# TRPV4 channel regulation and involvement in cell motility

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I dedicate this thesis to my better half Jean-donat,

For always believing in me and

For taking exceptional care of our son,

Allowing me to do all this

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## Abstract

The TRPV4 cation channel is expressed in a broad range of tissues where participates in the generation of a $Ca^{2+}$ signal. TRPV4 participation in osmo- and mechanotransduction contributes to important functions such as cellular and systemic volume homeostasis, among others. TRPV4 also responds to temperature and 4α-phorbol 12,13-didecanoate (4αPDD) thus showing multiple modes of activation. Besides, dozens of TRPV4 mutations have been linked to osteoarticular and neuromuscular diseases. However, little is known about the domains relevant for TRPV4 activation by different stimuli. This Thesis research aims to elucidate the participation of the N-terminal cytosolic tail in the gating of TRPV4 by physiological stimuli and the channel implication in cell motility. I provide evidences that activation of TRPV4 by hypotonic shocks and temperature requires $PIP_2$ binding within the sequence $^{121}$KRWRK$^{125}$ of the N-terminus tail and rearrangement of the cytosolic tails. My results also point to the participation of TRPV4 in cell migration by modulating the dynamics of trailing adhesions, a function that may require the interplay of TRPV4 with other cation channels present at the focal adhesion sites.



## Resumen

El canal TRPV4 es un canal catiónico capaz de generar señales intracelulares de $Ca^{2+}$ en diversos tejidos. La participación del canal TRPV4 en procesos de mecano-osmotransducción le implica en funciones tan importantes como la regulación del volumen celular y sistémico. El TRPV4 también se activa en respuesta a calor y al agonista sintético 4αPDD, lo que implica la presencia de varios modos de activación. Además, existen numerosas mutaciones en el TRPV4 que se han encontrado en pacientes que sufren de patología osteoarticular y neuromuscular. Sin embargo, aún se desconocen aspectos de su función relacionados con los mecanismos de activación. Mi trabajo de Tesis doctoral aborda el estudio de la región N-terminal del TRPV4, su participación en la activación del canal por estímulos fisiológicos y la relevancia del canal en proceso de migración celular. Esta Tesis doctoral proporciona evidencias de que el TRPV4 necesita unir $PIP_2$ a través de la secuencia $^{121}$KRWRK$^{125}$ de la cola N-terminal y que las colas se reorganicen para que el canal se abra en respuesta a estímulos osmóticos y de calor. Mis estudios también sugieren que el canal TRPV4 participa en la modulación de la adherencia de la cola durante el proceso de la migración celular, posiblemente interaccionando con otros canales presentes en las adhesiones focales.

# Prologue

This thesis is focused on the TRPV4 cationic channel that belongs to the large group of ion channels named transient receptor potential channels. These polymodally gated ion channels are recognized as universal sensors of environmental changes but also of changes at the cellular level. TRP channels are literally saving our lives, since it is thanks to these sophisticated molecular devices that we can sense the danger and find food. TRP channels are also enabling us, for example, to sense the sweetness of chocolate and they make chocolate sweeter when is warmer rather than cooler (TRPM5); they enable us to enjoy human touch and many other sensory processes. TRP channels are functionally very diverse and are implicated in a multitude of physiological processes, ranging from $Ca^{2+}$ and $Mg^{2+}$ homeostasis and regulation of the vascular tone to bone development, taste perception and temperature sensing. The importance of TRP channels in human health and disease is illustrated by the huge and growing number of monogenic human diseases that are caused by mutations in TRP channel genes, such as hypomagnesemia with secondary hypocalcemia (loss-of-function mutations in TRPM6), autosomal dominant brachyolmia (gain-of-function mutations in TRPV4), autosomal dominant focal segmental glomerulosclerosis (gain-of-function mutations in TRPC6), among many others. TRP channels are also implicated in complex pathophysiological conditions including neuropathic pain, cancer, asthma, urinary incontinence and cardiac hypertrophy. Therefore, TRP channel have a huge potential as novel therapeutic targets or diagnostic markers. But at this moment the molecular mechanisms of activation has been elucidated for only a few TRPs, as well as their cellular regulation and involvement in physiology and pathophysiology. Among TRP channels, TRPV4 is one of the most studied but also the most puzzling channel. It stands out as well for the fact that it causes more diseases than any other TRP channel. This is why it is urgent to elucidate the molecular mechanisms of its activation (especially by physiological activators) and its function-to-structure relationship. The work described in this thesis shed more light on these and other functions of TRPV4 channel.

# List of abbreviations

| | |
|---|---|
| $[Ca^{2+}]_i$ | intracellular calcium concentration |
| 3D | three-dimensional |
| 4α-PDD | 4α phorbol 12,13 didecanoate |
| aa | amino acid |
| AKAP | a kinase anchoring protein |
| ARD | ankyrin repeat domain |
| ATP | adenosine triphosphate |
| BAR | bin-amphiphysin-rvs |
| BBA | bisandrographolide a |
| BTEC | breast tumor-derived endothelial cells |
| C | carboxyl |
| CaM-BD | calmodulin binding domain |
| CANP | calcium-activated neutral protease |
| CHO | chinese hamster ovary |
| CNS | central nervous system |
| CTD | cytoplasmic domain |
| DAG | diacyl-glycerol |
| DMAPP | dimethylallyl pyrophosphate |
| DRG | dorsal root ganglion neurons |
| ECM | extracellular matrix |

| | |
|---|---|
| EET | epoxyeicosatrienoic acid |
| EGF | epidermal growth factor |
| ER | endoplasmic reticulum |
| F-actin | filamentous actin |
| FA | focal adhesion |
| FAK | focal adhesion kinase |
| FKBP | fk506 binding protein |
| FRB | fkbp rapamycin binding domain |
| FRET | fluorescence resonance energy transfer |
| GDP | guanosine diphosphate |
| GN11 | gonadotropin-releasing hormone-secreting neurons |
| GPCR | g protein coupled receptor |
| GSK | ((n-((1s)-1-{[4-((2s)-2-{[(2,4dichlorophenyl)sulfonyl]amino}-3-hydroxypropanoyl)-1 pipera-zinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide |
| GTP | guanosine triphosphate |
| HEK | human embryonic kidney |
| HeLa | henrietta lacks |
| HepG | hepatoblastoma |
| Inp54p | inositol polyphosphate 5-phophatase |
| IP3 | inositol triphosphate |
| IP3R | inositol triphosphate receptor |
| Lyn | Lck/Yes-related |

| | |
|---|---|
| MAP | microtubule-associated protein |
| MCT | monocarboxylate transporter |
| MLC | myosin light chain |
| N | amino |
| NBC | $Na^+$,$HCO_3^-$ cotransporter |
| NHE1 | sodium-hydrogen antiporter |
| NKCC1 | Na-K-Cl cotransporter |
| NO | nitric oxide |
| OTRPC | osmosensitive transient receptor potential channel |
| PACSIN | kinase c and casein kinase substrate in neurons |
| PAR | partitioning defective |
| PC | prostate carcinoma |
| PDZ | post synaptic density protein, drosophila disc large tumor suppressor, and zonula occludens-1 protein |
| PH | plecstrin homology |
| $pH_e$ | extracellular pH |
| $pH_i$ | intracellular pH |
| PI | phosphoinositide |
| $PIP_2$ | phosphatidylinositol-4,5-biphoshate |
| PKA | protein kinase a |
| PKC | protein kinase c |
| PLA | phospholipase a |

| | |
|---|---|
| PLC | phospholipase c |
| PRD | proline rich domain |
| Pky | proline-rich tyrosine kinase |
| S | transmembrane segment |
| SGK | serum and glucocorticoid-regulated kinase |
| SNP | single nucleotide polymorphism |
| TM | transmembrane |
| TMD | transmembrane domain |
| TRP | transient receptor potential |
| TRPA | transient receptor potential ankyrin |
| TRPC | transient receptor potential canonical |
| TRPM | transient receptor potential melastatin |
| TRPN | transient receptor potential NO-mechano-potential |
| TRPP | transient receptor potential polycystin |
| TRPV | transient receptor potential vanilloid |
| VR-OAC | vanilloid receptor-related osmotically activated channel |
| VRAC | volume regulated anion channel |
| VRL | vanilloid receptor-like |
| WNK | lysine deficient protein kinase |
| Wnt | wingless-related |

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# 1.INTRODUCTION

# 1. Ion channels

Ion channels are integral membrane proteins surrounded by a complex milieu of lipid molecules that, far from being just a passive barrier, play an active role in their structural and functional modulation. The basic function of an ion channel is to regulate the membrane permeability for a given ion species. At the simplest level, channels achieve this by forming a narrow transmembrane pore with an affinity for ions of a given charge. At the deeper level, channels are sophisticated devices of enormous complexity that allow about 10 million of ions to flow per second and even a subtle change in their sequence can cause tremendous consequences for the cell and a living beings life. There are over 300 types of ion channels in a living cell (Gabashvili et al., 2007) and they can be classified by the nature of their gating, the species of ions passing through those gates, the number of gates (pores) and localization of proteins.

## 1.1. TRP channels

TRP stands for Transient Receptor Potential that comes from a Drosophila mutant that had been discovered in 60's to have transient instead of sustained response to bright light (Cosens and Manning, 1969). In these flies, sustained light induced a transient instead of normal sustained, plateau-like increase in intracellular $Ca^{2+}$ signal, whose electrical equivalent is the receptor potential, and therefore the mutant was baptised *trp*, for transient receptor potential. Only two decades later the *trp* gene was cloned (Montell and Rubin, 1989) and few years later it was proposed that it coded for a six-transmembrane containing, $Ca^{2+}$ permeable cation channel, TRP, the founding member of the TRP family (Hardie and Minke, 1992). Currently, 27 TRP genes have been identified in various animals with the protein sequence homology among them ranging from 35 to 80%. Human TRP genes are diverse in length and range between 11 and 911 kb and the number of exons varying from minimum 11 up to 39. Based on protein homology, members of the TRP channel family can be subdivided into seven subfamilies (**Fig.1**). The transmembrane segments (S) tend to share the greatest homology within a particular subfamily. Within the six kingdoms of life, bacteria, protozoa, chromista, plantae, fungi and animalia, TRP-related genes seem to be found only in fungi and animalia. Interestingly during evolution, most vertebrates lost the mechanosensitive TRPN channels but almost doubled the number of TRPs involved in calcium and magnesium homeostasis, thermo- and chemosensing and calcium signaling (TRPCs, TRPVs and TRPM).

### 1.1.1. Structure

The basic structure of TRP channels is the same as that of voltage gated $K^+$ channels (Yellen, 2002). A typical TRP protein contains six putative

transmembrane segments (S1 to S6) with a pore-forming loop between S5 and S6 (Owsianik et al., 2006). Intracellular amino (N) and carboxyl (C) termini are variable in length and consist of a variety of domains. The amino termini of many TRPs contain ankyrin repeats, 33-residue motifs consisting of pairs of antiparallel α-helices connected by β-hairpin motifs. The number of repeats in the ankyrin repeat domain (ARD) can vary from 3 to 4 in TRPCs, 6 in TRPVs, 14 to 15 in TRPA1 up to 29 in TRPNs. Functionally, ARD seems to be important for channel tetramerization and interactions with ligands and protein partners (Gaudet, 2008). High resolution crystallographic data of ARD of TRPV channels is available (Gaudet, 2009;Jin et al., 2006), which fits with the recent full structure of TRPV1 resolved by electro cryo-microscopy (Liao et al., 2013). Subunits of different TRP channels can heteromerize (Ma et al., 2011) (**Fig.2 B,C**).

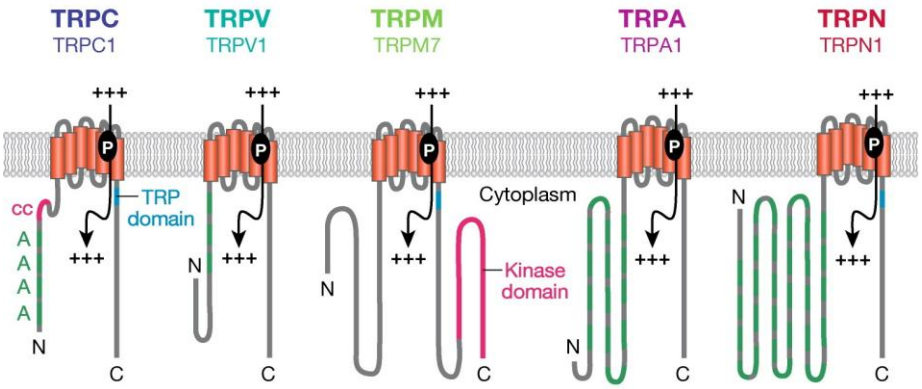


**Figure 1. Structural elements of the mammalian transient receptor potential cation channels.** TRP, transient receptor potential family (TRPA, TRPC, TRPM, TRPML, TRPP, TRPV). The canonical subfamily TRPC comprises closest homologs of Drosophila trp channels. TRPVs ('vanilloid') are named after a founding member vanilloid receptor 1 (now TRPV1) which can be activated by capsaicin, the pungent compound in hot chilli peppers. Capsaicin belongs to the vanilloids, compounds which possess a vanillyl chemical group. TRPMs were named after their first member discovered, melastatin-1 (tumor suppressor melastatin is a gene expressed in melanocytes). Unlike the TRPC and TRPV subfamilies, TRPM subunits do not contain N-terminal ankyrin repeat motifs but, rather, contain entire functional proteins in their C-termini. TRPM6 and TRPM7, for example, contain functional α-kinase segments. TRPAs were named after the protein that was found to contain many ankyrin repets. The TRPN subfamily is named after the 'NO-mechano-potential C' (NOMP-C) channel of Caenorhabditis elegans. The TRPPs are polycystins while, TRPMLs contain mucolipins. Image adopted from (Venkatachalam and Montell, 2007).

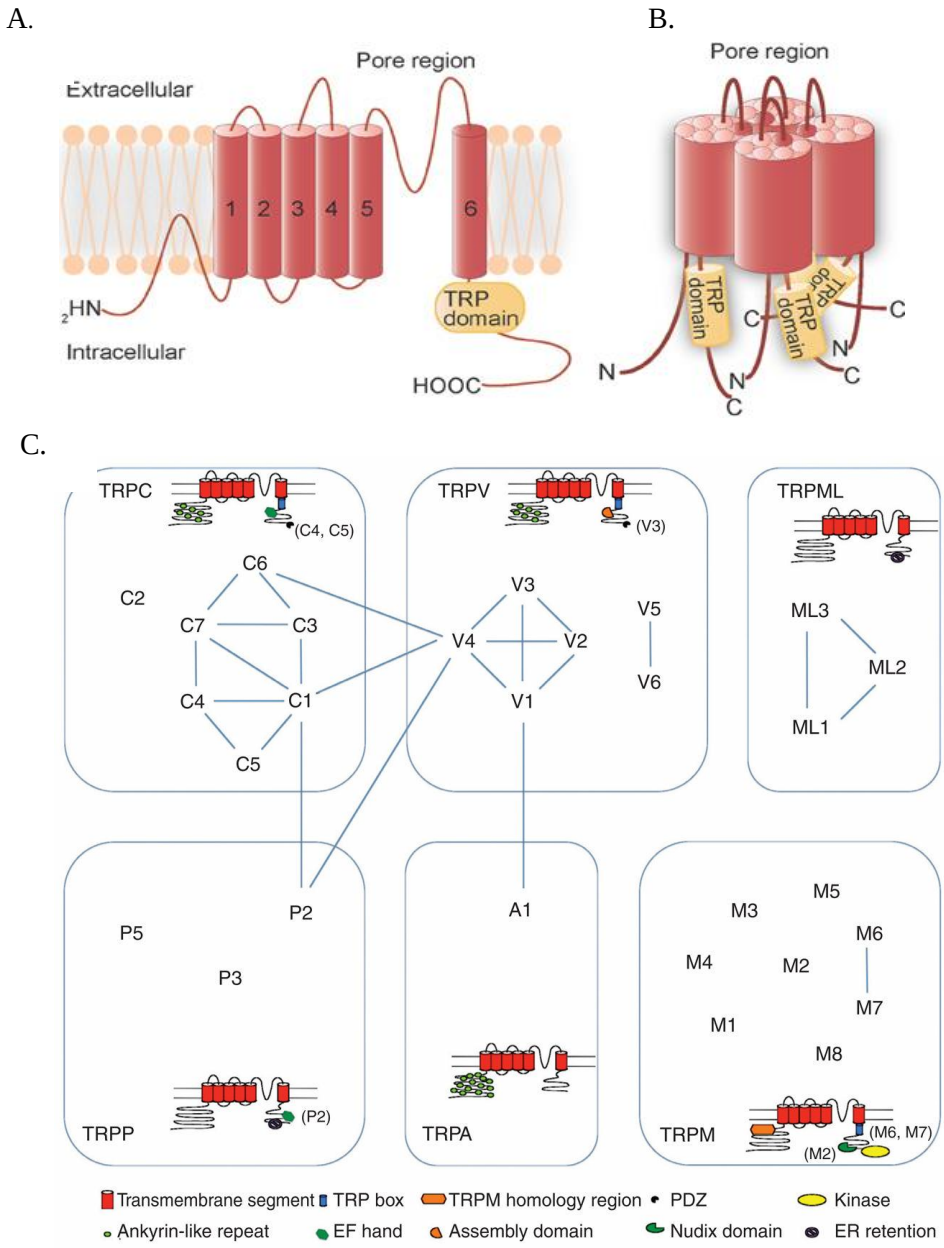


**Figure 2. Transmembrane topology of TRP channels and their tetramerization**. **A**.Transmembrane topology **B.** The quartenary structure of TRP channels. The TRP protein has six putative transmembrane domains, a pore region between the fifth and sixth transmembrane domains and a TRP domain in the C-terminal region. **C.** The TRP protein assembles into homotetramers or hetero-tetramers to form channels. Image adopted from Nobuaki Takahashi and (Zheng, 2013).

## 1.1.2. Polymodality

TRPs are polymodaly gated ion channels, *scilicet* they can be activated by many different stimuli and mechanisms. Such as:

I. Temperature. Temperature-dependent activation of TRP channels is a very peculiar gating process that has drawn intensive investigations since its discovery in TRPV1 in 1997 by the Julius group (Caterina et al., 1997). Temperature sensing is a basic and indispensable sensory form. Sensitive response of the nervous system to changes in temperature is of supreme importance for homeotherms to maintain a stable body temperature. Temperature cues are necessary to detect ambient environment, search for favorable conditions, and avoid harm. There are a number of TRP channels that are thought to be highly temperature sensitive (Caterina et al., 1997;Moqrich et al., 2005;Peier et al., 2002;Voets et al., 2004;Zheng, 2013) (**Fig. 3)**. Often they are referred to as thermoTRPs.

II. Calmodulin (CaM). A large number of TRP channels are known to be regulated by Calmodulin. Each CaM molecule contains four EF-hand motifs (helix-loop-helix structural domains) that differ in their $Ca^{2+}$ affinities. The existence of multiple $Ca^{2+}$- binding sites and cooperatively among them makes CaM a highly $Ca^{2+}$-sensitive regulator, while difference in $Ca^{2+}$-binding affinity at each sites allows it to function at a wide dynamic range of $Ca^{2+}$ concentrations occurring at the site of action. A large number of TRP channels are known to be regulated by CaM (Rosenbaum et al., 2004;Zhu, 2005).

III. Exogenous ligands. Most TRP channel activities are modulated by a large number of exogenous ligands. In particular, temperature-sensitive TRPs seem to be preferred targets for plant-derived chemicals. The classic example is heat-sensitive TRPV1, which is activated by structurally unrelated botanical compounds such as capsaicin (the pungent extract of hot pepper (Caterina et al., 1997), and camphor (the waxy substance with penetrating odor extracted from *Cinnamomum camphora* (Xu et al., 2005), which also activates TRPV3 (Moqrich et al., 2005). Other examples include TRPM8, a cold receptor directly activated by menthol (derived from the mint plant *Mentha piperita*) (McKemy et al., 2002) and eucalyptol (derived from the tree *Eucalyptus globulus*) (Peier et al., 2002), and TRPV4, which is activated by bisandrographolide (derived from the plant *Andrographis paniculata* (Smith et al., 2006b).

IV. Synthetic ligands. TRP channels respond also to a wide range of synthetic ligands, many of which are important pharmacological tools that can be used to modulate channel functions.

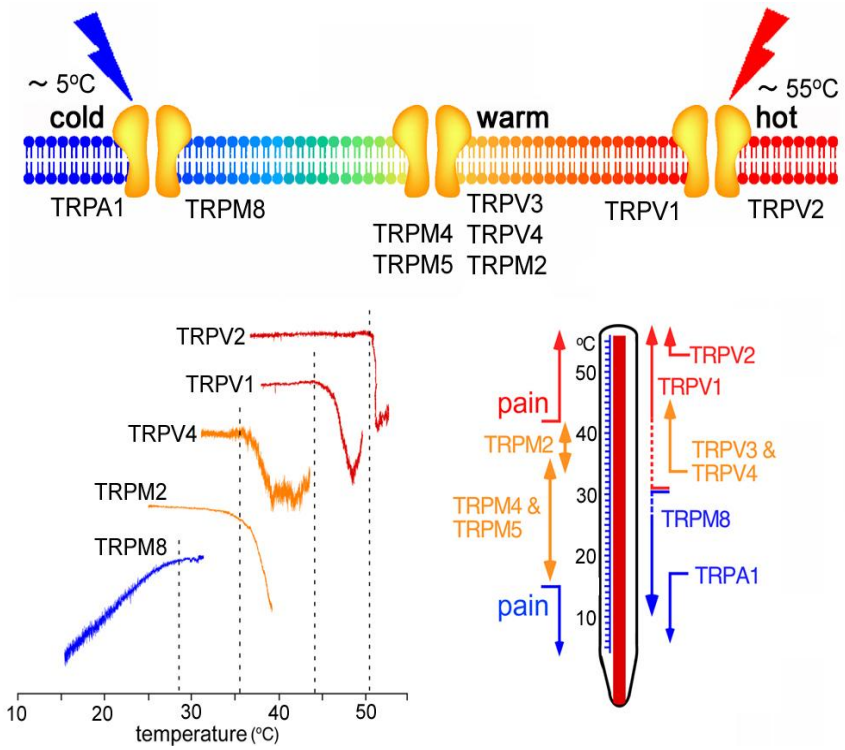


**Figure 3. Thermosensitive TRP channels**. TRPV1 starts to open when the temperature rises to near 40°C under otherwise normal conditions. Its homologs TRPV2, TRPV3, and TRPV4 are also heat activated but with distinct threshold temperatures. TRPV2 activates at more than 50°C, while TRPV3 and TRPV4 activates at lower temperatures around 30°C. TRPM2, TRPM4, and TRPM5 are also reported to be sensitive to high temperatures. Another heat-sensitive TRP channel is the TRPA1 channel of Drosophila fly and certain snake species. The best understood cold-sensitive TRP channel is TRPM8, which activates when temperature drops below about 20°C (McKemy et al., 2002;Peier et al., 2002;Zimmermann et al., 2011). Mammalian TRPA1 was thought to be a cold sensor with an extremely low threshold temperature below 10°C (Story et al., 2003). However, cold activation of TRPA1 remains controversial. Image adopted from Makoto Tominaga.

Some can activate more than one TRP channel, for example, 2-aminoethyl diphenylborinate activates TRPV1, TRPV2 and TRPV3 (Chung et al., 2004;Hu et al., 2004), whereas some are relatively highly selective for a particular TRP channel, such as olvanil for TRPV1 (Iida et al., 2003) and synthetic phorbol ester 4α-PDD, lumiphorbols, phorbol-hexonates and GSK 1016790A ((N-((1S)-1-{[4-((2S)-2-{[(2,4Dichlorophenyl)sulfonyl]amino}-3-hydroxypropanoyl)-1 pipera-zinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide) for TRPV4 (Klausen et al., 2009b;Thorneloe et al., 2008c;Watanabe et al., 2002c).

V. Endogenous compounds. Several TRP channels are receptors for endogenous compounds. TRPCs respond to diacyl glycerol (DAG)

(Kiselyov and Patterson, 2009). Arachidonic-acid related compounds are involved in gating TRPV1, such as arachidonoyl ethanolamide and 12,15-(*S*)-hydroperoxyeicosatetraenoic acid and in gating TRPV4 5',6'-epoxieicosatrienoic acid (EET) (Zygmunt et al., 1999), and sphingosine, a primary part of sphingolipids which activates TRPM3 (Grimm et al., 2005). However, the mechanisms of these gating behaviors of TRP channels are largly unknown.

VI. pH. A number of TRP channels are also known to be quite sensitive to environmental and intracellular pH. The effect of pH change on TRP channels can be either potentiation of the channels response to other stimuli, direct activation, or suppression of the single-channel current amplitude (Holzer, 2009;Zheng, 2013).

VII. Mechanical force. Several TRP channels are found to be sensitive to mechanical force either at the physiologically relevant level or with pathological significance. Interestingly, in several cases, TRP channels seem not to respond to the mechanical force *per se*, but instead to intracellular signaling molecules generated by mechanical stimuli to the cell. An ion channel can sense mechanical stimuli in several forms: transmembrane differences in osmolarity lead to cell swelling or shrinkage, resulting in changes in membrane tension. Tension can also be generated by pressure directly applied to the cell (Sachs, 2010). An important player in hypotonicity-induced cell swelling is TRPV4 and is described in detail later in this thesis. In addition to TRPV4, extracellular hypotonicity activates TRPM7 (Numata and Okada, 2008) and TRPV2 (Muraki et al., 2003) by increasing the channel's open probability. Membrane tension is also known to affect TRPM4 (Morita et al., 2007), TRPM7 (Numata and Okada, 2008;Wei et al., 2010), TRPC1 (Maroto et al., 2005), and TRPC6 (Inoue et al., 2009;Spassova et al., 2006). An N-terminal splice variant of TRPV1 found in arginine-vasopressin neurons of supraoptic nucleus was also reported to be activated by hypertonicity-induced cell shrinkage (Sharif et al., 2006). It is thought that activation of TRPV1 in organum vasculosum lamina terminalis neurons serves as the osmosensory transduction mechanism underlying thirst responses (Ciura and Bourque, 2006). Mechanical stimulus not only alters TRP channel functions while the force is present, but also it may induce changes in channel protein distribution. This is the case of TRPM7 (Oancea et al., 2006) and TRPV2 (Iwata et al., 2003).

VIII. Phosphatidylinositol-4,5-biphoshate ($PIP_2$). Interactions with $PIP_2$ have been reported for a large number of TRP channels. Positively charged residues at both N- and C- termini have been shown to be putative $PIP_2$ interacting domains (Nilius et al., 2008;Rohacs and Nilius, 2007;Suh and Hille, 2008). Rohacs and colleagues

demonstrate that the basic residues within the TRP domain are likely the putative-binding sites for TRPM8, TRPV5 and TRPM5 (Rohacs et al., 2005) as well as for TRPM6 and TRPM7 (Xie et al., 2011).

IX. Voltage. Large number of TRP channels has been reported to be voltage sensitive, but in case of TRP channels the voltage-dependent activation process exhibits a couple of unique features. First, the voltage sensitivity of most TRP channels is quite low in comparison to the classic voltage-gated $Na^+$, $K^+$, and $Ca^{2+}$ channels (Matta and Ahern, 2007). Second, the S4 segment of most TRP channels, with the exception of TRPPs, lacks the series of charged amino acids seen in the classic voltage-gated channels (Mochizuki et al., 1996). The lack of charged residues in TRP channels provides a simple explanation for their low voltage sensitivity, suggesting that the molecular mechanism underlying the observed voltage dependence in most TRP channels may have a different structural basis. Third, the voltage range in which TRP channels exhibit sensitivity often falls far beyond the physiological range a cell normally experiences. Despite the low voltage sensitivity that is often in a seemingly non-physiological range, response to changes in membrane potential by TRP channels is thought to be quite important for their functions. This is because the voltage response can be significantly affected by other channel stimuli, for example, agonists and temperature (Voets et al., 2004). The coupling between the weakly voltage-dependent activation gating and other gating processes shifts the voltage range at which the channel is responsive, making a voltage change in the physiological range relevant to channel function.

## 1.2. TRPV4

*TRPV4 could be compared to Proteus, a God of the sea in Greek mythology, who has characteristics of flexibility, versatility, mutability and adaptability, emerges in many shapes and can appear in frightening forms.*

— Bernd Nilius & Thomas Voets

TRPV4 was first described in 2000 (Liedtke et al., 2000a;Strotmann et al., 2000e;Wissenbach et al., 2000c) and received several different names before the current nomenclature was accepted: OTRPC4 (osmosensitive transient receptor potential channel), VR-OAC (vanilloid receptor-related osmotically activated channel), VRL-2 (vanilloid receptor-like) and TRP-12. The human TRPV4 gene is found in chromosome 12q23-q24.1 and presents 15 exons. Five splice variants (TRPV4-A-E) have been identified. Variants B, C and E

involve deletions in the N-terminal ankyrin repeat domains (ANK) that result in protein retention in the endoplasmatic reticulum, defective oligomerization and lack of channel activity (Arniges et al., 2006c;Vazquez and Valverde, 2006b). Compared to the vast knowledge obtained about TRPV4 channel regulation, little is known about the control of TRPV4 transcription.

### 1.2.1. Expression

TRPV4 is broadly expressed in heart, arteries, lung, skin, bone, brain, urinary bladder, kidney, intestine, liver, pancreas and female reproductive tract (Everaerts et al., 2010a) . TRPV4 is commonly found in the epithelial cells of the cornea (Mergler et al., 2010;Pan et al., 2008), bronchi (Fernandez-Fernandez et al., 2002;Fernandez-Fernandez et al., 2008;Li and Gundersen, 2008), trachea (Arniges et al., 2004;Lorenzo et al., 2008), intestine (d'Aldebert et al., 2011), urothelium (Everaerts et al., 2010b), larynx (Hamamoto et al., 2008), oviduct (Andrade et al., 2005), bile duct (Gradilone et al., 2007), epidermis (Sokabe et al., 2010), mammary gland (Jung et al., 2009) and endolympathic sac (Kumagami et al., 2009). TRPV4 is also found in the endothelium (Watanabe et al., 2002f), smooth (Earley et al., 2005d;Jia et al., 2004) and skeletal muscle (Kruger et al., 2008), sensorial and brain neurons (Alessandri-Haber et al., 2003;Shibasaki et al., 2007), immune cells (Kim et al., 2010), osteoclasts (Masuyama et al., 2008a), osteoblasts and chondrocytes (Muramatsu et al., 2007a), and pancreatic islets (Casas et al., 2008).

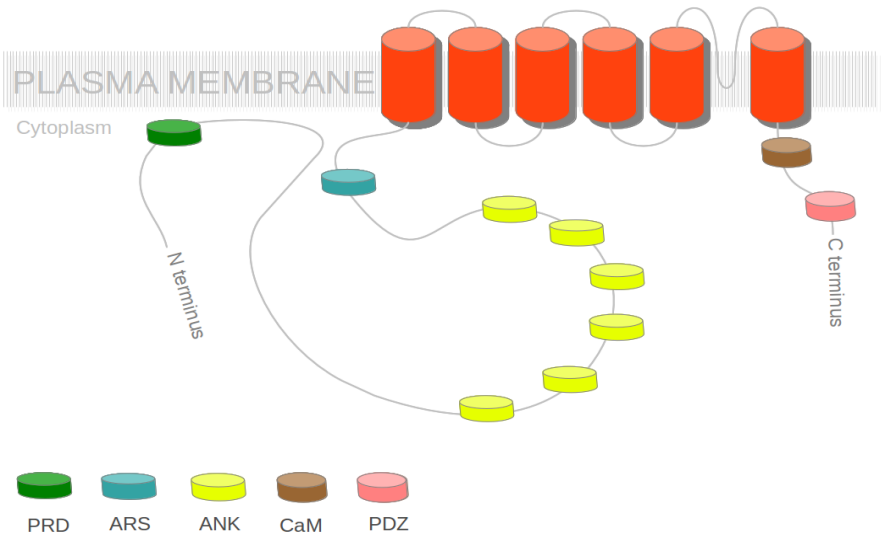


**Figure 4. Schematic rappresentation of TRPV4 channel.** On the plasma membrane (PM) are depicted 6 transmembrane domains (TM). The two cytoplasmic tails are containg several domains depicted in the scheme.

