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BRIEF COMMUNICATION

Ancient Origin of Four-Domain Voltage-gated Na^+ Channels Predates the Divergence of Animals and Fungi

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Abstract The four-domain voltage-gated Na^+ channels are believed to have arisen in multicellular animals, possibly during the evolution of the nervous system. Recent genomic studies reveal that many ion channels, including Na^+ channels and Ca^{2+} channels previously thought to be restricted to animals, can be traced back to one of the unicellular ancestors of animals, *Monosiga brevicollis*. The eukaryotic supergroup Opisthokonta contains animals, fungi, and a diverse group of their unicellular relatives including *M. brevicollis*. Here, we demonstrate the presence of a putative voltage-gated Na^+ channel homolog ($TtrNa_V$) in the apusozoan protist *Thecamonas trahens*, which belongs to the unicellular sister group to Opisthokonta. $TtrNa_V$ displays a unique selectivity motif distinct from most animal voltage-gated Na^+ channels. The identification of $TtrNa_V$ suggests that voltage-gated Na^+ channels might have evolved before the divergence of animals and fungi. Furthermore, our analyses reveal that Na_V channels have been lost independently in the amoeboid holozoan *Capsaspora owczarzaki* of the animal lineage and in several basal fungi. These findings provide novel insights into the evolution of four-domain voltage-gated ion channels, ion selectivity, and membrane excitability in the Opisthokonta lineage.

Keywords Channel evolution · Channel pore · Genomics · Na^+ channel · Protists · Selectivity motif

Voltage-gated Na^+ (Na_V) channels in animals initiate and propagate action potentials in many excitable cells such as neurons, myocytes, and neuroendocrine cells (Catterall et al. 2005a). Similar to animal voltage-gated Ca^{2+} (Ca_V) channels, Na_V channels possess four homologous domains, each of which contains six transmembrane segments (TMS) and a pore loop resembling the single-domain 6-TMS voltage-gated K^+ channels (Armstrong and Hille 1998; Cai 2008a; Hille 2001; Strong et al. 1993). It was hypothesized that primordial Ca_V channels were likely derived from an ancestral single-domain channel by two rounds of intragenic duplication (Armstrong and Hille 1998; Hille 2001; Strong et al. 1993). Na_V channels then diverged from some primitive Ca_V channels during the development of the nervous system and fast-conducting axons in ancestral multicellular animals (Fig. 1a) (Armstrong and Hille 1998; Hille 2001; Strong et al. 1993). Indeed, putative evolutionary intermediate two-pore channels (TPCs) with two homologous 6-TMS domains have recently been identified (Ishibashi et al. 2000) and characterized to be involved in Ca^{2+} signaling in animals (Brailoiu et al. 2009; Cai and Patel 2010; Calcraft et al. 2009).

The evolution of animal Na_V channels has been intensively studied because of its relevance not only for understanding the structure and function relationship of Na_V channels, but also for exploring the origin of membrane excitability and the nervous system (Arnegard et al. 2010; Goldin 2002; Lopreato et al. 2001; Plummer and Meisler 1999; Strong et al. 1993; Widmark et al. 2011; Zakon et al. 2006, 2011). However, little is known about

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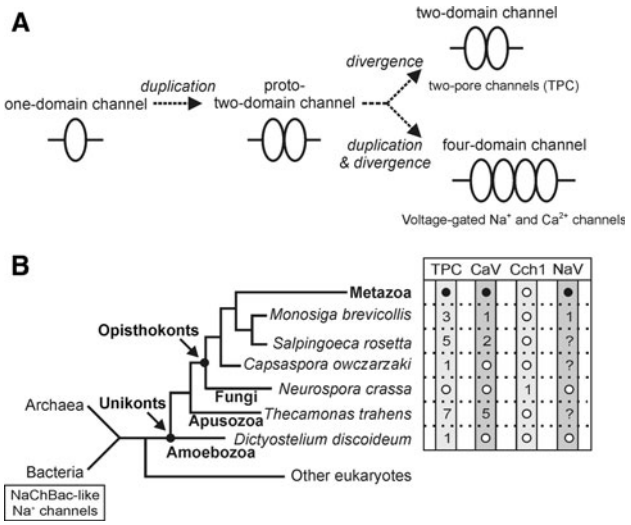

