UNIVERSITAT DE BARCELONA

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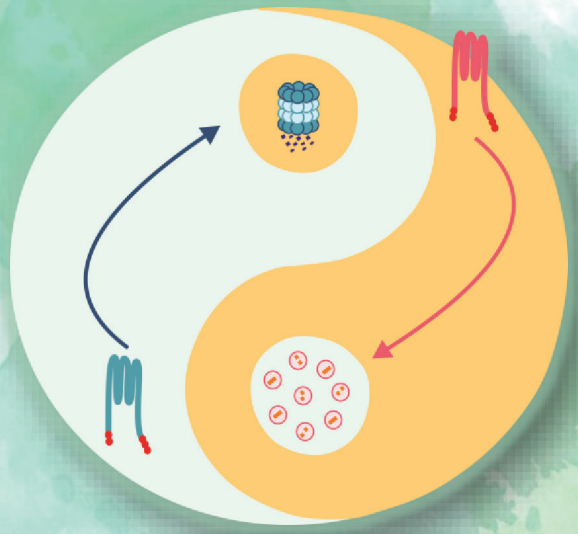

UNIVERSITAT DE BARCELONA

Protein kinase-dependent Kv1.3 biology

Dissertation presented by Irene Estadella Pérez, opting for the qualification of PhD by the University of Barcelona (UB).

This doctoral thesis was performed under the direction of Professor **Antonio Felipe Campo**, in the Molecular Physiology Laboratory in the Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, Institute of Biomedicine (IBUB), University of Barcelona.

Irene Estadella Pérez
PhD candidate

Antonio Felipe Campo
PhD Thesis director
and tutor

PhD Thesis
University of Barcelona
Faculty of Biology
Department of Biochemistry and Molecular Biomedicine

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Abstract

ABSTRACT

The voltage gated potassium channel Kv1.3 is a transmembrane protein that selectively drives potassium ions participating in the electrochemical gradient of cell membranes. This channel presents a wide distribution within the body, thereby playing an important role in several physiological processes, such as regulating the cell volume, proliferation and apoptosis, and leukocyte activation. Of particular interest in this dissertation is the role of Kv1.3 in the immune system, where its activity is crucial to initiate the immune response. Moreover, an increased and/or delocalized expression of the channel is observed at the onset of autoimmune diseases pointing Kv1.3 as a potential therapeutic target. In this contest, the study of the mechanisms involved in the modulation of the amount of Kv1.3 at the plasma membrane deserves considerable attention.

Kv1.3 activity mostly relies in its abundance and proper plasma membrane location, which is tightly regulated by a balance between the forward trafficking and the endocytic machinery. Thus, the control of channel internalization and degradation influences the inflammatory response. The endocytosis of Kv1.3 has been extensively studied in our laboratory and we claim that ubiquitination mediates the internalization and further lysosomal degradation of Kv1.3 via both PKC and EGFR activation. However, the specific residues, among all Kv1.3 intracellular lysines, which play a major role in channel turnover are still unknown. Moreover, although the ubiquitin ligase Nedd4-2 has been proposed to downregulate Kv1.3 activity, there is no clue about how the interaction takes place. In this context, adenosine (ADO), an endogenous key mediator in the immune response, triggers Kv1.3 endocytosis via PKC activation. However, via its A_1 and A_{2A} receptors, ADO not only activates PKC but also PKA. The ADO-dependent Kv1.3 modulation via such differential mechanisms had not been explored yet in immune cells. For that reason, in the present work we aimed to shed light to the mechanism involved in Kv1.3 turnover and thus, provide new knowledge of the molecular physiology of the immune system.

We have deciphered the molecular determinants involved in Ser/Thr kinase (PKC)- and Tyrosine kinase (EGF)-mediated Kv1.3 turnover. Activation of either pathway internalized the channel by the specific ubiquitination of the lysines K70, 84, 476, 498 and 519 which form two clusters at the amino and carboxy terminal domain of the channel. Moreover, our results suggested that these two clusters are also involved in the association between Kv1.3 and Nedd4-2. We confirmed that the Kv1.3-Nedd4-2 interaction is not direct but, a physical proximity (< 40 nm) between the proteins suggested the participation of adaptor proteins.

We have also characterized the PKA-mediated Kv1.3 downregulation which, unlike PKC, did not triggered the endocytosis of the channel targeting Kv1.3 to proteasomal degradation. Moreover, the activation of PKC and PKA pathways using specific ADO receptors agonists efficiently modulate Kv1.3-mediated leukocyte physiology. Thus, ADO exerts an efficient anti-inflammatory response by the activation of two complementary and synergic signalling pathways.

In conclusion, this thesis further expands the knowledge of the molecular mechanisms involved in Kv1.3 turnover. We provide an insight from the molecular determinants to the functional consequences of Kv1.3 downregulation controlling the immune response and the leukocyte physiology. Our results are of considerably physiological interest due to the combination of the multitherapeutic potential of Kv1.3 and ADO.

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List of abbreviations

LIST OF ABBREVIATIONS

8Bromo	8-Bromo-cAMP
ADA	Adenosine Deaminase
ADO	Adenosine
ARs	Adenosine Receptors
BafA1	Bafilomycin A1
BIM	Bisindolylmaleimide
BSA	Bovine serum albumin
CFP	Cyan Fluorescent Protein
CGS	2-[p-(2-carboxyethyl)phenylethylamino]-50 ethylcarboxamidoadenosine **C**
CHX	Cycloheximide
CIE	Clathrin independent endocytosis
CME	Clathrin-mediated endocytosis
CCV	Clathrin-coated vesicle
CPA	N6-Cyclopentyladenosine
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
DUB	Deubiquitin enzyme
EEs	Early endosomes
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmic reticulum
FRET	Förster Resonance Energy Transfer
HA	Hemagglutinin
IL-2	Interleukin-2
IB	Immunoblot
IP	Immunoprecipitation

K^+	Potassium ion
KCh	Potassium channel
Kv	Voltage-gated potassium channel
LEs	Late Endosomes
LPS	Lipopolysaccharide
MG132	Carbobenzoxy-Leu-Leu-leucinal
MgTx	Margatoxin
MOC	Manders' Overlap Coefficient
MVBs	Multy vesicular bodies
NECA	5′-(N-Ethylcarboxamido)adenosine
NEM	N-ethylmaleimide
PBS	Phosphate-buffered saline
PHA	Phytohemagglutinin
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PTM	Post Translational Modification
TBS	Tris-buffered saline
T_{EM}	T effector memory cell
TKR	Tyrosine Kinase Receptor
TGH	Tris-Glycerol-HEPES
UBQ	Ubiquitin
WGA	Wheat Germ Agglutinin
WT	Wild type
YFP	Yellow Fluorescent Protein

1. Introduction

1. INTRODUCTION

1.1. ION CHANNELS

Ion channels are transmembrane proteins that form pores driving selective ions through cell membranes. They are present in all cellular types and are essential for life [1]. From bacterial to complex eukaryotic organisms, membranes are crucial to isolate the inside of cells from the extracellular environment. Plasma membrane, due to its composition, it is selective permeable to hydrophobic and small polar molecules, which can diffuse through the lipid bilayer. However, it is impermeable to ions and large polar molecules, such as amino acids and sugars. Transporters and channels are specific proteins driving these molecules through membranes. Unlike active transporters, ion channels conserve signatures within the pore (selectivity filter) that confer selectivity to specific ions, preventing unspecific interactions, and preserving membrane permeation. Voltage, temperature and pH changes in the plasma membrane, as well as ligands, are some common triggers activating ion channels [2].

Ions (e.g., sodium, potassium, chloride, calcium, etc.) are distributed unevenly on both sides of the plasma membrane. Therefore, the ion composition of the intracellular and extracellular environment differs. Mostly, we have high Na^+ and Cl^- extracellular concentrations and high K^+ and very low Ca^{2+} intracellular concentrations, which along with intracellular organic anions (proteins and amino acids) generates a negative charged cytoplasm. Because the impermeability of the plasma membrane, an electrochemical gradient is generated determining the membrane potential (generally around -90 mV) [3]. The electrochemical gradient and, consequently, the membrane potential, is maintained by the activity of ion channels and the sodium-potassium pump (Na^+/K^+ ATPase). Variations in ion concentration through the membrane would generate changes in its electric charge, causing an immediate effect on membrane potential. In consequence, other channels could detect these membrane potential changes, activate and propagate the electric response [1].

Therefore, ion channels are crucial for excitable cells. In addition to electric impulse, ion channels also take part in other physiological processes such as cell proliferation and apoptosis and lymphocyte activation [4-6]. Their participation in such highly diverse processes highlights their crucial biological relevance.

Since 1952, when Hodgkin and Huxley demonstrated for the first time an ion channel activity using a giant squid axon [7], the knowledge in this field has grown enormously. Lots of specific ion channels blockers were discovered during the 60s, making possible to distinguish between K^+ and Na^+ channels. But it was not until 1976 when Neher and Sakmann developed the patch clamp technique, registering the activity of individual ion channels or, alternatively, the activity of the channels present in the plasma membrane [8, 9]. Later, in 1998, Mackinnon published the first crystalline structure of an ion channel with a 3.2 A resolution using an X-ray diffraction technique [10]. The crystalline structure boosted, in general, the knowledge and understanding of membrane

proteins and, particularly, the membrane insertion mechanisms, the ionic selectivity as well as the association with ancillary subunits and pore modulation. More recently, single particle cryo-electron microscopy (EM) has allowed structural determination of non-crystalline biomolecules in solution at high resolution. Cryo-EM has rapidly become a major tool in structural biology because, besides extending the possible size range for structure determination in solution, it is not a static structure and proteins can be trapped in structural sub-states offering functional information [11, 12].

1.2. POTASSIUM CHANNELS

1.2.1. Classification

Ion channels have been commonly classified according to their ion selectivity (Na^+, K^+, Cl^-, Ca^{2+} channels) and to the mechanisms that control their gating (opening and closure kinetics) [13]. Regarding their gating, the British Pharmacological Society (BPS) and the International Union of Basic and Clinical Pharmacology (IUPHAR) classified ion channels as: (i) voltage-gated, (ii) ligand-gated or (iii) channels using other gating mechanisms, including aquaporins, chloride channels and store-operated calcium channels.

Potassium channels (KCh) are the widest and most diverse group of ion channels, comprising around 90 members [14] (**Figure 1**, in red). KCh stabilize membrane potential bringing it closer to the potassium equilibrium potential, hyperpolarizing the cell, and transporting water and salts [1].

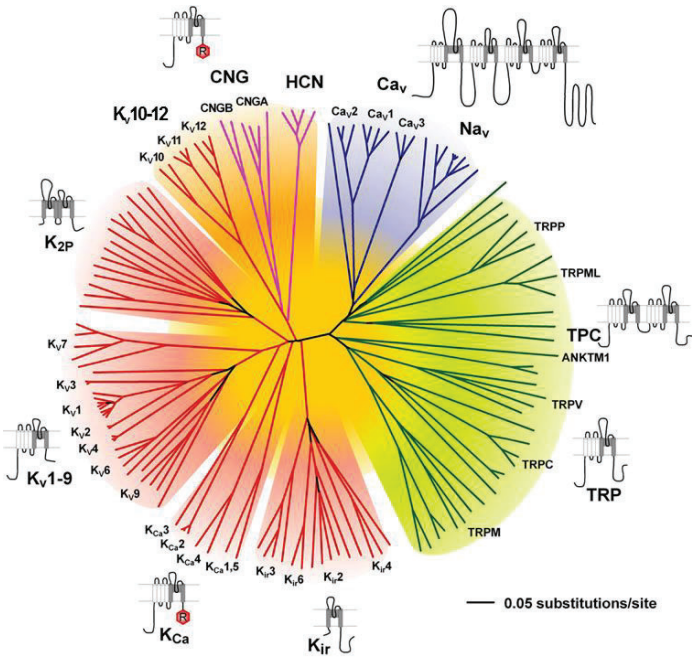

Figure 1. Diversity of ion channels. Unrooted phylogenetic tree representing the amino acid sequence relations of the minimal pore regions of the voltage-gated ion channel superfamily. In this classification, seven groups structurally related and their membrane topologies are underlined, and families are also shown. 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) [15].

According to their structure, KCh families are classified in [14] (**Figure 2**):

- **Inward-rectified channels (K_{ir})** (**Figure 2 A**) are structurally the simplest family. Tetramers are only formed by 2 transmembrane domains and 1 pore (2TM-1P). The pore region (P) is between the two TM domains. The group includes K_{ir} channels, the K_{ATP}, and the G-protein-coupled KCh.
- **Tandem pore domain channels (K2P)** (**Figure 2 B**) are formed by dimerization of two 4 transmembrane domains and 2 pores (4TM-2P). The two P regions are between the first and second TM and the third and fourth TM domains.
- **Voltage-dependent potassium channels (Kv)** (**Figure 2 C**) are tetramers with 6 transmembrane domains, confering voltage sensitivity, and 1 pore (6TM-1P). The P region, containing the K^+-conduction pathway, is between the fifth and sixth TM domain. This group includes the voltage gated KCh (Kv) and the small and intermediate conductance Ca^{2+}-activated KCh (K_{Ca})
- **Calcium-dependent potassium channels (K_{Ca})** (**Figure 2 D**) are formed by tetramerization. They also present 6, and sometimes 7, transmembrane domains and 1 pore (6/7TM-1P). Unlike the other groups, the N-terminus is extracellular. The P region is between the sixth and the seventh TM domain. Most of the members of this family show calcium sensitivity (K_{Ca}), but also include channels sensitive to other ions (such as Na^+ or Cl^-) and voltage-dependent members.

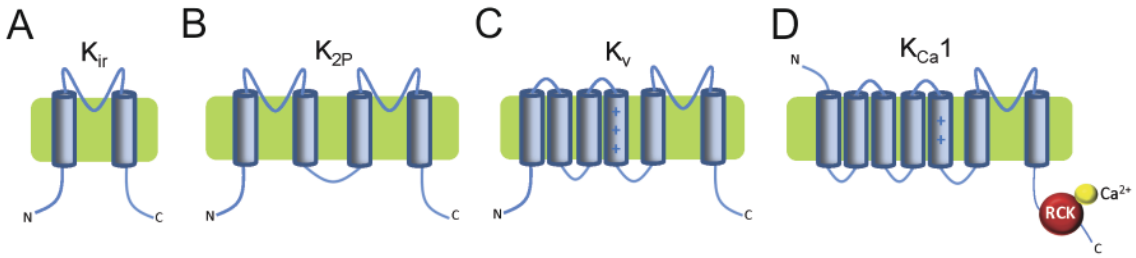

Figure 2. KCh structures. Schematic representation of each KCh family. **(A)** K_{ir} or 2TM-1P. **(B)** K_{2P} or 4TM-2P. **(C)** Kv or 6TM-1P. **(D)** K_{Ca} or 6-7TM-1P. Adapted from Prole and Marrion (2012) [16].

1.2.2. Voltage-gated potassium channels (Kv). Classification

The present work focuses on Kv channels, which are the largest family of KCh, comprising around 40 gens of the human genome. According to the International Union of Pharmacology (IUPHAR) they are classified in twelve families (Kv1-12) with different isoforms [15]. Phylogenetically, we can distinguish two groups: comprising either Kv1-9 and Kv10-12. This last group also presents sensitivity to cyclic nucleotides. Following a functional classification, they can be categorized in four groups:

- **Delayed rectifier channels** (I_{DR}) exhibit a delay before activation. 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. Consequently, the membrane repolarizes, reducing the duration of the nerve impulse. Although they are crucial in excitable cells, such as neurons and muscle, 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 (KCNQ) 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. Characterized by rapid inactivation, these channels open when depolarization occurs after hyperpolarization, and they increase the interval between action potentials. Therefore, I_A helps 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** 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, suggesting a tissue-specific function for the heterotetrameric channels.

- Some channels cannot be classified into any of the abovementioned groups according to their properties. One of them is Kv10.2 which is sometimes defined as a non-inactivating outward-rectifying potassium channel. On the other hand, Kv11.1, a member the hERG family, is a voltage-gated potassium channel but with inwardly rectifying properties. Finally, $K_{Ca}3.1$ channels are activated in response to voltage and Ca^{2+} changes.

1.2.3. Kv channels' structure and function

Kv channels have six transmembrane domains (TM, S1-S6), connected by intra or extracellular loops, and intracellular N- and C-terminal ends (**Figure 3**). The TMs domains are hydrophobic and span the phospholipid bilayers as coiled alpha-helixes. This polypeptide represents de alpha (α) subunit. The homo or heterotetramerization of four α subunits is needed to form a functional channel.

The first four TMs domains (S1-S4) form the voltage sensor domain. Specifically, the S4 domain contains one out three positive amino acids (usually arginine and lysine) spaced by pairs of hydrophobic residues [17]. These amino acids sequences are repeated between 4 and 8 times and it is highly conserved among all sodium, potassium, and calcium voltage-dependent channels, working as the voltage sensor [18].

Between the last two transmembrane spans (S5-S6) we find a short membrane re-entrant loop (P-loop) which contains a consensus sequence (TVGYG) that form the selectivity filter for K^+ when the four α subunits of the channel homo or heterotetramerize [19]. This sequence is highly conserved among all KCh and the permeability to K^+ is 1,000 times higher than to Na^+. The reason resides in the oxygen atoms of the side chains that are projected along the selectivity filter. Although the atomic radius of Na^+ (0.95 Å) is smaller than K^+ (1.33 Å) the oxygen atoms are not closer enough and the loss of water molecules is not energetic favourable. Consequently, several dehydrated K^+ ions cross co-ordinately, but passage of other ions is not favoured [10, 20] (**Figure 3**).

Overall, when there is a change in the electrical field, the S4 moves in response; upon depolarization, the positive amino acids will move within the membrane opening the pore of the channel. In this process, the interaction of the S4-S5 connector with the C-terminal part of the S6 induces the opening of the pore. These changes in intramolecular distances correlate with the voltage dependence of the charge movement. The positive charges of the S4 are stabilized by negative amino acids present in the S1-S3 regions [21-23].

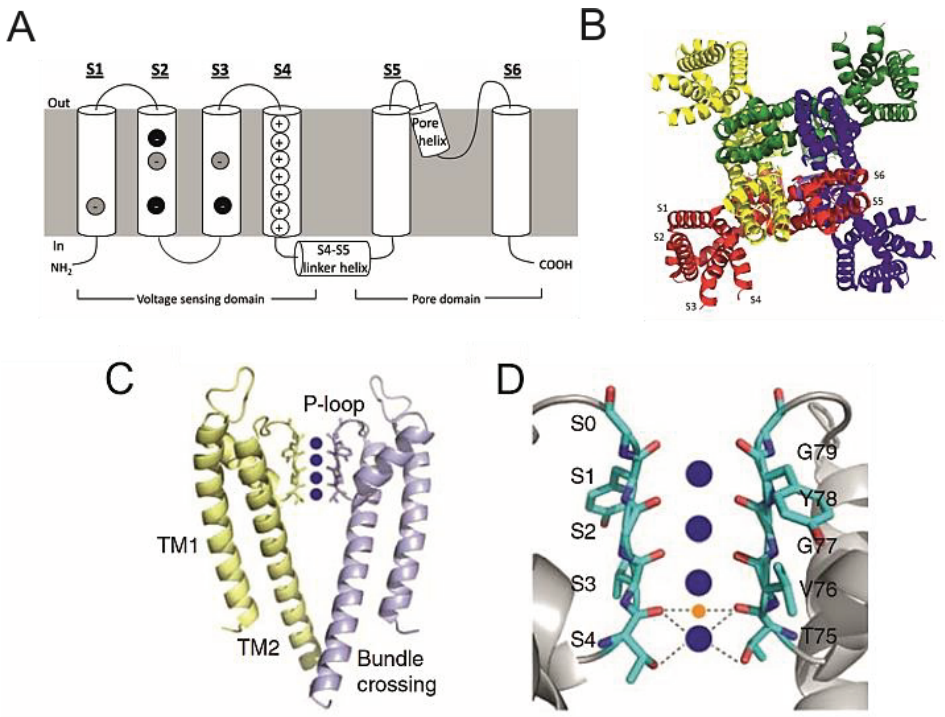

Figure 3. Structure of voltage-gated potassium channels. (A) Schematic representation of the membrane topology of a Kv channel α subunit, showing the voltage sensor domain (S1-S4), the pore domain (S5-S6 and pore helix) and the N- and C-terminal ends. In black, the negative charges evolutionary conserved in the whole family, whereas in grey only the ones present in some members. The positive amino acids in the S4 are shown in white. **(B)** Top view ribbon representation of a tetramerized Kv1.2 crystal structure. Each subunit is represented with a different colour. **(C)** KcsA channel viewed from the side with two opposing subunits removed for clarity. The P-loop contains the signature sequence TVGYG (shown in sticks) and form the selectivity filter. Four K^+ (blue spheres) are shown in the selectivity filter. **(D)** Detailed view of the selectivity filter of KcsA channel. Dashed lines depict coordination of the K^+ ion (blue sphere) in S4 and the oxygens. Na^+ is represent with an orange sphere. A and B from Cheng and Claydon (2012) [24]. C and D from Dorothy and Nimigean (2016) [25].

