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De novo mutations in HCN1 cause early infantile epileptic encephalopathy

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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 Ih 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 *HCN1* 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 *HCN1* mutations. The screening of follow-up cohorts comprising 157 patients in total identified four additional amino-acid substitutions. Patch-clamp recordings of Ih 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 HCN1* 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[1,2]. EEIEs typically result from *de novo* dominant mutations in a single autosomal gene, although autosomal recessive and X-linked forms also exist. Dravet syndrome, anintractable 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)[3]. Dravet syndrome is mostly caused by *de novo* mutations of *SCN1A,* encoding the neuronal voltage-gated sodium α1 ($Na_v1.1$) channel[4,5]. 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[6,7].

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 *HCN1* (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 *HCN1* 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 *HCN1,* 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 *HCN1* (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)[8-10]. We recently identified a deletion spanning exon 4 of *HCN1* in a female with sporadic ID and autism spectrum disorder (ASD) but no epilepsy[11]. 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[12-14]. In neurons, HCN1 is mainly localized in dendrites[15]. HCN subunits have six transmembrane domains and functional channels consist of four subunits. HCN channels are permeable to Na^+ and K^+ ions and are activated by membrane hyperpolarization. In the brain, they conduct the Ih current, which contributes to spontaneous rhythmic activity and the stabilization of neuronal membrane potential against excitatory or inhibitory inputs[16]. 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[14].

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 (V1/2) 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 Ih 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[13,14,17,18]. 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[19,20]. 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[14,16,21,22]. *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)[23,24]. 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[25-29], and an upregulation of HCN channel function by some antiepileptic drugs[30,31]. However, rare heterozygous deletions encompassing *HCN1* exons exist in non-epileptic individuals, and *Hcn1*$^{-/-}$ mice have motor learning and memory deficits[32] and a higher susceptibility to induced seizures[33,34], but display no spontaneous seizures[32]. 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[13]. 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*$^{-/-}$ mice display spontaneous absence seizures[35,36], and a recessive loss-of-function mutation in *HCN2* was recently identified in one patient with IGE[37]. 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[13,22]. 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 $Na_v1.1$ and Nav1.7[38,39].

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, hemiclonic, 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[40,41]. 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[40,41], or as follows: sequencing reads passing quality filtering were aligned to the human reference genome (hg19) with Burrows-Wheeler Aligner (BWA)[42]. GATK[43] 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[44] mpileup, GATK UnifiedGenotyper and Dindel[45]. For annotating, comparing and filtering the data the GenomeComb[46] program was used. For the calling of *de novo* variants the DeNovoGear[47] program by Conrad and colleagues was used and double-checked by GenomeComb analysis.

Screening of *HCN1* by pyrosequencing

Exons and intron-exon junctions of *HCN1* (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 *HCN1* 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 equimolarily 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 AmpFl 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^{TM} 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 x 10^5 cells / 40-mm Petri dish for electrophysiological experiments and 2.0 x 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_2, 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 $CaCl_2$,

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 $MgCl_2$, 1.8 mM $CaCl_2$, 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:

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

where *It* is the current amplitude of the tail current recorded for a given pre-pulse and *It(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 ± SEM.

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Table 1 Genetic and clinical characteristics of the patients with *HCN1* 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	?	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*
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
Status epilepticus	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

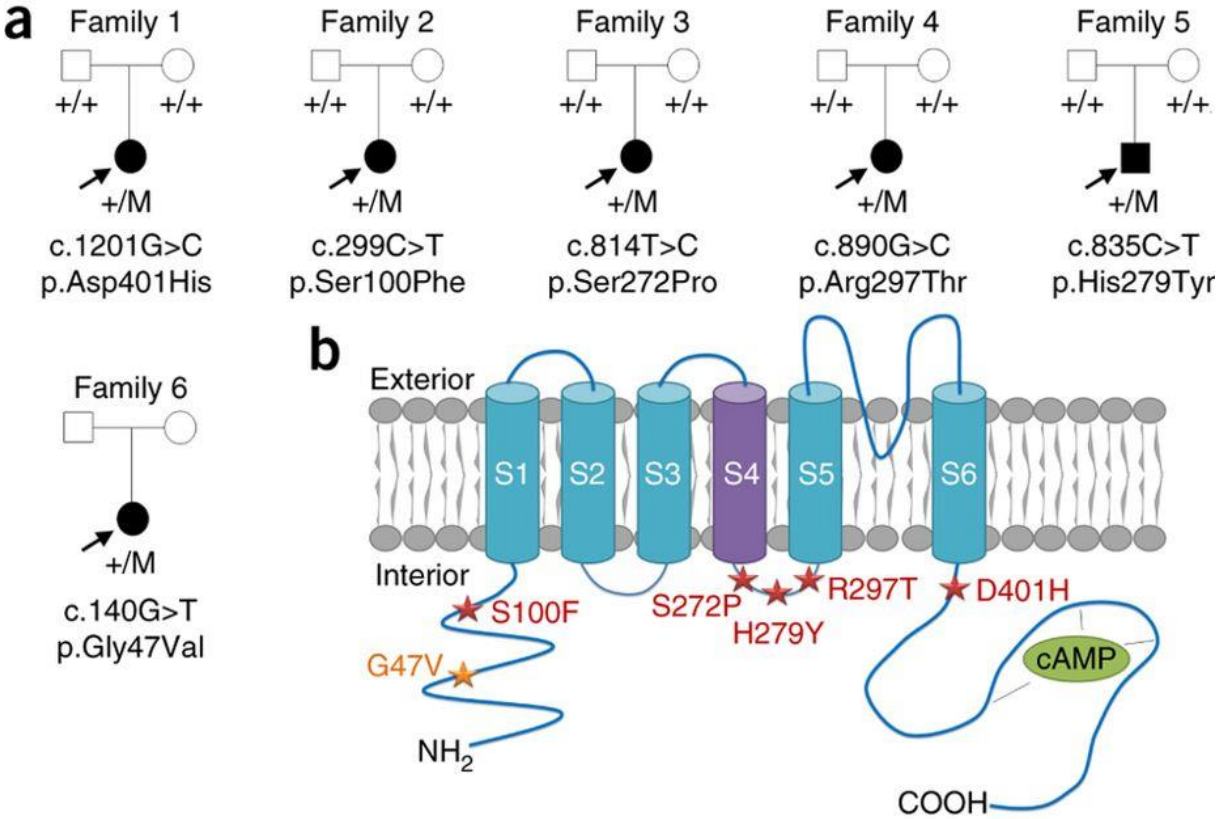

Figure 1 Identification of *HCN1* missense mutations. **(a)** Pedigrees and segregation analysis of the six *HCN1* missense variants identified in this study. The arrows indicate the probands. p.Ser100Phe, p.Ser272Pro, p.Arg297Thr, p.His279Tyr and p.Asp401His were absent from both parents, indicating 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).

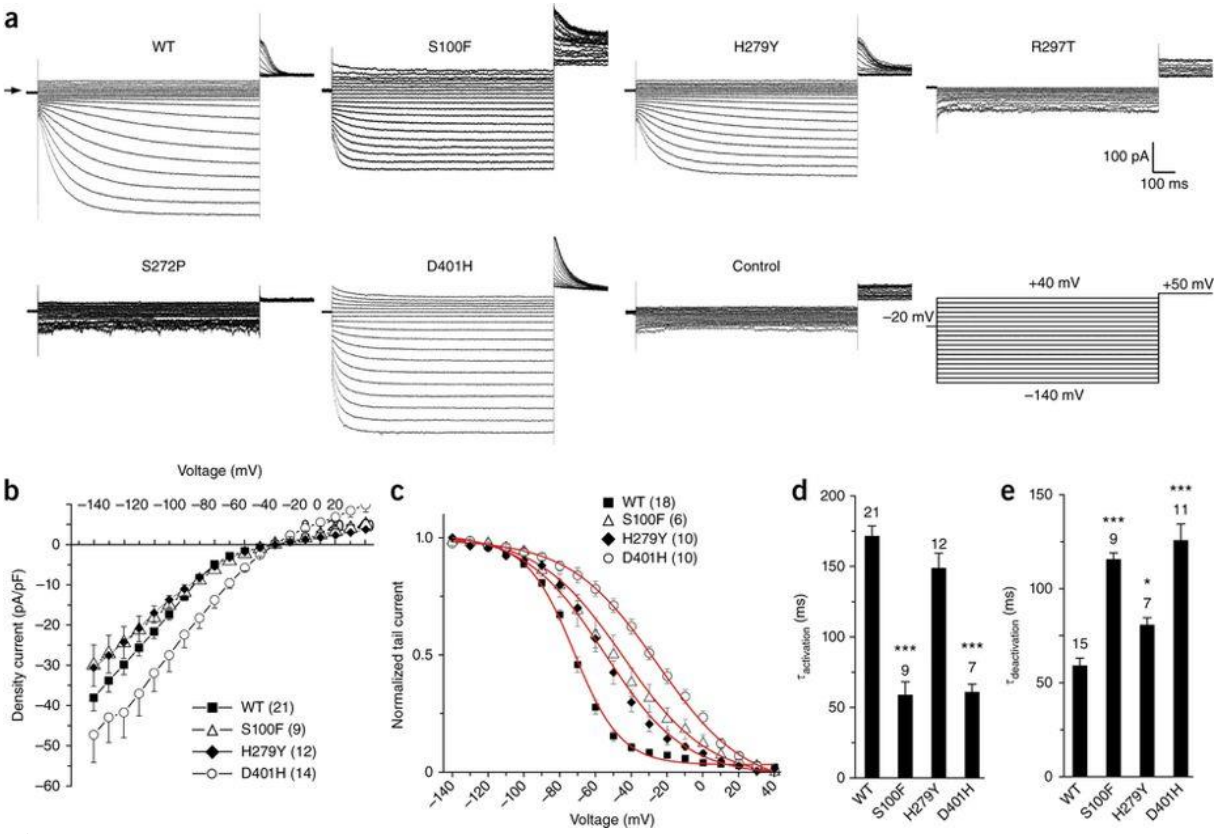

Figure 2 Patch-clamp analysis of the functional impact of the *de novo HCN1* mutations. **(a)** Representative traces of whole-cell currents recorded from CHO-K1 cells transfected with pEGFP, reflecting the endogenous current (control), WT, S100F, H279Y, R297T, S272P or D401H hHCN1 channels. The arrow 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 ± 0.73 mV, k = 13.1 ± 0.5 mV, S100F: -44.59 ± 1.91 mV, k = 23.9 ±1.3 mV; H279Y: -54.76 ± 1.49 mV, k = 22.4 ± 1.1 mV; D401H: -25.54 ± 0.96 mV, k = 24.5 ± 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 ± SEM, with the numbers of experiments indicated in brackets and SEM indicated by error bars.

