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## ***De novo mutations in HCN1 cause early infantile epileptic encephalopathy***

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## Summary

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels contribute to cationic I<sub>h</sub> current in neurons and regulate the excitability of neuronal networks. Studies in rodent models have shown that the *Hcn1* gene plays a key role in epilepsy, but clinical evidence implicating *HCNI* mutations in human epilepsy is lacking. We carried out exome sequencing for parent-offspring trios with fever-sensitive intractable epileptic encephalopathy, leading to the discovery of two *de novo* missense *HCNI* mutations. The screening of follow-up cohorts comprising 157 patients in total identified four additional amino-acid substitutions. Patch-clamp recordings of I<sub>h</sub> currents in cells expressing WT or mutant human HCN1 channels revealed that the mutations had striking, but divergent effects on homomeric channels. Patients with mutations had clinical features resembling those of Dravet syndrome, with progression toward atypical absences, intellectual disability and autistic traits. These findings provide clear evidence that *de novo* *HCNI* point mutations cause a recognizable early-onset epileptic encephalopathy in humans.

Early infantile epileptic encephalopathies (EIEEs) are mostly sporadic disorders characterized by recurrent seizures during the neonatal or infantile periods, with impaired cognitive and motor development. At least 15 different genetically determined forms of EIEE have been recognized<sup>1,2</sup>. EIEEs typically result from *de novo* dominant mutations in a single autosomal gene, although autosomal recessive and X-linked forms also exist. Dravet syndrome, an intractable epilepsy generally occurring during the first year of life, is the prototype condition: seizures are initially febrile and prolonged, and polymorphic afebrile seizures appear later in the course of the disease. Cognitive and motor development progressively slows, progressing toward intellectual disability (ID)<sup>3</sup>. Dravet syndrome is mostly caused by *de novo* mutations of *SCN1A*, encoding the neuronal voltage-gated sodium  $\alpha$ 1 (Na<sub>v</sub>1.1) channel<sup>4,5</sup>. This syndrome overlaps clinically with *PCDH19*-related epilepsy, an X-linked disorder also associating febrile and afebrile seizures and variable degrees of ID, but expressed only in heterozygous females<sup>6,7</sup>.

In this study, we carried out whole-exome sequencing on 39 parent-offspring trios including probands with EIEE resembling Dravet syndrome, without *SCN1A* and *PCDH19* mutations. Informed consent was obtained from the families and genetic studies were approved by local ethics committees. The analysis of exome data revealed heterozygous *de novo* mutations (c.299C>T, p.Ser100Phe and c.1201G>C, p.Asp401His) of *HCNI* (NM\_021072.2) in two female probands, one French and the other Italian. We then screened 95 additional patients with fever-sensitive EIEE (French cohort) for mutations in *HCNI* coding regions, by amplicon-based pyrosequencing. Three female patients were each found to have a previously unknown nonsynonymous variant (c.140G>T, p.Gly47Val; c.814T>C, p.Ser272Pro; c.890G>C, p.Arg297Thr) in the heterozygous state. The sequencing of available parental DNA showed that p.Ser272Pro and p.Arg297Thr also occurred *de novo*. In parallel, the sequencing of several genes, including *HCNI*, in a Dutch follow-up cohort comprising 62 patients identified the *de novo* c.835C>T (p.His279Tyr) substitution in a male patient. In total, six different heterozygous missense mutations, all confirmed by Sanger sequencing and absent from databases (HapMap, 1000 Genomes, dbSNP137, Exome variant server) were identified in *HCNI* (Fig. 1a). *De novo* occurrence was confirmed for the five mutations for which inheritance could be investigated. The pathogenicity of Gly47Val remained uncertain due to the unavailability of parental DNA.

All patients had similar clinical features including seizures beginning at ages of 4 to 13 months and the combination of febrile and afebrile polymorphic seizures, including hemiclonic and generalized seizures. These features were initially suggestive of Dravet syndrome, but with

a different progression over time. Atypical absences, with or without myoclonic jerks, and focal seizures became predominant in the oldest patients. All patients had mild to severe ID and major behavioral disturbances, including autistic traits (Table 1, Supplementary Table 1).

*HCN1* tolerates little functional variation, as only a few missense variants are present in control populations (Supplementary Fig. 1, Supplementary Table 2). Copy number variants (CNV) encompassing *HCN1* exons have been reported in healthy and intellectually disabled individuals, at similar low frequencies (Supplementary Table 3)<sup>8-10</sup>. We recently identified a deletion spanning exon 4 of *HCN1* in a female with sporadic ID and autism spectrum disorder (ASD) but no epilepsy<sup>11</sup>. Further analyses showed that this deletion was inherited from her asymptomatic father (Supplementary Fig. 2). No second pathogenic mutation was found in the proband, so an autosomal recessive disorder was unlikely. Intragenic *HCN1* deletions alone therefore seem to be insufficient to cause ID and ASD, although they possibly contribute to these disabilities. HCN1 dysfunction may therefore result in a spectrum of phenotypes, ranging from haploinsufficiency as an inherited risk factor for neurodevelopmental disorders to *de novo* mutations causing EIEE.

*HCN1* is one of four genes encoding hyperpolarization-activated cyclic nucleotide-gated channels (HCN) with different biophysical properties, expressed in the heart and brain<sup>12-14</sup>. In neurons, HCN1 is mainly localized in dendrites<sup>15</sup>. HCN subunits have six transmembrane domains and functional channels consist of four subunits. HCN channels are permeable to Na<sup>+</sup> and K<sup>+</sup> ions and are activated by membrane hyperpolarization. In the brain, they conduct the I<sub>h</sub> current, which contributes to spontaneous rhythmic activity and the stabilization of neuronal membrane potential against excitatory or inhibitory inputs<sup>16</sup>. The mutations identified here affect strongly conserved amino acids, except for glycine 47 and, to a lesser extent, serine 100, which are located in less well conserved domains of HCN1 (Supplementary Fig. 3). The amino acids affected are located in different parts of the channel, but all are intracellular, and four are located close to domains forming the channel pore (Fig. 1b), in particular in the S4-S5 linker, involved in voltage-dependent gating<sup>14</sup>.

We assessed the functional consequences of *de novo* mutations identified in EIEE patients, by carrying out patch-clamp recordings on CHO-K1 cells expressing WT or mutant human HCN1 channels. Voltage-dependent, slowly activating inward currents were recorded in cells expressing the WT, S100F, H279Y and D401H HCN1 proteins, consistent with the expression of functional channels (Fig. 2a and 2b). These three mutations had major effects on channel gating (Fig. 2c). The half-activation voltage (V<sub>1/2</sub>) for the S100F and H279Y channels was depolarized by ~27 mV and ~17 mV, respectively, with respect to the WT HCN1. D401H HCN1 was activated at higher positive voltages, due to a 46 mV shift of the activation curve. The S100F and D401H mutations also resulted in significantly faster activation than for WT HCN1 (Fig. 2d). Furthermore, all three mutations displayed a slower deactivation compared to the WT channel (Fig. 2e). Finally, the S100F mutation also significantly shifted the reversal potential to negative voltage (Supplementary Fig. 4). Altogether, these results indicate that S100F, H279Y and D401H lead to a gain of function (GoF) of homotetrameric HCN1 channels. By contrast, no I<sub>h</sub> current was recorded for cells expressing the S272P and R297T channels (Fig. 2a, Supplementary Fig. 5). These mutant channels were present in lower amounts in cell lysates and at the plasma membrane compared to the WT channels, but similar reduced protein expression, probably due to the instability of the mutated proteins, was also observed for other mutants retaining substantial channel activity, such as S100F (Supplementary Fig. 6), indicating that the loss of function (LoF) were specific to these amino acid changes.

To gain further insights into the mechanisms by which mutations with apparent divergent impacts cause similar phenotypes and to mimic the heterozygous state of the mutations in the patients, we performed co-expression of WT and mutant HCN1 proteins. Strikingly, R297T HCN1 and, to a lesser extent, S100F and S272P HCN1 but not D401H showed a dominant

negative effect on the WT form, by decreasing the current density of heteromeric channels (Fig. 3). These results support the hypothesis that the *de novo* HCN1 mutations identified in this study mostly lead to GoF or dominant negative effects rather than LoF.

Ih current regulates neuronal excitability and the dendritic integration of synaptic potentials in individual neurons and neuronal networks<sup>13,14,17,18</sup>. HCN1 and HCN2 are the main HCN isoforms expressed in the brain and contributing to this current. HCN1 is predominantly expressed in the neocortex and hippocampus, whereas HCN2 is expressed more evenly, but slightly more strongly in the thalamus than elsewhere<sup>19,20</sup>. Acquired Ih current dysfunction, due to abnormal *Hcn1* expression or distribution in particular, has been shown to play a crucial role in epileptogenic processes in rodents<sup>14,16,21,22</sup>. *HCN1* defects were then predicted to contribute to epilepsy in humans as well, and *HCN1* was screened for mutations in patients with idiopathic generalized epilepsy (IGE)<sup>23,24</sup>. Our results finally provide the first evidence implicating *HCN1* mutations in human epilepsy and show that the associated phenotype is more severe than in the patients with IGE that were previously screened.

Functional studies confirmed that the *de novo* mutations had major impacts on HCN1 function and mostly lead to GoF. However, the precise mechanisms by which mutations cause EIEE in humans remain to be clarified. The observations for LoF mutations were consistent with previous findings describing a decrease in *HCN1* expression occurring very early in epileptogenesis in seizure-induced or spontaneous epileptic rat models<sup>25-29</sup>, and an upregulation of HCN channel function by some antiepileptic drugs<sup>30,31</sup>. However, rare heterozygous deletions encompassing *HCN1* exons exist in non-epileptic individuals, and *Hcn1*<sup>-/-</sup> mice have motor learning and memory deficits<sup>32</sup> and a higher susceptibility to induced seizures<sup>33,34</sup>, but display no spontaneous seizures<sup>32</sup>. These observations suggest that *HCN1* haploinsufficiency, in contrast to point mutations altering channel function, can be functionally tolerated by the developing brain and that HCN1 deficiency promotes neuronal excitability but is insufficient *per se* for seizure development. Proteins with amino acid substitutions are predicted to be present in the cells of patients, with the HCN subunits assembling into functional homo- or heterotetramers<sup>13</sup>. Thus, missense mutations may have dominant negative effects, interfering with the function of the remaining *HCN1* allele, as demonstrated for R297T and S272P, but also with that of *HCN2* in neurons in which they are co-expressed. Consistent with this hypothesis, *Hcn2*<sup>-/-</sup> mice display spontaneous absence seizures<sup>35,36</sup>, and a recessive loss-of-function mutation in *HCN2* was recently identified in one patient with IGE<sup>37</sup>. Alternatively, both increases and decreases in Ih current may be pathological, since both the downregulation and upregulation of *HCN1* expression have been reported, depending on the epileptic rat model<sup>13,22</sup>. Finally, *HCN1* mutations may have opposite (*i.e.* LoF or GoF) effects depending on the physiological context and cells in which they are expressed, as observed for Nav1.1 and Nav1.7<sup>38,39</sup>.

In conclusion, this study provides further evidence of the crucial role of HCN1 and Ih current in human epilepsies. The phenotype of patients with *HCN1*-related encephalopathy, with initial fever-sensitive seizures progressing toward predominant absences and focal seizures, is consistent with HCN1 function and expression, and previous observations of HCN1 channelopathy in animal models of focal or absence epilepsies.

#### URLs.

HapMap, <http://hapmap.ncbi.nlm.nih.gov/>; 1000 Genomes, <http://www.1000genomes.org/>; dbSNP, <http://www.ncbi.nlm.nih.gov/SNP/>; Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (<http://evs.gs.washington.edu/EVS/>), <http://evs.gs.washington.edu/EVS/>; SIFT, <http://sift.jcvi.org/>; PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>.

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## AUTHOR CONTRIBUTIONS

**Clinical and genetic data.** *French cohort:* C.De. and C.N. analyzed whole-exome sequencing data. C.N., A.Ra., D.A., D.B., Y.M., C.V., N.E.H., and E.S. contributed to pyrosequencing and/or Sanger sequencing and sequence analysis. B.K. and C.N. contributed to CNV analysis. O.T. performed qPCR. C.C., D.V., R.N. and E.Ra. phenotyped and sampled the patients and provided clinical information. E.Ro. was involved in patient selection. A.B., A.N. and S.L. contributed to variant analysis in populations from the IPDGC. S.B, M.B. and A.B contributed to study design and discussions. C.De. and E.L.G. supervised the projects related to EIEE, including cohort collection. C.De, E.L.G. and T.H designed this study. *Dutch cohort:* B.P.C.K and C.G.F.K designed the study and analyzed sequencing data. B.P.C.K. supervised the study. E.H.B. phenotyped and sampled the patients. *Italian patient:* This patient was included in the EuroEPINOMICS RES consortium. A.S., I.H. and P.G. analyzed whole-exome sequencing data. P.S, A.Ro. and F.Z. contributed to validation of exome sequencing data and supervision of the study. G.G. phenotyped the patient and provided clinical information. P.D.J., I.H., A.S., S.W., and A-E.L. designed the study and/or coordinated projects in the EuroEPINOMICS RES Consortium. **Functional studies** C.N. and A.Ra. performed the site-directed mutagenesis, cell transfections, immunohistochemistry and plasma membrane enrichment experiments. C.Da. performed the electrophysiology analysis and wrote the sections relating to electrophysiology. C.De supervised the collaborative study and drafted the manuscript. All authors critically revised the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors have no competing financial interests to declare.

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## ONLINE METHODS

### Patients

We selected nine trios from the French cohort and 30 from the EuroEPINOMICS RES Consortium for exome sequencing. The inclusion criteria were: normal development before seizure onset, the presence of both febrile and afebrile seizures; the occurrence of polymorphic seizures (tonic-clonic, hemiconic, myoclonic, absence and/or focal seizures); drug-resistant seizures or *status epilepticus*; developmental delay after seizure onset. All probands had tested negative for *SCN1A* mutations by sequencing and MLPA or Multiplex Amplicon Quantification (MAQ). The follow-up cohorts included 95 additional patients (41 male and 54 female patients) with fever-sensitive epileptic encephalopathy referred for early genetic testing of Dravet syndrome (La Pitié-Salpêtrière, Paris, France) and 62 patients with refractory epileptic encephalopathy and seizure onset within the first four years of age selected from the Dutch cohort (Utrecht, the Netherlands). Informed written consent was obtained from each individual or his/her parents before blood sampling. All experiments were performed in accordance with French guidelines and legislation.

### Whole exome sequencing

Exome sequencing for patients N06 0565 and DRA-20 and their unaffected parents was performed by IntegraGen (Evry, France) or the Wellcome Trust Sanger Institute (Hinxton/Cambridge, UK), respectively, as described previously<sup>40,41</sup>. Exons were captured from fragmented genomic DNA samples using the SureSelect Human All Exon 50Mb (Agilent Technologies) exome kit, and paired-end 75-base massively parallel sequencing was carried out on an Illumina HiSeq2000, according to manufacturers' protocols.

### Analysis of whole exome data

Bioinformatics analyses were respectively done using the in-house pipeline developed by Integragen SA, as previously described<sup>40,41</sup>, or as follows: sequencing reads passing quality filtering were aligned to the human reference genome (hg19) with Burrows-Wheeler Aligner (BWA)<sup>42</sup>. GATK<sup>43</sup> was used to recalibrate base quality scores, realign around indels, and mark duplicate reads. Independent variant calling was performed on the mapped reads with SAMtools<sup>44</sup> mpileup, GATK UnifiedGenotyper and Dindel<sup>45</sup>. For annotating, comparing and filtering the data the GenomeComb<sup>46</sup> program was used. For the calling of *de novo* variants the DeNovoGear<sup>47</sup> program by Conrad and colleagues was used and double-checked by GenomeComb analysis.

