## Evolution of sodium channels predates the origin of nervous systems in animals

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Voltage-dependent sodium channels are believed to have evolved from calcium channels at the origin of the nervous system. A search of the genome of a single-celled choanoflagellate (the sister group of animals) identified a gene that is homologous to animal sodium channels and has a putative ion selectivity filter intermediate between calcium and sodium channels. Searches of a wide variety of animal genomes, including representatives of each basal lineage, revealed that similar homologs were retained in most lineages. One of these, the Placozoa, does not possess a nervous system. We cloned and sequenced the full choanoflagellate channel and parts of two placozoan channels from mRNA, showing that they are expressed. Phylogenetic analysis clusters the genes for these channels with other known sodium channels. From this phylogeny we infer ancestral states of the ion selectivity filter and show that this state has been retained in the choanoflagellate and placozoan channels. We also identify key gene duplications and losses and show convergent amino acid replacements at important points along the animal lineage.

eumetazoan | inactivation gate | pore motif

arly animals radiated explosively in the Precambrian (1). This radiation was facilitated by the previous evolution of genes for cell adhesion that presaged the evolution of multicellularity (2). Another key animal innovation was the nervous system, which is present in all but a few animals (i.e., sponges and placozoans). Rapid, specific, long-distance communication among excitable cells is achieved in bilaterian animals and a few jellyfish (cnidarians) through the use of action potentials (APs) in neurons generated by voltage-dependent sodium (Na<sub>v</sub>) channels. Voltagedependent calcium (Ca<sub>v</sub>) channels evolved in single-celled eukarvotes and were used for intracellular signaling. It has been hypothesized that Na<sub>v</sub> channels were derived from Ca<sub>v</sub> channels at the origin of the nervous system (3), thereby conferring the ability to conduct action potentials without interfering with intracellular calcium. This view was reinforced by the apparent lack of sodium currents in sponges (4).

To test this hypothesis, we searched newly available genome databases from two animals with simple nerve nets (the sea anemone *Nematostella vectensis* and the ctenophore *Mnemiopsis leidyi*), a placozoan with no nervous system (*Trichoplax adhaerens*), a sponge (*Amphimedon queenslandica*), a single-celled eukaryote (the choanoflagellate, *Monosiga brevicollis*), as well as fungi and additional single-celled eukaryotes for homologs of Ca<sub>v</sub> and Na<sub>v</sub> channels. We then verified the expression of these genes in *M. brevicollis* and *T. adhaerens* and examined amino acid changes in these genes throughout the history of animal evolution.

Choanoflagellates are widely distributed unicellular protists (5, 6) that form the sister group to the multicellular animals (7). Placozoans are an early-diverging animal lineage that has been proposed to be sister to the eumetazoans, that is, to all animals with nervous systems (8). However, phylogenetic placement of the basal animal lineages is not yet fully resolved (9–12), and many aspects of placozoan life cycles remain unknown (13–15). Choanoflagellates and placozoans have received considerable attention due to their pos-

session of numerous genes once thought to be exclusive to eumetazoans (2, 16–18).

Ca<sub>v</sub> and Na<sub>v</sub> channels have four domains, each of which has a pore loop (Fig. 1). A single amino acid at the deepest part of each pore loop is responsible for ion selectivity in the pore. Ca<sub>v</sub> channels have acidic residues (E and D) in the pore of domains I–IV (usually E/E/E or E/E/D/D). Selectivity for sodium, on the other hand, is based on the residues D/E/K/A in the pore. Sodium channels also have a cytoplasmic loop between their third and fourth domains that swings up and occludes the channel pore just milliseconds after activation (Fig. 1). This fast inactivation makes sodium signaling reliable on the millisecond time scale, and mutations at this region in human Nav channel genes cause many well-known pathologies (19). Calcium channels do not have a similar motif at the homologous region. Because of the differences in the amino acids responsible for ion selectivity, and because proteins are likely to be under strong evolutionary constraints along every point of their evolution (20), it has been suggested that channels with intermediate pore sequences may exist in extant taxa (3, 21), and some invertebrate channels have been proposed as representatives of these intermediate states (21, 22). The phylogenetic relationships of these channels are not clear however (22-24), and no suggestion of an ancestral metazoan pore state has been put forth.

