TRPV4-mediated channelopathies

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Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; 4αPDD, 4α Phorbol 12; 13-didecanoate; AD, association domain; AIP4, atrophin-1-interacting protein 4; ARD, ankyrin repeat domain; ATP, adenosine-5-triphosphate; CAM, calmodulin; CMT2C, charcot marie tooth disease type 2C; EM, electron microscope; ERAD, endoplasmic reticulum associated degradation; FRET, fluorescence resonance energy transfer; HECT, homologous to E6-AP carboxyl terminus; HEK cell, human embryonic kidney cell; HMSN2C, hereditary motor and sensory neuropathy type 2; MVB, multi vesicular body; NMR, nuclear magnetic resonance; OSM9, OSMotic avoidance abnormal family member 9; OS9, osteosarcoma amplified 9; PASCIN, protein kinase C and casein kinase substrate in neurons protein 1; PRD, proline-rich domain; RGA, recombinase gene activator; SEDM-PM2, spondylo-epiphysyeal dysplasia, maroteaux type (pseudo-morquio syndrome type 2); STAM, signal transducing adaptor molecule; SMA, spinal muscular atrophy; SMDK, spondylometaphyseal dysplasia kozlowski; SPSMA, scapuloperoneal spinal muscular atrophy; TM, transmembrane; TRP, transient receptor potential; TRPP2, transient receptor potential polycystin; TRPV, transient receptor potential vanilloid; TRPV1, transient receptor potential vanilloid sub type 1; TRPV4, transient receptor potential vanilloid sub type 4

Transient receptor potential vanilloid sub type 4 (TRPV4) is a member of non-selective cation channel that is important for sensation of several physical and chemical stimuli and also involved in multiple physiological functions. Recently it gained immense medical and clinical interest as several independent studies have demonstrated that mutations in the TRPV4 gene can results in genetic disorders like Brachyolmia, Charcot-Marie-Tooth disease type 2C, Spinal Muscular Atrophy and Hereditary Motor and Sensory Neuropathy type 2. Close analysis of the data obtained from these naturally occurring as well as other TRPV4 mutants suggest that it is not the altered channel activity of these mutants per se, but the involvement and interaction of other factors that seem to modulate oligomerization, trafficking and degradation of TRPV4 channels. Also, these factors can either enhance or reduce the activity of TRPV4. In addition, there are some potential signaling events that can also be involved in these genetic disorders. In this review, we analyzed how and what extent certain cellular and molecular functions like oligomerization, surface expression, ubiquitination and functional interactions might be affected by these mutations.

Introduction

In spite of sharing high degree of homology and identity, members of TRPV channels retain their uniqueness in terms of structure, function and ability to recognize different physical and chemical stimuli. Among all TRPV channels, TRPV4 is unique as it can be activated by temperature, pressure and also by specific chemical ligands. TRPV4 is functionally conserved throughout the evolution as mammalian TRPV4 can rescue the mechanosensitive defects observed in OSM9 mutants in C. elegans, suggesting that the functional and interacting network related to TRPV4 may be conserved to a large extent and TRPV4 functionality is important for some organism. Recent studies demonstrated that missense mutations result in either constitutively-active or constitutively-inactive TRPV4 channels, which leads to inheritable genetic disorders. To make it more complicated, point mutations at the same position generates different mutant TRPV4 channels that not only exhibit different electrophysiological properties in vitro but also reveals different level of surface expression. Based on the available data, we summarize the latest understanding of the structure–function relationship of TRPV4 and critically analyze how different mutations can affect the structure, function as well as the regulation of TRPV4 at the cellular level. Our analysis also indicates some other factors that may act as a missing link and probably also contributing in these genetic disorders and involved in TRPV4 functions.

Structure of TRPV4 and Different Interacting Proteins

At the functional level, four subunit of TRPV4 assemble in proper order to form a functional channel which can conduct ionic influx. At present, no crystallographic or nuclear magnetic resonance (NMR) data is available that can shed light on the fine atomic structure of functional TRPV4. However, recently, structure of Rat TRPV4 was analyzed at a resolution of 3.5 nm by cryo-electron microscopy. This electron microscopic (EM) study was conducted on His-tagged TRPV4 expressed in Baculovirus infected Sf9 cells, solubilized with detergents and further purified by several chromatography columns. This study reveals that

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functional TRPV4 forms a “hanging basket”-like structure, which is approximately 130 Å in length (from top to bottom) and 85 Å in width respectively. Approximately 30% volume of the functional channel lies in the plasma membrane and 70% of the total volume remains hanging from the plasma membrane. This 3D structure indicates that trans-membrane and/or membrane-integral proteins as well as several cytoplasmic proteins can interact with the TRPV4 and these interactions can modulate the structure–function relationship of TRPV4. As TRPV1 and TRPV4 share a high-degree (~41%) of sequence identity and functional TRPV1 also forms a similar “hanging basket”-like structure,\(^{10}\) it is justified to assume that TRPV4 and TRPV1 share similarity in structure–function relationship to some extent.