Additionally, the N- and C-terminal ends of Kv channels are intracellular, facing the cytoplasm. On one hand, the T1 domain, a region of about 100 amino acids located in the N-terminal, close to the S1 segment, is involved the tetramerization of most Kv channels [26]. It is not the case in KCNQ (Kv7) channels, where tetramerization takes places with the C-terminal. The resolution of the Kv1.2 structure together with the ancillary subunit Kvβ2.1 has shown that the T1 domain is also involved in the association with β-regulatory subunits of Kv channels [27-29].

On the other hand, the C-terminal is an intrinsically disordered domain and, due its flexibility, is the only region that its structure could not have been resolved with the rest of Kv1.2 associated with

Kvβ2.1 [29, 30]. Still today, with cryo-EM technology, this domain has not been fully resolved. The C-terminal domain can interact with different proteins such as PSD-95 [31, 32] and different amino acidic sequences have been described such as VXXXS(L/N), which is a forward trafficking signal (FTS) and it is necessary to facilitate the insertion of the channel in the membrane [33].

1.3. Kv1 CHANNELS

Kv1 channels, also named as *Shaker* for their homology with the Shaker channel of *Drosophila melanogaster* Kv1, were the first KCh cloned. A mutation which resulted in the shaking of the legs in the flies under anaesthesia suggested a function in the termination of nerve impulse [34]. Functional channels are, as abovementioned, homo or heterotetramers and generate outward delayed rectifying currents (except Kv1.4, which is an A-type current). The family is currently composed by six members (Kv1.1-Kv1.6), and each one plays a distinct physiological role. In the present work we will focus on member Kv1.3.

1.3.1. 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 [35-37]. Kv1.3 is expressed mainly in the nervous [38] and immune systems [39], but also in other tissues and in certain types of cancer [40, 41]. Kv1.3 is an outward delayed rectifier channel. Therefore, it activates in response of plasma membrane depolarization. The threshold potential for Kv1.3 is around -35 mV and the half activation, which is the voltage where half of the channels are open, is -25 mV [42]. Kv1.3 opens relatively fast, reaching the maximum peak of conductance in about 10 ms after a depolarizing stimulus and single channel conductance is around 14 pS [42]. Kv1.3 exhibits a marked C-type inactivation in front a sustained depolarization with a time constant inactivation (τ; tau) between 250 and 500 ms [35, 43] (**Figure 4 A**). Moreover, due a slow recover from inactivation, Kv1.3 is the only Kv1 channel that presents cumulative inactivation [42], which consists in a progressive decrease in the K^+ current after applying successive depolarizing pulse trains (**Figure 4 B**).

From its wide distribution, Kv1.3 has an important role in numerous physiological processes, both in excitable and non-excitable cells. Kv1.3 contributes to maintain the resting membrane potential, regulates the cell volume, participates in apoptosis and is highly relevant in leukocyte activation. Moreover, Kv1.3 may form heteromeric complexes with other members of the Kv1 family, shaping properties. Alterations in this channel have been related to autoimmune diseases such as multiple sclerosis [44], type I diabetes mellitus [45], rheumatoid arthritis [46], cancer, and obesity, among others [47, 48].

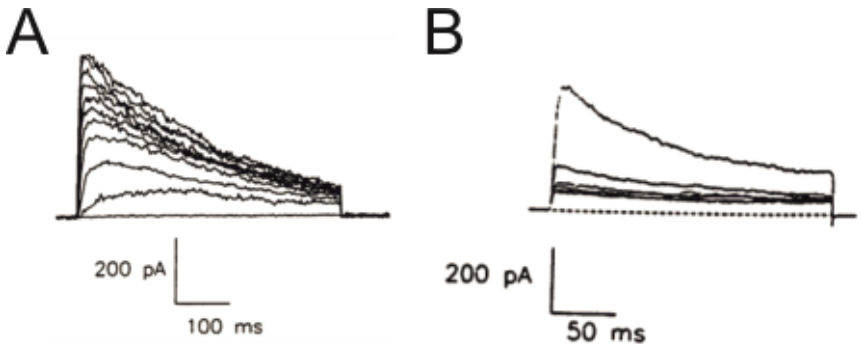

Figure 4. Kv1.3 inactivation properties. (A) Elicited currents in oocytes injected with Kv1.3 mRNA upon applying a series of depolarizing pulses from -50 to + 50 mV in 10 mV increments. C-type inactivation is visible. **(B)** Cumulative inactivation of Kv1.3 upon a train of 6 200 ms at +40 mV, applied at a frequency of 1 Hz. From Grissmer *et al.* (1990) [35].

1.3.2. Kv1.3 in the immune system

Kv1.3 is expressed, in combination with other channels, in immune cells and is crucial during leukocyte activation and proliferation. Specifically, Kv1.3 is the main Kv channel expressed in cytotoxic T lymphocytes (CTL), which are important in the destruction of tumour cells or infected by viruses. In T cells, Kv1.3 is reorganized surrounding the immunological synapse, within lipid rafts, and colocalizes with the T receptor complex (TCR) and CD3 [49]. Thus, Kv1.3 would take part in the cell signalling and regulation of the immunological synapse function having an effect over the immune response [50].

The T cell activation is triggered when the T lymphocyte recognizes an antigen presented by an antigen presenting cell (APC), such as dendritic cells or B lymphocytes. APC, through the MHC II (Mayor Histocompatibility Complex II) and T cells, through the TCR/CD3 complex, presents and detects the antigen, respectively. Then, tyrosine-kinases (TK) pathways are activated leading to phospholipase C (PLC) activation and production of inositol (1, 4, 5)-triphosphate (IP_3) and diacylglycerol (DAG). Consequently, a calcium-dependent signalling pathway in the T lymphocyte is generated. Ca^{2+} from endoplasmic reticulum stores is released and calcium release activated channels (CRAC) activate in response to Ca^{2+} store depletion. CRAC channels, encoded by ORAI1 and STIM1, promote the continuous entry of extracellular Ca^{2+}. Free Ca^{2+} activates calcineurin phosphatase which dephosphorylates the transcription factor NF-AT allowing its accumulation in the nucleus and the transcription of interleukin 2 gene (IL-2). Once IL-2 is produced, T cells proliferate. To sustain this Ca^{2+} signalling long enough to complete the activation of T lymphocytes, cation efflux through Kv1.3 (voltage-dependent) and $K_{Ca}3.1$ (calcium-dependent) leads to membrane hyperpolarization, providing the driving force for Ca^{2+} entry through CRAC channels [6] (**Figure 5**). For that reason, not only is Kv1.3 crucial for lymphocyte activation but also for the proliferation needed to initiate the immune response.

In mononuclear phagocytes, such as macrophages, microglia, Kv1.3 associates with Kv1.5 forming heterotetramers, with a variable composition. Thus, during a proinflammatory trigger the ratio of Kv1.3 increases, while in an anti-inflammatory state decreases [51]. In these cells, Kv1.3 expression is regulated by activation and proliferation stimulus whereas Kv1.5 abundance remains still [52-54]. Considering that Kv1.3 activity is key in proliferation and activation in macrophages [55, 56], and its

expression is tightly regulated, the presence of homo or heterotetramers would condition their level of activation and proliferation [53].

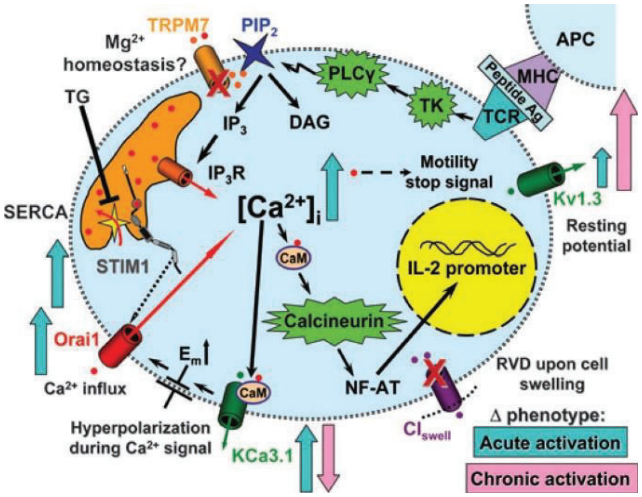

Figure 5. Schematic representation of the signaling pathway upon T lymphocyte activation. The signalling begins with the recognition of an antigen presented by the MHC II of an APC through the TCR/CD3 complex. Then, a calcium-dependent signalling pathway is triggered, resulting in an increase in the intracellular concentration of Ca^{2+} and culminates with the transcription of IL-2 gene. To maintain this signalling, the Ca^{2+} signal must be sustained. Kv1.3 and $K_{Ca}3.1$ channels allow K^+ output generating the driving force needed to the entry of Ca^{2+} through the CRAC channel. Arrows indicate the protein expression changes in acute (green) and chronic (pink) inflammation. From Cahalan and Chandy (2009) [57].

Moreover, Kv1.3 is also expressed in T-effector memory cells (T_{EM}), which are mediators of inflammatory autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. While the up regulation of Kv1.3 in human T cells after activation is limited in resting state and upon activation (around 200-400 Kv1.3 per cell). Following multiple rounds of antigen-stimulation, Kv1.3 is up-regulated and in T_{EM} cells we can find up to 1,500 Kv1.3 per cell. Moreover, in T_{EM}, KCh pattern resembles that of chronically activated T cells.

Therefore, considering all the processes in which Kv1.3 is involved during the immune response, the channel has been validated as a therapeutic target, especially against diverse autoimmune diseases [47]. In fact, NSAIDs (nonsteroidal anti-inflammatory drugs), such as diclofenac, and glucocorticoids, like dexamethasone, reduce Kv1.3 activity in immune cells [53, 58, 59]. Going a step further in this scenario, specific anti-Kv1.3 compounds have been demonstrated to alleviate multiple sclerosis, rheumatoid arthritis, and type I diabetes in rat models [60, 61]. Moreover, the use of specific Kv1.3-blockers would supress the proliferation, cytokine production and migration of T_{EM} without affecting naïve and central memory (T_{CM}) lymphocytes where $K_{Ca}3.1$ channel is up-regulated [39, 60, 62]. Thus, undesired side effects would be minimal, protecting the patient from other immunogenic challenges.

1.4. ION CHANNEL BIOGENESIS AND TRAFFIC

Kv channels are synthesized in the rough endoplasmic reticulum (ER) [63] but, unlike other membrane proteins, they do not have a canonical ER target signal. Instead, the TM2 functions as a signal sequence in Kv1.3 [64]. Once translocated into the ER lumen, subunit oligomerization takes place and, as above mention, tetramerization signatures can be located at either N- or C-terminal of the subunits. Misfolded proteins and aggregates are targeted to proteasomal degradation by the

ER-associated degradation (ERAD) pathway. Then, Kv tetramers exit the ER after a quality control checkpoint [65].

Concerning signal sequences, while RXR, KKX or KDEL promote the intracellular retention of Kv [66-68], VXXSL, HRETE or the non-canonical YMVIEE promote the anterograde transport [33, 69, 70]. In addition, the association of ancillary subunits and accessory proteins is important in determining protein fate because protein-protein interactions can expose or hide traffic motifs, as well as bend the structure of KCh, promoting specific targeting. In this context, KCNE4, a regulatory subunit that associates and retains Kv1.3 in ER by masking the forward trafficking signal (YMVIEE) of the channel [71]. Therefore, the forward trafficking of Kv channels depends on the equilibrium of several different inputs.

Most transmembrane proteins, including Kv channels, follow the canonical pathway from ER, through the ER-Golgi intermediate compartment (ERGIC) and Golgi compartments. However, some Kv channels follow unconventional pathways bypassing the Golgi apparatus [72] (Figure 6). Kv7.1/KCNE1 complexes are one example, which assemble at ER-plasma membrane junctions to each the plasma membrane [73].

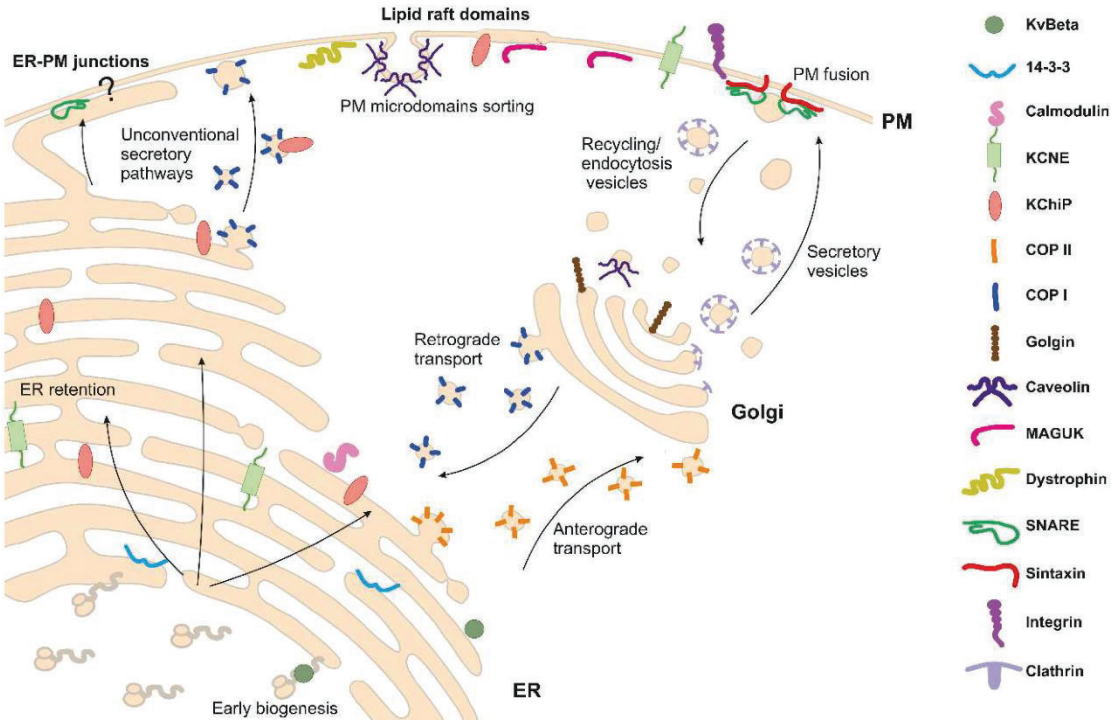

Figure 6. Schematic representation of traffic mechanisms and molecular associations of K^+ channels through secretory pathways. Different shapes represent molecules (see list on the right) known to associate with channels in different compartments. Arrows indicate the major anterograde and retrograde routes. Mechanisms could differ depending on the KCh and the interacting protein. From Capera *et al*. (2019) [72].

Moreover, channels undergo post-translational modifications (PTMs) which modulate their traffic. N-Glycosylation, which starts in the ER, is one of the main PTMs and promotes the surface expression of many KChs [74]. Another PTMs is the O-glycosylation which is mainly a Golgi

modification. Moreover, reversible PTMs, such as phosphorylation and ubiquitination can also take place in the Golgi apparatus. Other PTMs like redox modifications or lipidations have also been described. Acylation increases 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 surface expression [75] whereas it enhances $K_{Ca}1.1$ membrane targeting [76, 77].

1.5. PROTEIN TURNOVER

The activity of ion channels relies on their abundance, proper cell-surface localization, and intrinsic properties. Thus, cells can regulate ion channels activity quantitatively (the number of channels) or qualitatively (alterations to their biophysical properties) [78]. Quantitatively, changes in gene/protein expression as well as association with several ancillary subunits influence ion channel membrane abundance [72]. However, once inserted into the plasma membrane, ion channels can be internalized and either recycled or degraded. Therefore, the membrane abundance of ion channels relies on a balance between the secretory and the endosomal trafficking. Disruption of this balance is associated with diseases, especially those linked with aging and neurodegeneration [79-81]. Overall, the endocytic system has emerged as a crucial mechanism in the regulation of cell signaling and membrane dynamics, in addition to nutrient uptake and signal transduction initiated by cell surface stimuli, the regulation of cellular metabolism, and cell-to-cell communication.

1.6. ENDOCYTIC PATHWAYS

The endocytic network starts with the internalization of the cargo protein via clathrin-dependent and -independent mechanisms. Internalized vesicles fuse to early endosomes (EEs), which mature into late endosomes (LEs). Early and late endosomes sort cargo destined for one of two fates: they are recycled - by transport to the plasma membrane or to a secretory pathway compartment - or degraded in lysosomes [82, 83]. Moreover, cargo proteins recycle to the cell surface by two mechanisms: (i) the fast recycling pathway, directly from the EEs, and (ii) the slow recycling pathway, using specialized recycling endosomes (REs), which are frequently clustered in the perinuclear-localized endocytic recycling compartment (ERC) [83].

1.6.1. Clathrin-dependent endocytosis

Clathrin-mediated endocytosis (CME), the most studied endocytic pathway, is a common route of ion channels internalization [84-89]. CME involves the recruitment of transmembrane proteins (cargo) into small areas of the plasma membrane coated with clathrin on the cytoplasmic face of

the membrane (termed clathrin-coated pits, CCPs). Next, the coated membrane invaginates further until the clathrin-coated vesicle (CCV) carrying cargo is pinched off. The main component of CCPs and CCVs, clathrin, is a trimer of heavy chains (170 kDa), each associated with a light chain (25 kDa), to form a clathrin triskelion that polymerizes, forming a hexagonal coat covering the membrane [90]. Because clathrin cannot bind to the lipid bilayer adaptors are required to initiate the formation of CCPs. In fact, more than 50 ancillary cytosolic proteins are involved in CCP formation, invagination and CCV budding. Free CCVs are rapidly uncoated and fused with EEs [91-93].

Cargo recruitment is achieved by the recognition of internalization signals (linear sequence motifs, conformational determinants, and covalent modifications) predominantly located in the cytosolic region of cargo proteins. The AP-2 complex and other adaptors, named clathrin-associated sorting proteins (CLASPs), which are in the inner layer of clathrin coats, bind cargo [94, 95]. CME also requires the action of dynamin, which catalyses the constriction of the neck of membrane invagination, leading to the scission of a CCV [91-93] (**Figure 7**).

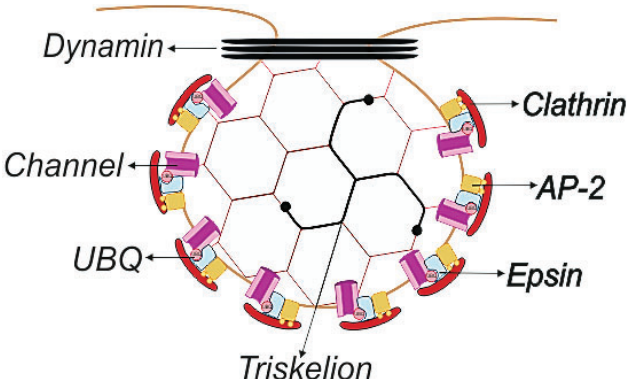

Figure 7. Schematic representation of clathrin-coated pit (CCP) structure. Clathrin, the major protein in CCPs, forms a triskelion that polymerizes to form a hexagonal coat that covers the membrane. The AP-2 complex links clathrin to the membrane and coordinates the assembly of the coat that covers the membrane. In addition, epsins couple ubiquitinated membrane proteins into CCPs and contribute to the membrane curvature during the formation of clathrin-coated buds. Finally, dynamin catalyzes the constriction of the "neck" of the membrane invagination and the scission of the clathrin-coated vesicles (CCVs) from the plasma membrane. Colour code: Red, clathrin; Orange, AP-2 complex; Blue, epsin; Black, dynamin; Light red, ubiquitin; Pink, ion channel. From Estadella *et al.* (2020) [96].

1.6.1.1. Linear sequences

Tyrosine-based motifs (YXXΦ) are involved in the CME of ion channels. One example is the voltage-dependent K^+ channel Kv7.1 which contains a C-terminal tyrosine signal (^{662}YEQL^{665}). The alteration of this motif prevents the AP-2- and clathrin-dependent internalization of Kv7.1 upon alpha-1-adrenergic receptor (α_1-AR) activation [86]. Another example is the ATP-sensitive K^+ (K_{ATP}) channel, which is involved in insulin secretion, and is composed of Kir6.2 and the sulfonylurea receptor (SUR1) subunits in pancreatic cells. K_{ATP} channels undergo rapid CME that is dependent on a tyrosine signal (^{330}YSKF^{333}) located in the C-terminus of Kir6.2. Disruption of this motif abolishes channel endocytosis, elevating channel surface expression and suppressing insulin secretion, thereby causing permanent neonatal diabetes mellitus [79]. On the other hand, the inward rectifier potassium channel Kir1.1 undergoes CME through an alternative tyrosine motif, [F/Y]XNPX[Y/F].

Kir1.1 is recruited into CCPs by phosphotyrosine-binding (PTB) domain-containing CLASPs, Disabled-2 (Dab2) and the autosomal recessive hypercholesterolemia (ARH) protein [97].

