UNIVERSITAT DE BARCELONA

Biology of the cardiovascular Kv7.1 functional complex

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Abstract

Voltage gated K^+ channels (Kv) are transmembrane proteins that allow the pass-thorugh of potassium ions, regulating the electrochemical gradient of the cell membrane. This way, they modulate several physiological processes, such as proliferation, migration or cell volume. Of particular interest in this dissertation is their role in excitable cells, were they control several key functions. The relevance of this ion channels is evidenced when mutations or alterations in the proper functioning of Kv channels causes severe pathologies, including cardiovascular or neuronal diseases, autoimmune affectations or cancer.

Kv channels are tetramers of 4 α subunits with 6 transmembrane segments each one, that associate to form the pore and generate a functional channel. The wide functional diversity of currents is due to a vast number of modulations: heterotetramerization of α subunits, splicing variants, post-translational modifications or the association with regulatory subunits. The last ones include KCNE family, which co-assemble with the channel and modulate its electrophysiological, pharmacological or physiological properties.

Kv7.1 associates with KCNE1 in cardiomyocytes to generate I_{Ks} cardiac repolarizing currents, in charge of finishing the cardiac action potential. Their assembly and traffic to the plasma membrane have been subject of discussion over the last years, with two opposite schools claiming an association early in the biogenesis versus a independent traffic to the plasma membrane, were both proteins would diffuse to assemble. We aimed in the present work to shed a light to this controversial topic. Kv channels have also been described in vascular smooth muscle, were they set the resting membrane potential and, therefore, control vascular tone. Kv7.1, Kv7.4 and Kv7.5 have been detected in different veins and arteries, were aberrations in their expression promote physiological alterations, but the specific role of each subunit remains unknown.

In this scenario, the proposed objectives for the current PhD dissertation included the study of Kv7.1-KCNE1 complex, its assembly and traffic mechanisms. We hypothesized an unconventional secretion for the complex and suggest ER-PM junctions as the potential trafficking system. Therefore, we aim to characterize this structures and their implication in Kv7.1 membrane targeting. Finally, due to its implication in proliferation, their importance in cardiovascular system and their known role in some cancers, we studied the changes in the expression of Kv channels in endothelial-derived vascular tumors.

We have been able to solve the traffic controversy of Kv7.1-KCNE1 complexes as they are not assembled early in their biogenesis. While KCNE1 is using the conventional secretion pathway, Kv7.1 takes an unconventional route that skips Golgi. Upon co-assembly, Kv7.1 redirects KCNE1 to this unconventional pathway. Moreover, we have proved that this non-conventional route are indeed ER-PM junctions, which also host the assembly of the complex. The molecular interactors of the channel during its ER-PM junction targeting have also been analysed during this PhD thesis, unravelling a complex and dynamic proteomic context. In addition, we have described for the first time the expression of Kv1.3, Kv1.5, Kv7.1 and Kv7.5 in endothelial cells of human veins and arteries. A remodelling of this composition is observed in different vascular cancers, related with the malignancy of the tumor in some of the cases.

2. Table of contents

1. General introduction 11
1.1. Ion channels 13
1.2. Classification 13
1.2.1. Potassium channels (KCh) 13
1.1.2. Voltage-gated potassium channels (Kv) 15
1.3. Structure and function 16
1.4. Kv channels 17
1.4.1. Kv7 channels 17
Kv7.1 18
Kv7.5 19
1.4.2. Kv1 channels 19
Kv1.3 19
Kv1.5 20
1.4.3. β-subunits regulation 20
Kv7.1-KCNE1 21
1.5. Ion channel traffic 22
1.5.1. Conventional secretory pathway 23
Endoplasmic reticulum 23
Golgi apparatus 25
Trans-Golgi network 26
1.5.2. Unconventional secretory pathway 26
1.5.3. Other traffic modulations 29
Post-translational modifications 29
Heterotetramerization 31
Subcellular/organelle targeting 31
1.6. Cardiovascular Kv7 channels physiology 32
1.6.1. The heart 32
Cardiomyocytes 32
Cardiac action potential 33
1.6.2. Cardiovascular system 35
1.7. Kv7 channels in disease 35
1.7.1 Cardiac channelopathies 35
LQTS 35
SQTS 36
1.7.2. Vascular smooth muscle disease 36
1.7.3. Ion channels and cancer 36
Kv channels and proliferation 38
Kv channels in apoptosis 40
Kv channels and cancer 41

2. Aims 45

3. Materials and methods 49
3.1. Cell culture 51
3.1.1. Cell lines 51
3.1.2. Cell lines culture 51
3.1.3. Cell line freezing and thawing 52
3.1.4. Transient cell transfection 52
3.1.5. Isolated cardiomyocytes 50
3.2. Molecular biology 53
3.2.1. Bacteria culture 53
3.2.2. DNA extraction 53
3.2.3. DNA purification from gel 53
3.2.4. PCR 54
3.2.5. Site directed mutagenesis 54
3.2.6. Sequencing 55
3.3. Biochemistry 55
3.3.1. Total protein extraction 55
3.3.2. Bradford assay 55
3.3.3. SDS-PAGE and Western Blot 56
3.3.4. Protein precipitation 57
3.3.5. Immunoprecipitation 57
3.3.6. Membrane protein biotinylation 57
3.3.7. Cell unroofing preparation 57
3.3.8. Lipid raft isolation 58
3.3.9. Glycosilation studies 58
3.3. Cell imaging 58
3.3.1. Immunocitochemistry 58
3.3.2. Confocal microscopy 59
3.3.3. FRET Acceptor Photobleaching 60
3.3.4. Cell unroofing preparations 60
3.3.5. 3D rendering 61
3.3.6. Electron microscopy 61
3.3.7. Super resolution microscopy 62
3.4. Tissue imaging 63
3.4.1. Immunohistochemistry (OCT embedded samples) 63
3.4.2. Immunohistochemistry (Parafin embedded samples) 64
3.4.3. Confocal microscopy 65
3.5. Electrophysiology 65
3.6. Proteomic studies 66
3.6.1. Immunoprecipitation 66
3.6.2. Protein digestion, nano-HPLC and mass spectrometry (MS) 66
3.6.3. Data analysis 66

4. Results 69
4.1. Endoplasmic Reticulum – Plasma membrane junctions hub the I_{Ks} complex 71
4.2. Deciphering the Kv7.1 traffic interactors 87

4.3. Kv channel expression in endothelial-derived vascular tumors 101

5. General discussion 115

6. Conclusions 131

7. References 135

1. General introduction

1. General Introduction

1.1. Ion channels

From bacteria to complex eukaryotic living systems, membranes are crucial for live. They isolate cells from the extracellular environment, but not in a hermetical way. Molecules flow through the membranes when necessary, and there are specific proteins in charge of this essential function: transporters and pores. Since the Greek postulated a theory that brain control muscle through the spinal cord or Hodgkin and Huxley first described ion channel existence in 1952 [1], until now, the knowledge in the field has grown enormously. Physics and biology interplay to explain the functional mechanisms of ion channels and the electrophysiological properties of these proteins.

Ion channels are transmembrane proteins that form hydrophilic pores on the lipid bilayer to allow ion movement from side to side of the membrane. Ions diffuse passively across them following their electrochemical gradient, at high speed (>10^6 ions/sec) and without metabolic cost. Ion channels also differentiate from active transporters as they present selectivity to specific ion channels depending on their nature, preventing unspecific interactions and preserving membrane permeation. Several channels share a conserved signature within the pore, known as selectivity filter, which allows for the selective passage of that specific ion. However, the signals that ion channels respond to open also depend on their class, showing high divergence among the sensing domain: voltage changes in the plasma membrane, temperature, ligand binding or pH are some of the most common stimulus that can activate ion channels [2].

Homeostasis stablishes the cellular ion asymmetric composition: high Na^+ and Cl^- extracellular concentration, together with high K^+ and low Ca^{2+} intracellular concentration. This ion distribution is maintained by active pumps and transporters, generating a negatively charge cytoplasm compared to the extracellular space. This electrochemical gradient generates what we call membrane potential. Because K^+ is more permeant that sodium or chloride, and also because its equilibrium reaches negative values, the cell membrane potential will be, generally, negative, around -90 mV. Changes in ion concentration through the membrane would generate changes in its electric charge, causing an immediate effect on membrane potential. Other channels can detect these changes in the membrane potential and become excited, propagating the electric response [3].

Therefore, ion channels are fundamental for excitable cells. Nevertheless, they are not only implicated in electrical excitation, but are also involved in cell functions such as proliferation, migration, cell volume and specific processes such as insulin release or muscular contractibility. Their participation in such highly diverse phenomena highlights a crucial biological relevance. This becomes evident when mutations and alterations of the normal function of these proteins trigger alterations in the organisms, as we will see in this dissertation.

1.2. Classification

1.2.1. Potassium channels

The British Pharmacological Society (BPS) and the International Union of Basic and Clinical Pharmacology (IUPHAR) classify ion channels as (i) voltage-gated ion channels, (ii) ligand-gated ion

channels, or (iii) channels using other gating mechanisms, including aquaporins, chloride channels, and store-operated calcium channels. Following these criteria, 141 members are included in the voltage-gated ion channel superfamily (**Figure 1**), making it one of the largest groups of signal transduction proteins [4,5].

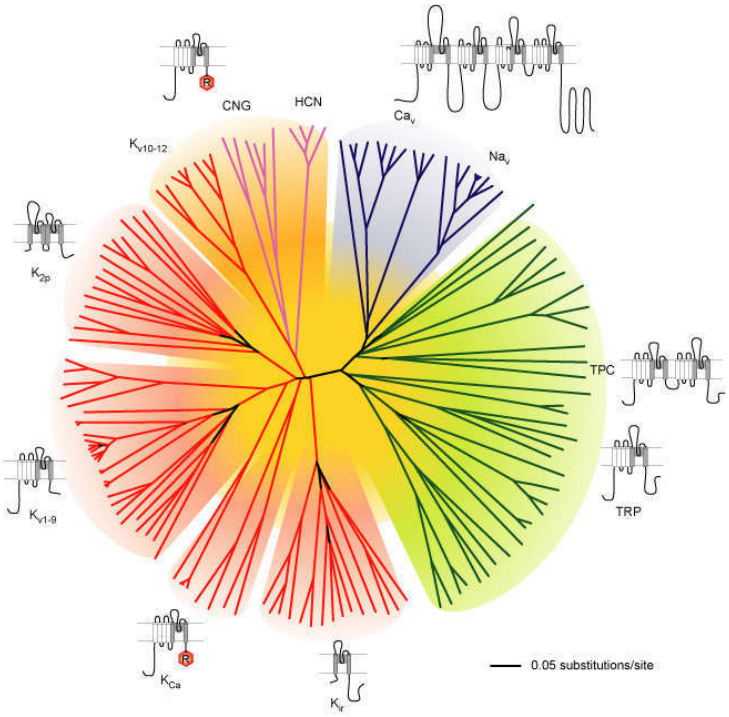


Figure 1. Diversity of ion channels. Unrooted phylogenetic tree representing the amino acid sequence relations of the minimal pore regions of the voltage-gated ion channel superfamily. This classification highlights the seven groups structurally related and their membrane topologies, and families are also indicated. Scale bar represents the tree distance corresponding to 0.05 substitutions per site in the sequence in the genetic code. From Yu and Catteral (2004) [4].

Potassium-selective channels (KCh) (**Figure 1**, in red) are the largest and most diverse group of ion channels expressed in both excitable and non-excitable cells. They can be classified following several criteria. From a structural point of view, KCh are classified into four groups, including four of the 11 families of the voltage-gated ion channel superfamily (**Figure 2**):

- 2TM/1P: Simplest form of KCh. Include the inward rectifier KChs (Kir), the K_{ATP}, and the G-protein-coupled KChs. These channels are tetramers formed by four 2TM/1P subunits. The pore region (P) is localized between the two TM domains.
- 4TM/2P: The K_2P family is formed by dimerization of two 4TM/2P subunits. Contain two P regions between the first and the second TM domain and between the third and the fourth TM domain.
- 6TM/1P: Tetramerizing peptides with an intracellular N- and C-terminus. The P region, containing the K^+-conduction pathway, is situated between the fifth and the sixth TM domain. This group includes the voltage-gated KCh (Kv) and the small and intermediate conductance Ca^{2+}-activated KCh (K_{Ca}).
- 7TM/1P: KCh are formed by tetramerization. Unlike the other groups, the N-terminus is extracellular. In this group, the P region is located between the sixth and seventh TM domain. This group includes the large-conductance members of the K_{Ca} family.

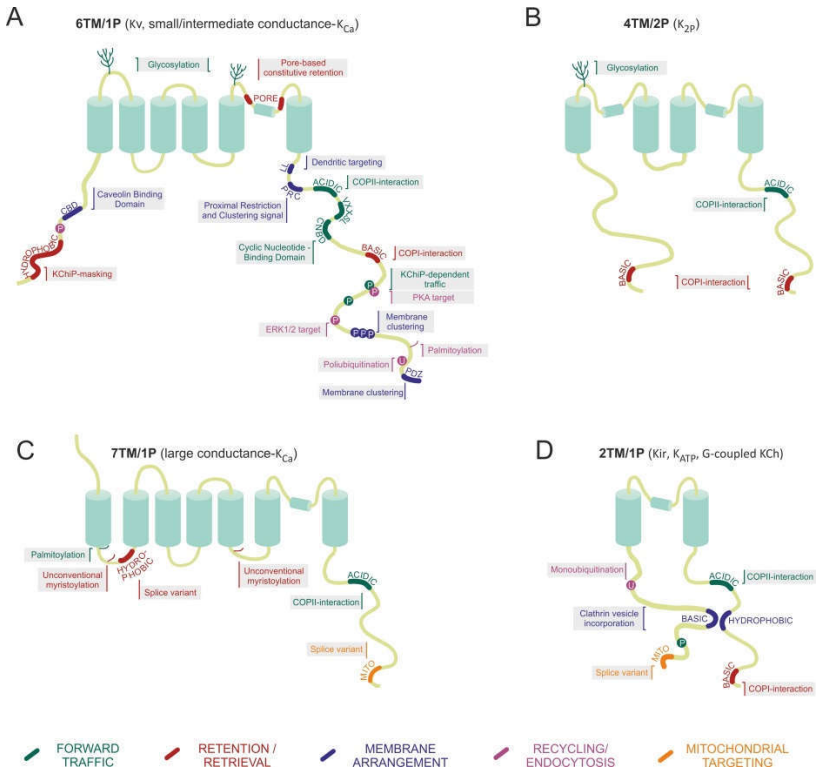


Figure 2. K⁺ channel structures. A schematic representation of each family of potassium channels. (A) 6TM/1P; (B) 4TM/2P; (C) 7TM/1P; and (D) 2TM/1P. Structural domains and post-translational modifications (PTMs) affecting traffic and the destination of different channels in each family are shown. Cartoons represent a compilation of signatures documented in each structural family. It is important to highlight that not all signatures are present in the same channel. The color code corresponds to the main dominant effect in traffic. Green, forward traffic. Red, retention/retrieval domains. Blue, membrane arrangement. Magenta, channel recycling and endocytosis. Orange, mitochondrial targeting. Basic, cluster of basic residues; Acidic, cluster of acid residues; Hydrophobic, hydrophobic clusters; LL, di-leucine motif; P, phosphorylation; U, ubiquitination; Mito, mitochondria; PDZ, domains. From Capera *et al.* (2019) [6].

1.2.2. Voltage-gated potassium channels (Kv)

The present work is focused on Kv channels, which present a 6TM/1P structure. The first potassium channel to be cloned was the *Drosophila* voltage-gated *Shaker* channel, responsible for a shaking-leg phenotype when mutated. Since then, up to 40 genes have been cloned, representing the largest family of KCh in the human genome. Their members are divided in 12 subfamilies, which following a functional classification, we can classify in 4 groups:

- Delayed rectifier channels (I_{DR}) exhibit a delay before activation (**Figure 3**). They generate an outward current of K^+ following membrane depolarization triggered by an influx of Na^+ ions inside the cell. To counteract this cation influx, I_{DR} channels allow the exit of K^+ ions from the cell. Therefore, the membrane repolarizes, shortening the duration of the nerve impulse. This is crucial in excitable cells such as neurons or muscle cells, but their presence is ubiquitous in the human body. This group includes members of the *Shaker*-related family (Kv1.1–Kv1.3, Kv1.5–Kv1.8), the *Shab*-related family (Kv2), some *Shaw*-related members (Kv3.1, Kv3.2), the Kv7 group and Kv10.1, from the ether-à-go-go (EAG) family.

- A-type channels (I_A) channels generate a transient-outward K+ current with little delay after depolarization (**Figure 3**). Characterized by rapid inactivation, these channels open when

depolarization occurs after hyperpolarization, and they increase the interval between action potentials. Thus, I_A help neuronal repetitive firing. This group includes members of the *Shaker* (Kv1.4), *Shaw*-related (Kv3.3, Kv3.4), and *Shal*-related (Kv4) families.

- Modifier/silencer subunits: Several groups have similar sequences and structures to those of some Kv families but are not functional in homotetrameric compositions. Instead, they mostly heterotetramerize with members of the Kv2 family, modulating their activity. This group includes the Kv5, Kv6, Kv8, and Kv9 families. These channels present a restricted tissue expression, indicating a tissue-specific function for the heterotetrameric channels.

- Some channels cannot be grouped into any of the abovementioned categories according to their properties. For example, Kv10.2 is sometimes defined as a noninactivating outward-rectifying potassium channel. In addition, Kv11.1, a member the hERG family, is a voltage-gated potassium channel with inwardly rectifying properties. Finally, KCa3.1 channels are activated in response to voltage and Ca^{2+} changes.

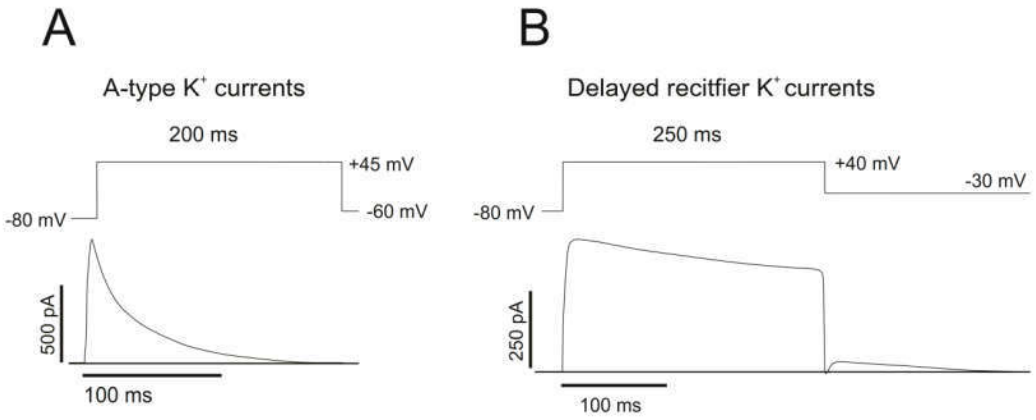


Figure 3. Representative outward A-type and delayed rectifier K^+ currents. Voltage-clamp records of Kv1.4 and Kv1.5 currents expressed in mouse L-cells. Results shown are traces obtained for depolarization test potentials as indicated. **A:** Kv1.4 currents rapidly inactivated during maintained depolarization. **B:** Kv1.5 displays fast activation and slow and only partial inactivation. From Serrano-Novillo *et al*. (2019) [7].

1.3. Structure and function

Each Kv channel gene encodes for a polypeptide of 6 transmembrane domains (TM, S1-S6), connected by intra or extracellular loops. These domains are hydrophobic and span the phospholipid bilayers as coiled alpha-helix. The sequence also encodes COOH and NH2-terminus with variable length facing the cytoplasm. This polypeptide represents the alpha subunit. The association of 4 α tetramers generates a functional channel when arranged around an aqueous central pore.

Between S5 and S6 loops we find a short membrane re-entrant loop (P loop) containing the signature TVGYG sequence for K^+ selectivity. This sequence is highly conserved among all types of K^+ channels. The oxygen atoms of the side chains are projected along the selectivity filter, substituting water molecules of the solvation shell surrounding K^+ ions in solution. This way, several dehydrated K^+ ions cross co-ordinately, while passage of other ions is not favoured [8,9].

The voltage sensor and gating machinery of the subunit also deserves mention. A string of charged amino acids, usually arginine and lysine spaced apart by pairs of hydrophobic residues, is located in the S4 and conserved among all sodium, potassium and calcium voltage-dependent channels. is suggested to work as the voltage sensor. This motif, repeated 5-7 times depending on the subfamily, is suggested to work as the voltage sensor [10]. When changes in the electrical field occur, S4 moves in response: the positively charged residues in S4 work as gating charges that will move within the membrane upon depolarization to open the pore of the channel. These changes in intramolecular distances correlate with the voltage dependence of the charge movement. Negative residues in the S1-S3 segments are required to stabilize the positively charged S4 inside the lipid bilayer [11,12,13] (**Figure 4**).

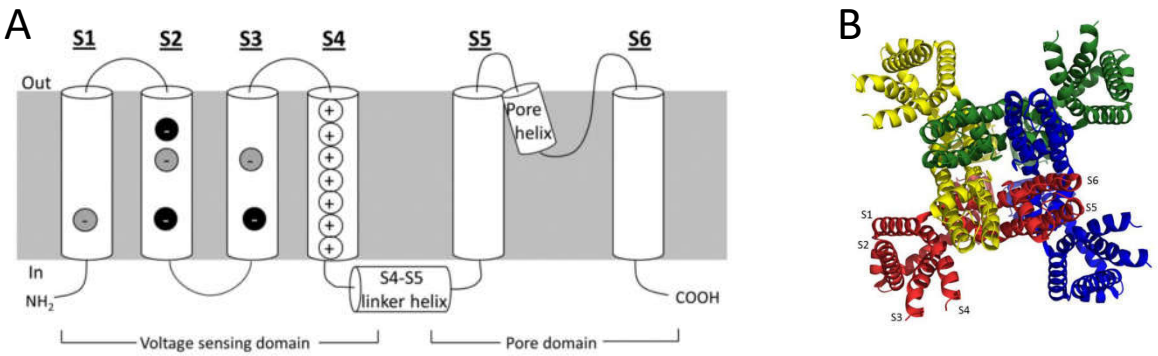


Figure 4. Topology of a voltage-gated potassium channel. Left: Cartoon representation of the membrane topology of a Kv channel α-subunit. Key charged residues within the voltage-sensing domain are highlighted. Negative charges highlighted in black are highly conserved. Right: Top view ribbon representation of the Kv channel tetrameric assembly based on the Kv1.2 crystal structure. Each subunit is highlighted in a different color. At the centre, the permeation pathway. From Cheng and Claydon (2012) [14].

