# Stim1 p.R304 mutations may cause constitutive puncta formation in murine cells

Ingvild Sveum Reinseth

Thesis submitted for the degree of
Master of Science in Molecular Bioscience
60 credits

Department of Bioscience
Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO

June / 2017

June 2017

Stim1 p.R304 mutations may cause constitutive puncta formation in murine cells

Ingvild Sveum Reinseth

http://www.duo.uio.no/

Trykk: Reprosentralen, Universitetet i Oslo

# Abstract

The stromal interaction molecule 1 (**STIM1)** controls the activation of the $Ca^{2+}$ release activated calcium channel ORAI1 in the plasma membrane. STIM1 induces store operated calcium entry (**SOCE**) by sensing reduced levels of $Ca^{2+}$ in the endoplasmic reticulum (**ER)** and activating ORAI1 allowing $Ca^{2+}$ to flow into the cell. Stormorken syndrome is an autosomal dominant disease caused by the *STIM1* p.R304W mutation. This gain-of-function mutation causes a constitutive activation of STIM1, resulting in a constitutively open ORAI1 channel. The symptoms in Stormorken patients include thrombocytopenia, mild bleeding tendency, headaches, dyslexia, asplenia, miosis and tubular aggregate myopathy.

This work presents the results of functional experiments in murine fibroblasts overexpressing constructs carrying the following mutations in Stim1: p.R304W, p.R304A, p.R304P, p.R304S, p.R304E, p.E296del and p.E296del+R304W. The aim of the work is to assess how the *Stim1* mutations affect the activation of the protein and the $Ca^{2+}$ levels of the cell.

**METHODS:** The Q5® mutagenesis system was used to produce constructs with *Stim1* mutations. All plasmids were verified for the mutations with Sanger sequencing. The constructs were transfected into murine fibroblast cells, using the PolyJet$^{TM}$ system and the expression was verified with Western blotting. Puncta formation was assessed by imaging transfected cells with and without TG treatment, with light that excites the fluorescent tags mCHERRY and YFP, which were introduced in the Orai1 and Stim1 constructs, respectively. $Ca^{2+}$ flux was measured with the ratiometric calcium indicator INDO-1 AM. Resting cytosolic $Ca^{2+}$ was measured in a 2mM $Ca^{2+}$ solution and $Ca^{2+}$ influx induced by TG was measured in a 10mM $Ca^{2+}$, 2µM TG containing solution.

**RESULTS:**

Sanger sequencing verified the presence of the mutations in the Stim1 vectors constructed, and microscopy and Western blotting results verified the expression of Stim1-YFP fusion proteins. Imaging results indicated constitutional puncta formation in cells ectopically expressing Stim1-p.R304W and Stim1-p.R304A. The results in cells expressing Stim1-p.E296del+R304W however, indicated induced puncta formation after thapsigargin treatment, similar to cells expressing Stim1-wildtype. Ratio-metric $Ca^{2+}$ indicators showed a tendency

for cells expressing Stim1-p.R304W to have a higher cytosolic $Ca^{2+}$ level and SOCE response compared to wildtype cells.

**CONCLUSIONS:**

The Stim1 constructs contain the mutations introduced and they express Stim1-YFP fusion proteins.

Assessment of images of transfected cells indicates constitutive puncta formation in cells ectopically expressing Stim1-p.R304W and Stim1-p.R304A. Cells ectopically expressing Stim1-p.E296del+R304W indicate puncta formation only with TG treatment. Additional $Ca^{2+}$ flux experiments are needed in order to conclude on the effect of the Stim1 mutations on puncta formation, resting $Ca^{2+}$ levels and SOCE.

# Acknowledgements

The work presented in this thesis was carried out at the Institute of Medical Genetics, Ullevål University Hospital, from December 2015 to June 2017.

I would like to thank

- My supervisor Professor Eirik Frengen for giving me the opportunity to work in his group, for his feedback and for the invaluable help during the writing process.
- I sincerely thank all the group members of the Frengen group
  Doriana Misceo for all her support with the experiments and help with results analysis.
  Thilini Gamage for always having a positive outlook, patiently answering all my questions and giving me invaluable guidance and help.
  Samudita Senaratne, Asbjørn Holmgren, Tuva Barøy, Dulika Sanjeewani Sumathipala and Emma Lengle.
- I greatly thank all collaborators at the institute for experimental medical research, William Louch, Michael Frisk and David Lipsett. Thank you for your help with the microscopes, other equipment, and your positivity.

- Thank you to my family and friends for your patience and understanding.
  A special thank you for your help with Balto, Odd Arne and Inger-Johanne.
  Thank you Balto for being nothing but joy, lighting up my days and showing me how easy life can be if you just think like a dog. I love you forever.
  Thank you Kristin and Malene for not giving up on me - I am back!

# Table of contents

# Abbreviations

**bp-** base pair(s)
**$Ca^{2+}$-** calcium
**$[Ca^{2+}]_i$-** intracellular calcium concentration
**CAD**- crac activation domain
**CRAC channel-** $Ca^{2+}$ release-activated calcium channel
**DAG-** diacylglyceride
**DMEM-** Dulbecco's modified Eagles medium
**ER-** endoplasmic reticulum
**HR-** homologous recombination
**HT-** Hepes Tyrade
**$IP_3$-** inositoltriphosphate
**$IP_3$-R-** inositoltriphosphate-receptor
**kDa-** Kilodalton (measuring unit)
**KI-** knock in
**KO-** knock out
**mCHERRY-** red fluorescent protein
**NHEJ**- Non-homologous end joining
**PBS-** Phosphate Buffered Saline
**PCR-** Polymerase chain reaction
**PLCβ-** phospholipase C-β
**$PIP_2$-** phosphatidylinositol-4,5-biphosphate
**PM**- plasma membrane
**PMCA-** plasma membrane associated calcium ATPase
**RNA-** ribonucleic acid
**SOAR**- STIM1-ORAI1 activating region
**SOCE-** store operated calcium entry
**SR-** sarcoplasmic reticulum
**STIM1-** stromal interaction molecule 1
**TAM-** tubular aggregate myopathy
**TG-** thapsigargin
**YFP**- yellow fluorescent protein

# 1 Introduction

This thesis presents the results of functional work performed in murine cells ectopically expressing *Stim1* p.R304W, which is the mutation causing Stormorken syndrome in humans. Stormorken syndrome is a rare genetic disorder, with only 13 patients so far described worldwide (Misceo et al., 2014, Morin et al., 2014, Nesin et al., 2014). The cause of the disease is a single base change in *STIM1*, which encodes the stromal interaction molecule 1. STIM1 is involved in regulating the calcium flow into most cell types (Stathopulos et al., 2012). Changes in $Ca^{2+}$ concentration are essential for a range of processes in all cells including synaptic signaling in neurons, muscle contraction and production of blood clots after injury (Varga-Szabo et al., 2009, Kraft, 2015, Clapham, 2007).

The genetic change in *STIM1* in patients with Stormorken syndrome, leads to an altered STIM1 protein function, which affects Store Operated Calcium Entry (**SOCE).** Therefore, this chapter will start by introducing SOCE and the cellular processes, causing depletion of endoplasmic reticulum (**ER)** $Ca^{2+}$ stores. STIM1 and ORAI1 are the proteins involved in SOCE. ORAI1 is located in the plasma membrane (**PM)** and is named after the keepers of the gates of heaven in Greek mythology (Feske et al, 2006), ORAI1 is a $Ca^{2+}$ release activated calcium (**CRAC)** channel which allows cellular $Ca^{2+}$ influx. An overview of the clinical presentation of patients with Stormorken syndrome follow with a summary of disease causing mutations in *STIM1* and *ORAI1*.

## 1.1 $Ca^{2+}$ is an important second messenger in cells

$Ca^{2+}$ is involved in many signaling processes in the cells, and fluctuations in $Ca^{2+}$ concentration is an intracellular signal involved in many pathways. This cation has an impact on nearly all aspect of cellular life. $Ca^{2+}$ is involved in for example cell excitability, motility, exocytosis as well as transcription (Stathopulos et al., 2012, Niemeyer 2016, Derler et al., 2016), and is important for example in the nervous system signaling pathways, the muscle contraction process as well as the coagulation system. The concentrations of $Ca^{2+}$ outside the cell is usually around 1,5mM, while the concentration on the inside of the plasma membrane is much lower, approximately 100nM. In addition there are intracellular stores of $Ca^{2+}$ in ER and the mitochondria (Clapham, 2007). It is important for the cell to have mechanisms to maintain the compartment concentrations after signaling processes are complete.

### 1.1.1 Depletion of endoplasmic reticulum $Ca^{2+}$ stores leading to SOCE

Store operated calcium entry (**SOCE)** is a mechanism to bring $Ca^{2+}$ into the cell. Several signaling pathways in several types of cells result in $Ca^{2+}$ release from ER stores (Clapham et al., 2007). Figure 1 illustrates one such pathway in which $Ca^{2+}$ is released from ER stores as a result of signaling by the second messenger $IP_3$. The ER $Ca^{2+}$ is released in response to signals conveyed from receptors at the plasma membrane. After such a signaling event the cell needs to return to its resting $Ca^{2+}$ concentrations. When calcium is released into the cytoplasm, some will be pumped back into the ER by the sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ pump, **SERCA**. Not all the released $Ca^{2+}$ will be transported back into the ER, some will be actively pumped out of the cell by channels on the plasma membrane e.g. the plasma membrane associated calcium ATPase, **PMCA**. The sodium $Ca^{2+}$ exchanger (**NCX**) may also contribute in $Ca^{2+}$ extrusion if the levels of $Ca^{2+}$ in the cytoplasm becomes high (Albarran et al., 2016, Clapham et al., 2007).

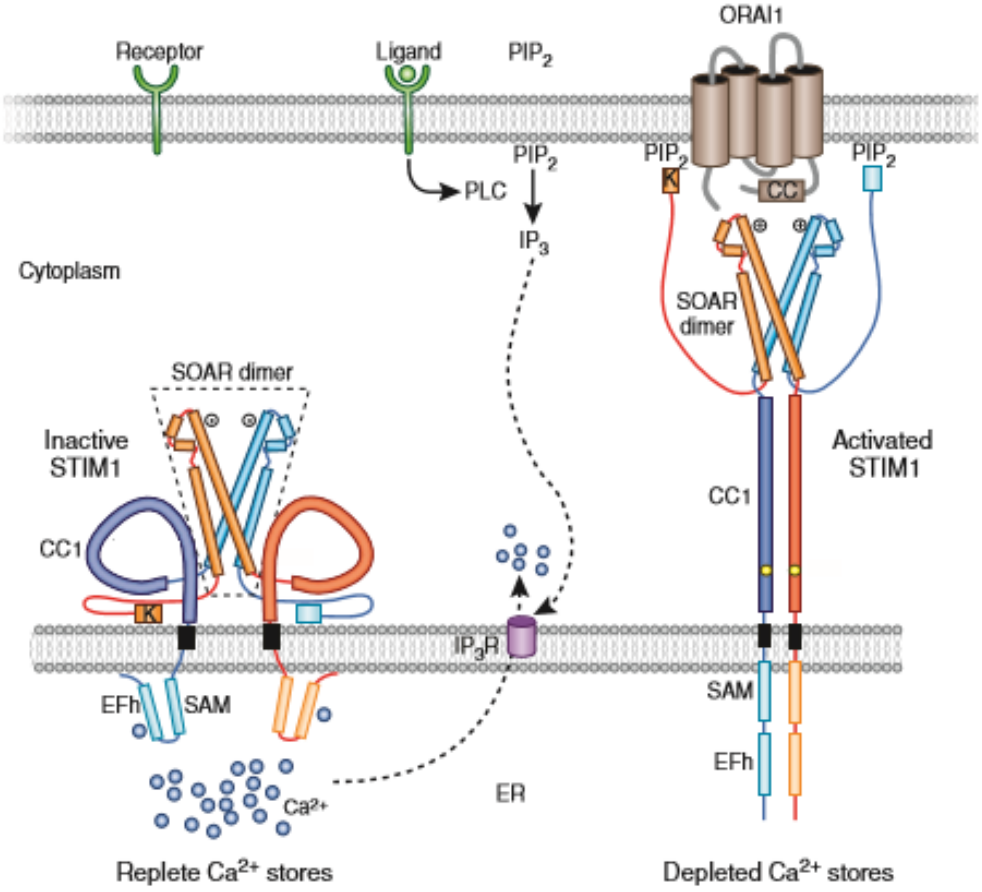



***Figure 1 The release of $Ca^{2+}$ from ER stores following $IP_3$ signaling results in SOCE activation*** *There are receptors in the cell membrane, for example the histamine receptor, which is bound to a G-protein that has a GDP molecule bound in its inactive state. Activation of the receptor starts a process that results in activation of*

*phospholipase C (**PLC**) which cleaves $PIP_2$ to diacylglyceride (**DAG**) and inositol-1,4,5-triphosphate (**$IP_3$**). $IP_3$ binds to $IP_3$ receptors on the ER membrane, which results in release of ER $Ca^{2+}$ into the cytoplasm. See main text for details of SOCE activation (Clapham, 2007, Albarran et al., 2016, Feske and Prakriya, 2013).*

The ER $Ca^{2+}$ release and depletion of the ER stores activates the SOCE pathway, which consists of sensors in the ER membrane, STIM1 that senses low ER $Ca^{2+}$ stores, which then activates the plasma membrane $Ca^{2+}$ channel ORAI1. As illustrated in figure 1; when ER $Ca^{2+}$ stores are reduced this causes a conformational change and multimerization of the ER $Ca^{2+}$ sensor STIM1. STIM1 multimers elongates to contact ORAI1 at plasma membrane-ER junctions. The contact allows STIM1 to activate ORAI1 and $Ca^{2+}$ passively diffuses through the ORAI1 channel into the cytoplasm (Feske and Prakriya, 2013).

### 1.1.2 STIM1 and ORAI1 in SOCE

The protein domains of STIM1 are shown in figure 2. The EF hand domain (**EF**), with a canonical and a hidden EF hand (**EF**) and the sterile alpha motif (**SAM**) domain, reside in the ER lumen. The transmembrane domain **(TM)** transverses the ER membrane, and the coiled coil 1 domain, (**CC1)**, and the CRAC activation domain/STIM1 ORAI1 activating region, (**CAD/SOAR)** are located in the cytoplasm. At the C-terminal, there is an ID region, serine/proline rich region (P/S) and a lysine rich region (K) that has been shown to interact with the plasma membrane (figure 1).

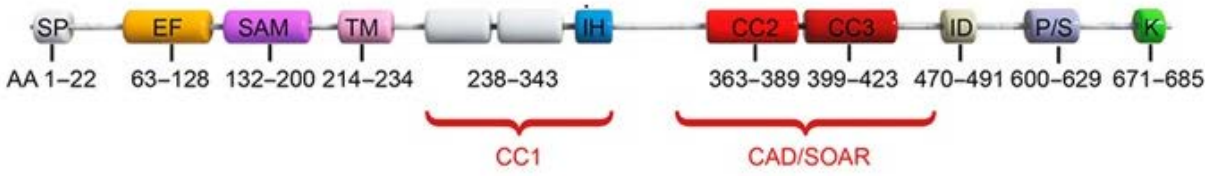



***Figure 2 The domains of the* STIM1 *protein.*** *The amino acid positions in each domain are indicated. See main text for details (Modified from Misceo et al., 2014).*

When the ER $Ca^{2+}$ stores are replete the STIM1 protein remains in an inactive state, characterized by a retracted protein conformation, and a partial dimerization with another STIM1 protein, as shown in figure 1 (Feske and Prakriya, 2013). In this state the $Ca^{2+}$ sensing EF domain, remains associated with the SAM domain. In addition to this, the CC1 domain forms a retracted loop, while the CAD/SOAR domain forms a V structure in association with the dimer partner (Feske and Prakryia, 2013). The CC1 region has been hypothesized as crucial for keeping the protein in an inactive conformation (Misceo et al., 2014).

The transition of STIM1 to the elongated activated state is accompanied by the dissociation of $Ca^{2+}$ from the EF hand and exposure of hydrophobic regions in the EF-SAM domain resulting in the multimerization and translocation of STIM1 to ER-PM junctions and activation of ORAI1 (Feske and Prakriya 2013).

The CRAC channel ORAI1 opens upon activation by STIM1. There are six functional domains in the ORAI1 protein; an N-terminal calmodulin binding domain, four transmembrane domains and a C-terminal coiled coil domain (figure 3). The first transmembrane domain (M1) is part of the pore that is the $Ca^{2+}$ channel, which consists of six M1 subunits (Cahalan, 2009). Both the cytoplasmic N- and C-terminal are critical for STIM1 binding (Feske et al., 2010, McNally et al., 2013).

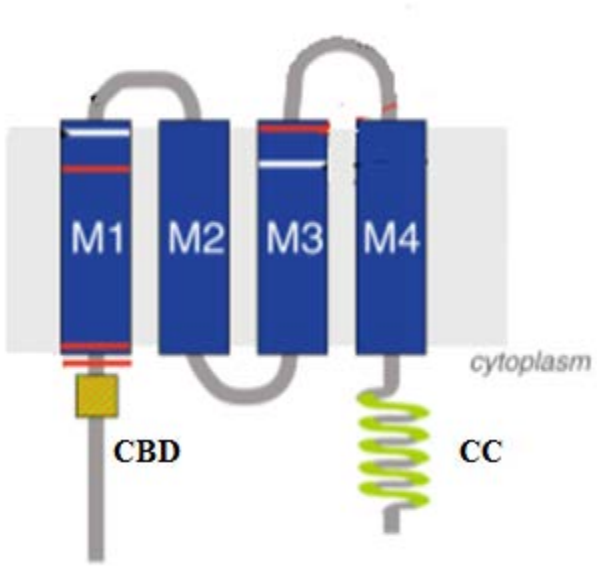



***Figure 3 The six recognized domains of the* ORAI1 *protein*** *The* ORAI1 *protein has 6 domains; an N-terminal calmodulin binding domain (**CBD**), four transmembrane domains (**M1-M4**) and a C-terminal coiled coil domain (**CC**) (Modified from Feske et al., 2010).*

Upon STIM1 interaction, the ORAI1 multimer undergoes a conformational change that opens the central pore of the CRAC channel through which $Ca^{2+}$ can flow. The ORAI1 pore has a $Ca^{2+}$ selectivity 1000 times higher than for $Na^{+}$. As the channel opens the $Ca^{2+}$ flows into the cytoplasm due to the electrochemical gradient across the plasma membrane (Derler et al., 2016).

After entry into the cytoplasm sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ activated (**SERCA**) pumps $Ca^{2+}$ into the ER (figure 4). SERCA is an ATPase exchanger, which uses energy from hydrolysis of ATP to exchange two $Ca^{2+}$ ions for three protons (Clapham, 2007).

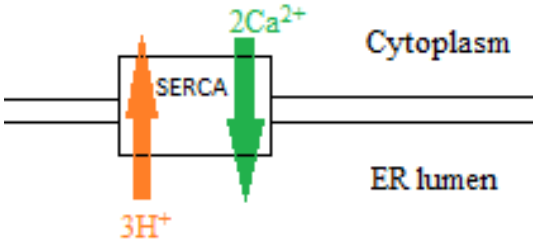



***Figure 4 Pumping of calcium and protons by SERCA*** *The SERCA protein pumps two calcium ions from the cytosol to the ER lumen in exchange for three protons from the ER lumen to the cytosol.*

It is possible to experimentally induce SOCE in vitro through the inhibition of SERCA. Thapsigargin (**TG**) is a non-competitive inhibitor of SERCA, extracted from the plant *Thapsia garganica*. When TG blocks the $Ca^{2+}$ pumping by SERCA, ER will become $Ca^{2+}$ depleted allowing secondary activation of plasma membrane $Ca^{2+}$ pumps and influx of $Ca^{2+}$. This mechanism is used for functionally evaluating SOCE in different cell types (Lytton et al., 1991).

### 1.1.3 The STIM1:ORAI1 interaction and puncta formation

The molecular interactions of STIM1 and ORAI1 are not completely understood, but STIM1 interacts with both the N- and C-terminal parts of ORAI1, with a stronger interaction between STIM1 and ORAI C-terminal than with the N-terminal (Derler et al., 2016). A STIM1-ORAI1 association pocket (**SOAP**) has been described in C-terminal ORAI1 (Derler et al., 2016), but no clear binding site has been established for the N-terminal. One hypothesis is that the C- and N-terminal ends of ORAI1 create a STIM1 binding site when in association with each other in the multimer, with the STIM1 CAD/SOAR domain as a bridge between the ORAI1 monomers. This can contribute to the change in the dimerization angle of the ORAI1 proteins, causing a conformational change in the opening of the channel. In addition, the C-terminal binding might occur before the STIM1 protein binds to the ORAI1 N-terminal (Derler et al., 2016).

The aggregates of STIM1 and ORAI1 proteins formed upon STIM1 activation are called puncta. Puncta are areas in the cell where the ER membrane, the anchor point of the STIM1 protein, is in close proximity to ORAI1 on the plasma membrane. The distance between the ER membrane and the plasma membrane is between 10-25 nanometers in these junctions (Cahalan, 2009). The reason for this specific accumulation is that the STIM1 protein needs to

still associate with the ER membrane, to which it is anchored, while it interacts with the ORAI1 pore in the plasma membrane (Derler et al., 2016). It is possible to visualize the localization of the STIM1 and ORAI1 proteins in the cell by tagging the proteins with fluorescent markers (figure 5).

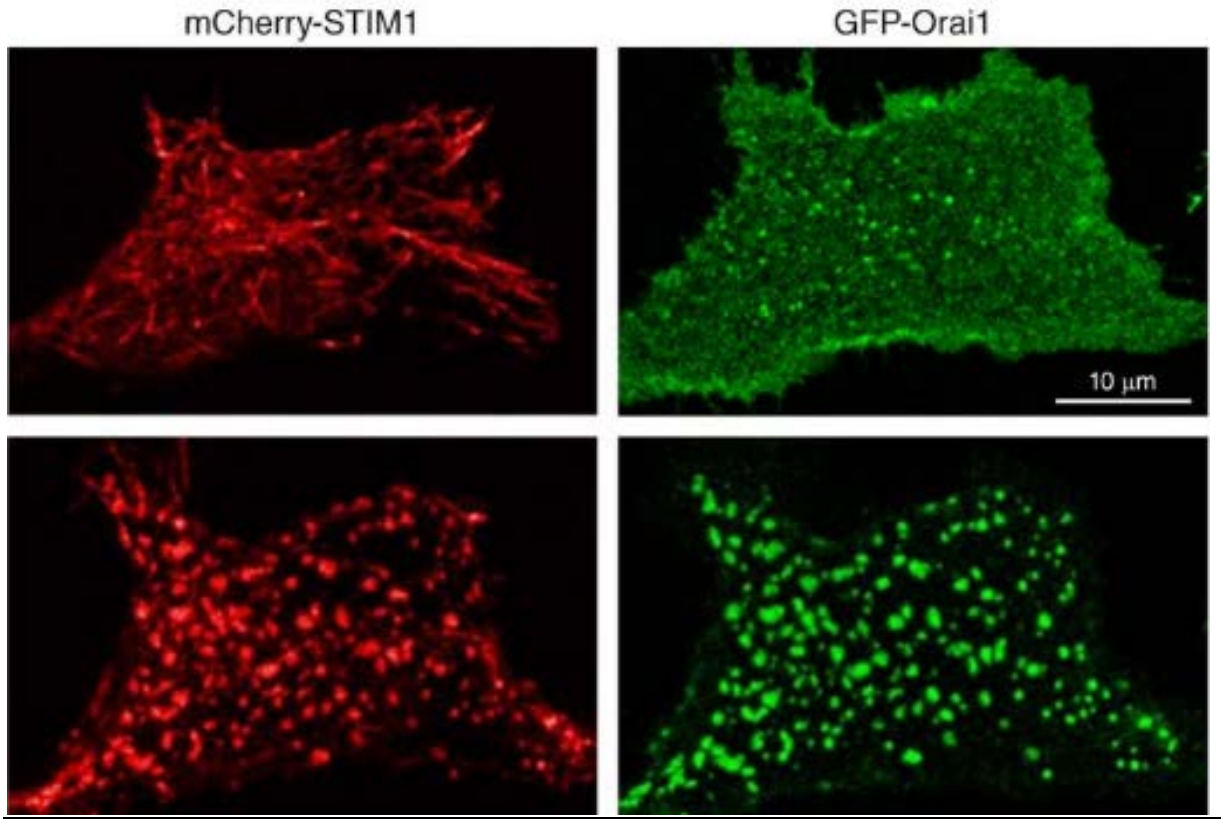



***Figure 5 Puncta formation*** *The ORAI1 protein, linked to a green fluorescent protein marker,* ***GFP****, and the* STIM1 *protein, linked to a red fluorescent protein marker,* ***mCHERRY****, shown without (top) and with thapsigargin (****TG****) treatment (bottom)* (Prakriya et al., 2015).

### 1.1.4 STIM1 and Stim1, ORAI1 and Orai1

The human *STIM1* and the murine orthologue *Stim1* show similar expression levels in most cell types (Prakriya et al., 2015). The *Stim1* homolog, *Stim2* has been implicated especially in brain tissues and capacitive $Ca^{2+}$ entry in neurons (Kraft 2015). STIM1 is expressed as two isoforms, one short and one long isoform (STIM1L). STIM1L is expressed in adult human muscle fibers and may play a specific role in these tissues (Niemeyer, 2016). The STIM1 and Stim1 proteins are conserved with 663 of 685 amino acids being identical between the two proteins as shown in figure 6.

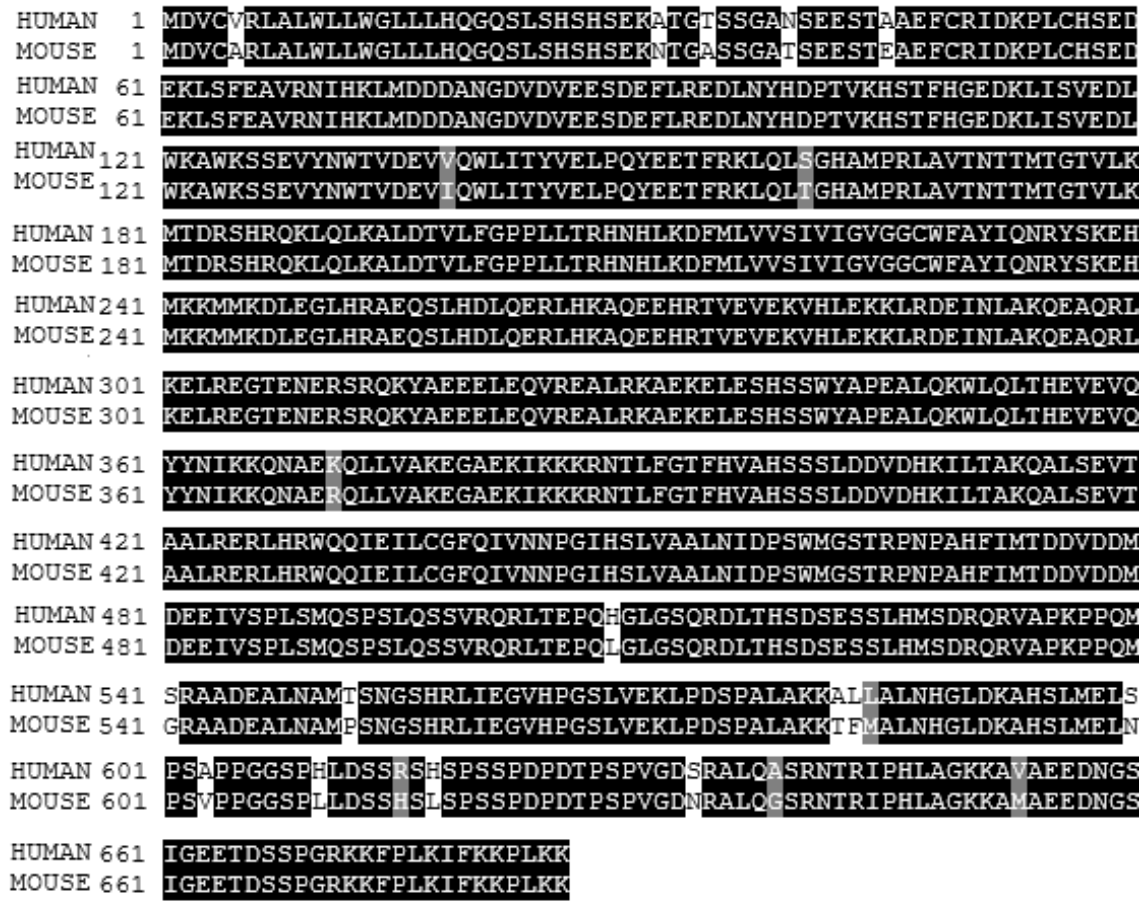



***Figure 6 Alignment of the human* STIM1 *and the mouse* Stim1 *protein*** *Shown in black are the amino acids that are identical, and deviations are illustrated in white or grey. Grey indicate positions where amino acids with similar side chain properties are located.*

## 1.2 Diseases caused by *STIM1* mutations

Three known conditions; Stormorken syndrome (STRMK, OMIM #185070), tubular aggregate myopathy, (**TAM1** OMIM #160565), and severe combined immunodeficiency, (**IMD10** OMIM #612783), are caused by mutations in STIM1. The positions of the dominant gain-of-function mutations in STIM1 that have been shown to cause Stormorken syndrome and TAM1, and the recessive loss-of-function mutations causing IMD10 are indicated in figure 7. The nine mutations causing TAM1 are all located in the EF hand domain (Reviewed in Lacruz and Feske, 2015), and the p.R304W mutation causes Stormorken syndrome. The effect of these dominant mutations will be described further.

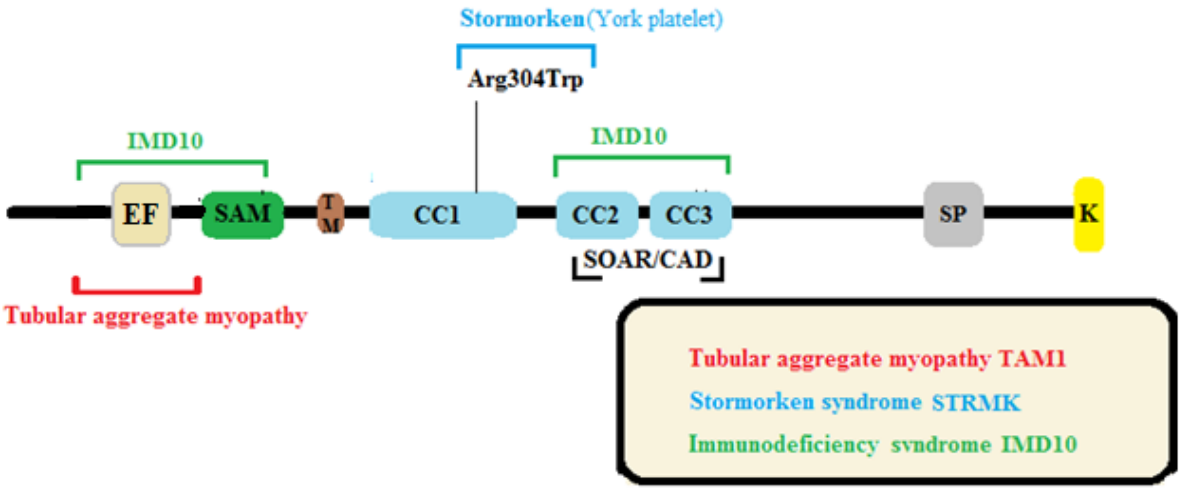



***Figure 7 Location of* STIM1 *mutations causing the diseases indicated*** *The mutations causing TAM1 are concentrated in the EFhand domain, marked in red. The mutation causing Stormorken syndrome, marked in blue, is in the CC1 domain of STIM1. The recessive loss of function mutations causing immunodeficiency are less concentrated in a specific domain, and affects both cytosolic and ER-lumen regions of* STIM1. *EF-EFhand domain. SAM – Sterile Alpha Motif. TM – transmembrane domain. CC1 – coiled coil domain 1-3. SOAR/CAD – Stim Orai activating region/CRAC activating domain. SP-serine and proline rich domain. K-poly-lysine domain.*

### 1.2.1 STORMORKEN SYNDROME, STRMK OMIM #185070

The mutation causing Stormorken syndrome is the gain-of-function *STIM1* p.R304W mutation located in the STIM1 CC1 domain. The dominant Stormorken syndrome mutation results in a constitutively active STIM1 causing constitutive store operated $Ca^{2+}$ entry (Nesin et al., 2014, Misceo et al., 2014).

Helge Stormorken described two patients with Stormorken syndrome in 1985 (Stormorken et al., 1985), and published a summary of the observed clinical features in 2002 (Stormorken et al., 2002). The index patient was brought to his attention because of a hematoma after a fall, and a mild bleeding tendency was established in the patient and his mother. Both had asplenia, as well as tubular aggregate myopathy. In addition, both had dyslexia, migraine-like headaches, ichthyosis and miosis (figure 8). Other traits include thrombocytopathy and thrombocytopenia as well as anemia (Stormorken et al., 2002). In 2014, Frengen's research group revealed the genetic cause of Stormorken syndrome, identifying the STIM1 missense mutation p.R304W in six patients from four independent families (Misceo et al., 2014). Another independent study discovered additional two patients with Stormorken syndrome with the same p.R304W mutation (Nesin et al., 2014). The findings were verified by a third

study documenting the p.R304W mutation in five more patients with the same clinical features (Morin et al., 2014).