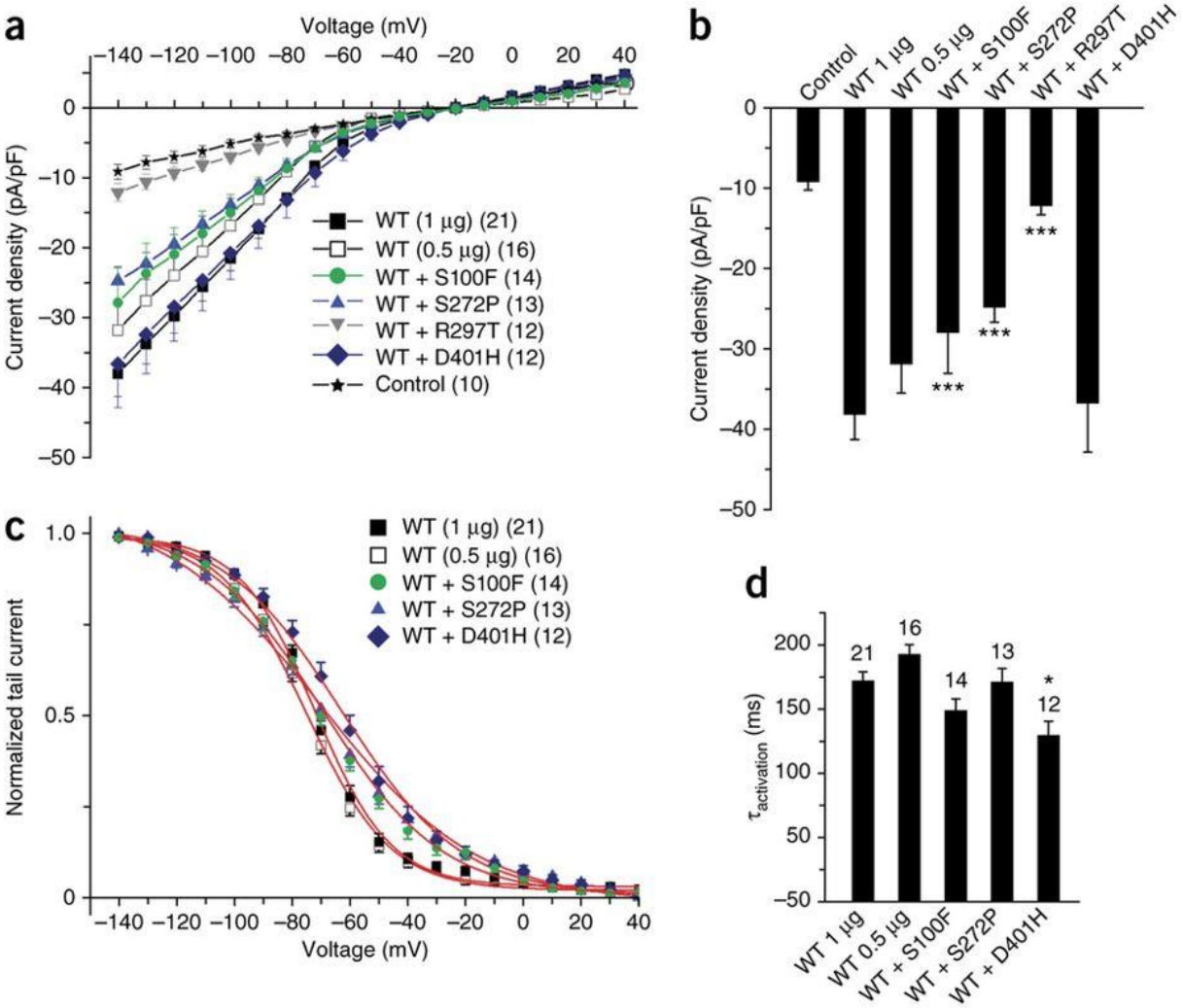

Figure 3 Data analysis of co-expression of WT and mutant hHCN1 channels with the patch clamp technique. **(a)** Plot of mean current density as a function of test voltage for CHO-K1 cells transfected with pEGFP, reflecting endogenous current (control), 0.5 µg WT, 1 µg WT, WT+S272P, WT+ R297T and WT+D401H hHCN1 channels. Identical amount of WT and mutant cDNA (i.e. 0.5 µg) were cotransfected. Current density of WT+S100F, WT+S272P, and WT+R297T hHCN1 channels were significantly different compared with current density of WT (1 µg) channels (two-way ANONA, *** $p<0.001$). **(b)** Bar graph of current densities for each experimental group at -140 mV. **(c)** Mean tail current activation curve for 0.5 µg WT, 1 µg 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 V1/2 and slope factor (k) for 1 µg WT: -72.06 ± 0.73 mV, k = 13.1 ± 0.5 mV; 0.5 µg WT: -75.22 ± 0.91 mV, k = 14.63 ± 0.7 mV; WT+S100F: -69.51 ± 0.99 mV, k = 20.7 ± 0.8 mV, WT+S272P: -70.38 ± 1.68 mV, k = 27.3 ± 1.4 mV; WT+D401H: -62.98 ± 1.33 mV, k = 20.8 ± 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 ± 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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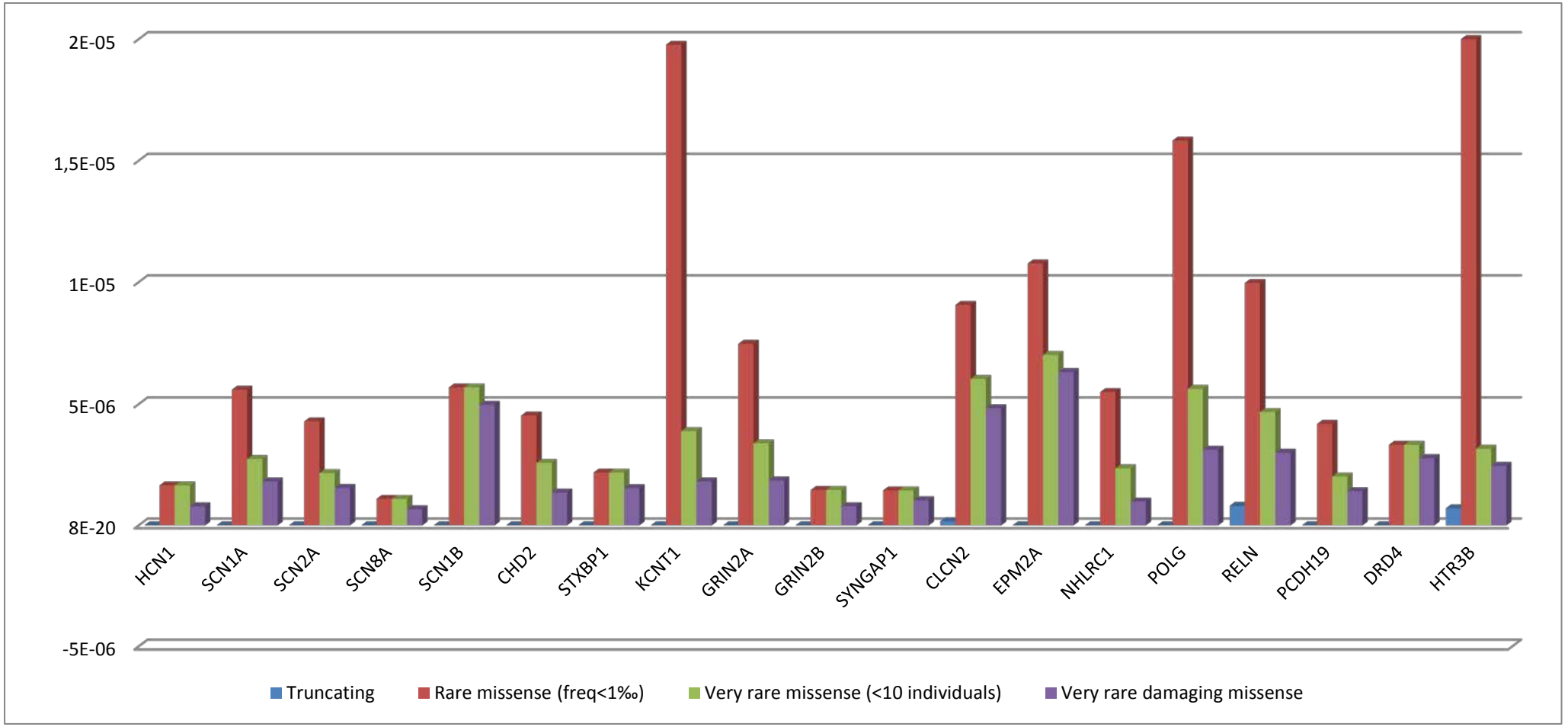