Furthermore, some di-leucine motifs [D/E]XXXL[L/I] are recognized by AP-2. The TWIK-related acid-sensitive K^+ (TASK, $K_{2P}3.1$) channel (belongs to a family of two-pore domain K^+ channels) contains two tyrosine-based motifs and one di-leucine ($EHRAL_{263}L$) motif. Nerve growth factor (NGF) triggers the CME of $K_{2P}3.1$, and in contrast to the tyrosine-based motifs, the di-leucine signal was partially responsible for the endocytosis [98].

1.6.1.2. Ubiquitination

This reversible posttranslational modification (PTM) involves the covalent attachment of ubiquitin (UBQ), a small 8 kDa protein, to lysine residues of a target protein. UBQ is a highly conserved 76 amino acid protein expressed in all eukaryotic cells. Ubiquitination can regulate a wide variety of processes, from protein degradation by proteasome, DNA repair and transcription, to membrane trafficking acting as an endocytic signal [99, 100]. Ubiquitination involves the sequential action of three families of enzymes: E1 or ubiquitin-activating, E2 or ubiquitin-conjugating and E3 or ubiquitin-ligase enzymes. The first step requires an ATP-dependent activation of the C-terminus of UBQ and its conjugation to the active site of an E1 enzyme through a thiol-ester bond. Next, UBQ is transferred to a similar cysteine residue located in the active site of an E2 enzyme. Finally, an isopeptide bond with the ε-amigo group of a lysine residue in substrate proteins is executed by E3 enzymes. UBQ can be removed by peptidases named deubiquitinating enzymes (DUBs) [101] (Figure 8).

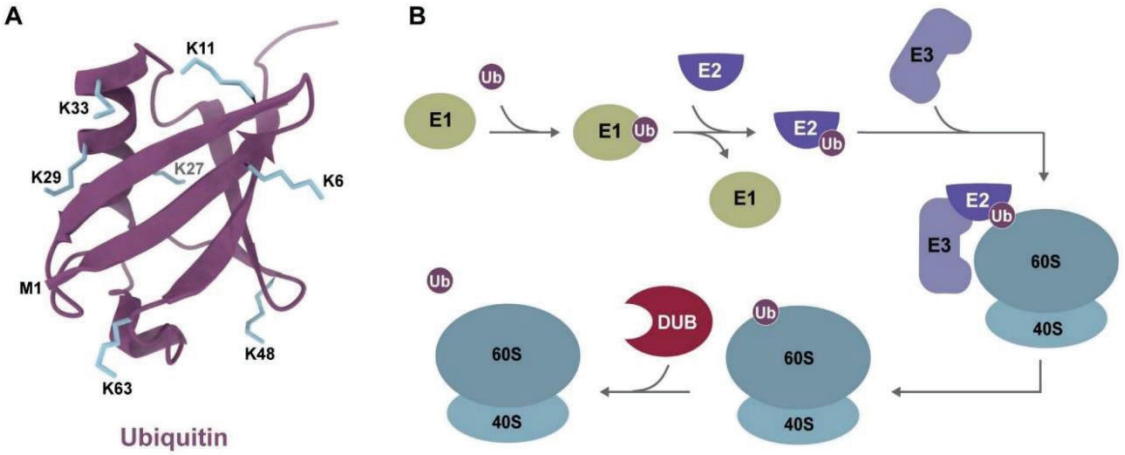

Figure 8. (A) Ubiquitin structure. Lysine residues (K) and the amino group of the first methionine residue (M1) are shown. **(B) Schematic representation of the ubiquitin enzymatic cascade.** The sequential action of E1 activating enzyme, E2 conjugating enzyme and E3 ligase are represented. Ubiquitin modifications can be reversed by deubiquitin enzymes (DUB). From Dougherty *et al.* (2020) [102].

Proteins can be differentially ubiquitinated: (i) monoubiquitination (attachment of one UBQ moiety to a single lysine residue), (ii) multi-monoubiquitination (attachment of one UBQ to several lysine

residues of a substrate) and (iii) polyubiquitination (attachment of additional UBQ molecules to UBQ(s) on a lysine residue to form a UBQ chain) [99, 100, 103, 104]. Moreover, polyubiquitin chains can be homotypic (one linkage type) or heterotypic (forming branched or mixed chains). Although UBQ contains 7 lysine residues for self-ubiquitination, Lys^{48}- and Lys^{63}-linked chains are the most abundant. Polyubiquitinated Lys^{48}-linked chains mainly play a role in proteasomal degradation. Polyubiquitinated Lys^{63}-linked chains target a protein for lysosomal degradation, induce the endocytosis of membrane proteins, and are involved in DNA damage repair, ribosomal function and NF-κB signaling [99, 104, 105].

In addition to its 7 lysine, an eighth chain type (Met1-linked) is generated when UBQ is attached to the N-terminus of a second UBQ [106]. Furthermore, not only can UBQ be ubiquitinated but can also be phosphorylated and acetylated, which provides an additional regulation in the ubiquitin system [107] (Figure 9).

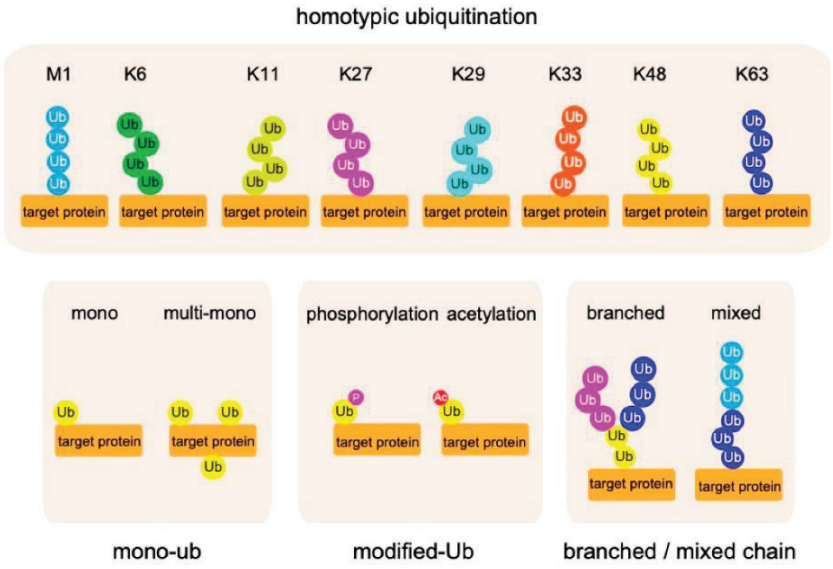

Figure 9. Schematic representation of the complexity in the ubiquitin code. Target proteins can be mono or polyubiquitinated. There are known eight types of homotypic ubiquitin linkages: M1, K6, K11, K29, K33, K48 and K63. Moreover, posttranslational modification of UBQ itself (P, phosphorylation; Ac, acetylation) and heterotypic UBQ chains containing different linkage types have been identified. From Fuseya and Iwai (2021) [108].

The UBQ signal is recognized by specific domains called ubiquitin-binding domains (UBDs) or ubiquitin-interacting motifs (UIMs) [109]. Among the adaptors that contain UBDs, Epsin1, 2 and 3 and their binding partners Eps15 and Eps15R deserve an especial mention. In general, Epsins and Eps15/R are adaptors that are believed to cooperative in the recruitment of ubiquitinated cargo to CCPs. Moreover, Epsins are thought to contribute to membrane curvature during CCV formation [105, 110]. The epithelial sodium channel (ENaC) is one of the most characterized ubiquitinated ion channels. CME and ubiquitination contribute to ENaC internalization. Channel subunits interact with Epsin, and both ENaC and Epsin interact with clathrin adaptors. The overexpression of Epsin downregulates ENaC activity in CHO cells and oocytes. This effect is dependent on the UBD of Epsin and ENaC ubiquitination and its interaction with the E3 ubiquitin ligase Nedd4-2 [111, 112].

1.6.2. Clathrin-independent endocytosis

Clathrin-independent endocytosis (CIE) comprises the other internalization pathways that require neither the clathrin-associated machinery nor a clathrin coat. This mechanism was first described for the entry of bacterial toxins. However, a variety of cargo proteins can enter cells by CIE, such as transporters, ion channels, cell adhesion molecules and immune cell receptors [113].

CIE facilitates two types of endocytosis: large micrometer-scale pathways (macropinocytosis and phagocytosis) and a spectrum of smaller (< 200 nm) scale processes [114]. The latter group can be further classified by whether dynamin, a large GTPase, participates – dynamin-dependent or dynamin-independent mechanisms – or whether other components of the endocytic machinery, such as small GTPases (RhoA, CDC42/ARF1 and ARF6-regulated), are involved [115] (**Figure 10**). In addition, evidence suggests that other, yet unidentified, CIE pathways might be involved.

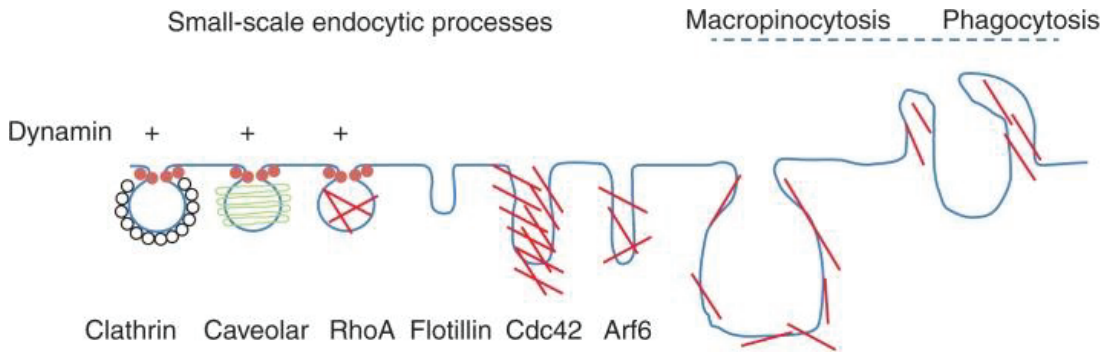

Figure 10. Schematic representation of the endocytic pathways. The scheme represents the multiple pathways by which a cargo located at the plasma membrane or in the extracellular medium is internalized. Dynamin-dependent pathways (+, red circles) are typically associated with small-scale endocytic processes. Contrary, the dynamin-independent pathways reflect a larger diversity of forms, ranging from small-scale (Flotillin, Cdc42 and Arf6-dependent pathways) to larger scale invaginations (micropinocytosis and phagocytosis). The main effectors of CIE pathways are indicated below their invaginations. Red bars represent actin filaments. From Mayor *et al*. (2014) [114].

1.6.2.1. Mechanisms dependent on caveolin

Caveolae-mediated endocytosis is the best characterized dynamin-dependent CIE pathway. Caveolae are specialized membrane invaginations (50-80 nm) marked by the presence of caveolin, an integral membrane protein (21 kDa) with cytosolic N- and C-terminal domains connected by a hydrophobic sequence. Caveolae, enriched in sphingolipids and cholesterol, concentrate signaling receptors and effectors [114, 115].

The Kir6.1 channel, the major vascular K_{ATP} isoform, is mainly localized in the caveolae of aortic smooth muscle cells. Caveolae disruption, with MβCD (Methil-b-cyclodextrin) or caveolin-1 siRNA, prevents the PKC-induced internalization of Kir6.1, suggesting that caveolae compartmentalization plays a functional role [116]. The transient receptor potential vanilloid 5 (TRPV5) channel is a gatekeeper for transepithelial Ca^{2+} reabsorption into the kidney. TRPV5 undergoes constitutive caveolin-dependent endocytosis, and PKC activation inhibits its caveolin-dependent internalization, leading to an increase in channel cell surface abundance [117].

The analysis of caveolar endocytosis is rather difficult because the same endocytic cargo may internalize by different mechanisms or may switch pathways under different conditions. In this context, the evidence indicates that TRPV5, which undergoes caveolin-dependent endocytosis, is also partially internalized by CME to enter a Ca^{2+}-controlled recycling pathway [118]. In this complex scenario, the renal outer medullary Kir1.1 channel, critical for K^+ secretion into cortical collecting ducts, is another example. As described above, the C-terminus of Kir1.1 contains the tyrosine-based NPXY internalization motif ([F/Y]XNPX[Y/F]) involved in CME. In addition, Kir1.1 interacts with clathrin and α-adaptin (an AP-2 subunit) [97, 119]. However, evidence suggests a CIE alternative. Thus, caveolin-1 decreases Kir1.1 abundance at the plasma membrane and inhibits channel activity. Deletion of the Kir1.1 clathrin-endocytosis motif fails to abolish the effect of caveolin-1 on channel activity. Moreover, the expression of microRNA 802, which suppresses caveolin-1, increases channel activity [120].

1.6.2.2. The RhoA-dependent mechanism

It is another internalization mechanism mediated by dynamin but dependent on the small GTPase RhoA. The RhoA-dependent mechanism was identified during the study of interleukin-2 receptor (IL-2R-β) internalization [115]. RhoA suppresses Kv1.2 currents by modulating channel endocytosis. This takes place via two different pathways: (i) clathrin-dependent and (ii) cholesterol-dependent mechanisms. Activation of Rho kinase (a RhoA effector, ROCK) via the lysophosphatidic acid (LPA) receptor triggers the CME of Kv1.2. This effect is blocked either by ROCK inhibition or clathrin RNA interference. However, constitutive Kv1.2 endocytosis is highly dependent on cholesterol levels and ROCK activity. The inhibition of ROCK and cholesterol alteration by filipin increases Kv1.2 membrane expression, whereas clathrin RNA interference shows no additive effect [121].

1.6.2.3. The ARF6-dependent pathway

The ARF6-regulated mechanism is a dynamin-independent CIE pathway. Some ion channels containing acidic clusters are recruited into the ARF6-regulated recycling pathway. The Kir3.4 channel, which has two types of acidic motifs (potassium acidic clusters, KACs), localizes into ARF6-positive vacuolar structures; however, a KAC-deleted mutant failed to enter an ARF6 compartment. Evidence suggests that cargo proteins entering an ARF6 structure can be either recycled to the cell surface or redirected to Rab5 endosomes. In this way, Kir3.4 inside an ARF6 compartment is recycled back to the plasma membrane rather than routed to Rab5 endosomes [122].

The hERG (human ether-a-go-go related gene) potassium channel (Kv11.1) is critical for the repolarization of the cardiac action potential. Reduced Kv11.1 cell surface expression correlates with long QT syndrome and an increased risk of acquiring ventricular arrhythmias [123]. The channel undergoes rapid internalization into endosomes, but neither dynamin inhibition nor dominant negative Rab5 prevent its internalization. However, cholesterol depletion by MβCD and inhibition of ARF6 activity significantly affect its endocytosis [124].

1.6.3. Stimulus induced endocytosis

1.6.3.1. Receptor-mediated internalization

Receptor activation triggers PTM of ion channels, such as phosphorylation and ubiquitination, which induce internalization. For example, Kv1.2 undergoes tyrosine phosphorylation and functional suppression through the activated M1 muscarinic ACh receptor (mAChR) and stimulated epidermal growth factor receptor (EGFR) [125]. The receptor-mediated tyrosine phosphorylation of Kv1.2 reduces its interaction with cortactin, which links the channel to the actin cytoskeleton, thereby leading to endocytosis and decreased Kv1.2 current [126]. Similarly, EGFR activation leads to Kv1.3 phosphorylation [127], but in contrast to Kv1.2 phosphorylation, Kv1.3 endocytosis is independent of this tyrosine phosphorylation. EGFR signals downregulate Kv1.3 via two complementary mechanisms: (i) tyrosine phosphorylation of the channel reduces the Kv1.3 current, and (ii) an unconventional ERK1/2 kinase-dependent mechanism triggers channel endocytosis [128]. In contrast to their effect on Kv1.3 and Kv1.2, the activation of tyrosine kinase receptors enhances, rather than suppresses, the Kv7.1 current [129]. Kv7.1, in association with the KCNE1 β-subunit, recapitulates the cardiac I_{Ks} current. Stimulation by α_1-AR suppresses the Kv7.1/KCNE1 current and triggers the ubiquitin-dependent CME by activating AMPK [86].

1.6.3.2. Drug-induced endocytosis

Kv11.1 (the hERG channel), which is critical for cardiac action potential repolarization (I_{Kr}), must be tested in preclinical safety assays for all potential drugs. Acquired long QT syndrome is the most common adverse cardiac effect caused by antidepressants. This pathology has been related to syncope, Torsade de Pointes arrhythmias and sudden cardiac death. Long QT syndrome might be caused by a direct blockade of Kv11.1 or by indirect inhibition of its trafficking [81, 130]. Desipramine, a tricyclic antidepressant, simultaneously blocks and indirectly inhibits the channel causing acquired long QT syndrome. Desipramine increases ubiquitin-dependent Kv11.1 internalization and degradation, as well as impedes channel trafficking from the endoplasmic reticulum [131].

On the other hand, quinidine, a class I antiarrhythmic drug, triggers Kv1.5 endocytosis. Kv1.5, participating in ultrarapid cardiac K^+ current (I_{Kur}), controls the duration of the atrial action potential. The treatment of human atrial myocytes with quinidine triggers Kv1.5 internalization and simultaneously blocks the channel. Moreover, acute quinidine-induced endocytosis is reversible, whereas chronic treatment results in channel proteasomal degradation [132].

1.6.3.3. Low K^+-induced endocytosis

A reduction in extracellular K^+ concentration (hypokalaemia) is a risk factor for long QT syndrome. Thus, low extracellular [K^+] triggers Kv11.1 channel degradation through the multivesicular body (MVB)/lysosomal pathway. Upon reduction of extracellular [K^+], Kv11.1 undergoes a conformational change, which results in channel degradation. Kv11.1 endocytosis is concomitant with the monoubiquitination of the channel and is dependent on caveolin [133]. However, as explained above, the study of caveolar endocytosis can be complex because of overlapping

alternative mechanisms. Thus, the ARF6-mediated mechanism might also be involved in the Kv11.1 recycling pathway [124]. In fact, Kv11.1 channels coimmunoprecipitate and colocalize with caveolin-1 (Cav1) under hypokalemic conditions. Moreover, the knockdown of Cav1 hampered the endocytosis of Kv11.1 channels in HEK-293 cells. Furthermore, knocking down caveolin-3 (Cav3) prevented the low K^+-induced reduction in Kv11.1 current in cultured neonatal rat ventricular myocytes [134].

1.7. COMPONENTS OF ION CHANNEL ENDOCYTOSIS

1.7.1. Ubiquitin ligases

The UBQ system regulates many cellular processes and is of major interest because of the ubiquitin-mediated degradation of ion channels. E3 ubiquitin ligases are crucial to target selection for ubiquitination. Ubiquitin ligases are structurally divided into 4 groups. The two main groups are (i) HECT-type E3 ligases, which contain a HECT (homologous to E6-AP COOH terminus) domain that forms a thioester bond with UBQ to ubiquitinate the target protein directly, and (ii) RING finger E3 ligases, which contain a RING (really interesting new gene) finger domain, acting as adaptor proteins for the E2 ligases and facilitating the transfer of UBQ from E2 directly to the substrate [103, 135, 136] (**Figure 11**).

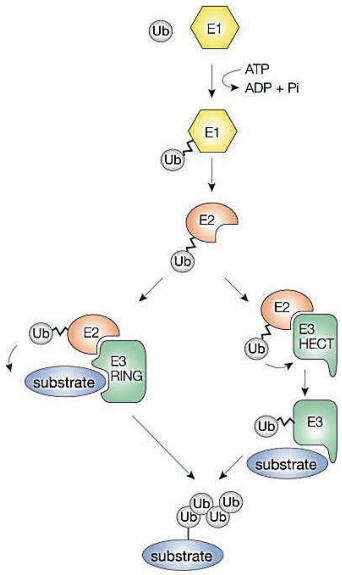

Figure 11. Schematic representation of the ubiquitination process. A hierarchical set of the three types of enzymes is required for substrate ubiquitination: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligase (E3) enzymes. The two major classes of E3 ligases are represented: (i) E3 HECT: a thioester bond with ubiquitin (Ub) is formed and ubiquitinates the substrate directly, and (ii) E3 RING: which act an adaptor protein for the E2, facilitating the transfer of ubiquitin from E2 directly to the substrate. From Woelk *et al* (2007) [99].

The Nedd4/Nedd4-like protein family consists of the major ion channel negative regulators. Kv1.3 [137], which is the member we will focus on this present work, and other ion channels such as ENaC [138], CLC-2 [139], some Nav (voltage-gated sodium channels) [140], Kv7.1 [141], Kv7.2/Kv7.3 [142] and Kv11.1 [137, 143] are regulated by Nedd4-2. The Nedd4/Nedd4-like family comprises 9 members in humans and belongs to the HECT-type ubiquitin ligase group [135, 144]. An altered Nedd4-2 function is implicated in hypertensive disorders such as Liddle's syndrome [145], epilepsy [146], neuropathic pain and long QT syndrome [147].