## 1.2.2. Structure

Although the crystal structure of the channel has not yet been fully determined, we do have some insight into its three-dimensional (3D) structure of the TRPV4 channel. A single TRPV4 subunit consists of 871 amino acids (aa) with 6 TM domains presenting both N- and C-terminal cytoplasmic tails (**Fig. 4**). The pore of the channel (aa 663 to 686) is found in the loop between TM5 and TM6. The 12 central amino acids of the pore are identical to those of TRPV1 and 2, the closest relatives of TRPV4 (Voets et al., 2002). Two key amino acids have been shown to regulate TRPV4 permeability: D672 and D682. Neutralization of both D672 and D682 greatly reduces permeability for calcium and rectification, and increases monovalent permeation, suggesting that these two negatively-charged residues are important for binding calcium ions inside the pore (Voets et al., 2002). Glycosylation of N651 is involved in the trafficking of TRPV4 (Xu et al., 2006). Mutation of E797 renders the channel constitutively opened (Watanabe et al., 2003a). The long N-terminal tail (aa 1-465) accounts for more than 50% of total TRPV4 length and contains 6 ANK (Phelps et al., 2010). The N-terminal tail plays a prominent role in channel regulation, a proline-rich domain (PRD) domain (aa 132-144) used for binding of and regulation by kinase C and casein kinase substrate in neurons 3 (PACSIN3) which affects channel activity by hypotonicity (Cuajungco et al., 2006) and an arachidonate- like recognition sequence (ARS-L) (aa 402-408) (Nilius et al., 2003). In addition, complete deletion of PRD (Garcia-Elias et al., 2008), renders the channel insensitive to all stimuli, including the sinthetic activator 4α-PDD, suggesting an important role of the N-tail in the gating of TRPV4. The C-terminal tail presents a calmodulin-binding domain (CaM-BD) (812-831 aa) (Strotmann et al., 2003), an oligomerization domain (Becker et al., 2008) and a PDZ-like domain (Post synaptic density protein, Drosophila disc large tumor suppressor, and Zonula occludens-1 protein) (Garcia-Elias et al., 2008;van de Graaf et al., 2006).The existence of a TRP box in the C-terminal tail has been suggested (Everaerts et al., 2010a) but remains unclear; the classic consideration is that the TRPV subfamily has no TRP box.

## 1.2.3. Related proteins

Several proteins have been reported to bind and/or affect TRPV4 activity or localization. Most of them are listed in the **Table 1**.

**Table 1.** TRPV4 and related proteins

| Protein | Short description | Relation to TRPV4 | Reference |
|---|---|---|---|
| PACSIN 3 | One of the F-BAR (Bin-Amphiphysin-Rvs) domain containing protein required to penetrate and remodel the plasma membrane. Participates in endocytic processes, neurotransmission, cell morphology and motility. | All members of the PACSIN family bind to the PRD of TRPV4 through their SH3 (SRC homology 3 SH3) domain, but only PACSIN3 appears to regulate TRPV4. | (Cuajungco et al., 2006;D'hoedt et al., 2008;Plomann et al., 2009). |
| OS-9 | Ubiquitous protein found in the cytoplasmatic site of the endoplasmatic reticulum (ER), plays a role in selecting substrates for degradation. | OS-9 (osteosarcomas 9 protein) interacts with TRPV4 monomers (aa 438-468 at the N-tail) retaining the monomers in the ER and reducing the amount of channel in the membrane, thereby protecting TRPV4 from polyubiquitination and premature proteosomal degradation. | (Wang et al., 2007) |
| AIP4 | Ubiquitin ligase | Atrophin-1-interacting protein (AIP4) binds TRPV4 (presumably to its N-tail) and promotes its endocytosis. | (Wegierski et al., 2006) |
| G protein coupled angiotensin receptor | Important for the signal transduction of the vasoconstricting stimulus of Angiotensin II. | In the absence of angiotensin, β-arrestin, does not bind TRPV4 and no AIP4-dependent internalization occurs. | (Shukla et al., 2010) |
| β-arrestin | Protein important for regulating signal transduction. | An adaptor between AIP4 and TRPV4. | (Shukla et al., 2010) |
| Caveolin-1 | The scaffolding protein, main component of the caveolae plasma membrane | TRPV4 location to lipid rafts containing caveolin-1 favors nitric oxide (NO) and endothelium-derived hyperpolarizing factor- | (Saliez et al., 2008b) |

| | | | |
|---|---|---|---|
| | | dependent vasodilatation. | |
| Annexin 2A | $Ca^{2+}$-dependent membrane-binding protein linked to vesicular trafficking and endosome formation. | Binds TRPV4 in dorsal root ganglia. The functional relevance of this interaction is presently unknown. | (Huai et al., 2012) |
| PKC (protein kinase C) and PKA (Protein kinase A) | Play important roles in several signal transduction cascades. Inolved in controlling the function of other proteins though the phosphorylation of hydroxyl groups of serine and threonine amino acid residues of these proteins. PKC are activated by signals such as an increase in DAG (diacyl-glycerol) or calcim ions. | Early studies on TRPV4 reported its activation and/or modulation by phorbol esters and PKC as well as by activators of PKA. Subsequent studies identified the residues involved in PKA (S824)- and PKC (S162, T175 and S189)-mediated modulation of TRPV4 | (Alessandri-Haber et al., 2006;Fan et al., 2009) |
| AKAP79 | A Kinase Anchoring Protein 79 | Plays a role in the optimization of TRPV4 phosphorylation by PKA and PKC. | (Fan et al., 2009) |
| WNK(lysine deficient protein kinase) 1and 4 | Cytoplasmic serine-threonine kinases expressed in distal nephron. | They down-regulate TRPV4 membrane location; hypertension-causing WNK mutants are not able to exert this effect. Experiments deleting the TRPV4 N-tail suggested the participation of this region in TRPV4 interaction with WNK kinases | (Fu et al., 2006) |
| CaM | Ubiquitous $Ca^{2+}$ -binding protein | CaM binds to TRPV4 within the second ANK domain (a binding site shared with adenosine triphosphate (ATP)) and at the C-tail (aa 812- 831), a site also used for binding to the IP3R (Inositol triphosphate receptor) | (Fernandes et al., 2008;Garcia-Elias et al., 2008;Phelps et al., 2010;Shin et al., 2012a;Strotmann et al., 2003) |

| | | | |
|---|---|---|---|
| | | and phosphorylation by the SGK-1 (serum glucocorticoid-induced protein kinase-1). Reported effects of CaM on TRPV4 activity range from a positive modulation to an inhibitory effect. | |
| MAP7 | Microtubule-associated protein | Enhances TRPV4 presence at the plasma membrane and, thereby increases TRPV4 activity. The binding of MAP7 to TRPV4 was proposed to be at its C-tail. | (Suzuki et al., 2003a) |
| F-actin | Linear polymer microfilament filamentous actin important for cell motility and contraction of cells during cell division and many other cell functions. | The interaction between TRPV4 and F-actin (filamentous actin) is essential to support channel activation following cell swelling. | (Becker et al., 2009) |
| Tubulin | Globular protein that makes up microtubules. | The interaction between TRPV4 and tubulin competes with actin for binding to the TRPV4 C-tail. | (Goswami et al., 2010a) |
| β-catenin, α-catenin and E-cadherin | The major components of the tight junctions in keratinocytes. | Interact with the proximal TRPV4 N-tail to maintain the integrity of the skin barrier. α-catenin coimmuniprecipitates with TRPV4 in urothelial cells | (Janssen et al., 2011) |
| Non-muscle myosin IIa | Molecular motor involved in cytokinesis, migration and control of cell morphology. | Non muscle myosin IIa interacts with TRPV4 and regulates TRPV4. | (Masuyama et al., 2012) |
| α2 integrin | Collagen/laminin receptor expressed on platelets, endothelial cells, fibroblasts and epithelial cells. | TRPV4 coimmunoprecipitates with α2 integrin in rat dorsal root ganglion (DRG) neurons. | (Alessandri-Haber et al., 2008) |

| | | | |
|---|---|---|---|
| Lyn kinase (Lck/Yes-related novel protein tyrosine kinase) | Member of Src family of protein kinases. | TRPV4 coimmunoprecipitates with Lyn kinase in rat DRG neurons | (Alessandri-Haber et al., 2008) |
| β1 integrin | Membrance receptor involved in cell adhesion, embryogenesis, tissue repair, immune response and metastasis. | TRPV4 participates in mechanical activation of β1 integrin. | (Matthews et al., 2010b) |
| TRPP2 | Polycystic kidney disease 2-like protein, also known as transient receptor polysictic protein. Involved in cell-cell/matrix nteractions. | Heteromeric channels are formed by TRPV4 interacting with TRPP2. | (Kottgen et al., 2008) |
| Aquaporin 4 | Integral membrane protein that conducts water through the cell membrane. Costitutively expressed in basolateral membrane of collecting duct of kidney. Also expressed in astrocytes. | Makes complex with TRPV4 which is essential for regularoty volume decrease in astrocytes. | (Benfenati et al., 2011) |
| Aquaporin 2 | Expressed in the apical cell membranes of the kidneys collecting duct and in intracellular vescicles. Reasorbs water from the urine. Regulated by vasopressin. | Cooperates with TRPV4 in activation of TRPV4 by hypotonicity and regulation of cellular response to osmotic stress | (Galizia et al., 2012) |
| TRPC1 | Transient potential receptor canonical channel 1 which permeates both sodium and calcium ions though the plasma membrane. | TRPC1 interacts physically with TRPV4 to form a complex which promotes flow induced calcium influx in vascular endothelial cells. | (Ma et al., 2010) |
| KCa2.3 | $Ca^{2+}$-activated $K^{+}$ | KCa2.3 and TRPV4 | (Ma et al., |

| | | | |
|---|---|---|---|
| | channel involved in endothelium-dependent hyperpolarization. | physically interact in rat mesenteric artery endothelial cells. | 2013) |
| IP 3 R | Inositol triphosphate receptor is a membrane glycoprotein complex acting as a $Ca^{2+}$ channel activated by inositol triphosphate. | Interacts with and modulates TRPV4 response, particularly under conditions of low level stimulation. | (Fernandes et al., 2008;Garcia-Elias et al., 2008) |

## 1.2.4. Gating

TRPV4 is a non-selective cationic channel with higher permeability to $Ca^{2+}$ and $Mg^{2+}$ than to $Na^{+}$ cations, thereby generates an influx of $Ca^{2+}$ following its activation under normal physiological conditions (Voets et al., 2002). Although TRPV4 also permeates monovalent cations in the absence of divalent ions, it discriminates very poorly between them. The sequence of permeation is $K^{+}>Cs^{+}>Rb^{+}>Na^{+}>Li^{+}$ (Nilius et al., 2001). Single-channel conductance of TRPV4 is larger at positive (80-100 pS) than at negative potentials (30-60 pS) and the current-voltage relationship of TRPV4 whole-cell currents presents outward rectification[1] (with a slight inward rectification at very negative voltages). This process depends on extracellular $Ca^{2+}$ ions that at the same time permeate and block TRPV4 (Everaerts et al., 2010a;Nilius et al., 2004;Voets et al., 2002;Watanabe et al., 2002c). TRPV4 is a polymodally gated TRP channel that can be activated by multifarious different stimuli.

I. TRPV4 responds to osmotic changes in the cell environment by increasing or decreasing its activity in hypotonic and hypertonic solutions, respectively (Liedtke et al., 2000a;Strotmann et al., 2000e;Wissenbach et al., 2000c), thereby contributing to cellular (Arniges et al., 2004;Fernandez-Fernandez et al., 2008) and systemic volume homeostasis (Liedtke and Friedman, 2003b;Mizuno et al., 2003).
II. TRPV4 also responds to mechanical stimuli such as shear stress (Gao et al., 2003a;Kohler et al., 2006) or high viscous loading (Andrade et al., 2005). Its osmotic (Vriens et al., 2004) and mechanical (Andrade et al., 2005;Fernandes et al., 2008) sensitivity depends on phospholipase A2 (PLA2) activation and the subsequent production of

[1] **Outwardly rectifying current** is a current that rectifies so that it passes more easily towards the exterior of a cell.

the arachidonic acid (AA) metabolites, EET, by the cytochrome P450.

III. A recent report has also claimed a direct and potent activation of TRPV4 by AA (Zheng et al., 2013). To date, however, it is not known how 5,6-EET mediates channel opening.

IV. TRPV4 is activated by membrane stretch in excised-patches from oocytes (Loukin et al., 2010), in apparent contradiction with early reports (Strotmann et al., 2000e) and responds to hypotonic stimuli in yeast, which do not contain AA (Loukin et al., 2009).

V. TRPV4 is activated by temperature (24-38$^{0}$C) ($Q_{10}$ between 10 to 20)[2] (Guler et al., 2002;Watanabe et al., 2002f). Early reports (Guler et al., 2002;Watanabe et al., 2002f) showed no channel response to heat in excised patches and until the work described in this thesis, it was unknown if TRPV4 is a real thermosensor. Mutation of the Y556 (Vriens et al., 2004;Vriens et al., 2007) impairs TRPV4 activation by heat.

VI. The non-PKC-activating, synthetic phorbol ester 4α-PDD ($IC_{50}$ ~ 400nM)[3] (Watanabe et al., 2002c) is widely used as a TRPV4 activator. 4α-PDD binds to a pocket formed between TM3 and TM4. Mutations of Y556, L584, W586 and M587 affect 4α-PDD-mediated responses (Klausen et al., 2009b;Vriens et al., 2007).

VII. Another potent activator of TRPV4 has been described, GSK2193874 ($IC_{50}$ ~2-100nM) with therapeutic potential against pulmonary edema (Thorneloe et al., 2012). However, it has been recently reported no activation of TRPV4 by GSK1016790A and TRPV4-independent, 4α-PDD-mediated $Ca^{2+}$ responses in DRG neurons (Alexander et al., 2013).

VIII. TRPV4 is activated by bisandrographolide A (BBA), extracted from Andrographis paniculata, a plant commonly used in Chinese traditional medicine, and mutation of L584, but not of Y556, prevents TRPV4 activation by BBA (Smith et al., 2006b). Apigenin, a plant-derived flavones, activates TRPV4 ($EC_{50}$ ~ 10μM) in heterologous systems as well as in cultured mesenteric artery endothelial cells (Ma et al., 2012). Plant cannabinoids also activate TRPV4 ($IC_{50}$ ~1-6 μM) (De et al., 2012). Two endogenous activators of TRPV4 have been identified. The endocannabinoid anandamide produces a robust TRPV4 activation via its metabolite AA and the formation of 5,6-EET (Watanabe et al., 2003b), and dimethylallyl pyrophosphate (DMAPP), a metabolite of the mevalonate pathway activates TRPV4 ($EC_{50}$ ~ 5μM) in heterologous expression systems,

[2] ***$Q_{10}$* temperature coefficient** is a measure of the rate of change of a biological or chemical system as a consequence of increasing the temperature by 10 °C.

[3] The **half maximal inhibitory concentration ($IC_{50}$)** is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function.

cultured sensory neurons and keratinocytes (Bang et al., 2012a).

IX. Calcium-dependent regulation of TRPV4 is complex. Extracellular $Ca^{2+}$ is responsible for the rectification of the whole-cell TRPV4 currents and intracellular $Ca^{2+}$, depending on its concentration, inhibits or potentiates TRPV4 channel activity. Intracellular $Ca^{2+}$-dependent inactivation ($EC_{50}$ ~ 400 nM) mediates the transient response of TRPV4 to many stimuli (Watanabe et al., 2002c;Watanabe et al., 2003a). Although the exact mechanisms of $Ca^{2+}$-dependent inactivation are not fully characterized, F707 in TM6 is involved in the extracellular $Ca^{2+}$-dependent inactivation (Watanabe et al., 2003a). Positive modulation of TRPV4 by $Ca^{2+}$ via a CaM-dependent mechanism has also been proposed (Strotmann et al., 2003).

X. Citral, a bioactive component of lemongrass commonly used as a taste enhancer and insect repellent, is a transient TRPV4 antagonist (Stotz et al., 2008).

XI. HC-067047 ($EC_{50}$ ~ 50-120 nM) has been shown to be a potent and reversible TRPV4 inhibitor that improved bladder function in animal models of cystitis but inhibited TRPM8 at higher concentrations (Everaerts et al., 2010b).

XII. Butamben (n-butyl-p-aminobenzoic acid), a local anesthetic for topical use known to affect voltage-gated channels, blocks also TRPV4 ($EC_{50}$ ~ 20 μM) and TRPA1 ($EC_{50}$ ~ 70 μM) (Bang et al., 2012a).

## 1.2.5. TRPV4 in physiology and pathology

Considering TRPV4s pollymodality and wide expression, it is not surprising that TRPV4 channel plays multiple roles in physiological processes as well as in pathological ones. In the brain, it is required as an osmosensor (Liedtke et al., 2000a). In motor, spinal ventral root and dorsal root ganglion neurons, it might have a role in nociception (Eid, 2011). Other prominent sites of TRPV4 expression, mainly with sensory functions, mechanosensing, volume regulation and osmosensing, are the bladder urothelium, kidney epithelium, vascular endothelium, inner ear, pulmonary aortic smooth muscle, cardiac fibroblasts, myocytes and skeletal muscle fibres. TRPV4 has been recognized also as a regulator of the skin barrier function of keratinocytes (Adapala et al., 2013;Ho et al., 2012;Shen et al., 2006) and of oxidative metabolism in adipocytes (Ye et al., 2012), and as an important player in the development of chondrocytes (Lewis et al., 2011;Muramatsu et al., 2007a). Our understanding of the physiological role of TRPV4 in cells and organs has increased significantly from the study of TRPV4-deficient mice. These mice are viable and fertile, but have various peculiar phenotypes, such as a larger bladder capacity due to an impaired stretch sensor in the bladder wall (Gevaert et al.,

2007a), thicker bones due to impaired osteoclast differentiation (Masuyama et al., 2008a), reduced water intake due to altered osmosensation (Liedtke, 2008;Liedtke and Friedman, 2003b), compromised vascular endothelial function (Loot et al., 2008;Marrelli et al., 2007;Saliez et al., 2008b;Sonkusare et al., 2012a;Vriens et al., 2005a) and some sensory defects, such as hearing alteration (Tabuchi et al., 2005), impaired pressure sensation (Suzuki et al., 2003c) and compromised pain sensing (Cortright and Szallasi, 2009).

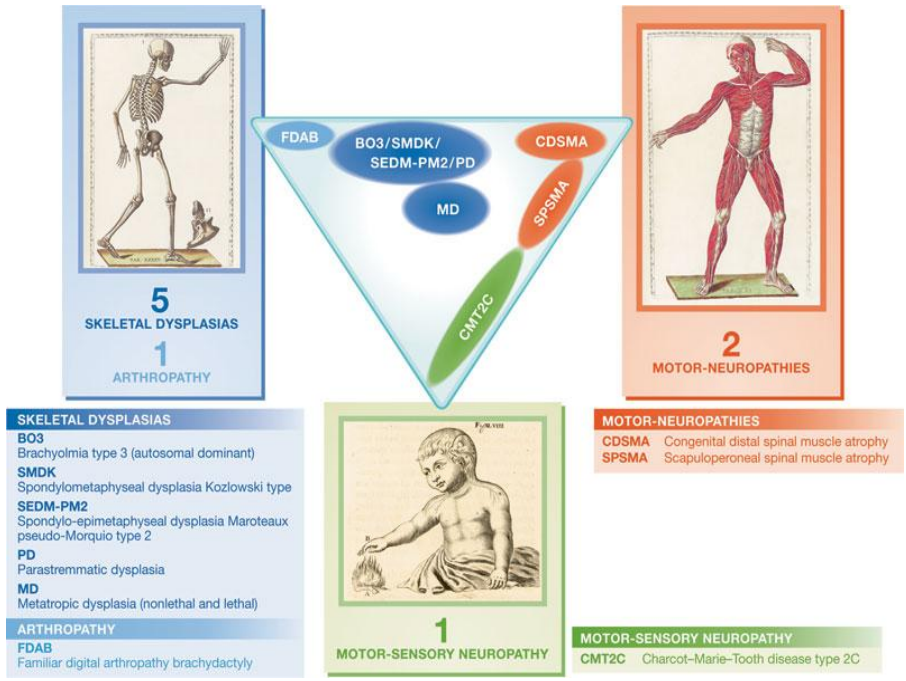


**Figure 5. Spectrum of TRPV4-pathies.** Qualitative description of the occurrence of skeletal, motor and sensory system dysfunctions in clinically distinct TRPV4-dependent diseases. Top: Bartholomeo Eustachi, Tabulae anatomicae, Rome, P. Junchus, 1783. Bottom: René Descartes, De homine figuris, Leiden, P. Leffen and F. Moyard, 1662. Illustrations are from the public domain of the US National Library of Medicine. Image adopted from (Nilius and Voets, 2013).

Considering that ion channels are highly specialized and sophisticated molecular devices, it is not surprising that any change to their structure might significantly modify their activity. Still, given the relatively mild nature of the phenotype of the TRPV4 knockout mice it is surprising that mutations in TRPV4 are the direct cause of several disabling or even lethal human diseases. Mutations in TRPV4 are causing mostly neuropathies, skeletal and neuromuscular diseases (**Fig. 5**) which is very puzzling considering that this channel is expressed almost ubiquitously and that the disease-causing mutations are spread all over the channel and in most of the cases are gain-of-function mutations. It is also intriguing that mutations within a single gene, in

some cases even within the same domain or the very same mutations can cause phenotypically distinct diseases, such as skeletal dysplasias and peripheral neuropathies. A big question mark is also what disposes the TRPV4 gene to such an exceptionally high number of disease-causing mutations compared with all other TRP superfamily members? These are probably the most puzzling open questions in the TRP channelopathies field at the present moment.

## 2. Cell migration

*If you can't fly then run, if you can't run then walk, if you can't walk then crawl, but whatever you do you have to keep moving forward.*

— Martin Luther King Jr.

Cell migration is a broad term that refers to those processes that involve the translation of cells from one location to another. Cells migrate in response to multiple situations they encounter during their lives. Some examples include: the need to feed; morphogenetic events that require the mobilization of precursors to generate new structures/layers/organs, sometimes at distant locations (during embryogenesis, organogenesis and regeneration); or the presence of environment cues that inform the cells of the need for their movement to accomplish a larger goal (*e.g.* wound healing or the immune response) (**Table 2.**). It is then fair to say that cell migration is accompanying us from conception to death.

**Table 2.** Several physiological situations in which cell migration play major roles.

| Process | Description | References |
|---|---|---|
| **Embryogenesis Organogenesis** | After the union of a flagellum-driven sperm and an oocyte into the zygote, this latter turns into a blastocyst that induces the formation of a placenta. During gastrulation, large groups of cells inside the blastocyst migrate to form layers and within these layers cells eventually migrate to specific locations along the developing embryo where they specialize to become components of extremities or internal organs. This cellular movement occurs either collectively (e.g., during | (Aman and Piotrowski, 2010;Cancelas and Williams, 2009;Kulesa et al., 2010;Vasilyev et al., 2009) |

| | | |
|---|---|---|
| | neural crest cell migration and kidney or mammary gland development) or as single cell migration which is typical for hematopoietic stem cells. | |
| **Neuronal migration in the postnatal brain** | Migration of neurons after birth is essential for the establishment, maintenance and modification of neural circuitry. | (Ghashghaei et al., 2007) |
| **Migration of enterocytes along the crypt-villus axis** | In the small intestine, cell migration occurs from the base of the crypt to the villi. This process occurs during the whole life span. | (Yen and Wright, 2006) |
| **Wound healing** | Wounding of the mucosa of the gastrointestinal track; migration of epithelial cells into the denuded area re-establishes the epithelial integrity. Wound healing of the skin requires the proliferation and migration of keratinocytes and fibroblasts. The wound triggers mechanical signals as well as the secretion of chemotactic molecules that attract cells into the wound. | (Blikslager et al., 2007;Dignass, 2001;Martin and Leibovich, 2005) |
| **Immune response** | Before immune cells can do their work reliably, they need to acquire the ability to distinguish between the body's own cells and foreign material. They gain this ability early on in their development as they migrate through the primary lymphoid tissues of the bone marrow and the thymus. While the wound is being closed immune cells migrate into the surrounding tissue to phagocytise bacteria. | (Silva, 2010;Silva, 2011) |
| **Angiogenesis** | The growth of new blood vessels relies on cell migration. | (Perruzzi et al., 2003;Wolf et al., 1998) |
| **Tumor formation** | Invasion of blood vessels that arise from the proliferation and migration of their component endothelium. | |
| **Metastasis** | In metastatic cancer, some tumor cells acquire the ability to migrate out of the initial tumor into the circulation and move to new locations where they form a secondary tumor. | |

The failure of cells to migrate or the migration of cells to inappropriate locations can result in abnormalities or have life-threatening consequences (**Fig. 6**).

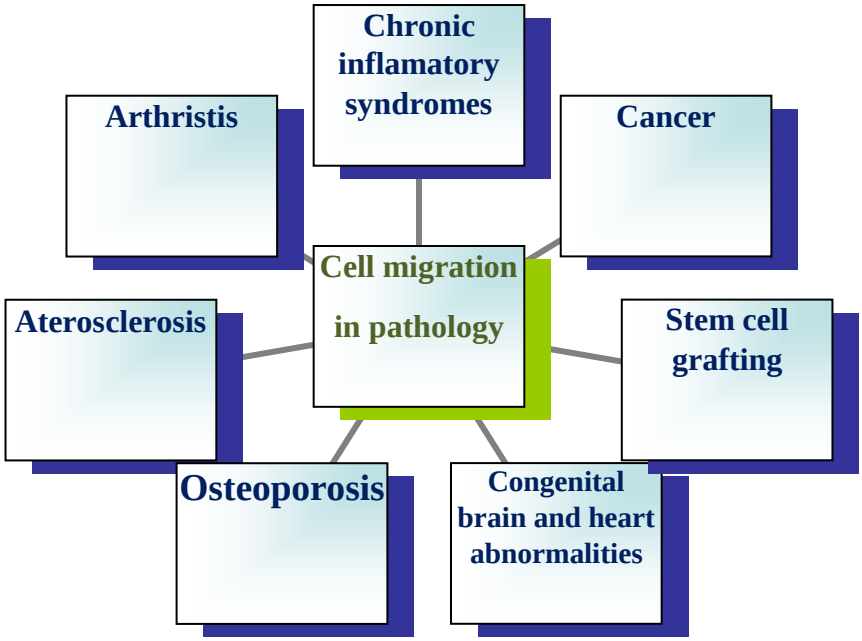


**Figure 6. Cell migration in pathology.** In pathology, production of abnormal migratory signals may induce the migration of the wrong cell type to the wrong place, which may have catastrophic effects on tissue homeostasis and overall health. Some examples include chronic inflammatory syndromes, such as asthma, rheumatoid arthritis, multiple sclerosis, psoriasis and Crohn′s disease which share a migratory component, i.e. the constant infiltration of immune cells into inappropriate places. Once these cells localize to their abnormal target tissues, they become activated and can cause massive damage and progressive deterioration of the tissue. Some therapies against multiple sclerosis and psoriasis are based on preventing immune cells from reaching their target tissues by counteracting receptors implicated in the abnormal homing to the central nervous system (CNS) and skin, respectively. Cell migration also contributes to the process of metastasis formation. Cancer cells migrate as single cells or in small groups to spread from the initial site of tumor growth. They acquire an invasive phenotype characterized by both the loss of cell-cell interactions and increased cell motility. These cells are able to enter the blood or lymph vessels (intravasation) and cross the vessel wall to exit the vasculature (extravasation) in distal organs where they can continue to proliferate forming a second tumor mass. Finally, the migration and proliferation of vascular smooth muscle cells is a key event in progressive vessel thickening leading to atherosclerosis and other vascular diseases. Vascular injury leads to endothelial dysfunction, which, in turn, promotes the expression of inflammatory markers and transendothelial leukocyte migration. Recruitment of leukocytes from the circulation into the vessel intima is a crucial step for the development of fibrous plaques. Cytokines are among the molecules known to upregulate endothelial cell adhesion molecules, recruit leukocytes and induce smooth muscle cell migration and proliferation. In development, aberrant migration may have catastrophic consequences: for example, brain and heart abnormalities and defective lymphopoiesis. In homeostasis, migratory impairment delays wound healing and/or

impair the immune response. Defective migration is a major caveat in cell-based therapy, such as, stem-cell grafting.

There are different modes of cell migration depending on the cell type and the context in which cells migrate. Cells can move as single entities, and the specifics of their motility depend on several factors, *e.g.*, adhesion strength and the type of substratum (including extracellular matrix ligands and other cells), external migratory signals and cues, mechanical pliability, dimensionality, and the organization of the cellular cytoskeleton. The intrinsic properties of the cell interact with the environment to produce a migratory mode or phenotype. For example, nimble, fast-moving and -turning cells, like immune cells, do not have a highly organized cytoskeleton and tend to adhere weakly; their motion is sometimes termed *amoeboid*. Some tumor cells can move by extending membrane blebs, and their actin cytoskeleton is not very organized, either. Fibroblasts and epithelial precursors lie at another extreme. They have elaborate cytoskeletal structures and adhesions, and their motion is generally slow. Some cell types can switch between these depending on their environment. Cells can also move in groups, including chains of cells and sheet-like layers. It is generally convenient to parse migration into a useful set of component processes, which are often regulated by the same effectors regardless of the cell type and the mode of migration. These processes include polarization, protrusion and adhesion, translocation of the cell body and retraction of the rear. These processes are coordinated and integrated by extensive transient, signalling networks.

## 2.1. Polarization

The centrepiece of cell migration is a functionally and morphologically polarized cell. Cell polarization refers to the tendency and capability of migrating cells to have distinct molecular processes at the front and the back (also known as rear or trailing edge). Eukaryotic cells can polarize spontaneously in the absence of pre-established asymmetric cues (Wedlich-Soldner and Li, 2003). Interestingly, migratory polarization shares many common effectors and adaptors with epithelial or embryonic polarity (Iden and Collard, 2008;Li and Gundersen, 2008). To migrate, cells must acquire a spatial asymmetry that enables them to turn intracellularly generated forces into net cell body translocation. Concentration gradients of stimuli are not required to elicit this response. Motile cell protrudes a lamellipodium at the front, where new focal adhesions are formed and traction force is exerted. This action gives rise to progressive stretching of the cell body. The polarity is reinforced and often even arises from environments that provide a directional cue. These directional cues can be chemotactic (induced by chemoattractants or morphogens), haptotactic (caused by varying concentrations of substrate), mechanotactic (breakdown of cell-cell contacts, as in wound healing), electrotactic (induced by electric fields), and durotactic, (due to differences in

pliability), or combinations of any of these (Iglesias and Devreotes, 2008;Swaney et al., 2010). The result is a defined cell front and a rear. The leading edge is usually characterized by intense actin polymerization that generates a protrusive structure, and by adhesion to the substratum. The trailing edge is characterized by stable bundles and by the release and disassembly of adhesions (**Fig.7**). Among the effectors that are asymmetrically recruited and activated by the membrane receptors are heterotrimeric G proteins, which activate, among other enzymes, phospholipase C (PLC) and different isoforms of PKC, inducing the local formation of second messengers DAG and IP3 and protein phosphorylation (Van Haastert and Devreotes, 2004). Among the many molecules important for cell polarity, a key player of polarity and master regulator that participates in every step of polarization is the small GTPase Cdc42 (Heasman and Ridley, 2008;Nobes and Hall, 1995;Osmani et al., 2006) reviewed in (Etienne-Manneville, 2004).

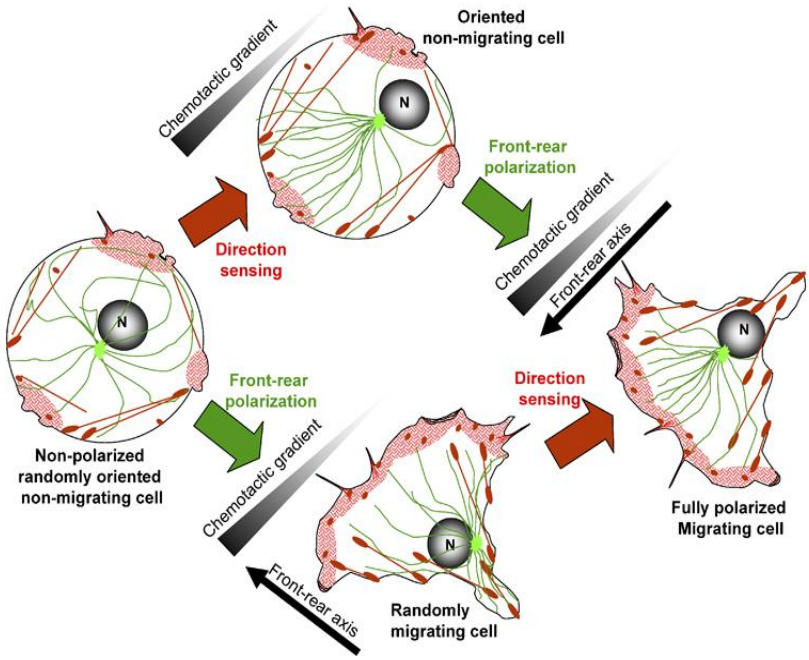


**Figure 7. Polarity in migrating cells.** In response to a chemotactic gradient, both front-rear polarization (green arrow) and direction sensing (red arrow) must occur to transform a non polarized and randomly oriented cell (on the left) into a fully polarized migrating cell (on the right). Front-rear polarization is required for migration *per se*, but is not sufficient for chemotaxis (lower drawing). In this case, cells undergo random migration. Cell orientation following direction sensing is crucial to promote directed (or oriented) migration, but is not sufficient to drive cell migration (upper drawing). Orientation of the nucleus-centrosome axis is a good indicator of cell orientation during directed migration of most cell types including

fibroblasts, astrocytes or epithelial cells. N stands for nucleus. Image taken from (Etienne-Manneville, 2008).