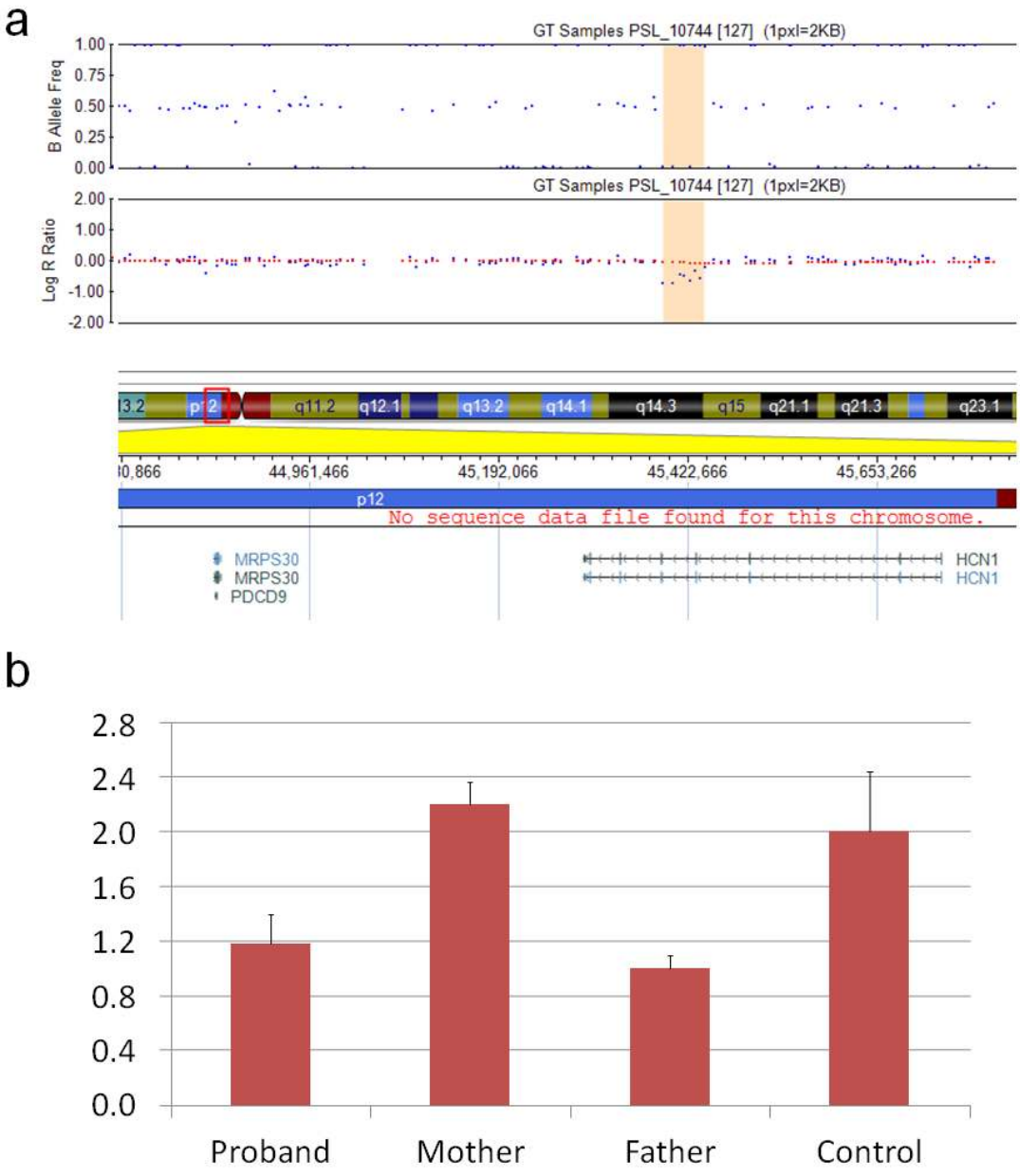
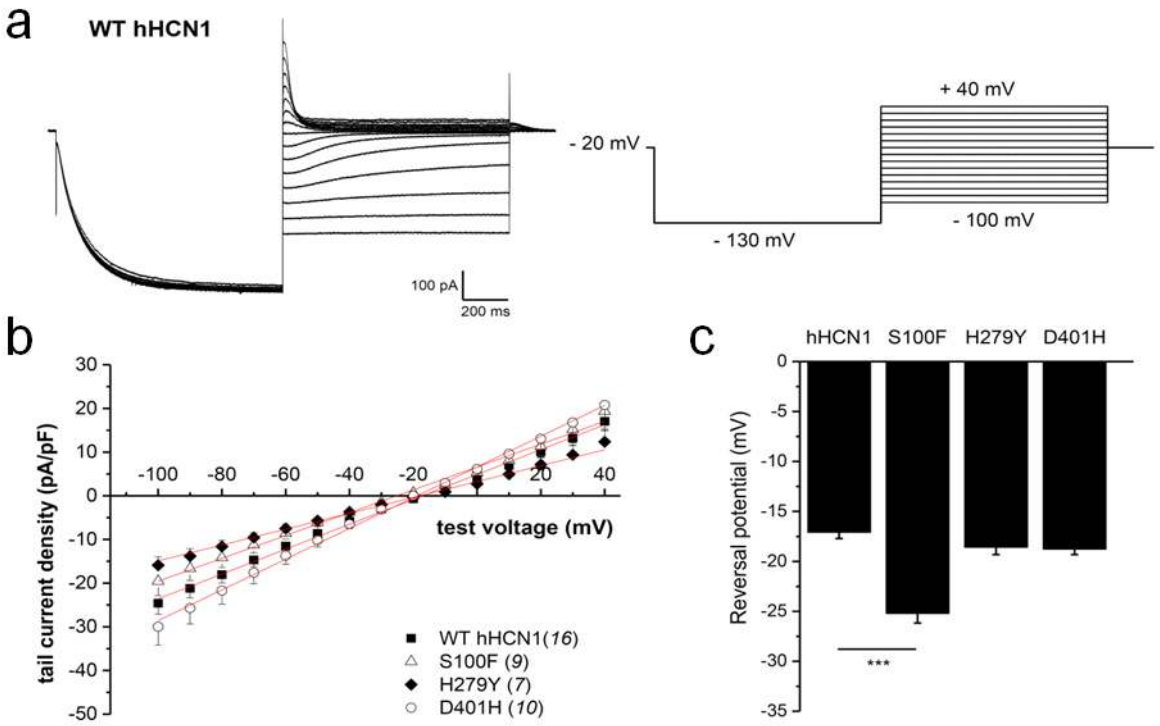


Supplementary Figure 1 Comparison of the mean number of rare variants per base and per individual between *HCN1* and selected genes expressed in the brain. The numbers of truncating variants (nonsense and splice site), rare missense variants (with MAF < 1‰), very rare missense variants (present in fewer 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/).

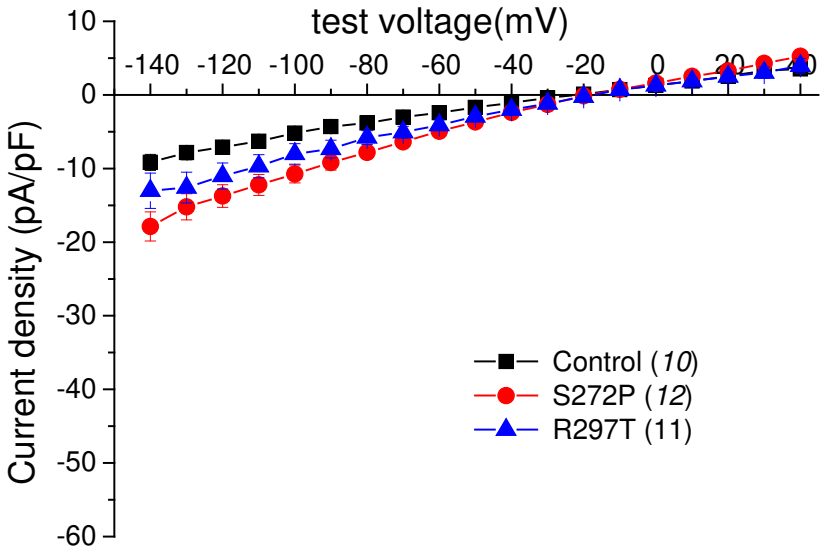


Supplementary Figure 2 Identification of a heterozygous deletion encompassing exon 4 of *HCN1* in a female subject with ID and ASD, inherited from her asymptomatic father. (**a**) Cyto-12 SNP array (Illumina) profiles of the patient with *HCN1* exon 4 deletion and details of the coding sequences included 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 µM of each primer and 12.5 µl of SYBR Green PCR master mix (Applied Biosystems) in a total volume of 25 µl. 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 $\Delta\Delta C_t = (C_{t\ \text{mutation}} - C_{t\ \text{RNaseP}})_{\text{ind tested}} - (C_{t\ \text{mutation}} - C_{t\ \text{RNaseP}})_{\text{ind ref}}$.

