CELLULAR Ca^{2+} HOMEOSTASIS IN THE PATHOPHYSIOLOGY OF CHRONIC RESPIRATORY DISEASES

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Per l'avi Fernando i l'avi Xicu,
per la iaia Cándida i la iaia Assumpció,
pels meus pares, Lluís i Loreto,
i pels meus germans, Elisabet i Agustí.

I, sobretot, per tu, Raquel,
perquè si em pregunten què és poesia…poesia ets tu.

"*You must keep sending work out; you must never let a manuscript do nothing but eat its head off in a drawer. You send that work out again and again, while you're working on another one. If you have talent, you will receive some measure of success - but only if you persist*."

Isaac Asimov

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Abstract

Calcium works as a second intracellular messenger in all cell types and its downstream signalling is a key pathway for many systemic functions. In the lungs, the majority of activating stimuli trigger intracellular calcium increase, which is indispensable for the normal functioning of the airways; thus, deregulation of this pathway leads to pathological conditions. This Thesis aims to understand the relationship of intracellular calcium homeostasis and chronic respiratory pathologies such as asthma. I have studied three different processes involved in calcium homeostasis and their role in asthma pathophysiology: 1) I have shown the genetic association of a defect in calcium entry via TRPV1 with wheezing and cough, which is one feature of asthma pathophysiology; 2) I have also demonstrated the product of the asthma associated ORMDL3 gene is a Ca^{++} homeostasis and UPR modulator; and 3) I have provided a new Ca^{++} dependent sorting mechanism for secretory cargoes that bind calcium.

Resum

El Calci és un segon missatger intracel·lular en tots els tipus cel·lulars i la cascada de senyalització generada pel calci és una via de senyalització cel·lular clau per moltes funcions sistèmiques. En els pulmons, la majoria d'estímuls activadors produeixen un increment del calci intracel·lular, el qual és indispensable pel funcionament correcte de les vies respiratòries; i, per tant, una desregulació d'aquesta via de senyalització porta a diferents situacions patològiques. Aquesta Tesi té com a objectiu entendre la relació entre l'homeòstasi del calci intracel·lular i les malalties respiratòries cròniques, com per exemple, l'asma. Hem estudiat tres processos diferents implicats en l'homeòstasi del calci i el seu rol en la fisiopatologia de l'asma: 1) Hem demostrat que hi ha una associació genètica entre un defecte en l'entrada de calci via TRPV1 i un dels trets característics de l'asma, la tos; 2) també hem trobat que l'ORMDL3, que havia estat associat amb l'asma, és un modulador de l'homeòstasi del calci i de la UPR; i 3) hem aportat un nou mecanisme de classificació en el Golgi depenent de Ca^{++} per a proteïnes que uneixen calci i que seran secretades.

Prologue

Bronchial Asthma and Chronic Obstructive Pulmonary Disease (COPD) are the two main chronic respiratory pathologies; being a growing global health and economical problem. Currently, the most effective therapy for asthma is a combination of inhalers; however, many asthmatic patients do not have their pathology adequately controlled. Therefore, there is a search for novel pharmacological treatments and for the identification of risk factors for asthma.

Calcium signalling is a good "place" to find new targets because it regulates, directly or indirectly, the asthma main pathophysiological characteristics: bronchoconstriction, airway hyperreactivity, airway inflammation, and mucus hypersecretion. Many proteins play a role in calcium signalling, and deregulation of any of them may produce a pathological condition; thus some of them could be important players in the pathophysiology of asthma.

This Thesis identifies new players related to calcium signalling that are either directly involved in the pathophysiology of asthma (TRPV1 and ORMDL3) or participate in the control of a cell biology process relevant to asthma (SPCA1), which may help in the search for novel pharmacological targets for the control of asthma.

Abbreviations

$[Ca^{++}]_{cyt}$	cytosolic calcium concentration
$[Ca^{++}]_{ER}$	endoplasmic reticulum calcium concentration
$[Ca^{++}]_{ext}$	external calcium concentration
$[Ca^{++}]_{mit}$	mitochondrial calcium concentration
aa	Aminoacids
AHR	Airway Hyperreactivity
ANK	Ankyrin
ARD	Ankyrin Repeat Domain
ATF6	Activating transcription factor 6
CaM	Calmodulin
CaM BD	Calmodulin Binding Domain
CBF	Ciliary Beat Frequency
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
COPI	Coatamer Protein I
DRG	Dorsal Root Ganglion
eIF2α	Eukaryotic translation-initiation factor 2α
ER	Endoplasmic Reticulum
GA	Golgi Apparatus
GC	Golgi Complex
hTRPV1	Human TRPV1
IP_3	Inostiol 1,4,5-triphosphate
IP_3Rs	IP_3 Receptors
IRE1α	Inositol requiring 1α
KD	Knock Down
KO	Knock Out
NCKX	Na^+/Ca^{++} - K^+ exchanger
NCX	Na^+/Ca^{++} exchanger
ORMDL3	ORM1-like 3
PERK	PKR -like ER kinase
PKR	Double-stranded RNA-dependent protein kinase
PM	Plasma Membrane
PMCA	Plasma-Membrane Ca^{++}-ATPase
PRD	Proline Rich Domain
rTRPV1	Rat TRPV1
RYRs	Ryanodine Receptors
SERCA	Sarco-Endoplasmic Reticulum Ca^{++}-ATPase

SNP	Single Nucleotide Polymorphism
SPCA	Secretory Pathway Ca^{++}-ATPase
SPT	Serine Palmitoyltransferase
SR	Sarcoplasmic Reticulum
TGN	Trans Golgi Network
Th-1/2	T-helper 1 or T-helper 2 cells
TM	Transmembrane
TRP	Transient Receptor Potential
UTR	Untranslated Region
VR1	Vanilloid Receptor 1
XBP1	X-box-binding protein 1

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INTRODUCTION

1. Intracellular Calcium Signalling

Calcium is the most important ion participating in cellular physiology. It works as a secondary messenger in all cell types [1] regulating multiple signalling cascades. Although the importance of calcium as a key element for signal transduction was already described by Sydney Ringer in 1883 when he demonstrated the role of calcium in muscle contraction [2], it was not until 70 years later that this main role of calcium was really accepted [3]. However, the big revolution in the calcium field came with the development of the patch-clamp technique [4] and the Ca^{++}- sensitive fluorescent indicators [5]. These new techniques have made possible to measure intracellular calcium gradients and transmembrane calcium fluxes with high resolution and precision in the last 30 years. Thus, we have now better knowledge about the cellular and molecular mechanisms participating in intracellular calcium signalling.

Nowadays, the role of calcium signalling in many different cellular reactions is well established, some of them promoting cell survival such as gene transcription [6]; but it is also known its role in apoptotic cell death due to large and prolonged calcium increases [7]. Moreover, calcium signalling works in a very wide temporal range, from milliseconds (e.g. neurotransmitter release [8]) to hours or even months (e.g. memory processes [9]).

The participation on so many distinct cellular processes is possible thanks to the characteristics of the calcium signals, which are: 1) effector promiscuity and 2) auto regulation. Indeed, the generation of calcium signalling is

limited to the work of a few protein families conserved among the cellular kingdom, including calcium channels, receptors, transporters and pumps as the most important players. Interestingly, most of these proteins are regulated by calcium itself, thus making calcium signalling a very versatile and adaptable system. Tight regulation of calcium signalling is mandatory, because any imbalance could lead to cell death and pathology.

2. Basic mechanism of Calcium Signalling

Although extracellular calcium concentration is high (mM range), resting cells maintain cytosolic calcium concentration $[Ca^{++}]_{cyt}$ at low levels (approximately 100nM) [10]. This is possible because Ca^{++} cannot pass by simple diffusion through the plasma membrane. However, when a specific stimulus arrives, there is a rapid Ca^{++} increase (µM range) which leads to a change in cellular activity. This increase can be maintained over a wide time scale (microseconds to hours) in order to regulate multiple cellular cascades. Thus, calcium signalling depends basically on the spatial-temporal dynamics of this ion.

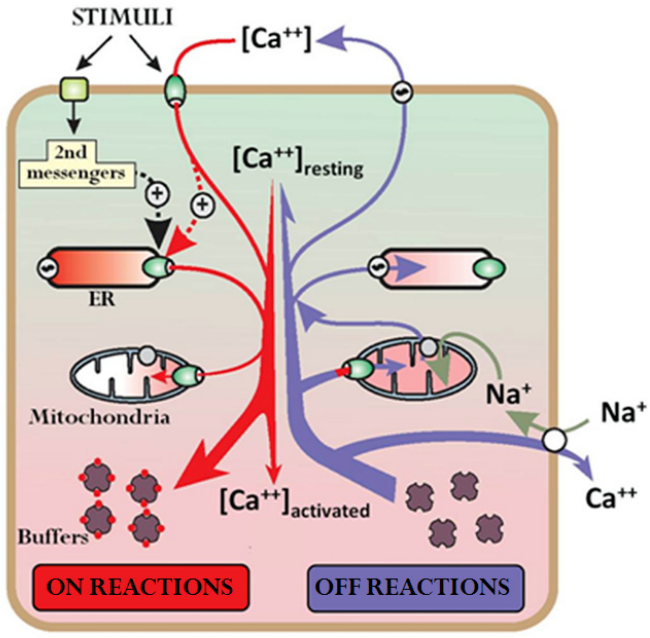

Figure 1: Basic mechanism of Ca^{++} signalling. External stimuli activate the Ca^{++} ON reactions, which increase $[Ca^{++}]_{cyt}$ through plasma membrane channels or from calcium internal stores. Later, Ca^{++} OFF reactions remove Ca^{++} from the cytoplasm to return to $[Ca^{++}]$ resting levels. Taken from www.cellsignallingbiology.org

Spatial-temporal calcium dynamics are controlled by Ca^{++} ON reactions, which increase cytosolic calcium levels, and Ca^{++} OFF reactions, which remove calcium from cytosol. Thus, Ca^{++} signals are transient, being the rising phase produced by the ON reactions; while falling phase depends on the OFF reactions [11]. Moreover, these ON and OFF reactions not only regulate Ca^{++} movements across plasma membrane, but also Ca^{++} interplay with internal stores such as endoplasmic reticulum (main dynamic calcium store) and mitochondria (Fig. 1).

Therefore, this system has multiple variations depending on the combination of Ca^{++} ON and Ca^{++} OFF reactions.

3. Calcium ON Reactions

There are several ways in which cells increase cytosolic Ca^{++} concentration ($[Ca^{++}]_{cyt}$) in response to particular stimulus to promote cell activation. Calcium will come from the extracellular medium or will be released from the intracellular calcium stores, mainly the endoplasmic reticulum (ER). Cells use a combination of these systems to create different calcium signals which will have distinct spatial-temporal features. Thus, two main subgroups can be distinguished in Ca^{++} ON reactions considering if Ca^{++} is coming from the extracellular medium or it is released from the internal stores.

Calcium entry through the plasma membrane is performed by different channels, which can be grouped according to their mechanism of activation:

A. Voltage Operated Channels (VOC)
B. Receptor Operated Channels (ROC)
C. Store Operated Channels (SOC)
D. Agonist Operated Channels (AOC)
E. Second Messenger Operated Channels (SMOC)

Ca^{++} release from the intracellular stores is carried out by different channels depending on cell type:

A: Ryanodine Receptors (RYRs)
B: IP_3 Receptors (IP_3Rs)
C: NAADP-regulated two pore channels (TPC)
D: Intracellular TRP channels

To date, several models have been postulated to explain how these channels located at the internal stores are activated after the arrival of a stimulus to the plasma membrane

1. **Protein-protein interaction**: Conformational changes in the plasma membrane channels after their activation are transmitted to the channels located in the internal stores by direct binding. This mechanism is very rapid and it is restricted to the skeletal muscle (and certain neurons). This is the case for Cav1.1 L-type channel (Voltage gated channel) and RYR1. [12]

2. **Calcium-induced Calcium Release (CICR)**: Ca^{++} entry through surface channels is also a potent activator of channels in the intracellular stores (RYR and IP_3R) [13,14]. This activation is very important to the development of Ca^{++} waves, which are essential for the correct functioning of cardiac cells [15].

3. **Calcium mobilizing diffusible factors**: After the activation of channels in the plasma membrane, PI3-kinase phosphorylates PIP_2 to form IP_3 second messenger. Then, IP_3 diffuses from the plasma membrane and activates IP_3R inducing a Ca^{++} release from the ER [16].

Most of the channels responsible for cytosolic calcium increase are strongly inactivated in order to avoid Ca^{++} overload, which could end in apoptosis and cell death. Once Ca^{++} ON reactions start decreasing by inactivation or because the stimulus is not longer present, Ca^{++} OFF reactions begin to pump calcium out of the cytosol to return to basal Ca^{++} levels.

4. Calcium OFF Reactions

Cells have multiple systems to remove Ca^{++} from cytosol after the activation of Ca^{++} ON reactions, producing a huge and fast increase in $[Ca^{++}]_{cyt}$. This increase is rapidly buffered and $[Ca^{++}]_{cyt}$ is returned to basal levels by Ca^{++} OFF reactions. These reactions control the shape and duration of Ca^{++} signalling by taking up Ca^{++} from the cytosol.

Ca^{++} OFF reactions involve Ca^{++} buffers, mitochondria, Ca^{++} pumps and exchangers. Although they all work to return $[Ca^{++}]_{cyt}$ to resting levels, they have different properties that help shaping Ca^{++} transient:

A. Cytosolic Ca^{++} buffers. Cytosolic Ca^{++} buffering is a fast process that occurs in a sub-second scale, acting as soon as Ca^{++} enters the cytosol. There are many buffers with different Ca^{++} binding and releasing kinetics that will determine the spatiotemporal Ca^{++} signalling in the cell. The composition and concentration of these buffers are distinct for each cell type. The main cytosolic Ca^{++} buffers are Calbindin D-28k, Calretinin and Parvalbumin.[17]

B. Ca^{++} buffers of the intracellular stores. Their main role is to bind the Ca^{++} entering the stores and to release it later to shape cytosolic Ca^{++} signalling. The major components of this group are Calreticulin (CRT) at the endoplasmic reticulum (ER)[18], Calsequestrin (CSQ) at the sarcoplasmic reticulum (SR)[19] and CALNUC, Cab45 and P54/NEFA at the Golgi apparatus (GA)[20].

C. Mitochondrion. This organelle operates rapidly taking up Ca^{++} after ON reactions.

D. Na^{+} - Ca^{++} exchangers. These exchangers are located at the plasma membrane. They have low affinity for Ca^{++}, but high capacity; thus, they work at the beginning of the Ca^{++} OFF reactions to remove high amounts of Ca^{++}.

E. Ca^{++} pumps. This group includes Sarco-Endoplasmic Reticulum Ca^{++}-ATPase (SERCA) (located at the ER), Plasma-Membrane Ca^{++}-ATPase (PMCA) (located at the plasma-membrane) and Secretory Pathway Ca^{++}-ATPase (SPCA) (located at the Trans-golgi network). They have high affinity for Ca^{++} but low capacity, so they can work when calcium levels are low, making them essential to maintain the basal cytosolic Ca^{++} levels and the Ca^{++} levels of the internal stores.