## 2.2. Protrusion

Protrusion involves the extension of cellular membranes. In most cells, a leading protrusion points in the direction of movement and is part of a polarity axis (**Fig.7**). Protrusion usually occurs in response to chemoattractive signals present in the microenvironment that are detected by the cell. However, some cells extend protrusions in a probing, exploratory manner in the absence of directional stimulation (Petrie et al., 2009). The formation of protrusions requires the integration of several cellular processes. The force for membrane deformation is provided by the dynamic polymerization of the actin cytoskeleton, which pushes the membrane forward. The membrane itself expands under tension, a process that requires membrane trafficking and the fusion of membrane-containing vesicles to support the increase in membrane surface. Finally, protrusions must adhere to the substrate. If they do not attach, protrusions are unproductive and tend to move rearward in waves in response to the tension generated in the cell, in a process known as *membrane ruffling*. In brief, protrusion results from active actin polymerization and reorganization, and targeted membrane extension.

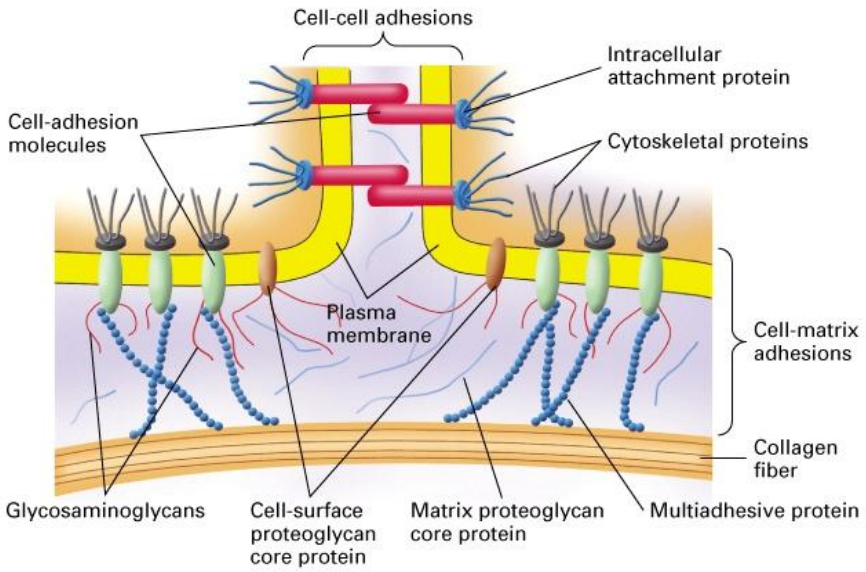


**Figure 8. Cell adhesion scheme**

## 2.3. Adhesion

Cell adhesion (the physical interaction of a cell with another cell or with the extracellular matrix) (**Fig.8**) involves a large multiprotein complexes that provide mechanical coupling as well as a means for cells to sense the chemical and physical properties of their environment and therefore is essential for cell migration and tissue integrity. Cell-cell adhesion maintains epithelial tissues, supports functional contacts between specialized cells, and can facilitate directed migration.

Adhesion plays key roles in protrusion: it not only provides traction for membrane ruffles to become stable, but also nucleates and regulates signalling components that control actin reorganization and membrane endocytosis and delivery. Cell-matrix adhesion is the best-studied form of adhesion that mediates cell migration. These adhesions were first observed in cultured cells (CURTIS, 1964;Heath and Dunn, 1978;Izzard and Lochner, 1976;Lazarides and Burridge, 1975).They are sites of convergence between the actin cytoskeleton and extracellular matrix fibrils. Owing to their highly localized nature, cell-matrix adhesions were initially called focal adhesions. Over the years, several types of cell-matrix adhesion have been described (**Table 3**.).

**Table 3.** Types of cell-adhesion matrix

| Adhesion type | Description | Reference |
|---|---|---|
| **Nascent adhesion** | First observable adhesive structures, emerging within the lamellipodium. Their formation and stability are linked to the dendritic actin. Nascent adhesions are small and highly transient, either maturing or disassembling (turning over), and are therefore not easily observed in every cell type. | (Alexandrova et al., 2008;Choi et al., 2008;Geiger, 1979) |
| **Focal complex** | Adhesions in the early stages of maturation. They were first observed in cells expressing a constitutively active form of Rac. They are larger than nascent adhesions, depend on myosin II for their formation and maintenance, and reside at the boundary of the lamellum and lamellipodium. Like nascent adhesions, they also tend to either disassemble (turnover) or grow and elongate into focal adhesions. The elongation occurs along a template of bundled actin. The presence of nascent adhesions and focal complexes is a marker of highly migratory cells; their fast appearance and turnover correlates directly with high velocities of protrusion and movement. | (Choi et al., 2008;Zaidel-Bar et al., 2003) |

| | | |
|---|---|---|
| **Focal adhesion** | Mature adhesions that evolve slowly over time. Being usually physically linked to large acto-myosin bundles, their appearance correlates inversely with motility. In terms of molecular composition, focal adhesions are similar to nascent adhesions and focal complexes. However, several adhesion proteins appear to change as focal adhesions evolve in response to tension, such as, zyxin and vinculin. | (Hotulainen and Lappalainen, 2006;Zaidel-Bar et al., 2003) |
| **Fibrillar adhesion** | They are extremely mature focal adhesions located towards the center of a cell and are believed to evolve from mature focal adhesions. They are bound specifically to fibronectin *via* $\alpha_5\beta_1$integrin and appear as long streaks or array of dots. Fibrillar adhesions are mainly composed of thin actin cables that are crosslinked by the actin binding protein, tensin, and lack linkage to stress fibres. They can be distinguished from focal adhesions by the high levels of tensin and low levels or absence of phosphotyrosine. They also lack attachment to stress fibres and do not diassemble when the force is relaxed. | (Katz et al., 2000;Pankov et al., 2000;Zaidel-Bar et al., 2003;Zamir et al., 1999;Zamir et al., 2000) |
| **Invadosomes** | Cell-matrix adhesion structures that are different from the other type of cell-matrix adhesion because these structures are capable to degrade components of the cell matrix and therefore playing a role in cell invasion. The term invadosomes comprise both podosomes and invadopodia, which are basically the same structures but in the different context, the first one being involved in physiological invasive contexts such as immune response while the second one in pathological invasive contexts such as metastasis. | (Albiges-Rizo et al., 2009;Block et al., 2008;Destaing et al., 2011) |

## 2.4. Cell-matrix adhesion components

### 2.4.1. Extracellular matrix

Extracellular matrix (ECM) serves as a substrate to which cells attach via cell-matrix adhesions. Cells secrete and remodel the ECM, and the ECM contributes to the assembly of individual cells into tissues, affecting this

process at both receptor and cytoskeletal levels (Hay, 1999). The ECM is highly diverse, ranging from loose connective tissue to densely packed tendons and sheets of basement membrane and, depending on type of matrix, the components of ECMs can vary widely.

### 2.4.2. Integrins

Integrins are receptors that constitute the physical link between the cell and the ECM. Since their recognition as a family in 1987 (DeSimone et al., 1987), they become the best understood cell adhesion receptors. All integrins are non-covalently-linked, heterodimeric molecules containing an α and a β subunit. Both subunits are type I transmembrane proteins, containing large extracellular domains and mostly short cytoplasmic domains (Springer and Wang, 2004). Many combinations of the α and β subunits exit giving rise to multiple heterodimers that interact with specific components of the ECM (Arnaout et al., 2005;Humphries et al., 2006). Integrins modulate the adhesiveness of a cell to the ECM in several ways. One way is by forming large clusters that increase the avidity of binding (Carman and Springer, 2003) or through a conformational change that creates a high-affinity state (Shattil et al., 2010). The conformational changes are induced by the binding of molecules such as talin or kindlin to the intracellular portion of the integrin receptor subunit, which causes the molecule to extend and expose the sites of ligand binding (*affinity modulation*, induced by so-called *inside-out* signalling) (Kim et al., 2003;Ye et al., 2010). Integrins also transmit chemical signalling into the cell that provide information about the cell's environment, a process termed *outside-in* signalling (Lau et al., 2004). Recently was demonstrated that integrin-dependent force transmission to the extracellular matrix by α-actinin triggers adhesion maturation (Roca-Cusachs et al., 2013).

### 2.4.3. Actin-ECM link

Some of the proteins that bind to the cytoplasmic tail of integrins also bind directly or indirectly to actin. These proteins include talin, vinculin, α-actinin and filamin (Bakolitsa et al., 2004;Hemmings et al., 1996;Horwitz et al., 1986;Otey et al., 1990). These linkages define a molecular clutch that shunts retrograde forces from actomyosin contraction or resistance generated by the leading edge membrane to the ECM (**Fig.9**). The efficiency of the clutch, that is, the degree of engagement (binding strength) between the components of the linkage (ECM proteins, integrins, actin-binding adaptors and the actin filaments), regulates the coupling between adhesion and protrusion.

### 2.4.4. Signalling molecules

Since more than 180 proteins participate in a complicated network of interactions within adhesion sites, this complex was given the specific term: the adhesome (Zaidel-Bar et al., 2007;Zaidel-Bar and Geiger, 2010). The discovery that under steady-state conditions, the bulk of tyrosine phosphorylation occurs, at the adhesion sites, it was suggested that adhesion sites may behave as *hubs* for intracellular signalling pathways.

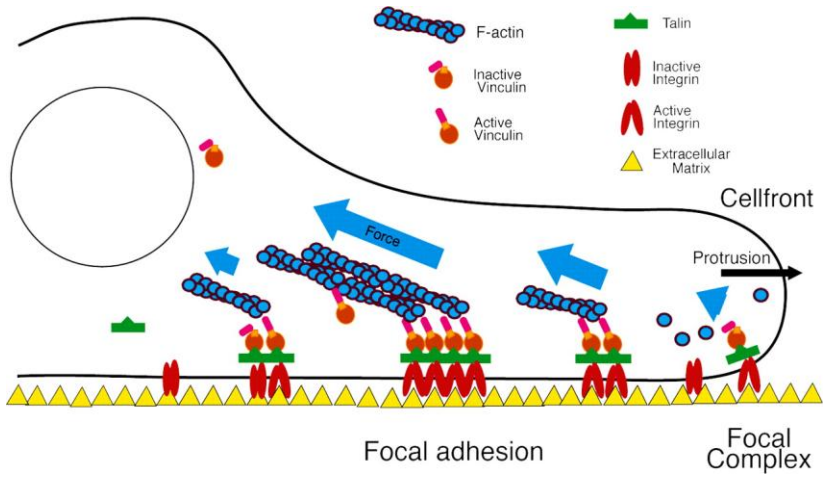


**Figure 9. Rappresentative scheme of Actin-ECM link.** Image take from (Humphries et al., 2007)

## I. FAK

In 2008, one research group identified a nonreceptor tyrosine kinase that associated constitutively with integrin-containing adhesion sites and was directly activated by integrin-mediated adhesion. Owing to its unique subcellular localization, the kinase was named the focal adhesion kinase (FAK) (Parsons et al., 2008). FAK is activated by autophosphorylation and Src family kinases (Parsons, 2003). The FAK-Src module is implicated in the disassembly of adhesion complexes at the cell front (Webb et al., 2004).

## II. Calpain 2

Another important signalling molecule is a $Ca^{2+}$ dependent protease Calpain which plays multiple roles in cell migration: adhesion and spreading; detachment of the rear, integrin- and growth-factor-mediated signalling, membrane protrusion and other (Franco and Huttenlocher, 2005) (**Fig.10**). There are 18 known isoforms of calpains; half of them are almost ubiquitously expressed (Farkas et al., 2003) and the other half are often expressed in many different cell types apart of the tissue in which they are abundantly expressed (Dear et al., 1999;Dear and Boehm, 1999). However the best studied ones are

the isoform 1 and 2. Different isoforms have different localizations within the cell, but interestingly calpain 2 is the only isoform that is localized within the adhesion complexes (Beckerle et al., 1987) and lipid rafts (Morford et al., 2002). The history of calpain originates in 1964, when calcium-dependent proteolytic activities caused by a calcium-activated neutral protease (CANP) were detected in brain, lens of the eye and other tissues (GUROFF, 1964).

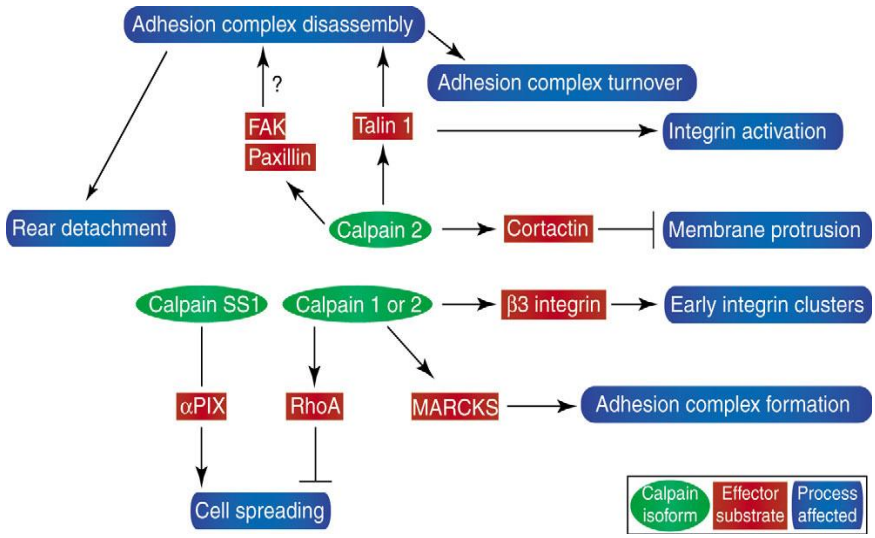


**Figure 10. Calpain in cell migration**. Calpain 2 can cleave adhesion complex proteins such as FAK, paxillin and talin 1, resulting in integrin activation, adhesion complex turnover or detachment of the cell rear. The isoforms required for proteolysis of integrins, RhoA and MARCKS remain to be determined, as do the processes affected by proteolysis of nearly 100 other calpain substrates. Image from (Franco and Huttenlocher, 2005).

When the sequence of this enzyme became known (Ohno et al., 1984), it was given the name *calpain*, to recognize it as a hybrid of two well known proteins at the time, the calcium regulated signalling protein, calmodulin, and the cysteine protease of papaya, papain. Shortly thereafter, the activity was found to be attributable to two main isoforms, μ(*mu*)-calpain and m-calpain (later named also calpain 1 and 2). These two isoforms are expressed ubiquitously in animal tissues and have been widely studied. Both are heterodimers, consisting of a homologous catalytic 80 kDa subunit and the identical regulatory 30 kDa subunit made up of distinct domains. The main parameter in which the two isoforms differ primarily is their calcium requirements in vitro. Their names reflect the fact that they are activated by micro- and nearly millimolar concentrations of $Ca^{2+}$ within the cell, respectively (Glass et al., 2002;Suzuki et al., 1981). Except under pathological conditions associated with cell death, such as neurodegeneration and tissue ischemia, the levels of calcium required to activate calpains *in vitro* (200-1000μM for calpain 2) do not exist within living cells. Actually, both calpains

are activated at physiological $Ca^{2+}$ leves of 100-300nM. This apparent paradox has led researchers towards the idea that other regulatory mechanisms can lower this requirement in vivo. Several different modes of regulation have been identified, although their contributions in vivo have not yet been determined:

a. The binding of phospholipids, in particular phosphatidylinositides decreases the calcium requirement for calpains *in vitro.* In the presence of phospholipids calpain 1 becomes fully active at μM or lower $Ca^{2+}$ concentrations but the presence of phospholipids is not sufficient to activate calpain 2 at the physiological $Ca^{2+}$ range. The *in vivo* relevance of this regulation is uknown (Arthur and Crawford, 1996;Melloni et al., 1996;Saido et al., 1992;Suzuki et al., 1992;Tompa et al., 2004).
b. Regulation by protein-protein interactions (such as Acyl-CoA-binding protein) also changes the calcium requirements of calpain (Melloni et al., 1998;Melloni et al., 2000a;Melloni et al., 2000b;Melloni et al., 2000c;Salamino et al., 1993) but their roles in activation are not clear.
c. Finally, calpains are regulated by their best-known interacting partner, the one and only specific endogenous calpain inhibitor calpastatin (Wendt et al., 2004).

Several explanations were proposed for this paradox. In 2004 Friedrich proposed that the calpain system developed this high requirement for calcium during evolution as a safety device to prevent potentially destructive hyperactivity of calpains, and that it is preferable for calpains to work at much less than half-maximal activity (Friedrich, 2004). It was also proposed that a very small number of calpain molecules, localized to a small region with a high local $Ca^{2+}$ concentration in the vicinity of $Ca^{2+}$ channels, is experiencing much higher $Ca^{2+}$ levels than the cytoplasmic average and is sufficient to fulfill calpain's functions. However, although these explanations got some pieces of evidence by structural studies, this question is not yet ultimately solved. Recent studies have linked calpain deficiencies or it's over-production with a variety of diseases, such as muscular dystrophies, gastropathy, diabetes, Alzheimer's and Parkinson's diseases, atherosclerosis and pulmonary hypertension (Sorimachi and Ono, 2012;Vosler et al., 2008;Yamada et al., 2009). Calpain cleaves target proteins in a restricted manner to modify their properties rather than digest the substrate proteins. In contrast to other proteolytic systems, calpains primarily elicit proteolytic processing, rather than degradation, to modulate or modify substrate activity, specificity, longevity, localization, and structure. Among many calpain target proteins some that are motility related are: Talin 1 (Carragher et al., 1999;Franco et al., 2004a;Franco et al., 2004b); Vinculin (Serrano and Devine, 2004); Paxillin (Carragher et al., 1999;Franco et al., 2004a); β-integrins (Du et al., 1995;Pfaff et al., 1999) and many others.

## III. Scaffold proteins

Not only kinases and proteases are signalling molecules of the adhesome but also molecules that function as scaffolds that recruit additional molecules to adhesions. A key signature of these proteins is their prominent phosphorylation on tyrosine residues (Ballestrem et al., 2006). Some of the most relevant of these phosphorilated scaffold proteins are:

A. Paxillin that through multiple phosphorylated tyrosine and serine/threonine residues (Burridge et al., 1992), creates binding sites that recruit other adhesion proteins and signaling regulators.

B. p130Cas which responds to mechanical force, becoming phosphorylated in response to stretching (Sawada et al., 2006).

C. Zyxin is also mechanoreactive, shuttling between actin/α-actinin and adhesions in response to stretching (Yoshigi et al., 2005)

D. Tensin is present only in very large, mature adhesions (Zaidel-Bar et al., 2003), and plays role in signalling through its interaction with protein phosphatases (Hall et al., 2009).

## 2.4.5. Rho GTPases

Other important players in cell migration processes are small GTPases. Small GTPases (~21 kDa) are a form of signalling G-proteins found in the cytosol which are homologous to the alpha subunit of heterotrimeric G-proteins, but unlike the alpha subunit of G proteins, a small GTPase can function independently to bind to and hydrolyze a guanosine triphosphate (GTP) to form guanosine diphosphate (GDP). Rho family of GTPases is a subfamily of the Ras superfamily of GTPases. In mammals, the Rho family contains 20 members (Boureux et al., 2007). Almost all research involves the three most common members of the Rho family: RhoA, Rac1 and Cdc42. RhoA was the first identified Rho GTPase, isolated serendipitously in 1985 (Madaule and Axel, 1985). RhoA is known to regulate actin cytoskeleton in the formation of stress fibers (Ridley and Hall, 1994). RhoA's functions in the cell are primarily related to cytoskeletal regulation. Microinjection of activated RhoA results in the formation of stress fibers, adhesion plaques and a contractile phenotype (Chrzanowska-Wodnicka and Burridge, 1996;Ridley and Hall, 1994). It was observed in some cell systems that activated RhoA is present at the cell tail and activated Rac1 at the leading edge (Kraynov et al., 2000;Wong et al., 2006). But more recent studies on fibroblast and cancer cells had demonstrated that small GTPases RhoA and Rac1 are not only spatially but also temporally regulated at significantly finer time-scales within regions that undergo cytoskeletal rearrangements including protrusions, retraction, ruffling or macropinocytosis (Spiering and Hodgson, 2011). Rac

was identified as the key mediator in the membrane ruffling response to growth factors, and soon after it was revealed that Rac controls protrusion formation (Ridley et al., 1992). Perhaps the best-studied effect of Rac is the dramatic induction of actin polymerization and the formation of dendritic (branched) actin (Machesky and Hall, 1997). Cdc42 is a critical regulator of polarity and also drives actin polymerization. An early insight into its function revealed that Cdc42 activation promotes filopodia formation (Nobes and Hall, 1995). In the last 10 years the study of small GTPase in cancer has become a trend. There is a vast number of studies suggesting roles that small GTPases play in cancer cell motility (Parri and Chiarugi, 2010;Reymond et al., 2012).

## 2.5. Cell body translocation

Cell body translocation immediately follows protrusion and is independent of actin polymerization (Anderson et al., 1996). In keratocytes, the cell body *rolls* behind the front protrusion. This movement is propelled by a coordinated contraction of the actomyosin cytoskeleton, and thus depends on myosin II (Svitkina et al., 1997). Myosin-mediated contraction and microtubule motors, such as dynein, also control the translocation of the nucleus (Gomes et al., 2005;Levy and Holzbaur, 2008).

## 2.6. Rear part retraction

For a cell to migrate, translocation of the cell body must be followed by retraction of the rear of the cell. Although for cells that migrate in the absence of any guidance cue the formation of the rear end may come first (Cramer, 2010). Rear retraction requires the coordinated contraction of the actin cytoskeleton and disassembly of the adhesions at the trailing edge. Several mechanisms converge to promote adhesion disassembly: actomyosin contraction, microtubule-induced adhesion relaxation, endocytosis of adhesion receptors, and proteolytic cleavage of focal adhesion proteins (Ezratty et al., 2009;Franco et al., 2004b;Kaverina et al., 1999;Worthylake et al., 2001). The actomyosin cytoskeleton (mediated predominantly by myosin II) promotes retraction of the rear of migrating cells (Chrzanowska-Wodnicka and Burridge, 1996;Crowley and Horwitz, 1995). The myosin IIA isoform acts downstream of the RhoA-ROCK axis and mediates retraction and adhesion disassembly in a variety of cell lines (Chrzanowska-Wodnicka and Burridge, 1996;Eddy et al., 2000;Even-Ram et al., 2007;Smith et al., 2003;Vicente-Manzanares et al., 2002;Vicente-Manzanares et al., 2007;Worthylake et al., 2001). Microtubule targeting stimulates focal adhesion dissociation (Efimov and Kaverina, 2009;Kaverina et al., 1999). Dynamin and FAK are also required for adhesion disassembly (Ezratty et al., 2005), but whether they mediate endocytosis only or deliver additional relaxation signal(s) is still unknown. Emerging evidence points to the

endocytosis and recycling of integrins and other receptors, such as GPCRs, as a key mechanism in regulating adhesion turnover (Gomes et al., 2005). More specifically, clathrin-mediated endocytosis has been shown to mediate disassembly of a specific subset of adhesions, near the nucleus, by promoting internalization and recycling of β integrins downstream of microtubules, FAK and dynamin (Ezratty et al., 2009), and is likely involved in adhesion disassembly during cell migration. The, previously mentioned calpain, is involved in retraction of the trailing edge of migrating cells (Huttenlocher et al., 1997). Several proteins that are present in cell-matrix adhesions have been identified as targets of these calcium-dependent enzymes. Cleavage of two of these, Talin and FAK, is critical for retraction of the rear of migrating cells (Chan et al., 2010;Franco et al., 2004b). Transient increases of $[Ca^{2+}]_i$ also play a role in rear part retraction (**Fig.11**). These $Ca^{2+}$ elevations coincide with phases of increased membrane tension. Membrane tension rises because the rear edge of the cell is stuck to the substratum while the lamellipodium keeps on protruding and thereby stretches the cell membrane. The increase in membrane tension activates a $Ca^{2+}$-permeable, stretch-activated cation channel(s). The resulting transient rise of $[Ca^{2+}]_i$ then triggers retraction of the rear edge of a migrating cell (Eddy et al., 2000;Lee et al., 1999). The stretch-activated cation channel thereby indirectly controls the cytoskeleton of a migrating cell. Molecular identity of the stretch activated channel until the present time was not elucidated and is a part of the work that this thesis is presenting.

## 2.7. Cell directionality

Despite all the research on cell motility, today it is still challenging to understand how the motility machinery is coupled to a steering mechanism that integrates environmental cues with polarized signalling, adhesion and cytoskeletal remodelling to promote directionally persistent migration. Conceptually, directional cell migration has two sources: intrinsic cell directionality and external regulation. Cells achieve directionally persistent migration by forming and stabilizing actin-rich protrusions or lamellipodia that maintain the orientation of the leading edge (Andrew and Insall, 2007;Arrieumerlou and Meyer, 2005). Multiple factors can influence this process, such as topography of the ECM, cell polarity and cell adhesion. Stable protrusions guide migration. New protrusions are characteristically generated from the pre-existing leading edge, rather than in different directions around the cell (Arrieumerlou and Meyer, 2005). Cell adhesion can guide the directionality of migration; for example, adhesion to the underlying substratum stabilizes lamellipodial protrusions during chemotaxis and chemokinesis (Bailly et al., 1998;Harms et al., 2005;Lo et al., 2000). The topography of the ECM can also regulate cell motility through physical cues that geometrically constrain adhesion sites to guide directional migration (Lo et al., 2000). Cells contain polarity signalling machinery that can influence

directional cell motility. This polarization influences the formation of the leading and trailing cell edges. The stability of the front–rear axis correlates with the extent of persistent directional cell movement (Iden and Collard, 2008). The intracellular signalling pathways at the leading edge that regulate actin cytoskeleton remodelling or adhesion formation to create or stabilize local protrusions are therefore contributing to directional migration (Bourne and Weiner, 2002;Ridley et al., 2003).

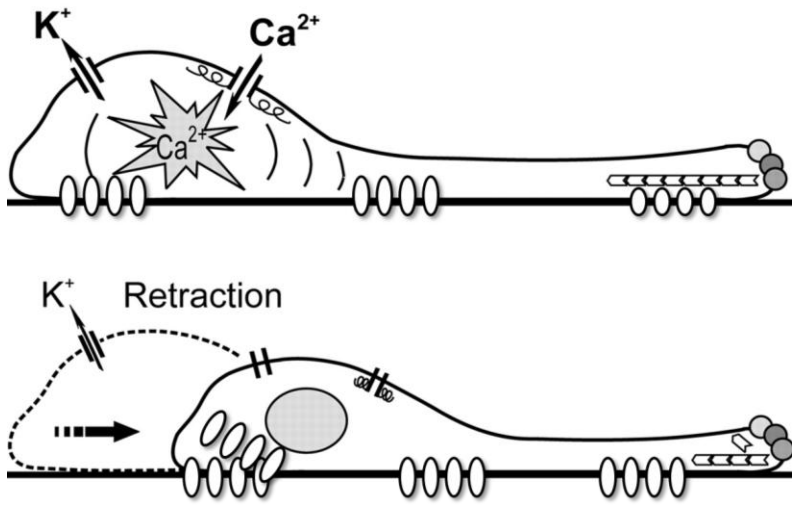


**Image 11. Rear part retraction** as example of importance of spatio-temporal fine tuning of $[Ca^{2+}]_i$ for the coordination of a concerted action of different effector proteins a rise of $[Ca^{2+}]_i$ triggers the phosphorylation of the myosin light chain (MLC) followed by the contraction of the actomyosin network (Yang and Huang, 2005), induces the calpain-mediated release of cell-matrix contacts (Franco and Huttenlocher, 2005), activates the calcium-regulated focal adhesion protein proline-rich tyrosine kinase-2 (Pyk2) as well as the effector proteins paxillin and p130(Cas) (Agle et al., 2010), and activates KCa3.1 channels thereby causing a local shrinkage of the rear part of migrating cells (Schneider et al., 2000). This view is supported by the observation that $[Ca^{2+}]_i$ transients conducted by stretch-activated $Ca^{2+}$ channels occur shortly before the retraction of the rear part of migrating neutrophils or keratinocytes (Eddy et al., 2000;Lee et al., 1999). Finally, a rise of $[Ca^{2+}]_i$ also triggers the release of vesicular and lysosomal contents (e.g., neurotransmitters, insulin) by fusion of the vesicular membrane with the plasma membrane (Jahn et al., 2003).

Basically, it is clear that if the protrusions and subsequent new adhesions formed by a polarized cell are themselves directionally persistent, the cell will move in a directionally persistent manner. However, understanding how these factors are integrated to regulate cell directionality remains incomplete. Some of the best studied molecular players that affect cell directionality are:

i. Rho small GTPases, RhoA, Rac1 and Cdc42 operate at each step of the cell motility cycle to promote directional migration by regulating leading edge formation (Etienne-Manneville, 2004).
ii. The Par (partitioning defective) complex connects Rho GTPase signalling, centrosome reorientation, microtubule stabilization and membrane trafficking to the regulation of directional persistence during intrinsic cell migration (Etienne-Manneville, 2008).
iii. Wnt[4] (Wingless-related integration site) signalling classically contributes to polarization of tissues in developing embryos and, more recently, has been shown to contribute to cell polarity and directional motility (Nishita et al., 2006;Nomachi et al., 2008;Schlessinger et al., 2007).
iv. Integrin trafficking might contribute to directional migration by facilitating the formation of new adhesions at the leading edge (Caswell and Norman, 2006).
v. The transmembrane proteoglycan syndecan 4 can sense ECM topography to control directional migration in 3D environments. Syndecan 4 cooperates with α5β1 integrin to bind fibronectin, form focal adhesions and support cell migration (Mostafavi-Pour et al., 2003;Saoncella et al., 1999).
vi. Recently was discovered a new player in cell directionality named Arpin (Arp2/3 inhibition protein) recruited and activated by Rac signalling at the lamellipodia tip. Arpin has an inhibitory effect on cell persistent directionality (Dang et al., 2013).
vii. Ions and ion channels (see the section *Ion channels in cell directionality*).

[4] The name Wnt was chosen because it is a combination, or portmanteau, of *int* and and *Wg* and stands for Wingless-related integration site.

# II.OBJECTIVES

## General objective

General objective of this thesis is to study the regulation of TRPV4 channel function by the cytosolic N-terminal tail, with special interest in gating of the channel by physiological stimuli and the role of TRPV4 in cell migration.

## Specific objectives

1. Characterization of the putative Phosphoinositide-interacting site in the TRPV4 N-terminal tail.

2. Evaluation of the $PIP_2$ effect on the TRPV4 channel gating.

3. Study of modulation of TRPV4 by PACSIN3.

4. Evaluation of the structural rearrangements induced by TRPV4 binding to $PIP_{2.}$

5. Study of the impact of non-conducting TRPV4 mutants on cell shape and migratory phenotype.

7. Study of the effect of TRPV4 on focal adhesions dynamic.

# III.RESULTS

CHAPTER 1

# Phosphatidylinositol-4,5-biphosphate-dependent rearrangement of TRPV4 cytosolic tails enables channel activation by physiological stimuli.

Garcia-Elias A *, **Mrkonjic S ***, Pardo-Pastor C, Inada H, Hellmich UA, Rubio-Moscardó F, Plata C, Gaudet R, Vicente R, Valverde MA.

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* S.M. and A.G.E. equally contributed to this work

Garcia-Elias A, Mrkonjic S, Pardo-Pastor C, Inada H, Hellmich UA, Rubio-Moscardó F et al. Phosphatidylinositol-4,5-biphosphate-dependent rearrangement of TRPV4 cytosolic tails enables channel activation by physiological stimuli. Proc Natl Acad Sci U S A. 2013 Jun 4; 110(23): 9553-8.