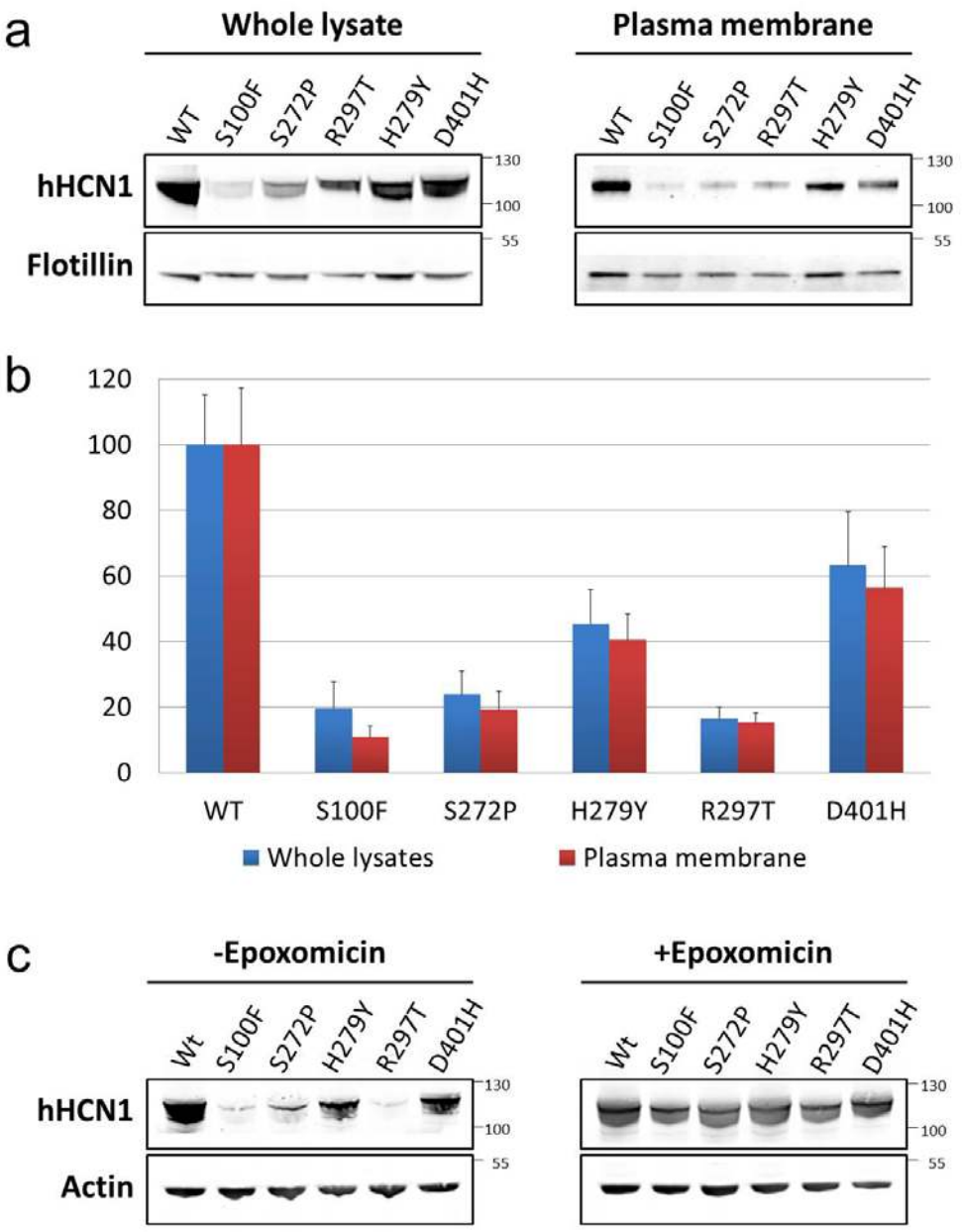
Supplementary Figure 3 Alignment of the regions flanking the six missense variants in orthologous proteins, showing the conservation of the altered amino acids. Multiple pairwise alignments were performed using ClustalW 2.1 (http://www.genome.jp/tools/clustalw/). The amino acids altered by the mutations 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 ANONA followed by a Dunnett's test, $^{***}P < 0.001$). Data are represented as mean values ± 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 ± 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 µg 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 ± 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 µM 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	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*
In silico 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.214-222delGGCGGCGGC, p.72delGGG; c.217-222delGGCGGC, p.73delGG) previously described in exon 1 of *HCN1*.[23,24]

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[48]. 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	CATTGCACAGTTGTTCATTGTAAA
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	TGAGAGTATTTCTTTCTGCTTTGACA

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

Supplementary Note Full alignments of HCN1 orthologous and paralogous sequences.

(a)

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HCN1_HUMAN          MEGGGKPNSSSNSRDDGNSVFPAKASATGAGPAAAEKRLGTPPGGGGAGAKEHGNSVCFK
HCN1_MACAQUE        MEGGGKPNSSSNSRDDGNSVFPAKASATGAGPAAAEKRLGTPPGGGGAGAKEHGNSVCFK
HCN1_ORANGUTAN      MEGGGKPNSSSNSRDDGNSVFPAKASATGAGPAAAEKRLGTPPGGGGAGAKEHGNSVCFK
HCN1_MARMOSET       MEGGGKSNSSSNSRDDGNSVFPAKASATGAGPAAAEKRLGTPPGGGGAGAKEHGNSVCFK
HCN1_MOUSE          MEGGGKPNSASNSRDDGNSVFPSKAPATGP---VAADKRLGTPPGGGAAGKEHGNSVCFK
HCN1_RAT            MEGGGKPNSASNSRDDGNSVYPSKAPATGP---AAADKRLGTPPGGGAAGKEHGNSVCFK
HCN1_SQUIRREL       ------------------------------------------------------XCFKVD
HCN1_HORSE          ------------------------------------------------------------
HCN1_COW            MEGGGKPNSSSNSRDDGNSVFPTKAPATGAGPAAAEKRLGTPPGGGGTGAKEHGNSVCFK
HCN1_GALAGO         MEGGGKPNSSSNSRDDGNSVFSAKAPAASAGPAGAEKRLGTPPGGGGAGAKEHGNSVCFK
HCN1_DOG            MEAGGKPNSASNSRDDGSSAFPGKAPATGAGPAAAEKRLGTPPGGGGAGAKELGNSVCFK
HCN1_PANDA          ------------------------------------------------------------
HCN1_RABIT          ------------------------------------------------------------
HCN1_PLATYPUS       ------------------------------------------------------------
HCN1_BIRD           -------------------------------------------GGGSGGVKEHGNSVCFK
HCN1_CHICKEN        ----------MEGGKRSSSPGSRDEGSANAFPGKQATPVEKAQSSPGGGAKEHGNSVCFK
HCN1_ANOLE          ---------MEGSKPSSSPGSREDGSPGANACFQGKAEKAPGSPGASGSLKEHANSVCFK
HCN1_FROG           ------------LPSMESKFNSSTNSSRDDGNNVLQGKAEKSLTASTSSVKEHGNSVCFK
HCN1_PIG            ----------------------MATASSPPRRPRRARGRQRPRSAWAPRRGAAGPARRS
HCN1_TILAPIA        ---------MEDKSNSFSSNKE-EKADGNNVFQRQDSIQKNNTGSQNMK--DHGNSVGFK
HCN1_PLATYFISH      ---------MEDKSNSFSSNKE-EKADGNNVFQRQDSIQKNNTGSQNTK--DHGNSVGFK
HCN1_TETRAODON      -------------------------------------------GSQNTK--EHSNSVGFK
HCN1_TROUT          ---------MEDKSNSFSSNKEGEKADGNNVFQRQDSIQKNNMGSQNMKGGDHGNSVGFK

HCN1_HUMAN          VDGGGGGGGGGGG--GEEPAGGFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_MACAQUE        VDGGGGGGG-------EEPAGGFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_ORANGUTAN      VDGGGGGEE---------PAGGFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_MARMOSET       VDGGGGGGGGGG----EEPAGGFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_MOUSE          VDGGG----------GEEPAGSFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_RAT            VDGGG----------GEEPAGSFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_SQUIRREL       GGGGG----------GEEPAGSFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_HORSE          ------------------------------------------------------------
HCN1_COW            VDGGGGGGE--------ESAGGFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_GALAGO         VDGGGGGAE--------ESAGGFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_DOG            VDGGGAGEE---------PAGGFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_PANDA          ---------------------------------GFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_RABIT          ----MATASSPPR--RPRRARGLEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_PLATYPUS       ------------------------------------------------------------
HCN1_BIRD           VDGGGGEEP----------AVGFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_CHICKEN        VDGGGAEEP----------VVGFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_ANOLE          VDGSGAAGEEPVAGLDDPDAAARRQQQQQQQQYGFMQRQFTSMLQPGVNKFSLRMFGSQK
HCN1_FROG           TDAADDPVVG-------------FEDAEGPSRPHGFMQRQFGSLMQPGVNKFSLRMFGSQK
HCN1_PIG            TATPCASRWTAAAAVAARSRPGASRTRRGPGGSTASCSGSHLYAAAWGQQILPPHVWEPE
HCN1_TILAPIA        GEREETMVG-------------FDDLEGASRQYGFMQRQFGAMMQPGVNKFSLRMFGSQK
HCN1_PLATYFISH      GEREEAMVG-------------FDDLDGASRQHGFMQRQFGAMMQPGVNKFSLRMFGSQK
HCN1_TETRAODON      GEREEAMVG-------------FDDLEGASRQHGFMQRQFGAMMQPGVNKFSLRMFGSQK
HCN1_TROUT          GDREEALVG-------------FDDIDGSGNRHGFMQRQFGAMMQPGVNKFSLRMFGSQK

