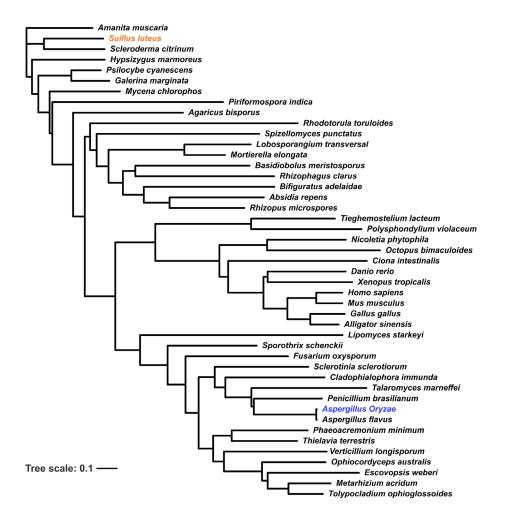
Voltage-gated proton channels from fungi highlight role of peripheral regions in channel activation

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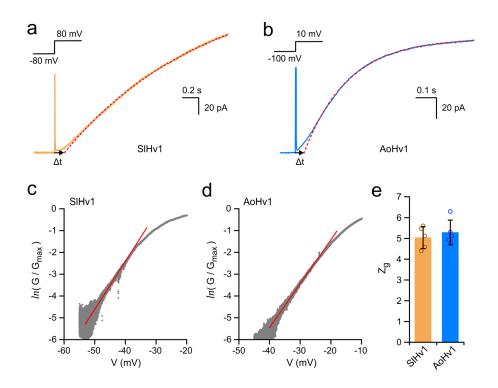
SUPPLEMENTARY FIGURES 1-6



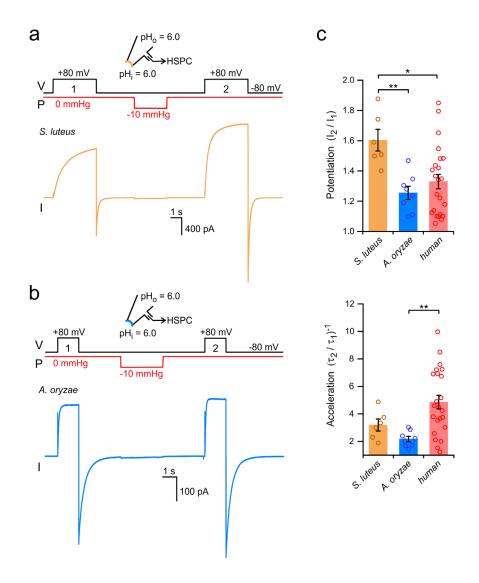
Supplementary Figure 1. Phylogenetic relationship between Hv1 channels from fungi and animals. Organisms from the kingdom Fungi includes representatives from *Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota and Glomeromycota* divisions (see "Methods" section for sequence IDs and details). Tree scale 0.1 = 10 % difference between sequences. The same animal species from the cladogram of Fig. 1a were included in the phylogenetic tree. Representatives of slime molds (*Tieghemostelium/Dictyostelium* and *Polysphondylium* species) are also included here. These organisms used to be considered part of the Fungi kingdom, but they are now classified as protists.