Our objective was to find voltage-gated ion-channel genes in basal animals and their close unicellular relatives, determine whether the genes are expressed in a few key species, and analyze the evolutionary history of the genes for Na<sub>v</sub> and Ca<sub>v</sub> channels. We examined pore motifs, inactivation gate sequence, and inactivation gate secondary structure and then mapped these states onto our phylogeny. This work provides a unique view of Na<sub>v</sub> and Ca<sub>v</sub> channel evolution and the evolution of excitable tissues in animals.

## Results

Sodium Channel Homologs in Early-Diverging Animals and Choanoflagellates. We found that the genomes of M. brevicollis, T. adhaerens, N. vectensis, and M. leidyi contain genes for ion channels that group with the  $Na_v$  family (Fig. 2), and we used these genomic sequences as references for further analyses. We found pairs of  $Na_v$  paralogs in Trichoplax, Nematostella, and Mnemiopsis, which we name  $\alpha$  and  $\beta$ . The genome of the sponge A. queenslandica did not contain  $Na_v$  homologs but did have one gene for a  $Ca_v$  channel. No  $Na_v$  homologs were found in the genomes of Aspergillus niger, Saccharomyces cerevisiae, or any other fungi in the Joint Genome Institute database. We sequenced the entire ORF of an mRNA

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Data deposition: Accession numbers and relevant databases are provided in Table S1. The sequences reported in this paper have been deposited in the GenBank database (accession nos. JF827087, JF905561, JF905562 and JF905563).

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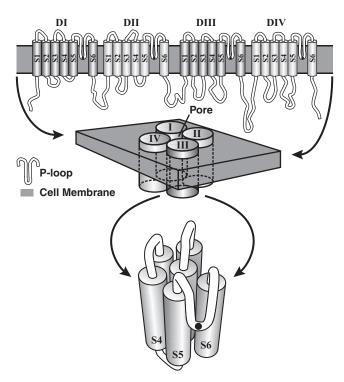


Fig. 1. Hypothetical secondary structure of a sodium-channel protein. (*Top*) Transmembrane domains (DI–DIV), their component segments (S1–S6), and their connecting loops (in white). The pore loops (P loop), which dip down into the membrane, form the ion-selectivity filter. The inactivation gate resides on the long loop between DIII/S6 and DIV/S1. (*Middle*) How the domains cluster to form the protein and its pore. (*Bottom*) Fine structure of one of the domains with the pore loop in the foreground. The black dots on the pore loops in the *Top* and *Bottom* represent the location of the amino acids, which makes up the pore motif.

transcript from *Monosiga* and partial transcripts from the two genes in *Trichoplax*, thereby demonstrating that these genes are expressed. The genes have a pore motif D/E/E/A that is intermediate between  $Ca_v$  and  $Na_v$  channels and is the same as some previously described invertebrate channels (21, 23).

For one of the paralogs,  $Trichoplax \ \beta$ , only three of the four domains typical to  $Ca_v$  and  $Na_v$  channels were found in the genome, likely due to a problem with the genome assembly. It is unlikely that a three-domain protein could function as an ion channel alone, but it is not yet known whether the genome sequencing effort simply missed part of the genome, whether our BLAST analysis misidentified the exons for the last domain, or whether it is actually a splice variant or some other regulatory transcript. The ctenophore  $Na_v$  homologs and the sponge  $Ca_v$  channel are missing amino acids in the putative pore regions, perhaps also due to incomplete assembly.

Four overlapping segments from choanoflagellate mRNA were compiled to yield 4,589 nucleotides, which we believe includes the whole ORF. This sequence was 93.4% identical to the reference sequence obtained with BLAST (*SI Materials and Methods* and Fig. S1).

Sequencing of the *Trichoplax* genes yielded 868 bp from the *Trichoplax*  $\alpha$  gene, which aligned to the reference with 92.7% identity, and 1,062 bp from the *Trichoplax*  $\beta$  gene, which aligned with 91.0% identity. Many of the mismatches in the *Trichoplax*  $\beta$  segment are from indeterminate nucleotides and may be due to the fact that this segment was sequenced directly from the PCR products rather than from cloned genes. Although further confirmation of the exact sequences is needed, the presence of these sequences in the mRNA demonstrates that both *Trichoplax* genes are indeed transcribed.