Hence, most of the Ca^{++} increase is rapidly taken up by Ca^{++} buffers or mitochondria. Then, Na^{+}-Ca^{++} exchangers extrude high amounts of Ca^{++} to the extracellular medium until $[Ca^{++}]_{cyt}$ is close to resting levels. Finally, Ca^{++} is pumped into the internal stores or to the extracellular medium by Ca^{++}-ATPases to achieve $[Ca^{++}]_{cyt}$ resting levels. In consequence, the combination of ON/OFF reactions produces a brief Ca^{++} transient. (Fig. 2)

OFF reactions not only work to return cytosolic Ca^{++} levels to basal, but also to maintain resting Ca^{++} levels within a small range. Ca^{++} OFF reactions are constitutively active as a dynamic system to reverse leak

pathways and passive Ca^{++} entry that could produce small increases in $[Ca^{++}]_{cyt}$.

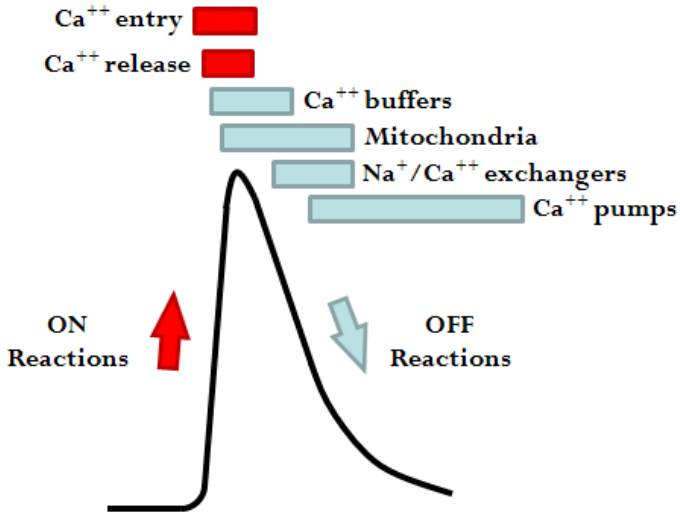

Figure 2: Calcium transient mechanism. The rising phase is due to Ca^{++} entry and Ca^{++} release (red bars), while the return to basal phase is a result of several Ca^{++} OFF reactions (blue bars).

In conclusion, Ca^{++} OFF reactions are capable to reduce the calcium increase produced by Ca^{++} ON mechanisms, generating the classic Ca^{++} transient characteristic of Ca^{++} signalling with different spatiotemporal aspects.

5. The Plasma Membrane

The plasma membrane (PM) delimits all cells, conferring them spatial identity and separating the intracellular medium from the extracellular space. The basic structure of the plasma membrane is the lipid bilayer model, described in 1925 by Gorter and Grendel [21]. However, cellular membranes are not only formed by lipids, but they are also rich in trans-membrane proteins [22]. Therefore, the plasma membrane is a three-dimensional solution of integral membrane proteins in a lipid bilayer solvent.

The plasma membrane is totally impermeable to calcium under basal conditions, although there is a large driving force for calcium entry ($[Ca^{++}]_{cyt}$ = 100nM vs $[Ca^{++}]_{ext}$ = 2mM). However, calcium can enter cells through diverse entry ion channels (voltage-gated Ca^{++} channels, TRP channels, Orai channels and many others) or it can be extruded against its gradient mainly by the Plasma Membrane Ca^{++}-ATPase (PMCA) or the Na^{+}-Ca^{++} exchangers (NCX and NCKX).

5.1 Extracellular Calcium Entry

The Ca^{++} increase produced by Ca^{++} ON reactions will partially come from the extracellular medium, consequently providing a huge and rapid Ca^{++} influx when a stimulus arrives to the cell.

The proteins in charge of producing this extracellular Ca^{++} entry are called Ion Channels. This entry can be directly through the ion channel (calcium

channel) or by an indirect mechanism. Thus, there are ion channels that do not permeate Ca^{++} (i.e. TRPM4 which permeates Na^{+}), but they can produce Ca^{++} entry by membrane depolarization and secondary activation of other channels that permeate Ca^{++}.

Among the different ion channels, Ca^{++} channels are a big super family that includes several protein families with many different properties and activators. Ca^{++} channels can be directly activated by voltage changes or intracellular signalling cascades to generate a Ca^{++} signal. In the next sections I will focus on a particular family of cationic channels, the TRP channels, which participate in the Ca^{++} entry in both excitable and non-excitable cells.

5.2 Transient Receptor Potential Channels

Transient Receptor Potential (TRP) cation channels are unique cellular sensors that permeate Ca^{++} to regulate a wide variety of cellular functions.

The first identified member of this superfamily, that names the whole family, was discovered in 1969 by Cosens and Manning when they isolated the *Drosophila melanogaster* TRP, named after the transient receptor potential phenotype that the fly mutant generated in light perception by photoreceptors [23]. Montell and Rubin cloned the *trp* gene in 1989 [24]. Since then, more than 50 different TRP channels have been described. Besides, these channels are widely expressed along phylogeny, from yeast or flies to mammals. Indeed, there are 28 members of this superfamily already described in mammals [25,26].

The TRP channel superfamily is divided into seven families that are classified in two groups based on sequence similarity. TRP channels from Group 1 have high sequence homology in their transmembrane domains and include the mammalian families TRPC (canonical, the first family to be described), TRPA (ankyrin), TRPV (vanilloid, named after the first member of this family), and TRPM (melastatin). The TRPN (named after *Drosophila melanogaster* NOMPC) is not present in mammals, but it is also a member of group 1 as it has a high sequence homology with the other members [26]. Moreover, four of these subfamilies - TRPA, TRPC, TRPN and TRPV - have ankyrin repeat motifs in their N-terminal cytosolic domains [27]. Group 2 of TRP channels are distantly related to group 1, and they all have a large extracellular loop between TM1 and TM2. This group includes TRPML family (mucolipin) and TRPP family (polycystin). They share some sequence homology and predicted topologies [26] (Fig. 3).

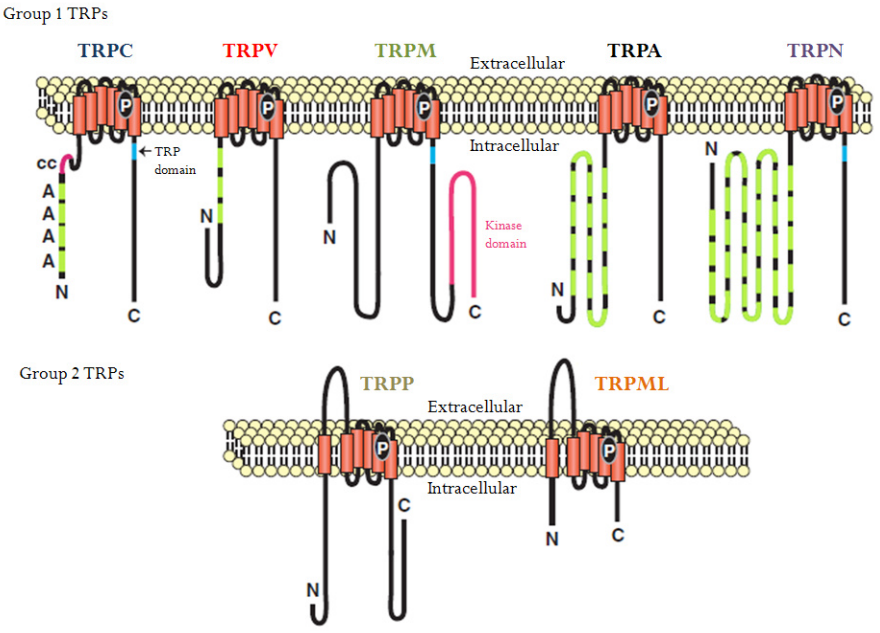

Figure 3: The seven TRP families. Group 1 TRPs are represented at the top and Group 2 at the bottom. Reproduced from Montell *et al.* 2005.[26]

TRP channels have non-selective cation properties, with different permeabilities for Ca^{++} and Na^{+} ($PCa^{++}/PNa^{+}<10$), with the exception of the Ca^{++} selective TRPV5, TRPV6 and TRPM3α2 ($PCa^{++}/PNa^{+}>100$) and the monovalent-selective TRPM4, TRPM5 and TRPM3α1 ($PCa^{++}/PNa^{+}<0.05$) [28].

TRP channels have rapidly gained importance because of their role in sensorial physiology (they transduce several environmental stimuli linked to pain, thermal, osmotic and mechanical sensations) and their involvement in pathophysiological processes.

5.2.1 Structure of TRP channels

One of the few characteristics that transient receptor potential (TRP) ion channel families share is the same basic structural organization.

All TRP channels have 6 transmembrane (TM) domains with N- and C-terminus facing the cytosol and the pore forming loop located in a short hydrophobic region between TM5 and TM6 (fifth - S5 - and sixth - S6 - segments). Most of the differences between the TRP families are found within the N- and C-terminal regions that participate in channel gating and regulation. In order to have a functional and complete pore, four channel subunits need to be oligomerized (Fig. 4). Besides, different members of the TRP ion channel superfamily are expressed simultaneously within the cell, and a few individual units can form heterotetramers [29-31].

Although TRP channels share some structural similarities with voltage gated channels, they are not considered voltage gated channels. This is because they have a few of the necessary charged amino acids for voltage sensitivity in the TM4 domain, but not all [32]. Nevertheless, TRP channels have voltage dependent modulation, but not direct voltage activation [33].

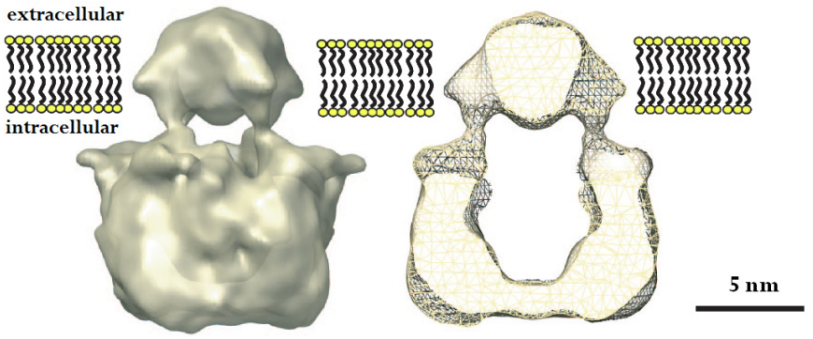

Figure 4: Proposed location of the 3D TRPV1 structure at the plasma membrane. The size and the symmetry of the structure revealed by electron cryomicroscopy confirm that TRPV1 is a tetramer. Reproduced from Moiseenkova-Bell *et al.*, 2008. [34]

In addition to TM domains, TRP channels share other common regions: the ankyrin (ANK) repeats, a 33 amino acid residues motif, which forms an anti-parallel helix-turn-helix structure (in TRPC, TRPV, TRPA and TRPN families); coiled-coil domains; the proline rich domain (PRD); or the TRP box, a unique highly conserved amino acid region present in some TRP channels. [26]

5.3 TRP Vanilloid Family

TRP vanilloid (TRPV) family takes its name from the vanilloid receptor (now known as TRPV1) discovered in 1997 [35]. Regarding the channel structure of this family, they have between 3-6 N-terminal ankyrin repeats that have been postulated to promote channel oligomerization [36,37] and to regulate channel activity [38,39].

There are six members of this family identified in mammals (TRPV1-TRPV6), which are usually divided into two subgroups based on sequence homology, functional similarities, and calcium selectivity [40]:

A. TRPV1-V4 subgroup: This subgroup is formed by the weakly Ca^{++}-selective cation channels TRPV1-V4 ($PCa^{++}/PNa^{+} < 10$), which are modulated by various intracellular signals (Ca^{++}, calmodulin and phosphoinositides) [41,42]. Channels in this group exhibit sensitivity to high temperatures, which added to their location in sensory nerves and epithelial tissues suggest a role for these channels in thermal sensing. However, they can be modulated by many other kinds of chemical and physical stimuli meaning they have more complex roles than being just thermal sensors.

B. TRPV5/TRPV6 subgroup: This subgroup includes the highly Ca^{++}-selective cation channels TRPV5 and TRPV6 ($PCa^{++}/PNa^{+} > 100$). These two channels share 74% sequence identities [43]. Unlike other TRPV members they are not heat-sensitive [40]. Besides, they tend to be activated at low cytosolic Ca^{++} levels and physiological membrane

potentials. Their activity at the plasma membrane is highly regulated by many different second messengers, such as Ca^{++} (which acts as a negative feedback regulator, promoting Ca^{++} inactivation), calmodulin (CaM), Mg^{++}, ATP, PIP_2 and protein kinases.[40,44-46]

Some TRP channels have been linked to several pathologies due to their function and location in the different organs [47]. Among them, TRPV1 and TRPV4 are key elements in the respiratory system, working as integrators of many different physical and chemical stimuli. Interestingly, they are expressed in sensory nerves, epithelia and smooth muscle cells, where they generate calcium signals that will contribute to airway reflexes and defence mechanisms. Therefore, their function and role in the respiratory pathology will be discussed in detail.

5.4 TRPV1 channel

The vanilloid receptor 1 (VR1) or TRPV1, also known as the Capsaicin Receptor, is the founding member of the mammalian TRPV family. This channel plays an important role in noxious sensing and pain perception. Besides, TRPV1 has been related to several symptoms in airway diseases, such as bronchoconstriction, mucus hypersecretion, airway irritation, and cough (reviewed in Lee and Gu, 2009 [48]). However, TRPV1 channel dysfunction has not been directly linked to any pathology yet.

5.4.1 History of TRPV1

Early in the XX century it was demonstrated that pain could be induced after exposing nociceptor neurons to many different stimuli, such as heat and mechanical or chemical stimuli [49]. One classical example of chemical irritants is capsaicin, which is a vanilloid derived from hot chilli peppers, capable to induce pain. However, the molecular nature of the receptors present in nociceptive terminals responsible for painful stimuli transduction remained unknown.

It was not until 1997 when the molecular identity of the capsaicin receptor was solved. Rat TRPV1 channel (rTRPV1) was identified by Caterina *et al.* using capsaicin for cloning experiments [35]. Few years later the human homolog was identified [50]. The human TRPV1 (hTRPV1) has a 92% homology at the amino acid level with the rTRPV1, being the N- and C-cytosolic terminal regions the most divergent. Indeed, both are activated not only by capsaicin, but also by heat (>43ºC) and pH [35]; thus TRPV1 channel transduces two of the main pain stimuli: chemical and thermal.

5.4.2 TRPV1: from gene to protein

Human *TRPV1* gene is located in chromosome 17p13.2 and presents 17 exons. There are 4 different transcripts coding for the same protein but with different 5'UTR (untranslated region) due to untranslated exon 1 that varies between transcripts. Nevertheless, during the last years, several *TRPV1* splice variants have been reported in many species. The hTRPV1b splice variant lacks exon 7 (corresponding to 60 aa in the N-terminal

region), it is expressed in dorsal root ganglion (DRG) neurons and in the central nervous system (CNS), where it seems to work as a dominant-negative variant [51,52]. Another known *TRPV1* splice variant is the rTRPV1 (VAR), which is a truncated form of TRPV1 and it is highly expressed in renal papillary lysates. It seems to modulate TRPV1 function although it is not functional by itself [53]. Finally, there is also the rat vanilloid receptor 5'-splice variant (VR.5'sv), which lacks major part of N-terminal region (including the ankyrin repeat domain) and can't form functional channels. It is expressed in the brain, DRG and peripheral mononuclear cells; and it inhibits TRPV1 activity acting as a dominant-negative variant [54,55].