Fig. 1 Schematic representation of ion channel evolution. **a** Schematic diagram illustrating the hypothesis of intragenic duplications resulting in two-domain two-pore channels (Ishibashi et al. 2000; Patel et al. 2010) and four-domain Na^+ and Ca^{2+} channels (Armstrong and Hille 1998; Strong et al. 1993). **b** Phylogeny of select species in Opisthokonta and its relationship within eukaryotes and with prokaryotes, inferred from the Tree of Life project (http://www.tolweb.org/) and recent references (Ruiz-Trillo et al. 2007; Sebe-Pedros et al. 2010). The number of protein homologs derived from currently available genomic databases is shown, while a *black dot* indicates the presence of protein homolog(s) and a *white circle* indicates the absence of related protein homolog. An interrogation mark "?" indicates the unknown status of protein homolog(s) in the genomes. Ca_V, voltage-gated Ca^{2+} channel; Cch1, fungal Ca^{2+} channel protein 1; NaV, voltage-gated Na^+ channel; TPC, two-pore channel

the evolution of Na_V channels outside of the metazoan lineage. A family of prokaryotic 6-TMS Na_V channels have been functionally characterized (Koishi et al. 2004), the first of which is the NaChBac channel isolated from the salt-loving bacterium *Bacillus halodurans* (Durell and Guy 2001; Ren et al. 2001). These prokaryotic Na_V channels appear to be missing in the currently available eukaryotic genomes. The apparent absence of Na_V channel homologues in the genomes of fungi, plants and many protists suggested that 24-TMS Na_V channels might be animal specific (Armstrong and Hille 1998).

Animals, fungi, and a diverse group of their unicellular relatives belong to the same eukaryotic supergroup Opisthokonta (Cavalier-Smith and Chao 2003; Rokas 2008; Ruiz-Trillo et al. 2007; Steenkamp et al. 2006). *Monosiga brevicollis*, one of the closest unicellular relatives of animals (Carr et al. 2008; Ruiz-Trillo et al. 2008; Steenkamp et al. 2006), has been shown to possess cell surface adhesion molecules, receptor tyrosine kinases, and several other signaling molecules previously thought to be restricted to animals (King and Carroll 2001; King et al. 2003, 2008; Li et al. 2008). We also demonstrated in *M. brevicollis* the presence of various ion channels and transporters involved in animal Ca^{2+} signaling (Cai 2008b). Interestingly, a putative 24-TMS Na_V channel homolog with an ion selectivity filter conserved in many invertebrates has recently been cloned in *M. brevicollis* (Liebeskind et al. 2011), which suggests that the evolution of 24-TMS Na_V channels occurred before the emergence of the nervous system in animals (Liebeskind et al. 2011).

Animals and fungi display lineage-specific diversifications in the components of many signaling pathways after having diverged from a common unicellular ancestor approximately 1 billion years ago (Rokas 2008; Ruiz-Trillo et al. 2007). The evolutionary origin of the integrin adhesion complex critical for intercellular communications in animals was recently shown to predate the divergence of Opisthokonta, with the key component being lost in fungi (Sebe-Pedros et al. 2010). We also showed that many components of the animal Ca^{2+} signaling machinery originated in the apusozoan protist *Thecamonas trahens* (Cai and Clapham 2012), which belongs to the putative unicellular sister group to Opisthokonta (Fig. 1b) (Cavalier-Smith and Chao 2010).

In this study, we aimed to identify ancestral Na^+ channel homologs in the Opisthokonta lineage by examining genomic data from *T. trahens* and several close relatives of animals and fungi. We show that a putative 24-TMS Na_V channel homolog is present in *T. trahens*, suggesting 24-TMS Na_V channels might have evolved before the divergence of animals and fungi, much earlier than previously thought.

