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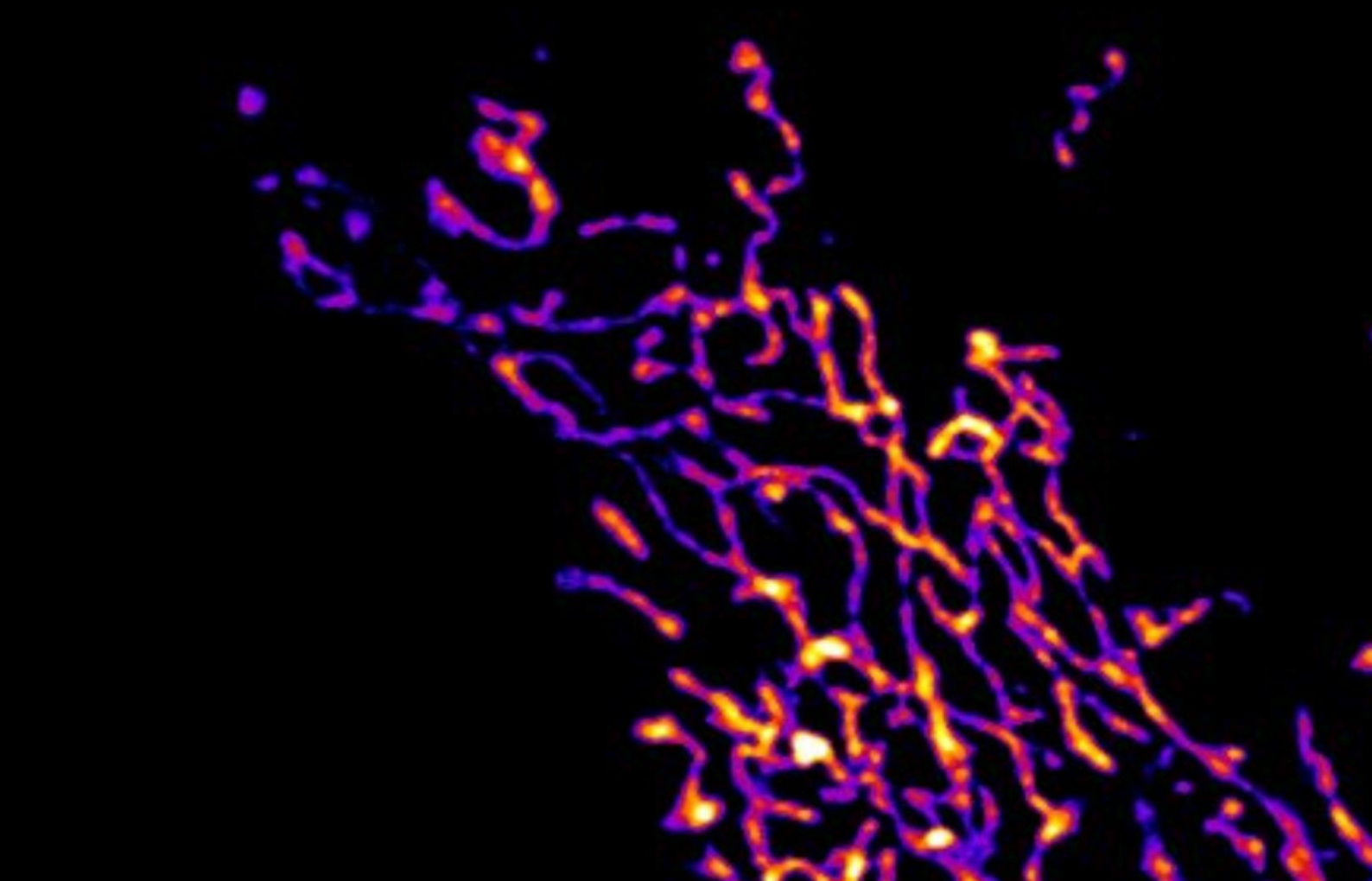
Barcelona, 2019



Functional implications of Kv1.3 spatial organization

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Doctoral programme in Biomedicine

Barcelona, 2019

ABSTRACT

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Kv1.3 is a voltage-gated potassium channel mainly expressed at the plasma membrane and at the inner mitochondrial membrane of different cell types. A dual role for the channel has been suggested: Plasma membrane channel is linked to proliferation and activation while mitochondrial channel controls apoptosis. In this thesis, we analyse in depth the duality of Kv1.3 function. We show that Kv1.3 orchestrates cellular physiology in a way even more complex than expected. The thesis is structured in two parts: *Plasma membrane Kv1.3* and *Mitochondrial Kv1.3*. Each part contains three contributions addressed to the understanding of Kv1.3 traffic and physiological implications of its localization. In the first part (*Plasma membrane Kv1.3*), we describe the caveolin-binding domain (CBD) of Kv1.3 as the determinant for the lipid raft targeting of the channel in cells expressing caveolin (Contribution I). The caveolar localization of the channel is required for proper insulin signalling in adipocytes. In these cells, Kv1.3 controls glucose uptake and insulin resistance. Kv1.3 is a target of Insulin Receptor activation via its localization at caveolae (Contribution II). Finally, we focus on the mechanisms of membrane arrangement of Kv1.3 in systems with limited Caveolin expression, such as lymphocytes, where the expression of Kv1.3 at the plasma membrane is also crucial. In this context, Kv1.3 palmitoylation resulted to be important for the Kv1.3 organization at the immunological synapse. This is the first time that Kv1.3 lipidation is reported and that Kv1.3 is visualized at the immunological synapse platform. Thus, our work expands our knowledge to define better therapeutic strategies in autoimmune diseases, where Kv1.3 is aberrantly expressed at the immunological synapse (Contribution III).

In the second part (*Mitochondrial Kv1.3)*, we first identify caveolin interaction as regulator of Kv1.3 pro-apoptotic activity. Kv1.3/Caveolin axis is an important regulator of cell apoptosis, with important consequences for chemotherapy resistance (Contribution IV). We also describe, for the first time, the mechanisms of Kv1.3 mitochondrial targeting, which involve an unconventional TIM/TOM pathway. We identify cytosolic chaperones as key regulators of Kv1.3 mitochondrial import and show how transmembrane domains cooperate to mediate such process (Contribution V). Finally, we show, for the first time, that mitochondrial Kv1.3 can also regulate proliferation through the control of mitochondrial fusion/fission equilibrium. This is a novel finding because reveals a novel function for mitochondrial Kv1.3 beyond apoptosis. At the same time, puzzle out Kv1.3 as an important component of the mitochondrial fusion/fission machinery (Contribution VI).

In conclusion, this thesis includes novel and relevant findings about Kv1.3 function linked to its localization in the cell.

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1. INTRODUCTION

1. INTRODUCTION

Ion channels are transmembrane (TM) proteins that form hydrophilic pores across the lipid bilayer driving specific inorganic ions down the electrochemical gradient in response to certain stimuli. Potassium channels (KCh) share a highly conserved signature within the pore, known as the selectivity filter, which allows the selective passage of K^+. However, the KCh superfamily shows a high divergence among the sensing domains. This feature permits KCh gating in response to a wide variety of signals [1].

From a structural point of view, KCh are classified in four groups. (i) KCh tetramers of 6TM/1P peptides, with both N and C terminal intracellular. The P region, which contains the K^+ conduction pathway, is situated between the 5th and the 6th TM. This group includes the voltage-gated KCh (Kv) and the small and intermediate conductance Ca^{2+}-activated KCh (K_{Ca}). (ii) The inward rectifiers KCh (K_{ir}), the K_{ATP} and the G protein-coupled potassium channels. These channels are tetramers formed by four 2TM/1P subunits. The P region localizes between the two TM domains. (iii) KCh assemble by tetramerization of 7TM/1P subunits and, unlike the other groups, the N terminus is extracellular. In this group, the P region is located between the sixth and seventh TM. This group includes the large-conductance members of the K_{Ca} family. Finally, (iv) the K_{2P} family. This group, formed by dimerization of 4TM/2P proteins, contains two P regions between the first and the second TM and between the third and the fourth TM [2].

KCh regulate many physiological processes, such as cell excitability [3], hormone secretion [4], proliferation [5] and apoptosis [6]. However, the unique expression of the channel is not enough to accomplish such effects. To do so, protein localization within specific membrane compartments or organelles is mandatory. An ever-growing list of examples is found in literature. Few examples given, lymphocytes concentrate Kv1.3 at the immune synapse to control Ca^{2+} influx during activation [7] or at the inner mitochondrial membrane to regulate apoptosis [8]. In addition, Kv1.3 is located in caveolae in adipocytes to participate in the insulin-dependent signaling [9]. In myocytes, Kv11.1 targets to the transverse tubular network, Kv7.1 localizes in the peripheral sarcolemma and T-tubules and, finally, Kv1.5 is enriched at the intercalated disks and in proximity to Z-lines [10 11]. Finally, neuronal Kv1 channels target to axons to modulate the action potential [12], whereas Kv4 are restricted to dendrites where they integrate and propagate excitatory impulses [13].

1.1 Potassium Channel Biogenesis

The nascent channel protein, containing a signal sequence, targets to the ER. Although scarce information is available about this step, the TM2 would function as signal sequence in Kv1.3 [14]. The formation of the translocon structure allows the KCh peptide to enter the ER lumen. Translocation, concomitantly with integration of the transmembrane segments into the lipid bilayer, defines the membrane topology of the channel. Coordinately, subunit oligomerization takes place. In Kv channels, the T1 domain, located at the N terminus, is responsible for the homo or heterotetramerization with members of the same family. In Kir channels, the C terminus is responsible for the channel assembly. Evidence also implicates transmembrane domains in the tetramer formation. Alongside, folding events configure the pore and sensor domains [15].

Nevertheless, channel biogenesis is the beginning of an odyssey that will finally deliver the channel to the ultimate destination. An impaired KCh traffic may lead to severe malfunctions, named channelopathies. For instance, mutations affecting the trafficking of Kv7.1 and Kv11.1 cause Long QT syndrome [16,17]. On the other hand, mutations altering the traffic of K_{ATP} trigger congenital hyperinsulinism and neonatal diabetes mellitus [18]. In addition to structural motifs, the association of auxiliary subunits and accessory proteins are also important determining the fate of a protein. Some ancillary peptides contain motifs that will govern the subcellular location of the functional complex. Protein-protein interactions can provide or hide traffic motifs as well as bend the structure of the KCh promoting specific targeting. Evidence, therefore, demonstrates that protein final destination is not just a one-signal phenomenon, but it depends on the equilibrium of several different inputs (motifs, accessory subunits, other partners, etc). This balance determinates the location of a channel for a proper role.

1.2 Potassium channel interactions

1.2.1. Regulatory subunits

As above mentioned, the functional diversity of KCh depends on the α-subunit conditions, but also the interaction with auxiliary β-peptides. These β-proteins can regulate both the cellular location and function. There are two kind of regulatory β-subunits: (i) cytosolic peptides, such as KCNAB (Kvβ), K^+ channel-associated protein (KChAP) and K^+ channel-interacting proteins (KChIP), and (ii) small transmembrane proteins (KCNE).

The KCNAB family (Kvβ) members are soluble proteins that may form an up to 4:4 complex with α-subunits. The association is via the T1 cytoplasmic regions of the α-subunit and the C-term of the Kvβ. Three genes encode Kvβ subunits in humans: Kvβ1, Kvβ2, and Kvβ3. Kvβ sequence alignment shows that Kvβ proteins display a highly conserved C-terminal with more than 80% sequence identity and variant NH_2-terminal sequences. Kvβ subunits are NADPH-dependent aldo-keto reductases. Thus, changes in the oxidoreductase activity may alter the gating of Kv channels. In addition, certain NH_2-termini confers rapid inactivation to otherwise non-inactivating Kv channels. Some Kvβ members contribute to the subcellular location of the channel complex [19]. However, the evidence that Kvβ subunits act as chaperones is not convincing. In fact, some Kvβs may either stimulate or attenuate Kv1 channel density at the plasma membrane. Concerning cellular locations, Kvβ subunits may help Kv1 channel driving to axonal compartments. Thus, Kv1.2 does not target to the axon, unless Kvβ2 is present. This scenario is only possible if Kv1.2 and Kvβ2, after assembly in the endoplasmatic reticulum, travel together through Golgi to reach their final destination [20]. However, work on Kvβ knockout mice argues against the chaperone role because both Kvβ2 knockout and Kvβ1/Kvβ2 double knockout mice express similar levels of Kv1.1 and Kv1.2 at the neuronal plasma membrane [21]. In summary, although Kvβ subunits may help Kv channel targeting to distinct cellular sites, this is still an open debate.

The KCNE family of K^+ channel subunits are short single transmembrane glycoproteins that have a regulatory function on the kinetics, voltage dependence and cellular distribution of the α-subunits of Kv channels. This family comprises five members (KCNE1-5) and their effect on the KCh activity has been widely studied. However, KCNE regulation of Kv traffic is tricky. Sometimes KCNE regulates the

channel, whereas in other situations the alpha subunit drives the KCNE location. For instance, all KCNE members associate with Kv7.1. Although KCNEs have limited cell surface expression and lipid raft microdomain targeting, Kv7.1 improves the KCNE1 cell surface expression. In addition, upon association with Kv7.1, KCNE1 and KCNE2 relocate partially in lipid rafts. Importantly, complexes located in these domains can contribute to the cardiac IKs current [22]. On the contrary, KCNE4 and KCN5 are responsible for the channel out of the lipid raft [22], and KCNE4 association impairs Kv1.3 membrane expression and caveolar lipid raft localization while retaining the channel in the ER, fine tuning channel surface abundance [23].

KChIP peptides, a class of EF-hand calcium-binding proteins, represent another family of auxiliary subunits. Alternative splicing of the four human KChIP genes generates a large number of isoforms (KChIP1–4) with a variant NH_2-terminal region and a conserved COOH-terminal domain containing four EF hands. KChIPs associate with Kv channels, mostly Kv4, and modulate their cell surface expression as well as the electrophysiological properties. They specifically interact with cytoplasmic domains of Kv4 α-subunits and promote a calcium dependent membrane targeting of the channel [24-26].

Finally, KChAP belongs to the protein inhibitor of activated signal transducer STAT 3 (PIAS3) gene family and is also called PIAS3 β. KChAP increases Kv1.3/2.1/2.2/4.3 functional expression and current amplitude in *Xenopus* oocytes and mammalian cells, without affecting channel kinetics and gating, suggesting a true chaperone function. KChAP binds transiently to the cytoplasmic N-terminus of Kvs. Moreover, KChAp and Kvβ subunits may interact. Kvβ1.2 inhibits the chaperone effects of KChAP on Kv2.1 and Kv4.3, indicating that KChAP's chaperone properties may be mediated indirectly by an interaction with Kvβ1.2, which further reveals a complex regulatory scenario for the auxiliary subunits [27,28].

1.2.2. Scaffolding proteins and other partners

KCh may associate to a number of miscellaneous proteins to finally reach their final destination. Scaffolding proteins are responsible for the tethering of oligomeric complexes, binding multiple members within a signalling pathway in discrete areas of the cell and thus helping the functionality of the Kv complex. They contain modular domain structures that recruit many proteins forming complexes. PDZ-domain containing proteins and SRC homology 3 domains (SH3) are the most notable. Therefore, these peptides connect KChs to the necessary signalling and trafficking molecules driving channels to target organelles. Scaffolding proteins and their partners show highly specific subcellular locations. For example, PSD-95 (postsynaptic density protein 95), also known as SAP-90 (synapse-associated protein 90), localizes in neuronal synaptic regions and drives Kv1.3 to those specific membrane spots [29]. Discs large homolog 1 (DLG1), also known as synapse-associated protein 97 or SAP97, is also a MAGUK (membrane-associated guanylate kinases) protein known to interact with Kv1.5 localizing in lipid raft domains, where it also interacts with caveolin 3 [30]. Caveolins are well-known scaffolding proteins reported to structure caveolar lipid rafts (caveolae) driving Kv channels into these domains. Direct interaction with the channel occurs via the N-terminal region of caveolin typically affecting the channel activity [31,32]. Miscellaneous scaffolding proteins are a wide and variable family. Some members as CASK (calcium/calmodulin dependent serine protein kinase) and some MAGUK proteins are directly involved in driving potassium channels to their final location. Thus, CASK associates with Kir2 channels in brain and heart [33]. Furthermlore, other scaffolding proteins, such as AKAP (A-kinase anchoring proteins), anchor channels to the membrane once Kv7.1 reaches the final destination [34,35].

A number of proteins, not included in a specific regulatory group, are widely involved in Kv channel trafficking and regulation will be mentioned in this review. Thus, integrins, glycoproteins involved in adhesion with the extracellular matrix, regulate channels such as Kv1.3. Calmodulin (CaM), a calcium-binding protein that regulates several cell processes in response to Ca^{2+} signals, governs the traffic of Kv7 channels. Vesicle proteins such as syntaxins and synaptogamins are related to the movements of neuronal channels along the traffic pathway, while modulating the adhesion or fusion to the membrane or other organelles. Regulatory molecules such as 14-3-3 will also deserve some mention in this review [30].

1.3 Potassium Channel Subcellular Targeting

1.3.1 Endoplasmic Reticulum

After translation, KCh locate into the ER exit sites (ERES) being packed into COPII-coated vesicles and transported via microtubules to the ER-Golgi intermediate compartment (ERGIC). Many motifs are essential for such forward trafficking. For instance, di-acidic signatures at the C-terminal of Kir channels are abundant. For instance, a VLSEVDETD motif in inward rectifier Kir1 channels, Kir2.1 (FYCENE) [36], Kir3.2A (ELETEEEE), Kir3.4 (NQDMEIGV) and Kir6.2 (DXE) channels [18]. Similar clusters are also present in other KCh, such as K_{2P}3.2 (EDE) [37], Kv1.3 (YMVIEE) [38] and the HRETE sequence of Kv1 channels, which exert great influence. Furthermore, an acidic extension of this HRETE in the C-terminus of the mouse Kv1.3 (ETEGE) [39] and a cluster of acidic residues (DDXXDXXX) in a splice variant of KCa1.1 [40] have been also implicated.

Another example is the VXXSL sequence, which is essential for the efficient Kv1.4 anterograde traffic. Variations of this domain are found in other members within the same family and explain the relative surface targeting efficiencies for each channel. Swapping such domain between channels, interchanges their forward trafficking efficiencies [41]**.** Finally, mutations at the C-terminal Cyclic-Nucleotide-Binding-Domain of Kv11.1 can also cause a defective channel anterograde trafficking with fatal consequences [42].

The association of Kvβ subunits has also a notable effect on the ER exit of the KCh. The interaction between Kv1 alpha and Kvβ subunits occurs in the endoplasmic reticulum early in channel biosynthesis. This assembly sometimes results in an increased traffic of channels to the plasma membrane. For example, the association between Kvβ2 and Kv1.2 occurs early in the channel biogenesis, because they interact co-translationally, even before the Kv1.2 is completely translated and translocated into the ER. The β-subunit also affects the N-glycosylation of the channel, mediating the proper folding and traffic of the channel throughout the ER journey [20].

In addition to forward trafficking motifs, ER quality controls prevent the anterograde traffic of misfolded channels. Defective proteins may undergo several rounds of folding before irreversibly targeted to ER-associated degradation (ERAD). This mechanism mediates the retrotranslocation of the channel protein back to the cytosol and a subsequent ubiquitin-dependent proteasomal degradation. The mechanism for ER retention mostly consists of the

exposure of retrieval motifs. Thus, di- or tri-basic motifs, recognized by COPI vesicle coats, retrieve the defective channel complex from Golgi back to the ER [43]. The RXR motif located at the C-terminal of K_{ATP} channels is an example [44]. Although many KChs also contain RXR domains, only in the case of Kv11.1 (ERG) [45] have been definitively confirmed. A similar basic C-terminal signal (KRR) in $K_{2P}3.2$ binds to COPI and drives the channel back to the ER [46]. Other ER retention signals might be less discrete but otherwise widespread. For instance, hydrophobic sequences situated at the N-term of Kv4.2 promote channel aggregation, misfolding and subsequent degradation [25].

ER retention can be physiological and not due to an unsatisfactory quality control. For instance, a pore-based retention mechanism has been described for Kv1 and Kv7. The pore region of Kv1.1 contains two critical residues for the typical ER retention phenotype this shaker channel [47]. Similarly, a pore residue in Kv7.3 is responsible for an inefficient forward trafficking [48]. Finally, a splice variant of KCa1.1 exhibits another retention/retrieval ER mechanism which implies a hydrophobic domain (CVLF) in the intracellular S0-S1 loop of the channel [49].

Many ancillary proteins alter the KCh surface targeting by associations to KCNE subunits. KCNE subunits are key regulators of KCh traffic in the immune system. As above mentioned, KCNE4 impairs Kv1.3 membrane targeting while retaining the channel in the ER by two mechanisms [23]. First, KCNE4 masks the forward trafficking motif (YMVIEE) at the C-terminus of Kv1.3, and second, KCNE4 contains a potent ER retention motif that further limits the surface expression of the complex [50].

KChIPs are also involved in both membrane targeting and retention of KCh. Co-expression of KChIPs1–3 causes a dramatic redistribution of Kv4.2, releasing intrinsic ER retention and improving the cell surface expression via a mechanism that may involve masking of a cytoplasmic trafficking and/or solubility determinant in excitable cells [25]. On the contrary, KChIP2 promotes ER retention when co-expressed with Kv1.5 [51].

Finally, Kv7 channels, such as Kv7.2, require CaM to properly fold and adopt an active conformation to exit the ER. Thus, CaM acting as a Ca^{2+} sensor confers Ca^{2+}dependence for the cell surface trafficking of the channel [52,53]. Similarly, the Kv7.2-Kv7.3 complex requires a Ca^{2+}/CaM dependent mechanism for targeting to the axonal surfacein neuronal cells [54,55].

1.3.2 Golgy Sorting

The Golgi apparatus is at the center of the secretory pathway. Golgi's unique structure of stacked flattened cisternae confers the proper environment for an accurate protein maturation. Proteins entering the ERGIC are transferred through the cis-, medial- and trans- cisternae to

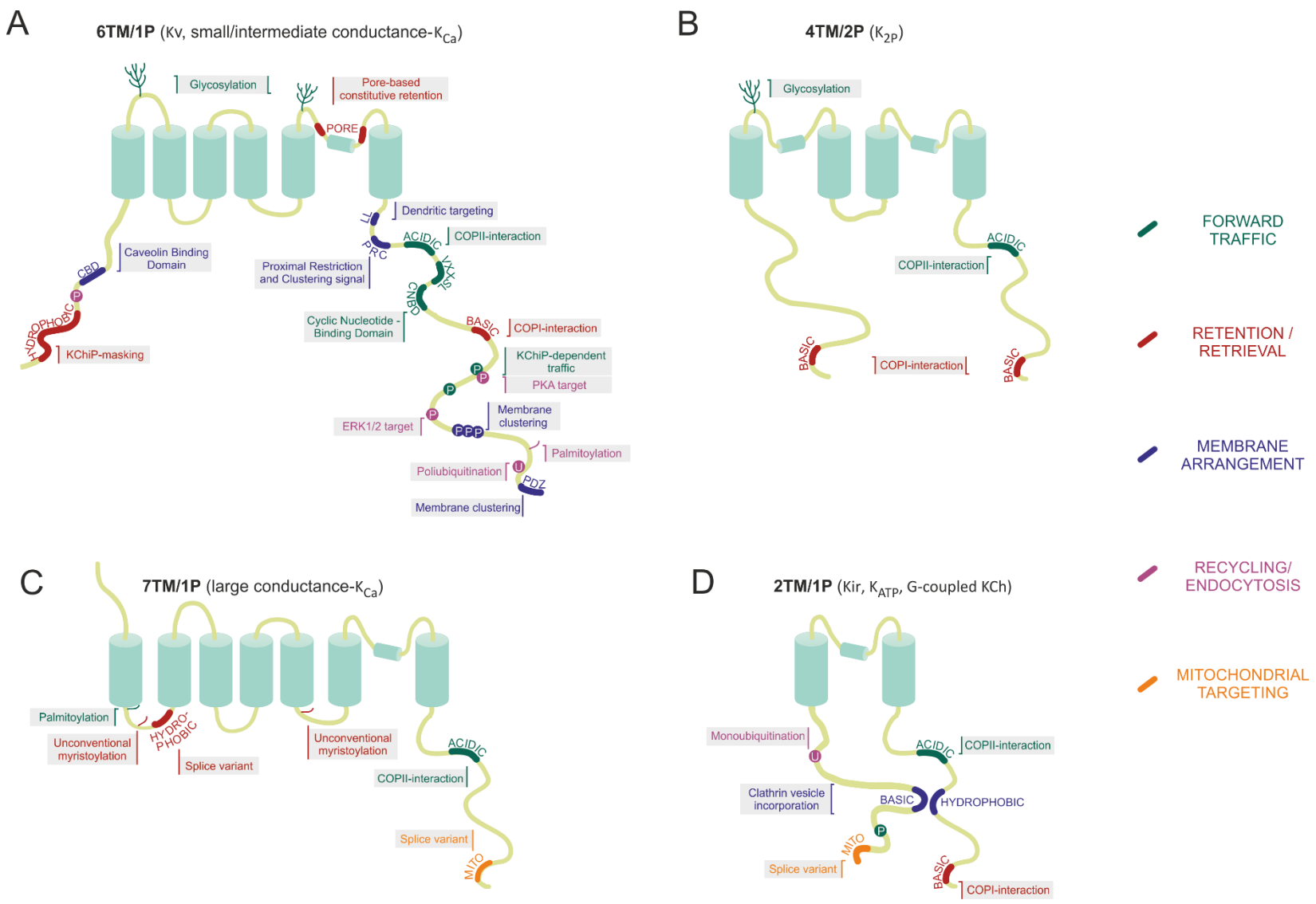

Figure 1| Traffic motifs and molecular interacting signatures within K+ channel structures. A schematic representation of each family of potassium channels. (**A**) 6TM/1P; (**B**) 4TM/2P; (**C**) 7TM/1P; and (**D**) 2TM/1P. Structural domains and post-translational modifications (PTMs) affecting traffic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

be further processed. The trans-Golgi network (TGN) organizes the sorting to the plasma membrane, where cargo proteins concentrate into specific pools of nascent clathrin-coated vesicles. Sorting signals, regulating such stages of KCh maturation, remain largely unexplored**.** Trans-Golgi export of Kir channels is mediated by a cluster of residues located within the confluence of the cytoplasmic N- and C-terminal domains. This creates a tertiary structure that contributes to the interaction with the adaptor protein complex 1 (AP1) driving the incorporation of Kir channels into clathrin-coated vesicles. This domain includes basic residues at the N-terminus and a hydrophobic cleft at the C-terminus [56]. Furthermore, the precise sorting of KCh to specific plasma membrane compartments is already defined in the TGN. For instance, neuronal Kv2.1 and Kv4.2 are sorted into distinct pools of transport vesicles specifically targeted to proximal (Kv2.1) or distal (Kv4.2) dendrite compartments. Kv2.1 vesicles traffic by a mechanism involving myosin and actin filaments, whereas Kv4.2 vesicles depend on kinesin and microtubules [57]. Several mutations, impairing the post-Golgi sorting process, misslocalize these channels in the neuronal membranes. Kv2.1 localizes in large surface clusters in soma and proximal dendrites thanks to a serine-enriched C-terminal motif, known as the proximal restriction and clustering (PRC) signal [58]. Disruption of this motif triggers incorrect post-Golgi sorting, causing Kv2.1 aberrant localization into distal dendrites. Similarly, the C-terminal di-leucine motif of Kv4.2 drives the dendritic targeting [59] and mutations cause an impaired vesicular-sorting of the channel [57].