1.4. Kv channels

1.4.1. Kv7 channels

Kv7 family is composed by 5 members (Kv7.1-Kv7.5), encoded by *KCNQ1-KCNQ5* genes, and all of them undergo outward delayed rectifying K^+ currents. While sharing the classical Kv topology explained above, they are characterized by an unusually long C-terminal domain, from 300 to 500 amino acids depending on the isoform. This region is considered as a multifunctional structure that acts as scaffold for many cytoplasmic regulatory molecules, such as phospholipid PIP2, calmodulin, anchoring protein AKAP, syntaxin 1A SNARE protein or the cytoskeleton-anchoring protein Ankyring G (**Figure 5**). It plays a crucial role in chanel physiology, with relevant roles in structure, electrophysiological properties and traffic [15].

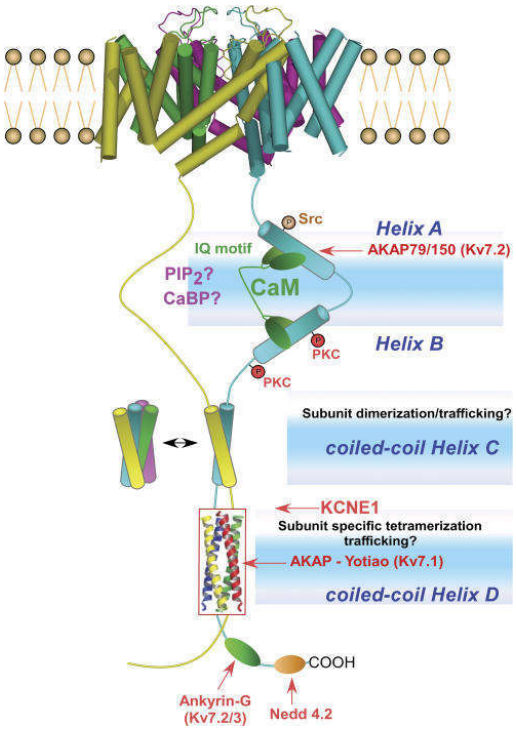


Figure 5. Structure of Kv7 channels and interaction sites on the carboxy-terminal tail. The transmembrane domains of the four subunits of the tetramer are shown in yellow, green, purple and blue. The C-termini of only two subunits are depicted for clarity. The Kv7 C-terminus exhibits 4 conserved α-helixes (A-D). I also exhibits conserved interaction sites with CaM (green) at helices A (IQ motif) and B (5-10 motif). It also shows putative interaction sites with PIP2 (purple) and specific interaction sites with the AKAP79/150, with the AKAP-yotiao , with ankyrin-G and with Nedd4-2 (red arrows). Helix A contains a tyrosine kinase phosphorylation site. Helix B is endowed with PKC phosphorylation sites. In addition to a potential role in channel trafficking, the coiled-coils C and D may correspond to subunit dimerization and tetramerization modules, respectively. From Haitin and Attali (2008) [15].

They have a central role in excitatory cells regulating their excitability and are responsible for well-established K^+ currents: the cardiac slow delayed rectifier currents (I_{Ks}), the slow subthreshold neuronal M-current (I_{KM}) and the low threshold K+ current in cochlear outer hair cells (I_{Kn}). They also contribute to potassium currents in some vascular smooth muscle cells. Moreover, some non-excitable cells require the expression of Kv channels to modulate physiological roles. They control cell homeostasis through the regulation of K^+ recycling or limit the insulin secretion in the pancreas, among others. Kv7 channels exert a crucial role for proper body functioning. This is clearly proved by the high number of mutations related with diseases found in this family, including arrhythmias and epilepsy or deafness [16].

Kv7.1

Kv7.1 is encoded by the *KCNQ1* gene, located in humans at the chromosome 11p15, and was the first Kv channel to be identified. The gene presents tissue-specific imprinting. This means that epigenetic chromosomal modifications that promote the preferential expression of a specific parental allele in the somatic cells of the offspring. In the case of Kv7.1, imprinting is mainly promoting maternal allele expression [17]. Moreover, alternative splicing gives rise to 2 isoforms: a full length isoform of 676 amino acids and a shorter isoform of 549 amino acids. The last one, however, generates non-functional channels that will supress Kv7.1 currents upon co-expression [18]. Kv7.1 is mainly a cardiac channel, where it is responsible for the I_{Ks} currents when associated with its regulatory subunit KCNE1 [19,20]. Nevertheless, it has also been detected in kidney, lung, placenta, testis or colon. Also in the inner ear, where it controls endolymph homeostasis through the regulation of K^+ recycling [21]. While it was typically believed that it was not expressed in central nervous system, Kv7.1-KCNE1 have been detected in many areas of the brain, where mutations of the channel may be involved in epileptic seizures [22].

The COOH terminus of Kv7.1 is especially different from the carboxy terminal of the other four members of the family. Therefore, no heterotramerization was believed for this channel. However, a few years ago our group described for the first time a Kv7.1-Kv7.5 association in blood vessels. The interaction causes spatial location modification of the subunits, thus, implicating a regulation of the heterotetramer properties versus the homomers and an increase in the diversity of structures that fine-tune vascular tissue [23].

Kv7.5

Kv7.5, first cloned in 2000, is encoded by the *KCNQ5,* located in the human chromosome 6q13, and translates to a protein of between 897 and 932 amino acids, depending on the isoform [24]. The gene has 3 splice variants: the full length is mostly expressed in brain; variants 2 and 3 are truncated forms expressed in skeletal muscle. The three isoforms show similar biophysical and pharmacological properties. However, truncations are located within the PIP2 and calmodulin binding domains. Consequently, the regulation by this molecules may differ between isoforms [25]. Kv7.5 has a crucial role in brain, where it overlaps with Kv7.2 and Kv7.3 to generate the I_{KM} currents [26]. In auditory central nervous system, where the other two Kv7 channels are less expressed, Kv7.5 controls pre-synaptic resting membrane potential. As synaptic signalling is a high-frequency signal, the properties of Kv7.5 are ideal to modulating the size, time and release probability of the fast axonal spiking associated with sound [27]. However, some authors claim that Kv7.5 localization is located post-synaptically rather than pre-synaptically [28]. Also expressed in lung, colon, uterus and skeletal and vascular smooth muscle. In vascular musculature modulates the contractile activity and participates mainly in vasodilation [29,30]. Interestingly, Kv7.5 is the only member of the family that has not been related yet with any human disease.

1.4.2. Kv1 channels

Also named *Shaker* because their homologous protein of *Drosophila melanogaster*, Kv1 channels were the first potassium channels to be cloned. A mutation in the flies which resulted in the shaking of the legs under anaesthesia suggested a function in the termination of nerve impulse [31]. Active channels are, as abovementioned, homotetramers and carry outward delayed rectifying currents (except Kv1.4, which is an A-type current). The family is now composed by 6 members (Kv1.1-Kv1.6), each one plays a distinct physiological role. We will focus on Kv1.3 and Kv1.5.

Kv1.3

Kv1.3 is encoded by the *KCNA3* gene, located in humans at the chromosome 12p13.32 and translates to a 575 amino acid protein [32,33,34]. Expressed mainly in nervous and immune system, Kv1.3 is found in several tissues, including brain, lungs, B and T lymphocytes, macrophages or microglia, among others [5]**.** As expected from its wide distribution, Kv1.3 has an important role in several physiological processes, both in excitable and non-excitable cells. In the brain, for instance, Kv1.3 modulates the firing rate, whereas in the immune system it controls the leukocyte physiology or participates in the immune synapse [35]. Moreover, it may form heteromeric complexes with other members of the Kv1 family, modulating their properties. Alterations in Kv1.3 expression lead to cell afectations such as apoptosis [36,37], as well as several pathologies such as diabetes, chronic inflammation or cancer [38].

Kv1.5

Kv1.5 is encoded by *KCNA5* gene, also located in humans at the chromosome 12p13.32 and translates to a 613 amino acid protein. Kv1.5 is mainly a cardiac channel. It is expressed in heart, where it is responsible for the ultrarapid-activating K^+ current in heart (IK_{ur}) that contributes to the ending of the action potential by repolarizing the membrane, and also in vascular musculature [39,40,41]. It is also abundantly expressed in immune system, especially in macrophages or B lymphocytes, maintaining a physiological balance against Kv1.3 [42], and other tissues including kidney, lung or brain [5]. When in association with other Kv1 channels, its trafficking and electrophysiological properties are modulated. Like Kv1.3, Kv1.5 is implicated in cell cycle regulation and it expressions changes during the cycle progression [43].

1.4.3. β-subunits regulation

In addition to pore-forming subunits of the K^+ channels (α subunits), they associate with several auxiliary subunits (β subunits), which increases the diversity of roles and implications of channels in health and disease. The diversity of α and β subunits– can regulate both cellular location and function. This, added to a wide range of pre- and posttranslational processes controlling protein expression, traffic, assembly, and/or function - explained further in this introduction -, configure the myriad of physiological roles and pathological dysfunctions. There are two kinds of regulatory β-subunits: (i) cytosolic peptides, such as KCNAB (Kvβ), K^+ channel-associated protein (KChAP), and K+ channel-interacting proteins (KChIPs); and (ii) small transmembrane proteins (KCNE). In this work, we are going to focus on KCNE.

Members of the KCNE family of K^+ channel subunits are short single-transmembrane glycoproteins (103-170 amino acids) that exhibit regulatory functions on the kinetics and cellular distribution of the α-subunits of Kv upon co-assembly. This family comprises five members (KCNE1–5), and their effect on KCh activity has been widely studied.

KCNEs show a wide repertoire of possible associations, being able to interact with different Kv α-subunits, and also to do it at the same time than other KCNE subtypes, with different consequences for each combination. Focusing on the main protein of this dissertation, Kv7.1 can interact with all the five members of the family, with different biophysical changes. For instance, Kv7.1 can co-assemble with KCNE1, that will increase the current to generate the IKs currents, or with KCNE2 and KCNE3, which will promote a loss of voltage dependence and, thus, a constitutively open channel. Contrary, KCNE4 and KCNE5 are inhibitory subunits of Kv7.1. Moreover, KCNEs can modulate the channel association with other interacting molecules, such as PIP_2 or AKAP9 [44,45].

KCNE regulation of Kv traffic is complex. Sometimes, KCNE regulates the channel, whereas, in other situations, the α-subunit drives the KCNE location. For instance, KCNE members associate with Kv7.1. Although KCNEs have limited cell surface expression and lipid raft microdomain targeting, Kv7.1 improves KCNE1 cell surface expression. In addition, upon association with Kv7.1, KCNE1 and KCNE2 relocate partially into specific membrane microdomains named lipid rafts. In contrast, KCNE4 and KCNE5 target Kv7.1 out of the rafts [46], and KCNE4 association impairs Kv1.3 membrane expression and caveolar lipid raft localization while retaining the channel in the ER, which fine-tunes channel surface abundance [47].

Kv7.1 and KCNE1

Kv7.1 association with KCNE1 has been the most deeply studied, and a high number of controversies arise around them, regarding their biogenesis, stoichiometry or subcellular localization. Kv7.1 alone generates a rapidly activating and inactivating current. Upon KCNE1 assembly, biophysical properties of the channel are profoundly modified: activation and deactivation kinetics slow down, activation shifts to more depolarized potentials, removes inactivation or increases unitary conductance, PIP_2 sensitivity and response to β-adrenergic stimuli. This way, the association generates the slow repolarizing IKs cardiac currents [48,49,50,51]. The physiological relevance of this complex is discussed below (see chapter 1.6).

Several regions of the two proteins are involved in their interaction. On one side, KCNE1 interacts with Kv7.1 carboxyl-terminal and some transmembrane regions (S1, S4 and S5-S6 linker). On the other side, the transmembrane segments and COOH-terminus of the regulatory subunit are the ones described to modulate Kv7.1 [52]. Together, these data suggests that KCNE1 is probably inserted in the plasma membrane between two adjacent voltage-sensor domains [53].

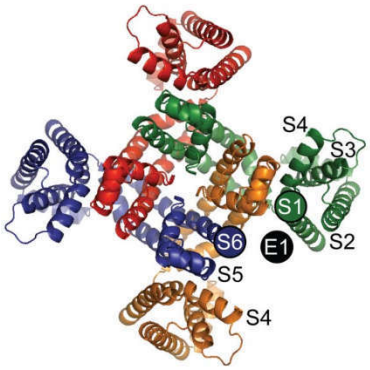


Figure 6. Placement of KCNE1 within the I_{Ks} channel complex. Extracellular view of a tetramer of Kv7.1 from Smith *et al* [54] with KCNE1 super imposed. Each subunit of the tetramer is highlighted in a different color. The extracellular ends of the S1 (green) and S6 (blue) helices of two different subunits are indicated by filled circles. The proposed location of KCNE1 is shown as a black circle. From Chung *et al*. (2009) [53].

Regarding stoichiometry, the number of KCNE1 subunits that associate with the Kv7.1 tetramers remains unclear. Accepting KCNE1 to lay between S1 and S6 transmembrane domains of different subunit, 4 regulatory subunits are able to associate within the tetrameric Kv7.1 structure. In this scenario, there is a debate between two possibilities: a fixed Kv7.1-KCNE1 stoichiometry of 4:2 [55,56] or multiple stoichiometries from 1 to 4 KCNE1 subunits, where the resulting current is functionally different depending on the Kv7.1:KCNE1 ratio. This would represent a new mechanisms for the electrophysiological properties based on KCNE1 expression levels [57,58]. However, contradictory results keep being obtained in different groups and there is no clear consensus yet on this issue [59]. Moroever, other KCNE subunits, such as KCNE2 or KCNE3, have been described to associate not only with the channel, but also with the Kv7.1-KCNE1, complicating even more the stoichiometry deciphering [60,58].

When it comes to biogenesis and assembly, we find similar controversy. Two main hypothesis are on the table. The first one, both subunits traffic separately to the cell surface, where they complexes are formed. The lone KCNE1 expression at the plasma membrane [61,62], the ability of KCNE1 to modify pre-existing Kv7.1 channels in the membrane [48] or the transient reversible interaction in a “kiss and go” manner [63] published by some groups are some of the arguments that support this idea. The second hypothesis supports that the interaction takes place early in the secretory pathway. KCNE1 is retained in the ER compartment until they co-assemble with Kv7.1 subunits. This association allows the complex progression through the secretory pathway until reaching the plasma membrane together [64,65]. Some of the results to reinforce this mechanism are the inhibition of the IKs currents using BFA, which inhibits forward traffic [66] or the presence of 3 arginine residues in the NH2-terminus of KCNE1 which are crucial for plasma membrane localization of the IKs complex, implying the complex assembly during the secretory pathway [67]. To better understand the complexity the Kv7.1-KCNE1

traffic pathway, a general vision on secretory routes might be useful. Therefore, an extensive chapter on ion channel traffic is included below.

1.5. Ion channel traffic

KChs cell functionality relies in their subcellular location and abundance. A wide repertoire of signatures regulate the proper targeting of the channel. These motifs can be part of the amino acidic sequence of the channel as well as result of the tertiary structure (Figure 2). Moreover, several ancillary subunits and other interacting partners can associate with the channel to form functional complexes and regulate both their activity and their traffic. Auxiliary subunits can expose or mask the channel inner signatures, bend the structure of the channel or they can add their own motifs to the signalling balance (Figure 7). Post-translational modifications have been also described to directly regulate traffic. Channel abundance at the membrane is important for cell physiology, thus, not only forward traffic has to be considered, but turnover is crucial. All these evidence suggest that the final destination of the channel is not a one-signal phenomena, but depends on the on the equilibrium of several different inputs that will condition the location of a channel for a specific role [6].

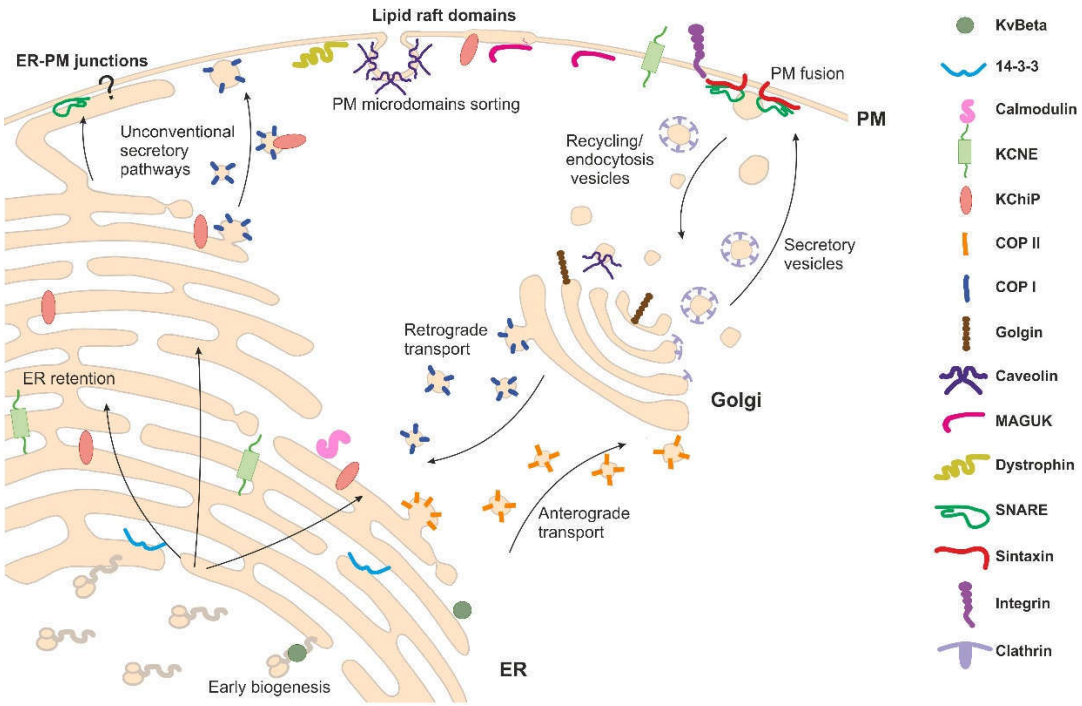


Figure 7. Schematic representation of traffic mechanisms and molecular associations of K+ channels through the secretory pathway. Different shapes represent molecules (see list on the right) known to associate with channels in different compartments. Channels are not depicted. Arrows represent major directions of either retrograde or anterograde routes. Newly synthetized peptides, bearing endoplasmic reticulum (ER) signatures, translocate to the rough ER where the association with Kvβ regulatory subunits takes place in early steps of the biogenesis. Different interactions and motifs balance between forward traffic or ER retention. Regulatory subunits, such as KCNEs, and accessory molecules, such K^+ channel-interacting protein (KChiP), 14-3-3, or calmodulin, interact with channels at this step. Conventional mechanisms dictate that regardless of whether anterograde traffic is promoted, cargo channels are processed along the COPII machinery driving to Golgi. Once in Golgi, channels either forward traffic to the cell surface or undergo retrograde transport to ER via the COPI pathway. Molecules, such as Golgin, caveolin, or clathrin facilitate channel routes to the surface. Accessory

molecules, such as MAGUK, dystrophin, syntaxin, integrins, or caveolins stabilize channels at the final destination (e.g., lipid raft microdomains and caveolae represented by a thicker part of the membrane and an invaginated omega-shaped structure, respectively). Turnover mechanisms, such as internalization, are mostly mediated by clathrin-coated pit-dependent endocytosis. Alternatively, unconventional pathways are also facilitated by auxiliary subunits assembling the channels. Thus, export from ER via COPI vesicles requires KChiP interaction. Direct contacts with the plasma membrane (PM) and further stabilization with SNARE at the ER–PM junctions are documented. However, most proteins implicated in this unconventional traffic remain unknown. It is important to highlight that mechanisms could differ depending on the KCh and the interacting protein. From Capera *et al*. (2019) [6].

1.5.1. Conventional secretory pathway

The conventional secretory route in eukaryotic cells is based on vesicular traffic to transport cargoes selectively through membranous compartments. A series of directed membrane translocation, budding or fusion take place between the compartments involved: endoplasmic reticulum (ER), Golgi apparatus (Golgi), trans-Golgi network (TGN), intermediate vesicular compartments and endosomes that will be fused with the plasma membrane or other membranous organelles such as lysosomes (**Figure 8**).

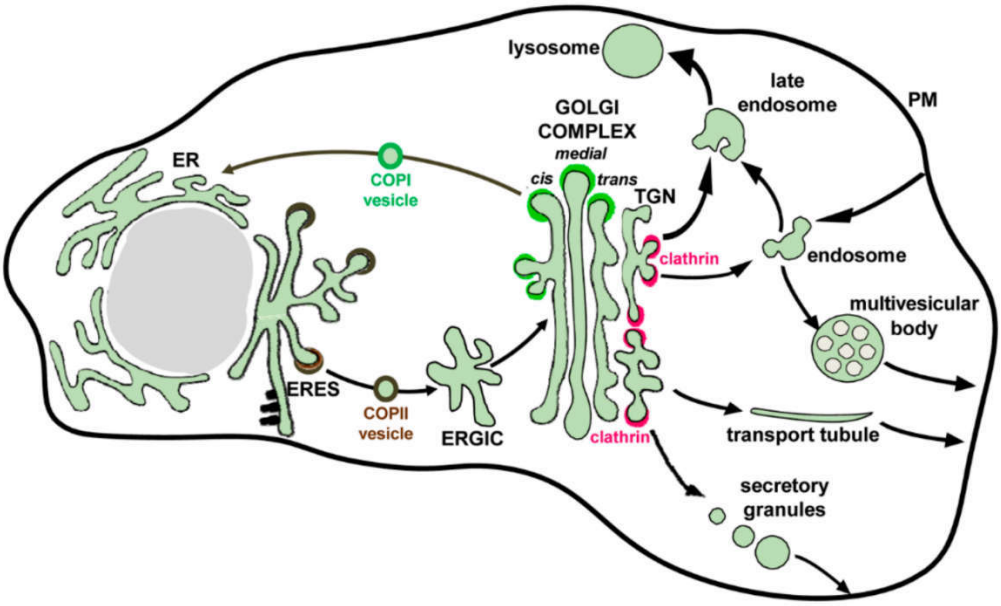


Figure 8. The conventional secretory pathway. Secretory cargoes or membrane proteins are synthesized in the ribosome-studded ER (Endoplasmic Reticulum), exit the ER at ERES in COPII-coated (brown) vesicles and are transported to the ER-Golgi compartment (ERGIC). There are then sorted into anterograde carriers that move them to the Golgi complex. After passage through the Golgi in the cis-to-trans direction, cargos are packaged at the TGN for delivery to the PM, early and late endosomes and in some cells to secretory granules. Sorting into endosomal compartments and secretory granules is mediated by clathrin-coated (red) vesicles. A COPI-mediated (green) recycling pathway retrieves escaped proteins from the ERGIC and the Golgi and returns them to the ER. From Sager *et al.* (2018) [68].

Endoplasmic reticulum

Nascent proteins contain a signal sequence targeting the ER. Co-translationally, they enter the ER lumen while integration of the transmembrane domains defines the membrane topology of the channel. The second transmembrane domain (TM2) has been described to function as a signal sequence in ion channels such as Kv1.3. The inside of the ER is equivalent to the extracellular space, and the transmembrane domains and flanking regions will determine the orientation of consecutive

transmembrane segments in opposite directions [69]. Oligomerization of subunits, folding and pore and sensor domains formation take place early in the biogenesis, but differently between KCh families [70]. Dimerization of dimers is the accepted mechanism for tetramerization. Kv1 channels tetramerize by their T1 domain at their N-terminus, which is properly fold very early in the biogenesis. Kv7 family, instead, does not possess a T1 tetramerization domain, but tetramerize between tandem coiled-coil domains (TCC) located at the C-terminus tail of the protein. Thus, it is unlikely that the assembly of the complex starts during protein synthesis. Most probably, tetramerization of Kv7 channels takes place once they are embedded in the ER membrane [71].

