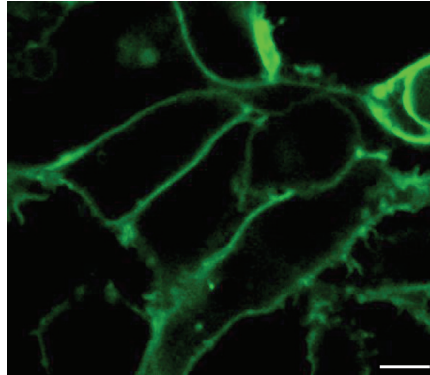


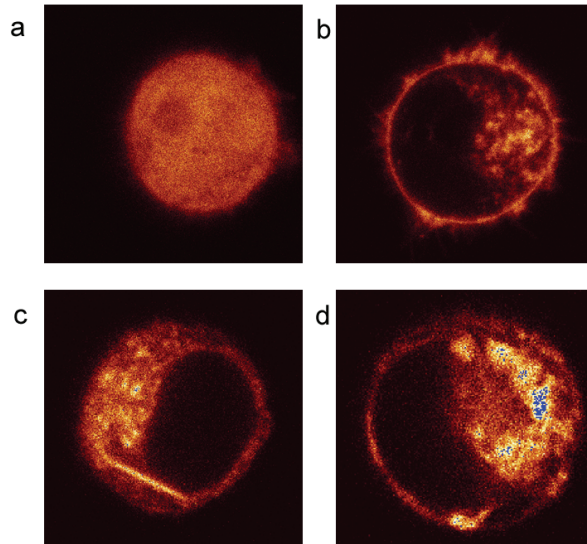
**Supplementary Figure S1. Phylogenetic relationship between the proton channel (H<sub>v</sub>1) and C15orf27 families and other VSD-containing proteins.**

Unrooted phylogram from a maximum likelihood analysis of 122 VSDs (Table S1) shows that H<sub>v</sub>1 sequences appear on a branch distinct from other VSDs. Sequences are colour coded: K<sub>v</sub> = voltage gated potassium channel; Na<sub>v</sub> = voltage gated sodium channel; Ca<sub>v</sub> = voltage gated calcium channel; VSP = voltage sensitive phosphatase. Notably, the VSD homologs separate into three main branches, indicating that the VSDs of the H<sub>v</sub>1/C15orf27/VSP group are phylogenetically distinct from VSDs of both K<sub>v</sub> and also Na<sub>v</sub> and Ca<sub>v</sub> channels. Phylogenetic analysis was performed on VSD sequences only, and did not include sequences of channel pores. Branches with likelihood support values (a measure of confidence in a branch's appearance in a tree) <0.50 were collapsed. The range of support values shown is representative of the full range of all (non-collapsed) branches. \*indicates hH<sub>v</sub>1 and C15orf27 sequence positions.

**Supplementary Figure S2. The c15orf27 protein localizes to the plasma membrane.**

Human C15orf27 cDNA was subcloned into pEGFPN1 vector (Clontech, CA) with GFP fused to the C-terminus of the cDNA. HEK cells were transfected with C15orf27-GFP and mid-plane images were obtained 24 hours post-transfection by Laser scanning confocal microscopy. Scale bar is 5  $\mu\text{m}$ .

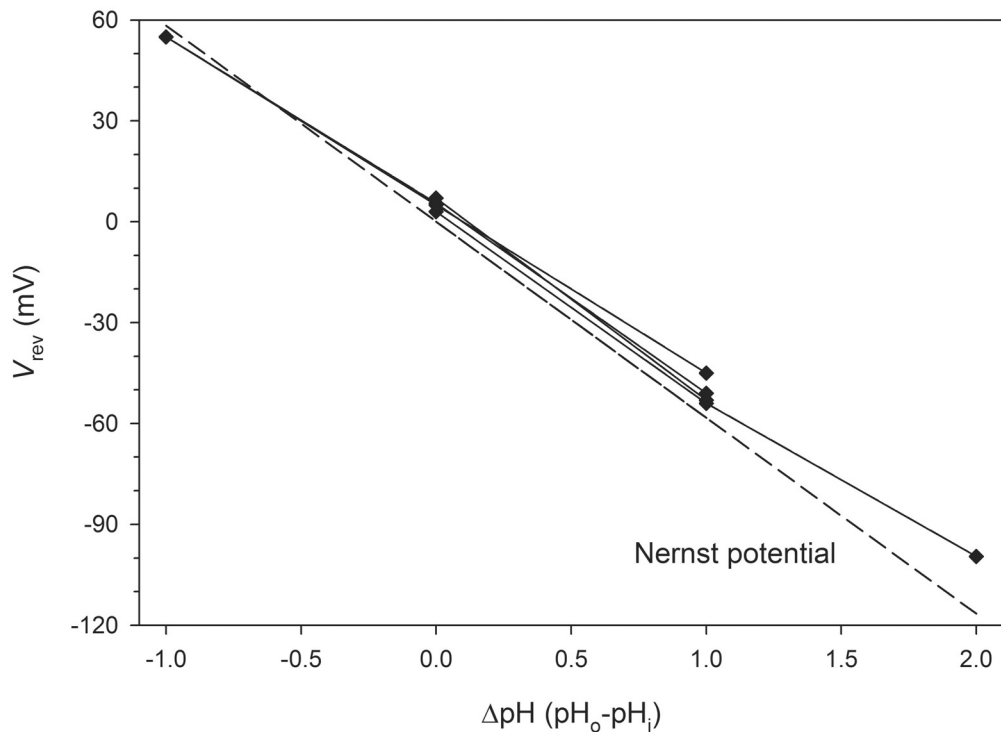
**Supplementary Figure S3. GFP-tagged constructs appear to localize to the plasma membrane.**



Pseudocolour images of GFP in representative COS-7 cells transfected with (a) GFP alone, (b) WT hH<sub>v</sub>1 with GFP tag, (c) D112S with GFP tag, or (d) D112V with GFP tag. GFP alone is diffusely distributed throughout the cytoplasm. GFP-tagged hH<sub>v</sub>1 constructs appear in the membrane and in intracellular compartments.

**Methods** - Transfected cells were cultured in 2 ml culture dishes in DMEM. The medium was removed by aspiration and the cells were detached by immersing monolayers in trypsin/EGTA solution (Sigma) for 3 min. Cells were washed in DMEM medium and suspended at a concentration of  $2 \times 10^6$  cells/ml. An aliquot of the cell suspension was added to a measuring chamber containing 300  $\mu$ l of Ringer's solution and cells were allowed to settle. Transfected cells were visualized by exciting at 488 nm and collecting emission at 490 - 560 nm using a Leica TCS SP2 confocal system (Leica Microsystems, Exton, PA, USA). Cells were imaged using the 40x water immersion lens and scanned at 400 Hz.

**Supplementary Figure S4. The elimination of  $Zn^{2+}$  sensitivity by the H140A/H193A mutation does not detectably impair proton selectivity.**

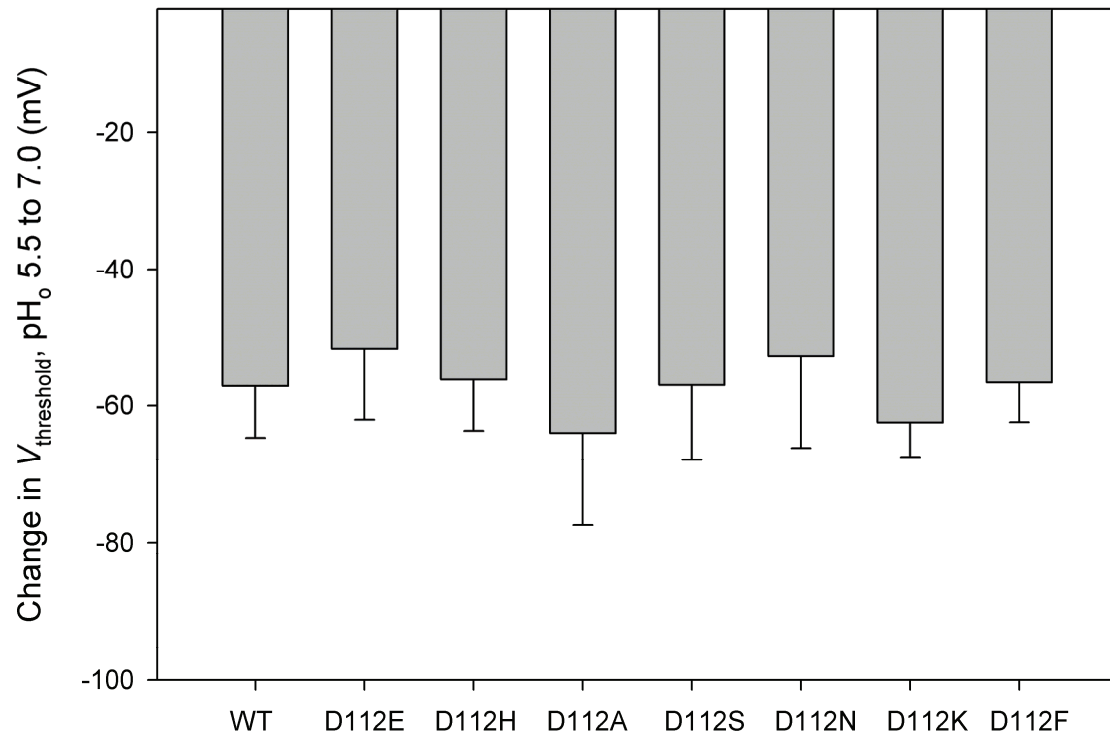


In inside-out patches of membrane from HEK-293 cells expressing H140A/H193A channels,  $V_{rev}$  was measured over a range of pH. Measurements in the same patch are connected by lines. This double mutant was used as a background for several of the Asp<sup>112</sup> mutants in order to validate that any currents detected were due to the mutant channel, rather than native proton currents. In the presence of 100  $\mu M$   $Zn^{2+}$ , WT proton current activation is slowed  $\sim 15$ -fold, and the  $g_H$ - $V$  relationship is shifted positively by  $\sim 60$  mV<sup>1</sup>. The H140A/H193A mutation nearly eliminates inhibition by  $Zn^{2+}$  (refs. 2,3).