CHAPTER 2

# TRPV4 participates in the establishment of trailing adhesions and directional persistence of migrating cells

**Sanela Mrkonjić** *, Anna Garcia-Elias *, Carlos Pardo, Elsa Bazellières, Xavier Trepat, Joris Vriens, Debapriya Ghosh, Thomas Voets, Rubén Vicente and Miguel A. Valverde

* S.M. and A.G.E. equally contributed to this work

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**TRPV4 participates in the establishment of trailing adhesions and directional persistence of migrating cells**

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**Abstract**

Calcium signaling participates in different cellular processes leading to cell migration. TRPV4, a non-selective cation channel that responds to mechano-osmotic stimulation and heat, is also involved in cell migration. However, the mechanistic involvement of TRPV4 in cell migration is currently unknown. We now report that expression of the mutant channel TRPV4-$^{121}$AAWAA (lacking the phosphoinositide-binding site $^{121}$KRWRK$^{125}$ and the response to physiological stimuli) altered HEK293 cell migration, first increasing followed by decreasing persistence directionality, and induced multiple long cellular protrusions. TRPV4-WT overexpressing cells showed almost complete loss of directionality with frequent turns, no progression and absence of long protrusions. Traction microscopy revealed higher tractions forces in the tail of TRPV4-$^{121}$AAWAA than in control cells. These results are consistent with a defective and augmented tail retraction in TRPV4-$^{121}$AAWAA and TRPV4-WT expressing cells, respectively. The activity of calpain, a protease implicated in focal adhesion (FA) disassembly, was decreased in TRPV4-$^{121}$AAWAA compared to TRPV4-WT expressing cells. Consistently, larger focal adhesions were seen in TRPV4-$^{121}$AAWAA compared to TRPV4-WT expressing HEK293 cells, a result that was also reproduced in T47D and U87 cells. The migratory phenotype obtained in HEK293 cells overexpressing TRPV4-$^{121}$AAWAA was mimicked by knocking-down TRPC1, a cationic channel that participates in cell migration. Together, our results point to the participation of TRPV4 in the dynamics of

trailing adhesions, a function that may require the interplay of TRPV4 with other cation channels present at the FA sites.

KEYWORDS

TRPV4, migration, focal adhesion, calcium, calpain

## Introduction

Cell migration is a cyclic process involving initial protrusion of the leading edge, formation of adhesive sites, contraction of the cell body, and release of adhesive sites at the cell rear [53]. This cell capability is of vital importance at different stages of our development, interaction with the environment or under pathological conditions [48]. The $Ca^{2+}$-dependency of the cell migratory activity is well established [50,78] and affects different aspects of cell migration: $Ca^{2+}$-dependent motor proteins [8], proteins involved in the establishment and disassembly of cell contacts with the extracellular matrix (e.g., calpains [26], integrin recycling [39]) and other signaling pathways (e.g., $Ca^{2+}$/calmodulin-dependent kinase [20]). Migrating cells present a front-to-rear rising gradient of intracellular [$Ca^{2+}$] [11,78] that can be locally modified to allow particular cell functions during migration such as steering in response to chemotactic cues [77]. Therefore, many ion channels either generating or modulating intracellular calcium signals are implicated in cell migration [56]. Transient receptor potential (TRP) channels [70] are among the channels that participate in the generation of such $Ca^{2+}$ signals. Members of the canonical/classical subfamily, TRPC1 [9,22,23,41,51,52,83], TRPC5 [12,80] and TRPC6 [13,18]; the melastatin subfamily, TRPM4 [6,57,76], TRPM7 [1,62,77] and TRPM8 [29,79,82] and the vanilloid subfamily, TRPV1 [30,72], TRPV2 [47] and TRPV4 (see below) have been reported to participate in cell migration.

The TRPV4 cationic channel is widely distributed (for a review see [21]) and participates in the transduction of both physical (osmotic [4,40,68], mechanical [3,27,37] and heat [28,32,75]) and chemical (endogenous [5,74], plant-derived [42,59] and synthetic ligands [67,73]) stimuli. In recent years, somewhat contradictory reports not fully resolved yet on the role of TRPV4 in cell migration have appeared. TRPV4 activation reduces migration of neuroendocrine cells [85] but mediates migration of pulmonary artery smooth muscle [44] and arachidonic acid-induced migration of endothelial cells [25]. In the present study we tested the impact of wild-type and non-functional TRPV4 mutants on cell shape and migration and further explored the role of TRPV4 in the dynamics of focal adhesions and rear retraction of migrating cells.

## Material and Methods

*Cell culture and transfection*

HEK-293, T49D breast cancer and U-87 glioma cells were cultured in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% streptomycin/ penicillin and grown in an incubator at 37ºC in an atmosphere of 95% air 5% $CO_2$. Cells were transiently transfected as previously described [24]. Small interference TRPC1 RNA (siTRPC1) was obtained from Life Technologies and transfected using Lipofectamine 2000 in optiMEM medium (Life Technologies). When necessary, coverslips were coated with 0.25

mg/ml of fibronectin (BD Bioscience). All experiments were performed 24-48 hours after transfection.

*Laser confocal immunodetection*

Cells were fixed with 4% paraformaldehyde, membranes were permeabilized with 0,1% triton (in PBS) and non-specific interactions were blocked with 2% FBS, 1% albumin and 0,05% triton in PBS. Cells were incubated at room temperature with a rabbit anti-TRPV4 antibody (1/1000) and a mouse anti-paxillin (BD Bioscience; 1/200) during two hours and then washed with PBS plus 0,05% triton. For immunodetection we used a goat anti-rabbit Alexa 555 and a mouse anti-mouse Alexa 488 antibodies (1/2000 diluted in the same solution used with the primary antibodies) for 1 hour at room temperature. Images were taken at RT with an inverted confocal microscope (SP2; Leica) using an HCX Pl APO 63 × 1.32 NA oil Ph3 confocal scanning objective and LCS confocal software (Leica). Original images were not further processed except for adjustments of brightness, contrast, and color balance. Identification and measurement of cell protrusions (> 5 μm) was carried out using the National Institutes of Health Image J software (http://rsb.info.nih.gov/ij/).

*Total Internal Reflection Fluorescence (TIRF)*

Expression of YFP-tagged TRPV4 proteins within a thin layer including plasma membrane and adjacent cytosol was evaluated by imaging cells using a 100x objective-based TIRF microscope (Zeiss Axio Observer.Z1 inverted microscope). TIFR was performed at 66º angle, recording about 10 nm of the evanescent field.

*Analysis of cell migration*

Time-lapse microscopy was used to monitor cell migration [23]. Cells were seeded on 24 well plates and imaged (30 min intervals) using a Zeiss Cell Observer microscope with a 10x objective over a 24h time period. Cell tracking was obtained using the "Manual Tracking" plugin (Fabrice Cordelières, Institut Curie, Orsay, France) and analysis of cell migration was carried out using the "Chemotaxis and Migration Tool" ImageJ plugin. All cell trajectories are transformed by setting each starting point to $(x_{start}, y_{start}) = (0,0)$. The euclidean distance (ED) for each cell was calculated as the length of the straight line between the cell start and end point. The accumulated distance (AD) for each cell is the result of the sum of all incremental movements measured in between all single images. Directional index (the cell's tendency to travel in a straight line) was calculated by dividing the euclidean distance by the accumulated distance for each cell [33] (see also Fig. 4c). A directional index of D=1 indicates a straight-line migration from start to endpoint.

*Traction force microscopy*

Traction force measurements were performed as previously reported [64,69]. Briefly, 12kPa polycrylamide gels contained 19% acrylamide, 8% bis acrylamide, 0.5% ammonium persulphate, 0.05% tetramethylethylenediamine, 0.64% of 200-nm-diameter red fluorescent carboxylate-modified beads and 2mg/ml$^{-1}$ NH-acrylate. After polymerization, gels were incubated with 0.1mg/mL of collagen I overnight. HEK293 cells were seeded 4 hours after transfection on the elastic substrate and allowed them to adhere and proliferate. Time lapse imaging was carried out using an automated inverted microscope (Nikon Eclipse Ti, objective 20x)) equipped with temperature, $CO_2$ and humidity control. Cells were monitored every 3 minutes. As cells migrate, they exert traction forces on the substrate resulting in the deformation of the polyacrylamide gels. Gel deformations between any experimental time point and a reference image obtained after seeding were computed by applying a home-made particle imaging velocimetry algorithm to images of the fluorescent beads embedded in the gels. From these deformations, traction forces are computed using Fourier transform traction microscopy with a finite gel thickness [69].

*Calpain activity*

The calpain fluorogenic substrate CMAC-tBOC (Life Technologies) was used to analyze the enzyme activity as previously described [54]. Briefly, cells were seeded in 24-well plates and transiently transfected with TRPV4

expressing pcDNA3 plasmids or siRNAs. 24h after transfection, cells were incubated with 30 µM CMAC-tBOC during 30 minutes at 37°C. Wells were washed twice with PBS and fluorescence was measured in a FLUOstar OPTIMA plate reader (BMG Labtech) using a combination of excitation (340±10 nm) and emission (470±10 nm) filters. Following calpain mediated cleavage of CMAC-tBOC the fluorescent signal increases, thereby correlating with increases in the enzyme activity. Transfected cells but not incubated with CMAC-tBOC were used to substract the background fluorescent signal.

*Statistics*

Data are expressed as the mean ± SEM. ANOVA or Student's *t* test was performed with Sigma Plot (Systat Software, Inc.) and SPSS (SPSS, Inc.) software to determine statistical significance. Bonferroni's test was used for post hoc comparison of means. $P < 0.05$ was considered significant.

**Results**

Expression of TRPV4 modifies cell shape and migration pattern.

We have recently reported that neutralization of the positive charges within the TRPV4 phosphoinositide-binding site (PIBS, $^{121}$KRWRK$^{125}$) prevents the N-tail interaction with plasma membrane $PIP_{2,}$ resulting in defective channel activation by physiological stimuli such as hypotonicity and warm temperature [28]. We now report that expression of the mutant TRPV4 channel (TRPV4-$^{121}$AAWAA) also induced a dramatic change in cell

morphology. Figure 1a shows HEK293 cells transiently transfected with TRPV4-WT or TRPV4-$^{121}$AAWAA and stained using an anti-TRPV4 antibody. The cell expressing TRPV4-$^{121}$AAWAA presented multiple long protrusions that were not present in either GFP (not shown) or TRPV4-WT expressing cells. Quantification of the mean number of cells presenting long protrusions and the length of the longest protrusion is shown in Fig. 1b-c. Analysis of TRPV4-WT and TRPV4-$^{121}$AAWAA expression at the plasma membrane by Total Internal Reflection Fluorescence (TIRF) confirmed the change in cell shape induced by overexpression of the mutant channel (Fig. 2a) and also revealed an increase in TRPV4-$^{121}$AAWAA presence at the plasma membrane (Fig. 2b).

The shape phenotype with multiple and long protrusions seen in TRPV4-$^{121}$AAWAA expressing cells may represent just a static, snapshot, view of the alteration of the more dynamic process that is cell migration. Migrating cells require a precise and coordinated pattern of $Ca^{2+}$ signals [78]. Considering that TRPV4-$^{121}$AAWAA channel shows defective activation by physiological stimuli, we reasoned that the cell shape phenotype observed may be related to a deficient $Ca^{2+}$ influx during cell migration. In order to check this possibility we first analyzed the impact of removing extracellular $Ca^{2+}$ on cell morphology. Figure 3 shows that in the presence of low extracellular [$Ca^{2+}$] (40 μM) the number of GFP and TRPV4-expressing cells with long protrusions (Fig. 3a) and the length of the protrusions (Fig. 3b) increased considerably (compared to Fig. 1b-c) while the phenotype of

TRPV4-$^{121}$AAWAA expressing cells (% of cell presenting protrusions and the length of protrusions) remained unaltered, consistent with an already impaired $Ca^{2+}$ influx in cells expressing the mutant channel.

To further investigate the function of TRPV4 in cell migration we carried out wound healing assays. A series of images taken at 30 min intervals (Fig. 4a) show that the TRPV4-$^{121}$AAWAA expressing cell developed a growing tail (marked by a circle) that remained attached to the starting point for almost 3h but finally detached (image taken at 210 min) while the control GFP-expressing cell moved steadily without generating long protrusions and the TRPV4-WT did not progress significantly. A clearer view of the differences in migratory patterns among GFP, TRPV4-WT and TRPV4-$^{121}$AAWAA expressing cells is shown in Fig. 4b. Individual representative trajectories show that control, GFP-transfected, cell (top trace) presented a tortuous trajectory with a clear directionality throughout the whole experiment. TRPV4-WT-expressing cell (middle trace) showed frequent turns and no progression throughout the whole duration of the experiment. TRPV4-$^{121}$AAWAA-expressing cell (bottom trace) showed a straighter trajectory at the initial stages of the experiment (while it remained anchored to the starting position through the long tail) that changed at later stages, becoming erratic, with frequent turns in the direction of the movement and unable to progress (similar to the behavior seen with TRPV4-WT expressing cells).

The directionality index of migration (Fig. 4d; with D=0 reflecting no directionality and D=1 reflecting maximum directionality) was quantified running ImageJ software on 24h time lapse videos. Calculations were obtained for two separate time periods (0-8h and 16-24h) corresponding to the two well differentiated migratory patterns of TRPV4-$^{121}$AAWAA expressing cells. Cells transfected with GFP showed an intermediate directionality (D~0.5) that did not change between the beginning (0-8h) and end (16-24h) of the experiment. TRPV4-WT transfected cells reduced their directionality both early and late times of the experiment while TRPV4-$^{121}$AAWAA showed opposing behaviors at the two time periods analyzed, almost maximal directionality at the beginning that was greatly reduced at the final stages of the migration experiments. Shape and migration patterns similar to that of TRPV4-$^{121}$AAWAA expressing cells were obtained in two other conditions that impaired normal TRPV4 channel activation: 1) coexpression of TRPV4-WT and the negative regulator PACSIN3 [16,17,28] (Suplem Fig. 1a, top images), a protein that contain a Bin-Amphiphysin-Rvs (BAR) domain required to penetrate and remodel the plasma membrane [71], and 2) overexpression of TRPV4-Δ100-130 (Suplem Fig. 1b), a deletion of 30 amino acids that include the PIBS [28]. Changes in persistence directionality in these two conditions (Suplem Fig. 1c-d) were undistinguishable from those obtained in cells overexpressing TRPV4-$^{121}$AAWAA. However, overexpressing TRPV4-WT with a PACSIN3 lacking the F-BAR domain (PACSIN3-ΔF-BAR), which does not affect TRPV4 channel activity [28],

reproduced the migratory pattern of cells overexpressing TRPV4-WT alone (Suplem Fig. 1c-d).

Together these experiments show that cells overexpressing non-functional TRPV4 channels presented an altered migratory pattern characterized first by an increase followed by a reduction in directionality. These results are consistent with a defective tail retraction at early stages followed by the coexistence of multiple cell projections in an attempt to redirect the movement of the cell. On the other hand, TRPV4-WT expressing cells also present alterations in the migratory pattern but in this case due to the almost complete loss of directionality with frequent turns and no progression.

Cell migration is tightly regulated at the level of focal adhesion (FA) turnover [49]. Thus, we evaluated whether the effect of mutant TRPV4 channels on tail retraction and cell migration may be related to changes in the activity of calpains, which are $Ca^{2+}$-dependent intracellular proteases implicated in spreading and locomotion of adherent cells by disassembling FA [26]. To measure calpain activity *in situ*, we used a fluorescent technique based on the detection of the cleavage of Boc-Leu-Met-CMAC peptide, a membrane-permeant fluorogenic substrate of calpain. We observed that the activity of calpain was decreased in TRPV4-$^{121}$AAWAA expressing cells (Fig. 5) compared to TRPV4-WT expressing cells. To confirm that the difference in calpain activity between TRPV4-WT and TRPV4-$^{121}$AAWAA expressing cells was due to a reduced intracellular [$Ca^{2+}$] in the latter, we

measured calpain activity in the presence of the $Ca^{2+}$ ionophore ionomycin, which supposedly induces equal increases in intracellular [$Ca^{2+}$] in both conditions. In the presence of ionomycin, calpain activity was increased in both TRPV4-WT and TRPV4-$^{121}$AAWAA expressing cells, without significant differences between them (Fig. 5).

*Knocking down TRPC1 mimics the TRPV4-$^{121}$AAWAA-induced migratory phenotype*

So far, we have shown that TRPV4-WT reduces persistence directionality, a phenotype that can be explained by the increased $Ca^{2+}$ entry induced by TRPV4 overexpression. On the other hand, expression of TRPV4-$^{121}$AAWAA induced a biphasic effect on persistence directionality, first increased and later on decreased, which may be interpreted as the result of a defective $Ca^{2+}$ signaling and calpain activity during cell migration. Therefore, we hypothesized that overexpression of TRPV4-$^{121}$AAWAA may somehow interfere with already existing $Ca^{2+}$ influx pathways that participate in HEK293 cell migration. Among such pathways we focused on the TRPC1 cation channel that is expressed in HEK93 cells [36], heteromerizes with TRPV4 [43] and is involved in cell migration [51].

We checked whether TRPC1 dysfunction may be responsible for the phenotype observed in TRPV4-$^{121}$AAWAA expressing cells by examining the migration of HEK293 cells in which TRPC1 was silenced using siRNA (Suplem Fig. 2). First, calpain activity of siTRPC1-treated HEK293 cells was evaluated and showed to be reduced compared to control siRNA treated cells (Fig. 6a). The migratory phenotype of siTRPC1 treated HEK293 cells was characterized by increased directionality without significant changes in the euclidean distance at early times followed by reduced persistence directionality and Euclidean distance at later stages (Fig. 6b-d). Thus, the migratory phenotype and the activity of the calpains involved in FA disassembly is similar in HEK293 cells in which TRPC1 has been silenced or TRPV4-$^{121}$AAWAA has been overexpressed. Together, these results suggest that TRPV4 most likely forms a functional complex with TRPC1 in the proximity of FA sites and that expression of TRPV4-$^{121}$AAWAA somehow exerts a dominant negative effect on such complex.

*TRPV4 modulates trailing adhesion*

A closer look at the process of tail retraction during cell migration was obtained using traction microscopy (Fig. 7). Migration of cells deforms the elastic substrates with embedded beads allowing the generation of maps of displacements. Figure 7a-b shows color-coded magnitudes of the bead

displacements mapping for GFP and TRPV4-$^{121}$AAWAA expressing cells. Tractions forces were much higher in the tail of TRPV4-$^{121}$AAWAA than in control GFP expressing cells. Mean traction forces at the trailing edge were about three-fold higher in TRPV4-$^{121}$AAWAA expressing cells than in control cells (Fig. 7c). This result indicates a stronger attachment of the trailing end of TRPV4-$^{121}$AAWAA expressing cells to the substrate, most likely as the result of the presence of more mature FA.

To further investigate the mechanism by which TRPV4 modulates trailing adhesions and cell migration, we studied the effect of TRPV4 on FA. Focal adhesions can be visualized by immunostaining for paxillin, an adaptor protein with a role in signal transduction at the FA [10]. Fig. 8a shows representative confocal microscopy images of TRPV4-WT (top left) and TRPV4-$^{121}$AAWAA (bottom left) as well paxillin (right). In TRPV4 expressing cells, paxillin staining showed a pattern of very few and small FA while larger and elongated FA were observed around the periphery of TRPV4-$^{121}$AAWAA expressing cells. Co-localization of overexpressed TRPV4 with endogenous paxillin was only partial in TRPV4-WT expressing cells but more intense in TRPV4-$^{121}$AAWAA expressing cells (right panels with higher magnification). Paxillin immunostaining was also used for quantification of different FA parameters: number (Fig. 8b), size (Fig. 8c; area) and relative intensity (Fig. 8d). TRPV4-WT expressing cells showed a marked reduction in the number, size and intensity of the FA compared to neighbor control (non-transfected, NT) cells whereas the size and intensity of

the FA, without a significant change in their number, were higher in TRPV4-$^{121}$AAWAA expressing cells. This is consistent with a higher and lower attachment to substrate of the FA from TRPV4-$^{121}$AAWAA and TRPV4-WT expressing cells, respectively.

To confirm these observations on different cell types we analyzed the FA in the breast cancer epithelial cell line T47D, which express endogenous TRPV4 [35], and in the glioma cell line U87. Decreased number of FA, size and intensity was observed in both T47D (Fig. 9) and U87 (Suplem Fig. 3) cells overexpressing TRPV4-WT while in TRPV4-$^{121}$AAWAA expressing cells increased FA area and intensity (mainly in U87 cells) without a change in FA number was observed. Together these results suggest that the stronger traction forces and attachment to the substrate of the trailing end of TRPV4-$^{121}$AAWAA expressing cells are due to the presence of large peripheral focal adhesions.

## DISCUSSION

Maintenance of cell polarity is required for directed migration [15]. Typically, the polarity of migrating cells is characterized by actin polymerization at the protruding front forming the filopodia, and lamelipodia, and the myosin-mediated contraction of actin fibers that retract the rear end. Other mechanisms involving actin cross-linking, actin filament depolimerization and

membrane tension are also implicated in rear retraction [14]. The different distribution of many signaling messengers and effectors, such as Rho GTPases, kinases and $Ca^{2+}$ ions also contribute to generate and maintain cell polarity [53,78]. The rear end dynamics acquires particular relevance in the migration of cells in the absence of guidance cues, with the distinctive creation of the cell rear first [15]. Besides, drag produced by an extended cell tail improved directionality of migrating cells and tail retraction precedes directional change [65]. On the other hand, short tail life-time increases the frequency of directional changes.

The moving cells are, therefore, continuously establishing (at the front edge where they serve as the anchorage points for cell locomotion) and disassembling (at the trailing edge) adhesions to the extracellular substrates [49,58]. Focal adhesions are typically mature forms of cell contacts with the substratum that contain a large number of proteins (the adhesome [84]). Among them it can be found proteins for binding to the extracellular matrix (integrins), binding to actin filaments (talin) and proteins that serve as anchoring and signaling elements (vinculin and paxillin). In the disassembly of FA, a requisite for cell migration, different $Ca^{2+}$-sensitive proteins participate including calpain proteases, myosin light chain kinase and focal adhesion kinase [49]. However, the transport systems generating the $Ca^{2+}$ signals required for FA disassembly, particularly at the rear end, are not fully characterized [56].

To date, very few $Ca^{2+}$ permeable channels and/or their regulatory molecules are claimed to play a role at the rear end of migrating cells: L-type voltage gated $Ca^{2+}$channels [81], TRPM7 [38], TRPC1 [23] and STIM1 [55], the latter probably working in conjunction with TRPC1 [52]. Knock-down of these channel proteins altered FA and results in long cellular protrusions [23,55,61], similar to those obtained in calpain-deficient cells [19].

Our study shows that overexpression of non-functional TRPV4 channels induced the presence of larger FA and increased traction forces at the tail of migrating cells. Our long-lasting migratory experiments revealed that cells overexpressing non-functional TRPV4 channels presented and altered migratory pattern characterized by an initial increased directional persistence due to the dragging effect of the long anchored tail followed by loss of persistent directionality with frequent turns. Cell morphology also changed towards one characterized by long protrusions. On the other hand, overexpression of TRPV4-WT resulted in smaller FA and traction forces at the rear end as well as frequent turns in migrating cells that prevented persistence directionality. Altogether, these results show that TRPV4 may regulate trail adhesion and directional persistence by coupling $Ca^{2+}$ entry via TRPV4 channels homotetramers or TRPV4/TRPC1 heterotetramers to calpain activity (shown in this study) or other $Ca^{2+}$-dependent proteins involved in FA turnover. While overexpression of TRPV4-WT would induce a $Ca^{2+}$ overload near FA sites facilitating FA disassembly, TRPV4-$^{121}$AAWAA overexpression, most likely exerting a dominant negative effect on TRPC1,

would reduce [$Ca^{2+}$] and FA disassembly. However, at present we can not discard that TRPV4 working as an adaptor/scaffold protein at or nearby the FA would also participate in the generation of the cell phenotype described here. In this sense, specific interactions of TRPV4 with the cytoskeleton have been described. TRPV4 interacts directly with microtubule-associated protein 7 [63], actin and tubulin [7,31]. Both α [34] and β-catenin as well as E-cadherin [60], major components of the tight junctions, and non-muscle myosin IIa [45] interact with TRPV4. TRPV4 coimmunoprecipitates with α2 integrin and Lyn kinase in rat dorsal root ganglion neurons [2] and participates in mechanical activation of β1 integrin in endothelial cells [66]. More relevant to this study is that mechanical forces applied to β1 integrin activate TRPV4 at focal adhesions [46]. All these previous studies along with our description of the role of TRPV4 in trailing adhesions and persistence directionality illustrate the cross-talk between TRPV4 and structures involved in mechanotransduction and cell migration.

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# Figure 1.

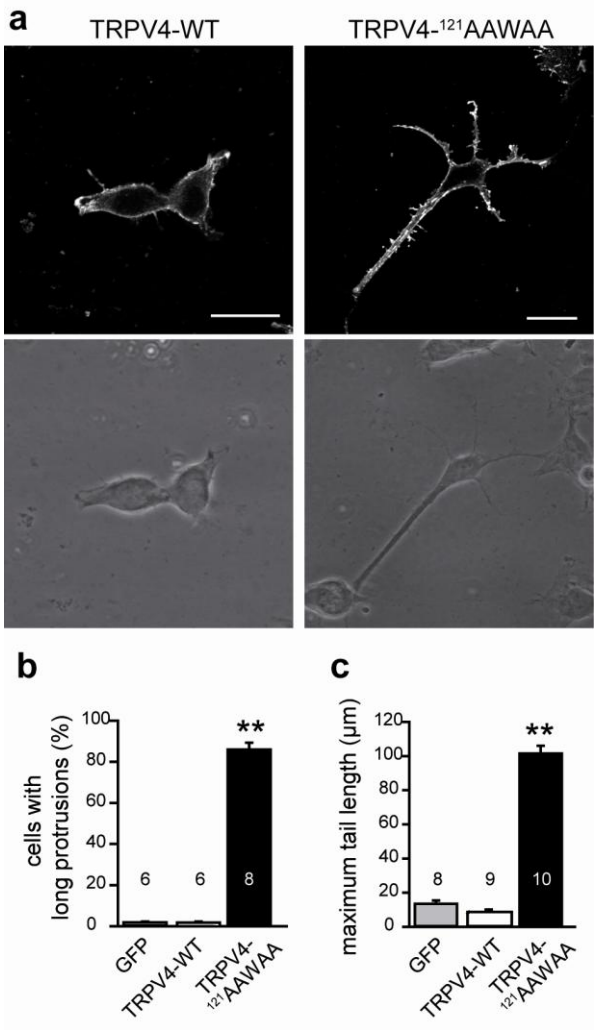



**Fig. 1. Expression of TRPV4 proteins and cell morphology.** a, Immunofluorescence localization of TRPV4-WT and TRPV4-$^{121}$AAWAA proteins in transiently transfected HEK293 cells. Corresponding phase-contrast images are shown at the bottom. Scale bar 20 µm. b, Percentage of cells presenting long protrusions (>10 µm) in GFP (2/865), TRPV4-WT (2/860) and TRPV4-$^{121}$AAWAA (841/914) expressing cells. Number of coverslips analysed is provided for each bar. c, Maximum tail length measured in representative cells.

## Figure 2.

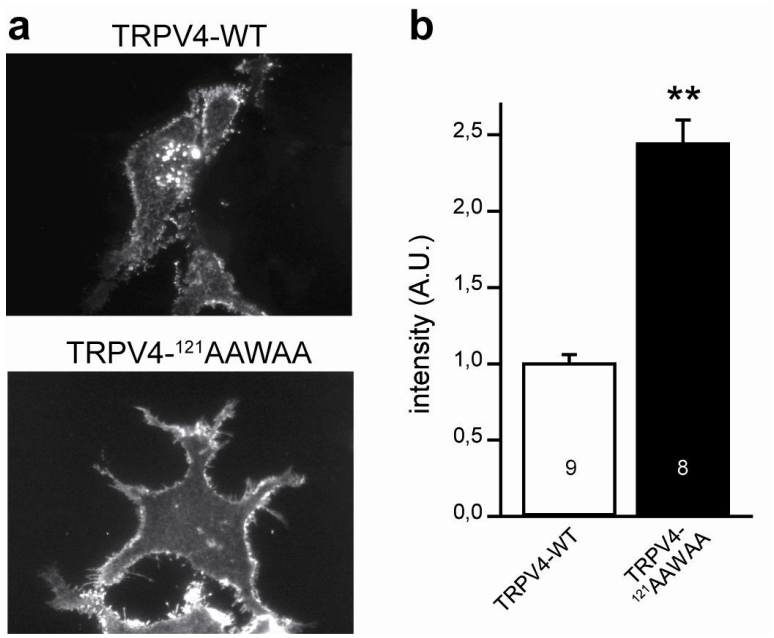



**Fig. 2. Membrane expression of TRPV4 proteins**. a, TIRF images of YFP fluorescence from HEK293 cells overexpressing TRPV4-WT-YFP and TRPV4-$^{121}$AAWAA-YFP. b, Intensity of the membrane YFP signal. Number of cells analysed is shown for each condition. ** $P$<0.01.

## Figure 3.

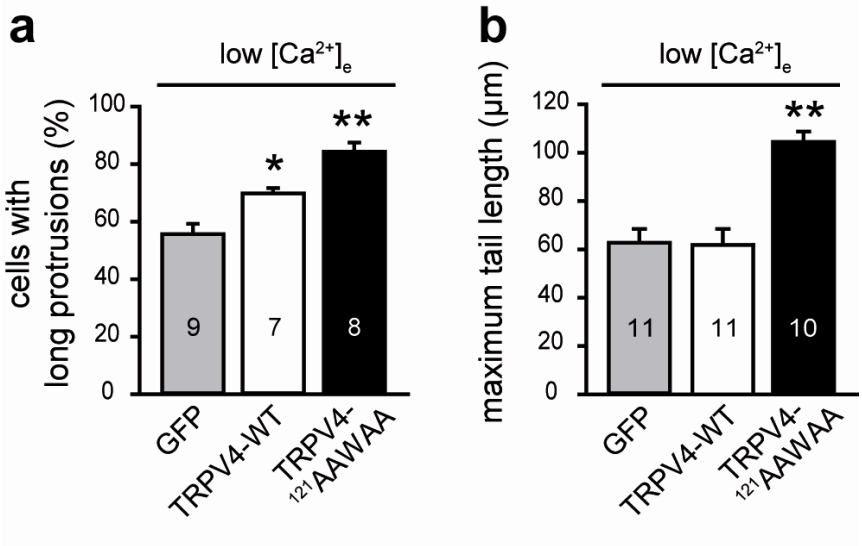



**Fig. 3. Effect of extracellular calcium removal on cell morphology.** a, Percentage of cells presenting long protrusions (>10 µm) in GFP (567/978), TRPV4-WT (533/762) and TRPV4-$^{121}$AAWAA (675/844) expressing HEK293 cells. Number of coverslips analysed is provided for each bar. b, Maximum tail length measured in representative cells.