Phylogenetic Analyses. We performed maximum likelihood (ML) analyses on a data set consisting of our sequenced choanoflagellate gene, a putative Ca<sub>v</sub> gene from *Monosiga*, and Na<sub>v</sub> and Ca<sub>v</sub> genes from all major animal lineages and two fungal species, Aspergillus and Saccharomyces (Fig. 2). The phylogenetic placement of the ion channel genes agrees with the well supported parts of the phylogeny for animals, choanoflagellates, and fungi (8, 9, 12), and the topology was robust to analyses on other platforms and removal of taxa (SI Materials and Methods). The placement of the Amphimedon Ca<sub>v</sub> channel as basal to Monosiga Ca<sub>v</sub> (Fig. 2) is probably an artifact due to long-branch attraction (LBA). This seems likely because the Amphimedon branch is long, and Monosiga Cav is a partial sequence. The placement of these two sequences within Ca<sub>v</sub> channels is not consistent with an LBA artifact, however, and is strongly supported by bootstrap analysis, indicating that these are true Ca<sub>v</sub> channels. The fungal Ca<sub>v</sub> channels were resolved as the sister group to all animal and choanoflagellate channels. These results support the hypothesis that Na<sub>v</sub> genes evolved from Ca<sub>v</sub> genes, because the Na<sub>v</sub> family emerges from within animal and fungal Ca<sub>v</sub> channels.

Our phylogeny supports the view that placozoans, which have the simplest animal body plan, branched off the animal stem after ctenophores and are therefore likely to be secondarily simplified. This scenario was found in both Na<sub>v</sub> and N/P/Q type Ca<sub>v</sub> genes (*SI Materials and Methods*).

Bootstrapping scores indicate strong support for critical nodes of the  $\text{Ca}_{\text{v}}/\text{Na}_{\text{v}}$  gene phylogeny. The position of the choanoflagellate  $\text{Na}_{\text{v}}$  channel gene at the base of animal  $\text{Na}_{\text{v}}$  channel genes was supported in 100% of the bootstrap replicates. The bootstrapping analysis also provides strong support for the monophyly of known groups of  $\text{Ca}_{\text{v}}$  and  $\text{Na}_{\text{v}}$  channel genes, including the bilaterian  $\text{Na}_{\text{v}}$  1 clade and the three major groups of  $\text{Ca}_{\text{v}}$  channels. The clades containing channels with pore motifs D/E/E/A in both Cnidaria and Bilateria were less well supported in the bootstrap analysis (50–65% of replicates).

**Secondary Structure.** Analysis of secondary structure of the inactivation gate revealed conservation of two important  $\alpha$ -helices across the  $Na_v$  channel family (Fig. S2). This structure was absent in  $Ca_v$  channels, including the yeast  $Ca_v$  channel.

## Discussion

**Rooting the Na\_{v} and Ca\_{v} Gene Families.** The choanoflagellate M. brevicollis and the placozoan T. adhaerens express ion-channel genes that group phylogenetically with previously described sodium channels (Fig. 2) and have key molecular signatures of sodium channels (Fig. 3). Others have proposed that Na<sub>v</sub> channels evolved from an ancient Ca<sub>v</sub> channel resembling the T-type channels (3) and that there may therefore be extant channels that have properties midway between Ca<sub>v</sub> and Na<sub>v</sub> channels (21). Candidates for such channels have been proposed (21, 22), but the origin and genetic history of Na<sub>v</sub> channels have remained obscure. Our phylogenies show that the Na<sub>v</sub> ion channel family originated not only before the advent of the nervous system, but probably even before the advent of multicellularity. These results support the idea that Na<sub>v</sub> channels arose from Ca<sub>v</sub> channels, but push back this divergence date to at least the common ancestor of animals and choanoflagellates. This demonstrates that complex systems like excitable tissues can evolve by coopting existing genes for new functions, rather than by de novo evolution of new genes.