In addition to the polarized traffic, the packing of KCh into specialized membrane microdomains takes place at the TGN. For instance, the Kv1.3 lipid raft targeting depends on a caveolin interaction through its N-terminal caveolin-binding domain (QRQVWLLF). Such interaction could be functional already at the TGN, where lipid rafts begin to structure concentrating cholesterol [60]. Altogether, TGN could not only direct the journey of KCh nascent vesicles but also the channel affinity for structured plasma membrane domains.

Anterograde traffic of several proteins through the Golgi require specific Golgin molecules. Thus, Kir1.1, Kv1.5 and Kv4.3 channels need Golgin-160 to reach the plasma membrane, even though there is no obvious sequence similarity in the cytoplasmic tails of these KCh. Kir1.1 membrane traffic is also promoted by GM130. Contrary, GM130 is responsible for the HERG cardiac channel retention at this traffic step [61]. Furthermore, Kir2.1 is recognised by Golgin-97, recognizing the channel as an export cargo to be deliveredinto the TGN [62]. Next, some Kir2.1 N-terminal motifs are required to exit Golgi reaching the membrane [63].

1.3.3 Plasma Membrane Arrangement

Eventually, KCh are inserted into the plasma membrane. Mechanisms to maintain KCh at their target destination are needed in polarized cells. A major mechanism for protein retention involves the KCh anchoring to the cytoskeleton. For instance, neuronal Kv7 channels are retained at the axonal initial segment by anchoring to the actin cytoskeleton through a C-terminal ankyrin-G binding motif (I/LAXGES/TDXE/D) [64,65]. Likewise, Kv1.3 is immobilized by interaction with cortactin through its SH3 C-terminal domain (PQTP). Such interaction occurs only at the immunological synapse, suggesting that the polarization of the T cell would promote Kv1.3 trapping at active sites by the actin network [66]. Other arrangement mechanisms include co-clustering by protein-protein interactions. For instance, a PDZ-binding motif at the C-terminal end of Kv1 channels (S/TXV) facilitates Kv1 immobilization by PSD-95 interaction [67]. Effects of protein-lipid interactions on membrane organization of KCh remain elusive although are considerably expectable, given the number of KCh targeting lipid raft microdomains [68,69].

Kvβ subunits modulate cell surface expression of many Kv1 channels. Kvβ members not only promote Kv1.2 membrane targeting, but they also need their $NADP^+$ binding pocket to do so. Defects on the cofactor binding pocket reduced axonal targeting of the channel in hippocampal neurons [70]. In arterial myocytes, Kvβ2 takes a chaperone role when facilitating Kv1.5 trafficking and membrane localization [71].

SNARE proteins, syntaxines and integrines are important helping channels in reaching the membrane. SNARE proteins are membrane integrated proteins responsible for vesicle associated exocytosis processes, but they can also act as a recruitment signal for proteins that must reach the membrane. KAT1 is a plant *Shaker* family-related member that shares a number of structural features with the voltage-sensitive Kv superfamily of K^+ channels of eukaryotes. KAT1 binds directly and selectively with the plasma membrane protein SYP121, a Q-SNARE protein, through a conserved RYxxWE motif located at the cytosolic surface of the voltage sensor domain [72]. K_{2P} and Kv2.1, as well as many others Kv channels, interact with SNARE and syntaxins, which shape the intracellular itinerary of the channels. However, mechanisms by which SNARE proteins affect vesicular transport are unknown [73,74].

Scaffolding proteins are also very important for membrane trafficking and protein stabilization at specific locations. The C-terminal PDZ domains of the Kir channels interact with several partners that regulate intracellular trafficking. Members from the MAGUK family, including SAP91, PSD-95, Chapsyn-110, SAP102, as well as Veli or actin-binding LIM proteins, are scaffolding proteins related with the membrane expression of Kir channels. Thus, PSD-95 facilitates Kir2.3 channel clustering on the plasma membrane [75] and SAP97 regulates Kir2 channel traffic [76]. In addition, dystrophin-associated complexes are also involved in the anchoring and stabilization of these channels at the plasma membrane [33].

In addition to the conventional secretory pathway, which involves COPII vesicles to exit the ER and reach the membrane, some proteins use unconventional routes to target the cell surface. These non-classical pathways are complex and usually comprise cargo molecules with an unknown a signature [77,78]. For instance, Kv4.2 uses COP-I vesicles, instead of COP-II, but only in co-expression with KChIP1 [26]. The CFTR chloride channel uses the ER-PM junctions, which bypass the Golgi apparatus to reach membrane [79]. Concerning KCh, Kv2.1 targets and stabilises these domains. In fact, Kv2.1 provides platforms for delivery and retrieval of multiple membrane proteins [80,81]. The Golgi bypass is recently under the scope. Thus, unknown combinations of SNARE proteins and/or specific molecules yet to be identified would drive KCh to the membrane unconventionally.

Spatially, KCh can locate in specific membrane microdomains, such as lipid rafts. Rafts domains are membrane fractions enriched in sphingolipids and cholesterol that serve as scaffolding regions where signal transductions pathways interface. Proteins reach these domains via different mechanisms, such as lateral diffusion, the formation of lipid “shells” surrounding the channel, which increase the affinity for rafts, protein-lipid interactions such as palmitoylation (see below) or protein-protein interactions [68]. In this last scenario, caveolin acts as a scaffolding protein driving ion channels to raft domains. Caveolins, by impairing lateral diffusion, enlarge the time that KCh are located in these platforms. Moreover, a N-terminal motif for caveolin-1 interaction (FQRQVWLL) has been recently described for Kv1.3,

responsible for the channel trafficking to caveolar rafts [60]. In addition, KChIP proteins, also govern Kv4.3 targeting to lipid raft domains [82]. Finally, PSD-95 is partially responsible of the lipid raft targeting of Kv1.4 and protects Kv1.3 against PMA-induced ubiquitination and endocytosis [29,83]. Kv2.1, Kv1.5 or Kir3.1 are also found in these domains [68], but not direct interaction with scaffolding or auxiliary proteins has been described for such location.

1.4 Post-Translational Modifications Regulating the Traffic

1.4.1 N-glycosylation

N-glycosylation promotes the surface expression of many KCh, probably by reducing protein dynamics and thus increasing stability. N-glycosylation consists in the addition of oligosaccharide chains on a luminal N residue of the N-X-S/T/C consensus sequence (where X can be any aminoacid except P). This process starts in the ER, where a preassembled polymannose oligosaccharide is co-translationally transferred to the N residue. Once in Golgi, sequential enzymatic reactions increase the complexity of the initially added glycan tree [84]. N-glycosylation at the S1-S2 loop favors the surface expression of some *Shaker* channels, such as Kv1.4 [85], Kv1.2 [86] and Kv1.3 [87]. With similar consequences, Kv12.2 and Kv10.1 are glycosylated at the S5-P loop [88,89] and K_{2P}3.1 at the first loop [90]. More controversial is the effect of N-glycosylation on the Kv11.1 traffic [91,92]**.** In fact, N-glycosylation regulates gating rather than surface expression of Kv1.1 [85]**.** Interestingly, although Kv3.1 N-glycosylation is not essential for plasma membrane targeting [93], N-glycan structures guide the spatial distribution of the channel once at the plasma membrane, with major effects on cell adhesion, migration and cell-cell interaction [94,95].

Post-Translational Modifications (PTM) of regulatory subunits or scaffolding proteins can also modify the traffic of KCh. For instance, glycosylation of some β regulatory proteins such as KCNE1, undergoing one or two N-glycan trees, drives the biogenic process of the Kv7.1-KCNE1 complex. KCNE1 can also be O-glycosylated, and altered isoforms have deleterious effects on the biogenesis of the peptide as well as on the Kv7.1-KCNE1 complex [96].

1.4.2 Phosphorylation

Phosphorylation is a reversible and dynamic PTM consisting the transfer of the γ-phosphate group of ATP to the hydroxyl group on the side chains of Ser, Thr or Tyr residues. Usually, the addition of the phosphate group causes significant changes in the protein

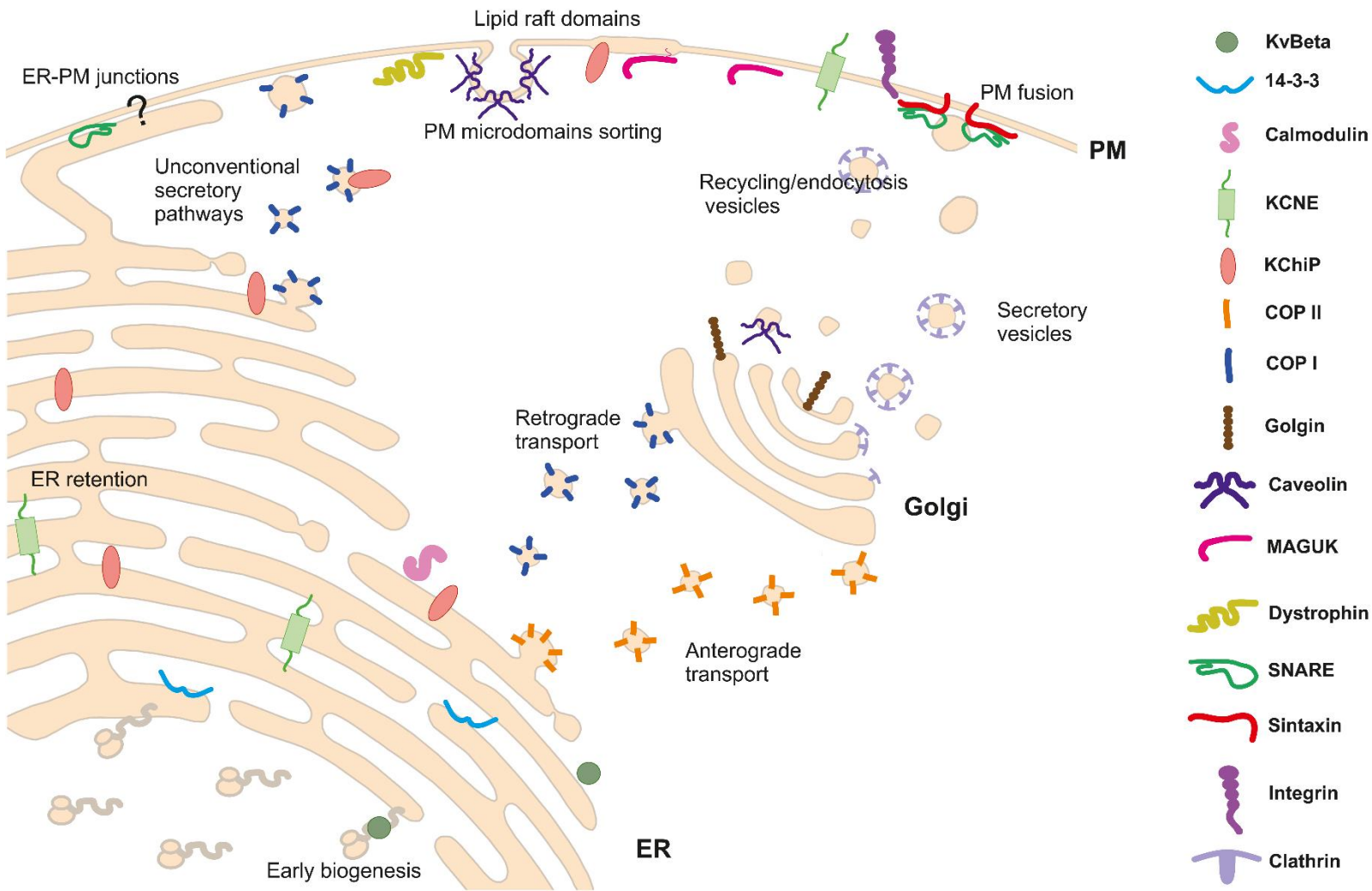

Figure 2| A schematic representation of traffic mechanisms and molecular associations of K+ channels through the secretory pathway. Different shapes represent molecules (see list on the right) known to associate with channels in different compartments. Channels are not depicted. Arrows represent major directions of either retrograde or anterograde routes. Newly synthetized peptides, bearing endoplasmic reticulum (ER) signatures, translocate to the rough ER where the association with Kv□ regulatory subunits takes place in early steps of the biogenesis. Different interactions and motifs balance between forward traffic or ER retention. Regulatory subunits, such as KCNEs, and accessory molecules, such K+ channel-interacting protein (KChiP), 14-3-3, or calmodulin, interact with channels at this step. Conventional mechanisms dictate that regardless of whether anterograde traffic is promoted, cargo channels are processed along the COPII machinery driving to Golgi. Once in Golgi, channels either forward traffic to the cell surface or undergo retrograde transport to ER via the COPI pathway. Molecules, such as Golgin, caveolin, or clathrin facilitate channel routes to the surface. Accessory molecules, such as MAGUK, dystrophin, syntaxin, integrins, or caveolins stabilize channels at the final destination (e.g., lipid raft microdomains and caveolae represented by a thicker part of the membrane and an invaginated omega-shaped structure, respectively). Turnover mechanisms, such as internalization, are mostly mediated by clathrin-coated pit-dependent endocytosis. Alternatively, unconventional pathways are also facilitated by auxiliary subunits assembling the channels. Thus, export from ER via COPI vesicles requires KChiP interaction. Direct contacts with the plasma membrane (PM) and further stabilization with SNARE at the ER–PM junctions are documented. However, most proteins implicated in this unconventional traffic remain unknown. It is important to highlight that mechanisms could differ depending on the KCh and the interacting protein. See text for further details.

conformation inducing new protein-protein interactions. Phosphorylation is enzymatically catalysed by protein kinases and reversed by phosphatases. The existence of thousands of phosphosites and hundreds of protein kinases and phosphatases makes phosphorylation a highly versatile PTM with many different implications in the activity and, to our concern, the trafficking and partitioning of KCh.

For instance, phosphorylation of a N-terminal Ser in Kir1.1 promotes export of the channel overriding ER-retention signals [97]. By contrast, ERK1/2-dependent phosphorylation of C-terminal T495 of Kv1.3 mediates the EGF-dependent endocytosis of the channel [98]. Similarly, PKA-induced phosphorylation of S552 of Kv4.2 causes internalization [99]. Intriguingly, the same S552 requires phosphorylation for an efficient KChIP-dependent Kv4.2 surface targeting [100].This fact supports that phosphorylation of the same residue may trigger opposed effects depending on the life-stage of the protein. Another example of the versatility of phosphorylation is Kv1.2. Phosphorylation at a C-terminal Ser in Kv1.2 enhances the cell-surface expression of the channel [101] whereas phosphorylation of a N-terminal Tyr promotes rapid internalization [102]. Finally, phosphorylation not only regulates both forward trafficking and turnover of KCh but can also determine their arrangement within the membrane. Exempli gratia, dephosphorylation of a large Ser/Thr-rich domain in Kv2.1 C-terminal tail dramatically disperses the somatodendritic clusters of the channel uniformly throughout the membrane of the neuron [103].

14-3-3 is an ubiquitous protein that acquires scaffolding roles. This protein participates in intracellular trafficking of membrane proteins by recognizing phosphoserine-containing motifs in the target. 14-3-3 participates in the surface expression of K_{2P} channels such as $K_{2P}3.1$ or $K_{2P}9.1$. $K_{2P}5.1$ associates to 14-3-3 at the early stages of the biogenesis in the ER. This association facilitates the anterograde traffic of the channel to reach the plasma membrane [104].

1.4.3 Redox modifications

The formation of reactive oxygen and nitrogen species (ROS/RNS) can induce the posttranslational oxidative modification of proteins, usually affecting Cys residues due to their highly reactive thiol group. Redox mechanisms affecting KCh trafficking remain highly unexplored yet are very promising; especially for the understanding of the progression of many pathologies such as cancer or cardiovascular diseases [105]. For instance, oxidative stress reduces surface expression of the cardiac Kv1.5 via sulfenic acid modification of a C-terminal Cys [106]. Likewise, hypoxia disrupts Kv1.3 clathrin-mediated forward trafficking from trans-Golgi to the plasma membrane in T-lymphocytes [107].

1.4.4 Lipidation

Protein lipidation is an emerging field regulating different properties of ion channel function. Lipidation increases protein hydrophobicity. Therefore, this process may regulate the affinity of membrane proteins for specialized membrane microdomains..

S-acylation is the attachment of a fatty acid to an intracellular Cys via a thioester linkage. Unlike other lipidations, S-acylation is reversible due to the lability of the thioester bond. Therefore, proteins can undergo several rounds of acylation/deacylation during their lifetime, leading to multiple regulatory scenarios. In most cases, the identity of the acyl chain attached to the protein is unknown. However, palmitoylation has been reported to modulate the trafficking of some KCh. Palmitoylation of a single Cys residue at the C-terminus of Kv1.5 decreases the surface expression of the channel, probably raising the rate of internalization [108]. The large-conductance KCa1.1 is palmitoylated, by different palmitoyl acyltransferases at different residues. Palmitoylation of the S0-S1 Cys enhances the surface expression of the channel. Interestingly, palmitoylation also regulates the ER exit but, depalmitoylation of KCa1.1 by APT1 largely retains the channel at the TGN, suggesting a major checkpoint for forward trafficking at the Golgi exit [109,110]. Palmitoylation of PSD-95 increases Kv1.4 targeting to lipid rafts. However, when manipulating PSD-95 no apparent modifications in Kv4.2 membrane targeting are observed. These result support that different subpopulations of lipid rafts may require specific signals or ancillary associations [83]. Similarly, evidence supports that KChIP participate in lipid raft targeting only when palmitoylated [82].

Myristoylation also affects the traffic of KCh. N-Myristoylation is the attachment of a myristoyl fatty acid to the N-terminal Gly of a target protein, usually co-translationally, via an amide bond. Like other protein lipidations, myristoylation can regulate protein affinity for specific membrane localizations, while controlling protein-protein and protein-lipid interactions. Oddly, myristoylation also takes place post-translationally on internal residues via ester or amide bonds. KCa1.1 undergoes myristoylation on Ser and Thr residues of intracellular loops 1 and 3. This lipidation modulates KCa1.1 traffic to the plasma membrane [111].

1.4.5 Ubiquitination

Ubiquitination is the covalent attachment of an ubiquitin to a Lys residue of a target protein. Strikingly, ubiquitin itself can be ubiquitinated at any Lys, which increases the complexity and regulatory potential of this PTM. Monoubiquitination mostly triggers endocytosis and lysosomal degradation. For instance, Kir1.1 is endocytosed in response to monoubiquitination at the N-terminus [112]. By contrast, polyubiquitination targets Kv11.1 to proteasomal degradation [113]. Nedd4-2 is an ubiquitin ligase that targets KCh. Thus, Kv7.1 undergoes ubiquitin-dependent degradation via interaction with Nedd4-2 at the C-terminal PY motif of the channel (PPXY) [114]**.** However, Kv1.3, with no apparent PY motif, undergoes Nedd4-2-mediated ubiquitin-dependent endocytosis [29].

1.5. Mechanisms for Organelle Targeting

1.5.1 Lysosomes

Two types of KCh have been identified in lysosomes. TMEM175, a novel KCh with a non-canonical structure that regulates lysosomal membrane potential and pH, and BK channels, which regulate lysosomal Ca^{2+} movements. While lysosomal targeting mechanisms of the former are unknown, dileucine motifs at the C-terminal of the latter have been identified. Dysfunction of lysosomal KCh relates to a variety of neurodegenerative disorders [115,116].

1.5.2 Nucleus

Some KCh have been identified at the nuclear envelope of different cell types. A putative role has been postulated controlling

nuclear $\Delta\Psi$, nucleoplasmic calcium movements and, ultimately, gene expression. The targeting mechanisms and specific localization within the nuclear envelope are unclear, as it is the case for K_{ATP} [117], Kir2.2 [118], KCa3.1 [119], BK [120], or Kv1.3 [121].

The outer nuclear membrane (ONM) is an extension of the ER. Despite that, it contains a distinct protein composition; hence the existence of specific targeting mechanisms from the ER is obvious. The inner nuclear membrane (INM) connects the ONM through the lateral channels of the nuclear pore complex (NPC). Integral proteins may travel from the ONM across such lateral channels to finally locate at the INM. Channels can do so by free lateral diffusion or accompanied by importins, which can recognize nuclear localization sequences (NLS) within the cytosolic domains of transmembrane proteins. Once at the INM, proteins can be retained by interactions with chromatin and/or laminins [122]. NLS can be monopartite (a short basic sequence) or bipartite (two short basic sequences separated by a linker). Although the oncogenic potassium channel Kv10.1 contains a bipartite C-terminal NLS, its removal does not affect the INM localization. Interestingly, a mechanism by which plasma membrane Kv10.1 and not ER-localized Kv10.1 relocate to the INM has been postulated [123].

1.5.3 Mitochondria

An increasing number of KCh are reported in the inner mitochondrial membrane (IMM). Although the physiological and pathophysiological role for mitochondrial channels is not completely understood, intense work has been performed pointing to a major role in the regulation of energy metabolism [124], apoptosis [125] and autophagy [126].However, little is known about the mechanisms governing the mitochondrial targeting of KCh. The inclusion of a splice exon at the C-terminal end of KCa1.1 finely sorts the channel into mitochondria [127]. Similarly, a short isoform of Kir1.1 contains a canonical N-terminal mitochondrial targeting motif, which drives the channel to the IMM [128]. For the rest of mitochondrial KCh, the sorting mechanisms can only be speculated. To date, the list includes KCa3.1 [129], KCa2.2 [130], Kv7.4 [131], K_{2P}3.2 [132], KNa1.2 [133] and Kv1.3 [8].

Mitochondrial KCh are encoded by the nuclear genome, because no obvious gene for a KCh is found in the mitochondrial genome. The channel protein is synthetized by cytosolic ribosomes and, probably, translocated into mitochondria through the TOM/TIM system. Although the above-mentioned Kir1.1 presents a mitochondrial targeting sequence at the N-terminus, this is far to be a general mechanism, such as it has been demonstrated for BK channels. In the case of Kv1.3, an even more complex scenario is hypothesized. Thus, the plasma membrane channel could be transferred to mitochondria via ER-mitochondria contact sites, the so-called MAM structures [6]. This idea is reinforced by the fact that N-glycosylated proteins can be transported from ER to chloroplast [134] and by the discovery of direct transfer of viral proteins between ER and mitochondria [135]. However, a very interesting study about the mitochondrial sorting of a viral KCh supports that the channel is translocated into mitochondria through the canonical TIM/TOM system, guided by structural information encoded by the C-terminus of the channel[42,136].

1.6 Heterotetramerization

In addition to the influence of ancillary molecules on KCh traffic, heterotetrameric associations, mostly with other members of the same family, raise a number of potential re-routings. Kv1.3 associates with Kv1.1, Kv1.2 and Kv1.4 in brain, and Kv1.3/Kv1.5 hybrids are found in macrophages. While homomeric Kv1.5 does not target to lipid raft domains, heterotetrameric channels with Kv1.3 redirects the Kv1.5 to caveolar domains. Moreover, different stoichiometry within the complex shows different cell distribution and electrophysiological properties [137]. Furthermore, heteromeric assembly of neuronal channels generates variable phenotypes. Kv1.1 is mainly localised in the ER, Kv1.4 at the plasma membrane, while Kv1.2 is found in both locations. Kv1.1. and 1.2 increase their membrane expression when assembled with Kv1.4, in a dose-dependent manner. However, when Kv1.1 is saturated, the complex formed with Kv1.4 reduced its surface targeting significantly. This again suggests an effect of the α-subunit stoichiometry on the targeting of the complexes [138,139].

Kv7.2/Kv7.3 generates the M-current, which controls neuronal excitability. Kv7.3 does not form functional homotetramers on the membrane, but needs the association of Kv7.2 in order to escape the ER and reach the cell surface [48]. Furthermore, functional Kv7.2/Kv7.3 heterotetramers associate with ankyrin-G targeting the axon initial segment [65].

Even further complex heteroteoligomerization can also happen between different ion channels. For instance, Kir2.1 and Nav1.5 form macromolecular complexes participating in the cardiac excitability by interacting with several other partners at early stages of their secretory pathway. The complex pre-assembles during early forward traffic and exits the Golgi apparatus being recognized by AP1. Moreover, traffic alterations of one member affect the whole complex secretion. Thus, retention of Kir2.1 at Golgi impairs Nav1.5 membrane targeting [140,141].