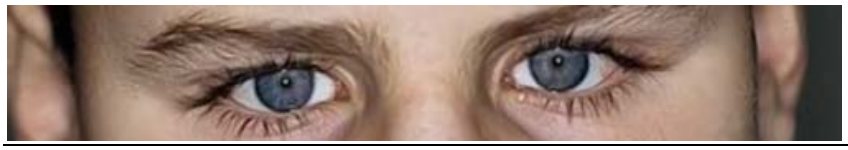


***Figure 8 Miosis in a Stormorken syndrome patient of Norwegian origin*** *The patient has miosis, a constant contraction of the pupil and showed no reaction when treated with mydriatics (Stormorken, 2002).*

The common clinical features present in all 13 known Stormorken patients are thrombocytopenia, miosis, and increased creatine kinase levels (Morin et al., 2014). In 2015 Markello et al., described patients with similar clinical features as the Stormorken patients, and showed that they also had the p.R304W mutation. Four out of seven of the patients described had similar symptoms, but carried the EFhand mutation p.L115F. However, these patients were given the diagnosis York Platelet Syndrome (Markello et al., 2015). Stormorken and York Platelet Syndrome have in common clinical features such as increased bleeding tendency connected to thrombocytopenia and platelet defects, and muscle defects such as weakness and TAM (Markello et al., 2015, Misceo et al., 2014, Nesin et al., 2014, Morin et al., 2014). Although the clinical spectrum of the patients with the p.R304W mutation is similar, modifier genes and allelic variation may determine the phenotype (Reviewed in Lacruz and Feske, 2015).

### 1.2.2 TUBULAR AGGREGATE MYOPATHY, TAM1, OMIM #160565

Tubular aggregate myopathy 1 (TAM1) is a muscular disease characterized by tubular aggregates in muscle. Tubular aggregates are found in several muscle disorders as a secondary feature, but can also have a genetic cause (Bohm et al., 2013). TAM1 can be caused by mutations in the EF hand of *STIM1*, causing a constitutive SOCE activation (Bohm et al, 2013). There are several mutations detected in the EF hand domain that cause TAM1, but they all have the same mechanism of disrupting the $Ca^{2+}$ binding to the EF hand and destabilizing the association with the SAM domain. TAM is one of the symptoms found in Stormorken syndrome, also caused by a constitutionally activating mutation in *STIM1* (Misceo et al., 2014, Nesin et al., 2014, Morin et al., 2014).

$Ca^{2+}$ signaling in muscle cells is involved in contraction of muscle, but also growth and differentiation of muscle cells. Bohm et al. described the tubular aggregates as arrays of

membranes that naturally accumulate in muscle with age. It was postulated that problems with the sarcoplasmic reticulum (**SR**) structure as well as defective SOCE cause TAM, because $Ca^{2+}$ is involved in membrane repair in skeletal muscle (Bohm et al., 2013). Nesin et al. postulated that the cause of the tubular aggregate formation in the muscle is that there is a hostile environment in the SR of the muscle cells in the patients, due to an abnormal resting $Ca^{2+}$ concentration, negatively affecting protein folding and consequently leading to formation of the tubular aggregates. The hostile environment was postulated to be caused by the sustained entry of $Ca^{2+}$ due to the constitutively active STIM1 causing constitutive SOCE (Nesin et al., 2014).

TAM1 patients lack the other clinical symptoms present in Stormorken syndrome. In contrast to Stormorken syndrome caused by one mutation, there are nine known mutations, all located in conserved residues in the EF hand that cause TAM1 (Bohm et al., 2013, Bohm et al., 2014, Walter et al., 2015).

## 1.3 Diseases caused by mutations in *ORAI1*

There are two known conditions caused by mutations in *ORAI1*; immunodeficiency (**IMD9,** OMIM #612782), and tubular aggregate myopathy (**TAM2**, OMIM #615883). TAM2 is an autosomal dominant disorder and the patients have a similar phenotype as TAM1 patients. The overlapping clinical presentation is caused by the constitutive SOCE regardless if the mutation is in *STIM1* or *ORAI1*. IMD9 and IMD10 also have overlapping clinical presentation of immunodeficiency syndromes, due to loss of SOCE caused by LOF-mutations in ORAI1 or STIM1 respectively, regardless of which gene is mutated (Endo et al., 2015, Shahrizaila et al., 2004, Nesin et al., 2014).

## 1.4 Selected Stim1 mouse models

Over the years, many mouse models have been created to investigate the effects of alterations in *Stim1*. Of relevance to this thesis is specifically the Sax mouse model, carrying the Stim1 p.D86G mutation (Grosse et al., 2007). This mutation is located in the EFhand, causes constitutive SOCE, and therefore indicates the effect of a constitutive mutation in a murine setting.

### 1.4.1 The Saxcoburggotski Sax mouse model

Grosse et al. described a mouse model created by random mutagenesis using N-ethyl-N-nitrosurea (Grosse et al. 2007). One of the resulting mouse lines showed dominant inheritance of reduced platelet count and elevated mean platelet volume caused by a missense mutation in S*tim1*, an A to G transition at position c.444. This gives a p.Asp84Gly amino acid substitution in the EFhand. This mutation has also been detected in patients with TAM1 (Bohm et al., 2013). Homozygous $Stim1^{Sax/Sax}$ mice show embryonal lethality due to severe bleeding. The platelet phenotype of $Stim1^{Sax/+}$ mice resulted in prolonged bleeding time as determined by tail bleeding experiments. The intracellular $Ca^{2+}$ concentration (**$[Ca^{2+}]_i$)** had a 3-fold increase in T-cells from the $Stim1^{Sax/+}$ mouse and the platelets showed constitutive $Ca^{2+}$ influx (figure 9), compared to WT controls. The mutant mouse platelets were in a pre-activated state and the platelets showed reduced TG evoked influx indicating defective SOCE (Grosse et al., 2007).

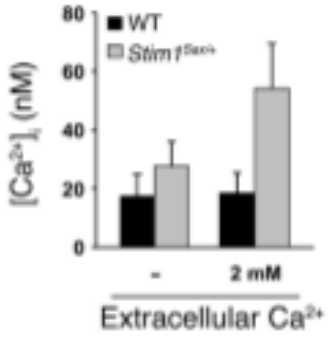



***Figure 9 Basal levels of intracellular $Ca^{2+}$ in platelets from the Sax mouse model*** *The concentration of intracellular $Ca^{2+}$ is higher in platelets from the Sax mouse line (grey), than in the wild type (black) in cells in the absence of $Ca^{2+}$ in the media. The intracellular concentration of $Ca^{2+}$ is approximately three times elevated in platelets in the presence of 2mM $Ca^{2+}$ extracellular concentration (Grosse et al., 2007).*

### 1.4.2 Stim1 knockout models.

Many mouse models have been made to study the effects of Stim1 knockout in different cellular processes, such as oxidative stress and immune responses (Braun et al., 2009, Henke et al., 2012). Global knock-out of Stim1 results in approximately 70% postnatal lethality, and the surviving $Stim1^{-/-}$ animals have a marked growth retardation compared to their WT littermates. In Stim1 deficient platelets from chimeric animals having Stim1 knocked out in

platelets, TG induced store operated $Ca^{2+}$ entry was almost absent, which could indicate that Stim1 is necessary for SOCE (Varga-Szabo et al., 2008).

## 1.5 Knock in mouse model expressing the Stim1 p.R304W mutation

Thilini Gamage (Frengen's group, UIO) used zinc finger technology (**ZFN**) to create a mouse model expressing the *Stim1* p.R304W mutation on a mixed CBAxc57bl/6 background (Thilini Gamage, personal communication).

Of the 117 mice born after two sets of ZFN injections, five mice had the knock-in-allele on target. Of these five mice, breeding was started on one mouse expressing the p.R304W-mutation and one mouse carrying a p.R304W+E296del double mutant allele. Founder animals were backcrossed, with c57Bl6 animals, and intercrossed to establish the two mouse colonies.

Phenotypic characterizations of the two mouse models are currently ongoing. The Stim1 p.R304W model shows homozygous lethality. $Stim1^{p.R304W/+}$ animals show severe degeneration of skeletal muscles and reduced endurance on treadmill. However, the model shows no bleeding phenotype (unpublished data).

In contrast, the $Stim^{p.R304W+E296del/p.R304W+E296del}$ mice do not show lethality or growth retardations and has a normal muscle phenotype.

SOCE measurements have also been performed on fibroblast from neonatal $Stim^{p.R304W/p.R304W}$ (**HOM KI**), wildtype (**WT**) and $Stim1^{p.R304W/+}$ (**HET KI**) mice. It demonstrated slightly increased SOCE in cells from homozygous $Stim1^{p.R304W}$ mice compared to wildtype animals (figure 10).

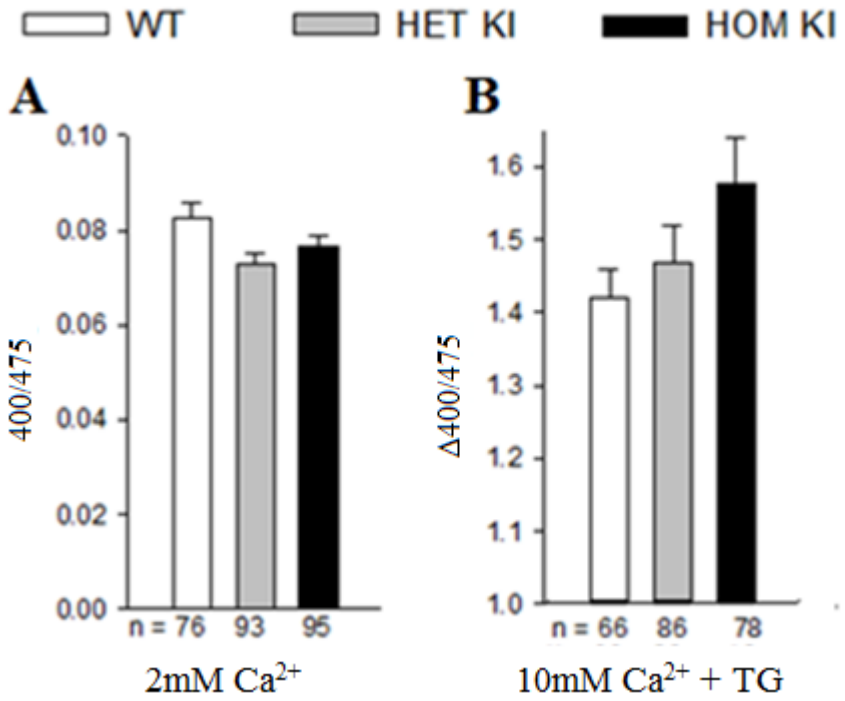



***Figure 10 SOCE measurements in neonatal mouse fibroblasts from homozygous (HOM KI) and heterozygous (HET KI),* Stim1$^{p.R304W}$ *mice, compared to cells from wild type mice (WT)*** *A. The levels of intracellular $Ca^{2+}$ at resting physiological conditions. B. TG induced SOCE response with a 10mM $Ca^{2+}$ extracellular solution (Thilini Gamage, unpublished data). n= number of cells analyzed*

When SOCE measurements were performed in platelets from patients with Stormorken syndrome, the resting $Ca^{2+}$ levels were higher in the patient cells than in the control cells whereas SOCE was lower in patient cells than in control cells (Misceo et al., 2014).

# 2 Aims of the work

The aim of this work was to introduce the following seven Stim1 mutations; p.R304W, p.R304A, p.R304P, p.R304S, p.R304E, p.E296del and p.E296del+R304W, and evaluate their effect on puncta formation and intracellular $Ca^{2+}$ concentration. This therefore entailed:

- Construct plasmids expressing mouse Stim1 with a C-terminal yellow fluorescent protein (**YFP**) tag containing the seven mutations.
- Design and procure plasmid DNA of constructs expressing mouse Orai1 with an N-terminal red fluorescent protein (**mCHERRY**) tag.
- Co-transfect the *Stim1-YFP* plasmid-constructs with mCHERRY-*Orai1* into a murine embryonic fibroblast cell line (NIH 3T3) and evaluate co-localization of the fluorescent proteins to detect puncta formation.
- Co-transfect the *Stim1-YFP* plasmid-constructs with mCHERRY-*Orai1* into mouse cells (NIH 3T3) and measure cytosolic $Ca^{2+}$ concentrations at physiological levels and SOCE induced by thapsigargin.

# 3 Materials and methods

## 3.1 Sanger sequencing

### 3.1.1 Polymerase Chain Reaction

The Polymerase Chain Reaction (**PCR**) utilizes a DNA polymerase enzyme to make many copies of a DNA sequence. DNA polymerases have the ability to create a double helix by elongating a primer sequence, using a DNA strand as a template. The primer sequence is a short DNA sequence complementary to the template DNA that anneals and elongates by incorporating dNTPs complementary to the template DNA. Many cycles are performed, resulting in a doubling of each strand at every cycle as illustrated in figure 11 below (Brown, 2007).

The PCR reactions were performed with 30 cycles, each starting with 30 seconds on 94°C to denature the DNA double helix, followed by 58°C for 30 seconds for annealing of the primers to the template. Then 78°C for 1,5 min for elongation. Before the 30 cycles the denaturing temperature, 94°C, is kept for 2 minutes for initial denaturation, and to conclude the PCR there is a 7 minute final extension at 78°C.

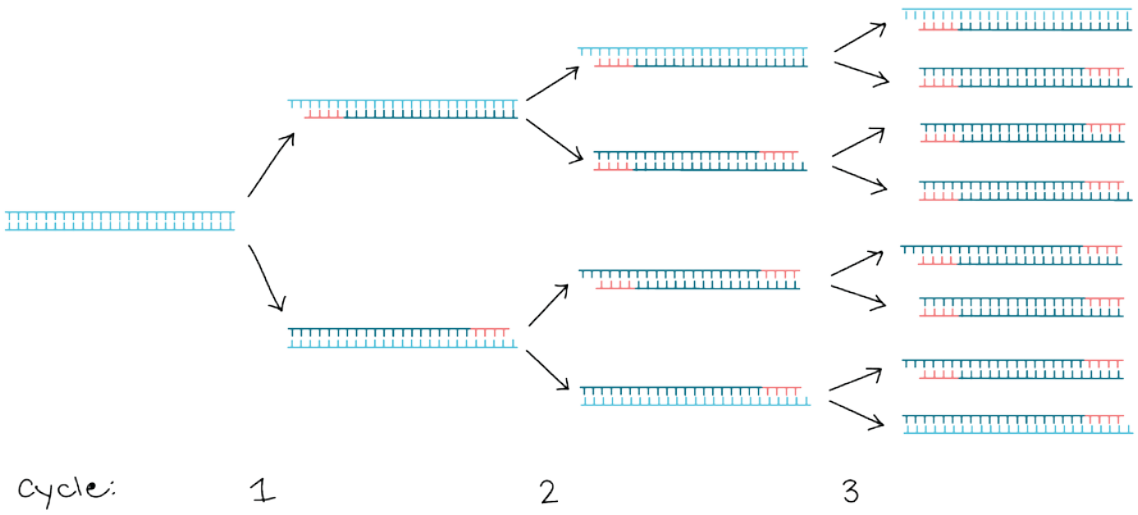



***Figure 11 Polymerase Chain Reaction*** *A DNA template is mixed with a sample of dNTPs, primers(red), buffer and DNA polymerase enzyme. The DNA polymerase elongates the primer and for each PCR cycle the number of DNA molecules are doubled (Creative commons licence 3.0).*

**PCR procedure**

The following reagents were assembled to a total of 25µl

Buffer (-$MgCl_2$) 2,5µl
dNTP 0,5µl (0,2 mM)
$MgCl_2$ 0,75µl (1,5 mM)
Primer forward 0,5µl (5ng/µl)
Primer reverse 0,5µl (5ng/µl)
Polymerase taq platinum 0,25µl (0,5U)
Template DNA 1µl (50ng/µl)
Nuclease free $H_2O$ 19µl

### 3.1.2 Sequencing reaction

Sanger sequencing is based on producing different length DNA fragments each with a different ddNTP base at the last position. The DNA fragments are created in a PCR reaction containing normal dNTPs as well as labelled ddNTPs that will terminate the reaction due to the lack of a 3'-hydroxyl group. Each of the four types of ddNTP is labelled with a specific fluorochrome excited by a laser to identify the corresponding nucleotide. From this a complete read out sequence can be assembled by separating the fragments in a capillary gel based on the length and the corresponding ddNTP based on the fluorescence signal detected. The recording of the different fluorochromes give rise to the coloured peaks of the chromatograph and the sequence can be determined (figure 12).

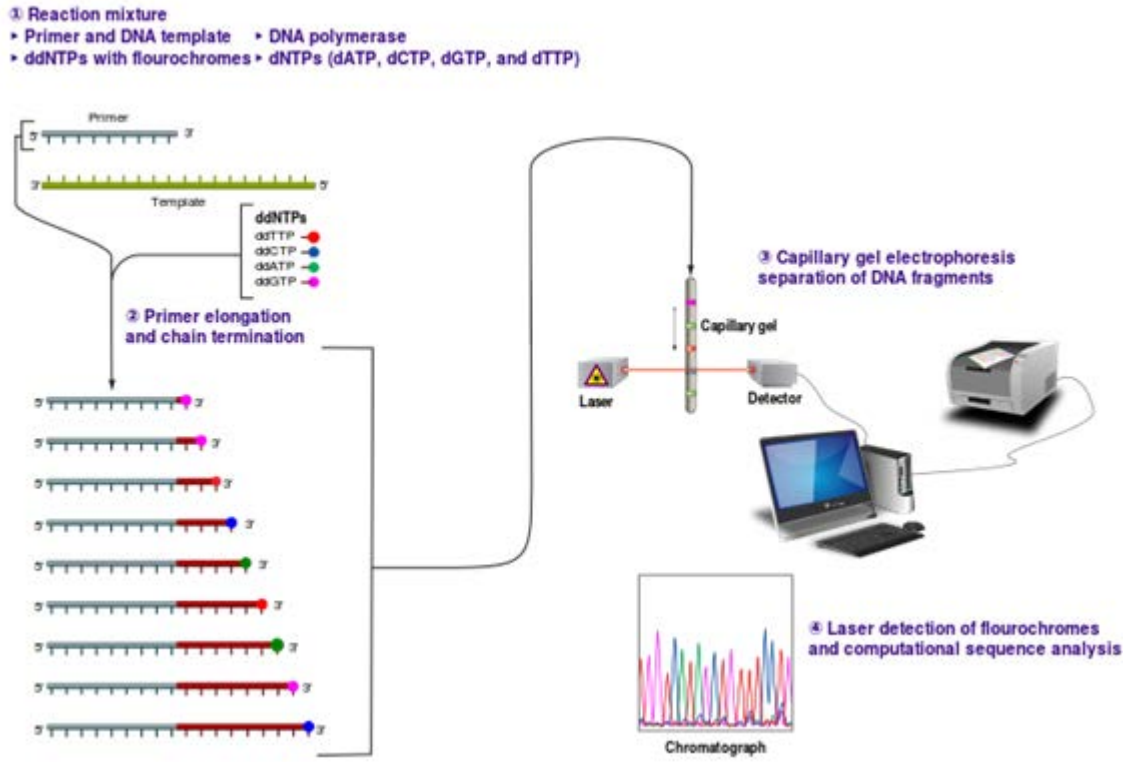



***Figure 12 Process of Sanger sequencing*** *A PCR reaction is performed with dNTPs and a smaller amount of fluorochrome tagged ddNTPs that will terminate the elongation reaction. This produces different length DNA fragments with a fluorescent 3' end. The fluorochromes are read by a laser according to the length of the fragments and the sequence is elucidated from this information (Creative Commons license BY-SA 3.0).*

**PCR procedure**

The following reagents were assembled

Plasmid DNA 2µl

5x sequencing buffer 2µl

Primer 1µl

$H_2O$ 4,75µl

Big Dye terminator v 3.1 0,25µl

The sequencing buffer contains amongst others energy molecules for the enzyme, as well as the ddNTP's and dNTP's. The Big Dye Terminator is the polymerase enzyme performing the elongation reaction. The thermocycler conditions are specified in chapter 8.3.2.

### 3.1.3 *Stim1* primers

| PCR-primer | Name | Sequence |
|---|---|---|
| Sanger sequencing | R1 | AGTCCCTGTCATGGTGGTGT |
| | R2 | AACTGGAGATGGTGTGTCTGG |
| | F3 | TGTGGATGACATGGATGAGG |
| | F7 | TGGAGCTGCCACAGTATGAG |

***Table 3 Stim1 primers*** *All primers were synthesized by Eurofins®, Luxemburg.*

### 3.1.4 *Orai1* sequencing

| PCR-primer | Name | Sequence |
|---|---|---|
| Sanger sequencing | Forward1(F1) | CTGTGAGCAACGTCCACAAC |
| | Reverse1 (R1) | GTGCCCGGTGTTAGAGAATG |
| | Reverse2 (R2) | CCTGGTGGGTAGTCATGGTC |
| | CMV_promoter (CMV) | CGCAAATGGGCGGTAGGCGTG |
| | CHERRY_tag (CHERRY) | TTGGTCACCTTCAGCTTGG |

***Table 4 Orai1 primers*** *All primers were synthesized by Eurofins®*

The following illustration shows the primers annealing locations on the mCHERRY-Orai1 plasmid DNA. The primers cover the CMV promoter region, the mCHERRY tag sequence and the Orai1 gene.

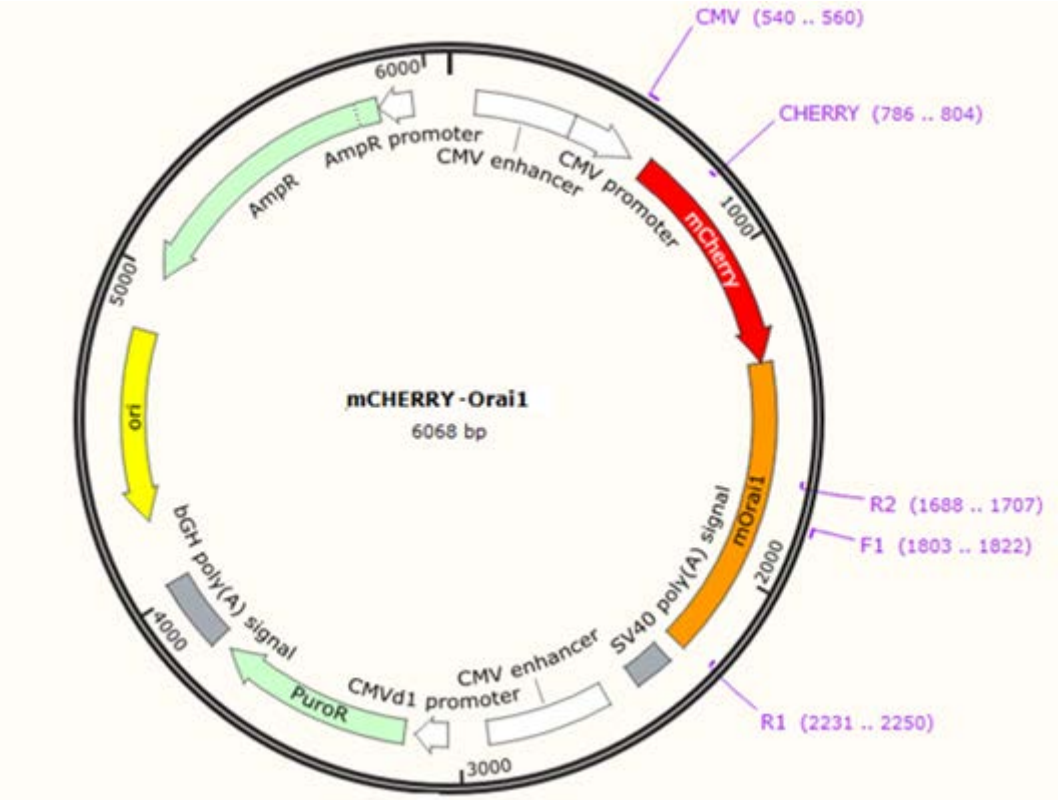



***Figure 13 The positions of Orai1 sequencing primers*** *There are five primers included; 1. CMV anneals in the end of the CMV promoter and sequences towards the mCHERRY tag. 2. The CHERRY primer anneals in the mCHERRY tag and sequences toward the CMV promoter. 3. The R2 primer anneals close to the middle of the Orai1 gene and sequences toward the start of the gene. 4. The F1 primer binds in the middle of the Orai1 gene*

*and sequences toward the end of the gene. 5. The R1 primer anneals in the end of the Orai1 gene and sequences toward the beginning of the gene. Map created with SnapGene software (snapgene.com).*

## 3.2 Mutagenesis

In order to construct vectors expressing Stim1 with amino acid substitutions at the p.R304 position, as well as a deletion of p.E296 the Q5 mutagenesis system was utilized. The template used was Stim1-WT-YFP and Stim1-p.R304W-YFP, which was a gift from professor Leonidas Tsiokas (University of Oklahoma Health Sciences Center, USA), figure 14 illustrates the plasmid map.

The primers used were corresponding to the New England Biolabs recommendations for the Q5 mutagenesis kit (www.NEB.com) and were as listed in table 5.

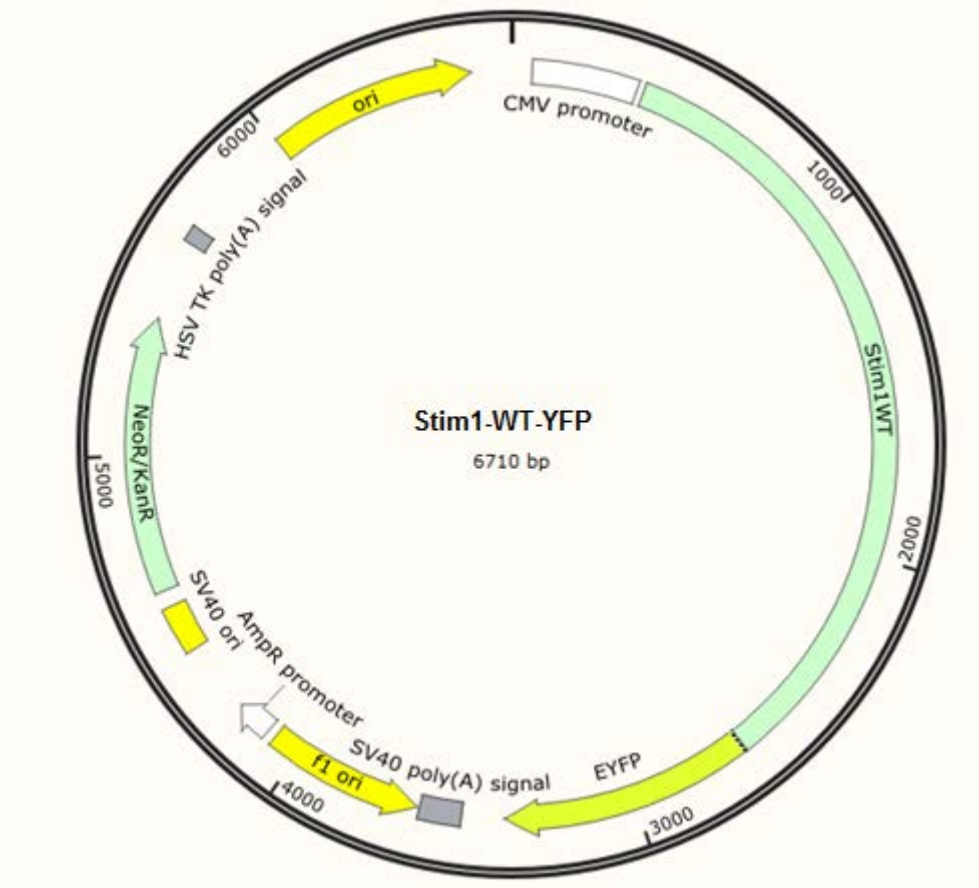



***Figure 14 Stim1-WT-YFP vector*** *with the Stim1 cDNA inserted in frame with the gene encoding the yellow fluorescent protein. The map was constructed using SnapGene software (snapgene.com).*

### 3.2.1 Mutagenesis

The mutagenesis process consists of three steps: the mutagenesis PCR reaction, the Kinase Ligase DpnI (**KLD**) reaction and the transformation step. The first round of PCR for

mutagenesis will result in complexes where one DNA strand in the double helix contains the mutation and the other does not. In the next round, that molecule will result in one complex where both strands contain the mutation and one complex where only one does. In the subsequent rounds, the new DNA strands containing the mutation will increase, and the number of un-mutated plasmid stays the same. To remove the original template the enzyme DpnI was added. DpnI preferentially degrades methylated DNA, a modification the newly synthetized DNA will not have as bacteria performs this modification upon cell-cycle replication (Brown, 2007).

**Mutagenesis PCR** The following reagents were assembled

Q5 Hot Start High-Fidelity 2X Master Mix 5 µl
10 µM forward primer 1µl
10 µM reverse primer 1µl
Template DNA 1µl (25 ng/µl)
Nuclease free water 2µl
To a total of 10 µl

**KLD reaction** The following regents were assembled and incubated at room temperature for 5 minutes.

PCR product 1µl (from mutagenesis PCR)
2x KLD reaction buffer 5 µl
10x KLD enzyme mix 1 µl
Nuclease free water 3 µl

**Transformation** The DNA constructs were transformed into NEB 5-alpha Competent E. coli cells. The cells were thawed on ice. 5 µl of the KLD reaction added and incubated on ice for 30 minutes. The cells were then heat-shocked at 42 °C for 30 seconds, and incubated on ice for 5 minutes before 950 µl of Super Optimal Broth with Catabolite repression (**SOC)** was added. The cells were incubated at 37°C with shaking for one hour. The cells were plated on an LB agar plate with 50 µg/ml kanamycin, and allowed to grow overnight in an incubator at 37°C.

### 3.2.2 Mutagenesis primers

| | | |
|---|---|---|
| Mutagenesis | R304A_forward | GAAGGAGCTGGCGGAGGGTACTGAGAATGAG |
| | R304P_forward | GAAGGAGCTGCCGGAGGGTACTGAGAATGAG |
| | R304S_forward | AGGAGCTGAGCGAGGGTACTGAG |
| | R304E_forward | GAAGGAGCTGGAGGAGGGTACTGAGAATGAG |
| | E296del_forward | CTCAGCGGCTGAAGGAGC |
| | R304A/P/E_reverse | AGCCGCTGAGCTTCCTGC |
| | R304S_reverse | TCAGCCGCTGAGCTTCCT |
| | E296del_reverse | CCTGCTTGGCAAGGTTGATC |

***Table 5 Stim1 primers*** *All primers were synthesized by Eurofins®, Luxemburg.*

## 3.3 Plasmid DNA extraction.

### 3.3.1 *Stim1* plasmid

Single colonies were selected from the LB agar plate, and cultured overnight in 50 ml LB, media with 50 μg/ml kanamycin. The plasmid DNA was extracted using either a Quantum Prep® Plasmid Miniprep kit (BioRad) or a QIAGEN® plasmid Midiprep kit. The concentration was measured on a NanoDrop™ 1000 Spectrophotometer.

**Quantum Prep® Plasmid miniprep kit**

Bacteria in 4 ml of culture was spun down in a table-top centrifuge at 13000 rpm and the LB removed.

200μl of Resuspension solution was added and the bacterial pellet resuspended by pipetting up and down.

250μl of Cell Lysis solution was added and the tube carefully inverted 10 times.

250μl of Neutralization solution was added and the tube carefully inverted 10 times.

The tube was spun in a table-top centrifuge at 13000 rpm for 5 minutes to pellet cell debris.

The supernatant was added to a spin filter placed in a collection tube.

200μl of matrix solution was added and mixed well with the supernatant by pipetting up and down.

The tubes was spun in a table-top centrifuge at 13000 rpm for 30 seconds.

500μl of wash solution was added two times and the tubes spun after each time, first for 30 seconds and then for 2 minutes, both in a table top centrifuge at 13000 rpm.

The DNA was eluted in 90μl nuclease free water.

**QIAGEN plasmid Midiprep kit**

The bacterial pellet from 50 ml culture was harvested by 15 minutes centrifugation at 4°C and 4500 rpm in a table-top centrifuge.

The pellet was resuspended in 4 ml buffer P1; containing RNase.

The P2 buffer was added for lysis to occur and the tube was gently inverted.

P3 buffer was added for precipitation of proteins and cell debris.

The sample was centrifuged at approximately 17000 rpm at 4°C for 30 minutes. The supernatant containing plasmid DNA was quickly transferred to a new tube.

The centrifugation was repeated for 15 minutes.

A QIAGEN-tip 100 was equilibrated with QBT buffer that reduces surface tension and allows flow through the column.

The supernatant was added to the column and as it flowed through the plasmid DNA attached to the membrane

The QIAGEN-tip 100 was washed two times with 10 mL QC buffer. This wash removes contaminants like carbohydrates from the column.