### Screening of *HCNI* by pyrosequencing

Exons and intron-exon junctions of *HCNI* (NM\_021072.2) were analyzed by universal tailed amplicon sequencing (454 GS Junior System, Roche), with the exception of the amplicons 3 of exon 8 (8-3), which was screened by Sanger sequencing. Primer pairs (Supplementary Table 4) were designed for exon amplification by PCR; a second PCR was performed to incorporate a multiplex identifier and 454 adaptors, and emulsion PCR was carried out as described in the emPCR Amplification Method Manual (Roche).

### Screening of candidate genes including *HCNI* by targeted capture and sequencing

Target enrichment of 340 selected candidate genes, including known epileptic encephalopathy genes and brain-expressed ion channels, was performed using glass slides (Agilent). Barcoded fragment libraries were equimolarly pooled and enriched using multiplexed targeted genomic enrichment. Sequencing was done on a SOLiD 5500 (Applied Biosystems). Alignment of reads on the human reference was performed with BWA, and additional bioinformatics steps including filtering for novel coding variants, were done using an in-house pipeline.

### **Sanger sequencing**

Mutations identified by exome sequencing and pyrosequencing were validated by Sanger sequencing with the same primers as for pyrosequencing. Mutations found in patients were directly searched for in available parents by sequencing the corresponding exon. The amplicon 8-3, not covered by pyrosequencing, and exons covered less than 5X by pyrosequencing in some patients were screened by Sanger sequencing. Sequencing reactions were performed on G-50-purified PCR products, with the the BigDye Terminator kit. Sequencing products were run on an ABI Prism 3730 DNA Analyzer (Applied Biosystems) and the sequences obtained were analyzed with SeqScape 2.6 software (Applied Biosystems).

### **Parental testing**

Parental testing was carried out with the AmpFI STR SGM plus Kit (Applied Biosystems), to exclude false paternity and DNA inversion, and to check that the mutation had occurred *de novo*.

### ***In silico* analyses**

The effects of mutations were interpreted with Alamut 2.2 (Interactive Biosoftware). The effects of predicted amino-acid substitutions were assessed with SIFT (<http://sift.jcvi.org/>) and PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>).

Protein sequences of orthologs and paralogs of human HCN1 were retrieved from Uniprot (<http://www.uniprot.org/uniprot/>) and aligned using slow/accurate pairwise alignment defaults parameters with Clustalw (<http://www.genome.jp/tools/clustalw/>).

### **HCN1 expression plasmids**

The plasmid containing the human *HCN1* cDNA was kindly provided by Dr. Juliane Stieber (Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany). The S100F, S272P, R297T, H279Y and D401H mutations were introduced into the cDNA with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). All constructs were sequenced to ensure that no additional mutations were introduced.

### **Cell culture and transfection**

CHO-K1 cells (CCL-61, ATCC) were cultured in HAM's F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were transiently cotransfected with cDNAs encoding the WT or a mutant human HCN1 (hHCN1) channel and pEGFP, with the Neon<sup>TM</sup> transfection system (Invitrogen), according to the manufacturer's instructions (medium without antibiotic): 1 µg of plasmid cDNA per dish was used for cotransfection, at a ratio of 50:1 (HCN1:pEGFP) for test experiments or with pEGFP alone for control experiments. For coexpression experiments, an equal ratio of WT and mutant (0.5µg of each plasmid) was used in the same conditions. Cells were plated at a density of  $5.0 \times 10^5$  cells / 40-mm Petri dish for electrophysiological experiments and  $2.0 \times 10^6$  cells / 100-mm Petri dish for western blot analysis. Cells were incubated for 24 h at 37°C, under an atmosphere containing 5% CO<sub>2</sub>, before use.

### **Patch-clamp technique**

Currents were recorded from GFP-expressing cells, with an Axopatch 200B amplifier and a Digidata 1440 analog/digital interface. Pipette resistance was 2-3 MΩ when filled with the intracellular solution containing 120 mM K aspartate, 10 mM NaCl, 10 mM KCl, 1 mM CaCl<sub>2</sub>,

10 mM EGTA, 2 mM MgATP and 10 mM HEPES, adjusted to pH 7.2 with KOH. The extracellular solution contained 130 mM NaCl, 15 mM KCl, 0.5 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 10 mM glucose and 5 mM HEPES adjusted to pH 7.4 with NaOH. Series resistance was typically between 3 and 5 MΩ. Capacitive currents were cancelled and series resistance was compensated by 70 to 80 %. The liquid junction potential was not corrected. Whole-cell currents were low-pass Bessel-filtered at 1 kHz and digitized at 10 kHz. Data were acquired and analyzed with pClamp10 software. Electrophysiological recordings were carried out at room temperature (21-23°C). Whole-cell voltage-clamp mode was used to investigate the rate and voltage-dependence of channel activation. A series of test pulses, ranging from -140 to +40 mV in 10 mV increments from a holding potential of -20 mV, was applied to the cells for 1 second. Currents were measured in steady state at the end of the test pulses and were normalized with respect to cell capacitance. To obtain voltage-dependent activation curves, tail currents were measured at the fixed voltage of +50 mV immediately after each test pulse, normalized to maximum amplitude tail currents and then plotted against test voltage. Activation curves were fitted with a Boltzmann function:

$$I_t = I_t(\max) / [1 + \exp (V - V_{1/2})/k],$$

where  $I_t$  is the current amplitude of the tail current recorded for a given pre-pulse and  $I_t(\max)$  is the maximum current amplitude of the tail current,  $V$  is voltage of the pre-pulse,  $V_{1/2}$  is the half activation voltage and  $k$  is the slope factor.

Activation and deactivation time constant were obtained by fitting with a monoexponential function the steady-state current obtained on hyperpolarization at -110mV and the tail current at +10 mV following a conditioning prepulse at -130mV, respectively. Some currents presenting complex kinetic behaviour were not used for analysis. Analysis of raw data, graphical representations and statistical tests were performed with Origin software (OriginLab Corporation), using one-way ANOVA followed by Dunnett's *post hoc* test or two-way ANOVA followed by Bonferroni's post-hoc test for multiple comparison. The significance level was set at  $p < 0.05$ . All data are reported as means  $\pm$  SEM.

#### **Additional references**

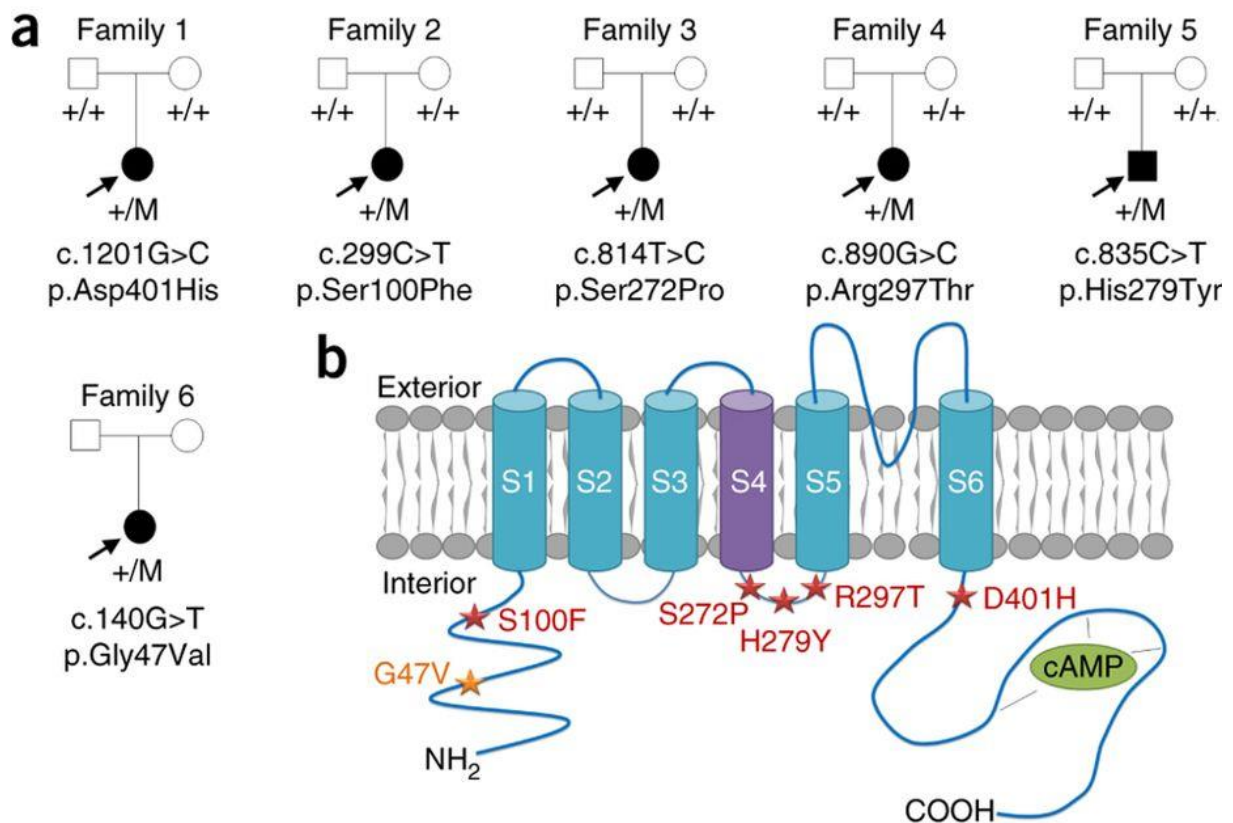
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**Table 1 Genetic and clinical characteristics of the patients with *HCNI* mutations identified in this study**

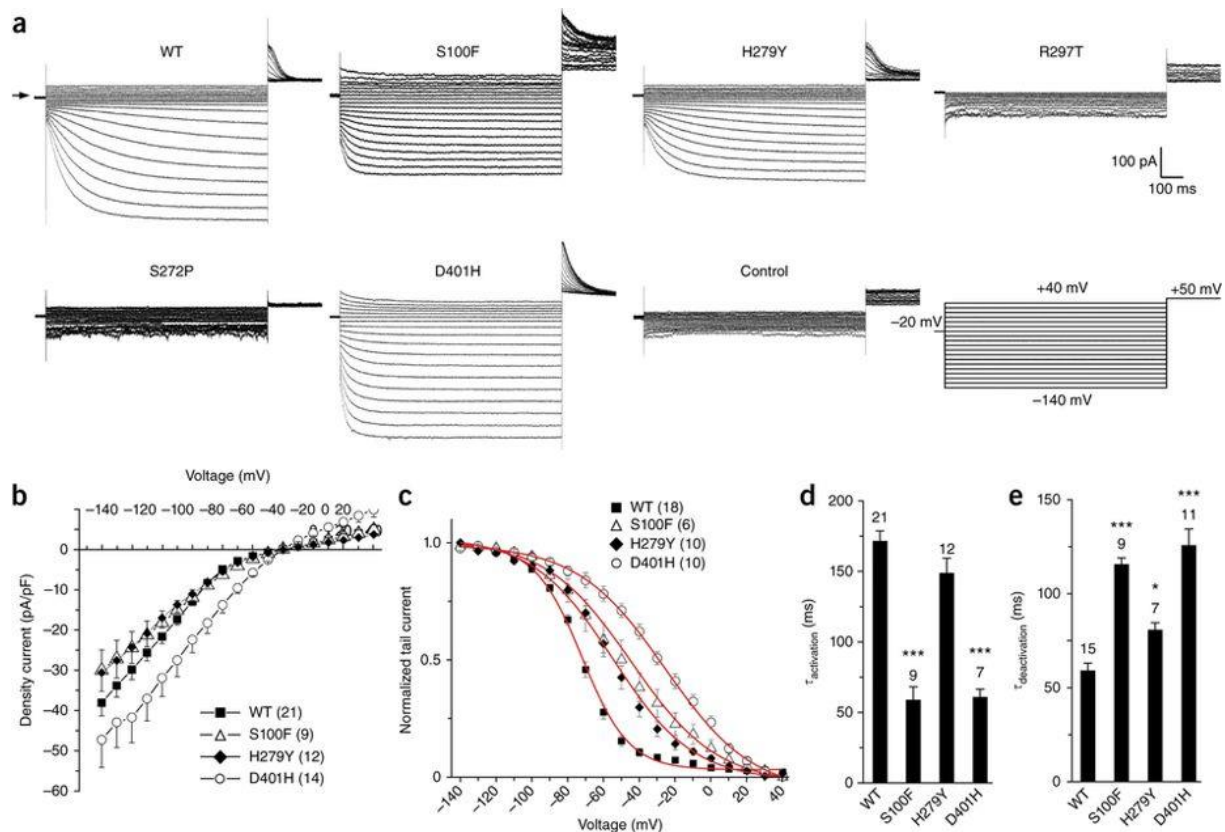
Family number	6	2	3	5	4	1
Patient origin	France	Italy	France	The Netherlands	France	France
Sex	Female	Female	Female	Male	Female	Female
Base change	c.140G>T	c.299C>T	c.814T>C	c.835C>T	c.890G>C	c.1201G>C
Amino acid change	p.Gly47Val	p.Ser100Phe	p.Ser272Pro	p.His279Tyr	p.Arg297Thr	p.Asp401His
Exon	1	1	2	2	3	4
Inheritance	?	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>
Current age (years)	13	6	16	12	15	18
Age at seizure onset (months)	7	10	8	13	8	4
Seizure types	FS, TCS, absences, focal, myoclonic seizures	FS, TCS, CS, absences, focal, myoclonic seizures	FS, CS, focal seizures, absences	FS (atypical), CS, TCS, absences, myoclonic seizures	FS, focal seizures, absences	FS, TCS, absences, focal, myoclonic seizures
<i>Status epilepticus</i>	yes	no	yes	no	yes	yes
ID	Moderate/severe	Moderate	Severe	Mild	Moderate/severe	Moderate/severe
MRI	NA	Normal	Normal	Normal	Normal	Normal
Pharmacoresistance	yes	yes	yes	yes	yes	yes
Behavior and language	Absence of language	Autistic features	Behavioral disturbances, autistic features	Behavioral disturbances; ADHD	Behavioral disturbances, autistic features	Behavioral disturbances, autistic features
Other features	Ataxia	None	Motor delay	Truncal ataxia	None	Polyphagia

FS: febrile seizures, TCS: tonic-clonic seizures, CS: clonic seizures, ID: intellectual disability, ADHD: attention deficit hyperactivity disorder; NA: unavailable