The channel complex contains several motifs to drive its journey throughout the vesicular pathway. Cooperatively, association with auxiliary subunits and other interacting partners helps the channel to reach the final destination. These associations can mask or expose channel motifs which contain traffic signals. A heteromeric composition of the channel protein can also modulate the traffic. Additionally, channels can undergo several PTM with direct effect on their traffic. Mechanisms of forward traffic will favour the ER exit driving the channel to further quality controls. KCh can be either retained or retrieved into ER. Once in Golgi, the channel will be further matured and sorted within specific vesicles. At the plasma membrane, the channel distribution can be further spatially organized into specific microdomains. Evidences support unconventional pathways including direct arrival to the plasma membrane by-passing Golgi. Specific mechanisms can also drive the channel to other destinations within the vesicular pathway, such as lysosomes or the nuclear membrane. Additionally, KCh can reach organelles outside the anterograde vesicular pathway by alternative mechanisms, such as the mitochondrial targeting. The amount of channel at the destination is of great relevance for the cellular physiology. Thus, a fine regulation of turnover is crucial.

Considering all this, the traffic regulation shows a huge amount of possibilities in order to accomplish the channels proper function. A certain variable in the whole process can trigger differential effects, not only at the cellular level, but also at the whole-body homeostasis.

2. AIMS

AIMS

Ion channels are the greatest example in nature of how protein structure determines function, and *vice versa*. Because cellular phenomena are 4D processes, the spatiotemporal context is also intrinsically related to protein function.

In this thesis, we will study the relevance of Kv1.3 distribution within cellular compartments during different biological processes.

The voltage-gated potassium channel Kv1.3 is a multifunctional protein with relevant implications in the pathophysiology of different diseases. The channel has been linked to autoimmune diseases, such as multiple sclerosis or rheumatoid arthritis. It is also involved in cancer progression and chemotherapy resistance. In addition, Kv1.3 is at the onset of many metabolic disorders, such as obesity and diabetes.

Kv1.3 has been classically studied as a plasma membrane-resident protein, whose function is limited to its conducting properties.

Interestingly, growing number of evidences indicate that Kv1.3 can exert cellular functions beyond its conducting properties. In addition, Kv1.3 in mitochondria has emerged as a key regulator of cell fate.

In this context, our aims in this doctoral thesis are:

1. To study the mechanisms of Kv1.3 arrangement at the plasma membrane and the physiological implications of its membrane organization.

2. To elucidate the mechanisms of Kv1.3 mitochondrial targeting and delve into the physiological consequences of this localization.

3. RESULTS

3.1 PART 1: PLASMA MEMBRANE Kv1.3

3.1.1 CONTRIBUTION I

Caveolin interaction governs Kv1.3 lipid raft targeting

Mireia Perez-Verdaguer[1,2] · Jesusa Capera[1,2] · Ramón Martínez-Mármol[1,3] · Marta Camps[2] · Núria Comes[1,4] ·Michael M. Tamkun[5] · Antonio Felipe[1,2]

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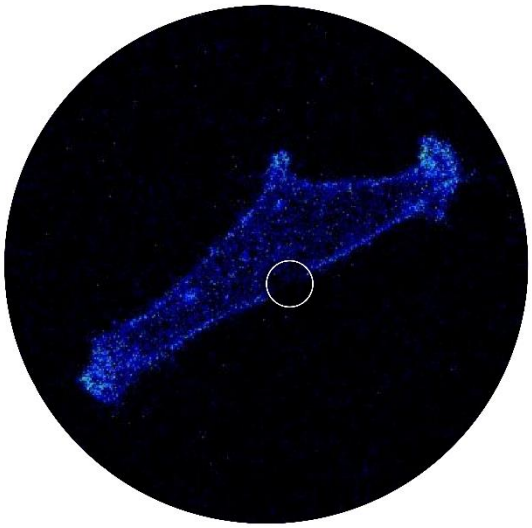

Report of the PhD student participation in the article:

Caveolin interaction governs Kv1.3 lipid raft targeting

Published in *Scientific Reports; 2016, march, 6:22453. doi: 10.1038/srep22453*

Impact factor (2018): 4.525

Jesusa Capera participated in the realization of all main and supplemental figures containing lipid raft isolations, co-immunoprecipitation assays and half-life studies.

Prof. Antonio Felipe
Thesis director

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Caveolin interaction governs Kv1.3 lipid raft targeting

Mireia Pérez-Verdaguer[1,2], Jesusa Capera[1,2], Ramón Martínez-Mármol[1,3], Marta Camps[2], Núria Comes[1,4], Michael M. Tamkun[5] & Antonio Felipe[1,2]

Received: 16 December 2015
Accepted: 15 February 2016
Published: 02 March 2016

The spatial localization of ion channels at the cell surface is crucial for their functional role. Many channels localize in lipid raft microdomains, which are enriched in cholesterol and sphingolipids. Caveolae, specific lipid rafts which concentrate caveolins, harbor signaling molecules and their targets becoming signaling platforms crucial in cell physiology. However, the molecular mechanisms involved in such spatial localization are under debate. Kv1.3 localizes in lipid rafts and participates in the immunological response. We sought to elucidate the mechanisms of Kv1.3 surface targeting, which govern leukocyte physiology. Kv1 channels share a putative caveolin-binding domain located at the intracellular N-terminal of the channel. This motif, lying close to the S1 transmembrane segment, is situated near the T1 tetramerization domain and the determinants involved in the Kvβ subunit association. The highly hydrophobic domain (FQRQVWLLF) interacts with caveolin 1 targeting Kv1.3 to caveolar rafts. However, subtle variations of this cluster, putative ancillary associations and different structural conformations can impair the caveolin recognition, thereby altering channel's spatial localization. Our results identify a caveolin-binding domain in Kv1 channels and highlight the mechanisms that govern the regulation of channel surface localization during cellular processes.

Subcellular localization of ion channels is essential for proper cell physiology. Lipid raft microdomains, which are enriched with highly packed sphingolipids and cholesterol, have emerged as specific membrane platforms where ion channels converge with signaling molecules, thereby regulating cellular responses[1]. In this context, caveolae, specialized omega-shaped lipid raft microdomains, are abundant in differentiated cell lines such as adipocytes, pneumocytes, endothelial and muscle cells[2–4]. These structures participate in endocytosis, membrane compartmentalization, mechanosensing and mechanoprotection, cell signaling and lipotoxicity protection[5]. Therefore, it is not surprising that an impaired caveolae expression results in extensive physiological dysfunctions[6]. Caveolae are folded by structural proteins called caveolins (Cav), a protein family that is composed of three members of 18–24 KDa (Cav1, Cav2 and Cav3)[7]. Cav participates not only in caveolae biogenesis but also in protein-protein and protein-lipid interactions evoking part of the caveolae properties and functions. The structural associations of Cav during cell signaling are mediated via a Caveolin Scaffolding Domain (CSD) located at the N-terminus of the protein, which interacts with putative Caveolin Binding Domains (CBD) located in target proteins. The CSD of Cav1 and Cav3 recognize a canonical CBD consensus sequence ΦxΦxxxxΦxxΦ, with slight variations (where Φ is an aromatic residue and x can be any amino acid)[8].

K^+ channels play an essential role in many cellular functions in both excitable and nonexcitable cells. Voltage dependent potassium channels (Kv) participate in controlling repolarization and resting membrane potential. The mammalian Kv1 family (*Shaker*) comprises 8 members (Kv1.1–Kv1.8) which are involved in nerve and muscle physiology[9]. Kv1.3, the third member of the *Shaker* family, is mainly expressed in the nervous and immune systems[10,11] and participates in multiple cellular functions such as the maintenance of the resting membrane potential, immune cell activation, proliferation, cell volume control and apoptosis. Altered Kv1.3 expression is associated with autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis and diabetes[12], as well as changes in sensory discrimination[13]. In leukocytes, Kv1.3 associates with Kv1.5, another *Shaker* isoform that is crucial for myoblast proliferation[14–16]. Heteromeric channels present specific biophysical and pharmacological properties, as well as different cell surface expressions[14]. Although Kv1.3 and Kv1.5 share lipid raft

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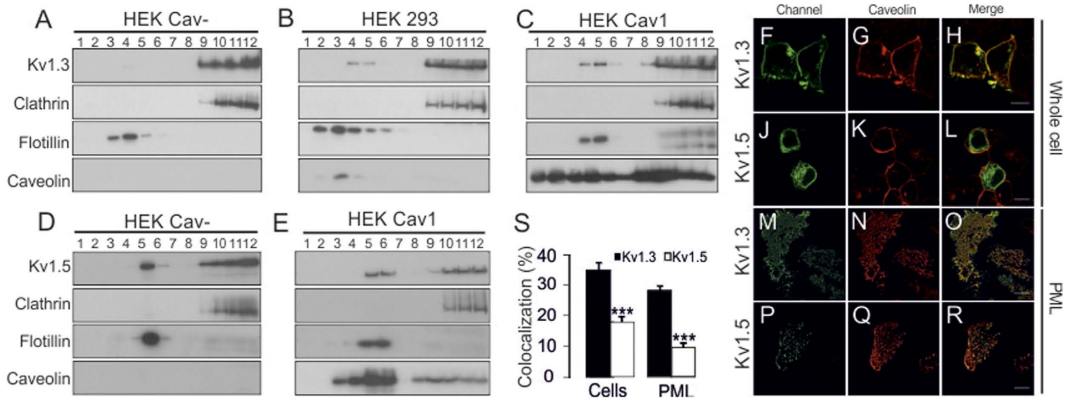

Figure 1. Kv1.3, but not Kv1.5, targeted to lipid raft microdomains in a caveolin-dependent manner. Sucrose-density gradients were performed with total lysates from HEK Cav- (**A**), HEK 293 (**B,D**) and HEK Cav1 (**C,E**) expressing Kv1.3YFP (**A–C**) and Kv1.5YFP (**D,E**). Clathrin and Flotillin were used as non-lipid raft and lipid raft markers, respectively. Note that the overexpression of Cav1 (HEK Cav1) partially targeted caveolin to floating (enriched in lipid raft) and non-floating fractions. Colocalization of Kv1.3 (F_H and **M–O**) and Kv1.5 (**J–L**,**P–R**) with caveolin in whole HEK Cav1 cells (**F–L**) and plasma membrane lawns (PML) (**M–R**). Green: channel; Red: caveolin; Merge: colocalization in yellow. Bar scale 10 μm. (**I**) Histogram of the colocalization (%) between channel and caveolin. Closed bars, Kv1.3; open bars, Kv1.5. Data are the mean ± SE of whole-cell (>15 cells) and PML (>35 cells). ***$p < 0.001$ vs Kv1.3 (Student's t-test).

localization, membrane dynamics suggest distinct membrane microdomain targeting[14,16–18]. In this context, differential protein-protein interactions, which influence cell surface expression, have been suggested[17,18]. In fact, the Kv1.5-caveolin association is under debate[19,20]. In this context, we have previously described the importance of protein interactions influencing Kv1.5 lipid raft targeting and postulate that several partners could compete to determine channel localization[18].

Here, we study the influence of Cav on the lipid raft targeting of Kv1 channels by analyzing the function of a putative CBD motif conserved in the *Shaker* family. Both Kv1.3 and Kv1.5 target lipid rafts, but only Kv1.3 efficiently interacted with Cav via the CBD where this association is essential for the channel localization in these domains. Moreover, Kv1.3 behavior and activity was conditioned by the presence of Cav1. Therefore, the presence of a CBD near the T1 of Kv1.3 has important functional consequences for Kv1.3 channel physiology.

Results

Differential caveolin dependence of Kv1.3 and Kv1.5 lipid raft partitioning. Voltage-dependent potassium channels (Kv) from the Kv1 (*Shaker*) family share several structural features that govern channel expression at the cell membrane. To analyze the microdomain targeting of Kv1 channels, three different HEK 293 cell lines exhibiting differential Cav1 expression levels, named HEK Cav-, HEK 293 and HEK Cav1, were used (Supplementary Fig. S1). Thus, Kv1.1-Kv1.5 channels were expressed in HEK Cav- and HEK Cav1 cells. While Kv1.1, Kv1.2 and Kv1.4 showed no relevant lipid raft localization, neither in the presence nor in the absence of Cav1 (Supplementary Fig. S2), Kv1.3 and Kv1.5 partially targeted to raft microdomains (Fig. 1). The steady augmentation of Cav1 increased the targeting of Kv1.3 to floating fractions, which are enriched in lipid raft microdomains and identified by the presence of flotillin (Fig. 1A–C). Unlike Kv1.3, different Cav1 expression levels did not alter Kv1.5 raft association (Fig. 1D,E). As we previously described[17], while Kv1.3 efficiently targets to the membrane surface (Fig. 1F–H), Kv1.5 was mostly retained intracellularly. In this scenario the presence of Cav1 did not alter this behavior (Fig. 1J–L). In addition, the colocalization between Kv1.3 and Cav1 was higher than that of Kv1.5 (39.5 ± 2.5% and 17.8 ± 1.8%, respectively, $p < 0.001$, Fig. 1S). To gain further insight into the plasma membrane targeting of Kv1.3 and Kv1.5 and their colocalization with Cav1, we isolated plasma membrane lawns (PML) (Fig. 1M–R). In these preparations, Kv1.3 also showed higher Cav1 colocalization than did Kv1.5 (28.1 ± 1.4% and 9.5 ± 1.4%, respectively, $p < 0.001$, Fig. 1S). These results suggest that Kv1.3 and Cav1 share spatial localizations at the membrane surface. To understand the extent to which this Kv1.3-Cav1 relationship is physiologically relevant, we analyzed lipid rafts of bone marrow-derived macrophages (BMDM) from Cav1 null mice ($Cav1^{-/-}$). Although Kv1.3 partially localized in low-buoyant fractions in macrophages from both wild-type and $Cav1^{-/-}$ animals, the amount of Kv1.3 in rafts was clearly lower in BMDM from $Cav1^{-/-}$ mice than in those from wt (Supplementary Fig. S3).

Caveolin 1 associates with Kv1.3 altering channel membrane dynamics, stability and activity. The Kv1.3 Cav1-dependent lipid raft localization suggested a putative interaction between Kv1.3 but not Kv1.5 and Cav1. Caveolin interacts with many different signaling proteins such as eNOS (endothelial nitric oxide synthase), Src kinases or PKC, thereby recruiting them into caveolae platforms[21–23]. To directly address this point, we performed coimmunoprecipitation and Förster Resonance Energy Transfer (FRET) experiments. Low-buoyant Cav1 and flotillin-enriched fractions (see Fig. 1) were subjected to caveolin immunoprecipitation. Kv1.3, but not Kv1.5, was coimmunoprecipitated with Cav1 (Fig. 2A,B). Similar results were obtained from clathrin-enriched non-raft fractions (data not shown). Furthermore, FRET results corroborated a molecular interaction between

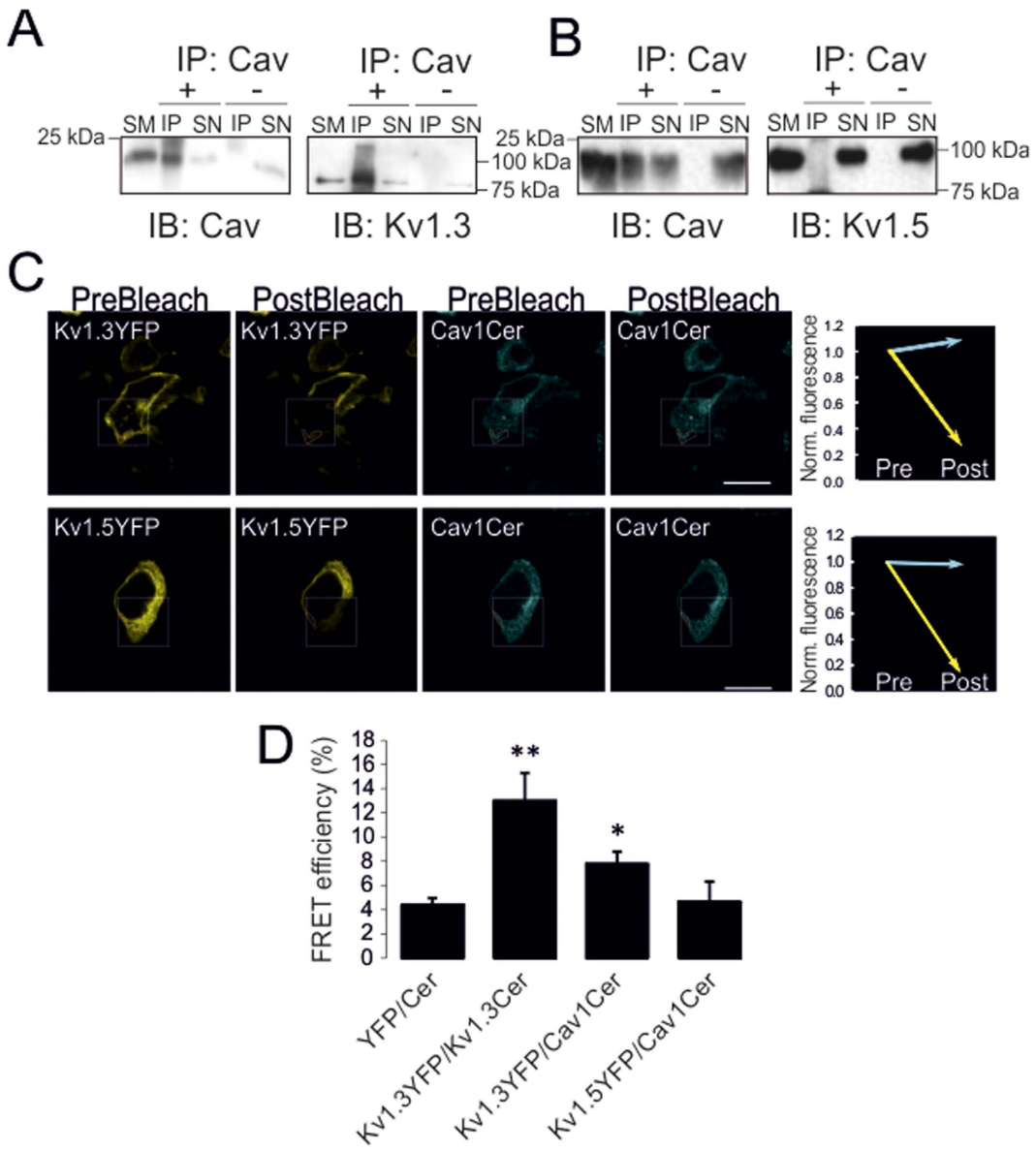

Figure 2. Molecular association of Kv1.3 with caveolin. HEK Cav1 cells were transfected with Kv1.3 (**A**) and Kv1.5 (**B**) and, after lipid raft isolation, floating fractions were subjected to caveolin immunoprecipitation (IP: Cav) in the presence (+) and the absence (−) of caveolin antibody. Blots were analyzed for the presence of Kv1.3 (IB: Kv1.3), Kv1.5 (IB: Kv1.5) and Cav1 (IB: Cav). SM: starting material; SN: Supernatant; IP: immunoprecipitate. (**C**) Representative FRET images of HEK Cav- cotranfected with Cav1-Cerulean and Kv1.3YFP (top panels) or Kv1.5YFP (bottom panels). Images from left to right show acceptor (Channel-YFP) prebleach and postbleach and donor (Cav1-Cerulean) prebleach and postbleach images. Squares insets indicate the bleached zone containing the quantified red limited areas. The line graphs at the right show changes in donor (cyan) and acceptor (yellow) fluorescence after bleach (**D**) Histogram with the FRETeff quantification (%) of YFP-Cer (negative control), Kv1.3YFP-Kv1.3Cer (positive control), and samples from (**C**). Data are the mean ± SE ($n > 35$). *$p < 0.05$; **$p < 0,01$ vs YFP-Cer (Student's t-test).

Kv1.3 and Cav1. Thus, when Kv1.3-YFP, but not Kv1.5-YFP, and Cav1-Cer were coexpressed the post-bleching intensity of Cav1-Cer increased (Fig. 2C,D).

The caveolin 1 expression induces *de novo* formation of caveolae structures in caveolin-null cells, thereby increasing the plasma membrane structuration[24,25]. Moreover, caveolae appear as rigid structures in which caveolins show a reduced mobility[26]. Because Kv1.3 and Cav1 physically interact, the caveolae targeting and the membrane dynamics of Kv1.3 were tested in HEK Cav- and HEK Cav1. Electron micrographs indicated that Kv1.3 was recruited into caveolae-like structures in the presence of Cav1 (Fig. 3A,B). Whether Kv1.3 membrane lateral diffusion was altered in the presence of Cav1 was studied by fluorescence recovery after photobleaching (FRAP) analysis (Fig. 3C–G). The Kv1.3YFP fluorescence recovery was monitored over time in HEK Cav- and HEK Cav1, until a steady state was achieved (Fig. 3E). While the mobile fraction (0.56 ± 0.04 vs 0.48 ± 0.04, for HEK

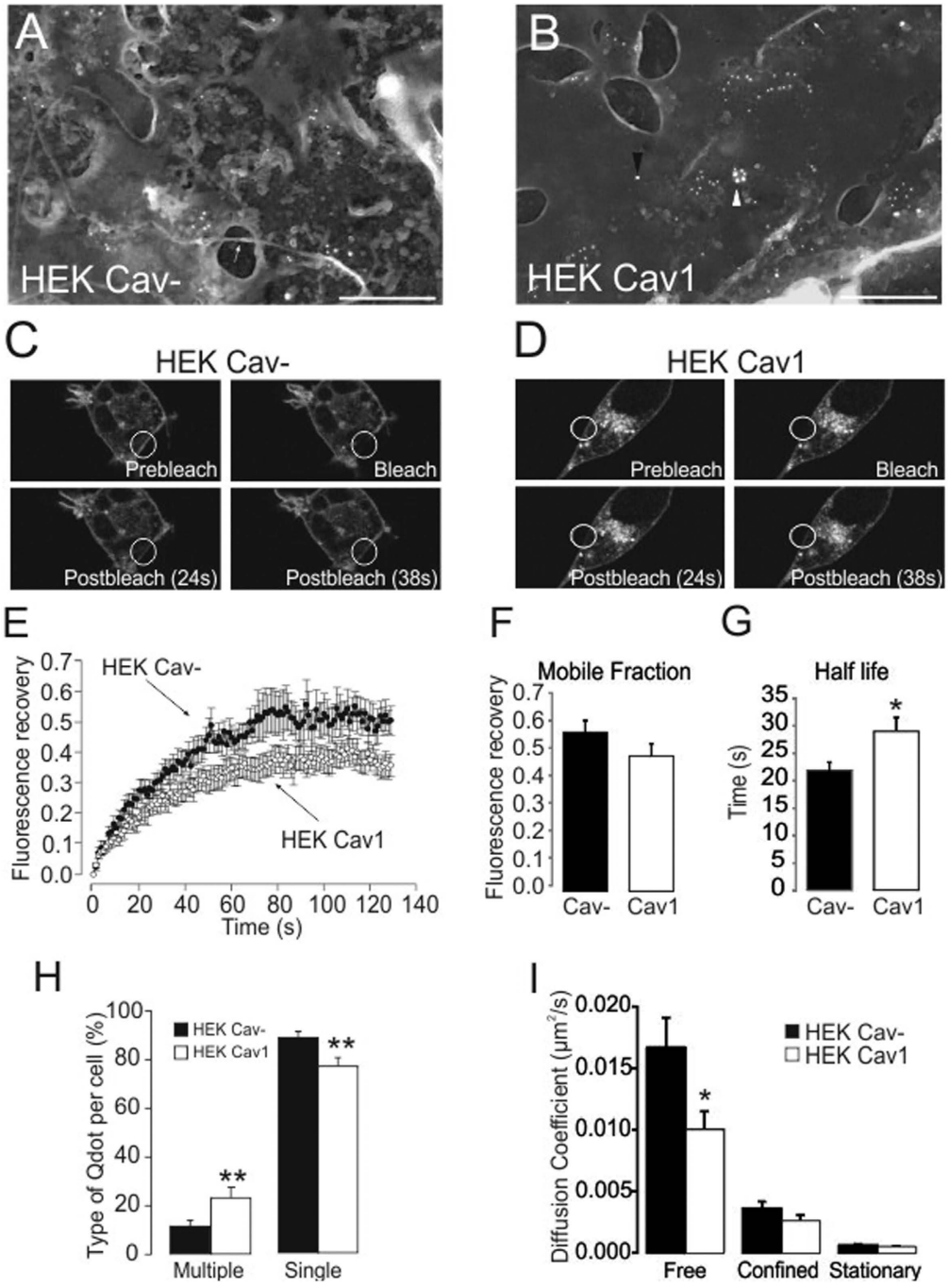

Figure 3. Caveolin expression induces aggregation and slows the lateral diffusion of Kv1.3. (**A,B**) Electronic micrographs of PML from HEK Cav- (**A**) and HEK Cav1 (**B**) expressing Kv1.3. Cav1 and Kv1.3 were immunolocalized with anti-Kv1.3 monoclonal and anti-caveolin polyclonal antibodies and a mixture of 10 nm and 15 nm colloidal gold conjugated secondary antibodies tagging Kv1.3 and caveolin, respectively. Bar Scale 500 nm. White arrows point at microtubules. White arrowhead highlights caveolae. Black arrowhead points at caveolin outside caveolae like structures. (**C–G**) FRAP performed 24 h after Kv1.3YFP transfection in HEK Cav- (**C**) and HEK Cav1 (**D**). Representative images are before (prebleach), during (bleach) and 24 or 38 s after bleach (postbleach). White circles highlight the bleached membrane. (**E**) Fluorescence recovery. (**F**) Mobile fraction and (**G**) half live recovery (the mean ± SE, n = 10). (**H,I**) SPT analysis of HEK Cav- and HEK Cav1 expressing Kv1.3LoopBAD together with BirA for 24 h and tagged for 5 min with Qdots-Streptavidin. (**H**) Qdots were classified as single or multiple units. Histogram displays the relative abundance of both in HEK Cav- and HEK Cav1 (the mean ± SE, n ≥ 15). (**I**) Single Qdots were further classified according to its MSD shape as being free: Brownian movement (linear plot); confined: region restricted movement (decreasing slope plot); or stationary: motionless (diffusion coefficient bellow 0.001 $\mu m^2/s$). Histogram displaying the diffusion coefficient of the different types of single Qdots. Closed bars, HEK Cav-; open bars, HEK Cav1. Data are the mean ± SE (n ≥ 9). *$p < 0.05$, **$p < 0.01$ vs HEK Cav- (Student's t-test).