а		S 0	S1				
	F. Oxysporum S. sclerotiorum C. immunda T. marneffei A. oryzae P. brasilianum A. muscaria S. luteus H. marmoreus P. cyanescens G. marginata H. sapiens	SRSYVRSFLST LRHSLQHWFSS FRAKTRSFLMS WRRAARDFLSS WRRSARDFLNS LRDKAIQLLES WRENISCILES WRTKTEHLLES YRIKTAHFLEH CRAKTAHFLDH	KFGHYSVLTLVSLDVL RGQHYTVLLLVACDLI KWGHYFVILLVSADIC KWGHYLVLLLVAVDVA RRGHYLVLLLVSVDVA RWGHYLVLLLVTIDVC NTFHTFVIALIAIDAS RRFHTFVIALIVIDAT RRFHIIVIVLIALDAA PRLHKTVITLITIDAI PKLHKIVIALITTDAI HRFQVIIICLVVLDAL O	GIFADIIINLY(CIFADFLISLH CSFANFLIELRY CTFADFLIELHY CSFSEFLIQLHY CVVADLIYTLL CVVADLIYTLL CVLADLGYTVL CVLADLGYTVL CVLADLAYSFL CVLADLAYSFL	QCDNDK 92 ICEHAG 110 VCELRE 163 VCELKQ 70 VCELKQ 70 P-QDCT 68 S-DGCA 73 YADQCS 71 S-PNCA 71 S-PTCE 71	2	
			S2				
	F. Oxysporum S. sclerotiorum C. immunda T. marneffei A. oryzae P. brasilianum A. muscaria S. luteus H. marmoreus P. cyanescens G. marginata H. sapiens	KGSDWDLALEILGSVSLVFSCLFVVELIASVWAFGWKYFN132E-GKTDPIWNEVRVGLGIAGLVFSCLFMLELIASVWAFGWS131E-KGFN-LRAWEQANDVLGYASLVFSCLFMLELIASVVAFGRYFN160R-HQTPIDRRWSLAQETLGLLGLIFSCLFMLELIASVLSFGLSYFR207-HGSHVAIGWGVTQKVLAIVGLVFSCLFMLELMVTVFSFGKGYFS124-NGYKVGHEWAVIEETLGIAGLVISCLFMVELIVSTLSFGMGYFS114PDQPMGDVPAWLEVLSHLSLTITTLFLLEIPLAVWAFGPRYYNPSGTVPH118P-PDEGPEWLEVLAHISLSITSLFLVEIPVSLWAFGLEHFNPFGAVIH118P-PG-EDSPQWLEVLSHISLAITTLFLIEIPLNLWAFGPQFMNPLGPVAH119P-PGGEDVPAWLEVLSHISLAITTLFLVEIPLNLWAFGFQFMNPFGPVPH120PDKNNYAAMVFHYMSITILVFFMMEIIFKLFVFRLEFFH167**					
			\$3		S4	CCD	
	F. Oxysporum S. sclerotiorum C. immunda T. marneffei A. oryzae P. brasilianum A. muscaria S. luteus H. marmoreus P. cyanescens G. marginata H. sapiens	-KFHCFDATVI NSFHIFDALVI LKFHTFDALVI SKFHVFDALVI TWFHVFDSIVI ASLHLFDAFII AALHLFDTAII AGLHLFDALII AGLHAFDSVII AGLHAFDSIII	IAGFITDVALRGIIE- VAGFVVDVLLHGIVE- IAAFVIDVLLRGPLE- VLAFVLDVSLRGIVE- IVAFGVDVALHGIEE- LVAFIIQVSLRGVEE- LATFTLEAVLKGKER- VTTFVLEFVLKGRQR- VTTFILEVALKGQER- LTTFILEVVLRGKER- LTTFILEAILRGKER- VVSFILDIVLLFQEHQ *	-EVASLVIVLR -EAGSLVVVLR -ELGSLIVVLR -ELGSLIVVLR -EVGSLVIVLR -ELAGLLIVFR -ELAGLLIILR -ELAGLLIILR -ELAGLLVILR -ELAGLLVIR	LWRFFKII LWRVFKII LWRVFKII LWRVFQII LWRIVKLVGGIAV LWRLVKLVGGIAV LWRLVKLVGGVAV LWRLVKLVGGVAV	EEFSVGAQ EEFSSGAE EEMSEVSA EELQSANE EELKSASE GAGELEEEDA GAGELEEETA GAGELEEETA GAGELEEETA DAGELEEETL	184 183 212 259 176 166 177 179 177 178 179 231
	S. luteus A. oryzae H. sapiens	КІІ	AELGEETAQELEDT -EELQSANEDTLEEY IRSERQLLRLKQMNVQ	EHEIER	LRQENTYLRQRI	LNVSLSNADPMD	200 182 273

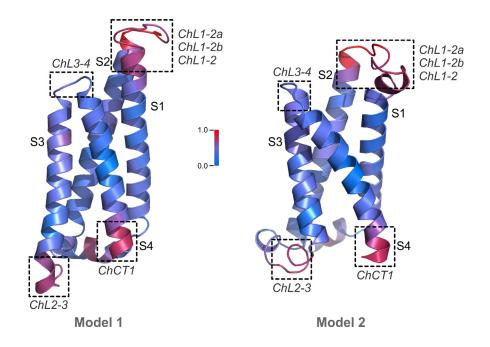
Supplementary Figure 2. Sequence alignment of proton channels from multiple fungal species. a) Comparison focused on VSDs of Hvs from *Ascomycetes* and *Basidiomycetes* in relation to human Hv1. ^OAspartate residue known to be part of the selectivity filter of hHv1. *Histidine residues proposed to coordinate Zn²⁺ in hHv1. •I127: when a cysteine is introduced at this position in hHv1, it forms a spontaneous intersubunit disulfide bond. Endogenous cysteines in the S1-S2 loops of fungal Hvs are highlighted in yellow. b) Highlighted in green are regions predicted to form a CCD in SIHv1 and AoHv1 in relation to the CCD of hHv1 (estimated from 3VMX).