The functional TRPV1 protein has 839 amino acids and, similar to other TRPs, consists of a transmembrane region with N- and C- terminal regions facing the cytosol (Fig. 5). The transmembrane region is formed by residues 433-684 and consists of six transmembrane domains (named S1 to S6) and a p-loop between S5 and S6. Indeed, the p-loop plus both surrounding TM domains are thought to form the ion pore and the selectivity filter based on homology with other ion channels [35,56]. Furthermore, there is a cholesterol binding Motif in the S5 Helix that mediates the inhibition by cholesterol of the TRPV1 response to capsaicin and temperature [57]. The N- and C-terminal regions represent the rest of the protein (over 70% of the sequence).

The N-terminal region has six ankyrin repeats located at residues 101-364, each consisting of a pair of antiparallel α helices followed by a ''finger'' loop [58]. This TRPV1-ARD (ankyrin repeat domain) binds ATP and interacts with Ca^{++}-CaM, being an important region for channel regulation[58].

The C-terminal region includes the TRP box (which is important for channel gating) close to the sixth TM and a Ca^{++} Independent CaM binding domain (residues 767-800) [59,60]. The latter region, which has eight positively charged residues, has been also identified as a PIP_2 binding site (residues 777-820) [61]. In addition, C-terminal region determines TRPV1 thermosensation [62].

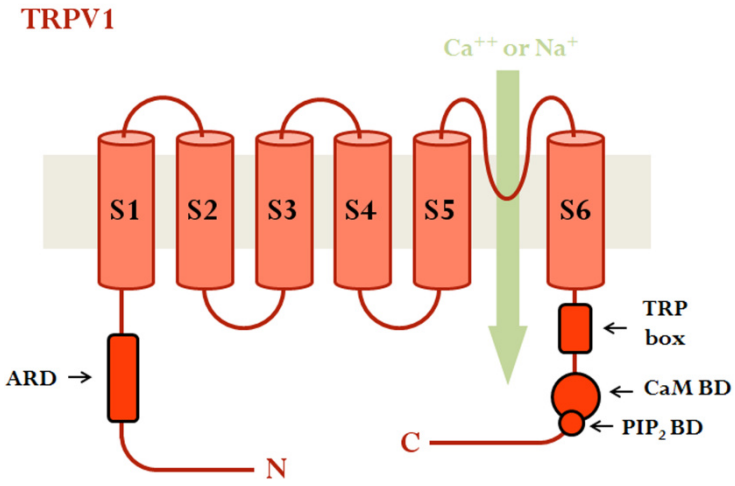

Figure 5: Schematic view of TRPV1 structure. TRPV1 channels present 6 TM domains (S1-S6) with the p-loop between S5 and S6. Several domains are represented: Ankyrin Repeat Domain (ARD), TRP box, Ca^{++}-Calmodulin binding domain (CaM BD), and PIP_2 binding domain (PIP_2 BD).

Like other TRP channels, TRPV1 is only functional as a tetramer. TRPV1 low resolution 3D structure was recently solved proving that the predominant form of TRPV1 is an homotetramer [34]. However, TRPV1 is thought to be able to form heterooligomers with TRPV3, another heat sensitive channel [63].

5.4.3 Activation and regulation of TRPV1

TRPV1 is a nonselective cation channel with a high permeability to Ca^{++} (permeability sequence $Ca^{++} > Mg^{++} > Na^{+} \cong K^{+} \cong Cs^{+}$) [64,65]. It is activated by a diverse range of stimuli: chemical stimuli (capsaicin and other vanilloids such as resiniferatoxin and anandamide); physical stimuli (heat, temperature between 42ºC and 53ºC); arachidonic acid derivates; and direct phosphorylation by protein kinase C (PKC) [35,66,67]. Moreover, TRPV1 is also activated (directly and indirectly) by many neuroinflammatory mediators [68]. Besides, TRPV1 can be sensitized by various endogenous mediators (i.e. bradykinin, substance P, glutamate, prostaglandins, hydroperoxy fatty acids, and ATP) [69].

Regarding its expression, TRPV1 is found in all sensory ganglia (i.e. DRG, trigeminal ganglia, and vagal nerves) and in small sensory C-and Aδ fibres, although it is expressed at low levels in a wide variety of tissues [50]. TRPV1 is also found at the Central Nervous System (CNS) and in non-neuronal tissues such as keratinocytes, mast cells, hair follicles, smooth muscle, bladder, liver, kidney, spleen and lungs [70-78].

5.4.4 TRPV1 physiological and pathophysiological role

TRPV1 channel has been implicated in many cellular and physiological processes, such as noxious physical and chemical stimuli sensing. Due to its desensitization in response to some agonists, which can result in a lower response of TRPV1 to further noxious stimuli, this channel can be a promising target for treating pain [68]. Furthermore, multiple inflammatory

signals converge on TRPV1, which will be activated in sensory neurons generating pain perception.

For instance, neurogenic inflammation is characterized by oedema, thermal and mechanical hyperalgesia and inflammatory pain (caused by overstimulation of peripheral nociceptor terminals after injury, mainly due to TRPV1 activation) [79]. This overstimulation will produce an increased release of neurotransmitters and pro-inflammatory peptides, and, in case of tissue damage, release of protons from injured cells. Besides, many inflammatory diseases like asthma, allergic dermatitis or pancreatitis include a neurogenic component due to the release of neuropeptides (i.e. substance P, calcitonin gene-related peptide, and neuropeptide Y) [80-82]. Also other proinflammatory mediators will be secreted, such as nerve growth factor (NGF), ATP, histamine and cytokines. Indeed, many of these released elements will activate TRPV1 channels, directly or indirectly, contributing to the development of neurogenic inflammation [47,83]. Moreover, the increased expression of TRPV1 correlates with inflammatory hyperalgesia [84].

Therefore, changes in TRPV1 expression or function could lead to a pathological profile, making this channel a novel target for the identification of risk factors as well as for therapeutic interventions.

5.4.4.1 TRPV1 in the respiratory system

TRPV1 is expressed in lung bronchial smooth muscle cells and in C-fibres that innervate the respiratory system [85-87]. The activation of sensorial fibres

in the airways will produce bronchoconstriction and mucus secretion, as well as cough and airway irritation [88-90]. Interestingly, asthma and Chronic Obstructive Pulmonary Disease (COPD) are characterized by bronchoconstriction, mucus secretion, airway hyperreactivity and cough.[91,92]

It is known that chronic cough is a symptomatic manifestation of airway hyperresponsiveness and characteristic also of other respiratory diseases (such as bronchitis and common cold) [93]. A growing body of evidence shows that TRPV1 could play an important role in the genesis of cough. First, airway sensory nerves expressing TRPV1 channels are involved in cough reflexes [94,95]. Experimental cough reflex can be induced by inhalation of capsaicin, citric acid or anandamide (all are typical TRPV1 activators) [96-101] and TRPV1 is a key player for the sensory regulation of cough reflex in animals [95,102,103]. Second, some endogenous inflammatory mediators such as prostaglandin E2 (PGE2), bradykinin and histamine (which upregulate TRPV1 sensitivity) also enhance cough sensitivity [104-106]. Third, TRPV1 is upregulated in patients with chronic cough [107,108] and there is a positive correlation between the capsaicin induced cough sensitivity and the density of TRPV1 expressing terminals in the mucosa of COPD patients [107]. Furthermore, capsaicin induced cough reflex is increased in patients with asthma, bronchitis and COPD [109]. Altogether, TRPV1 has an important role in airway inflammation, airway hyperresponsiveness and cough, linking this channel with many respiratory diseases.

5.5 TRPV4 channel

TRPV4 is another member of TRPV family, which is also known as the mammalian osmosensor, although the channel does not seem to sense osmotic gradients across cell membranes *per se*. This channel has also an important role in thermo and mechanosensation. Besides, its dysfunction has been associated with several pathologies [110,111], remarking its importance in normal cell physiology.

5.5.1 History of TRPV4

The TRPV4 channel was identified by two different groups in 2000 [112,113]. Since then, TRPV4 has been given several names: OTRPC4 (Osmosensitive Transient Receptor Potential Channel) [112], VR-OAC (Vanilloid Receptor-Related Osmotically Activated Channel) [113], VRL2 (Vanilloid Receptor-Like) [114] and TRP12 [115]. Finally, TRPV4 was accepted as the universal name for this channel, and it was considered as the mammalian osmosensor. However, the discovering of new activators showed a much more complex function of TRPV4 channel.

5.5.2 TRPV4: from gene to protein

Human *TRPV4* gene is located in chromosome 12q23-q24.1 and contains 15 exons [116]. This gene undergoes alternative splicing resulting in 5 variants (TRPV4-A-E). Only two variants are capable to form the functional channel, the isoform TRPV4-A (this is the full-length sequence) and the isoform TRPV4-D (which presents a short deletion inside exon 2). All

other isoforms (TRPV4-B, C, E) present trafficking problems and are retained in the ER. [36]

Functional TRPV4 protein has 871 aminoacids (aa) and has a similar structure to TRPV1: it consists of six transmembrane domains (containing a pore forming loop, the p-loop, between S5 and S6) with N- and C-terminal regions facing the cytosol. The N-terminal region represents over 50% of the gene sequence. This region presents six ankyrin repeats (residues 148 to 398) [39], a Proline Rich Domain (PRD) located just before the ARD (132-144aa) and an archidonate-like recognition sequence (ARS-L) (402-408aa) [117] (Fig 6).

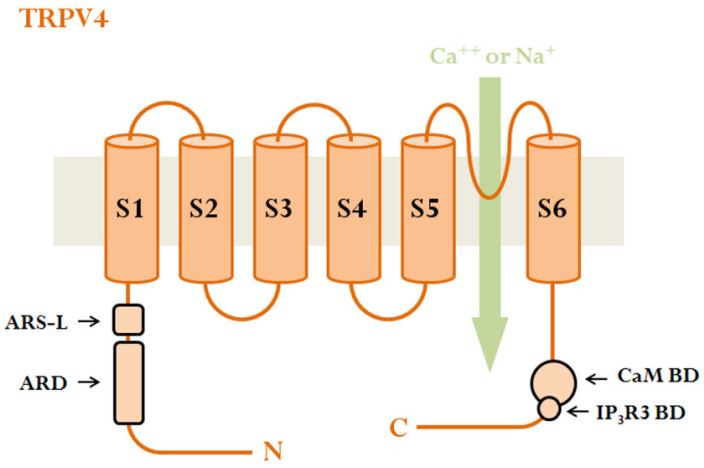

Figure 6: Schematic view of TRPV4 structure. TRPV4 channels present 6 transmembrane (TM) domains (S1-S6) with p-loop between S5 and S6. Several domains are represented: Ankyrin Repeat Domain (ARD), archidonate-like recognition sequence (ARS-L), Ca^{++}-Calmodulin binding domain (CaM BD), and IP_3R3 binding domain (IP_3R3 BD).

The proline rich domain is involved in the channel gating[118]. Indeed, the presence of the non-synonymous single nucleotide polymorphism (SNP) rs3742030 (which results in a Proline (Pro) to Serine (Ser) substitution in

residue 19, located at the beginning of N-terminal domain), impairs channel response to mild hypotonicity and it is linked to human hyponatremia [119].

The C-terminal region presents a calmodulin binding domain (CaM BD) (812-831) [120] which also binds IP_3R3 in a Ca^{++}-independent manner to sensitize the channel to osmotic stimuli [118]. Unlike TRPV1, it is not clear whether there is a TRP box at the TRPV4 C-terminus [121].

5.5.3 Activation and regulation of TRPV4

TRPV4 is a non selective cation channel with high affinity for divalent cations such as Ca^{++} and Mg^{++} ($PCa^{++}>PMg^{++}$) [122]. This channel also permeates monovalent cations in the absence of divalent cations with poor discrimination between them ($PK^{+}>PCs^{+}>PRb^{+}>PNa^{+}>PLi^{+}$) [123].

The TRPV4 channel is known to be activated by a wide range of stimuli, including physical (channel activity is strongly enhanced at physiological temperatures, between 24-37ºC [124]) and chemical stimuli (4α-phorbol esters, like 4αPDD [125], and the natural agonist bisandrographolide, BAA[126]). In addition, it is activated by hypotonicity (via a PLA2-arachidonic acid-5,6-EET dependent pathway [127,128]) and inhibited by hypertonicity [123]. Consequently, it has been considered as the mammal osmosensitive TRP channel. Moreover, TRPV4 is also activated by mechanical stimuli like shear stress [129] or high viscous loading [130].

Furthermore, TRPV4 can be modulated by many different factors (intracellular Ca^{++} concentration [131] and ATP [39]) and regulatory proteins such as calmodulin [120] and IP_3R3 [118,132]. Another way to regulate TRP channels is by phosphorylation that will affect either channel activity [133] or channel trafficking [134]. There are different kinases capable to phosphorylate TRPV4 channel, such as Src kinase [135], PKA [136] and PKC [137]. Other proteins regulate the TRPV4 channel activity by direct binding (i.e. PACSIN3 [138], aquaporins [139], OS-9 [140], MAP7 [141], actin and tubulin [142]).

Regarding its distribution, TRPV4 is widely expressed and there are only few tissues where it cannot be found. In the respiratory system it is expressed in epithelial cells [114], ciliated cells [143], smooth muscle cells [144] and most probably in sensory nerve endings.

5.5.4 TRPV4 in the ciliated epithelium

In ciliated epithelial cells, TRPV4 channels not only control the shear stress[145] or the regulatory volume decrease (RVD) response (by coupling calcium entry to activation of calcium sensitive K^+ channels) [146], but also the ciliary beat frequency (CBF). Cilia are the first line of defence against allergens or pathogens and their movement is essential for mucus clearance[147]. TRPV4 channel mediated calcium influx in ciliated epithelial cells couples to increases in CBF. Therefore, TRPV4-KD ciliated cells show a reduced calcium entry and CBF [148].

5.6 Calcium Extrusion

There are two different groups of proteins that expulse Ca^{++} from the cytosol to the external medium: the Ca^{++} pumps and the Ca^{++} exchangers[149].

Plasma membrane Ca^{++} pumps are P-type ATPases called PMCAs (Plasma Membrane Ca^{++} ATPases) [150]. There are four human PMCA isoforms encoded by separate genes (PMCA1, 2, 3, 4) with many alternatively spliced forms [151]. These splice isoforms show tissue and cell specificity, and they are differently expressed during development and differentiation [152]. The PMCA isoforms 1 and 4 are widely expressed, while isoforms 2 and 3 are mainly found in the brain and the skeletal muscle [151]. Some evidences show that changes in $[Ca^{++}]_{cyt}$ might affect the expression of these isoforms[153]. Each PMCA isoform shows specific regulation of activity and kinetics suggesting that they are functionally diverse and optimized for cell specific demands. For instance, PMCA3f (expressed in the skeletal muscle) and PMCA2a (found in stereocilia) are the fastest, while PMCA4b (expressed in Jurkat cells) is the slowest [153]. Therefore, PMCAs shape Ca^{++} signals with high spatial and temporal resolution by regulating Ca^{++} clearance from the cytosol.

