**A synthetic peptide that prevents cAMP regulation in mammalian Hyperpolarization-activated Cyclic Nucleotide-regulated (HCN) channels**

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**Abstract**

Binding of TRIP8b to the cyclic nucleotide binding domain (CNBD) of mammalian HCN channels prevents their regulation by cAMP. Since TRIP8b is expressed exclusively in the brain, we envisage that it can be used for orthogonal control of HCN channels beyond the central nervous system. To this end, we have identified by rational design a 40-aa long peptide (TRIP8b$_{nano}$) that recapitulates affinity and gating effects of TRIP8b in HCN isoforms (human hHCN1, mHCn2, rbHCN4) and in the cardiac current $I_f$ in rabbit and mouse sinoatrial node cardiomyocytes. Guided by a NMR-derived structural model that identifies the key molecular interactions between TRIP8b$_{nano}$ and HCN CNBD, we further designed a cell-penetrating peptide (TAT-TRIP8b$_{nano}$) which successfully prevented b-adrenergic activation of mouse $I_f$ leaving the stimulation of the L-type calcium current ($I_{CaL}$) unaffected. TRIP8b$_{nano}$ represents a novel approach to selectively control HCN activation, which yields the promise of a more targeted pharmacology compared to pore blockers.

## Introduction

Hyperpolarization-activated cyclic nucleotide-regulated (HCN1-4) channels are the molecular correlate of the $I_f/I_h$ current, which plays a key role in controlling several higher order physiological functions, including dendritic integration and intrinsic rhythmicity both in cardiac and neuronal cells (Robinson & Siegelbaum, 2003). Unique among the voltage-gated ion channel superfamily, HCN channels are modulated by the direct binding of cAMP to their cyclic nucleotide binding domain (CNBD). Binding of the cyclic nucleotide increases the channel open probability upon hyperpolarization via conformational changes in the CNBD that are propagated to the pore through the C-linker domain (DiFrancesco & Tortora, 1991; Wainger, DeGennaro, Santoro, Siegelbaum, & Tibbs, 2001; Weissgraeber, Saponaro, Thiel, & Hamacher, 2017; Zagotta et al., 2003).

In addition to cAMP, HCN channels are regulated by TRIP8b, a brain-specific auxiliary (β) subunit, which modulates two independent features of the channel namely trafficking and gating (Bina Santoro et al., 2009; Zolles et al., 2009). For this dual regulation TRIP8b binds HCN channels in two distinct sites: *via* the tetratricopeptide repeat (TPR) domain, which interacts with the last three amino acids (SNL) of HCN channels and regulates their trafficking; and *via* the TRIP8b$_{core}$ domain, which interacts with the CNBD and antagonizes the effect of cAMP on the voltage dependency of the channel (Bankston, DeBerg, Stoll, & Zagotta, 2017; Deberg et al., 2015; Han et al., 2011;  Hu et al., 2013).

Here we focus our attention on the specific action of TRIP8b in preventing cAMP regulation of HCN channels. Given the brain-specific localization of TRIP8b, we posit  that a TRIP8b–derived peptide drug, able to reproduce the effect of the full length protein on HCN channel gating, can be developed for orthogonal selective regulation of HCN in cells/tissues in which TRIP8b is not expressed. cAMP dependent modulation of HCN channels underlies distinct roles of cAMP in heart rate regulation (DiFrancesco, 1993) and

68 development of peripheral neuropathic pain (Emery, Young, & McNaughton, 2012;

69 Herrmann et al., 2017) which can be dissected by using a TRIP8b-based tool. In this

70 regard, peptide-based drugs (2-50 aa long) are emerging as a fascinating application area

71 as they open new therapeutic possibilities with an advantage over small molecules in

72 terms of specificity and affinity for the target (Fosgerau & Hoffmann, 2015; Henninot,

73 Collins, & Nuss, 2017). To this end, we searched for the minimal peptide that binds to the

74 CNBD and recapitulates the gating effect of full length TRIP8b in three HCN isoforms

75 (HCN1, HCN2 and HCN4) and in the native $I_f$ current. In previous studies we identified the

76 core portion of TRIP8b (TRIP8b$_{core,}$ 80 aa long) that interacts with HCN CNBD and

77 prevents cAMP modulation in full length channels (Hu et al., 2013; B. Santoro et al., 2011).

78 A recent paper (Lyman et al., 2017) reported an even shorter binding sequence of TRIP8b

79 (37 aa). However, this peptide, which was identified by progressive truncation of

80 TRIP8b$_{core}$, failed to reproduce the binding affinity of the starting construct. Moreover,

81 evidence for activity of this peptide on HCN currents is lacking. In the present work, we

82 adopted a structure-driven rational desing approach to engineer a 40 aa long peptide,

83 TRIP8b$_{nano}$, that efficiently prevents cAMP regulation of HCN channels. The rational

84 design of this peptide, based on secondary structure predictions and on NMR data of

85 TRIP8b$_{core}$, was supported by an NMR-based 3D model structure of the complex formed

86 by the TRIP8b$_{nano}$ peptide and the CNBD of human HCN2 channel isoform. This structural

87 information identifies crucial interactions between the two partners and explains both direct

88 (Bankston, DeBerg, Stoll, & Zagotta, 2017; Deberg et al., 2015; Han et al., 2011) and

89 indirect (allosteric) (Bankston et al., 2017; Han et al., 2011; Hu et al., 2013; Saponaro et

90 al., 2014) modes of competition between TRIP8b and cAMP for binding to the CNBD. The

91 evidence that TRIP8b$_{nano}$ establishes all relevant interactions with the CNBD is reflected

92 by the finding that, contrary to shorter core sequences (Lyman et al., 2017), it binds to the

93 isolated CNBD with identical affinity to TRIPb$_{core}$ and acts with even higher efficacy than

94     $TRIP8b_{core}$ in preventing cAMP modulation of full length HCN channels (Hu et al., 2013). In

95     pacemaker myocytes of the sino-atrial node (SAN), $TRIP8b_{nano}$ equally preventes cAMP

96     stimulation of native f-channels leading to a 30% reduction in spontaneus firing rate.

97     To develop $TRIP8b_{nano}$ as a membrane permeable drug, we linked it with the positively

98     charged TAT sequence (Herce, Garcia, & Cardoso, 2014). $TAT-TRIP8b_{nano}$ was tested in

99     SAN pacemaker myocytes where its addition to the extracellular buffer prevented

100    adrenergic stimulation of the $I_f$ current leaving the activation of the L-type calcium current

101    ($I_{CaL}$) unaffected. Our study opens the possibility of selective *in vivo* control of the cAMP-

102    dependent facilitation of HCN channel opening, by local supply of $TAT-TRIP8b_{nano}$ peptide.

103

104    **Results**

105    We have previously shown that $TRIP8b_{core}$ (residues 223 - 303 of mouse TRIP8b splice

106    variant 1a4, hereafter TRIP8b) interacts with two elements of the isolated CNBD protein

107    fragment from HCN channels (residues 521 - 672 of human HCN2, hereafter CNBD): the

108    C-helix and the N-bundle loop, a sequence connecting helix E' of the C-linker with helix A

109    of the CNBD (Saponaro et al., 2014). Biochemical assays confirmed that each of these

110    two elements, i.e. the N-bundle loop and C-helix, is necessary but not sufficient for binding

111    (Saponaro et al., 2014).

112    To understand the interaction in atomic details, we used solution NMR spectroscopy to

113    characterize the structural properties of the CNBD - $TRIP8b_{core}$ complex. However, the

114    NMR spectra of $TRIP8b_{core}$ showed very few signals. In order to improve the quality of the

115    NMR spectra, we reduced the length of the TRIP8b fragment by progressively removing

116    residues at the N- and C-termini with no predicted secondary structure. The truncated

117    peptides were then tested for CNBD binding activity by isothermal titration calorimetry

118  (ITC). We thus identified a 40-aa peptide (TRIP8b$_{nano}$, comprising residues 235 – 275 of

119  TRIP8b, *Figure 1A*) with a binding $K_D$ of 1.5 ± 0.1 µM, a value similar to the $K_D$ of 1.2 ± 0.1

120  µM obtained with TRIP8b$_{core}$ (*Figure 1B*). TRIP8b$_{nano}$ was therefore employed for all

121  subsequent NMR experiments, resulting in a remarkable improvement in the spectral

122  quality and sample stability.

**Structural characterization of TRIP8b$_{nano}$ bound to CNBD**

124  The comparison of the $^1$H-$^{15}$N HSQC spectra of TRIP8b$_{nano}$ with and without CNBD bound

125  shows that the peptide folds upon interaction with the CNBD. Thus, the $^1$H-$^{15}$N HSQC

126  spectrum of TRIP8b$_{nano}$ without CNBD shows a limited $^1$H resonance dispersion,

127  characteristic of unstructured proteins (Dyson & Wright, 2004), while a larger number of

128  well-dispersed amide signals appear in the spectrum of the CNBD-bound form (*Figure*

129  *1C*). Importantly, we were now able to assign the backbone chemical shift resonances of

130  TRIP8b$_{nano}$ bound to the CNBD. The φ and ψ dihedral angles obtained from the NMR

131  assignment indicate that the peptide displays two α-helices (stretch $L_{238}$-$E_{250}$ named helix

132  N and stretch $T_{253}$-$R_{269}$ named helix C) when bound to CNBD. The helices are separated

133  by two amino acids; three and six residues at the N- and C- termini, respectively, are

134  unstructured (*Figure 1D*).

**Structural characterization of CNBD bound to TRIP8b$_{nano}$**

136  NMR-analysis of the CNBD fragment bound to TRIP8b$_{nano}$ revealed that the interaction

137  with the peptide does not affect the overall fold of the protein. Thus, the CNBD adopts the

138  typical fold of the cAMP-free state, in line with previous evidence that this is the form

139  bound by TRIP8b (Deberg et al., 2015; Saponaro et al., 2014). More specifically, the

140  secondary structure elements of the cAMP-free CNBD are all conserved in the

141  TRIP8b$_{nano}$–bound CNBD (*Figure 2*). This finding generally agrees with a previous double

142  electron-electron resonance (DEER) analysis of the CNBD - TRIP8b interaction, which

showed that TRIP8b binds to a conformation largely similar to the cAMP-free state (Deberg et al., 2015). Despite the overall agreement with the DEER study, the NMR data also reveal a new and unexpected feature of TRIP8b binding to the CNBD. Indeed, our results show that TRIP8b$_{nano}$ binding to the CNBD induces a well-defined secondary structure of the distal region of the C-helix (*Figure 2*). This means that the distal region of the C-helix (residues 657-662), which is unstructured in the free form of the CNBD (Lee & MacKinnon, 2017; Saponaro et al., 2014), extends into a helical structure upon ligand binding irrespectively of whether the ligand is cAMP (Lee & MacKinnon, 2017; Puljung & Zagotta, 2013; Saponaro et al., 2014) or TRIP8b (*Figure 2*). In contrast, and very differently from cAMP, which directly contacts the P-helix in the Phosphate Binding cassette (PBC) and causes its folding (Lee & MacKinnon, 2017; Saponaro et al., 2014), the NMR data show that TRIP8b$_{nano}$ binding to the CNBD does not induce P-helix formation (*Figure 2*).

**Modelling the CNBD - TRIP8b$_{nano}$ complex**

Despite the significant improvement in sample stability and NMR spectra quality achieved upon TRIP8b$_{nano}$ binding, we were still unable to assign the side chains of both proteins in the complex and thus could not solve the solution structure of the complex by the canonical NMR procedure. We therefore built a model (*Figure 3*) of the CNBD - TRIP8b$_{nano}$ complex by docking the two NMR-derived structures described above using the Haddock program (a detailed description of how the respective structures were generated is provided in Materials and Methods and *Figure 3-source data 1*).

In order to define the active residues (ambiguous interaction restraints) on the CNBD we used the chemical shift perturbation values as described in *Figure 3-figure supplement 1*. For TRIP8b$_{nano}$, we defined as active a stretch of residues, $E_{239}$-$E_{243}$, previously identified as critical for the interaction (B. Santoro et al., 2011; Bina Santoro et al., 2009). Output

168  clusters of this first molecular docking calculation (settings can be found in Material and

169  Methods) were further screened for TRIP8b$_{nano}$ orientations in agreement with a previous

170  DEER analysis, which placed TRIP8b residue A$_{248}$ closer to the proximal portion and

171  TRIP8b residue A$_{261}$ closer to the distal portion of the CNBD C-helix (Deberg et al., 2015).

172  Remarkably, in all clusters selected in this way, residues E$_{264}$ or E$_{265}$ in TRIP8b were

173  found to interact with residues K$_{665}$ or K$_{666}$ of the CNBD (*Figure 3-figure supplement 2*).