The Nedd4-like family exhibits a catalytic HECT domain at the C-terminus; a C2 domain at the N-terminus, which binds to phospholipids in a calcium-dependent manner; and from 2 to 4 tandem WW (tryptophan) domains, which mediate binding to specific substrates. Two WW domains specifically interact with conserved PY motifs (PPXY or LPXY sequences) or proline-rich regions in proteins [148] (**Figure 12**).

ENaC is clearly the best-studied example of a ubiquitinated ion channel by the Nedd4-like family. The C-terminal regions of each channel subunit contain a PY motif (PPXYXXΦ), which overlaps with a tyrosine-based motif (YXXΦ) related to CME. The mutation of the ENaC PY motifs causes Liddle's syndrome, an inherited hypertension disorder triggered by increased channel activity. The PY motif of ENaC recruits Nedd4-2 and downregulates channel surface expression via ubiquitination. In Liddle's disease, the loss of the PY-binding motif, where Nedd4-2 attaches, reduces the ubiquitination of ENaC and, consequently, increases the number of channels at the plasma membrane [149, 150]. More interestingly, the serum and glucocorticoid-regulated kinase (SGK), a downstream mediator of aldosterone, reduces the binding of Nedd4-2 to ENaC by phosphorylating Nedd4-2 and thus also increases ENaC cell surface expression and regulates epithelial Na^+ absorption [151].

Nedd4-2 also targets Nav channels, which are critical for maintaining the action potential in most excitable cells. Among the 10 isoforms of Navs channels, 7 contain a PY motif in the C-terminus [140] (**Table 1**). Laedermann and colleagues, by using the spread nerve injury (SNI) model of traumatic nerve injury-induced neuropathic pain, showed a reduction in Nedd4-2 expression in dorsal root ganglion (DRG) cells associated with an increase in Nav1.7 and Nav1.8 expression and function. In addition, the nociceptive DRG neuron-specific knockout of Nedd4-2 triggered the dysregulation of Nav1.7 and Nav1.8 expression, similar to that obtained with the SNI model. Moreover, animals show an altered nociceptive pain phenotype, which is a feature of peripheral neuropathic pain, characterized by the dysregulation of Nav channels [152].

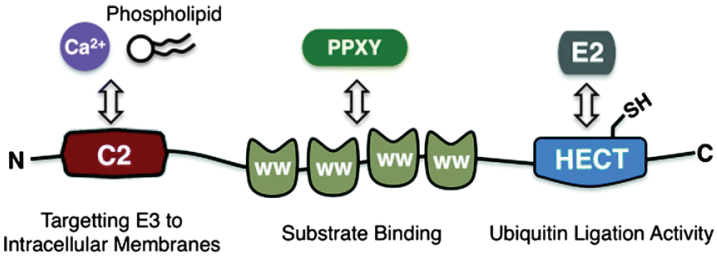

Figure 12. Schematic structure of the Nedd4-like E3 ligase family. Nedd4 family members have 3 domains: (i) an N-terminal C2 domain which binds Ca^{2+} and phospholipids and targets the ligase to intracellular membranes; (ii) from 2 to 4 WW (Trp-Trp) domains that bind the conserved sequence PPXY (PY motif) on substrates; (iii) catalytic HECT domain which forms a thioester bound with ubiquitin. From Heeseon *et al.* (2014) [148].

Nevertheless, the presence of a PY motif is not always mandatory for Nedd4-like function. For example, Kv1.3 [85, 137, 153] and Kv1.5 [154] have no canonical PY motifs. This evidence suggests either alternative mechanisms of binding or the presence of yet undiscovered intermediate adaptors that facilitate the interaction of a ubiquitin ligase and a target.

In addition to ubiquitin ligases acting at the plasma membrane, other E3 enzymes can be recruited into the early endosomes (EEs) and ubiquitinate cargo proteins prior to sorting. The sorting in the

EEs is mediated by the endosomal sorting complex required for transport (ESCRTs). ESCRT-0 contacts the ubiquitinated cargo, and ubiquitination is necessary for sorting. Whether the cargo is ubiquitinated before EE formation the target can be degraded [155]. c-Cbl is a member of the RING finger E3 ligase family with an N-terminal tyrosine kinase-binding domain (TKB) and an extended C-terminal tail with proline-rich motifs and a UBD. c-Cbl regulates CFTR by two mechanisms: (i) acting as an adaptor protein facilitating CFTR endocytosis by a UBQ-independent mechanism and (ii) ubiquitinating CFTR in EEs to promote lysosomal degradation [156].

Protein Name	Gene Name	Accession Code (UniProtKB)	PY Motif	Position
NEDD4-1	NEDD4	P46934	LPPY	L1292–Y1295
NEDD4-2	NEDD4L	Q96PU5	LPPY	L948–Y951
NDFIP1	NDFIP1	Q9BT67	PPPY	P39–Y42
			PPSY	P64–Y67
NDFIP2	NDFIP2	Q9NV92	PPPY	P148–Y151
			PPPY	P174–Y177
SGK1	SGK1	O00141	PPFY	P295–Y298
SGK2	SGK2	Q9HBY8	PPFY	P292–Y295
SGK3	SGK3	Q96BR1	PPFY	P359–Y362
αENaC	SCNN1A	P37088	PPAY	P641–Y644
βENaC	SCNN1B	P51168	PPNY	P617–Y620
γENaC	SCNN1G	P51170	PPKY	P624–Y627
Nav1.1	SCN1A	P35498	PPSY	P1983–Y1986
Nav1.2	SCN2A	Q99250	PPSY	P1972–Y1975
Nav1.3	SCN3A	Q9NY46	PPSY	P1967–Y1970
Nav1.5	SCN5A	Q14524	PPSY	P1974–Y1977
Nav1.6	SCN8A	Q9UQD0	LPSY	L1944–Y1947
Nav1.7	SCN9A	Q15858	PPSY	P1955–Y1958
Nav1.8	SCN10A	Q9Y5Y9	PPSY	P1918–Y1921
Kv7.1	KCNQ1	P51787	LPTY	L659–Y662
Kv7.2	KCNQ2	O43526	*APPY*	A667–Y670
Kv7.3	KCNQ3	O43525	*EPPY*	E694–Y697
Kv11.1 (hERG)	KCNH2	Q12809	PPAY	P1075–Y1078
ClC-5	CLCN5	P51795	LPPY	L669–Y672
ClC-Ka/Barttin; ClC-Kb/Barttin	BSND	Q8WZ55	*QPPY*	Q95–Y98
Cx43	GJA1	P17302	PPGY	P283–Y286

Table 1. List of Human Nedd4 substrates containing the PY motif or a tentative PY motif. Detailed protein and gene names, accession Code (UniProtKB), PY motif (highlighted in green) and its position. Letters in italics denote the tentative PY motifs. From Lamothe and Zhang (2016) [157].

1.7.2. Deubiquitin (DUB) enzymes

Moreover, there is dynamic interplay among ubiquitinating and DUB enzymes, which fine-tune the ubiquitin cascade by cleaving UBQ from substrates and editing UBQ chains, processing ubiquitin precursors and inhibiting E2 and E3 enzymes. Proteasome-related DUBs contribute to the

prevention of ubiquitinated protein degradation, whereas lysosome-associated DUBs are crucial in receptor degradation and recycling. DUBs are classified according to their sequences and domain conservation in 6 families, with the largest family consisting of ubiquitin-specific proteases (USPs) [158]. As explained above, ENaC is ubiquitinated by Nedd4 family members, a process that can be reversed by USP2-45. USP2-45, induced by aldosterone, deubiquitinates ENaC and increases channel abundance [159]. USP2-42 also interacts with ENaC to moderate increases in channel surface expression and activity [160]. On the other hand, USP2 isoforms also regulate Kv7.1. Krzystanek and colleagues described the antagonism between USP2-45 and USP2-63 on Nedd4-2-dependent Kv7.1 ubiquitination, showing the restoration of the channel localization at the plasma membrane. Evidence suggests that the balance between the ubiquitination/deubiquitination of Kv7.1 ultimately controls the duration of the cardiac action potential and the potassium flux across the membrane of epithelial cells [161]. Finally, USP10, located in EEs, regulates the deubiquitination of CFTR, facilitating the recycling of the channel to the plasma membrane. USP10 decreases the ubiquitination of CFTR, whereas the knockdown of USP10 promotes the ubiquitination and reduction of CFTR at the cell surface [162].

1.7.3. Ubiquitin ligases' adaptors proteins

1.7.3.1. β-*Arrestins*

β-Arrestin 1 and 2 are clathrin adaptors that recognize cargo protein, ubiquitously expressed in mammalian cells. β-Arrestins desensitize members of the G-protein coupled receptor (GPCR) family [163, 164]. In addition, β-arrestins act as multifunctional adaptors mediating trafficking and signal transduction, through GPCRs and other receptors and ion channels [165]. β-Arrestins, via interactions with clathrin and AP-2, act as adaptors for the agonist-induced endocytosis of many GPCRs, promoting the accumulation of cargo protein in CCVs [166].

The inward rectifying potassium channel 3 (Kir3) controls neuronal excitability in response to GPCR activation. Kir3.1/3.2 subunits form signaling complexes with the delta opioid receptor (DOR). Upon receptor stimulation, the complex provides a platform for β-arrestin 2 association with receptors and channels, which drives their internalization by CME [167]. Similarly, sustained angiotensin II stimulation of angiotensin receptor type 1 (AT1R) on the Cav1.2 calcium channel induces β-arrestin 1 recruitment to the channel complex, causing internalization of Cav1.2 in T-tubules. Therefore, L-type calcium currents decrease by ∼60 %, and calcium transient amplitude and action potential duration decrease [168].

Moreover, β-arrestins act as adaptors for ubiquitin ligases mediating the ubiquitination and degradation of targets [165]. One example is the TRPV4, a member of the vanilloid subfamily of the transient receptor potential family, which is present in cardiovascular tissues and epithelial cells. TRPV4-mediated entry of Ca^{2+} into endothelial cells seems to be important for nitric oxide production, vasoconstriction, and vasodilatation of peripheral blood vessels. AT1R forms a complex with TRPV4 in vascular smooth muscle cells. Upon angiotensin stimulation, TRPV4 is ubiquitinated via β-arrestin 1, which interacts with the E3 ligase AIP4, leading to channel internalization. Annihilation of β-arrestin 1 impairs ubiquitination and angiotensin-induced TRPV4 internalization [169]. The presence of β-arrestin 1 is also essential for Na^+/H^+ exchanger 1 (NHE1) ubiquitination

by Nedd4-1. siRNAs against either Nedd4-1 or β-arrestin 1 reduce NHE1 ubiquitination and endocytosis, resulting in an increase in NHE1 plasma membrane abundance. Because NHE1 lacks the PY motif and β-arrestins are scaffolding proteins for ubiquitin ligases, it is likely that β-arrestin 1 recruits Nedd4-1 to the C-terminus of NHE1 for subsequent ubiquitination [170].

1.7.3.2. Ndfips

Nedd4 family-interacting proteins (Ndfip) possess a PY motif acting as recruiters and adaptors of Nedd4 family ligases to facilitate the binding of Nedd4 to its target substrate. There are two Ndfip protein forms identified: Ndfip1 (N4WBP5) and Ndfip2 (N4WBP5A). Both isoforms have three transmembrane domains and two PY motifs in their cytoplasmic N-terminal end (**Table 1**). These proteins are localized to the Golgi, endosomes and multivesicular bodies (MVBs) [171-173]. Ndfip proteins can induce ubiquitination in two ways: (i) they bind to substrates which do not possess a typical PY motif and recruit ligases to them [174] and (ii) they activate the catalytic activity of the ligases, allowing them to ubiquitinate proteins that are in the immediate surrounding area [175].

As previously described, there are substrates of Nedd4 family that do not contain the PY motif which allows the direct binding of the ubiquitin ligase to the substrate (**Figure 13**). Among Nedd4 targets, Ndfip 1 and 2 promote the Nedd4 member WWP2-mediated degradation of the divalent metal transporter 1 (DMT1) [174]. Similarly, Ndfip1 and 2 specifically interact with water-channel aquaporin 2 (AQP2) enabling its ubiquitination and degradation by Nedd4-1 and Nedd4-2 ligases [176]. Moreover, although Nedd4-2 ubiquitinates hERG via a PY motif located in the C-terminus of the channel (**Table 1**), Ndfip1 and 2 recruit and activate Nedd4-2 in the Golgi apparatus to mediate the degradation of hERG proteins during channel trafficking to the plasma membrane [177]. On the one hand, Ndfips adaptors can prevent the effect of Nedd4-2 on different substrates such as ENaC [172] and EGFR [173].

1.7.3.3. 14-3-3

14-3-3 proteins are a ubiquitous family of small, acidic proteins that functioning as adaptors, scaffolds, and chaperones, bind more than 200 targets and thus, regulate a huge variety of signaling pathways such as cell cycle and apoptosis, signal transduction, metabolism and protein trafficking [178]. There are 7 isoforms expressed in mammals: β, γ, ζ, ε, η, τ, and Θ; α and δ are phosphorylated forms of β and ζ. Although they are express ubiquitously in mammals (except τ and Θ which are endothelial and T-cell specific, respectively), they can also have isoform and tissue specific roles.

14-3-3 monomer is composed of nine α-helixes and usually homo- or hetero-dimer with their N-terminal domain. Each dimer contains two binding pockets, so each monomer is able of binding with a phosphoSer/Thr motifs. There are two consensus sequences able to bind 14-3-3 when phosphorylated: RSXpS/TXP (mode I) and RXY/FXpSXP (mode II) (where X is not proline and pS/T represents phosphorylated Ser or Thr) [179, 180]. Therefore, phosphorylation of the associated protein controls its binding to 14-3-3 proteins. Moreover, there are “suboptimal” motifs in the majority of 14-3-3 targets, changing by 1-3 amino acids from the canonical sequence. 14-3-3 also interact in a phosphorylation-independent manner with some targets [181].

As above mention, ENaC is ubiquitinated by the E3 ligase Nedd4-2 and the serum and glucocorticoid-regulated kinase (SGK) reduces the binding of Nedd4-2 to ENaC by phosphorylating Nedd4-2 [151]. This phosphorylation in insufficient to fully inhibit the interaction between the ligase and ENaC until 14-3-3 protein binds to phosphorylated Nedd4-2. In this way, 14-3-3 maintains Nedd4-2 in a phosphorylated form and blocks the binding between the ligase and ENaC [182]. Moreover, 14-3-3 inhibits the interaction between the WW domains of Nedd4.2 with the PY motif of ENaC [182]. On the other hand, 14-3-3 facilitated Nedd4-2 mediated ubiquitination of Kir4.1 channel [183].

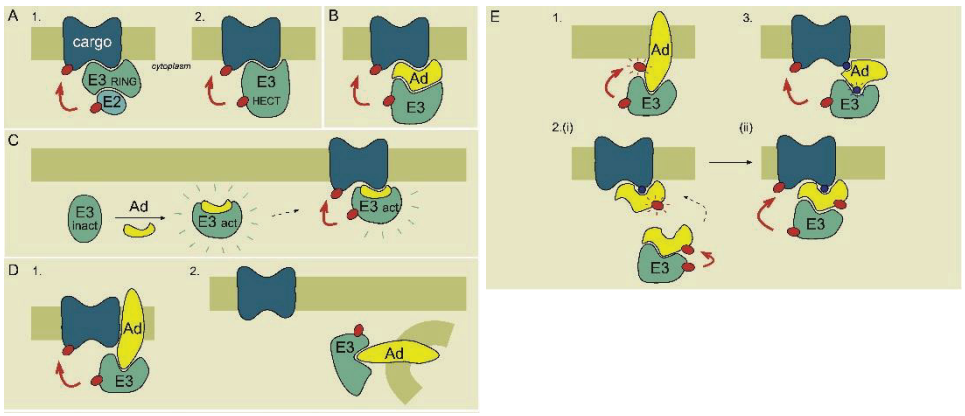

Figure 13. Schematic representation of the regulation of E3 ligases by adaptor proteins. (A) Direct recognition of the target by the E3 (1-RING E3; 2-HECT E3). **(B)** The adaptor mediates the interaction between the E3 and the substrate. The adaptor could also be a membrane protein. **(C)** The adaptor activates the E3 ligase. **(D)** Adaptors can regulate the E3 subcellular localization. (D1) Adaptors can recruit the substrate or (D2) can regulate the E3 availability by titrating the E3 away from the substrate. **(E)** Adaptors can have PTMs. (E1) Most adaptors are ubiquitinated by the E3 and that influences its localization or affects its stability. (E2) In other cases adaptors ubiquitination is required for their fuction. (E3) Adaptor phosphorylation could be required for E3 recruitment. From León and Haguenauer-Tsapis (2009) [184].

1.8. PROTEIN KINASES ON Kv CHANNELS

Protein kinases are proteins that catalyse the covalent addition of phosphate to target proteins. This process is named phosphorylation. Protein kinases form a superfamily with more than 500 protein kinases identified in the human genome [185].

Phosphorylation is a reversible and dynamic PTM which consists of the transfer of the γ-phosphate group of ATP to the hydroxyl group on the side chain of serine (Ser, S), threonine (Thr, T) and tyrosine (Tyr, Y) residues. Eukaryotic organisms possess two types of protein kinases: (i) Ser/Thr kinases, which transfer the phosphate to serine and threonine residues and (ii) Tyr kinases, which transfer the phosphate to tyrosine residues [186]. Frequently, the addition of the phosphate group causes significant changes in protein conformation, generating new protein-protein interactions. Phosphorylation is reversed by phosphatases. The phosphorylation and dephosphorylation of

proteins is very dynamic and regulates hundreds of cell processes, such as proliferation, gene expression, metabolism, motility, membrane transport, differentiation and apoptosis.

Moreover, protein kinases can be positively upregulated by phosphorylation and dephosphorylation, as well as protein cleavage, translocation, second-messenger or ion binding, dimerization or oligomerization, activation of subunit interaction, and protein-protein or protein-lipid interactions. Similarly, protein kinases can be downregulated to attenuate or end the kinase activity and then the induced downstream signals. Some examples of negative feedback include ligand-induced receptor tyrosine kinase signaling, antagonistic ligands, heterooligomerization with truncated receptors, phosphorylation and dephosphorylation, endocytosis, protein degradation and reduction of receptor mRNA [187].

Overall, the massive number of protein kinases and phosphatases, as well as phosphosites, makes phosphorylation an extremely versatile PTM, with hundreds of implications in activity and, to our concern, the turnover of Kv channels.

1.8.1. Protein kinase C (PKC)

Protein kinase C (PKC) is a multifunctional, cyclic nucleotide-independent family of Ser/Thr protein kinases that includes 11 isoenzymes. PKC is involved in diverse signaling transduction pathways, such as activation, proliferation, differentiation, apoptosis, and autophagy. All PKC enzymes are single polypeptide with an N-terminal regulatory domain and a C-terminal catalytic region. The PKC family can be broadly divided into three groups that differ in their cofactor requirements: (i) conventional PKC isoforms (α, β_I, β_{II} and γ) which require Ca^{2+}, diacylglycerol (DAG) and phosphatidylserine to activate; (ii) novel PKC isoforms (δ, ε, η, Θ and μ) which only require DAG and phosphatidylserine to activate; and (iii) atypical PKC isoforms (ζ and ι or λ, which is the mouse ortholog of human PKC- ι) that require neither Ca^{2+} nor DAG [188-190] (**Figure 14 A**).

Due the presence of the cysteine rich C1 domain, both conventional and novel PKCs isoforms, can be activated by DAG and the biologically active phorbol esters, which mimic the interaction of DAG with the enzyme. Phorbol esters, such as PMA (phorbol-12-myristate-13-acetate), are stable in the cell and their effects last longer, helping in the study of PKC signaling *in vitro* and *in vivo* [191].

Activated PKC regulates many cellular functions such as receptor desensitization and internalization, transcription, immune responses, cell growth and, importantly to our study, endocytosis and trafficking [192]. Although the precise mechanisms are still unclear, PKC appears to function as a central hub in controlling vesicular pathways of receptors, transporters, channels, and plasma membrane proteins [193]. PMA-induced PKC downregulation is observed in most cell types. Concerning our studies, Kv1.3 is target of PKC, and PMA-induced PKC produces a ubiquitination and clathrin-dependent endocytosis of Kv1.3 [85].

1.8.2. Protein kinase A (PKA)

Protein kinase A (PKA) is a cAMP-dependent Ser/Thr protein kinase. At low cAMP concentration, inactive PKA exists as a tetrameric holoenzyme composed of two regulatory (RI and RII) and two

catalytic (C) subunits (**Figure 14 B**). Activation of PKA occurs in the presence of cAMP, when two cAMP molecules bind cooperatively to each regulatory subunit. The binding of cAMP results in a conformational change, promoting the dissociation of the PKA holoenzyme into two active catalytic subunits, which phosphorylate downstream targets, and a dimer of regulatory subunits [194].

