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Ratio of Hydrophobic–Hydrophilic and Positive–Negative Residues at Lipid–Water-Interface Influences Surface Expression and Channel Gating of TRPV1

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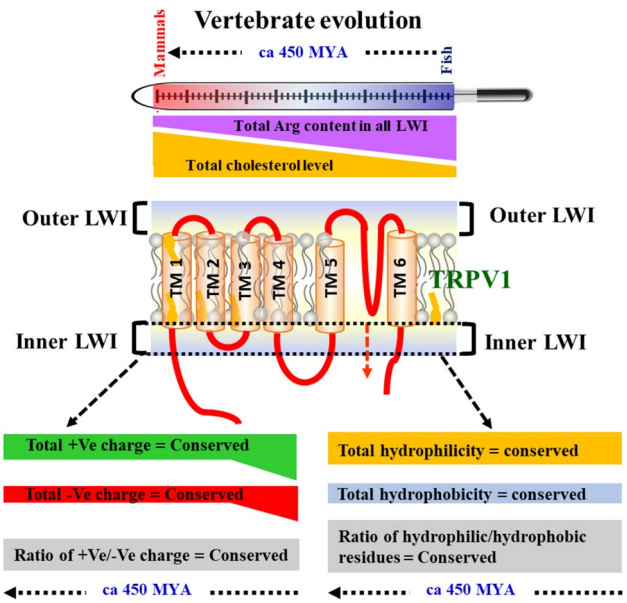
Abstract

During evolution, TRPV1 has lost, retained or selected certain residues at Lipid–Water-Interface (LWI) and formed specific patterns there. The ratio of "hydrophobic–hydrophilic" and "positive–negative-charged" residues at the inner LWI remains conserved throughout vertebrate evolution and plays important role in regulating TRPV1 trafficking and localization. Arg575 is an important residue as Arg575Asp mutant has reduced surface expression, co-localization with lipid raft markers, cell area and increased cell lethality. This lethality is most likely due to the disruption of the ratio between positive–negative charges caused by the mutation. Such lethality can be rescued by either using TRPV1-specfic inhibitor 5′-IRTX or by restoring the positive–negative charge ratio at that position, i.e. by introducing Asp576Arg mutation in Arg575Asp backbone. We propose that Arg575Asp mutation confers TRPV1 in a "constitutive-open-like" condition. These findings have broader implication in understanding the molecular evolution of thermo-sensitive ion channels and the micro-environments involved in processes that goes erratic in different diseases.

Graphical Abstract

The segment of TRPV1 that is present at the inner lipid–water-interface (LWI) has a specific pattern of amino acid combinations. The overall ratio of +ve charge /−ve charge and the ratio of hydrophobicity/hydrophilicity remain constant throughout the vertebrate evolution (ca 450 million years). This specific pattern is not observed in the outer LWI region of TRPV1.



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Keywords Lipid–Water-Interface · Amino acid pattern · Capsaicin · Channelopathy · Molecular evolution · Lipid raft

Introduction

Ion channels have unique abilities to sense different physical and chemical stimuli and conduct influx of specific ions. These are involved in a plethora of complex cellular functions, including regulation of volume and ionic homeostasis, signal transduction as well as propagation of action potential (Jentsch et al. 2004). Notably, function/s of ion channels are heavily dependent on the membrane environment, i.e. change of pH, voltage difference across membrane, binding of a ligand and temperature, and also by the composition of lipid bilayers, the physico-chemical properties, surface charges, etc. (Yi et al. 2001; Moreau et al. 2014; Chowdhury et al. 2014; Hempling 1995; Ciardo and Ferrer-Montiel 2017). Within the lipid bilayer, ion channels undergo multistep conformational changes and this switching between closed and open states is generally referred to as channel gating (Zhou and McCammon 2010). However, the complex interplay of these different stimuli, the molecular mechanism of gating in response to these stimuli, and how such factors contribute in the channel's functions are poorly understood and often differ in case-by-case basis. Slight alterations in such complex system lead to non-functionality of ion channels leading to pathological conditions and diseases commonly known as channelopathies (Kim 2014). Escalation in the number of channelopathies has triggered the need to understand structure–function relationship of ion channels, their gating mechanisms, influence of membrane microenvironments in channel functions, and importance of ion channels in physiology and pathophysiology. Such understanding will enable designing of new drugs and/or effective gene therapy for treating pathological conditions (Imbrici et al. 2016).

TRPV1, also known as the "Capsaicin receptor" is the founding member of TRPV subfamily and has been well characterized in the context of different physiological and sensory functions (Smutzer and Devassy 2016). TRPV1 act as a polymodal channel which gets activated by a plethora of exogenous and endogenous stimuli, like capsaicin, different endogenous compounds, low pH, temperature > 43 °C, etc. (Elokely et al. 2016). In human population, TRPV1 has large number of genetic variations (Ghosh et al. 2016). However, there has been no reported case of any disease or channelopathy that has been linked with point mutations in TRPV1 (Okamoto et al. 2018). At least one copy of TRPV1 gene is present in all vertebrates and in different tissues ranging from haploid gametes to peripheral sensory neurons (Majhi et al. 2013; Sardar et al. 2012; De Toni et al. 2016). Therefore, involvement of TRPV1 in sensory and physiological functions are most likely to provide certain adaptive and physiological benefits to the individuals (Storozhuk et al. 2019). For this reason, molecular changes in TRPV1 sequence during vertebrate evolution can be correlated well with its structure and function/s. Such changes or no changes in the amino acids in key positions of TRPV1 can provide important clue for channel functions in different membrane micro-environments that differ in different organisms, physiological conditions, and also often in ecological niches.

Several studies suggest that membrane components like different lipids, PIP_2, DAG, as well as cholesterol regulate TRPV1 channel function to a large extent (Ciardo and Ferrer-Montiel 2017). Interestingly, inverse relationship of cholesterol in TRPV1 channel function has been suggested by several groups (Morales-Lázaro and Rosenbaum 2019). Cholesterol interacts with Arg residues present in the inner lipid–water-interface region of TRPV1 in closed conformation only (Saha et al. 2017).

The availability of TRPV1 sequences from diverse species, the high-resolution structure of TRPV1 in open and closed conformation, human genome data offer unique opportunity to analyse the importance of individual amino acids in channel functions, especially the amino acids present in the lipid–water-interface region, i.e. approximately 6–10 Å (or even less) thin layer of micro-environment present on both sides of the lipid bilayer. This work suggests that TRPV1 has evolved with unique combinations of amino acids that maintain a critical ratio of hydrophobic–hydrophilic nature. We propose that the conserved ratio of positive–negative-charged amino acids on the inner LWI region of TRPV1 is a critical parameter that regulates the channel's surface expression as well as functions.

Materials and Methods

Generation of Frequency Plots

Vertebrate TRPV1 sequences were retrieved from NCBI as previously described (Saha et al. 2017). LWI residues were determined and percentage contents of all amino acids were separately calculated using MEGA 5.1 (Saha et al. 2017). Natural average frequency of all amino acids in nature is obtained from available literature and sources (source: http://www.tiem.utk.edu/~gross/bioed/webmodules/aminoacid.htm). In addition to this, calculations were made for the following groups: Positively charged, negatively charged, hydrophobic and hydrophilic amino acids. Each LWI region comprised a stretch of 5 amino acids on either side of the lipid bilayer. Frequency calculations were done for LWI on the inside (cytoplasmic side, for 6 positions; total 30 amino

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acids), outside (extracellular side, for 6 positions; total 30 amino acids), as well as for the overall (i.e. for a total of 60 amino acids) residues for each species. These values were then plotted using GraphPad Prism7 and/or GraphPad Prism8 (http://www.graphpad.com/).

Calculation of Absolute Hydrophobicity and Hydrophilicity at Lipid–Water-Interface

Whole-residue interfacial hydrophobicity scale was used for obtaining transfer free energies of each amino acid where contributions of the peptide bonds as well as sidechains were taken into consideration (Wimley and White 1996). Whole-residue scales for POPC bilayer interfaces and for n-octanol using two families of peptides: host–guest pentapeptides of the form AcWL-X-LL (for determining side-chain hydrophobicities) and the homologous series AcWLm ($m = 1$ to 6) (for determining peptide bond hydrophobicities) was used. The values used for calculating the hydrophobicity and hydrophilicity were derived from free energies of transfer of AcWL-X-LL peptides from bilayer interface to water as described before (Wimley and White 1996). The decision level for selection of hydrophobic amino acid was taken as $\Delta G \geq 0$ and Hydrophilic as $\Delta G < 0$.

Site-Directed Mutagenesis and Construct Preparation

The Arg557Ala, Arg557Asp, Arg557His, Arg557Lys, Arg575Ala, Arg575Asp, Arg575His Arg575Lys, and Arg575Asp-Asp576Arg mutants of TRPV1 were prepared using site-directed mutagenesis kit (Agilent Technologies) with specific primer sets. In all cases, full-length rTRPV1 (Rat TRPV1) cloned in pCDNA3.1 was used as a template. TRPV1-Arg575Asp-Asp576Arg mutant was generated using TRPV1-Arg575Asp as the template. In this study, all these mutants will be henceforth collectively referred to as LWI mutants.

Full-length rTRPV1-WT and all LWI mutants were cloned into pSGFP2-C1 (Addgene) or pmCherryC1 (Takara) using 5′-CCAGGAATTCTATGGAACAACGGGCTAGC-3′ and 5′-CCAGGTCGACTTATTTCTCCCCTGGGACC-3′ primer sets having EcoR1 and SalI site, respectively. All these constructs were verified by restriction digestion and DNA sequencing.

