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# Insights into the early evolution of animal calcium signaling machinery: A unicellular point of view

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## Abstract

The basic principles of $Ca^{2+}$ regulation emerged early in prokaryotes. $Ca^{2+}$ signaling acquired more extensive and varied functions when life evolved into multicellular eukaryotes with intracellular organelles. Animals, fungi and plants display differences in the mechanisms that control cytosolic $Ca^{2+}$ concentrations. The aim of this review is to examine recent findings from comparative genomics of $Ca^{2+}$ signaling molecules in close unicellular relatives of animals and in common unicellular ancestors of animals and fungi. Also discussed are the evolution and origins of the sperm-specific CatSper channel complex, cation/$Ca^{2+}$ exchangers and four-domain voltage-gated $Ca^{2+}$ channels. Newly identified evolutionary evidence suggests that the distinct $Ca^{2+}$ signaling machineries in animals, plants and fungi likely originated from an ancient $Ca^{2+}$ signaling machinery prior to early eukaryotic radiation.

### Keywords

Animals; Calcium channels; Calcium signaling; Choanoflagellates; Evolution; Genomics

## 1. Introduction

Binding of the divalent metal calcium ion ($Ca^{2+}$) to alter charge and hence protein conformation is one of the most extensively employed signal transduction mechanisms in life [1]. $Ca^{2+}$ modulates nearly every aspect of cellular function in bacteria [2], protists [3], plants [4], fungi [5] and animals [1,6]. Owing to the ~10,000-20,000-fold $Ca^{2+}$ concentration gradient across the plasma membrane, an intricate $Ca^{2+}$ signaling machinery

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is required to maintain an extremely low cytosolic $Ca^{2+}$ concentration at ~100 nM in resting conditions, and to raise cytosolic $Ca^{2+}$ concentration to several hundred nM or higher upon the activation of ion channels and transporters [1,7]. The evolution of primitive $Ca^{2+}$ binding molecules that shaped the basic principles of $Ca^{2+}$ signaling occurred very early in the life processes of prokaryotic organisms [7-9]. In eukaryotes, intracellular organelles such as mitochondria, lysosomes and the endoplasmic reticulum emerged as intracellular $Ca^{2+}$ compartments to likely allow better spatial and temporal regulation of $Ca^{2+}$ concentration in response to more complex environmental stimuli [1,7-10].

In multicellular organisms, the need for intercellular communication is believed to have driven the development of versatile $Ca^{2+}$ signaling systems to facilitate the communication and coordination between cells through chemical and electrical signals [7,8]. Different cell types possess distinct sets of $Ca^{2+}$ signaling molecules to carry out specific physiological functions. Neurotransmitters activate ionotropic receptors at the postsynaptic membrane, inducing $Ca^{2+}$ influx critical in the nervous system, heart, skeletal muscle, and secretory organs. Immune cells sense invading organisms and adapt via $Ca^{2+}$-mediated changes in gene expression. The cation channels of sperm (CatSpers), a class of sperm-specific $Ca^{2+}$ channels at the sperm flagella, trigger hyperactivated motility [11,12]. Selective expression of molecules from a large repertoire of the animal $Ca^{2+}$ signaling machinery has evolved to accommodate animal development and physiology at the cell and organ system levels.

The transition to multicellularity observed in animals, plants and fungi likely originated independently from multiple distinct ancestral unicellular lineages [13,14]. Animals [1,6], plants [3,4,15,16] and fungi [5] exhibit marked differences in terms of the components of their $Ca^{2+}$ signaling machineries. Animal multicellularity is generally more complex, reflected in the diversity of different cell types and intercellular communications [17,18]. Similarly, animals appear to possess a complex $Ca^{2+}$ signaling machinery while plants and fungi seem to have adopted more simplified $Ca^{2+}$ signaling cascades [4,5,15]. $Ca^{2+}$ signaling might have developed through independent evolution or from the same ancient $Ca^{2+}$ signaling network in the early evolution of eukaryotes followed by subsequent lineage-specific expansion, innovation and losses.

Recent comparative genomics of animals, fungi and plants and their unicellular sister groups has illuminated the origin and early evolution of the eukaryotic $Ca^{2+}$ signaling machinery [3,5,15,19-25]. In this review, we address the dynamic evolutionary pattern and origin of ancestral $Ca^{2+}$ signaling molecules in close unicellular relatives of animals and in common unicellular ancestors of Opisthokonta, a clade that contains animals, fungi and their unicellular relatives (Fig. 1). For detailed evolutionary review of $Ca^{2+}$ signaling in plants and other photosynthetic eukaryotes [3,22], see review by Edel and Kudla in the same issue [26].

## 2. The eukaryotic tree of life and the origins of multicellularity

The evolutionary root of eukaryotes appears to lie between Unikonta, the eukaryotic supergroup composed of Amoebozoa and Opisthokonta (animals and fungi), and Bikonta, the eukaryotic supergroup containing plants and algae (Fig. 1) [27-29]. Poriferans (sponges),

placozoans, and cnidarians (anemones) are the morphologically simplest animal phyla, with sponges being the earliest branching lineage of animals [30]. Indeed, sponges lack a nervous system and gut, both of which are present in eumetazoans - cnidarians and bilaterians (flies, worms, sea squirts, and humans). Nevertheless, comparative analysis of the *Amphimedon queenslandica* genome with eumetazoan genomes revealed that sponges already developed a wide array of signaling molecules and transcription factors critical for eumetazoan development and physiology, including cell cycle regulation, cell specification, cell adhesion and immunity [30]. The *Amphimedon* genome also encodes homologs of the core components of post-synaptic proteins [31] and neural regulatory proteins important for neural development and nerve cell function in eumetazoans, predating the development of the nervous system in animals [30].

### 2.1. Close unicellular relatives of animals – choanoflagellates and filastereans

Multicellular animals likely emerged from single-celled ancestors more than six hundred million years ago [14,32]. Choanoflagellates, a group of single-celled and colony-forming eukaryotes, display striking structural and functional similarities to flagellated collar cells (choanocytes) in sponges [33]. Choanoflagellates have a spherical or ovoid cell body with a single apical flagellum surrounded by a collar of actin-filled microvilli (Fig. 2) [34]. Phylogenomics studies have shown that choanoflagellates are the closest unicellular relatives of animals; sponges and other metazoans share a common unicellular ancestor with choanoflagellates (Fig. 1) [35-37]. The filasterean *Capsaspora owczarzaki* is a unicellular amoeboid symbiont of the pulmonate snail *Biomphalaria glabrata*. *C. owczarzaki* is among the closest known unicellular relatives of animals besides choanoflagellates [38]. Ancestral homologs of various signaling molecules previously thought to be restricted to animals, such as receptor tyrosine kinases and transcription factors crucial for animal multicellularity and development, are present in the genomes of two choanoflagellates *Monosiga brevicollis* and *Salpingoeca rosetta* and the filasterean *C. owczarzaki* [33,38-40].

In response to environmental cues, *S. rosetta* and *C. owczarzaki* can differentiate into different cell types and form multicelled colonies (or aggregative multicellularity) as part of their life cycle, which may resemble a transition state in the early evolution of animal multicellularity [41,42].

### 2.2. A putative unicellular progenitor of Opisthokonta - the apusozoan Thecamonas trahens

The complex multicellularity in the lineages leading to animals and fungi appears to have evolved independently from lineage-specific unicellular relatives. Apusozoa (apusomonads), a group of biflagellate and gliding protists, is believed to branch as a sister group to Opisthokonta (Fig. 1) [29,43]. The phylogenetic position of apusomonads between Amoebozoa and common ancestors of Opisthokonta makes them ideal candidates for comparative studies into the origin and early evolution of animals and fungi. The apusozoan protist *Thecamonas trahens* (previously known as *Amastigomonas sp.*) was chosen for genome sequencing as a representative outgroup to the entire opisthokont clade [44].

*T. trahens* was recently shown to harbour the core components of the integrin-mediated cell adhesion complex, a signaling machine critical for intercellular communication in animals [45]. Moreover, several components of the integrin adhesion complex have been lost independently in fungi and choanoflagellates. The absence of many of the integrin components in *M. brevicollis* and *S. rosetta* indicates the importance of a broad taxonomic sampling in comparative genomics of cellular signaling pathways [45].