**Voltage-Gated Ion Channels and the Animal Phylogeny.** The phylogenetic placement of basal animal lineages (sponges, ctenophores, placozoans, and cnidarians) is not yet fully clear, although some placements are less controversial than others. The placement of sponges as sister to all other animals, and of cnidarians as sister to bilaterians, are fairly consistent results (12). The placements of ctenophores and placozoans, however, are less certain

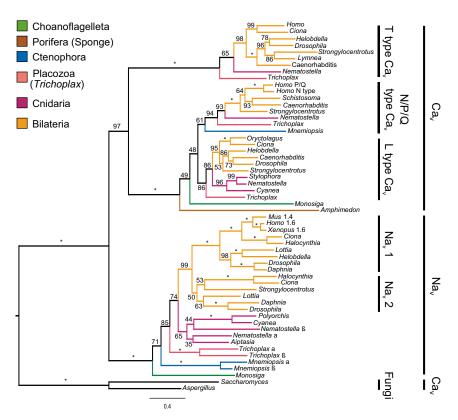


Fig. 2. Maximum likelihood phylogeny of Na<sub>v</sub> and Ca<sub>v</sub> channels. Bootstrap scores are indicated on branches, with stars indicating scores of 100%. Clades corresponding to major ion channel groups are detailed on the Right.

(SI Materials and Methods). Our results are consistent with the traditional phylogenetic placement of sponges and cnidarians, but place ctenophores, which have a nervous system, outside of the placozoans, cnidarians, and bilaterians (Figs. 2 and 4). This would suggest that placozoans have lost their nervous system or, much less likely, that the nervous system evolved twice in ctenophores and the cnidarian-bilaterian ancestor. Although our analysis has relatively strong bootstrap support, it has sparse taxon sampling, which has been shown to meaningfully affect phylogenetic inference (11, 25) and cannot therefore be considered a decisive species phylogeny.

Also interesting is the apparent loss of Na<sub>v</sub> homologs in the sponge Amphimedon, an event that may reflect the sedentary lifestyle of these animals. Electrical impulse conduction has not been shown in demosponges, the group that includes Amphimedon, but it has been shown in a hexactinellid sponge (4). Hexactinellids differ drastically from demosponges in terms of morphology; further analysis of hexactinellids will be needed to determine whether Na<sub>v</sub> homologs have been retained in this group.

Genetic History—Bilateria. Our results help clarify the diversity of pore states observed in animal Na<sub>v</sub> channels. The topology of our tree suggests that D/E/E/A is the ancestral pore sequence of the Na<sub>v</sub> gene family and that genes with this motif have been retained in every metazoan lineage that we examined, except for sponges, vertebrates, and the cnidarian subgroup Medusozoa (Figs. 3 and 4). The topology of the Na<sub>v</sub> 1 and Na<sub>v</sub> 2 clades supports the hypothesis that a gene duplication occurred around the time of the bilaterian radiation and before the split of protostomes and deuterostomes (24). The Na<sub>v</sub> 1 duplicate evolved a pore motif D/E/K/A and underwent further duplications in early tetrapods, creating the genes for Na<sub>v</sub> 1.1-1.9 in mammals (26). The other duplicate retained the ancestral pore motif and was lost in vertebrates.

Genetic History—Cnidaria. Cnidarians diverged before the bilaterian gene duplication and do not have D/E/K/A channels, but the medusozoans have an amino acid substitution in the second domain pore loop, resulting in a clade of channels with the pore motif D/K/E/A. Although the topology of cnidarian channels with glutamic acid (E) in the second domain was not well supported, the clade of D/K/E/A channels was repeatedly found to represent a derived state and was monophyletic with 100% support. In species-tree analyses, the medusozoans share a common ancestor that is not shared with the anthozoans (8–12). The medusozoan subgroups represented here are Hydrozoa (Polyorchis) and Scyphozoa (Cyanea), both of which have D/K/E/A in the pore, whereas the anthozoan representatives (Aiptasia and Nematostella) both have D/E/E/A channels (Fig. 3). Our Na<sub>v</sub> tree is therefore consistent with proposed species trees and suggests a lysine (K) substitution in the common ancestor of medusozoans (Fig. 4). There is also a Nematostella channel whose pore sequence D/E/E/T is unique among sampled ion channels.