Materials and Methods

Database Searches

Protein sequences of Na_V and Ca_V channels from *Homo sapiens* were used as queries to perform initial BlastP and TBlastN searches (Altschul et al. 1997) against the Origins of Multicellularity Database (http://www.broadinstitute.org/annotation/genome/multicellularity_project/MultiHome.html), the Broad Institute Genomic Database (http://www.broadinstitute.org/scientific-community/data), and the National Center for Biotechnology Information Genome Database (http://www.ncbi.nlm.nih.gov/blast/). In order to identify potential distantly related homologs in nonmetazoan organisms that might not be detected by using *H. sapiens* query proteins, repeated BLAST searches using hit sequences from the first round of searches were also performed against the above databases. In addition, PHMMER searches (HMMER 3.0, http://hmmer.janelia.org/) were also performed against protein data sets downloaded from the Origins of Multicellularity Database.

Multiple Sequence Alignments and Phylogeny Reconstruction

Nonredundant protein sequences were aligned by MAFFT (Katoh and Toh 2008) or PRANK (Loytynoja and Goldman 2010) programs and were subsequently manually edited to improve alignments displayed with the Blosum62 Similarity Scoring Table in Genedoc (Nicholas et al. 1997). Poorly aligned sites from the multiple sequence alignments were eliminated by using Gblocks (Talavera and Castresana 2007), and unambiguous sequence alignments were then exported to files in PHYLIP format. Next, ProtTest (Darriba et al. 2011) was used to select the best-fit evolution model and parameter estimates for the phylogeny reconstruction.

Maximum likelihood phylogenies were estimated by PHYML 3.0 (Guindon et al. 2010), with 100 resampled data sets obtained by SEQBOOT (PHYLIP package, v. 3.69) (Felsenstein 1996), the LG amino acid substitution matrix (Le and Gascuel 2008), the four-category discrete-gamma model and empirical amino acid frequencies (LG + G + F) selected by ProtTest. Bootstrap is a commonly used method to assess confidence in phylogenetic analyses by resampling sites from the multiple sequence alignment with replacements and assigning a bootstrap value for each clade of the reconstructed tree (Felsenstein 1985). A bootstrap value of $\geq$70 is considered to define a true clade in the phylogenetic tree with a probability of $\geq$95% (Hillis and Bull 1993). An outgroup is a protein sequence that is outside the sequences of interest (ingroup protein sequences) but is also closely related to ingroup proteins. The outgroup sequence provides a reference point for the determination of the evolutionary relationships among ingroup proteins. The TREEVIEW program (v. 1.6.6) (Page 1996) were used to display the phylogenetic trees.

Results and Discussion

Identification of a Putative 24-TMS Na_V Channel Homolog in the Apusozoan *Thecamonas trahens*

The apparent absence of 24-TMS Na_V channels in fungi suggests that 24-TMS Na_V channels might have evolved to modulate membrane excitability specifically in the lineage leading to animals, possibly as early as in the choanoflagellate *M. brevicollis* (Liebeskind et al. 2011). Alternatively, Na_V channels could have been developed in the common ancestors of animals and fungi but were subsequently lost in fungi following the animal–fungal divergence. To better understand the evolutionary origin of Na_V channels, we searched for Na_V channel homologs in several genomes at the Origins of Multicellularity Database (Ruiz-Trillo et al. 2007; Sebe-Pedros et al. 2010), including the apusozoan *T. trahens,* the amoeboid holozoan *Capsaspora owczarzaki,* the choanoflagellate *Salpingoeca rosetta*, the basal chytridiomycete fungi *Allomyces macrogynus*, and *Spizellomyces punctatus*, as well as other select genomes in the NCBI genomic databases.