# Figure 4.

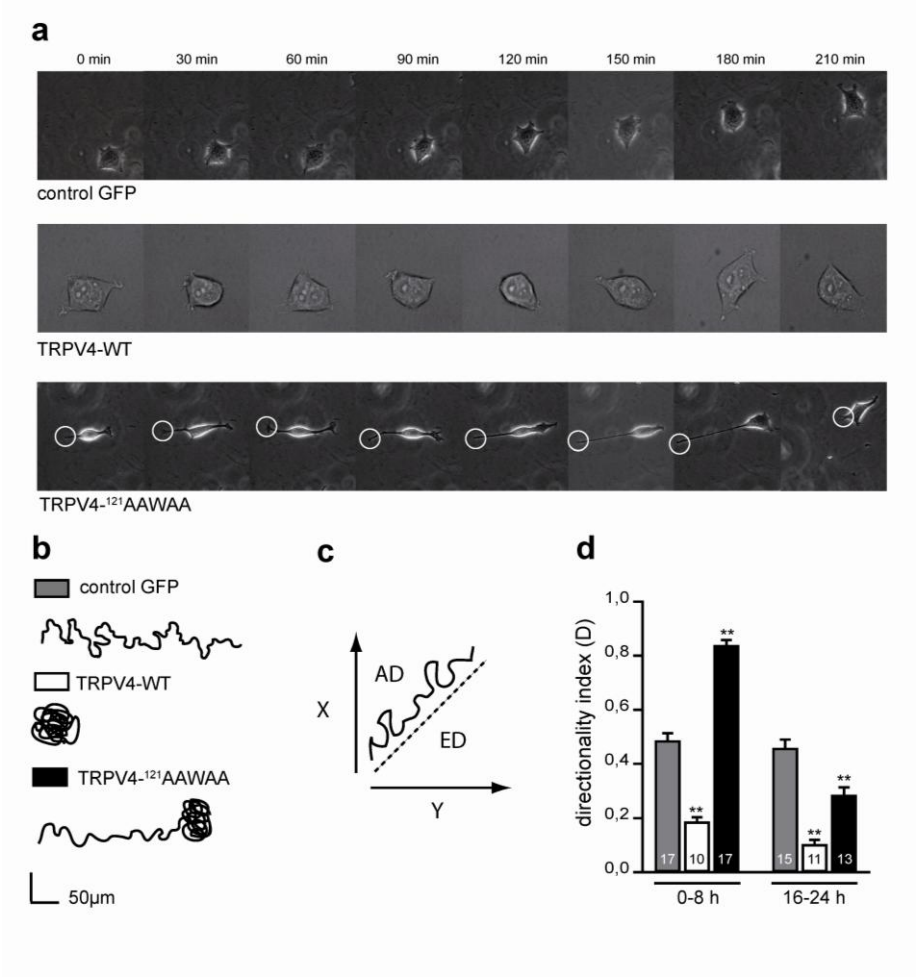



**Fig. 4. Cell migration is affected in TRPV4 transfeted cells**. a, Images of time lapse movies with 30-min intervals of control GFP (top), TRPV4-WT (middle) and TRPV4-$^{121}$AAWAA (bottom) expressing HEK293 cells. b, Representative trajectories over a 24h recording period of GFP, TRPV4-WT and TRPV4-$^{121}$AAWAA expressing cells. c, Schematic representation of the analysis of directional migration. The directionality index (D) is the ratio of AD/ED. d, Directionality index (D) of GFP, TRPV4-WT and TRPV4-$^{121}$AAWAA expressing cells at two time intervals after generation of the wound (0-8h and 16-24h). ** $P$<0.01 TRPV4-WT and TRPV4-$^{121}$AAWAA *vs* control GFP, one way ANOVA and Bonferroni *post hoc.*

# Figure 5.

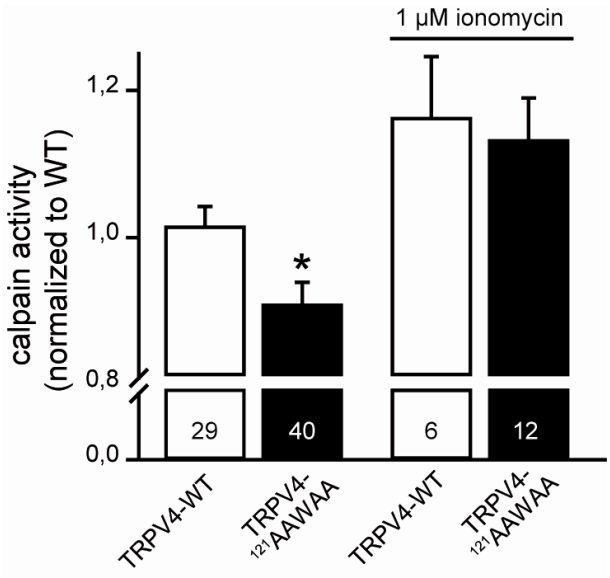



**Fig. 5. Calpain activity is reduced by TRPV4-$^{121}$AAWAA expression**. Calpain activity was measured using a permeant non-fluorescent substrate of calpain that becomes fluorescent upon calpain-mediated cleavage. Calpain activity was measured in TRPV4-WT and TRPV4-$^{121}$AAWAA expressing cells in the absence (left) and in the presence of 1 µM ionomycin (right). * $P$<0.05.

**Figure 6.**

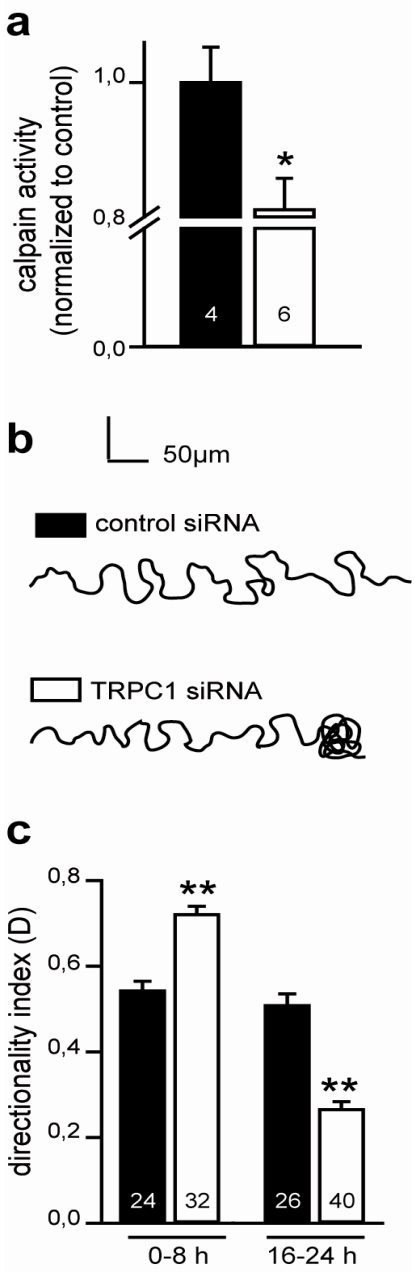



**Fig. 6. Calpain activity and cell migration in TRPC1-interfered HEK293 cells.** a, Calpain activity was measured in siCtrl and siTRPC1 treated cells. b, Representative trajectories over a 24h recording period of siCtrl and siTRPC1 treated cells. c, Directionality index (D) of siCtrl and siTRPC1 treated cells cells at two time intervals after generation of the wound (0-8h and 16-24h). * $P<0.05$ and ** $P<0.01$.

# Figure 7.

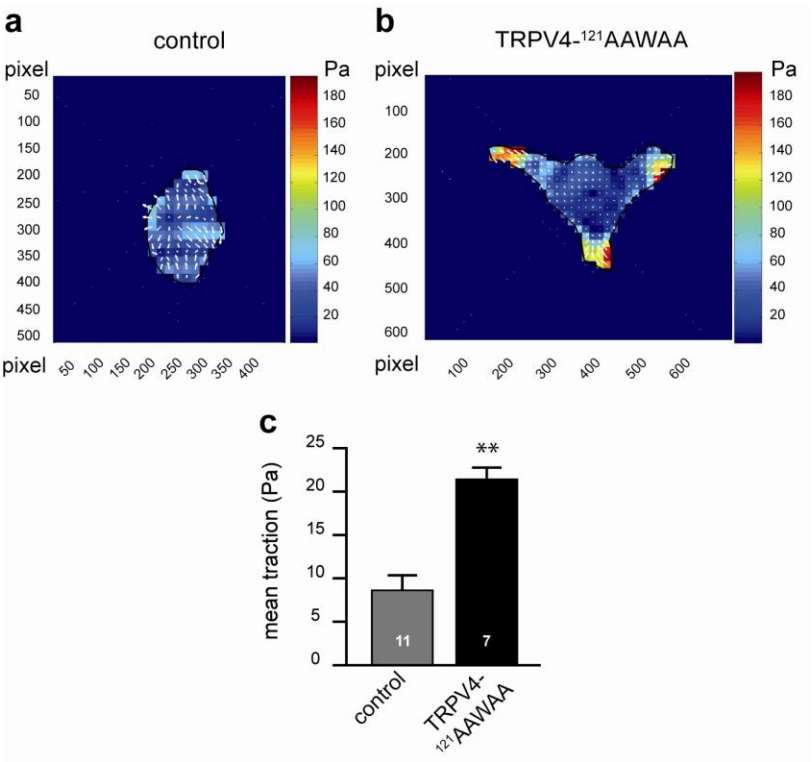



**Fig. 7. Traction forces are affected by expression of TRPV4-$^{121}$AAWAA**. Images of elastic substrate traction mapping of a control (a) and TRPV4-$^{121}$AAWAA (b) expressing HEK293 cells. Color bars indicate relative traction force values. c, Calculation of the total traction force generated by cells. ** $P$<0.01 TRPV4-$^{121}$AAWAA *vs* control GFP.

## Figure 8.

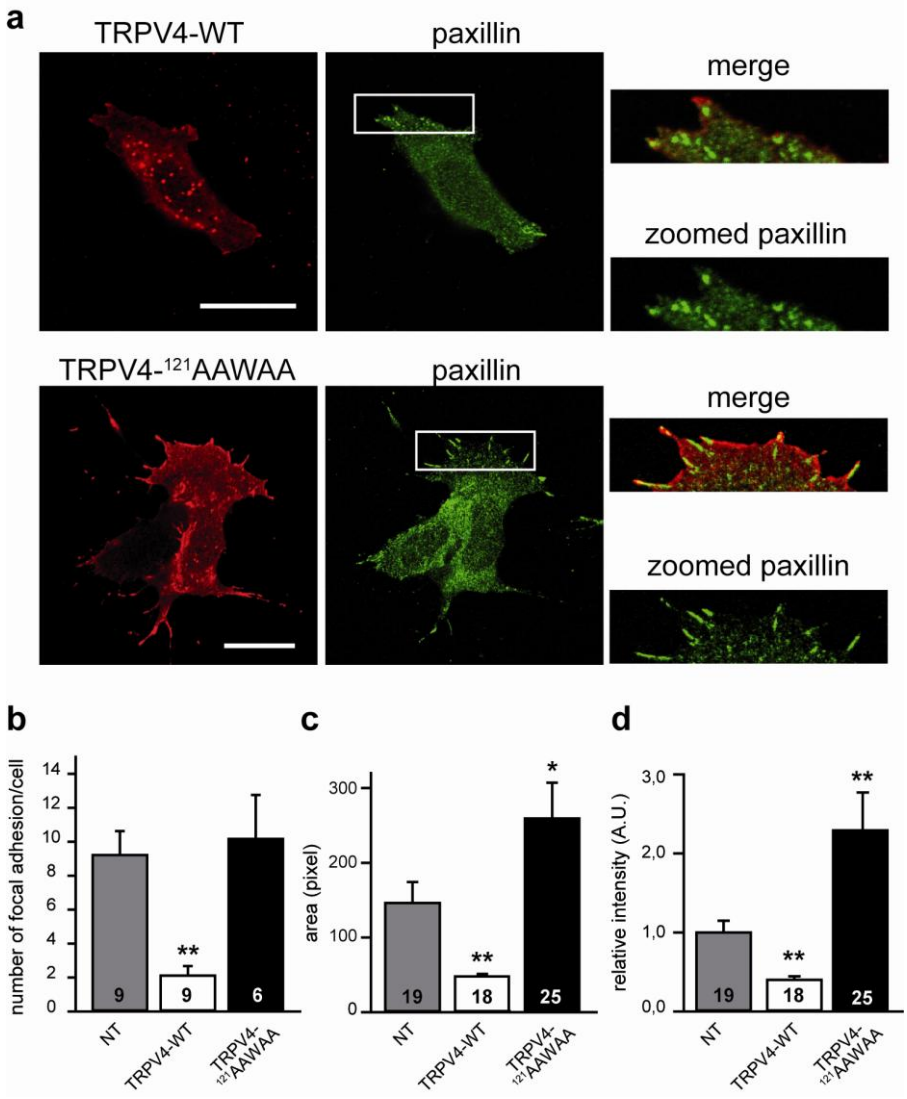



**Fig. 8. TRPV4 expression affects focal adhesions in HEK293 cells.** a, TRPV4 (red) and paxillin (green) staining in HEK293 cells. A magnified view of the respective boxes with or without merged images is shown on the right. Scale bar = 20 μm. b-d, Quantification of number (b), area (c) and intensity (d) of focal adhesions in non-transfected, TRPV4-WT and - TRPV4-$^{121}$AAWAA expressing cells. The number of cells analysed is indicated for each condition. * $P$<0.05 and ** $P$<0.01 *vs* NT cells, one way ANOVA and Bonferroni *post hoc*

## Figure 9.

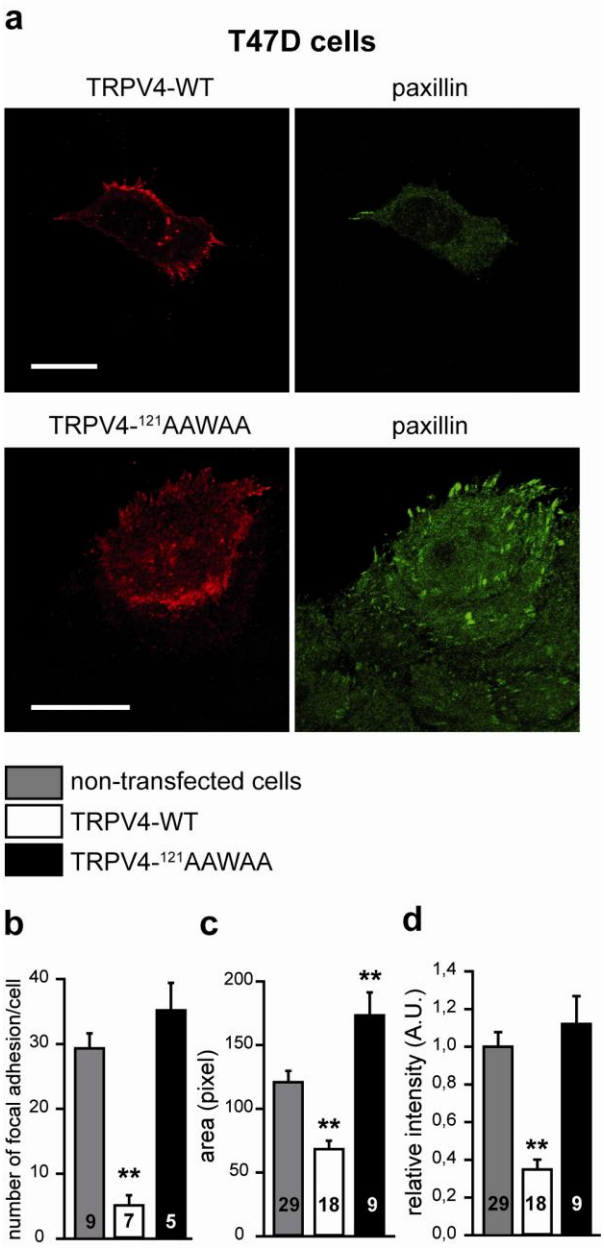
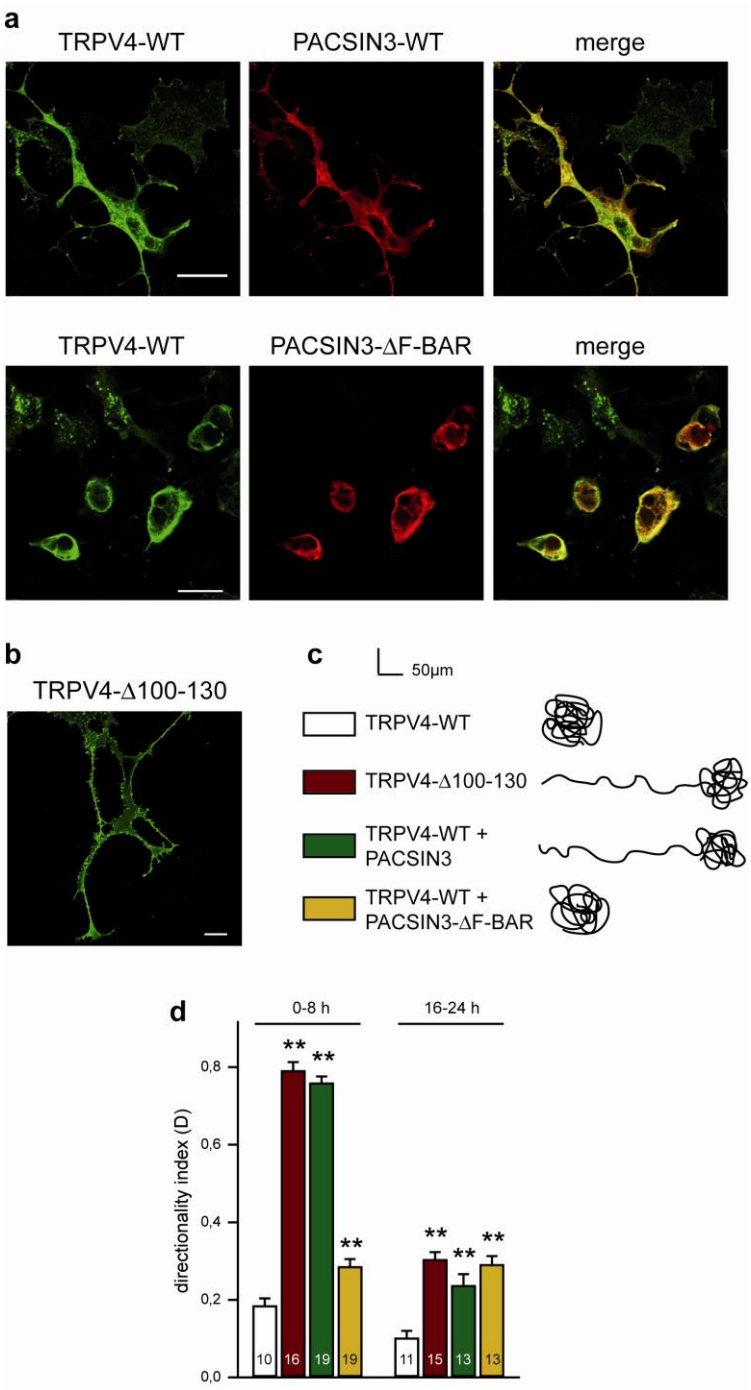



**Fig. 9. TRPV4 expression affects focal adhesions in T47D cells.** a, TRPV4 (red) and paxillin (green) staining in T47D cells. Scale bar = 20 μm. b-d, Quantification of number (b), area (c) and intensity (d) of focal adhesions in non-transfe°1 cted, TRPV4-WT and - TRPV4-$^{121}$AAWAA expressing cells. The number of cells analysed is indicated for each condition. * $P$<0.05 and ** $P$<0.01 *vs* NT cells, one way ANOVA and Bonferroni *post hoc*

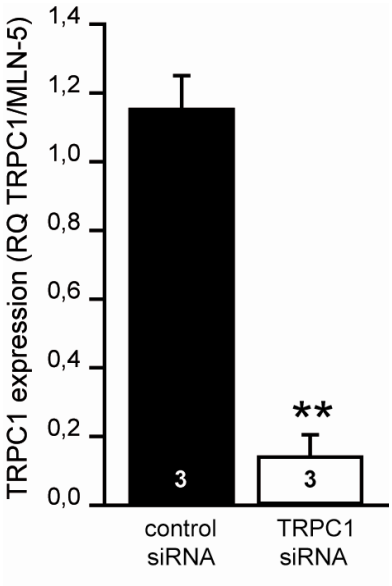
## Supplemental Figure S1.




**Supplemental Fig. S1.** a, Immunofluorescence localization of TRPV4-WT and PACSIN3 (top) or PACSIN3-ΔF-BAR (bottom) proteins in transiently transfected

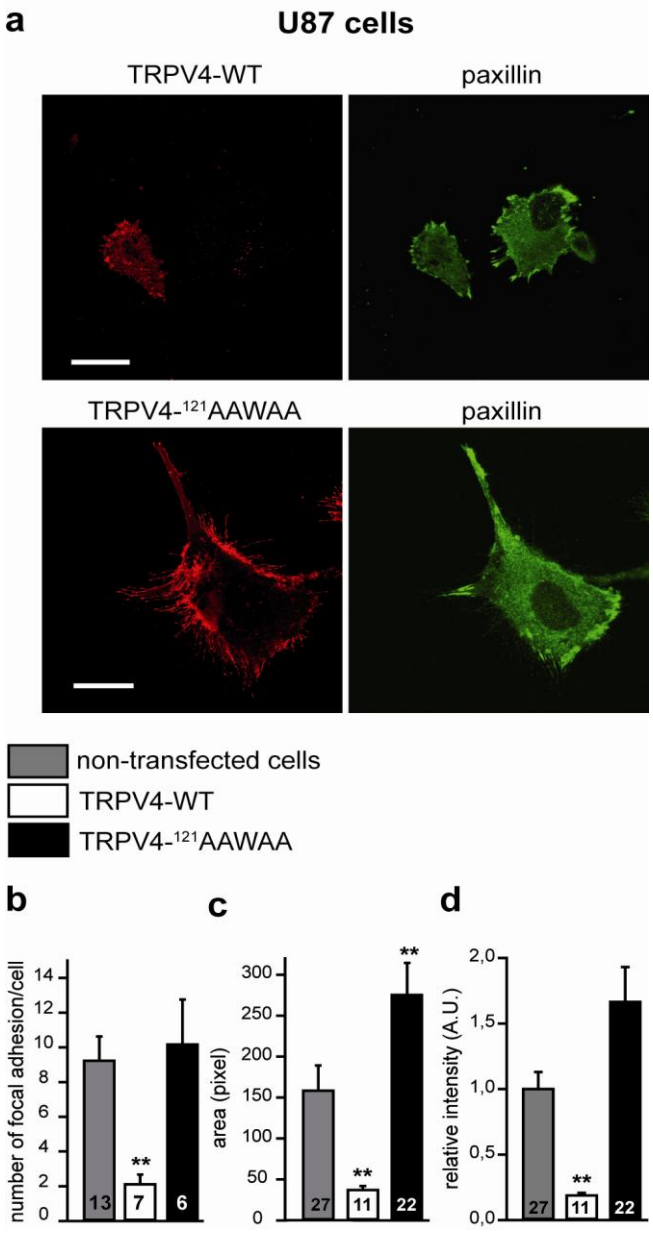
HEK293 cells. Scale bar 20 μm. b, Immunofluorescence localization of TRPV4-Δ100−130. c, Representative trajectories over a 24h recording period of TRPV4-WT, TRPV4-Δ100−130, TRPV4+PACSIN3 and TRPV4+ PACSIN3-ΔF-BAR expressing cells. d, Directionality index (D) of TRPV4-WT, TRPV4-Δ100−130, TRPV4+PACSIN3 and TRPV4+ PACSIN3-ΔF-BAR expressing cells at two time intervals after generation of the wound (0-8h and 16-24h). ** $P<0.01$ one way ANOVA and Bonferroni *post hoc*.

## Supplemental Figure S2.




**Supplemental Fig. 2.** Quantitative RT-PCR demonstrating downregulation of TRPC1 in HEK293 cells transfected with TRPC1 siRNA, relative to control siRNA transfected cells. MLN-5 was use as a house-keeping reporter. ** $P<0.01$

## Suplemental figure S3.




**Supplemental Fig. 3. TRPV4 expression affects focal adhesions in U87 cells.** a, TRPV4 (red) and paxillin (green) staining in U87 cells. Scale bar = 20 μm. b-d, Quantification of number (b), area (c) and intensity (d) of focal adhesions in non-transfected, TRPV4-WT and - TRPV4-$^{121}$AAWAA expressing cells. The number of cells analysed is indicated for each condition. * $P$<0.05 and ** $P$<0.01 *vs* NT cells, one way ANOVA and Bonferroni *post hoc*.

# IV. DISCUSSION

# Discussion

## 1. TRPV4 domains required for channel gating by different stimuli

The importance of TRPV4 channel in a plethora of physiological and pathological processes is unquestionable, but there is still limited knowledge about the specific domains responsible for channel gating by different stimuli. It is known the interaction of the TRPV4 channel with its activator, phorbol ester 4α-PDD (Vriens et al., 2007) through transmembrane domains 3 and 4. In my research work we focused on the N-terminal cytosolic tail because it is exactly in the N terminal tail where it was found the P19S single nucleotide polymorphism (SNP) associated with hyponatremia that generates a channel with reduced response to hypotonic cell swelling (Tian et al., 2009). The only other amino acid whose neutralization was reported to reduce TRPV4 response to hypotonicity is arginine 584 located in the transmembrane 4 (Vriens et al., 2007). More evidences for the relevance of the N-terminal tail to the channel gating by hypotonicity are available. In addition to the previously mentioned P19S in the N terminal tail of TRPV4, there is also a PRD domain that binds PACSIN3, an F-BAR containing protein known to bind and modulate TRPV4 activity (Cuajungco et al., 2006). These facts led us to believe that the N terminal tail of TRPV4 could be important for the channel gating by physiological stimuli, so we further screened this region by generating different deletions. First we generated three mutants: TRPV4-Δ1-30 (lacking the first 30aa), TRPV4-Δ1-130 (lacking first 130aa) and TRPV4-Δ100-130 (lacking 30aa in between 100 and 130aa). We evaluated the sensitivity of TRPV4 to hypotonic conditions using whole-cell patch-clamp recording and intracellular calcium imaging with Fura-2[5]. Deleting the first 30 amino acids of TRPV4 channel greatly reduced the transduction of mild hypotonicity without affecting the 4α-PDD or high hypotonic response, mimicking the changes induced by the P19S polymorphism. When we investigated the gating of the channel with a longer deletion including the first 130 amino acids, we observed that the hypotonic response was completely abolished, and unlike the shorter deletion, this one had affected also the response to 4α-PDD. More interestingly, the deletion of 30 amino acids in

[5] **Fura-2**, an aminopolycarboxylic acid, is a ratiometric fluorescent dye which binds to free intracellular calcium. It was the first widely used dye for calcium imaging, and remains very popular. Fura-2 is excited at 340nm and 380nm of light, and the ratio of the emissions at those wavelengths is directly correlated to the amount of intracellular calcium. Regardless of the presence of calcium, Fura-2 emits at 510 nm of light. The use of the ratio automatically cancels out confounding variables, such as variable dye concentration and cell thickness, making Fura-2 one of the most appreciated tools to quantify calcium levels.

between the amino acid 100 and amino acid 130 has also showed a greatly reduced response to 30% hypotonicity and pinpointed the region of interest. We evaluated if the effect of the truncation on channel gating was due to the incapability of the channel to reach the plasma membrane correctly. Using two different approaches we revealed that these truncated channels presented no traffic problem but a real gating problem. Curiously, we observed that the truncated channel was reaching the membrane significantly better than the TRPV4-WT channel indicating that this region could be important also for channel trafficking. We also revealed that TRPV4 protein is present constitutively in the membrane without the need of a previous stimulation. An important fact related to the 100-130 aa region that caught my attention was the accumulations of 11 positive charges (6 of them being lysines), with a tryptophan in the middle. This sequence resembled the putative Pleckstrin homology (PH) like domain or, more specifically, a phosphoinositide binding site, which was proposed to be present in the TRPV4 N-terminal tail by the Nilius lab (Nilius et al., 2008). We decided to neutralize to alanine four of these positive charges around the tryptophan $^{121}$KRWRK (obtaining a mutant named TRPV4-$^{121}$AAWAA) and to test the hypothesis that this aminoacid sequence will be a true phosphoinositide (PI) binding domain. From that moment on all my work was focused around this mutant who brought us several discoveries, a few of them quite unexpected. First, we tested the gating properties of TRPV4-$^{121}$AAWAA mutant channel and we confirmed that the neutralization of just 4 positive charges in this region greatly reduced channel response by 30% hypotonicity, but curiously it did not alter currents of the channel when stimulated with the synthetic agonist 4α-PDD. At this point we hypothesised that this domain could be important not only for swelling induced physiological stimulus but also for another physiological stimulus such as temperature. Actually, several TRPs are "thermo TRPs", sensitive to different ranges of temperatures (**Fig.3**). TRPV4 is activated by moderate heat (above 25°C) (Guler et al., 2002;Watanabe et al., 2002f) although the mechanism of its temperature sensitivity is not fully understood. Temperature sensitivity was also significantly reduced in TRPV4-$^{121}$AAWAA mutant, pointing out that this region is relevant for the channel response by both physiological stimuli, swelling induced hypotonicity and temperature, but not by the synthetic activator 4α-PDD. Considering that this region resembled the putative PI binding site, we hypothesized that our amino acids of interest $^{121}$KRWRK may form a PI-binding site required for the $PIP_2$ binding to the TRPV4 channel and that this binding could be required for a proper channel gating by physiological stimuli.

## 2. $PIP_2$ relation with TRP ion channels

Many ion channels are in one way or another affected by $PIP_2$. $PIP_2$ is a small but very important molecule amongst macromolecular giants. $PIP_2$ belongs to phoshoinositides - acidic phospholipids of cell membranes with myo-inositol

($C_6H_{12}O_6$) in their head group (**Fig. 13**). The parent compound phosphatidylinositol can become phosphorylated on the positions 3, 4, and 5 of the inositol ring in every combination, giving rise to the seven low-abundance polyphosphoinositides. $PIP_2$ shot into fame in the early 80's when this molecule was found to be the precursor of two important secondary messengers, DAG and IP3 (de Chaffoy et al., 1984;Sasaguri et al., 1985). $PIP_2$ is required for many cellular processes such as endocytosis, autophagy, remodeling of the actin cytoskeleton, cell growth, and proliferation.

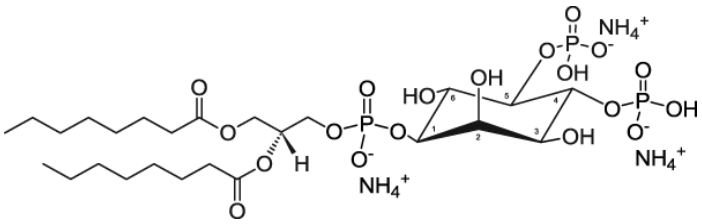



**Figure 13. Chemical structure of $PIP_2$**

Although it is the most abundant polyphosphoinositide in the plasma membrane, it still comprises only 1% of the acidic lipid in the whole cell (Hilgemann, 2007;McLaughlin et al., 2002;Xu et al., 2003a). The total amount of $PIP_2$ would be equivalent to a 4–10μM solution if dissolved in cytoplasm and to 5000–10,000 molecules $\mu m^{-2}$ of plasma membrane. Thus, if multiplicity of function is a fair measure of biological importance, $PIP_2$ is the most important lipid in the cytoplasmic leaflet of the plasma membrane. Several experimental approaches show that most cellular $PIP_2$ is located in the plasma membrane (Hammond et al., 2009;Szentpetery et al., 2009;Watt et al., 2002). There has also been accumulating support for models in which $PIP_2$ (and other lipids) are clustered into small plasma membrane microdomains (James et al., 2008;Lingwood and Simons, 2010;van den et al., 2011). However the most enigmatic discovery about the PI localization is the existence of a small lipid pool in the nuclear matrix (Barlow et al., 2010;Cocco et al., 1987;Irvine, 2003;Watt et al., 2002). The existence of this and other nuclear phosphoinositide pools are known since late 1980s but their functions are still unclear. $PIP_2$ has three phosphate groups (**Fig. 6**), and it is negatively charged. Its net charge depend on a number of factors such as local pH and its interactions with proteins, and it can be -3, -4, or -5. For example, at pH 7 the net charge of $PIP_2$ would be -4 (McLaughlin et al., 2002). Negative charges allow $PIP_2$ to interact electrostatically with positively charged residues on membrane proteins and with other cationic molecules. Many cytoplasmic proteins bind phosphoinositides in cell membranes using a collection of basic residues for major electrostatic interactions and additional hydrophobic and hydrogen-bonding residues. In addition, specialized PIP interactions sites have been identified, such as the pleckstrin homology

domains, which function as a more specific PIP binding pocket. Interactions with $PIP_2$ have been reported for a large number of TRP channels. Positively charged residues at both N- and C- termini have been shown to be putative $PIP_2$ interacting domains (Nilius et al., 2008;Rohacs and Nilius, 2007;Suh and Hille, 2008). Rohacs and colleagues demonstrate that the basic residues within the TRP domain are likely the putative-binding sites for TRPM8, TRPV5 and TRPM5 (Rohacs et al., 2005) as well as for TRPM6 and TRPM7 (Xie et al., 2011). Very early on following its cloning, it was realized that the gating of TRP in the Drosophila photoreceptor cells is tightly coupled to $PIP_2$ metabolism. In these cells, TRP and the closely related TRPL (TRP-like) (Phillips et al., 1992) function as receptor-operated channels that are activated downstream of the G-protein-coupled receptor rhodopsin. Light-induced activation of rhodopsin leads to the activation of PLC, which mediates hydrolysis of $PIP_2$ into DAG and IP3 (Hardie et al., 2001). Most TRP channels are regulated by $PIP_2$ (Nilius et al., 2008;Rohacs and Nilius, 2007). $PIP_2$ activates or is required by a large number of TRP channels for activation, including TRPM4 (Nilius et al., 2006), TRPM5 (Liu and Qin, 2005), TRPM6 (Xie et al., 2011), TRPM7 (Runnels et al., 2002) and TRPM8 (Liu and Qin, 2005;Rohacs et al., 2005). $PIP_2$ modulates TRPA1 by preventing its desensitization (Karashima et al., 2008). $PIP_2$ is required for TRPM6 and TRPM7 activation, as depletion of $PIP_2$ prevents channels activity. The $Ca^{2+}$ dependence of TRPM4 and TRPM5 is changed by $PIP_2$ levels. In the case of TRPM8, menthol and cold enhance the ability of $PIP_2$ to open the channel (Rohacs et al., 2005). In case of TRPV channels, $Ca^{2+}$ entry induced $PIP_2$ hydrolysis is responsible for $Ca^{2+}$-dependent inactivation of TRPV6 (Thyagarajan et al., 2008), and $Ca^{2+}$ dependent desensitization of TRPV2 (Mercado et al., 2010). $PIP_2$ also activates TRPV5 (Lee et al., 2005b). Although many studies have investigated TRPV1 relation with $PIP_2$ it is still an ongoing contraversy if $PIP_2$ is an inhibitor or an activator of TRPV1 (Cao et al., 2013;Klein et al., 2008;Liu et al., 2005;Lukacs et al., 2007;Yao and Qin, 2009). $PIP_2$ hydrolysis potentiates voltage and temperature-dependent TRPV3 activation (Doerner et al., 2011). While the effect of $PIP_2$ on TRPM channels are well established, effects of $PIP_2$ on TRPC channels are complicated by the metabolites of $PIP_2$ (Rohacs and Nilius, 2007). It has been shown that after $PIP_2$ hydrolysis, DAG activates TRPC3, TRPC6 and TRPC7 (Hofmann et al., 1999). It is conceivable that $PIP_2$ exhibits inhibitory effects so that hydrolysis of $PIP_2$ and production of DAG activates TRPC channels (Large et al., 2009).
To test if $PIP_2$ affects TRPV4 activity thought its binding to the sequence $^{121}$KRWRK we recorded TRPV4-dependent $Ca^{2+}$ entry using Fura-2 in rapamycin-induced translocatable 5-phosphatase system[6] which depletes $PIP_2$.

