanodiscs to improve their stability. The kinetics between these ligands and A2aR will be determined using SPR biosensor technology.

1.3 Calcium Release-Activated Calcium (CRAC) Channels

1.3.1 Calcium Signalling by GPCR Activation

Ca^{2+} is an important secondary messenger necessary for the transmission and transduction of signals generated from cell surface receptors to their respective targets in the cell [82]. The most common Ca^{2+} signalling pathway is the phospholipase C pathway. In this Ca^{2+} signalling process, GPCRs are first activated by their ligands e.g. dopamine, glutamate [83]. The binding of the G-proteins to the GPCRs results in the stimulation and activation of Phospholipase C, an enzyme that hydrolyses phosphatidylinositol 4, 5-bisphosphate (PIP₂) into diacyl-glycerol (DAG) and inositol-1, 4, 5-triphosphate (IP₃) [84]. DAG remains bound to the membrane due to its hydrophobic properties whereas IP₃, being highly soluble, diffuses into the cytosol [84].

Diacyl-glycerol (DAG) functions as a second messenger signalling lipid and is involved in the activation of protein kinase C (PKC). The binding of DAG to the C1 domain of PKC results in the activation and regulation of the kinase enzyme. Production of PKC leads to G-protein receptor desensitisation and regulation of other cellular processes [85].

Additionally, IP₃ binds to the Ins3P receptor (IP₃R) that is located on the surface of the endoplasmic reticulum (ER). IP₃R is coupled to a ligand-gated Ca^{2+} channel and its subsequent activation and opening results in the release of Ca^{2+} from the endoplasmic reticulum into the cytosol [84].

The CRAC channels located and embedded in the plasma membrane are activated by a sudden increase in cytosolic Ca^{2+} levels [86]. The end result is an influx of Ca^{2+} into the cytosol and subsequent replenishment of ER Ca^{2+} stores. This ubiquitous Ca^{2+} entry pathway is referred to as store operated Ca^{2+} entry (SOCE). SOCE is important for the regulation of all types of physiological processes that are Ca^{2+} dependent, including gene transcription, progression of cell cycle and apoptosis [87]. In the human immune system, B and T cells are activated when there is an influx of extracellular Ca^{2+} into the cytosol (Figure 6).

Figure 6. Illustration of a GPCR mediated Ca^{2+} signaling cascade. When the GPCR is activated by a ligand, it leads to the activation of the G-protein. The $\text{G}\alpha$ -subunit of the G-protein causes the activation of phospholipase C β ($\text{PLC}\beta$), which brings about the hydrolysis of PIP_2 into IP_3 and DAG. The IP_3 binds to and activates IP_3R located at the ER membrane. This releases Ca^{2+} from the ER lumen into the cytosol resulting in the depletion of Ca^{2+} from the ER lumen. This depletion of Ca^{2+} activates STIM1 located in the ER membrane which then interacts with and opens the Orai1 Ca^{2+} channel located at the plasma membrane. The opening of Orai1 results in an influx of extracellular Ca^{2+} into the cytosol resulting in a significant increase in intracellular Ca^{2+} concentration.

1.3.2 Activation and Deactivation of CRAC Channels

The activation and function of T cells in both innate and adaptive immunity systems are highly dependent on the CRAC channels. Inactivation of the CRAC channels by SKF96365, an imidazole compound, results in T cells being unable to produce interleukin-2 (IL-2) in the presence of an antigen, rendering the T cells inactive [88]. Other studies have shown that blocking of the CRAC channels by La^{3+} causes non-production of CD25 and CD69 in T cells when stimulated with either CD3 or thapsigargin [89]. Most importantly, immune-compromised patients were found to have mutated CRAC channel proteins that are unable to function properly [90].

A CRAC channel is made up of two transmembrane proteins: the Ca^{2+} sensor stromal interacting molecule 1 (STIM1) [91] and the Ca^{2+} channel Orai1 [92]. When Ca^{2+} depletion of ER stores occurs, STIM1 forms oligomers and aggregates. This is followed by the migration of STIM1 towards the puncta at the plasma membrane where the Orai1 Ca^{2+} channel is located [93]. The channel activating domain (CAD) of STIM1 binds to the C-terminus of Orai1 [94], which results in the opening of the Orai1 Ca^{2+} channel. The opening of the Orai1 channel results in the influx of extracellular Ca^{2+} into the cytosol which leads to the activation of various enzymatic reactions within the cells.

Subsequent replenishment of ER Ca^{2+} stores causes the STIM1 oligomer to dissociate from Orai1, closing the Ca^{2+} channel and completing the cycle. CRAC channels have three defining characteristics [95-99]:

- (1) High selectivity towards Ca^{2+} over other monovalent cations like Li^{+} and Na^{+} ;
- (2) Activation only upon the depletion of ER Ca^{2+} stores
- (3) Fast inactivation which is dependent only on Ca^{2+} concentration

1.3.3 Stromal Interacting Molecule 1 (STIM1)

STIM1 is a multi-domain integral transmembrane protein localised in the ER. It is a 77.4 kDa protein with its N-terminus located within the ER lumen and a cytosolic C-terminus (Figure 7). Knockdown of the STIM1 gene dramatically reduces the store operated calcium entry in cells like HEK 293, *Drosophila* S2, HeLa cells and Jurkat T cells [100]. STIM1 is also found to be involved in growth inhibition and cell degeneration [101-103]. A key component of the N-terminus is the two EF-hands (A67-L96) that sense Ca^{2+} concentration in the ER lumen. The EF-hand is a helix-loop-helix structural domain that binds to Ca^{2+} and is in the family of calcium binding proteins like STIM1 and calmodulin [104]. Located next to the EF-hands is the presence of a sterile α -motif (SAM) protein-protein interaction domain (W132-F200), which is involved in both STIM1 oligomerization and clustering [105]. STIM1 is embedded within the ER membrane by virtue of a single helical domain (M215-N234).

A distinctive structural feature of the STIM1 C-terminus is that it contains three coiled-coil domains which are localised in the cytosol [105]: first (L248 to Y342), second (I364 to N388) and third (S399 to L423) (Figure 7). The CAD region (Y342-H448) is located within the second and third coiled-coil domains and is able to activate the CRAC channels [94, 106, 107].

The structure of the CAD region showed that the formation of a CAD dimer was necessary for the activation of Orai1 [108]. The CAD construct used was mutated at three residues: L374M, V419A and C437T. These mutations improved the expression and stability of the CAD construct, allowing the elucidation of the structure as the expression of wild type CAD was low [108]. Disruption of the dimerization of CAD into a monomer resulted in the absence of STIM1 binding to Orai1. A positively charged patch present in STIM1 containing five residues, Lys382, Lys384, Lys385, Lys386, and Arg387, was found to be located at the surface of CAD [108]. Any mutations or removal of these five residues resulted in the loss of interaction of STIM1 with Orai1 [108].

Following the coiled-coil domain is the location of a serine/proline-rich domain (S600-P629). This region contains sites of N-linked glycosylation and phosphorylation located on serines and threonines [109]. The phosphorylation of these sites is believed to be involved in the inhibition of STIM1 clustering and SOCE activity [110]. Lastly, a lysine-rich domain (K672-K685) is present at the end of the C-terminus. This domain is involved in the anchoring of STIM1 to the lipid rafts that are embedded in the plasma membrane [111].

When Ca^{2+} concentration is high (above 300 μM) in the ER, STIM1 is distributed evenly throughout the ER membrane [112]. This is due to the binding of Ca^{2+} to the EF-hands, which in turn prevents the oligomerization and aggregation of STIM1 [91, 100]. Release of Ca^{2+} from the ER stores leads to the dissociation of Ca^{2+} from the EF-hands resulting in the clustering of STIM1. The STIM1 oligomer translocate to the puncta located at the plasma membrane and interacts with Orai1, leading to the activation of the CRAC channels [112-114]. The translocation of STIM1 to the puncta occurs before the activation of the CRAC channels [115].

Figure 7. Pictorial representation of wild type STIM1. Beginning from the left (N-terminus of STIM1) are the domains present in the ER lumen. They consist of two Ca^{2+} binding EF-hands and the SAM domain. On the right of the transmembrane layer is the C-terminus located solely in the cytosol. The three coiled-coil domains (CC1, CC2 and CC3) are known to be involved in STIM1 interaction with Orai1. Towards the end are the serine/proline-rich region and the lysine-rich region.

1.3.4 Calcium Release-Activated Calcium Channel Protein 1 (Orai1)

Orai1 is a highly conserved ion channel involved in selective Ca^{2+} permeation and gating [102, 116]. Orai1 is a 33 kDa plasma membrane embedded protein with four transmembrane domains (Figure 8). Both its N- and C- termini are localised within the cytosol. The N-terminus of Orai1 is made up of a proline/arginine-rich region [117]. The C-terminus consists of a single coiled-coil domain that binds to STIM1 during Ca^{2+} store depletion. Orai1 functions essentially as the pore-forming subunit of the CRAC channel [92, 118, 119] and its two glutamate residues (E106 and E190) located on the first and third transmembrane domain of Orai1 are the Ca^{2+} binding sites inside the channel.

The crystal structure of Orai1 shows that the Ca^{2+} channel is made up of six Orai1 subunits that are arranged around a central axis with a six-fold symmetry within the channel's transmembrane region [120]. Each Orai1 subunit contains four transmembrane α -helices with an additional helix that extends into the cytosol. The Ca^{2+} pore is at the centre of the channel and the Orai1 transmembrane helices are arranged in three concentric rings. The Ca^{2+} pore consists of a ring of six E178 side chains (from each Orai1 subunit) that point towards the centre of the pore, creating a negative electrostatic potential at its entrance which is responsible for Orai1 selectivity towards Ca^{2+} [120].

Figure 8. Pictorial representation of wild type Orai1. Orai1 has four transmembrane domains with its N- and C- termini located within the cytosol. The N-terminus contains a proline/arginine-rich region whereas the C-terminus contains a single coiled-coil domain. The Ca^{2+} binding residues, E106 and E190, are found in the first and third transmembrane domains of Orai1 respectively.

1.3.5 STIM1 Coupling with Orai1

The STIM1-Orai1 coupling is fully reversible upon replenishment of the ER Ca^{2+} stores [107]. The CAD region (Y342-H448) is known to induce CRAC currents and have high expression at the plasma membrane when co-expressed with Orai1 [94]. Immuno-precipitation experiments have shown direct interactions between STIM1 and Orai1 which support the model that the activation of the CRAC channels occurs through a physical interaction between Orai1 and STIM1 [121].

The oligomerization and relocation of STIM1 to the puncta upon Ca^{2+} store depletion is believed to be linked to the Orai1 relocation into the puncta, resulting in the activation of CRAC channels [115, 122, 123]. The distance between the STIM1 oligomer and the plasma membrane was calculated to be approximately 10–25 nm [122].

Removal of the Orai1 C-terminus region results in both the loss of CRAC channel activity and Orai1 relocation to the puncta [107, 123]. Mutations of certain hydrophobic amino acids in the C-terminus region of Orai1 result in the disruption of the STIM1-Orai1 coupling [124]. Additionally, a single point mutation (L273S) which disrupts the single coiled-coil domain structure of Orai1 was found to inhibit its interaction with STIM1 [107].

The role of the Orai1 N-terminus in the activation process is unknown. Orai1 constructs without the N-terminus are still able to co-localise at the puncta together with STIM1 upon store depletion. However, no Orai1 channel activity was detected [107, 123].

1.3.6 Diseases Related to CRAC Channels

The immunity disease, severe combined immunodeficiency (SCID) syndrome, is associated with loss of CRAC channel activity [102]. SCID occurs when a point mutation in the Orai1 first transmembrane domain at arginine 91 is substituted for tryptophan (R91W). This change will lead to a loss of function for the CRAC channels in the immune cells. Patients suffering from SCID are unable to produce fully functional B and T cells resulting in a highly compromised and weakened immune system.

Patients suffering from SCID are susceptible to all types of infectious diseases like meningitis and pneumonia [125]. They are usually required to live in highly controlled environment due to their weakened body condition and any infection may prove fatal. SCID is treated by either bone marrow transplant or gene therapy but these methods have low success rates and are prone to complications [125].

The absence of CRAC channel activity for patients suffering from SCID has also been shown to be responsible for many other immunity-related diseases. Most immune cells are dependent on the CRAC channels for either their function or maturation (Table 1). A detailed understanding regarding the interaction between STIM1 and Orai1 will lead to the development of better treatments for immunity diseases dependent on the activation of the CRAC channels.

Table 1. Pathways in the immune system cells that are dependent on CRAC channels

Immune Cell Type	Pathway to activation of the CRAC channel proteins	Functions related to activation of the CRAC channels	Related disease due to absence of CRAC channel activity
B Cells	B cell receptor $\Rightarrow$ Phospholipase $C\gamma 2 \Rightarrow$ Inositol 1,4,5,-tris-phosphate receptor $\Rightarrow$ CRAC channel proteins [84]	B cell activation and maturation [84, 126]	X-linked agammaglobulinemia [127], Common variable immunodeficiency [128]
	B cell receptor $\Rightarrow$ cyclic ADP Ribose $\Rightarrow$ Ryanodine receptor 3 $\Rightarrow$ CRAC channel proteins [129]		
Macrophages	Fc γ receptor $\Rightarrow$ Phospholipase $C\gamma \Rightarrow$ Inositol 1,4,5,-tris-phosphate receptor $\Rightarrow$ CRAC channel proteins [130]	Expression of TNF, and cytokines [130]	Not discovered yet
	Chemokine receptors $\Rightarrow$ CRAC channel proteins [131]		
Mast Cells	Fc ϵ receptor $\Rightarrow$ Phospholipase $C\gamma 1 \Rightarrow$ Inositol 1,4,5,-tris-phosphate receptor $\Rightarrow$ STIM1 $\Rightarrow$ CRAC channel proteins [132]	Release of histamine and degranulation [132]	Not discovered yet

Immune Cell Type	Pathway to activation of the CRAC channel proteins	Functions related to activation of the CRAC channels	Related disease due to absence of CRAC channel activity
Natural Killer Cells	CD16 $\Rightarrow$ Phospholipase $C\gamma 2 \Rightarrow$ Inositol 1,4,5,-tris-phosphate receptor $\Rightarrow$ CRAC channel proteins [133-135]	Cytosolic function [133-135]	Not discovered yet
Neutrophils	Fc γ RIIa/Fc γ RIIIb $\Rightarrow$ Phospholipase $C\gamma \Rightarrow$ Inositol 1,4,5,-tris-phosphate receptor $\Rightarrow$ CRAC channel proteins [136]	Phagocytosis [136]	Not discovered yet
	IL-8 $\Rightarrow$ Phospholipase $C\gamma \Rightarrow$ Inositol 1,4,5,-tris-phosphate receptor $\Rightarrow$ CRAC channel proteins [137]		
T cells	T-Cell Receptor $\Rightarrow$ Phospholipase $C\gamma 1 \Rightarrow$ Inositol 1,4,5,-tris-phosphate receptor $\Rightarrow$ CRAC channel proteins [102, 138]	Activation of T-cell [102, 138]	Severe combined immunodeficiency [102, 138], Compromised immune system [102, 138]
	Chemokine receptor $\Rightarrow$ Phospholipase $C\beta \Rightarrow$ Inositol 1,4,5,-tris-phosphate receptor $\Rightarrow$ CRAC channel proteins [139]		

1.3.7 Objectives of the STIM1 Study

Although STIM1 is known to interact with Orai1, there have been no reports of affinity values for the interactions of STIM1 with the Orai1 N- and C- termini. The expression of wild type human STIM1 *in vitro* has been shown to be low and its structure was elucidated after it was stabilized by mutations at multiple sites [108]. However, the drawback is that the functionality and structure of a thermo-stabilized STIM1 may be compromised after the mutations. Therefore, there is a requirement to produce stable STIM1 proteins in large amounts with minimal mutations of residues.

Different constructs were designed in order to try to create a more stable STIM1 protein with a high expression. The oligomeric state of each construct was analysed to determine which residues are necessary for STIM1 aggregation. Thermo-stability assays were done to identify constructs that have high stability. Most importantly, this library was used to investigate the interaction between various key STIM1 domains and Orai1 N- and C- termini.

Surface plasmon resonance biosensor technology was used to monitor the kinetics of the interaction of Orai1 with STIM1 constructs and determine the affinity of the interaction. This multi-construct approach permits the identification of the key regions involved in the interaction between these two key CRAC proteins.

2. Materials and Methods

2.1 Cloning of STIM1

A library of different lengths of human STIM1 constructs was designed and produced for this study. The cloning of the STIM1 constructs cDNA sequence into their plasmids was done by the Protein Production Platform (PPP) group, School of Biological Sciences, Nanyang Technological University. A detailed protocol regarding the cloning process is described in How et al [140] (Appendix A). Briefly, the cDNA sequence of the STIM1 constructs was inserted into the vector pNIC28-Bsa1 that contains an N-terminal hexa-histidine tag (His-tag) using ligation-independent cloning. These STIM1 plasmids were transformed and expressed into *E. coli* BL21-DE3 Rosetta cells (DE3).

2.2 Small Scale Expression Screening of STIM1

STIM1 cells were incubated overnight at 37°C and then diluted into 1 mL of terrific broth medium (TB) (Formedium, United Kingdom) to an optical density of 0.07-0.1 at wavelength 600 nm (OD_{600}). OD_{600} was measured with the use of the Infinite 200 PRO (Tecan, Switzerland). This was followed by incubation of the cell culture at 37°C and 220 rpm until OD_{600} was 0.8.

Protein expression was induced with 0.2 mM of isopropyl- β -D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation at $3200 \times g$ for 5 min after overnight incubation at 18°C. Harvested cells were re-suspended in lysis buffer (20 mM Na^+ HEPES, pH 8.0, 150 mM NaCl, 30 mM Imidazole) supplemented with protease inhibitors (Roche, Switzerland), 1 mg/mL lysozyme (Sigma-Aldrich, USA) and 10 units/mL benzonase (Merck, USA). These cells were then lysed by three cycles of freezing at -80°C and thawing at 25°C.

The lysate was clarified by centrifugation at $3200 \times g$ for 15 min through a 96-well filter plate (0.45 μ m) (Millipore, USA). The filtrate was then transferred to a 96-well filter plate containing Ni-NTA agarose resin (Invitrogen) for His-tag purification. After 15 min of incubation at 4°C, the unbound material was removed by centrifugation at $100 \times g$ for 30 sec. The wells were then washed with 10 column volumes (CV) of wash buffer (20 mM Na^+ HEPES, pH 8.0, 150 mM NaCl, 30 mM imidazole) by centrifugation at $100 \times g$ for 1 minute.

The bound recombinant proteins were then recovered in 30 μ L of elution buffer (20 mM Na^+ HEPES, pH 8.0, and 150 mM NaCl, 300 mM imidazole) by centrifugation at $100 \times g$ for 1 minute. The eluted material was analysed by SDS-PAGE using Nu-PAGE 4%–12% Bis-Tris gels (Invitrogen) and stained with SimplyBlue Coomassie stain (Invitrogen). Purified protein constructs were subsequently loaded onto a Superdex 200 5/150 size exclusion column (GE-Healthcare) connected to an Akta-Micro controlled by Unicorn version 5.20 (GE-Healthcare). The running buffer used was 20 mM Na^+ HEPES, pH 8.0 and 150 mM NaCl. The retention volume and the size of the protein peaks for each construct were recorded.

2.3 Thermo-stability Screening of STIM1

The thermo-stability of each STIM1 construct was studied by the use of differential static light scattering described previously by Senisterra et al [141] using a multi-well instrument (Stargazer, Harbinger Biotech, Toronto, Canada). STIM1 constructs with a concentration of 30 μM in 50 μL of buffer (20 mM Na^+ HEPES, pH 7.5, 300 mM NaCl, 300 mM) were heated from 25°C to 80°C at a rate of 1 °C/min.

The aggregation of the STIM1 constructs was monitored through the recording of scattered light using a CCD camera and images of the plate was taken with an interval of 0.5°C. The intensities calculated from each well were integrated to obtain an approximate value representative of the total amount of scattered light in that area. The total intensities were then plotted against temperature and fitted to the Boltzmann equation by non-linear regression. Melting temperature (T_m) of the STIM1 constructs is defined as the temperature it will take for 50% of the sample proteins to be denatured.

2.4 Oligomerization Screening of STIM1

Six molecular weight markers containing proteins ranging from 1350 to 670000 Daltons were added into a SEC column (Superdex 200 5/150) to calibrate the column. The running buffer used was 20 mM Na⁺ HEPES, pH 8.0 and 150 mM NaCl. The molecular markers used were ovalbumin (43 kDa), conalbumin (75kDa), aldolase (158 kDa), ferritin (440 kDa), thyroglobulin (669 kDa) and blue dextran (2000 kDa). A calibration curve was obtained by plotting the logarithms of the molecular weight markers against the respective ratios of their elution volume to the column's void volume.

STIM1 constructs were injected into the same column using the same running buffer used for the calibration. The elution volume from each gel filtration profile was recorded. The molecular weight of the STIM1 constructs was then determined by comparing its elution volume with those of the known protein standards using the calibration curve.