In addition to this EM structure, certain structural information can also be extracted on the basis of characterizations performed on smaller fragments of TRPV4. The mammalian [based on Rat (gi62901120), Mice (gi62901468) and Human (gi62901470) sequence] TRPV4 polypeptide is 871 amino acid long with both the N- and C-terminal domains located at the cytoplasmic side. The N-terminal cytoplasmic domain contains six Ankyrin Repeat Domains (ARD) that are specifically located between amino acid residues 132–383.\(^5\) These ARDs are predicted to be involved in the interaction with several proteins (discussed later). These ARDs are also the key molecular determinants assumed to be important for subunit assembly and interaction with different molecules.\(^{11,12}\)

Apparently, the C-terminal cytoplasmic region of TRPV4 does not have any specific domain or motifs that can impart some special structure-function prediction related to TRPV4. However, the C-terminal cytoplasmic domain of TRPV4 contains a conserved TRP-motif, which is a specific characteristic signature present in many other TRP channels.\(^{13}\)

At present, only few proteins have been identified which are known to interact with TRPV4. The C-terminus of TRPV4 is known to interact with IP3 receptor and Calmodulin.\(^{14,15}\) The Calmodulin interaction seems to regulate the self-interaction of N- and C-terminal of TRPV4 in a Ca\(^{2+}\)-dependent manner.\(^{16}\) In addition, C-terminus of TRPV4 shares a high homology with the tubulin-binding motif sequence of TRPV1 which suggests that TRPV4 can also be a part of microtubule cytoskeleton.\(^{17}\) Indeed, MAP7, a microtubule binding protein and soluble tubulin as well as polymerized microtubule interact with TRPV4, especially at the C-terminal region of TRPV4.\(^{18,19}\) In a similar manner, close proximity of TRPV4 and actin filaments has been demonstrated by using fluorescence resonance energy transfer (FRET).\(^{20}\)

As TRPV4 is involved in the Charcot-Marie-Tooth disease type 2 (CMT2), it suggests that TRPV4 is genetically linked with other genes that are also involved in the same disease and thus TRPV4 share a special genotype-phenotype correlation with these gene products.\(^{21}\) Thus, several proteins, namely Kif1b, Neurofilament L, Mfn2, Rab7a, Lamin A, Med25, GarS, heat shock protein 27, MPZ, GDAP1 and heat shock protein 22, which are also involved in CMT2 disease, are actually potential TRPV4 interacting partners.\(^{21}\) Indeed, our recent study confirmed that Neurofilament protein physically interacts with TRPV4.\(^{22}\) In the same context, Kif1B, which is also involved in the CMT2 disease and/or peripheral neuropathic pain development, may be responsible for cellular transport and surface expression of TRPV4.\(^{22}\)

Naturally Occurring TRPV4 Mutants and Genetic Disorder

Recently, few naturally occurring TRPV4 mutants have been identified. Interestingly, most of these miss-sense and nonsense point mutations are linked with the development of genetic disorders in human and a detailed list of naturally occurring TRPV4 mutations and related disease is documented (Table 1). Here we briefly discuss some of these mutations gained importance in terms of genetic disease.

**Brachyolmia.** Using a linkage analysis and candidate gene sequencing, Rock et al. have identified that some patients affected with brachyolmia have missense mutation in TRPV4, specifically at position R616Q or V620L respectively.\(^7\) These mutations are located at the 5\(^{th}\)-transmembrane region which is a part of the functional pore. Each of these two mutations increases basal level activity when compared to the wild type TRPV4. Also the response to 4αPDD (a TRPV4 specific agonist) is more in the mutants when compared with the wild type. This result also indicates that these two mutations preferably stabilize TRPV4 in its “open stage” resulting in constitutive activity of the channel.

