

**Supplemental Figure 1.** Species tree of taxa used in this study, after (Parfrey et al. 2011) and (Porter et al. 2011). Soft polytomies added to convey grouping that are not agreed upon in the literature.

		D1	D2
	<i>Mus</i> Na <sub>v</sub> 1.4	<b>R</b> TF <b>R</b> VL <b>R</b> AL <b>K</b> TIT	<b>r</b> sf <b>r</b> ll <b>r</b> vf <b>k</b> la <b>k</b>
NT	Homo $P/Q(Ca_2.1)$	<b>r</b> tl <b>r</b> av <b>r</b> vl <b>r</b> pl <b>k</b>	<b>r</b> al <b>r</b> ll <b>r</b> if <b>k</b> vt <b>k</b>
	Homo	<b>r</b> ip <b>r</b> plimi <b>r</b> af <b>r</b>	TYFQVL <b>r</b> vv <b>r</b> li <b>k</b>
	Caenorhabditis	<b>R</b> SI <b>R</b> PFIII <b>R</b> LIP	TYFQTF <b>r</b> ll <b>r</b> li <b>k</b>
T.	Drosophila	<b>R</b> AP <b>R</b> PLIMI <b>R</b> FL <b>R</b>	TYFQVL <b>r</b> vv <b>r</b> li <b>k</b>
	Nematostella	<b>R</b> AP <b>R</b> ALIMV <b>R</b> VF <b>K</b>	AIFHVM <b>r</b> vl <b>r</b> lig
N	Trichoplax		TVFAVL <b>R</b> IL <b>R</b> IV <b>R</b>
	Amphimedon	SVTSAA <b>k</b> lfipl <b>k</b>	VVFQAL <b>r</b> lp <b>r</b> li <b>r</b>
F	Saccharomyces	<b>K</b> PLAIL <b>R</b> IL <b>R</b> LVN	SIFHIS <b>r</b> fy <b>r</b> vii
U	Aspergillus	SMLSCL <b>R</b> IL <b>R</b> LLN	TLFQIL <b>r</b> vy <b>r</b> vvl
N	Phycomyces	<b>K</b> MLSTLILL <b>R</b> LLN	TGFQVL <b>r</b> iy <b>r</b> vvv
G	Mucor	<b>K</b> MLSALILL <b>R</b> LLN	TGFQVL <b>r</b> iy <b>r</b> lvv
ΙI	Allomyces	<b>R</b> GMAAL <b>R</b> VF <b>R</b> LLS	TGFQLA <b>r</b> tn <b>k</b> lvt
	Thecamonas3	<b>R</b> AF <b>R</b> AL <b>R</b> PM <b>R</b> AL <b>K</b>	<b>R</b> VF <b>R</b> VL <b>R</b> IT <b>R</b> LLV
		D3	D4
	<i>Mus</i> Na,1.4	D3 kslrtlralrplr	D4 RLARIGRVLRLIR
	<i>Mus</i> Na <sub>v</sub> 1.4 <i>Homo</i> P/Q(Ca <sub>v</sub> 2.1)	D3 kslrtlralrplr kslrvlrvlrplk	D4 RLARIGRVLRLIR RLFRAARLIKLLR
N	Mus Na <sub>v</sub> 1.4 Homo P/Q(Ca <sub>v</sub> 2.1) Homo	D3 KSLRTLRALRPLR KSLRVLRVLRPLK LMVLRCLRPLR	D4 RLARIGRVLRLIR RLFRAARLIKLLR ACVIVFRFFSICG
N   A	Mus Na <sub>v</sub> 1.4 Homo P/Q(Ca <sub>v</sub> 2.1) Homo Caenorhabditis	D3 KSLRTLRALRPLR KSLRVLRVLRPLK LMVLRCLRPLR LMICRAMRPLR	D4 RLARIGRVLRLIR RLFRAARLIKLLR ACVIVFRFFSICG YLVVILRFFTIAS
N A T.	Mus Na <sub>v</sub> 1.4 Homo P/Q(Ca <sub>v</sub> 2.1) Homo Caenorhabditis Drosophila	D3 KSLRTLRALRPLR KSLRVLRVLRPLK LMVLRCLRPLR LMICRAMRPLR LMILRCVRPLR	D4 RLARIGRVLRLIR RLFRAARLIKLLR ACVIVFRFFSICG YLVVILRFFTIAS FMVVILRFFTITG
N A L C	Mus Na <sub>v</sub> 1.4 Homo P/Q(Ca <sub>v</sub> 2.1) Homo Caenorhabditis Drosophila Nematostella	D3 KSLRTLRALRPLR KSLRVLRVLRPLK LMVLRCLRPLR LMICRAMRPLR LMILRCVRPLR LMIFRCLRPLR	D4 RLARIGRVLRLIR RLFRAARLIKLLR ACVIVFRFFSICG YLVVILRFFTIAS FMVVILRFFTITG VVIIIFRFLTLSG