### 2.3. Bikonta - the eukaryotic supergroup containing plants and algae

Comparative genomics of $Ca^{2+}$ signaling molecules in bikont species, the eukaryotic supergroup including plants, algae and diatoms [27-29,46], has been reported previously [3,15,20,22,47,48] and is reviewed in this issue [26]. A brief discussion on the conservation of key $Ca^{2+}$ signaling molecules will be presented based on a recent report of genomic analysis of a marine bikont protist *Aurantiochytrium limacinum* and a basally diverging alga *Cyanophora paradoxa* [25].

## 3. Evolution of the $Ca^{2+}$ signaling machinery in close unicellular ancestors of animals

The core components of the $Ca^{2+}$ signaling machinery in animals comprise proteins that control $Ca^{2+}$ influx and extrusion across plasma or organellar membranes [1,6]. In resting cells, low cytosolic $Ca^{2+}$ levels are maintained by plasma membrane $Ca^{2+}$ ATPases (PMCAs) and sarco/endoplasmic reticulum $Ca^{2+}$ ATPases (SERCAs), $Na^{+}/Ca^{2+}$ exchangers (NCXs), and cytosolic $Ca^{2+}$ binding proteins and buffers. In response to appropriate environmental stimuli, cytosolic $Ca^{2+}$ signals mainly arise from 1) $Ca^{2+}$ entry across the plasma membrane through $Ca^{2+}$ channels and/or the “reverse mode” of NCXs; 2) $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores such as the endo/sarcoplasmic reticulum (ER/SR), through the intracellular $Ca^{2+}$ release channels inositol 1,4,5-trisphosphate receptors ($IP_3Rs$) and/or ryanodine receptors (RyRs); or 3) the combination of both $Ca^{2+}$ entry and $Ca^{2+}$ release, such as excitation-contraction coupling in cardiac muscle and store-operated $Ca^{2+}$ entry (SOCE) in many nonexcitable cells. In addition, endolysosomes, mitochondria and other intracellular membrane-bound compartments are important regulators of $Ca^{2+}$ homeostasis [1,6,10].

Multicellular complexity is reflected by the presence of numerous cell types with distinct physiological functions, for example, approximately ∼200 somatic cell types in hominids [49]. Many components of the animal $Ca^{2+}$ signaling machinery show tissue- and/or cell-type-specific expression. Several lines of evidence indicate that homologs of a wide range of signaling molecules required for animal multicellularity and development are present in unicellular ancestors of animals [13,14]. These findings prompted us to determine the evolutionary pattern and origin of the animal $Ca^{2+}$ signaling machinery in the genomes of choanoflagellates *M. brevicollis* and *S. rosetta* (Fig. 2) and the filasterean *C. owczarzaki* [21,23].

### 3.1. $Ca^{2+}$ entry across the plasma membrane

The choanoflagellates *M. brevicollis* [21] and *S. rosetta* [23] have all 5 modes of regulated $Ca^{2+}$ entry across the plasma membrane identified in animals [50] — the $Ca^{2+}$ release-activated $Ca^{2+}$ channel (Orai) and the ER $Ca^{2+}$ sensor protein stromal interaction molecule (STIM), ligand-gated channels (nicotinic acetylcholine receptor and P2X purinergic receptor), voltage-gated $Ca^{2+}$ channels (CaV; similar to dihydropyridine-sensitive L-type $Ca^{2+}$ channels), second messenger-gated channels (cyclic nucleotide-gated), and transient receptor potential (TRP) channels (Fig. 2). The $Ca^{2+}$-permeable mechanosensitive Piezo channels are also present in *M. brevicollis* and *S. Rosetta* [25]. Furthermore, *M. brevicollis* contains NCXs [51].

Most TRP channels are $Ca^{2+}$ permeable, but the degree of $Ca^{2+}$ selectivity varies widely [52,53]. TRP channels appear to be polymodal cell sensors responding to environmental signals such as chemical compounds, changes in temperature, and pH. Homologs of 5 mammalian TRP channel families - TRPC, TRPV, TRPM, TRPML and TRPA, but not TRPP, are identified in *M. brevicollis* and *S. rosetta* [21,23]. Although TRP-like channel sequences are identified in several bikont protists [3,22,25], TRP channel homologs in choanoflagellates show higher degree of sequence conservation and are phylogenetically grouped with known animal TRP subfamilies [21,23]. It is not clear if TRPML and TRPP are on intracellular or specialized membranes as they are in higher organisms.

SOCE is a major $Ca^{2+}$ influx pathway in nonexcitable cells, but is also present in excitable cells such as skeletal muscle [50]. ER $Ca^{2+}$ depletion is sensed by ER-membrane spanning STIM proteins, which then bind and gate highly $Ca^{2+}$-selective Orai channels on the plasma membrane. Orai and STIM homologs in M. brevicollis and S. rosetta possess highly conserved motifs, intragenic repeat patterns and critical residues identified in their animal counterparts [54,55]. Orai and STIM homologs are also present in *C. owczarzaki* [23]. SOCE may represent a primordial $Ca^{2+}$ entry pathway in unicellular organisms. Distantly related Orai-like sequences can be occasionally found in few bikont protists [56], but these bikont protists generally lack STIM homologs and often $IP_3$Rs, two integral parts of animal SOCE. Therefore, Orai and STIM-mediated $Ca^{2+}$ entry in response to ER $Ca^{2+}$ depletion likely evolved in the ancestral animal lineages as early as the amoeboid holozoan *C. owczarzaki*, after the animal–fungi split. SOCE is also involved in exocytosis in Paramecium following $Ca^{2+}$ release from alveolar sacs mediated by polyamine, caffeine or 4-chloro-meta-cresol [57-59]. However, Orai/STIM homologs are absent in *Paramecium tetraurelia* [56]. The molecular components of SOCE in Paramecium remain to be identified.

$Ca_V$ channel homologs are found in choanoflagellates *M. brevicollis* [21] and *S. rosetta* [23]. In addition, cyclic nucleotide-gated channel homologs are present in *M. brevicollis*. A homolog of hyperpolarization-activated, cyclic nucleotide-regulated channel, which has been shown to be $Ca^{2+}$ permeant [60], is found in *S. rosetta* [61]. In contrast, none of these channels are present in *C. owczarzaki* [23]. Moreover, P2X receptor channels are found in all vertebrates, many invertebrates, basal fungi, and certain protists. Modern fungi, land plants and some invertebrate species generally lack P2X receptor channel homologs [62,63],

but P2X receptors are present in the three unicellular ancestors of animals discussed here [21,23].

### 3.2. $Ca^{2+}$ channels at the ER/SR and other organellar membranes

$Ca^{2+}$ release from the ER/SR $Ca^{2+}$ store through $IP_3Rs$ and/or RyRs is a common feature of almost all animal cell types [24]. $IP_3Rs$ are ubiquitously distributed, while RyRs are enriched in skeletal and cardiac muscles and neurons [64]. The first protozoan $IP_3R$ was characterized from the ciliate protist *P. tetraurelia* [65], which possess 34 $IP_3R$/RyR-like homologs [59,66,67]. Multiple copies of $IP_3R$ homologs are found in *M. brevicollis*, *S. rosetta* and *C. owczarzaki* [21,23]. Compared with *P. tetraurelia* $IP_3Rs$, *M. brevicollis* $IP_3Rs$ show overall higher sequence identity and similarity to animal $IP_3Rs$ [21].

RyR homologs with SPRY structural domains and moderate sequence identity/similarity with animal RyRs are present in *C. owczarzaki* and *S. rosetta* [23], but not in *M. brevicollis* [21]. Prototype RyRs, bearing the critical protein domains such as the SPRY domain and key residues conserved in animal RyRs, likely emerged in the unicellular lineages leading to animals [23,68]. Ancestral $IP_3R$/RyR-like homologs are found in *P. tetraurelia* and several other protists [48,59,66-68]. Late incorporation of SPRY and RyR domains into ancestral homologs may have led to the innovation of animal RyRs [68].

Among the three unicellular ancestors of animals discussed here, only *S. rosetta* contains both $Ca_V$ channel and RyR homologs, which are known to be functionally coupled in excitable cells of animals [64]. Four-domain voltage-gated $Na^+$ ($Na_V$) channel homologs have also been found in choanoflagellates [69,70]. Whether an ancestral form of functional coupling between $Ca_V$ channel and RyR homologs by membrane depolarization had evolved in *S. rosetta* is unknown.