**Spinal Muscular Atrophy (SMA).** SMA is a heterogeneous disorder of peripheral nervous system. Patients with SMA have been reported to have several missense mutations in the TRPV4, namely at R316C, R269H and R315W.\(^4\) These mutations are located at the ARD of TRPV4. These (mutants R316C, R269H and R315W) reveal loss of function when challenged by hyposmotic solution and 4αPDD.\(^4\)

**Hereditary Motor and Sensory Neuropathy type 2 (HMSN2C).** Charcot-Marie-Tooth disease type 2C (CMT2C) and Scapuloperoneal Spinal Muscular Atrophy (SPSMA) are also known as HMSN2C. SPSMA patients are characterized by weakness of scapular muscle and bone abnormalities. CMT2C leads to weakness of distal limbs, vocal cords and often impairs hearing and vision. Genetic analyses of these patients have shown the presence of missense mutations at the TRPV4, especially at the R269H, R315W and R316C positions.\(^9\)

**Spondylometaphyseal dysplasias (SMDK) and metatropic dysplasia.** SMDK is an autosomal dominant dysplasia. Genetic mapping of the patients affected with this disease have shown missense mutation in TRPV4, either at R594H, D333G or A716S.\(^27\) Any of these mutations seems to alter the basal level activity. In addition, I331F and P799L mutations are known to induce metatropic dysplasia.\(^27\)

As all these above mentioned mutants are naturally occurring, these mutants are not embryonically lethal (as most lethal mutants will be naturally excluded from the population). It is also important to note that none of these mutants show complete loss of their prime function, i.e., the ionic conductivity. Indeed, experimental results suggest that some of these mutants even have enhanced channel opening.\(^7\) As most of the patients are heterozygous, it can be concluded that it is not only the ionic conductivity of the TRPV4 per se, but also the signaling events that...
A better and further understanding of TRPV4 structure, interaction and function can also be derived from studies that have generated and characterized several artificial TRPV4 mutations (Table 2). All these mutations mostly alter single amino acids or cause deletion of specific regions. The change (or loss) of these residues (or regions) correlates well with the change in pathophysiology. This is in agreement with the observation that TRPV4 knock-out animals do not reveal embryonic lethality but develop some pathophysiological disorders like hearing loss, impaired pressure sensation, reduced osmoregulation, defective bladder function and impaired release of antidiuretic hormone.2,28-31