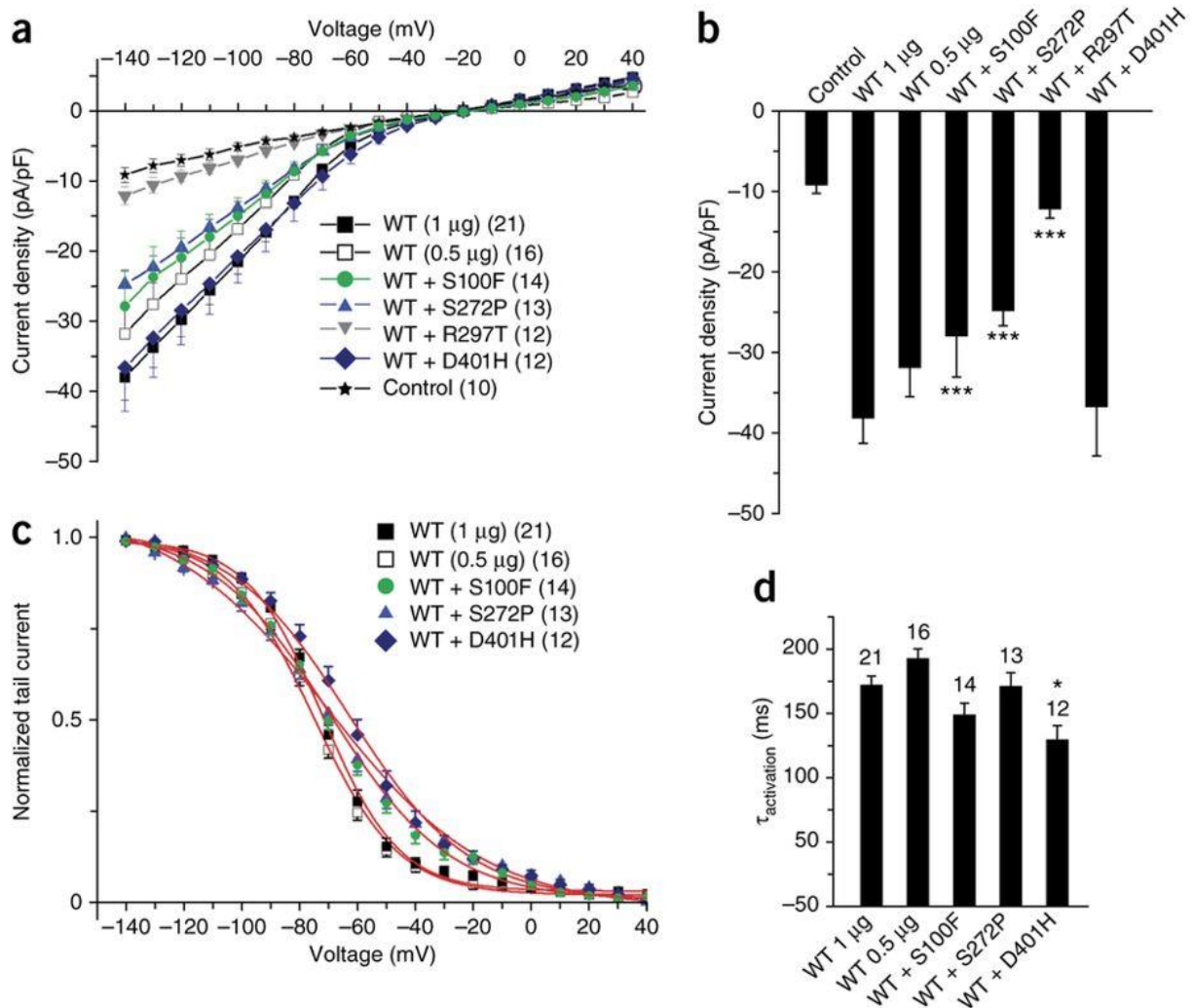
## FIGURE LEGENDS



**Figure 1** Identification of *HCN1* missense mutations. **(a)** Pedigrees and segregation analysis of the six *HCN1* missense variants identified in this study. The arrows indicate the probands. p.Ser100Phe, p.Ser272Pro, p.Arg297Thr, p.His279Tyr and p.Asp401His were absent from both parents, indicating that they occurred *de novo* in the probands. Segregation analysis could not be performed for the p.Gly47Val variant, because parental DNA samples were not available. **(b)** Schematic diagram of the HCN1 channel, showing the location of the amino acids affected by the missense mutations identified in this study (stars).



**Figure 2** Patch-clamp analysis of the functional impact of the *de novo* hHCN1 mutations. **(a)** Representative traces of whole-cell currents recorded from CHO-K1 cells transfected with pEGFP, reflecting the endogenous current (control), WT, S100F, H279Y, R297T, S272P or D401H hHCN1 channels. The arrow indicates the zero current level. **(b)** Plot of mean current density as a function of test voltage for WT, S100F, H279Y and D401H hHCN1 channels. Current densities did not differ significantly between the WT and mutant channels ( $p > 0.05$ ), except for the D401H mutant (two-way ANOVA,  $* p < 0.05$  for voltage  $-140$  mV to  $-60$  mV). **(c)** Mean tail current activation curve for the WT, S100F, H279Y and D401H hHCN1 channels. Red lines show fits of a Boltzmann function providing the half-activation voltage  $V_{1/2}$  and slope factor ( $k$ ) for the WT:  $-72.06 \pm 0.73$  mV,  $k = 13.1 \pm 0.5$  mV, S100F:  $-44.59 \pm 1.91$  mV,  $k = 23.9 \pm 1.3$  mV; H279Y:  $-54.76 \pm 1.49$  mV,  $k = 22.4 \pm 1.1$  mV; D401H:  $-25.54 \pm 0.96$  mV,  $k = 24.5 \pm 0.8$  mV. **(d)** The D401H and S100F mutant channels had higher activation time constants than the WT hHCN1 channel (one-way ANOVA,  $*** p < 0.001$ ). **(e)** The three mutant channels displayed enhanced deactivation time constants compared with those of the WT hHCN1 (one-way ANOVA,  $* p < 0.05$  and  $*** p < 0.001$ ). Data are presented as means  $\pm$  SEM, with the numbers of experiments indicated in brackets and SEM indicated by error bars.



**Figure 3** Data analysis of co-expression of WT and mutant hHCN1 channels with the patch clamp technique. **(a)** Plot of mean current density as a function of test voltage for CHO-K1 cells transfected with pEGFP, reflecting endogenous current (control), 0.5  $\mu$ g WT, 1  $\mu$ g WT, WT+S272P, WT+ R297T and WT+D401H hHCN1 channels. Identical amount of WT and mutant cDNA (i.e. 0.5  $\mu$ g) were cotransfected. Current density of WT+S100F, WT+S272P, and WT+R297T hHCN1 channels were significantly different compared with current density of WT (1  $\mu$ g) channels (two-way ANOVA, \*\*\*  $p < 0.001$ ). **(b)** Bar graph of current densities for each experimental group at -140 mV. **(c)** Mean tail current activation curve for 0.5  $\mu$ g WT, 1  $\mu$ g WT, WT+S272P, WT+R297T, and WT+D401H hHCN1 channels. Red lines show fits of a Boltzmann function (online methods) providing the half-activation voltage  $V_{1/2}$  and slope factor ( $k$ ) for 1  $\mu$ g WT:  $-72.06 \pm 0.73$  mV,  $k = 13.1 \pm 0.5$  mV; 0.5  $\mu$ g WT:  $-75.22 \pm 0.91$  mV,  $k = 14.63 \pm 0.7$  mV; WT+S100F:  $-69.51 \pm 0.99$  mV,  $k = 20.7 \pm 0.8$  mV, WT+S272P:  $-70.38 \pm 1.68$  mV,  $k = 27.3 \pm 1.4$  mV; WT+D401H:  $-62.98 \pm 1.33$  mV,  $k = 20.8 \pm 0.9$  mV. **(d)** The WT+D401H hHCN1 channels had higher activation time constants than the WT channels (one-way ANOVA, \*  $p < 0.05$ ). Data are presented as means  $\pm$  SEM, with the numbers of experiments given in brackets and SEM indicated by error bars.

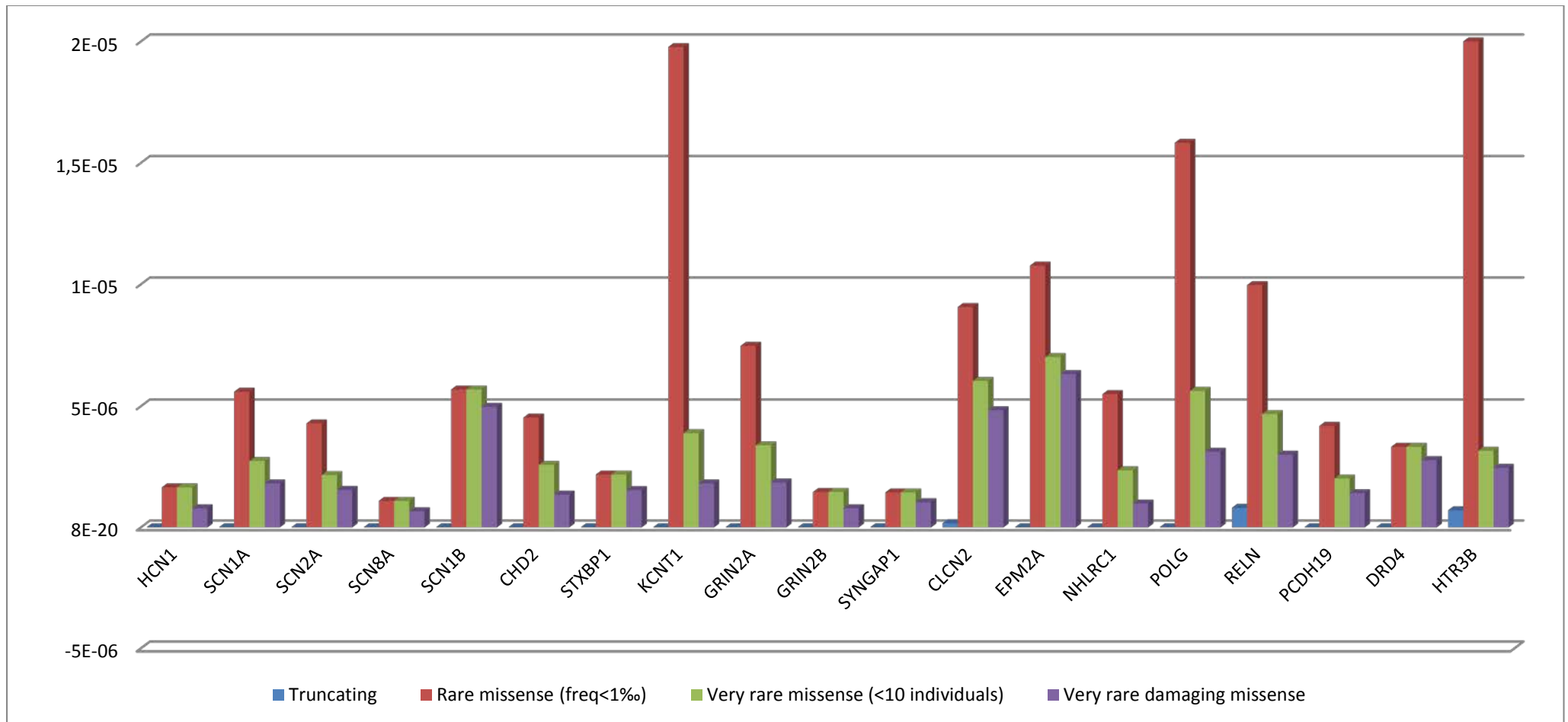


## Supplementary information

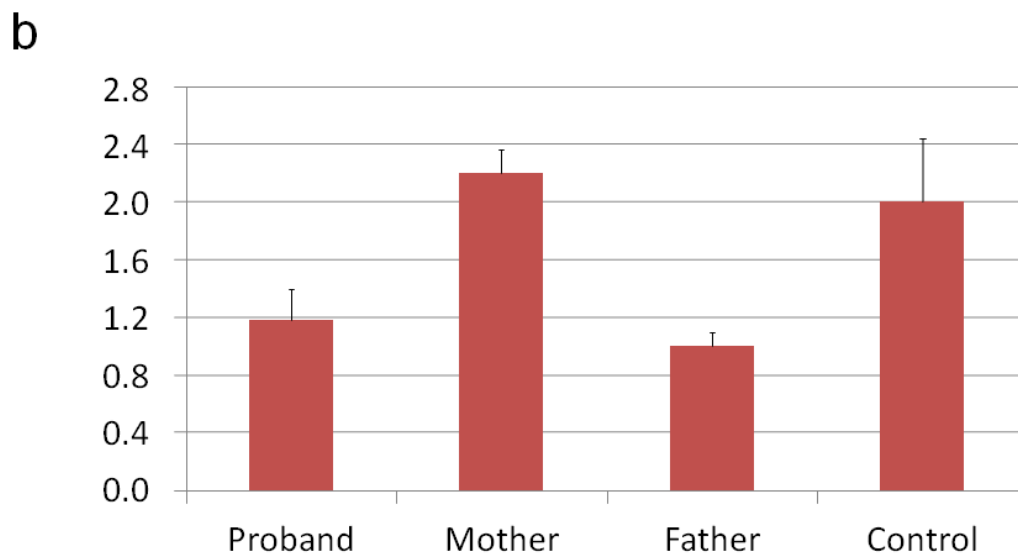
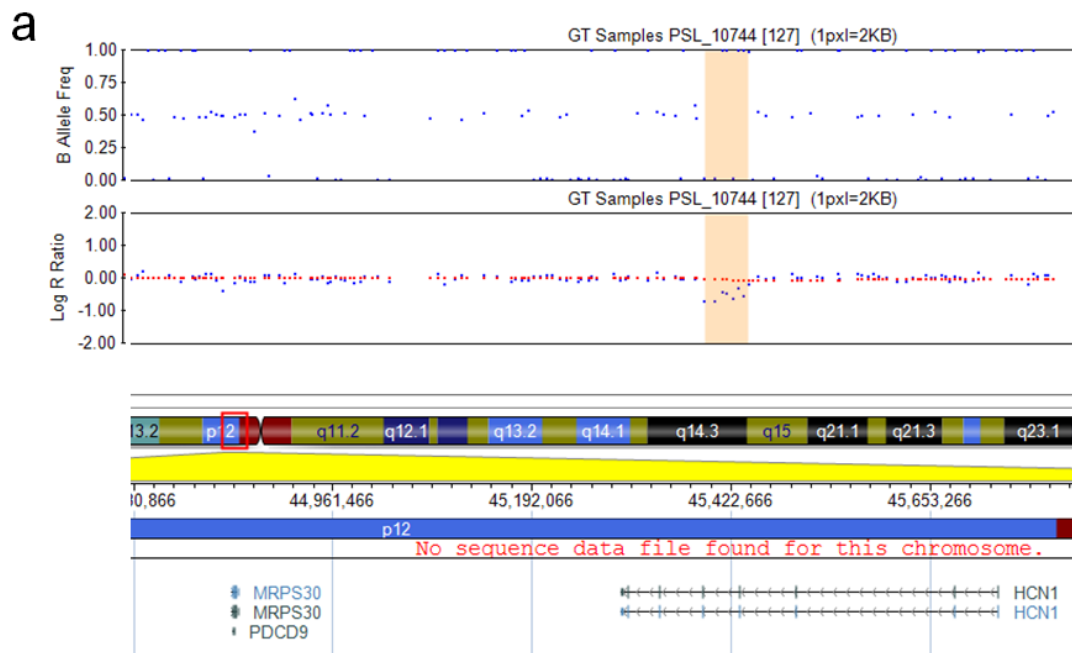
### **De novo mutations in *HCN1* cause early infantile epileptic encephalopathy**

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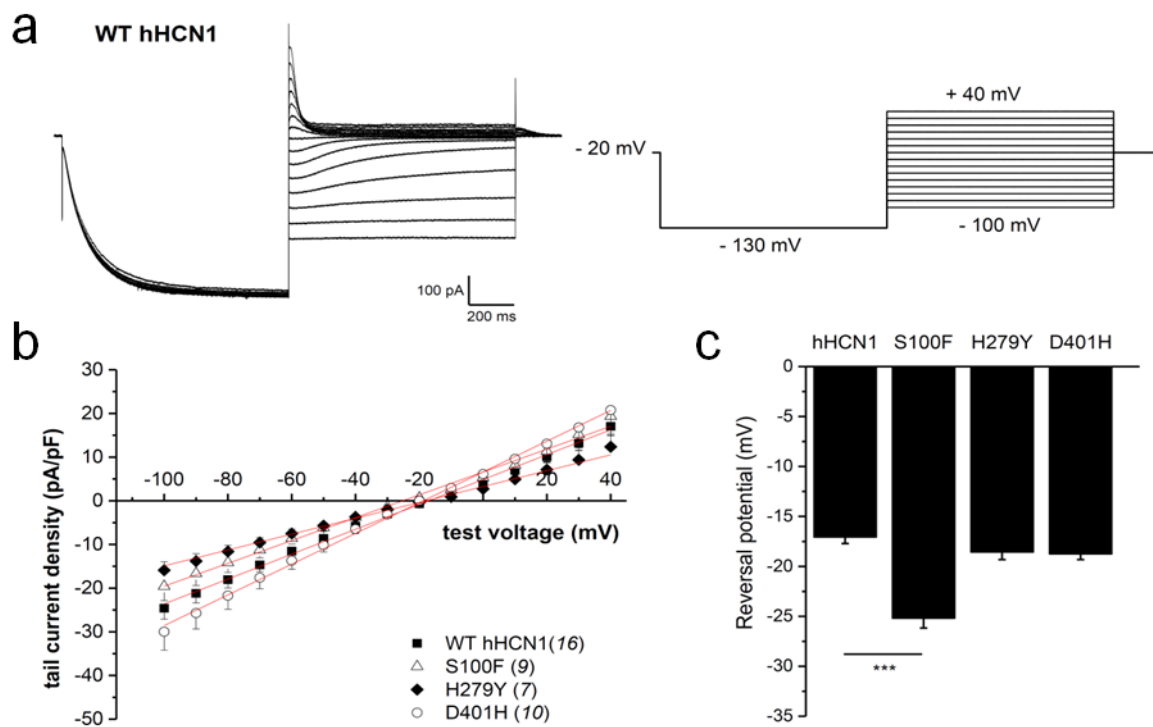
**Supplementary Figure 1** Comparison of the mean number of rare variants per base and per individual between *HCN1* and selected genes expressed in the brain. The numbers of truncating variants (nonsense and splice site), rare missense variants (with MAF < 1‰), very rare missense variants (present in fewer than ten individuals) and very rare missense variants predicted to be possibly or probably damaging by Polyphen-2 per base and per individual have been calculated for selected genes expressed in the brain, associated with autosomal dominant epilepsies or epileptic encephalopathies (*SCN1A*, *SCN2A*, *SCN8A*, *SCN1B*, *CHD2*, *STXBP1*, *KCNT1*, *GRIN2A*, *GRIN2B*, *SYNGAP1*), autosomal recessive various phenotypes (*CLCN2*, *EPM2A*, *NHLRC1*, *POLG*, *RELN*) and X-linked epileptic encephalopathy (*PCDH19*) and for genes associated with complex phenotypes (*DRD4*) or no known disorder in humans (*HTR3B*), from data of the European ESP population (Exome Variant Server, <http://evs.gs.washington.edu/EVS/>).