Cav- and HEK Cav1, respectively, n = 10) was similar, the half-life recovery increased in HEK Cav1 (21.77 ± 1.49 s and 28.87 ± 2.49 s for HEK Cav- and HEK Cav1, respectively, $p < 0.05$, n = 10) (Fig. 3F), where a lower motion of Kv1.3 in the presence of caveolin was observed. This could be explained not only by the major recruitment of the channels in rigid structures, such as caveolae, but also by an increase of liquid-ordered domains structuring the membrane. Therefore, the Kv1.3 membrane dynamics were also analyzed by single particle tracking (SPT) using Qdots (Fig. 3H,I). Single Kv1.3 molecules at the cell surface were tagged with Qdots and monitored by total internal reflection fluorescence (TIRF) imaging with a temporal resolution of 0.5 s (Supplementary video 1). Qdots were classified according to their behavior in single or multiple units (Fig. 3H and Supplementary video 1). The last were defined when more than one Qdot motion agroupated for more than 10 s, thereby suggesting aggregated channels. While the abundance of multiple Qdots increased in HEK Cav1 cells (11.15 ± 2.24% vs 23.84 ± 3.26%, $p < 0.05$), single Qdots decreased (88.85 ± 2.24% vs 76.15 ± 3.26%, $p < 0.01$), which suggests an aggregated distribution of Kv1.3 channels in the presence of Cav1. Moreover, the trajectories of single Qdots were analyzed by plotting the mean square displacement (MSD) against time[27]. Three types of motion were observed: (i) simple Brownian diffusion (free), (ii) confined diffusion (confined) and (iii) stationary diffusion (stationary) (Fig. 3I). In this context, the diffusion coefficient of free diffusing channels in the presence of Cav1 decreased (0.016 ± 0.002 vs 0.010 ± 0.001 $\mu m^2/s$ for HEK Cav- and HEK Cav1, respectively, $p < 0.05$). Thus, SPT results suggested both the aggregation of Kv1.3 and reduced channel mobility in the presence of caveolin.

Glucose transporter type 4 (Glut4) and insulin receptor (IR) are recruited in caveolae[28], and Cav1 participates in Glut4 and IR stability. Thus, Cav1 depletion reduces Glut4 and IR protein abundance by their faster degradation[29]. Therefore, we next studied whether Cav1 association altered Kv1.3 stability. Time-course experiments performed in Cav- and Cav1 HEK cells demonstrated that, similar to Glut4 and IR, Kv1.3 persisted for a longer period of time in the presence of Cav1 (Fig. 4A,B). In this context, Cav1 can also affect channel activity[1]. Therefore, for these experiments, Cav1 was reintroduced into HEK Cav- cells, and Kv1.3 electrophysiological properties were analyzed. While the threshold of activation was similar, the presence of Cav1 increased the Kv1.3 current density (Fig. 4C,D). Slow C-type inactivation is a characteristic of Kv1.3. It involves conformational changes of the channel that result in closure of the external mouth of the pore with probable cooperativity between subunits[30]. In this context, the Cav1 interaction enhanced the C-type inactivation of Kv1.3. Thus, the current at the end of a 5 s pulse (+60 mV) was lower in the presence than in the absence of Cav1 (Fig. 5E,F).

Caveolin 1 interacts with Kv1.3 via a CBD signature located at the N-terminal of the channel. The N-terminus of Kv1 channels contains important signatures involving tetramerization and regulatory subunit association. Although caveolin uses a CSD to interact with substrates via a CBD[7,8,31], this model has been compromised by structural and bioinformatic analysis[32]. In this context, the N-terminus of Kv1.1-Kv1.5 contains putative CBDs lying next to the first transmembrane segment (amino acids 166 to 174 in rKv1.3), right after the T1 tetramerization domain and the Kvβ subunit association signature (Supplementary Fig. S2G). This CBD is represented by a ΦxxxxΦxxΦ consensus sequence, where Φ is a hydrophobic residue. Our data indicates that Kv1.3 and Kv1.5 localized significantly in rafts but only Kv1.3 directly interacted with Cav1. Therefore, we next focused on whether the Kv1.3 CBD molecular determinant was involved in Cav1 interaction. To do so, we performed coimmunoprecipitation assays with different Kv1.3 mutants and Kv1.3-Kv1.5 chimerical channels (Figs 5 and 6). While Kv1.3ΔCt (no C-terminal domain) coimmunoprecipitated with Cav1, Kv1.3ΔNt (no N-terminal domain) did not. In this scenario, to rule out the effect of an altered tetramer formation on traffic and subcellular localization of the Kv1.3 truncated channels, we analyzed Kv1.3/Kv1.5 chimeras that preserved the full integrity of the channel. Chimeras, containing the Kv1.3 N-terminal domain coimmunoprecipitated with Cav1 to a greater extent than did chimers that contained the N-terminus of Kv1.5 (Fig. 5B,C). To further understand this specific Kv1.3 signature, the putative CBD of Kv1.3 and Kv1.5 was mutated. Thus, aromatic amino acids were substituted by alanine or glycine-generating CBD mutants (Kv1.3: ^{166}FQRQVWLLF^{174} to ^{166}AQRQVGLLA^{174}; Kv1.5: ^{232}FQRQVWLIF^{240} to ^{232}AQRQVGLIA^{240}). While the Kv1.3 mutant (Kv1.3CBD) showed a reduced lipid raft partitioning with no Cav1 dependency (Fig. 6A), the Kv1.5CBD showed no lipid raft targeting alterations (Fig. 6B). Moreover, the Kv1.3CBD did not coimmunoprecipitate with Cav1, thereby highlighting the importance of the CBD integrity for Kv1.3 interactions with Cav1 (Fig. 6C).

We found no interactions between Kv1.5 and Cav1; however, by converting the putative Kv1.5 CBD to that of Kv1.3 (Kv1.5I239L), we observed a positive Cav1 coimmunoprecipitation Fig. S4). Furthermore, evidence suggests that Kv1.5 could interact indirectly with caveolins by the formation of supramolecular complexes, including SAP97[33,34]. SAP97 (synapse-associated protein 97) is a member of the membrane associated guanylate kinase (MAGUK) family that also includes PSD95 (postsynaptic density protein 95). Therefore, we expressed Kv1.5 in the presence and absence of PSD95 in HEK Cav1 cells. In this scenario, Kv1.5 coimmunoprecipitated with Cav1, only in the presence of PSD95 (Fig. S4). Therefore, our data suggest that, while the full integrity of the Kv1.3 CBD is sufficient to interact with Cav1, Kv1.5 requires ancillary proteins.

Discussion

Evidence demonstrates that Kv1.3 targets specific membrane localizations[35,36], and location displacements entail pathological consequences[37]. Our study highlights the main mechanism of Kv1.3 channel membrane surface partitioning. We report here that, among Kv1 (*Shaker*) channels, only Kv1.3 and Kv1.5 targeted significantly to lipid rafts. However, while the Kv1.5 floatability was independent of the caveolin expression, Kv1.3 lipid raft targeting increased in a caveolin dose-dependent manner. This is of physiological relevance because this was confirmed in BMDM from Cav1$^{-/-}$ mice. Furthermore, caveolin-channel colocalization was higher with Kv1.3 than with Kv1.5. Finally, we have clearly identified a CBD that is located at the N-terminal domain of Kv1.3 and is the responsible element for Cav1 interaction and lipid raft localization of the channel. Because lipid raft targeting has been proposed as a mechanism for ion channel regulation[1,38], our results contribute to this expanding field.

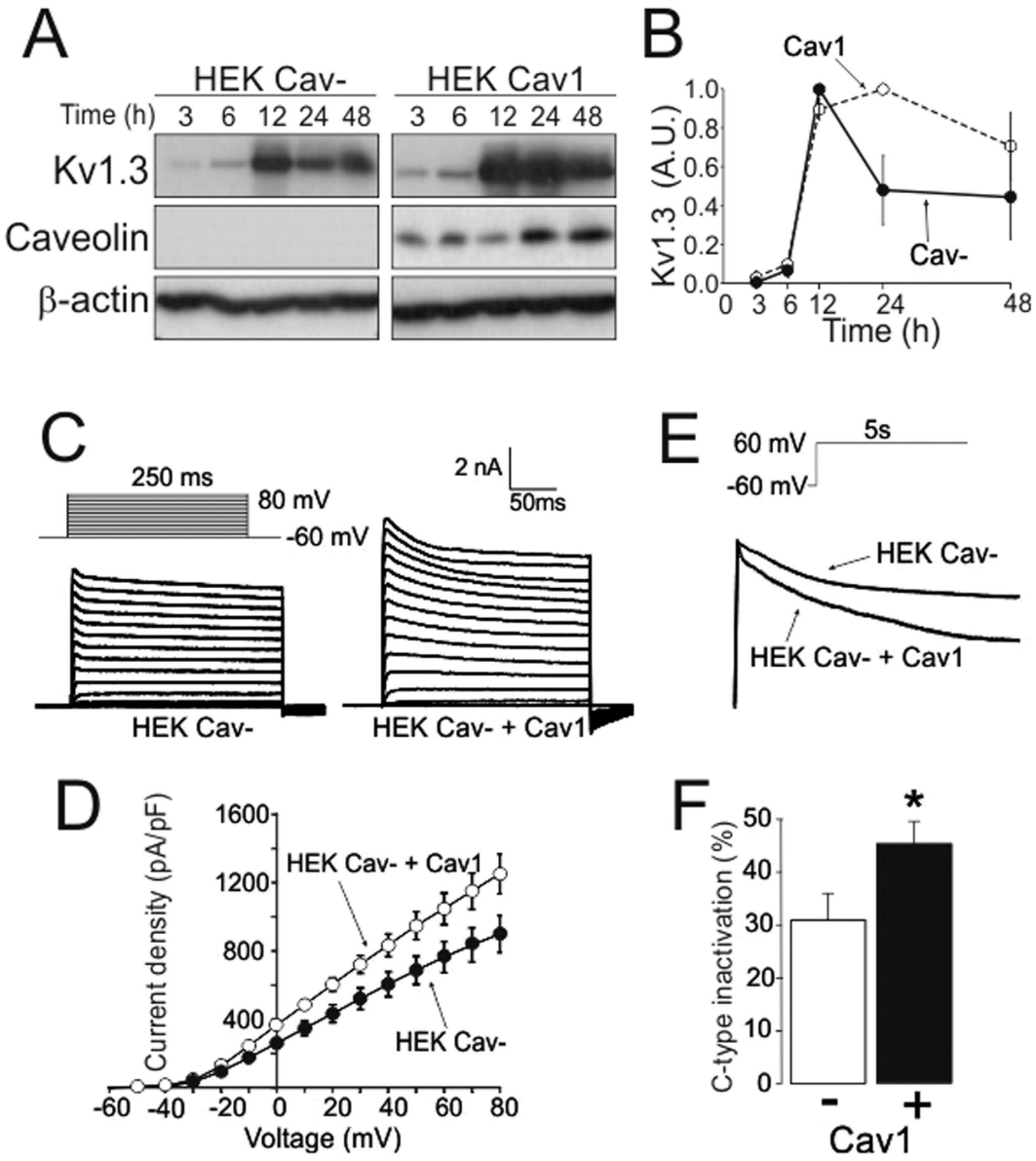

Figure 4. Caveolin 1 stabilized Kv1.3 and modulated current density and inactivation. HEK Cav- and HEK Cav1 cells were transfected with Kv1.3YFP. (**A**) Time course experiments were performed extracting proteins 3, 6, 12, 24 and 48 hours after transfection. (**A**) Representative experiment is shown. Total protein extracts were separated by SDS-PAGE and immunoblotted using Kv1.3, β-actin, and caveolin antibodies. Kv1.3 expression values were corrected by β-actin levels and normalized with the maximum. (●) HEK Cav- cells; (○) HEK Cav1 cells. (**B**) Results are the mean ± SE of two independent experiments. (**C–E**) HEK Cav- cells were transfected with Kv1.3 in the absence (HEK Cav-) or the presence (HEK Cav- +Cav1) of Cav1. (**C**) K^+ currents were elicited by 250 ms voltage sweeps ranging from −60 mV to +80 mV in 10 mV increments. (**D**) Current density-voltage relationship of HEK Cav- (○) and HEK Cav- +Cav1 (●) cells (mean ± SE of n = 15 and 9 cells, respectively). (**E,F**) C-type inactivation. (**E**) Representative traces evoked by a 5 s pulse from −60 mV to +60 mV. (**F**) Remaining current density (%) at the end of the pulse shown as the mean ± SE of six cells both in the HEK Cav- (Cav1 -) and the HEK Cav- +Cav1 (Cav1+). *$p < 0.05$ vs Cav- (Student's t-test).

Kv1.3 redistribution within the plasma membrane is critical for lymphocyte physiology[37]. Upon activation, T cells spatially reorganize membrane proteins to form the immunological synapse (IS), where lipid rafts accumulate and recruit TCR (T-cell receptor), CD3 (cluster of differentiation protein 3) and Kv1.3[10]. Caveolin is crucial for the IS reorganization of CD8 T cells[39]. Thus, the Kv1.3-caveolin interaction described here could participate in the recruitment of Kv1.3 into the IS orchestrated by caveolin. Moreover, the cell membrane composition and lipid raft integrity regulates Kv1.3 activity[40]. This is in agreement with the functional consequences that are observed when Kv1.3 rearranges into the IS[41]. Similarly, Kv1.3 activity was altered in the presence of caveolin. Caveolins also regulate the activity of other channels such as Nav1.5[42]. In addition, caveolin 3 also coimmunoprecipitates with cardiac Kv11.1[43,44]; however, a direct interaction between caveolins and channels is not a unique

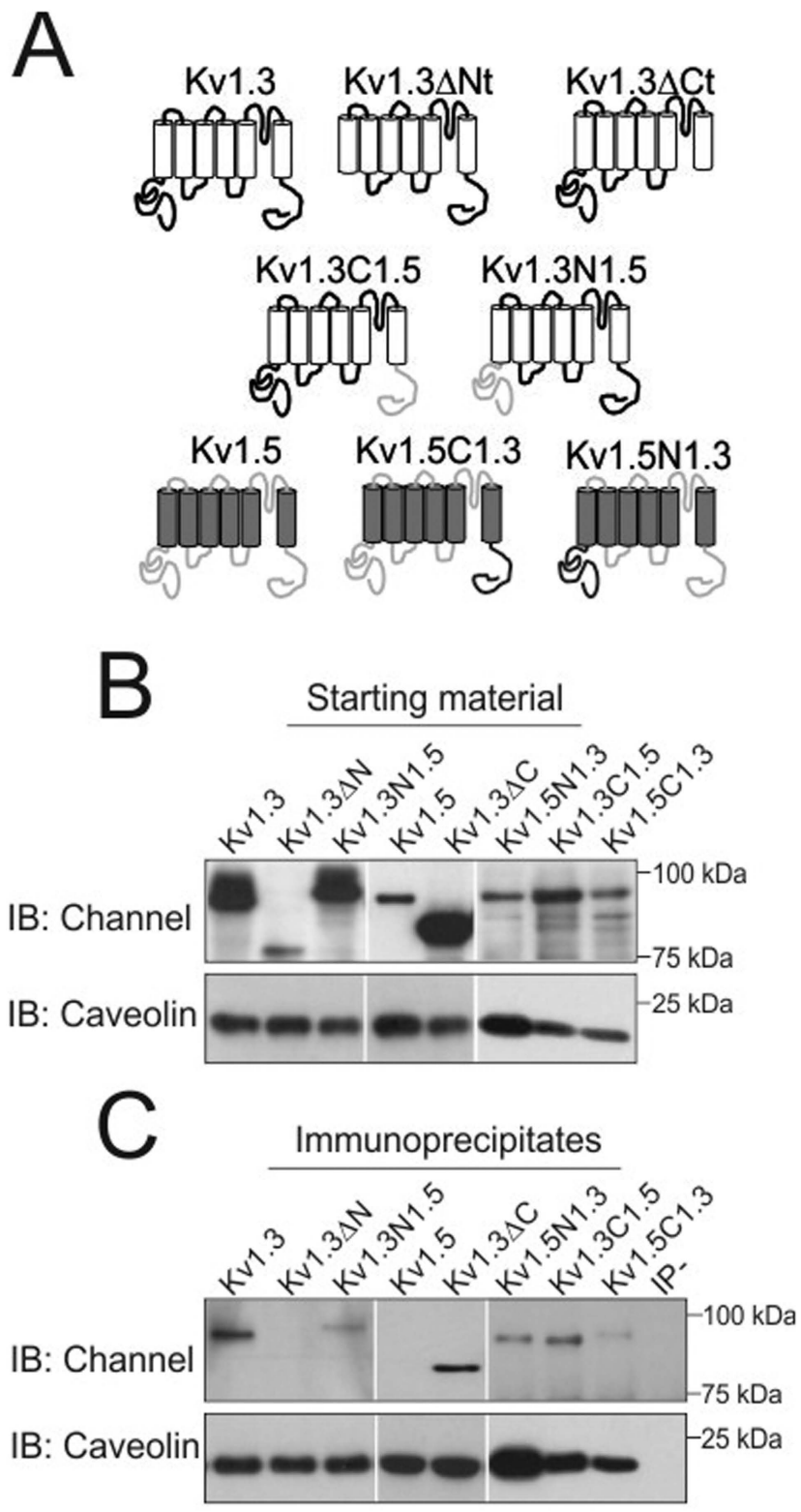

Figure 5. The N-terminal domain of Kv1.3 is essential for the interaction with caveolin. Total protein lysates of HEK Cav1 obtained 24 h after transfection with the indicated channel (tagged with YFP) were subjected to immunoprecipitation against caveolin. Samples separated by SDS-PAGE and immunoblotted (IB) against GFP (channel) and caveolin antibodies. (**A**) Schematic diagrams of Kv1.3 truncated channels and Kv1.3-Kv1.5 chimeras. Kv1.3 domains: white barrels and black lines. Kv1.5 domains: gray barrels and gray lines. (**B**) Starting materials. Top panel, filters were immunobloted against GFP (Channels). Bottom panel, filters were probed against Cav to demonstrate Cav immunoprecipitation. (**C**) Immunoprecipitates. No immunoprecipitation was observed in the absence of antibody (IP-).

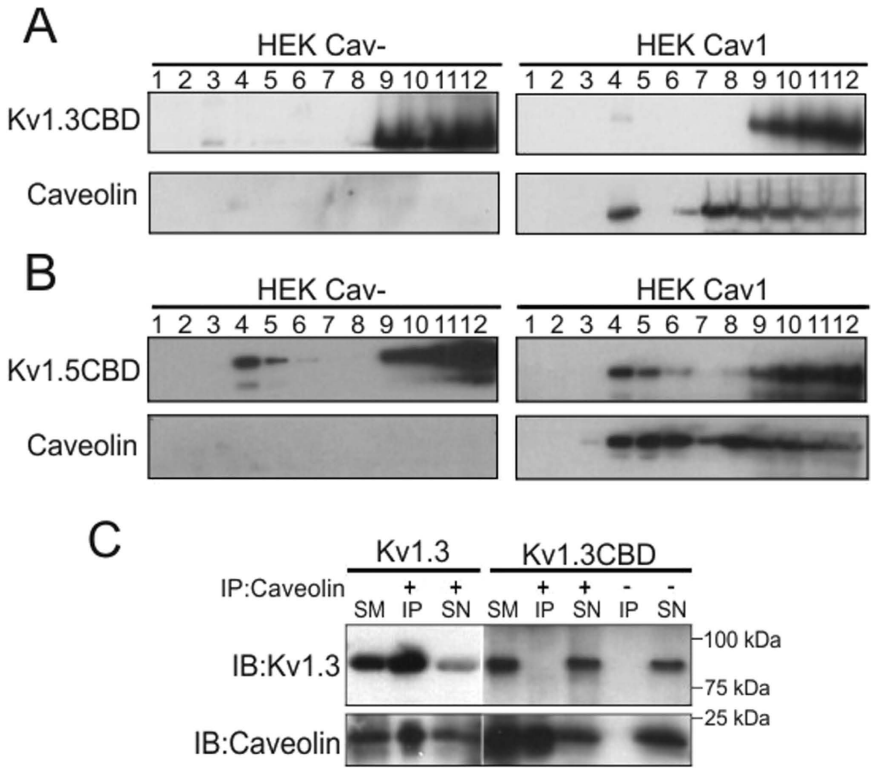

Figure 6. The CBD at the N-terminal domain is responsible for the targeting of Kv1.3 to lipid raft and the association with Cav1. Lipid raft isolations performed in HEK Cav- (left panels) and HEK Cav1 (right panels) cells expressing Kv1.3CBD (**A**) and the Kv1.5CBD (**B**) mutant channels ([166]FQRQVWLLF[174] to AQRQVGLLA and [232]FQRQVWLIF[240] to AQRQVGLIA, respectively). (**C**) HEK Cav1 expressing either Kv1.3 or Kv1.3CBD were subjected to immunoprecipitation against caveolin (IP: Cav1) in the presence (+) and the absence (−) of caveolin antibody. Samples were separated by SDS-PAGE and immunoblotted against Kv1.3 (IB:Kv1.3) and caveolin (IB:Caveolin) SM: starting material; SN: Supernatant; IP: immunoprecipitate.

way to target channels to raft domains. In this context, our Kv1.5 data are in the same line of evidence as that described for Kv1.4. The location of Kv1.4 in caveolar domains is uncertain, but the presence of PSD95 increases the targeting to rafts microdomains[45]. Conversely, the raft localization of Kv2.1 and Kv4.2 seems independent of the presence of auxiliary scaffolding proteins such as caveolins or PDZ-containing proteins[45,46]. In this scenario, much work must be conducted to decipher different partnership associations, thereby conforming specific cell channelosomes, which allows for the spatial localization of channels and the regulation of physiological response.

Evidence suggests an increasing number of ion channels, mostly cardiac, are in caveolar rafts[47]. However, our results confirmed, for the first time, that Kv1.3 lipid raft targeting occurs via a direct interaction wherein caveolin recruits the channel inside caveolae structures, thereby restricting the channel's lateral diffusion. The molecular determinant of Kv1.3 that is involved in such interaction is a CBD located at the N-terminus of the channel in close proximity to the T1 tetramerization domain and the Kvβ subunit interaction signature. Although other Kv1 members share similar motifs, none displayed a caveolin-dependent behavior or major lipid raft targeting. Our results demonstrated that few, single point differences within the CBD signature and/or impaired CBD accessibility due to bulky intracellular domains, may impair this interaction. Interestingly, coimmunoprecipitation studies using chimeric Kv1.3/Kv1.5 and Kv1.5(I239L) mutant channels suggest both. Thus, while Kv1.5Nt, containing a CBD motif, was not enough for Kv1.5-Cav1 coimmunoprecipitation, a fairly positive association was observed when the bulky C-terminal of Kv1.5 was substituted by the C-terminal of Kv1.3. In addition, the introduction of a di-leucine motif, within the CBD of Kv1.5 (Kv1.5 I239L), triggered Cav1 co-immunoprecipitation. Our data suggest that the balance of other interacting motifs within Kv1.5 could mask the CBD accessibility and/or effectiveness[18], which is similar to what has been previously reported for other forward trafficking signals, such as VxxSL or YMVIEE[48,49]. In this sense, Kv1.2, containing the same CBD of Kv1.3, lacks strong trafficking signals and exhibits endoplasmic reticulum retention[50]. Unlike Kv1.3 that colocalizes with caveolin in Golgi[17], Kv1.2 and caveolin do not share intracellular compartments what would impair the association. However, Kv1.2 cell surface is promoted by PSD95 and Kvβ subunits[51]. It is tempting to speculate that different structural tertiary configurations of bulky domains could condition protein–protein interactions with MAGUK proteins, such as PSD95 or SAP97, that interact differently with Kv channels[37–39]. This could be explained by supramolecular complexes formed by Kv1.5, Cav1 and SAP97, which further supports our Kv1.5 and PSD95 data in HEK Cav1 cells[33,52,53].

In summary, our results help to elucidate the mechanisms that target Kv1 channels to specific surface microdomains that participate in fine-tuning the cellular responses. Unlike other neuronal Kv1 channels, Kv1.3 interacts with caveolin through a CBD placed at the N-terminal domain of the channel adjacent to the first transmembrane

segment and in close proximity to the T1 domain and the Kvβ binding site. This association targets Kv1.3 to caveolar structures that regulate both the channel membrane dynamics and activity.

Methods

Expression plasmids and site-directed mutagenesis. Rat Kv1.3 in pRcCMV was provided by T.C. Holmes (University of California, Irvine, CA). Rat Kv1.1 and Kv1.4 in pGEM7 and human Kv1.5 in pBK constructs were subcloned into pEYFP-C1 and pECerulean-C1 (Clontech). Kv1.3/Kv1.5 chimeras were generated in the pEYFP-rKv1.3 and pEYFP-hKv1.5 channels by inserting BglII and EcoRI sites in the N- and C-terminal domains of the channels. Mutants were generated using the QuikChange site-directed mutagenesis kits (Stratagene). LoopBAD (BAD, Biotin acceptor domain) sequence was inserted in the first extracellular loop of pEYFP-Kv1.3 within a preexisting NruI site for rKv1.3LoopBAD construct. *E. coli* Biotin ligase containing construct pBtac_BirA was used as previously described[54]. Rat Cav 1 into pECerulean-C1 was provided from J.R. Martens (University of Florida Medical School). Cav1 was inserted into pcDNA3 by digestion of Cav-pECerulean (HindIII-BamHI). Constructs were verified by sequencing.

Cell culture, transient transfections and raft isolation. HEK 293 cells were grown in DMEM containing 10% FBS and 100 U/ml penicillin/streptomycin (Gibco). Transient transfection was performed using MetafecteneTM Pro (Biontex) at nearly 80% confluence. Murine bone marrow derived macrophages were isolated, as previously described[55]. All of the experiments and surgical protocols were performed in accordance with the guidelines approved by the ethical committee of the Universitat de Barcelona following the European Community Council Directive 86/609 EEC.