2.5 Large Scale Purification of STIM1

Transformed Rosetta (DE3) strains containing the STIM1 gene were grown overnight at 37°C in 10 mL of TB media supplemented with 50 µg/mL kanamycin. The overnight culture was diluted 1:500 in 1 L of TB (Formedium, United Kingdom). The cells were then grown at 37°C.

When the OD600 was between 0.6 - 1.0, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added, resulting in a final concentration of 0.2 mM. The expression was continued overnight at 18°C. The cell culture was harvested by centrifugation at 10000 × g for 10 min and the pellets were re-suspended in lysis buffer supplemented with protease inhibitors (Roche, Switzerland), 1 mg/mL lysozyme (Sigma-Aldrich, USA) and 10 units/mL benzonase (Merck, USA).

The cells were lysed by three cycles of freezing at -80°C and thawing at 25°C. Unlysed cells and cell debris were removed by centrifugation at 30000 × g for 20 min. The sample of lysed cells was then loaded onto 1 mL of Ni-NTA agarose beads (Invitrogen, USA) and washed with 10 CV of wash buffer.

The bound recombinant membrane proteins were then eluted with elution buffer. Purified protein constructs were then injected onto a Superdex 200 10/300 column size exclusion column (GE-Healthcare) connected to an Akta-Explorer controlled by Unicorn 5.20 (GE-Healthcare) and the fractions were analysed by SDS-PAGE using Nu-PAGE 4%–12% Bis-Tris gels (Invitrogen) and stained with SimplyBlue Coomassie stain (Invitrogen). The purest and most homogeneous fractions were then pooled and used for further studies.

2.6 Production and Sequence of the N- and C- Termini Orai1 Peptides

Orai1 peptides were synthesised by Peptide 2.0 (USA) and delivered in a lyophilised state.

The sequences of the Orai1 peptides are as follow:

Orai1 N-terminus: HSMQALSWRKLYLSRAKLKC

Orai1 C-terminus:

CRSLVSHKTDRQFQELNELAEFARLQDQLDHRGDHPLTPGSHYA

The lyophilised peptides were then dissolved in running SPR buffer (20 mM Na⁺ HEPES, pH 7.5, 150 mM NaCl) and were injected onto a Superdex peptide 10/300 size exclusion column (GE-Healthcare) connected to an Akta-Micro controlled by Unicorn 5.20 (GE-Healthcare). The fractions were analysed by SDS-PAGE using Nu-PAGE 4%–12% Bis-Tris gels (Invitrogen) and stained with SimplyBlue Coomassie stain (Invitrogen). The purified peptides were then stored in 100 µL aliquots at -20°C.

2.7 SPR Study of STIM1

Binding of different STIM1 constructs and Orai1 peptides (synthesized by Peptide 2.0) was assessed by SPR on a BIAcore T200 instrument (BIAcore AB, Uppsala, Sweden). Orai1 peptide was immobilized on a sensor chip by a disulphide bond via a cysteine residue which was added to either the C-terminus end of the Orai1 N-terminus or the N-terminus end of the Orai1 C-terminus to ensure the peptides were immobilized at an orientation relative to the plasma membrane.

The CM5 sensor chip was preconditioned with the injection of three 1-minute pulses of immobilization buffer (10 mM Na⁺ HEPES, pH 7.5, 150 mM NaCl) at a flow rate of 10 µL/min. The surface was then equilibrated using immobilization buffer (10 mM Na⁺ HEPES, pH 7.5, 150 mM NaCl) for about 15 min.

50 µL of a 1:1 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) with a flow rate of 5 µL/min were injected through a CM5 sensor chip to activate the surface. This was followed by a 20 µL injection with a flow rate of 5 µL/min of 80 mM PDEA (2-(2-pyridinyldithio) ethaneamine hydrochloride) in 0.1 M borate buffer pH 8.5 to introduce the reactive disulphide groups onto the surface.

The Orai1 peptides (1 mg/mL) were then injected at a flow rate of 5 µL/min to reach around 1000 RU. Deactivation of the unreacted sulfide groups was performed with the injection of 20 µL 50 mM L-cysteine, 1 M NaCl in 0.1 M formate buffer pH 4.3 at a flow rate of 5 µL/min.

For the screening assay, STIM1 constructs with concentration of 10 µM were injected over the sensor chip surfaces diluted in running buffer (20 mM Na⁺ HEPES, pH 7.5, 150 mM NaCl) at a flow rate of 30 µL/min. Lysozyme was injected as a negative control. Between experiments, 10 mM glycine-HCl pH 3.5 was used to regenerate the surface. The maximum response level (RU) of the interaction between the STIM1 constructs and the Orai1 peptides was recorded. These RU value were then normalised to their respective molecular weights.

For the kinetic assay of STIM1, four STIM1 constructs, with concentrations ranging from 3 to 20 μM were injected over the sensor chip surfaces diluted in running buffer (20 mM Na^+ HEPES, pH 7.5, 150 mM NaCl) at a flow rate of 30 $\mu\text{L}/\text{min}$. Lysozyme was injected as a negative control. The interactions between the ligand and the analyte were measured over a six minute time period. 10 mM glycine-HCl pH 3.5 was used to regenerate the surface after each construct injection.

Ten injections of running buffer were performed before the injection of the STIM1 constructs for the kinetics experiment. These ten blank injection responses were averaged and then used for the referencing of the binding data. The STIM1 constructs were diluted four times in a two-fold dilution using the running buffer resulting in five different concentrations for each STIM1 construct. The experiment for each STIM1 construct was repeated 3 times. Binding data were fitted with a 1:1 Langmuir binding kinetic model using the Scrubber software (v2.0c, Biologic, Australia).

2.8 Cloning of the Human A2aR-BRIL

A detailed protocol regarding the cloning process is described in Liu et al [142]. Briefly, the gene of wild type human A2aR was synthesized with the insertion of the encoding region of apocytochrome b562RIL (BRIL) from *E. coli* [143]. The resulting translational product comprised the residues Ala1 to Leu106 from the BRIL fusion protein inserted into the third intracellular loop region (ICL3) of A2aR, between Lys209 to Gly218. Additionally, the residues 317-412 of A2aR were also truncated. The A2aR gene was then cloned into the expression vector, pFastBac1-830400 (Invitrogen), which includes the encoding regions for a FLAG tag at the N-terminus and a 10 × His-tag at the C-terminus.

2.9 Expression and Purification of the Human A2aR-BRIL

High-titer recombinant baculovirus of around 10^8 viral particles per mL was obtained using the Bac-to-Bac Baculovirus Expression System (Invitrogen). The recombinant baculovirus was then used to infect *Sf9* cells at a cell density of $2-3 \times 10^6$ cells/mL with a MOI (multiplicity of infection) of 5. Cells were harvested by centrifugation following 48 hours of post infection and the insect cell pellet was stored at -80°C until use.

Insect cell membranes were subsequently broken by thawing the frozen pellets in a hypotonic buffer containing 10 mM Na^+ HEPES, pH 7.5, 10 mM MgCl_2 and 20 mM KCl and protease inhibitor (Roche). Repeated washings of the thawed cell pellets were performed using a combination of dounce homogenization and ultracentrifugation at $150000 \times g$ in the same hypotonic buffer for three times. This was followed by a similar washing process in a high osmotic buffer (10 mM Na^+ HEPES, pH 7.5, 10 mM MgCl_2 , 20 mM KCl, 1.0 M NaCl) for three times to remove any proteins peripherally bound to the plasma membranes.

The purified membranes were then re-suspended in a suspension buffer (10 mM Na^+ HEPES, pH 7.5, 10 mM MgCl_2 , 20 mM KCl, 4 mM theophylline (Sigma), 2.0 mg/mL iodoacetamide (Sigma) and protease inhibitor) for 30 min at 4°C . The membranes were then solubilized with the addition of 0.5% (w/v) n-dodecyl- β -D-maltopyranoside (DDM) (Anatrace) and 0.1% (w/v) cholesteryl hemisuccinate (CHS) (Sigma) and incubated for 2.5-4 h at 4°C .

The insolubilized cell material was subsequently removed by centrifugation at $150000 \times g$ for 45 min. The supernatant was then incubated with TALON resin (Clontech) overnight at 4°C . The TALON resin was washed with 10 CV of wash buffer 1 (50 mM Na^+ HEPES, pH 7.5, 800 mM NaCl, 10% (v/v) glycerol, 25 mM imidazole, 0.1% (w/v) DDM, 0.02% (w/v) CHS, 10 mM MgCl_2 , 8 mM ATP (Sigma) and 4 mM theophylline).

This was followed by 10 CV of wash buffer 2 (50 mM Na^+ HEPES, pH 7.5, 800 mM NaCl, 10% (v/v) glycerol, 50 mM imidazole, 0.05% (w/v) DDM, 0.01% (w/v) CHS and 4 mM theophylline). The receptors bound to the resin

were then eluted in wash buffer 2 containing 220 mM imidazole. The monodispersity of the purified receptors was analyzed by size exclusion chromatography in 50 mM Na⁺ HEPES, pH 7.5, 800 mM NaCl, 10% (v/v) glycerol, 0.05% (w/v) DDM, and 0.01% (w/v) CHS and 4 mM theophylline.

2.10 Expression and Purification of MSP1D1

The expression and purification of the membrane scaffolding protein MSP1D1 containing a His-tag at the N-terminus followed by TEV protease cleavage site were performed according to the previously described method [57]. Briefly, transformed *E. coli* cells were grown at 37°C until an OD₆₀₀ of 0.7-0.8 was reached.

The expression was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside and continued for 3h at 37°C. The cells were harvested by centrifugation at 8000 × g for 15 min. The harvested cells were lysed by sonication in the presence of 1 mg/mL lysozyme in 50 mM Tris-HCl pH 8.0, 300 mM NaCl and 1% (v/v) Triton X-100 supplemented with DNase I and a protease inhibitor cocktail.

The lysate was centrifuged for 30 min at 30000 × g to remove unbroken cells and debris. The clarified supernatant was loaded on a TALON column and the flow-through was discarded. The column was then washed with 20 mM Tris-HCl pH 8.0 and 300 mM NaCl in three steps: 1) 0.1% (v/v) Triton X-100, 20 mM imidazole, 2) 50 mM sodium cholate, 30 mM imidazole, 3) 50 mM imidazole. Finally, MSP1D1 was eluted in 20 mM Tris-HCl pH 8.0, 300 mM NaCl and 300 mM imidazole. The eluted protein was treated with TEV protease overnight and His-tag cleaved protein was separated from His-tagged proteins on a fresh TALON column.

2.11 Reconstitution of A2aR into Nanodiscs

The purified A2aR was reconstituted into nanodiscs following the protocol as described previously [57]. Briefly, solubilized receptors were mixed with phosphocholine and His-tag cleaved MSP1D1 in a molar ratio of 1:700:10 (receptor: lipid: MSP1D1) and incubated for 1h at 4°C. This mixture was incubated overnight at 4°C with biobeads (Sigma). The reconstituted nanodiscs with His-tagged A2aR were then mixed with 1 mL of TALON resin (Clontech) and incubated for 30 min at 4°C. The TALON resin was washed with 10 CV of wash buffer 3 (50 mM Na⁺ HEPES, pH 7.5, 800 mM NaCl, 10% (v/v) glycerol, 50 mM imidazole).

The A2aR-nanodiscs were then eluted using 50 mM Na⁺ HEPES, pH 7.5, 800 mM NaCl, 10% (v/v) glycerol, and 220 mM imidazole. Eluted nanodiscs were subsequently desalted into a buffer of 50 mM Na⁺ HEPES, pH 7.5, 300 mM NaCl, 40% Glycerol to remove the imidazole and flash frozen in -70°C for storage.

Analysis of receptor purity and mono-dispersity was performed using both SDS-PAGE and size exclusion chromatography. Nanodiscs with no A2aR (“empty” nanodiscs) were prepared using the same method described above except for the replacement of GPCRs with an equal volume of elution buffer.

Two monoclonal antibodies, A2aR mAb and MSP mAb, were provided by the Ray Stevens group. The first monoclonal antibody, A2aR mAb, binds to the active form of the A2aR. The second antibody, MSP mAb, binds only to the MSP1D1 protein on the nanodisc.

The antibodies were covalently immobilized onto the flow cells on a CM5 chip using amine coupling. 70 µL of a 1:1 mixture of 0.4 M EDC and 0.1 M NHS with a flow rate of 10 µL/min were injected into a CM5 sensor chip to activate the surface. The receptors (1 mg/mL) were then injected at a flow rate of 5 µL/min to reach above 5000 RU, using a BIAcore T200 (GE Healthcare). Injection of 70 µL of 1 M ethanolamine at a flow rate of 10 µL/min was performed to deactivate the remaining reactive groups. Three different samples, purified A2aR, empty nanodiscs and reconstituted A2aR-nanodiscs, were then

injected into the flow cells at a flow rate of 30 $\mu\text{L}/\text{min}$. The running buffer used for the purified A2aR was 10 mM Na^+ HEPES, pH 7.5, 150 mM NaCl and 0.01% DDM. The running buffer used for the empty nanodiscs and reconstituted A2aR-nanodiscs was 10 mM Na^+ HEPES, pH 7.5 and 150 mM NaCl.

2.12 Immobilization of A2aR for SPR Analysis

The NTA sensor chip was preconditioned by three 1-minute pulses of 350 mM EDTA at a flow rate of 10 μ L/min. This was followed by a 1-minute injection of 500 μ M NiCl₂ at a flow rate of 10 μ L/min. The freshly charged surfaces were then equilibrated by immobilization buffer (10 mM Na⁺ HEPES, pH 7.5, 150 mM NaCl).

For the CM5 chips, only immobilization buffer (10 mM Na⁺ HEPES, pH 7.5, 150 mM NaCl) was used for pre-conditioning. The solubilized A2aR and the A2aR-nanodiscs were then immobilized on various sensor chip surfaces using three different methods:

1) Nickel affinity only: the purified receptors (1 mg/mL) were injected at a flow rate of 5 μ L/min onto a Ni²⁺-NTA sensor chip. An immobilization level of more than 7000 RU was targeted and the injection was terminated once this level was met.

2) Nickel affinity with amine coupling: the NTA sensor chip was pre-activated with the injection of 60 μ L of a 1:1 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) with a flow rate of 10 μ L/min. The receptor (1 mg/mL) was subsequently injected at a flow rate of 5 μ L/min to achieve a level of more than 7000 RU. The remaining reactive groups were deactivated with the subsequent injection of 60 μ L of 1 M ethanolamine at a flow rate of 10 μ L/min.

3) Amine coupling only: 70 μ L of a 1:1 mixture of 0.4 M EDC and 0.1 M NHS with a flow rate of 10 μ L/min were injected into a CM5 sensor chip to activate the surface. The receptors (1 mg/mL) were then injected at a flow rate of 5 μ L/min to reach above 10000 RU, using a BIAcore T200 (GE Healthcare). Injection of 70 μ L of 1 M ethanolamine at a flow rate of 10 μ L/min was performed to deactivate the remaining reactive groups.

The reference surface was prepared with the immobilization of empty nanodiscs using either nickel affinity or amine coupling, depending on how the receptor was immobilized, to exclude any non-specific binding that may occur. For the study of the solubilized A2aR, the reference surface was prepared using

purified MSP1D1 instead of empty nanodiscs using the same method as mentioned above.

2.13 Kinetics of the Interaction between A2aR and its Ligands

Taking into account the unstable nature of A2aR, titration kinetics was performed to eliminate the need for a regeneration step [144]. The regeneration step often uses conditions that can denature or deactivate the immobilized ligand irreversibly [145, 146]. In titration kinetics, the analyte was injected from a low to a high concentration with short dissociation times in between injections with a long dissociation time at the end [144].

Titration kinetic experiments were performed at 25°C. Detergent-solubilized A2aR or A2aR-nanodiscs were serially diluted two-fold in the respective running buffers (10 mM Na⁺ HEPES, pH 7.5, 150 mM NaCl, 0.01% DDM, 1% DMSO or 10 mM Na⁺ HEPES, pH 7.5, 50-500 mM NaCl, 1% DMSO). For a single titration kinetic experiment, small molecule ligands were injected sequentially in the order of increasing concentrations over both the immobilized receptors and the reference surfaces. The highest concentrations of the ligands used are as follows: (A) ZM 241395: 100nM, (B) UK 432097: 1μM, (C) CGS 21860: 10μM, and (D) NECA: 2.5μM.

At the start of each kinetic experiment, ten injections of running buffer were performed. These ten blank injection responses were averaged and then used to reference the binding data. DMSO solvent correction was performed injecting 8 different concentrations of DMSO ranging from 0.5% - 2% to correct for the excluded volume effect contributed by DMSO which has a very high refractive index. The kinetic curves obtained were evaluated using the T200 Bia-evaluation software.

3. Results of STIM1 Study

3.1 Design of STIM1 Constructs

The low expression and lack of stability of wild type human STIM1 have made it difficult to study the protein *in vitro* [108, 147]. In order to increase STIM1 expression, multiple thermo-stabilizing mutations are usually required [108]. These modifications to STIM1 might change its structure or affect its function. With the aim of improving the expression and stability of human STIM1 without mutations, a library of STIM1 constructs was designed and sorted into six groups (Figure 9) (Table 2).

Group 1: Constructs that include a short linker region Q233-M245 (QNRYSKEHMKMM) which connects the coiled-coil domains of STIM1 to the transmembrane region.

Group 2: Constructs that exclude the short linker region Q233-M245.

Group 3: Constructs that exclude the first coiled-coil domain of STIM1.

Group 4: Constructs that exclude the first and second coiled-coil domains of STIM1.

Group 5: Constructs that exclude the entire coiled-coil domain of STIM1.

Group 6: Constructs that include the N-terminus region of STIM1. Within this group are the transmembrane constructs of STIM1 and soluble STIM1 constructs that contain only the STIM1 N-terminus residues without the transmembrane residues.

These constructs were studied using four different assays: 1. Expression assay to identify residues that affect STIM1 expression; 2. Thermo-stability assay to identify residues that affect STIM1 melting temperature; 3. Oligomerization assay to identify residues that contribute to STIM1 oligomerization; 4. SPR assay to characterize the interaction between STIM1 and Orai1 N- and C-termini.

Figure 9. Pictorial representation of STIM1 constructs.

Table 2. Grouping of STIM1 constructs.

Group	Key Feature	Comments on the construct design
1	Contains the short linker region connecting STIM1 to the transmembrane	<ul style="list-style-type: none">• Constructs designed with Q233 as starting residue
2	Contains the first coiled-coil domain of STIM1 (exclusion of the short linker region)	<ul style="list-style-type: none">• Coiled-coil region of STIM1 starts at K246• Focus of construct design is on the how the linker region Q233-M245 affects its expression
3	Contains the second and third coiled-coil domains (exclusion of first coiled-coil domain)	<ul style="list-style-type: none">• Second coiled region starts at S340• Focus of construct design is on the expression of STIM1 without the presence of the first coiled-coil domain
4	Contains the third coiled-coil domain (exclusion of first and second coiled-coil domains)	<ul style="list-style-type: none">• Third coiled region starts at S388• Focus of construct design is on the expression of STIM1 without the presence of the first and second coiled-coil domains
5	No coiled-coil region domain	<ul style="list-style-type: none">• Focus of construct design is how the removal of the entire coiled-coil domain affects STIM1 expression
6	Contains the N-terminus region of STIM1	<ul style="list-style-type: none">• Focus of construct design is on the N-terminus region of STIM1 with some soluble constructs made up of residues located only at the ER lumen

3.2 Overview of STIM1 Expression Study

The level of expression of the STIM1 constructs was determined through a combination of two experimental techniques: SDS-PAGE (to determine molecular weight and the relative concentration) and size exclusion chromatography (the relative percentage of the main peak area was compared to the entire chromatogram). The scoring system is described as follows:

High expression (denoted in green):

- High expression of target protein (above 1 mg/L of purified protein yield)
- Target protein molecular weight in gel matches theoretical size
- Single peak present in gel filtration profile (more than 80% of the entire peak area)

Medium expression (denoted in yellow):

- Medium expression of target protein (0.1 - 1 mg/L of purified protein yield)
- Target protein molecular weight in gel matches theoretical size
- Presence of a smaller peak together with a dominant main peak (above 60% but below 80% of the entire peak area) present in gel filtration profile

Low expression (denoted in red):

- Expression of target protein is low (below 0.1 mg/L of purified protein yield)
- Target protein molecular weight in gel matches theoretical size
- Presence of multiple peaks in gel filtration profile

No expression (denoted in grey):

- No expression of target protein observed in gel
- Size of target protein does not match with theoretical size in gel
- Presence of multiple peaks in gel filtration profile

The use of size exclusion chromatography allows the identification of STIM1 constructs that are homogeneous and with high purity. Expression scores from all the 211 STIM1 constructs were consolidated and tabulated for easier reference (Table 3 and Appendix B).

Table 3. Expression scores of the STIM1 library. A total of 211 different STIM1 constructs were expressed and purified. These constructs were sorted by their groups with scores ranging from high to low expression.