HCN1_HUMAN          AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_MACAQUE        AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_ORANGUTAN      AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_MARMOSET       AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_MOUSE          AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_RAT            AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_SQUIRREL       AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_HORSE          ----------------------RFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_COW            AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_GALAGO         AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIVF
HCN1_DOG            AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_PANDA          AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_RABIT          AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_PLATYPUS       ----------------------------MLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_BIRD           AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_CHICKEN        AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_ANOLE          AVEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_FROG           AVEKEQERVKTAGVWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_PIG            GGEKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
HCN1_TILAPIA        AVEKEQERVQTAGYWIIHPYSDFRFYWDLIMLIMMMGNLIIIPVGITFFSEQTTTTWLVF
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HCN1_PLATYFISH      AVEKEQERVQTAGYWIIHPYSDFRFYWDLIMLIMMMGNLIIIPVGITFFSEQTTTTWLVF
HCN1_TETRAODON      AVEKEQERVQTAGYWIIHPYSDFRFYWDLIMLIMMMGNLIIIPVGITFFSDQNTTTWLVF
HCN1_TROUT          AVEKEQERVQTAGYWIIHPYSDFRFYWDLVMLVMMMGNLIIIPVGITFFSEQTTTTWLIF
                                          **:**:***:*********::*.**.*::*

HCN1_HUMAN          NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_MACAQUE        NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_ORANGUTAN      NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_MARMOSET       NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_MOUSE          NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_RAT            NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_SQUIRREL       NVASDTIFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_HORSE          NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_COW            NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_GALAGO         NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_DOG            NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_PANDA          NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_RABIT          NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_PLATYPUS       NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_BIRD           NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKIIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_CHICKEN        NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_ANOLE          NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_FROG           NVASDTVFLFDLFMNFRTGIVIEDNSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_PIG            NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLI
HCN1_TILAPIA        NVASDTIFLVDLVMNFRTGIVNEESSEIILDPKVIKMNYLKSWFVVDFLSSIPVDYIFLI
HCN1_PLATYFISH      NVASDTIFLVDLVMNFRTGIVNEESSEIILDPKVIKMNYLKSWFVVDFLSSIPVDYIFLI
HCN1_TETRAODON      NVASDTIFLVDLVMNFRTGIVNEESSEIILDPKVIKMNYLKSWFVVDFLSSIPVDYIFLI
HCN1_TROUT          NVASDTIFLVDLVMNFRTGIVNEESSEIILDPKVIKMNYLKSWFVVDFLSSIPVDYIFLI
                    ******:**.**.****** * *:.********:***************:***********

HCN1_HUMAN          VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_MACAQUE        VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_ORANGUTAN      VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_MARMOSET       VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_MOUSE          VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_RAT            VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_SQUIRREL       VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_HORSE          VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_COW            VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_GALAGO         VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_DOG            VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_PANDA          VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_RABIT          VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_PLATYPUS       VEKGMDSDVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_BIRD           VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_CHICKEN        VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_ANOLE          VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_FROG           VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_PIG            VEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_TILAPIA        VEKGFDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_PLATYFISH      VEKGFDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_TETRAODON      VEKGFDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN1_TROUT          VEKGFDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
                    ****:**:**************************************************

HCN1_HUMAN          FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_MACAQUE        FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_ORANGUTAN      FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_MARMOSET       FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_MOUSE          FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_RAT            FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_SQUIRREL       FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNKMVNDSWGKQYSYALFKAMSHMLC
HCN1_HORSE          FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_COW            FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_GALAGO         FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_DOG            FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_PANDA          FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_RABIT          FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_PLATYPUS       FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNGMVNNFYGTGYALVVFKMCFFMVP
HCN1_BIRD           FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNGMVNDSWGKQYSYALFKAMSHMLC
HCN1_CHICKEN        FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNGMVNDSWGKQYSYALFKAMSHMLC
HCN1_ANOLE          FNLIGMMLLLCHWDGCLQFLVPLLQDFPQDCWVSLNGMVNDSWGKQYSYALFKAMSHMLC
HCN1_FROG           FNLIGMMLLLCHWDGCLQFLVPLLQEFSPLYWVSLYLLVNDSWGKQYSYALFKAMSHMLC
HCN1_PIG            FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
HCN1_TILAPIA        FNLIGMMLLLCHWDGCLQYLVPLLQDFPPDCWVSLNGMVNVSWGKQYSYALFKAMSHMLC
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HCN1_PLATYFISH      FNLIGMMLLLCHWDGCLQYLVPLLQDFPPDCWVSLNGMVNVSWGKQYSYALFKAMSHMLC
HCN1_TETRAODON      FNLIGMMLLLCHWDGCLQYLVP-LQDFPPDCWVSLNGMVNVSWGKQYSYALFKAMSHMLC
HCN1_TROUT          FNLIGMMLLLCHWDGCLQFLVPLLQDFPQDCWVSLNGMVNDSWGKQYSYALFKAMSHMLC
                    ******************:*** **:*.   ****  :**  :*. *: .:**   .*:

HCN1_HUMAN          IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_MACAQUE        IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_ORANGUTAN      IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_MARMOSET       IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_MOUSE          IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_RAT            IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_SQUIRREL       IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_HORSE          IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_COW            IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_GALAGO         IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_DOG            IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_PANDA          IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_RABIT          IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_PLATYPUS       VKCLLPASVSEGSVLLTTLSALHTPPFFLYDSSFLGHSWGWLSNLENSEKEFNIKEYKQV
HCN1_BIRD           IGYGARAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_CHICKEN        IGYGARAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_ANOLE          IGYGARAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_FROG           IGYGARAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_PIG            IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_TILAPIA        IGYGARAPVSMSDLWVTMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_PLATYFISH      IGYGARAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_TETRAODON      IGYGARAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
HCN1_TROUT          IGYGARAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD----SSRRQYQEKYKQV
                    :     *.** ..: :* ** :  .. :    ..       *.    *.:.   ::****

HCN1_HUMAN          EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_MACAQUE        EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_ORANGUTAN      EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_MARMOSET       EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILSELNDPLREEIVNFNCRKLVATMP
HCN1_MOUSE          EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILSELNDPLREEIVNFNCRKLVATMP
HCN1_RAT            EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILSELNDPLREEIVNFNCRKLVATMP
HCN1_SQUIRREL       EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_HORSE          EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_COW            EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_GALAGO         EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLREXXXNFNCRKLVATMP
HCN1_DOG            EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_PANDA          EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_RABIT          EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_PLATYPUS       EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEDNILNELNDPLREEIVNFNCRKLVATMP
HCN1_BIRD           EQYMSFHKLPAEMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_CHICKEN        EQYMSFHKLPAEMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_ANOLE          EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEDSILNELNDPLREEIVNFNCRKLVATMP
HCN1_FROG           EQYMSFHKLPSDMRQKIHDYYEHRYQGKIFDEDNILNELNDPLREEIVNFNCRKLVATMP
HCN1_PIG            EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMP
HCN1_TILAPIA        EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILSELNDPLKEEIVNFNCRKLVATMP
HCN1_PLATYFISH      EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEDNILSELNDPLKEEIVNFNCRKLVATMP
HCN1_TETRAODON      EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEDNILSELNDPLKEEIVNFNCRKLVATMP
HCN1_TROUT          EQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEDNILSELNDPLKEEIVNFNCRKLVATMP
                    **********::*********************:.**.******:*   ************

HCN1_HUMAN          LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_MACAQUE        LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_ORANGUTAN      LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_MARMOSET       LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_MOUSE          LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_RAT            LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_SQUIRREL       LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_HORSE          LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_COW            LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_GALAGO         LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_DOG            LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_PANDA          LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_RABIT          LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_PLATYPUS       LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_BIRD           LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSNKELKLTD
HCN1_CHICKEN        LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSNKELKLTD
HCN1_ANOLE          LFANADPNFVTAMLSKLKFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_FROG           LFANADPNFVTAMLSKLKFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_PIG            LFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTD
HCN1_TILAPIA        LFANADPNFVTGMLSKLKFEVFQPNDYIIREGTVGKKMYFIQHGVASVITKFNKEMKLTD
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HCN1_PLATYFISH      LFANADPNFVTGMLSKLKFEVFQPNDYIIREGTVGKKMYFIQHGVVSVITKFNKEMKLTD
HCN1_TETRAODON      LFANADPNFVTGMLSKLKFEVFQPNDYIIREGTVGKKMYFIQHGVASVITKSNKEMKLTD
HCN1_TROUT          LFANADPNFVTGMLSKLKFEVFQPNDYIIREGTVGKKMYFIQHGVASVITKLNKEMKLTD
                    ***********.*****:******.*******:************..**** .**:****

HCN1_HUMAN          GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_MACAQUE        GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_ORANGUTAN      GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_MARMOSET       GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_MOUSE          GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_RAT            GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_SQUIRREL       GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_HORSE          GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_COW            GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_GALAGO         GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_DOG            GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_PANDA          GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_RABIT          GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_PLATYPUS       GSYFGAEICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_BIRD           GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_CHICKEN        GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_ANOLE          GSYFG-EICLLTKGRRTASVKADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_FROG           GSYFG-EICLLTKGRRTASVRAETYCRLYSLSVENFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_PIG            GSYFG-EICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_TILAPIA        GSYFG-EICLLTKGRRTASVRADTYCRLFSLSVDHFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_PLATYFISH      GSYFG-EICLLTKGRRTASVRADTYCRLFSLSVDHFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_TETRAODON      GSYFG-EICLLTKGRRTASVRADTYCRLFSLSVDHFNEVLEEYPMMRRAFETVAIDRLDR
HCN1_TROUT          GSYFG-EICLLTKGRRTASVRADTYCRLFSLSVDHFNEVLEEYPMMRRAFETVAIDRLDR
                    ***** ***************:*:*****:****::*************************