Low density, Triton-insoluble complexes were isolated, as previously described[18,20]. Cells were homogenized in 1 ml of 1% Triton X-100, and sucrose was added to a final concentration of 40%. A 5–30% linear sucrose gradient was layered on top and further centrifuged (39,000 rpm) for 20–22 h at 4 °C in a Beckman SW41 rotor. Gradient fractions (1 ml) were collected from the top and analyzed by Western blot.

Protein extraction, co-immunoprecipitation and western blot analysis. Cells, washed in cold PBS, were lysed on ice with NHG solution (1% Triton X-100, 10% glycerol, 50 mmol/L HEPES pH 7.2, 150 mmol/L NaCl) supplemented with 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin and 1 mM of phenylmethylsulfonyl fluoride to inhibit proteases. Homogenates were centrifuged at 16,000 × g for 15 min, and the protein content was measured using the Bio-Rad Protein Assay.

For immunoprecipitation, samples were precleared with 30 μl of protein A-Sepharose beads for 2 h at 4 °C with gentle mixing as part of the co-immunoprecipitation procedures. The beads were then removed by centrifugation at 1,000 × g for 30 s at 4 °C. Samples were incubated overnight with the anti-caveolin antibody (4 ng/μg protein) at 4 °C with gentle agitation. Thirty microliters of protein A-Sepharose were added to each sample and incubated for 4 h at 4 °C. The beads were removed by centrifugation at 1,000 × g for 30 s at 4 °C, washed four times in NHG, and resuspended in 100 μl of Laemmli SDS buffer.

Protein samples (50 μg), raft fractions (50 μl) and immunoprecipitates were boiled in Laemmli SDS loading buffer and separated by 10% SDS-PAGE. Next, samples were transferred to PVDF membranes (Immobilon-P, Millipore) and blocked with 5% dry milk-supplemented 0.05% Tween 20 PBS. The filters were then immunoblotted with specific antibodies: anti-GFP (1/1,000, Roche), anti-caveolin (1/2,500, BD Biosciences), anti-Kv1.3 (1/500, Neuromab), anti-clathrin (1/1,000, BD Biosciences), anti-flotillin (1/1,000, BD Biosciences). Finally, filters were washed with 0.05% Tween 20 PBS and incubated with horseradish peroxidase conjugated secondary antibodies (BioRad).

Immunocytochemistry, plasma membrane lawns (PML) and transmission electron microscopy. HEK cells seeded on poly-D-lysine-treated coverslips were used 24 h after transfection (Metafectene Pro). Cells were washed in PBS (phosphate-buffered saline without K^+) and fixed with 4% paraformaldehyde for 10 min at room temperature (RT). To detect Cav 1, cells were permeabilized using 0.1% Triton X-100 for 10 min. After a 60 min in blocking solution (10% goat serum (Gibco), 5% non-fat dry milk, PBS), cells were treated with rabbit anti-caveolin (1/100, BD Biosciences) antibody in 10% goat serum, 0.05% Triton X-100 and again incubated for 1 h. After 3 washes, preparations were incubated for 45 min with Alexa-Fluor-555 conjugated antibody (1:500; Molecular Probes), washed and mounted in Mowiol (Calbiochem). All procedures were performed at RT.

PML preparations were obtained via osmotic shock with minor modifications[29]. Briefly, cells were cooled on ice for 5 min and washed twice in PBS. Next, cells were incubated for 5 min in 1/3 KHMgE (70 mM KCl, 30 mM HEPES, 5 mM MgCl2, 3 mM EGTA, pH 7.5) and gently washed with non-diluted KHMgE to induce the hypotonic shock. Busted cells were removed from the coverslip by pipetting up and down. After two washes with KHMgE buffer only membrane sheets remained attached. PML were fixed with fresh 4% paraformaldehyde for 10 min at room temperature and mounted in Mowiol mounting media.

For transmission electron microscopy, PML were treated as performed for immunocytochemistry, but visualized with different secondary antibodies. Kv1.3 was recognized by a mouse anti-Kv1.3 antibody (1/20, Neuromab). Goat anti-mouse and anti-rabbit secondary antibodies, conjugated to 10 nm and 15 nm gold particles, recognized Kv1.3 and Cav1, respectively. Briefly, processed samples were further fixed with 2.5% glutaraldehyde in PBS for 30 min at RT. Next, samples were subjected to freeze-drying, washed and cryoprotected with 10% methanol. Samples were then cryofixed using slam-freezing (BAF-060, Bal-Tec) for 90 min at −90 °C and 10–7 mbar pressure. Replicas were obtained by rotationally (136 rpm) evaporating 1 nm platinum through electron cannon (at an angle of 23°). This was reinforced by evaporating 10 nm carbon (at an angle of 75°). Replicas were separated from the sample using 30% fluorhydric acid. Finally, samples were washed and mounted over Formvar coated grilles.

Föster resonance energy transfer (FRET). FRET was performed in the acceptor photobleaching configuration. Samples were imaged with a Leica SP2 confocal microscope. Images were acquired before and after YFP bleach using 63 × oil immersion objective at zoom 4. Excitation was via the 458 and 514 nm lines of the Ar laser, and 473–495 and 535–583 bandpass emission filters were used. FRET efficiency (FRETeff) was calculated using the equation:

$$[(F_D\text{after} - F_D\text{before})/F_D\text{before}] \times 100$$

where, $F_D$after: donor fluorescence (Cerulean) after and $F_D$before before acceptor (YFP) bleach. Analysis was performed using ImageJ.

Fluorescence recovery after photobleaching (FRAP) and Single particle tracking (SPT). Experiments were performed as previously described[46,54,56]. For FRAP experiments, an Olympus FV1000 microscope was used. Briefly, YFP was bleached during 250 ms with a 515 nm line Ar laser at 30% and was fluorescence monitored before and after bleach with a PLAPO 60x NA 1:1,40 oil objective at zoom 4 acquiring every 1.108 s. Acquisition was performed with the 515 nm Ar laser line at 1% and a 525–560 nm bandpass emission filter.

SPT analysis was performed as previously described[46]. Briefly, cells co-expressing Kv1.3LoopBAD and BirA for 24 h were incubated for 5 min at RT with 0.1 nM Streptavidin Qdots655 (Invitrogen, Oregon) in 1% BSA HIS (146 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2 \times 2H_2O$, 0.6 mM $MgSO_4 \times 7H_2O$, 0.15 mM $NaH_2PO_4 \times 2H_2O$, 0.1 mM ascorbic acid, 8 mM Glucose, 20 mM HEPES, pH 7.4) and washed five times with HIS at RT. Cells were imaged in the following hour at 37 °C in a 5% CO_2 atmosphere. Imaging was performed with Nikon Eclipse Ti PerfectFocus equipped TIRF (Total internal reflection fluorescence) microscope with a 100xPlanApoTIRF, 1.49 NA, oil objective. YFP was excited with 488 nm line Ar laser at 2% and Qdots with 561 nm laser at 20%. Emission was collected through a Sutter Lambda 10–3 filter wheel and recorded with an Andor iXon EMCCD DUD897 camera. For TIRF acquisition, the incident angle was 63.3°. Imaging acquisition was approximately 10 MHz. Videos were processed using Volocity (PerkinElmer Software). SPT was performed manually. The tracks were then analyzed using Sigmaplot to obtain mean square displacement (MSD) and the Diffusion Coefficient.

Electrophysiology. Patch–clamp whole-cell configuration experiments were performed, as performed in[49]. To evoke voltage-gated currents, cells were stimulated with 250 ms square pulses ranging from −60 to +80 mV in 10 mV steps. C-type inactivation was studied by applying a long pulse of 5 s at +60 mV. The peak amplitude (pA) was normalized using the capacitance values (pF). Data analysis was performed using FitMaster (HEKA) and Sigma Plot 10.0 software (Systat Software). All recordings were performed at RT.

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Acknowledgements

Supported by the Ministerio de Economia y Competitividad (MINECO, Spain) grants (BFU2014-54928-R and CSD2008-00005). MPV and JC hold fellowships from the MINECO and the Fundación Tatiana Pérez de Guzmán el Bueno, respectively. NC and RMM were supported by the Juan de la Cierva program (MINECO). Authors thank Dr. C. López-Iglesias (CCiTUB, Universitat de Barcelona) for her help in electronic microscopy, Dr. C. Dart (University of Liverpool) for the kind gift of the HEK Cav1 cell line and Dr. A. Pol (Universitat de Barcelona) for the Cav1$^{-/-}$ mice. The English editorial assistance of the American Journal Experts is also acknowledged.

Author Contributions

M.P.V., J.C., R.M.M., M.C. and N.C. performed the experiments. M.P.V., M.C., M.M.T. and A.F. designed the experiments. A.F. directed the study. All authors participated in writing the manuscript.

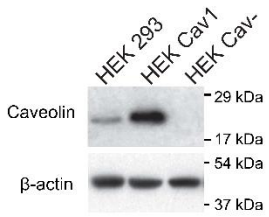
Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

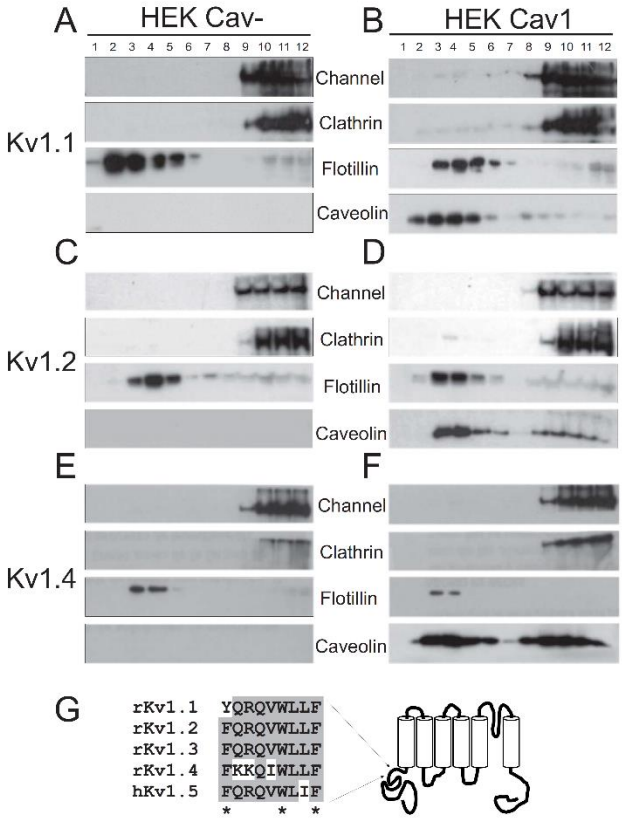
Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Pérez-Verdaguer, M. *et al.* Caveolin interaction governs Kv1.3 lipid raft targeting. *Sci. Rep.* **6**, 22453; doi: 10.1038/srep22453 (2016).

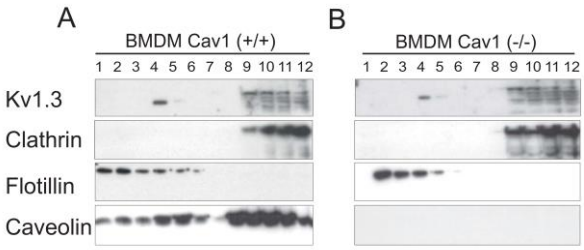
SUPPLEMENTARY MATERIALS



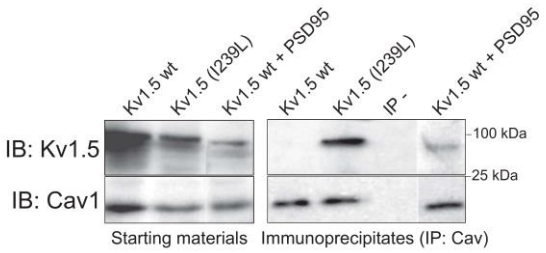
Supplementary Figure 1. Caveolin expression in different HEK 293 cell lines. Representative western blot of total protein lysates from HEK 293 cells (regular HEK cell line), HEK Cav1 (stably overexpressing caveolin 1) and HEK Cav- (lentiviral ablation of Cav1) and. β-actin was used as a loading control.



Supplementary Figure 2. Lipid raft distribution of Kv1 channels. rKv1.1, rKv1.2 and rKv1.4 YFP-tagged channels were transfected in HEK Cav- (left panels) and HEK Cav1 (right panels). Lipid rafts were isolated using a sucrose gradient and the channel distribution was analyzed by western blot against GFP. (A, B) Kv1.1; (C, D) Kv1.2; (E, F) Kv1.4. While caveolin was used as a raft marker, clathrin indicated non-raft fractions. Flotillin highlighted raft fractions, despite the absence of caveolin in HEK Cav- cells. (G) Putative CBD sequence in the N-terminal domain of Kv1.1-Kv1.5 channels. Identities appear highlighted in gray. * denotes hydrophobic residues of the putative ΦXXXXΦXXΦ motif.



Supplementary Figure 3. Lipid raft distribution of Kv1.3 in macrophages. (A-B) Murine bone marrow derived macrophages (BMDM) were isolated from wild-type C57BL/6 (BMDM Cav1+/+) and Cav null (BMDM Cav1-/-) mice. Total lysates from BMDM were subjected to lipid raft isolation. Note that while flotillin was situated in low-buoyant density fractions in both cases, Cav1 was only expressed in wild type animals.



Supplementary Figure 4. The Kv1.5 (I239L) mutant and the presence of PSD95 trigger the co-immunoprecipitation of Kv1.5 and caveolin. Total protein lysates of HEK Cav1 were obtained 24 h after transfection with Kv1.5 wt, in the presence or the absence of PSD95, and Kv1.5(I239L). See Supplementary Fig. S2G for details. Samples were immunoprecipitated against caveolin (IP: Cav) and immunoblotted against Kv1.5 (IB:Kv1.5) and caveolin (IB: Cav1) antibodies. Left panels, Starting materials (SM). Right panels, Immunoprecipitates (IP). IP- negative control in the absence of the anti-Cav antibody.

Supplementary video 1. Representative TIRF video of HEK Cav- cells 24 h after cotransfection with Kv1.3LoopBAD and BirA. Cells were tagged for 5 min with Qdots. Qdots were classified as single or multiple units (see Fig. 4). Several Qdots with independent motions were classified as single, while only a few moved together in small groups and were classified as multiple.

Supplementary video 2. Representative TIRF video of HEK Cav1 cells 24 h after cotransfection with Kv1.3LoopBAD and BirA. Cells were tagged for 5 min with Qdots. Qdots were classified as single or multiple units (see Fig. 4). Several Qdots with independent motions were classified as single, while only a few moved together in small groups and were classified as multiple. White arrows point at some multiple Qdots.

3.1.2 CONTRIBUTION II

Caveolar targeting links Kv1.3 with the insulin-dependent adipocyte physiology

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Report of the PhD student participation in the article:

Caveolar targeting links Kv1.3 with the insulin-dependent adipocyte physiology

Published in *Cellular and Molecular Life Sciences; 2018, November* 75(21):4059-4075.
doi: 10.1007/s00018-018-2851-7
Impact factor (2018): 7.014

Jesusa Capera performed tissue handling and primary cultures. Performed and analysed all the PCR and qPCR experiments in adipocytes, participated in the co-immunoprecipitation and glucose uptake experiments in adipocytes and performed all the experiments with 3T3-L1 cells, except for the transmission electron microscopy.

Prof. Antonio Felipe
Thesis director

Cellular and Molecular Life Sciences (2018) 75:4059–4075
https://doi.org/10.1007/s00018-018-2851-7

ORIGINAL ARTICLE

Caveolar targeting links Kv1.3 with the insulin-dependent adipocyte physiology

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Received: 15 December 2017 / Revised: 14 May 2018 / Accepted: 5 June 2018 / Published online: 11 June 2018

Abstract

The voltage-dependent potassium channel Kv1.3 participates in peripheral insulin sensitivity. Genetic ablation of Kv1.3 triggers resistance to diet-induced weight gain, thereby pointing to this protein as a pharmacological target for obesity and associated type II diabetes. However, this role is under intense debate because Kv1.3 expression in adipose tissue raises controversy. We demonstrated that Kv1.3 is expressed in white adipose tissue from humans and rodents. Moreover, other channels, such as Kv1.1, Kv1.2, Kv1.4 and especially Kv1.5, from the same *Shaker* family are also present. Although elevated insulin levels and adipogenesis remodel the Kv phenotype, which could lead to multiple heteromeric complexes, Kv1.3 markedly participates in the insulin-dependent regulation of glucose uptake in mature adipocytes. Adipocyte differentiation increased the expression of Kv1.3, which is targeted to caveolae by molecular interactions with caveolin 1. Using a caveolin 1-deficient 3T3-L1 adipocyte cell line, we demonstrated that the localization of Kv1.3 in caveolar raft structures is important for proper insulin signaling. Insulin-dependent phosphorylation of the channel occurs at the onset of insulin-mediated signaling. However, when Kv1.3 was spatially outside of these lipid microdomains, impaired phosphorylation was exhibited. Our data shed light on the putative role of Kv1.3 in weight gain and insulin-dependent responses contributing to knowledge about adipocyte physiology.

Keywords Adipose tissue · Potassium channels · Caveolae · Differentiation · Insulin

Introduction

Adipose tissue (AT) is crucial for buffering nutrient availability and demand by storing excess calories and preventing the toxic accumulation of surplus nutrients in non-adipose tissues. Because of the high incidence of obesity in industrialized countries, AT has attracted many investigations. White adipose tissue (WAT), even in a lean person, represents approximately 20% of body weight, and it can increase by more than 50% in morbidly obese patients. The primary function of WAT is to store lipids coming from the diet or synthetized from carbohydrate precursors and to release free fatty acids in response to various neural and hormonal stimuli. Moreover, WAT actively communicates with body organs, secreting lipid and protein factors that mediate local and systemic effects in nutrient intake, metabolism and energy expenditure at numerous levels, immunological responses and vascular physiology. In addition, WAT is regulated by sympathetic innervation and hormones such as insulin, catecholamine, thyroid and steroid hormones [1].

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00018-018-2851-7) contains supplementary material, which is available to authorized users.

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Insulin and catecholamines modulate the membrane potential in adipocytes, eliciting intracellular signaling [2]. Therefore, the role of ion channels in adipocytes is under investigation. Voltage-dependent K^+ (Kv) currents in human and rat WAT present an activation threshold between − 20 and − 30 mV and are responsive to tetraethylammonium (TEA) [3, 4]. WAT and brown adipose tissue (BAT) share a Kv channel repertoire [3–5], with Kv1.3 being a putative candidate [6]. This is further supported by the obesity-resistant phenotype displayed by Kv1.3-null mice and the anti-obesity effect of the Kv1.3 blocker Shk-186 [7, 8]. In this context, Kv1.3 inhibition increases insulin sensitivity via GLUT4 translocation [9, 10]. However, the role of Kv1.3 in peripheral insulin responses has been highly questioned in skeletal muscle and AT [11]. Thus, the role of other Kv isoforms, mainly Kv1.5, in adipocyte glucose signaling is reported [12]. However, a main participation of Kv1.3 in insulin signaling is localized in the olfactory bulb, and strong evidence from BAT studies situates this channel as the most encouraging potential target for diabetes and obesity [13, 14].

Kv1.3 partially localizes in lipid raft microdomains whose lipid environment regulates channel activity [15–17]. Certain ion channels localize into lipid rafts, serving as targets for adjacent signaling molecules [18]. The adipocyte membrane is enriched in omega-shaped lipid raft structures named caveolae [19]. Caveolae, crucial for adipocytes physiology, serve as organizing centers, participating in signaling transduction, mechanosensing and mechanoprotection, lipotoxicity protection, endocytosis and lipid regulation [19–21]. Caveolin (Cav) is the major protein-mediating caveolae biogenesis and structure. Cav forms a family of three integral membrane proteins (Cav 1, Cav 2 and the muscular isoform Cav 3) [21]. Cav has a caveolin scaffolding domain (CSD) that can bind to other proteins via a caveolin-binding domain (CBD) [22]. We have recently identified a CBD at the N-terminal domain of Kv1.3 that is responsible for Cav association and lipid raft targeting of the channel [23]. This interaction is physiologically relevant in lymphocytes because, upon activation, Kv1.3 situates in the immunological synapse (IS), which is also enriched in rafts [24].

Taking all of this into account, we investigated the association of Kv1.3 with Cav 1 in adipocytes and whether this interaction affects localization and insulin signaling of the channel in adipocyte physiology. We found that although Kv1.3 is not the only Kv in WAT, the expression of the channel increases during adipogenesis, recruiting new channels into caveolae. In addition, our results demonstrated that mislocation of Kv1.3 in rafts impaired insulin-dependent phosphorylation of the channel. The role of ion channels in the AT physiology is an open debate and our data clearly situate Kv1.3 as a target in this complex scenario.

Methods

Cell culture, tissue handling and sample preparation

Rat adipocytes were isolated from epididymal WAT extracted immediately after euthanasia with CO_2. Animals were moistened with 70% ethanol before the abdominal cavity was opened and WAT extracted. The tissue was immediately washed in dissociation media (in mM: 120 NaCl, 5 KCl, 1 $CaCl_2$, 5 HEPES, 5 glucose, 3% albumin fraction V, pH 7.4) and digested in collagenase solution (0.5 mg/ml collagenase type II, 0.5 mg/ml DNase type I in dissociation buffer) for 30 min at 37 °C under agitation (60–70 rpm). Next, the suspension was filtered through a 100 µm Millipore filter and collected in a centrifuge tube. Dissociation buffer was added to fill the tube, and the cell suspension was centrifuged (400×*g* for 10 min). The resulting floating fad pat containing mature adipocytes was saved in a new tube. The pellet, containing the stromal vascular fraction (SVF), was subjected to erythrolysis for 5 min at 37 °C in 1 ml erythrolysis buffer (in mM: 155 NH_4Cl, 5.7 K_2HPO_4, 0.1 EDTA). Afterwards, dissociation buffer was added and cells were centrifuged to stop erythrolysis (400×*g* for 5 min). Finally, the pellet was resuspended in adipocyte medium (DMEM supplemented with 10% FBS, 25 mM HEPES, 10,000 U/ml penicillin G and 10 mg/ml streptomycin), and 250,000 cells were plated in 35 mm diameter dishes containing poly-lysinated coverslips. Cells were cultured in adipocyte media supplemented with 500 µIU/ml of insulin. Three drops of the floating fat pad containing mature adipocytes were added. The medium was replaced after 2 days with 10 or 100 µIU/ml insulin supplemented with complete medium, as indicated. Differentiated preadipocytes were used 4–7 days after culture (fresh medium was added every 2 days). All products were from Sigma-Aldrich except FBS, which was purchased from Linus.

Subcutaneous human fat tissue was obtained from normal-weight subjects (BMI < 27 kg/m^2) undergoing abdominal surgery or surgical mammary reduction. Fresh human adipose tissue was immediately processed as above to obtain differentiated adipocytes for immunocytochemistry. Human breast biopsies were obtained and treated as previously described for immunohistochemistry [25]. All human procedures were in accordance with current local guidelines and the Declaration of Helsinki. Experiments and surgical protocols were performed in accordance with the guidelines approved by the ethical committee of the Universitat de Barcelona following the European Community Council Directive 86/609 EEC.

3T3-L1 pre-adipocytes were cultured in DMEM containing 10% NCS at 37 °C in a 7% CO_2 atmosphere. To

induce differentiation, post-confluent 3T3-L1 pre-adipocytes were treated with 0.5 mM IBMX, 10 mg/ml insulin and 1 µM dexamethasone in DMEM containing 10% FBS for 2 days. Then, cells were transferred into DMEM with 10 mg/ml insulin and 10% FBS for 2 additional days. Afterwards, cells were maintained in DMEM containing 10% FBS, and fresh medium was added every other day until day 9, when the cells were fully differentiated into adipocytes.

HEK 293 cells were grown in DMEM containing 10% FBS and 100 U/ml penicillin/streptomycin (Gibco). Jurkat T-lymphocytes were cultured in RPMI containing 10% FBS and supplemented with 100 U/ml penicillin/streptomycin and 2 mM L-glutamine (Gibco).

RNA isolation, RT-PCR analysis and real-time PCR

Total RNA from cells and rat WAT and brain was isolated using a NucleoSpin RNA II (Macherey-Nagel, Germany). RNA was treated with DNase I, and PCR controls were performed in the absence of retrotranscriptase. cDNA synthesis was performed using transcriptor RT (Roche Applied Science) with random hexanucleotide and oligo dT primers, according to the manufacturer's instructions. Once cDNA was synthesized, the conditions were set for further PCR using the Phire Hot Start II DNA polymerase (Finnzymes, Thermo Scientific), following the manufacturer's instructions, for 35 cycles. The final PCR reactions (20 µl) were electrophoresed in a 1% agarose TBE gel (in mM: 40 Tris, 20 acetic acid, 1 EDTA, pH 8.0). Primers, accession numbers, annealing temperatures and the amplicon lengths for every gene are shown in Table 1.

Real-time PCR (RT-PCR) was performed using a 7500 real-time system machine with TaqMan Universal Maxter Mix II, no uracil *N*-glycosylase, according to the manufacturer's instructions (Applied Biosystems). Validated rat TaqMan probes (Applied Biosystems) were used on cDNA retrotranscribed from 0.5 µg RNA, as described above.

Electrophysiology

Ionic currents were recorded using the whole-cell configuration of the patch-clamp technique as described previously [3, 4]. K^+ currents were recorded using an EPC-10 (HEKA), and the appropriate software was used for data recording and analysis. Ionic currents were capacitance and leakage current compensated by a P/4 protocol sampled at 10 kHz (Digidata 1440; Molecular Devices) and filtered at 2.9 kHz. Patch electrodes of 2–4 MΩ were fabricated in a vertical puller (L/M 3P-A, List-Medical) from soda glass (Deltalab). The composition of the external control solution was (in mM): 140 NaCl, 2.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 5 glucose; pH 7.4. When indicated, a high K^+ external solution was used (in mM: 62.7 NaCl, 80 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 5 glucose; pH 7.4). The standard solution used to fill the electrodes contained (in mM): 90 K^+-glutamate, 55 KCl, 2 $MgCl_2$, 10 HEPES and 5 EGTA; pH 7.4. To evoke voltage-gated currents, cells were stimulated with 250 ms square pulses ranging from −60 either to +60 or to +80 mV in 10 mV steps from a holding potential of −80 mV. The peak amplitude (pA) was normalized using the capacitance values (pF). The activation voltage dependence was analyzed using the same protocol in high K^+ external solution and the inward peak data was measured. The activation curve was fitted with the Boltzmann equation:

$$y = 1/\{1 + \exp[-(V - V_{1/2})/k]\},$$

where k represents the slope factor, V is the test potential and $V_{1/2}$ is the potential at which the conductance was half maximal. To analyze the cumulative inactivation, currents were elicited by a train of seven depolarizing voltage steps of 250 ms from −60 to +60 mV once every 400 ms.