Group	Expression Score			
	High	Medium	Low	Failed
1	1	19	19	5
2	43	10	2	2
3	28	7	12	0
4	0	4	3	0
5	0	10	7	1
6	23	9	3	3

3.3 Analysis of STIM1 Expression of Groups 1 and 2

The constructs designed in group 1 contained the short linker region (QNRYSKEHMKMM) which connected the coiled-coil domains of STIM1 with the ER membrane. The expression of these constructs was observed to be low and most of the constructs had a protein yield lower than 0.1 mg/L (Table 4, Appendix B). Their low protein yields made them unsuitable for functional assays.

The constructs in group 2 included the first coiled-coil domain with the exclusion of the linker region. The expression of the STIM1 constructs from group 2 had higher levels of expression compared to the STIM1 constructs from group 1 (Table 4).

It was observed that group 2 constructs excluding the region H395-L450 do not have high expression. The region H395-L450 contains the entire third coiled-coil domain with an additional region R423-L450.

Table 4. Expression scores of groups 1 and 2 STIM1 constructs. The STIM1 constructs from these two groups differ by the presence of the linker region Q233-M245 in group 1 but not in group 2. A pictorial representation of these constructs can be found in Appendix A (Page 129).

Group	Starting amino acid	Ending amino acid	Expression	Purified protein yield [mg/L]	Molecular weight [kDa]	Included coiled-coil domain
1	233	360	Low	0.09	18.22	First
2	246	360	High	1.68	16.52	First
1	233	395	Low	0.03	22.34	First and Second
2	246	395	High	1.74	20.64	First and Second
1	233	463	Low	0.06	29.83	Entire coiled-coil domain
2	246	463	High	1.34	28.13	Entire coiled-coil domain
1	233	490	Low	0.05	32.88	Entire coiled-coil domain
2	246	490	High	1.56	31.19	Entire coiled-coil domain
1	233	685	Low	0.05	53.65	Entire coiled-coil domain
2	246	685	High	1.43	51.96	Entire coiled-coil domain

Table 5. Expression scores of selected group 2 STIM1 constructs. STIM1 constructs from group 2 were sorted based on their ending amino acid to observe the change in expression when residues were added or removed. A pictorial representation of these constructs can be found in Appendix A (Page 129).

Group	Starting amino acid	Ending amino acid	Expression	Purified protein yield [mg/L]	Molecular weight [kDa]	Included coiled-coil domain
2	246	345	High	1.96	14.72	First
2	246	360	High	1.68	16.52	First
2	246	390	High	1.85	20.05	First and Second
2	246	399	Medium	0.57	21.04	First and Second
2	246	414	Medium	0.61	22.69	First and Second
2	246	425	Medium	0.57	23.83	First and Second
2	246	440	Low	0.04	25.74	First and Second
2	246	455	High	1.59	27.25	Entire coiled-coil domain
2	246	500	High	1.67	32.26	Entire coiled-coil domain
2	246	685	High	1.43	51.96	Entire coiled-coil domain

3.4 Analysis of STIM1 Expression of Groups 3 and 4

The three coiled-coil domains of STIM1 are made up of the following residues: K246-Y342 (first coiled-coil domain), I364-N388 (second coiled-coil domain) and S399-L423 (third coiled-coil domain). Located at the second and third coiled-coil domains is CAD (Y342-H448) which is needed for STIM1 to interact with Orai1 for the activation of the CRAC channel.

Two groups (3 and 4) of STIM1 constructs were designed to investigate the effect of the first and second coiled-coil domains on its expression. Group 3 constructs contain both the second and third coiled-coil domains (excluding the first coiled-coil domain). Group 4 constructs contain STIM1 constructs excluding the first and second coiled-coil domains.

When comparing constructs from group 3 and 4 with identical ending residues, constructs from group 3 have high expression whereas the group 4 constructs have low expression (Table 6). The difference between these two constructs is the region S340-N388 that includes the entire second coiled-coil domain (I364-N388).

Group 3 STIM1 constructs were found to have high expression when it includes the region S340-R500 (Table 6). The region S340-R500 contains the entire second and third coiled-coil domains with an additional region R423-R500. The construct S340-E425 is the smallest construct in group 3 that contains both the second (I364-N388) and third coiled-coil domains (S399-L423). The low expression observed for this construct showed that the second and third coiled-coil domains alone are not sufficient for high STIM1 expression.

Table 6. Expression scores of groups 3 and 4 STIM1 constructs. The STIM1 constructs from these two groups differ by the presence of the second coiled-coil domain in group 3 but not in group 4. A pictorial representation of these constructs can be found in Appendix A (Page 130).

Group	Starting amino acid	Ending amino acid	Expression	Purified protein yield [mg/L]	Molecular weight [kDa]	Included coiled-coil domain
3	340	425	Low	0.05	12.55	Second
3	340	450	Low	0.09	15.50	Second and third
3	340	474	Low	0.07	18.11	Second and third
4	388	480	Low	0.05	13.06	Third
3	340	500	High	1.24	20.97	Second and third
4	388	500	Low	0.08	15.23	Third
3	340	520	High	1.00	23.23	Second and third
4	388	520	Low	0.05	17.49	Third
3	340	580	High	1.83	29.62	Second and third
4	388	580	Low	0.03	23.88	Third

3.5 Analysis of STIM1 Expression of Groups 5 and 6

Beyond the coiled-coil domains of STIM1 is a stretch of residues from V442 to K685 that are involved in the anchoring of STIM1 to lipid rafts [111]. The STIM1 constructs in group 5 contain residues after the coiled-coil domains of STIM1 to observe how the coiled-coil domains affect STIM1 expression. None of the STIM1 constructs in group 5 have high levels of expression (Table 7).

Group 6 constructs were designed to include the N-terminus region of STIM1, with 33 of them containing a transmembrane region. Most of the transmembrane constructs were found to have high expression with the exception of construct S58-H395 (Table 7). This construct has a truncated CAD region which was similar to the construct K246-H395 from group 2. Both these constructs had poor expression, indicating that the STIM1 expression will be low whenever the CAD region (Y342-H448) is truncated.

The soluble STIM1 constructs in group 6 contain the EF-hands and the SAM domain. It was observed that most of these constructs have low expression (Table 7). Truncation of residues from the SAM domain resulted in low expression for the STIM1 constructs.

Table 7. Expression scores of groups 5 and 6 STIM1 constructs. The constructs from group 5 do not contain the coiled-coil domains of STIM1. Constructs from group 6 include soluble STIM1 constructs that do not contain the transmembrane region (ending amino acid before 215). A pictorial representation of these constructs can be found in Appendix A (Pages 128 and 130).

Group	Starting amino acid	Ending amino acid	Expression	Transmembrane region	Purified protein yield [mg/L]	Molecular weight [kDa]	Included coiled-coil domain
5	448	650	Low	Not included	0.02	24.36	None
5	461	685	Medium	Not included	0.88	26.81	None
6	58	193	Low	Not included	0.03	18.65	None
6	58	206	Medium	Not included	0.68	19.99	None
6	58	209	High	Not included	1.01	20.40	None
6	58	360	High	Included	1.29	38.46	First
6	58	395	Low	Included	0.02	42.58	First and Second
6	58	456	High	Included	1.74	49.31	Entire coiled-coil domain
6	58	600	High	Included	1.26	63.33	Entire coiled-coil domain
6	58	685	High	Included	1.35	73.84	Entire coiled-coil domain

3.6 Thermo-stability Study of STIM1

Higher melting temperature correlates to an increase in the stability of the protein. A light scattering assay was performed on the purified STIM1 constructs to identify their respective melting temperatures. In this assay, STIM1 constructs at concentrations of 30 μM were heated from 25°C to 80°C at a rate of 1°C/min. Aggregation of proteins was determined through the recordings of scattered light using a CCD. The total intensities of scattered light for each STIM1 construct were plotted against temperature and fitted by a Boltzmann equation formula using nonlinear regression [141]. Melting temperature (T_m) of the STIM1 construct is defined as the temperature it will take for 50% of the sample proteins to be denatured. The end result of this thermo-stability assay was the generation of a melting temperature library for the STIM1 constructs that were previously designed for the generation of the expression library.

When comparing STIM1 constructs from groups 2 and 3, it was observed that constructs from group 2 had higher T_m compared to constructs from group 3 (Table 8, Figure 10). The difference between these two groups is the presence of the first coiled-coil domain. This result shows that the presence of the first coiled-coil domain will contribute to the stability of STIM1.

Table 8. Melting temperature (T_m) of STIM1 constructs. Differences in the melting temperatures of constructs for group 2 and 3 are tabulated in the last column. Constructs in group 2 (shaded grey) containing the first coiled-coil domain have higher T_m than constructs in group 3 (unshaded).

Group	Starting amino acid	Ending amino acid	Included coiled-coil domain	Melting temperature (T_m / °C)	T_m difference between constructs with or without the first coiled-coil domain (°C)
2	246	516	1-3	56.7	9.7
3	340	516	2-3	47.0	
2	246	514	1-3	54.4	7.2
3	340	514	2-3	47.2	
2	246	500	1-3	53.7	7.0
3	340	500	2-3	46.7	
2	246	490	1-3	53.4	6.6
3	340	490	2-3	46.8

Figure 10. Thermodenaturation profiles of STIM1. Constructs on the left side of the green line are from group 2 that includes the first coiled-coil domain. Constructs on the right side of the green line are from group 3 that excludes the first coiled-coil domain. All these constructs contain the second and third coiled-coil domains.

3.7 Oligomerization Study of STIM1

One of the main functional characteristics of STIM1 is its oligomerization upon activation after depletion of Ca^{2+} in the ER. For the oligomerization study, STIM1 constructs were injected into a SEC column and their elution volume was recorded. The molecular weight of each STIM1 construct was determined by comparing its elution volume with those of known protein standards using a calibration curve. By comparing the calculated STIM1 constructs' molecular weights obtained from their SEC profiles to their molecular weights from their protein sequence, the oligomeric state of the STIM1 constructs can be approximated (Table 9, Figure 11).

For constructs containing the first and second coiled-coil domains, the oligomeric state of STIM1 was found to be a dimer. Constructs containing the second and third coiled-coil domains only also resulted in the formation of a dimer. However, when all three coiled-coil domains were present, the STIM1 constructs were found to become a tetramer. The removal of the three coiled-coil domains resulted in the formation of a monomer indicating that the three coiled-coil domains are necessary for STIM1 oligomerization into either a dimer or tetramer.

Table 9. Oligomeric states of STIM1 constructs. The molecular weights of the STIM1 constructs calculated from their SEC profiles were compared with their molecular weights to determine the oligomeric state.

Group	Start region	End region	Included coiled-coil domain	Molecular weight / kDa	SEC calculated molecular weight / kDa	Calculated oligomeric state
2	246	345	1	14.72	30.23	2
2	246	395	1-2	20.64	44.95	2
2	246	520	1-3	34.51	141.51	4
2	246	600	1-3	43.04	173.54	4
3	340	520	2-3	23.23	53.76	2
3	340	600	2-3	30.10	65.60	2
4	461	685	None	26.81	30.23	1

Molecular Weight Markers

Figure 11. SEC profiles of STIM1 constructs used in the oligomerization study. A calibration curve was obtained by plotting the logarithms of the molecular weight markers against the respective ratios of their elution volume to the column's void volume. The molecular weight of each STIM1 construct was determined by comparing its elution volume with the calibration curve.

3.8 Screening of the Interactions between STIM1 and Orai1 N- and C-termini using SPR Biosensors

SPR assays were conducted using the STIM1 constructs as the injected samples. Peptides representing Orai1 C- and N- termini were used as immobilized ligands on the surface of the sensor chip.

Both the Orai1 C-terminus and N-terminus peptides were synthesized based on the wild type Orai1 sequence. An extra cysteine group was added to either the N-terminus (Orai1 C-terminus peptide) or the C-terminus (Orai1 N-terminus peptide). The additional cysteine residue present at each end enabled the immobilization of the Orai1 N- and C- termini peptides on the SPR sensor chip surface by thiol coupling. This covalent immobilization of the peptides represents the *in vivo* topology of the soluble Orai1 terminus domains in the cytoplasm (Figure 12). A check on the Orai1 wild type sequence shows the absence of cysteine residues at either side of the cytosolic domains. This means that the addition of a cysteine residue will not result in the formation of unwanted disulphide bridges and also ensures that the orientation of the immobilized Orai1 samples will be favoured in an outward direction.

The response levels (RU) of the interaction between the STIM1 constructs (concentration of 10 μ M) and the Orai1 peptides were recorded. These RU values were then normalised by dividing the constructs' masses by their respective molecular weights (kDa) as the SPR signal is proportional to the mass on the sensor surface.

Lysozyme was used in the SPR assay to serve as negative control. Lysozyme is a stable generic protein with no reported interaction with either STIM1 or Orai1. The SPR response of lysozyme with Orai1 was recorded and subtracted from the STIM1 constructs responses (Figure 13, Appendix C). STIM1 constructs that have a positive response have interaction with the immobilized Orai1 peptide.

Figure 12. Orientation of the Orai1 N- and C- termini peptides. Immobilized Orai1 peptides present its natural orientation in the cell. The Orai1 C- and N- termini were synthesized with the introduction of a cysteine residue at their ends to facilitate thiol coupling to the sensor chip surface.

Figure 13. SPR responses of STIM1 constructs interacting with either the Orai1 N- or C- termini. STIM1 constructs at a concentration of 10 μ M were injected over surfaces with immobilized the Orai1 N- and C- termini peptides, and their responses were recorded. The normalized response of each STIM1 construct was plotted against its molecular weight. STIM1 constructs that showed a positive response with Orai1 were labelled using different colours.

3.9 STIM1 Interaction with the Orai1 N- and C-Termini

Constructs containing only the first coiled-coil domain of a STIM1 (K246-Y342) interact with the Orai1 N-terminus but not with the Orai1-C-terminus (Figure 13, Appendix C).

All STIM1 constructs that showed interaction with both the Orai1 N- and C-termini contained the entire CAD region (Y342-H448) (Figure 13, Appendix C). Constructs that contained a partially truncated CAD region (Y342-H448) do not have any interaction with the Orai1 C- terminus but these same constructs were observed to have interaction with the Orai1 N- terminus. This indicates that the entire CAD region is important for STIM1 interaction with the Orai1 C- terminus but not with the Orai1 N-terminus.

The N-terminus STIM1 constructs (group 6) showed no interaction with neither the Orai1 N- nor C-termini. These constructs can be taken as a negative control as they are not expected to interact with Orai1.

3.10 Purification of STIM1 Constructs for SPR Study

By combining the results from the three assays (expression, thermo-stability and oligomerization), an extensive STIM1 library was created containing information regarding each STIM1 construct. Various well-expressing STIM1 constructs containing different combinations of the coiled-coil domains were chosen from this library to be used for the determination of the affinity between STIM1 and Orai1. These constructs were classified into 4 distinct groups (A, B, C, and D):

- A. STIM1 containing only the first coiled-coil domain
- B. STIM1 containing both the first and second coiled-coil domains
- C. STIM1 containing both the second and third coiled-coil domains
- D. STIM1 containing all the coiled-coil domains

For every group class, three constructs were chosen and expressed on a larger scale (one liter of TB media). Observations were then made on the characteristics of these protein constructs based on three criteria (Table 10, Figure 14):

1. Stability of the purified protein constructs with focus on the amount of precipitation, if any, in the sample after 24 hours
2. Gel filtration profile after 24 hours incubation
3. Average amount of purified protein yield

Interestingly, all the STIM1 constructs that contained only the first and second coiled-coil domains (group B) were unstable after incubation at room temperature. This instability was different from what was observed for the other groups (A, C and D) which remained stable after incubation of 24 hours at room temperature. Additionally, the protein yield from group B was significantly lower than the rest of the groups, indicating protein instability upon large scale expression.

The most stable constructs with high levels of expression from each of the four groups were then chosen to be used for affinity determination of their interactions

with the Orai1 peptides using SPR. The chosen constructs from each group are as follows: (A) K246-Q360; (B) K246-L390; (C) S340-D520; (D) K246-K520. These constructs were then expressed in larger amounts to be used for further SPR studies.

Table 10. Observed characteristics of STIM1 constructs from Group A to D

Group	Constructs	Conditions		
		Stability at room temperature	Homogeneity of sample in SEC	Average amount of purified protein obtained per one litre of TB media (mg/L)
A	K246-E345	Good	Single Peak	3.4
	K246-E356	Good	Single Peak	3.5
	K246-Q360	Good	Single Peak	3.8
B	K246-I383	Precipitates within a day	Double Peaks	1.2
	K246-L390	Precipitates within a day	Double Peaks	1.6
	K246-H395	Precipitates within an hour	Double Peaks	0.8
C	S340-M490	Precipitates within a day	Single Peak	1.7
	S340-D520	Precipitates within a day	Single Peak	2.1
	S340-E570	Precipitates within a day	Single Peak	1.8
D	K246-D520	Good	Single Peak	3.9
	K246-S600	Good	Single Peak	3.4
	K246-K685	Good	Single Peak	3.7

Group A

Group B

Group C

Group D

Figure 14: SDS-PAGE gel and size exclusion chromatography profiles of STIM1 constructs from Groups A to D. The protein ladder is labelled for identification of the STIM1 constructs molecular weights. The size exclusion chromatography profile was generated from the injection of the STIM1 constructs into a Superdex 200 10/300 column.

3.11 Determination of the Affinity of STIM1 Interaction with the Orai1 C-Terminus

An SPR assay was performed on two constructs for the determination of the affinity of their interactions with Orai1: S340-D520 (second and third coiled-coil domains) and K246-D520 (all three coiled-coil domains). The assay was not done on the constructs K246-Q360 (first coiled-coil domain only) and K246-L390 (first and second coiled-coil domains) as they showed no interaction with the Orai1 C-terminus in the previous SPR study (Section 3.7). A series of four two-fold dilutions for each STIM1 construct were prepared and injected onto the immobilized Orai1 C-terminus peptide sensor surface. The respective affinity values of each STIM1 construct were calculated using the equation for steady state 1:1 binding using Scrubber 2.0 software (Table 11).

The S340-D520 construct showed the strongest interaction with the Orai1 C-terminus (26 μ M) (Table 11 and Figure 15). This was consistent with previous reports showing that the CAD region of STIM1 interacts primarily with the Orai1 C-terminus to open the Orai1 channel [94, 148]. The STIM1 construct (K246-D520) containing both the CAD region and the first coiled-coil domain had a weaker binding affinity of 41 μ M compared to the STIM1 construct of S340-D520 which contained only the CAD region (excluding the first coiled-coil domain).

Table 11. Binding affinity of the interaction between STIM1 and the Orai1 C-terminus. Two different constructs were used: S340-D520 and K246-D520. Lysozyme was used as a negative control. Affinity values were calculated using the steady state equilibrium model from Scrubber 2.0.

Interaction with the Orai1 C-terminus					
STIM1 constructs	K246-Q360	K246-L390	S340-D520	K246-D520	Lysozyme
Affinity value, K_D	N.A	N.A	$26 \pm 1 \mu\text{M}$	$41 \pm 2 \mu\text{M}$	N.A

Figure 15. SPR sensorgrams of STIM1 constructs interacting with the Orai1 C-terminus. The data were fitted using a steady-state equilibrium model from Scrubber 2.0 to obtain the affinity value of the interaction between STIM1 and the Orai1 C-terminus.

3.12 Determination of the Affinity of STIM1 Interaction with the Orai1 N-Terminus

From the SPR screening study of STIM1 interaction with the Orai1 N-terminus (Section 3.8), it was observed that constructs containing only the first coiled-coil domain (L248-Y342) have interaction with the Orai1 N-terminus but not with the C-terminus. This raises an interesting possibility for the role of the first coiled-coil domain in STIM1-Orai1 coupling as no studies had previously shown that the first coiled-coil domain have an interaction with Orai1.

To investigate this observation of the interaction between STIM1 and Orai1 N-terminus, a SPR experiment was performed using the four constructs chosen (Section 3.9): K246-Q360 (first coiled-coil domain only), K246-L390 (first and second coiled-coil domains), S340-D520 (second and third coiled-coil domains) and K246-D520 (all three coiled-coil domains). The experimental procedures used were the same as in the Orai1 C-terminus study (Table 12 and Figure 16).

All the constructs were found to interact with the Orai1 N-terminus. The binding affinity for the S340-D520 construct (exclusion of first coiled-coil domain) was observed to be the weakest. The negative control, lysozyme, showed no interaction with the Orai1 N-terminus.

Table 12. Binding affinity of the interaction between STIM1 and the Orai1 N-terminus. Lysozyme was used as a negative control. Affinity values were calculated using the steady state equilibrium model from Scrubber 2.0.

Interaction with the Orai1 N-terminus					
STIM1 constructs	K246-Q360	K246-L390	S340-D520	K246-D520	Lysozyme
Affinity value, K_D	$9 \pm 3 \mu\text{M}$	$11 \pm 4 \mu\text{M}$	$35 \pm 6 \mu\text{M}$	$14 \pm 6 \mu\text{M}$	N.A

Figure 16. SPR sensorgrams of STIM1 constructs interacting with the Orai1 N-terminus. The sensorgrams were fitted using a steady-state equilibrium model Scrubber 2.0 to obtain the affinity value of the interaction between STIM1 and the Orai1 N-terminus.