12-TMS TPC channels are widely distributed among eukaryotes except in fungi (Fig. 1b) (Brailoiu et al. 2009; Galione et al. 2009). In contrast, 24-TMS Ca_V channel homologs are present in animals, fungi (Cch1 channels), and some protists (Fig. 1b). We found the presence of a 24-TMS Na_V channel homolog in the colonial choanoflagellate *S. rosetta* (Fig. 2), further confirming that Na_V channels had evolved in Choanoflagellata. However, the amoeboid holozoan *C. owczarzaki*, one of the unicellular lineages branching close to choanoflagellates and animals (Ruiz-Trillo et al. 2007, 2008), does not possess Na_V channel homologs. Ca_V channels also appear to be lost in *C. owczarzaki* (Cai and Clapham 2012), and therefore, the absence of Na_V channel homologs in *C. owczarzaki* could be related to lineage-specific gene loss.

We next examined the genome of the apusozoan *T. trahens* for potential Na_V channel homologs that might exist before the divergence of the animal and fungal lineages. Indeed, a putative 24-TMS Na_V homolog with a unique selectivity filter was detected in *T. trahens* ($TtrNa_V$) (Figs. 2, 3; Supplemental Figs. S1, S2). Na_V and Ca_V channels display a certain degree of structural and sequence similarity and are speculated to share common evolutionary origins (Hille 2001; Strong et al. 1993). Nevertheless, robust phylogenetic analyses coupled with strong bootstrap support can be utilized to identify critical clades for the Na_V/Ca_V channel phylogeny and thus provide evolutionary evidence to distinguish between Na_V and Ca_V homologs, as shown previously in analyzing channel homologs from the cnidarian jellyfish *Cyanea capillata* (Anderson et al. 1993) and the choanoflagellate *M. brevicollis* (Liebeskind et al. 2011). To delineate the evolutionary relationships of $TtrNa_V$ with other Na_V and Ca_V channels from select animal species and two choanoflagellates, maximum likelihood analyses were performed by using three different outgroups that are closely related to animal 24-TMS voltage-gated Na^+ and Ca^{2+} channels but possess distinct evolutionary histories and functional properties: (1) 24-TMS channels—two fungal Ca^{2+}-selective Cch1 channels (Fig. 2a), presumably voltage independent (Hong et al. 2010), and a 24-TMS voltage-independent and nonselective cation channel NALCN (Lu et al. 2007) (Supplemental Fig. S1); (2) a 12-TMS TPC channel (Fig. 2b); and (3) 6-TMS channels, two prokaryotic voltage-dependent Na^+ channels (Supplemental Fig. S2). In all four phylogenetic trees with different outgroups