Some TRP channels like TRPML are primarily localized in intracellular compartments such as lysosomes [53,71]. These intracellular TRP channels might contribute to organellar $Ca^{2+}$ release. TRPML channel homologs are found in *M. brevicollis*, *S. rosetta* and *C. owczarzaki* [23].

Two-pore channels (named for having two pore *domains*, not for having two pores; TPCs) localise to acidic organelles. They are found in land plants, animals and many protists, but are absent in fungi and many algae [3,72]. TPCs are present in the three unicellular ancestors of animals presented here [21,23].

In mitochondria, cytosolic $Ca^{2+}$ readily diffuses through large pores on the outer membrane, whereas $Ca^{2+}$ flux across the inner membrane is tightly regulated by ion channels and transporters [1]. The mitochondrial $Ca^{2+}$ uniporter (MCU and MICU subunits) proteins are widely distributed in animals, plants, basal fungi and many protists, but are absent in certain protozoan and fungal lineages [23,73]. Homologs of MCU and MICU as well as the mitochondrial $Ca^{2+}/H^+$ exchanger (Letm 1) and the mitochondrial $Na^+/Ca^{2+}$ exchanger (NCLX) are all found in *M. brevicollis*, *S. rosetta* and *C. owczarzaki* [23].

### 3.3. Removal of cytosolic $Ca^{2+}$ signals

To prevent $Ca^{2+}$ overload and maintain $Ca^{2+}$ homeostasis, $Ca^{2+}$ signals must be removed from the cytosol, either to the extracellular environment or to intracellular $Ca^{2+}$ stores. All three unicellular ancestors of animals discussed here contain PMCA and SERCA pumps and $Na^+/Ca^{2+}$ exchangers that can function to maintain low cytosolic $Ca^{2+}$ [21,23]. Both $K^+$-independent $Na^+/Ca^{2+}$ exchangers (NCXs) and $K^+$-dependent $Na^+/Ca^{2+}$ exchanger (NCKX) are present in *M. brevicollis*, whereas *S. rosetta* contains only NCXs and *C. owczarzaki* has one NCKX.

## 4. Evolution of the $Ca^{2+}$ signaling machinery in the apusozoan *T. trahens*

Animals and fungi diverged from a common unicellular ancestor of Opisthokonta approximately one billion years ago [44]. Therefore, analyzing the genome of the apusozoan protist *T. trahens*, a putative unicellular progenitor of Opisthokonta, would likely reveal the common components of the distinct $Ca^{2+}$ signaling machineries in animals and fungi. We recently identified a complex ancestral $Ca^{2+}$ signaling network in *T. trahens* [23]. Not surprisingly, *T. trahens* contain not only many components of the animal $Ca^{2+}$ signaling machinery, but also fungal proteins that are absent in the three unicellular ancestors of animals, including a $Ca^{2+}/H^+$ exchanger (CAX) and putative stretch-activated $Ca^{2+}$ channel Mid1 homologs. CatSper channels are also present in *T. trahens*. Interestingly, our analysis revealed that the basal chytridiomycete fungi *Allomyces macrogynus* and *Spizellomyces punctatus* had retained P2X receptors [62], which had long been thought to have been lost in the fungal lineage [63].

*Dictyostelium discoideum*, a soil-living amoeba, lacks many components of the $Ca^{2+}$ signaling system indentified in *T. trahens*; *D. discoideum* does not have homologs of SERCA and SPCA pumps, $Na^+/Ca^{2+}$ exchangers, $Ca_V$ channels and CNG channels [56] or CatSpers. Amoebozoa and Apusozoan species are placed at the base of Unikonta (Fig. 1) [29]. Thus, to further explore the evolutionary origins of the animal $Ca^{2+}$ signaling machinery requires extensive comparative genomics analyses of species in Bikonta. Previous genomics studies of plant, algal and other bikonts have provided detailed comparison of many $Ca^{2+}$ signaling molecules between various bikont species and animals [3,15,20,22,47]. In the following section, we will review our recent findings on the early evolution of the CatSper channel complex and three classes of cation/$Ca^{2+}$ exchangers [25], and briefly discuss the evolution of four-domain $Ca_V$ channels.

## 5. Evolution of the CatSper complex, cation/$Ca^{2+}$ exchangers, and four-domain $Ca_V$ channels

### 5.1. The CatSper complex

Flagella and cilia are believed to have arisen from the last common ancestor of all eukaryotes [74]. Flagella and motile cilia are structurally similar organelles in eukaryotes, projecting from the cell surface with a typical “9+2” axoneme. Primary cilia lack the central-pair microtubules and dyneins, and thus, are immotile [74]. In humans, primary cilia are present in almost every cell type, whereas flagella are only found in sperm cells, and motile

cilia are selectively expressed in few epithelial cell types. Recent electrophysiological and molecular studies revealed that distinct $Ca^{2+}$ signaling molecules function in these compartments – CatSpers in sperm flagella (Fig. 3A) [75] and TRPP channels in primary cilia [76,77].

The mammalian CatSper complex comprises four pore-forming α subunits and at least three auxiliary subunits – CatSper-β, CatSper-γ, and CatSper-δ, all of which are sperm-specific transmembrane proteins (Fig. 3A) [78]. The identification of the CatSper channel complex in *T. trahens* suggests that the protein complex critical for mammalian sperm hyperactivation might have an ancient role in regulating flagellar motility in protists, predating the divergence of animals and fungi. CatSper homologs were not found in previously sequenced fungal genomes [79]. The basal fungus *A. macrogynus* produces motile gametes with flagella. Similar to progesterone-activated $Ca^{2+}$ entry in human sperm cells, a pheromone released by female gametes can induce $Ca^{2+}$ influx in *A. macrogynus* sperm cells and modulate sperm motility [80]. Indeed, the CatSper $Ca^{2+}$ channel complex is present in *A. Macrogynus* [23]. CatSpers have not been found in bikont protists such as *Chlamydomonas* and *Paramecium*, algae, and plants. However, many lower plant and algal species also have motile gametes [3], which prompted the search for CatSper channel homologs in bikont species.

Recent extensive searches of bikont genomes revealed the presence of CatSpers in *Aurantiochytrium limacinum* [25], a common marine thraustochytrid protist within one of the earliest diverging lineages in the stramenopile phylum [81]. Both *A. limacinum* and *T. trahens* possess the same components of the CatSper channel complex – four pore-forming α subunits, auxiliary β and γ subunits and a distantly related homolog of the δ subunit [25]. Further analysis of the preliminary *Cyanophora paradoxa* algal genome also identified the presence of the CatSper complex. *C. paradoxa* is considered to be a basally diverging alga in the lineage leading to green plants and red algae [82]. The conservation of CatSpers in at least two bikont species suggests that the CatSper channel complex likely belonged to an ancestral $Ca^{2+}$ signaling network before the divergence of Unikonta and Bikonta. Alternatively, although much less likely, the presence of the CatSper complex in the two bikonts might be due to horizontal gene transfer from unikont species. Loss of either one of the four α subunits [83] or an auxiliary subunit [84] by gene knockout results in male infertility and degradation of the whole CatSper protein complex in mice. Assuming a similar degradation pathway in these bikonts, horizontal gene transfer would require at least seven genes encoding the CatSper complex, possibly located on different genomic regions, to be simultaneously transferred to host species. Interestingly, similar to CatSpers, TRPP channel homologs are found in both *T. trahens* and *A. limacinum* [25]. Thus, the regulatory role of CatSpers in flagellar motility and TRPP in primary cilia might have evolved at the root of early eukaryotic evolution.

$Ca^{2+}$ signaling is critical to initiate complex flagellar activities such as capacitation, chemotaxis, and hyperactivation [78]. However, regular beating patterns of flagella depend on ATP hydrolysis by axonemal dyneins; neither CatSpers nor $Ca^{2+}$ is required. The loss of the entire CatSper complex in several metazoan lineages likely reflects a species-dependent manner in modulating flagellar motility [79]. Hyperactivation might not be required for

sperm function, which eventually led to the relaxation of selective pressure on CatSper-induced $Ca^{2+}$ influx in sperm cells of these lineages. Similar cases of evolutionary degeneration have been documented in the loss of two other ion channels in primate evolution – TRPC2 [85] and TPC3 [86].