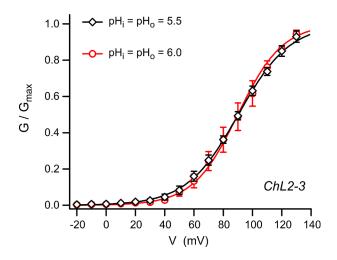
Supplementary Figure 3. Voltage-dependent opening of fungal Hvs. a-b) Examples of the initial phase of the timecourse of activation for SIHv1 (a) and AoHv1 (b) in response to the indicated voltage steps (pH_i = pH_o = 6.0). Single exponential fits of the current traces, after the initial sigmoidal phase, are shown as red dotted lines. Black arrows indicate $\Delta t = t_e - t_o$, where t_e is the time at which the current extrapolated from the fit is zero, and t_o is the time of the transition in membrane potential. A $\Delta t > 0$ indicates that the channel spends time transitioning through closed states before opening. In these examples, Δt was 123 ms for SIHv1, and 40 ms for AoHv1. **c-d**) Representative plots for the determination of the gating charge (z_g) based on the limiting slope method. $In(G/G_{max})$ was calculated as described in the "Methods" section. The linear fit, showed as red line, was performed between the ordinate range -5 to -4. Measurements were carried out in inside-out patch configuration, pH_i = 5.5, pH_o = 6.5 for SIHv1; pH_i = pH_o = 6.0 for AoHv1. **e**) Quantification of the gating charge (z_g) associated with voltage-dependent activation of the indicated channels. z_g values were derived from the slope of linear fits like those shown in (c) and (d), see "Methods". Each bar represents a mean value from five independent measurements. Error bars are SD.



Supplementary Figure 4. Hv1s from *S. luteus* and *A. oryzae* are sensitive to mechanical stimulation. a-b) Examples of proton currents elicited by membrane depolarization for SlHv1 (a) and AoHv1 (b) before (step 1) and after mechanical stimulus (step 2). Change in membrane tension was induced via negative pressure applied to the patch pipette. The mechanical stimulus was delivered at resting membrane potential to inside-out patches. c) Averaged increases in current (potentiation) and activation rate (acceleration) caused by the mechanical stimulus ($\Delta P = -10 \text{ mmHg}$) in fungal Hv1s compared to human channel. Current values I₁ and I₂ were measured at the end of depolarization steps 1 and 2, respectively. Time constants from mono-exponential fits of current traces were used to calculate acceleration in channel activation (τ_2/τ_1)⁻¹. Each bar represents the mean of at least 6 independent measurements ± SEM. One-way ANOVA with Tukey's post-hoc tests were used for statistical analysis: *p < 0.05, **p < 0.01. Reference values for hHv1 are from Pathak et al. 2016 (⁵¹).



Supplementary Figure 5. Alternative structural models for the VSD of SIHv1. Divergence in sequence homology between SIHv1 and AoHv1 mapped on two alternative models of the SIHv1 VSD. Model 1 is based on the structure of mHv1cc (3WKV:A). Model 2 is based on the structure of CiVSP-VSD (4G80:I). Color gradient varies from minimal divergence (blue) to maximal divergence (red) (same as Fig. 5a). Dashed boxes indicate regions with the largest sequence divergence which were targeted by chimeragenesis. In both models these regions include: the S1-S2 loop and the outermost portions of helices S1 and S2, the part of the S2-S3 loop closer to helix S3, the S3-S4 loop, and the innermost portion of helix S4. Local differences between the two models can be seen in all the divergent regions; the most noticeable involves the transition between the S2-S3 loop and helix S3 (region targeted in the ChL2-3 chimera).



Supplementary Figure 6. Reduced pH sensitivity of the G-V relationship of chimera ChL2-3 under symmetrical conditions ($\Delta pH = 0$). G-Vs under the indicated pH conditions represent the mean of 5 independent measurements. Error bars are SEM. The following G-V parameters were derived from Boltzmann fits of the data: $V_{1/2} = 90.2 \pm 1.6$ mV, slope = 17.7 ± 1.8 mV for pH_i = pH_o = 5.5 (n = 5), and $V_{1/2} = 89.9 \pm 3.9$ mV, slope = 15.3 ± 1.7 mV for pH_i = pH_o = 6.0 (n = 5). The negligible change in $V_{1/2}$ is to be compared to the corresponding change observed with SlHv1 WT ($\Delta V_{1/2} \sim 7.6$ mV) from Fig. 3j.