Sodium-based APs have been reported in both Cyanea (27) and *Polyorchis* (28), whereas APs in anthozoans and ctenophores seem to be carried mostly by calcium (29, 30). The pore motif D/K/E/A has been shown to be less selective for sodium than the D/E/K/A pore but more so than the D/E/E/A pore (21, 31–33). Channels with D/E/E/A have a higher affinity for calcium than sodium. The convergence to lysine in different domains of medusozoan and bilaterian ion channels may therefore have resulted from similar evolutionary pressure for sodium selectivity, as this would allow for less disruption of calcium homeostasis because Ca<sup>2+</sup> is used for intracellular signaling in eukaryotes (3). Some medusozoans have concentrated nerve clusters and complex sense organs, which likely emerged convergently with the bilaterian central nervous system, as such nerve concentration is absent in anthozoans (34). It is not known whether the Na<sub>v</sub> genes function in these organs.

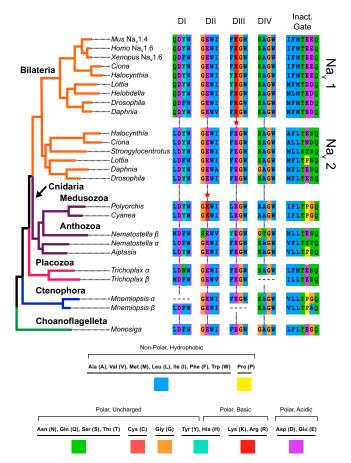


Fig. 3. Phylogeny of  $Na_{\nu}$  channels with key amino acid sequences mapped to their corresponding taxa. Taxa are color coded the same as in Fig. 2. The amino acids are alignments of the pore loops of all four domains (DI–DIV) and the critical inactivation particle on the inactivation gate. The critical amino acids in the pore are indicated by the vertical lines, and there are red stars next to convergent lysines (red K). Note the functional conservation of the hydrophobic triplet called the "inactivation particle" (first three amino acids on the inactivation gate).

Evolution of Sodium Selectivity and Fast Inactivation. The pore sequence D/E/E/A is intermediate between  $Ca_v$  channel and  $Na_v$  channel pore motifs. It may also have an intermediate selectivity between calcium and sodium. The function of D/E/E/A channels in such a wide range of organisms and the reason for their apparent loss in medusozoans and vertebrates remains unknown. Mutation studies of the DSC1 channel (called *Drosophila*  $Na_v$  2 here) showed an effect in olfactory behavior in flies (35), but no function for these channels has been suggested in other organisms. The widespread retention of these channels suggests that they probably have important, yet possibly divergent, functions (e.g., not all lineages with D/E/E/A channels have olfaction). The sea urchin *Strongylocentrotus purpuratus* is only known to have an  $Na_v$  2 ortholog (24).

Hydrophobic sites on the domain III/IV linker that are critical for inactivation (19) are functionally conserved in all of the sodium channels that we investigated here, albeit with a wide range of different amino acid combinations at homologous sites (Fig. 3). Secondary structure of the inactivation gate is also relatively conserved. Two helices on either side of the hydrophobic triad that forms the "inactivation particle" have been predicted before and may act to stabilize and direct the inactivation particle as it swings up and binds to the channel (36, 37). These two helices are present across the Na<sub>v</sub> family, but not in the Ca<sub>v</sub> families (Fig. S2). These findings suggest

that all of the Na<sub>v</sub> homologs presented here may include an inactivation gate, even in the single-celled choanoflagellate.

Na<sub>v</sub> Channels in the Animal Genetic Repertoire. This study adds to the growing evidence that much of the genetic repertoire for animal development, cell signaling, and even the nervous system was already present in the common ancestor of choanoflagellates and animals. Choanoflagellates have genes for cell-adhesion proteins (2, 38), tyrosine kinases and related proteins (2), proteins related to the postsynaptic density of neurons (18), and a remarkable complement of calcium signaling proteins (17). Some choanoflagellate species have a colonial life stage (7), and these genes may function in colony maintenance.