### 5.2. Cation/$Ca^{2+}$ exchangers

A defining feature of cation/$Ca^{2+}$ exchangers is the presence of two highly conserved α-repeat regions in the two transmembrane domains, separated by a relatively large intracellular loop [51]. NCX, NCKX and CAX are three major eukaryotic branches of the cation/$Ca^{2+}$ exchanger superfamily [19]. Biochemical [87,88] and crystallographic [89,90] evidence indicates that these exchangers share the same overall structure with 10-TM segments, two inverted α-repeats and two key acidic residues essential for ion binding and transport (Fig. 3B). NC(K)Xs and CAXs were previously shown to be present in animals and in bacteria, plants, and fungi, respectively [19,91].

The apusozoan *T. trahens* contains homologs of both NCKX and CAX exchangers [23]. CAX is absent in the three unicellular relatives of animals described above and NC(K)X is not found in the two basal fungi [23], suggesting that the loss of CAX in the animal lineage and NC(K)X in the fungal lineage occurred soon after the animal/fungal divergence. *A. limacinum* possesses all three classes – NCKXs, NCXs and CAXs (Fig. 3C). Consistent with a recent report on the evolution of cation/$Ca^{2+}$ exchangers in plants and algae [92], the presence of NCX, NCKX and CAX exchangers in *A. limacinum* suggests the common origin of these three classes of exchangers in ancestral protists prior to the Unikonta/Bikonta split. In the lineage leading to green plants, NCX and NCKX were often lost and CAX exchangers were retained as early as in the basally diverging freshwater alga *C. paradoxa* [25]. The presence of NCKX in many marine protists, but not in freshwater protists, may correlate with the adaptation of NCKX to regulate cytosolic $Ca^{2+}$ in the high $Na^{+}$, $Ca^{2+}$, and $K^{+}$ environment of seawater [92].

### 5.3. Relationship of CatSpers and cation/$Ca^{2+}$ exchangers to their prokaryotic counterparts

The NaChBac $Na^{+}$ channel [93] and the YRBG exchanger [51] are speculated to be the prokaryotic counterparts of the CatSper pore-forming α subunits and the cation/$Ca^{2+}$ exchangers, respectively. Gene duplication events may have led to the expansion of four unique CatSper channel pore-forming α subunits and three classes of exchangers – NCKXs, NCXs and CAXs. YRBG-like exchangers in prokaryotes can mediate $Na^{+}/Ca^{2+}$ exchange activity with kinetic characteristics similar to those of NCXs [89,94]. NaChBac-type channels may be involved in regulating flagellar motility and chemotaxis in certain bacteria, although more basic functions in processes such as metabolism are likely [95]. While YRBG-like exchangers have yet to be found in eukaryotes [92], NaChBac-type channels with divergent P-loop sequences and presumably higher $Ca^{2+}$ selectivity are present in two diatom genomes (Fig. 3C) [3]. Indeed, NaChBac-type bacterial channels appear to be phylogenetically closer to CatSper channels [96].

### 5.4. Evolution of four-domain voltage-gated $Ca^{2+}$ channels

Four-domain $Ca_V$ channels are an integral part of the animal $Ca^{2+}$ signaling machinery. Similar to four-domain $Na_V$ channels in animals, the ion-conducting pores of four-domain $Ca_V$ channels reside in the centre of the large polypeptide that spans the plasma membrane [97]. Lined by the transmembrane (TM) segments (S) S5 and S6 and the intervening 'Pore (P)-loop', each domain contributes to the central pore, with the four voltage-sensing modules (S1–S4 TM helices) symmetrically arranged around the central pore. The four-domain $Ca_V$ channels probably arose by two sequential gene duplication events from a single six-TM voltage-gated channel at the root of eukaryotes [98]. The four-domain $Na_V$ channels likely evolved from ancestral $Ca_V$ or $Na_V$ channels in protists since $Na_V$ homologs have already been identified in *T. trahens* [69] and *M. brevicollis* [70]. Similarly, $Na^+$-selective NaChBac-type channels are speculated to have evolved independently from ancestral non-selective, or $Ca^{2+}$-selective channels in bacteria [96]. It appears that all voltage-gated $K^+$ ($K_V$), $Ca_V$, and $Na_V$ channels likely arose from a single voltage sensor domain coupled to an S5/S6 pore domain. $K_V$ channels are widely distributed in all organisms from bacteria to humans. Presumably early in eukaryotic evolution, a six-TM single-domain ancestral channel gave rise to ancient two-domain ion channels by duplication (TPCs), which then further duplicated to form evolutionary precursors of modern four-domain ion channels [96,98]. Single-domain channels such as $K_V$, CatSper, transient receptor potential (TRP), and NaChBac-type channels have been phylogenetically placed outside the individual domain clades of four-domain $Ca_V$ and $Na_V$ channels [96], raising the possibility that ancestral forms of four-domain ion channels in early eukaryotic evolution might have been lost or functionally diversified to mask their ancestral state.

The crystal structures of NaChBac-type bacterial $Na_V$ channels revealed that the side groups of key Glu residue (E/E/E/E, one from each 6-TM subunit) coordinate with other residues in the P-loop to form the strongly negatively charged portion in the selectivity filter [99,100]. Mutagenesis studies have shown that a point mutation of Glu to Asp (D/D/D/D) yields a mutant NaChBac channel permeable to both $Ca^{2+}$ and $Na^+$, and the incorporation of two additional Asp residues in the P-loop region converts NaChBac from a highly $Na^+$-selective channel to a relatively $Ca^{2+}$-selective channel [101]. In addition, mutations in the selectivity filter of the animal four-domain $Ca_V$ and $Na_V$ channels also alter their ion selectivity [102,103]. Therefore, the P-loops of ancestral four-domain $Ca_V$ and $Na_V$ channels likely underwent evolutionary selection pressures to modulate ion selectivity in the lineages leading to fungi and animals. Recent phylogenetical and pharmacological analyses of TPCs and TPC-related proteins in unicellular organisms demonstrated that modifications in ion selectivity within four-domain $Ca_V$ and $Na_V$ channels may have occurred before intragenic duplication of an ancient two-domain ancestor [104].

Finally, analysis of four-domain voltage-gated ion channel sequences from Opisthokonta and *T. trahens* suggests that fungal $Ca^{2+}$ channels and animal $Na^+$ leak- nonselective channels, both of which are voltage-insensitive, diverged from $Ca_V$ and $Na_V$ channels before the animal-fungal split [105]. Furthermore, in contrast to the analysis of several putative $Na_V/Ca_V$-like four-domain channel sequences in *T. trahens* [69,105], all $Na_V$ and $Ca_V$ channel homologs from choanoflagellates *M. brevicollis* and *S. rosetta* form clear

branching patterns with their animal counterparts [21,23]. These findings further support the lineage-specific modulation of P-loop sequence and ion selectivity in the animal and fungal lineages after divergence.

## 6. Conclusion

Comparative genomics of recently sequenced genomes of unicellular organisms have provided evolutionary evidence that the distinct $Ca^{2+}$ signaling machineries in animals, plants and fungi share a common origin in ancestral protists prior to the eukaryotic radiation (Fig. 4) [3,22-25]. These mechanisms appear to be comprehensive, as shown in the very few protists such as *T. trahens* and *A. limacinum*, and are often conserved in the lineage leading to animals with further diversification and expansion. Even though most modern protists, plants and fungi tend to exhibit simplified $Ca^{2+}$ signaling networks, traces of relatively conserved $Ca^{2+}$ signaling systems remain in some basally divergent species, for instance, the basal chytridiomycete fungus *A. macrogynus* [23] and the basally diverging alga *C. paradoxa* [25].

Questions of current interest include functional characterization of these ancestral homologs and investigation of their physiological roles in the unicellular lineages leading to animals. Further studies on the extensive network of $Ca^{2+}$ signaling molecules in unicellular organisms will provide novel evolutionary and mechanistic insights into the formation of distinct $Ca^{2+}$ microdomains in a single cell to mediate coordinated interaction and spatial and temporal patterns of different $Ca^{2+}$ signals.