#### References

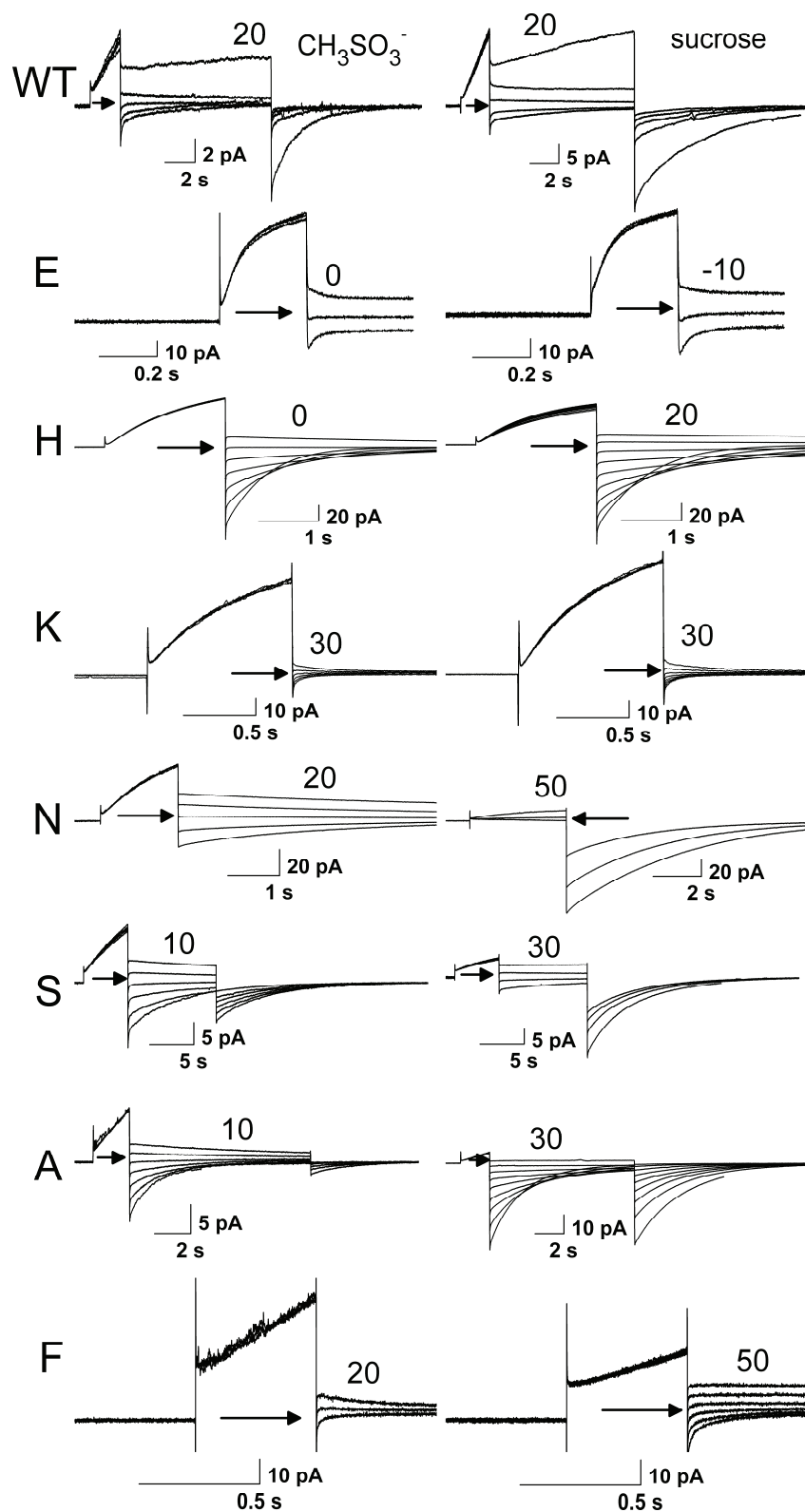
1. Cherny, V. V. & DeCoursey, T. E. pH-dependent inhibition of voltage-gated  $H^+$  currents in rat alveolar epithelial cells by  $Zn^{2+}$  and other divalent cations. *J Gen Physiol* **114**, 819-38 (1999).
2. Musset, B. et al. Zinc inhibition of monomeric and dimeric proton channels suggests cooperative gating. *J Physiol* **588**, 1435-49 (2010).
3. Ramsey, I. S., Moran, M. M., Chong, J. A. & Clapham, D. E. A voltage-gated proton-selective channel lacking the pore domain. *Nature* **440**, 1213-6 (2006).

**Supplementary Figure S5. Mutation of Asp<sup>112</sup> does not eliminate the  $\Delta$ pH dependence of gating.**



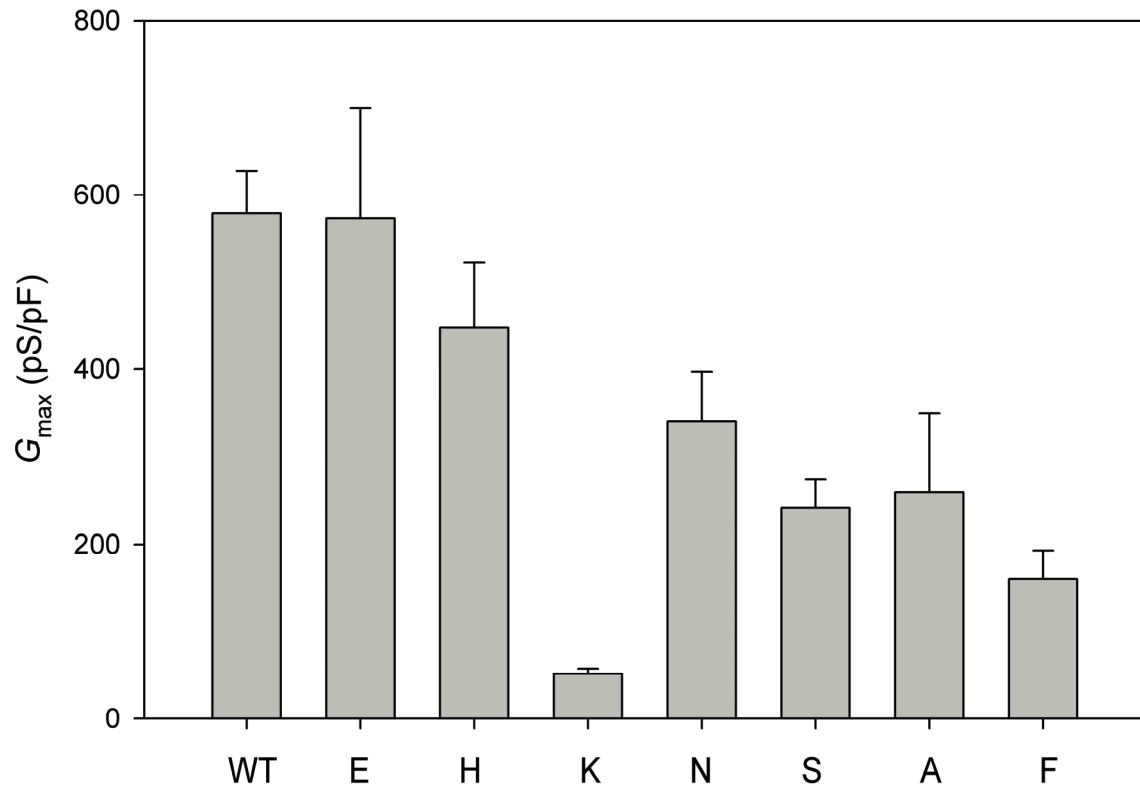
In whole-cell measurements like those in Fig. 2,  $V_{\text{threshold}}$  was estimated as the voltage at which distinct conductance was activated, usually determined from tail currents, which are more sensitive. The graph shows the mean  $\pm$  SD shift of  $V_{\text{threshold}}$  when pH<sub>o</sub> 5.5 and 7.0 are compared, both at pH<sub>i</sub> 5.5. Numbers of cells range 3 to 11. None of the shifts for D112x mutants differed significantly from that in WT ( $p > 0.28$  for each).

**Supplementary Figure S6. Dilution of ionic strength with isotonic sucrose reveals that most Asp<sup>112</sup> mutants are anion selective.**