The plasmid DNA was eluted with 5 mL QF buffer.

Plasmid DNA was precipitated with 3,5 mL isopropanol and centrifuged at approximately 15000 rpm at 4°C for 30 minutes.

The pellet was washed with 2 mL 70% ethanol and centrifuged at the same conditions for 10 minutes to remove the isopropanol and precipitated salt.

The pellet was air-dried and the DNA dissolved in 500µl TE buffer.

### 3.3.2 *Orai1* plasmid

An *Orai1* plasmid with the gene in frame with an N-terminal mCHERRY fluorescent tag was designed and was received as a stab culture (Vector Builder, Santa Clara California). The stab culture was streaked out on an LB plate with 100µg/ml ampicillin. The plate was incubated at 37°C over night. A single colony was picked for over-night culturing in 150ml of LB media with 100µg/ml ampicillin. The culture was spun down at 15000 rpm for 15 minutes and the media removed.

**ZymoPURE™ Plasmid Maxiprep**

14 ml of ZymoPURE™ P1 buffer was added to the bacterial pellet and vortexed for complete resuspension
14 ml of ZymoPURE™ P2 buffer was added and the tube was immediately and carefully inverted 6 times.
The lysis was allowed to proceed for 2-3 minutes at room temperature.
14 ml of ZymoPURE™ P3 buffer was added and the tube was gently inverted for neutralization to occur
The contents of the tube was added to the ZymoPURE™ Syringe Filter and left at room temperature for 5-8 minutes
The solution was pushed through the filter into a 50ml canonical tube and the filtrate was saved
14 ml of ZymoPURE™ binding buffer was added and the tube was inverted 10 times
The solution was added to the Zymo-Spin™ V-P Column assembly and centrifuged at 500 x g for 2 minutes
The solution was washed once with 5 ml wash buffer 1 and twice with 5 ml wash buffer 2 with 10 000 x g centrifugation for 1 minute in between.
The filter was moved from the reservoir to a 2 ml collection tube and spun at 13 000 rpm for 1 minute to remove residual wash buffer
The DNA was eluted with 400µl of ZymoPURE™ Elution Buffer

**Glycerol stock**

Prior to pelleting the bacterial cells, a glycerol stock was created for each mutant plasmid for long-term storage.

500µl of bacterial culture
500µl of 50% glycerol

This was stored at -80°C

## 3.4 Transfection

Transfection is a method to introduce DNA constructs into eukaryotic cells.
The experiments were performed with the PolyJet™ (SignaGen®) transfection reagent, to introduce plasmids into murine NIH 3T3 fibroblast cells (Sigma Aldrich®).

### 3.4.1 Cell culturing

**Cell growth**

The cells were grown in Dulbecco's Modified Eagle Medium (**DMEM**) with high glucose (4500 mg/L, Sigma Aldrich), supplemented with 10% Bovine Calf serum (**BCS**, Thermo Fisher®), 10 ml Penstrep (10,000 U/mL, Thermo Fisher®) and 5 ml L-glutamine (200mM, Thermo Fisher®), in a 37°C, 5% $CO_2$ incubator. See supplements 8.5.2 for full media composition.

**Seeding cells**

To detach the NIH 3T3 cells 2ml of 0,05% trypsin-EDTA (Life Technologies) was added and incubated for 2-5 minutes. 10 ml of DMEM supplemented with 10% BCS was added and the cells were pelleted in a table-top centrifuge at 1200 rpm for 7 minutes. The cell pellet was resuspended in 2 ml DMEM and counted on the Nucleocounter® (Chemometec). The Nucleocounter® requires addition of equal parts buffer A, that lyses the cells, and buffer B, that stabilizes pH and contains a dye that binds to nucleic acid. This visualizes the nuclei and the number of cells was estimated based on the number of nuclei.

Approximately 400 000 cells were seeded to glass coverslips pre-coated with gelatin/fibronectin. After growing for 48 hours in 1ml DMEM, the cells were transfected.

### 3.4.2 PolyJet™

The PolyJet™ transfection reagent (SignaGen® Laboratories) consists of a transfection solution that forms complexes with the provided plasmid DNA and delivers it to the cell through endocytosis. Cationic lipids in the transfection reagents helps to bind the complex to the plasma membrane. The coated vesicle is pinched off into the interior of the cell and the

plasma membrane coat as well as the PolyJet™ reagent is degraded in the cytoplasm. The construct is brought into the nucleus and expressed in the cell (SignaGen® Laboratories).

Transfections were performed according to the protocol suggested by the manufacturer. For the six well plate the surface area is 10 $cm^2$ and the amount of DNA added to each well should be 1 μg per construct. Therefore, the Stim1-YFP and mCHERRY-Orai1 constructs are co-transfected 1:1, or 1000 ng: 1000 ng.

**Cell transfection**

Prior to transfection, the cells were supplemented with 1ml fresh supplemented DMEM (3.4.1).

1000ng DNA was diluted in 100μl high glucose media without BCS, antibiotics or L-glutamine.

8μl of PolyJet™ transfection reagent was diluted in 100μl high glucose media without BCS, antibiotics or L-glutamine.

The PolyJet™ dilution was added to the DNA dilution. The reaction was mixed by pipetting up and down 4 times.

The reaction was incubated at room temperature for 10-15 minutes and added dropwise to the cells.

After 8 hours incubation at 37°C with 5% $CO_2$ the media was exchanged with 1ml fresh supplemented DMEM. The cells were incubated at the same conditions for another 16 hours.

### 3.4.3 Whole cell protein lysates

To investigate the expression of the transfected plasmids in the transfected cells, protein needs to be isolated. Whole cell proteins lysates were therefore prepared and Western blotting was performed with the protein lysates to detect Stim1 expression. The lysates were prepared with radioimmunoprecipitation assay (**RIPA**) buffer (SigmaAldrich). The protein concentration of the sample was measured with a Pierce™ bicinchoninic acid (**BCA**) Protein Assay kit according to manufacturer's instructions. This system is based on a standard curve of absorption, which the sample concentration is calculated from.

**Cell lysis and protein extraction**

The cells were scraped to loosen them from the growth surface and pipetted into a 15 ml tube.

The cells were centrifuged in a table-top centrifuge at 1200 rpm for 7 minutes and the cell-pellet washed with 1x Phosphate Buffered Saline, pH 7,4 (**PBS**).
The washing was repeated two times.
RIPA**,** buffer with 0,1% Proteinase A, protease and phosphatase inhibitor, was used to re-suspend the cell pellet.
The cells were rotated for 15 minutes at 4°C to lyse the cells.
The cell debris was spun down at 14 000 rpm at 4°C for 15 minutes.
The supernatant was transferred to a new tube.
The protein concentrations were measured and the lysates were stored at -80°C.

**Composition of RIPA buffer**

25mM Tris•HCl pH 7.6
150mM NaCl
1% NP-40
1% sodium deoxycholate
0.1% SDS

**Pierce™ BCA Protein Assay**

A standard curve was prepared by using Bovine Serum Albumin as a protein standard at six different concentrations; 2 mg/ml, 1 mg/ml, 0,5mg/ml, 0,25 mg/ml, 0,125 mg/ml and 0,063 mg/ml.
The samples were diluted 1:3 with RIPA buffer.
The standard curves and samples were added in triplets in a 96 well plate.
200µl of kit WR reagent was added to each well and the samples mixed on a plate mixer.
The plate was incubated at 37°C for 30 minutes, protected from light.
The absorption at 562 nm is recorded and the concentration back-calculated with the standard curve.

### 3.4.4 Imaging

YFP fluorescence was assessed with a Nikon eclipse Ts2 and illuminated at 470 nm. All of the images were captured with a 20x objective.

## 3.5 Western blotting

| Antibody | Origin |
|---|---|
| Stim1-C-terminal | Gift from Professor Stefan Feske, NYUMC New York |
| Orai1-N-terminal | Abcam, Cambridge |
| GADPH | Abcam, Cambridge |
| Secondary α-rabbit | Thermo Fisher, Waltham |

***Table 6 Antibodies for western blotting***

Western blotting was performed on whole cell lysates of 10µg total protein in a total volume of 16µl ($H_2O$) and with 8µl 3x loading buffer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**) separates 10µg protein on a BIO RAD® TGX$^{TM}$ any KDa® 10 well precast gel. The gel was run for approximately 50 minutes at 185V.
The proteins were wet-blotted onto a nitrocellulose membrane. The blotting was performed on ice with magnetic stirring, at 110V for exactly 20 minutes.

The proteins transferred to the nitrocellulose membrane were blocked with 5% Bovine Serum Albumin (BSA) in Tris-Buffered Saline with Tween20 (**TBST**) for 1 hour at room temperature. Then it was washed with TBST for 10 minutes three times.
The nitrocellulose membrane was incubated overnight at 4°C with the primary antibody. After the first incubation, the membrane was treated with an anti-rabbit IgG secondary antibody conjugated with HRP for 1 hour at room temperature and the membrane was developed with Amersham ECL Primer Western Blotting Detection Reagent (GE Healthcare Life Sciences).

The membrane was re-blocked in TBST with 5% BSA for 1 hour at room temperature. Afterwards it was incubated with an anti-GAPDH primary antibody and a HRP conjugated α-rabbit IgG secondary antibody for 1 hour at room temperature as a control antibody. The membrane was developed with the same reagents, the Amersham ECL Primer Western Blotting Detection Reagent (GE Healthcare Life Sciences).

## 3.6 Imaging of transfected cells

The imaging of the transfected cells was performed on a Zeiss LSM 800 microscope, and captured with the ZEN image-processing program. The ZEN lite imaging-processing program

was used for analysis. The mCHERRY tag was excited at 560 nm and fluorescence was recorded at an interval of 580-700 nm (figure 15, red). The YFP tag was excited at 490 nm and fluorescence was recorded at an interval of 470-580 nm (figure 15, green).

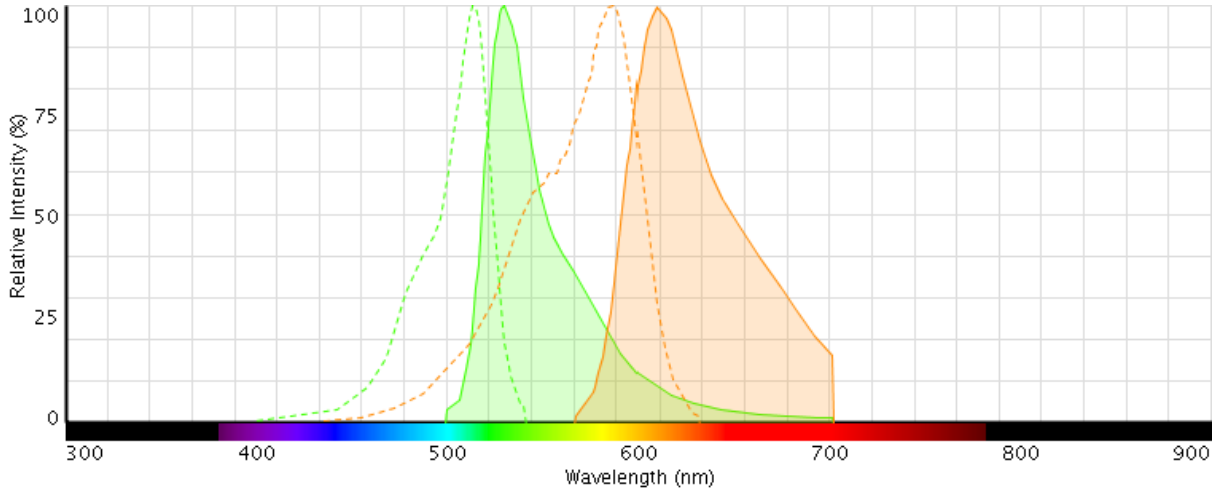



***Figure 15 Excitation and emission of the YFP tag and mCHERRY tag*** *The YFP tag is excited at 490nm and emission was recorded between 470 and 580nm. The mCHERRY tag is excited at 560 nm and emission was recorded between 580 and 700 nm.*

### 3.6.1 Fluorescence microscopy

Fluorescence microscopy is a powerful technique for visualization of cell organelles, specific proteins after transfections of vectors expressing fluorescently tagged proteins, immunofluorescence, and much more. The principle of fluorescence is the absorption and emission of light according to the principles of electrons moving orbits. When energy in the form of light is applied to an atom, electrons in the orbits that correspond to the amount of energy will be excited into a higher orbit. However, this is an unstable state for the atom and the electron will fall back into the original orbit, sending out light with a particular amount of energy. This energy corresponds to a wavelength of light and is known as fluorescence (Spring and Davidson, 2017).

### 3.6.2 Imaging

**Before induction**

Viable cells were located with phase contrast light microscopy, according to the morphological characteristics belonging to living healthy fibroblast cell cultures, as shown in figure 16. Unhealthy cells with a round shape, without elongated attachment structures were not imaged. The cells were kept in 500µl clear physiological $Ca^{2+}$ solution (2mM $Ca^{2+}$) and

were imaged with the Z-stacked function of the ZEN imaging processing software and with the appropriate settings to detect the two fluorescent tags (YFP-tag and mCHERRY) as described above.

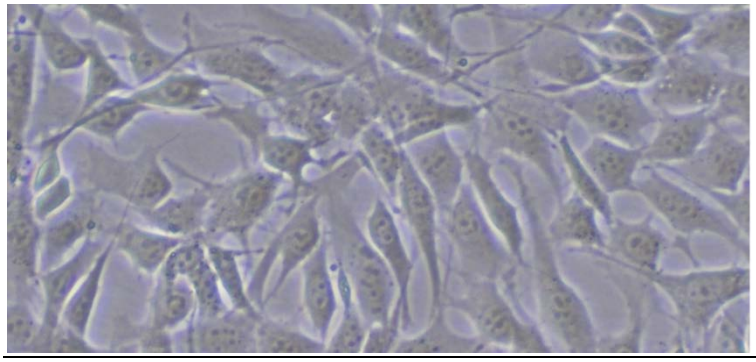


***Figure 16 Morphologically healthy fibroblasts*** *Cells with an elongated cell structure, growing attached to the surface and with a clear nuclear structure.*

**Thapsigargin induction**

1µl thapsigargin was added to the cells and the cells were left for 2-5 minutes before resuming imaging. The imaging was performed with the same settings as before SOCE induction.

## 3.7 Whole cell $Ca^{2+}$ measurements

Cells that were seeded on cover slips 2 days prior and transfected as described in chapter 3.4, were loaded with INDO-1 AM dye (10ng/µl for 20 minutes in dark, Molecular Probes, Life Technologies) and then mounted on the stage of an inverted Zeiss microscope equipped for fluorescence. A single transfected cell, confirmed by the presence of YFP-fluorescence representing Stim1, as well as red fluorescence representing Orai1, was isolated in the pinhole of the microscope. This cell was used to measure $Ca^{2+}$ fluxes based on whole cell fluorescence.

### 3.7.1 INDO-1 AM dye

The cell was excited with 350 nm UV-light from a laser source. INDO-1 AM emits light at a peak of 400 nm when it is bound to $Ca^{2+}$, and light at a peak of 475 nm when it is not bound to $Ca^{2+}$ (Molecular Probes, 2011). Data in these experiments are represented as a ratio of fluorescence at the two wave lengths.

2µg of INDO-1 AM (Life Technologies) was added to the cell chamber. As the wavelengths emitted were detected, we can measure how much intracellular $Ca^{2+}$ that was present when

the cell was perfused with different concentrations of extracellular $Ca^{2+}$ with or without thapsigargin. The INDO-1 AM molecule can freely diffuse across the hydrophobic cell membrane due to the presence of an acetoxy methyl (R) group, but as illustrated in figure 17, once the indo-dye enters the cytoplasm, esterases will cleave off the R-group trapping the dye inside the cell (Molecular Probes, 2011).

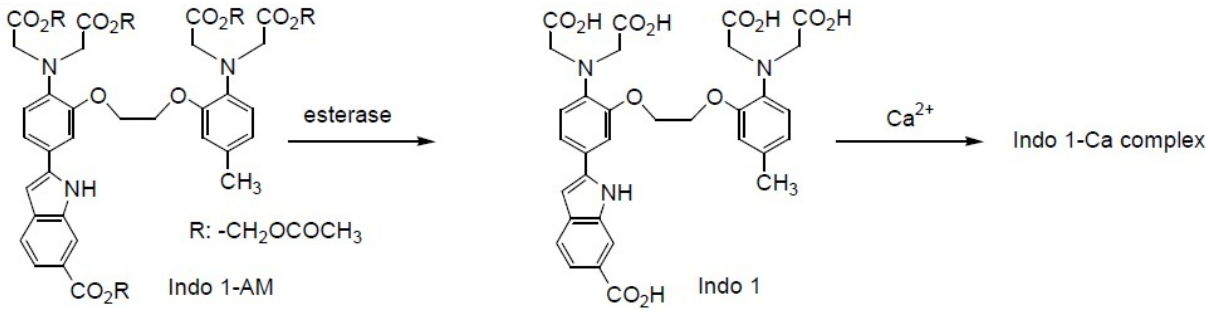



***Figure 17 Cleavage of the $CH_2OCOCH_3$ group from the INDO-1 AM molecule*** *The $CH_2OCOCH_3$ group is cleaved off the INDO-1 AM molecule by esterases that are present in the cytoplasm of the cell. This makes the INDO-1 AM molecule unable to diffuse across the cell-membrane ensuring that it stays inside the cell (Dojindo Molecular Technologies, Inc.).*

### 3.7.2 Solutions

| Solution | | HT buffer | $CaCl_2$ [1,0M] | Thapsigargin(**TG**) [1mM] |
|---|---|---|---|---|
| A | 2 mM $Ca^{2+}$ | 50 ml | 100 µl | |
| B | 0 $Ca^{2+}$ + TG | 50 ml | | 100 µl |
| C | 10 mM $Ca^{2+}$ + TG | 50 ml | 500 µl | 100 µl |

***Table 7 Solutions in functional calcium flux experiments****. See supplements for HT buffer composition*

**Recordings**

The coverslip with the transfected cells was placed in a metal frame fixed to a perfusion setup with a flow rate of 0,6 ml/min. Suction removes fluid from the chamber setup so that the volume is kept at 500 µl.

The cell was perfused with solution A, B and C and the fluorescence was recorded for 10 seconds, 5 minutes and 5 minutes respectively.
Solution B contains thapsigargin and this should induce SOCE.
Solution C contains thapsigargin as well as 10mM $Ca^{2+}$.
The pinhole was changed to a nearby place on the coverslip that contains no cell and the fluorescence was measured. This defines the background fluorescence that was subtracted from the cellular fluorescence.

### 3.7.3 Microscope and analysis

**Microscope details**

The microscope system used was an Carl Zeizz inverted light microscope (Zeiss, Oberkochen). Viable cells were located with phase contrast light microscopy and illuminated at 435 nm wavelengths so that the YFP tag can fluoresce, as well as 561 nm light for the mCHERRY tag. This to verify co-transfection of the constructs. A single cell was adjusted to the pinhole of the microscope. The cell was illuminated with 350 nm UV-laser light for recording the INDO-1 AM emission. The emission was recorded at 400 nm and 475 nm, which represents $Ca^{2+}$ bound and unbound respectively, in the Clampfit 10.4 program (Molecular Devices).

**Clampfit analysis**

In the Clampfit 10.4 program, data analysis was performed by selecting three data points in the recorded fluorescence.

The first data point was the resting level under perfusion of solution A.

The second was the stable base level before switching to solution C. This was 5 minutes after solution B was introduced.

The third point was the peak after perfusion of solution C. This was the peak of $Ca^{2+}$ influx.

The background measured was deducted and the ratio of the two emissions was presented.

# 4 Results

## 4.1 Construction of vectors with mutant Stim1

In this thesis, vectors were constructed for the expression of various Stim1-mutants where the arginine (R) at position p.304 was substituted with the amino acids shown in figure 18.

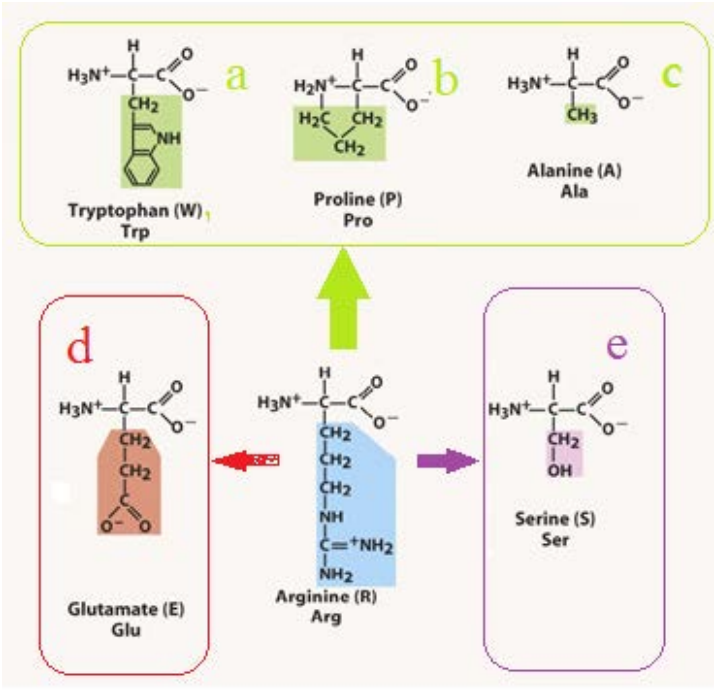



***Figure 18 Amino acid substitutions performed at Stim1 p.304 in this thesis*** *Arginine is the amino acid at the p.304 position in the wild type* Stim1 *protein (Middle).* STIM1 *p.R304W causes constitutive SOCE in Stormorken patients (Misceo et al., 2014).*

| Amino acid | Charge |
|---|---|
| Arginine | Positive |
| Tryptophan | Nonpolar, bulky |
| Serine | Uncharged, polar |
| Glutamate | Negative |
| Alanine | Nonpolar, small |
| Proline | Nonpolar |

***Table 8 Functional properties of the amino acids introduced in the p.304 position of* Stim1 *in this thesis***

These substitutions were chosen due to their differing characteristics in charge and structure from the arginine present in wild-type Stim1 (Figure 18 and table 8).

PCR based mutagenesis was performed with the NEB Q5 mutagenesis kit as described in chapter 3.2. The primers used for the mutagenesis (Table 5, page 21) anneal to the template

and introduce base changes in the subsequent cycles of PCR because of the mismatches in the primer as illustrated in figure 19 for the p.R304A construct.

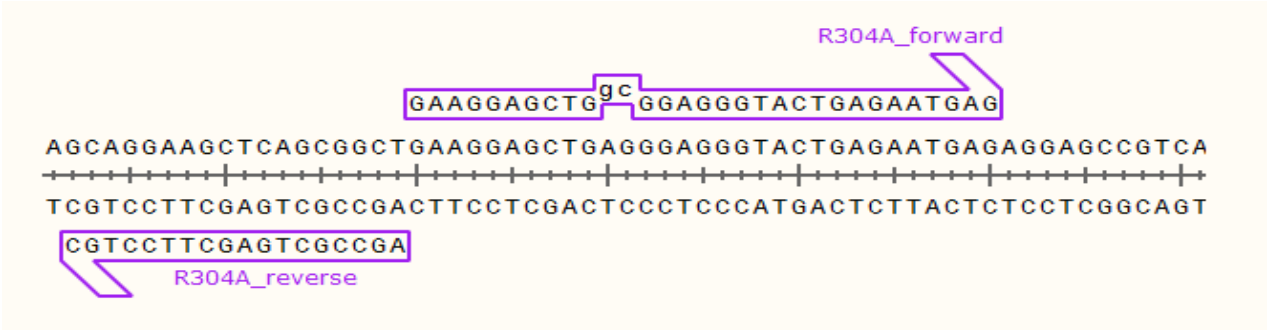



***Figure 19 p.R304A construct template annealing*** *The R304A _forward and the R304A/P/E_reverse primers anneal to the template sequence and elongate in opposite directions. The forward primer introduces changes to bases in the sequence; c.910A>G and c.911G>C resulting in expression of Stim1 with the p.R304A change from the resulting construct. The figure was constructed using SnapGene software (snapgene.com).*

In addition, deletion constructs were made using primers that anneal to the template three base pairs apart to delete the p.296 codon from the Stim1 WT and Stim1-p.R304W plasmid constructs (figure 20).

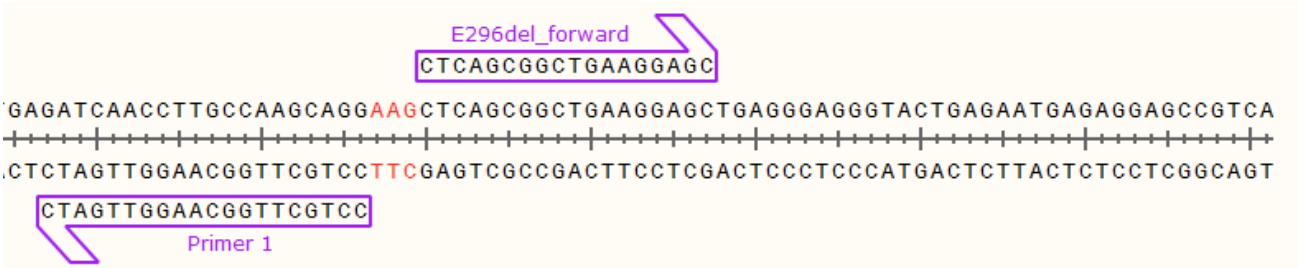



***Figure 20 p.E296del construct template annealing*** *The primers E296del_forward and E296del_reverse anneal to the template three base pairs apart. Therefore, codon 296 (red) is deleted in the resulting construct, creating the p.E296del construct. The figure was constructed using SnapGene software (snapgene.com).*

### 4.1.1 Verifying the presence of the Stim1 mutations

The mutagenesis products were transformed into E. coli (materials and methods 3.2.1), which were grown over night at 37°. Direct PCR was performed on DNA from five colonies per plate and subsequent sequencing covered the mutated region and assessed whether the colony contained a construct with only the intended mutation. The sequences were analyzed using ‘Blat’, aligning the sequence to the database *Stim1* gene, GRCm38/mm10, as shown for the p.R304A-construct in the example in figure 21.

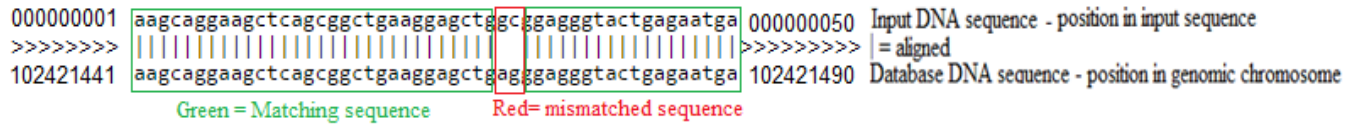



***Figure 21 Blat alignment of an example sequence*** *In this figure the red square indicates mismatched bases when aligning the Sanger read (input) to the sequence from murine chromosome 7 below.*

Overnight cultures from one colony from each construct verified to contain the correct mutation were used for plasmid DNA isolation (3.3.1). The extracted plasmid DNA was Sanger sequenced with primers R1, R2, F3 and F7 (figure 22) resulting in the average sequencing read of approximately 600 bp per sequencing reaction as indicated in the figure.

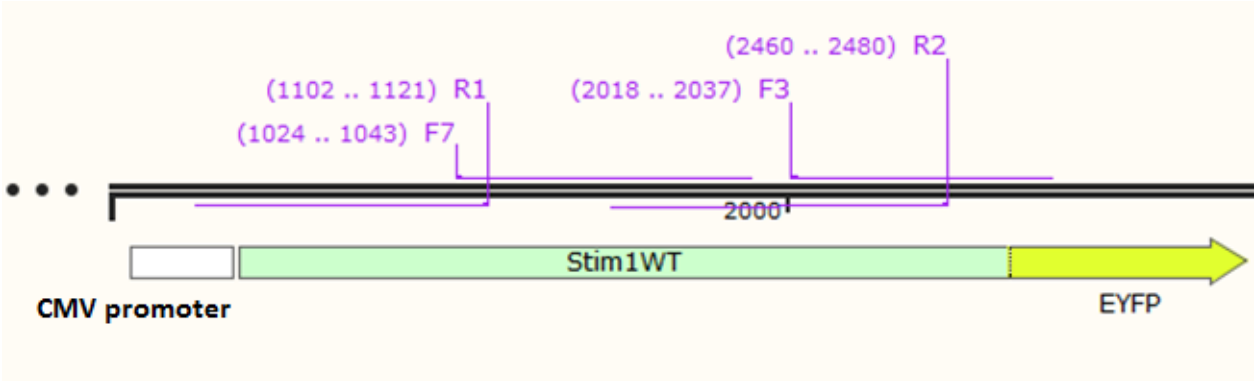



***Figure 22 Primers for Stim1 sequencing and approximate read length obtained by the Sanger sequencing*** *The primers used for sequencing are R1, R2, F3 and F7. The elongated purple lines indicate the average sequencing read length for each primer.*

Figure 23 shows the introduced mutations in an alignment of the mutated region in the clones sequenced in plasmid DNA extracted from over-night cultures. The rest of the Stim1 sequence acquired from Sanger sequencing contained no deviations when aligned to the mouse reference genome (Data not shown).

The complete data from Sanger sequencing are shown in supplements 8.8.

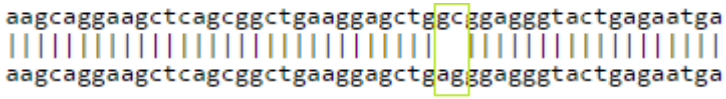
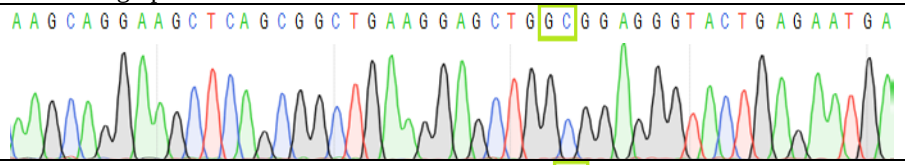
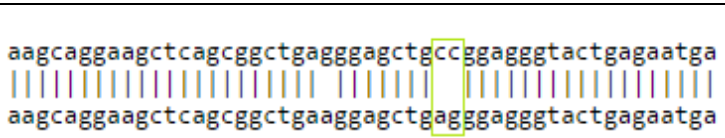
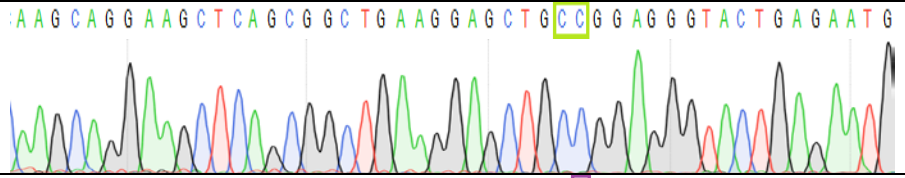
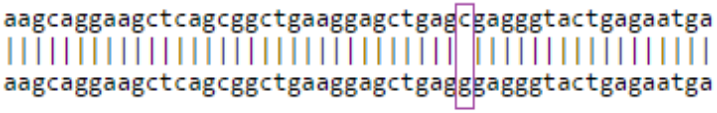
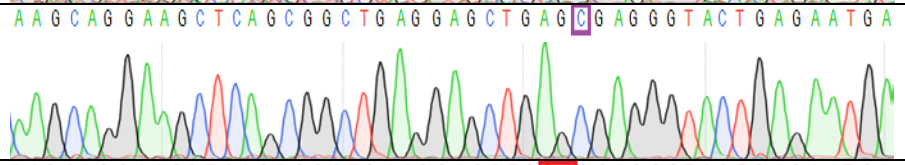
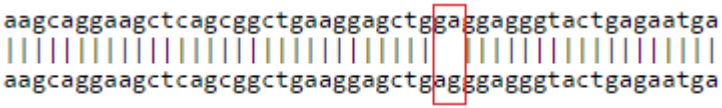
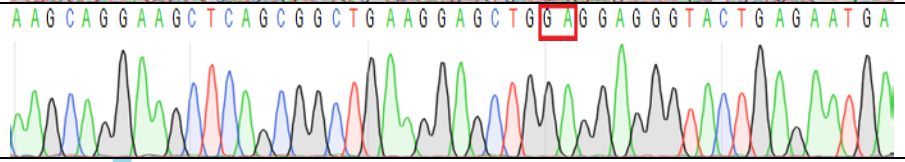
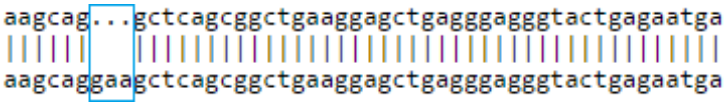
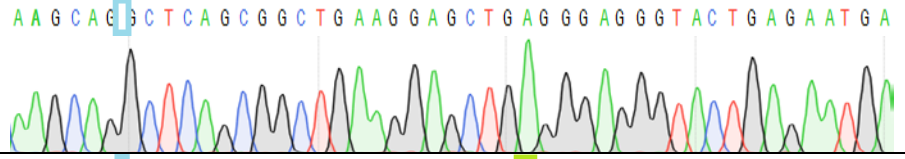
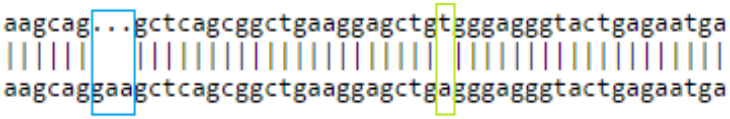
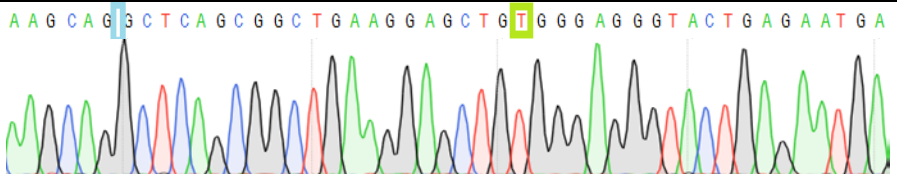
| Mutation | Codon change | | Alignment | Chromatograph |
|---|---|---|---|---|
| p.R304A | AGG | GCG |  |  |
| p.R304P | AGG | ACG |  |  |
| p.R304S | AGG | AGC |  |  |
| p.R304E | AGG | GAG |  |  |
| p.E296del | Deletion GAA at p.296 | |  |  |
| p.E296del +R304W | Deletion GAA at p.296 Template carries p.R304W | |  |  |

***Figure 23 Alignment of plasmid DNA sequence using Blat*** *The upper sequences are the DNA sequences from the plasmid DNA from the colonies, acquired from Sanger sequencing. The lower sequences are the reference sequences. The coloured squares represents where the plasmid DNA sequence differs from the reference, resulting in amino acid changes at position p.304 or p.296. To the right are the corresponding Sanger sequence chromatograms.*

Clones carrying the verified mutated Stim1 constructs were identified as indicated in Table 9.