The two PKA isozymes differ in their regulatory subunits: (i) PKA type I (PKA-I) containing RIa or RIb and (ii) PKA type II (PKA-II) containing RIIa or RIIb. The regulatory isoforms differ in their localization and expression level and the importance of the relative cellular ratio between PKA-RI and PKA-RII has already been reported, supporting the idea that specific functions can be assigned to PKA isozymes mediating the distinct effects of cAMP in cellular processes such as growth and differentiation [195, 196].

The cAMP/PKA signaling system constitutes an inhibitory pathway in T cells [197]. As above mentioned, Kv1.3 plays an important role in T-cell activation, and its inhibition suppresses T-cell activation and proliferation. It has been described that PKA modulates Kv1.3 activity and downregulates T cell function [198]. PKAI and PKAII are two types of PKA isoforms expressed in human T cells, but PKAI has been shown to inhibit T-cell activation via suppression of the tyrosine kinase Lck [199]. Moreover, 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), a cell-permeable cAMP analogue nonselective activator of PKA, inhibited Kv1.3 currents both in primary human T cells [198].

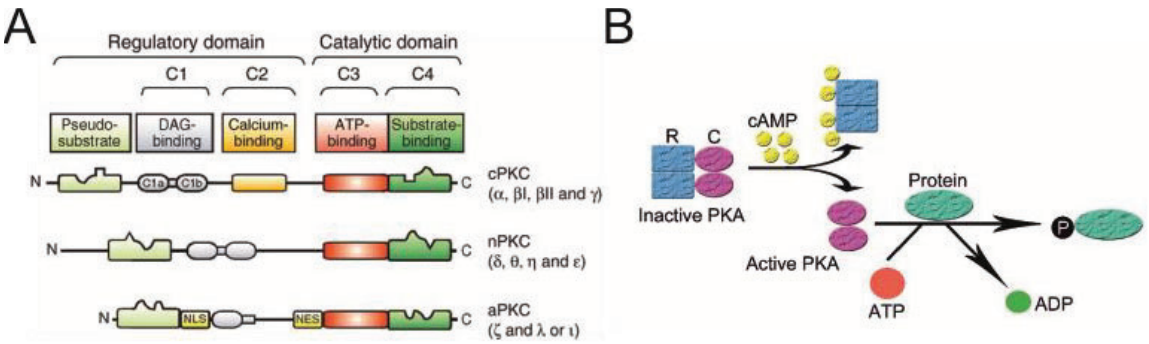

Figure 14. (A) Classification and structural characteristics of PKC isoforms. The catalytic domain is conserved among all isoforms. The differences between the tree groups reside in their regulatory domains. The classical PKC (cPKC) isoforms have the autoinhibitory pseudosubstrate motif (light green), two DAG-binding C1 domains (C1a and C1b, blue) and the calcium-binding C2 domain (yellow). Novel PKC (nPKC) isoforms lack de calcium-binding motif but are still regulated by DAG. Atypical PKC (aPKC) isoforms are independent of DAG and Ca^{2+}. From Spitaler and Cantrell (2004) [189]. **(B) Schematic representation of PKA activation.** In the absence of cAMP the holoenzyme is composed of two regulatory (R) and two catalytic (C) subunits. In the presence of cAMP, each R subunit binds two molecules of cAMP at separate allosteric binding sites, leading to a decrease in the affinity between the R and C subunits and the dissociation of the PKA holoenzyme into a dimer of the regulatory subunits and two monomeric catalytic subunits. The C subunits become active and phosphorylate their substrates in Ser/Thr residues. From Liu *et al.* (2020) [200].

1.8.3. ERK1/2

The extracellular signal-regulated kinase 1 and 2 (ERK1/2) are related protein-Ser/Thr kinases that participate in the Ras-Raf-MEK-ERK signal transduction cascade. This pathway is involved in the

regulation of a large variety of processes such as cell adhesion, cell cycle progression, cell migration, proliferation, differentiation, cell survival, metabolism, and transcription. ERK1/2 is activated by phosphorylation at Tyr204/187 and Thr202/185, by the dual-specificity kinase MEK1/2, which in turn is activated by the Raf kinase. Scaffold proteins play a key role in ERK1/2 activation by binding multiple components of the Raf-MEK-ERK module to promote signal transduction, amplification, and specificity. While Raf and MEK kinases have limited substrate specificity, ERK1/2 phosphorylates hundreds of cytoplasmic and nuclear substrates (**Figure 15**). ERK1/2 are proline-directed kinases that preferentially phosphorylate substrates with a proline motif PXS/TP [201]. Indeed, activation of the epidermal growth factor receptor (EGFR) triggers the endocytosis of Kv1.3 via an unconventional ERK1/2-dependent mechanism that phosphorylates Kv1.3 Thr495 residue (^{493}PQTP^{496}) [128].

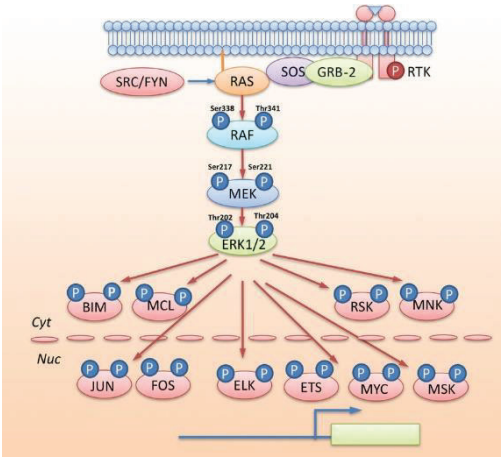

Figure 15. Schematic representation of MAPK patway. RAS-RAF-MEF-ERK1/2 transduction cascade and the major downstream targets of ERK1/2 MAPK pathway are indicated. ERK regulates both cytosolic substrates and nuclear transcription factors, promoting proliferation, survival, and other malignant phenotypes. From Liu *et al.* (2018) [202].

1.8.4. Receptor tyrosine kinase (RTK)

Receptor tyrosine kinases (RTKs) comprise over half of the 90 tyrosine kinases in the human genome [185] and many of them have emerged as key regulators in different cellular processes, such as proliferation and differentiation, cell survival and metabolism, cell migration and cell cycle control [203, 204]. Alteration or abnormal activation of RTKs have been recurrently observed and recognised as a contributing factor in the progression of different cancers [205]. All 58 known RTKs, divided into twenty families, have a ligand-binding region in the extracellular domain, a single transmembrane helix, and a cytoplasmic region that contains the protein Tyr kinase (TK) domain plus additional C-terminal and juxtamembrane regulatory regions. Most of them bind to specific protein ligands, such as growth factors and cytokines, via their extracellular domain, which results in activation of the cytoplasmic catalytic domain upon ligand-mediated dimerization and phosphorylation of cytoplasmic proteins, thus transducing extracellular signals across the plasma membrane [187] (**Figure 16**).

In the present thesis, among all TRKs we focus on the epidermal growth factor receptor (EGFR) family. EGFR family tyr kinases has four members: EGFR (or Erb1/HER1), ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. EGFR, ErbB3 and ErbB4 bind ligands in the EGF family (7 different activating ligands, being EGF itself one of them). For ErbB2, no soluble ligand has been identified yet and is

generally assumed only to be regulated by heterodimeriation with the other ErbB family receptors [206]. EGFR, ErbB2 and ErbB4 are active kinases, whereas ErbB3 lacks catalytic activity. Upon ligand binding, these receptors can heterodimerize, thus generating distinct intracellular signals depending on the combination. For instance, ligand-induced interaction of any of the three active receptors in combination with ErbB3 results in activation of the PI3K pathway [204].

Endocytosis is the mayor regulator of signaling from RTKs, which undergo rapid internalization upon ligand-induced activation. Thus, endocytosis is a potent mechanism to reduce the number of receptors present on the cell surface and the cellular response to the extracellular ligand is attenuated [207, 208]. All surface RTKs undergo constitutive internalization and recycling from endosomes back to the cell surface. But, once ligand binding is produced, the rate of RTKs endocytosis increase several folds and culminates in receptor lysosomal degradation. EGFR endocytosis, the most popular system for the study of ligand-induced endocytosis, is triggered by EGF binding to EGFR dimers at the plasma membrane [209]. Stabilization of EGFR dimers promotes EGFR activation and trans-phosphorylation. Active EGFR is ubiquitinated by the E3 RING ligase Cbl. Both clathrin-dependent [210] and clathrin-independent [211-213] pathways contribute to EGFR endocytosis.

Moreover, activation of EGFR mediates inhibition of Kv1.3 [127]. Upon EGFR activation, Kv1.3 is phosphorylated directly, resulting in the current suppression and modulation of channel kinetics [214, 215]. More recently, our laboratory demonstrated that EGF-mediated tyrosine phosphorylation not only decreases Kv1.3 current amplitude but also mediates channel endocytosis via a novel ERK1/2-dependent Thr phosphorylation mechanism [128].

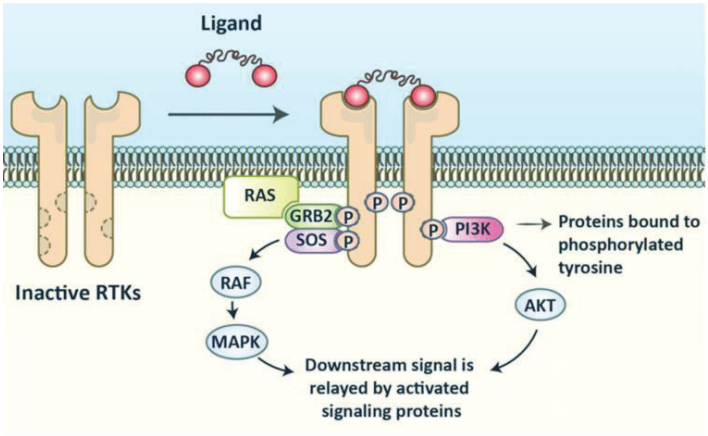

Figure 16. Schematic representation of receptor tyrosine kinase (TRK) domains and activation process. A specific ligand binds to inactive RTKs dimers. Then RTKs activates, self-phosphorylate, and phosphorylate proteins that interact to the intracellular protein binding domain of phosphorylated-RTKs, thus initiating a downstream signaling cascade. From Azad *et al* (2020) [216].

1.8. ADENOSINE

Adenosine (ADO) is an endogenous nucleoside that maintains cellular and tissue homeostasis in pathological and stress conditions. ADO exerts several physiological functions in a large variety of tissues, such as modulation of the neurotransmitter release [217], synaptic plasticity [218],

neuroprotection in ischemic, hypoxic and oxidative stress events [219], vasodilatation and vasoconstriction [220], as well as T cell proliferation and cytokine production [221, 222]. ADO exerts its biological effects through four receptors subtypes: A_1, A_{2A}, A_{2B} and A_3AR [223]. While A_1 and A_{2A}AR have a high affinity for ADO (0.3-3 and 1-20 nM, respectively), A_{2B} and A_3AR possess a lower affinity (μM range) [224, 225]. ADO receptors are transmembrane G protein-coupled receptors (GPCRs); A_1 and A_3 are coupled to Gi proteins and A_{2A} and A_{2B}ARs to Gs. Thus, their activation inhibits and stimulates cAMP production, decreasing and increasing PKA activity, respectively.

ADO is primarily produced, either intracellularly or extracellularly, from the metabolism of adenosine triphosphate (ATP). Under physiological conditions, ADO is intracellularly produced from the hydrolysis of AMP. However, under stress conditions, the intracellular conversion of ATP to ADO is increased. In addition, adenine nucleotides (AMP, ADP, ATP) are released into the extracellular space where they are dephosphorylated to ADO. Once ADO is in the extracellular space, (i) activate adenosine receptors before being deaminated to inosine by adenosine deaminase (ADA), (ii) is captured at the intracellular level by specific nucleoside transporters (ENT1 and ENT2 and CNTs), and then phosphorylated to AMP by adenosine kinase, or deaminated to inosine [226] (**Figure 17**).

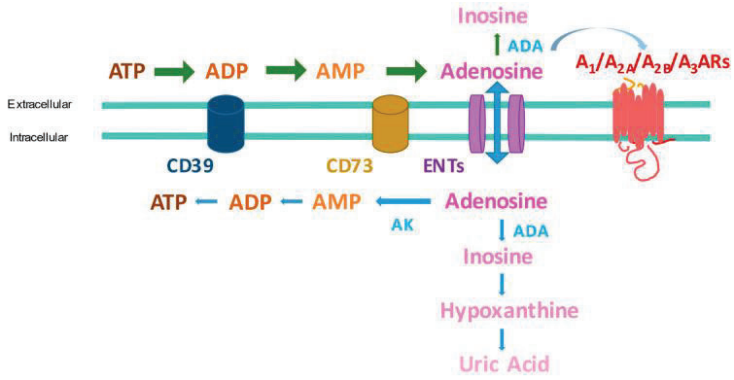

Figure 17. ADO metabolism and transport in the intracellular and extracellular space. ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ADA, adenosine deaminase; AK, adenosine kinase; ENTs, equilibrative nucleoside transporters; AR, adenosine receptors. Extracted from Pasquini *et al* (2021) [227].

Adenosine receptors (ARs) have a unique pharmacological profile and tissue distribution and are ubiquitous in almost every cell in the body. A_1AR subtype is expressed in the central nervous system (CNS) as well as heart arteria, kidney, adipose tissue, pancreas, airway epithelia and smooth muscle cells [228-232]. Besides decreasing cAMP levels, A_1AR induces phospholipase C (PLC)-β activation, increasing inositol 1,4,5-triphosphate (IP_3) and intracellular Ca^{2+} levels, which stimulates Ca^{2+}-dependent PKC and other Ca^{2+}-binding proteins. Moreover, at the neuronal and myocardial level, A_1AR stimulates ATP-sensitive potassium channels (K_{ATP}), while reducing Q-, P-, and N-type Ca^{2+} channels. In addition, it has been reported the involvement of A_1AR in the MAPK cascade, including ERK1/2 [233, 234].

A_{2A}AR is mainly expressed in striatum, the olfactory tubercle, and the immune system. It has a great presence in astrocytes, microglia, and oligodendrocytes, as well as leukocytes, platelets and the vasculature [235, 236]. cAMP dependent PKA is the usual effector activated by A_{2A}AR. Moreover, it has been suggested that A_{2A}AR is involved in the modulation of MAPK signalling [228] (**Figure 18**).

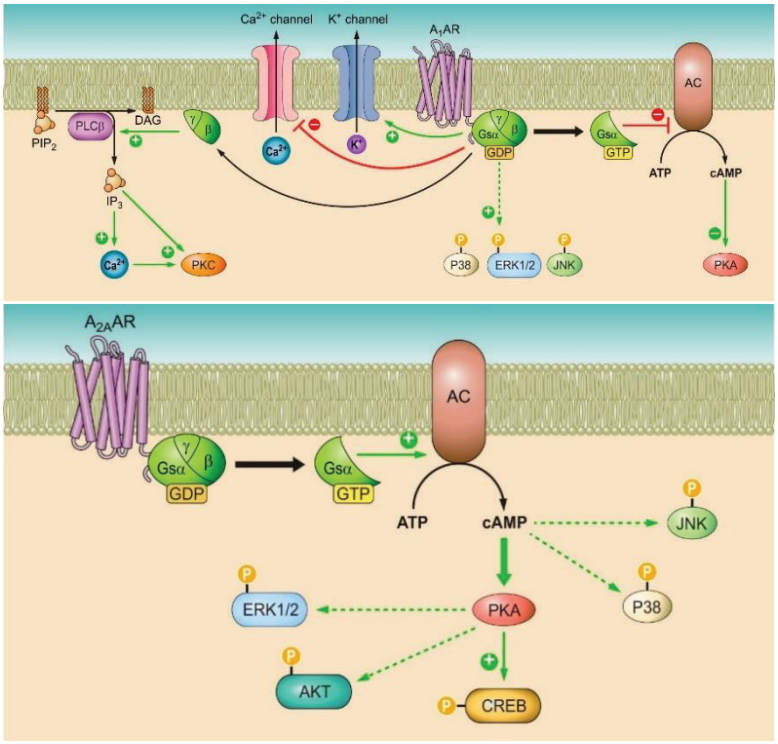

Figure 18. Overview of A_1 (top) and $A_{2A}AR$ (bottom) intracellular pathways. Top: Activation of A_1AR decreases adenylate cyclase (AC) activity and cAMP production, hence inhibiting PKA. Concomitantly, PLC-β and Ca-dependent PKC are activated. K^+ and Ca^{2+} channels are opened and close, respectively. By A_1AR enrolment, phosphorylation of MAPK p38, ERK1/2, and JNK1/2 are induced. **Bottom:** Activation of $A_{2A}AR$ increases AC activity, cAMP production and thus PKA activation and CREB phosphorylation. Activation of AKT and MAPK p38, ERK1/2 and JNK1/2 are followed by $A_{2A}AR$ recruitment. From Borea *et al* (2018) [226].

1.8.1. ADO in the immune response

Concerning this thesis project, among all physiological processes where ADO is involved, we will focus on its role in inflammation. Under physiological conditions, ADO extracellular concentrations are low due the rapid metabolism and uptake [237]. However, upon inflammation as well as hypoxia or tissue injury, ADO levels increase. ADO exerts mainly anti-inflammatory effects, mediating against tissue damages and stress conditions. Nevertheless, prolonged ADO signal may hamper antitumoral and antibacterial immunity, facilitating cancer progression and sepsis, respectively [238].

ARs are expressed in all immune cells where they participate in regulating the immune response, mainly with anti-inflammatory effects [227]. A_1, A_{2A} and A_3ARs are expressed at low levels in quiescent monocytes, increasing their levels over differentiation intro macrophages [239]. The ADO anti-inflammatory effects are supported by A_{2A}, A_{2B} and A_3AR activation, which block the release of proinflammatory cytokines such as TNF-α, IL-6, IL-12 and nitric oxide (NO_2) [240, 241]. Moreover, A_{2A} and $A_{2B}AR$ activation enhances the release of the anti-inflammatory cytokine IL-10 [242]. On dendritic cells, antigen-presenting cells which trigger adaptive immunity, A_1 and A_3AR are mainly expressed in immature cells, whereas $A_{2A}AR$ is mostly expressed in mature cells, leading to a decrease in proinflammatory cytokines [243]. Moreover, in CD4+ T cells and Th1 and Th2 cells, $A_{2A}AR$ activation blocks IL-4 and IFN-γ production [238]. Additionally, regulatory T cells (Treg), which control and supress autoreactive T cells and, thus prevent autoimmunity, produce ADO which, in turn, activates $A_{2A}AR$ reducing the proinflammatory cytokine release through nuclear factor-κB activation (NF-κB) [244]. The fact that AR can selectively regulate leukocyte functions make ARs a promising target for pharmacological approaches in a wide range of diseases such as rheumatoid arthritis [245], asthma [246, 247] and psoriasis [248].

2. Aims

2. AIMS

The voltage gated potassium channel Kv1.3, which is ubiquitously expressed and involved in many physiological processes, plays a special role in the immune response participating in the activation and proliferation of leukocytes. Because an increased and/or delocalized expression of Kv1.3 has been observed at the onset of autoimmune diseases, in the present dissertation we focused on the study of the mechanism involved in the modulation of the Kv1.3 abundance at the plasma membrane.

By balancing the forward trafficking and the endocytic mechanisms, cells fine-tune the plasma membrane Kv1.3 expression. Changes in the homo-/heterotetrameric composition, as well as the association with ancillary subunits and posttranslational modifications, determine Kv1.3 traffic and proper surface targeting. However, once inserted in the plasma membrane, the recycling and endocytic machinery regulates Kv1.3 membrane abundance. An excess of functional channel at the cell surface triggering a long-sustained signal will end with chronic inflammation and pathology. Therefore, the control of channel internalization and degradation effectively influence the inflammatory response.

The present dissertation aims to understand the link between Kv1.3 downregulation and physiological adenosine immunosuppression effects. We tackle the molecular mechanisms involved in Kv1.3 turnover and the functional consequences derived from the downregulation of the channel in the leukocyte physiology.