Table 1. Naturally occurring TRPV4 mutations

| Mutation | Residue | Change in charge | Domain/motif affected | Effects on ion conductivity | Genetic disorder | Ref |
|----------|---------|------------------|-----------------------|-----------------------------|-----------------|-----|
| 1        | -       | P19S             | Nonpolar to polar     | N-terminal                  | Less conductivity | hyponatermia | 23 |
| 2        | C366T (exon 2) | T89I             | Polar (uncharged) to nonpolar | N-terminal                  | Not done          | Metatropic dysplasia | 24 |
| 3        | G547A (exon 3) | E183K             | Negative to plus      | ARD1                        | Not done          | SEDM-PM2 | 25 |
| 4        | A590G (exon 4) | K197R             | Plus to plus          | ARD2                        | Not done          | Metatropic dysplasia | 24 |
| 5        | -       | L199F             | Nonpolar to aromatic  | ARD2                        | Not done          | Metatropic dysplasia | 26 |
| 6        | G806A (exon 5) | R269H             | Plus to plus          | ARD3                        | Less conductivity | SMA | 4 |
| 7        | G806A (exon 5) | R269H             | Plus to plus          | ARD3                        | More conductivity | CMT2C, SPSMA | 6 |
| 8        | G806A (exon 5) | R269H             | Plus to plus          | ARD3                        | More conductivity | CMT2C | 5 |
| 9        | G806A (exon 5) | R269C             | Plus to polar uncharged | ARD3                        | More conductivity | CMT2C | 5 |
| 10       | -       | E278K             | Negative to plus      | ARD3                        | Not done          | SMDK | 26 |
| 11       | -       | T295A             | Polar (uncharged) to nonpolar | ARD4                        | Not done          | Metatropic dysplasia | 26 |
| 12       | C943T (exon 6) | R315W             | Plus to aromatic      | ARD4                        | Less conductivity | HMSN2C | 4 |
| 13       | C946T (exon 6) | R316C             | Plus to polar (uncharged) | ARD4                        | Less conductivity | HMSN2C | 4 |
| 14       | A1080T (exon 6) | I331F             | Nonpolar to aromatic  | ARD5                        | Not done          | Metatropic dysplasia | 27 |
| 15       | -       | I331T             | Nonpolar to polar (uncharged) | ARD5                        | Not done          | Metatropic dysplasia | 26 |
| 16       | A992G (exon 6) | D333G             | Negative to nonpolar  | ARD4                        | More conductivity | SMDK | 27 |
| 17       | -       | V342F             | Nonpolar to aromatic  | ARD5                        | Not done          | Metatropic dysplasia | 26 |
| 18       | -       | FS92L             | Aromatic to nonpolar  | TM4                         | Not done          | Metatropic dysplasia | 26 |
| 19       | G1781A (exon 11) | R594H             | Plus to plus          | TM4                         | More conductivity | SMDK | 27 |
| 20       | A1805G (exon 11) | Y602C             | Aromatic to polar     | TM4-TMS                     | Not done          | SEDM-PM2 | 25 |
| 21       | C1812G (exon 11) | I604M             | Nonpolar to nonpolar  | TM4-TMS                     | Not done          | Metatropic dysplasia | 24 |
| 22       | G1847A (exon 12) | R616Q             | Plus to polar uncharged | TMS, pore region            | More conductivity | Brachylomia | 7 |
| 23       | C1851A (exon 12) | F617L             | Aromatic to nonpolar  | TMS, pore region            | Not done          | Metatropic dysplasia | 24 |
| 24       | T1853C (exon 12) | L618Q             | Nonpolar to polar (uncharged) | TMS, pore region            | Not done          | Metatropic dysplasia | 24 |
| 25       | G858A (exon 12) | V620I             | Nonpolar to nonpolar  | TMS, pore region            | More conductivity | Brachylomia | 7 |
| 26       | -       | M625I             | Nonpolar to nonpolar  | TMS, pore region            | Not done          | SMDK | 26 |
| 27       | -       | L709M             | Nonpolar to nonpolar  | TMS, pore region            | Not done          | SMDK | 26 |
| 28       | C2146T (exon 13) | A716S             | Nonpolar to polar     | Cytoplasmic side of TM6     | Same as wild type | SMDK | 27 |
| 29       | -       | R775K             | Plus to plus          | C-terminal region           | Not done          | Metatropic dysplasia | 26 |
| 30       | -       | C777Y             | Polar (uncharged) to aromatic | C-terminal region           | Not done          | SMDK | 26 |
| 31       | -       | E797K             | Negative to plus      | C-terminal region           | Not done          | SEDM-PM2 | 26 |
| 32       | -       | P799R             | Nonpolar to plus      | C-terminal region           | Not done          | Metatropic dysplasia | 26 |
| 33       | -       | P799S             | Nonpolar to polar (uncharged) | C-terminal region           | Not done          | Metatropic dysplasia | 26 |
| 34       | -       | P799A             | Nonpolar to non polar | C-terminal region           | Not done          | Metatropic dysplasia | 26 |
| 35       | C2396T (exon 15) | P799L             | Nonpolar to nonpolar  | C-terminal region           | Not done          | SMDK | 27 |
| No | Mutation | Domain | Change | Effects | Species | Refs |
|----|----------|--------|--------|---------|---------|------|
| 1  | P142A    | Proline-rich domain | Nonpolar to nonpolar | PASCIN binding abolished | Murine | 32   |
| 2  | P143L    | Proline-rich domain | Nonpolar to nonpolar | PASCIN binding abolished | Murine | 32   |
| 3  | R151D    | Proline-rich domain | Plus to negative | No change in response to 4αPDD | Murine | 33   |
| 4  | R151A    | Proline-rich domain | Plus to nonpolar | No change in response to 4αPDD | Murine | 33   |
| 5  | R151Q    | Proline-rich domain | Plus to polar (uncharged) | No change in response to 4αPDD | Murine | 33   |
| 6  | R151K    | Proline-rich domain | Plus to plus | No change in response to 4αPDD | Murine | 33   |
| 7  | P152A    | Proline-rich domain | Nonpolar to nonpolar | No effect | Murine | 32   |
| 8  | K178A    | ARD 2 | Plus to nonpolar | Reduced binding to CAM and ATP | Chicken | 34   |
| 9  | K183A    | ARD2 | Plus to nonpolar | Reduced binding to CAM and ATP | Chicken | 34   |
| 10 | K185A    | ARD2 | Plus to nonpolar | Reduced binding to CAM and ATP | Chicken | 34   |
| 11 | Y253F    | TM3 | Aromatic to aromatic | No response in Ca\textsuperscript{2+}-permeability to hypotonic swelling | Mouse | 35   |
| 12 | N456H    | TRP box | Polar (uncharged) to plus | Marginal response to APB | Mouse | 36   |
| 13 | Y55A     | TM3 | Aromatic to nonpolar | Strongly impaired permeability, lower basal level | Mouse | 35   |
| 14 | Y55F     | TM3 | Aromatic to nonpolar | Same as wild type | Mouse | 35   |
| 15 | S556A    | TM3 | Polar to nonpolar | Lower basal level activity | Mouse | 35   |
| 16 | Y566A    | TM4 | Aromatic to nonpolar | Increased basal level Activity | Murine | 37   |
| 17 | L584M    | TM4 | Nonpolar to nonpolar | Increased basal level Activity | Murine | 37   |
| 18 | W586A    | TM4 | Aromatic to nonpolar | Increased basal level Activity | Murine | 37   |
| 19 | M587A    | TM4 | Nonpolar to nonpolar | Marginal response | Murine | 37   |
| 20 | N588A    | TM4 | Polar (uncharged) to polar | Marginal response | Murine | 37   |
| 21 | Y591A    | TM4 | Aromatic to nonpolar | Marginal response | Murine | 37   |
| 22 | R594Q    | TM4 | Plus to polar (uncharged) | No response | Murine | 37   |
| 23 | R594A    | TM4 | Plus to nonpolar | No response | Murine | 37   |
| 24 | R594K    | TM4 | Plus to plus | Increased response to 4αPDD | Murine | 37   |
| 25 | L619P    | TMS | Nonpolar to nonpolar | Increase in response to 4αPDD (Gain of function) | Rat | 38   |
| 26 | L623P    | TMS | Nonpolar to nonpolar | Not done | Rat | 38   |
| 27 | N651Q    | TM5-TM6, pore region | Polar (uncharged) to polar (uncharged) | Increase surface expression and increased Ca\textsuperscript{2+} influx in response to hypotonicity | Murine | 39   |
| 28 | D672A    | Pore region | Negative to nonpolar | Reduced Ca\textsuperscript{2+}-permeability | Mouse | 40   |
| 29 | D672K    | Pore region | Negative to plus | Decreased osmotic response | Rat | 2    |
| 30 | K675A    | Cytoplasmic pore region | Plus to nonpolar (uncharged) | No change | Mouse | 40   |
| 31 | M680A    | Pore region | Nonpolar to nonpolar | Decreased Ca\textsuperscript{2+}-selectivity | Mouse | 40   |
| 32 | M680K    | Pore region | Nonpolar to plus | Lack of channel activity | Rat | 2    |
| 33 | M680D    | Pore region | Nonpolar to negative | Complete loss of Ca\textsuperscript{2+}-selectivity | Mouse | 40   |
| 34 | D682A    | Pore region | Negative to nonpolar | Decreased Ca\textsuperscript{2+}-selectivity | Mouse | 40   |
| 35 | D682K    | Pore region | Negative to plus | Decreased osmotic response | Rat | 2    |
| 36 | F707A    | TM6 | Aromatic to nonpolar | Increased response to 4αPDD | Murine | 33   |
| 37 | F707K    | TM6 | Aromatic to plus | Resulted in non-functional channel | Murine | 33   |
| 38 | F707D    | TM6 | Aromatic to negative | Resulted in non-functional channel | Murine | 33   |
| 39 | M713V    | TM6 | Nonpolar to nonpolar | Not done | Rat | 38   |
| 40 | M713I    | TM6 | Nonpolar to nonpolar | Gain of function | Rat | 38   |
| 41 | W733R    | C-terminal region | Aromatic to plus | Not done | Rat | 38   |
| 42 | W737R    | TRP-BOX | Aromatic to plus | Increased sensitivity to APB (Gain of function) | Mouse | 36   |
Can These Mutations Affect TRPV4 Oligomerization?