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## References

1. Clapham DE. Calcium signaling. Cell. 2007; 131:1047–1058. [PubMed: 18083096]
2. Dominguez DC. Calcium signalling in bacteria. Mol Microbiol. 2004; 54:291–297. [PubMed: 15469503]
3. Verret F, Wheeler G, Taylor AR, Farnham G, Brownlee C. Calcium channels in photosynthetic eukaryotes: implications for evolution of calcium-based signalling. New Phytol. 2010; 187:23–43. [PubMed: 20456068]
4. Kudla J, Batistic O, Hashimoto K. Calcium signals: the lead currency of plant information processing. Plant Cell. 2010; 22:541–563. [PubMed: 20354197]
5. Zelter A, Bencina M, Bowman BJ, Yarden O, Read ND. A comparative genomic analysis of the calcium signaling machinery in Neurospora crassa, Magnaporthe grisea, and Saccharomyces cerevisiae. Fungal Genet Biol. 2004; 41:827–841. [PubMed: 15288019]
6. Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol. 2003; 4:517–529. [PubMed: 12838335]
7. Case RM, Eisner D, Gurney A, Jones O, Muallem S, Verkhratsky A. Evolution of calcium homeostasis: From birth of the first cell to an omnipresent signalling system. Cell Calcium. 2007; 42:345–350. [PubMed: 17574670]
8. Kazmierczak J, Kempe S, Kremer B. Calcium in the Early Evolution of Living Systems: A Biohistorical Approach. Current Organic Chemistry. 2013; 17:1738–1750.

9. Verkhratsky A, Parpura V. Calcium signalling and calcium channels: Evolution and general principles. Eur J Pharmacol. 2014; 739C:1–3. [PubMed: 24291103]
10. Patel S, Docampo R. Acidic calcium stores open for business: expanding the potential for intracellular $Ca^{2+}$ signaling. Trends in Cell Biology. 2010; 20:277–286. [PubMed: 20303271]
11. Strunker T, Goodwin N, Brenker C, et al. The CatSper channel mediates progesterone-induced $Ca^{2+}$ influx in human sperm. Nature. 2011; 471:382–386. [PubMed: 21412338]
12. Lishko PV, Botchkina IL, Kirichok Y. Progesterone activates the principal $Ca^{2+}$ channel of human sperm. Nature. 2011; 471:387–391. [PubMed: 21412339]
13. Rokas A. The origins of multicellularity and the early history of the genetic toolkit for animal development. Annu Rev Genet. 2008; 42:235–251. [PubMed: 18983257]
14. Richter DJ, King N. The genomic and cellular foundations of animal origins. Annu Rev Genet. 2013; 47:509–537. [PubMed: 24050174]
15. Nagata T, Iizumi S, Satoh K, et al. Comparative analysis of plant and animal calcium signal transduction element using plant full-length cDNA data. Mol Biol Evol. 2004; 21:1855–1870. [PubMed: 15215322]
16. McAinsh MR, Pittman JK. Shaping the calcium signature. New Phytol. 2009; 181:275–294. [PubMed: 19121028]
17. Bell G, Mooers AO. Size and complexity among multicellular organisms. Biol J Linn Soc. 1997; 60:345–363.
18. Rokas A. The molecular origins of multicellular transitions. Curr Opin Genet Dev. 2008; 18:472–478. [PubMed: 18926910]
19. Cai X, Lytton J. The cation/$Ca^{2+}$ exchanger superfamily: phylogenetic analysis and structural implications. Mol Biol Evol. 2004; 21:1692–1703. [PubMed: 15163769]
20. Nagamune K, Sibley LD. Comparative genomic and phylogenetic analyses of calcium ATPases and calcium-regulated proteins in the apicomplexa. Mol Biol Evol. 2006; 23:1613–1627. [PubMed: 16751258]
21. Cai X. Unicellular $Ca^{2+}$ signaling 'toolkit' at the origin of Metazoa. Mol Biol Evol. 2008; 25:1357–1361. [PubMed: 18385221]
22. Wheeler GL, Brownlee C. $Ca^{2+}$ signalling in plants and green algae--changing channels. Trends Plant Sci. 2008; 13:506–514. [PubMed: 18703378]
23. Cai X, Clapham DE. Ancestral $Ca^{2+}$ signaling machinery in early animal and fungal evolution. Mol Biol Evol. 2012; 29:91–100. [PubMed: 21680871]
24. Plattner H, Verkhratsky A. $Ca^{2+}$ signalling early in evolution--all but primitive. J Cell Sci. 2013; 126:2141–2150. [PubMed: 23729741]
25. Cai X, Wang X, Clapham DE. Early evolution of the eukaryotic $Ca^{2+}$ signaling machinery: conservation of the CatSper channel complex. Mol Biol Evol. 2014; 31:2735–2740. [PubMed: 25063443]
26. Edel KH, Kudla J. Calcium signalling in plants. Cell Calcium. 2014 in the same issue.
27. Stechmann A, Cavalier-Smith T. Rooting the eukaryote tree by using a derived gene fusion. Science. 2002; 297:89–91. [PubMed: 12098695]
28. Richards TA, Cavalier-Smith T. Myosin domain evolution and the primary divergence of eukaryotes. Nature. 2005; 436:1113–1118. [PubMed: 16121172]
29. Derelle R, Lang BF. Rooting the eukaryotic tree with mitochondrial and bacterial proteins. Mol Biol Evol. 2012; 29:1277–1289. [PubMed: 22135192]
30. Srivastava M, Simakov O, Chapman J, et al. The Amphimedon queenslandica genome and the evolution of animal complexity. Nature. 2010; 466:720–726. [PubMed: 20686567]
31. Sakarya O, Armstrong KA, Adamska M, et al. A post-synaptic scaffold at the origin of the animal kingdom. PLoS One. 2007; 2:e506. [PubMed: 17551586]
32. Knoll AH. The Multiple Origins of Complex Multicellularity. Annu Rev Earth Planet Sci. 2011; 39:217–239.
33. King N, Westbrook MJ, Young SL, et al. The genome of the choanoflagellate *Monosiga brevicollis* and the origins of metazoan multicellularity. Nature. 2008; 451:783–788. [PubMed: 18273011]
34. King N. Choanoflagellates. Curr Biol. 2005; 15:R113–114. [PubMed: 15723775]