The other players that rapidly extrude Ca^{++} from the cytoplasm are the Na^{+}/Ca^{++} exchangers. There are two families of Na^{+}/Ca^{++} exchangers: Na^{+}/Ca^{++} exchanger (NCX) and Na^{+}/Ca^{++} - K^{+} exchanger (NCKX) families [154]. The first exchanger discovered was the Na^{+}/Ca^{++} exchanger (NCX), which expulses Ca^{++} from the cell in exchange for Na^{+}. Energy

from Na^+ gradient across the plasma membrane is used to extrude Ca^{++} out of the cell against its large electrochemical gradient. NCX can work in two ways, depending on the electrochemical potential that determines the Na^+ flux. In the forward mode, Ca^{++} is extruded from the cell, while in the reverse mode, Ca^{++} enters the cell [155]. The Na^+/Ca^{++} - K^+ exchangers (NCKXs), which are the other family, are found in both excitable and non-excitable cells. The main functional difference with NCX is that NCKXs extrude both Ca^{++} and K^+ in exchange for Na^+.[156]

6. The Intracellular Calcium Stores

Intracellular calcium stores are able to accumulate large amounts of Ca^{++}, which can be released, being a major calcium source in Ca^{++} signalling. The main Ca^{++} store is located in the Endoplasmic Reticulum (ER), which is a large network inside the cell. In muscle, this network has been specialized and forms the Sarcoplasmic Reticulum (SR). Nevertheless, there are other intracellular stores, such as the Golgi Apparatus and the Mitochondrion, which are also very important in intracellular Ca^{++} signalling.

Intracellular Ca^{++} stores have Ca^{++} channels to release Ca^{++} upon a particular type of stimulation. There are two families of channels that release Ca^{++} from the ER or the SR, Ryanodine receptors (RYRs) and Inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$ or IP_3Rs). Recently, the Trans Golgi Network (TGN) has also been described to participate in calcium release and not only in calcium storage, since it presents functional Ryanodine Receptors (RYRs) [157].

Moreover, each compartment also has distinct ways to take up calcium from the cytosol during OFF reactions. The protein in charge to pump calcium into the ER or the SR is the Sarco/endo-plasmic reticulum Ca^{++}-ATPase (SERCA). In a similar way, another pump is transferring calcium from the cytosol to the Trans-Golgi Network, the Secretory-pathway Ca^{++}-ATPase (SPCA). Mitochondria use a different mechanism to accumulate Ca^{++}, the voltage-dependent anion channel (VDAC) which not only permeates Ca^{++} through the Outer Mitochondrial Membrane (OMM) but also small molecules and other ions [158]. Moreover, the main transporter at

the Inner Mitochondrial Membrane (IMM) involved in the uptake of Ca^{++} into mitochondrial matrix is the Mitochondrial Calcium Uniporter (MCU), with Ca^{++}/H^{+} exchanger LETM1 and Na^{+}/Ca^{++} exchanger NCLX also playing a role.

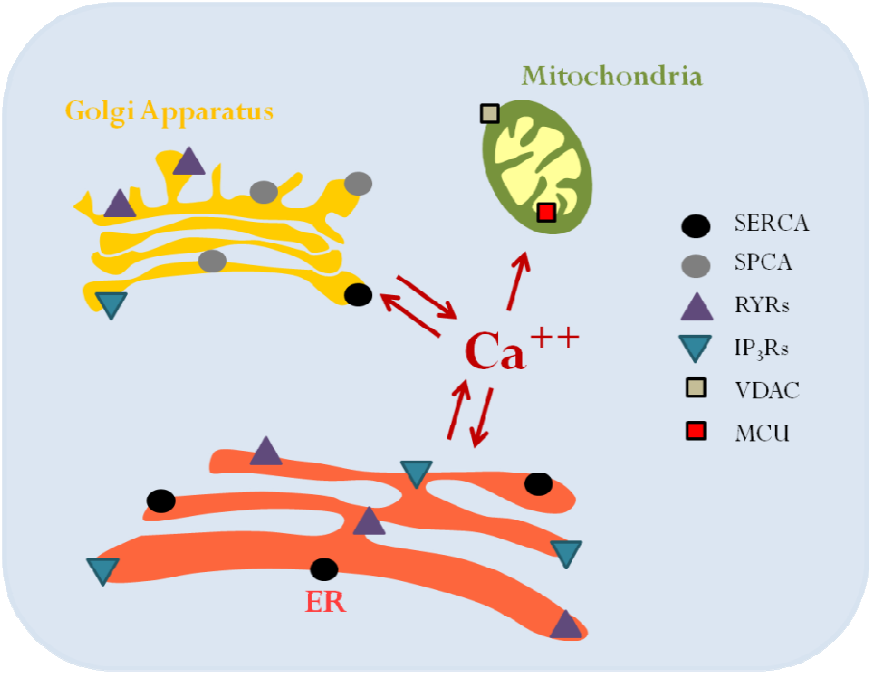

Figure 7: Main pumps and channels of the intracellular calcium stores. The Endoplasmic Reticulum (ER) is shown in red, the Golgi Apparatus in orange, and the Mitochondrion in green.

6.1 The Endoplasmic Reticulum

The endoplasmic reticulum (ER) is a huge membrane system with different domains that perform several functions including Ca^{++} signalling; generation, folding and modification of proteins; and lipid synthesis for new cellular membranes and steroids [159-162].

The ER consists of a three-dimensional network of endomembranes, which has two different structures: microtubules and cisternae. The ER, which is considered the largest intracellular organelle, has been historically divided in two types depending on its histology: the smooth ER and the rough ER (with ribosomes). The rough ER (RER) usually has a tubular appearance, while the smooth ER (SER) is often more convoluted [163]. Another way to characterize ER is morphologically, distinguishing between the Nuclear ER and the Peripheral ER. The nuclear ER, also known as the nuclear envelope (NE), consists of two sheets of membranes with a lumen surrounding the nucleus. The peripheral ER is a network of interconnected tubules that extends throughout the cell [164]. Other morphological characteristics of the ER are its extreme plasticity and its remodelling ability [165].

The ER is indispensable in intracellular calcium signalling because it can storage a huge amount of Ca^{++} via SERCA pumps (micromolar range); and, under a specific stimuli, it can release this calcium through RYRs or IP_3Rs channels.

6.1.1 Ryanodine Receptors

There are three different ryanodine receptor (RYR) isoforms, encoded by different genes (*ryr1*, *ryr2* and *ryr3*): RYR1, RYR2 and RYR3 [166,167]. All isoforms have the same function; releasing Ca^{++} from the internal stores (the ER and the SR). They are named ryanodine receptors because ryanodine (an alkaloid) binds and activates them at low concentrations (although high concentrations inhibit the channel) [168]. The RYRs are very large proteins (more than 5000 aminoacids) and they form homotetramers to be functional channels [166].

Although the RYRs are mainly expressed in the skeletal muscle cells, they can be also found in the endoplasmic reticulum of neurons, exocrine cells, smooth-muscle cells, epithelial cells and lymphocytes [166].

Ryanodine receptor 1 (RYR1) is predominantly expressed in the skeletal muscle (where its main function is in excitation-contraction coupling and gets activated directly by Cav1.1 L-type channels), however, it is also found at lower densities in cerebellar Purkinje cells, gastric smooth muscle cells and B lymphocytes. Ryanodine receptor 2 (RYR2) is the major isoform expressed in cardiac muscle cells. Furthermore, it is also found in smooth muscle cells, many neuronal cells and adrenal chromaffin cells. Finally, ryanodine receptor 3 (RYR3) seems to have an important role during development, although it is also found in mature cells; for example in the diaphragm, epithelial cells, brain and smooth muscle cells (reviewed in Capes *et al.*, 2011 [169]).

6.1.2 Inositol 1,4,5-trisphosphate receptors

The Inositol 1,4,5-trisphosphate receptors (IP_3Rs) are the main Ca^{++} release channels in the ER of most cell types. There are three mammal IP_3R encoded by different genes: IP_3R1, IP_3R2 and IP_3R3, which have similar primary structures and properties. However, cells express them in different proportions and cellular locations, showing that there are some differences between IP_3R isoforms. Nevertheless, a functional IP_3R is form by four subunits (about 310 kDa each) as homo- or hetero- tetramers.

Structurally, each subunit has cytosolic N- and C- terminal regions, and six transmembrane domains, with the pore loop located between TM5 and TM6. The luminal loops of the pore are glycosylated. The IP_3 binding domain is located at the N-terminal domain, as well as a regulatory domain[170]. Indeed, the IP_3Rs can be modulated directly by IP_3 and Ca^{++} itself (although it can also modulate the IP_3R indirectly through calmodulin (CaM)) [14]. Other modulators are different kinases that phosphorylate the channel, such as Ca^{++}/CaM-dependent kinase II (CaMKII), cGMP-dependent protein kinase (PKG), protein kinase C (PKC), and cAMP-dependent protein kinase (protein kinase A, PKA) [171].

6.1.3 Sarco/endoplasmic reticulum Ca^{++}-ATPase

Sarco/endoplasmic reticulum Ca^{++}-ATPase (SERCA) is the pump that transports Ca^{++} from the cytosol to the ER/SR. SERCA pump is a single polypeptide (110 kDa) localized in the ER/SR membrane which belongs to the family of P-type ATPases, characterized by coupling the hydrolysis of

ATP to the movement of ions across the membrane. Indeed, SERCA pump uses the energy resulted from the hydrolysis of one molecule of ATP to transport two Ca^{++} ions across the ER membrane.

SERCA pump has been identified in both prokaryotic and eukaryotic cells (from yeast to mammals). In vertebrates, there are more than 10 SERCA isoforms of the three paralogous SERCA genes (*ATP2A1-3*) [172]. These isoforms (produced by alternative splicing of the transcripts, which mainly affects COOH terminal domain) have different physiological properties and expression profiles, showing tissue and developmental specificity.

SERCA1 is expressed in fast-twitch skeletal muscle and it has two isoforms: SERCA1a (994 aa) and SERCA1b (found in foetal tissues, 1011 aa) [173,174]. SERCA2 encodes three isoforms: SERCA2a, SERCA2b and SERCA2c. SERCA2a isoform (997 aa) is mainly expressed in cardiac and slow-twitch skeletal muscle [175,176]. SERCA2b isoform (1042 aa) is also found in cardiac cells and at low levels in all cell types (including muscle and non-muscle cells) [177-179]. SERCA2c isoform (999 aa) has been identified in cardiac muscle and hematopoietic cells [180,181]. SERCA3 is expressed in non-muscle cells, although it is also found as a minor form in the muscle [182,183]. There are six known isoforms for SERCA3 in humans, from 3a to 3f (999 aa - 1052 aa), which have been detected at the mRNA levels in multiple cell types [184-186]. SERCA3 isoforms are expressed at high levels in the hematopoietic cell lineages, platelets, epithelial cells, fibroblasts, and endothelial cells [183-185,187]. However, only SERCA3a, 3b and 3c have been detected at protein level.

SERCA activity controls different cell functions such as muscle contraction or lymphocyte activation, so it needs to be regulated in order to adapt SERCA pump function to changing circumstances. There are two major modulators of SERCA pump activity in a tissue-specific manner: phospholamban (PLB, 52 aa) and sarcolipin (SLN, 31 aa), which is only expressed in cardiac and skeletal muscles [188-190].

6.1.4 Endoplasmic Reticulum Stress

The ER needs a high Ca^{++} concentration not only to shape intracellular Ca^{++} signalling, but also to fold, modify and assemble new proteins; Ca^{++} is a key element for the translocation of those proteins to the Golgi. Thus, deregulation of Ca^{++} homeostasis results in the accumulation of misfolded proteins that triggers the Unfolded Protein Response (UPR) and ER stress. The UPR is necessary to rapidly restore protein folding by altering both the transcription and the translation of proteins, although prolonged UPR will activate pro-apoptotic pathways [191]. In mammals, the UPR is characterized by transcriptional activation of chaperone and protein-folding genes, regulation of growth arrest and apoptosis genes, and repression of global protein synthesis [192].

It is known that pathophysiological conditions or some pharmacological reagents that alter Ca^{++} levels can trigger the UPR, as well as an oxidizing environment or the glycosylation machinery in the ER [161,193]. Moreover, several physiological cell activities that increase the need for protein folding (i.e. antibody production) or stimuli affecting protein folding reactions creates an imbalance between the capacity of the ER and the amount of

proteins to be fold. This may generate an accumulation of misfolded or unfolded proteins, which will finally trigger ER stress. [194]

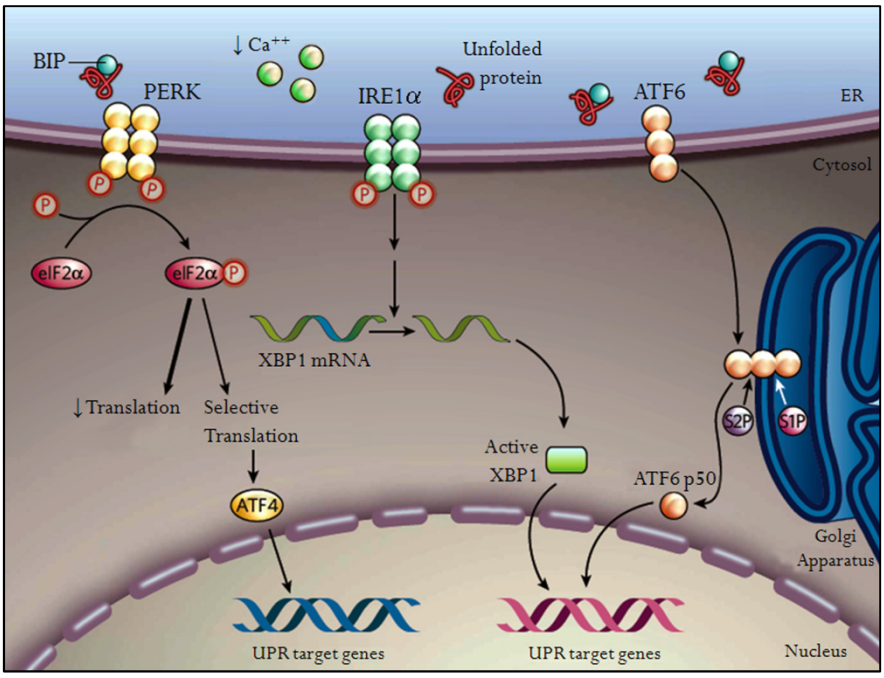

Figure 8: The mammalian Unfolded Protein Response (UPR) pathways. A decrease in $[Ca^{++}]_{ER}$ or accumulation of unfolded proteins can trigger the different pathways of UPR or ER stress. Reproduced from Zhang and Kaufman, 2008 [194]

In mammalian cells, there are three main ER-localized protein sensors that trigger the UPR signalling cascade. They are: 1) IRE1α (Inositol-requiring 1α), 2) PERK (double-stranded RNA-dependent protein kinase (PKR)-like ER kinase), and 3) ATF6 (activating transcription factor 6) [191,195]. All these proteins are transmembrane proteins with an ER-luminal domain that senses unfolded proteins, an ER retention sequence, and a cytosolic domain which initiates the UPR cascade. IRE1α has protein-kinase and endoribonuclease (RNase) activities. PERK also has protein-kinase activity and functions phosphorylating the α-subunit of the eukaryotic translation-

initiation factor 2α (eIF2α). Finally, ATF6 is a transcription factor which belongs to the CREB family (cyclic-AMP responsive element binding protein) and the ATF family of transcription factors; and it presents a bZIP containing domain (basic region and leucine zipper) (Fig. 8).