Recently a number of studies have characterized the assembly and oligomerization of TRP channels. Members of TRP family can also form homo- or hetero-tetramer. It has been also postulated that the assembly and oligomerization can occur in phases to get functional conformation of the channel. For example, in TRPC, tetramerization occurs through interaction of association domain 1 (AD1) (N-terminal region) followed by interaction with AD2 (putative pore region S4-S5 and C-terminal region). However, in spite of several studies, the molecular mechanism underlying the assembly of TRPV monomers into functional tetramer is still at infancy. Thus cells expressing A and D isotypes can form functional channels. Thus cells expressing A and D isotypes can form functional channels. This conclusion is drawn from the reduced Ca²⁺-peaks observed after activation α-PDD in cells expressing TRPV4 mutants. Cells expressing -47-50 This conclusion is drawn on the basis of the fact that TRPV4 splice variants namely B, C and E isotypes cannot assemble into functional channel. These isotypes lack regions located at the N-terminal region, are sequestered in endoplasmic reticulum and thus cannot reach to the plasma membrane. In contrast, A and D isotypes are transported to the membrane and can form functional channels. Thus cells expressing A and D isotypes can respond against TRPV4-specific stimuli. This result is in line with another study which demonstrated that the N-terminal region of TRPV4 is responsible for homotetramer formation. In the agreement with the role of ARDs in the oligomerization and surface expression, mutations namely R269H, R315W and R316C result in loss of function due to reduced surface expression, at least when expressed in HeLa cells. This conclusion is drawn from the reduced Ca²⁺-peaks observed after activation with 4α-PPD in cells expressing TRPV4 mutants. Cells expressing these mutants also contain aggregated patches all over the cytoplasm. As all these mutations are located at the ARDs, these results strongly suggest that ARDs located at the N-terminal region play important role in subunit assembly and act as prime site for binding auxiliary proteins.