174  This finding was notable, because we previously identified K$_{665}$/K$_{666}$ as being critical for

175  TRIP8b interaction in a biochemical binding assay (Saponaro et al., 2014). We thus

176  proceeded to individually mutate each of these four positions, and test their effect on

177  binding affinity through ITC. As expected, reverse charge mutations K$_{665}$E or K$_{666}$E

178  (CNBD) as well as E$_{264}$K or E$_{265}$K (TRIP8b$_{nano}$) each strongly reduced the

179  CNBD/TRIP8b$_{nano}$ binding affinity (*Figure 3-figure supplement 3*).

180  Based on these observations, we performed a second molecular docking calculation,

181  including E$_{264}$ and E$_{265}$ as additional active residues for TRIP8b$_{nano}$. This procedure

182  resulted in the model shown in *Figure 3*, which represents the top-ranking cluster for

183  energetic and scoring function (*Figure 3-source data 2*) and was fully validated by

184  mutagenesis analysis as described below. Scrutiny of the model shows that TRIP8b$_{nano}$

185  binds to both the C-helix and the N-bundle loop (*Figure 3A*). Binding to the C-helix is

186  mainly guided by electrostatic interactions between the negative charges on TRIP8b$_{nano}$,

187  and the positive charges on the CNBD (*Figure 3A*). As shown in *Figure 3B*, the model

188  highlights a double saline bridge (K$_{665}$ and K$_{666}$ of CNBD with E$_{265}$ and E$_{264}$ of TRIP8b$_{nano}$)

189  in line with the ITC results described above (*Figure 3-figure supplement 3*). Of note, the

190  contribution of residue R$_{662}$ to the binding is also consistent with previous experiments

191  showing residual TRIP8b interaction in a CNBD deletion mutant ending at position 663

192  (Saponaro et al., 2014). Our modelling data suggest that, upon folding of the distal portion

193  of the C-helix, the side chains of residues $R_{662}$ and $K_{665}$ face to the inside when contacting

194  cAMP, but face to the outside when binding TRIP8b (*Figure 3-figure supplement 4*). This

195  indicates that cAMP and TRIP8b directly compete for the binding to the distal region of C-

196  helix.

197  In addition to clarifying the role of residues in the distal portion of the CNBD C-helix, the

198  model also highlights a second important cluster of electrostatic interactions with $R_{650}$ in

199  the proximal portion of the CNBD C-helix contacting $E_{240}$ and $E_{241}$ in helix N of $TRIP8b_{nano}$

200  (*Figure 3C*). To confirm the contribution of these residues, we reversed charges and tested

201  each residue mutation for binding in ITC. The results in *Figure 3-figure supplement 3* show

202  that $R_{650}E$ caused a more than six-fold reduction in binding affinity for $TRIP8b_{nano}$, with

203  smaller but significant effects seen also for $E_{240}R$ and $E_{241}R$.

204  A third important contact highlighted by the model is the interaction between $N_{547}$ in the N-

205  bundle loop of the CNBD and $D_{252}$ in the link between helix N and helix C of $TRIP8b_{nano}$

206  (*Figure 3D*). We tested this potential interaction by disrupting the expected hydrogen bond

207  between $N_{547}$ and the carboxyl group of the negative residue ($D_{252}$) in $TRIP8b_{nano}$. The

208  asparagine in CNBD was mutated into aspartate ($N_{547}D$) to generate an electrostatic

209  repulsion for $D_{252}$, and the carboxyl group in $D_{252}$ of $TRIP8b_{nano}$ was removed by mutation

210  into asparagine ($D_{252}N$). As predicted, $N_{547}D$ greatly reduced binding to TRIP8b in ITC

211  assays (*Figure 3-figure supplement 3*), with a smaller but significant effect observed also

212  for $D_{252}N$ (*Figure 3-figure supplement 3*). These results confirm and extend our previous

213  finding that the N-bundle loop contributes in a substantial manner to the binding of TRIP8b

214  (Saponaro et al., 2014). In search for the allosteric effect of TRIP8b on cAMP binding,

215  already postulated on the basis of electrophysiological and structural data (Hu et al., 2013;

216  Saponaro et al., 2014), we have further tested by ITC the affinity of $N_{547}D$ CNBD mutant

217  for cAMP. Somewhat surprisingly, the affinity of the mutant is much lower than that of the

218    wt ($N_{547}D$ $K_D$ = 5.5 ± 0.4 µM (n=3) *vs.* wt $K_D$ = 1.4 ± 0.1 µM (n=3)). Moreover, we

219    measured a reduced sensitivity to cAMP also in patch clamp experiments where addition

220    of 5 µM cAMP caused a right shift in the $V_{1/2}$ of the mutant HCN2 channel of only 5 mV

221    while the wt channel shifted by 12 mV (*Figure 3-figure supplement 5*). To exclude that the

222    $N_{547}D$ mutation affects the overall structure of the CNBD, we performed the NMR ($^{1}H$-$^{15}N$

223    HSQC spectrum) analysis of the $N_{547}D$ CNBD mutant. Our data show that the protein is

224    appropriately folded (*Figure 3-figure supplement 6*). In conclusion, since the N-bundle loop

225    does not directly contact any of the residues of the cAMP binding pocket, these findings

226    underscore a previously unaddressed role of the N-bundle loop in allosterically modulating

227    cAMP binding to the CNBD (see Discussion).

228

229    **TRIP8b$_{nano}$ as a tool for the direct regulation of native HCN currents**

230    Next, we asked whether the relatively short TRIP8b$_{nano}$ could be used to block cAMP-

231    dependent modulation of HCN channels by delivering the peptide to full length channels.

232    To this end, we dialyzed TRIP8b$_{nano}$ into the cytosol of HEK 293T cells transfected either

233    with HCN1, HCN2, or HCN4 channels. The peptide was added (10 µM) in the recording

234    pipette together with a non-saturating concentration of cAMP (5 µM for HCN2, 1 µM for

235    HCN4) expected to induce a ~10 mV rightward shift in the half-activation potential ($V_{1/2}$) of

236    the channels (*Figure 4*). No cAMP was added in the case of HCN1, because, in HEK 293T

237    cells, this isoform is already fully shifted to the right by the endogenous cAMP and does

238    not respond further (*Figure 4-figure supplement 1*). Still, it is possible to induce a ~10 mV

239    left shift in HCN1 $V_{1/2}$ by introducing the mutation R549E that prevents cAMP binding to

240    the CNBD (*Figure 4-figure supplement 1*).

*Figure 4A-C* show representative currents recorded at four given voltages, in control solution, + 5 $\mu$M cAMP and + 5 $\mu$M cAMP + 10 µM TRIP8b$_{nano}$ in the patch pipette (HCN4 and HCN2) or + 10 µM TRIP8b$_{nano}$ (HCN1). Already from a visual comparison of the most positive voltage at which the current appears measurable, it is evident that TRIP8b$_{nano}$ counteracts the activating effect of cAMP on the voltage-dependent gating. In the case of HCN1, the effect of TRIP8b$_{nano}$ can be observed without added cAMP for the aforementioned reasons. *Figure 4D-F* show the mean channel activation curves obtained from the above and other experiments. Fitting the Boltzmann equation to data (solid and dashed lines of *Figure 4D-F*, see Methods for equation) yielded the half-activation potential values ($V_{1/2}$) plotted in *Figure 4G.* The addition of TRIP8b$_{nano}$ prevents the cAMP-induced right shift of about 13 mV in HCN4 ($V_{1/2}$ = -102.8, -89.2, -102.1 mV, for control, + cAMP and + cAMP + TRIP8b$_{nano}$, respectively), and of about 11 mV in HCN2 ($V_{1/2}$ = -93.7, -83.5, -94.5 mV, for control, + cAMP and + cAMP + TRIP8b$_{nano}$, respectively). In HCN1, TRIP8b$_{nano}$ induced a left shift of about 10 mV in $V_{1/2}$ (from – 72.8 to -82 mV) that is comparable to that induced by the R549E mutation (from -72.7 to -80.4 mV) (*Figure 4-figure supplement 1*).

Figure 4G also shows the result of a control experiment performed on HCN4 where 10 µM TRIP8b$_{nano}$ was added to the extracellular medium (TRIP8b$_{nano}$ bath) in order to test if the peptide was able to pass the cell membrane (current traces and activation curves not shown). The $V_{1/2}$ value, which is similar to that of cAMP alone (-91.7 vs. -89.2 mV), confirmed that TRIP8b$_{nano}$ peptide affects channel gating only if added to the intracellular solution presumably because it does not diffuse through the cell membrane.

It is worth noting that TRIP8b$_{nano}$ prevents other related aspects of cAMP activation in HCN, such as the acceleration of activation kinetics (Wainger et al., 2001) and, for HCN2 only, the increase in maximal current (Craven & Zagotta, 2004; Wainger et al., 2001). For

266    example, the activation kinetics ($\tau_{on}$) of HCN4 measured at -120 mV was: control = 2 ± 0.2

267    s, + cAMP = 1.2 ± 0.1 s, + cAMP + TRIP8b$_{nano}$ = 2 ± 0.3 s (*Figure 4-figure supplement 2*).

268    Moreover, Figure 4B clearly shows that TRIP8b$_{nano}$ fully prevented the increase in maximal

269    current in HCN2.

270    From these results, we reckoned the peptide may be employed as a regulatory tool for

271    native I$_f$/I$_h$ currents. As proof of principle, we tested whether TRIP8b$_{nano}$ can modulate the

272    frequency of action potential firing in SAN myocytes. In these cells I$_f$ is key contributor of

273    the diastolic depolarization phase of the pacemaker action potential cycle. Moreover, the

274    autonomic nervous system modulates the frequency of action potential firing by changing

275    intracellular cAMP levels, which in turn acts on f-HCN channel open probability

276    (DiFrancesco, 1993). We thus recorded the native I$_f$ current in acutely isolated rabbit SAN

277    myocytes with and without 10 µM TRIP8b$_{nano}$ in the pipette solution (*Figure 5A*). *Figure 5B*

278    shows that the averaged I$_f$ activation curve measured in presence of TRIP8b$_{nano}$ is

279    significantly shifted to hyperpolarized voltages compared to the control. This indicates that

280    the peptide is displacing the binding of endogenous cAMP to native HCN channels.

281    Moreover, when the experiment was repeated in the presence of 1 µM cAMP, TRIP8b$_{nano}$

282    prevented the typical cAMP-dependent potentiation of the native I$_f$ current (*Figure 5B*). In

283    light of these results, we tested whether TRIP8b$_{nano}$ is also able to modulate cardiac

284    automaticity by antagonizing basal cAMP. The data in *Figure 5C* show that TRIP8b$_{nano}$

285    indeed significantly decreased the rate of action potential firing in single SAN cells.

286    Strikingly, the observed 30% decrease in action potential rate corresponds to the effect

287    induced by physiological concentrations of acetylcholine (DiFrancesco, Ducouret, &

288    Robinson, 1989).

289    To conclusively prove that the inhibition of the native I$_f$ current was specifically due to

290    TRIP8b$_{nano}$ rather than caused by the dilution of the cellular content followed by whole cell

291 configuration, we created a TAT version of TRIP8b$_{nano}$ (hereafter TAT-TRIP8b$_{nano}$). Indeed,

292 the TAT sequence allows the entry of biomolecules into a cell via endocytosis and/or direct

293 translocation across the plasma membrane, thus leaving the cytosolic content unaltered

294 (Guidotti, Brambilla, & Rossi, 2017).

295 We thus tested whether both TRIP8b$_{nano}$ and TAT-TRIP8b$_{nano}$ were able to selectively

296 inhibit the β-adrenergic stimulation of I$_f$ current, while leaving the potentiation of L-type

297 Ca$^{2+}$ current (I$_{Ca,L}$) unaltered. To this end, we recorded either the native I$_f$ or I$_{Ca,L}$ current

298 from cardiomyocytes acutely isolated from mouse sinoatrial node (SAN) in the presence

299 and in the absence of 10 μM TRIP8b$_{nano}$ or TAT-TRIP8b$_{nano}$, before and after stimulation

300 with 100 nM isoproterenol (ISO), a β-adrenergic receptor agonist (*Figure 6*). Strikingly,

301 TRIP8b$_{nano}$ prevented the isoproterenol-induced increase of I$_f$ current density, both when

302 the peptide was added in the recording pipette solution (*Figure 6A and 6B*), and when it

303 was used in the TAT version (*Figure 6A and 6C*). The specificity of TRIP8b$_{nano}$ for I$_f$ current

304 was confirmed by the absence inhibition of basal I$_{Ca,L}$ (*Figure 6D*). In addition, we failed to

305 record a significant difference in the isoproterenol-stimulated increase of the I$_{CaL}$ current

306 density between the control condition and 10μM TRIP8b$_{nano}$ (*Figure 6E*) or TAT-

307 TRIP8b$_{nano}$ (*Figure 6F*) conditions. To test whether the TAT-TRIP8b$_{nano}$ effect described

308 above was exclusively due to TRIP8b$_{nano}$ peptide, we repeated the experiments with a

309 scrambled version of the peptide  (TAT- (SCRAMBLED) TRIP8b$_{nano}$) to exclude that the

310 effect could be due to the TAT sequence (*Figure 6-figure supplement 1*). We failed to

311 observe a significant reduction in the responsiveness of I$_f$ to isoproterenol in the presence

312 of TAT-  (SCRAMBLED) TRIP8b$_{nano}$ confirming that prevention of cAMP induced f- current

313 stimulation was specific of the TRIP8b$_{nano}$ sequence.

314

315

## Discussion

### TRIP8b-CNBD complex

In this work we have identified the minimal binding peptide that reproduces the effects of TRIP8b on HCN channel gating. The peptide is 40 aa long and binds the HCN CNBD with high affinity ($K_D$ =1.4 $\mu$M). By solving the NMR structures of TRIP8b$_{nano}$ and HCN CNBD in the bound form, we generated a structural model of their complex. The model provides detailed information on this protein - protein interaction at the atomic level with implications on their physiological function. The data show that the minimal binding unit of TRIP8b, TRIP8b$_{nano}$, folds in two helices upon binding and suggest that this region is intrinsically disordered when it is not bound. The model structurally validates previous indirect evidences, which suggested that TRIP8b binds to two discrete elements of the CNBD: the N-bundle loop and the C-helix (Saponaro et al., 2014). The complex forms by electrostatic interactions, which are spread throughout the contact surface. As a consequence of the interaction with TRIP8b$_{nano}$, the C-helix of CNBD increases in length, a behavior previously observed in the case of cAMP binding (Puljung & Zagotta, 2013). This portion of C-helix includes the two residues $R_{662}$ and $K_{665}$ engaged in salt bridge formation with respectively $E_{250}$/$D_{257}$ and $E_{264}$ of TRIP8b$_{nano}$. Important to note is that the two cationic residues are also involved in cAMP binding (Lolicato et al., 2011; Zagotta et al., 2003; Zhou & Siegelbaum, 2007). The finding that TRIP8b and cAMP share the same binding sites on the C-helix provides a solid molecular explanation for functional data, which imply a competition between the two regulators (Bankston et al., 2017; Deberg et al., 2015; Han et al., 2011). Another study however has indicated that a direct competition model does not fully explain the mutually antagonistic effect of the two ligands (Hu et al., 2013). Specifically, the fact that the inhibitory effect of TRIP8b on channel activity persists even at saturating cAMP concentrations advocated an allosteric component in the regulation

mechanism. Our data, showing that N547D mutation in the N-bundle loop controls cAMP affinity in the binding pocket support the conclusion that the N-bundle loop allosterically controls cAMP binding. This is not surprising, given its crucial role of mechanically transducing to the pore the cAMP binding event within the CNBD (Saponaro et al., 2014).