The function of sodium channel homologs in choanoflagellates or placozoans is unknown. They may create calcium-based APs, as suggested by the presence of such APs in ctenophores (30), but there are other possibilities. Both organisms can inhabit coastal marine areas with abundant fresh water runoff (5, 13). Trichoplax is restricted to warm coastal waters and is known to be sensitive to lowered salinity (13). It is possible that the channels act as osmosensors or osmoregulators in these organisms. Choanoflagellates have a long flagellum that they use to swim and to capture prey, and Trichoplax has a ciliated ventral layer that it uses for gliding across surfaces. It is possible that the channels control flagellar or ciliary beating through the influx of calcium, which triggers actin, or sodium, which is known to mediate flagellar motors in bacteria (39). Trichoplax has a layer of contractile fiber cells that form a syncytium and seem to function as muscle and a nervous system simultaneously (40). It is possible that the channels function in this dual purpose tissue.

Functional assays of  $Na_v$ -channel homologs will shed light on their biological function and on the evolution of  $Na_v$  channels as a whole. Determining the ion selectivity of these channels is critical to understanding how sodium selectivity can evolve from calcium selectivity by sequential mutations. Gaining insight into the function of these channels will not only enlighten the history of this protein's "adaptive walk" (20), it will also help elucidate the evolution of the nervous system.

## **Materials and Methods**

**Sources of RNA.** *M. brevicollis* and *T. adhaerens* were cultured in the laboratory using previously described and publicly available protocols (41). Placozoans were provided by Andreas Heyland. Choanoflagellate cells were fed on the bacteria present in the inoculum, and the placozoans were fed *Cryptomonas* sp. (LB 2423) from the University of Texas at Austin collection of algae. To extract RNA from *M. brevicollis*, we mixed and centrifuged 2 mL of the culture medium at 4 °C. Whole RNA was extracted using a RNA STAT-60 kit (Tel-Test) and then stored at –20 °C. The same protocol was used to isolate and store RNA from 15 *T. adhaerens* individuals that had been kept in algae-free seawater for 2 d to reduce the chance of contamination with algal RNA.

Gene Amplification and Sequencing. Specific primers were designed from the BLAST sequences for RT and PCR reactions. RT reactions were conducted with a SuperScript II kit (Invitrogen) using both specific and poly-T primers to prevent bacterial RNA contamination. Primers are reported in SI Materials and Methods. PCR reactions were carried out with the following cycle for 39 repetitions: Denaturation at 94° (30 s), annealing at a primer-specific temperature (30 s), and elongation at 72° (1 min/kb). This cycle was preceded by an initial denaturation at 94° for 3 min 10 s and followed by a final elongation at 72° for 7 min. PCR products were visualized and purified with gelectrophoresis and then cloned using a TOPO cloning kit (Invitrogen) and One Shot Top 10 (Invitrogen) chemically competent Escherichia coli. We sequenced the M. brevicollis gene in four overlapping segments using vector-specific primers after cloning.

**Sequence Analysis.** We performed a maximum likelihood phylogenetic analysis using the translated mRNA sequence from *M. brevicollis* and amino acid sequences from online databases for the other organisms. The latter were obtained either from catalogued, known channels or from BLAST searches of available genomes (for accession numbers, see Table S1). Amino

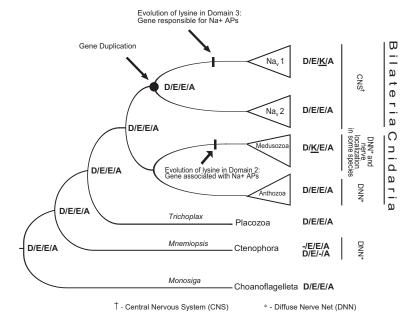


Fig. 4. Simplified gene tree of the Na<sub>v</sub> family showing inferred ancestral states of the pore motifs. The gene duplication leading to the bilaterian Na<sub>v</sub> 1 and Na<sub>v</sub> 2 clades is noted, as are the points where we reconstruct fixation of lysines (K) in pore loops. Taxonomic information and information about the nervous system is also given. The Nematostella eta and Trichoplax eta genes have been left out for simplicity, but their addition would not change the proposed ancestral states. Pore states for both Mnemiopsis genes are shown because neither has a complete pore motif.

acid sequences were aligned using the E-INS-I strategy in MAFFT (42). We used the Guidance algorithm available on the Guidance server to remove columns that had a score below 0.377 from the alignment (43). Maximum likelihood phylogenetic analysis and bootstrapping were performed in Garli (44), using a model of amino acid replacement selected using the Akaike Information Criterion in ProtTest (45). The model of protein evolution selected in the ProtTest analysis was WAG + I + G + F (Whelan and Goldman model, with invariant sites, parameter for gamma-distributed rate heterogeneity, and amino acid frequencies matched to the observed data). The maximum likelihood tree was obtained using Garli set to use the WAG + I + G + F model. The full amino acid alignment was analyzed for four search repetitions operating across 5 million generations each. A total of 100 bootstrap samples were collected using a halved topological termination

condition, as recommended in the Garli manual, and a stop time of 1 million generations. All bootstrap outputs were analyzed in PAUP (46).