Primers Used for Site-Directed Mutagenesis

1	TRPV1-Arg557Ala	Forward Primer: 5′- CCAACATGCTCT ACTATACCGCAG GATTCCAGCAGA TGGGC -3′ Reverse Primer: 5′- GCCCATCTGCTG GAATCCTGCGGT ATAGTAGAGCAT GTTGG -3′
2	TRPV1-Arg557Asp	Forward Primer: 5′- CCAACATGCTCT ACTATACCGACG GATTCCAGCAGA TGGGC -3′ Reverse Primer: 5′- GCCCATCTGCTG GAATCCGTCGGT ATAGTAGAGCAT GTTGG -3′
3	TRPV1-Arg557His	Forward Primer: 5′- CCAACATGCTCT ACTATACCCATG GATTCCAGCAGA TGGGC -3′ Reverse Primer: 5′- GCCCATCTGCTG GAATCCATGGGT ATAGTAGAGCAT GTTGG -3′
4	TRPV1-Arg557Lys	Forward Primer: 5′- CCAACATGCTCT ACTATACCAAAG GATTCCAGCAGA TGGGC -3′ Reverse Primer: 5′- GCCCATCTGCTG GAATCCTTTGGT ATAGTAGAGCAT GTTGG -3′
5	TRPV1-Arg575Ala	Forward Primer: 5′-GATTGAGAAGAT GATCCTCGCAGA CCTGTGCCGGTT TATG-3′ Reverse Primer: 5′- CATAAACCGGCA CAGGTCTGCGAG GATCATCTTCTC AATC-3′

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6	TRPV1-Arg575Asp	Forward Primer: 5′- GATTGAGAAGAT GATCCTCGACGA CCTGTGCCGGTT TATG-3′ Reverse Primer: 5′- CATAAACCGGCA CAGGTCGTCGAG GATCATCTTCTC AATC -3′
7	TRPV1-Arg575His	Forward Primer: 5′- GATTGAGAAGAT GATCCTCCACGA CCTGTGCCGGTT TATG-3′ Reverse Primer: 5′- CATAAACCGGCA CAGGTCGTGGAG GATCATCTTCTC AATC-3′
8	TRPV1-Arg575Lys	Forward Primer: 5′- GATTGAGAAGAT GATCCTCAAAGA CCTGTGCCGGTT TATG-3′ Reverse Primer: 5′- CATAAACCGGCA CAGGTCTTTGAG GATCATCTTCTC AATC -3′
9	TRPV1-Arg575Asp-Asp576Arg	Forward Primer: 5′-AAGATGATCCTC **GACCG**CCTGTG CCGGTTTATG-3′ Reverse Primer: 5′- CATAAACCGGCA CAGGCGGTCGAG GATCATCTT -3′

Cell Culture, Transfection and Imaging

F11 cells were cultured in nutrient mixture F-12 Ham media supplemented with 10% Foetal Bovine Serum, L-glutamine (2 mM), Streptomycin, and Penicillin (100 mg/ml each, HiMedia, Bangalore, India). Cells were maintained in a humidified atmosphere at 5% CO_2 and 37 °C. F11 cells were transiently transfected with TRPV1-WT-GFP, all LWI mutants and Flotillin-1-RFP (for co-localization studies) using Lipofectamine (Invitrogen). Cells were fixed with 4% PFA 36-h post-transfection. Fixed cells were then washed twice with 1X PBS (Himedia), treated with DAPI (Invitrogen, 1:1000 dilution) and then mounted using Fluoromount-G (Southern Biotech). Cells were subsequently observed using Zeiss LSM-800 or Olympus FV3000 Confocal Microscope. For surface expression study, cells were transiently transfected with TRPV1-WT and LWI mutants in pmCherryC1 (Takara) and post-fixation, non-permeabilized cells were stained with TRPV1-ATTO-488 antibody (Alomone) that was directed against the 3rd extracellular loop of TRPV1. Length, breadth, area and perimeter of F11 cells transiently transfected with TRPV1-WT-GFP, TRPV1-Arg575Asp-GFP and TRPV1-Arg575Asp-Asp576Arg-GFP were measured using Fiji Software. The same analysis was performed for F11 cells expressing TRPV1-WT-GFP and TRPV1-Arg575Asp-GFP and treated with or without 5′-IRTX (1 µM, Tocris), Capsazepine (10uM, Sigma) and Ruthenium Red (100uM, Sigma).

Ca^{2+} Imaging

F11 cells were cultured in nutrient mixture F-12 Ham media supplemented with 10% Foetal Bovine Serum, L-glutamine (2 mM), Streptomycin and Penicillin (100 mg/ml each, HiMedia, Bangalore, India). Cells were maintained in a humidified atmosphere at 5% CO_2 and 37 °C as mentioned before. F11 cells were transiently transfected with two plasmids TRPV1-WT-pmCherryC1, or TRPV1-Arg575Asp-pmCherryC1 (R575D), or TRPV1-Arg575Asp-Asp576Arg-pmCherryC1 (R575D-D576R) along with plasmid pGP-CMV-GCaMP6f encoding the ultrasensitive Ca^{2+} sensor protein (Addgene) (Chen et al. 2013). Approximately 24 h after transfection, doubly transfected cells were imaged using Olympus FV3000 confocal microscope for 200 frames whereby cells were activated by adding 100 nM RTX (Sigma-R8756) at the 30th frame. Snaps of doubly transfected cells were acquired using 488 nm and 561 nm laser. Time series of live cells were conducted using 0.4% 488 nm laser and images were acquired for total 217 s at a rate of 1 frame/1.085 s.

Quantification of Ca^{2+} Imaging

Change in intensity of the ultrasensitive protein calcium sensor pGP-CMV-GCaMP6f was quantified over time throughout 200 frames. Each cell was considered to be a single ROI and then the change in intensity was quantified using Fiji software. The initial value was considered to be 1 and accordingly the changes were calculated and the graphs were plotted using GraphPad Prism 8 software.

Statistical Test

Length, breadth, area and perimeter of transfected cells were calculated as described by Fiji software. Column graphs having just one variable were plotted for each construct from these values using GraphPad Prism 7 or GraphPad Prism 8. Statistical significance was analysed using One-way ANOVA (and non-parametric) which compared the mean of each column with the mean of every other column and the *P* values were assessed using Tukey's multiple comparison test. All the intensity measurements were performed with Fiji software and the

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raw intensity values were plotted as arbitrary units in GraphPad Prism.

Results

Analysis of Molecular Selection and Exclusion of Amino Acids in the Lipid–Water-Interface Regions of TRPV1 Throughout Vertebrate Evolution

A total of 37 mammalian, 7 avian, 3 reptilian, 3 amphibian and 4 piscean TRPV1 sequences were considered. The frequency of occurrence of all 20 different amino acids at the lipid–water-interface (LWI) region was calculated. The same calculation was also performed for the outer as well as inner LWI region separately (Fig. 1). Analysis reveals that the frequency of occurrence of positively charged amino acids in inner LWI remains mostly constant during vertebrate evolution, however with different values. Amongst all the positively charged amino acids, Arg (having the highest pI of 13.5 for the side group) occurs most frequently (10%) in the inner LWI region of TRPV1 and this percentage remains conserved from amphibians to mammals. The other positively charged amino acids, Lys ($pI = 10.4$ for the side group) also show a conserved frequency of occurrence (6.67%) across evolution on the inner leaflet, especially from amphibians to mammals. However, frequency of occurrence of another positively charged amino acid, namely His ($pI = 6.8$ for the side group) remains conserved, yet at a very low value or at zero value suggesting that His residue is never selected in the inner LWI region, whilst other positively charged residues were selected there. Notably, the comparative frequency of Arg, Lys and His in the inner LWI region also suggests that positively charged residues with high pI values (for side group) are only selected and retained in the inner LWI region (discussed later).

For negatively charged residues at the inner LWI region, higher the *pI*, lower is the frequency of occurrence. Asp residue (*pI* of 4 for the side group) occurs more frequently on the inner leaflet as compared to Glu residue (*pI* of 4.4, for the side group). Moreover, the % frequency of Asp residue on the inner LWI has increased from fishes to amphibians (indicative of positive selection) and subsequently remained conserved throughout the evolution (indicative of stabilization). On the other hand, frequency of Glu has decreased from nearly 10% in fishes to almost 0% in amphibians (indicative of negative selection) and remains at 0% value in mammals suggesting gradual exclusion of this amino acid in inner LWI region. However, in the outer LWI region, frequency of occurrence of Glu and Asp residues are random and remain at a relatively high frequency.

Aromatic amino acids, namely Tryptophan, Tyrosine and Phenylalanine, have relatively low frequency of occurrence across evolution in the inner LWI. Both Tryptophan and Tyrosine remain at less than their natural frequency of 1.3% and 3.3%, respectively, yet remain conserved at the inner LWI region throughout the vertebrate evolution (indicative of critical function played by these amino acids). Frequency of occurrence of Phenylalanine remains random in the inner as well as outer LWI region. Out of these three aromatic amino acids, only tyrosine (*pI* of 9.6 for the side group) retains a higher frequency of occurrence (13%) at the outer LWI.

Helix-breaking amino acid Proline has almost equal distribution throughout evolution both in outer (3.33%) and inner LWI (3.33%) region, especially in the higher vertebrates. Another helix-breaking amino acid, namely Gly, has been retained at less than 7.4% frequency at the inner LWI region, but mostly remain conserved throughout the vertebrate evolution. Hydrophobic amino acids, namely Isoleucine, Leucine, Valine and Alanine, are retained at different percentages of frequency of occurrence and conservation. Notably, Isoleucine remains conserved in outer LWI. Amongst other amino acids, Met remains conserved in the inner LWI region. At inner LWI region, the frequency of occurrence for Cys is low in all vertebrates and eventually excluded in the higher vertebrates. Taken together, the analysis suggest selection, retention or exclusion of specific amino acids at the inner LWI region and therefore provides important clue of TRPV1 function in different species (discussed later).

In contrast to the LWI regions, barring a few exceptions, there is no specific pattern of amino acids in the TM regions or in the pore region of TRPV1 in majority of the vertebrate phyla (Fig S1). These data strongly suggest the importance of amino acids present in the LWI region, especially in the inner LWI region and in the context of overall channel function and regulation.

Comparison of Amino Acids Present at LWI of TRPV1 from Warm-Blooded and Cold-Blooded Animals

As TRPV1 acts as a temperature-sensitive ion channel, we compared the frequency of amino acids between warm-blooded and cold-blooded animals (Fig S2). In case of total LWI, we observed that frequency of Lys, Asn and Val has increased from cold-blooded animals to warm-blooded animals, indicating positive selection (i.e. increase in frequency from cold-blooded animals to warm-blooded animals). There is a trend of positive selection for Asp, His, Ala and Phe, although the values (with the n number available at present) remain non-significant. In contrast, frequency of Glu, Pro and Gly has declined from cold-blooded animals to warm-blooded animals, indicating true-negative selection. There is a trend of negative selection for Gln, Thr and Ser, although the values remain non-significantly different (with the n number available at present). Frequency of Arg, Met,