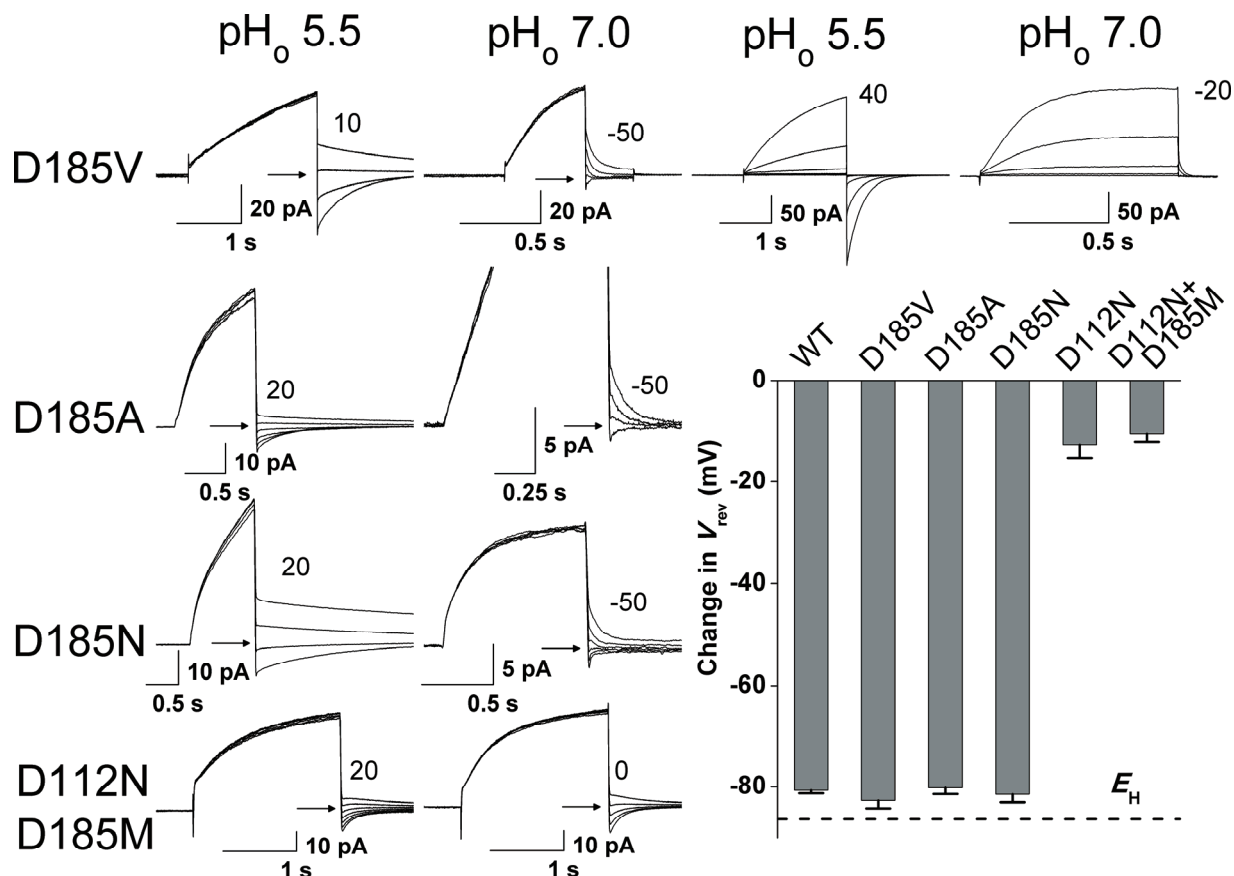


Measurement of  $V_{\text{rev}}$  by tail currents (or direct reversal of test current in D112N in sucrose) at pH 5.5//5.5 ( $\text{CH}_3\text{SO}_3^-$  solutions) is shown in the first column, and in the same cell after 90% dilution of the bath by isotonic sucrose in the second column. Arrows indicate zero current. Letters indicate the amino acid substituted at position 112. The most positive voltage in each series is given, without correction for liquid junction potentials.  $V_{\text{rev}}$  of WT or D112E channels did not change, consistent with proton selectivity. For other mutants, except D112K,  $V_{\text{rev}}$  shifted positively, indicating anion selectivity.  $V_{\text{hold}}$  was -40 mV, or -20 mV for F.  $V_{\text{pre}}$  for control, sucrose was 30, 30 mV (WT); 20, 10 mV (E); 60, 60 (H); 140, 140 mV (K); 60 mV (N); 50, 40 mV (S); 50, 50 mV (A); and 100, 90 mV (F). The lack of a shift for D112K in  $\text{CH}_3\text{SO}_3^-$  at pH<sub>o</sub> 5.5 is anomalous, because sucrose did produce a positive shift at pH<sub>o</sub> 5.5 in  $\text{Cl}^-$  solution, and at pH<sub>o</sub> 7.0 in both  $\text{Cl}^-$  and  $\text{CH}_3\text{SO}_3^-$  solutions (Fig. 3).

**Supplementary Figure S7. Maximum conductance of Asp<sup>112</sup> mutants expressed in COS-7 cells, at pH 5.5/5.5, normalized to capacity.**



The maximum chord conductance  $G_{\max}$  was calculated from the maximum current measured in each cell, using  $V_{\text{rev}}$  measured in the same solution. Mean of 8-14 cells for each mutant, with s.e. bars.

Supplementary Figure S8. Mutation of Asp<sup>185</sup> does not impair proton selectivity.

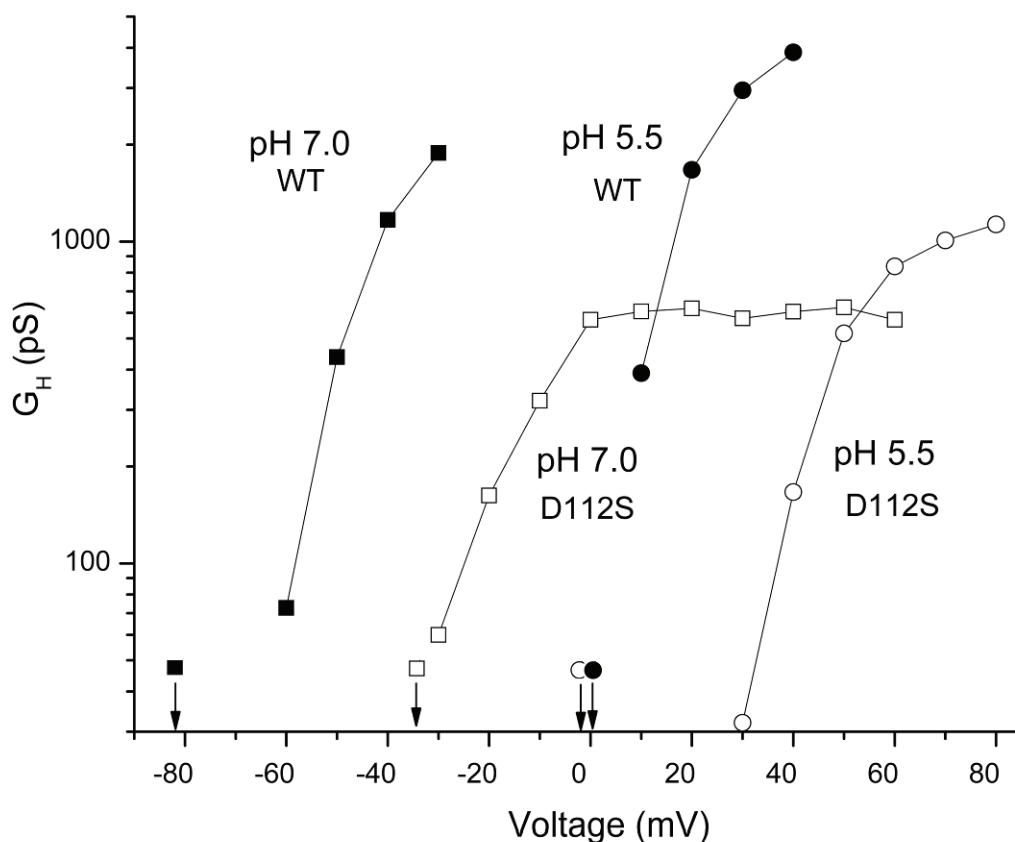
In whole-cell measurements like those in Fig. 2,  $V_{rev}$  was determined from tail currents at pH<sub>o</sub> 5.5 and pH<sub>o</sub> 7.0, both at pH<sub>i</sub> 5.5, as illustrated here. Families of currents are shown for D185V at pH<sub>o</sub> 5.5 and 7.0. The most positive voltage for tail currents or for families of pulses in 10 mV increments is labelled. For D185V tail currents,  $V_{hold}$  was -40 mV and  $V_{pre}$  was +30 mV at pH<sub>o</sub> 5.5,  $V_{hold}$  was -60 mV and  $V_{pre}$  was -30 mV at pH<sub>o</sub> 7.0. For families,  $V_{hold}$  was -40 mV and -70 mV at pH<sub>o</sub> 5.5 or pH<sub>o</sub> 7.0, respectively. For D185A,  $V_{hold}$  was -60 mV for both pH and  $V_{pre}$  was +75 or +15 mV, respectively, for pH<sub>o</sub> 5.5 and pH<sub>o</sub> 7.0. For D185N,  $V_{hold}$  was -60 mV for both pH and  $V_{pre}$  was +45 or -31 mV, respectively, for pH<sub>o</sub> 5.5 and pH<sub>o</sub> 7.0.

To determine whether the addition of Asp<sup>185</sup> neutralization to an anion selective Asp<sup>112</sup> mutant might produce additional effects, we also studied the double mutant D112N/D185M. Tail currents at pH<sub>o</sub> 5.5 and pH<sub>o</sub> 7.0 are shown, with  $V_{hold}$  -40 mV and  $V_{pre}$  +100 or +60 mV at pH<sub>o</sub> 5.5 and pH<sub>o</sub> 7.0, respectively.

The bar graph shows the change in  $V_{rev}$  when pH<sub>o</sub> was changed from 5.5 to 7.0 for these Asp<sup>185</sup> mutants. For comparison, values from WT and D112N channels from Fig. 2b are replotted here. There is no difference between WT and D185V ( $n = 4$ ), D185A ( $n = 4$ ), or D185N ( $n = 4$ ) or between D112N and D112N/D185M ( $n = 6$ ).



**Supplementary Figure S9. Dissociation of the relationship between  $V_{\text{rev}}$  and the  $g$ - $V$  relationship in Asp<sup>112</sup> mutants.**



For representative cells expressing WT hH<sub>v</sub>1 (solid symbols) and D112S channels (open symbols), the conductance was calculated from the current amplitude extrapolated from a rising exponential fit, using  $V_{\text{rev}}$  measured in the relevant solution at pH<sub>o</sub> 5.5 or pH<sub>o</sub> 7.0, all at pH<sub>i</sub> 5.5. The arrows indicate measured  $V_{\text{rev}}$  values. In both D112S and WT, the  $g$ - $V$  relationship shifted negatively by  $\sim 60$  mV at pH<sub>o</sub> 7.0 compared to its position at pH<sub>o</sub> 5.5, but  $V_{\text{rev}}$  shifted much less in D112S than in WT. In WT hH<sub>v</sub>1, there is a linear relationship between  $V_{\text{rev}}$  and  $V_{\text{threshold}}$  (or the  $g_H$ - $V$  relationship) with a slope of  $\sim 40$  mV/unit increase in  $\Delta\text{pH}$ <sup>1</sup>.

<sup>1</sup> Musset, B. *et al.* Detailed comparison of expressed and native voltage-gated proton channel currents. *J. Physiol.* **586**, 2477-2486(2008).