Therefore, the specific objectives of the PhD thesis were:

- To characterize the molecular determinants involved in PKC- and EGF-mediated Kv1.3 ubiquitination
- To determine the molecular interaction between Kv1.3 and the E3 ligase Nedd4-2
- To study the functional consequences of Adenosine-mediated PKC and PKA activation on the Kv1.3 leukocyte physiology

3. Materials and Methods

3. MATERIALS AND METHODS

3.1. CELL CULTURE

3.1.1. Cell lines

The experiments were performed using 5 different cell lines:

- **HEK293** (Human Embryonic Kidney): Derived from human embryonic kidney cells grown in culture and generated by the transformation with adenovirus 5 DNA [249]. They are adherent cells with an epithelial/fibroblastic phenotype. Even it has been suggested, its neuronal origin could not be concluded [250]. As they are deficient in voltage gated potassium channels and β regulatory subunit expression, they are a good cell model for heterologous expression [251].
- **HEK293 stable expressing Kv1.3-YFP (HEKe):** HEK293 cells stably expressing Kv1.3 WT attached to the yellow fluorescent protein (YFP). The cell line was previously generated in the laboratory. Briefly, cells were transfected with a plasmid that codifies Kv1.3-YFP and a resistance gene to the antibiotic geneticin (G-418 sulphate, Gibco). Next day, cells are cultured with selection medium (DMEM with 500 µg/mL Geneticin G-418). Therefore, only the cells expressing the plasmid survived. After a few weeks, the clones were isolated by flow cytometry. The correct expression of Kv1.3-YFP was regularly check it.
- **HeLa** (Henrietta Lacks): Derived from human cervix adenocarcinoma. They are also adherent cells with fibroblastic morphology and although they have a similar use in cell biology as HEK293 cells, we used HeLa cell line because they endogenously express the Epidermal Growth Factor Receptor (EGFR) [252].
- **CY15:** Immortalized cell line isolated from murine histiocytic tumour from a BALB/c IFN$\gamma^{-/-}$ mouse. Both morphology and cell surface marker phenotype studies suggest that CY15 cells resemble immature dendritic cells. They can respond to interleukin 4 (IL-4), IFNγ and lipopolysaccharide (LPS) and are able to take up antigens as well as stimulate T-lymphocytes but less efficiently than their normal counterparts [253].
- **Jurkat:** Immortalized human T-lymphocytes cells derived from an acute lymphocytic leukaemia from the peripheral blood of a 14-year-old boy. It is commonly used as a model to study T cell gene expression and signalling [254].

3.1.2. Cell lines culture

HEK293 and HeLa cell lines were cultured in DMEM (Dulbecco's modified Eagle's Medium, Gibco) containing 4.5 g/L glucose and L-Glutamine and supplemented with 10 % FBS (Fetal Bovine Serum, Nibco) and antibiotics (10,000 U/mL Penicillin G and 10 mg/mL Streptomycin, Gibco). In HEK293e Kv1.3- 250 µg/mL Geneticin were added. Growth conditions were 37°C in a 5 % CO_2 atmosphere.

For regular maintenance, cells were washed with PBS 1x and then incubated with 1 mL of trypsin (Gibco) until they detach (2-5 minutes). Trypsin was inactivated with supplemented cultured media and suspended cells were precipitated by centrifugation at 400 g for 4 min. Next, cells were resuspended in culture media and seeded in new dishes.

CY15 and Jurkat cell lines were cultured in RPMI-1640 (Roswell Park Memorial Institute, Lonza) containing L-Glutamine and supplemented with 10 % FBS and antibiotics (10,000 U/mL Penicillin G and 10 mg/mL Streptomycin). Growth conditions were 37°C in a 5 % CO_2 atmosphere.

For regular maintenance, CY15 cells were washed with PBS 1x and then gently detached with culture media using a cell scraper. Next, cells were seeded in new dishes. As Jurkat are non-adherent cells, to be precipitated, they were placed in a tube and centrifuged for 400 g for 4 min. Then, they were resuspended and seeded in new flasks.

3.1.3. Cell lines freezing and thawing

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

- Thawing: To thaw cells, 1 mL of supplemented media was added to the cryogenic vial. The cell suspension was mixed with additional 9 mL of supplemented media and precipitated by centrifugation at 400 g for 4 min. Then, precipitated cells were washed with PBS 1x and precipitated by centrifugation at 400 g for 4 min. Precipitated cells were resuspended with culture media and seeded in a new dish. Culture media was replaced the following day.

3.1.4. Cell counting

CY15 cells were detached by gentle scrapping with complete RPMI, whereas Jurkat cells were precipitated by centrifugation and resuspended with 5-8 mL of complete medium. Then, 6 µL Trypan Blue stain (Invitrogen) were mixed with 6 µL of cells. 10 µL of the mixture were added to the cell counter chamber (Invitrogen) and cell viability was measured using a Countess™ automatic cell counter (Invitrogen) by means of trypan blue exclusion.

3.1.5. Transient cell transfection

For immunocytochemistry, biochemistry, and molecular biology experiments, HEK293 and HeLa cells were transfected with Lipotransfectin® (AttendBio Research). Lipotransfectin® is a transfection reagent based in cationic lipids with colipids in suspension. For immunocytochemistry, 500 ng of Kv1.3 DNA (WT and mutants) and 5 µL of Lipotransfectin® were mixed separately with 50 µL of DMEM. After 5 min incubation, the DNA mix was added drop by drop to the Lipotransfectin® mix, and mixed 3 times in the same way. Then, the mix was incubated for 20 min and added dropwise to the dish with DMEM for 4 h. After that time, the media was replaced for supplemented media and, after 4-5 h cells were washed with PBS 1x and starved overnight with DMEM + 0.2% BSA. The experiments were performed 24 h after the transfection. For biochemistry and molecular biology experiments, the workflow is similar than abovementioned but 4 µg of Kv1.3 DNA (WT and mutants) and 12 µL of Lipotransfectin® were mixed separately with 700 µL of DMEM.

For experiments performed at Prof. Sorkin's laboratory, HEK293 and HeLa cells were transfected with Lipofectamine® 2000 (Invitrogen). For immunocytochemistry, every µg of DNA was mixed with 1 µL of Lipofectamine® 2000. Then, 500-1,000 ng of DNA (specific for every protein) and 0.5-1 µL of Lipofectamine® 2000, respectively, were mixed separately with 150 µL OPTIMEM (Glibco). After 5 min of incubation, the DNA mix was added to the Lipofectamine® 2000 mix drop by drop and mixed 3 times in the same way. Then, the mixture was incubated for 20 min and added dropwise to the well with supplemented media. After 4-6 h, the media was replaced for supplemented or starving media (DMEM + 0.2% BSA). The experiments were performed 24 h after the transfection. For biochemistry and molecular biology experiments, 4 µg of Kv1.3 DNA (WT and mutants) and 25 µL of Lipofectamine® 2000 were mixed separately with 750 µL of OPTIMEM.

3.1.6. siRNA transfection for gene silencing

- siRNA for 14-3-3 gamma subunit (14-3-3γ) (SantaCruz).
- siRNA for human Nedd4-2 (DharmaFECT™): siGENOME SMARTPool.
- siRNA for human Ndfip1 (DharmaFECT™): siGENOME SMARTPool.
- mock siRNA: scrambled sequence control siRNA-A (SantaCruz) and siGENOME Non-Targeting Control siRNA Pool #2 (DharmaFECT™) were used.

The transfection reagent used was Lipotransfectin® for 14-3-3 γ siRNA and DarmaFECT™1 for Nedd4.2 siRNA.

For 14-3-3γ, 3.3 nmol of lyophilized siRNA were resuspended in 330 µL RNase free H_2O obtaining 10 µM stock. Control siRNA-A was prepared at the same concentration.
In a 6-well multiwell plate or 100 mm dish we used 60 nM siRNA working concentration. For that purpose, 5-30 µL of siRNA and 6-36 µL of Lipotransfectin®, respectively, were mixed separately with up to 100-600 µL of siRNA Transfection Media (SantaCruz) and incubated for 5 min. Then, the siRNA mix was added drop by drop to the Lipotransfectin® mix and incubated for 45 min. After that time, 0.8-3.8 mL of siRNA Transfection Media were added to the mixture, mixed gently, and overlay the washed cells. After 7 h of incubation, without removing the transfection mixture, supplemented media was added to reduce toxicity. The experiment was performed 24-36 h after transfection.

For Nedd4-2, 10 nmol of lyophilized siRNA were resuspended in 200 µL RNase free H_2O, obtaining a 50 µM stock. siCONTROL™ non-targeting siRNA (NT-siRNA) was also prepared at 50 µM. In a 60 mm dish, for 25 nM siRNA working concentration, 2 µL of siRNA/NTsiRNA and 8 µL DarmaFECT™1 reagent were separately mixed with up to 400 µL OPTIMEM and incubated for 5 min. Then, siRNA/NTsiRNA mix was added drop by drop to DarmaFECT1 mix, incubated for 20 min. Finally, 3.2 mL of antibiotic-free complete medium (DMEM + 10 % FBS) was added for a total volume of 4 mL transfection medium. The next day, cells were transfected with Kv1.3-YFP and the experiment was performed 24 h after transfection.

3.1.7. Activation of protein kinases signalling pathways

In the present thesis, we have used different drugs to study the mechanisms associate to the turnover of Kv1.3. As abovementioned, after transfection cells were starved overnight with DMEM

supplemented with 0.2% BSA. When protein kinases inhibition was required, before the incubation with the agonist, inhibitors were pre-incubated for 15 min (1 h for Erbstatin) at 37°C.

In order to degrade the adenosine present in the culture media, before activating adenosine receptors, 30 min pre-incubation at 37°C with 0.2 U/mL adenosine deaminase (ADA, Roche) was performed.

Reagent	Function	Stock Solution	Working Solution	Timing (37 ºC)	Company
Phorbol-12-myristate-13-acetate (PMA)	Phorbol ester, PKC signalling pathway activator	1 mM (in DMSO)	10 µM	30 min – 4 h	Alomone
Epidermal growth factor (EGF)	Stimulates cell growth, proliferation, and differentiation via receptor (EGFR)	100 µg/mL (in DMEM + 1% BSA + 2 mM NaN3)	10 ng/mL	30 min – 4 h	Molecular Proves (Invitrogen)
8-Br-cAMP	Cell-permeable cyclic AMP (cAMP) analogue, PKA signalling pathway activator	200 mM (in Milli-Q® H_2O)	0.5 mM	2 – 4 h	SantaCruz
Adenosine	Purine adenine and sugar ribose	10 mM (in Milli-Q® H_2O)	200 µM	48 h	Sigma
N^6-Cyclopentyladenosine (CPA)	Selective agonist of adenosine A_1 receptor.	100 µM - 100 mM (in DMSO)	50 nM – 100 µM	30 min – 48 h	Merck Millipore
CGS 21680	Xanthin derivative, agonist of adenosine A_{2A} receptors	200 µM (in DMSO)	200 nM	30 min – 48 h	Sigma-Aldrich
5'-N-Ethylcarboxamidoadenosine (NECA)	Non-selective adenosine receptor agonist.	14 µg/mL (in DMSO)	10 nM-	24 h	SantaCruz
Bisindolylmaleimide I (BIM)	Inhibitor of PKC signalling pathway	10 mM (in DMSO)	1 µM	15 min pre-incubation	Sigma-Aldrich
H-89	Inhibitor of PKA signalling pathway	10 mM (in DMSO)	10 µM	15 min pre-incubation	Cell Signaling
U0126 ethanolate	Inhibitor of MEK1 and MEK2	10 mg/mL (in DMSO)	10 µM	15 min pre-incubation	Sigma-Aldrich
Erbstatin	Inhibitor of tyrosine kinase	25 mM	25 µM	1 h pre-incubation	Sigma-Aldrich

Table 2 (previous page). Chemical compounds and growth factors used for activating protein kinase signalling pathways. Detailed reagent, use, stock and working solution, time of incubation and brand.

3.1.8. CY15 morphology studies

The capacity of dendritic cells (DC) to regulate the immunity relies on their maturation state. Bacterial-derived antigens, such as the lipopolysaccharide (LPS) trigger the maturation of DC [255]. CY15 cells were grown in 6-well multiwell plates or 100-150 mm dishes (depending on the experiment). Twenty-four h after the incubation with or without LPS and adenosine receptor agonists, we obtained 4-5 images of every experimental condition. Images were acquired using a Leica DM IL inverted optical microscope equipped with a Moticam 5.0 MP camera controlled with Motic Images Plus software. Cells were imaged using a total magnification of 800x. Images were analysed using FIJI distribution of ImageJ freeware.

To measure the dendrite length, we consider the distance between the closest part to the soma to the distal point of the dendrite (**Figure 17**). The distance obtained was in pixels and to convert the measure from pixels to micrometres we take a photo of a Neubauer chamber using the same microscope settings described above. The Neubauer chamber has a standardized proportion, and the smaller squares of the central square measure 50 µm length (**Figure 17**). With FIJI software we measured the distance of the smaller square, that was 364 pixels. Therefore, the conversion from pixels to micrometres for the CY15 cell images was 0.137 µm/pixel.

The total number of dendrites per cell was another morphological analysis. We considered two possible options (**Figure 19**):

- **Number of dendrites:** Every dendrite that emerged from the soma was counted, independently of the number of sub-branches generated.
- **Number of prolongations:** Dendrites and the sub-branches produced were counted.

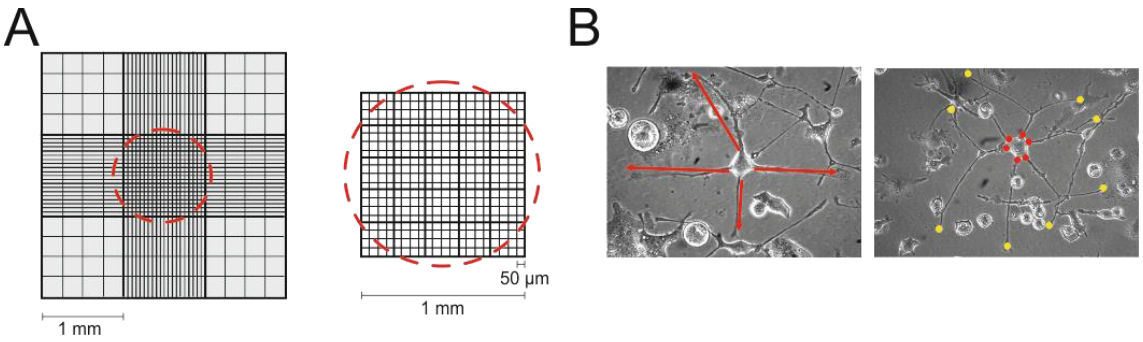

Figure 19. Representation of the criteria used to quantify the number and spike length. (A) Schematic representation of a Neubauer chamber and the standardized size of each square of the centre (50 µm). Using the same microscope, we took a picture of the Neubauer chamber. With FIJI we measured the 50 µm distance, which was 364 pixels. Therefore, the conversion from pixels to micrometres was 0.137 µm/pixel. **(B)** Schematic representation of the criteria used to quantify the length of the spikes (left; red arrows) and the number of spikes (right; red spots) and subspikes (right; yellow spots).

3.2. MOLECULAR BIOLOGY

3.2.1. Bacteria culture

Bacteria, used to amplify DNA, were grown in Lysogeny Broth (LB) culture plates or media or Super Optimal Broth (SOB) culture media and incubated at 37°C for 24 h.

- **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 it at 60°C for 1-2 h before adding antibiotics (Kanamycin: 30 µg/mL; Ampicillin: 100 µg/mL) and pour.
- **LB bacterial culture media:** 5 g tryptone, 2.5 g yeast extract, 5 g NaCl up to 500 mL with water. Autoclave and aliquot in 50 mL tubes.
- **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 2M $MgSO_4x7H_2O$.

3.2.2. DNA extraction

To extract DNA from bacteria, GenElute™ Plasmid kits (Miniprep and MidiPrep, Sigma-Aldrich-Aldrich) were used following the manufacturer's protocol. Briefly, bacterial cells were obtained from an overnight 37°C incubation in LB media and antibiotics (Kanamycin: 30 µg/mL; Ampicillin: 100 µg/mL) and centrifuged to discard the supernatant. Then, pellets were resuspended using the Resuspension Solution until full disaggregation. Next, Lysis Solution was added (no more than 5 min) and mixed gently by inversion. Neutralization Solution was added and mixed by gentle inversion, and the mixture was centrifuged at 15,000 g for 15 min at 4°C. Meanwhile, the binding columns were activated with the Column Preparation Solution. Once centrifuged, the clear lysate was transferred to the column and after two washes with the Optional Wash Solution and the Wash Solution, the column was placed in a new collection tube. DNA was eluted using pre-warmed Milli-Q® H_2O.

3.2.3. DNA purification from agarose gel

DNA was loaded in TAE agarose gels (1 % agarose gel in presence of 1:15,000 SyberSafe (LifeTechnologies)), observed under UV exposure and the selected DNA-containing regions were cut. To purify the DNA from the gel, QIAquick® Gel Extraction Kit (Qiagen) was used following the manufacturer protocol. Briefly, to dissolve the gel, 3 volumes of Buffer QG were added and incubated at 50°C for 10 min, vortexing every 2-3 min. Then, 1 volume of isopropanol was added and mixed. To bind the DNA, the sample was transferred to a column and centrifuged. After a wash with Buffer PE, the column was placed into a clean microcentrifuge tube and the DNA was eluted with 50 µL Buffer EB.

3.2.4. Site and multi-site-directed mutagenesis

To study the molecular determinants involved in Kv1.3 ubiquitination and endocytosis, amino acids potentially involved were changed to alanine or arginine. Site and multi-site-directed mutagenesis were performed using QuickChange™ Site-Directed Mutagenesis Kit and QuickChange™ Multi Site-

Directed Mutagenesis Kit (Agilent Technologies), respectively. In site directed mutagenesis we designed for each reaction specific oligonucleotides pairs (forward and reverse), whereas for multi-site, only forward or reverse single oligonucleotides were used for each reaction. In both cases, oligonucleotides were designed with Clone Manager suite v7.1 (Scientific & Educational Software) and the mutagenesis was performed following the manufacturer's protocol.

Briefly, template DNA was amplified by a PCR reaction catalysed by Pfu Ultra High-Fidelity DNA polymerase or PfuTurbo DNA polymerase (site and multi-site, respectively) with mutagenesis oligonucleotides including the target mutation. Then, the product of the PCR was digested at 37°C with DpnI, a specific DNase for methylated DNA that cleaves the parental DNA strands (PCR generated chains are not methylated). After digestion, DNA was introduced in *E. coli* XL10-Gold ultracompetent bacteria.

The transformation protocol consisted in mixing 45 µL of bacteria with 2 µL of β-mercaptoethanol for 10 min in ice. Then, 1.5-2 µL of the mutagenesis reaction (multi-site and site, respectively) was added, mixed gently, and kept in ice for 30 min. Next, to introduce the DNA inside the bacteria, a thermal shock of 30 s at 42°C was applied, followed by 2 min on ice. After that, bacteria were recovered for 1 h at 37°C in 500 µL of SOB and plated on LB agar plates with the specific antibiotic. After an overnight 37°C incubation, positive clones were selected, grown in liquid LB, extracted the DNA and verified by sequencing.

Table 4. Plasmids used. See Addendum.

Table 5. Oligonucleotides sequences used. See Addendum.

3.2.5. Sequencing

We used the BigDye™ Terminator V3.1 Kit (Applied Biosystems).

Mixture (final volume 10 µL):

- 3 µL BigDye™ Terminator Buffer
- 1 µL DNA
- 0.35 µL primer 10 µM
- 1 µL BigDye™ Ready Reaction Mix
- 4.65 µL Milli-Q® H_2O

Reaction:

Initial denaturalization	96°C	1 min	
Denaturalization	96°C	10 sec	x 28
Annealing	50°C	5 min	
Extension	60°C	4 min	
	4°C	hold	

Samples were processed by the analysed cytometer ABI3730 in the *Centres Científics i Tecnològics (CCiT)* from the University of Barcelona. Clone Manager suite v7.1 was used to analyse the results.

3.3. BIOCHEMISTRY

3.3.1. Total protein extraction

Total protein extraction was performed to analyse the protein expression in different samples. The experiment starts with confluent cells seeded in 100 mm diameter dishes for HEK293 and HeLa cells, 150 mm for CY15 cells, and 75 cm^2 flasks for Jurkats cells. All the steps were developed in cold, incubating all dishes and tubes in ice or at 4°C.

Cells were washed twice with cold 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 homogenized by incubating the samples in an orbital at 4°C for 10 min and then, centrifuged at 15,000 g for 15 min. Supernatant was collected and the pellet, containing cell debris and non-lysed cells, was discarded. To determine the protein concentration, Bradford assay was performed. The protein samples were stored at -20°C.

As Jurkat are non-adherent cells, the content of the flask was transferred to 50 mL tubes and centrifuged for 10 min at 600 g at 4°C. The supernatant media was discarded and two washes with cold PBS were performed. The pelleted cells were resuspended with Lysis Buffer and, to disaggregated them, a 25 G needle was used 10 times. Then, the lysates were incubated 10 min at 4°C and centrifuged as the rest of the cells.

To study the ubiquitination of Kv1.3, after the two PBS washes, the dishes were frozen overnight. The next day, for extracting the protein, the Protein Lysis Buffer (150 mM NaCl, 50 mM HEPES, 0.1 % Glycerol, 0.1 % triton X-100, pH 7.4) was supplemented with 10 nM N-ethylmaleimide (NEM), 0.2 mM MG-132, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 1 mM Na_3VO_4, 2 mM DTT and 1 % triton X-100. In this protocol, the cells were incubated with the Lysis Buffer for 20 min.

3.3.2. Bradford assay

Bradford assay was used to determine the 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, 5, 10, 15 and 20 µL of 0.1% BSA (Bovine Serum Albumin) as a standard in semimicro cuvettes. After mixing, the absorbance was measure at 595 nm.

3.3.3. SDS-PAGE and western blot

50 – 100 µg of protein samples were mixed in 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_2O, pH 6.75) with 10 % β-mercaptoethanol, up to a final volume of 50 µL. To denaturalize the proteins, the samples were boiled for 10 min and then separated in a SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis).