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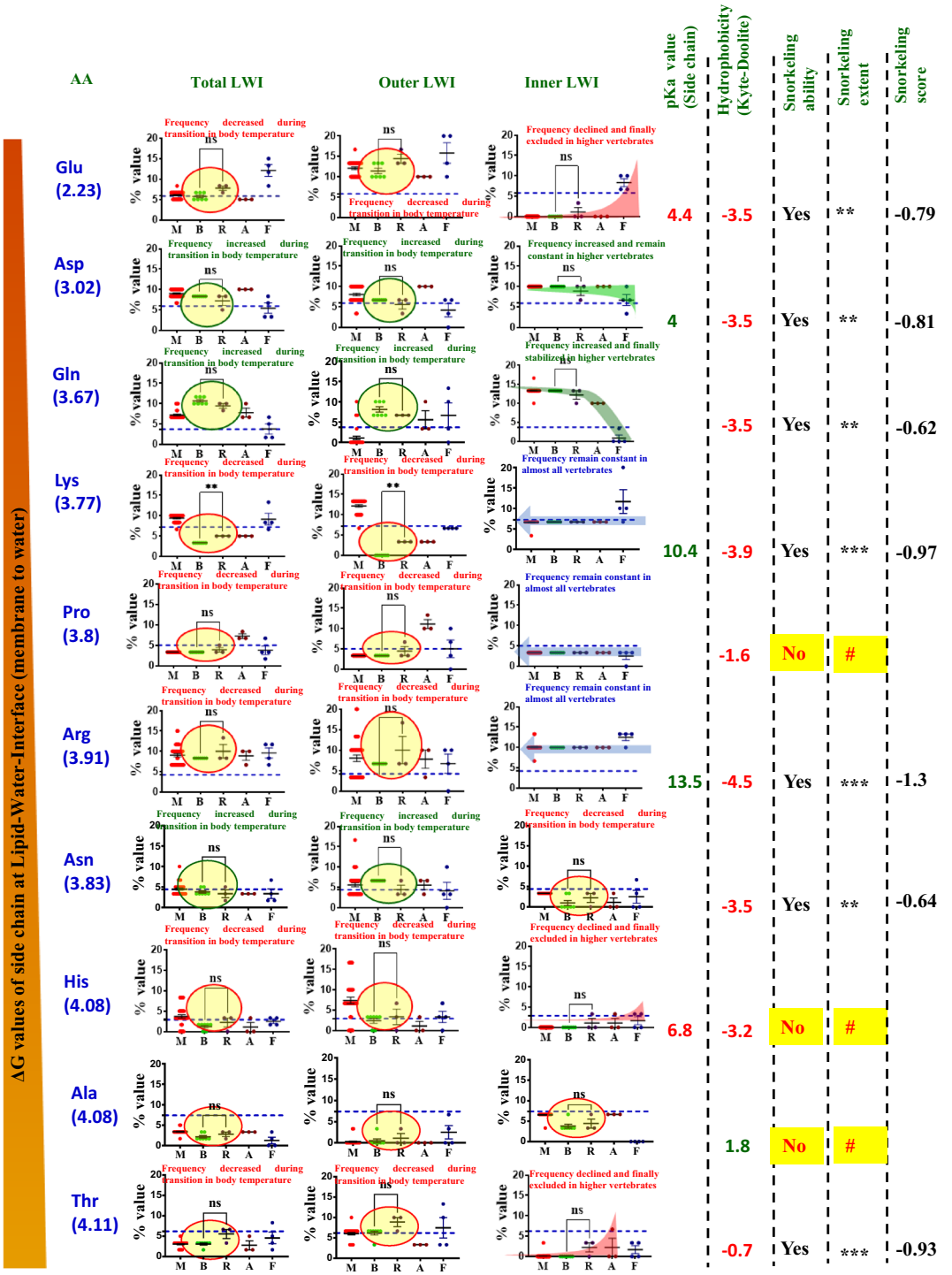

Fig. 1 "Frequency-of-occurrence" analysis for different amino acids at the lipid–water-interface (LWI) of TRPV1 in different phylogenetic groups. The "frequency-of-occurrence" of all 20 different amino acids across different vertebrate species ranging from fishes (F), amphibians (A), reptiles (R), birds (B) and mammals (M) at inner LWI (right-most row), at outer LWI (middle row), and total (combining inner and outer LWI, left-most row) have been shown separately. The amino acids were arranged according to the low to high ΔG values of the side-chain values (Wimley and White 1996). In cases where percentage of amino acids remains constant are indicated by blue arrow as back ground. Similarly, positively selected and negatively selected (or excluded) are marked with green or red background. In right side, the snorkelling extent of each amino acid is represented in *** (high), ** (moderate), * (low) and not at all (#). The circles with green (for positive selection) or red (for negative selection) lines indicate the changes in frequency of occurrence during transition from reptiles to birds as a result of change in body temperature. The statistical significance values are as follows: ns = non-significant. P value > 0.05, * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001, respectively. The dotted blue line indicates the observed average frequency of all amino acids in nature

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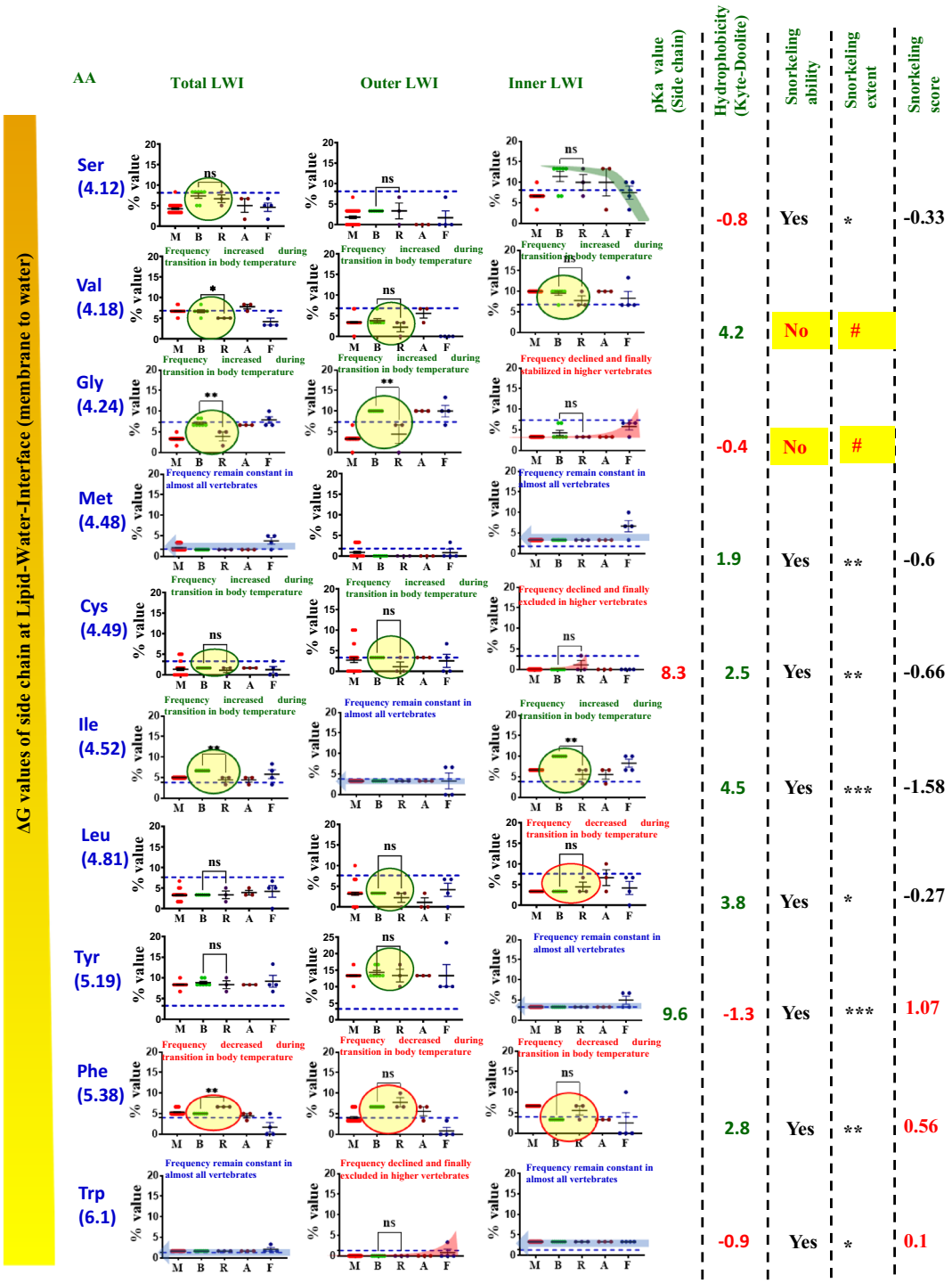

Fig. 1 (continued)

Cys, Ile, Leu, Tyr and Trp remains almost identical in both groups and thus indicates no change during the vertebrate evolution.

In case of outer LWI, positive selection has been observed for Lys, His and Val. In the same notion, the trend for positive selection has been observed for Asn and Cys (although the values remain non-significant with the n number available at present). In contrast, true-negative selection has been observed for Gln, Pro and Gly. In the same notion, the trend for negative selection has been observed for Glu, Asp and Ala (although the values remain non-significant with the n number available at present). In the outer LWI, frequency

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of Arg, Thr, Ser, Met, Ile, Leu, Tyr, Phe and Trp remains almost identical in both groups.

In case of inner LWI, a large number of amino acids show discrete and/or significant changes. Positive selection has been observed for Asp, Gln, Asn, Ala Val and Phe. In contrast, negative selection has been observed for Glu, Lys, Arg, His, Thr, Ser, Gly, Met, Leu and Tyr. In the outer LWI, frequency of Pro, Cys, Ile and Trp remains almost identical in both groups indicating no change during vertebrate evolution. Notably, Cys at the inner LWI is excluded in both groups. Met and Trp at the outer LWI are excluded in both warm-blooded and cold-blooded animals. Ala at the outer LWI and Glu, His, as well as Thr at the inner LWI are excluded in all warm-blooded animals. These exclusions largely indicate the misfit nature of these amino acids in these positions.

Ratio of Positive–Negative Amino Acids in the Inner LWI Region of TRPV1 Remains Constant Throughout Vertebrate Evolution

All positively charged amino acids (Arg, Lys, His) have a higher frequency of occurrence at the outer LWI region in mammals but its pattern of occurrence is not conserved throughout evolution. Although the frequency of occurrence of positively charged amino acids is less on the inner LWI its frequency of occurrence has remained conserved throughout vertebrate evolution from amphibians to mammals (Fig. 2a). Similarly, negatively charged amino acids have a higher frequency of occurrences on the outer LWI, but is more conserved on the inner LWI. Whilst frequency of occurrence for individual amino acids is variable, the ratio of the total number of positively charged amino acids and total number of negatively charged amino acids at the inner LWI shows a ratio of 1.67:1 and this ratio remains conserved in the entire vertebrate evolution. This ratio is not conserved in case of outer LWI. These findings strongly suggest that during vertebrate evolution, this conserved ratio at the inner LWI has played a strong selection pressure in TRPV1 function (discussed later).

TRPV1 Retains Unique Combination of Hydrophobic and Hydrophilic Amino Acids in Its Inner Lipid–Water-Interface

Hydrophilic amino acids (Ala, Asp, Glu, Gly, His, Lys, Asn, Pro, Gln, Arg, Ser, Thr, Val) have a higher incidence of occurrence (~73.14% in total) on the inner LWI region as compared to the hydrophobic residues (Cys, Phe, Ile, Leu, Met, Trp, Tyr, Total ~26.85%). This difference in distribution of hydrophobic and hydrophilic amino acids at the inner LWI region remains conserved across evolution from fishes to mammals. However, when the ratio of occurrence of all hydrophobic and hydrophilic amino acids is considered, the ratio remains conserved in the inner LWI (Fig. 2b). These findings strongly suggest that during vertebrate evolution, the conserved ratio of hydrophilic versus hydrophobic amino acids at the inner LWI region of TRPV1 has also played a strong selection pressure in TRPV1 function (discussed later).

As different amino acids have different hydrophobicity index, we explored if "frequency" can reflect reliably for the "absolute total hydrophobicity or hydrophilicity". For that purpose, we calculated the absolute hydrophobicity or hydrophilicity in each species and plotted these values. The analysis suggests a fairly good conservation in the total hydrophobicity or hydrophilicity values (considering the side chains and peptide bond contribution of the amino acids as mentioned in total, outer and inner LWI regions (Wimley and White 1996) (Fig. 2c). The absolute hydrophobicity or hydrophilicity values correlate well with the frequency of occurrence of these amino acids also (Fig. 2c). These suggests that the frequency of occurrence of different amino acids in the LWI region can reliably be used as a bio-physical parameter that is relevant for understanding the structure–function relationship of ion channels.