The structural model also explains why a previously identified peptide selected by Lyman et al (Lyman et al., 2017) failed to reproduce the binding affinity of the TRIP8b$_{core}$ for the CNBD. This 37 aa long fragment is lacking one important contact residue, namely $E_{240}$ that, in our model, forms a salt bridge with $R_{650}$ of the CNBD. The loss of one crucial interaction is presumably the cause for such a major decrease in affinity (about 20 times lower) reported for this peptide.

**TRIP8b$_{nano}$ as a tool for modulating native I$_f$ currents**

In functional assays we showed that TRIP8b$_{nano}$ binds the HCN channel CNBD with high affinity and fully abolishes the cAMP effect in all tested isoforms (HCN1, 2 and 4).

Given the small size of the peptide (<5kDa), TRIP8b$_{nano}$ is a good candidate for an *in vivo* delivery into intact cells. As a proof of concept, we fused TRIP8b$_{nano}$ to an internalization sequence, the TAT peptide (YGRKKRRQRRRGG). This arginine-rich Cell Penetrating Peptide (CPP) from HIV has been used in several studies as a vehicle for the delivery of large molecules across the plasma membrane (Guidotti et al., 2017). In our case, the challenge was to construct a TAT-fusion protein, which is efficiently delivered in the cell without compromising TRIP8b$_{nano}$ function. Indeed, covalent conjugation of a CPP may negatively affect both the function of the cargo, and the cell-penetrating efficacy of the CPP-peptide chimera (Kristensen, Birch, & Morck Nielsen, 2016). The design of the construct was greatly supported by the detailed knowledge of the electrostatic interactions with the target protein CNBD, provided by the NMR model structure. This structure suggested that the polycationic TAT sequence would be best linked to the N-terminus of

366 TRIP8b$_{nano}$ to avoid interference with the cationic residues of CNBD, mainly located in the

367 distal region of C-helix, which are crucial for the binding of the peptide. From test

368 experiments with the TAT-TRIP8b$_{nano}$ peptide in SAN myocytes we can conclude that this

369 strategy was successful in that: i) the peptide is efficiently delivered inside the cells; ii) it is

370 kept in its active conformation; iii) the TAT sequence did not destroy cell membranes and

371 did not interfere with the basic features of I$_f$ and I$_{Ca,L}$ currents; iv) the modification did not

372 affect the proteolytic stability of the TRIP8b$_{nano}$ peptide at least in the time frame of our

373 experiments (30 min to 1 hour).

374 In conclusion, we successfully used the miniaturized TRIP8b$_{nano}$ peptide to selectively

375 control native I$_f$ currents and the rate of spontaneous firing in SAN myocytes. Unlike

376 channel blockers, which inhibit ionic currents, the peptide only interferes with the cAMP-

377 based regulation of HCN channels, while leaving basal HCN functions unaltered. In

378 addition and in contrast to even the most selective blockers, it is selective for HCN and it

379 does not interfere with other cAMP-modulated channels present in the SAN, such as L-

380 type Ca$^{2+}$ channels. Collectively, this makes TRIP8b$_{nano}$ a promising tool in targeted

381 therapeutic interventions.

382

383 **Materials and Methods**

384 **Key resources table**

| Reagent type. Species | Designation | Source or Reference | Identifiers | Additional information |
|---|---|---|---|---|
|  |  |  |  |  |
| gene (human) | HCN1 | Xention Ltd. (Cambridge, UK) | NM_021072.3 |  |
| gene (mouse) | HCN2 | other | NM_008226.2 | Laboratory of Steven A. |

| | | | | Siegelbaum |
|---|---|---|---|---|
| gene (rabbit) | HCN4 | PMID: 10212270 | NM_001082707.1 | |
| gene (mouse) | TRIP8b | other | NM_001163516.3 | Laboratory of Steven A. Siegelbaum |
| strain, strain background (E. coli) | DH5α | Thermo Fisher Scientific | | |
| strain, strain background (E. coli) | Stbl2 | Thermo Fisher Scientific | | |
| strain, strain background (Mus musculus) | either male or female mice | The Jackson Laboratory | RRID: C57BL6/J | |
| strain, strain background (Oryctolagus cuniculus) | New Zealand white female rabbits | Envigo | RRID: HsdOkd:NZW | |
| cell line (human) | HEK 293T | ATCC | RRID: CRL-3216™ | Tested negative for mycoplasma |
| biological sample (Mus musculus) | Isolated adult Sinoatrial node (SAN) cardiomyocytes | PMID: 11557233 | | |
| biological sample (Oryctolagus cuniculus) | Isolated adult Sinoatrial node (SAN) cardiomyocytes | PMID: 2432247 | | |
| recombinant DNA reagent | pET-52b (plasmid) | EMD Millipore | | |
| recombinant DNA reagent | modified pET-24b (plasmid) | Laboratory of Daniel L. Minor, Jr. | | |
| recombinant DNA reagent | pcDNA 3.1 (plasmid) | Clontech Laboratories | | |
| recombinant DNA reagent | pCI (plasmid) | Promega | | |
| recombinant DNA reagent | TRIP8bnano (cDNA) | This paper | | Made by PCR and cloning; see Constructs |
| recombinant DNA reagent | TRIP8bnano (E240R) (cDNA) | This paper | | Made by site-directed mutagenesis of TRIP8bnano wt; see Constructs |

| recombinant DNA reagent | TRIP8bnano (E241R) (cDNA) | This paper | | Made by site-directed mutagenesis of TRIP8bna no wt; see Constructs |
|---|---|---|---|---|
| recombinant DNA reagent | TRIP8bnano (E264K) (cDNA) | This paper | | Made by site-directed mutagenesis of TRIP8bna no wt; see Constructs |
| recombinant DNA reagent | (E265K) (cDNA) | This paper | | Made by site-directed mutagenesis of TRIP8bna no wt; see Constructs |
| recombinant DNA reagent | TRIP8bnano (D252N) (cDNA) | This paper | | Made by site-directed mutagenesis of TRIP8bna no wt; see Constructs |
| recombinant DNA reagent | TRIP8bcore (cDNA) | PMID: 25197093 | | |
| recombinant DNA reagent | human HCN2 CNBD (cDNA) | PMID: 25197093 | | |
| recombinant DNA reagent | human HCN2 CNBD (N547D) (cDNA) | This paper | | Made by site-directed mutagenesis of human HCN2 CNBD wt; see Constructs |
| recombinant DNA reagent | human HCN2 CNBD (K665E) (cDNA) | This paper | | Made by site-directed mutagenesis of human HCN2 CNBD wt; see Constructs |
| recombinant DNA reagent | human HCN2 CNBD (K666E) (cDNA) | This paper | | Made by site-directed mutagenesis of human HCN2 CNBD wt; see Constructs |

| recombinant DNA reagent | human HCN2 CNBD (R650E) (cDNA) | This paper | | Made by site-directed mutagenesis of human HCN2 CNBD wt; see Constructs |
|---|---|---|---|---|
| recombinant DNA reagent | human HCN1 (cDNA) | Xention Ltd. (Cambridge, UK) | | |
| recombinant DNA reagent | TRIP8b (1a4) (cDNA) | This paper | | Made by PCR and cloning, see Constructs |
| recombinant DNA reagent | mouse HCN2 (cDNA) | Laboratory of Steven A. Siegelbaum | | |
| recombinant DNA reagent | rabbit HCN4 (cDNA) | PMID: 10212270 | | |
| recombinant DNA reagent | mouse HCN2 (N520D) (cDNA) | This paper | | Made by site-directed mutagenesis of mouse HCN2 wt; see Constructs |
| sequence-based reagent | human HCN1 (R549E) (cDNA) | This paper | | Made by site-directed mutagenesis of human HCN1 wt; see Constructs |
| peptide, recombinant protein | TAT-TRIP8b$_{nano}$ (YGRKKRRQR RRGG-NHSLEEEFERA KAAVESTEFW DKMQAEWEE MARRNWISEN) | CASLO ApS | | |
| peptide, recombinant protein | TAT-(SCRAMBLED) TRIP8b$_{nano}$ (YGRKKRRQR RRGG-RNEAEAAEVA QKDMINERAR THEFEWESWE MWENLSESFK) | CASLO ApS | | |

| | | | | |
|---|---|---|---|---|
| commercial assay or kit | QuikChange Lightning Site-Directed Mutagenesis Kit | Agilent | | |
| commercial assay or kit | Thermo Scientific TurboFect Transfection Reagent | Thermo Fisher Scientific | | |
| chemical compound, drug | Adenosine 3',5'-cyclic monophosphate (cAMP) | SIGMA | | |
| software, algorithm | Clampfit 10.5/10.7 | Molecular Devices | RRID:SCR_011323 | |
| software, algorithm | CYANA-2.1 | L. A. Systems, Inc. | | |
| software, algorithm | AMBER 12.0 | http://pyenmr.cerm.unifi.it/access/index/amps-nmr | | |
| software, algorithm | HADDOCK2.2 | www.wenmr.eu | | |

385

## Constructs

The cDNA fragment encoding residues 235 – 275 (TRIP8b$_{nano}$) of mouse TRIP8b (splice variant 1a4) was cloned into pET-52b (EMD Millipore) downstream of a Strep (II) tag sequence, while the cDNA fragment encoding residues 521–672 of human HCN2 (HCN2 CNBD) was cloned, in a previous study, into a modified pET-24b downstream of a double His$_6$-maltose-binding protein (MBP) (Saponaro et al., 2014). The cDNA encoding full-length human HCN1 channel and mouse TRIP8b (1a4) were cloned into the mammalian expression vector pcDNA 3.1 (Clontech Laboratories), while mouse HCN2 channel and rabbit HCN4 channel were cloned into the mammalian expression vector pCI (Promega). Mutations were generated by site-directed mutagenesis (QuikChange site-directed mutagenesis kit; Agilent Technologies) and confirmed by sequencing.

## Preparation of proteins

The HCN2 CNBD WT and mutant proteins, as well as the TRIP8b$_{core}$ and TRIP8b$_{nano}$ proteins (WT and mutants) were produced and purified following the procedure previously described (Saponaro et al., 2014).

20

**Structure calculation of the cAMP-free human HCN2 CNBD in complex with TRIP8b_nano and vice versa**

NMR experiments were acquired on Bruker Avance III 950, 700 and 500 MHz NMR spectrometers equipped with a TXI-cryoprobe at 298 K. The acquired triple resonance NMR experiments for the assignment of backbone resonances of cAMP-free HCN2 CNBD (CNBD hereafter) in complex with TRIP8b_nano and vice versa are summarized in *Figure 3-source data 1*. $^{15}N$, $^{13}C'$, $^{13}C_{\alpha}$, $^{13}C_{\beta}$, and $H_{\alpha}$ chemical shifts were used to derive $\phi$ and $\psi$ dihedral angles by TALOS+ program (Cornilescu, Delaglio, & Bax, 1999) for both CNBD and TRIP8b_nano. For TRIP8b_nano, CYANA-2.1 structure calculation (Guntert & Buchner, 2015) was performed using 68 $\phi$ and $\psi$ dihedral angles and 40 backbone hydrogen bonds as input. For CNBD, CYANA-2.1 structure calculation was performed using 108 $\phi$ and $\psi$ dihedral angles, combined with the NOEs obtained in our previous determination of the cAMP-free form of the CNBD (Saponaro et al., 2014) for those regions not affected by the interaction with TRIP8b_nano. The 10 conformers of TRIP8b_nano and CNBD with the lowest residual target function values were subjected to restrained energy minimization with AMBER 12.0 (Case et al., 2012) (http://pyenmr.cerm.unifi.it/access/index/amps-nmr) and used as input in docking calculations.

**Docking calculations**

Docking calculations were performed with HADDOCK2.2 implemented in the WeNMR/West-Life GRID-enabled web portal (www.wenmr.eu). The docking calculations are driven by ambiguous interaction restraints (AIRs) between all residues involved in the intermolecular interactions (Dominguez, Boelens, & Bonvin, 2003). Active residues of the CNBD were defined as the surface exposed residues (at least 50% of solvent accessibility), which show chemical shift perturbation upon TRIP8b_nano binding.

The assignment of the CNBD bound to TRIP8b_nano allowed to highlight the residues of CNBD whose backbone featured appreciable Combined Chemical Shift Perturbation (CSP) (*Figure 3-figure supplement 1*). The combined CSP ($\Delta_{HN}$) is given by the equation $\Delta_{HN}=\{((H_{Nfree}-H_{Nbound})^2+((N_{free}-N_{bound})/5)^2)/2\}^{\frac{1}{2}}$ (Garrett, Seok, Peterkofsky, Clore, & Gronenborn, 1997).

Passive residues of CNBD were defined as the residues close in space to active residues and with at least 50% solvent accessibility.