HCN1_HUMAN          IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIAP---------------
HCN1_MACAQUE        IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIAP---------------
HCN1_ORANGUTAN      IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIAP---------------
HCN1_MARMOSET       IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIAP---------------
HCN1_MOUSE          IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIPP---------------
HCN1_RAT            IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIPP---------------
HCN1_SQUIRREL       IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIAP---------------
HCN1_HORSE          IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIAP---------------
HCN1_COW            IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIPP---------------
HCN1_GALAGO         IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIAP---------------
HCN1_DOG            IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIAP---------------
HCN1_PANDA          IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIAP---------------
HCN1_RABIT          IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIAP---------------
HCN1_PLATYPUS       IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQTTAP---------------
HCN1_BIRD           IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQTIAP---------------
HCN1_CHICKEN        IGKKNSILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQTIAP---------------
HCN1_ANOLE          IGKKNSLLLQKFQKDLNTGVFNNQESEILKQIVKHDREMVQTVGP---------------
HCN1_FROG           IGKKNSILQQRFQKDLNTGIFNNQESEILKQMLTQDREMVEGHPS---------------
HCN1_PIG            IGTLLSFL-----------------------------------------------------
HCN1_TILAPIA        IGKKNSLLLQKFQKDLNAGVFNTQENELLKQIIRQDREMVMMVDRKQSVT----------
HCN1_PLATYFISH      IGKKNSLLLQKFQKDLNAGVFNTQENEILKQIIRQDREMVMMVDRKQSVT----------
HCN1_TETRAODON      IGKKNSLLLQKFQKDLNAGVFNTQENEMLKQIIRQDR------------------------
HCN1_TROUT          IGKKNSLLLQKFQKDLNAGVFNTQENEILKQIIRQDREMVMMVDRKQSVTGMSVTGMSVT
                    **.  *:*

HCN1_HUMAN          ---------------------------------------INYPQMTTLNSTSSTTTPTSRM
HCN1_MACAQUE        ---------------------------------------INYPQMTTLNSTSSTTTPTSRM
HCN1_ORANGUTAN      ---------------------------------------INYPQMTTLNSTSSTTTPTSRM
HCN1_MARMOSET       ---------------------------------------INYPQMTTLNSTPSTTTPTSRM
HCN1_MOUSE          ---------------------------------------INYPQMTALNCTSSTTTPTSRM
HCN1_RAT            ---------------------------------------INYPQMTALNCTSSTTTPTSRM
HCN1_SQUIRREL       ---------------------------------------INYPQMTALNSTSSTTTPTSRM
HCN1_HORSE          ---------------------------------------INYPQMTALNSTSSTTTPTSRM
HCN1_COW            ---------------------------------------LNYPQMTALNSTSSTTTPTSRL
HCN1_GALAGO         ---------------------------------------INYPQMTALNSTASTTTPTSRV
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           ---------------------------------------VSYQQLPALN---SSTSSSLRM
HCN1_PIG            -------------------------------------------------------------
HCN1_TILAPIA        GMNSTPMSGNSIINSPAQPPFSTAFGTTQLQQSSVPLTYSASAIANASAARMMPAAAVAA
```

```
HCN1_PLATYFISH      GMNSTPMSGNSIINSPAQPPYSTAFGAAQLQQSSVPMTYSASAIANASVARMLPVAAAAA
HCN1_TETRAODON      ---------------------------EMLQQASAPMTYSASAIANASAARMLPVAAAAA
HCN1_TROUT          GMNTTPISGNSIINSPAQPPYTTALGNNQFQQSATSLTYSASAVTAPSSAATARILPASA

HCN1_HUMAN          RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPVQSPLAA-RTFHY
HCN1_MACAQUE        RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPVQSPLAA-RTFHY
HCN1_ORANGUTAN      RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPVQSPLAA-RTFHY
HCN1_MARMOSET       RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPVQSPLAA-RTFHY
HCN1_MOUSE          RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPIQSPLAT-RTFHY
HCN1_RAT            RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPIQSPLAT-RTFHY
HCN1_SQUIRREL       RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPVQSPLTT-RTFHY
HCN1_HORSE          RTQSPPVYSATSLSHSNLHSPSPSTQTPQSSAILSPCSYTTVVCSPPVQSPLAT-RTFHY
HCN1_COW            RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPVQSPLAT-RTFHY
HCN1_GALAGO         RTQSPPVYTAPSLSHGNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPVQSPLAS-RTFHY
HCN1_DOG            RTQSPPVYSAPSLSHGNLHSPSPGTQTPQPSAVLSPCSYTTAVCSPPVQSPLAA-RTFHY
HCN1_PANDA          RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPVQSPLAT-RTFHY
HCN1_RABIT          RTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPVQSPLAT-RTFHY
HCN1_PLATYPUS       RTQSPPVYTAPNLSHGNLHSPTPSTQPPPQATILSPCSYTTAVCSPPVQSPLAG-RTFQY
HCN1_BIRD           RTQSPPVYSTSSLSHTNLHSPSPSTQPPQQAVILSPCSYTTAVCSPPVQSPLAG-RTFQY
HCN1_CHICKEN        RTQSPPVYTAGSLSHGNLHSPSPSTQTPQQAVILSPCSYTTAVCSPPVQSPLAG-RTFQY
HCN1_ANOLE          RTQSPPVYSMTSLSHGSLNSPTPSTQTPQQAAIFSPCSYSSAVCSPPVQSPLAAGRTFQY
HCN1_FROG           RTRSPPVYNANSLSHGNLQSPTPSPQTPQQAVVFSPSSFTSAACSPSVQSPLAG-RTFQY
HCN1_PIG            ------------------------------------------------------------
HCN1_TILAPIA        AAAQGLYQTQSG---TLNSSQT---PLQQQGAIMSPCSFTAAMCSPPVQTP-LANRSFQY
HCN1_PLATYFISH      AVAQGGFPTSSLSQVNLNSSSQ---TALQQGAIMSPCSFTAAMCSPPVQTP-LASRSFQY
HCN1_TETRAODON      AAAQAGYPPPSLSHSSLNSSLTNPQGALQQAAIMSPCSFTAAMCSPPVQTP-LANRSFQY
HCN1_TROUT          QGVYPVPSVIHGNLNSSSPVPQTPLSLHQQGSIMSPVSFTTAVCSPPVQTPGLAGRSFQY

HCN1_HUMAN          ASPTASQLSLMQQ---------------------------------QPQQQVQQSQPPQTQ
HCN1_MACAQUE        ASPTASQLSLMQQ---------------------------------QPQQQVQQSQPPQTQ
HCN1_ORANGUTAN      ASPTASQLSLMQQ---------------------------------QPQQQVQQSQPPQTQ
HCN1_MARMOSET       ASPTASQLSLMQQ---------------------------------QPQQQVQQSQAPQTQ
HCN1_MOUSE          ASPTASQLSLMQQPQQQLPQSQVQQTQTQTQQQQQQQQQQQQQQQQQQQQQQQQQQQQQ
HCN1_RAT            ASPTASQLSLMQQPQPQLQQSQVQQTQTQTQQQQQQQQPQPQPQQPQQQQQQQQQQQQQ
HCN1_SQUIRREL       ASPTASQLSLMQQ---------------------------QQQQQQQQLQQSQAPQTQPQ
HCN1_HORSE          ASPTASQLSLMQQ-----------------------------------QQQLQQSQPPQ
HCN1_COW            ASPTASQLSLIQQ-----------------------------------QQVQQPPQPQ
HCN1_GALAGO         ASPTASQLSLMQQ-----------------------------------QLQSQPPPQT
HCN1_DOG            ASPTASQLSLAQQPQP-------------------------AQPPQPPQPAQPAQPPQPQ
HCN1_PANDA          ASPTASQLSLTQQQQ---------------------------------QQLQQPPPPQ
HCN1_RABIT          ASPTASQLSLMPQ-----------------------------------QQQQPQAPQTQ
HCN1_PLATYPUS       VSPTASQLSLMQQ-----------------------------------------------Q
HCN1_BIRD           ASPTASQLSLMQQQQQ---------------------------QQQQGGGGGGGGGGG
HCN1_CHICKEN        ASPTASQLSLMQQQ----------------------------------------------
HCN1_ANOLE          ATPTASQLSLLQQ-----------------------------------------------
HCN1_FROG           SSLTASQLSLIQQQQQ--------------------------------------------
HCN1_PIG            ------------------------------------------------------------
HCN1_TILAPIA        ASPTASQLSLIQQPIA-------------------------QPSLSTQQLSQQPAAPTA
HCN1_PLATYFISH      GSPTASQLSLIQQPIG-------------------------QPSLPTQQQQLGQQPAAS
HCN1_TETRAODON      GSRTASQLSLIQQPTG-------------------------QPTLPTQQPP---------
HCN1_TROUT          GSPTASQLSLIQQPLP-------------------------TALPPQQPLPQPQQPGGA