Combination of amino acids offering glycosylation or phosphorylation does not show any trend, neither in inner LWI nor in outer LWI regions (Fig S3). Therefore, the data clearly suggest that ratio of hydrophilic–hydrophobic amino acids as well as ratio of positive–negative charge plays important selection pressure, at least in the inner LWI region of TRPV1.

TRPV1 is Localized in the Cholesterol-Enriched Lipid Rafts and Arg575Asp Mutation Induces Cell Lethality

TRPV1 in closed conformation interacts with cholesterol mediated by the arginine residues present at 557 and 575 positions (Saha et al. 2017). Substitution of arginine by a negatively (Aspartic Acid) or neutrally charged (Alanine) amino acid resulted in altered localization of TRPV1 in lipid rafts. In this work, we explored if presence of arginine in these positions is critical or if substitution of these Arg residues with any other positively charged residue like Lys or His is capable of retaining same properties. For this reason, we generated 4 more substitution mutants: Arg557His, Arg557Lys, Arg575His and Arg575Lys. All these mutants (henceforth referred as LWI mutants) were subsequently transfected in DRG neuron-derived F11 cell line to analyse the localization of these mutants with respect to TRPV1-WT-GFP (Fig S4).

In an over-expression system, when these GFP-tagged constructs were co-transfected along with other lipid raft markers, such as Flotillin-1-RFP (Fig S5), we obtained

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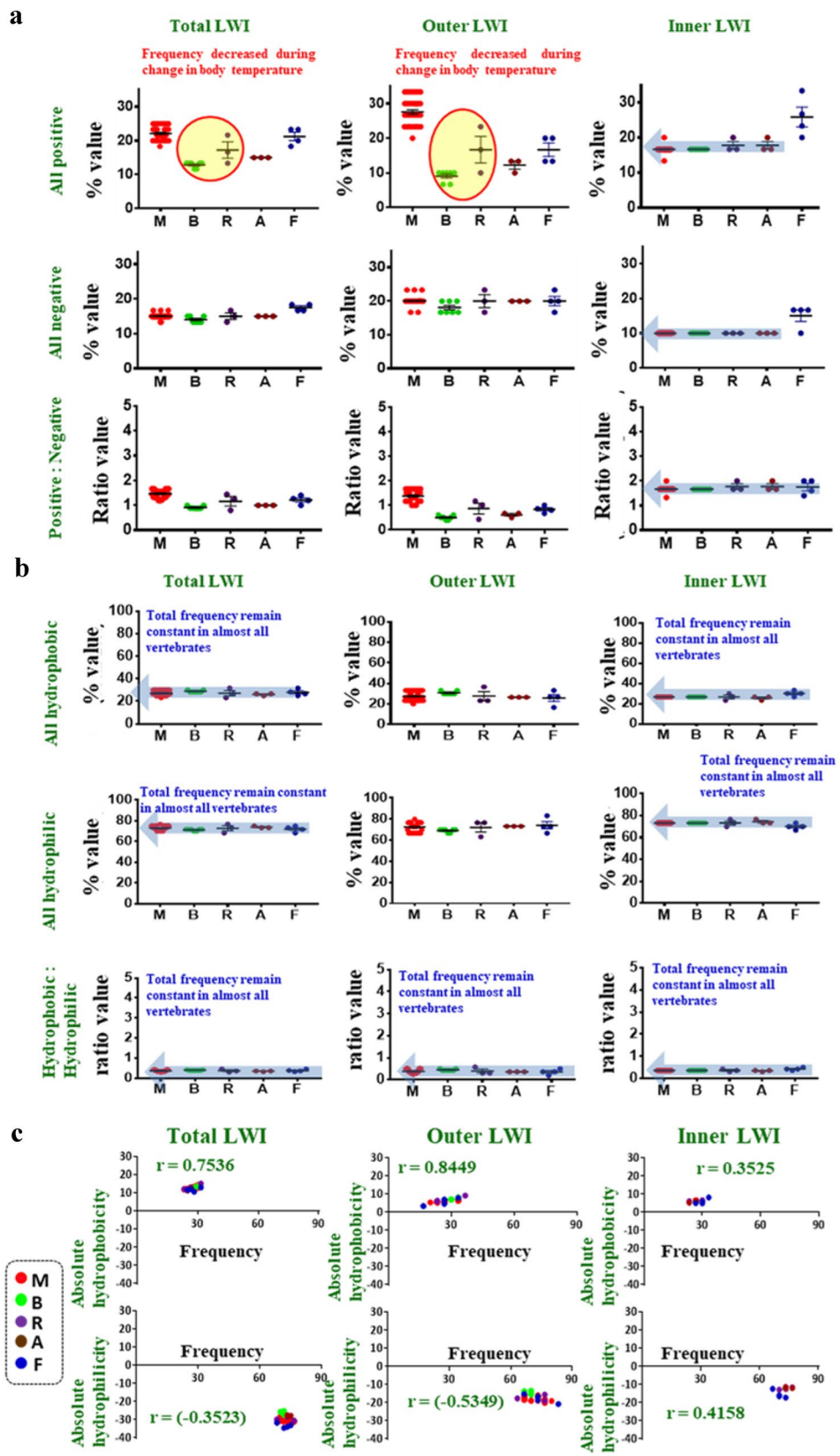

Fig. 2 Ratio of positive/negative amino acids and ratio of hydrophobic/hydrophilic amino acids remain constant at the inner lipid–water-interface of TRPV1. Frequency of occurrence of **a** positively and negatively charged residues and **b** hydrophobic and hydrophilic amino acids at the lipid–water-interface of TRPV1 during vertebrate evolution are shown. **a** Positively charged amino acids (Arginine, Lysine, Histidine) and negatively charged amino acids (Aspartic Acid and Glutamic Acid) at the lipid–water-interface region of TRPV1 across different vertebrate species ranging from fishes, amphibians, reptiles, birds and mammals has been calculated. Negatively charged amino acids on the inner leaflet appear to be more conserved across evolution. The ratio of positively charged to negatively charged amino acids on the inner leaflet at a value of 1.67:1 remains conserved throughout vertebrate evolution. **b** The frequency of occurrence of hydrophobic amino acids (Cys, Phe, Ile, Leu, Met, Trp, Tyr), hydrophilic amino acids (Ala, Asp, Glu, Gly, His, Lys, Asn, Pro, Gln, Arg, Ser, Thr, Val) and their ratios are shown. The ratio of hydrophobic/hydrophilic/amino acids at the inner leaflet remains conserved at a value of 0.36:1 throughout the vertebrate evolution. **c** The frequency (plotted in the *X*-axis) of hydrophobic or hydrophilic amino acids correlates with absolute hydrophobicity or hydrophobicity (plotted in *Y*-axis) at the LWI regions. The values for Pearson correlation coefficient (*r*) are provided along with the graphs

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similar results. Flotillin-1 co-localized with TRPV1-WT-GFP but not with the LWI mutants which exhibited little or no co-localization. The correlation values (r^2) also suggest that Wt-TRPV1 has the highest efficacy ($r^2 > 0.6$) with respect to other mutants ($r^2 < 0.1$) tested in this work (data not shown). This suggests that arginine at positions 557 and 575 is important for proper localization of TRPV1-WT in the lipid rafts. All the TRPV1-LWI mutants exhibit membrane trafficking problems, yet none of these are lethal for the cells except for Arg575Asp mutant. Most of the F11 cells expressing TRPV1-Arg575Asp tend to assume a rounded morphology soon after transfection (in ~24 h), exhibit no neurites or filopodial structures and were often observed to detach from the surface on which they were grown. We conclude that TRPV1-Arg575Asp mutant imparts lethality to cells expressing them.

Arg575Asp Lethality Can be Rescued by Maintaining Overall Positive–Negative Charge Ratio

As this Arg575Asp mutation disrupts the positive–negative charge ratio at the inner LWI (especially in the TM5 region which regulates pore opening), this perturbation could be one of the plausible cause leading to constitutive channel opening and thus lethality observed in the cells. In order to explore this possibility, we mutated the adjacent residue having a conserved Aspartic Acid at 576 position to arginine in the TRPV1-Arg575Asp (R575D) mutant. Notably, the ratio of positive- and negative-charged residue in this double mutant is same as in wild-type TRPV1. Interestingly, F11 cells expressing the double mutant, i.e. TRPV1-Arg575Asp/Asp-576Arg-GFP (R575D/D576R-GFP), retain their normal morphology. Therefore, the lethality observed in case of TRPV1-Arg575Asp can be rescued by introducing positive charge at 576th position (Fig. 3a). When the length, breadth, area and perimeter of F11 cells transfected with TRPV1-WT-GFP, TRPV1-Arg575Asp-GFP and TRPV1-Arg575Asp/Asp576Arg-GFP were calculated, we found a significant increase in the length, perimeter and area of cells transfected with TRPV1-Arg575Asp/Asp576Arg-GFP as compared to cells expressing TRPV1-Arg575Asp-GFP (Fig. 3b). This particular phenotype attributed by TRPV1-Arg575Asp was not specific to F11 cells. In SaOS (cell with osteogenic properties) and HaCaT (keratinocyte) cells too, the cells attained rounded morphology when TRPV1-Arg-575Asp (R575D) was expressed. This was effectively rescued by TRPV1-Arg575Asp/Asp576Arg (R575D/D576R) double mutant. This data indicates that the phenotype observed is independent of cell types (Fig S6).

TRPV1-Arg575Asp Exhibits Impaired Surface Expression that is Partly Rescued by TRPV1-Arg575Asp-Asp576Arg

In order to check the surface expression of full-length Rat TRPV1-Arg575Asp (R575D) and TRPV1-Arg575Asp-Asp576Arg (R575D/D576R) with respect to TRPV1-WT, all three constructs in pmCherryC1 vector were transiently transfected in F11 cells and subsequently the non-permeabilized cells were stained with TRPV1-ATTO-488 antibody directed against the 3rd extracellular loop of TRPV1. TRPV1-WT shows discrete surface expression, whereas TRPV1-Arg575Asp exhibited inconspicuous level of surface expression. Although, TRPV1-Arg575Asp-Asp576Arg is capable of rescuing several defects observed in case of TRPV1-Arg575Asp mutant, the double mutant is also not observed in surface explicitly. However, TRPV1-Arg-575Asp/Asp576Arg mutant is detected on the surface by the extracellular antibody (Fig. 3c). We also quantified the fluorescence intensity of surface-labelled TRPV1, total TRPV1 and their ratio. The results suggest that TRPV1-Wt has best and Arg575Asp has the least surface expression comparatively, which is partially rescued by Arg575Asp-Asp576Arg mutant (Fig. 3d).