In the case of TRIP8b$_{nano}$, the conserved stretch E$_{239}$-E$_{243}$, located in helix N, was defined as active region in a first docking calculation, while all the other solvent accessible residues of the peptide were defined as passive. This docking calculation generated several clusters. A post-docking filter step allowed us to select those clusters having an orientation of TRIP8b$_{nano}$ bound to CNBD in agreement with a DEER study on the CNBD - TRIP8b$_{nano}$ interaction (Deberg et al., 2015). The selected clusters grouped in two classes on the basis of the orientation of helix N of TRIP8b$_{nano}$ (N) relative to CNBD (*Figure 3-figure supplement 2*. A second docking calculation was subsequently performed introducing also residues E$_{264}$-E$_{265,}$ located in helix C of TRIP8b$_{nano}$ as active residues. The active residues for CNBD were the same used for the first calculation. For this second HADDOCK calculation 14 clusters were obtained and ranked according to their HADDOCK score. Among them only four clusters showed both an orientation of TRIP8b$_{nano}$ bound to CNBD in agreement with the DEER study (Deberg et al., 2015) and the involvement of E$_{239}$-E$_{243}$ stretch of TRIP8b$_{nano}$ in the binding to CNBD. These clusters were manually analyzed and subjected to a per-cluster re-analysis following the protocol reported in http://www.bonvinlab.org/software/haddock2.2/analysis/#reanal. From this analysis, it resulted that the top-ranking cluster, i.e. the one with the best energetic and scoring functions, has a conformation in agreement with mutagenesis experiments (*Figure 3-figure supplement 3*). Energy parameters (van der Waals energy, electrostatic energy, desolvation energy, and the penalty energy due to violation of restraints) for this complex model are reported in *Figure 3-source data 2.*

Both docking calculations were performed using 10 NMR conformers of both the CNBD and the TRIP8b$_{nano}$ structures calculated as described above. In the TRIP8b$_{nano}$ structures the unfolded N- and C-terminal regions were removed, while in the CNBD structures only the unfolded N-terminal region was removed. This is because the C-terminal region of the CNBD is known to comprise residues involved in TRIP8b$_{nano}$ binding (Saponaro et al., 2014). Flexible regions of the proteins were defined based on the active and passive residues plus two preceding and following residues. The residue solvent accessibility was calculated with the program NACCESS (Hu et al., 2013). In the initial rigid body docking calculation phase, 5000 structures of the complex were generated, and the best 400 in terms of total intermolecular energy were further submitted to the semi-flexible simulated annealing and a final refinement in water. Random removal of the restraints was turned off. The number of flexible refinement steps was increased from the default value of 500/500/1000/1000 to 2000/2000/2000/4000. The final 400 structures were then clustered

using a cutoff of 5.0 Å of RMSD to take into consideration the smaller size of protein-peptide interface.

**Electrophysiology of HEK 293T cells**

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (Euroclone) supplemented with 10% fetal bovine serum (Euroclone), 1% Pen Strep (100 U/mL of penicillin and 100 µg/ml of streptomycin), and stored in a 37°C humidified incubator with 5% $CO_2$. The plasmid containing cDNA of wild-type and mutant HCN1, HCN2 and HCN4 channels (1 µg) was co-transfected for transient expression into HEK 293T cells with a plasmid containing cDNA of Green Fluorescent Protein (GFP) (1.3 µg). For co-expression with TRIP8b (1a-4), HEK 293T cells were transiently transfected with wild-type (wt) and/or mutant human HCN1 cDNA (1 µg), wt TRIP8b (1a-4) cDNA (1 µg) and cDNA of Green Fluorescent Protein (GFP) (0.3 µg).

One day after transfection, GFP-expressing cells were selected for patch-clamp experiments in whole-cell configuration. The experiments were conducted at R.T. The pipette solution in whole cell experiments contained: 10 mM NaCl, 130 mM KCl, 1 mM egtazic acid (EGTA), 0.5 mM $MgCl_2$, 2 mM ATP (Mg salt) and 5 mM HEPES–KOH buffer (pH 7.4). The extracellular bath solution contained 110 mM NaCl, 30 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5 mM HEPES–KOH buffer (pH 7.4).

TRIP8b$_{nano}$ was added (10 µM) to the pipette solution. cAMP was added at different concentration to the pipette solution depending on the HCN isoform used: 0 µM for HCN1, 5 µM for HCN2 and 1 µM for HCN4.

Whole-cell measurements of HCN channels were performed using the following voltage clamp protocol depending on the HCN isoform measured: for HCN1, holding potential was –30 mV (1s), with steps from –20 mV to –120 mV (10 mV interval, 3.5 s) and tail currents recorded at –40mV (3 s); for HCN2, holding potential was –30 mV (1 s), with steps from –40 mV to –130 mV (10 mV interval, 5 s) and tail currents recorded at -40mV (5 s); for HCN4, holding potential was –30 mV (1s), steps from –30 mV to –165 mV (15 mV interval, 4.5 s) and tail currents were recorded at -40mV (5 s). Current voltage relations and activation curves were obtained by the above activation and deactivation protocols and analysed by the Boltzmann equation, see data analysis.

**Isolation and electrophysiology of rabbit sinoatrial node cells**

Animal protocols conformed to the guidelines of the care and use of laboratory animals established by Italian and European Directives (D. Lgs n° 2014/26, 2010/63/UE). New Zealand white female rabbits (0.8–1.2 kg) were anesthetized (xylazine 5mg/Kg, i.m.), and euthanized with an overdose of sodium thiopental (i.v.); hearts were quickly removed, and the SAN region was isolated and cut in small pieces. Single SAN cardiomyocytes were isolated following an enzymatic and mechanical procedure as previously described (DiFrancesco, Ferroni, Mazzanti, & Tromba, 1986). Following isolation, cells were maintained at 4 °C in Tyrode solution: 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM D-glucose, 5 mM HEPES-NaOH (pH 7.4).

For patch clamp experiments cells were placed in a chamber on an inverted microscope and experiments were performed in the whole-cell configuration at 35 ± 0.5 °C. The pipette solution contained: 10 mM NaCl, 130 mM KCl, 1 mM egtazic acid (EGTA), 0.5 mM $MgCl_2$, and 5 mM HEPES–KOH buffer (pH 7.2). The $I_f$ current was recorded from single cells superfused with Tyrode solution with 1 mM $BaCl_2$, and 2 mM $MnCl_2$.

$I_f$ activation curves were obtained using a two-step protocol in which test voltage steps (from -30 to -120 mV, 15 mV interval) were applied from a holding potential of -30 mV and were followed by a step to -125 mV. Test steps had variable durations so as to reach steady –state activation at all voltages. Analysis was performed with the Boltzmann equation (see data analysis).

In current-clamp studies, spontaneous action potentials were recorded from single cells superfused with Tyrode solution, and rate was measured from the interval between successive action potential. When indicated cAMP (1 µM) and/or nanoTRIP8b (10 µM) were added to the pipette solution.

**Isolation and electrophysiology of mouse sinoatrial node cells**

Mice were killed by cervical dislocation under general anesthesia consisting of 0.01 mg/g xylazine (2% Rompun; Bayer AG), 0.1 mg/g ketamine (Imalgène; Merial) and 0.04mg/g of Na-pentobarbital (Euthanasol VET, Laboratoire TVM, Lempdes, France), and beating hearts were quickly removed. The SAN region was excised in warmed (35°C) Tyrode's solution containing: 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Hepes-NaOH (pH = 7.4), and 5.5 mM D-glucose and cut in strips. Strips were then transferred into a "low-$Ca^{2+}$-low-$Mg^{2+}$" solution containing: 140 mM NaCl; 5.4 mM KCl, 0.5 mM $MgCl_2$,

529 0.2 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 50 mM taurine, 5.5 mM D-glucose, 1 mg/ml bovine serum

530 albumin (BSA), 5 mM Hepes-NaOH (pH = 6.9).

531 Tissue was digested by adding Liberase TH (0.15 mg/ml, Roche Diagnostics GmbH,

532 Mannheim, Germany), elastase (1.9 U/ml, Worthington, Lakewood, USA). Digestion was

533 carried out for a variable time of 15–18 minutes at 35°C. Tissue strips were then washed

534 and transferred into a modified "Kraftbrühe" (KB) medium containing: 70 mM L-glutamic

535 acid, 20 mM KCl, 80 mM KOH, (±) 10 mM D- b-OH-butyric acid; 10 mM KH$_2$PO$_4$, 10 mM

536 taurine, 1mg/ml BSA and 10 mM Hepes-KOH (pH = 7.4).

537 Single SAN cells were isolated by manual agitation in KB solution at 35°C for 30–50

538 seconds.

539 Cellular automaticity was recovered by re-adapting the cells to a physiological extracellular

540 Ca$^{2+}$ concentration by addition of a solution containing: 10 mM NaCl, 1.8 mM CaCl$_2$ and

541 normal Tyrode solution containing BSA (1 mg/ml). The final storage solution contained:

542 100 mM NaCl, 35 mM KCl, 1.3 mM CaCl$_2$, 0.7 mM MgCl$_2$, 14 mM L-glutamic acid, (±) 2

543 mM D-b-OH-butyric acid, 2 mM KH$_2$PO$_4$, 2 mM taurine, 1 mg/ml BSA, (pH = 7.4). Cells

544 were then stored at room temperature until use. All chemicals were from SIGMA (St

545 Quentin Fallavier, France).

546 For electrophysiological recording, SAN cells in the storage solution were harvested in

547 special custom-made recording plexiglas chambers with glass bottoms for proper cell

548 attachment and mounted on the stage of an inverted microscope (Olympus IX71) and

549 perfused with normal Tyrode solution. The recording temperature was 36°C. We used the

550 whole-cell variation of the patch-clamp technique to record cellular ionic currents, by

551 employing a Multiclamp 700B (Axon Instruments Inc., Foster USA) patch clamp amplifier.

552 Recording electrodes were fabricated from borosilicate glass, by employing a WZ DMZ-

553 Universal microelectrode puller (Zeitz-Instruments Vertriebs GmbH, Martinsried,

554 Germany).

555 I$_f$ was recorded under standard whole-cell configuration during perfusion of standard

556 Tyrode's containing 2 mM BaCl$_2$ to block I$_{K1}$. Patch-clamp pipettes were filled with an

557 intracellular solution containing: 130 mM KCl, 10 mM NaCl, 1 mM EGTA, 0.5 mM MgCl$_2$

558 and 5 mM HEPES (pH 7.2).

559 For recording of L-type Ca$^{2+}$ currents, pipette solution contained: 125 mM CsOH, 20 mM

560 tetraethylammonium chloride (TEA-Cl), 1.2 mM CaCl$_2$, 5 mM Mg-ATP, 0.1 mM Li$_2$-GTP, 5

mM EGTA and 10 mM HEPES (pH 7.2 with aspartate). 30 µM TTX (Latoxan, Portes lès Valence, France) to block INa was added to external solution containing: 135 mM tetraethylammonium chloride (TEA-Cl), 4 mM $CaCl_2$,10 mM 4-amino-pyridine, 1 mM $MgCl_2$, 10 mM HEPES and 1 mg/ml Glucose (pH 7.4 with TEA-OH).

Electrodes had a resistance of about 3 MΩ. Seal resistances were in the range of 2–5 GΩ. 10µM TRIPb8$_{nano}$ was added to pipette solution. 10µM TAT-TRIPb8$_{nano}$ was added in cell storage solution for at least 30 min before patch clamp recording.

**TAT-peptides**


TAT-TRIP8b$_{nano}$   (**YGRKKRRQRRRGG**-NHSLEEEFERAKAAVESTEFWDKMQAEWEEMARRNWISEN, TAT sequence is shown in bold type) and TAT-(SCRAMBLED)TRIP8b$_{nano}$ (**YGRKKRRQRRRGG**-RNEAEAAEVAQKDMINERARTHEFEWESWEMWENLSESFK, TAT sequence is shown in bold type) were purchased from CASLO ApS. TAT-peptides were dissolved in Milliq water (1.5 mM) and added to the petri dish at the final concentration (10 µM) 30 minutes before current recordings. During the patch clamp experiments, cells were perfused with standard Tyrode with 2 mM $BaCl_2$ (see above) without the peptides. Recordings from the same petri dish were performed over a time window of 10 to 60 minutes in peptide-free solution


**Data analysis**

Data were acquired at 1 kHz using an Axopatch 200B amplifier and pClamp10.5 or 10.7 software (Axon Instruments). Data were analyzed off-line using Clampfit 10.5 or 10.7 (Molecular Devices) and Origin 2015 or 16 (OriginLab Corp., Northampton MA). Activation curves were analysed by the Boltzmann equation, $y=1/\{1+\exp[(V-V_{1/2})/s]\}$, where y is fractional activation, V is voltage, $V_{1/2}$ half-activation voltage, and s the inverse slope factor (mV) (DiFrancesco, 1999). Mean activation curves were obtained by fitting individual curves from each cell to the Boltzmann equation and then averaging all curves obtained. Activation time constants ($\tau_{on}$) were obtained by fitting a single exponential function,

$I=I_0 \exp(-t/\tau)$ to current traces recorded at the indicated voltages.

**Ethics statement**

591 Experiments on rabbit SAN cells were performed using left-over cells obtained during
592 experiments approved by the Animal Welfare Body of the University of Milan and by the
593 Italian Ministry of Health (license n.1127/2015-PR). Animal procedures were conformed to
594 the guidelines of the care and use of laboratory animals established by Italian and
595 European Directives (D. Lgs n° 2014/26, 2010/63/UE).