- Polyacrylamide gels:
    - Running: 7.5 – 10 % acrylamide, 375 mM Tris-HCl (pH 8.8), 0.1 % SDS, 0.1 % APS (ammonium persulphate) and 0.04% TEMED (tetramethyletilendiamine).
    - Stacking: 5 % acrylamide, 125 mM Tris-HCl (pH 6.8), 0.4 % SDS, 0.4 % APS and 0.01 % TEMED.

After the electrophoresis (100-120 V), gel content was transferred to PVDF membranes (Polyvinylidene fluoride, Millipore) for 2 h at 375 mA in cold temperature or overnight at 4°C and 35 V. In Sorkin's laboratory, the gel content was transferred to Nitrocellulose membranes (LI-COR) for 4 h at 500 mA in cold temperature.

Then, membranes were incubated in blocking solution (5 % non-fat milk in PBS-0.05 % Tween or TBS-0.05% Tween in Sorkin's laboratory) for 1 h at room temperature (RT). Next, blocked membranes were washed 4 times with PBS/TBS-T and incubated overnight at 4°C with a primary antibody (diluted in PBS/TBS-T with 0.02 % sodium azide). Primary antibodies used and dilutions are detailed in Addendum (Table 6). Following 3 washes with PBS/TBS-T, membranes were incubated for 1-2 h with a secondary antibody (diluted in PBS/TBS-T) specific for the species of the primary antibody and conjugate to HRP (Horseradish peroxidase) or far-red fluorescent dyes. After 3 washes with PBS/TBS-T, membranes were ready to be scanned. To have a higher signal, nitrocellulose membranes were dried for more than 1 h:

- HRP conjugate secondary antibodies (1/10,000 for Goat anti-Mouse and 1/3,000 for Goat anti-Rabbit, Bio-Rad) were detected using ECL* (enhanced chemiluminescence), a peroxidase substrate containing luminol, and expose to LAS-3000 machine (Fujifilm) to detect the signal. Images of different times exposures were taken to ensure that intense not-saturated images were gotten. Quantifications were performed using Fiji (Fiji is just ImageJ).
- Far-red fluorescent dyes (IRDye-680 and -800, LI-COR) secondary antibodies were detected using Odyssey LI-COR system. 1/20,000 for Goat anti-Mouse IgG1 and IgG2a and Goat anti-Rabbit secondary antibodies. Quantifications were performed using Fiji.

Secondary antibodies used and dilutions are detailed in Addendum (Table 7).

*ECL: mixture 1:1 of ECL1 and ECL2

- 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. Half-life studies

To study the half-life of Kv1.3 upon agonists activation, cells transfected with Kv1.3-WT or MSmut were pre-incubated for 3 h with 100 µg/mL cycloheximide (CHX, Sigma-Aldrich) to inhibit protein synthesis. Also, 60 nM Bafilomycin A1 (BafA1, Sigma-Aldrich) or 5 µM MG132 (Sigma-Aldrich) was added in some conditions to inhibit lysosomal or proteasomal degradation, respectively. Then, CHX remained in the culture media in the next incubations. 1 µM PMA, 10 ng/mL EGF or 0.5 mM 8-Br-cAMP for activating PKC, EGFR or PKA, respectively, was added for 2 and 4 h. After the incubations, protein extraction and 10 % SDS-PAGE were performed as above described.

3.3.5. Immunoprecipitation and co-immunoprecipitation

To study Kv1.3 ubiquitination and protein interaction immunoprecipitation (IP) and co-immunoprecipitation were performed, respectively. In both protocols, experiments on transfected HEK293 or HeLa cells were performed 24 h after transfection. Protein extraction was executed as abovementioned. Then, 1,000-2,000 µg of protein samples were pre-cleaned with 30 µL of protein A-Sepharose™ 4 Fast Flow beads (GE Healthcare) for 1 h at 4°C with gentle mixing. Beads were then removed by centrifugation at 1,000 g for 30 s. At this point, IP and coIP protocols differ:

For IPs, samples were incubated overnight in Micro Bio-Spin™ Chromatography Columns* (Bio-Rad) with 50 µL of protein A-Sepharose beads and anti-GFP (4 ng antibody/µg protein) (GenScript) at 4°C with gentle agitation. The next day, samples were centrifuged at 1,000 g for 30 s and the eluted supernatant was collected. After 5 washes with washing solution (150 mM NaCl, 50 mM HEPES, 10 % glycerol, Triton 0.1 % X-100; pH 7.5) the IP was collected in 100 µL 0.2 M glycine pH 2.5. loading buffer containing β-mercaptoethanol was added, and samples were boiled for 5 min.

For coIPs, antibodies were incubated with Protein A/G-Sepharose beads for 2-3 h at 4°C with gentle agitation. Then, the supernatant collected from the pre-clean was added and incubated O/N at 4°C with soft agitation. The complex protein-antibody-protein A/G was formed and precipitated by centrifugation at 1,000 g for 30 s. After 3 washes with lysis buffer, beads were resuspended with 100 µL Laemmli buffer (1x) with β-mercaptoethanol and boiled for 5 min.
At this point, samples were ready to be loaded in SDS-PAGE.

**Column preparation:* 50 µL Protein A-Sepharose beads were incubated with anti-GFP antibodies for 1 h at RT with gentle agitation. Then, beads were washed twice with Borate Buffer (0.2 M Boric Acid, pH 9) and, to covalently link the antibody-beads complexes, 20 mM dimethyl pimelimidate (DMP, ThermoFischer Scientific) with Borate Buffer was added and incubated for 30 min at RT. The crosslinking reaction was quenched by adding 0.2 M Glycine (pH 2.5). After 4 washes with glycine and 3 wases with TBS, columns were ready to be used. Negative columns were prepared the same way, except they did not contain the antibody. Columns were used for 3 times and between uses, were stored at 4°C with PBS 1x + 0.02 % Sodium Azide.

3.3.6. Membrane protein biotinylation

To biotinylate and isolate cell surface proteins, the Pierce Cell Surface isolation kit (ThermoFisher Scientific) was used. Briefly, after two rapid washes with cold PBS 1x, cells were labelled with 6 mL of EZ-Link-Sulfo-NHS biotin in PBS (0.25 mg/mL) for 30 min at 4°C under soft agitation. To quench the labelling reaction, 350 µL of quenching solution (provided with the kit) was added to each dish. Then, cells were washed two times with TBS 1x solution. Next, cells were lysed 10 min with 200 µL lysis solution (150 mM NaCl, 50 mM HEPES, 10 % glycerol, 0.1 % Triton X-100; pH 7.5) supplemented with 1 µg/µL aprotinin, 1 µg/µL leupeptin, 1 µg/µL pepstatin and 1 mM PMSF as protease inhibitors. Total lysates were centrifuged at 16,200 g for 10 min at 4°C. Protein concentration was measured using Bradford method.

Next, 2,000 µg of protein sample was incubated overnight in columns (provided with the kit) with 167 µL of NeutrAvidin agarose beads (provided with the kit) at 4°C with gentle agitation. Samples were centrifuged at 1,000 g for 1 min and the eluted supernatant was collected. After four washes with washing solution (provided with the kit), samples were resuspended in 100 µL loading buffer (1M DTT, Laemmli buffer supplemented with 2 % β-mercaptoethanol and water up to 100 µL) and boiled for 5 min. Centrifugated supernatant was ready to be loaded in SDS-PAGE.

3.3.7. Cell unroofing preparations (CUPs)

Cell Unroofing Preparations (CUPs) are membrane sheets obtained by an osmotic shock. The CUP assay was performed in transfected HEK293 cells cultured in 100 mm dishes and 8 dishes were used for each condition. Twenty-four h after transfection, cells were incubated with or without 0.5 mM 8-Br-cAMP for 4 h at 37°C. Then, cells were washed twice with cold PBS 1x and incubated for 5 min in KHMgE buffer (70 mM KCl, 30 mM HEPES, 5 mM $MgCl_2$, 3 mM EGTA, pH 7.5) diluted threefold. To produce the hypotonic shock, cells were gently washed with non-diluted KHMgE buffer. The dish was divided in four sections and using a 5 mm cut 200 µL micropipette tip attached to a Pasteur pipette, cells were removed by 8 up and down pipetting. After two washes with non-diluted KHMgE buffer, only the membrane sheets remained attached at the dish surface. Then, protein lysis was proceeded as above mention and samples were prepared for loading an SDS-PAGE.

3.3.8. Nitrite colorimetric assay

Nitric oxide (NO) concentration in CY15 cell culture media was measured using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman). Specifically, the nitrite (NO_2^-) concentration was measured, which is a stable product of the NO synthesis catalyzed by the inducible nitric oxide synthase (iNOS). Nitrite medium quantification is optimal after 24 h of the stimulus. Moreover, under those experimental conditions, at 24 h post-stimulus, the majority of NO has already been converted into nitrite instead of nitrate [256].

Briefly, 200,000 cells/well were seeded in a 6-well multiwell plate. The next day, cells were incubated at 37°C with or without 100 ng/mL LPS, 10 nM Margatoxin (MgTx) and adenosine receptor agonists A_1 (50 nM CPA) and A_{2A} (200 nM CGS). Twenty-four h after the treatment, 100 µL of culture media were added to a 96-well multiwell plate. Then, 50 µL of Griess Reagent R1 and 50 µL of Griess Reagent R2 were added to each of the wells (standards and samples). Griess reaction is a colorimetric assay that converts nitrite into a deep purple azo compound. After 10 min, the color developed and the absorbance was read at 540 nm. A nitrite standard curve (up to 200 µM $NaNO_2$) was performed. Both, standard and samples were analyzed in duplicate.

3.3.9. IL-2 colorimetric ELISA assay

In order to analyze IL-2 production in Jurkat T lymphocytes, 12,000 cells/well were seeded in a 96-well multiwell plate. Jurkat cells were activated for 48 h using 80 nM PMA and 5 mg/mL phytohematoxylin (PHA, Sigma-Aldrich). In some conditions, adenosine receptor A_1 and A_{2A} were activated with 50 nM CPA and 200 nM CGS, respectively. Briefly, cell culture was centrifuged 10

min at 1,200 g to pellet cells. Supernatant was collected and 100 µL were added to the appropriate wells and incubated for 2 h. Reactions were performed in capture antibody-coated 96-well microplate. All incubations were performed at 37°C in a humid environment. After 4 washes with 1x Wash Buffer (provided with the kit), 100 µL of 1x Detection Antibody solution was added to each well and incubated for 1 h. Then, 4 washes were repeated and 100 µL of 1x HRP-conjugated antibody were added to each well and incubated for 40 min. Washes were repeated and 100 µL of TMB substrate solution were added to each well and incubated for 15 to 20 min in the dark. A positive reaction was indicated by the color blue. Finally, to quench the color development, 100 µL of Stop Solution were added. The color, then, changes from blue to yellow. Results were immediately read at 450 nm. An IL-2 standard was prepared by serial dilutions of the standard stock (2,000 pg/mL). Both, standard and samples were assay in duplicate.

3.4. CELL IMAGING

3.4.1. Subcellular structure's identification

- **Plasma membrane labelling:** To stain plasma membrane we used Wheat Germ Agglutinin (WGA) conjugated with Alexa fluor 555. In this protocol is crucial to keep the cells cold to avoid the internalization of the WGA. Cells were cooled down changing culture media for fresh one at 4°C and putting them on ice for 2 min. Then, cells were quickly washed with cold PBS K^+ free (PBS-PF) and incubated for 5 min in ice with WGA diluted 1,500 times in binding solution (cold DMEM-30 mM HEPES). Cells were washed for 1 min 3 times with cold PBS-PF. At this point, the protocol continues as a regular immunocytochemistry (see 3.4.2).
- **Endocytic vesicles labelling:**
  - Early endosomes: EEA1 antibody (Early Endosomes Antigen 1)
  - Late endosomes and lysosomes: Lysotracker™ Red DND-99

3.4.2. Immunocytochemistry

HEK293 and HeLa cells were seeded over Poly-D-Lysine (Sigma-Aldrich Aldrich) treated coverslips inside a 6-well multiwell plate. The experiment was performed 24 h after transfection and the incubation with agonists/antagonists to study protein kinase signalling pathways. Multiwell plates were covered with tinfoil to prevent the fluorophores to fade. CY15 cells were seeded in coverslips inside a 6-well multiwell plate without Poly-D-Lysine. Samples were prepared after the incubation with LPS and adenosine agonists.

Coverslips were quickly washed 3 times with 1 mL of pre-warmed PBS-PF (1.54 M NaCl, 88.5 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.4) and fixed for 10 min at RT with 1 mL 4 % paraformaldehyde (PFA, Sigma-Aldrich). The absence of K^+ in the solutions throughout the successive media changes helps cells, that tend to easily detach, to adhere. Then, coverslips were washed 3 times for 5 min with PBS-PF to remove the excess of PFA. In case we only want to image transfectable fluorophores, coverslips

were mounted over slides with homemade Mowiol mounting solution (9.6 g Mowiol in 24 g Glycerol in 48 mL Tris-HCl 0.2 M pH 8.5).

To study the subcellular distribution of native or non-fluorophore transfectable proteins, we performed the immunocytochemistry assay after fixation. When necessary, depending on the antibody and the target protein, cells were permeabilized by incubating the coverslips for 10 min at RT with 0.1 % Triton X-100 and 20 mM Glycine in PBS-PF. On cells permeabilized, 0.05 % Triton X-100 was added to the following solutions. After 3 washes with PBS-PF, we proceed to reduce the unspecific binding of the antibodies incubating the cells for 1 h at RT with blocking solution (5 % non-fat milk, 10 % goat serum, 20 mM Glycine in PBS-PF). Next, after 3 washes with PBS-PF, coverslips were incubated 1 h at RT or O/N at 4°C with the primary antibody (diluted in 10 % Goat serum and 20 mM Glycine in PBS-PF). The concentration of the antibody varies according to manufacturer's instructions. See Addendum. Table 6). Then, coverslips were washed trice with PBS-PF and incubated for 1-2 h at RT with fluorescent-conjugated secondary antibody (1/500; diluted in 1 % BSA and 20 mM Glycine in PBS-PF, Invitrogen. See Addendum. Table 7). Finally, coverslips were mounted over slides with Mowiol mounting solution.

In Prof. Sorkin's Lab, the protocol was similar with minor buffer modifications:

- Washes with PBS Calcium and Magnesium Free (PBS-CMF).
- Permeabilization solution: 0.1 % Triton X-100, 0.5 % BSA in PBS-CMF.
- Blocking solution: 0.1 % BSA, 0.05 % Triton X-100 in PBS-CMF.
- Primary and secondary antibody solution: 0.1 % BSA, 0.05 % Triton X-100 in PBS-CMF.
- Mounting media: ProLong™Gold Antifading mounting media (Invitrogen).

3.4.3. Confocal microscopy

Images were acquired with an inverted Zeiss LSM880 laser scanning confocal microscope, equipped with an argon multiline laser (458, 488, 514 nm) a DPSS 561 nm laser and a helium-neon laser (633 nm); a 63x Plan Apo PH NA 1.4 oil immersion objective; all controlled by Zen Black 2.3 SP1 FP3 software.

Images of the CFP fluorophore were obtained using the 458 nm line. Those with Alexa Fluor 488 were acquired with 488 nm laser line; those with YFP fluorophore, were obtained using the 514 nm laser line, those with WGA-555, Cy3, or Lysotracker™ Red were acquired with 561 nm laser line, and those with Alexa Fluor 647, 660, or Cy5 were obtained with 633 laser line. Double dichroic (488/561 or 458/514) and triple dichroic filters (488/561/633) were used accordingly. Images were acquired at a resolution of 1504 x 1504 pixels and scanning with a pinhole aperture of 1 airy unit (1 AU) at zoom 1x. All offline image analyses were performed using FIJI distribution of ImageJ freeware.

For FRET experiments images were acquired with a Leica TCS-SP5 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). Images of the CFP fluorophore were obtained using the 458 nm line, whereas those of YFP were obtained using 514 nm laser line. Double dichroic filter (458/514) was used accordingly. Images were acquired at a resolution of 512

x 512 pixels using a 63x oil immersion objective lens (NA 1.32) and scanning with a pinhole aperture of 1.8 AU at zoom 1x.

In Prof. Sorkin's lab, images were acquired with a spinning-disk Marianas system based on a Zeiss Axio Observer Z1 inverted fluorescence microscope, equipped with CSU-W1 Yokogawa spinning disk; 405-, 436-, 488-, 515-, 561-, and 640 nm lasers; a 63x Plan Apo PH NA 1.4 oil immersion objective; an Evolve EM-CCD camera (Photometrics), a piezo stage controller, a temperature and CO_2 controller chamber, all controlled by SlideBook6 software (Intelligent Imaging Innovation). Typically, a 512 x 512 resolution z-stack of 13-18 confocal images was acquired for HEK293 cells, at 0.3 µm z-steps.

Colocalization analysis was carried out using Manders' overlapping coefficient (MOC) which is based on global analysis of pixel intensity distributions. The coefficient ranges from 0 to 1, being 0 non-overlapping images and 1 the entirely colocalization between both images. When colocalization, for example between Kv1.3 and Nedd4.2 was assessed, the MOC reflected the proportion of Kv1.3 signal coincident with Nedd4.2 signal.

3.4.4. Antibody feeding assay

This technique consists in an immunocytochemistry assay to study the endocytosis in individual cells (**Figure 20**). HEK293 were grown over Poly-D-Lysine treated coverslips inside a 24-well multiwell plate and were transfected with Kv1.3-HA (HA is an extracellular epitope YPYDVPDYA). Twenty-four h after transfection, cells were incubated with 2 µg/mL HA.11 antibody (Covance, stock 1 mg/mL) in binding solution (0.1 % BSA diluted in DMEM) for 1 h at RT. To keep pH, 10 mM HEPES was added to the binding solution, and the multiwell was kept in an air-blown bag to keep some CO_2. Then, cells were washed with binding solution and incubated at 37°C with DMEM with or without 1 µM PMA and 0.5 mM 8-Br-cAMP (30 min and 2 h, respectively). After incubation, cells were washed three times with PBS-CMF and fixed for 15 min with 4 % PFA in PBS-CMF at RT. Then, cells were washed thrice with PBS-CMF and stained with 5 µg/mL Donkey anti Mouse-Cy5 (Invitrogen, Stock 500 µg/mL) for 1 hour at RT. The concentration of this secondary antibody must be saturating in order to occupy all the extracellular epitopes. Next, cells were washed three times with PBS-CMF + BSA 0.1 % and fixed again with 4 % PFA. After that, cells were washed and permeabilized 5 min with permeabilizing solution (0.1 % Triton X-100, 0.5 % BSA diluted in PBS-CMF). Then, cells were stained with 1 µg/mL Donkey anti Mouse-Cy3 (Invitrogen, Stock 700 µg/mL) for 1 h at RT. After three washes with PBS-CMF + BSA 0.1 %, coverslips were mounted using ProLong™Gold Antifading mounting media (Invitrogen).

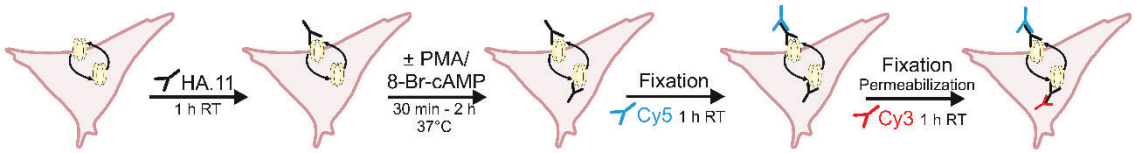

Figure 20. Antibody feeding assay. Schematic representation of the individual endocytosis assay. In yellow it is represented the protein we want to detect (Kv1.3 in the present thesis). This protein can be localized either at the plasma

membrane or in intracellular compartments. The primary antibody used (HA.11) is a monoclonal antibody that specifically recognise the HA epitope. The Kv1.3 construction used has the HA tag in an extracellular domain of the channel (concretely, in the extracellular loop between the transmembrane domains S3 and S4). Therefore, without permeabilization conditions, the HA.11 antibody will recognize the channel that is at the plasma membrane. Cy5 and Cy3 are donkey's secondary antibodies that recognize mouse IgG and are labelled with cyanin 5 and 3, respectively. Fixation: 4 % PFA for 15 min; Permeabilization: cells were permeabilised for 5 min with 0.1 % Triton X-100, 0.5 % BSA diluted in PBS-CMF.

3.4.5. 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 (< 10 nm). 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. Therefore, there are two main requirements for FRET to take place:

- The emission spectra of the donor must overlap with the excitation spectra of the acceptor.
- 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. For that reason, 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 thesis, we performed FRET Acceptor Photobleaching using the CFP-YFP pair, where we measured fluorescence intensity increases of the donor (CFP) when the acceptor (YFP) is bleached. This intensity is known as FRET energy transfer efficiency (FRET efficiency).