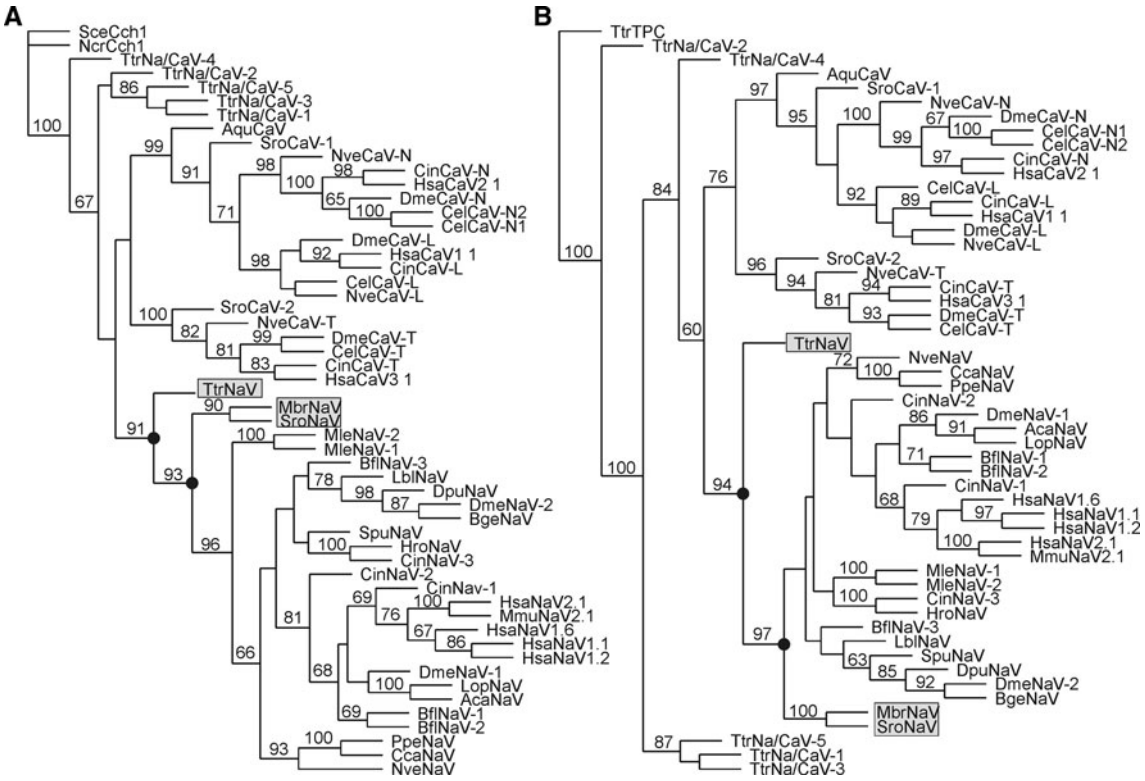

Fig. 2 Phylogenetic analysis of Na_V and Ca_V channel homologs by using two four-domain fungal Ca^{2+} channels (**a**) or a two-domain TPC Ca^{2+} channel (**b**) as the outgroup. The phylogenetic tree was built by using the maximum likelihood approach, as described in Section "Materials and Methods". *Bootstrap values* greater than 60 are shown at the nodes. The Na_V branches containing protist Na_V channel homologs ($TtrNa_V$, $MbrNa_V$, and $SroNa_V$) are highlighted by *black circles*. Abbreviations used for species: *Aplysia californica* (Aca); *A. queenslandica* (Aqu); *Branchiostoma floridae* (Bfl); *Blattella germanica* (Bge); *Caenorhabditis elegans* (Cel); *Cyanea capillata* (Cca); *Ciona intestinalis* (Cin); *Drosophila melanogaster* (Dme); *Daphnia pulex* (Dpu); *Halocynthia roretzi* (Hro); *H. sapiens* (Hsa); *Loligo bleekeri* (Lbl); *Loligo opalescens* (Lop); *M. brevicollis* (Mbr); *Mnemiopsis leidyi* (Mle); *Mus musculus* (Mmu); *Neurospora crassa* (Ncr); *N. vectensis* (Nve); *Polyorchis penicillatus* (Ppe); *Saccharomyces cerevisiae* (Sce); *Strongylocentrotus purpuratus* (Spu); *S. rosetta* (Sro); *T. trahens* (Ttr)

(Fig. 2; Supplemental Figs. S1, S2), $TtrNa_V$ is consistently grouped with the Na_V channel protein family including the two choanoflagellate Na_V channel homologs ($MbrNa_V$ and $SroNa_V$), not with the Ca_V channel protein family in the animal lineage. TtrNaV is placed at the base of the Na_V protein family in the animal lineage (strong bootstrap values ranging from 88 to 97 depending on the outgroup used). In addition, the phylogeny of Na_V and Ca_V channels is well correlated with the known evolutionary history of the species analyzed here. Thus, the identification of a putative 24-TMS Na_V channel homolog in *T. trahens* suggests that Na_V channels might have originated not only before the evolution of animal multicellularity and the nervous system (Liebeskind et al. 2011), but also before the divergence of the animal and fungal lineages.

Interestingly, five 24-TMS channel homologs from *T. trahens* are not grouped with either animal Na_V or Ca_V protein families in the trees using 24-TMS and 12-TMS channel outgroups (Fig. 2a, b; Supplemental Fig. S1) and are therefore named as Na_V and Ca_V-like homologs ($TtrNa/Ca_V$-1–5). Indeed, if only based on sequence homology and BLASTP (Altschul et al. 1997) searches, $TtrNa/Ca_V$ homologs show high similarity not only to animal Ca_V channels and fungal Cch1 Ca^{2+} channels, but also to a lesser extent to animal Na_V channels. $TtrNa/Ca_V$ channel homologs could represent ancestral Ca^{2+}-permeable ion channel homologs from which Na_V channels had evolved, as proposed previously (Armstrong and Hille 1998; Hille 2001; Strong et al. 1993).