Once the ion channel is assembled, proper folding allows them to overcome the first quality control and depart from the ER if they are supposed to. ER exit sites (ERES) are specific ER domains characterized by the absence of ribosomes and the concentration of cytosolic COPII coat members that will promote the formation of COPII vesicles. KChs are packed into these COPII coated vesicles and transported via microtubules to the ER-Golgi intermediate compartment (ERGIC). COPII coat vesicles are composed by 5 members: Sar1, Sec23, Sec24, Sec13 and Sec31. First inserted in the ER membrane thanks to the GTPase Sar1, it associates with cargoes that need to be trafficked to Golgi. This direct interaction with ion channels occurs mainly via Sec24, both directly or indirectly. Budding of all the members of the complex and membrane curvature is required before the Sar1's GFP hydrolysis takes place and the vesicle is released from the ER [72,73].

Contrary, some proteins reside in the ER instead of following the secretory pathway. Retrograde traffic vesicles formed by the COPI coat complex maintain ER resident enzymes and chaperones there and will be in charge of returning ER retention-tagged proteins again to the reticulum when they escape [74]. COPI vesicles are composed by coatomers, which are heptameric complexes composed by two subcomplexes: a trimeric complex forming the outer layer (α, β' and ε) and a tetrameric complex forming the inner core (δ, ζ and β). Their recruitment and uncoating is activated by ARF1 GTPase (ADP Ribosylation Factor 1), a different GTPase than the one for COPII but with a similar mechanism, and it is inactivated by Brefeldin A (BFA). BFA is a fungal toxin that inhibits the ARF1's GFP hydrolysis and impairs COPI vesicle retrograde transport. Golgi collapses after few minutes of treatment with BFA [75]. This dynamic cycling process is highly conserved in yeast and eukaryotic cells and regulates cargoes to reach the Golgi and follow the anterograde direction or to remain in the early secretory compartments.

Acidic motifs, as well as hydrophobic or aromatic motifs are essential for forward trafficking (for example DxE Kir6.2 channels [76], YMVIEE for Kv1.3 [77] or HRETE for Kv1 [78]. When misfolded, lacking these ER to Golgi forward traffic sequences or when expressing ER retention signals, channels can be retained in this compartment. It is the case of Kv1.1 or Kv7.3: basic domains such as di-lysines or arginine based signals are responsible for an inefficient forward trafficking [79,80,74]. Many ancillary subunits may regulate ER exiting. KCNE4 retains Kv1.3 in the ER by masking the above-mentioned forward trafficking motif YMVIEE and by exposing its own retention motive that further limits the surface expression of the complex [81] and KChIP2 promotes ER retention when coexpressed with Kv1.5 [82]. By contrast, Kv4.2 improves the cell surface expression dramatically when coexpressed with KChIPs1-3 [83]. Some Kv7 channels require an accessory subunit, calmodulin (CaM), to properly fold and exit the ER in a calcium dependent manner [84,85,86]. Also 14-3-3 associates with many ion channels such as Kv11.1 or $K_{2P}5.1$ to ensure their proper folding before exiting the ER [6]. This regulatory molecule acts as a scaffolding protein and is important for stabilizing the complex and promoting its surface expression. 14-3-3 has higher affinity for oligomerized complexes rather than monomers, becoming a quality checkpoint for unassembled channels. Moreover, monomer subunits may expose ER retention signals that will become a second quality control to keep them trapped in the ER [87,88].

Golgi Apparatus

Before entering the Golgi, vesicles cross a vesiculo-tubular "compartment" (VTC) adjacent to ERES, also known as the ER-Golgi Intermediate Compartment (ERGIC), with a biochemical composition different from both the ER and the Golgi. Whether it is a stable compartment or only a transient structure that appears when ER vesicles maturate and fuse with each other to form the *cis*-Golgi cisterna is still an open debate [89,90]. ERGIC works as a sorting site for two fate decisions: anterograde or retrograde traffic and Golgi-dependent or independent forward traffic. It is directly communicated with endosomes and microtubules, as it is located close to the centrosome: minus-end-directed toward the Golgi [91] and plus-end-directed toward the plasma membrane and backward to the ER [92,93] (**Figure 9**). This intermediate pre-Golgi compartment presents two subdomains: peripheral intermediate compartment and pericentrosomal intermediate compartment (pcIC). The last one is described to be involved in the communication with endosome compartments to secrete proteins bypassing Golgi and also to take part in the unfolded protein control by helping to the ER-associated degradation (ERAD) [92].

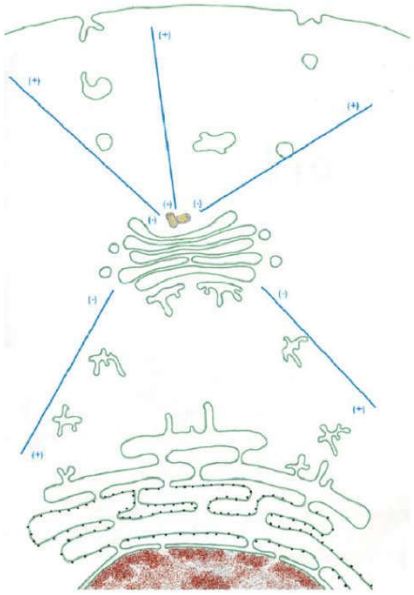


Figure 9. Relationship of the secretory pathway to the microtubule cytoskeleton. Golgi apparatus is located near the centrioles and surrounded by minus-ends of microtubules. This orientation directs the transport from and to the Golgi. COPII vesicles exit the ER and reach the Golgi following minus-end-directed microtubules (e.g.: dynein). The ERGIC is located in between, next to the Golgi and the minus ends of microtubules, sorting the anterograde or retrograde trafficking. Plus end-directed microtubules (e.g.: kinesin) connect Golgi to the plasma membrane or back to the ER. From Murshid *et al*. (2004) [93].

Once the conventional secretory pathway has been determined at the ERGIC, cargoes reach the Golgi apparatus. Golgi consists of stacked cisternae with a ribosome-free surrounding environment that can be divided in three sub-compartments: *cis*, medial and *trans*, in order of forward move and maturation [94]. Recycling between compartments using COPI-mediated traffic is used to maintain the normal structure and composition of the Golgi complex [75].

Proteins are here exposed to another quality control as glycosylation maturation occurs in the Golgi. Sugar chain processing must occur in coordination with protein trafficking and sorting. Other modifications such as sulfation and phosphorylation also take place in Golgi. All these maturations, being glycosylation the most common, impact protein folding, stability, interactions, activity or traffic. Glycosylation defects have been linked to numerous human diseases like diabetes, cancer or cystic fibrosis, revealing the importance of an exquisite regulation of these processes in Golgi. Stacking of Golgi cisternae is described to be the main feature of the maturation quality control. When Golgi stack formation is disrupted, protein traffic is accelerated. Contrary to what was though, the close spatial arrangement of cisternae and the local tethering proteins that were supposed to facilitate vesicle fusion do no increase the efficiency of protein trafficking. Instead, GRASP (Golgi ReAssembly

Stacking Protein) and golgins work together to maintain the structure and function of the compartment, and limit vesicle budding and fusion to the rims of the cisternae. This delays the traffic process, which allows the protein to remain sufficient time in each compartment to ensure an accurate maturation process [95].

Trans-Golgi network

When in the further cisternae of the Golgi, newly synthesized proteins are sorted to their final destination: plasma membrane, endosomal compartments, lysosomes or other membranous compartments. TGN specific microdomains are assembled together with the sorting machinery coming from the cytosol, to encapsulate the cargo proteins. Cargo adaptors are required to form the vesicles, and adaptor protein complexes (AP) are the best characterized at the TGN. They are composed by four subunits (β, γ, μ and σ) that will bind ARF proteins, cargo molecules, phospholipids and clathrin, the main component of the vesicles. Other classes of clathrin-associated cargo adaptors are Epsin and Golgi-localized γ-ear-containing ARF-binding proteins (GGAs). Cargo adaptors recognize tyrosine-based (Yxxϕ, ϕ is an hydrophobic residue) and di-leucine ([DE]xxxL[LI]) sorting sequences in the cytoplasmic domains of transmembrane cargo proteins, and also motifs that appear when the tertiary structure of the proteins is folded, representing an additional folding quality control [96,97].

Furthermore, TGN vesicles should contain not only the cargo molecules and adaptors, but also specific SNARE to their destination. SNARE proteins are specialized membrane fusion proteins that allow the passage of the cargo from one compartment to another. Two components are required: v-SNARE, carried by the vesicle (e.g.: VAMPs (vesicle-associated membrane proteins) or sinaptobrevines), and t-SNARE, located in the target membrane (e.g.: syntaxins or SNAPs (synaptosome-associated proteins). When they associate, both membranes face tightly together triggering membrane fusion. Very few associations between SNARE proteins and ion channels has been described. Kv2.1 has been related to VAMP-2 during synaptic vesicle docking and fusion, and Syntaxin-1A seems important for membrane insertion of Kv1.1, Kv2.1 or Kv4.3 in brain [98].

As we have said, clathrin vesicles can target directly to the plasma membrane, but also to endosomal compartments. These structures can be the destination of the proteins, but they can as well be part of the traffic pathway before their surface delivery, revealing a second role for the endosome system a part from endocytosis. Their traffic role is especially important in polarized cells, which possess specific endosomal compartments for the trafficking to apical or to the basolateral membranes of the epithelial cell [99,100].

1.5.2. Unconventional secretory pathway

There are proteins lacking those peptide-bearing signals or having still unknown targeting tags that cannot be recognised by the ER membrane translocation machinery. This way, they by-pass Golgi and other conventional compartments to reach the cell surface using other mechanisms. A large and growing number of proteins, both transmembrane and soluble, can undergo unconventional secretion, reaching the plasma membrane or exiting the cell to the extracellular media by unusual trafficking systems. This systems can be vesicle-dependent or independent, being the first one the one used by ion channels [101].

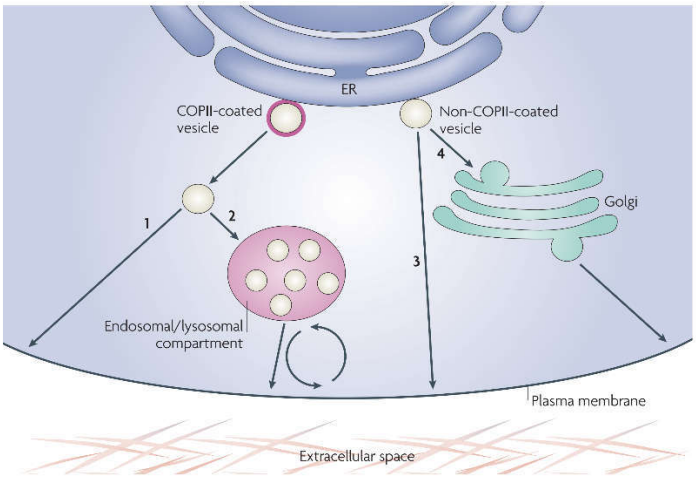


Figure 10. Unconventional traffic mechanisms. Different possible non-conventional secretory pathways for proteins that target the plasma membrane. 1: Following endoplasmic reticulum (ER) translocation, these proteins are packaged into COPII-coated vesicles that fuse directly with the plasma membrane. 2. Alternatively, they can fuse with an endosomal or lysosomal compartments that will fuse with the plasma membrane. 3. Proteins can also be packaged into non-COPII-coated vesicles than can fuse directly with the plasma membrane. In the case of transmembrane proteins, they can be targeted to the Golgi apparatus in a COPII-independent manner before reaching the plasma membrane.

Focusing on transmembrane proteins and following the conventional pathway, there are several steps at which proteins can deviate from this classical secretion. Avoiding COPII-coated vesicles is one of the earliest examples, and SNARE proteins are usually involved in this process. For example, Kv4.2, only upon association with its regulatory subunit KChIP1, exits the ER using COPI vesicles instead [102,103]. In this scenario, proteins can still reach the Golgi by this unconventional system, or they can follow a particular and "quicker" route reaching directly the cell surface. Metabolic enzymes or heat shock proteins, such as EDEM1 or Hsp150, are also related with COPII-independent pathways [101].

Another possibility for the unconventional protein secretion, and of special interest in this dissertation, is the Golgi bypass. Several vesicular or membranous mechanisms have been described for this group. Transmembrane proteins can use COPII-coated vesicles to reach organelles different from the Golgi, such as lysosomes or late endosomes that will then fuse directly with the PM or they can fuse directly with the membrane without any intermediates. COPII-independent vesicles can as well be used to avoid Golgi. Proteins such as SNARE and GRASP have been implicated with these processes. However, not much is known with regarding the machinery that mediates a Golgi bypass [104,105].

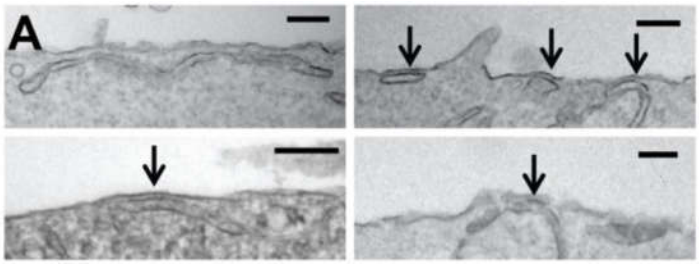


Figure 11. Electron micrograph of ER-PM junctions (Endoplasmic reticulum – plasma membrane junctions) in HEK293 cells indicated by black arrows. Scale bars: 200 nm. From Fox *et al.* (2015) [106].

A particular Golgi bypass mechanisms are the ER-PM junctions. These are contact sites between the ER and the plasma membrane (**Figure 11**), which structure the membrane components, participate in the lipid transference, and help in the localization of some proteins in the cell surface. To maintain both structures close enough for the different processes and cell signalling that take place, several protein tethers have been described to participate in their connection, including junctophilins, STIM or syntaxins, among others [107,108] (**Figure 12**). Also ion channels have been related. Kv2.1 targets and stabilizes these domains. In fact, Kv2.1 provides platforms for delivery and retrieval of multiple membrane proteins [106,109]. Unknown combinations of SNARE proteins and/or specific molecules, yet to be identified, would drive KChs to the membrane unconventionally using these contact sites [6].

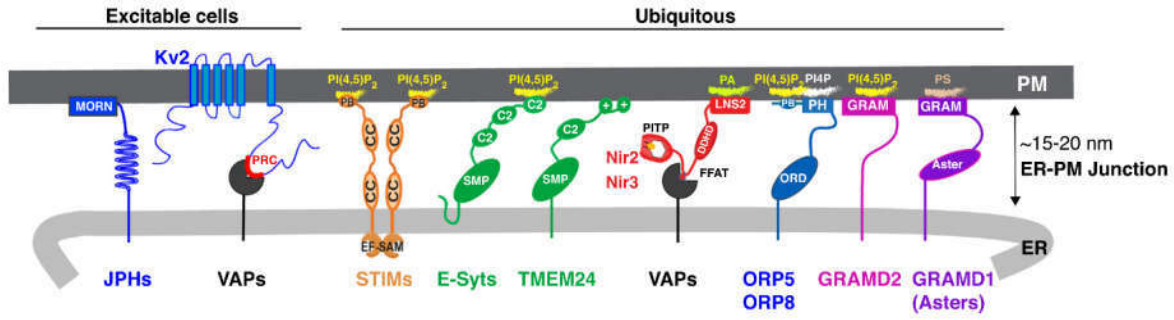


Figure 12. Proteins enriched at mammalian ER–PM junctions. JPH, Junctophilin; MORN, membrane occupation and recognition nexus motif; PRC, proximity restriction and clustering domain; EF-SAM, EF hand and sterile alpha motif; CC, coiled-coil domain; PB, polybasic domain; SMP, synaptotagmin-like mitochondrial-lipid binding protein domain; PITP, phosphatidylinositol transfer protein domain; FFAT, two phenylalanines (FF) in an acidic tract motif; DDHD, domain characterized by these conserved residues; LNS2, Lipin/Ned1/Smp2 domain; ORD, OSBP-related domain; PH, pleckstrin homology domain; GRAM, glucosyltransferases, Rab-like GTPase activators and myotubularins domain; ASTER, START (StAR-related lipid-transfer)-like domain. From Chen *et al.* (2019) [107].

While most of the proteins are unconventionally secreted by mammalian cells constitutively, some are activated after stress conditions. In this scenario, COPII vesicles are driven to the formation of a new and stress-dependent organelle: the phagophore. Under critical conditions, in order to recycle essential amino acids and other elements, the cell will use autophagy. While COPII-coated vesicles are now redirected to this membranous compartment, proteins must bypass Golgi as now the COPII machinery is not available [110]. Moreover, some of them are associated with pathologies. Mutated or affected proteins, whose associated trafficking defects to the cell surface cause inherited genetic disorders, can alternatively reach the plasma membrane unconventionally. This is the case of CFTR, probably the most documented cargo bypassing Golgi. When the trafficking mutant responsible for cystic fibrosis (ΔF508-CFTR) interacts with GRASP proteins, the association rescues the surface targeting. In other cases, the inhibition of the unconventional traffic is the cause of the disease. Immature Mpl or the M2 mutant of Smoothened, for example, are related to cancer in (**Figure 13**). Therefore, understanding these mechanisms is important not only for understanding intracellular trafficking, but because it has severe implications in health and disease [104].

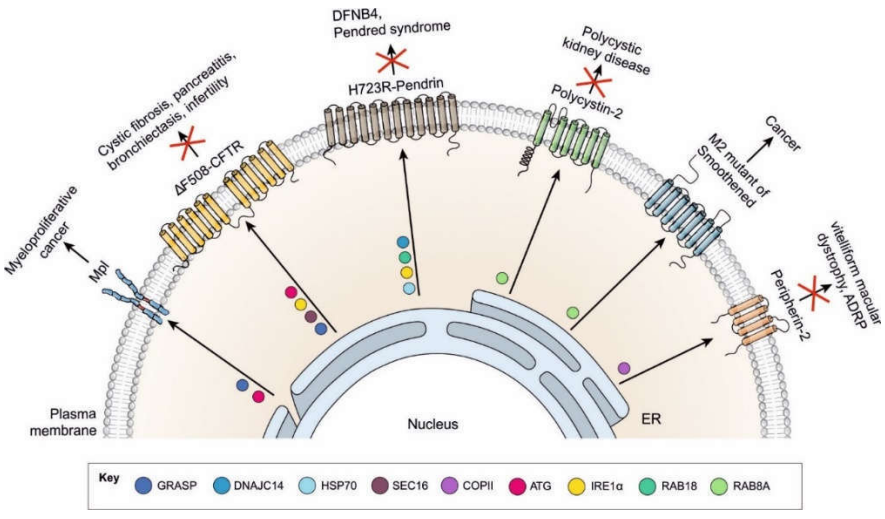


Figure 13. Molecular arrangements involved in unconventional protein secretion of transmembrane proteins bypassing Golgi. Known examples of altered trafficking processes in transmembrane proteins resulting in the avoidance of Golgi while reaching the plasma membrane. Mutated or affected proteins, whose associated trafficking defects to the cell surface cause inherited genetic disorders, can alternatively reach the plasma membrane unconventionally. In other

cases, the inhibition of the unconventional traffic is the cause of the disease. Different mechanisms of avoiding Golgi are here shown. Immature Mpl involved in myeloproliferative cancer reaches the plasma membrane by a GRASP55- and ATG5-mediated pathway. ΔF508-CFTR, which causes cystic fibrosis because of a trafficking defect, can be transported to the plasma membrane by a pathway involving GRASP, ATGs, SEC16A and IRE1α. The substitution mutant H723RPendrin, which causes congenital hearing loss, can reach the plasma membrane by a route that is mediated by HSP70, DNAJC14, RAB18 and IRE1α. Polycystin-2, whose mutations cause autosomal-dominant polycystic kidney disease, is transported by RAB8A-mediated secretion. The M2 mutant of Smoothened, which can induce cancer, is transported to the plasma membrane by RAB8A-mediated secretion. Peripherin 2, whose mutations cause ophthalmic diseases, can be transported to the plasma membrane by COPII-mediated secretion. Modified from Kim *et al*. (2018) [104].

1.5.3. Other traffic modulations

Not only sequences and motifs (linear or embedded in the secondary or tertiary structures) can regulate ion channel traffic. Other modulations can add a further dimension to the traffic regulation, so that the fate of a channel does not depend on the gene sequence but on the balance of many other factors.

Post-translational modifications

Channels can also undergo post-translational modifications (PMT) with direct effects on their traffic. N-Glycosylation is the first and one of the main PTMs that some Kv channels suffer. It promotes the surface expression of many KChs probably by reducing protein dynamics and thus, increasing stability. The process starts in the ER lumen, where a preassembled polymannose oligosaccharide is co-translationally transferred to the asparagine residue of a Nx[STC] consensus sequence (x can be any amino acid except P). Once in Golgi, sequential enzymatic reactions will maturate the oligosaccharide by trimming and elongating the glycan tree [111] (**Figure 14**). Also O-glycosylation can occur when an acetylglucoseamine (GlcNAc) is added to a serine or a threonine residue without a consensus amino acid sequence. It can be started at the ER or the cytoplasm, but it is mainly a Golgi modification. S1-S2 loop N-glycosylation favour surface expression of some Kv1 and K_2P channels. Kv7.1, similarly to Kv10.1, Kv11.1 or Kv12.1, contains asparagine suitable for glycosylation in the S5-P loop [112,6]. Regulatory subunits biogenic process is also driven by glycosylation. It is the case of KCNE1, bearing up to two N-glycan trees and also O-glycosylations [113].

