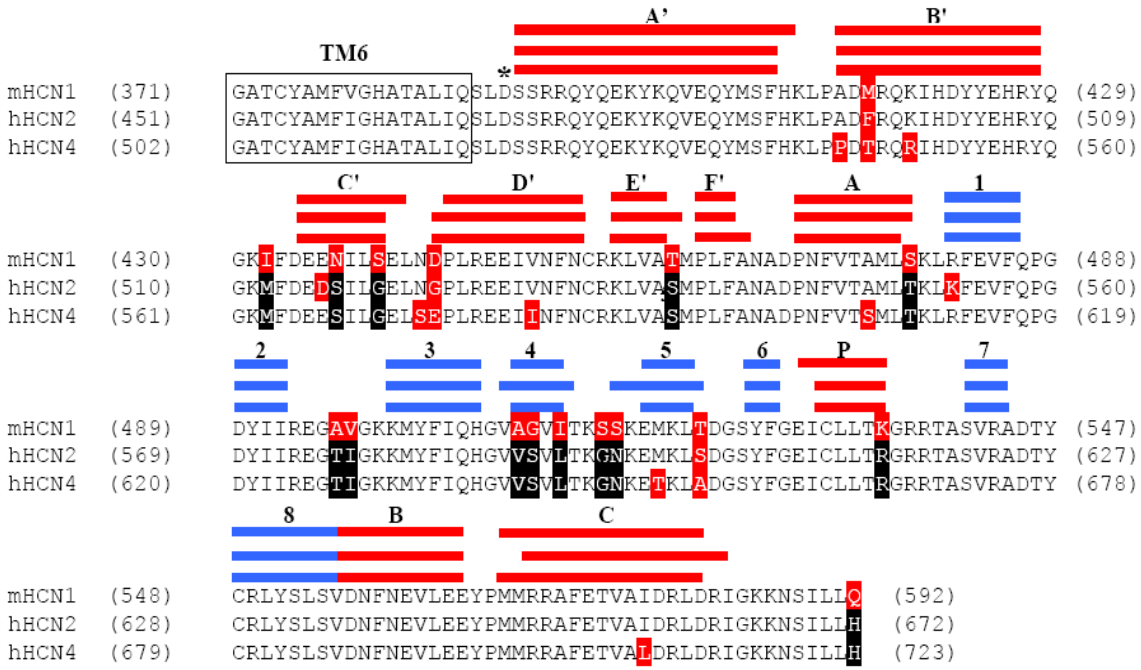
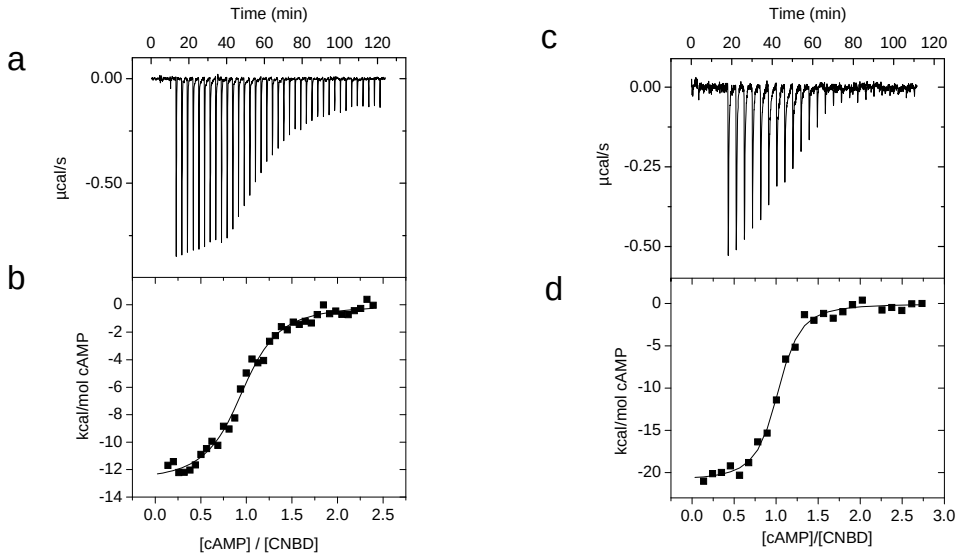