Cell Lethality Due to TRPV1-Arg575Asp Can be Partly Rescued by Long-Term Channel Inhibition

We hypothesized that one of the reasons for Arg575Asp lethality might be constitutive channel opening. In order to test this hypothesis, F11 cells were transiently transfected with TRPV1-WT-GFP and TRPV1-Arg575Asp-GFP, treated with 1 μM 5′-IRTX for 36 h and then fixed with 4% PFA. Images of treated as well as untreated transfected cells were acquired and the length, breadth, area and perimeter of cells for each condition were measured. Constitutive application of TRPV1-specific channel blocker 5′-IRTX prevented lethality of cells expressing TRPV1-Arg575Asp suggesting that the cell lethality is mainly due to the excess channel function per se (Fig. 4). To check whether this rescue phenomenon is specific to 5′-IRTX, the cells were also treated with Capsazepine, i.e. a competitive inhibitor of TRPV1 and Ruthenium red which is a non-competitive inhibitor of TRPV1 channel. Like 5′-IRTX, application of either Capsazepine or Ruthenium red leads to partial rescuing of the mutant phenotype (Fig. 5).

Taken together, the results suggest that charge distribution at the inner LWI region, especially at the TM5 is critical for channel function and regulation.

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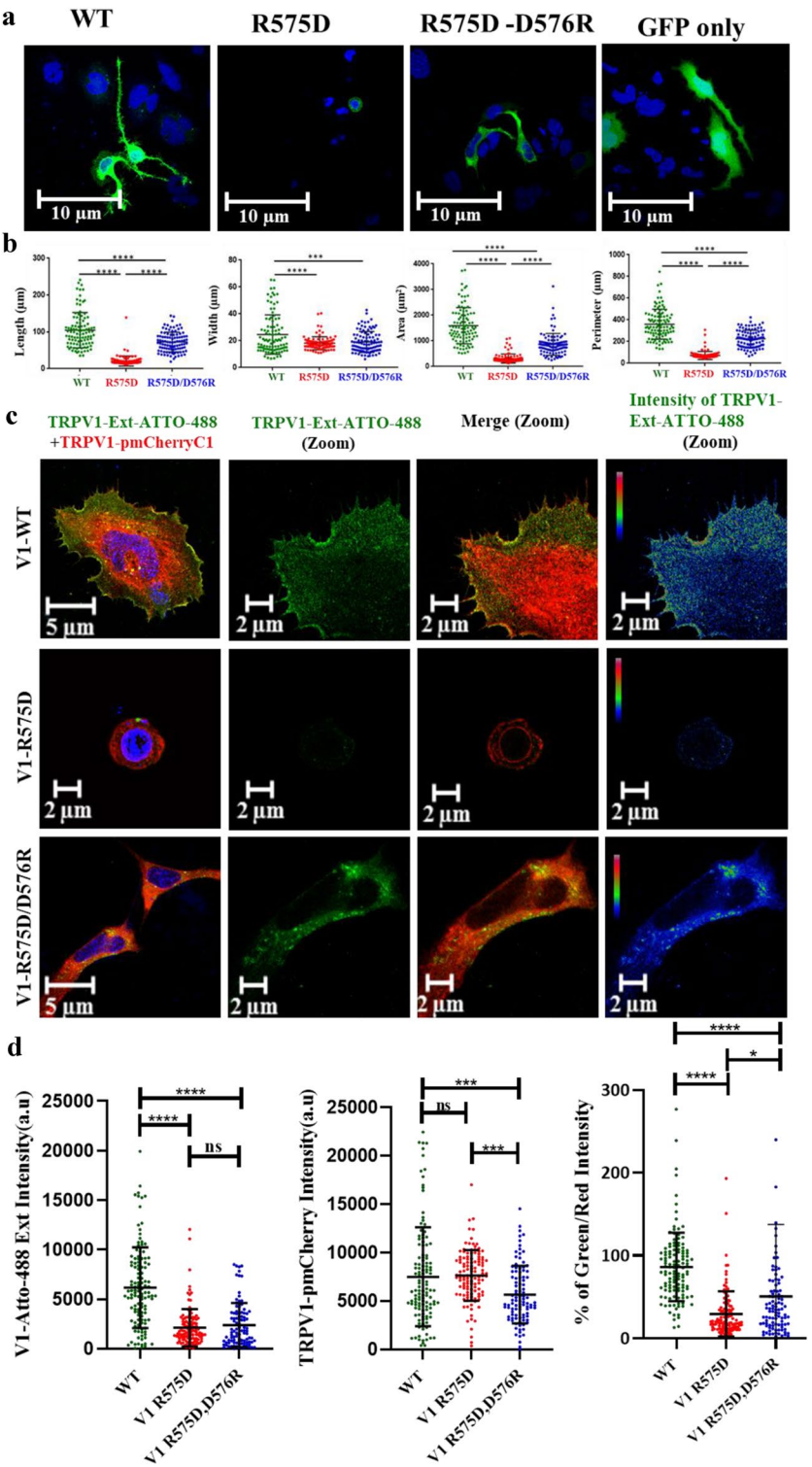

Fig. 3 Cell lethality due to Arg-575Asp mutation can be rescued by introducing a positive charge next to it. **a** Confocal images of TRPV1-WT, TRPV1-Arg575Asp and the rescue mutant TRPV1-Arg575Asp/Asp576Arg in GFP vector expressed in F11 cells. Cells expressing Arg-575Asp mutation tend to assume a circular morphology and decreased surface adhesion behaviour. **b** Quantification of different morphology parameters such as length, width, area and perimeter suggest that Arg575Asp mutant induces reduction in cell size and introduction of Asp576Arg on Arg575Asp rescues all these parameters. In all cases, minimum 100 cells were quantified. The **** ($p < 0.0001$) and *** ($p < 0.0003$) indicate values that are significantly different. **c** TRPV1-Arg575Asp shows reduced surface expression that is partly rescued by TRPV1-Arg575Asp-Asp576Arg. Confocal images of F11 cells transiently transfected with TRPV1-WT, TRPV1-Arg575Asp and TRPV1- Arg575Asp-Asp576Arg (all in pmCherryC1 vector) and subsequently stained with TRPV1-extracellular-ATTO-488 antibody (green) and DAPI (blue). TRPV1-WT shows distinct surface expression, a phenomenon which is almost negligible in case of TRPV1-Arg575Aspand is partly restored in case of TRPV1-Arg575Asp-Asp576Arg. **d** Quantification of the total surface expression of TRPV1 (Left), total expression of TRPV1-mCherry (middle) and percentage expression at surface (surface labelling/total expression) of TRPV1 (right most) as detected by confocal microscopy are shown. Amount of TRPV1 present in the surface is quantified by calculating intensity of TRPV1 in non-permeabilized cells detected by extracellular loop-specific antibody raised against TRPV1. Minimum 20 region of interest (ROI) was taken per cells of at least 5 cells in each group were considered and plotted. The p values are represented by * (p value < 0.1), *** (p value ≤ 0.001), **** (p value < 0.0001)

TRPV1-Arg575Asp Mutant Forms a RTX-Insensitive Channel that is Rescued by Arg575Asp-Asp576Arg

F11 cells were transiently co-transfected with full-length Rat TRPV1-WT-pmCherryC1 along with a Ca^{2+} sensor pGP-CMV-GCaMP6f. The same was repeated for TRPV1-Arg575Asp (R575D) and TRPV1-Arg575Asp-Asp576Arg (R575D-D576R) mutants (both present in pmCherryC1 vector). Approximately 24-h post-transfection, live cell imaging of only doubly transfected cells was executed (Fig. 6).

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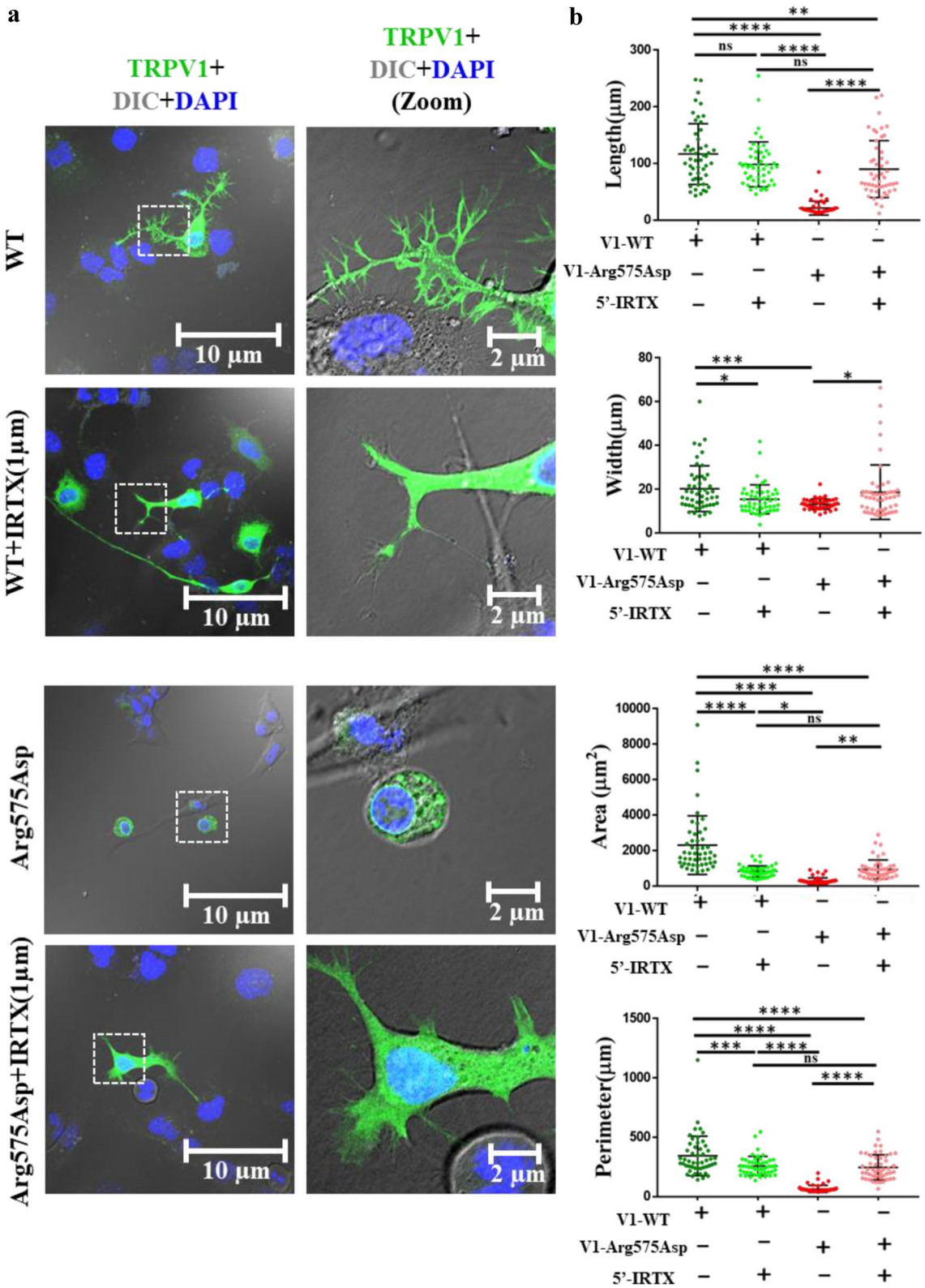