HCN1_HUMAN          PQQPSPQPQTP-----------GSSTPKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE
HCN1_MACAQUE        PQQPSPQPQTP-----------GSSTPKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE
HCN1_ORANGUTAN      PQQPSPQPQTP-----------GSSTPKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE
HCN1_MARMOSET       PQQQSPQPQTP-----------GSSTPKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE
HCN1_MOUSE          QQQQQQQPQTP-----------GSSTPKNEVHKSTQALHNTNLTKEVRPLSASQPSLPHE
HCN1_RAT            QQQQQQQPQTP-----------GSSTPKNEVHKSTQALHNTHLTREVRPLSASQPSLPHE
HCN1_SQUIRREL       QQQPPQQPQTP-----------SSSTPKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE
HCN1_HORSE          PQQQPQQPQTP-----------SSSTPKNDVHKSTQALHNTSLTRDVRPLSASQPSLPHE
HCN1_COW            Q--PPQPPQTP-----------GSSTPKNEVHKSTQALHNTSLTREVRPLSASQPSLPHE
HCN1_GALAGO         QQPPPPPPQTP-----------GSSTPKNEVHKSTQALHNASLTREVRPLSASQPSLPHE
HCN1_DOG            PPQPQPQPPQPPQPPQPPQTPGGGSTPRNDVHRSTQALHNASLTREVRPLSASQPSLPHE
HCN1_PANDA          PPQPPPQPQTP-----------STSTPRNDVHRSTQALHNTSLTREVRPLSASQPSLPHE
HCN1_RABIT          PQQPPQQPQTP-----------GSATPKNEVHRSTQALPNTSLTREVRPLSASQPSLPHE
HCN1_PLATYPUS       QPPSQQQPQPP-----------PSSAQKNEVHKSTQALHNTNLTREARPLSASQPSLPHE
HCN1_BIRD           GQQQPQAAQQP-----------PGAAQKNEVHKSTQALHNTNLTREVRPLSASQPSLPHE
HCN1_CHICKEN        ----PQAPQLP-----------PGTAPKNEVHKSTQALHNTNLSREVRPLSASQPSLPHE
HCN1_ANOLE          QQLQQQQPQQP-------LQQMPSASQKSDVHKSTQALHNASLTREVRPLSASQPSLPHE
HCN1_FROG           ----------------------ASGASQIHEVHKSAQALANTNLTREVRPLSASQPSLPHE
HCN1_PIG            ------------------------------------------------------------
HCN1_TILAPIA        ATSSAAQQQQQQQQASPQQQQVPSPQRGD-IHKSTQALQSGSLSRDVRHLSASQPSLPHD
```

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HCN1_PLATYFISH      TAAAAS----------AAQQQVPSPQRGD-IHKSTQALQSGSLSRDVRHLSASQPSLPHD
HCN1_TETRAODON      ---------------ASATASAPSPQRGD-IPKGAQALQSGSLSRDVRHLSASQPSLPHE
HCN1_TROUT          AASSATQQ-------PQQQQQVPSPQRSDSLHKASHALQSGSLSRDVRHLSASQPSLPHD


HCN1_HUMAN          VSTLISRPHPTVGESLASIPQPVTAVPGTGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGV
HCN1_MACAQUE        VSTLISRPHPTVGESLASIPQPVTAVPGTGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGV
HCN1_ORANGUTAN      VSTLISRPHPTVGESLASIPQPVTAVPGTGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGV
HCN1_MARMOSET       VSTLISRPHPTVGESLASIPQPVTAVPGTGLQAGGRSTVPQRVTLFRQMSSGAIPPNRGV
HCN1_MOUSE          VSTLISRPHPTVGESLASIPQPVAAVHSTGLQAGSRSTVPQRVTLFRQMSSGAIPPNRGV
HCN1_RAT            VSTMISRPHPTVGESLASIPQPVATVHSTGLQAGSRSTVPQRVTLFRQMSSGAIPPNRGV
HCN1_SQUIRREL       VSTLISRPHPTVGESLASIPQPVAAVHGTSLQAGGRGTVPQRVTLFRQMSSGAIPPNRGV
HCN1_HORSE          VSTLISRPHPTVGESLASIPQPVTAVHGTGLQAGGRGTVPQRVTLFRQMSSGAIPPNRGV
HCN1_COW            VSTLISRPHPTVGESLASIPQPVTTVHGSGLQAGGRGTVPQRVTLFRQMSSGAIPPNRGV
HCN1_GALAGO         VSTLISRPHPTVGESLASIPQPVTAVPGPGLQAAGRSSVPQRVTLFRQMSSGAIPPNRGV
HCN1_DOG            VATLMSRPHPTVGESLASIPQPVTAVHGPGLQAGGRGTVPQRVTLFRQMSSGAIPPNRGA
HCN1_PANDA          VSTLISRPHPTVGESLASIPQPVSAVHGPGLQAGGRGTVPQRVTLFRQMSSGAIPPNRGA
HCN1_RABIT          VSTLISRPHPTVGESLASIPQPVAAVHSAGLQAAGRSTVPQRVTLFRQMSSGAIPPNRGV
HCN1_PLATYPUS       VSTPISRPHPTVGESLASLPQPASGAQAAGFQAGSRGSVPPRVTLFRQMSSGAIPPHRGV
HCN1_BIRD           ISTLIARPHPTVGESLASLPQPAPGP---GVPPGGRAGVPQRVSLFRQMSSGAIPPHRAA
HCN1_CHICKEN        ISTLIARPHPTVGESLASLPQPAPGT---SVPPASRATVPQRVSLFRQMSSGAIPPNRGA
HCN1_ANOLE          ISMMIARPHPTVGESLASIPQPLSGFQSTGLQAGNKGTVPQRVALFRQMSSGAIPPTRGA
HCN1_FROG           ISTLGSKPHPTVGESLASLPQPISNVQAAGTQSGSRSNIPPRVALFRQMSSGALPPARVG
HCN1_PIG            ------------------------------------------------------------
HCN1_TILAPIA        TSLGPRVHPTSGDSLASIVPPTAAVIQGMNLQSGIRTTVPQRVNLFRQMSSGALPPVRSA
HCN1_PLATYFISH      TSLGPRAHPASGESLASMVPPTAAAIQGISLQGGIRTTVPQRVSLFRQMSSGALPPVRSA
HCN1_TETRAODON      ASLGPRAHPASGDSLASIAPPTAAAIQGMSLQGGIRTTVPQRVNLFRQMSSGALPPVRSA
HCN1_TROUT          TSLGPRAHPAASGDSLASIAPPVAAVQGMGIQSGLRTTVPQRVNLFRQMSSGALPPVRAV


HCN1_HUMAN          PPAPPPPAAALPRES-----------SSVLNT--DPDAEKPRFASNL
HCN1_MACAQUE        PPAPPPPAAALPRES-----------SSVLNT--DPDAEKPRFASNL
HCN1_ORANGUTAN      PPAPPPPAAALPRES-----------SSVLNT--DPDAEKPRFASNL
HCN1_MARMOSET       PPAPPPPAAALPRES-----------SSVLNT--DPDAEKPRFASNL
HCN1_MOUSE          PPAPPP-PAAVQRES-----------PSVLNT--DPDAEKPRFASNL
HCN1_RAT            PPAPPP-PAAVQRES-----------PSVLNK--DPDAEKPRFASNL
HCN1_SQUIRREL       PPAPPPPAAALQREP-----------SSVLNT--DPDAEKPRFASNL
HCN1_HORSE          PPAPPPPAAALPRES-----------SSVLST--DPDAEKPRFASNL
HCN1_COW            PPAPPPPAAAHPREA-----------PSVLTT--DSEAEKPRFASNL
HCN1_GALAGO         PPAPPPPAAALQREA-----------SSVLNT--DPEAEKPRFASNL
HCN1_DOG            PPAPPPPAAALPRES-----------SSVLTT--DPEAEKPRFASNL
HCN1_PANDA          PPAPPPPAAALPRES-----------SSVLTT--DPEAEKPRFASNL
HCN1_RABIT          PPAPPPPAAPLQREA-----------SSVLNT--DPEAEKPRFASNL
HCN1_PLATYPUS       VPPGPPTGAPLQRDT-----------SGVLNA--EPEGDKPRFASNL
HCN1_BIRD           APPAAPLQKIGRAVQ-----------QECRDRYRMPEGDKPRFASNL
HCN1_CHICKEN        VPPAAPLPRDSSAVL-----------STE------PEGDKPRFASNL
HCN1_ANOLE          APSAATLQRDSSTVL-----------NTELEG------DKPRFASNL
HCN1_FROG           ATTQPSPAPPTKDSS-----------TVSSTDT---DTDKLKYASNL
HCN1_PIG            ------------------------------------------------
HCN1_TILAPIA        AAAAAAAAVAAASSSSTQHRDSPGSRRDSVLSSTETEQDKMRFASNL
HCN1_PLATYFISH      ASAVAAVAAASSSTS-TQHRESPGSRRDSVLSSTETEQDKMRFASNL
HCN1_TETRAODON      AAAAAAAAAASSVSSS-------------STQQSTETEQDKLRFSSNL
HCN1_TROUT          SSAAQHRDSTGSRRD---------SRRDSTLSSTETEQDKMRFASNL
```