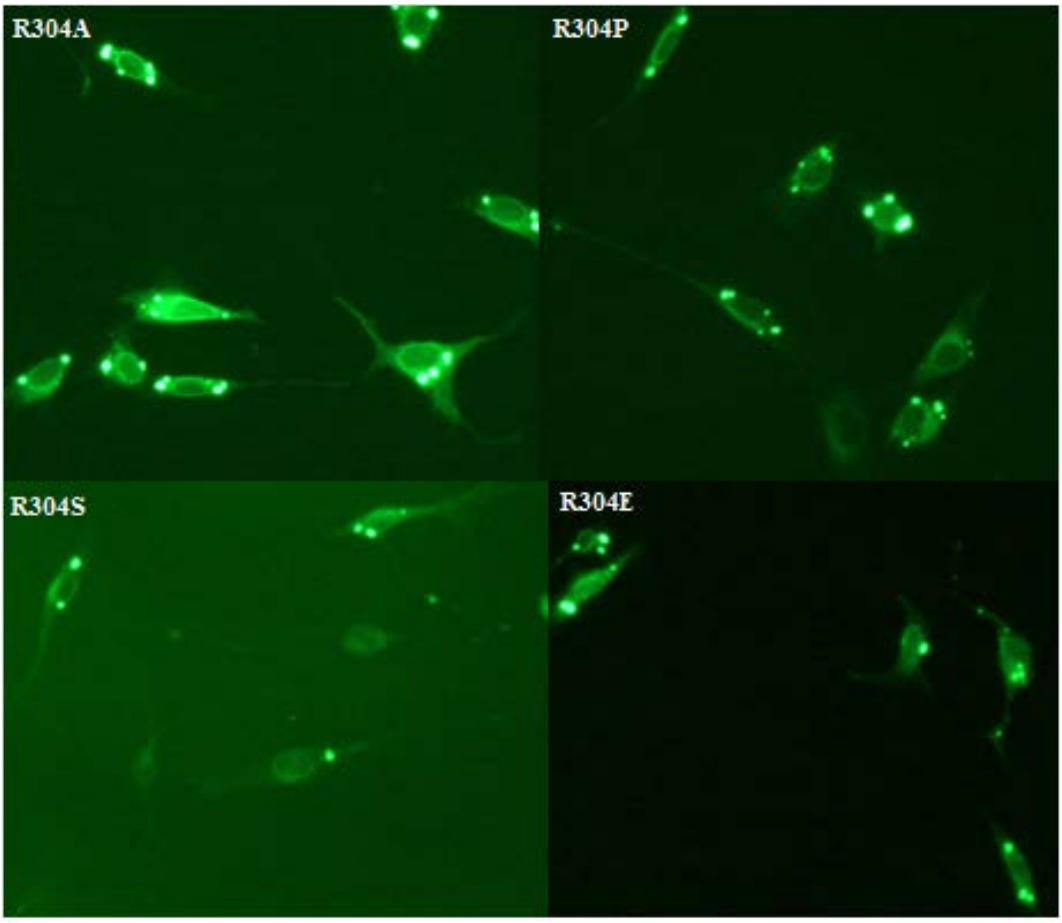
| Construct name | Amino acid substitution in Stim1 | Primers used for mutagenesis | Template used in the mutagenesis | Number of colonies tested | Colonies with substitution | Colonies without other alterations from reference | Mutation | Codon change | |
|---|---|---|---|---|---|---|---|---|---|
| Stim1-R304A-YFP | p.R304A | R304A_forward R304A/P/E_reverse | Stim1-WT-YFP | 5 | 2 | 2 | c.910A>G and c.911G>C | AGG | GCG |
| Stim1-R304P-YFP | p.R304P | R304P_forward R304A/P/E_reverse | | 5 | 3 | 1 | c.911G>C | AGG | ACG |
| Stim1-R304S-YFP | p.R304S | R304S_forward R304S_reverse | | 5 | 2 | 1 | c.912G>C | AGG | AGC |
| Stim1-R304E-YFP | p.R304E | R304E_forward R304A/P/E_reverse | | 5 | 3 | 1 | c.910A>G and c.911G>A | AGG | GAG |
| Stim1-E296del-YFP | p.E296del | E296del_forward E296del_reverse | | 5 | 3 | 1 | c.del886-888 | Deletion of codon 296 (GAA) | |
| Stim1-E296del+R304W-YFP | p.E296del+ R304W | E296del_forward E296del_reverse | Stim1-R304W-YFP | 5 | 2 | 1 | c.del886-888 | Deletion of codon 296 (GAA); R304W mutation present in template | |

**Table 9** The expression vectors constructed by Q5 mutagenesis

### 4.1.2 Expression of the YFP marker

All six constructs were transfected into the murine NIH 3T3 fibroblast cell line to assess fluorescence. The transfected cells were inspected in a fluorescence microscope after incubation at 37°C with 5% $CO_2$ for 24 hours. The cells that were transfected with each of the six Stim1-YFP constructs demonstrated fluorescence when illuminated with 470nm light, (figure 24) verifying the expression of the YFP marker.

**A.**



B.

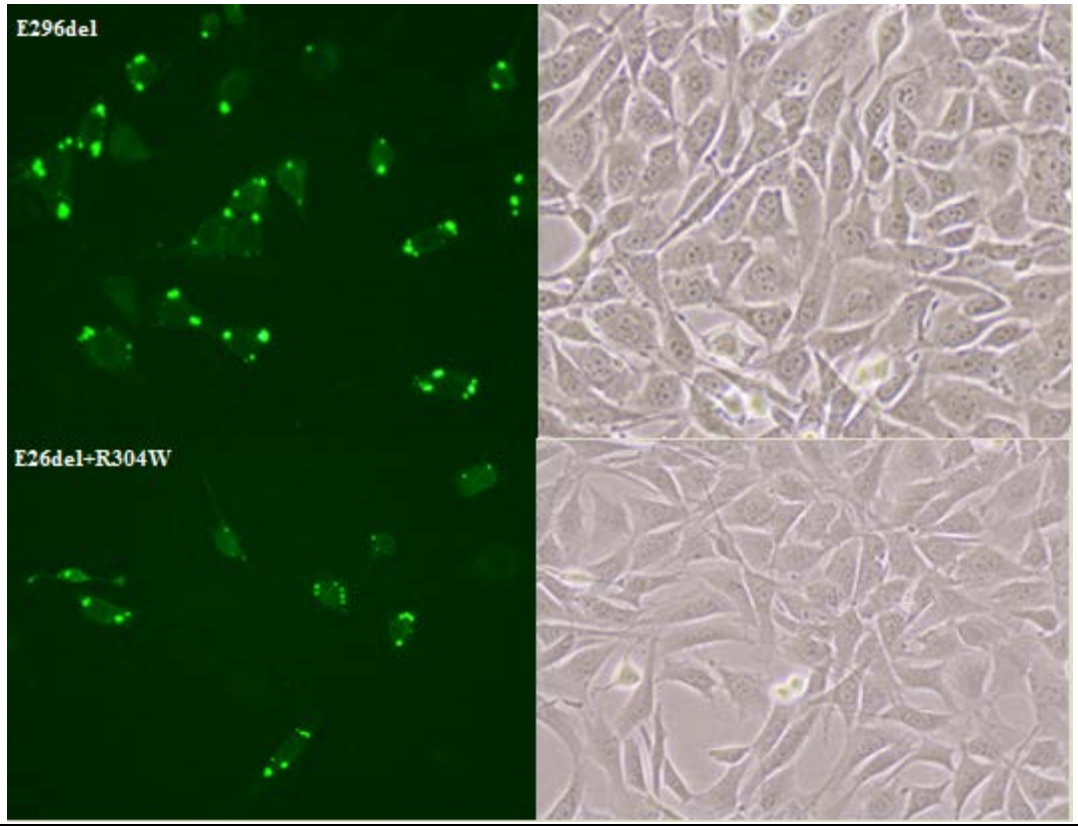



***Figure 24 Detection of fluorescence from transfected cells*** *A. The four Stim1 constructs indicated gave fluorescence signals after transfection into NIH 3T3 cells. B. A fraction of the cells transfected with the constructs expressing the deletion mutants p.E296del and p.E296del+R304W as indicated show fluorescence (left) when comparing to the same field captured with bright-field (right).*

### 4.1.3 Expression of Stim1-YFP fusion proteins

Western blotting was performed to investigate if the Stim1-YFP fusion gene is expressed in cells transfected with the vectors constructed. The transfections and Western blotting were performed as described in materials and methods. The nitrocellulose membrane was treated with a Stim1 antibody (table 6).

Western blotting was also performed with whole cell lysates from cells transfected with a construct expressing an un-tagged Stim1.

The band corresponding to the endogenous Stim1 protein of 90 kDa was detected in all cells analyzed. The western blot results in figure 25 show a band corresponding to the Stim1-YFP fusion protein, in cells transfected with all six vectors. This band, which corresponds to 117 kDa because the YFP tag adds approximately 27 kDa, was not detected in un-transfected cells and cells transfected with the construct expressing untagged Stim1.

These results support the conclusion that all six constructs express a Stim1-YFP fusion protein.

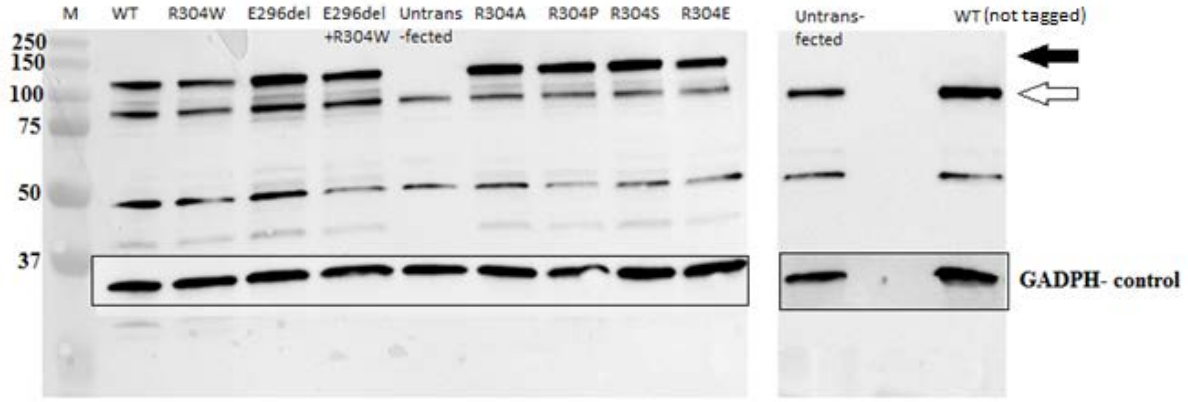



***Figure 25 Western blots of transfected and un-transfected NIH 3T3 cells*** *A signal is detected with the Stim1 antibody in all lanes transfected with the Stim1-YFP constructs indicated (Black arrow). Endogenous* Stim1 *expression is detected in all lanes (White arrow). The signal obtained with the control antibody detecting GADPH indicated equal protein loading. M- Precision plus protein ladder kDa (BIO-RAD).*

Western blotting was also performed to investigate the endogenous expression of Orai1 in the murine NIH 3T3 fibroblast cell line. The nitrocellulose membrane was treated with an anti-Orai1 antibody (Abcam, Cambridge). A signal of the expected size was detected on the membrane (figure 26).

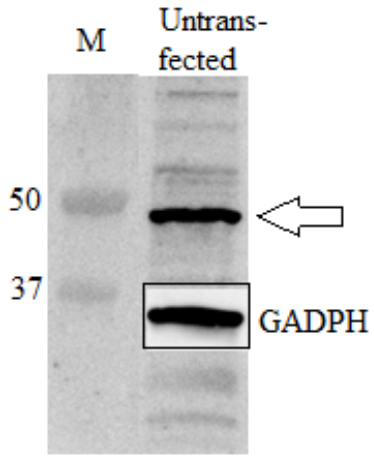



***Figure 26 Endogenous* Orai1 *expression in NIH 3T3 cells*** *A signal is detected with the Orai1 antibody in NIH 3T3 cells (White arrow). This corresponds to endogenous Orai1 expression. M- Precision plus protein ladder kDa (BIO-RAD)*

## 4.2 Assessment of puncta formation

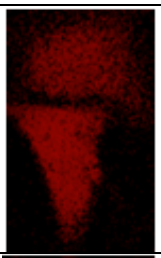
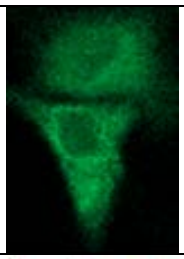
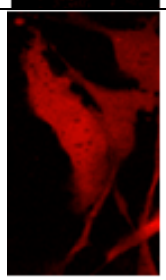
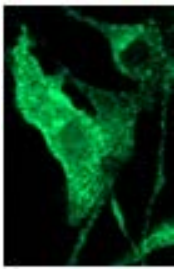
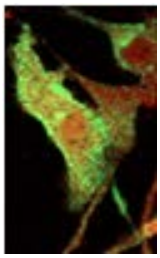
### 4.2.1 WT transfected cells – puncta formation

Imaging experiments were performed to assess puncta formation, which may be detected by co-localization of Orai1 and Stim1 in transfected cells. The transfection was performed with PolyJet$^{TM}$ (3.4.2). After 24 hours of incubation at 37°C in 5% $CO_2$ the cells were inspected in a Zeiss LSM 800 microscope using 560 nm excitation for the mCHERRY tag and 490 nm light for the YFP tag (3.6).

In two independent experiments transfections were performed with 1,0 µg Stim1-WT-YFP and 1.0 µg mCHERRY-Orai1 (3.4.2). Each experiment was performed as four parallel transfections. Approximately 20 cells/groups of cells were imaged in each transfection, and all images were included in analysis. Figure 27 shows representative transfected cells with or without TG treatment.

The cells that are not treated with thapsigargin show both the red and yellow fluorescence evenly distributed in the cells.

TG treated cells transfected with the Stim1-WT-construct show a speckled distribution of the YFP signal, which differs from the even cytoplasmic distribution in cells not treated with TG, thus indicating puncta formation (figure 27). The mCHERRY-Orai1 signal show even distribution in the cell both with and without TG treatment.

| Thapsigargin | Red channel | Yellow channel | Merge |
|---|---|---|---|
| - |  |  |  |
| - |  |  |  |

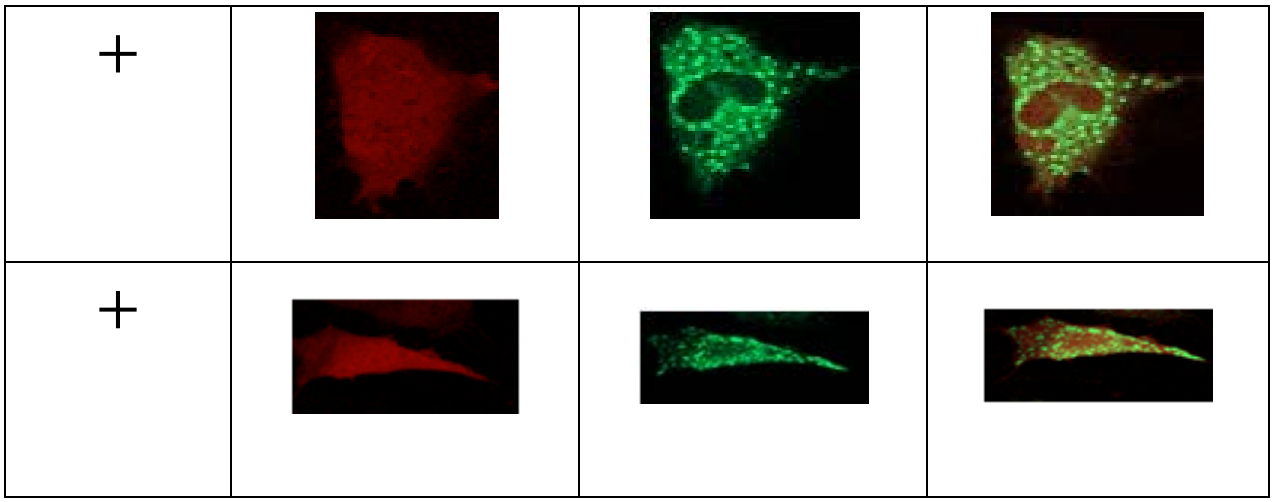


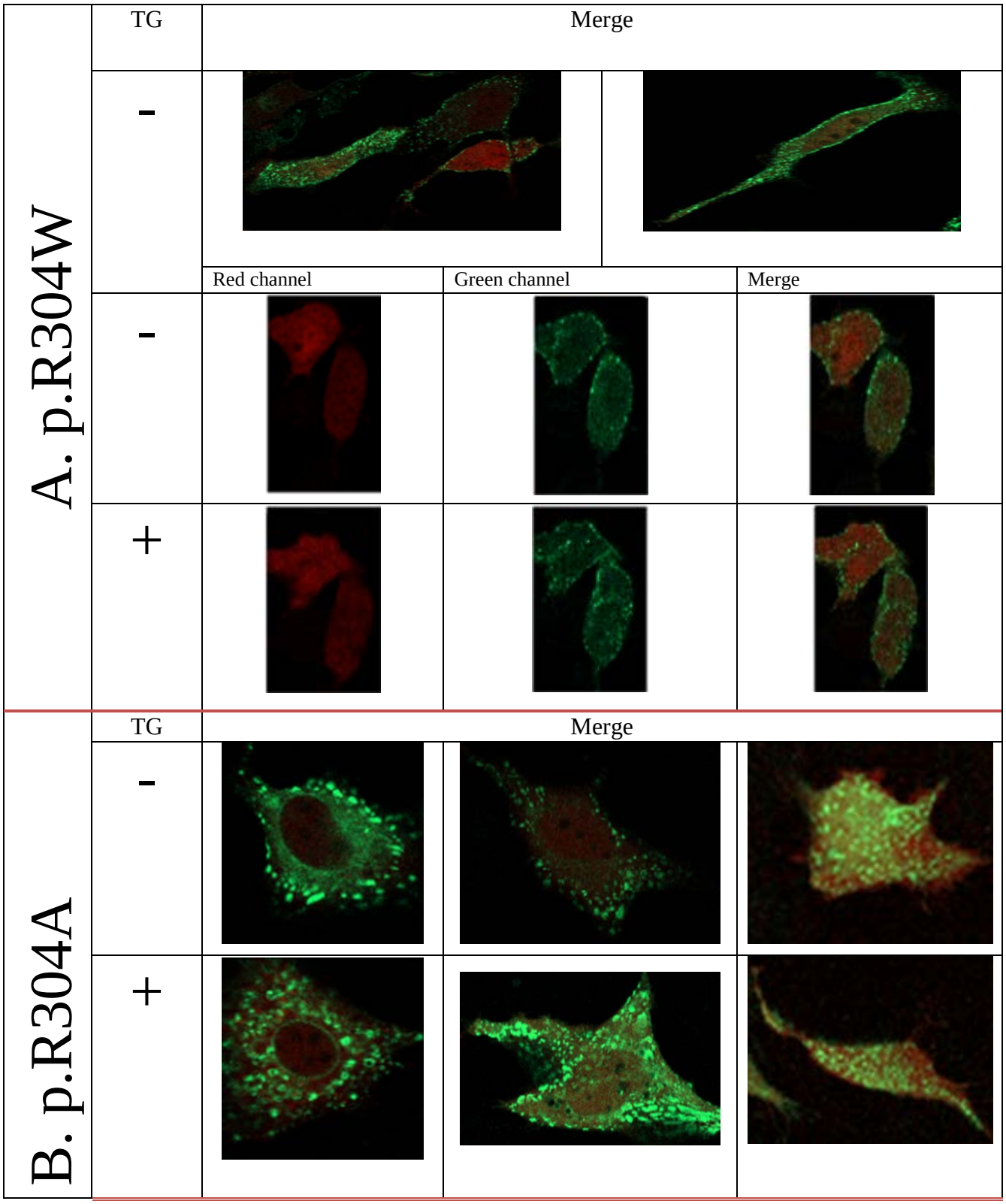
***Figure 27 Stim1-WT transfected cells before and after TG treatment*** *The red channel detects the mCHERRY-Orai1 expression and the yellow channel detects the Stim1-YFP expression. The emission signal of the YFP tag resides in the shorter wavelengths, close to green, and therefore the tag may appear green.*

### 4.2.2 Puncta formation in cells with ectopic expression of mutated Stim1

In order to assess if differences in signal distribution can be detected upon TG treatment in cells ectopically expressing mutant Stim1, two independent experiments were performed with four parallel transfections using constructs expressing Stim1-p.R304W, Stim1-p.R304A, Stim1-p.R304P, Stim1-p.R304S, Stim1-p.R304E, Stim1-p.E296del and Stim1-p.E296del+R304W.

Representative images demonstrating speckled signal distribution with and without TG treatment of cells ectopically expressing Stim1-p.R304W and Stim1-p.R304A are shown in figure 28 A and B. These results may indicate that puncta were formed without TG treatment in these cells, indicating a constitutively activated Stim1.

The cells transfected with Stim1-p.E296del+R304W showed an even distribution of YFP in cells not treated with TG, but the TG treated cells showed that the YFP signals tend to aggregate at specific areas indicating puncta formation (figure 28C).


A. p.R304W
TG
Merge
-
Red channel
Green channel
Merge
-
+
B. p.R304A
TG
Merge
-
+

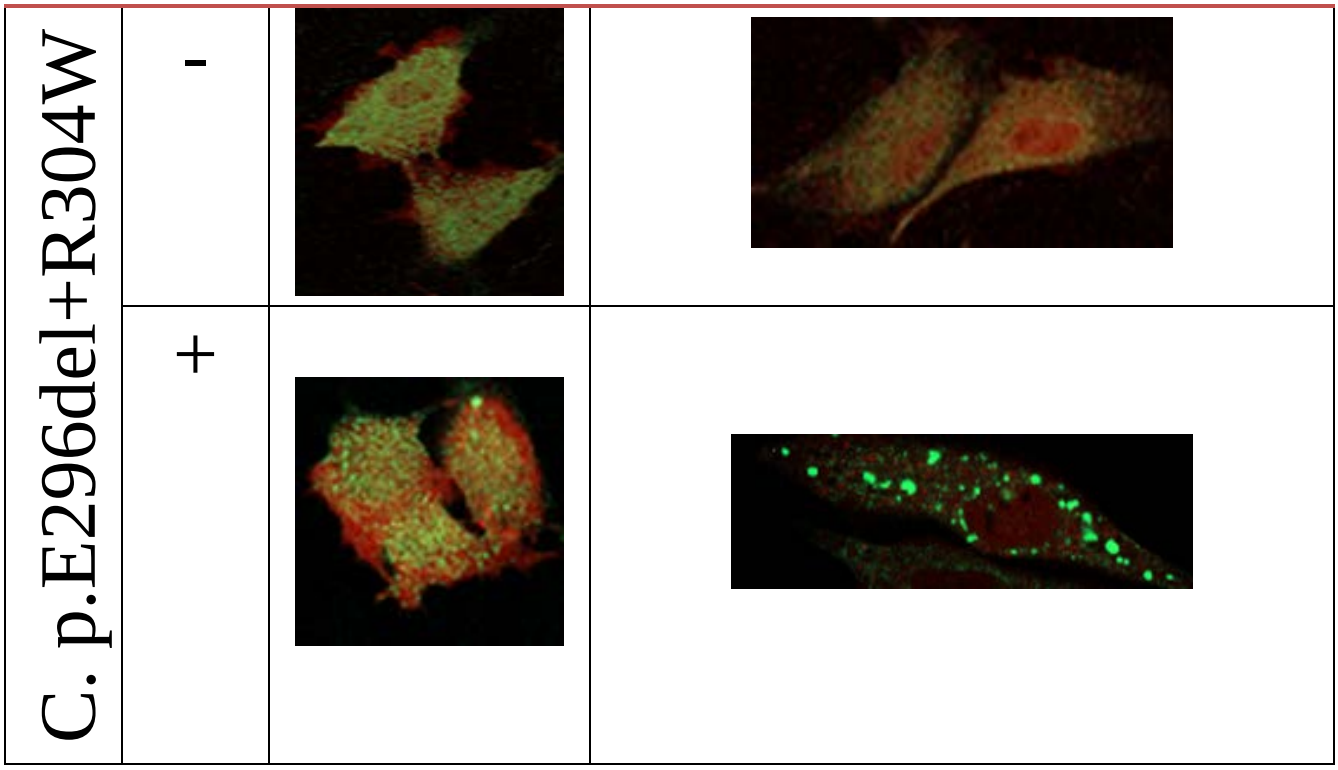
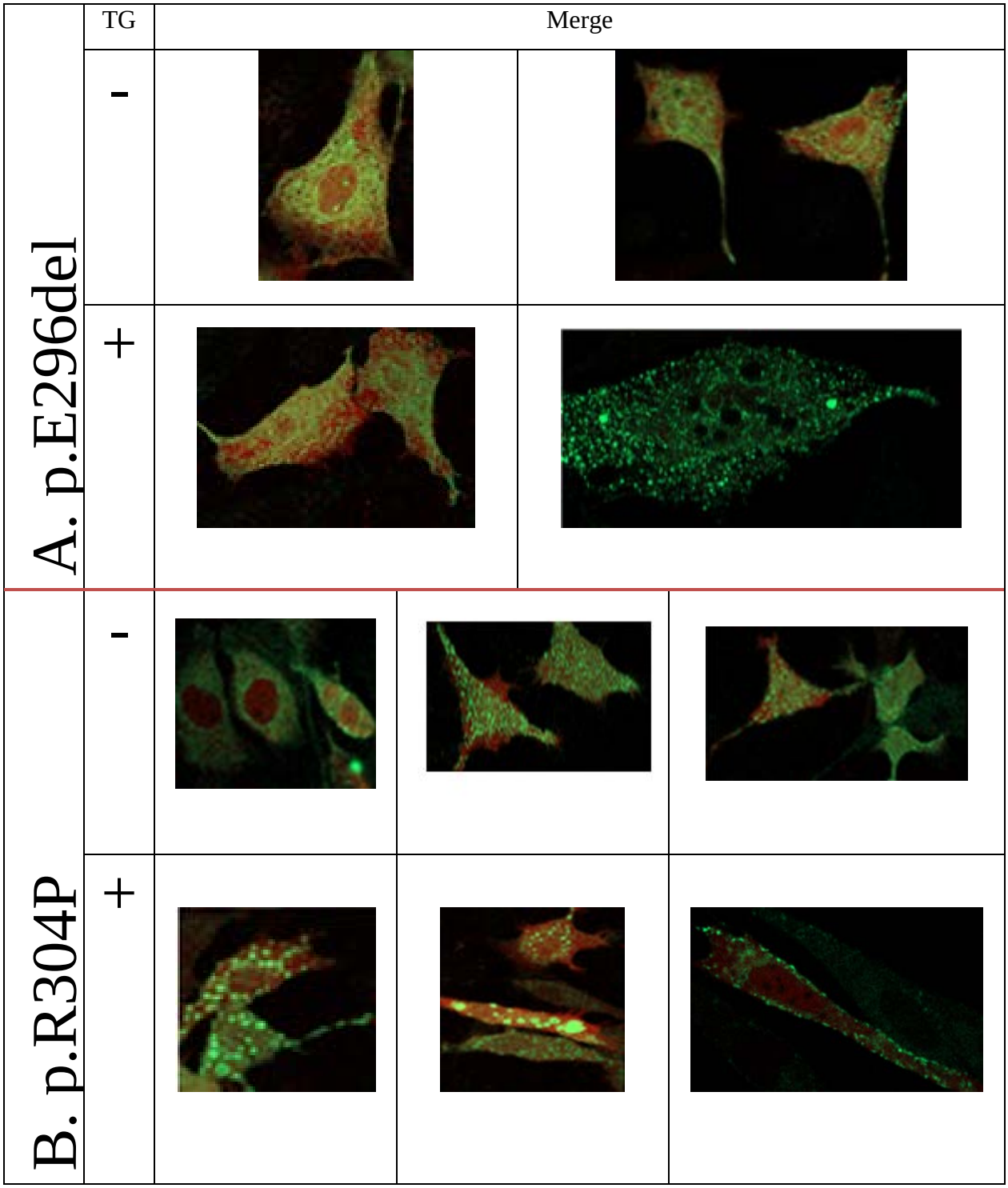



***Figure 28 Ectopic expression of Stim1-constructs*** *A. Stim1-p.R304W B. Stim1-p.R304A C. Stim1-p.E296del+R304W. Representative murine NIH 3T3 fibroblast cells co-expressing Stim-YFP variants (green) and mCHERRY-Orai1 (red) presented as a merge of the two signals. Stim1-p.R304W and Stim1-p.R304A show puncta formation with and without TG treatment. Stim1-p.E296del+R304W show puncta formation only with TG treatment.*

The constructs Stim1-p.E296del, Stim1-p.R304P and Stim1-p.R304S, gave variable results, some cells showed an even distribution of the YFP signal and some cells gave a speckled YFP pattern. The variable results were detected in both untreated and TG treated cells (figure 29).


A. p.E296del
B. p.R304P
TG
Merge
-
+
-
+

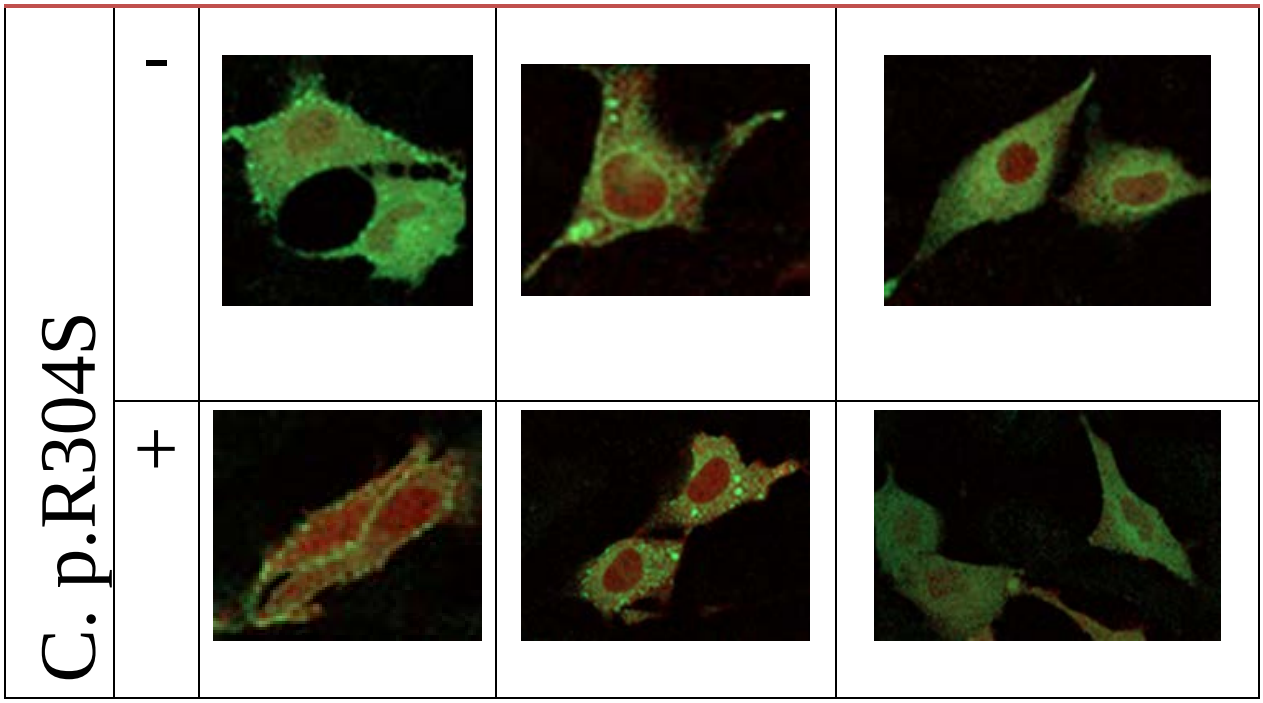



***Figure 29 Ectopic expression of mutant Stim1*** *A. Stim1-p.E296del B. Stim1-p.R304P C. Stim1-p.R304S. Representative murine NIH 3T3 fibroblast cells co-expressing Stim-YFP variants (green) and mCHERRY-Orai1 (red) presented as a merge of the two signals. All constructs show large variation both with and without TG treatment and it is difficult to determine puncta formation.*

In the wells with cells transfected with Stim1-YFP-p.R304E, there were many living cells without YFP or mCHERRY signals. The cells that showed both fluorescence signals had an appearance indicating that they were dead or dying cells, as demonstrated with examples in figure 30. They showed an elongated shape and the red signal showed ‘blobbing’ in the few cells that had the morphological appearance of living murine NIH 3T3 fibroblast cells.