Na_V channel homologs are absent in the basal fungi *A. macrogynus,* S. *punctatus*, *Rhizopus oryzae*, and *Batrachochytrium dendrobatidis*. Similar to the evolution of the integrin adhesion complex (Sebe-Pedros et al. 2010), Na_V channels appear to have been subsequently lost in the fungal lineage after its divergence from animals (Liebeskind et al. 2011).

Selectivity Filter Motif of Na_V Channels

The ion selectivity of Na_V and Ca_V channels is primarily determined by the pore loops, particularly a ring of four key residues that form the selectivity filter motif—

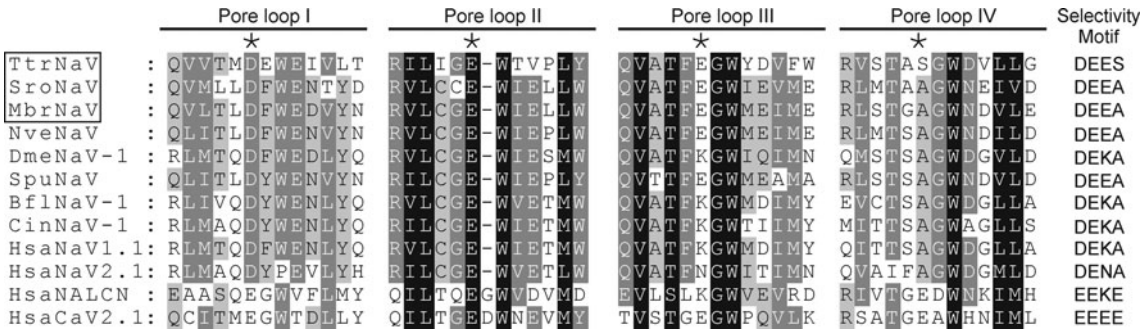

Fig. 3 Evolution of the pore loop region and the selectivity filter motif in Na_V channels. The amino acid sequences of the pore loop regions from each homologous domain of select Na_V channels and the human Ca_V 2.1 and NALCN channels are aligned, with the four key residues of the selectivity motif indicated by an *asterisk* and listed on the *right side* of the alignment

D/E/K/A for Na_V channels and E/E/E/E for Ca_V channels (Heinemann et al. 1992). Although these selectivity motifs are generally conserved in animals, variations have been observed in some species, for example, D/E/E/A in the α isoform of the cnidarian *Nematostella vectensis* and the choanoflagellates *M. brevicollis* (Liebeskind et al. 2011) and *S. rosetta* (Fig. 3), and D/K/E/A in the cnidarian jellyfish *C. capillata* (Anderson et al. 1993). Surprisingly, the selectivity motif in $TtrNa_V$ is D/E/E/S, which contains an unusual Ser residue in the fourth loop, instead of an Ala residue conserved in most Na_V channels. So far, there are only two Na_V channel homologs shown to possess a hydrophilic residue in the fourth loop—D/E/E/T in *N. vectensis* (β isoform) (Liebeskind et al. 2011) and D/E/E/S in *T. trahens* (Fig. 3).