Fig. 4 Cell lethality due to Arg575Asp mutation can be rescued by TRPV1 channel blocker. **a** Confocal images of TRPV1-WT-GFP and TRPV1-Arg575Asp-GFP expressed in F11 cells and grown in absence and presence of 5′-IRTX, a specific inhibitor of TRPV1. F11 cells expressing TRPV1-Arg575Asp-GFP become much elongated and produce neurites and filopodia in presence but not in the absence of 5′-IRTX. **b** Quantification of different morphology parameters such as length, width, area and perimeter of cells mentioned above. In all cases, minimum 50 cells for each condition were quantified. The ** ($p = 0.0098$), **** ($p < 0.0001$) and *** ($p = 0.0006$) indicate values that are significantly different whilst ns indicate values that are non-significantly different

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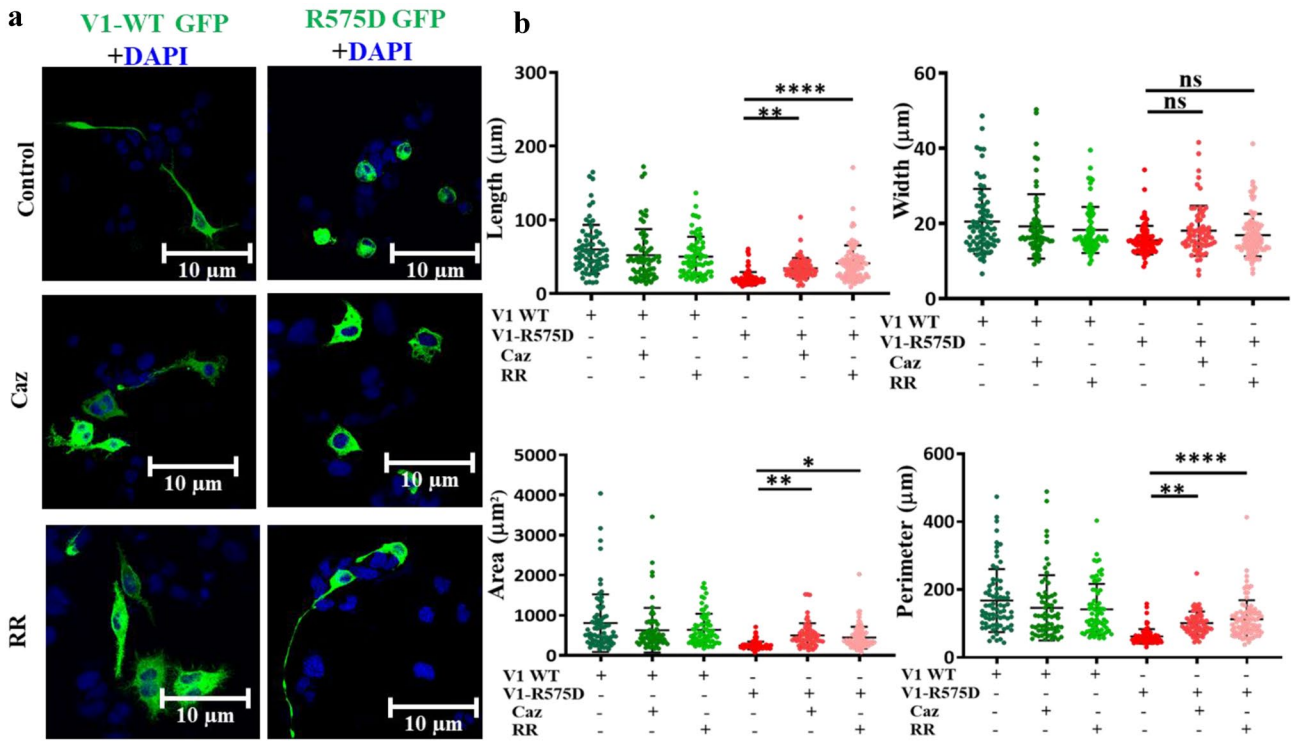

Fig. 5 Cell lethality due to Arg575Asp mutation can be rescued by non-specific TRPV1 channel blockers. **a** Confocal images of TRPV1-WT-GFP and TRPV1-Arg575Asp-GFP expressed in F11 cells and grown in absence and presence of capsazepine, a specific inhibitor of TRPV1and ruthenium red, a non-specific inhibitor of TRPV1. **b** Quantification of different morphology parameters such as length, width, area and perimeter of cells mentioned above. In all cases, minimum 50 cells for each condition were quantified. The values are: $* \leq 0.05$, $** \leq 0.01$; $**** < 0.0001$) and indicate values that are significantly different whilst "ns" indicate values that are non-significantly different

TRPV1-WT exhibited a rapid increase in Ca^{2+} level upon addition of RTX, whereas cells expressing TRPV1-Arg575Asp (R575D) show no visible change in fluorescence intensity upon RTX application, suggesting that TRPV1-Arg-575Asp expressing cells are mostly insensitive to RTX. On the other hand, TRPV1-Arg575Asp-Asp576Arg (R575D-D576R) expressing cells exhibited similar ligand sensitivity like that of TRPV1-WT. Quantification of pGP-CMV-GCaMP6f fluorescence intensities from doubly transfected cells show that both TRPV1-WT and TRPV1-Arg575Asp-Asp576Arg exhibit immediate response to RTX application (at the 30th frame). Taken together, the data suggest that the inner LWI region of TRPV1 has a conserved pattern that maintains a fixed ratio of positive–negative charges as well as hydrophobic–hydrophilic residues. Changes in such pattern may impact the surface expression, lipid raft localization and ligand sensitivity of TRPV1 and thereupon affect cell viability.

Discussion

In this work we analysed the conservation of different amino acids in the lipid–water-interface of TRPV1 and evaluated the importance of critical residues. Such understanding provides critical information about functionality in different ion channels which are of importance.

Mutation-Induced Channelopathies and Channel Gating

So far several diseases have been identified that are linked with malfunctioning of ion channels, collectively termed as "channelopathies". The most common cause of such impairment is mutation in genes encoding ion channels. Such mutations affect different properties, such as channel folding, proper localization, relevant interactions with lipids and/or proteins, post-translational modifications, and often channel gating behaviour (Verma et al. 2010). As TRPV1 is a non-selective cation channel and polymodal in nature (i.e. it gets activated by different physico-chemical stimuli), changes in TRPV1 sequence are indicative of suitability of amino acids in micro-environments at the lipid bilayer suitable for proper channel gating. As TRPV1 is involved in thermosensory functions and other physiological functions, improper channel gating (i.e. "constitutive-opening" or "constitutive-closing" due to different mutations) is expected to provide either disadvantages or remain neutral (i.e. at least provide no adaptive advantages either). Therefore,

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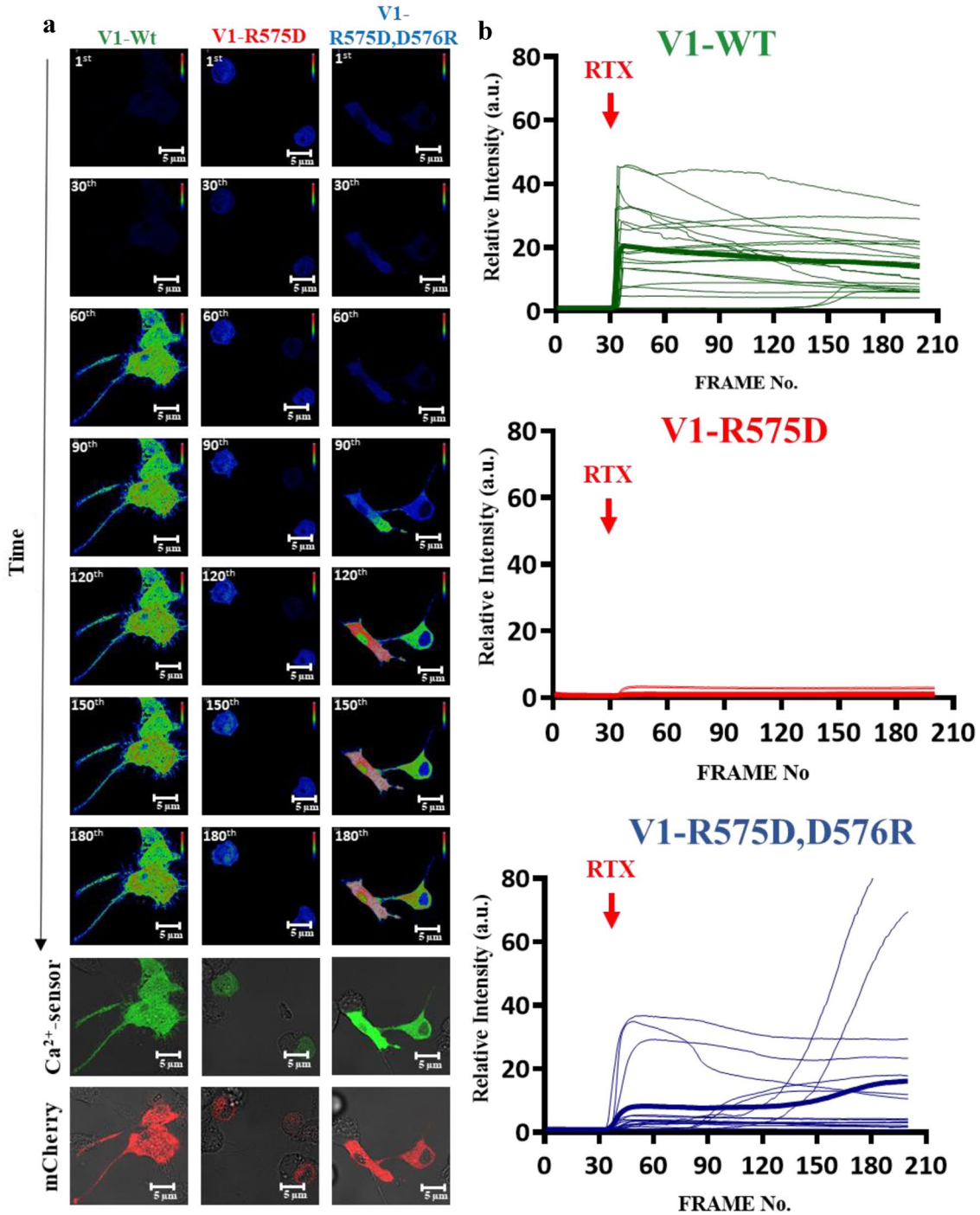

Fig. 6 Insensitivity of TRPV1-Arg575Asp mutant to RTX can be rescued by introducing Asp576Arg mutation. F11 cells expressing either TRPV1-WT, or Arg575Asp, or Arg575Asp-Asp576Arg along with Ca^{2+} sensor pGP-CMV-GCaMP6f were used for Ca^{2+}-influx assay. TRPV1-specific agonist resiniferatoxin (RTX, 100 nM) was added at 30th frame and Ionomycin (2 µM) was added at 120th frame. **a** TRPV1-WT and TRPV1-Arg575Asp-Asp576Arg show rapid Ca^{2+} influx upon RTX addition. The change in fluorescence intensity of pGP-CMV-GCaMP6f before and after agonist stimulation has been depicted in pseudo colour mode (blue indicating low and red indicating high levels of Ca^{2+}). TRPV1-Arg575Asp remained insensitive to RTX addition. **b** Graphical representation of the change in GCaMP6f intensities upon addition of RTX is shown. TRPV1-WT ($n=21$ cells), TRPV1-Arg575Asp ($n=22$ cells), and TRPV1-Arg575Asp/Asp576Arg ($n=17$). In all cases, the initial value is normalized as 1. The thick line in each intensity plot demarcates the average intensity of each construct

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uncontrolled spontaneous opening of TRPV1 is expected to cause lethality at the level of cells, tissues and also in individual. Our results confirm that TRPV1 retains a specific ratio of hydrophobic–hydrophilic residues (0.36:1) as well as positive–negative-charged residues (1.67:1) in its inner LWI region throughout vertebrate evolution. In spite of the variation in "frequency-of-occurrence" in individual amino acids, the conservation in these ratios are highly significant and shed important information about the structure–function relationship of TRPV1. Notably, these values remain constant in all vertebrates. In fact, there is no change in these ratio values in reptiles as well as in birds (indicating transition from cold-blooded animals to warm-blooded animals), and the same values exist in early vertebrates too suggesting that these values actually indicate functions that are independent of body temperature. We propose that these ratio values indicate suitability of amino acids in specialized micro-environments required for "channel-gating" and not for "thermo-gating" per se as the values remain same in cold-blooded as well as in warm-blooded animals.