35. Lang BF, O'Kelly C, Nerad T, Gray MW, Burger G. The closest unicellular relatives of animals. Curr Biol. 2002; 12:1773–1778. [PubMed: 12401173]
36. Steenkamp ET, Wright J, Baldauf SL. The protistan origins of animals and fungi. Mol Biol Evol. 2006; 23:93–106. [PubMed: 16151185]
37. Ruiz-Trillo I, Roger AJ, Burger G, Gray MW, Lang BF. A phylogenomic investigation into the origin of metazoa. Mol Biol Evol. 2008; 25:664–672. [PubMed: 18184723]
38. Suga H, Chen Z, de Mendoza A, et al. The Capsaspora genome reveals a complex unicellular prehistory of animals. Nat Commun. 2013; 4:2325. [PubMed: 23942320]
39. Sebe-Pedros A, de Mendoza A, Lang BF, Degnan BM, Ruiz-Trillo I. Unexpected repertoire of metazoan transcription factors in the unicellular holozoan Capsaspora owczarzaki. Mol Biol Evol. 2011; 28:1241–1254. [PubMed: 21087945]
40. Fairclough SR, Chen Z, Kramer E, et al. Premetazoan genome evolution and the regulation of cell differentiation in the choanoflagellate Salpingoeca rosetta. Genome Biol. 2013; 14:R15. [PubMed: 23419129]
41. Dayel MJ, Alegado RA, Fairclough SR, et al. Cell differentiation and morphogenesis in the colony-forming choanoflagellate Salpingoeca rosetta. Dev Biol. 2011; 357:73–82. [PubMed: 21699890]
42. Sebe-Pedros A, Irimia M, Del Campo J, et al. Regulated aggregative multicellularity in a close unicellular relative of metazoa. Elife. 2013; 2:e01287. [PubMed: 24368732]
43. Cavalier-Smith T, Chao EE. Phylogeny and evolution of apusomonadida (protozoa: apusozoa): new genera and species. Protist. 2010; 161:549–576. [PubMed: 20537943]
44. Ruiz-Trillo I, Burger G, Holland PW, et al. The origins of multicellularity: a multi-taxon genome initiative. Trends Genet. 2007; 23:113–118. [PubMed: 17275133]
45. Sebe-Pedros A, Roger AJ, Lang FB, King N, Ruiz-Trillo I. Ancient origin of the integrin-mediated adhesion and signaling machinery. Proc Natl Acad Sci U S A. 2010; 107:10142–10147. [PubMed: 20479219]
46. Burki F, Pawlowski J. Monophyly of Rhizaria and multigene phylogeny of unicellular bikonts. Mol Biol Evol. 2006; 23:1922–1930. [PubMed: 16829542]
47. Chan CX, Reyes-Prieto A, Bhattacharya D. Red and green algal origin of diatom membrane transporters: insights into environmental adaptation and cell evolution. PLoS One. 2011; 6:e29138. [PubMed: 22195008]
48. Plattner H. Calcium regulation in the protozoan model, Paramecium tetraurelia. J Eukaryot Microbiol. 2014; 61:95–114. [PubMed: 24001309]
49. Valentine JW, Collins AG, Meyer CP. Morphological complexity increase in metazoans. Paleobiology. 1994; 20:131–142.
50. Parekh AB, Putney JW Jr. Store-operated calcium channels. Physiol Rev. 2005; 85:757–810. [PubMed: 15788710]
51. Philipson KD, Nicoll DA. Sodium-calcium exchange: a molecular perspective. Annu Rev Physiol. 2000; 62:111–133. [PubMed: 10845086]
52. Wu LJ, Sweet TB, Clapham DE. International Union of Basic and Clinical Pharmacology. LXXVI. Current progress in the mammalian TRP ion channel family. Pharmacol Rev. 2010; 62:381–404. [PubMed: 20716668]
53. Gees M, Colsoul B, Nilius B. The role of transient receptor potential cation channels in $Ca^{2+}$ signaling. Cold Spring Harb Perspect Biol. 2010; 2:a003962. [PubMed: 20861159]
54. Cai X. Molecular evolution and structural analysis of the $Ca^{2+}$ release-activated $Ca^{2+}$ channel subunit, orai. J Mol Biol. 2007; 368:1284–1291. [PubMed: 17400243]
55. Cai X. Molecular evolution and functional divergence of the $Ca^{2+}$ sensor protein in store-operated $Ca^{2+}$ entry: stromal interaction molecule. PLoS ONE. 2007; 2:e609. [PubMed: 17622354]
56. Collins SR, Meyer T. Evolutionary origins of STIM1 and STIM2 within ancient $Ca^{2+}$ signaling systems. Trends Cell Biol. 2011; 21:202–211. [PubMed: 21288721]
57. Klauke N, Blanchard M, Plattner H. Polyamine triggering of exocytosis in Paramecium involves an extracellular Ca(2+)/(polyvalent cation)-sensing receptor, subplasmalemmal Ca-store mobilization

and store-operated Ca(2+)-influx via unspecific cation channels. J Membr Biol. 2000; 174:141–156. [PubMed: 10742458]

58. Mohamed I, Klauke N, Hentschel J, Cohen J, Plattner H. Functional and fluorochrome analysis of an exocytotic mutant yields evidence of store-operated Ca2+ influx in Paramecium. J Membr Biol. 2002; 187:1–14. [PubMed: 12029373]
59. Ladenburger EM, Sehring IM, Korn I, Plattner H. Novel types of $Ca^{2+}$ release channels participate in the secretory cycle of Paramecium cells. Mol Cell Biol. 2009; 29:3605–3622. [PubMed: 19380481]
60. Yu X, Duan KL, Shang CF, Yu HG, Zhou Z. Calcium influx through hyperpolarization-activated cation channels (I(h) channels) contributes to activity-evoked neuronal secretion. Proc Natl Acad Sci U S A. 2004; 101:1051–1056. [PubMed: 14724293]
61. Cai X. Evolutionary genomics reveals the premetazoan origin of opposite gating polarity in animal-type voltage-gated ion channels. Genomics. 2012; 99:241–245. [PubMed: 22326743]
62. Cai X. P2X receptor homologs in basal fungi. Purinergic Signal. 2012; 8:11–13. [PubMed: 21887491]
63. Fountain SJ, Burnstock G. An evolutionary history of P2X receptors. Purinergic Signal. 2009; 5:269–272. [PubMed: 19015952]
64. Amador FJ, Stathopulos PB, Enomoto M, Ikura M. Ryanodine receptor calcium release channels: lessons from structure-function studies. FEBS J. 2013; 280:5456–5470. [PubMed: 23413940]
65. Ladenburger EM, Korn I, Kasielke N, Wassmer T, Plattner H. An Ins(1,4,5)P3 receptor in Paramecium is associated with the osmoregulatory system. J Cell Sci. 2006; 119:3705–3717. [PubMed: 16912081]
66. Ladenburger EM, Plattner H. Calcium-release channels in paramecium. Genomic expansion, differential positioning and partial transcriptional elimination. PLoS One. 2011; 6:e27111. [PubMed: 22102876]
67. Plattner H. Calcium signalling in the ciliated protozoan model. Paramecium: Strict signal localisation by epigenetically controlled positioning of different Ca-channels. Cell Calcium. 2014
68. Mackrill JJ. Ryanodine receptor calcium release channels: an evolutionary perspective. Adv Exp Med Biol. 2012; 740:159–182. [PubMed: 22453942]
69. Cai X. Ancient origin of four-domain voltage-gated $Na^{+}$ channels predates the divergence of animals and fungi. Journal of Membrane Biology. 2012; 245:117–123. [PubMed: 22258316]
70. Liebeskind BJ, Hillis DM, Zakon HH. Evolution of sodium channels predates the origin of nervous systems in animals. Proc Natl Acad Sci U S A. 2011; 108:9154–9159. [PubMed: 21576472]
71. Cheng X, Shen D, Samie M, Xu H. Mucolipins: Intracellular TRPML1-3 channels. FEBS Lett. 2010; 584:2013–2021. [PubMed: 20074572]
72. Brailoiu E, Churamani D, Cai X, et al. Essential requirement for two-pore channel 1 in NAADP-mediated calcium signaling. J Cell Biol. 2009; 186:201–209. [PubMed: 19620632]
73. Bick AG, Calvo SE, Mootha VK. Evolutionary diversity of the mitochondrial calcium uniporter. Science. 2012; 336:886. [PubMed: 22605770]
74. Mitchell DR. The evolution of eukaryotic cilia and flagella as motile and sensory organelles. Adv Exp Med Biol. 2007; 607:130–140. [PubMed: 17977465]
75. Ren D, Navarro B, Perez G, et al. A sperm ion channel required for sperm motility and male fertility. Nature. 2001; 413:603–609. [PubMed: 11595941]
76. DeCaen PG, Delling M, Vien TN, Clapham DE. Direct recording and molecular identification of the calcium channel of primary cilia. Nature. 2013; 504:315–318. [PubMed: 24336289]
77. Delling M, DeCaen PG, Doerner JF, Febvay S, Clapham DE. Primary cilia are specialized calcium signalling organelles. Nature. 2013; 504:311–314. [PubMed: 24336288]
78. Lishko PV, Kirichok Y, Ren D, Navarro B, Chung JJ, Clapham DE. The control of male fertility by spermatozoan ion channels. Annu Rev Physiol. 2012; 74:453–475. [PubMed: 22017176]
79. Cai X, Clapham DE. Evolutionary genomics reveals lineage-specific gene loss and rapid evolution of a sperm-specific ion channel complex: CatSpers and CatSperb. PLoS ONE. 2008; 3:e3569. [PubMed: 18974790]