596 Mouse primary pacemaker cells were isolated from adult C5B6/J mice as previously
597 described (Mangoni and Nargeot, Cardiovasc Res 2001), in accordance with the Guide for
598 the Care and Use of Laboratory Animals (eighth edition, 2011), published by the US
599 National Institute of Health and European directives (2010/63/EU). The protocol was
600 approved by the ethical committee of the University of Montpellier and the French Ministry
601 of Agriculture (protocol N°: 2017010310594939).

602

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612

613 **Conflict of interest:** none

614

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616

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730    **Figure 1.** Functional and structural characterization of TRIP8b$_{nano}$. (**A**) Primary sequence

731    of TRIP8b$_{nano}$. Amino acid numbering refers to full length mouse TRIP8b (1a4). (**B**) Binding

732    of TRIP8b$_{core}$ and TRIP8b$_{nano}$ to purified His$_6$-MBP-CNBD measured by Isothermal titration

733    calorimetry (ITC). Upper panel, heat changes (μcal/sec) during successive injections of 8

734    μL of the corresponding TRIP8b peptide (200 μM) into the chamber containing His$_6$-MBP-

735    CNBD (20 μM). Lower panel, binding curve obtained from data displayed in the upper

736    panel. The peaks were integrated, normalized to TRIP8b peptide concentration, and

737    plotted against the molar ratio (TRIP8b peptide / His$_6$-MBP-CNBD). Solid line represents a

738    nonlinear least-squares fit to a single-site binding model, yielding, in the present examples,

739    a $K_D$ = 1.2 ± 0.1 μM for TRIP8b$_{core}$ and $K_D$ = 1.4 ± 0.1 μM for TRIP8b$_{nano}$. (**C**) Evidence for

740    TRIP8b$_{nano}$ folding upon CNBD binding based on the superimposition of the [$^1$H, $^{15}$N]

741    heteronuclear single quantum coherence (HSQC) NMR spectrum of CNBD-free

742    TRIP8b$_{nano}$ (black) and CNBD-bound TRIP8b$_{nano}$ (red). The latter experiment was

743 performed at the molar ratio ([CNBD]/[TRIP8b$_{nano}$]) = 3. The backbone amide (HN) signals

744 of the residues of CNBD-bound TRIP8b$_{nano}$ are labelled in red. (**D**) (Top) Ribbon

745 representation of the 10 lowest energy conformers of TRIP8b$_{nano}$ bound to CNBD used for

746 *in silico* modelling of CNBDTRIP8b$_{nano}$ complex. The unfolded regions at the N- and C-

747 termini of the construct (residues 235–237 and 270 – 275) are omitted for clarity. (Bottom)

748 Chemical Shift Index (CSI, calculated using TALOS+) plotted as a function of the residue

749 number of TRIP8b$_{nano}$ bound to CNBD. Positive values represent helical propensity.

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753 **Figure 2.** NMR structure of CNBD bound to TRIP8b$_{nano}$. (**A**) (Top) comparison of

754 secondary structure elements of cAMP-free CNBD (Saponaro et al., 2014), cAMP-bound

755 CNBD (Zagotta et al., 2003) and cAMP-free CNBD bound to TRIP8b$_{nano}$ (this study).

756 Secondary structure elements are indicated by arrows ($\beta$-strands) and cylinders ($\alpha$-

757 helices) and labelled. The loop between $\beta_6$ and $\beta_7$ constitutes the Phosphate Binding

758 Cassette (PBC). The elements that fold upon binding of cAMP and TRIP8b$_{nano}$ are shown

759 in red. (Bottom) Chemical Shift Index (CSI, calculated using TALOS+) plotted as a function

760 of the residue number of CNBD bound to TRIP8b$_{nano}$. Positive values represent helical

761 propensity, while negative values represent strands. (**B**) Ribbon representation of the 10

762 lowest energy conformers of CNBD bound to TRIP8b$_{nano}$ used for *in silico* modelling of

763 CNBD - TRIP8b$_{nano}$ complex. Secondary structure elements are coloured in light gray and

764 labelled. Loop regions are coloured in dark gray. The distal region of the C-helix (residues

765 657-662), which is unfolded in the free form of the CNBD (Saponaro *et al*, 2014) and folds

766 upon TRIP8b$_{nano}$ binding, is coloured in red. The unfolded regions at the N- and C-termini

767 of the construct (residues 521–532 and 663 – 672 respectively) are omitted for clarity.

768

769 **Figure 3.** Structural model of CNBD – TRIP8b$_{nano}$ complex. (**A**) Ribbon representation of

770 the complex where CNBD is in gray and TRIP8b$_{nano}$ is in orange. Helix N (N) and helix C

771 (C) of TRIP8b$_{nano}$ are labelled. C-helix of CNBD ($\alpha$C) is labelled, while N-bundle loop is

772 coloured in yellow. Positively charged residues of C-helix CNBD (blue) and negatively

773 charged residues of TRIP8b$_{nano}$ (red) involved in salt bridges are shown as sticks and

774 labelled. N$_{547}$ of the N-bundle loop (yellow) and D$_{252}$ of TRIP8b$_{nano}$ (orange) are shown as

sticks and labelled. (**B**) Close view of $K_{665}$ and $K_{666}$ of CNBD that interact respectively with $E_{265}$ and $E_{264}$ of TRIP8b$_{nano}$. C-helix ($\alpha$C) of CNBD, and Helix C (C) of TRIP8b$_{nano}$ are labelled. (**C**) Close view of $R_{650}$ of CNBD that is positioned between $E_{240}$ and $E_{241}$ of TRIP8b$_{nano}$. C-helix ($\alpha$C) of CNBD, and Helix N (N) of TRIP8b$_{nano}$ are labelled. (**D**) Close view of $N_{547}$ of N-bundle loop that forms a hydrogen bond (red dashed line) with $D_{252}$ of TRIP8b$_{nano}$. Helix E' ($\alpha$E') and C-helix ($\alpha$C) of CNBD, and Helix N (N) of TRIP8b$_{nano}$ are labelled.


**Figure 4.** TRIP8b$_{nano}$ abolishes cAMP effect on HCN channel gating. (**A**) Representative whole-cell HCN4, HCN2 and HCN1 currents recorded, at the indicated voltages, with control solution or with cAMP (1 μM for HCN4 and 5 μM for HCN2) or cAMP + 10 μM TRIP8b$_{nano}$ in the patch pipette (for HCN1 we added only 10 μM TRIP8b$_{nano}$). (**B**) Mean activation curves measured from HCN4, HCN2 and HCN1 in control solution (open circles), cAMP (filled circles), cAMP + TRIP8b$_{nano}$, or TRIP8b$_{nano}$ only in the case of HCN1(filled triangles). Solid and dashed lines indicate Boltzmann fitting to the data (see Material and Methods). (**C**) Half activation potential ($V_{1/2}$) values of HCN4 (blue), HCN2 (black) HCN1 (black) in control solution (open circle), cAMP (filled circle) and cAMP + TRIP8b$_{nano}$, or TRIP8b$_{nano}$ only in the case of HCN1 (filled triangle). HCN4, control = -102.8 ± 0.3 mV; HCN4 + 1μM cAMP = -89.2 ± 0.6 mV; HCN4 + 1μM cAMP + 10 μM TRIP8b$_{nano}$ = -102.1 ± 0.6 mV, HCN4 + 1μM cAMP in the patch pipette + 10 μM TRIP8b$_{nano}$ in the bath solution= -91.7 ± 0.3 mV; HCN2, control = -93.7± 0.3 mV; HCN2 + 5 μM cAMP = -83.5 ± 0.3 mV; HCN2 + 5 μM cAMP + 10 μM TRIP8b$_{nano}$ = -94.5 ± 0.6 mV; HCN1, control = -72.8 ± 0.2 mV; HCN1 + 10 μM TRIP8b$_{nano}$ = -82 ± 0.5 mV. Data are presented as mean ± SEM. Number of cells (N) ≥ 11. There is no significant difference between the controls and the addition of TRIP8b$_{nano}$ with (HCN4, HCN2) or without (HCN1) cAMP in the pipette. No significant difference was also observed for the addition of TRIP8b$_{nano}$ in the bath. Statistical analysis performed with two-way ANOVA, followed by post-hoc Tukey test ($P < 0.001$).


**Figure 5.** Effects of TRIP8b$_{nano}$ on voltage-dependent activation of I$_f$ and spontaneous rate in rabbit sinoatrial node (SAN) myocytes. (**A**) Representative whole-cell I$_f$ currents

807     recorded, at the indicated voltages, in the control solution and in the presence of 10 µM

808     TRIP8b$_{nano}$, without (top) and with 1 µM cAMP in the pipette (bottom). (**B**) Mean I$_f$

809     activation curves were measured using a two-step protocol (see Material and Methods) in

810     control (filled circles) or in the presence of: 1 µM cAMP (open circles); 10 µM TRIP8b$_{nano}$

811     (filled squares); 1 µM cAMP + 10 µM TRIP8b$_{nano}$ (open squares). Ligands were added in

812     the patch pipette. Half activation potential (V$_{1/2}$) of I$_f$ activation curves measured in control

813     = -64.1 ± 0.4 mV or in the presence of: 1 µM cAMP = -59.9 ± 0.4 mV; 10 µM TRIP8b$_{nano}$ =

814     -67.7 ± 0.4 mV; 1 µM cAMP + 10 µM TRIP8b$_{nano}$ = -67.6 ± 0.7 mV. Data are presented as

815     mean ± SEM. Number of cells (N) was ≥ 15. V$_{1/2}$ values are significantly different between

816     each other's whit the exception of V$_{1/2}$ obtained in the presence TRIP8b$_{nano}$ and cAMP +

817     TRIP8b$_{nano}$. Statistical analysis performed with two-way ANOVA, followed by post-hoc

818     Bonferroni test (*, P < 0.05) (**C**) (Left) Representative recordings of single SAN cell

819     spontaneous activity in control and in the presence of 10 µM TRIP8b$_{nano}$. (Right) Mean

820     spontaneous rate (Hz) recorded in control solution = 3.65 ± 0.29 Hz and in the presence of

821     10 µM TRIP8b$_{nano}$ added to the pipette = 2.69 ± 0.27 Hz. Data are presented as mean ±

822     SEM. Number of cells (N) was ≥ 7. Statistical analysis performed with t test (*, P < 0.05).

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824     **Figure 6.** Effect of TRIP8b$_{nano}$ and TAT-TRIP8b$_{nano}$ on I$_f$ and I$_{Ca,L}$ in mouse sinoatrial node

825     (SAN) myocytes. (**A**) Representative examples of I$_f$ recordings at -95 mV in control

826     conditions (top), in 10 µM TRIP8b$_{nano}$ dialysed cell (middle) and in cells perfused with 10

827     µM TAT-TRIP8b$_{nano}$ (bottom), before (black trace) and after (red trace) application of ISO

828     100 nM. The voltage-clamp protocol used for recordings is shown above current traces.

829     (**B**) Mean normalised I$_f$ current density recorded at -95 mV in absence and in presence of

830     10 µM TRIP8b$_{nano}$ in the patch pipette, before (open bars) and after (filled bars) 100 nM

831     ISO perfusion. Data are presented as mean ± SEM. Number of cells (N) ≥ 6. Statistical

832     analysis performed with two-way ANOVA test, followed by Sidak multiple comparisons test

833     (**, P < 0.01). (**C**) Mean normalised I$_f$ current density recorded at -95 mV in control solution

834     or in the solution containing 10 µM TAT-TRIP8b$_{nano}$, in absence (open bars) and in the

835     presence (filled bars) of 100 nM ISO. Data are presented as mean ± SEM. Number of cells

836     (N) ≥ 8. Statistical analysis performed with two-way ANOVA test, followed by Sidak

837     multiple comparisons test (****, P < 0.0001). (**D**) Representative examples of I$_{Ca,L}$

838     recordings at -10 mV in control conditions (top), in 10µM TRIP8b$_{nano}$ dialysed cell (middle)

839     and in cells perfused with 10 µM TAT-TRIP8b$_{nano}$ (bottom), before (black trace) and after

840  (red trace) application of ISO 100 nM. The voltage-clamp protocol used for recordings is

841  shown above current traces. (**E**) Mean normalised $I_{Ca,L}$ current density recorded at -10 mV

842  in absence and in presence of 10 µM TRIP8b$_{nano}$ in the patch pipette, before (open bars)

843  and after (filled bars) 100 nM ISO perfusion. Data are presented as mean ± SEM. Number

844  of cells (N) ≥ 8. Statistical analysis performed with two-way ANOVA test, followed by Sidak

845  multiple comparisons test (***, P < 0.001; ****, P < 0.0001). (**F**) Mean normalised $I_{Ca,L}$

846  current density recorded at -10 mV in control solution or in the solution containing 10 µM

847  TAT-TRIP8b$_{nano}$, in absence (open bars) and in the presence (filled bars) of 100 nM ISO.

848  Data are presented as mean ± SEM. Number of cells (N) ≥ 7. Statistical analysis

849  performed with two-way ANOVA test, followed by Sidak multiple comparison test (**, P <

850  0.01; *** P < 0.001).


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852  **Figure 3 - figure supplement 1.** CNBD residues involved in TRIP8b$_{nano}$ binding.