Secondary structure of the inactivation gate region was examined using the online server PsiPred (47), the results of which are reported in Fig. S2.

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- 1. Rokas A, Krüger D, Carroll SB (2005) Animal evolution and the molecular signature of radiations compressed in time. Science 310:1933-1938.
- 2. King N, et al. (2008) The genome of the choanoflagellate Monosiga brevicollis and the origin of metazoans, Nature 451:783-788.
- 3. Hille B (1984) Ionic Channels of Excitable Membranes (Sinauer, Sunderland, MA), pp 720-722.
- 4. Leys SP, Mackie GO, Meech RW (1999) Impulse conduction in a sponge. J Exp Biol 202: 1139-1150
- 5. King N (2005) Choanoflagellates. Curr Biol 5:113-114.
- 6. Caron DA, Worden AZ, Countway PD, Demir E, Heidelberg KB (2009) Protists are microbes too: A perspective. ISME J 3:4-12.
- 7. Carr M, Leadbeater BSC, Hassan R, Nelson M, Baldauf SL (2008) Molecular phylogeny of choanoflagellates, the sister group to Metazoa. Proc Natl Acad Sci USA 105: 16641-16646.
- 8. Philippe H, et al. (2009) Phylogenomics revives traditional views on deep animal relationships. Curr Biol 19:706-712.
- 9. Dunn CW, et al. (2008) Broad phylogenomic sampling improves resolution of the animal tree of life. Nature 452:745–749.
- Hejnol A, et al. (2009) Assessing the root of bilaterian animals with scalable phylogenomic methods. Proc Biol Sci 276:4261-4270.
- 11. Pick KS, et al. (2010) Improved phylogenomic taxon sampling noticeably affects nonbilaterian relationships. Mol Biol Evol 27:1983-1987
- 12. Philippe H, et al. (2011) Resolving difficult phylogenetic questions: Why more sequences are not enough. PLoS Biol 9:e1000602.
- 13. Pearse VB, Voight O (2007) Field biology of placozoans (Trichoplax): Distribution, diversity, biotic interactions. Integr Comp Biol 47:677-692.
- 14. Signorovitch AY, Dellaporta SL, Buss LW (2005) Molecular signatures for sex in the Placozoa. Proc Natl Acad Sci USA 102:15518-15522.
- 15. Eitel M, Schierwater B (2010) The phylogeography of the Placozoa suggests a taxonrich phylum in tropical and subtropical waters. Mol Ecol 19:2315-2327.

- 16. Srivastava M, et al. (2008) The Trichoplax genome and the nature of placozoans. Nature 454:955-960.
- 17. Cai X (2008) Unicellular Ca2+ signaling 'toolkit' at the origin of metazoa. Mol Biol Evol 25.1357-1361
- 18. Alié A, Manuel M (2010) The backbone of the post-synaptic density originated in a unicellular ancestor of choanoflagellates and metazoans. BMC Evol Biol 10:34.
- 19. Goldin AL (2003) Mechanisms of sodium channel inactivation, Curr Opin Neurobiol 13: 284-290
- 20. Maynard-Smith J (1970) Natural selection and the concept of a protein space. Science 225.563-564
- 21. Zhou W, Chung I, Liu Z, Goldin AL, Dong K (2004) A voltage-gated calcium-selective channel encoded by a sodium channel-like gene. Neuron 42:101-112.
- 22. Spafford JD, Spencer AN, Gallin WJ (1998) A putative voltage-gated sodium channel alpha subunit (PpSCN1) from the hydrozoan jellyfish, Polyorchis penicillatus: Structural comparisons and evolutionary considerations. Biochem Biophys Res Commun 244: 772-780.
- 23. Nagahora H, et al. (2000) Diversity of voltage-gated sodium channels in the ascidian larval nervous system. Biochem Biophys Res Commun 275:558-564.
- 24. Hill AS, et al. (2008) Ion channel clustering at the axon initial segment and node of Ranvier evolved sequentially in early chordates. PLoS Genet 4:e1000317.
- 25. Hedtke SM, Townsend TM, Hillis DM (2006) Resolution of phylogenetic conflict in large data sets by increased taxon sampling. Syst Biol 55:522-529.
- 26. Zakon HH, Jost MC, Lu Y (2011) Expantion of voltage-dependent Na+ channel gene family in early tetrapods coincided with the emergence of terrestriality and increased brain complexity. Mol Biol Evol 28:1415-1424.
- 27. Anderson PA. Schwab WE (1983) Action potential in neurons of motor nerve net of Cyanea (Coelenterata). J Neurophysiol 50:671-683.
- 28. Spencer AN, Satterlie RA (1981) The action potential and contraction in subumbrellar swimming muscle of Polyorchis penicillatus (Hydromedusae). J Comp Physiol 144: 401-407.