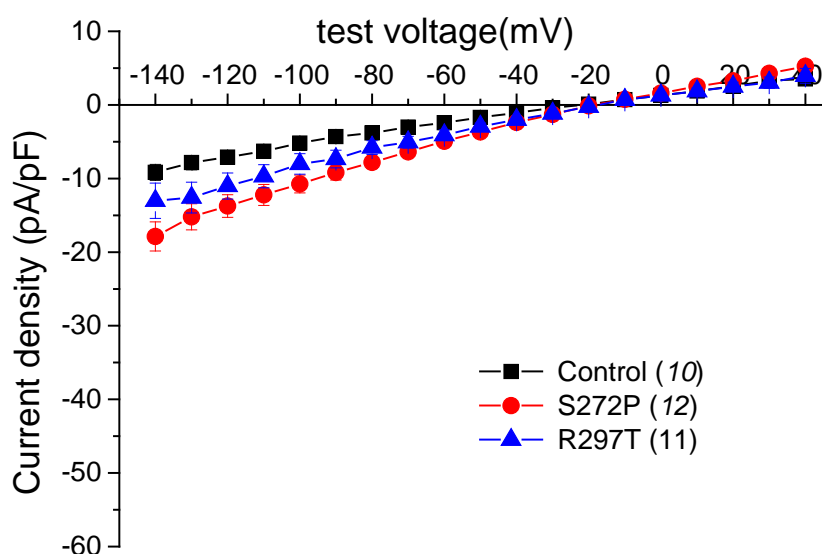
**Supplementary Figure 2** Identification of a heterozygous deletion encompassing exon 4 of *HCN1* in a female subject with ID and ASD, inherited from her asymptomatic father. **(a)** Cyto-12 SNP array (Illumina) profiles of the patient with *HCN1* exon 4 deletion and details of the coding sequences included the deletion: the y axis indicates the B allele frequency (above) and the log R ratio (below), and the x axis indicates the position on chromosome 5. The deletion spans ~33 kb and theoretically leads to an in-frame deletion of 73 amino acids (p.Asn338\_Lys410del) in the extracellular loop between the S5 and S6 domains containing the pore region. **(b)** Confirmation of the presence of the *HCN1* heterozygous deletion in the proband and her father by quantitative PCR using primers located on exon 4 (forward: 5'-CCACCTGCTATGCCATGTTT-3'; reverse: 5'-ATACTGCCGCCTCGAAGsAAT-3'). RT-PCR experiments were performed using 10 ng of genomic DNA, 0.8  $\mu$ M of each primer and 12.5  $\mu$ l of SYBR Green PCR master mix (Applied Biosystems) in a total volume of 25  $\mu$ l. RNase P (RNase P Control Assay, Applied Biosystems) was used as the reference amplicon. Each sample was run in triplicate on an ABI PRISM 7700 Detection System (Applied Biosystems), and three different experiments were used for final quantification. Relative ratios were calculated using the formula  $r = 2^{-\Delta\Delta C_t}$  with  $\Delta\Delta C_t = (C_{t \text{ mutation}} - C_{t \text{ RNaseP}})_{\text{ind tested}} - (C_{t \text{ mutation}} - C_{t \text{ RNaseP}})_{\text{ind ref}}$ .

	47		100
HCN1_HUMAN	PPGGGGAGAKEHGNSVCFK	HCN1_HUMAN	QRQFTSMLQPGVKNKFSLRMFGSQK
HCN1_MACAQUE	PPGGGGAGAKEHGNSVCFK	HCN1_MACAQUE	QRQFTSMLQPGVKNKFSLRMFGSQK
HCN1_ORANGUTAN	PPGGGGAGAKEHGNSVCFK	HCN1_ORANGUTAN	QRQFTSMLQPGVKNKFSLRMFGSQK
HCN1_MARMOSET	PPGGGGAGAKEHGNSVCFK	HCN1_MARMOSET	QRQFTSMLQPGVKNKFSLRMFGSQK
HCN1_MOUSE	PPGGG-AAGKEHGNSVCFK	HCN1_MOUSE	QRQFTSMLQPGVKNKFSLRMFGSQK
HCN1_RAT	PPGGG-AAGKEHGNSVCFK	HCN1_RAT	QRQFTSMLQPGVKNKFSLRMFGSQK
HCN1_COW	PPGGGGTGAKEHGNSVCFK	HCN1_COW	QRQFTSMLQPGVKNKFSLRMFGSQK
HCN1_GALAGO	PPGGGGAGAKEHGNSVCFK	HCN1_DOG	QRQFTSMLQPGVKNKFSLRMFGSQK
HCN1_DOG	PPGGGGAGAKELGNSVCFK	HCN1_PANDA	QRQFTSMLQPGVKNKFSLRMFGSQK
		HCN1_RABBIT	QRQFTSMLQPGVKNKFSLRMFGSQK
		HCN1_BIRD	QRQFTSMLQPGVKNKFSLRMFGSQK
		HCN1_CHICKEN	QRQFTSMLQPGVKNKFSLRMFGSQK
		HCN1_FROG	QRQFGSLMQPGVKNKFSLRMFGSQK
		HCN1_TETRAODON	QRQFGAMMQPGVKNKFSLRMFGSQK
	272 279	297	401
HCN1_HUMAN	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_MACAQUE	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_ORANGUTAN	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_MARMOSET	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_MOUSE	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_RAT	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_COW	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_DOG	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_PANDA	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_RABBIT	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_BIRD	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_CHICKEN	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_FROG	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK
HCN1_TETRAODON	LLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLI	GMMML	GHATALIQSLDSSRRQYQEKYK

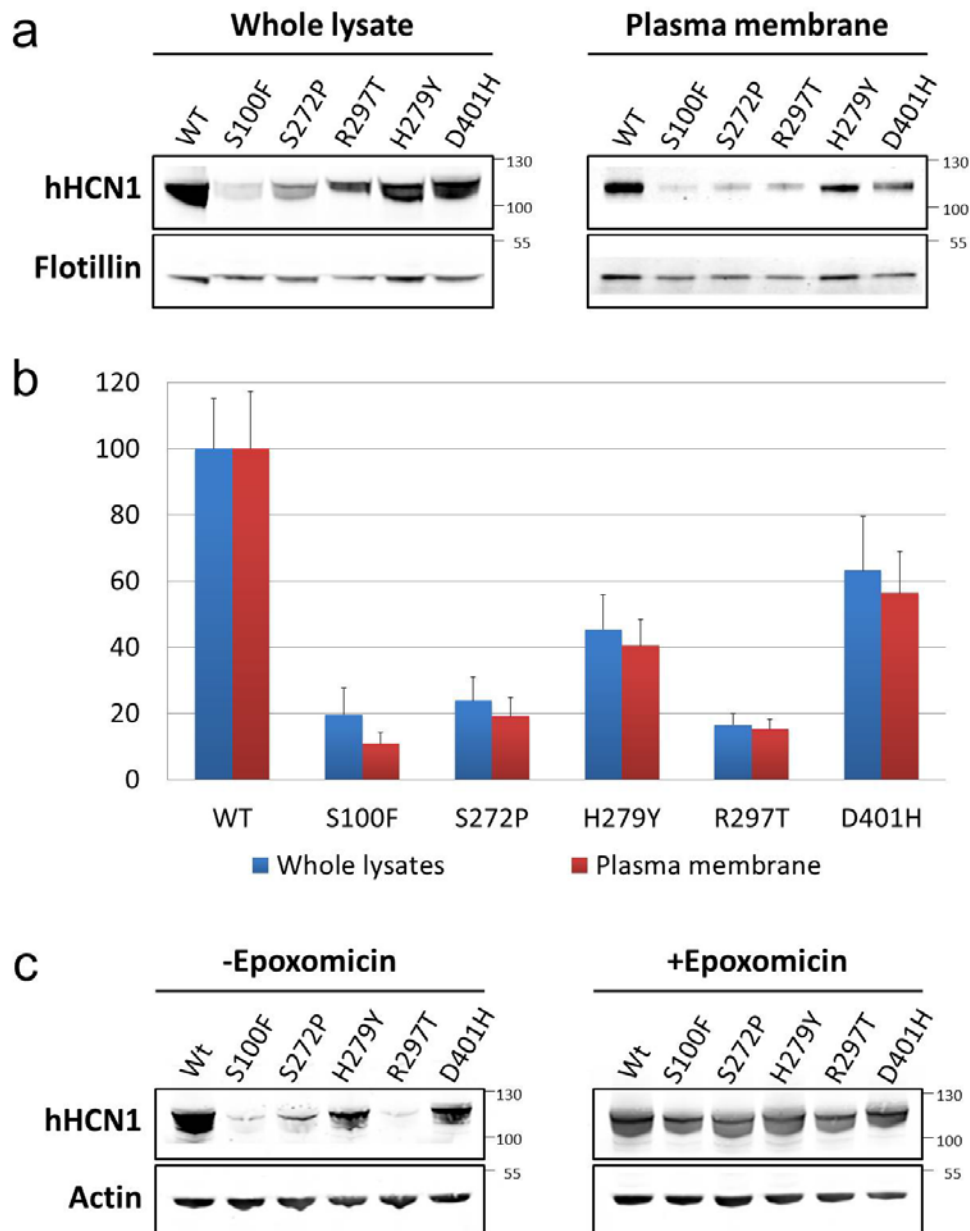
**Supplementary Figure 3** Alignment of the regions flanking the six missense variants in orthologous proteins, showing the conservation of the altered amino acids. Multiple pairwise alignments were performed using ClustalW 2.1 (<http://www.genome.jp/tools/clustalw/>). The amino acids altered by the mutations are highlighted. Full alignments including all species are provided as a Supplementary Note.



**Supplementary Figure 4** Reversal potential ( $E_{rev}$ ) of gain-of-function mutations affecting the hHCN1 channel. **(a)** Representative whole-cell current trace recorded from a CHO-K1 cell transfected with construct for wild-type (WT) hHCN1 channel. For determination of  $E_{rev}$ , currents were fully activated by a prepulse to  $-130$  mV for 1 s from a holding potential of  $-20$  mV, and test pulses were then applied from  $-100$  mV to  $+40$  mV in 10-mV increments, with tail current measured immediately after the prepulse. **(b)** Plot of mean tail current density as a function of test voltage for WT, S100F, H279Y and D401H hHCN1 channels. The number of cells for each condition is indicated in parentheses. The red line represents a linear regression through the mean values for the tail current density and is used to obtain  $E_{rev}$ . **(c)** Bar graph of  $E_{rev}$  for WT hHCN1 and the three mutant channels (one-way ANOVA followed by a Dunnett's test,  $***P < 0.001$ ). Data are represented as mean values  $\pm$  s.e.m. with numbers of experiments given in parentheses.



**Supplementary Figure 5** Data analysis of loss-of-function mutations affecting the hHCN1 channel identified with the patch clamp technique. Plot of mean current density as a function of test voltage for control cells (cells transfected only with pEGFP plasmid, reflecting the endogenous current) and cells expressing S272P and R297T mutant channels. The number of cells for each condition is indicated in parentheses. Currents were elicited by test pulses ranging from  $-140$  mV to  $+40$  mV in 10-mV increments from a holding potential of  $-20$  mV and were normalized to cell capacitance (representative traces in Fig. 2a). Current densities of both mutant channels were not significantly different compared with endogenous currents from control cells (one-way ANOVA followed by a Dunnett's test,  $P > 0.05$ ). Data are presented as means  $\pm$  s.e.m. with numbers of experiments given in parentheses.



**Supplementary Figure 6** Impact of *de novo* HCN1 mutations on protein expression and localization at the plasma membrane. **(a)** Semiquantitative analysis of WT and mutant (S100F, S272P, R297T, H279Y or D401H) HCN1 protein expression in whole-cell lysates and at the plasma membrane by protein blot. CHO-K1 cells were transiently cotransfected with 12  $\mu$ g of WT or mutant HCN1 expression plasmid using the Neon electroporation system (Invitrogen); 24 h after transfection, proteins present at the plasma membrane were isolated using the Cell Surface Protein Isolation kit (Pierce), following the manufacturer's recommendations. Whole-cell lysates were kept before isolation of membrane protein from the same experiments. Proteins present in both fractions were resolved by SDS-PAGE on 4–12% gradient gels (Invitrogen) and electrotransferred onto nitrocellulose membranes. HCN1 was probed using a monoclonal mouse anti-HCN1 antibody (ab84816, Abcam; 1:10,000 dilution), and the signal was visualized with enhanced chemiluminescence (Pierce). Membranes were probed with anti-Flotillin-1 antibody (610820, BD Biosciences; 1:1,000 dilution) for normalization. The image shows the result of a representative experiment. **(b)** Semiquantitative measurement of WT and mutant HCN1 proteins present in whole-cell lysates (blue) and at the plasma membrane (red) from three independent experiments using the ImageJ program (<http://rsb.info.nih.gov/ij/>). The values obtained for WT and mutant HCN1 proteins were corrected for the intensity of the corresponding flotillin bands. Data are presented as means  $\pm$  s.e.m. Note that, although the expression of S100F, S272P and R297T HCN1 channels was similarly decreased, an  $I_h$  current was recorded for S100F but nor for S272P and R297T. In addition, the S272P and R297T mutants showed a dominant-negative effect on the WT-mutant heteromeric channels, suggesting that mutant homomeric channels are less stable than WT-mutant heteromeric channels. **(c)** Effect of proteasome inhibition on mutant (S100F, S272P, R297T, H279Y or D401H) HCN1 protein expression in whole-cell lysates. CHO-K1 cells were transiently cotransfected with WT or mutant HCN1 expression plasmids; 24 h after transfection, half of the cells was treated with 1  $\mu$ M epoxomicin (E3652, Sigma) for 16 h. Proteins were then resolved by SDS-PAGE on 4–12% gradient gels (Invitrogen) and electrotransferred onto nitrocellulose membranes. HCN1 was probed using the monoclonal mouse anti-HCN1 antibody, and the signal was detected by enhanced direct near-infrared fluorescence detection system (LI-COR Biosciences). Membranes were probed with anti-actin antibody (ab3280, Abcam; 1:4,000 dilution) for normalization. The image shows the result of a representative experiment.