How the N-terminal region contributes and regulates all these properties is currently unknown. The ARDs can possibly act as scaffold or adaptor and help the interaction of TRPV4 with other proteins. In a bigger context, these results suggest that oligomerization of TRPV4 is important not only for its trafficking but also for its function.
and surface expression but also for functional properties like selectivity and gating mechanisms. Like all other membrane proteins, assembly of TRPV4 also occurs in endoplasmic reticulum and it undergoes diverse modifications like disulfide bond formation and glycosylation. Coiled-coil domain (protein oligomerization unit), ARDs and transmembrane domains of TRPV4 seem to be important for tetrameric assembly. Thus, any mutation in these regions can potentially result in abnormal channel assembly and/or activity. Within endoplasmic reticulum, proper folding of TRPV4 and further tetramer formation seems to be facilitated by interaction with OS-9 which has chaperone-like activity. Though the exact position where OS-9 interacts with monomeric TRPV4 is not known, it is known to interact at the N-terminus of TRPV4 and especially within amino acid 40–235. This interaction can prevent misfolding of TRPV4 and further polyubiquitination of misfolded monomeric TRPV4. Therefore this interaction can potentially reduce the degradation of TRPV4 by endoplasmic reticulum-associated degradation (ERAD) pathway and/or by 26S proteasome.

### How These Mutations Can Affect Function and Surface Expression of TRPV4?

Previously it has been shown that deletion of extreme C-terminal 16 amino acid residues does not alter the surface expression as truncated TRPV4 having 1–855 amino acids can exported to the membrane and this surface expression is equivalent to the wild type TRPV4 (amino acid 1–871). However, the same study revealed that the deletion of 16 amino acids at the region of 828–844 results in accumulation of TRPV4-∆828-844 in the ER. Another report demonstrate that deletion of amino acid residue 132–144 (located at the N-terminal cytoplasmic domain), i.e., deletion of Proline-rich domain (PRD) results in loss of channel function despite having proper trafficking at the membrane. It has also been demonstrated that interaction of PACSIN 3, a cytoskeletal protein to the N-terminal region of TRPV4 enhances the membrane localization. Though all these studies characterized the trafficking of TRPV4 to a large extent, the understanding of surface expression of TRPV4 and actual regulation/s underlying this is still fragmented. As both N- and C-terminal regions seems to be important, the reported self-interaction between N- and C-terminal of TRPV4 mediated by Calmodulin and Ca²⁺ might play an important role here.