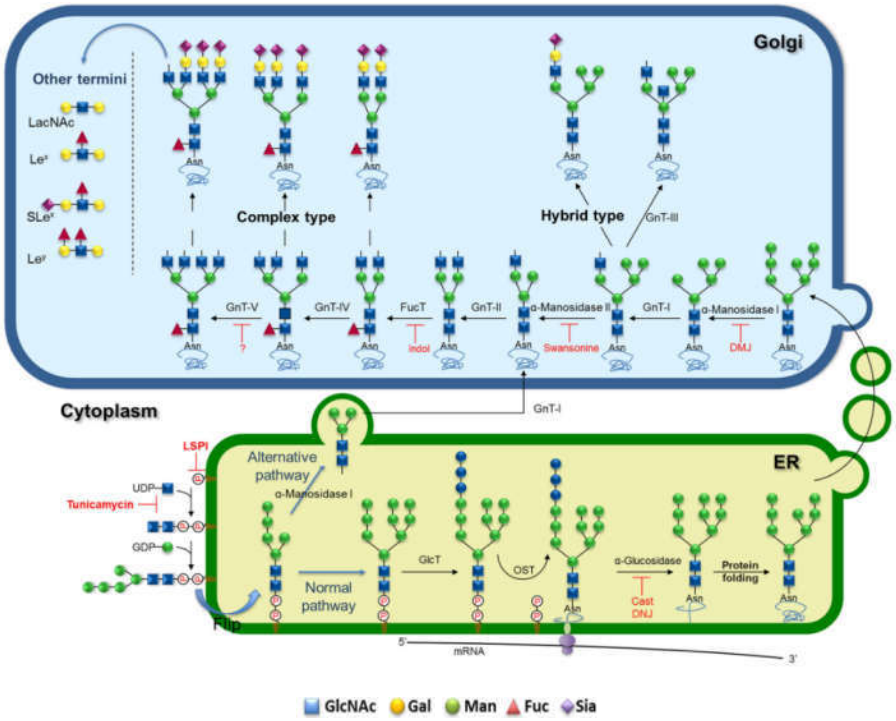


Figure 14. Schematic representation of biosynthesis and processing of N-linked oligosaccharides. Enzymes that mediate glycan processing in the Golgi apparatus are segregated into distinct cisternae to ensure that glycosylation occurs in a step-wise fashion. Glycosylation process initiates at the lumen of the ER, with the addition of a polymannose oligosaccharide. After initial trimming in the ER, the glycoprotein is trafficked to the Golgi, where Golgi mannosidase I removes multiple mannose sugars. Glycans that do not undergo further glycosylation are called high-mannose oligosaccharides. Further sugar addition and removal yields a common core oligosaccharide onto which multiple glycosyltransferases add different sugars to generate the highly variable complex oligosaccharides. Glycans can be high-mannose, complex or a combination of both (i.e., hybrid oligosaccharide). The scheme also shows known inhibitors and key targets for inhibition: Inhibitors of the lipid-linked saccharide pathway (LSPi), tunicamycin, castanopermine (Cast), 1-deoxynojirimycin (DNJ), 1-deoxymannojirimycin (DMJ), swansonine, and indolizidine (Indol). From Vasconcelos-dos-Santos *et al.* (2015) [114].

As we have said before, glycosylation involves a quality control mechanism. In the ER, if glycosylation is impaired many proteins undergo misfolding and fail to reach their native conformation, driving them to degradation at the ERAD: proteins will be ubiquitin-dependent degraded by the proteasome [115]. In the case of Kv channels, N-glycosylation has folding promoting effects by stabilizing structures and binding the new structures to ER lectins such as calnexin or calreticulin that will act as chaperons to promote the channel proper folding that will pass now the quality control. Association with calnexin regulates Kv1 channel family traffic [116] and explains the ER retention of KCNE1 subunit [64]. Also non-lectin chaperons are important, such as Hsp70, which associates with Kv7.1 and Kv11.1 [117]. Glycan trees guide the spatial distribution of the channels and subunits not only until the membrane, but as well once at there, regulating cell adhesion, migration or cell-cell interactions [118].

Phosphorylation is another PTM to consider. It consists in the transfer of a γ-phosphate group of ATP to the hydroxyl group on the side chains of serine, threonine or tyrosine residues. It is a reversible and dynamic modification and usually causes significant changes in the protein conformation. This can induce new protein-protein interaction and regulate ion channel trafficking and turnover. Serine phosphorylation in the N-terminus of Kir1.1 promotes export of the channel from the ER, avoiding its intrinsic ER-retention signals [119]. Contrary, Kv1.3 phosphorylation in its C-terminal mediates EGF-dependent endocytosis of the channel [120]. In the case of Kv2.1, phosphorylation at a C-terminal serine enhances the cell surface expression of the channel, whereas a tyrosine in the N-terminus promotes rapid internalization when phosphorylated. Moreover, this channel has a large serine/threonine rich domain that, when dephosphorylated, modifies the classic cluster pattern of Kv2.1 in neurons [121,122,123].

Ubiquitination is the covalent attachment of an ubiquitin to a lysine residue. This lysines can be itself ubiquitinated at any of their own lysines, which increases the possibilities and complexity of the regulatory potential of this PTM. Monoubiquitination is mostly related to endocytosis and lysosomal degradation. Polyubiquitination, instead, triggers proteasomal degradation. Nedd4-2 is an ubiquitin ligase that targets potassium channels, such as Kv7.1 or Kv1.3 [124,125].

Other PTMs, such as redox modifications or lipidations have also been related to KChs. Palmitoylation and other acylations increase protein hydrophobicity and may regulate the affinity of membrane proteins for specialized membrane microdomains. Palmitoylation of a single cysteine residue at the C-terminus of Kv1.5 decreases its surface expression [126], but enhances K_{Ca}1.1 membrane targeting [127]. Palmitoylation of the PSD-95 (postsynaptic density protein 95) increases Kv1.4 targeting to lipid raft membrane microdomains [128]. The formation of reactive oxygen and nitrogen species (ROS/RNS) can induce the post-translational oxidative motification of proteins, usually affecting the highly reactive thiol group of cysteine residues. Redox mechanisms affecting KCh trafficking remain highly unexplored but appear as promising features in the understanding of the progression of many

pathologies such as cancer or cardiovascular diseases (Redox regulation of ion channels – Bogeski – 2014).

Heterotetramerization

Some KCh channels have the ability to associate with other channels, mostly with members of the same family, causing a potential rerouting. Kv1.3 associates with Kv1.1, Kv1.2 and Kv1.4 in the brain, and Kv1.3-Kv1.5 hybrids are found in macrophages. All of them regulate their cell localization depending on the stoichiometry within the oligomers [129,130]. Kv7 family can form oligomers between different members of the group. Kv7.3 is only functional when heterotetramerizing with Kv7.2. Together, they can escape the ER and reach the cell surface, where the complex generates the M-current, which controls neuronal excitability [80]. Kv7.1, who was thought to be the only member of the family unable to combine with the other channels, was described recently to associate with Kv7.5 [23]. Furthermore, complex hetero-oligomerizacion can also happen between diferent ion channels. It is the case of Kir2.1 and Nav1.5, which form macromolecular complexes crucial for cardiac excitability and need to assemble early during their forward traffic to be able to reach the membrane [131].

Subcellular/organelle targeting

Stabilization at the PM

Once the channels are inserted into the plasma membrane, sophisticated machinery exists to retain them there. A major mechanism involves Kch anchoring to the cytoskeleton, as it happens with Kv7 channels in the axonal initial segment by being anchored through the abovementioned Ankyrin-G binding domain [132]. Other mechanisms to avoid diffusion into the lipid bilayer are ion channel agglomeration in larger complexes or anchoring to scaffolding proteins. PDZ-domains are present in the C-terminus of several Kv channels to bind a number of PDZ-domain scaffolding proteins such as PSD-95 or SAP97.

Lipid raft microdomains

Spatially, KChs can be located in specific membrane microdomains, such as lipid rafts. Raft domains are membrane fractions enriched in sphingolipids and cholesterol that serve as scaffolding regions where signal transduction pathways interface. Proteins reach these domains via different mechanisms, such as lateral diffusion, the formation of lipid “shells” surrounding the channel, which increase the affinity for rafts, protein-lipid interactions, such as palmitoylation, or protein-protein interactions [133]. In this last scenario, caveolin acts as a scaffolding protein driving ion channels to raft domains. For instance, an N-terminal motif for the caveolin-1 interaction (FQRQVWLL) has been described in Kv1.3. Caveolins, by impairing lateral diffusion, increase the time that KChs are located in these platforms [134]. In addition, KChIP proteins also govern Kv4.3 targeting to lipid raft domains [135]. Finally, PSD-95 is partially responsible for the lipid raft targeting of Kv1.4 and protects Kv1.3 against phorbol 12-myristate 13-acetate (PMA)-induced ubiquitination and endocytosis [125,128]. Kv2.1, Kv1.5, or Kir3.1 are also found in these domains [133], but no direct interaction with scaffolding or auxiliary proteins has been reported.

Polarized cells

Polarized distribution of proteins is sorted in TGN, in which apical and basolateral proteins will exit using different carriers. Epithelial cells are the best example of polarized cells. Basolateral epithelial sorting is directed by tyrosine or di-leucine motifs in the primary structure of target proteins [99]. K^+ channels are located in basolateral membranes to stabilize the membrane potential and create the driving force for sodium and chloride transport. Kv7.1, which presents two basolateral-localization signals in its NH_2-terminus, is one of the best characterized K^+ channels in epithelial tissue, and is mainly expressed in basolateral membranes of stomach, colon, kidney or inner ear [136].

Neurons, another example of polarized cells, segregate the cell between the axon and the dendrites. Proteins are addressed to each of them, again, from the TGN, where the sorting of specifically coated vesicles takes place. Kv1.1 or Kv2.1 show particular distributions within the neuron architecture [137,138].

1.6. Cardiovascular Kv7 channels physiology

1.6.1. The heart

Cardiomyocytes

Cardiomyocytes are extremely differentiated cells, responsible for the excitation-contraction coupling (ECC). This is, the transduction from an excitatory electrical signal into a mechanical response. In the heart, the result of this mechanisms is the contraction, and thus, heart beating. To do so, cardiomyocytes present a particular anatomy with one major functional unit of contraction: the sarcomere. It is composed by three cytoskeletal filaments: actin, myosin and titin. This functional unit is limited by flanking Z lines, where sarcolemma (the specialized plasma membrane of cardiomyocytes) invaginates to form the T-tubules. These membrane projections face closely the adjacent sarcoplasmic reticulum (SR, the specialized ER). By surrounding the sarcolemma, it forms short intermembrane clefs known as dyads (equivalent to the ER-PM junctions abovementioned) that will ensure the proper calcium signalling to enable ECC (**Figure 15**).

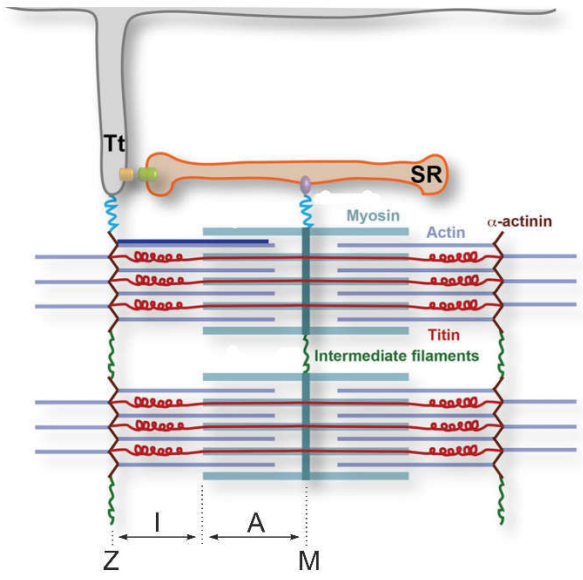


Figure 15. A schematic view of the sarcomeric cytoskeleton. T-tubules (Tt) link the sarcolemma to the sarcoplasmic reticulum (SR) at the dyads. The figure shows myosin (turquoise), actin (light blue), α-actinin Z-links (brown), M-band crosslinks (green), titin (red). Sarcomeres are linked by other extra-sarcomeric cytoskeletal structures at the Z-disk and M-band, comprising desmin, synemin, plectin and nesprin (summarised as green wavy lines). Principal sarcomere regions are marked by Z, I, A and M. Adapted from Gautel and Djinović-Carugo (2016) [139].

In cardiomyocytes, contraction is initiated by depolarization-mediated Ca^{2+} entry via sarcolemmal L-type Ca^{2+} channels (LTCCS, $Ca_v1.2$), which triggers Ca^{2+} release from the SR via the Ryanodine receptors (RyR2). This is only possible thanks to the juxtaposition of LTCCs and the RyR in the dyads. Once the intracellular calcium has increased in what is known as calcium-induced calcium release (CICR), myosin will be able to bind actin and pull the filament toward the centre of the sarcomere, generating muscle contraction. Relaxation will occur when calcium is removed from the cytoplasm. To do so, RyRs close and calcium is pumped back to the SR by SERCA (SR Ca-ATPase) and to the extracellular space by the sodium-calcium exchange (NCX). To ensure the propagation of this signal, T-tubules and dyadic structures are repeated periodically along the cardiomyocyte to ensure the proximity of the actin and myosin and reduce response time (**Figure 16**).

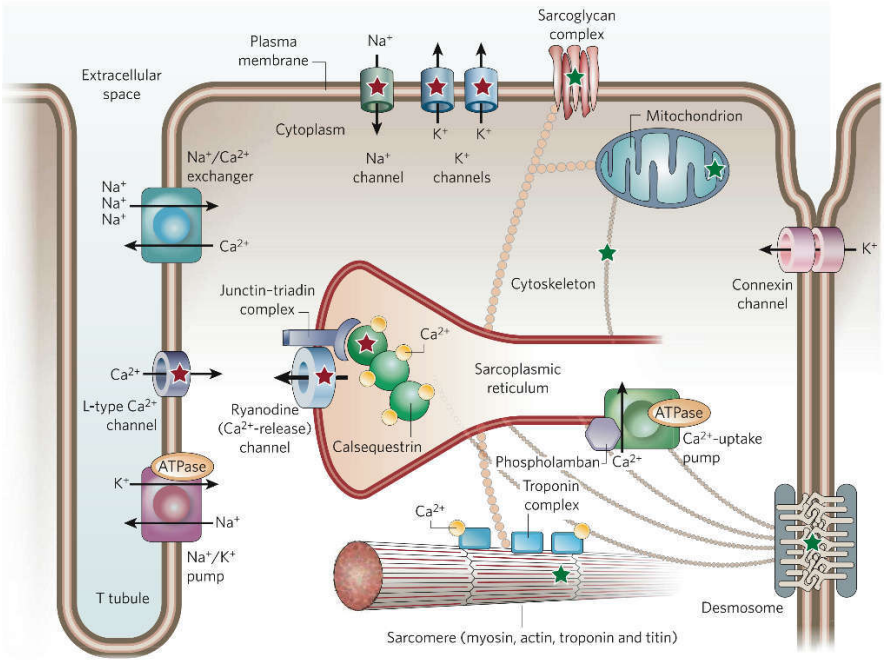


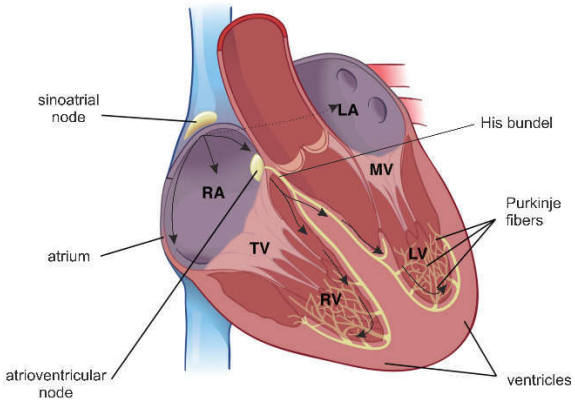
Figure 16. Ventricular cardiomyocytes electric-contraction coupling (ECC). Membrane depolarization arrives from neighbour cells through tight junctions. Na+ and, then, Ca2+ channels open. The increase in intracellular calcium activates the sarcoplasmic reticulum calcium release, which will now bind troponin and activate the contractile apparatus (Sarcomere, at the bottom). Cellular relaxation occurs after removal of Ca2+ from to the cytosol again to the SR (by SERCA) or to the extracellular media (by Na+Ca2+ exchangers). Intracellular Na+ homeostasis is achieved by the Na+/K+ pump. From Knollmann and Roden (2008) [140].

Cardiac action potential

This ionic movements translate into a cardiac action potential. Sodium, calcium and potassium influx and efflux will coordinate in the myocardium, which works as an individual unit of contraction thanks to the electrical coupling of cardiomyocytes via gap junctions. The signal travels through the heart using ion movements to propagate the action potential until reach the ventricle (This mechanism is explained briefly in **Figure 17**).

Figure 17. The cardiac action potential propagation. The initial impulse is started at sinoatrial node, a cluster of excitatory, which spontaneously generates action potentials. Upon hyperpolarization, HCN channels (Hyperpolarization-activated Cyclic Nucleotide-gated channels) will open, the membranes slowly depolarize until the activation threshold potential for Cav1 channels, and the action potential is triggered. Moreover, HCN channels are also activated by cAMP molecules,

therefore receive inputs from the nervous system during exercise. This activation is faster, ensuring heart acceleration in required situations [141]. From the sinoatrial node, the action potential is spread through myocytes to the atria, generating the atrial action potential and, consequently, the contraction of these two chambers. Parallel, the action potential will also reach the atrioventricular node where the propagation is slowed down to ensure that atria are fully contracted previous to ventricle stimulation. From there, the potential will diverge into His bundles and then to the Purkinje fibres. The last ones are responsible for the ventricular cardiomyocyte stimulation. Adapted from Munshi (2012) [142].



In ventricular myocytes, the resting potential (-90 mV) is maintained by the Na^+/K^+ ATPase and the conductance I_{K1} by Kir1.2. Once the depolarizing wave arrives, I_{Na} inward currents flow through $Na_v1.5$ (Phase 0). In response to this depolarization, partial repolarization takes places after $Na_v1.5$ inactivation and Kv4 channels aperture (I_{to}) (Phase 1). Then, Ca_v1 channels open and the inward Ca^{2+} movement, together with the delayed outward movement of K^+ ions by I_K, prolong the action potential duration in a so-called "plateau" phase (Phase 2). This time is crucial to ensure that myocytes cannot be re-excited while in this phase, and all cells contract at the same time. The Ca^{2+} entry triggers the release of SR calcium by RyR at the dyads as abovementioned. This calcium spike provokes the ventricle contraction or systole. When calcium channels are inactivated, the I_K is now able to restore the negative electrochemical gradient (Phase 3), finishing the action potential. The I_K has two components: the rapid and major component, I_{Kr}, and the slow, I_{Ks}. The I_{Ks}, generated by the Kv7.1-KCNE1 complex, is crucial to limit the action potential prolongation when the repolarization reserve is compromised. Once this two components close, I_{K1} is the responsible for maintaining the resting potential (Phase 4), allowing muscle relaxation or diastole. Now the heart is ready for a new action potential [143] (**Figure 18**).

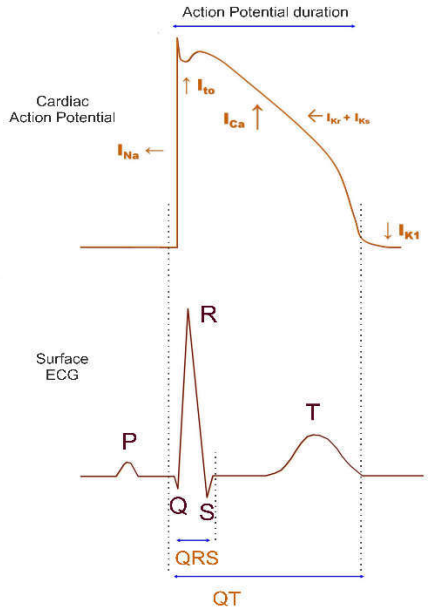


Figure 18. Chronological relationship between ventricular action potential and surface electrocardiogram (ECG). The ion fluxes taking place during action potential are indicated in the top panel, in orange. Bottom panel translates the action potential into the electrical currents detected in an ECG. From Kaczorowski *et al.* (2011) [144].

The action potential generates electrical currents which can be "read" in a classical electrocardiogram (ECG). It presents a typical tracing of cyclic repetitions of 3 electrical entities: the P wave represents atrial depolarization; the QRS complex, the depolarization of the ventricles; the T wave, the repolarization of the ventricles. Beyond these waves, an important measurement of the ECG results is the length between them, being the QT the most relevant for Kv7 channels. Measured from the beginning of the QRS complex to the end of the T wave, is mainly determined by the duration of the action potential in ventricular myocytes. Severe channelopaties are associated with alterations in this interval, explained below (see 1.7.1).

1.6.2. Cardiovascular system

Kv7.1, Kv7.4 and Kv7.5 show a consistent expression in smooth muscle. Kv7.4 and Kv7.5 seem to play a clear role as regulators of contractility in vascular smooth muscle cells. For example, upon vessel constriction signalling in rat aorta or mesenteric arteries, PKC will supress Kv7.5 currents. This will depolarize the membrane, opening Ca^{2+} channels and promoting muscle contraction [145]. Kv7.4 is also expressed in this and other vessels, and is able to form heterotetramers with Kv7.5 to regulate vasodilation [30]. The use of Kv7 channel activators results in vasodilation, whereas inhibitors were found to contract the vessels [16] **(Figure 19)**.

A variety of blood vessels, either veins or arteries, express Kv7.1. This suggests a possible role on smooth muscle contraction. However, studies have not reached a clear conclusion on this issue. While outward currents have been linked to the channel in murine veins [146] and the expression has been confirmed in human arteries, specific pharmacology against Kv7.1 did not alter vascular tone [147]. Some authors attribute the absence of Kv7.1 functional contribution to the presence of auxiliary KCNE subunits, as KCNE4 and KCNE5, which inhibit Kv7.1 currents, are often co-expressed with the channel in blood vessels [148]. Another hypothesis is the heterotetramerization of the channel. Our group has published the Kv7.1-Kv7.5 association in vascular myocytes, which could imply altered biophysical properties for the channel [23].

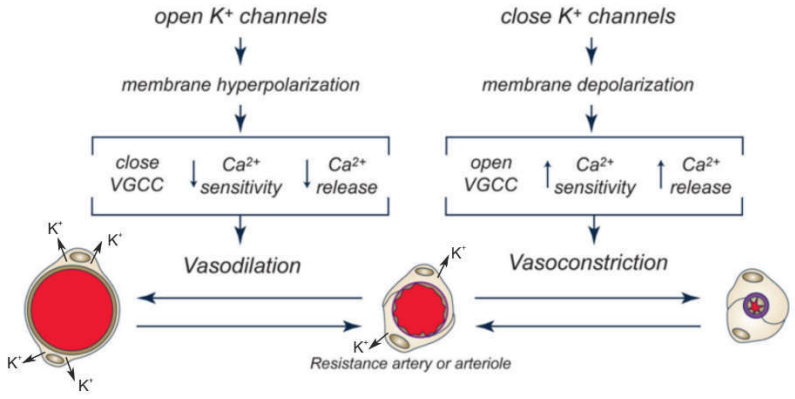


Figure 19. Physiological role of Kv7 channels is vascular smooth muscle. Effects of K^+ channel opening and closing on the membrane potential of vascular smooth muscle cells, which, in turn, generate calcium movements and lead to relaxation or contraction (vasodilation or vasoconstriction, respectively). VGCC: Voltage-gated Ca^{2+} channels. Adapted from Jackson (2017) [149].

1.7. Kv7 channels in disease

1.7.1. Cardiac channelopathies

LQTS

Cardiac channelopathies are responsible for about half of sudden cardiac death cases. The congenital LQTS (Long QT Syndrome) is an inherited heart condition in which a delayed repolarization of the cardiac action potential generates arrhythmias that can lead to palpitations, fainting or sudden

cardiac death. From the 15 genes that have been related to this syndrome, 70% of the cases are generated by 3 cardiac ion channels: Kv7.1, Kv11.1 (gain of function) and $Na_v1.5$ (loss of function).