Importance of Membrane Composition in Channel Functions

Importance of different membrane components in channel localization and function is well established. In this context, it is important to mention that TRPV1 activity is modulated by various exogenous and endogenous lipid molecules (Morales-Lázaro et al. 2017). For example, mutations that abolish TRPV1-PIP_2 interaction result in TRPV1 having lower chemical and thermal activation thresholds (Prescott and Julius 2003). PIP_2 helps TRPV1 to stabilize in a closed state. Depletion of membrane cholesterol by MβCD treatment results in decreased expression and activity of TRPV1 in Rat DRG neuronal membrane (Liu et al. 2006). Cholesterol depletion also affects the pore size and dilation of TRPV1 (Jansson et al. 2013). This accords well with studies demonstrating that TRPV1 is localized in cholesterol-enriched lipid rafts and that its channel properties are altered by membrane cholesterol (Sághy et al. 2015). In fact TRPV1 has at least one cholesterol-binding site in the S5-helix and interaction of TRPV1 cholesterol results in inhibition of channel opening (Picazo-Juárez et al. 2011). This aspect matches well with other TRPV-induced channelopathies. For example, mutation (R616Q) causing loss of interaction with cholesterol can result in formation of constitutively open TRPV4 channel which causes physiological disorders (Das and Goswami 2019). Previously it has been shown that arginine residues present in the inner LWI region of TRPV1, especially at 557 and 575 positions, form H bonds with cholesterol in the closed but not in open conformation (Saha et al. 2017). Accordingly, substitution of Arg with any other neutral (Ala) or negatively charged (Asp) amino acid resulted in lack of TRPV1 localization at membranes and with lipid raft markers (Saha et al. 2017). Inhibition of spontaneous opening of TRPV1 by membrane components seem to be logical and physiologically relevant as TRPV1 has a relatively large pore which is permeable to several different ions (including Ca^{2+}) and even large molecules (Munns et al. 2015; Hellwig et al. 2004; Meyers et al. 2003; Binshtok et al. 2007; Jansson et al. 2013). This fact is supported by studies that show that under hypocalcemic conditions, persistent agonist application induces a time-dependent dilation of TRPV1 pore and therefore allow the penetration of large cations (Jansson et al. 2013). However, under the same conditions reduction of cellular cholesterol by ~54% inhibits such pore dilation of TRPV1 and its subsequent permeability to large cations, like NMDG (Jansson et al. 2013). Taken together, it is apparent that cholesterol and other membrane components play important regulatory function relevant for TRPV1 localization and seems to regulate its gating property. However, the underlying mechanisms are not clear. In this context, it is important to note that selection of amino acids in the LWI regions and also in the TM regions may suggest a complex coevolution process influenced by the presence of certain membrane components, such as cholesterol (Kumari et al. 2015).

LWI Region of TRPV1 and Importance of Positively Charged Amino Acids

Lipid–water-interface provides a special micro-environment on both sides of the membrane where bulk water level is low (MacCallum et al. 2008). Notably, the protonation and deprotonation of amino acid side chains are expected to be different in LWI regions than what is considered in standard aqueous solution and thus *pI* values of side groups (also impact on their pH-dependent titration) of amino acids are different in LWI regions (Wimley and White 1996). In many cases, the transmembrane helices of the integral membrane proteins consist of apolar amino acids flanked by charged amino acids at the LWI region which allow stable insertion of these TMs in the lipid bilayer (Lee 2003). These interfacial regions are often demarcated by charged residues (like lysine, arginine) or other snorkelling amino acids (such as Tyrosine and Tryptophan). Amongst positively charged residues, arginine and lysine have better "snorkelling" ability depending on the availability of free water in such LWI regions (Strandberg and Killian 2003). The long-charged side chains of arginine and lysine can stretch themselves to place the charged moiety in the polar lipid–water interfacial regions whilst keeping the hydrophobic part of their side chains in the hydrophobic core of the lipid bilayer. This kind of arrangement has been observed in several other ion channels and other proteins too. Membrane-inserted arginine residues has been found in several ion channels (such as in case

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of Mg^{2+}-channels) (Payandeh et al. 2013), anti-microbial peptides (Chan et al. 2006), pore-forming peptides (Herce et al. 2009) and in case of cell-penetrating peptides too (Allolio et al. 2018). Insertion of the most hydrophilic amino acid arginine (pK_a of the side group 13.5) into the highly hydrophobic core requires sufficient amount of energy. However, it has been suggested that the guanidinium group present in the side chain of arginine is capable of forming as many as six hydrogen bonds and hence its occurrence in a membrane protein is mainly localized to the interfacial region where it can interact with both water molecules and polar lipid moieties (Hristova and Wimley 2011). Considering that surface of lipid bilayer is enriched with negative charge (due to head groups of phospholipids), positioning of highly positively charged residues in the LWI region fits well there, especially for the purpose of minimizing lateral movement of TM regions. The distributions of positively charged residues at LWI regions is often coupled with the specific arrangements of lipid/cholesterol-interacting motifs suitable for proper stabilization of the TM regions in lipid bilayers and also for the thermodynamic events experienced during conformational changes required for channel opening (Fantini and Barrantes 2013).

Importance of Decoding the Amino Acid Distribution in LWI

The frequency of occurrence of Arg, Lys, Pro, Trp and Tyr remains constant in inner LWI of TRPV1 throughout vertebrate evolution. Notably, Cys is completely excluded in inner LWI throughout vertebrate evolution, suggesting that Cys residue is misfit there. Residues such as His, Glu and Thr gradually declined and ultimately got excluded in mammals, suggesting that these amino acids are not suitable in the inner LWI regions, especially in warm-blooded animals with respect to the channel functions'. Whilst positive-charged amino acids such as Arg and Lys are selected at high frequency, exclusion of His in the inner LWI is highly suggestive. High frequency of Arg and Lys accords well with their ability to snorkel as well as their ability to form bonds with membrane components (Caputo and London 2003; Killian and Von Heijne 2000). For example, Arg and Lys are able to form bonds with the –OH group of cholesterol at the LWI region, whilst His residue is unable to form such bonds. Also, considering the protonation–deprotonation possibilities in physiological pH at the inner LWI, His appears to be a misfit amino acid there (Iyer et al. 2018). Reduction in frequencies of Phe, Asn, Ala and Leu and increment in frequencies of Ile, Val and Gln in the LWI correlate with the transition from cold-blooded animals to warm-blooded animals (reptiles to birds). Therefore, these changes are more -likely to accommodate the bio-physical changes that took place in lipid membrane at low body temperature to warm temperature. The frequency of occurrence of helix-breaking amino acids, namely Proline and Glycine, remains conserved throughout vertebrate evolution, especially in the inner LWI region. The frequency of occurrence of hydrophobic and hydrophilic amino acids correlates well with actual hydrophobic–hydrophilic values (of the side groups) at the LWI regions. Aromatic amino acids such as Trp and Tyr remain conserved in the inner LWI region throughout vertebrate evolution. Other aromatic amino acid, namely Phe, is positively selected in mammals in inner LWI. In this context, it is important to mention that the aromatic residues determine the preferred rotational and dynamics of membrane-spanning segments relevant for the function of membrane proteins (Sparks et al. 2014).

Frequency at LWI: Importance of Certain Amino Acids in Channel Functions

Our analysis sheds important information about the structure–function relationship of TRPV1. Notably, in inner LWI region, amino acids with different side groups have followed three distinct trajectories. Either such amino acids were retained at a conserved frequency, such as Asp or Lys residues in the inner LWI region, or such amino acids were gradually excluded. For example, Glu residues have been gradually excluded from the inner LWI regions. On the other hand, some amino acids with titratable side group, such as Arg, have been positively selected. From our data it becomes evident that the amino acids that have retained a high frequency of occurrence (~ 10%, and at constant values) during vertebrate evolution in the inner LWI are arginine and aspartic acid. In fact, Arg and Asp share 1:1 ratio at the inner LWI region, especially in higher vertebrates. Data suggest that Arg at 575th position may play important role in the channel gating, possibly by altering the conformation of TM5 and thus the lower gate of TRPV1 (Zheng and Wen 2019). It seems that Arg575 is involved in the interaction of other negatively charged residues in order to regulate channel opening and thus involved in channel gating. For example, Arg575 can form salt bridges with Glu692 at physiological temperature (Wen and Zheng 2018). Similarly, Asp576 forms salt bridge with Thr685 at physiological temperature and also at higher temperature as a result of heat deactivation (Wen and Zheng 2018). Based on molecular simulation data, charge neutralization of Arg575, Arg579, and Lys694 by PIP_2 has also been suggested (Díaz-Franulic et al. 2016). Based on sequence conservation and experimental evidence, we propose that it is possible that at physiological pH, the net charge at the inner LWI of TM5 is neutral [due to presence of a positive (Arg575) and negative charge (Asp576)] and ability of the Arg residue to interact with cholesterol may allow TM5 to be "fixed" in a conformation that is equivalent to "closed state", yet flexible enough for opening

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in presence of proper stimuli (Fig. 7). Considering that the inner surface of the lipid bilayer is intrinsically negatively charged (due to the presence of large number of phospho groups), when the inner LWI region of the TM5 is fixed with more negative charge, the TM5 may shift further down from the bilayer resulting in "locking of mutant channel" in either "open" or at least "open-like" or in a "dilated" state. This is more relevant, as TRPV1 become sensitized (equivalent to more spontaneous opening) upon phosphorylation in its Ser residues, which introduces more negative charge in its loop regions (Numazaki et al. 2002).