Table 1 Gene name, accession number, primer sequence (F: forward; R: reverse), annealing temperature and amplicon length (bp) of the Kv channels analyzed

Gene	Accession number	Sequence	Annealing (°C)	Amplicon (bp)
Kcna1	NM_173095	F: 5′ GTCATGGTCATCCTCATCTCCAT 3′ R: 5′ ACAATGACAGGTACGGGCAGGGCA 3′	60.9	703
Kcna2	NM_012970	F: 5′ TCCCTGGGCACCCACAGGAC 3′ R: 5′ TGCTGGCCTTGCTGAGCGTC 3′	62.5	794
Kcna3	NM_019270	F: 5′ CTCATCTCCATTGTCATCTTCTGA 3′ R: 5′ TTGAATTGGAAACAATCAC 3′	61	718
Kcna4	NM_012971	F: 5′ GACCTGATGCCCAGTGGCTC 3′ R: 5′ TGTGCCCTGAGTTCTCCAGGTG 3′	63	777
Kcna5	NM_012972	F: 5′ GGATCACTCCATCACCAG 3′ R: 5′ GGCTTCCTCCTCTTCCTTG 3′	61	334

Steady-state inactivation was studied by applying a train of 500 ms pulses, ranging from − 120 to + 60 mV in 10 mV steps (prepulse), followed by a 500 ms pulse at + 60 mV in high K^+ external solution. The pulse peak amplitude was collected to obtain the inactivation curve fitted with the Boltzmann equation as follows:

$$(I - \mathrm{I_{max}})/(\mathrm{I_{max}} - I_c) = 1/[1 + \exp(V - V_{1/2})/k],$$

where I_{max} is the peak current measured after the most hyperpolarizing prepulse, and I_c is the smallest non-zero peak current obtained after a depolarizing prepulse.

Data analysis was performed using FitMaster (HEKA) and Sigma Plot 10.0 software (Systat Software). All recordings were performed at room temperature (RT). Toxins, TEA, Bupivacaine and Psora-4, diluted in H_2O or DMSO, respectively, were applied and the inhibition of the peak current at + 60 mV calculated.

Glucose uptake

Adipocytes were prepared from rat epididymal adipose tissue. Tissue was minced and digested with collagenase in Krebs–Ringer–HEPES (KRH) buffer (in mM: 137 NaCl, 4.7 KCl, 1.15 $MgSO_4$, 1.18 KH_2PO_4, 2.5 $CaCl_2$, 20 HEPES, pH 7.4). Digested sample was filtered, and the infranatant was removed, and the floating layer of adipocytes was washed four times with fresh buffer. Adipocytes were incubated in KRH buffer in the presence or absence of 10 µM insulin for 30 min at 37 °C. When indicated, cells were treated with 100 nM Margatoxin 15 min before insulin treatment. Glucose uptake started by adding 5 mM 2-deoxyglucose and 0.1 mCi/ml 2-deoxy[H^3]glucose for 15 min. Next, transport was stopped by adding cold STOP solution containing 50 mM D-glucose in PBS. Cells were lysed for 1 h in 0.1 N NaOH, 0.1% SDS and radioactivity was measured. Uptake is expressed per mg of protein, determined using the Bradford assay.

Immunocytochemistry, plasma membrane lawns (PML) and transmission electron microscopy

Adipocytes and 3T3-L1 cells were seeded on poly-D-lysine-treated coverslips. Cells were washed in phosphate-buffered saline without K^+ (PBS-K^+) and fixed with 4% paraformaldehyde for 10 min. Cells were permeabilized using 0.1% Triton X-100, Gly 20 mM in PBS-K^+ for 10 min at room temperature (RT). After 60 min in blocking solution (1% BSA, 20 mM Gly, 0.05% Triton X-100, PBS-K^+), cells were treated with either mouse anti-Kv1.3 (1/20, Neuromab) or rabbit anti-Kv1.3 (1/20, Alomone), rabbit anti-Kv1.5 (1/20, Alomone), rabbit anti-caveolin and rabbit anti-clathrin heavy chain (1/100, BD Biosciences) antibody in 1% BSA, 20 mM Gly, 0.05% Triton X-100 in PBS-K^+ and again incubated for 90 min. After 3 washes, preparations were incubated for 60 min with Alexa-Fluor-488 or with Cy5 conjugated antibody (1:200; Molecular Probes), washed and mounted in Mowiol (Calbiochem). All procedures were performed at RT.

Plasma membrane lawns preparations from isolated adipocytes and 3T3-L1 adipocytes were obtained via osmotic shock, as previously described, with minor modifications [26]. Briefly, cells were washed twice in PBS-K^+ and cooled on ice for 5 min. Next, cells were incubated for 5 min in 1/3 KHMgE (in mM: 70 KCl, 30 HEPES, 5 $MgCl_2$, 3 EGTA, pH 7.5) and gently washed with non-diluted KHMgE to induce hypotonic shock. Burst cells were removed from the coverslip by pipetting up and down. After two washes with KHMgE buffer, only membrane sheets remained attached. PML were fixed with fresh 4% paraformaldehyde for 10 min at RT and mounted in Mowiol mounting media.

For transmission electron microscopy, PML were treated as performed for immunocytochemistry, but visualized with different secondary antibodies. Kv1.3 and caveolin were recognized by anti-Kv1.3 monoclonal (1/20, Neuromab) and rabbit anti-caveolin (1/100, BD Biosciences) antibodies. Goat anti-mouse and anti-rabbit secondary antibodies, conjugated to 10 nm and 15 nm gold particles, recognized Kv1.3 and Cav1, respectively. Briefly, samples were further fixed with 2.5% glutaraldehyde in PBS for 30 min at RT. Next, samples were subjected to freeze-drying, washed and cryoprotected with 10% methanol. Samples were then cryofixed using slam-freezing (BAF-060, Bal-Tec) for 90 min at − 90 °C and 10–7 mbar pressure. Replicas were obtained by rotationally (136 rpm) evaporating 1 nm platinum through an electron cannon (at an angle of 23°). This was reinforced by evaporating 10 nm carbon (at an angle of 75°). Replicas were separated from the sample using 30% hydrofluoric acid. Finally, samples were washed and mounted over Formvar coated grilles.

Double-labeling indirect immunofluorescence to measure fluorescence resonance energy transfer (FRET)

We performed indirect double-labeling immunofluorescence combined with conventional confocal laser scanning microscopy to measure FRET as previously described [27]. Briefly, adipocytes were immunolabelled as detailed above. Kv1.3 was tagged with the donor fluorophore (A488) and clathrin or caveolin with the acceptor (Cy3 or A546, respectively). Samples were imaged with a Leica SP2 confocal microscope. Images were acquired [donor: Ar laser at 7% and emission filter at (525–560 nm); acceptor: He/Ne laser at 5% and emission filter (640–670 nm)] before and after A488 bleach (3 frames with laser 514 nm at 100%). FRET efficiency was calculated using the equation:

$(FD_{after} - FD_{before})/FD_{before} \times 100$,

where FD_{after} is the donor fluorescence (A488) after and FD_{before} is the donor fluorescence before acceptor (Cy3 or A546) bleach. Analysis was performed using ImageJ.

Oil Red O staining

3T3-L1 cells, washed twice in PBS and fixed in 4% paraformaldehyde at RT for 10 min, were stained with Oil Red O (stock solution: 0.5 g/100 ml dissolved in isopropanol; working solution: 60% Oil Red O stock solution and 40% distilled water) at RT for 1 h. Next, cells were washed with water, and the stained fat droplets in the cells were visualized by light microscopy.

Protein extraction, co-immunoprecipitation and western blot analysis

Cells, washed in cold PBS, were lysed on ice with lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl, pH 7.5) supplemented with 1 µg/ml of aprotinin, 1 µg/ml of leupeptin, 1 µg/ml of pepstatin and 1 mM of phenyl-methylsulfonyluoride to inhibit proteases. Homogenates were centrifuged at 16,000*g* for 15 min at 4 °C, and the protein content was measured using Bradford assay.

For immunoprecipitation, samples were precleared with 50 µl of protein A or G-Sepharose beads for 2 h at 4 °C with gentle mixing as part of the co-immunoprecipitation procedure. The beads were then removed by centrifugation at 1000×*g* for 30 s at 4 °C. Samples were incubated overnight with Sepharose beads previously coated with anti-caveolin (BD transduction), anti-Kv1.3 (Neuromab), anti-Kv1.5 (Alomone) or anti-phosphotyrosine (Sigma) antibody (4 ng/µg protein) at 4 °C with gentle agitation. The beads were removed by centrifugation at 1000×*g* for 30 s at 4 °C, washed four times in wash buffer (0.1% Triton X-100, 10% Glycerol, 150 mM NaCl, 50 mM HEPES, pH 7.4), and resuspended in 100 µl of Laemmli SDS buffer.

Protein samples (50 µg), raft fractions (50 µl) and immunoprecipitates were boiled in Laemmli SDS loading buffer and separated by 10% SDS-PAGE. Next, samples were transferred to PVDF membranes (Immobilon-P, Millipore) and blocked with 5% dry milk supplemented with 0.05% Tween 20 in PBS. The filters were then immunoblotted with specific antibodies: anti-caveolin (1/200, BD Biosciences), anti-Kv1.3 (1/200, Neuromab), anti-Kv1.3 (1/200, Alomone), anti-clathrin (1/1000, BD Biosciences), anti-flotillin (1/1000, BD Biosciences), anti-βactin (1/50.000, Sigma), anti-Glut4 (1/500, OSCRX), anti-Insulin Receptor β (1/500, BD Transduction Laboratories). Finally, filters were washed with 0.05% Tween 20 PBS and incubated with horseradish peroxidase-conjugated secondary antibodies (BioRad).

Raft isolation

Low density, Triton-insoluble complexes were isolated, as previously described [15, 16]. Briefly, cells were homogenized in 1 ml of 1% Triton X-100 MBS (150 mM NaCl, 25 mM 2-morpholinoethanesulfonic acid 1-hydrate (MES), pH 6.5). After three washes in PBS, sucrose in MBS was added to a final concentration of 40%. A 5–30% linear sucrose gradient was layered on top and further centrifuged (39,000 rpm) for 20–22 h at 4 °C in a Beckman SW41 rotor. Gradient fractions (1 ml) were collected from the top and analyzed by Western blot.

Statistics

The results are expressed as the mean ± SE. Student's *t* test, Paired *t* test or one-way ANOVA and Tukey's post hoc test were used for statistical analysis (GraphPad PRISM v5.01). $p < 0.05$ was considered statistically significant.

Results

Adipocytes exhibit multiple voltage-dependent K^+ current phenotypes

Potassium currents in adipocytes have been reported for more than 20 years [4]. However, the identification and role of the molecular candidates is still an open debate. In this context, rat adipocytes were differentiated from SVF and voltage-dependent K^+ currents were elicited (Fig. 1). Following 250 ms pulses from −60 to +60 mV and based on the C-type inactivation of Kv currents, adipocytes exhibited three different phenotypes: (1) currents with almost no inactivation (< 10%), (2) intermediate inactivation (10–30%) and (3) a pronounced (> 30%) inactivation (Fig. 1a–c). All three groups showed similar current densities and activation thresholds were around −40 mV (Fig. 1d). This rather negative threshold could be due to the high external potassium in which these experiments were conducted [28]. Steady-state activation and inactivation parameters were analyzed (Fig. 1d–g). Half-activation voltages steadily shifted to more depolarized potentials concomitantly with minor inactivating phenotypes (Table 2). $V_{0.5}$ was −18.6 ± 2.6, −15.3 ± 1.6 and −11.7 ± 0.8 mV for > 30, 10–30 and < 10%, respectively (Fig. 1d, e). No differences in the k slope were found. In addition, steady-state inactivation showed similar behavior (Fig. 1f, g). Thus, $V_{0.5}$ were −27.4 ± 1.0, −24.9 + 0.5 and −21.2 ± 0.6 for > 30, 10–30 and < 10%, respectively, whereas *k* slopes were again similar (Table 2).

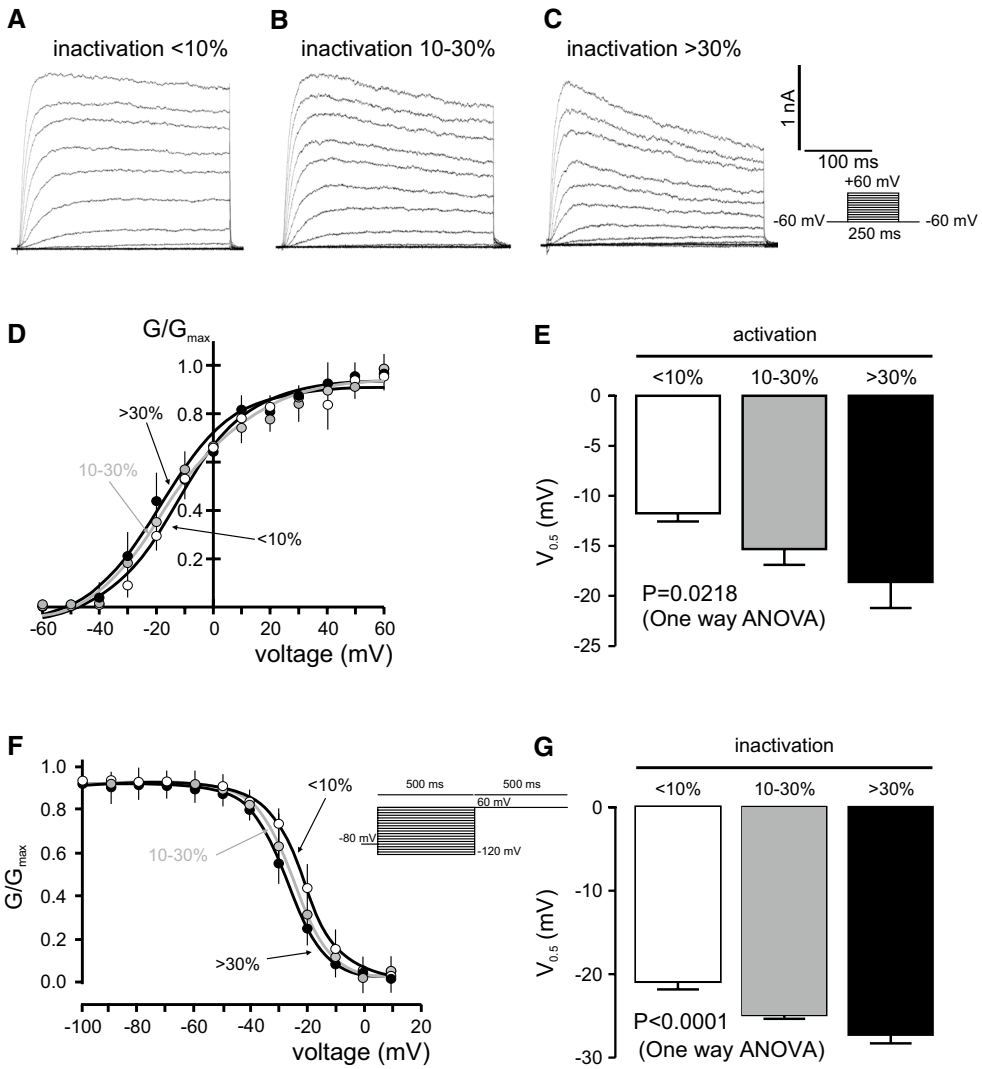


Fig. 1 Multiple voltage-dependent K^+ current phenotypes in adipocytes. Adipocytes were isolated from rat epididymal SVF and Kv currents were elicited by applying 250 ms pulses from −60 to +60 mV in 10 mV steps. **a** Kv currents with poor inactivating characteristics (< 10%). **b** Kv currents with intermediate inactivation (10–30%). **c** Kv currents with pronounced inactivation (> 30%). **d** Steady-state activation, measured in high K^+ external solution, of three phenotypes observed in adipocytes. **e** Half-maximal activation ($V_{0.5}$) of Kv currents. **f** Steady-state inactivation of Kv currents observed in the three groups of adipocytes. **g** Half-maximal inactivation ($V_{0.5}$) of Kv currents. Circles and columns: white, < 10%; gray, 10–30%; black, > 30%. Statistical analysis was performed by one-way ANOVA ($n = 4$–8 independent cells for each group). For detailed post hoc analysis, see Table 2

Adipocytes express different Kv1 channels

Although evidence indicates that not only Kv1.3 but also Kv1.5 could notably contribute to Kv currents in adipocytes, others claim no expression [10–12]. Unlike Kv1.5, Kv1.3 shows pronounced current inactivation and a greater negative half-activation voltage [29]. In addition, during microglia proliferation, macrophage activation and vascular muscle hyperplasia, shifts between Kv1.3 and Kv1.5 are documented [29–31]. However, other isoforms, forming homo- or heteromeric structures with Kv1.3 and Kv1.5, could contribute to the heterogeneous phenotype observed in Fig. 1 [32–34]. In this scenario, we characterized the molecular entities contributing to the Kv currents in adipocytes. A complex repertoire containing Kv1.1–Kv1.5 isoforms was confirmed by an PCR analysis (Fig. 2a). Because adipocytes from different anatomical depots are intrinsically different as a result of genetic or developmental events [35] we also analyzed whether different fat depots shared Kv genetic background. The mRNA expression of members of the Kv1 family, further extended to other channels documented in WAT (http://www.proteinatlas.org/humanproteome) such as Kv2.1, KCa1.1 [36, 37], are shown in Fig. 2b. While denoting

Table 2 Steady-state activation and inactivation of the three differentiated voltage-dependent K^+ current phenotypes

Group	Activation		Inactivation	
	$V_{0.5}$ (mM)	k	$V_{0.5}$ (mM)	k
Poor inactivation < 10%	−11.7 ± 0.8	12.8 ± 0.8	−21.2 ± 0.6	6.8 ± 0.5
Moderate inactivation 10–30%	−15.3 ± 1.6	17.9 ± 1.5	−24.9 ± 0.5	6.5 ± 0.4
Pronounced inactivation > 30%	−18.6 ± 2.6	12.9 ± 2.3	−27.4 ± 1.0	6.8 ± 0.8

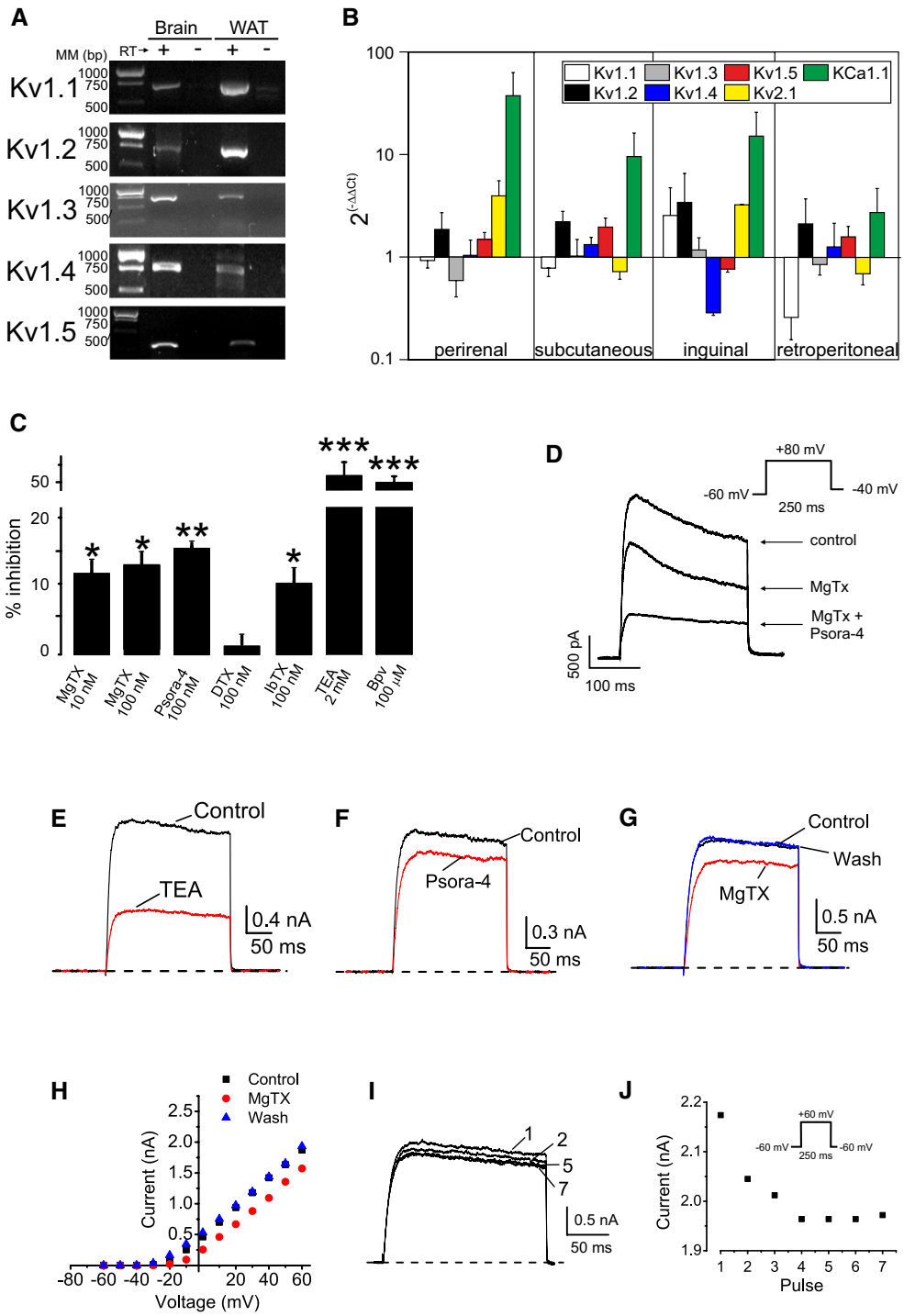
Adipocytes were clearly differentiated in poor, moderate and pronounced for < 10, 10–30 and > 30% of inactivation, respectively. Values are mean ± SE from 4 to 12 different cells. Statistics: $p = 0.0218$ and $p < 0.0001$ for half-activation and half-inactivation voltages, respectively (one way ANOVA). Post-hoc Tuckey test: $p < 0.05$, < 10 vs > 30% (activation); $p < 0.001$, < 10 vs 10–30 and < 10 vs > 30% (inactivation)

some differences, adipocytes from several fat depots shared similar K^+ channel expression profile.

K^+ currents were recorded in the presence of different toxins and chemicals (IC_{50} and K_D shown in Supplementary Table 1). Differentiated adipocytes exhibited Kv currents that were partially sensitive to MgTx (10–100 nM), which blocks Kv1.1, Kv1.2 and Kv1.3 (Fig. 2c). The presence of 100 nM Psora-4, which also blocks Kv1.3, Kv1.2 and Kv1.5, also inhibited Kv currents (Fig. 2c). The pharmacological profile of Kv currents was further extended by additional chemicals and toxins. While 2 mM TEA inhibited the currents by 50%, 100 nM α-DTX did not. IbTx (100 nM) revealed a minimal KCa1.1 contribution in the presence of 5 mM EGTA. Although this profile could suggest a complex Kv scenario in adipocytes, higher TEA concentrations (3–60 mM) inhibited Kv currents from 50 to 80% supporting Kv1.3. MgTx and Psora also suggested that Kv1.3, and possibly Kv1.2, could be involved. The effect of 100 nM Psora-4 would indicate the presence of Kv1.5 and also Kv1.2. TEA and MgTx would also suggest Kv1.1 and Kv1.2, but the limited effect of α-DTX gives to these channels a relative minor role [38, Supplementary Table 1]. While TEA would also support a role for Kv2.1, in this scenario 100 µM Bupivacaine would reinforce the contribution of Kv1.5. Our results could suggest the presence of not only homotetrameric but also multiple heteromeric Kv1 forms with mixed pharmacology, as previously described [29, 34]. To further demonstrate the participation of Kv1.3, we analyzed specific biophysical and pharmacological properties. Kv blockers were applied in cells exhibiting pronounced and poor inactivating Kv currents. Inactivating Kv currents were partially sensitive to 20 nM MgTx and the additional presence of 100 nM Psora-4, which also blocks Kv1.5, further inhibited Kv currents (Fig. 2d). In addition, TEA, Psora-4 and MgTx partially blocked poorly inactivating (< 10%) Kv currents (Fig. 2e–h). Interestingly, the MgTX effect was more notable in cells exhibiting pronounced (> 30%) inactivation. Finally, following a train of depolarizing pulses a cumulative inactivation, a characteristic of Kv1.3, was apparent in cells with poor inactivating phenotype (Fig. 2i, j).