**Supplementary Table 1. FASTA-formatted alignment of voltage sensor domain sequences.**

This alignment was used to generate the phylogenetic tree in Fig. S1. Sequences are numbered as in the tree. One sequence from a protein that responds to hyperpolarizing potentials (109) was included. All other sequences were from protein families known to respond to depolarizing potentials, or from C15orf27 homologs. Accession numbers used are from NCBI (gi), Uniprot (uniprot or sp), or PDB (pdb) sequence databases.

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KTIVGALIQSVKK
>40 canis_SCN_repeat1 gi|74004456|
IKILVH-----SLFNVLIMCTILTNCVFMTMSN-----
-----PPDW-----TKNVEYFTFTGIYT
FESLIKILARGFCL-----EDFTFLRDPWNWLDFTVITFAYVTEFVDLG-----
-----NVSALRTFRVLRALKTISVI-----PGL-
KTIVGA-----
>41 danio_SCN8AA_repeat1 sp_Q9DF53
IKILIH-----SVFSMFIMCTILTNCVFMTFSN-----
-----PPEW-----SKQVEYFTFTGIYT
FESAVKIIARGFCI-----DGFTFLRDPWNWLD FFMVISMAYVTEFVDLG-----
-----NVSALRTFRVLRALKTISVI-----PGL-
KTIVGALIQSVKK
>42 mouse_SCN8A_repeat1 sp_Q9WTU3
IKILIH-----SVFSMIIMCTILTNCVFMTFSN-----
-----PPEW-----SKNVEYFTFTGIYT
FESLVKIIARGFCI-----DGFTFLRDPWNWLD FSVIMMAYVTEFVDLG-----
-----NVSALRTFRVLRALKTISVI-----PGL-
KTIVGALIQSVKK
>43 canis_SCNAA_repeat1 sp_Q46669
IKVSVH-----SWFSLFITVTILVNCVGMTQTE-----
-----LPD-----RIEYVFTVIYT
FEALIKILARGFCL-----NEFAYLRDPWDWLD FSVITLAYIGEATALR-----
-----GISGLRTFRVLRALKTVSVI-----PGL-
KVIVGALIH SVRK
>44 homo_SCN7A_repeat1 sp_Q01118
IKVLVH-----PFFQLFILISVLIDCVFMSLTN-----
-----LPKW-----RPVLENTLLGIYT
FEILVKLFARGVWA-----GSFSFLGDPWNWLD FSVTVFEVIIRYSPLD-----
-----FIPTLQTARTLRILKIIPLN-----QGL-
KSLVGVLIIHCLKQ
>45 rabbit_CAC1C_repeat1 sp_P15381
ISIVEW-----KPF EIIILLTIFANCVALAIYIPFPED-----
-----DSNATNSN-----LERVEYLFLII FT
VEAFLKVIAYGLLF-----HPNAYLRNGWNLLDFIIVVVGLFSAILEQATK-AD-
-----GANALGGKGAGF-DVKALRAFRVLRPLRLVSGV-----PSL-
QVVLNSIIKAMV-
>46 mouse_CAC1S_repeat1 sp_Q02789
ISIVEW-----KPFETIILLTIFANCVALAVYLPMPED-----
-----DNNTLNLG-----LEKLEYFFLIVFS
IEAAMKIIAYGFLF-----HQDAYLRSGWNVLD FIIIVFLGVFTVILEQVNI IQT-
-----NTAPMSSKGAGL-DVKALRAFRVLRPLRLVSGV-----PSL-
QVVLNSIFKAML-
>47 mouse_CAC1F_repeat1 sp_Q9JIS7
ISIVEW-----KPF DILILLTIFANCVALGVYIPFPED-----
-----DSNTANHN-----LEQVEYVFLVI FT
VETVLKIVAYGLVL-----HPSAYIRNGWNLLDFIIVVVGLF SVLLEQGPGRPG-
-----DAPHTGGKPGGF-DVKALRAFRVLRPLRLVSGV-----PSL-
HIVVNSIMKALV-
>48 gallus_CAC1D_repeat1 sp_Q73700
ISLVEW-----KPF DIFILLSIFANCVALAVYIPFPED-----
-----DSNSTNHN-----LEKVEYAFLLI FT
VETFLKIIAYGLLL-----HPNAYVRNGWNLLDFVIVVVGLF SVILEQLTKETE-
-----GGSHSGGKPGGF-DVKALRAFRVLRPLRLVSGV-----PSL-
QVVLNSIIKAMV-
>49 homo_CACN_repeat1 gi|193788728|
ISIVEW-----KPF EIIILLTIFANCVALAIYIPFPED-----
-----DSNATNSN-----LERVEYLFLII FT

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VEAFLKVIAYGLLF-----HPNAYLRNGWNLDFIIVVVGLFSAILEQATKADG-
-----ANALGGKGAGF-DVKALRAFRVLRPLRLVSGV-----PSL-
QVVLNSIIKAMVP
>50 drosophila_CAC1D_repeat1 sp_Q24270
IRIVEW-----KPFEFLLILLTIFANCIALAVYTPYPGS-----
-----DSNVTNQT----LEKVEYVFLVIIFT
AECVMKILAYGFVL-----HNGAYLRNGWNLDFIIVVIGAISTALSQMK----
-----DAF-DVKALRAFRVLRPLRLVSGV-----PSL-
QVVLNSILKAMV-
>51 homo_CAC1A_repeat1 sp_O00555
KKITEW-----PPFEYMILATIIANCIVLALEQHLPDD-----
-----DKTPMSER----LDDTEPYFIGIFC
FEAGIKIIALGFAG-----HKGSYLRNGWNVMDFVVVLTGILATVGTEF-----
-----DLRTLRAVRVLRPLKLVSGI-----PSL-
QVVLKSIMKAMIP
>52 homo_CAC1B_repeat1 sp_Q00975
KRITEW-----PPFEYMILATIIANCIVLALEQHLPDG-----
-----DKTPMSER----LDDTEPYFIGIFC
FEAGIKIIALGFVF-----HKGSYLRNGWNVMDFVVVLTGILATAGTDF-----
-----DLRTLRAVRVLRPLKLVSGI-----PSL-
QVVLKSIMKAMV-
>53 rat_SCN11A_repeat3 sp_O88457
YQIVKH-----SWFESFIIIFVILLSSGALIFEDVNLPS-----
-----RPQVEKL----LRCTDNIFTFIFL
LEMILKWVAFGF-----RRYFTSAWCWLDLIVVVSVLSLMNLP-----
-----SLKSFRTLRLRPLRALSQF-----EGM-
KVVVYALISAIPA
>54 mouse_SCN11A_repeat3 sp_Q9R053
YQIVKH-----SWFESFIIIFVILLSSGALIFEDVNLPS-----
-----RPQVEKL----LKCTDNIFTFIFL
LEMILKWVAFGF-----RKYFTSAWCWLDLIVVVSGLSLTNLP-----
-----NLKSFRLRLRPLRALSQF-----EGM-
KVVVNALMSAIPA
>55 rat_SCN9A_repeat3 sp_O08562
YRIVEH-----SWFESFIVLMILLSSGALAFEDIYIEK-----
-----KKTIIKII----LEYADKIFTYIFI
LEMLLKWVAYGY-----KTYFTNAWCWLDLIVDVSLVTLVANTLGYSDLG
-----PIKSLRTLRLRPLRALSFR-----EGM-
RVVVNALIGAIPS
>56 rabbit_SCN9A_repeat3 sp_Q28644
YRIVEH-----SWFESFIVLMILLSSGALAFEDIYIEK-----
-----KKTIIKII----LEYADKIFTYIFI
LEMLLKWVAYGY-----KTYFTNAWCWLDLIVDVSLVTLVANTLGYSDLG
-----PIKSLRTLRLRPLRALSFR-----EGM-
RVVVNALIGAIPS
>57 mouse_SCN9A_repeat3 uniprot_B7ZWN
YRIVEH-----SWFESFIVLMILLSSGALAFEDIYIEK-----
-----KKTIIKII----LEYADKIFTYIFI
LEMLLKWVAYGY-----KTYFTNAWCWLDLIVDVSLVTLVANTLGYSDLG
-----PIKSLRTLRLRPLRALSFR-----EGM-
RVVVNALIGAIPS
>58 mouse_KCNH1 sp_Q60603
-----TWDWIILILITFYTAILVPYNVFSK-----
-----TRQNNVA----WLVDVSDIVDVIIFL
VDIVLNFHTTFVGPAGEVISDPKLIRMNYLK-TWFVIDLLSCLPYDVINAFENVDEVSFAF
MGDPGKIGFADQIPPLEGRESQGISSLFS-SLKVVRLRLRGRVARKLDHY-----IEY-
GAAVLV-----
>59 mouse_KCNH8 sp_P59111
-----GWDWLILLATFYVAVTVPYNVCFIGN-----
-----EDLSTTRS----TTVSDIAVEILFI
IDIILNFRTTYVSKSGQVIFEARSICIHVVT-TWFIIDLIAALPFDLLYAFNVTV-----