Focusing on the IKs currents, 500 mutations in Kv7.1 and at least 36 in KCNE1 have been identified and related with LQT1 and LQT5 syndromes respectively. IKs currents are crucial in the heart due to its slow inactivation kinetics. Moreover, Kv7-1-KCNE1 complexes generate the only repolarizing current upregulated by PKA in the heart. Therefore, mutations in both of these subunits are extremely dangerous, as exercise or emotional stress will trigger arrhythmias in LQT1 or LQT5 patients. Mutations described to generate this pathologies are usually single amino acid substitutions that affect traffic to the PM [150,65], assembly of the complex [62] or its electrophysiological properties [151].

SQTS

Short QT Syndrome (SQTS) is also an inherited arrhythmia disorder associated with sudden death, in this case generated by atrial and ventricular fibrillation caused by extremely short refractory periods. Contrary to LQTS, SQTS is not affected by increased heart rate in exercise or emotional stress situations. Gain-of-function mutations in the cardiac repolarizing currents I_{Kr}, I_{Ks} and I_{K1}, generated by Kv11.1, Kv7.1 and Kir2.1 respectively are associated to this disease, as they will reduce the duration of the plateau phase in the cardiac action potential. Similar effects are triggered if the initial depolarization is lengthened by loss-of-function mutations in $Na_v1.5$ or $Ca_v1.2$ (I_{Na} and I_{Ca}, respectively).

In the case of the Kv7.1-KCNE1 complex, a left shift on the activation potential [152], an acceleration on the kinetics [153] or mutations that promote voltage-independent currents and, thus, constitutively open channels are the main causes of SQT2 [154].

1.7.2. Vascular smooth muscle disease

Smooth muscle contractibility modulates important physiological mechanisms such as blood preassure or gastrointestinal motility. When contraction is impaired or excessive, clinical disorders such as ischemic heart disease, cerebral strokes, urinary incontinence or migraine can occur. Because contractibility depends on voltage-dependent Ca^{2+} influx, the maintenance of the resting membrane potential has a pivotal role in modulating the vascular smooth muscle reactivity. Therefore, alterations in ion channel expression or function lead to important smooth muscle pathologies.

Kv7 channels are suggested as key mediators to control the resting potential. As we have seen, Kv7.1, Kv7.4 and Kv7.5 are expressed in all types of vascular smooth myocytes and have been related to vascular diseases. Hypertension can be caused by Kv7.4 downregulation. Diabetes has been widely related with Kv7.1. One of the mechanisms refers to hyperglycaemia in diabetic rats as the cause of Kv7.1 reduced activity, expression and vasodilatory function. Obesity, arrhythmias or autoimmune diseases, among others, may also be explained by alterations in vascular Kv channels [155,156,16].

1.7.3. Ion channels and cancer

Cancer is a group of diseases involving abnormal cell growth and proliferation within the same tissue or spreading to other parts of the body. The main properties of cancer are well known thanks to Hanahan and Weinberg “Hallmarks of cancer”. They include sustaining proliferative signalling,

evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming cellular metabolism and evading immunological destruction while promoting inflammation at the same time [157].

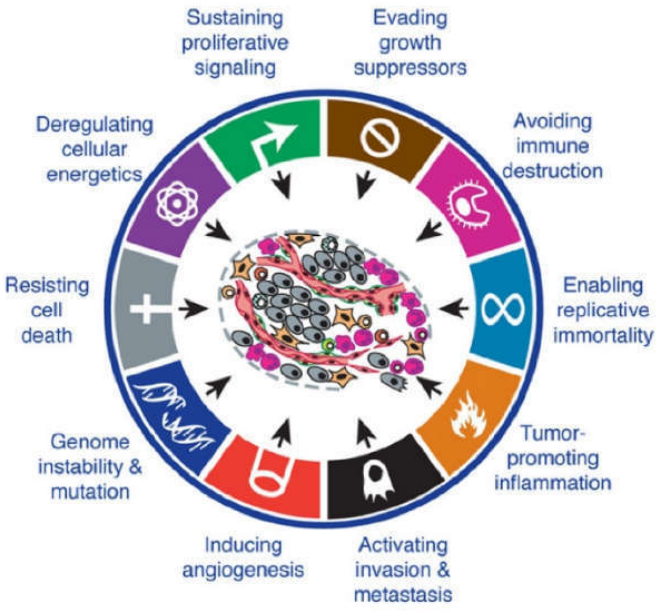


Figure 20. The Hallmarks of cancer. Adapted from Hanahan and Weinberg (2011) [157].

Cancers are classified according to the location in the body where the first developed (topographical classification) or from a histological standpoint (including carcinoma, myeloma, leukaemia, lymphoma, myeloma, sarcoma or mixed types) [158]. This complexity leads to more than 100 types of cancer. They share the main hallmarks, but present also specific genetic or protein aberrations that allow deeper detailed classifications.

Cancer is a multifactorial process. Cells acquire atypical phenotype cause by genetic and/or protein aberrant expression. Suppression of anti-apoptotic genes or overexpression of oncogenes leads to miss-regulated proliferation and tumorigenesis. But cancer cells studies should also include the tumoral microenvironment, which contains mesenchymal, endothelial and immune cells, as well as extracellular matrix proteins and soluble factors. This microenvironment plays and important role in tumor progression, being responsible for cell-cell interactions or cell-matrix signals. Sometimes, the relationship between cancer and the immune system response against the tumor stimulates a favourable environment for tumor progression. Some tumor cells become resistant, escaping from the attack of leukocytes. Thus, the tumor loses immunogenicity and stimulates the production of antiapoptotic cells. This generates an immunosuppressive system in the microenvironment that leads the immune system to a failure in tumor growth control [159,160].

Altered ion channel expression leads to modifications that could favour tumor progression. Evidence has documented a close relationship between Kv channels and cancer, supporting a pivotal role for K^+ channels in cancer therapy. The specific point where ion channels are involved in tumorigenesis remains unclear, as does how K^+ channels remodel under neoplastic cell proliferation [161,162]. Highly proliferative cells are more depolarized than differentiated or quiescent cells. However, transient hyperpolarization is needed for progression during the first stages of the cell cycle (G_1/S). Therefore, a change in the membrane potential must occur for cell cycle progression, as well as during cell

migration and adhesion and cytokine production against the tumor. These phenomena require the participation of ion channels, including voltage-gated potassium channels (Kv). Evidence has suggested that Kv control a check point around the initial stages of the cycle, fitting with the change in the membrane potential, cell volume control, and other ion channel regulation, such as Ca^{2+}-dependent ones. However, it is important to highlight that, although Kv are involved in proliferation, only few trigger clear oncogenic effects [163,164,165,166,42,167].

Kv channels and proliferation

Progression of cell cycle promotes cell growth. Several mechanisms finely control cell proliferation and cell cycle progression in all cell types. A set of molecular events operate through a checkpoint system, which organize the cell cycle in different phases (**Figure 21**). These phases include G_1 (gap 1), S (DNA synthesis), G_2 (gap 2) and M (mitosis). After mitosis, a cell can re-enter the cell cycle in G_1 phase, or enter a quiescent state (G_0). Transition between phases is regulated by the cyclic activation and inactivation of cyclin-dependent kinases (CDKs) by cyclins and CDK inhibitors (CDKIs), respectively. Uncontrolled cell division or propagation of damaged DNA can contribute to genomic instability and tumorigenesis [168,169]. Membrane potential has also been linked to control of the progression through the cell cycle. Membrane potential is not constant during the cell cycle. While the causal relationship is not clear, evident suggest a bioelectric control of the cell cycle [170]. Kv channels can control the upstream biochemical events leading to cell cycle progression by regulating biophysical properties such as the membrane potential and cell volume, or protein-protein interaction mechanisms which converge in tight regulation of calcium oscillations.

An increase in K^+ permeability hyperpolarizes the cell at the end of the G_1 phase. By contrast, depolarization is found at the G_2 M border. These changes in the membrane potential are gradual rather than instantaneous and have been proven to be essential for the proliferation of many cell types. Thus, inhibition of Kv channels activity produces cell cycle arrest, typically by hindering G_2/S transition [171,172,162]. Interestingly, although cell cycle-dependent fluctuations in the membrane potential are observed under both physiological and pathological conditions, cells with a high proliferative phenotype tend to be more depolarized at each step than their normal analogues. Therefore, malignant cancerous cells show a depolarized phenotype, and depolarization itself can induce cancerous transformation. Indeed, depolarization has been suggested as a cellular hallmark of cancer [172]. Growing evidence is unravelling a complex scenario where not only the type of current but also the molecular identity of the potassium channel is important to regulate a function that, in most cases, is time and place dependent.

For instance, Kv1.3 and Kv1.5 are transcriptionally upregulated during G1 phase of the cell cycle. Kv1.5 activity is important for myoblast proliferation through a mechanism involving CDKIs $p21^{cip1}$ and $p27^{kip1}$ [173].In oligodendrocyte progenitors, a similar increase in Kv1.3 and Kv1.5 expression is found at the G1 phase. However, Kv1.3 activity, rather than Kv1.5 activity, is involved in G_1 progression in these cells. Similar to myoblasts, this mechanism involves the accumulation of CDKIs [174,175]. Kv1.3 and Kv1.5 expression changes have also been linked to the proliferation of astrocytes, microglia or macrophages. Kv7.1 and Kv7.5 are both upregulated during proliferation. However, only Kv7.5 is involved in G1 phase progression in myoblasts. Many other examples of fluctuations in K+ channels expression and activity along the cell cycle are reported. For example, Kv1.2 and Kv2.1 mRNA are decreased from early to late G1, while KCa3.1 increases in mesenchymal stem cells from the bone marrow [176]. In spinal cord astrocytes, the downregulation of inwardly rectifying K^+ currents is important for G1/S transition, whereas blockade of delayed outwardly rectifying currents causes G1

arrest. Conversely, a recovery of K_{ir} currents is critical for mitosis. Furthermore, S-phase cell cycle arrest accumulates delayed outwardly rectifying currents [177].

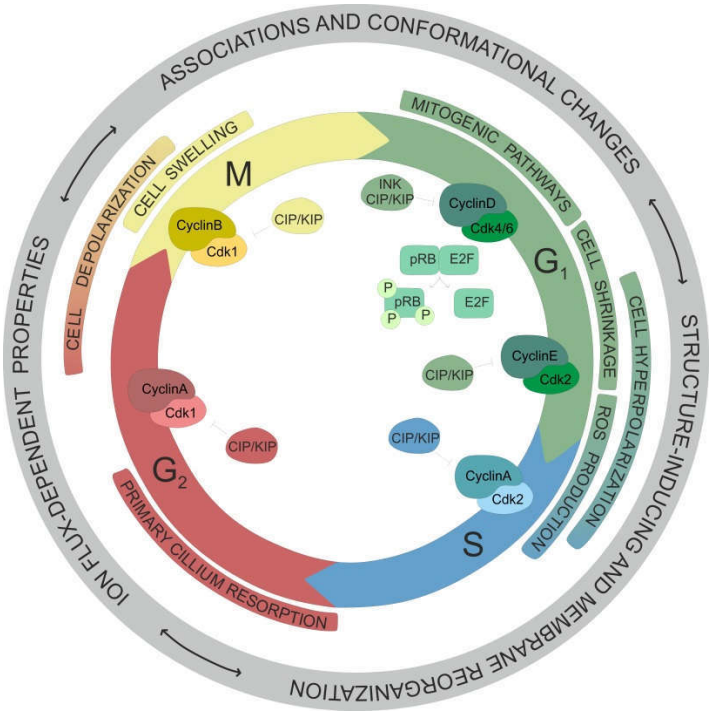


Figure 21. Participation of potassium channels on the control of the cell cycle progression. Outer grey circle: Physical and biochemical properties of ion channels affecting cell cycle progression: (i) Ion flux-dependent properties due to K^+ conduction; (ii) Kv conformational changes may associate with other down-stream signalling partners; and (iii) Kv channels can also induce membrane reorganization phenomena and promote the formation of subcellular structures. These connected events, related to no specific phase, contribute to the regulation of Ca^{2+} oscillations leading to cell cycle progression. Inner colored circles: Events regulated by Kv channels in specific phases of the cell cycle. Colors represent phases and gradients transitions. Kv channels regulate membrane potential, cell volume, mitogen-dependent signal transduction pathways, and other processes involved in cell cycle progression, such as the primary cilium resorption and mitochondrial ROS production. Cell cycle representation: M (yellow) and S (blue) phases of the cell cycle are separated by G1 (green) and G2 (red) gap phases. Several CDK-Cyclin complexes and CDK-inhibitors regulate transitions between phases. In the inner circle, colored complexes are active at specific stages of the cell cycle. From Capera *et al.* (2019) [6].

Kv channels also participate in cell cycle progression by arranging its localization. Kv2.1 is an intriguing example. This channel, which clusters at ER-PM junctions during mitosis, diffusely distributes during interphase. Such Kv2.1 transient localization is dependent on the phosphorylation state, which increases at M phase [178]. When in these membrane contact sites, Kv2.1 stabilises and enhances their formation. Therefore, the channel indirectly regulates localized Ca^{2+} movements and the composition of lipidic microenvironments, suggesting a structural role for Kv2.1 during mitosis. Similarly, Kv10.1 is located at the centrosome and primary cilium. Disregarded for many years, the primary cilium is assembled at the plasma membrane of nearly all quiescent cells. Increasing evidence has pointed to the primary cilium as an important organelle for the transduction of extracellular information. However, the mechanism (either mechanical, chemical, or both) is still unclear. The primary cilium consists of a microtubule-based protrusion whose basal body derives from the mother centriole. Upon cell cycle entry, the primary cilium resorbs, and the centriole is redistributed to form the microtubule-organizing center (MTOC). Transient Kv10.1 expression is transcriptionally induced during G_2/M and the channel is then located at the basal primary cilium membrane where it promotes cilium resorption. The hypothesized mechanism postulates that local membrane hyperpolarization,

due to Kv10.1 activity, would lead to increased Ca^{2+} influx and PIP_2 dispersion from the basal cilium membrane; both events are necessary for primary cilium retraction [179,180,181]. Further examples of the importance of K+ channels localization for cell cycle regulation include Kv1.3. Inhibition of Kv1.3 activity at the plasma membrane blocks G1/S transition in many cell types. However, a recent study has shown that specific blockade of mitochondrial Kv1.3 slightly favours proliferation, most likely by a mechanism involving mitochondrial ROS production [182].

Regulation of cell volume is also linked to cell cycle control, by intrinsically affecting membrane potential. Hyperpolarization via Kv channel activation favours chloride exit by increasing its electrical driving force. The consequent leakage of KCl implies cell shrinkage by osmotic water loss, which favours Ca^{2+} oscillations driving cell proliferation. For instance, Kv11.1 channels, in combination with Cl^- channels, influence proliferation, as well as migration, hormone release and gene expression [183,184].

In addition to flux-dependent activities, K^+ channels can also regulate the cell cycle by non-conducting properties of the channel. As transmembrane proteins, Kv channels can contribute to the initiation of many biochemical events that could involve direct protein-protein interactions. For instance, Kv10.1 induces proliferation through the activation of the mitogen-activated protein kinase (MAPK) cascade or the association with calmodulin. Kv1.3 exposes a C-terminal docking domain which contains phosphorylation sites essential to induce proliferation. Interaction with proliferation effectors such as 14-3-3, Src or TNF-α receptor have also been described for Kv11.1 [185,186,187,171].

Kv channels in apoptosis

Apoptosis is a tightly controlled process of cell death characterized by nuclear condensation, cell shrinkage, loose of membrane integrity and DNA fragmentation, which occurs in physiological and pathological conditions. Apoptosis can be triggered by mitochondrial damage (intrinsic pathway) or by death-promoting molecules (extrinsic pathway) [161]. Defects can occur at any point along these pathways, leading to malignant transformation or tumor metastasis. However, apoptosis plays also an important role in treatment strategies and is a potential target for anticancer drugs. Cell shrinkage is an apoptosis feature resulting from changes in intracellular ions, with the particular loss of intracellular K^+. Activation of K^+ channels during apoptosis is much more pronounced than during proliferation, causing a decrease in K^+ concentration and ionic strength. This reduction plays a critical role in two apoptotic steps: (i) the downstream activation of the apoptotic machinery including and (ii) the inhibition of the proliferative activity, because many of the growth and mitosis related enzymes require a minimal K^+ concentration. In addition, another important factor could be Ca^{2+} signalling. A steady Ca^{2+} increase appears to be needed for apoptotic enzymes activity, while proliferation was associated with oscillatory Ca^{2+} rises [188,43].

Several potassium channels have been described to be involved in cell death physiology, mainly involving cell shrinkage. Kv2.1 is involved in oxidant apoptosis in neurones and mediates K^+ current increase during apoptosis, involving MAPK and CamKII in the process [189]. An increase in BK channels activity in vascular smooth muscle cells is related to apoptosis when mitochondrial homeostasis is affected. Also in these cells, overexpression of Bcl-2, an initiator of the apoptotic volume decrease, downregulated the mRNA expression of Kv1.1, Kv1.5 and Kv2.1 [190,191]. Mitochondrion plays a pivotal role in cell metabolism and, therefore, mitochondrial Kv channels contribute to apoptosis regulation in many cell types. Kv1.3 plays an outstanding role in these mechanisms. MitoKv1.3 mediates apoptosis in lymphocytes via the pro-apoptotic protein Bax and releasing cytochrome C due to the

production of reactive oxygen species (ROS). Moreover, inhibitors of mitoKv1.3 have been reported to induce cancer cell apoptosis [192,193].

Kv channels and cancer

Cancer cells present aberrant Kv channel expression in various cancer and tumour types, and it has been increasingly documented that Kv channels have an oncogenic potential. Several Kv channels have been associated with cancer, such as Kv1.1, Kv1.3, Kv1.5, Kv2.1, Kv3.4, Kv7.1, Kv10.1, Kv10.2, Kv11.1, and it has been suggested that a certain grade of malignancy correlates with the expression of Kv channels in many cases. However, there is no clear pattern that can explain these changes in the different cancer. Kv channels appear as over- or underexpressed depending on the tissue and cancer type, unravelling a high complexity regarding their regulation in carcinogenesis (**Figure 22**).

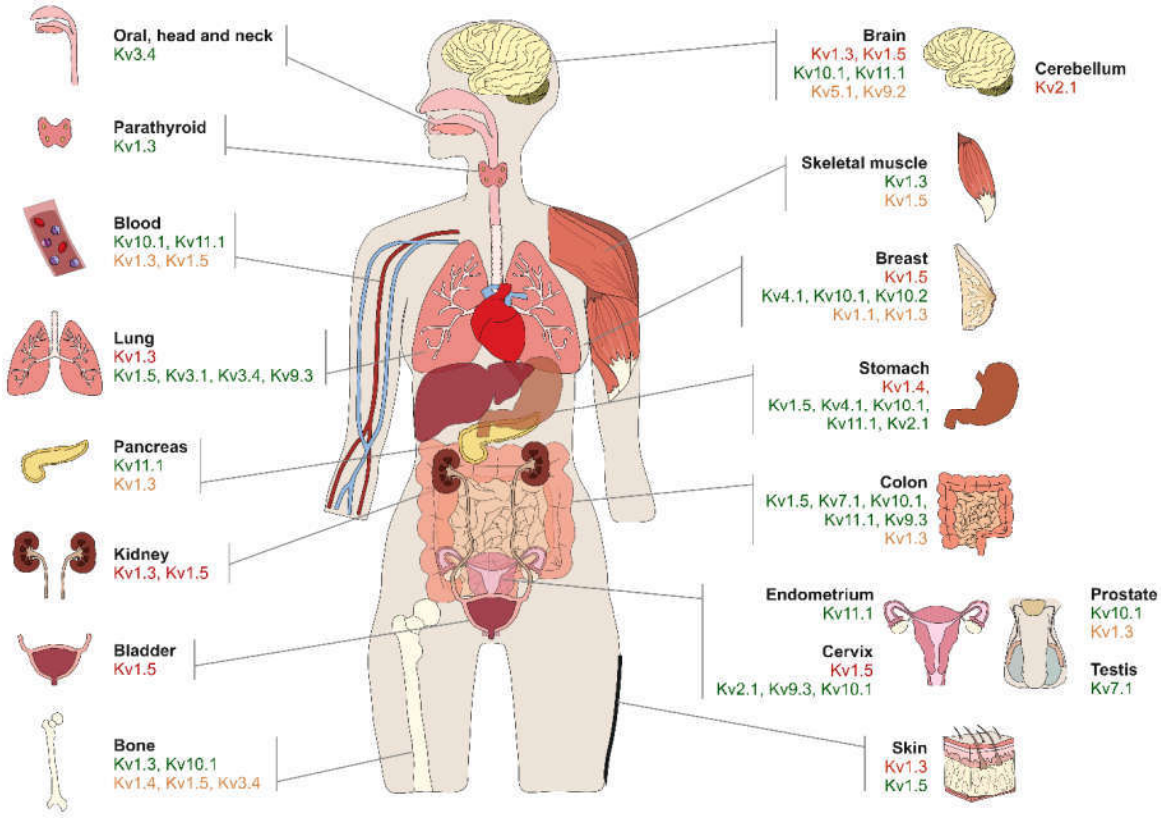


Figure 22. Remodeling of voltage-gated K+ channels (Kv) channel expression in human cancers. Schematic representation of the human body highlights the Kv distribution in tumors. Colors represent differential levels of expression: Red, down-regulation; green, up-regulation; orange, altered expression (evidence claim opposite effects in the Kv channel abundance). From Serrano-Novillo *et al.* (2019) [7].

Alterations in Kv channels expression in tumors is usually related with the physiology of these ones. Because Kv control crucial functions suchs as cell proliferation, activation, migration or apoptosis, it is not surprising that cancer cells remodel these channels. Their role is highly variable: they are differentially expressed in various tumors and can be pro- or anti-apoptotic. Focusing on the channels studied in these thesis, Kv1.3, Kv1.5, Kv7.1 have been described aberrantly expressed in tumor

samples and cancer cell lines. Kv7.5, even though contributes to myoblast proliferation as abovementioned, has not been linked to any cancer previously.

Kv1.3, one of the most documented channels in solid tumors, has been assigned with pro- and anti-proliferative properties. It is differentially remodelled in breast, colon, lung, glioma, muscle, brain, or prostate cancers [43,194]. Its role in tumor progression is not clear, and different implications are described depending on the cancer. Sometimes Kv1.3 expression is aberrant and related to proliferation and apoptosis [195,196,182,197], whereas only cell migration and adhesion are altered in others [198].

Similarly, some examples inversely correlate Kv1.5 and malignancy, linking the channel with apoptosis and impairing cancer progression [43,199]. However, Kv1.5 is overexpressed in some malignant and aggressive neoplasia, such as gastric, bone or colon cancers, where it participates in tumor proliferation and calcium homeostasis [200,201,202]. Furthermore, Kv1.5 is overexpressed in muscle sarcoma and is related to tumor malignancy [203,204]. By contrast, and similarly to lymphomas, a Kv1.5 abundance is inversely correlated with the degree of malignancy in gliomas [205,206]. Moreover, the methylation of Kv1.3 and Kv1.5 promoters silences channel expression in some neoplastic phenotypes, which supports their roles as tumor suppressors [207,208].