Family number/ Patient ID	6 / N09 2300	2/ DRA-20	3/ N 07 1339	5/ 2008D01179	4/ N10 0713	1/ N06 0565
Patient origin	France	Italy	France	The Netherlands	France	France
Sex	Female	Female	Female	Male	Female	Female
Base change	c.140G>T	c.299C>T	c.814T>C	c.835C>T	c.890G>C	c.1201G>C
Amino acid change	p.Gly47Val	Ser100Phe	p.Ser272Pro	p.His279Tyr	p.Arg297Thr	p.Asp401His
Exon	1	1	2	2	3	4
Inheritance	Unknown	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>
<i>In silico</i> predictions (SIFT / Polyphen-2)	Deleterious / Benign	Deleterious / Possibly damaging	Deleterious / Possibly damaging	Deleterious / Possibly damaging	Deleterious / Possibly damaging	Deleterious / Probably damaging
Age at time of analysis (years)	13	6	16	12	15	18
Age at seizure onset (months)	7	10	8	13	8	4
Seizure type at onset	Febrile, TCS	Febrile and afebrile, TCS	Febrile, hemiclonic	Febrile seizures (atypical)	Febrile, hemiclonic (left / right) seizures	TCS, cyanosis
Seizure types during disease course	TCS, atypical absences with or without myoclonic jerks; focal seizures, seizures in clusters	Clonic seizures, TCS, atypical absences, myoclonic jerks, focal (frontal?) seizures	Clonic seizures, atypical absences, focal seizures	Clonic seizures, TCS, atonic, atypical absences, myoclonic jerks, nocturnal seizures	Hemiclonic seizures, atypical absences, nocturnal frontal seizures, focal seizures, drop attacks	TCS, myoclonic jerks, atypical absences, nocturnal frontal seizures
Febrile seizures	yes	yes	yes	yes	yes	yes
Status epilepticus	yes	no	yes	no	yes	yes



Frequency of seizures	Several per month before 3 years, several per year thereafter	Several per week, clusters	Several per month	Several per week at onset; several per year later on; absences and myoclonic jerks: several days per week	Several per week at onset; seizure-free between the ages of 2 and 6 yrs, with STP,VPA CLB; Rare seizures between 6 and 10 yrs of age; several seizures per week from the age of 10 yrs	Several per week at onset; several absences per day and several nocturnal seizures per month later on
ID	Moderate/severe	Moderate	Severe	Mild	Moderate/severe	Moderate/severe
MRI	NA	Normal	Normal	Normal	Normal	Normal
EEG	NA	GSW	Normal then multifocal spikes, slowing	Diffuse (poly)peakwave complexes; later on: multifocal epileptic discharges with frontocentral maximum	8 months: normal 9 months: diffuse PSW, IPS+ ; 9 yrs: frontal synchronous spike and wave with activation during sleep	PSW
Pharmacoresistance	yes	yes	yes	yes	yes	yes
AEDs	VPA, CLB	LTG, CLB, VPA, PB	VPA, CLB, STP	ETX, LTG	VPA, CLB, STP, TPM, ZNS, LVT, LTG, ketogenic diet, VNS	VPA, CLB, TPM
Behavior and language	Absence of language	Autistic features	Behavioral disturbances, autistic features, stereotypies	Behavioral disturbances; agitation, attention deficit disorder	Behavioral disturbances, autistic features	Autistic features, agitation, aggressiveness, stereotypies
Other features	Ataxia	None	Motor delay	Truncal ataxia	None	Polyphagia

**Supplementary Table 1** Detailed genetic and clinical characteristics of the patients with *HCN1* mutations identified in this study.

TCS: tonic-clonic seizures, yrs: years, ID: intellectual disability, AED: antiepileptic drug, VPA: sodium valproate, CLB: clobazam, STP: stiripentol, TPM: topiramate, ZNS: zonisamide, LVT: levetiracetam, LTG: lamotrigine, ETX: ethosuximide, VNS: Vagus Nerve stimulation; GSW: generalized spike waves, PSW: polyspike waves, IPS: intermittent photic stimulation, NA: unavailable.

Population	Chrom	Position	Ref base	Sample base	Gene	Transcript	Transcript Variant	Protein Variant	Number of ind (EA/AA)	Impact	SIFT Prediction	PolyPhen-2 Prediction	RsNumber
PD	5	45696129	C	A	HCN1	NM_021072.3	c.67G>T	p.Ala23Ser	1	missense	Tolerated	Benign	
PD	5	45695967	G	T	HCN1	NM_021072.3	c.229C>A	p.Pro77Thr	1	missense	Tolerated	Benign	
ESP	5	45695964	C	T	HCN1	NM_021072.3	c.232G>A	p.Ala78Thr	0/1	missense	Tolerated	Benign	
ESP	5	45695937	G	A	HCN1	NM_021072.3	c.259C>T	p.Pro87Ser	0/1	missense	Deleterious	Benign	
ESP	5	45695900	G	A	HCN1	NM_021072.3	c.296C>T	p.Thr99Ile	1/0	missense	Tolerated	Benign	rs143865339
ESP	5	45645537	T	C	HCN1	NM_021072.3	c.599A>G	p.Asn200Ser	1/0	missense	Tolerated	Benign	
ESP	5	45645433	T	C	HCN1	NM_021072.3	c.703A>G	p.Ile235Val	1/0	missense	Deleterious	Benign	
ESP	5	45462005	T	G	HCN1	NM_021072.3	c.954A>C	p.Leu318Phe	1/0	missense	Deleterious	Possibly damaging	
ESP	5	45461997	A	G	HCN1	NM_021072.3	c.962T>C	p.Leu321Pro	1/0	missense	Deleterious	Benign	rs149434809
ESP	5	45396611	G	A	HCN1	NM_021072.3	c.1213C>T	p.Arg405Trp	1/0	missense	Deleterious	Probably damaging	
ESP	5	45353327	T	G	HCN1	NM_021072.3	c.1252A>C	p.Met418Leu	1/0	missense	Deleterious	Possibly damaging	
ESP	5	45353314	T	G	HCN1	NM_021072.3	c.1265A>C	p.Lys422Thr	1/0	missense	Deleterious	Probably damaging	rs140166527
ESP	5	45353231	G	A	HCN1	NM_021072.3	c.1348C>T	p.Leu450Phe	1/0	missense	Deleterious	Probably damaging	rs146123836
PD	5	45303809	G	A	HCN1	NM_021072.3	c.1510C>T	p.Arg504*	1	stop gain			
CI	5	45303797	C	T	HCN1	NM_021072.3	c.1522G>A	p.Val508Met	1	missense	Deleterious	Benign	
CI, PD	5	45262760	T	A	HCN1	NM_021072.3	c.1936A>T	p.Thr646Ser	1, 1	missense	Tolerated	Benign	
PD	5	45262715	T	A	HCN1	NM_021072.3	c.1981A>T	p.Arg661Trp	1	missense	Deleterious	Possibly damaging	
ESP	5	45262714	C	T	HCN1	NM_021072.3	c.1982G>A	p.Arg661Lys	0/1	missense	Deleterious	Benign	rs143224211
ESP	5	45262688	C	T	HCN1	NM_021072.3	c.2008G>A	p.Ala670Thr	1/0	missense	Deleterious	Benign	
ESP	5	45262687	G	A	HCN1	NM_021072.3	c.2009C>T	p.Ala670Val	2/1	missense	Deleterious	Benign	rs142280884
ESP	5	45262675	G	A	HCN1	NM_021072.3	c.2021C>T	p.Ser674Phe	0/1	missense	Deleterious	Benign	rs138171983
ESP	5	45262619	T	C	HCN1	NM_021072.3	c.2077A>G	p.Ile693Val	1/0	missense	Tolerated	Benign	
ESP	5	45262591	G	A	HCN1	NM_021072.3	c.2105C>T	p.Ala702Val	3/0	missense	Deleterious	Possibly damaging	rs147007826
ESP	5	45262564	G	A	HCN1	NM_021072.3	c.2132C>T	p.Pro711Leu	0/2	missense	Deleterious	Possibly damaging	
ESP	5	45262526	C	T	HCN1	NM_021072.3	c.2170G>A	p.Ala724Thr	0/6	missense	Deleterious	Possibly damaging	rs141383188
ESP	5	45262387	G	A	HCN1	NM_021072.3	c.2309G>A	p.Ser770Asn	0/1	missense	Deleterious	Benign	
ESP	5	45262375	A	G	HCN1	NM_021072.3	c.2321T>C	p.Leu774Pro	0/2	missense	Deleterious	Possibly damaging	rs149702217
ESP	5	45262321	G	A	HCN1	NM_021072.3	c.2375C>T	p.Ser792Leu	0/1	missense	Deleterious	Probably damaging	rs140758934
PD	5	45262306	A	C	HCN1	NM_021072.3	c.2390T>G	p.Val797Gly	1	missense	Tolerated	Benign	
ESP	5	45262234	C	T	HCN1	NM_021072.3	c.2462C>T	p.Ala821Val	1/0	missense	Tolerated	Benign	
PD	5	45262211	C	A	HCN1	NM_021072.3	c.2485G>T	p.Ala829Ser	1/0	missense	Tolerated	Benign	
ESP	5	45262189	C	T	HCN1	NM_021072.3	c.2507C>T	p.Pro836Leu	1/0	missense	Deleterious	Possibly damaging	
ESP	5	45262137	C	G	HCN1	NM_021072.3	c.2559C>G	p.Asn853Lys	1/0	missense	Tolerated	Benign	rs140186173
ESP	5	45262106	C	T	HCN1	NM_021072.3	c.2590G>A	p.Ala864Thr	0/1	missense	Deleterious	Benign	

ESP	5	45262090	C	T	HCN1	NM_021072.3	c.2606G>A	p.Arg869Lys	0/1	missense	Tolerated	Benign
CI	5	45262037	C	T	HCN1	NM_021072.3	c.2659G>A	p.AlaA887Thr	1	missense	Tolerated	Benign

**Supplementary Table 2** Summary of *HCN1* variants present in the ESP population and in cases with Parkinson's disease (PD,  $n = 1,407$ ) and control individuals (CI,  $n = 530$ ) included in the IPDGC study.

A total of 27 missense variants, all in the heterozygous state, in 38 individuals (Frequency of heterozygotes  $38/6503=0.0058$ ) are present in the ESP population. This list does not include the frequent polymorphic in-frame deletions in the glycine stretch (c.199-207delGGTGGCGGC, p.67delGGG; c.214-222delGGCGCGGC, p.72delGGG; c.217-222delGGCGGC, p.73delGG) previously described in exon 1 of *HCN1*.<sup>23,24</sup>

The frequency of *HCN1* heterozygous variants is identical in two other populations (530 control individuals and 1407 subjects with Parkinson disease) from the International Parkinson's Disease Genomics Consortium (IPDGC) study:  $3/530=0.0056$ ;  $7/1407=0.005$ , indicating that this observation is reliable. Considering only the variants predicted "possibly or probably damaging" by Polyphen-2, and the regions of *HCN1* that are sufficiently covered by exome sequencing *i.e.* (excluding positions c.1 to c.239, insufficiently covered), the number of heterozygotes in the ESP, PD and control populations is respectively 20, 1 and 0. These numbers are significantly lower than the number of *HCN1* damaging missense variants found in epileptic patients (*i.e.*  $5/196$ ,  $p= 0.00067$ ,  $p= 0.0001$  and  $p= 0.001$ ; Exact Fisher test).

Note that one PD patient has a *HCN1* nonsense mutation, further supporting the hypothesis that haploinsufficiency of *HCN1* does not cause EIEE.

Several missense variants present in these control populations are comparable, in terms of predictions, to the mutations identified in patients with EIEE. The existence of apparently or even well-characterized deleterious mutations in individuals from the ESP population is not specific of *HCN1* and is observed for other genes, in particular those involved in human epilepsies. For example, 51 different missense variants predicted to be deleterious are present in *SCN1A* in EVS, including the R1596C mutation in one individual<sup>48</sup>. These observations have several possible grounds: 1) the clinical status of individuals included in the ESP population, in particular the occurrence of epilepsy in subjects with possibly deleterious variants, is unavailable; 2) the presence of the variants/mutations has not been validated and some of these variants might be false-positives or mosaic (*i.e.* present in the blood but not in the brain); 3) rare subjects might have constitutive deleterious mutations without expressing any symptom, possibly due to genetic buffering or other mechanisms.

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Study	platform	CNV detection	population	Sample	DNA source	Genome version	Chr	CNV type	Gene	Number of ind	start	end	length	Localization/ effect on HCN1	Phenotype
Itsara et al, 2009	Illumina 650Y	HMM	hgdp	HGDP01238	lymphoblastoid cell lines	Hg19	5	Del	HCN1	1	45339429	45445657	106228	encompasses exons 4 and 5	control subject
Itsara et al, 2009	Illumina 550K	HMM	NINDS-317+240	222	lymphoblastoid cell lines	Hg19	5	Del	HCN1	1	45361672	45506180	144508	encompasses exons 3 to 5	control subject
Itsara et al, 2009	Illumina 317K	HMM	parc-prince	X	peripheral blood	Hg19	5	Del	HCN1	1	45361672	46322869	961197	encompasses exons 1 to 5	control subject
Itsara et al, 2009	Illumina 550K	HMM	NINDS-550K	1780862470_A	lymphoblastoid cell lines	Hg19	5	Del	HCN1	1	45400632	45492834	92202	encompasses exon 3	control subject
Itsara et al, 2009	Illumina 317K	HMM	parc-prince	X	peripheral blood	Hg19	5	Del	HCN1	1	45400632	45506180	105548	encompasses exon 3	control subject
Itsara et al, 2009	Illumina 317K	HMM	parc-prince	X	peripheral blood	Hg19	5	Dup	HCN1	1	45442078	46264090	822012	encompasses exons 1 to 3	control subject
Pinto et al, 2011	Illumina 1M	PCNV	5 healthy subjects	NA10851	lymphoblastoid cell lines	Hg19	5	Del	HCN1	1	45363063	45383084	20022	in intron 4	control subject
Pinto et al, 2011	Illumina 1M	PCNV	5 healthy subjects	NA10851	lymphoblastoid cell lines	Hg19	5	Del	HCN1	1	45363063	45373943	10881	in intron 4	control subject
Pinto et al, 2011	Illumina 1M	PCNV	5 healthy subjects	NA15510	lymphoblastoid cell lines	Hg19	5	Del	HCN1	1	45363063	45383084	20022	in intron 4	control subject
Pinto et al, 2011	Illumina 1M	QSNP	5 healthy subjects	NA15510	lymphoblastoid cell lines	Hg19	5	Del	HCN1	1	45363063	45406321	43259	encompasses exon 4	control subject
Pinto et al, 2011	Illumina 1M	cnvPart	5 healthy subjects	NA18517	lymphoblastoid cell lines	Hg19	5	Del	HCN1	1	45363063	45409900	46838	encompasses exon 4	control subject
Pinto et al, 2011	Illumina 1M	PCNV	5 healthy subjects	NA18517	lymphoblastoid cell lines	Hg19	5	Del	HCN1	1	45366788	45378207	11420	in intron 4	control subject
Pinto et al, 2011	Illumina 1M	PCNV	5 healthy subjects	NA18517	lymphoblastoid cell lines	Hg19	5	Del	HCN1	1	45363063	45390738	27676	in intron 4	control subject
Pinto et al, 2011	Illumina 1M	PCNV	5 healthy subjects	NA18980	lymphoblastoid cell lines	Hg19	5	Del	HCN1	1	45363063	45409900	46838	encompasses exon 4	control subject