In addition, IRE1α, PERK and ATF6 are regulated by BiP/GRP78 (immunoglobulin-heavy-chain binding protein/glucose-regulated 78kDa protein), which has an important role in the Ca^{++} storage in the ER (1-2 mols of Ca^{++} / mole of BiP/GRP78) [196]. In basal conditions, all these ER-stress sensors are inactivated by the association with BiP/GRP78. However, when there is a decrease in $[Ca^{++}]_{ER}$, BiP/GRP78 disassociates from PERK and IRE1α activating them [197,198]; and it also disassociates from ATF6, triggering its translocation to the Golgi where it is processed and activated [199,200]. Then, IRE1α and ATF6 play a main role in mediating transcriptional regulation, while PERK represses protein synthesis.

Specifically, after BiP disassociates from the three ER-stress sensors, the faster response to ER stress is the homodimerization and trans-phosphorylation of PERK. Then PERK phosphorylates eIF2α, which inhibits the assembly of the 80S ribosome and, therefore, the protein synthesis. This response is essential to promote cell survival by preventing the production of more polypeptides into the ER lumen which is already highly saturated [201]. The phosphorylated eIF2α is necessary for the translation of mRNAs with a regulatory sequence with several possible open reading frames at the 5' UTR (untranslated region), like mRNA encoding for the transcription factor ATF4 [202,203]. ATF4 induces the transcription of UPR target genes involved in aminoacid biosynthesis and

transport, oxidative stress response and ER-stress induced apoptosis [204]. Another response to ER stress is mediated by IRE1α which is autophosphorylated after BiP disassociation. Once IRE1α is phosphorylated and its RNase function activated, it removes a 26-base intron from the X-box-binding protein 1 (XBP1) mRNA in order to produce the active XBP1 isoform, which has strong activity as a transcription factor [191,195]. At the same time, ATF6 translocates to the Golgi apparatus after being released from BiP. There, ATF6 is cleft by the site-1 and site-2 proteases (S1P and S2P), releasing a functional fragment of ATF6 into the cytosol. This fragment, called ATF6 p50, migrates to the nucleus where it activates the transcription of several UPR related genes [205,206]. Indeed, ATF6 p50 and the active XBP1 work in parallel promoting the transcription of chaperones and enzymes necessaries for protein folding, maturation, secretion and ER-associated protein degradation (ERAD) [207,208].

Nevertheless, if the UPR is not enough to solve problems in protein folding and to restore homeostasis in the ER, it triggers apoptosis to protect the organism [191]. This ER-stress induced apoptosis is basically mediated by CHOP (a C/EBP homologous transcription factor) which is downstream of two UPR pathways: the PERK-eIF2α-ATF4 pathway and the ATF6-ATF6p50 pathway [209].

6.2 The Golgi Apparatus

The Golgi Apparatus (GA) or Golgi Complex (GC) is well known for its function in the processing and sorting of lipids and proteins [210]. However,

the Golgi apparatus also plays a role in Ca^{++} signalling, acting together with the ER as an intracellular Ca^{++} store [211].

The Golgi complex (GC) is an organelle in the endomembrane system and has an ancient origin (it appeared along with the first eukaryotic organisms) [212]. Its basic structure is a stack of flattened membrane structures (cisternae) ordered in a polarized way (cis-to-trans). However, this basic structure varies between organisms; for example, in mammalian cells the stacks are laterally linked to form a ribbon-like membrane system [213], while in invertebrates these stacks exist as isolated entities [214]. It has been postulated that this difference in the GC organization could be linked with its functions; so more complexity of the GC structure is related with more complex functions of the organelle [215].

In particular, the mammalian GC is divided into three main polarized subcompartments: the cis-golgi, the medial-golgi, and the trans-golgi; each one with distinctive structural and functional characteristics [216]. From the trans-golgi (TG) emanates a network of tubular reticular membranes that form what is known as the trans-golgi network (TGN) [217]. The TGN is classically considered as the main sorting node of protein and lipid biosynthetic pathways.

Regarding Ca^{++} homeostasis, the Golgi apparatus is capable to release calcium into the cytoplasm during agonist stimulation. Ca^{++} release is triggered by the binding of IP_3 to the IP_3R on the cis-Golgi [211] or via RYR at the trans-Golgi of neonatal cardiac myocytes [157,218]. Moreover, the Golgi apparatus has a role in calcium removal from the cytosol via two types of

Ca^{++}-ATPases, the Sarco-endoplasmic reticulum Ca^{++}-ATPase (SERCA) at the proximal Golgi, and the secretory pathway Ca^{++}-ATPase1 (SPCA1)[20,219] (Fig. 7). Besides, there are different Ca^{++} binding proteins that buffer this Ca^{++} entering into the GC, such as CALNUC [220], Cab45 [221] and P54/NEFA[222]. Altogether, the Golgi apparatus also contributes to the duration and pattern of intracellular Ca^{++} signalling (Fig. 9).

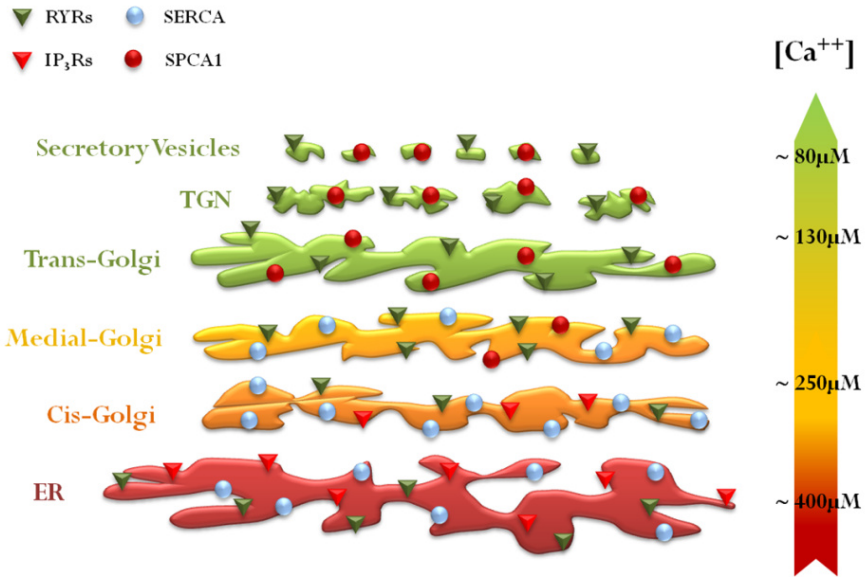

Figure 9: Proteins regulating Ca^{++} concentration at the secretory pathway. SERCA pumps and IP_3Rs are localized at the ER and cis-Golgi, RYRs are expressed in all compartments of the secretory pathway, and SPCA1 pumps are restricted to the trans-golgi, TGN and secretory vesicles. Reproduced from Pizzo *et al.*, 2010 [223]

6.2.1 Secretory Pathway Ca^{++}-ATPase

Secretory Pathway Ca^{++}-ATPases (SPCAs) are responsible of pumping Ca^{++} into the Golgi stacks [224,225]. The SPCA pumps belong to the family of P-type ATPases, and they are located at the membrane of the GC [226]. SPCA is an

integral membrane protein with a large cytosolic head and 10 transmembrane helices (reviewed in Missiaen *et al.*, 2007 [219]).

There are two genes coding for human SPCAs, *ATP2C1* (SPCA1) and *ATP2C2* (SPCA2). Hu *et al*. and Sudbrak *et al*. described the *ATP2C1* gene[227,228], which undergoes alternative splicing to produce four SPCA1 proteins with the C-terminal region differing in length and specific amino-acid sequence (SPCA1a-d) [229]. The human *ATP2C2* gene was independently described by two groups in 2005 [226,230]. In contrast to *ATP2C1*, the *ATP2C2* does not suffer alternative splicing.

Although both SERCA and SPCA contribute to Ca^{++} pumping into the Golgi, they have several differences in distribution and activity. Regarding distribution, SERCA pump is found in the early parts of the Golgi (those closer to the ER), while SPCA pump is restricted to the trans-Golgi and trans-Golgi Network [231]. In contrast to SERCA pumps, the SPCA only permeates one Ca^{++} ion per each ATP hydrolyzed, but is able to pump also Mn^{++}.[232] Moreover, SPCA are less sensitive to thapsigargin than SERCA[233]. Interestingly, mutations affecting the SPCA protein expression cause Hailey-Hayle disease, an autosomal dominant skin disorder [229].

6.2.2 Protein Sorting at the TGN

After their synthesis in the ER and maturation at the Golgi complex, different proteins (cargoes) are sorted at the Trans-Golgi-Network (TGN). Indeed, the cargo sorting can start in other compartments of the Golgi; but in the TGN is where sorting machinery is more complex. Thus, the TGN

controls multiple different pathways to direct proteins into many different acceptor compartments, such as: apical plasma membrane (PM), basolateral PM, endosomes, Golgi stacks, or secretory granules [234]. In addition, lipids are also transferred, inserted and their synthesis is completed in the TGN membrane. These lipids are then exported to the PM or the endosomal system, where they have an important role on the composition of these membranes.

In order to understand these complex tasks, the TGN dynamic structure was first studied revealing a composite tubular membrane network emerging from Golgi cisternae [235]. Later, the sorting signals and the molecular requirements for the different TGN exit pathways were investigated [236]. Some of these signals have been identified. There are two identified signals for the apical sorting, the protein-sugar interactions (i.e. Lectins, such as galectin-3, aggregate N-linked and O-linked glycans of proteins or lipids [237]) and the lipid-lipid interactions (direct glycosyl-phosphatidyl-inositol-anchored proteins to lipid rafts [238]). The mechanisms underlying the basolateral and the endosomal sorting are now well known. They are essentially involve epithelial specific AP-1B, AP-4 and clathrin which recognize tyrosine-based or dileucine-based sorting motifs present in the cytosolic domains of some transmembrane proteins [239]. Another classical signal is the mannose-6-phosphate (M6P), which is only added to the N-linked oligosaccharides of lysosomal soluble hydrolases, and signals them to be sorted to the endosomal/lysosomal compartment [240]. There is also the COPI (coatamer protein I) pathway, which recycles material from post-ER membranes back to the ER [241].

Nevertheless, the sorting pathways for the majority of secreted proteins remain unknown. Von Blume and collaborators postulated that molecular sorters for these secreted proteins could be different forms of ion transporters (such as ion pumps or channels) that could modulate pH and calcium gradients of the TGN [242]. Thus, Ca^{++} stored by the Golgi apparatus could be essential for the sorting of some secretory proteins.

6.3 The Mitochondrion

Mitochondria are important components of the Ca^{++} OFF reactions because they can shape amplitude [243] and spatio-temporal characteristics of the Ca^{++} signalling [244,245]. Mitochondrial Ca^{++} uptake is important in the regulation of many cellular functions, such as ATP production or apoptosis. Mitochondrial Ca^{++} increase will activate several dehydrogenases and carriers, incrementing the respiratory rate and the ATP production. However, a prolonged increase in mitochondrial calcium concentration ($[Ca^{++}]_{mit}$) will produce the opening of the mitochondrial permeability transition pore (PTP), which leads to apoptosis [246].

The main proteins involved in the mitochondrial calcium uptake through the Inner Mitochondrial Membrane (IMM) are: 1) the recently identified Mitochondrial Uniporter Channel (MCU), which is characterized by a low Ca^{++} affinity and only take Ca^{++} at the micromolar range [247,248]; 2) mitochondrial calcium uptake 1 (MICU1), a two EF hand protein that regulates MCU [249]; 3) and the high-affinity Ca^{++}/H^{+} exchanger called leucine zipper-EF-hand containing transmembrane protein 1 (LETM1), which is able to import Ca^{++} in the nanomolar range [250]. Another key

protein involved in Mitochondrial Calcium Homeostasis is Voltage Dependent Anion Channel (VDAC) at the Outer Mitochondrial Membrane (OMM), which is permeable to molecules up to 5 kDa, thus small molecules like Ca^{++} can cross the OMM through its pore [251-253].

7. Respiratory Diseases

During the last years, there has been a large increase in the prevalence of respiratory diseases, being a growing social, medical and economical problem [254]. There are two main chronic airway diseases, the bronchial asthma and the Chronic Obstructive Pulmonary Disease (COPD). Both diseases share some pathophysiological characteristics like inflammatory changes in the airways; but reversibility of the inflammatory process, cell types involved and genetics differ between them [255].

7.1 Chronic Obstructive Pulmonary Disease pathophysiology

The Chronic Obstructive Pulmonary Disease (COPD) is characterized by airflow obstruction (defined as decreased FEV_1, forced expiratory volume in 1 s, compared to FVC, forced vial capacity), which is not completely reversible; persistent inflammation of the airways; and potential presence of noxious stimuli [256]. Moreover, most COPD patients have a chronic obstructive bronchiolitis (with fibrosis and obstruction of small airways); emphysema; and mucus hypersecretion [257].

However, although COPD is an increasing global health problem, very little is known about it, and there are no therapies to reduce the development of the disease. Recently, COPD has been considered also as a chronic inflammatory disease, which has focused research on the abnormal inflammatory response.

7.2 Asthma pathophysiology

Asthma is a multifactorial and chronic inflammatory disorder of the airways with heritability near to 60% [258]. Although there are many effective therapies for asthma, its chronic course and its prevalence (about 5-10% of adult population) suppose a high cost for society [259].

Aetiology of asthma is complex and involves both environmental and genetic factors. A key element in asthma pathophysiological process is the imbalance between the T-helper 1 and the T-helper 2 lymphocytes (Th-1 and Th-2). Th-2 cells are responsible for chronic inflammation, smooth muscle contraction and airway remodelling, which are some of the main pathophysiological features of asthma [260,261]. However, not only lymphocytes are important in the asthma process, but also other cell types, such as epithelial cells, sensory nerves, other immune cells like mast cells, and smooth muscle cells, are involved in asthma pathophysiology [262].

The main characteristics of asthma pathophysiology are: reversible (or partially reversible) airflow obstruction, airway inflammation, persistent airway hyperreactivity (AHR), and airway remodelling. [261]

Ca^{++} signalling regulates the activity of all cell types having a role in asthma pathophysiology. Ca^{++} controls the activation of resident cells (such as macrophages, mast cells, epithelial cells and ASM) and also of migrating cells during inflammation (i.e. eosinophils, lymphocytes and neutrophils). Indeed, Ca^{++} signalling controls ASM contraction [263]; in lymphocytes it regulates degranulation and cell proliferation [264]; and it is essential for

Ciliary Beat Frequency (CBF) in epithelial cells (to provide good mucus clearance) [265].

Altogether, malfunction of proteins (by mutations, downregulation, upregulation…) that control Ca^{++} signalling will produce a loss of Ca^{++} homeostasis, which could end in a pathophysiological process. Thus Ca^{++} homeostasis may be an interesting regulatory process to study in the context of asthma pathophysiology and, possibly, in the identification of new pharmacological targets.