[6] The immunosuppressant macrolide, **Rapamycin**, induces the dimerization of two naturally occurring protein domains: FKBP12 with the FRB. Two components are used: a plasma membrane-anchored FRB domain and a cytosolic $PIP_2$-specific phosphatase fused to FKBP12. In cell lines transfected with both of these components, rapamycin promots dimerization of the FRB domain with FKBP12, and

Depletion of $PIP_2$ with this system prevented TRPV4 activation by physiological stimuli, hypotonicity and temperature, while the response by 4α-PDD was unaffected. This evidence of $PIP_2$-dependent TRPV4 gating was further confirmed in primary cultures of ciliated epithelial cells obtained from oviduct and trachea that express functional TRPV4 channels. Depletion of $PIP_2$ by purinergic stimulation of epithelial cells with ATP, which has been shown to activate the PLC-DAG-PKC leads to $PIP_2$ hydrolysis and its depletion, and generation of an IP3 mediated $Ca^{2+}$ signal. TRPV4 response by hypotonicity was abrogated after the activation of PLC with ATP. The temperature response was not abolished but it was significantly reduced in these cells after ATP stimulation. The ability of ciliated epithelial cells to maintain a small response to temperature suggests that in native cells the temperature response is complex and involves other $Ca^{2+}$ permeable channels in addition to TRPV4.

## 3. Is TRPV4 a real thermosensor?

One of the widely used and extremely important tools to study phopshoinositedes regulation of ion channels is the inside-out[7] excised patch configuration of the patch clamp technique. After the excision of the membrane patch, the activity of $PIP_2$ dependent ion channels decreases, a phenomenon referred to as run-down. For many $PIP_2$-sensitive channels the mechanism underlying run-down is the decrease in $PIP_2$ concentration in the patch due to the action of membrane-bound lipid phosphatases present in the patch membrane. The velocity of the run-down of the activity of a given channel generally correlates with its apparent affinity for $PIP_2$. Channels with higher $PIP_2$ affinity run down slower than channels with lower affinity. One can also apply $PIP_2$ chelating agents, such as $PIP_2$ antibody or poly-Lysine to excised patches to accelerate run-down. Perhaps the most direct way to study the effects of $PIP_2$ on ion channels is to apply phosphoinositides directly to the cytoplasmic surface of excised inside-out patches after current rundown. Phosphoinositides with various lipid side chains are available for these experiments; $PIP_2$ from natural sources has mainly arachidonyl-stearyl side chains, while synthetic $PIP_2$ usually contains two palmitoyl side chains. These long acyl chain lipids accumulate in the patch membrane, thus it is difficult to control their effective concentration. After activation with these $PIP_2$ analogues most ion channels run down quite slowly upon cessation of the application of the lipid, making repeated application of the these analogues

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induces a rapid translocation of the phosphatase to the plasma membrane which locally hydrolyses $PIP_2$.

[7] **The excised patch** is called an inside-out patch because allows one to expose the cytoplasmic side of the cell to defined solutions.

impractical (Rohacs et al., 2002). Short acyl chain, water soluble (e.g. DiC8-$PIP_2$ ) analogues of $PIP_2$ on the other hand activate most ion channels quickly and reversibly, presumably because they diffuse out the membrane easily upon washout (Rohacs et al., 1999). DiC8 phosphoinositides are water soluble, whereas long acyl chain phosphoinositides are found in micelles in aqueous solutions. Activity of $PIP_2$-regulated channels typically decreases in excised inside-out patches and recovers after the addition of exogenous $PIP_2$ such as for example in case of TRPM8. TRPM8 clearly requires $PIP_2$ for activity. Its activity runs down in excised patches, and application of $PIP_2$ reactivates the channel (Liu and Qin, 2005;Rohacs et al., 2005). The activating effect is isomer specific; $PIP_2$, is more effective than PtdIns(3,4)P2, PtdIns(3,4,5)P3 or PtdIns(4)P (Rohacs et al., 2005). $PIP_2$ chelating agents, such as PIP2 antibody, or poly-Lysine also inhibit TRPM8 in excised patches (Liu and Qin, 2005;Rohacs et al., 2005). The activity of the purified TRPM8 reconstituted into lipid bilayers depends on the presence of $PIP_2$ with a similar phosphoinositide specificity profile as in excised patches, providing a strong evidence for direct activation of the channel by $PIP_2$ (Zakharian et al., 2010). Before our study published in 2013 (Garcia-Elias et al., 2013) nothing was reported about possible regulation of TRPV4 by $PIP_2$. We have found that $PIP_2$ was not able to activate TRPV4 in excised patches which may indicate loss of another, yet unidentified modulator required for channel activity after patch excision. Hypotonicity-mediated activation of TRPV4 can not be directly evaluated in excised patches. Instead, the osmotransducing cytosolic messenger EET has been used (Vriens et al., 2004;Watanabe et al., 2003b). We report that in excised patches TRPV4 is activated with EET in the presence of $PIP_2$ while its activation is greatly reduced in the absence of $PIP_2$.
TRPV4 responds to warm temperature and its expression in sensory neurons, keratinocytes and in the hypothalamus point to its role in thermosensation and thermoregulation. Initial studies with $Trpv4^{-/-}$ mice revealed the contribution of TRPV4 in detecting warm temperatures (Lee et al., 2005a;Todaka et al., 2004) and chemically-induced hyperalgesia (Todaka et al., 2004), but more recent studies from the same laboratories showed no thermal response differences between $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice (Huang et al., 2011). Until now it was an open question if TRPV4 is a real thermosensor or a downstream effector of a yet unknown body temperature sensor. Actually, when stimulated with heat in excised patches TRPV4 activity was lost, indicating that temperature response of TRPV4 channel needs another player to be able to sense temperature stimulus. We showed that in the presence of $PIP_2$, TRPV4-WT channel activity was detected within seconds after application of warm solution bringing us to the conclusion that TRPV4 is a real thermosensor but only in presence of phospholipid $PIP_2$.

# 4. PACSIN3

PACSIN3 belongs to a highly conserved protein family PACSINS (also named Syndapins) that can discern the curvature of a membrane by offering curved, crescent-shaped membrane interaction surfaces (Kessels and Qualmann, 2004;Peter et al., 2004) by virtue of the presence of Fer-CIP4 homology (EFC)/FCH-BAR Bin-Amphiphysin-Rvs (F-BAR) domains (Aspenstrom et al., 2006;Chitu and Stanley, 2007;Dawson et al., 2006). The F-BAR domain is present on the N-terminal of PACSINs (Itoh and De Camilli, 2006;Tsujita et al., 2006). The F-BAR domain plays a role in dimerization and membrane phospholipid binding (Tsujita et al., 2006). Three pairs of basic residues are conserved in the F-BAR domain, which bind lipids and mediate membrane tubulation activity (Tsujita et al., 2006). The F-BAR domain binds specifically to certain kinds of lipids. This is in contrast to the BAR domain, which does not have such lipid preferences. The F-BAR domain has high affinity for $PIP_2$, such as in case of F-BAR containing proteins formins (Kovar, 2006;Tsujita et al., 2006) moderate affinity for $PIP_3$ and phosphatidylserine, and does not bind lysophosphatidic acid, lysophosphocholine or sphingosine-1-phosphate (Tsujita et al., 2006). The F-BAR domain tabulates the membrane in vitro and in vivo (Itoh et al., 2005;Tsujita et al., 2006). The F-BAR domain induces a gently curved helical-bundle dimer with a length ~200 fitting into a ~600 Å tubulated membrane (Shimada et al., 2007). For some members of the F-BAR family such as PACSIN1, the FCH domain has been shown to represent a self-association interface within a larger module, the extended F-BAR domain (eF-BAR), which is responsible for self-association *in vivo* (Kessels and Qualmann, 2006). PACSINs also contain a SH3 domain on their C terminal important for protein to protein interactions. Among other proteins implicated in vesicle trafficking PACSIN3 interact also with the large GTPase dynamin (Itoh et al., 2005;Modregger et al., 2000). PACSIN3 differs from the other family members in having a short proline-rich region and lacking asparagine-proline-phenylalanine motifs. In contrast to the neurospecific PACSIN1 and the ubiquitously expressed PACSIN2, PACSIN3 is mainly detected in lung and muscle tissues. The diversity of BAR proteins might reflect different types of membrane interfaces and biological functions. PACSINs play roles in endocytosis, actin regulation and signaling (Itoh 2006). In 2006 Cuajungco et al found that all three murine PACSIN isoforms can bind to the amino terminus of rodent TRPV4. In their work co-expression of PACSIN3, but not PACSINs 1 and 2, shifted the ratio of plasma membrane-associated versus cytosolic TRPV4 toward an apparent increase of plasma membrane-associated TRPV4 protein. A similar shift was also observable when they blocked dynamin-mediated endocytotic processes, suggesting that PACSIN3 specifically affects the endocytosis of TRPV4, thereby modulating its subcellular localization. Mutational analysis shows that the interaction of the two proteins requires both a TRPV4-specific PRD domain upstream of the ankyrin repeats of the channel and the carboxyl-terminal SH3 domain of PACSIN3. Such a functional interaction could be important in cell types that

show distribution of both proteins to the same subcellular regions such as renal tubule cells where the proteins are associated with the luminal plasma membrane (Cuajungco et al., 2006). Two years later D'hoedt et al. reported that that PACSIN3 strongly inhibits the basal activity of TRPV4 and its activation by hypotonicity and heat, but it does not affect 4α-PDD response (D'hoedt et al., 2008). This scenario mimics the gating properties of $^{121}$-AAWAA mutant and considering that PACSIN3 also binds TRPV4 just next to this region, we decided to investigate the nature of this interaction. Two hypotheses were investigated: that binding of membrane-bound PACSIN3 to the PRD of TRPV4 may either promote or physically block the interaction of the PI-binding site with membrane $PIP_2$. To test these hypotheses, we generated a PACSIN3 lacking the F-BAR domain. A similar deletion in PACSIN1 renders the protein unable to interact with the lipids of the plasma membrane (Plomann et al., 2009). Although the F-BAR domain of PACSIN3 comprises half of the protein, it is not required for interaction with TRPV4 (Cuajungco et al., 2006). Accordingly, we detected interaction of PACSIN3-ΔF-BAR with TRPV4. Coexpression of TRPV4 with PACSIN3-ΔF-BAR, unlike coexpression with PACSIN3, did not affect channels gating by hypotonicity. These results are consistent with the hypothesis that PACSIN3 interferes with the interaction of TRPV4 with $PIP_2$, an effect lost when a membrane-unbound PACSIN3-ΔF-BAR was used. Considering that PACSIN3 is endogenously expressed in HEK-293 cells and that TRPV4 may be constitutively expressed in plasma membrane, perhaps this protein could be acting as a cellular regulator of calcium influx through TRPV4. If TRPV4 would operate without any control, the calcium influx achieved by the channels basal activity could be deleterious for the cell. Intriguingly, both PACSIN3 and TRPV4 are expressed highly selectively within the notochord during the process of segmentation in zebrafish embryonic development (Mangos et al., 2007). In zebrafish, *pacsin3* silencing leads to a penetrant, severe developmental phenotype consistent with a primary failure in notochord differentiation (Edeling et al., 2009). Interestingly, general overexpression of wild-type mouse TRPV4 in zebrafish embryos induced abnormalities, including dysplasia-like phenotypes such as a curved and shortened body axis, and also malformations of the eye and brain (Wang et al., 2007). It could be that by silencing PACSIN3, a TRPV4 regulatory protein, the TRPV4 activity cannot be properly regulated and it affects notochord differentiation consequently causing severe developmental defects.

## 5. Is $PIP_2$ directly binding TRPV4?

Although the presence of a PH like domain was suggested within TRPV4 N-terminus, nobody ever investigated it or the possible binding of TRPV4 to $PIP_2$. We carried out limited proteolysis assays on the purified TRPV4 N-terminal region (residues 1–397), which includes the N-terminal tail and the

ankyrin repeats. Papain[8] digestion led to cleavage at four positions within the N-tail. Quantification of the bands showed that proteolysis of the TRPV4 N-terminus is reduced in the presence of $PIP_2$, but not in the presence of PI. $PIP_2$-dependent proteolysis protection was not observed with the isolated TRPV4 ankyrin repeats (residues 136–397), the TRPV1 ankyrin repeats, or the TRPV4-$^{121}$AAWAA N-terminal region, ruling out non-specific inhibition of papain by $PIP_2$. Thus, these biochemical data support a direct interaction of $PIP_2$ with the N-tail region of TRPV4-WT.

## 6. Structural changes upon $PIP_2$ binding

We are currently far from understanding the structural rearrangements that occur upon PIP binding and TRP channel gating, but on the basis of the available results, four main mechanisms of action can be distinguished**.** In the first and most straightforward model, specific intracellular parts of the channel are attracted towards the plasma membrane due to the direct interaction with $PIP_2$, leading to a stabilization of the open state. This model is similar to what has been proposed for $PIP_2$-dependent gating of inward-rectifier $K^+$ channels, where results from structural, functional and molecular modeling studies suggest that $PIP_2$ exerts tangential force on the N- and C-termini to open the channel (Logothetis et al., 2007;Suh and Hille, 2008). In the second mechanism, modulation of a TRP channel occurs through a competition between PIPs and other accessory proteins for binding to the same site on the TRP channel. This model is based on results obtained for TRPC6, where binding of calmodulin to a C-terminal site inhibits the channel, whereas removal of calmodulin by PIPs results in channel activation (Kwon et al., 2007). The third mechanism depicts the situation where PIPs interact indirectly with the TRP channel, through a PIP-binding accessory protein, such as in the case of the Pirt-dependent modulation of TRPV1 by $PIP_2$ (Kim et al., 2008). The fourth mechanism might exploit TRP-channel interaction with an agonist delivering enzyme. Such a mechanism can be hypothesized for TRPC3 (van Rossum et al., 2005), where interaction between channel and $PIP_2$ involves an intermolecular PH like domain, with the N-terminal part provided by PLCγ1 and the C-terminal part provided by TRPC3. The close association between TRPC3, PLCγ1 and $PIP_2$ may be considered as a signalplex, causing localized production of the channel agonist DAG and controlling the surface expression of the channel (van Rossum et al., 2005). We investigated a question that has been addressed for the first time almost simultaneously by our group and David Julius group: Does $PIP_2$ binding affect the structural conformation of a TRP channel? Cao et al. (2013) demonstrated by a chemical strategy that $PIP_2$ binding to TRPV1 brings the channel tail

[8] **Papain**, also known as papaya proteinase I, is a cysteine protease enzyme present in papaya (*Carica papaya*) and mountain papaya (*Vasconcellea cundinamarcensis*).

towards the membrane (Cao et al., 2013). We approached this question by studying the impact of TRPV4 deletions and mutations on the conformation of cytosolic tails by microscopy techniques. For this purpose, we evaluated the proximity of the intracellular C-tails of CFP- and YFP-tagged TRPV4 proteins, which we assumed formed a random population of heteromeric channels, by Fluorescence Resonance Energy Transfer (FRET) method. We tagged C-tails, which remained unmodified in all of the TRPV4 deletions/mutations generated, to avoid possible FRET artefacts generated by the different lengths of the N-tails. The relative CFP and YFP fluorescence intensities in the plasma membrane were determined for every single cell and used to calculate FRET efficiencies in transiently transfected HEK-293 cells. TRPV4-WT generated a FRET ratio similar to that reported previously (Arniges et al., 2006c), whereas TRPV4-Δ1–130 and TRPV4-121AAWAA doubled the FRET ratio, indicating a more compacted tail conformation. Similarly, coexpression of TRPV4-WT with PACSIN3 markedly increased the FRET signal, an effect lost when coexpressed with PACSIN3-ΔF-BAR. We reasoned that the increased FRET observed with mutant TRPV4 proteins or coexpression with PACSIN3 was related to the inability of TRPV4 to interact with membrane $PIP_2$. To test this hypothesis, we studied how the reduced $PIP_2$ levels affected the FRET efficiency of TRPV4-WT. We overexpressed CFP- and YFP-tagged TRPV4-WT channels in HEK-293 cells engineered with tetracycline-inducible expression of 5-phosphatase IV (Nilius et al., 2006). Induction of this enzyme depleted $PIP_2$ from the plasma membrane and significantly increased the FRET ratio. This finding further supports the hypothesis that conditions that prevented the N-tail access to membrane $PIP_2$ (by deletion/mutation of the PI-binding site or overexpression of PACSIN3) or depleted $PIP_2$ from the plasma membrane rearranged the cytosolic TRPV4 tails into a more compacted conformation (i.e., increased FRET ratio). Thus, in the presence of $PIP_2$ and an intact PI-binding site, the intracellular tails appeared to have an expanded conformation.

In 2011, for the first time, the crystal structure of an ion channel (Kir 2.2 channel) was obtained in complex with $PIP_2$, which provided an atomic view, especially in the interfacial region between the transmembrane domain (TMD) and the cytoplasmic domain (CTD), where $PIP_2$ binds to a well-defined pocket in the channel and exerts its regulatory function (**Fig. 14.)**. $PIP_2$ binding leads to a 6Å translational movement that brings the TMD and CTD closer, which is predicted to be a key molecular motion mediating PIP-dependent gating (Hansen et al., 2011). The present thesis suggests that, similar to $PIP_2$-regulated $K^+$ channels, the $PIP_2$–TRPV4 channel interaction rearranges the cytosolic domains. Whether the intracellular tail rearrangement occurring upon binding of $PIP_2$ to TRPV4 facilitates the access of stimuli-generated messengers (e.g., EET) to their binding sites or favours the stimulus-dependent opening of the gates themselves remains to be elucidated in the future studies.

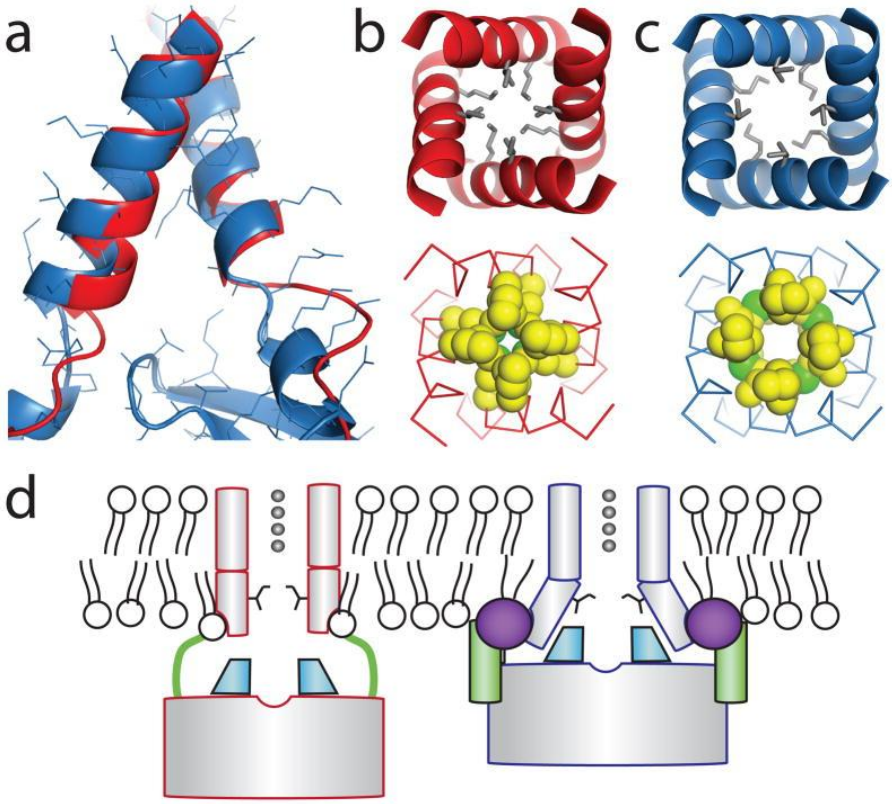


**Figure 14. A proposed mechanism of Kir2.2 activation by $PIP_2$ A**) Superposition of the TMD inner helices of the $PIP_2$-bound (blue ribbon) and apo- (red ribbon ) Kir2.2 structures. $PIP_2$ binding results in a widening of the helices near the helix bundle activation gate. **B) and C)**, comparison of the inner helix bundle gate in PPA bound Kir2.2 (**b**) and $PIP_2$ bound Kir2.2 (**c**) viewed from the extracellular side. Side chains of the residues in the bundle crossing are represented as either grey sticks. **D)**, A proposed mechanism for Kir2.2 activation by $PIP_2$. $PIP_2$ (purple sphere) binds at an interface between the TMD (grey cylinder) and the CTD (grey rectangle) and induces a large conformational change: a flexible linker (green line) contracts to a compact helical structure (green cylinder), the CTD translates towards and becomes tethered to the TMD, the G-loop (cyan wedge) inserts into the TMD, and the inner helix activation gate opens. Image adopted from (Hansen et al., 2011).

# 7. Ion channels in cell migration

The research on cell migration has documented the substantial importance of plasma membrane and its transport molecules in all cell migratory processes (Komuro and Rakic, 1998;Schwab et al., 2007). Ions are essential for the proper functioning of many, motility-related, physiological processes such as sperm migration, beating of cilia and nerve growth cone motility (**Figure 12**.)

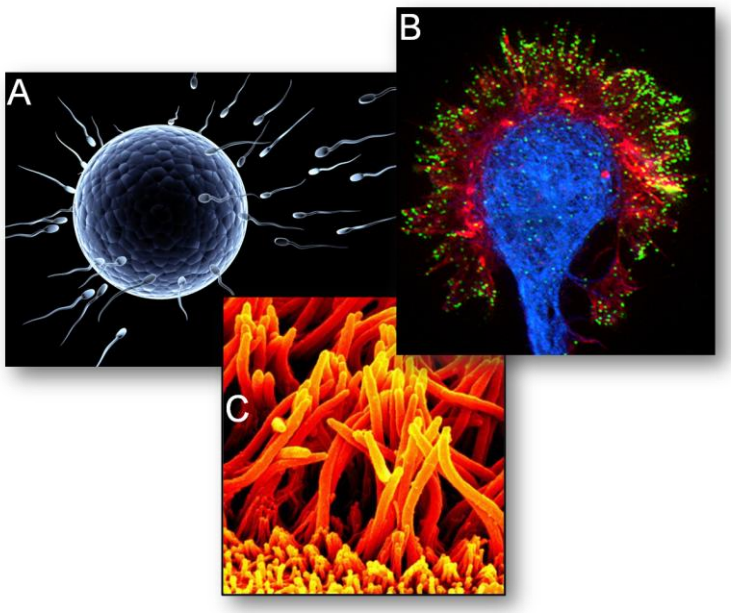


**Figure 12. Importance of ions in physiological motility related process. A.** In sperm migration process, ion channels play important roles. Several voltage-dependent $Ca^{2+}$ channels (VDCCs and Cavs) have been detected in sperm from different species (Darszon et al., 2005). TRPC2 is present at the head of the mouse sperm and has been proposed to participate in the acrosome reaction (Jungnickel et al., 2001). TRPM8 is present in membranes of the head and the flagellum of human sperm (De Blas et al., 2009). TRPM8-mediated increase in intracellular $Ca^{2+}$ levels seems to be involved in signalling events resulting in thermotaxis or chemotaxis of human sperm. **B.** $Ca^{2+}$ transients are also involved in regulating nerve growth cone motility (Gomez and Spitzer, 1999), a process very similar to the protrusion of the lamellipodium of a migrating cell. **C.** Ions are playing role also in the cilia function. The speed and the direction of movement of the ciliary/flagellar beating, are regulated by the local $Ca^{2+}$ concentration inside the cilium/flagellum (Naito and Kaneko, 1972;Pernberg and Machemer, 1995).

There are many ion channels and transporters that play different roles in cell motility such as:

I. **K+ channels** (Kv1.1, Kv1.3, Kv1.5, Kv1.7, Kv2.1, Kv3.1, Kv4.2 , Kv1.5 ,Kv1.7, Kv2.1, Kv3.1, Kv4.2 ,Kv7.1, Kv10.1, Kv11.1) (Rao et al., 2002;Schwab et al., 1999;Soroceanu et al., 1999;Wei et al., 2008;Williams et al., 2007)

II. **$Ca^{2+}$-sensitive $K^+$ channels** ($K_{Ca1}$.1 ,$K_{Ca}$ 2.3 ,$K_{Ca}$ 3.1) (Cruse et al., 2006;Kawasaki et al., 2004;Schwab et al., 1994;Sciaccaluga et al., 2010;Wu et al., 2008)

III. **Inward-rectifying $K^+$ channel Kir4.2** (deHart et al., 2008)

IV. **$Na^+$ channels** Nav1.2, Nav 1.6, Nav 1.7, Nav 1.5(Black et al., 2009;Fraser et al., 2005;Fulgenzi et al., 2006) ENaC (Drummond et al., 2006); ASIC ASIC 1, ASIC1.3, ASIC2 (Kapoor et al., 2009;Vila-Carriles et al., 2006)

V. **Orai/STIM1** (Bisaillon et al., 2010;Schaff et al., 2010;Yang et al., 2009a)

VI. **L-type $Ca^{2+}$ channels** Cav1.2 (Chao et al., 2011;Gui et al., 2006)

VII. **P2X Receptors** (P2X1,P2X4,P2X7) (Lecut et al., 2009;Ohsawa et al., 2007)

VIII. **Proton channels** (VSOP/Hv1) (El et al., 2010)

IX. **Cl− Channels** (VRAC,CFTR;ClC3,CLIC1,CLIC4,CLCN4,GABAA receptor) (Mao et al., 2007;Soroceanu et al., 1999)

X. **Aquaporins** AQP1,AQP3,AQP4,AQP5,AQP9 (Hayashi et al., 2009;Huebert et al., 2010;Papadopoulos et al., 2008)

XI. **$Na^+/H^+$ exchanger** NH1 (Denker et al., 2000;Denker and Barber, 2002a)

XII. **$Na^+$,$HCO3^-$ cotransporter** NBC1 (Schwab et al., 2005)

XIII. **Monocarboxylate transporter** MCT4 (Gallagher et al., 2007)

XIV. **Plasma membrane (V-)$H^+$-ATPase** (Rojas et al., 2006),

XV. **Gastric H+/K+-ATPase** (Ritter et al., 1998)

XVI. **$Na^+$,$K^+$,$2Cl^-$ cotransporter** NKCC1 (Bereiter-Hahn and Voth, 1988)

XVII. **$Na^+/Ca^{2+}$ exchanger** NCX1 (Sakamoto et al., 2009)

XVIII. **$Na^+/K^+$-ATPase** (Senner et al., 2003)

XIX. **TRP channels**

The roles of the ion channels and transporters in cell migration are various and multifarious. There is a detailed review that comprise all of the functions of ion channels in cell migration and all the recent discoveries in the field (Stock et al., 2013). In this thesis I will focus on the role of TRP channels in cell migration (**Table 3**.) and, especially, the involvement of TRPV4 in cell migration (see the section TRPV4 in cell migration).

## 8. How do ion channels affect cell motility?

### 8.1. Regulating the intracellular ion concentration of $Ca^{2+}$

Cell migration is a $Ca^{2+}$-dependent process (Freisinger et al., 2008;Komuro and Kumada, 2005;Pettit and Fay, 1998;Zheng and Poo, 2007). The migratory machinery includes many $Ca^{2+}$-sensitive effector molecules such as myosin II (Betapudi, 2010;Betapudi et al., 2010;Blaser et al., 2006), calpain (Franco and Huttenlocher, 2005;Pettit and Fay, 1998;Pettit and Hallett, 1996), calcineurin ($Ca^{2+}$calmodulin-activated protein phosphatase 2B ) (Conklin et al., 2005), gelsolin (actin binding protein) (McGough et al., 2003), integrins and S100 proteins (actin regulators) (Jung et al., 2010). Therefore, a spatiotemporal fine tuning of $[Ca^{2+}]_i$ is required for the coordination of a smooth, concerted action of different effector proteins in all the processes of cell migration, for example during rear part retraction (**Fig.6.**). Locally different concentrations of intracellular $Ca^{2+}$ (Brundage et al., 1991;Fabian et al., 2008;Hahn et al., 1992;Schwab et al., 1997), allow spatially localized regulation of $Ca^{2+}$ dependent components of cell migration machinery such as calpains (Franco and Huttenlocher, 2005;Pettit and Fay, 1998;Pettit and Hallett, 1996). In a migrating cell there is a gradient of $[Ca^{2+}]_i$ along the direction of movement. In general, $[Ca^{2+}]_i$ is higher at the rear part than at the front of migrating cells (Brundage et al., 1991;Hahn et al., 1992;Schwab et al., 1997). Recent studies have provided an interesting refinement of this view in that at the lamellipodial leading edge of fibroblasts, zones of short-lived *$Ca^{2+}$ flickers* of localized $Ca^{2+}$ transients can be observed (Conklin et al., 2005;Wei et al., 2009). These local $Ca^{2+}$ gradients favour directional migration by modulating cell adhesion via calcineurin (Lawson and Maxfield, 1995). In addition to these spatial variations of $[Ca^{2+}]_i$ and short-lived $Ca^{2+}$ flickers, there are also transient, global elevations of cytosolic $Ca^{2+}$ that are required for migration of neutrophils (Hendey and Maxfield, 1993), vascular smooth muscle cells (Scherberich et al., 2000), transformed epithelial cells (Schwab et al., 1997), and neuroblasts (Komuro and Kumada, 2005).

## 8.2. Regulating the intracellular ion concentration of $H^+$

Uneven distribution of $H^+$ ion concentration (Rojas et al., 2006) permits spatially confined regulation of $H^+$ dependent components of the migratory machinery (Denker and Barber, 2002a;Denker and Barber, 2002b;Stock and Schwab, 2009). The accumulation of the $Na^+/H^+$ exchanger NHE1 at the cell front contribute to the polarity of migrating cells (Grinstein et al., 1993;Krahling et al., 2009;Martin et al., 2011). Irrespective of both the orientation of the gradient and the ion species involved, the generation of a gradient always requires a fairly uneven, sometimes even patchy, spatial distribution, and/or locally different regulation of the contributing ion transporters and channels (Schwab et al., 1995) reviewed in (Schwab, 2001)). The $Na^+/H^+$ exchanger NHE1, for example, is one of the transporters involved in maintaining the polarity of migrating cells. NHE1-deficient cells fail to migrate directionally (Denker and Barber, 2002a;Dieterich et al., 2008). A precise pH regulation by transport proteins that move acidic or alkaline

equivalents across the plasma membrane is obligatory for all cells because the ionization state of cellular and extracellular proteins, and thus their function including the resultant physiological processes, is affected by the ambient pH. That way, also those proteins that constitute or affect the cellular migration apparatus are regulated by pH. Accordingly, it has been clearly shown that intra- and extracellular pH ($pH_i$ and $pH_e$) are critical determinants of efficient cell migration (Busco et al., 2010;Denker and Barber, 2002a;Hayashi et al., 2008;Krahling et al., 2009). The interrelation of pH and cell migration is of particular pathophysiological importance in tumor metastasis and innate immune cell function.