Some members of the KCNQ (Kv7) family are also related to cell proliferation and cancer. Kv7.1 remodels in some tumors, and channel inhibition reduces cell proliferation. Kv7.1 is increased in colon cancer as well as in seminoma and germinal cell line tumors [209,210].

2. Aims

2. Aims

Voltage-dependent potassium channels play a key role in regulating repolarization and resting membrane potential in excitable cells, regulating neuronal and muscular excitability. In addition, they control muscle tone of skeletal and smooth muscle and play a crucial role in K^+ homeostasis in non-excitable cells.

However, the unique expression of the channel is not enough to accomplish the wide variety of functions Kv channels are responsible for. To do so, protein localization within specific membrane compartments or organelles is mandatory. A wide variety of signatures, auxiliary and regulatory subunits, post-translational modifications or other regulations fine-tune the balance that determines traffic and location. Kv7.1 is electrophysiological modulated by its regulatory subunit KCNE1. Together, they generate the cardiac I_{Ks} repolarizing currents, and their expression in myocyte membranes is essential for the proper function.

Considering this, traffic regulation shows a large number of possibilities. A certain variable in the whole process can trigger differential effects, not only at the cellular level but also at the level of whole-body homeostasis. Traffic defects can lead to mild affectations such as , to letal conditions such as sudden death or cancer.

The present dissertation aims to understand the traffic regulations that ensure the proper membrane expression of the Kv7.1-KCNE1 complex and the biology of their assembly and secretory pathway. Moreover, Kv channels are important for blood vessel contraction, but their endothelial expression and/or role remains unknown. As ion channels have been widely involved with neoplastic processes and Kv channels are important for vascular function, we wondered if there was a relation between them two.

Therefore, the specific objectives of the PhD thesis were:

- To analyse the Kv7.1-KCNE1 complex assembly and traffic.
- To characterize the ER-PM junctions as the non-conventional structures hosting Kv7.1 traffic and identify the protein interactors implicated in its membrane targeting.
- To study the expression of Kv channels in endothelial layers of blood vessels and their possible implication in endothelial-derived vascular tumors.

3. Materials and Methods

3. Materials and Methods

3.1. Cell culture

3.1.1. Cell lines

- *HEK293 (Human Embryonic Kidney):* Derived from human embryonic kidney cells grown in culture and generated by the transformation with adenovirus 5 DNA, Resulting in a hypotriploid human cell line incorporating 4,5 kb of viral genome into its chromosome 19 (19q13.2). They are adherent cells with an epithelial/fibroblastic phenotype. Even it has been suggested its neuronal origin 683 it could not be concluded. They are deficient in voltage gated potassium channels and β regulatory subunit expression [211,212].
- *COS-7 (CV-1 in Origin with SV40 genes):* Derived from African Green Monkey kidney cells (CV-1). Cells were transformed and immortalized with an SV40 mutant virus that produces large T-antigen but does not replicate correctly. They are fibroblast-like cells and are highly adherent in glass and plastic surfaces [213,214].

3.1.2. Cell lines culture

Both HEK293 cells and COS-7 cells were cultured in DMEM (Dulbecco's modified Eagle Medium, Lonza) supplemented with 10% FBS (Fetal Bovine Serum, Nibco) and antibiotics (10.000 U/mL Penicillin G and 10 mg/mL streptomycin, Gibco). Growth conditions were 37°C and 5% CO_2.

For passing, cells were washed with PBS 1x and then incubated with 1 mL until they detach (2-5 minutes). Trypsin was inactivated with culture media and suspended cells were precipitated by centrifugation at 400 g for 4 minutes. Precipitated cells were resuspended in culture media and seeded in new flasks or dishes.

3.1.3. Cell line freezing and thawing

Freezing

Dishes cultured at 80% confluence were washed once with PBS 1x and then incubated with 1 mL until they detach (2-5 minutes). Trypsin was inactivated with culture media and suspended cells were precipitated by centrifugation at 400 g for 4 minutes. Precipitated cells were resuspended in 1 mL freezing media (90% FBS, 10% DMSO). Cells were collected in cryogenic vials and placed in a room temperature pre-chilled cooler that was stored at -80°C for 2-3 days and then finally stored in liquid N_2.

Thawing

To thaw cells, 1 mL of supplemented media was added to the cryogenic vial were cells were stored. The cell suspension was mixed with additional 8 mL of supplemented media and precipitated by centrifugation at 400 g for 4 minutes. Precipitated cells were resuspended with culture media and seeded in a new dish. Culture media was replaced the following day.

3.1.4. Transient cell transfection

For immunohistochemistry, biochemistry and molecular biology experiements, HEK293 cells were transfected with Lipotransfectin® (AttendBio Research). Lipotransfectin® is a transfection reagent based in cationic lipids with colipids in suspension. 3-6 µg of DNA (specific for every protein) and 12 µL of Lipotransfectin® are mixed separately with 700 µL of DMEM. After 10 minute incubation, the DNA mix was added to the Lipotransfecti® mix drop by drop, and mixed 3 times in the same way. The mix was incubated 20 minutes, and then added dropwise to the dish with DMEM for 4-6 hours. Then, the media was replaced for supplemented media. 48 hours after the transfection, experiments were performed.

For electrophysiological experiments, FuGENE® was used. It is a non-liposomal formulation designed to transfect DNA into a wide variety of cells lines with high efficiency and low toxicity. 1,5 mL of FuGENE® were mixed with DMEM up to a final volume of 100 mL minus the volume of DNA and incubated for 5 minutes. On an additional microcentrifuge tube, 300 ng of DNA were added and FuGENE® mix was later combined. After an additional incubation of 20 minutes, the mix was added to the 35 mm of 6-well multiwell were cells were cultured. In this protocol, it is not advisable the replacement of the media.

Plasmids use are referred in Addendum I.

3.1.5. Isolated cardiomyocytes

Hearts were freshly obtained from wild-type male Wistar rats according to a protocol approved by the Animal Ethics Approval Committee of the University of Exeter (UK). Briefly, rats (250-350 g, 7-8 weeks old) were terminally anaesthetised via 100 mg/kg pentobarbitone sodium intraperitoneal injection. Animals were dispatched via cervical dislocation followed by exsanguination. Once observed reflexes were lost, rats underwent cervical dislocation. The chest cavity was immediately opened and the heart resected, thus confirming kill via exsanguination. The heart was briefly placed in Ca^{2+}-free Tyrode's solution before the aorta was cannulated and the heart retroperfused. The hearts was perfused at 37°C on a Langendorff apparatus at a constant flow rate for a period of 4 minutes with Ca^{2+}-free Tyrode's solution. Finally, the heart was exposed to isolation solution (containing 1 mg/mL collagenase (Worthington, USA), and 1mg/mL protease (Sigma, USA) and 200 µM $CaCl_2$) for 15 mins. The heart was then dissected at the atrio-ventricular septum. The ventricles were diced and triturated to liberate individual cardiomyocytes in 150µM $CaCl_2$ containing modified Tyrode's solution.

For attached cell experiments, cardiomyocytes were allowed to settle for 2 hours at 37°C onto precleaned (brief wash in saturated NaOH in methanol and excessive washing in MilliQ filtered water) number 1,5 laminin coated coverslips mounted into open chambers prior to fixation. Isolated cardiomyocytes were immediately fixed in 2% formaldehyde for 10 minutes by 1:1 addition of 4% formaldehyde in PBS to Tyrode's containing isolated cardiomyocytes at the end of the pharmacological incubation period. Cardiomyocytes were pelleted and washed for 15 mins in PBS before being pelleted and re-suspended in storage solution (75 µM Bovine Serum Albumen, 15 mM NaN3, in 1x PBS).

Hearts destined for tissue sectioning were fixed in 2% formaldehyde in PBS for 1 hour. Whole hearts were then washed for 15 mins before being incubated in 10% (1 hour), 20% (2hours), and 30% (overnight) sucrose containing PBS (w/v) and subsequently flash frozen in methyl butane cooled liquid

nitrogen. Tissue was then placed at -80°C for long term storage embedded in O.C.T (Tissue Tek). Sectioning was performed at -16°C on a Leica CM 3050 cryostat at a thickness of 10 µm. Sections were oriented to either longitudinal or cross-sectional views and attached to 0,05% poly-L-lysine (Sigma) cleaned number 1,5 coverslips.

Cells destined for Western blot assay were similarly obtained. Hearts were digested with collagenase and homogenized until cell dissociation, and the homogenates were then centrifuged for 1 min at 750 rpm. Lysis buffer (150 mM NaCl, 50 mM TRIS, 1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 1% Triton X-100, pH 7,2) was added, and the samples were mixed, incubated for 20 min until complete disaggregation at 4°C and centrifuged at 12000 g for 10 min at 4°C. The supernatants were kept as protein extract and prepared for western blot analysis and immunoprecipitation.

3.2. Molecular biology

3.2.1. Bacteria culture

Bacteria were used to amplify DNA. Cells were grown in LB bacterial culture plates or SOB bacterial culture media, and incubated at 37°C for 24 hours.

- *LB bacterial culture plates*: 5 g tryptone, 2,5 g yeast extract, 5 g NaCl, 7,5 g agar up to 500 mL with water. Autoclave and keep at 50°C during 2 h previous to add antibiotics* and pour.
- *SOB bacterial culture media*: 10 g tryptone, 2,5 g yeast extract, 0,3 g NaCl, 0,09 g KCl up to 500 mL with water. Autoclave and add 2,5 M $MgCl_2x6H_20$ and 2,5 mL of 2 M $MgSO_4x7H_20$.

*Kanamycin: 30 µg/mL or Amplicilin: 100 µg/mL

3.2.2. DNA extraction

To extract DNA from bacteria, GenElute™ Plasmid kits (Miniprep and Midiprep, Sigma-Aldrich) were used and manufacturer instructions were followed. Briefly, cells were obtained from an overnight 37 °C incubation in liquid LB media and centrifuged to discard the supernatant. Pellets were resuspended in Resuspension Solution until full disgregation. Then, Lysis solution was added and gently mixed. After a maximum of 5 min, Neutralization solution was added and mixed by gentle inversion. The mix was centrifuged at 15000 g for 15 min at RT. In the meantime, binding columns were prepared with Column Preparation Solution and, when centrifuged, the clear lysate was transferred to the column. After being washed with Wash Solution and the column was transferred to a new collection tube, DNA was eluted using Elution solution and a 1 min spin.

3.2.3. DNA purification from gel

DNA was loaded in agarose gels (1 % agarose gels in the presence of 1:15000 SyberSafe (Life Technologies), observed under UV exposure and the selected DNA-containing regions were excised. To purify the DNA from the gel, ATP™ gel/PCR DNA fragment extraction Kit (ATP biotech Inc) was used. Manufacturer instructions were followed. Briefly, transfer up to 300 mg of the gel slice into a microcentrifuge tube, add 500 µL of DF buffer and mix by vortexing. Incubate at 60 °C for 10-15 minutes until it completely dissolves. Apply 800 µL of the same mixture into a DF column with a 2 mL

collection tube, and centrifuge at 10000 x for 30 seconds. Add 600 µL of Wash buffer into the DF column and centrifuge again. Discard the flow-through and place the DF column in the collection tube. Centrifuge again for 3 minutes. Transfer dried column in a new microcentrifuge tube and add 15-50 µL of Elution buffer. Centrifuge at full speed for 2 minutes to elute purified DNA.

3.2.4. PCR

Polymerase chain reactions (PCR) were used to amplify DNA, detect or identify DNA presence or add small fragments to specific regions. Specific oligonucleotides or primers are used for each DNA.

PCR mix:

50 µg DNA
5 µL Buffer 5x
1 µL dNTPS
1.25 µL primer forward (F_)
1.25 µL primer reverse (R_)
1 µL Phire/Phusion

Up to 60 µL with H_20

Reaction:

	Initial denaturalization	95 °C	2 min
x18	Denaturalization	95 °C	20 sec
	Annealing	60 °C	10 sec
	Extension	68 °C	30 sec/Kb plasmid
	Final extension	68 °C	5 min
		4 °C	

3.2.5. Site directed mutagenesis

To study molecular determinants implicated in the processes studied in this thesis, amino acids potentially involved were changed to alanine/glycine/serine. Site directed mutagenesis was performed using QuickChange™ Site-Directed. Mutagenesis Kit (Agilent Technologies). For each reaction specific oligonucleotide pairs were designed with CloneManager Suite v7.1 (Scientific & Educational Software). Mutagenesis was performed following manufacturer's instructions.

Briefly, template DNA was amplified by a PCR reaction catalysed by Pfu DNA polymerase with mutagenesis oligonucleotides including the target mutation. The product of this PCR was digested with DpnI, a specific DNase for methylated DNA that cleaved the parental DNA strands (PCR generated chains are not methylated). The digested DNA was next introduced in *E.coli* XL10-Gold ultracompetent bacteria.

45 µL of bacteria were mixed with 2 µL of β-mercaptoethanol, mixed for 5 minutes, and then 2 µL of the mutagenesis reaction were added and kept in ice for 30 minutes. Next, a thermal shock of 30 sec at 42 °C was applied, followed by 2 min on ice. Bacteria were recobered for 1 h in 500 µL of SOB and plated on LB agar plates with the specific antibody. After an overnight 30 °C incubation, positive clones were selected.

When multiple and distant amino acids were mutated, we used QuickChange™ Multi Site-Directed Mutagenesis Kit (Agilent Technologies). In this case, only single oligonucleotides were used for each mutation, binding all the same DNA template.

Finally, in both cases, some clones were selected, grown in liquid and verified by sequencing.

For Oligonucleotids used, see Addendum II.

3.2.6. Sequencing

We used the BigDye™ terminator v3.1 Kit (Applied Biosystems).

Mixture:	Reaction:			
3 µL Big Dye™ Terminator Buffer	Initial denaturalization	96 °C	1 min	
1 µL DNA	Denaturalization	96 °C	10 sec	x28
0.35 µL primer 10 µM	Annealing	50 °C	5 min	
1 µL Big Dye™ Ready Reaction mix	Extension	60 °C	4 min	
Up to 10 µL with H_20		4 °C		

Samples were processed by the analyser cytometer ABI3730 in the *Centres Cientifics i Tecnològics (CCiT)* from the Universitat de Barcelona. BioEdit, a Tom Hall developed software, and Clone Manager Suite v7.1 were used to analyse the results.

3.3. Biochemistry

3.3.1. Total protein extraction

Total protein extraction was performed to analyse protein expression in different samples. The experiment starts with cells plated in 10 cm diameter dishes and is developed in all its stages in cold by incubation of all dishes and tubes in ice.

Plated cells were washed twice with PBS and scrapped in 0.5 mL of Protein Lysis Buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1% triton X-100, pH 7.5) with protease inhibitors added (1 µg/mL aprotinin, 2 µM leupeptin, 1 µM pepstatin, 1 mM PMSF). Lysates were homogenated by incubation in an orbital at 4ºC for 10 minutes and centrifuged at 16000 g for 10 minutes. Supernatant was collected, while the pellet was discarded as it contained cell debris and non-lysed cells.

For rat tissue, including rat brain, heart and skeletal muscle, and several human blood vessels, two methodologies were used, one for the extraction or membrane proteins, and another for the extraction of DNA, RNA and protein. For the membrane protein extraction, samples were kept embedded in OCT. Prior to the extraction, stock pieces were allowed to defreeze in a saline solution. 250-500 mg of each tissue were cutted and homogenized in 5 mL HES (20mM HEPES, 1mM EDTA, 255 mM sacarose, pH 7.4) with protease inhibitors with the help of a Polytron® homogenizer. Mixes were ultracentrifuged in a SW41 Ti Swinging-Bucket Rotor at 15000 g for 20 min at 4 °C. Supernatant was kept, and the whole procedure was repeated to re-homogenize the pellet. The second supernatant was also kept, and mixed with the first one and KCl 0.8 M was added. Mixes were incubated for 30 min at 4 °C and them centrifuged at 200000 g for 1,5 h at 4 °C. Supernatant was then discarded, and pellet was resuspended in Lysis buffer (5mM EDTA, 5 mM EGTA, 1% Triton X-100) with a 25 G needle.

3.3.2. Bradford assay

Bradford assay was used to determine protein concentration in samples. 1 mL of the Bradford reagent (Protein Assay Dye Reagent Concentrate, Bio-Rad) was mixed with 1-2 µL of sample or 2-20 µL of

0.1% BSA (Bovine Serum Albumin) as a standard in semimicro cuvettes. After mixing and incubating for 10 minutes, absorbance was measured at 595 nm. Samples were processed by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) or stored at -20ºC.

3.3.3. SDS-PAGE and Western Blot

Samples were prepared by mixing 50 µg of total protein with protein loading buffer up to a final volume of 50 µL and heated at 65 ºC for 10 minutes. Denaturalized proteins were separated by SDS-PAGE in a 10% polyacrylamide gel which was polymerized by preparing both running and stacking fractions of the gel.

Buffers:

- Protein loading buffer: Laemlli buffer/β-mercaptoethanol (9:1)
- Laemmli Buffer: 1.51 g Tris, 5 g SDS, 23 mL glycerol, 0.5 mL bromophenol blue up to a final volume of 36 mL with H_20, pH 6.75)
- Polyacrylamide gel:
  - Running: 8-10% acrylamide, 375 mM Tris (pH 8.8), 0.1% SDS (sodium dodecyl sulphate), 0.1% APS (ammonium persulphate), 0.04-0.08% TEMED (tetramethyletilendiamine).
  - Stacking: 5% acrylamide, 125 mM Tris (pH 6.8), 0.4% SDS (sodium dodecyl sulphate), 0.4% APS (ammonium persulphate), 0.01% TEMED (tetramethyletilendiamine).

After the electrophoresis (120 V), gel content was transferred to PVDF (Polyvinylidene fluoride) membranes for 2 hours at 360 mA in cold temperature. Membranes were then incubated in blocking solution (5% milk in PBS-T) for 1 hour at room temperature. Then, blocked membranes were incubated overnight at 4ºC with a primary antibody and washed four times for 5 minutes with PBS-T. They were incubated for 1 hour with a secondary antibody specific for the species and conjugated to HRP (Horseradish peroxidase) of the primary antibody and washed again. Finally, membranes were incubated with ECL (enhanced chemiluminescence), a peroxidase substrate containing luminol, and exposed to a LAS 3000 machine (Fuji) to detect the signal. Images of different time exposures were taken to ensure that intense not-saturated images were gotten. In case any experiment was to be quantified, it was performed using Fiji (Fiji is just ImageJ).

Buffers:

- PBS: 140 mM NaCl, 2.7 mM KCl, 1.46 mM KH_2PO_4, 8 mM Na_2HPO_4.
- PBS-T: 0.05% Tween in PBS.
- Blocking solution: 5% nonfat powder milk in PBS-T.
- Primary antibody solution: 5% nonfat powder milk in 4 mL PBS-T with 10 µL sodium azide. Add 1/100 – 1/1000 primary antibody following manufacturer recommendations. For used antibodies and dilutions, see Addendum III.
- Secondary antibody solution: 4 mL PBS-T. Add 1/10000 for mouse secondary antibody (BioRad) or 1/3000 for rabbit (BioRad).
- ECL: ECL1/ECL2 (1:1)
  - ECL1: 100 mM Tris-HCL pH 8.5, 2.5 mM luminol, 396 µM p-Coumaric acid.
  - ECL2: 100 mM Tris-HCL pH 8.5, 5.632 M H_2O_2.

3.3.4. Protein precipitation

When required, total lysates were precipitated to concentrate proteins. Thus, 500 µl of sample and 750 µL of methanol were mixed. Next, 187.5 µL of chloroform and 562.5 µL of water were added. The mixture (vortexed) was then centrifuged at 16000 g for 1 min. Proteins appeared within a white layer (the more protein the thicker the layer) in the interface between methanol-H_2O and chloroform phases. The supernatant (methanol-H_2O) was discarded and 562.5 µL of methanol were added. Centrifugation was repeated to get the protein pellet that was dried up in a heater to remove organic solvents. Pellet was finally resuspended in 50 µL of 1x Laemmli buffer with 2% β-mercaptoethanol and boiled prior to be SDS-PAGE processed.

3.3.5. Immunoprecipitation

Immunoprecipitation experiments on transfected HEK293 cells were performed 48 h after transfection. The cells were washed twice with ice-cold PBS and lysed in 1% Triton X-100, 10% glycerol, 5 mM HEPES pH 7.2 and 150 mM NaCl supplemented with protease inhibitors (1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin and 1 mM PMSF) to isolate total protein samples. The homogenates were centrifuged at 12000 g for 15 min, and the total protein content of each supernatant was determined using a Bradford assay. Protein A-Sepharose beads (GE Healthcare) were incubated with anti-KCNE1 or anti-Kv7.1 antibodies (Alomone) for 1 h at RT. Once bead-antibody complexes were formed, they were covalently bound with dimethyl pimelimidate (DMP) (Pierce). The bead-antibody complexes were incubated with 20 mM DMP for 30 min at RT. The cross-linking reaction was quenched by adding 0.2 M glycine (pH 2.5). The cell lysates were precleared with protein A-Sepharose beads for 1 h at 4°C. Equal amounts of protein samples were incubated overnight with the bead-antibody complexes at 4°C. After 5 washes, the proteins bound to the antibody-bead complexes were eluted in 100 µl of 0.2 M glycine (pH 2.5).

To study traffic pathways, in some experiments, cells were treated with brefeldin A (BFA) (Sigma-Aldrich) at 5 µg/mL for 4 h to ensure the complete turnover of Kv7.1 and Kv7.1-KCNE1 complexes. Tunicamycin was used to inhibit N-Glycosilation. Cells treated treated with 5 µg/mL tunicamycin (Sigma-Aldrich) during the whole transfection process.

3.3.6. Membrane protein biotinylation

Cell surface protein biotinylation was carried out with a Pierce Cell Surface Protein Isolation Kit (Pierce). Briefly, cells were incubated with EZ-Link Sulfo-NHS-SS-Biotin (sulfosuccinimidyl-2-[biotinamido]ethyl-1,3-dithiopropionate) for 30 min, and surplus free reagent was then quenched following the manufacturer's instructions. Lysates, processed as above, were incubated overnight at 4°C in NeutrAvidin agarose columns. After 3 washes, the biotinylated surface proteins were eluted in a DTT SDS-PAGE sample buffer.

3.3.7. Cell unroofing preparations (CUP)

To purify proteins in CUPs, prior to lysis, the CUP protocol was performed in transfected HEK293 cells cultured in 100-mm dishes. The whole CUP protocol is explained in 3.4.4.

3.3.8. Lipid raft isolation

Lipid rafts are microdomains within plasma membrane that are enriched in cholesterol and tightly packed sphingolipids. Thus, lipid rafts are resistant to non-ionic detergent solubilisation, contrary to the rest of the plasma membrane. Starting from that principle, rafts can be biochemically isolated from the rest of the cell as a non-dissolved membrane in nonionic detergent. Protocol was entirely performed in cold temperatures by leaving tubes and dishes over ice and with cold buffers.