TRPV4-R269H mutant mostly accumulated in the cytoplasm, indicating that amino acid R269 located at the 3rd ARD is important for the surface expression, at least in case of HeLa cells. However, using a different cellular system, namely HEK cell, another group has reported that the same TRPV4-R269H mutant has proper cell surface expression. A similar study revealed that a different mutation at the same position, i.e., TRPV4-R269C does not have an altered surface expression in HEK cells. In contrast, R316C reveals a loss of function (compared to the wild type) when expressed in HEK cells while the same mutation reveals a gain of function in HEK cells. Though, these results appear as contradicting to each other, these data reveal important clues about the surface expression of TRPV4 and also raise an important and interesting question: how the surface expression of TRPV4 is regulated? As the R residues at the position of 269 and 315–316 cannot act as potential phosphorylation sites, altered functions of the relevant mutations cannot be explained by direct phosphorylation-mediated regulation on TRPV4. Preliminary bioinformatic analysis (AGADIR prediction algorithm, available at http://agadir.crg.es/) with human TRPV4 sequence (NCBI number gi62901470) indicate that substitution of R to H (R269H) or R to C (R269C) at the position of 269 and substitution of R to W (R315W) or R to C (R316C) at the position of 315–316 can potentially change the structure of these regions, especially the alpha-helical propensity and/or helical percentage of that region to some extent. These changes might be important if compared in case of permissive temperature (T = >37°C to 45°C, a temperature range in which TRPV4 can be activated) within non-permissive temperature (T = <37°C to 15°C, a temperature range in which TRPV4 should not be activated). R to K substitutions (R269K and R315K) in these positions is known to rescue the functions of the mutants (R269H and R315C) respectively. Interestingly, the bioinformatic analysis reveals that these rescue mutants may have helical properties in these regions that are similar to the wild-type. However, at present, the true helicity of these two important regions are not known and further experimental proof is required to confirm if these mutations can indeed alter the helicity/secondary structure of these regions.

Interestingly, R269 is located within the ARD-helix and brings a positive charge at the surface of the ARD3 and thus may be crucial for interaction with other protein. Though R269K mutation rescue the effect of R269H mutation, critical analysis of relevant mutations reveal few important clues: first, a specific positive charge at the position of 269 is neither very important nor plays the prime determining factor. This is due to the fact that substitution of polar R (10.75) with another polar residue H (7.59, though having a different side chain and size) results in abnormal trafficking and accumulation of mutant TRPV4 at the cytoplasm. Secondly, substitution of polar R (10.75) with a non-polar residue C (5.07) does not alter the surface expression. Also substitution of R with K (9.74) can rescue the effect. Thus, a change in the size and/or at the side chain of the residues at 269 and mutation-induced local yet minute change in the structure seems to be more important than the positive charge. The contradictory results from Auer-Grumbach et al. and Deng et al. may also suggest a differential interaction and/or regulation of surface expression of TRPV4 even in different cell lines of Human origin (HeLa and HEK). As most of the point mutations are observed in the N-terminal region, specifically at the position of ARDs (R269H, R315W, R316C), a loss or at least a different route of trafficking seems to be relevant. Considering that constitutive activation of any ion channel is harmful and in general, most of the ion channels stay in their closed state, it seems logical to assume that proteins interacting with TRPV4 may act as inhibitory complex and thus stabilize TRPV4 in its closed state, thermodynamically most stable state. Thus, it is tempting to speculate that substitution of R with other amino acids (like H, C and W) at the position of 269 and 315–316 might result in
either weak or strong interaction of at least one molecular component that works as an inhibitory complex for TRPV4 (Fig. 1).

Significant information can be imparted from these results: How is the surface expression of TRPV4 regulated? Generally, surface expression of TRP channels is an important aspect regulated in several manners. Depending on the signaling events, new functional channels are recruited to the plasmamembrane when required. The pre-existing channels will be either recycled by endocytosis and exocytosis or internalized and degraded by 26S proteasomal pathway if not required. These regulations are essential and form the basis of channel homeostasis at the plasma membrane. In that context, multivesicular body (MVB) pathway is important as it regulates the level of surface expression by degrading the internalized transmembrane protein at lysosome. Surface expression of transmembrane proteins can also be regulated by Ubiquitin, a 76 amino acid long chain that serves as a degradation signal in ubiquitin-mediated proteasomal degradation pathway. Ubiquitin attachment is carried out by three enzymes E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) and internalized ubiquitinated proteins are known to be degraded at lysosome. However, recent studies demonstrated that ubiquitin can also be used as a sorting signal for MVB pathway.