These results may indicate that a cell death process was induced in cells co-transfected with Stim1-p.R304E and Orai1, but additional experiments are needed in order to explore if ectopic Stim1-p.R304E and Orai1 expression may be toxic to the NIH 3T3 cells.

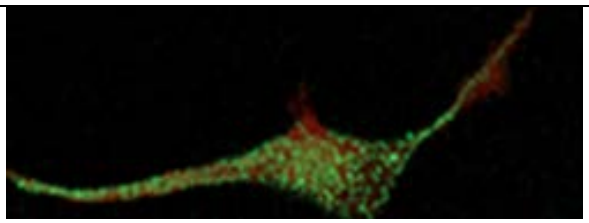
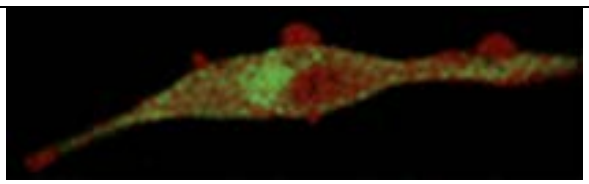
| | |
|---|---|
| R304E –TG Experiment 1 |  |
| R304E –TG Experiment 2 |  |

***Figure 30 Appearance of Stim1-p.R304E-YFP and m-CHERRY-Orai transfected cells.*** *Representative murine fibroblast 3T3 cells co-expressing Stim-YFP variants (green) and mCHERRY-Orai1 (red) presented as a merge of the two signals. The cells that are transfected are either dead or dying and the cells that still have an elongated cell shape, indicate red 'blobbing'.*

## 4.3 Measuring $Ca^{2+}$-levels in transfected cells

In order to measure $Ca^{2+}$-flux in cells ectopically expressing mutant Stim1, the cells were transfected with constructs expressing Stim1-WT, Stim1-p.R304W, Stim1-p.E296del and Stim1-p.E296del+R304W in co-transfection with mCHERRY-Orai1. The next day cells were treated with the dye INDO-1 AM, as described in material and methods and the whole cell fluorescence is presented as emission ratios in graphs in figure 32.

Figure 32 shows the results from measurements in cells co-transfected with mCHERRY-*Orai1* and constructs expressing Stim1-WT, Stim1-p.R304W, Stim1-p.E296del or Stim1-p.E296del+R304W. Figure 32A shows the resting $Ca^{2+}$ levels in cells incubated in medium with 2mM $Ca^{2+}$ without TG. In these measurements the Stim1-p.R304W-, Stim1-p.E296del- and Stim1-WT- expressing cells show similar resting $Ca^{2+}$ level, although Stim1-p.R304W has a slight tendency to be increased. The cells expressing Stim1-p.E296del+R304W show a tendency for lower resting $Ca^{2+}$ level than the Stim1-WT expressing cells.

Figure 32B shows the change in the fluorescent ratio, and therefore the influx of $Ca^{2+}$, after TG treatment in medium containing 10mM $Ca^{2+}$. The levels in cells expressing Stim1-p.R304W and Stim1-p.E296del+R304W are comparable to cells expressing Stim1-WT although there may be a tendency for a slight increase. There is a tendency for an increase in the cells expressing Stim1-p.E296del. Additional experiments are required to get sufficient data to obtain conclusive results.

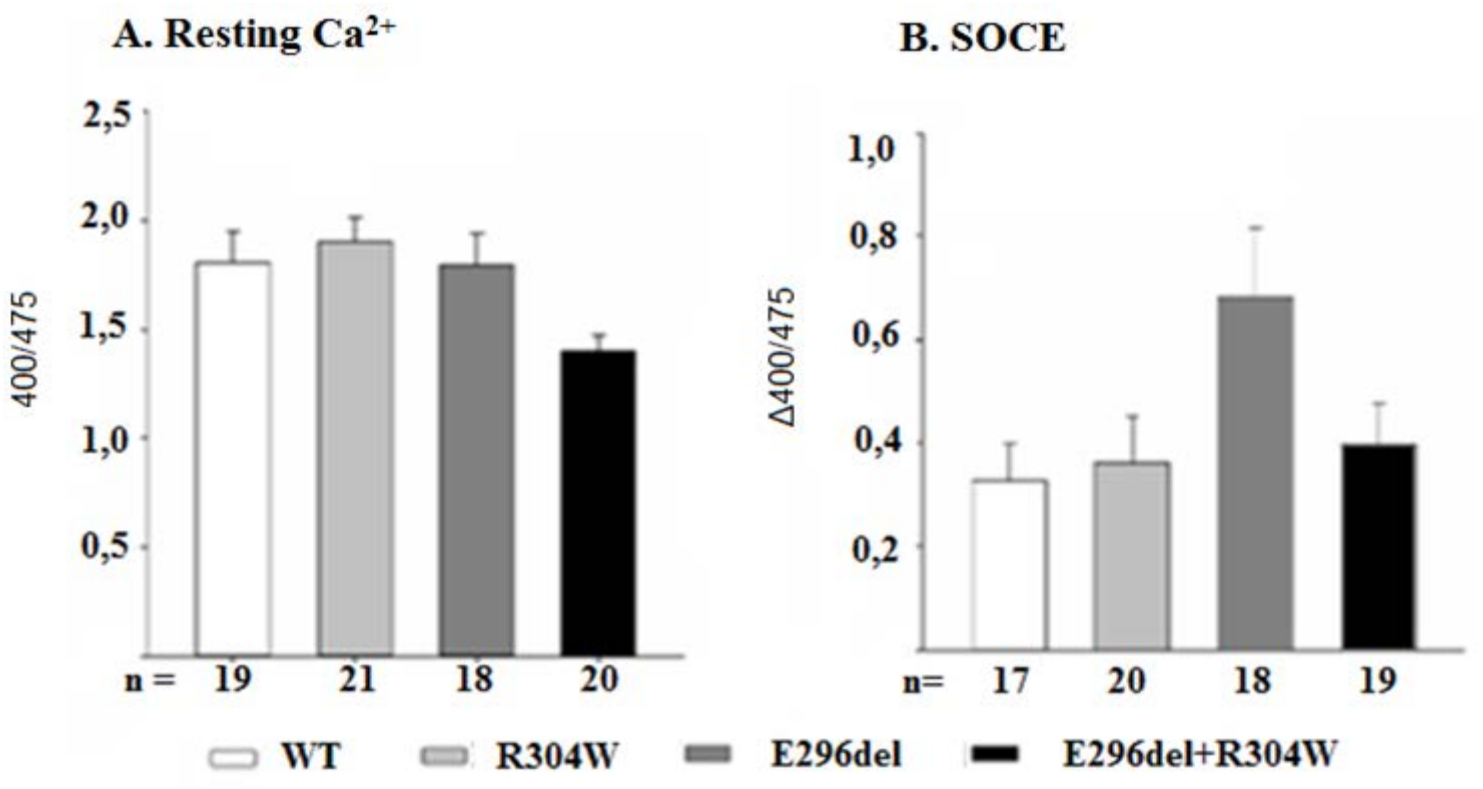



***Figure 32 Results in cells ectopically expressing mCHERRY-Orai1 together with the mutant Stim1-YFP indicated*** *A. Resting intracellular $Ca^{2+}$ levels at physiological extracellular $Ca^{2+}$ (2mM) B. Intracellular $Ca^{2+}$ level after TG treatment in a 10mM $Ca^{2+}$ solution. n = number of cells recorded. The scale of the Y-axis represents the ratio between $Ca^{2+}$ bound INDO-1 AM and unbound INDO-1 AM. The standard error is indicated in the bar.*

# 5 Discussion

The STIM1-p.R304W substitution causes Stormorken syndrome as a result of a constitutively active STIM1 protein, which has been shown to cause elevated platelet resting-$Ca^{2+}$ concentrations in the patients (Misceo et al., 2014). In neonatal skin fibroblasts from the $Stim1^{p.R304W}$ mouse model, the resting-$Ca^{2+}$ levels were lower compared to cells from control mice (table 10). The set up presented in the current work aimed to investigate how ectopic expression of mutant Stim1 affects $Ca^{2+}$-flux. The Stim1-WT and the Stim1-p.R304W proteins were ectopically expressed in the murine NIH 3T3 fibroblast cell line to compare their effect on intracellular $Ca^{2+}$ levels and on puncta formation. In addition to this, two deletion constructs were investigated within the same experimental set up, because the mouse model expressing a $Stim1^{p.E296del+R304W}$ double mutant allele show no obvious differences in phenotype compared to the wild type litter mates. In these investigations, the constructs ectopically expressing Stim1-p.R304A, Stim1-p.R304P, Stim1-p.R304S and Stim1-p.R304E were also included. The results with these constructs may give an indication whether these changes of the amino acid at position p.304 are significant for the Stim1 activation state.

## 5.1 Analysis of the Stim1-constructs

The constructed Stim-1 expression vectors were analyzed using Sanger sequencing. The results demonstrated that the constructs contained the expected base substitutions after mutagenesis (figure 23). In addition, the rest of the sequence obtained, covering the transition between the CMV promoter and Stim1 as well as Stim1 and the transition to the YFP tag, did not have any deviations from reference sequence (section 8.8). Thus, the results indicate that the open reading frame for the Stim1-YFP fusion protein is intact and the introduced mutations were verified in all the vectors constructed.

The expression of Stim1 in the NIH 3T3 cells was assayed using Western blotting. Cells transfected with all constructs gave a band that corresponds to the size of the Stim1 fusion protein, which was not seen in the un-transfected cells (Figure 25). The functionality of the YFP tag was also assayed by transfecting cells followed by imaging in 470nm light. All of the transfected cells demonstrated fluorescence as shown (figure 24).

In summary, all the Stim1 expression vectors constructed were concluded to express a Stim1-YFP fusion protein with the amino acid substitution at the p.304 position as expected, in

addition to the deletion of codon p. E296 in two of the constructs; one with the p.R304W substitution and one with the wildtype p.R304.

## 5.2 The mCHERRY-Orai1 construct

The construct with the N-terminal tagged Orai1 plasmid was purchased from VectorBuilder. We designed the fusion protein with the mCHERRY tag as an N-terminal fusion. Current research suggests that both the N- and C-terminals of Orai1 are involved in Stim1 interaction (see section 1.1.3). The possibility of tag interference with Stim1-Orai1 interaction is a caveat that should be noted when results with this construct is interpreted.

The construct was received as a stab culture and the extracted plasmid DNA was Sanger sequenced across the promoter-, tag- and Orai1-regions showing no deviations from the reference Orai1 WT sequence (8.8.7).

Western blotting demonstrated endogenous expression of Orai1 in the NIH 3T3 cells (figure 26). Thus, the endogenous Orai1 expression in the NIH 3T3 cells may be sufficient for the $Ca^{2+}$ measurements performed. In this thesis transfections were performed as co-transfections with the mCHERRY-Orai1 construct in order to obtain similar levels of the two proteins as they both were expressed from the strong CMV-promoter.

## 5.3 Puncta formation in NIH 3T3 cells with ectopic expression of mutant Stim1

Many previous studies performed transfections with human STIM1 wildtype and STIM1 variants in human cell lines to investigate different aspects of puncta formation (Gwozdz et al., 2008, Klejman et al., 2009, McNally et al., 2013, Nesin et al., 2014). However, to our knowledge the current study is the first transfecting a vector expressing the murine Stim1-p.R304W into a murine cell line. Although the amino acid sequences of STIM1 and Stim1 show 97% identity (1.1.3), the clear difference in resting $Ca^{2+}$ and SOCE between cells from patients with Stormorken syndrome compared to cells from $Stim1^{p.R304W}$ mice may indicate differences in effect of this mutation between man and mouse.

The choice of an appropriate cell line could facilitate a comparison between the previous work of Thilini Gamage on fibroblast cells from the $Stim1^{p.R304W}$ mouse model, see section

1.5, and the experiments in the current work. The cell line chosen in these experiments was the NIH 3T3 cell line, which is an immortalized murine embryonic fibroblast cell line.

Two independent transfection experiments were performed with all of the eight Stim1 constructs including the Stim1-WT construct. In each experiment, four parallel transfections were performed as a control of the transfection conditions. The Stim1 constructs were from different plasmid extractions in the two independent experiments. All plasmid extracts were Sanger sequenced prior to transfection.

The mCHERRY-Orai1 signal did not re-locate in the cells treated with TG, in contrast to Stim1-WT in these experiments (figure 27), and also contrasting Orai1 signals documented in previously described experiments (Prakriya et al., 2015, Klejman et al., 2009, Gwozdz et al., 2008). Klejman et al. demonstrated that if Orai1 was overexpressed without co-expression of Stim1, Orai1 failed to redistribute into puncta (Klejman et al., 2009). They concluded that STIM1 is necessary for ORAI1 puncta formation. These experiments were performed with transfection of an untagged ORAI1 using immunofluorescence with an antibody specific to a stretch in the N-terminal of ORAI1. Gwozdz et al. (2008) transfected STIM1 together with an ORAI1 with a C-terminal GFP fusion into HEK293 cells and observed redistribution of both proteins after 5μM TG treatment for five minutes. As previously mentioned it cannot be excluded that the mCHERRY N-terminal tag interfered with Stim1 binding explaining the lack of redistribution of Orai1 in the current work.

In the results presented in this thesis, the cells that ectopically expressed the Stim1-WT construct showed a speckled pattern after TG treatment (figure 27). These results were reproducible in both individual experiments and in all parallel transfections. It was therefore concluded that it is possible to demonstrate puncta formation in transfected cells after TG treatment (figure 27).

Likewise, the cells ectopically expressing Stim1-p.E296del+R304W showed a speckled pattern only after TG treatment. These results indicate that the Stim1-p.E296del+R304W double mutant does not cause constitutive puncta formation, and that puncta formation was induced by TG treatment similar to the results in cells expressing Stim-WT (figure 28C). This is in agreement with the observation that Stim1$^{p.E296del+R304W}$ double mutant mice show no phenotypic differences compared to wild type littermates.

The cells ectopically expressing Stim1-p.R304W and Stim1-p.R304A showed a speckled pattern both with and without TG treatment. This is consistent with constitutive puncta formation (figure 28A and B).

The cells ectopically expressing the remaining constructs: Stim1-p.R304P, Stim1-p.R304S and Stim1-p.E296del gave varying results (figure 29). Additional experiments are required to quantify the number of puncta per cell in order to assess if these mutations cause a more subtle effect on the puncta formation.

The cells ectopically expressing the Stim1-p.R304E construct were different from the rest of the constructs. The change from the wild type arginine, a positive amino acid, to the negative glutamate, may be the largest functional change for the protein function. In all individual and parallel experiments, most of the cells transfected with the construct expressing Stim1-p.R304E showed morphological signs of cell-death. There were several cells present in each well that were clearly alive, but they did not show fluorescence and therefore were not transfected. Also in the few cells that were transfected, and still had an elongated cell shape, red fluorescence had leaked out of the cell (figure 30). It is possible that this is an indication that the cell membrane was compromised. Considering that the cell has endogenous expression of wildtype Stim1 it is not likely that the p.R304E mutation causes the apoptotic effect by loss of Stim1 function. It is interesting to speculate whether a further increase of Stim1 function occurs in this variant, and thereby is responsible for an apoptotic effect. More experiments are required to investigate whether these initial findings are correct, and further what mechanism causes the effect.

The results with the construct expressing Stim1-p.R304A indicated constitutive puncta formation, and considering that alanine has similar charge properties as tryptophan (figure 18), it is possible to speculate that the charge of the amino acid in position p.304 plays a role in determining the ability of the CC1 domain to keep Stim1 inactive. However, further investigation of the other amino acid substitutions, Stim1-p.R304S, Stim1-p.R304P and Stim1-p.R304E are needed to investigate the effect of other charge properties.

Quantification of puncta formation is required to verify the results from this analysis as well as investigate the various constructs further to assess if more subtle variations can be documented. This can be achieved by analyzing images of a larger number of cells using programs like ImageJ.

Previous transfection studies have shown that the co-expression of STIM1-WT and STIM1-p.R304W in a transfection setting suppressed the effect of the STIM1-p.R304W mutation on $Ca^{2+}$ flux (Nesin et al., 2014). This was shown in a transfection state where the WT and p.R304W variants were expressed in equal amounts in HEK293 cells. The possibility of endogenous Stim1 expression in the murine NIH 3T3 fibroblast cell line interfering with the effect of the ectopically expressed Stim1 mutants in these experiments needs to be taken into consideration. If endogenous Stim1 either forms multimers on its own or participates in hetero-multimers with Stim1 variants, it could hide the effect of mutations, because it is possible that it can keep the dimers in an inactive state, even if the other constituents are in an active state. In this way, the endogenous expression could possibly hide the effect of the Stim1 variants. Western analysis may not give a correct measure of the ratio between endogenous and fusion protein expression levels in the transfected cells (figure 25). This is because in the preparation of cell lysates for Western blotting all of the cells from a well is included. Many of the cells included are not transfected, see figure 24B, which results in a diluting effect on the amount of fusion protein measured compared to the endogenous expression and not give a correct representation of the expression relationship in a single transfected cell.

## 5.4 $Ca^{2+}$ measurements in NIH 3T3 cells ectopically expressed mutant Stim1

The transfections were performed with the PolyJet™ system, which show a high transfection efficiency in NIH 3T3 cells (SignaGen® laboratories), but will also result in many un-transfected cells. In this thesis, $Ca^{2+}$ was directly measured in single cells identified by the tagged fusion proteins, thus measuring only cells that are transfected. Therefore, higher transfection efficiencies were not necessary for the work presented and the transfection efficiency achieved was not calculated.

The $Ca^{2+}$ measurements were performed over a few days as independent experiments, with a mean cell number measure of approximately three per construct per day. All four constructs were measured in each independent experiment and the solutions were made from the same stock preparation and the pH adjusted to 7,4 before $CaCl_2$ and/or TG were added (see section 3.7.2), minimizing technical variation.

Previous experiments where intracellular $Ca^{2+}$ levels were measured have used $Ca^{2+}$-indicators such as Fluo4 (work of Thilini Gamage, see section 1.5) or Fura-2 (Korzeniowski et al., 2010). The results obtained in platelets from patients with Stormorken syndrome were recorded with INDO-1 AM (Misceo et al., 2014). INDO-1 AM was chosen as the most suitable $Ca^{2+}$ indicator in the experiments in the current work because the cells are transfected with both a red fluorescent protein and a yellow fluorescent protein. The wavelengths of both of these proteins emission can interfere with the emission from of Fluo4 or Fura-2 $Ca^{2+}$-indicators. INDO-1 AM emits light at 400nm and 475nm, which does not interfere with either YFP (emission peak at approximately 510nm) or mCHERRY (emission peak at approximately 610nm). The different $Ca^{2+}$ indicators used in the previous experimental set ups can potentially be a source of variation when comparing the $Ca^{2+}$ level results presented.

Previous experiments have indicated that the $Ca^{2+}$ influx was affected according to the level of ORAI1 expression (Gwozdz et al., 2008). Therefore, the transfection experiments were performed with co-expression of Orai1 with Stim1 when measuring the $Ca^{2+}$ flux.

### 5.4.1 Stim1-WT and Stim1-p.R304W

Even though more experiments are needed before firm conclusions can be drawn, the results presented in this thesis, with ectopic expression of the Stim1-p.R304W indicated a tendency for a slightly higher resting $Ca^{2+}$- and SOCE-level compared to cells ectopically expressing Stim1-WT (figure 32). Higher $Ca^{2+}$ levels would correspond to the results previously presented in human cells (some observations summarized in table 10). Morin et al. (2014) documented significantly increased resting $Ca^{2+}$ levels in HEK293T cells expressing STIM1-p.R304W and a significant increase in TG-activated SOCE when compared to cells expressing STIM1-WT. Nesin et al. (2014) obtained similar results when they expressed the STIM1-p.R304W variant in HEK293T cells and measured induced SOCE with electrophysiology (Nesin et al., 2014).

Nesin et al. (2014) also measured intracellular $Ca^{2+}$ in skin fibroblasts from a patient with Stormorken syndrome. They found an increased SOCE in cells expressing STIM1-p.R304W compared to control cells. This correlates to the findings in transfected HEK293T cells (Morin et al., 2014, Nesin et al., 2014).

The primary cells from the murine model Stim1$^{p.R304W/p.R304W,}$ however, demonstrated decreased resting $Ca^{2+}$ level compared to the Stim1$^{WT}$ cells (Thilini Gamage, personal communication). The measurements in the Stim1$^{p.R304W/p.R304W}$ cells after TG treatment demonstrated an increased SOCE response compared to the Stim1$^{WT}$ cells. These results are different from the results observed in human cells expressing the STIM1-p.R304W variant. The transfected human cells showed increased resting $Ca^{2+}$, while the primary cells from the murine model showed decreased resting $Ca^{2+}$. The resting $Ca^{2+}$ level of the fibroblasts from patients with Stormorken syndrome was not measured (Nesin et al., 2014). Both the primary cells from the murine model and the human transfected cells showed increased SOCE $Ca^{2+}$ levels.

Additional experiments are needed before the results from transfected murine cells can be compared to the results obtained in the primary murine model cells.

| Sample | $Ca^{2+}$ indicator used | SOCE inducer used | Difference in resting $Ca^{2+}$ level, comparing STIM1p.R304W or Stim1-p.R304W to STIM1-WT or Stim1-WT | SOCE, STIM1-p.R304W/Stim1-p.R304W compared to STIM1/Stim1-WT ($Ca^{2+}$ concentration used) |
|---|---|---|---|---|
| Murine **NIH 3T3** cells co-transfected (this thesis) | 10µM INDO-1 AM | 2µM TG | Increased | Increased (10mM $Ca^{2+}$) |
| Transfected **HEK293T** cells (Morin et al., 2014) | 3µM FURA-2 | 2µM TG | Increased | Increased (10mM $Ca^{2+}$) |
| Transfected **HEK293T** cells (Nesin et al., 2014) | Electrophysiology | 10mM EGTA | Increased | Increased |
| **Skin fibroblasts** from Stormorken patients (Nesin et al., 2014) | 2µM FURA-2 | 2µM TG | Not tested | Increased (2mM $Ca^{2+}$) |
| Murine Stim1$^{R304W/R304W}$ **fibroblast** (Thilini Gamage, personal communication) | Fluo4 | 2µM TG | Decreased | Increased (10mM $Ca^{2+}$) |
| **Platelets** from Stormorken patients (Misceo et al., 2014) | 10µM INDO-1 AM | 1µM TG | Increased | Decreased (1mM $Ca^{2+}$) |

***Table 10 Comparison of the intracellular $Ca^{2+}$ levels*** *recorded in cells with the STIM1-p.R304W/Stim1-p.R304W mutation*

Different $Ca^{2+}$ indicators were utilized in several of the experiments described (table 10). These may have an influence on different aspects of the analysis. Results from ratio-metric $Ca^{2+}$ indicators are more robust because the $Ca^{2+}$ level is estimated from the ratio of two wavelengths, which are equally affected by photo-bleaching. However, the variations recorded in the experiments with the STIM1/Stim1-variants are reproducible and should not be affected significantly by the difference in $Ca^{2+}$ indicator.

There are differences between the measurements performed in human fibroblast cells expressing the STIM1-p.R304W variant and human platelets from patients with Stormorken syndrome. Measurements from the platelets showed decreased SOCE and measurements from fibroblasts showed increased SOCE (Misceo et al., 2014, Nesin et al., 2014). The measurements from the fibroblasts from patients with Stormorken syndrome were more similar to the measurements in primary cells from the murine model; both showing increased SOCE (Thilini Gamage, personal communication). This could indicate that the SOCE response is cell type specific.

The results from the only experiments performed where $Stim1^{WT}$ is not present, the murine $Stim1^{R304W/R304W}$ fibroblasts, show a decreased resting $Ca^{2+}$-level (figure 10). The $Ca^{2+}$-levels were increased after TG treatment in these cells, indicating that SOCE is functional (Thilini Gamage, personal communication). It is possible to speculate whether the presence of the wildtype allele affects $Ca^{2+}$-flux. However, the primary cells from the heterozygous $Stim1^{R304W/+}$ mice also showed decreased resting $Ca^{2+}$, which do not support this hypothesis.

Increased resting $Ca^{2+}$ levels have been detected in fibroblasts from patients with Stormorken syndrome (Thilini Gamage, personal communication), and also in transfection studies of human HEK293T cells (Morin et al., 2014 and Nesin et al., 2014), but the murine $Stim1^{R304W/R304W}$ fibroblasts showed decreased resting $Ca^{2+}$. There are two possible explanations for these results. First, that there is a species-specific effect of the Stim1-p.R304W variant, which manifests in a decreased resting $Ca^{2+}$ level in primary cells from the murine model, which has so far not been detected in the current work. Second, that the ectopic expression of the Stim1-variant is not an accurate representation of the physiological process. However, the increased SOCE measurements in fibroblasts from patients with Stormorken syndrome does not correspond to the decreased SOCE measurements in platelets from patients with Stormorken syndrome (Nesin et al., 2014, Misceo et al., 2014), but this

difference may conceivably be caused by the differences in $Ca^{2+}$-concentrations used in the SOCE experiments, 2mM and 1mM respectively.

In summary, the differences seen between the measurements done in transfected cells and patient/model cells in the different species (mouse/human) may be due to technical differences, ectopic expression giving a different representation compared to the physiological condition, cell-type specific effects, species-specific effects or a combination of these. Obtaining more results from the experiments presented in the current work could increase the knowledge and help determine the effect of the Stim1-p.R304W variant in mice.

All of the studies mentioned here (Misceo et al., 2014, Nesin et al., 2014 and Morin et al., 2014) conclude that STIM1-p.R304W is constitutively active. Additional experiments are required before conclusions can be drawn concerning the effect of Stim1-p.R304W in mouse cells.

### 5.4.2 Stim1-p.E296del and Stim1-p.E296del+R304W

In addition to the experiments with constructs expressing Stim1-WT and Stim1-p.R304W, the two constructs expressing Stim1-p.E296del and Stim1-p.E296del+R304W were included in the experiments measuring $Ca^{2+}$-levels. To our knowledge no other experiments have been performed with these constructs. The motivation for the investigation of these constructs was the observation that $Stim1^{p.R304W+E296del}$ double mutant mice, showed no difference in phenotype compared to $Stim1^{WT}$ animals.

In the current work no difference in the resting $Ca^{2+}$ were detected in cells ectopically expressing Stim1-p.E296del compared to cells ectopically expressing Stim1-WT, but a tendency for increased SOCE was observed. The cells ectopically expressing Stim1-p.E296del+R304W showed a tendency towards a decreased resting $Ca^{2+}$-concentration compared to cells ectopically expressing Stim1-WT. Additional experiments are needed before conclusions can be drawn concerning how the deletion of p.E296 affects the Stim1 function.

# 6 Conclusion

The aim of this thesis was to perform mutagenesis in order to create mutated Stim1-YFP fusion proteins. These constructs were to be used to assess puncta formation and to measure differences in $Ca^{2+}$ flux. The experiments were performed to elucidate if the mutations have an impact on the activation state of Stim1. The main findings from this work were:

- The constructs; Stim1-p.R304A-YFP, Stim1-p.R304P-YFP, Stim1-p.R304S-YFP, Stim1-p.R304E-YFP, Stim1-p.E296del-YFP and Stim1-p.E296del+R304W-YFP, were shown to contain the mutations introduced, and expression of Stim1-YFP fusion proteins were verified by Western blot and microscopy.

- Microscope imaging of cells transfected with wild type constructs showed speckled YFP pattern only when treated with TG, suggesting that puncta formation was detected in these experiments.

- The results obtained in cells ectopically expressing Stim1-p.R304W-YFP and Stim1-p.R304A-YFP indicate constitutive puncta formation.

- Cells transfected with Stim1-p.E296del+R304W showed normal induction of puncta similar to cells transfected with Stim1-WT constructs.

Further experiments and objective quantifications of the images are required in order to detect small changes in puncta formation efficiency.

The $Ca^{2+}$ flux experiments performed so far are inconclusive, but may indicate that cells ectopically expressing the Stim1-p.R304W construct have a slightly increased resting $Ca^{2+}$ level and SOCE compared to cells expressing Stim1-WT. Higher $Ca^{2+}$ levels have been interpreted as constitutive Stim1 in other studies (Morin et al., 2014, Nesin et al., 2014).

Additional experiments are required in order to obtain enough results to document the effects and to exclude outliers.

# 7 Future perspectives

Assessment of puncta formation

- Quantify the puncta formation to objectively determine puncta formation efficiency.
- Perform additional and independent imaging experiments with all the vectors constructed and investigate puncta formation efficiencies.

It would also be of interest to repeat the transfection with Stim1-p.R304E-YFP with mCHERRY-Orai1, since the results presented in the current work might suggest a toxic effect of the co-expression of this variant with Orai1 in the NIH 3T3 cells.

SOCE measurements

- Perform additional $Ca^{2+}$-flux measurements aiming to obtain conclusive results.
- Measure resting $Ca^{2+}$ levels in cells transfected with the Stim1-R304A-YFP, Stim1-R304P-YFP, Stim1-R304S-YFP and Stim1-R304E-YFP constructs to investigate whether these results correlate with the results from the imaging experiments.
- Perform $Ca^{2+}$-flux measurements in a human fibroblast cell line transfected with the Stim1 constructs and Orai1 in order to compare with the results obtained in the murine cell line.

# 8 Supplements

## 8.1 Primer determined temperatures for thermocycler Q5 mutagenesis

| Primer | Annealing temperature |
|---|---|
| R304A_forward | 68°C |
| R304A/P/E_reverse | 68°C |
| R304P_forward | 68°C |
| R304E_forward | 68°C |
| R304S_forward | 66°C |
| R304S_reverse | 66°C |
| E296del_forward | 67°C |
| E296del_reverse | 67°C |

***Table S1.*** *Temperatures for the different primers in the Q5 mutagenesis reaction.*

## 8.2 Standard PCR reaction

### 8.2.1 Reaction

| Reagent | Amount (µl) |
|---|---|
| Buffer (-$MgCl_2$) | 2,5 |
| dNTP | 0,5 |
| $MgCl_2$ | 0,75 |
| Primer forward | 0,5 |
| Primer reverse | 0,5 |
| Polymerase taq platinum | 0,25 |
| Template | 1 |
| Nuclease free $H_2O$ | 19 |
| Total | 25 |

***Table S2****. Standard reaction assembled for amplification of DNA with the PCR method.*

### 8.2.2 Thermocycler

| Step | | Temperature °C | Duration |
|---|---|---|---|
| Initial denaturation | | 94° | 2 minutes |
| Denaturation<br>Annealing<br>Elongation | 30 cycles | 94°<br>58°<br>72° | 30 seconds<br>30 seconds<br>1 minute 30 seconds |
| Final extension | | 72° | 7 minutes |
| Hold | | 4° | ∞ |

***Table S3****. Fluctuations and duration of temperature in the PCR reaction, allowing the different replication steps to take place.*

## 8.3 Standard sequencing reaction

### 8.3.1 Reaction

| Reagent | Amount (µl) |
|---|---|
| Mq-$H_2O$ | 4,75 |
| PCR product | 2 |
| 5x sequencing buffer | 2 |
| Primer | 1 |
| BigDye terminator 3.1 | 0,25 |
| Total | 10 |

***Table S4.*** *Standard reaction assembled for sequencing of a DNA strand with the Sanger sequencing method.*

### 8.3.2 Thermocycler

| Step | | Temperature °C | Duration |
|---|---|---|---|
| Initial denaturation | | 96° | 1 minute |
| Denaturation<br>Annealing<br>Elongation | 25 cycles | 96°<br>50°<br>60° | 10 seconds<br>5 seconds<br>4 minutes |
| Hold | | 10° | ∞ |

***Table S5****. Fluctuations and duration of temperature in the PCR reaction creating the DNA strands read by the Sanger sequencing machine.*

### 8.3.3 CleanSEQ

Before the Sanger sequencing PCR reaction can be measured it must be purified. This was achieved with the CleanSEQ system from Beckman Coulter. It consists of magnetic beads that bind the sequencing product and thereby separate it from the rest of the solution. Magnets pull the beads to the walls of the tube and the solution containing contaminants was extracted. The sequencing product was eluted from the magnetic beads and can be pipetted out while the beads again were held to the walls of the tube.