1. 4 dishes per condition were washed twice with 5 mL of PBS and scraped in 2 mL of PBS twice to recover the maximum number of cells.
2. Suspension was precipitated by centrifugation at 600 g for 25 minutes at 4ºC. Supernatant was discarded.
3. The pellet, which contains cells and membrane fragments, was resuspended in 1 mL of non-ionic lysis buffer (0.01% Triton X-100 in MBS (150 nM NaCl, 250 µM MES (2-(N-morpholino)-ethanesulfonic acid), pH 6.5).
4. Resuspension was then physically homogenized with a Dounce all-glass tissue grinder (Kimble®) and the homogenate was transferred to an ultracentrifuge tube.
5. In the ultracentrifuge tube, a sucrose gradient was formed by using 3 different sucrose solutions (5%, 30% and 53.28% in MBS 1x). Tube was ultracentrifuged in a SW41 Ti Swinging-Bucket Rotor at 39000 rpm (revolutions per minute) for 24 hours at 4 ^{o}C. This centrifugation speed would equal to around 250000 g force at maximum radius (r_{max}) and 110000 g force at minimum radius (r_{min}).
6. After centrifugation, tube content was gently extracted millilitre per millilitre, stored separately. Samples were prepared by combining 80% of fraction and 20% of protein loading buffer 5x and were analysed by western blotting. Clathrin was used as non-lipid raft marker, while caveolin served as lipid raft marker.

3.3.9. Glycosilation studies

Tunicamycin inhibits N-glycosylation. Cells treated with 5 µg/mL tunicamycin (Sigma-Aldrich) were washed twice with PBS, lysed and processed for western blot analysis. Furthermore, a sequential deglycosylation protocol to remove all N-linked and many common O-linked glycans from glycoproteins was also used. Transfected HEK cells were lysed as described above, and Kv7.1 and KCNE1 were immunoprecipitated. Sequential enzymatic reactions for 1 h at 37°C followed by heat inactivation were performed following the manufacturer's instructions (New England Biolabs). Briefly, 50 µg of immunoprecipitates was sequentially treated for EndoH, PNGase F and β1-4 galactosidase digestion. Control samples were incubated with reaction buffers in the absence of glycosidases. Finally, the samples were subjected to SDS-PAGE for western blot analysis.

3.4. Cell imaging

3.4.1. Immunocitochemistry

Cells were seeded over poly-D Lysine (Sigma-Aldrich) treated coverslips inside a 6-well multiwell plate. 24-48 h after transfection, samples were prepared. Protocol was conducted at room temperature unless stated otherwise and the multiwell plates were covered by tinfoil to prevent the

fluorophores to fade. For the preparation of samples, coverslips were washed thrice with 1 mL of PBS(-K^+) for 5 min. Then, coverslips were treated for 10 minutes with 1 mL of PFA solution (paraformaldehyde) to fixate the cells and washed thrice with K^+-less PBS to clean them of the PFA.

In case no immunocytochemistry is needed (as in the case of transfectable fluorophores), coverslips were mounted over slides with Mowiol mounting solution.

In some cases, transfectable fluorophores are not available or are not the preferable approach. In these cases, immunocytochemistry was performed after the post-fixation washes. Immunocytochemistry is performed at room temperature conditions and avoiding the exposure of the samples to light. Depending on the antibody and the nature of the target protein, the immunocytochemistry was performed with permeabilizing or not-permeabilizing buffers. Briefly, coverslips were washed and fixated as abovementioned. If permeabilization was required, cells were treated for 10 min with permeabilization solution (In this case, 0.05% Triton X-100 was added to the following solutions). After washing the coverslips with PBS(-K^+), they were treated with blocking solution for 1 hour and washed again. Coverslips were treated with primary antibody solution overnight at 4 ^oC and washed with PBS(-K^+). Next, coverslips were treated with fluorescence-conjugated secondary antibody solution for 2 h at room temperature and washed again. Finally, they were mounted over the slides with handmade Mowiol mounting solution.

Buffers:

- PBS(-K^+): 154 mM NaCl, 8.85 mM NaH_2PO_4, pH 7.4.
- PFA: 4% paraformaldehyde (Sigma-Aldrich) in PBS(-K^+).
- Permeabilization solution: 0.1% Triton X-100 in PBS(-K^+).
- Blocking solution: 5% nonfat powder milk, 10% goat serum in PBS(-K^+).
- Primary antibody solution: 10% Goat serum in PBS(-K^+). Varying concentration of antibody according to manufacturer's indications. See Addendum III.
- Secondary antibody solution: 1% BSA in PBS(-K^+). Varying concentration of antibody according to manufacturer's indications.
- Mowiol: 9.6 g of Mowiol in 24 g of glycerol and 48 mL of Tris HCl 0.2 M pH 8.5. Store at -20 ^oC.

3.4.2. Confocal microscopy

Cell preparations were imaged under a Leica SP2 laser-scanning confocal spectral microscope (Leica Microsystems GmbH) equipped with an argon multiline laser (458, 488 and 514 nm), a DPSS 561 nm laser and a helium–neon laser (633 nm). Images of the YFP fluorophore were obtained using the 514 nm laser line, those of CFP were obtained using the 458 nm line, those of DsRed were obtained using the 561 nm line, and those of Cy5 were obtained using the 633 nm line. Double dichroic (458/514) and triple dichroic filters (488/568/633) were used accordingly. Images were acquired at a resolution of 1024 x 1024 pixels using a 63X oil immersion objective lens (NA 1.32) and scanning with a pinhole aperture of 1 Airy unit at zoom 4 following the Nyquist theorem to achieve maximal lateral optical resolution. Scanning was performed sequentially for observation of each fluorescent protein.

Colocalization analysis was carried out using Manders' coefficient (M) based on global analysis of pixel intensity distributions. The coefficient varies from 0 to 1, being 0 non-overlapping images and 1 the 100% colocalization between both images. When colocalization between Kv7.1 and KCNE1 was assessed, the M coefficient reflected the proportion of Kv7.1 signal coincident with KCNE1 signal. Alternatively, we estimated the colocalization between either Kv7.1 or KCNE1 and specific subcellular

markers. In that case, the M coefficient showed the overlapping distribution of a given subunit (Kv7.1 or KCNE1) with the evaluated subcellular compartment.

3.4.3. FRET Acceptor Photobleaching

The Förster Resonance Energy Transfer (FRET) technique is used to assess whether a pair of proteins are directly interacting with each other. It is based in the quantification of the energy transferred from a donor fluorophore (D), in its electronic excited state, to an acceptor fluorophore (A) through non-radiative dipole-dipole coupling. As the efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor, FRET is extremely sensitive to small distances. This makes FRET efficiency measurements useful to determine whether two fluorophores are within a certain distance. Only directly interacting proteins bring fluorophores close enough to transfer energy. Thus there are two main requirements for FRET to take place:

i. The emission spectra of the donor must overlap with the excitation spectra of the acceptor.
ii. The distance between fluorophores cannot be larger than 10 nm.

Moreover, this electrodynamic phenomenon also depends on other parameters such as the quantum yield of the donor and the relative orientation of the donor and acceptor transition dipoles. That is why with this approximation the distance could not be calculated but different situations could be compared. Fluorophores are excitable molecules with the ability to absorb the energy of a certain wavelength electromagnetic wave and use this energy to change from a resting state to an excited state. The excited state is unstable and tends to go back to the resting state by dissipating the excitation energy as temperature or fluorescence (emission in a larger wavelength). When a FRET pair fluorophores are close enough part of the donor excitation energy is transferred to the acceptor instead of being dissipated as donor fluorescence. In this situation there is an increase in acceptor and a decrease in donor fluorescence, also changing fluorophore lifetime and polarization. In the present work, we performed FRET Acceptor Photobleaching using the CFP-YFP pair, where we measure the fluorescence intensity increase of the donor when the acceptor is bleached. This intensity is known as FRET energy transfer efficiency (FRET efficiency).

We measured FRET efficiency 48 h after transfection. Cells were fixed as explained in section 3.4.1, and confocal images were taken. Three scans of the region of interest (ROI) using the 514 nm line of an argon laser at 100% power intensity were performed. Before and after photobleaching, CFP and YFP images were collected. All images were sampled at 512 x 512 pixels with a 12-bit resolution. FRET efficiency was calculated following the formula:

$$FRET\ efficiency = \frac{Dpost - Dpre}{Dpost}$$

where D is the CFP fluorescence intensity before (Dpre) or after (Dpost) photobleaching normalized by the CFP fluorescence intensity outside the ROI to monitor CFP photobleaching due to imaging. Alternatively, qualitative FRET ratio images are presented in some figures. Analysis was performed using ImageJ.

3.4.4. Cell unroofing preparations

Cell unroofing preparations (CUPs) are membrane sheets obtained through osmotic shock. HEK293 cells were seeded on poly-D-lysine-treated glass coverslips. Forty-eight hours after transfection, the

cells were cooled on ice for five minutes and washed twice in PBS. Next, the cells were incubated for five minutes in KHMgE buffer (70 mM KCl, 30 mM HEPES, 5 mM $MgCl_2$, 3 mM EGTA, pH 7.5) diluted threefold and then gently washed with undiluted KHMgE to induce hypotonic shock. Lysed cells were removed from the coverslips by intensively pipetting up and down. After two washes with KHMgE buffer, only the membrane sheets remained attached. We also used a milder modified protocol based on a gentler burst to prepare mCUPs. In mCUPs, a fraction of the ER compartment remains connected to the PM by the ER-PM junctions (**Figure 23**). The preparations were fixed with fresh 4% PFA in PBS for 10 min at room temperature and mounted with homemade Mowiol mounting medium.

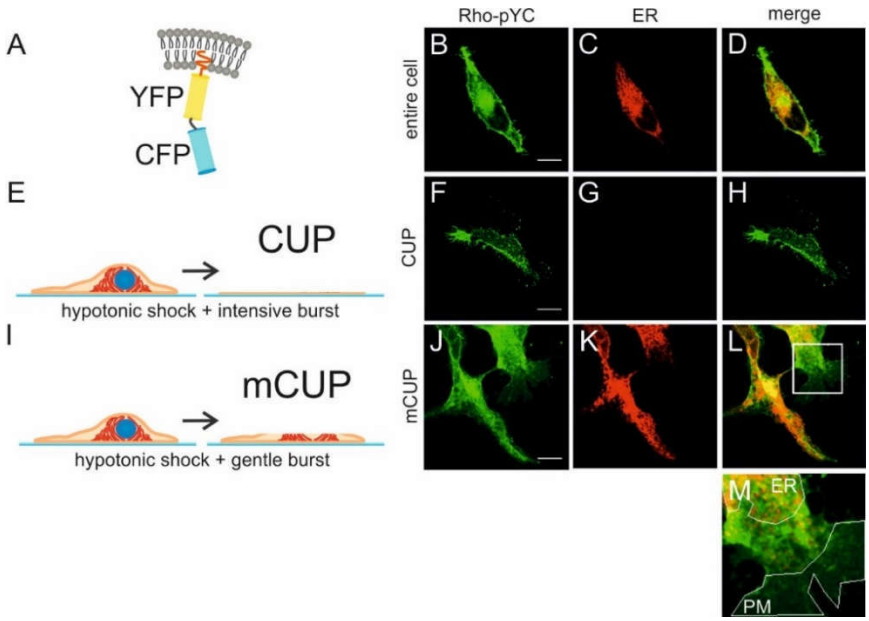


Figure 23. Characterization of cell unroofing preparations (CUPs) and modified CUPs (mCUPs). HEK293 cells were transfected with Rho-pYC and ER-DsRed to identify the plasma membrane and ER, respectively. **A:** Cartoon of Rho-pYC, a membrane-localized CFP-YFP tandem, used as a membrane marker. **B-D:** Rho-pYC (green) and ER-DsRed (red) colocalization. **E:** Cartoon of the protocol used to obtain CUPs. **F-H:** Rho-pYC (green) and ER-DsRed (red) colocalization in CUP. Note that the ER (G) is absent. **I:** Cartoon of the protocol used to obtain ER-preserved CUPs (mCUPs). **J-L:** Rho-pYC (green) and ER-DsRed (red) colocalization in mCUP. **M:** Magnified view of the highlighted area in mCUPs. The ER and PM are delineated with white lines. Scale bar, 10 µm.

3.4.5. 3D rendering

For 3D reconstructions, we obtained z-stack images of whole cell volumes. Acquisitions were performed following the Nyquist theorem in the x, y and z axes. Next, the confocal images were deconvoluted using Huygens Essential software (Scientific Volume Imaging B. V.) to compensate for the optical distortion during acquisition. Employing Imaris software (Bitplane), we reconstructed the optical sections into a 3D representation. The volume data from the 3D reconstructions were rendered to represent the anatomical surfaces of the particular objects of interest. This information is shown in the figures as surface renders.

3.4.6. Electron microscopy

HEK293 cells (control and transfected) were cultured for 48 h before being were fixed for 1 h with 4% PFA and 0.1% glutaraldehyde in 0.1 M PBS and then in 2% paraformaldehyde for 30 min at RT.

High-pressure freezing cryofixation with liquid N¬2 and cryosubstitution, Lowicryl resin embedding, polymerization of blocks and ultrathin sectioning at a 60 nm thickness were performed in collaboration with the *Unitat de Criomicroscòpia Electrònica* (CCiT, Universitat de Barcelona). The samples were mounted over Formvar-coated grilles, and the sections were finally contrasted with 2% uranyl acetate for 15 min and immunogold-labeled. Proteins were labeled using polyclonal primary antibodies (see Addendum III). Goat anti-rabbit (GoRa) and goat anti-mouse (GoMo) secondary antibodies conjugated to 12 nm and 18 nm gold particles, respectively, were used to next. The samples were observed using a Tecnai Spirit 120kV microscope. Morphometric analysis was performed using ImageJ.

3.4.7. Super resolution microscopy

Superresoltion microscopy allows high resolution image acquisition. Spatial distances of 10-50 nm are distinguishable, solving the confocal microscopy limitations. Direct Stochastic Optical Reconstruction microscopy (dSTORM) is based on the bleaching properties of fluorophores. Whole sample is excited, but only discrete fluorophores will activate at a time (the signal is detected) and then inactivate. Constant excitation allows all fluorophores of the sample to be excited and detected. The addition of all discrete signals gives the reconstruction of a high resolution image (Figure 24).

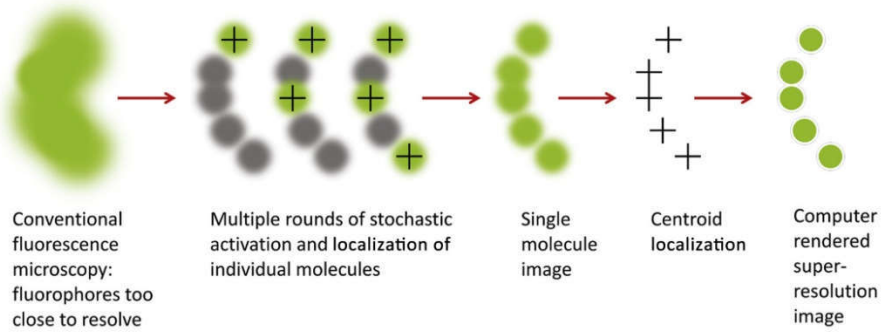


Figure 24. The process of super-resolution image generation by dSTORM. Through iterative identification of the centroids of individual fluorophores, super-resolution images can be reconstructed. From Thorley *et al.* (2014) [215].

Frozen rat heart sections 10 µm thick were cut using a Leica CM 3050 cryostat and collected onto precleaned coverslips coated with 0.1% poly-L-lysine (Sigma). The sections were blocked using an Image-iT FX signal enhancer (Thermo Fisher) for 1 h at RT. Primary antibodies against Kv7.1 (Santa Cruz), KCNE1 (Alomone) and RyR2 (Thermo Scientific) were applied overnight at 4°C. Next, the samples were incubated for 2 h at RT with secondary antibodies conjugated to Alexa Fluor 647 or Alexa Fluor 700 (Thermo Fisher). Imaging was performed in switching buffer (90% glycerol and 100 mM 2-mercaptoethylamine in PBS). For image acquisition, dSTORM images were obtained with a modified Ti-Eclipse inverted fluorescence microscope (Nikon, Japan). Image processing, event localization and grayscale rendering were performed using custom-written Python Microscopy Environment software (https://bitbucket.org/david_baddeley/python-microscopy).

3.5. Tissue imaging

3.5.1. Immunohistochemistry (OCT embedded samples)

Human tumor and healthy samples, from *Banc de Teixits de Bellvitge,* Human vein and arteries healthy control vessels (not associated to neoplastic processes), were obtained from a 79 year old man with dyslipidemia and diabetes. Vessels used for this work include carotid arteria, aorta, subclavian vein and inferior cava vein. Tumor samples correspond to patients (age and sex not provided) with three types of highly malignant vascular cancers: cutaneous angiosarcoma, epithelioid haemangioendothelioma and angiosarcoma derived from a testicular teratome.

Samples were given as tumor pieces embedded in OCT (Optimal Cutting Temperature) (Sakura) and were later cut in 10 µm sections using a Leica CM 3050 S cryostat. Slices were placed in previously poly-L lysinated coverslips and kept at -80 ^{o}C until immunolabelling. Slices used were consecutive in order to ensure the preservation of the same structures observed among conditions.

1. Ensure complete dryness of sections by incubating overnight at 37 ^{o}C.
2. Rehydrate the slice with a drop of PBS-G.
3. Fixation: 4% PFA, 60 mM sacarose in PBS-G for 15 min.
4. Wash thrice with PBS-G for 5 min.
5. Permeabilize slices with 0.3% Triton X-100 in PBS-G for 1h at RT.
6. Wash thrice with PBS-G for 5 min.
7. Blocking solution 1 h at RT.
8. Incubate with primary antibody solution with each Kv channel antibody (Ra), overnight at 4 ^{o}C. Varying concentration of antibody according to manufacturer's indications. See Addendum III.
9. Wash thrice with PBS-G for 5 min.
10. Incubate with GoRa Cyanine 5-conjugated antibody in secondary antibody solution (1:200) for 2 h at RT.
11. Wash thrice with PBS-G for 5 min.
12. Repeat blocking solution 1 h at RT.
13. Incubate with α-actin antibody (Mo) in primary antibody solution (1:100) overnight at 4 ^{o}C, to label muscular structures.
14. Wash thrice with PBS-G for 5 min.
15. Incubate with GoMo Alexa 488-conjugated antibody in secondary antibody solution (1:100) for 2 h at RT.
16. Wash thrice with PBS-G for 5 min.
17. Labell nuclear structures with DAPI 1:10000 in PBS-G for 20 min at 37 ^{o}C.
18. Mount using a coverslip and handmade Mowiol mounting solution.

Buffers:

- PBS-G: 20 mM glycine, 0.05% Triton X-100 in PBS(-K^+) 1x.
- PBS(-K^+): 154 mM NaCl, 8.85 mM NaH_2PO_4, pH 7.4.
- Fixation solution: 4% paraformaldehyde (Sigma-Aldrich), 60 mM sacarose in PBS-G.
- Permeabilization solution: 0.3% Triton X-100 in PBS-G
- Blocking solution: 5% nonfat powder milk, 10% goat serum, 0.3% Triton X-100 in PBS-G.
- Primary antibody solution: 10% Goat serum, 0.05% Triton X-100 in PBS(-K^+). Varying concentration of antibody according to manufacturer's indications. See Addendum III.

- Secondary antibody solution: 1% BSA, 0.05% Triton X-100 in PBS(-K^+). Varying concentration of antibody according to manufacturer's indications. See Addendum III.
- Mowiol: 9.6 g of Mowiol in 24 g of glycerol and 48 mL of Tris HCl 0.2 M pH 8.5. Store at -20 ^oC.

3.5.2. Immunohistochemistry (Paraffin embedded samples)

Tumor samples embedded in paraffin were also kindly given by Dr. Enric Condom from *Departament d'Anatomia Patològica de l'Hospital Universitari de Bellvitge*, according to the ethic committee of clinical experimentation. In this case, samples were already sliced and attached to coverslips. Samples were kept at RT until immunolabelling.

1. Ensure complete dryness of sections by incubating overnight at 56 ^oC.
2. Deparafination:
   i. Xylene, 5 min (x2).
   ii. Ethanol absolute, 3 min (x2).
   iii. 95% ethanol, 3 min (x2).
   iv. 70% ethanol, 3 min.
   v. 30% ethanol, 3 min.
   vi. PBS-G, 5 min (x2)
3. If required, Antigen Retrieval*
   Temper gently at RT by letting the slices in the solution overnight.
4. Fixation: 4% PFA, 60 mM sacarose in PBS-G for 15 min.
5. Wash thrice with PBS-G for 5 min.
6. Permeabilize slices with 0.3% Triton X-100 in PBS-G for 1h at RT.
7. Wash thrice with PBS-G for 5 min.
8. Blocking solution 1 h at RT.
9. Incubate with both primary antibodies at the same time in primary antibody solution, overnight at 4 ^oC. For Kv channels (Ra), varying concentration of antibody according to manufacturer's indications. See Addendum III. For α-actin (Mo), use dilution 1:500.
10. Wash thrice with PBS-G for 5 min.
11. Incubate with both secondary antibodies at the same time in secondary antibody solution (GoRa Cyanine 5-conjugated antibody (1:200) and GoMo Alexa 488 (1:500)) for 2 h at RT.
12. Wash thrice with PBS-G for 5 min.
13. Label nuclear structures with DAPI 1:10000 in PBS-G for 20 min at 37 ^oC.
14. Mount using a coverslip and handmade Mowiol mounting solution.

**Antigen Retrieval*

Paraffin can mask the epitopes of peptides that become unrecognisable for antibodies. Therefore, antigen retrieval is required before immunolabelling the samples. Because the tumoral samples were limited, first controls to stablish the protocol for paraffin embedded samples were performed in colon control samples, which are highly irrigated. Thus, we could determine the best treatment for these samples regarding deparaffination, antigen retrieval (AgR) and immunohistochemical conditions for each channel in the blood vessel found in colon. Several AgR treatments were tested for each protein studied.

Sodium Citrate Antigen retrieval
10 mM sodium citrate in dH_20, pH 9.
Incubate 40 min at 98 ^oC.

Citric acid Antigen retrieval
10 mM citric acid in dH_20, pH 6.
Microwave at maximum power for 12 min.

EDTA Antigen retrieval
EDTA pH 8
Autoclave 10 min at 110 ^oC.

For each condition, the best treatment was assessed:
Kv7.1: No antigen retrieval.
Kv7.5: No antigen retrieval.
Kv1.3: Sodium citrate antigen retrieval.
Kv1.5: Sodium citrate antigen retrieval.

Moreover, primary and secondary antibody controls were performed in colon samples.