853  Combined chemical shift variations of NMR signals between CNBD unbound and

854  TRIP8b$_{nano}$-bound state. Combined chemical shift variations are calculated from the

855  experimental $^{1}$H and $^{15}$N chemical shift changes (Δδ($^{1}$H) and Δδ ($^{15}$N), respectively)

856  between the corresponding peaks in the two forms, through the following equation (Garrett

857  et al., 1997): $\Delta\partial^{combined} = \sqrt{\dfrac{\left(\Delta\partial(1_H)\right)^2 + \frac{1}{25}\left(\Delta\partial(15N)\right)^2}{2}}$


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860  Residues experiencing intermediate exchange regime (whose NMR signal becomes broad

861  beyond detection upon addition of TRIP8b$_{nano}$) are shown in grey. The horizontal dotted

862  line indicates the average value plus one standard deviation. Residues above the line

863  were set as "active" in the docking calculation described in the text (see Materials and

864  Methods).

865  Garrett DS, Seok YJ, Peterkofsky A, Clore GM & Gronenborn AM (1997) Identification by NMR of the binding
866      surface for the histidine-containing phosphocarrier protein HPr on the N-terminal domain of enzyme I
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**Figure 3 - figure supplement 2.** Representative families of clusters obtained from the first docking calculation. The clusters obtained can be grouped in two classes, shown here, on the basis of the position of helix N of TRIP8b$_{nano}$ (N) relative to the CNBD. In the left representative structure, helix N is oriented in a way that allows E$_{241}$ to interact with R$_{650}$ of C-helix. In the right representative structure, E$_{240}$ - E$_{241}$ interact with N$_{547}$ of CNBD. In all clusters K$_{665}$ or K$_{666}$ of CNBD establish a contact with either E$_{264}$ or E$_{265}$ of TRIP8b$_{nano}$. The structural elements of CNBD are labelled: αE', αA and αC. Helices N and C of TRIP8b$_{nano}$ are labelled.

**Figure 3 - figure supplement 3.** Biochemical validation of CNBD – TRIP8b$_{nano}$ complex. Dissociation constant (K$_D$) of the interaction between the indicated CNBD and TRIP8b$_{nano}$ peptides were measured by means of Isothermal Titration Calorimetry (ITC). CNBD WT - TRIP8b$_{nano}$ WT (black filled circle) = 1.4 ± 0.1 µM; CNBD K$_{665}$E - TRIP8b$_{nano}$ WT (grey filled circle) = 6 ± 0.3 µM; CNBD K$_{666}$E - TRIP8b$_{nano}$ WT (grey filled circle) = 9.9 ± 0.8 µM; CNBD WT - TRIP8b$_{nano}$ E$_{64}$K (purple filled circle) = 4.8 ± 0.2 µM; CNBD WT - TRIP8b$_{nano}$ E$_{65}$K (purple filled circle) = 6 ± 0.5 µM; CNBD R$_{650}$E - TRIP8b$_{nano}$ WT (red filled circle) = 9.6 ± 0.7 µM; CNBD WT - TRIP8b$_{nano}$ E$_{40}$R (green filled circle) = 5 ± 0.3 µM; CNBD WT - TRIP8b$_{nano}$ E$_{41}$R (green filled circle) = 3.8 ± 0.2 µM; CNBD N$_{547}$D - TRIP8b$_{nano}$ WT (blue filled circle) = 11 ± 0.9 µM; CNBD WT - TRIP8b$_{nano}$ D$_{52}$N (orange filled circle) = 3.4 ± 0.1 µM. Data are presented as mean ± SEM. Number of experiments (N) ≥ 3.  K$_D$ values of all tested combinations are statistically different from the K$_D$ of CNBD WT for TRIP8b$_{nano}$ WT (*, P ≤ 0.05; **, P < 0.01). Statistical analysis performed with ANOVA, followed by post-hoc Tukey test. Dashed black vertical line indicates the K$_D$ of WT peptides.

**Figure 3 - figure supplement 4.** Different orientation of R$_{662}$ and K$_{665}$ in the cAMP-bound and TRIP8b$_{nano}$-bound conformation of the CNBD. Residues R$_{662}$ and K$_{665}$ of CNBD C-helix (αC) which interact with cAMP (left, (Lolicato et al., 2011) and TRIP8b$_{nano}$ (right, this study) are represented as blue sticks and labelled. TRIP8b$_{nano}$ residues E$_{250}$ and D$_{257}$ interacting with R$_{662}$ and residue E$_{265}$ interacting with K$_{665}$ are shown in red sticks and labelled.

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904    channels. *J. Biol. Chem.* **286:** 44811–44820. Retrieved from doi: 10.1074/jbc.M111.297606.

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908    **Figure 3 - figure supplement 5.** Mutation $N_{520}D$ affects cAMP affinity in full-length HCN2

909    channel. Note that in mouse HCN2 $N_{520}D$ corresponds to $N_{547}D$ in human HCN2 (used in

910    our structural studies). (**A**) Representative whole-cell HCN2 currents recorded, at the

911    indicated voltages (-70, -80, - 90, -130 mV) in HEK 293T cells transfected with WT and

912    $N_{520}D$ mutant, in the absence and presence of 5 µM cAMP in the pipette. (**B**) Mean

913    activation curves obtained from the tail currents in (A) and other recordings in control

914    solution (filled circle) and in the presence of cAMP (open circles), with HCN2 wt (black)

915    and $N_{547}D$ mutant. Data were fitted to a Boltzmann equation (solid and dotted lines) (see

916    Methods) . (**C**) Half activation potential ($V_{1/2}$) values derived from Boltzmann equation

917    fitting. HCN2 WT (black filled circle) $V_{1/2}$ = -95.9 ± 0.3 mV; HCN2 WT + 5 µM cAMP (black

918    open circle) $V_{1/2}$ = 85.2 ± 0.4 mV; HCN2 $N_{520}D$ (red filled circle) $V_{1/2}$ = -96.8 ± 0.2 mV;

919    HCN2 $N_{520}D$ + 5 µM cAMP (red open circle) $V_{1/2}$ = -91.2 ± 0.2 mV. Data are presented as

920    mean ± SEM. Number of experiments (N) ≥ 6. Statistical analysis performed with two-way

921    ANOVA, followed by post-hoc Tukey test ( P < 0.001) showed that wt and mutant channel

922    are identical and that the cAMP-induced shifts are different.

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925    **Figure 3 - figure supplement 6.** Structural characterization of $N_{547}D$ CNBD protein.

926    Superimposition of the [$^1$H, $^{15}$N] HSQC spectra of CNBD wt (black) and CNBD $N_{547}D$

927    mutant (red).

928

929    **Figure 3- source data 1.** Acquisition parameters for NMR experiments performed on

930    cAMP-  free human HCN2 CNBD in complex with TRIP8b$_{nano}$ and vice-versa.

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932    **Figure 3- source data 2.** Docking calculation.

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**Figure 4 - figure supplement 1.** Comparison of half activation potentials ($V_{1/2}$) of HCN1 WT and of $R_{549}E$ mutant (Chen, Wang, & Siegelbaum, 2001). (A) Currents were measured at the indicated voltages  (- 50, -60, -70, -120 mV), from HEK 293T cells with control solution or with 15 µM cAMP in the patch pipette. (B) Mean activation curves obtained from tail currents were fitted to a Boltzmann equation (see Material and Methods). (C) Half activation potential ($V_{1/2}$) determined from Boltzmann fitting. HCN1 WT (black filled circle) = -72.7 ± 0.2 mV; HCN1 WT + cAMP (black open circle) = -73.1 ± 0.3 mV; HCN1 $R_{549}E$ (red filled circle) = -80.4 ± 0.2 mV; HCN1 $R_{549}E$ + cAMP (red open circle) = -81 ± 0.3 mV. Data are presented as mean ± SEM. Number of experiments (N) ≥ 8. Wt values are significantly different from mutant. Statistical analysis performed with two-way ANOVA, followed by post-hoc Tukey test (P < 0.001).

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**Figure 4 - figuresupplement 2.** Voltage-dependency of activation time constant ($\tau_{on}$) of HCN4 channels in control solution (open circles), 1 µM cAMP (filled circles), 1 µM cAMP + 10 µM TRIP8b$_{nano}$ (filled triangles). Data are presented as mean ± SEM. Number of cells (N) = 5.

**Figure 6 - figure supplement 1.** Effect of TAT- (SCRAMBLED) TRIP8b$_{nano}$ on $I_f$ in mouse sinoatrial node (SAN) myocytes. (A) Representative examples of $I_f$ recordings (at -95mV) in TAT-(SCRAMBLED)TRIP8b$_{nano}$ (top) or in TAT-TRIP8b$_{nano}$ (bottom) incubated cells. The voltage-clamp protocol used for recordings is shown above current traces. (**B**) Mean normalised $I_f$ current density recorded at -95 mV in the presence of 10 µM TAT-(SCRAMBLED) TRIP8b$_{nano}$ or TAT-TRIP8b$_{nano}$, before (open bars) and after (filled bars) 100 nM isoprenaline (ISO) perfusion. Data are presented as mean ± SEM. Number of cells (N) ≥ 11. Statistical analysis performed with two-way ANOVA test, followed by Sidak multiple comparisons test (****, P < 0.0001).

967

968

**A**

```
235           245           255           265           275
N HSLEEEFER A KAAVESDTE F WDKMQAEWE E MARRNWISE N
```

**B**

CNBD - TRIP8b_core

CNBD - TRIP8b_nano

Time (min)

Time (min)

μcal/sec

μcal/sec

kcal/mole
of injectant

kcal/mole
of injectant

Molar Ratio

Molar Ratio

**C**

$^{15}$N ppm

E250,W272
K246
E243
Q260
S237
W256
E254
N235
E265
F242 A261
E274
L238
E240
N270
T253
F244,V249
S251
R269
I272
E264
A247
A248
M259
D252
W263
D257
H236
A245
N275,E262