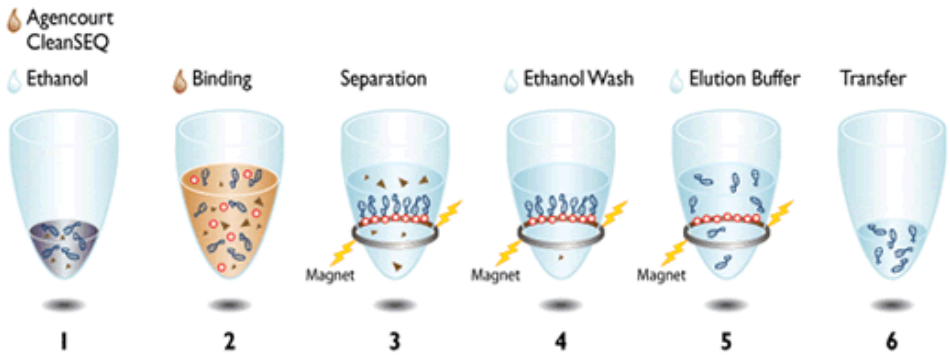



***Figure S1 CleanSEQ procedure*** *CleanSEQ was added to the sequencing reaction solution and the PCR-product binds to the magnetic beads. A magnet pulled the beads with the product bound to the sides of the tube and the contaminants was extracted with the solution. The beads were washed with ethanol and the product was eluted*

*from the magnetic beads. Finally, the product was transferred without the magnetic beads and the product was thereby purified (BeckmanCoulter, 2016).*

## 8.4 Cell counting

Cell counting was performed on the NucleoCounter® machine delivered by Chemometec. The system is based on a dye that binds to nucleic acid and has a fluorescent capacity. There are two buffers, buffer A that lysis the cells and buffer B that stabilizes the pH and stops the nucleic acids from degrading. The solution was added to the nucleo-casette where the machine counts the number of fluorescent signals. It therefore counts the number of nuclei, hence the name, and gives the number in cells per ml. After this one needs to compensate for the dilutions with the solutions A and B as well as how many milliliters the cells were resuspended in. This is the total number of cells in the sample.

## 8.5 Buffer and media composition

### 8.5.1 Buffer composition QIAGEN® Plasmid Kit Midi prep

| Buffer | Components |
|---|---|
| P1 (resuspension) | 50 mM Tris-Cl, pH 8,0<br>10 mM EDTA<br>100µg/ml RNase A |
| P2 (lysis) | 200 mM NaOH, 1% SDS |
| P3 (precipitation, neutralization) | 3.0 M potassium acetate, pH 5,5 |
| QBT (equilibration) | 750 mM NaCl<br>50 mM MOPS, pH 7,0<br>15% isopropanol<br>0,15%Triton® X-100 |
| QC (wash) | 1.0 M NaCl<br>50 mM MOPS pH 7.0<br>15% isopropanol |
| QF (elution) | 1.25 M NaCl<br>50 mM Tris-Cl pH 8.5<br>15% isopropanol |
| TE | 10 mM Tris-Cl, pH 8,0<br>1mM EDTA |

**Table S6 Buffer composition** for QIAGEN® Plasmid Kit

### 8.5.2 NIH 3T3 media composition

The growth media for the cells is DMEM delivered by SIGMA-ALDRICH, D6546. This is high glucose media with 4500 mg/L glucose, 110 mg/L sodium pyruvate and sodium bicarbonate. It does not contain L-glutamine and has exchanged pyridoxal for pyridoxine for

stability of the media. Before use 50 ml (10%) fetal calf serum, 5 ml L-glutamine and 10 ml penstrep is added making the composition as below (SIGMA-ALDRICH, 2016).

| | | |
|---|---|---|
| 4500 mg/L | 2250 mg | Glucose |
| 110 mg/L | 55 mg | Sodium pyruvate |
| 110 mg/L | 55 mg | Sodium bicarbonate |
| 10% | 50 ml | FCS |
| 1% | 5 ml | L-glutamine |
| 2% | 10 ml | PenStrep (penicillin and streptomycin) |

***Table S7****. Contents of the growth media for NIH 3T3 cells.*

### 8.5.3 Hepes Tyrade (**HT**) buffer composition 500 ml

| mM | Component | Amount (gram) |
|---|---|---|
| 140 | NaCl | 4,00 |
| 5 | Hepes | 0,60 |
| 5,4 | KCl | 0,20 |
| 0,5 | $MgCl_2$ $6H_2O$ | 0,05 |
| 5,5 | Glucose | 0,55 |
| 0,4 | $NaH_2PO_4H_2O$ | 0,03 |

***Table S8.*** *HT buffer composition*

## 8.6 Symptoms table, Stormorken syndrome

| Sex | M | M | F | F | F | M | F | F | M | M | F | M | M | 7M/6F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age at report | 33 | 6 | 19 | 2.5 | 65 | 42 | 65 | 36 | 43 | 44 | ? | ? | 52 | |
| Miosis | Marked | Moderate | Marked | Moderate | Marked | Marked | Severe | Severe | Present | Severe | Present | Present | Severe | 13/13 |
| Short stature (cm) | 157 | 101.5 | 160 | 85 | 159 | 172 | ? | 146 | 171 | 163 | ? | ? | ? | 5/9 |
| Low body weight (kg) | 57.6 | 13.6 | 49 | ? | 53 | 73 | ? | 31.5 | 61 | ? | ? | ? | ? | 3/7 |
| Ichthyosis | + | Urticaria | Urticaria | + | + | ++ | + | + | + | ? | ? | ? | + | 10/10 |
| Dyslexia | ? | ? | + | ? | + | ++ | ? | ? | ? | ? | ? | ? | ? | 3/3 |
| Learning difficulties | + | + | + | ? | ? | + | + | + | ? | ? | ? | ? | ? | 6/6 |
| Headache | − | − | + | − | ++ | + | ND | + | + | ND | ND | ND | + | 6/9 |
| Muscular weakness and cramps | +/− | + | + | − | ++ | ++ | + | + | + | + | + | + | ? | 11/12 |
| Asplenia | + | − | + | − | + | + | Hypoplasia | Hypoplasia | + | Splenectomy | + | + | + | 8/13 |
| Bleeding tendency | − | − | − | − | + | + | + | + | + | + | + | + | ? | 8/12 |
| Thrombocytes $10^9$/l | 97–146 | 28–207 | 40–120 | <10 | 75–90 | 70–95 | 18 | 64 | 60–74 | 140–150 | 99 | 83 | 83 | 13/13 |
| Bleeding time (Ivy) (min) | 7.5 | 15 | + | ? | 16–20 | 14–18 | ND | ND | ND | ND | ND | ND | ND | 5/5 |
| Increased creatine kinase level (U/L) | 4,412 | 769 | 919 | 491 | + | + | 327 | 1,923–4,017 | 820–1,718 | 1,834–3,540 | 1,668 | 3,819 | 3,819 | 13/13 |
| Muscle biopsy | TAM | Mild TAM | ND | ND | ND | TAM | ND | TAM | ND | TAM | ND | ND | ND | 5/5 |
| Hypocalcemia | + | + | +/− | ND | + | + | ND | − | + | + | ? | ? | + | 8/9 |
| ($Ca^{2+}$) (mmol/l) | 1.05 | 1.1 | 1.26 | ND | 1.1 | 1.01–1.16 | ND | ND | 0.95–1 | ND | ND | ND | ND | |

***Table S9**. Symptoms of described patients with Stormorken syndrome (Morin et al., 2014).*

## 8.7 Reference sequence

Sequences collected from the ensembl database for mouse;

http://www.ensembl.org/Mus_musculus/Info/Index, and human GRCh37;

http://grch37.ensembl.org/Homo_sapiens/Info/Index

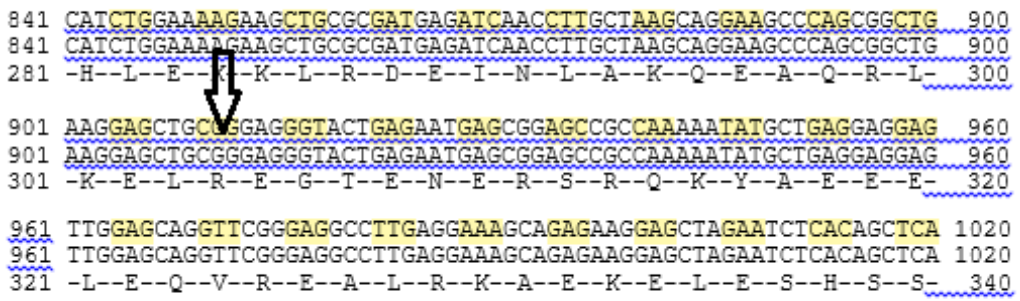
### 8.7.1 Human *STIM1*

```
  1 ATGGATGTATGCGTCCGTCTTGCCCTGTGGCTCCTCTGGGGACTCCTCCTGCACCAGGGC   60
  1 ATGGATGTATGCGTCCGTCTTGCCCTGTGGCTCCTCTGGGGACTCCTCCTGCACCAGGGC   60
  1 -M--D--V--C--V--R--L--A--L--W--L--L--W--G--L--L--L--H--Q--G-   20

 61 CAGAGCCTCAGCCATAGTCACAGTGAGAAGGCGACAGGAACCAGCTCGGGGGCCAACTCT  120
 61 CAGAGCCTCAGCCATAGTCACAGTGAGAAGGCGACAGGAACCAGCTCGGGGGCCAACTCT  120
 21 -Q--S--L--S--H--S--H--S--E--K--A--T--G--T--S--S--G--A--N--S-   40

121 GAGGAGTCCACTGCAGCAGAGTTTTGCCGAATTGACAAGCCCCTGTGTCACAGTGAGGAT  180
121 GAGGAGTCCACTGCAGCAGAGTTTTGCCGAATTGACAAGCCCCTGTGTCACAGTGAGGAT  180
 41 -E--E--S--T--A--A--E--F--C--R--I--D--K--P--L--C--H--S--E--D-   60

181 GAGAAACTCAGCTTCGAGGCAGTCCGTAACATCCACAAACTGATGGACGATGATGCCAAT  240
181 GAGAAACTCAGCTTCGAGGCAGTCCGTAACATCCACAAACTGATGGACGATGATGCCAAT  240
 61 -E--K--L--S--F--E--A--V--R--N--I--H--K--L--M--D--D--D--A--N-   80

241 GGTGATGTGGATGTGGAAGAAAGTGATGAGTTCCTGAGGGAAGACCTCAATTACCATGAC  300
241 GGTGATGTGGATGTGGAAGAAAGTGATGAGTTCCTGAGGGAAGACCTCAATTACCATGAC  300
 81 -G--D--V--D--V--E--E--S--D--E--F--L--R--E--D--L--N--Y--H--D-  100

301 CCAACAGTGAAACACAGCACCTTCCATGGTGAGGATAAGCTCATCAGCGTGGAGGACCTG  360
301 CCAACAGTGAAACACAGCACCTTCCATGGTGAGGATAAGCTCATCAGCGTGGAGGACCTG  360
101 -P--T--V--K--H--S--T--F--H--G--E--D--K--L--I--S--V--E--D--L-  120

361 TGGAAGGCATGGAAGTCATCAGAAGTATACAATTGGACCGTGGATGAGGTGGTACAGTGG  420
361 TGGAAGGCATGGAAGTCATCAGAAGTATACAATTGGACCGTGGATGAGGTGGTACAGTGG  420
121 -W--K--A--W--K--S--S--E--V--Y--N--W--T--V--D--E--V--V--Q--W-  140

421 CTGATCACATATGTGGAGCTGCCTCAGTATGAGGAGACCTTCCGGAAGCTGCAGCTCAGT  480
421 CTGATCACATATGTGGAGCTGCCTCAGTATGAGGAGACCTTCCGGAAGCTGCAGCTCAGT  480
141 -L--I--T--Y--V--E--L--P--Q--Y--E--E--T--F--R--K--L--Q--L--S-  160

481 GGCCATGCCATGCCAAGGCTGGCTGTCACCAACACCACCATGACAGGGACTGTGCTGAAG  540
481 GGCCATGCCATGCCAAGGCTGGCTGTCACCAACACCACCATGACAGGGACTGTGCTGAAG  540
161 -G--H--A--M--P--R--L--A--V--T--N--T--T--M--T--G--T--V--L--K-  180

541 ATGACAGACCGGAGTCATCGGCAGAAGCTGCAGCTGAAGGCTCTGGATACAGTGCTCTTT  600
541 ATGACAGACCGGAGTCATCGGCAGAAGCTGCAGCTGAAGGCTCTGGATACAGTGCTCTTT  600
181 -M--T--D--R--S--H--R--Q--K--L--Q--L--K--A--L--D--T--V--L--F-  200

601 GGGCCTCCTCTCTTGACTCGCCATAATCACCTCAAGGACTTCATGCTGGTGGTGTCTATC  660
601 GGGCCTCCTCTCTTGACTCGCCATAATCACCTCAAGGACTTCATGCTGGTGGTGTCTATC  660
201 -G--P--P--L--L--T--R--H--N--H--L--K--D--F--M--L--V--V--S--I-  220

661 GTTATTGGTGTGGGCGGCTGCTGGTTTGCCTATATCCAGAACCGTTACTCCAAGGAGCAC  720
661 GTTATTGGTGTGGGCGGCTGCTGGTTTGCCTATATCCAGAACCGTTACTCCAAGGAGCAC  720
221 -V--I--G--V--G--G--C--W--F--A--Y--I--Q--N--R--Y--S--K--E--H-  240

721 ATGAAGAAGATGATGAAGGACTTGGAGGGGTTACACCGAGCTGAGCAGAGTCTGCATGAC  780
721 ATGAAGAAGATGATGAAGGACTTGGAGGGGTTACACCGAGCTGAGCAGAGTCTGCATGAC  780
241 -M--K--K--M--M--K--D--L--E--G--L--H--R--A--E--Q--S--L--H--D-  260
```

```
 781 CTTCAGGAAAGGCTGCACAAGGCCCAGGAGGAGCACCGCACAGTGGAGGTGGAGAAGGTC  840
 781 CTTCAGGAAAGGCTGCACAAGGCCCAGGAGGAGCACCGCACAGTGGAGGTGGAGAAGGTC  840
 261 -L--Q--E--R--L--H--K--A--Q--E--E--H--R--T--V--E--V--E--K--V-  280

 841 CATCTGGAAAAGAAGCTGCGCGATGAGATCAACCTTGCTAAGCAGGAAGCCCAGCGGCTG  900
 841 CATCTGGAAAAGAAGCTGCGCGATGAGATCAACCTTGCTAAGCAGGAAGCCCAGCGGCTG  900
 281 -H--L--E--K--K--L--R--D--E--I--N--L--A--K--Q--E--A--Q--R--L-  300

 901 AAGGAGCTGCGGGAGGGTACTGAGAATGAGCGGAGCCGCCAAAAATATGCTGAGGAGGAG  960
 901 AAGGAGCTGCGGGAGGGTACTGAGAATGAGCGGAGCCGCCAAAAATATGCTGAGGAGGAG  960
 301 -K--E--L--R--E--G--T--E--N--E--R--S--R--Q--K--Y--A--E--E--E-  320

 961 TTGGAGCAGGTTCGGGAGGCCTTGAGGAAAGCAGAGAAGGAGCTAGAATCTCACAGCTCA 1020
 961 TTGGAGCAGGTTCGGGAGGCCTTGAGGAAAGCAGAGAAGGAGCTAGAATCTCACAGCTCA 1020
 321 -L--E--Q--V--R--E--A--L--R--K--A--E--K--E--L--E--S--H--S--S-  340

1021 TGGTATGCTCCAGAGGCCCTTCAGAAGTGGCTGCAGCTGACACATGAGGTGGAGGTGCAA 1080
1021 TGGTATGCTCCAGAGGCCCTTCAGAAGTGGCTGCAGCTGACACATGAGGTGGAGGTGCAA 1080
 341 -W--Y--A--P--E--A--L--Q--K--W--L--Q--L--T--H--E--V--E--V--Q-  360

1081 TATTACAACATCAAGAAGCAAAATGCTGAGAAGCAGCTGCTGGTGGCCAAGGAGGGGGCT 1140
1081 TATTACAACATCAAGAAGCAAAATGCTGAGAAGCAGCTGCTGGTGGCCAAGGAGGGGGCT 1140
 361 -Y--Y--N--I--K--K--Q--N--A--E--K--Q--L--L--V--A--K--E--G--A-  380

1141 GAGAAGATAAAAAAGAAGAGAAACACACTCTTTGGCACCTTCCACGTGGCCCACAGCTCT 1200
1141 GAGAAGATAAAAAAGAAGAGAAACACACTCTTTGGCACCTTCCACGTGGCCCACAGCTCT 1200
 381 -E--K--I--K--K--K--R--N--T--L--F--G--T--F--H--V--A--H--S--S-  400

1201 TCCCTGGATGATGTAGATCATAAAATTCTAACAGCTAAGCAAGCACTGAGCGAGGTGACA 1260
1201 TCCCTGGATGATGTAGATCATAAAATTCTAACAGCTAAGCAAGCACTGAGCGAGGTGACA 1260
 401 -S--L--D--D--V--D--H--K--I--L--T--A--K--Q--A--L--S--E--V--T-  420

1261 GCAGCATTGCGGGAGCGCCTGCACCGCTGGCAACAGATCGAGATCCTCTGTGGCTTCCAG 1320
1261 GCAGCATTGCGGGAGCGCCTGCACCGCTGGCAACAGATCGAGATCCTCTGTGGCTTCCAG 1320
 421 -A--A--L--R--E--R--L--H--R--W--Q--Q--I--E--I--L--C--G--F--Q-  440

1321 ATTGTCAACAACCCTGGCATCCACTCACTGGTGGCTGCCCTCAACATAGACCCCAGCTGG 1380
1321 ATTGTCAACAACCCTGGCATCCACTCACTGGTGGCTGCCCTCAACATAGACCCCAGCTGG 1380
 441 -I--V--N--N--P--G--I--H--S--L--V--A--A--L--N--I--D--P--S--W-  460

1381 ATGGGCAGTACACGCCCCAACCCTGCTCACTTCATCATGACTGACGACGTGGATGACATG 1440
1381 ATGGGCAGTACACGCCCCAACCCTGCTCACTTCATCATGACTGACGACGTGGATGACATG 1440
 461 -M--G--S--T--R--P--N--P--A--H--F--I--M--T--D--D--V--D--D--M-  480

1441 GATGAGGAGATTGTGTCTCCCTTGTCCATGCAGTCCCCTAGCCTGCAGAGCAGTGTTCGG 1500
1441 GATGAGGAGATTGTGTCTCCCTTGTCCATGCAGTCCCCTAGCCTGCAGAGCAGTGTTCGG 1500
 481 -D--E--E--I--V--S--P--L--S--M--Q--S--P--S--L--Q--S--S--V--R-  500

1501 CAGCGCCTGACGGAGCCACAGCATGGCCTGGGATCTCAGAGGTTGGTAGAGGGCGAGGCT 1560
1501 CAGCGCCTGACGGAGCCACAGCATGGCCTGGGATCTCAGAGGTTGGTAGAGGGCGAGGCT 1560
 501 -Q--R--L--T--E--P--Q--H--G--L--G--S--Q--R--L--V--E--G--E--A-  520

1561 GGCCACTTCTTGACAAGCCGGGTATCTCTGCGGCGAATGCGCAGCCTTTCATCTGGACAG 1620
1561 GGCCACTTCTTGACAAGCCGGGTATCTCTGCGGCGAATGCGCAGCCTTTCATCTGGACAG 1620
 521 -G--H--F--L--T--S--R--V--S--L--R--R--M--R--S--L--S--S--G--Q-  540

1621 TCTTTCAGTTCTGAAGGCTACGGGACCAGCTCTCCATCTGCCTCTGCTGCTGCTTCTTGC 1680
1621 TCTTTCAGTTCTGAAGGCTACGGGACCAGCTCTCCATCTGCCTCTGCTGCTGCTTCTTGC 1680
 541 -S--F--S--S--E--G--Y--G--T--S--S--P--S--A--S--A--A--A--S--C-  560

1681 TCCTCTTCCATCACCACCATCACCACTACCACCACCACCACCACCACCTTCACCACCGTC 1740
1681 TCCTCTTCCATCACCACCATCACCACTACCACCACCACCACCACCACCTTCACCACCGTC 1740
 561 -S--S--S--I--T--T--I--T--T--T--T--T--T--T--T--T--T--F--T--T--V-  580

1741 CATGTCCACCCTGTTTATTACCACCACAGCACTTCCTATTTCCTCCAGATGGAGCCCTAC 1800
1741 CATGTCCACCCTGTTTATTACCACCACAGCACTTCCTATTTCCTCCAGATGGAGCCCTAC 1800
```

```
 581 -H--V--H--P--V--Y--Y--H--H--S--T--S--Y--F--L--Q--M--E--P--Y-  600

1801 CCTGACACACCCCCTTCTGACAGCACCGCTGTGATGCCTGGGCATTCAGAGAGCTTGGGG 1860
1801 CCTGACACACCCCCTTCTGACAGCACCGCTGTGATGCCTGGGCATTCAGAGAGCTTGGGG 1860
 601 -P--D--T--P--P--S--D--S--T--A--V--M--P--G--H--S--E--S--L--G-  620

1861 GATTTGACCCATTCCGATTCGGAGTCCTCCCTCCACATGAGTGACCGCCAGCGTGTGGCC 1920
1861 GATTTGACCCATTCCGATTCGGAGTCCTCCCTCCACATGAGTGACCGCCAGCGTGTGGCC 1920
 621 -D--L--T--H--S--D--S--E--S--S--L--H--M--S--D--R--Q--R--V--A-  640

1921 CCCAAACCTCCTCAGATGAGCCGTGCTGCAGACGAGGCTCTCAATGCCATGACTTCCAAT 1980
1921 CCCAAACCTCCTCAGATGAGCCGTGCTGCAGACGAGGCTCTCAATGCCATGACTTCCAAT 1980
 641 -P--K--P--P--Q--M--S--R--A--A--D--E--A--L--N--A--M--T--S--N-  660

1981 GGCAGCCACCGGCTGATCGAGGGGGTCCACCCAGGGTCTCTGGTGGAGAAACTGCCTGAC 2040
1981 GGCAGCCACCGGCTGATCGAGGGGGTCCACCCAGGGTCTCTGGTGGAGAAACTGCCTGAC 2040
 661 -G--S--H--R--L--I--E--G--V--H--P--G--S--L--V--E--K--L--P--D-  680

2041 AGCCCTGCCCTGGCCAAGAAGGCATTACTGGCGCTGAACCATGGGCTGGACAAGGCCCAC 2100
2041 AGCCCTGCCCTGGCCAAGAAGGCATTACTGGCGCTGAACCATGGGCTGGACAAGGCCCAC 2100
 681 -S--P--A--L--A--K--K--A--L--L--A--L--N--H--G--L--D--K--A--H-  700

2101 AGCCTGATGGAGCTGAGCCCCTCAGCCCCACCTGGTGGCTCTCCACATTTGGATTCTTCC 2160
2101 AGCCTGATGGAGCTGAGCCCCTCAGCCCCACCTGGTGGCTCTCCACATTTGGATTCTTCC 2160
 701 -S--L--M--E--L--S--P--S--A--P--P--G--G--S--P--H--L--D--S--S-  720

2161 CGTTCTCACAGCCCCAGCTCCCCAGACCCAGACACACCATCTCCAGTTGGGGACAGCCGA 2220
2161 CGTTCTCACAGCCCCAGCTCCCCAGACCCAGACACACCATCTCCAGTTGGGGACAGCCGA 2220
 721 -R--S--H--S--P--S--S--P--D--P--D--T--P--S--P--V--G--D--S--R-  740

2221 GCCCTGCAAGCCAGCCGAAACACACGCATTCCCCACCTGGCTGGCAAGAAGGCTGTGGCT 2280
2221 GCCCTGCAAGCCAGCCGAAACACACGCATTCCCCACCTGGCTGGCAAGAAGGCTGTGGCT 2280
 741 -A--L--Q--A--S--R--N--T--R--I--P--H--L--A--G--K--K--A--V--A-  760

2281 GAGGAGGATAATGGCTCTATTGGCGAGGAAACAGACTCCAGCCCAGGCCGGAAGAAGTTT 2340
2281 GAGGAGGATAATGGCTCTATTGGCGAGGAAACAGACTCCAGCCCAGGCCGGAAGAAGTTT 2340
 761 -E--E--D--N--G--S--I--G--E--E--T--D--S--S--P--G--R--K--K--F-  780

2341 CCCCTCAAAATCTTTAAGAAGCCTCTTAAGAAGTAG                          2376
2341 CCCCTCAAAATCTTTAAGAAGCCTCTTAAGAAGTAG                          2376
 781 -P--L--K--I--F--K--K--P--L--K--K--*-                          2376
```

*STIM1*
p.R304W
c.910C>T



### 8.7.2 Mouse *Stim1*

```
   1 ATGGATGTGTGCGCCCGTCTTGCCCTGTGGCTTCTTTGGGGGCTCCTTCTGCATCAGGGC   60
   1 ATGGATGTGTGCGCCCGTCTTGCCCTGTGGCTTCTTTGGGGGCTCCTTCTGCATCAGGGC   60
   1 -M--D--V--C--A--R--L--A--L--W--L--L--W--G--L--L--L--H--Q--G-   20

  61 CAGAGTCTCAGCCATAGTCACAGTGAAAAGAATACAGGAGCTAGCTCCGGGGCGACTTCT  120
  61 CAGAGTCTCAGCCATAGTCACAGTGAAAAGAATACAGGAGCTAGCTCCGGGGCGACTTCT  120
  21 -Q--S--L--S--H--S--H--S--E--K--N--T--G--A--S--S--G--A--T--S-   40

 121 GAAGAGTCTACCGAAGCAGAGTTTTGCCGAATTGACAAGCCCCTGTGCCACAGTGAGGAT  180
 121 GAAGAGTCTACCGAAGCAGAGTTTTGCCGAATTGACAAGCCCCTGTGCCACAGTGAGGAT  180
  41 -E--E--S--T--E--A--E--F--C--R--I--D--K--P--L--C--H--S--E--D-   60

 181 GAGAAGCTCAGCTTTGAGGCCGTCCGAAACATCCATAAGCTGATGGATGACGATGCCAAT  240
 181 GAGAAGCTCAGCTTTGAGGCCGTCCGAAACATCCATAAGCTGATGGATGACGATGCCAAT  240
  61 -E--K--L--S--F--E--A--V--R--N--I--H--K--L--M--D--D--D--A--N-   80

 241 GGTGATGTGGATGTGGAAGAAAGTGATGAGTTCCTAAGGGAAGACCTCAATTACCATGAC  300
 241 GGTGATGTGGATGTGGAAGAAAGTGATGAGTTCCTAAGGGAAGACCTCAATTACCATGAC  300
  81 -G--D--V--D--V--E--E--S--D--E--F--L--R--E--D--L--N--Y--H--D-  100

 301 CCAACAGTGAAACATAGCACCTTCCATGGTGAGGATAAGCTTATCAGCGTGGAGGACCTG  360
 301 CCAACAGTGAAACATAGCACCTTCCATGGTGAGGATAAGCTTATCAGCGTGGAGGACCTG  360
 101 -P--T--V--K--H--S--T--F--H--G--E--D--K--L--I--S--V--E--D--L-  120

 361 TGGAAGGCGTGGAAGTCATCAGAAGTGTACAACTGGACTGTGGATGAGGTGATACAGTGG  420
 361 TGGAAGGCGTGGAAGTCATCAGAAGTGTACAACTGGACTGTGGATGAGGTGATACAGTGG  420
 121 -W--K--A--W--K--S--S--E--V--Y--N--W--T--V--D--E--V--I--Q--W-  140

 421 CTCATTACGTATGTGGAGCTGCCACAGTATGAGGAAACCTTCCGGAAGTTGCAGCTTACT  480
 421 CTCATTACGTATGTGGAGCTGCCACAGTATGAGGAAACCTTCCGGAAGTTGCAGCTTACT  480
 141 -L--I--T--Y--V--E--L--P--Q--Y--E--E--T--F--R--K--L--Q--L--T-  160

 481 GGCCACGCCATGCCAAGGCTAGCAGTAACCAACACCACCATGACAGGGACTGTACTGAAG  540
 481 GGCCACGCCATGCCAAGGCTAGCAGTAACCAACACCACCATGACAGGGACTGTACTGAAG  540
 161 -G--H--A--M--P--R--L--A--V--T--N--T--T--M--T--G--T--V--L--K-  180

 541 ATGACAGATCGGAGCCACAGGCAGAAGCTGCAGCTGAAGGCCCTGGACACAGTGCTGTTT  600
 541 ATGACAGATCGGAGCCACAGGCAGAAGCTGCAGCTGAAGGCCCTGGACACAGTGCTGTTT  600
 181 -M--T--D--R--S--H--R--Q--K--L--Q--L--K--A--L--D--T--V--L--F-  200

 601 GGGCCTCCTCTCTTGACTCGGCATAATCACCTGAAGGACTTCATGCTGGTGGTGTCTATC  660
 601 GGGCCTCCTCTCTTGACTCGGCATAATCACCTGAAGGACTTCATGCTGGTGGTGTCTATC  660
 201 -G--P--P--L--L--T--R--H--N--H--L--K--D--F--M--L--V--V--S--I-  220

 661 GTTATTGGTGTGGGTGGCTGCTGGTTTGCCTATATCCAGAACCGTTACTCTAAGGAGCAC  720
 661 GTTATTGGTGTGGGTGGCTGCTGGTTTGCCTATATCCAGAACCGTTACTCTAAGGAGCAC  720
 221 -V--I--G--V--G--G--C--W--F--A--Y--I--Q--N--R--Y--S--K--E--H-  240

 721 ATGAAGAAAATGATGAAGGATCTGGAAGGGTTACACCGGGCTGAGCAGAGTCTGCATGAC  780
 721 ATGAAGAAAATGATGAAGGATCTGGAAGGGTTACACCGGGCTGAGCAGAGTCTGCATGAC  780
 241 -M--K--K--M--M--K--D--L--E--G--L--H--R--A--E--Q--S--L--H--D-  260

 781 CTTCAGGAAAGGCTGCACAAGGCCCAGGAGGAGCACCGAACTGTGGAAGTAGAGAAGGTC  840
 781 CTTCAGGAAAGGCTGCACAAGGCCCAGGAGGAGCACCGAACTGTGGAAGTAGAGAAGGTC  840
 261 -L--Q--E--R--L--H--K--A--Q--E--E--H--R--T--V--E--V--E--K--V-  280

 841 CACCTGGAGAAGAAGCTGCGAGATGAGATCAACCTTGCCAAGCAGGAAGCTCAGCGGCTG  900
 841 CACCTGGAGAAGAAGCTGCGAGATGAGATCAACCTTGCCAAGCAGGAAGCTCAGCGGCTG  900
 281 -H--L--E--K--K--L--R--D--E--I--N--L--A--K--Q--E--A--Q--R--L-  300

 901 AAGGAGCTGAGGGAGGGTACTGAGAATGAGAGGAGCCGTCAAAAATATGCTGAGGAAGAG  960
 901 AAGGAGCTGAGGGAGGGTACTGAGAATGAGAGGAGCCGTCAAAAATATGCTGAGGAAGAG  960
 301 -K--E--L--R--E--G--T--E--N--E--R--S--R--Q--K--Y--A--E--E--E-  320

 961 CTGGAGCAGGTTCGGGAGGCCTTGAGGAAAGCAGAGAAGGAGCTGGAATCACACAGCTCA 1020
```

```
 961 CTGGAGCAGGTTCGGGAGGCCTTGAGGAAAGCAGAGAAGGAGCTGGAATCACACAGCTCA 1020
 321 -L--E--Q--V--R--E--A--L--R--K--A--E--K--E--L--E--S--H--S--S-  340

1021 TGGTATGCTCCTGAGGCCCTGCAGAAGTGGCTGCAGCTGACCCATGAGGTGGAGGTGCAG 1080
1021 TGGTATGCTCCTGAGGCCCTGCAGAAGTGGCTGCAGCTGACCCATGAGGTGGAGGTGCAG 1080
 341 -W--Y--A--P--E--A--L--Q--K--W--L--Q--L--T--H--E--V--E--V--Q-  360

1081 TACTACAACATCAAGAAGCAAAATGCAGAGAGGCAGCTGCTGGTGGCCAAGGAGGGGGCT 1140
1081 TACTACAACATCAAGAAGCAAAATGCAGAGAGGCAGCTGCTGGTGGCCAAGGAGGGGGCT 1140
 361 -Y--Y--N--I--K--K--Q--N--A--E--R--Q--L--L--V--A--K--E--G--A-  380

1141 GAGAAAATAAAAAAGAAGAGAAACACGCTTTTTGGTACCTTCCATGTGGCCCACAGCTCT 1200
1141 GAGAAAATAAAAAAGAAGAGAAACACGCTTTTTGGTACCTTCCATGTGGCCCACAGCTCT 1200
 381 -E--K--I--K--K--K--R--N--T--L--F--G--T--F--H--V--A--H--S--S-  400

1201 TCCCTGGATGATGTGGATCATAAAATCCTAACTGCTAAGCAAGCTCTGAGTGAGGTGACA 1260
1201 TCCCTGGATGATGTGGATCATAAAATCCTAACTGCTAAGCAAGCTCTGAGTGAGGTGACA 1260
 401 -S--L--D--D--V--D--H--K--I--L--T--A--K--Q--A--L--S--E--V--T-  420

1261 GCGGCACTGAGGGAGCGCCTGCACCGGTGGCAGCAGATCGAGATCCTCTGCGGTTTCCAG 1320
1261 GCGGCACTGAGGGAGCGCCTGCACCGGTGGCAGCAGATCGAGATCCTCTGCGGTTTCCAG 1320
 421 -A--A--L--R--E--R--L--H--R--W--Q--Q--I--E--I--L--C--G--F--Q-  440

1321 ATTGTCAATAACCCCGGCATCCACTCCTTGGTGGCTGCTCTCAACATCGACCCCAGCTGG 1380
1321 ATTGTCAATAACCCCGGCATCCACTCCTTGGTGGCTGCTCTCAACATCGACCCCAGCTGG 1380
 441 -I--V--N--N--P--G--I--H--S--L--V--A--A--L--N--I--D--P--S--W-  460

1381 ATGGGCAGCACCCGCCCTAACCCCGCCCACTTCATCATGACTGACGATGTGGATGACATG 1440
1381 ATGGGCAGCACCCGCCCTAACCCCGCCCACTTCATCATGACTGACGATGTGGATGACATG 1440
 461 -M--G--S--T--R--P--N--P--A--H--F--I--M--T--D--D--V--D--D--M-  480

1441 GATGAGGAGATTGTGTCGCCCTTGTCCATGCAGTCCCCCAGCCTGCAGAGCAGTGTCCGG 1500
1441 GATGAGGAGATTGTGTCGCCCTTGTCCATGCAGTCCCCCAGCCTGCAGAGCAGTGTCCGG 1500
 481 -D--E--E--I--V--S--P--L--S--M--Q--S--P--S--L--Q--S--S--V--R-  500

1501 CAGCGCCTGACGGAGCCACAGCTTGGCCTGGGATCTCAGAGGGATTTGACCCATTCCGAT 1560
1501 CAGCGCCTGACGGAGCCACAGCTTGGCCTGGGATCTCAGAGGGATTTGACCCATTCCGAT 1560
 501 -Q--R--L--T--E--P--Q--L--G--L--G--S--Q--R--D--L--T--H--S--D-  520

1561 TCGGAGTCCTCCCTCCACATGAGTGACCGCCAGCGTGTGGCCCCCAAGCCTCCTCAGATG 1620
1561 TCGGAGTCCTCCCTCCACATGAGTGACCGCCAGCGTGTGGCCCCCAAGCCTCCTCAGATG 1620
 521 -S--E--S--S--L--H--M--S--D--R--Q--R--V--A--P--K--P--P--Q--M-  540

1621 GGCCGTGCTGCAGATGAAGCTCTCAATGCCATGCCTTCCAATGGCAGCCATCGGCTGATT 1680
1621 GGCCGTGCTGCAGATGAAGCTCTCAATGCCATGCCTTCCAATGGCAGCCATCGGCTGATT 1680
 541 -G--R--A--A--D--E--A--L--N--A--M--P--S--N--G--S--H--R--L--I-  560

1681 GAGGGGGTCCATCCAGGATCTCTGGTGGAGAAACTGCCTGACAGCCCTGCTCTGGCCAAG 1740
1681 GAGGGGGTCCATCCAGGATCTCTGGTGGAGAAACTGCCTGACAGCCCTGCTCTGGCCAAG 1740
 561 -E--G--V--H--P--G--S--L--V--E--K--L--P--D--S--P--A--L--A--K-  580

1741 AAGACATTTATGGCGTTGAACCATGGCCTAGACAAGGCCCACAGCCTGATGGAGCTGAAC 1800
1741 AAGACATTTATGGCGTTGAACCATGGCCTAGACAAGGCCCACAGCCTGATGGAGCTGAAC 1800
 581 -K--T--F--M--A--L--N--H--G--L--D--K--A--H--S--L--M--E--L--N-  600

1801 CCCTCAGTCCCACCTGGTGGCTCCCCACTTTTGGATTCTTCCCATTCTCTTAGCCCCAGT 1860
1801 CCCTCAGTCCCACCTGGTGGCTCCCCACTTTTGGATTCTTCCCATTCTCTTAGCCCCAGT 1860
 601 -P--S--V--P--P--G--G--S--P--L--L--D--S--S--H--S--L--S--P--S-  620

1861 TCCCCAGACCCAGACACGCCATCTCCAGTTGGGGACAACCGAGCTCTGCAGGGTAGCCGA 1920
1861 TCCCCAGACCCAGACACGCCATCTCCAGTTGGGGACAACCGAGCTCTGCAGGGTAGCCGA 1920
 621 -S--P--D--P--D--T--P--S--P--V--G--D--N--R--A--L--Q--G--S--R-  640

1921 AACACACGAATTCCCCACTTGGCTGGCAAGAAGGCAATGGCTGAGGAGGATAATGGTTCC 1980
1921 AACACACGAATTCCCCACTTGGCTGGCAAGAAGGCAATGGCTGAGGAGGATAATGGTTCC 1980
 641 -N--T--R--I--P--H--L--A--G--K--K--A--M--A--E--E--D--N--G--S-  660
```

```
1981 ATTGGTGAGGAGACAGACTCCAGTCCAGGCAGGAAGAAGTTTCCTCTCAAAATTTTTAAG 2040
1981 ATTGGTGAGGAGACAGACTCCAGTCCAGGCAGGAAGAAGTTTCCTCTCAAAATTTTTAAG 2040
 661 -I--G--E--E--T--D--S--S--P--G--R--K--K--F--P--L--K--I--F--K-  680

2041 AAGCCTCTTAAGAAGTAG                                           2058
2041 AAGCCTCTTAAGAAGTAG                                           2058
 681 -K--P--L--K--K--*-                                           2058
```