N A L C N	Mus Na <sub>v</sub> 1.4 Homo P/Q(Ca <sub>v</sub> 2.1) Homo Caenorhabditis Drosophila Nematostella Trichoplax	D3 KSLRTLRALRPLR KSLRVLRVLRPLK LMVLRCLRPLR LMICRAMRPLR LMILRCVRPLR LMIFRCLRPLR LAVLRCLRPLR	D4 RLARIGRVLRLIR RLFRAARLIKLLR ACVIVFRFFSICG YLVVILRFFTIAS FMVVILRFFTITG VVIIIFRFLTLSG
N A L C N	Mus Na <sub>v</sub> 1.4 Homo P/Q(Ca <sub>v</sub> 2.1) Homo Caenorhabditis Drosophila Nematostella Trichoplax Amphimedon	D3 KSLRTLRALRPLR KSLRVLRVLRPLK LMVLRCLRPLR LMICRAMRPLR LMILRCVRPLR LMIFRCLRPLR IVLMGVRALRPLH	D4 RLARIGRVLRLIR RLFRAARLIKLLR ACVIVFRFFSICG YLVVILRFFTIAS FMVVILRFFTITG VVIIIFRFLTLSG
N   A   C   F	Mus Na <sub>v</sub> 1.4 Homo P/Q(Ca <sub>v</sub> 2.1) Homo Caenorhabditis Drosophila Nematostella Trichoplax Amphimedon Saccharomyces	D3 KSLRTLRALRPLR KSLRVLRVLRPLK LMVLRCLRPLR LMICRAMRPLR LMILRCVRPLR LMIFRCLRPLR IVLMGVRALRPLH RIFKGLTALRALR	D4 RLARIGRVLRLIR RLFRAARLIKLLR ACVIVFRFFSICG YLVVILRFFTIAS FMVVILRFFTITG VVIIIFRFLTLSG ILKCLKAML GFFLLVIFLFIIP
N   A   C   F   U	Mus Na <sub>v</sub> 1.4 Homo P/Q(Ca <sub>v</sub> 2.1) Homo Caenorhabditis Drosophila Nematostella Trichoplax Amphimedon Saccharomyces Aspergillus	D3 KSLRTLRALRPLR KSLRVLRVLRPLK LMVLRCLRPLR LMICRAMRPLR LMILRCVRPLR LMIFRCLRPLR IVLMGVRALRPLH RIFKGLTALRALR RAIGAFKALRALR	D4 RLARIGRVLRLIR RLFRAARLIKLLR ACVIVFRFFSICG YLVVILRFFTIAS FMVVILRFFTITG VVIIIFRFLTLSG ILKCLKAML GFFLLVIFLFIIP KLFLVSITLLIIP
N   A   C N   F   N	Mus Na <sub>v</sub> 1.4 Homo P/Q(Ca <sub>v</sub> 2.1) Homo Caenorhabditis Drosophila Nematostella Trichoplax Amphimedon Saccharomyces Aspergillus Phycomyces	D3 KSLRTLRALRPLR KSLRVLRVLRPLK LMVLRCLRPLR LMICRAMRPLR LMILRCVRPLR LMIFRCLRPLR IVLMGVRALRPLH RIFKGLTALRALR RAIGAFKALRALR	D4 RLARIGRVLRLIR RLFRAARLIKLLR ACVIVFRFFSICG YLVVILRFFTIAS FMVVILRFFTITG VVIIIFRFLTLSG ILKCLKAML GFFLLVIFLFIIP KLFLVSITLLIP KLFMTALCFKLVQ
N   A   C N   F   N G	Mus Na <sub>v</sub> 1.4 Homo P/Q(Ca <sub>v</sub> 2.1) Homo Caenorhabditis Drosophila Nematostella Trichoplax Amphimedon Saccharomyces Aspergillus Phycomyces Mucor	D3 KSLRTLRALRPLR KSLRVLRVLRPLK LMVLRCLRPLR LMICRAMRPLR LMIFRCLRPLR LMIFRCLRPLR IVLMGVRALRPLH RIFKGLTALRALR RAIGAFKALRALR RGFRAFKALRALR	D4 RLARIGRVLRLIR RLFRAARLIKLLR ACVIVFRFFSICG YLVVILRFFTIAS FMVVILRFFTITG VVIIIFRFLTLSG ILKCLKAML GFFLLVIFLFIIP KLFLVSITLLIIP KLFMTALCFKLVQ KLFMTALCFKLVQ
N A L C N F U N G I	Mus Na <sub>v</sub> 1.4 Homo P/Q(Ca <sub>v</sub> 2.1) Homo Caenorhabditis Drosophila Nematostella Trichoplax Amphimedon Saccharomyces Aspergillus Phycomyces Mucor Allomyces	D3 KSLRTLRALRPLR KSLRVLRVLRPLK LMVLRCLRPLR LMICRAMRPLR LMILRCVRPLR LMIFRCLRPLR IVLMGVRALRPLH RIFKGLTALRALR RAIGAFKALRALR RVFRAFKALRALR GVLRLMRSLRPLR	D4 RLARIGRVLRLIR RLFRAARLIKLLR ACVIVFRFFSICG YLVVILRFFTIAS FMVVILRFFTITG VVIIIFRFLTLSG ILKCLKAML GFFLLVIFLFIIP KLFLVSITLLIP KLFMTALCFKLVQ KLFMTALCFKLVQ KLVLIGYALRIAR