FRET efficiency was measured 24 h after transfection. Cells were fixed as explained in section 3.4.2. Four scans of the region of interest (ROI) using 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) YFP photobleaching normalized by the CFP fluorescence intensity normalized by the CFP fluorescence intensity outside the ROI to monitor CFP photobleaching due to imaging. Analysis was performed using Image J.

3.4.6. *In situ* proximity ligation assay (PLA)

The *in situ* Proximity Ligation Assay (PLA) identifies physical closeness of proteins with a distance < 40 nm [257]. In this strategy, the *in situ* target detection depends on the binding by pairs of oligonucleotide-conjugated antibodies, given rise to circular DNA strands that are then amplified by rolling circle amplification (**Figure 21**). This technique is useful to identify interacting proteins in cells and tissues as well as post translational modifications.

HEK293 cells were transfected with WT Kv1.3-YFP. The next day, cells were quickly washed three times with PBS-PF and fixed with 2 % PFA for 10 min at RT. After 3 washes with 20 mM Gly in PBS-PF, cells were permeabilized for 5 min with 0.1 % Triton X-100, 20 mM Gly in PBS-PF. Washes were repeated and then cells were blocked for 1 h at RT with blocking solution (5 % BSA + 0.3 % Triton X-100 in PBS-PF). After 3 washes with 20 mM in PBS-PF, cells were incubated O/N at 4°C with monoclonal anti-GFP (1/100, Roche) and polyclonal anti-Nedd4.2 (1/100, Abcam) primary antibodies diluted in Duolink blocking solution 1x (Sigma-Aldrich-Aldrich). Next, after 3 quick washes, cells were incubated for 1 h at 37°C with secondary PLA antibodies (for 100 µL: 20 µL Duolink *in situ* PLA probe anti-Mouse PLUS, 20 µL Duolink *in situ* PLA probe anti-Rabbit MINUS and 60 µL Duolink antibodies diluent 1x, Sigma-Aldrich). Then, cells were washed five times and incubated for 30 min at 37°C with ligation-PLA-solution (for 100 µL: 20 µL ligation buffer 5x, 2.5 µL ligase and 77.5 µL Milli-Q® water; Duolink Ligase, Sigma-Aldrich). After 5 washes, cells were incubated for 3 h at 37°C in the dark with amplification-PLA-solution (for 100 µL: 20 µL amplification buffer 5X, 1.25 µL polymerase and 78.75 µL Milli-Q® water; Duolink Far Red amplification, Sigma-Aldrich). Next, cells were washed 4 more times and incubated 2 h at RT with fluorescent secondary antibody Goat anti Rabbit Alexa 568 (1/500, Invitrogen) diluted in 1 % BSA and 20 mM Glycine in PBS-PF in order to label endogenous Nedd4.2. Finally, after 3 washes, coverslips were mounted over slides with Mowiol mounting solution.

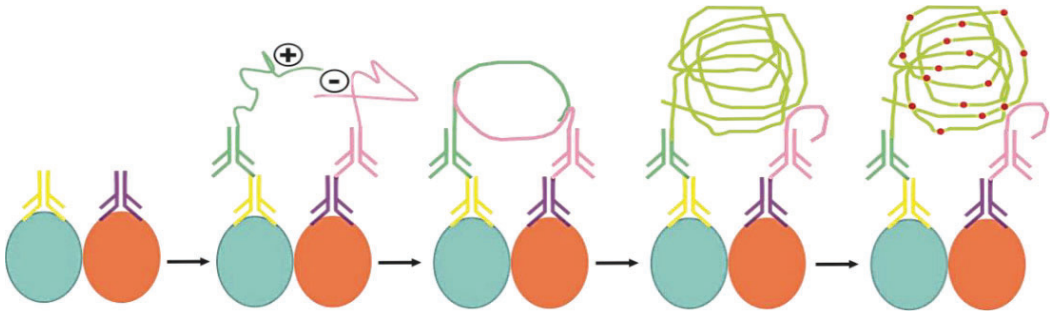

Figure 21. *in situ* Proximity Ligation Assay. Schematic representation of PLA. In blue and orange there are represented the two proteins we want to assess if they are in close proximity (< 40 nm; in our case were Kv1.3 and Nedd4.2). First, our sample is incubated with primary antibodies against our proteins of interest (the two primary antibodies must be from different species, e.g., Mouse and Rabbit). Then, a pair of oligonucleotide-conjugated antibodies PLUS (+) and MINUS (-) bind to the primary antibodies. If the PLA probes are in close proximity, the oligonucleotides will be ligated and give rise a circular DNA strand that will be amplified by rolling circling amplification. The complementary detection oligos, couple to fluorochromes (in our case in FarRed), hybridize to repeating sequences in the amplicons. Therefore, as a result of positive PLA, discrete red spots will be detected by confocal microscopy.

3.5. ELECTROPHYSIOLOGY

HEK293 cells were transfected with WT or MSmut Kv1.3-YFP. Twenty-four h later, cells were trypsinized and replated on a perfusion chamber. In case of PMA treatment, cells were first incubated 30 min at 37°C with PMA and then trypsinized and replated on a perfusion chamber. After 15 min, cells were extensively washed with extracellular solution (in mM: 145 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES and 10 Glucose, pH 7.4 adjusted with NaOH).

Borosilicate electrodes were fabricated from glass capillaries (1.2 OD x 0.944 x 100 L mm, Harvad Aparatus, Holliston, MA, USA) with a P-97 puller (Sutter Instruments, Novato, CA, USA) and fire-polished with a MF-830 Microforge (Narishige) to achieve an average resistance of 2-5 MΩ. The intracellular pipette filling solution for HEK293 cells contained (in mM) 80 AspK, 42 KCl, 10 mM KH_2PO_4, 5 EGRA-K and 5 HEPES-K, pH 7 adjusted with KOH, 3 phosphocreatine and 3 ATP-Mg.

Cells were selected by fluorescence using a Motic AE31 inverted microscope. Gigaseal formation was achieved by suction and the membrane patch was ruptured with an additional brief suction. Cells were clamped at a holding potential of -80 mV. Recording of potassium currents were performed at RT (between 22-25°C) using the whole-cell configuration of the patch clamp technique with an EPC-10 amplifier (Harvard Bioscience, Holliston, MA, USA), waiting 45 s between pulses to allow the complete recovery of Kv1.3 from inactivation. Cells were stimulated with 250 ms square pulses ranging from +60 to -80 mV in 10 mV steps to elicit voltage-gated currents. Peak current was relativized by cell capacitance to obtain current density measures.

3.6. PROTEOMIC STUDIES

3.6.1. Immunoprecipitation, protein precipitation and sample preparation

For Mass Spectrometry analysis, immunoprecipitation (IP) experiments were performed as above described (see 3.3.5), but in this case, 25-30 IPs were performed for each condition. Then, all the immunoprecipitation elution's were concentrated by methanol-chloroform precipitation. Briefly, 750 µL of methanol were added to 500 µL sample and vortexed. Then, 187.5 µL chloroform was added to the mixture and vortexed. Next, 562 µL Milli-Q® H_2O was added and vortexed. After mixing all, samples were centrifuged for 2 min at 15,000 rpm. Proteins were located within a white interface layer between methanol-H_2O and chloroform phases. The aqueous supernatant (methanol- H_2O) was discarded and 562 µL of methanol was added to the mixture and vortexed. Centrifugation was repeated for 2 min at 15,000 rpm to get the protein pelleted. The supernatant was discarded, and the pellet was dried at 37°C and resuspended in 35 µL of resuspension buffer (50 mM Tris, 4 % SDS, pH7.4) and Laemmli buffer without β-mercaptoethanol. Without boiling (to avoid denaturalization), samples were loaded in 1 mm SDS-PAGE in order to have less surrounding acrylamide within the band. Then, polyacrylamide gel was stained overnight at RT with bromophenol blue (provided by Mass Spectrometry and Proteomics facility). To remove the excess

of staining, several washes with Milli-Q® H_2O were performed, and the gel was process by the Mass Spectrometry and Proteomics facility (Parc Científic de Barcelona).

3.6.2. Protein digestion, liquid chromatography, and mass spectrometry

The gel bands were cut in small pieces and washed with 50 mM NH_2HCO_3 (ABC) and acetonitrile (ACN), reduced with 10 mM dithiothreitol (DTT) and alkylated with 50 mM iodoacetamide (IAA). Next, samples were digested with trypsin (Sequencing Grade Modified Trypsin, Promega) and peptides were extracted using 100 % ACN and 5 % formic acid. Tryptic peptides solutions were dried in SpeedVac, reconstituted in 30 µL of 50 mM ABC and redigested with chymotrypsin for 6 h at 37°C. Digestion was stopped with 1 % formic acid and dried in SpeedVac. Final redigested samples were reconstituted in 20 µL 3 % ACN, 1 % formic acid in aqueous solution for mass spectrometry (MS) analysis.

The nano liquid chromatography (LC)-MS set up was as follows. Briefly, peptides were separated using C18 analytical column (NanoEase MZ HSS T3 column, 75 µm x 250 mm, 100 Å, waters) with a 90 min run, comprising three consecutive steps with linear gradients, followed by isocratic elution at 85 % in 0.1 % formic acid in CH_3CN in 5 min and stabilization to initial conditions. The column outlet was directly connected to an Advion Triversa Nanomate (Advion BioSciences, Ithaca, USA) fitted on an Orbitrap Fusion Lumos™ Tribrid (Thermo Scientific). The mss spectrometer was operated in a data-dependent acquisition (DDA) mode. Survey MS scans were acquired in the Orbitrap with the resolution set to 120,000. The highest state ions per scan were fragmented in the HDC cell and detected in the Orbitrap. The ion count target value was 400,000 for the survey scan and 50,000 for the MS/MS scan. Target ions already selected for MS/MS were dynamically excluded for 15 s. Spray voltage in the NanoaAte source was set to 1.6 kV. The spectrometer was working in positive polarity mode and singly charge state precursors were rejected for fragmentation.

3.6.3. Database searches

A database search was performed with Proteome Discover software v2.3.0.480 (Thermo) using Sequest HT and Amanda 2.0 search engine and KCNA3 and contaminants database. Also, a second search with MaxQuant software v1.6.2.6a with Andromeda search engine was made in order to get more confident results. Searches were run against targeted and decoy database to determine the false discovery rate (FDR). Search parameters included no enzyme (PD) and ubiquitin (GG). Peptide mass tolerance was 10 ppm and the MS/MS tolerance 0.02 Da. Peptides with a FDR < 1 % were considered as positive identifications with a high confidence level. Sites detected with the three search engines were considered as high confident while sites detected with two or one search engine should be considered as low confident hits. KCNA3 protein was detected with around 70-80% sequence coverage.

The modification ratio (r) for each PTM site (p-site) was computed counting the number of modified (N_{Mod}) specific peptide spectrum matches (PSMs) and the number of non-modified (N_{Nomon}) PSMs, from which then r was computed as follows:

$$r = \frac{NMod}{NMod + NNoMod}$$

3.6.4. Kv1.3 interactome bioinformatic analysis

Kv1.3 interactome in HEK293 cells was previously obtained in our laboratory. Cystoscape v3.9.1 software was used for the proteomic analysis of the Kv1.3 interactome [258, 259]. The network of protein–protein interactions was retrieved using STRINGapp with a confidence of 95% and the Allegro weak-clustering force-directed layout was used for visualization. Functional enrichment analysis was performed using Reactome Pathways, GO Function and GO Process databases. Manual annotation of interactions described in the literature was performed when indicated.

3.7. OTHER CHEMICAL COMPOUNDS USED

Compound	Function	Company
Adenosine Deaminase (ADA)	Deamination of adenosine to inosine	Roche
Bafilomycin A1	Lysosomal degradation inhibitor by blocking the vacuolar-type H^+-ATPase pump	Sigma
Cycloheximide	Protein synthesis inhibitor	Sigma
Lipopolysaccharide (LPS)	Activates the immune response in dendritic cells CY15	Sigma
Lectin (PHA)	Together with PMA is used to activate T-cells immune response.	Sigma
Margatoxin (MgTx)	Kv1.3 inhibitor peptide	Sigma
Z-Leu-Leu-Leu-al (MG132)	Proteasome inhibitor	Sigma

Table 3. Chemical compounds used. Name, function, and source company are detailed.

3.8. STATISTICS

Values are expressed as mean ± standard error. Significance has been stablished through t-test and ANOVA (PRISM 5.0 GraphPad) for two or more variables, respectively. A p value < 0.05 was considered significant. For statistical analysis, every experiment has been repeated independently for a minimum of three times.

4. Results

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5. Discussion

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6. Conclusions

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Addendum

ADDENDUM

Plasmids

Name	Vector	Insert	Restriction enzymes	Comments
YFP-Kv1.3	pEYFP	rKv1.3	BglII-HindIII	YFP fluorophore
YFP-Kv1.3 Kless	pEYFP	rKv1.3	BglII-HindIII	Lys70, 84, 146, 270, 342, 467, 476, 498, 519Arg, Lys520Ala
YFP-Kv1.3 $Kless_{MS}$	pEYFP	rKv1.3	BglII-HindIII	Lys70, 84, 476, 498, 519Arg
YFP-Kv1.3 Nt	pEYFP	rKv1.3	BglII-HindIII	Lys70, 84, 146Arg
YFP-Kv1.3 K70, 84R	pEYFP	rKv1.3	BglII-HindIII	Lys70, 84Arg
YFP-Kv1.3 Ct	pEYFP	rKv1.3	BglII-HindIII	Lys467, 476, 498, 519, 520Arg
YFP-Kv1.3 K476, 498, 519R	pEYFP	rKv1.3	BglII-HindIII	Lys476, 498, 519Arg
YFP-Kv1.3 Midd	pEYFP	rKv1.3	BglII-HindIII	Lys270, 342Arg
YFP-Kv1.3 ΔN	pEYFP	rKv1.3	BglII-HindIII	New BglII target
YFP-Kv1.3 E442Stop	pEYFP	rKv1.3	BglII-HindIII	Phe442Stop
HA-Kv1.3	pRcCMV	rKv1.3	HindIII-HindIII	Extracellular S3-S4 HA epitope
CFP-Nedd4-2	pECFP	hNedd4.2	BglII-HindIII	CFP fluorophore

Table 4. Plasmids used. Detailed given name, type of vector, insert and restriction enzymes needed to extract the insert and some comments.

Oligonucleotides primer sequences

Name	Sequence 5' – 3'	Comments
F_Kv1.3_K146R	AGGAGGCTATGGAGAgGTTCCGTGAGGACGAGGGCTTCCTG	Mutation K146R
R_Kv1.3_K146R	CTCGTCCTCACGGAACcTCTCCATAGCCTCCTCAC	Mutation K146R
F_Kv1.3_K270R	TGGTGCGATTCTTTGCTTGCCCCAGTAgAGCCACCTTCTCCAG	Mutation K270R
R_Kv1.3_K270R	TATTTCTGGAGAAGGTGGCTcTACTGGGGCAAGCAAAGAATCGC	Mutation K270R
F_Kv1.3_K342R	CAGATCCTGGGACAGACACTGAgGGCTTCCATGCGAGAG	Mutation K342R
R_Kv1.3_K342R	CCAGCTCTCGCATGGAAGCCcTCAGTGTCTGTC	Mutation K342R
F_Kv1.3_K467R	CTTCAGCAGAGGAGCTCCGAAgAGCCCGGAGTAACTCCAC	Mutation K467R
R_Kv1.3_K467R	AGTGGAGTTACTCCGGGCTcTTCGGAGCTCCTCTGCTG	Mutation K467R
F_Kv1.3_K476R	AGCCCGGAGTAACTCCACTCTGAGTAgGTCGGAGTATATG	Mutation K476R
R_Kv1.3_K476R	CCTCTTCGATCACCATATACTCCGACcTACTCAGAGTGGAGTTAC	Mutation K476R
F_Kv1.3_K498R	CCCCAGACCCCCTTCAgAACGGGCAACTCCAC	Mutation K498R
R_Kv1.3_K498R	AGTGGAGTTGCCCGTTcTGAAGGGGGTCTGG	Mutation K498R
F_Kv1.3_R213K	GAGTTTCGCGACGAGAaGGACTATCCC	Mutation R213K
F_Kv1.3_A519R, R520K	CAACTCCTGTGTCAACATCcgAAAGATATTCACTGATGTC	Mutation A519R, R520K
F_Kv1.3_R70K	TCGAGACACAGCTCAAGACCCTCTGCCAGTTC	Mutation RK70K
F_Kv1.3_R80K	CTAGGCGACCCCAAGCGGCGCATGCGATAC	Mutation R84K
F_Kv1.3_R469A (14.3.3)	GAGGAGCTCCGAAAAGCCgcGAGTAACTCCACTCTG	Mutation R469A
R_Kv1.3_R469A (14.3.3)	GTGGAGTTACTCgcGGCTTTTCGGAGCTCCTCTGC	Mutation R469A
F_Kv1.3 S470,472,475A	GAAAAGCCCGGgcTAACgCCACTCTGgcTAAGTCGGAGTATATG	Mutation R470, 472, 475A

R_Kv1.3 S470,472,475A	CATATACTCCGACTTAgcCAGAGTGGcGTTAgcCCGGGCTTTTC	Mutation R470, 472, 475A
F_Kv1.3_S470, 472A	GCTCCGAAAAGCCGCGgcTAACgCCACTCTGAGTAAGTCG	Mutation R470, 472A
R_Kv1.3_S470, 472A	CGACTTACTCAGAGTGGcGTTAgcCGCGGCTTTTCGGAGC	Mutation R470, 472A
F_Kv1.3_S475A	GGCTAACGCCACTCTGgcTAAGTCGGAGTATATGGTG	Mutation R475A
R_Kv1.3_S475A	CCATATACTCCGACTTAgcCAGAGTGGCGTTAGCC	Mutation R475A

Table 5. Oligonucleotide primer sequences used. Detailed given name, primer sequence from 5' to 3' and comments.

Primary antibodies

ANTIGEN RECOGNIZED	SPECIE	WORKING SOLUTION				COMPANY
		WB	ICC	PLA	IP	
pan14-3-3	Rabbit	1/200				SantaCruz (K-19)
14-3-3 γ	Mouse	1:100				SantaCruz (D-6)
β-actin	Mouse	1:50,000				Sigma (AC-15)
Calnexin	Mouse	1:500				BD Transduction (610523)
Calreticulin	Rabbit	1:1,000				Enzo (ADI-SPA-600)
CREB	Mouse	1:500				Santa Cruz (D-12)
p-CREB	Mouse	1:500				Santa Cruz (10E9)
EEA1	Mouse		1:500			BD Transduction (610456)
GFP	Mouse	1:200		1:100		Roche
GFP	Rabbit	1:200			4 µg/mg prot	GenScript
iNOS (NOS2)	Mouse	1:100				SantaCruz (C-11)
Kv1.3	Mouse	1:200				Antibodies Incorporated. Neuromab (L23/27)
Kv1.3	Rabbit		1:150			Alomone (APC-101)
K48-linkage Specific Polyubiquitin	Rabbit mAb	1:1,000				Cell Signaling Technology (D9D5)
Poly-ubiquitin Antibody K63-linkage	Mouse	1:1,000				Novus Biologicals (HWA4C4)
Nedd4-2	Rabbit	1:200		1:100	1:30-1:50	Abcam (whole antiserum)
Ndfip1	Rabbit	1:200				Abcam
Ndfip1	Mouse	1:500			4 µg/mg prot	Santa Cruz (D-4)
p44/42 MAPK (Erk1/2)	Rabbit	1:1,000				Cell Signaling Technology
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit	1:1,000				Cell Signaling Technology
PKC	Rabbit	1/500				SantaCruz (A-3)
p-PKCε	Rabbit	1/200				Abcam

Sodium Potassium ATPase	Mouse	1:1,000				Developmental Studies Hybridoma Bank (The University of Iowa)
Ubiquitin	Mouse	1:500				Santa Cruz (P4D1)

Table 6. Primary antibodies. Primary antibodies used for WB, immunocytochemistry (ICC), PLA and immunoprecipitation (IP).

Secondary antibodies

ANTIGEN RECOGNIZED	WORKING SOLUTION		COMPANY
	WB	ICC	
Goat anti-Mouse HRP-conjugated	1:10,0000		Bio-Rad
Goat anti-Rabbit HRP-conjugated	1:3,000		Bio-Rad
IRDye® 680LT Goat anti-Mouse IgG2a	1:20,0000		LI-COR
IRDye® 800CW Goat anti-Mouse IgG1	1:20,000		LI-COR
IRDye® 680RD Goat anti-Rabbit	1:20,000		LI-COR
Alexa 568 Goat anti-Rabbit		1:500	Provided by CCiTUB
Alexa 660 Goat anti-Mouse		1:500	Provided by CCiTUB
Alexa 647 Goat anti-Rabbit		1:500	Provided by CCiTUB
Cy3 Donkey anti-Mouse		1:1,000	Jackson ImmunoResearch
Cy5 Donkey anti-Mouse		1:100	Jackson ImmunoResearch

Table 7. Secondary antibodies. Secondary antibodies used for WB and immunocytochemistry (ICC).