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-----VSLVH-LLKTVRLLRLLRLLQKLDRY-----SQH-
STIVLTLLMSM--
>60 homo_KCNH3 sp_Q9ULD8
-----TWDFGILLATLYVAVTVPVYVVCVSTA-----
-----REPSAARGP----PSVCDLAVEVLFI
LDIVLNFRTTFVSKSGQVVFAPKSI CLHYVT-TWFLLDVIAALPFDLLHAFKVVN-----
-----YFGAH-LLKTVRLLRLLRLLPRLDRY-----SQY-
SAVVL-----
>61 homo_CAC1G_repeat4 sp_O43497
HHLCTS-----HYLDLFIITGVIGLNVVVTMAMEHYQQ-----
-----PQILDEA----LKICNYIFTVIFV
LESVFKLVAFG-----FRRFFQDRWNQLDLAIVLLSIMGITLEEIEVNA--
-----SLPINPT-IIRIMRVLRIARVLKLLKMA-----VGM-
RALLDVTVMQALPQ
>62 mouse_SCN11A_repeat4 sp_Q9R053
FDLVTS-----QVFDVIILGLIVTNMIIMMAESEGO-----
-----PNEVKKI----FDILNIVFVVI FT
VECLIKVFALR-----QHYFTNGWNLFDCV VVLSIISTLVSGLENSN--
-----VFPPT-LFRIVRLARIGRILRLVRAA-----RGI-
RTLLFALMMSLPS
>63 rat_SCN9A_repeat4 sp_O08562
FDLVTN-----QAFDITIMVLI CLNMVTMMVEKEGO-----
-----TEYMDYV----LHWINMVFIILFT
GECVLKLLISLR-----HYYFTVGWNIFDFV VVILSIVGMFLAEMIEKY--
-----FVSPT-LFRVIRLARIGRILRLIKGA-----KGI-
RTLLFALMMSLPA
>64 rat_SCN11A_repeat4 sp_O88457
FDLVTS-----QVFDVIILGLIVLNMIMMAESADQ-----
-----PKDVKKT----FDILNIAFVVI FT
IECLIKVFALR-----QHYFTNGWNLFDCV VVLSIISTLVSRLESD--
-----ISFPPT-LFRVVRLARIGRILRLVRAA-----RGI-
RTLLFALMMSLPS
>65 humo_CAC1G_repeat2 sp_O43497
RKIVDS-----KYFGRGIMIAIILVNTLSMGIEYHEQ-----
-----PEELTNA----LEISNIVFTSLFA
LEMLLKL LVYG-----PFGYIKNPYNIFDGVIVVISVWEIVGQQGG-----
-----GLSVLRTFRLMRVLKLVRF-----PAL-
QRQLVVLMTMDN
>66 homo_CACNA1E_repeat_4 sp_Q15878
WHFVVS-----PSFEYTIMAMIALNTVVLMMKYISA-----
-----PCTYELA----LKYLNIAFTMVFS
LECVLKVIAFG-----FLNYFRDWNIFDFITVIGSITEI ILTDSKLVN--
-----TSGF-NMSFLKLFRAARLIKLLRQG-----YTI-
RILLWTFVQS FKA
>67 drosophila_CAC1A_repeat_4 sp_P91645
WRIVVS-----TPFEYFIMMLIVFNTLLMMKYHNQ-----
-----GDMYEKS----LKYINMGFTGMFS
VETVLKIIGFG-----VKNFFKDPWNIFDLITVLGSIVDALWMEFGHDD--
-----SNSI-NVGFLRLFRAARLIKLLRQG-----YTI-
RILLWT-----
>68 homo_KCNV2 sp_Q8TDN2
WNLMEKPFSSVA AKAIGVASSTFVLVSVVALALNTVEEMQ-----QHSG
-----QEGE----GPDLRPI----LEHVEMLCMGFFT
LEYLLRLASTP-----DLRRFARSALNLVDLVAILPLYLQLLLECFTEGHQ
-----RGQTVGSVGVKVGQ-VLRVMRLMRIFRILKLARHS-----TGL-
RAFGFTLRQCYQQ
>69 homo_KCNF1 sp_Q9H3M0_KCNF1
WKFLEKPESSCPARVVAVLSFLLIILVSSVVMCMGTIPELQ-----VLD-
-----AEG----NRVEHPT----LENVETACIGWFT
LEYLLRLFSSP-----NKLHFALSF MNIVDVLAILPFYVSLTLTHLGAR--
-----MMELTNVQQ-AVQALRIMRIARIFKLARHS-----SGL-

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QTLTYALKRSFKE  
 >70 homo\_KCNB1 sp\_Q14721  
 WDLLEKPNSSVAAKILAIISIMFIVLSTIALSLNTPPELQ-----SLD-  
 -----EFG----QSTDNPQ----LAHVEAVCIAWFT  
 MEYLLRFLSSP-----KKWKFFKGPLNAIDLLAILPYYVTIFLTESNKS---  
 -----VLQFQNVRR-VVQIFRIMRILRILKLARHS-----TGL-  
 QSLGFTLRRSYNE  
 >71 canis\_KCNB2 sp\_Q95167  
 RDLEKPNSSVAAKILAIIVSNLFIVLSTIALSLNTPPELQ-----EMD-  
 -----EFG----QPNDNPQ----LAHVEAVCNAWFT  
 MEYLLRFLSSP-----NKWKFFKGPLNVIDLLAILPYYVTIFLTESNKS---  
 -----VLQFQNVRR-VVQIFRIMRILRILKLARHS-----TGL-  
 QSLGFTLRRSYNE  
 >72 drosophila\_KCNAB sp\_P17970  
 WELLEKPNTSFAARVIAVISILFIVLSTIALTLNTPQLQ-----HIDN  
 G-----TPQDNPQ----LAMVEAVCITWFT  
 LEYILRFSASP-----DKWKFFKGGLNIIDLLAILPFVSLFLETNKN---  
 -----ATDQFQDVRV-VVQVFRIMRILRVLKLARHS-----TGL-  
 QSLGFTLRNSYKE  
 >73 pongo\_KCNV1 sp\_Q5RC10  
 WNILEKPGSSSTAARIFGVISIIIFVVVSIINMALMSAEL-----  
 -----SWLDLQL----LEILEYVCISWFT  
 GEFVLRFLCVR-----DRCRFLRKVPNIIDLLAILPFYITLLVESLSGSQ--  
 -----TTQELENVGR-IVQVLRLLRMLKLGRHS-----TGL-  
 RSLGMTITQCYEE  
 >74 homo\_KCNS3 sp\_Q9BQ31  
 WIRMENPAYCLSAKLIAISSLSVVLASIVAMCVHSMSEFQ-----NED-  
 -----GEVDDPV----LEGVEIACIAWFT  
 GELAVRLAAAP-----CQKKFWKNPLNIIDFVSIIPFYATLAVDTKEEE---  
 -----SEDIENMGK-VVQILRLMRIFRILKLARHS-----VGL-  
 RSLGATLRHSYHE  
 >75 squirrelmonkey\_KCNS1 sp\_A4K2X4  
 WLTMENPGYSLPSKLFSCVSIISVVLASIAAMCIHSLPEYQ-----AREA  
 AAAVA-----AVAAGRSAE----GVRDDPV----LRRLEYFCIAWFS  
 FEVSSRLLLAP-----STRNFFCHPLNLIIDIVSVLPFYLTLLAGAALGDQG-  
 -----GTGGKEFGHLGK-VVQVFRMLRIFRVLKLARHS-----TGL-  
 RSLGATLKHYSYRE  
 >76 gallus\_KCNG2 sp\_O73606  
 RDMVENPHSGIPGKIFACISISFVAITAVSLCISTMPDVR-----EEE-  
 -----DRGE----CSQKCYD----IFVLETVCVAWFS  
 FEFLLRISIQAE-----NKCAFLKTPLNIIIDILAILPFYISLIVDMASKNNS  
 KP-----GGGAGNKYLERVGL-VLRFLRALRILYVMRLARHS-----LGL-  
 QTLGLTVRRCTRE  
 >77 homo\_KCNG4 sp\_Q8TDN1  
 REMVENPQSGLPKVFACLSILFVATTAVSLCVSTMPDLR-----AEE-  
 -----DQGE----CSRKCY----IFIVETICVAWFS  
 LEFCLRFVQAQ-----DKCQFFQGPLNIIDILAI SPYVSLAVSEPPEDGE  
 -----RPSGSSYLEKVGL-VLRVLRALRILYVMRLARHS-----LGL-  
 QTLGLTVRRCTRE  
 >78 rat\_KCNC3 sp\_Q01956\_KCNC3  
 WALFEDPYSSRAARYVAFASLFFILISITTFCLETHEGFI-----HISN  
 KTVTQASP-----IPGAPPENITNV----EVETEPF----LTYVEGVCVVWFT  
 FEFLMRVTFPCP-----DKVEFLKSSLNIIDCVAILPFYLEVGLSGLSSK---  
 -----AAKDVLG-FLRVVRFVRILRIFKLTRHF-----VGL-  
 RVLGHTLRASTNE  
 >79 homo\_KCNC2 sp\_Q96PR1  
 WALFEDPYSSRAARFIAFASLFFILVSIITTFCLETHEAFN-----IVKN  
 KTEPV-----INGTSVVLQY----EIETDPA----LTYVEGVCVVWFT  
 FEFLVRIVFSP-----NKLEFIKNLLNIIDFVAILPFYLEVGLSGLSSK---  
 -----AAKDVLG-FLRVVRFVRILRIFKLTRHF-----VGL-  
 RVLGHTLRASTNE