7.3 Genetics of Asthma

To date, more than 100 genes have been reported to be associated with asthma or related phenotypes. Nevertheless, very little is known for most of these candidate genes and their relation with asthma pathophysiology, and only a few have been tested at the functional level. Among them, it is worth mentioning those with an strong association with the disease: Filaggrin (important in the formation of the protective skin barrier); Interleukin-13 (cytokine secreted by the Th-2 cells); Interleukin-17F (cytokines produced by activated mast cells, CD4+ T cells, and basophils); Cysteinyl leukotriene receptors (receptors for bronchoconstrictors and proinflammatory mediators); and ORMDL3 (an ER protein) [266].

There are also several genes with important functions in the correct functioning of the airways that have been postulated as candidate genes, thus mutations on these genes could lead to asthma pathophysiology. Ion channels are key elements in airways physiology because they regulate many

functions in different cell types [262]. Hence, they have been studied over the last 30 years to understand their contribution to asthma pathophysiology.

Currently, the TRP channels superfamily has focused all attention as they work as integrators of several irritant stimuli and inflammatory response[267]. For instance, TRPC1 channel contributes to ASM proliferation [263]; and TRPC3 and TRPC6 are related to ASM contraction [268,269]. Therefore, the TRP channels have been postulated as good target genes in asthma.

7.4 ORM1-like 3 protein and Asthma

A Genome-Wide Association Study (GWAS) performed by Moffatt *et al.* showed strong association of one SNP (rs7216389) with childhood asthma[270]. Interestingly, this polymorphism was controlling transcript levels of ORM1-like 3 (ORMDL3), and no other transcript levels were associated with this marker. Thus, changes in *ORMDL3* gene expression are associated with childhood asthma [270].

The ORM1-like 3 protein (ORMDL3) is a member of the ORMDL family, which is highly conserved along phylogeny (from yeast and plants to mammals) (Fig.10). *ORMDL* genes encode for transmembrane proteins located at the ER. In mammals, ORMDL family includes ORMDL1, ORMDL2 and ORMDL3, all of them widely expressed, although ORMDL3 is highly expressed in cells that participate in the inflammatory response like T-lymphocytes [270,271]. However, ORMDL3 function or its relation with asthma pathophysiology remained unknown at the beginning of my Thesis work.

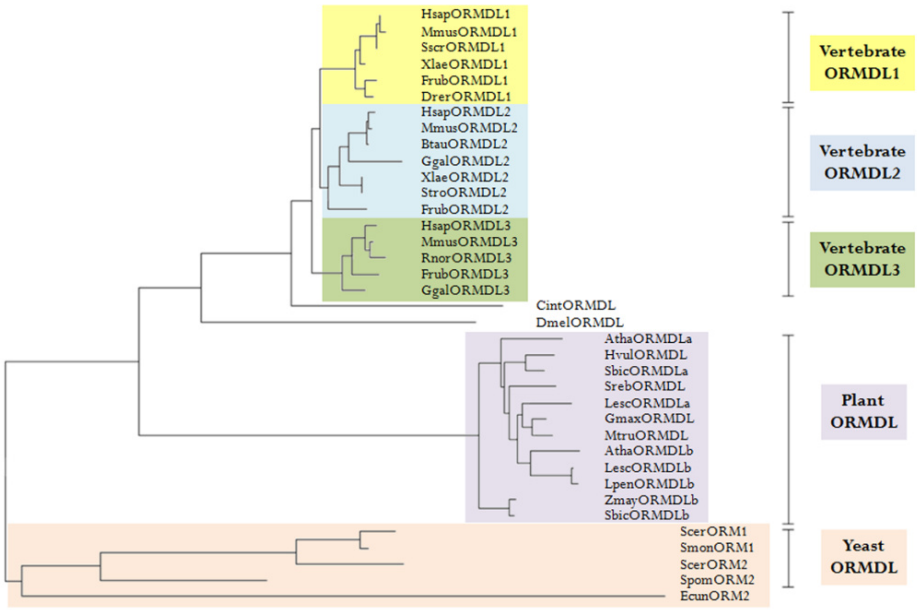

Figure 10: Unrooted phylogenetic tree of ORMDL aminoacid sequences. Vertebrate ORMDL1 are highlighted in yellow; vertebrate ORMDL2 in blue, vertebrate ORMDL3 in green, plant ORMDL in purple, and yeast ORMDL in pink. Species abbreviations are as follows: Hsap, human; Mmus, mouse; Rnor, rat; Sscr, pig; Btau, cow; Ggal, chicken; Xlae, *Xenopus laevis*; Stro, *Silurana tropicalis*; Frub, *Takifugu rubripes* (pufferfish); Drer, *Danio rerio* (zebrafish); Cint, *Ciona intestinalis*; Dmel, *Drosophila melanogaster*; Atha, *Arabidopsis thaliana*; Hvul, *Hordeum vulgare* (barley); Sbic, *Sorghum vulgare*; Sreb, *Stevia rebaudiana*; Lesc, *Lycopersicon esculentum* (tomato); Gmax, *Glycine max* (soybean); Mtru, *Medicago truncatula*; Lpen, *Lycopersicon pennellii*; Zmay, *Zea mays* (maize); Scer, *Saccharomyces cerevisiae*; Smon, *Saccharomyces monacensis*; Spom, *Schizosaccharomyces pombe*; Ecun, *Encephalitozoon cuniculi*. Adapted from Hjelmiq *et al.*, 2002 [271]

II. OBJECTIVES

General objectives

The general objective of this Thesis research is to study the relationship between calcium homeostasis and cellular functions relevant to the pathophysiology of chronic respiratory diseases.

Specific objectives

1. The study the genetic association of TRPV1 and TRPV4 non synonymous SNPs with asthma or any of its characteristics.

2. The evaluation of the functional effect of TRPV1 and TRPV4 non synonymous SNPs that associate with asthma.

3. The study of the location and structure of the asthma associated ORMDL3.

4. The study of the functional implication of ORMDL3 in HEK cells and immune cells, focusing on calcium homeostasis and unfolded protein response.

5. The study of the role of calcium in ADF/Cofilin sorting mechanism, using a fluorescence resonance energy transfer (FRET)–based Ca^{++} sensor (Go-D1cpv) to measure Golgi calcium concentration.

6. The evaluation of the relationship between ADF/Cofilin and SPCA1, and their role in the sorting of secretory cargoes that bind calcium.

III. RESULTS

CHAPTER 1

Loss of Function of Transient Receptor Potential Vanilloid 1 (TRPV1) Genetic Variant Is Associated with Lower Risk of Active Childhood Asthma

Gerard Cantero-Recasens, Juan R. Gonzalez, César Fandos, Enric Duran-Tauleria, Lidwien A. M. Smit, Francine Kauffmann, Josep M. Antó, and Miguel A. Valverde

J Biol Chem. 2010 Sep 3;285(36):27532-5. Epub 2010 Jul 18.

PMID: 20639579

CHAPTER 2

The asthma-associated ORMDL3 gene product regulates endoplasmic reticulum-mediated calcium signaling and cellular stress

Gerard Cantero-Recasens, César Fandos, Fanny Rubio-Moscardó, Miguel A. Valverde, and Rubén Vicente

Hum Mol Genet. 2010 Jan 1;19(1):111-21. Epub .

PMID: 19819884

CHAPTER 3

ADF/cofilin regulates secretory cargo sorting at the TGN via the Ca^{2+} ATPase SPCA1.

Julia von Blume J, Anne-Marie Alleaume, **Gerard Cantero-Recasens**, Amy Curwin, Amado Carreras-Sureda A, Timo Zimmermann, Josse van Galen, Yuichi Wakana, Miguel A. Valverde MA, and Vivek Malhotra.

Dev Cell. 2011 May 17;20(5):652-62.

PMID: 21571222

IV. DISCUSSION

1. TRPV1, but not TRPV4, is associated with asthma symptoms

The Transient receptor potential vanilloid (TRPV) family of channels have been postulated as candidate genes participating in the pathogenesis of several airway diseases such as asthma and COPD. They are expressed in different lung cell types: epithelial ciliated cells, sensory nerves, vascular endothelial cells, submucosal glands and immune cells [85,143,272]. Among the TRPV family, two members have been shown to play an important role in the regulation of airway normal function, TRPV1 and TRPV4.

TRPV1 channels are expressed mainly in sensory nerves in the airways. There, they promote Ca^{++} influx when activated by different stimuli (like neuropeptides, increasing temperature to noxious heat, protons and capsaicin [35]). This Ca^{++} increase activates reflex responses and local release of neuropeptides; resulting in cough, airway irritation, reflex bronchoconstriction and neurogenic inflammation in the airways [272].

TRPV4 channels are expressed in airway epithelial [148] and smooth muscle cells [144]. They produce Ca^{++} entry when they are activated by mechanical, osmotic [132] and/or other stimuli (heat, acidic pH and arachidonic acid metabolites [127]).

However, although there is increasing evidence that TRPV1 and TRPV4 have important roles in airway physiology, no genetic analysis of their importance to asthma pathophysiology existed when this Thesis was initiated.

One of the main aims of this Thesis has been to test if there is any genetic association between TRPV1 and TRPV4 channels and asthma.

We studied the association of two functional Single Nucleotide Polymorphisms (SNPs): I585V for TRPV1 (rs8065080) and P19S for TRPV4 (rs3742030), with childhood asthma. Although none of the studied SNPs changed the risk of suffering from asthma, carriers of the TRPV1-I585V variant showed a lower risk of current wheezing (characteristic of active asthma) or cough. Moreover, our functional Ca^{++} analysis of TRPV1-I585V demonstrated a reduced channel activity in response to heat and capsaicin of the TRPV1-585 Val variant compared to WT variant (585 Ile)[273].

1.1 TRPV1-I585V and TRPV4-P19S

Our experiments showed that the Val variant of TRPV1-I585V (rs8065080) produced a 20-30% loss of channel function (depending on stimuli). Aminoacid 585 is located at the transmembrane S5, which is thought to be part of the channel pore and the selectivity filter. The protein sequence of the transmembrane S5 is highly conserved in mammals and birds (fig. 11), although residue at position 585 varies between species. For example, in birds and most mammals (like rodents) it is a Leucine (Leu, L); in most primates aminoacid 585 is a Valine (Val, V); and in humans there are two natural variants, Isoleucine (Ile, I) and Valine. We have considered TRPV1-585 Ile as the reference sequence because it is the most frequent allele in humans (61%). In our population of study, we found that 34% subjects were homozygous for Isoleucine, 51% were heterozygous

(Ile/Val), and 15% were homozygous for Valine. Nevertheless, although all three variants (Leu, Val and Ile) are aminoacids with hydrophobic side chains, meaning a conservative change; they produce different effects on the channel activity [57,273].

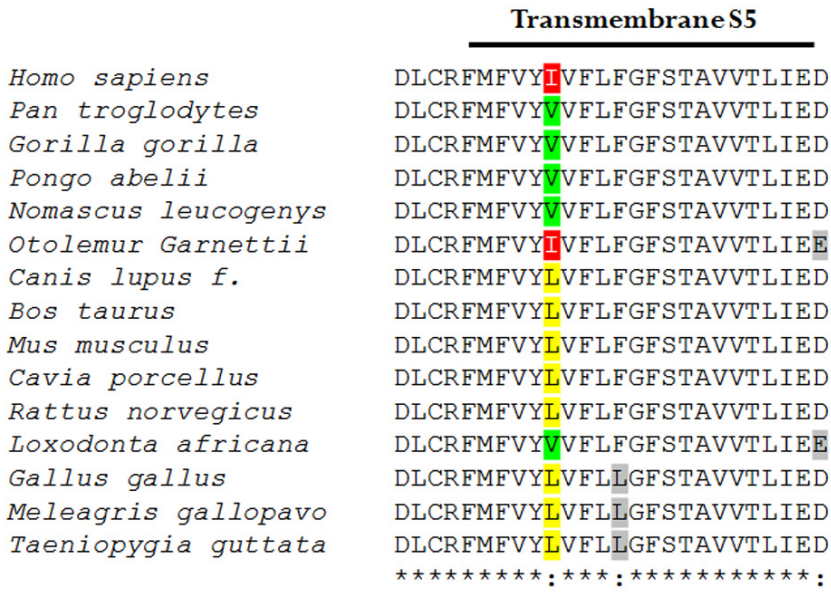

Figure 11: TRPV1 transmembrane S5 alignment of mammals and birds. Position 585 is highlighted in different colours depending on the aa; Isoleucine is shown in red, Valine in green, and Leucine in yellow. Changes in other positions are coloured in grey. Alignment was performed using Clustalw.

This Ile to Val substitution at position 585 reduces TRPV1 channel response to both capsaicin and heat response. Several regions have been postulated to be capsaicin binding sites; such as Tyr 511 and Ser 512, located at the transition between the second intracellular loop and the third TM domain [274]; Leu 547 and Tyr 550 in human and rabbit located at the S4 also contribute to vanilloid binding [275]; and Arg 114 and Glu 761 in the N- and C-termini [276]. On the other hand, the distal half of the C-terminus is involved in TRPV1 thermal sensitivity [62,277]. More experiments are needed to know how a single point mutation in the S5 transmembrane segment is

able to affect two different stimuli with completely different binding domains. However, a possible explanation is via the cholesterol binding domain located at the transmembrane S5. Picazo-Juárez *et al.* showed that enrichment with cholesterol markedly decreased capsaicin and heat evoked currents in the hTRPV1 585 Val variant (as well as the rTRPV1 585 Leu); however, the hTRPV1 585 Ile was insensitive to cholesterol addition (also the rTRPV1 585 Ile variant) [57]. Altogether, it is possible that the TRPV1 585 Val has a decreased response to heat and capsaicin because it is inhibited by cholesterol; whereas the Ile variant does not present this inhibition.

Regarding TRPV1 association with asthma, our results have shown that carriers of the 585 Valine variant present lower risk of current wheezing or cough. It is known that TRPV1 is a key element for extracellular Ca^{++} entry in response to many irritants and endogenous activators, which will trigger bronchoconstriction (wheezing) and cough[48]. Therefore, a decreased Ca^{++} entry will reduce all asthma symptoms triggered by Ca^{++} (besides those already mentioned above, it is worth to include airway hyperreactivity and mucus secretion, as possible Ca^{++} - dependent cellular processes [48]). Consistent with this hypothesis, the TRPV1-585 Valine variant, which has a decreased response to capsaicin and heat (thus, less Ca^{++} entry), is associated with a lower risk of presenting wheezing or cough in asthmatic kids [273]. It would be interesting to check if other polymorphisms such as I315M or P91S, which increase the channel response to capsaicin [278], have the opposite effect on asthma pathophysiology.

We also studied the role of TRPV4 on asthma pathophysiology by evaluating if there was any association between TRPV4-P19S (rs3742030) and childhood asthma or with any asthma symptoms. Although TRPV4 channel activity may be involved in the triggering of many of the pathophysiological symptoms of asthma (TRPV4 channels produce Ca^{++} entry after osmotic and/or mechanical stimuli such as an increase in mucus density, resulting in bronchoconstriction due to ASM contraction and increased CBF) there was no association between TRPV4-P19S and asthma (or its symptoms: cough and wheezing). However, the TRPV4-P19S (which produces a loss of channel function in response to mild hypotonic stimuli[119]) has been related to COPD [279] and hyponatremia [119]. The association of TRPV4-P19S with COPD but not with asthma (or any of its symptoms) could be explained because even though these two diseases of the airways share several characteristics (like inflammation and bronchoconstriction); they differ in the reversibility of the process and the affected cell types [255]. Another explanation could be that our study may not have enough statistical power to detect the real impact of P19S on asthma pathophysiology due to the low incidence of this polymorphism.