Insulin remodels Kv currents in adipocytes

Kv channels participate in insulin-dependent regulation of body weight. Insulin promotes adipogenesis as well as enhances K^+ currents in adipocytes [39]. In addition, high plasma insulin levels are associated with obesity and type II diabetes and Kv1.3 has been implicated in these processes [8, 9]. Therefore, we further characterized whether insulin remodeled Kv currents in differentiated adipocytes. In this scenario, SVF-derived adipocytes were cultured in physiologically low (10 µIU/ml) and high (100 µIU/ml) insulin media, mimicking plasma concentrations from healthy and obese humans, respectively. As expected, high insulin increased Kv currents (Fig. 3a). At + 60 mV depolarizing pulses, high insulin rose currents by 83% (1.2 ± 0.1 vs 2.2 ± 0.1 nA for 10 and 100 µIU/ml, respectively). Concomitantly, an increase in cell capacitance, most likely due to cell size augmentation by lipid deposition under high insulin treatment, was associated with 100 µIU/ml insulin (Fig. 3b). In this context, Kv current density was still elevated under high insulin, despite the cell growth (34.5 ± 2.1 and 47.9 ± 1.9 pA/pF for 10 and 100 µIU/ml, respectively at + 60 mV) (Fig. 3c). Interestingly, while 100 µIU/ml insulin notably reduced the C-type inactivation of Kv currents by 30% (Fig. 3d) slightly increased the percentage of cells that exhibited currents with cumulative inactivation (Table 3). Analyzing further, this specific electrophysiological feature, we found that high insulin levels remodeled the Kv current phenotype (Fig. 3e). Thus, while 100 µIU/ml insulin almost doubled the frequency of poor inactivating cells (20.5 vs 38.2%), it markedly decreased the abundance of pronounced inactivating cells (38.6 vs 22.4% of > 30%), with no major changes in intermediate (40.9 vs 39.5% of 10–30%) inactivation. Furthermore, the percentage of cumulative inactivation of the Kv currents remained similar (Table 3). These data indicates a remodeling of Kv channels under high insulin stimulation in WAT cells. Concomitantly, the mRNA expression of Kv1.1–1.5, Kv2.1, KCa1.1 and Kvβ1–3 regulatory subunits, which affect the inactivation properties of Kv1 channels, was differentially regulated (Fig. 3f). While high insulin (100 µIU/ml) increased the mRNA of Kv1.3, Kv2.1 and KCa1.1, the expression of Kv1.5 decreased. In this context, the mRNA of different



Kvβ regulatory subunits was differentially regulated upon high insulin treatment. Our results indicate that multiple K^+ channel subunits are expressed in adipocytes and their expression was remodeled under high insulin condition. In addition, the formation of not only homotetrameric but also variable heteromeric structures with altered pharmacology should not be discarded, as previously described [29, 34].

Kv1.3 is present in WAT and participates in the glucose metabolism

Multiple Kv currents suggested a complex scenario in white adipocytes but the C-type and cumulative

◂**Fig. 2** Adipocytes express a repertoire of Kv1 channels. **a** Kv1.1–Kv1.5 mRNA expression in rWAT. Total RNA was isolated from epididymal WAT and PCR was performed and products were run in a 1% TBE agarose gel. Brain was used as a positive control. (+) Presence or (−) absence of retrotranscriptase (RT) reaction. *MM* molecular markers in base pairs (bp). **b** mRNA expression of Kv1.1–Kv1.5, Kv2.1 and KCa1.1 in different rat fat depots. RT-PCR was performed as described in methods and relative expression calculated by applying $2^{(-\Delta\Delta Ct)}$. 18s ribosomal RNA was used as a loading control and epididymal data was taken as reference (value of 1). Color code is indicated at the inset. **c** Kv currents were elicited in adipocytes from SVF by applying 250 ms pulses from −60 to +80 mV and back to −60 mV. Pharmacological profile of Kv currents of adipocytes. K^+ currents were measured and 10–100 nM MgTx, 100 nM Psora-4, 100 nM α-DTX, 100 nM IbTx, 2 mM TEA, and 100 μM Bupivacaine (Bpv) were added to the bath in independent experiments. The percentage of inhibition was calculated as the current blocked respect to the initial current. Values are the mean ± SE, $n = 6–8$. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control (no additions) Paired *t* test. **d**–**j** Kv currents were elicited in adipocytes differentiated from epididymal SVF by applying the indicated protocol. Kv currents with pronounced inactivation (> 30%, **d**) and poor inactivation (< 10%, **e**–**j**) were analyzed. **d** Currents were elicited 250 ms pulses from −60 to +80 mV and back to −40 mV. **e**–**j** Currents were obtained by 250 ms pulses from −60 to +60 mV and back to −60 mV. Poorly inactivating currents were inhibited by **e** 2 mM TEA, **f** 100 nM Psora-4 and **g**, **h** 20 nM MgTx. **i**, **j** Cumulative inactivation of Kv currents was obtained by a train of seven depolarizing voltage steps of 250 ms from −60 to +60 mV once every 400 ms. **i** Representative current traces. **j** Intensity of the current vs the applied pulse

inactivation (Figs. 1, 2, 3), the pharmacological profile (Fig. 2) and relevant bibliography pointed to an important contribution of Kv1.3. Although the physiological role of Kv1.3 in AT is an issue of controversy, we confirmed the presence of Kv1.3 in several WAT sources from human and rodents (Fig. 4). RNA was obtained from rat epididymal WAT and the expression of Kv1.3 was detected, as well as in Jurkat T-lymphocytes that were used as a positive control (Fig. 4a). The protein expression of Kv1.3 in rWAT and rat-isolated adipocytes was confirmed (Fig. 4b). Opposite evidence claim either the presence or the absence of Kv1.3 in human adipose samples [8, 11, 12]. However, we unequivocally characterize the channel expression in fat tissue from different human depots. Therefore, we performed immunohistochemistry in human adipose tissue from the mammary gland (Fig. 4c) and immunocytochemistry in isolated adipocytes from rat epididymal and human subcutaneous adipose tissue biopsies (Fig. 4d, e). In this scenario, all our samples positively stained for Kv1.3.

Furthermore, the role of Kv channels, and specifically Kv1.3, on insulin-dependent glucose uptake is controversial [8, 10–12, 14, 40]. Therefore, we next analyzed whether Kv1.3 participates in insulin signaling (Fig. 4f). MgTx was used because α-DTX discarded the participation of Kv1.1 and Kv1.2 (Fig. 2c, Supplementary Table 1). In adipocytes, 100 nM MgTx significantly reduced insulin-induced (10 μM insulin) glucose transport with no major effects on basal uptake, which points to the participation of Kv1.3 during glucose influx upon insulin signaling.

Caveolin interaction governs localization and insulin-dependent phosphorylation of Kv1.3

Kv1.3 participates in insulin signaling and channel subcellular localization is crucial in cell physiology [9, 10, 41, 42]. The insulin pathway inducing glucose uptake initiates in caveolae. The insulin receptor (IR) interacts with Cav and insulin-dependent GLUT4 translocation recruits the carrier to caveolae. Thus, an altered Cav 1/caveolae expression affects insulin signaling in adipose cells [43]. MgTx pointed to Kv1.3 participating in insulin-dependent glucose uptake. Moreover, Kv1.3 localizes in lipid rafts via interacting with Cav 1 [23]. Therefore, we wanted to study caveolar Kv1.3 targeting and whether an altered localization could affect the insulin signaling in AT. WAT express high levels of Cav 1 and caveolae is an important component of the adipocyte membrane [43]. Plasma membrane lawn preparations (PML) demonstrated that omega-shaped caveolae are abundant membrane structures of the adipocyte (Fig. 5a). Because insulin-mediated signaling initiates in caveolae and the turnover of the channel involves a clathrin-mediated endocytosis [44, 45], we analyzed the colocalization of Kv1.3 with both structures (Fig. 5b–g). PML indicated that Kv1.3 colocalized with Cav 1 almost threefold higher than with clathrin (Fig. 5h). As expected, Kv1.3 coimmunoprecipitated with Cav 1 (Fig. 5i) and clathrin (not shown). Moreover, Kv1.5, also expressed in WAT, coimmunoprecipitated with Kv1.3 and Cav 1. Although Kv1.5 does not interact with Cav 1, this result is not surprising because Kv1.5 may form heterotetrameric complexes with Kv1.3 [29]. To further demonstrate that Kv1.3 physically associated with Cav 1 in adipocytes, we performed FRET immunoassays (Fig. 5j–r). Kv1.3 physically interacted with Cav 1 but not with clathrin (Fig. 5s).

Insulin is an important trophic factor in adipocyte differentiation. To decipher whether Kv1.3 targeting to caveolae is important for adipogenesis, we used the pre-adipocyte 3T3-L1 cell line [43, 46]. In the presence of 10 mg/ml insulin, 3T3-L1 cells differentiated to adipocytes as demonstrated by morphological changes, which include the formation of fatty body droplets (Fig. 6a). Adipocyte differentiation was further documented by an increase in Cav 1 and GLUT4 expression. In this scenario, the expression of Kv1.3 augmented by almost threefold (Figs. 6b and see also 7a, b). The localization of Kv1.3 was also analyzed by immunocytochemistry (Fig. 6c–k). As expected, Kv1.3 targeted to the cell surface. Electron micrographs further demonstrated that the destination of Kv1.3 in 3T3-L1 adipocytes was mostly caveolae, because of its presence in characteristic omega-shaped structures that stained positively for Cav 1 (Fig. 6l–n).

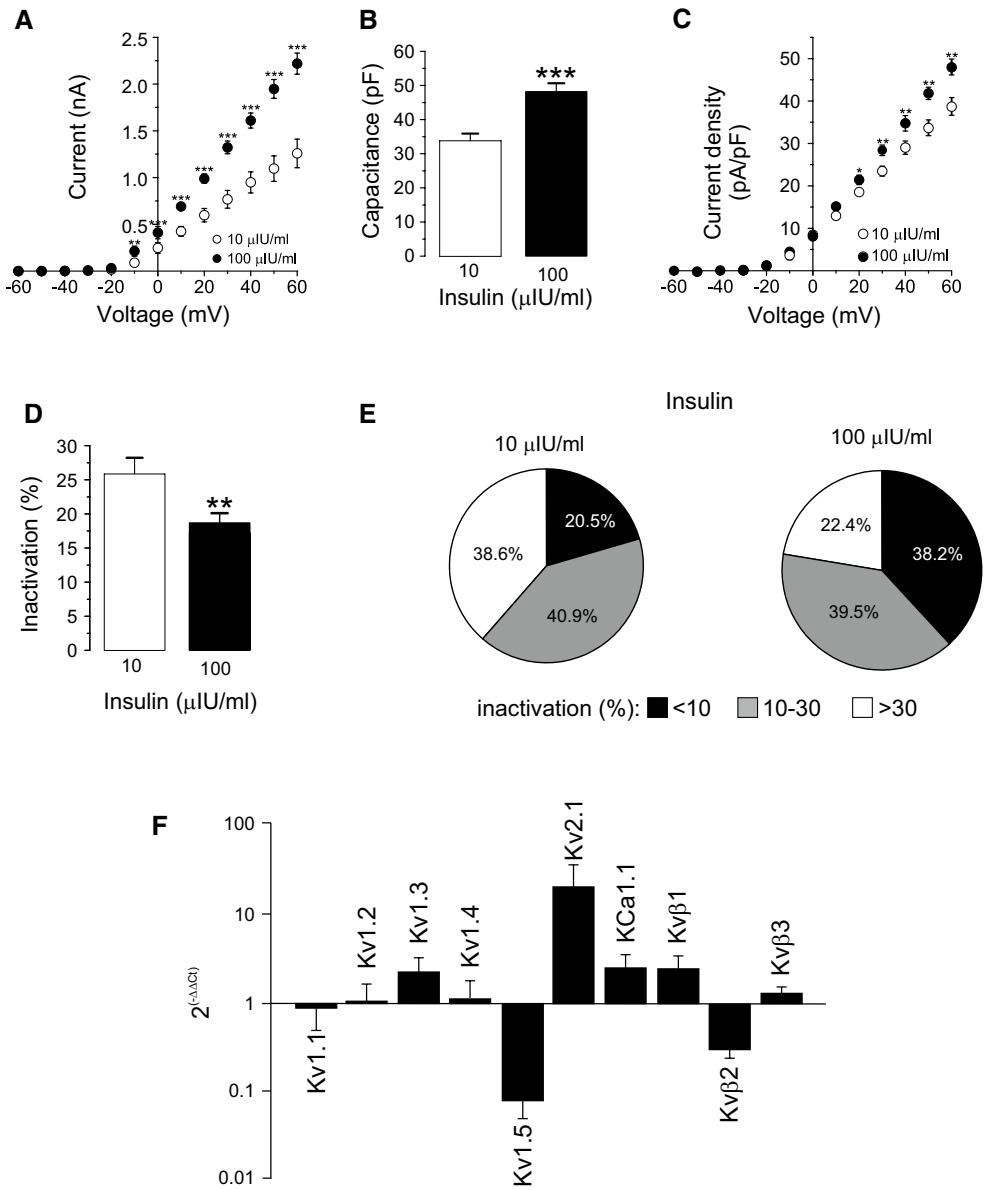


Fig. 3 High insulin concentrations remodeled Kv currents in adipocytes. SVF-derived adipocytes were incubated 4–7 days in low (10 µIU/ml) or high (100 µIU/ml) insulin concentrations following what described in the methods section. From a holding potential of −60 mV, Kv currents were elicited by applying 250 ms pulses from −60 to +60 mV in 10 mV steps. **a** Current–voltage relationship in both conditions. **b** Cell capacitance in pF. **c** Current density (pA/pF) vs voltage (mV) plot of K^+ currents in both conditions. **d** Percentage of inactivation at +60 mV. Data are the mean ± SE of 44 (10 µIU/ml) and 76 (100 µIU/ml) cells. Circles and columns: white, 10 µIU/ml; black, 100 µIU/ml insulin. Statistical significance is denoted as $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs 10 µIU/ml Student's *t* test. **e** Distribution of cells according to their inactivation percentage at +60 mV: poor (< 10%), intermediate (10–30%) or pronounced (> 30%) inactivation, at each insulin concentration. A high insulin concentration triggers a notable switch in the Kv phenotype. Black, < 10%; gray; 10–30%; white, > 30%. **f** RT-PCR was performed from mRNA purified from isolated epididymal adipocytes as described in methods and the relative expression was calculated by applying $2^{(-\Delta\Delta Ct)}$. 18s ribosomal RNA was used as a loading control and 10 µIU/ml data was taken as reference (value of 1). Adipocytes were incubated in the presence of 10 and 100 µIU/ml for 3 days. Data are the mean ± SE of three independent rats each performed in triplicate

To further demonstrate that the newly synthetized Kv1.3 targeted to caveolar lipid rafts, these microdomains were isolated from pre-adipocytes and differentiated fatty 3T3-L1 cells. Adipogenesis notably increased the abundance of caveolar lipid rafts (Fig. 7a, b) with no apparent changes in clathrin. In this context, Kv1.3 augmented its localization into high-buoyancy fractions in adipocytes (Fig. 7f). The depletion of Cav 1 in 3T3-L1 adipocytes (3T3-L1 cav1$^-$) reduced the maximal insulin response, diminishing the abundance of IR and GLUT4 at the cell surface [43]. Therefore,

Table 3 Cumulative inactivation of Kv currents in adipocytes

Group of cells	Insulin (μIU/ml)	Percentage of cells with cumulative inactivation	Percentage of cumulative inactivation
<10% inactivation	10	50	11.7 ± 0.4 (2)
	100	54.5	16.1 ± 3.1 (6)
10–30% inactivation	10	60	11.5 ± 2.9 (6)
	100	70	14.2 ± 1.9 (14)
>30% inactivation	10	41.2	15.4 ± 5.2 (7)
	100	46.7	13.9 ± 3.5 (7)

Adipocytes were differentiated in poor (< 10%), moderate (10–30%) and pronounced (> 30%) inactivation of Kv currents. SVF-derived adipocytes were incubated 4–7 days in low (10 μIU/ml) or high (100 μIU/ml) insulin concentrations following what described in the methods section. The cumulative inactivation was measured applying a train of seven depolarizing voltage steps of 250 ms from − 60 to + 60 mV once every 400 ms. The percentage of inactivation was calculated from the remaining current at the end of the last pulse vs the first. Values are mean ± SE. Number of cells indicated in brackets

we used this Cav 1-deficient 3T3-L1 cell line to analyze whether Kv1.3 localization was affected (Fig. 7c, d). The expression of Kv1.3 was augmented in 3T3-L1 cav1$^-$ adipocytes, but to a lesser extent than in WT cells (Fig. 7e). Concomitantly, Kv1.3 targeting to lipid rafts was clearly impaired by 50% (Fig. 7f). Kv1.5 was not located in lipid rafts microdomains as previously described [15] and, as expected, no changes were observed in 3T3-L1 cav1$^-$ cells (Supplementary Fig. 1). Evidence demonstrates that insulin-dependent signaling initiates in caveolar rafts [43], Kv1.3 targets to raft microdomains [15] and Kv1.3 is phosphorylated by the IR upon insulin stimulation [47, 48]. Therefore, we finally analyzed whether the depletion of Cav 1, which impaired Kv1.3 raft localization, could affect insulin-dependent Kv1.3 phosphorylation in adipocytes. Thus, the phosphorylation of IR and Kv1.3 was clearly diminished in Cav 1$^-$ depleted cells (Fig. 7g). Our results clearly demonstrated that (1) Kv1.3 targets caveolae due to an association with caveolin, (2) adipocyte differentiation increases raft localization and channel expression and (3) this emplacement is important for proper insulin-dependent signaling, which in turn would have important consequences in WAT physiology.

Discussion

The function of Kv channels in the physiology of WAT is under investigation. Contradictory evidence is well documented [3, 4, 8, 10, 12, 13, 39, 40]. The main subject of the debate is the participation of Kv1.3 because Kv1.3 null mice exhibit a lean phenotype and present an altered insulin sensitivity, which points to this channel as a target in type II diabetes and obesity [6, 8–13, 40]. In this complex scenario, our work documents several important facts: (1) Kv1.3 is undoubtedly expressed in WAT from human and rodents; (2) although other isoforms, specially Kv1.5, are also present and although insulin remodeled the Kv phenotype of adipocytes, the pharmacological and biophysical properties pointed to Kv1.3 as an important contributor to Kv currents; and finally, (3) the abundance of Kv1.3 increased during adipogenesis in mouse 3T3-L1 cells, and this channel, via a physical interaction with Cav 1, was targeted to caveolar lipid rafts for proper insulin-dependent phosphorylation.

A decrease in Kv1.3 function facilitates a lean phenotype by enhancing insulin sensitivity, increasing GLUT4 translocation and glucose uptake via Ca^{2+} signaling in adipocytes [9, 10]. However, these results have been debated [8, 11, 12, 40]. In fact, from a metabolic point of view, any increase in glucose influx would enhance lipogenesis rather than generating a lean phenotype. Our data would support this because under high insulin, similar to type II diabetes and obesity, cell size was augmented, and adipogenesis increased Kv1.3 expression. In this complex scenario, while evidence demonstrates that Kv1.3 is expressed in WAT and that channel inhibition increases glucose transport [9], other findings support neither expression nor glucose uptake affectation in adipocytes [8, 11, 12]. The fact that Kv1.3$^{-/-}$ (KO) mice fed a high-fat diet gain less weight and are less obese than littermate controls is at the top of this debate [6]. Moreover, KO animals remain euglycemic with low blood insulin levels [9]. However, for the latter it was demonstrated that although meal regime was similar between Kv1.3 KO and wild type animals, basal metabolic rate was higher in the KO mice [7]. This was further investigated in a double gene-targeted deletion of Kv1.3 and melanocortin-4 receptor (MC4R) [7]. Thus, Kv1.3 abrogation in this genetic model of obesity triggers reductions in adiposity and body weight due to increased locomotor activity and energy expenditure [7, 14]. The last evidence provided by Chandy and coworkers described that the lean phenotype resulting from

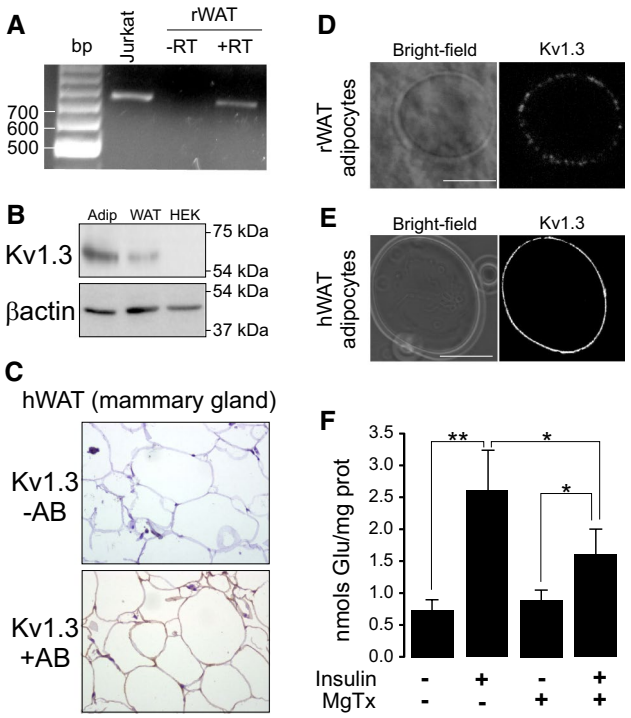


Fig. 4 Human and rat WAT express Kv1.3 participating in insulin-activated glucose uptake in adipocytes. **a** RNA was extracted from rat WAT and RT-PCR was performed in the absence (−) or the presence (+) of retrotranscriptase (RT). Human Jurkat T-lymphocytes were used as a positive control. **b** Protein extracts from isolated rat adipocytes and WAT and human HEK 293 cells were analyzed for the expression of Kv1.3. β-actin was used as a loading and transfer control. **c** Human WAT from mammary gland biopsies was analyzed for the Kv1.3 staining in the absence (−) or the presence (+) of Kv1.3 antibody (AB). **d** Rat adipocytes from SVF were isolated and analyzed by immunohistochemistry for the presence of Kv1.3. **e** Adipocytes from subcutaneous tissue of human fat were stained for the expression of Kv1.3. Bars represent 50 µm. **f** Kv1.3 participates in insulin-dependent glucose uptake augmentation in adipocytes. Adipocytes were incubated with (+) or without (−) of 10 µM insulin for 30 min and glucose uptake was measured in the presence (+) or the absence (−) of 100 nM Margatoxin as described in the methods section. Values represent the mean ± SE, $n = 4–6$. $^{*}p < 0.05$, $^{**}p < 0.01$ Student's *t* test

inhibiting Kv1.3 with ShK-186 is most likely due to an increase in BAT activity rather than WAT participation [8]. Along these lines, other channels such as TRPV2 or TASK-1 play a role in BAT thermogenesis and could also be a target for human obesity therapy [49, 50]. In fact, WT mice fed with a hypercaloric-fat diet reduced the number of mitochondria in mitral cells from the olfactory bulb, whereas this is not observed in Kv1.3 KO mice [51]. Note that sensory neurons from the olfactory bulb are a hot spot for the Kv1.3 function in the nervous system controlling food intake [14]. In this complex scenario, we found that selective inhibition of Kv1.3 diminished, rather increased, the insulin-dependent glucose uptake, which could be, in a sense, a reduction of fuel intake, which in turn would generate less fat deposition. This open controversy could have a surprising explanation. AT is a very complex endocrine tissue. In addition to the origin, metabolic status, age, sex and endocrine situation of the sample, the implication of beige adipose tissue may shed some light on this debate. BAT adipocytes appear upon thermogenic insults in WAT locations. Such brown cells, derived from precursors, are similar to classical white adipocytes. This BAT is often named inducible, beige, or bright. The thermogenic function of beige adipocytes, which differs from that of classical BAT, is not known, but some data suggest a major role in protection against obesity [52]. It is tempting to speculate that this heterogeneous composition would merge evidence. To generate a further complex and exciting scenario, our and other's data suggest that the formation of heterotetrameric structures with other shaker channels, such as Kv1.5, as well as the contribution of other Kv1 isoforms that can be remodeled under several physiological situations, such as insulin resistance and obesity, should be contemplated [29, 33, 34]. We found that exposure to high insulin concentrations, similar to those observed in type II diabetes, triggered a switch in the K^+ current phenotype in adipocytes. Our data would support that formation of hybrid complexes is highly possible. Similar remodeling has been documented in macrophages, microglia and smooth muscle [29–31]. Our work would incorporate adipocytes, under hyperinsulinemia, in this ever-growing list.