- 29. White GB, et al. (1998) Structure of a putative sodium channel from the sea anemone Aiptasia pallida. Invert Neurosci 3:317–326.
- Bilbaut A, Nicaise MH, Meech RW (1989) Evolution of the First Nervous Systems, ed Anderson PAV (Plenum, New York), pp 299–314.
- Heinemann SH, Terlau H, Stühmer W, Imoto K, Numa S (1992) Calcium channel characteristics conferred on the sodium channel by single mutations. *Nature* 356: 441–443.
- Lipkind GM, Fozzard HA (2008) Voltage-gated Na channel selectivity: The role of the conserved domain III lysine residue. J Gen Physiol 131:523–529.
- Schlief T, Schönherr R, Imoto K, Heinemann SH (1996) Pore properties of rat brain II sodium channels mutated in the selectivity filter domain. Eur Biophys J 25:75–91.
- 34. Watanabe H, Fujisawa T, Holstein TW (2009) Cnidarians and the evolutionary origin of the nervous system. *Dev Growth Differ* 51:167–183.
- Kulkarni NH, Yamamoto AH, Robinson KO, Mackay TFC, Anholt RRH (2002) The DSC1 channel, encoded by the smi60E locus, contributes to odor-guided behavior in Drosophila melanogaster. Genetics 161:1507–1516.
- 36. Sirota FL, Pascutti PG, Anteneodo C (2002) Molecular modeling and dynamics of the sodium channel inactivation gate. *Biophys J* 82:1207–1215.
- 37. Catterall WA (2000) From ionic currents to molecular mechanisms: The structure and function of voltage-gated sodium channels. *Neuron* 26:13–25.

- 38. King N, Hittinger CT, Carroll SB (2003) Evolution of key cell signaling and adhesion protein families predates animal origins. *Science* 301:361–363.
- Fukuoka H, Wada T, Kojima S, Ishijima A, Homma M (2009) Sodium-dependent dynamic assembly of membrane complexes in sodium-driven flagellar motors. Mol Microbiol 71:825–835.
- 40. Rassat R, Ruthmann A (1979) *Trichoplax adhaerens* F.E. Schulze (Placozoa) in the scanning electron microscope. *Zoomorphologie* 93:59–72.
- The King Lab Resources. Available at http://kinglab.berkeley.edu/resources/; accessed on February 19, 2010.
- 42. Katoh K, Kuma K, Toh H, Miyata T (2005) MAFFT version 5: Improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res* 33:511–518.
- Penn O, et al. (2010) Guidance: A web server for assessing alignment confidence scores. Nucleic Acids Res 38:W23–W28.
- 44. Zwickl DJ (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. PhD dissertation (University of Texas, Austin, TX).
- 45. Abascal F, Zardoya R, Posada D (2005) ProtTest: Selection of best-fit models of protein evolution. *Bioinformatics* 21:2104–2105.
- 46. Swofford DL (2003) PAUP\*: Phylogenetic Analysis Using Parsimony (\*and Other Methods) (Sinauer, Sunderland, MA), Version 4.
- Bryson K, et al. (2005) Protein structure prediction servers at University College London. Nucleic Acids Res 33:W36–W38.