4. STIM1 Discussion

4.1 Removal of Linker Region Results in High Expression of Soluble STIM1 Constructs

From the comparison of STIM1 constructs from group 1 and 2, it is observed that the presence of amino acids from Q233-M245 (QNRYSKEHMKMM) resulted in lower levels of expression. Q233 and N234 have been observed to undergo structural changes upon STIM1 activation which indicate the flexible nature of the residues [149]. Proteins that have highly flexible regions typically have low expression due to their structure instability [150, 151].

The flexibility of a protein sequence can be predicted using computational models like FlexPred that compares the protein sequence with a database of macromolecular movements to predict the flexibility of a given set of residues [152, 153]. Three different protein sequences, S58-K685 (representing the entire transmembrane STIM1 construct), Q233-K685 (representing the sequence of STIM1 C-terminus excluding the transmembrane region) and K246-K685 (exclusion of the linker region) were uploaded to the FlexPred server to determine the predicted flexibility of the sequences.

The sequence Q233-K685 was observed to have a high degree of flexibility in the region Q233-Y236 (Table 13). As expected, this degree of flexibility is not observed for the sequence S58-K685 in which the STIM1 C-terminus is attached to the transmembrane region and its expression was high (Table 7).

Comparison of the first ten amino acids of sequences Q233-K685 and K246-K685 showed that K246-K685 was less flexible (Table 13) and its expression was high (Table 4). Based on these observations, low expression can be correlated to the high flexibility of the linker and it is recommended to remove it when expressing soluble STIM1 constructs.

Table 13. Probability of flexibility scores of STIM1 sequences. The probability of flexibility scores of the STIM1 residues were obtained using the FlexPred modelling system [152, 153]. Residues with flexibility scores of above 0.75 (highlighted green) were predicted by the model to be flexible.

Sequence S58-K685		Sequence Q233-K685		Sequence K246-K685	
Amino Acid	Flexibility Score	Amino Acid	Flexibility Score	Amino Acid	Flexibility Score
Q233	0.4935	Q233	0.7276	K246	0.7815
N234	0.6171	N234	0.775	D247	0.592
R235	0.6268	R235	0.8281	L248	0.6576
Y236	0.6314	Y236	0.7834	E249	0.5286
S237	0.6203	S237	0.7398	G250	0.6944
K238	0.5428	K238	0.5753	L251	0.5628
E239	0.6677	E239	0.62	H252	0.54
H240	0.5663	H240	0.4918	R253	0.6267
M241	0.4813	M241	0.359	A254	0.5956
K242	0.5342	K242	0.4358	E255	0.4298

4.2 Regions that are Essential for High Expression of STIM1

The main distinguishing feature of the STIM1 cytosolic region is the three coiled-coil domains: first (K246-Y342), second (I364-N388) and third (S399-L423). Most importantly, the CAD region (Y342-H448) includes parts of the second and third coiled-coil domains [154].

STIM1 constructs K246-Q440 (entire coiled-coil domain) and S340-E425 (second and third coiled-coil domains) were observed to have low expression while K246-N455 (entire coiled-coil domain) and S340-R500 (second and third coiled-coil domains) have high expression (Tables 5 and 7). From these results, it was concluded that the region R423-L450 is important for high STIM1 expression. A probable explanation for this observation can be derived from the structure of the STIM1 construct E345-N444 (PDB_3TEQ) [108]. It is observed that residue T354 of the first STIM1 monomer forms a hydrogen bond with R429 of the second STIM1 monomer (Figure 17). There are also multiple hydrophobic interactions between the residues L347, W350 and L351 of the first monomer with L436, W430 and I433 respectively of the second monomer (Figure 17). The residues from the second STIM1 monomer are present in the region R423-L450 and their removal destabilizes the STIM1 structure resulting in a drop of expression observed in groups 2 and 3. The reduction of STIM1 expression is also seen in group 4 constructs when the second coiled-coil domain (containing residues L347, W350, L351 and T354) is removed.

The thermo-stability assay showed that the first coiled-coil domain contributed to an improvement in the thermo-stability of STIM1 (Table 8). From the structure of the *Caenorhabditis elegans* (*C.elegans*) STIM1 construct (Q256-F391)(PDB_3TER) [108], it was observed that hydrogen bonds were formed between E264, N265, Q269, and R276 of the first monomer with R295, Q291, L288 and A286 respectively of the second monomer (Figure 18). Sequence alignment between *C.elegans* and human STIM1 showed that these residues involved in the hydrogen bonding were mostly conserved and it corresponded to the human STIM1 region E322-Q352 (Figure 18). This region is located within the

first coiled-coil domain of STIM1 and the hydrogen bonds helps to stabilise the structure of these constructs.

From the results, it is concluded that multiple factors affect the expression and stability of STIM1 constructs and these are summarised below (Table 14):

1. The first coiled-coil domain improves STIM1 thermo-stability when included in constructs containing the second and third coiled-coil domains.
2. The second coiled-coil domain needs to be expressed with either the first or the third coiled-coil domain for high STIM1 expression.
3. The expression of STIM1 constructs containing the entire coiled-coil domain is dependent on the region R423-L450 which is a sequence of amino acids after the third coiled-coil domain.

Table 14. Regions located within the coiled-coil domains that affect STIM1 expression.

Coiled-coil domain	STIM1 expression	Additional factors
First	high	none
First and second	high	none
Second	low	none
Second and third	high	must include region R423-R500
Third	low	none
All three	high	must include region R423-L450

Figure 17. Interactions between the second and third coiled-coil domains of STIM1. Pymol was used to visualise the structure of STIM1 E345-N444 (PDB_3TEQ) [108]. T354 of STIM1 monomer formed a hydrogen bond with R429 of monomer 2. Three hydrophobic interactions were also observed between the two STIM1 monomers: W350 (monomer 1) and W430 (monomer 2); L351 (monomer 1) and I433 (monomer 2); L347 (monomer 1) and L436 (monomer 2).

Sequence alignment of STIM1 human with STIM1 *C.elegans*

Q13586	STIM1_HUMAN	322	EQVREALRKAKELES	HS-SWYAPEALQKWLQLTHE
G5EF60	STIM1_CAEEL	264	ENLRVQLEEAE	RRLANSNGSQAPLALQPLLRRRTCE
			:	*:*:*:*:*:*:*:*:*:*:*:*
			↑↑	↑

Figure 18. Interactions between the first and second coiled-coil domains of STIM1. Pymol was used to visualise the structure of *C.elegans* STIM1 Q256-F391 (PDB_3TER)[108]. Four hydrogen bonds were observed: E264 (monomer 1) and R295 (monomer 2); N265 (monomer 1) and Q291 (monomer 2); Q269 (monomer 1) and L288 (monomer 2); R276 (monomer 1) and A286 (monomer 2). Located below is the sequence alignment analysis between human and STIM1 *C.elegans*. The black arrows indicate the residues that are highlighted above.

4.3 Oligomerization of STIM1 Constructs into Tetramers

The STIM1 constructs S340-D520 (containing second and third coiled-coil domains) and K246-E345 (containing first coiled-coil domain only), were found to be dimers in the oligomerization study (Table 9). However, the construct K246-D520 (containing the entire coiled-coil domain) was found to be a tetramer. Based on the information from the expression and oligomerization study, a model is proposed for the formation of the STIM1 tetramer (Figure 19).

The first coiled-coil domain residues from STIM1 monomer 1 and 2 form hydrogen bonds with the first coiled-coil domain residues of STIM1 monomer 4 and 3 respectively. The residues from the second coiled-coil domain of STIM1 monomer 1 and 2 will interact with the third coiled-coil domain of STIM1 monomer 3 and 4 respectively. This will result in the formation of STIM1 tetramer.

In this tetramer model, each monomer is bound to two different monomers. Removal of either the first or the third coiled-coil domain would disrupt the hydrogen bonds between the monomers resulting in the formation of a dimer as observed in the oligomerization study. For example, when the first coiled-coil domain is removed, monomer 1 loses its interaction with monomer 4 but is still bound to monomer 3 resulting in the formation of a STIM1 dimer (Figure 19).

Figure 19. Model of the formation of STIM1 tetramer. STIM1 tetramer is formed from two different interactions: 1. First coiled-coil domain of monomer 1 and 2 interacts with monomer 4 and 3 respectively; 2. Second coiled-coil domain of monomer 1 and 2 interacts with monomer 3 and 4 respectively. Double-sided arrows represent interaction between residues. Removal of either the first or third coiled-coil domain will result in the formation of a STIM1 dimer. The monomer 2 (far left) of the STIM1 tetramer is repeated at the right to show the interaction between the same monomer 2 and monomer 4.

4.4 First Coiled-Coil Domain Hinders STIM1 Interaction with the Orai1 C-Terminus

The STIM1 construct (K246-D520) which contains the entire coiled-coil domain has a weaker binding affinity (41 μ M) to the Orai1 C-terminus as compared to that of the S340-D520 construct (26 μ M) which contains only the second and third coiled-coil domains (Table 11). This shows that the presence of the first coiled-coil domain contributes to a reduction in affinity binding for the coupling of STIM1 to the Orai1 C-terminus.

A positively charged patch of residues (K382, K384, K385, K386 and R387) located at the CAD region of STIM1 is important for the activation and binding of STIM1 to Orai1 [108, 155, 156]. From the structure of the *C.elegans* STIM1 construct (Q256-F391) (PDB_3TER) [108], a hydrogen bond is formed between R276 of monomer 1 and C382 of monomer 2 (Figure 20). Hydrophobic interactions are also observed between E272 (monomer 1) and G383 (monomer 2). Sequence alignment between *C.elegans* and human STIM1 showed that E272, R276, C382 and G383 of *C.elegans* STIM1 correspond to K330, E334, C437 and G438 of human STIM1 (Figure 20).

The interaction of STIM1 residues from the first coiled-coil domain (K330-E334) to both C437 and G438 partially inhibited the positively charged patch K382-R387 of STIM1. This account for the reduction in affinity values between K246-D520 (entire coiled-coil domain) and S340-D520 (second and third coiled-coil domains, no first coiled-coil domain).

Sequence alignment of STIM1 human with STIM1 *C.elegans*

Q13586	STIM1_HUMAN	329	RKAEKELESHS-SWYAPEALQKWLQLTHE
G5EF60	STIM1_CAEEL	271	EEAERRLEANSNGSQAPLALQPLLRRTCE
			..**:.**:.* . ** ** *
		↑ ↑	
Q13586	STIM1_HUMAN	437	.CGFQIVNNPGIHSLSVAALNIDPSW---MG
G5EF60	STIM1_CAEEL	382	.CGFPLLYLNETEHINRSIASSHFYNKSHEG
			*** :: . : : : *
		↑↑	

Figure 20. First coiled-coil domain hinders STIM1 interaction with the Orai1 C-terminus. Pymol was used to visualise the structure of *C.elegans* STIM1 Q256-F391 (PDB_3TER)[108]. A hydrogen bond was formed between R276 (monomer 1) and C382 (monomer 2). Hydrophobic interactions was observed between E272 (monomer 1) and G383 (monomer 2). The sequence alignment analysis between human and *C.elegans* STIM1 is provided below. The black arrows indicate the residues that are highlighted above.

4.5 First Coiled-Coil Domain of STIM1 Interacts with the Orai1 N-Terminus

The first coiled-coil domain of STIM1 was found to interact with the Orai1 N-terminus (Table 12). From the structure of drosophila Orai1 (PDB_4HKR) [120], there is a positively charged region (R155, K157, K159 and K163) located at the Orai1 N-terminus (Figure 21). Using sequence alignment, these residues correspond to R83, K85, K87 and R91 of human Orai1. The first coiled-coil domain of STIM1 has a negatively charged patch E318-E322 that is conserved (Figure 21) across other STIM1 species (mouse, bovin and rat). One can speculate that this negatively charged region interacts with the positively charged region (R83, K85, K87 and R91) of Orai1 N-terminus differently from the interaction between STIM1 and the Orai1 C-terminus where the negatively charged region is located on the Orai1 C-terminus and the positively charged region is located on STIM1.

In patients suffering from SCID, Orai1 was found to have the mutation R91W which results in the loss of CRAC channel activity [8]. Substitution of the positively charged residue arginine 91 with tryptophan disrupts the binding of STIM1 to Orai1 N-terminus [8].

From these results, a model is proposed in which the first coiled-coil domain binds and docks STIM1 to the Orai1 N-terminus, facilitating the binding of CAD to Orai1 C-terminus (Figure 22). When STIM1 is inactive, the first coiled-coil domain is bound to the residues C437 and G438 (located at the CAD region). Upon depletion of Ca^{2+} in the ER lumen, the elongation of activated STIM1 results in the first coiled-coil domain dissociating from C437 and G438 and exposing the CAD region. STIM1 tetramer is formed from the interactions between the coiled-coil domains of four STIM1 monomers. At the plasma membrane, the positively charged region (R83, K85, K87 and R91) of Orai1 N-terminus provides an interaction site for the first coiled-coil domain of STIM1 which allows the docking of the STIM1 to Orai1. This facilitates the binding of the exposed CAD region with the Orai1 C-terminus, resulting in the activation of the CRAC channels and the influx of extracellular Ca^{2+} into the cytosol.

B

Sequence alignment of Orai1 human with Orai1 drosophila

Q9U6B8	CRCM1_DROME	154	.SRAKLKASSKTSALLSGFAMVAMV
Q96D31	CRCM1_HUMAN	82	.SRAKLKASSRTSALLSGFAMVAMV

			↑↑↑↑

C

Sequence alignment of STIM1 human with other species

Q13586	STIM1_HUMAN	301	KELREGTENERSRQKYAEELEQVREALRKAKELESHSSWYAPEALQKWLQLTHEVEVC
P70302	STIM1_MOUSE	301	KELREGTENERSRQKYAEELEQVREALRKAKELESHSSWYAPEALQKWLQLTHEVEVC
Q58CP9	STIM1_BOVIN	299	KELREGTENERSRQKYAEELEQVREALRKAKELESHSSWYAPEALQKWLQLTHEVEVC
P84903	STIM1_RAT	301	KELREGTENERSRQKYAEELEQVREALRKAKELESHSSWYAPEALQKWLQLTHEVEVC

			↑↑↑↑

Figure 21. Electrostatic surface representation of the positively charged region of Orai1 N-terminus. (A) Pymol was used to visualise the structure of drosophila Orai1 (PDB_4HKR)[120]. The surface is coloured as follows: red indicates negative charge, blue indicates positive charge, yellow indicates the Orai1 N-terminus residues and orange indicates the Orai1 transmembrane region. (B) The sequence alignment analysis between human and drosophila Orai1. The black arrows indicate the residues located at positively charged region of Orai1 N-terminus. (C) The sequence alignment analysis between human STIM1 with other species. The black arrows indicate the negatively charged region, E318-E322, of STIM1.

Figure 22. First coiled-coil domain of STIM1 binds and docks at the Orai1 N-terminus to facilitate CAD binding to Orai1 C-terminus. When STIM1 is inactive, the first coiled-coil domain is bound to the CAD region. Upon STIM1 activation, elongation of STIM1 occurs resulting in the exposure of the CAD region. STIM1 oligomerizes into a tetramer from the interaction between its coiled-coil domains. At the plasma membrane, the first coiled-coil domain binds and docks at the Orai1 N-terminus. This will facilitate the binding of CAD to the Orai1 C-terminus.

4.6 Overview of STIM1 Study

The production of STIM1 constructs was known to be difficult [140] and mutations were necessary for the high expression of STIM1 [41, 157]. The drawback with mutations on wild type STIM1 is the possibility of loss of its function and a change to its structure. The aim of this study was to produce high expressing and stable STIM1 constructs without any mutations and use the protein for SPR studies of the interaction between STIM1 and Orai.

In this study, a total of 211 STIM1 constructs were designed and expressed. These constructs were studied using four different assays (expression, thermostability, oligomerization and SPR). The key observations from the STIM1 study are as follows:

1. The first coiled-coil domain contributes greatly to the expression level and oligomerization of STIM1.
2. The linker region (Q233-M245) connecting the coiled-coil domains of STIM1 with the transmembrane decreases overall expression of the STIM1 constructs.
3. STIM1 is a tetramer when it contains the entire coiled-coil domain but it becomes a dimer when either the second or third coiled-coil domain is removed. A new model is proposed that explains the formation of the STIM1 tetramer.
4. The first coiled-coil domain of STIM1 interacts with Orai1 N-terminus and also inhibits CAD interaction with Orai1 C-terminus. A new model is proposed where the function of the first coiled-coil domain is to bind and dock to Orai1 N-terminus, facilitating the interaction of CAD to Orai1 C-terminus.

5. Results of GPCR Study

5.1 Verification of Successful Expression of A2aR

Purified A2aR was reconstituted into nanodiscs by mixing the A2aR with phosphocholine and MSP1D1 in a molar ratio of 1:700:10 (receptor: lipid: MSP1D1). Analysis of the reconstituted A2aR-nanodisc purity and monodispersity was performed using both SDS-PAGE and size exclusion chromatography that do not provide any indication on the folding of the protein. The A2aR in the nanodiscs may become misfolded resulting in potential false negative results when used in subsequent biophysical and biochemical assays. To ensure that the A2aR in the nanodiscs are well folded, two monoclonal antibodies that bind to either the nanodiscs or A2aR were used. A2aR mAb binds to the well folded active form of the A2aR. MSP mAb binds to the MSP1D1 protein which is located at the nanodisc surface.

Antibodies were covalently immobilized on the surface of two different flow cells of a CM5 chip using amine coupling. Three different samples were then injected through the flow cells: purified A2aR, empty nanodiscs and reconstituted A2aR-nanodisc. The respective sensorgrams for each sample are shown in Figure 23. Purified A2aR binds only to the A2aR mAb. Empty nanodiscs were shown to bind only to the MSP mAb. The reconstituted A2aR-nanodisc was found to bind to both antibodies, indicating the successful reconstitution of A2aR into the nanodisc.

Figure 23. Sensorgrams showing results of antibody tests to determine A2aR folding. A2aR mAb and MSP mAb were immobilized by amine coupling onto two different flow cells. Three types of samples were then injected into both the flow cells: 1. Purified A2aR (colored blue) 2. Denatured A2aR (colored orange) 3. Purified A2aR reconstituted into nanodisc (colored green). The sensorgrams were double referenced with the subtraction of the reference flow cell (flow cell one) and buffer injections.

5.2 Difference in Stability between Solubilized A2aR and A2aR-nanodiscs

A series of experiments was performed to investigate whether the reconstitution of A2aR into nanodiscs brought about increased stability for binding experiments compared to detergent-solubilized receptors. A2aR (in either detergent micelles or nanodiscs) was captured onto a NTA chip using nickel affinity and left to stabilize for two hours with flowing buffer at a rate of 30 $\mu\text{L}/\text{min}$.

A series of five injections of running buffer was performed to compare the relative stability of the two surfaces. The sensorgrams obtained from the injection of buffer through the A2aR-nanodiscs were stable whereas the curves from the detergent-solubilized receptors (Figure 24) showed a negative drift that indicates possible removal of receptors from the surface.

A series of titration kinetics experiments were performed using the A2aR antagonist ZM 241385 with either the detergent-solubilized A2aR or A2aR-nanodiscs. The quality of the sensorgrams obtained from the detergent-solubilized receptors was poor, which made the analysis difficult and no convincing kinetic data could be obtained (Figure 25).

In contrast, the sensorgrams generated from the A2aR-nanodisc titration experiments were of higher quality (Figure 25). Despite the low level signal obtained from the antagonist binding to A2aR-nanodiscs, the higher quality of the sensorgrams enabled kinetic analysis using the 1:1 binding model. Altogether, the A2aR-nanodiscs showed greater stability and better response compared to the detergent-solubilized receptors. Therefore, subsequent kinetics studies were performed using A2aR-nanodiscs.

Figure 24. Stability of detergent-solubilized A2aR vs. A2aR-nanodiscs. Five buffer injections were performed onto NTA-surfaces using either detergent-solubilized A2aR (blue) or A2aR-nanodiscs (black). The sensorgrams were double referenced with the subtraction of the reference flow cell (flow cell one) and buffer injections.

Figure 25. Sensorgrams of a titration kinetics experiment depicting the interaction of (A) detergent-solubilized A2aR or (B) A2aR-nanodiscs, with the A2aR antagonist, ZM 241385. Receptors were immobilized onto a NTA sensor chip by means of nickel affinity immobilization. The analyte used for the assays is the A2aR antagonist, ZM 241385, injected in a 3x dilution series with the highest concentration at 100 nM. The black lines are the responses of ZM 241385 with A2aR-nanodiscs and the orange lines are the generated fitting curves of the 1:1 Langmuir model using the Biacore T200 software. The sensorgrams were double referenced with the subtraction of the reference flow cell (flow cell one) and buffer injections. DMSO solvent correction was done to account for the excluded volume effect contributed by the DMSO used to solubilize ZM 241385.