*Stim1*
p.R304W
c.910A>T

```
841 CACCTGGAGAAGAAGCTGCGAGATGAGATCAACCTTGCCAAGCAGGAAGCTCAGCGGCTG  900
841 CACCTGGAGAAGAAGCTGCGAGATGAGATCAACCTTGCCAAGCAGGAAGCTCAGCGGCTG  900
281 -H--L--E--K--K--L--R--D--E--I--N--L--A--K--Q--E--A--Q--R--L-  300

901 AAGGAGCTGAGGGAGGGTACTGAGAATGAGAGGAGCCGTCAAAAATATGCTGAGGAAGAG  960
901 AAGGAGCTGAGGGAGGGTACTGAGAATGAGAGGAGCCGTCAAAAATATGCTGAGGAAGAG  960
301 -K--E--L--R--E--G--T--E--N--E--R--S--R--Q--K--Y--A--E--E--E-  320

961 CTGGAGCAGGTTCGGGAGGCCTTGAGGAAAGCAGAGAAGGAGCTGGAATCACACAGCTCA 1020
961 CTGGAGCAGGTTCGGGAGGCCTTGAGGAAAGCAGAGAAGGAGCTGGAATCACACAGCTCA 1020
321 -L--E--Q--V--R--E--A--L--R--K--A--E--K--E--L--E--S--H--S--S-  340
```

### 8.7.3 Human *ORAI1*

```
  1 ATGAGCCTCAACGAGCACTCCATGCAGGCGCTGTCCTGGCGCAAGCTCTACTTGAGCCGC  60
  1 ATGAGCCTCAACGAGCACTCCATGCAGGCGCTGTCCTGGCGCAAGCTCTACTTGAGCCGC  60
  1 -M--S--L--N--E--H--S--M--Q--A--L--S--W--R--K--L--Y--L--S--R-  20

 61 GCCAAGCTTAAAGCCTCCAGCCGGACCTCGGCTCTGCTCTCCGGCTTCGCCATGGTGGCA 120
 61 GCCAAGCTTAAAGCCTCCAGCCGGACCTCGGCTCTGCTCTCCGGCTTCGCCATGGTGGCA 120
 21 -A--K--L--K--A--S--S--R--T--S--A--L--L--S--G--F--A--M--V--A-  40

121 ATGGTGGAGGTGCAGCTGGACGCTGACCACGACTACCCACCGGGGCTGCTCATCGCCTTC 180
121 ATGGTGGAGGTGCAGCTGGACGCTGACCACGACTACCCACCGGGGCTGCTCATCGCCTTC 180
 41 -M--V--E--V--Q--L--D--A--D--H--D--Y--P--P--G--L--L--I--A--F-  60

181 AGTGCCTGCACCACAGTGCTGGTGGCTGTGCACCTGTTTGCGCTCATGATCAGCACCTGC 240
181 AGTGCCTGCACCACAGTGCTGGTGGCTGTGCACCTGTTTGCGCTCATGATCAGCACCTGC 240
 61 -S--A--C--T--T--V--L--V--A--V--H--L--F--A--L--M--I--S--T--C-  80

241 ATCCTGCCCAACATCGAGGCGGTGAGCAACGTGCACAATCTCAACTCGGTCAAGGAGTCC 300
241 ATCCTGCCCAACATCGAGGCGGTGAGCAACGTGCACAATCTCAACTCGGTCAAGGAGTCC 300
 81 -I--L--P--N--I--E--A--V--S--N--V--H--N--L--N--S--V--K--E--S- 100

301 CCCCATGAGCGCATGCACCGCCACATCGAGCTGGCCTGGGCCTTCTCCACCGTCATCGGC 360
301 CCCCATGAGCGCATGCACCGCCACATCGAGCTGGCCTGGGCCTTCTCCACCGTCATCGGC 360
101 -P--H--E--R--M--H--R--H--I--E--L--A--W--A--F--S--T--V--I--G- 120

361 ACGCTGCTCTTCCTAGCTGAGGTGGTGCTGCTCTGCTGGGTCAAGTTCTTGCCCCTCAAG 420
361 ACGCTGCTCTTCCTAGCTGAGGTGGTGCTGCTCTGCTGGGTCAAGTTCTTGCCCCTCAAG 420
121 -T--L--L--F--L--A--E--V--V--L--L--C--W--V--K--F--L--P--L--K- 140

421 AAGCAGCCAGGCCAGCCAAGGCCCACCAGCAAGCCCCCCGCCAGTGGCGCAGCAGCCAAC 480
421 AAGCAGCCAGGCCAGCCAAGGCCCACCAGCAAGCCCCCCGCCAGTGGCGCAGCAGCCAAC 480
141 -K--Q--P--G--Q--P--R--P--T--S--K--P--P--A--S--G--A--A--A--N- 160
```

```
481 GTCAGCACCAGCGGCATCACCCCGGGCCAGGCAGCTGCCATCGCCTCGACCACCATCATG 540
481 GTCAGCACCAGCGGCATCACCCCGGGCCAGGCAGCTGCCATCGCCTCGACCACCATCATG 540
161 -V--S--T--S--G--I--T--P--G--Q--A--A--A--I--A--S--T--T--I--M- 180

541 GTGCCCTTCGGCCTGATCTTTATCGTCTTCGCCGTCCACTTCTACCGCTCACTGGTTAGC 600
541 GTGCCCTTCGGCCTGATCTTTATCGTCTTCGCCGTCCACTTCTACCGCTCACTGGTTAGC 600
181 -V--P--F--G--L--I--F--I--V--F--A--V--H--F--Y--R--S--L--V--S- 200

601 CATAAGACTGACCGACAGTTCCAGGAGCTCAACGAGCTGGCGGAGTTTGCCCGCTTACAG 660
601 CATAAGACTGACCGACAGTTCCAGGAGCTCAACGAGCTGGCGGAGTTTGCCCGCTTACAG 660
201 -H--K--T--D--R--Q--F--Q--E--L--N--E--L--A--E--F--A--R--L--Q- 220

661 GACCAGCTGGACCACAGAGGGGACCACCCCCTGACGCCCGGCAGCCACTATGCCTAG    717
661 GACCAGCTGGACCACAGAGGGGACCACCCCCTGACGCCCGGCAGCCACTATGCCTAG    717
221 -D--Q--L--D--H--R--G--D--H--P--L--T--P--G--S--H--Y--A--*-    717
```

### 8.7.4 Mouse *Orai1*

```
  1 ATGCATCCGGAGCCTGCCCCGCCCCCGAGTCACAGCAATCCGGAGCTTCCCGTGAGCGGC  60
  1 ATGCATCCGGAGCCTGCCCCGCCCCCGAGTCACAGCAATCCGGAGCTTCCCGTGAGCGGC  60
  1 -M--H--P--E--P--A--P--P--P--S--H--S--N--P--E--L--P--V--S--G-  20

 61 GGCAGCAGCACTAGCGGCAGCCGCCGGAGCCGCCGCCGCAGCGGGGACGGGGAGCCCTCG 120
 61 GGCAGCAGCACTAGCGGCAGCCGCCGGAGCCGCCGCCGCAGCGGGGACGGGGAGCCCTCG 120
 21 -G--S--S--T--S--G--S--R--R--S--R--R--R--S--G--D--G--E--P--S-  40

121 GGGGCCCCACCGCTGCCGCCGCCGCCACCCGCCGTCAGCTACCCGGACTGGATCGGCCAG 180
121 GGGGCCCCACCGCTGCCGCCGCCGCCACCCGCCGTCAGCTACCCGGACTGGATCGGCCAG 180
 41 -G--A--P--P--L--P--P--P--P--P--A--V--S--Y--P--D--W--I--G--Q-  60

181 AGTTACTCCGAGGTGATGAGCCTCAACGAGCACTCGATGCAGGCGCTGTCCTGGCGCAAG 240
181 AGTTACTCCGAGGTGATGAGCCTCAACGAGCACTCGATGCAGGCGCTGTCCTGGCGCAAG 240
 61 -S--Y--S--E--V--M--S--L--N--E--H--S--M--Q--A--L--S--W--R--K-  80

241 CTCTACTTAAGCCGCGCCAAGCTCAAAGCTTCCAGCCGGACCTCGGCTCTGCTCTCCGGC 300
241 CTCTACTTAAGCCGCGCCAAGCTCAAAGCTTCCAGCCGGACCTCGGCTCTGCTCTCCGGC 300
 81 -L--Y--L--S--R--A--K--L--K--A--S--S--R--T--S--A--L--L--S--G- 100

301 TTCGCCATGGTAGCGATGGTGGAAGTCCAGCTGGACACAGACCATGACTACCCACCAGGG 360
301 TTCGCCATGGTAGCGATGGTGGAAGTCCAGCTGGACACAGACCATGACTACCCACCAGGG 360
101 -F--A--M--V--A--M--V--E--V--Q--L--D--T--D--H--D--Y--P--P--G- 120

361 TTGCTCATCGTCTTTAGTGCCTGCACCACAGTGCTAGTGGCCGTGCACCTGTTTGCCCTC 420
361 TTGCTCATCGTCTTTAGTGCCTGCACCACAGTGCTAGTGGCCGTGCACCTGTTTGCCCTC 420
121 -L--L--I--V--F--S--A--C--T--T--V--L--V--A--V--H--L--F--A--L- 140

421 ATGATCAGCACCTGCATCCTGCCCAACATCGAGGCTGTGAGCAACGTCCACAACCTCAAC 480
421 ATGATCAGCACCTGCATCCTGCCCAACATCGAGGCTGTGAGCAACGTCCACAACCTCAAC 480
141 -M--I--S--T--C--I--L--P--N--I--E--A--V--S--N--V--H--N--L--N- 160

481 TCGGTCAAAGAGTCACCCCACGAGCGCATGCATCGCCACATCGAGCTGGCCTGGGCCTTC 540
481 TCGGTCAAAGAGTCACCCCACGAGCGCATGCATCGCCACATCGAGCTGGCCTGGGCCTTC 540
161 -S--V--K--E--S--P--H--E--R--M--H--R--H--I--E--L--A--W--A--F- 180

541 TCCACGGTCATCGGGACGCTGCTTTTCCTAGCAGAGGTCGTGCTGCTCTGCTGGGTCAAG 600
541 TCCACGGTCATCGGGACGCTGCTTTTCCTAGCAGAGGTCGTGCTGCTCTGCTGGGTCAAG 600
181 -S--T--V--I--G--T--L--L--F--L--A--E--V--V--L--L--C--W--V--K- 200

601 TTCTTACCTCTCAAGAGGCAAGCGGGACAGCCAAGCCCCACCAAGCCTCCCGCTGAATCA 660
601 TTCTTACCTCTCAAGAGGCAAGCGGGACAGCCAAGCCCCACCAAGCCTCCCGCTGAATCA 660
201 -F--L--P--L--K--R--Q--A--G--Q--P--S--P--T--K--P--P--A--E--S- 220
```

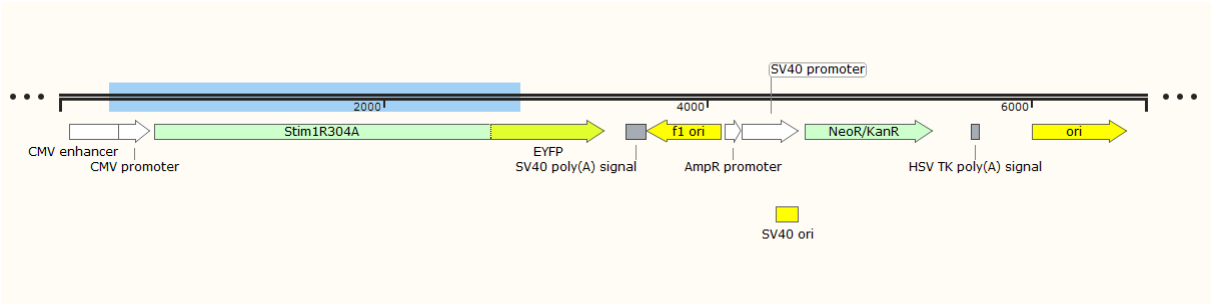
```
661 GTCATCGTCGCCAACCACAGCGACAGCAGCGGCATCACCCCGGGTGAGGCGGCAGCCATT 720
661 GTCATCGTCGCCAACCACAGCGACAGCAGCGGCATCACCCCGGGTGAGGCGGCAGCCATT 720
221 -V--I--V--A--N--H--S--D--S--S--G--I--T--P--G--E--A--A--A--I- 240

721 GCCTCCACCGCCATCATGGTTCCCTGTGGCCTGGTTTTTATCGTCTTTGCTGTTCACTTC 780
721 GCCTCCACCGCCATCATGGTTCCCTGTGGCCTGGTTTTTATCGTCTTTGCTGTTCACTTC 780
241 -A--S--T--A--I--M--V--P--C--G--L--V--F--I--V--F--A--V--H--F- 260

781 TACCGCTCCCTGGTCAGCCATAAGACGGACCGGCAGTTCCAGGAGCTCAATGAGCTGGCC 840
781 TACCGCTCCCTGGTCAGCCATAAGACGGACCGGCAGTTCCAGGAGCTCAATGAGCTGGCC 840
261 -Y--R--S--L--V--S--H--K--T--D--R--Q--F--Q--E--L--N--E--L--A- 280

841 GAGTTTGCCCGCTTGCAGGACCAGCTGGACCACAGAGGGGACCATTCTCTAACACCGGGC 900
841 GAGTTTGCCCGCTTGCAGGACCAGCTGGACCACAGAGGGGACCATTCTCTAACACCGGGC 900
281 -E--F--A--R--L--Q--D--Q--L--D--H--R--G--D--H--S--L--T--P--G- 300

901 ACCCACTATGCCTAA                                               915
901 ACCCACTATGCCTAA                                               915
301 -T--H--Y--A--*-                                               915
```

## 8.8 Constructs

| Number | Construct | Origin |
|---|---|---|
| 1 | Stim1-WT-YFP (clone 3) | Gift from professor Leonidas Tsiokas (Nesin et al., 2014). Transformed into competent E. coli bacteria, plated on LB agar plates and mini/midi prep. |
| 2 | Stim1-p.R304W-YFP (clone 2) | |
| 3 | Stim1-p.E296del-YFP | In-house Q5 mutagenesis and mini prep |
| 4 | Stim1-p.E296del+R304W-YFP | |
| 5 | Stim1-p.R304A-YFP | In-house Q5 mutagenesis and mini prep. |
| 6 | Stim1-p.R304P-YFP | |
| 7 | Stim1-p.R304S-YFP | |
| 8 | Stim1-p.R304E-YFP | |
| 9 | mCHERRY-Orai1 | Acquired from Vector Builder. Plated on agar plates and maxi prep. |

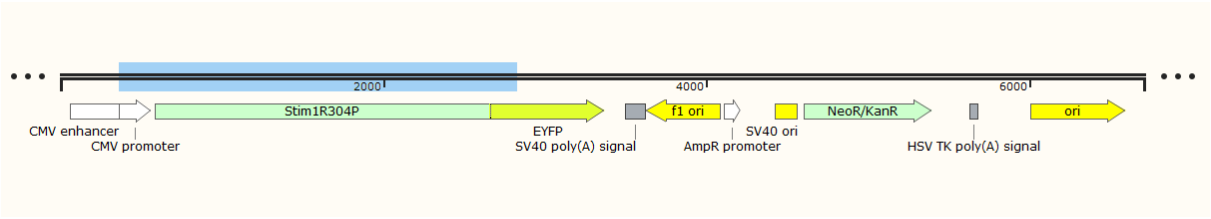
***Table S10**. Overview of constructs created and used in this thesis.*

### 8.8.1 Sequence acquired of Stim1-p.R304A-YFP, ID 5



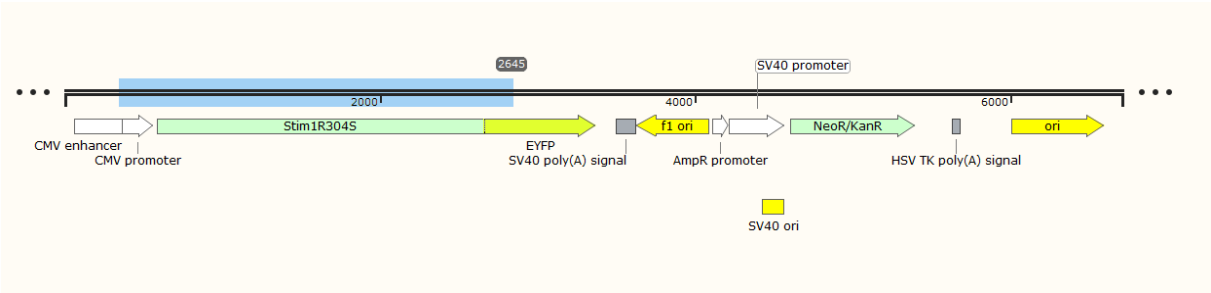

>Stim1-R304A-YFP
GGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTG
GCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGA
CGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGC
CCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTAGT
GAACCGTCAGATCCATGGATGTGTGCGCCCGTCTTGCCCTGTGGCTTCTTTGGGGGCTCCTTCTGCA
TCAGGGCCAGAGTCTCAGCCATAGTCACAGTGAAAAGAATACAGGAGCTAGCTCCGGGGCGACTT
CTGAAGAGTCTACCGAAGCAGAGTTTTGCCGAATTGACAAGCCCCTGTGCCACAGTGAGGATGAG
AAGCTCAGCTTTGAGGCCGTCCGAAACATCCATAAGCTGATGGATGACGATGCCAATGGTGATGTG
GATGTGGAAGAAAGTGATGAGTTCCTAAGGGAAGACCTCAATTACCATGACCCAACAGTGAAACA
TAGCACCTTCCATGGTGAGGATAAGCTTATCAGCGTGGAGGACCTGTGGAAGGCGTGGAAGTCATC
AGAAGTGTACAACTGGACTGTGGATGAGGTGATACAGTGGCTCATTACGTATGTGGAGCTGCCACA
GTATGAGGAAACCTTCCGGAAGTTGCAGCTTACTGGCCACGCCATGCCAAGGCTAGCAGTAACCAA
CACCACCATGACAGGGACTGTACTGAAGATGACAGATCGGAGCCACAGGCAGAAGCTGCAGCTGA
AGGCCCTGGACACAGTGCTGTTTGGGCCTCCTCTCTTGACTCGGCATAATCACCTGAAGGACTTCAT
GCTGGTGGTGTCTATCGTTATTGGTGTGGGTGGCTGCTGGTTTGCCTATATCCAGAACCGTTACTCT
AAGGAGCACATGAAGAAAATGATGAAGGATCTGGAAGGGTTACACCGGGCTGAGCAGAGTCTGCA
TGACCTTCAGGAAAGGCTGCACAAGGCCCAGGAGGAGCACCGAACTGTGGAAGTAGAGAAGGTCC
ACCTGGAGAAGAAGCTGCGAGATGAGATCAACCTTGCCAAGCAGGAAGCTCAGCGGCTGAAGGAG
CTGGCGGAGGGTACTGAGAATGAGAGGAGCCGTCAAAAATATGCTGAGGAAGAGCTGGAGCAGGT
TCGGGAGGCCTTGAGGAAAGCAGAGAAGGAGCTGGAATCACACAGCTCATGGTATGCTCCTGAGG
CCCTGCAGAAGTGGCTGCAGCTGACCCATGAGGTGGAGGTGCAGTACTACAACATCAAGAAGCAA
AATGCAGAGAGGCAGCTGCTGGTGGCCAAGGAGGGGGCTGAGAAAATAAAAAAGAAGAGAAACA
CGCTTTTTGGTACCTTCCATGTGGCCCACAGCTCTTCCCTGGATGATGTGGATCATAAAATCCTAAC
TGCTAAGCAAGCTCTGAGTGAGGTGACAGCGGCACTGAGGGAGCGCCTGCACCGGTGGCAGCAGA
TCGAGATCCTCTGCGGTTTCCAGATTGTCAATAACCCCGGCATCCACTCCTTGGTGGCTGCTCTCAA
CATCGACCCCAGCTGGATGGGCAGCACCCGCCCTAACCCCGCCCACTTCATCATGACTGACGATGT
GGATGACATGGATGAGGAGATTGTGTCGCCCTTGTCCATGCAGTCCCCCAGCCTGCAGAGCAGTGT
CCGGCAGCGCCTGACGGAGCCACAGCTTGGCCTGGGATCTCAGAGGGATTTGACCCATTCCGATTC
GGAGTCCTCCCTCCACATGAGTGACCGCCAGCGTGTGGCCCCCAAGCCTCCTCAGATGGGCCGTGC
TGCAGATGAAGCTCTCAATGCCATGCCTTCCAATGGCAGCCATCGGCTGATTGAGGGGTCCATCC
AGGATCTCTGGTGGAGAAACTGCCTGACAGCCCTGCTCTGGCCAAGAAGACATTTATGGCGTTGAA
CCATGGCCTAGACAAGGCCCACAGCCTGATGGAGCTGAACCCCTCAGTCCCACCTGGTGGCTCCCC
ACTTTTGGATTCTTCCCATTCTCTTAGCCCCAGTTCCCCAGACCCAGACACGCCATCTCCAGTTGGG
GACAACCGAGCTCTGCAGGGTAGCCGAAACACACGAATTCCCCACTTGGCTGGCAAGAAGGCAAT
GGCTGAGGAGGATAATGGTTCCATTGGTGAGGAGACAGACTCCAGTCCAGGCAGGAAGAAGTTTC
CTCTCAAAATTTTTAAGAAGCCTCTTAAGAAGTAGCGCCACCATGGTGAGCAAGGGCGAGGAGCTG
TTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTG
TCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGG
CAAGCTGCCCGTGCCCTGGCCCACCCTCGTGAC

8.8.2 Sequence acquired of Stim1-p.R304P-YFP, ID 6



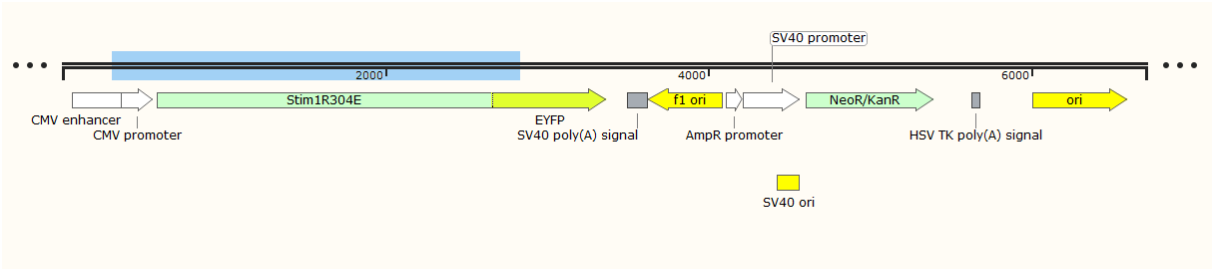

>Stim1-p.R304P-YFP
GGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAG
TCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGT
CGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGC
AGAGCTGGTTTAGTGAACCGTCAGATCCATGGATGTGTGCGCCCGTCTTGCCCTGTGGCTTCTTTGG
GGGCTCCTTCTGCATCAGGGCCAGAGTCTCAGCCATAGTCACAGTGAAAAGAATACAGGAGCTAG
CTCCGGGGCGACTTCTGAAGAGTCTACCGAAGCAGAGTTTTGCCGAATTGACAAGCCCCTGTGCCA
CAGTGAGGATGAGAAGCTCAGCTTTGAGGCCGTCCGAAACATCCATAAGCTGATGGATGACGATG
CCAATGGTGATGTGGATGTGGAAGAAAGTGATGAGTTCCTAAGGGAAGACCTCAATTACCATGAC
CCAACAGTGAAACATAGCACCTTCCATGGTGAGGATAAGCTTATCAGCGTGGAGGACCTGTGGAA
GGCGTGGAAGTCATCAGAAGTGTACAACTGGACTGTGGATGAGGTGATACAGTGGCTCATTACGTA
TGTGGAGCTGCCACAGTATGAGGAAACCTTCCGGAAGTTGCAGCTTACTGGCCACGCCATGCCAAG
GCTAGCAGTAACCAACACCACCATGACAGGGACTGTACTGAAGATGACAGATCGGAGCCACAGGC
AGAAGCTGCAGCTGAAGGCCCTGGACACAGTGCTGTTTGGGCCTCCTCTCTTGACTCGGCATAATC
ACCTGAAGGACTTCATGCTGGTGGTGTCTATCGTTATTGGTGTGGGTGGCTGCTGGTTTGCCTATAT
CCAGAACCGTTACTCTAAGGAGCACATGAAGAAAATGATGAAGGATCTGGAAGGGTTACACCGGG
CTGAGCAGAGTCTGCATGACCTTCAGGAAAGGCTGCACAAGGCCCAGGAGGAGCACCGAACTGTG
GAAGTAGAGAAGGTCCACCTGGAGAAGAAGCTGCGAGATGAGATCAACCTTGCCAAGCAGGAAGC
TCAGCGGCTGAAGGAGCTGCCGGAGGGTACTGAGAATGAGAGGAGCCGTCAAAAATATGCTGAGG
AAGAGCTGGAGCAGGTTCGGGAGGCCTTGAGGAAAGCAGAGAAGGAGCTGGAATCACACAGCTC
ATGGTATGCTCCTGAGGCCCTGCAGAAGTGGCTGCAGCTGACCCATGAGGTGGAGGTGCAGTACTA
CAACATCAAGAAGCAAAATGCAGAGAGGCAGCTGCTGGTGGCCAAGGAGGGGGCTGAGAAAATA
AAAAAGAAGAGAAACACGCTTTTTGGTACCTTCCATGTGGCCCACAGCTCTTCCCTGGATGATGTG
GATCATAAAATCCTAACTGCTAAGCAAGCTCTGAGTGAGGTGACAGCGGCACTGAGGGAGCGCCT
GCACCGGTGGCAGCAGATCGAGATCCTCTGCGGTTTCCAGATTGTCAATAACCCCGGCATCCACTC
CTTGGTGGCTGCTCTCAACATCGACCCCAGCTGGATGGGCAGCACCCGCCCTAACCCCGCCCACTT
CATCATGACTGACGATGTGGATGACATGGATGAGGAGATTGTGTCGCCCTTGTCCATGCAGTCCCC
CAGCCTGCAGAGCAGTGTCCGGCAGCGCCTGACGGAGCCACAGCTTGGCCTGGGATCTCAGAGGG
ATTTGACCCATTCCGATTCGGAGTCCTCCCTCCACATGAGTGACCGCCAGCGTGTGGCCCCCAAGC
CTCCTCAGATGGGCCGTGCTGCAGATGAAGCTCTCAATGCCATGCCTTCCAATGGCAGCCATCGGC
TGATTGAGGGGGTCCATCCAGGATCTCTGGTGGAGAAACTGCCTGACAGCCCTGCTCTGGCCAAGA
AGACATTTATGGCGTTGAACCATGGCCTAGACAAGGCCCACAGCCTGATGGAGCTGAACCCCTCAG
TCCCACCTGGTGGCTCCCCACTTTTGGATTCTTCCCATTCTCTTAGCCCCAGTTCCCCAGACCCAGA
CACGCCATCTCCAGTTGGGGACAACCGAGCTCTGCAGGGTAGCCGAAACACACGAATTCCCCACTT
GGCTGGCAAGAAGGCAATGGCTGAGGAGGATAATGGTTCCATTGGTGAGGAGACAGACTCCAGTC
CAGGCAGGAAGAAGTTTCCTCTCAAAATTTTTAAGAAGCCTCTTAAGAAGTCGCCACCATGGTGAG
CAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACG
GCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAG
TTCATCTGCACCACCGGCAAGCTGCCCGTGCCC