In addition to this intense debate that deserves further research, our and other's glucose transport data strongly support that Kv1.3 is somehow involved in insulin sensitivity in the AT [9]. Indeed, a T-1645C polymorphism in *KCNA3* (the human Kv1.3 gene) is associated with impaired insulin sensitivity and altered glucose tolerance [53]. This channel, which is crucial for the immunological response, concentrates in lipid raft structures upon IS formation and leukocyte activation [24]. We have recently described that this targeting is mostly mediated via an association with caveolin [23]. In adipocytes, insulin activates and recruits IR into caveolae [46]. IR phosphorylates Kv1.3 in tyrosines, which inhibits the channel activity [47, 48, 54]. Concomitantly, an insulin-dependent Kv1.3-mediated GLUT4 translocation to the membrane triggers an increase in glucose uptake [10]. All this endocrine regulation is spatially located within caveolae in AT [46]. We demonstrated that, similar to Cav 1 and GLUT4, Kv1.3 increases during adipogenesis, and these newly synthetized channels are properly located in caveolae. We found that Kv1.3 targeting to these raft domains in adipocytes is through physical interactions with Cav 1. An important part of our contribution is focused on the localization of Kv1.3 and the importance of this spatial regulation for insulin-dependent signaling. We demonstrated, using a

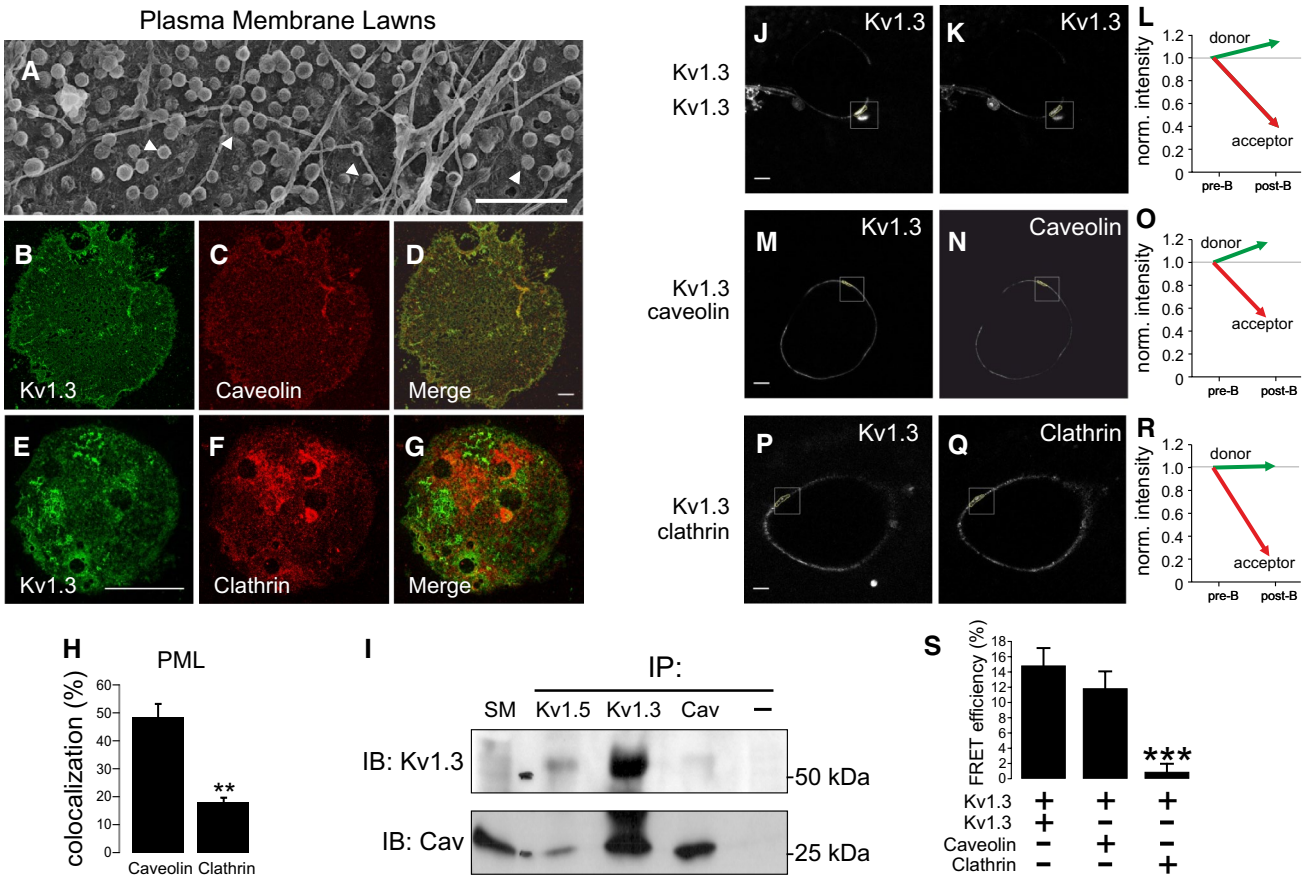


Fig. 5 Kv1.3 targets to caveolae in adipocytes. Kv1.3 physically interacted with caveolin 1 in plasma membrane lawn preparations (PML) from adipocytes. **a** Electron micrograph of a representative PML from rat adipocytes. Multiple omega-shaped structures, recognized as caveolae, highlighted by white arrowheads, are widely distributed throughout the adipocyte plasma membrane. Bar represents 250 nm. **b–h** High colocalization of Kv1.3 with caveolin but not clathrin in PML. **b–d** Kv1.3 and caveolin; **e–g** Kv1.3 and clathrin. Color code: green, Kv1.3 (**b**, **e**); red, caveolin (**c**) and clathrin (**f**); Merge channel in yellow (**d**, **g**). Scale bars: 10 µm (**b–d**), 50 µm (**e–g**). **h** Kv1.3 colocalization with caveolin and clathrin in PML. A pixel by pixel analysis was performed and the percentage of colocalization was analyzed. Values are the mean ± SE of $n > 20$ cells. **i** Kv1.3 coimmunoprecipitated with caveolin 1 and Kv1.5 in isolated adipocytes. Total protein extracts were immunoprecipitated (IP) against Kv1.5, Kv1.3 and Cav. Next, samples were immunoblotted (IB) against Kv1.3 and Cav. SM, starting material; –, immunoprecipitated in the absence of antibody. **j–s** Kv1.3 associates with caveolin 1, but not clathrin, in adipocytes. **j–l** Immunofluorescent FRET analysis of the tetrameric Kv1.3 channels. Kv1.3 was immunodetected with anti-C-terminal polyclonal (**j**) and monoclonal (**k**) Kv1.3 antibodies and further immunostained with Alexa-Fluor-488 (donor) or Cy3-conjugated (acceptor) conjugated antibodies, respectively. **l** Normalized intensity prior to (pre-B) and after photobleaching (post-B). **m–o** Kv1.3 associated with caveolin. **m** Kv1.3 was immunolabelled with monoclonal anti-Kv1.3 and stained with A488. **n** Caveolin with polyclonal anti-Cav and Cy3. **o** Normalized intensity. **p–r** Kv1.3 did not associate with clathrin. **p** Kv1.3 was immunolabelled with polyclonal anti-Kv1.3 and stained with A488. **q** Clathrin with monoclonal anti-clathrin and A546. **r** Normalized intensity. **s** Efficiency of the indirect immunofluorescence FRET analysis. Bars represent 10 µm. ***$p < 0.001$ vs Kv1.3/Kv1.3 Student's t test ($n > 10$ cells)

Cav 1 deficient 3T3-L1 cell line, that Kv1.3 targeting away from lipid rafts and insulin-dependent phosphorylation of the channel, which is crucial for proper insulin-dependent signaling, is impaired in Cav 1^- adipocytes.

Caveolae act as signaling platforms approximating effector molecules and their targets. The importance of Cav 1 in adipocyte physiology is well known because Cav depletion reduces GLUT4 and IR levels, decreasing their stability, and affecting glucose transport [46]. The effect of Cav depletion in the insulin-signaling cascade leading to reduced insulin-enhanced glucose transport could be aggravated by targeting Kv1.3 away from lipid raft structures. In this scenario, although Kv1.3 could be normally expressed, the channel would undergo less phosphorylation and thereby, as previously demonstrated for insulin and epidermal growth factor, the function would be mostly preserved [44, 54]. It is tempting to speculate that if Kv1.3 inhibition improves insulin sensitivity by abrogating the inflammatory state similar to what observed in leukocytes [55], an altered localization of the channel would maintain

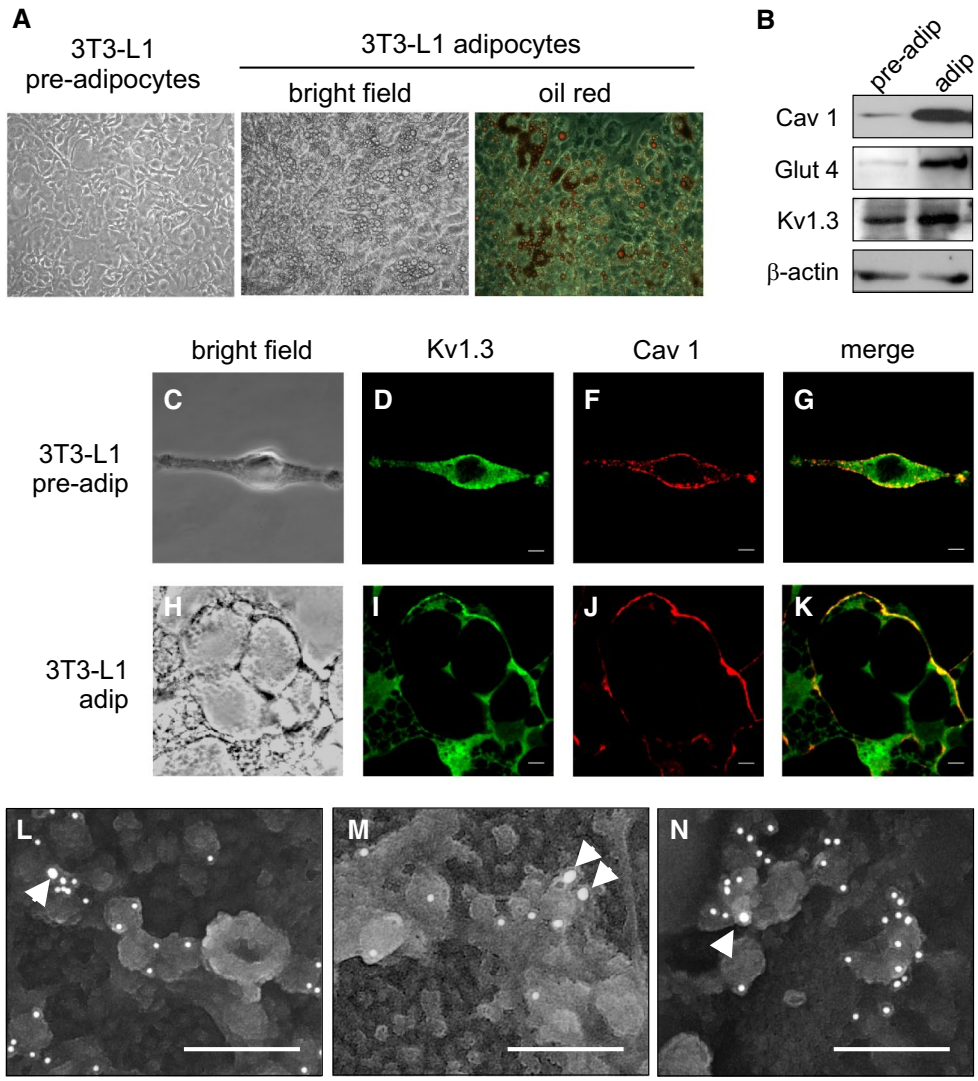


Fig. 6 Adipocyte differentiation increases Kv1.3 and targets the channel to caveolae. 3T3-L1 pre-adipocytes were differentiated to adipocytes. **a** Oil red O staining showed an increased adiposity of 3T3-L1 adipocytes. **b** 3T3-L1 adipogenesis elevated the expression of Cav1, Glut 4 as well as Kv1.3, although to a lesser extent. **c**–**k** Representative confocal images of Kv1.3/Cav 1 colocalization in 3T3-L1 pre-adipocytes (**c**–**g**) and adipocytes (**h**–**k**). **c**, **h** Bright field; **d**, **i** Kv1.3 in green; **f**–**j** Cav 1 in red; **g**–**k** merge in yellow. Bars represent 10 μm. **l**–**n** Electron micrographs of Kv1.3 located in caveolae stained with Cav 1 in 3T3-L1 adipocytes. Arrowhead points to Kv1.3 tagged with 15 nm gold particles. Cav 1 stained with 10 nm gold particles. Bars represent 200 nm

the function, thereby impairing the insulin-dependent response. Moreover, in a metabolic context, reduced channel inhibition would counteract elevated mitochondrial function, facilitating an obese phenotype, insulin resistance and the appearance of type II diabetes.

Obesity and related metabolic disorders are currently a global epidemic which requires novel and reliable pharmacological strategies. The medical and social implications derived from these diseases will represent a significant cost to healthcare systems. Kv1.3 participates in peripheral insulin sensitivity and is being considered as a new target for obesity treatment. Therefore, our results are of relevance because it clearly contributes to knowledge of adipocyte physiology.

Acknowledgements Supported by the Ministerio de Economia y Competitividad (MINECO, Spain) Grants (BFU2014-54928-R and BFU2017-87104-R) and Fondo Europeo de Desarrollo Regional (FEDER). MPV and JC contributed equally and hold fellowships from the MINECO and the Fundación Tatiana Pérez de Guzmán el Bueno, respectively. Authors thank Dr. C. López-Iglesias (CCiTUB, Universitat de Barcelona) for her help in electronic Microscopy and to Dr. J. Peinado-Onsurbe for the access to human samples. The English editorial assistance of the American Journal Experts is also acknowledged.

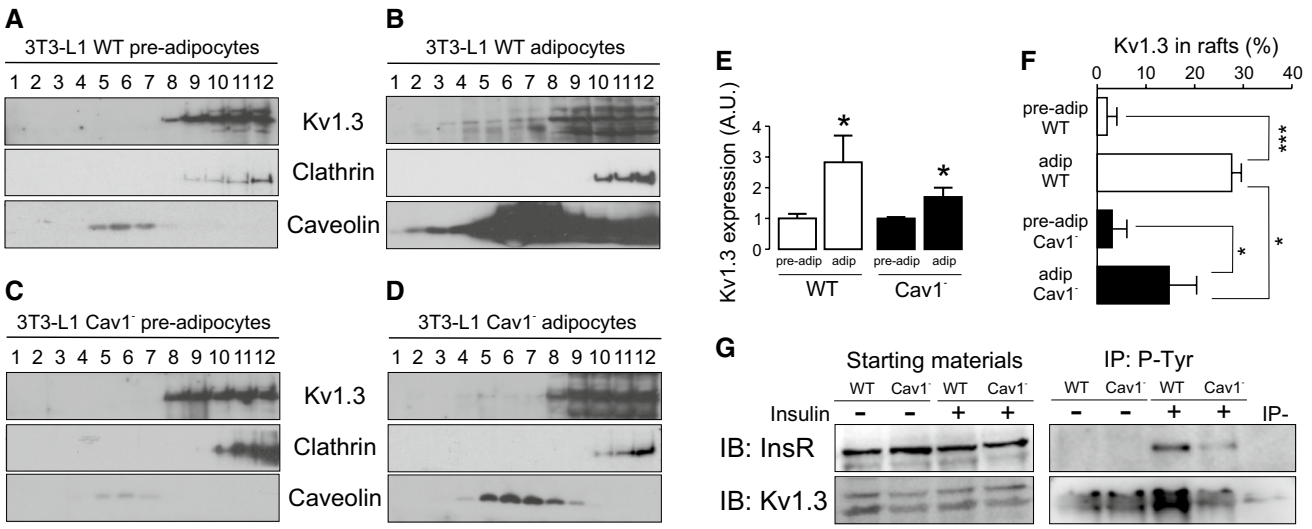


Fig. 7 The depletion of Cav 1 in 3T3-L1 adipocytes mistargeted Kv1.3 lipid raft localization as well as impaired insulin-dependent phosphorylation of the channel. Lipid rafts were isolated from 3T3-L1 pre-adipocytes and adipocytes. A sucrose gradient, from low (1)- to high (12)-density fractions was applied, and the expression of Kv1.3, clathrin (non-raft marker) and caveolin (lipid raft marker) was analyzed. In addition, the 3T3-L1 Cav 1$^-$ cell line, with a silenced expression of Cav 1, was used. **a** Low expression of Kv1.3 in lipid rafts from 3T3-L1 wild type (WT) pre-adipocytes. **b** Increased expression of Kv1.3 targeting to rafts in 3T3-L1 WT adipocytes. Note the exacerbated augmentation of Cav 1 due to adipocyte differentiation. **c** Low expression of Kv1.3 in lipid rafts from 3T3-L1 Cav1$^-$ pre-adipocytes. **d** A minor increase of Kv1.3 is concomitant with reduced channel localization in rafts in 3T3-L1 Cav 1$^-$ adipocytes. Note the limited augmentation of Cav 1 due to adipocyte differentiation in this cell line. **e** Increase in Kv1.3 expression during adipocyte differentiation in 3T3-L1 WT and Cav 1$^-$ cells. *$p<0.05$ vs pre-adipocytes (Student's t test, $n=3$-5). **f** Percentage of Kv1.3 in lipid rafts. Kv1.3 expression in caveolin-positive floating fractions was analyzed and relativized to the total amount of Kv1.3. *$p<0.05$, ***$p<0.001$ (Student's t test, $n=3$–5). White columns represent WT, black columns represent Cav 1$^-$ cells. **g** The reduction of Cav 1 in Cav 1$^-$ 3T3-L1 cell line impairs the insulin-dependent phosphorylation of Kv1.3. WT and Cav 1$^-$ cells were incubated with (+) or without (−) insulin, as described in methods. Total cell lysates were immunoprecipitated (IP) against phosphotyrosines (P-Tyr) and immunoblotted (IB) against the insulin receptor (InsR) and Kv1.3. Note an impairment in the phosphorylation of InsR and Kv1.3 in Cav 1$^-$ cells in the presence (+) of insulin

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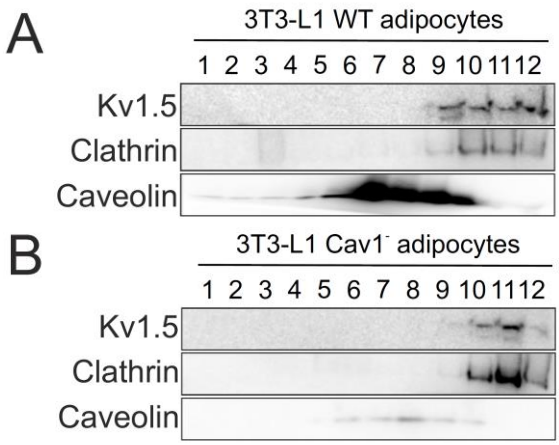
SUPPLEMENTARY MATERIAL



Supplementary Fig. 1. Kv1.5 did not target to lipid rafts. Lipid rafts were isolated from 3T3-L1 Wt and 3T3-L1 Cav 1^{-} adipocytes. A sucrose gradient, from low (1) to high (12) density fractions was applied, and the expression of Kv1.5, clathrin (non-raft marker) and caveolin (lipid raft marker) was analysed. (A) Expression of Kv1.5 in sucrose fractions from (A) 3T3-L1 wild type (WT) and (B) 3T3-L1 Cav 1^{-} adipocytes. Kv1.5 distribution, out rafts, was independent of the expression of Cav 1. Note the limited augmentation of Cav 1 due to adipocyte differentiation in this cell line.

Supplementary Table 1. Pharmacological profiles of Kv1 and KCa1.1 channels potentially expressed in adipocytes.

Drug	Target	IC_{50} or K_D	Reference
Margatoxin (MgTX)	Kv1.1	1.7 nM	[1]
	Kv1.2	11.7 nM	[1]
	Kv1.3	30 pM	[2]
Dendrotoxin (DTX)	Kv1.1	12 nM	[3]
	Kv1.2	4 nM	[3]
	Kv1.3	> 600 nM	[3]
Iberiotoxin (IbTX)	$K_{Ca}1.1$	1.16 nM	[4]
Psora-4	Kv1.1	62 nM	[5]
	Kv1.2	49 nM	[5]
	Kv1.3	3 nM	[5]
	Kv1.4	202 nM	[5]
	Kv1.5	7.7 nM	[5]
Tetraethylammonium (TEA)	Kv1.1	600 µM	[3]
	Kv1.2	129 mM	[3]
	Kv1.3	11 mM	[6]
	Kv1.4	>100 mM	[3]
	Kv1.5	330 mM	[7]
	$K_{Ca}1.1$	35.4 µM	[12]
Bupivacaine (Bpv)	Kv1.1	240 µM	[8]
	Kv1.2	210 µM	[8]
	Kv1.3	20 µM	[9]
	Kv1.4	100 µM	[10]
	Kv1.5	45 µM	[11]
	$K_{Ca}1.1$	324 µM	[13]

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3.1.3 CONTRIBUTION III

Effects of palmitoylation on Kv1.3 organization at the immunological synapse

Jesusa Capera[1] · María Navarro-Pérez[1] · James H Felce[2] · Silvia Cassinelli[1] · Michael L Dustin[2] · Antonio Felipe[1]

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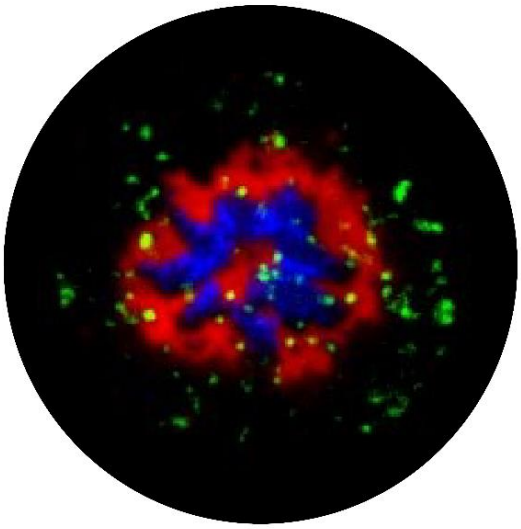

Report of the PhD student participation in the article:

Effects of palmitoylation on Kv1.3 organization at the immunological synapse

Will be submitted to *Proceedings of the National Academy of Sciences*

Impact factor (2018): 9.580

Jesusa Capera performed and analysed all the experiments (except for TIRF-SIM microscopy)

Prof. Antonio Felipe
Thesis director

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3.2 PART 2: MITOCHONDRIAL Kv1.3

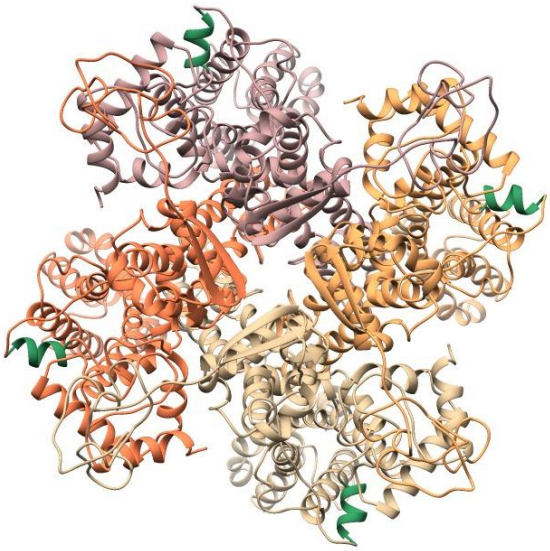
3.2.1 CONTRIBUTION IV

The mitochondrial Kv1.3-caveolin axis in the control of cell survival and apoptosis

Jesusa Capera[1*] · Mireia Pérez-Verdaguer[1*] · Roberta Peruzzo[2] · Juan Martinez-Pinna[3] · Andrés Morales[3] · Antonio Felipe[1]

[1] Molecular Physiology Laboratory, Dpt. de Bioquímica i Biomedicina Molecular, Institut de Biomedicina (IBUB), Universitat de Barcelona, Barcelona, Spain.
[2] Dpt. of Biology, University of Padova, Padova, Italy.
[3] Dpt, de Fisiología, Génetica y Microbiología, Universidad de Alicante, Alicante, Spain.

*JC and MPV contributed equally to this work



Report of the PhD student participation in the article:

The mitochondrial Kv1.3-caveolin axis in the control of cell survival and apoptosis

Will be submitted to *EMBO Journal*
Impact factor (2018): 11.2

Jesusa Capera performed and analysed all experiments with confocal imaging on HEK293 cells (excluding FRET experiments), non-correlative electron microscopy, lipid raft isolation, protein glycosylation, mitochondria purification, analysis of protein expression in Jurkat and 3T3-L1 cells and apoptosis quantification on HEK293 Cav^- cells, in addition to site-directed mutagenesis of the Kv1.3 pore mutants.

Prof. Antonio Felipe
Thesis director

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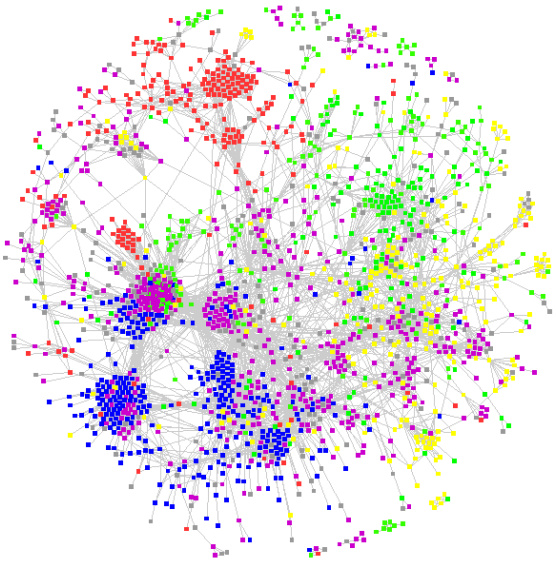
3.1.3 CONTRIBUTION III

Transmembrane domains cooperate to mediate Kv1.3 unconventional targeting into mitochondria

Jesusa Capera[1] · María Navarro-Pérez[1] · Anne-Stine Strøm-Moen[1] · Antonio Felipe[1]

[1]Molecular Physiology Laboratory, Universitat de Barcelona, 08028 Barcelona, Spain. [2]Institut de Biomedicina (IBUB). Departament de Bioquímica i Biologia Molecular. Universitat de Barcelona, 08028 Barcelona, Spain.



Report of the PhD student participation in the article:

Transmembrane domains cooperate to mediate Kv1.3 unconventional targeting into mitochondria

Will be submitted to *Science Signalling*

Impact factor (2018): 6.481

Jesusa Capera performed and analysed all the experiments.

Prof. Antonio Felipe
Thesis director

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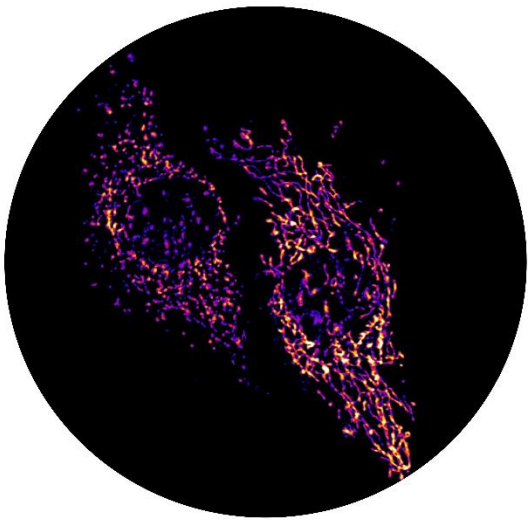
3.2.3 CONTRIBUTION VI

Kv1.3 regulates mitochondrial dynamics during cell cycle progression

Jesusa Capera[1] · Maria Navarro-Pérez[1] · Mireia Pérez-Verdaguer[1,2] · Albert Vallejo-Gracia[1,3] · Antonio Felipe[1]

[1] Molecular Physiology Laboratory, Dpt. de Bioquímica i Biomedicina Molecular, Institut de Biomedicina (IBUB), Universitat de Barcelona, Barcelona, Spain.
[2] Dpt. of Cell Biology, School of Medicine, University of Pittsburgh, Pittsburgh, USA.
[3] Gladstone Institute for Virology and Immunology, San Francisco, USA.



Report of the PhD student participation in the article:

Kv1.3 regulates mitochondrial dynamics during cell cycle progression

Will be submitted to *Cell Reports*

Impact factor (2018): 7.815

Jesusa Capera performed and analysed all the experiments (expect generation of the shRNA cell lines).

Prof. Antonio Felipe
Thesis director

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4. DISCUSSION

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5. CONCLUSIONS

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