5.3 Comparison of Different Immobilization Techniques

Three different types of immobilization techniques were tested to immobilize the A2aR-nanodiscs on the sensor surface: 1) nickel affinity, 2) nickel affinity combined with amine coupling (NTA chip) and 3) amine coupling (CM5 chip). The maximum immobilization level observed for the two nickel affinity techniques was around 7000 RU while the immobilization using amine coupling resulted in an immobilization level of around 15000 RU.

Upon immobilization, the receptors were left to stabilize for two hours by flowing buffer at a rate of 30 $\mu\text{L}/\text{min}$. During this step, the rate of dissociation of the A2aR-nanodiscs immobilized through nickel affinity was observed to be much higher compared to the other two techniques where amine coupling was used. This dissociation of nanodiscs from the surface complicates future ligand binding experiments as the signal obtained is the sum of association and dissociation at any point of time.

A series of titration kinetics experiments were performed using ZM 241385 to establish whether the amine coupling affected the activity of receptors. The reproducibility and similarity of kinetics for each type of immobilization indicated that the amine coupling modification did not negatively impact the binding kinetics compared to NTA immobilization (Table 15) (Figure 26). The immobilization levels when only amine coupling was used is much higher than the other two immobilization techniques, indicating more receptors on the surface. The immobilization of more A2aR-nanodiscs will lead to larger signals during kinetic experiments. As amine coupling was identified to be the most robust method and it did not interfere negatively with the activity of the receptor, this method was used as the main immobilization technique for subsequent ligand binding studies.

Table 15. Kinetic constants of A2aR-ZM 241385 interaction. A2aR-nanodiscs were immobilized onto the sensor surface using: NTA immobilization only; NTA immobilization combined with amine coupling; and amine coupling only. As this experiment was conducted only once, the standard error was not calculated.

Immobilization technique	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_D (nM)
Nickel affinity only	3.30×10^7	1.80×10^{-2}	0.56
Nickel affinity with amine coupling	1.70×10^5	1.30×10^{-2}	0.76
Amine coupling only	3.08×10^7	1.90×10^{-2}	0.50

Figure 26. Sensorgrams of A2aR-ZM 241385 interaction. A2aR-nanodiscs were immobilized onto the sensor surface using: NTA immobilization only; NTA immobilization combined with amine coupling; and amine coupling only. The red lines are the responses of ZM 241385 with A2aR-nanodiscs and the black lines are the generated fitting curves of the 1:1 Langmuir model using the Biacore T200 software. The sensorgrams were double referenced with the subtraction of the reference flow cell (flow cell one) and buffer injections. DMSO solvent correction was done to account for the excluded volume effect contributed by the DMSO used to solubilize ZM 241385.

5.4 Kinetics of A2aR Interactions with Ligands

After establishing the initial working parameters, all SPR experiments to study ligand-receptor interactions used A2aR-nanodiscs immobilized through amine coupling. The kinetics of the interactions between the antagonist ZM 241385 and three different agonists, UK 432097, CGS 21860 and NECA, and A2aR-nanodiscs were investigated.

The ligands were injected over the immobilized A2aR-nanodiscs in a series of two-fold dilutions (five different concentrations) with 60 sec injection time for each, followed by a dissociation time of 600 sec at the end of the last ligand injection. The highest concentration of each ligand was as follows: (A) ZM 241395: 100nM, (B) UK 432097: 1 μ M, (C) CGS 21860: 10 μ M, and (D) NECA: 2.5 μ M. The curves obtained were fit with the 1:1 binding model for all the ligands (Figure 27).

The association and dissociation rates of the antagonist and all the agonists to and from A2aR nanodiscs are shown in Table 16. The antagonist ZM 241385 had the highest association rate and binding affinity among all the ligands, with 2-4 orders of magnitude difference.

The agonists tested were found to have similar or slower dissociation rate as compared to the antagonist ZM 241385. UK 432097 showed the slowest dissociation rate among all the ligands (around 50 times slower than ZM 241385). The affinity values obtained from the titration kinetics experiments correlate well with the affinity values (K_i) reported from other studies using radio-labeled ligands, although CGS 21860 and NECA showed lower affinities (Table 16).

Table 16. Kinetic constants of A2aR interactions with its ligands. Four different ligands were used in the experiments: ZM 241385 (antagonist); UK 432097 (agonist); CGS 21860 (agonist); and NECA (agonist). Results are the average of three independent experiments.

Ligand	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_D (nM)	K_i from radio-labeled assays (nM)
ZM 241385	$2.98 \pm 2.64 \times 10^7$	$1.47 \pm 0.72 \times 10^{-2}$	0.49 ± 0.1	$0.3^{[41]}$
UK 432097	$5.58 \pm 2 \times 10^4$	$2.91 \pm 0.98 \times 10^{-4}$	5.21 ± 2.32	$4.75^{[158]}$
CGS 21860	$6.21 \pm 0.31 \times 10^3$	$1.81 \pm 0.05 \times 10^{-3}$	291 ± 18	$83^{[159]}$
NECA	$1.03 \pm 0.07 \times 10^5$	$1.24 \pm 0.05 \times 10^{-2}$	120 ± 8	$84.7^{[160]}$

Figure 27. Sensorgrams of agonists and antagonists binding to A2aR-nanodiscs. Titration kinetics experiments were performed on A2aR-nanodiscs using (A) ZM 241385 (antagonist) (B) UK 432097 (agonist) (C) CGS21860 (agonist), and (D) NECA (agonist). All ligands were prepared and injected using a 3x dilution series. The highest concentration of each ligand is as follows: (A) ZM 241395: 100nM, (B) UK 432097: 1 μ M, (C) CGS 21860: 10 μ M, and (D) NECA: 2.5 μ M. The colored lines (yellow, orange, green and purple) are the responses of the ligands to A2aR-nanodiscs and the black lines are the generated fitting curves of the 1:1 Langmuir model using the Biacore T200 software. The sensorgrams were double referenced with the subtraction of the reference flow cell (flow cell one) and buffer injections. DMSO solvent correction was done to account for the excluded volume effect contributed by the DMSO used to solubilize the ligands.

5.5 Analysis of Na⁺ Effect on the Kinetics of Ligand Binding to A2aR-nanodiscs

During the optimization of buffers in the SPR experiments between A2aR-nanodiscs and the agonist, UK 432097, it was observed that the affinity value of UK 432097 increased when the buffer NaCl concentration increased. Previous studies had reported that Na⁺ can negatively affect agonist binding to A2aR [161, 162]. In order to investigate the effect of Na⁺ on ligand binding to A2aR, a series of SPR experiments were performed with increasing concentrations of NaCl in the buffer from 50 mM to 500 mM. No interaction between A2aR-nanodiscs and their ligands was observed when the NaCl concentration was below 50 mM (in all SPR experiments it is important to have salt in the sample buffer to avoid non-specific interactions with the negatively charged surface).

The running buffer for these assays was 10 mM Na⁺ HEPES, pH 7.5, 1% DMSO with varying concentrations of NaCl. No change was observed in the affinity of the antagonist ZM 241385 towards A2aR-nanodiscs with increasing Na⁺ concentrations (Table 17, Figure 28).

All the agonists were shown to have reduction of binding affinity between the agonist and A2aR at higher NaCl concentration (Tables 17-20) (Figure 28). The decrease in the affinity for the agonists seemed to be related to an increase in the dissociation rate and a decrease in the association rate.

Table 17. Na⁺ concentration effect on the kinetics of ZM 241385 binding to A2aR. Results are the average of three independent experiments.

[NaCl]	k_a (M ⁻¹ s ⁻¹)	k_d (s ⁻¹)	K_D (nM)
50 mM	$2.98 \pm 2.64 \times 10^7$	$1.47 \pm 0.72 \times 10^{-2}$	0.49 ± 0.1
150 mM	$3.48 \pm 1.7 \times 10^7$	$1.53 \pm 0.71 \times 10^{-2}$	0.44 ± 0.07
300 mM	$3.33 \pm 2.25 \times 10^7$	$1.86 \pm 1.17 \times 10^{-2}$	0.56 ± 0.12
500 mM	$4.24 \pm 1.86 \times 10^7$	$1.69 \pm 0.3 \times 10^{-2}$	0.40 ± 0.17

Table 18. Na⁺ concentration effect on the kinetics of UK 432097 binding to A2aR. Results are the average of three independent experiments.

[NaCl]	k_a (M ⁻¹ s ⁻¹)	k_d (s ⁻¹)	K_D (nM)
50 mM	$5.58 \pm 2 \times 10^4$	$2.91 \pm 0.98 \times 10^{-4}$	5.21 ± 2.32
150 mM	$2.61 \pm 1.2 \times 10^4$	$3.83 \pm 0.23 \times 10^{-4}$	14.7 ± 31
300 mM	$1.20 \pm 0.56 \times 10^4$	$9.19 \pm 3.99 \times 10^{-4}$	76.6 ± 171
500 mM	$9.63 \pm 7.35 \times 10^3$	$6.65 \pm 3.95 \times 10^{-4}$	69.1 ± 192

Table 19. Na⁺ concentration effect on the kinetics of CGS 21860 binding to A2aR. Results are the average of three independent experiments.

[NaCl]	k_a (M ⁻¹ s ⁻¹)	k_d (s ⁻¹)	K_D (nM)
50 mM	$6.21 \pm 0.31 \times 10^3$	$1.81 \pm 0.05 \times 10^{-3}$	291 ± 18
150 mM	$5.02 \pm 0.21 \times 10^3$	$2.07 \pm 0.02 \times 10^{-3}$	412 ± 16
300 mM	$4.96 \pm 0.39 \times 10^3$	$2.49 \pm 0.21 \times 10^{-3}$	502 ± 17
500 mM	$1.61 \pm 0.67 \times 10^4$	$8.91 \pm 3.7 \times 10^{-3}$	553 ± 48

Table 20. Na⁺ concentration effect on the kinetics of NECA binding to A2aR. Results are the average of three independent experiments.

[NaCl]	k_a (M ⁻¹ s ⁻¹)	k_d (s ⁻¹)	K_D (nM)
50 mM	$1.03 \pm 0.07 \times 10^5$	$1.24 \pm 0.05 \times 10^{-2}$	120 ± 8
150 mM	$1.11 \pm 0.02 \times 10^5$	$3.15 \pm 0.06 \times 10^{-2}$	284 ± 3
300 mM	$1.31 \pm 0.17 \times 10^5$	$3.91 \pm 0.53 \times 10^{-2}$	298 ± 3
500 mM	$1.42 \pm 0.01 \times 10^5$	$4.24 \pm 0.05 \times 10^{-2}$	296 ± 5

Figure 28. Sensorgrams of the Na⁺ effect on agonists and antagonists binding to A2aR-nanodiscs. Titration kinetics experiments were performed on A2aR-nanodiscs using (A) ZM 241385 (antagonist) (B) UK 432097 (agonist) (C) CGS21860 (agonist), and (D) NECA (agonist) with different NaCl concentrations (50 mM, 150 mM, 300 mM and 500 mM). The respective kinetics data can be found in Table 17-20. The colored lines (red, blue, yellow, orange, green and purple) are the responses of the ligands and the black lines are the generated fitting curves of the 1:1 Langmuir model using the Biacore T200 software. The sensorgrams were double referenced with the subtraction of the reference flow cell (flow cell one) and buffer injections. DMSO solvent correction was done to account for the excluded volume effect contributed by the DMSO used to solubilize the ligands.

6. GPCR Discussion

6.1 Nanodiscs: An Alternative to Thermo-Stabilizing Mutations

Biochemical and biophysical studies of GPCRs suffer from their relatively high instability after they have been extracted from the membranes using detergents [163]. This instability is caused by the loss of membrane potential or essential lipids, e.g. cholesterol, which is a problem among expression of GPCRs and other membrane proteins [164].

One method to improve the stability of isolated GPCRs is to introduce thermo-stabilizing mutations into its sequence. Although this method has proven to be successful for maintaining protein stability, mutations might affect the GPCR function and structure [165]. The fact that binding studies of agonists to thermo-stabilized GPCRs have not yet been reported can be an indication of a conformation of these mutants that is biased towards antagonists.

The reconstitution of A2aR-nanodiscs is quick and easy and the A2aR was observed to be well-folded (Figure 31). Moreover, the positive results obtained from the nanodisc study showed that good kinetic data can be obtained with no loss of A2aR function.

With the use of receptor-incorporated nanodiscs, the presence of detergents was excluded from the binding experiments. As a result, artificial interference of detergent micelles with the experimental procedures was removed. Additionally, the mixture of lipids and proteins found in these nanodiscs replicates the natural lipid bilayer environment for the embedding of A2aR [166, 167].

Elimination of detergents removes the need for detergent screening that is both costly and time-consuming [168]. The replacement of detergent micelles surrounding the receptor by lipids has other advantages. Studies have shown that the binding of arrestins and other intracellular proteins to GPCRs require the presence of specific types of lipids which can be used when creating the nanodiscs [64, 169].

Using nanodiscs to stabilize the A2aR was shown to be a successful and novel strategy for measuring the interaction of the receptors with their potential binding partners. This strategy may be successful for other GPCRs as there was no need for thermo-stabilizing mutations, keeping the native sequence of the GPCRs intact.

6.2 Optimization of SPR Assay to Measure Agonist-A2aR Interactions

The slow dissociation rates of the agonists UK 432097 and CGS 21860 (Table 16), made the regeneration of the A2aR-nanodiscs difficult as it required the use of stringent and harsh conditions, which may lead to inactivation of the A2aR. This might also explain the absence of kinetics in regards to agonist-A2aR interactions as the regeneration step would have damaged the A2aR. To overcome the regeneration problem, the experiments were performed using titration kinetics which does not require a regeneration step [144].

One of the main obstacles faced in this study was to obtain sufficient amounts of active immobilized receptors on the surface of the SPR chip that will provide a high enough signal when monitoring small molecules interactions. From the binding results obtained using the three different immobilization strategies (Ni-NTA only, Ni-NTA with amine coupling and amine coupling only), it was observed that amine coupling immobilization was the optimal strategy (Table 15). In the amine coupling immobilization of A2aR, the N-terminus residue and the ϵ -amino groups of lysine residues of either A2aR or MSP1D1 is attached covalently to the sensor chip surface. The drawback of using amine coupling immobilization is that the covalent attachment might alter the structure of A2aR that would affect its function. Comparison of the ZM 241385 kinetics between the Ni-NTA immobilization and the amine coupling immobilization showed no significant difference (Figure 26 and Table 15). This indicates that the use of amine coupling immobilization for the A2aR-nanodisc did not negatively affect its interaction with ZM 241385.

The affinity values of the interactions of both the antagonist ZM 241385 and the agonist UK 432097 with A2aR obtained in the experiment (Section 5.4) were in full agreement with previously reported data [41, 50], validating the proposed SPR setup to be robust and reliable.

6.3 Analysis of A2aR-agonist Interactions

The agonists, UK 432097 and CGS 21860, have a very slow dissociation rate (Table 16) compared to both NECA (agonist) and ZM 241385 (antagonist). The agonists UK 432097, NECA and CGS 21860 were all developed as potential drugs for the treatment of chronic obstructive pulmonary disease [170]. In physiological situations, the duration of the effect produced by a drug is directly related to the lifetime of the drug-target complex; the slower the dissociation time of the drug, the longer the drug effect lasts [72].

The low dissociation rate of UK 432097 makes it best suited for treatments where prolonged activation of A2aR is desired. From the structure of A2aR co-crystallized with UK 432097, a total of 11 hydrogen bonds were observed between UK 432097 and the ligand binding cavity of A2aR [50] which would explain the high affinity and slow dissociation rate of UK 432097. The slower dissociation rate of agonist NECA would make it suitable for treatments that require a shorter drug effect, especially in situations when prolonged activation of A2aR will lead to adverse effects [171, 172].

In drug discovery, large libraries of ligands are screened against a target and the affinity value of the ligand has been regarded as an indicator of its drug efficacy [70]. Studies have shown that the affinity value is sometimes insufficient and drug screening based on association and dissociation rates can be more effective [71, 72]. The strategy used in this study to determine the kinetic constants for both agonist and antagonist with A2aR will be useful to identify potential drug candidates depending on the treatment required.

6.4 Effect of Sodium on Agonist Binding to A2aR

When there is an increase in the Na^+ concentration, a corresponding decrease in the affinity of the agonists was observed. This drop in affinity was found to be correlated to the drop of the association rate and a rise in the dissociation rate of the agonist. For binding of the antagonist ZM 241385 to A2aR, the addition of Na^+ had no effect on the affinity values with both association and dissociation rates almost unchanged.

The main structure difference between the agonists and the antagonist ZM 241385 is the presence of a ribose group in the agonists [55]. In the structure of A2aR co-crystallized with the agonist UK 432097, the ribose group forms multiple hydrogen bonds with S277 and H278 located inside the binding cavity. The structure of A2aR-antagonist shows the presence of a highly conserved sodium binding site [142]. In this sodium binding site, a sodium ion is surrounded by water molecules that form multiple hydrogen bonds with residues D52, S91, N241, W246, N280, N284 and Y288. In the A2aR-agonist structure, this sodium binding site has a smaller surface area of 70 Å compared to 200 Å in the A2aR-antagonist structure.

From the SPR results, one can speculate that when Na^+ binds to A2aR at the sodium binding site, it causes a conformational change that disrupts the interaction of the agonist ribose group with S277 and H278 of A2aR. The agonists bind to A2aR at a slower rate leading to a reduction to their affinity value. This phenomenon of Na^+ negatively affecting agonist binding to GPCRs was also observed in previous studies [62, 173-175], which validate the results of these experiments.

6.5 Overview of GPCR Study

The kinetic constants and affinity values of agonist interactions with A2aR were determined with SPR using the titration kinetics method developed by BIAcore to eliminate the need for a regeneration step which might denature A2aR. The determination of kinetic constants for the interaction between A2aR and its agonist will be useful for the screening of high efficacy drugs that can activate A2aR.

The concentration of Na^+ was shown to have an inverse correlation with the binding affinity of the agonists with A2aR. Conversely, the binding of antagonist to A2aR was shown to be unaffected by the concentration of Na^+ .

The A2aR used in this study did not contain any thermo-stabilizing mutations which were a requirement in other SPR studies involving A2aR, minimizing the risk of activity loss and structural changes through mutations.

This study has introduced a new strategy with the use of reconstituted A2aR-nanodisc (for increased A2aR stability) coupled to titration kinetics assays (overcoming the requirement of a regeneration step) for the determination of kinetics for agonist-A2aR interactions. This strategy can be used for the study of other GPCRs and membrane proteins for the determination of their kinetics with their ligands.

7. Conclusions

Studies involving membrane proteins have always proven to be challenging due to difficulties in expression and lack of stability of the target membrane protein. Thermo-stabilizing mutations are often used to increase membrane protein stability but this procedure might potentially change the proteins' structure and function. In this study, new strategies have been introduced to improve the expression and stability of two membrane protein targets: STIM1 and A2aR.

A library of STIM1 constructs was designed and the assays used to study these constructs are as follows:

1. Expression assay: Regions affecting STIM1 expression were identified.
2. Thermo-stability assay: First coiled-coil domain was found to improve the stability of STIM 1.
3. Oligomerization assay: STIM1 was found to be a tetramer when the entire coiled-coil domain is present. A new model is proposed to explain the formation of the STIM1 tetramer.
4. SPR assay: The affinity values of the interaction between STIM1 and Orai1 N- and C- termini were determined.

The first coiled-coil domain of STIM1 was found to bind to the Orai1 N-terminus. A new model is proposed where the binding of the first coiled-coil domain to Orai1 N-terminus will facilitate the interaction of CAD to the Orai1 C-terminus.

The GPCR study used the incorporation of A2aR into nanodiscs as a strategy to improve its stability without the need for any thermo-stabilizing mutations. Titration kinetics, which removes the requirement of a regeneration step, was used to determine the kinetic constants between A2aR and its ligands.

The kinetic constants of agonists (UK 432097, CGS 21860 and NECA) interaction with A2aR were determined which would provide vital information for the screening of high efficacy drugs. By changing the Na⁺ concentration in the SPR assays, it was

observed that there was an inverse correlation between the Na^+ concentration and the binding affinity of the agonist with A2aR.

Overall, this thesis study has established novel strategies for improving the expression and stability of STIM1 and A2aR. The strategies introduced will facilitate the study of interactions between membrane proteins and their binding partners.

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Appendix A. Comprehensive analysis and identification of the human STIM1 domains for structural and functional studies. PLoS one, 2013. 8(1): p. e53979

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Comprehensive Analysis and Identification of the Human STIM1 Domains for Structural and Functional Studies

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Abstract

STIM1 is a Ca^{2+} sensor within the ER membrane known to activate the plasma membrane store-operated Ca^{2+} channel upon depletion of its target ion in the ER lumen. This activation is a crucial step to initiate the Ca^{2+} signaling cascades within various cell types. Human STIM1 is a 77.4 kDa protein consisting of various domains that are involved in Ca^{2+} sensing, oligomerization, and channel activation and deactivation. In this study, we identify the domains and boundaries in which functional and stable recombinant human STIM1 can be produced in large quantities. To achieve this goal, we cloned nearly 200 constructs that vary in their initial and terminal residues, length and presence of the transmembrane domain, and we conducted expression and purification analyses using these constructs. The results revealed that nearly half of the constructs could be expressed and purified with high quality, out of which 25% contained the integral membrane domain. Further analyses using surface plasmon resonance, nuclear magnetic resonance and a thermostability assay verified the functionality and integrity of these constructs. Thus, we have been able to identify the most stable and well-behaved domains of the hSTIM1 protein, which can be used for future *in vitro* biochemical and biophysical studies.