>80 *drosophila\_KCNAW* sp\_P17972  
 WSLFDEPYSSNAAKTIGVSVVFFICISILSFCLKTHPDMR-----VPIV  
 RNITVKT-----ANGSNGWFLDKT----QTNAHIA----FFYIECVNAWFT  
 FEILVRFISSP-----NKWEFIKSSVNIIDYIATLSFYIDLVLQRFAS----  
 -----HLENAD-ILEFFSIIRIMRLFKLTRHS-----SGL-  
 KILIQTFRASAKE  
 >81 *homo\_KCNA1* sp\_Q09470  
 WLLFEYPESSGPARVIAIVSVMVILISIVIFCLETLPPEL-----DDKD  
 F----TGT-----VHRIDNTTVIYN-----SNIFTDP----FFIVETLCIIWFS  
 FELVVRFFACP-----SKTDFFRNIMNFIDIVAIIPYFITLGTETIAEQE-G-  
 -----NQGGEQATSLA-ILRVIRLVRVFRIFKLSRHS-----KGL-  
 QILGQTLKASMRE  
 >82 *rat\_KNCA6* sp\_P17659  
 WLLFEYPESSGPARGIAIVSVLVILISIVIFCLETLPQFRADGRGGSNEGSGTRMSPASR  
 GSHEEEDDEDSDYAFPGSIPSGGLGTGGTSSFFSTLGGSFFTDP----FFLVETLCIVWFT  
 FELLVRFSAACP-----SKAAFFRNIMNIIDLVAIFPYFITLGTETLVQRHEQQ  
 PV-----SGGSGQNROQAMSLA-ILRVIRLVRVFRIFKLSRHS-----KGL-  
 QILGKTLQASMRE  
 >83 *homo\_KCNA5* sp\_P22460  
 WLIFEYPESSGSARAIIVSVLVILISIIIFCLETLPPEFRDEREL-----LRHPPAPHQ  
 PPAPAPGANGS-----GVMAPPSGPTVAPLL----PRTLADP----FFIVETTTCVIWFT  
 FELLVRFSAACP-----SKAGFSRNIMNIIDVVAIFPYFITLGTETLAEQQPGG  
 G-----GGGQNGQQAMSLA-ILRVIRLVRVFRIFKLSRHS-----KGL-  
 QILGKTLQASMRE  
 >84 *rat\_KCNA3* sp\_P15384  
 WLLFEYPESSGPARGIAIVSVLVILISIVIFCLETLPPEFR-----DEKD  
 YPASPSQDV-----FEAANNSTSGASSG----ASSFSDP----FFVETLCIIWFS  
 FELLVRFSAACP-----SKATFSRNIMNLIDIVAIIPYFITLGTETLAERQ---  
 -----GNGQQQAMSLA-ILRVIRLVRVFRIFKLSRHS-----KGL-  
 QILGQTLKASMRE  
 >85 *canis\_Kv1.3* gi|57088651|  
 WLLFEYPESSGPARGIAIVSVLVILVSVIVIFCLETLPPEFR-----DDKD  
 YAAAAQEQ-----PEAARNGTSGPPA----AAGFADP----FFVETLCIIWFS  
 FELLVRFSAACP-----SKATFSRNIMNLIDIVAIIPYFITLGTETLAERQ---  
 -----GNGQQQAMSLA-ILRVIRLVRVFRIFKLSRHS-----KGL-  
 QILGQTLKASMRE  
 >86 *bovine\_KCNA4* sp\_Q05037  
 WLLFEYPESSSPARGIAIVSVLVILISIVIFCLETLPPEFR-----DDR  
 LIMALSTGGHG-----GLLNDTSAPHPENSG----HTIFNDP----FFIVETVCIVWFS  
 FEFVVRFCFACP-----SQALFFKNIMNIIDIVSILPYFITLGTDLAQQQGG-  
 -----GNGQQQQAMSF A-ILRIIRLVRVFRIFKLSRHS-----KGL-  
 QILGHTLRASMRE  
 >87 *homo\_KCA10* sp\_Q16322  
 WLLFEYPESSSAARAVAVSVLVVVISITIFCLETLPPEFR-----EDRE  
 LKVVRD-----PNLNMSKTVLS----QTMFTDP----FFMVESTCIVWFT  
 FELVLRVFCFACP-----SKTDFFRNIMNIIDIIISIIIPYFATLITELVQETE--  
 -----PSAQQNMSLA-ILRIIRLVRVFRIFKLSRHS-----KGL-  
 QILGQTLKASMRE  
 >88 *rat\_Kv1.2\_2R9R\_b\_vs* gi|16087779|  
 WLLFEYPESSGPARIIAIVSVMVILISIVSFCLETLPPIFR-----DENE  
 DMHGGGVT-----FHTYSQSTIGYQQ----STSFTDP----FFIVETLCIIWFS  
 FEFLVRFSAACP-----SKAGFFTNIMNIIDIVAIIPYVVTI--FLTESN---  
 -----KSVLQFQNVRRVQIFRIMRILRIFKLSRHS-----KGL-  
 Q-----  
 >89 *homo\_Kv* gi|4826782|  
 WLLFEYPESSGPARIIAIVSVMVILISIVSFCLETLPPIFR-----DENE  
 DMHGGGVT-----FHTYSNSTIGYQQ----STSFTDP----FFIVETLCIIWFS  
 FEFLVRFSAACP-----SKAGFFTNIMNIIDIVAIIPYFITLGTETLAEKPED-  
 -----AQQQQQAMSLA-ILRVIRLVRVFRIFKLSRHS-----KGL-  
 QILGQTLKASMRE  
 >90 *rat\_Kv* pdb:2A79\_chainb

WLLFEYPESGPARIIAIVSVMVILISIVSFCLETLPFR-----DENE  
 DMHGGGV-----FHTYSNSTIGYQQ----STSFTDP----FFIVETLCIIWFS  
 FEFLVRRFFAC-----SKAGFTNIMNIIDIVAIIPYFITLGTAEKPED-  
 -----AQQGQQAMSLA-ILRVIRLVRVFRIFKLSRHS-----KGL-  
 QILGQTLKASMRE  
 >91 canis\_KCNA2 sp\_Q28293  
 WLLFEYPESGPARIIAIVSVMVILISIVSFCLETLPFR-----DENE  
 DMHGGGV-----FHTYSNSTIGYQQ----STSFTDP----FFIVETLCIIWFS  
 FEFLVRRFFAC-----SKAGFTNIMNIIDIVAIIPYFITLGTAEKPED-  
 -----AQQGQQAMSLA-ILRVIRLVRVFRIFKLSRHS-----KGL-  
 QILGQTLKASMRE  
 >92 drosophila shaker\_Kchannel gi|288442|  
 WLLFEYPESQAARVVAIISVFVILLSIVIFCLETLPFR-----HYKV  
 FNTTT-----NGTKIEDE-----VPDITDP----FFLIETLCIIWFT  
 FELTVRFLACP-----NKLNFCRDVMNVIDIIAIIIPYFITLATVVAEEEDTL  
 NLPK-----APVSPQDKSSNQAMSLA-ILRVIRLVRVFRIFKLSRHS-----KGL-  
 QILGRTLKASMRE  
 >93 rabbit\_KCND3 sp\_Q9TTT5  
 WRAFENPHTSTLALVFYYVTGFFIAVSVITNVVETVPCGT-----VPGS  
 -----KELPC----GERYSVA----FFCLDTACVMIFT  
 VEYLLRLFAAP-----SRYRFIRSVMIIIDVVAIMPYYIGLVMTNNE-----  
 -----DVSG-AFVTLRVFRVFRIFKFSRHS-----QGL-  
 RILGYTLKSCASE  
 >94 hum\_CACNA1E repeat\_2 sp\_Q15878  
 RHMVKS-----QVFWIVLSLVALNTACVAIVHHNQ-----  
  
 -----PQWLTHL----LYYAEFLFLGLFL  
 LEMSLKMYGM-----PRLYFHSSFNCFDFGVTVGSIFEVVAIFRP-----  
 -----GTSF-GISVLRALRLLRIFKITKYW-----ASL-  
 RNLVVSLMSSMK  
 >95 drosophila\_CAC1A repeat\_2 sp\_P91645  
 RHTVKT-----QWFYWFVIVLVFLNTVCVAVEHYGQ-----  
 -----PSFLTEF----LYYAEFIFLGLFM  
 SEMFIKMYALG-----PRIYFESSFNRFDCVVISGSIFEVIWSEVK-----  
 -----GGSF-GLSVLRALRLLRIFKVTKYW-----SSL-  
 RNLVISLLNSMRS  
 >96 mouse\_SCN11A repeat2 sp\_Q9R053  
 QTIMTD-----PFTELAITICIIIVNTVFLAMEHHNM-----  
 -----DNSLKI----LKIGNWVFTGIFI  
 AEMCLKIIALD-----PYHYFRHGWNIFDSIVALVSLADVLFHKLSK-----  
 -----NLSFLASLRVLRVFKLAKSW-----PTL-  
 NTLIKIIGHSVGA  
 >97 rat\_SCN11A repeat2 sp\_O88457  
 RTIMTD-----PFTELAITICIIINTVFLAVEHHNM-----  
 -----DDNLKTI----LKIGNWVFTGIFI  
 AEMCLKIIALD-----PYHYFRHGWNVFDSDIVALLSLADVLYNTL-----  
 -----SDN-NRSFLASLRVLRVFKLAKSW-----PTL-  
 NTLIKIIGHSVGA  
 >98 rat\_SCN9A repeat2 sp\_O08562  
 YFIVMD-----PFVDLAITICIVLNTLFLMAMEHHPM-----  
 -----TEEFKNV----LAVGNLIFTGIFA  
 AEMVLKLIAMD-----PYEYFQVGWNIFDSLIVTSLIE-LFLADVE-----  
 -----GLSVLRSFRLLRVFKLAKSW-----PTL-  
 NMLIKIIGNSVGA  
  
 >99 ornitho\_C15orf27 |gi\_149410687|  
 WQVFLLS-----ASLNSFLVACVILVVILLTLELLIDIK-----LLQ-  
 -----FSS----ASQFASV----VHWISLIILSVFF  
 TETILRIVVLG-----IWDYIENKIEVFDGAVIILSLAPMVASTVANGP--  
 -----SSPWD-AISLIITLRIWRVKRIIDAYVLPVKVEM-