## 8.3. Modifying volume of migrating cells

During cell migration, cells undergo rapid changes in their shape due to the rapid formation and retraction of protrusions such as lamellipodia or filopodia. These rapid changes in cell shape are accompanied by cell volume changes based on water flowing into and out of the cell (Schwab et al., 2007). In migrating cells, the physiological significance of local volume consists in supporting protrusion and retraction of front and rear parts, respectively. Cells that migrate through a netted three-dimensional extracellular matrix may need to perform local volume changes, that is, shrinkage, to pass through constrictions, such as malignant glioblastoma through the particularly narrow extracellular space in brain tissue (Ransom et al., 2001;Soroceanu et al., 1999;Thorne and Nicholson, 2006). In many cell types osmotic shrinkage leads to a net increase in actin polymerization whereas osmotic swelling is accompanied by a net decrease in actin polymerization (Hallows et al., 1991;Levitan et al., 1995;Pedersen et al., 1999;Rizoli et al., 2000;Schwab et al., 1999). By adjusting the cell volume, ion channels, and transporters have an important impact on the organization of the actin cytoskeleton. Representative examples include Volume regulated anion channel (VRAC) (Mao et al., 2007;Schneider et al., 2008), KCa3.1 (Cruse et al., 2006;Schilling and Eder, 2009;Schwab et al., 1994;Sciaccaluga et al., 2010), Potassium voltage gated channel 1.3 (Kv1.3) (Matheu et al., 2008), various TRP channels (Waning et al., 2007;Wei et al., 2009), Sodium-hydrogen antiporter 1 (NHE1) (Denker and Barber, 2002a;Klein et al., 2000), Na-K-Cl cotransporters (NKCCs) (Schwab et al., 1994), and Aquaporins (AQPs) (Papadopoulos et al., 2008). An important volume sensitive regulator of the actin cytoskeleton is $PIP_2$ whose concentration rapidly increases after osmotic shrinkage and decreases after osmotic swelling (Nielsen et al., 2007). Many of the ion channels and transporters involved in cell volume regulation have been found to be part of the cellular migration machinery as well.

## 8.4. Modifying membrane potential

The membrane potential affects cell motility indirectly as a result of a variety of common housekeeping and signaling functions. It contributes to the regulation of cell volume, cytosolic and cell surface pH, and plays a particularly important role in regulating the intracellular $Ca^{2+}$ concentration either modifying the driving force for $Ca^{2+}$ movement or gating VDCCs. Besides of the triggering of individual voltage-dependent membrane proteins the transmembrane voltage also modulates the interaction between membrane proteins such as that of KV11.1 channels with β1-integrins (Olivotto et al., 1996). In addition to its indirect impact on cell migration, the membrane potential exerts a direct modulatory effect on the cytoskeleton: plasma membrane depolarization stimulates Rho and induces its peripheral translocation eventually regulating the MLC phosphorylation via the Rho-ROCK pathway (Chifflet et al., 2003;Szaszi et al., 2005;Waheed et al., 2010). This could result in an enhanced actin-myosin interaction and increased myosin ATPase activity (Somlyo and Somlyo, 2003).

### 8.5. Thought $PIP_2$

$PIP_2$ is a particularly interesting regulator of the cytoskeleton since it is also a major regulator of many ion channels and transporters involved in cell migration. In general, an increase in cellular $PIP_2$ levels drives actin polymerization whereas a decrease causes actin disassembly (Ling et al., 2006).

### 8.6. Interacting directly with the components of the cytoskeleton

For example KCa1.1 channels (Tian et al., 2006), KV1.2 channels (Williams et al., 2007), CaV1.2 channels (Chao et al., 2011;Gui et al., 2006), or NHE1 (Denker et al., 2000) interact directly with the components of the cytoskeleton leading to an interactive regulation. Different types of $K^+$ channels (e.g., KCa1.1, KV1.3, and KV11.1) were shown to interact directly or indirectly with integrins in endothelial, tumor, epithelial, and neuronal cells as well as lymphocytes. αVβ3 or α5β1 integrin ligand binding (vitronectin or fibronectin) activate KCa1.1 channels in endothelial and arteriolar smooth muscle cells, respectively (Kawasaki et al., 2004;Wu et al., 2008). It was also found that activation of FAK depends on its interaction with KV2.1 channels (Wei et al., 2008), and that TRPM8 channels inactivate FAK in PC-3 prostate carcinoma cells (Yang et al., 2009b).

## 9. How do the ion channels get the polarized distribution?

The polarized distribution of ion channels and transporters can be established by trafficking of vesicles that carry newly synthesized membrane proteins to the leading edge of the lamellipodium (Schmoranzer et al., 2003). An alternative mechanism allowing the confined distribution of transport proteins

in the plasma membrane is their presence in lipid rafts. Depending on the cell type, lipid rafts were described to be present either at the front (Manes et al., 1999) or at the rear part of migrating cells (Gomez-Mouton et al., 2001;Sitrin et al., 2010). Several ion channels and transporters involved in cell motility are integrated in lipid rafts. Stimulation of glioblastoma cells with epidermal growth factor (EGF) results in TRPC1 channel localization to the leading edge, and biochemical assays show that the caveolar raft fraction of the membrane is enriched in TRPC1 colocalizing with the lipid raft marker-proteins caveolin-1 and cholera toxin β-subunit (Bomben et al., 2011). In Chinese hamster ovary (CHO) cells lipid rafts mediate the targeting and spatio-functional coupling of integrin αIIbβ3, the NHE1 and the NCX1 to the plasma membrane, triggering very local $Ca^{2+}$ oscillations (Yi et al., 2009).

**Table3.** The main roles of TRP channels in cell migration

| TRP channel | Function | Reference |
|---|---|---|
| **TRPC1** | • contributes to cell polarity<br>• is essential for chemotaxis<br>• promotes myoblast migration | (Bomben et al., 2011;Fabian et al., 2008;Louis et al., 2008) |
| **TRPC3** | • guidance of nerve growth cones | (Li et al., 2005;Shim et al., 2005) |
| **TRPC5** | • contributes to neurite outgrowth<br>• impairs migration when incorporated into the plasma membrane<br>• promotes cell migration | (Chaudhuri et al., 2008;Greka et al., 2003;Greka and Mundel, 2011) |
| **TRPC6** | • is involved in vascular endothelial growth factor (VEGF) -stimulated migration<br>• causes lysophosphatidylcholine (LPC)-induced inhibition of migration<br>• contributes to macrophage inflammatory protein 2 (MIP2)-induced chemokinetic migration | (Chaudhuri et al., 2008;Damann et al., 2009;Ge et al., 2009) |
| **TRPV1** | • accelerates migration<br>• initiates and regulates filopodia<br>• interacts with β-tubulin<br>• activation induces | (Goswami and Hucho, 2007;Waning et al., 2007) |

| | | |
|---|---|---|
| | disassembly of microtubules and growth cone retraction | |
| **TRPV2** | • promotes migration upon stimulation with lysophospholipids<br>• which is required for chemotaxis | (Monet et al., 2009;Nagasawa et al., 2007) |
| **TRPV4** | • its activation inhibits migration and chemotaxis<br>• ultrafast activation upon mechanical stimulation of β1-integrins<br>• mediates reorientation upon cyclic strain<br>• interacts with actin and microtubules | (Goswami et al., 2010a;Matthews et al., 2010b;Ramadass et al., 2007;Thodeti et al., 2009a;Zaninetti et al., 2010) |
| **TRPM4** | • involved in migration by regulation of $Ca^{2+}$-dependent actin rearrangements<br>• mediates chemokine-induced migration to lymph nodes<br>• promotes motility of T helper cells 2 (TH2)<br>• impairs TH1 lymphocyte | (Barbet et al., 2008;Shimizu et al., 2009;Weber et al., 2010) |
| **TRPM7** | • required for polarized cell movement ;<br>• regulates smooth muscle cells migration<br>• contributes to platelet-derived growth factor (PDGF)-induced migration;<br>• controls cell adhesion and migration | (Callera et al., 2009;Clark et al., 2006;Clark et al., 2008;Su et al., 2011;Wei et al., 2009) |
| **TRPM8** | • its inhibition reduces cancer progression<br>• stimulates migration | (Gkika et al., 2010;Wondergem et al., 2008) |
| **TRPA1** | • its activation impairs gastric epithelial monolayer restitution | (Hayashi et al., 2007) |

## 10. Ion transport in cell directionality

The emerging roles of membrane ion transport proteins in the control of directionally persistent cell migration have been first reported for the voltage-gated $Na^+$ channel in rat prostate cancer cells (Djamgoz et al., 2001), followed by other studies concerning TRPP2 cation channels in directional sperm movement (Gao et al., 2003b), aquaporins in astroglial cell migration (Auguste et al., 2007), potassium channels in the invasiveness of embryonic stem cells (Morokuma et al., 2008), TRPM7 channels in embryonic lung fibroblast motility (Wei et al., 2009), and voltage-gated calcium channels in the electrotaxis of osteoblast cells (Ozkucur et al., 2009). More recently, the involvement of the $Na^+/K^+$ ATPase, NHE3 and NHE1 in cellular directedness during electrotaxis has been demonstrated (Ozkucur et al., 2011). It has been known for almost 20 years that there is a gradient of $[Ca^{2+}]_i$ along the direction of movement. In general, $[Ca^{2+}]_i$ is higher at the rear part than at the front of migrating cells (Brundage et al., 1991;Hahn et al., 1992;Schwab, 2001), but short-lived *$Ca^{2+}$ flickers* or localized $Ca^{2+}$ transients thought TRPM7 can be observed at the leading edge (Conklin et al., 2005;Wei et al., 2009). These local $Ca^{2+}$ signals superimpose the global front-rear $Ca^{2+}$ gradient and promote directional migration by modulating cell adhesion via calcineurin (Lawson and Maxfield, 1995). The ubiquitously expressed NHE1 is also targeted to lamellipodia and locally modulates intracellular pH to promote directional migration of fibroblasts thought pH sensitive, actin-severing protein, cofilin (Caldieri et al., 2009). Ion channels operating on the leading edge are much more studied that those on the rear part. Although it has been known for more than 10 years that mechanosensitive ion channels on the rear part play roles in cell migration and directionality, the identity of these channels are still not elucidated (Eddy et al., 2000;Lee et al., 1999). To date, very few $Ca^{2+}$ permeable channels and/or their regulatory molecules are claimed to play a role at the rear end of migrating cells: L-type voltage gated $Ca^{2+}$channels (Yang and Huang, 2005), TRPM7 (Kuras et al., 2012), TRPC1 (Fabian et al., 2008) and STIM1 (Schafer et al., 2012), the latter probably working in conjunction with TRPC1 (Rao et al., 2010). Knock-down of these channel proteins altered focal adhesions (FA) and results in long cellular protrusions (Fabian et al., 2008;Schafer et al., 2012;Su et al., 2011), similar to those obtained in calpain-deficient cells (Dourdin et al., 2001).

## 11. TRPV4 in cell migration

TRPV4 channel is an interesting emerging player in cell migration. TRPV4 is activated by changes in cell morphology: during cell swelling (Arniges et al., 2004) and shear stress (Everaerts et al., 2010; Vriens et al., 2004). More recently the molecular mechanism underlying ultrafast TRPV4 activation by shear stress has been investigated: mechanical force applied through β1 integrin induces a near instantaneous and localized transient TRPV4 mediated

Ca2+ influx in intact capillary endothelial cells expressing both native and genetically engineered integrin receptors. The ultra rapid response of the Ca2+ signal (within 4 msec), observed using whole cell Ca2+ imaging, strongly suggests that TRPV4 channels are activated in the absence of second messengers, and are directly mechanosensitive (Matthews et al., 2010b) although TRPV4 activation by cell swelling seems to require intracellular signalling (Fernandez-Fernandez et al., 2008;Vriens et al., 2004). Such findings make TRPV4 channels an attractive candidate for the mechanosensitive Ca2+ channel playing a role in cell migration. Zaninetti et al. (2011) published a very elegant and careful study showing that tonic activation of TRPV4 channels impairs migration in immortalized gonadotropin-releasing hormone-secreting neurons (GN11). In this study, after TRPV4 activation, lamellipodia were retracted and chemotaxis and directionality were reduced (Zaninetti et al., 2010). In another study in HepG2 hepatoblastoma cells, stimulation of TRPV4 channels with their activator 4α-PDD, on the contrary, increases lamellipodial dynamics (Waning et al., 2007). This obviuous controversy is suggesting that the cell type, the cellular context and probably the surrounding tissue define the TRPV4 dependent lamellipodial dynamics. However, both studies coincide in the observation that TRPV4 activation leads to inefficient migratory phenotype and reduction of cell directionality. Similarly to the study in HepG2 hepatoblastoma cells, also in F11 cells (neuroblastoma×DRG neuron hybrid cells) transfected with TRPV4, 4α- PDD treatment causes, in this case, growth cone retraction. These effects may be attributed to a direct interaction of TRPV4 channels with the actin and microtubule cytoskeleton (Goswami et al., 2010a;Ramadass et al., 2007). TRPV4 role in migration has been also studied in breast tumor-derived endothelial (BTEC) cells. Knockdown of TRPV4 expression completely abolished arachidonic acid-induced BTEC migration, suggesting that TRPV4 mediates the pro-angiogenic effects promoted by arachidonic acid in these cells (Fiorio et al., 2012c). In the work described in this thesis we reported that cells overexpressing non-functional TRPV4 channels present an altered migratory pattern characterized by an initial increased directional persistence due to the dragging effect of the long anchored tail followed by loss of persistent directionality with frequent turns. Similar to the reports of Zanineti and Waning using GN11 and HepG2 cell lines, we observed that Human embryonic kidney 293 cells (HEK-293) overexpressing TRPV4-WT loose directionality. We report for the first time that this loss of directionality is linked to the increased influx of calcium and altered focal adhesion dynamics. The non-functional TRPV4 channels revert the migratory phenotype and during several hours present greatly incresead persistent directionality. This increase in directionality is directly correlated also with the increased polarization, which results in the altered cell shape presented by long protrusions. We bring evidences that the increased polarization and

persistent directionality in these cells is due to the trailing edge retraction incapability. We report that TRPV4 therefore plays role in the rear part retraction, most likely collaborating with other players important for the rear part retraction such as TRPC1 (Fabian et al., 2008) with whom TRPV4 forms tetramers, a hypothesis grounded in the observation that interfering TRPC1 leads to the same migrational pattern as the overexpression of the non-functional TRPV4 channel. While overexpression of TRPV4-WT would induce a $Ca^{2+}$ overload near FA sites facilitating FA disassembly, TRPV4-$^{121}$AAWAA ovexpression, most likely exerting a dominant negative effect on TRPC1, would reduce $[Ca^{2+}]_i$ and FA disassembly. It is also worth mentioning the importance of single cell dynamic tracking over a long time in cell motility experiments rather than the final euclidean distance measurement (the distance between the initial and end positions). It has been the individual dynamic cell tracking that has allowed us to identify differences in the migratory behavior among the conditions analysed.

## 12. TRPV4 and focal adhesion dynamics

Trailing edge retraction is directly linked to focal adhesion dynamics because the migrating cell relies on proper focal adhesion attachment and disassembly during its body translocation and retraction of the trailing edge. TRPV4 has been previously reported to be present at focal adhesion in the study that showed that mechanical forces applied to β1 integrin can activate TRPV4 channel within focal adhesions (Matthews et al., 2010b). I now report that overexpression of TRPV4-WT channel significantly decreases the number, the size and the intensity of the focal adhesions in several cell lines. This correlates well with the loss of directionality of the cells overexpressing TRPV4-WT, since smaller FAs indicate that the dynamics of FA are increased which leads to altered migratory pattern. On the other hand, the non-functional mutant presented greatly increased size and intensity of the FAs indicating that $Ca^{2+}$ influx though TRPV4 plays roles in FA dynamics. This view was confirmed also by traction force microscopy that showed increased tractions at the trailing edges of the cells expressing non-functional TRPV4-$^{121}$AAWAA channel. However, at present we can not discard that TRPV4 working as an adaptor/scaffold protein at or nearby the FA would also participate in the generation of the cell phenotype. In this sense, specific interactions of TRPV4 with the cytoskeleton have been described. TRPV4 interacts directly with microtubule-associated protein 7 (Suzuki et al., 2003a), actin and tubulin (Becker et al., 2009;Goswami et al., 2010a). TRPV4 also interacts with α- and β-catenin (Janssen et al., 2011) as well as E-cadherin (Sokabe et al., 2010), major components of the tight junctions, and non-muscle myosin IIa (Masuyama et al., 2012). TRPV4 coimmunoprecipitates with α2 integrin and Lyn kinase in rat dorsal root ganglion neurons

(Alessandri-Haber et al., 2008) and participates in mechanical activation of β1 integrin in endothelial cells (Thodeti et al., 2009a). More relevant to this study is that mechanical forces applied to β1 integrin activate TRPV4 at focal adhesions (Matthews et al., 2010b). All these previous studies along with our description of the role of TRPV4 in trailing adhesions and persistence directionality illustrate the cross-talk between TRPV4 and structures involved in mechanotransduction and cell migration.

# V.CONCLUSIONS

# Conclusions

1. The N-terminal tail of TRPV4 is required for proper transduction of the hypotonic and heat stimuli. The region within the sequence $^{121}$KRWRK$^{125}$ is required for TRPV4 response to hypotonicity and heat, while the first 30 aminoacids are important only for the channel response to mild hypotonicity. The region $^{121}$KRWRK$^{125}$ it is not necessary for the channel activation by the synthetic agonist 4α-PDD.

2. Depletion of $PIP_2$ levels in transfected HeLa cells as well as in native TRPV4-expressing epithelial ciliated cells prevents channel response to hypotonicity and heat while it does not affect the response to 4α-PDD.

3. $PIP_2$ allows TRPV4 activation by heat in inside-out patches, thereby suggesting that TRPV4 is *bona fide* thermosensitive channel.

4. PACSIN3 negatively regulates TRPV4 response by hypotonicity and heat while it does not affect the 4α-PDD response, most likely interfering with the TRPV4-$PIP_2$ interaction, since the membrane-unbound PACSIN3ΔF-BAR mutant is not affecting the activity of the channel.

5. $PIP_2$ binds TRPV4 within the sequence $^{121}$KRWRK$^{125}$.

6. When $PIP_2$–TRPV4 channel interaction is prevented, the channel rearranges its cytosolic domains into a more compacted conformation

7. Expression of non-functional TRPV4 channel alters cell motility due to a defect in trailing edge retraction and directional persistence of the migrating cell in two phases, first by increasing it and then by the loss of directionality.

8. The alteration in trailing edge retraction results from the altered TRPV4-dependent $Ca^{2+}$ influx (via TRPV4 homo or heterotetramers), reduced $Ca^{2+}$-dependent calpain activity and altered dynamics of focal adhesions.

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# VII. ANNEX

ANNEX

## THE TRPV4 CHANNEL

Book chapter in Handbook of Experimental Pharmacology. TRP channels

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# THE TRPV4 CHANNEL

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**Abstract.** The widely distributed TRPV4 cationic channel participates in the transduction of both physical (osmotic, mechanical and heat) and chemical (endogenous, plant-derived and synthetic ligands) stimuli. In this chapter we will review TRPV4 expression, biophysics, structure, regulation and interacting partners as well as physiological and pathological insights obtained in TRPV4 animal models and human genetic studies.

## 1. Gene

The TRPV4 channel was first described in 2000 (Liedtke et al., 2000b;Strotmann et al., 2000d;Wissenbach et al., 2000a) and received several different names before the current nomenclature was accepted: OTRPC4 (osmosensitive transient receptor potential channel), VR-OAC (vanilloid receptor-related osmotically activated channel), VRL-2 (vanilloid receptor-like) and TRP-12. The human TRPV4 gene is found in chromosome 12q23-q24.1 and presents 15 exons. Five splice variants (TRPV4-A-E) have been identified. Variants B, C and E involve deletions in the N-terminal ankyrin repeat domains (ANK) that result in protein retention in the endoplasmatic

reticulum, defective oligomerization and lack of channel activity (Arniges et al., 2006b;Vazquez and Valverde, 2006a).

Compared to the vast knowledge obtained about TRPV4 channel regulation, little is known about the control of TRPV4 transcription. Progesterone receptor mediates repression of TRPV4 transcription in epithelial and vascular smooth muscle cells (Jung et al., 2009). Downregulation of TRPV4 expression by micro-RNA 203 in condylar cartilage of the temporomandibular joint (Hu et al., 2012) and by probiotic bacteria strains in the colon (Distrutti et al., 2013) has also been reported. Inflammatory signals such as interleukin 1β and interleukin 17 increase TRPV4 mRNA levels in dorsal root ganglia (DRG) neurons (Segond von et al., 2013) and nerve growth factor (NGF) increases TRPV4 expression in the urothelium (Girard et al., 2013). Hypoxia/ischemia increases TRPV4 expression and function in astrocytes (Butenko et al., 2012) and in pulmonary arterial smooth muscle cells of mice exposed to chronic hypoxia-induced pulmonary hypertension (Xia et al., 2013).

## 2. Expression

TRPV4 is broadly expressed in heart, arteries, lung, skin, bone, brain, urinary bladder, kidney, intestine, liver, pancreas and female reproductive tract (for a review see (Everaerts et al., 2010a)). TRPV4 is commonly found in the epithelial cells of the cornea (Mergler et al., 2010;Pan et al., 2008), bronchi (Fernandez-Fernandez et al., 2002;Fernandez-Fernandez et al., 2008;Li et al., 2011), trachea (Arniges et al., 2004;Lorenzo et al., 2008), intestine (d'Aldebert et al., 2011), urothelium (Everaerts et al., 2010b), larynx (Hamamoto et al., 2008), oviduct (Andrade et al., 2005), bile duct (Gradilone et al., 2007), epidermis (Sokabe et al., 2010), mammary gland (Jung et al.,

2009) and endolympathic sac (Kumagami et al., 2009). TRPV4 is also found in the endothelium (Watanabe et al., 2002f), smooth (Earley et al., 2005c;Jia et al., 2004) and skeletal muscle (Kruger et al., 2008), sensorial and brain neurons (Alessandri-Haber et al., 2003;Li et al., 2013;Shibasaki et al., 2007), glia (Benfenati et al., 2007;Konno et al., 2012), immune cells (Kim et al., 2010), osteoclasts (Masuyama et al., 2008c), osteoblasts and chondrocytes (Muramatsu et al., 2007c)) and pancreatic islets (Casas et al., 2008).

## 3. Protein

The TRPV4 protein consists of 871 amino acids (aa) with 6 transmembrane (TM) domains presenting both N- and C-terminal cytoplasmic tails (Fig. 1). The pore of the channel (aa 663 to 686) is found in the loop between TM5 and TM6. The 12 central amino acids of the pore are identical to those of TRPV1 and 2, the closest relatives of TRPV4 (Voets et al., 2002). Two key amino acids have been shown to regulate TRPV4 permeability: D672 and D682. Neutralization of both D672 and D682 greatly reduces permeability for calcium and rectification, and increases monovalent permeation, suggesting that these two negatively-charged residues are important for binding calcium ions inside the pore (Voets et al., 2002). D682 also participates in ruthenium red block. M680 residue strongly affects $Ca^{2+}$ permeation; K675 does not contribute significantly to the properties of the pore. Glycosylation of N651 is involved in the trafficking of TRPV4 (Xu et al., 2006). Mutation of E797 renders the channel constitutively opened (Watanabe et al., 2003a).

The long N-terminal tail (aa 1-465) accounts for more than 50% of total TRPV4 length and contains 6 ANK (Phelps et al., 2010) that participate in channel oligomerization (Arniges et al., 2006a). The N-terminal tail plays a prominent role in channel regulation, having a phosphoinositide binding site (PIBS, aa 121-125) required for channel activation by physiological stimuli, hypotonicity and heat (Garcia-Elias et al., 2013); a proline-rich domain (PRD) domain (aa 132-144) used for binding of and regulation by kinase C and

casein kinase substrate in neurons 3 (PACSIN3) (Cuajungco et al., 2006) and an arachidonate-like recognition sequence (ARS-L) (aa 402-408) (Nilius et al., 2003). In addition, complete deletion of PRD (Garcia-Elias et al., 2008), aa 1-130 or 100-130 renders the channel insensitive to all stimuli, including the sinthetic activator 4α-phorbol 12, 13 didecanoate (4α-PDD), suggesting an important role of the N-tail in the gating of TRPV4 (Fig. 2). The C-terminal tail presents a calmodulin-binding domain (CaM-BD) (812-831 aa)(Strotmann et al., 2003), an oligomerization domain (Becker et al., 2008) and a PDZ-like domain (Garcia-Elias et al., 2008;van de Graaf et al., 2006). The existence of a TRP box in the C-terminal tail has been proposed for TRPV1 (Garcia-Sanz et al., 2007) although its existence in TRPV4 has not been thoroughly studied. Heteromerization of TRPV4 with other TRP channels is discussed in Section 4.4.

## 4. Interacting and regulatory proteins

A detailed database of TRPV4 channel protein-protein interactions (Chun et al., 2013;Shin et al., 2012b) is available at http://trpchannel.org/summaries/TRPV4.

### 4.1.

### Proteins modifying TRPV4 location on the plasma membrane.

In addition to being trigger by activating stimuli, TRPV4 channel activity in the plasma membrane is affected at several different levels: targeting of the channel protein to its final destination, post-translational modification and lysosomal degradation.

TRPV4 location on the plasma membrane and channel response to hypotonicity and warm temperature is regulated by PACSIN3 (Cuajungco et al., 2006;D'hoedt et al., 2008). PACSIN3 belongs to a family of three proteins with the Bin-Amphiphysin-Rvs (BAR) domain required to penetrate and

remodel the plasma membrane and to participate in endocytic processes, neurotransmission, and cell morphology and motility (Plomann et al., 2009). All members of the PACSIN family bind to the PRD of TRPV4 through their SRC homology 3 (SH3) domain; however, only PACSIN3 appears to regulate TRPV4.

A few more proteins affect the presence of TRPV4 at the plasma membrane. OS-9, a ubiquitous protein found in the cytoplasmatic site of the endoplasmic reticulum (ER), plays a role in selecting substrates for degradation. It interacts with TRPV4 monomers (aa 438-468 at the N-tail) retaining the monomers in the ER and reducing the amount of channel in the membrane, thereby protecting TRPV4 from polyubiquitination and premature proteosomal degradation (Wang et al., 2007). The ubiquitin ligase AIP4 binds TRPV4 (presumably to its N-tail) and promotes its endocytosis (Wegierski et al., 2006). Intimately linked to TRPV4 ubiquitination in vascular smooth muscle is the complex formed by the G protein-coupled angiotensin receptor, β-arrestin and TRPV4 (Shukla et al., 2010). In the absence of angiotensin, β-arrestin (an adaptor between AIP4 and TRPV4) does not bind TRPV4 and no AIP4-dependent internalization occurs. Another protein binding to and modulating TRPV4 presence and function at the plasma membrane is caveolin-1 (Saliez et al., 2008a). TRPV4 location to lipid rafts containing caveolin-1 favors nitric oxide (NO) and endothelium-derived hyperpolarizing factor-dependent vasodilatation. Annexin 2A, a calcium-dependent membrane-binding protein that is linked to vesicular trafficking and endosome formation, also binds TRPV4 in dorsal root ganglia (Huai et al., 2012), although the functional relevance of this interaction is presently unknown.

### 4.2.
### Signaling molecules

Early studies on TRPV4 reported its activation and/or modulation by phorbol esters and protein kinase C (PKC) (Watanabe et al., 2002b;Xu et al., 2003b) as well as by activators of protein kinase A (PKA)(Alessandri-Haber et al., 2006). Subsequent studies (Fan et al., 2009) identified the residues involved in PKA (S824)- and PKC (S162, T175 and S189)-mediated modulation of TRPV4 and the role of the A Kinase Anchoring Protein (AKAP79) in the optimization of TRPV4 phosphorylation by PKA and PKC.

TRPV4 regulation by tyrosine kinases is controversial. The proposed role of Y253 in the hypotonicity-mediated activation of TRPV4 (Xu et al., 2003c) was not observed by others (Vriens et al., 2004). Y110 has been shown to participate in the sensitization of TRPV4 response to heat and hypotonicity but not to 4α-PDD (Wegierski et al., 2009). However, preincubation with the tyrosine kinase inhibitors PP1 (Vriens et al., 2004) or PP2 (Fig. 3) does not affect TRPV4-WT activation by hypotonicity.

The "with no lysine" (WNK) kinases WNK1 and WNK4 down-regulate TRPV4 membrane location; hypertension-causing WNK mutants are not able to exert this effect. Experiments deleting the TRPV4 N-tail suggested the participation of this region in TRPV4 interaction with WNK kinases, without providing evidence for a diret interaction between WNK proteins and TRPV4 (Fu et al., 2006).

Calmodulin (CaM) binding to TRPV4 has been identified within the second ANK domain (a binding site shared with ATP) (Phelps et al., 2010) and at the C-tail (aa 812-831)(Strotmann et al., 2003), a site also used for binding to the inositol 1,4,5-trisphosphate (IP3) receptor **(**$IP_3R$) (Fernandes et al., 2008;Garcia-Elias et al., 2008) and phosphorylation by the serum glucocorticoid-induced protein kinase-1 (SGK-1) (Shin et al., 2012a). However, the reported effects of CaM on TRPV4 activity range from a positive modulation (Strotmann et al., 2003) to an inhibitory effect (Phelps et al., 2010). The other molecules interacting with these sites, ATP and $IP_3R$ are positive modulators of TRPV4 channel activity.

**4.3.**

**Cytoskeletal proteins**

The first reported link between TRPV4 and the cytoskeleton came with the observation that microtubule-associated protein 7 (MAP7), which also interacts with actin microfilaments, enhances TRPV4 presence at the plasma membrane and, thereby increases TRPV4 activity (Suzuki et al., 2003a). The binding of MAP7 to TRPV4 was proposed to be at the channel C-tail.

TRPV4 interacts directly with actin and tubulin (Becker et al., 2009;Goswami et al., 2010b). The interaction between TRPV4 and F-actin is essential to support channel activation following cell swelling (Becker et al., 2009), and tubulin competes with actin for binding to the TRPV4 C-tail. The interplay between these three molecules exerts a two-way modulation of cytoskeletal dynamics and TRPV4 activity (Fiorio et al., 2012b;Goswami et al., 2010b) that may contribute to the mechanical allodynia reported in mice models of neuropathic pain (Huai et al., 2012;Wei et al., 2013). Key molecules that connect the actin cytoskeleton with structures that maintain the barrier function in epithelia also interact with TRPV4. Both β-catenin and E-cadherin, the major components of the tight junctions in keratinocytes, interact with the proximal TRPV4 N-tail to maintain the integrity of the skin barrier (Sokabe et al., 2010). Another study showed coimmunoprecipitation of TRPV4 with α catenin in urothelial cells but not with β catenin (Janssen et al., 2011). TRPV4 also interacts with and is regulated by non-muscle myosin IIa (Masuyama et al., 2012).

TRPV4 coimmunoprecipitates with α2 integrin and Lyn kinase in rat dorsal root ganglion (DRG) neurons (Alessandri-Haber et al., 2008) and participates in mechanical activation of β1 integrin (Thodeti et al., 2009b). Moreover, mechanical forces applied to β1 integrin activate TRPV4 at focal adhesions (Matthews et al., 2010a), another illustration of the cross-talk

between TRPV4 and cytoskeletal structures involved in mechanotransduction.

### 4.4.

### Channel proteins.

Heteromeric channels are formed by TRPV4 interacting with TRPP2 (Kottgen et al., 2008), aquaporin 4 (Benfenati et al., 2011), aquaporin 2 (Galizia et al., 2012), TRPC1 (Ma et al., 2010) or calcium-activated potassium channel ($K_{Ca}$2.3 cells) (Ma et al., 2013). $IP_3R3$ interacts with and modulates TRPV4 response, particularly under conditions of low level stimulation (Fernandes et al., 2008;Garcia-Elias et al., 2008).

## 5. TRPV4 biophysics and activation

### 5.1.

### Basic biophysical properties

TRPV4 is a non-selective cationic channel with higher permeability to $Ca^{2+}$ and $Mg^{2+}$ than to $Na^{+}$ cations, thereby, which generates an influx of $Ca^{2+}$ following its activation under normal physiological conditions (Voets et al., 2002). Although TRPV4 also permeates monovalent cations in the absence of divalent ions, it discriminates very poorly between them. The sequence of permeation is $K^{+}>Cs^{+}>Rb^{+}>Na^{+}>Li^{+}$ (Nilius et al., 2001).