Cooper et al, 2011	CGH	HMM	15,767 children with ID	ND	peripheral blood	Hg19	5	Del	HCN1	3	NA	NA	NA	encompassing coding sequences: 13	Individuals with ID or DD +/- craniofacial defects (n=1)
Cooper et al, 2011	Illumina SNP arrays	HMM	8,329 control subjects	ND	peripheral blood	Hg19	5	Del	HCN1	13	NA	NA	NA		control subjects
Cooper et al, 2011	CGH	HMM	15,767 children with ID	ND	peripheral blood	Hg19	5	Dup	HCN1	9	NA	NA	NA	encompassing coding sequences: 5	Individuals with ID or DD and ASD (n=1) or craniofacial defects (n=4)
Cooper et al, 2011	Illumina SNP arrays	HMM	8,329 control subjects	ND	peripheral blood	Hg19	5	Dup	HCN1	5	NA	NA	NA		control subjects
Nava et al, 2013	Illumina SNP arrays	cnvPart	194 subjects with ASD	PSL_10744	peripheral blood	Hg19	5	Del	HCN1	1	NA	NA	NA	encompasses exon 4	Individual with ID and ASD

**Supplementary Table 3** Copy number variants (CNVs) encompassing *HCN1* reported in the literature.

NA: unavailable

HCN1-ex1_F1	TACCCTCTCGGCTACGTGTC
HCN1-ex1_R1	CGTCCACCTTGAAGCACAC
HCN1-ex1_F2	GTCTAACAGCCGGGACGAT
HCN1-ex1_R2	CGGAGGGAGAATTTGTTGAC
HCN1-ex1_F3	TTCATGCAGAGGCAGTTCAC
HCN1-ex1_R3	GAGTGGAGCCTGCTTAGCC
HCN1-ex2_F1	GGGGGATAGGCTGGTTATTTT
HCN1-ex2_R1	GGATGAGATGAAGTCAACCACA
HCN1-ex2_F2	GGACCCCAAAGTGATCAAGA
HCN1-ex2_R2	CATTGCACAGTTGTTTCATTGTA
HCN1-ex3_F	TGCTTACGCTACACAACATCAA
HCN1-ex3_R	TTTTGGCACAACGTTGAAAA
HCN1-ex4_F	GCCATCCTAATGGCTCAGTC
HCN1-ex4_R	CTGGTTAAAGACATTGGCGATA
HCN1-ex5_F	TGATTTCTGATACACCCTAACAATG
HCN1-ex5_R	AGGTTTTTCTTTAGAGTAACGTGGA
HCN1-ex6_F1	TTCAGCATGTTTTTCTTCCAGA
HCN1-ex6_R1	TTTTACCCACGGCTCCTTC
HCN1-ex6_F2	CGGAAACTGGTGGCTACAAT
HCN1-ex6_R2	CTGACATGCTGACATCTCCAA
HCN1-ex7_F	TCAAACAAGTCATTCTTCTTTGTTG
HCN1-ex7_R	ATGAGTCACCACTCCCCACT
HCN1-ex8_F1	GCGTTGTCATCAGTCATTGG
HCN1-ex8_R1	TGGAGATTGTGTCCTCATGC
HCN1-ex8_F2	TGACAACCCTGAATTCCACA
HCN1-ex8_R2	GACTGCTGTACCTGCTGCTG
HCN1-ex8_F3	CCGCTCGAACTTTCCACTATG
HCN1-ex8_R3	TCACGGGTTGAGGGATGG
HCN1-ex8_F4	CTCTGATTTCCAGACCTCATCC
HCN1-ex8_R4	TGAGAGTATTTCTTTCTGCTTGACA

**Supplementary Table 4** Primers used to amplify the coding regions of *HCN1*.

Supplementary Note Full alignments of HCN1 orthologous and paralogous sequences.

(a)

HCN1\_HUMAN MEGGGKPNSSNSRDDGNSVFPKASATGAGPAAAEKRLGTPPGGGGAGAKEHGNSVCFK  
 HCN1\_MACAQUE MEGGGKPNSSNSRDDGNSVFPKASATGAGPAAAEKRLGTPPGGGGAGAKEHGNSVCFK  
 HCN1\_ORANGUTAN MEGGGKPNSSNSRDDGNSVFPKASATGAGPAAAEKRLGTPPGGGGAGAKEHGNSVCFK  
 HCN1\_MARMOSET MEGGGKSNSSNSRDDGNSVFPKASATGAGPAAAEKRLGTPPGGGGAGAKEHGNSVCFK  
 HCN1\_MOUSE MEGGGKPNASNSRDDGNSVFPKAPATGP---VAADKRLGTPPGGGGAAGKEHGNSVCFK  
 HCN1\_RAT MEGGGKPNASNSRDDGNSVFPKAPATGP---AAADKRLGTPPGGGGAAGKEHGNSVCFK  
 HCN1\_SQUIRREL -----XCXFKVD  
 HCN1\_HORSE -----  
 HCN1\_COW MEGGGKPNSSNSRDDGNSVFPKAPATGAGPAAAEKRLGTPPGGGGTGAKEHGNSVCFK  
 HCN1\_GALAGO MEGGGKPNSSNSRDDGNSVFSKAPAAASAGPAGAEKRLGTPPGGGGAGAKEHGNSVCFK  
 HCN1\_DOG MEAGGKPNASNSRDDGSSAFP GKAPATGAGPAAAEKRLGTPPGGGGAGAKELGNSVCFK  
 HCN1\_PANDA -----  
 HCN1\_RABIT -----  
 HCN1\_PLATYPUS -----  
 HCN1\_BIRD -----GGGSGGVKEHGNSVCFK  
 HCN1\_CHICKEN -----MEGGKRSSSPGSRDEGSANAFP GKQATPVEKAQSSPPGGGAKEHGNSVCFK  
 HCN1\_ANOLE -----MEGSKPSSSPGSRDEGSPGANACFQGKAEKAPGSPGASGLKEHANSVCFK  
 HCN1\_FROG -----LPSMESKFNSSTNSSRDDGNNVLQGKAEKSLTASTSSVKEHGNSVCFK  
 HCN1\_PIG -----MATASSPPRRRRARGRQRPRSAWAPRRGAAGPARRS  
 HCN1\_TILAPIA -----MEDKSNSFSSNKE-EKADGNNVFQRQDSIQKNNTGSQNMK--DHGNSVGFK  
 HCN1\_PLATYFISH -----MEDKSNSFSSNKE-EKADGNNVFQRQDSIQKNNTGSQNTK--DHGNSVGFK  
 HCN1\_TETRAODON -----GSQNTK--EHSNSVGFK  
 HCN1\_TROUT -----MEDKSNSFSSNKEGKADGNNVFQRQDSIQKNMGSQNMKGGDHGNSVGFK

HCN1\_HUMAN VDGGGGGGGGGG--GEEPAGGFEDAEGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_MACAQUE VDGGGGGGGG--GEEPAGGFEDAEGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_ORANGUTAN VDGGGGGEE-----PAGGFEDAEGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_MARMOSET VDGGGGGGGGGG--GEEPAGGFEDAEGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_MOUSE VDGGG-----GEEPAGSFEDAEGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_RAT VDGGG-----GEEPAGSFEDAEGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_SQUIRREL GGGGG-----GEEPAGSFEDAEGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_HORSE -----  
 HCN1\_COW VDGGGGGGGE-----ESAGGFEDAEGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_GALAGO VDGGGGGAE-----ESAGGFEDAEGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_DOG VDGGGAGEE-----PAGGFEDAEGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_PANDA -----GFMQRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_RABIT ----MATASSPPR--RPRRARGLEDAEGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_PLATYPUS -----  
 HCN1\_BIRD VDGGGGGEEP-----AVGFEDAEGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_CHICKEN VDGGGAEEP-----VVGFEDEAGPRRQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_ANOLE VDGGGAAGEEVPVAGLDDPDAARRQQQQQQYGFMRQFTSMLQPGVNFSLRMFGSQK  
 HCN1\_FROG TDAADDPVVG-----FEDAEGPSRPHGFMQRQFGSLM QPGVNFSLRMFGSQK  
 HCN1\_PIG TATPCASRWTA AAAVAARSRPGASRRTRRPGGSTASC SGSHLYAAAWGQQLPPHVWEPE  
 HCN1\_TILAPIA GEREEAMVG-----FDDLEGASRQYGFMRQFGAMMQPGVNFSLRMFGSQK  
 HCN1\_PLATYFISH GEREEAMVG-----FDDLDGASRQHGFMRQFGAMMQPGVNFSLRMFGSQK  
 HCN1\_TETRAODON GEREEAMVG-----FDDLEGASRQHGFMRQFGAMMQPGVNFSLRMFGSQK  
 HCN1\_TROUT GDREEALVG-----FDDIDGSGNRHGFMRQFGAMMQPGVNFSLRMFGSQK

HCN1\_HUMAN AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_MACAQUE AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_ORANGUTAN AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_MARMOSET AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_MOUSE AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_RAT AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_SQUIRREL AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_HORSE -----RFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_COW AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_GALAGO AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIVIF  
 HCN1\_DOG AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_PANDA AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_RABIT AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_PLATYPUS -----MLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_BIRD AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_CHICKEN AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_ANOLE AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_FROG AVEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_PIG GGEKEQERVKTAGFWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIIF  
 HCN1\_TILAPIA AVEKEQERVQTAGYWI IHPYSDFRFYDWLIMLIMMVGNLVIIPVGITFFTEQTTTPWLVF

HCN1_PLATYFISH	AVEKEQERVQTAGYWI IHPYSDFRFYDWLIMLIMMMGNLIIIPVGITFFSEQTTTTWLVF
HCN1_TETRAODON	AVEKEQERVQTAGYWI IHPYSDFRFYDWLIMLIMMMGNLIIIPVGITFFSQTTTTWLVF
HCN1_TROUT	AVEKEQERVQTAGYWI IHPYSDFRFYDWLVLVMMGNLIIIPVGITFFSEQTTTTWLIF
	***:***:***:*****:*.**.*:.*
HCN1_HUMAN	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_MACAQUE	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_ORANGUTAN	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_MARMOSET	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_MOUSE	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_RAT	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_SQUIRREL	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_HORSE	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_COW	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_GALAGO	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_DOG	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_PANDA	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_RABIT	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_PLATYPUS	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_BIRD	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_CHICKEN	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_ANOLE	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_FROG	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_PIG	NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_TILAPIA	NVASDTIFLVDLVMNFRTGTVNEESSEIILDPKVIKMNYLKSWFVVDFLSSIPVDYIFLI
HCN1_PLATYFISH	NVASDTIFLVDLVMNFRTGTVNEESSEIILDPKVIKMNYLKSWFVVDFLSSIPVDYIFLI
HCN1_TETRAODON	NVASDTIFLVDLVMNFRTGTVNEESSEIILDPKVIKMNYLKSWFVVDFLSSIPVDYIFLI
HCN1_TROUT	NVASDTIFLVDLVMNFRTGTVNEESSEIILDPKVIKMNYLKSWFVVDFLSSIPVDYIFLI
	*****:*.**.*:***** * *:*:*****:*****:*****:*****
HCN1_HUMAN	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_MACAQUE	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_ORANGUTAN	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_MARMOSET	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_MOUSE	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_RAT	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_SQUIRREL	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_HORSE	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_COW	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_GALAGO	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_DOG	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_PANDA	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_RABIT	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_PLATYPUS	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_BIRD	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_CHICKEN	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_ANOLE	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_FROG	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_PIG	VEKGMDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_TILAPIA	VEKGFDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_PLATYFISH	VEKGFDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_TETRAODON	VEKGFDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_TROUT	VEKGFDSVYKKTARALRIVRFTKILSLLRLLRSLRLIRYIHQWEEIFHMTYDLASAVVRI
	****:*.**.*:*****:*****:*****:*****:*****:*****
HCN1_HUMAN	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_MACAQUE	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_ORANGUTAN	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_MARMOSET	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_MOUSE	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_RAT	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_SQUIRREL	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_HORSE	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_COW	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_GALAGO	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_DOG	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_PANDA	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_RABIT	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_PLATYPUS	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_BIRD	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_CHICKEN	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_ANOLE	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_FROG	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_PIG	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_TILAPIA	FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC



HCN1_PLATYFISH	FNLIGMMLLLCHWDGCLQYLVLPLQDFPPDCWVSLNGMVNVSWGKQYSYALFKAMSHMLC
HCN1_TETRAODON	FNLIGMMLLLCHWDGCLQYLVP-LQDFPPDCWVSLNGMVNVSWGKQYSYALFKAMSHMLC
HCN1_TROUT	FNLIGMMLLLCHWDGCLQFLVPLQDFPQDCWVSLNGMVNDSWGKQYSYALFKAMSHMLC *****:*** **:* . **** :** :* . :** .**
HCN1_HUMAN	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_MACAQUE	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_ORANGUTAN	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_MARMOSET	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_MOUSE	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_RAT	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_SQUIRREL	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_HORSE	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_COW	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_GALAGO	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_DOG	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_PANDA	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_RABIT	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_PLATYPUS	VKCLLPASVSEGSVLLTTLSALHTPPFFLYDSSFLGHSWGWSNLENSEKEFNIKEYKQV
HCN1_BIRD	IGYGARAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_CHICKEN	IGYGARAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_ANOLE	IGYGARAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_FROG	IGYGARAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_PIG	IGYGAQAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_TILAPIA	IGYGARAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_PLATYFISH	IGYGARAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_TETRAODON	IGYGARAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV
HCN1_TROUT	IGYGARAPVMSDDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEYKQV : * . ** . . : : * ** : . . : . . * . * . : : : ****
HCN1_HUMAN	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_MACAQUE	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_ORANGUTAN	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_MARMOSET	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILSELNDPLREEIVNFNCRKLVATMP
HCN1_MOUSE	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILSELNDPLREEIVNFNCRKLVATMP
HCN1_RAT	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILSELNDPLREEIVNFNCRKLVATMP
HCN1_SQUIRREL	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_HORSE	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_COW	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_GALAGO	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILNELNDPLREXXXNFNCRKLVATMP
HCN1_DOG	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_PANDA	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_RABIT	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_PLATYPUS	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_BIRD	EQYMSFHKLPAEMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_CHICKEN	EQYMSFHKLPAEMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_ANOLE	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEEDSILNELNDPLREEIVNFNCRKLVATMP
HCN1_FROG	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEDNILNELNDPLREEIVNFNCRKLVATMP
HCN1_PIG	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_TILAPIA	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEENILSELNDPLKEEIVNFNCRKLVATMP
HCN1_PLATYFISH	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEDNILSELNDPLKEEIVNFNCRKLVATMP
HCN1_TETRAODON	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEDNILSELNDPLKEEIVNFNCRKLVATMP
HCN1_TROUT	EQYMSFHKLPADMRQKIHDIYEHRYQGKIFDEDNILSELNDPLKEEIVNFNCRKLVATMP *****:*****: . ** *****: * *****
HCN1_HUMAN	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_MACAQUE	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_ORANGUTAN	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_MARMOSET	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_MOUSE	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_RAT	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_SQUIRREL	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_HORSE	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_COW	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_GALAGO	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_DOG	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_PANDA	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_RABIT	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_PLATYPUS	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_BIRD	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSNKELKLT
HCN1_CHICKEN	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSNKELKLT
HCN1_ANOLE	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_FROG	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_PIG	LFANADPNFVTAMLSKLRFEVFPQGDYI IREGAVGKKMYF IQHGVAGVITKSSKEMKLT
HCN1_TILAPIA	LFANADPNFVTGMSKLRFEVFPQNDYI IREGTVGKKMYF IQHGVASVITKFNKEMKLT