### 8.8.3 Sequence acquired of Stim1-p.R304S-YFP, ID 7



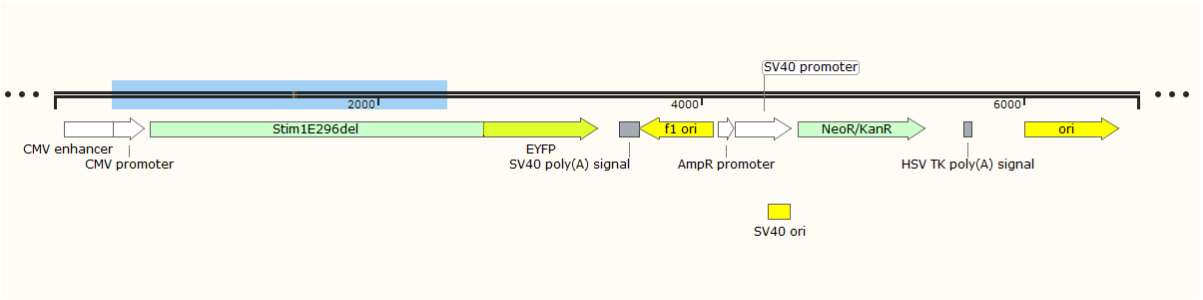

>Stim1-p.R304S-YFP
TTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTG
ACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATC
AACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTAC
GGTGGGAGGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAGATCCATGGATGTGTGCGCCCGT
CTTGCCCTGTGGCTTCTTTGGGGGCTCCTTCTGCATCAGGGCCAGAGTCTCAGCCATAGTCACAGTG
AAAAGAATACAGGAGCTAGCTCCGGGGCGACTTCTGAAGAGTCTACCGAAGCAGAGTTTTGCCGA
ATTGACAAGCCCCTGTGCCACAGTGAGGATGAGAAGCTCAGCTTTGAGGCCGTCCGAAACATCCAT
AAGCTGATGGATGACGATGCCAATGGTGATGTGGATGTGGAAGAAAGTGATGAGTTCCTAAGGGA
AGACCTCAATTACCATGACCCAACAGTGAAACATAGCACCTTCCATGGTGAGGATAAGCTTATCAG
CGTGGAGGACCTGTGGAAGGCGTGGAAGTCATCAGAAGTGTACAACTGGACTGTGGATGAGGTGA
TACAGTGGCTCATTACGTATGTGGAGCTGCCACAGTATGAGGAAACCTTCCGGAAGTTGCAGCTTA
CTGGCCACGCCATGCCAAGGCTAGCAGTAACCAACACCACCATGACAGGGACTGTACTGAAGATG
ACAGATCGGAGCCACAGGCAGAAGCTGCAGCTGAAGGCCCTGGACACAGTGCTGTTTGGGCCTCC
TCTCTTGACTCGGCATAATCACCTGAAGGACTTCATGCTGGTGGTGTCTATCGTTATTGGTGTGGGT
GGCTGCTGGTTTGCCTATATCCAGAACCGTTACTCTAAGGAGCACATGAAGAAAATGATGAAGGAT
CTGGAAGGGTTACACCGGGCTGAGCAGAGTCTGCATGACCTTCAGGAAAGGCTGCACAAGGCCCA
GGAGGAGCACCGAACTGTGGAAGTAGAGAAGGTCCACCTGGAGAAGAAGCTGCGAGATGAGATC
AACCTTGCCAAGCAGGAAGCTCAGCGGCTGAAGGAGCTGAGCGAGGGTACTGAGAATGAGAGGA
GCCGTCAAAAATATGCTGAGGAAGAGCTGGAGCAGGTTCGGGAGGCCTTGAGGAAAGCAGAGAA
GGAGCTGGAATCACACAGCTCATGGTATGCTCCTGAGGCCCTGCAGAAGTGGCTGCAGCTGACCCA
TGAGGTGGAGGTGCAGTACTACAACATCAAGAAGCAAAATGCAGAGAGGCAGCTGCTGGTGGCCA
AGGAGGGGGCTGAGAAAATAAAAAAGAAGAGAAACACGCTTTTTGGTACCTTCCATGTGGCCCAC
AGCTCTTCCCTGGATGATGTGGATCATAAAATCCTAACTGCTAAGCAAGCTCTGAGTGAGGTGACA
GCGGCACTGAGGGAGCGCCTGCACCGGTGGCAGCAGATCGAGATCCTCTGCGGTTTCCAGATTGTC
AATAACCCCGGCATCCACTCCTTGGTGGCTGCTCTCAACATCGACCCCAGCTGGATGGGCAGCACC
CGCCCTAACCCCGCCCACTTCATCATGACTGACGATGTGGATGACATGGATGAGGAGATTGTGTCG
CCCTTGTCCATGCAGTCCCCCAGCCTGCAGAGCAGTGTCCGGCAGCGCCTGACGGAGCCACAGCTT
GGCCTGGGATCTCAGAGGGATTTGACCCATTCCGATTCGGAGTCCTCCCTCCACATGAGTGACCGC
CAGCGTGTGGCCCCCAAGCCTCCTCAGATGGGCCGTGCTGCAGATGAAGCTCTCAATGCCATGCCT
TCCAATGGCAGCCATCGGCTGATTGAGGGGTCCATCCAGGATCTCTGGTGGAGAAACTGCCTGAC
AGCCCTGCTCTGGCCAAGAAGACATTTATGGCGTTGAACCATGGCCTAGACAAGGCCCACAGCCTG
ATGGAGCTGAACCCCTCAGTCCCACCTGGTGGCTCCCCACTTTTGGATTCTTCCCATTCTCTTAGCC
CCAGTTCCCCAGACCCAGACACGCCATCTCCAGTTGGGGACAACCGAGCTCTGCAGGGTAGCCGAA
ACACACGAATTCCCCACTTGGCTGGCAAGAAGGCAATGGCTGAGGAGGATAATGGTTCCATTGGTG
AGGAGACAGACTCCAGTCCAGGCAGGAAGAAGTTTCCTCTCAAAATTTTTAAGAAGCCTCTTAAGA
AGTAGCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGA
GCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCT
ACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCG
TGACC

### 8.8.4 Sequence acquired of Stim1-p.R304E-YFP, ID 8



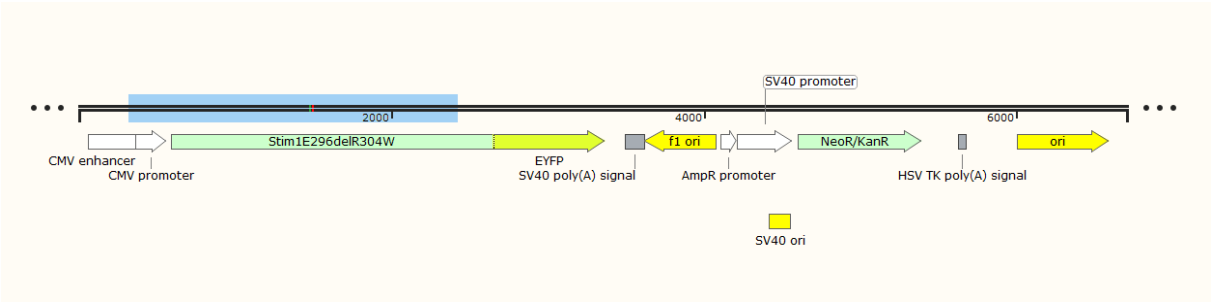

>Stim1-p.R304E-YFP
TATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTT
TTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCAT
TGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTC
CGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTT
AGTGAACCGTCAGATCCATGGATGTGTGCGCCCGTCTTGCCCTGTGGCTTCTTTGGGGGCTCCTTCT
GCATCAGGGCCAGAGTCTCAGCCATAGTCACAGTGAAAAGAATACAGGAGCTAGCTCCGGGGCGA
CTTCTGAAGAGTCTACCGAAGCAGAGTTTTGCCGAATTGACAAGCCCCTGTGCCACAGTGAGGATG
AGAAGCTCAGCTTTGAGGCCGTCCGAAACATCCATAAGCTGATGGATGACGATGCCAATGGTGATG
TGGATGTGGAAGAAAGTGATGAGTTCCTAAGGGAAGACCTCAATTACCATGACCCAACAGTGAAA
CATAGCACCTTCCATGGTGAGGATAAGCTTATCAGCGTGGAGGACCTGTGGAAGGCGTGGAAGTC
ATCAGAAGTGTACAACTGGACTGTGGATGAGGTGATACAGTGGCTCATTACGTATGTGGAGCTGCC
ACAGTATGAGGAAACCTTCCGGAAGTTGCAGCTTACTGGCCACGCCATGCCAAGGCTAGCAGTAAC
CAACACCACCATGACAGGGACTGTACTGAAGATGACAGATCGGAGCCACAGGCAGAAGCTGCAGC
TGAAGGCCCTGGACACAGTGCTGTTTGGGCCTCCTCTCTTGACTCGGCATAATCACCTGAAGGACTT
CATGCTGGTGGTGTCTATCGTTATTGGTGTGGGTGGCTGCTGGTTTGCCTATATCCAGAACCGTTAC
TCTAAGGAGCACATGAAGAAAATGATGAAGGATCTGGAAGGGTTACACCGGGCTGAGCAGAGTCT
GCATGACCTTCAGGAAAGGCTGCACAAGGCCCAGGAGGAGCACCGAACTGTGGAAGTAGAGAAG
GTCCACCTGGAGAAGAAGCTGCGAGATGAGATCAACCTTGCCAAGCAGGAAGCTCAGCGGCTGAA
GGAGCTGGAGGAGGGTACTGAGAATGAGAGGAGCCGTCAAAAATATGCTGAGGAAGAGCTGGAG
CAGGTTCGGGAGGCCTTGAGGAAAGCAGAGAAGGAGCTGGAATCACACAGCTCATGGTATGCTCC
TGAGGCCCTGCAGAAGTGGCTGCAGCTGACCCATGAGGTGGAGGTGCAGTACTACAACATCAAGA
AGCAAAATGCAGAGAGGCAGCTGCTGGTGGCCAAGGAGGGGGCTGAGAAAATAAAAAAGAAGAG
AAACACGCTTTTTGGTACCTTCCATGTGGCCCACAGCTCTTCCCTGGATGATGTGGATCATAAAATC
CTAACTGCTAAGCAAGCTCTGAGTGAGGTGACAGCGGCACTGAGGGAGCGCCTGCACCGGTGGCA
GCAGATCGAGATCCTCTGCGGTTTCCAGATTGTCAATAACCCCGGCATCCACTCCTTGGTGGCTGCT
CTCAACATCGACCCCAGCTGGATGGGCAGCACCCGCCCTAACCCCGCCCACTTCATCATGACTGAC
GATGTGGATGACATGGATGAGGAGATTGTGTCGCCCTTGTCCATGCAGTCCCCCAGCCTGCAGAGC
AGTGTCCGGCAGCGCCTGACGGAGCCACAGCTTGGCCTGGGATCTCAGAGGGATTTGACCCATTCC
GATTCGGAGTCCTCCCTCCACATGAGTGACCGCCAGCGTGTGGCCCCCAAGCCTCCTCAGATGGGC
CGTGCTGCAGATGAAGCTCTCAATGCCATGCCTTCCAATGGCAGCCATCGGCTGATTGAGGGGGTC
CATCCAGGATCTCTGGTGGAGAAACTGCCTGACAGCCCTGCTCTGGCCAAGAAGACATTTATGGCG
TTGAACCATGGCCTAGACAAGGCCCACAGCCTGATGGAGCTGAACCCCTCAGTCCCACCTGGTGGC
TCCCCACTTTTGGATTCTTCCCATTCTCTTAGCCCCAGTTCCCCAGACCCAGACACGCCATCTCCAG
TTGGGGACAACCGAGCTCTGCAGGGTAGCCGAAACACACGAATTCCCCACTTGGCTGGCAAGAAG
GCAATGGCTGAGGAGGATAATGGTTCCATTGGTGAGGAGACAGACTCCAGTCCAGGCAGGAAGAA
GTTTCCTCTCAAAATTTTTAAGAAGCCTCTTAAGAAGTAGCGCCACCATGGTGAGCAAGGGCGAGG
AGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCA
GCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACC
ACCGGCAAGCTGCCCGTGCCCTG

8.8.5 Sequence acquired of Stim1-p.E296del-YFP, ID 3



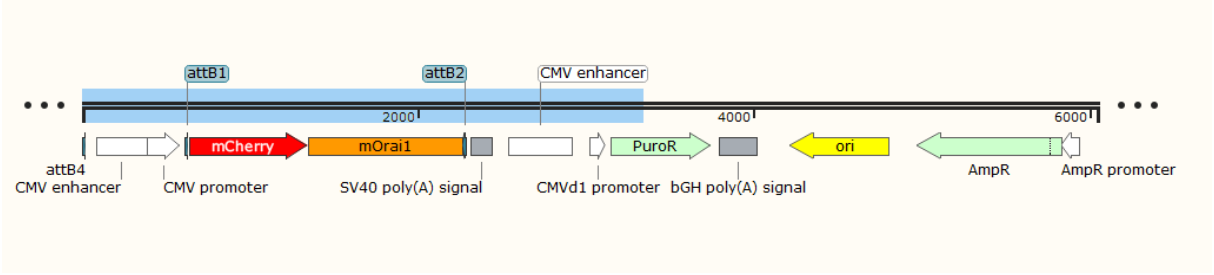

>Stim1-p.E296del-YFP
TCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTC
ACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACG
GGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTG
GGAGGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAGATCCATGGATGTGTGCGCCCGTCTTG
CCCTGTGGCTTCTTTGGGGGCTCCTTCTGCATCAGGGCCAGAGTCTCAGCCATAGTCACAGTGAAA
AGAATACAGGAGCTAGCTCCGGGGCGACTTCTGAAGAGTCTACCGAAGCAGAGTTTTGCCGAATTG
ACAAGCCCCTGTGCCACAGTGAGGATGAGAAGCTCAGCTTTGAGGCCGTCCGAAACATCCATAAG
CTGATGGATGACGATGCCAATGGTGATGTGGATGTGGAAGAAAGTGATGAGTTCCTAAGGGAAGA
CCTCAATTACCATGACCCAACAGTGAAACATAGCACCTTCCATGGTGAGGATAAGCTTATCAGCGT
GGAGGACCTGTGGAAGGCGTGGAATCATCAGAAGTGTACAACTGGACTGTGGATGAGGTGATACA
GTGGCTCATTACGTATGTGGAGCTGCCACAGTATGAGGAAACCTTCCGGAAGTTGCAGCTTACTGG
CCACGCCATGCCAAGGCTAGCAGTAACCAACACCACCATGACAGGGACTGTACTGAAGATGACAG
ATCGGAGCCACAGGCAGAAGCTGCAGCTGAAGGCCCTGGACACAGTGCTGTTTGGGCCTCCTCTCT
TGACTCGGCATAATCACCTGAAGGACTTCATGCTGGTGGTGTCTATCGTTATTGGTGTGGGTGGCTG
CTGGTTTGCCTATATCCAGAACCGTTACTCTAAGGAGCACATGAAGAAAATGATGAAGGATCTGGA
AGGGTTACACCGGGCTGAGCAGAGTCTGCATGACCTTCAGGAAAGGCTGCACAAGGCCCAGGAGG
AGCACCGAACTGTGGAAGTAGAGAAAGTCCACCTGGAGAAGAAGCTGCGAGATGAGATCAACCTT
GCCAAGCAGGCTCAGCGGCTGAAGGAGCTGAGGGAGGGTACTGAGAATGAGAGGAGCCGTCAAA
AATATGCTGAGGAAGAGCTGGAGCAGGTTCGGGAGGCCTTGAGGAAAGCAGAGAAGGAGCTGGA
ATCACACAGCTCATGGTATGCTCCTGAGGCCCTGCAGAAGTGGCTGCAGCTGACCCATGAGGTGGA
GGTGCAGTACTACAACATCAAGAAGCAAAATGCAGAGAGGCAGCTGCTGGTGGCCAAGGAGGGG
GCTGAGAAAATAAAAAAGAAGAGAAACACGCTTTTTGGTACCTTCCATGTGGCCCACAGCTCTTCC
CTGGATGATGTGGATCATAAAATCCTAACTGCTAAGCAAGCTCTGAGTGAGGTGACAGCGGCACTG
AGGGAGCGCCTGCACCGGTGGCAGCAGATCGAGATCCTCTGCGGTTTCCAGATTGTCAATAACCCC
GGCATCCACTCCTTGGTGGCTGCTCTCAACATCGACCCCAGCTGGATGGGCAGCACCCGCCCTAAC
CCCGCCCACTTCATCATGACTGACGATGTGGATGACATGGATGAGGAGATTGTGTCGCCCTTGTCC
ATGCAGTCCCCCAGCCTGCAGAGCAGTGTCCGGCAGCGCCTGACGGAGCCACAGCTTGGCCTGGG
ATCTCAGAGGGATTTGACCCATTCCGATTCGGAGTCCTCCCTCCACATGAGTGACCGCCAGCGTGT
GGCCCCCAAGCCTCCTCAGATGGGCCGTGCTGCAGATGAAGCTCTCAATGCCATGCCTTCCAATGG
CAGCCATCGGCTGATTGAGGGGGTCCATCCAGGATCTCTGGTGGAGAAACTGCCTGACAGCCCTGC
TCTGGCCAAGAAGACATTTATGGCGTTGAACCATGGCCTAGACAAGGCCCACAGCCTGATGGAGCT
GAACCCCTCAGTCCCACCTGGTGGCTCCCCACTTT

### 8.8.6 Sequence acquired of Stim1-p.E296del+R304W-YFP, ID4




>Stim1-p.E296del+R304W-YFP
ACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCA
ATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGA
GTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGC
AAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAG
ATCCATGGATGTGTGCGCCCGTCTTGCCCTGTGGCTTCTTTGGGGGCTCCTTCTGCATCAGGGCCAG
AGTCTCAGCCATAGTCACAGTGAAAAGAATACAGGAGCTAGCTCCGGGGCGACTTCTGAAGAGTC
TACCGAAGCAGAGTTTTGCCGAATTGACAAGCCCCTGTGCCACAGTGAGGATGAGAAGCTCAGCTT
TGAGGCCGTCCGAAACATCCATAAGCTGATGGATGACGATGCCAATGGTGATGTGGATGTGGAAG
AAAGTGATGAGTTCCTAAGGGAAGACCTCAATTACCATGACCCAACAGTGAAACATAGCACCTTCC
ATGGTGAGGATAAGCTTATCAGCGTGGAGGACCTGTGGAAGGCGTGGAAGTCATCAGAAGTGTAC
AACTGGACTGTGGATGAGGTGATACAGTGGCTCATTACGTATGTGGAGCTGCCACAGTATGAGGAA
ACCTTCCGGAAGTTGCAGCTTACTGGCCACGCCATGCCAAGGCTAGCAGTAACCAACACCACCATG
ACAGGGACTGTACTGAAGATGACAGATCGGAGCCACAGGCAGAAGCTGCAGCTGAAGGCCCTGGA
CACAGTGCTGTTTGGGCCTCCTCTCTTGACTCGGCATAATCACCTGAAGGACTTCATGCTGGTGGTG
TCTATCGTTATTGGTGTGGGTGGCTGCTGGTTTGCCTATATCCAGAACCGTTACTCTAAGGAGCACA
TGAAGAAAATGATGAAGGATCTGGAAGGGTTACACCGGGCTGAGCAGAGTCTGCATGACCTTCAG
GAAAGGCTGCACAAGGCCCAGGAGGAGCACCGAACTGTGGAAGTAGAGAAGGTCCACCTGGAGA
AGAAGCTGCGAGATGAGATCAACCTTGCCAAGCAGGCTCAGCGGCTGAAGGAGCTGTGGGAGGGT
ACTGAGAATGAGAGGAGCCGTCAAAAATATGCTGAGGAAGAGCTGGAGCAGGTTCGGGAGGCCTT
GAGGAAAGCAGAGAAGGAGCTGGAATCACACAGCTCATGGTATGCTCCTGAGGCCCTGCAGAAGT
GGCTGCAGCTGACCCATGAGGTGGAGGTGCAGTACTACAACATCAAGAAGCAAAATGCAGAGAGG
CAGCTGCTGGTGGCCAAGGAGGGGGCTGAGAAAATAAAAAAGAAGAGAAACACGCTTTTTGGTAC
CTTCCATGTGGCCCACAGCTCTTCCCTGGATGATGTGGATCATAAAATCCTAACTGCTAAGCAAGCT
CTGAGTGAGGTGACAGCGGCACTGAGGGAGCGCCTGCACCGGTGGCAGCAGATCGAGATCCTCTG
CGGTTTCCAGATTGTCAATAACCCCGGCATCCACTCCTTGGTGGCTGCTCTCAACATCGACCCCAGC
TGGATGGGCAGCACCCGCCCTAACCCCGCCCACTTCATCATGACTGACGATGTGGATGACATGGAT
GAGGAGATTGTGTCGCCCTTGTCCATGCAGTCCCCCAGCCTGCAGAGCAGTGTCCGGCAGCGCCTG
ACGGAGCCACAGCTTGGCCTGGGATCTCAGAGGGATTTGACCCATTCCGATTCGGAGTCCTCCCTC
CACATGAGTGACCGCCAGCGTGTGGCCCCCAAGCCTCCTCAGATGGGCCGTGCTGCAGATGAAGCT
CTCAATGCCATGCCTTCCAATGGCAGCCATCGGCTGATTGAGGGGGTCCATCCAGGATCTCTGGTG
GAGAAACTGCCTGACAGCCCTGCTCTGGCCAAGAAGACATTTATGGCGTTGAACCATGGCCTAGAC
AAGGCCCACAGCCTGATGGAGCTGAACCCCTCAGTCCCACCTGGTGGCTCCCCACTTTTGGAT

8.8.7 Sequence acquired from Sanger sequencing of mCHERRY-*Orai1*, ID9




>mCHERRY-*Orai1*
CAACTTTGTATAGAAAAGTTGTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCA
TATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCC
CGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGT
CAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGT
ACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTA
TGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTT
GGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTG
ACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCG
CCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTAG
TGAACCGTCAGATCCAAGTTTGTACAAAAAAGCAGGCTGCCACCATGGTGAGCAAGGGCGAGGAG
GATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGC
CACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCT
GAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGG
CTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGG
CTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTC
CCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCC
CGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCG
CCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTC
AAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGTT
GGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCC
ACTCCACCGGCGGCATGGACGAGCTGTACAAGATGCATCCGGAGCCTGCCCCGCCCCCGAGTCACA
GCAATCCGGAGCTTCCCGTGAGCGGCGGCAGCAGCACTAGCGGCAGCCGCCGGAGCCGCCGCCGC
AGCGGGGACGGGGAGCCCTCGGGGGCCCCACCGCTGCCGCCGCCGCCACCCGCCGTCAGCTACCC
GGACTGGATCGGCCAGAGTTACTCCGAGGTGATGAGCCTCAACGAGCACTCGATGCAGGCGCTGTC
CTGGCGCAAGCTCTACTTAAGCCGCGCCAAGCTCAAAGCTTCCAGCCGGACCTCGGCTCTGCTCTC
CGGCTTCGCCATGGTAGCGATGGTGGAAGTCCAGCTGGACACAGACCATGACTACCCACCAGGGTT
GCTCATCGTCTTTAGTGCCTGCACCACAGTGCTAGTGGCCGTGCACCTGTTTGCCCTCATGATCAGC
ACCTGCATCCTGCCCAACATCGAGGCTGTGAGCAACGTCCACAACCTCAACTCGGTCAAAGAGTCA
CCCCACGAGCGCATGCATCGCCACATCGAGCTGGCCTGGGCCTTCTCCACGGTCATCGGGACGCTG
CTTTTCCTAGCAGAGGTCGTGCTGCTCTGCTGGGTCAAGTTCTTACCTCTCAAGAGGCAAGCGGGA
CAGCCAAGCCCCACCAAGCCTCCCGCTGAATCAGTCATCGTCGCCAACCACAGCGACAGCAGCGG
CATCACCCCGGGTGAGGCGGCAGCCATTGCCTCCACCGCCATCATGGTTCCCTGTGGCCTGGTTTTT
ATCGTCTTTGCTGTTCACTTCTACCGCTCCCTGGTCAGCCATAAGACGGACCGGCAGTTCCAGGAGC
TCAATGAGCTGGCCGAGTTTGCCCGCTTGCAGGACCAGCTGGACCACAGAGGGGACCATTCTCTAA
CACCGGGCACCCACTATGCCTAAACCCAGCTTTCTTGTACAAAGTGGTGATGGCCGGCCGCTTCGA
GCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATG
CTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTT
AACAACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGC
AAGTAAAACCTCTACAAATGTGGTACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATCA
ATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGC
CCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTA

ACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCA
GTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCC
TGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCA
TCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCAC
GGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGG
ACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGG
AGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCGCCACCATGACCGAGTACAA
GCCCACGGTGCGCCTCGCCACCCGCGACGACGTCCCCAGGGCCGTACGCACCCTCGCCGCCGCGTT
CGCCGACTACCCCGCCACGCGCCACACCGTCGATCCGGACCGCCACATCGAGCGGGTCACCGAGCT
GCAAGAACTCTTCCTCACGCGCGTCGGGCTCGACATCGG

# 9 References


Albarran L, Lopez JJ, Salido GM, Rosado JA. 2016. Historical Overview of Store-Operated Ca(2+) Entry. Adv Exp Med Biol 898:3-24.

Bohm J, Chevessier F, Koch C, Peche GA, Mora M, Morandi L, Pasanisi B, Moroni I, Tasca G, Fattori F and others. 2014. Clinical, histological and genetic characterisation of patients with tubular aggregate myopathy caused by mutations in STIM1. J Med Genet 51(12):824-833.

Bohm J, Chevessier F, Maues De Paula A, Koch C, Attarian S, Feger C, Hantai D, Laforet P, Ghorab K, Vallat JM and others. 2013. Constitutive activation of the calcium sensor STIM1 causes tubular-aggregate myopathy. Am J Hum Genet 92(2):271-278.

Braun A, Gessner JE, Varga-Szabo D, Syed SN, Konrad S, Stegner D, Vogtle T, Schmidt RE, Nieswandt B. 2009. STIM1 is essential for Fcgamma receptor activation and autoimmune inflammation. Blood 113(5):1097-1104.

Brown TA. 2007. Genomes 3. New York: Garland Science Pub. xxii, 713 p. p.

Cahalan MD. 2009. STIMulating store-operated Ca(2+) entry. Nat Cell Biol 11(6):669-677.

Clapham DE. 2007. Calcium signaling. Cell 131(6):1047-1058.

Derler I, Jardin I, Romanin C. 2016. Molecular mechanisms of STIM/Orai communication. Am J Physiol Cell Physiol 310(8):C643-662.

Endo Y, Noguchi S, Hara Y, Hayashi YK, Motomura K, Miyatake S, Murakami N, Tanaka S, Yamashita S, Kizu R and others. 2015. Dominant mutations in ORAI1 cause tubular aggregate myopathy with hypocalcemia via constitutive activation of store-operated Ca(2)(+) channels. Hum Mol Genet 24(3):637-648.

Feske S. 2010. CRAC channelopathies. Pflugers Arch 460(2):417-435.

Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A. 2006. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature 441(7090):179-185.

Feske S, Prakriya M. 2013. Conformational dynamics of STIM1 activation. Nat Struct Mol Biol 20(8):918-919.

Grosse J, Braun A, Varga-Szabo D, Beyersdorf N, Schneider B, Zeitlmann L, Hanke P, Schropp P, Muhlstedt S, Zorn C and others. 2007. An EF hand mutation in Stim1 causes premature platelet activation and bleeding in mice. J Clin Invest 117(11):3540-3550.

Gwozdz T, Dutko-Gwozdz J, Zarayskiy V, Peter K, Bolotina VM. 2008. How strict is the correlation between STIM1 and Orai1 expression, puncta formation, and ICRAC activation? Am J Physiol Cell Physiol 295(5):C1133-1140.

Henke N, Albrecht P, Pfeiffer A, Toutzaris D, Zanger K, Methner A. 2012. Stromal interaction molecule 1 (STIM1) is involved in the regulation of mitochondrial shape and bioenergetics and plays a role in oxidative stress. J Biol Chem 287(50):42042-42052.

Klejman ME, Gruszczynska-Biegala J, Skibinska-Kijek A, Wisniewska MB, Misztal K, Blazejczyk M, Bojarski L, Kuznicki J. 2009. Expression of STIM1 in brain and

puncta-like co-localization of STIM1 and ORAI1 upon depletion of Ca(2+) store in neurons. Neurochem Int 54(1):49-55.
Korzeniowski MK, Manjarres IM, Varnai P, Balla T. 2010. Activation of STIM1-Orai1 involves an intramolecular switching mechanism. Sci Signal 3(148):ra82.
Kraft R. 2015. STIM and ORAI proteins in the nervous system. Channels (Austin) 9(5):245-252.
Lacruz RS, Feske S. 2015. Diseases caused by mutations in ORAI1 and STIM1. Ann N Y Acad Sci 1356:45-79.
Lytton J, Westlin M, Hanley MR. 1991. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. J Biol Chem 266(26):17067-17071.
Markello T, Chen D, Kwan JY, Horkayne-Szakaly I, Morrison A, Simakova O, Maric I, Lozier J, Cullinane AR, Kilo T and others. 2015. York platelet syndrome is a CRAC channelopathy due to gain-of-function mutations in STIM1. Mol Genet Metab 114(3):474-482.
McNally BA, Somasundaram A, Jairaman A, Yamashita M, Prakriya M. 2013. The C- and N-terminal STIM1 binding sites on Orai1 are required for both trapping and gating CRAC channels. J Physiol 591(11):2833-2850.
Misceo D, Holmgren A, Louch WE, Holme PA, Mizobuchi M, Morales RJ, De Paula AM, Stray-Pedersen A, Lyle R, Dalhus B and others. 2014. A dominant STIM1 mutation causes Stormorken syndrome. Hum Mutat 35(5):556-564.
Morin G, Bruechle NO, Singh AR, Knopp C, Jedraszak G, Elbracht M, Bremond-Gignac D, Hartmann K, Sevestre H, Deutz P and others. 2014. Gain-of-Function Mutation in STIM1 (P.R304W) Is Associated with Stormorken Syndrome. Hum Mutat 35(10):1221-1232.
Nesin V, Wiley G, Kousi M, Ong EC, Lehmann T, Nicholl DJ, Suri M, Shahrizaila N, Katsanis N, Gaffney PM and others. 2014. Activating mutations in STIM1 and ORAI1 cause overlapping syndromes of tubular myopathy and congenital miosis. Proc Natl Acad Sci U S A 111(11):4197-4202.
Niemeyer BA. 2016. Changing calcium: CRAC channel (STIM and Orai) expression, splicing, and posttranslational modifiers. Am J Physiol Cell Physiol 310(9):C701-709.
Prakriya M, Lewis RS. 2015. Store-Operated Calcium Channels. Physiol Rev 95(4):1383-1436.
Shahrizaila N, Lowe J, Wills A. 2004. Familial myopathy with tubular aggregates associated with abnormal pupils. Neurology 63(6):1111-1113.
Spring KR, Davidson MW. 2017. Introduction to Fluorescence Microscopy. www.microscopyu.com: Nikom.
Stathopulos PB, Seo MD, Enomoto M, Amador FJ, Ishiyama N, Ikura M. 2012. Themes and variations in ER/SR calcium release channels: structure and function. Physiology (Bethesda) 27(6):331-342.
Stormorken H. 2002. [Stormorken's syndrome]. Tidsskr Nor Laegeforen 122(30):2853-2856.

Stormorken H, Sjaastad O, Langslet A, Sulg I, Egge K, Diderichsen J. 1985. A new syndrome: thrombocytopathia, muscle fatigue, asplenia, miosis, migraine, dyslexia and ichthyosis. Clin Genet 28(5):367-374.

Varga-Szabo D, Braun A, Kleinschnitz C, Bender M, Pleines I, Pham M, Renne T, Stoll G, Nieswandt B. 2008. The calcium sensor STIM1 is an essential mediator of arterial thrombosis and ischemic brain infarction. J Exp Med 205(7):1583-1591.

## 9.1 Web resources:

The URLs for data presented herein are as follows

Ensembl sequences, http://www.ensembl.org/index.html

Blat, https://genome.ucsc.edu/cgi-bin/hgBlat?command=start

Online Mendelian Inheritance in Man, OMIM®. McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD), World Wide Web URL: https://omim.org/