3.5.3. Confocal microscopy

Two systems were used, depending on the simple type (due to system availability on the facilities). Paraffin embedded samples were imaged under Leica TCS SP5 II (2 photon) laser-scanning confocal spectral microscope (Leica Microsystems GmbH) equipped with an argon multiline laser (458, 488 and 514 nm), a DPSS 561 nm laser and a helium–neon laser (633 nm). To screen the whole size of the sample, Matrix HCS-A Software module, a system that performs High Content Screening. Briefly, a small augment (10X) lens was used to take consecutive images of the whole sample size, which were stitched together to generate a single image. This image was used to identify regions of interest (ROI) including healthy and tumor blood vessels. ROIs were indicated and, individually, set to the focal plane to avoid the sample thickness variations. Using a large augment lens (63X oil immersion objective lens (NA 1.32), a new screening of each ROI was performed, and consecutive images were overlapped to obtain single of the area selected.

OCT embedded samples were imaged under LSM880 Zeiss confocal laser scanning microscope (Carl Zeiss Microscopy). The process of image acquisition was similar, but in this case, no addition software was required, as the software has a function that allows the capture of consecutive images and the automatic stitching to generate a single image.

In both cases, 10X augment images were taken at low resolution (256 x 256), 600 Hz speed, bidirectional mode scanning with a pinhole aperture of 2 Airy unit at zoom 1. Scanning was performed sequentially for observation of each fluorescent protein by grouping UV and proximal red in one scan (DAPI and Cy3, 405 nm and 561 nm laser lines, respectively), and green and far red channels in another (Alexa 488 and Cy5, 458 nm and 633 nm line, respectively).

3.6. Elecrophysiology

Currents were recorded from HEK293 and COS-7 cells using the perforated (amphotericin B) patch-clamp technique with an Axopatch 200B amplifier (Axon Instruments), as previously described (41). The intracellular pipette filling solution contained (in mM): 80 K-aspartate, 50 KCl, 3 phosphocreatine, 10 KH_2PO_4, 3 MgATP, 10 HEPES-K, and 5 EGTA and was adjusted to pH 7.25 with KOH. The bath

solution contained (in mM): 130 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-Na, and 10 glucose and was adjusted to pH 7.40 with NaOH. Amphotericin B (20 mM, Sigma-Aldrich) was prepared in DMSO and added to the internal solution to a final concentration of 0.5 mg/mL, as reported (41).

The currents were filtered at 1 kHz (with a 4-pole Bessel filter) and sampled at 2 kHz. Micropipettes were pulled from borosilicate glass capillary tubes (Narishige, GD-1) on a programmable horizontal puller (Sutter Instruments Co.) and heat-polished with a microforge (Narishige). The micropipette resistance was 1-3 MΩ. pClamp version 9 software (Axon Instruments) was used for data acquisition and analysis. The currents were recorded at room temperature (21-23°C) at a stimulation frequency of 0.03 Hz. The Microcal Origin 2018 (Microcal Software) and Clampfit 10.8 programs were used to perform least-squares fitting and for data presentation.

3.6. Proteomic studies

3.6.1. Immunoprecipitation

For proteomic analysis, co-immunoprecipitation experiments were performed with with Dynabeads-Protein A® (Life Technologies). Briefly, 2 mg of protein lysate were brought to 500 µL of lysis buffer supplemented with protease inhibitors. Samples were incubated for 2 h at room temperature or overnight at 4 ^{o}C with Dynabeads® cross-linked to 7.5 mg of anti-GFP antibody (GeneScript). For antibody cross-linking, BS^3 reagent was used following manufacturer's instructions. Immunoprecipitates were washed three times and eluted with the elution buffer provided by the manufacturer. A total of 124 immunoprecipitations were independently performed and stored at 20 ^{o}C. Elutions were concentrated together by methanol/chloroform precipitation and the protein pellet was used for HPLC-MS analysis.

3.6.2. Protein digestion, nano-HPLC and mass spectrometry (MS).

The sample was digested with Trypsin, following standard protocols [216]. Briefly, sample was reduced with 0.5 mM TCEP /50mM Tris pH 7.4 for 30 min at 37 ^{o}C and alkylated for 30 min with IAA in the dark. Then, digestion was performed with trypsin (0.1 µg/µL) in 25 mM Tris pH 7.4 0.1% SDS at 37 ^{o}C overnight. The digestion was stopped by adding formic acid. Peptides were extracted with 100% acetonitrile (ACN) and completely evaporated. Samples were reconstituted in 9 µL of 3% ACN and 1% formic acid aqueous solution for MS analysis. Liquid chromatography was performed using a nano-HPLC Eksigent system. HPLC separation was performed with a gradient from 3% to 35% ACN in 0.1% formic acid during 120 min. Peptides were analysed with the Velos LCQ-Orbitrap mass spectrometer (Thermo Fisher Scientific). Data was processed using Xcalibur 2.1 (Thermo Fisher Scientific) and submitted to the SEQUEST software for further analysis with the HUMAN UniProt SwissProt database. This procedures were performed in collaboration with *Unitat de Proteòmica* (CCiT, Universitat de Barcelona).

3.6.3. Data analysis

For proteome analysis of Kv1.3 interactome, Cytoscape v3.7.1 software was used. Functional enrichment analysis was performed using Gene Ontology, Reactome Pathways and KEGG Pathways databases.

4. Results

4. Results

4.1. Endoplasmic reticulum - Plasma membrane junctions hub the I_{Ks} complex

4.1.1. Kv7.1-KCNE1 association recapitulates the I_{Ks} current

Cardiac Kv7.1 and KCNE1 participate in the repolarization of the action potential contributing to functional I_{Ks} currents in cardiomyocytes. The importance of such functional complex for the heart physiology deserves massive attention. One controversial issue is the specific location of their interaction. Many information about trafficking mechanisms and sorting of ion channels come from heterologous expression, which support heart physiology. Thus, to study the co-assemblage and traffic of the Kv7.1-KCNE1 complex, we employed heterologous system expression of Kv7.1-CFP and KCNE1-YFP in HEK293 and COS-7 cells. First, we corroborated the basic electrophysiological features of the cardiac Kv7.1-KCNE1 complex in our models. Our findings were validated in both cell lines independently of their genetic background. As expected, Kv7.1-CFP and KCNE1-YFP timely formed functional heteromeric channels at the PM that conduct a slowly activating outward K+ current resembling the cardiac I_{Ks} in COS-7 (**Figure 25**) cells.

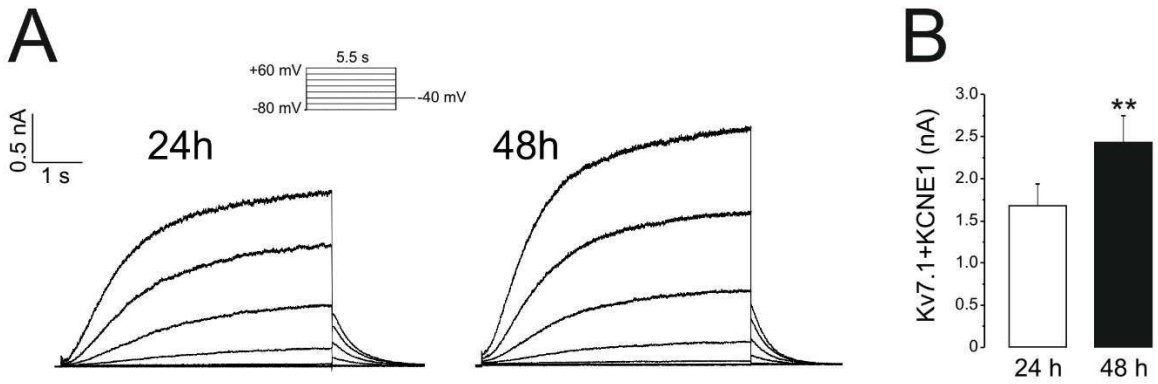


Figure 25. Kv7.1 and KCNE1 recapitulate cardiac I_{Ks} currents. A: Co-expression of Kv7.1 and KCNE1 in COS-7 cells recapitulates I_{Ks} currents. COS-7 cells were cotransfected with Kv7.1 and KCNE1 during 24 and 48 h. Cells were held at -40 mV and 5.5 s depolarizing pulses were applied from -80 mV to +60 mV. **B:** Maximal current intensity of currents at +60 mV. White column, cells transfected during 24 h; Black column, currents recorded after 48 h expression. Values are mean ± SEM of 6-8 cells. **, p<0.01 vs 24h Student's t test.

4.1.2. Kv7.1-KCNE1 complex is fully co-assembled at the plasma membrane

Confocal imaging was used to define the complex localization (**Figure 26**). While the α-subunit Kv7.1 was notably retained in intracellular compartments, the regulatory subunit KCNE1 was mostly present at the cell surface. Colocalization of both proteins was restricted to the plasma membrane.

Cell unroofing preparations (CUP) discriminate between the cell surface and intracellular compartments. In these preparations, we burst the cell and keep only the membranes attached to

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4. 2. Deciphering the Kv7.1 traffic interactors

The complex Kv7.1-KCNE1 uses an unconventional secretory pathway to reach the cell surface via ER-PM junctions. Therefore, we next aimed to decipher further the details on this specific mechanism. To this purpose, we will dedicate the next chapter to the study of the molecular architecture and composition of the junctions used by the I_{Ks} complex. As abovementioned, Kv7.1 is major driving the complex through the Golgi-independent pathway thereby we will focus in this protein.

4.2.1. Kv7.1 and cardiac ER-PM junctions

As abovementioned, Kv7.1 and KCNE1 generate the I_{Ks} current in cardiomyocyte t-tubules. These domains are direct contacts between the sarcoplasmic reticulum and the sarcolemma membranes. Also known as ER-PM junctions, they are present in many cell types with different and differential characteristics. One of the main features among different types of ER-PM junctions is the structural protein composition.

Kv2.1 and junctophilin (JPH) enhance and stabilize the formation of ER-PM junctions in different excitable cell types. Kv2.1 is expressed in neurons, forming patches that function as traffic hubs for other proteins to reach these specific domains of the membrane. In addition, Kv2.1 is also involved in the cardiac I_{Ks} currents. JPH is crucial for the generation of dyads in cardiac tissue, for neuronal signalling and activation of the T cells. While JPH1-2 are cardiac isoforms, JPH3-4 are expressed in brain. JPHs require the presence of other proteins to generate these membranous contact domains (e.g. JPH2 and Cav1.2 [222,223], whereas JPH4, the isoform used in this work, generates contacts between the ER and the PM by itself.

Similar to the I_{Ks} complex, both Kv2.1 and JPH are expressed in heart and are related to the cardiac action potential. Thus, both could be implicated in the Kv7.1 sarcolemma targeting. Contrarily, JPH1 is expressed in HEK293 cells (**Figure 37**). Therefore, we will use Kv2.1 and JPH4, to promote ER-PM junctions in a heterologous system.

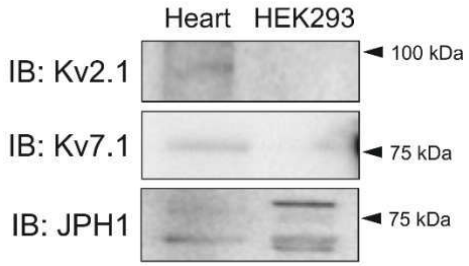

Figure 37. Expression of Kv2.1, Kv7.1 and JPH1 in rat heart samples and HEK293 cells. Total crude protein extracts from rat ventricular cardiomyocytes (Heart) and control (no transfected) HEK293 cells (HEK293) were analysed to determine endogenous protein expression.

4.2.2. Kv2.1 and JPH4 enhance Kv7.1 membrane targeting

Membrane expression of Kv7.1 was analysed by confocal microscopy in transfected HEK293 (**Figure 38**). As previously mentioned in Chapter I, Kv7.1-CFP targets to the membrane and forms timely functional heteromeric channels with KCNE1 recapitulating cardiac I_{Ks} currents. Thus, we studied PM

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4.3. Kv channel expression in endothelial-derived vascular tumors

Chapters 4.1 and 4.2 addressed the importance of targeting and association of the cardiovascular Kv channels for proper physiological functions. Alterations in Kv channel expression lead to several pathologies including cancer. It has been mentioned throughout the work that the malfunction of Kv7.1 triggers cardiac dysfunctions mostly related with the heart physiology. However, the role of Kv7.1 is wider than just cardiac action potential. Kv7.1 and, its vascular partner, Kv7.5 control the vascular smooth muscle tone. In addition, both channels are implicated in the myocyte growth. Therefore, we wanted to analyse whether Kv7.1 and Kv7.5 were somehow remodelled/implicated in vascular tumors. In addition, we wanted to extend our analysis to other Kv channels that have been related with neoplastic processes, including vascular hyperplasia. Kv1.3 and Kv1.5, two isoforms from the Kv1 *Shaker* family, participate in cell proliferation, may form heteromers between them, similar to Kv7.1 and Kv7.5, and are differentially expressed in vascular muscle hyperplasia.

In this chapter we analysed vascular and endothelial derived tumor samples. Vascular musculature and blood vessel walls express ion channels to regulate contraction and relaxation facilitating blood circulation. Specifically, Kv1 and Kv7 channels regulate vascular tone and myogenic function [147,240-242]. Thus, we wanted to study whether some of these ion channels could be remodelled in the development or progression of vascular tumors.

4.3.1. Ion channel expression in blood vessels

First, we analyzed the expression of Kv1 and Kv7 channels in veins and arteries. Before studying the localization of the channels in blood vessel's anatomy, we first confirmed the protein expression of Kv1.3, Kv1.5, Kv7.1 and Kv7.5 in several cardiovascular tissues (**Figure 49**). Rat brain and muscle tissues were used as positive controls. Human blood vessels, including arteries (aorta and carotid) and veins (inferior vena cava and subclavian), expressed all channels. Slight differences in molecular weight are due to isoform variabilities between tissues and species.

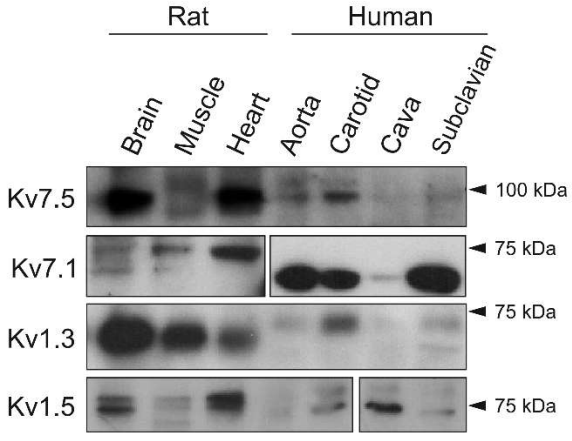

Figure 49. Protein expression of Kv1.3, Kv1.5, Kv7.1 and Kv7.5 in cell membrane extractions from human arteries and veins. Membrane protein extracts were obtained from human blood vessels (aorta and carotid arteries, and inferior cava and subclavian veins) and rat samples as controls (brain, muscle and heart). Protein extracts were loaded in a SDS-PAGE western blot gel to determine the expression.

Once the expression of the four potassium channels was confirmed, we next check controls for the immunohistochemistry analysis. Thus, autofluorescence (**Figure 50**), absence of primary antibody (**Figure 51**) and the presence of antigen peptide controls (**Figure 52**) were performed.

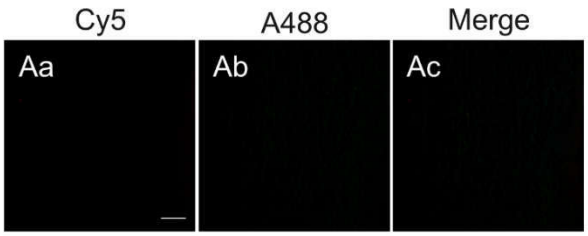

Figure 50. Autofluorescence control in human blood vessel. Fresh human samples were obtained and processed as described in materials and methods. This control is performed before each replica of the experiment. Muscular α-actin was used to identify muscle structures (central, in green). DAPI staining was used to identify cell nucleus (in blue). Right panels should show merge between red and green with nuclear DAPI staining. Carotid artery was chosen as representative. Bar represents 50 µm.

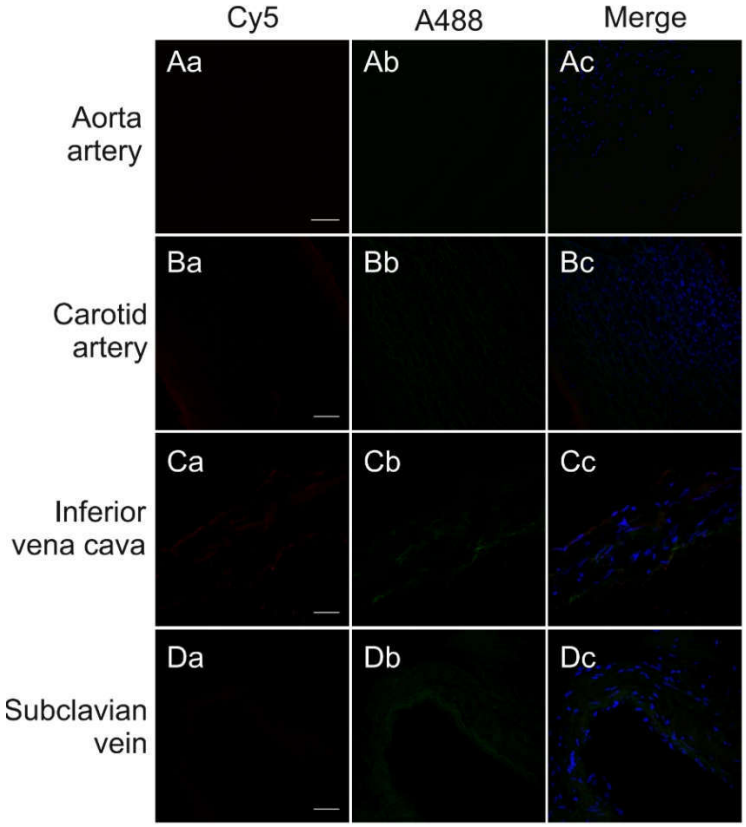

Figure 51. Evaluation of non-specific fluorescence of secondary antibodies in human veins and arteries. Fresh human samples were obtained and processed as described in materials and methods. Slices were stained with the secondary antibodies in the absence of primary antibody. Left panels, Cy5 anti rabbit (in red). Central panels, Alexa488 anti-mouse (in green). Right panels show merge between red and green with nuclear DAPI staining (used to identify cell nucleus, in blue). Scale bars: 50 µm in inferior vena cava; 100 µm in carotid artery and subclavian vein; 150 µm in aorta artery.

Confocal microscopy was used to assess the distribution of Kv1.3, Kv1.5, Kv7.1 and Kv7.5 in the different layers of the vessels. Both arteries and veins were included in the study to further define the distribution in the distinct morphologies of the human cardiovascular system. Representative images of the immunofluorescent labelling are shown in Figure 53. Kv channels studied are differentially expressed in smooth vascular muscle. The expression in the tunica intima showed a great variability. All channels stained aorta and carotid tunica intima, being the endothelial layer predominant over the subendothelial one. Only Kv1.3 was highly expressed in the subendothelial layer. Because cava and subclavian veins are narrow and thin, the distinction of the different layers, especially between the tunica intima and media was difficult. We hardly define between the endothelial or the muscular cells. On the contrary, adventitia or tunica externa is thick and stained

by all channels. However, the abundance in this layer appears lower compared to the muscular or intima.

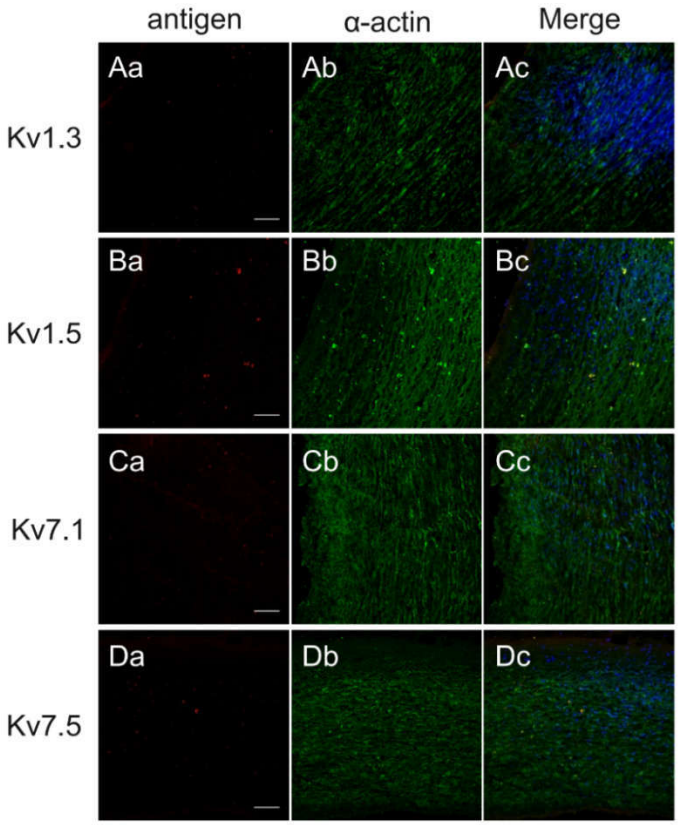

Figure 52. Controls of negative staining for Kv1.3, Kv1.5, Kv7.1 and Kv7.5 in human veins and arteries. Fresh human samples were obtained and processed as described in materials and methods. This control is performed before each replica of the experiment. Slices were incubated in the presence of the antigen peptide provided by the manufacturer for Kv1.3, Kv1.5, Kv7.1 or Kv7.5 (left, in red). Muscular α-actin was used to identify muscle structures (central, in green). Right panels show merge between red and green with nuclear DAPI staining (used to identify cell nucleus, in blue). Scale bars: 100 µm.

4.3.2. Kv channel expression in vascular tumors and malformations

Veins and arteries from the human cardiovascular system express Kv1.3, Kv1.5, Kv7.1 and Kv7.5. In addition, the distribution in wall's vessel was defined. Therefore, we next studied tumor samples. Eight different vascular tumors were analysed and divided into two groups: moderate tumors and highly malignant tumors. The first group included cavernous angioma or hemangioma, capillary angioma or hemangioma, Kaposi's sarcoma, angiosarcoma and glomic tumors. First four are derived from endothelial cells and present different grades of malignancy, from benign or indolent to highly proliferative, respectively. Glomus tumor derives from glomus cells, surrounding the vessels walls. All samples were embedded in paraffin. The second group included three malignant, highly metastatic and very rare tumors: cutaneous angiosarcoma, epithelioid hemangioendothelioma and testicular teratome-derived angiosarcoma. In this case, samples were embedded in OCT compound (Optimal cutting temperature compound). The sample availability was very limited for this group and only one replica was possible. Considering the distinct processing required for their different embedding and the limitation of the sampling and analysis in the second group, the two groups were analysed separately and not compared between them.

We will focus now on the first group. Samples were stained with hematoxilin-eosin to identify the morphological characteristics:

Cavernous haemangioma: "Bening vascular tumor or malformation". Located in the spleen. In the white pulp we find lymphoid aggregates; in the red pulp, sinusoid vascular structures. Angiogenic new blood vessels, also known as congestive vessels, generated by the tumour are highly dilated

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5. General discussion

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6. Conclusions

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