HCN1\_PLATYFISH LFANADPNFVTGMLSKLKFVFPNDYI IREGTVGKKMYFI QHGVSIVITKFNKEMKLT  
HCN1\_TETRAODON LFANADPNFVTGMLSKLKFVFPNDYI IREGTVGKKMYFI QHGVASVITKSNKEMKLT  
HCN1\_TROUT LFANADPNFVTGMLSKLKFVFPNDYI IREGTVGKKMYFI QHGVASVITKLNKEMKLT  
\*\*\*\*\* . \*\*\*\*\* : \*\*\*\*\* . \*\*\*\*\* : \*\*\*\*\* . \*\*\*\*\* . \*\*\*\*\* . \*\*\*\*\* : \*\*\*\*\*

HCN1\_HUMAN GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_MACAQUE GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_ORANGUTAN GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_MARMOSET GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_MOUSE GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_RAT GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_SQUIRREL GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_HORSE GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_COW GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_GALAGO GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_DOG GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_PANDA GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_RABIT GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_PLATYPUS GSYFGAEICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_BIRD GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_CHICKEN GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_ANOLE GSYFG-EICLLTKGRRITASVKADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_FROG GSYFG-EICLLTKGRRITASVRAETYCRLYSLSVENFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_PIG GSYFG-EICLLTKGRRITASVRADTYCRLYSLSVDNFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_TILAPIA GSYFG-EICLLTKGRRITASVRADTYCRLFSLSVDHFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_PLATYFISH GSYFG-EICLLTKGRRITASVRADTYCRLFSLSVDHFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_TETRAODON GSYFG-EICLLTKGRRITASVRADTYCRLFSLSVDHFNEVLEEYPMRRRAFETVAIDRLDR  
HCN1\_TROUT GSYFG-EICLLTKGRRITASVRADTYCRLFSLSVDHFNEVLEEYPMRRRAFETVAIDRLDR  
\*\*\*\*\* : \*\*\*\*\* : \*\*\*\*\* : \*\*\*\*\* : \*\*\*\*\* : \*\*\*\*\* : \*\*\*\*\* : \*\*\*\*\*

HCN1\_HUMAN IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_MACAQUE IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_ORANGUTAN IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_MARMOSET IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_MOUSE IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_RAT IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_SQUIRREL IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_HORSE IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_COW IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_GALAGO IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_DOG IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_PANDA IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_RABIT IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_PLATYPUS IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_BIRD IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_CHICKEN IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_ANOLE IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_FROG IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_PIG IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_TILAPIA IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_PLATYFISH IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_TETRAODON IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
HCN1\_TROUT IGKKNSILLQKFQKDLNTGVFNQENE ILKQIVKHDREMVQAIAP-----  
\*\* . \* : \*

HCN1\_HUMAN -----INYPQMTTLNSTSSTTTPTSRM  
HCN1\_MACAQUE -----INYPQMTTLNSTSSTTTPTSRM  
HCN1\_ORANGUTAN -----INYPQMTTLNSTSSTTTPTSRM  
HCN1\_MARMOSET -----INYPQMTTLNSTSSTTTPTSRM  
HCN1\_MOUSE -----INYPQMTALNCTSSSTTTPTSRM  
HCN1\_RAT -----INYPQMTALNCTSSSTTTPTSRM  
HCN1\_SQUIRREL -----INYPQMTALNCTSSSTTTPTSRM  
HCN1\_HORSE -----INYPQMTALNCTSSSTTTPTSRM  
HCN1\_COW -----LNYPQMTALNCTSSSTTTPTSRM  
HCN1\_GALAGO -----INYPQMTALNSTASTTTPTSRM  
HCN1\_DOG -----INYPQMTALNSTSSTATPTCRA  
HCN1\_PANDA -----INYPQMTALNSASSTTTPTSRM  
HCN1\_RABIT -----ISYPQMTALNSTSSTATPTSRM  
HCN1\_PLATYPUS -----ANYSQMPSLNSSASTTLQSSRL  
HCN1\_BIRD -----VSLQQMPALN--SSTAA--SSRG  
HCN1\_CHICKEN -----VSLQQMPALNSSSSTTS--SSRV  
HCN1\_ANOLE -----VLLQQMPALN----SATASAM  
HCN1\_FROG -----VSYQQPALN---SSTSSSLRM  
HCN1\_PIG -----  
HCN1\_TILAPIA GMNSTPMSGNSIINSPAQPPFSTAFGTTQLQQSSVPLTYSASAIANASAARMMPAAAVAA

HCN1\_PLATYFISH GMNSTPMSGNSI INSPAQPPYSTAFGAAQLQQSSVPMTYSASAIANASVARMLPVAAAAA  
 HCN1\_TETRAODON -----EMLQQASAPMTYSASAIANASAARMLPVAAAAA  
 HCN1\_TROUT GMNTPISGNSI INSPAQPPYTALGNQFQQSATSLSLYSASAVTAPSSAATARILPASA  
  
 HCN1\_HUMAN RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPPCSYTTAVCSPPVQSPPLAA-RTFHY  
 HCN1\_MACAQUE RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPPCSYTTAVCSPPVQSPPLAA-RTFHY  
 HCN1\_ORANGUTAN RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPPCSYTTAVCSPPVQSPPLAA-RTFHY  
 HCN1\_MARMOSET RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPPCSYTTAVCSPPVQSPPLAA-RTFHY  
 HCN1\_MOUSE RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPPCSYTTAVCSPPVQSPPLAT-RTFHY  
 HCN1\_RAT RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPPCSYTTAVCSPPVQSPPLAT-RTFHY  
 HCN1\_SQUIRREL RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPPCSYTTAVCSPPVQSPPLTT-RTFHY  
 HCN1\_HORSE RTQSPPVYSATSLSHSNLHSPSPSTQTPQSSAILSPPCSYTTVVCSPPVQSPPLAT-RTFHY  
 HCN1\_COW RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPPCSYTTAVCSPPVQSPPLAT-RTFHY  
 HCN1\_GALAGO RTQSPPVYTAPSLSHGNLHSPSPSTQTPQPSAILSPPCSYTTAVCSPPVQSPPLAS-RTFHY  
 HCN1\_DOG RTQSPPVYSAPSLSHGNLHSPSPSTQTPQPSAVLSPCSYTTAVCSPPVQSPPLAA-RTFHY  
 HCN1\_PANDA RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPPCSYTTAVCSPPVQSPPLAT-RTFHY  
 HCN1\_RABIT RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPPCSYTTAVCSPPVQSPPLAT-RTFHY  
 HCN1\_PLATYPUS RTQSPPVYTAPNLSHGNLHSPSTQPPQATILSPCSYTTAVCSPPVQSPPLAG-RTFQY  
 HCN1\_BIRD RTQSPPVYSTSSLSHNLHSPSPSTQPPQAVILSPCSYTTAVCSPPVQSPPLAG-RTFQY  
 HCN1\_CHICKEN RTQSPPVYTAGSLSHGNLHSPSPSTQTPQAVILSPCSYTTAVCSPPVQSPPLAG-RTFQY  
 HCN1\_ANOLE RTQSPPVYSMTSLSHGSLNSPTQTPQAAIFSPCSYSSAVCSPPVQSPPLAAGRTFQY  
 HCN1\_FROG RTRSPPVYNANSLSHGNLQSPPTSPQTPQAVVFPSSFTSAACSPSVQSPPLAG-RTFQY  
 HCN1\_PIG -----  
 HCN1\_TILAPIA AAAQGLYQTSQSG---TLNSSQT---PLQQGAIMSPCSFTAAMCSPPVQTP-LANRSFQY  
 HCN1\_PLATYFISH AVAQGGFPTSSLSQVNLNSSQ---TALQQGAIMSPCSFTAAMCSPPVQTP-LASRSFQY  
 HCN1\_TETRAODON AAAQAGYPPPSLSHSSLNSSLTNPQALQQAAIMSPCSFTAAMCSPPVQTP-LANRSFQY  
 HCN1\_TROUT QGVYVPSVVIHGNLNSSSPVQTPPLSLHQQGSIMSPVFTTAVCSPPVQTPGLAGRSFQY  
  
 HCN1\_HUMAN ASPTASQLSLMQ-----QPQQVQQSQPPQTQ  
 HCN1\_MACAQUE ASPTASQLSLMQ-----QPQQVQQSQPPQTQ  
 HCN1\_ORANGUTAN ASPTASQLSLMQ-----QPQQVQQSQPPQTQ  
 HCN1\_MARMOSET ASPTASQLSLMQ-----QPQQVQQSQAPQTQ  
 HCN1\_MOUSE ASPTASQLSLMQPPQQQLPQSQVQQTQTQTQQQQQQQQQQQQQQQQQQQQQQQQQQQ  
 HCN1\_RAT ASPTASQLSLMQPPQPQLQSSQVQQTQTQTQQQQQQQQQPQPQPQQPQQQQQQQQQQQQQ  
 HCN1\_SQUIRREL ASPTASQLSLMQ-----QQQQQQQLQSSQAPQTQPQ  
 HCN1\_HORSE ASPTASQLSLMQ-----QQQLQPSQSPQ  
 HCN1\_COW ASPTASQLSLIQ-----QQVQPPQPQ  
 HCN1\_GALAGO ASPTASQLSLMQ-----QLSQPPQT  
 HCN1\_DOG ASPTASQLSLAQPPQP-----AQPPQPQPAPQPPQPQ  
 HCN1\_PANDA ASPTASQLSLTQQQ-----QQLQPPPPQ  
 HCN1\_RABIT ASPTASQLSLMPQ-----QQQPQAPQTQ  
 HCN1\_PLATYPUS VSPTASQLSLMQ-----Q  
 HCN1\_BIRD ASPTASQLSLMQQQQ-----QQQGGGGGGGGGGG  
 HCN1\_CHICKEN ASPTASQLSLMQQ-----  
 HCN1\_ANOLE ATPTASQLSLLQ-----  
 HCN1\_FROG SSLTASQLSLIQQQQ-----  
 HCN1\_PIG -----  
 HCN1\_TILAPIA ASPTASQLSLIQPIA-----QPSLSTQQLSQPAAPTA  
 HCN1\_PLATYFISH GSPTASQLSLIQPIG-----QPSLPTQQQLGQQAAS  
 HCN1\_TETRAODON GSRTASQLSLIQPTG-----QPTLPTQQPP-----  
 HCN1\_TROUT GSPTASQLSLIQPLP-----TALPPQPPLPQPQPQGGG  
  
 HCN1\_HUMAN PQQPSPQPQTP-----GSSTPKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE  
 HCN1\_MACAQUE PQQPSPQPQTP-----GSSTPKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE  
 HCN1\_ORANGUTAN PQQPSPQPQTP-----GSSTPKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE  
 HCN1\_MARMOSET PQQPSPQPQTP-----GSSTPKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE  
 HCN1\_MOUSE QQQQQQPQTP-----GSSTPKNEVHKSTQALHNTNLTKEVRPLSASQPSLPHE  
 HCN1\_RAT QQQQQQPQTP-----GSSTPKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE  
 HCN1\_SQUIRREL QQQPPQPQTP-----SSSTPKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE  
 HCN1\_HORSE PQQPQPQTP-----SSSTPKNDVHKSTQALHNTSLTRDVRPLSASQPSLPHE  
 HCN1\_COW Q---PPQPQTP-----GSSTPKNEVHKSTQALHNTSLTREVRPLSASQPSLPHE  
 HCN1\_GALAGO QPPPPPPQTP-----GSSTPKNEVHKSTQALHNASTREVRPLSASQPSLPHE  
 HCN1\_DOG PPQPQPQPQPQPQPQPQPQPQPQPQGGGSTPRNDVHRSTQALHNASTREVRPLSASQPSLPHE  
 HCN1\_PANDA PPQPQPQPQTP-----STSTPRNDVHRSTQALHNTSLTREVRPLSASQPSLPHE  
 HCN1\_RABIT PQQPPQPQTP-----GSATPKNEVHRSTQALPNTSLTREVRPLSASQPSLPHE  
 HCN1\_PLATYPUS QPPSQQPQPP-----PSSAQKNEVHKSTQALHNTNLTREARPLSASQPSLPHE  
 HCN1\_BIRD GQQQPQAAQQP-----PGAAQKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE  
 HCN1\_CHICKEN ---PQAPQLP-----PGTAPKNEVHKSTQALHNTNLSREVRPLSASQPSLPHE  
 HCN1\_ANOLE QQLQQQPQPQ-----LQQMPSASQKSDVHKSTQALHNASTREVRPLSASQPSLPHE  
 HCN1\_FROG -----ASGASQIHEVHKSAQALANTNLTREVRPLSASQPSLPHE  
 HCN1\_PIG -----  
 HCN1\_TILAPIA ATSSAAQQQQQQQASPPQQQVPSQRGD-IHKSTQALQSGSLSRDVRHLSASQPSLPHE

HCN1\_PLATYFISH TAAAAS-----AAQQQVPSQQRGD-IHKSTQALQSGSLSRDVRHLSASQPSLPHD  
HCN1\_TETRAODON -----ASATASAPSPQQRGD-IPKGAQALQSGSLSRDVRHLSASQPSLPHE  
HCN1\_TROUT AASSATQQ-----PQQQQVPSQQRSDSLHKASHALQSGSLSRDVRHLSASQPSLPHD

HCN1\_HUMAN VSTLISRPHPTVGESLASIPQPVTAVPGTGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGV  
HCN1\_MACAQUE VSTLISRPHPTVGESLASIPQPVTAVPGTGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGV  
HCN1\_ORANGUTAN VSTLISRPHPTVGESLASIPQPVTAVPGTGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGV  
HCN1\_MARMOSET VSTLISRPHPTVGESLASIPQPVTAVPGTGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGV  
HCN1\_MOUSE VSTLISRPHPTVGESLASIPQPVAAVHSTGLQAGSRSTVPQRVTLFRQMSSGAIPPNRGV  
HCN1\_RAT VSTMISRPHPTVGESLASIPQPVAAVHSTGLQAGSRSTVPQRVTLFRQMSSGAIPPNRGV  
HCN1\_SQUIRREL VSTLISRPHPTVGESLASIPQPVAAVHSTGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGV  
HCN1\_HORSE VSTLISRPHPTVGESLASIPQPVTAVHGTGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGV  
HCN1\_COW VSTLISRPHPTVGESLASIPQPVTAVHGTGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGV  
HCN1\_GALAGO VSTLISRPHPTVGESLASIPQPVTAVPGPGLQAAGRSSVPQRVTLFRQMSSGAIPPNRGV  
HCN1\_DOG VATLMSRPHPTVGESLASIPQPVTAVHGPGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGA  
HCN1\_PANDA VSTLISRPHPTVGESLASIPQPVS AVHGPGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGA  
HCN1\_RABIT VSTLISRPHPTVGESLASIPQPVA AVHSTGLQAAGRSTVPQRVTLFRQMSSGAIPPNRGV  
HCN1\_PLATYPUS VSTPISRPHPTVGESLASLPPASGAQAAGFQAGSRGSPVPRVTLFRQMSSGAIPPHRGV  
HCN1\_BIRD ISTLIARPHPTVGESLASLPPAPGP---GVPPGGRAGVPQRVSLFRQMSSGAIPPHRAA  
HCN1\_CHICKEN ISTLIARPHPTVGESLASLPPAPGT---SVPPASRATVPQRVSLFRQMSSGAIPPNRGA  
HCN1\_ANOLE ISMMIARPHPTVGESLASIPQPLSGFQSTGLQAGNKGTVPQRVALFRQMSSGAIPPTRGA  
HCN1\_FROG ISTLGSKPHPTVGESLASLPPISNVQAAGTQSGSRSNIPPRVALFRQMSSGALPPARVG  
HCN1\_PIG -----  
HCN1\_TILAPIA TSLGPRVHPTSGDSLASIVPPTAAVIQGMNLQSGIRTTVPQRVNLFRQMSSGALPPVRS  
HCN1\_PLATYFISH TSLGPRAPASGESLASMVPPTAAAIQGISLQGGIRTTVPQRVSLFRQMSSGALPPVRS  
HCN1\_TETRAODON ASLGPRAPASGDSLASIA PPTAAAIQGMNLQGGIRTTVPQRVNLFRQMSSGALPPVRS  
HCN1\_TROUT TSLGPRAPAAASGDSLASIA PPTAAAVQMGIQSGLRTTVPQRVNLFRQMSSGALPPVRAV