**Supplemental Figure 2.** Alignment of the voltage sensing S4 segments from Na<sub>v</sub>, Ca<sub>v</sub>, NALCN, fungal calcium channels and an apusozoan outgroup (*Thecamonas* 3). D1-4 are the constituent domains and the voltage sensing residues, arginine (R) or lysine (K), are in bold. NALCN and fungal channels have reduced numbers of sensors relative to animal Na<sub>v</sub>, Ca<sub>v</sub>, and apusozoan channels



Supplemental Figure 3. A.) Addition of four-domain ion channels from non-opisthokont eukaryotes to the tree in Figure 2. A majority consensus tree from Bayesian analysis (PhyloBayes) is presented along with posterior probabilities of branch support. The placements of added sequences have poor support, and several branches that were well supported in Figure 2 lose support after addition of these sequences (bolded and underlined support values).
B.) Two possible rooting placements for opisthokont four-domain ion channels. Both are equally parsimonious with regards to loss of channels in the fungal lineage, but are more parsimonious than rooting with either voltage-gated clade (1 loss rather than 2). The upper topology is consistent with the midpoint rootings in Figure 1, and in A. In this scenario, *Thecamonas* 3 is the sister channel to the voltage-insensitive clade, but in the lower scenario apusozoans have no homolog of this group.

	Pore State	Inactivation Particle
<i>Mus</i> Na <sub>v</sub> 1.4	D/E/K/A	IFMTEEQ
<i>Homo</i> Na <sub>v</sub> 1.6	D/E/K/A	IFMTEEQ
<i>Drosophila</i> Na <sub>v</sub> 1	D/E/K/A	MFMTEDQ
Micromonas	E/E/E/E	LFVTEEQ *
Chlamydomonas	E/E/E/E	VFVTPQQ
Volvox	S/E/S/E	VDLTPAQ
Ectocarpus	E/E/E/E	VLVTEEQ *
Phytophthora 2	E/E/E/E	YLLSHIQ
Thalassiosira	E/E/E/E	GLMTQAQ
Paramecium	E/E/E/E	FYMTLEQ
Ostreococcus 2	E/E/E/E	TIMSESQ
Emiliana	E/E/E/E	AMMTDAQ
Ostreococcus 1	E/E/D/E	KLLTERQ
Phytophthora 1	D/D/K/C	) EILTESQ
<i>Homo</i> P/Q Ca <sub>v</sub>	E/E/E/E	YSLEKNE

**Supplemental Figure 4.** Pore motifs and inactivation particle motifs of non-opisthokont channels. Well characterized  $Na_v$  channels from *Mus*, *Homo*, and *Drosophila* and *Homo* P/Q-type  $Ca_v$  are given for comparison. Several non-opisthokont channels have Cav-like pore motifs and Nav-like motifs at the region that is putatively homologous to the Nav inactivation particle. The channels from the green alga Micromonas and the brown alga Ectocarpus (stars) are the most similar to bilaterian Nav channels in the inactivation particle region.