Citation: How J, Zhang A, Phillips M, Reynaud A, Lu SY, et al. (2013) Comprehensive Analysis and Identification of the Human STIM1 Domains for Structural and Functional Studies. PLoS ONE 8(1): e53979. doi:10.1371/journal.pone.0053979

Editor: Karl-Wilhelm Koch, University of Oldenburg, Germany

Received: October 25, 2012; **Accepted:** December 4, 2012; **Published:** January 8, 2013

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Funding: This work was supported by funding from Nanyang Technological University, the National Research Foundation grant NRF-CRP4-2008-02, and the Biomedical Research Council. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

A primary route for calcium ion (Ca^{2+}) entry across the plasma membrane is through store-operated channels that are widespread yet primordial among Ca^{2+} permeable ion channels [1]. The prototypic store-operated channel, as characterized in T-lymphocytes and mast cells, is the Ca^{2+} release-activated Ca^{2+} (CRAC) channel [2]. This channel is activated by the depletion of intracellular Ca^{2+} stores within the endoplasmic reticulum (ER) lumen caused by the release of the second messenger inositol-1,4,5-trisphosphate (IP_3). STIM1 has been identified as the Ca^{2+} sensor and regulator of Ca^{2+} influx and CRAC channel function [2–4] and is an ER membrane protein with a single transmembrane helix. STIM1 consists of an N-terminal region, which contains two EF-hand Ca^{2+} -binding motifs, that is located in the ER lumen; a sterile alpha motif (SAM) known to be involved in protein-protein interactions; one transmembrane domain; and a cytosolic C-terminus containing three coiled-coil regions, a Ser/Pro region and a Lys-rich domain. STIM1 senses the Ca^{2+} concentration inside the ER lumen through its EF-hand motif. Upon depletion of Ca^{2+} from the ER lumen, STIM1 loses its bound Ca^{2+} , oligomerizes and interacts with the plasma membrane Ca^{2+} channel, Orai1, thereby activating the channel [1]. The activation of Orai1 is believed to occur through the interactions of residues 339–448, also known as the channel activation domain (CAD), within the coiled-coil region of the human STIM1 (hSTIM1) with the C-terminus of Orai1 [5–7]. Recently, the crystal structure of the CAD domain was reported,

which was the first evidence that confirmed its coiled-coil nature [8]. The structures of the CAD and the N-terminal region of hSTIM1 [9] are the only high-resolution structures of this protein available to date. In fact, most of the current knowledge concerning the function of hSTIM1 is based on *in vivo* studies. This limited knowledge can be attributed to the nature of hSTIM1; it is a transmembrane protein with a large coiled-coil region (approximately 150 amino acids) and has evolved to oligomerize in order to reach its active state. In an attempt to produce large quantities of different domains of hSTIM1 using both an *Escherichia coli* (*E. coli*) and a *Saccharomyces cerevisiae* (*S. cerevisiae*) expression system, we noticed that many of the constructs were very unstable and either heavily aggregated or degraded. This result would further explain the lack of information available regarding large-scale preparation of hSTIM1 that hampers the detailed biochemical, biophysical and characterization studies of hSTIM1 using *in vitro* systems.

Challenges in the production of recombinant proteins may be correlated to the amino acid composition of the primary sequence. Designing multiple constructs of a single protein often leads to the production of large quantities of stable protein once the region of the amino acid sequence that yields high expression is determined [10]. In this approach, each protein domain is maintained intact and minor changes are made to the length of the construct at either the N and/or C-termini. Here, we present a comprehensive high-throughput study using over 200 hSTIM1 constructs to identify the key domains and boundaries that must be maintained to obtain a high yield of stable and homogeneous hSTIM1. This

multi-construct approach has also revealed constructs that were not expressed properly and therefore should be avoided. Finally, to validate the integrity of high quality constructs, we have performed surface plasmon resonance (SPR), nuclear magnetic resonance (NMR) and thermostability analyses, which demonstrate the functionality of the resulting constructs.

Results and Discussion

Expression of hSTIM1

A total of 211 hSTIM1 constructs were designed based on the key regions of wild-type hSTIM1. Within each key region, a number of highly similar constructs were designed that differ only slightly in their lengths (1–2 amino acids difference) to identify the exact regions that could yield stable and homogeneous protein constructs for further biophysical characterization and crystallization. Table 1 summarizes the results for the screen of the expression of these constructs. A total of 196 constructs were successfully cloned and expressed of which 30 contained both the N-terminal and the transmembrane region. All the constructs that contained the N-terminal region were truncated and began from S58 because this truncation was previously shown to yield the most stable N-terminal constructs [9]. The successfully cloned constructs were further sorted by scoring them from 1–4 based on their observed expression level, with '4' indicating a high expression level, '3' good expression, '2' low expression and '1' very low or no expression. The expression levels were determined empirically through the estimation of the observed protein level by SDS-PAGE followed by Coomassie staining (Fig. 1). Using this scoring system, over 70% of the constructs yielded good or high levels of expression (Table 1). Nevertheless, some of the constructs were found to contain high levels of impurities and possible degradation products (Fig. 1).

Expression analysis of the transmembrane-containing constructs

The expression level of the transmembrane-containing constructs was determined by SDS-PAGE analysis, following small-scale extraction and purification with the detergent Fos-Choline-12 (FC12). This detergent was previously shown to be very efficient for the extraction and subsequent identification of low-expressing membrane proteins [11–13]. Because this strong detergent can solubilize even unfolded membrane proteins, which might result in false positive data [14], the quality of any membrane protein purified with FC12 must be further verified with less harsh detergents. As shown previously, a detergent screen is typically required to identify the optimal extraction and purification condition for the target membrane protein [12]. Such detailed analysis of the transmembrane-containing constructs of hSTIM1 was excluded from this study. Hence, the scoring of such constructs is solely based upon the level of the small-scale FC12-extracted construct with the correct molecular weight, as judged by SDS-PAGE. The relative expression levels of the transmembrane protein constructs are shown in Figure 2. Most of the transmembrane-containing constructs were found to express very well. The main interesting observation from this expression analysis was the decreased expression of the constructs containing the S600-K685 region, which is the Ser/Pro and Lys-rich domain (S600-K685).

Constructs beginning at K246 yield a high level of expression

Two similar sets of hSTIM1 C-terminal constructs, one beginning at Q233 and the other beginning at K246, were

Table 1. Expression scores for the hSTIM1 constructs.

Constructs	Expression Score				
	4	3	2	1	Failed
Transmembrane	23	6	1	0	3
Soluble	70	55	17	28	8
Total	93	61	18	28	11
Percentage	44%	29%	9%	13%	5%

An expression score of '4' indicates high expression, '3' indicates good expression, '2' indicates low expression and '1' indicates very low expression. Overall, majority of soluble hSTIM1 constructs expressed well with 73% having good to high expression.

doi:10.1371/journal.pone.0053979.t001

cloned, and their expression levels were analyzed. Each set consisted of seven constructs that differed in their lengths. Q233 is predicted to be the first amino acid on the cytosolic side, whereas K246 corresponds to the beginning of the first predicted coiled-coil region. The expression and quality studies of these fourteen constructs revealed that all the constructs beginning at K246 yielded a very high level of expression, in contrast to those beginning at Q233 (Fig. 3). We expanded the number of constructs beginning at K246, and the majority showed very high levels of expression (Fig. 4). Thus, constructs beginning at K246 yield high levels of C-terminal hSTIM1 fragments. Currently, it is unclear why the Q233-constructs demonstrated much less expression compared with those beginning at K246. It is possible that the Q233-K246 region makes the protein more flexible or less stable, which leads to higher levels of inclusion bodies or severe degradation of the hSTIM1 constructs. Most of the reported C-terminal constructs have been designed to begin at position Q233, but the expression was performed in eukaryotic cells that typically resulted in fairly low levels of expression [15]. Note that we were able to obtain the expression of all constructs beginning at Q233, but the level of the expression was low in comparison to the K246 constructs. Therefore, it would be interesting to investigate whether C-terminal constructs beginning at K246 would also yield much higher expression levels in eukaryotic cells.

The first coiled-coil domain is required for stable hSTIM1 expression

The large cytosolic domain of hSTIM1 is distinguished by the high content of coiled-coil forming helical regions. Three coiled-coil domains have been predicted within the regions K246-Y342, I364-N388 and S399-L423. The channel activating domain (CAD) [5] and the inhibitory domain are found within this region [5,16,17]. Therefore, this region is of special interest when studying the mechanism of hSTIM1 function. As outlined in Figure 4, nearly all constructs containing the entire coiled-coil region demonstrated a high level of expression. However, there was a clear decrease in the expression level when the constructs were made slightly shorter than the coiled-coil region. To investigate the effect of the absence/presence of each coiled-coil domain on the expression level of hSTIM1 fragments, new constructs were designed beginning from either coiled coil 2 (S340) or coiled coil 3 (N388). The latter had a detrimental effect on the expression level of the constructs (Fig. 5). The constructs beginning at S340 yielded a high level of expression only when an extended region beyond the coiled-coil domains was included. Compared with the constructs beginning at K246, the overall yield of the two sets of coiled-coil constructs was much lower. Complete removal of

Figure 1. Coomassie stained gel images of selected hSTIM1 constructs. The hSTIM1 constructs are scored from a range of 1 to 4: An expression score of '4' indicates high expression of target protein relative to gel background with the appropriate MW; '3' indicates high expression of target protein relative to gel background with the appropriate MW; '2' indicates medium to low expression of target protein relative to gel background with the appropriate MW; '1' indicates either the absence of expression of target protein or an incorrect MW representation. doi:10.1371/journal.pone.0053979.g001

the coiled-coil domains yielded proteins with good expression levels (Fig. 5). Hence, we could conclude that to obtain stable hSTIM1 C-terminal fragments, the presence of an intact coiled-coil region, especially coiled coil 1, is necessary. This conclusion was further strengthened by the increased stability of constructs containing coiled coil 1 compared with those lacking it, as shown by the results of the thermostability shift assay (Table 2).

Identification of high quality hSTIM1 constructs

Next, we identified hSTIM1 constructs that are of high quality and useful for biochemical and biophysical studies. To achieve this goal, we used analytical size exclusion chromatography (ASEC) to ascertain the promising constructs. ASEC has proven to be a highly reliable technique to verify the quality of protein preparations, which is typically scalable [18,19]. Thus, samples that were

originally scored as 3 or 4 were further analyzed by ASEC. Here, the chromatograms were the main factors in determining the protein quality. As shown in Figure 6, high quality samples are referred to as highly homogenous samples judged by both SDS-PAGE and ASEC, whereas low/medium quality samples contain impurities and are heterogeneous. After analyzing the constructs, the high quality constructs were identified and are summarized in Table 3.

SEC at low ionic strength improves the quality of purified hSTIM1 constructs

For constructs lacking coiled coil 1, we noticed that the increased instability and decreased solubility of the protein correlated with increased impurities. Hence, we included ion exchange chromatography (IEX) as a subsequent step to immobilized metal affinity chromatography (IMAC) and size

Figure 2. The relative expression levels of various transmembrane hSTIM1 constructs. Most of the transmembrane constructs expressed very well. The amino acid identity of both the start and end residues are displayed at the end of each color bar. Green indicates an expression score of 4; Yellow indicates an expression score of 3; Red indicates an expression score of 2. Grey indicates an expression score of 1. A graphical representation (not to scale) of wild-type hSTIM1 protein is embedded at the top for easier reference to the key domains with the approximate amino acid numbers listed: EF hand (EF), the sterile- α motif (SAM) domain, transmembrane region (TM), three coiled-coil domains (CC1, CC2 and CC3), serine/proline rich region (S/P) and the lysine rich region (K). doi:10.1371/journal.pone.0053979.g002

Figure 3. Comparison of expression scores for hSTIM1 constructs starting with either Q233 or K246. Same representation as in Fig. 2. Note that constructs starting from K246 have better expression compared to those starting from Q233 when having the same end residue. doi:10.1371/journal.pone.0053979.g003

exclusion chromatography (SEC). When purifying the CAD-containing construct (S340-M490), two peaks were separated by IEX, with the peak that eluted with a higher salt concentration (Peak 2) as the most prominent (Fig. 7A). SDS-PAGE analysis of the protein content of Peak 2 revealed that the hSTIM1 construct was eluted together with a prominent portion of impurities (Fig. 7B). The target protein appeared to elute with a conductivity of approximately 30 mS/cm (equivalent to approximately 300 mM NaCl), i.e., at relatively high ionic strength. Taking into consideration the coiled-coil rich nature of this construct, it is possible that the protein would precipitate in a high ionic condition as a result of promoted protein-protein hydrophobic interactions. In this particular case, the impurities may associate

with the target protein via hydrophobic interactions. To test this hypothesis, the eluate from Peak 2 was subjected to SEC at a low ionic strength (50 mM NaCl). SDS-PAGE analysis of the eluates from this low salt SEC step (Fig. 7C) reveals a clear improvement in the purity of the hSTIM1 construct compared with those that were obtained from IEX. Additionally, the solubility of this construct increased more than twenty fold from 70 μ M to 1.6 mM, which indicates increased stability. The introduction of a similar low salt SEC step into the purification of other hSTIM1 constructs lacking coiled coil 1 resulted in increased purity and solubility as well (data not shown).

The coiled coil 1 domain has been reported to be involved in the autoinhibition mechanism of hSTIM1 activation [16,17].

Figure 4. Comparison between hSTIM1 constructs that have identical starting points with different end regions. Same representation as in Figs. 2 and 3. Note that expression score dropped once there is a truncation of residues within the coiled-coil 2 and coiled-coil 3 regions. doi:10.1371/journal.pone.0053979.g004

Figure 5. Relative expression scores of various soluble hSTIM1 constructs. Same representation as in Figs. 2, 3, 4. Note that lower expression levels were observed for these hSTIM1 constructs that do not contain any coiled-coil regions compared to constructs with the entire coiled-coil domain region.
doi:10.1371/journal.pone.0053979.g005

Through this autoinhibition mechanism, a weak electrostatic interaction between the CAD and the coiled coil 1 domain of hSTIM1 appears to mask the CAD when hSTIM1 is in a resting state. Hence, activation of hSTIM1 would require unmasking the CAD region, which could be achieved by a conformational change in hSTIM1 in response to Ca^{2+} loss. Therefore, constructs lacking coiled coil 1 would contain an exposed CAD, possibly mimicking

the active state of hSTIM1. With the CAD domain exposed, the probability of binding other contaminant proteins could also increase. In contrast, constructs containing the coiled coil 1 domain would mimic hSTIM1 in its resting state, in which coiled coil 1 masks the CAD domain. Hence, the increased stability and purity of constructs containing coiled coil 1 can be attributed to

Table 2. hSTIM1 melting temperature (T_m) comparison between constructs with different start regions and identical end points.

Start residue	End residue	Coiled-coil regions	Melting Temperature (T_m)/°C	T_m Difference*
246	516	1–3	56.7	9.7
340	516	2–3	47.0	
246	514	1–3	54.4	7.2
340	514	2–3	47.2	
246	500	1–3	53.7	7.0
340	500	2–3	46.7	
246	490	1–3	53.4	6.6
340	490	2–3	46.8	

The difference in their melting temperatures is tabulated at the end and is colored green. Constructs were found to have a higher T_m when the first coiled-coil region (246–342) was present.
*between constructs with identical end points.

Figure 6. Analytical Size Exclusion chromatograms and SDS-Page gel electrophoresis photos of selected hSTIM1 constructs. The scoring of the chromatograms is differentiated into High, Medium and Low quality. The chromatograms are plotted as relative absorbance (mAU) against retention volume (mL). Chromatograms of high quality constructs have a dominant peak area of $>80\%$ of the entire peak area. Chromatograms of medium quality constructs quality have a dominant peak area of $>60\%$ but $<80\%$ of the entire peak area. Chromatograms of low quality constructs have multiple peaks present. Their corresponding SDS-Page gel photos are placed next to the chromatograms. doi:10.1371/journal.pone.0053979.g006

Table 3. List of hSTIM1 constructs that have both the highest expression score and quality out of the 211 constructs that have been cloned and expressed.

Number	Start residue	End residue
1	58	520
2	58	557
3	58	580
4	58	600
5	246	345
6	246	360
7	246	490
8	246	500
9	246	520
10	246	556
11	246	561
12	246	567
13	246	571
14	246	575
15	246	580
16	246	587
17	246	595
18	246	600
19	246	632
20	246	650
21	246	660
22	246	667
23	246	685
24	340	490
25	340	520
26	340	556
27	340	571
28	340	575
29	340	580
30	340	685

Expression score was determined based on protein expression in SDS-Page gel electrophoresis analysis. Quality score was determined based on Analytical Size exclusion chromatogram profile.
doi:10.1371/journal.pone.0053979.t003

the masking of CAD by coiled coil 1 that would prevent the interaction between hSTIM1 and impurities.

Monitoring the hSTIM1-Orai1 interaction with surface plasmon resonance (SPR)

To verify the functionality of the high quality samples, we performed SPR studies to monitor the interactions between the hSTIM1 constructs with the Orai1 C-terminus. The Orai1 C-terminus was synthesized with the addition of an extra Cys at the N-terminus to immobilize it on a carboxymethylated dextran matrix via thiol coupling. Such coupling leaves a free C-terminal end that mimics the *in vivo* topology of the Orai1 C-terminus. We monitored the interactions between the immobilized Orai1 C-terminus using three different hSTIM1 constructs: coiled coil 1 only (K246-Q360), the CAD (S340-M490) and K240-D520 in which the entire coiled-coil domain, including the CAD, were

Figure 7. Ion exchange (IEX) chromatogram and SDS-PAGE photos of hSTIM1 S340-M490 purification. **A:** The UV signal at 280 nm (UV₂₈₀ nm) is plotted relative absorbance (mAU) against retention volume (mL) as shown in blue. The corresponding salt concentration is plotted as conductivity (mS/cm) against retention volume (mL) as shown in green. On UV₂₈₀ nm plot, two peaks were separated. Fractions from Peak1 and Peak2 were analyzed by SDS-PAGE as shown in **B**. The most prominent band represents protein of interest. 1, protein marker; 2, injected sample before IEX; 3, empty; 4 and 5, fractions from peak 1; 6–9, fractions from peak 2. Fractions from peak 2 were pooled and subsequently purified by size exclusion chromatography (SEC) with low salt (50 mM NaCl) buffer. SDS-PAGE analysis of the protein content after SEC is shown in **C**. 1, protein marker; 2–9, fractions of elution peak from SEC.
doi:10.1371/journal.pone.0053979.g007

present. These constructs were chosen as their overall electronegativity and hydrophobicity are rather similar. Therefore, under identical experimental conditions (i.e., a constant level of immobilized Orai1 C-terminus in all experiments, an identical concentration of hSTIM1 constructs and an identical buffer condition) any significant differences in the interactions with the Orai1 C-terminus could reflect specific interactions. At this point, SPR was only used to probe the proximity interaction between the Orai1 C-terminus and hSTIM1 constructs. As shown in Figure 8, no interactions between the coiled coil 1 (lacking the CAD) and the Orai1 C-terminus were observed, whereas the CAD-contain-

ing hSTIM1 constructs and the Ora1 C-terminus demonstrated strong responses. Lysozyme was used as a negative control and, similar to the coiled coil 1 (K246-Q360) construct, demonstrated no response when injected over the identical immobilized Ora1 surface. Hence, the observed interactions between the CAD-containing hSTIM1 constructs and the Ora1 C-terminus are specific protein-protein interactions. These results strongly suggest that the hSTIM1 constructs are indeed functional, as the Ora1 C-terminus has been shown to interact only with CAD-containing constructs [20,21].

Characterization of hSTIM1 with nuclear magnetic resonance (NMR)

To characterize the binding of hSTIM1 coiled-coil domains with the peptides derived from the Ora1 C-terminal region, we recorded the ^1H -TROSY spectrum of hSTIM1 (K246-S600) and monitored the chemical shift perturbations of hSTIM1 resonances upon titration with the Ora1-derived peptide. Complete residue specific assignment of the F resonances was not achieved due to significant line broadening on a majority of the resonances. This finding is presumably a result of pervasive conformational exchange. However, resonances in TROSY spectra were still observed and tentatively assigned to the terminal residues of hSTIM1. Upon titration with an Ora1-derived peptide, changes in the position of cross-peaks of hSTIM1 were observed (Fig. 9). The majority of the resonances stemming from the Ora1 peptide were significantly perturbed compared to its free state, indicating the acquisition of secondary structure in the otherwise unstructured peptide. Figure 10 illustrates the spectral perturbations caused by the addition of the peptide, indicating interactions between the CAD-containing hSTIM1 and the Ora1 peptide.