In case of TRPV4, surface expression seems to be regulated by ubiquitination, at least in parts. Atrophin-1-interacting protein 4 (AIP4) is a member of HECT ligases which acts as an E3 ligase. AIP4 is known to add ubiquitin on TRPV4, especially within amino acid residues 411–437 located at the N-terminal cytoplasmic domain. It has been demonstrated that AIP4 actually promotes endocytosis and thus increases the intracellular pool of TRPV4. These internalized TRPV4 containing vesicles efficiently recycles to the plasma membrane. However, it has been observed that in spite of tagged with ubiquitin, some of the TRPV4 does not get degraded, but becomes accumulated beneath plasma membrane. Thus, AIP4 seems to plays a role which is not only relevant in the context of ubiquitination of the TRPV4 but also can direct the TRPV4 under regulation by MVB pathway. As AIP4 binds to the N-terminal region of the TRPV4, mutations in this region can leads to altered ubiquitination. This altered ubiquitination might affect the surface expression and Ca\(^{2+}\)-influx via TRPV4 that is relevant in the context of cellular function (Fig. 2).
Figure 2. A hypothetical model depicting how surface expression of TRPV4 can be regulated. (A) Surface expression of wild-type TRPV4. OS9 protein with a chaperone-like activity assists TRPV4 monomer (indicated by red) to form tetramer. Misfolded TRPV4 is targeted for ubiquitin-dependent 26S Proteasomal degradation pathway (ERAD, shown in steps 1a–d). Perfectly folded TRPV4 tetramer are inserted in vesicles and recruited to the plasma membrane (steps 2a and b). Most of the membrane inserted TRPV4 can be recycled (steps 3a–d). A fraction of the membrane inserted TRPV4 is regulated by AIP4, an E3 ligase and become monoubiquitinated (step 4a). This monoubiquitinated TRPV4 can be either degraded by lysosomes by multivesicular body pathway (MVB pathway, indicated in steps 4a–d) or they get recycled back and become part of the recyclable vesicular pool located just beneath the plasma membrane. (B) Reduced surface expression of TRPV4 mutant. In case of mutant (for example R269H, R316C and R315W in HeLa cell, indicated by yellow), a major fraction of total synthesized TRPV4 is misfolded and thus degraded by ERAD pathway as a quality control mechanism (steps 1a–d). Only a minor fraction of synthesized TRPV4, which is fully assembled and functional will be transported by vesicles and recruited to the plasma membrane (steps 2a and b). The other steps related to MVB pathway (steps 4a–d) and recycling of vesicles (steps 3a–d) are expected to be operational in case of mutants also. But for simplicity it is not shown in (B).
Sequence analysis also shed light on the differential distribution of the wild-type and mutant TRPV4. Both carboxyl-terminal dileucine KKKX motif that interacts with coat protein I (Copl complex) and the internally positioned RXR motif regulate the retention of any protein within endoplasmic reticulum.\(^7\) Apparently, TRPV4 polypeptide contains four RXR motifs, two on the N-terminal region (684–721, which is highly conserved in all TRP membrane but remain nonfunctional. For example, substitution and functionality of TRP channels is poorly understood and at the N-terminal domain and thus regulate the surface expression significantly. However, this possibility needs to be verified experimentally in future.

**Future Direction and Conclusion**

The relation between assembly, trafficking, surface expression and functionality of TRP channels is poorly understood and seems to be specific for each TRP channel. In many cases the TRPV channel can assemble and be expressed at the plasma membrane but remain nonfunctional. For example, substitution of TRP domain (684–721, which is highly conserved in all TRP family and known as TRP box) from TRPV1 to TRPV2-TRPV6 results assembly of TRP channels and proper surface expression also. However, these TRPV chimera remain nonfunctional.\(^7\) In case of TRPV4, OS-9 seems to be important for its surface expression. In this regard it is important to mention that ectopic expression of TRPV4 causes deformities in body and bone in zebrafish embryos.\(^42\) Interestingly, expression of OS-9 can rescue these TRPV4-mediated defects in zebrafish.\(^42\) Therefore, similar lethal phenotypes observed in patients suffering from Brachymya and in the zebrafish embryos largely suggest that the fine regulation of the basal level activity of TRPV4 is extremely important for normal bone function.\(^7\) This similarities in human as well as zebrafish may also hint that the OS-9-mediated regulation of TRPV4 is conserved throughout the evolution.\(^7\) However, further experimental proof is needed to validate this.

The proteins which are actually involved in insertion of TRPV4-containing vesicles to the plasma membrane and for recognizing ubiquitin-tagged TRPV4 as sorting signal has not been identified yet. In this regard, recent studies on other TRP channels indicate that membrane trafficking of TRPs are complex and different Rab-GTPase, dynamin, 80KH, annexins and kinesins might be involved.\(^7\) Based on the structural information available and existing sequence homology among other TRPs, especially with TRPV members and the manner by which the surface expression of TRP channels are regulated, it can be speculated that proteins like Signal Transducing Adaptor Molecule (STAM), Hrs (which downregulate TRPP2 in *C. elegans*) and Recombinase Gene Activator (RGA, which regulates cell surface expression of TRPV2) may also be involved in the surface expression of TRPV4.\(^7\) However, further detailed studies are needed to confirm if these proteins really interact and are involved in the surface expression of TRPV4.

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