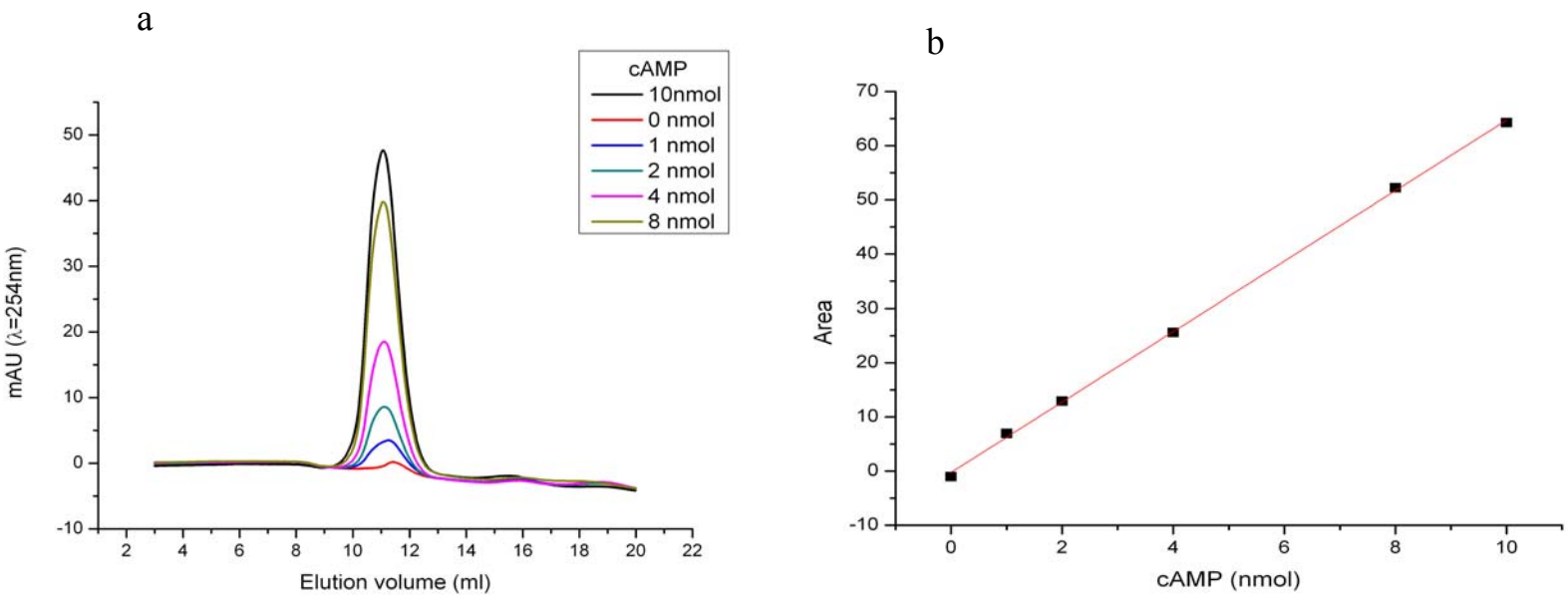