HCN1\_HUMAN PPAPPPAAALPRES-----SSVLNT--DPDAEKPRFASNL  
HCN1\_MACAQUE PPAPPPAAALPRES-----SSVLNT--DPDAEKPRFASNL  
HCN1\_ORANGUTAN PPAPPPAAALPRES-----SSVLNT--DPDAEKPRFASNL  
HCN1\_MARMOSET PPAPPPAAALPRES-----SSVLNT--DPDAEKPRFASNL  
HCN1\_MOUSE PPAPP--PAAVQRES-----PSVLNT--DPDAEKPRFASNL  
HCN1\_RAT PPAPP--PAAVQRES-----PSVLNK--DPDAEKPRFASNL  
HCN1\_SQUIRREL PPAPPPAAALQREP-----SSVLNT--DPDAEKPRFASNL  
HCN1\_HORSE PPAPPPAAALPRES-----SSVLST--DPDAEKPRFASNL  
HCN1\_COW PPAPPPAAAHPREA-----PSVLTT--DSEAEKPRFASNL  
HCN1\_GALAGO PPAPPPAAALQREA-----SSVLNT--DPEAEKPRFASNL  
HCN1\_DOG PPAPPPAAALPRES-----SSVLTT--DPEAEKPRFASNL  
HCN1\_PANDA PPAPPPAAALPRES-----SSVLTT--DPEAEKPRFASNL  
HCN1\_RABIT PPAPPPAAALQREA-----SSVLNT--DPEAEKPRFASNL  
HCN1\_PLATYPUS VPPGPPTGAPLQRDT-----SGVLNA--EPEGDKPRFASNL  
HCN1\_BIRD APPAAPLQKIGRAVQ-----QECRDRYRMPEGDKPRFASNL  
HCN1\_CHICKEN VPPAAPLPRDSSAVL-----STE-----PEGDKPRFASNL  
HCN1\_ANOLE APSAATLQRDSSTVL-----NTELEG-----DKPRFASNL  
HCN1\_FROG ATTQPSAPPTKDSS-----TVSSTDT---DTDKLKYASNL  
HCN1\_PIG -----  
HCN1\_TILAPIA AAAAAAAAAVAASSSSTQHRDSPGSRSDSVLSSTETEQDKMRFASNL  
HCN1\_PLATYFISH ASAVA AVAAASSSTS-TQHRESPGSRSDSVLSSTETEQDKMRFASNL  
HCN1\_TETRAODON AAAAAAAAAASSVSSS-----STQQSTETEQDKLRFSSNL  
HCN1\_TROUT SSAAQHRDSTGSRD-----SRRDSTLSSTETEQDKMRFASNL

**(b)**

HCN3\_HUMAN -----  
HCN4\_HUMAN MDKLPPSMRKRLYSLPQQVGAKAWIMDEEEDAE EEGAGGRQDPSRRSIRLRPLPSPSPSA  
HCN2\_HUMAN -----MDARGGGGRPGESPGATPAPGPPPPPPAPPQQQP  
HCN1\_HUMAN -----

HCN3\_HUMAN -----  
HCN4\_HUMAN AAGGTESSALGAADSEGPARGAGKSSSTNGDCRRFRGSLASLGSRRGGSGGTGSGSSHG  
HCN2\_HUMAN PPPPPAPP PGPAPPQHPPRAEALPPEAADEGGPRGLRSDSSCGRPGTPGAASTAK  
HCN1\_HUMAN -----MEGGGKPNSSNSRDDGNSVFPKASATG

HCN3\_HUMAN -----MEAEQRPAAGASEGATPGLEAVPPVAPPATAASGPIPKSGP  
HCN4\_HUMAN HLHDSAEERLLIAEGDASPGEDRTPPGLAAEPERPGASAQPAASPPPPQPPQPPASASCE  
HCN2\_HUMAN GSPNGECGRGEPQCSAPGEPARGPKVFSFCRGAASGPAPGPPAE EAGSEEAGPAGEP  
HCN1\_HUMAN AGPAAA EKR LGT PPGGGGAGAKA EHGNSVCFKVDGGGGGGGGGGGEEEPAGGFEDAEGPRR

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HCN3_HUMAN      EP-----KRRHLLGTTLLQPTVKNKFSLRVFGSHKAV
HCN4_HUMAN      QPSVDTAIKVEGGAAAGDQILPEAEVRLGQAGFMQRQFGAMLQPGVKNKFSLRMFGSQKAV
HCN2_HUMAN      RGS-----QASFMQRQFGALLQPGVKNKFSLRMFGSQKAV
HCN1_HUMAN      QYG-----FMQRQFTSMLQPGVKNKFSLRMFGSQKAV
               :*: :*: **
               .

HCN3_HUMAN      EIEQERVKSAGAWIIHPYSDFRFYWDLIMLLLMVGNLIVLPVGITFFKEENSPPWIVFNV
HCN4_HUMAN      EREQERVKSAGAWIIHPYSDFRFYWDLTMLLLMVGNLIIIPVGITFFKDETTWPVIVFNV
HCN2_HUMAN      EREQERVKSAGAWIIHPYSDFRFYWDFTMLLFMVGNLIIIPVGITFFKDETTAPWIVFNV
HCN1_HUMAN      EKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIFNW
* * * * * : * * * * * : * * * * * : * * * * * : * * * * * : * * * * *
               . . . . .

HCN3_HUMAN      LSDTFFLLDLVLFNRFTGIVVEEGAEILLAPRAIRTRYLRWFLVDLISSIPVDYIFLIVE
HCN4_HUMAN      VSDTFFLIDLVLNFRFTGIVVEDNTEIILDPRQIKMKYLKSWFMVDFISSIPVDYIFLIVE
HCN2_HUMAN      VSDTFFFLMDLVLNFRFTGIVIEDNTEIILDPEKIKKKYLRTWFVDFVSSIPVDYIFLIVE
HCN1_HUMAN      ASDTVFLLDLIMNFRFTGTVNEDSSEIILDPKVIKMNLYKSWFVDFISSIPVDYIFLIVE
               * * . * * : * * : * * * * * : * * : * * : * * : * * : * * : * *

HCN3_HUMAN      LEPRLDAEYVKTARALRIVRFTKILSLRLLRLSRLIRYIHWQEEIFHMTYDLASAVVRI
HCN4_HUMAN      TR--IDSEYVKTARALRIVRFTKILSLRLLRLSRLIRYIHWQEEIFHMTYDLASAVVRI
HCN2_HUMAN      KG--IDSEYVKTARALRIVRFTKILSLRLLRLSRLIRYIHWQEEIFHMTYDLASAVMRI
HCN1_HUMAN      KG--MDSEYVKTARALRIVRFTKILSLRLLRLSRLIRYIHWQEEIFHMTYDLASAVVRI
               : * : * * * * * * * * * * * * * * * * * * * * * * * * * * * *

HCN3_HUMAN      FNLI G M L L L C H W D G C L Q F L V P M L Q D F P D C W V S I N H M V N H S W G R Q Y S H A L F K A M S H M L C
HCN4_HUMAN      V N L I G M M L L L C H W D G C L Q F L V P M L Q D F P D C W V S I N N M V N N S W G K Q Y S Y A L F K A M S H M L C
HCN2_HUMAN      C N L I S M M L L L C H W D G C L Q F L V P M L Q D F P R N C W V S I N G M V N H S W S E L Y S F A L F K A M S H M L C
HCN1_HUMAN      F N L I G M M L L L C H W D G C L Q F L V P L L Q D F P D C W V S L N E M V N D S W G K Q Y S Y A L F K A M S H M L C
               * * * . * * * * * * * * * : * * * * * : * * * * * : * * . * * . . * * . * * * * * * * * *

HCN3_HUMAN      IGYGQQAPVGM P D V W L T M L S M I V G A T C Y A M F I G H A T A L I Q S L D S S R R Q Y Q E K Y K Q V E Q Y M
HCN4_HUMAN      IGYGRQAPV G M S D V W L T M L S M I V G A T C Y A M F I G H A T A L I Q S L D S S R R Q Y Q E K Y K Q V E Q Y M
HCN2_HUMAN      IGYGRQAPESMTDIWLTMSMIVGATCYAMF IGHATALIQSLDSSRRQYQEKYKQVEQYM
HCN1_HUMAN      IGYGAQAPVMSDLWITMSMIVGATCYAMFVGHATALIQSLDSSRRQYQEKYKQVEQYM
               * * * * * * * . * . * : * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

HCN3_HUMAN      SFHKL PADTRQRIHEY EYEHRYQGMFDEESILGELSEPLREEIINFTCRGLVAHMPLFAN
HCN4_HUMAN      SFHKLPPDTRQRIHDY EYEHRYQGMFDEESILGELSEPLREEIINFNCRKLVASMPLFAN
HCN2_HUMAN      SFHKL PADFRQKIHDIY EYEHRYQGMFDEESILGELNGPLREEIVNFNCRKLVASMPLFAN
HCN1_HUMAN      SFHKL PADMRQKIHDIY EYEHRYQKIFDEENILNELNDPLREEIVNFNCRKLVATMPLFAN
               * * * * * . * * : * * : * * * * * * * * * * * : * * . * * . * * * * * : * * . * * * * * * * * * :

HCN3_HUMAN      ADPNFVTVAVLTKLRFEV F Q P G D L V V R E G S V G R K M Y F I Q H G L L S V L A R G A R D T R L T D G S Y F
HCN4_HUMAN      ADPNFVTSMLTKLRFEV F Q P G D Y I I R E G T I G K K M Y F I Q H G V V S V L T K G N K E T K L A D G S Y F
HCN2_HUMAN      ADPNFVTAMLTKLRFEV F Q P G D Y I I R E G T I G K K M Y F I Q H G V V S V L T K G N K E M K L S D G S Y F
HCN1_HUMAN      ADPNFVTAMLSKLRFEV F Q P G D Y I I R E G A V G K K M Y F I Q H G V A G V I T K S S K E M K L T D G S Y F
               * * * . * * * : * * : * * : * * * * * * * * * : * * * * * : * * * * * : * * : . . : : * * : * * * * *

HCN3_HUMAN      GEICLLTRGRRTASVRADTYCRLYSLSDVDFNAVLEEFPMRRAFETVAMDRLLRIGKKN
HCN4_HUMAN      GEICLLTRGRRTASVRADTYCRLYSLSDVDFNEVLEEYPMRRAFETVALDRDLRIGKKN
HCN2_HUMAN      GEICLLTRGRRTASVRADTYCRLYSLSDVDFNEVLEEYPMRRAFETVAIDRLDRIGKKN
HCN1_HUMAN      GEICLLTKGRRTASVRADTYCRLYSLSDVDFNEVLEEYPMRRAFETVAIDRLDRIGKKN
               * * * * * : * * * * * * * * * * * * * * * * : * * * * * : * * * * * * * * * : * * * * * * * * *

HCN3_HUMAN      SILQRKRSEPSPG---SSGIMEQHVLVQHDRDMARGVRGRAPSTGAQLSGKPVLWEPLV
HCN4_HUMAN      SILLHKVQHDLNSGVFNQENEI IQQIVQHDREMAHCAHRVQAAAATPTPTPIWTPV
HCN2_HUMAN      SILLHKVQHDLNSGVFNQENAI IQEIVKYDREMVQQAELGQRVGLFPFPPPPPPVQVTSAI
HCN1_HUMAN      SILLQKFQKDLNTGVFNQENEILKQIVKHDREMVQAIAPINYPQMTTLNSTSS' TTPTS
               * * * : * . . . . . : : . : * * : * * : * . : . .

HCN3_HUMAN      HAPLQAAA V T S N V A I A L T H Q -----
HCN4_HUMAN      QAPLQAAA A T T S V A I A L T H H P R L P A A I F R P P P G S G L G N L G A G Q T P R H L K R L Q S L I P S A L G
HCN2_HUMAN      A T L Q Q A A M S F C P Q V A R P L V G -----
HCN1_HUMAN      R M R T Q S P P V Y T A T S L S H S N L H S P S P S T Q T P Q S A I L S P C S Y T T A V C S P P V Q S P L A A R T F H
               * : . . . : : .

HCN3_HUMAN      -----
HCN4_HUMAN      SASPASSPSQVDT P S S S S F H I Q Q L A G F S A P A G L S P L L P S S S S P P P G A C G S P S A P T P S A G
HCN2_HUMAN      -----
HCN1_HUMAN      Y A S P T A S Q L S L M Q Q Q P -----

HCN3_HUMAN      -----
HCN4_HUMAN      V A A T T I A G F G H F H K A L G G S L S S D S P L L T P L Q P G A R S P Q A A Q P S P A P P G A R G G L G L P E H F
HCN2_HUMAN      -----

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HCN1_HUMAN -----
HCN3_HUMAN -----RGPLPLSPDSPATLLARSAWRSAGSPASPLV
HCN4_HUMAN LPPPPSSRSPSSSPGQLGQPPGELSLGLATGPLSTPETPPRQPEPPSLVAGASGGASPVG
HCN2_HUMAN -----PLALGSPRLVRRPPPGPAPAAAASPGPPPP
HCN1_HUMAN -----QQVQQSQPPQTQPQQPSPQPQTPGSSTPKN
                               : . : . . *

HCN3_HUMAN PVRAGPWASTSRLPAPPARTLHASLSRAGRSQVSLLGPPP-----
HCN4_HUMAN FTPRGGLSPPGHSPGPPRTFPSAPPRASGSHGSLLLPPASSPPPPQVPQRRGTPPLTPGR
HCN2_HUMAN ASPPGAPASPRAPRTSPYGGLPAAPLAGPALPARRLSRAS-----
HCN1_HUMAN EVHKSTQALHNTNLTREVRPLSASQPSLPHEVSTLISRPHPTVG-----
          . : * . : .

HCN3_HUMAN -----GGGGRRLGPR
HCN4_HUMAN LTQDLKLISASQPALPQDGAQTLRRASPHSSGESMAAFPLFPRAGGGSGGSGSSGGLGPP
HCN2_HUMAN -----RPLSASQP
HCN1_HUMAN -----ESLASIQPVTA VPGTGL

HCN3_HUMAN GRPLSASQPSLPQRATGDGSPGRKGSERLPPSGLLAKPPRTAQPPRPPVPEPATPRGL
HCN4_HUMAN GRPYGAIPGQHVTLPKRTSSGSLPPLSLFGARATSSGGPPLTAGPQREPGARPEPVRSK
HCN2_HUMAN SLPHGAPGPAASTRPASSSTPRLGPTPAARAAAAPSPDRRDSASPGAAGGLDPQDSARSRL
HCN1_HUMAN QAGGRSTVPQRVTLFRQMSSGAIPPNRGVPPAPPPPAALPRES SSVLNTDPDAEKPRFA
          : . : . . . . .

HCN3_HUMAN QLSANM
HCN4_HUMAN LPSNL-
HCN2_HUMAN SSNL--
HCN1_HUMAN SNL---

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Multiple pairwise alignments of HCN1 orthologous (a) and paralogous (b) sequences were performed using Clustalw 2.1 (<http://www.genome.jp/tools/clustalw/>). The amino acids altered by the mutations are highlighted.