Single-channel conductance of TRPV4 is larger at positive (80-100 pS) than at negative potentials (30-60 pS) and the current-voltage relationship of TRPV4 whole-cell currents presents outward rectification (with a slight inward rectification at very negative voltages). This process depends on extracellular $Ca^{2+}$ ions that at the same time permeate and block TRPV4 (Everaerts et al., 2010a;Nilius et al., 2004;Voets et al., 2002;Watanabe et al., 2002a).

**5.2.**

**Activation by osmotic and mechanical stimuli**

TRPV4 responds to osmotic changes in the cell environment by increasing or decreasing its activity in hypotonic and hypertonic solutions, respectively (Liedtke et al., 2000c;Strotmann et al., 2000c;Wissenbach et al., 2000b), thereby contributing to cellular (Arniges et al., 2004;Fernandez-Fernandez et al., 2008) and systemic volume homeostasis (Liedtke and Friedman, 2003c;Mizuno et al., 2003). TRPV4 also responds to mechanical stimuli such as shear stress (Gao et al., 2003a;Kohler et al., 2006) or high viscous loading (Andrade et al., 2005). Its osmotic (Vriens et al., 2004) and mechanical (Andrade et al., 2005;Fernandes et al., 2008) sensitivity depends on phospholipase $A_2$ activation and the subsequent production of the arachidonic acid (AA) metabolites, epoxyeicosatrienoic acids (EET), by the cytochrome P450. A recent report has also claimed a direct and potent activation of TRPV4 by AA (Zheng et al., 2013). To date, however, it is not known how EETs mediate channel opening. In any case, whether it binds TRPV4 or is related to changes in the lipid environment, EET-mediated activation of TRPV4 requires the binding of $PIP_2$ to a PIBS at the N-tail (Garcia-Elias et al., 2013). Alternatively, EET-independent mechanisms have also been reported: TRPV4 is activated by membrane stretch in excised-patches from oocytes (Loukin et al., 2010), in apparent contradiction with early reports (Strotmann et al., 2000b) and responds to hypotonic stimuli in yeast, which do not contain AA (Loukin et al., 2009).

**5.3.**

**Activation by temperature**

Moderate heat (24-38º C) activates TRPV4 ($Q_{10}$ between 10 to 20) in heterologous expression systems and native tissues (Garcia-Elias et al., 2013;Guler et al., 2002;Watanabe et al., 2002f), although other studies claimed no role of TRPV4 in mouse thermosensation (Huang et al., 2011).

Early reports (Guler et al., 2002;Watanabe et al., 2002f) showed no channel response to heat in excised patches but it has recently been demonstrated that the reported lack of activation is fully recovered in the presence of $PIP_2$, which suggests that TRPV4 is a *bona fide* thermosensitive channel (Garcia-Elias et al., 2013). Mutation of the PIBS (Garcia-Elias et al., 2013) or Y556 (Vriens et al., 2004;Vriens et al., 2007) impairs TRPV4 activation by heat.

**5.4.**

**Activation by chemicals**

The non- PKC-activating, synthetic phorbol ester 4α-PDD ($EC_{50}$ ~400 nM) (Watanabe et al., 2002d) is widely used as a TRPV4 activator. 4α-PDD binds to a pocket formed between TM 3 and TM4. Mutations of Y556, L584, W586 and M587 affect 4α-PDD-mediated responses (Klausen et al., 2009a;Vriens et al., 2007). Another potent channel activator is GSK1016790A ($EC_{50}$ ~10 nM) (Dunn et al., 2013;Thorneloe et al., 2008a). However, it has been recently reported no activation of TRPV4 by GSK1016790A and TRPV4-independent, 4α-PDD-mediated $Ca^{2+}$ responses in DRG neurons (Alexander et al., 2013).

TRPV4 is activated by bisandrographolide A (BBA, $EC_{50}$ ~800 nM) extracted from *Andrographis paniculata*, a plant commonly used in Chinese traditional medicine, and mutation of L584 and W586, but not of Y556, prevents TRPV4 activation by BBA (Smith et al., 2006a;Vriens et al., 2007). Apigenin, a plant-derived flavones, activates TRPV4 ($EC_{50}$ ~10 μM) in heterologous systems as well as in cultured mesenteric artery endothelial cells (Ma et al., 2012). Plant cannabinoids also activate TRPV4 ($EC_{50}$ ~1-6 μM) (De et al., 2012).

Two endogenous activators of TRPV4 have been identified. The endocannabinoid anandamide produces a robust TRPV4 activation via its metabolite AA and the formation of 5,6-EET (Watanabe et al., 2003b), and dimethylallyl pyrophosphate (DMAPP), a metabolite of the mevalonate

pathway activates TRPV4 ($EC_{50}$ ~5 μM) in heterologous expression systems, cultured sensory neurons and keratinocytes (Bang et al., 2012b).

**5.5.**

**Regulation by calcium**

Calcium-dependent regulation of TRPV4 is complex. Extracellular $Ca^{2+}$ is responsible for the rectification of the whole-cell TRPV4 currents and intracellular $Ca^{2+}$, depending on its concentration, inhibits or potentiates TRPV4 channel activity. Intracellular $Ca^{2+}$-dependent inactivation ($IC_{50}$ ~400 nM) mediates the transient response of TRPV4 to many stimuli (Watanabe et al., 2002e;Watanabe et al., 2003a). Although the exact mechanisms of $Ca^{2+}$ -dependent inactivation are not fully characterized, F707 in TM 6 is involved in the extracellular $Ca^{2+}$ -dependent inactivation (Watanabe et al., 2003a). Positive modulation of TRPV4 by $Ca^{2+}$ via a CaM-dependent mechanism has also been proposed (Strotmann et al., 2003).

**5.6.**

**Modulation by $PIP_2$ and the phospholipase C (PLC)-$IP_3R$ pathway**

Modulation of TRPV4 by the purinergic receptor (P2Y2)-PLC-$IP_3R$ pathway was first described in ciliated epithelial cells and heterologous expression systems (Fernandes et al., 2008;Garcia-Elias et al., 2008;Lorenzo et al., 2008) and later in kidney cells (Mamenko et al., 2011) and astrocytes (Dunn et al., 2013). TRPV4 and many other TRP channels are regulated by $PIP_2$. The interaction of the N-tail PIBS with plasma membrane $PIP_2$ favors an expanded conformation of the intracellular tails as well as channel activation by hypotonicity and heat (Garcia-Elias et al., 2013). Conditions such as mutations in the PIBS, coexpression with PACSIN3 or reduced $PIP_2$ levels interfere the interaction of TRPV4 with $PIP_2$ and promote a compacted tail conformation and prevent channel activation. Following the activation of P2Y2 receptor, the sensitization of TRPV4 activity to low mechanical/osmotic stimulation may be counteracted by depletion of $PIP_2$ due to PLC activation.

The meaning of this dual and antagonistic TRPV4 regulation by the $PIP_2$-PLC-$IP_3R$ pathway remains unclear at present.

### 5.7.

### TRPV4 antagonists

Three blockers have been classically used for inhibition of TRP channels, although none of them are specific: ruthenium red, gadolinium and lanthanum (Nilius et al., 2004). Citral, a bioactive component of lemongrass commonly used as a taste enhancer and insect repellent, is a transient TRPV4 antagonist (Stotz et al., 2008). HC-067047 ($IC_{50}$ ~50-120 nM) has been shown to be a potent and reversible TRPV4 inhibitor that improved bladder function in animal models of cystitis but inhibited TRPM8 at higher concentrations (Everaerts et al., 2010b). GSK2193874 ($IC_{50}$ ~2-100 nM) has been identified as a TRPV4 inhibitor with therapeutic potential against pulmonary edema (Thorneloe et al., 2012).

Butamben (n-butyl-p-aminobenzoic acid), a local anesthetic for topical use known to affect voltage-gated channels, blocks TRPV4 ($IC_{50}$ ~20 μM) and TRPA1 ($IC_{50}$ ~70 μM) (Bang et al., 2012a). RN-1747 and RN-1734 have both agonist ($EC_{50}$ = 700 nM) and antagonist activity ($IC_{50}$ ~2-6 μM), respectively (Vincent et al., 2009). Both compounds affect TRPV1, TRPV3 and TRPM8 channels at higher concentrations (Vincent et al., 2009).

## 6. Physiological functions of TRPV4

### 6.1.

### Osmoregulation and mechanotransduction.

When exposed to hypotonic solutions, cells rapidly swell. The regulatory response to this increase in cell volume is called regulatory volume decrease (RVD), which is normally associated with changes in intracellular $Ca^{2+}$

concentrations, particularly in epithelial cells, and typically activates $K^+$ and $Cl^-$ channels, permiting the passive loss of inorganic ions and osmotically obliged water ((Arniges et al., 2004) and references within). TRPV4 provides the $Ca^{2+}$ signal required to activate $Ca^{2+}$-dependent potassium channels and the subsequent RVD in epithelial cells (Arniges et al., 2004;Fernandez-Fernandez et al., 2002). TRPV4 also acts in astrocyte RVD (Benfenati et al., 2011) and participates in the maintenance of systemic osmoregulation (Liedtke and Friedman, 2003e;Mizuno et al., 2003). TRPV4 is expressed in primary osmosensory neurons in the brains' organum vasculosum (Liedtke et al., 2000d) and kidney epithelium (Berrout et al., 2012;Tian et al., 2004), although the exact mechanism by which TRPV4 participates in systemic osmoregulation is not yet known.

Mechanical and osmotic activation of TRPV4 triggers ATP release from many different epithelial cells (Gevaert et al., 2007c;Seminario-Vidal et al., 2011;Ueda et al., 2011) and increases ciliary beat frequency (CBF) in ciliated epithelia (Andrade et al., 2005). ATP-induced increase in $Ca^{2+}$ and CBF acceleration are also favored by TRPV4 (Lorenzo et al., 2008), which may generate a positive feedback mechanism between ATP- and TRPV4-mediated responses.

TRPV4 channels activated by AA, EET and shear stress are coupled to the calcium-dependent potassium channels in the endothelium (Bagher et al., 2012;Kohler et al., 2006;Sonkusare et al., 2012b;Vriens et al., 2005b) and in vascular smooth muscle (Earley et al., 2005b), thereby favoring vasodilation. TRPV4 is essential to endothelial reorientation in response to mechanical forces, which is required to shape vascular growth and development (Thodeti et al., 2009c). Excessive activation of TRPV4 also bears harmful vasculature consequences due to increased endothelial permeability and circulatory collapse (Thorneloe et al., 2012;Willette et al., 2008). For a recent review on TRPV4 and the control of vascular tone, see (Filosa et al., 2013).

TRPV4 is highly expressed in the kidneys, particularly in the apical water-impermeant regions of the nephron (Delany et al., 2001;Strotmann et al., 2000a) although discrepancies exist on its polarized membrane location (Berrout et al., 2012;Tian et al., 2004). It also has functions in the sensing of flow and osmolality (Wu et al., 2007), RVD (Galizia et al., 2012), ATP release (Silva and Garvin, 2008) and, more importantly, in flow dependent salt reabsortion and potassium secretion (Taniguchi et al., 2006). A heteromeric TRPV4-TRPP2 channel in the primary cilium of collecting duct cells is required for the calcium cascade involved in flow sensing (Du et al., 2012;Kottgen et al., 2008)

TRPV4 is also highly expressed in the bladder urothelium where it participates in the sensing of intravesical mechanical pressure (bladder filling) and ATP release (Birder et al., 2007;Everaerts et al., 2010b;Gevaert et al., 2007e). TRPV4-KO mice manifest an incontinent phenotype with a lower frequency of voiding contractions (Gevaert et al., 2007d).

**6.2**.

**Thermoregulation**

TRPV4 activates at normal body temperatures (see 5.3.), thereby participating in cell functions ranging from regulating neuronal excitability (Shibasaki et al., 2007) and possibly thermogenesis (Guler et al., 2002) to maintaining epithelial barrier function (Sokabe and Tominaga, 2010) and vasodilation (Earley et al., 2005a;Watanabe et al., 2002f). However, direct evidence of TRPV4-mediated vasodilation in response to heat is lacking. Peripheral temperature sensing at the level of keratinocytes, corneal epithelium and sensory neurons has been also associated with TRPV4 (Chung et al., 2003;Lee et al., 2005a;Mergler et al., 2010) but challenged by other studies (Huang et al., 2011).

**6.3.**

**Nociception and neuroinflammation**

TRPV4 is expressed in peripheral nociceptive neurons and has been involved in hyperalgesia. Hypotonic stimuli trigger pain-related behavior by activating TRPV4 channels in dorsal root ganglion neurons (Alessandri-Haber et al., 2003), and TRPV4-KO mice have a lower sensitivity to harmful pressure on the tail (Suzuki et al., 2003b). TRPV4 is sensitized by PKC, PKA and Src phosphorylation (Alessandri-Haber et al., 2008); proteases (Grant et al., 2007); and serotonin, histamine and neurogenic inflammation (Cenac et al., 2010;Vergnolle et al., 2010). This can lead to hypersensitivity.

6.4.

TRPV4 in the central nervous system

In the brain, TRPV4 expression and function has been seen in both neurons and glial cells. Activation of microglia by lipopolysaccharide (LPS) is suppressed following activation of TRPV4 (Konno et al., 2012). TRPV4-mediated $Ca^{2+}$ entry into astrocytic endfeet leads to parenchymal arteriole dilation (Dunn et al., 2013) and in hippocampal CA1 pyramidal neurons potentiates NMDA response and the excitotoxicity associated with cerebral ischemia (Li et al., 2013). Together with TRPV1, TRPV4 is involved in the glucocorticoid-mediated regulation of feeding-related neuroendocrine cells (Boychuk et al., 2013).

**6.5.**

**TRPV4 in cell migration and motility**

In recent years, somewhat contradictory reports on the role of TRPV4 in cell migration have appeared. TRPV4 activation reduces migration of neuroendocrine cells (Zaninetti et al., 2011) but mediates migration of pulmonary artery smooth muscle (Martin et al., 2012) and AA-induced

migration of endothelial cells (Fiorio et al., 2012a). At present no clear explanation exists for these apparent discrepancies.

## 7. Lessons from knock-out mice

Many different studies have made use of TRPV4 knock-out models (*Trpv4*$^{-/-}$). In this section we focus on studies offering novel insights into the physiological role of TRPV4 that have not been introduced in other sections or reporting results that conflict with previous cell-based experiments. Two different *Trpv4*$^{-/-}$ mice models have been generated through neo-replacement of exon 4 (Mizuno et al., 2003) and lox-cre mediated excision of exon 12 (Liedtke and Friedman, 2003d), a fact to be considered in view of contradictory information when comparing functional responses between the two *Trpv4*$^{-/-}$ models.

### 7.1. Thermosensation

Initial studies with *Trpv4*$^{-/-}$ mice revealed the contribution of TRPV4 in detecting warm temperatures (Lee et al., 2005a;Todaka et al., 2004) and chemically-induced hyperalgesia (Todaka et al., 2004). However, more recent studies from the same laboratories showed no thermal response differences between *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice (Huang et al., 2011).

### 7.2. Systemic osmoregulation

In vivo analysis of *Trpv4*$^{-/-}$ mice has produced conflicting results, showing increased (Liedtke and Friedman, 2003a) or unaffected serum osmolarity (Mizuno et al., 2003). In other reports, *Trpv4*$^{-/-}$ mice have no defect in the response to tonicity or mechanical stimulation by the primary osmosensory neurons in the organum vasculosum lamina terminalis (Ciura et al., 2011) but defective responses in peripheral osmosensory neurons (Lechner et al., 2011).

### 7.3.

### Epithelia

Mechanically-induced ATP release and bladder function are strongly impaired in *Trpv4*$^{-/-}$ mice (Gevaert et al., 2007b). Moreover, the development of cystitis-induced bladder dysfunction is lessened in *Trpv4*$^{-/-}$ mice (Everaerts et al., 2010b). TRPV4 activity and ATP release from oesophageal keratinocytes are also reduced in *Trpv4*$^{-/-}$ mice (Mihara et al., 2011). In *Trpv4*$^{-/-}$ mice, the response to different TRPV4-activating stimuli in tracheal ciliated cells displays a reduced $Ca^{2+}$ entry and CBF (Lorenzo et al., 2008). Activation of TRPV4 disrupts the alveolar barrier and activates macrophages, both leading to acute lung injury (Alvarez et al., 2006;Hamanaka et al., 2010).

### 7.4.

### Osteoarticular and muscular systems

Bone resorption defects due to disrupted osteoclast function have been reported for *Trpv4*$^{-/-}$ mice (Masuyama et al., 2008b;Mizoguchi et al., 2008). Normal cartilage physiology also depends greatly on TRPV4 function. Chondrocyte differentiation requires TRPV4 (Muramatsu et al., 2007b) and responses to hypotonic and 4α-PDD are reduced in *Trpv4*$^{-/-}$ mice (Clark et al., 2010).

**7.5**.

**Metabolism**

Knock-out of *Trvp4* induces compensatory increases in TRPC3 and TRPC6, elevation of calcineurin activity affecting energy metabolism in skeletal muscle, and protection from diet-induced obesity in mice (Kusudo et al., 2012). *Trpv4*$^{-/-}$ mice have elevated thermogenesis and protection from diet-induced obesity, adipose inflammation and insulin resistance, highlighting the role of TRPV4 in metabolic disorders (Ye et al., 2012).

**7.6**

Vascular function

The development of pulmonary hypertension, right heart hypertrophy, and vascular remodeling was significantly delayed and suppressed in hypoxic *Trpv4−/−* mice, suggesting that TRPV4 serves as a signal pathway crucial for the development of hypoxia-induced pulmonary hypertension (Yang et al., 2012). TRPV4 plays also a role in blood pressure control. Although portal osmolality decreases after water ingestion in both wild-type and *Trpv4*$^{-/-}$ mice, only the wild-type animals show a pressure response (McHugh et al., 2010).

## 8. TRPV4 in hereditary and acquired diseases

The participation of TRPV4 in disease has been documented at different levels ranging from disease-causing mutations (Fig. 4) and single nucleotide polymorphisms (SNP) to abnormal responses to pathological stimuli. Further research is required to address the intriguing questions that remain.

## 8.1.

## Causal mutations

A puzzling question about the pathophysiological consequences of TRPV4 dysfunction is why the clinically relevant TRPV4 mutations mainly affect osteoarticular and peripheral nervous systems despite wide tissue distribution of TRPV4. Also surprising is the very mild phenotype of *Trpv4*$^{-/-}$ mice under normal conditions. Together, these observations may indicate that the cellular environment is essential to determining TRPV4 function and regulation. Cells from different tissues most likely present different protein networks that modulate the final outcome of TRPV4 functions.

### 8.1.1. Osteoarticular disorders.

The first disease-causing TRPV4 mutations were identified in patients with autosomal dominant brachyolmia (ADB), a rather mild type of skeletal dysplasia (Rock et al., 2008). TRPV4-R616Q and TRPV4-V620I were identified as causal gain-of-function mutants, and 33 other TRPV4 mutations have been linked to different skeletal dysplasias. Due to space restrictions we cannot cite all original studies on TRPV4-causing mutations and, instead, refer the reader to excellent reviews (Dai et al., 2010;Nilius and Voets, 2013). All these skeletal dysplasias form part of a heterogeneous group of bone disorders ranging from mild to lethal. Patients may present abnormalities in vertebrae and tubular bones as well as cartilage, resulting in severe scoliosis, short trunk, and extremities and craniofacial defects. Although phenotypes may differ widely, they all share defects in bone ossification. Furthermore, the same mutation may be found in patients presenting widely different phenotypes.

Three TRPV4 mutations have been found in familial digital arthropathy-brachydactyly (FDAB), an inherited arthropathy in hands and feet

with a related severe osteoarthritis (OA)(Lamande et al., 2011). These three mutations presented increased baseline but decreased stimuli-dependent channel activity. The mechanism by which these mutations lead to OA is not known. Previous studies in animal models had shown that TRPV4 was responsible for the hypotonic responses seen in articular chondrocytes and that TRPV4 KO mice had an age- and sex-dependent progression to OA (Clark et al., 2010). Still unanswered is the question of how TRPV4 mutations lead to osteoarticular pathology: Is the cause of the disease related to changes in TRPV4 channel activity and/or TRPV4 interaction with other proteins?

### 8.1.2. Neuropathies.

TRPV4-linked neuropathies were first described in 2010 (Auer-Grumbach et al., 2010;Deng et al., 2010;Landoure et al., 2010). Although very heterogeneous, all of the disorders lead to a degeneration of peripheral nerves. They may occur alone or with sensory-associated phenotypes such as vocal cord paresis (Chen et al., 2010) or hearing defects (Zimon et al., 2010). Wide variability in phenotype or in age at onset is observed, even between families that share the same causal mutation. Genetic and environmental factors are likely responsible for such variation, but further studies should clarify this point. As seen with the skeletal disorders, most of the neuropathy-related TRPV4 mutations generate gain-of-function and the same mutation can produce different phenotypes (Nilius and Voets, 2013). Single-channel analysis of skeletal (Loukin et al., 2011) and neuronal (Fecto et al., 2011) disease-causing mutations revealed increased basal open probability of mutant channels.

### 8.1.3. Mixed skeletal and neuromuscular disorders.

A few TRPV4 mutations have been associated with both skeletal and neuromuscular disorders. Patients with motor neuropathies have a short trunk (Chen et al., 2010;Cho et al., 2012), although patients with the mild forms of

skeletal dysplasias rarely have any neuropathy except in metatropic dysplasia with fetal akinesia (Unger et al., 2011).

## 8.2.

## Single nucleotide polymorphisms and abnormal TRPV4 responses associated with disease

The rs3742030 polymorphism (P19S) generates a TRPV4 channel with reduced response to mild hypotonic shocks and is associated with higher risk of hyponatremia (Tian et al., 2009) and, chronic obstructive pulmonary disease and forced expiratory volume in 1 second ($FEV_1$) (Zhu et al., 2009) but not with cough in asthmatic children (Cantero-Recasens et al., 2010) or healthy/asthmatic adults (Smit et al., 2012). Dysregulation of TRPV4 has been described in cystic fibrosis epithelium (Arniges et al., 2004). TRPV4 mRNA and protein is increased in sinus mucosal biopsies from chronic rhinosinusitis patients (Bhargave et al., 2008). TRPV4 participates in the inflammatory signaling pathways leading to neurogenic inflammation and pancreatitis (Ceppa et al., 2010;Zhang et al., 2013), intestinal chronic inflammation (d'Aldebert et al., 2011;Fichna et al., 2012) and mastication-associated pain at the temporomandibular joint (Chen et al., 2013).

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**Figure 1.**

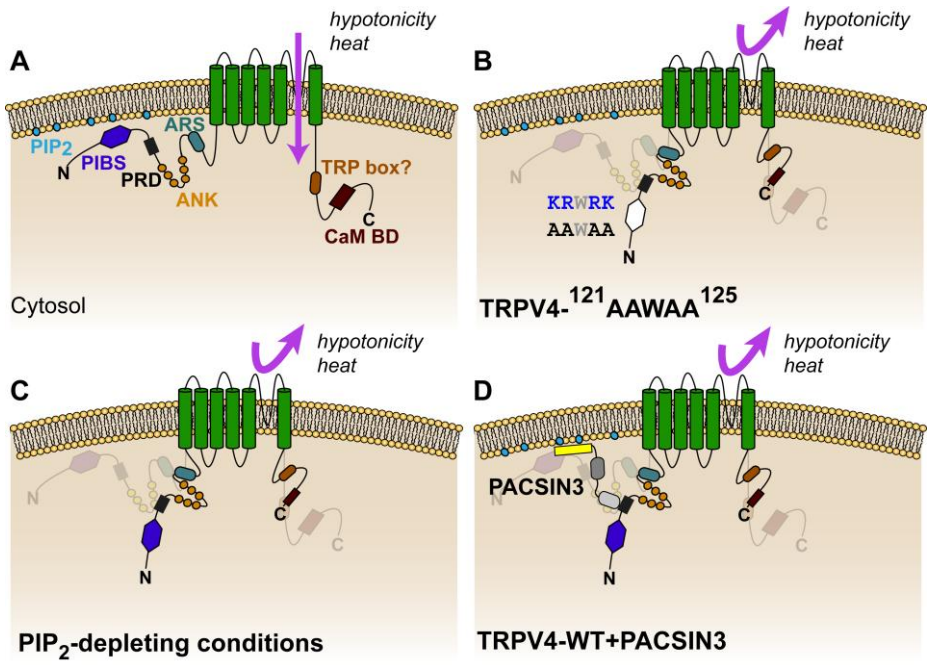


**Figure 1. Domain structure and $PIP_2$-dependent functional rearrangement of TRPV4**. **A**, cartoon of a single TRPV4 protein in its expanded conformation due to interaction of the N-tail with $PIP_2$. Phosphoinositide binding site (PIBS), proline-rich domain (PRD), ankyrin domains (ANK), archidonate-like recognition sequence (ARS), the six transmembrane domains, a questioned TRP box and the CaM binding domain (CaM BD). Intracellular tail rearranges into a more compacted form upon neutralization of positive charges in the PIBS (**B**), depleting $PIP_2$ from the plasma membrane (**C**) or coexpression of TRPV4 with PACSIN3 (**D**). The expanded conformation is required for TRPV4 response to hypotonic and heat stimuli.

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**Figure 2.**

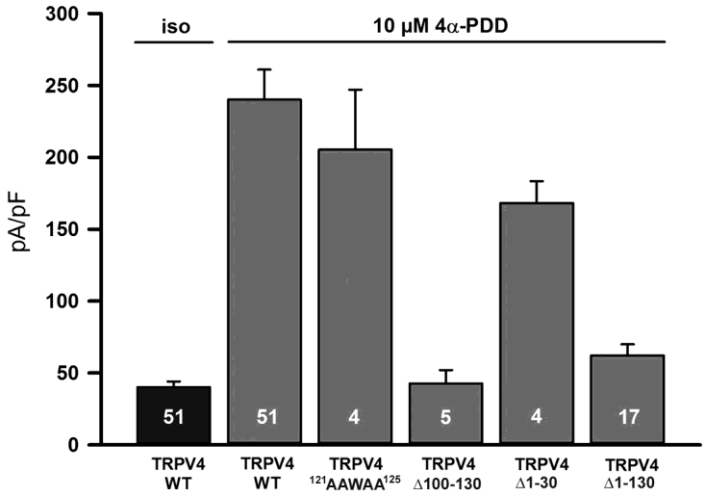


**Figure 2. Response to 4α-PDD of N-terminal truncations and mutations of TRPV4.** Mean current responses to 4α-PDD (10 µM) stimulation in cells transfected with TRPV4-WT, TRPV4-$^{121}$AAWAA$^{125}$, TRPV4-Δ1-30, TRPV4-Δ1-130, TRPV4-Δ100-130. Number of cells recorded is shown for each condition. Iso: isotonic.

# Figure 3.

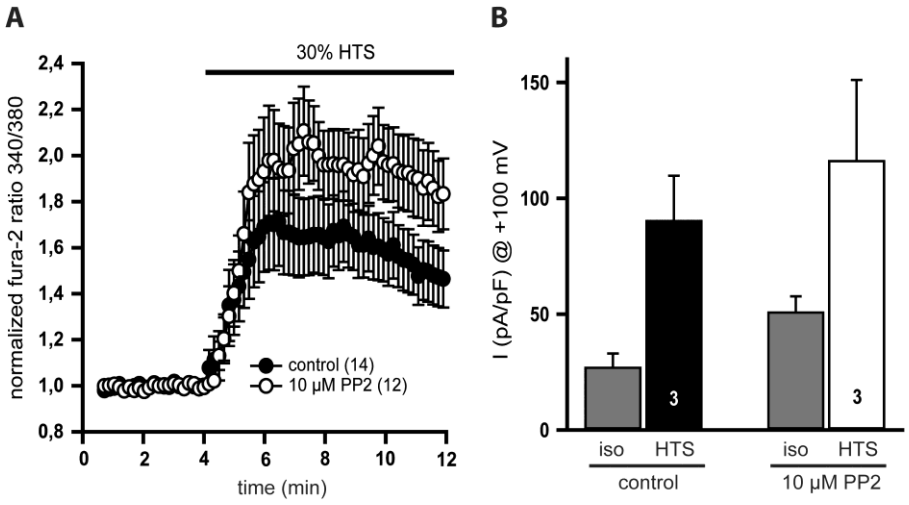


**Figure 3. Tyrosine phosphorylation and TRPV4 activation**. Calcium signals (A) and whole-cell currents (B) obtained in HeLa cells transfected with TRPV4-WT and exposed to vehicle (control) or the tyrosine kinase inhibitor PP2 (10 μM). Iso: isotonic; HTS: 30% hypotonic solution.

## Figure 4.

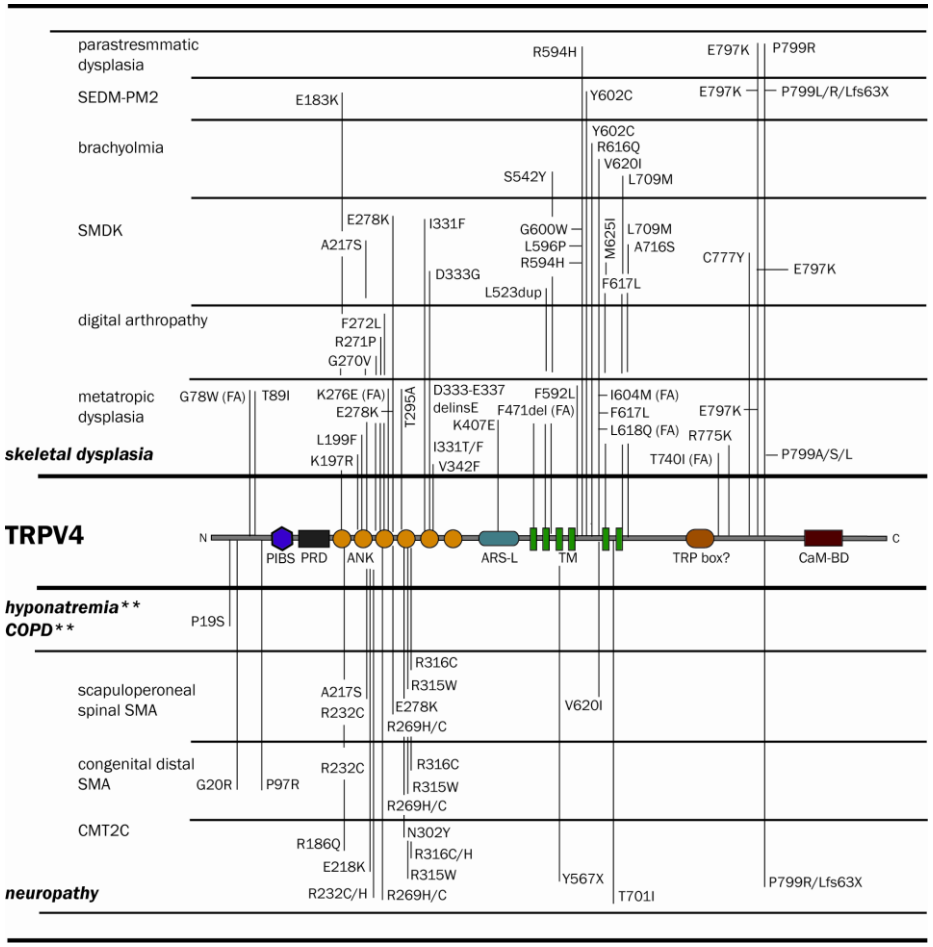


**Figure 4. TRPV4 mutations related to human diseases.** TRPV4 mutations and SNPs associated to different skeletal dysplasias, neuropathies, hyponatremia and COPD are shown. Each mutation is positioned over the schematic representation of the channel. **TRPV4-P19S SNP, although not causal, has been associated with hyponatremia and COPD. *PRD, proline-rich domain; ANK, ankyrin repeats; ARS-L, arachidonate recognition sequence-like; TM, transmembrane segments; CaM-BD, calmodulin-binding domain; CMT2C, hereditary motor and sensory neuropathy 2C (Charcot-Marie-Tooth 2C disease); SMA, spinal muscular atrophy; COPD, chronic obstructive pulmonary disease; SMDK, spondylometaphyseal dysplasia Kozlowski type; SEDM-PM2, spondyloepimetaphyseal dysplasia Maroteaux pseudo-Morquio type 2. Adapted from Dai et al. (2010).*