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EMVIQQYEKA---
>100 danio_c15orf27 |gi_123703002|
WQVCLLS-----AGFNCFLVACVILVVLLTLELLIDTK-----LLQ-
-----FNN----AFQFACI----IHWISLVILSVFF
TETVFRIVVLG-----IWDYIENKVEVFDGAVIVLSLAPMVASTVANGP--
-----SSPWD-AISLIITLRIWRVKRIIDAYVLQVKVEM-
ELEIQQYEKS---
>101 monodelphis_C15orf27 gi|12627230|
WQVFLLS-----ASLNSFLVACVILVVILLTLELLIDIK-----SLQ-
-----FSN----SSQFAGV----SHWISLVILSVFF
SETILRIVVLG-----IWDYIENKIEVFDGAVIILSLAPMVASTIANGP--
-----SSPWD-AISLTIALRIWRVKRIIDAYVLPVKVEL-
EMVIQQYEKA---
>102 sus_C15orf27 gi|194039682|
WQVFLLS-----ASVNSFLVACVILVVILLTLELLIDIK-----LLQ-
-----FSS----AFQFAGV----IHWISLVILSVFF
SETVLRIVVLG-----IWDYIENKIEVFDGAVIILSLAPMVASTVANGP--
-----RSPWD-AISLIIMLRIWRVKRVIDAYVLPVKVEM-
EMVIQQYEKA---
>103 homo_C15orf27 |gi_118442841|
WQVFLLS-----ASLNSFLVACVILVVILLTLELLIDIK-----LLQ-
-----FSS----AFQFAGV----IHWISLVILSVFF
SETVLRIVVLG-----IWDYIENKIEVFDGAVIILSLAPMVASTVANGP--
-----RSPWD-AISLIIMLRIWRVKRVIDAYVLPVKLEM-
EMVIQQYEKA---
>104 pan_C15orf27 |gi_114658268|
WQVFLLS-----ASLNSFLVACVILVVILLTLELLIDIK-----LLQ-
-----FSS----AFQFAGV----IHWISLVILSVFF
SETVLRIVVLG-----IWDYIENKIEVFDGAVIILSLAPMVASTVANGP--
-----RSPWD-AISLIIMLRIWRVKRVIDAYVLPVKLEM-
EMVIQQYEKA---
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SETVLRIVVLG-----IWDYIENKIEVFDGAVIILSLAPMVASTVANGP--
-----RSPWD-AISLIIMLRIWRVKRVIDAYVLPVKVEM-
EMVIQQYEKA---
>106 mus_C15orf27 gi|27370422|
WQVFLLS-----ASLNSFLVACVILVVILLTLELLIDTK-----LLQ-
-----FSN----AFQFAGV----IHWISLVILSVFF
SETVLRIVVLG-----IWDYIENKIEVFDGAVIILSLAPMVASTVANGP--
-----RSPWD-AISLIIMFRIWRVKRVIDAYVLPVKLEM-
EMVTQQYEKA---
>107 rat_C15orf27 gi|157817759|
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-----FSS----AFQFAAV----IHWISLVILSVFF
SETILRIVVLG-----IWDYIENKIEVFDGAVIILSLAPMVASTVANGP--
-----RSPWD-AISLIIMFRIWRVKRVIDAYVLPVKLEM-
EMVTQQYEKA---
>108 ciona_C15orf gi|198433556|
RKILHS-----VAFYIYILISTFIVTLLLLAELLIDVG-----VINI
PSSPDTVVL-----NASALSTLKVQTP----AQKTSTI----LHWISFSFSLSLFF
IEIMFRLYAWK-----LNIIRSIVSVFDCSIVTMAIATNLAATLAAGS--
-----TSPFD-AISLLIILRFIRIHSLIQRCVSDSKQEIR
EKLTKTECS----
>109 methanococcus_hyperpol_Kv_sp_Q57603
KKI-----MEVLSLIFTFEIVASFILSTY-----
-----NPPYQDL----LIKLDYISIMFFT
FEFIYNFYVED-----KAKFFKDIYNIVDAIVVIAFLLYSLQVIFY-SKA--
-----FLGLR-VINLLRILVLLRIIKLRKL-----EEN-
QALIN-----

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>110 ornitho_vsp gi|149635858|
-----LT-----TKTEIFGVSLIFVDVALLIVILVTTTSK-----
-----SIRIPFA-----YRVVSLLIALLFFL
FDVLLRIFAEG-----FRNYFSIKLNILDAFIVVGTLMIDIVYIYVNTG--
-----GVKQIPRLAI-LLRPLRIIILIRIFRLAVQK-----KQL-
EKVTRRMVSENKR
>111 xenopus_t_vsp |gi_62859843|
SPFVMS-----FGFRVFGVVLIIIVDFVLVIVDLSVSTQ-----
-----SSGASTA-----ISSISLSISFFFL
IDVLLHIFVEG-----FRQYFSSKLNIFDAVIVIVTLLVTLVYAF'TDFS--
-----GASNIPRMVN-FLRALRIIILIRILRLASQK-----RQL-
EKVTRRLVSENKR
>112 gallus_vsp gi|118084924|
SPFVMS-----FGFRVFGVVLIIIVDIIIVVIVDLAISEK-----
-----KRGIREI-----LEGVSLAIALFFL
VDVLMRVFVEG-----FKNYFRSCLNLTDAVIVVGTLLINMTYSFSDLA--
-----ATDQMPRMVT-LLRVLRIVILIRIFRLASQK-----KQL-
EVVTRRMVSENKR
>113 danio_vsp gi|70887553|
TPFVMS-----FGFRVFGVVLIIILDIIMVIVDLSLSEK-----
-----SRDVGGA-----PETVSLVISFFFL
IDVLLRVYVEG-----FKVYFSSKLNIVDACIVVITLVVTMIYAFSDFS--
-----GASLIPRVVT-FLRSLRILILVRIFRLASQK-----REL-
EKVTRRMVSENKR
>114 xenopus_vsp gi|148230800|
SPFVMS-----FGFRVFGVVLIIIVDFVLVIVDLSVIDK-----
-----SREATA-----ISSISLAISFFFL
IDVLLHIFVEG-----FRQYFSSKLNIFDAAIVIVTLLVTLVYAF'TDFS--
-----GATNIPRLVN-FLRGLRIIILVRILRLASQK-----RQL-
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-----NIYIPLE-----YRAISLAIALFFL
VDVLLRVYVEG-----RQRYFSDVLTLDVAVVIGVTVLVAVIYTYDKQ--
-----FLRNIPRLAV-LLRPLRLLILVRILQLAHQK-----RQL-
EKLTRQLVSGNKR
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GILVSS-----VAFRIFGIFLVILDVFLVVVDLNVSEK-----
-----KIYIPLD-----YRSISLAIALFFL
VDILLRVSVEG-----RRRYFSDVLTLDVAVVIGVTVVVAVIYALYDKH--
-----FLRDIPRLAV-LLRPLRLLILIRILQLAHQK-----RQL-
ERLTRKLVSGNKR
>117 dog_vsp gi|73993164|
GSSLVSPGHN---TNNRIFGILLIFVDLSLIITDLLFTER-----
-----TMHIPLD-----YRSISLAIALFFF
FDVLLRVYVEG-----IQRYFSDILNYLDAVIVVTLLIDIIYMFYDFK--
-----FLKTIPRLTI-LFRPLRLIILIRVFHLAHQK-----RHL-
EMLTRRMVSGNKR
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HSIVSS-----FAFGIFGVFLVLLDVTLTLLADLIFTDS-----
-----KLYIPLE-----YRSISLAIGLFFL
MDVLLRVFVEG-----RQQYFSDLFNILDTAIIVIPLLVDVIYIFFDIK--
-----LLRNIPRWTH-LVRLRLRIIILIRIFHLLHQK-----RQL-
EKLMRRLVSENKR
>119 homo_vsp_gamma gi|40549435|
HSIVSS-----FAFGLFGVFLVLLDVTLILADLIFTDS-----
-----KLYIPLE-----YRSISLAIALFFL
MDVLLRVFVER-----RQQYFSDLFNILDTAIIVILLVVDVYIYFFDIK--
-----LLRNIPRWTH-LLRLLRLIILIRIFHLFHQK-----RQL-
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>120 ciona_vsp gi|76253898|

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RAVIDH-----LGMRVFGVFLIFLDIILMIIDLSLPGK-----  
-----SESSQSF----YDGMALALSICYFM  
LDLGLRIFAYG-----PKNFFTNPWEVADGLIIVVTFVVTIFYTVLDEY--  
-----VQE--TGADG-LGRLVVLARLLRVVRLARIF-----YSH-  
QQMKASSRRTISQ  
>121 Aeropyrum_Kv_PDB_1ORS_c  
-DVMEH-----PLVELGVSYAALLSVIVVVVEYTMQL-----  
-----SGEYLVR----LYLVDLILVIILW  
ADYAYRAYKSG-----DPAGYVKKTLYEIPALV--PAGLLALIEGHLA----  
-----GLGLFRLVRLLRFLRILLII-----SRG-  
SKFLSAIAD----  
>122 homo_BK_gi|119574982|  
GVMISA-----QTLTGRVLVVLVVFALSIGALVIYFIDSSN-----  
-----PIESC-----QNFYKDF----TLQIDMAFNVFFL  
LYFGLRFIAAN-----DKLWFVLEVNSVVDFFTVPVVFVSVYLNRSWL----  
-----GLRFLRALRLIQFSEILQF-----L-  
NILKTSN---SIK  
>123 mouse_BK_mslo_gi|4639628|  
GVMISA-----QTLTGRVLVVLVVFALSIGALVIYFIDSSN-----  
-----PIESC-----QNFYKDF----TLQIDMAFNVFFL  
LYFGLRFIAAN-----DKLWFVLEVNSVVDFFTVPVVFVSVYLNRSWL----  
-----GLRFLRALRLIQFSEILQF-----L-  
NILKTSN---SIK
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**Supplementary Table S2. Gating kinetics of Asp<sup>112</sup> mutants at pH 5.5//5.5 (mean  $\pm$  s.e. (*n*)).**

	$\tau_{\text{act}}$ at 40 mV (s)	$\tau_{\text{act}}$ at 60 mV (s)	$\tau_{\text{act}}$ at 80 mV (s)	$\tau_{\text{tail}}$ (s)
WT hHv1	1.1 $\pm$ 0.4 (7)	1.4 $\pm$ 0.6 (4)		0.81 $\pm$ 0.07 (6)
D112E	0.2 $\pm$ 0.09 (3)	*0.26 $\pm$ 0.07 (6)	0.28 $\pm$ 0.22 (3)	<sup>†</sup> *6.0 $\pm$ 0.4 (4) 0.069 $\pm$ 0.008 (4)
D112H		2.78 $\pm$ 0.40 (8)	1.19 $\pm$ 0.37 (4)	0.69 $\pm$ 0.06 (14)
D112S		2.76 $\pm$ 0.41 (9)	1.17 $\pm$ 0.17 (5)	*3.35 $\pm$ 0.25 (15)
D112A/A/A		3.03 $\pm$ 0.99 (4)	2.47 $\pm$ 0.18 (4)	*2.46 $\pm$ 0.23 (8)
D112N/A/A		3.36 $\pm$ 0.57 (7)	1.73 $\pm$ 0.07 (5)	*2.46 $\pm$ 0.21 (7)
D112K/A/A		1.06 $\pm$ 0.19 (3)	1.46 $\pm$ 0.29 (6)	*0.18 $\pm$ 0.03 (7)
D112F/A/A		2.22 $\pm$ 0.03 (3)	1.67 $\pm$ 0.23 (8)	*0.028 $\pm$ 0.0005 (6)