Supplementary Figure 1. Sequence alignment of the C terminal part of human HCN2 (NP_001185.3), human HCN4 (NP_005468.1) and mouse HCN1 (NP_034538.2) proteins including the sixth transmembrane domain (TM6) (boxed), the C-linker and the CNBD. Red lines indicate the alpha helices in the C-linker (A'-F') and CNBD (P,B,C); blue lines the beta sheets in the CNBD (1-8) for the three isoforms as determined by the crystal structures described in this work. Substituted residues are highlighted in red, while in black are highlighted the 14 residues that are conserved among HCN2 and HCN4 but not in HCN1. Asterisk marks the start of the expressed fragments.



Supplementary Figure 2. Isothermal titration calorimetry of cAMP binding to the purified protein fragments $HCN2_{CB}$ and $HCN4_{CB}$ linked to the maltose binding (MBP). (a) Heat changes during successive injections of 1 µl of cAMP (900 µM) to the MBP-$HCN2_{CB}$ protein (75 µM). (b) Binding curve obtained by data in (a). The peaks have been integrated, normalized to the cAMP concentration and plotted against the molar ratio of cAMP to the protein. The solid line represents a nonlinear least-square fit to a single–site binding model. The K_D value was 3.3 µM and the binding stoichiometry was 0.97. (c,d) Similar experiment as in (a,b) performed with MBP-$HCN4_{CB}$ (35 µM) and successive 1.25 µl injections of cAMP (600 µM). The K_D value was 0.6 µM and the binding stoichiometry was 0.99.



Supplementary Figure 3 Determination of cAMP content.

Cyclic AMP was released by boiling the protein in solution and the cAMP content measured by monitoring absorbance at λ=254 nm (standard absorbance shown in (a)) followed by data interpolation with the calibration curve (shown in (b)). In the control experiment, cAMP content was not affected by prolonged boiling (30').

Supplementary Table I. Data collection and refinement statistics of HCN_{CB} in complex with cAMP

	HCN1-cAMP	HCN2-cAMP	HCN4-cAMP
Data collection			
Space group	*I*4	$P42_12$	*P*4
Cell dimensions (Å)	*a* = 97.7	*a* = 96.7	*a* = 88.3
	b = 97.7	*b* = 96.7	*b* = 88.3
	c = 113.2	*c* = 50.8	*c* = 57.8
Resolution limits (Å)	43.80-2.90	35.03-2.30	62.44-2.50
(outer shell)	(3.06-2.90)	(2.42-2.30)	(2.64-2.50)
Observations	78,275	106,430	74,883
Unique reflections	11,818	11,143	15,615
Completeness (%)	100 (100)[a]	99.8 (100)	100 (100)
R-merge[b] (%)	12.0 (48.5)	12.8 (42.7)	13.2 (41.9)
I/σ(I)	12.5 (4.6)	14.3 (6.7)	10.0 (4.3)
Multiplicity	6.6 (6.3)	9.6 (9.7)	4.8 (4.9)
Refinement			
R-factor[c]/R-free[d] (%)	20.5 / 27.5	20.2 / 27.1	19.3 /27.7
N° of residues:	198 (390-588 chain A)	202 (443-644)	198 (521-718 chain A)
	198 (390-588 chain B)		202 (521-722 chain B)
Mean B-factors (Å^2):	38.4 (chain A)	25.5	19.5 (chain A)
	38.3 (chain B)		21.9 (chain B)
N° of cAMP:	1 (chain A)	1	1 (chain A)
	1 (chain B)		1 (chain B)
Mean B-factors (Å^2):	28.6 (chain A)	11.1	11.6 (chain A)
	28.3 (chain B)		17.5 (chain B)
N° of water molecules:	49	87	148
Mean B-factors (Å^2):	36.5	33.8	22.6
N° of glycerol molecules	–	–	6
Mean B-factors (Å^2):	–	–	43.0
R.m.s.d. from ideality			
bond lengths (Å)	0.016	0.021	0.015
bond angles (°)	1.71	1.89	1.75
Ramachandran plot			
most favored regions (%)	92.3	99.0	97.2
additional allowed regions (%)	7.9	0.5	2.6

[a] Values in parentheses are for highest-resolution shell.

[b] R-merge = $\Sigma_h\Sigma_i \mid I_{hi} - \langle I_h \rangle \mid / \Sigma_h\Sigma_i I_{hi}$.

[c] R-factor = $\Sigma_h \mid\mid F_{obs}\mid - \mid F_{calc}\mid\mid / \Sigma \mid F_{obs}\mid$ where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively.

[d] R-free is calculated on 5% of the diffraction data, which were not used during the refinement.

Supplementary Table II. Protein-cAMP interactions in HCN1, HCN2, HCN4. Polar contacts are reported in detail and Van der Waals contacts are indicated as a total number. Contacts conserved among isoforms are shown on the same line.