80. Pommerville JC, Strickland JB, Harding KE. Pheromone interactions and ionic communication in gametes of aquatic fungus *Allomyces macrogynus*. Journal of Chemical Ecology. 1990; 16:121–131. [PubMed: 24264902]
81. Riisberg I, Orr RJ, Kluge R, et al. Seven gene phylogeny of heterokonts. Protist. 2009; 160:191–204. [PubMed: 19213601]
82. Price DC, Chan CX, Yoon HS, et al. Cyanophora paradoxa genome elucidates origin of photosynthesis in algae and plants. Science. 2012; 335:843–847. [PubMed: 22344442]
83. Qi H, Moran MM, Navarro B, et al. All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. Proc Natl Acad Sci U S A. 2007; 104:1219–1223. [PubMed: 17227845]
84. Chung JJ, Navarro B, Krapivinsky G, Krapivinsky L, Clapham DE. A novel gene required for male fertility and functional CATSPER channel formation in spermatozoa. Nat Commun. 2011; 2:153. [PubMed: 21224844]
85. Liman ER, Innan H. Relaxed selective pressure on an essential component of pheromone transduction in primate evolution. Proc Natl Acad Sci U S A. 2003; 100:3328–3332. [PubMed: 12631698]
86. Cai X, Patel S. Degeneration of an intracellular ion channel in the primate lineage by relaxation of selective constraints. Mol Biol Evol. 2010; 27:2352–2359. [PubMed: 20463046]
87. Cai X, Zhang K, Lytton J. A novel topology and redox regulation of the rat brain $K^+$-dependent $Na^+/Ca^{2+}$ exchanger, NCKX2. J Biol Chem. 2002; 277:48923–48930. [PubMed: 12377762]
88. Ren X, Philipson KD. The topology of the cardiac $Na^+/Ca^{2+}$ exchanger, NCX1. J Mol Cell Cardiol. 2013; 57:68–71. [PubMed: 23376057]
89. Liao J, Li H, Zeng W, Sauer DB, Belmares R, Jiang Y. Structural insight into the ion-exchange mechanism of the sodium/calcium exchanger. Science. 2012; 335:686–690. [PubMed: 22323814]
90. Waight AB, Pedersen BP, Schlessinger A, et al. Structural basis for alternating access of a eukaryotic calcium/proton exchanger. Nature. 2013; 499:107–110. [PubMed: 23685453]
91. Shigaki T, Rees I, Nakhleh L, Hirschi KD. Identification of three distinct phylogenetic groups of CAX cation/proton antiporters. J Mol Evol. 2006; 63:815–825. [PubMed: 17086450]
92. Emery L, Whelan S, Hirschi KD, Pittman JK. Protein Phylogenetic Analysis of $Ca^{2+}$/cation Antiporters and Insights into their Evolution in Plants. Front Plant Sci. 2012; 3:1. [PubMed: 22645563]
93. Clapham DE, Garbers DL. International Union of Pharmacology. L. Nomenclature and structure-function relationships of CatSper and two-pore channels. Pharmacol Rev. 2005; 57:451–454. [PubMed: 16382101]
94. Besserer GM, Nicoll DA, Abramson J, Philipson KD. Characterization and purification of a $Na^+/Ca^{2+}$ exchanger from an archaebacterium. J Biol Chem. 2012; 287:8652–8659. [PubMed: 22287543]
95. Fujinami S, Terahara N, Krulwich TA, Ito M. Motility and chemotaxis in alkaliphilic Bacillus species. Future Microbiol. 2009; 4:1137–1149. [PubMed: 19895217]
96. Liebeskind BJ, Hillis DM, Zakon HH. Independent acquisition of sodium selectivity in bacterial and animal sodium channels. Curr Biol. 2013; 23:R948–949. [PubMed: 24200318]
97. Armstrong CM, Hille B. Voltage-gated ion channels and electrical excitability. Neuron. 1998; 20:371–380. [PubMed: 9539115]
98. Strong M, Chandy KG, Gutman GA. Molecular evolution of voltage-sensitive ion channel genes: on the origins of electrical excitability. Mol Biol Evol. 1993; 10:221–242. [PubMed: 7680747]
99. Payandeh J, Scheuer T, Zheng N, Catterall WA. The crystal structure of a voltage-gated sodium channel. Nature. 2011; 475:353–358. [PubMed: 21743477]
100. Zhang X, Ren W, DeCaen P, et al. Crystal structure of an orthologue of the NaChBac voltage-gated sodium channel. Nature. 2012; 486:130–134. [PubMed: 22678295]
101. Yue L, Navarro B, Ren D, Ramos A, Clapham DE. The cation selectivity filter of the bacterial sodium channel, NaChBac. J Gen Physiol. 2002; 120:845–853. [PubMed: 12451053]

102. Heinemann SH, Terlau H, Stuhmer W, Imoto K, Numa S. Calcium channel characteristics conferred on the sodium channel by single mutations. Nature. 1992; 356:441–443. [PubMed: 1313551]

103. Yang J, Ellinor PT, Sather WA, Zhang JF, Tsien RW. Molecular determinants of $Ca^{2+}$ selectivity and ion permeation in L-type $Ca2^{+}$ channels. Nature. 1993; 366:158–161. [PubMed: 8232554]

104. Rahman T, Cai X, Brailoiu GC, Abood ME, Brailoiu E, Patel S. Two-pore channels provide insight into the evolution of voltage-gated $Ca^{2+}$ and $Na^{+}$ channels. Sci Sigal. 2014; 7:ra 109.

105. Liebeskind BJ, Hillis DM, Zakon HH. Phylogeny unites animal sodium leak channels with fungal calcium channels in an ancient, voltage-insensitive clade. Mol Biol Evol. 2012; 29:3613–3616. [PubMed: 22821012]

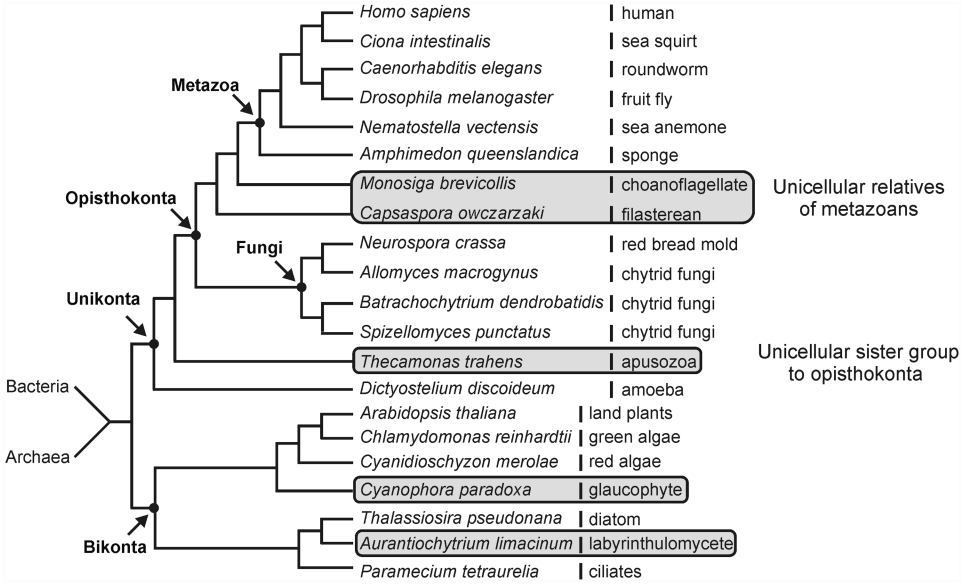


**Fig. 1. Schematic representation of the eukaryotic tree of life illustrating the evolutionary relationships of select species in Unikonta and Bikonta**

The tree is inferred from the Tree of Life project (http://www.tolweb.org/) and recent studies of the eukaryotic tree [29,46]. Highlighted are the close unicellular relatives of animals, *M. brevicollis* and *C. owczarzaki*, the putative unicellular progenitor of Opisthokonta *T. trahens*, the marine thraustochytrid protist *A. limacinum*, and the basally diverging alga *C. paradoxa* [82].