In conclusion, the presented report based on the comprehensive analyses of hSTIM1 regions indicates three key factors for obtaining large quantities of stable hSTIM1 constructs: 1) K246 is the most suitable initial amino acid for soluble cytosolic constructs, 2) the first coiled-coil region is required to obtain stable constructs, and 3) SEC using a buffer of low ionic strength improves the quality of the obtained constructs. In addition, the wealth of various constructs produced and characterized in this study identifies the suitable constructs, which we have shown to be functional by various biophysical assays, for in-depth structural and functional investigations of this protein.

Materials and Methods

Cloning of hSTIM1 constructs

The hSTIM1 constructs were cloned into the expression vector pNIC-28-*Bsa*I by ligation independent cloning (LIC) to introduce an additional DNA encoding for an N-terminal hexa-histidine tag paired together with a TEV protease cleavage site (MHHHHHSSGVDLGTEFLYFQSM), as previously described [22]. The genes were amplified by PCR with an equal mixture of both PhusionTM High-Fidelity DNA Polymerase (Finnzymes) and Platinum[®] Pfx DNA Polymerase (Invitrogen) following the manufacturer's recommendation.

The PCR products were subsequently purified using the PureLink Pro 96 PCR Purification Kit (Invitrogen) and treated with T4 DNA polymerase in the presence of dCTP to generate sticky ends. The pNIC-28-*Bsa*I vector was linearized using the restriction enzyme *Bsa*I and further treated with T4 DNA polymerase in the presence of dGTP to generate sticky ends complementary to the PCR products. This step was followed by an annealing step and transformation into *E. coli* MACH1 cells. Colonies were subsequently grown in Luria-Bertani broth (LB)

agar plates containing 50 $\mu\text{g}/\text{mL}$ kanamycin and screened for correctly sized inserts by PCR using the BIOTAQTM Red DNA Polymerase (Bioline). Plasmids from the positive colonies were transformed into *E. coli* Rosetta cells (DE3). Colonies were then grown on LB agar plates containing 50 $\mu\text{g}/\text{mL}$ kanamycin and 34 $\mu\text{g}/\text{mL}$ chloramphenicol.

Small-scale expression screen

Overnight cultures were diluted 1:100 in 1 mL of Terrific Broth (TB) medium (Formedium, United Kingdom) and grown at 37°C in 96-well deep-well plates by shaking at 220 rpm until the OD₆₀₀ reached 0.8. Protein expression was induced with 0.2 mM of isopropyl- β -D-thiogalactopyranoside (IPTG), and the cultures were further grown overnight at 18°C. The cells were then harvested by centrifugation at 3200 $\times g$ for 5 minutes. The cells pellets were resuspended in lysis buffer (20 mM HEPES, pH 8.0, 150 mM NaCl, 30 mM imidazole) supplemented with a cocktail of protease inhibitors (Roche, Switzerland), 1 mg/mL lysozyme (Sigma-Aldrich, USA) and 10 units/mL Benzonase (Merck, USA). The cells were then lysed by three cycles of freezing at -80°C and thawing at 25°C. The lysate was clarified by centrifugation at 3200 $\times g$ for 15 minutes through a 96-well filter plate (0.65 μm) (Millipore, USA). The filtrate was then transferred to a 96-well filter plate containing Ni-NTA agarose resin (Invitrogen) for purification using IMAC. After 15 min of incubation at 4°C, the unbound material was removed by centrifugation at 100 $\times g$ for 30 s. The wells were then washed with 10 column volumes (CV) of wash buffer (20 mM HEPES, pH 8.0, 150 mM NaCl, 30 mM imidazole) by centrifugation at 100 $\times g$ for 1 min. The bound recombinant proteins were then recovered in 50 μL of elution buffer (20 mM HEPES, pH 8.0, 150 mM NaCl, 300 mM imidazole) by centrifugation at 100 $\times g$ for 1 min. The eluted material was analyzed by SDS-PAGE using NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and stained with SimplyBlue Coomassie stain (Invitrogen). Purified protein constructs were subsequently loaded onto a Superdex 200 5/150 analytical size exclusion column (GE Healthcare) connected to an AKTAmicro chromatography system controlled by Unicorn software version 5.20 (GE Healthcare).

Large-scale protein purification

The overnight cultures were diluted 1:500 in 1 L of TB (Formedium, United Kingdom). The cells were then grown further at 37°C by shaking at 220 rpm until the OD₆₀₀ reached 0.6–0.7. Protein expression was induced by the addition of 0.2 mM IPTG and grown overnight at 18°C. The cell cultures were harvested by centrifugation at 10,000 $\times g$ for 10 min, and the pellets were resuspended in lysis buffer supplemented with a cocktail of protease inhibitors (Roche, Switzerland), 1 mg/mL lysozyme (Sigma-Aldrich, USA) and 10 units/mL Benzonase (Merck, USA). The cells were lysed by three cycles of freezing at -80°C and thawing at 25°C. Unbroken cells and cell debris were removed by centrifugation at 30,000 $\times g$ for 20 minutes. The supernatant was loaded onto 1 mL of Ni-NTA agarose beads (Invitrogen, USA), washed with 10 CV of wash buffer and then eluted with elution buffer. Purified protein constructs were then injected onto either a Superdex 75 or Superdex 200 16/60 size exclusion column (GE Healthcare). Prior to IEX, the samples were desalted using a PD-10 desalting column (GE Healthcare) into an initial buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM TCEP). The desalted samples were subsequently injected into a pre-equilibrated 1 mL HiTrap Q HP column (GE Healthcare, USA). A linear NaCl gradient from 50 mM to 1 M NaCl in an identical buffer was used for the elution of the target protein. All

Figure 8. SPR sensorgrams with their corresponding ASEC and gel images of selected hSTIM1 constructs. A: All hSTIM1 constructs injected have a concentration of 10 μ M. The Orai1 peptide was immobilized at 1000 RU for all runs. Lysozyme is included as negative control. SPR sensorgrams are plotted as response units (RU) against time (seconds). B–D: Analytical Size Exclusion chromatograms and SDS-Page gel electrophoresis of K246-Q360 (B); S340-M490 (C); K246-D520 (D). Chromatograms are plotted as relative absorbance (mAU) against retention volume (mL).
doi:10.1371/journal.pone.0053979.g008

IEX and SEC were performed using an AKTAexplorer FPLC chromatography system controlled by Unicorn software version 5.20 (GE Healthcare), and the fractions were analyzed by SDS-PAGE using NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and stained with SimplyBlue Coomassie stain (Invitrogen).

Production and sequence of the Orai1 C-terminal domain

The Orai1 C-terminal domain was synthesized by Peptide 2.0 (USA) and delivered in a lyophilized state. The sequence of the Orai1 C-terminal peptide is as follows: CRSLVSHKTDTRQFQELNELAEFARLQDQLDHRGDHPLTPGSHYA.

A Cys was added to the N-terminus of the Orai1 peptide to enable immobilization via thiol coupling to a CM5 chip (GE Healthcare). The lyophilized peptides were dissolved in acidic buffer (10 mM sodium acetate, pH 6.0) and injected onto a Superdex Peptide 10/300 size exclusion column (GE Healthcare) connected to an AKTAmicro controlled by Unicorn software

version 5.20 (GE Healthcare). The quality of the fractions was analyzed by SDS-PAGE using NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and stained with SimplyBlue Coomassie stain (Invitrogen). The purified peptides (approximately 100 μ M) were then stored in 100 μ L aliquots at -20°C .

SPR analysis of hSTIM1 constructs

The binding of different hSTIM1 constructs to Orai1 peptides (synthesized by Peptide 2.0) was assessed on a Biacore T200 (GE Healthcare). Orai1 peptide was immobilized on a CM5 sensor chip (GE Healthcare) by ligand thiol coupling. The surface was activated using 20 μ L of a 1:1 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) with a running flow rate of 10 μ L/min. Reactive disulfide groups were introduced to the surface with an injection of 80 mM 2-(2-pyridinyldithio)ethanamine (PDEA) in 0.1 M sodium borate, pH 8.5, at a running flow rate of 10 μ L/min for 4 min. The Orai1 peptide (approximately 100 μ M) was

Figure 9. Superimposed ^{15}N , ^1H -TROSY spectra of free hSTIM1 and hSTIM1 bound to Orai1 C-terminal derived peptide. The free hSTIM1 is colored blue and the Orai C-terminal peptide is red. The spectra were collected at 298 K in 20 mM TRIS, 300 mM NaCl, 0.1 mM TCEP at pH 8.0.
doi:10.1371/journal.pone.0053979.g009

Figure 10. Superimposed ^{15}N , ^1H -HSQC spectra of Orai1 derived C terminal peptide. ^{15}N , ^1H -HSQC of free peptide (colored blue) contains specifically ^{15}N labeled Leu. ^{15}N , ^1H -HSQC of peptide with unlabeled hSTIM1 K246-S600 is colored red. The spectra were collected at 298 K in 20 mM HEPES, 300 mM NaCl, 0.1 mM TCEP at pH 8.0.
doi:10.1371/journal.pone.0053979.g010

injected at a flow rate of 10 $\mu\text{L}/\text{min}$ until an immobilization of 1,000 RU was obtained. Twenty microliters of 50 mM cysteine and 1 M NaCl in 0.1 M sodium acetate buffer, pH 4.3, at a running flow rate of 5 $\mu\text{L}/\text{min}$ was subsequently injected for 4 min to deactivate any remaining reactive groups. Blank immobilization was performed on the first flow cell, which was used as a reference flow cell for subsequent analysis. Various hSTIM1 constructs at concentrations of 10 μM were injected over the sensor surfaces with HES-EP buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Polysorbate 20) at a flow rate of 30 $\mu\text{L}/\text{min}$ for 1 min, allowing 4 min for the dissociation before regeneration. The interactions between the ligand and the analyte were measured over a 6-min time period allowing for a dissociation time of four minutes. Between injection cycles, 10 mM glycine-HCl pH 3.5 was used to regenerate the surface with a contact time of 30 sec. Lysozyme (approximately 10 μM) was injected as a negative control. SPR data were analyzed using Scrubber software (v2.0c; Biologic, Australia).

Protein Expression and Purification for NMR studies

Constructs of hSTIM1 containing the domains coiled coil 1, 2 and 3 with an extension (K246-S600) were used for this study. ^{13}C , ^{15}N -isotope labeled hSTIM1 protein was obtained by growing *E. coli* cells in M9 minimal media containing isotope labeled glucose and NH_4Cl (Cambridge Isotope Laboratories). Induction was performed using 0.5 mM IPTG for approximately 18 hours at 18°C and purified using identical chromatographic procedures as described previously. The Orai1 C-terminus derived peptide (China Peptides) contained an identical sequence used previously except for the presence of six ^{15}N -labeled Leu.

NMR Sample Preparation

The sample used for the NMR study consisted of 0.3 mM ^{15}N -labeled hSTIM1 in 90% $\text{H}_2\text{O}/10\%$ D_2O . All samples used to record the two-dimensional TROSY were prepared in 20 mM Tris-HCl and 300 mM NaCl adjusted to pH 8.0. Identical buffer conditions were used for the binding studies of hSTIM1 with the Orai1 C-terminus-derived peptide. The peptide was prepared as a 50 mM stock solution in an identical buffer as that for the hSTIM1 protein. The addition of the Orai1 C-terminus-derived peptide to the peptide-free hSTIM1 sample was performed stepwise to achieve a final concentration of Orai1 peptide: hSTIM1 protein with a ratio of 1:1. The hSTIM1 sample without any Orai1 peptide was used as the reference for observing any chemical shifts. All NMR experiments were conducted at 298 K.

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NMR Spectroscopy

All NMR experiments were performed on a Bruker AVANCE II 600 MHz and 700 MHz NMR Spectrometer equipped with four RF channels and a 5 mm Z-gradient TCI CryoProbe. All NMR experiments were performed at 298 K, and sweep widths for ^1H and ^{15}N were 9804 and 2412 Hz, respectively. Two-dimensional ^{15}N , ^1H -TROSY was performed for each sample and binding experiments. Data were processed using Topspin 2.1, and the chemical shifts were referenced directly (^1H) to the frequency of DSS. Peak picking and spectral analysis was performed using CARRA.

Thermal shift assay

The thermostability of the hSTIM1 constructs was studied using differential static light scattering as described by Hong et al. [23] using a multiwell instrument (Stargazer, Harbinger Biotech, Toronto, Canada). Protein samples with concentrations of 30 μM in 50 μL of buffer (20 mM HEPES, pH 7.5, 300 mM NaCl) were heated from 25–30°C at a rate of 1°C/min. The aggregation of protein was determined through the recording of scattered light using a CCD camera and images of the plate were taken at every 0.5°C interval. Using proprietary software, pixel intensities of selected wells were integrated, thereby obtaining an approximate value that is representative of the total amount of scattered light in that particular region. The total intensities were then plotted against temperature and fitted to the Boltzmann equation by nonlinear regression. The temperature it takes for 50% of the studied protein to achieve the aggregation state was defined as the melting temperature, T_m .

Acknowledgments

The authors would like to thank Newsha Sahaf and Martina Nilsson for their technical assistance in this work, and Greg Taylor for his help with preparing the final draft of the manuscript.

Author Contributions

Conceived and designed the experiments: JH AZ MP KP SGS SE. Performed the experiments: JH AZ MP AR SYL SE. Analyzed the data: JH AZ MP LXP HTH YHY AG KP SGS SE. Contributed reagents/materials/analysis tools: LXP HTH. Wrote the paper: JH AZ MP KP SGS SE.

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Appendix B. Expression scores of STIM1 constructs

S/N	Group	Starting amino acid	Ending amino acid	Expression	His-tag terminus	Purified protein yield [mg/L]	Molecular weight [kDa]	Extinction coefficient [mL/(mg*cm)]	Peak area [mAU × mL]	Total area of all peaks [mAU × mL]	Percentage of peak area against total area [peak area/total area]
1	6	58	193	Low	N	0.03	18.65	29450	0.24	0.74	32.74
2	6	58	198	Low	N	0.08	19.15	29450	0.37	0.79	47.38
3	6	58	201	Low	N	0.08	19.47	29450	0.71	1.42	50.32
4	6	58	206	Medium	N	0.68	19.99	29450	0.80	1.24	64.01
5	6	58	209	High	N	1.01	20.40	29450	1.65	2.02	81.86
6	6	58	360	High	N	1.29	38.46	51910	17.27	17.99	95.99
7	6	58	395	Low	N	0.02	42.58	54890	1.36	2.29	59.45
8	6	58	450	High	N	1.54	48.67	60390	6.68	8.17	81.77
9	6	58	452	High	N	1.86	48.90	60390	6.66	7.62	87.43
10	6	58	454	High	N	1.31	49.08	60390	3.30	3.86	85.31
11	6	58	456	High	N	1.74	49.31	60390	10.02	10.79	92.79
12	6	58	458	Failed	N	0.00	49.52	60390	0.00	0.00	0.00
13	6	58	459	High	N	1.41	49.61	60390	7.74	8.46	91.51
14	6	58	462	High	N	1.37	49.98	65890	6.00	6.69	89.66
15	6	58	464	High	N	1.43	50.17	65890	12.73	13.49	94.39
16	6	58	466	High	N	1.99	50.42	65890	6.79	7.50	90.60
17	6	58	468	High	N	1.74	50.63	65890	12.21	13.02	93.78
18	6	58	470	High	N	1.15	50.84	65890	6.77	7.39	91.61
19	6	58	472	High	N	1.13	51.10	65890	9.93	10.71	92.73

S/N	Group	Starting amino acid	Ending amino acid	Expression	His-tag terminus	Purified protein yield [mg/L]	Molecular weight [kDa]	Extinction coefficient [mL/(mg*cm)]	Peak area [mAU × mL]	Total area of all peaks [mAU × mL]	Percentage of peak area against total area [peak area/total area]
20	6	58	474	High	N	1.27	51.27	65890	4.28	4.84	88.52
21	6	58	474	High	C	1.62	49.68	64400	5.83	6.53	89.24
22	6	58	500	Medium	N	0.56	54.20	65890	2.46	3.20	76.94
23	6	58	520	High	N	1.85	56.46	65890	3.84	4.45	86.23
24	6	58	520	High	C	1.65	54.80	64400	8.25	9.12	90.42
25	6	58	530	Medium	N	0.34	57.59	65890	2.04	2.61	78.28
26	6	58	557	High	C	1.48	58.77	64400	5.46	6.23	87.50
27	6	58	570	Medium	N	0.63	61.82	65890	2.06	2.62	78.65
28	6	58	580	High	C	1.76	61.18	64400	4.13	4.95	83.48
29	6	58	600	High	C	1.26	63.33	64400	5.06	5.82	86.95
30	6	58	629	Failed	N	0.00	67.82	65890	0.00	0.00	0.00
31	6	58	629	High	N	1.47	67.82	65890	2.69	3.16	85.10
32	6	58	670	Medium	N	0.35	72.02	65890	1.43	2.16	66.29
33	6	58	673	High	N	1.23	72.43	65890	2.26	2.64	85.70
34	6	58	677	Failed	N	0.00	0.00	65890	0.00	0.00	0.00
35	6	58	680	Medium	N	0.58	73.31	65890	1.26	1.84	68.60
36	6	58	684	High	N	1.42	73.77	65890	5.60	6.21	90.25
37	6	58	685	High	N	1.35	73.84	65890	5.01	5.42	92.38
38	6	58	685	Medium	C	0.81	72.24	64400	3.81	4.79	79.60
39	1	233	345	Medium	N	0.45	16.41	11460	1.74	2.23	78.19
40	1	233	349	Medium	N	0.57	16.85	11460	1.66	2.14	77.67

S/N	Group	Starting amino acid	Ending amino acid	Expression	His-tag terminus	Purified protein yield [mg/L]	Molecular weight [kDa]	Extinction coefficient [mL/(mg*cm)]	Peak area [mAU × mL]	Total area of all peaks [mAU × mL]	Percentage of peak area against total area [peak area/ total area]
41	1	233	356	Medium	N	0.68	17.76	16960	2.20	2.95	74.67
42	1	233	360	Low	N	0.09	18.22	16960	0.86	1.48	58.24
43	1	233	365	Failed	N	0.00	18.90	19940	0.00	0.00	0.00
44	1	233	390	Low	N	0.05	21.75	19940	0.51	1.01	50.72
45	1	233	395	Low	N	0.03	22.34	19940	0.28	0.74	38.03
46	1	233	399	Low	N	0.03	22.73	19940	0.33	0.82	40.24
47	1	233	425	Failed	N	0.00	25.52	19940	0.00	0.00	0.00
48	1	233	425	Low	N	0.00	25.52	19940	0.44	0.84	51.93
49	1	233	429	Low	N	0.05	26.09	19940	0.31	0.80	38.57
50	1	233	429	Low	N	0.03	26.09	19940	0.42	1.06	39.70
51	1	233	448	Low	N	0.05	28.28	25440	0.22	0.50	44.40
52	1	233	463	Low	N	0.06	29.83	30940	0.32	0.90	35.99
53	1	233	474	Low	N	0.04	31.09	30940	0.13	0.37	36.19
54	1	233	474	Failed	N	0.00	31.09	30940	0.00	0.00	0.00
55	1	233	474	Low	C	0.00	29.43	29450	0.86	2.11	40.63
56	1	233	485	Low	N	0.09	32.36	30940	0.62	1.10	56.33
57	1	233	490	Low	N	0.05	32.88	30940	0.43	0.83	51.57
58	1	233	498	Low	N	0.07	33.69	30940	0.39	0.92	41.79
59	1	233	520	Low	C	0.06	34.55	29450	1.51	2.71	55.54
60	1	233	557	Medium	C	0.79	38.53	29450	2.69	4.33	62.01
61	1	233	580	Low	C	0.09	40.93	29450	1.91	3.39	56.17

S/N	Group	Starting amino acid	Ending amino acid	Expression	His-tag terminus	Purified protein yield [mg/L]	Molecular weight [kDa]	Extinction coefficient [mL/(mg*cm)]	Peak area [mAU × mL]	Total area of all peaks [mAU × mL]	Percentage of peak area against total area [peak area/total area]
62	1	233	600	Medium	C	0.36	43.08	29450	1.13	1.87	60.23
63	1	233	629	Failed	N	0.00	47.57	30940	0.00	0.00	0.00
64	1	233	685	Failed	N	0.00	53.65	30940	0.00	0.00	0.00
65	1	233	685	Low	N	0.05	53.65	30940	0.33	0.95	34.77
66	1	233	685	Low	C	0.00	52.00	29450	0.97	1.94	49.98
67	1	234	557	Medium	N	0.46	40.06	30940	1.20	1.87	63.98
68	1	235	514	Medium	N	0.77	35.30	30940	1.15	1.64	69.83
69	1	235	557	Medium	N	0.37	39.94	30940	1.16	1.72	67.65
70	1	237	514	Medium	N	0.24	34.98	29450	3.49	4.64	75.26
71	1	237	557	Medium	N	0.45	39.62	29450	1.73	2.25	76.92
72	1	238	557	Medium	N	0.44	39.54	29450	1.27	1.68	75.66
73	1	239	514	Medium	N	0.68	34.76	29450	1.63	2.29	71.16
74	1	239	557	Medium	N	0.52	39.41	29450	1.09	1.61	67.68
75	1	240	514	Medium	N	0.47	34.63	29450	1.59	2.43	65.43
76	1	240	557	Medium	N	0.76	39.28	29450	0.64	0.94	67.36
77	1	241	450	Low	N	0.01	27.44	23950	0.15	0.40	38.36
78	1	242	514	Medium	N	0.77	34.36	29450	1.75	2.41	72.81
79	1	242	557	Medium	N	0.58	39.01	29450	1.62	2.21	73.41
80	1	244	490	Medium	N	0.44	31.45	29450	2.61	3.45	75.73
81	1	245	514	High	N	1.59	33.98	29450	9.94	11.91	83.47
82	1	245	557	Medium	N	0.79	38.62	29450	5.75	7.57	75.90