HCN1-cAMP	**HCN2-cAMP**	**HCN4-cAMP**
Polar contacts (<3.5 Å):	Polar contacts (<3.5 Å):	Polar contacts (<3.5 Å):
Thr539 N - cAMP O1P 2.9 Å	Thr619 N - cAMP O1P 3.0 Å	Thr670 N - cAMP O1P 3.0 Å
Thr539 Oγ - cAMP O1P 2.7 Å	Thr619 Oγ - cAMP O1P 2.8 Å	Thr670 Oγ - cAMP O1P 2.9 Å
Arg538 NH1 – cAMP O2P 2.8 Å	Arg618 NH1 - cAMP O2P 2.9 Å	Arg669 NH1 - cAMP O2P 2.9 Å
Cys531 N - cAMP O2P 2.8 Å	Cys612 N - cAMP O2P 3.0 Å	Cys662 N - cAMP O2P 3.0 Å
Cys531 N - cAMP O1P 3.3 Å		
Gly528 N - cAMP O2* 2.8 Å	Gly609 N - cAMP O2* 3.1 Å	Gly659 N - cAMP O2* 2.6 Å
		Gly659 N - cAMP O2P 3.4 Å
Glu529 Oε1 - cAMP O2* 2.5 Å	Glu610 Oε1 - cAMP O2* 2.5 Å	Glu660 Oε1 - cAMP O2* 2. 8 Å
		Glu660 Oε2 - cAMP O2* 3.1 Å
Arg579 O - cAMP N6 3.2 Å	Arg659 O - cAMP N6 2.9 Å	
Arg582 NH1 - cAMP N1 3.3 Å		
		Glu649 0ε1 - cAMP N6 3.2 Å
		Thr650 Oγ - cAMP N1 3.2 Å
van der Waals contacts (<4 Å): 83	van der Waals contacts (<4 Å): 76	van der Waals contacts (<4 Å): 72

Supplementary Table III. Sedimentation coefficients**.**

(a) Calculated sedimentation coefficients (s) obtained with HYDROPRO (25) using the coordinates from the crystal structures described in this work for $HCN1_{CB}$, $HCN2_{CB}$ and $HCN4_{CB}$, and from pdb file 1FQD for E.coli maltose binding protein (MBP, m.w. 41 kDa). The solution parameters calculated with SEDENTERP were: 293K, density 1.02872 and viscosity 0.013284. **(b)** Experimentally determined sedimentation coefficients (s) (see Figure 3d,e,f).

a

	$HCN1_{CB}$	$HCN2_{CB}$	$HCN4_{CB}$	MBP
monomer	1.503	1.57	1,516	2.276
dimer	2.263	2.292	2,386	
tetramer	3.792	3.859	3,913	

b

		s	%	s	%	s	%
$HCN2_{CB}$	0 cAMP	1.52	84				
$HCN2_{CB}$	0.3mM cAMP	1.43	52			3.6	21
$HCN1_{CB}$	0 cAMP	1.59	62			3.2	15
$HCN1_{CB}$	0.3mM cAMP	1.7	32			3.5	54
$HCN4_{CB}$	0 cAMP	1.32	46				
$HCN4_{CB}$	0.3mM cAMP	1.45	7	2.24	9	3.63	55
MBP		2.1					

Supplementary Table IV. Dynamic light scattering experiments performed on HCN_{CB} proteins. Values of radius are net of the dehydratation shell. The hydrodynamic radius calculated from the crystal structures are (in nm): 2.3 for the monomer, 3.1 for the dimer and 3.7 for the tetramer. The expected M.W. for monomer, dimer and tetramer are (in kDa): 24, 48 and 96 for the wt; 18, 36 and 72 for the ΔC-linker. cAMP concentration 1 mM. M=monomer, D=dimer, T=tetramer.

		Radius (nm)	Polydispersion (%)	M.W. Kda	Molecular species
$HCN1_{CB}$	-	3.8	24	79	D + T
	+ cAMP	4.3	23.4	105	T
$HCN2_{CB}$	-	3.1	9.6	49	D
	+ cAMP	4.3	15.6	105	T
$HCN4_{CB}$	-	3.2	17.2	53	D
	+ cAMP	4	15	91	T
ΔClinker-$HCN1_{CB}$	-	2.8	15.4	39	D
	+ cAMP	2.8	16.3	39	D