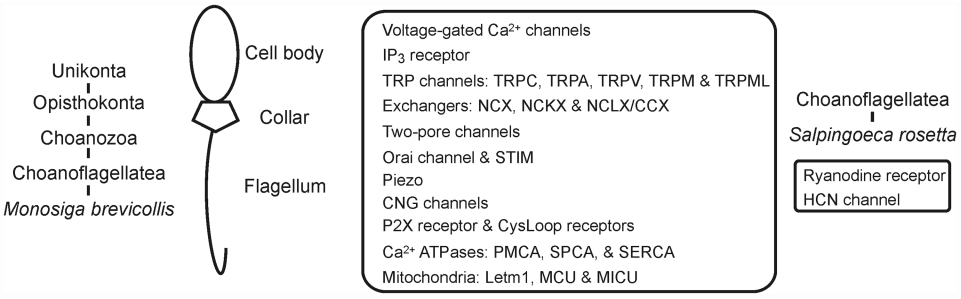


**Fig. 2. Components of the $Ca^{2+}$ signaling machineries in choanoflagellates *M. brevicollis* and *S. rosetta***

The evolutionary relationship of choanoflagellates is inferred from the Tree of Life project (http://www.tolweb.org/) and recent references [36,37]. The schematic diagram depicts a choanoflagellate cell with a spherical cell body, a collar-like ring of microvilli, and an apical flagellum [34]. Compared with *M. brevicollis*, *S. rosetta* contains two more $Ca^{2+}$ signaling molecules – ryanodine receptors and HCN channels. *Abbreviations*: *CNG*, cyclic nucleotide-gated channel; CysLoop receptors, cysteine-loop ligand-gated receptor; *HCN channel*, hyperpolarization-activated cyclic nucleotide-gated channel; *$IP_3$ receptor*, inositol 1,4,5-trisphosphate receptor; *Letm1*, leucine zipper-EF-hand containing transmembrane protein 1; *MCU*, mitochondrial $Ca^{2+}$ uniporter; *MICU*, mitochondrial EF hand $Ca^{2+}$ uniporter regulator; *NC(K)X*, $Na^+/Ca^{2+}$ ($K^+$-dependent) exchanger; NCLX/CCX; $Na^+/Li^+/Ca^{2+}$ exchanger or cation/$Ca^{2+}$ exchanger; *P2X receptor*, P2X purinergic receptor channel; *PMCA*, plasma membrane $Ca^{2+}$ ATPase; *SERCA*, sarco/endoplasmic reticulum $Ca^{2+}$ ATPase; *SPCA*, secretory pathway $Ca^{2+}$ ATPase; *STIM*, stromal interaction molecule; *TRP*, transient receptor potential channel.

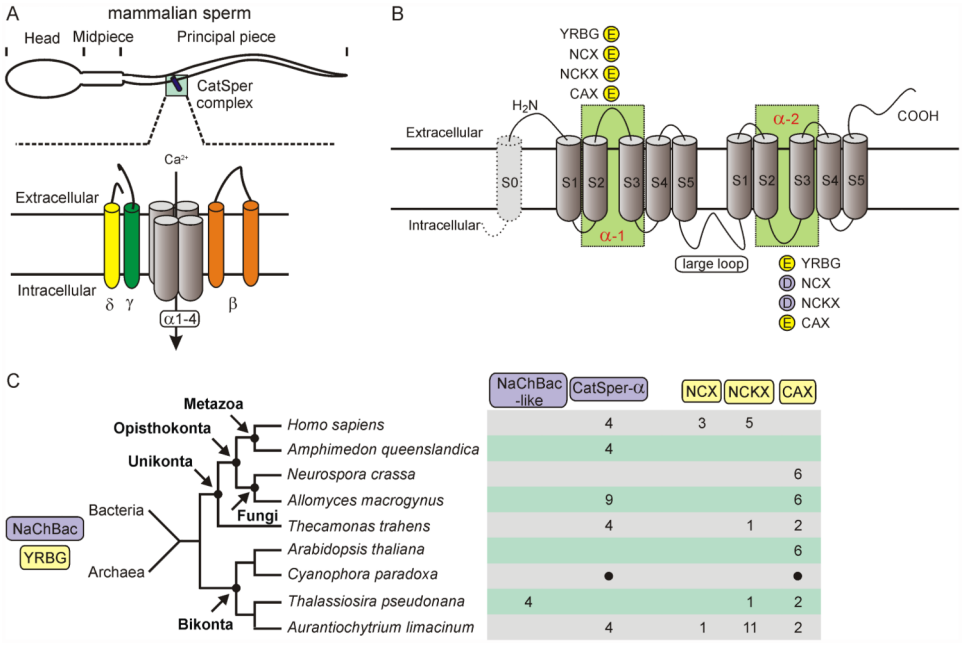


**Fig. 3. Evolution of the CatSper channel complex and cation/$Ca^{2+}$ exchangers**

(**A**) Schematic representation showing the head, midpiece and principal piece regions of mammalian spermatozoa. Located at the principle piece, the CatSper channel complex mediates $Ca^{2+}$ influx that is critical for sperm hyperactivation. The mammalian CatSper complex is composed of four pore-forming α subunits and at least three auxiliary subunits – CatSper-β, CatSper-γ, and CatSper-δ [78]. (**B**) Topological illustration of cation/$Ca^{2+}$ exchangers. NCX, NCKX, CAX, and YRBG exchangers all contain two transmembrane domains, each with 5-TM segments and one alpha-repeat. An extra N-terminal TM segment (*S0*) often serves as the signal peptide or targeting sequence of NCX, NCKX and CAX in eukaryotes. The key acidic residues crucial for ion exchange activity, one each in the two alpha repeats, are labelled for each exchanger class. (**C**) Distribution of NaChBac-like proteins, CatSpers, and cation/$Ca^{2+}$ exchangers in select unikont and bikont species. Due to incomplete genome assembly, the exact numbers of homologs in *C. paradoxa*, shown as *filled circles*, remain to be determined. Abbreviations: *CAX*, cation/$H^+$ exchanger; *NaChBac*, $Na^+$ channel from *Bacillus halodurans*; *NCKX*, $K^+$-dependent $Na^+/Ca^{2+}$ exchanger; *NCX*, $K^+$-independent $Na^+/Ca^{2+}$ exchanger; *YRBG*, an $Na^+/Ca^{2+}$ exchanger in *Escherichia coli*.

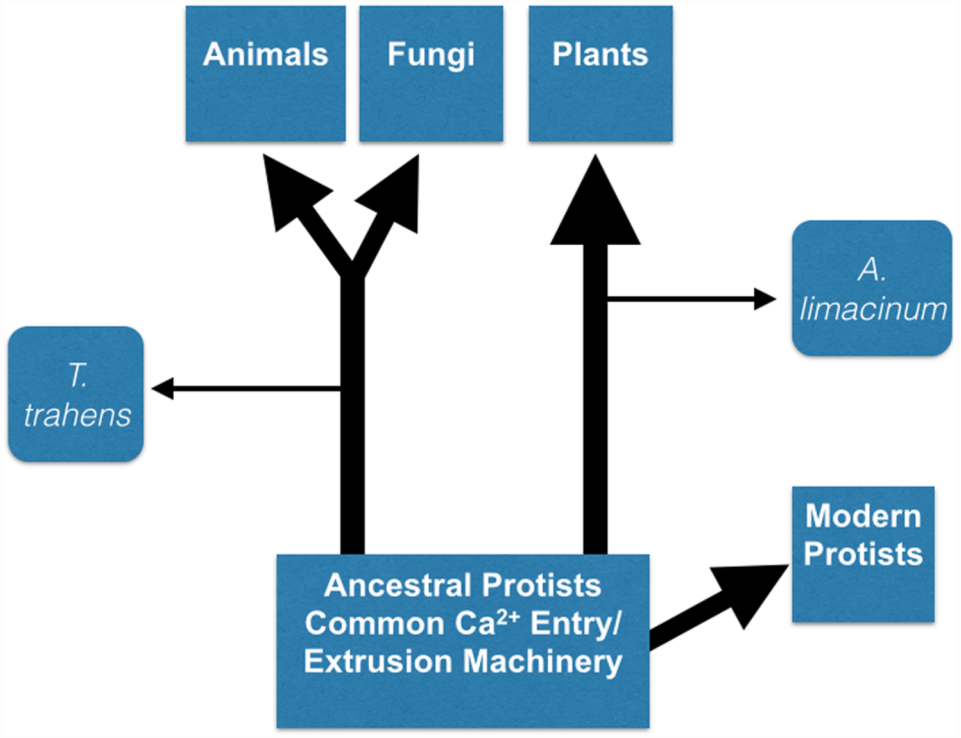


**Fig. 4. Schematic representation of the evolutionary history of $Ca^{2+}$ signaling machineries in eukaryotes**

A comprehensive $Ca^{2+}$ signaling machinery likely had evolved early at the root of eukaryotes before the divergence of major eukaryotic supergroups. In the lineage leading to animals, the ancestral $Ca^{2+}$ signaling machinery is often highly conserved. In contrast, the $Ca^{2+}$ signaling machineries in most modern protists, plants and fungi exhibit substantial gene losses. However, evidence of the presence of an extensive ancestral $Ca^{2+}$ signaling machinery can still be found in few protists such as *A. limacinum* and *T. trahens*.