1.2 Calcium Entry and Asthma

The airways are in constant contact with many substances and stimuli that activate a huge amount of cellular pathways. Indeed, some of these stimuli produce Ca^{++} entry, which is a key messenger for several functions related with the correct working of the airways. During the last years, Ca^{++} entry and Ca^{++} homeostasis have been postulated to play an important role in the pathophysiology of respiratory diseases [262,280]. Besides, we have shown the

first genetic evidence for the involvement of TRPV1 channel in asthma pathophysiology and the possible link between a functional defect in Ca^{++} entry and respiratory diseases [273].

The involvement of other members of the TRP superfamily in respiratory pathologies has been also tested in population genetic studies. Among TRP channels, TRPA1 has also been postulated as a key mediator of the airway inflammatory response. TRPA1 is considered as a major irritant sensor in the airways and it is selectively expressed by a subpopulation of TRPV1-expressing nociceptive neurons and is activated by many pungent compounds (i.e. allicin, mustard oil…), and environmental irritants (i.e. acrolein, which is present in air pollution) [281-284]. In addition, the TRPA1 activation produces cough in animal models and humans [285]; while TRPA1-KO showed impaired inflammatory response in an ovalbumin model of asthma [286] (For further information on ion channels and asthma pathophysiology, see annex 1).

The involvement of *TRPV1* and *TRPV4* genes in asthma symptomatology has also been evaluated more recently on European adults. This study evaluated the association of SNPs in *TRPV1*, *TRPV4*, and *TRPA1* genes with cough symptoms and the conclusion of this study also pointed to TRPV1 as an important gene modulating cough [287]. In European adult population, *TRPV1* I585V was also associated with a lower risk of nocturnal cough in one adult population with asthma (OR 0.62 [0.40-0.96]), but the association was not statistically significant in the pooled analysis of the two populations studied. This study also provided an interesting observation, the lack of an association between common *TRPA1* and *TRPV4* variants and cough, which

in the case of *TRPA1* was particularly surprising, since many environmental irritants have been shown to activate TRPA1 receptors to cause cough.

2. ORMDL3 regulates ER Ca^{++} Homeostasis and ER stress

ORMDL proteins are a conserved family of ER transmembrane proteins (they are found in yeast, microsporidia, plants, Drosophila, urochordates and vertebrates) (Fig. 10). There are three members in mammals (ORMDL1, ORMDL2 and ORMDL3), that are expressed ubiquitously in adult and fetal tissues. Based on experiments with the yeast orthologs (Orms), it was first postulated that the ORMDL family could be involved in protein folding in the ER [271].

In 2007, Moffatt *et al*. associated a common intronic SNP (rs7216389), controlling *ORMDL3* expression, with childhood asthma [270]. To date, many studies have replicated *ORMDL3* association with asthma (and some of its characteristics) [288-292]. Interestingly, *ORMDL3* has been associated to many autoimmune and inflammatory diseases; such as primary biliary cirrhosis [293], ulcerative colitis [294], type 1 diabetes [295,296] and Crohn's disease [297]. Besides, *ORMDL3* has been associated with total number of leukocytes [298,299]. However, the function and the mechanism of ORMDL3 in asthma and other immune mediated inflammatory diseases were unknown.

Therefore, considering that: 1) asthma is a chronic inflammatory disease of the airways [92,260]; 2) ORMDL3 has been associated with several immune mediated inflammatory diseases; 3) ORMDL3 is highly expressed in lymphocytes [270,271]; 4) it is an ER transmembrane protein; 5) and ER is a central organelle in Ca^{++} signalling and protein folding; we postulated that ORMDL3 could regulate the ER Ca^{++} homeostasis and, consequently, the

unfolded protein response (UPR); two key pathways in cells involved in asthma pathophysiology, specially lymphocytes [264,300].

First, our results showed that human ORMDL3 has two transmembrane domains with a large intrareticular loop (which is the most conserved region among different members of its family) (Fig. 14) and the N- and C-terminal regions facing the cytosol. Interestingly, the N- terminal domain is about 20-60 aa larger in the yeast homologues Orm1 and Orm2 compared to the plant and animal ORMDLs. This region, lacking in human ORMDL3, contains an Ypk-1 phosphorylation site necessary for Orm1 and 2 regulation (Fig. 13) [301]. Besides, there is a putative ER retention motif in the C-terminal domain. However, our deletion lacking the last nine aa, ORMDL3-Δ145-153 (which involves the putative retention domain), showed a distribution similar to ORMDL3-WT; thus the ER retention motif in human ORMDL3 does not appear to be located at the end of the C-terminus.

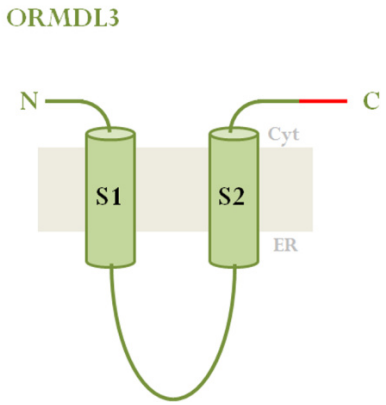

Figure 12: Schematic view of ORMDL3 structure. ORMDL3 presents two transmembrane domains with N- and C- regions facing cytosol. Region lacking in our mutant ORMDL3-Δ145-153 is coloured in red.

Second, our data demonstrated that ORMDL3 regulates ER Ca^{++} homeostasis by inhibiting SERCA2b activity. Intrareticular $[Ca^{++}]_{ER}$ was

decreased in cells overexpressing ORMDL3 protein; whereas ORMDL3 Knock Down (KD) cells had increased $[Ca^{++}]_{ER}$. In addition, our ORMDL3-Δ145-153 mutant does not affect $[Ca^{++}]_{ER}$ but still interacts with SERCA2b. We concluded that the C-terminal region is important for the ORMDL3 inhibitory effect on SERCA pump, although it is not necessary for SERCA-ORMDL3 interaction [280]. In order to know whether this effect on Ca^{++} homeostasis was cell type dependent, we repeated these experiments in Jurkat cell line (T cell), observing the same results as with HEK293 cells. Thus, ORMDL3 also regulates ER Ca^{++} homeostasis in lymphocytes, which are key players in the pathophysiology of asthma. Activation of T cells depends on calcium entry through CRAC channels (ORAI1) which is triggered following the antigen binding to the T-cell receptor (TcR), the generation of IP_3, and the depletion of ER Ca^{++} stores through IP_3Rs [264]. Therefore, ORMDL3 could modulate the activation of T-lymphocytes by decreasing $[Ca^{++}]_{ER}$.

Hence, if increased ORMDL3 expression is associated with asthma, and overexpression of ORMDL3 produced a decrease in $[Ca^{++}]_{ER}$; we should expect asthmatics to have less Ca^{++} in the ER. Consistent with that, moderately severe asthmatics have decreased levels of SERCA2 in airway smooth muscle (ASM) cells, which produced a lower ER Ca^{++} release and delayed ER refill [302]. Furthermore, it is well known that many characteristics of asthma are regulated by $[Ca^{++}]_{cyt}$, such as airway remodelling [263], inflammation [264] and ciliary beat frequency [265]; and it is demonstrated that ORMDL3 is expressed in almost all cell lines involved in asthma pathophysiology [270,271]. Consequently, deregulation of ER Ca^{++} signalling produced by ORMDL3 overexpression may affect many different

cell types. However a puzzling question we have not resolved yet is the link between a reduced ER-mediated Ca^{++} response, which is found in cells overexpressing ORMDL3, and asthma pathophysiology.

Third, this decrease in $[Ca^{++}]_{ER}$ is not only affecting Ca^{++} signalling but can also produce problems in protein assembly and folding, triggering UPR [193]. Indeed, it has been postulated that UPR can initiate inflammation; and this crosstalk in some cells (such as immune cells) could be essential in the genesis of immune mediated inflammatory diseases, among them asthma [194]. Thus we investigated if UPR is affected in ORMDL3 overexpressing cells.

Our experiments showed that ORMDL3 overexpression facilitates the UPR. We checked two of the three main UPR pathways: PERK and IRE1α. Particularly, ORMDL3 overexpression induced higher basal eIF2α phosphorylation (which is part of PERK pathway), whereas ORMDL3 KD produced lower eIF2α phosphorylation. However, when we checked XBP1 cleavage, which is downstream of IRE1α, there were no differences between control and ORMDL3 overexpression. McGovern *et al*. studied the effect of ORMDL3 on XBP1 induced transcription using an UPRE (unfolded protein response element) reporter in epithelial cells. The UPRE reporter contains a consensus motif that is recognized by XBP1(S). Using this luciferase reporter UPRE, they showed that ORMDL3 overexpression decreased UPRE transcription in both basal and after inducing ER stress with Thapsigargin (an inhibitor of SERCA pump) and Tunicamycin (an ER-stress-inducing chemical) [294]. Recently, Miller *et al*. examined all three UPR pathways in ORMDL3 overexpressing airway epithelial cells. They claimed the only effect of ORMDL3 overexpression is on the ATF6

pathway which is increased; while the other pathways are not affected. These differences with our results could be due to the use of different techniques or cell types [303]. Nevertheless, what is common to all studies is that ORMDL3 affects UPR.

Altogether, these experiments suggest that ORMDL3 activates the PERK pathway, which triggers the phosphorylation of eIF2α. Besides, ORMDL3 decreases the activation of the IRE1α pathway and activates the ATF6 pathway. On one hand, the phosphorylation of eIF2α inhibits protein synthesis, only allowing mRNA with many open reading frames in the 5'UTR to be translated, such as ATF4 (which will produce transcription of several genes). On the other hand, a reduced activation of IRE1α produces less transcription of mRNAs encoding for chaperones, enzymes and other proteins necessary for the correct folding and secretion of mature proteins; but this could be bypassed by the activation of ATF6 which is also a transcription factor for these elements. This mechanism, activating some UPR pathways and inactivating others, mimics the mechanism that some viruses use to block the induction of UPR-responsive genes, hence preventing the synthesis of host cells proteins while viral proteins are translated [304].

Moreover, several authors have shown that ORMDL3 is an inducible gene after some proinflammatory stimuli like pathogens (in normal human lung fibroblasts) [305], or allergens and cytokines (in airway epithelial cells) [303]. Thus, ORMDL3 could have an important role in immune mediated inflammatory diseases.

Yeast Orms (Orm1 and Orm2) inhibit Serine Palmitoyltransferase (SPT), which is the first enzyme of the sphingolipid synthesis pathway, therefore decreasing sphingolipid synthesis [306,307]. Breslow *et al*. also showed that human ORMDL3 interacts with SPT. These authors postulated that the possible mechanism of ORMDL3 on asthma physiopathology is through sphingolipid metabolism; based on some studies supporting the role of sphingolipids (ceramides and sphingosine-1-phosphate, S1P) in airway hyperresponsiveness and immune-cell trafficking [308,309]. SERCA pumps are inhibited by gangliosides (glycosphingolipid) [310], thus, ORMDL3 effect on $[Ca^{++}]_{ER}$ could be due to its putative role on sphingolipid synthesis.

```
Hsap ORMDL3    ------------------------------------------------------------
Scer Orm1      MTELDYQGTAEAA--STSYSRNQTDLKPFPSAGSASSSIKTTEPVKDHRRRRSSSIISHVEPE
Scer Orm2      --MIDRTKNESPAFEESPLTPNVSNLKPFP-----SQSNKISTPVTDHRRRRSSSVISHVEQE

Hsap ORMDL3    --MNVGTAHSEVNPNTRVMNSRGIWLSYVLAIGLLHIVLLSIPFVSVPVVWTLTNLIHNMGMY
Scer Orm1      TFEDENDQQLLPNMNATWVDQRGAWIIHVVIIILLKLFYNLFPGVTTEWSWTLTNMTYVIGSY
Scer Orm2      TFEDENDQQMLPNMNATWVDQRGAWLIHIVVIVLLRLFYSLF-GSTPKWTWTLTNMTYIIGFY
                  : .  :   * *:  ::.** *: ::: * **::.   :   :    *****: : :* *

Hsap ORMDL3    IFLHTVKGTPFETPDQGKARLLTHWEQMDYGVQFTASRKFLTITPIVLYFLTSFYTKYDQIHF
Scer Orm1      VMFHLIKGTPFDFNG-GAYDNLTMWEQIDDETLYTPSRKFLISVPIALFLVSTHYAHYDLKLF
Scer Orm2      IMFHLVKGTPFDFNG-GAYDNLTMWEQINDETLYTPTRKFLLIVPIVLFLISNQYYRNDMTLF
               :::* :*****:  . *    ** ***::  . :*.:**** .**.*::::. * : *   *

Hsap ORMDL3    VLN-TVSLMSVLIPKLPQLHGVRIFGINKY------
Scer Orm1      SWNCFLTTFGAVVPKLPVTHRLRISIPGITGRAQIS
Scer Orm2      LSNLAVTVLIGVVPKLGITHRLRISIPGITGRAQIS
                 *  :: :  :: ***   * :**   .
```

Figure 13: Alignment of Human ORMDL3 with yeast Orms. Ypk1 phosphorylation site predicted for Orms (bold red) is not conserved in ORMDL3. Predicted transmembrane domains are shown in green on the sequence, and the deletion of the last 8 aa on ORMDL3 is highlighted in blue. Abbreviations: Hsap, Human; Scer, Saccharomyces cerevisiae. This alignment was performed using Clustalw.

However, the putative effect of ORMDL3 on sphingolipid synthesis is to decrease their levels, therefore SERCA pump should be less inhibited, producing more calcium entry within the ER; which is the contrary to what we observe in our results. Besides, preliminary data from our lab show that ORMDL3 overexpression has no effect on sphingolipid synthesis (see

Annex 2: ORMDL3 effect on sphingolipid synthesis). Likewise, similar to the results of Breslow *et al*. and Siow *et al.* showing that only the triple KD (ORMDL1, ORMDL2 and ORMDL3) affects lipid synthesis, our data showed no effect of ORMDL3 KD on lipid synthesis [306,311]. These authors propose that it may be due to compensatory effects of the other family members; because individual KD of different members of the ORMDL family is not enough to increase ceramide levels. Indeed, Siow *et al.* postulate that ORMDL1 and ORMDL2 may be more important for this role, as their double KD slightly increases sphingolipid levels. Other plausible explanation is that the role in lipid metabolism is not conserved in human ORMDL3. Indeed, the regulatory domain of Orms implicated in the control of sphingolipid synthesis has been assigned to the N-terminal region, a stretch of the 60 aa that is not present in animals or plants (Fig. 13). So the involvement of ORMDL3 in sphingolipid metabolism, unlike Orms, is not fully characterised. Interestingly, Han *et al*. also demonstrated that yeast Orm proteins have a central role in the protein quality control[307], a function that is preserved in the human ORMDL3.

```
Hsap ORMDL1      MNVGVAHSEVNPNTRVMNSRGMWLTYALGVGLLHIVLLSIPFFSVPVAWTLTNIIHNLGM 60
Hsap ORMDL2      MNVGVAHSEVNPNTRVMNSRGIWLAYIILVGLLHMVLLSIPFFSIPVVWTLTNVIHNLAT 60
Hsap ORMDL3      MNVGTAHSEVNPNTRVMNSRGIWLSYVLAIGLLHIVLLSIPFVSVPVVWTLTNLIHNMGM 60
                 ****.****************:**:* : :*****:*******.*:**.*****:***:.

Hsap ORMDL1      YVFLHAVKGTPFETPDQGKARLLTHWEQLDYGVQFTSSRKFFTISPIILYFLASFYTKYD 120
Hsap ORMDL2      YVFLHTVKGTPFETPDQGKARLLTHWEQMDYGLQFTSSRKFLSISPIVLYLLASFYTKYD 120
Hsap ORMDL3      YIFLHTVKGTPFETPDQGKARLLTHWEQMDYGVQFTASRKFLTITPIVLYFLTSFYTKYD 120
                 *:***:**********************:***:***:*****:.*:**:**:*:*******

Hsap ORMDL1      PTHFILNTASLLSVLIPKMPQLHGVRIFGINKY 153
Hsap ORMDL2      AAHFLINTASLLSVLLPKLPQFHGVRVFGINKY 153
Hsap ORMDL3      QIHFVLNTVSLMSVLIPKLPQLHGVRIFGINKY 153
                   **::**.**:***:**:**:****:******
```

Figure 14: Alignment of the different members of human ORMDL family. The putative transmembrane domains are shown in green, and changes between members are shown in bold red. This alignment was performed using Clustalw.