The structure of a prokaryotic 6-TMS Na_V channel from *Arcobacter butzleri* has recently been determined (Payandeh et al. 2011), which showed that the four acidic side chains of E/E/E/E (one from each 6-TMS subunit) coordinate to form the strongly negatively charged and the narrowest part of the ion conduction pathway. Previous studies have demonstrated that a single mutation of Glu to Asp converts NaChBac from a highly Na^+-selective channel to a Ca^{2+}- and Na^+-permeable channel (D/D/D/D) and incorporating two additional Asp residues in the pore loop region of NaChBac results in a highly Ca^{2+}-selective channel (Yue et al. 2002). Conversely, mutations in the selectivity filter of the 24-TMS Na_V and Ca_V channels in animals also modulate the Na^+ and Ca^{2+} ion selectivity (Heinemann et al. 1992; Yang et al. 1993). Therefore, it is conceivable that in the four homologous channel domains of ancestral 24-TMS Ca_V and Na_V channels, the selectivity filters and the pore loop regions underwent evolutionary selection pressures to modulate ion selectivity. Furthermore, Ca_V and Na_V channels are likely also subject to changes in other conserved regions in the channel domains, which are often related to their different functional properties and pharmacological characteristics (Catterall et al. 2005a, 2005b). By taking into account not only the pore loop/selectivity filter regions but also other conserved regions in the channel domains, our phylogenetic analyses with three evolutionarily distinct outgroups (Fig. 2; Supplemental Figs. S1, S2) distinguish the ancestral Na_V channel homolog from Ca_V channels by consistently grouping the $TtrNa_V$ homolog with the animal Na_V family.

It should be noted that phylogenetic analyses used to reconstruct the evolutionary relationships of ion channel families can provide strong support for, but may not necessarily define, the nature of their functional properties, especially for these ancestral channel homologs. None of these putative protist Na_V channel homologs from *T. trahens* and the choanoflagellates *M. brevicollis* (Liebeskind et al. 2011) and *S. rosetta* have been functionally characterized so far. It remains possible that these ancestral Na_V channel homologs could represent the putative channel intermediates between Ca_V and Na_V channels as proposed previously (Hille 2001; Liebeskind et al. 2011), or display previously undescribed properties, as shown in recent characterization of two protist P2X receptors (Fountain et al. 2007, 2008). Sequence alignment, domain assignment and phylogenetic analyses support the grouping of these protist P2X receptors with their animal counterparts (Cai 2011; Fountain and Burnstock 2009). In animals, P2X receptors sense extracellular ATP and induce the flux of cations across the plasma membrane (Khakh and North 2006). In contrast, P2X receptors in the dictyostelid social amoeba *Dictyostelium discoideum* (Fountain et al. 2007) and the green alga *Ostreococcus tauri* (Fountain et al. 2008) appear to serve functional roles on intracellular organelle membranes with distinct properties, presumably sensing the changes in intracellular ATP.

Conclusion

Together with the seminal report on the first cloning and phylogenetic characterization of a protist 24-TMS Na_V channel homolog from the choanoflagellates *M. brevicollis*

(Liebeskind et al. 2011), the identification of $TtrNa_V$ from the apusozoan protist *T. trahens* with a unique pore sequence provides an exciting opportunity to study the evolution of ion selectivity in voltage-gated ion channels and the role of ion channels in the eukaryotic evolution leading to multicellular organisms. Little is known about the biology of the phylum Apusozoa, including *T. trahens* (Cavalier-Smith and Chao 2003, 2010). *T. trahens* are unicellular and biflagellate eukaryotes living in soils and aquatic environments, where they glide on the surface and feed on bacteria. Flagellar movement promotes motility in search for food, which is ingested by pseudopodia-like locomotion. Presumably, the emergence of a 24-TMS Na_V channel could provide a new means of controlling membrane excitability and enabling Ca^{2+} to function more specifically as a signaling molecule to regulate diverse cellular processes (Clapham 2007). Indeed, *T. trahens* had acquired a set of Ca^{2+} signaling molecules conserved in the animal lineage such as voltage-gated CatSper Ca^{2+} channels and Na^+/Ca^{2+} exchangers (Cai and Clapham 2012).

Determining the biophysical properties and ion selectivity of these protist Na_V homologs will no doubt broaden our current knowledge on the evolution of the electrophysiological properties in protists and in excitable cells such as neurons and cardiomyocytes (Armstrong and Hille 1998; Rosati and McKinnon 2009). Further evolutionary systems biology studies (Medina 2005) of ion channel protein families will also shed novel evolutionary and mechanistic insights into signaling pathways mediated by different ion channels.

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