$^1$H ppm

**D**

helix N          helix C

CSI

Residue Number

**A**



**B**
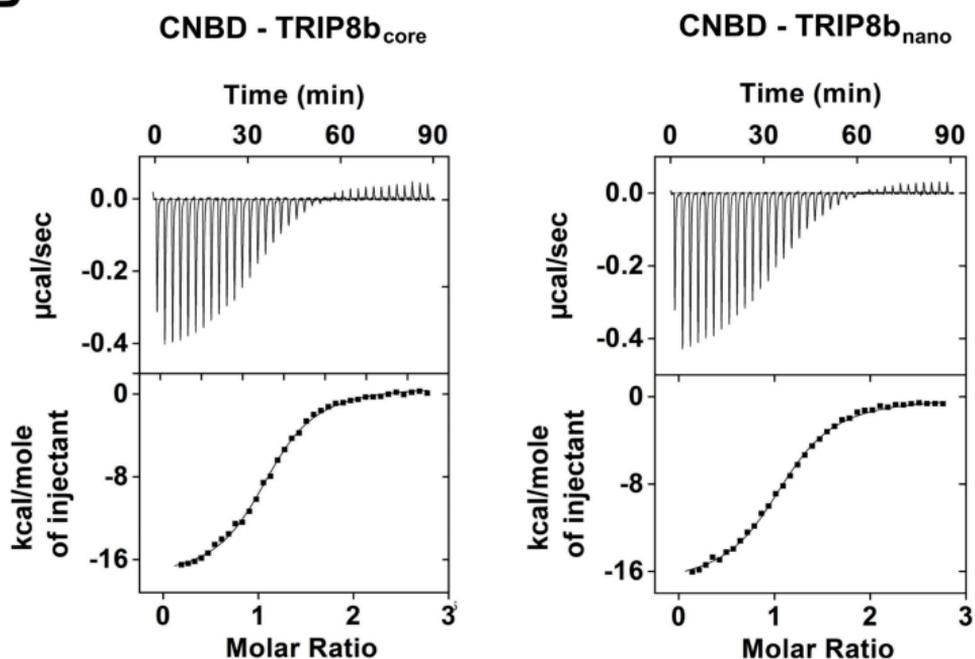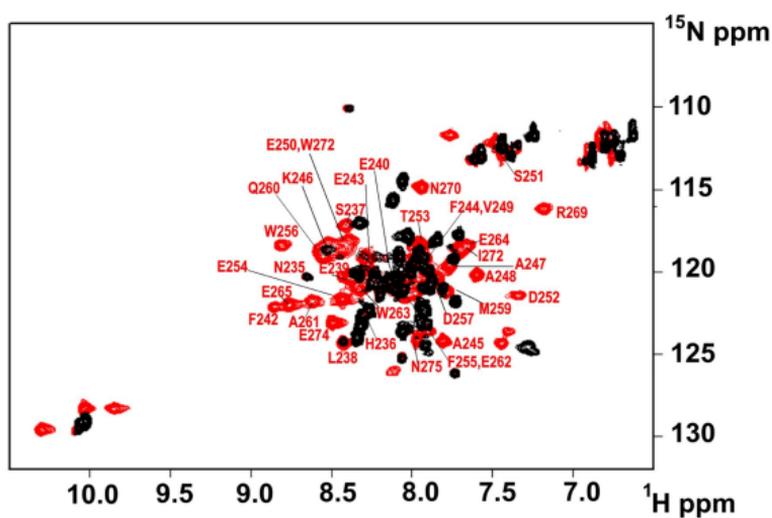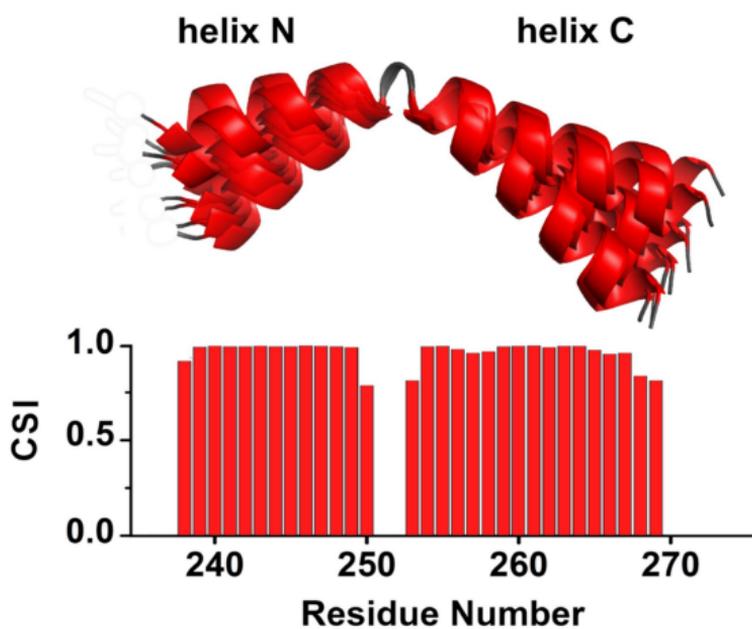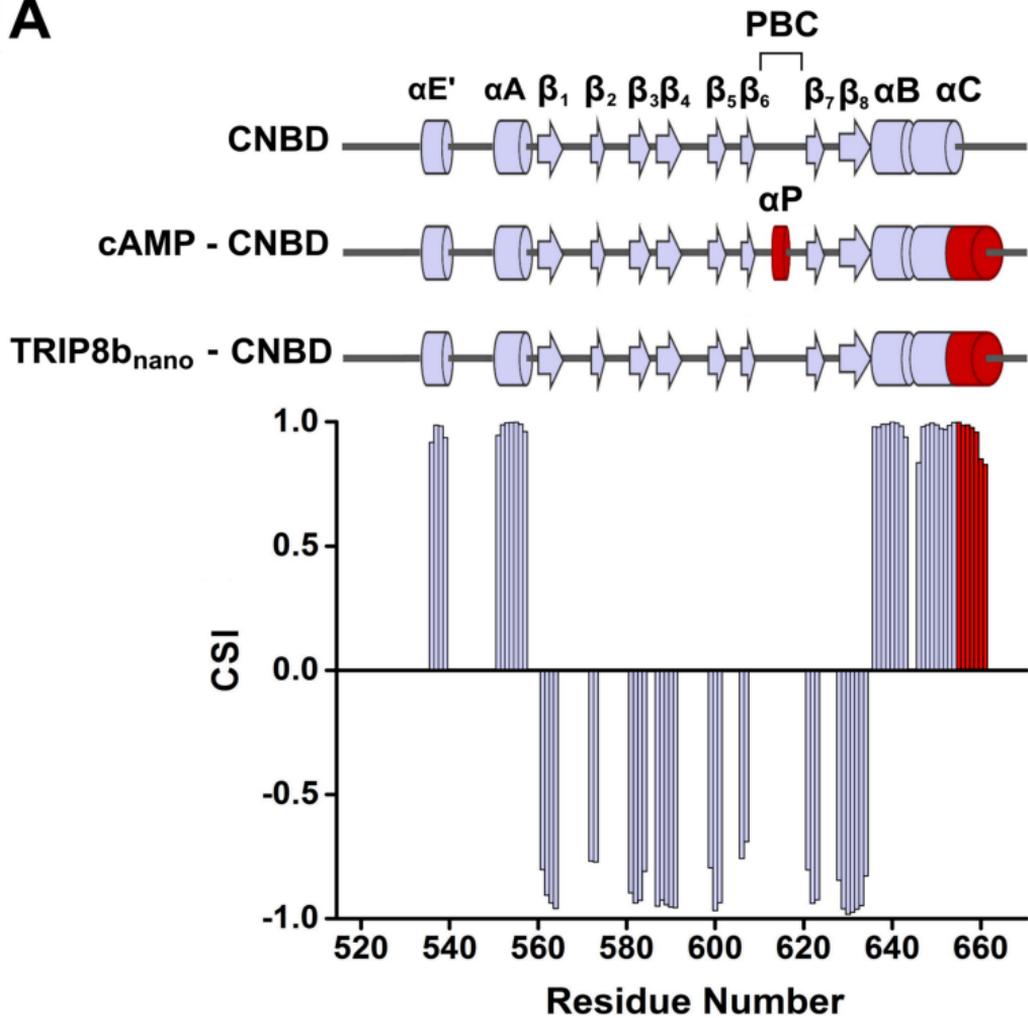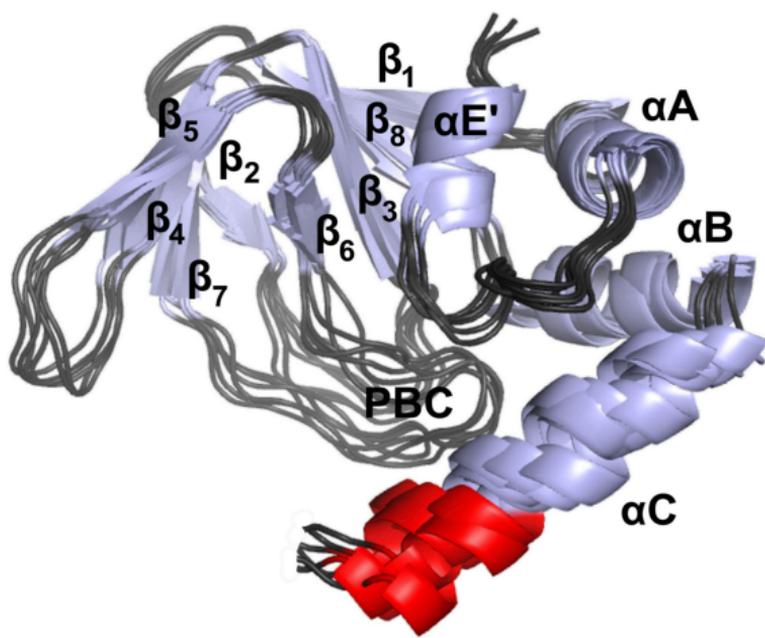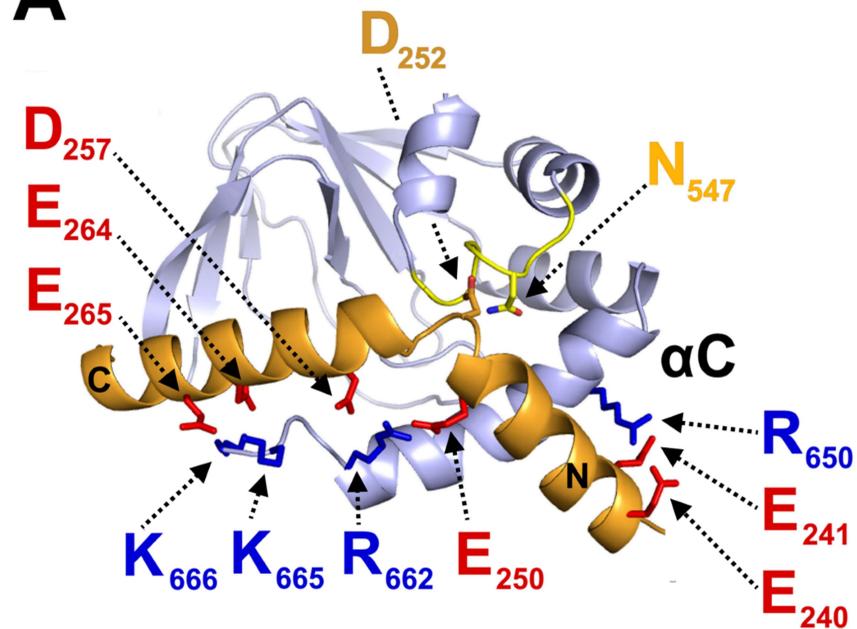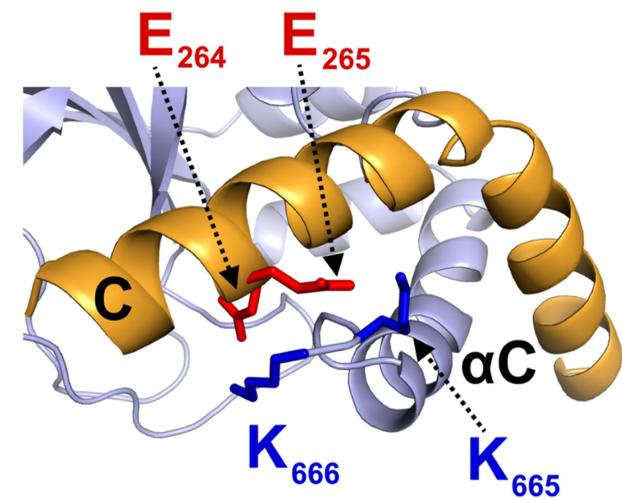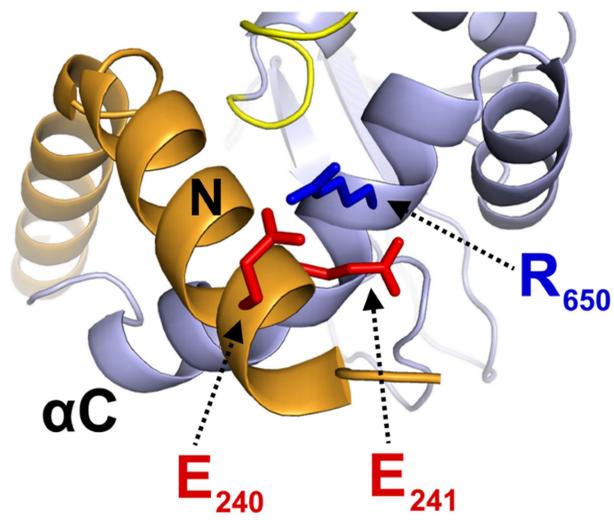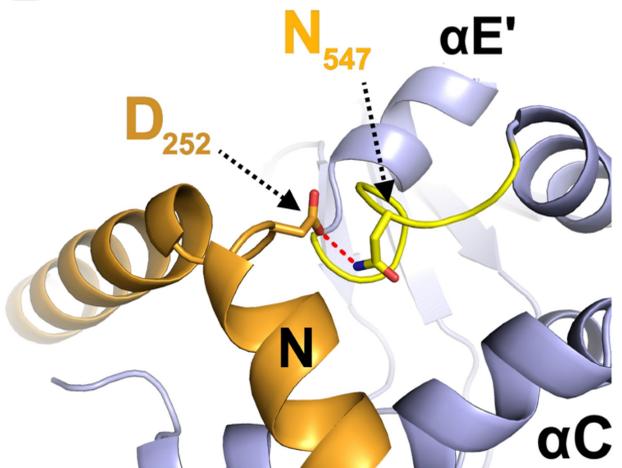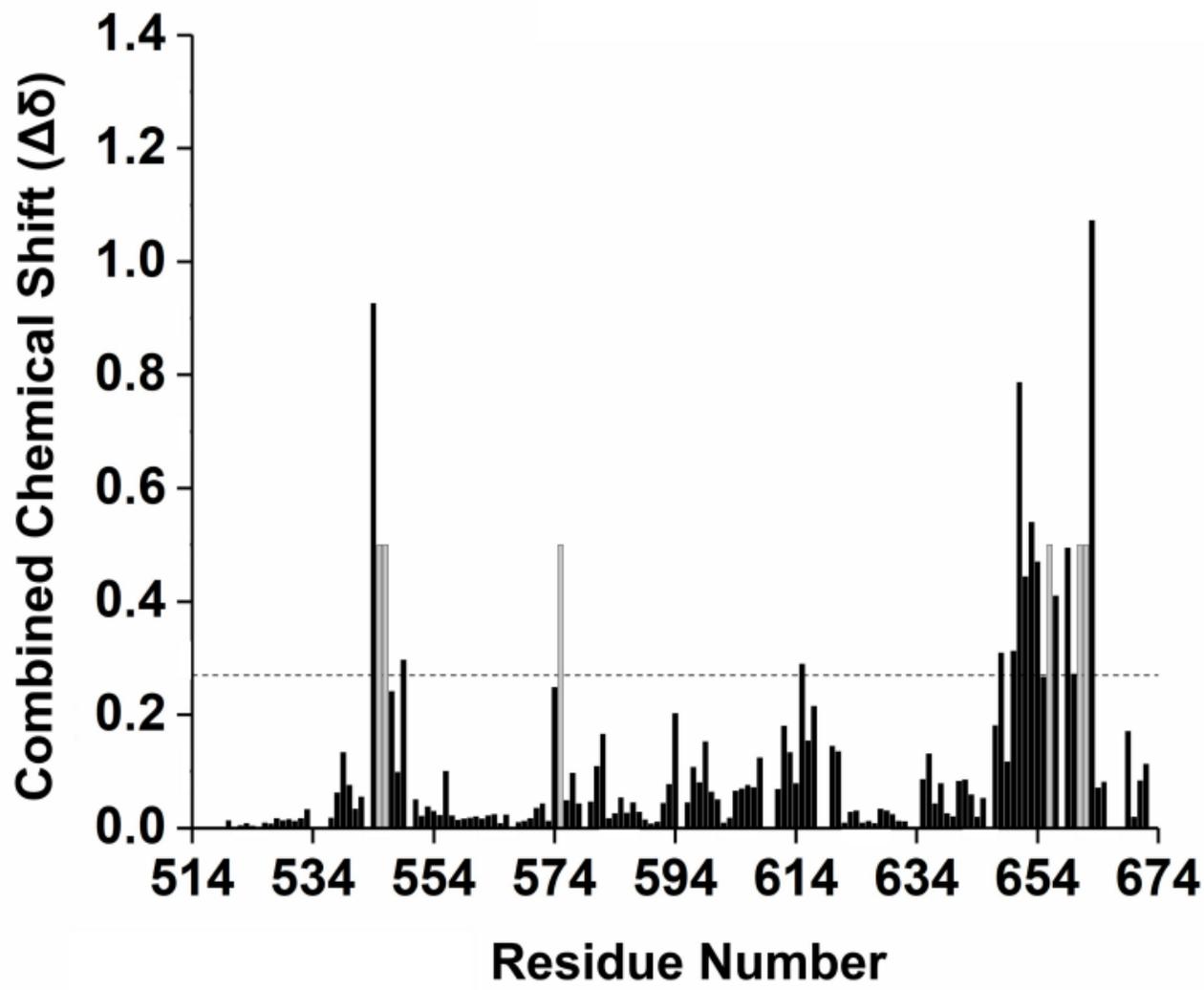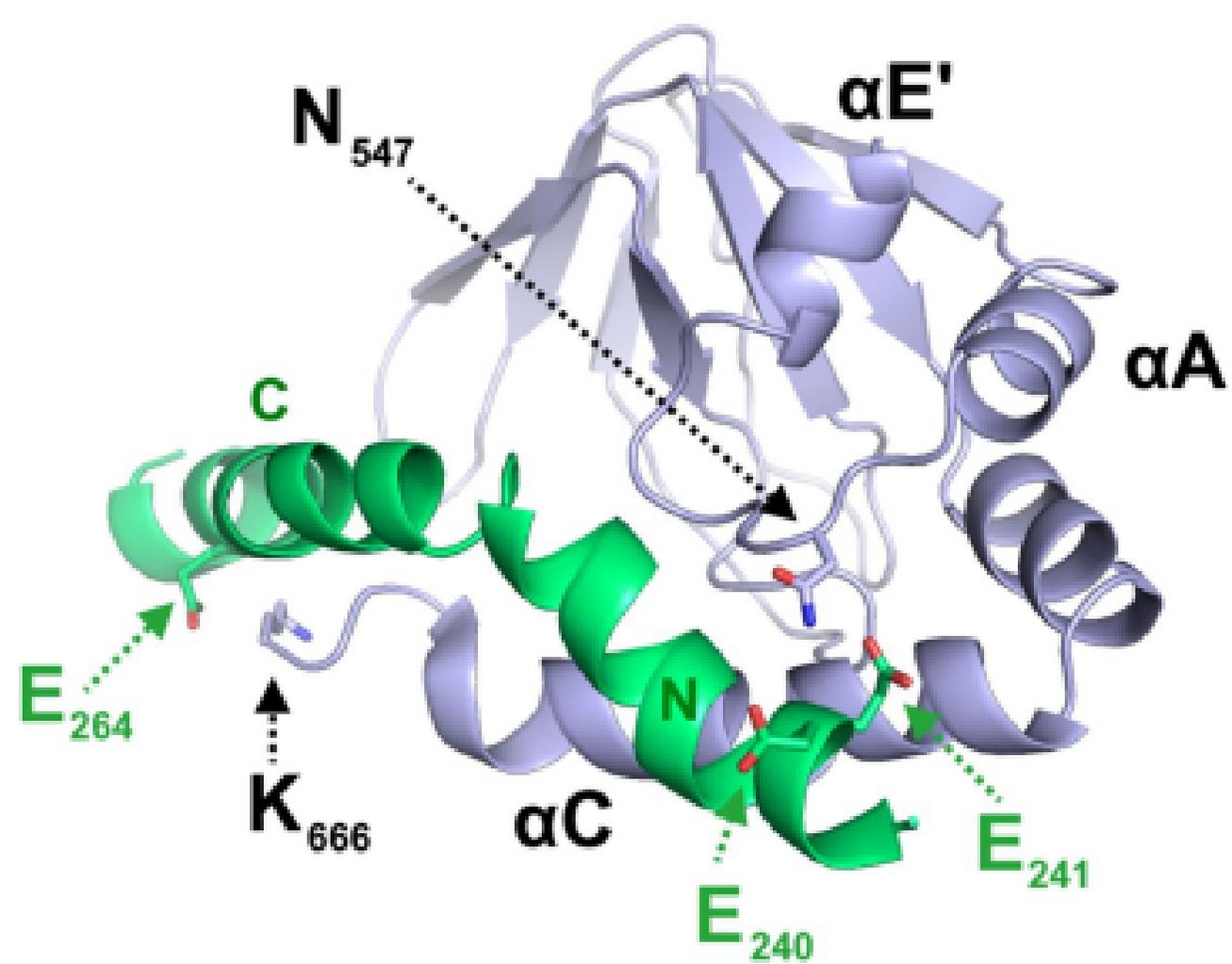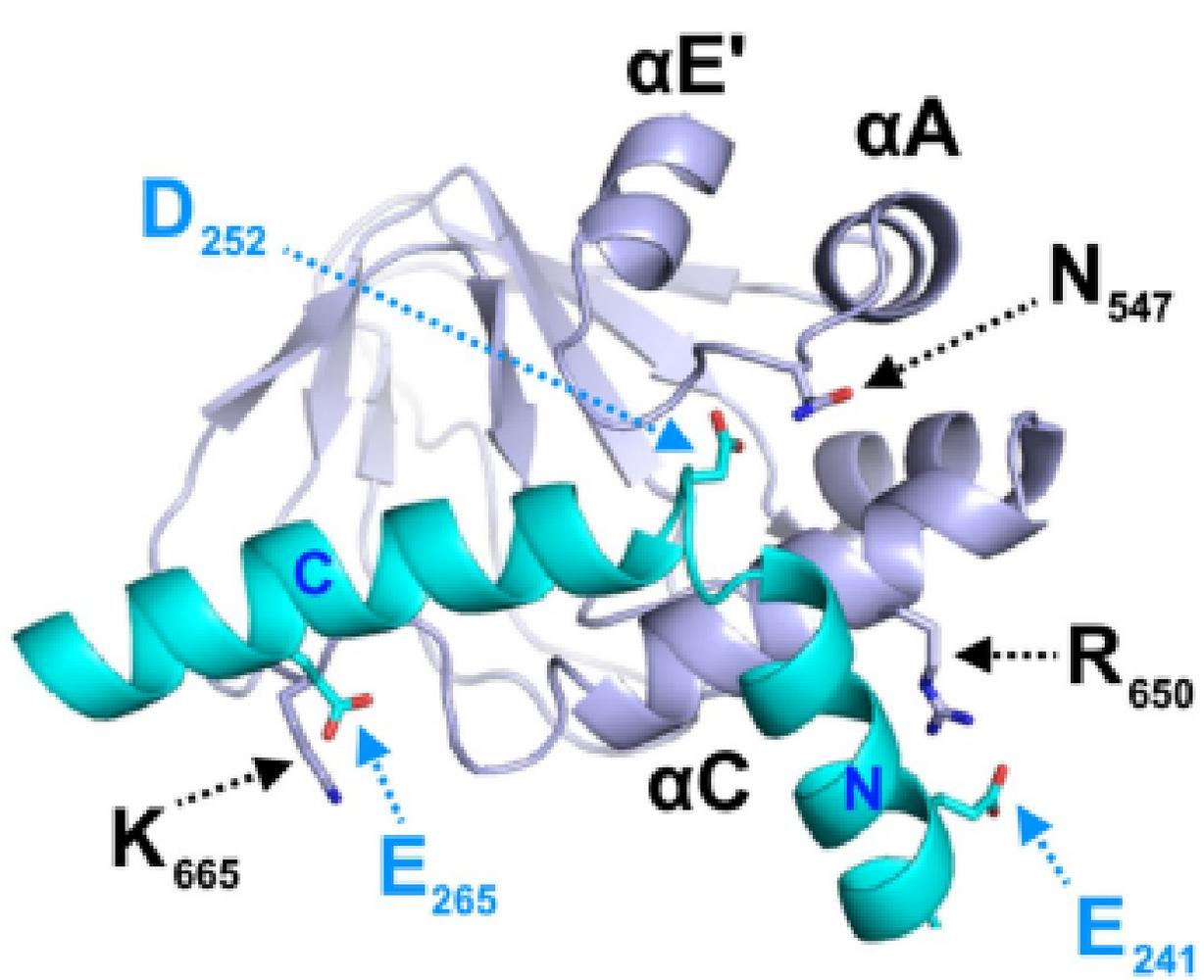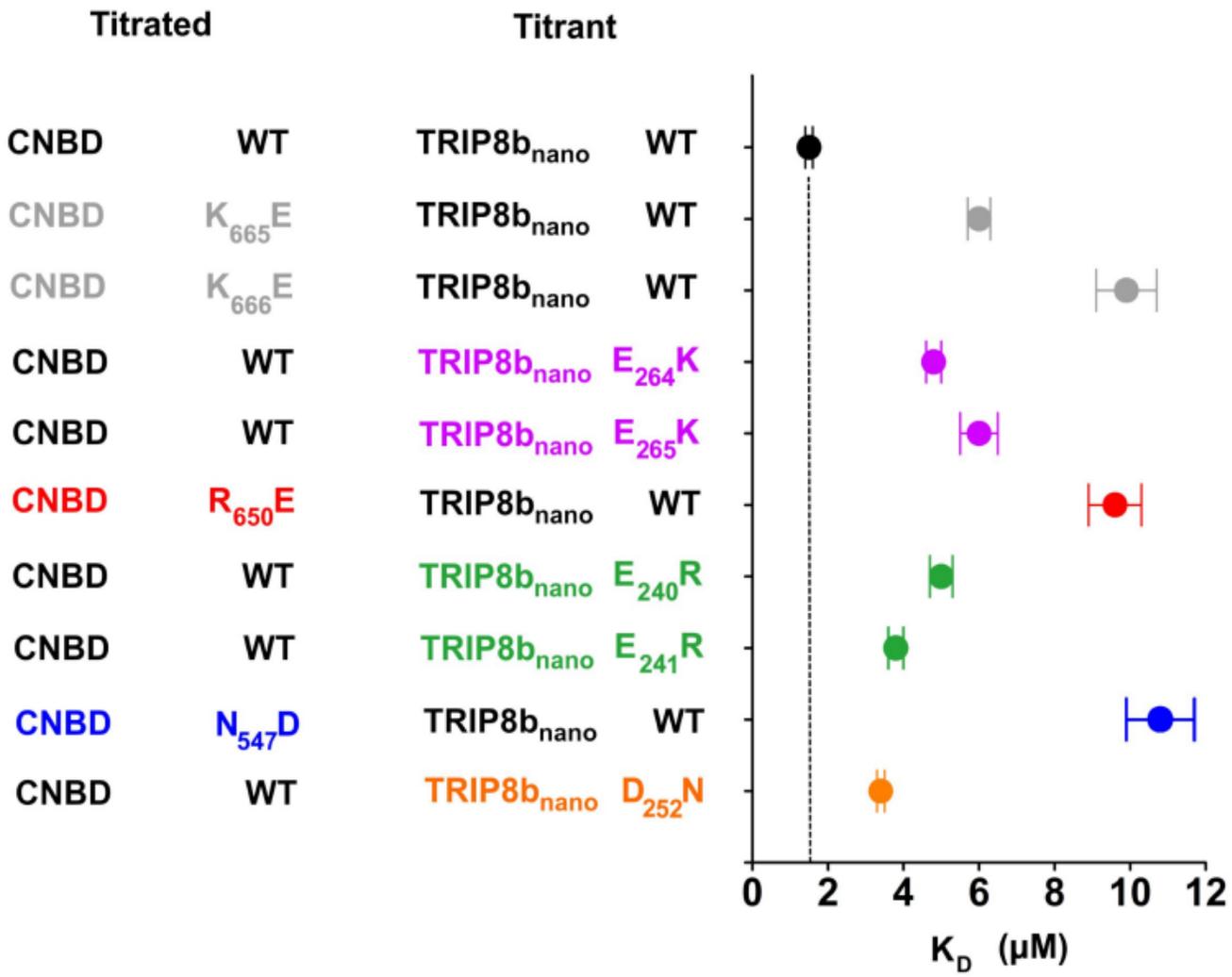
**cAMP-bound CNBD**