Our working hypothesis is that each member of ORMDL family has different activities regarding Ca^{++} homeostasis, UPR regulation and sphingolipid metabolism. It is possible that, unlike ORMDL3, ORMDL1 and/or ORMDL2 have a role in sphingolipid metabolism. This is interesting because the three human ORMDLs share 80% of protein sequence (fig. 14) and single point mutations could be used to map the functional domains of ORMDLs involved in lipid metabolism.

A further question that remains unsolved is why only ORMDL3 is associated with inflammatory diseases, but not the other members of this family. Again a reasonable explanation could be that each member of the family has a different function, being ORMDL3 more related to Ca^{++} and UPR whereas the others are more related to sphingolipid metabolism. We cannot rule out that the three members of the ORMDL family form a bigger complex, involving SERCA pump and SPT; being ORMDL3 a regulatory subunit for SERCA, whereas the other ORMDL members regulate SPT and/or ORMDL3. ORMDL1 has been postulated as a good candidate in blood pressure which is the major cardiovascular disease risk factor[312], and it is part of the region deleted in one hereditary Aortic dilatation/dissection (AD) [313]. Moreover, ORMDL1 and ORMDL2 are associated with presenilin expression and gamma-secretase function; thus they may have some role in the development of Alzheimer's Disease (AD)[314].

In conclusion, increased expression of ORMDL3, which is involved in the ER Ca^{++} signalling and UPR, affects lymphocytes and epithelial cell physiology. Therefore, although the molecular mechanism underlying

ORMDL3 association with asthma is still unknown, our work provides more information about the role of ORMDL3 in the pathogenesis of this disease.

3. Calcium regulates sorting of some Secretory Cargo

Mucus and mucociliary clearance are the first defence lines in the airway epithelia. They protect us against the almost 25 million particles (i.e. dust, microbes, allergens…) we inhale each hour [315]. Mucus is a viscoelastic gel that forms a thin film on the surface of epithelia and protects it from damage by inhaled irritants, bacteria and viruses. Under normal conditions, these particles are removed from airways by a ciliary movement, which is called mucociliary clearance [316]. However, excessive production of mucus, also known as mucus hypersecretion; changes in the biophysical properties of the mucus; or altered ciliary beat frequency can impair mucociliary clearance producing an accumulation of mucus in the lungs, a condition characteristic of asthma [317] and COPD [318].

Airway mucus is an aqueous solution containing lipids, glycoconjugates and many secreted proteins; such as enzymes and anti-enzymes, endogenous antibacterial secretions and mucins. In normal conditions, almost 2% of the mucus weight is due to mucins [319]. There are 2 main classes of mucins (with distinct function and structure): 1) membrane-associated mucins and 2) secreted mucins. Mucins are synthesized at the ER, where they oligomerize and travel to the Golgi for glycosylation. After maturation, mucins are sorted in the TGN to the secretory granules called mature mucin granules, waiting in the cytoplasm to be secreted in response to some stimuli [320]. One of the hallmarks of asthma is mucus hypersecretion due to abnormal secretion of stored mucin and the overproduction of new mucin proteins[316]. However, although mucin secretion process as well as the proteins involved

have been extensively studied [320], the mechanisms implicated in the sorting of mucin and other secretory cargoes remain unknown.

Using a genome-wide screen to identify new components of the secretory pathway in *Drosophila melanogaster*, Bard *et al*. identified a protein called twinstar (ADF/cofilin in mammals), which regulates actin polymerization, as a new component of this pathway [321]. Later, von Blume *et al*. found that inactivation of ADF/cofilin impaired the sorting of a subset of both soluble and integral membrane proteins at the trans-Golgi network (TGN). The authors suggested that actin organizes the sorters, which are the ones that bind secretory cargo; hence, actin is important for the sorting of secretory cargo. Moreover, they postulated that the molecular identity of these sorters could be different proteins involved in Ca^{++} transport [242].

ADF/cofilin are required for the sorting of secretory cargo at the TGN in mammalian cells although the mechanism whereby these cytoplasmic proteins interact with the cargoes in the lumen of the TGN was unknown. Thus, the last objective of this Thesis was to identify and characterize this TGN sorting mechanism. Our current model proposes that ADF/cofilin depolymerizes actin filaments, which triggers the activation of SPCA1. Then, SPCA1 pumps Ca^{++} into the lumen of the TGN for the sorting of secretory Ca^{++} binding cargo [322].

First we shown by immunoprecipitation and mass spectrometry (MS) analysis that ADF/Cofilin interact with SPCA1, which is the Ca^{++} pump of the TGN. We realized that many of the missorted secretory cargoes in ADF/cofilin KD were Ca^{++} binding proteins (i.e. Cab45), so we tested if

Ca^{++} import into the TGN by SPCA1 was important for the sorting of secretory proteins. We measured Ca^{++} concentration in the TGN using a fluorescence resonance energy transfer (FRET)–based Ca^{++} sensor (Go-D1cpv) that is targeted to the TGN [157]; to directly study SPCA1 role in the TGN Ca^{++} homeostasis. Finally, we demonstrated that SPCA1 KD causes cargo missorting and impairs Ca^{++} influx into the TGN.

Second, our Ca^{++} experiments showed that ADF/Cofilin knockdown affects Ca^{++} import into the TGN. Besides, despite cofilin is a cytoplasmic protein, there is a pool of cofilin located at the TGN and this localization is dependent on actin. Indeed, cofilin located at the TGN binds to SPCA1, although dynamic actin is required for this interaction. Altogether, our data indicate that Ca^{++} import into the TGN is mediated by the interaction of ADF/cofilin with SPCA1. Thus, SPCA1 is the postulated molecular sorter[242] connecting secretory cargoes with cytoplasmic ADF/cofilin. Therefore, we provided a new TGN sorting mechanism, the Ca^{++} dependent sorting of secretory cargo that binds Ca^{++} [322].

This mechanism could be the sorting process for secretory mucins. Indeed, mucins are polyanionic, and they are highly condensed within the granules due to high concentrations of Ca^{++} (Fig. 15). Moreover, without Ca^{++} present in the mucin matrix, packaging in granules could not occur [323]. Therefore, it is possible that mucins oligomers, which are negatively charged, could bind Ca^{++}; and they could be sorted by the Ca^{++} dependent sorting of secretory cargo that we recently identified.

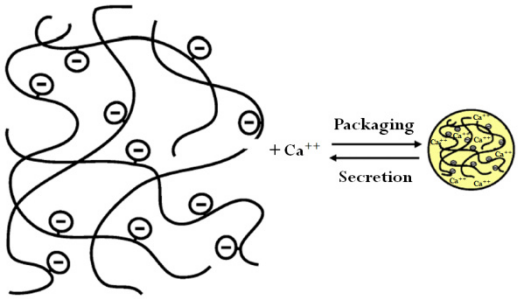

Figure 15: Calcium is necessary for mucin packaging. Mucin are polyanionic proteins, thus when Ca^{++} is present, they can be highly condensed within the granules. Taken from Rogers D., 2007 [320]

Moreover, changes in Ca^{++} homeostasis, such as those described in asthmatic patients [302], could affect [Ca^{++}] in the TGN. As a consequence, this could produce missorting of secretory Ca^{++} binding proteins, among them, mucins; so changes in Ca^{++} homeostasis could trigger mucus hypersecretion, another characteristic of asthma pathophysiology [261]. Hence, Ca^{++} could have a role in mucus hypersecretion not only by regulating ciliary beat frequency (and mucociliary clearance) [148], but also by regulating the sorting of mucins.

4. Calcium homeostasis and asthma pathophysiology

Altogether, we have shown several mechanisms linking deregulation of Ca^{++} signalling with different hallmarks of asthma pathophysiology; like bronchoconstriction, inflammation and mucus hypersecretion. Moreover, we provided new target genes involving different levels of Ca^{++} signalling: 1) TRP channels controlling Ca^{++} entry; 2) ORMDL3 that regulates ER Ca^{++} homeostasis and ER stress; and 3) SPCA1 that pumps Ca^{++} into the TGN, necessary for the sorting of secretory proteins.

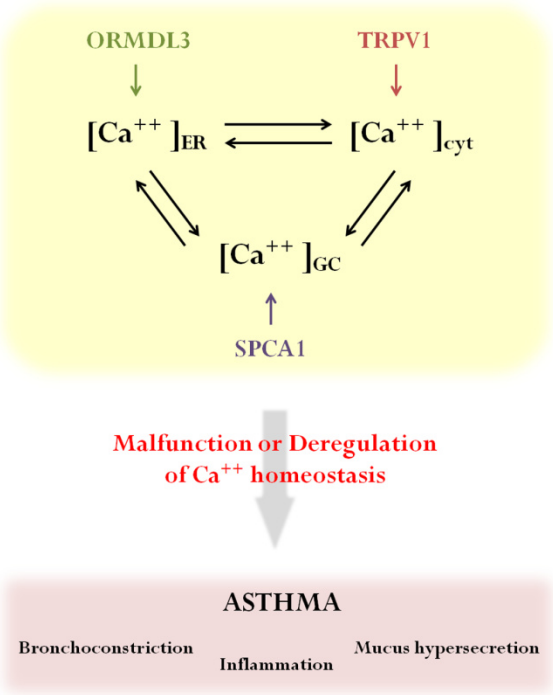

Figure 16: Summary of the different mechanisms studied in this Thesis and their relationship with asthma pathophysiology. Abbreviatons: ER, Endoplasmic Reticulum; cyt, cytosol; and GC, Golgi Complex.

V. CONCLUSIONS

1. Population-based genetic analysis of Spanish kids has identified a loss-of-function genetic variant of the sensorial TRPV1 channel associated with less severe symptoms of asthma (cough and wheezing)

2. A loss-of-function SNP of TRPV4 is not associated with asthma symptoms in the same population.

3. The ORMDL3 protein, product of the *ORMDL3* gene associated with childhood asthma is a two transmembrane domain protein present in the ER with N- and C-tail facing the cytosol.

4. ORMDL3 levels directly correlate with cytosolic Ca^{++} concentration and inversely correlate with ER Ca^{++} concentration.

5. ORMDL3 interacts with and modulates SERCA activity in the ER. Besides, increased expression of ORMDL3 negatively regulates SERCA activity.

6. The last 9 aminoacids of ORMDL3 are required to modulate SERCA activity.

7. ORMDL3 affects the unfolded protein response at the ER.

8. SPCA1 and Ca^{++} are required for the correct sorting at the TGN. Actin dynamics modulate the activity of SPCA1, and, hence, protein sorting.

9. The research carried out in this Thesis has provided different pieces of evidence showing that genetically determined changes in cellular Ca^{++} homeostasis may occur in asthma pathophysiology.

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VII. ANNEXES

ANNEX 1

Review: Ion channels in asthma.

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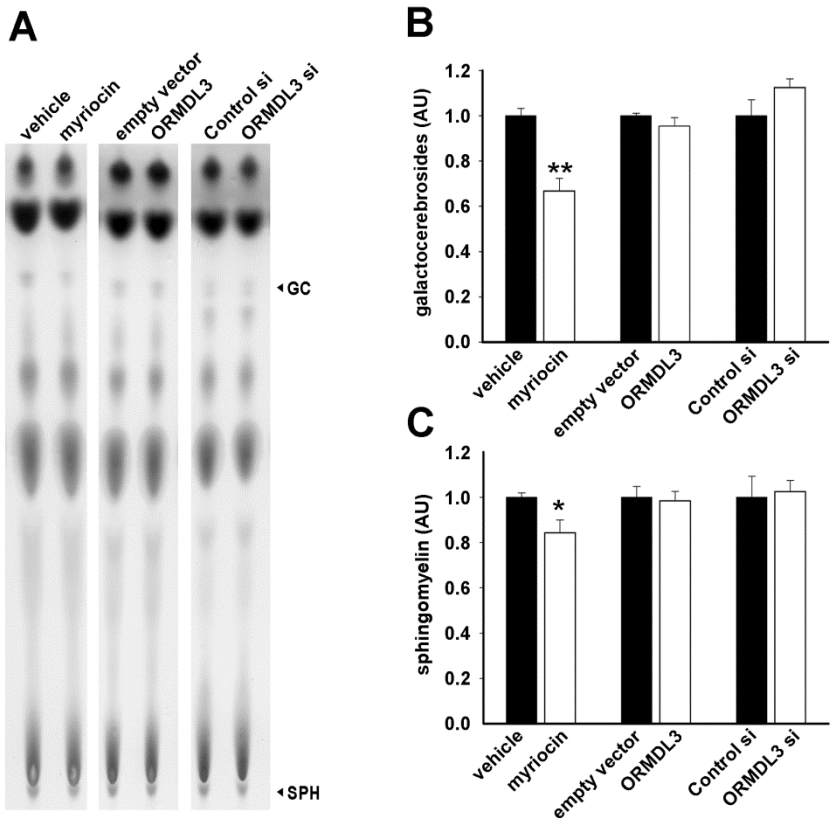
ANNEX 2

ORMDL3 effect on sphingolipid synthesis

Protocol: Cells were transfected with ORMDL3, empty vector, ORMDL3 siRNA and Control siRNA or treated for 24h with myriocine (an inhibitor of SPT enzyme) (10μg/ml) or vehicle (DMSO). Lipid extraction was performed using a mixture of hexane:isopropanol (3:2, v/v). Lipid extracts from samples were diluted in chloroform and loaded in a TLC plate. Using the same procedure a mixture of known lipid standards was loaded. The identification of the different types of lipids was based on the comparison with the mobility, shape and color of the spots of pure standards.



Annex 2: Lipid evaluation. **A**, Representative TLC lanes under myriocin treatment (an inhibitor of serine Palmitoyltransferase (right), ORMDL3 overexpression (center), and ORMDL3 siRNA (left). Arrows indicate the position of galactocerebrosides (GC) and sphingomylelin (SPH). **B**, Relative quantification of galactocerobrosides normalized by total lipid amount. **C**, Relative quantification of sphingomylelin normalized by total lipid amount.