Mean  $\pm$  s.e. (*n*) values of  $\tau_{\text{act}}$  at several voltages 5 and  $\tau_{\text{tail}}$  at -40 mV, all at pH 5.5//5.5 at room temperature (21°C). Because the  $g_{\text{H}}-V$  relationship was shifted positively in most D112x mutants, as reported previously<sup>1</sup>, fittable currents were not present at all voltages. <sup>†</sup>Tail currents in D112E had two components that were measured at -60 mV, because at -40 mV channel closing was impracticably slow. For all mutants  $\tau_{\text{tail}}$  differed significantly from WT ( $p < 0.0001$ ) by Student's t-test. The time constant of activation ( $\tau_{\text{act}}$ ) of proton current during depolarizing pulses to +60 mV was obtained by fitting the current with a single rising exponential. The deactivation (channel closing, tail current) time constant ( $\tau_{\text{tail}}$ ) was obtained by fitting the tail current upon repolarization to a single decaying exponential. The D112E mutant had fast and slow components of tail current decay; we give the time constant of the dominant slower component. \*Differs significantly from WT at  $p < 0.05$  ( $\tau_{\text{act}}$ ) by Student's t-test.

#### References

1. Ramsey, I. S. *et al.* An aqueous H<sup>+</sup> permeation pathway in the voltage-gated proton channel Hv1. *Nat. Struct. Mol. Biol.* **17**, 869-875 (2010).

**Supplementary Table S3. Comparison of relative permeability values obtained from the GHK equation assuming  $\text{OH}^-$  or  $\text{H}^+$  permeation in D112x mutants.**

	$V_{\text{rev}}$ pH 7.0 (mV)	$V_{\text{rev}}$ pH 5.5 $\text{Cl}^-$ (mV)	$P_{\text{CH}_3\text{SO}_3}/P_{\text{Cl}}$ ( $P_{\text{H}} = 0$ )	$P_{\text{OH}}/P_{\text{Cl}}$ ( $P_{\text{H}} = 0$ )	$P_{\text{CH}_3\text{SO}_3}/P_{\text{Cl}}$ ( $P_{\text{OH}} = 0$ )	$P_{\text{H}}/P_{\text{Cl}}$ ( $P_{\text{OH}} = 0$ )
WT hH <sub>v</sub> 1	-80.9	-0.3	-	-	-	-
D112E	-80.7	-0.1	-	-	-	-
D112H	-58.4	-37.5	0.14	$3.0 \times 10^6$	0	$1.6 \times 10^4$
D112K	-51.7	-20.6	0.35	$4.6 \times 10^6$	0.04	$2.6 \times 10^4$
D112N	-12.5	-33.1	0.23	$0.3 \times 10^6$	0.13	$0.5 \times 10^4$
D112S	-25.9	-40.8	0.15	$0.5 \times 10^6$	0.04	$0.6 \times 10^4$
D112A	-33.8	-28.8	0.27	$1.4 \times 10^6$	0.07	$1.3 \times 10^4$
D112F	-36.4	-33.5	0.20	$1.2 \times 10^6$	0.04	$1.1 \times 10^4$

Column 2 is the mean change in  $V_{\text{rev}}$  when  $\text{pH}_o$  was changed from 5.5 to 7.0, in  $\text{CH}_3\text{SO}_3^-$  solutions (Fig. 2b). The third column is the change in  $V_{\text{rev}}$  measured when  $\text{Cl}^-$  replaced  $\text{CH}_3\text{SO}_3^-$  at  $\text{pH}_o$  5.5 (Fig. 3g). Columns 4 and 5 show  $P_{\text{CH}_3\text{SO}_3}/P_{\text{Cl}}$  and  $P_{\text{OH}}/P_{\text{Cl}}$  values obtained from the GHK equation (Eq. 1) by fitting the data in columns 2 and 3, assuming that the shift in  $V_{\text{rev}}$  in Column 2 is due mainly to  $\text{OH}^-$  permeation ( $P_{\text{H}} = 0$ ). Columns 6 and 7 show analogous results, but assuming that the shift in  $V_{\text{rev}}$  in Column 2 is due mainly to  $\text{H}^+$  permeation ( $P_{\text{OH}} = 0$ ).

Although the data can be fitted assuming that either  $\text{H}^+$  or  $\text{OH}^-$  is permeant, distinct predictions apply to sucrose dilution experiments at different  $\text{pH}_o$ . Sucrose effects should be larger at  $\text{pH}_o$  5.5 than 7.0 if  $\text{OH}^-$  is permeant, because  $[\text{OH}^-]$  is 32 times larger at  $\text{pH}_o$  7.0, and  $[\text{OH}^-]$  remains constant as other anions are diluted. The term  $P_{\text{OH}}[\text{OH}^-]_o$  (in Eq. 1) will have a greater effect on  $V_{\text{rev}}$  at  $\text{pH}_o$  7.0, and because  $E_{\text{OH}}$  does not change,  $V_{\text{rev}}$  will change less.



Consistent with  $\text{OH}^-$  permeability, sucrose produced a larger shift of  $V_{\text{rev}}$  at  $\text{pH}_o$  5.5 than at  $\text{pH}_o$  7.0 for all mutants except D112K (Fig. 3e & 3f). By similar reasoning, substituting  $\text{Cl}^-$  for  $\text{CH}_3\text{SO}_3^-$  should shift  $V_{\text{rev}}$  more at  $\text{pH}_o$  5.5 than at  $\text{pH}_o$  7.0 if  $\text{OH}^-$  is permeant. Fig. 3g shows that this occurred in all six anion selective mutants. The data consistently point to a high  $\text{OH}^-$  permeability in D112x mutants.

Outward  $\text{H}^+$  flux and inward  $\text{OH}^-$  flux both likely occur by a Grotthuss mechanism in a single-file channel, a more efficient permeation mechanism than diffusion used by other ions.  $\text{H}^+$  moves in water by hopping from  $\text{H}_3\text{O}^+$  to  $\text{H}_2\text{O}$ , whereas  $\text{OH}^-$  conduction occurs when  $\text{OH}^-$  extracts a proton from a nearby  $\text{H}_2\text{O}$ . The latter process involves proton transfer between neutral and negatively charged species, and thus seems more probable for an anion selective channel. Although we cannot rule out the possibility that  $\text{H}^+$  carries some current, the sucrose dilution results indicate a distinct preference for anions, suggesting that  $\text{OH}^-$  permeation is more likely than  $\text{H}^+$  permeation in the D112x mutant channels.

That WT  $\text{hH}_v1$  conducts  $\text{H}^+$  rather than  $\text{OH}^-$  is based mainly on the unitary conductance increasing at low  $\text{pH}_i$ . Lowering  $\text{pH}_i$  from 6.5 to 5.5 to 5.0 to 4.1 increased the conductance from 37 to 139 to 220 to 400 pS, respectively<sup>1</sup>. In contrast, changing  $\text{pH}_o$  from 7.5 to 6.5 had no effect. These results are consistent with increased conductance by increasing permeant ion concentration  $[\text{H}^+]$  on the proximal side of the membrane. That deuterium reduced the conductance by 50% also supports  $\text{H}^+$  permeation<sup>2</sup> through WT  $\text{H}_v1$  channels.

## References

1. Cherny, V. V., Murphy, R., Sokolov, V., Levis, R. A. & DeCoursey, T. E. Properties of single voltage-gated proton channels in human eosinophils estimated by noise analysis and by direct measurement. *J Gen Physiol* **121**, 615-28 (2003).
2. DeCoursey, T. E. & Cherny, V. V. Deuterium isotope effects on permeation and gating of proton channels in rat alveolar epithelium. *J Gen Physiol* **109**, 415-34 (1997).

**Supplementary Table S4. Monovalent cation substitution does not change  $V_{\text{rev}}$  in anion selective Asp<sup>112</sup> mutant channels.**

<b>Mutant</b>	<b>Ion</b>	<b>Raw <math>\Delta V_{\text{rev}}</math> (mV)</b>	<b><math>V_{\text{jet}}</math> correction (mV)</b>	<b>Corrected <math>\Delta V_{\text{rev}}</math> (mV)</b>
Various	Na <sup>+</sup>	+0.5 ± 0.6 (6)	+1.3	+1.8
D112N/A/A	Na <sup>+</sup>	-1.8 ± 0.6 (4)	+1.3	-0.5
D112N/A/A	TEA <sup>+</sup>	+1.8 ± 0.6 (6)	-3.4	-1.6
D112N/A/A	K <sup>+</sup>	-5.3 ± 1.7 (4)	+4.8	-0.5
D112N/A/A	NMDG <sup>+</sup>	+6.6 ± 0.6 (4)	-6.0	+0.6

Mean ± s.e. (*n*) values of the change in  $V_{\text{rev}}$  relative to standard TMA<sup>+</sup> solution, when TMA<sup>+</sup> was replaced by the indicated cation (all at ~130 mM), all at pH 5.5//5.5 at room temperature (21°C). TEA<sup>+</sup> is tetraethylammonium<sup>+</sup>, NMDG<sup>+</sup> is N-methyl-D-glucamine<sup>+</sup>. Many COS-7 cells had endogenous K<sup>+</sup> conductances that prevented  $V_{\text{rev}}$  measurement. “Various” includes 1 D112H, 1 D112A, 1 D112S, and 3 D112N/D185M cells. The raw measured values are given (column 2), the correction for measured liquid junction potential differences (Column 3), and the corrected  $V_{\text{rev}}$  values (Column 4). The net values are of the same magnitude as the junction potential corrections, and well within the error of the measurements.