S/N	Group	Starting amino acid	Ending amino acid	Expression	His-tag terminus	Purified protein yield [mg/L]	Molecular weight [kDa]	Extinction coefficient [mL/(mg*cm)]	Peak area [mAU × mL]	Total area of all peaks [mAU × mL]	Percentage of peak area against total area [peak area/ total area]
83	2	246	345	High	N	1.96	14.72	9970	11.32	12.03	94.10
84	2	246	349	High	N	1.93	15.16	9970	11.60	12.13	95.63
85	2	246	356	High	N	1.56	16.07	15470	11.83	12.29	96.21
86	2	246	360	High	N	1.68	16.52	15470	11.96	12.50	95.68
87	2	246	365	Medium	N	0.63	17.21	18450	0.85	1.29	66.10
88	2	246	370	High	N	1.59	17.78	18450	8.55	9.45	90.48
89	2	246	375	Low	N	0.07	19.18	18450	4.31	8.29	51.96
90	2	246	383	High	N	1.21	18.36	18450	4.24	4.74	89.40
91	2	246	390	High	N	1.85	20.05	18450	7.67	9.44	81.27
92	2	246	395	High	N	1.74	20.64	18450	11.32	12.39	91.34
93	2	246	399	Medium	N	0.57	21.04	18450	3.77	5.01	75.28
94	2	246	414	Medium	N	0.61	22.69	18450	3.97	5.12	77.51
95	2	246	425	Medium	N	0.57	23.83	18450	1.91	2.59	73.65
96	2	246	429	High	N	1.34	24.39	18450	2.53	3.04	83.39
97	2	246	440	Low	N	1.43	25.74	23950	3.45	6.46	53.45
98	2	246	455	High	N	1.59	27.25	23950	5.24	6.03	86.98
99	2	246	463	High	N	1.34	28.13	29450	13.69	14.65	93.41
100	2	246	474	High	C	1.59	27.74	27960	1.82	2.43	74.98
101	2	246	480	High	N	1.38	30.09	29450	12.33	13.06	94.37
102	2	246	490	High	N	1.56	31.19	29450	28.71	29.56	97.13
103	2	246	500	High	N	1.67	32.26	29450	11.03	11.87	92.91

S/N	Group	Starting amino acid	Ending amino acid	Expression	His-tag terminus	Purified protein yield [mg/L]	Molecular weight [kDa]	Extinction coefficient [mL/(mg*cm)]	Peak area [mAU × mL]	Total area of all peaks [mAU × mL]	Percentage of peak area against total area [peak area/ total area]
104	2	246	514	High	N	1.75	33.85	29450	8.77	10.10	86.83
105	2	246	516	High	N	1.59	34.07	29450	12.45	13.73	90.63
106	2	246	518	High	N	1.73	34.31	29450	12.50	13.42	93.18
107	2	246	520	High	N	1.79	34.51	29450	12.82	14.28	89.79
108	2	246	520	High	C	1.12	32.86	27960	3.29	4.09	80.46
109	2	246	554	High	N	1.13	38.21	29450	10.41	11.85	87.87
110	2	246	555	High	N	1.80	38.27	29450	9.01	10.63	84.74
111	2	246	556	High	N	1.77	38.35	29450	11.02	12.63	87.27
112	2	246	557	High	N	1.93	38.49	29450	10.98	12.57	87.41
113	2	246	557	High	C	1.16	36.83	27960	9.36	10.33	90.64
114	2	246	561	High	N	1.52	39.00	29450	16.62	19.63	84.69
115	2	246	567	High	N	1.45	39.54	29450	15.54	17.87	86.98
116	2	246	571	High	N	1.04	40.01	29450	18.26	20.58	88.73
117	2	246	575	High	N	1.25	40.42	29450	20.28	23.09	87.80
118	2	246	580	High	N	1.49	40.90	29450	10.60	12.72	83.30
119	2	246	580	Medium	C	0.54	39.24	27960	2.40	3.15	76.43
120	2	246	587	High	N	1.54	41.62	29450	9.01	10.94	82.30
121	2	246	591	High	N	1.43	42.05	29450	11.00	13.24	83.12
122	2	246	592	High	N	0.57	42.17	29450	13.02	15.57	83.67
123	2	246	593	High	N	1.60	42.25	29450	10.56	12.95	81.55
124	2	246	594	High	N	0.68	42.38	29450	11.44	14.05	81.37

S/N	Group	Starting amino acid	Ending amino acid	Expression	His-tag terminus	Purified protein yield [mg/L]	Molecular weight [kDa]	Extinction coefficient [mL/(mg*cm)]	Peak area [mAU × mL]	Total area of all peaks [mAU × mL]	Percentage of peak area against total area [peak area/ total area]
125	2	246	595	High	N	1.01	42.47	29450	12.69	14.72	86.24
126	2	246	596	Medium	N	0.74	42.58	29450	5.60	7.51	74.63
127	2	246	597	Medium	N	0.61	42.71	29450	6.84	9.41	72.63
128	2	246	598	Medium	N	0.54	42.84	29450	10.93	14.12	77.41
129	2	246	599	Low	N	0.00	42.94	29450	1.54	3.06	50.26
130	2	246	600	High	N	1.14	43.04	29450	9.53	10.74	88.72
131	2	246	600	High	C	1.65	41.39	27960	2.50	3.03	82.37
132	2	246	612	Failed	N	0.00	44.16	29450	0.00	0.00	0.00
133	2	246	632	High	N	1.23	46.15	29450	7.46	8.83	84.46
134	2	246	639	Medium	N	0.60	46.86	29450	8.08	10.33	78.20
135	2	246	650	High	N	1.05	48.11	29450	10.87	11.83	91.91
136	2	246	660	High	N	1.01	49.11	29450	9.68	11.53	83.97
137	2	246	667	High	N	1.98	49.84	29450	9.09	10.55	86.19
138	2	246	685	High	N	1.43	51.96	29450	6.10	7.44	82.00
139	2	246	685	Failed	N	0.00	48.36	94990	0.00	0.00	0.00
140	3	336	485	High	N	1.65	19.83	27960	3.18	3.93	81.05
141	3	340	425	Low	N	0.05	12.55	16960	0.30	0.81	36.95
142	3	340	450	Low	N	0.09	15.50	22460	0.15	0.38	38.72
143	3	340	474	Low	N	0.01	18.11	27960	0.14	0.33	43.22
144	3	340	474	Low	N	0.07	18.11	27960	0.27	0.86	31.33
145	3	340	474	Low	C	0.04	16.45	26470	0.32	0.77	41.81

S/N	Group	Starting amino acid	Ending amino acid	Expression	His-tag terminus	Purified protein yield [mg/L]	Molecular weight [kDa]	Extinction coefficient [mL/(mg*cm)]	Peak area [mAU × mL]	Total area of all peaks [mAU × mL]	Percentage of peak area against total area [peak area/total area]
146	3	340	490	Medium	N	0.75	19.90	27960	4.85	6.29	77.04
147	3	340	500	High	N	1.24	20.97	27960	3.97	4.87	81.49
148	3	340	508	Medium	N	0.54	21.96	27960	4.84	6.23	77.77
149	3	340	510	High	N	1.96	22.13	27960	7.89	9.54	82.69
150	3	340	512	High	N	1.96	22.28	27960	8.37	9.89	84.67
151	3	340	514	High	N	1.65	22.56	27960	10.05	11.66	86.18
152	3	340	516	High	N	1.64	22.79	27960	14.51	16.39	88.54
153	3	340	518	High	N	1.90	23.03	27960	8.44	9.97	84.69
154	3	340	520	High	N	1.00	23.23	27960	10.39	12.15	85.49
155	3	340	520	High	C	1.03	21.57	26470	11.31	11.88	95.22
156	3	340	530	Low	N	0.05	24.36	27960	0.31	0.91	34.48
157	3	340	550	High	N	1.83	26.49	27960	9.62	11.37	84.56
158	3	340	556	High	N	1.07	27.07	27960	5.82	6.69	86.98
159	3	340	557	High	C	1.11	25.55	26470	7.09	8.30	85.38
160	3	340	558	High	N	1.83	27.36	27960	12.40	13.86	89.48
161	3	340	560	High	N	1.72	27.59	27960	10.96	12.52	87.55
162	3	340	562	High	N	1.12	27.78	27960	6.99	8.58	81.43
163	3	340	564	Medium	N	0.81	28.01	27960	1.56	2.08	74.70
164	3	340	566	Medium	N	0.77	28.17	27960	6.76	8.65	78.13
165	3	340	568	Medium	N	0.44	28.37	27960	5.74	7.20	79.68

S/N	Group	Starting amino acid	Ending amino acid	Expression	His-tag terminus	Purified protein yield [mg/L]	Molecular weight [kDa]	Extinction coefficient [mL/(mg*cm)]	Peak area [mAU × mL]	Total area of all peaks [mAU × mL]	Percentage of peak area against total area [peak area/total area]
166	3	340	570	Low	C	0.11	28.60	27960	0.26	0.63	40.68
167	3	340	570	High	N	1.11	28.60	27960	7.35	8.40	87.54
168	3	340	571	High	N	1.14	28.72	27960	12.79	14.16	90.37
169	3	340	572	High	N	1.64	28.84	27960	13.91	15.27	91.13
170	3	340	573	High	N	1.37	28.93	27960	5.73	6.65	86.10
171	3	340	574	High	N	1.96	29.05	27960	12.65	13.97	90.51
172	3	340	575	High	N	1.94	29.14	27960	14.69	16.21	90.67
173	3	340	576	High	N	1.72	29.23	27960	12.85	14.17	90.65
174	3	340	577	High	N	1.92	29.30	27960	9.07	10.18	89.06
175	3	340	578	High	N	1.46	29.42	27960	8.79	9.91	88.70
176	3	340	579	High	N	1.77	29.49	27960	2.70	3.35	80.62
177	3	340	580	High	N	1.83	29.62	27960	5.45	6.39	85.19
178	3	340	580	High	C	1.23	27.96	26470	11.73	12.29	95.43
179	3	340	600	Low	C	0.04	31.76	27960	0.25	0.45	55.01
180	3	340	600	High	N	1.46	30.10	26470	12.40	14.20	87.33
181	3	340	629	Medium	N	0.68	34.59	27960	0.92	1.45	63.48
182	3	340	649	Low	N	0.07	36.69	27960	0.13	0.37	34.69
183	3	340	670	Low	N	0.09	38.80	27960	0.35	0.80	43.27
184	3	340	685	Low	N	1.84	40.68	27960	0.54	1.51	35.62
185	3	340	685	High	C	0.04	39.02	26470	5.22	6.14	84.94
186	3	340	685	Low	N	0.10	40.74	27960	0.46	0.81	57.12
187	4	388	480	Low	N	0.05	13.06	12490	0.85	1.44	59.12

S/N	Group	Starting amino acid	Ending amino acid	Expression	His-tag terminus	Purified protein yield [mg/L]	Molecular weight [kDa]	Extinction coefficient [mL/(mg*cm)]	Peak area [mAU × mL]	Total area of all peaks [mAU × mL]	Percentage of peak area against total area [peak area/ total area]
188	4	388	490	Low	N	0.09	14.16	12490	0.68	1.51	45.02
189	4	388	500	Low	N	0.08	15.23	12490	0.92	1.59	57.86
190	4	388	520	Low	N	0.05	17.49	12490	1.04	1.78	58.30
191	4	388	580	Low	N	0.03	23.88	12490	1.04	2.21	46.87
192	4	388	650	Medium	N	0.32	31.08	12490	2.23	3.19	69.94
193	4	388	685	Medium	N	0.35	34.94	12490	0.86	1.40	61.77
194	5	425	650	Medium	N	0.31	27.11	12490	0.49	0.80	61.08
195	5	431	560	Medium	N	0.34	17.00	6990	2.53	3.62	69.86
196	5	431	580	Medium	N	0.23	19.02	6990	1.03	1.62	63.53
197	5	431	650	Low	N	0.06	26.23	6990	1.13	2.16	52.51
198	5	431	685	Low	N	0.02	30.08	6990	0.76	1.29	58.76
199	5	432	650	Medium	N	0.23	26.10	6990	1.94	2.57	75.58
200	5	440	650	Low	N	0.02	25.20	6990	0.72	1.64	43.72
201	5	448	650	Low	N	0.02	24.36	6990	0.76	1.82	41.65
202	5	455	650	Medium	N	0.24	23.67	6990	1.05	1.51	69.80
203	5	461	560	Low	N	0.09	13.72	1490	1.12	2.03	55.16
204	5	461	580	Medium	N	0.25	15.75	1490	0.86	1.34	64.19
205	5	461	650	Medium	N	0.17	22.96	1490	0.96	1.37	70.04
206	5	461	655	Failed	N	0.00	23.45	1490	0.00	0.00	0.00
207	5	461	658	Low	N	0.08	23.81	1490	0.65	1.35	48.19
208	5	461	665	Medium	N	0.15	24.49	1490	2.14	2.78	77.02
209	5	461	667	Low	N	0.06	24.69	1490	0.91	1.83	49.47

S/N	Group	Starting amino acid	Ending amino acid	Expression	His-tag terminus	Purified protein yield [mg/L]	Molecular weight [kDa]	Extinction coefficient [mL/(mg*cm)]	Peak area [mAU × mL]	Total area of all peaks [mAU × mL]	Percentage of peak area against total area [peak area/total area]
210	5	461	670	Medium	N	0.16	24.93	1490	1.84	2.46	74.65
211	5	461	685	Medium	N	0.88	26.81	1490	11.84	15.23	77.75

Appendix C. SPR-normalized response scores of soluble STIM1 constructs

S/N	Starting amino acid	Ending amino acid	Normalized response with the Orai1 C-terminus peptide	Normalized response with the Orai1 N-terminus peptide	His-tag terminus
1	58	193	0	0	N
2	58	198	0	0	N
3	58	201	0	0	N
4	58	206	0	0	N
5	58	209	0	0	N
6	233	345	0	0	N
7	233	349	0	0	N
8	233	356	0	0	N
9	233	360	0	0	N
10	233	365	0	0	N
11	233	390	0	0	N
12	233	395	0	0	N
13	233	399	0	0	N
14	233	425	0	0	N
15	233	425	0	0	N
16	233	429	0	0	N
17	233	429	0	0	N
18	233	448	0	0	N
19	233	463	0	0	N
20	233	474	0	0	N
21	233	474	0	0	N
22	233	474	0	0	C
23	233	485	0	0	N
24	233	490	0	0	N
25	233	498	0	0	N
26	233	520	0	0	C
27	233	557	0	0	C
28	233	580	0	0	C
29	233	600	0	0	C
30	233	629	0	0	N
31	233	685	0	0	N
32	233	685	0	0	C
33	233	685	0	0	N
34	234	557	0	0	N

S/N	Starting amino acid	Ending amino acid	Normalized response with the Orai1 C-terminus peptide	Normalized response with the Orai1 N-terminus peptide	His-tag terminus
35	235	514	0	0	N
36	235	557	0	0	N
37	237	514	0	0	N
38	237	557	0	0	N
39	238	557	0	0	N
40	239	514	0	0	N
41	239	557	0	0	N
42	240	514	0	0	N
43	240	557	0	0	N
44	241	450	0	0	N
45	242	514	0	0	N
46	242	557	0	0	N
47	244	490	0	0	N
48	245	514	0	0	N
49	245	557	0	0	N
50	246	345	0	105.33	N
51	246	349	0	109.78	N
52	246	356	0	111.63	N
53	246	360	0	152.36	N
54	246	365	0	136.84	N
55	246	370	0	110.25	N
56	246	375	0	146.23	N
57	246	383	0	122.47	N
58	246	390	0	108.71	N
59	246	395	0	157.6	N
60	246	399	0	133.6	N
61	246	414	0	118.84	N
62	246	425	0	135.54	N
63	246	429	0	164	N
64	246	440	0	91.2	N
65	246	455	4.35	95.51	N
66	246	463	5.92	93.96	N
67	246	474	17.18	98.65	C
68	246	480	13.82	95.38	N
69	246	490	7.31	60.21	N
70	246	500	7.07	63.11	N
71	246	514	15.28	98.77	N

S/N	Starting amino acid	Ending amino acid	Normalized response with the Orai1 C-terminus peptide	Normalized response with the Orai1 N-terminus peptide	His-tag terminus
72	246	516	7.69	58.81	N
73	246	518	12.17	96.87	N
74	246	520	7.9	77.88	N
75	246	520	12.75	77.34	C
76	246	554	9.56	77.74	N
77	246	555	10.11	74.1	N
78	246	556	6.38	52.18	N
79	246	557	10.75	69.87	N
80	246	557	9.63	64.2	C
81	246	561	10.15	70.19	N
82	246	567	13.63	92.19	N
83	246	571	8.13	56.97	N
84	246	575	7.4	54.4	N
85	246	580	13.84	91.93	N
86	246	580	7.32	55.66	C
87	246	587	17.84	97.78	N
88	246	591	7.22	55.82	N
89	246	592	7.97	55.77	N
90	246	593	9.81	72.46	N
91	246	594	16.33	95.85	N
92	246	595	8.03	54.17	N
93	246	596	9.61	65.89	N
94	246	597	11.65	77.67	N
95	246	598	10.56	69.14	N
96	246	599	3.9	40.33	N
97	246	600	7.4	53.02	C
98	246	600	2.77	27.92	N
99	246	612	5.74	40.64	N
100	246	632	8.53	63.02	N
101	246	639	7.99	55.66	N
102	246	650	8.38	62.03	N
103	246	660	5.35	41.81	N
104	246	667	9.43	65.69	N
105	246	685	4.71	39.96	N
106	246	685	4.57	44.73	N
107	336	485	5.59	92.76	N
108	340	425	0	150.3	N

S/N	Starting amino acid	Ending amino acid	Normalized response with the Orai1 C-terminus peptide	Normalized response with the Orai1 N-terminus peptide	His-tag terminus
109	340	450	0	140.74	N
110	340	474	14.8	151.69	N
111	340	474	17.96	130.62	C
112	340	474	16.73	67.46	N
113	340	490	26.14	151.88	N
114	340	500	16.33	93.16	N
115	340	508	22.11	132.56	N
116	340	510	30.19	155.35	N
117	340	512	22.66	116.29	N
118	340	514	30.9	164.18	N
119	340	516	25.4	124.34	N
120	340	518	25.38	157.99	N
121	340	520	35.9	152.41	N
122	340	520	20.63	104.66	C
123	340	530	36.44	162.44	N
124	340	550	25.26	117.46	N
125	340	556	18.39	81.78	N
126	340	557	12.06	82.9	C
127	340	558	26.26	137.5	N
128	340	560	35.38	154.54	N
129	340	562	25.89	149.95	N
130	340	564	19.09	94.99	N
131	340	566	15.44	100.33	N
132	340	568	29.33	132.27	N
133	340	570	10.47	78.6	N
134	340	570	7.73	50.05	N
135	340	571	18.17	102.85	N
136	340	572	28.26	124.44	N
137	340	573	12.46	81.71	N
138	340	574	26.14	127.5	N
139	340	575	19.04	98.71	N
140	340	576	12.35	87.9	N
141	340	577	25.02	118.62	N
142	340	578	26.47	130.61	N
143	340	579	24.53	119.22	N
144	340	580	16.57	100.42	N
145	340	580	12.59	74.97	C

S/N	Starting amino acid	Ending amino acid	Normalized response with the Orai1 C-terminus peptide	Normalized response with the Orai1 N-terminus peptide	His-tag terminus
146	340	600	5.61	72.66	C
147	340	600	10.81	34.87	N
148	340	629	15.57	75.3	N
149	340	649	7.49	56.87	N
150	340	670	25.04	109.46	N
151	340	685	28.34	98.93	N
152	340	685	10.3	54.76	C
153	340	685	11.7	28.33	N
154	388	480	0	0	N
155	388	490	0	0	N
156	388	500	0	0	N
157	388	520	0	0	N
158	388	580	0	0	N
159	388	650	0	0	N
160	388	685	0	0	N
161	425	650	0	0	N
162	431	560	0	0	N
163	431	580	0	0	N
164	431	650	0	0	N
165	431	685	0	0	N
166	432	650	0	0	N
167	440	650	0	0	N
168	448	650	0	0	N
169	455	650	0	0	N
170	461	560	0	0	N
171	461	580	0	0	N
172	461	650	0	0	N
173	461	655	0	0	N
174	461	658	0	0	N
175	461	665	0	0	N
176	461	667	0	0	N
177	461	670	0	0	N
178	461	685	0	0	N