**TRIP8b$_{nano}$-bound CNBD**

**A**

HCN2 WT     HCN2 WT + cAMP     HCN2 N$_{520}$D     HCN2 N$_{520}$D + cAMP

-70

-80
-90

500 pA

500 ms

-130

**B**

Fractional activation

Voltage (mV)

**C**

HCN2 WT

+ cAMP

HCN2 N$_{520}$D

+ cAMP

$V_{1/2}$ (mV)

**A**

Control       TRIP8b$_{nano}$

-30
-60
-90
-120
-125

300 pA
2 S

Control + cAMP     TRIP8b$_{nano}$ + cAMP

**B**

Fractional activation

Voltage (mV)

**C**

Control       TRIP8b$_{nano}$

20 mV

0.2 S

Rate (Hz)

*

Control    TRIP8b$_{nano}$

**A** $I_f$

250 pA

CTRL

TRIP8b$_{nano}$

TAT-TRIP8b$_{nano}$

250 ms

0 mV
−35 mV
−95 mV
+5 mV

**D** $I_{Ca,L}$

500 pA

ISO    0nM
ISO 100nM

25 ms

0 mV
−55 mV
−10 mV

**B**

$I_{f(-95mV)}$ (pA/pF)

0
−5
−10
−15
−20

**

0                    10
TRIP8b$_{nano}$ (μM)

**E**

$I_{Ca,L(-10mV)}$ (pA/pF)

0
−10
−20
−30

****              ***

0                    10
TRIP8b$_{nano}$ (μM)

**C**

$I_{f(-95mV)}$ (pA/pF)

0
−5
−10
−15
−20

****

0                    10
TAT-TRIP8b$_{nano}$ (μM)

**F**

$I_{Ca,L(-10mV)}$ (pA/pF)

0
−10
−20
−30

**                ***

0                    10
TAT-TRIP8b$_{nano}$ (μM)

**A**

TAT- (SCR) TRIP8b$_{nano}$

ISO 0nM
ISO 100nM

TAT- TRIP8b$_{nano}$

500 pA

250 ms

0 mV         +5 mV
-35 mV
-95 mV

**B**

I$_{f(-95mV)}$ (pA/pF)

****

TAT- TRIP8b$_{nano}$
(SCR)

TAT- TRIP8b$_{nano}$
(SCR)