Does Arg575Asp Mutation Makes it a Constitutive Open Ion Channel?

Occurrence and positioning of a positively charged residue followed by negatively charged residues in specific LWI sequences can be of high importance (Fig S8). Indeed, the Arg residue at 575 position, Asp residue in 576 position and Arg residue at 579 position are actually present in a cluster and these three residues are highly conserved throughout vertebrate evolution (Fig S9). Such arrangements have also been observed in other channels, such as in Shaker Kv channels which contain Arg followed by Glu amino acid in the inner LWI region of its TM5 (Zhang et al. 2017).

Notably Arg at 575 position in TRPV1 seems to be very critical for proper localization of the channel at lipid rafts and also for maintaining interaction with cholesterol, at least in the closed state. As these two residues, namely Arg575 and Asp576, have not changed during the transition from reptiles (cold blooded) to birds (warm blooded), it can be proposed that both Arg575 and Asp576 are not involved in "thermo-gating" property per se, but is actually involved in "channel gating" in standard vertebrate membrane containing cholesterol (and/or other lipids). However, Arg575 is a crucial amino acid for overall channel gating and Arg-575Asp mutation brings double negative charge to the end of TM5 located at LWI regions. Such strong negative charge is expected to displace TM5 further down from LWI region due to strong repulsion by negatively charged surface and lack of bond formation with membrane cholesterol may happen. Such aspects may cause "permanent locking" of Arg575Asp mutant in a constitutive open-like conformation (Fig. 7). However, this behaviour of Arg575Asp mutant may vary in other membrane conditions, such as altered membrane tension, membrane fluidity and/or altered cholesterol level, which needs further investigation.

Indeed, there are several reasons that prompt us to propose that Arg575Asp mutation leads to a "constitutive open-like" TRPV1 channel. Firstly, expression of Arg-575Asp mutant leads to cell lethality (~within 24 h) after its expression in F11 cell. The same lethality is also observed in other cell types such as SaOS and HaCaT suggesting that Arg575Asp mutation is universally lethal in different types of cells (Fig S6). Second, most of the cells expressing Arg575Asp mutant are insensitive to RTX as well as other TRPV1 agonists, like Capsaicin, suggesting that a majority of the cells are already in an activated stage where further activation by agonists may not be possible (data not shown). Lastly and most notably, the lethality due to Arg575Asp can be rescued by a TRPV1-specific blocker 5′-IRTX or a general non-specific blocker Ruthenium red. Also, Ca^{2+} imaging experiments suggest that even prolonged application of RTX or Capsaicin does not facilitate further entry of Ca^{2+} ions into the cells expressing Arg575Asp mutant (data not shown). All these results suggest that Arg575Asp mutation most likely induces TRPV1 to form a constitutively open channel. This possibility has been supported by the fact that introducing Asp576Arg mutation in the background of Arg575Asp rescues cell lethality, although the double mutant shows defects in the lipid raft localization (data not shown). Non-transfected cells do not show any staining for TRPV1 at the surface (Fig S7a) and cells transfected with only pmCherryC1 vector backbone do not show any response to TRPV1-specific stimuli (Fig S7b). Ca^{2+} imaging results suggest that Arg575Asp/Asp576Arg is capable of rescuing the channel gating function to some extent, but not the lipid raft localization fully. In that case non-annular interaction of TRPV1 cholesterol might be relevant. FACS and immunocytochemistry of TRPV1-WT, Arg575Asp, and Arg575Asp/Asp576Arg suggest that although Arg575Asp mutation induces cell lethality but does not totally impair the channels' expression at the cell surface (data not shown). It could be possible that the cell lethality (followed by detachment of cells from culture surface) is initiated when a threshold amount of Arg575Asp mutant reaches to the cell surface. This unique characteristic of inducing cell lethality is only exhibited by TRPV1-Arg575Asp because when the adjacent Aspartate at 576 position in TRPV1 was mutated to Arginine (i.e. TRPV1-Asp576Arg), no altered cellular morphology was observed (data not shown). However, Asp576Arg alone exhibited different levels of agonist sensitivity. Asp576Arg mutant show sensitivity to RTX, but is largely insensitive to Capsaicin (data not shown).

In this context it is worth mentioning that TRPV1 is highly permeable to Ca^{2+} and it shows Ca^{2+}-dependent desensitization, which effectively allows TRPV1 channel to become insensitive to stimuli when applied repetitively (Vyklický et al. 2008). Notably, intracellular Ca^{2+} can interact with phosphatidylserine in physiological concentration and cause physico-chemical changes, altered hydration in the lipid–water-interface. This in turn alters lipid packing and may slow down interfacial dynamics (Valentine et al. 2018). Such changes might be useful for the Ca^{2+}-dependent desensitization event of wild-type TRPV1. However, Arg575Asp mutant may have differences in that aspect.

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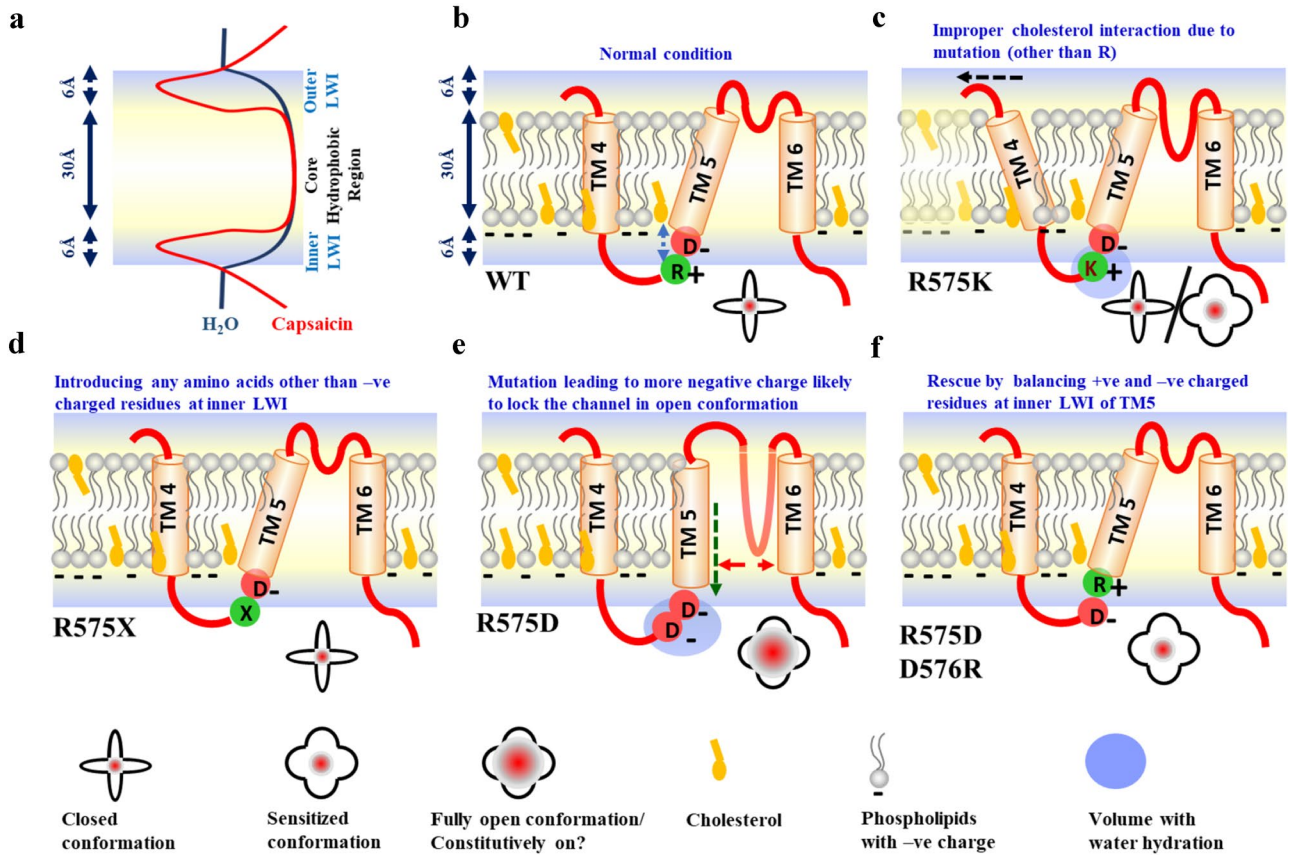

Fig. 7 A plausible model depicting the importance of positive- and negative-charged amino acids in inner LWI region of TM5 in channel functions. **a** The transmembrane helices and unstructured loops are mostly occupied in the core hydrophobic lipid bilayer (~thickness of 30 Å) and both side lipid–water-interface regions (~thickness of 6A), respectively. **b**–b Shown are the schematic representation of TM5-P-loop-TM6 of TRPV1 in different scenarios. Whilst R residues interact with –OH group of the cholesterol effectively, positive and negative charge at the inner LWI region of TM5 neutralize each other in order to position TM5 properly in an ideal confirmation suitable for channel gating. Alteration in negative–positive charge distribution there alters the positioning and conformation of TM5 as well as its possibility to interact with cholesterol. Introducing more negative charge in the inner LWI region of TM5 possibly leads to "locking" of the TM5 in a more open-like conformation and pore dilation resulting cell lethality. Accordingly, introducing a positive charge next to 575th position rescue this lethality

Notably, in human population, a large number of variations including missense mutations exist for all TRPV channels, yet the pathogenicity of these variations differ from different channels (Ghosh et al. 2016). While a large number of point mutations have been identified in human population for TRPV4 and TRPV3 (which are associated with diseases), so far no point mutation has been identified for TRPV1 in human population. In that context, Arg575Asp largely represent a "lab-made mutation" for TRPV1. Therefore, it might be possible that this mutation is wiped out from the population due to its lethality or is present in undetectable and/or in low frequency. Nevertheless, Arg575Asp and Asp576Arg mutations of TRPV1 offer interesting candidates where further and detail investigation is required.

Conclusion

In this work we have "decoded" a molecular pattern in the lipid–water-interface of the membrane protein. We also decoded the significance of certain amino acids, their patterns and their combinations in the lipid–water-interface regions of TRPV1. We term this molecular pattern as "Lipid–Water-Interface Pattern Theory" (LWI-PT). We experimentally demonstrate that such pattern plays important role in channel functions and changing such code can induce cell lethality. Notably, restoring such codes can also rescue channel functions. This understanding will allow to analyse the other thermosensory and non-thermosensory channels and their functions in the light of LWI-PT. Such understanding may also help us to dissect the impact of different point mutations that induce pathophysiological disorders.

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Author Contributions CG conceived the idea and designed all the experiments. RD1, D., NT, RD2 and SM performed all in silico analyses. RD1, DV, NT, AK, SM and CG analysed all the in silico results. SS performed all the construct preparation. SS and SM did all the cell culture, sample preparation, all the microscopic experiments, image processing, quantification and their statistical analysis. AT performed some functional experiments related to mutant and wild-type channels. CG wrote the paper. The model has been prepared by CG. All authors contributed towards manuscript editing.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interests The authors declare that they have no competing interests.

Ethical Approval This manuscript has been prepared following highest level of scientific ethics concerned.

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