(b)

```
HCN3_HUMAN      ------------------------------------------------------------
HCN4_HUMAN      MDKLPPSMRKRLYSLPQQVGAKAWIMDEEEDAEEEGAGGRQDPSRRSIRLRPLPSPSPSA
HCN2_HUMAN      -------------------------MDARGGGGRPGESPGATPAPGPPPPPPPAPPQQQP
HCN1_HUMAN      ------------------------------------------------------------


HCN3_HUMAN      ------------------------------------------------------------
HCN4_HUMAN      AAGGTESRSSALGAADSEGPARGAGKSSTNGDCRRFRGSLASLGSRGGGSGGTGSGSSHG
HCN2_HUMAN      PPPPPPAPPPGPGPAPPQHPPRAEALPPEAADEGGPRGRLRSRDSSCGRPGTPGAASTAK
HCN1_HUMAN      -------------------------------MEGGGKPNSSSNSRDDGNSVFPAKASATG


HCN3_HUMAN      ------------------MEAEQRPAAGASEGATPGLEAVPPVAPPPATAASGPIPKSGP
HCN4_HUMAN      HLHDSAEERRLIAEGDASPGEDRTPPGLAAEPERPGASAQPAASPPPPQQPPQPASASCE
HCN2_HUMAN      GSPNGECGRGEPQCSPAGPEGPARGPKVSFSCRGAASGPAPGPGPAEEAGSEEAGPAGEP
HCN1_HUMAN      AGPAAAEKRLGTPPGGGGAGAKEHGNSVCFKVDGGGGGGGGGGGGEEPAGGFEDAEGPRR
                                                . .   .     .
```

```
HCN3_HUMAN      EP------------------------------KRRHLGTLLQPTVNKFSLRVFGSHKAV
HCN4_HUMAN      QPSVDTAIKVEGGAAAGDQILPEAEVRLGQAGFMQRQFGAMLQPGVNKFSLRMFGSQKAV
HCN2_HUMAN      RGS-------------------------QASFMQRQFGALLQPGVNKFSLRMFGSQKAV
HCN1_HUMAN      QYG-----------------------------FMQRQFTSMLQPGVNKFSLRMFGSQKAV
                .                                 :*:: ::*** *******:***:***

HCN3_HUMAN      EIEQERVKSAGAWIIHPYSDFRFYWDLIMLLLMVGNLIVLPVGITFFKEENSPPWIVFNV
HCN4_HUMAN      EREQERVKSAGFWIIHPYSDFRFYWDLTMLLLMVGNLIIIPVGITFFKDENTTPWIVFNV
HCN2_HUMAN      EREQERVKSAGAWIIHPYSDFRFYWDFTMLLFMVGNLIIIPVGITFFKDETTAPWIVFNV
HCN1_HUMAN      EKEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIFNV
                * ******:** ***************: **::*****:::*******.::.:.***:***

HCN3_HUMAN      LSDTFFLLDLVLNFRTGIVVEEGAEILLAPRAIRTRYLRTWFLVDLISSIPVDYIFLVVE
HCN4_HUMAN      VSDTFFLIDLVLNFRTGIVVEDNTEIILDPQRIKMKYLKSWFMVDFISSIPVDYIFLIVE
HCN2_HUMAN      VSDTFFLMDLVLNFRTGIVIEDNTEIILDPEKIKKKYLRTWFVVDFVSSIPVDYIFLIVE
HCN1_HUMAN      ASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVDYIFLIVE
                 ***.**:**::***** * *:.:**:* *. *: .**::**:**::**********:**

HCN3_HUMAN      LEPRLDAEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN4_HUMAN      TR--IDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
HCN2_HUMAN      KG--IDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVMRI
HCN1_HUMAN      KG--MDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRI
                    :*:**************************************************:**

HCN3_HUMAN      FNLIGMMLLLCHWDGCLQFLVPMLQDFPPDCWVSINHMVNHSWGRQYSHALFKAMSHMLC
HCN4_HUMAN      VNLIGMMLLLCHWDGCLQFLVPMLQDFPDDCWVSINNMVNNSWGKQYSYALFKAMSHMLC
HCN2_HUMAN      CNLISMMLLLCHWDGCLQFLVPMLQDFPRNCWVSINGMVNHSWSELYSFALFKAMSHMLC
HCN1_HUMAN      FNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLC
                 ***.*****************:***** :****:* ***.**.. **.***********

HCN3_HUMAN      IGYGQQAPVGMPDVWLTMLSMIVGATCYAMFIGHATALIQSLDSSRRQYQEKYKQVEQYM
HCN4_HUMAN      IGYGRQAPVGMSDVWLTMLSMIVGATCYAMFIGHATALIQSLDSSRRQYQEKYKQVEQYM
HCN2_HUMAN      IGYGRQAPESMTDIWLTMLSMIVGATCYAMFIGHATALIQSLDSSRRQYQEKYKQVEQYM
HCN1_HUMAN      IGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLDSSRRQYQEKYKQVEQYM
                **** *** .*.*:*:***************:****************************

HCN3_HUMAN      SFHKLPADTRQRIHEYYEHRYQGKMFDEESILGELSEPLREEIINFTCRGLVAHMPLFAH
HCN4_HUMAN      SFHKLPPDTRQRIHDYYEHRYQGKMFDEESILGELSEPLREEIINFNCRKLVASMPLFAN
HCN2_HUMAN      SFHKLPADFRQKIHDYYEHRYQGKMFDEDSILGELNGPLREEIVNFNCRKLVASMPLFAN
HCN1_HUMAN      SFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDPLREEIVNFNCRKLVATMPLFAN
                ******.* **:**:*********:***:.**.**. ******:**.** *** *****:

HCN3_HUMAN      ADPSFVTAVLTKLRFEVFQPGDLVVREGSVGRKMYFIQHGLLSVLARGARDTRLTDGSYF
HCN4_HUMAN      ADPNFVTSMLTKLRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGNKETKLADGSYF
HCN2_HUMAN      ADPNFVTAMLTKLKFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGNKEMKLSDGSYF
HCN1_HUMAN      ADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKKMYFIQHGVAGVITKSSKEMKLTDGSYF
                ***.***::*:**:******** ::***::*:********: .*::::. :: :*:*****

HCN3_HUMAN      GEICLLTRGRRTASVRADTYCRLYSLSVDHFNAVLEEFPMMRRAFETVAMDRLLRIGKKN
HCN4_HUMAN      GEICLLTRGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVALDRLDRIGKKN
HCN2_HUMAN      GEICLLTRGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDRIGKKN
HCN1_HUMAN      GEICLLTKGRRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDRIGKKN
                *******:**********************:** ****:***********:*** ******

HCN3_HUMAN      SILQRKRSEPSPG----SSGGIMEQHLVQHDRDMARGVRGRAPSTGAQLSGKPVLWEPLV
HCN4_HUMAN      SILLHKVQHDLNSGVFNYQENEIIQQIVQHDREMAHCAHRVQAAASATPTPTPVIWTPLI
HCN2_HUMAN      SILLHKVQHDLNSGVFNNQENAIIQEIVKYDREMVQQAELGQRVGLFPPPPPPPQVTSAI
HCN1_HUMAN      SILLQKFQKDLNTGVFNNQENEILKQIVKHDREMVQAIAPINYPQMTTLNSTSSTTTPTS
                *** :* ..         . . : :.:*::**:*.:               .    .

HCN3_HUMAN      HAPLQAAAVTSNVAIALTHQ-------------------------------------------
HCN4_HUMAN      QAPLQAAAATTSVAIALTHHPRLPAAIFRPPPGSGLGNLGAGQTPRHLKRLQSLIPSALG
HCN2_HUMAN      ATLQQAAAMSFCPQVARPLVG-----------------------------------------
HCN1_HUMAN      RMRTQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPVQSPLAARTFH
                    *:..      :: .

HCN3_HUMAN      ------------------------------------------------------------
HCN4_HUMAN      SASPASSPSQVDTPSSSSFHIQQLAGFSAPAGLSPLLPSSSSSPPPGACGSPSAPTPSAG
HCN2_HUMAN      ------------------------------------------------------------
HCN1_HUMAN      YASPTASQLSLMQQQP----------------------------------------------


HCN3_HUMAN      ------------------------------------------------------------
HCN4_HUMAN      VAATTIAGFGHFHKALGGSLSSSDSPLLTPLQPGARSPQAAQPSPAPPGARGGLGLPEHF
HCN2_HUMAN      ------------------------------------------------------------
```

```
HCN1_HUMAN      ------------------------------------------------------------


HCN3_HUMAN      -----------------------------RGPLPLSPDSPATLLARSAWRSAGSPASPLV
HCN4_HUMAN      LPPPPSSRSPSSSPGQLGQPPGELSLGLATGPLSTPETPPRQPEPPSLVAGASGGASPVG
HCN2_HUMAN      --------------------------------PLALGSPRLVRRPPPGPAPAAASPGPPPP
HCN1_HUMAN      -----------------------------QQQVQQSQPPQTQPQQPSPQPQTPGSSTPKN
                                             :            .    : . ..*

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

HCN3_HUMAN      ------------------------------------------------GGGGRRLGPR
HCN4_HUMAN      LTQDLKLISASQPALPQDGAQTLRRASPHSSGESMAAFPLFPRAGGGSGGSGSSGGLGPP
HCN2_HUMAN      ----------------------------------------------------RPLSASQP
HCN1_HUMAN      ---------------------------------------ESLASIPQPVTAVPGTGL


HCN3_HUMAN      GRPLSASQPSLPQRATGDGSPGRKGSGSERLPPSGLLAKPPRTAQPPRPPVPEPATPRGL
HCN4_HUMAN      GRPYGAIPGQHVTLPRKTSSGSLPPPLSLFGARATSSGGPPLTAGPQREPGARPEPVRSK
HCN2_HUMAN      SLPHGAPGPAASTRPASSSTPRLGPTPAARAAAPSPDRRDSASPGAAGGLDPQDSARSRL
HCN1_HUMAN      QAGGRSTVPQRVTLFRQMSSGAIPPNRGVPPAPPPPAAALPRESSSVLNTDPDAEKPRFA
                     :            .:       .   . .      .  . .     .

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

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.